Preparation of Chromosomes from Cryopreserved Oyster Larvae

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Introduction

Unlike studies in biomedical fields, research in aquatic species is often hindered by the lack of pre-existing reagents, tools and methods. Research tools such as cell lines, DNA probes and tagged antibodies that are readily available, indeed taken for granted, for work in mice or humans, do not exist for aquatic species. This is especially true for genetic analysis of even commercially important aquaculture species. For example, it is well established that the quality of chromosome preparations is directly related to the quality of source material. For this reason, reproducible cell lines are often used as a source for high quality chromosomes. In mollusks such as oysters, there is no single cell type that possesses all traits required for use in chromosome studies (Zhang et al. 1999). Somatic tissues such as mantle and gill of adult oysters are available year-round and provide chromosomes with minimal background. However, most adult tissues of oysters have low mitotic activity, and treatments for stimulation of mitotic activity in oysters are not available (Cornet 1993).

For such reasons, it has been extremely difficult to establish cell lines to support studies such as karotyping (Buchanan et al. 1999), and only a single cell line has ever been developed for mollusks (Hansen 1976). Meiotic chromosomes prepared from gonad tissue of oysters during spawning season are helpful for identification of individual chromosomes due to the presence of unique structures (chromomeres), however these chromosomes are not useful for karyotyping because of their diffuse and overlapping appearance (Figure 1). The embryos of oysters can provide actively dividing cells, and chromosomes prepared from these cells have morphology useful for mapping of gene location. Chromosomes at different phases of division can be found in these cells, and the elongated prophase and pro-metaphase chromosomes are most suited for application in high-resolution genome mapping (Table 1).

However, embryonic cells are only available during spawning season. Recent studies have shown that larvae of the eastern oyster *Crassostrea virginica* can be cryopreserved and remain viable after thawing (Paniagua-Chavez et al. 1998 and pp. 230-239, this volume), circumventing the problem of seasonal availability of embryos. In channel catfish, we have used cryopreservation to store primary cultures of leukocytes for chromosome analysis after thawing (Zhang and Tiersch 1995). Our goal in this study was to develop procedures for obtaining chromosomes from cryopreserved larvae of the eastern oyster and to establish a year-round source of material for preparation of chromosomes for physical genome mapping despite the lack of cell lines.

Materials and Methods

Cryopreservation

Eastern oysters were obtained from Grand Isle, Louisiana, and were maintained in an indoor recirculating system until use (Buchanan et al. 1998). Fifteen ripe male and female oysters were used for production of larvae by artificial fertilization which were

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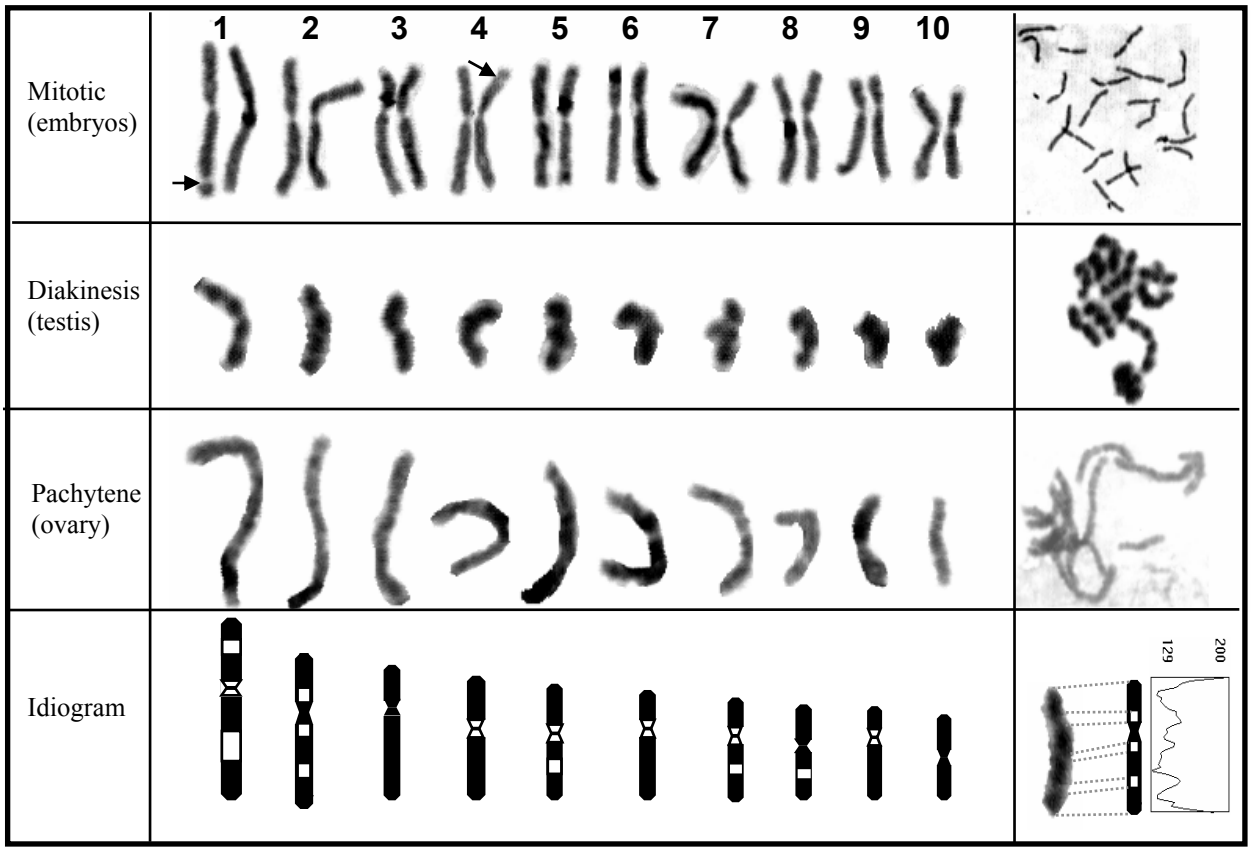

Figure 1. Karyotypes developed from different tissues of the eastern oyster. Arrowheads indicate secondary constrictions. The insets to the right for mitotic, diakinesis and pachytene chromosomes were the original spreads used for karyotyping. To the right of the idiogram is a demonstration of the method of microdensitometry (Zhang and Tiersch 1998). Reprinted with permission of the National Shellfisheries Association (Zhang et al. 1999).

frozen according to the methods described by Paniagua-Chavez et al. (1998) (Paniagua-Chavez et al., pp. 230-239, this volume). Larve were held at room temperature (~21 ºC) in 12-L buckets of artificial sea water (ASW) at 15 ppt until most had reached the trochophore stage (7 to 11 hr after fertilization). The larvae were concentrated and incubated for 15 min in ASW containing 10% propylene glycol, were frozen in 5-mL macrotubes at a rate of -2.5 °C per min to -30 ºC, and were stored in liquid nitrogen (LN_2) for at least 2 wk before thawing.

Preparation of Chromosomes from Frozen Larvae

Samples were thawed at 70 °C for 15 sec, were rinsed in ASW (15 ppt) for 30 min and were incubated in 0.01% colchicine (final concentration) for 40 min. The larvae were rinsed and incubated in 0.075 M KCl for 40 min, were fixed 3 times for 30 min each using cold methanol and acetic acid (3:1), and were stored in this fixative overnight. Chromosomes were prepared on glass microscope slides using standard air-drying procedures.

Table 1. Suitability of chromosomes derived from different tissues of the eastern oyster for cytogenetic analysis and physical genome mapping.

Characters	Tissue			
	Embryo	Gill (or mantle)	Testis	Ovary
Ploidy	2N	2N	1N, 2N	1N, 2N
Availability	spawning season	year-round	spawning season	spawning season
Division stage	mitotic metaphase	Mitotic metaphase	mostly diakinesis	mostly patchytene
Spreads per slide	>30	<5	*	*
Elongation	long	Short	intermediate	very long
Dispersal	separated	Separated	less separated	Overlapping
Resolution	high	Low	high	Highest
Centromeres identified	yes	Yes	no	No
Chromosome bands	no	No	yes	Yes
Chromosome pairs identified	7	3-5	10	10
Potential for mapping	suitable	not suitable	suitable	Suitable

* Cell division dependent on season.

Results and Discussion

The general morphology of chromosomes obtained from cryopreserved larvae (Figure 2a) was not different from that obtained from fresh larvae (Figure 2b). Survival and viability of the thawed larvae largely determined the success of chromosome preparations. Although optimal conditions for incubation after thawing remain to be determined, this study indicates that mitotic activity of trochophore larvae was recovered within 110 min after thawing (the time until fixation).

In previous studies, embryonic cells of the eastern oyster were found to be the best choice of source material for cytogenetic studies (Zhang et al. 1999). However, the limitations of using embryonic cells included seasonal availability and poor visibility of chromosomes due to adherent materials. This study demonstrated that cryopreservation is an effective tool for overcoming the first problem. We also found that chromosomes prepared from trochophore larvae were not obscured by adherent materials as seen for embryos.

Cryopreservation of oyster larvae has provided standardized test animals for analysis of chemical and biological pollution (McFadzen and Cleary 1994). Clearly cryopreservation of gametes, embryos and larvae can open the door to significant advances in a wide array of research areas. Thus, as stated elsewhere in this volume, the value and utility of cryopreserved samples is governed by labeling and record-keeping, especially for effective use in rapidly evolving fields such as molecular genetics where the range of tests is constantly expanding.

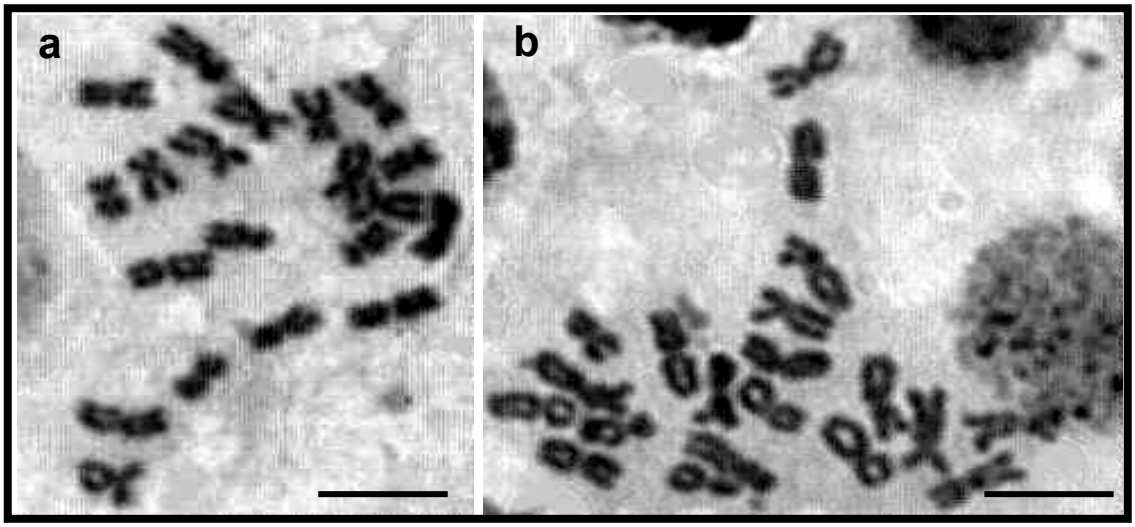

Figure 2. Mitotic chromosomes produced from cryopreserved (a) and non-frozen (b) trochophore larvae of the eastern oyster. Bar = 10 μm.

Acknowledgments

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