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A short-term culture method for chromosome preparation from somatic tissues of adult mussel (*Mytilus edulis*)

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Abstract. In order to improve the efficiency of mussel chromosome preparation, a tissue culture procedure has been developed. Mantle and foot explants were grown in tubes in media composed of Eagle's Basal Medium supplemented either with salts or seawater, enriched with egg yolk, adjusted to pH 7.50, and containing penicillin and streptomycin. After 4 days of incubation at 18 °C, antibiotics were renewed and after 6–7 days, cultures were ready for harvesting and preparation of microscopical slides. The cultures were a source of actively dividing cells and consistent metaphase spreads were obtained. Evidence from BrdU incorporation suggested that cells could undergo several rounds of replication. The chromosome spreads were good enough for karyotyping and to successfully silver stain the nucleolar organizer regions.
Key words. Chromosomes; karyotype; tissue culture; Mollusca; Bivalvia.

Bivalve cytogenetics has developed rapidly following the establishment of the direct 'cell suspension' method for metaphase chromosome preparation, in the mid 1970s[1]. The number of species studied cytogenetically has grown from less than 40 before 1974 (from Nakamura's data[2]) to about 170 today (out of nearly 8000 in the class Bivalvia). However, there is one major limitation to this technique: it gives highly irregular results so a great number of slides is required to obtain adequately well-spread metaphases for chromosome counting and, in the best cases, for measuring chromosome length. This may account for the scarcity both of karyological information and of successful serial banding of bivalve chromosomes, in spite of the fact that detailed karyotyping allows intra- and interspecific comparisons to be made. To develop banding procedures, a reliable source of actively dividing cells must be available[3]. Cell or tissue culture in defined conditions offers such a possibility and, for this reason, is universally used in vertebrate chromosome analysis.

In the bivalves, this method is poorly developed even though a number of growth media have been developed to support prolonged growth of cells, tissues or organs in vitro[4–17]. In some cases, mitotic activity was reported[4,8,10,14,15,17], especially in cultures derived from embryonic tissues[12,16]. However, the majority of the procedures described came from studies of bivalve pathogens, were somewhat complex and were not followed by cytogenetical applications. In contrast, several investigations have shown that explants from different tissues support the outward migration of individual cells for long periods (up to 6 months[12]) and the occurrence of mitoses within the explants themselves was suggested[7–9,12,14]. We took advantage of this peculiarity to develop a short-term, reliable culture technique using explants from somatic tissues of adult mussel, already described in a preliminary form elsewhere[18]. In the present paper, details about the technique and additional results are given.

Methods

Adult specimens of *Mytilus edulis* were collected from a natural population in Cap Ferret, Bay of Arcachon (French Atlantic coast), and laboratory-reared with artificial feeding as previously described by Cornet and Soulard[19].

Culture media. Two media were used. They were composed of Eagle's Basal Medium and Hanks' salts and L-glutamine (powdered BME, Sigma), which contains inorganic salts, vitamins, amino acids, and glucose, but must be modified to sustain cell development. First, since this medium is designed for mammalian cell culture, the osmotic pressure needs to be adjusted for growing marine organism tissues. Thus, osmolarity was increased to a level close to that of seawater either with additional salts (Medium A: 275.02 mM/l NaCl, 6.47 mM/l KCl, 8.17 mM/l $CaCl_2$, $2H_2O$, 23.37 mM/l $MgCl_2$, $6H_2O$, 24.22 mM/l $MgSO_4$, 5.0 mM/l $NaHCO_3$) or with seawater (Medium B: 700 ml/l of 0.45 µm filtered, 35‰ seawater). Then, both media were supplemented with Hepes (15.0 mM/l) and fortified with 6% egg yolk. The pH was adjusted to 7.50 with 1M NaOH and the medium was sterilized by filtration through a 0.22-µm membrane filter (Millex GS, Millipore) under positive pressure. Finally, penicillin and streptomycin were added shortly before use to give a final concentration of 100 U/ml and 100 µg/ml respectively.

Initiation of cultures. After thorough scrubbing of the shell, one valve was carefully removed. Several tissues were tested: gills, labial palps, digestive gland, foot, and mantle. The tissue selected for culture was dissected out with micro-scissors, washed in 3 changes (20 min each) of medium without egg yolk but with 400 U/ml penicillin, 400 µg/ml streptomycin and 1 µg/ml amphotericin B, and rinsed in the same medium without antibiotics or antimycotic. Tissues could either be processed immediately or stored in the latter medium at 4 °C for up to 3 weeks before processing. Each piece of tissue was transferred onto a plastic Petri dish, in a drop of medium, where it was cut up in small fragments of about 1 mm^2 with a scalpel; twenty of these explants were then placed in a 15-ml disposable culture tube (Corning) with 3 ml of complete medium. Tubes, with caps tightly screwed, were incubated at an angle in darkness, at 18 °C (±1 °C). The pH and possible contamination were checked daily using the indicator dye (phenol red) included in the medium. Penicillin and streptomycin were renewed after four days. In order to test the mitotic activity, 5-bromo-2′-deoxyuridine (BrdU) was added to several culture tubes four days before harvesting, to a final concentration of 5×10^{-5} M. From the fifth day, but preferably after six to seven days, cultures were ready for harvesting.

Harvesting. To the 3 ml of medium contained in each tube, 1.8 ml of a 280 µg/ml colchicine solution in distilled water was added. This stage arrested mitotic activity in metaphase (final colchicine concentration: 105 µg/ml) and also gave a preliminary hypotonic treatment (medium dilution: 38%). In order to mix the liquids, tubes were gently shaken, which also released the explants and migrating cells from the plastic surface where they were not firmly attached. The resulting suspension was left to incubate for 1.5 h at 18 °C. Then the contents of each tube were transferred to a conical-based tube centrifuged at 400 × g for 10 min (as in all further centrifugations). The supernatant was discarded and the pellet carefully resuspended with a Pasteur pipette in 5 ml of 0.8% tri-sodium citrate. After incubation at room temperature for 1 h, the tubes were centrifuged. The hypotonic solution was removed and replaced with 5 ml of cold freshly prepared first fixative (6 parts of methanol:3 parts of chloroform:1 part of acetic acid). The suspension was immediately centrifuged and the pellet was resuspended in 5 ml of the second fixative (3 parts of methanol:1 part of acetic acid) for at least 1 h at 4 °C; cells and explants could be stored at this temperature for several days before slide preparation.

Slide preparation and staining. Slide preparation was the same as in the direct method[19]. After centrifugation, fixative was thoroughly removed and explants were dissociated with 400 µl of a 50% acetic acid solution. The cell suspension obtained was dropped 100 µl at a time onto hot microscope slides (40–45 °C). Slides were air-dried, rinsed in 100% ethanol, dried under a warm air stream and stained for 20 min in a 4% Giemsa R solution in phosphate buffer at pH 6.8. Several slides were treated to stain the nucleolar organizer regions (NORs) according to Howell and Black's method[20], with Gold and Ellison's modification[21], but without Giemsa counterstaining. Slides of BrdU-exposed cultures were differentially stained using the Hoechst 33258 plus Giemsa technique[22] (FPG).

Results

No appreciable differences were found between the two culture media employed. Altogether, 77 cultures were performed in tubes with the different tissues mentioned above, giving a total of 236 slides which were qualitatively examined. Foot and mantle explant cultures were found to yield best results in terms of mitotic cell index. 61 slides were analysed quantitatively. They provided 3380 mitotic figures (i.e. an average of about 55 per

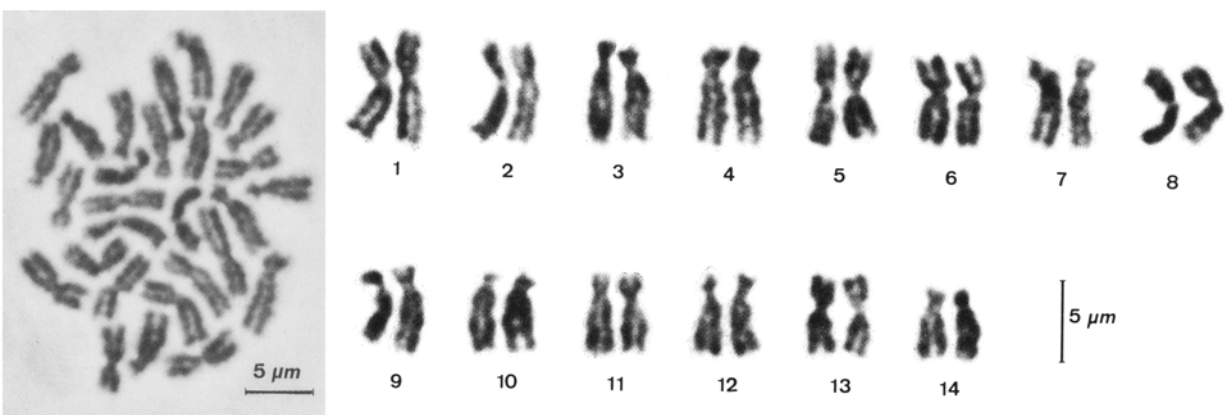

Figure 1. Mitotic metaphase spread of *Mytilus edulis*, with a diploid number of 2n = 28 chromosomes, obtained from mantle explant culture (Giemsa staining), and the corresponding karyotype. The 14 chromosome pairs were arranged in order of decreasing size and, on the basis of centromere positions, could be classified in metacentrics (pairs 1, 2, 5, 6, 8, 13), submetacentrics (pairs 4, 7, 9, 11, 12, 14), and subtelocentrics (pairs 3, 10).

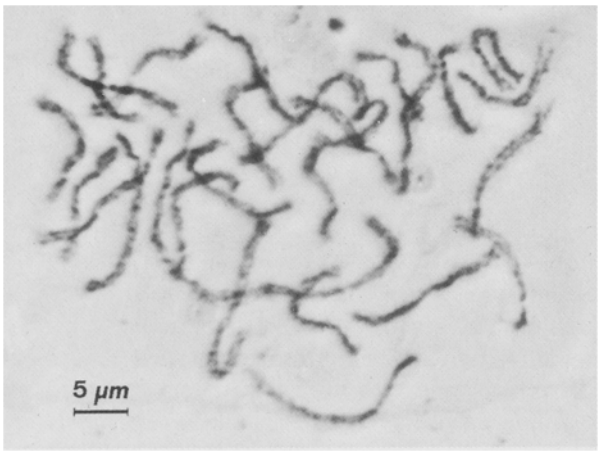

Figure 2. Mitotic late prophase of *Mytilus edulis* obtained from mantle tissue grown according to the procedure described in text. The 28 chromosomes appear to be well-defined enough for banding and karyotyping.

slide), 495 (i.e. 14.6%) of which were of sufficient quality for chromosome counting and karyotyping (fig. 1). A modal diploid number of $2n = 28$ was determined, confirming previous chromosome counts in *Mytilus edulis*, a species of bivalve whose cytogenetics are best characterized (see review in Nakamura[2]). Figure 1 shows an example of metaphase with 28 chromosomes and the corresponding karyotype which is composed of 6 metacentric, 6 submetacentric and 2 subtelocentric chromosome pairs.

Mitotic figures were essentially early and mid metaphases (fig. 1), but late prophases (fig. 2) and prometaphases were also present (about 8–10%). Polyploid complements were not frequent (0.4%); in contrast, in more than 20% of the metaphase spreads, one or two chromosomes were absent.

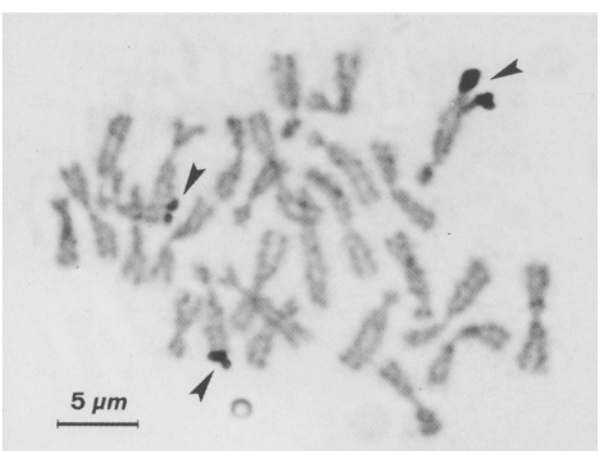

Figure 3. Silver-stained mitotic metaphase of *Mytilus edulis* obtained from mantle explant culture. Three nucleolar organizer regions (NORs) are shown as black dots in terminal position on submetacentric and subtelocentric chromosomes (arrows).

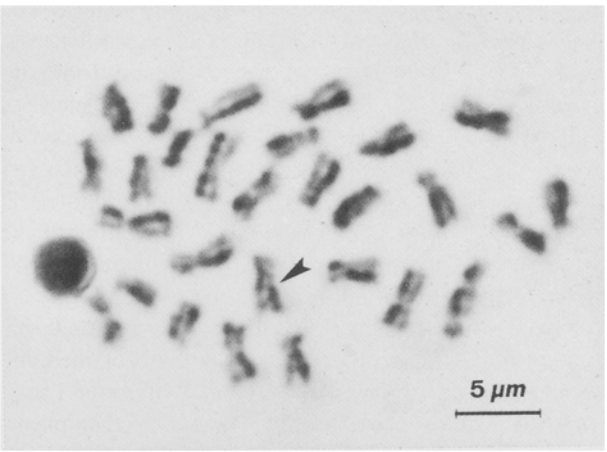

Figure 4. Mitotic metaphase of *Mytilus edulis* obtained from mantle explant grown with 5-bromo-2′-deoxyuridine (BrdU) in the culture medium for 96 h before harvesting: chromosomes are clearly BrdU-labelled. To induce such a sister chromatid differentiation (SCD), two successive cell cycles are necessary. Arrow: sister chromatid exchange (SCE).

The staining of the NORs was very easy to achieve either on unstained or on de-stained (with fixative) slides. In the same individual, the NORs varied from one to four in number, and they were always located at the end of the long arm of submetacentric and/or subtelocentric chromosomes (fig. 3).

Cultures exposed to BrdU yielded metaphases with sister chromatid differentiation (SCD) and, rarely, with sister chromatid exchanges (SCE) (fig. 4).

Discussion

When compared with the direct method of chromosome preparation, tissue culture is necessarily more costly in terms of time, money and equipment required. In order to minimize this disadvantage, our main concern was to keep the protocol simple as shown by the following examples.

The best results were provided by the foot and mantle. As it is more readily available, however, only the latter is currently used routinely, explants being easily obtained by cutting out a fine grid of palleal velum. Gills and labial palps, usually employed as a source of mitoses in the direct method[19,23,24], showed a very low mitotic activity in our cultures (less than 10 mitotic figures per slide). It was impossible to obtain metaphases from digestive tissues because of strong bacterial contamination.

Since a cell monolayer was not necessary, explants were grown in plastic tubes. This technique is simple and cheap but tubes could not be examined with an inverted microscope for cell growth, and whether mitoses occurred in the cells that migrated from explants as found by several authors,[8,9,14] or within the explants themselves as suggested by others[7,12], was not determined.

Complete media were easy to obtain from commercially available BME, especially medium B. However, because of the inclusion of seawater the composition of the latter is not so well defined as medium A. A Hepes-buffered system was used, which allowed incubation in closed vessels and did not require a controlled gas mixture enriched with CO_2.

Medium enrichment with 6% egg yolk was sufficient to induce and maintain cell proliferation, as demonstrated by the formation of SCD which need two mitotic cycles to appear. In fact, some cells underwent a minimum of three rounds of replication since metaphases with their two chromatids bifilary substituted with BrdU were also found. It is noteworthy that the time of BrdU exposure (96 h) is the same as for in vivo incorporation[25]. Other additives such as a serum substitute, whole egg ultrafiltrate, and homologous tissue extracts, intended to provide nutrients and growth factors, were empirically tested in a range of concentrations. They did not improve significantly the results and in some cases were found to be harmful. To induce mitogenic activity, lectins (i.e. phytohemagglutinin C and concanavalin A) were used but, as already reported on several occasions[8,12], no effect was observed. This is not surprising as the cultured tissues did not contain specific target cells. Moreover, it was found that mitotic activity was better if cells remained in the original medium for prolonged periods. Tripp et al.[8] reported a similar situation when growing oyster cells. Therefore, no medium change was performed.

Microbial contamination was the major problem encountered, a problem already emphasized by earlier researchers who studied methods of culturing bivalve tissues[4,7,12]. Owing to the fact that palleal tissues of mussels are in continuous direct contact with seawater, they carry bacteria which are very difficult to remove. The extent of contamination could be significantly reduced by washing the pieces of tissues before explantation in a solution containing high concentrations of antibiotics (penicillin and streptomycin)[7]. Then the multiplication of the bacteria which have not been eliminated by washing, could be restricted by adding low concentrations of antibiotics to the culture medium. These antibiotics are unstable and need to be renewed after 4 days of culture. In such conditions, the contamination level fell below 10%. Fungal contamination was much easier to control. Addition of amphotericin B to the washing medium and, above all, rigorous aseptic manipulation prevent it completely. Consequently, no fungicide had to be added to culture media; the disadvantages of this practice have been discussed[16].

As in the direct method of chromosome preparation[19,23,24], we consider that chromosome losses were due to the slide preparation technique rather than the culture-induced aneuploidies. In this respect, treatment with 50% acetic acid solution appeared to be the most critical phase. The use of a less drastic method to release cell nuclei would probably result in reducing the abnormal metaphase number.

The method as described provides a new way for preparing consistent metaphase spreads in which chromosomes are of sufficiently good quality for karyotyping and for specific banding, as suggested by the NOR staining. It is easier to perform than earlier techniques of bivalve cell culture because enzymatic cell dissociation is not needed for culture initiation or for culture harvesting. Moreover, since somatic adult tissues are used and not embryonic ones, problems related to in vitro fertilization are removed. Another advantage is that somatic tissues can be readily sampled, even in individuals of small size.

We believe that tissue culture has the potential to provide a reliable source of dividing cells in the bivalves as in other taxa. Therefore, efforts should be made to use the technique for other species.

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