Chromosomal markers in three species of the genus *Mytilus* (Mollusca: Bivalvia)

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Heredity, volume 74**,** issue 4, pages 369-375, 01 april 1995
Received 26 may 1994, first published 01 april 1995

How to cite:
Martínez-Lage, A., González-Tizón, A. & Méndez, J. Chromosomal markers in three species of the genus *Mytilus* (Mollusca: Bivalvia). *Heredity* **74**(4), 369–375 (1995). DOI:10.1038/hdy.1995.55

Abstract

The analysis of C-banding, NOR and fluorochrome staining was carried out in three species of European mussel, *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus*. The results obtained allow us to detect changes in the constitutive heterochromatin within the genus *Mytilus*. The existences of chromosomal markers permit us to identify and distinguish, at the cytogenetical level, these three types of mussel.

Keywords: C-bands; chromosome markers; CMA3 bands; heterochromatin; *Mytilus*; NORs.

Introduction

The genus *Mytilus* is the subject of an important controversy about the systematic status of the different forms within it. In this genus, taxonomic studies were initially developed in order to establish a systematic relationship between *M edulis* and *M. galloprovincialis* and, later, among other forms of mussels. At first, the studies were focused on morphological criteria and morphometric parameters (internal and external shell characteristics, the anterior adductor muscle features, etc.). However, as pointed out by Seed (1968), such characters are enormously plastic, being influenced by factors such as the age of the mussels, the density of the population, the tidal level and habitat type, and even then they have not allowed identification of the different types of mussels. Later, samples of mussels from different geographical regions in the northern and southern hemispheres were electrophoretically analysed. Different loci were studied, but none of them was truly diagnostic; namely none allowed assignment of a particular sample to a particular species. However, the results obtained after studying several loci per individual and a great number of individuals per population have allowed us to characterize different populations and to discriminate between different mussel types within the genus *Mytilus* (Koehn, 1991; Gosling, 1992).

According to these data, it is considered that there are three species of mussel within the genus *Mytilus* distributed along European coasts. *M. edulis* is distributed along the Atlantic coast of Europe; *M. galloprovincialis* appears to be distributed along the Mediterranean coast, the French Atlantic coast and along various coastal regions of the British Isles. Subsequently, in France, Britain and Ireland, *M. galloprovincialis* has been found to be intermixed with *M. edulis*, producing hybrid forms (Skibinski & Beardmore, 1979). *M. trossulus* is distributed in the Baltic Sea. In the Danish straits, *M. trossulus* is intermixed with *M. edulis* (Väinölä & Hvilsom, 1991).

On the other hand, studies using *Mytilus* cytogenetics have shown that the diploid number is 28 chromosomes (Ahmed & Sparks, 1970; Ieyama & Inaba, 1974; Thiriot-Quiévreux & Ayraud, 1982;

Moynihan & Mahon, 1983; Dixon & Flavell, 1986; Pasantes et al., 1990), and were confined to the descriptions of the nucleolar organizer regions and the location of heterochromatin in *M. edulis* (Dixon et al., 1986; Dixon & McFadzen, 1987). In *M. galloprovincialis* a 2 x SSC banding pattern has been induced by Méndez et al. (1990) and NORs were described by Martínez-Expósito et al. (1994) in four populations of La Coruña (Galicia, NW Spain) and cytogenetic characterization by means of C-banding, fluorochromes and restriction endonucleases has been carried out by Martínez-Lage et al. (1994).

In the present paper we describe the differences among *M. edulis*, *M. galloprovincialis* and *M. trossulus* on the basis of C-, NOR and fluorescence banding in order to detect possible chromosome markers which will allow us to identify, at the individual level, the different mussel types.

Materials and methods

Taking into account the distribution of the genus *Mytilus* along the European coasts, we have collected samples from places where hybrid forms have not been detected. So, individuals of *M. edulis* were collected from Zoutelande (Zeeland, Holland), individuals of *M. galloprovincialis* from Ría de Betanzos (La Coruña, Spain) and individuals of *M trossulus* from Dahme (Mecklenburger Bay, Germany). The process of fertilization and culture was carried out as described by Martinez-Lage et al. (1994), from six females and five males of *M. edulis*, 10 females and 10 males of *M. galloprovincialis*, and five females and seven males of *M. trossulus*.

The C-banding method (Sumner, 1972) was used for constitutive heterochromatin localization; metaphases were stained with acridine orange (AO) in Sorensen's buffer (0.06 M, pH 6.5) for 5 min. Silver nitrate staining was performed according to Howell & Black (1980). Fluorescence analysis was carried out using quinacrine (Cassperson et al., 1968); chromomycin A3 (CMA3), dystamicin (DA) and 4'-6 diamidine-phenyl indole (DAPI) according to the methods developed by Schweizer (1976, 1980).

Metaphase chromosomes were observed and photographed with a Nikon microphot AFX microscope. For fluorescence, the Nikon filters employed were UV1A (AD/DAPI), BV-lA (CMA3) and B-2A (AO).

Results

Giemsa karyotype

The karyotypes of *M edulis*, *M. galloprovincialis* and *M. trossulus* comprise six pairs of metacentric and eight pairs of submeta-subtelocentric chromosomes (Fig. 1).

C-banding

In these three species, some differences exist in the C-bands distribution. In *M. galloprovincialis*, as previously reported by Martínez-Lage et al. (1994), C-bands appear on chromosomes 1, 3, 5, 6, 7, 9, 12 and 13. *M edulis* and *M. trossulus* show the same location of C-bands; these are located on chromosomes 1, 3, 5, 6, 7, 8, 12 and 13. Table 1 shows the localization of the C-bands in these species. We observed that the main differences involved chromosomes 1, 3, 6, 8 and 9 (Fig. 2; Table 1). These differences are: (i) the presence of one centromeric and two telomeric bands in chromosome 1 of *M. edulis* and *M trossulus*, which are replaced by an interstitial one in *M. galloprovincialis*; (ii) chromosome 3 from *M edulis* and *M. trossulus* shows telomeric C-bands on the p arm, which are absent in *M. galloprovincialis*; (iii) the intercalary C-band of chromosome 6 fails to appear in *M edulis* and *M. trossulus*; (iv) the telomeric C-band of chromosome 8

appears in *M. edulis* and *M. trossulus* but not in *M galloprovincialis*; and (v) *M. galloprovincialis* shows a telomeric C-band on chromosome 9, which is absent in the other species.

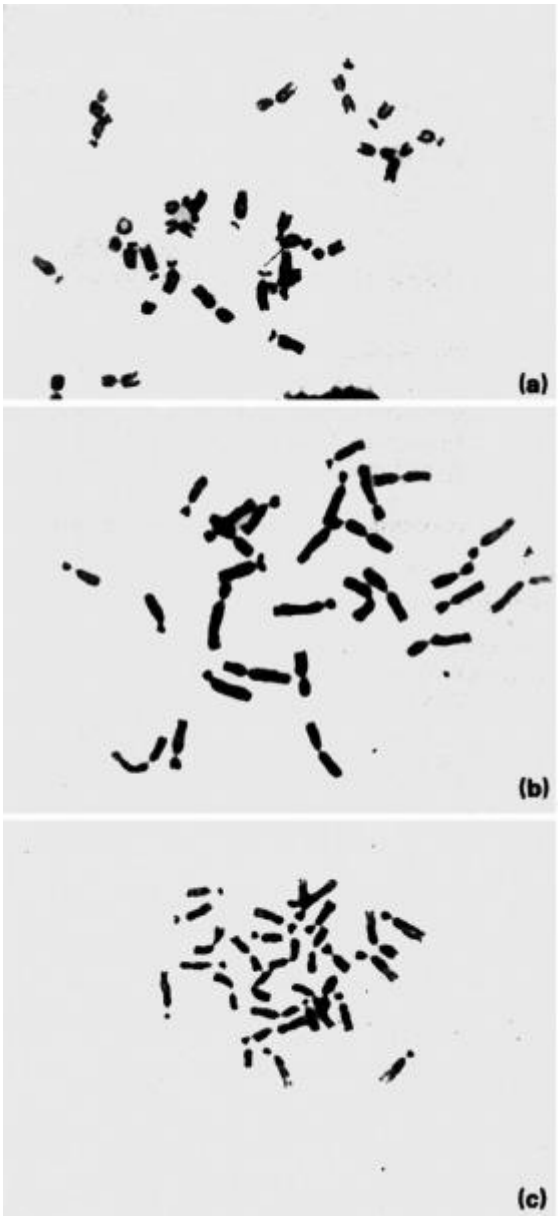

Figure 1. Giemsa metaphase plates from *Mytilus edulis*, *M. galloprovincialjs* and *M. trossulus*.

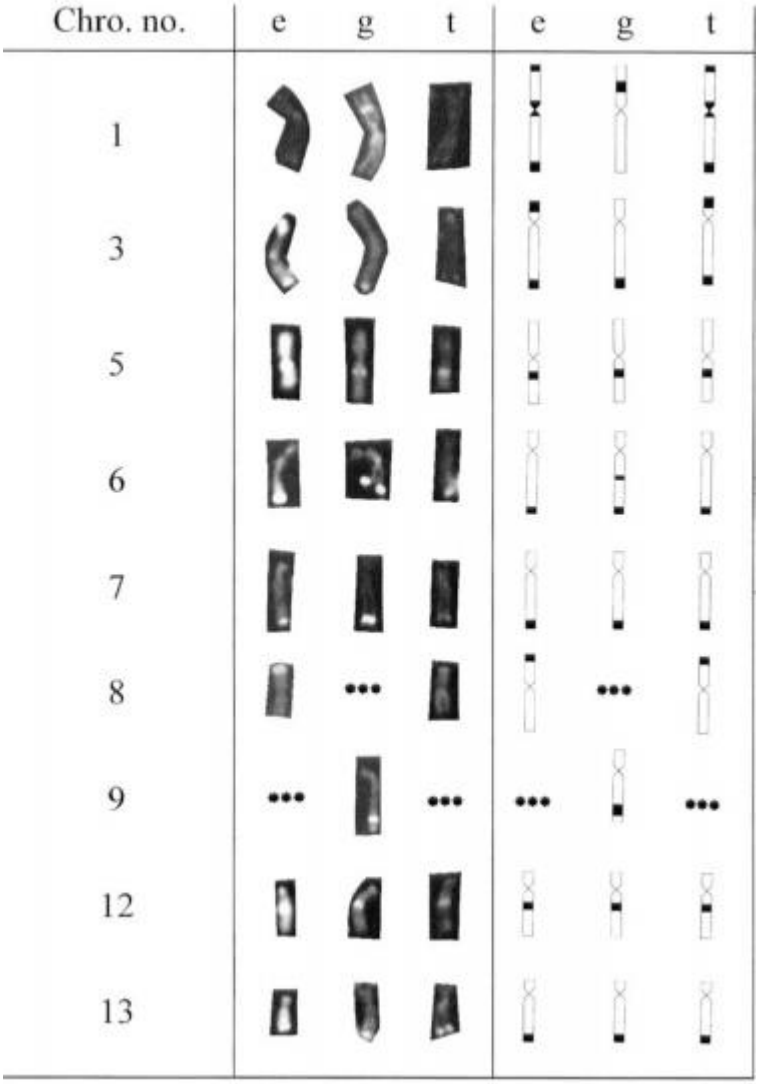

Figure 2. C-banding and comparative idiograms of the three European mussel species. **e**, *Mytilus edulis*; **g**, *M. galloprovincialis*; **t**, *M. trossulus*; **chro. no**., chromosome number; *******,absence of banding.

NORsilver staining

Forty larval metaphases of each mussel species were analysed. We have detected a variable number of AgNORs, which varied from two to four in *M. edulis* and *M galloprovincialis*, and from two to five in *M trossulus*. The silver-stained karyotypes of the three species show chromosomal pairs 6 and 7 (submeta-subtelocentrics) with telomeric NORs (Fig. 3a, c, e). In *M trossulus*, a third additional NOR appears located on the p arm of metacentric chromosome 8 (Fig. 3e, Table 2).

Table 1. Distribution of C-bands in mussel species

C. no.	*M. edulis*	*M. galloprovincialis*	*M. trossulus*
1p, t	*	--	*
1 p, i	--	***	--
1c	*	--	**
1q, t	*	--	*
3 p, t	***	--	*
3 q, t	**	**	*
5 q, i	***	**	**
6 q, i	--	**	--
6 q, t	***	***	**
7 q, t	**	***	*
8 p, t	**	--	*
9 q, t	--	***	--
12 q, i	***	***	**
13 q, t	**	**	**

*******, strong bands; ****** intermediate bands; ***** dull bands; -- no banding; **p**, band on p arm; **t**, telomeric band; **c**, centromeric band; **i,** interstitial band; **C. no**., chromosome number.

Fluorochrome staining

Telomeric CMA3 bands were found on chromosomes 6 and 7 in each one of the three mussel species (Fig. 3b, f, g; Table 2). In *M galloprovincialis*, a third telomeric CMA3 band appears located on the p arm of chromosome 3 (Fig. 3d).

DA/DAPI produced a negative response on the chromosomes, which was accompanied by a decrease of the staining in the telomeric regions of chromosomes 6 and 7 from the three species (Fig. 3g,h). Similarly, Q staining (Fig. 3i) revealed a negative response in each of the species.

Discussion

The genus *Mytilus* is a complex of closely related species which share the same basic karyotype in terms of external chromosome morphology. When we examined metaphases from *M. edulis*, *M. galloprovincialis* and *M. trossulus*, we observed an uniformity of chromosome number, relative chromosome size and centromere position.

According to our previous report on *M. galloprovincialis* (Martínez-Lage et al., 1994), C-banding analysis confirms that these three species possess small amounts of constitutive heterochromatjn which is located, mainly, in the telomeres and interstially, and very rarely located on centromeres. After C-banding, chromosomes from *M. edulis* and *M. trossulus* exhibit 11 blocks or regions of constitutive beterochromatin, and *M. galloprovincialis* only shows nine heterochromatin regions (Fig. 2; Table 1). The more remarkable differences in C-bands among these species are related to chromosome 1 from *M. galloprovincialis*, which only shows an interstitial C-band, whereas *M. edulis* and *M. trossulus* show two telomeric and one centromeric C-band (Fig. 2; Table 1). Furthermore, in *M. galloprovincialis*, the loss of the heterochromatic C-bands on the p arms of chromosomes 3 and 8 and the gain of the interstitial C-band on chromosome 6 and the telomeric one on chromosome 9 reflect some changes in the heterochromatin of these species in the course of divergence from *M edulis*.

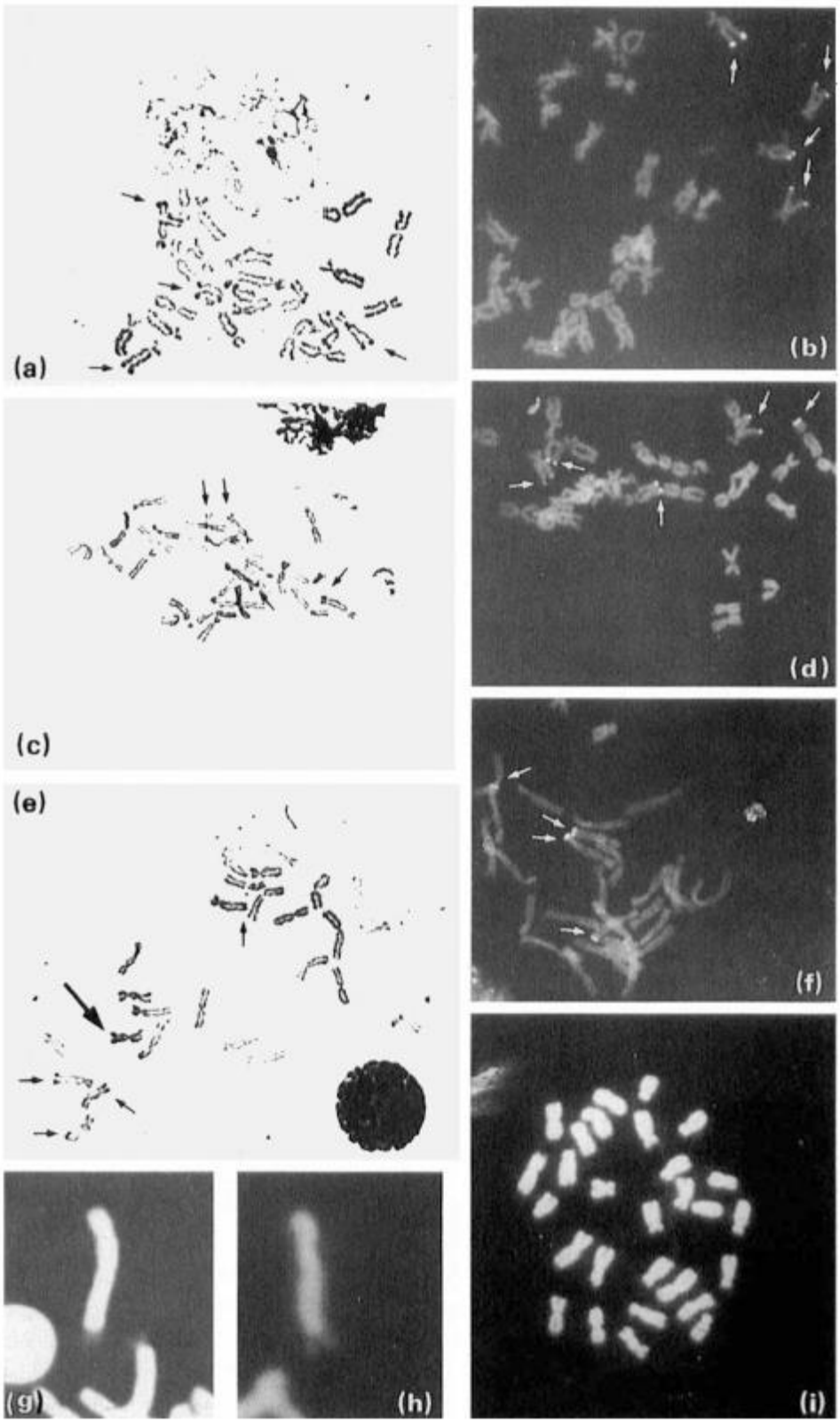

Figure 3. NORs and CMA3 bands in *Mytilus edulis* (**a,b**), *M. galloprovincialis* (**c,d**) and *M. trossulus* (**e,f**). In (**3e)** the wide arrow identifies the additional single NOR. (**g,h**) absence of staining on the telomeric regions of chromosomes 6 and 7 from *M. edulis* after DA/ DAPI staining. (**i)** metaphase from *M. galloprovincialis* after quinacrine staining.

Table 2. Distribution of NORs and CMA3 bands in mussel species

	M. edulis		*M. galloprovincialis*		*M. trossulus*	
C. no.	**NOR**	**CMA3**	**NOR**	**CMA3**	**NOR**	**CMA3**
3 p, t	--	--	--	*	--	--
6 q, t	*	*	*	*	*	*
7 p, t	*	*	*	*	*	*
8 q, t	--	--	--	--	*	--

*****, presence of band; —, absence of band; **C. no**., chromosome number; **p**, band on p arm; **q**, band on q arm; **t**, telomeric band

It is assumed that the difference in the amount and location of constitutive heterochromatin is a mechanism of karyotype differentiation. In this sense, some models have been proposed to explain these changes or variations in heterochromatin. King (1980) and, later, other authors predicted that the differences in C-bands could be attributed to the addition or loss of heterochromatin; this process may be accompanied by chromosomal rearrangements such as fusions or fissions which would alter the morphology of the chromosome complement. Such types of changes have been described by Mayr et al. (1985) in mammals and by John et al. (1985) in grasshoppers. However, in mussels, we have not observed differences in the chromosome length or morphology between the species studied; our results suggest that this mechanism is not involved in the differentiation of the karyotypes. The second mechanism postulates the transformation of heterochromatin to euchromatin (or the reverse), which would involve internal transformation changes. This process seems to be uncommon, but it has been described in rats by Yosida & Sagai (1975), in orthopterans by Camacho et al. (1981) and in crocodiles by King et al. (1986). Initially, our results suggest that the differences in the C-banding patterns between *M. edulis—M trossulus* and *M. galloprovincialis* could be produced by a heterochromatin—euchromatin transformation process. As we have pointed out, there are changes in the heterochromatin but chromosomal rearrangements do not exist.

On the other hand, the C-bands of the *Mytilus* species analysed in this paper do not show centromeric location (except for chromosome 1 from *M. edulis* and *M. trossulus*). To explain the location and distribution of the heterochromatic regions, Macgregor & Sessions (1986) propose that the centromeres are C-band initiation sites from which heterochromatin transference takes place towards the telomeres and, therefore, karyotypes with more telomeric heterochromatin have an older phylogenetic status. In this sense, the presence of more telomeric bands in *M. edulis* than in *M. galloprovincialis* corroborates the phylogenetic data which indicate that *M edulis* has an older evolutionary status.

The analysis of NOR regions revealed the functional location on the telomeres of chromosomes 6 and 7 in each one of the mussel species. In *M. trossulus* a third additional single NOR was located on the telomere of metacentric chromosome 8. A clear coincidence between C-banding and NORs is detected in chromosomes 6 and 7 from the three species and in chromosome 8 from *M. trossulus*. Furthermore, CMA3 staining reveals a positive response on the NOR regions of chromosomes 6 and 7 in the three species. A third positive CMA3 band appears on the short arm of chromosome 3 in *M galloprovincialis*, but it does not appear to be an active NOR. The additional NOR detected in *M. trossulus* appears to be CMA3 negative.

Some possible mechanisms have been postulated to explain the appearance of new NORs in species. One of the most common suggests the activation of latent NORs, which take over the primary nucleolar function under certain conditions or in certain genomes (King, 1980; Verma & Raina, 1981; Fernández-Piqueras et al., 1983; Cabrero et al., 1987). However, we do not think that the additional NOR of *M. trossulus* constitutes a latent NOR, because this region is shown to have a different heterochromatin from the NORs of *M. edulis* and *M galloprovincialis* (chromosomes 6 and 7 are CMA3 positive, whereas chromosome 8 from

M. trossulus is CMA3 negative). In this sense, we hope that *in situ* hybridization analysis will clarify this situation.

The existence of an additional NOR in *M trossulus* allows us to distinguish between this species and *M. edulis*. As both species show the same location and distribution of C- and CMA3 bands, let us suppose that they have the same evolutionary status. The NOR of *M. trossulus* has a different heterochromatin status from the other NORs; this is therefore evidence of its uniqueness in this species. The additional CMA3 band detected in *M. galloprovincialis* and the additional NOR in *M trossulus* can be considered as chromosomal markers which allow us to identify and distinguish the three types of mussels.

On the other hand, staining with AD/DAPI reveals an absence of banding on the chromosomes of the genus *Mytilus*. The negative staining observed in the telomeric areas of chromosomes 6 and 7 (Fig. 3g, h) had already been observed by Dixon & Flavell (1986) in *M. edulis* when they treated the chromosomes with Giemsa staining and/or borate buffer treatment. The existence of these areas, which are coincident with the locations of active NORs, led us to suppose that a correlation between chromatin decondensation and transcriptional activity must exist in these organisms. Furthermore, the fact that these telomeric regions were shown to be C positive/CMA3 positive/NOR positive/AD/DAPI negative, confirms the absence of AT-rich base pairs, in opposition to the data reported by Dixon & McFadzen (1987) from *M edulis* that C positive/NOR positive regions were AT-rich. The negative response after Q banding seems to reflect the nonexistence of AT-rich regions along the chromosome structure of these organisms. However, taking into account the results obtained after *in situ* digestion with restriction endonucleases (unpublished data), we could suppose that AT sequences are interspersed in the genomes of these species and, consequently, there could exist a certain degree of genome compartmentalization.

In conclusion, the distribution of C-bands and the existence of chromosomal markers allow us to distinguish these mussel species, and open up new possibilities to clarify the systematic status of the genus *Mytilus*. We consider that the karyotypic changes which have taken place in the evolution of this genus result from qualitative modifications in the constitutive heterochromatin. We hope that studies of satellite DNA and in situ hybridization will help us to elucidate the role of the constitutive heterochromatin and NORs in the course of karyotypic divergence of the *Mytilus* species studied in this paper.

Acknowledgements

We are very grateful to Dr H. Hummel, who permitted us to use his laboratory at Delta Instituut voor Hydrobiologisch Onderzoek of Yerseke (Holland), and to Miss B. Carril for her technical assistance. This work has been supported by a D.G.I.C.Y.T. 90/0323 (Spain).

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