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Molecular Markers Reveal Reduced Genetic Diversity in Romanian Populations of Brown Trout, *Salmo trutta* L., 1758 (Salmonidae)

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Abstract: Various technologies along Romanian mountain rivers are destructive and affect aquatic habitats and communities. The salmonids are one of the affected fish families and a decrease in their populations is being reported throughout Europe. In order to mitigate the reduction of numbers and even loss of populations as a result of human impact, frequent restocking activities are being applied. Often these activities are not preceded by any genetic analysis aiding the selection of the needed biological material. Our study aimed to evaluate the genetic diversity of four brown trout populations from stream habitats, which are heavily modified owing to micro-hydropower plants, small dams on the riverbed and permanent water extractions. Based on nine microsatellite loci, the mean values of the Inbreeding Coefficient Index (Fis) showed heterozygote deficit for two populations. The mean number of migrants suggested the existance of a gene flow between three pairs of the analysed brown trout populations, while the factorial correspondence analysis grouped the individuals in three clusters. In conclusion, the genetic diversity of the four brown trout populations, assessed by using nuclear markers, was high for only two of them, with a degree of overlapping between the other two.

Keyords: salmonids, Carpathians, nuclear markers, genetic diversity

Introduction

Worldwide, the representatives of the family Salmonidae, which are often reered in aquaculture systems, have an important market potential because of their high quality meat (Bănăduc *et al.* 2012; FAO 2015; MBIE 2015; USFDA 2015). For instance, in Romania high interest in fish farming of brown trout has been manifested since 1890 (Georgescu *et al.* 2011). On the other hand, a series of studies have reported human activities, such as dams or hydropower plants constructions on some Romanian mountain rivers that could be too destructive for the aquatic and riverine habitats and communities (Bănăduc 1999, 2005; Curtean–Bănăduc *et al.* 2007; Burghelea *et al.* 2013). Although Romania has had an extensive plan for developing national hydropower resources since 1951, with more than 1600 water storages built for this purpose, there are still no official national reports from the authorities concerning their direct or indirect ecological impacts (Dăscălescu, Kleps 2010). River flow changes may occur naturally, but can also be one of the main effects that hydropower plants generate. According to the review by Warren

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et al. (2015), all developing stages of salmonids can be severely affected by low or high flow conditions and unnatural variations.

TroutConcert is an European scientific network that aims to promote the identification, management and exploitation of genetic resources of brown trout. Unfortunately, Romania is not a member of this network and the genetic analyses of Romanian salmonids are still at the beginning. A report of the TroutConcert network highlights the presence of river dams in Finland and Sweden, acid rains in Norway and contamination throughout Europe's waters among the stressors affecting the genetic diversity of the brown trout populations. Additionally, climate change also affects wildlife populations and in order to assess its impacts, various tools are proposed (Wilsey *et al*, 2013).

Re-population of *Salmo trutta* is used to mitigate the reduction of the population size as well as local extinction, frequently with non-native or hybrid individuals from aquaculture.Several studies highlight the consequences of releasing fisheries salmonids into rivers (Rand *et al.* 2012), and one of them, authored by Naman & Sharpe (2012), concludes that predation by hatchery yearling salmonids on wild ones is an important negative aspect that is present in almost all rivers where this kind of activities occur. Quinones *et al.* (2014) found that hatchery salmonids could replace wild ones, at least in California, where the study took place. Salmonidae populations in Romania are included in Natura 2000 network for both marine and alpine sites that are, however, affected by overfishing and deterioration of the habitats by diverse anthropogenic activities (Bănăduc 2001; Zaharia *et al.* 2012).

Maric *et al.* (2012) found Atlantic brown trout instead of representatives of the Danubian lineage in Serbian rivers, explaining their presence with escapes from nearby fish farms. Tosic *et al.* (2014) propose restocking activities in Serbian waters with brown trout individuals that should take place only after detailed genetic analyses in order to avoid non-native individual presence. They also report that some of the so-called Danubian brown trout are actually non-native and their presence is due to hazardous restocking. Horvath *et al.* (2014) reveals that the only brown trout fish farm in Hungary, that is supposed to be involved in restocking with Danubian brown trout has, in fact, Atlantic brown trout, thus the risk of non-specific individuals being present in Hungarian rivers is increased. The same issue can be found in Slovenia, as highlighted by Maric *et al.* (2010).

Hatcheries not only provide the fish market with various types of individuals, lowering the pressure upon wild individuals, but they prove to be helpful for local surrounding communities, as highlighted by Teuşdea & Puşcaş (2012) concerning the potential of hydroenergetic systems that rely on fisheries water catchments. Thus, management measures that deal with the awareness of limited natural resources are one of the most important directions that sustainable fisheries should focus on (Golumbeanu *et al.* 2014).

Studies on the phylogeny of Salmonidae family fish by using mitochondrial 16s and 12s rRNA as well as D-loop molecular markers have been assessed, with samples from various Romanian rivers (Dudu *et al.* 2011; Khalaf *et al.* 2014). Kohout *et al.* (2013) analysed several Romanian brown trout samples from Timiş River (also an Olt River tributary) and reported that they belonged to the Danubian lineage based on molecular marker analysis. Another study that concerned the status of *Salmo trutta* in several Western Romanian Rivers focused on evaluating the meristic characteristics and suggested different ecotypes and the impact of various anthropic activities regarding the brown trouts' populations number (Bud *et al.* 2009). Several other papers that presented Romanian brown trout focused mainly on the role of macroinvertebrates in fish diets (Ureche *et al.* 2010) or on ecological studies for determining the structure and diversity of fish assemblage for the needs of fish management (Bănăduc & Curtean-Bănăduc, 2013).

Given this complex context, our study aims to analyse the genetic diversity of some Romanian brown trout populations by using nuclear molecular markers and data from a previous study that involved the D-loop mitochondrial region (Popa *et al.* 2013).

Material and Methods

Sampling sites

The samples, represented by small anal fin fragments, were collected from 118 wild individuals of *Salmo trutta* from four streams in the Northern side of Făgăraş Mountains (Romanian Carpathians), tributaries of Olt River (Fig. 1) in the Danube River Basin: Ucea (31 individuals), Cârţişoara (26 individuals), Porumbacu (29 individuals) and Avrig (32 individuals). These habitats are known to be modified due to the micro hydropower plants, small dams on the riverbed and permanent water extractions. The samples were grouped in four populations, depending on the rivers where they were collected from, N1

corresponding to Ucea River, N2 – Cârţişoara River, N3 – Porumbacu River and N4 – Avrig River.

DNA extraction and amplification

The DNA extraction was done using a phenol-chloroform protocol (Taggart *et al.* 1991), followed by amplification of nine microsatellite loci (Table 1; the sequences of the primers are the ones presented on the TroutConcert website).

Initially, we optimised the PCR reaction conditions by varying the annealing temperature between 50 and 62°C (Table 1). The PCR program that we used for amplification was as follows: denaturation at 95°C for 10 minutes, 35 cycles of: denaturation at 95°C for 30 seconds, annealing at 50-62°C for 30 seconds and extension at 72°C for one minute, with final extension at 72°C for 20 minutes.

The PCR reactions were done using GeneAmp 9700 PCR System (Applied Biosystems) in a final volume of 25 µl with 1X PCR Buffer, 1.5 mM of $MgCl_2$, 0.8 mM of dNTPs, two pmol/µl of each primer, one unit of AmpliTaq Gold DNA polymerase, nuclease free water and 50 ng of DNA template. The nine pairs of primers were grouped based on their optimal annealing temperature in: two 3-Plex reactions (for Str73, Str15 and Str60, and for OmyFgt1, Ssa197 and Ssa85), one 2-Plex (for Strutta12 and Str543) and one monoplex (BS131). After the PCR reactions, for each sample, one µl of the amplicon, along with 0.5 µl Gene-Scan500 LIZ Size Standard (Applied Biosystems) and 12.5 µl formamide (Applied Biosystems) were mixed, denatured and loaded in the ABI Prism 310 Genetic Analyzer.

Data analysis

The resulted genetic profiles were analysed with: GeneMapper (Applied Biosystems) in order to visualise the genetic profile for each individual; GENETIX (Belkhir *et al.* 2004) was used for determining the mean values of observed (H_O) and expected (H_E) heterozygosity; gene flow (Nm – number of migrants parameter; see Wright 1969) and factorial correspondence analysis (FCA), in order to determine the relationship between the four analysed populations and to identify clusters of individuals with similar genotypes: FSTAT (Goudet 1995) was used for determining the allelic richness values; GENEPOP (Rousset 2008) was used for determining the Rho(st) statistical index (Slatkin 1995) following standard ANOVA analogous to Weir & Cockerham (1984), and Arlequin (Excoffier & Lischer 2010) was used for determining the Fis and Fst statistical indices (Weir & Cockerham 1984), along with their statistical significance (p-value) following the implemented permutation method (10100 permutations). In order to infer the presence of distinct populations, the assignment of individuals to populations and migrants' identification was done in Structure (Pritchard *et al.* 2000) with a burn-in value of 50.000, 100.000 repetitions, 100 iterations, and a K interval of 1-10. The maximum number of clusters K was chosen as the K with the highest L(K) and DK, as described by Evanno *et al.* (2005) with Structure Harvester (Earl & Vonholdt 2014).

Results

We used Fst and Rho(st) statistical indices in order to evaluate the genetic diversity of the four populations (Table 2). The obtained matrix showed that the highest statistically significant genetic differentiation was obtained for the population pair N3 and N1 (Fst = 0.34566, $p < 0.05$; Rho(st) = 0.3737), while the lowest value was obtained for the N3 and N4 populations (Fst = 0.08921, $p < 0.05$; Rho(st) = 0.0176).

The Fis, also known as inbreeding coefficient, showed mean positive values, which were statistically significant only for N2 (Fis = 0.24255, $p < 0.05$) and N3 (Fis = 0.07936, $p < 0.05$) groups (Table 3), suggesting a heterozygote deficit for these two popu-

Table 1. The characteristics of the primers used for the amplification of nine microsatellite loci from *Salmo trutta*

Name	Repetitive motifs	Estimated product size (bp)	Hybridization temperature
Str73	GT	138-162	57ºC
Str15	CT	193-225	
Str60	GT	87-111	
Omy Fgt1	GT	187-263	54ºC
Ssa197	GTCA(+GT)	107-177	
Ssa85	CT	104-120	
Strutta12	GT	124-216	57 ºC
Str543	CT	119-169	
BS131	GT	149-177	50 ºC

lations. The number of migrants parameter (Nm) was the highest for the pairs of populations N2 and N3 (Nm = 2.1), N2 and N4 (Nm = 1.73) and N3 and N4 (Nm = 2.72), while the lowest values where obtained when the N1 populations was part of the pairing (Table 4).

By analysing the allelic richness parameter (AR) per locus and per populations, we obtained the values presented in Table 5, while the mean values for observed (H_O) and expected (H_E) heterozygosity are presented in Table 6. The maximum AR value was 19.321, found for Strutta12, followed by the OmyFGT1 locus. The minimum value, AR = 1, was obtained for Str15 (in N3 population), Ssa85 (in N2 and N3 populations) and Str73 in all the four analysed populations.

Table 2. The values of the Fst (above) and Rho(st) (below) statistical indices; *p < 0.05

Population	N1	N2	N3	N4
N1	-	0.3037 *	0.3456 *	0.326 *
N2	0.2946	-	0.1086 *	0.1338 *
N3	0.3737	0.1156	-	0.0892 *
N4	0.2398	0.065	0.0176	-

Table 3. The values of the mean Fis values, grouped on the four populations; * p<0.05

Population	FIS	P (Rand FIS>=Obs FIS)
N1	0.0173	0.436238
N2	0.24255*	0
N3	0.07936*	0.023564
N4	0.05083	0.091386

Table 4. The values for the Nm (number of migrants) parameter

Population	N2	N3	N4
N1	0.60	0.49	0.55
N2	-	2.1	1.73
N3	-	-	2.72

In order to determine the relationship between the four analysed populations and to identify clusters of individuals with similar genotypes, we performed a factorial correspondence analysis (FCA) using GENETIX (Fig. 2) along with a bar plot representation of the populations following the method implemented in Structure software (Fig. 4). The results of the bar plot representation, for the highest delta K determined with Structure Harvester showed a value of 130.375590 corresponding to K = 3, while the second highest value was 64.549002 corresponding to K = 2 (Fig. 3). Both graphical displays suggested that all individuals from the four ecologically defined populations can be genetically grouped in three different populations, with the N3 and N4 populations overlapping.

Discussion

By using nuclear markers we managed to assess the genetic diversity considering the Fst, Rho(st) and Fis statistical indices and the Nm parameter. The pairings that involved the N1 population resulted in Fst > 0.3, values that suggest a very high degree of genetic differentiation considering the scale proposed by Wright (1978). However, the pairings that involved N2, N3 and N4 gave lower Fst values, which correlated with the correspondent Nm parameter values higher than 1 and could suggest the existent of migrants between these three populations (Frankham *et al.* 2010). Finally, the Fis index suggested that the N2 and N3 population have a statistically significant heterozygote deficit.

The maximum AR value of 19.321, found for Strutta12, suggested that this locus had the highest polymorphism degree. Also, the AR value showed that Str73 was a fixed allele. If this result is confirmed by further studies on other populations of brown trout from Romania, Str73 might be considered a useful locus for molecular diagnostic of Romanian *S. trutta*. Consequently, we observed

Table 5. The values for the allelic richness parameter

Locus	Population N1	Population N2	Population N3	Population N4	All (mean)
Str60	1.839	2	1.999	2.999	**3.199**
Str15	2	2	1	1.995	**2.727**
Str73	1	1	1	1	**1**
OmyFGT1	6.673	18	12.463	14.888	**19.151**
Ssa85	2.997	1	1	8.024	**5.376**
Ssa197	5.814	8	13.465	15.330	**14.479**
Str543	3.677	4	4.887	5.928	**6.280**
Strutta12	6.513	13	16.351	13.792	**19.321**
BS131	1.976	8	5.897	7.560	**7.199**

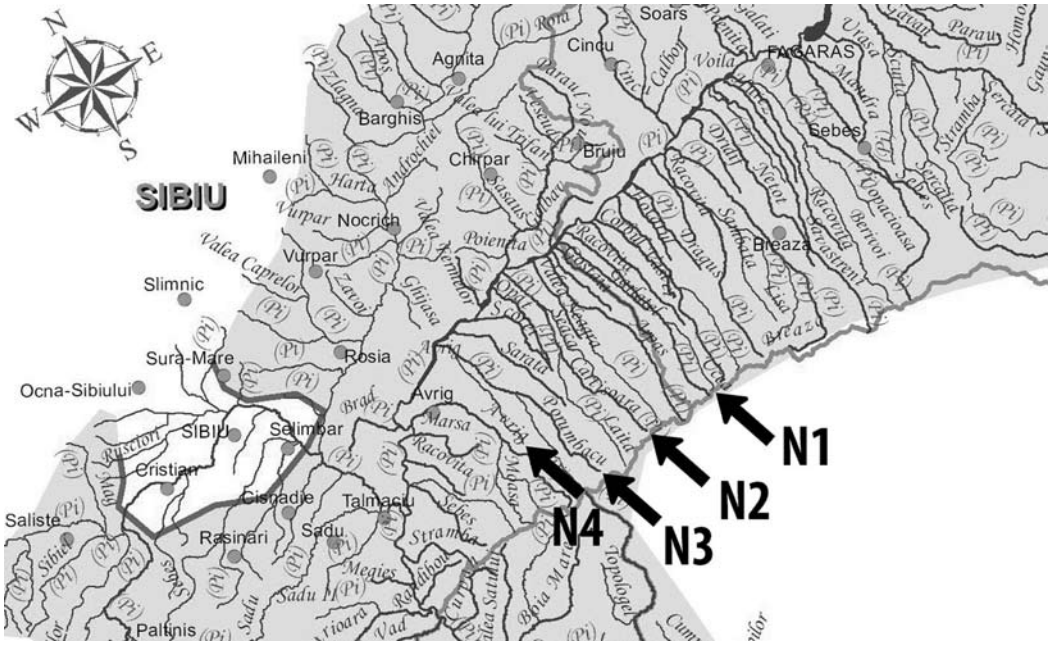

Fig. 1. The sampling locations of the four brown trout populations; map edited from Georgescu *et al.* (2011)

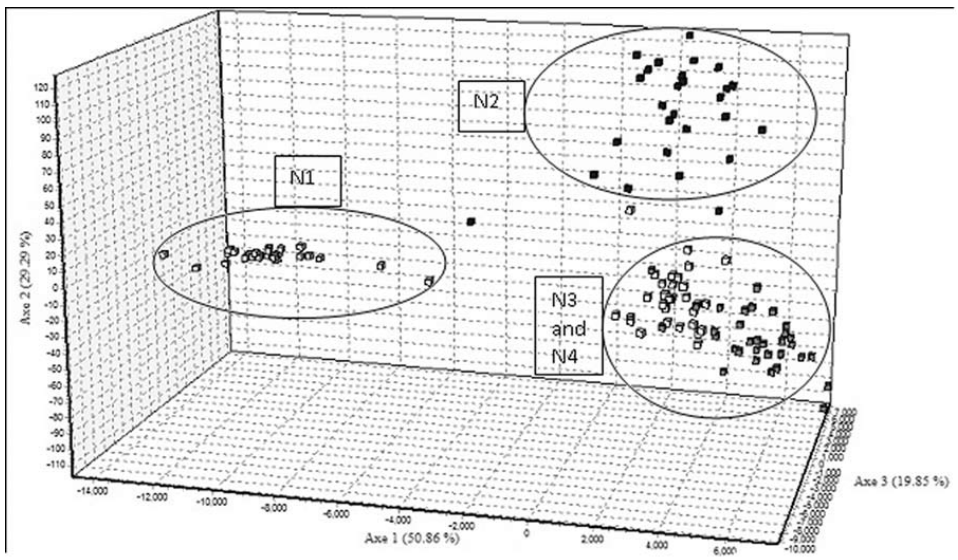

Fig. 2. FCA for the four populations. N1, N2, N3 and N4 – the *S. trutta* populations, each cube represents individuals and the circles represent the grouping resulted from the analysis

that the N3 and N4 populations presented a degree of overlapping, suggesting that some individuals from these populations were genetically similar and the presence of two distinct and non-overlapping groups (N1 and N2). Given the fact that the N4 population was from the Avrig River tributary of the corresponding river for N3 population (the Porumbacu River), we could explain the graphical displays of the genetic clusters with the presence of some lotic sectors connected by a lotic continuum. The individuals from the studied populations were previously analysed based on the mitochondrial control region sequence in order to determine their affiliation to the Danubian lineage of brown trout, as expected for Romania, and our results showed that all the individuals belong to the Danubian lineage (Popa *et al.* 2013). Since there are no official reports regarding restocking activities in the studied area, we might consider that either there were no such activities on the four analysed rivers, either the activities were done with individuals that were from neighbouring areas. Still, the N2 and N3 heterozygote deficit could suggest some degree of physical isolation of the two populations.

The problem of fish migration was reported from Norway owing to a conflict between their national management scheme for *Salmo salar* and private projects for micro hydropower plant constructions and other related exploitative activities. Fortunately, the anthropic influence was limited by promoting a new acceptance system for the water exploitation plans (Vollestad *et al.* 2014). According to the new regulation system the applicants for new construction sites should manage environmental studies in their targeted area in order to evaluate the real consequences of their proposed project, thus reducing the number of those projects that were considered too menacing for the environment. The same fish migration problem in areas with dams and other types of barriers exist in the Romanian riverbeds too and were addressed in various scientific articles, one of them emphasising on the need for considerable investment in developing strategies that include studies of fish behaviour when regarding river dams (Luca 2011). The author presented the need of regulations for monitoring the fish ladders proposed as solution for facilitating fish migration and the state of the dams before and after 1990. This proposed monitoring scheme should have a genetic variability-monitoring dimension too.

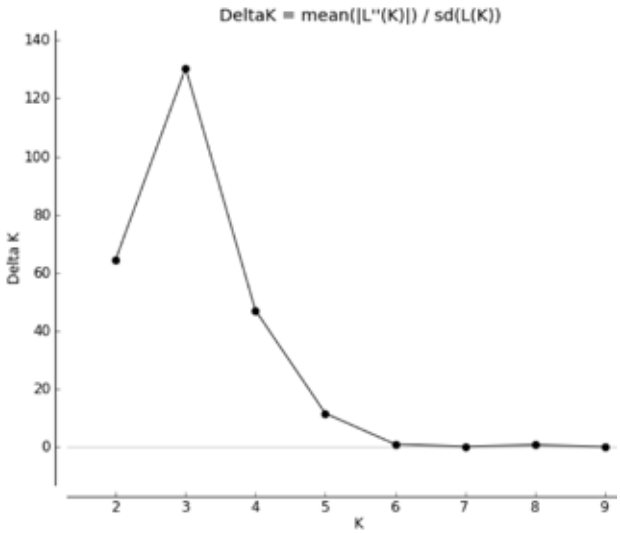

Fig. 3. The Delta K values along with the corresponding K (number of clusters)

Table 6. Mean values for the observed (Ho) and expected heterozygosity (He) for different populations

Population	H_e	H_o
N1	0.3294	0.3226
N2	0.4987	0.3889
N3	0.4493	0.4215
N4	0.5649	0.5451

Conclusions

Based on molecular marker assessments of the genetic diversity of brown trout, we can conclude that there was a high degree of genetic differentiation between the analysed populations, especially when comparing the Ucea River brown trout population (N1) to the other three rivers. In addition, we found two populations that presented inbreeding, namely the Cârțișoara River (N2) and Porumbacu River (N3) populations. Further studies on other populations of brown trout from Romania will be designed, Str73 might be considered a useful locus for molecular diagnostic of Romanian *S. trutta.*

The increasing river fragmentation (due to water abstraction, dams and micro-hydropower plants) of these four Olt River tributaries can reduce in the future the number of individuals and their genetic variability. Ongoing transformation of their collector, the Olt River, in a series of anthropogenic lakes with high dams and no fish ladders creates an important series of barriers which stops the fish individuals switch between its tributaries, which also

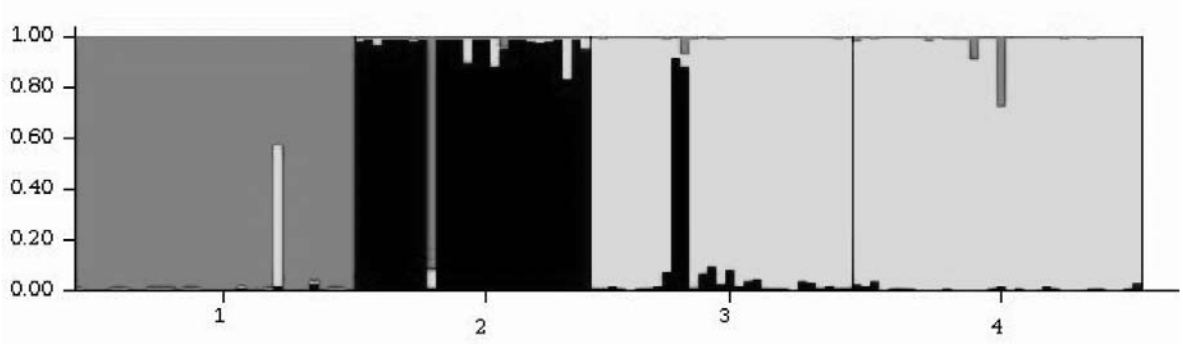

Fig. 4. The clusters resulted from Structure analysis of the four populations. Each bar corresponds to each individual and K = 3. 1, 2, 3, 4 – populations N1, N2, N3 and N4

will increase this populations genetic isolation. The results highlight the need of similar studies and encourage the extension of this type of research at the Carpathian level.

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