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LACTATE DEHYDROGENASE GENE GENOTYPIZATION FOR SPECIES IDENTIFICATION IN A FISH FARM ON THE RIVER NERETVA

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SUMMARY

There are severale Salmonid species, found in the river Neretva basin, among which S. trutta and S. obtusirostris. Also, natural hybrids such as S. obtusirostris x S. trutta have been observed. In one fish farm on the river Neretva, S. trutta and S. obtusirostris were decided to breed separately. Parental fishes were separated phenotypicaly on the basis of the morphological signs. PCR-RFLP analysis of the exon 3 to exon 4 part of the lactate dehydrogenase (LDH) C1* gene with restriction endonuclease RsaI was employed to identify the presence of other species representatives or intercrosses in two groups of juvenille fishes. Using this method, we were able to identify two S. trutta representatives in the S. obtusirostris group.

Key-words: Lactate dehydrogenase gene, PCR-RFLP, S. trutta, S. obtusirostris

INTRODUCTION

There are severale Salmonid species, found in the river Neretva basin, among which *S. trutta* (brown trout), *Salmo marmoratus* (marble trout), *Salmo obtusirostris* (softmouth trout) *Salmo farioides* and *Salmo dentex*.

Salmo obtusirostris also known as the Adriatic trout, Adriatic salmon, and softmouth trout, is a species of salmonid fish endemic to the rivers of Western Balkans. It is found naturally in four drainages of the Adriatic Sea, in Croatia, Bosnia and Herzegovina and Montenegro: the Neretva-Vrljika system, the Jadro, the Morača-Zeta system and the Krka river drainages. Morphological different characteristics for different softmouth trout populations from different river-systems resulted in the description of three putative subspecies: *Salmo obtusirostris oxyrhynchus* from the River Neretva, Bosnia and Herzegovina, *Salmo obtusirostris salonitana* from the river Jadro, Croatia, and *Salmo obtusirostris krkensis* from the River Krka, Croatia.

In the river Neretva basin, natural hybrids such as *S. obtusirostris x S. trutta* have been observed and reported (Vuković, 1982). The hybridization between autochthonous species was confirmed also in an experiment performed in the fish farm located in the river Buna, a tributary of the river Neretva (Kosorić and Vuković, 1969). Introduction of non-native brown trout has also been practised in the river Neretva and stocking activities have never been well documented.

Lactate dehydrogenase (LDH) C1* gene containing parts of exons 3 and 4 with intermediate intron has been found, firstly in brown trout (Mc Meel et al, Ferguson, 2001), and after that also in *S. obtusirostris* (Snoj et al., 2002) proven as an informative genetic marker.

A mutation in the intron 3 of the (LDH) C1* gene (G/C substitution) was connected with the disruption of the *Rsa*I restriction enzyme recognition site, being specific for *S. obtusirostris* (Razpet, 2004; Razpet, 2007).

The same mutation was found in *Salmo obtusirostris salonitana and in Salmo obtusirostris oxyrhynchus (Odak, 2004).*

In one fish farm on the river Neretva, *S. trutta* and *S. obtusirostris* were decided to breed separately.

Parental fishes were separated phenotypically on the basis of the morphological signs. The aim of this work was, based on the LDH genotype, to find intercrosses or representatives of the other species in two groups of juvenille fishes. In the first group, there should

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be only representatives of *S. trutta*, while in the second one only representatives of *S. obtusirostris.*

MATERIAL AND METHODS

In the fish farm, juvenille fishes were raised in two groups. In the first group, there were juvenille fishes of *S. trutta*, and in the second juvenille fishes of *S. obtusirostris*. Fin clips were taken from 21 individuals from both groups (together 42 samples) and stored in 96% ethanol. In the lab, genomic DNA was isolated from the preserved fin clips using Bio Basic EZ-10 Spin Column Animal DNA Mini-Preps Kit (Bio Basic Canada Inc.), following the supplier's instructions.

PCR of the lactate dehydrogenase (LDH) C1* gene containing parts of exons 3 and 4 with intermediate intron has been performed as described by Mc Meel et al. (2001). Twenty μl PCR contained 100 ng of genomic DNA, 1,5 mM $MgCl_2$, 0,2 mM of each dNTP, 1 μM of each primer (Ldhxon4R and Ldhxon3F), and 1U of *Taq* DNA polymerase (Fisher scientific). Amplification was performed in the thermal cycler (*Eppendorf*) as described before (Mc Meel et al., 2001).

PCR products were cleaved by *Rsa*I restriction endonuclease, with a recognition site gt/ac. The amplified part of the (LDH) C1* gene in *S. trutta* contained two *Rsa*I recognition sites, leading to three bands after restriction in the lenght of 74, 135 and 166 bp (genotype NP). On the other hand, PCR product of *S. obtusirostris* posessed only one restriction site because of the disruption of the second recognition site due to a G/C transformation (MP genotype), leading to only two bands in the length of 74 and 300 bp after restriction, respectively (Figure 1). Products of restriction reactions were checked on the 2% agarose gel.

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CLUSTAL 2.1 multiple sequence alignment


S.obtusirostris      TGTTACCACGACGATACGAGAGTTCGCCGTCACAGAGTAGTCTGACCGTGGGAGAACAAT 60
S.trutta             TGTTACCACGACGATACGAGAGTTCGCCGTCACAGAGTAGTCTGACCGTGGGAGAACAAT 60
                     ************************************************************

S.obtusirostris      CAATCAATGAGAGTACTGTGTATCATTTGTGTCTAGTATTTCTTCAGTAATTTGTCATAT 120
S.trutta             CAATCAATGAGAGTACTGTGTATCATTTGT--CTAGTATTTCTTCAGTAATTTGTCATAT 118
                     ******************************  ************************** 

S.obtusirostris      CATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTAGGATTTCAGAAATTGCTTT 180
S.trutta             CATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTGGGATTTCAGAAATTGCTTT 178
                     ************************************ ******************** 

S.obtusirostris      GAGAAACTTCATTCATACATTTCCCTTTCACCCTTTTCCCCCCCATCTCCCTTTCATACA 240
S.trutta             GAGAAACTTCATTCATACATTTCCCTTTCACCC------CCCCCATCTCCCTTTCATACA 232
                     ********************************      ********************** 

S.obtusirostris      CTTCCCCTCTCAGAGAGACTACTTCATTTAACACACAGACATTTGACATGCAGATGGTGT 300
S.trutta             CTTCCCCTCTCAGAGAGAGTACTTCATTTAACACACAGACATTTGACATGCAGATGGTGT 292
                     ***************** **************************************** 

S.obtusirostris      CCATCTTGGTTTTCTGGTTTCCTGGTGCCCAATTGCTACACACTCACCTTTGCTGGCGAC 360
S.trutta             CCATCTTGGTTTTCTGGTTTCCTGGTGCCCAATTGCTACACACTCACCTTTGCTGGCGAC 352
                     ************************************************************

S.obtusirostris      TATCTTGGGCGTTTTGAGGAA 381
S.trutta             TATCTTGGGCGTTTTGAGGAA 373
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Figure 1. *Clustal 2.1* alignment of the PCR-amplified part of the (LDH) C1* gene in *S. trutta* and *S. obtusirostris* with underlined *Rsa*I restriction enzyme recognition sites

RESULTS AND DISCUSSION

In 21 fishes analyzed from the first group (*S. trutta*), PCR-RFLP analysis of the exon 3 to exon 4 part of the lactate dehydrogenase (LDH) C1* gene with restriction endonuclease *Rsa*I revealed presence of only NP genotype, specific for *S. trutta*, with two *Rsa*I restriction sites and tree bands on the agarose gel after restriction (Figure 2).

In the second group of juvenille fish, there should be softmouth trutt individuals, with characteristic MP genotype of the lactate dehydrogenase (LDH) C1* gene. However, after PCR amplification and subsecvent restriction of the PCR product with *Rsa*I restriction endonuclease, 19 samples had MP genotype and two ones had NP genotype (Figure 2), being not speciffic for *S. obtusirostris*.

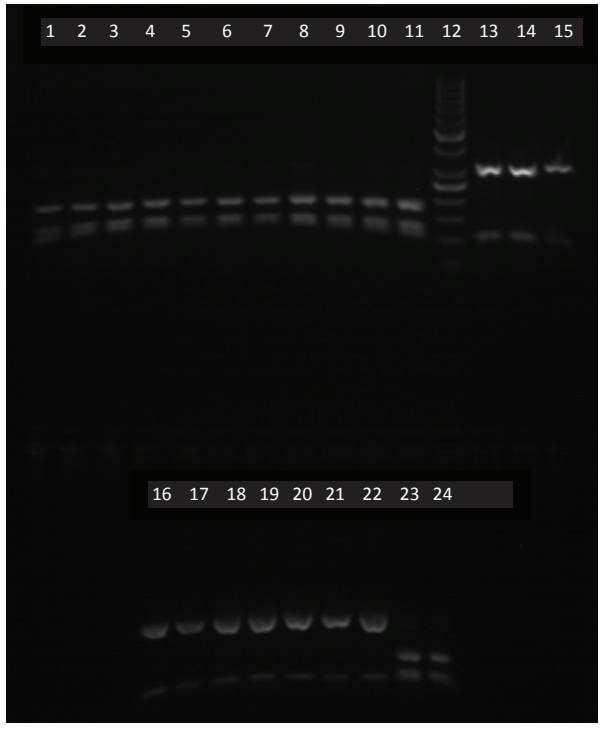

Figure 2. PCR-RFLP results on agarose gel. Samples 1-11 are from the *S.trutta* group and they are all of NP genotype with tree bands after restriction. Samples 13-24 are from the *S.obtusirostris* group. While samples 13-22 posess MP genotype, specific for *S. obtusirostris*, samples 23 and 24 have NP genotype. Sample 12 is 50bp marker

In the both analized groups no intercrosses of MP and NP genotypes were found. The assumption is that the presence of the NP genotype in the group of juvenille softmouth trutt is either consequence of the presence of pure *S. trutta* fish in the *S. obtusirostris* parental group, or juvenille fish were accidentally mixed during handling. In the fish fry morphological signs, characteristic for adult fishes of both species, were not well defined and distinguishing between representatives of both species is difficult. Based on the results, the suggestion was to first devide adult fishes into two groups, based on morphological signs. Further, parental lines of both species should be marked and sampled for genetic analysis for detection of intercrosses or representatives of other species in parental groups. Combination of morphological signs and genetic methods was allready proven to be very powerfull method in distinguishing between *S. marmoratus* and hybrids with alien *Salmo* species in the Soča river basin (Delling et al., 2000).

Disadvantage of the McNeel's method is that it could be used only to elucidate *S. obtusirostris* representatives from other *Salmo* species, found in the river Neretva. While the MP genotype of the (LDH) C1* gene is a characteristic only for *S. obtusirostris*, the NP genotype is not present only in *S. trutta*, but also in *S. marmoratus* and other *Salmo* species, found in the river Neretva basin (Razpet, 2006). Other genetic methods like microsatellite genotyping, mitochondrial DNA analysis and SNP genotyping were described in different phylogenetic studies of *Salmo* species in the river Neretva (Snoj et al., 2002; Razpet et al., 2007; Pustovrh et al., 2014). The described methods could also be used for species identification in fish farms. The problem is that these methods are much more expensive than PCR-RFLP genotyping and therefore they are financially not suitable for the fish farm owners.

CONCLUSIONS

PCR-RFLP analysis of the exon 3 to exon 4 part of the lactate dehydrogenase (LDH) C1* gene was employed to identify the presence of other species representatives or intercrosses in the two groups of salmonides (*S. trutta* and *S. obtusirostris* group), raised in a fish farm on the river Neretva. Using this method, we were able to identify two *S. trutta* representatives in the *S. obtusirostris* group. There is also a great interest of fish farmeres to raise other salmonides from the river Neretva with a help of genetic methods. Due to financial limitation, there is a need for developing the low-cost genetic methods distinguishing between other commercially interesting salmonides from the river Neretva.

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