Characterization of different chromatin types in *Mytilus galloprovincialis* L. after C-banding, fluorochrome and restriction endonuclease treatments

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Abstract

In this study, we have carried out the cytogenetical characterization of *Mytilus galloprovincialis* L. (2*n* = 28) using conventional staining and banding techniques such as fluorochromes and restriction endonucleases treatment. Chromosome digestion with trypsin enzyme resulted in a G-banding pattern which allowed us to clearly identify and classify the chromosome pairs of *M. galloprovincialis*. C-banding and chromomycin A3 staining confirmed the existence of small amounts of constitutive heterochromatin. The treatment of samples with *Alu*I, *Hae*III, *Dpn*I, *Msp*I, *Hpa*II and *Hinf*I restriction endonucleases produced specific banding patterns which demonstrate the potential of endonucleases for chromosome banding in mussels. The results obtained allow us to describe six different types of chromatin in *M. galloprovincialis*. The type is determined by the response of the chromosomes to the different treatments. Differential digestion by the enzyme pair *Hpa*II-*Msp*I of specific C-band positive heterochromatic areas in some of the chromosomes suggests the presence of methylation.

Keywords: heterochromatin differentiation; *Mytilus galloprovincialis*; restriction endonucleases.

Introduction

Up to the present time, most studies on bivalves' chromosomes have focused on morphological and quantitative criteria, i.e. chromosome number and the length and arm ratios of chromosomes. However, cytogenetical data on the chromosome banding in these marine organisms are minimal, perhaps owing to the technical problems involved in working with chromosomes from bivalves. To our knowledge, most work on the cytogenetic features of these organisms is limited to the description of karyotypes, studies of nucleolar organizer regions (NORs), and some data on G- and C-bands. These include chromosome data on karyotypes of *M. edulis* (Thiriot-Quiévreux & Ayraud, 1982; Moynihan & Mahon, 1983; Dixon & Flavell, 1986), *M. galloprovincialis* (Thiriot-Quiévreux, 1984; Dixon & Flavell, 1986; Pasantes et al., 1990), *Cerastoderma edule, Venerupis pullastra* and *Venerupis rhomboides* (Insua & Thiriot-Quiévreux, 1992) among others, as well as the description of NORs in *M. edulis* (Dixon et al., 1986) and in three oyster species, *Crassostrea gigas, Ostrea edulis* and *Ostrea denselamellosa* (Thiriot-Quiévreux & Insua, 1992). C-bands for *O. denselamellosa* were also obtained in this last study, while in the case of *Crassostrea virginica* the only chromosomal bands obtained were 'G'-bands (Rodríguez-Romero et al., 1979). In *M. edutis*, Moore et al. (1986) showed an idiogram for G- and C-banding patterns and in *M. galloprovincialis* the only bands described was a 2 x SSc-banding pattern (Méndez et al., 1990). In the last decade, the combined use of

different cytogenetic techniques of fluorescence, in situ digestion with restriction endonuclease and C-banding has allowed an extensive study of heterochromatin in the chromosomes of a great number of species. This has been very useful in the analysis of heteromorphisms and/or the analysis of the existence and distribution of different heterochromatic types (Babu & Verma, 1986; Bianchi et at., 1990; Juan et al., 1990; Sanchez et al., 1991). We have applied this powerful set of techniques on fixed metaphase chromosomes to determine the heterochromatin differentiation in *M. galloprovincialis*.

The results described in this paper are the first to provide extensive information about the cytogenetical characterization and heterochromatin differentiation of mussel chromosomes. Moreover, the existence and distribution of different heterochromatin regions is described. Finally, we must point out that as with other species, the use of fluorochromes and restriction enzymes has been essential for this chromosomal analysis.

Materials and methods

Fertilization

Adult mussels (5-10 cm length) were collected from the Ria de Betanzos (La Coruña, N.W. Spain) from September 1991 to May 1992. Once in the laboratory, each mussel was placed in a separate beaker containing 25 1µm-filtered seawater at 27°C (Harrison & Jones, 1982). Upon spawning, the sex of each sample was identified and the fertilization process was carried out by mixing ova and sperm from each sample (in a proportion of 10,000 spermatozoa: 1 ovum, approximately). To avoid bacterial contamination, 500 U/1µ1 of penicillin and streptomycin antibiotics were added. Twenty hours after fertilization, colchicine 0.125mM was added for 4 h at 18-20°C. In order to obtain metaphase chromosomes, the seawater (containing the veliger larvae) was centrifuged at 225 *g* for 5 min; the supernatant was discarded and KCl (0.56 per cent) was added to the pellet for 10 min at room temperature and then centrifuged again. Cells were fixed in ethanol: acetic acid (3:1) for 10 min at 4°C and chromosome spreads were made on cleaned slides.

G- and C-banding

G-banding was carried out as described by Seabright (1971) with some slight modifications. Briefly, the samples were treated with 0.01 per cent trypsin for 5-20 s, then incubated in 5 per cent fetal calf serum for 8 s and washed in PBS. Metaphases were stained with 4 per cent Giemsa for 10 min. The C-banding method developed by Sumner (1972) was employed. The modifications introduced were the following: incubation in HCl 1N for 5 min at room temperature; incubation in 5 per cent $(OH)_2Ba$ for 5 min at 60°C and 15 min in 2 x SSC at 60°C. Metaphases were stained with 0.01 per cent (g ml^{-1}) acridine orange (A.O.) in Sorensen's buffer (0.06 M, pH 6.5) for 5 min and, finally, washed and mounted in the same buffer.

Chromomycin A_3 staining (CMA 3)

The method developed by Schweizer (1976, 1980) was applied, although metaphases were stained for 1 h.

Digestion with restriction enzymes (REs)

Enzymes were supplied by Boehringer Mannheim laboratories. Once the REs were suspended in the appropriate buffer, digestions were induced by placing a drop of each enzymatic solution on a slide and covering with a coverslip. The concentration of each enzyme (AluI, DpnI, HaeIII, Hinfl, HpaII and MspI) varied from 0.3 to 1.0 U/1µl, depending on its activity. Slides were incubated in a moist chamber at 37°C for 6 h, washed with distilled water and, finally, stained with 4 per cent Giemsa for 5-10 min.

Photography

Metaphase chromosomes were observed and photographed with a Nikon microphot AFX microscope.

Results

Chromosome identification

The G-banding pattern obtained after digestion with trypsin (Figs 1 and 2a) allowed us to classify the chromosome complement in *M. galloprovincialis.*

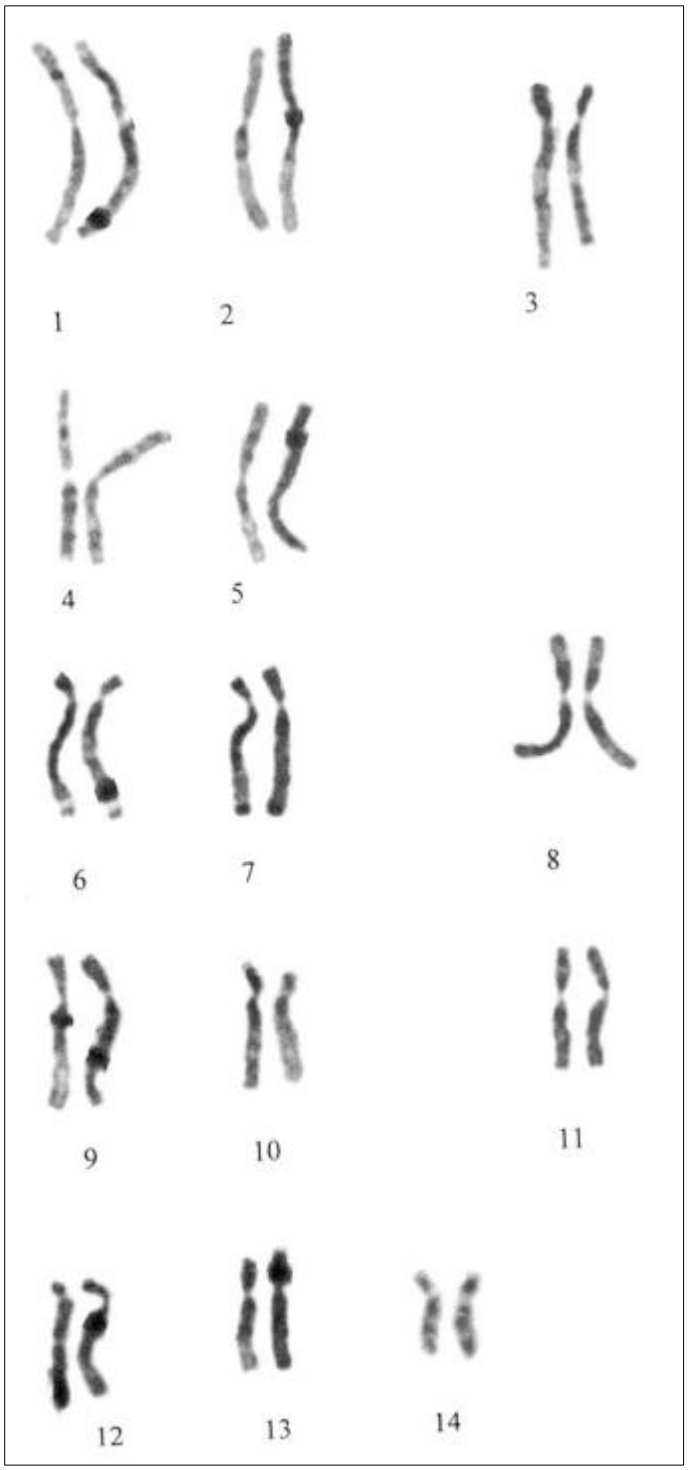

Fig.1. G-banded karyotype of *M. galloprovincialis* (2*n*=28) after digestión with trypsin.

C-bands

Our results demonstrate that *M. galloprovincialis* possesses small amounts of constitutive heterochromatin and no centromeric constitutive heterochromatin. Positive C-band regions are always located on the telomeres and/or appear intercalarily placed along chromosomal arms and only chromosome nos. 1, 3, 5, 6, 7, 9, 12 and 13 show C-bands (Figs 2b and 3). In Fig. 3 we can observe that euchromatic segments show a homogeneously weak fluorescence while C-bands can be divided into three groups: (i) bright fluorescent C-bands: chromosome nos. 1, 3, 7, 9 and the telomere of chromosome 6; (ii) intermediate fluorescent C-bands: chromosome nos. 5, 12 and the intercalary band of chromosome 6; (iii) dull fluorescent C-bands: chromosome no. 13. C-bands are intercalarily located on chromosomes 1, 5 and 12. Chromosome nos. 3, 7, 9 and 13 possess telomeric C-bands and, finally, chromosome 6 shows intercalary and telomeric C-bands.

CMA3 staining

Chromosome nos. 3 and 6 reveal a bright stain when treated with CMA3 and chromosome 7 shows intermediate fluorescence (Fig. 3). The positive CMA3 bands are located terminally on the telomeres; on p arm in chromosome no. 3 and on q arm in chromosome nos. 6 and 7. Positive C- and CMA3- bands coincide in the case of chromosomes 6 and 7 and appear in both members of homologous chromosomes.

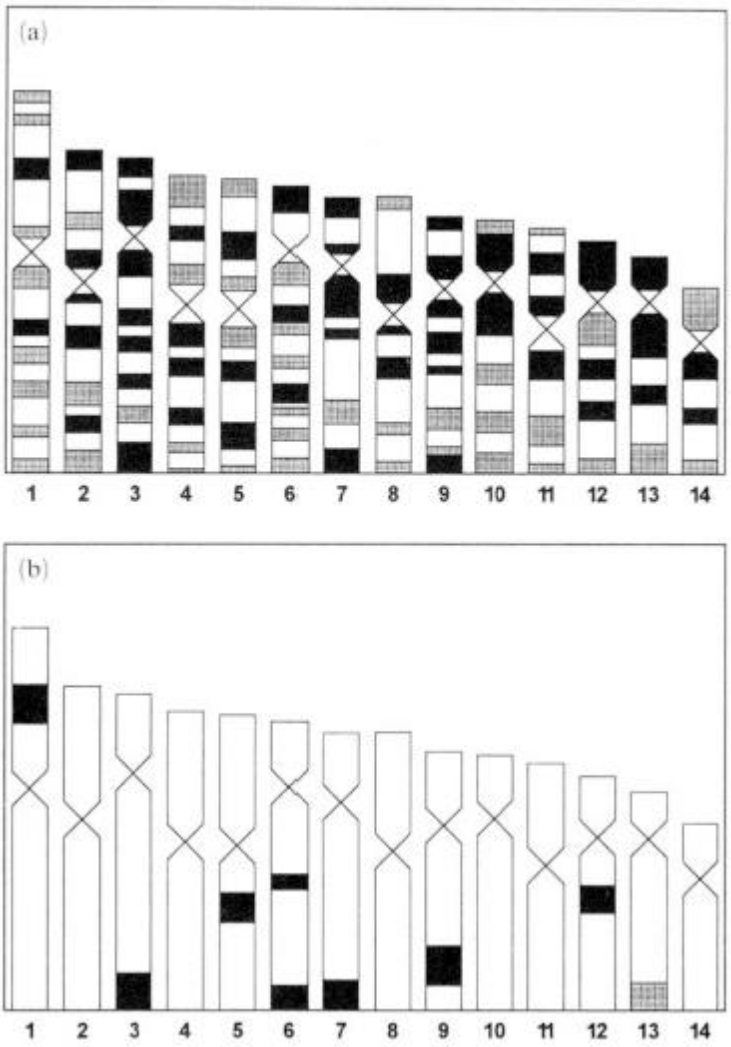

Fig. 2. Idiograms of (a) G- and (b) C-banding of *M. galloprovincialis*

Restriction endonuclease banding

All the restriction endonucleases tested in this study yield specific banding patterns (Fig. 3). The activity of each enzyme is described separately for a more accurate description of the results, using the C-banding pattern as reference. The results summarized in Table 1 represent the distribution of chromosomal bands in *M. galloprovincialis.*

*AIu*I *(AG/CT).*
This enzyme produced positive intercalary and terminal bands and, in general, centromeres and telomeres are clearly well defined. A comparison with CB reveals no difference in the positive C-hands. The heterochromatin of C-bands from chromosomes 1, 3, 5, 6, 7, 9, 12 and 13 appears undigested and well stained after *Alu*I treatment. Centromeres are well differentiated.

Table 1. Distribution of C-, CMA3 and restriction bands in *M. galloprovincialis*

Chromosome no.	C-band	CMA3	*Alu*I	*Hae*III	*Msp*I	*Hpa*II	*Hinf*I	*Dpn*I
1	**	-	**	-	*	-	*	**
3 (p arm)	-	***	**	-	-	***	*	***
3 (q arm)	***	-	***	*	*	**	*	*
5	**	-	**	*	-	-	*	*
6 (interc.)	**	-	**	*	**	*	**	-
6 (telom.)	***	***	***	**	**	-	**	*
7	***	**	***	*	**	-	**	**
9	***	-	**	*	*	*	**	-
12	**	-	**	*	**	**	*	*
13	*	-	**	-	**	-	**	**

***: bright bands; **: intermediate bands; *: dull bands; -: no bands

*Hae*III *(GG/CC).*
Treatment with this endonuclease shows that centromeres and telomeres are well defined and that there are positive bands on chromosomes from *M. galloprovincialis. Hae*III activity causes shorter bands than those produced after C-banding as we can observe on chromosome nos. 3, 5, 6, 7, 9 and 12 (partially digested). C-bands appear totally digested in chromosome nos. 1 and 13 (no banding).

*Hpa*II and *Msp*I *(C/CGG).*
These isoschizomers show different activities in *M. galloprovincialis.* Firstly, treatment with *Hpa*II has no effect on centromeres, which appear undigested and stained, or on the C-band of chromosome 12. *Hpa*II activity results in the partial digestion of the C-band on chromosome 3, and on the intercalary bands of chromosomes 6 and 9, and in the total digestion of the C-band of chromosomes 1, 5, 6 (telomeric), 7 and 13.
After being treated with *Msp*I, centromeres appear unstained. The C-bands on chromosome nos. 1, 3, 6 (telomeric), 7 and 9 are partially digested. The intercalary C-band on chromosomes 6 and 12 is undigested and, finally, the C-band on chromosome no. 5 fails to appear (totally digested).

*Hinf*I *(G/ANTC).*
Chromosomes digested with *Hinf*I show positive bands that were produced as a result of a partial digestion of chromosomes. The *Hinf*I-induced band on chromosome no. 13 is greater than the one produced with the C-banding method. Centromeres are observed to be undigested and unbanded whereas telomeric regions are clearly differentiated.

*Dpn*I *(G^mA/TC).*
Centromeres appear highly decondensed after treatment with this endonuclease, while telomeres appear well-differentiated. *Dpn*I activity causes the partial digestion of C-bands on chromosome nos. 1, 3, 5, 7, 12, 13 as well as the partial digestion of the telomeric band on chromosome no. 6. In contrast, the C-band of chromosome no. 9 is totally digested, as is the intercalary C-band of chromosome no. 6.

Discussion

The G-banding obtained after digestion with trypsin allows the classification and identification of the chromosome complement of mussel. Our results show some differences from those obtained by Méndez et al. (1990). Basically, we visualize, in all the chromosomes, that the centromeres always appear unbanded and the telomeric areas well-banded. The q arm of chromosome no.7 possess a strong clear band and the centromeric region on p arm of chromosome 11 shows a dark band. Furthermore, we do not observe the intercalary bands of chromosome 8 and the proximal centromeric band of chromosome 1. We suppose that these differences between our G-banding pattern and those obtained by Méndez et al. could be caused: (i) by the different chromosomal condensation; (ii) by the different banding methods employed in each case; and (iii) because the chromosomes from the gills are spread on a hot-plate (at 43°C), which can induce the chromosomal contraction.

Comparing the G-banding results we obtained for *M. galloprovincialis* with those reported earlier by Moore et al. (1986) for *M. edulis*-species complex from S.W. England, we must point out that the differences are very significant. These authors presented only a diagram showing what they describe as the 152 band pro-metaphase stage in *M. edulis*, but gave no indication of the method used to visualize these bands (note, trypsin; D. R. Dixon, 1993, personal communication). In another paper Dixon et al. (1986) present a photograph of the banding pattern produced using hot borate buffer which shows only a pale (i.e. G-band negative) staining region associated with the telomere on the q arm of chromosome pair 8 of their karyotype.

The analysis of fixed metaphase chromosomes from *M. galloprovincialis* larvae treated with the C-banding method, CMA3 fluorochrome and restriction enzymes reveals remarkable facts about the nature of heterochromatin and provides information about the existence of different specific classes of highly repetitive DNA in these marine organisms. Firstly, we must clarify why we employ acridine orange to stain C-banded chromosomes. It is known that the C-banding method causes an extensive extraction of DNA (Holmquist, 1979; Burkholder & Duczek, 1982). Consequently, it is difficult to obtain well-stained chromosomes with conventional Giemsa staining and some C-bands are not even distinguishable (Lozano et al., 1990). Staining with fluorochromes allows chromosomes to be observed more clearly, distinctly and selectively; for example Sato (1988) employed acridine orange staining after the C-banding procedure and showed that in plants the NOR-associated with the heterochromatic segments could be differentiated from other segments of heterochromatin.

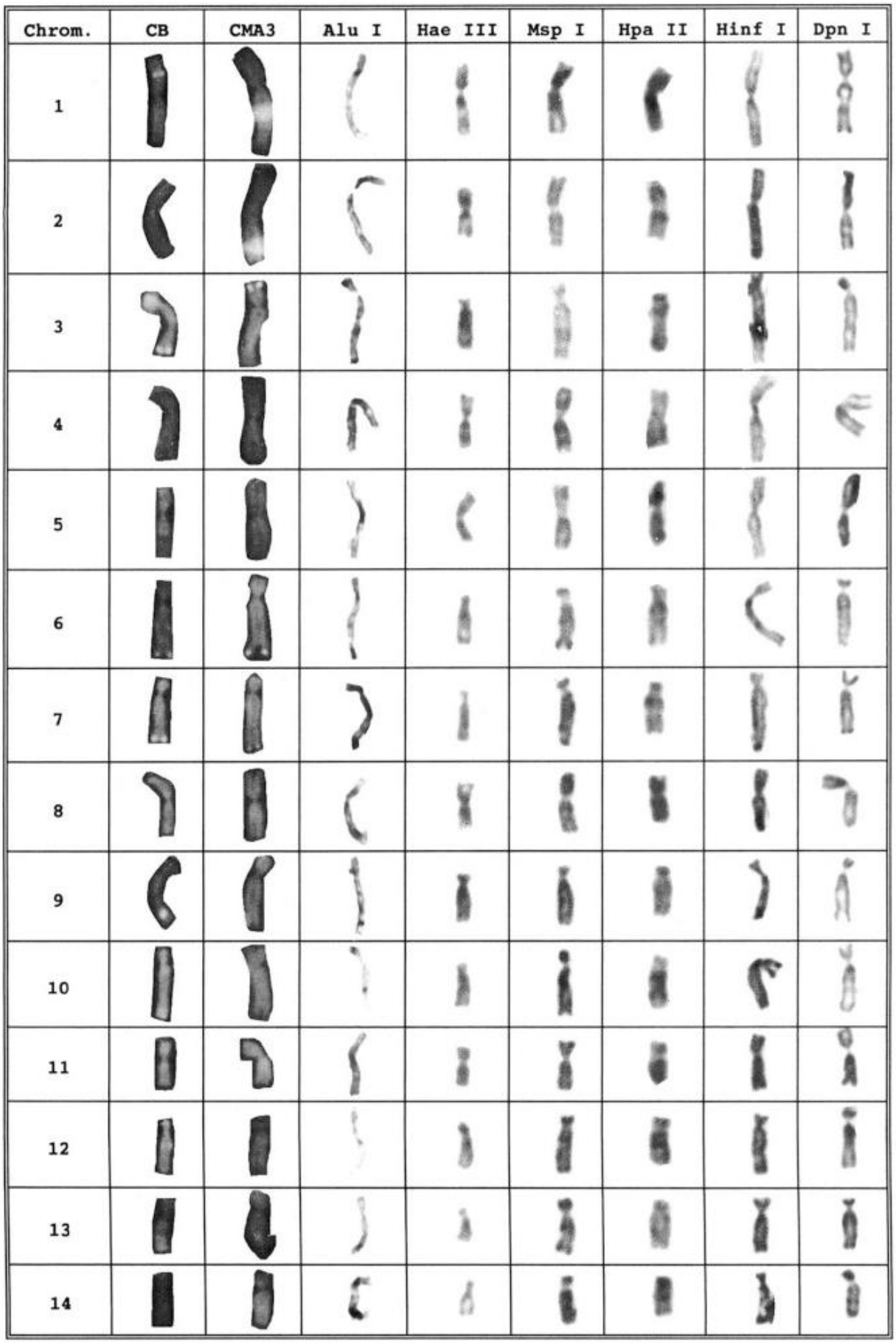

Fig. 3. Haploid karyotype of *M. galloprovincialis* after C-banding, CMA3 staining and *in situ* digestion with *Alu*I, *Hae*III, *Msp*I, *Hpa*II, *Hinf*I and *Dpn*I

According to our results, the C-banding technique reveals that *M. galloprovincialis* possesses small amounts of constitutive heterochromatin and that this type of chromatin: (i) is only observed in some of the chromosomes of the complement, and (ii) is located at the telomeres and/or is also placed intercalarily along the chromosomal arms. We do not observe any telomeric band on metacentric chromosomes, such as pointed out by Dixon et al. (1986), although there are positive telomeric C-bands in three acrocentric chromosomes. The higher number of bands showed by us could be the consequence of the technical problems indicated above. We have also not found that these positive C-bands were negative G-bands as described by these authors. This may indicate significant cytogenetic difference between these two closely related species of mussels (Thiriot-Quiévreux and Ayraud, 1982), which deserves further investigation.

Similarly, treatment with CMA3, a fluorochrome that specifically stains chromosomal areas of GC-rich DNA (Schmid, 1982), results in positive bands on the telomeres of chromosomes 3, 6 and 7. In the case of fish and amphibians it has been proven that with the CMA3 technique the NORs are stained regardless of the activity (Schmid, 1982; Amemiya & Gold, 1986; Cau et al., 1988; Phillips et al., 1988; Martínez et al., 1991). Taking these data into account, we can asume that the positive CMA3 bands which are observed on the chromosomes of mussel are CMA3-stained NORs. These results suggest that the DNA heterochromatin of the heterochromatic telomeric block of chromosomes 3, 6 and 7: (i) is characterized by the presence of repetitive sequences composed of GC base pairs (GC-rich DNA); (ii) that these telomeric blocks are the chromosomal sites where the rDNAs involved in the NORs are cytologically located (not functionally located; see for review, Babu & Verma, 1987); and (iii), it is possible to infer that heterochromatin from C-bands, CMA3-bands and NORs are associated or overlap and that, consequently, these regions share a similar molecular structure.

The combined use of C-banding, staining with fluorochromes and chromosomal digestion with restriction endonucleases has revealed specific banding patterns and allowed us to differentiate C-heterochromatic regions in humans (Miller et at., 1983; Bianchi et al., 1985; Ferrucci et al., 1986; Ludeña et al., 1990), plants (Lozano et al., 1990), insects (Bianchi et al., 1986; Gosálvez et al., 1987; Sentís et al., 1988), fish (Cau et al., 1988; Sánchez et al., 1990) and other different species. However, until now, no cytogenetical study employing these methods had been carried out in mussel. The results obtained when *M. galloprovincialis* chromosomes are treated with restriction enzymes indicate that each one of the endonucleases tested acts differentially and also determines specific banding patterns on the chromosome complement. The presence of residual intercalary bands after treatment with these enzymes (more outstanding after *Alu*I digestion), which are absent after C-banding, indicates that euchromatin is susceptible to banding.

The analysis with REs allowed us to detect C-heterochromatin heterogeneity in mussel chromosomes. Table 2 shows that the heterochromatin could be divided into at least six different types (results from C-banding and CMA3 are also considered). The results obtained suggest that the DNA located at the C-bands contains few, if any, *Alu*I *(AG/CT)* and *Hinf*I *(G/ANTC)* recognition sites but does contain a relatively large number of *Hae*III and *Hpa*II recognition sites *(GG/CC* and *C/CGG*, respectively). This leads us to the conclusion that the highly repeated DNA in *M. galloprovincialis* is GC-rich. The differences in the digestion patterns of the isoschizomers *Msp*I and *Hpa*II could be attributed to the existence of a certain amount of methylation in the cytosine residues of *CCGG* sequences. The *Dpn*I-restriction banding pattern can result from a low concentration of restriction sites or from adenine methylation. This enzyme needs the methylation of adenine residues for its activity and only digests $G^{m}ATC$ sequences. DNA methylation of *M. galloprovincialis* requires a more extensive study which we are currently undertaking.

In conclusion, despite the technical problems and limitations of working with mussel chromosomes, our results indicate that the staining with fluorochromes and *in situ* digestion with restriction enzymes are both particularly useful techniques in the analysis of the nature and distribution of heterochromatin in *M. galloprovincialis*. Five of the six enzymes used produced specific banding patterns that differed from conventional C-bands. Such results prove that some chromosomes or some chromosomal regions belong to the same heterochromatin type, while others are unique (for example, the telomere of chromosome 3 and the intercalary band of chromosome 5) and this allows us to subdivide these regions based on the presence or the absence of the restriction sites within the respective DNA as well as to detect C-heterochromatin heterogeneity in mussel chromosomes.

Table 2. Different chromatin types in *M. galloprovincialis*

Types	C-band	CMA3	*Alu*I	*Hae*III	*Msp*I	*Hpa*II	*Hinf*I	*Dpn*I	Chromosome location
1	*	*	*	*	*	-	*	*	Telomere q arm C6, C7
2	*	-	*	*	*	*	*	-	Telomere q arm C9
									Intercalary q arm C6
3	-	*	*	-	-	*	*	*	Telomere p arm C3
4	*	-	*	-	*	-	*	*	Telomere q arm C13
									Intercalary p arm C1
5	*	-	*	*	-	-	*	*	Intercalary q arm C5
6	*	-	*	*	*	*	*	*	Telomere q arm C3
									Intercalary q arm C12

*: presence of banding; -: abscence of banding; C: chromosome

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