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Endonuclease banding reveals that atrazine-induced aneuploidy resembles spontaneous chromosome loss in Crassostrea gigas

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Abstract: Aneuploidy has previously been observed in the Pacific oyster, Crassostrea gigas, and shown to be negatively correlated with growth. Moreover, a significant impact of atrazine exposure has been described in C. gigas, and persistence of that effect has been observed between generations. Evidence of differential chromosome loss has been demonstrated in aneuploid karyotypes of C. gigas using the G-banding technique. Pairs 1, 5, 9, and 10 are characterized by the loss of 1 chromosome. As restriction enzyme (RE) digestion chromosome banding allows a better identification of chromosome pairs, we used this technique to identify which chromosomes are affected when aneuploidy is increased by exposure to atrazine. The progeny of oysters contaminated by atrazine were analysed using the restriction enzyme HaeIII. The study of 26 RE-banded aneuploid karyotypes showed that the same chromosome pairs (1, 5, 9, and 10) were affected by the loss of 1 chromosome (61%, 15%, 42%, and 42%, respectively). Further investigation is required to enable a better understanding of aneuploidy in oysters, especially with respect to why some chromosomes are more easily lost than others, and why cells tolerate the loss of these chromosomes.

Keywords: aneuploidy, atrazine, restriction enzyme digestion chromosome banding, Pacific oyster, Crassostrea gigas

Introduction

Aneuploidy is a phenomenon commonly observed in oysters (e.g. Leitão et al. 2001a). In the Pacific oyster, *Crassostrea gigas*, the alteration of the normal diploid chromosome number (2n = 20) leads to hypodiploid cells with 2n = 19, 18 or 17 (Thiriot-Quiévreux et al. 1992). The fact that somatic aneuploidy is negatively correlated to growth rate in this species (Leitão et al. 2001a) is of increasing concern for this economically important bivalve species. Moreover, a positive correlation between aneuploidy and atrazine (a selective herbicide widely used in agriculture) has been experimentally described in juvenile and adult *C. gigas* (Bouilly et al. 2003). This has environmental implications as *C. gigas* is primarily reared in estuarine areas, subject to various recurring pollutants from agricultural or industrial wastes. The most concern is the persistence of atrazine impact on Pacific oyster aneuploidy in time within and between generations (Bouilly et al., in press).

Evidence of differential chromosome loss has been demonstrated in aneuploid karyotypes of *C. gigas* using the G-banding technique, and has shown that four (1, 5, 9, and 10) out of the ten chromosome pairs of *C. gigas* are affected by the loss of one homologous chromosome (Leitão et al. 2001b). Application of the restriction endonuclease (RE) digestion chromosome banding technique to bivalves has yielded some success in mussels (Martínez-Lage et al. 1994), scallops (Gajardo et al. 2002), and in oysters (Leitão et al., in press). The digestion of bivalve chromatin with restriction enzymes produces specific chromosome bands (Martínez-Lage et al. 1994), and thus this technique may be useful in the understanding of the processes affecting chromosome loss. In this paper, we investigate which chromosomes are affected when aneuploidy is induced by exposure to an environmental factor (atrazine), compared to spontaneous aneuploidy with the *in situ* restriction enzyme technique.

Materials and Methods

The studied animals are progeny of oysters exposed to different atrazine doses (10 and 100 µg l^{-1}) for two months as described in Bouilly et al. (in press). Two controls with seawater from Marennes-Oléron Bay (Charente-Maritime, France) were used in this previous experiment and atrazine concentrations in seawater were validated by the Institut de Recherche pour l'Ingénierie de l'Agriculture et de l'Environnment (Bordeaux, France) throughout the treatment period. At the size of 30-40 mm, four and a half month post-fertilisation, the progeny was incubated for 7-8 h in seawater containing 0.005% colchicine. The gills were then dissected in seawater, treated for 40 min in 0.9% sodium citrate and fixed in a freshly prepared mixture of absolute alcohol/glacial acetic acid (3:1) with two 10-min changes followed by two 20-min changes. Slides were made from a piece of an individual gill following the air-drying technique of Thiriot-Quiévreux and Ayraud (1982). Air-dried slides from 14 animals were aged at 65°C for 6 h and then treated overnight with restriction endonuclease digestion. The restriction enzyme used for chromosome digestion was *Hae*III, diluted in the buffer recommended by the manufacturer (Invitrogen, Life Technologies), to a final concentration of 30U of enzyme per 100 µl (Leitão et al., in press). The restriction enzyme solution (100 µl) was evenly dispersed on the preparations by use of a coverslip. Digestion was carried out in a moist chamber at 37°C overnight. After incubation, the slides were washed two times with water at room temperature and air-dried. The preparations were then stained for 20 min with Giemsa (1%). A Zeiss Axioplan 2 Imaging microscope containing an Axiocam digital camera and Axio Vision software were used to visualise and photograph chromosome preparations. Digitised photos were printed from Adobe Photoshop (version 5.0) using only contrast, overlay and colour optimisation functions that affected the whole of the image. Chromosomes preparations were subjected to karyotype analysis by the standard measurements of chromosomes pairs (measurements of size and centromeric index) and restriction enzyme digestion chromosome banding technique. We analysed 26 RE-banded

aneuploid karyotypes and the same number of standard aneuploid karyotypes. A χ^2 test was used to compare the percentage of loss found in a population originating from hatchery crosses which was not exposed to atrazine (that "natural" population is considered as a control) (Leitão et al. 2001b) and the one that we found in the population contaminated indirectly by atrazine. The χ^2 test is a suitable test to compare our observed percentages and the published ones as this test measures the random deviation between observed percentages and theoretical percentages (values from Leitão et al. 2001b).

Results and Discussion

An increase of 32% was observed in the level of aneuploidy of the progeny originating from contaminated oysters (15,97%) in comparison with the control (12,11%). It was suggested that atrazine has an adverse effect on mitotic spindle fibre formation leading to aberrant chromosome loss in oysters. The analysis of all aneuploid karyotypes of the studied oysters showed that only 4 of the 10 chromosome pairs (1, 5, 9, and 10) were affected by chromosome loss. Chromosome loss from either one pair (Fig. 1A) or more than one pair (Fig. 1B) per karyotype was observed. The loss of both homologues of one pair was not observed in any of the samples. Percentages of chromosome loss, independently calculated for each pair in the 26 RE-banded aneuploid karyotypes analysed, were 61, 15, 42, and 42% for pairs 1, 5, 9, and 10 respectively (Table 1). Among the 26 observed metaphases, 42 chromosomes were missing as we analysed 13, 10, and 3 metaphases with 2n = 19, 18, and 17 chromosomes respectively (Fig. 2). It is interesting to note that the chromosome pair 1 was preferentially lost alone in comparison with pairs 9 and 10, and that chromosome pair 5 was not lost alone. To complete the analysis, we studied 26 standard karyotypes. The percentages of chromosome loss for these metaphases were 46, 4, 15 and 46% for pairs 1, 5, 9, and 10 respectively (Table 1). However, the identification of these chromosome pairs could not be done as precisely as with the banding, especially with regard to pairs 5, 9 and 10 which are difficult to differentiate with precision.

Figure 1: Banded aneuploid karyotypes with the restriction enzyme HaeIII in Crassostrea gigas contaminated indirectly by atrazine. (A) Chromosome loss in pair 9. (B) Chromosome loss in pairs 1 and 10. Scale bar = 5 µm

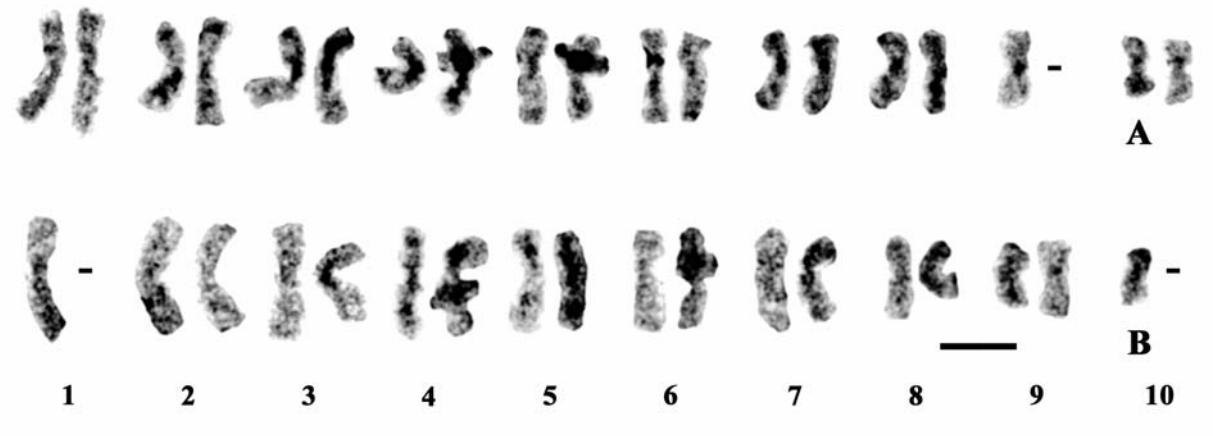

Figure 2: Frequency of missing chromosomes in 26 RE-banded aneuploid karyotypes realised from progeny of oysters contaminated by atrazine.

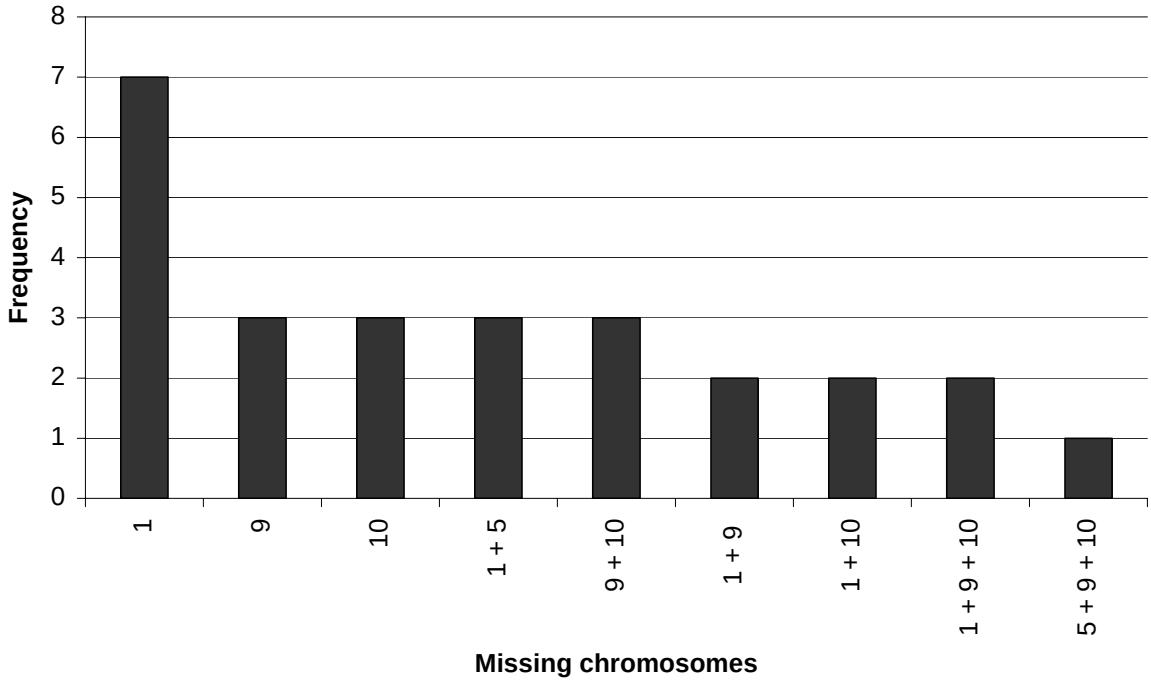

Table 1. Percentage of chromosome loss in Crassostrea gigas metaphase cells for each lost pair with three methods of karyotype analysis (Restriction enzyme RE-banding, standard method, and G-banding).

Technique	Number of metaphases	Number of missing chromosomes	Percentage of chromosome loss in pair: 1	5	9	10
RE-banding (this study)	26	42	61	15	42	42
Standard (this study)	26	29	46	4	15	46
G-banding (Leitão et al. 2001b)	95	143	56	19	33	43

The *in situ* restriction enzyme procedure revealed clear and characteristic bands, so it was easier to identify each chromosome pair. This enabled the precise identification of each chromosome pair by comparison with standard karyotype. The percentage of chromosome loss observed for pair 5 was significantly lower than those for pairs 1, 9, and 10. Therefore, pairs 1, 9, and 10 can be considered to be those predominantly affected by exposure to atrazine. These results corroborate those of Leitão et al. (2001b) who observed in 95 G-banded aneuploid karyotypes 56, 19, 33, and 43% of chromosome loss for pairs 1, 5, 9, and 10 respectively (Table 1). These results obtained using the G-banding technique are close to the ones we obtained with RE-banding and a χ^2 test revealed that there are no significant differences between these two populations of karyotypes. RE-banding is more reliable than G-banding because this latter classical banding technique presents some disadvantages such as: limited repeatability, large time investment required and the fact the banding is lost during fluorescent *in situ* hybridisation (FISH) procedure (Leitão et al., in press). Moreover, the RE-banding technique allows a better preservation of the chromosome morphology representing an additional advantage for chromosome identification of oysters.

Such a phenomenon of preferentially loss of chromosomes has already been observed in other species. Cieplinski et al. (1983) showed a statistically significant dependency among patterns of loss of specific chromosomes (both within and between cell lines from human-mouse myeloma), with certain chromosomes preferentially retained and others lost more often than expected under the assumption of randomness of segregation. Although several studies have showed that aneuploidy can be induced by chemicals especially in humans and in rodents (e.g. Leopardi et al. 1993; Güerci et al. 2000), there are few studies on identification of lost chromosomes following chemical exposure. In humans, metabolites of benzene are highly effective in inducing loss of all or part of chromosomes 5 and/or 7 that are involved in the development of myeloid leukemia (Zhang et al. 1998). In invertebrates, no studies have yet reported the identification of chromosome pairs lost as a consequence of a contaminated environment, furthermore, there are also very few studies on chromosome identification in aneuploid karyotypes in this group of animals (Leitão et al. 2001b).

Relatively few studies have examined the biochemical and physiological effects caused by atrazine on hormonal systems. Atrazine can elevate the plasma thyroid hormones T_4 concentrations in salmonids (Waring and Moore 2004) and in salamanders (Larson et al. 1998). The mode of action for atrazine on oysters is poorly known but this herbicide may interact with the mitotic spindle to cause aneuploidy. It was shown that herbicides of several classes act by inhibiting mitosis through direct interaction with tubulin (Duke 1990). Moreover, Grossmann et al. (2001) suggested that some triazines in algae lead to mitotic

disruption by a loss of spindle and inhibiting microtubule formation. Further experiments are needed to detail alternative mode of action for atrazine and its specific action in oysters.
In conclusion, there is no effect of atrazine on specific chromosome pairs lost in aneuploid karyotypes of the Pacific oyster, *Crassostrea gigas*. It would be of great interest to know why some chromosomes are more easily lost than others, and why the gill cells of *C. gigas* tolerate the loss of these specific chromosome pairs, to enable a better understanding of the aneuploidy phenomenon in *C. gigas*.

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