Ecology, Population Genetics, Evolution and Taxonomy of Ponto-Caspian Trout

Levan Ninua

The doctoral thesis is submitted to the Faculty of Natural Sciences and Medicine, Ilia State University to obtain the degree of Doctor of Philosophy in Ecology

Faculty of Natural Sciences and Medicine

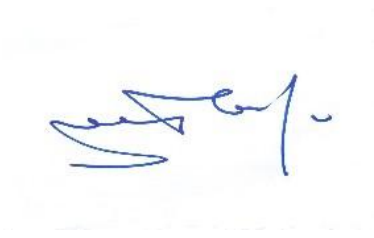
Supervisors: Natia Kopaliani, PhD and Davit Tarkhnishvili, PhD

Ilia State University

Tbilisi 2025

Statement

As an author of this doctoral thesis, I confirm that the thesis is my original work and does not include the materials of other authors that have already been published, accepted for publication or submitted for a degree, which have not been cited in an accepted manner.

Levan Ninua  **09.06.2025**

აბსტრაქტი

პონტო-კასპიურ რეგიონში, მურა კალმახის (*Salmo trutta*) კომპლექსის მრავალი სახეობაა აღწერილი, თუმცა მათ შორის მორფოლოგიურ და გენეტიკურ სხვაობებზე ცოტა რამაა ცნობილი. შავი და კასპიის ზღვის აუზების კალმახის პოპულაციებს შორის გენეტიკური ცვალებადობის დონესა და გენთა ნაკადის შესახებ ცოდნაც მწირია.

კვლევის მიზანი იყო პონტო-კასპიური კალმახის ფილოგეოგრაფიის შესწავლა და როგორც ნომინალურ სახეობებს შორის, ასევე, სახეობის ფარგლებში პოპულაციებს შორის გენეტიკური ცვალებადობისა და გენთა ნაკადის შეფასება. აღნიშნული საკითხების შესასწავლად გავაანალიზე შავ ზღვასა და საქართველოს 9 ძირითადი მდინარის აუზიდან მოპოვებული კალმახების მიტოქონდრიული დნმ-ს (ციტოქრომ b) სექვენსები და 10 მიკროსატელიტური ლოკუსი. თეზისში წარმოდგენილია ასევე სრული მიტოგენომებისა და RAD სექვენირების შედეგად მიღებული 20,272-ზე მეტი ნუკლეოტიდური პოლიმორფიზმის (SNP) ანალიზის წინასწარი შედეგები და კალმახის მორფოლოგიისა და ეკოლოგიის კვლევის შედეგები.

კვლევის შედეგად ვასკვნით:

- ✓ პონტო-კასპიური კალმახების გენეტიკური მრავალფეროვნება ყველაზე კარგად აიხსნება გეოლოგიურ წარსულში აუზების ფრაგმენტაციით; პონტო-კასპიურ რეგიონში გავრცელებული ყველა კალმახი მონოფილეტურ მიტოქონდრიულ კლადას მიეკუთვნება, რომელიც გამოეყო სხვა კალმახებს ადრეული პლეისტოცენიდან. კლადის შიგნით კი გენეტიკური მრავალფეროვნების ჩამოყალიბება პლეისტოცენის გამყინვარების ტალღებთანაა დაკავშირებული.
- ✓ პონტო-კასპიური კლადის გენეტიკური დივერსიფიკაცია დიდი ალბათობით მოხდა შავი ზღვის აუზის სახმრეთ-აღმოსავლეთ ნაწილში. აღნიშნული კლადა

გენეტიკურად ყველაზე ახლოს დგას სამხრეთ ანატოლიურ კლადასთან. კასპიის ზღვის აუზის კალმახის პოპულაციები რეციპროკულად მონოფილეტურია.

- ✓ შავი და კასპიის ზღვების აუზების კონსპეციფიკურ პოპულაციებს შორის გენეტიკური მანძილი აღემატება იმავე აუზის პოპულაციებს შორის არსებულ გენეტიკურ მანძილს. თითოეული აუზის ფარგლებში, გენეტიკური მანძილი გეოგრაფიულ მანძილთან დადებითად კორელირებს. შავი ზღვა თავისთავად არ წარმოადგენს ბარიერს გენების ნაკადისთვის იმ წყალშემკრებ აუზებს შორის, რომლებიც შავ ზღვაში ჩაედინება; ადგილობრივი *Salmo rizeensis*-ის გარდა, ყველა გენეტიკური ხაზი წარმოქმნის ანადრომულ ფორმებს.
- ✓ *S. rizeensis*-ის შემთხვევაში ანადრომიის არარსებობა შესაძლოა დაკავშირებული იყოს მიტოქონდრიულ ND4 (NADH დეჰიდროგენაზას სუბერთეული 4) გენში სამი ნუკლეოტიდის დელეციასთან.
- ✓ აღწერილი მორფოლოგიური განსხვავებები სახეობებს შორის არ არის საკმარისი დიაგნოსტიკისთვის, ზოგიერთი ადრე აღწერილი განსხვავება სხეულის ზომაზეა დამოკიდებული. მტკნარი წყლისა და ზღვის ფორმებს შორის არსებული მორფოლოგიური განსხვავებები სხვადასხვა გენეტიკურ ხაზებს შორის განსხვავებებს აღემატება.
- ✓ მდინარეებში კალმახის უმრავლესობა *(ნომინალური S. labrax, S. caspius, S. ciscaucasicus*) სქესმწიფობას აღწევს სიცოცხლის მეოთხე წელს. ყველა ნომინალური სახეობის კალმახის სიცოცხლის ხანგრძლივობა (*S. rizeensis*-ისთვის არ შეფასებულა) 5 წლამდეა.
- ✓ ზაფხულში, კალმახის საკვები ბაზა მრავალფეროვანია და მასში Ephemeroptera, Trichoptera, Diptera, Hymenoptera, Orthoptera და Amphipoda რიგების სახეობები დომინირებენ. თუმცა, მათი თანაფარდობა რაციონში პოპულაციებს შორის განსხვავდება.
- ✓ კვლევისას გამოვლინდა კალმახების დაავადების გამომწვევი რამოდენიმე პათოგენი. ცამეტიდან ერთ-ერთი პოპულაციის (ქვაბისხევი) ყველა ინდივიდი *Pomphorhynchuss* sp.-ით იყო ინფიცირებული .

მდინარე ქვაბისხევის კალმახში აღინიშნებოდა *Acanthocephal*a-ს მაღალი პრევალენტობა, რაც საკვებ რაციონში Amphipoda-ს მაღალი წილით აიხსნება.

კალმახის ველური პოპულაციებისა და აკვაკულტურისთვის მიქსოზოური პათოგენების (*Tetracapsuloides bryosalmonae* და *Mixobolus cerebralis*) მდინარის ეკოსისტემებში ცირკულაცია შესაძლოა მნიშვნელოვან საფრთხეს წარმოადგენდეს.

ამ კვლევის შედეგები მნიშვნელოვან წვლილს შეიტანს მურა კალმახის კომპლექსის, განსაკუთრებით პონტო-კასპიური კალმახის ეკოლოგიის, ევოლუციასა და ტაქსონომიაზე არსებული ცოდნის გაღრმავებაში. კვლევის შედეგებზე დაყრდნობით შესაძლებელია მეცნიერულად დასაბუთებული კონსერვაციის და მართვის ღონისძიებების შემუშავება.

Abstract

There are multiple species of *Salmo trutta* complex described in the Ponto-Caspian region. However, it is not entirely clear, to what extent some species are distinct genetically and morphologically. Specifically for brown trout from the Black and Caspian Sea basins, in spite of some previous publications, it remains unclear the level of genetic variation and gene flow among the populations. The main goal of my study was to explore phylogeography and species boundaries in freshwater and anadromous trout from the drainages of the Black and the Caspian Seas; to evaluate genetic variation and gene flow among the populations within and between both the nominal taxa and local populations. We analyzed mitochondrial DNA sequences and genotypes at 10 microsatellite loci of fish caught in the Black Sea and from nine river catchments in Georgia, flowing into either the Black or Caspian seas. The thesis also comprises preliminary

results of 20,272 SNPs harvested from the RAD sequences and complete mitogenome analyses for fish from the Black Sea populations. Genetic analysis has been supplemented by the analysis of external morphology and ecological observations.

The results of the studies suggest that:

- The genetic diversity of trout from the Ponto-Caspian region is best explained with the fragmentation of catchments in geological past; all trout species from Ponto-Caspian belong to a monophyletic mitochondrial clade, separated from the other trout since early Pleistocene; genetic diversification within the Ponto-Caspian clade is related to Pleistocene glacial waves.
- The southeastern Black Sea area is the most likely place of diversification of this clade, which is closely related to the clades from southern Anatolia; The populations of brown trout from the Caspian Sea drainage are reciprocally monophyletic.
- The genetic distance between conspecific populations from the Black and Caspian Sea basins exceeds that among the populations within the same basin. Moreover, within drainages, genetic distance correlates with the geographic distance; the Black Sea itself is not a barrier to gene flow among the watersheds draining into the Black Sea. Except for the resident *Salmo rizeensis*, all the lineages produce anadromous forms.
- Absence of anadromy in *S. rizeensis* maybe associated with triplet deletion in mitochondrial ND4 gene.
- Described morphological differences between the species are not fully diagnostic, and some earlier described differences depend on body size; the differences between freshwater and marine forms exceed those between the different lineages.
- Majority of trout in rivers (nominal *S. labrax, S. caspius, S. ciscaucasicus*) attain sexual maturity in the fourth year of life. Longevity for all nominal species (for *S. rizeensis* was not assessed) trout is up to 5 years.

- Trout diet in summer is diverse, dominated by insects (Ephemeroptera, Trichoptera, Diptera, Hymenoptera, Orthoptera) and Crustaceans (Amphipoda). However, the diet composition strongly varies among individual locations.
- All individuals from one out of 13 populations were infected with *Pomphorhynchuss* sp. High prevalence of Acanthocephala in the Kvabiskhevi trout is explained by the high proportion of Amphipoda in their diet.
- Circulation of Mixozoan pathogens (*Tetracapsuloides bryosalmonae* and *Mixobolus cerebralis*) in river ecosystems potentially represent a serious threat for wild trout populations and aquaculture.

The knowledge generated by this study is a valuable contribution to our understanding of the ecology, evolution, and taxonomy of the brown trout complex, particularly the Ponto-Caspian trout. The results will provide a strong basis for implementing evidence-based conservation and management measures for trout populations in the region.

Acknowledgements

I am deeply grateful to my supervisors, Professor Natia Kopaliani and Professor Davit Tarkhnishvili, for their professional and moral support and their invaluable contribution to my dissertation. They created a comfortable environment that allowed me to conduct most research activities independently, while always being available when needed.

I also wish to thank Professor Davit Tarkhnishvili and Professor Cort Anderson for obtaining and leading projects that provided funding for this research. Thanks to Professor Cort Anderson for sharing his experience in fish pathogen research.

Special thanks are extended to Ilia State University professors:

- Marine Murtskhvaladze for her support in laboratory activities and the analysis of obtained data (especially NGS analysis).
- Zurab Javakhishvili for his useful advices and fruitful discussions related to research topics.

I am very thankful to my friends and colleagues for their meaningful support across various trout research topics: Davit Dekanoidze, Ivane Daraselia, Nana Devidze, Elguja Gvazava, Armen Seropian, Nika Paposhvili, Niko Kerdikoshvili, Nika Melikishvili, and Sopio Kiknavelidze.

My dearest family and friends for daily support and encouragement through my doctoral studies.

Finally, I thank the Dean of the Ilia State University Faculty of Natural Sciences and Medicine, Professor Elene Zhuravliova, the faculty office, especially Marta Taktakishvili and Tamar Khocholava for technical support throughout my doctoral studies.

This research was made possible through financial support from:

- Ministry of Environment Protection and Agriculture of Georgia (Project title: *Baseline study of trout and salmon in Georgia for the purpose of developing a monitoring program*).

- Shota Rustaveli National Science Foundation of Georgia (Project title: *Salmonids in the Southern Caucasus: Conservation Status, Taxonomy, and Fisheries Management Implications*, Grant # NFR-18-6953).
- Caucasus Nature Fund (CNF) (Project title: *Monitoring of Brown Trout (Salmo trutta s.l) in Selected Protected Areas*, Contracts # CNF/2021/TAGA-GEO-170; CNF/2022/TAGA-GEO-226; CNF/2023/TAGA-GEO-302; CNF/2024/TAGA-GEO-380).

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Abbreviations

CPUE -Catch Per Unit Effort

Cyt b – Cytochrome b

ND4 - NADH dehydrogenase 4

PCA – Principal Component Analysis

PCR – Polymerase Chain Reaction

SNP – Single Nucleotide Polymorphism

Introduction

Family Salmonidae is a best studied group of fish in the world for their economic, social and cultural importance. It is a diverse group naturally inhabiting the cool and temperate waters of the northern hemisphere. The Family comprises 3 subfamilies (Coregoninae, Thymallinae, Salmoninae), 11 genera (*Brachymystax, Hucho, Oncorhynchus, Salmo, Parahucho, Salvethymus, Salvelinus, Coregonus, Prosopium, Stenodus* and *Thymallus*) and 66 species. The most diverse subfamily is Salmoninae, with seven genera distributed worldwide. The species of only three out of seven genera: *Salmo, Salvelinus* and *Hucho* are naturally distributed in Europe (Dysin et al., 2022). The species of *Salmo* (Atlantic salmon *S. salar* and brown trout species complex, formerly unified under the name *S. trutta*) are the most widespread salmonids throughout the western Eurasia. The natural distribution range for brown trout (*Salmo trutta* species complex) extends over West Asia and from West Siberia to Atlantics (Maitland & Linsell, 2006). There has always been much discussion regarding the taxonomy of brown trout; and still, a reasonable number of questions are left open. It is unclear how different some species are from one another in terms of morphology and genetics. In old treatises (Sabaneev 1875), the names *Salmo salar*, *Salmo trutta* and *Salmo fario* were used for marine, anadromous and freshwater forms of brown trout respectively. Later, *Salmo salar* was assigned to Atlantic salmon, *Salmo trutta* remained for both, anadromous and resident brown trout and *Salmo fario* lost its taxonomic meaning. In the last two decades, under the *Salmo trutta* complex, ichthyologists distinguished a number of species, previously assumed as geographic populations or subspecies of brown trout (Kottelat & Freyhof 2007; Turan et al., 2009). Later, the number of newly described species under the brown trout species complex increased up 47 (Segherloo et al., 2021). Before initiating this study there were at least nine species mentioned in different sources potentially distributed in the Ponto-Caspian region. However, genetic and/or morphological and/or geographic distinctiveness was not demonstrated sufficiently well for some of these species.

Theoretically, speciation of brown trout should follow geological patterns. That means that catchments separated later should have more closely related trout lineages which coincides with described taxonomic diversity assuming the presence of more than one species in the same catchments. Apart from taxonomic and evolutionary questions, it remained unclear how the distribution of genetic variation and gene flow is partitioned among taxa, and whether differentiation among local trout populations scales with geographic distance. The old studies describe both anadromous and resident life histories in Ponto-Caspian trout, although it is unclear if populations with different life modes are genetically differentiated, if anadromy is an inherent feature or switching to anadromy is facultative. Turan et al. (2009) described two, matrilineally different, anadromous and resident populations coexisting in the same river of northeastern Turkey.

Aiming to clear up these taxonomic and evolutionary puzzles; to infer which nominal species of brown trout present throughout the Caucasus Ecoregion, to evaluate more precisely gene flow among the populations and the geographic distribution of genetic variation, we analyzed mitochondrial DNA (cytochrome *b*) and genotypes at 10 microsatellite loci.

haplotypes were analyzed in tandem with 10 microsatellite markers. We had four working hypotheses: (1) the populations from the Black and Caspian Sea drainages are isolated. (2) There is isolation by distance among populations from the same river drainage, reflected in the correlation between the genetic and geographic (riverine) distances; (3) the coastal area of the Black Sea is the barrier for migration from one river drainage to another; and (4) there are resident populations in the Black Sea basin, genetically distinct from the anadromous populations of the same drainage, that is, different species, as previously suggested by Turan et al. (2009).

The thesis also comprises preliminary results of 20,272 SNPs harvested from the RAD sequences and complete mitogenome analyses for fish from the Black Sea populations. Genetic analysis has been supplemented by the analysis of external morphology and ecological observations.

Additionally, we investigated the ecology of some Ponto-Caspian trout populations: for the first time in Georgia, we did first assessments of trout relative abundance in 13 populations by using

standardized sampling protocol. We also assessed the size and age structure; identified the main prey taxa in trout diet and screened different populations for some micro and macro parasites.

The knowledge generated by this study may be a valuable contribution to our understanding of the ecology, evolution, and taxonomy of the brown trout complex, particularly the Ponto-Caspian trout. The results will provide a strong basis for implementing evidence-based conservation and management measures for trout populations in the region

Literature review

Brown trout taxonomy

In old treatises Sabaneev (1875), used the names *Salmo salar*, *Salmo trutta* and *Salmo fario* for marine, anadromous and freshwater form of brown trout respectively. Later, Salmo salar was assigned to Atlantic salmon, Salmo trutta remained for both, anadromous and resident brown trout and Salmo fario lost its taxonomic meaning. Until late 20th century, number of species/subspecies were described (based on morphology) in the brown trout complex (Fishbase.se). Since when (early 1990s) using molecular methods in the field of taxonomy became more intensive, the number of "brown trout species" have gradually increased (Sanz 2017). By analyzing 641nt mitochondrial control region sequences from 24 populations Bernatchez et al. (1992) identified 5 evolutionary lineages under brown trout complex: Adriatic (AD), Mediterranean (ME), Marmoratus (MA), Atlantic (AT) and Danubian (DA). Intensive studies in the field in subsequent years resulted in adding two more lineages – Tigris – TI from Turkey (Bardakci et al., 2006) and Duero – DU from Duero River in Spain (Vera et al., 2010). Farther genetic studies described new species or supported species previously described just based on meristic features (Guinard et al., 2021). Genetic differentiation was shown for several nominal species from Mediterranean and Atlantic basins: *S. akairos* and *S. pellegrini* from Morocco

(Doadrio et al., 2015); *S. ohridanus* from isolated Ohrid Lake (on the border of Albania and North Macedonia); *S.platycephalus* (from eastern Mediterranean basin), *S. obtusirostris* , S. marmoratus, *S. carpio* from Adriatic basin; (Crête-Lafrenière et al., 2012; Segherloo et al., 2021); *S. cettii* (Sicily); *S. lumi* (Lake Ohrid), *S. peristericus* (Lake Prespa) and *S. trutta* s.s (Segherloo. Et al., 2021). Before taxonomic revision by Ninua et al. (2018) there were at least 9 species mentioned in different sources potentially distributed in Ponto-Caspian region: *S. caspius* (Kessler, 1877 syn. *S. trutta caspius*) and *S. ciscaucasicus* (Dorofeeva, 1967 syn. *Salmo trutta caspius*), the two species, inhabiting Southern and northern drainages of Caspian Sea respectively; *S. labrax* (Pallas, 1814) –"Black Sea Salmon" an anadromous fish from tributaries of the Black Sea (Kottelat & Freyhof, 2007); *S. ischchan* (Kessler, 1877), native for Lake Sevan in Armenia (Berg, 1962); *S. ezenami* (Berg, 1962; Syn *S. trutta ezenami*) in inhabiting Lake Kezenoi-Am in the northeastern Caucasus (Bogutskaya & Naseka, 2002).; *S. coruhensis* and *S. rizeensis* (Turan et al., 2009) – anadromous and freshwater species from the drainages of Eastern and Southeastern Black Sea; and at last, *S. trutta* s.s (Linneaus, 1758)-European brown trout inhabiting some rivers of Black and Caspian Sea basins (Svetovidov, 1984; Schenekar et al., 2014). Genetic and/or morphological and/or geographic distinctiveness is not demonstrated sufficiently well for some of these species. Ninua et al. (2018), suggested presence of at list 4 nominal species in the Ponto-Caspian region based on 841nt mitochondrial Cyt b sequence analysis: *S. ciscaucasicus* and *S. caspius* in the Caspian Sea and *S. labrax* and *S. rizeensis* in the Black Sea basins. These results were later supported with nuclear markers too (Ninua et al., 2023). Segherloo et al. (2021) performed genomic analyses on a large sample obtained from all over brown trout species distribution range. Authors suggested the presence of 47 extant nominal species, most of them exhibiting some level of genetic differentiation (Segherloo et al., 2021). They synonymized *S. labrax, S. coruhensis* and *S. rizeensis* (Black Sea); S. caspius and S. ciscaucasicus; (Caspian Sea) in addition to the results of Ninua et al. (2018), from Ponto-Caspian trout they confirmed the separated species status for *S.abanticus* (from Abant Lake, Turkey) and *S. oxianus* (Aral Sea) and separated two unnamed *Salmo* spp. from Danube River and from rivers draining into the southern Caspian.

Brown trout ecology

Brown trout (*Salmo trutta* species complex) is one of the most wide-spread salmonid species complex, naturally distributed in West Asia, throughout Europe and North Africa (Figure 1). ince 19th century it has been widely introduced in Asia, Australia, New Zealand, and Americas (McIntosh et al., 2012). Preferable habitats are cool lakes and rivers with moderate to fast current velocity (Elliot & Elliot 2010).

Figure 1. Distribution map of brown trout (*S. trutta*) in its natural (dark grey) and invasive (light grey) range (from McIntosh et al., 2012, modified).

Habitat use in rivers and lakes is depended on multiple factors: habitat type, food preference and availability (Huntingford 1993), habitat changes due to variable meteorological condition (Davenport 1992), ontogenetic stage of fish, sex (Jonsson 1989), fish density, variable substrate, shelter abundance and distribution, life history (Jonsson 1989; Jonsson & Jonsson 2011), although the main variables affecting the habitat use in rivers are: water depth, current velocity, substrate structure and composition and shelter availability (Jenkins and Keeley 2010).

After hatching, alevins (newly hatched fish with yolk sac) spend a certain time hidden in a nest (gravel bed made by female). Their emergence from the gravel is associated with switching to feed on external food sources from the environment. This is the critical period characterized with high mortality. At that stage trout is already called parr. Parr chose shallow riversides with slow current. Deep pools in the central part of the river are becoming preferable with increasing age and size (Heggenes et al. 1999). The tolerance to the fast current (Mäki-Petäys et al. 1997), distance from the bank and from the bottom (Bremset and Berg 1999) also increases with fish size. Brown trout can dwell on a wide variety of substrates starting from fine gravel to big boulders; however it prefers large stone substrate, which allows to save energy through reducing current velocity at the bottom, provides shelter, and reduces stress caused by limiting visual contact among hidden fish (Heggenes 1988). Overhead cover is an important factor significantly affecting habitat use. Brown trout are presented in higher densities in the areas of dense overhead cover than in less covered areas (Johansen et al., 2005; Ucarli 2011). Artificial overhead covering of trout rearing tanks was reducing stress and improving growth in aquaculture (Krebs et al., 2016), this finding additionally emphasizes the importance of overhead cover for brown trout. Territoriality and aggressive behavior related to it are other important factors affecting habitat use. Territorial behavior is stronger expressed during feeding in younger rather than older individuals (Heggenes et al. 1999). It is shown that habitat use can be associated with food preferences, life history, daily and seasonal environmental changes such as water temperature (Jonsson & Jonsson 2011).

The optimal temperature range for growth and development of brown trout is 13-19◦C and is closely related to food type (fish or invertebrates). Increase of the water temperature above 20-22 ◦C causes thermal stress in all age brown trout. An ultimate critical temperature is above 25◦C. (Elliot & Elliot 2010). The age at maturity is meaningfully influenced by temperature. It changes with latitude and is lowest in southern populations. Although temperature above a certain threshold might impair maturation and reproduction in general. In smolts the growth rate negatively correlates with age at maturity. The growth rate is higher in warmer habitats, but life

longevity decreases with decreasing latitude. Males usually mature earlier than females, although male age at maturity is more variable than that of the females (Jonsson & Jonsson 2011).

Spawning time for brown trout varies from September to December. Spawning date is genetically defined in salmonids (Carlson & Seamons 2008) and is strongly connected to dates of hatching, subsequent alevin emergence from gravel and feeding from environment. So, proper spawning date is connected to the availability of proper size food for alevins when they start feeding from the environment. The survival rate meaningfully decreases if alevins emerge earlier or later than the proper time (Cushing 1982).

Female brown trout appear at spawning grounds just when they are ready for spawning activity. Before that, they stay in shelters (deep pools, undercut banks) close to the spawning area. Males generally appear at the spawning ground earlier. Both males and females express aggressive behavior against the individuals of same sex during spawning. Males show breeding behavior (vibrating body next to female) next to aggressive behavior against other competing males.

Female chooses a gravel substrate and digs the nest by fast movement of the belly and tail. Some females (large ones) distribute their eggs among several nests dug up to one meter interval in a row. The majority of eggs are fertilized by large dominant males, but sometimes smaller parr male also contribute in fertilization (even in presence of large males). Females cover the nest with stones after the spawning completes.

Life history variation

Brown trout exhibits a partial anadromy, which means that variable proportion (population depended) of fish in certain population at different stage of their life may undergo physiological and biochemical changes and gain ability (the process called smoltification) to adapt to saline water (anadromous trout), while the others spend entire life in fresh waters (resident trout). The resident and anadromous morphs differ in adult mass (Jonsson, 1985); age at maturity (Jonsson &

Jonsson, 2006); and egg size (Olofsson & Mosegaard, 1999). Anadromous trout undergo smoltification and start downstream migration towards the marine habitat generally at age of 1-3 years and rarely migrate far from shallow (mostly ≤5m) estuarine area of their home river (Jonsson & Jonsson 2011). The age of smoltification changes with latitude and decreases from north to south. In warmer climates brown trout start seaward migration at an earlier stage than in colder climates (L'Abée-Lund et al. 1989). Anadromy has its costs and benefits. Migration towards the marine habitats (and back) and acclimation to the salt water are energy demanding processes that increase energetic requirements for anadromous trout; migration to the sea increases risks of predation on smolt by other species and exposure to new pathogens in changed environment. The benefits of anadromous life history are: Fast growth rate associated with increased water temperature and feeding opportunities; and increased fecundity (Ferguson 2006). It seems that costs and benefits are in a balance (Ferguson 2006) and the trade-off in different environmental conditions triggers the switch between the life histories (Ferguson 2006; Jensen et al., 2019).

It is not entirely clear whether the anadromous life history is an inherent feature of certain populations of trout, or whether switching to anadromy is facultative. For *S. trutta* sensu stricto, existing analyses show very different results, suggesting either a facultative switch to anadromous life history, or population-dependent life history (Jonsson & Jonsson, 1993), perhaps dependent on both, environmental conditions and certain genetic factors (Jonsson & Jonsson, 2006). Ferguson (2019) described a variety of life histories presented in Figure 2.

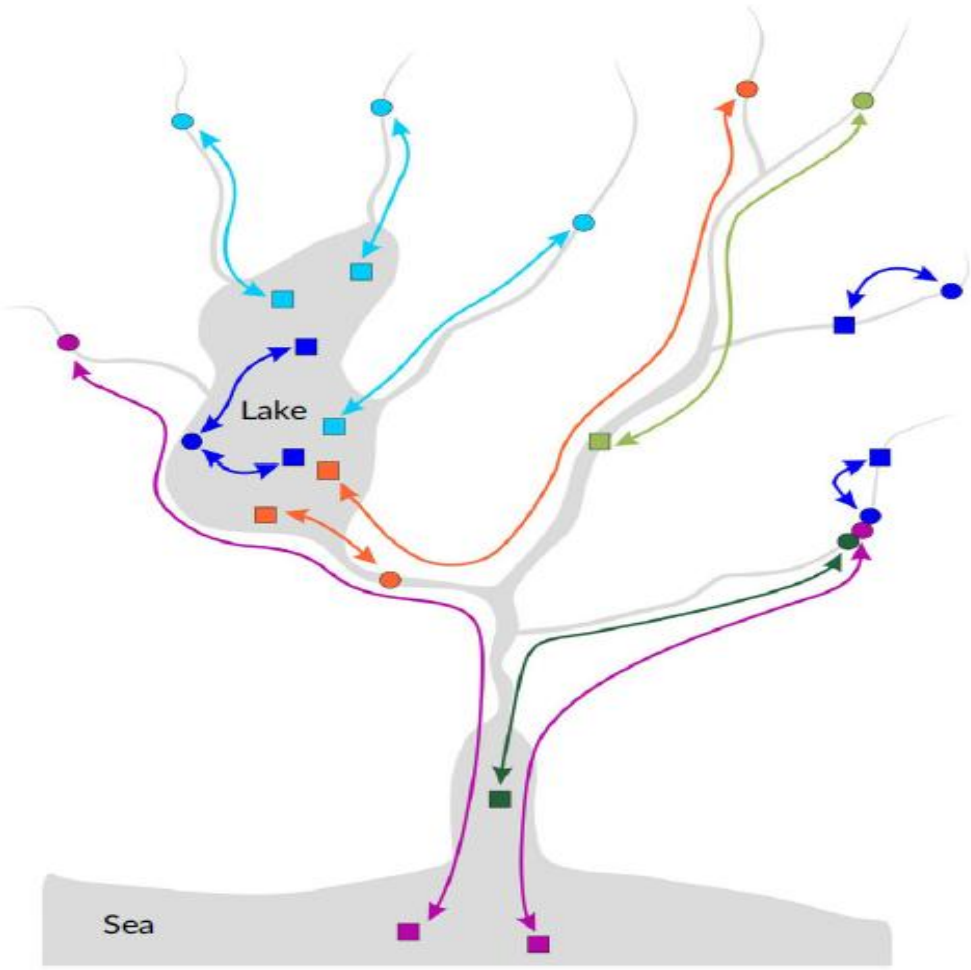

Figure 2. Potential life-history diversity of *Salmo trutta* in atypical catchment with a lake ●, pawning locations; □, adultfeeding sites. (—) Lake- or river-resident, (—) Fluvial–adfluvial, (—) Lacustrine–adfluvial, (—) Allacustrine, (—) Semi-anadromous, and (—) Anadromous (from Ferguson et al., 2019).

According to these authors, choosing each of the shown life histories is defined by a combination of physiological, genetic and environmental factors.

Knowledge about mechanisms of anadromy in Ponto-Caspian trout is even more obscure. Earlier studies on *S. labrax (*Barach, 1962) suggest a facultative nature of anadromy in this species. By contrast, Turan et al. (2009), based on the analysis of mitochondrial DNA and phenotypic

features, suggest the presence of resident forms of the trout from the Chorokhi (Coruh) basin in the south-eastern drainage of the Black Sea, coexisting with anadromous individuals.

The genetic basis of anadromy is better studied in rainbow trout (*O. mykiss*) from the Pacific Ocean; in this species, multiple genes are identified that influence life history; anadromy can be present or absent within the same polymorphic populations, depending upon unique constellations of alleles aggregating in individuals (Hecht et al., 2013; Le Bras et al., 2011; Nichols et al., 2008). Based on complete genome analysis, Collins at al. (2022) found strong association of anadromy to two genes (COP9 signalosome complex subunit 6 (CSN6) and NACHT, LRR, and PYD domain–containing protein 3 (NLRP3)) on Omy12 chromosome. However, this genomic region has only a minor effect on male *O. mykiss* anadromy. The candidate genes of major effect on anadromy are still not identified (Collins at al., 2022).

Trout infectious diseases

A variety of micro- and macroparasites (including viruses, bacteria, fungi, and helminths) affect wild and farmed salmonids globally, leading to severe ecological and economic consequences (Bruno et al., 2013). Among viruses, infectious hematopoietic necrosis virus (IHNV), piscine orthoreovirus (PRV), infectious pancreatic necrosis virus (IPNV), and infectious salmon anemia virus (ISAV) are the most common significant pathogens of brown trout and other salmonid species (Tapia et al., 2025). *Flavobacterium branchiophilum*-(causative agent of Bacterial gill disease), *Flavobacterium columnare* – (causative agent of Columnaris disease/Saddle back disease), *Flavobacterium psychrophilum* (causative agent of bacterial coldwater disease), *Renibacterium salmoninarum* (causative agent of Bacterial kidney disease), *Yersinia ruckeri,* (causative agent of Enteric red mouth disease), *Lactococcus garvieae* (causative agent of Lactococcosis), *Aeromonas salmonicida* (causative agent of Furunculosis), *Mycobacterium marinum* (causative agent of Mycobacteriosis [fish tuberculosis]), *Photobacterium damselae* (causative agent of Photobacteriosis) are important bacterial pathogens for freshwater salmonids

and other fish species (Ahmed & Sayed 2025). However, there is limited information about the prevalence and pathogenesis of each listed bacterial species in wild brown trout populations. Two bacterial pathogens *Renibacterium salmoninarum* and *Mycobacterium* sp. with low prevalence were found in wild brown trout in rivers of Austria. However, bacterial infections were not associated with major histological changes in affected fish tissues (Delghandi et al., 2020).

Various helminth species are identified in brown trout. The species of genera *Crepidostomum, Eubothrium, Diphyllobothrium, Diplostomum, Discocotyle, Phyllodistomum, Proteocephalus, Cyathocephalus, Dibothriocephalus,* (Platyhelminthes)*, Capillaria, Salmonema, Raphidascaris, Eustrongylides* (Nematoda) *Echinorhynchus, Neoechinorhynchus, Acanthocephalus and Pomphorhinchus* (Acanthocephala) are the most common helminth species infecting brown trout in Europe. No obvious morbidity was observed in any trout infected with these species (Lewisch et al., 2020; Borgstrøm et al., 2021; Couso-Pérez et al., 2023).

The most devastating pathogens causing high morbidity and mortality in wild brown trout populations in Europe is Myxozoa *Tetracapsuloides bryosalmonae* , a causative agent of proliferative kidney disease (PKD) (Lewisch et al., 2018; Waldner et al., 2020). The infection caused by *T. bryosalmonae* in salmonids is temperature-dependent. The severity positively correlates with temperature (Sudhagar et al., 2019). *T. bryosalmonae* belongs to Class Malacosprea, Sub-phylum Myxozoa and Phylum Cnidaria. Similarly to most myxozoan pathogens T. bryosalmonae needs two hosts (fish and Bryozoa) to complete its life cycle. Species of Genera *Fredericella* and *Plumatella* are the main definitive host and salmonids – intermediate host for *T. bryosalmonae* (Okamura & Wood 2002).

Another significant myxozoan pathogen is *Myxobolus cerebralis* (Class: Myxosporea). M. cerebralis infection in brown trout is generally subclinical (Eszterbauer et al., 2015). However, it causes whirling disease, characterized by high mortality in rainbow trout (*O. mykiss*), and has the potential for epizootics both in fish farms and wild environments. The definitive host for the pathogen is the oligochaete worm, Tubifex tubifex (Grabner & El-matbouli, 2010).

Materials and Methods

Standardized sampling protocol for assessing trout population parameters for future monitoring

We standardized the sampling (Bonar et al., 2009) in order to make data collected on the first year of trout population assessment comparable to data of subsequent years. Before starting sampling, we previously defined, mapped, and gave individual codes to all sampling locations. These locations will be permanent for population assessment in following years. The two assessments we've already done at each river were conducted at relatively the same period of the year and same period of a day (for each location). At each sampling event we used the same gear with the same electric parameters. Sampling effort is fixed - 10 minutes for each location. Fishing always took place when water was clear and there was no flooding in the last two weeks.

We used to collect the following information at each sampling site: (1) Fishing site code; (2) Date of fishing, the start and the end time of fishing; (3) Number of fish caught; (4) Length of each fish in millimeters (fork length).

To avoid damage, fish were permanently in the water while measurements were taken.

Fish sampling

Fish were caught by electrofishing with a handheld portable device EFGI 650 (Bretschneider Spezialelektronik: http://www.elect ric-fishing.de/index_e.html) and cast netting (mesh size – 1.5cm). The permits were obtained from the Ministry of Environmental Protection and Agriculture of Georgia (permit # 4029, 21/07/2014; 4288/01, 30/04/2019; N 3233, 28/07/2022; ბ32.8622185001, 04/07/2022; N 2784, 24/07/2023; ბ32. 8624226001, 13/08/2024). During the study 4749 wild trout were caught in total. Additionally, we collected: 59 rainbow trout (*Oncorhynchus mykiss*) from 6 fish farms, 24 individuals of introduced European cisco *(Coregonus albula*) from Tabatskuri Lake (captured with gill net by local fisherman) and 27 Kura

Loach (*Oxynoemacheilus brandtii*) in order to screen them on two most dangerous myxozoan pathogens (*Tetracapsuloides bryosalmonae* and *Myxobolus cerebralis*) of salmonid species. The majority of wild trout samples were taken under the project: "Monitoring of Brown Trout (*Salmo trutta*) in Selected Protected Areas in Georgia" (Ninua, Daraselia & Devidze, 2023). 422 wild trout were euthanized for morphology, ecological and genetic studies in total. The rest of the fish were returned back to their natural habitats. The first assessment of population parameters was performed for 13 trout populations from 6 protected areas (assessments were performed just on protected sections of rivers).

Tissue samples of trout for genetic studies were fixed in 95% ethanol until the DNA extraction. 238 individual fish from 19 locations were analyzed for genetic studies: 13 in the Black and six locations from the Caspian Sea basin. The 13 locations from the Black Sea basin were small rivers (1) Chvana and (2) Akavreta (Chorokhi (Coruh) drainage); (3) Chakvistskali; (4) Kintrishi; (5) coastal area of the Black Sea; rivers (6) Shareula, (7) Krikhula, and (8) Sakraula (Rioni drainage); (9) Tsentskali (Khobistskali drainage); rivers (10) Magana, (11) Khuberi, (12) Nenskra, and (13) Dolra (drainage of Enguri). The 6 locations from the Caspian Sea basin were (14) Snostskali (Tergi (Terek) drainage); (15) Khiso's Alazani (Sulak drainage); (16) rivers Aragvi, (17) Ilto, (18) Iori, and (19) Ktsia (drainage of the river Mtkvari (Kura)). 4–21 samples (12.5 on average) were collected from each location (Figure 3).

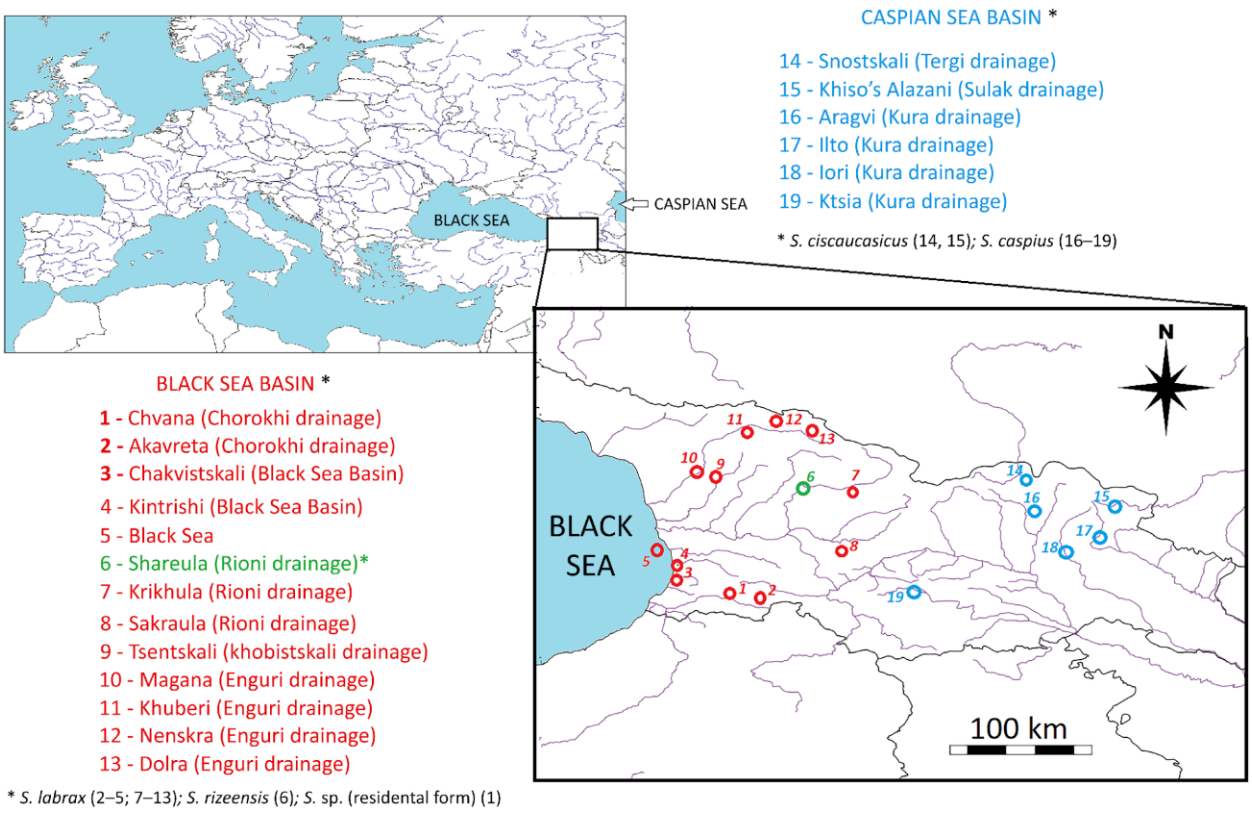

Figure 3. Sampling locations of brown trout: Colors show ultimate destination of drainage, red indicates Black Sea, blue indicates Caspian Sea. Population 6, shown in green, indicates the location of fish whose mitochondrial DNA clustered with *Salmo rizeensis* (Turan et al., 2009).

In addition, we analyzed 44 sequences of brown trout downloaded from GenBank (Appendix 1).

Bryozoa and Oligochaete sampling

We used the same approach for sampling Bryozoa and Oligochaetes at each sampling location. We sampled Bryozoa by wading 100 meters along the river and checked tree roots and undersides of large stones (Waldner et al., 2020). For oligochaetes we sieved (steel mesh size 2mm) 10 areas of 0.25m^2 bottom sediment surface at each river. Sampling locations are shown on Figure 4.

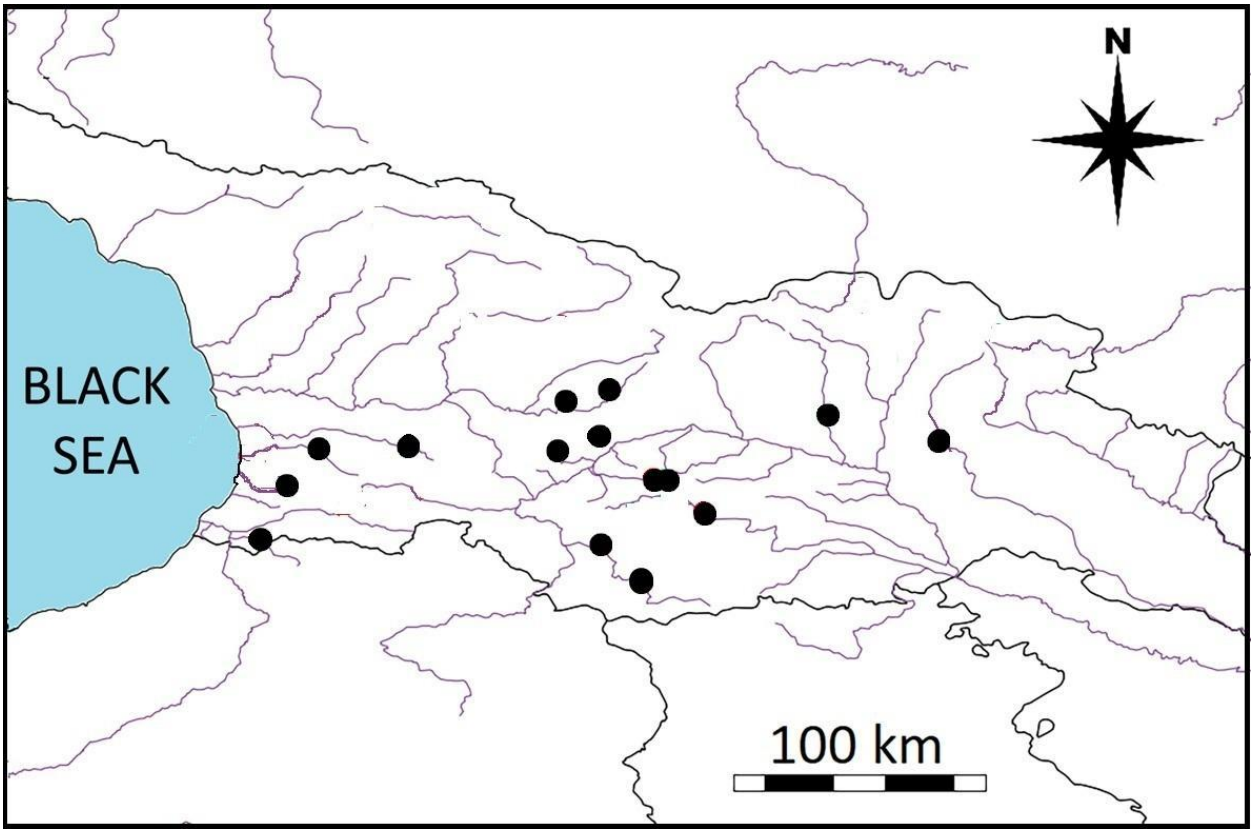

Figure 4. Sampling locations for Bryozoa and Oligochaetes are shown in black dots

Morphometry

Fish used in morphometry were photographed from the lateral side. The images were used for scoring the 28 conventional distances among the 14 landmarks (shown in Figure 5), using software ImageJ (Rasband, 2016). The distances were log-transformed to control for allometric effects. Subsequently, the regression of each of the 27 distances on the first distance (landmarks 1–8, reflecting body length of an individual) was calculated and the standardized residuals on the regression line (*size-removed* body proportions) were used for final calculations as recommended by Thorpe and Leamy (1983), instead of the original 28 distances. Principal component analysis (PCA) was applied for extracting principal components with loadings exceeding unity, and for calculating the individual scores. The individual scores on PCA loadings 1–4 were used for

ordination of the individuals and inferring possible differences among the species, which revealed genetic groups, and life forms (riverine vs. marine). All calculations were conducted using SPSS 21.0 for Windows. River trout from Georgia was included in the analysis along with the marine form ("Black Sea salmon"), and two individuals of North American rainbow trout (*Onychorhynus mykiss*), an introduced/invasive species in Georgia's rivers and lakes. Besides this morphometric study, we counted (1) the number of rays in dorsal fins, (2) the number of main rays in pectoral fins, and (3), the number of gill rakers at the first gill arch (Andersson et al., 2016; Kara et al., 2011).

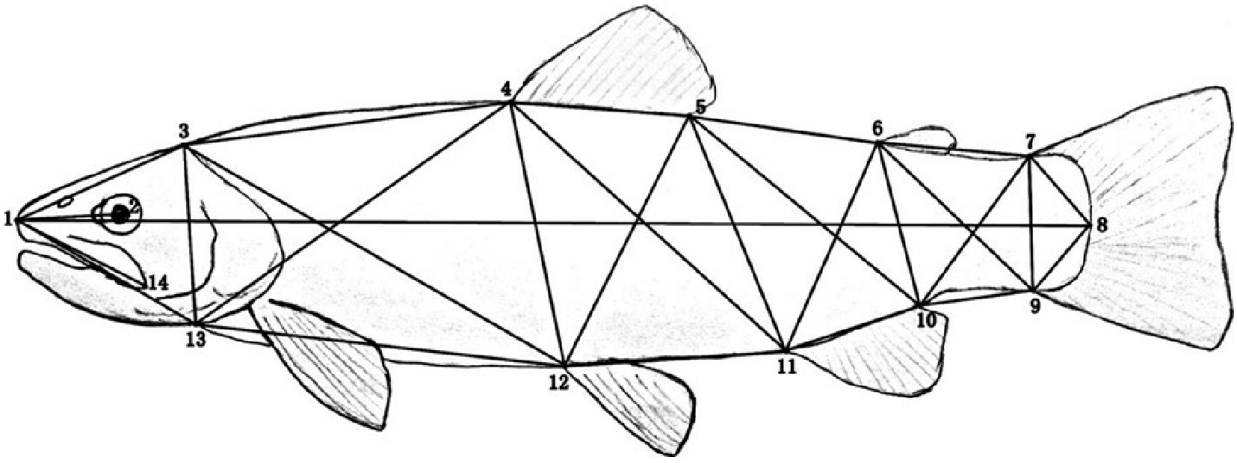

Figure 5. Landmarks (1–14) and the distances between the landmarks using in the morphometric analysis of brown trout and salmon used in the analysis (from Ninua et al., 2018).

Catch per unit effort (CPUE) and size/age structure

CPUE was calculated by dividing the total number of fish caught, on units of time (10 minutes) used for fishing at each river (Paragamian, 1989).

Frequencies of each size fish (measurements were rounded from decimal to the nearest whole number) were calculated for total catch from each river.

We defined an age for fish of different sizes to see the relationship between the length and age in order to translate the population size structure to the age structure. For that purpose, we've collected and euthanized around 30-55 fish from each river. We tried to obtain different length classes with relatively the same frequency in the sample. The age for each fish was defined based on growth rings on otoliths (Figure 6). Extracted otoliths were photographed under the microscope (40X magnification). Ages were defined by tree specialists independently. The results were additionally confirmed by comparing it to scale growth ring data (Jonsson 1976).

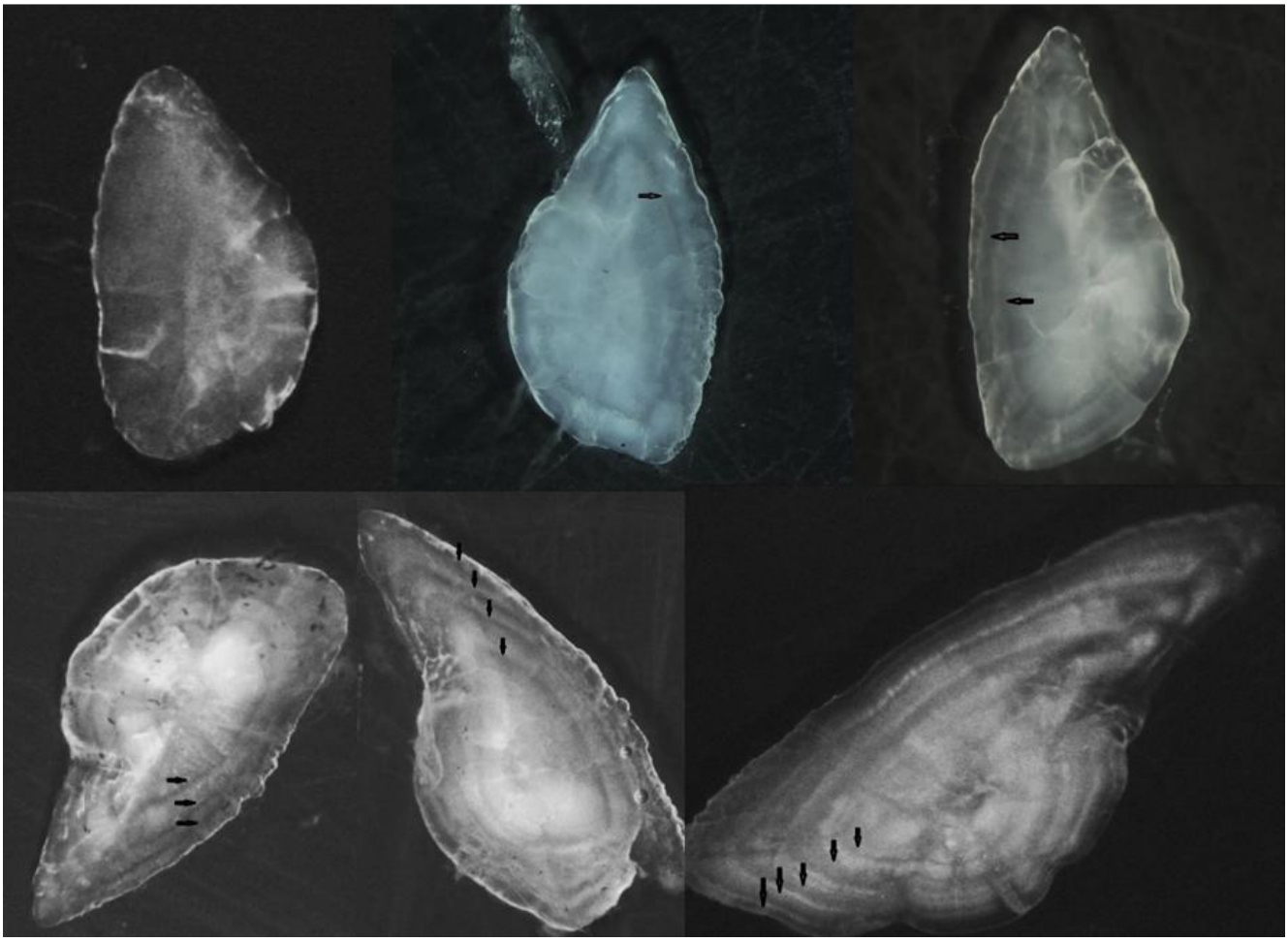

Figure 6. Growth rings on otoliths of 0+,1+,2+,3+,4+ and 5+ years old trout.

Identifying state of maturity

State of maturity in both sexes were assessed with macroscopic observation. Fish was defined as adult female, when it had large vitellogenic oocytes and adult male, when gonads were white colored and large. In mature individuals of both sex, gonads occupied a significant part of the fish body cavity (Pavlov et al., 2020).

Identifying trout prey taxa

We checked the stomach content of all trout euthanized for morphology and age determination. Along with almost intact invertebrates, we also collected body parts (legs, wings etc.) that could help us to identify animals on at least Order level (Figure 7). Invertebrates from stomach content were identified under stereomicroscope by an experienced entomologist.

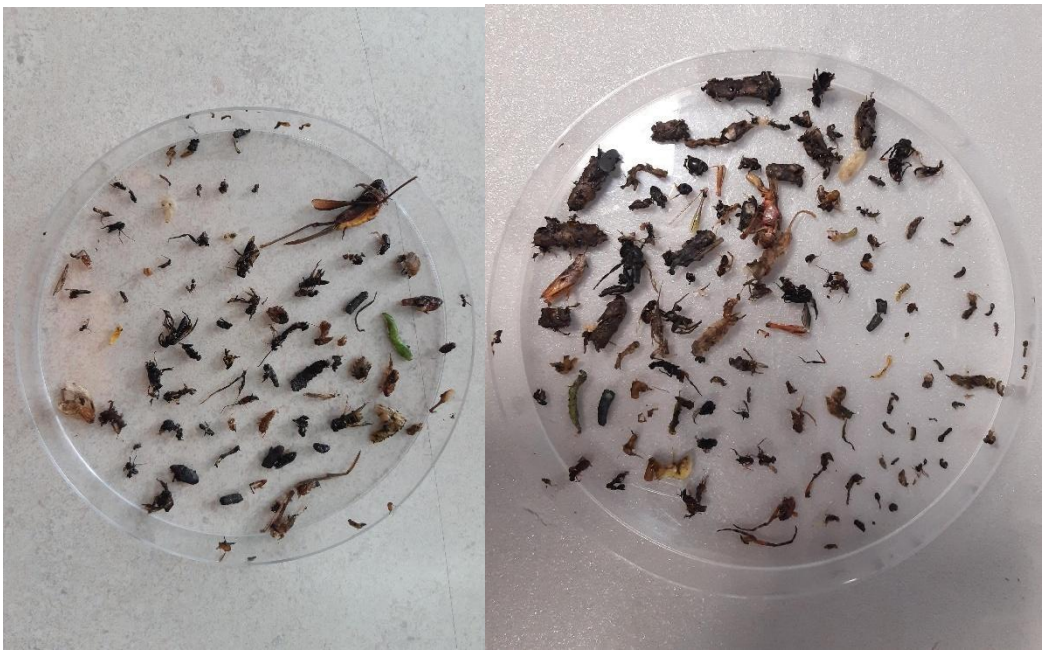

Figure 7. Aquatic and terrestrial invertebrates extracted from trout stomach.

DNA extraction, PCR and sequencing of trout samples

DNA was extracted from clipped adipose fins using the Qiagen DNeasy Blood & Tissue kit according to the manufacturer's instructions. Mitochondrial cytochrome *b* fragments were amplified using the following primers: nSsaL14437 (5′-GCTAA TGA CGC ACT AGTCG-3′, Warheit & Bowman, 2008) and StrCBR (5′-GGGGGCGA GRA CTA GA AGAT-3′ (Turan et al., 2009). The primers BrtD-F20 (5′-GAGATTTTAACTCCCACCCT-3′) and BrtD-R20 (5′-TAGGGTCCATCTTAACAGCT-3′) (Segherloo et al., 2012) were used in order to amplify 505-bp-long control region fragment (26 samples). Amplification conditions were the same for both mitochondrial fragments. Twenty microliters of PCR reactions contained 3 µl of template DNA, 1U Promega Tag polymerase, 1X Promega buffer, 2.5 mmol/L MgCl2, 0.1 mmol/L of each dNTPs, and primer concentrations 0.1 µmol/L. Thermal profile was as follows: 3-min initial denaturation at 94°C, followed by 35 cycles at 94°C for 40 s, 50°C for 1 min, and 72°C for 2 min, and 10 min at 72°C for final extension. 5 µl from each PCR was run on 1% agarose gel to visualize the DNA fragments. The amplicons were sequenced on an ABI 3130 *XL* automated sequencer. PCR fragments were sequenced in both directions using the same PCR primers and Big Dye Terminator 3.1, as per manufacturer's protocol. Sequences were aligned and edited with Geneious (ver.8.1.9) and the *unique* sequences of the mitochondrial cytochrome *b* gene and that of the mitochondrial control region from our samples were deposited to GenBank accession # MG029536–MG029553 and OP849668–OP849680 for Cyt-*b* and MG214765–MG214775 for the control region).

For phylogenetic analyses, an 841-bp-long region of cytochrome *b* was used. The sequences were obtained from 83 individuals collected in Georgia, and aligned with the 44 published homologous sequences of brown trout from western Eurasia, including those from the Ponto-Caspian basin (Ninua et al., 2018; Turan et al., 2009); the total number of sequences from each drainage (river basin is presented in Appendix 2) with accession numbers in Appendix 1.

DNA extraction and sequencing of complete mitochondria through NGS (Illumina)

We isolated mitochondrial fraction from two fish (one *S. rizeensis* and one *S. labtax*) muscle tissue according to Guderley & Johnston 1996 in order to increase mitochondrial DNA yield by reducing the proportion of nuclear DNA.

DNA from the obtained mitochondrial fraction was extracted by using the Qiagen DNeasy Blood & Tissue kit according to the manufacturer's instructions.

Sequencing libraries were prepared with 50 ng of DNA, randomly fragmented at 550 bp using a Covaris M220 Focused ultrasonicator (Covaris inc, Woburn MA). NEBNext ultra II DNA kit was used for library preparation according to the manufacturer's instructions. Adaptor-ligated DNA molecules were enriched by 10 cycles of PCR. The enriched libraries were purified by magnetic beads followed by eluting with Resuspension Buffer. Libraries were pooled and normalized to the 2nM final mix, 2N NaOH was added to equal volumes of the normalized DNA libraries. 1% PhiX DNA was used as internal control to observe the efficiency of DNA incorporation during DNA sequencing. Multiplexed two read libraries were sequenced on Illumina Miseq platform with Reagent Kit v2, 500-cycle. Fluorescent images were analyzed according to the Illumina base-calling pipeline 1.4.0 for obtaining FASTQ-formatted sequence data.

Quality control analysis was performed using FastQC v. 0.11.5 software (Andrews 2010), NGS data trimming and adapter clipping with Trimmomatic version 0.33 (Bolger et al., 2014) Quality trimmed reads de-novo assembled with CLC-Bio Genomics Workbench v 8.0 Consensus sequences were exported with at least 10X coverage (www.qiagen.com/us/search/clc-genomics-workbench). When the coverage did not meet the threshold, N (ambiguity) was inserted. At positions with disagreements in base calls between reads (minimum 10%), an appropriate ambiguous nucleotide symbol was inserted. Sequence assembly (De-novo and Reference guide) was performed with Geneious Prime (https://www.geneious.com) software.

DNA extraction, PCR and sequencing for molecular identification of myxozoan pathogens (Tetracapsuloides bryosalmonae & Myxobolus cerebralis)

DNA for *Myxobolus cerebralis* and *Tetracapsuloides bryosalmonae* diagnostic studies was extracted from cartilages of ventral calvarium (Kelley et al., 2004) and kidney tissues of fish respectively with DNA extraction kit (OxGEn) acccording to the manufacturer's instructions. from 18S ribosomal DNA fragment of *M. cerebralis* (causative agent of whirling disease) was amplified by nested PCR using TR5-16/TR3-16>TR5-17/TR3-17 primer pairs (Andree 1998). The first primer pair (TR5-16/TR3-16) amplifies 1300bp and the second one 415bp fragments of the gene. 640bp fragments of 18S ribosomal gene of *T. bryosalmonae* (causative agent of proliferative kidney disease) were amplified by Malacosporean-specific primer pairs mala-f and mala-r (Grabner & El-Matbouli, 2010).

Table 1. The primers used for myxozoan pathogen amplification and sequencing

Primer	Primer sequence	Annealing t	Reference
TR5-16	5'GCATTGGTITACGCTGATGTAGCGA-3'	57 °C	Andree 1998
TR3-16	5'-GAATCGCCGAAACAATCATCGAGCTA-3'	57 °C	Andree 1998
TR5-17	5'-GCCCTATTAACTAGTTGGTAGTATAGAAGC-3'	56 °C	Andree 1998
TR3-17	5'-GGCACACTACTCCAACACTGAMTTG-3'	56 °C	Andree 1998
Mala-f	5'- AAA CGA RTA AGG TCC AGG TC -3'	58 °C	Grabner & El-matbouli, 2010
Mala-r	5'- CAC CAG TGT AKC CCG CGT- 3'	58 °C	Grabner & El-matbouli, 2010

PCR amplification was performed on the thermal cycler "BioRad Tetrad 2". PCR conditions were the same for all primer pairs. 20 µL PCR reaction mix contained: (5x) Green GoTaq Flexi Buffer (Promega) 1X; 2mM $MgCl_2$; 0.5 µM each primer; 0.3mM dNTP mix; 1.5U GoTaq polymerase (Promega); 2µL DNA (10-35ng/µL); and nuclease free water filled up to 20 µl. The cyclic conditions were as follows: Initial denaturation for 5 mins at 95 °C , followed by 40 cycles of 30 secs at 95, 30secs at X °C (annealing temperature for each primer pair see in Table 1) and 1 min at 72 °C . Final elongation step was at 72 °C for 5 minutes followed by incubation at 10 °C for 5 minutes. PCR products were visualized on 1% agarose gel. We used 1 Kb Plus DNA Ladder (INVITROGEN) for defining the lengths of amplicons. The amplicons were sequenced on an ABI 3130 *XL* automated sequencer.

Microsatellite analysis

For investigating population genetic structure, we amplified 10 microsatellite loci for 238 samples of trout collected from the sampled locations (Figure 3, Appendix 2). Each locus was amplified at least twice for each sample to ensure reproducibility. Microsatellite loci were amplified in three multiplex reactions using fluorescently labeled forward primers (Table 2). The 10-µL final volume PCR mix contained 1 U of GoTaq Flexi DNA polymerase Promega), 1× Colorless Flexi 5× buffer, and 2.5 mM final concentration of MgCl2.

Amplification conditions were the same for all multiplex reactions: 3 min of initial denaturation at 95°C, followed by 30 cycles at 95°C for 20 s, 53°C with 0.2°C touchdown at each cycle for 30 s, and 72°C for 1 min. PCR products were mixed with formamide and LIZ 500 size standard (ThermoFisher). After 3 min denaturation at 95°C, samples were sequenced on ABI 3031 *xl* genetic analyzer. For analysis, we used GeneMapper vers. 5.0 (ThermoFisher). We used the software MICRO-CHECKER ver. 2.2.3 (Van Oosterhout et al., 2004) to check microsatellite data for large allele dropout and null alleles, separately for each of the study populations and each of the 10 studied loci.

Table 2. List of microsatellite locus names along with: repeat motif, multiplex group, primer melting temperature (Tm), primer concentration (PC; µmol), fluorescent dye (Dye), primer sequences, and references (Ninua et al., 2023)

Locus	Motif	Multiplex	Tm	Final conc. (µmol)	Dye	Sequences (5`-3`)	Reference
Ssa85	(CA)x(CG)x(CA)x	1	59	0.15	FAM	ACCCGCTCCTCACTTAATC	O'Reilly et al. (1996)
				0.15		AGGTGGGTCCTCCAAGCTAC	
Ssa408Uos	(GACA)x	1	56	0.15	VIC	CTCTTGTGCAGGTTCTTCATCTGT	Cairney et al. (2000)
				0.15		AATGGATTACGGGTACGTTAGA	
SsaD237	(TAGA)x	1	51	0.25	NED	CCTCTATCCATACAACACATGC	King et al. (2005)
				0.25		CAATGATGGAGTGGGAATTATC	
543AE	(CT)x	2	52	0.15	FAM	CTTTCTCTTGCGATAGTACGG	Estoup et al. (1998)
				0.15		GTTTCTACAGTCAGCACAAGTC	
SsoSL417	(GT)x	2	57	0.125	VIC	TTGTTCAGTGTATATGTGTCCCAT	Slettan et al. (1995)
				0.125		GATCTTCACTGCCACCTTATGACC	
SSsp2201	(GATA)x	2	60	0.125	VIC	TTTAGATGGTGGGATACTGGGAGGC	Paterson et al. (2004)

				0.125		CGGGAGCCCCATAACCCTACTAATAAC	
BG935388	(CAAT)x	2	54	0.125	NED	TGACCCCACCAAGTTTTTCT	Vasemaegi et al. (2005)
				0.125		GTTTAAACACAGTAAGCCCATCTATTG	
Ssa197	(GTGA)x(GT)x	3	57	0.2	FAM	TGGCAGGGATTTGACATAAC	O'Reilly et al. (1996)
				0.2		GGGTTGAGTAGGGAGGCTTG	
Ssa410Uos	(CAGA)x	3	54	0.25	FAM	CTACAATCTGGACTATCTTCTTCA	Cairney et al. (2000)
				0.25		GGAAAATAATCAATGCTGCTGGTT	
SSsp2216	(TAAC)x	3	60	0.2	NED	GCCAACAGCAGCATCTACACCCAG	Paterson et al. (2004)
				0.2		GGCCCAGACAGATAAACAAACACGC	

Phylogenetic analyses

We used three methods for the analysis of cytochrome *b* sequences: (1) Minimum spanning network was constructed only for the trout from Mediterranean basin and the rivers drawing into Gulf of Persia the Black and the Caspian Seas (Ponto-Caspian Basin) using NETWORK 10.2.0 (Bandelt et al., 1999). (2) Maximum likelihood (ML) tree was built and bootstrap support was estimated using software MEGA 7.0.21 (Kumar et al., 2016). In this analysis, besides the fish from

the Ponto-Caspian drainage, the fish from the drainage of the Indian and the Atlantic Oceans and Mediterranean (nominal *S. trutta*, *S. marmoratus*, *S. platycephalus,* and *S. akairos*) were included along with Balkan species *S. ohridanus* and *S. obtusirostris* (Crête- lafrenière et al., 2012), and Atlantic salmon (*S. salar*) was added to the analysis as the outgroup. For this analysis, only one haplotype/location was included if more than one individual from the same location had the same haplotype. The best-fit model (that with the lowest Bayesian Information Criterion, BIC, as recommended in Nei & Kumar, 2000) was identified with MEGA 7.0.21 using ML method (3) Bayesian inference (BI) tree was built using the same taxa included in the ML analysis, for validating the tree topology using different approach, counting posterior probability of the branches,

and inferring split times using BEAST v. 2.6.3 (Drummond et al., 2012). Settings for BEAST for Bayesian analysis included: relaxed lognormal clock, Yule model, random distribution of the offspring number among the individuals; MCMC = 100,000,000; nucleotide substitution models were selected using MEGA software (Kumar et al., 2018). The Bayesian analysis was initiated from random starting trees, assuming an uncorrelated log-normal relaxed clock model and a coalescent model with constant population size. Posterior distributions of parameters were approximated using Markov chain Monte Carlo with chain length set at 100,000,000 to provide sufficient sample size for each parameter (i.e., effective sample size $\gg$ 100). The same substitution model was used as in the ML analysis.

We inferred the best-fit model and conducted ML analysis for the obtained and downloaded sequences of the control region. These sequences did not show much informative variability among the studied taxa, and we did not apply BI for this dataset. The software used was MEGA 7.0.21. We used BEAST v. 2.6.3 (the uncorrelated log-normal relaxed molecular clock model) to account for variable rates of evolution among the lineages and to infer the 95% HPD intervals for the node ages, based on cytochrome *b* gene variation. For calibrating the tree, we used an estimated time of split between the reference cytochrome *b* sequences included in our analysis, drawn from multiple publications (www.timetree.org; Hedges et al., 2015). The divergence time

between *S. ohridanus* and *S. obtusirostris*, in one clade, and *S. marmoratus*, *S. platycephalus*, and *S. trutta* in the other was set as a time of the initial split. We also used the alternative scaling by Schenekar et al. (2014), not considered by www.timetree.org, and based on the sequence analysis of two distant lineages ("Atlantic" vs. "Black Sea" lineages) from Austria (Schenekar et al., 2014), which suggests a much later split between the lineages than other authors (Alexandrou et al., 2013; Crête-Lafrenière et al., 2012; Macqueen & Johnston, 2014).

Population genetic analyses

Individual microsatellite profiles were subjected to principal component analysis (PCA), to visualize clustering of the populations in PCA space. The software used was SPSS 23.0 for Windows (IBM corp., 2015). We used STRUCTURE ver. 2.3.4 (Pritchard et al., 2000) to evaluate the genetic structure across the Ponto-Caspian populations. In the analyses we used an admixture model, assumed independent allele frequencies, and 50,000 iterations as burn-in, with a total of 500,000 iterations. The number of potential population clusters (K) was set from 1 to 19 with five independent runs for each. The most probable K was chosen using the online tool Structure Harvester (Earl & VonHoldt, 2012). The number of alleles, allelic richness, allele frequencies, and deviations from Hardy–Weinberg equilibrium (HWE) were evaluated using Arlequin ver. 3.5 (Excoffier & Lischer, 2010). The same program was applied to calculate the pairwise fixation index *F*ST (1) between geographic populations of the Black Sea versus Caspian Sea basins and (2) among individual populations within each of the two sea basins, Caspian and Black, separately. In addition, we calculated the pairwise *R*ST value a modification of the fixation index for microsatellite loci; Gaggiotti et al., 1999; Slatkin, 1995) among the same populations. Sequential Bonferroni correction (Rice, 1989) was applied to the respective p values. Finally, population genetic structure was evaluated by analysis of molecular variance (AMOVA) using ARLEQUIN ver. 3.5.2.2 (Excoffier & Lischer, 2010). We applied locus-by-locus analysis of genetic differentiation between the Black and Caspian Sea basins, among the populations from each of

those basins, and among individuals of the same population (no individual level included), in order to infer whether the differences between the sea basins exceed the differences among the populations. We used 16,000 permutations for testing the significance of the obtained values; the distance matrices were computed based on different allele numbers (*F*ST-like) and the sum of squared differences (*R*ST-like), for validating the concurrence of the estimates based on these two metrics. We used the models comparing two groups (Black and Caspian Sea basins) and six groups (population outliers from Chvana and Shareula, the rest of the populations from the Black Sea basin, populations from Kura, Tergi, and Sulak drainages).

Inferring isolation by distance

We used simple Mantel tests (Manly, 1986) to test whether isolation by distance best explains genetic structure of the sampled populations. We calculated correlation coefficients *Rxy* and the significance level *p* between two distance matrices: geographic riverine distances among the studied locations and genetic distances calculated as *F*ST (among-population component of departure of genotype frequencies from Hardy–Weinberg equilibrium not taking account of allelic differences) and *R*ST (the departure while accounting for distances among alleles). Since populations from the Black and the Caspian basins appear to be effectively isolated, even if they are geographically close (Ninua et al., 2018), we separated analysis for the populations from rivers draining into these two seas. The riverine distances among the locations in the rivers independently flowing into the Black Sea (e.g., Chorokhi, Rioni, and Enguri) were calculated as the sum of the distances from the location to the respective river mouth, plus the distance along the Black Sea coast between the mouths of the two rivers. The same approach was used for the rivers draining into the Caspian Sea basin. To avoid possible nonlinear effects, prior to running Mantel tests, we log-transformed the geographic distances. The software for running Mantel tests was zt (Bonnet & Van de Peer, 2002).

RAD sequencing and data analysis

The ddRAD-seq libraries were prepared following a protocol described by Brelsford et al. (2016), which included (i) digestion of the genomic DNA with PstI and MseI restriction enzymes; (ii) the ligation of indexed adapters to the digested fragments; (iii) the amplification of double indexed libraries, and (iv) purification to remove the leftover adapters and short DNA fragments. The individual libraries were pooled in equimolar concentrations for sizeselection in a 350–550 bp window on a Pippin Prep instrument (Sage Science, Beverly, MA, USA). The final pool was outsourced to a commercial provider Macrogen-Europe (Amsterdam, The Netherlands) for sequencing on a single flow cell of an Illumina HiSeq X platform with 2 × 150 paired-end reads.

The sequence data were demultiplexed using process radtags and suspected PCR duplicates were removed using clone filter commands in STACKs 2.62 (Rochette et al., 2019). The reads were then aligned to the chromosome-level reference genome assembly of *Salmo trutta* (NCBI acc.no. GCA_901001165.2) using Bowtie2 (Langmead & Salzberg 2012), with the conservative global alignment option (end-to-end). The reads with QC < 30 were discarded using SAMtools (Danecek et al., 2021). The ref_map.pl program in STACKs was used to build a catalog of RAD loci and assemble the SNP datasets.

Two SNP datasets were produced (in populations program in STACKs) for different types of subsequent analyses: (A) all ddRAD loci from the core sample set included without imposing any population representation filters (B) SNPs were additionally subjected to LD-pruning in PLINK 1.9 (Chang et a., 2015) by applying the --indep-pairwise 50 5 0.5 filter, the resulting MAP/PED files were formatted to 'snpRdata' object in snpR package Hemstrom et al., 2023)

SNP Set (A) was analyzed using RADpainter and fineRADstructure software version 0.3.2 (Malinsky et al., 2018)

Results

Trout distribution and population status

During this study up to 5000 fish were caught from more than 70 localities. The predicted distribution of brown trout nominal species (based on 120 locations with confirmed trout presence) is shown on Figure 8.

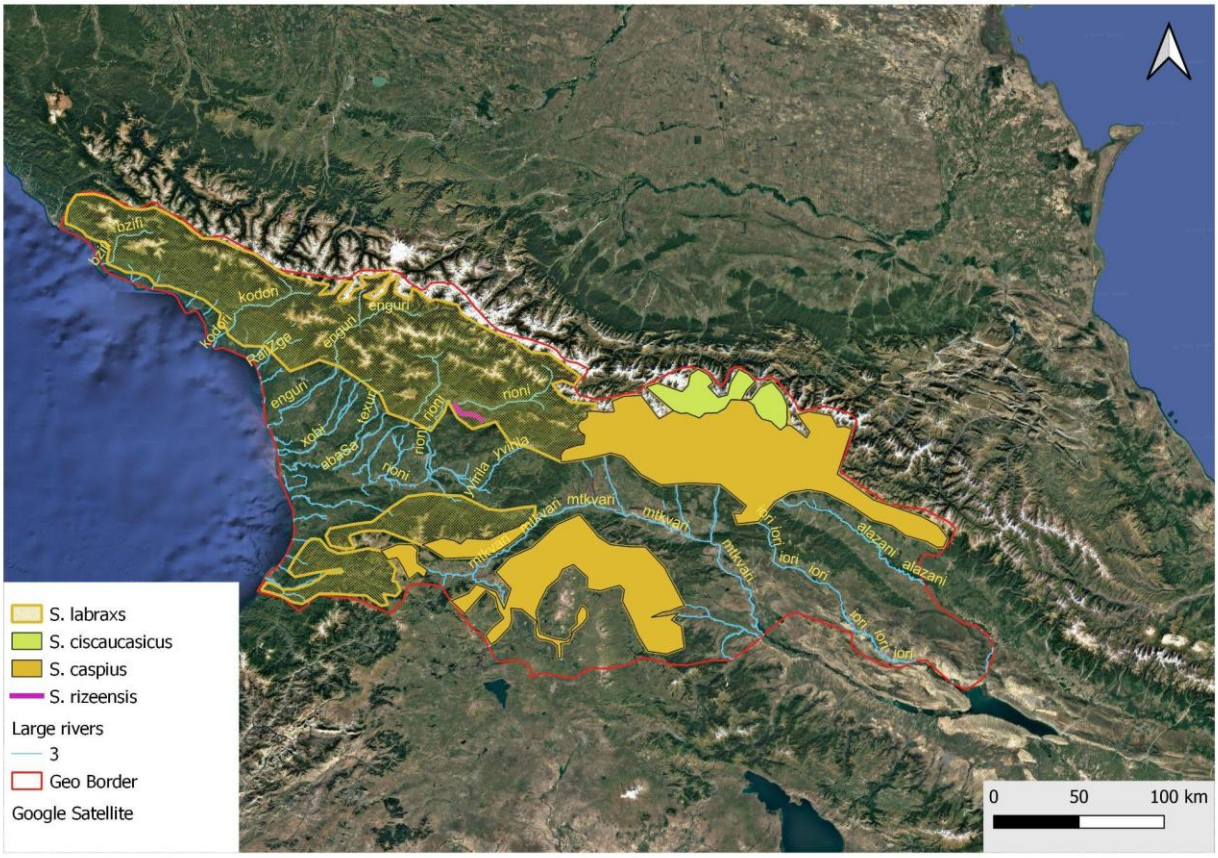

Figure 8. Distribution of nominal trout species in Georgia.

Data collected during the last 10 years indicate that all nominal species (will be discussed later) of trout except nominal *S. rizeensis* (only found in one stream in Georgia) are wide spread on their historical distribution areas in Georgia. Despite the wide distribution, the population trends for most of the watercourses are decreasing. However, we don't have scientific evidence for that due to the absence of a long-term monitoring system. This is mainly based on empirical data of

fishermen and scientists working in this field (including my experience of working on trout over the last ten years).

Relative abundance, age structure, age at maturity and longevity

The first assessment of trout populations on 13 rivers of 6 protected areas was conducted in 2021-2022. The catch per unit effort (CPUE) for each population is compared on Figure 9.

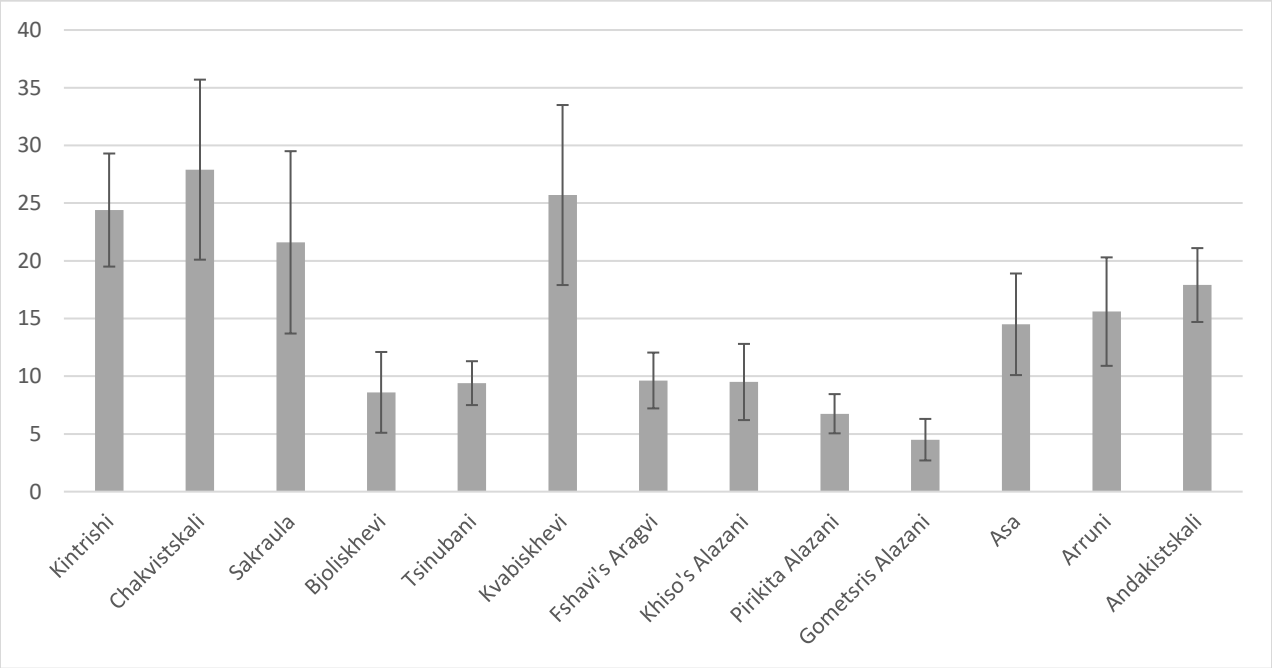

Figure 9. CPUE of 13 river trout populations from 6 protected areas. Error bars are 95% confidence intervals (two times the standard error).

The frequencies of each length class (size structure) for Kintrishi river (taken as an example) in 2021 is shown on Figure 10.

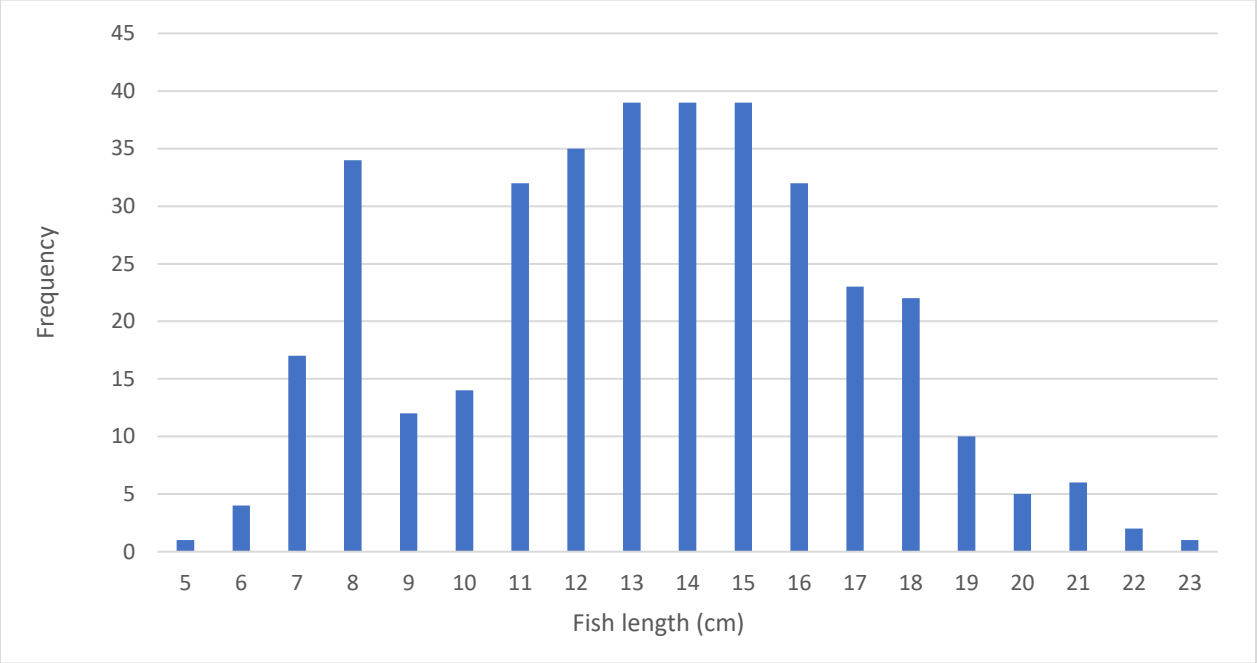

Figure 10. The size structure of Kintrishi River trout population in 2023.

The size structure for each population was converted to an age structure based on the ages defined for a group of fish from each length class in the sub-sample (Figure 11).

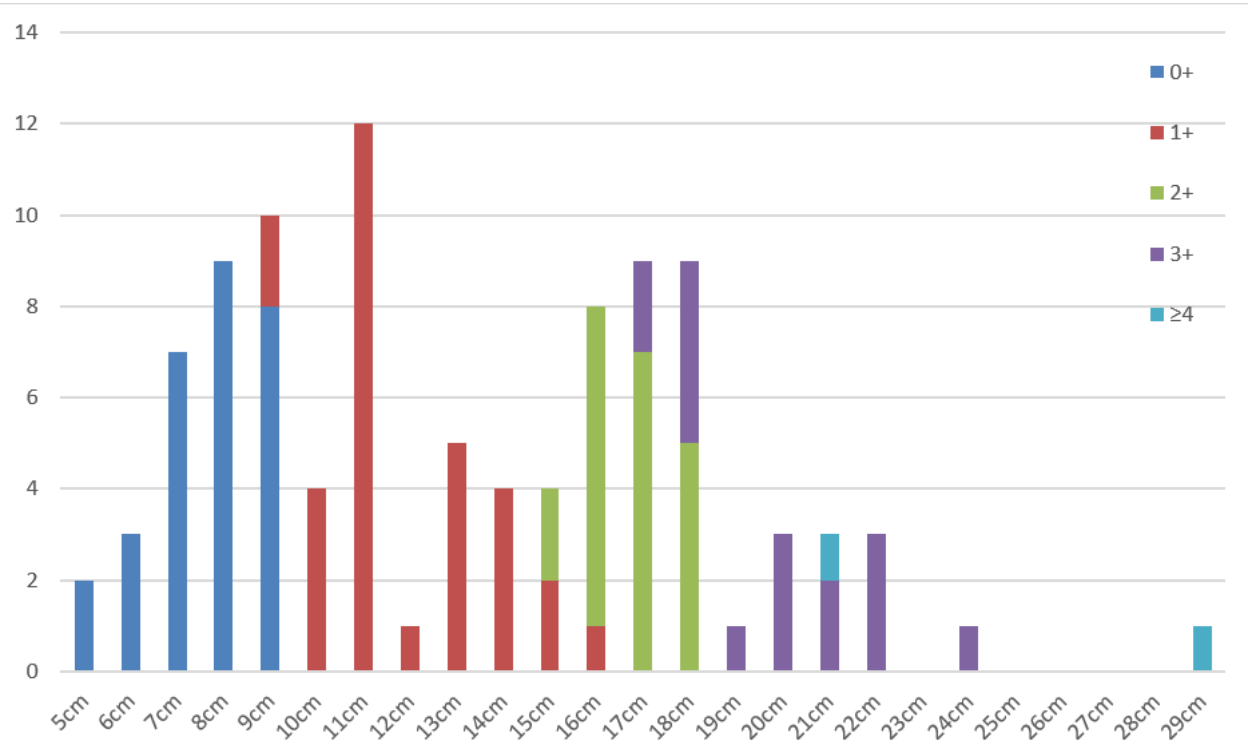

Figure 11. The frequency of different age fish in each length class (based on mixed samples from Kintrishi and Chakvistskali).

A total of 100 fish were analyzed. From these data, age classes were defined as follows: 0+ for 5-9 cm fish, 1+ for 10-14 cm fish, 2+ for 15-18 cm fish, 3+ for 18-24 cm fish, and 4+ for trout exceeding 24 cm. (age was defined for each population or for a group of populations geographically close to each other). The proportion of each age class in each population is shown on Figure 12.

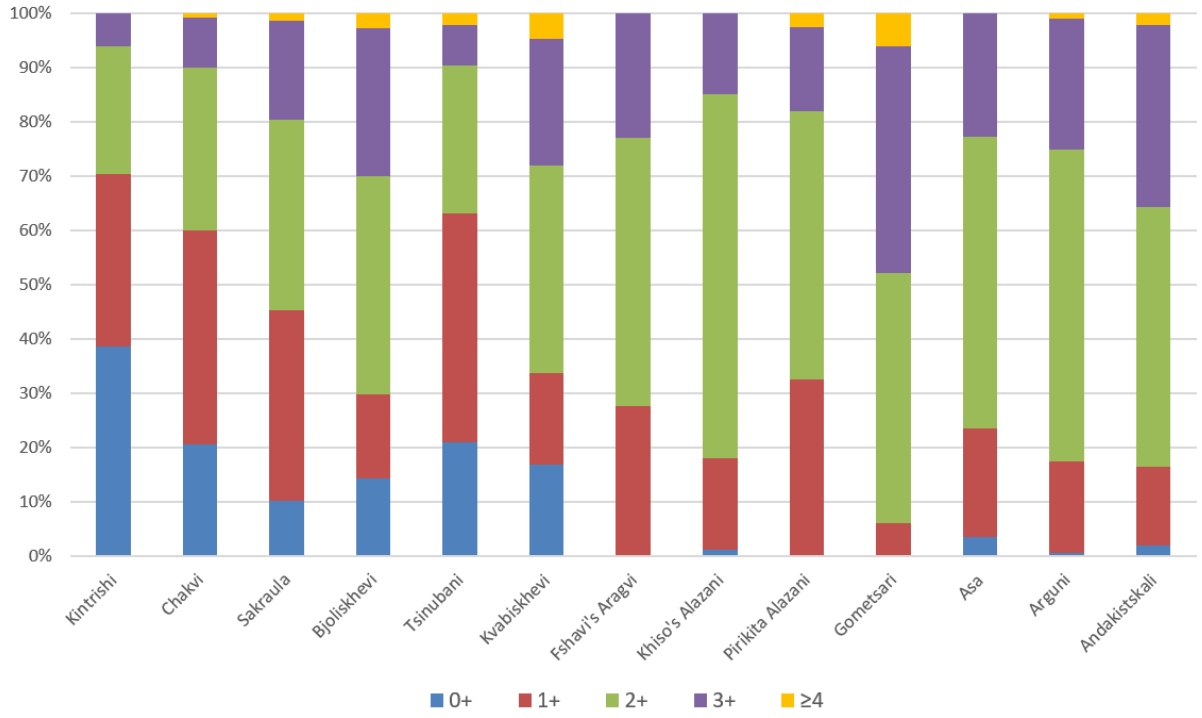

Figure 12. The proportion of each age class in each population studied population

Longevity of trout in analyzed populations is up to 5 years. The age of the largest fish (25-35cm) based on otolith studies was a maximum 4+ years. 5+ was defined for only one, 29cm individual from Tsinubnistskali River. The majority of studied populations were mainly represented with 4 age classes (from 0+ to 3+). The proportion of 4+ year old fish in different population samples varied in 1- 6% range. There was a difference between the population groups from Lesser (first

six populations on figure 12) and Greater Caucasus (last six populations on figure 12). Age 0+ is almost absent in the sample from rivers flowing from the Greater Caucasus.

The youngest mature trout were in their third year (2+). 10 males and 2 females out of 98 trout from all sampled populations were mature at age 2+. 61 % of trout were mature at age 3+ (N=62). The sex ratio in 3+ years old fish was 27/35 (females/males). All fish (n=10) of age 4+ were mature.

The distribution of sampling locations and trout relative densities assessed based on relative abundance are shown on Figure 13.

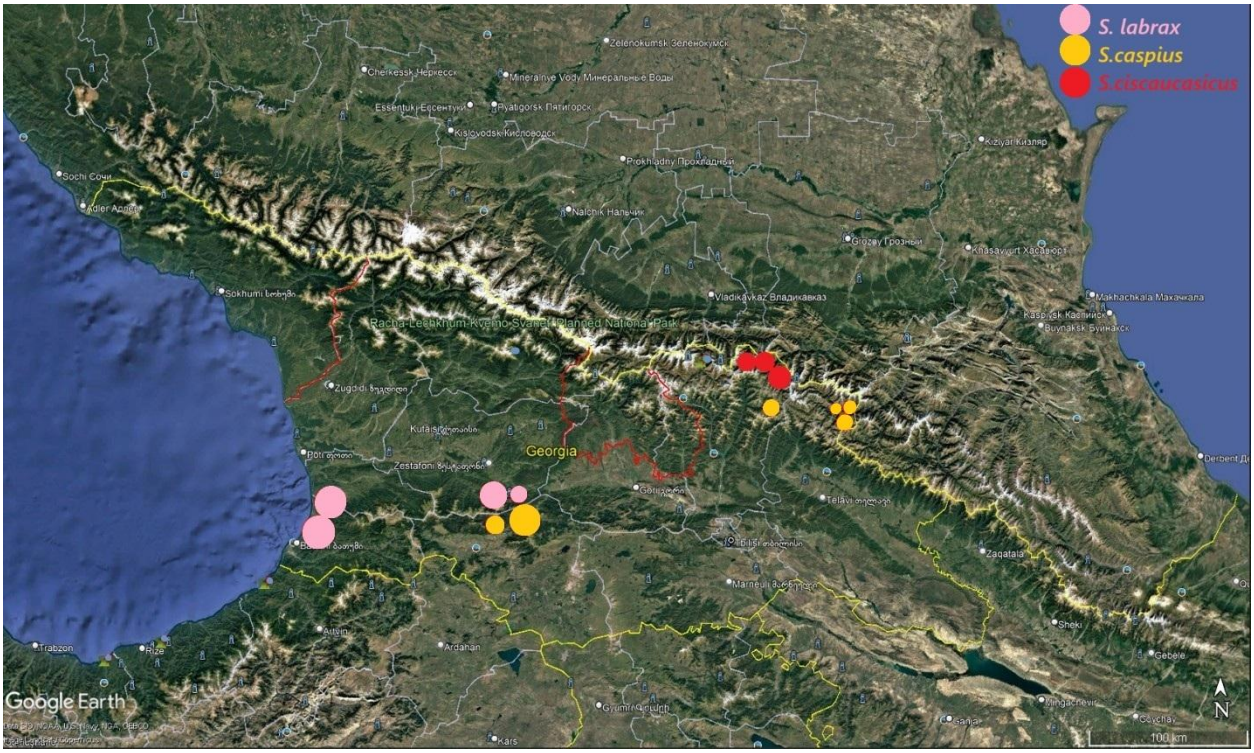

Figure 13. The distribution of sampling locations. The color of circles indicates nominal species and the size reflects the relative density (based on relative abundance) of fish at each sampling location in 2022

Trout prey taxa

19 Orders, 60 Families and 33 Genera of aquatic and terrestrial invertebrates were identified from stomach content of 181 trout from 13 populations (kintrishi, Chakvistskali, Kvabiskhevi,

Tsinubnistskali, Bjoliskhevi, Sakraula, Fshavi's Aragvi, Asa, Arguni, Andakistskali, Khiso's Alazani, Gometsris Alazani and Pirikita Alazani). Trout diet in studied populations from North Eastern Georgia (Fshavi's Aragvi, Asa, Arguni, Andakistskali, Khiso's Alazani, Gometsris Alazani and Pirikita Alazani) were dominated by following orders: Hymenoptera (mainly Formicidae), Trichoptera, Diptera (mainly Blephariceridae) და Orthoptera. For the majority of fish (irrespective of their size) around 60-70% of stomach contents were represented with Formicidae (ants). Some families of Diptera (especially Blephariceridae) were found mainly in small size fish while Orthopterans (Tettigoniidae) only in large fish (>23cm).

More than 90% of sampled trout population diet from central and West Georgia were represented with Orders: Ephemeroptera, Trichoptera, Diptera, Hymenoptera and Amphipoda. Although, the proportion of each taxon differed by the river. In the case of Kintrishi and Chakvistskali over 80% of identified individuals were Ephemeroptera and Trichoptera. The proportion of those two Orders in Sakraula, Bjolistskali and Kvabiskhevi trout diet was up to 55%, 65% and 19% respectively. On the other hand, we found Amphipoda almost exclusively in Kvabiskhevi trout where it represented 45% of the whole diet, while less than 1% in the diet of other trout populations (Figure 14).

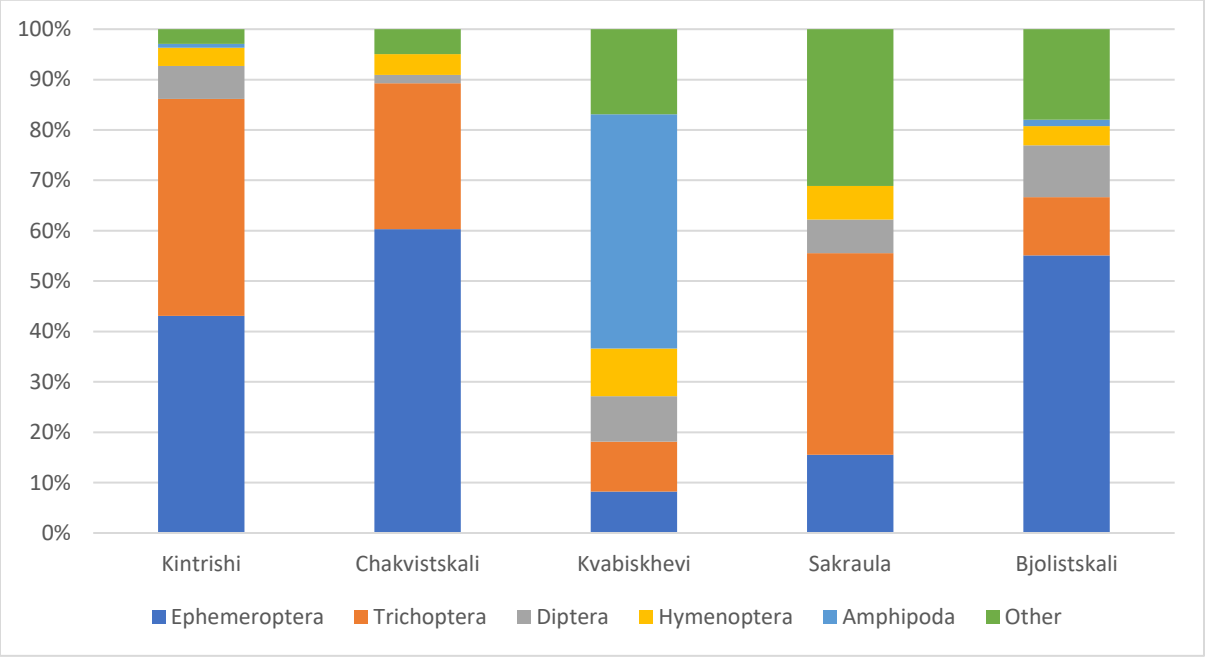

Figure 14. The proportion of most abundant taxa in diet of different trout populations

Distribution of Bryozoa in trout rivers in Georgia

Two Genus of Bryozoa were found on five locations, in 4 out of 13 sampled watercourses (Figure 17). Colonies of *Fredericella* sp. were found in Aragvi River, downstream of Jinvali reservoir and in Tabatskuri Lake in southern Georgia (Figures 15 & 16).

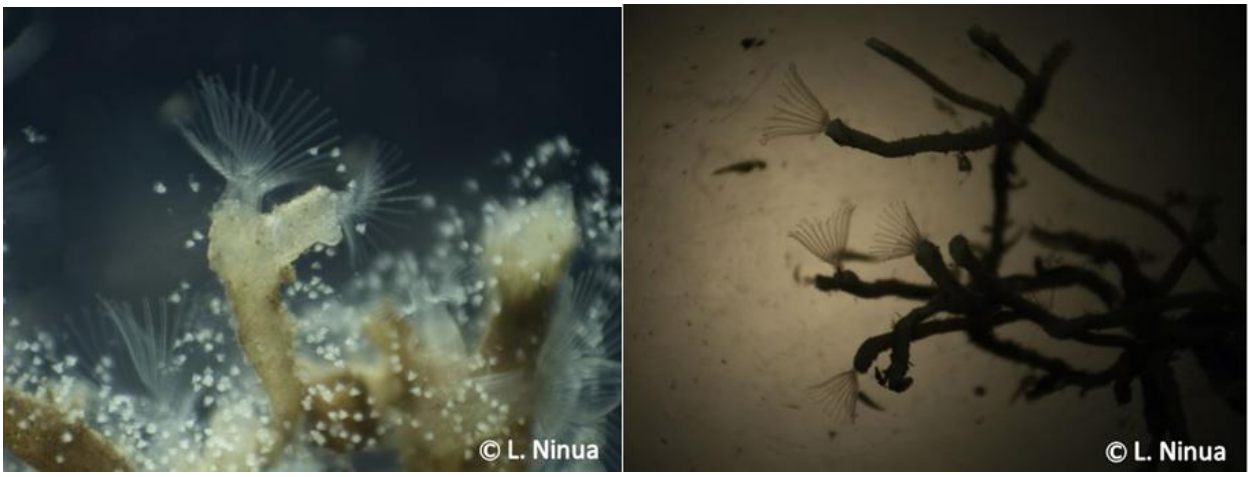

Figure 15. *Hyalinella punctata* (left) and *Fredericella* sp. (right)

COI sequence from that specimen showed 89.25% and 88.96% identity with undetermined species (Family: Fredericellidae) from GenBank and BOLD DNA databases respectively (indicating the absence of identified Bryozoa sequences in the genetic databases).

Figure 16. *Hyalinella punctata* colonies from Bugdasheni River

Second Bryozoa species identified in Tabatskuri Lake was *Hyalinella punctata* (97% COI sequence identity with the closest sequence from BOLD and GenBank repositories). *H. punctata* (Figures 15 & 16) was identified on 3 other locations on Javakheti Plateau in southern Georgia.

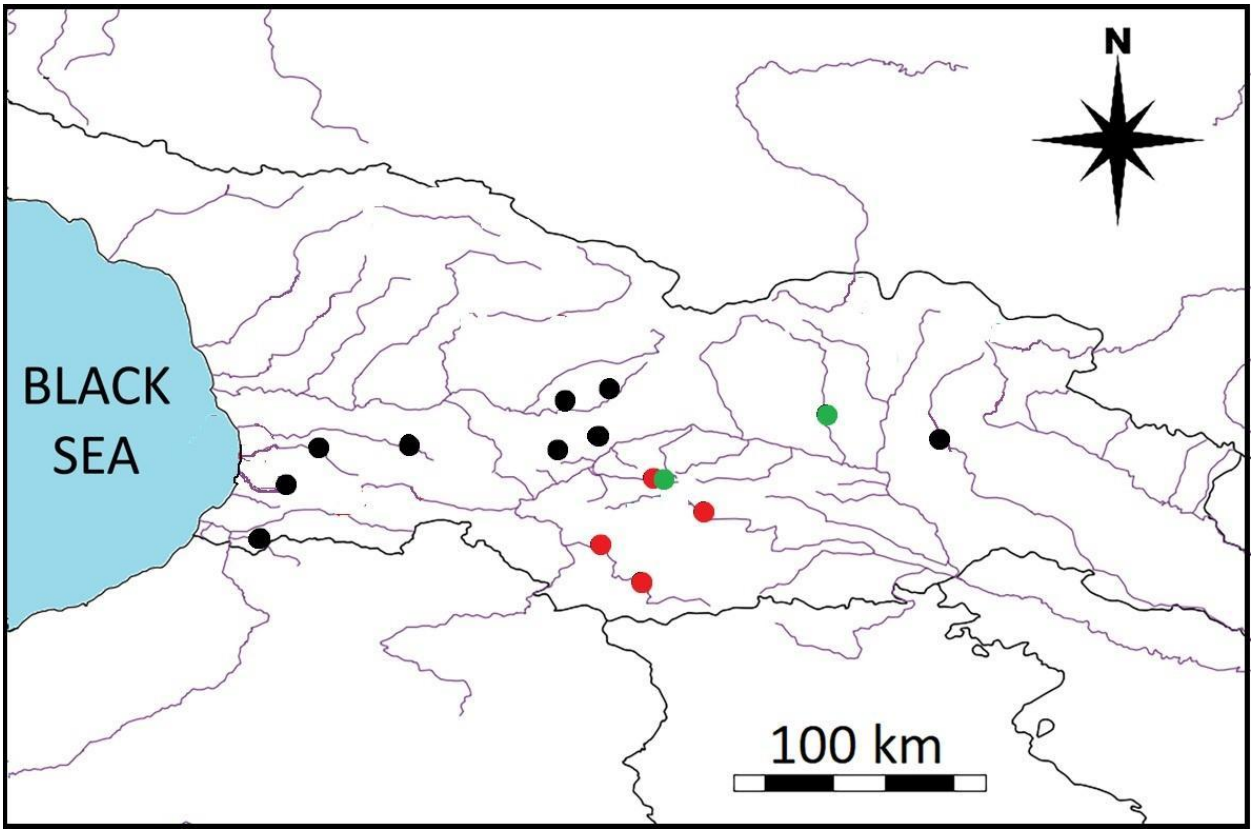

Figure 17. Bryozoa sampling locations are shown with circles. Green and red circles show locations where *Fredericella* sp. and *Hyalinella punctata* were found respectively.

Proliferative kidney disease (PKD)

229 kidney tissue samples including fish from 10 (4 *S.labrax* and 6 *S. caspius* populations) wild trout populations (119, mean sample size = 11.9); 6 rainbow trout fish farms (59, mean =9.8) Tabatskuri lake population of introduced European cisco (24), Kura Loach (*Oxynoemacheilus brandtii*) and 13 Bryozoa colonies (7 from *H. punctata* and 6 from *Fredericella* sp.) were screened for Malacosporeans. None of the salmonid species were positive for target myxozoans. At the same time two *H. punctata* but none of *Fredericella* sp. were positive on Malacosporean targeting PCR. Sequencing of PCR amplicons revealed that all positive Bryozoa was infected with *Buddenbrockia plumatellae* (but not *T. bryosalmonae*), one of the few known Malacosporean

species infecting kidney tubules of Cyprinid fish. Interestingly, *T. bryosalmonae* was accidentally found in Kura loach (*Oxynoemacheilus brandtii*) while investigating the prevalence of *B. plumatellae* in different cyprinid species in the same river system *B. plumatellae* positive Bryozoa were found (Figure 18). Kura loach was only one out of 5 Cyprinid species from Bugdasheni river which was exclusively infected by *T. bryosalmonae*. This might indicate that Kura loach is a true host for the pathogen.

Figure 18. Maximum likelihood tree based on 18S ribosomal gene sequences of Malacosporeans. Bootstrap support shown above the nodes. Two species of Malacosporeans identified in fish and Bryozoa during this study (*Tetracapsuloides bryosalmonae* in green and *Buddenbrockia plumatellae* in grey).

Whirling disease

78 wild trout specimens from 6 trout populations (two *S. labrax* and four *S. caspius*) and 59 (49 rainbow trout and 10 native trout) from 4 trout farms were screened for *Myxobolus cerebralis*. Pathogen was identified in any wild populations with the highest prevalence in Chakvistskali population - 80% (12/15), followed by Kintrishi - 55% (12/22), Kvabiskhevi – 40% (6/15), Iori 17% (2/12) and Ktsia – 8% (1-12). Only symptomatic fish found in Khiso's Alazani River was positive on *M. cerebralis* (Figure 19).

Figure 19. Trout with spinal deformities associated with *M.cerebralis* infection from Khiso's Alazani River (left) and rainbow trout from fish farm (right).

Trout in two out of four fish farms had the symptoms of either acute (in juveniles) or chronic infection (in adults). One farm had 20% (7000/35000) mortality of juvenile fish in three weeks. In the second farm we just found several size and age fish with symptoms of previous infection (Figure 19) kept in separate pool. We tested oligochaetes (definitive host) from outlet channel of trout fish farm on *M.cerebralis* in order to assess the probability of pathogen persistence in the system and the risk of future outbreaks. *M.cerebralis* infection in oligochaetes was confirmed by PCR.

We did not manage to find oligochaetes in the rivers where wild trout were infected by *M.cerebralis*.

Acanthocoephala in wild trout

An investigation of 302 trout of 13 populations (mean sample size = 23) on digestive system macroscopic parasites revealed high Acanthocephala infestation rate in Kvabiskheri River population (Figure 20). The prevalence of Pomphorhynchus sp. in Kvabiskhevi trout was 100% (the sample included 5 age classes) while there was no Acanthocephala found in any other trout populations.

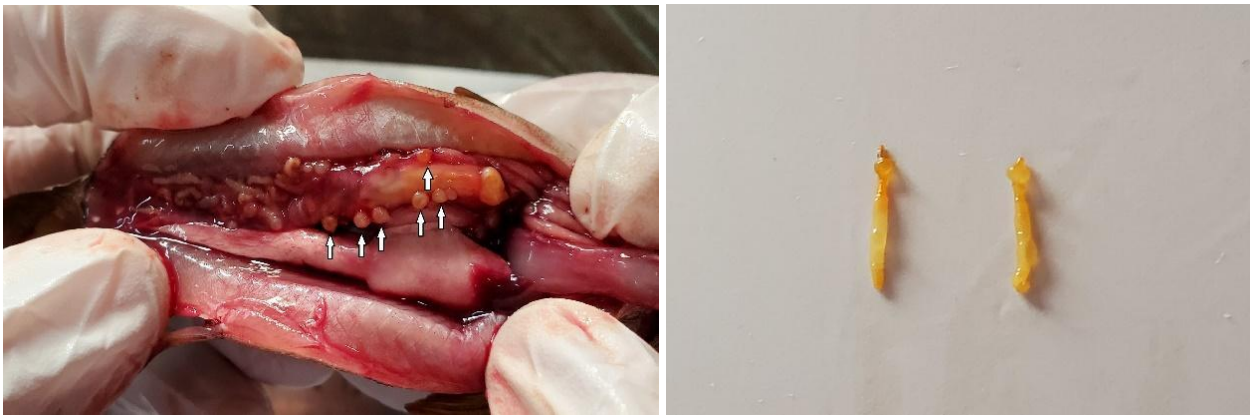

Figure 20. The pathogen species of *Pomphorhynchus* genus isolated from Kvabiskhevi River trout. (right). The rounded proboscis of the pathogen visible after the penetration of trout gut wall (left).

In the same study, we collected the stomach content of every euthanized trout and identified arthropod species in order to investigate the local trout food base. Obtained data shows that the dominant taxon (<45%) in Kvabiskhevi trout diet is *Gammarus* sp. (Amphipoda) while in most of the other populations Gammarus represent less than 1% of trout diet (Figure 14).

The growth rate of trout in Kvabiskhevi river seemed slower than in any other populations which can be associated with Acanthocephala infection (Figure 21).

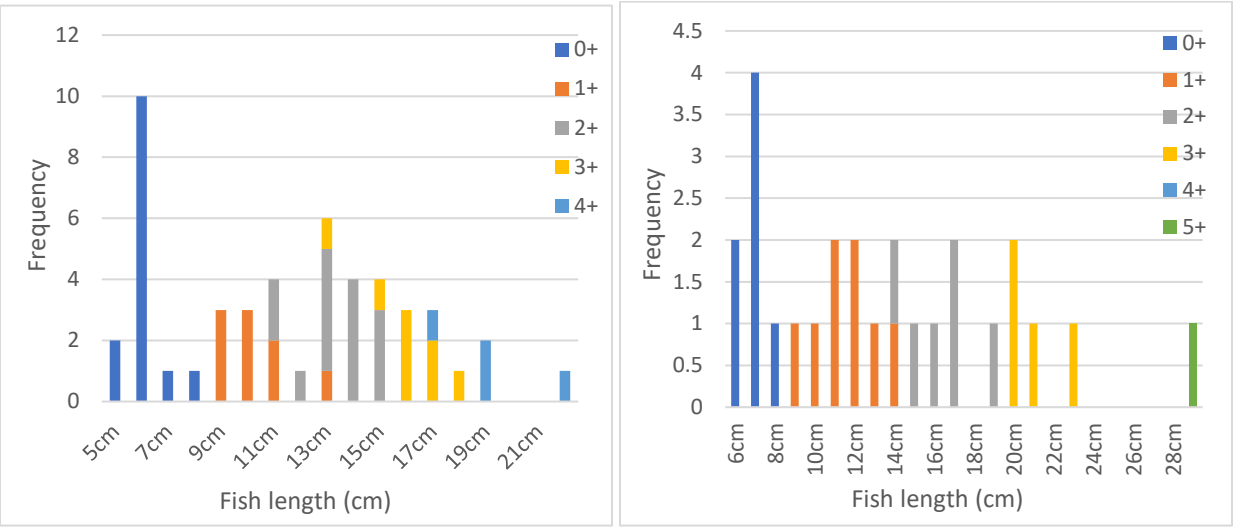

Figure 21. Frequency of different age fish in each length classes (Kvabiskevi population (left) and adjacent Tsinubnistskali population (right).

Other threats trout facing in Georgia

Barriers on the way to migration

We identified artificial barriers on six out of 13 rivers. We think those barriers either completely block or seriously impair trout (and other fish species) migration on that section of the river.

Hydroelectric power station on Kintrishi River

Two kilometers downstream from the border of Kintrishi protected areas there is a dam supporting the Kintrishi hydropower station with water. The majority of river water collected by this dam flows into the pipe and supplies turbines in the main station building almost 3 km downstream the dam. The dam has a fish passage. There is a small waterfall on the last part of the vertical slot fish ladder. Water from the last part of the fish ladder directly drops on a large piece

of rock. There is no pool under the waterfall deep enough for fish to pass the barrier. I think this is the most difficult part of passing the passage (Figure 22).

Figures 22. Fish passage (vertical slot fish ladder) on Kintrishi hydropower station dam.

Another problem related with this dam could be, there is no special construction that would direct fish, migrating downstream, to the fish passage or protect them from turbines. The only barrier for fish on the way to turbines is the set of iron plates. The main function of this iron construction is to protect turbines from wood debris brought by the river. The distance between the plates is 4 centimeters which is large enough space to pass even the largest fish we caught during our fieldwork.

Chakvistskali dam

Chakvistskali dam is located on Chakvistskali River 700-800 meters downstream from the Mtirala national park border (Figure 23). The dam is used to collect drinking water for Batumi (city). There is no fish passage on the dam. I think that the waterfall cascade flowing from the top of the dam is unpassable for fish migrating upstream.

Figure 23. Dam on Chakvistskali River

Chakvistskali and Kintrishi are rivers where anadromous populations of trout still exist. Most of the smolts included in genetic studies are from estuarine areas of those rivers.

Hydroelectric power stations on Arguni river

There are micro hydroelectric (run-off-river) power stations on Asa and Arguni Rivers. However, according to their scale and construction type, they should be much less harmful to trout populations than e.g. hydropower plant on Kintrishi River, which direct more than 80% of river water in turbines (described in 2021 report). The main difference from Kintrishi hydropower plant is that micro hydroelectric power stations in Khevsureti use less than 20% of whole river water

(Figure 24) and the distance between the metal plates making a sieve for gravel and wood debris on the way to the turbine is 2cm (4cm in case of Kintrishi station). 2cm is enough space to pass 1 year and possibly up to 2 years old fish towards the turbine. However, regarding the scale of hydroelectric power stations, and proportion of water diverted, trout in Khevsureti Rivers (compare to Kintrishi) have higher probability to avoid the turbines.

Figure 24. Hydroelectric power stations on Arguni River

Small dam on the tributary of Tsinubnistkali River

Tsinubnistskali dam is located in Borjom-Kharagauli national park on the small tributary of Tsinubnistskali River (Figure 25). The dam is built to collect drinking water for adjacent villages. There is no fish passage on the dam. It seems that most of the year only a little water is flowing

over the dam which we think makes it unpassable for trout. According to the high density of small size fish, we caught during fieldworks, this tributary should be an important spawning and nursery ground for trout. It is not assessed yet if this dam affects the trout migration.

Figure 25. Tsinubnistskali tributary and small dam on it

Poaching

Indications of poaching were obvious on some rivers even in protected areas:

1. CPUE for those rivers were much lower compared to the rivers of same size (or even smaller) and morphology from the same protected areas.
2. We caught almost no fish on the same rivers even a few hundred meters outside the border of protected areas.
3. The highest number of fish on those rivers we caught on sampling points near the ranger stations.

Taxonomy and evolutionary history of Ponto-Caspian trout

In order to solve the uncertainty in Ponto-Caspian trout taxonomy along with 44 brown trout sequences downloaded from GenBank we analyzed 841bp Cytochrome b sequences for 78 trout derived from 19 rivers of Black and Caspian Sea basins along with 44 brown trout sequences downloaded from GenBank.

Both trees constructed by Maximum likelihood and Bayesian algorithms, supported the monophyly of Ponto-Caspian trout. All trout sequences (obtained during the study and downloaded from GenBank) except S. obtusirostris and S. ohridanus form two monophyletic clades (Figure 26, see also Appendix 4) One includes the sequences of fish from the rivers flowing into the Atlantic Ocean and the other – fish from Mediterranean, Black and Caspian seas basins and Euphrates River (Persian Gulf). This suggests that the basal genetic diversification of brown trout from the Ponto-Caspian region occurred in the northern and eastern Anatolia, where the split between the Mediterranean and Ponto- Caspian lineages most likely occurred.

The Ponto-Caspian clade comprises five monophyletic well-supported subclades: one encompasses the fish from the Caspian Sea basin, second – from the Danube River basin and least three – Eastern Black Sea basin. The one out of the three is represented by nominal S. rizeensis and trout sequences from Shareula (Rioni basin) forming a separate monophyletic clade. The clades from the eastern Black Sea drainage, except *S. rizeensis* clade, are not geographically distinct. Each of them includes the specimens from at least two of the main river basins (Chorokhi, Enguri, Rioni) and small rivers - Chakvistskali and Kintrishi. Trout from the coastal area of the Black Sea are distributed in two well supported clades. Additionally, the trout individuals identified as *S. coruhensis* by Turan et al. (2009) were present in both clades. Based on these we can conclude that anadromous life mode is typical for all trout lineages from the eastern Black Sea drainage, with the exception of *S. rizeensis*. The same is true for the clade from the Caspian Sea drainage, which along with the trout from Lake Sevan (nominal *S. ischchan*) contains at least one anadromous form captured in the Caspian Sea. This clade contains,

additionally, a well-supported subclade of fish from the basin of the River Tergi (nominal *S. ciscaucasicus*).

Bayesian inference could not resolve (posterior probability < .5) the relative positions of the five described clades, suggesting a bush-like pattern of evolution.

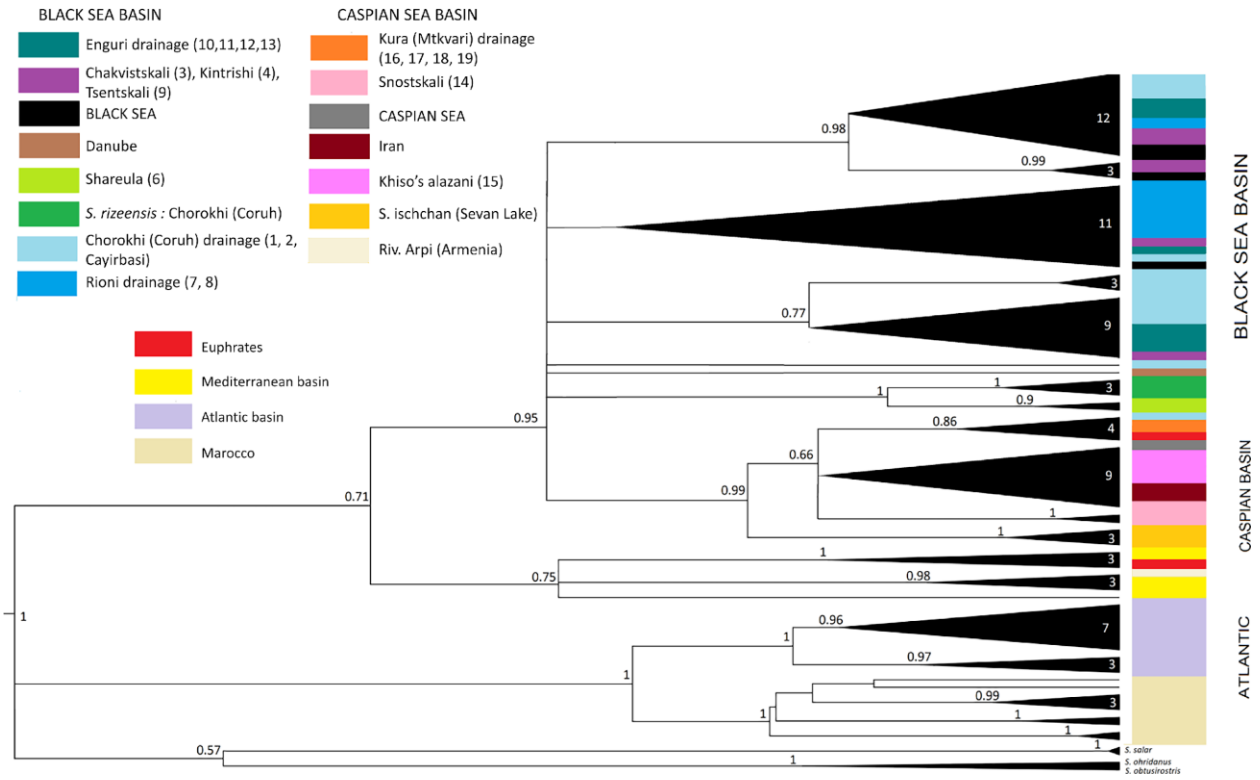

Figure 26. The Bayesian inference tree showing mitochondrial (cyt *b*) haplotype phylogeny in our samples and other published sequences of brown trout. The numbers on the triangles show the numbers of unique sequences from each cluster (Ninua et al 2023).

Control region

The T92 model (Kumar et al., 2016) was identified as the best-fitting substitution model for the control region of the ingroup samples. Both sequences obtained in this study and downloaded

from GenBank showed minimal genetic divergence across the analyzed segment and failed to distinguish between Black Sea, Caspian Sea, and Aral Sea evolutionary lines (Figure 27). However, the tree was not able to resolve phylogenetic relations among the subclades within the Pontocaspian clade, possibly due to saturation of the variable sites.

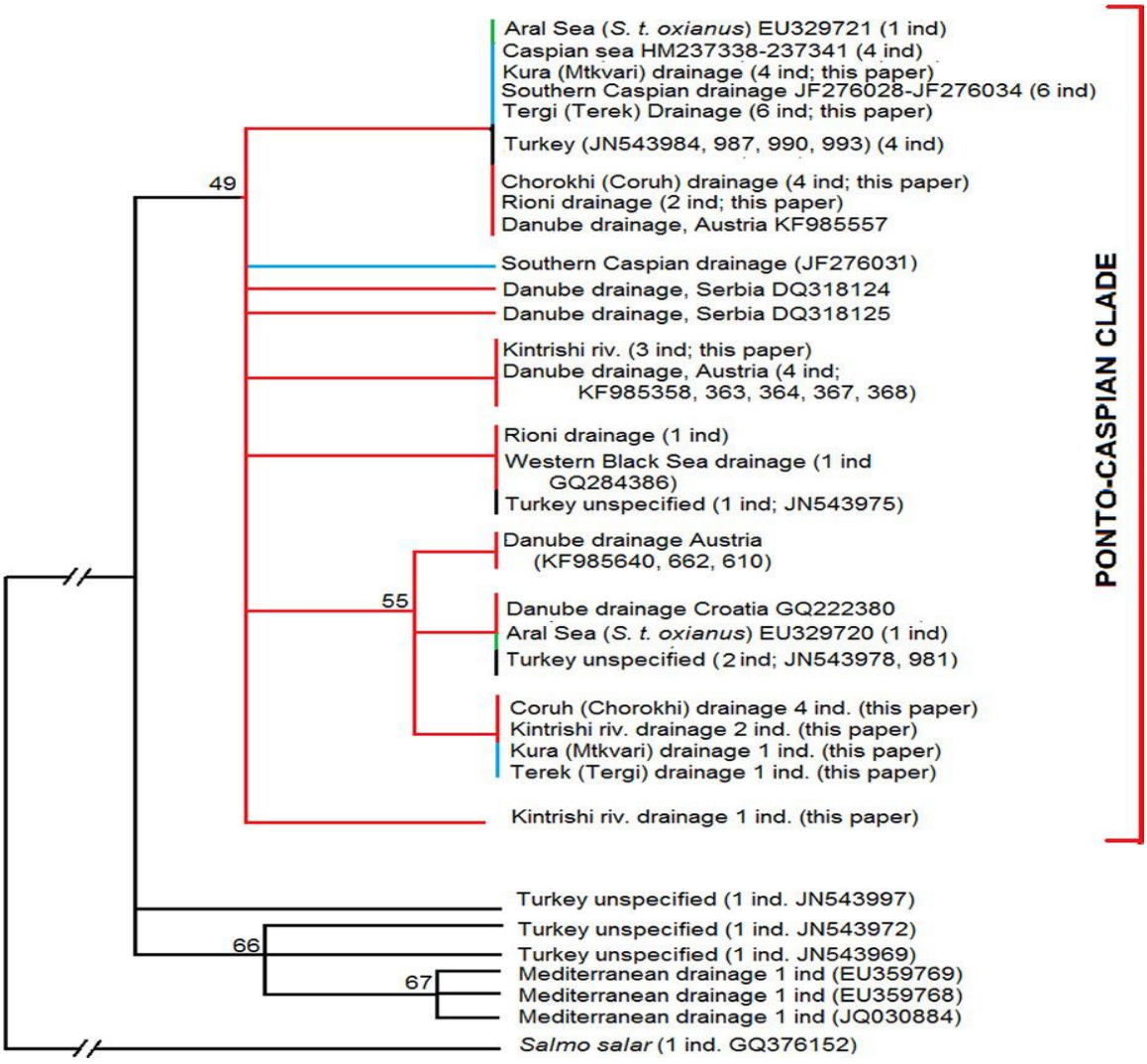

Figure 27. Maximum likelihood tree based on the control region sequences of *Salmo*. Bootstrap support shown above the nodes. Identical haplotypes shown separately if found at more than one location. Clades with bootstrap support below 49 shown as polytomies. Red branches—samples from the drainage of the Black Sea; blue branches—samples from the drainage of the Caspian Sea; green branches—samples from the drainage of the Aral Sea (Ninua et al., 2018).

Minimum spanning network

The minimum spanning network gives even better visualization of results based on Cyt-*b* sequence analysis. It shows the distribution of twenty-three haplotypes of the same Cyt-*b* sequences (obtained from our samples and those downloaded from Genbank) used in Bayesian analysis (Figure 28). The Caspian Sea drainage is represented with haplotypes from basins of Terek ((Tergi) (rivers: Terek and Snostskali)), Kura ((Mtkvari) (Sevan Lake from Armenia, rivers: Ktsia and Aragvi,)), Sulak (river Khiso's Alazani) and southern coast of Caspian Sea. The haplotypes from Terek basin and Lake Sevan (nominal *S. ischchan*) occupy separate positions, distinct from the haplotype comprising the rest of the samples from the Caspian Sea drainages. The haplotypes from the Black Sea drainage are derived from the basins of rivers flowing into it from the east. The three most common haplogroups are shared among the specimens from the Black Sea and main rivers: Enguri, Rioni, Chorokhi. Additionally, there are nine rare haplotypes different from the "main" ones with up to three mutated positions. The most deviated cluster of haplotypes from the Black Sea drainage are downloaded sequences of nominal *S. rizeensis* from the Chorokhi drainage and closely related haplotype from Shareula (Rioni basin). The single sequence from Arpi (Armenia) is close to the haplotype from the Mediterranean drainage. That would not be a surprise if we assumed that translocation of trout is very popular for recreation fishing.

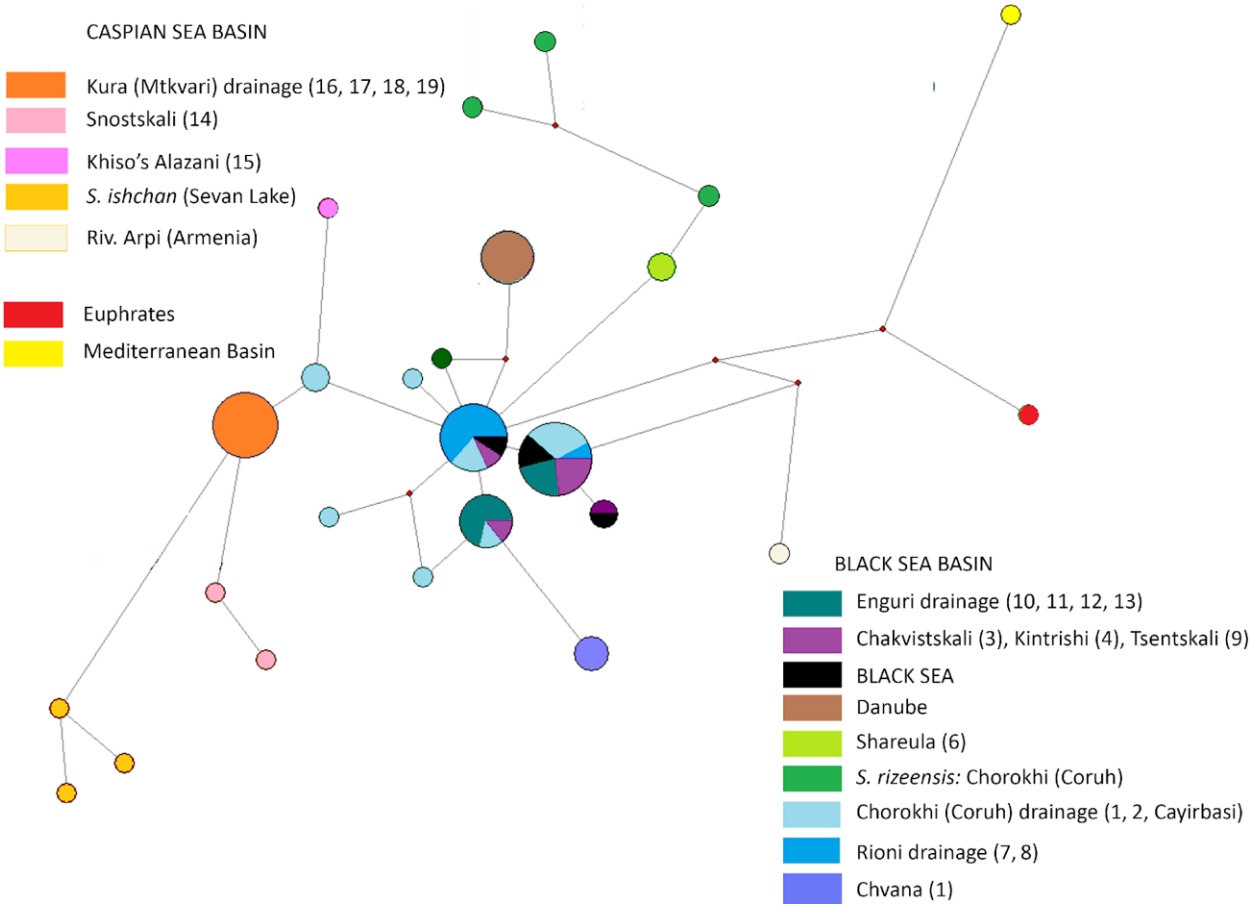

Figure 28. Median-joining network linking the haplotypes of brown trout from the Caspian, Black Sea, and Mediterranean basins. The sampling locations of brown trout from the individual river basins marked with different colors ([see the legend on the figure] From Ninua et al., 2023).

Morphometry

Principal component analysis based on the standardized residuals of 27 size-removed body measurements extracted seven principal components with eigenvalues exceeding one. Cumulative weight of the components 1–4 exceeded 68%. Measurements showing relative height of fish bodies had the highest loadings on the first PC. Measurements of the head (relative length of snout) had the highest loading on the second PC. The distances in the hind part of the body

had the highest loading on the third PC. Relative distances between the dorsal and adipose fins had the highest loadings on the fourth PC.

The first PCA axis separated river trout (including rainbow trout) from the trout caught in the coastal area of the Black Sea ("Black Sea Salmon"). The second axis separated the fish from the basin of Terek (Tergi; nominal *S. ciscaucasicus*) from the rest of the samples (Figure 29). The third axis did not add any valuable information to the analysis, and the fourth axis separated rainbow trout (*Onychorhynchus mykiss*) from *Salmo* spp (result not shown). Simultaneously, the first and the second axes partly separated river trout from the Black and the Caspian (excluding Terek) watercourses (Figure 29).

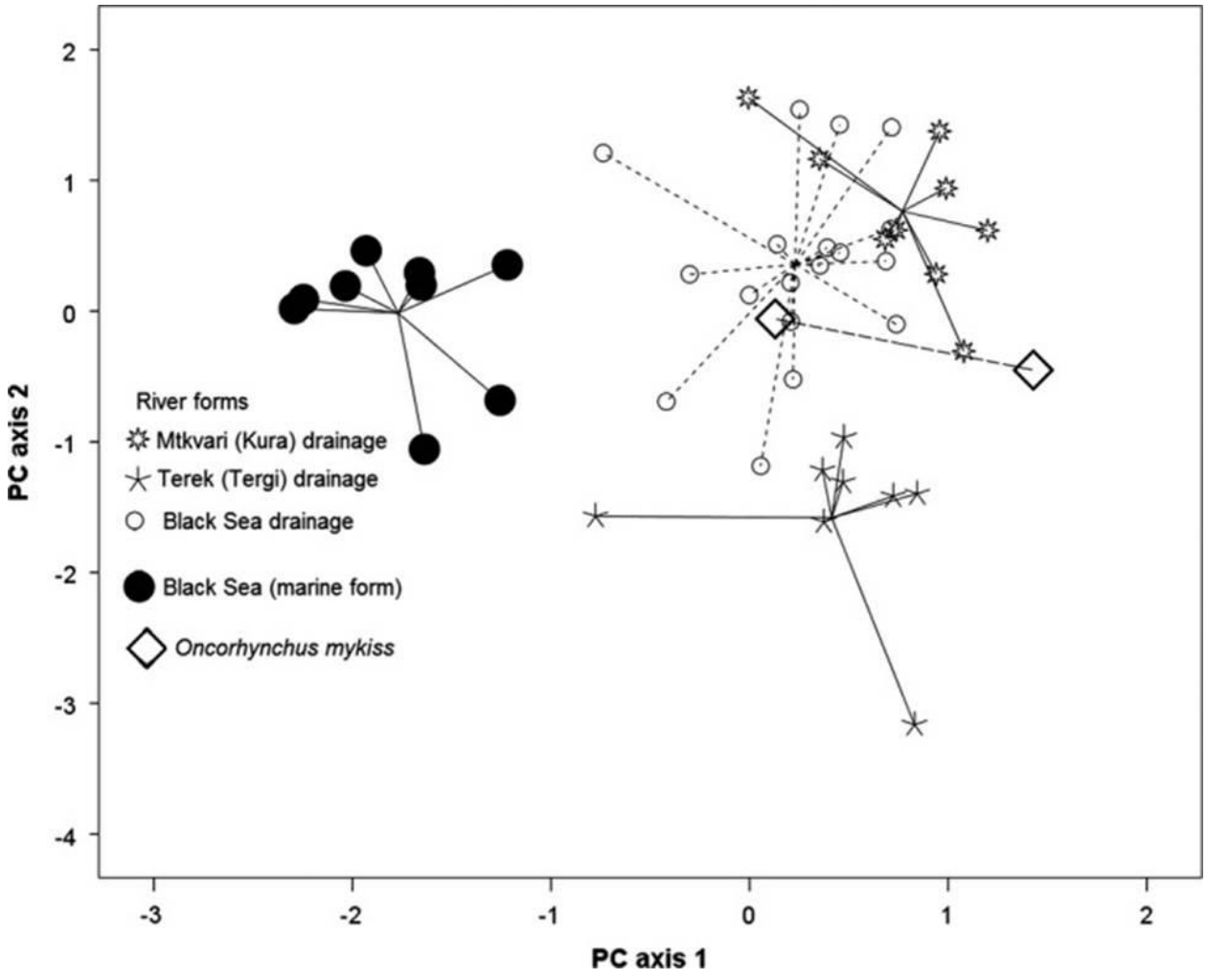

Figure 29. Individual scores of riverine and marine forms of trout and rainbow trout from rivers and Black Sea coastline of Georgia and the first and second principal component axes relayed on 27-size removal body measurements (Ninua et al., 2018).

The number of gill rakers at the first gill arch varied with fish size (between 14 and 22) and did not show stable differences between the individuals from different populations. Even *Salmo* and *Oncorynchus* individuals were not possible to distinguish with the same trait. Correlation between body length and the number of rakers was significant ($r^2 = .81$, $n = 32$, $p < .01$).

Out of 32 studied individuals, the majority had 10 rays in dorsal fins, and three individuals (two from the Caspian, and one from the Black Sea catchment) had nine rays. The number of rays in the pectoral fin was either 12 (12 individuals) or 13 (20 individuals), without respect to body size, genetics, or river catchment.

There are more or less stable differences in color pattern between the marine and freshwater forms of trout (Figure 30). The background was silver in marine form but yellowish in the freshwater trout. The individuals from Terek (Tergi) River were gray rather than yellowish. Red spots on the lateral side are either paler or absent in marine forms.

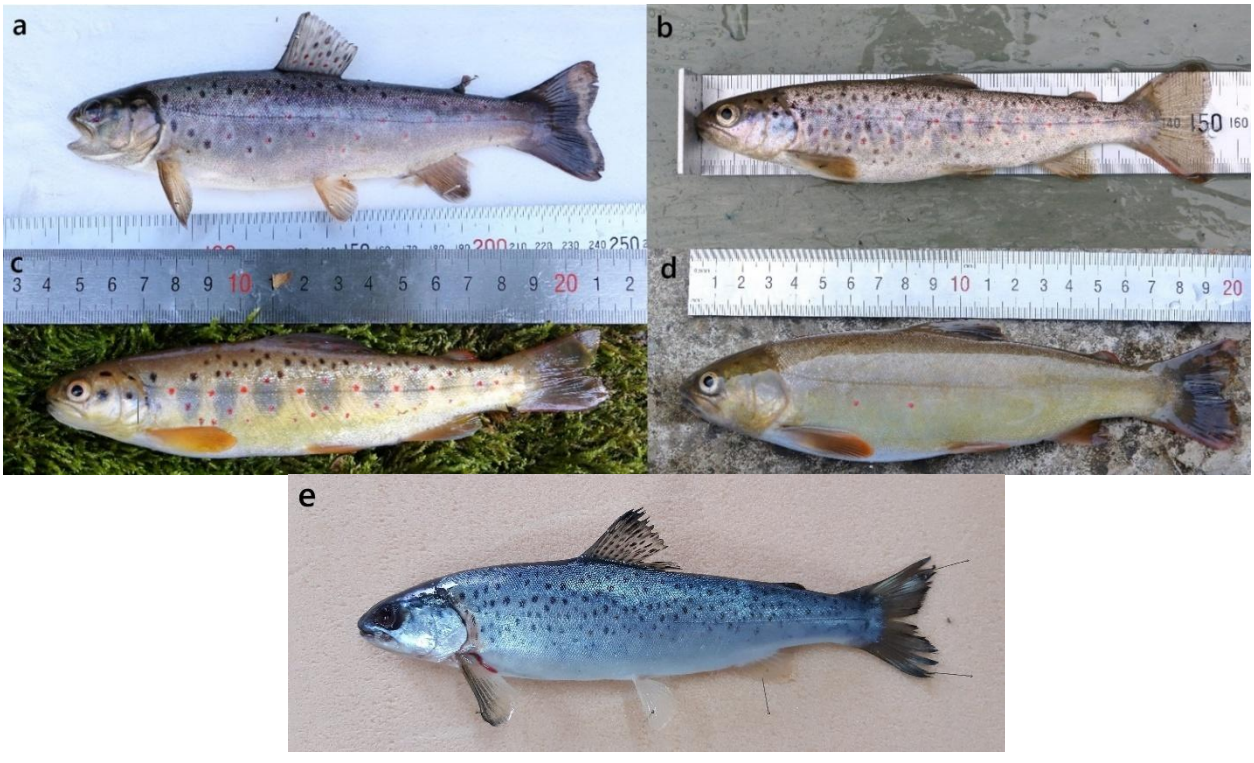

Figure 30. Nominal trout species from the Black and Caspian Sea basins: a) *S. caspius*, b) *S. ciscaucasicus*; c) *S. labrax* and d) S. rizeensis; e) *S. labrax* smolt.

Inferred time of split among the clades

According to different authors the divergence time between *S. ohridanus* and *S. obtusirostris* versus *S. marmoratus*, *S. platycephalus*, and *S. trutta* is 5.6 mya (www.timetree.org; Hedges et al., 2015). If this date is set as the initial split, the estimated time of divergence between the brown trout lineages inhabiting the Atlantic drainage and Anatolian/Mediterranean/Ponto-Caspian lineage is dated to 3.5 mya (with a range of 2.1–5.4 mya); between the Mediterranean/Euphrates basin and the Ponto-Caspian lineage - 2.4 (1.9–3.6) mya and the split between the five subclades of the Ponto-Caspian brown trout—1.5 (0.9–2.4) mya (Figure 31). Different dates are offered by Schenekar et al. (2014) based on the 1%–2% per MY divergence rate for *Thymalus* spp. (Koskinen et al., 2002; Weiss et al., 2002). According to the authors, divergence between the Black Sea and Atlantic lineages of brown trout in Austria occurred 320–745 kya. Applying the calibration rates used by Schenekar et al. (2014) would change the previously offered dates. The average divergence time among the subclades of the Ponto-Caspian clade is reduced to approximately 200 kya. This does not affect the main conclusion: The earliest genetic diversification of brown trout from the Ponto-Caspian Basin occurred at the southeastern coast of the Black Sea and the rivers located in this area and the divergence of Ponto-Caspian trout clades was associated to waves either in early or late Pleistocene.

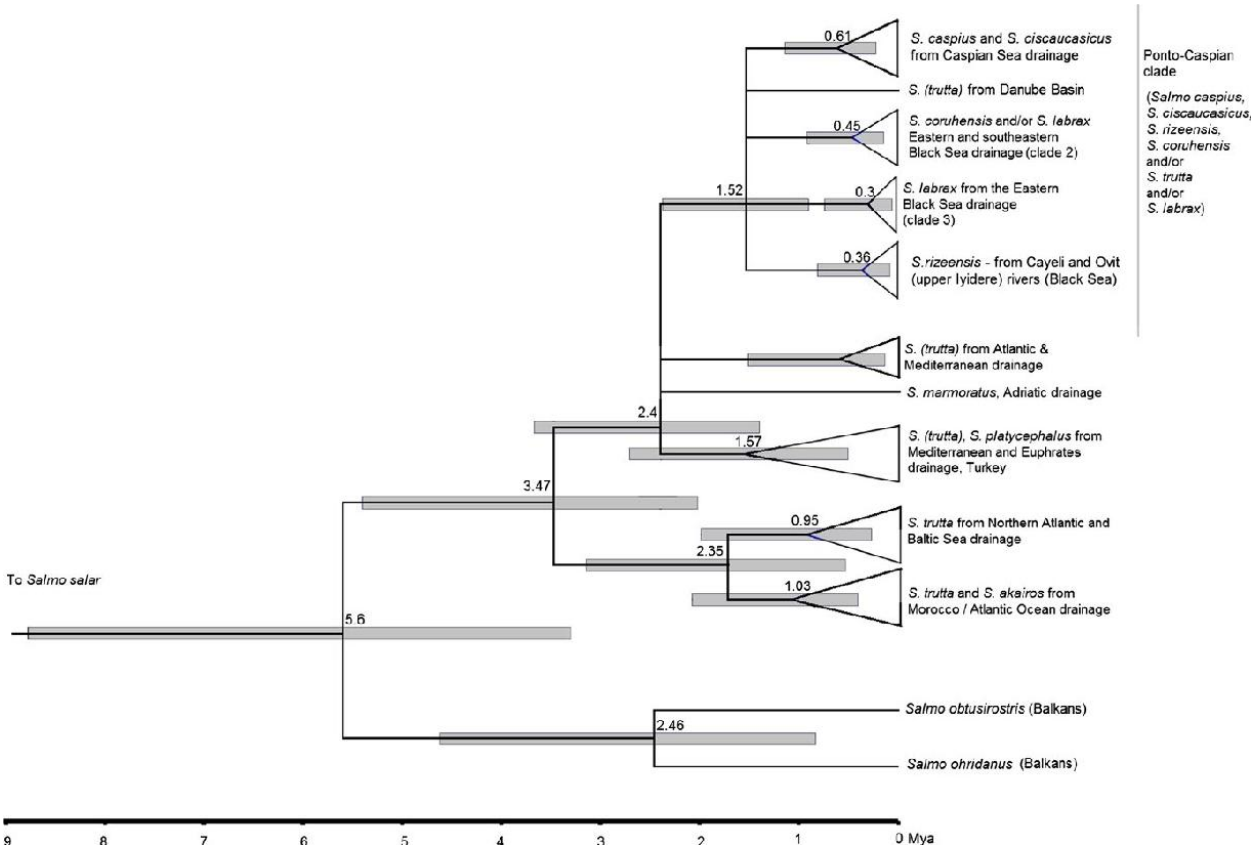

Figure 31. Timescale for the divergence of the clades according to the consensus calibration of www.timetree.org. The numbers at the nodes—estimated divergence time (millions of years, mya); gray horizontal bars—95% HPD intervals (Ninua et al., 2018).

Complete mitogenomes of anadromous S. labrax and resident S. rizeensis

The length of *S. labrax* and *S. rizeensis* mitogenomes were 16,677 bp and 16,674 respectively. Each of them includes 13 protein-coding sequences, 2 rRNA genes, 22 tRNA genes and a control region (1014 bp) (Figure 32). Nucleotide composition for *S. labrax* was A - 28.0%, C - 29.4%, G - 16.5% and T - 26.0% and for *S. rizeensis* - A - 27.9%, C - 29.5%, G - 16.6% and T - 26.0%. The start codon was AGT for all protein-coding regions except COX1 and ND6, where alternative codons GTG and CTA were observed respectively. COX2, ND3, ND4, Cyt B had incomplete stop codon T and COX3 – TA.

S. *rizeensis* has 3 nucleotide deletions at position 10491-93 causing loss of amino acid Serine in the NADH dehydrogenase subunit 4 (ND4) gene. This character is unique for *S. rizeensis* compared to sequences of any other *S. trutta* s.l. available on GenBank. Sequencing depth at the mutated position was 117 (Appendix 5). The deletions do not cause a shift of a reading frame.

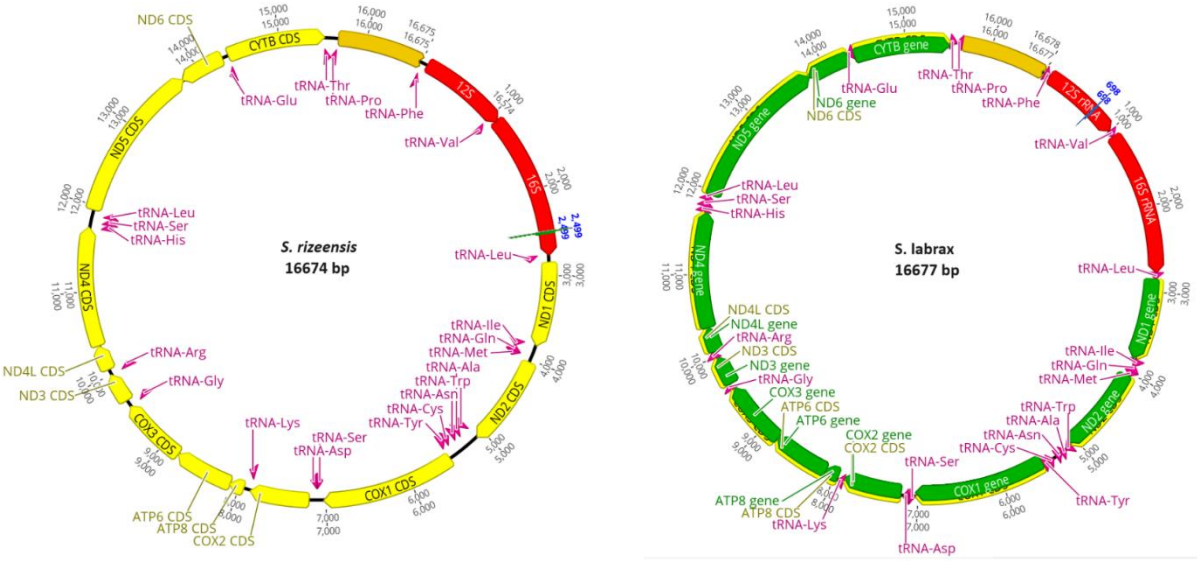

Figure 32. Circular map of the *S. rizeensis and S. labrax* mitochondrial genomes (GenBank accession numbers: PP700288-PP700289)

Genetics structure of Ponto-Caspian trout population

In order to evaluate the geographic distribution of genetic variation of Ponto-Caspian trout and assess the gene flow among the populations, we analyzed genotypes at 10 microsatellite loci for 238 trout samples derived from 9 catchments of the Black and Caspian seas.

There was no obvious evidence of null alleles or large allele dropout in any population or locus, according to microsatellite genotype analysis. The lowest allele diversity (6) was found at locus BG935388 and the highest (34) – at SSsp2201. The mean allelic richness for populations varied

from 2.8 (for Chvana with sample size 10) to 10.7 (for Coastal area of the Black Sea with sample size 14). Within the Black Sea basin populations, allelic richness exhibited a negative correlation (Pearson Rxy = –.69, p ~ .02) with increasing riverine distance from the Black Sea coast. Genotype frequencies in none of the studied populations were significantly deviated from Hardy–Weinberg equilibrium expectations.

STRUCTURE analysis

Based on STRUCTURE analysis, the most probable K = 11 and delta K = 13 (Figure 33).

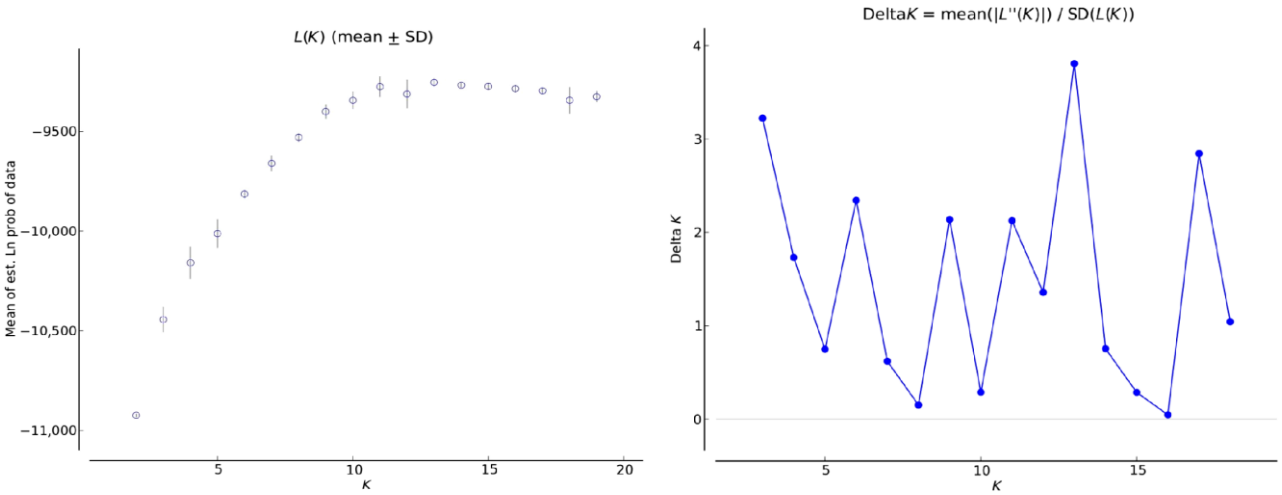

Figure 33. Dependence of mean likelihood and delta *K*, as suggested by Evanno et al. (2005) calculated using the online application Structure Harvester (Earl & VonHoldt, 2012) for the studied samples of brown trout (from Ninua et al., 2023).

On Figure 34 is shown the assignment of individual fish to the inferred clusters with K meaning from 2 to 13 in order to show the clusters that are separated at the lowest values of K. The population from the Black and Caspian seas created separate clusters at K=2. When K=5, populations from drainages of Tergi and Sulak (Snostskali and Khiso's Alazani) separated from Mtkvari (Kura) drainage. At the same time, population 6 from river Shareula (*S. rizeensis*

mitochondrial haplogroup) separated from any other populations ([keeps separated position until K=13] Figure 34).

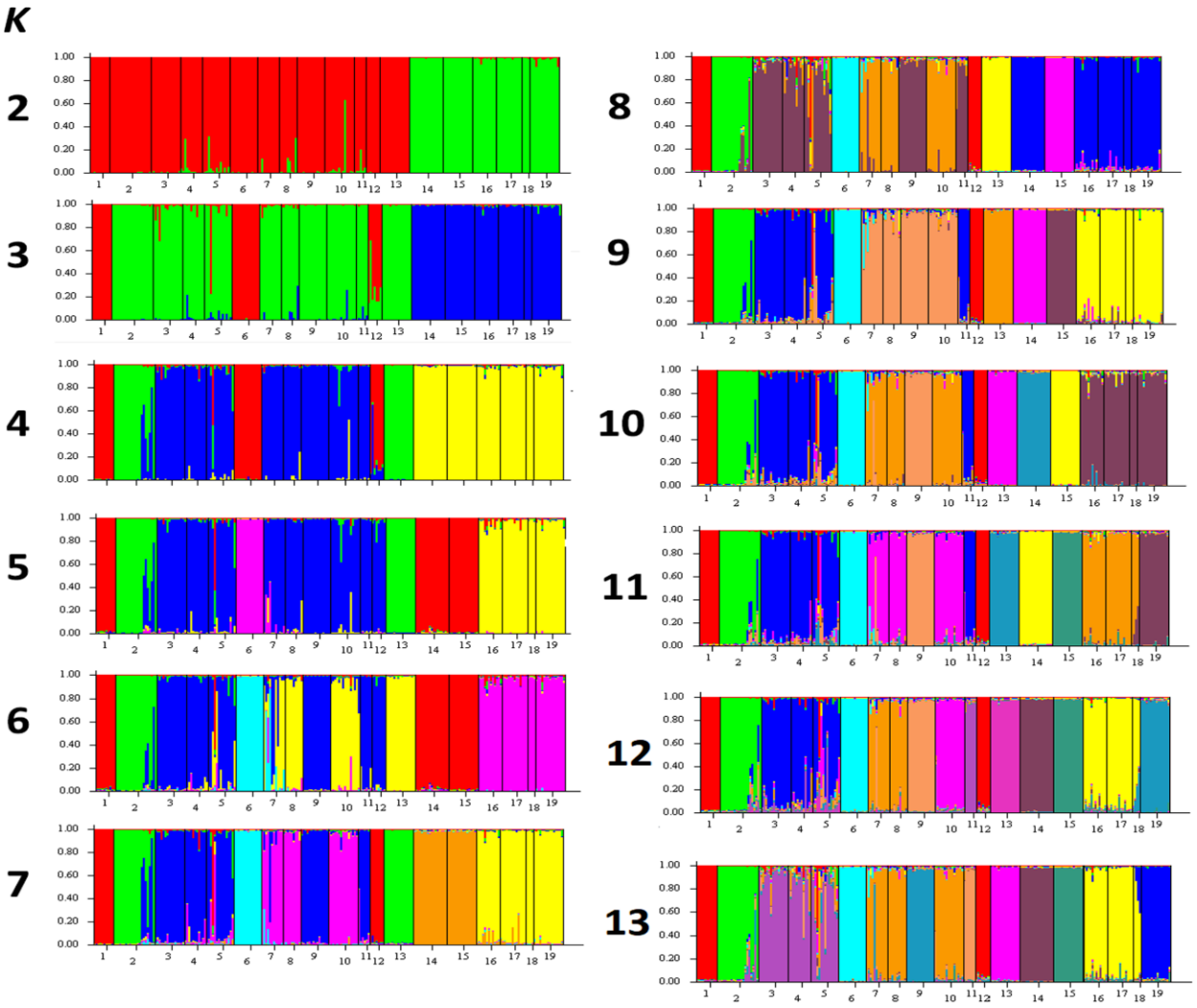

Figure 34. The allocation of the studied individuals to the clusters (*K*) inferred with STRUCTURE algorithm (considering $1 < K < 14$). For the location numbers, see Appendix 2 (Ninua et al., 2023).

Surprisingly, the majority of individuals from all studied populations are almost fully assigned to one or another cluster, indicating the genetic distinctiveness of groups within the studied geographic area (Figure 35). These separations remain until $K = 11$. With $K = 11$, the clusters identifying the populations from the Black and the Caspian Sea drainages are allocated in two

distinct groups, and the cluster of location 6 is the most distant from the rest of the clusters representing Black Sea drainage (Figure 35).

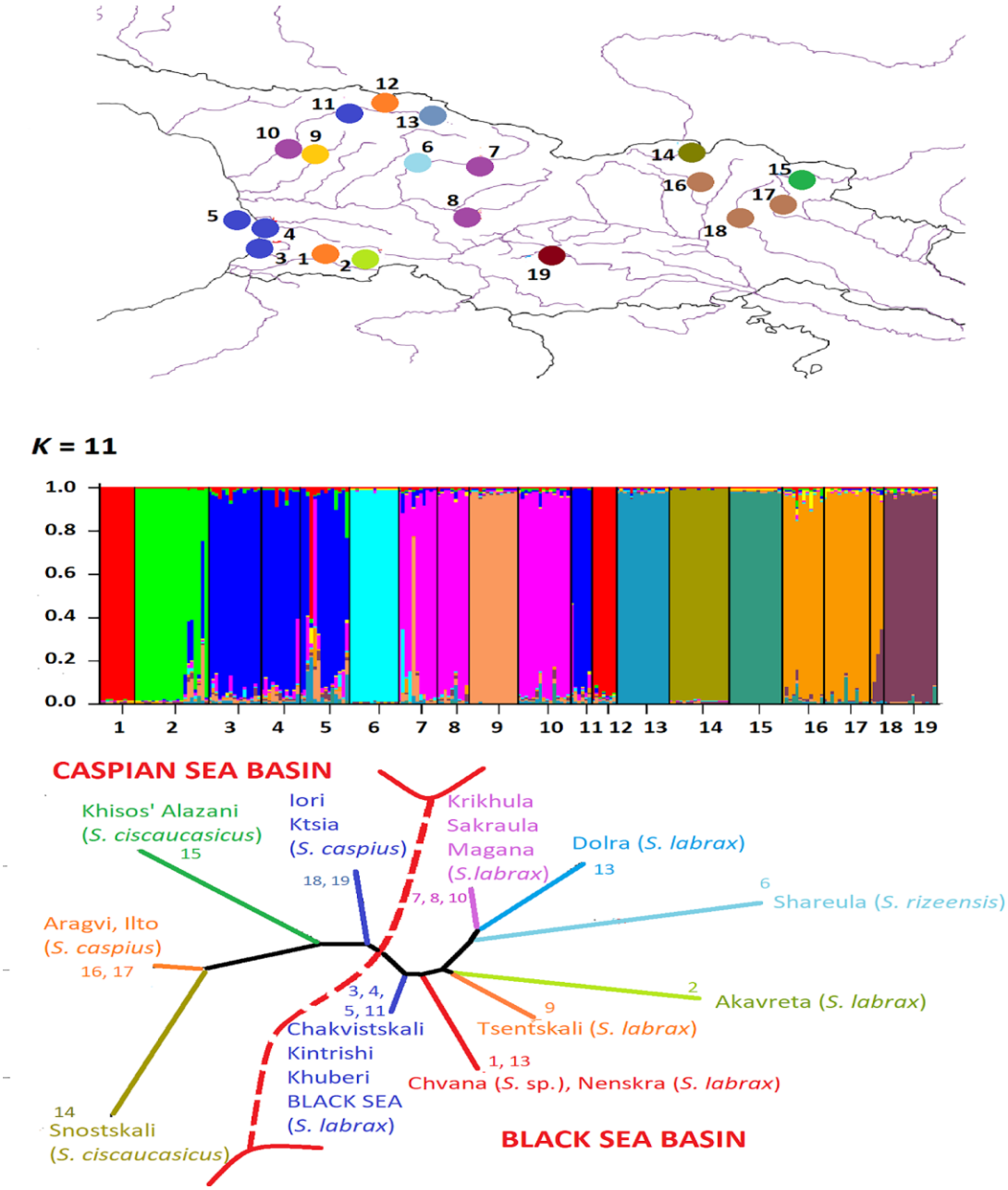

Figure 35. The allocation of the studied individuals to the 11 clusters (K) showing the highest delta log likelihood, inferred with STRUCTURE algorithm (considering $1 < K < 14$). The non-rooted tree reflects genetic distances among the inferred clusters (colored numbers) dominating in the locations marked with the black numbers. Colored dots on map are showing the origin of populations grouped in each cluster (from Ninua et al., 2023).

Principal component analysis (PCA)

Two first PCA axes explained 35.5% of the total variation in microsatellite alleles. Ordination along these axes showed (1) separation of the individuals from the Black and Caspian Sea basins along the first PCA axis, with the highest loadings on loci Ssa408Uos and BG935388; (2) the second axis, with the highest loading on the locus Ssa410Uos, separated genotypes of fish from the drainage of Tergi (location 14), Sulak (15) and Kura (16–19); and (3) the second axis separated genotypes from Shareula (location 6), Chvana (location 1), Akavreta (location 2), and Dolra (location 13) from the genotypes from other locations of the Black Sea basin (Figure 36).

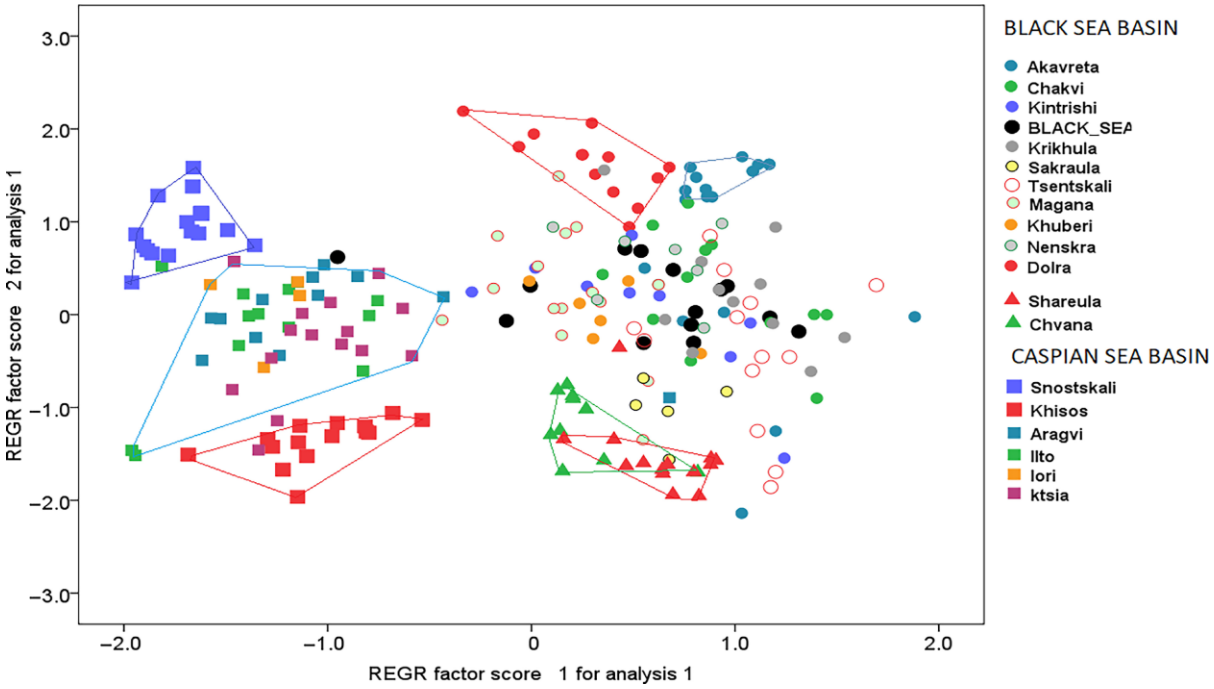

Figure 36. Ordination of the individual microsatellite genotypes along the first and the second principal component analysis axes. The first axis separates the individuals from the Black and the Caspian Sea basins, the second—individual populations within each of the two basins, including resident populations from Shareula and Chvana, as well as Akavreta and Dolra from the other populations of the Black Sea basin; and, populations from three river drainages independently flowing into the Caspian Sea (Ninua et al., 2023).

The PCA based on 20,272 SNPs partly supported the PCA results obtained from 10 microsatellite loci, however, the differences between three distinct groups were more prominent. The first PCA axis separated resident fish from the Shareula population (nominal *S. rizeensis*) from the rest of the populations from the both Black and Caspian Sea basins. The second PCA axis separated the Black Sea cluster from the Caspian and populations from Sakraula and Dolra (upper currents of the river Rioni and Enguri) from each other and from the rest of the Black Sea populations (Figure 37).

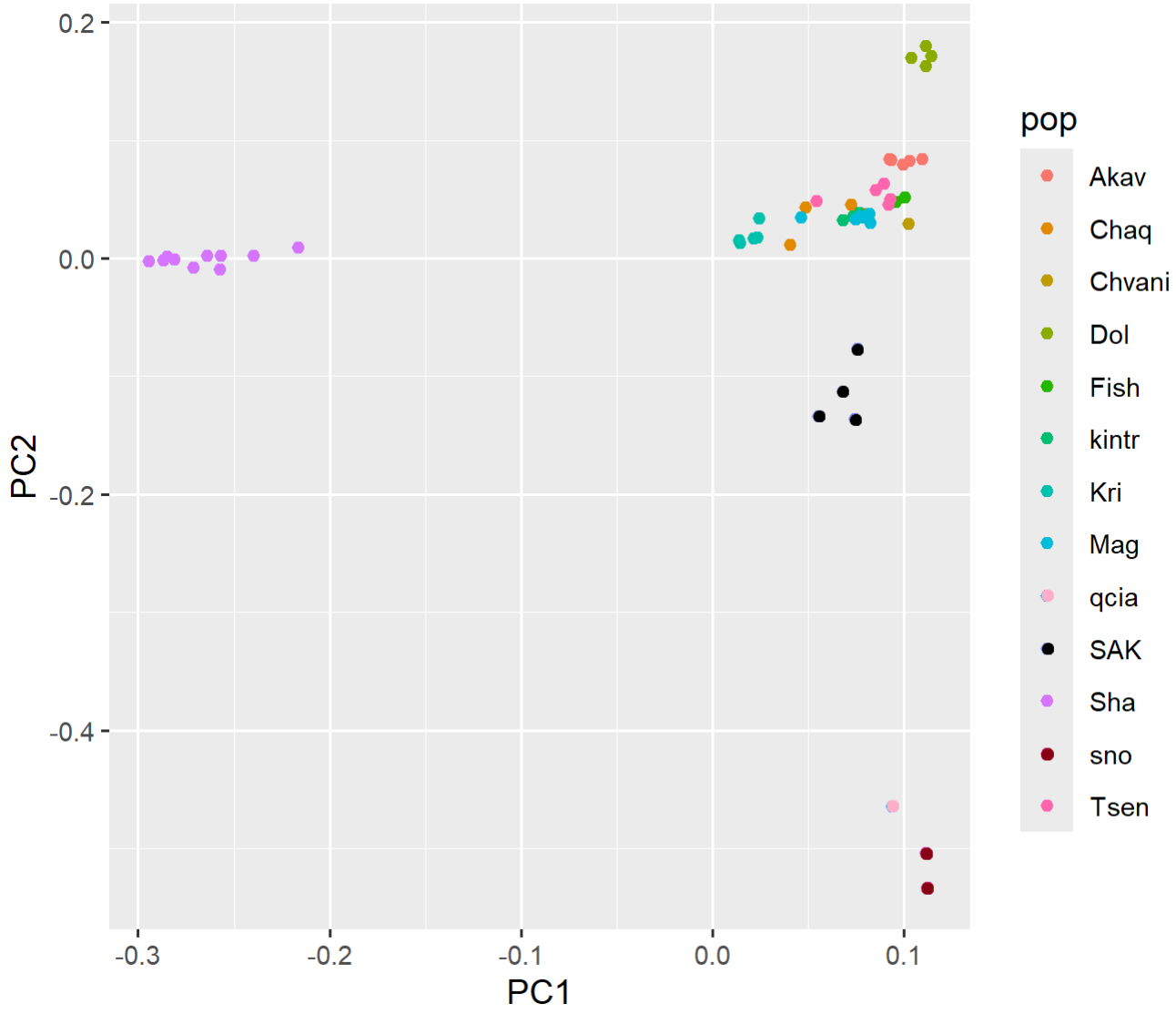

Figure 37. Ordination of the groups of SNP genotypes (in total 20,272SNPs) along the first and the second principal component analysis axes.

Genetic variation (Pairwise FST and RST)

Pairwise *F*ST and *R*ST values based on microsatellite genotypes are presented in Figure 38. Mean pairwise *F*ST and *R*ST values within the populations of the Black Sea basin were 0.236 and 0.300, respectively, while these values for the populations within the Caspian Sea drainage were 0.221 and 0.304. Average pairwise *F*ST and *R*ST values among the populations from the different sea basins were 0.286 and 0.395. The average values of the pairwise *F*ST and *R*ST among the populations from the different sea basins were significantly higher than these values calculated for the populations from the same sea basin (*t*-test, $p < .001$). When the populations from Shareula and Chvana, which showed the highest genetic differentiation from the other populations of the Black Sea basin, were excluded from the Black Sea cluster, the pairwise *F*ST and *R*ST values dropped to 0.189 and 0.239, and when the populations of nominal *S. ciscaucasicus* were excluded from the Caspian Sea cluster, the pairwise *F*ST and *R*ST values dropped to 0.145 and 0.140. In most cases, the differences among the local populations are significant after stepwise Bonferroni correction is applied (*p* values < .05; Figure 38). Pairwise *F*ST, was not significantly above zero only between Chakvistskali and Black Sea populations; pairwise *R*ST values between populations from the Black Sea and from Chakvistskali, Krikhula, Kintrishi, Magana, and Khuberi varied between 0.02–0.06, suggesting close genetic relations between these rivers and Black Sea stock. Other insignificant values are shown in Figure 38.

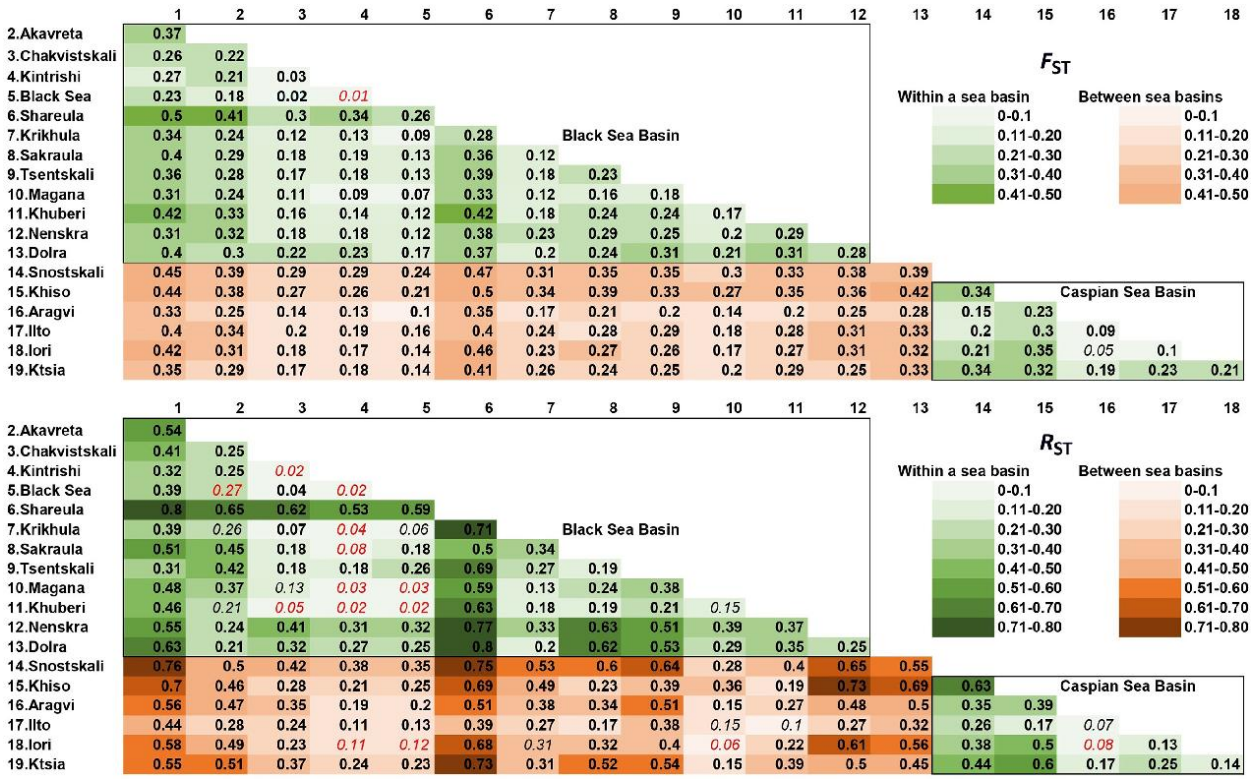

Figure 38. Pairwise measures of genetic differentiation for Ponto-Caspian trout populations. Genetic distances within the same basin are shown with gradation of green and between sea basins with gradation of red. Upper panel—pairwise *F*ST values; lower panel—pairwise *R*ST values. For the most of the population pairs, *F*ST p values < .01. Those where .01 < p < .05 shown in Italics. For insignificant values, red font is used (Ninua et al., 2023).

The unrooted neighbor-joining tree based on the pairwise *F*ST values among the studied populations is shown in Figure 39. The tree shows distinct clusters coinciding with the Black and Caspian Sea basins. However, the populations from individual river drainages flowing into the Black and Caspian Seas are not distinct. Some populations from Enguri, Rioni, and Chorokhi river drainages were genetically closer to the populations from the other and not the same river drainages. The fish collected in the Black Sea were closest genetically to the populations from the small rivers entering the sea (#3 and #4 in Figure 39), and to one population from the Enguri drainage (Magana). The other finding of note is the high genetic distance of the population of Shareula from the rest of the Black Sea drainage populations, including those from the other tributaries of the same river (Krikhula and Sakraula).

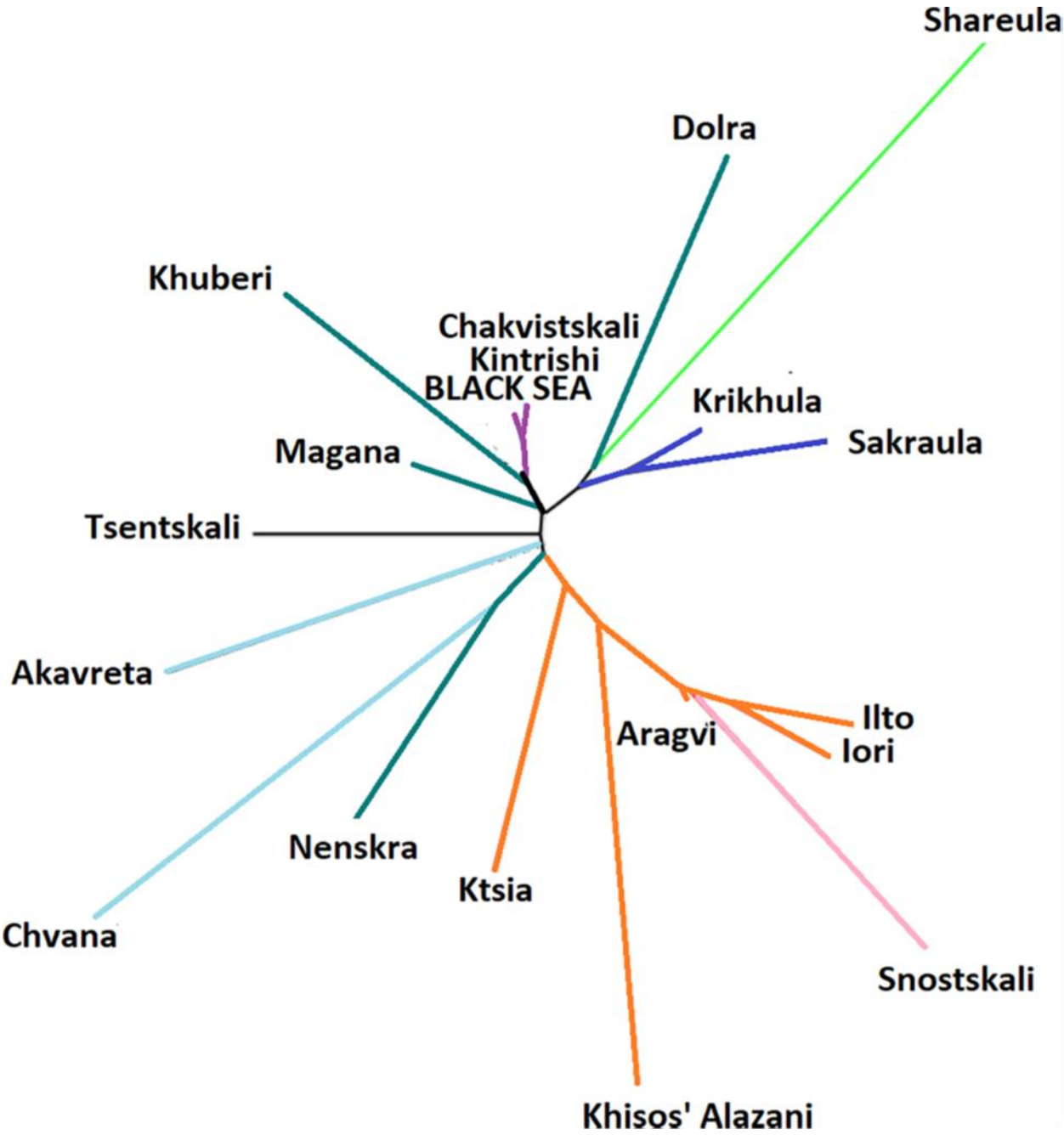

Figure 39. Neighbor-joining unrooted tree based on pairwise *F*ST of all sampled populations. The average distance (*F*ST estimates) between this population and the other populations from the Rioni Basin was 0.300, which substantially and significantly exceeded that for most of the other populations. Remarkably, fish from Shareula had a haplotype clustering with that of the nominal resident *S. rizeensis*. There was also another population (Chvana) quite distant from the other populations of the Black Sea drainage (average *F*ST = 0.347).

Analysis of Molecular Variance (AMOVA)

AMOVA analysis results are shown in Table 3. Out of the total genetic variance of the studied samples, 8% is accounted for the variance between the Black and Caspian Sea basins (Fixation coeficient FST). If the distance between STR alleles is considered (RST mcoeficient) this portion encreases to 20%) this was lower than the variance between populations of the samebasin. (Table 3). We repeated the analysis after removal from the Black Sea basin population cluster the populations from Shareula and Chvana, showing distinct genetic position from the populations of the same basins (as shown by Bayesian inference), pairwise *F*ST calculation, and (in case of Shareula) clustered with mitochondrial haplogroup of *S. rizeensis*. In this case, the component of the between-basin variation *F*CT increased from 0.077 to 0.083 (*F*ST) and from 0.209 to 0.252 (*R*ST).

Table 3. The output of AMOVA analysis based on the microsatellite profiles of the studied populations.

Source of variation	Sum of squares	Variance components	Percentage variation
Two-group design, FST			
Between the sea basins	107.469	0.357	7.708
Among locations within a basin	512.318	1.098	23.695
Within locations	1439.359	3.179	68.597
Average F-statistics over all loci	Fixation indices		
Fixation indices	Significance (p)		
FST: 0.31403	<.00001		
FSC: 0.25674	<.00001		
FCT: 0.07708	<.00001		
Two-group design, RST			
Between the sea basins	27,445.52	106.572	20.882
Among locations within a basin	82,411.49	189.252	37.083
Within locations	97,001.78	214.517	42.034
Average F-statistics over all loci	Fixation indices		

Fixation indices	Significance (p)
FST: 0.57966	<.00001
FSC: 0.46871	<.00001
FCT: 0.20882	<.00001

Geographic isolation versus genetic distances

There was significant positive correlation between the ln-transformed geographic (riverine) distances and pairwise *F*ST values for the populations from the Black Sea drainage: (Mantel test, *Rxy* = .325, *p* = .007). For the Caspian Sea drainage, correlation was higher (Mantel test, *Rxy* = .510, *p* = .047). Correlation of *R*ST values with geographic distance was lower (*Rxy* = .267, *p* = .008) than the correlation of *F*ST with geography for the Black Sea drainage, but higher (*Rxy* = .599, *p* = .024) for the Caspian Sea drainage. Significant correlation between the geography and genetics for those locations that are not in the same river drainage means that the Black Sea is not an impenetrable barrier to migration among populations from different rivers. Moreover, the genetic distance between the fish found in the Black Sea or that from the locations of different rivers close to the sea was commonly smaller than the genetic distances among distant populations from within the same river basin. In other words, isolation by distance appears to be a stronger driver of genetic differentiation than water salinity/transit through the Black Sea.

Discussion

Trout population assessment in Georgia: First steps towards long-term monitoring

This study presents the first-ever results of a trout population assessment conducted with a standardized method in Georgia. These findings represent the initial steps toward building a long-

term monitoring system crucial for the effective conservation and management of trout populations in the future.

While comparing trout densities among different river populations based on Catch Per Unit Effort (CPUE) introduces a reasonable amount of bias affecting catchability and leads to rough rather than precise comparisons, the relative abundance and size/age structure assessed for 13 populations provide valuable data. This data is comparable to information collected at the same locations, using the identical standardized method, in subsequent years. Consistent sampling protocols, while not correcting for bias, can permit meaningful population inferences if effort and catchability remain constant across sampling events, as the size and direction of the bias tend to remain consistent (Pope et al., 2010).

Age at maturity, longevity, and diet of trout populations

Few mature trout were observed at age 2+. It appears that the majority of trout in the sampled populations mature in their fourth or fifth year of development. Given that trout longevity in any of the studied populations rarely exceeds five years, most females likely reproduce only once in their lifetime. Data related to age at maturity and longevity could be biased if extrapolated to entire populations, as the sample was collected in August, primarily from the middle and upper sections of rivers within protected areas. If life histories other than river residency (Ferguson et al., 2019) are typical for any of the studied populations, larger and older individuals might spend most of their time in lower river sections, appearing in the studied headwater areas just before spawning.

Data from analyzing the stomach content of trout from 13 populations indicate a diverse trout diet in summer. The full stomachs of almost all sampled trout suggest high food availability during this period. The species/taxa composition in the diet differed among some (but not all) populations (Figure 14).

Parasitic Infections in trout populations

The unexpectedly high prevalence of Acanthocephala in only one (Kvabiskhevi) of the 13 studied populations can be explained by the dominance of Amphipods in the Kvabiskhevi trout population's diet. The complex life cycle of Acanthocephala is linked to amphipods (Genus *Gammarus*), which serve as intermediate hosts for the pathogen, representing an essential (obligatory) step before infecting fish (the definitive host). Acanthocephala reproduce sexually and complete their life cycle in fish (Grabner et al., 2023). This parasite impairs food digestion and absorption by damaging the gut wall, causing inflammation, and affecting neuromodulators in the intestine (Dezfuli et al., 2002). The lower growth rate of trout in Kvabiskhevi compared to other rivers can be attributed to Acanthocephala infection. However, the smaller size of the river and relatively high fish density should also be considered (Jonsson & Jonsson 2011).

Other pathogens identified in trout in this study were Myxozoa. *Myxobolus cerebralis* and *Tetracapsuloides bryosalmonae* are significant myxozoan pathogens affecting both wild and farmed salmonid species (Banu & Rathinam 2023). The molecular identification of these myxozoan pathogens in this study provides the first evidence of their presence in natural and artificial aquatic systems in Georgia.

The prevalence of *M. cerebralis* was much higher in *S. labrax* populations (80% and 55% in Chakvistskali and Kintrishi, respectively) compared to *S. caspius* (40%, 17%, and 8% in Kvabiskhevi, Iori, and Ktsia, respectively). None of the infected fish from these rivers showed clinical symptoms. A single symptomatic fish infected with the pathogen was caught during trout population assessment in Khiso's Alazani. A whirling disease epizootic was confirmed in rainbow trout (*O. mykiss*) farms. The infection was also detected in tubificid worms collected from the outlet channel of one of the fish farms, likely indicating that the pathogen is maintained in the system (many fish farms draw water from and release waste back into the same river). This finding was not surprising given the high prevalence of the pathogen in wild brown trout populations.

There is no effective treatment for whirling disease in wild and farmed trout; disease management is the most effective way to minimize pathogen spread (Turner et al., 2014).

Another myxozoan pathogen of interest was *Tetracapsuloides bryosalmonae*, which more severely affects brown trout than rainbow trout (Bailey et al., 2019). *T. bryosalmonae* is assumed to be a reason for wild brown trout population decline in some European countries (Lewisch et al., 2018; Waldner et al., 2020). Bryozoa species of the *Fredericella* and *Plumatella* genera are assumed to be the main definitive hosts for *T. bryosalmonae* worldwide (Okamura & Wood 2002). We collected *Hyalinella punctata* and *Fredericella* sp. from four watercourses during this study. None of the Bryozoa colonies tested positive for *T. bryosalmonae*. However, two colonies (out of seven) of *H. punctata* were positive for another Malacosporean pathogen, *Buddenbrockia plumatellae*. *B. plumatellae* was identified in Cyprinid fish from the same river. Interestingly, *T. bryosalmonae* was exclusively found in one of the Cyprinid species—*Oxynoemacheilus brandtii* (Kura loach).

The newly identified pathogen is closely related to *T. bryosalmonae* isolated from trout but occupies a separate position on the ML tree (based on 1148nt of 18S ribosomal DNA) from the *T. bryosalmonae* cluster (Figure 18). However, the bootstrap value is low (55). The presence of *T. bryosalmonae* solely in loach (among five Cyprinid species) probably indicates that the Kura Loach is a true intermediate host (the pathogen produces spores). However, it is unclear if the Malacosporean identified in loach has the ability to infect salmonid species as well.

Historically, brown trout were abundant in almost all rivers and lakes of the Javakheti upland (Barach 1964 in Russian; Elanidze 1983 in Russian). However, we did not manage to catch even a single brown trout with electrofishing during six fishing events. We observed high water temperatures (23^{0}C) in the Bugdasheni River, while the highest daily temperature varied between 15-20^{0}C during the same period (August) in other trout rivers. Malacosporean infection in salmonids is temperature-dependent, with the severity of infection positively correlating with temperature (Sudhagar et al., 2019). It would be speculative to directly link these data to the extreme decrease of trout in Javakheti rivers; however, the issue warrants further investigation.

Brown trout from the Black and the Caspian Sea drainages comprises a monophyletic evolutionary lineage (matrilineal clade) distinct from brown trout from other parts of West Eurasia, including those from the drainages of Atlantic, Mediterranean, and the Indian Ocean. This lineage separated from other clades native to the rivers of Anatolia. Most of the subclades within this clade produce both riverine and anadromous forms, or may spontaneously switch between these two life modes. There are some differences in body proportions between different lineages of brown trout, although morphology should be used very carefully while differentiating among the nominal species: Our analyses suggest that body proportions and number of gill rakers, commonly used in taxonomy, may be associated with highly variable body size of the fish.

Diversification of brown trout and geological past

Basal diversification within *Salmo*, separating Atlantic salmon (*S. salar*) from the brown trout lineage, according to different authors, occurred from 9.6 to 15.4 mya (Alexandrou et al., 2013; Campbell et al., 2013; Crête-Lafrenière et al., 2012; Ma et al., 2015; Shedko, Miroshnichenko, & Nemkova, 2012). The split between the widely distributed European *S. trutta* and recently described species *S. ohridanus* and *S. obtusirostris* occurred 3 to 9 Mya, in the late Miocene or Pliocene (Crête-Lafrenière et al., 2012; Macqueen & Johnston, 2014; see http://www.timetree.org/; Hedges et al., 2015 for review). If these dates are accepted, the Ponto-Caspian lineage has been separated from the populations of the Anatolian rivers draining into the Persian Gulf and Mediterranean Sea ca. 2.4 mya, shortly after early Pleistocene glaciations began (Subcommission on Quaternary Stratigraphy, 2017). Consequently, all trout populations from the Black, Caspian, and Aral Sea drainages, from Austria to Iran and Kazakhstan, descend from this lineage dispersed during the Pleistocene, probably in Pleistocene glacials, from the rivers of the northeastern Anatolia. Studies on Black and Caspian history based on paleontological and geochemical methods suggests the positive freshwater balance in those systems during most of the last glacial periods (Esin et al., 2016; Hoyle et al., 2021). Less saline and cold water in seas

most probably helped anadromous trout to disperse. Schenekar et al. (2014) suggested that the divergence time between brown trout from the Atlantic and Black Sea basins in Austria occurred 320,000–745,000 years ago, based on the 1%–2% per MY divergence rate for *Thymalus* spp. (Koskinen, et al., 2002; Weiss et al., 2002). If the scale used by these authors is applied, the separation of the Ponto-Caspian lineage should have happened only ca. 200 kya, hence in late Pleistocene. However, this dating would suggest the revision of the inferred divergence time for the entire genus *Salmo*, and we suggest there is not yet enough evidence for such revision. It is likely that the divergence of Ponto-Caspian trout was associated with glacial cycles. The landscape model of the last glacial maximum (Gavashelishvili & Tarkhnishvili, 2016) suggests that the river courses throughout the most of the Caucasus and Asia Minor were shorter than in present time, which means that the upper currents of these rivers would likely not be suitable for trout reproduction. Additionally, the Black Sea was permanently influenced by both Caspian and Mediterranean seas resulting in fluctuations of water salinity during Pleistocene glacial waves (Badertscher et al., 2011). A large continuous refugium was located at the southeastern coast of the Black Sea (Gavashelishvili & Tarkhnishvili, 2016; Van Andel & Tzedakis, 1996) and, hence, the brown trout populations could have survived there from constantly changing environmental conditions during glacial waves. The trout habitats of the southeastern coast of the Black Sea (mostly in the basin of Chorokhi [Coruh] and other rivers draining into the Black Sea along its southeastern coast) were least affected by climatic fluctuations. This is the area of the highest genetic diversity of the Ponto-Caspian trout, home also to river resident taxon - *S. rizeensis*. The split between this lineage and the Euphrates/Persian Gulf lineage happened in the early Pleistocene. It is possible that some parts of the upper reaches of the Euphrates and Coruh (Chorokhi) changed their courses, causing isolation of some mountain populations, hence reinforcing the divergence of fish from the drainages of the Black Sea and the drainage of the Euphrates; a similar process explains the presence of two distinct lineages of trout in upper reaches of the Danube (Schenekar et al., 2014). Indeed, only the largest rivers of northern Eurasia retained their courses through the entire Pleistocene (Shahgedanova, 2002). The populations from the Black Sea were captured during the consequential glacial cycles in small isolated streams

flowing into the southeastern part of the sea, and in glacial periods, probably used the sea as a transit area, dispersing through different rivers of the same drainage. One of these lineages might gain some morphological/genetic peculiarities and lose ability to switch to anadromy, the form recently described as *S. rizeensis* (Turan et al., 2009). Other four major lineages have apparently maintained this ability. One of those dispersed into the Caspian drainage (and probably into the Aral Sea drainage), and three others remained in the Black Sea drainage. The most recent conjunction between the Black and Caspian Seas, through the lowland drainages north of the Caucasus Mountains, could have happened as late as 160 kya (Gelembiuk et al., 2006) and even 9 kya (Reid & Orlova, 2002). However, over millions of years of isolation between the Black Sea lineages and the Caspian lineage of brown trout (according to a more likely estimate) suggests that the conjunction periods have been strongly reduced since earlier times. Although there are differences between average body proportions of trout from the Black and Caspian catchment, the morphological overlap is high. Morphological differences between the fish from the basin of Tergi (Terek) and Mtkvari (Kura) are stronger and fixed. The split between the four Black Sea clades, including the Danube clade, occurred shortly after the initial split and could be associated with increasing time of glacial cycles and decreasing temperature during the glacial maxima in mid-Pleistocene (Imbrie et al., 1993). In conclusion, Pleistocene glacial cycles and the latest transgressions between the Black and Caspian Seas are the main reasons of separation and genetic diversification of Ponto-Caspian lineages of brown trout.

Taxonomic inference

The trout from the Caspian Sea basin (Caspian trout) is a monophyletic lineage. Nominal *S. caspius* from the drainage of Mtkvari (Kura) and southern Caspian, on one hand, and nominal *S. ciscaucasicus* from the Terek (Tergi) River are monophyletic. Moreover, the river forms of this fish are morphologically distinct from each other: Nominal *S. ciscaucasicus* has a shorter snout, and this feature is nearly diagnostic. Black Sea trout have on average shorter trunk than nominal

S. caspius, and narrower body, although these differences cannot be used for diagnosis between the two lineages. We suggest using the valid names *S. caspius* for brown trout from the rivers flowing into the Caspian Sea from the south and southwest, and *S. ciscaucasicus* for those found in the Terek River and other rivers flowing into the Caspian from the north and northwest. These results contradict with findings of Segherloo et al. (2021). Based on genomic study (analyzed over 15000 SNPs) authors suggested synonymizing of *S. ciscaucasicus* and *S. caspius*, which do not contradict our results based on the mitochondrial sequences. Although microsatellite profiles of the fish from the drainage of the river Tergi are distinct to the fish from Kura drainage; besides, these forms are phenotypically more distinct than some species recognized by Segherloo et al (2021). Indeed, this nominal species deserves to be discussed as a separate unit for conservation and management.

The anadromous individuals from the Black Sea catchment, described as *S. coruhensis* by Turan et al. (2009), were shown not to represent a monophyletic lineage with respect to both the riverine and anadromous fish from Georgia, as well as to the fish from the Danube drainage. Turan et al. (2009) showed some morphological differences between the anadromous fish from Chorokhi (Coruh) catchment and *S. labrax* from the northern part of the Black Sea drainage. However, the latter were not investigated genetically, and their characteristic features (lower number of gill rakers and different body measurements) may be associated with body size of fish, smaller than that of other nominal species. The number of rakers may increase with the body size of fish (Ross et al., 2006). The same pattern was observed in our samples. Moreover, running PCA without excluding size would obviously harvest the first component dependent largely on body size (Thorpe & Leamy, 1983), an extremely variable characteristic in fish. Hence, there is not sufficient evidence of morphological separation between *S. labrax* and *S. coruhensis*. Consequently, we suggest a conservative approach, and all individuals from the watercourses of Coruh (Chorokhi), Kintrishi, Rioni, Enguri, Danube, and those caught in the Black Sea (except *S. rizeensis*, a narrow-ranged purely riverine form) we consider conspecific at this stage, and use the priority name *S. labrax* (Black Sea salmon). These results were supported by genomic study of Segherloo et al. (2021). However, the authors also synonymized river resident *S. rizeensis* with

S. labrax and *S. coruhensis*. Our results contradict with this conclusion. The species status of *S. rizeensis*, which Turan et al. (2009) described as a resident species, reflects real genetic differentiation; *S. rizeensis* shares a mitochondrial haplogroup with trout from the disjunct location Shareula (Rioni River drainage; Figure 26), which genetic distance from other populations of the Black Sea basin, based on the analysis of microsatellite multilocus genotypes, exceeds the genetic distance between the trout from the Black and Caspian Sea basins. We suggest that trout from this location, along with resident fish from northeastern Turkey, belong to a distinct species, *S. rizeensis*. According to our data (Cyt-*b*) lacustrine trout *S. ischchan* from Lake Sevan (Armenia) form a well-supported subclade of Caspian clade, in accordance with finding of Levin et al., (2018).

Segherloo et al. (2021) additionally proposed four more species from the Ponto-Caspian area: *Salmo abanticus* from an isolated Abant Lake and *Salmo* sp. from Danube Basin (see also Schenekar et al., 2014), the fish from the rivers flowing into the Caspian Sea from the south (*Salmo* sp.) and *Salmo oxianus* from the Aral Sea drainage. Meanwhile, the taxonomic position of lacustrine *S. ezenami* needs further analysis.

Life histories and their effect on the genetic structure of Ponto-Caspian populations

There is lack of conclusive evidence to what extent anadromy is an inheritable feature specific for individual evolutionary lineages of brown trout. Even in better studied American rainbow trout, there are multiple gaps of knowledge concerning this question (Kendall et al., 2014; Collins at al., 2022). Berg (1959) suggested that an individual trout may occasionally change the riverine life mode to an anadromous one during its life cycle. Elliot (1994) showed that resident and anadromous forms of brown trout coexist (and, probably interbreed) in many rivers draining into the Atlantic. Jonsson and Jonsson (2006) suggest that the resident versus anadromous life history may depend on the presence of physical barriers separating river habitat from the sea, or simply on the distance from the particular location to marine habitat; they also can co-occur in the same

river. Simultaneously, Jonsson and Jonsson (2006) suggest the presence of genetic differences between the resident and anadromous morphs based on some differences in heritable characters, although environmental conditions can also lead to switching between the life histories. In particular, water level in a river may trigger change to and from anadromy (Jonsson et al., 2018). Findings of King et al. (2021) showed that commercially bred brown trout, stocked into a natural habitat of anadromous *S. trutta*, remain genetically distinct for at least 13 years after the stocking. This indirectly supports the presence of genetic differences between the non-migratory and anadromous populations of trout. The other species of the genus *Salmo*, Atlantic salmon (*S. salar*), is usually anadromous and rarely produces resident forms; however, resident forms of *S. salar* are landlocked and isolated from the anadromous fish, most likely, since early Holocene (Berg, 1985). There is evidence for different gene pools in resident and anadromous forms of *S. salar*, including a higher allelic richness in populations of the latter form and stronger genetic differences between the resident populations (Ozerov et al., 2012; Tonteri et al., 2007). To conclude, the switch between the anadromous and resident life histories in brown trout is a multifactorial process that may occur both between conspecific populations, as well as among the individuals of the same population, and is determined simultaneously by genetic and environmental factors.

Much less is known about the variation in life history and reproductive strategies of brown trout from the Ponto-Caspian drainages, including wide-spread *S. labrax* and *S. caspius*, than in the *S. trutta* from the Atlantic drainage. Sabaneev (1875) considered Black Sea salmon and brown trout from the Black Sea drainage as two different species, and only in the 20th century it was shown that they are two forms of the same species (Barach, 1962). Turan et al. (2009) was the first author who adduced two genetically distinct groups of fish in the south-eastern drainage of the Black Sea: anadromous *S. coruhensis* (synonymized later with *S. labrax*) and resident *S. rizeensis*, marked with a distinct mitochondrial haplogroup. We showed that this haplogroup is not less distant from the other haplogroups of the Black Sea trout (nominal *S. labrax*) than reciprocally monophyletic brown trout haplogroups from the Black and the Caspian/Aral Sea drainages. These observations and the analysis of recombinant alleles reported in this study support the maintenance of genetic differences between resident and anadromous trout. The present study

identified two populations, in the drainages of the geographically distant Chorokhi and Rioni (Chvana and Shareula), showing high genetic distances, both from each other and from other trout populations from the Black Sea drainage. The pairwise *F*ST of each of these populations to the Black Sea population is 0.23–0.26, whereas the distance of the other 10 river populations to the Black Sea population varies between 0.01 and 0.18, depending on the distance from the sea coast. These distances are comparable with the genetic distance between *S. labrax* and *S. caspius*. One of these populations (Shareula) is marked with a mitochondrial haplotype that clusters with *S. rizeensis* (Turan et al., 2009). These facts drive the conclusion that Shareula from the Rioni drainage (and, perhaps, Chvana from the Chorokhi drainage) are effectively isolated from the rest of the populations from the Black Sea basin, and are probably genetically determined residential forms similar to that described as *S. rizeensis* by Turan et al. (2009). Considering multiple evidence of spontaneous switching between anadromous and residential life histories in Atlantic brown trout (*S. trutta*) and the differences between rainbow trout and steelhead (*O. mykiss*), the presence of a similar pattern in *S. labrax* is not surprising. The present study suggests that divergence between *S. rizeensis* and "*S. coruhensis*" ((described by Turan et al. (2009)), the forms with different life histories occurred in the early to middle Pleistocene. Another challenge is the sister status of *S. rizeensis* and our population from Shareula, based on mitochondrial DNA sequencing. This indicates that the divergence between the nominal *S. rizeensis* and *S. labrax* may precede their isolation as a result of their different life histories. Haplogroups from Chorokhi and Rioni River drainages, marking the *S. rizeensis* lineage, likely represent a monophyletic taxon that once could migrate from river to river through the coastal area of the Black Sea. Considering that during the glacial periods, the salinity of the Black Sea was substantially lower than now (Soulet et al., 2010) this could well happen. The questions remain—what was the trigger of separation between the two lineages, both of which could migrate through the coastal zone; and why might one of these lineages, in different parts of its range, turn from anadromous to residential life history (one of the potential ways is discussed below).

In fact, there are at least three almost equidistant mitochondrial lineages in the Black Sea trout, including *S. rizeensis*, and those lineages could emerge in case of temporary impenetrability of

the Black Sea area, perhaps during interglacials when Black Sea water was brackish and oxygen deficient due to impact of Mediterranean water inflow (Hoyle et al., 2021; Wegwerth et al., 2019). The cause for the switch to an exclusively freshwater life history in *S. rizeensis* remains unclear. This turns us to a question of sympatric speciation, which according to multiple recent publications is thought to be a speciation mode not less common than the "classical" pattern, wherein geographic isolation triggers early stages of speciation (Miles et al., 2018; Nunes et al., 2022).

In conclusion, this study shows that brown trout from the studied area comprises several distinct genetic clusters, coinciding with both separation of the sea basins and the differentiation of life histories. Fish from the Caspian Sea basin shows higher genetic differentiation among the river catchments, explained with greater average geographic distance among sampled populations compared to the Black Sea. In the Black Sea basin anadromous *S. labrax* coexists with resident form, sharing a mitochondrial haplogroup with *S. rizeensis* from northeastern Turkey.

A better understanding of the genetic background and palaeoecological factors that triggered the divergence between the resident and anadromous trout in this area can be achieved with further genomic studies of the group.

A mutation in the ND4 gene might explain the restriction of Salmo rizeensis to residency

"We sequenced and analyzed the complete mitogenomes of anadromous *S. labrax* and resident *S. rizeensis*. The analysis revealed a triplet deletion in the mitochondrial ND4 gene of *S. rizeensis*, which causes the loss of the amino acid serine. The ND4 gene product is a core protein of respiratory chain NADH dehydrogenase complex I and is essential for its normal functioning. It plays a key role in the assembly of other mtDNA-encoded proteins of respiratory complex I in humans (Hofhaus & Attardi, 1993), and several human mitochondrial diseases are associated with

mutations in the mt-ND4 gene. All of these are associated with dysfunction of the mitochondrial respiratory chain (Jiang et al., 2015; Lin et al., 2020; Mahalaxmi et al., 2021).

The acclimation to saltwater in anadromous trout is associated with a number of metabolically demanding physiological and biochemical changes (Brijs et al., 2017). For example, an upregulation of Na+/K+-ATPase (NKA) is crucial in maintaining osmotic homeostasis in Brown trout (*S. trutta*) and Rainbow trout (*Oncorhynchus mykiss*) after exposure to saltwater (Madsen et al., 1995; Seidelin et al., 2000; Brijs et al., 2017). Increased NKA activity is accompanied by increased mitochondrial coupling and ATP synthesis to satisfy elevated energetic demands related to osmotic stress. In rainbow trout, the relative contribution of complex I to electron transport increased approximately nine times and was still five-fold higher than that of freshwater-acclimated trout on the 35th day of seawater acclimation, while the results were opposite for complex II (Brijs et al., 2017).

The missing amino acid in the core protein of electron transport complex I could potentially be the reason why *S. rizeensis* cannot adapt to saltwater and is exclusively found in freshwater. If this is true, *S. rizeensis* could only have spread from its area of origin to other drainages when seawater was almost fresh.

Until recently, it was assumed that mitochondrial genes were not associated with life histories. However, Wang et al. (2022), by analyzing the complete mitogenomes of 18 freshwater and 31 anadromous salmonid species, found significantly higher dN/dS ratios in most of the mitochondrial protein-coding genes of the anadromous group than in the resident group. This finding likely further emphasizes the role of mitochondrial genes in anadromy.

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Appendix 1. Accession numbers of Cyt-b sequences of Ponticaspian brown trout used in this study (both obtained and downloaded from GenBank).

Accession #	*Species*	Basin	Drainage, Country	Reference
FJ608987	*S. Choruhensis*	Black Sea	Coruh drainage, Turkey	Turan et al. 2009
FJ608988	*S. Choruhensis*	Black Sea	Coruh drainage, Turkey	Turan et al. 2009
FJ608995	*S. Choruhensis*	Black Sea	Iyidere drainage, Turkey	Turan et al. 2009
FJ608994	*S. Choruhensis*	Black Sea	Iyidere drainage, Turkey	Turan et al. 2009
FJ608999	*Salmo sp.*	Persian Gulf	Euphrates drainage, Turkey	Turan et al. 2009
FJ608991	*S. riseensis*	Black Sea	Iyidere drainage, Turkey	Turan et al. 2009
FJ608993	*S. riseensis*	Black Sea	Cayeli River, Turkey	Turan et al. 2009
FJ608992	*S. riseensis*	Black Sea	Cayeli River, Turkey	Turan et al. 2009
FJ608997	*Salmo sp.*	Persian Gulf	Euphrates drainage, Turkey	Turan et al. 2009
EU492282	*S. sp*	Baltic Sea	Baltic Sea, Sweden	Noren et al. 2008
KF985737	*S. trutta*	Black Sea	Danube drainage, Austria	Schenekar et al.2014
KF985738	*S. trutta*	Black Sea	Danube drainage, Austria	Schenekar et al.2014
FJ655773	*S. caspius*	Caspian Sea	Caspian Sea, Iran	Jamshidi & Kalbassi
EU492348	*S.trutta trutta*	Baltic Sea	Baltic Sea, Sweden	Noren et al. 2008
FJ608989	*S. Choruhensis*	Black Sea	Coruh drainage, Turkey	Turan et al. 2009
JN995186	*S. trutta fario*	Caspian Sea	Caspian Basin, Iran	Rezaei & Akhshabi 2011
JX960835	*S. trutta*	Mediterranean Sea	Göksu River, Turkey	Crete-Lafreniere et al. 2012
LC011387	*S. trutta caspius*	Caspian Sea	Caspian Sea, Iran	Rezaei et al. 2014

JX960837	*S. trutta*	Caspian Sea	Arpa River, Armenia	Crete-Lafreniere et al. 2012
JX960839	*S. trutta*	Adriatic Sea	Voidomatis River, Greece	Crete-Lafreniere et al. 2012
JX960836	*S. trutta*	Atlantic Ocean	Leksa River, Norway	Crete-Lafreniere et al. 2012
JX960764	*S. ohridanus*	Lake Ohrid	Lake Ohrid, Republic of Macedonia	Crete-Lafreniere et al. 2012
JX960841	*S. obtusirostris*	Adriatic Sea	Buna River, Bosnia-Herzegovena	Crete-Lafreniere et al. 2012
JX960842	*S. platycephalus*	Mediterranean Sea	Soğuksu Stream, Turkey	Crete-Lafreniere et al. 2012
JX960838	*S. marmoratus*	Adriatic Sea	Zala River, Slovenia	Crete-Lafreniere et al. 2012
KX594755	*S. trutta*	Unknown river	India	Singh et al. 2016
EU492108	*S. trutta*	North Sea	North Sea, Netherlands	van Pelt-Heerschap & Stein
EU492280	*S. salar*	Baltic Sea	Baltic Sea, Sweden	Noren et al. 2008
JX960834	*S. salar*	Atlantic Ocean	Ims, Norway	Crete-Lafreniere et al. 2012
KT279188	*S. trutta*	Atlantic Ocean	Dades River, Morocco	Doadrio et al. 2015
KT279196	*S. trutta*	Atlantic Ocean	Tensift Basin, Morocco	Doadrio et al. 2015
KT279174	*S. trutta*	Atlantic Ocean	Oum er Rbia, Morocco	Doadrio et al. 2015
KT279171	*S. trutta*	Isli Lake	Isli Lake, Morocco	Doadrio et al. 2015
KT279195	*S. trutta*	Mediterranean Sea	Moulouya Basin, Morocco	Doadrio et al. 2015
KT279191	*S. trutta*	Atlantic Ocean	Oum er Rbia, Morocco	Doadrio et al. 2015
KT279184	*S. trutta*	Atlantic Ocean	Ziz Basin, Morocco	Doadrio et al. 2015
KT279179	*S. trutta*	Atlantic Ocean	Oum er Rbia, Morocco	Doadrio et al. 2015
KT279176	*S. akairos*	Ifni Lake	Ifni Lake, Morocco	Doadrio et al. 2015
KT279177	*S. akairos*	Ifni Lake	Ifni Lake, Morocco	Doadrio et al. 2015
KX594732	*S. trutta fario*	Unknown river	India	Singh et al. 2016
KX594730	*S. trutta fario*	Unknown river	India	Singh et al. 2016
KX594740	*S. trutta fario*	Unknown river	India	Singh et al. 2016
KX594724	*S. trutta fario*	Unknown river	India	Singh et al. 2016
NC_037399	*S. ischchan*	Lake Sevan	Caspian Basin, Armenia	Ninua et al. 2018
MG551591	*S. ischchan*	Lake Sevan	Caspian Basin, Armenia	Ninua et al. 2018
MG940853	*S. ischchan*	Lake Sevan	Caspian Basin, Armenia	Ninua et al. 2018
MG029536	*S. labrax*	Black Sea	Black Sea, Georgia	Ninua et al. 2018
MG029537	*S. labrax*	Black Sea	Black Sea, Georgia	Ninua et al. 2018
MG029538	*S. labrax*	Black Sea	Black Sea, Georgia	Ninua et al. 2018
MG029539	*S. labrax*	Black Sea	Black Sea, Georgia	Ninua et al. 2018

MG029540	*S. labrax*	Black Sea	Coruh (Chorokhi) drainage, Georgia	Ninua et al. 2018
MG029541	*S. labrax*	Black Sea	Coruh (Chorokhi) drainage, Georgia	Ninua et al. 2018
MG029542	*S. labrax*	Black Sea	Coruh (Chorokhi) drainage, Georgia	Ninua et al. 2018
MG029543	*S. labrax*	Black Sea	Enguri drainage, Georgia	Ninua et al. 2018
MG029544	*S. labrax*	Black Sea	Enguri drainage, Georgia	Ninua et al. 2018
MG029545	*S. labrax*	Black Sea	Kintrishi River, Georgia	Ninua et al. 2018
MG029546	*S. labrax*	Black Sea	Kintrishi River, Georgia	Ninua et al. 2018
MG029547	*S. labrax*	Black Sea	Kintrishi River, Georgia	Ninua et al. 2018
MG029548	*S. caspius*	Caspian Sea	Kura (Mtkvari) drainage, Georgia	Ninua et al. 2018
MG029549	*S. caspius*	Caspian Sea	Kura (Mtkvari) drainage, Georgia	Ninua et al. 2018
MG029550	*S. labrax*	Black Sea	Rioni drainage, Georgia	Ninua et al. 2018
MG029551	*S. labrax*	Black Sea	Rioni drainage, Georgia	Ninua et al. 2018
MG029552	*S. ciscaucasicus*	Caspian Sea	Terek (Tergi) drainage, Georgia	Ninua et al. 2018
MG029553	*S. ciscaucasicus*	Caspian Sea	Terek (Tergi) drainage, Georgia	Ninua et al. 2018
OP849668	*S. labrax*	Black Sea	Coruh (Chorokhi) drainage, Georgia	Ninua et al., 2023
OP849669	*S. labrax*	Black Sea	Chakvistskali, Georgia	Ninua et al., 2023
OP849670	*S. labrax*	Black Sea	Chakvistskali, Georgia	Ninua et al., 2023
OP849671	*S. labrax*	Black Sea	Coruh (Chorokhi) drainage, Georgia	Ninua et al., 2023
OP849672	*S. labrax*	Black Sea	Enguri drainage, Georgia	Ninua et al., 2023
OP849673	*S. labrax*	Black Sea	Enguri drainage, Georgia	Ninua et al., 2023
OP849674	*S. riseensis*	Black Sea	Rioni drainage, Georgia	Ninua et al., 2023
OP849675	*S. labrax*	Black Sea	Rioni drainage, Georgia	Ninua et al., 2023
OP849676	*S. labrax*	Black Sea	Khobistskali drainage, Georgia	Ninua et al., 2023
OP849677	*S. labrax*	Black Sea	Enguri drainage, Georgia	Ninua et al., 2023
OP849678	*S. caspius*	Caspian Sea	Kura (Mtkvari) drainage, Georgia	Ninua et al., 2023
OP849679	*S. caspius*	Caspian Sea	Kura (Mtkvari) drainage, Georgia	Ninua et al., 2023
OP849680	*S. caspius*	Caspian Sea	Sulak drainage, Georgia	Ninua et al., 2023

Appendix 2. The number of samples from each location and species used for the analysis of mitochondrial Cyt-b gene and microsatellite genotypes (STR) at 10 loci (Ninua et al., 2023)

Watercourse	Drainage	Location #	Species	CytB	STR
Our results					
Chvana	Chorokhi (Coruh) (BS)	1	*S. labrax (?)*	3	10
Akavreta	Chorokhi (Coruh) (BS)	2	*S. labrax*	9	21
Chakvistskali	Chakvistskali (BS)	3	*S. labrax*	3	15
Kintrishi	Kintrishi (BS)	4	*S. labrax*	10	11
Black Sea	Black Sea	5	*S. labrax*	8	14
Shareula	Rioni (BS)	6	*S. sp. (rizeensis)*	3	14
Krikhula	Rioni (BS)	7	*S. labrax*	3	11
Sakraula	Rioni (BS)	8	*S. labrax*	7	9
Tsentskali	Khobistskali (BS)	9	*S. labrax*	3	14
Magana	Enguri (BS)	10	*S. labrax*	3	15
Khuberi	Enguri (BS)	11	*S. labrax*	4	6
Nenskra	Enguri (BS)	12	*S. labrax*	5	7
Dolra	Enguri (BS)	13	*S. labrax*	3	15
Snostskali	Tergi (Terek) (CS)	14	*S. ciscaucasicus*	6	17
Khiso's Alazani	Sulak (CS)	15	*S. caspius*	4	15
Aragvi	Kura (Mtkvari) (CS)	16	*S. caspius*	2	12
Ilto	Kura (Mtkvari) (CS)	17	*S. caspius*	3	13
Iori	Kura (Mtkvari) (CS)	18	*S. caspius*	0	4
Ktsia	Kura (Mtkvari) (CS)	19	*S. caspius*	4	15
Downloaded from GenBank					
Cayirbasi	Chorokhi (Coruh)(BS)	-	*S. coruhensis*	3	-
Kendirli	Iyidere (BS)	-	*S. coruhensis*	2	-
Sirli Stream	Euphrates (PG)	-	*Salmo sp.*	1	-
Ovit Stream	Iyidere (BS)	-	*S. rizeensis*	1	-
Cayeli	Cayeli (BS)	-	*S. rizeensis*	2	-
Rizekent Stream	Euphrates (PG)	-	*Salmo sp.*	1	-
Baltic Sea	Baltic Sea	-	*S. trutta*	3	-
Baltic Sea	Baltic Sea	-	*S. salar*	1	-

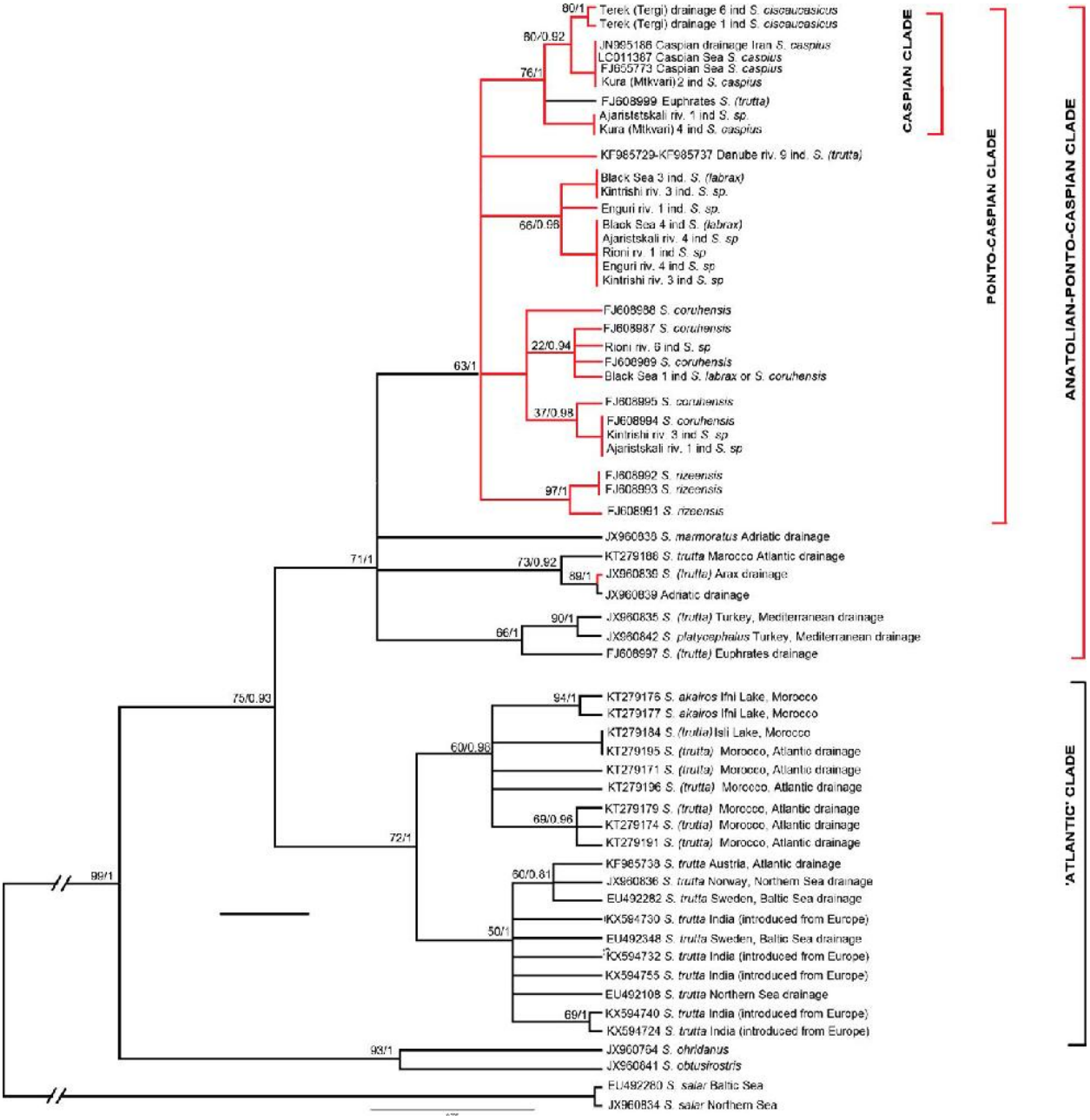
Lohnbach	Danube (BS)	-	*S. trutta*	2	-
Unknown	Iran (CS)	-	*S. caspius*	1	-
Göksu	Göksu (MS)	-	*S. trutta*	1	-
Caspian Sea	Caspian Sea	-	*S. caspius*	2	-
Arpa	Kura (Mtkvari) (CS)	-	*S. trutta*	1	-
Voidomatis	Aoös (MS)	-	*S. trutta*	1	-
Leksa	Leksa (AO)	-	*S. trutta*	1	-
Lake Ohrid	Lake Ohrid	-	*S. ohridanus*	1	-
Buna River	Buna River	-	*S. obtusirostris*	1	-
Soğuksu	Soğuksu (Md)	-	*S. platycephalus*	1	-
Zala	Zala (Ad)	-	*S. marmoratus*	1	-
N/A	India	-	*S. trutta*	5	-
North Sea	North Sea	-	*S. trutta*	1	-
Ims	Ims (AO)	-	*S. salar*	1	-
Dades	Dades (AO)	-	*S. trutta*	1	-
Tensift	Tensift (AO)	-	*S. trutta*	1	-
Oum er Rbia	Oum er Rbia (AO)	-	*S. trutta*	3	-
Isli Lake	Isli Lake AO)	-	*S. trutta*	1	-
Moulouya	Moulouya (MS)	-	*S. trutta*	1	-
Ziz	Ziz (AO)	-	*S. trutta*	1	-
Ifni Lake	Ifni Lake (AO)	-	*S. akairos*	2	-

Appendix 3. Diversity of taxa identified from the stomach content of 181 trout from 13 populations

Order	Family	Genus	Species
Hymenoptera	Formicidae Tenthredinidae Ichnemonidae Vespidae Apidae Crabronidae	*Vespula*	*V. germanica*
Heteroptera	Pentatomidae	*Carpocoris*	
Diptera	Blephariceridae Tipulidae		

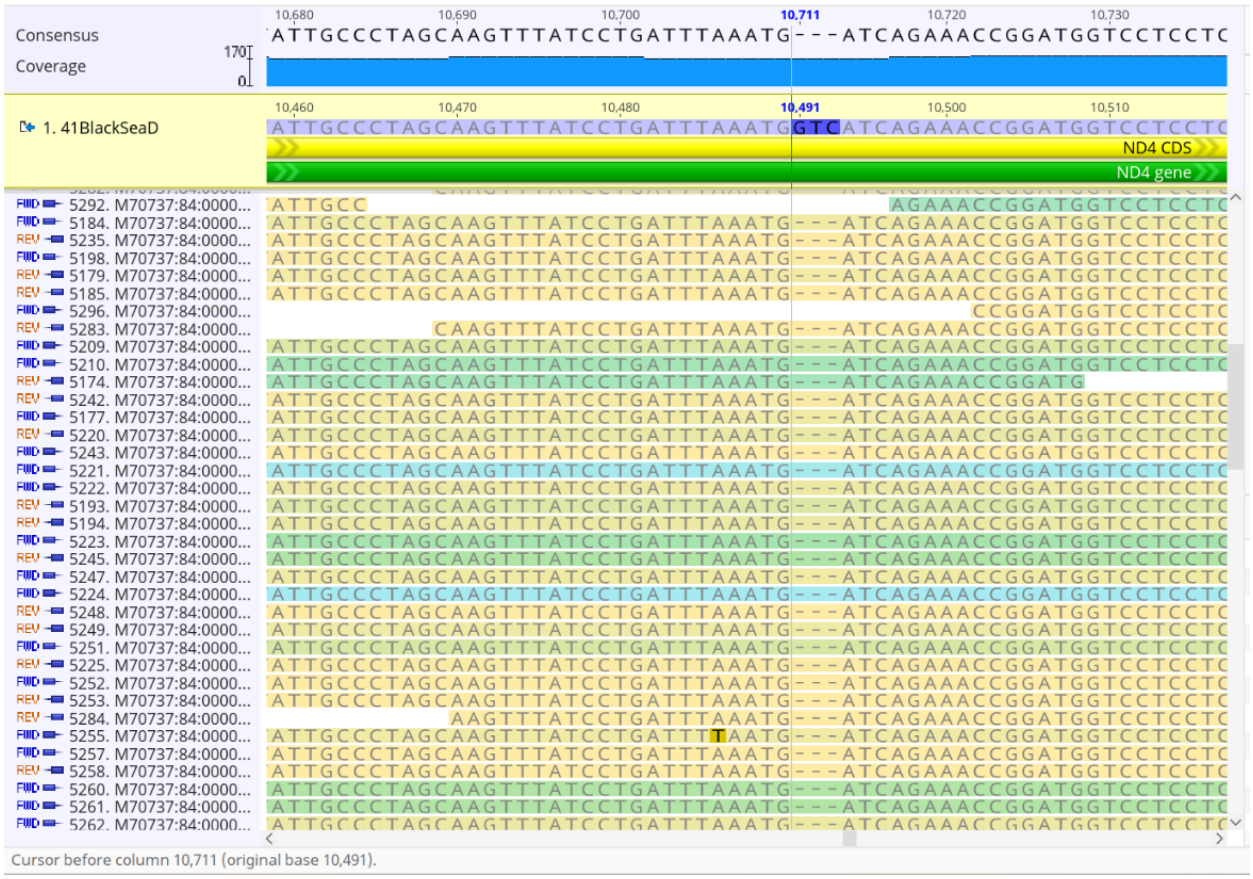
	Syrphidae	*Syritta*	*S. pipiens*
		Chrysotoxum	
	Simuliidae		
	Scathophagidae		
	Faniidae		
	Chironomidae		
	Bibionidae	*Bibio*	*Bibio hortulanus*
	Asilidae		
	Culicidae		
	Dolichopodidae		
	Conopidae	*Sicus*	
	Tephritidae	*Rhagoletis*	
	Empididae		
Orthoptera	Acrididae	*Chorthippus*	
	Tettigoniidae	*Bicolorana*	*Bicolorana bicolor*
		Psorodonotus	*P. venosus*
		Isophya	
		Parapholidoptera	
		Stenonemobius	
Coleoptera	Carabidae	*Harpalus*	
	Cerambycidae		
	Coccinellidae		
	Dytiscidae	*Agabus*	
	Tenebrionidae		
	Staphilinidae		
	Curculionidae		
	Elmidae		
	Leiodidae		
	Gnaphosidae		
	Scarabaeidae		
	Meloidae		
Hemiptera	Acanthosomatidae	*Elasmucha*	*Elasmucha grisea*
	Cicadellidae	*Aphrodes*	*A. makarovi*
		Eupteryx	
	Pyrrhocoridae	*Pyrrhocoris*	*P. apterus*

	Phsilidae Issidae	*Mycterodes*	
Trichoptera	Philopotamidae	*Wormaldia*	
Ephemeroptera	Heptageniidae		
Plecoptera	Unidentified		
Odonata	Libellulidae		
Opiliones	Trogulidae Nemastomatidae	*Histricostoma*	*H. caucasicum*
Neuroptera	Chrysopidae Osmylidae	*Osmylus*	
Lepidoptera	Noctuidae Geometridae		
Mecoptera	Panorpidae	*Paborpa*	
Araneae	Thomisidae	*Ebrechtella* *Xysticus* *Synema*	*E. tricuspidata*
	Araneidae	*Araneus*	
	Dictynidae		
	Philodromidae	*Philodromus*	
	Clubionidae	*Clubiona*	
	Gnaphosidae	*Gnaphosa*	
Amphipoda	Gammaridae	*Gammarus*	
Gastropoda	Planorbidae	*Ancylus*	
Isopoda			
Pseudoscorpiones	Neobisiidae		



Appendix 4. Phylogeny of the brown trout/salmon from the Ponto-Caspian basin, Anatolia/basin of the Indian Ocean, basin of Atlantic Ocean, and the Balkans, based on the mitochondrial cytochrome b gene sequences. The topology inferred using Bayesian analysis (BI). Left to the nodes: bootstrap support of the respective clade inferred using maximum likelihood (ML) algorithm before the slash, posterior probability as inferred by BI after the slash. Identical haplotypes shown separately if found at more than one location. Clades with bootstrap support below 50 and PPs below 0.5 shown as polytomies. Red branches—samples

from the drainage of the Black Sea; blue branches—samples from the drainage of the Caspian Sea; green branches—samples from the Atlantic, Northern, and Baltic Sea drainages (Ninua et al., 2018).



Appendix 5. NGS reads of S. rizeensis mapped on ND4 gene of smoltified S. labrax showing 3 nucleotide deletion in S. rizeensis.