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Genetic differentiation and demographics of brown trout (*Salmo trutta*) in tributaries of a sub-arctic watercourse affected by fragmentation and stocking

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Preface

When approaching this task, I had little knowledge about electrofishing, brown trout population dynamics and genetic theory. This has been a process of learning, and one that wouldn't have been possible without help. I would like to thank my supervisor at NMBU, Thrond Oddvar Haugen, for supervision in the lab, help with statistics, and general feedback. At NIBIO Svanhovd I would like to thank Snorre B. Hagen for help with organizing the project and for feedback during the process. I would like to thank Cornelya F. C. Klütsch for help with genetic analysis and tests, as well as great feedback and help during the whole process. Paul Eric Aspholm was a great support during the field work period, thank you. Many thanks also to everyone contributing to the DNA-analysis at the lab in Svanhovd. Lastly, the data sampling was crucial for the whole project. My assistant Juho Vuolteenaho made this go smoothly, with his patience and dedication to the project.

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Abstract

The Pasvik river in north-eastern Norway harbours the only piscivorous brown trout (*Salmo trutta*) population in Finnmark County. Throughout the river, seven hydroelectric power dams were constructed between 1951 and 1978. This caused a decline in the brown trout population and the amount of spawning habitat. A stocking programme has been implemented for the last few decades and 5000 >25 cm brown trout are released into the watercourse annually. Fragmentation and stocking have led to alteration and homogenization of the genetic structure in the main river trout population. The focus in this study has been on the brown trout populations inhabiting the tributaries within the watercourse, specifically addressing if there is evidence of geneflow among tributaries and if there is geneflow between the main river and tributaries. Effects of dams and distance in the genetic structuring of brown trout in the watercourse was explored, as well as bottleneck events and the effect of admixture from stocked individuals on population densities.

Sampling by electrofishing was conducted in 10 tributaries during 2018. A total of 320 brown trout were captured. In addition, 287 samples from the main river were also used, mostly collected by anglers. Tissue for genetic analysis (MSATs) was collected from all samples, while otoliths and scales for age determination were only collected from tributary samples. Further, following the methods of Bohlin et al. (1989), data for density estimates were captured in the tributaries.

Nine out of 10 tributaries harboured brown trout, and age classes of 0+ to 3+ were present in all, meaning natural recruitment occurs every year. Genetic admixture with main river individuals in several of the tributaries strongly indicate that main river trout use these tributaries for spawning. However, some tributaries were strongly genetically differentiated from the main river and other tributaries. There was no clear evidence of the effect of barriers (hydroelectric dams) on genetic structure in the tributaries, but there were signs of isolation-by-distance. Brown trout density in the tributaries decreased with the proportion of individuals admixed with the main river/stocked trout. Signs of recent bottleneck events were detected in several tributaries, mostly at the sampling station level. Lastly, the genetic diversity was found to be higher in the tributaries combined, than in the main river.

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1 Introduction

Today, almost no large river systems on Earth are unaffected by human made installations, such as hydroelectric power dams (Hall et al. 1991). Although providing close to emission-free energy, negative effects of hydroelectric power dams include the removal of free-flowing river habitat, prohibiting fish migration and reduced water quality in reservoirs and downstream river reaches (Jager & Smith 2008). Alternation of flow regimes in rivers is often claimed to be one of the largest and continuous threats to river ecosystems and wetlands connected to them (Sparks 1995).

The disturbance and isolation of fish populations within a watercourse are unavoidable results following the construction of a hydroelectric power dam. Although installations to encourage fish movement are common, hydroelectric power dams are still condition-dependent (e.g., water level, water flow), and in many cases permanent barriers for several fish species (Junge et al. 2013). Fish movement and migration works as mechanisms for gene flow (Heggenes et al. 2006), while fragmentation may lead to genetic drift (Willi et al. 2006). Downstream gene flow in river systems has been found to have a positive effect on the genetic diversity in fish populations, although resulting in the populations upstream losing diversity. Nevertheless, upstream movement in free-flowing waterways is more frequent and consistent than in river systems with hydroelectric dams (Junge et al. 2013).

The dynamics of large, complex biological systems are often more stable through space and time than their components (Schindler et al. 2010, 2015). This portfolio effect averages out temporal and spatial changes in the dynamics within and among system components (Dey & Joshi 2006, Schindler et al. 2010, 2015). Diversity in nature is a sign of robustness, whether it be species diversity or genetic diversity within a population. Although primarily applied in ecological contexts (Lhomme et al. 2001), increasing evidence points to similar stabilizing (portfolio) effects of genetic diversity and differentiation. Isolation of a fish population over time can lead to loss of genetic diversity (Schmidt et al. 2017), which in turn provides raw material for evolution through natural selection (Fisher 1930). Therefore, reduced genetic diversity in a population may lead to the population being less adaptable and less resilient to new environmental conditions (Hanski & Gaggiotti 2004) (e.g., climate change, new diseases or pathogens).

The Eurasian brown trout (*Salmo trutta* L. 1758) is widespread around the northern hemisphere (Jonsson & Jonsson 2006), and indigenous to Europe, North Africa and western

Asia (MacCrimmon 1970). The brown trout is considered one of the world's most invasive species (Budy et al. 2013). Being a socio-economically important freshwater fish, it has been introduced by man to almost every corner of the world (Townsend 1996). Hence, the brown trout can by no means be called a threatened species. However, when considering natural genetic diversity in brown trout populations, things become more complex. The brown trout has experienced significant alterations of its genetic diversity and population structure throughout its natural distribution range due to overexploitation, translocations, and stocking (Klütsch et al. 2019).

The Pasvik river, shared between Norway, Russia and Finland, is the largest river system in the northern part of Fennoscandia (Dauvalter & Rognerud 2001). Owing to the large-bodied piscivorous brown trout in this river system, the Pasvik river is an attractive fishing destination for anglers. Throughout the river, seven hydroelectric power dams were built between 1951 and 1978, destroying as much as 70 % of the brown trout's spawning habitat (Klütsch et al. 2019).

Stocking of salmonids to mitigate negative impacts of anthropogenic intervention, such as hydropower regulation, has been an important measure for decades (Wollebæk 2010, Vøllestad & Hesthagen 2001). As the natural recruitment potential for the brown trout was considerably reduced after the implementation of the power dams in the Pasvik river, 5000 stocked brown trout > 25 cm are released into the watercourse annually. There is evidence that stocking may have both stabilizing and destabilizing effects on metapopulation persistence in brown trout. Stocking may be essential to keep subpopulations intact; for instance, stocking can counter the isolation effect of hydroelectric dams. Further, stocking may alleviate strong harvest pressures and, in this manner, contribute to subpopulation persistence. However, studies show that stocked fish may lose genetic diversity compared to the wild population over time (Allendorf & Phelps1980, Blanchet et al. 2008, Christie et al. 2012, Valiquette et al. 2014), and homogenize genetic diversity in heavily stocked salmonid populations (Marie et al. 2010), with potential fitness effects even after just one generation of stocking (Cristie et al 2016). In the Pasvik River, wild brood fish are captured each year to tackle this issue. However, the brood fish are captured at the same spot every year and have been for more than 30 years. This practice suggests that genotypes from one part of the watercourse are overrepresented in the stocking programme. In addition, incidents of reusing the same individuals to brood several year classes of stocked fish have occurred. Brown trout is a species with high fecundity and strong sexual selection, hence, a few individuals can

contribute to large proportions of an age class in the wild (Serbezov et al. 2010). However, Christie et al. (2012) found genetic adaption in steelhead trout (*Oncorhynchus mykiss*) to captivity after only one generation. Steelhead trout with the highest fitness in a captive environment produced offspring that performed poorly under wild conditions (Christie et a. 2012). A study focusing on the diet and growth of the Pasvik brown trout concluded that more than 80 % of fish caught throughout the watercourse originated from stocked fish (Haugland 2014). This is also in compliance with a recent genetic study, which found that the genetic diversity in the main river trout is heavily influenced by stocking (Klütsch et al. 2019). This indicates that the natural genetic diversity in this brown trout population is under threat, adding it to the pile of other genetically inflicted stocked trout populations around the world (Araguas 2009).

Several tributaries of variable size exist throughout the Pasvik watercourse. In light of both stocking and hydroelectric dams, there is now an increased interest in the tributaries' role and importance for natural recruitment in the wild part of the trout population. Brown trout inhabiting dendritic river systems are known to use main waterways to grow and mature (Jonsson & Jonsson 1993, Forseth et al. 1999) and smaller tributaries as spawning-and nursery grounds (Crisp 1996, Armstrong et al. 2003). This study aims to assess the genetic differentiation and demographics of brown trout within the Pasvik watercourse, with special focus on a selection of tributaries in the Norwegian part of this transnational and fragmented river system. Although much research has been done regarding piscivorous trout populations in southern Norway, less has been done in the sub-arctic (see Jensen et al. 2008, Haugland 2014, Klütsch et al. 2019). By looking at age structure and densities of brown trout in the tributaries, the aim is to illuminate potential demographical differences between the tributaries. A comparison of genetic structure from the tributaries with structure from the main river, which is influenced by both stocking and hydroelectric regulation, may give an insight into the tributaries' role in the genetic stability of this system. To this aim, the focus was on testing the following set of hypotheses:

Hypotheses:

A) If brown trout uses the tributaries as spawning grounds, then the expectation is to find individuals of lower age classes (i.e., 0+ - 2+ year olds) in the tributaries.

B) If dams constitute major migration barriers to gene flow, then brown trout populations in tributaries within the same closed-off section of the main river will be more genetically similar to each other than to populations in tributaries located in different sections of the river that are separated by dams.

C) If main river brown trout uses tributaries as spawning grounds, then genetically mixed ancestry of brown trout in the tributaries would indicate offspring of tributary and main river brown trout which would be evidence for natural recruitment in the tributaries.

D) If brown trout uses the tributaries as spawning grounds, and since the tributaries are variable in both spatial structuring, size, biotic and abiotic factors, then densities of brown trout should also vary. In addition, tributaries in stocked sections of the main river should have higher densities as they are potentially used by main river brown trout.

E) If stocking leads to reduction in genetic diversity (Klütsch et al. 2019) and stocking does not occur in the tributaries, it can be expected that higher genetic diversity is found there compared to the main river, based on the assumption that they support natural recruitment.

2 Methods

2.1 Study species

The brown trout spawns in running water and prefers a river bed composed of stone and gravel (Scott & Irvine 2000). Incidents of lake spawning in brown trout populations do occur, but rarely (Brabrand et al. 2002). Reproduction takes place in autumn or winter, earlier at higher altitudes and latitudes as temperatures here are lower, resulting in the egg incubating period being longer (Klemetsen et al. 2003). Large individuals can utilize relatively small stream systems for spawning (Jonsson & Jonsson 2011, Jonsson et al. 2001). Brown trout may to a large extent be sedentary in rivers, but in many cases also seem to move around frequently (Crisp 1993). After hatching and when the alevins (yolk-sac larvae) reach a size of around 20 mm, they will start feeding around the spawning area. Successful individuals will disperse as they grow larger with expanded needs for food and space (Klemetsen et al. 2003). Brown trout occurring in lakes or rivers connected to the sea often form anadromous populations (Klemetsen et al. 2003). The dispersal of brown trout occurs mainly in their first years of life. In South-Eastern Norway, a study concluded that brown trout in tributaries of Lake Femund mainly migrated to the lake at age 2 (40 %), or 3 (27 %). Time of migration in total varied among ages 1-8 (Jonsson et al. 1999). Another study from the same area found that faster-growing individuals migrated earlier from the stream than slower-growing individuals. 2+ year old migrants were significantly larger than the ones remaining in the stream, and 3+ year old migrants were significantly larger than 2+ year old migrants (Forseth et al. 1999). The brown trout is an opportunistic generalist, but different individuals seem to temporarily specialize on certain types of food (Klemetsen et al. 2003). In streams, insect larvae appear to be important for young individuals, while littoral zoobenthos is the main food source for many lake-dwelling populations. Prey fish is important for large trout (Klemetsen et al. 2003). Both in Lake Femund and Lake Fyresvatnet, Southern Norway, the majority of brown trout had a piscivorous diet at approx. 30 cm of length (Jensen et al. 2012, Næsje et al. 1998). This manifest itself, among other things, in different life history strategies (Höjesjö et al. 2011). Resident brown trout remain in the river/tributary their entire life (Dodson et al. 2013; Jonsson & Jonsson 2006; Laikre 1999). Anadromous brown trout migrate from natal rivers to the sea until they reach sexual maturity, upon which they return to their native streams to spawn, while lake-dwelling brown trout travel from natal rivers to lakes and back. The different life history strategies (Dodson et al. 2013; Nielsen et al. 2003) may partially

explain the intricate population-genetic structure and diversity patterns seen in this species (Kraabøl et al. 2009), including regional genetic differentiation patterns that might be indicative of local adaptions to specific environments (Fraser et al. 2011; Meier et al. 2011).

2.2 The fish community in the Pasvik watercourse

Commercial fishing and fishing for private consumption have long traditions in the Pasvik River (Schaanning 1916). Throughout the watercourse, 15 species of fish have been recorded. The most commonly occurring species are perch *(Perca fluviatilis)*, pike *(Esox lucius)*, whitefish *(Coregonus lavaretus)*, burbot *(Lota lota)* and brown trout (Amundsen et al. 1999). Vendace (*Coregonus albula*) was observed for the first time in 1989 and has since colonized the entire watercourse (Amundsen et al. 1999). Originating from introductions to tributaries of lake Inari in the 1960s (Mutenia & Salonen 1992), vendace is now considered one of the most important prey fish for the main river-dwelling Pasvik trout (Jensen 2008). The trout population in the Pasvik watercourse is a piscivorous one. There are 165 such populations in Norway, but the Pasvik population is the only piscivorous brown trout population in Finnmark (Dervo et al. 1996). These populations are considered a result of the brown trout's phenotypical plasticity, where the combination of prey availability and the physical environment play important roles. A report from 2018 presented a twofold definition; *A piscivorous brown trout population is naturally reproducing with a regular occurrence of individuals preying on smaller fish, and where the transition to a fish-based diet results in A) change in growth rate or B) persistent growth* (Museth et al. 2018). Many of historically described populations are now extinct, and many of those remaining are under threat from human activity. Utilizing rivers for hydropower production is considered the biggest threat to piscivorous brown trout populations in Norway (Kraabøl 2010). However, fishing and overharvest can also have a negative impact, as large trophy individuals of brown trout are a sought catch among anglers (Dervo et al. 1996). The latter is also what makes these populations so valuable in an anthropogenic perspective. Large individuals are important in a vital salmonid population. For example, large females produce more eggs (Foote 1989) and are able to dig deeper nests than smaller females, thereby increasing the survival rate of the eggs (Steen & Quinn 1999).

2.3 Location and sampling areas

The Pasvik valley is located in north-eastern Norway (Figure 1). Originating from lake Inari in northern Finland, the Pasvik river runs into Russia before functioning as a border between Norway and Russia for about 110 kilometres. The total area of the Norwegian-Russian part of the river is 142 km^2, with a mean annual water flow of 175 m^3/s. Tributaries connected to the main river are located both in the Norwegian and Russian part. Due to the regulations, most of the rapid waterfalls have disappeared, and the watercourse now consists of lakes, reservoirs and sections of slow-flowing river stretches. Dam construction started in 1932 and ended in 1956 in the upper Russian part (sections A-C, Figure 1). In the Norwegian-Russian part the dam constructions unfolded between 1956 and 1978 (sections D-J). The Pasvik river has a water catchment of 18287 km^2 (NVE).

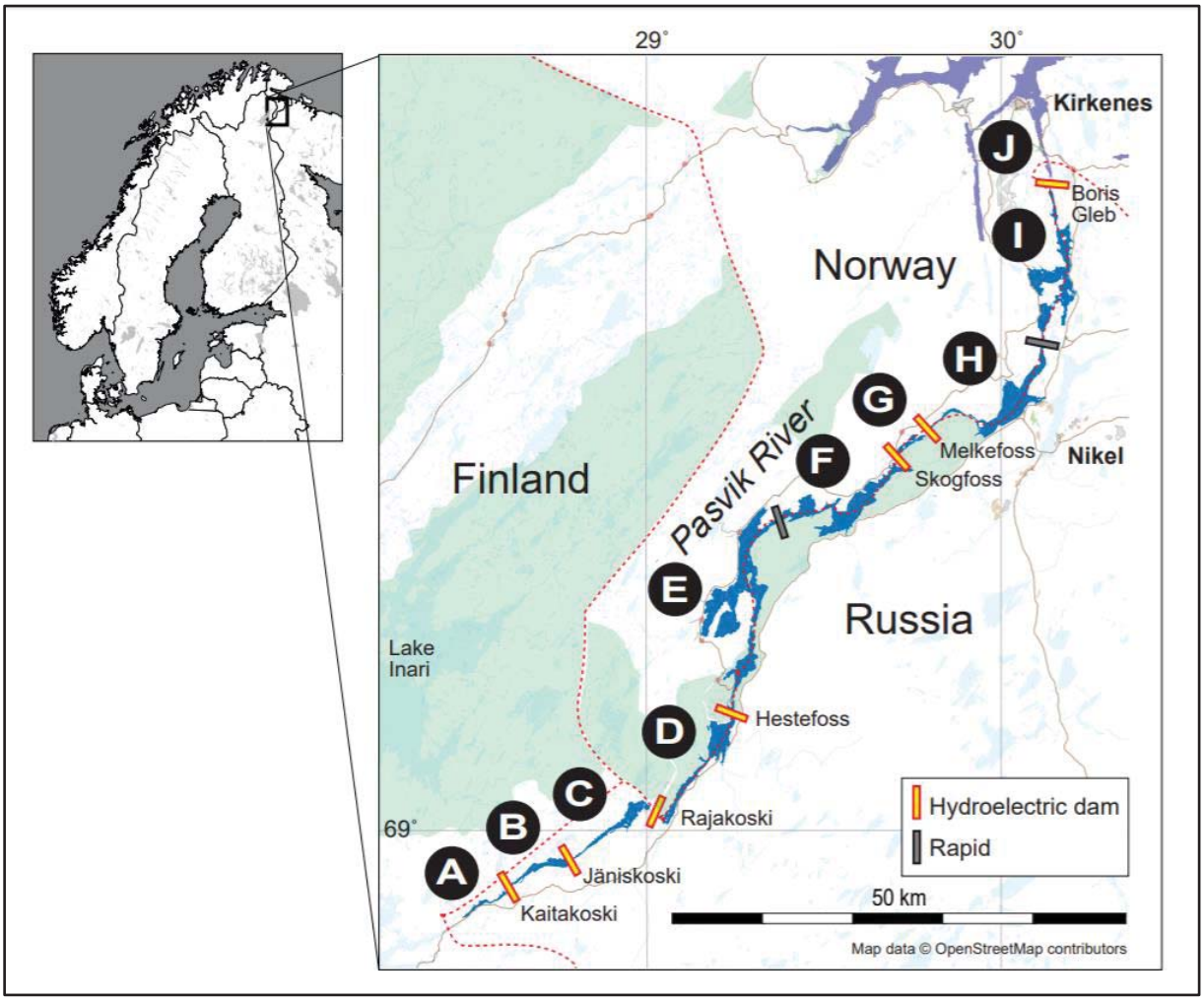


Figure 1. *The Pasvik valley is located in the eastern part of Finnmark. with Russia to the east and Finland to the west. The Pasvik river makes up the border between Norway and Russia. The river has been divided into sections to make management easier. Map retrieved from Klütsch et al. (2019).*

It was a goal to investigate streams connected to all the closed-off sections of the main river. Four sections are closed off by hydroelectric dams, with no effort made to enable fish to pass. On the Norwegian side, 10 tributaries were considered important for spawning within the wild brown trout population. These are marked in red in figure 2. The closed-off sections from upstream to downstream is Rayakoski-Hestefoss (section D) Hestefoss-Skogfoss (section E-F) Skogfoss-Melkefoss (section G) and Melkefoss-Boris Gleb (section H-I). The tributaries investigated from south to north were Ødevassbekken (DOB), Gjeddebekken (DGB), Gjøkbekken (EGB), Ellenelva (EE), Steinbekken (ESTB), Spurvbekken (ESB), Skjellbekken (FSB), Samentielva (GSE), Ørretbekken (IOB) and Boddibekken (IBB). From here on, only the abbreviations will be used. Section D is the only section never to have been stocked, whereas section G has not been stocked for the past decade. The other sections are stocked with approximately 5000 brown trout from the breeding facility, annually.

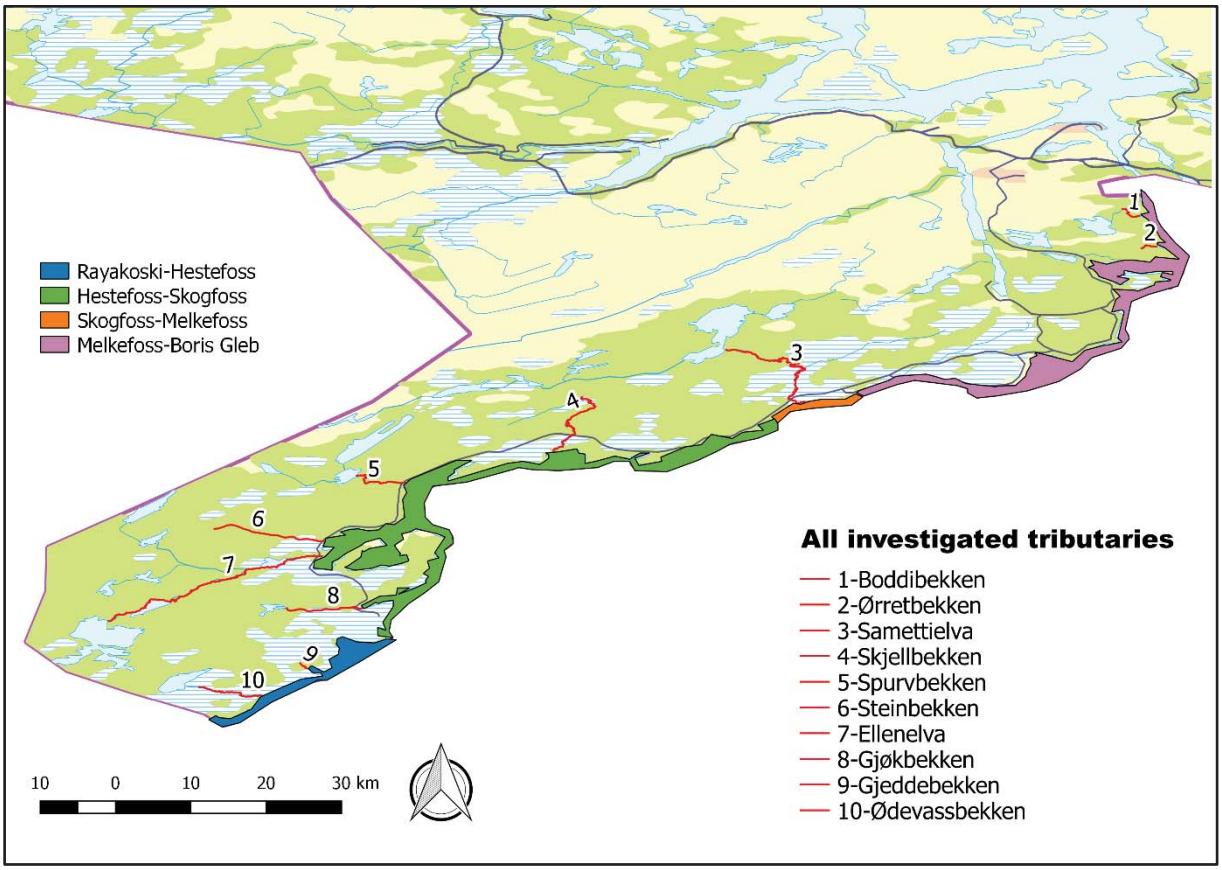


Figure 2. *Tributaries investigated in the Norwegian part of the Pasvik watercourse, with different colour codes used to denote the different main-river sections closed off by hydroelectric dams.*

2.4 Field sampling

Fishing was conducted in the period between 28th of August and 13th of September 2018. The fish were captured by electro fishing, using portable backpack electroshocking gear (Steinar Paulsen: 1983 FA2 No. 7, 700/1400 volt, 35-70 Hz, pulsed-DC). Fishing was done walking upstream, and electroshocks were given in pulses of up to 30 seconds.

Several factors have been proven to affect catchability of fish by electrofishing. Water conductivity may vary between streams and affect the voltage obtained when fishing, and thereby the catchability of fish (Bohlin et al. 1989). Catchability also improves exponentially with fish size, since surface area increases as the fish gets bigger. High water levels and strong current reduces catchability, as there will be less fish per area unit and visibility will be lower (Bohlin et al. 1989). The weather can also influence the detection rate of stunned fish. Polaroid glasses were used to increase visibility.

The aim was a total sample size of 40 brown trout per stream. In some streams the number caught was slightly lower, due to different factors such as the stream size, amount of brown trout habitat, or low density of fish. In other streams, the number of caught fish was higher, due to some being damaged by electrocution; those were therefore dispatched and not released.

Locations for fishing in each stream were selected based on where brown trout could be expected to be present, typically in more rapid water. Depth is also decisive for whether electrofishing is possible or not. The landscape in the Pasvik valley is generally flat, with many of the streams floating through mires where they are too deep, and the current is slow.

Every stream was divided into two or three stations, depending on the stream length and amount of rapid water. Fishing was started in one spot and coordinates were registered using GPS. After a certain distance or a certain amount of time, fishing was ended and coordinates were registered again, making up one station. Effort was made to cover as much of the streams in every station as possible, and the percentage of coverage was estimated after fishing ended. Factors that limited the coverage percentage were depth, current or tributary width. The river width was estimated on site, while the length of the stretch fished was measured in a map based on the GPS coordinates (QGIS, version 2.18.24).

While fishing, caught fish were stored in a bucket, which was carried by the person carrying the dip net. The bucket had a small opening in the lid preventing the fish from jumping out. The lid also functioned to minimize visual stress for the fish.

Fish were put in separate bags and dispatched either by a snap with the finger or by a big nail. They were then stored in a styrofoam box with ice (Figure 3). In some cases, excess fish beyond the 40 samples needed were released back into the stream, assuming they were undamaged.

Figure 3. Fish were stored in a styrofoam box with ice.

2. 5 Sample size

A total of 320 brown trout were caught during the three-week sampling period. Out of 10 investigated tributaries, DGB was the only one where no trout was detected. In the other nine, sample sizes varied between eight and 44 individuals (Table 1). The aim was to collect around 40 samples per stream, but different factors made this difficult in some locations. In addition, samples from the main river from both 2017 (presented in Klütsch et al. 2019) and 2018 were included in this study for comparison of genetic structure, allelic richness and (private) allelic richness. These samples were mainly collected by local anglers, but some originate from the brood fish used in the stocking programme.

Table 1. *Sample size from the tributaries of the Pasvik watercourse (this study) and from the main river (Klütsch et al. 2019).*

Tributary	N	Main river	N
DOB	8	Section A	2
DGB	0	Section B	53
EGB	27	Section C	32
EE	41	Section D	3
ESTB	43	Section E	24
ESB	42	Section F	17
FSB	37	Section G	88
GSE	40	Section H	31
IOB	38	Section I	36
IBB	44	Section J	1
Total	320		287

2.6 Density estimates

To estimate fish density in the sampling sections, a three-pass approach was applied with a 30-minute break between each pass (Bohlin et al 1989). Waiting at least 30 minutes between each round of fishing is important, as fish already exposed to electricity will have a higher tolerance for some time afterwards. All caught fish were put in a bucket, and the number of fishes stunned but not retrieved, were counted and registered as "missed" fish. Estimates of fish density were calculated using the Zippin removal method (Zippin 1958; Bohlin et al 1989).

2.7 DNA-sampling and biological measurements

To illuminate ecological questions, genetic analysis is becoming increasingly important. Microsatellites have emerged as a popular and versatile marker type for ecological applications (Selkoe & Toonen 2006), as it allows researchers to assess genetic diversity at a fine scale, measured by allelic variation at distinct loci (Estoup et al. 1998).

In the lab, each fish was weighed and measured. The adipose fin were used for the DNA-sample for most fish, as this fin was of suitable size on fish >7 cm (1+). For fish <7 cm (0+), the tail fin was used. DNA-samples were stored in 96 % ethanol until analysis. Furthermore, scale samples were retrieved from every fish from the area between the lateral line and the adipose fin (corresponding to the area where the first scales are formed on the fish). Otolith samples were also retrieved from fish >8 cm. All scale and otolith samples were put together in individual envelopes. A scalpel was used for the cutting, and every tool, the cutting board and gloves used were disinfected with 95 % alcohol between the sampling of each fish. This was done mainly to prevent contamination of the DNA-samples. Gloves and scalpel blades were changed regularly during the process.

Prior to age determination, original scales were separated from regenerated scales using a microfilm reader. A few scales from each fish were mounted between two glass plates that were glued together. This was done to preserve the samples in an orderly way, but also to make sure that the scales were flattened, facilitating the reading process.

A stereoscopic microscope (Leica MS5, 16x magnification) with a mounted digital camera (Leica DFC320, 0.63x magnification) was used to take a picture of every scale and otolith. To assess the amount of winter zones in each scale, an image editing program (Image-Pro Express version 6.3.0.531 for Windows XP/Vista (Media Cybernetics, Inc.).) was used. This program measures the scale radius and distance between winter zones, while the placement of the zones is done manually. The yearly growth of the fish was identified by high and small inter-circuli distances within the scale, representing summer and winter growth, respectively. Together they make up one year of growth (Jonsson 1976) (Figure 4). Brown trout is assumed to grow proportionally with the annuli in the scale (Jonsson & Stenseth 1976, Borgstrøm & Qvenild 2000).

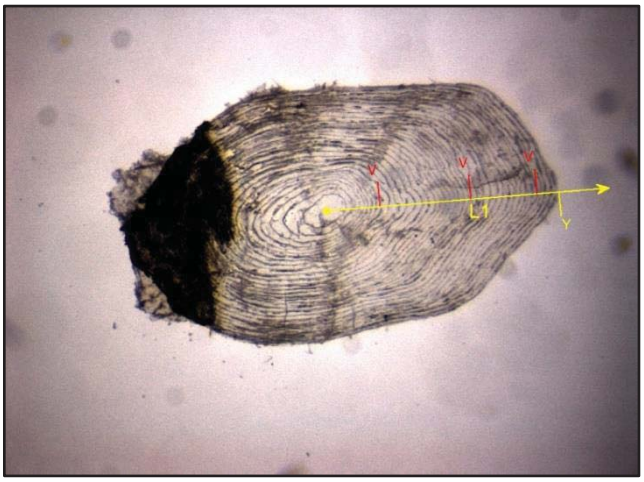


Figure 4. *A scale with three visible winter zones.*

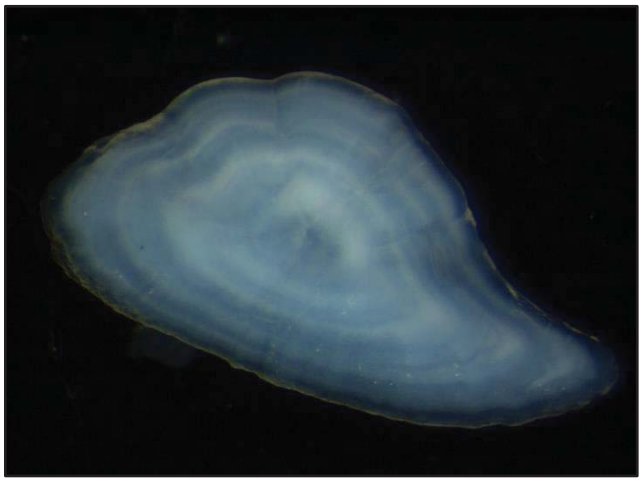


Figure 5. *Otolith after 2-3 hours in 96 % ethanol.*

Otoliths were put in propandiol and studied in the microscope before the picture was taken. Age was estimated based on the number of winter zones (dark bands) visible in the otoliths. Some otoliths that had blurry zones or were otherwise hard to read, were put in 96 % ethanol for two-three hours (Figure 5). This makes zones clearer and age determination more accurate. Some otoliths were also cut in half and burned, making zones more visible. This was done especially with older fish, as an increasing number of zones can be hard to tell apart by just studying the whole otolith.

2.8 DNA extraction and multiplex PCR-STR analysis

The DNA extraction, multiplex PCR-STR analyses and analyses of genetic variation, structure and bottlenecks all follow the same methods applied in Klütsch et al. (2019).

By using a DNeasy Blood & Tissue kit (Qiagen), genomic DNA was isolated from the brown trout tissue samples (fin-clips preserved in 96 % ethanol) and genotyped at 16 microsatellite loci. Polymerase chain reactions (PCR) amplifications were performed in five novel multiplexes, ensuring that each reaction contained 5.0 µL 2x Multiplex PCR Master Mix (Qiagen, USA), 1.0 µL 10x primer mix, 0.05 µL BSA, and 2.95 µL RNase-free water. The PCR cycling profile included a 10-minute initial denaturation step at 95 °C, followed by 28 cycles including 30 s of denaturing at 94°C, 30 s of annealing at 55ºC/58 °C (depending on multiplex, followed by an extension step at 72°C for 1 minute. Finally, an extension at 72 °C for 45 minutes made the reaction complete.

Fluorescently-labeled products were separated on an Applied Biosystems 3730xl Genetic Analyzer (Applied Biosystems, UK), sized and scored using GenMapper 5.0 (Applied Biosystems, USA), and manually verified. To check for scoring errors and null alleles (microsatellite alleles that do not amplify during PCR and therefore lead to increased homozygosity values), MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) was used – testing the quality of the loci.

2.9 Analyses of genetic variation

The software GENEPOP 4.7 (Rousset 2008) was used to test for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium. Using GenAlEx 6.51 (Peakall & Smouse 2012), genetic summary statistics (observed and expected heterozygosity and inbreeding coefficient) for all stations in the side rivers as well as the eight sampled sections in the main river were calculated. In addition, GenAlEx was used to provide an estimate of pairwise population genetic differentiation based on G_{ST} (Nei and Chesser 1983) and Jost's D (Jost 2008) and to test their significance based on 9999 random permutations. To correct for multiple testing, the modified False Discovery Rate method of Benjamini and Yekutieli (2001) was used. Furthermore, the R Adegenet package (Jombart et al. 2010) was used for conducting a Discriminant Analysis of Principal Components (DAPC) to illustrate genetic differentiation between populations.

To avoid over-fitting, the cross-validation function was applied with 100 replicates to identify the optimal number of principal components to be retained with randomly generated training sets. The number of principal components (PCs) associated with the lowest 'root mean squared error' (RMSE) value was selected and results were displayed as a scatterplot to visualize genetic differentiation between tributaries.

Allelic richness and private allelic richness were calculated with ADZE 1.0 (Szpiech et al. 2008) based on a standardized sample size of 16.

2.10 Genetic structure and bottleneck analysis

To characterize the spatial population structure in brown trout between the tributaries and the main river, the Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000) was used, identifying individuals of potentially admixed ancestry and detecting presence of distinct genetic clusters. With correlated allele frequencies, the admixture model (Falush et al. 2003) was run twice. One run was done once using the LocPrior option, and one without. 40 replicates were carried out for each K from 1 to 10, with a burn-in period of 100,000 and 1,000,000 MCMC steps. To consider whether additional subtle population genetic structure could be detected, the LocPrior option was chosen. Including information on the sampling location of individuals has been shown to improve clustering without resulting in the detection of non-existing population genetic structure (Hubisz et al. 2009). Four recently proposed estimators were used to estimate the number of genetic clusters within the data (Puechmaille 2016: the median of means (MedMeaK), maximum of means (MaxMeaK), median of medians (MedMedK), and maximum of medians (MaxMedK) with the program STRUCTURESELECTOR (Li and Liu 2018) to account for uneven sample sizes in the data set. To visually summarize results from the separate STRUCTURE runs, the program CLUMPAK (Kopelman et al. 2015) was used.

The program BOTTLENECK 1.2.02 (Piry et al. 1999) was applied to look for recent reductions in effective population sizes (i.e., genetic bottlenecks). The algorithm in BOTTLENECK assumes allelic diversity being lost more rapid than heterozygosity and, therefore, tests for an excess of heterozygosity compared to what would be expected at mutation-drift equilibrium (Cornuet and Luikart 1996).

Two mutation models were assessed, the infinite-alleles-model (IAM) and the two-phase-model (TPM). The TPM allows different proportions of microsatellites to follow either the IAM or the stepwise mutation model (SMM). The TPM model was run three times for each population, assuming that the percentage of stepwise mutations was 20%, 50, and 70%, respectively. The 1-way Wilcoxon sign-rank test (Luikart et al., 1997) was applied to assess significance.

2.11 Statistical analysis

To explore if the number of hydroelectric dams or isolation by distance influenced genetic differentiation, candidate linear models were fitted. Additive and multiplicative effects of waterway distance and number of dams were used as effects and F_{ST} as the response variable.

The statistical software R (version 3.5.2) was used for the statistical analysis and the visual presentation of the data (R Development Core Team 2018). For statistical analysis on the effect of number of barriers (i.e., number of dams, *nB*) and waterway distance (*D*) on the various pairwise (*i*) F_{ST}-values, candidate linear models were fitted. The fully factorial candidate model was expressed as:

$$FST_i = \alpha_0 + \beta_1 D_i + \beta_2 nB_i + \beta_3 D_i nB_i + \varepsilon_i$$

where α_0 is the global intercept and $ß_n$ constitute different slope, estimates associated with effects of *D* and *nB*. ε_i is the residual random variation assumed to be ~N (0, σ^2) distributed. Model selection was based on the n-corrected version of Akaike's Information Criterion, AICc (Anderson 2007).

To study the effect grade of admixture had on the genetic differentiation between pairs of tributaries, the following equations were used:

$$\Delta_{adm} = \left| q_{Adm_i} - q_{Adm_j} \right|$$

$$rel\Delta_{adm} = \frac{\Delta_{adm}}{\overline{q_{Adm_{ij}}}}$$

where Δ_{adm} is difference in admixture between population *i* and j, while q_{Adm_i} is fraction of admixed individuals in tributary i. Further, $rel\Delta_{adm}$ is the relative difference in admixture between tributaries i and j, and finally, $\overline{q_{Adm_{ij}}}$ is the mean fraction of admixed individuals in the two tributaries.

3 Results

3.1 Age distribution

All collected brown trout individuals were within the 0+ to 5+ age groups (Figure 6). There was a clear bias towards younger age classes, especially within ages 0+, 1+ and 2+. Only nine and four individuals of the sample size of 320 were determined to be age 4+ and 5+, respectively.

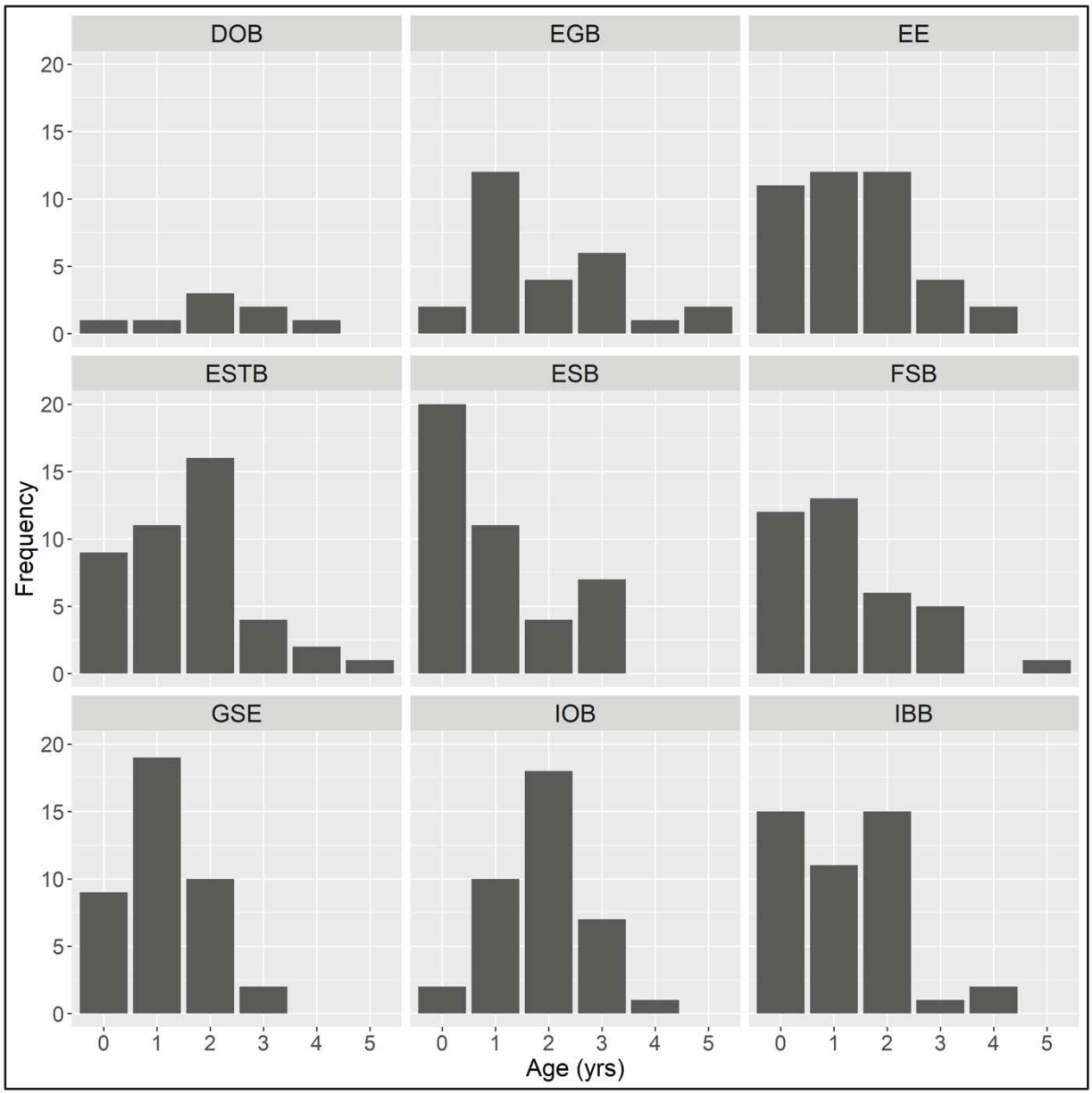


Figure 6. *Age distribution of brown trout within the different tributaries sampled in Pasvik during 2018.*

3.2 Length at age

Length at age varied among tributaries. For the tributaries combined, the average length at age was 4.8 cm for 0+ year olds, 8.9 cm for 1+ year olds, 12.5 cm for 2+ year olds, 14.8 cm for 3+ year olds, 17.8 cm for 4+ year olds and 22.7 cm for 5+ year old brown trout (Figure 7). Sample sizes varied between age classes in total and within each tributary.

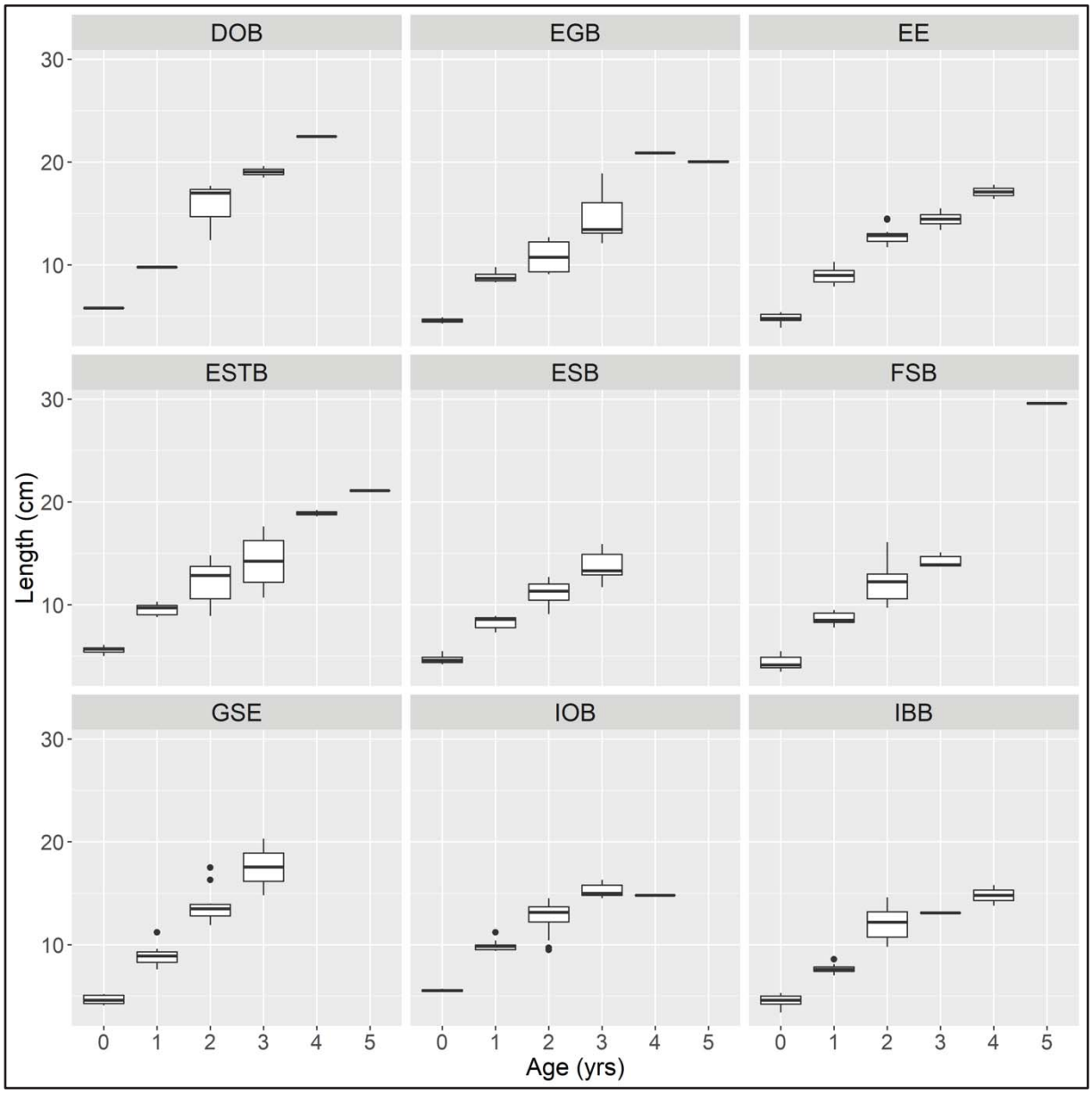


Figure 7. *Age at length plotted for every brown trout age class within tributaries of the Pasvik watercourse sampled in 2018.*

3.3 Juvenile density estimates

The density of brown trout in different stations varied from 1.1 to 80.2 individuals/100m^2 (Table 2). The tributary with the lowest overall fish density was DOB, with 1.9 individuals/100m^2. The tributaries with the highest density of brown trout were IBB and IOB, with an overall density of 45.5 and 44.0 individuals/100m^2, respectively.

Table 2. *Size, coverage (fished area of total station area), catchability (est catch), and estimated fish/100m^2 in each location, with coordinates (decimal degree).*

Station	Length (m)	Width (m	Coverage %	p	SE(p)	N/100m^2	se(N)	Latitude	Longitude
DOB1	52	5	7	1.00		2.7	0.0	69.04670	29.07625
DOB2	75	7	75	0.67	0.15	1.1	0.4	69.04668	29.08813
EGB1	45	5	85	0.38	0.08	17.7	10.0	69.14917	29.12527
EGB2	40	3	95	0.26	0.24	22.0	14.7	69.14902	29.12435
EE1	50	10	40	0.58	0.11	15.6	1.3	69.21350	29.14223
EE2	34	2.5	95	0.78	0.15	10.0	0.4	69.18463	29.03627
EE3	45	11	90	0.07	0.24	26.7	92.2	69.16612	28.99540
ESTB1	45	8	70	0.47	0.16	9.3	1.7	69.23093	29.15837
ESTB2	28	6	80	0.59	0.13	18.4	1.7	69.23238	29.15068
ESB1	50	10	40	0.24	0.20	19.5	12.4	69.30032	29.25303
ESB2	52	12	50	0.44	0.23	4.3	1.2	69.30123	29.21378
FSB1	30	8	75	0.63	0.21	4.1	0.5	69.36068	29.46078
FSB2	90	5	80	0.63		4.0		69.36463	29.45948
FSB3	75	5	80	0.63		11.1		69.38228	29.46277
GSE1	90	15	45	0.16	0.20	10.5	11.3	69.39992	29.71880
GSE2	65	12	40	0.83	0.03	2.3	0.2	69.44995	29.70775
GSE3	100	12	30	0.50	0.15	6.7	1.0	69.44780	29.70303
IOB1	25	1.2	95	1.00		31.6	0.0	69.58267	30.15177
IOB2	65	1.2	90	0.23	0.10	80.2	98.7	69.58238	30.14868
IOB3	120	1	95	0.69	0.03	20.4	2.7	69.58382	30.14172
IBB1	27	3	95	0.51	0.12	47.2	5.5	69.61852	30.13143
IBB2	41	2	95	0.41	0.15	43.8	9.5	69.61813	30.12565

3.4 General genetic variation

There were no signs of allele dropout or scoring errors and no systematic significant tests for null alleles in any of the 16 loci used in this study. Microsatellite locus D157-1 showed one significant binominal test (0.01) for null alleles in a population with small sample size (3 individuals – population D1). MST73-1 (0.025) in population IOB1 (N =10). In population EE1, two markers, SsoSL85-1 (0.05) and Ssa85-1 (0.01) showed significance for null alleles. However, since none of these markers showed statistical significance for null alleles in more than one population (out of 26 populations for which enough data existed to perform the tests), it is feasible to conclude that none of the markers showed strong indications for null alleles. Further, no significant linkage disequilibrium could be detected after Bonferroni correction for multiple testing. Out of 1866 tests, 116 were significant at the 0.05 level. Finally, Hardy-Weinberg deviations could be found in 37 out of 455 tests. None of them remained significant after Bonferroni correction.

3.5 Patterns of genetic diversity

Observed and expected heterozygosity levels (Appendix, Table 1) were similar among sampling stations with a few notable exceptions. All sampling stations within side rivers EGB and ESTB displayed lower values in these two metrics in comparison with other sampling stations and main river sections. In addition, sampling stations DOB2 in section D and IOB1 had low observed and expected heterozygosity values as well. Further, most inbreeding coefficients suggested that inbreeding is absent or low as indicated by F_{IS} values that were either negative or not significantly different from zero when considering standard error. Exceptions to this pattern could be observed in section D and one of its tributary stations, DOB2. However, in these cases, sample size was very low and therefore, the estimates are likely not reliable. Allelic richness (A_R) and private allelic richness (A_{PR}) varied among tributaries (Table 3). Some tributaries have low levels of both A_R and A_{PR} (EGB and ESTB) while IOB only have low levels of A_{PR}. Contrastingly, tributary ESB have levels of both A_R and A_{PR} comparable to values found in the main river sections. Further, when combining values from all tributaries against the main river sections combined and the Russian part of the main river combined, the tributaries were found to have the highest levels of both A_R and A_{PR} (Table 4).

Table 3. *Genetic summary statistics for tributaries (sampling stations combined) and genetic clusters detected in the main river sections (Klütsch et al. 2019). N = number of individuals. Allelic richness (A_R) and private allelic richness (A_{PR}) was not calculated for very small sampling sizes. For individual values in every sampling site, including observed and expected heterozygosity levels and inbreeding coefficient, see table 1 in the appendix.*

	N	A_R	SE (A_R)	A_{PR}	SE(A_{PR})
BCD (=CL1)	88	6.233	0.959	0.337	0.176
EF (=CL3)	41	6.561	1.041	0.463	0.172
EE	43	5.541	0.816	0.191	0.080
EGB	27	2.437	0.203	0.022	0.022
ESB	42	6.071	0.961	0.419	0.204
ESTB	43	3.454	0.586	0.055	0.039
FSB	37	4.930	0.597	0.288	0.221
GSE	40	5.364	0.693	0.068	0.035
GHI (=CL2)	155	6.100	0.878	0.176	0.068
IBB	44	5.265	0.728	0.144	0.076
IOB	38	4.081	0.455	0.066	0.032

Table 4. *Genetic summary statistics for tributaries (combined), Norwegian main river sections (combined) and the Russian part of the main river (combined). The tributaries seem to contain more allelic richness and private alleles than the rest of the river system.*

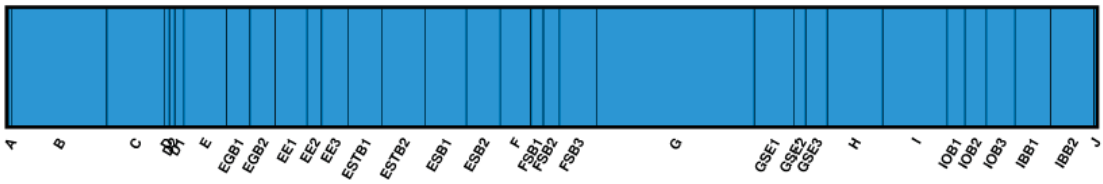
	N	A_R	SE (A_R)	A_{PR}	SE (A_{PR})
Russia	87	7.429	1.249	0.761	0.306
Main river	199	8.061	1.347	0.835	0.274
Tributaries	320	8.262	1.493	1.300	0.533

3.6. Population genetic structure

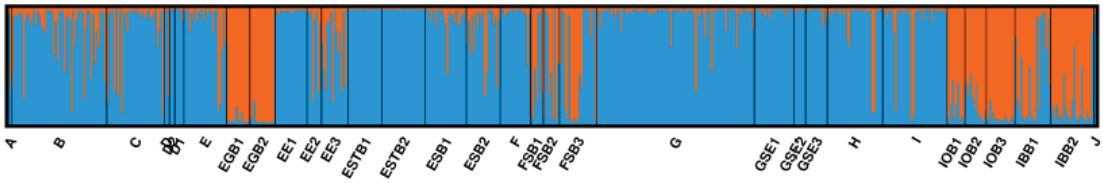
Since STRUCTURE runs with and without LocPrior were rather consistent, the runs without using LocPrior and the lowest number of supported K were used to describe the main patterns in the data set. In some runs, additional subtle genetic structure was indicated to be potentially present; however, due to the complexity of the data set and the hypotheses that were tested, it was considered more important to capture the main genetic patterns than describing weak genetic structure that might be the result of stochastic effects. For more subtle genetic structure not shown in the results chapter, see figure 1b, 2b, and 4b in the appendix.

Considerable genetic structure was detected in the tributaries of the Pasvik watercourse. Data from the main river (published in Klütsch et al. 2019) was run together with data from the tributaries. STRUCTURESELECTOR (Li and Liu 2018) found support for 8-11 genetic clusters within this entire data set (Appendix, Figure 1a). Here, At K= 3, signs of genetic differentiation from the rest of the system is visible in tributaries EGB, ESTB, IOB and IBB. At K = 4, genetic structuring from the Russian non-stocked part of the main river is separated, as found in Klütsch et al. (2019). Some of this structure is found throughout the Norwegian-Russian part of the watercourse, and in stations EE2 and EE3 (in tributary EE). Importantly, we tried to organize the data in a way that the tributary stations that are closest to the main river sections are next to those (e.g., station EE1 is geographically closer to the main river than EE2). At K = 5, all differentiated tributaries are assigned to unique genetic clusters, apart from IOB and IBB, which appears to be assigned to the same cluster. EE, ESB and GSE seem to have the most admixture with the main river/stocked cluster. At K = 6, FSB is assigned to a unique genetic cluster, and seems to be a mix of this cluster and the main river cluster. At K = 7, GSE1 is assigned to a cluster also found in ESB. GSE2 and GSE3 remain assigned to the main river cluster. At K8, some further structuring occurs in the green “Russian” cluster (Figure 8). These results suggest that side rivers are generally differentiated from the main river and from each other.

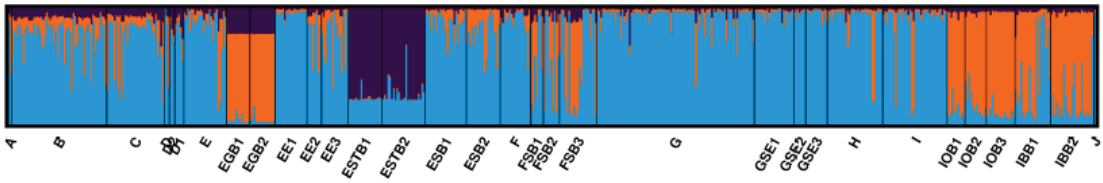
K=1



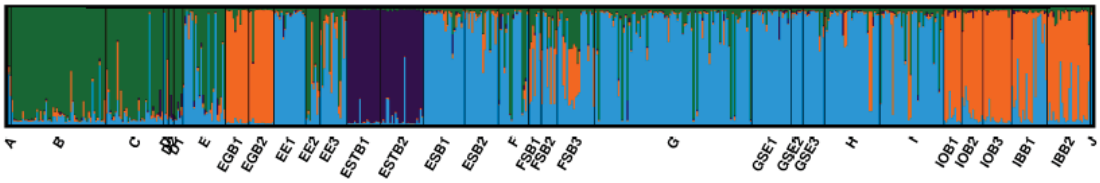
K=2



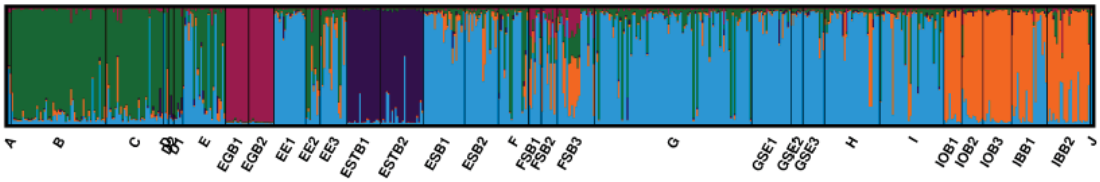
K=3



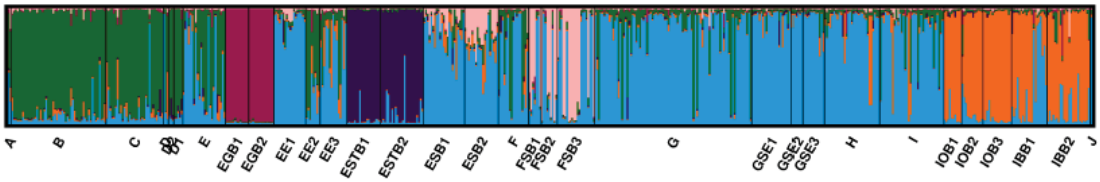
K=4



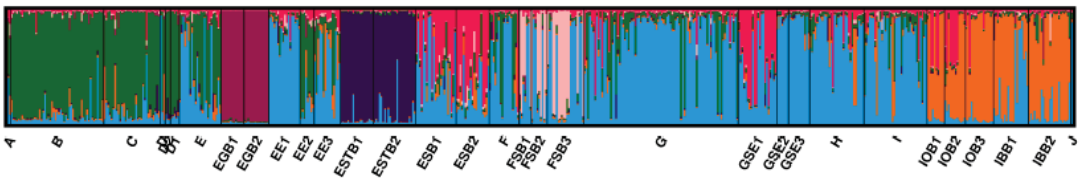
K=5



K=6



K=7



K=8

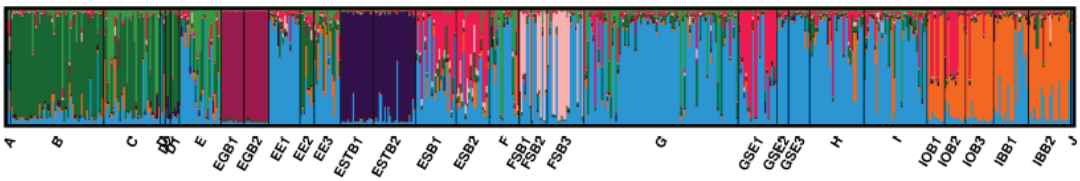
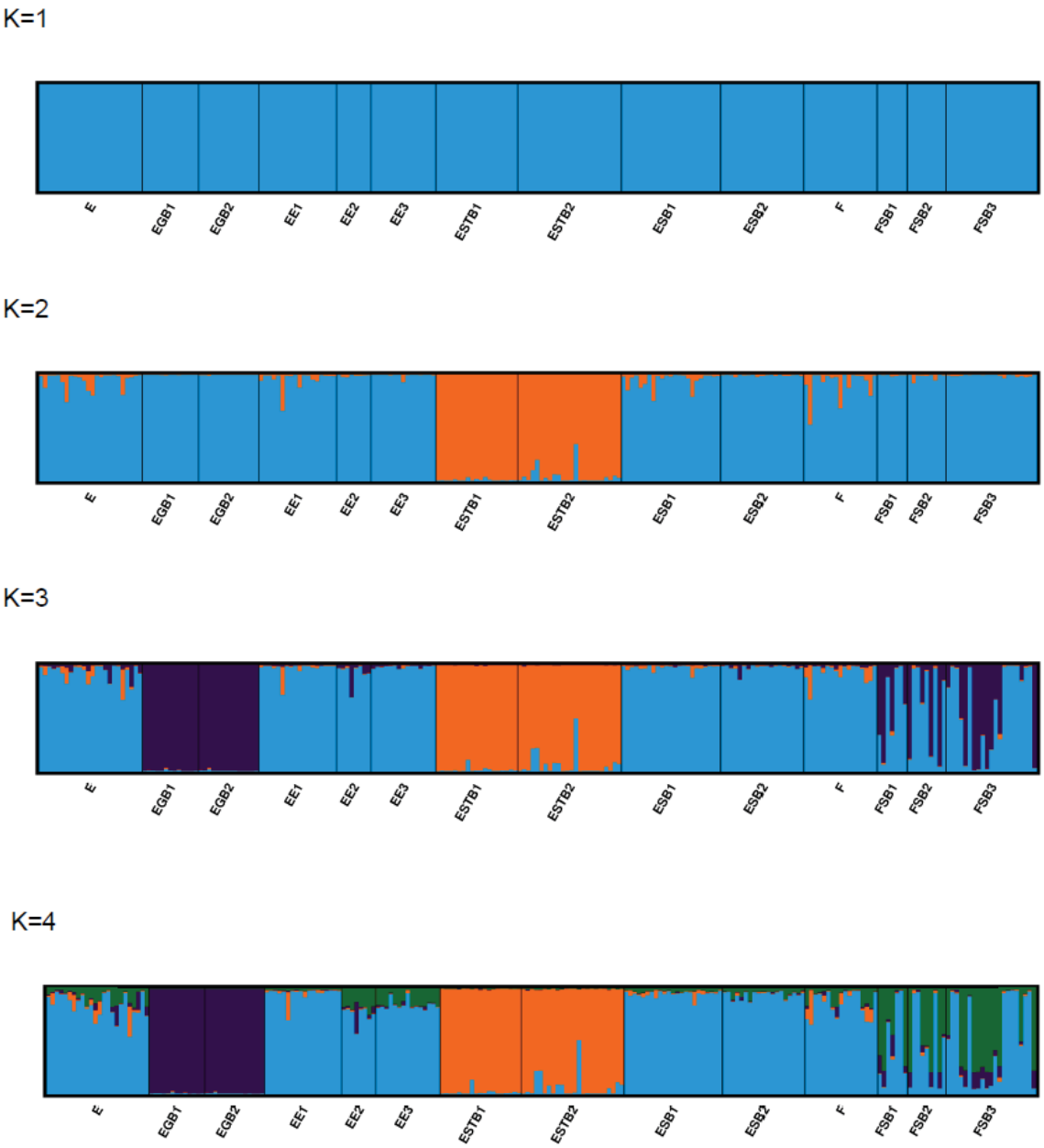
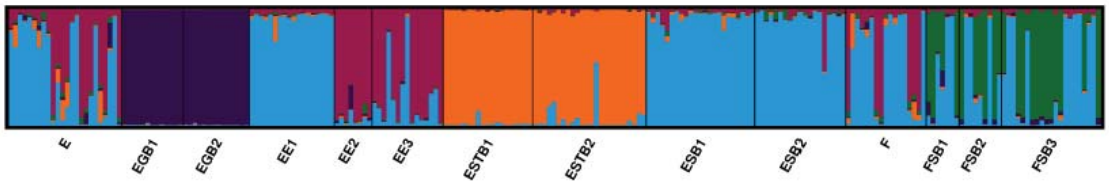


Figure 8. *Structure bar plot showing genetic structure in the Russian and Norwegian part of the Pasvik river, and tributaries investigated in the Norwegian part of the watercourse.*

To look for more detail, continuous sections of the main river (with no impassable barriers) with belonging tributaries, were run separately in STRUCTURESELECTOR. When running section E and F with tributaries, the pattern seen in the first run is largely confirmed. Support for 6-8 genetic clusters in the data set was found (Appendix, Figure 2a). At K=2, ESTB is differentiated, followed by EGB at K=3, and to some extent FSB. At K=5, station EE2 and EE3 starts showing substructure, similar to some of the structure found in section E and F. At K=6, ESB stands out. In total, tributary EE and ESB largely show structure similar to that of the main river/stocked trout. FSB shows admixture with the main river, but also has substructure. EGB is completely differentiated, even more so than ESTB (Figure 9).



K=5



K=6

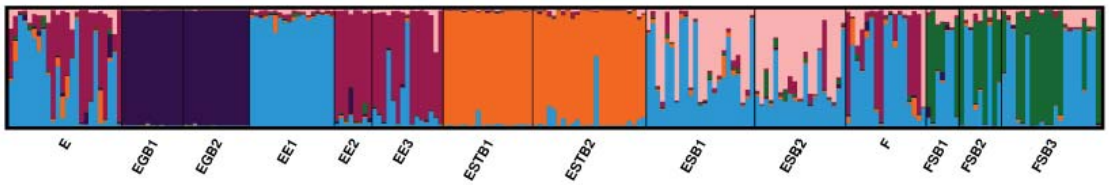


Figure 9. *Structure bar plot showing genetic structure found in section E and F of the Pasvik river, and the belonging tributaries (EGB, EE, ESTB, ESB and FSB).*

In addition, section G with tributary GSE was run separately in STRUCTURESELECTOR, which found support for two clusters in this data set (Appendix, Figure 3). Station GSE1 seems to be genetically differentiated from the main river and the other stations in the tributary. Station GSE2 and GSE3 appears to be genetically more similar to the main river, while being geographically further away than GSE1 (Figure 10).

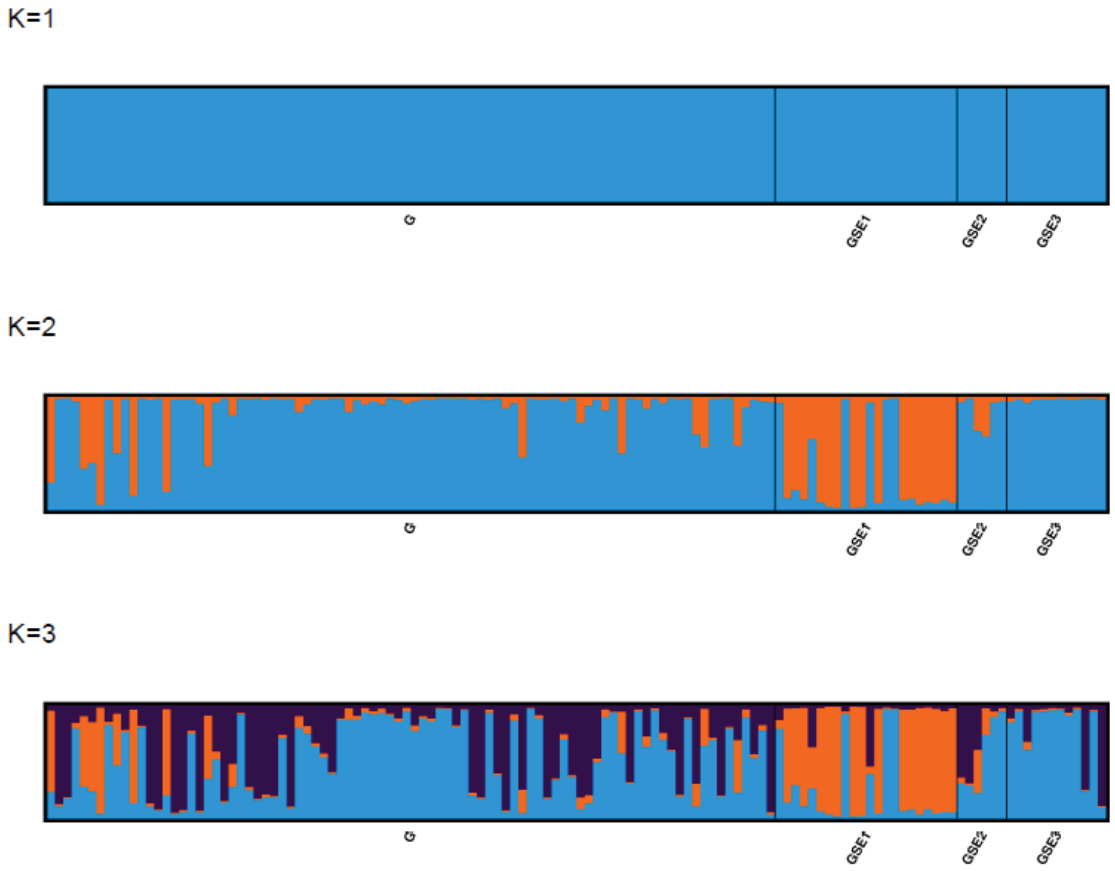
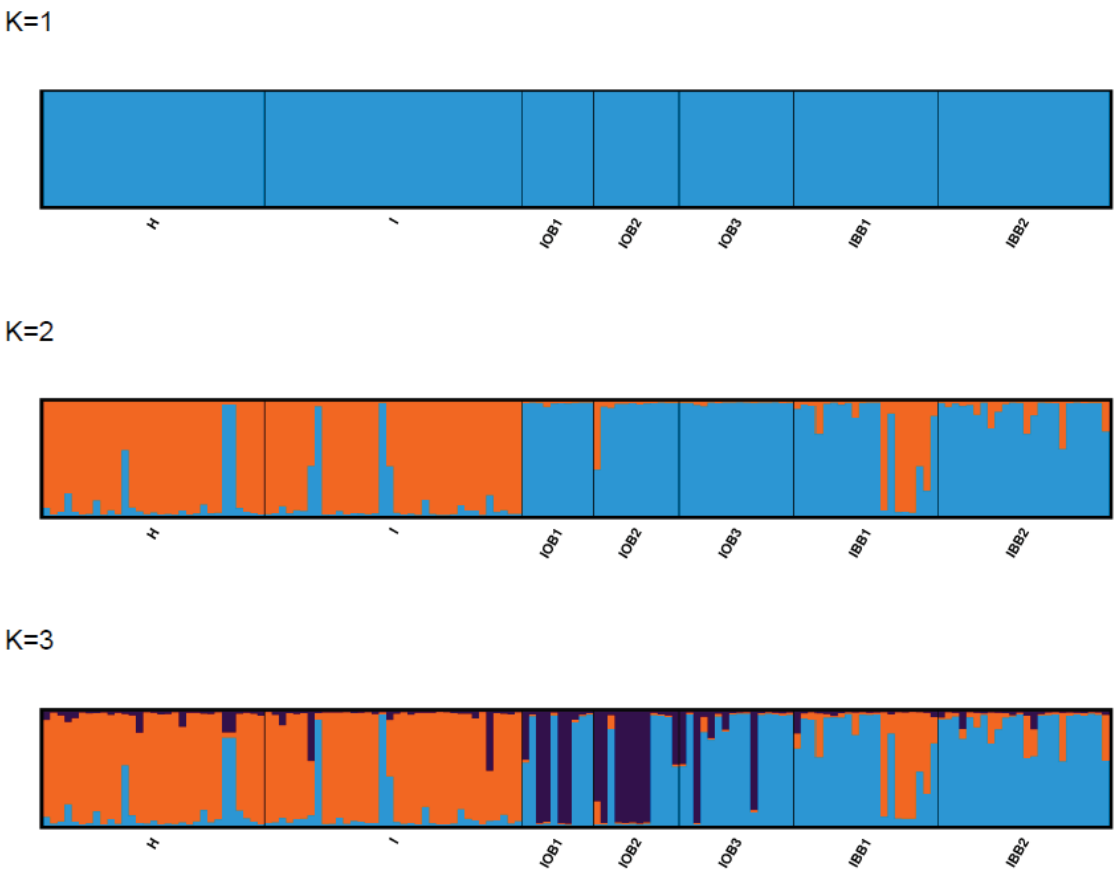
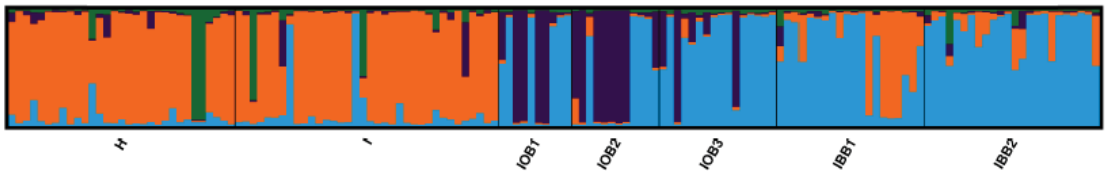


Figure 10. *Structure bar plot showing genetic structure found in section G of the Pasvik river and belonging tributary GSE.*

Finally, data from sections H and I, including tributaries IOB and IBB, also had a separate run (Figure 11). Three out of four STRUCTURESELECTOR estimators support K = 3 as the most likely number of clusters in this part of the system (Appendix, Figure 4a). Looking at K = 2, two groups are found, separating the main stream sections H and I from the tributary sections. Being geographically closer to the main river than IBB2, it seems that IBB1 is a mix of main river and potentially resident individuals. Going further into the tributaries, less mixing of individuals seems to occur. At K = 3 to K = 5, several other potential groups appear. Specifically, it seems that side river IOB shows substructure.



K=4



K=5

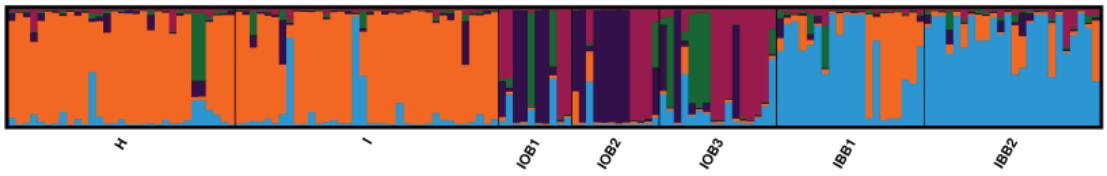


Figure 11. *Structure bar plot showing genetic structure found in section I and H of the Pasvik river, with belonging tributaries IOB and IBB.*

3.7 Demographic history

Signatures of recent demographic bottlenecks were found in several sampling sites (Table 5). For main river sections, bottleneck tests in the stocked Norwegian-Russian river sections G, H, and I were generally significant regardless of mutation model considered. One test in main river section G was non-significant when using the TPM_70 model that assumes a high proportion of microsatellites that follow the stepwise mutation model. However, this model consistently yielded the highest P-values, indicating that it might be the worst fitting model. This is also supported by the fact that the infinite allele model usually retrieved the lowest P values, suggesting that this model is the best-fitting model for the microsatellite set used in this study.

Concerning tributaries, the results provided evidence that recent demographic changes have occurred in some of them, but not in others. In sections E and F that are not separated by a dam, several tributaries or sampling sites within tributaries displayed bottleneck signatures. Specifically, for both sampling stations in tributary EGB bottleneck tests were significant in all four tests. Similarly, sampling stations EE1 and EE3 in tributary EE showed signatures of recent demographic decline. Lastly, two out of three sampling stations (i.e., FSB2 and FSB3) within tributary FSB in section F exhibited signs of recent bottlenecks. In section G, only sampling station GSE1 within tributary GSE had consistent bottleneck signatures. Finally, sampling station IBB1 in tributary IBB and sampling station IOB3 in tributary IOB in section I displayed reliable significant bottleneck tests.

Table 5. *Tests for genetic bottlenecks in the main river sections, and in the sampling stations in the tributaries, using BOTTLENECK (Piry et al. 1999).*

Location	IAM	TPM_70	TPM_50	TPM_20	Location	IAM	TPM_70	TPM_50	TPM_20
B	**0.01677**	0.53006	0.17419	0.06487	F	**0.01932**	0.26408	0.14893	0.07193
C	**0.00775**	0.35286	0.16125	**0.04672**	FSB1	0.09641	0.18773	0.17419	0.13722
D	0.91232	0.88185	0.88185	0.88185	FSB2	**0.01450**	0.05833	**0.04672**	**0.02533**
DOB2	0.75134	0.89185	0.89185	0.89185	FSB3	**0.00042**	**0.03270**	**0.01932**	**0.00381**
DOB1	0.75565	0.87381	0.84860	0.78940	G	**0.00314**	0.07193	**0.01677**	**0.00459**
E	**0.01248**	0.43013	0.24771	0.09641	GSE1	**0.00459**	**0.04672**	**0.02884**	**0.01450**
EE1	**0.00105**	**0.01450**	**0.00912**	**0.00655**	GSE2	0.21313	0.38043	0.35742	0.33490
EE2	0.64014	0.83487	0.77286	0.71930	GSE3	**0.02884**	0.20187	0.09641	0.07193
EE3	**0.00014**	**0.00912**	**0.00381**	**0.00105**	H	**0.00549**	**0.04672**	**0.02533**	**0.01248**
EGB1	**0.00038**	**0.00134**	**0.00084**	**0.00084**	I	**0.00258**	**0.01677**	**0.00775**	**0.00381**
EGB2	**0.00076**	**0.00168**	**0.00168**	**0.00101**	IBB1	**0.00001**	**0.00004**	**0.00002**	**0.00002**
ESB1	**0.04672**	0.29829	0.23187	0.16125	IBB2	**0.00381**	0.09641	**0.04163**	**0.01677**
ESB2	0.35286	0.90359	0.82581	0.66573	IOB1	**0.02396**	0.17957	0.09381	0.06027
ESTB1	0.44519	0.59802	0.57654	0.46704	IOB2	**0.04730**	0.19470	0.10388	0.08441
ESTB2	0.68066	0.96350	0.89612	0.83487	IOB3	**0.00107**	0.06027	**0.02396**	**0.00754**

3.8 Genetic assignment and highlight of main river/stocked ancestry in the tributaries

Based on genetic assignment by STRUCTURESELECTOR at K=8 (Figure 8), maps were created in QGIS (version 2.18.24), (Figure 12, 13 and 14). The threshold was set to 80 %. Individuals that were assigned to a genetic cluster by less than 80 % were categorized as "admixed". The exception was brown trout of main river/stocked ancestry, where two categories were made; one for fish assigned to this cluster at the same level as the overall threshold (80 %), and then an additional one for fish with 25-80 % main river/stocked ancestry. This was done to visualize what tributaries seem to be used by the main river/stocked trout. The size of the diagrams indicates the fish density in each station. As density estimates in the main river are not available, sizes of pie charts are based on the average density in all the tributaries. The location of the pie charts in the main river is also random, as the samples were mainly captured by local anglers at different sites.

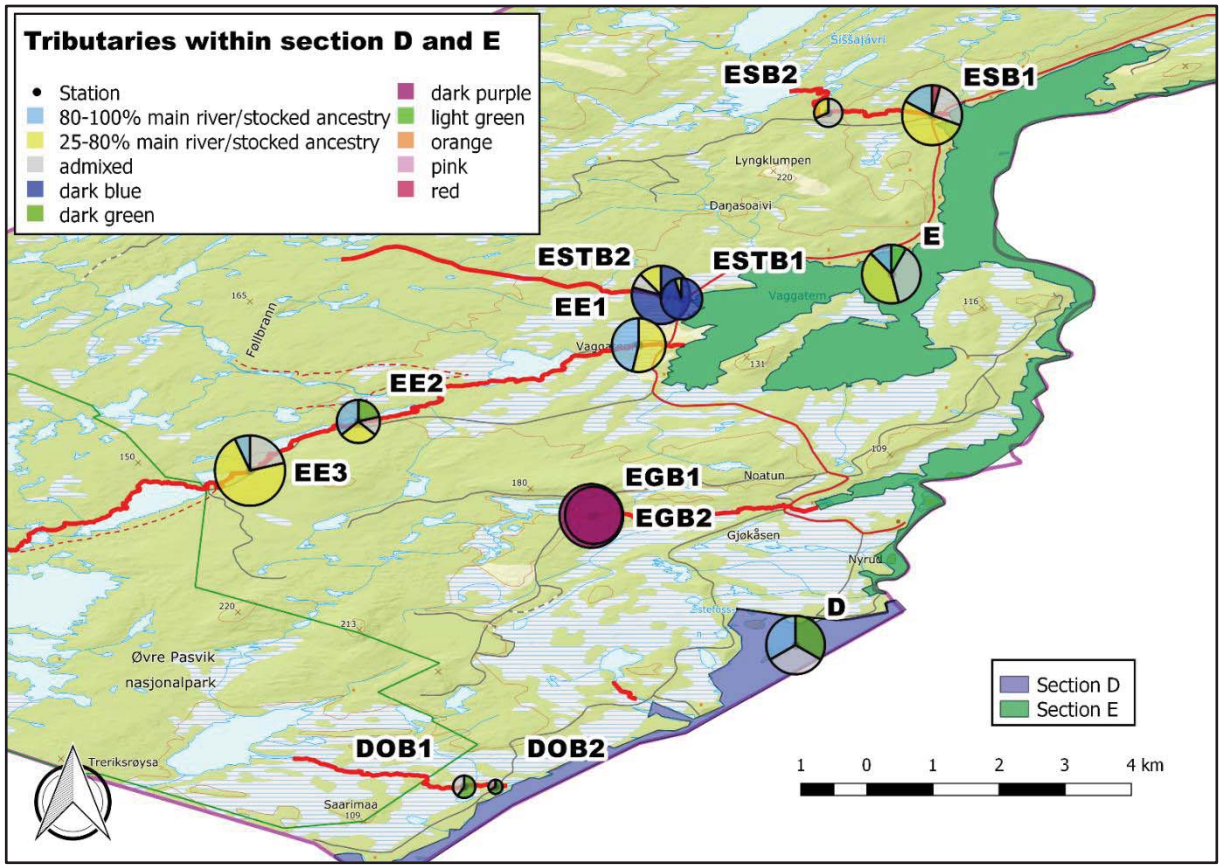


Figure 12. *Genetic assignment for brown trout in tributaries DOB, EGB, EE, ESTB and ESB within sections D and E of the Pasvik river. Section E and D is separated by a hydroelectric dam.*

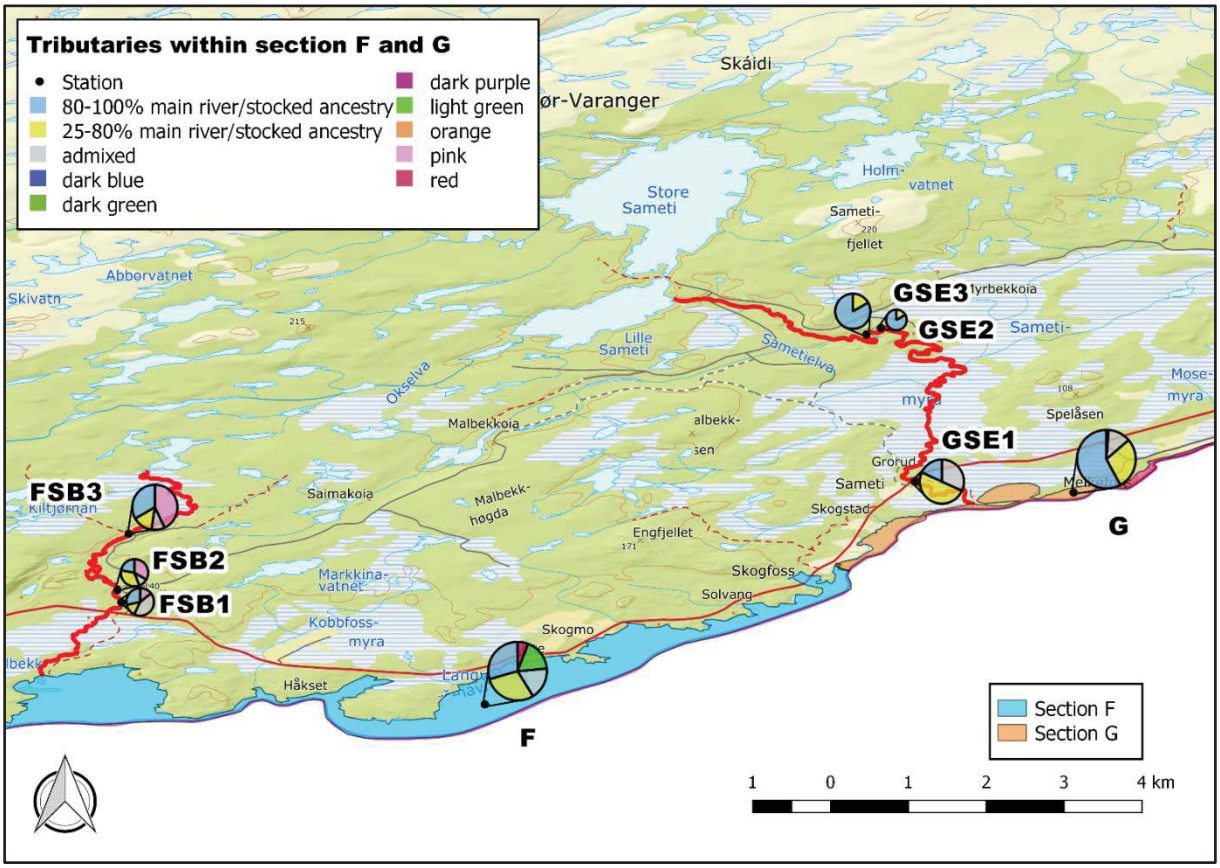


Figure 13. *Genetic assignment for brown trout in tributaries FSB and GSE within section F and G of the Pasvik river. Section F and G is separated by a hydroelectric dam.*

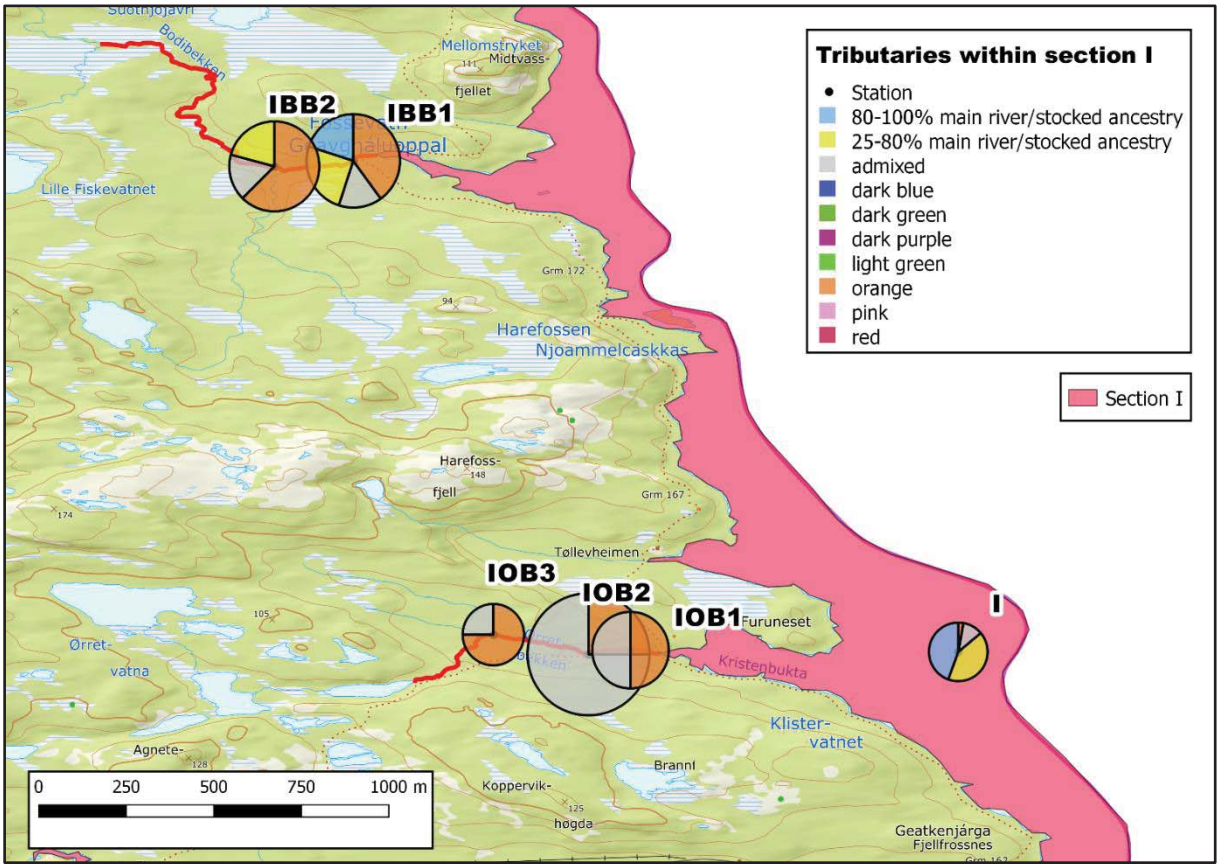


Figure 14. *Genetic assignment for brown trout in tributaries IOB and IBB within section I of the Pasvik river. A hydroelectric dam separates section H and I from section G.*

3.9 Effect of admixture-grade on densities

There was a significant negative effect of the grade of admixture with main river/stocked cluster in individuals on brown trout density in the tributaries (Table 6). This was true for both categories of main river/stocked ancestry (80 % threshold and 25-80 % threshold) (Figure 15a and b). Tributary DOB was left out, because there is no stocking taking place in section D which makes the effect of admixture from the main river/stocked cluster on DOB minimal.

Table 6. *Parameter estimates and corresponding ANOVA-test on the effect of admixture with the main river/stocked cluster (80 % and 20-80 % threshold) on brown trout density in the tributaries.* R^2*=0.31 (FractAdm80) and 0.21 (FractAdm25).*

Parameter estimates (80 % threshold)			
Term	Estimate	SE	p
Intercept	3.0823	0.2039	>0.001
FractAdm80	-1.7798	0.6323	0.0115

Parameter estimates (25-80 % threshold)			
Term	Estimate	SE	p
Intercept	3.1795	0.2661	>0.001
FractAdm25	-1.0669	0.4832	0.0405

Anova test					
Effect	**df**	**SS**	**MSS**	**F**	**p**
Admixed 80 %	1	4.113	4.113	7.923	0.011*
Admixed 25-80 %	1	2.868	2.867	4.874	0.040*

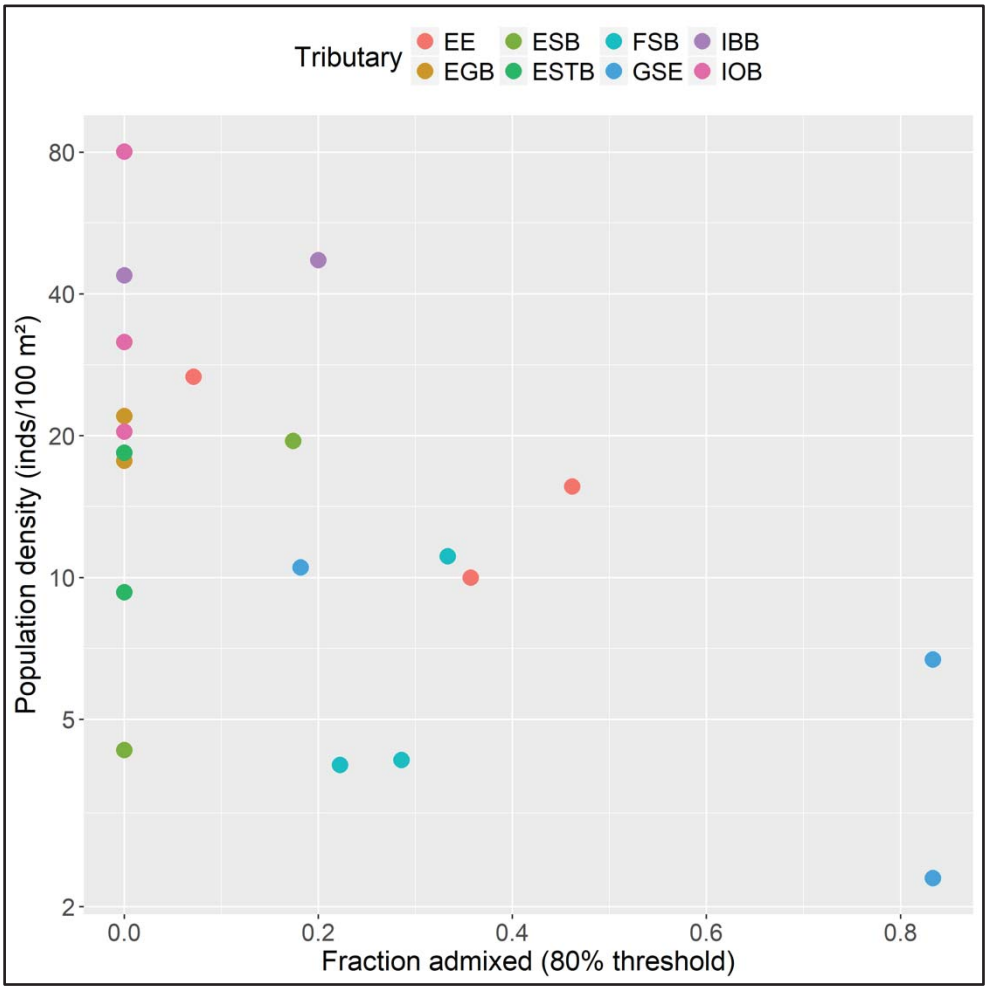


Figure 15a. *Scatterplot of the relationship between fraction of main river/stocked fish (80 % threshold) and population density in eight tributaries in the Pasvik watercourse.*

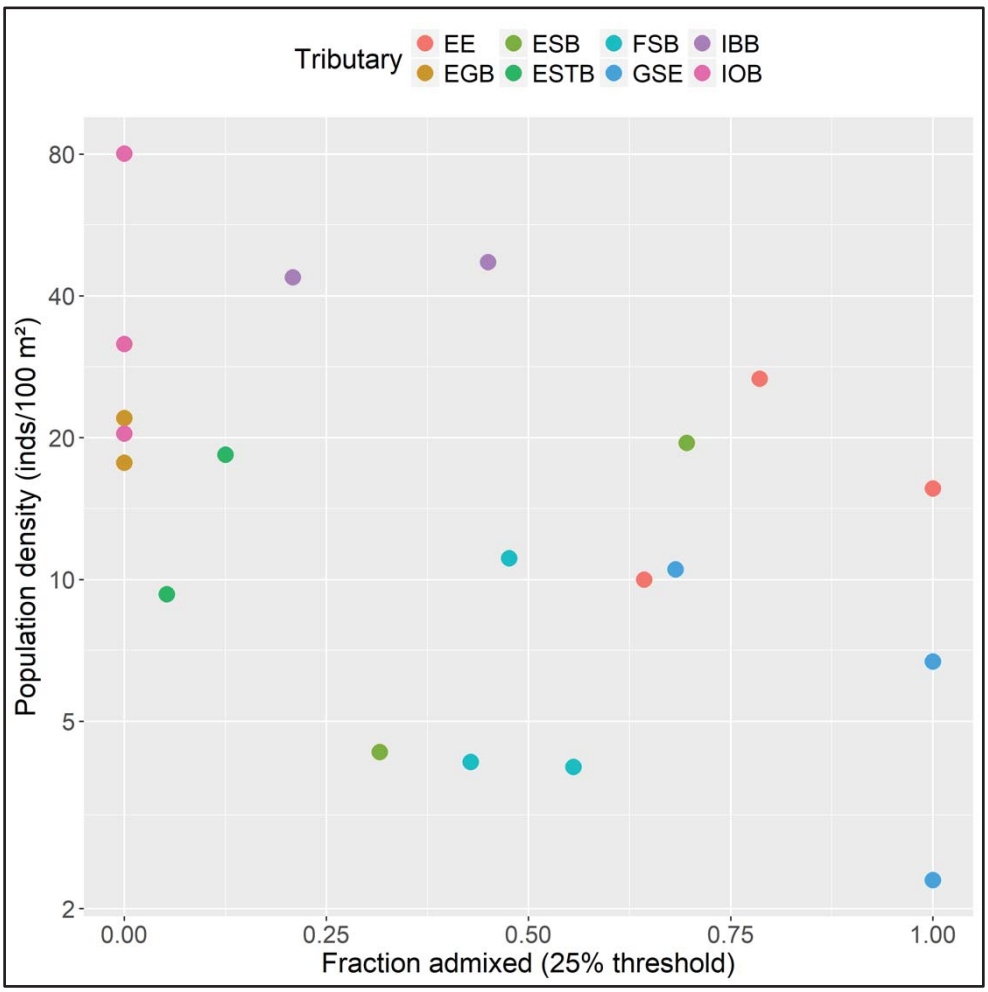


Figure 15b. *Scatterplot of the relationship between fraction of main river/stocked fish (25-80 % threshold) and population density in eight tributaries in the Pasvik watercourse.*

3. 10 Effect of distance and barriers on genetic differentiation

The model selection among candidate models fitted to estimate effects of water-way distance and number of barriers on pairwise F_{ST}-values yielded highest AICc-support to an additive effect between the two predictors (Table 7). The most supported model's parameter estimates are presented in table 8 and its predictions in figure 16. The model predicted pairwise F_{ST}-values to increase with distance to the compared tributary. There was no over-all effect of number of barriers, but a post-hoc contrast test revealed distance-corrected estimated F_{ST}-values to be significantly higher between population pairs with no barriers between them compared to those with more than one barrier between them (Tukey HSD: p= 0.027).

Table 7. *Model selection testing explanatory variables for high F_{ST}-values. Model 2 yielded highest AICc support. NDams = number of dams between tributaries, dist = distance between pop1 and pop2.*

Candidate models	K	AICc	Delta_AICc	ModelLik	AICcWt	LL
NDams+dist	4	-108.680	0.000	1.000	0.177	58.985
$rel\Delta_{adm}$	3	-108.407	0.273	0.872	0.154	57.578
$rel\Delta_{adm}$***Same or different section**	5	-108.025	0.655	0.721	0.128	60.012
($rel\Delta_{adm}$**)²**	4	-107.007	1.674	0.433	0.077	58.148
Barriers+dist	5	-106.679	2.001	0.368	0.065	59.340
$rel\Delta_{adm}$**+Same or different section**	4	-106.612	2.068	0.356	0.063	57.951
$rel\Delta_{adm}$**+dist**	4	-106.251	2.429	0.297	0.053	57.771
NDams*dist	5	-106.068	2.612	0.271	0.048	59.034
Same or different section+dist	4	-105.291	3.389	0.184	0.033	57.291
$rel\Delta_{adm}$***dist**	5	-105.013	3.668	0.160	0.028	58.506

Table 8. *Parameter estimates and corresponding ANOVA-test on the effect of barriers and distance on F_{ST} value between pairs of the Pasvik brown trout populations. R^2= 0.20.*

Parameter estimates				
Term	Group	Estimate	SE	p
Intercept	>one barrier	-0.007	0.043	0.87
Barrier	One barrier	0.058	0.029	0.011
Barrier	No barrier	0.100	0.037	0.058
Distance between streams		1.045	0.394	0.012

Anova test					
Effect	df	SS	MSS	F	p
Barrier	2	0.002	0.001	0.478	0.625
Distance between streams	1	0.017	0.017	7.021	0.012

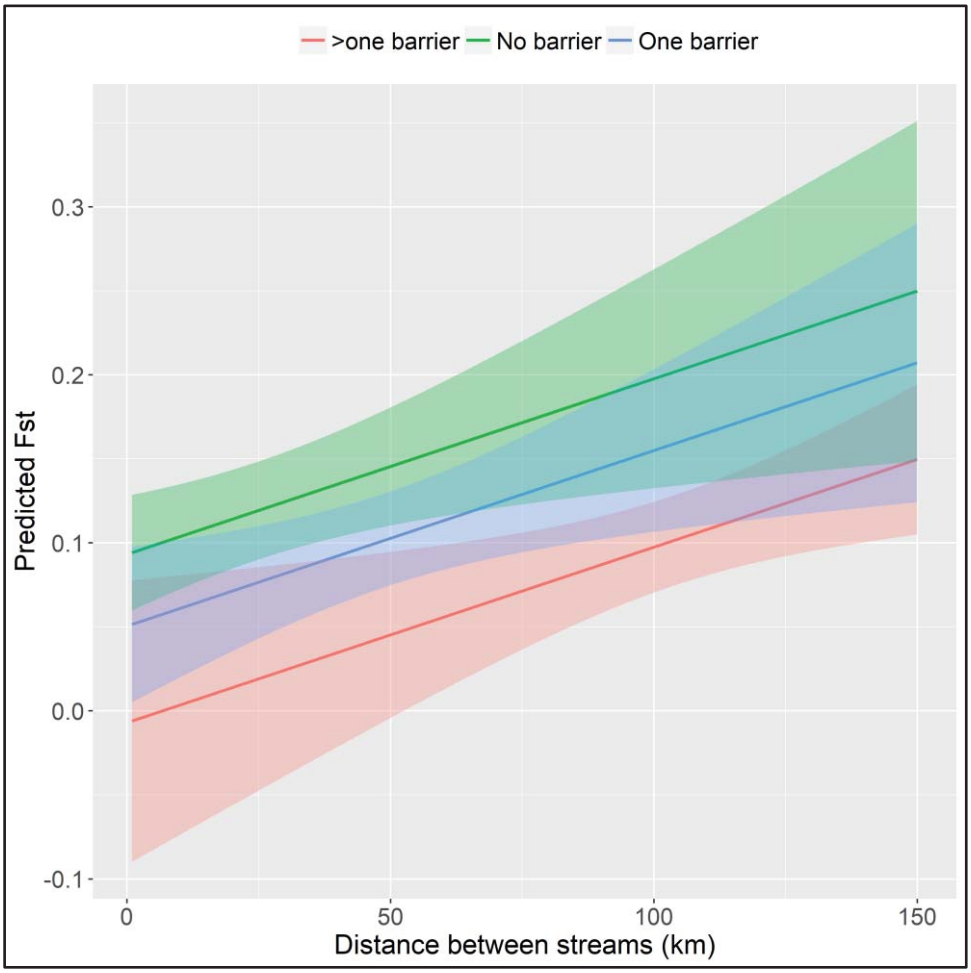


Figure 16. *The predicted effect of distance between streams and number of barriers on F_{ST}-value with corresponding 95 % confidence interval (shaded areas). Estimates were retrieved from the linear model presented in table 8.*

4 Discussion

The field sampling in this study revealed that brown trout are present in nine out of 10 investigated tributaries, which had not been confirmed before. This confirmation establishes the baseline for further investigation into these populations, and their role in the Pasvik watercourse. Moreover, the majority of trout caught were smaller than 25 cm, indicating natural recruitment as a contributor along with the stocking programme to the brown trout population in the watercourse.

4. 1 Age composition in the tributaries

Age determination based on otoliths and scales revealed six age classes in the sample material. The most abundant age class was 1+ year olds, although the age composition varied between tributaries. Since brown trout is a species with high fecundity, one could assume that 0+ individuals should be most abundant. However, various factors may have played a role in the age composition in the data set. Trout fry has been found to have a density-dependent mortality rate in the first three months of life (Mortensen 1977), but there is also evidence that the density of one age class of brown trout, affects the density of subsequent age classes (Nordwall 2001). As mentioned in the methods chapter, catchability while electrofishing increases with the size of the fish (Bohlin 1989). Inter-annual environmental stochasticity and the selection of sample sites may also have played a role. Nevertheless, all nine tributaries where brown trout was caught had individuals in the age classes 0+ - 3+ (Figure 6 & 7), suggesting that natural recruitment is taking place every year. The hypothesis that tributaries supporting natural recruitment should have individuals of lower age classes can be confirmed.

4. 2 The effect of dams on genetic structure

Results showed high spatial genetic differentiation within the study system and that tributaries were mostly significantly differentiated from one another and to the main river. This was true even among tributaries within main river sections not separated by a dam, indicating that other isolating mechanisms than the man-made barriers are responsible for the observed pattern. Fine-scale population structure within brown trout populations, with significant genetic differentiation found over relatively short geographical distances, has been found in other river systems (Carlsson et al. 1999, Lehtonen et al. 2009). In addition, genetic differentiation in brown trout may evolve in few generations (Heggenes et al. 2006). Despite this, examples of genetically interconnected salmonid populations in dendritic river systems are also present. In New Hampshire, a study on brook char (*Salvelinus fontinalis*) found that most mobile adult fish caught in mainstream rivers were genetically similar to those found in

tributaries without waterfalls. The only genetically differentiated subpopulations found in tributaries were *above* waterfalls (Kelson et al. 2015), suggesting that natural barriers within tributaries were responsible for genetic differentiation from the main river and other tributaries. Brook trout may, however, not be comparable with brown trout. In Pasvik, natural barriers in tributaries can explain some, but not all, the differentiated populations. Apart from tributary DOB, the tributary EGB is the only tributary without any structure from the main river/stocked cluster and no admixture with other tributaries (Figure 12). The situation in DOB can be explained by no stocking taking place in section D, combined with a very low sample size from both DOB and section D.

In 2013, Norway's state highways authority conducted an examination of migration barriers for brown trout where the road crossed tributaries in Pasvik (Statens Vegvesen 2013). EGB was then found to have a migration barrier, namely rocks in the river mouth. This barrier may explain why EGB is completely differentiated, together with the geographical distance from the main river to the stations where samples were collected in EGB. Interestingly, trout from ESTB are also very differentiated from trout in other tributaries and the main river (Figure 8). Here, the state highways authority found no migration barriers, and sampling stations were situated close to the main river, meaning that the differentiation cannot be linked to geographical distance either. On top of that, the adjacent tributary EE contains a lot of genetic admixture and is especially admixed with trout from the main river. Other factors than barriers must therefore be responsible for the significant genetic differentiation between these seemingly comparable tributaries. EE is however a considerably larger tributary. Nevertheless, when looking at densities of trout (N/100m^2), the estimates for EE and ESTB are comparable (Table 2, Table 10). In a boreal forest stream in central Sweden, Carlsson et al. (2000) found that differentiation in brown trout occurred along a continuous stream flow without geomorphological or further structuring of the stream that could interfere with movement of the fish. This suggests barriers to be less important factors for genetic differentiation in this species. Other studies point to limited dispersal of younger age classes of brown trout in forest streams. A mark-recapture study in south-eastern Norway found that smaller individuals in the same cohort of brown trout dispersed further than larger individuals (Vøllestad et al. 2012). However, about 70 % of tagged individuals were always observed at the same sampling location on consecutive sampling sessions (spring and autumn). Vøllestad et al. (2012) also found a clear signal of isolation-by-distance for 1+ and 2+ year old brown trout.

Further, genetic structure in tributaries IOB and IBB also stands out from the rest of the system. IBB has some admixture with the main river, but a substantial part in both tributaries are assigned to the same orange cluster (Figure 14), and this cluster cannot really be detected anywhere else throughout the watercourse (Figure 8). This is atypical, as linear river systems usually have asymmetrical gene flow, from upstream to downstream (Junge et al. 2013). Also, no barriers in these tributaries were detected. Moreover, since the same genetic cluster is found in both tributaries it would seem logical to find more of it in the main river sections I and H, but that is not the case. Possible explanations are that stocking in the main river has shifted genetic diversity there as suggested by Klütsch et al. (2019), or that samples from the main river section I and H originate far away from tributary IOB and IBB, which are situated far away from roads and other infrastructure.

Finally, the populations in remaining tributaries EE, ESB, FSB and GSE (Figure 12 & 13) are all partly genetically differentiated from the stocked/main river cluster, but also harbour a lot of main river genetic structure. F_{ST}-values (metric on the genetic variance within a subpopulation relative to the total genetic variance) were positively associated with distance (Figure 16), in compliance with other studies already mentioned (Carlsson et al. 2000, Carlsson et al. 1999, Lehtonen et al. 2009, Vøllestad et al. 2012). The effect of barriers (hydroelectric dams) were however absent (Table 8), opposite of what was anticipated. The hypothesis that tributaries within closed-off sections of the main river are more genetically similar to each other than to tributaries in other sections, beyond the general isolation-by-distance effect found in the data, therefore has little support.

Brown trout show strong homing behaviour and can home accurately to their natal streams for spawning (Jonsson & Jonsson 2011). Also, after artificial displacement in streams over considerable distances, brown trout will often return to the capture site (its “home range”) regardless of being displaced upstream or downstream (Armstrong & Herbert 1997, Halvorsen et al. 1990). Stocked trout originating from a breeding facility will not have a natal home range in the river system they are released into. This factor may impact the genetic structure observed in the tributaries of the Pasvik watercourse. In other dendritic river systems, the spatial distribution of captive-bred brown trout genotypes has been found to not be homogenous (Saint-Pé et al. 2018), but rather overrepresented in some tributaries. Perhaps low densities of native brown trout in some tributaries prior to stocking make them more attractive for colonisation by stocked fish. Nevertheless, strong homing behaviour in brown

trout is a likely cause for the complex genetic structure in the tributaries of the Pasvik river observed in this study.

4. 3 Tributaries as spawning grounds

Another objective was to understand the role of tributaries as spawning grounds for main river stocked fish and to gain a more detailed understanding of how offspring of stocked fish are distributed in the river system. The extent of suitable trout spawning habitat in the main river is unknown, but probably plays a role in natural recruitment in some areas. Historically, before the construction of the dams, spawning in the main river was occurring on a much bigger scale (70 % spawning habitat lost after dam constructions (Klütsch et al. 2019)). Therefore, it is adequate to assume that tributaries have become more important for spawning. The stocking programme has homogenized the genetic diversity in the main river trout population (Klütsch et al. 2019), which in turn makes it possible to identify main river brown trout in the tributaries. Several tributaries were found to contain individuals with high assignment probability (>80 % threshold and 80-25% threshold) to the main river/stocked brown trout cluster. These were EE, ESB, FSB, GSE and to some extent IBB (Table 9). Individuals with 80+ % assignment to the main river are either offspring from two main river trout that spawned in the tributary, or migrants from the main river. Homing behaviour (Jonsson & Jonsson 2011) and strong isolation-by distance at a young age in brown trout (Vøllestad et al. 2012) are, however, making the possibility that they are migrants seem unlikely. The admixed individuals (25-80 % assignment) must be a result of a mating event between main river and tributary individuals.

Table 9. *Proportion of individuals assigned to the main river/stocked cluster by either 80 or 25-80 % in every investigated tributary.*

Tributary	80+ % assignment	25-80 % assignment	Total
DOB	0 %	0 %	0 %
EGB	0 %	0 %	0 %
EE	29 %	51 %	80 %
ESTB	0 %	9 %	9 %
ESB	10 %	43 %	52 %
FSB	30 %	19 %	49 %
GSE	48 %	35 %	83 %
IOB	0 %	0 %	0 %
IBB	9 %	23 %	32 %

The admixed individuals of lower age classes indicate natural recruitment in the tributaries to different extents. In addition, main river fish clearly use the tributaries as spawning grounds to different levels with the exception of highly isolated tributaries such as EGB and ESTB. IOB is also isolated in this regard, but has more admixture with other tributaries, mainly IBB. Thus, the hypothesis that main-river fish use tributaries as spawning grounds seems supported by the data.

However, there were spatial differences in the tributaries in the proportions of potential migrants from the main river and proportions of admixed individuals indicating that main river trout use tributaries for reproduction. It is noteworthy that fish from tributaries appear to be migrating or reproducing in the main river to a far lower extent as suggested by the largely missing individuals with admixed ancestry in the main river. Therefore, the tributaries appear crucial for natural recruitment for both main river and local tributary brown trout populations.

4. 4 Density of brown trout in the tributaries

The tributaries vary in size and spatial structure. Density estimates for each tributary also varied considerably (Table 2, Table 10). Since brown trout density was only calculated at the sampling stations, large areas in many of the larger tributaries were not sampled. In addition, bigger tributaries varied more in structure throughout their course as well. Therefore, estimates here may not be very reliable, but they give an indication. These tributaries include EE, ESTB, ESB, FSB and GSE. The remaining tributaries DOB, DGB, EGB, IOB and IBB were of such small size and so uniform that estimates here are more reliable. Interestingly, tributaries DOB and DGB in the non-stocked section D of the main river had very little trout. DGB was fished throughout its entire length without any sign of fish, suggesting it to be empty of trout. DOB was also fished thoroughly but revealed very little trout and had the lowest density out of all the tributaries investigated apart from DGB.

Table 10. *Densities of brown trout in the tributaries of the Pasvik watercourse at the sampling station level and total average densities within each tributary.*

Tributary	Station 1	Station 2	Station 3	Total average density
DOB	2.7	1.1		1.9
DGB	0	0	0	0
EGB	17.7	22		19.9
EE	15.6	10	26.7	17.4
ESTB	9.3	18.4		13.9
ESB	19.5	4.3		11.9
FSB	4.1	4	11.1	6.4
GSE	10.5	2.3	6.7	6.5
IOB	31.6	80.2	20.4	44
IBB	47.2	43.8		45.5

The anticipation was that stocking led to more brown trout overall, in both the main river and tributaries. However, brown trout densities in the tributaries were significantly lower in tributaries with higher proportions of individuals assigned to the main river stocked cluster (Table 6, Figure 15a & b). Similar tendencies are seen in other salmonids. Stocking may significantly reduce a wild populations fitness during supportive breeding (Ford 2002). Fish with high fitness in captive environments perform poorly under wild conditions, as well as their offspring (Christie et al. 2012). These findings also resemble the situation with escapee versus wild Atlantic salmon (*Salmo salar*) introgression in Norway – persisting geneflow from slightly domesticated individuals to wild ones may have fitness consequences after just a few generations (Bolstad et al. 2017). Based on the data presented here, the hypothesis predicting tributaries in stocked sections of the main river to have higher densities of brown trout than tributaries in non-stocked sections has little support, and the opposite seems to be the case. However, one season of data is a limited basis for drawing conclusions.

4. 5 Genetic diversity and demography

At the sampling station level in the tributaries (Appendix, Table 1), genetic diversity estimates provided little support to the hypothesis that tributaries harbour higher levels of genetic diversity than the main river. Instead, local differences in genetic diversity levels could be observed. First, (private) allelic richness was lowest in EGB, ESTB, and IOB whereas main river sections generally showed higher (private) allelic richness. However, some tributaries showed (private) allelic richness values similar to the main river (e.g., ESB, Table 3). Concerning the main river, sections G, H, and I, which have been found to have undergone bottlenecks (this study and Klütsch et al.2019), also showed the lowest (private) allelic

richness values of the main river sections indicating that genetic diversity has been altered by stocking here. This was in contrast to non-stocked Russian river sections and some sampling stations in the tributaries (Appendix, Table 1). Other brown trout populations subject to stocking have also been found to undergo bottlenecks, with genetic diversity being lost at a negative rate since the founding event (Aho et al. 2006). Interestingly, two of the most strongly differentiated tributaries (i.e., ESTB and EGB) also showed the lowest (private) allelic richness estimates.

However, when combining all tributaries and comparing this to stocked and non-stocked river sections, then (private) allelic richness was found to be higher in the tributaries (Table 4). These results suggest that tributaries hold considerable unique genetic diversity but that local differences in genetic diversity exist. Potential explanations for this pattern include different levels of main river ancestry among the tributaries that might have influenced these estimates because of mixing of stocked and non-stocked populations.

Further, results showed that recent bottleneck events were present in six out of nine tributaries. However, within tributaries, some sampling stations showed significant bottleneck effects while others did not (Table 5). An example of where a significant bottleneck was detected is in one of the most differentiated tributaries, EGB. This tributary is highly isolated based on the genetic differentiation values and it shows low genetic diversity and has recently undergone a genetic bottleneck. Since the tributaries are non-stocked, other factors like small population size and barriers to gene flow may offer explanations. The population also shows very low admixture with main river populations so that poor spawning grounds may be one possible explanation for low genetic diversity observed in this tributary. Contradicting to this, EGB has a rather high population density (Table 10) compared to many other tributaries. Further, the other highly differentiated tributary, ESTB, showed low levels of genetic diversity but no recent signs of bottlenecks. Therefore, recent demographic changes cannot explain the low genetic diversity estimates in this population. Long-standing isolation and subsequent genetic drift may be a more likely explanation in this case. More generally, there was relatively weak alignment between significant bottleneck signatures and lowest genetic diversity levels. This is explainable by different levels of genetic diversity before the bottleneck (i.e., more genetic diversity is retained in populations that had higher genetic diversity levels before the bottleneck) and possibly the strength and duration of the bottleneck event that will affect the genetic diversity level left after the bottleneck event. This points to a highly dynamic system with different local underlying causes for genetic patterns seen. The

hypothesis that genetic diversity is higher in the tributaries can be strengthened, but local differences in both the tributaries and main river are present.

4.6 Limitations of the study

One uncertainty regarding main river structure in the tributaries is the stocking history in some of the larger lakes in the Pasvik valley. Although now ceased, this stocking practice likely influenced genetic structure in tributaries connecting the lakes with the Pasvik river (e.g., Thaulow et al 2013). Although details are lacking at this point, there are indications that stocking has occurred in Ellenvann (connected to EE), Store Spurvvatn (connected to ESB) and Store Sameti (connected to GSE). Fish from all these tributaries show strong admixture with the main river, but a part of this might as well be admixture with the corresponding lakes. This factor is an important part of the picture, since these tributaries as a result likely have been more heavily exposed to the stocked/main river genotype than the other tributaries in the system.

Another current limitation of this study is the lack of tributary data from Russia – several tributaries on the Russian side of the border should be investigated, as they are probably equally important to the natural recruitment in the system as the Norwegian ones. Getting genetic data from these tributaries would give a more complete picture of the demographics of the Pasvik brown trout, so more transnational cooperation is needed. This might be especially interesting because no stocking occurs in the Russian part of the main river (sections A-C, Figure 1). Hence, this would allow for a comparative analysis of genetic differentiation of main river and tributary populations to aid in the understanding of the effects of stocking and natural recruitment.

4.7 Conclusion and the way forward

This study confirms the presence of natural recruitment and fine-scale genetic differentiation in brown trout in a sub-arctic, dendritic river system, namely in the tributaries of the Pasvik watercourse. This is adding to the findings by Klütsch et al. (2019), who proved that genetic diversity in the main river brown trout population has been altered by fragmentation and stocking. The effect of barriers (hydroelectric dams) on genetic diversity and structure in the tributaries was of less significance than anticipated. Genetic admixture with the main river in several of the tributaries proves that main river brown trout use the tributaries for spawning – some tributaries were however strongly genetically differentiated from the main river and other tributaries. Admixture of main river/stocked trout in the tributaries was found to have a

negative effect on fish density. The tributaries combined were found to harbour more genetic diversity than the main river.

Signs of recent bottlenecks in many of the tributary populations are concerning, as individuals are being lost. Further, the unique genetic structure in many tributaries are under threat from stocked main river genotypes. If the ongoing practice with stocking at the same scale continues, it is likely that this structure will be lost over time. Stocked brown trout also seem to bring genes into the wild tributary populations that may be unfavourable for fitness traits and local adaption, and thereby decrease population density. Little genetic structure from the tributaries are found in the main river, which can mean either that tributary populations are very sedentary, or that the stocking program has such a large impact that genotypes from the tributaries are "swamped". Another possibility is that tributary individuals are outcompeted by the stocked fish in the main river.

The natural recruitment potential in the population might be bigger than previously thought. Maybe, by reducing the number of stocked brown trout released into the main watercourse, recruitment in the tributaries will have an increased contribution to the system compared with today. Stocked fish are spawning in the wild, and naturally recruited brown trout as a result of strong sexual selection would be a healthy addition to the stocking programme. This may also give room to more genetic diversity in the population. A genetically diversified brown trout population will be better positioned when facing environmental stochasticity, diseases, pathogens or a changing climate. Therefore, enhancing this genetic portfolio-effect and striving to protect as much of the genetic diversity as possible is key to the long-term persistence of the population.

Moving forward, the genetic stability in the system needs to be evaluated. At this point it is uncertain if the current genetical structure data resembles a stable picture, or if it's just a snapshot. Additional sampling seasons would shed more light on this.

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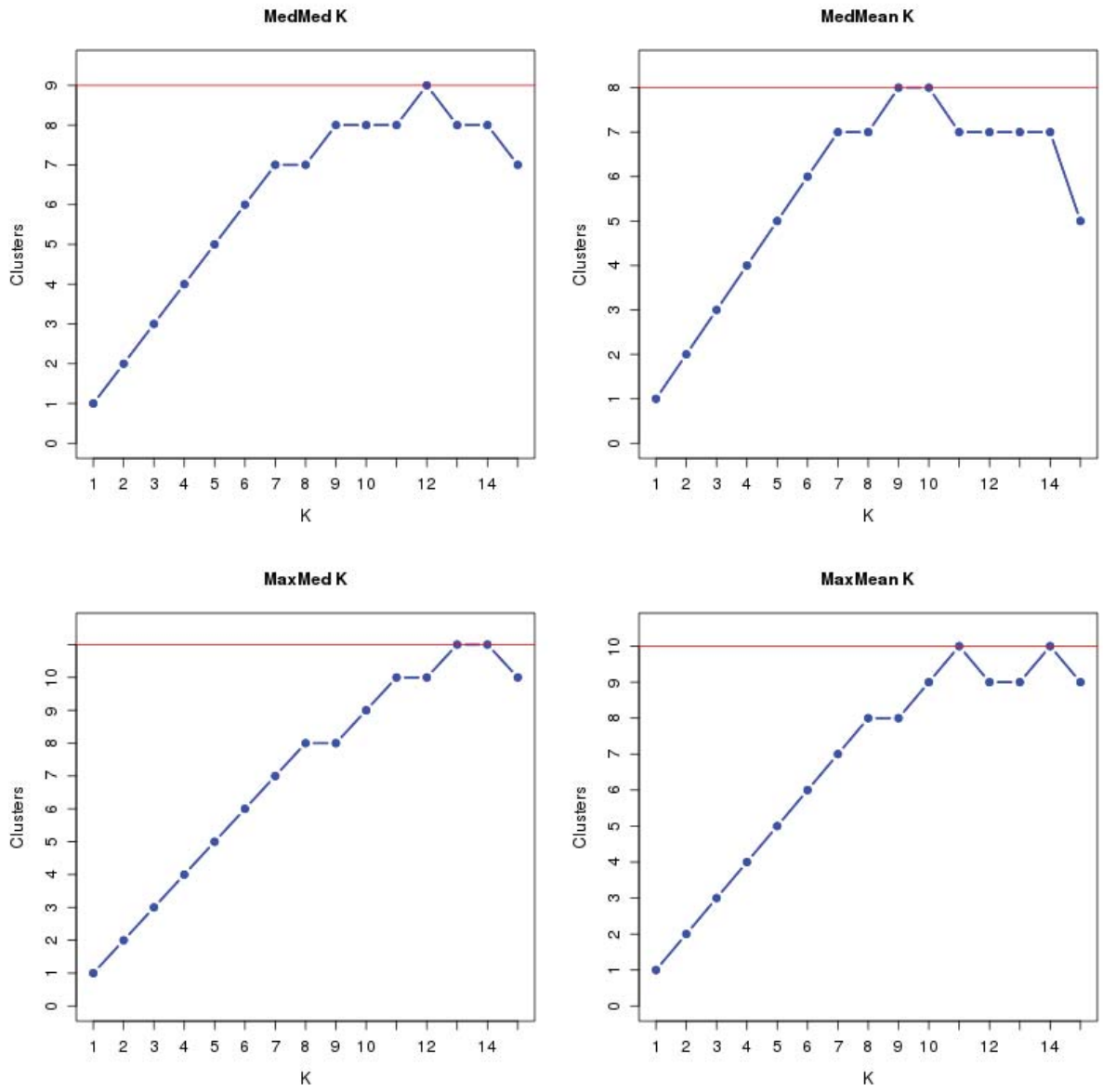
6 Appendix

Table 1. *Genetic summary statistics for all tributary sampling stations and main river sections. N = number of individuals. H_o (SE) = observed heterozygosity with standard error, H_E (SE) = expected heterozygosity with standard error, F_{IS} (SE) = inbreeding coefficients with standard error. Allelic richness and private allelic richness were not calculated for very small sampling sizes.*

	N	HO (SE)	HE (SE)	FIS (SE)	AR	APR
A	2	0.563 (0.09)	0.445 (0.062)	-0.282 (0.096)		
B	53	0.583 (0.052)	0.590 (0.054)	0.007 (0.023)	3.892 (0.432)	0.121 (0.068)
C	32	0.565 (0.048)	0.606 (0.053)	0.066 (0.022)	4.019 (0.429)	0.116 (0.044)
D	3	0.646 (0.048)	0.545 (0.037)	-0.213 (0.070)	3.697 (0.402)	0.126 (0.076)
DOB2	3	0.313 (0.077)	0.42 (0.064)	0.251 (0.130)		
DOB1	5	0.5 (0.068)	0.488 (0.053)	-0.029 (0.082)		
E	24	0.606 (0.055)	0.619 (0.045)	0.038 (0.035)	4.040 (0.424)	0.112 (0.053)
EE1	18	0.604 (0.068)	0.558 (0.058)	-0.084 (0.041)	3.436 (0.320)	0.009 (0.008)
EE2	8	0.546 (0.072)	0.486 (0.061)	-0.133 (0.064)	3.340 (0.345)	0.119 (0.065)
EE3	15	0.624 (0.041)	0.614 (0.037)	-0.027 (0.040)	3.724 (0.362)	0.102 (0.060)
EGB1	13	0.462 (0.061)	0.422 (0.049)	-0.095 (0.065)	2.271 (0.176)	0.014 (0.014)
EGB2	14	0.426 (0.072)	0.393 (0.054)	-0.051 (0.097)	2.142 (0.178)	0.000 (0.000)
ESB1	23	0.590 (0.059)	0.606 (0.053)	0.023 (0.043)	3.881 (0.425)	0.081 (0.049)
ESB2	19	0.622 (0.039)	0.587 (0.041)	-0.077 (0.035)	3.913 (0.325)	0.143 (0.071)
ESTB1	19	0.342 (0.067)	0.321 (0.058)	-0.055 (0.057)	2.150 (0.287)	0.001 (0.001)
ESTB2	24	0.380 (0.065)	0.356 (0.061)	-0.064 (0.035)	2.502 (0.325)	0.043 (0.034)
F	17	0.590 (0.052)	0.644 (0.044)	0.099 (0.042)	4.249 (0.431)	0.169 (0.076)
FSB1	7	0.643 (0.051)	0.582 (0.044)	-0.118 (0.045)	3.716 (0.316)	0.010 (0.008)
FSB2	9	0.597 (0.040)	0.593 (0.035)	-0.028 (0.061)	3.700 (0.387)	0.064 (0.056)
FSB3	21	0.545 (0.037)	0.612 (0.043)	0.094 (0.030)	3.726 (0.299)	0.042 (0.033)
G	88	0.627 (0.050)	0.641 (0.050)	0.021 (0.016)	4.069 (0.435)	0.065 (0.022)
GSE1	22	0.652 (0.033)	0.627 (0.038)	-0.058 (0.035)	3.771 (0.305)	0.051 (0.024)
GSE2	6	0.531 (0.078)	0.454 (0.061)	-0.187 (0.079)	2.938 (0.266)	0.034 (0.025)
GSE3	12	0.641 (0.059)	0.600 (0.049)	-0.063 (0.041)	3.805 (0.326)	0.007 (0.004)
H	31	0.654 (0.047)	0.651 (0.047)	-0.018 (0.045)	4.161 (0.406)	0.056 (0.021)
I	36	0.644 (0.045)	0.651 (0.048)	0.005 (0.028)	4.120 (0.437)	0.050 (0.034)
IBB1	20	0.65 (0.043)	0.651 (0.040)	-0.008 (0.043)	3.875 (0.397)	0.030 (0.017)
IBB2	24	0.599 (0.055)	0.588 (0.052)	-0.022 (0.025)	3.524 (0.329)	0.046 (0.023)
IOB1	10	0.469 (0.061)	0.502 (0.052)	0.058 (0.075)	3.057 (0.272)	0.000 (0.000)
IOB2	12	0.573 (0.064)	0.500 (0.050)	-0.142 (0.067)	2.981 (0.270	0.011 (0.009)
IOB3	16	0.566 (0.050)	0.567 (0.053)	-0.028 (0.051)	3.368 (0.307)	0.031 (0.016)

Figure 1a. *STRUCTURESELECTOR found support for 8-11 genetic clusters within the entire data set (tributaries, main river and Russia).*



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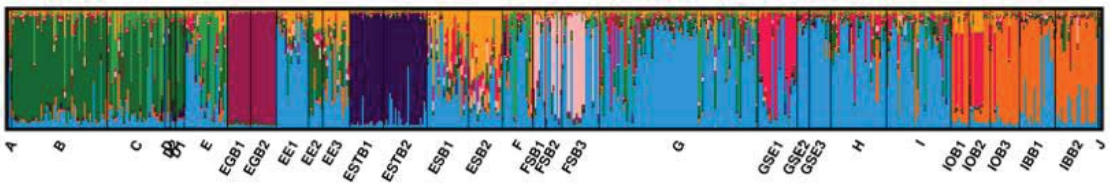

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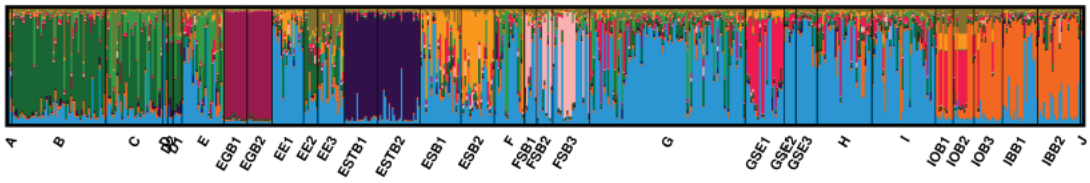

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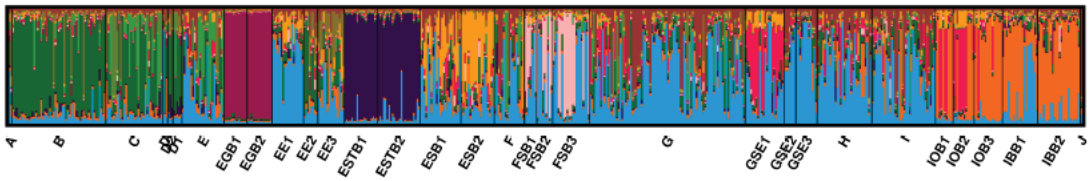
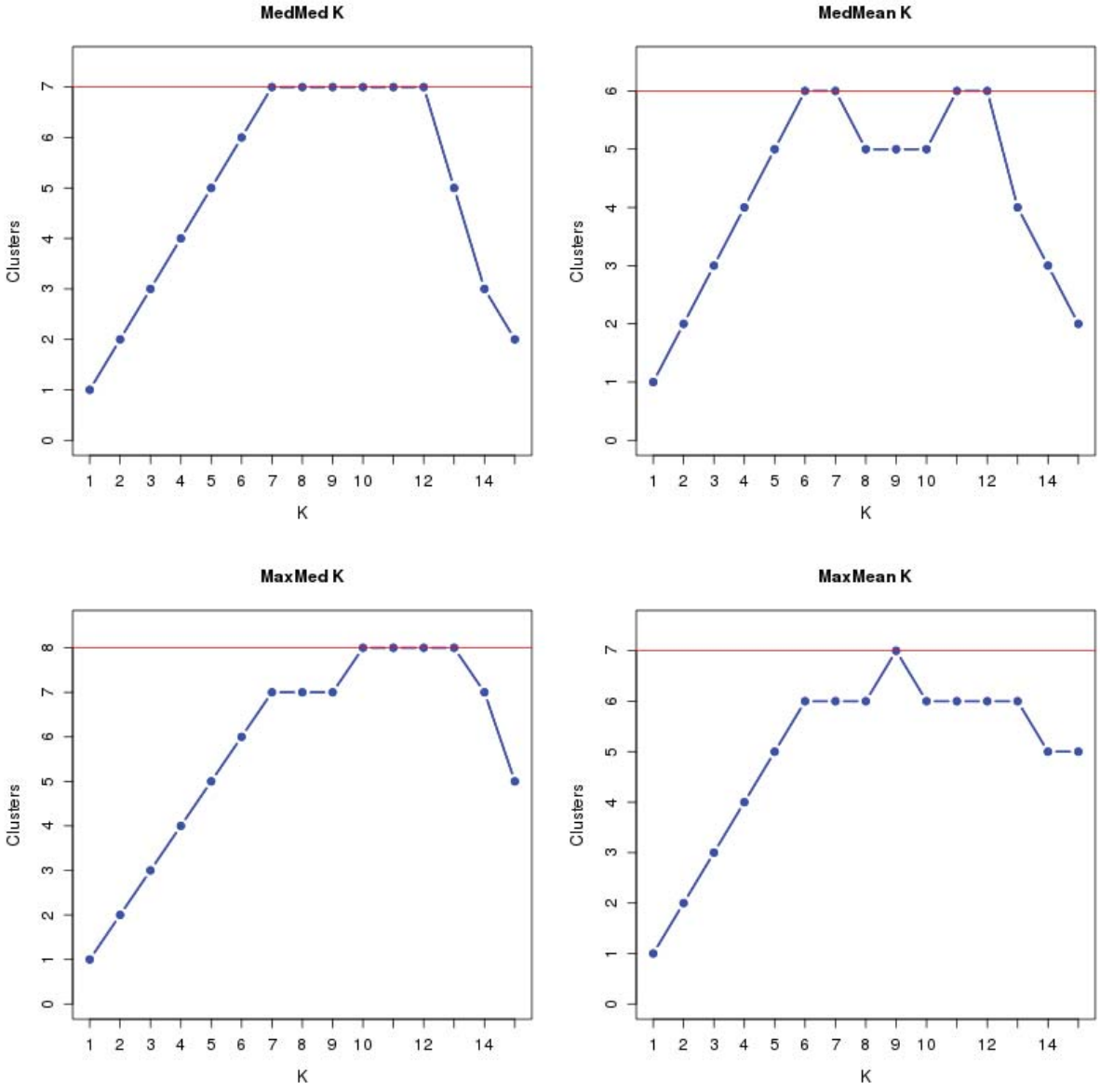
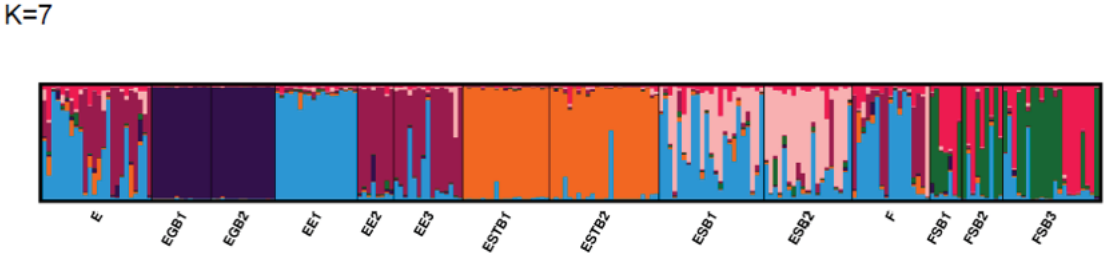


Figure 1b. *Additional structure found throughout the watercourse, not presented in the results chapter.*

Figure 2a. *STRUCTURESELECTOR found support for 6-8 genetic clusters within sections E and F of the main river and tributaries EGB, EE, ESTB, ESB and FSB.*





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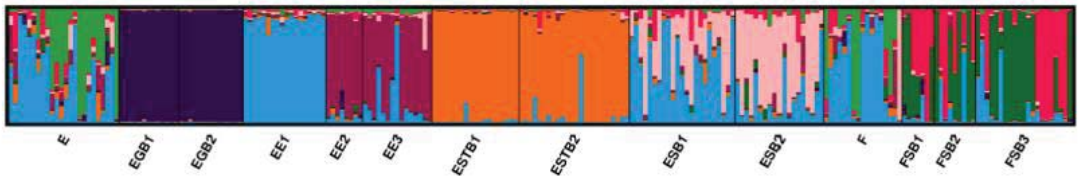

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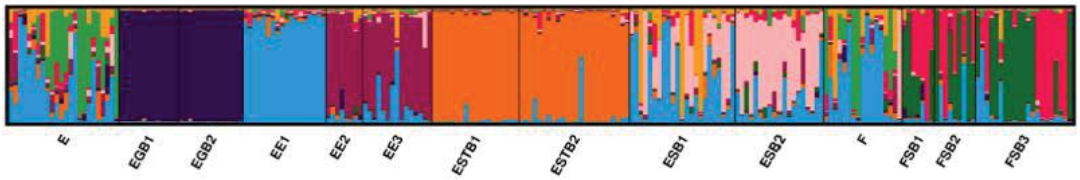

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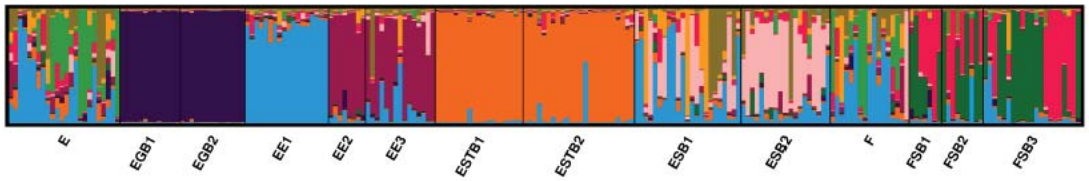

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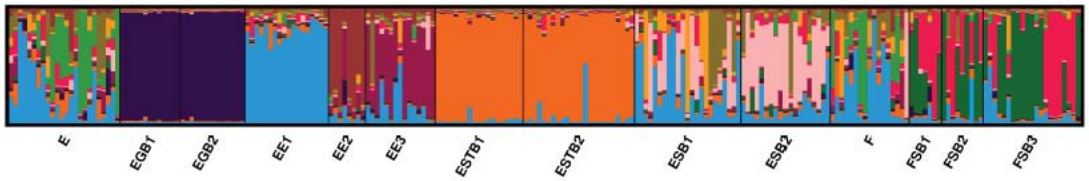

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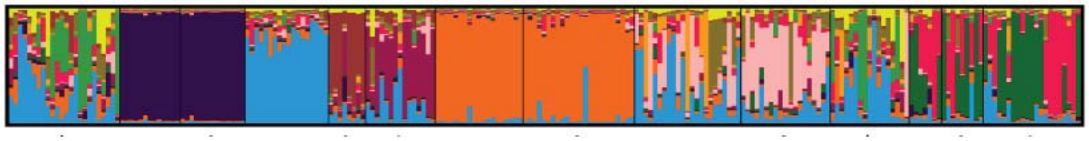


K=13

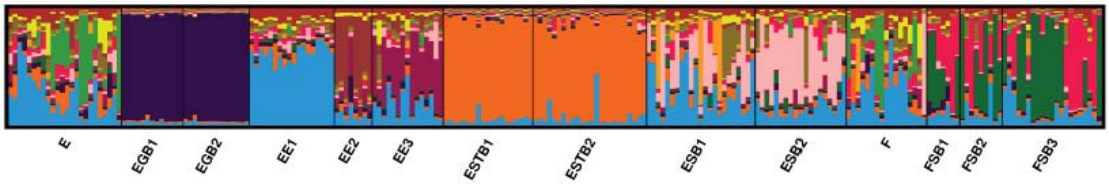


Figure 2b. *Additional structure found throughout section E+F, not presented in results the chapter.*

Figure 3. *All four STRUCTURESELECTOR estimators found support for two genetic clusters within section G with belonging tributary GSE.*

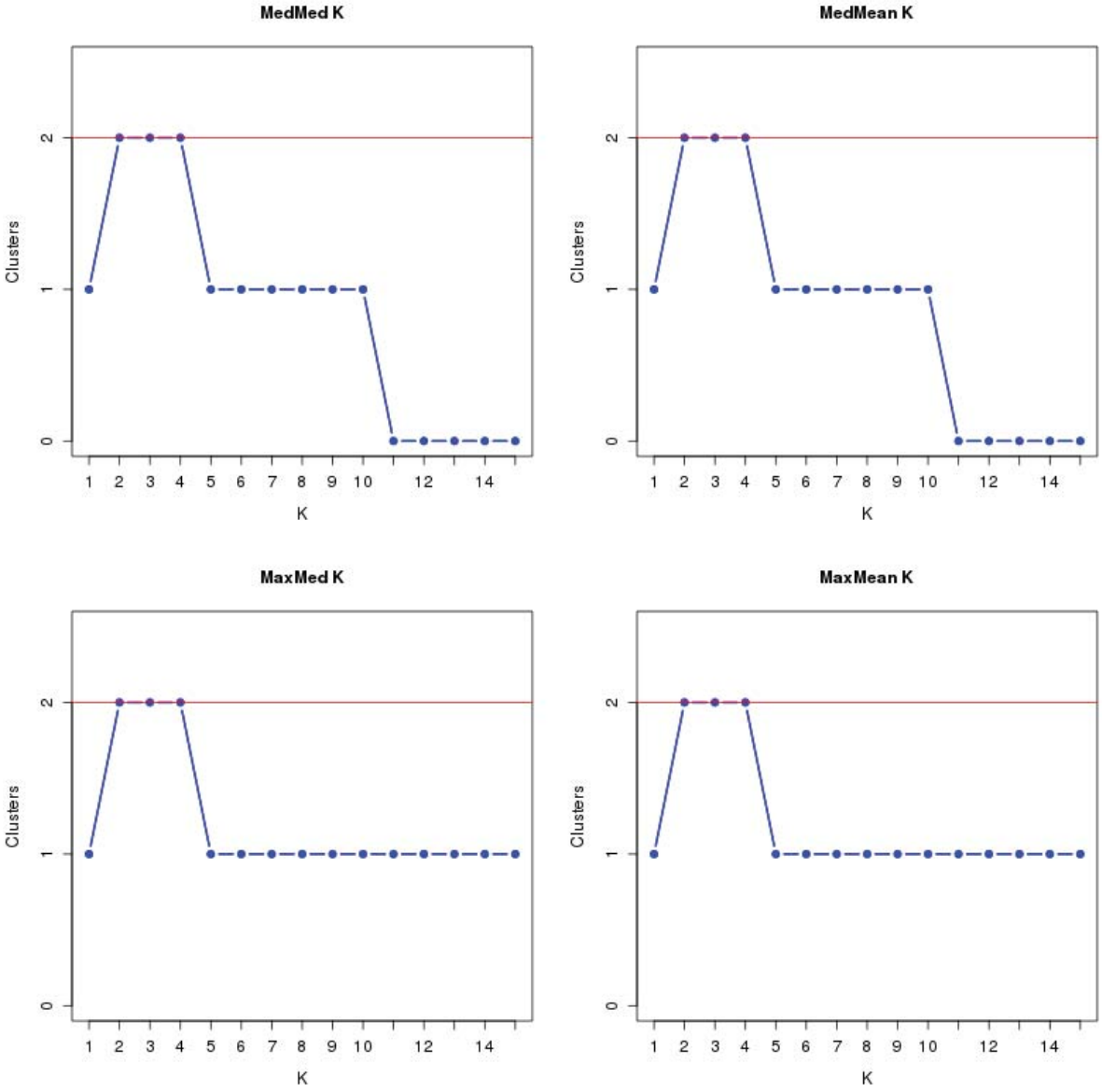
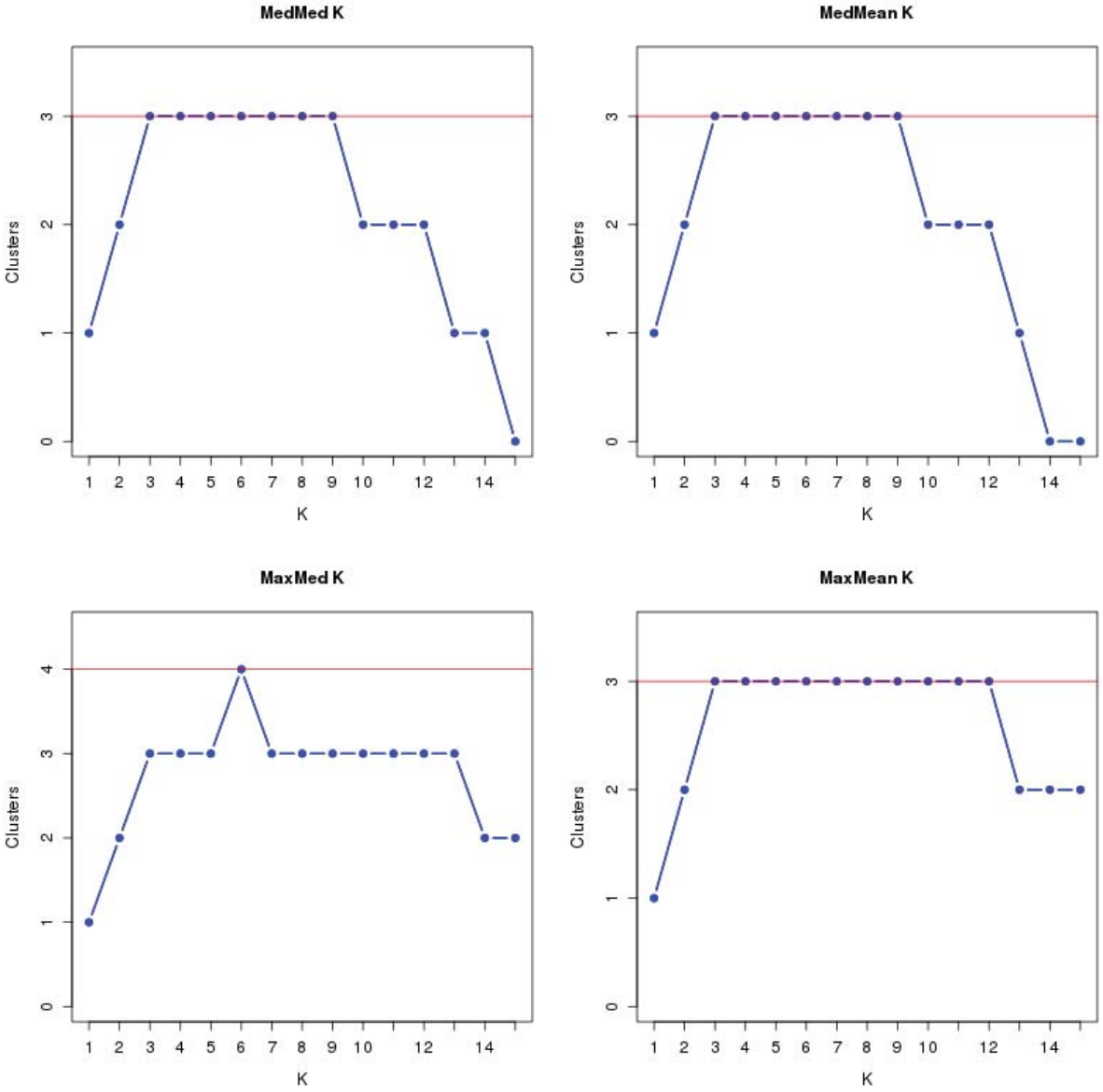
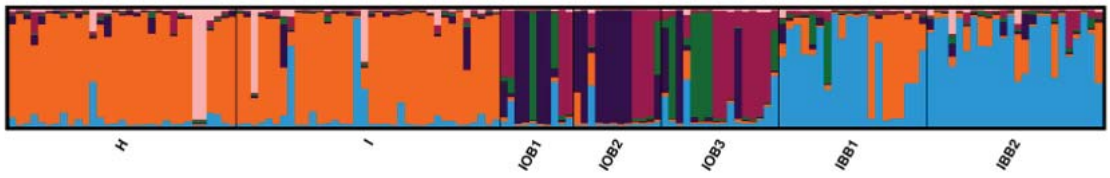


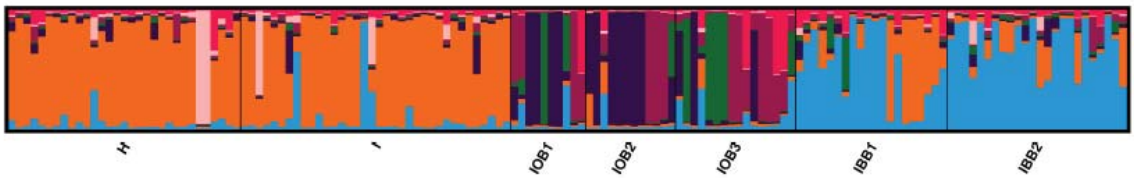
Figure 4a. *Three out of four estimators in STRUCTURESELECTOR found support for three genetic clusters in section H and I of the main river and tributaries IOB and IBB.*



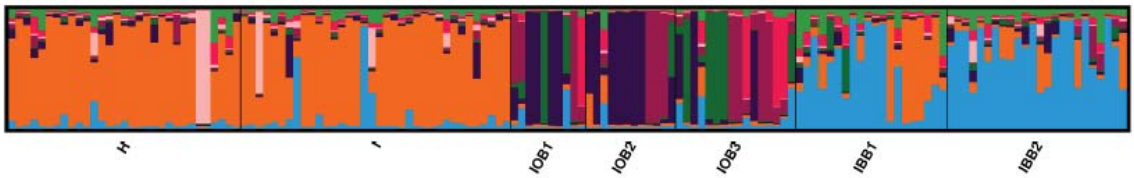
K=6



K=7



K=8



K=9

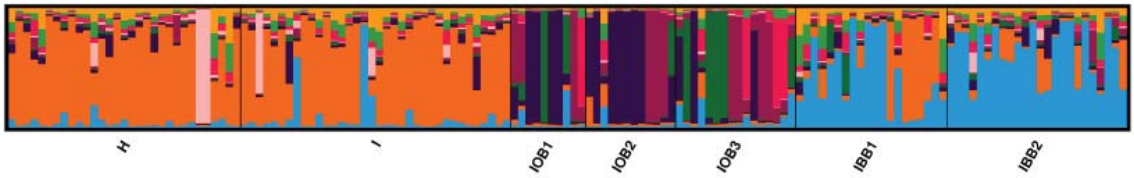


Figure 4b. *Additional structure found throughout section H and I, not presented in the results chapter.*

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