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CHARACTERIZATION OF THE GENETIC STRUCTURE OF THE BROWN TROUT (*SALMO TRUTTA*) FROM "BRADULJICA" FISH FARM, SERBIA

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Abstract: Due to the ecological concerns and preservation of genetic resources, the characterization of genetic structure of the brown trout (*Salmo trutta*) was carried out on the "Braduljica" fish farm. DNA was isolated from fin clips of 10 individuals, and after that molecular PCR-RFLP methods were used for distinguishing between Atlantic and Danubian lineages based on control region of the mitochondrial DNA (CR mtDNA) and lactate dehydrogenase gene of the nuclear DNA (LDH nDNA). Based on phenotypic characteristics it was estimated that out of 10 individuals included in this study five belonged to the allochtonous Atlantic lineage and remaining five belonged to the native Danubian lineage of brown trout. However, results of molecular analyses showed a high percentage of allochthonous genes among the individuals, which confirms the hybridization between these two lineages. Also, the results showed that the selection based on the phenotype is not adequate. In order to continue with proper broodstock management, it is necessary to eliminate allochtonous individuals of the Atlantic lineage from the broodstock.

Key words: *Salmo trutta*, CR mtDNA, LDH nDNA, PCR, RFLP, selection.

Introduction

Brown trout (*Salmo trutta*) is one of the most important and widely distributed freshwater fish, which inhabits the waters of Eurasia and North Africa as an autochthonous salmonid species *(Behnke, 1986; Elliott, 1994*). It inhabits cold waters, usually the upper river sections, although it can also be found in plain rivers and lakes with clean and cold water.

In the last decades, based on genetic analyses, it has been concluded that the trout of a specific geographical area show significant similarities at the genetic level. The presence of five main phylogenetic groups i.e. lineages of the brown trout were determined: the Danubian (Da), the Atlantic (At), the Adriatic (Ad), the Mediterranean (Me) and the Marmoratus (Ma). Statement made by *Bernatchez et al. (1992)* is still relevant: "for the purpose of clarification of the phylogeographical structure of the brown trout populations, the results have not been completed, as some parts of the areal have not been sufficiently examined". One of such insufficiently explored territories is the Balkan Peninsula, which, in addition to the Apennine and the Pyrenees, was of great importance as a refugium during the Ice Ages in Pleistocene *(Hewitt, 1996; Hewitt, 1999).* Ichthyofauna of the Balkan Peninsula is very complex, because each river basin, lake or mountain stream has its own distinctive character *(Banarescu, 2004).* In the area of the Balkan Peninsula, perhaps the largest phenotypic diversity in the brown trout population is present *(Kottelat, 1997).*

Genetic research of the brown trout shows the presence of three phylogeographical lineages on the territory of Serbia: the Danubian (Da), the Adriatic (Ad) and the Atlantic (At) (*Marić et al., 2006*). Haplotypes of Da lineage are autochthonous for the locations of the Black Sea basin, while haplotypes of Ad lineage are autochthonous for the locations of the Adriatic and Aegean basins. However, haplotypes of At lineage are of allochthonous origin. It is assumed that the At lineage was introduced into waters in Serbia through an anthropogenic factor *(Marić et al., 2006)*. According to research by *Bernatchez et al. (1992)* and *Weiss et al. (2001)* the Atlantic lineage is autochthonous only in the upper parts of the Danube River basin.

In Serbia, out of the total area under fish farms (13500-14000 ha), only 0.1% is under the trout fish farms, and the rest is used for common carp (*Cyprinus carpio*) fish farms. Trout fish farms are located south of the Sava and the Danube rivers in the mountainous regions *(Marković et al., 2009).*

Brown trout is farmed in a small percentage for consumption, and more for the fish stocking of open waters. Some of the problems in open water aquaculture are: inadequate introduction of fish into waters (mainly using fish from other basins and allochthonous species), illegal fishing/poaching, destruction of habitats and absence of regular monitoring of fish resources. These processes lead to the extinction of local populations, hybridization and loss of genetic variability within populations *(Ryman et al., 1995; Laikre and Ryman, 1996).*

It is well established that hereditary basis and proper selection are among the most important factors for successful agricultural production. The selection is mainly conducted on the basis of phenotypes and production data for the given animal, but in some animal species, this is not possible. In the case of aquaculture, specifically in breeding of brown trout, the individuals of different lineages are very similar in the phenotypic view, and the hybridization between lineages can

occur. In theory, the brown trout of certain lineage has a characteristic phenotype, however this is not always the rule, so the colour and shape of the body can also depend on the ecological characteristics of aquatic environment in which fish live. In such cases, for the proper selection, a more precise method is needed, i.e. it is necessary to determine genetic structure, because only in this way the phylogenetic lineages can be precisely distinguished.

The aim of this paper is to use such a method for the characterization of the genetic structure of brown trout originating from the "Braduljica" fish farm in the vicinity of Ivanjica, using PCR (Polymerase Chain Reaction) and RFLP (Restriction Fragment Length Polymorphism) methods. In the fish farm, breeding of brown trout has been routinely performed in the last 30 years, exclusively by phenotypic selection, without precise data on the phylogenetic origin of the parent material.

The initial hypothesis tested in this study is whether the phenotype is sufficient and reliable indicator of the origin of brown trout.

Materials and Methods

DNA sampling and isolation

Sampling of fish was done at the trout fish farm "Braduljica", which is located on the river Braduljica in the vicinity of Ivanjica. River Braduljica is the left tributary of the River Studenica, Ibar River basin. Annually, around 200,000 brown trouts are grown/spawned in this fish farm, and subsequently introduced into rivers Moravica, Studenica and the surrounding rivers. There is a mixture of different genotypes of brown trout on the farm because part of the fish are of allochthonous origin (At lineage), and part of the autochthonous (fish collected from nearby streams). Based on the phenotype characteristics, five individuals were considered to be originating from the At lineage (samples marked A1 to A5) and five samples originating from the nearby river and considered to be originating from the Da lineage (samples marked from P1 to P5). All sampled brown trout individuals were three years old and were used as a broodstock. Before sampling, anesthetization of fish with several drops of clove oil was carried out, which was added to a vessel with 10 liters of water. Then, the small piece of the anal fin was cut and placed in 96% ethanol, which is the standard method of tissue sampling for the isolation of the fish DNA, without sacrificing the animal. Sampling was carried out in July 2015, and laboratory processing of DNA samples was done in October of the same year.

DNA isolation was performed using the specialized kit Zymo Research Genomic DNA™ -Tissue MiniPrep (Irwin, USA). Following the DNA extraction,

DNA concentration checks of samples were performed using the nanodrop apparatus Implen P300 (Munich, Germany).

Polymerase Chain Reaction (PCR)

Polymerase chain reaction began with the preparation of PCR Mix, from the Taq PCR Kit produced by Kapa Biosystems (Wilmington, USA) whose ingredients are: 171,5 µl MilliQ H_2O, 25 µl buffer A, 12,5 µl $MgCl_2$, 10 µl dNTP, 10 µl of primer F solution, 10 µl of primer R solution, 1.5 µl Taq polymerase. This amount was enough for 10 analysis. Once the mix was made, the ingredients were evenly mixed on the vortex. Subsequently, 24 µl of PCR mix was added to 1 µl of the eluted DNA sample to a separate volume vessel (200 µl). The amplification of the desired fragments was performed in Eppendorf Mastercycler Nexus GSX1 (Hamburg, Germany) according to the protocol by *Marić et al., (2010).*

Control region of mitochondrial DNA: Step 1: 94°C - 3 min; Step 2: 94°C - 45 sec; Step 3: 54°C - 45 sec; Step 4: 72°C - 1 min 20 sec; Step 5: 72°C - 10 min; Step 6: 10°C - ∞; Steps 2-4 are repeated 32 times.

Lactate dehydrogenase of nuclear DNA gene: Step 1: 94°C - 3 min; Step 2: 94°C - 45 sec; Step 3: 62°C - 45 sec; Step 4: 72°C - 1 min; Step 5: 72°C - 10 min; Step 6: 10°C - ∞; Steps 2-4 are repeated 32 times.

The oligonucleotide sequences used in the amplification of CR mtDNA and LDH nDNA gene are listed in Table 1.

Table 1. Sequences of primers used in PCR method

Primer	Sequence (5'-3')
Ldhxon3F	GGCAGCCTCTTCCTCAAAACGCCCAA
Ldhxon4R	CAACCTGCTCTCTCCCTCCTGCTGACGAA
28RIBa	CACCCTTAACTCCCAAAGCTAAG
cytR	GTGTTATGCTTTAGTTAAGC

The first two oligonucleotide sequences were designed for partial amplification of the nuclear LDH (LDH-C1) gene in the study of *McMeel et al. (2001),* while the other two were used for the amplification of CR mtDNA (*Snoj et al., 2000; Bernatchez and Danzmann 1993).*

Restriction Fragment Length Polymorphism (RFLP)

For the RFLP method, two endonucleases were used:

1. For amplified CR mtDNA, *SatI* (Thermo Fisher Scientific) was used, which specifically cuts the following nucleotide sequence:

5' G C↓N G C 3'
3' C G N↑C G 5'

2. For cutting the LDH gene, *BseLI* (Thermo Fisher Scientific) was used, which specifically cuts the following nucleotide sequence:

5' C C N N N N N↓N N G G 3'
3' G G N N↑N N N N N C C 5'

Both endonucleases were used according to the manufacturer's specification, according to the following protocols:

1. Endonuclease *SatI*: PCR product CR - 10 μL, MilliQ water - 18 μL, 10X G buffer - 2 μL, *SatI* - 2 μL. This mixture was gently stirred (without the use of a vortex) and incubated for 3 hours at 37°C.

2. Endonuclease *BseLI*: PCR product LDH - 10 μL, MilliQ water - 18 μL, 10X Tango buffer - 2 μL, *BseLI* - 2 μL. This mixture was gently stirred (without the use of a vortex) and incubated for 3 hours at 55°C.

RFLP fragments were separated by electrophoresis on agarose gel. A reference marker of 3 kbp was used to determine the length of the RFLP fragments. The voltage on the electrophoresis device was constantly at 120 V. The power supply used was the Serva BluePower 500 and the BlueMarine 200 tub (Heidelberg, Germany). RFLP fragments stained with ethidium bromide were visualized using ultraviolet light on the Vilber Lourmat EBox VX5 (Marin la Vale, France).

Results and Discussion

The *SatI* restriction enzyme cut the amplified CR mtDNA of the At lineage at a specific site C_{434}, while the control region of the Da lineage remained uncut. The mtDNA control region of 1088 bp length of At lineage was cut into two fragments of length 654 and 434 bp. The *BseLI* restriction enzyme cut an amplifed nDNA fragment i.e. LDH gene at the position G_{353} on allele LDH-C1*90, specific for the At lineage. After cutting, the LDH gene of the At lineage was divided into two fragments of 353 and 75bp lengths. Allele LDH-C1*100 of Da lineage remains uncut. The analysis of CR mtDNA and LDH nDNA gene showed that both brown trout lineages were present, as well as their hybrids (Figures 1a, 1b, 2a, and 2b).

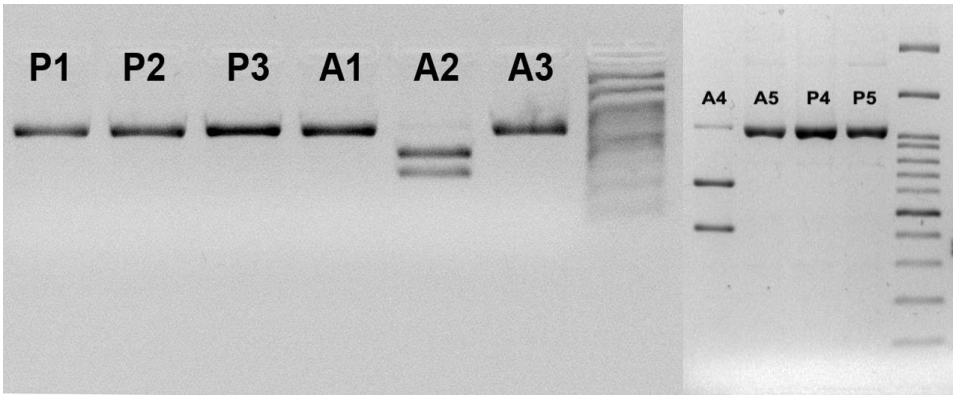

Figure 1. 1a (left) and 1b (right): Restriction of mtDNA control region with *Sat I* enzyme.

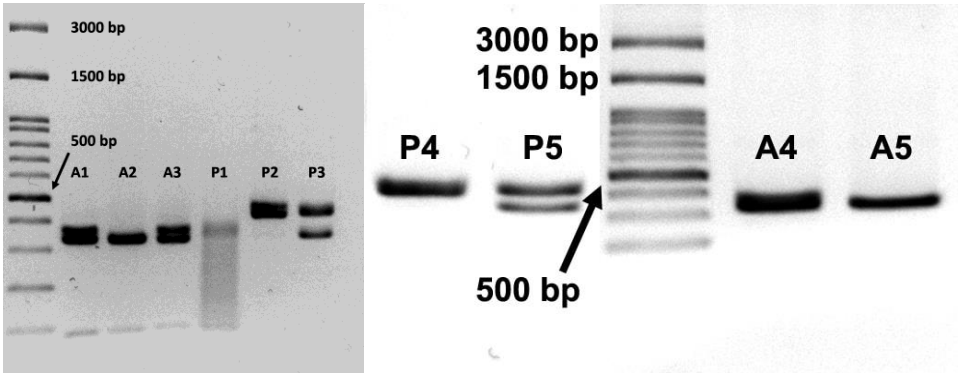

Figure 2. 2a (left) and 2b (right): Restriction of nDNA partial LDH gene with *BseLI* enzyme

The analysis of CR mtDNA showed that only two individuals belonged to the At lineage, both A2 and A4 (Figures 1a and 1b). Since mtDNA is inherited exclusively through the maternal lineage, nuclear LDH gene inherited biparentally was used to obtain more precise results. An analysis of the LDH gene revealed that eight individuals had an allochtonous LDH-C1*90 allele characteristic for the At lineage (A1, A2, A3, A4, A5, P1, P3 and P5) (genotypes LDH-C1*90/90 and LDH-C1*90/100) (Figures 2a and 2b). Six individuals were homozygous for LDH-C1*90 allele, while two individuals (P3 and P5) were heterozygous (LDH-C1*90/100). Four individuals (P2, P3, P4 and P5) had the autochthonous LDH-C1*100/100 allele characteristic for the Da lineage with only two individuals homozygous for LDH-C1*100/100 allele (P2 and P4) (Table 2).

Table 2. Frequency of alleles and genotypes of 10 brown trout speciemens originating from fish farm "Braduljica"

Genetic markers	Number of specimens	Frequency
At mtDNA	2/10	20%
Da mtDNA	8/10	80%
LDH-C1*90	16/20	80%
LDH-C1*100	4/20	20%
Genotypes		
LDH-C1*90/90	6/10	60%
LDH-C1*90/100	2/10	20%
LDH-C1*100/100	2/10	20%

By crossing the At and Da lineages, the F_2 generation can have mtDNA of one lineage and nuclear alleles of the other lineage. The degree of hybridization between the At and the Da lineages can be more accurately determined using multiple genetic markers, but even with the use of mtDNA and a single nuclear marker, it can be concluded that the degree of hybridization is very high in the fish farm "Bladuljica", with only two individuals (P2 and P4) suitable for the broodstock. If these results are compared with the study conducted in the fish farm "Bled" in Slovenia, it is noticeable that the structure is somewhat different, since the LDH-C1*90/90 genotype is predominant in the "Braduljica" fish farm, with a frequency of 60%, while in the "Bled" fish farm, the dominant genotype is hybrid LDH-C1*90/100 (55%) *(Marić et al., 2010).* Similarly, by comparing mtDNA, the Da lineage (80%) is dominant in the fish farm "Braduljica" while in the fish farm "Bled" the percentage of individuals belonging to the autochthonous Da lineage is 57.5% *(Marić et al., 2010).* The result obtained from the "Bled" fish farm can be considered more reliable than ones from this study, because the study was done on a larger sample (40 fish, 20 males and 20 females). Fish stocking of natural aquatic ecosystems is one of the basic conservation activities, which helps in restoration of water ecosystems, however, it must be taken into account which broodstock material will be used. In order for the fish farm "Braduljica" to continue with adequate selection and broodstocking, it is necessary to eliminate individuals of the At lineage from the broodstock.

The hybridization with Atlantic brown trout in the Danube basin was also indicated according to the research by *Kohout et al. (2013)* in the eastern Balkans.

The PCR-RFLP method is often used to determine the population structure of different fish species in freshwater and marine ecosystems around the globe. A study very similar to this one is the discovery of genetic differences between Atlantic and Pacific herring at several locations in the Atlantic and Pacific oceans (Norwegian Sea, Iceland, Barents Sea and near Vancouver; *Shaw et al., 1999*). The PCR-RFLP method was also used for the identification of hybrid species (*Hashimoto et al., 2010*). For example, in South America, two species *Leporinus macrocephalus* and *Leporinus elongatus* can give hybrid offsprings. In the study by

Hashimoto et al. (2010), partial gene sequences of the mitochondrial 16S subunit of ribosomal RNA and for nuclear α-tropomyosin were used. After RFLP analysis of the endonuclease *NsiI*, the 300 bp fragment remains uncut in the *L. elongatus* species, whereas mentioned endonuclease cuts the amplified fragment into two smaller fragments of approximately 190 and 110 bp, in the species *L. macrocephalus* (*Hashimoto et al., 2010*). Hybrid, after restriction, has all three fragments, which confirms that it has inherited one allele from *L. elongatus*, which is not cut by restriction endonuclease and one allele from *L. macrocephal*, which is cut into two fragments. It is interesting that after the restriction of the mitochondrial gene, *L. elongatus* may be distinguished from the species *L. macrocephalus*, but not the specimen created by crossing the hybrid crossbreeding and *L. macrocephalus*, because endonuclease cuts both types so that two fragments of the same length appear. In fish farms in Denmark, the PCR-RFLP method has been used for the genotyping of different brown trout lineages *(Hansen et al., 1997).* Namely, samples were taken from 11 fish farms in which the individuals had already gone through the selection process because there was a suspicion that fish from fish farms that inadvertently escape or are deliberately used to introduce them into river waterways can affect (and disrupt) the genetic structure of wild populations, so that samples from fish farms were compared with wild populations from eight large Danish rivers. The study used 11 different endonucleases that cut mtDNA into two segments, and it was concluded that in all 11 fish farms the populations had suffered a significant loss of genetic variability, compared to wild populations.

The informativeness of the PCR-RFLP method is evident also from the study of *Wolf et al. (2000)* in which 23 fish species were detected.

Conclusion

The initial hypothesis, that the phenotypic selection was reliable and that of the 10 sampled brown trout, five belonged to the Atlantic (At) and five to the Danubian (Da) lineages was rejected. The hypothesis was based on the differences in the phenotypic appearance between these two lineages. Based on genetic analysis, it was found that eight individuals contained allochthonous genes related to the Atlantic lineage. Although it is a small sample, it can be said that the hybridization of these two lineages is very high, which is not adequate and acceptable, because in this fish farm brown trout is bred for the purpose of conservation of genetic resources as well as to introduce fish stock in the surrounding rivers. In this way, the allochthonous genes are spread and the genetic structure of the native Danubian lineage is disturbed.

Eventhough we can distinguish to which lineage one individual/specimen belongs based on phenotype, from the given research it is obvious that it is not

always accurate. In order to improve fisheries and aquaculture in our country, it is necessary to continue research in other fish farms as well as to increase the size of the sample and the number of markers analyzed in order to obtain higher precision of the results. With mtDNA, the use of large number of microsatellite nuclear loci would be very useful because they are very precise markers for determining the genetic structure of the population.

Presently, genetic analysis is increasingly available and can be used in other livestock production. These methods are extremely useful in conserving animal genetic resources and applying adequate selection methods. They could also be used in the detection of the individuals used for breeding/reproduction that carries lethal and semi-lethal genes, which would reduce the losses and increase the economic benefits.

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Određivanje genetičke strukture potočne pastrmke (*Salmo trutta*) iz ribnjaka „Braduljica“, Srbija

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Rezime

U cilju zaštite životne sredine i genetičkih resursa potočne pastrmke (*Salmo trutta*) u radu je ispitivana genetička struktura ove vrste na ribnjaku “Braduljica” u blizini Ivanjice. DNK je izolovana iz odsečaka peraja 10 jedinki, a nakon toga molekularne PCR-RFLP metode su korišćene za razlikovanje između atlantske i dunavske linije na bazi kontrolnog regiona mitohondrijalne DNK (KR mtDNK) i jedarnog gena za laktat dehidrogenazu (LDH nDNK). Na osnovu fenotipskih karakteristika procenjeno je da je pet jedinki pripadalo alohtonoj atlantskoj liniji, a preostalih pet autohtonoj dunavskoj liniji potočne pastrmke. Međutim, rezultati molekularnih analiza pokazali su prisustvo visokog procenta alohtonih gena u analiziranom uzorku, što potvrđuje hibridizaciju između ove dve linije. Takođe, rezultati ukazuju na to da selekcija na osnovu fenotipa nije adekvatna. Kako bi ribnjak nastavio da se bavi gajenjem potočne pastrmke za potrebe poribljavanja okolnih reka, neophodno je da se eliminišu jedinke atlantske linije iz matičnog fonda.

Ključne reči: *Salmo trutta*, KR mtDNK, LDH nDNK, PCR, RFLP, selekcija

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