The Genetic Profiles of two Salmonid Populations from Romania Obtained through Nuclear Markers Analysis

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Abstract
The *Salmonidae* fish family is well represented in Romanian fauna, with a total of six species in the wild and reared in fish farms. Among them, the brown trout (*Salmo trutta fario*) can be found in all major Romanian river basins. However, anthropogenic activities might disrupt salmonids' habitats, so that inbreeding and genetic isolation might easily occur in the wild populations. We analyzed two wild brown trout populations from rivers targeted by anthropogenic activities, by using nuclear markers and genotyping in order to observe their genetic structure. We analyzed nine microsatellites and we observed their alleles frequencies, number of private alleles, observed and expected heterozygosity, as well as their population structure. The two populations are not in Hardy-Weinberg equilibrium for most of the loci and the inbreeding coefficient for both populations suggests a heterozygote deficit. Further sequencing data are needed in order to have a better view upon their complete genetic structure

Keywords: salmonids, wild populations, microsatellites, genotyping.

1. Introduction

Salmonids are fish species with economic, ecologic and cultural importance, natively distributed in the Northern Hemisphere, especially in Europe and North Africa and they remark by their long-distance migration skills [1-3]. *Salmo trutta fario* (brown trout), member of the *Salmonidae* fish family, prefers the habitats with clean and cold water, being one of the six salmonid species found in Romania, along with *S. labrax, Salvelinus fontinalis, Hucho hucho, Thymallus thymallus* and *Onchorynchus mykiss* [2]. During Pleistocene (18,000 years ago) many salmonid populations have become extinct, but the Danubian River has not been affected, therefore its watercourse has been populated since then [4]. Unfortunately, Romania's main rivers, along with their main tributaries, are targeted by anthropogenic activities like overfishing, hydroelectric plants or dams, activities that have negative effects on the life cycle of *Salmo trutta fario* [1, 5]. Thus, native brown trout populations are rare and consist of a small number of individuals [6, 7].
The aim of this study was to determine the genetic diversity of two brown trout populations from two Romanian rivers (Topolog and Sebeșel) by using nuclear markers

2. Materials and methods

a. Sampling
Fin clips were collected from 58 wild individuals of *Salmo trutta fario.* 28 individuals (S5 population) were sampled from Toplog River, located in the Southern side of Făgăraș Mountains,

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tributary of Olt River and 30 individuals (Sb population) were sampled from Sebeşel River, located in the Western side of Meridionali Carpathian Mountains, tributary of Sebeş River, both in the Danube River Basin. Both river basins are known to be modified due to the micro hydropower plants, small dams on the riverbed and permanent water extractions [8, 9, 10]. The samples were deposited in 96% ethanol until DNA extraction.

b. DNA extraction and amplification

The DNA extraction was done using the phenol-chloroform protocol [11], followed by multiplex PCR of nine microsatellite loci: Str60, Str73, Str15, Ssa197, OmyFGT1, Ssa85, Strutta12, Str543, BS131 [12]. The amplification program that we used for amplification was: denaturation at 95°C for 2 minutes, 35 cycles of: denaturation at 95°C for 30 seconds, annealing at 50-57°C for 30 seconds (depending on the primers pairs) and extension at 72°C for one minute, withfinal extension at 72°C for 60 minutes. The PCR reactions were done using a Verity PCR System (Applied Biosystems) in a final volume of 25 µl with 1X PCR Buffer, 1.5 mM of MgCl2, 0.4 mM of dNTPs, six pmol of each primer, 0.5 units of AmpliTaq 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 (for BS131). After amplification, the PCR products were mixed with Gene-Scan500 LIZ Size Standard (Applied Biosystems) and formamide, denatured and then loaded in the ABI Prism 310 Genetic Analyzer.

c. Data analysis

The resulted genetic profiles were visualised with GeneMapper (Applied Biosystems) and analysed with GeneAlex [13] for determining the values of observed (Ho) and expected (He) heterozygosity, the number of private alleles and the Hardy-Weinberg equilibrium. GENETIX [14] was used for factorial correspondence analysis (FCA), in order to determine the relationship between the two analysed populations and to identify clusters of individuals with similar genotypes. Structure [15] was used to infer the presence of distinct populations using a Bayesian approach, the assignment of individuals to populations and migrants' identification, with a burn-in value of 50.000, 100.000 repetitions, 100 iterations, and a K interval of 1-5. The maximum number of clusters was chosen as the K with the highest L(K) and DK, as described [16] with Structure Harvester [17].

3. Results and discussion

For the nine microsatellites, the values for the number of alleles varied from 3 (for the Str60 locus in population S5, and for the Str15 locus for the Sb population) to 20 (for the OmyFGT1 locus in population S5), with a mean of 9.556 (±2.082) and 7.333 (±1.509), respectively (Table 1). These results show that three loci have the highest polymorphic degree (OmyFGT1, Sssa197 and Strutta 12) in the S5 population, while for the Sb population, only two loci (OmyFGT1 and Strutta12) showed high polymorphic degree.

The values for the observed (Ho) and expected heterozygosity (He) were the lowest for the Str60 locus in the population S5 and the highest for the same population, but for the locus OmyFGT1; the two populations deviate from the Hardy-Weinberg equilibrium for the majority of the loci (table 1).

Concerning the number of private alleles, for the S5 population we identified 4 private alleles, while for the Sb population only 2. The mean value for the inbreeding coefficient index (Fis) was 0.14276 (95% CI 0.04731 - 0.19229) for the S5 population and 0.40145 (95% CI 0.30784 - 0.46337), both statistically significant, suggesting that there is a heterozygote deficit in both brown trout populations. These results are in concordance with a previous study focused on the Făgăraș Mountains area [18].

The FCA analysis revealed two different clusters, one for each sampled population, with non-overlapping members suggesting that the two populations are genetically distinct (Figure 1). However, the population assignment based on the Bayesian approach, with a D(K) value of 9.236 for K = 2, showed that six individuals from the Sb population share similar genetic structure with the S5 population (Figure 2). This might be a result of previous restocking activities with related individuals or a reminiscence of old populations in the Carpathian area.

Table 1. Summary statistics for genetic variation at the nine surveyed microsatellite loci. ns=not significant, * P < 0.05, ** P < 0.01, *** P < 0.001

Population	Locus	N	Na	Ho	He	HWE (Significance)
S5	Str60	28	3.000	0.107	0.103	ns
	Str15		4.000	0.214	0.510	***
	Str73		6.000	0.429	0.598	***
	OmyFGT1		20.000	0.964	0.909	*
	Ssa85		6.000	0.214	0.545	***
	Ssa197		14.000	0.964	0.823	ns
	Str543		6.000	0.643	0.595	ns
	Strutta12		18.000	0.750	0.923	*
	BS131		9.000	0.750	0.749	ns
	Mean (±SE)		9.556 (±2.082)	0.560 (±0.110)	0.639 (±0.085)	
Sb	Str60	30	4.000	0.200	0.242	ns
	Str15		3.000	0.033	0.415	***
	Str73		4.000	0.467	0.518	**
	OmyFGT1		15.000	0.600	0.888	***
	Ssa85		8.000	0.133	0.399	***
	Ssa197		9.000	0.567	0.787	*
	Str543		4.000	0.467	0.682	*
	Strutta12		14.000	0.500	0.886	***
	BS131		5.000	0.172	0.306	***
	Mean (±SE)		7.333 (±1.509)	0.349 (±0.071)	0.569 (±0.083)	

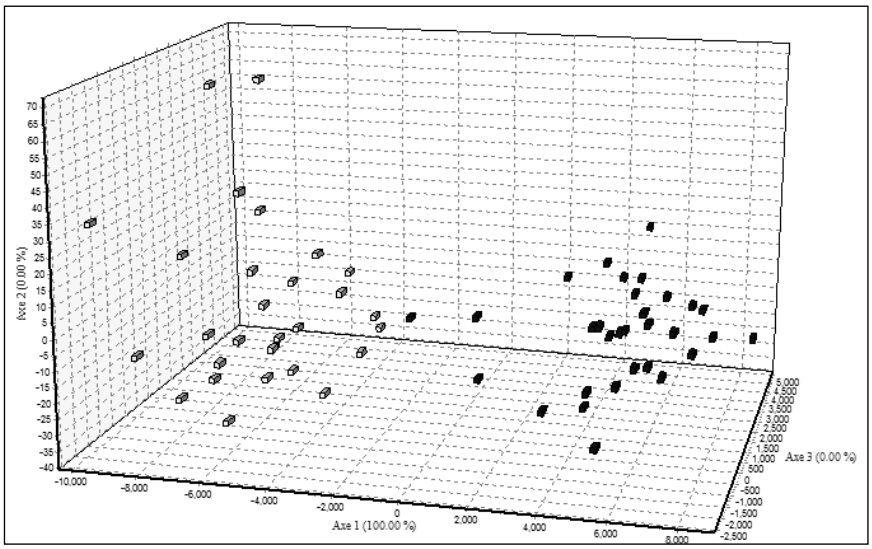
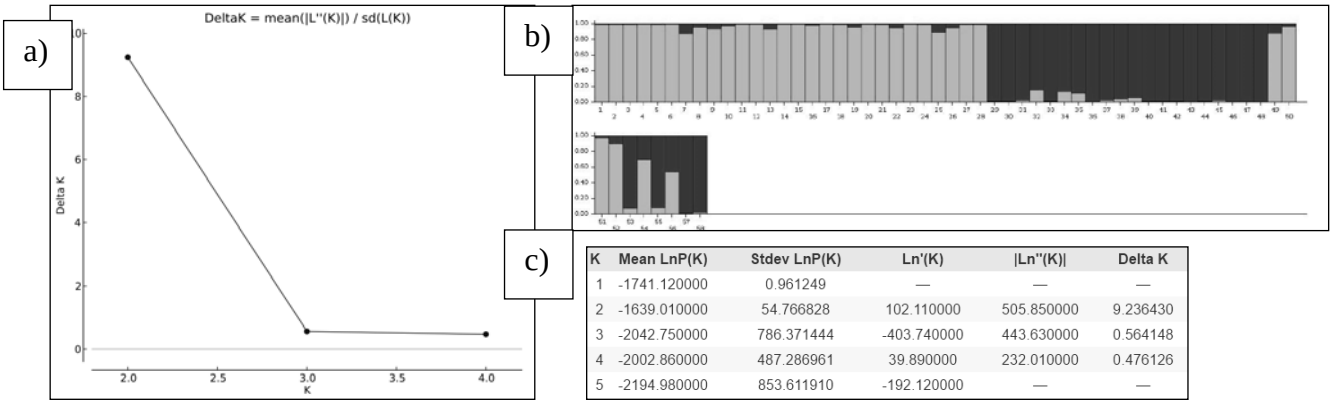


Figure 1. FCA analysis of two brown trout populations. The white cubes depict the members of the S5 (Topolog) population, while the black ones depict the Sb (Sebeșel) population members.



K	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	-1741.120000	0.961249	—	—	—
2	-1639.010000	54.766828	102.110000	505.850000	9.236430
3	-2042.750000	786.371444	-403.740000	443.630000	0.564148
4	-2002.860000	487.286961	39.890000	232.010000	0.476126
5	-2194.980000	853.611910	-192.120000	—	—

Figure 2. Delta K values along with the corresponding K (number of clusters) - a) and c); clusters resulted from Structure analysis of the two populations - b) (each bar corresponds to each individual, 1-28: members of S5 population, 29-58: members of Sb population).

4. Conclusions

Since there are no official reports regarding restocking activities in the studied areas, we might consider that either there were no such activities on neither of the two analysed rivers (Topolog or Sebeşel), either the activities were done with individuals that were from neighbouring areas. Still, the heterozygote deficit could suggest some degree of physical isolation of both populations, maybe due to river damming. While the anthropogenic activities and their effects on the Olt River brown trout populations were previously documented, the case of the Sebeşel River populations is still to be analysed.

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