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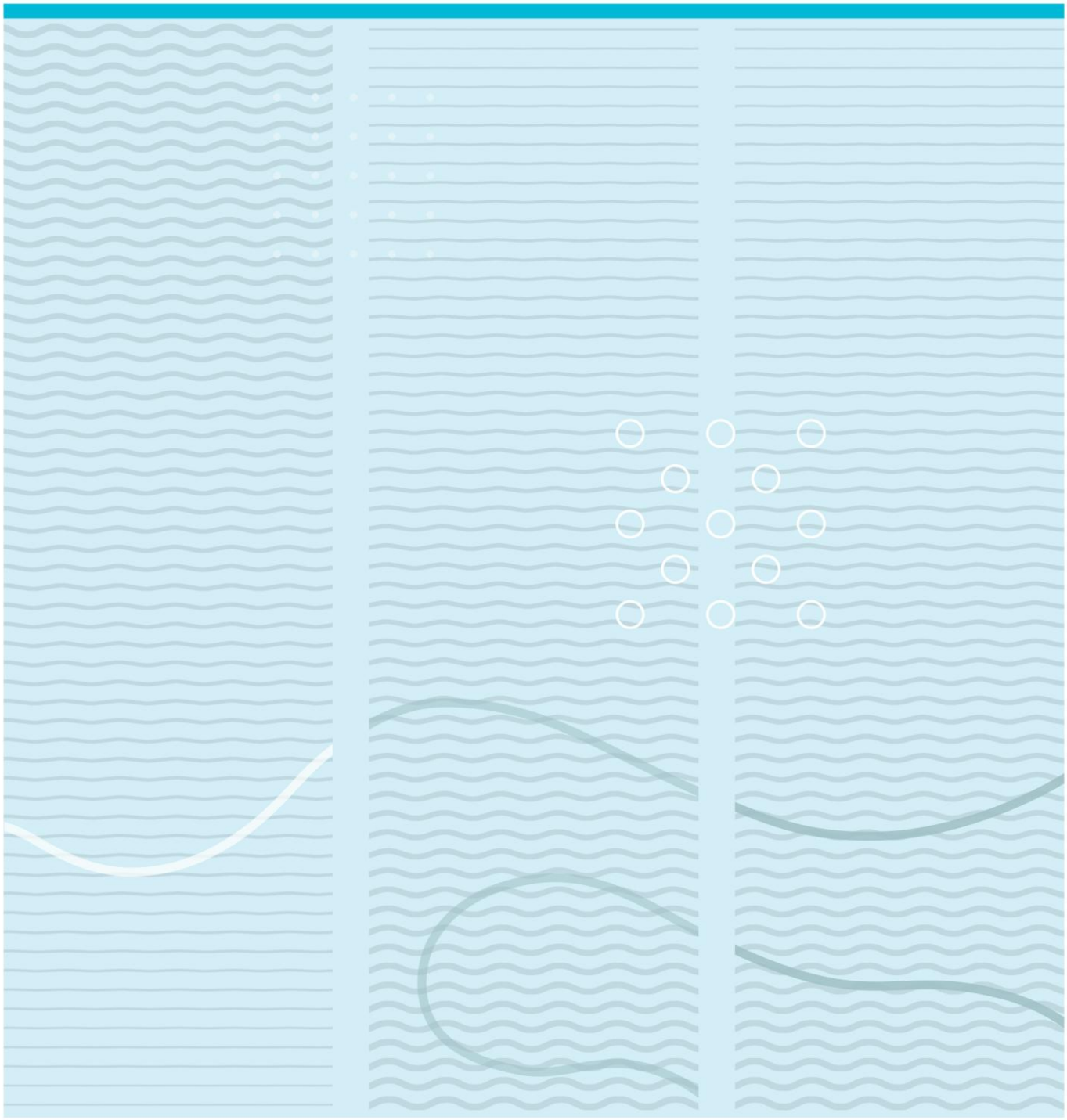
Population structure of brown trout (*Salmo trutta*), a host of freshwater pearl mussel (*Margaritifera margaritifera*) in Lake Norsjø tributaries



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This thesis is worth 60 study points

Abstract

Brown trout (*Salmo* trutta) is one of a few suitable host species for endangered freshwater pearl mussel (*Margaritifera margaritifera*). Freshwater pearl mussel larvae (glochidia) are obligate parasites. Glochidia, must attach to the gills of a suitable host fish, which in our study area is brown trout only. Microsatellite markers have been proven to express levels of genetic variation. We used 13 DNA microsattelite markers to evaluate population structure of brown trout (*Salmo trutta*) in three tributaries of Lake Norsjø, Telemark County, Norway. Genetic differentiation between rivers was found. It was indicated by our results that the river populations are composed of smaller subpopulations with gene flow strongly influencing the genetic variation, and recommended to treat each rivers as one population. We compared genetic diversity and population structure of brown trout and freshwater pearl mussel. Convergence between genetic diversity was found, as less diversity was found in upstream parts of rivers. Because the freshwater pearl mussel showed high genetic variability, compared to previous studies, it may be possible that high gene flow in host populations is beneficial.

Key words: *Brown trout, Freshwater pearl mussel, population structure, host-parasite relations, microsatellites*

Contents

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Bø i Telemark/14.5.2018
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1. Introduction

Genetic variation is recognized as fundamental for evolutionary processes and is directly linked with biodiversity (Laikre et al., 2010). Genetic marker such as microsatellites has been proven to express levels of genetic variation (Paetkau & Strobeck, 1994; Putman & Carbone, 2014). Such variation is considered an essential component of, and basis for conservation management program (O`Connell & Wright, 1997). Microsatellites can further be used to estimate population differentiation, connectivity and therefore population structure (Balloux & Lugon-Moulin, 2002; Hoshino et al., 2012)

Brown trout (*Salmo trutta)* is a genetically highly structured species (Ferguson, 1989; Skaala, 1992) and its differentiation into subpopulations is proven at both large (Hansen et al., 2007) and small geographical scales (Heggenes & Røed, 2006; Linlokken at al., 2014; Wollebaek at al., 2018). Brown trout also shows extensive variability in its life cycle, and can be of lake dwelling or stream resident forms. Lake dwelling trout migrate between lakes and spawning sites in rivers, whereas stream resident trout stays in the river throughout life and usually spawns in smaller tributaries nearby (Elliott, 1994). With rivers as its main habitat, trout may be genetically more or less isolated by natural migration (semi)barriers like waterfalls. These barriers allow downstream movement in most cases, but depending on size, can restrict upstream migration to a minimum or none at all (Carlsson et al., 1999). It is also known that brown trout is one of a few host species for the freshwater pearl mussel (*Margaritifera margaritifera*), during the obligate parasitic phase as a young larvae in its life cycle (Young & Williams, 1984), which is very different from the life cycle of brown trout in nearly all aspects.

Freshwater pearl mussel is an endangered species (IUCN, 2017), and even though the estimated number of populations in Norway is one of the highest in Europe, there is still a serious decline, mostly in the southern parts of the country (Geist, 2010). Freshwater pearl mussels larvae (glochidia), must attach to the gills of a suitable host fish, which in our study area is brown trout only. Atlantic salmon (*Salmo Salar*) the other potential host, is partly distributed in lower parts of the same waters, but there are indications that freshwater mussel glochidia are species specific (Clements et al., 2018). They stay encysted in their host gills for up to 10 months in order to complete their metamorphosis and are therefore considered an obligatory parasite (Geist, 2010; Geist & Kuehn, 2008, Geist et al. 2006). Freshwater pearl mussel and brown trout represent very different life histories and reproductive strategies, yet mussels directly depend on brown trout for their spatial distribution, and thus indirectly also for their population differentiation, connectivity and populations structure.

The aim of this study is to evaluate brown trout genetic diversity and population structure, while considering also the effects on freshwater pearl mussel populations present in the same ecosystem. Brown trout is the main host of freshwater pearl mussel in the area, as Atlantic salmon can only ascend to lower parts in two of our study drainages (Heggenes et al., unpublished manuscript). According to Geist & Kuehn (2008) it is necessary to simultaneously consider both freshwater pearl mussel and its host species for conservation purposes. An insight to natural brown trout population structure in rivers where freshwater pearl mussel occurs naturally can be used to test links between their genetic variances. This may be important for purposes of future ecosystem functioning in case of introduction (Preston et al., 2007; Jones et al., 2006), as advised by Geist (2010).

2. Materials & methods

2.1. Study area

Our study area was compounded of three drainages in Telemark County, Norway. The Hjartdøla, Bøelva and Eidselva Rivers where sampling sites were located, are all tributaries to the Lake Norsjø, 15 m.a.s.l. (Figure 1), from where both brown trout and freshwater mussel naturally colonized the rivers after the last ice age when Lake Norsjø was a fjord. Natural populations of brown trout dominate in all three rivers. All together, there were 8 sampling sites spread across the study drainages (Figure 1), depending on spatial connectivity, i.e. occurrence of waterfalls. Sampling sites within rivers were separated by waterfalls of different heights, but all impassable. Individual river information with respective waterfall heights are in Table1

Table 1. Drainage information showing length of the river, its mean flow, height of waterfall separating lower sampling sites within river (WF 1-2) and upper sampling sites within river (WF 2-3). (Heggens et al., unpublished manuscript)

River	length (km)	mean flow (m^3s^{-1})	WF 1-2 (m)	WF 2-3 (m)
Hjartdøla	34.5	23	20	8
Bøelva	35	18	24	11
Eidselva	23.5	107	2x 2	

Three sites were located in Hjartdøla River (Hj), three on Bøelva River (Bo) and two on Eidselva River (Lu). The lowermost site is fully connected to Lake Norsjø and have a natural populations of Atlantic salmon, whereas the second and third sites were above upstream waterfalls currently impassable for upstream migrating fish. The exception was Eidselva River which does not have Atlantic salmon at the lower site because of a downstream dam. Sites were numbered within rivers in ascending order as they were further upstream (e.g. lowest site on Hjartdøla : Hj1, uppermost site on Bøelva : Bo3). All sampling sites used for data collection in this study were previously

sampled and used for a population genetic divergence study of freshwater pearl mussels (Heggenes et al., unpublished manuscript)

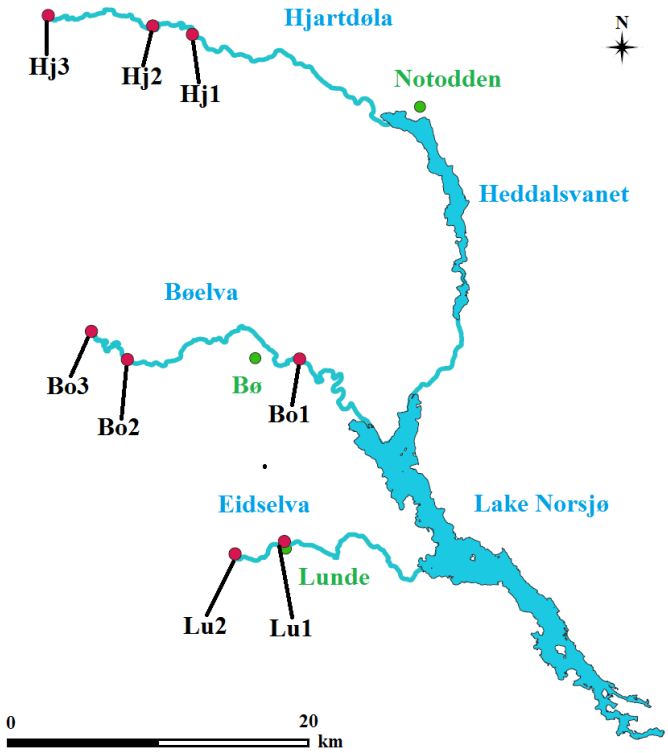

Figure 1. Study area map, with marked sampling sites (red dots), and nearby cities (green dots**)**

2.2. Sampling

Fish were caught using electro-fishing equipment (backpack electroshocker produced by Geomega AS and constructed by Paulsen Engineering, Trondheim, Norway, delivering condensator pulses with about 1600 V and frequency 80 Hz). Each brown trout total length was measured to discriminate small young fish (0+) in our data set, and tissue was sampled by adipose fin clips, placed in 96% ethanol for transport and storage. Exception was made for 9 brown trout (3 per sampling site Bo3, Bo2, Lu1) where, for supplementary samples, fish smaller than 60mm were sampled by caudal fin clipping. Fish were then released unharmed back in the river at the same sampling site. Fin samples were stored in room temperature until the DNA extraction. Target sample size per site was 30 brown trout. All samples of brown trout were collected during a

period of two years, ranging from summer 2014 to summer 2016. Within two sampling sites, namely Bo1 and Lu2, samples were collected during more than one sampling run and more than a year apart, as sample size was insufficient after the first sampling run. The rest of the sampling sites contained samples from one sampling run.

2.3. DNA & microsatellites

DNA was isolated from each adipose fin with spin-column DNeasy Blood & Tissue Kit (Qiagen), following extraction protocol of the manufacturer. We used the whole adipose fin clips for lysis with proteinase K. Genetic variation was analyzed using 13 DNA microsatellite markers: Str15, Str60, Str73 (Estoup et al. 1993), Strutta-12, Strutta-58 (Poteaux et al. 1999), SsOSL417 (Slettan et al. 1995), SsOSL438 (Slettan et al. 1996) Bru07, Bru09, Bru13, Bru14, Bru22, Bru25 (registered as BHMS135, BHMS102, BHMS155, BHMS111, BHMS206 and BHMS362, respectively; www.salmongenome.no›maps› markerinfo›all marker). This set of markers has been used in several previous studies (Wollebaek et al., 2010; Kraabøl et al., 2015). We divided the markers into eight primer mixes (1: Str15, str60; 2: Bru07, Bru14; 3: Bru25; 4: Bru13, Bru22; 5: Bru09; 6: Strutta-58; 7: Strutta-12; 8: Str73, SsOSL417, SsOSL438) used for Polymerase chain reaction (PCR) with forward primers labeled with florescent dyes (FAM, HEX, NED). Total PCR volume was 12 µl with 2 µl of template DNA, with 0.4 µM concentration in reaction of each forward and reverse primer, 1x concentration of Type-it Multiplex PCR Master Mix (Qiagen, Cat. No. 206241), and filled up to total volume with ultra pure water. We included negative control samples in PCR to check for contamination, and prepared the PCR in separate room. The thermal cycler (Eppendorf Mastercycler® gradient thermal cycler) was configured for initial denaturation at 95°C for 5 minutes, followed by 29 cycles of 95°C denaturation for 30s, 57°C annealing for 90s and 73°C extension for 30s. The last

elongation was set for 30min at 60°C. Genotyping was done using 1 µl of PCR product mixed with 9.25 µl formamide and 0.25 µl GeneScan 500LIZ (Applied Biosystems, Cat. No. 4322682). We used 3130xl Genetic Analyzer (Applied Biosystems) for genotyping and GeneMapper v5.0 software (Applied Biosystems) to read allele sizes. Readings were checked manually and repeatedly to ensure consistency of readings. To check for genotyping error, we did PCR & genotyping twice on 20% of samples.

2.4. Data analysis

We checked data for presence of null alleles, allelic dropout and stutter using MICRO-CHECKER v.2.2.3 (Van Osterhout, 2004). Departure from Hardy-Weinberg equilibrium was tested in GENEPOP v.4.7.0 (Rousset, 2008), with Markov chain parameters set to 10000 dememorization number and 300 batches with 30000 iterations per batch, with separate one-tailed tests for both heterozygote deficiency and excess. Same program and Markov chain parameters were used to test for linkage disequilibrium between microsatellite markers. BOTTLENECK v.1.2.02 software (Piry et al., 1999) was used to detect recent population bottlenecks by Wilcoxon sign rank test using two phase model with 90% proportion of stepwise mutation and 10000 iterations. Program Fstat 2.9.3.2 (Goudet, 2002) was used to obtain number of alleles and allelic richness based on minimum sample size for each loci*population combination. Observed and expected heterozygosity were obtained in R (R Core Team, 2017) by using a "summary" function in adegenet 2.1.1 package (Jombart, 2008; Jombart & Ahmed, 2011). We used Kruskal-Wallis test, conducted by "kruskal.test" base function in R to address differences in number of alleles, allelic richness, observed and expected heterozygosity between sampling sites.

We conducted hierarchical analysis of molecular variance (AMOVA) using R package poppr (Kamvar et al., 2014) utilizing ade4 AMOVA (Dray & Dufour, 2007).

Hierarchical levels used for analysis were sampling site and river according to samples. We tested the components of covariance using 999 permutations as described in Excoffier et al. (1992) using the "randtest" function from ade4 R package (Dray & Dufour, 2007). All R functions were conducted using integrated development environment Rstudio1.0.153 (Rstudio team, 2016).

In order to look at population differentiation, we used Fstat 2.9.3.2 (Goudet, 2002) to calculate Weir & Cockerham`s (1984) pairwise Fst estimate (θ). In this study, if Fst is mentioned, it is always representing the Fst estimate (θ) by Weir & Cockerham (1984). We standardized the pairwise Fst by calculating maximum value that Fst can obtain in RECODEDATA v0.1 (Meirmans, 2006), which adjusts alleles to be population-specific. Further, we divided the original Fst values by the estimated maximum values as described in Meirmans (2006). When temporal samples were present at sampling site, we separated them from the rest and calculated pairwise Fst again to check for temporal differentiation. We used POPTREE2 (Takezaki et al., 2010) to calculate Nei`s Da genetic distance measure (Nei et al., 1983) and construct a phylogenetic tree using neighbor joining method (Saitou and Nei 1987). Graphical presentation of the tree was then produced in MEGA7 software (Kumar et al., 2016).

We used QGIS v3.0.1(Open Source Geospatial Foundation Project) geographic information system software to measure geographic distances between sampling sites through water, using equidistance coordinate system (ESPG: 3044). Test for isolation by distance (IBD) was carried out by using Mantel test (Mantel, 1967; Sokal, 1979) between distance matrices of geographic distance between sites and genetic distance of Fst/(1-Fst), using R package vegan (Oksanen et al., 2012) with 999 permutations. Same package and number of permutations was used to further look at the effect of isolation by distance by conducting mantel correlogram for distance classes. Number of classes for the correlogram was determined by Sturge`s rule (Sturges, 1926) and range of

classes was varying, so each class would contain similar number of observations (Legendre and Legendre, 1998). Holm correction (Holm, 1979) method was used to account for multiple testing.

We used STRUCTURE v2.3.4 (Pritchard, 2000; Falush et al. 2003) to look at population structure, assuming number of clusters under Bayesian model (using likelihood of samples belonging to predefined number of clusters, assuming Hardy-Weinberg/Linkage equilibrium within clusters). We ran the algorithm without prior sample information using Admixture ancestry model and correlated allele frequency model for number of clusters ranging from 1-8 K, with 15 runs per each K. Monte carlo markov chain (MCMC) parameters were set to 50000 burn-in period and 80000 MCMC reps To estimate K, we used estimated Ln prob of data and method described in Evanno et al. (2005) using Structure harvester web based program (Earl & vonHoldt, 2012). We further used CLUMPP software (Jakobsson & Rosenberg, 2007) to consolidate output from STRUCTURE and used it to create an image of cluster assignments in Distruct software (Rosenberg, 2004). We also addressed structuring in our data by discriminant analysis of principal components (DAPC) by Jombart et al. (2010), a model-free method in R package adegenet (Jombart & Ahmed, 2011) which does not assume the populations to be panmictic. We used grouping of samples based on original sampling site. During computations, we retained 75 PCA axes conserving 95% of variance, and all 7 DA axes.

For assignment test, we used Geneclass2 software (Piry et al., 2004), using Frequencies-based method of Paetkau et al. (1995), with probabilities calculated using monte carlo resampling simulation algorithm for exclusion method by Cornuet et al. (1999) with 1000 simulated individuals. Individuals were assigned to population showing highest probability. We tested assignment to correct sampling site and correct river using methods above.

3. Results

3.1. Genetic data quality and equilibrium

The genotyping error based on the 20% of samples which were genotyped twice, showed an overall error rate of 0.3%, with highest error rate of 1.6% for marker SsoSL438. There were no missing data, except in samples from two sampling sites, and very limited. At site Bo3 there was only 0.3% missing data and at Lu2 it was 1% of data.

MICRO-CHECKER software suggested null allele/stuttering presence in two out of 104 tests, specifically at Hj1(str73) and Lu2(bru7). As proportion of positive tests was 0.019, and they did not show any consistency across loci or population, it was found unlikely to affect the results. Testing for linkage equilibrium initially showed 8.3% of tests with $p<0.05$. However, there were no significant results after sequential Bonferonni correction (Rice, 1989). Tests for Hardy-Weinberg equilibrium were significant for heterozygote deficiency in 6.7% (7out of 104) of tests ($p<0.05$), as expected because of Type II error, but none were significant after sequential Bonferonni correction (Rice, 1989). Tests of heterozygote excess were significant in 3.8% (4 out of 104) of tests, and again none were significant after sequential Bonferonni correction. Test for recent population bottleneck showed only one significant result, for site Hj2 ($p= 0.0266$).

3.2. Genetic variation within sites

All 13 loci were polymorphic with number of alleles ranging from 2 (str60, bru23) to 35 (bru25), making a total of 169 alleles across all samples and loci (mean= 13, SD ±10.08). Within group genetic diversity (Table 2), specifically number of alleles (Nall) (mean = 6.87, SD ± 4.44), observed (Ho) and expected (He) heterozygosity (both

Ho & He mean= 0.64, SD ±0.22) and allelic richness (for n = 22) based on minimum sample size (Ar) (mean= 6.4, SD ±3.95) did not differ significantly between sampling sites. (Kruskal-Wallis test; Nall: H = 3.4, p = 0.846; He: H = 3.08, p = 0.877; Ho: H = 3.69, p = 0.815; Ar: H = 3.82, p = 0.8).

Table 2. Within group genetic diversity desriptive statistics based on sampling site. Number next to the sampling site name corresponds to the number of samples obtained. (* = P<0.05 HWE significance before Bonferroni correction)

	Hj3 (37)					**Hj2 (29)**					**Hj1 (34)**				
Loci	**N**	**Nall**	**He**	**Ho**	**Ar**	**N**	**Nall**	**He**	**Ho**	**Ar**	**N**	**Nall**	**He**	**Ho**	**Ar**
str15	3	4	0.557	0.67	3.566	29	4	0.665	0.79	3.724	34	6	0.719	0.67	5.565
str60	3	2	0.078	0.08	1.923	29	2	0.285	0.20	2	34	2	0.251	0.29	2
bru7	3	7	0.783	0.83	6.133	29	5	0.727	0.75	4.927	34	9	0.792	0.88	8.02
bru14	3	3	0.513	0.51	2.969	29	3	0.499	0.51	2.724	34	4	0.59	0.5	3.851
bru25	3	16	0.89	0.86	12.76	29	14	0.858	0.93	12.584	34	19	0.918	0.94	16.16
bru13	3	9	0.814	0.78	8.063	29	10	0.815	0.82	9.497	34	13	0.855	0.79	11.87
bru23	3	2	0.456	0.54	2	29	2	0.383	0.31	2	34	2	0.403	0.38	2
bru9	3	9	0.61	0.51	7.322	29	6	0.561	0.51	5.705	34	14	0.685	0.70	10.63
bru58	3	8	0.723	0.67	7.546	29	8	0.776	0.82	7.629	34	10	***0.824**	0.73	9.243
bru12	3	11	0.747	0.67	9.328	29	9	0.806	0.93	8.356	34	12	0.867	0.91	11.19
str73	3	3	0.537	0.45	2.923	29	3	0.64	0.62	3	34	3	0.605	0.5	3
ssosl417	3	6	*0.792	0.67	6.791	29	9	0.825	0.93	8.367	34	10	0.828	0.76	8.747
ssosl438	3	3	0.558	0.64	2.995	29	4	0.663	0.75	3.724	34	4	***0.697**	0.52	3.998
	Bo3 (30)					**Bo1 (24)**					**Bo2 (31)**				
Loci	**N**	**Nall**	**He**	**Ho**	**Ar**	**N**	**Nall**	**He**	**Ho**	**Ar**	**N**	**Nall**	**He**	**Ho**	**Ar**
str15	3	4	0.664	0.73	3.914	24	6	***0.726**	0.70	5.749	31	3	***0.649**	0.80	3
str60	3	2	0.339	0.3	2	24	2	0.43	0.45	2	31	2	0.35	0.38	2
bru7	3	6	0.77	0.7	5.973	24	8	***0.780**	0.95	7.846	31	6	0.758	0.67	5.869
bru14	3	3	0.659	0.66	3	24	3	***0.603**	0.83	3	31	3	***0.633**	0.48	3
bru25	2	16	0.917	0.86	14.518	24	18	0.918	0.95	17.093	31	18	0.921	0.90	16.27
bru13	3	7	0.794	0.8	6.398	24	12	0.867	0.91	11.375	31	8	0.796	0.71	7.322
bru23	3	2	0.433	0.36	2	24	2	0.457	0.29	2	31	2	0.437	0.32	2
bru9	3	7	0.703	0.66	6.651	24	8	0.6	0.70	7.61	31	11	0.72	0.71	8.577
bru58	3	7	0.663	0.73	6.352	24	9	0.804	0.87	8.733	31	8	***0.744**	0.90	8.115
bru12	3	9	0.806	0.76	8.226	24	12	0.852	0.83	11.25	31	12	0.826	0.77	10.27
str73	3	3	0.566	0.46	2.998	24	3	0.484	0.5	2.875	31	3	0.54	0.58	2.991
ssosl417	3	5	0.369	0.36	4.59	24	6	***0.765**	0.62	5.874	31	4	0.535	0.61	3.899
ssosl438	3	2	0.255	0.23	2	24	4	0.562	0.5	3.999	31	3	0.093	0.09	2.577
	Lu1 (30)					**Lu2 (22)**					**all (237)**				
Loci	**N**	**Nall**	**He**	**Ho**	**Ar**	**N**	**Nall**	**He**	**Ho**	**Ar**	**N**	**Nall**	**He**	**Ho**	**Ar**
str15	3	5	0.434	0.46	4.4	21	5	0.424	0.47	5	23	7	0.709	0.67	5.163
str60	3	2	0.206	0.23	2	21	2	0.387	0.33	2	23	2	0.29	0.27	2
bru7	3	6	0.762	0.76	5.694	22	7	0.762	0.81	6.909	23	12	0.848	0.79	8.629
bru14	3	5	0.661	0.76	4.694	22	3	***0.594**	0.31	3	23	6	0.615	0.57	3.64
bru25	3	17	0.911	0.86	15.432	21	15	0.908	0.95	15	23	35	0.945	0.90	19.82
bru13	3	12	0.852	0.96	11.101	22	13	0.869	0.86	12.858	23	21	0.887	0.82	12.42
bru23	3	2	0.124	0.13	1.994	22	2	0.087	0.09	1.999	23	2	0.382	0.32	2
bru9	3	14	0.691	0.63	11.979	22	12	0.838	0.72	11.859	23	21	0.699	0.64	11.21
bru58	3	9	0.849	0.83	8.588	22	10	0.846	0.86	9.864	23	22	0.861	0.79	12.12
bru12	3	9	0.722	0.7	8.495	22	11	0.846	0.77	10.816	23	20	0.89	0.79	12.73
str73	3	3	0.602	0.66	3	22	3	0.6	0.59	3	23	3	0.584	0.54	2.991
ssosl417	3	10	0.834	0.83	9.416	22	9	0.827	0.72	8.908	23	12	0.816	0.69	8.236
ssosl438	3	4	0.647	0.53	3.994	22	5	0.685	0.63	4.955	23	6	0.602	0.48	4.084

3.3. Genetic variation between sites

AMOVA results (Table 3) with grouping factors based on the sampling site and corresponding river indicated that most of the allelic variation resided within samples. Among group allelic variation was greater between rivers than between sites within rivers, both being significant after 999 permutations.

Table 3. Components of covariance from AMOVA

components	% of variation	Sigma	p-value
between rivers	6.694285	0.314102	0.001
between sites within rivers	2.380299	0.1116858	0.001
between samples within sites	2.232966	0.1047728	0.027
within samples	88.69245	4.1615308	0.001

Pairwise Fst values were significant after 560 permutations for all population pairs using sequential bonferroni correction (Rice, 1989) and were further standardized (Table 4).

Table 4. Pairwise Nei`s Da genetic distance (Nei, 1983) above the diagonal and pairwise Fst estimate (Weir & Cockerham, 1987) standardized by method of Meirmans (2006) bellow the diagonal.

	Hj1	Hj2	Hj3	Bo1	Bo2	Bo3	Lu1	Lu2
Hj1		0.077	0.108	0.179	0.232	0.241	0.153	0.152
Hj2	0.0261		0.07	0.232	0.275	0.271	0.195	0.213
Hj3	0.1244	0.0871		0.226	0.312	0.308	0.226	0.236
Bo1	0.1253	0.1821	0.2828		0.102	0.145	0.233	0.242
Bo2	0.2472	0.2968	0.3917	0.0705		0.068	0.265	0.263
Bo3	0.2446	0.2862	0.3831	0.1174	0.0353		0.280	0.257
Lu1	0.1376	0.1650	0.2805	0.2306	0.3018	0.3362		0.106
Lu2	0.1308	0.2009	0.3111	0.2323	0.2925	0.2966	0.0598	

Temporal structuring was found unlikely to affect our data, as pairwise Fst values were nonsignificant when comparing temporal samples with the rest of samples within site (θ: -0.0269; -0.0096).

3.4. Isolation by distance

Mantel test for isolation by distance (IBD) between genetic distance (Fst/1-fst) and geographical distance showed significant result after 999 permutations (r = 0.7439, p = 0.001). Follow-up Mantel correlogram (Figure 2) showed significant positive autocorrelation for first (range 0-15km, r = 0.59, p = 0.002) and fourth (59-71km, r = 0.31, p = 0.036) distance classes, and significant negative autocorrelation for the third (39-59km, r = -0.38, p = 0.026) and sixth (78.5-94km, r = -0.55, p = 0.008). After correction for multiple testing (Holm, 1979), third and fourth distance classes were marginally non-significant (p= 0.052; p= 0.078)

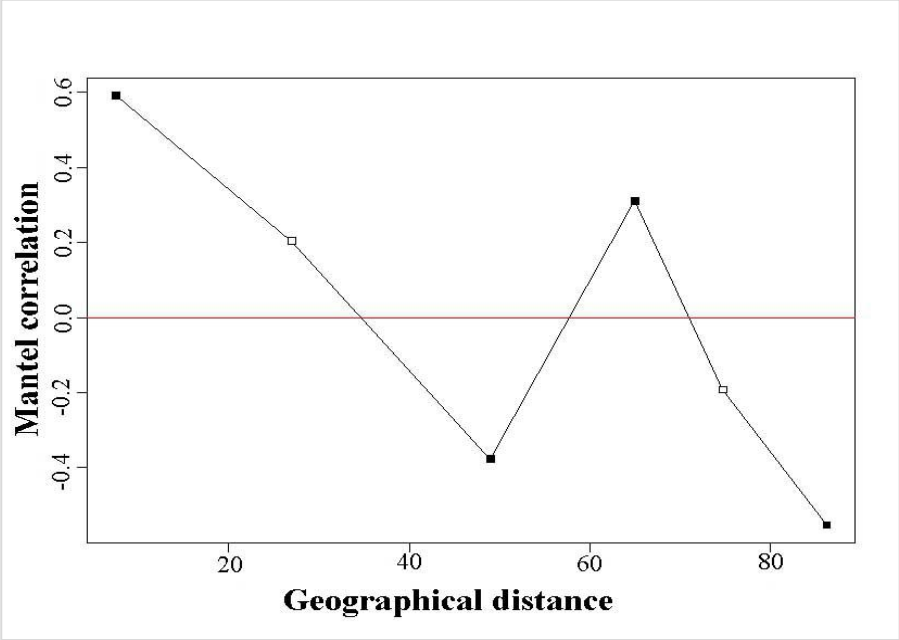

Figure 2. Mantel correlogram based on six distance classes. Classes 2 and 3 contained 4 pairs of observations, rest of classes contained 5 pairs of observations. Distance class index is in km. (Filled squares represent significant autocorrelation, before correction for multiple testing.)

3.5. Population structure

Estimating number of clusters based on mean linear probability of data (LnP(D)) suggested k=3 (LnP(D) = -9371, SD= 1.678) (Figure 3a). Results for number of clusters using ΔK suggested k=2 (Figure 3b), and thus separated sampling sites in

Bøelva River from the rest when considering population assignment to cluster (Figure 4). We further estimated number of clusters for separate datasets of Bøelva R. sampling sites and pooled Hjartdøla and Eidselva Rivers sampling sites

The Bøelva dataset showed no indications of clustering under a Bayesian model, as LnP(D) values remained similar for range from k1 to k4 (k1= -3226, k2= -3208, k3 = -3243, k4= -3176) and dropped continuously as k increased. Assignment of individuals to clusters in Q-matrix exceeded the value of 0.9, i.e. strongly assigned individuals, only in 15 individuals (17.6%) considering k2. The proportion of strongly assigned individuals (q>0.9) was decreasing further with increasing k for Bøelva data.

Pooled samples from Hjartdøla and Eidselva R.`s indicated further clustering. Both LnP(D) (figure 3c) and ΔK (figure 3d) indicated k2.

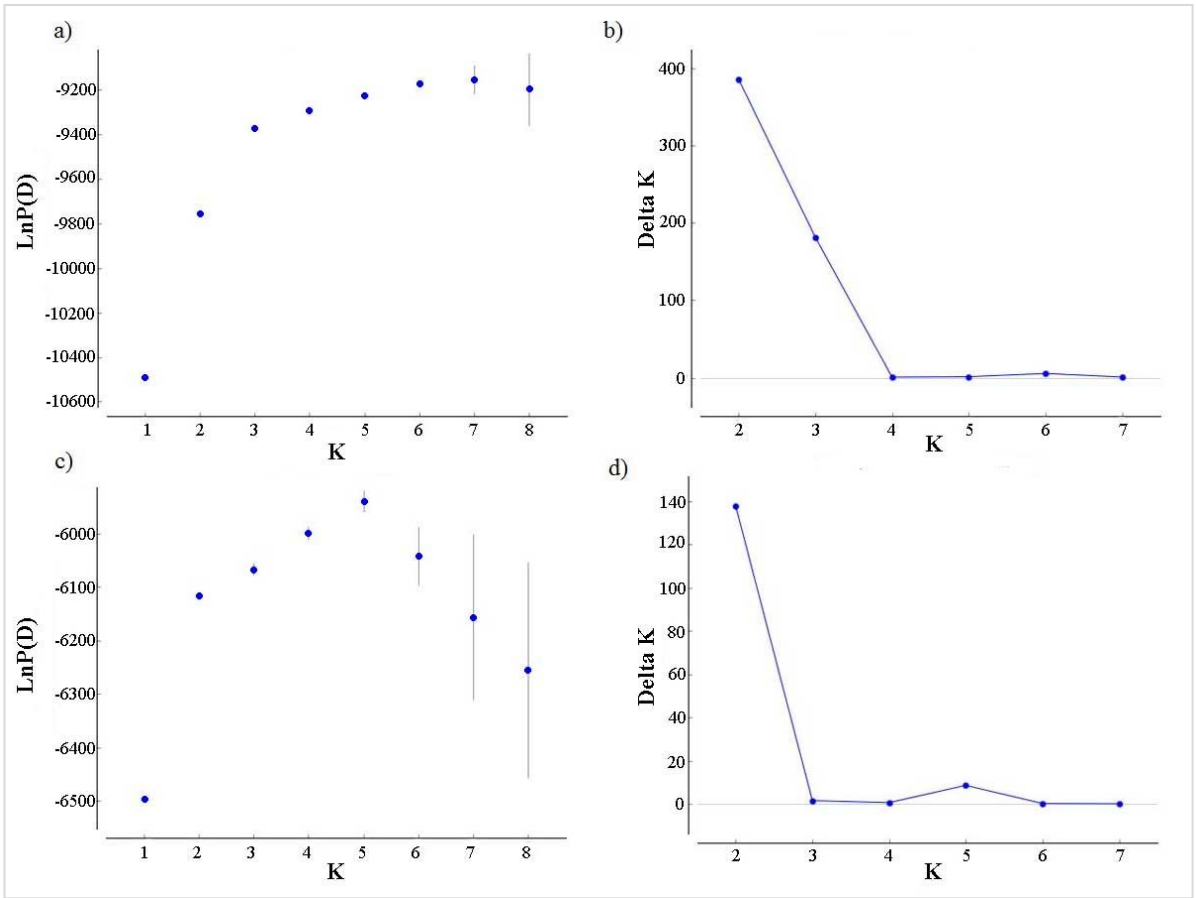

Figure 3. Estimating number of clusters based on Bayesian model. Figure a) shows mean LnP(D) for all samples b) ΔK for all samples c) mean LnP(D) for data without Bøelva river samples d) ΔK for data without Bøelva river samples. Numbers on x-axis correspond to K (number of assumed clusters) on all four graphs.

Considering assignment of individuals to clusters under a Bayesian model (Figure 4), strongly assigned individuals (q>0.9) were present in all clusters, when number of clusters was 4 or less. For k5, one of the clusters was lacking strongly assigned individuals, and was therefore considered uninformative. As total number of clusters further increased so did the proportion of clusters with weak individual assignments.

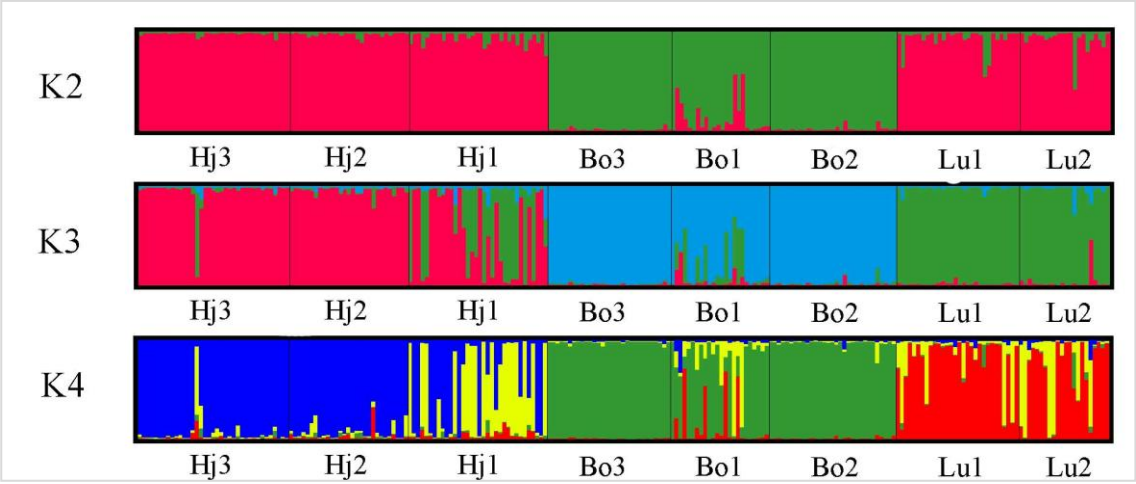

Figure 4. STRUCTURE results plot of Q estimates for k2, k3 and k4. Different colors represent clusters. Thin black vertical lines separate sampling sites (name of the sampling site underneath). Plots are composed of colored, thin vertical lines, each representing an individual sample.

Discriminant analysis of principal components (DAPC) with grouping factors based on sampling site (Figure 5) showed clear segregation between rivers, but not between any sites within rivers, as they overlapped.

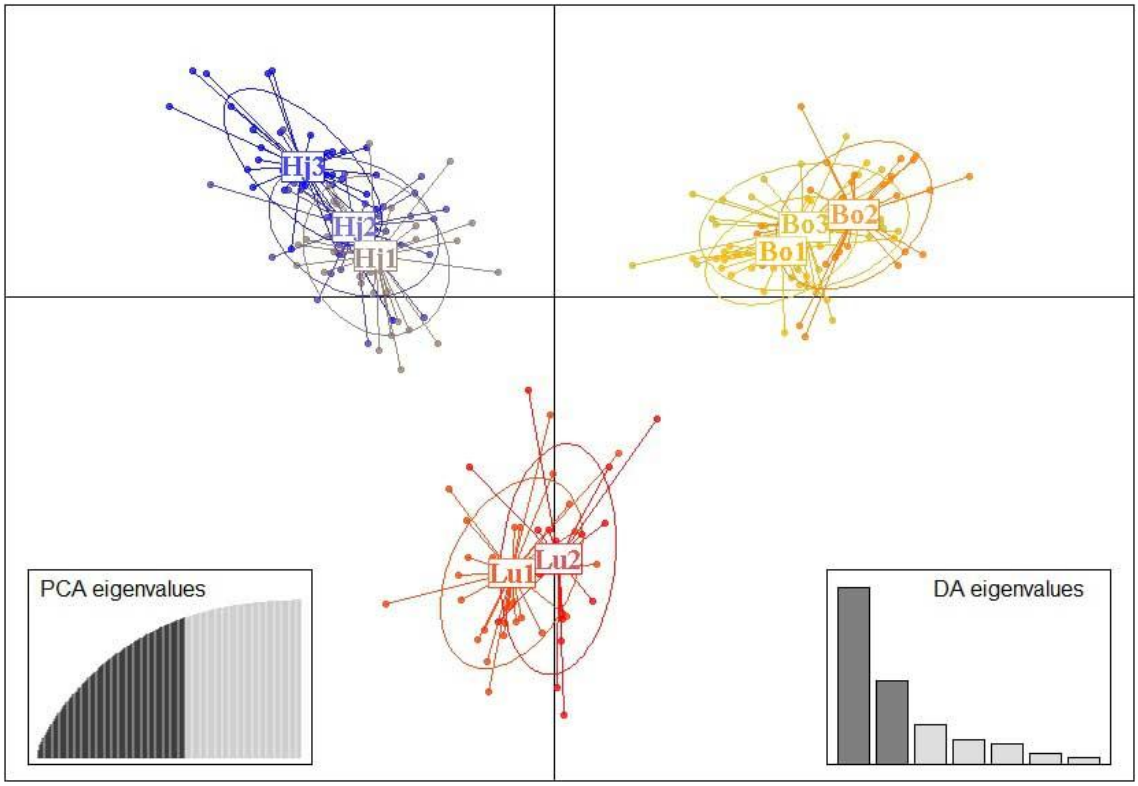

Figure 5. DAPC scatter plot, showing first and second discriminant axes.

Phylogenetic tree (Figure 6) shows clear segregation between Bøelva and rest of the samples, and milder one between Eidselva and Hjartdøla.

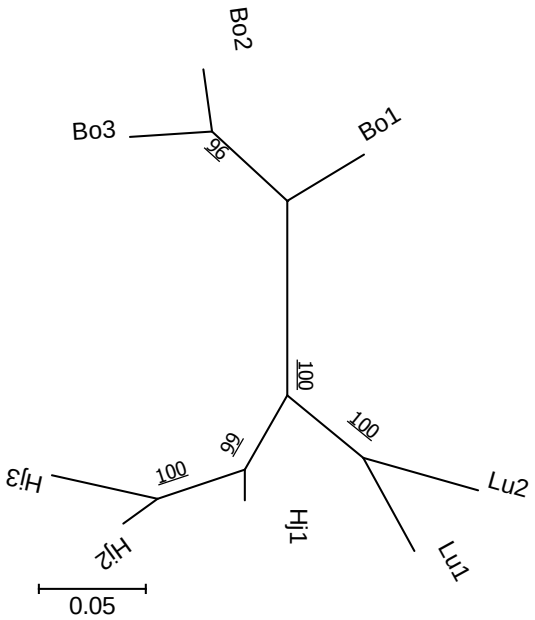

Figure 6. Unrooted neighbor joining phylogenetic tree based on Nei Da genetic distance with scale. Underlined numbers next to nodes are bootstrap values (%) after 1000 iterations.

3.6. Assignment tests

Assignment tests using frequency based method resulted in 58.2% of correctly assigned individuals to site where they were sampled, and 92.4% were assigned to sampling site within correct river. Results for individual sampling sites and rivers are in Table 5. Only 13.9% of individuals had assignment probability larger than 0.8 (20 at Hjartdøla, 7 at Bøelva and 6 at Eidselva).

Table 5. Results of assignment test using exclusion method by Cornuet et al. (1999) . Showing number of correct assignments (c.a.) for individual sites, and correct assignment of individual to river. Total number of samples (N) and percentage of correct assignments (%c.a.) are also presented.

Site/river	c.a.	N	% c.a.
Hj1	31	34	94.1
Hj2	7	29	24.1
Hj3	15	37	40.6
Bo1	17	24	70.8
Bo2	13	31	41.9
Bo3	19	30	63.3
Lu1	24	30	80
Lu2	12	22	54.5
Hjartdola	98	100	98
Boelva	79	85	92.9
Eidselva	42	52	80.8

4. Discussion

4.1. Main findings

Based on our results, we conclude there are three populations of brown trout present in our study area, one within each river. These populations seem to be composed by smaller subpopulations, where gene flow is a crucial factor for genetic variation. Geist et al. (2006) suggest that low density of host fish is not a limiting factor for freshwater pearl mussel populations, but suitable substrate is. During the post-parasitic phase of life cycle, freshwater pearl mussels are completely dependent on stable, high sediment quality substrate, where they spend a period of 5 years (Geist, 2010). We therefore conclude that our results are of relevance, as population connectivity within rivers provides larger dispersal area for freshwater pearl mussel glochidia than isolated populations. This results in access to more areas with suitable substrate for freshwater pearl mussel post-parasitic juveniles. Heggenes et al. (unpublished manuscript) reported genetic diversity of freshwater pearl mussel in our study area, expressed by allelic richness to be very high compared to previous studies. It might suggest that high gene flow of host population is favorable for freshwater pearl mussel. This may be valuable for freshwater pearl mussel conservation management plans, and choosing adequate site for introduction purposes in future.

4.2. Data quality

Overall genotyping error rate of 0.3%, witnessed in this study is acceptable for microsatellite data according to Tiedemann et al. (2012), so we can consider our data informative. Reported null allele/stuttering occurrence for Hj1(str73) and Lu2(bru7) was found unlikely to affect our results. Stuttering was found unlikely, as no signs of consistency across loci were present. Considering null alleles, reported reduction of

power to assign individuals correctly is 0.2% for Bayesian algorithm in STRUCTURE (Pritchard, 2000) and 2.4% for GENECLASS (Piry et al., 2004) assignment test and should not alter the overall outcome, but can affect Fst values (Carlsson, 2008). We therefore used adjusted allele frequencies (Brookfield, 1996) for loci*site combinations with indications of null allele presence to calculate second pairwise Fst matrix. The maximum difference between Fst for unadjusted and adjusted allele frequencies was 0.0034, thus concluding that null alleles presence is not altering our results.

4.3. Genetic variation

Genetic variation observed in our study was comparable with previous studies using same set of markers. Regarding markers, Wollebaek et al. (2010) reports lower total number of alleles observed (155 alleles; mean 11.9; SD ±8.9) whereas Kraabøl et al. (2015) reports slightly higher (172 alleles; mean 13.2; SD ±8.8) compared to our study (169 alleles; mean= 13; SD ±10.08). Within site allelic richness and observed heterozygosity witnessed in our study (Ar mean= 6.4, SD ±3.95; Ho mean= 0.64, SD ±0.22) were both in between values of Kraabøl et al. (2015) (Ar mean= 5.5, SD±3.0; Ho mean= 0.502, SD±0,213) and Wollebaek et al. (2010) (Ar mean= 7.1; SD±4.2; Ho mean= 0.65, SD±0.226). This suggests that genetic variation of brown trout within sites in our study is not exceeding previously reported values. Comparison of our results to genetic structure of freshwater pearl mussel in the same ecosystem (Heggenes et al., unpublished manuscript) showed convergence of genetic diversity within sites. Uppermost sites showed less genetic diversity (although not significantly in our study) for both species. This is in contradiction with results of Geist & Kueahn (2008), reporting genetic diversity being negatively correlated for this host-parasite system. Results of continuous populations of freshwater pearl mussel within river from our study area can be also linked to our findings, supporting our assumption of strong gene

flow. Site Bo3 was reported to differ significantly within river for freshwater pearl mussel data, but this was not observed in this study. It can be explained by the fact, that no further upstream populations of freshwater pearl mussel are present, while brown trout populations are, therefore potential for genetic drift is not as high for brown trout.

4.4. Population differentiation

Population differentiation between sampling sites based on Fst values showed a considerable amount of differentiation within rivers (Fst: 0.0261 – 0.1244) and even stronger differentiation (Fst: 0.1253 – 0.3917) between rivers. Population differentiation was supported by results from AMOVA. Mantel test for IBD showed significant results. IBD was already observed for brown trout (Carlsson & Nilsson, 2000; Linløkken et al., 2014). We addressed the IBD further by mantel correlogram, because global mantel test can be biased (Diniz-Filho et al., 2013). Also occurrence of waterfalls in study drainages, which is most relevant in our study, has been reported to affect the global mantel test for IBD in brown trout genetic structure study (Carlsson et al., 1999). In mantel correlogram (figure 2), positive autocorrelation in fourth class included two pairs of sampling sites approachable through lake and three pairs with one waterfall. This indicated that with lack of sample site pairs separated by waterfall/s, geographical distance alone is not increasing with genetic distance in such a strong manner, as supposed by the global IBD mantel test. Therefore we cannot conclude that isolation by distance is present, as isolation by migration barriers could be interfering with genetic distances, suggested by positive autocorrelation for pairs of sites without waterfall in between.

4.5. Clustering

Segregation of our samples into clusters was addressed by several methods. Phylogenetic tree based on Nei`s Da genetic distance (Nei et al., 1983), which is

preferable for constructing correct topology of the tree (Takezaki & Nei, 1996), showed clear separation into two clusters, separating Bøelva river samples from others, and a milder separation between Eidselva and Hjartdøla river and between Bo1 site and rest of Bøelva sites (figure 6). DAPC (Jombart et al., 2010) showed clear separation of clusters between rivers, with no indication of structuring within them (figure 5). Using STRUCTURE software (Pritchard, 2000; Falush et al. 2003), the linear probability of data suggested three clusters, while method by Evanno et al. (2005) suggested two clusters. It was found, that Evanno method frequently reports k=2, even when more clusters are present (Janes et al., 2017) and further segregation of our data based on assignment to the two clusters, indicated structuring in one of them, resulting in k=3. Also it is recommended to report results for a range of clusters using structure, as biological interpretation can be valuable for more than a single k (Meirmans, 2015). Results of individual assignment to clusters based on a bayesian algorithm (figure 4), shows clear segregation of Bøelva river from the rest for k=2, and is supported by the Nei`s Da (Nei et al., 1983) neighbor-joining (Saitou and Nei 1987) phylogenetic tree topology. When k=3, rivers got separated into clusters, with admixture present at Bo1 and Hj1. Further increase in number of clusters to k=4 showed clear separation of rivers, while two upstream sites at Bøelva and Hjartdøla, remained clearly separated, rest of sites showed admixture, being mostly site Hj1. Results from the assignment test in Geneclass2 software (Piry et al., 2004), showed an average of 58.2 % of correct assignments to sampling site, and average of 92.4% for correct assignment to river, suggesting continuous populations within rivers, and supported by previous results. Site Hj1 showed 94.1% of correct assignments of individuals to site, indicating that separate population may be present, and supported by admixture presence considering Structure results, but is not supported by pairwise Fst with neighboring site within river (Hj2), showing lowest observed Fst value (θ: 0.0261) of all pairs. Based on our clustering

results, we concluded that there are three populations, one within each river, but based on AMOVA, within river pairwise Fst, mantel correlogram and admixture in structure result, we suspect that they are composed by smaller subpopulations where gene flow is of most importance considering genetic variation. Similar cases of structuring were previously reported for brown trout populations (Palm et al., 2003, Jensen et al., 2006). Studies also proved population differentiation in smaller scale, such as within lakes (Wollebaek et al., 2018) and within rivers (Carlsson & Nilsson, 2000). We recommend to treat rivers as separate populations, for management and conservation purposes, as was suggested by Hansen et al. (2007).

4.6. Reccomendations for further research

Recent bottleneck suggested for site Hj2, also supports our assumption of small populations, with high genetic drift, but should be investigated by further sampling at this site, to confirm our results and account for possible sampling error based on only one sampling session. Further research is also recommended for higher resolution population structure study in our study area, as underlying structers are likely to be found within rivers. As exhaustive sampling is expected, we recommend to limit the research to one of the drainages. Preferably Hjartdøla or Bøelva River.

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