Protocol I - Preparation of metaphasic chromosomes [Pratical guide of protocols: methods of verification of the ploidy]

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I - Introduction

The uses of chromosome cytologic information are many, including karyotypic evolution, cytotaxonomy and phylogenetic relationships in plants, insects, and mammals among other organisms. In fish, chromosome analysis based on variations in chromosome number and morphology are typically used to conduct evolutionary and genetic questions such as stock identification, study of hybrids or induced polyploidy. Most fishes possess relatively large numbers of small chromosomes that can be easily viewed with a light microscope at the metaphase stage of mitosis.

The techniques for chromosome preparation are based on obtaining sources of dividing cells to produce high quality metaphase spreads with good chromosome definition. These procedures use specific chemicals, one of the most used reagents is colchicine, to inhibit the formation of the mitotic huse and thus, to block dividing cells at metaphase. Subsequently, a hypotonic treatment is used to separate the metaphase chromosomes from each other in the cells. Next, cells are fixed for staining. Commonly used methods of chromosome preparations are based on direct chromosome preparation from solid-tissues or cell cultures (Thorgaard and Disney, 1990). Accordingly, mitotic chromosome preparations can be successfully made using several tissues collected at different life stages of fish (Baksi and Means, 1988). These include embryos and fish fry and alternatively, the head and gill arches. If adult fish are used for chromosome preparation and analysis, dividing cells are obtained from sources showing a high proportion of actively dividing cells. These are head kidneys, gills, intestines, spleens, testes, scale epithelium or regenerating fin tissues. The methods for collecting adult tissues are the same as those used for fish fry, except antimitotic chemical is injected directly into the adult fish. Of all these adult sources, scale epithelium and regenerating fin tissues are collected without sacrificing the fish, although biopsies of other tissues are also possible.

Direct chromosome preparation techniques are simple and inexpensive to perform in the laboratory and do not require special equipment (light microscope) or much experience. Nevertheless, solid-tissue preparations sometimes yield little information or poor-spread metaphases with inconsistent condensed chromosomes from cell to cell. Thus, cell culture system is an alternative approach that improves the quality of chromosome preparations. On the other hand, it is more costly and time consuming and requires more equipment (inverted microscope, incubator and laminar flow hood) and handling experience (Amemiya *et al.*, 1984).

Accordingly, blood leukocyte culture is a method by which chromosomes can be successfully obtained without killing the fish. It is considered the best method for chromosome preparation and analysis from adult fish when one wants the fish to survive. Leukocyte culture has been described in several fish species including common carp, channel catfish, goldfish, several neotropical fish and three species of the genus *Leporinus*. Nevertheless, it should be noted that this procedure needs to be previously tested in the laboratory to assess successful application for cytogenetic studies of other teleosts (Heckman and Brubaker, 1970). Primary cell cultures can also be performed for making chromosome preparations from whole embryos and adult fish (spleen, kidney, swim bladder, liver and gonads), although with the sacrifice of the animal.

Today, evaluation with karyology is the most common technique used in experiments involving chromosome set manipulation in fish. Polyploidy induction in fish requires the use of methods to corroborate the yield of these manipulations. Karyotyping is a direct method for the verification and identification of triploid fish. Although gynogenetic fish can be putatively identified by chromosome karyotyping, alternative and more accurate methods are required to corroborate

the uniparental inheritance in these types of fish with exclusively maternal inheritance (see Protocol *L*). For practical considerations, chromosome karyotyping in triploid fish are based on solid-tissue preparations, although cell culture techniques are used, if necessary. Although karyotyping is often used for rapid screening to identify triploid individuals, it is time consuming and sometimes requires sacrifice of the fish. Thus, the quantification of the nuclear DNA content by flow cytometry is an alternative, direct methodology for triploid screening. Although it requires equipment that may not be available in all laboratories (Ihssen *et al*., 1990; Komen and Thorgaard, 2007). Several papers have demonstrated that triploid fish can also be accurately identified using indirect methods, including the counting of the number of the nucleolar organizer regions (NORs) in interphase nuclei by using a silver staining procedure (see Protocol *J*) or by the erythrocyte, cellular and nuclear, measurements after a blood smear and staining by conventional means (see Protocol *L*).

The method developed in the present Protocol has been designed for obtaining chromosome plates from newly-hatched sea bass larvae according to the Protocol previously described by Kligerman and Bloom (1977).

II - Purpose

The objective of this Protocol is to demonstrate that chromosome preparation is an accurate method to corroborate triploidy condition. Determination of ploidy is based on counting the number of chromosomes in colchicine-arrested metaphase spreads from the newly-hatched sea bass larvae with different ploidy levels.

III - Procedure

(i) Place the newly hatched larvae (n~12) into a 250-ml beaker containing 0.007% colchicine solution prepared in seawater and allow them to swim for 4 hours. Seawater may be aerated, if necessary.

(ii) Chop each larvae individually into 2-3 mm small pieces using a clean razor bladder.

(iii) Expose the pieces to a hypotonic solution of 0.4% KCl for 20-30 minutes.

(iv) Discard the hypotonic solution and fix the tissues by washing the chopped pieces twice in a freshly made cold mixture of 3:1 ethanol:acetic acid for at least 30 minutes each wash. Tissue may be stored in fixative for several months at 4ºC.

(v) Take the pieces out from the fixative and dry the excess fixative on a paper towel.

(vi) Place the pieces on an excavated micro slide and add 2-3 drops of 50% acetic acid.

(vii) Chop the pieces until a cell suspension is formed.

(viii) Clean micro slides using a mixture of 1:1 ether:ethanol and warm them on a heat plate at 40-50ºC.

(ix) Take the cell suspension with a pasteur pipette and let this suspension fall from a distance of ~12 cm on a precleaned and prewarmed micro slide.

(x) Repeat this process several times forming 2-3 rings of cells of ~1 cm of diameter.

(xi) Allow chromosome preparation to dry for 10-15 minutes.

(xii) Stain chromosome preparation with 15% Giemsa for 45 minutes.

(xiii) Wash chromosome preparation with distilled water and allow it to air dry for 10-15 minutes.

(xiv) Wash it with xylen for 10 minutes and add a cover glass using 2-3 drops of DPX.

(xv) Use a light microscope to view the chromosome preparation. The use of a 20x objective is recommended for a fast localization of chromosome metaphases that usually appear in the periphery of the rings formed.

IV - Materials and equipment

- Razor blades for chopping larvae
- Tweezers
- Excavated glass micro slides
- Clean glass slides
- Paper towel
- Heat plate
- Glass pasteur pipette
- Cover glass
- Light microscope
- Gloves and lab coat

V - Reagents and solutions

- 0.007% colchicine solution prepared in seawater. Colchicine is highly toxic and it should be handled with care. The operator should wear gloves during the procedure since colchicine is a hazardous chemical that may cause cancer and heritable genetic damage.
- Hypotonic solution of 0.4% KCl prepared in distilled water
- Fixer solution of 3:1 ethanol: acetic acid
- Staining solution of 15% Giemsa prepared in phosphate buffer (0.01 M, pH 7.0)
- Distilled water
- Xylen
- DPX

VI - Results and discussion

Karyotyping is frequently used to verify triploidy in fish. It is a direct method based on the counting of metaphasic chromosomes from solid-tissues or cell culture preparations. The use of antimitotic agents, such as colchicine, colcemid or vinblastine sulfate, are required to inhibit the formation of the mitotic huse and thus, blocking mitotic division at the metaphase stage when the chromosomes can be visually and individually identified. As shown in Fig. I.1, the above method using 0.007% colchicine solution resulted in good metaphases from newly hatched sea bass larvae, allowing positive identification of different ploidy levels in this species.

The diploid sea bass larvae showed 2n = 48 chromosome number (Fig. I.1 A) whereas cold-shocked triploid larvae showed 3n = 72 (Fig. I.1 B). Haploid larvae showed 1n = 24 chromosomes (Fig. I.1 C) and meiogynogenetic diploids 2n = 48 (Fig. I.1 D) (Felip *et al*., 1997, 1999ab). Chromosomes were observed condensed and well-spread, thus facilitating their counting. However, in some cases a reduced number of metaphases can be observed with intensely condensed chromosomes with spread metaphases apparently showing one out of two separate filaments (chromatids) (Fig. I.1 B-D). Although the number of chromosomes can be

determined for identification of the ploidy, it should be noted that the quality of these spread metaphase chromosomes could be improved controlling the concentration of the antimitotic reagent used and the duration of the treatment.

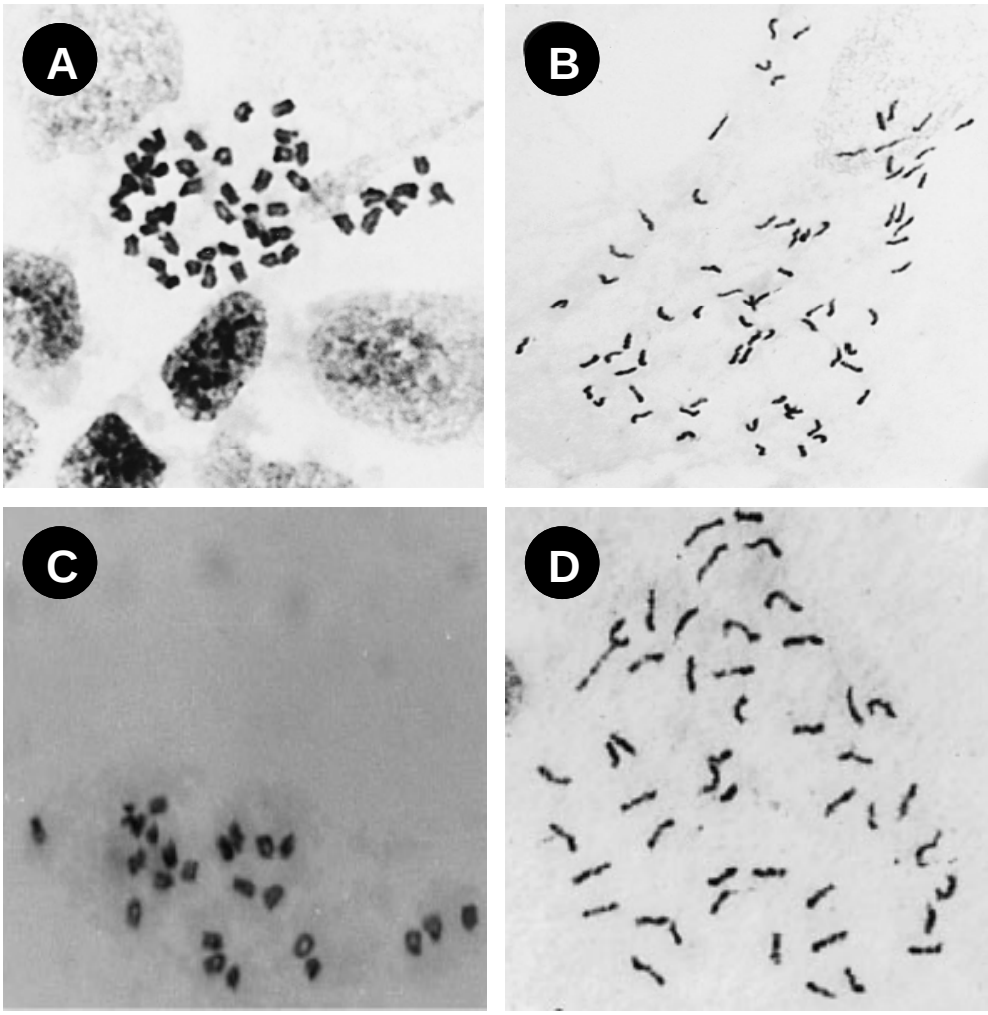

Fig. I.1. Metaphase spreads in sea bass. (A) diploid. (B) triploid. (C) haploid. (D) gynogenetic. Modified from Felip *et al*. (1999a,b).

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