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A preliminary study on the determination of triploidy by chromosome analysis at the different stages of development in rainbow trout, *Oncorhynchus mykiss*.

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ABSTRACT

There are several methods for ploidy determination of fish, those have been developed by many researchers until to date. For these methods, the cost and the applicability of each stage of fish development are very important. The aim of this study was to compare the applicability of chromosome preparation in rainbow trout (*Oncorhynchus mykiss*) at the different stages of development, including the early and late stages of embryo, pre-larvae, post-larvae and fry stages. Chromosome preparations were modified from previous studies and successfully used in order to determine the ploidy in *O. mykiss*. At the end of applications, the best result for the applicability of chromosome preparation was obtained from the embryos at late stage. Also, it was determined that the chromosome numbers in diploid and triploid *O. mykiss* were 2n=56 and 3x=84, respectively, that were selected from the well-spred metaphase chromosomes.

Introduction

O. mykiss is a very important aquaculture species in the world on account of its economic value and scientific interest. Artificially-induced triploidy of *O. mykiss* is popular in aquaculture (Benfey 1996) because triploidy potentially could avoid the diminished growth and survival losses associated with sexual maturity in normal fish (Gjedrem 1976; Small and Benfey 1987).

Triploidy induction is a technique which based on the principle of genetic manipulation of chromosome number. Many researchers interested regarding its possible detection of polyploidy so they applied many several methods for ploidy determination, such as erythrocyte measurements (Woznicki and Kuzminski 2002; Kenanoğlu et al. 2012), electrophoresis of proteins and examination of morphology (Liu et al. 1978), erythrocyte nuclear volume measurements (Johnson et al. 1984), silver staining of nucleolar organizing regions (Phillips et al. 1986), number of active nucleoli per cell (Kucharczyk et al. 1999) and cytological karyotyping (Thorgaard and Disney 1990).

Chromosome preparation is the most precise method for determining polyploidy it provides to determine chromosome numbers truly and the information of essential necessity for cytogenetic research. Also, cytogenetic studies provide analysis of chromosomal behavior in the organization and transmission of genetic information (Shao et al. 2010). There are several techniques for chromosome preparation, such as the blood leukocyte (Al-Sabti 1985) and the lymphocyte culture techniques (Fujiwara et al. 2001), air drying technique (Bertollo et al. 1978), squashing technique of the testis (Roberts 1964) or embryonic tissues (Yamada 1967), cell suspension technique from gill epithelium (Mcphail and Jones 1966), scales (Ojima et al. 1972), fin tissue (Hussain and McAndrew 1994) and kidney (Chakraborty and Kagwade 1989).

Determining polyploidy is very important so as to plan the applications such as triploidy on fish at the beginning of

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the development. In this study, method of chromosome preparation was modified partly or completely, from the previous methodologies for teleost fishes which have been reported by several researchers (Yamada 1967; Bertollo et al. 1978 and Shao et al. 2010) and was used for determining of triploidy in *O. mykiss*.

Material and methods

Triploid Fish

Cultured *O.mykiss* were maintained in freshwater (10°C) in outdoor concrete raceway system prior to stripping. Eggs and milt were taken from 3 females and 2 males then, the eggs were fertilized in 10°C natural spring water. After several minutes, the fertilized eggs were rinsed in fresh water and waited for 10 min for egg swelling. Eggs were thermal-shocked (26°C) at 15 min after the 15 min post fertilization (PF) (Kenanoglu et al. 2012).

Different Development Stages

For chromosome preparation, it was benefitted from different development stages in *O. mykiss*, such as the early stage of embryo (14 day PF, n=30), the late stage of embryo (22-27 day PF, n=30), the larvae stage with 4, 6 and 20 day after hatching (DAH); n=30 for each stages and the fry stage (33 DAH, n= 30) (Figure 1).

Chromosome Preparations

In this method, healthy embryo, larvae or fry individuals were selected and placed in 0.02% colchicine (Sigma). Embryo, pre-larvae and larvae waited at 2 h, post-larvae and fry stages waited at 6-7 h in colchicine solution. Then, each embryo or larvae were transferred into the watch glass. The chorion of the embryo (Figure 2) or head and yolk of the larvae were removed with the use of forceps and lancet. Additionally, the gills of fry fish at the 33 DAH were used for preparation.

Each sample which was obtained by dissection at different development stages rinsed with an isotonic solution of 0.9% sodium chloride (NaCl). Previous studies reported that optimal concentration and duration of treatment with KCl are crucial factors in improving the chromosome spreads (Völker and Kullmann 2006; Pradeep et al. 2011). Thus, in this study samples were minced with the lancet into the KCl (0.075 M). For hypotonic treatment, we tested incubation times ranging 20-60 min at 24°C.

The tissue components of each sample were separately transferred into the 15 ml falcon tubes with 5 ml of KCl solution (0.075 M). After the hypotonic treatment, 2 drops of freshly prepared Carnoy's fixative (3:1 methanol: acetic acid) was added into the hypotonic solution for prefix. Then, the samples were briefly vortexed and centrifuged 10 min at 2000 rpm. Once the centrifugation was finished, the supernatant (KCl solution and fixative) was removed and the cell pellet resuspended with 5 ml ice-cold fixative by vortexing and waiting for 15 min and then a new centrifugation period of 10 min has started. The procedure was repeated twice or three times. At the end of this process the pellet was resuspended in about 1-2 ml fixative.

Preparation of Slides

Slides were cleaned with 1% HCl for 20 min at room temperature. Using a pasteur pipette, the cell suspension was dropped from a height of 5-40 cm onto a clean glass slides that had been kept in cold (4°C) with 95% ethanol and then allowed to dry at room temperature.

Chromosome Analysis

Slides were stained in 5% Giemsa (prepared with phosphate buffer at pH 6.8) for 20 min at room temperature. The slides were rinsed in distilled water, air dried and mounted with D.P.X.. Slides were observed using a Novel microscope equipped with a camera at 100X.

At the end of this process, all samples were analysed at least 10 metaphase plates from each specimen.

Results

The result of chromosome preparation showed that, the diploid *O. mykiss* has 2n=56 chromosomes (Figure 3) and the triploid *O. mykiss* has 3x=84 chromosomes (Figure 4).

The comparisons of chromosome preparation at different stages of development for easiest applicability showed that the late stage embryos are more suitable than

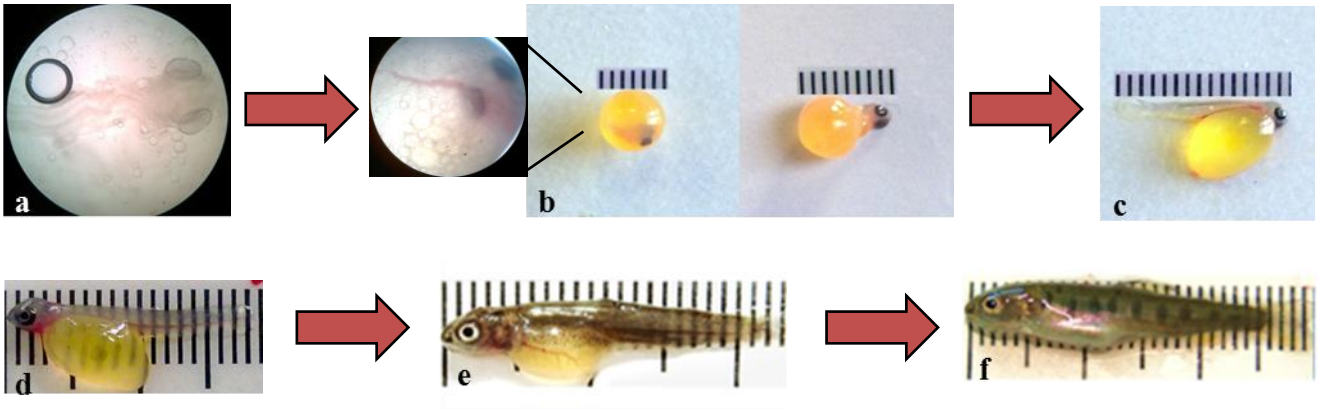

Figure 1. Developmental stages of *O. mykiss* a) Early stage (14 day PF) b) Late stage (22 and 27 days PF, respectively) c) Pre-larvea (4 DAH) d) Pre-larvae (6 DAH) e) Larvae (20 DAH) f) Fry (33 DAH).

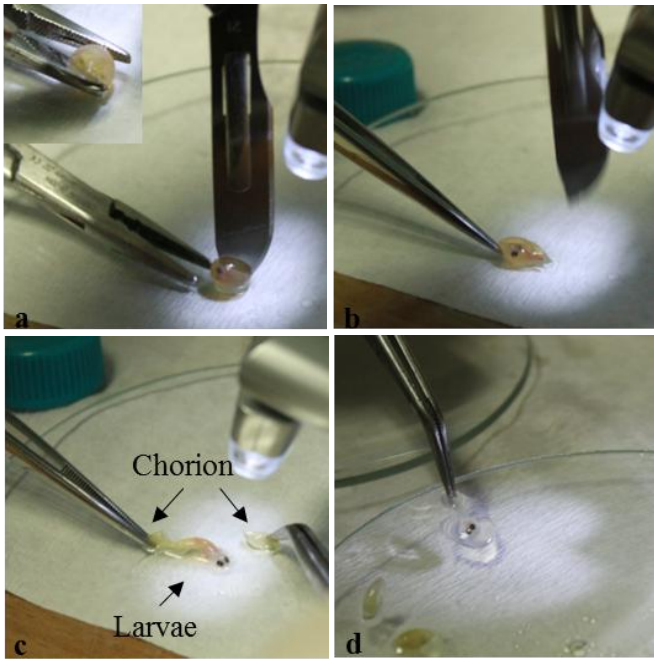

Figure 2. a-c) Steps of dissection in *O. mykiss* embryos d) Larvae cleaning with NaCl.

other stages for good preparations. So, the samples of early stages provided not enough cell material. In contrast, samples of other development stages have tissue construction which is more difficult to homogenize than late stage embryos.

The fixation process with the duration of implementation and the repeats of application were increased with the growth. So it was determined that the application of centrifugation which was repeated just once was enough for clear metaphase from early stages to larval stages. This was probably due to the tissues and organs becoming complex with the maturation of fish.

According to the results of the experimental applications, hypotonic treatment with 0.075M KCl at 35-40 min (24°C) was found to have the best timing for good spreading of chromosomes. In addition, in the present method, dropping of the cell solution from the height between 25-30 cm on a cold slide has given a better result for the spread of metaphase chromosomes.

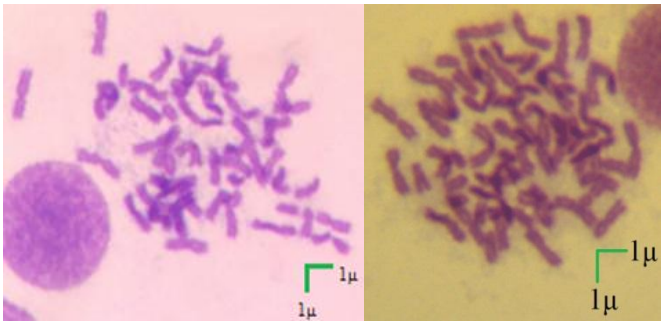

Figure 3. Chromosome of metaphase for diploid *O. mykiss* (2n=56).

Discussion

According to the results of the study, it was determined that diploid chromosome number of *O.mykiss* was 2n=56. Oliveira et al. (1995; in review) showed that this number ranged from 2n=56 to 2n=68 for *O. mykiss*. Additionally, Colihueque et al. (2001) determined that diploid chromosome number of *O. mykiss* changed from 58 to 63. Similarly Ulupınar and Okumuş (2001) found that for *O. mykiss*, diploid number was 2n=58-64, and 2n=60 and 2n=62 are prevalent diploid chromosome numbers in Northeast Black Sea. Inokuchi et al. (1994) have reported diploid chromosome numbers between 60 and 62 in *O. mykiss* as a result of their banding operations with bromodeoxyuridine (BrdU). These changing chromosome numbers were due to Robertsonian translocations, where two acrocentric chromosomes join to make a metacentric one (Inokuchi et al. 1994; Ulupınar and Okumuş 2001).

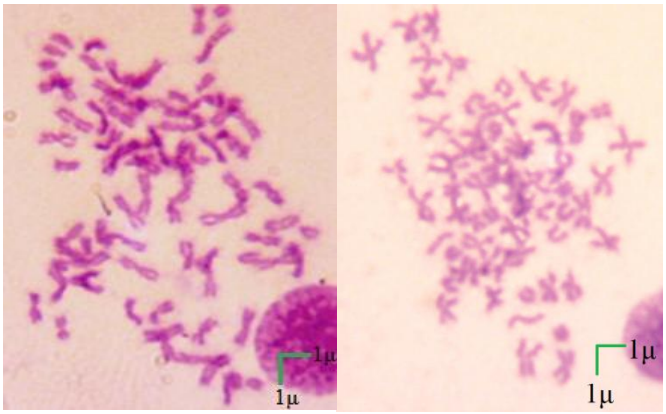

Figure 4. Chromosome of metaphase for heat shock applied triploid *O. mykiss* (3x=84).

This investigation describing triploid individuals was to be focused on the late stage embryos due to the suitability of chromosome preparation for not only cell material but also high mitotic index. Previous studies about deciding optimum developmental stages of fish for chromosome preparation was reported various results. Shao et al. (2010) determined that the mitotic index of embryo or larvae was higher than juvenile fish. Völker and Kullmann (2006) reported that, with the beginning of the eyed stage the mitotic index started to decrease. They also stated that the first days of embryonic development was suitable. Harrell et al. (1998) explained this situation as young fish being more suitable than older fish for chromosome analysis, because growth rate and number of cell at the metaphase stage decreases with aging. According to our results, the late stage embryos are more suitable than early stage because there is not enough cell material at the early stage of embryo. In contrast to the situation, Shao et al. (2010) were succeeded in making chromosome preparation from the larvae of *C. semilaevis* at blastula stage and they attained large numbers of well-spread metaphase chromosomes with method of the single embryo.

In this study, it was advised that the concentrations for colchicine and KCl were 0.02% colchicine for 2 h and 0.075M KCl for 40 min (24°C), on fish at embryonic stage. However, some previous studies showed different parameters for the chemical treatment. For example; Bencsik et al. (2011) applied 0.005% colchicine solution for 6 h and they did not use KCl for hypotonic treatment in embryos of *O. mykiss*. Ladygina and Wakamatsu (1999) treated 2-5 days embryos of medaka (*Oryzias latipes*) with 0.001% colchicine solution

for 4-5 h and KCl (0.075 M) for 30 min (26°C). Völker and Kullmann (2006) advised 0.02% colchicine for 8-9 h or 0.05% colchicine for 4-5 h. and KCl (0.075M) for 35-50 min (23°C) in killifishes (*Chromaphyosemion* sp.) embryos ranging from 2 to 8 days of age for optimum mitostatic treatment.

Inokuchi et al. (1994) treated early embryos of *O.mykiss* with 0.012-0.0025% colchicine for 6-12 h and 0.8% tri-sodium citrate dihydrate for 15-30 min at room temperature. Shao et al. (2010) applied 0.02% colchicine solution for 1-2 h which is parallel to our study, and they also incubate post-blastula stage embryos of tongue sole (*Cynoglossus semilaevis*) with KCl (0.075 M) at room temperature for 30 min. Furthermore, some researchers also successfully used tri-sodium citrate (Chourrout and Happe 1986) and saline solution (Hussain and McAndrew 1994) for hypotonic treatment.

In this study, embryos chorion was removed after the colchicine treatment. Because, we centrifuged samples we needed purified cell suspension before dropping. However, some researchers removed the vitelline membrane of the embryo after the fixation step (Yamada 1967; Inokuchi et al. 1994; Shao et al. 2010).

Some advantages of providing increase in mitotic index with earlier stages of fish development were known from the previous studies. In this study, results showed that the chromosome analysis made at the late stage of embryos are useful compared to other development stages for determining triploidy in *O. mykiss*.

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