Aquaculture
May 2014, Volumes 428–429, Pages 223–225
http://dx.doi.org/10.1016/j.aquaculture.2014.03.017

Karyotype variation in neoplastic cells associated to severity of disseminated neoplasia in the cockle *Cerastoderma edule*

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Abstract:

Disseminated neoplasia (DN) is characterized by an abnormal cell proliferation, usually resulting in death of the affected mollusks. In the present study we performed, for the first time, karyotypes of two different stages of DN in the cockle *Cerastoderma edule.* Analysis of moderate (N2) and high (N3) severity neoplasic metaphases displayed a chromosome number ranging from 57 to 317, instead of the normal diploid number of 38. Cockles with N2 showed a lower percentage of microchromosomes and a higher ploidy than those with N3. The comparison of both cytogenetic profiles contributes to better understand the evolution of DN in cockles.

Highlights

► First karyotypes of disseminated neoplasia metaphases of the cockle *C. edule*. ► Karyotype variation in neoplastic cells associated to severity of DN in *C.edule.* ► Progression of neoplasia reveals an increase of the percentage of microchromosomes.

Keywords : Karyotype ; Disseminated neoplasia ; *Cerastoderma edule* ; microchromosomes

1. Introduction

Disseminated neoplasia (DN) is characterized by cellular changes that lead to an overgrowth or abnormal cell proliferation (Peters, 1988; Barber, 2004). Since the initial description by Farley (1969), in the oysters *Crassostrea virginica* and *C. gigas*, DN has been observed in several mollusk species, such as the clams *Mya arenaria* (Reno et al., 1994) and *Macoma balthica* (Thiriot-Quiévreux and Wolowicz, 2001; Smolarz et al., 2005), in which the chromosomal abnormalities associated to DN have been mainly associated with abnormal ploidy. Hiperploidy and hipoploidy were indeed detected in various mollusk species, through the analysis of DNA content of neoplastic cells by flow cytometry (Moore et al.1991; da Silva et al., 2005; Smolarz et al., 2005; Delaporte et al., 2008; Le Grand et al., 2010; Díaz et al., 2013). In the edible cockle, *Cerastoderma edule*, DN was described for the first time in 1982 in Cork Harbour (Ireland) (Twomey and Mulcahy, 1984, 1988), and in Brittany (France) (Poder and Auffret, 1986) and, more recently, in Galicia (Spain) (Carballal et al., 2001; Villalba et al., 2001; Ordas and Figueras, 2005). Cockle disseminated neoplasia is transmissible (Collins and Mulcahy, 2003) and retroviral etiology was suggested (Romalde et al., 2007). The cockle is a dominant species in many Galician bivalve beds and it has an important economical value (Carballal et al, 2001). *Cerastoderma edule is* a valuable natural resource in Europe and in recent years there has been an on growing interest on its use in integrated land-based multi-trophic aquaculture systems (Batista *et al*, 2012) Previous studies conducted by Díaz et al. (2010), allowed classifying cockles according to a scale of disease severity; non-affected (N0); low severity (N1), when individuals showed proportion of neoplasic cells lower than 15% in the haemolymph cell monolayers; moderate severity (N2), when the proportion ranged from 15% to 75%; and high severity (N3), when the proportion was higher than 75%. More recently, differences in DNA content between normal and DN affected cells through ploidy analysis by flow cytometry were put in evidence and a preliminary cytogenetic evaluation in only one of the neoplasic stages, N3, was performed (Díaz et al, 2013). In the N3 observed metaphases, a chromosome number ranging from 41 to 145 was found as well as the presence of numerous microchromosomes. In abnormal metaphases of *Macoma balthica* affected with DN, Smolarz et al. (2005) had also observed the presence of microchromosomes in all abnormal metaphases and a general increase in the amount of the genetic material. The main objective of this study was to perform, for the first time, the karyotypes of DN metaphases of two different severity stages

in order to accomplish a sound cytogenetic characterization of this phenomenon and contribute to enlighten the evolution of DN in cockles.

2. Material and methods

Cockles affected with DN were collected in Cambados, Galicia (NW, Spain). Thirty four specimens of *C. edule* were processed, sixteen N2 and eighteen N3. Whole animals were incubated for 7 h in a 0.005% solution of colchicine in seawater. Gills were dissected and treated for 30 min in 0.9% sodium citrate in distilled water and fixed in a freshly prepared mixture of absolute ethanol 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 an air-drying technique (Thiriot-Quiévreux and Ayraud, 1982) and directly stained with Giemsa (4%, pH 6.8) during 10 min. Images of metaphases affected with N2 and N3 were acquired using a Nikon Eclipse 80i imaging microscope, with an image acquisition Nikon DS-Fi1camera incorporated. Adobe Photoshop (version 5.0) was used for the treatment of digitized photos, using only contrasting optimization functions that affected the whole of the image. Karyotypes of metaphases were organized according to relative length and centromeric position, and the nomenclature followed that of Levan et al. (1964). Chromosomes that presented a reduced size when compared to the normal diploid complement were designated as microchromosomes.

3.Results

The analysis of 35 N2 and 32 N3 metaphases revealed a large number of chromosomes, reaching 317 in N2 and 230 in N3, instead of the normal diploid number of 38 (Fig. 1). Three aneuploid peaks were observed in N2, the first ranged from 3.1n to 3.8n, the second from 7.3n to 10.3n, and the last peak observed at 15.2n. The aneuploid peak in N3 cockles ranged from 5.1n to 8.9n. The standard karyotype of *C. edule* consists of 12 submetacentric, 4 subtelocentric and 3 telocentric chromosomal pairs (Insua and Thiriot-Quiévreux, 1992). The performance of the karyotypes of N2 and N3 metaphases revealed in N2 a higher percentage of chromosomes, which probably have an homologous in the normal diploid complement, while a higher percentage of microchromosomes was detected in N3 (Fig. 2). An example is given in Fig. 2, in which a characteristic N2 metaphase with 210

chromosomes, presents 40% of microchromosomes, while a N3 metaphase with 163 chromosomes, showed 45%. Considering all the examined metaphases, 28% of chromosomes corresponded to microchromosomes in N2, while in N3 that value reached 39%.

4. Discussion

In this study karyotypes of disseminated neoplasia metaphases of the cockle *C. edule* were performed for the first time. Karyotipycal analysis revealed chromosomal alterations in neoplasic cells of DN-affected cockles, such as a high number of chromosome and multiple microchromosomes. Differences between moderate (N2) and high severity (N3) stages of the disease were also noticed. With the progression of neoplasia, an increase of the percentage of microchromosomes was observed. The performance of the standard Giemsa stained karyotypes (based on relative length and centromeric index measurements) revealed that, in the N2 methaphases, there were a higher percentage of copies of the homologous chromosome pairs of the normal diploid complement than in N3 metaphases. The future characterization of the neoplasic karyotypes with differential chromosome banding techniques will allow the accurate determination of the number of copies of each chromosomal pair of the normal diploid complement as well as the identification of structural chromosomal aberrations. Alterations in normal ploidy values in other DN affected mollusks have been previously detected. For instance, in neoplasic cells of surf clams *Mya arenaria*, Reno et al, (1994) observed hyperdiploid chromosome numbers, ranging from 44 to 80, whereas normal cells had 34; more recently Smolarz et al. (2005) also reported abnormal ploidy values, ranging from 59 to 105 in hyperdiploid cells of Baltic clams *M. balthica*, while the normal value is 38. However, in our study, the range of the chromosomal number was much higher (57 – 317) than the previously observed in other bivalve species affected with DN. The ploidy values obtained ranged from 3.1n to 15.2n in N2 and from 5.1n to 8.9n in N3. In a previous study of the ploidy of haemolymph cells using flow cytometry, Díaz et al. (2013) found ploidy levels ranging from 1.3n to 5.3n in N2 and two peaks in N3, the first ranging from 1.6n to 4.5n and the second from 7.5n to 8.9n. The differences observed in the ploidy levels determined by flow cytometry by Díaz et al. (2013) and the ones determined by karyotype analysis in this study can be due to differences in the sample size used in karyotyping and in flow cytometry approaches and/or to the DN karyotypes composition found, with different percentages of microchromosomes and

chromosomes which might present a homologous on the normal haploid complement of the species. Indeed, the reduced size of microchromosomes influences the quantity of DNA present in cells. The mechanism of formation of microchromosomes is frequently unclear and their evolutionary relevance remains not well understood. Moreover, microchromosomes tend to be mitotically unstable, consequently varying in number in different cells of the same individual (e.g. Schmid et al. 2010). Microchromosomes have previously been observed associated with virus-induced sarcoma in mice (*e.g.* Donner and Bubenik 1968) and also in some human tumors, such as: neuroblastoma and metastatic carcinoma (*e.g.* Levan et al. 1968; Spriggs et al. 1962). More recently, in the fish species *Poecilla formosa*, the molecular characterization of the observed B microchromosomes revealed an unidentified locus that conferred a higher susceptibility to tumor formation (Lamatsch et al 2011). Pierre et al. (1970) have also suggested that microchromosomes could be marker chromosomes to identify clone or clones of leukemia cells, and their presence was also higher with the advance severity of the disease. Although additional studies are needed to further characterize by differential chromosomal banding techniques the neoplasic karyotypes and observed microchromosomes and the wide range of ploidy present in neoplasic cells of *C. edule*, this study highlights the importance of the performance of karyotypes on DN metaphases.

ACKNOWLEDGEMENTS

This study was supported by the project PTDC/MAR/72163/2006: FCOMP-01-0124-FEDER-007384. We are also grateful to João Maurício Teixeira and Raquel Alves for the excellent technical assistance.

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FIGURE LEGENDS

Figure 1: Example of neoplasic metaphases in the cockle *Cerastoderma edule*, a) metaphase of a cockle diagnosed with moderate severity disseminated neoplasia (N2), with 317 chromosomes, and b) metaphase of a cockle diagnosed with high severity disseminated neoplasia (N3), with 169 chromosomes.

Figure 2: Example of karyotypes of *Cerastoderma edule* affected with disseminated neoplasia a) karyotype of a N2 metaphase with 210 chromosomes, from which 40% are microchromosomes, and b) karyotype of a N3 metaphase with 163 chromosomes, from which 45% are microchromosomes

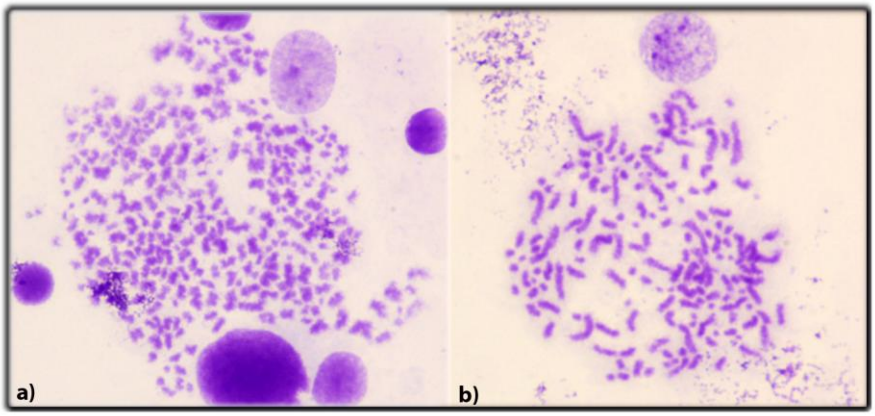

Figure 1

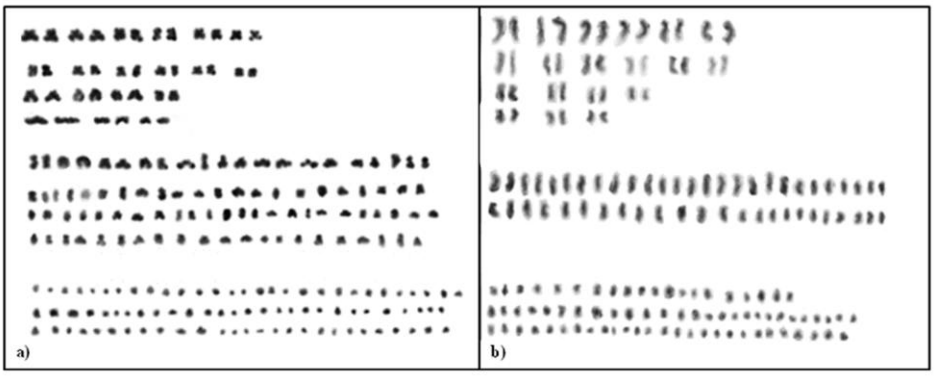

Figure 2