Acta Veterinaria (Beograd), Vol. 56, No. 5-6, 423-430, 2006.

DOI: 10.2298/AVB0606423M UDK 619:639.21:597.552.512

GENETIC DIFFERENTIATION OF TROUT (*SALMO* SPP.) POPULATIONS IN SERBIA ASCERTAINED USING RFLP TECHNIQUE ON PCR AMPLIFIED CONTROL REGION OF MITOCHONDRIAL DNA

MARIĆ S*, SNOJ A**, NIKOLIĆ VERA* and SIMONOVIĆ P*

**Faculty of Biology, Institute of Zoology, Belgrade*
***Biotechnical Faculty, Department of Animal Science, University of Ljubljana, Domžale, Slovenia*

(Received 11. October 2006)

Genetic variability of trout populations from 13 localities situated in all three sea basins (Black, Aegean and Adriatic) of Serbia was ascertained using the restriction endonuclease Alu I, i.e., by RFLP (Restriction Fagment Length Polymorphism) technique on PCR (Polymerase Chain Reaction) amplified control region of the mtDNA. Restriction endonuclease Alu I cut the control region of the mtDNA at two characteristic profiles featured by populations of trout from the the Black Sea basin and those from basins of Aegean and Adriatic Seas ("southern" populations), respectively. This revealed a strong correlation between the geographic situation and the genetic differentiation of trout populations.

Key words: Salmo, Serbia, PCR, mtDNA, RFLP, genetic differentiation

INTRODUCTION

Brown tout *Salmo trutta* L., 1758 shows very high level of genetic differentiation in its dispersal area (Largiadèr and Scholl, 1995). Analyzes accomplished so far revealed that great part of brown trout's intraspecific variability was lost, whereas the rest of it is strongly jeopardized (Laikre *et al*., 1999). The loss of intraspecific variability comes as an outcome of human activities of three general types: habitat degradation, overfishing and fish stocking (Allendorf, 1988; Laikre and Ryman, 1996).

Estimation of the loss of genetic variability was accomplished using genetic markers: allozymes (Morizot and Schmidt, 1990; May, 1992), microsatellites (Poteaux *et al*., 1999; Hansen *et al*., 2000) and mtDNA. mtDNA is an important genetic marker used for investigations of the genetic structure of populations, reconstruction of phylogenetic relationships between taxa, as well as of migration, introduction rates and speciation (Bernatchez *et al.*, 2001; Weiss *et al*., 2001; Duftner *et al*., 2003; Cortey *et al*., 2004). Bernatchez *et al*. (1992) used sequencing of the mtDNA control region to desribe five phylogenetic lineages of European trout: Mediterranean, Adriatic, Danubian, Atlantic and marmoratus that mainly

correspond to the specific geographic locations where the samples originated from. Since sequencing is both an expensive and complex method, the RFLP (Restriction Fagment Length Polymorphism) analyis of particular regions of mtDNA (Berg and Ferris, 1984; Dovč *et al*., 2004) was often used to ascertain inter- and intraspecific genetic variability of geographically distinct populations that belong either to the same, or to different sea basins.

The territory of Serbia, being an important refuge during the Pleistocene glaciations (Hewitt *et al*., 1999), contains a plenty of phylogeographic information. It is a "hydrographic node" of the Balkans, i.e., it contains the watershed of three great drainages: those of Black, Aegean and Adriatic Sea basins (Gavrilović *et al*., 2002). The hydrographic diversity of Serbia is a derivative of paleogeographic, paleoclimatological and geotectonic events (Stevanović, 1982) that determined the occurrence of separated, locally specific populations of brown trout. Thus, four original brown trout taxa were reported from waters in Serbia: *Salmo labrax* (Pallas, 1814) in river drainages of the Black Sea basin (Janković, 1963; Simonović, 2001), *Salmo macedonicus* (Karaman, 1924) in the Dragovištica River drainage of the Aegean Sea basin (Marić *et al*., 2004), as well as *Salmo marmoratus* (Cuvier, 1829) and *Salmo farioides* (Karaman, 1937) in the Beli Drim River drainage of the Adriatic Sea basin (Šorić, 1990).

The aim of this paper is to ascertain the genetic differentiation between trout populations from drainages of all three sea basins of Serbia using the *Alu I* restriction endonuclease in the PCR and RFLP analysis on the control region of mtDNA and to compare it with their current taxonomic status.

MATERIAL AND METHODS

Samples and DNA isolation

A total of 60 trout individuals from 13 locations across southern Serbia were collected by electrofishing and fly-fishing from 1997 until 2004. Twenty six of these individuals came from 6 sample sites distributed across the tributaries in Serbia feeding the Danubian drainage (Black Sea basin), 24 from five tributaries of the Vardar and Struma Rivers (Dragovištica River drainage) in the Aegean Sea basin and the remaining 10 from two upper stretches of the Beli Drim River drainage of the Adriatic Sea basin (Fig. 1).

Total DNA was isolated from fin clips preserved in 96% ethanol using the Wizard Genomic DNA Purification Kit (Promega).

DNA amplification and Restriction fragment length polymorphism - RFLP

PCR amplification of the entire control region (mtDNA CR) (ca. 1050 bp) was performed using primers 28RIBa (Snoj *et al*., 2000) and HN20 (Bernatchez *et al*., 1993). The conditions for PCR were: initial denaturation (95^{o}C, 3 min) followed by 30 cycles of strand denaturation (94^{o}C, 45 s), primer annealing (52^{o}C, 45 s) and DNA extension (72^{o}C, 2 min). All PCR amplifications were performed in a programmable thermocycler GeneAmp® PCR System 9700 (AB Applied Biosystems). A total PCR volume of 30 ìl was used, containing 1 μM of each

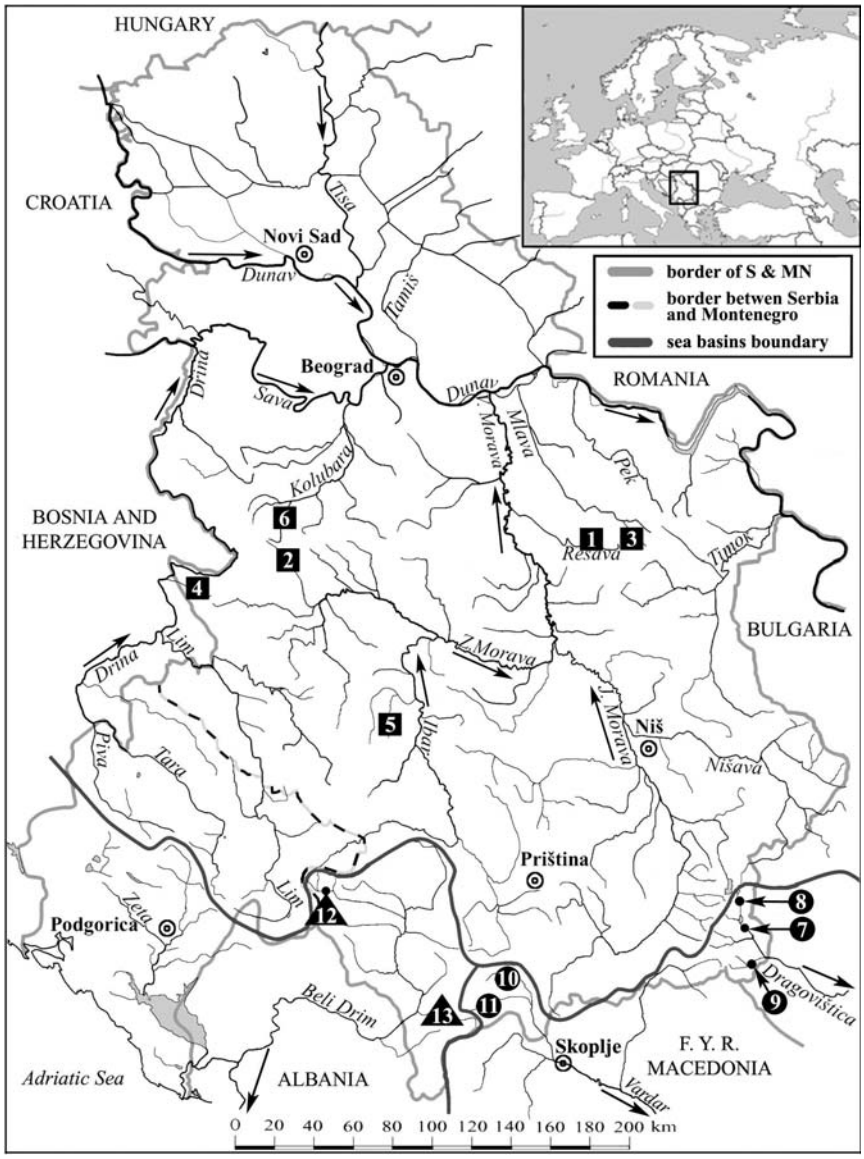

Figure 1. Sampling locations in Serbia (names and sample sizes (N)). Three main drainages are marked (■ Danubian drainage, ● Aegean drainage, ▲ Adriatic drainage).
1. Resava (N=4), 2. Godljevačka River (N=4), 3. Buk (N=5), 4. Crni Stream (N=5), 5. Brevina (N=4), 6. Gradac (N=4), 7. Božica (N=6), 8. Dejanov Stream (N=3), 9. Brankovačka River (N=5), 10. Tisova River (N=5), 11. Čerenačka River (N=5), 12. Pećka Bistrica (N=5), 13. Prizrenska Bistrica (N=5).

primer, 0.2 μM dNTP, 1.5 μM $MgCl_2$, 1 x PCR buffer, 1 U *Taq* polymerase (PE Applied Biosystems) and 100 ng of genomic DNA. To chech the efficacy of amplified DNA, fragments were run on a 1.5% agarose gel.

The amplified segments were subsequently screened for polymorphism with the endonuclease *Alu I*. To a 0.5 mL microcentrifuge tube the following were added: 5 μL PCR product, 2 μL digestion buffer, 0.5 μL (5 U) restriction enzyme (*Alu I*) and 12.5 μL autoclaved distilled water, which totals 20 μL for the restriction reaction. The samples were digested at the appropriate incubation temperature 37°C for 3h. The total restiction reaction was loaded on to 1.5% agarose gel with 0.5 x TBE electrophoresis baffer, stained with ethidium bromide and run 5 min on 80 V, and jet 10 min at 120 V. The gel was observed by UV light (302 nm) and documented photographically. For molecular weight size standard a 1kb ladder (Pharmacia) was used. The exact length of fragments derived from *Alu I* endonuclease use was ascertained from the positions of the characteristic sequence ag/ct on the complete sequence of the mtDNA control region of brown trout available in Gene Bank (Accession No. X93586), with the reference to the characteristic polymorphisms of samples from the Danubian and Adriatic Sea basins (Corty *et al*., 2004).

RESULTS AND DISCUSION

RFLP techique, i.e., Alu I restriction enzyme provided preliminary data on the presence of mtDNA lineages (Bernatchez *et al*., 1992) of brown trout in drainages of all three sea basins in Serbia. The *Alu I* restriction enzyme cut the mtDNA control region at a length of about 1050 bp at the sequence ag/ct on fragments of characteristic lengths for two different restriction profiles. In all samples from drainages of the Black Sea basin, *Alu I* cut the control region on four places and formed five fragments of lengths of 464, 311, 252, 37 and 4 bp. Samples from drainages of the Aegean Sea basin and Adriatic Sea basin, where cut by the same enzyme on three places and formed four fragments of lengths 563, 464, 37 and 4 bp. The fragments 4 i 37 bp long were too small to be visible on gels (Fig. 2). Thus, it provided the efficient discrimination of the Black Sea basin brown trout Salmo labrax from "southern" trout (Salmo macedonicus, Salmo farioides i Salmo marmoratus) populations from drainages of seas (Adriatic and Aegean) that belong to the Mediterranean Sea basin.

In spite of that *Alu I* can also provide efficient discrimination of Salmo marmoratus within the "southern" trout group (Dovč *et al*., 2004), the third profile was not recorded on this occassion due to the lack of samples of *Salmo marmoratus* from the Miruša River, the only habitat reported for Serbia (Šorić, 1990). It is also possible that there is no third profile due to the lack of a close relationship between marble trout of Bosnia and Herzegovina, Montenegro and Serbia on one, and that of the Soča River drainage on the other side. In marble trout from the Neretva, Zeta and Cijevna Rivers no marmoratus mtDNA haplotypes were discovered, opposite to marble trout from the Soča River drainage (Aleš Snoj, pers. comm. – unpublished data). There are yet no relevant data on the mtDNA haplotype of marble trout from the Miruša River, a tributary of

the Beli Drim River of Serbia that is the only reported locality of this species, which is now inaccessible.

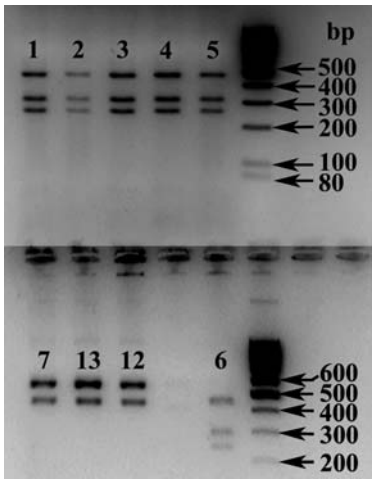

Figure 2. Photograph of RFLP of the control region mtDNA after restriction with the endonuclease *Alu I*. The numbers above profiles represent sampling locations. The marker used is a 1kb

Previous investigations revealed that haplotypes of the Ad lineage occur the in Aegean Sea basin, as well as that they are identical in both Aegean and Adriatic Sea basins, e.g., haplotype Ad1 (Apostolidis *et al*., 1997). That hindered *Alu I* to reveal the occurrence of genetic differentiation between "southern" trout taxa of Aegean (Salmo macedonicus) and Adriatic (Salmo farioides) Sea basins.

The applicative significance of *Alu I* endonuclease use is in the successful detection of aboriginality of trout populations in Serbia, especially considering frequent translocations that occurred mainly on watersheds between particular drainages. The use of RFLP tecnique (*Alu I* endonuclease) is a simple, non-invasive way to ascertain the genetic identity of trout populations. That provides a quick and efficient establishment of a system for biological conservation included into the sustainable fisheries utilization of trout stocks of Serbia. In addition to the conservation at the species level, it is important to conserve the differences in genetic structure occuring within and between local populations. Since in all investigated localities the aboriginal populations of trout occur yet as revealed in this paper, it is possible to restore and conserve the original trout diversity in all drainages. This is to be accomplished by conservation of trout habitats and of the original genetic structure of populations within them. The use of RFLP technique is necessary for the establishment of brood stocks for the production of autochtonous stocking material, when stocking is necessary. However, it should be cautious about mtDNA as a genetic marker regarding its maternal inheritance which disables its use in differentiation of hybrids in populations subject to

introgressions, when the use of more informative nuclear genetic markers is recomended (Ferris and Berg, 1988).

ACKNOWLEDGEMENT:
This work was supported by Ministry of Science and Environment Protection of the Republic of Serbia (Grant No. ON 143040).

Address for correspondence:
Dr Saša Marić
Faculty of Biology, Institute of Zoology,
Studentski trg 3,
11000 Belgrade, Serbia
e-mail: sasa@bf.bio.bg.ac.yu

REFERENCES

1. *Allendorf FW,* 1988, Conservation biology of fishes, *Conserv Biol*, 2, 145-8.
2. *Apostolidis AP, Triantaphyllidis C, Kouvatsi A, Economidis, PS,* 1997, Mitochondrial DNA sequence variation and phylogeography among *Salmo trutta* L (Greek brown trout) populations, *Mol Ecol*, 6, 531-42.
3. *Berg WJ, Ferris SD,* 1984, Restriction endonuclease analysis of salmonid mitochondrial DNA, *Can J Fish Aquat Sci*, 41, 1041-47.
4. *Bernatchez L,* 2001, The evolutionary history of brown trout (*Salmo trutta* L.) interred from phylogenetic, nested clade, and mismatch analyses of mitochondrial DNA variation, *Evolution*, 55, 2, 351-79.
5. *Bernatchez L, Danzmann RG,* 1993, Congrugence in control - region sequence and restriction – site variation in mitochondrial DNA of brook charr (*Salvelinus fontinalis* Mitchill), *Mol Biol Evol*, 10, 1002-14.
6. *Bernatchez L, Guyomard R, Bonhomme F,* 1992, DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations, *Mol Ecol*, 1, 161-73.
7. *Cortey M, Pla C, García-Marín JL,* 2004, Historical biogeography of Mediterranean trout, *Mol Phylogenet Evol*, 33, 831-44.
8. *Cuvier G*, 1829, Le Règne Animal, distribué d'après son organisation, pour servir de base à l'histoire naturelle des animaux et d'introduction à l'anatomie comparée, *Règne Animal*, (ed. 2) i-xv + 1-406.
9. *Dovč P, Sušnik S, Snoj A,* 2004, Expirience from Lipizzan horse and salmonid species endemic to the Adriatic river system Examples for the application of molecular markers for preservation of biodiversity and management of animal genetic resource, *J Biotech*, 113, 43–53.
10. *Duftner N, Weiss S, Medgyesy N, Sturmbauer C,* 2003, Enhanced phylogeographic information about Austrian brown trout populations derived from complete mitochondrial control region secuences, *J Fish Biol*, 62, 427-35.
11. *Ferris SD, Berg WJ,* 1988, The utility of mitochondrial DNA in fish genetics and fishery managment, V: *Population genetics and fishery management*, Seattle, Washington press, 277-99.
12. *Gavrilović LJ, Dukić D,* 2002, Reke Srbije, Zavod za udžbenike i nastavna sredstva, Beograd, 3-10.
13. *Hansen MM, Ruzzante DE, Nielsen EE, Mensberg K-L D,* 2000, Microsatellite and mitochondrial DNA polymorphism reveals life – history dependent interbreeding between hatchery and wild brown trout (*Salmo trutta* L.), *Mol Ecol*, 9, 583-94.
14. *Hewitt GM,* 1999, Post-glacial re-colonization of European biota, *Biol J Linnean Soc*, 68, 87-112.
15. *Janković D,* 1963, Blatnjača iz Plavskog jezera, *Arch Biol Sci Belgrade*, 15, 1-2.
16. *Karaman S*, 1924, Pisces Macedoniae, Derzeit am institut Z. Erforschung und Beksampfung D. Malaria, Trogir (Dalmatien) Split, 1-90.

17. *Karaman S*, 1937, Prilog poznavanju slatkovodnih riba Jugoslavije, *Glasnik Skopskog naučnog društva*, 18, 131-9.
18. *Laikre L, et al.,* 1999, Conservation genetic management of brown trout (*Salmo trutta*) in Europe. Report by the concerted action on identification, management and exploitation of genetic resources in the brown trout (*Salmo trutta*), "TROUTCONCERT"; EU FAIR CT97-3882. Silkeborg, Danmarks fiskeriundersrgelser, 91.
19. *Laikre L, Ryman N,* 1996, Effects on intraspecific biodiversity from harvesting and enhancing natural populations, *Ambio*, 25, 504-9.
20. *Largiadèr CR, Scholl A, 1995,* Effects of stocking on the genetic diversity of brown trout populations of the Adriatic and Danubian drainages in Switzerland, *J Fish Biol*, 47, 209-25
21. *Marić S, Hegediš A, Nikolić V, Simonović P,* 2004, Conservation status of two eastern Balkan endemic fish species in Serbia and proposal for their protection, *Acta Zool Bulg*, 56, 213-22.
22. *May B,* 1992, Starch gel electrophoresis of allozymes, In Hoelzel, A.R. (ed.), Molecular Genetic Analysis of Populations, *A Practical Approach*, IRL Press, Oxford, 1-27.
23. *Morizot DC, Schmidt ME,* 1990, Starch gel electrophoresis and histochemical visualization of proteins, In Whitmore, D.H. (ed.), *Electrophoretic and Isoelectric Focusing Techniques in Fishery Management*, CRC Press, Boca Raton, Ann Arbor, 23-80.
24. *Pallas PS,* 1814, Zoographia rosso-asiatica, sistens omnium animalium in extenso Imperio Rossico et adjacentibus maribus observatorum recensionem, domicilia, mores et descriptiones anatomen atque icones plurimorum. Vol. 3. *Animalia monocardia seu frigidi sanguinis imperii rosso-asiatici recensente,* Supplendis quirusdam ranarum descriptionibus et locupletavit Guil. Theophil. Tilesius. Academia Scientiarum, Petropolis, 422.
25. *Poteaux C, Bonhomme F, Berrebi P,* 1999, Microsatellite polymorphism and genetic impact of restocking in Mediterranean brown trout (*Salmo trutta* L.), *Heredity*, 82, 645-53.
26. *Simonović P,* 2001, Ribe Srbije, NNK Internacional, Zavod za zaštitu prirode Srbije, Biološki fakultet, Beograd, 101-2.
27. *Snoj A, et al.,* 2000, Mitochondrial and mirosatellite DNA analysis of marble trout in Slovenia, *J Fish Biol,* 29, 5-11.
28. *Stevanović MP,* 1982, Istorijska geologija, Rudarsko – Geološki fakultet Univerziteta u Beograda, Beograd, 604.
29. *Šorić V,* 1990, Salmonids in the Ohrid-Drim-Skadar system, *Acta Soc Zool Bohemoslov*, 54, 4, 305-19.
30. *Weiss S, Schlötterer C, Waidbacher H, Jungwirth M,* 2001, Haplotype (mtDNA) diversity of brown trout *Salmo trutta* in tributaries of the Austrian Danube: massive introgresion of Atlantic basin fish – by man or nature, *Mol Ecol*, 10, 1241-6.

UTVRĐIVANJE GENETIČKE DIFERENCIJACIJE POPULACIJA PASTRMKE (*SALMO* SPP.) NA TERITORIJI SRBIJE, UPOTREBOM RFLP TEHNIKE NA PCR AMPLIFIKOVANOM KONTROLNOM REGIONU MITOHONDRIJSKE DNA

MARIĆ S, SNOJ A, NIKOLIĆ VERA i SIMONOVIĆ P

SADRŽAJ

Utvrđivanje genetičke varijabilnosti populacija potočne pastrmke sa 13 lokaliteta iz sva tri sliva (crnomorskog, egejskog i jadranskog) na teritoriji Srbije izvedeno je upotrebom restrikcijske endonukleaze *Alu I*, odnosno RFLP (Restriction

Fagment Length Polymorphism) tehnike na PCR (Polymerase Chain Reaction) amplifikovanom kontrolnom regionu mitohondrijske DNA. Restrikcijska endonukleaza *Alu I* rezala je kontrolni region mtDNA na dva restrikcijska profila, od kojih jedan karakteriše populacije potočne pastrmke crnomorskog sliva, a drugi populacije egejskog i jadranskog sliva ("južne" populacije). Upotrebom endonukleaze *Alu I*, uočena je korelacija između geografskog porekla populacija potočne pastrmke i njihove genetičke diferenciranosti.