


Triploid Black Sea Trout (*Salmo labrax* Pallas, 1814) Induced by Heat Shock and Evaluation of Triploidy with Different Techniques

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Abstract

Triploid fish production has become important in the aquaculture sector, especially in the culturing and breeding of the big sized Salmonid species. Black Sea trout (*Salmo labrax*) is one of the promising Salmonid species for these purposes considering increased demand for this species in the aquaculture sector. In this research, triploid Black Sea trout were treated with heat shocks during reproduction and triploidy of obtained trouts were identified with different techniques. For this reason, heat shocks were applied at 26.5°C, 28°C and 36°C temperatures for 10 minutes during 30th minute of fertilization and triploidy was determined with fragment analysis, flow cytometry and classic hematological methods. Results indicate that all three temperatures were suitable for the successful reproduction of Black Sea trout. However, 26.5°C gives better results considering both the triploidy success and survival rates. Besides, all three determination techniques especially erythrocyte size measurements and flow cytometry can be used in evaluating of triploidy in Black Sea trout with precise results.

Introduction

Triploidy applications are being used for several economically viable fish species in the world (Maxime, 2008). Induced triploidy is valuable for aquaculture sector (Thorgaard, 1986) and is an effective method for mass production of reproductively sterile salmonids (Benfey, 2001). Triploid fish production is commonly used for quick breeding caused by suppression of gonadal development (Lincoln & Scott, 1984), since gonadal development of fish slows down after a certain size and age (Tiwarly, Kirubakaran, & Ray, 2004). Salmonid production has increased dramatically over the last 30 years (Asche & Bjørndal, 2014) and associated with this growth, triploidy applications have become more commonly used methods in important salmonid species just like Atlantic salmon (*Salmo salar* L., 1758) and rainbow trout (*Oncorhynchus mykiss*

Walbaum, 1792) (Gray, Evans, & Thorgaard, 1993). Besides these species, Black Sea trout (*Salmo labrax* PALLAS, 1814) which is an endemic salmonid species for Eastern Black Sea (Okumuş, Kurtoğlu, & Atasaral, 2004) is culturing widely nowadays and it becomes more popular day by day in turkey (Çakmak, Çankırılıgil, & Özel, 2018; TSI, 2015). In spite of this popularity and increasing usage of triploid applications, only few studies (Akhan, Delihasan Sonay, Okumuş, Köse, & Yandi, 2011; Akhan, Serezli, & Delihasan Sonay, 2011) were carried out about on triploidy of Black Sea trout.

Triploid organisms have been identified by various methods including measuring the red blood cell size (Onozato, Torisawa, & Kusama, 1983; Sezaki, Kobayasi, & Nakamura, 1977), karyotyping (Kim, Nam, & Park, 2005; Kunitake, Nakashima, Mori, & Tanaka, 1998), flow cytometry (Normark, 1996; Lamatsch, Steinlein, Schmid, & Schartl, 2000) and isozyme analyses (Arai & Mukaino,

1998). Although the analysis of the red blood cell size and karyotyping are the most straightforward methods to assess ploidy level, they are time-consuming, labor-intensive and not able to differentiate genotypes/clonal lines. In some fish species, to obtain karyotypes are extremely difficult due to the smaller and similar sizes of different chromosomes, copious numbers of them or the absence of suitable staining techniques. Newly developed analytical tools such as flow cytometry and multiallelic molecular markers (microsatellites) help to determine ploidy levels rapidly in closely related taxa (Besnard et al., 2007). The first studies of polymorphism on microsatellite locus belongs to Tautz (1989) and Weber & May (1989). Usage of microsatellites has become popular due to such features such as easy amplification, high polymorphism, and accuracy of allele characterization. Microsatellites are considered as ideal indicators of several applications in the fisheries and aquaculture research (Dunham, 2011). Flow cytometry has been used to estimate ploidy level by assessing the total DNA content of cells in comparison with a known control (Callis & Hoehn, 1976). This method based on evaluation of signals obtained from suspended cells or particles with inducing laser (Karaboz, Kayar, & Akar, 2008). Flow cytometry can be used to analyze rapidly the DNA content of large numbers of interphase cells with accuracy exceeding that of other quantitative (Callis & Hoehn, 1976; Riccardi, C., & Nicoletti, I. 2006; Doležel, J., Greilhuber, J., & Suda, J. (2007).

In light of such information, the main purpose of this study is to get triploid Black Sea trout induced by heat shock at different temperatures such as 26.5°C, 28°C, 36°C and to determine triploidy of them with contemporary triploidy determination methods.

Material and Methods

Triploid Induction

In trout production, three male and three female individuals having 1824.32 ± 92.07 g mean weight and 55.61 ± 0.90 cm mean length were used as broodstocks. Fertilized eggs of Black Sea trout were (5.73 ± 0.03 mm in diameter) incubated in recirculating aquaculture system (RAS) at 10°C. In the 30th minute of fertilization, fertilized eggs were treated with heat shock at three different temperatures such as 26.5°C, 28°C and 36°C for 10 minutes which are determined in previous studies. In fertilization, approximately 200 g fertilized eggs were used for each replicate group. In addition, one group was separated as control group to not be shocked (diploid) and all four group were evaluated with different approaches. All trials were carried out with three replicates. Besides, survival rate of diploid and triploid trouts were determined according to an equation that suggested by Kritsanapuntu, Chaitanawisuti, & Santaweek (2013):

$$SR: 100 \times (n_1/n_0)$$

(n_1 : Final living fish quantity, n_0 : starting living fish quantity)

Fragment Analysis

Larvae samples were collected (12 from each group) and DNA isolations were carried out from the samples via spin column according to handbook of Qiagen (2016). Quantity and quality of DNA was evaluated by means of spectroscopy (Termo Seintific, ND8000) and agarose gel 1.5% in TBE buffer 1X (composed of: Trizma base 89 mM (Sigma), Boric acid 89 mM (Sigma) and EDTA 2 mM pH = 8 (Sigma)). The obtained DNA samples were loaded to agarose gel electrophoresis in the ratio of loading dye (1ul): DNA (4ul) and density of DNA was specified via spectrophotometer (Termo Seintific, ND8000). Known four locus of trout species (Str543, Bs131, Str58 and Ssa410) were amplified with polymerase chain reaction (PCR) via Termo Seintific, Veriti Termal Cycler. The ingredients of this reaction comprise of 1μl DNA, 0.5μl each primer, 10μl 2x multipleks mastermix and 7μl purified water. Forward primers were colour-labelled at the 5'-end with FAM, VIC or NED. In the following, prepared reaction tubes were transferred to polymerase chain reaction (PCR) device. PCR cycle (30 cycle) was determined as at 95°C for 5 minutes (first denaturation), at 95°C for 30 second and at 60°C for 30 seconds. The elongation was carried out at 72°C for 30 minutes. The obtained PCR outputs were crosschecked via horizontal gel electrophoresis (Max Fill, CHU20 model). Finally, diluted PCR outputs were denatured with LIZ SIZE Standard (0.5 ul) and formamid (9.5 ul) at 95°C for 5 minutes. After that, sizes of alleles were determined for each locus separately in ABI 3500 sequence analyzer. The acquired data were evaluated with GenMapper (ABI) software. Microsatellite characteristics and primers sequences that used shown in Table 1.

Measurements of Erythrocytes

Thereafter, healthy fish larvae were selected as 30 individuals for each groups with 3 replicate (total 270 individuals) and they were kept in RAS system at 12°C with monthly measurements until the completion of possible gonadal development, which is approximately 3 years. In the end of this period, blood samples were taken from the caudal vein of all fish individually considering ethical amendments of ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010). Some of the samples were reserved for blood smear examination, while others were separated for flow cytometry in EDTA tubes. In blood smear examination, blood samples were transferred to microscope slides

and they were stained with Giemsa and May-Grünwald (Blaxhall & Daisley, 1973). Ultimately, obtained sections were examined under the light microscope and some measurements of erythrocytes were done (Benfey, Sutterlin, & Thompson, 1984). In measurements; 50 erythrocytes from each 30 fish individuals were selected and cell length, cell width, nucleus length, nucleus width, cell area and nucleus area of cells were measured. The obtained data were analyzed with one-way Anova procedure by SPSS 21 package program (Zar, 1999).

Flow Cytometry

In flow cytometry analysis, the blood samples stored in EDTA were used. After the one milliliter of the samples was centrifuged (12.000g for 5 minutes), 10^6 cell/ml of the samples were transferred to 1 milliliter staining suspensions containing 0.1% sodium citrate, 0.1% triton and $50\mu\text{g}/\text{ml}^{-1}$ propidium iodide and stored 15 minutes in the dark environment till the analysis. Blood cells of the all individuals which are stimulated with laser were analyzed via flow cytometry (BD accuri™) within 5 minutes. Besides, same procedure was executed for the set up with non-stained samples. Finally, cells selected and gated for the analysis. In the following, DNA content of the blood samples were stained with propidium iodide and fluorescent peaks were specified by using yellow fluorescent filter (FL2). Finally, total cellular DNA amounts of diploid and triploid trouts were compared each other by using fluorescent

peaks (Yu, Abbas, Wang, & Zhou, 2014). Samples were illuminated with laser argon ion at 488nm and detection was done in 650nm via fluorescence detector (FL2). Ultimately, the obtained data was evaluated with Lizis II software (Ritz, Tholozan, Federighi, & Pilet, 2002).

Results and Discussion

According to results, polymorphism was observed in three locus among four known locus. In the trial carried out in 36°C polymorphism was observed in Str543, Bs131, Str58 locus, whereas polymorphism was observed only in Bs131 and Str58 locus in other two groups. No polymorphism was detected in Ssa410 locus. It is clear that the determination of the exact locus that provides best discrimination could not be determined due to limited number of loci that were used. However, among four loci, Bs131 having more polymorphic allele is more convenient for this study. In further, this locus should be re-evaluated with positive and negative samples with the aim of achieve more precise results. Results of the fragment analysis were shown in Table 2. The achieved peak of Bs131 locus in Gene Marker was shown in Figure 1.

A total of 7 alleles (124-126-132-138-140-146-148) were determined for the all groups in the locus of Str543. Polymorphic alleles were not observed in triploid individuals at temperatures of 26°C (3N) and 28°C (3N), whereas in 2 individuals at 36°C (3N) triple structuring was observed. In the BS131 locus, a total of 8 alleles (138-152-144-148-152-156-154-166) were

Table 1. Microsatellite characteristics, structures and primer sequences

Locus	Primer Sequencing	Repeat Motif	Annealing Temp. °C	Allel Size Range (bp)
Strutta-58	F:AACAATGACTTTCTCTGAC R:AAGGACTTGAAGGACGAC	(GT)40	55	135–205
BS131	F:CACATCATGTTACTGCTCC R:CAGCCTAATTCTGAATGAG	(TG)6-(TG)18	50	160–186
Str543INRA	ATT CTT CGG CTT TCT CTT GC ATC TGG TCA GTT TCT TTA TG	(CT) _x	60	100-170
Ssa410Uos	GGA AAA TAA TCA ATG CTG CTG GTT CTA CAA TCT GGA CTA TCT TCT TCA	(CAGA) _x	55	172-320

Table 2. Results of fragment analysis

Locus	26.5°C		28°C		36°C	
	Allele size range (bp)	Polyploid samples	Allele size range (bp)	Polyploid samples	Allele size range (bp)	Polyploid samples
Str543INRA	124-126-140	-	124-126-140	-	124-126-132-138-140-146-148-	2-12
Bs131	138-144-148-152-156-166	9-15-14	138-144-148-152-156-166	1-9-13	138-152-144-148-152-156-154-166-	5-9-11-14-15
Strutta-58	112-114-120-122-124-144-168-172	15	114-120-122-124-132-144-162-166-172-	4	120-122-124-132-144-172-174-176-178-180	16-15-14
Ssa410Uos	235-237	-	235-237	-	235-237	-

determined for the all groups. Polymorphism was determined as 3 for 26°C (3N), 3 for 28°C (3N) and 5 for 36°C (3N). In the Str58 locus, a total of 15 alleles (112-114-120-122-124-132-144-162-166-168-172-174-176-178-180) were determined. In addition, while the one polymorphism was determined for 26°C (3N) and 28°C (3N) groups, three polymorphism was determined for 36°C (3N) group. Finally, in Ssa410 locus, a total of 2 alleles (235-237) were identified and no polymorphic regions were found in this locus (Table 1). Polyploidy determination techniques based on the analysis of microsatellites which are codominant marker are among the most used methods in many studies (Bai, Liu, Li, & Yue, 2011; Christiansen, 2005). Due to the fact that the limited loci were tested in this study, it was not possible to determine the best discriminating locus. Among the 4 loci, Bs131 locus showed better polymorphism results than others. In order to make more concise distinctions in this area, it is necessary to evaluate more loci which have shown polymorphic structures.

Results of measurements of diploid and triploid trout cells were shown in Table 3 and figure 2. According to results, cell lengths were measured as $16.11\pm 0.18\mu\text{m}$, $21.62\pm 0.52\mu\text{m}$, $21.24\pm 0.38\mu\text{m}$, $21.3\pm 0.44\mu\text{m}$; nucleus lengths were measured as $7.33\pm 0.48\mu\text{m}$, $9.54\pm 0.27\mu\text{m}$, $9.75\pm 0.36\mu\text{m}$, $9.63\pm 0.31\mu\text{m}$ for control, 26.5°C, 28°C and 36°C groups, respectively. Besides; cell length, cell width, nucleus length, cell area and nucleus area of cells

were found higher in triploid samples than diploids statistically ($P<0.05$). However, nucleus width was determined as statistically same in all groups ($P>0.05$). Along with these, erythrocytes of triploid trouts were observed as more elliptic compared to diploid ones and number of erythrocytes of diploids was observed as denser in terms of number than triploid ones. Survival of triploid trouts were calculated as $94.75\pm 2.13\%$ for diploid, $88.17\pm 3.75\%$ for 26.5°C, $87.32\pm 1.35\%$ for 28°C and $80.42\pm 2.13\%$ for 36°C. Success of triploidy was calculated as 91% in 26.5°C, 93% in 28°C and 98% in 36°C. According to Thorgaard, Jazwin, & Stier (1981) the best triploidy application in rainbow trout is 36°C for 1 minutes 10 minutes after fertilization considering both survival rate and triploidy success similar to our research. Measurements of diploid and triploid erythrocytes of Black Sea trout under the light microscope was shown in Figure 2.

In flow cytometry analysis, total DNA amounts of diploid and triploid individuals evaluated. According to analysis, total DNA amounts (Mean FL2-A) are determined as 393.3 ± 4.5 , 548.5 ± 4.1 , 553.6 ± 5.6 , 571.30 ± 5.6 for control, 26.5°C, 28°C and 36°C groups respectively, similar with results obtained from erythrocytes measurements. By using this feature, total amount of cellular DNA can be specified easily via flow cytometry in triploid fish species. However, control groups should be known to be triploid and diploid to get accurate results from analyses. In a study based on fish

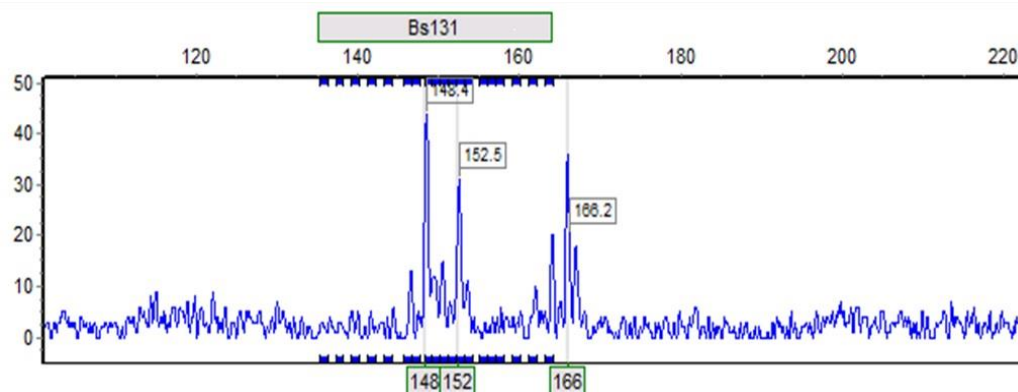


Figure 1. Fragments analyze results of Bs131 locus obtained from triploid trout individuals in Gene Marker.

Table 3. Measurements of diploid and triploid erythrocytes of Black Sea trout

Parameters	Diploid (control)	Triploid (26.5°C)	Triploid (28°C)	Triploid (36°C)
Cell length (μm)	16.11 ± 0.18^b	21.62 ± 0.52^a	21.24 ± 0.38^a	21.30 ± 0.44^a
Cell width (μm)	9.72 ± 0.22^b	11.98 ± 0.35^a	12.07 ± 0.63^a	12.09 ± 0.51^a
Nucleus length (μm)	7.33 ± 0.48^b	9.54 ± 0.27^a	9.75 ± 0.36^a	9.63 ± 0.31^a
Nucleus width (μm)	3.96 ± 0.81^a	3.92 ± 0.17^a	4.11 ± 0.23^a	4.02 ± 0.43^a
Cell area (μm^2)	128.12 ± 3.52^b	188.81 ± 4.24^a	190.38 ± 5.31^a	196.22 ± 5.17^a
Nucleus area (μm^2)	24.69 ± 1.34^b	31.15 ± 1.29^a	31.52 ± 1.51^a	32.61 ± 1.14^a

*Values are expressed as mean \pm SD (n = 1500), mean values in line with different superscripts were significantly different ($P<0.05$).

and shellfish ploidy were described using of flow cytometry in the ploidy determination (Thorgaard et al., 1982; Allen, 1983; Brown, Schultz, & White (2000). According to this study, the mean ratio of trout red blood cell was found as 2.031 ± 0.026 for diploids and 3.051 ± 0.088 for triploid ones. Reconstruction experiments showed that as low as 0.2% diploid cells could have been identified within a triploid majority. In another study, heat shocks were applied to wild rainbow trout eggs for 10 min, starting at 1 and 40 min after fertilization and at temperatures ranging from 24 to 30°C. Analysis of blood samples by flow cytometry revealed triploid induction ranging from 10 to 100% depending on the temperature and timing of the treatment (Solar, Donaldson, & Hunter, 1984). Flow cytometry profiles of cellular DNA content of diploid and triploid Black Sea trout were shown in Figure 3.

Conclusion

In conclusion, all three heat shocks worked on Black Sea trout (*Salmo labrax* Pallas, 1814). However, 36°C heat shock is more successful than others. However, this group has less survival compared to others. Considering survival and triploidy success together, the group of 26.5°C is more convenient than others. Besides, all three determination techniques

especially erythrocyte size measurements and flow cytometry are practicable with showing strong similarities. On the other hand, it is clear that discrimination of diploid and triploid fishes by using flow cytometry is more convenient in terms of accuracy and standardization coupled with reducing measurements errors caused by human factor and also it is much more time saving than blood smears due to the quick usage of cytometer. Despite the results of fragment analysis indicates to polymorphism of alleles that observed in triploid fish, the results are not as clear as the other methods.

In this research, triploid Black Sea trout was achieved by heat shock and triploidy was specified with different methods. Our research will be contributed with further works and these preliminary results can be attractive to researchers that are interested with polyploidy studies.

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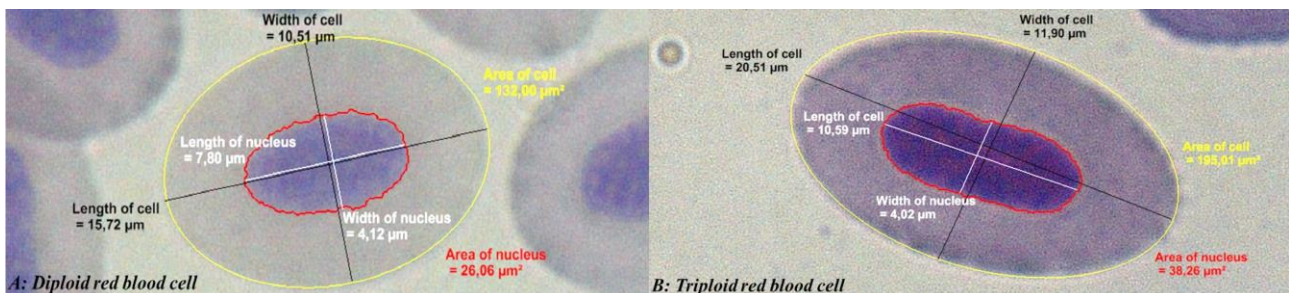


Figure 2. Measurements of diploid and triploid erythrocytes of Black Sea trout under the light microscope via NIS-Elements Advance Research software.

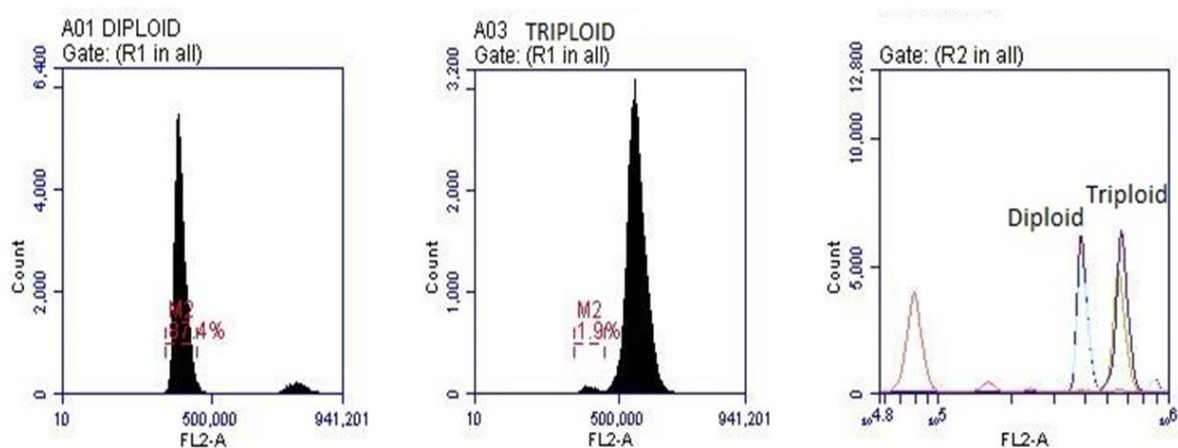


Figure 3. Flow cytometry profiles of cellular DNA content of the diploid and triploid trout individuals.

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