A Simple Technique for Chromosome Preparation from Embryonic Tissues of teleosts for Ploidy Verification

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Abstract — The modified preparation technique developed in the present study has provided an excellent chromosome metaphase spread from the whole body of larvae of the red hybrid tilapia Oreochromis mossambicus (Peters, 1852) × Oreochromis niloticus (Linnaeus, 1758). Unlike other chromosome preparation methods, this modified technique showed a reduction in cell loss while suspending cells during the dropping process. This modified technique can therefore be widely applied not only to various tilapias but also to other teleostean fishes for preparing chromosome spreads. The best treatment parameters for preparing good chromosome spreads from red hybrid tilapia were optimized as 0.01% concentration of colchicine for 4-6 hours, hypotonic 40 minutes treatment, fixation with carnoy solution at 3:1 ratio and a concentration of 10% Giemsa for 20 minutes.

Key words: Chromosome; Diploids, Hybrid tilapia; Metaphase spread; Triploids.

INTRODUCTION

Cytogenetical studies on fishes have valuable importance in evolutionary studies, taxonomy, mutagenesis and in aquaculture, especially for ploidy determination and fish stock management (CHOURROUT and HAPPE 1986; FENOCCHIO and BERTOLLO 1988; FORESTI et al. 1993; DEMIROK and UNLU 2001). Cytological investigations are necessary also for quality control. In fact modern biotechnological techniques involve the manipulation of entire sets of chromosomes, including gynogenesis, androgenesis and ploidy manipulation (LUTZ 2006). The advancement in cytogenetical studies of teleostean fishes has been followed by a variety of karyotypic techniques, including tissue cultures (ROBERTS 1964), squashing technique of the testis (ROBERTS 1964; OHNO et al. 1965), embryonic tissues or haematopoetic materials (SIMON 1963; YAMADA 1967), smearing technique from gill epithelium (MCPHAIL and JONES 1966; STEWART and LEVIN 1968), solid tissues like kidney (OJIMA et al. 1972; ARAI 1973; UENO and OHMA 1977), from regenerating fin tissue (CATTIN and FERREIRA 1989; VOLKER et al. 2005) and air drying techniques (EICHER 1966; FUKUOKA 1972; BERTOLLO et al. 1978; THODE et al. 1988), together with colchicine treatment (YAMAZAKI 1971).

In most of the previous studies, dropping method was used to disperse cells from various tissues for chromosome preparation. Squash technique is the oldest method for spreading and flattening metaphase chromosomes (DENTON 1973). However, air drying method is the most widely adopted method developed for preparing animal chromosomes (EVANS et al. 1964). CHOURROUT and HAPPE (1986) reported that the conventional techniques of chromosome preparations by air drying after colchicine injection in young fishes resulted in adequate metaphase...
spreads (McPhail and Jones 1966; Kligerman and Bloom 1977). Although these methods gave some results, a large amount of cells has been found to be lost during dropping of the cells. Moreover this method requires quite high technical skill to drop the cells exactly on the preheated slides. Some researchers have also attempted to drop cells from a given height on frozen slides for preparing the chromosome spreads (Ojima et al. 1964; Idà et al. 1978). Researchers even tried to use hot steam and metal plates with temperature gradient across its surface for chromosome preparations (Henegariu et al. 2001).

Our investigation is centered on developing a chromosome preparation technique which is easy and accurate particularly for determining ploidy in red hybrid tilapia larvae. In order to increase the yield of usable chromosome spreads of red hybrid tilapia, the chopping method of Yamazaki et al. (1981) was modified in the present investigation. The larval tissues of red hybrid tilapia were used for the development of the present technique.

**MATERIALS AND METHODS**

In the present study, tissues were collected from one day old larvae of artificially bred red hybrid tilapia, for chromosome preparation. Firstly we attempted to optimize the colchicine concentration to obtain the maximum metaphase spreads. A series of trial experiments were conducted for this purpose. For each experiment a duplicate trial with twenty larvae per batch was undertaken. Three different concentrations of colchicine, i.e. 0.005, 0.01 and 0.1% were used in three separate petri dishes. Twenty larvae (one day old) for each concentration were immersed into colchicine. We tested each colchicine concentration, for 2, 4-6 and 10 hours, respectively. In all the trial experiments, freshly prepared colchicine (Sigma Ltd.) was used and the samples were kept undisturbed at room temperature (25°C). After the desired duration of 2, 4-6 and 10 hours, all the larvae from different batches of colchicine treatment were taken out from the petri dishes and transferred to an isotonic solution of 0.7% chilled sodium chloride to anaesthetize the larvae.

The yolk sac and debris of the larvae were carefully removed using a sharp scalpel. In continuation with our previous attempts to optimize the concentrations of colchicine and the duration of its treatment, an attempt was further made to optimize the duration of hypotonization. For this reason the whole body was hypotonized in 0.56% potassium chloride (0.075 M) for 20, 30, 40 and 50 minutes respectively at room temperature (25°C) to find out the most effective hypotonic treatment.

Similarly, in order to find out the best ratio for carnoy solution, 3 different ratios of methanol and acetic acid (2:1, 3:1 and 4:1) of carnoy’s fixative were prepared and tried. The whole body of the larvae was then stored in a small glass vial containing 15 ml of freshly prepared carnoy’s solution, with the different ratios, and kept separately at 4°C. The fixative was changed twice, at an interval of 20 minutes, during the initial hour in the same vial. Afterwards, the larvae were stored with fixative at 4°C for a minimum duration of 6 hours inside the same glass vial. The slides and cover slips for the chromosome preparation were cleaned thoroughly with an overnight treatment of 95% ethanol and distilled water (Yu et al. 1981; Sofy et al. 2008), followed by swabbing of slides by soft tissue paper. After the fixation, the whole body of the larvae was taken out from the vials and used for slide preparation. A drop of distilled water was put on the larva to prevent drying and for proper dissociation of the embryonic cells. Then we applied the chopping method as suggested by Yamazaki et al. (1981). The effectiveness of the method was tried with both distilled water and 50% acetic acid as described by other researchers (Hussain et al., 1991; Hussain and M'candrew 1994).

The samples were chopped thoroughly using a sharp scalpel, finally obtaining a whitish suspension. Then we added 30 µl of Carnoy’s solution onto the chopped suspension to facilitate proper spreading of cells on the slide. The cells were then spread by using the edge of another microscope slide. Immediately, the slide with the spread cells was warmed over the flame of an alcohol lamp till the liquid evaporated completely. The slides were then rinsed in acetone solution to remove the oil droplets. All slides were again air dried for 10-15 minutes and then stained with freshly prepared 10% Giemsa stain (prepared in 0.01 M phosphate buffer at pH 7) as in Hussain and M’candrew (1994). We searched also for the ideal concentrations of Giemsa staining for achieving the best images of chromosomes on the slide. We examined different concentrations of Giemsa stain (5, 10 and 20%) at different exposure times: 10, 20 and 40 minutes respectively. After staining, slides were dipped in xylene for 10 minutes. Following the xylene wash, slides
were subsequently rinsed in distilled water, air dried and mounted with DPX. The metaphase spreads were photographed under 400X and 1000X (oil immersion) using a Nikon Esclipse 80i microscope (Nikon, Japan). We counted the chromosome spreads that were clear enough to allow a proper counting of the total number of 44 chromosomes in red hybrid tilapia. As many countable chromosome spreads as possible were counted on each slide and were compared. The aim was to select the most appropriate preparation parameters for getting the best results intended as the highest number of counted chromosome spreads. Chromosome spreads that were dispersed or clumsy were excluded during the counting of countable chromosome spreads. The batch showing the highest number of clear metaphase spreads among all the trial experiments was selected for further experiment.

The next experiment was designed to prepare chromosome spreads from both diploid and triploid red hybrid tilapia larvae using the above optimized parameters. For producing the triploid red hybrid larvae, the optimized heat shock induction protocol of Pradeep et al. (2010) was applied. Well spread 20 countable metaphase chromosomes from a single slide were considered as the maximum (100%) and accordingly the percentage of metaphase chromosome spreads in each slide were decided.

The data obtained for the different colchicine concentrations and durations of the treatment were tested statistically by applying TWO WAY-ANOVA (SPSS 16. for windows). Differences among the means between the groups and within the groups (multiple comparisons) were tested by ‘Tuckey’s test’.

RESULTS

In this experiment, the whole body of the larva was taken, since in the embryonic stage of the fish, very little transparent tissue was available for making the spread. Results of the improved method showed that good chromosome spreads

![Fig. 1 — Metaphase chromosomes of red hybrid tilapia A: Diploid (2n = 44) and B: Triploid (3n = 66).](image)

| Ploidy   | Age (days after hatch) | Slides with metaphase (%) | Countable metaphase/slide |
|----------|------------------------|---------------------------|---------------------------|
|          |                        |                           | Maximum | Minimum |
| Diploid  | 1                      | 100                       | 25      | 15      |
| Triploid | 1                      | 95-100                    | 23      | 11      |

Table 1 — Percentage of chromosome spreads in each slide and yield of maximum and minimum countable metaphase from embryonic tissue of red hybrid tilapia.
could be made from the whole body of red hybrid tilapia larvae (Fig. 1). Slides with diploid and triploid larvae showed a high percentage of chromosome spread ranging from 95-100% in all the three experimental attempts done with different batches of the larvae (Table 1). Secondly the improved technique eliminated the dropping of cell suspension onto pre-heated or frozen slides. Instead, the chopping method was developed to avoid the dropping of cells suspension where all steps of preparations were carried out only on slides with a shorter duration. The results of the experiment done for determining the optimum concentration and duration of colchicine treatment for red hybrid tilapia fish larvae showed that the best concentration and timing were 0.01% for 4-6 hours of treatment, respectively (P < 0.05) (Table 2). From the experimental trials, treatment duration of 40 minutes with potassium chloride was found to be the best timing for hypotonic treatment in red hybrid tilapia larvae (P < 0.05) (Fig. 2). Compared to the other ratios of Carnoy’s solutions, 3:1 ratio was found very effective in fixing the cells of the larvae. The experimental trial conducted to determine the best concentrations of Giemsa stain and treatment duration, revealed that the best concentration and exposure time was: 10% Giemsa stain, prepared in 0.01 M phosphate buffer at pH 7, for 20 minutes.

**DISCUSSION**

For karyotypic analysis, every single step including the preparation of tissues and slides are critically important for obtaining large number of well spread metaphases and hence affordable results. Generally, a cell culture population has a mixture of cells at all stages of cell division cycle at any given time. However, chromosome preparations could be effective only when the cell culture population has enough mitotic cells. For enriching the amount of mitotic cells, the drug colchicine is used, allowing the arrest of the chromosome division at metaphase stage of the cell division, thanks to the disruption of spindle microtubules (Chiarugi 1950; Zhong and Bowen 2006). Optimal concentration and duration of treatment with colchicine are critically important for getting better results. Thus, the initial step in this technique was based on colchicine treatment of the red hybrid tilapia larvae for arresting the cell division. In the present study the optimal colchicine concentration for red hybrid tilapia fish larvae was determined as 0.01% for 4-6 hours of treatment. Hussain and McAndrew (1994) have already reported that inadequate or over concentration of both colchicine treatments can lead to many unburst cells having uncountable and overlapping chromosomes in the preparation. The present study also showed many unburst cells that resulted in quite frequent clumsiness of cells while changing the colchicine concentration from 0.005% to 0.1%. Colchicine treatments of 2 and 10 hours, respectively, produced limited chromosome spreads and more clumsy cells. The colchicine stored at a temperature of 4°C was not found as effective as compared to freshly prepared colchicine during the present experiments.

Hypotonic treatment is an important and crucial factor in improving the chromosome spreads. This treatment helps in removal of lipid and denatures proteins. Hypotonic treatment allows the swelling of the cell, which facilitates cell disruption and the dispersion of chromosomes when the cell contents are spread on slides. Ida et al. (1978) reported that the use of potassium chloride showed the best chromosome spreads as compared to other two hypotonic solutions of sodium citrate and distilled water. In this technique, the hypotonic treatment with potassium chloride was standardized for 40 minutes, while a comparison with treatment times of 20, 30, 40 and 50 minutes respectively, were used.

| Table 2 — Average number of metaphase spreads at each concentration and treatment duration of colchicine (n=20). |
|-----------------------------------------------|------------------|------------------|
| Colchicine duration (hours) | Colchicine concentrations (%) |
|-----------------------------------------------|------------------|------------------|
| 0.005                                        | 0.01 | 0.1 |
| 2                                             | 4    | 10   | 10 |
| 4-6                                           | 13   | 21*  | 11 |
| 10                                            | 10   | 12   | 11 |

*significant changes with means (P < 0.05)
A time span of less than 40 minutes resulted in more sub-burst cells, whereas most of the chromosomes were found overlapping at 50 minutes of exposure. Choubrout and Happe (1986) reported that the chromosome spreading was insufficient at 0.56% KCl for hypotonic treatment at a lower temperature in the rainbow trout. However, the same concentration of KCl showed slightly better results when the experiments were performed at ambient temperature. According to the same author trisodium citrate as hypotonic treatment gave significant improvement in chromosome spreading. However, in the present improved technique, 0.56% KCl for hypotonic treatment, with an appropriate duration at room temperature produced good results.

In the present technique, a fixative solution of carnoy’s at the ratio of 3:1 was more effective as compared to both 2:1 (Ida et al. 1978) and 4:1 ratio (Hussain and McAndrew 1994). The Carnoy’s fixative allows to preserve the internal structure of the cells for a better staining of the chromosomes (Comings 1978). In the present study we found that the tissues can be stored in Carnoy’s solution for a time longer than one month. In our method, the steps followed by Klingerman and Bloom (1977) and Hussain and McAndrew (1994) of maceration and dissociation of cells with 50% and 60% acetic acid respectively, was avoided. Instead, distilled water (2-4 µl) was used for preventing cells from drying when kept on the slides for chopping and was found most effective in the present study. We tried also 50% acetic acid during the chopping of tissues, but simple distilled water, produced better suspensions. In the modified technique, different durations of staining along with different concentrations of Giemsa stain were also tried. A concentration of 5% Giemsa stain for 20 minutes of treatment as described by Bayat and Wozniki (2006) was not very effective. Moreover, counting of the chromosomes was found difficult at a concentration of 20% as suggested by Don and Avtalion (1986). Changing timing and concentrations of Giemsa stain significantly affected the visibility and brightness of the spreads on the slides. A concentration of 10% Giemsa stain prepared in 0.01 M phosphate buffer of pH 7 for 20 minutes, as described by Hussain and McAndrew (1994) was also tried in the present study. Such Giemsa concentration was very effective in obtaining clear images.

One of the main advantages of the present method is that, it showed minimal loss of tissues during each process of slide preparation, which is an advantage for smaller samples like embryonic tissues. Another benefit is that instead of using preheated or frozen slides, direct flame from an alcoholic lamp was used for drying the cells. In conclusion the technique allows to prepare good metaphase spreads with or without the aid of karyotyping software’s.

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