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Research Article

Genetic analysis of populations of brown trout (*Salmo trutta* L.) from the Romanian Carpathians

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Abstract – The Carpathian Mountains are one of the most complex orogenetic areas of Europe, with unique fauna, including the brown trout (*Salmo trutta*). In this study we performed population genetic analysis of 12 different *S. trutta* populations using two types of molecular markers: nine microsatellites and mitochondrial D-loop sequences. The following working hypothesis was considered: the Romanian Carpathians and their surrounding lowlands can be key relief units based on which the *S. trutta* genetic diversity, spread, distribution, connectivity, relative isolation and genetic divergence can be at least partially explained. The phylogenetic analysis revealed that the majority of sequences were grouped in the Danubian clade. The high haplotype diversity of the 12 analyzed brown trout populations can be explained by the high nucleotide diversity. The microsatellite analysis revealed an inbreeding event for all the loci and for the populations analyzed. The Romanian Carpathians' shape and geographic orientation play a zoogeographical key role driving force in respect to the *S. trutta* populations.

Keywords: Brown trout / Carpathians Mountains / genetic diversity / microsatellites / mitochondrial DNA

1 Introduction

The brown trout (*Salmo trutta,* Linnaeus, 1758) is a member of the *Salmonidae* family; the only family currently placed in the order Salmoniformes (Behnke, 2002; Klemetsen et al., 2003). Members of the *Salmonidae* are fish species that rely on a multitude of habitats (resting, sheltering, feeding, spawning, nursery, etc.) since in this family are included both migratory and resident fish species. Moreover, these habitats, often differ from one age class to another (Elliott, 1994; Crisp, 2000). For the conservation of this fish species, the connection between the spawning and maturation habitats, alongside the habitats themselves must be preserved (Schwartz et al., 2007). In consequence, there is a need for regional management strategies that rely on supporting genetic diversity, and therefore on the adaptability potential of the populations (Reed and Frankham, 2003). Increased genetic diversity is also associated with greater resilience in the face of exploitation (Hilborn et al., 2003; Schindler et al., 2010).

S. trutta is a common fish species in the Romanian Carpathians and the most important salmonid species for angling, being found in many streams including in our area of interest. During the reproduction period the brown trout migrates upstream in the rivers and their tributaries. In general, the genetic diversity studies of brown trout populations show a decreasing trend of within population genetic diversity from Western to Eastern Europe (Kohout et al., 2013), which is clear in Romania when the few studies available are being considered.

All developmental stages of salmonids can be severely affected by environmental changes like low or high flow conditions and different alterations due to anthropic interventions (Warren et al., 2015). Therefore, genetic diversity is needed by the populations for adequate adaptive/evolutionary potential (Frankel and Soule, 1981). One of the ways to preserve genetic diversity is admixture, which takes place

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when spawners migrate haphazardly from one small tributary to another (Ostergren and Nilsson, 2012). This scenario occurs more often when the tributaries are spatially close, thus the genetic differences of the local populations are correlated with the geographical distance within the river system (Carlsson and Nilsson, 2000). Furthermore, anthropogenic activities and disastrous natural events can cause a reduction in population size, which increases inbreeding and genetic drift (Cunjak and Power, 1986). A series of studies have reported dams or hydropower plants constructions on some Romanian mountain streams and rivers that could have negative impacts on the aquatic and riverine habitats and fish communities (Bănăduc, 1999; Burghelea et al., 2013).

Non-indigenous Atlantic brown trout can hybridise with local populations, a phenomenon which took place between the Atlantic and Danubian lineages in the Upper/Middle Danube River basin (Hansen, 2002; Duftner et al., 2003; Sanz et al., 2006; Simonović et al., 2017). There are studies that highlight translocation of non-indigenous brown trout individuals from former Czechoslovakia to Bulgaria, and sporadic stocking in Serbia (Marić et al., 2006; Kohout et al., 2013). Attention must be drawn to the fact that the genetic variability between the brown trout populations is lost in central European parts, of the North, Black and Baltic Sea basins due to various restocking activities with individuals of unknown genetic diversity (Wenne, 2001; Wlodarczyk and Wenne, 2001; Was and Wenne, 2003; Kohout et al., 2012; Schenekar et al., 2014; Wenne et al., 2016; Osz et al., 2018).

The Carpathians represent an area where frequent chaotic stocking and restocking of *S. trutta*. These activities were carried out without considering the status of the population and the origin (lineage) of the individuals used for restocking. In this context, the genetic studies are more than necessary in respect to understanding this fish species' past zoogeographical paths and mechanisms, the present ecological status trends and the support for an appropriate management strategy (Curtean-Bănăduc et al., 2015). The necessity for research in this field is necessary, especially in the context of no public reports stating previous stocking activities (Didenko et al., 2011, 2014). Only few genetics studies were conducted on brown trout populations from the Romanian Carpathians (Popa et al., 2013, 2016; Nechifor et al., 2017) and a more complex analysis is necessary.

Given this context, our study aims to analyse the genetic diversity of some Romanian Carpathians brown trout populations by using nuclear and mitochondrial molecular markers. The study presented here was conducted in aleatory chosen rivers from the Romanian Carpathian Mountains, on their East to West axis. This paper's working hypothesis is that the Carpathians and their surrounding lowlands can be key relief units based on which the *S. trutta* genetic diversity, spread, distribution, connectivity, relative isolation and genetic divergence can be at least partially explained.

2 Materials and methods

2.1 Sampling and laboratory analyses

A total of 362 samples of brown trout was collected between 2012 and 2017. The individuals originated from 12 river drainages of the Danubian basin (Fig. 1). Fin clips were preserved in 96% ethanol and stored at 4 °C. The genomic DNA was extracted using the standard method with phenol/chloroform (Taggart et al., 1991).

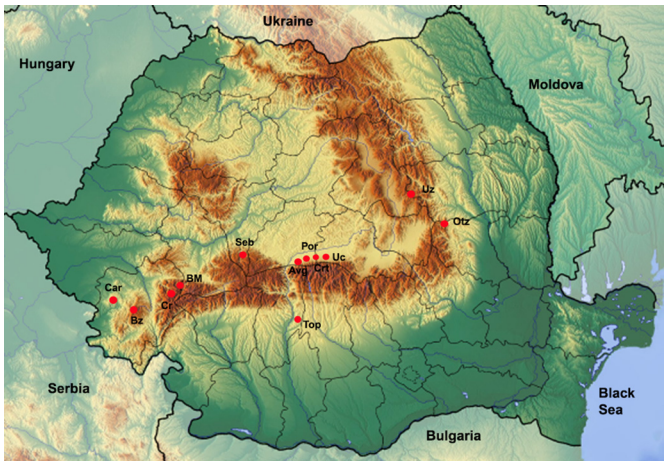

Fig. 1. Map of locations. (A) Sampling sites: Uz – Uz River, Otz – Oituz River, Uc – Ucea River, Por–Porumbacu River, Avg – Avrig River, Crt – Cârțișoara River, Top – Topolog River, BM – Bistra Mărului River, Sb – Sebeşel River, Cr – Craiului River, Bz – Bârzava River, Car – Caraş River. Rivers Porumbacu, Avrig, Cârțișoara, Ucea, Sebeşel discharge to the Northern/inner slope, while Toplog River discharge to the Southern/outer slope of the mountain ridge. (B) Map of region showing the locations of 12 sampling sites.

A fragment of the mtDNA control region (partial D-loop region) of 1003 bp was amplified using the PST-FST primer pair (Cortey and Garcia-Marin, 2002) with the following PCR conditions: 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Amplified fragments were sequenced on ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Sequences were revised using BIOEDIT (Hall, 1999) and aligned using MAFFT online v7 (https://mafft.cbrc.jp/alignment/software/). For microsatellite analyses, we used nine primer pairs (Tab. S1) grouped in two 3-plexes (I and II), one duplex (III) and one monoplex (IV). The PCR conditions for the microsatellite amplification were: 95 °C for 10 min, 35 cycles at 95 °C for 30 s, group specific hybridisation temperature (Tab. S1) for 30 s and 72 °C for 1 min, followed by final extension at 72 °C for 60 min. The amplification reactions were done using a Veriti Thermal Cycler (Applied Biosystems) in a final volume of 25 µl with 1X PCR Buffer, 1.5 mM of $MgCl_2$, 0.8 mM of dNTPs, 20 pmol of each primer, 1 unit of AmpliTaq Gold DNA polymerase, nuclease free water and 50 ng of DNA template. Amplified fragments were separated on an ABI Prism 310 Genetic Analyzer and the allele size was determined relative to the LIZ-500 Size Standard (Applied Biosystems) size standard using GENEMAPPER 4.0 (Applied Biosystems).

2.2 Data analyses

For mtDNA, the number of haplotypes and the haplotype diversity indices were computed using DNASPv5 (Librado and Rozas, 2009). To identify new haplotypes and to reveal their phylogenetic relationships, all sequences of the brown

Table 1. The number of D-loop sequences along with the number of haplotypes and genotypes obtained for each population. The abbreviation for each population name is in the brackets.

River/Population	No. of samples	No. of D-loop sequences	No. of haplotypes	No. of genotypes
Avrig (Avr)	32	30	2	32
Bârzava (Bz)	26	26	2	26
Bistra Mărului (BM)	27	21	14	27
Caraș (Car)	21	8	1	21
Cârțișoara (Crt)	26	21	5	26
Craiului (Cr)	30	27	9	30
Oituz (Otz)	20	19	4	20
Porumbacu (Por)	29	21	7	29
Sebeșel (Sb)	30	28	8	30
Topolog (Top)	28	26	11	28
Ucea (Uc)	31	28	4	31
Uz (Uz)	62	61	13	62

trout control region of appropriate length available from GenBank were included in the analysis. These sequences were belonging to the following evolutionary lineages: Atlantic – At, Danubian-Da, Mediterranean – Me, and Adriatic – Ad (Bernatchez, 2001). Sequences from a separate lineage designated as *marmoratus* – Ma corresponding to marble trout (*S. marmoratus*) were also included in the data set despite the controversy about its taxonomic status (Pustovrh et al., 2014). The marble trout is considered either a separate species (Berrebi et al., 2000; Fumagalli et al., 2002; Splendiani et al., 2006) or a member of the *S. trutta* complex (Bernatchez, 2001; Meraner et al., 2007).

The AF133701–*Salmo salar* mitochondrial region 15662–16669 bp was used as outgroup (Tab. S2). The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model with a set of 141 D-loop sequences (the unique haplotypes identified in the 12 analysed populations along with sequences downloaded from GenBank, including the outgroup *S. salar*) with MEGA6 (Tamura et al., 2013) and the statistical support for branching patterns was estimated by 1000 bootstrap replications. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G parameter =0.2809). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 946 positions in the final dataset. For an accurate phylogenetic analysis of the data set the extremely variable homopolimeric T region of D-loop was cut out as it might be lead to a phylogenetic pattern that not reflect the true phylogenetic relationships. The phylogenetic tree was visualized with FIGTREE v1 (http://tree.bio.ed.ac.uk/software/figtree/).

For microsatellites, we tested all loci with Micro-Checker (Van Oosterhout et al., 2004). The Polymorphic Information Content (*PIC*) values for each locus, alongside tests for deviation from Hardy-Weinberg proportions were computed with POWERMARKERv3.25 (Liu and Muse, 2005). Allele frequencies, F_{ST} values between pairs of populations and values of F_{IS}, along with the *Nm* parameter values were computed with ARLEQUIN (Excoffier and Lischer, 2010) and tested for significance using 1023 nonparametric permutations. GENALEXv6.5 (Peakall and Smouse, 2006) was used to find private alleles in each population and in the 12 rivers. The same software was used to determine the PCoA based on Nei's genetic distance (*D*) between the individuals, along with the observed (H_O) and expected (H_E) heterozygosity. To explore indications of recent bottleneck events, the Garza–Williamson index was calculated across loci with the Arlequin software. This index is a ratio calculated by using the number of alleles and the range in allele size, based on the assumption that the number of alleles declines faster than the range in allele size during a bottleneck, while a value *M* lower than 0.68 can mean that a population has gone through a recent reduction in size (Garza and Williamson, 2001). The Bayesian-based clustering method in STRUCTUREv2.3.4 (Pritchard et al., 2000) was applied to infer the population structure and to reveal potential admixture between populations, without *a priori* assigned individuals to populations. The most probable number of genetic clusters (*K*) was estimated based on the posterior probability of the data for a given *K* and clarified using STRUCTURE HARVESTER (Earl and Von Holdt, 2012). For the estimation, genotypes were assigned into one to 20 groups and 100 iterations with 50.000 burn-in and 100.000 repetitions were applied for each *K*.

3 Results

3.1 Mitochondrial DNA

Out of the 362 samples processed for sequencing, we have obtained 316 usable D-loop sequences (Tab. 1, Tab. S3), this probably due to the various polymorphisms in this mitochondrial control region (Consuegra et al., 2015).

Among the sequences obtained for the 12 sampling sites, 80 haplotypes were revealed. The number of haplotypes per site varied between 1 for Caraș River and 14 for Bistra Mărului River (Tab. 1). The maximum number of sequences (106) belonged to one haplotype group which was composed of individuals that came from Avrig, Oituz, Uz, Topolog, Cârţişoara, and Porumbacu rivers populations. The haplotype diversity within the populations varied from 0.22 ± 0.10 (Bârzava River) to 0.94 ± 0.03 (Bistra Mărului River), with the most numerous polymorphic sites (46) being found in the Sebeşel population. The most numerous polymorphic sites

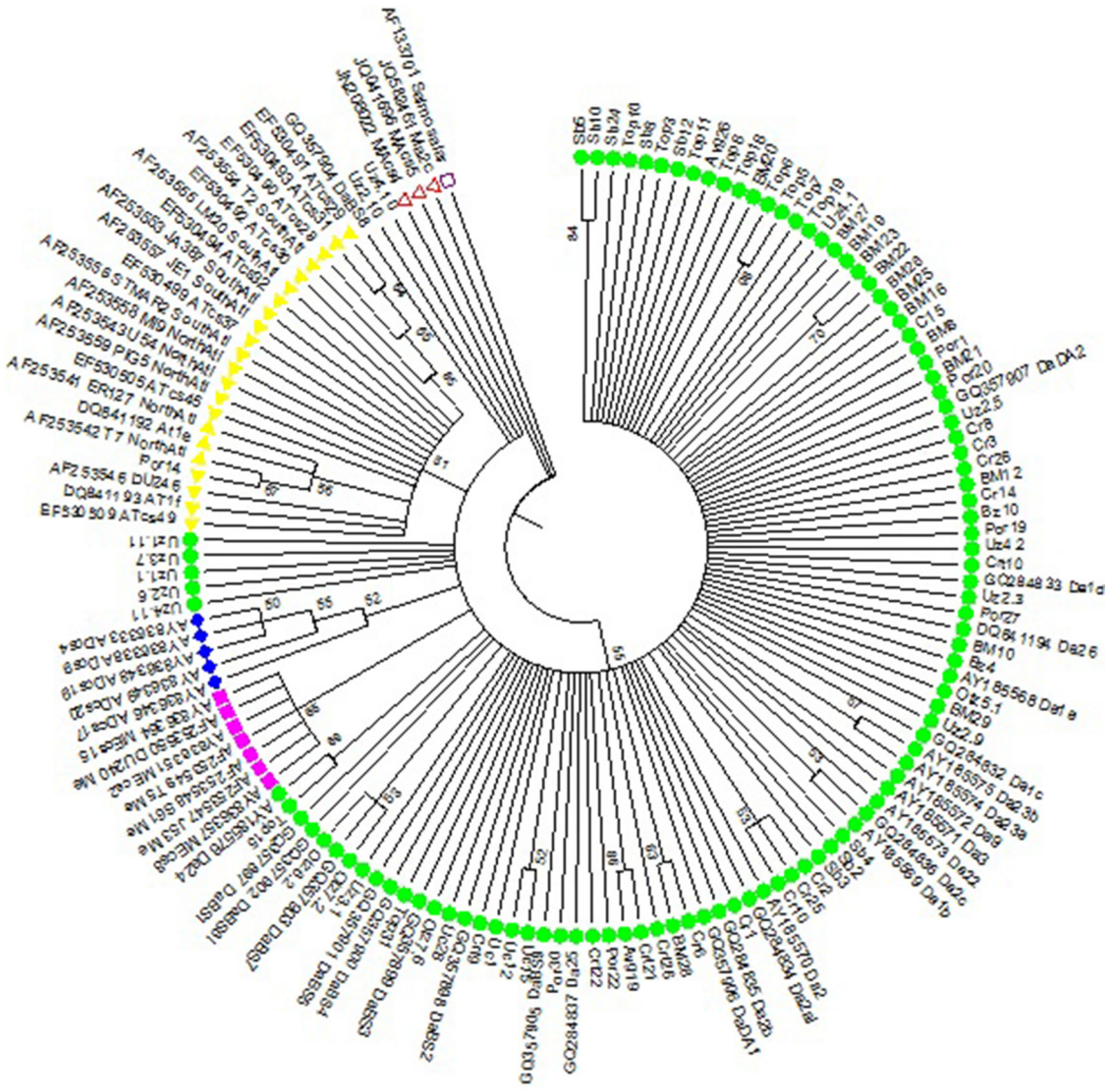

Fig. 2. Phylogenetic tree of D-loop sequences obtained with the Maximum Likelihood method based on the Tamura 3-parameter model with 1000 bootstrap replicates. The bootstrap values higher than 50 are displayed. Green circles – Danubian clade, yellow triangles – Atlantic clade, pink squares – Mediterranean clade, blue diamonds – Adriatic clade, red triangles – *S. marmoratus*.

were found in the first 500 bp of the D-loop sequence. The phylogenetic analysis revealed that the majority of sequences were grouped together in the Danubian clade, with the exception of Por14 (Porumbacu River), included in the Atlantic clade. Some sequences from Uz River (Uz1.1, Uz1.11, Uz2.6, Uz3.7 and Uz4.11) cluster separately. Also, the sequences Uz2.10 and Uz4.10 were grouped separately in a distinct clade together with a sequence from GenBank classified as Danubian haplotype (Fig. 2).

3.2 Microsatellites

For the nine microsatellites, a minimum number of alleles were identified for the inner Carpathian arch northern river basins (Cârţişoara, Porumbacu, Ucea, and Avrig) populations (Tab. S4). According to the results of Micro-checker test, we did not detect evidence for genotype inferring errors due to stuttering, neither for large allele dropout, nor for a high frequency of null alleles. A maximum number of alleles were identified for OmyFGT1 locus (24 alleles) in the Uz population (Tab. S4). Most of the loci were not in Hardy-Weinberg equilibrium ($p < 0.05$), and the *PIC* parameter varied from 0.03 for Str15, Str60 and Str73 (for Bistra Mărului, Ucea or Bârzava populations) to 0.90. The Garza–Williamson index had values from 0.20 ± 0.13 (Oituz population) to 0.36 ± 0.21 (Porumbacu population), while the observed and expected heterozygosity varied from 0.03 to 0.90 and 0.20 to 0.93, respectively (Tab. S4). Private alleles were recorded in all populations, for all loci, and the mean number of private alleles varied from 0.11 to 2.78 (Tab. S4). The mean coefficient of inbreeding F_{IS} within populations was significant in Cârţişoara, Bistra Mărului, Bârzava, Oituz, Uz, Craiului, Caraş, and Topolog populations, reflecting a homozygote excess (Tab. 2).

The F_{ST} values across all Romanian brown trout populations varied from 0.08 (population pairs Porumbacu–Avrig, Topolog–Craiului, Topolog–Caraş) to 0.44 (population

Table 2. The inbreeding coefficient (F_{IS}) values for the nine loci. *$p < 0.05$, tested with 1000 bootstrap replications. Abbreviated are used for the sampled populations.

Population	Str60	Str15	Str73	Omy FGT1	Ssa85	Ssa197	Str543	Strutta12	BS131	Average F_{IS} value
Ucea	0	–0.03	–	0.14	–0.36	0.19	0.02	0.07	–	0.01
Cârțișoara	–0.13	–0.19	–	–0.06	–	0.25	0.89 *	0.34*	0.13	0.24*
Porumbacu	–0.03	–	–	0.14	–	0.002	–0.05	0.13	0.15	0.07
Avrig	–0.73	–	–	0.18*	0.33	0.08	0.15	0.37*	–0.17	0.05
Bistra Mărului	–0.009	0	–0.04	0.53*	0.56*	0.02	0.29*	0.50*	0.12	0.31*
Bârzava	–0.07	–0.02	0	0.16	0.66*	0.2	–0.19	0.58*	0.33*	0.24*
Oituz	–0.35	–0.02	–0.1	0.35*	0.73*	0.26	0.24	0.62*	0.27	0.25*
Uz	0.21	0.49	0.01	0.55*	0.89*	0.01	0.005	0.61*	0.26*	0.31*
Craiului	–0.01	1*	0.35*	0.33*	0.24	0.03	0.26*	0.52*	0.53*	0.33*
Caraș	–0.58	0.3	–0.06	0.07	0.92*	0.02	0.2	0.41*	0.33*	0.21*
Topolog	–0.02	0.59*	0.29*	–0.04	0.61*	0.15	–0.06	0.20*	0.01	0.14*
Sebeșel	0.18	0.92*	0.11	0.33*	0.67*	0.29*	0.33*	0.44*	0.45	0.40*

Table 3. F_{ST} values (above the diagonal) and *Nm* values (under the diagonal) per population. *$p < 0.05$, tested with 1023 permutations. 1–Ucea, 2–Cârțișoara, 3–Porumbacu, 4–Avrig, 5–Bistra Mărului, 6–Bârzava, 7–Oituz, 8–Uz, 9–Craiului, 10–Caraș, 11–Topolog, 12–Sebeșel.

Population	2	3	4	5	6	7	8	9	10	11	12
1	0.30*	0.34*	0.32*	0.43*	0.32*	0.37*	0.31*	0.41*	0.41*	0.35*	0.44*
2	–	0.10*	0.13*	0.25*	0.12*	0.16*	0.12*	0.22*	0.25*	0.20*	0.27*
3	2.11	–	0.08*	0.23*	0.12*	0.14*	0.12*	0.24*	0.26*	0.19*	0.27*
4	1.65	2.58	–	0.25*	0.12*	0.12*	0.13*	0.22*	0.21*	0.18*	0.28*
5	0.72	0.79	0.73	–	0.25*	0.26*	0.24*	0.14*	0.14*	0.13*	0.15*
6	1.71	1.69	1.72	0.71	–	0.16*	0.11*	0.23*	0.21*	0.19*	0.28*
7	1.3	1.45	1.73	0.69	1.25	–	0.13*	0.21*	0.22*	0.19*	0.27*
8	1.77	1.74	1.57	0.78	1.84	1.67	–	0.17*	0.19*	0.14*	0.25*
9	0.86	0.78	0.86	1.51	0.8	0.92	1.22	–	0.10*	0.08*	0.15*
10	0.72	0.7	0.9	1.47	0.91	0.85	1.01	2.03	–	0.08*	0.17*
11	0.99	1.04	1.11	1.58	1.03	1.02	1.42	2.79	2.6	–	0.11*
12	0.66	0.67	0.64	1.32	0.62	0.65	0.73	1.32	1.22	1.9	–

pair Ucea–Sebeşel), all values being statistically significant ($p < 0.05$). A high degree of genetic differentiation ($F_{ST} > 0.25$; $p < 0.05$) was found for the following population pairs: Ucea-all populations; Cârţişoara–Bistra Mărului; Caraş-Sebeşel; Porumbacu-Caraş and Sebeşel; Avrig–Bistra Mărului and Sebeşel; Bistra Mărului-Bârzava and Oituz; Bârzava-Sebeşel; Oituz-Sebeşel, and Uz–Sebeşel (Tab. 3).

The Bayesian analysis in STRUCTURE revealed that the 12 populations can be genetically grouped into four clusters ($K = 4$), due to maximum $L(K)$, based on the nine analyzed microsatellites (Fig. 3, Fig. S1). The first cluster is made from all Ucea populations members; the second one represents the Cârţişoara, Porumbacu, Avrig and Bârzava individuals, with one individual genetically closer to the first cluster. The third group is composed of the individuals coming from the Uz and Oituz rivers, while the fourth one grouped the Bistra Mărului, Craiului, Caraş, Sebeşel and Topolog individuals.

Another type of analysis used to investigate the genetic structure of the populations, based on the genetic distance between the individuals, is the PCoA (Fig. 4). The individuals that are genetically close tend to cluster together, and the results from the Structure analysis support this representation: Ucea individuals are separated from the others, followed by a group of populations made of individuals from Cârţişoara and a part of the individuals from Porumbacu, Avrig, Bârzava, Oituz and Uz, and, finally a group formed by individuals from Sebeşel, Topolog, Caraş, Craiului and Bistra Mărului.

4 Discussion

4.1 Mitochondrial DNA analysis

The high haplotype diversity of the 12 analysed brown trout populations can be explained by the high nucleotide diversity within the D-loop mitochondrial region. Analysing the nucleotide position at which polymorphic sites were found, we observed that the most polymorphic region is at the 5′ end of the D-loop, but also close to the homopolimeric T region, which is situated after the first 400 bp of the D-loop as it has been reported in literature (Dunner et al., 2000).

The presence of the Danubian lineage might reflect the existence of distinct groups of ancient populations in this part of Europe (Kohout et al., 2013). Past cyclic glacial events and vast changes in the interconnectivity of the Black Sea with

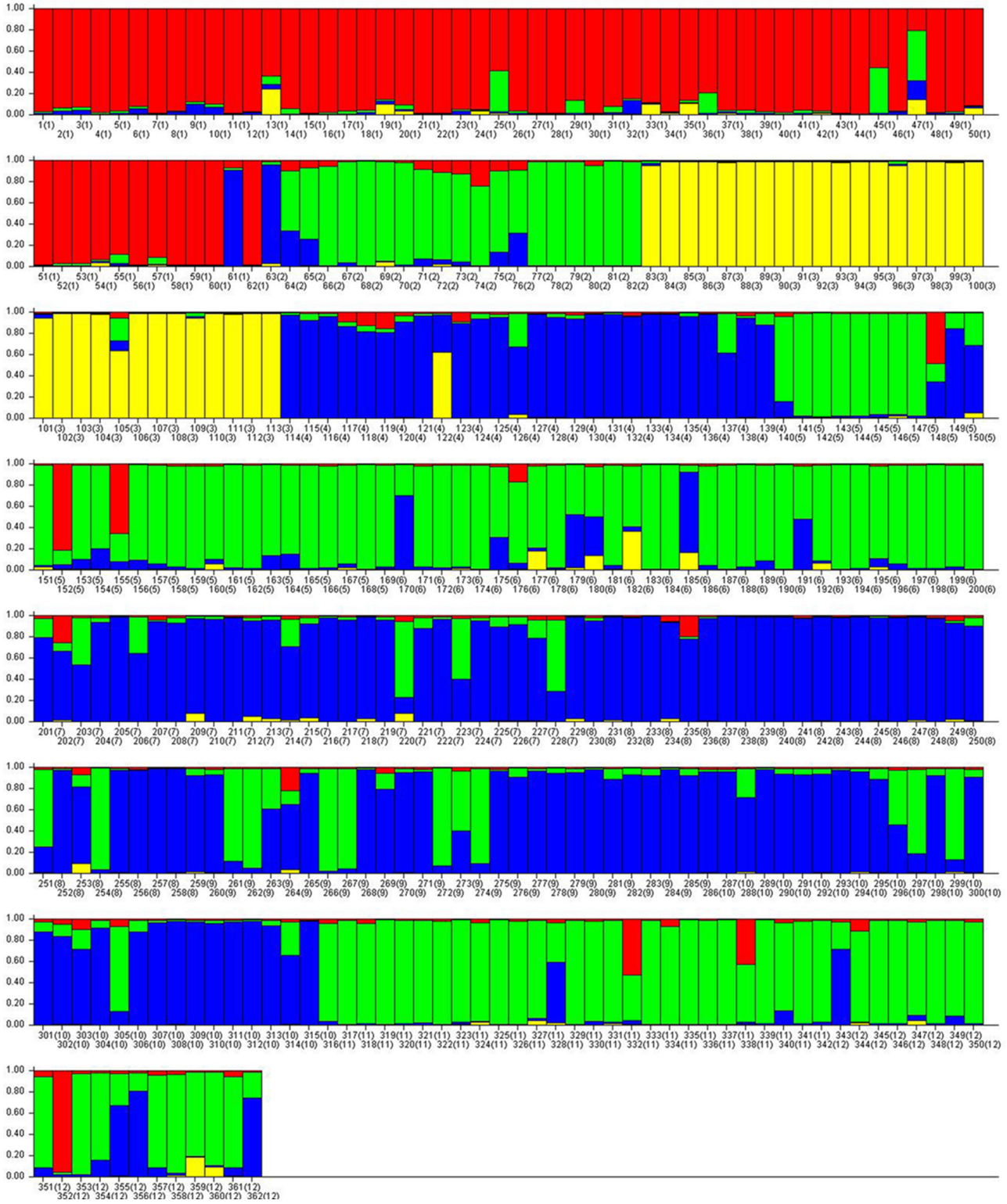

Fig. 3. Bayesian Structure results showing the individual membership of the samples from 12 Romanian rivers populations ($K = 4$). Each individual is represented by a vertical line partitioned into segments according to the proportion of the genome assigned to each of the identified clusters. Vertical line-the membership coefficient (Q), horizontal line-individuals. The populations are grouped from east to west as follows: (1) – Uz, (2) – Oituz, (3) – Ucea, (4) – Cârțișoara, (5) – Porumbacu, (6) – Avrig, (7) – Topolog, (8) – Sebeșel, (9) – Bistra Mărului, (10) – Craiului, (11) – Bârzava, (12) – Caraș.

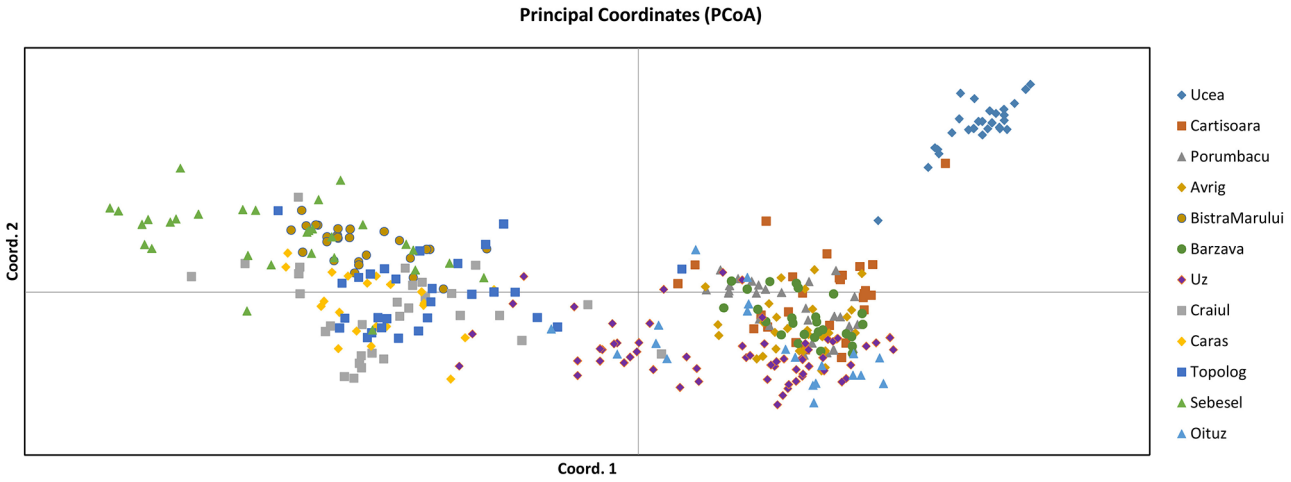

Fig. 4. PCoA (Principal Coordinate Analysis) for the 12 brown trout populations based on Nei's genetic distance (D) for the nine microsatellites. The two axes (Coord. 1 and Coord. 2) explain 11.72% and 6.28% of the variation, respectively.

Caspian and Aral basins have also impacted the populations (Bernatchez, 2001). The easternmost (Uz and Oituz Rivers) and westernmost (Bârzava and Caraş Rivers) populations have the highest genetic diversity. This observation could be explained by the climate and relief variability on this Eastern and Western limits of the studied Romanian Carpathians (Posea, 2006). Furthermore, the populations belonging to the northern oriented mountainous slopes basins (except Cârţişoara population), have shown a lower genetic variability. The shorter lengths of the inner Carpathians Rivers are favourable lotic sectors for *S. trutta* and the significant human impact pressure in the downstream sectors (Bănăduc, 1999) can offer a second category of arguments in explaining the upper described special case of the inner Carpathian arch populations.

In the Romanian Carpathians there still are pure Danubian *S. trutta* populations. The post-glacial colonization (Hamilton et al., 1989) and peri-alpine dispersion of relict populations of pure Danubian lineage *S. trutta* suggest that they settled headwater streams post-glacially earlier than the appearance and spread of the Atlantic lineage (Lerceteau-Kohler et al., 2013). The presence of the Atlantic individuals can be a result of some chaotic stocking and restocking with *S. trutta* in the Romanian Carpathian streams and rivers in the 19th and 20th centuries situation which is continuing in the 21st century too. Few individuals from the Atlantic clade from stocked rivers were found in the southern Danube Serbian mountain areas, but not from the Mediterranean clade (Marić et al., 2006). We should take into consideration also that single sequences retrieved from GenBank can be assigned erroneous to a taxon, so this aspect should be carefully treated.

For the populations with exclusively Danubian lineage haplotypes, a significant overall genetic diversity was highlighted. This might be the effect of the relatively low human impact for the whole area of alpine headwater rivers or at least in the upstream lotic sectors. So, this area can be considered in this context as a regional genetic pool of remnant populations of the Danubian clade of *S. trutta.* The exceptions represented by the Porumbacu river populations can be an effect of more accentuated and more chaotic stockings and restocking activities in the past and present. Furthermore, in the north of the Romanian Carpathians, in Ukraine, there are no data about *S. trutta* individuals of other clades, except for the Danubian one. In addition, the natural spread of fish from Upper Danube in the Lower Danube basin may be improbable since 1972, the year when the Iron Gate dam on the Danube was finished (Bănăduc et al., 2014).

4.2 Microsatellites analysis

It is easily observed that the genetic clusters resulted from the analysis of the 12 populations are not entirely related to their geographic closeness. It was observed that the groups were formed by individuals coming from streams of the Meridional and Western Carpathians (Cârţişoara–Porumbacu–Avrig–Bârzava populations group, followed by the Bistra Mărului–Craiului–Caraş–Topolog–Sebeşel), with the exception of the streams from the Eastern Carpathians (the Uz–Oituz populations group) which showed a more homogenous genetic structure. Thus, the very complex genetic structure of these clusters may be a result of the existence of ancient refugees on the Romanian Carpathians that allowed later propagation with original individuals which had a genetic structure adapted to the specific environmental conditions.

The microsatellites data reveals signals of bottleneck and inbreeding events. Therefore, the patterns of reduced genetic variability in some of the analysed populations like Cartisoara, Porumbacu, Ucea and Avrig represent probably an artefact of the described past bottleneck effect. However, other factors could be contributing to patterns of genetic diversity and this includes the effect of gene flow. Connected populations are expected to show higher genetic diversity than isolated populations. Likewise, effective population size can also explain patterns in genetic diversity. Populations with low effective population sizes are expected to have lower genetic diversity due to stronger genetic drift (Freeland et al., 2011). Furthermore, lower diversity may suggest a population was recently formed by a small number of individuals (Allendorf et al., 2012).

The maximum number of alleles identified in the extreme East Uz River population (Trotuş River basin) can be an effect of the possible stockings and restocking activities in that river. Ucea, Porumbacu and Avrig form again a separate group of

inner Carpathians arch rivers (Cârţişoara is again an exception from this group) this time due to their extremely low coefficient of inbreeding, their close geographical locations allowed spawners to haphazardly migrate from one stream to another. The base for this high degree of genetic differentiation among different *S. trutta* populations can be based on the geographical induced lack of connectivity. These resulted clusters of intra- and interrelationships suggest that the zoogeographic paths were eased by the relief and hydrography evolution along time.

Regarding the number of private alleles for each population, based on the total number of alleles, we observed that the highest number of private alleles is found in the Uz population, followed by the population of Bistra Mărului, while the populations of Porumbacu and Avrig show the lowest number of private alleles (Figs. 3 and 4). This result suggests that isolated populations might be important as genetic reservoirs of metapopulations (Linløkken et al., 2014), as suggested by the high genetic diversity of brown trout from several small sized streams. Concerning the average F_{IS} index, all populations show positive values ($p < 0.05$ for 10 out of the 12 populations), suggesting an inbreeding event for the nine nuclear loci analysed in this study. However, by analysing the same values per locus and per populations, we observed that for the Str60 locus 10 out of the 12 populations showed a heterozygote excess, as also suggested by the negative not statistically significant F_{IS} values.

Furthermore, the *Nm* parameter (number of migrants) has values >1 for the following population pairs (Tab. 3). This suggests that there are migrants between the analysed populations, and that they are not genetically isolated (Frankham et al., 2009). So, the individuals may have been part of an ancient, isolated metapopulation, from which further migration into large rivers and into their tributaries took place, or sporadic restocking activities with similar genetic structure individuals were done, or the current structure is an effect of local adaptations to similar habitats. Also, the highly F_{ST} values might be a consequence of the low genetic within population diversity.

However, a profound understanding of the phenomenon of genetic dispersion, implicitly the existence of a gene flow, is more difficult since its environmental implications are observed on a broader time scale compared to a short-term period in which preservative management measures of natural populations are taken (Palumbi, 2003). Thus, measuring the gene flow and the immigrants flow through the *Nm* parameter, closely related to the F_{ST} parameter, may prove difficult for species with high dispersal rates, as it might be the case for the brown trout.

At the same time, data on the process of colonization with brown trout are not detailed for Romania, which makes it difficult to understand the current structure and its causes of the natural trout populations. More information is known about the distribution of the Atlantic lineage that appears to have existed in the post-glacial era, since the northern part of the Atlantic was covered by ice (Apostolidis et al., 1996). However, the existence of trout populations in regions not covered by ice is due to the existence of isolated populations in refuges (Ferguson and Fleming, 1983). Studies focused on allozymes and DNA analysis have shown that there may have been several stages of European colonization with brown trout: from a Mediterranean-Caspian refuge, from an Iberian one or from a refuge in the proximity of the English Channel (Ferguson and Fleming, 1983; Simonović et al., 2017). The influence of the Black Sea Basin on this hypothesis is not excluded, but this is still a controversy about this complex colonization process (Weiss et al., 2000) through one of the most complex historical ichthyofauna areas of convergence in the Lower Danube-Danube Delta-North West Black Sea (Bănăduc et al., 2016).

5 Conclusions

To summarize, the shape and geographical orientation of the inner Carpathians can play a "protective/conservative" role in this situation. The easternmost and westernmost populations have a higher genetic variability by comparing with the populations belonging to the northern oriented mountainous slopes basins. The results suggests that the complex structure of the analysed brown trout populations could be explained by an "inner/outer" Carpathians pattern alongside the anthropic intervention and past events of bottleneck and inbreeding.

Supplementary material

Table S1 The characteristics of the primers used for the amplification of nine microsatellite loci from *S. trutta*.
Table S2 The GenBank sequences used in the phylogenetic analysis.
Table S3 Polymorphic sites distribution within D-loop analyzed region. PoS – polymorphic sites, SS – singleton sites, PaS – parsimonic sites.
Table S4 The nuclear markers analyzed for the 12 brown trout populations. Na – number of alleles, HO – observed heterozygosity, HE – expected heterozygosity, PIC – polymorphic information content, HW – exact test for testing the deviation from Hardy-Weinberg proportions, $^{*}p < 0.05$, NS – non significant, number of private alleles per population, M – mean Garza-Williamson index, SD – standard deviation.
Figures S1. The Delta *K* parameter values (vertical axis) for each *K* (horizontal axis).

The supplementary materials are available at
http://www.alr-journal.org/10.1051/alr/2019021/olm

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