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Supernumerary chromosomes on Southern European populations of the cockle *Cerastoderma edule*: Consequence of environmental pollution?

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A b s t r a c t

Cerastoderma edule (Cardiidae) has a diploid chromosome number of 2n ¼ 38, its karyotype consisting of 12 submetacentric, 4 subtelocentric and 3 telocentric chromosome pairs. Hyperdiploid cells had pre- viously been observed in two populations of the Northern Galician coasts (northwest of Spain). The supernumerary chromosomes being easily distinguished by their reduced differentiated size and by their intra- and inter-individual variability. After the recent observation of 35% of cells with supernumerary chromosomes in a population of the Southern Galician coasts (Vigo) and 15% of cells with supernumerary chromosomes in a population of the south of Portugal (Ria Formosa, Algarve), we attempted, in this paper, an elucidation of the nature of these supernumerary chromosomes, by differential banding technique with restriction enzymes on these hyperdiploid cells. Analysis of the restriction enzyme banding of the 2n > 38 karyotypes led us to propose the occurrence of a chromosomal fission event involving the largest submetacentric chromosome pair. This study represents the first description of the occurrence of a possible chromosomal fission in marine bivalves. Different levels of environmental pollution are suggested as possible explanation for the differences observed on the proportion of hyperdiploid cells between the Southern Portugal population and the three Galician ones.

1. Introduction

A chromosome complement of 2n ¼ 38 has been reported for *Cerastoderma edule* (Cardiidae) (Insua and Thiriot-Quié vreux, 1992; Koulman and Wolff, 1977), standard karyotype consisting of 12 submetacentric, 4 subtelocentric and 3 telocentric chromosomal pairs (Insua and Thiriot-Quié vreux, 1992). Five chromosomal pairs are carriers of a cluster of 5S rDNA at the telomeres of the long arm (Insua et al., 1999).

However, in Insua and Thiriot-Quié vreux (1992) observed the presence of 1–3 supernumerary chromosomes in addition to those of the standard complement in 43% (15 cells out of 35) in one Northern Galician (northwest of Spain) population (Baldayo). They were easily distinguished by their reduced differentiated size, which was much smaller than that of the smallest chromosomal pairs of the standard complement. In another Northern Galician population (Ria del Pasaje), the same authors (unpublished data), observed 153 metaphases (taken from 10 animals), and identified in 57 cells (37%) what that they presumed to be B chromosomes. Recently, during a restriction enzyme banding study (Leitão et al., 2006) of two veneroid bivalves including this species, made by our group, we also noticed (unpublished data) the occurrence of hyperdiploid cells (2n > 38) in a population of the south of Portugal (Ria Formosa, Algarve) and in a population of the Southern Galician Coasts (Vigo).

Supernumerary chromosomes, also called accessory or B chromosomes in order to distinguish them from the standard A chromosomes (normal diploid complement), were first detected by Wilson (1906) in the karyotype of a hemipteran insect. B chromosomes can be originated intraspecifically from the standard A complement or interspecifically as a result of interspecies mating (Camacho et al., 2000). B chromosomes could either be a by-product of chromosomal rearrangements or a by-product of in- jured chromosomes. Until recent years, most supernumerary chromosomes went unclassified because their small size did not allow their identification using banding techniques. However, in humans, the combination of banding methods, has allowed their complete identification in most cases, since almost all of the

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were derived from A chromosomes (Fuster et al., 2004). Among Crustacean Decapoda, numerical chromosome variability is frequent, and it has been hypothesised that the presence of supernumerary chromosomes account for this variability. Thanks to the improvement of cytogenetic analysis by chromosomal banding, supernumerary B chromosomes have been demonstrated in Nephrops norvegicus, Homarus americanus, Palinurus elephas and Palinurus mauritanicus, belonging to several different families (Collucia et al., 2004). In all four species Chromosomes B were variable in number, mainly heterochromatic and undigested by various endonucleases. In the catfish, Iheringichthys labrosus, B microchromosomes appeared totally heterochromatic and were not digested by two different restriction endonucleases (Carvalho and Dias, 2005).

In marine bivalves, besides the previously mentioned study by Insua and Thiriot-Quiévreux (1992), supernumerary chromosomes have also been observed in a variable number on several strains of the bivalve genus Lasaea (e.g. O'Foighil and Thiriot-Quiévreux, 1991) where odd ploidy numbers were also observed and in Sphaerium corneum (Petkeviciute et al., 2006). In the last case, B chromosomes were one of the causes of observed inter- and intra- individual variation in the diploid chromosome number. Characterization of the supernumerary chromosomes observed by Insua and Thiriot-Quiévreux (1992 and unpublished) was never performed. One of the main problems that might have prevented a better understanding of this phenomenon (and also can explain the limited studies existing in chromosomes of *Cerastoderma edule* or other veneroid bivalves) was the difficulty to get good quality banding patterns in chromosomes of this species. In situ digestion with restriction endonucleases (REs), which cleave DNA at specific target sequences, has been shown to produce consistent banding patterns, for instance, in fixed mammalian (e.g. Chaves et al., 2002), fishes (e.g. Carvalho and Dias, 2005) and insect chromosomes (e.g. Marchi and Mezzanote, 1988). In bivalves it has already been successfully applied to mussels (Martinez-Lage et al., 1994), scallops (Gajardo et al., 2002), oysters (Leitão et al., 2004; Bouilly et al., 2005; Cross et al., 2005) and more recently to two species of veneroids, the clam Ruditapes decussatus and the cockle C. edule (Leitão et al., 2006). In all cases, specific chromosome bands were obtained after digestion with REs, allowing the unambiguous identification of all chromosome pairs as well as the establishment of precise karyotypes.

Hence, in this study, we applied the restriction enzyme banding technique in order to try to characterize the hyperdiploid metaphases of Cerastoderma edule.

2. Material and methods

2.1. Biological material

Fifteen specimens of *Cerastoderma edule* were intertidally collected at Almargem (Ria Formosa lagoon, Algarve, south of Portugal) and 15 at Isla de San Simón (Baia de Vigo, south of Galicia, Spain). Before processing, the animals of both populations were acclimated at the IPIMAR-Tavira hatchery for 1 week..

2.2. Chromosome preparation

Whole juvenile animals (ca. 1.5 cm length) were incubated for 7 h in a 0.005% solution of colchicine in seawater. Then the gills were dissected and treated for 30 min in 0.9% sodium citrate in distilled water. The material was fixed in a freshly prepared mixture of absolute alcohol and acetic acid (3:1) with three changes of 20 min each. Fixed pieces of gill from each individual were dissociated in 50% acetic acid with distilled water solution. Slides were prepared following and air-drying technique (Thiriot-Quiévreux and Ayraud, 1982). The slides were kept at 20 C until further used.

2.3. In situ restriction endonuclease digestion

Slides were aged during 6 h, in a dry incubator at 65 C, before the restriction endonuclease treatment. The restriction enzyme used: HaeIII (GG/CC) was diluted in the buffer indicated by the manufacturer (Invitrogen, Life Technologies), and final concentrations of 30 U were obtained per 100 ml (following Leitão et al., 2006). Then 100 ml of this solution were placed on each slide and covered with coverslips. These slides were incubated in a humid chamber for 16 h at 37 C. The slides were then washed in distilled water, air dried and stained with Giemsa (1% solution, diluted in phosphate buffer at pH 6.8).

2.4. Microscopy and image processing

Images of metaphases of *Cerastoderma edule* from both the Ria Formosa and the Vigo populations banded with the restriction endonuclease HaeIII were acquired with a CCD camera (Axiocam, ZEISS) coupled to a ZEISS Axioplan 2 Imaging microscope. Digitised photos were printed from Adobe Photoshop (version 5.0) using only contrast optimisation functions that affected the whole of the image.

2.5. Karyotypes organization

The karyotypes of the banded hyperdiploid metaphases were organized by relative length, centromeric position and also following the pattern of the HaeIII restriction enzyme banding established for *Cerastoderma edule* (Leitão et al., 2006).

3. Results

In Fig. 1 are presented examples of a 2n ¼ 40 hyperdiploid metaphase (Fig. 1a) and respective karyotype (Fig. 1b) of Cerastoderma edule banded with the RE HaeIII.

The analysis of the RE banding pattern of 81 metaphases of *Cerastoderma edule* from the south of Portugal population showed the presence of 15% (12 out of the 81) of hyperdiploid metaphases, observed in 8 of the 15 animals studied. These corresponded to 10 metaphases with 2n ¼ 40 and 2 metaphases with 2n ¼ 39. The analysis of the RE banding pattern of 77 metaphases of C. edule from the Vigo population (Southern Galician one) showed the presence of 35% (27 out of the 77) of hyperdiploid metaphases, observed in 11 of the 15 animals studied. These corresponded to 24 metaphases with 2n ¼ 40 and 3 metaphases with 2n ¼ 39. We did not observe any hyperdiploid metaphase with 2n ¼ 41, as previously observed by Insua and Thiriot-Quiévreux (1992) study.

The analysis of the corresponded karyotypes suggested the presence of one rearrangement involving the largest submetacentric pair and the ''supernumerary small chromosomes''. Restriction enzyme banding pattern indicates that homology exists between the largest submetacentric chromosome pair number one and the small ''supernumerary chromosomes'' present in the hyperdiploids cells, more precisely between these last ones and the final/distal part of the short arm of the largest chromosomal pair number one as place in evidence in Fig. 2.

4. Discussion

The analysis of the hyperdiploid metaphases of the studied Southern European populations of Cerastoderma edule, banded with the restriction enzyme, led us to suggest the hypothesis of the occurrence of a fission event involving the largest submetacentric

chromosome pair number one of the A chromosome set. Following this hypothesis the 2n > 38 metaphases should have originated from the 2n ¼ 38 through chromosomal fission.

Karyological polymorphisms are often best explained by the fission theory even in non-mammalian animal taxa (Kolnicki, 2000). Feldberg et al. (2004) suggested as one of the possible scenarios for the origin of B chromosomes in Amazon cichlid species, chromosomal breakdowns, by lagging chromosome fragments during cell division. Chromosomal fission events have also already been suggested in marine invertebrate species. Dixon et al. (1994) proposed that the five pairs of metacentric chromosomes of the 2n ¼ 26 karyotype of the gastropod species Nucella lapillus con- tributed to the polymorphism of the 2n > 26, through Robertsonian chromosomal fission event. Later Pascoe (2006), showed that the karyotype was not always the same for a given chromosome number, and that more than five pairs, mentioned in previous studies, were polymorphic in this species. This study is, however, to our knowledge the first description of the occurrence of a possible chromosomal fission in marine bivalves. Chromosomal fissions provide indeed a fertile playground of karyotypic and genetic var- iation for selection pressures to act upon. A meticulous analysis of individual chromosomal cases, using molecular cytogenetic tech- niques could elucidate the role of this phenomenon in karyotypic polymorphisms and evolution.

The discrepancy of the percentage of hyperdiploid cells ob- served between the Ria Formosa population with 15% and the 3 Galician populations with 35%, 37% and 43% could probably be due to: (a) interpopulation genetic variability among the different *Cerastoderma edule* populations, as found for instance, in different populations geographically distant of Pecten maximus (Heipel et al., 1998), Haliotis rubra (Huang et al., 2000) and Mytilus chilensis (Toro et al., 2004); (b) to the impact of different environmental conditions to which these populations have been subjected. There are not presently any comparative studies on population genetics in C. edule. In order to check the last hypothesis on differential envi- ronmental impact, we searched for existing bibliographic data in order to examine if there were different levels of environmental adversity that could be the cause of this discrepancy. Ria Formosa is a shallow mesotidal lagoon located in a Natural park between land and open sea. Although subjected to high anthropogenic pressures derived from domestic sewage (major tourist area) and agriculture effluents, it has never suffered significant industrial impact or any oil spill. On the other hand the Galician coast has suffered several major oil spills in the last 30 years: in 1970 the Norwich tank ''Polycommander'' with 50,000 tons of heavy fuel oils, in 1976 the Spanish ''Unquiola'' released into the ''La Coruna'' bay 100,000 tons of oil, in 1978 the Greek tank ''Andros Patnia'' released 50,000 tons, and in 1992 another Greek tank ''Mar Egeu'' created a massive marine pollution over more than 200 km of the Galician coast. And more recently, the ''Prestige'' oil spill in November 2002 that sunk 270 km of the Galician coast releasing 30,000 tons of oil.

Effects of pollutants are usually displayed first at the bio- chemical and molecular levels (Veldhuizen-Tsoerkan et al., 1991). This then leads to genetic changes that become cytological visible, especially in the tissues of organisms which are good pollutant bio accumulators, such as molluscs. A relationship has in fact been suggested between hyperdiploid metaphases and effects of envi- ronmental mutagenic factors, such as industrial pollution (Giagia et al., 1985).

Due to their sedentary character and relatively long life-span, benthic faunal organisms, specifically bivalves are relatively susceptible to environmental changes in the ecosystem which occur in space and over time (Sokolowski et al., 2004), which makes them suitable for studies on environmental pollution.

In a study by Baršiene (1994), in 80% of the bivalve specimens studied a positive correlation between heavy metal, aromatic hydrocarbon or radionuclide bioaccumulation and chromosome set disturbances was put in evidence. Exposure of mussels to environmental contamination gave rise to DNA damage comprising chromosomal aberrations (AlSabti and Kurelec, 1985), sister-chromatid exchange (Dixon et al., 1985), strand-breaks and cross links (Vukmirovic et al., 1994) and higher coefficient of variation (CV) in DNA fluorescence which is indicative of increased chromosomal damage (Bickman, 1990), and could be reflective of aneuploid mosaicism or of chromosomal fragmentation phenomena (Lowcock et al., 1997). Cytogenetic analysis showed the presence of disseminated neoplasia in gill tissue of the Baltic clam Macoma balthica, with high-accumulated tissue concentrations of trace metals (As, Ag, Cd, Pb, Cu and Zn) from the Gulf of Gdansk, Poland (Sokolowski et al., 2004). Pesticides like atrazine have also shown to have a significant impact by enhancing the aneuploidy level of the Pacific oyster Crassostrea gigas (Bouilly et al., 2003) and also the persistence of atrazine impact on the aneuploidy level in time, within and between generations of this oyster species (Bouilly et al., 2004).

In what specifically concerns pollution by oil spills, higher micronuclei frequencies were found in mussels collected on the Baltic Sea (Palanga) following an accidental oil spill in November 2001 (Barsˇiene_ et al., 2004). More recently the release of polycyclic aromatic hydrocarbons into the marine environment after the ''Erika'' ship wreck along the coats of France, mussels (Mytilus sp.) were affected by the oil slick in various degrees (Lemiere et al., 2005).

We can then suggest, as a hypothesis, that the important differences on the proportion of hyperdiploid cells, cells with the occurrence of the fission event, between the Southern Portugal population and the Galician ones, could possibly be explained by the important pollution by polycyclic aromatic hydrocarbons due to several major oil spills suffered by the Galician populations. We believe that it would be interesting to develop an ecotoxicological study in order to test this hypothesis.

The fact that the Southern Portugal population, which has suffered pollution from agricultural effluents and domestic sewage, but has never suffered major industrial impact or oil spill, presents nevertheless 15% of cells with occurrence of the fission event on the distal part of the largest submetacentric chromosome short arm, could suggest that this chromosome site represents a ''hotspot'' for chromosome breakage on the karyotype of Cerastoderma edule. Hence, in a recent study, Webber and Ponting (2005) proposed the occurrence of ''hotspots'' of mutation and breakage in dog and human chromosomes.

The observation by Insua and Thiriot-Quie´ vreux (1992) of sometimes hyperdiploid metaphases of 2n ¼ 41 (2n ¼ 38 þ 3), which were not observed in this study, might suggest the possible existence of another ''hotspot'' for chromosome breakage on the karyotype of this species.

The results of this study could then sustain the use of cytogenetic damage in pollution control measurement studies, atypical cytogenetic features in bivalve species could be in this way con- sidered as alerting indicators of poor environment health.

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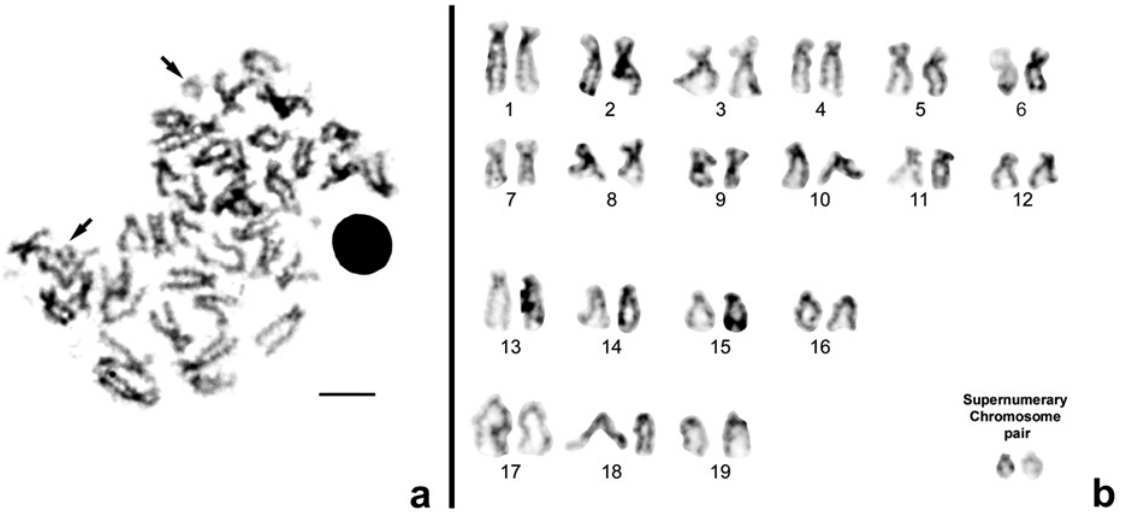

Fig. 1. Examples of a 2n ¼ 40 hyperdiploid C. edule metaphase (a) and respective karyotype (b) banded with the RE HaeIII. Arrows indicate the ''supernumerary chromosomes''. Scale bar ¼ 5 mm.

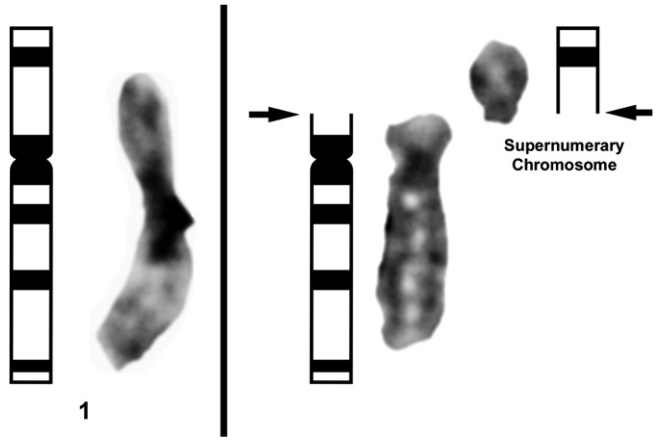

Fig. 2. Detail of the largest submetacentric chromosome pair number one from a 2n ¼ 38 (left) restriction enzyme banded karyotype and from the 2n ¼ 40 (right) restriction enzyme banded karyotype of Fig. 1b. To put in evidence the banding ho- mology of the final/distal part of the short arm of the largest submetacentric pair number one and the ''supernumerary'' small chromosome pair, supporting the chro- mosomal fission hypothesis.