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Genetic and morphological diversity in brown trout (*Salmo* spp.) in the Aaos/Vjosa River drainage in Greece and Albania: a comparison of genetic population structure with other brown trout populations in Mediterranean catchments in the southwestern Balkans

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Abstract: Three phylogenetic lineages of brown trout have been discovered in the Aaos/Vjosa River based on mtDNA control region (in this study the name Aaos (also Aoös) refers only to the Greek section of the Vjosa (also Vjosë) River in Albania). In addition to the previously detected Mediterranean (ME) lineage, the Adriatic (AD) and *marmoratus* (MA) lineages have also been found. While the AD lineage is widespread in the Mediterranean basin of the Balkans, the ME lineage is known to exist in only a few rivers, such as the Krka River (Croatia), Mati and Izvori rivers (Albania), and Aaos/Vjosa and Aliakmon River systems (Greece/Albania). The MA lineage, which in the Balkan rivers is by no means identical to marble trout (*Salmo marmoratus*), also occurs in the Krka River (Croatia), in the Bistrica River (Albania) and at least four other river systems in Greece (Acheloos, Arachthos, Mornos and Aliakmon). The Balkan cluster can be considered a distinct phylogenetic lineage, closest to the AD lineage. Some Balkan cluster haplotypes are characteristic of southern populations of marble trout. In the Aaos River, there are two phenotypes of trout in terms of the coloration and spotting pattern. In the Voïdomatis, a tributary of the Aaos, trout not only display a separate phenotype but also two different morphological forms with regard to snout shape, which appear to reflect dietary differences. Factorial Correspondence Analysis (FCA) based on microsatellite genotypes, clearly separated brown trout from the Aaos/Vjosa drainage and the Izvori, a short coastal stream south of the Vjosa estuary in Albania, from the six other Albanian river populations studied. In both Albania and Greece, native trout are endangered by uncontrolled fishing. However, the trout populations of the Aaos and Voïdomatis are among the few in Greece that have not been drastically or completely decimated by fishing pressure. In addition to uncontrolled fishing, hydropower is currently the greatest threat to the ecosystem of the entire Vjosa River system. The conservation of native trout stocks in this region is therefore closely linked with fishing restrictions and preventing further expansion of hydroelectric power generation.

Introduction

The Vjosa River, known as the Aaos River in Greece, springs in the Pindus Mountains east of the city of Ioannina in northwestern Greece and flows over 80 km of Greek territory and about 270 km of Albanian territory before draining into the Adriatic Sea. As one of the last living, free-flowing wild rivers in Europe, the Vjosa and its tributaries offers a dynamic, near-natural ecosystem with a species diversity that is yet to be fully explored. To date, 31 species of fish fauna have so far been recorded. Of these, 27 are indigenous species and eight are endemic to the Balkan Peninsula (Shumka et al. 2018a, 2018b; Schiemer et al. 2020). The Vjosa is part of the last major river system in Albania that is not over polluted by wastewater from industry and households (Cullaj et al., 2005). The use of hydropower is currently the greatest threat to this river system. The Ministry of Energy has plans to build eight dams along the Albanian section of the Vjosa and at least 23 at its tributaries. So far,

dams have only been constructed on two tributaries in the entire Albanian catchment area of the Vjosa. In the Greek section, there is a large dam near to the source of the Aaos (Pigos-Aaos hydropower station) since 1984, and the Greek government is planning another dam project to withdraw around 70 million cubic meters of water from the river for irrigation purposes every year (Vjosa fact sheet, 2016; U. Eichelmann, RiverWatch, pers. comm.). An additional serious threat is the sale of rights to exploit oil and natural gas by the Albanian government to Shell in the mountainous regions through which the Vjosa flows. Concerned nature conservationists and organizations are working to combat these projects at both the national and international levels. A coalition of environmentally oriented NGOs, such as RiverWatch and EuroNatur, has launched the campaign entitled “Save the blue heart of Europe” to draw attention to the impending wave of dams planned on the Balkan Peninsula and to save the most valuable rivers and river sections from destruction. According to environmental organizations, i.e. EcoAlbania, the entire catchment area of the Vjosa in Albania should be declared a national park in to protect this still intact ecosystem from future encroachments (Lovgren 2021). Recently, the entire course of the Vjosa in Albania was recently declared a nature park, as an important step towards the first “wild river national park” in Europe. Finally, in summer 2022, the Albanian government officially committed to establishing this area as a national park.



Map: Balkan Peninsula. *Black line:* continental divide between the basins of the Mediterranean Sea and Black Sea.
Red numbers – sampling sites for genetic analyses: 1 Cemi (Cijevna), 2 Shala, 3 upper Valbona, 4 lower Valbona, 5 Nikaj, 6 Sina (Crni Drim/Black Drin), 7 Izvori, 8 Bënça, 9 Aaos.
Black numbers – Other mentioned rivers: 10 Voïdomatis, 11 Drinos, 12 Shushica, 13 Bistrica, 14 Sarandaporos, 15 Venetikos, 16 Mati, 17 Krka, 18 Louros, 19 Arachthos, 20 Acheloos, 21 Mornos, 22 Beli Drim/White Drin, 23 Radika.

Distribution and current state of brown trout in the Vjosa drainage

In the Albanian catchment of the Vjosa River (Fig. 2), native trout are known to inhabit three left tributaries (Drinos, Bënça, and Shushica) that flow into the middle and lower course of the main river. The small remaining population in the Drinos is restricted to the last section of the river before the confluence with the Vjosa (about 3 km), where trout can survive at the entrance of springs and in deep pools. There is an issue with water quality, due to the inflow of domestic sewage and presence of waste landfills further upstream at the town of Gjirokastra, and due to water abstraction for irrigation, which dries up large parts of the upper reaches in the summer months (S. Shumka, pers. comm.). The 15 km long Bënça River (Fig. 3) is still in very good ecological condition, though there are plans for up to eight hydropower plants; none are currently under construction (U. Eichelmann, pers. comm.). In addition to cyprinids, trout are also found in large numbers in this river, with the age groups 0+ and 1+ (6–13 cm TL) predominating. Specimens with 18–20 cm TL are rare and larger ones have not been seen (S. Shumka, pers. comm.; author observations). The apparent shortage of large trout is likely due to fishing pressures. Most of the 80 km long Shushica River is still in excellent condition. However, gravel extraction from the river bed for road construction destroyed a considerable section of the upper reaches, and five dams are planned along the entire river (U. Eichelmann, pers. comm.). The Shushica is rich in cyprinids, and according to local residents, trout have never been common, which could be due to the relatively high water temperatures. In summer, the water temperature in the middle course can reach 20°C, while in the upper course and in the tributaries it reaches 16–18°C. The water stays cooler year round only in the headwaters. In a fish population survey carried out by Albanian ichthyologists in 2010, only two brown trout were caught in the middle course, and one was caught here in spring 2021 by scientists of BOKU Vienna (Universität für Bodenkultur Wien) (S. Shumka, pers. comm.). This team also caught brown trout in two small tributaries of the lower reaches of the Vjosa (upstream of the projected Kalivaç dam; work was started but has been stopped), while no trout were found in the main river itself despite intensive electro-fishing from a boat (P. Meulenbroek, pers. comm.). However, since one of the tributaries in which trout were caught does not have water year round and the second is only 100 m long, it can be assumed that trout also occur in the main river. On Greek territory, two large rivers flow towards the Aaos: the Sarandaporos River (50 km long) and the Voïdomatis from the Vikos Gorge north of Ioannina, which is only 15 km long, but richer in water.

Trout native to the Vjosa River drainage are usually assigned to two taxa common in the Western Balkans: *Salmo farioides* and *S. dentex* (Karakousis et al. 1991; Delling 2003; Kottelat & Freyhof 2007). From the entire river system, genetic studies have only been published on trout from the Voïdomatis to date, confirming two haplotypes of the ME lineage (Apostolidis 1997; Bernatchez 2001).

Genetic characteristics of trout in the Mediterranean basin of the Balkan Peninsula

Based on the mitochondrial DNA, the trout of the “*Salmo trutta* complex” that are native to the Mediterranean basin of the Balkan Peninsula belong to four phylogenetic lineages: the Adriatic (AD), the Balkan cluster, the Mediterranean (ME) and the *marmoratus* (MA) lineage. In addition, softmouth trout (*S. obtusirostris*) and the belvica trout (*S. ohridanus*) from Lake Ohrid are found here. The predominant and most widespread haplotypes found in the trout populations of this region are those of the AD lineage. Even some populations (subspecies) of softmouth trout show AD haplotypes (Sušnik et al. 2007a, 2007b). However, all softmouth trout in the Neretva drainage and the belvica trout have their own species-specific haplotypes (Snoj et al. 2002, 2008; Sušnik et al. 2006). Some haplotypes of the Balkan cluster are also characteristic for brown trout and marble trout (*S. marmoratus*) from the Neretva and Skadar–Drim River systems (Marić et al. 2006, 2017; Sušnik et al. 2007a; Snoj et al. 2009, 2010; Pustovrh et al. 2012, 2014). The distribution of the Balkan cluster haplotypes corresponds well with the distribution of the questionable taxon *S. farioides*. Balkan cluster haplotypes appear not particularly closely related to haplotypes from the AD lineage, indicating independent and successive colonization events into the Adriatic drainage (Marić et al. 2006; Razpet et al. 2007; Sušnik et al. 2007a; Snoj et

al. 2009). Trout of the ME mtDNA phylogenetic lineage can only be found in a few waters of the Balkans; either as the only present (fixed) haplotype, as in the Mati River in northern Albania (Snoj et al. 2009), or together with haplotypes of other lineages. In the Bistrice River, which flows into the Ionian Sea in southern Albania, only one haplotype of the MA lineage was detected (Snoj et al. 2009). In Greece, MA and AD haplotypes were found in the Acheloos and Arachthos Rivers, while only one MA haplotype was found in the Mornos River (Bernatchez 2001; Apostolidis et al. 2008). It should be also noted that trout from these Balkan rivers having a MA haplotype are not phenotypically similar to the marble trout of the northern Adriatic basin, characterised by a marbled skin pattern. The phylogenetic lineage MA (*marmoratus*) was originally established for the marble trout of the northern Adriatic basin (Bernatchez et al. 1992), which exhibit exclusively MA haplotypes.

The common occurrence of three lineages typical to the Mediterranean region (AD, ME, MA) was described from the Krka River in Croatia (Jadan et al. 2015)*. However, it is unclear to what extent this is due to stocking measures. The presence of a haplotype of the non-native Atlantic lineage (AT1a), often found in fish farms, is evidence that stocking has occurred. The occurrence of haplotypes of the three Mediterranean region lineages and one haplotype of the Danubian lineage (DA) in the Aliakmon drainage in Greece can partly be traced back to anthropogenic causes, since the rivers in this area were almost exclusively restocked with trout from the Acheloos, which mainly show MA and AD haplotypes (Apostolidis et al. 1997, 2008; Economidis et al. 2000; Bernatchez 2001). It cannot be determined with certainty whether the presence of a DA haplotype in the Venetikos River, a tributary of the Aliakmon, is due to natural immigration from the Black Sea basin or whether it is also anthropogenic. The presence of the ME haplotype in the Venetikos River is still unclear, since only AD haplotypes in the Balkans and DA haplotypes in northwestern Turkey occur naturally in the Aegean basin. The origin of the Venetikos River is close to the source of the Aaos River, which could explain the immigration of trout from the Aaos into the Venetikos with help of stream captures in postglacial times. However, human influence (translocation) cannot be completely ruled out.

*) Shared occurrences of the three Mediterranean lineages in the broader sense (AD, ME, MA) are also known from several rivers in Italy, such as the tributaries of the Chienti River in the Central Apennines (Splendiani et al. 2006) or the drainage of the Pellice in the Po Basin (Splendiani et al. 2020).

Material

In 2015, the Leeway collective organized a 270 km kayak descent along the Vjosa River to protest the planned hydropower plant construction on this last undammed river in Europe. As part of this campaign, brown trout sampling was also performed in highly isolated river stretches that are only accessible by kayak (Fig. 4). Five additional specimens were caught by fly fishing in the Aaos River: four specimens (Fig. 5 and 6) were caught about 40 km upstream from the Aaos-Voidomatis confluence, while the fifth (specimen no. 5; Fig. 7) was caught 10 km further upstream. In September 2021, J. Schöffmann collected 19 samples from the Bënça River. In both rivers, the fish were immediately released at the place of capture after a small piece of fin was cut off for DNA analysis. In 2019, J. Schöffmann received three dead trout from the Izvori River from a local fisherman. This approximately 4.5 km long coastal stream flows 36 km south of the Vjosa estuary into the Gulf of Vlora. The samples of brown trout populations from the drainage of the Skadar-Drim-System (see map, 1-6) used for comparative studies were mostly collected by the authors or by others (S. Shumka, D. Ulqini, R. Beaumont). The entire sample-set (see Table 1) was used for both phenotypic characterization and genetic analysis. In addition, trout from the Voidomatis River were used for phenotypic characterization.

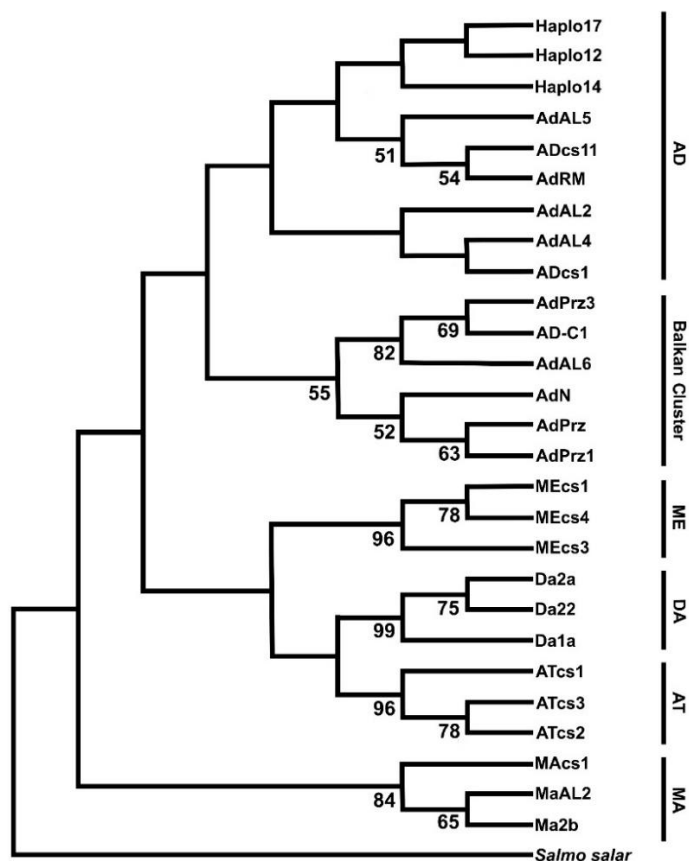


Figure 1: Evolutionary relationships of taxa/haplotypes.

Methods

Phenotypic characterization

To record morphological characteristics (snout and body shape and spotting pattern, here designated as phenotype), in 1991 and 1993 J. Schöffmann collected 15 brown trout (17–36 cm TL) in the Voïdomatis River (Figs. 7–9) and in 2001 one brown trout (27 cm TL) in the upper Aaos River (Fig. 8) and photographed them in an aquarium. The stomach contents of several specimens were also examined. Photographs in the aquarium (locations according to the map, 1-5 and 8) or outside the water (6 and 7) were also taken of brown trout from most of the other genetically studied populations.

Genetic analyses

Total DNA was isolated from fin tissue preserved in 96% ethanol following the protocol of Medrano et al. (1990).

Mitochondrial DNA

The complete mitochondrial control region (mtDNA CR, approx. 1100bp) was amplified in sampled individuals and sequenced in both directions, following the protocol in Marić et al. (2012) using primers LRBT-25 and LRBT-1195 (Uiblein et al. 2001). Sequences were aligned using Clustal X (Thomson et al. 1997) implemented in MEGA version 6 (Tamura et al. 2013) and blasted to find identical previously described haplotypes in GenBank database. Haplotypes without 100% identical hit in GenBank were described as new haplotypes.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa/haplotypes analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa/haplotypes clustered together in more than 50% bootstrap test (500 replicates) are shown below the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair. There was a total of 990 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013) (see figure 1).

Microsatellite DNA

Twelve microsatellite loci were amplified in all sampled individuals (Table 1) using fluorescently labelled forward primers as described in Lerceteau-Köhler & Weiss (2006). Aliquots of amplified fluorescently labelled DNA were mixed with formamide and GENESCAN-500 ROX Size Standard (Applied Biosystems) and genotyped on the ABI Prism 3130xl with GeneMapper® Software v4.0 (Applied Biosystems).

Factorial Correspondence Analysis (FCA; Benzécri, 1973) implemented in the GENETIX 4.04 (Belkhir et al., 2004) was used to visualize genetic distance and relatedness between the tested individuals and to check for genetically homogenous groups within the entire sample-set (Fig. 21 and 22).

Location	Coordinates	N	mtDNA haplotypes
Cemi (Cijevna)	42.467444, 19.565508	22	ADcs11 [19] (AY836340), AdPrz3 [2], AdRM [1]
Shala	42.388324, 19.779559	4	no data
Upper Valbona, with tributary	42.453085, 19.894170 42.458246, 19.902924 42.436332, 19.978927	24	Haplo14 [3] (AY926571), ADcs1 [8] (AY836330), ADcs11 [6] (AY836340), AdAL2 [5], AdAL4 [2]
Lower Valbona	42.331699, 20.085008	5	ADcs11 [2] (AY836340), AdPrz [1] (KU667310), AdAL2 [1], AdPrz1 [1]
Nikaj	42.289440, 19.939825	2	Haplo14 [1] (AY926571), AdPrz [1] (KU667310)
Sina (Crni Drim)	41.677320, 20.327978	16	Haplo14 (AY926571)
Izvori	40.327772, 19.484980	3	MEcs1 [1] (AY836350), AdAL6 [2]
Bënça	40.249119, 19.974091	19	MEcs1 (AY836350)
Aoos	40.007664, 21.003361 40.028831, 20.760392	5	AdAL5 [2], MaAL2 [2], MEcs1 [1] (AY836350)

Table 1: Sample description - location, coordinates (... north, ... east), number of analysed samples (N), mtDNA haplotypes detected in locations and their respective number in square brackets, accession number for previously described haplotypes in parentheses.

Results

Morphological characteristics

In the Aaos River, two different phenotypes can be distinguished based on the six examined specimens:

- a) With round red, light-rimmed spots along the flanks and a few black spots on the upper sides of the body; yellowish-brown background (Fig. 5, 6, 8).
- b) With numerous, irregularly shaped, black spots except for a few reddish-brown spots along the posterior part of the lateral line; silvery-gray background. This colouration and spotting pattern was seen in only one specimen (Fig. 7) caught in the lower section of the Aaos, i.e. nearest to the mouth of the Voïdomatis.

The trout of the Voïdomatis have the following appearance: The sides are covered with small to medium-sized black and red spots of irregular shape. In fish larger than 22 cm TL, four dark transverse bands are visible on the sides; one behind the head, one between the dorsal and pelvic fins, one above the anal fin and one in front of the caudal fin. These markings are particularly intense in large specimens (>30 cm TL; fig. 11) and can also be clearly seen from outside the water. The trout of the Aaos also have the four dark bars, albeit less pronounced (Fig. 6). Trout from both rivers keep their parr marks up to 20–24 cm TL.

With regard to the shape of the snout, there are two extremes in the trout of the Voïdomatis:

- A) Long and pointed snout with a maxillary extending far behind the eye; strong teeth (Figs. 10 and 11).
- B) Short and blunt snout with a maxillary that extends only to the posterior edge of the eye; lower jaw set back slightly; small teeth (Fig. 9).

The different snout shape appears to reflect the diet of the fish. Based on examinations of stomach contents of a few specimens, it was found out that group A trout feed mainly on large terrestrial insects (e.g., cicadas, beetles), while group B trout specialize in small aquatic insects (e.g., stonefly larvae). It has not yet elucidated whether these two forms belong to two different, reproductively separated populations or whether there are variations within a single population. The obvious presence of intermediate forms, however, tends to favour the latter option.

The trout from the other populations examined in this study all show a pointed snout shape, with the exception of two specimens from the Bënça River and the Sina River, a small tributary of the Crni Drim (Black Drin), both of which have a slightly blunter snout and a relatively high body (Figs. 12 and 18). All juvenile specimens (N = 19; 6–12 cm TL) from the Bënça River and the one pictured individual from the Sina River show several medium-sized red spots along the flanks and smaller black spots on the upper sides of the body (Figs. 12 and 19).

Trout with a comparatively blunt snout, short upper jaw and often slightly inferior mouth can also be found in the two coastal streams Izvori and Bistrica in Albania (Schöffmann 2019) and in the Louros River in Greece (Delling 2003). In addition, these fish all have a relatively high body and short head. The latter trout population has even been described as a separate species: *Salmo lourosensis* Delling, 2010. Two phenotypes appear in the three specimens from the Izvori: Two particularly high-bodied individuals (28 and 31 cm TL) show irregularly shaped black spots, more numerous on the anterior half of the body, while red spots dominate on the posterior half (Fig. 20). The smaller, less high-bodied individual (20 cm TL) has slightly larger red spots evenly distributed along the sides, and smaller black spots located mainly on the front flanks (see Schöffmann, 2019: Fig. 10). However, these phenotypic differences could also be due to the two different age classes of the fish.

What all populations have in common is that four more or less clearly visible transverse bands appear in adults. These markings gradually form from the parr marks in fish from 17–25 cm in total length (Figs. 8, 15 and 18 show different transition stages). The Izvori trout appear to be an exception as neither parr marks nor four

transverse bands were clearly visible in the three specimens (20, 28, 31 cm TL) examined (Fig. 20). However, these fish were purchased from a local harpoon poacher and were already dead at the time of photographing. The four transverse bands or four dark areas on the sides are not only observed in brown trout in the Mediterranean region, but also in some populations of the Atlantic basin from Morocco to the Iberian Peninsula (Schöffmann, 2021). This feature is often (not always) present in marble trout but never in softmouth trout.

Genetic analysis

Aligned sequences of the 1004 bp mtDNA CR, obtained from 100 individuals, grouped into 13 haplotypes (Table 1). PCR-amplification failed in five specimens, all four from the Shala and one from the Nikaj.

Vjosa drainage, including Izvori: Three haplotypes were found in the Aaos samples, representing three mtDNA brown trout evolutionary lineages: Adriatic (AdAL5), *marmoratus* (MaAL2) and Mediterranean (MEcs1). In the Bënça, only the latter was found. Of these haplotypes, two (AdAL5 and MaAL2) have not been previously described. In the Izvori, MEcs1 and another undescribed Balkan cluster haplotype (AdAL6) were found.

Skadar-Drim drainage: In Cemi, three Adriatic/Balkan haplotypes were found, of which ADcs11 predominated; the other two haplotypes AdRM and AdPrz3 (considering 563 bp of the 5' end of the mtDNA CR as sequenced in Sušnik et al. 2007) correspond to haplotypes previously described in the lower, Montenegrin part of the Cemi (Cijevna) River as ADcs11 and AdPrz (Sušnik et al., 2007). In the Valbona River, haplotype diversity was highest consisting of seven Adriatic/Balkan haplotypes. The haplotype distribution was not the same between the upper and lower sections of the river. For example, haplotype 14 (Haplo14 for short), otherwise characteristic of the Ohrid letnica (Sušnik et al. 2007), was found only in the upper section, while the AdPrz haplotype, characteristic of marble trout from the Skadar drainage in Montenegro (Snoj et al. 2010), appeared only in the lower section. These two haplotypes were both found in the Nikaj River, and Haplo14 was also found in the Sina River, a tributary of the Crni Drim (Table 1).

As shown on the FCA graph (Fig. 21), the sample set is roughly divided into four genetic groups that primarily correspond with the geographically separated populations: Izvori, Skadar-Drim drainage, Bënça and Aaos, though one of the Aaos specimens (no. 5; Fig. 7) tends to the group Bënça, while the other four form an independent group, though the genetic distances between them are still considerable.

When removing the most distant group Izvori from the analysis (Fig. 22), three genetic subgroups emerge in the Skadar-Drim drainage, coinciding with Sina, Cemi/Shala and Valbona; samples from Nikaj fall within the Valbona and Sina subgroups. It should be noted that the distances among these subclusters are comparable to or even smaller than the distances among the specimens of the Aaos genetic group.

Discussion

Phenotypic differences can be observed both within and between the studied trout populations. Phenotypic traits reflect what is happening at the genetic level; they can be affected by the presence or absence of certain genetic variants (alleles), some of which are advantageous in one environment and some in another, or could be affected by direct influence of the environment (epigenetics). Genes that influence phenotype interact with the environment and are therefore not neutral. Genes that are not affected by the environment are said to be neutral. In principle, this also includes microsatellites that are used to determine genetic relationships between individuals or populations. The studied trout populations, although all stemming from the Adriatic basin in Albania, also show considerable genetic differences.

The trout of the upper Aaos differ significantly in coloration and spotting pattern from the trout of the lower reaches and the tributary Voïdomatis. The spotting pattern of the specimen of the lower reaches of the Aaos (no. 5; Fig. 7) corresponds most closely to that of the Voïdomatis trout (Fig. 9–11). In addition to morphological differences, trout from Aaos are also characterized by genetic heterogeneity: in five specimens we found as many as three haplotypes representing three phylogeographic lineages of trout. This suggests that the individuals are not descendants of a single ancestor, but that they came to Aaos independently (naturally via several colonization events or/and by human mediated translocations). Their dissimilarity is also suggested by the large mutual distances on the FCA graph. It is interesting that specimen no. 5 is not only morphologically but also genetically very different from the other four Aaos specimens. These four specimens were sampled about 40 km upstream of the confluence of the Aaos and Voïdomatis, while specimen no. 5 was sampled only 10 km upstream from the confluence. The sampled locations are separated by a large canyon, where the Aaos has a considerable elevation drop, many siphons, and a number of sharp rapids and drops of approx. 1–2 m, which is assumed to hinder upstream trout migration and gene-flow. Specimen no. 5 shares the same mitochondrial haplotype (MEcs1) with trout from Bënça (a tributary of the Vjosa in Albania, some 100 km downstream from the confluence with the Voïdomatis) and considering the distribution of the samples on the FCA graph, specimen no. 5 is closest to the Bënça trout, yet not identical with them. Morphological similarities between specimen no. 5 and the trout from the Bënça could not be determined because the latter were too small for adequate phenotypic examination. Unfortunately, we do not have samples from the Voïdomatis, so we could not check the microsatellite-based similarity of these trout with the remaining samples from the Vjosa river system. Regardless, ME haplotypes (and a phenotype similar to that of specimen no. 5) are also characteristic of Voïdomatis trout (i.e., MEs2r1, MEs3r1; Bernatchez, 2001).

Based on these findings, we hypothesise that perhaps the lower Aaos – approximately from the point where specimen no. 5 was sampled – and the Albanian Vjosa drainage are inhabited by a different trout population that the upper part of the Aaos above the canyon, where morphologically and genetically different and more heterogeneous trout were found. Given they are characterised by AD and MA haplotypes, and that trout with this mitochondrial profile have previously been stocked in nearby rivers (e.g., in the Aliakmon River system; see Introduction), it is possible that they also arrived in the upper Aaos in the same way. However, due to the small sample size examined here, their native origin cannot be ruled out.

The Izvori River is 1.5 km long and flows directly into the sea, approx. 35 km south of the Vjosa estuary. Given its proximity, one might expect the genetic makeup of trout to be similar. Genetic analysis indeed shows the presence of the MEcs1 haplotype, which is also present in the Vjosa drainage, as well as a previously undescribed haplotype from the Balkan cluster (AdAL6). Only three samples were analysed, showing microsatellite based genetic similarity, though at the same time two quite distinct mtDNA haplotypes, implying that this population was stocked. Genetic data of trout from hatcheries used for stocking in Albania could help unravel whether this is the original population naturally present here or these are stocked individuals.

Phenotypic differences, especially in spotting pattern, were also observed within the studied trout populations/rivers. For example, the trout from the upper Valbona show medium-sized, rounded, black and red spots with a light border (Fig. 14 and 15), while those from the lower Valbona have a large number of either small to medium-sized, irregularly shaped (Fig. 16) or particularly small spots (Fig. 17). The latter characteristic is also found in the trout of the Nikaj, which flows into the Drim only a few kilometers west of the Valbona (see Schöffmann, 2019: Fig. 2). However, it must be taken into account that the number of spots can increase with age, as can be seen from the Bënça specimens (Figs. 12 and 13). In addition, genetic differences between the upper and lower Valbona have been observed; not only on microsatellites (Fig. 22), but also on mtDNA. For instance, haplotype AdPrz (and its as yet undescribed derivate AdPrz1) were found in the Valbona only in its lower part. AdPrz belongs to a separate haplogroup, the so-called Balkan cluster, which is rather divergent from the remaining AD haplotypes (e.g., ADcs11, Haplo14, ADcs1) of the AD clade (Snoj et al. 2009), and was found to be typical for all *S. marmoratus* from the Skadar Lake drainage (Zeta and Cijevna) in Montenegro sequenced

so far (n=12) (Snoj et al. 2010). This haplotype was also found there in putative hybrids between marble and brown trout (Snoj et al. 2010; Mrdak et al. 2012). It should also be emphasized that the Balkan cluster, first mentioned by Marić et al. (2006), can be considered as a distinct phylogenetic lineage, since its haplotypes are less closely related to those of the Adriatic lineage than previously assumed (Fig. 1; Snoj et al. 2009).

Marble trout is known to inhabit the lower Valbona, where some individuals were last personally witnessed by J. Schöffmann in 1993. Moreover, *S. marmoratus* is known to inhabit (or to have inhabited) the Drim River and its tributaries, including the lower reaches of the Shala (Poljakov et al., 1958), the Beli Drim with its tributaries Istočka River and Miruša (Marković, 1962; Šorić, 1990; Grapci-Kotor et al. 2010) and the Radika River (Georgiev, 2011), a tributary of the Crni Drim. In many of these rivers or their tributaries previously studied, trout are also characterized with the AdPrz mtDNA haplotype, such as Shala (Snoj et al. 2009), Radika (Marić et al. 2017), Prizrenska Bistrica-tributary to Beli Drim (Marić et al. 2006). In the present study, the haplotype AdPrz was found not only in the lower Valbona but also in the lower Nikaj. The confirmed existence of hybrids (Mrdak et al., 2012) and the presence of the AdPrz haplotype together with a phenotype that differs from other brown trout (e.g., in the upper Valbona) could indicate that in the lower Valbona and Nikaj introgression has occurred between brown and marbled trout. Whether this assumption is correct, and whether putative introgression was of natural or anthropogenic cause, is difficult to say until the original Drim marbled trout are tested; however, this is unlikely to ever happen as it appears that marbled trout have since become extinct here. Unfortunately, the two samples of marble trout from the Valbona that J. Schöffmann collected in 1993 and sent to L. Bernatchez can no longer be found.

Trout from the Shala (Fig. 18) and the Cemi (Cijevna) Rivers are characterized by numerous small to medium sized, but always irregularly shaped, black and red spots. These two are also genetically very similar as shown by the FCA graph (Fig. 22) – even more than trout from Shala and Valbona, even though Shala is connected to Valbona directly via Drim, and to Cemi via Lake Skadar (see Map), making this similarity surprising. Data for CR mtDNA for Shala samples are missing, but according to previous study (Snoj et al. 2009), haplotypes ADcs11 and AdPrz were reported there. AdPrz has never been found in the Albanian part of Cemi (Snoj et al. 2009; present study), only the closely related haplotype AdPrz3. In contrast, ADcs11 is very common in the Skadar-Drim-system (Marić et al. 2006; Sušnik et al. 2007; Snoj et al. 2009) and also the prevailing haplotype in Cemi in Albania. In the Montenegrin part of Cemi (Cijevna) AdPrz also occurs, but only in marble trout (Snoj et al. 2010). The cause of the morphological resemblance and the genetic similarity of trout from Shala and Cemi, detected by microsatellites, remains elusive and could be due to natural process (possibly via river capture) or human mediated translocation. Since the headwaters of Shala and Cemi are very close, both options are possible.

The influence of the environment of the morphology of trout is well known. A good example are the trout of the upper Valbona and one of its tributaries (nine samples, included in the sampling site “upper Valbona”), which could not be genetically distinguished. Compared to the trout from the main river, the specimens from the short side stream that flows through a plateau show particularly corpulent and high body structure and more rounded fin tips (Fig. 15). This peculiarity is likely due to the special habitat (low current) and a rich food supply. Another feature, though not environmental, is a single red dot in the adipose fin in individuals from about 14 cm TL, whereas the trout of the main river (upper and lower Valbona) only have red-lined adipose fins. The population of the small tributary is isolated from the trout of the other sampling sites by waterfalls just above the most upstream sampling site in the main river. Gene flow would only be possible in one direction (downstream). Nevertheless, individuals from the tributary and from the upper Valbona are genetically very similar. There are no barriers between the other sampling sites in the river, but as explained above, the trout of the upper and lower Valbona differ from each other both phenotypically and genetically.

This study showed again that we can infer their relatedness through the phenotypes of individuals, though this assessment is not always reliable, because even unrelated individuals living in similar environments can develop similar phenotype due to similar selection, or vice versa. By evaluating the genetic distances obtained through neutral genetic markers, the determination of kinship is more reliable. The combined approach,

including genetic markers and morphology, is even more reliable due to the complementarity of both approaches and mutual verification, and provides useful information, through which we can determine how reliably certain taxa can be distinguished based on their external appearance.

Conservation status

Agricultural, industrial and urban effluents are considered the main sources of river pollution worldwide. Their impacts on water quality include the increase of phosphates and ammonium and the decrease of oxygen concentrations in the water. Such influences, which are particularly evident in the more densely populated regions and agricultural areas of Albania and Greece, impair the habitat of trout and other species. In both countries, the catchment area of Vjosa has so far been largely spared from harmful environmental influences of this kind (Cullaj et al. 2005; Stefanidis et al. 2019). Here, however, water extraction for field irrigation in summer can lead to declining water levels and increased water temperatures, as observed in the Shushica and Drinos Rivers. In the southern Balkans, native trout are mainly endangered by uncontrolled fishery. Fishing with cast nets is still widespread, especially in the Albanian rivers. Occasionally, explosives are also used, and recently more often using harpoon and diving equipment. The trout stocks of the Aaos and the Voïdomatis are among the few in Greece that have not yet been drastically decimated or even wiped out by uncontrolled fishing. In the inaccessible canyons in particular, the fish have so far escaped the pursuit of anglers. In addition, all fishing in the heart of the Vikos-Aaos National Park is prohibited by law. One can only hope that these unique trout populations will be preserved in the future and not be genetically contaminated by stocking with domesticated hatchery fish or threatened by further restrictions on their habitats.

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Figure 2: Middle course of the Vjosa near Tepelena.



Figure 3: Bënça River, a tributary of the Vjosa River near Tepelena.



Figure 4: Upper reaches of the Aoös River, sampled in 2015.



Figure 5: Trout from the upper Aoös River; 24 cm TL, MA haplotype.



Figure 6: Trout from the upper Aoös River; 23 cm TL, AD haplotype.



Figure 7: Trout from the lower Aoös River; 22.5 cm TL, ME haplotype.



Figure 8: Trout from the upper Aoös River; 27 cm TL, unknown haplotype.



Figure 9: Trout with blunt snout from the Voïdomatis River; 22 cm TL.



Figure 10: Trout with pointed snout from the Voïdomatis River; 23 cm TL.



Figure 11: Trout from the Voïdomatis River; 36 cm TL.



Figure 12: Trout from the Bënça River; 12 cm TL.



Figure 13: Trout from the Bënça River; 20 cm TL. Photo: S. Shumka.



Figure 14: Trout from the upper Valbona River; 21 cm TL.



Figure 15: Trout from a tributary of the upper Valbona River; 22 cm TL.



Figure 16: Trout from the lower Valbona River; 23 cm TL.



Figure 17: Trout from the lower Valbona River; 24 cm TL.



Figure 18: Trout from the Shala River; 22 cm TL.



Figure 19: Trout from the Sina River; 19 cm TL. Photo: S. Shumka.



Figure 20: Trout from the Izvori River; 31 cm TL.

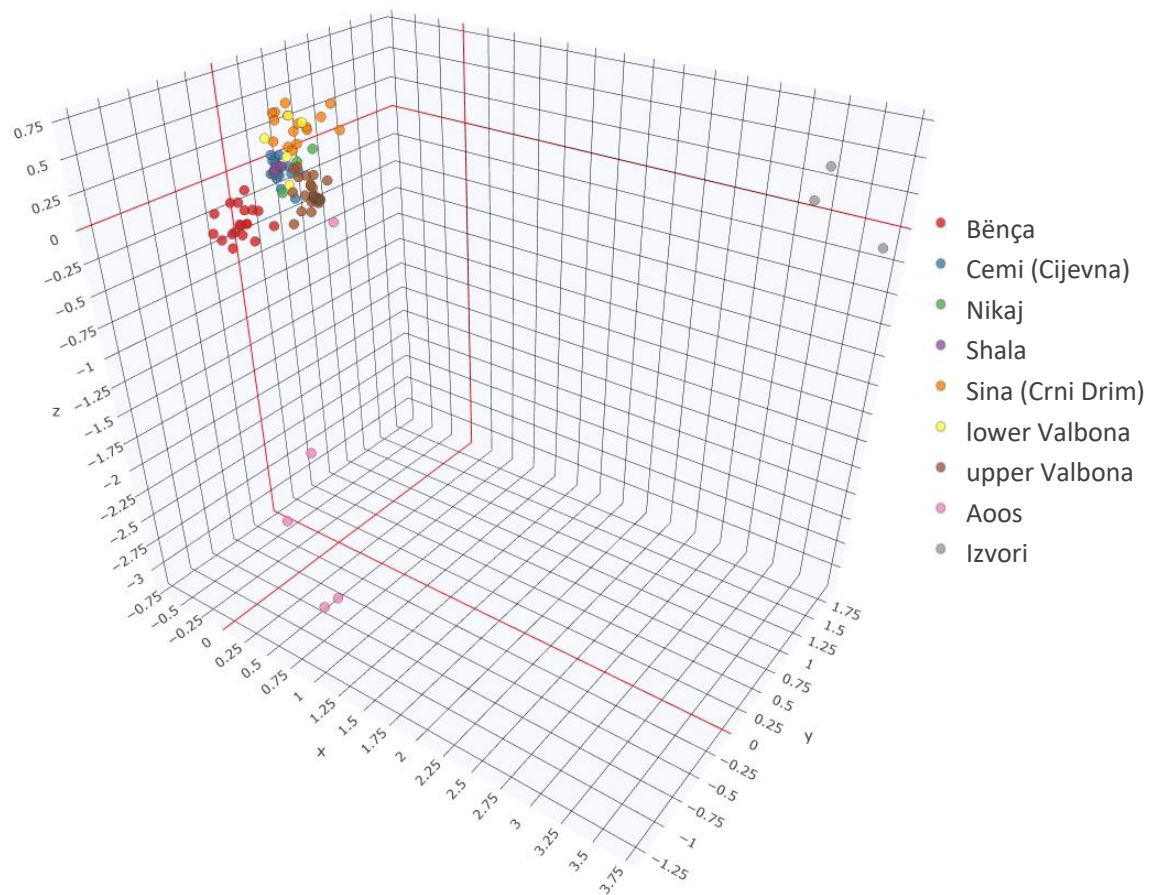


Figure 21: FCA of all trout genotyped on 12 microsatellite loci. Populations are colour coded. The first, second and third axes explain 6.35%, 5.37% and 4.63% of the variance, respectively.

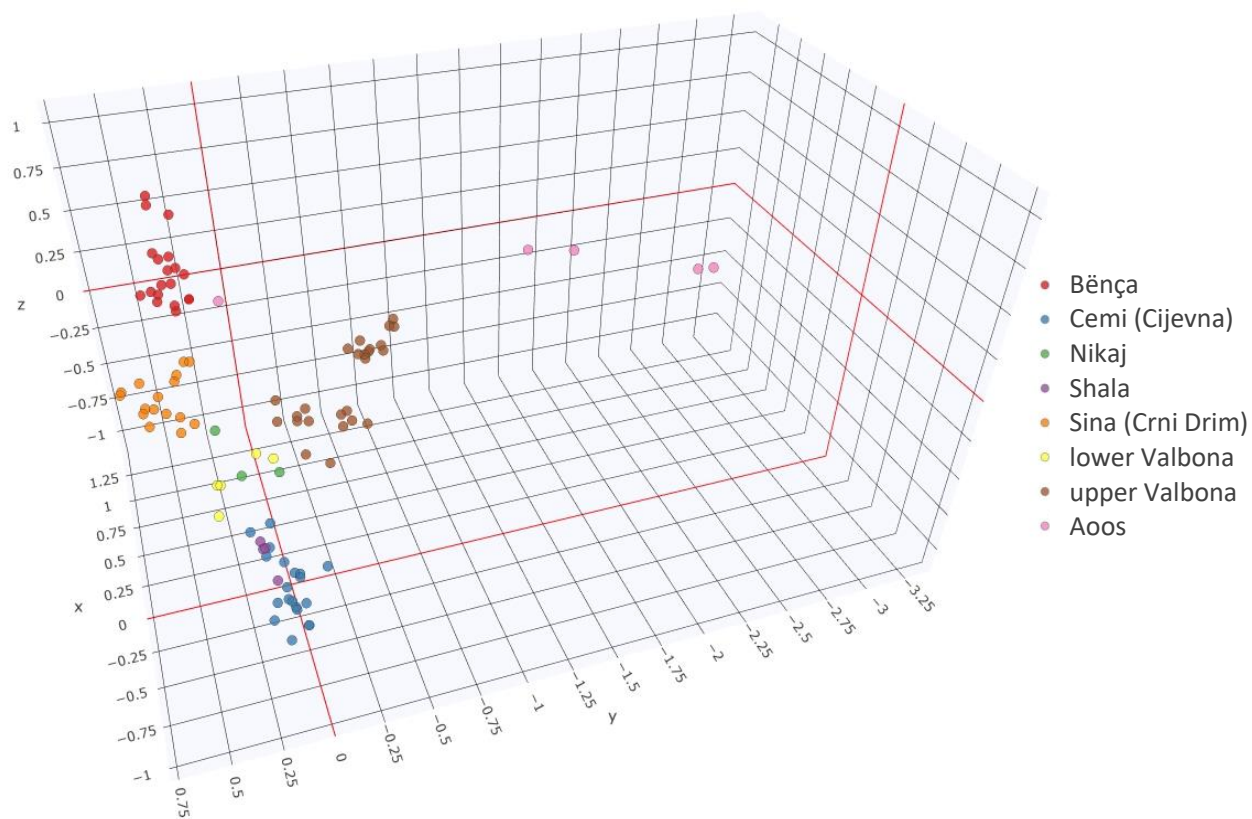


Figure 22: FCA of trout without individuals from Izvori, genotyped on 12 microsatellite loci. Populations are colour coded. The first, second and third axes explain 6.11%, 5.07% and 4.62% of the variance, respectively.

References

- Apostolidis, A. P., C. Triantaphyllidis, A. Kouvatsi & P. S. Economidis, 1997. Mitochondrial DNA sequence variation and phylogeography among *Salmo trutta* L. (Greek brown trout) populations. *Molecular Ecology*, 6:531-542.
- Apostolidis, A. P., D. Loukovitis & C. S. Tsigenopoulos, 2008a. Genetic characterization of brown trout (*Salmo trutta*) populations from the southern Balkan using mtDNA sequencing and RFLP analysis. *Hydrobiologia*, 600:169-176.
- Apostolidis, A. P., M.-J. Madeira, M. M. Hansen & A. Machordom, 2008b. Genetic structure and demographic history of brown trout (*Salmo trutta*) populations from the southern Balkans. *Freshwater Biology*, 53:1555-1566.
- Belkhir, K., P. Borsa, L. Chikhi, N. Raufaste & F. Bonhomme, 1996-2004. GENETIX 4.04, Logiciel sous Windows TM Pour la Genetique des Populations. Laboratoire Genome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier, France.
- Benzécri, J. P., 1973. L'analyse des données: Tome 2, L'analyse des correspondances. Dunod, Paris, France.
- Bernatchez, L., R. Guyomard & F. Bonhomme, 1992. DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations. *Molecular Ecology*, 1: 161-173.
- Bernatchez, L., 2001. The evolutionary history of brown trout (*Salmo trutta* L.) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation. *Evolution*, 55:351-379.
- Cullaj, A., A. Hasko, A. Miho, F. Schanz, H. Brandl & R. Bachofen, 2005. The quality of Albanian natural waters and human impacts. *Environment International*, 31: 133-146.
- Delling, B., 2003. Species diversity and phylogeny of *Salmo* with emphasis on southern trouts (Teleostei, Salmonidae). Doctoral thesis. Stockholm University, Stockholm, Sweden.
- Delling, B., 2010. Diversity of western and southern Balkan trout, with the description of a new species from the Louros River, Greece. *Ichthyological Exploration of Freshwaters*, 21:331-344.
- Economidis, P. S., E. Dimitriou, R. Pagoni, E. Michaloudi & L. Natsis, 2000. Introduced and translocated fish species in the inland waters of Greece. *Fisheries Management and Ecology*, 7:239-250.
- Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39: 783-791.
- Georgiev, S., 2011. Study of *Salmo* genus (Pisces, Salmonidae) on the Balkan Peninsula. *Ribarstvo*, 69: 11-20.
- Grapci-Kotor, L., F. Zhushi-Etemi, H. Sahiti, A. Gashi, R. Škrijelj & H. Ibrahim, 2010. The ichthyofauna of Drini I Bardhë River (Kosovo). *Ribarstvo*, 68: 149-158.
- Jadan, M., I. Strunijak-Perović, N. Topić Popović & R. Čoš-Rakovac, 2015. Three major phylogenetic lineages of brown trout (*Salmo trutta* Linnaeus, 1758) in the Krka River (Croatia) revealed by complete mitochondrial DNA control region sequences. *Journal of Applied Ichthyology*, 31:192-196.
- Karakousis, Y., C. Triantaphyllidis & P. Economidis, 1991. Morphological variability among seven Greek populations of brown trout (*Salmo trutta*). *Journal of Fish Biology*, 38:807-817.
- Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16: 111-120.
- Kottelat, M. & J. Freyhof, 2007. Handbook of European freshwater fishes. Kottelat, Cornol, Switzerland & Freyhof, Berlin, Germany, 646 pp.
- Lerceteau-Köhler, E. & S. Weiss, 2006. Development of a multiplex PCR microsatellite assay in brown trout *Salmo trutta*, and its potential application for the genus. *Aquaculture*, 258: 641-645.
- Lovgren, S., 2021. Will dams spoil one of Europe's last wild rivers? National Geographic.
<https://www.nationalgeographic.com/environment/article/will-dams-spoil-one-of-europe-last-wild-rivers>

- Marić, S., S. Sušnik, P. Simonović & A. Snoj, 2006. Phylogeographic study of brown trout from Serbia, based on mitochondrial DNA control region analysis. *Genetics, Selection, Evolution*, 38: 411-430.
- Marić, S., S. Sušnik Bajec, J. Schöffmann, V. Kostov & A. Snoj, 2017. Phylogeography of stream-dwelling trout in the Republic of Macedonia and molecular genetic basis of revision of the taxonomy proposed by S. Karaman. *Hydrobiologia*, 785: 249-260.
- Marić, S., B. Kalamujić, A. Snoj, A. Razpet, L. Lukić-Bilela, N. Pojskić & S. Sušnik Bajec, 2012. Genetic variation of European grayling (*Thymallus thymallus*) populations in the Western Balkans. *Hydrobiologia*, 691: 225-237.
- Marković, T., 1962. Ribolovne vode Srbije – Vodič. Turistička štampa.
- Medrano, F. J., E. Aasen & L. Sharrow, 1990. DNA extraction from nucleated red blood cells. *Biotechniques*, 8: 43.
- Mrdak, D., V. Nikolić, A. Tošić & P. Simonović, 2012. Molecular and ecological features of the soft-muzzled trout *Salmo obtusirostris* (Heckel, 1852) in the Zeta River, Montenegro. *Biologia*, 67: 222-233.
- Poljakov, G. D., N. Filipi, K. Basho & A. Hysenaj, 1958. Peshqit e Shqiperise. Universiteti Shtetëror i Tiranës, 286 pp.
- Pustovrh, G., S. Sušnik Bajec & A. Snoj, 2011. Evolutionary relationship between marble trout of northern and southern Adriatic basin. *Molecular Phylogenetics and Evolution*, 59:761-766.
- Pustovrh, G., A. Snoj & S. Sušnik Bajec, 2014. Molecular phylogeny of *Salmo* in the western Balkans, based upon multiple nuclear loci. *Genetics Selection Evolution*, 46:7. <https://doi.org/10.1186/1297-9686-46-7>.
- Razpet, A., S. Sušnik, T. Jug, A. Snoj, 2007. Genetic variation among trout in the River Neretva basin, Bosnia and Herzegovina. *Journal of Fish Biology*, 70: 94-110.
- Saitou, N. & M. Nei, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4: 406-425.
- Schiemer, F., S. Beqiraj, A. Drescher, W. Graf, G. Egger, F. Essl, T. Frank, C. Hauer, S. Hohensinner, A. Miho, P. Meulenbroek, W. Paill, U. Schwarz & S. Vitecek, 2020. The Vjosa River corridor: a model of natural hydromorphodynamics and a hotspot of highly threatened ecosystems of European significance. *Landscape Ecology* 35: 953-968.
- Schöffmann, J., 2013. Die Forellen der Gattung *Salmo* – Diversität und Verbreitung. AquaTech Publications, Kitzbühel, 234 pp.
- Schöffmann, J., 2019. Zur Situation der heimischen Forellen (*Salmo* spp.) Albaniens. [On the situation of the native trout (*Salmo* spp.) in Albania.] *Österreichs Fischerei*, 72:108-115.
- Schöffmann, J. 2021. Trout and salmon of the genus *Salmo*. American Fishery Society, Bethesda, Maryland, USA, 303 pp.
- Shumka, S., P. Meulenbroek, F. Schiemer & R. Šanda, 2018a. Fishes of the River Vjosa – an annotated checklist. *Acta ZooBot Austria*, 155: 163-176.
- Shumka, S., F. Bego, S. Beqiraj, A. Papparisto, L. Kashta, A. Miho, O. Nika, J. Marka & L. Shuka, 2018b. The Vjosa chatchment – a natural heritage. *Acta ZooBot Austria*, 155: 349-376.
- Snoj, A., E. Melkić, S. Sušnik, S. Muhamedagić & P. Dovč, 2002. DNA phylogeny supports revised classification of *Salmothymus obtusirostris*. *Biological Journal of the Linnaean Society*, 77: 399-411.
- Snoj, A., I. Bogut & S. Sušnik, 2008. Evidence of genetically distinct population of Vrljika softmouth trout *Salmo obtusirostris* Heckel evolved by vicariance. *Journal of Fish Biology*, 72: 1945-1959.
- Snoj, A., S. Marić, P. Berrebi, A. J. Crivelli, S. Shumka & S. Sušnik, 2009. Genetic architecture of trout from Albania as revealed by mt DNA control region variation. *Genetics Selection Evolution*, 41(1):22.
- Snoj, A., B. Glamuzina, A. Razpet, J. Zablocki, I. Bogut, F. Lerceteau-Köhler, N. Pojskić & S. Sušnik, 2010. Resolving taxonomic uncertainties using molecular systematics: *Salmo dentex* and the Balkan trout community. *Hydrobiologia*, 651: 199-212.

- Šorić, V., 1990. Salmonids in the Ohrid-Drim-Skadar system. *Acta Societatis Zoologicae Bohemoslovacae*, 54(4): 305-319.
- Splendiani, A., M. Giovannotti, P. Nisi Cerioni, M. L. Caniglia & V. Caputo, 2006. Phylogeographic inferences on the native brown trout mtDNA variation in central Italy. *Italian Journal of Zoology*, 73(2): 179-189.
- Splendiani, A., P. Berrebi, C. Tougard, T. Righi, N. Reynaud, T. Fioravanti, P. Lo Conte, G. B. Delmastro, M. Baltieri, L. Ciuffardi, A. Candiotto, A. Sabatini & V. Caputo Barucchi, 2020. The role of the south-western Alps as a unidirectional corridor for Mediterranean brown trout (*Salmo trutta* complex) lineages. *Biological Journal of the Linnean Society*, 131(4): 909-926.
- Stefanidis, K., G. Papaioannou, V. Markogianni & E. Dimitriou, 2019. Water quality and hydromorphological variability in Greek rivers: a nationwide assessment with implications for management. *Water*, 11, 1680; doi:10.3390/w11081680.
- Sušnik, S., I. Knizhin, A. Snoj & S. Weiss, 2006. Genetic and morphological characterization of a Lake Ohrid endemic, *Salmo (Acantholingua) ohridanus*, with a comparison to sympatric *Salmo trutta*. *Journal of Fish Biology*, 68: 2-23.
- Sušnik, S., A. Snoj, I. E. Wilson, D. Mrdak & S. Weiss, 2007a. Historical demography of brown trout (*Salmo trutta*) in the Adriatic drainage including the putative *S. letnica* endemic to Lake Ohrid. *Molecular Phylogenetics and Evolution*, 44:63-76.
- Sušnik, S., S. Weiss, T. Odak, B. Delling, T. Treer & A. Snoj, 2007b. Reticulate evolution: ancient introgression of the Adriatic brown trout mtDNA in softmouth trout *Salmo obtusirostris* (Teleostei: Salmonidae). *Biological Journal of the Linnean Society*, 90:139-152.
- Tamura, K., G. Stecher, D. Peterson, A. Filipski & S. Kumar, 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30: 2725-2729.
- Thompson, J. D., D. G. Higgins & T. J. Gibson, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22: 4637-4680.
- Uiblein, F., A. Jagsch, W. Honsig-Erlenburg & S. Weiss, 2001. Status, habitat use, and vulnerability of the European grayling in Austrian waters. *Journal of Fish Biology*, 59: 223-247.
- Vjosa fact sheet, 2016. Europe's wild jewel – The Vjosa River in Albania. RiverWatch, EcoAlbania, EuroNatur. https://balkanrivers.net/sites/default/files/pictures/Vjosa-Aoos_FactSheet_PM_FINAL.pdf