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Comparison of different ploidy detection methods in *Oncorhynchus mykiss*, the rainbow trout

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Abstract

The objective of this study was to determine a simple and reliable ploidy identification protocol for the rainbow trout (RT), *Oncorhynchus mykiss*, in the field condition. To evaluate the ploidy level and compare different detection protocols, triploid RT and gynogenesis were induced by UV irradiation and/or heat shock. The hatching rate at day 30 was 85.2% and the survival rate at day 90 was 69.4% (fingerling). The sex ratio of female RT was 93.75% in the gynogenesis group, illustrating that the UV irradiation inactivated the sperm DNA. The hatching rate and survival rate were 82.0 and 74.7%, respectively, in the triploid-induced group. The triploid induction rate by heat shock procedure was 73.9%. Cytogenetic protocols for ploidy identification such as chromosome counting, erythrocyte nuclear size comparison, and analysis of nucleolar organizing regions (NORs) by silver staining were compared. Silver nitrate staining showed the greatest success rate (22/23 and 32/32 for the triploid-induced group and gynogenesis group, respectively), followed by erythrocyte nuclear size comparison (16/23 and 19/32 for the triploid-induced group and gynogenesis group, respectively) and, lastly, chromosome preparation (2/23 and 6/32 for the triploid-induced group and gynogenesis group, respectively) with the lowest success rate. Based on our findings, silver staining for RT ploidy identification is speculated to be highly applicable in a wide range of research conditions, due to its cost-effectiveness and simplicity compared to other numerous ploidy detection protocols.

Keywords: Rainbow trout, Nucleolar organizing regions, Silver staining, Triploid, Gynogenesis

Background

It has been 50 years since the domestication of rainbow trout (RT) in South Korea, reaching a production of more than 3000 tons per year (Ministry of Ocean and Fisheries 2016). However, the lack of a systematic control of brood stock, recessive growth due to inbreeding, and the increased male ratio are causing the overall productivity of RT to slump (Hwang 2012). In the global aquaculture industry, the induction of numerous artificial triploid fish species is already an important subject of study (Felip et al. 1997; Gjedrem et al. 2012; Maxime 2008). The usage of triploid fish for industrial purposes has numerous advantages as it contains three sets of chromosomes and is genetically sterile. Above all, these types of fish have reduced gonadal development (Cal et al. 2006; FAO 2005), meaning that instead of sexual maturation, the energy is directed towards the development of flesh quality and somatic growth (Felip et al. 2001; Kizak et al. 2013; Piferrer et al. 2009). These characteristics have drawn the attention of people for the preference of triploid fish over diploid.

Although diploid and triploid fish are morphologically equal throughout their life cycle, they are cytologically different. Hence, there are many ways, direct or indirect, to identify the ploidy of a fish (Maxime 2008; Tiwary et al. 2004). Among those are the measurement of nuclear and cellular size (Alcantar-Vazquez 2016; Thomas and Morrison 1995), electrophoresis of proteins (Liu et al. 1978; Shimizu et al. 1993), nuclear and cell size measurement of erythrocyte (Olele and Tiguiri 2013; Pradeep et al. 2011), chromosome counting (Thogaard 1983; Tiwary et al. 1997), DNA content determination with flow cytometry (Alcantar-Vazquez et al. 2008; Lamatsch et al. 2000), and staining of nucleoli with silver nitrate (Howell and Black 1980; Porto-Foresti et al.

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2002). Yet, regardless of type, it is believed that an easy, simple, and inexpensive method for ploidy identification is most advantageous and productive. The silver staining method for nucleoli identification, nuclear and cell size measurement of erythrocyte, and chromosome counting meet the criterion mentioned above since they are functional and have the capacity for a hasty identification of ploidy level, whereas most other methods require specific equipments and expensive materials (Carman et al. 1992). In this study with RT, we have preferentially focused on silver staining over chromosome counting and erythrocyte nuclear size comparison because of two main reasons: first is randomness. Chromosome preparation is known to be very random (Deng et al. 2003). There are too many factors to consider such as relative humidity (Spurbeck et al. 1996), cell dropping height (Barch et al. 1997; Hlics et al. 1997), and *flame vs. air* drying method of the slide (Karami et al. 2015). Still, even taking into account all these aspects, getting a well-spread metaphase is overly time consuming and not always rewarding. This is not an exception with the erythrocyte nuclear size comparison method. Although it is widely used, as mentioned in reports by Felip et al. (2001) and Caterina et al. (2014), the nuclear size of red blood cells is not always ~ 1.5 times bigger and it depends on the type of anticoagulant used while collecting the blood samples, as well as the preservation time of samples and slide preparation conditions. Second is inconsistency in chromosome numbers. Due to the Robertsonian translocation in RT chromosome (Inokuchi et al. 1994; Jankun et al. 2007), the change in number is unavoidable. The numbers range from $2n$ = 56 (Kenanoglu et al. 2013), $2n$ = 56 to 68 (Oliveira et al. 1995), and $2n$ = 58 to 63 (Colihueque et al. 2001), making chromosome preparation less reliable. To the contrary, considering there is a direct relationship between the numbers of nucleolar organizing regions (NORs) per chromosome pair (Jankun et al. 2007; Phillips et al. 1986) in RT, silver staining is a more reliable method of ploidy identification.

In order to identify polyploidy of the samples (gynogenetic diploid females and presumed triploid RT), three different ploidy detection methods were compared. Furthermore, hatching rate, survival rates, sex ratio determination, and triploid induction rate were also measured.

Methods

Fish

RT were randomly selected from Dong Gang Aquaculture located in Pyeongchang. Males (n = 5, length 63.4 ± 2.3 cm; body weight 3415 ± 576.8 g) and females (n = 19, length 58.6 ± 4.2 cm; body weight 3519 ± 835.7 g) were anesthetized by MS-222 (Tricaine methane sulfonate, 25 mg/l) in a 50-l container. All eggs and milt used in this experiment were obtained by abdominal massage. Egg quality was evaluated by visual inspection. By calculating the average mass of an RT egg (~ 0.6 g), the total number of eggs collected was calculated based on the mass of the container (35.7 l), giving a total of approximately 59,500 eggs. A total of about 47 ml of milt was collected from five males and divided into two for the treatment of gynogenesis and triploid production.

Gynogenesis and triploid production

Milt stripped from males was diluted (1:10) with saline solution and transferred to Petri dishes, 10 cm in diameter, forming a thin layer of sperm. The Petri dishes were exposed to UV (Phillips 6 W UV lamp) for 15 min on ice for the inactivation of sperm DNA (Fernandez-Diez et al. 2016). The eggs were divided into two groups, and each group was treated with normal intact milt (triploid-induced group) and UV-irradiated milt (gynogenesis group) for 2 min and stirred with a feather. For every ~ 3000 eggs, 1 ml of milt was used. After 10 min of fertilization, eggs were exposed to heat shock at 28 °C for 20 min to prevent extrusion of the second polar body. Hatching rate was calculated 30 days post fertilization, and survival rate was determined as the fish reached 90 days post fertilization. To further confirm ploidy by means of erythrocyte nuclear size, chromosome counting, and NOR identification, RT fingerlings (n = 23, age 3 months old; body weight 1.5–2 g) were randomly selected and kept alive while being transported to the lab in a *1-gal dispensing bag* connected to an air pump.

In addition, gonadal tissue slices obtained from the pool of gynogenesis group fingerlings (n = 32) were set onto a slide and gently squashed using a cover glass for sex ratio determination by histological examination under a microscope.

Detection of NORs by silver staining

Small pieces of fin tissue were obtained without sacrificing the samples (triploid-induced group), then sheared on a pre-cleaned slide with few drops of 50% acetic acid and finally let dry in air at room temperature. Samples were stained with silver nitrate following the procedures proposed by Howell and Black (1980) with a modification to remove silver residue precipitation. The first solution, solution A (Sol A), was made with 0.5 g of gelatin, 25 ml of double-distilled water, and 0.25 ml of formic acid containing formaldehyde (2% final concentration). An aqueous solution, solution B (Sol B), was a mix of 5 g of silver nitrate and 10 ml of double-distilled water. Both Sol A and Sol B were covered with aluminum foil and stored in the dark to avoid photoreaction. As for the staining of the slide, 50 μl of Sol A and 100 μl of Sol B were dropped on the slide and the solutions were gently mixed using the side of a pre-cleaned 3-ml disposable pipette. Next, the slide was placed on a hot plate (60 °C) that was covered well to provide as much darkness as possible for the stain to take place. As the solution became golden brown, the slide was removed from the hot plate, gently washed under running double-distilled water, and let dry in air.

Chromosome preparation

Fingerling samples were prepared as described by Kligerman and Bloom (1977) but modified to suit our experiment. To intercept cell division by interrupting the polymerization of microtubules, the fish were transferred into a 2-l glass beaker and then treated with 0.005% colchicine for 3 h. After colchicine treatment, the fish were sacrificed, and fins and gills were collected and placed in individual 1.5-ml Eppendorf tubes. Immediately after, samples were treated with 0.075 M potassium chloride (KCl) hypotonic solution for 20 min at room temperature twice. Samples were centrifuged at 3000 rpm for 2 min, supernatant was removed, and Carnoy's fixative solution (3:1 methanol/acetic acid) was added twice, each lasting 20 min. At the end of the last fixation procedure, samples were stored at 4 °C until assay. Each sample was placed on a slide with two to three drops of 50% acetic acid. Tissues were gently minced into tiny pieces using a 14-gauge needle attached to a 1-ml syringe under a dissecting microscope. Afterwards, 7 μl of the minced solution was pipetted and dropped onto a pre-cleaned slide at a height of 30~40 cm and air-dried. The slide was then stained with 5% Giemsa for 20 min at room temperature, washed with running double-distilled water, and let dry in air before observing under the microscope.

Erythrocyte nuclear size comparison

Due to the difficulty of blood withdrawal from fingerling (3 months old), fish were sacrificed and blood samples were aspirated using a 14-gauge needle in a 1-ml syringe coated with EDTA solution, while preparing the samples for chromosome preparation. On a pre-cleaned slide, 20 μl of blood was placed and smeared using a cover glass. The smeared blood was then stained with 0.22% Coomassie blue stain (composed of 220 mg Coomassie blue in 50 ml methanol, 10 ml acetic acid, and 40 ml double-distilled water) for 3 min, washed with double-distilled water, and let dry in air.

Microscope and camera equipment

All slides were observed using a Zeiss Axiovert 200 inverted microscope with a magnification of × 600, × 900, and × 1000, and photographs were taken using a Canon PowerShot G9 digital camera connected to the microscope via a Soligor adapter tube.

Results and discussion

Hatching rate, survival rate, and sex ratio determination

The average hatching and survival rates were calculated from 250 randomly selected samples of each group. The hatching rate of triploid-induced group and gynogenesis group was 85.2% (n = 212) and 82.0% (n = 205), respectively. The survival rate for each group was 69.6% (n = 174, triploid-induced group) and 74.4% (n = 186, gynogenesis group) at 90 days post fertilization (Table 1).

Based on the gonadal tissue examination (Fig. 1), the female sex ratio of gynogenesis group was 93.75% (30:32), indicating a fairly high induction of female. The histological section of female gonadal tissue showed corrugated structural morphology with signs of immature oocytes (Fig. 1a). On the other hand, the male testis showed an overall silky surface with immature spermatogonial development (Fig. 1b).

Triploid induction rate

The triploid induction rate measured by silver staining was 73.9% (17/23, Fig. 2). Throughout our experiments, we encountered samples with four NORs (Fig. 2e, f), which show similar patterns to the previous results reported by Flajshans et al. (1992) on the existence of four NORs in the course of triploid fish production.

Ploidy identification

The success rate for ploidy identification of each method was recorded. Chromosome preparation, erythrocyte nuclear size comparison, and silver nitrate staining methods were performed in all samples (gynogenesis group and triploid-induced group). The results of each method are shown in Table 2.

Chromosome preparation showed a very poor success rate of 6/32 and 2/23 for the gynogenesis group and triploid-induced group, respectively (Table 2). Attaining a clear image for chromosomal count was very random (Fig. 3a, b). After many experimental attempts, in which we tried our best to maintain a uniform working condition, we were occasionally able to obtain a justifiable spread of chromosomes. An approximate of 60 chromosomes, a characteristic of a diploid cell, was observed (Fig. 3b). Incomplete spread of metaphase chromosome, disturbing the viewer while performing chromosomal count, is shown in Fig. 3a.

Erythrocytes of gynogenesis group and triploid-induced group are shown in Fig. 4. The difference of nuclear length of triploid samples from those of diploid was at the major axis as mentioned by Jankun et al. (2007). However, the majority of the samples had the tendency to display a minor length difference showing

Table 1 Hatching and survival rate of induced gynogenesis and triploid rainbow trout

Group	Hatching rate	Survival rate
	Days[a]	
	30	90
Gynogenesis group (%)	82.0 (205/250)[b]	74.4 (186/250)
Triploid-induced group (%)	85.2 (212/250)	69.6 (174/250)

[a]Days after fertilization
[b]Counted no. of samples/total no. of samples (n = 250)

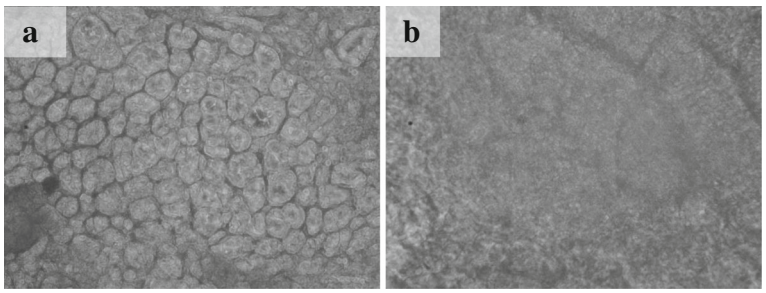

Fig. 1 Histological examination of gonadal tissue from rainbow trout. **a** Section of squeezed female gonadal structure showing signs of primordial oocytes (× 600). **b** Section of squeezed male gonadal structure showing signs of primordial spermatogonia (× 600)

difficulties in ploidy detection. Additionally, although an anticoagulant (EDTA) was used to prevent aggregation of erythrocytes, some samples showed signs of coagulation while others displayed signs of hemorrhage (data not shown). Overall, the success rate for ploidy detection in erythrocyte nuclear size comparison method was of 19/32 and 16/23 for the gynogenesis group and triploid-induced group, respectively (Table 2).

Phillips and Ihssen (1985) and Phillips et al. (1986) reported that *Oncorhynchus* species have only one NOR per chromosome pair. Therefore, if the samples from the triploid-induced group were triploids, the cells would be expected to have a maximum of three NORs. Ploidy detection using silver nitrate was the most successful (Table 2) compared to the other two methods. The results were 32/32 in the gynogenesis group and

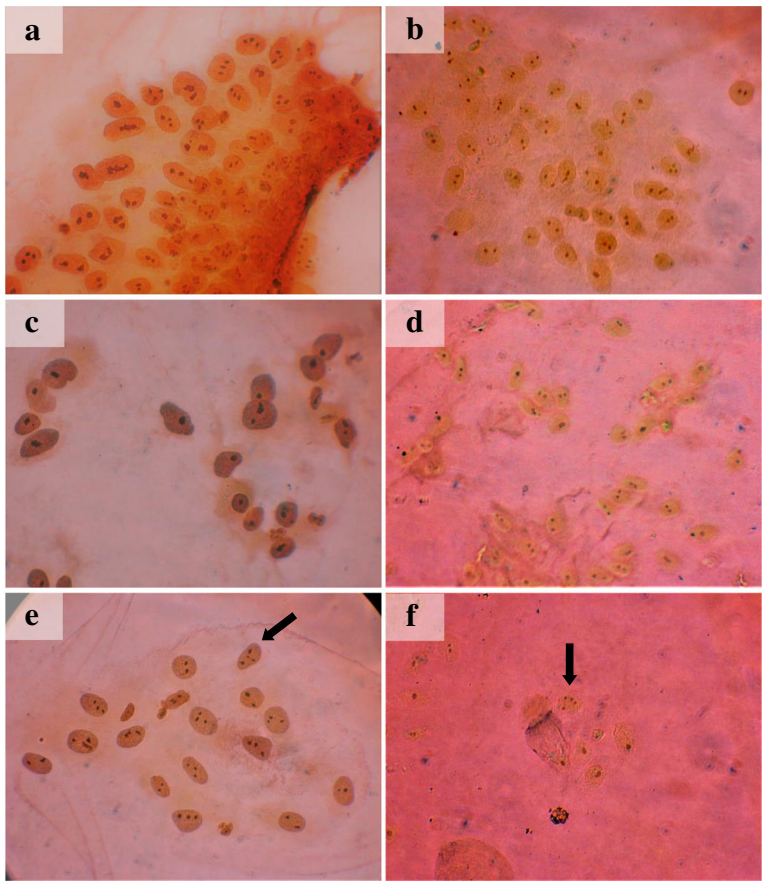

Fig. 2 Interphase nuclei from rainbow trout stained with silver nitrate. **a** Triploid *O. mykiss* sample containing up to three NORs. The excess number of cells but identifiable (× 600). **b** Ideal triploid sample with a maximum of three NORs (× 600). **c**, **d** Diploid *O. mykiss* sample containing one and two NORs, respectively (× 600). **e**, **f** Samples with a maximum of four NORs shown in arrows (× 900 and × 600, respectively)

Table 2 Number of successful ploidy detection experiments from three different ploidy detection methods

Ploidy detection method[a]	Group	
	Gynogenesis (n = 32)	Triploid-induced (n = 23)
Chromosome preparation	6/32[b]	2/23
Erythrocyte nuclear size comparison	19/32	16/23
Silver nitrate staining	32/32	22/23

[a]All ploidy detection methods were tested under identical samples
[b]Ploidy-identified sample/total no. of samples

22/23 in the triploid-induced group. Moreover, as previously mentioned by Kavalco and Pazza (2004), silver debris precipitation in the conventional silver nitrate staining procedure is responsible for false positive results, giving difficulties to the viewer when identifying the ploidy of a sample. Nonetheless, our results show clearer stains with few or no silver debris reason being the filtration (0.45 μm) of the staining solution before usage. A difference in coloration of stain can be seen in Fig. 2, which is due to the amount of time exposed to the silver nitrate stain. As reported by Howell and Black (1980), within 30 s, the stain turns yellow, and within 2 min, it turns golden brown. Because the time taken for the stain to transform into golden brown was not always the same, avoiding the stain to become too dark was critical. Through our study, we recommend that the optimal staining time should be less than 90 s, because longer exposure to the stain would negatively affect the imaging of the sample.

Through our study, we have compared and demonstrated three different but easily approachable methods for ploidy detection in RT and, hereinabove, presented the results (Table 2).

It can be denoted from our results that all three methods have their advantages. However, the most field-applicable, easy, and rapid method of ploidy identification funnels down to silver nitrate staining for NOR identification. Although chromosome counting is precise and excels to identify different ploidy levels, chromosome analysis requires technically sophisticated skill. Furthermore, our study shows that the erythrocyte nuclear size comparison method is, in fact, faster when compared to chromosome counting with higher success rate. Yet, we speculated that nuclear size comparison from fish blood cells was, to some degree, subjective and an inaccurate ploidy detection protocol since it depended on numerous factors such as the anticoagulant used, sample preservation time, and preparation conditions (Felip et al. 2001; Caterina et al. 2014).

Despite the fact that ploidy identification using silver nitrate in fish specimen is not as widely used as in animals, plants, and insects, silver staining for ploidy identification is fast and, at the same time, easy and very reliable since neither special skills nor expensive equipments are necessary. There are also several advantages when identifying the ploidy in RT; for instance, in place of sacrificing the specimens, samples could be obtained by cutting small pieces of fin from different yearlings and applying the staining method directly in the field without the inconvenience of returning to the laboratory. Moreover, this method could be applied in the early embryonic stage and therefore obviate the high raising cost and waste of time until being fully grown for ploidy identification. According to Phillips et al. (1986), the majority of these rapidly dividing embryonic cells are composed of their maximum number of nucleoli, thus making silver staining possible for the identification of triploids in the early developmental stage of fish.

Furthermore, the trial to induce triploid RT from our study [diploid 26.1%; triploid (including those with four NORs) 73.9%] is somewhat different from the previously reported studies (Hwang 2012). This may be due to the contributed experimental condition's discrepancies, such as temperature applied to eggs and the prevention timing of the second polar body extrusion.

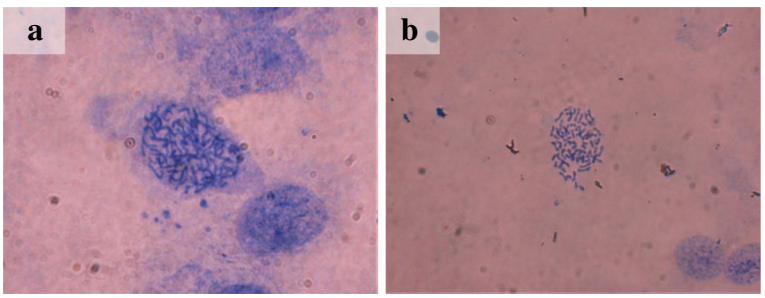

Fig. 3 Metaphase chromosome from rainbow trout stained with Giemsa. **a**, **b** Triploid and diploid chromosomes stained with 5% Giemsa stain under × 900 and × 600, respectively

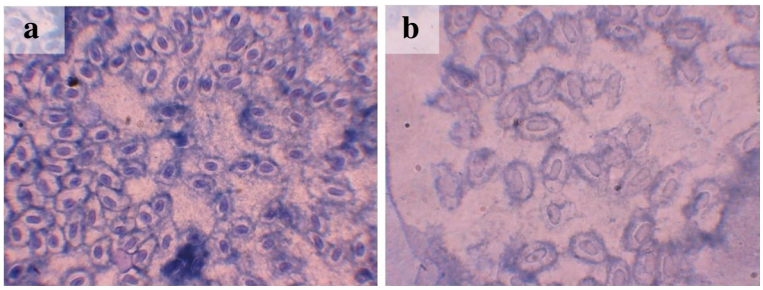

Fig. 4 Erythrocyte size comparison. Samples stained with 0.22% Coomassie blue. **a** Diploid sample (× 1000). **b** Triploid sample (× 1000)

Conclusions

According to the data obtained in this study, it is speculated that silver staining is a suitable ploidy detection method in RT not only for technically unsophisticated farms but also to fish research personnel. We hope that this silver staining method is useful to those who seek to produce an all-female and/or triploid brood in RT and/or other fish species.

Abbreviations

NORs: Nucleolar organizing regions; RT: Rainbow trout

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Availability of data and materials

All data analyzed during the study are available on reasonable request from the corresponding author.

Authors' contributions

HSK and JHS carried out the collection of the samples and interpreted the results altogether with KHC. HSK, JHS, and KHC designed the experiment. HSK and JHS performed the experiment and prepared the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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