Genetic Evaluation of the Breeding Structure of the Atlantic Salmon Population of the Farrar River

Project Report, Years 1-3

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Background and Objectives

Contemporary molecular methods enable us to examine the population genetic characteristics of Atlantic salmon in detail. These methods have revealed, for example, that salmon stocks in larger rivers may be subdivided into genetically distinct, locally adapted sub-populations, and that a small number of genes may have large effects on salmon life histories (e.g. *vgll3*, associated with age-at-maturity and *six6* associated with seasonal migration timing; Pritchard *et al.* 2018). The patchy distribution of salmon spawning habitat, and the possibility that adult salmon might return to the exact spawning grounds means that there is potential for salmon populations to be sub-structured even at a very fine geographic scale.

These molecular genetic methods also make it possible to infer the number of adult salmon that have produced a sample of juvenile salmon taken from the river, by identifying siblings and half-siblings among the juveniles. This approach can be applied to estimate the relative differences in the sizes of the spawning stocks in different parts of a river and to monitor how they change from year to year, and may provide a more consistent and accurate assessment of variations in spawner abundance than can be achieved using fish counters or catch data. It also enables estimation of 'effective population size' (N_e) – an important parameter which is related to a population's ability to maintain its viability and genetic diversity over different time frames (Waples 2024).

The aim of this project is to examine the population genetic structure, number of breeders, and distribution of potentially functional genetic variation in the River Farrar, a tributary of the River Beauly in north eastern Scotland. The Farrar supports a salmon population mostly consisting of grilse (1SW salmon: adults that mature early and return to spawn after only one year at sea), with a range of seasonal return timing. The Farrar is divided by natural and hydro induced habitat heterogeneity into a number of distinct and potentially biologically different spawning habitats.

Specific objectives of the project are:

- To assess the number of spawning groups in the Farrar, the relative number of spawners present in each section of the river and in each spawning group, and the effective population size of Farrar salmon.
- To assess whether long-term genetic sub-structuring is present in the population and how this associates with different postulated spawning areas. Of particular interest is whether the hydropower dam at Braulen structures the breeding population.
- To assess the population variability at known functional genes associated with age at maturity and adult migration timing.

- To use the information to define a framework for genetic monitoring of the health of Farrar Atlantic salmon.

Sampling

Tissue samples were collected from juvenile Atlantic salmon in the Farrar by the Beauly District Fishery Board and associates of the landowners, following a previously developed sampling plan. All electrofishing and tissue collection was performed following standard protocols under required licences and permissions. Briefly, 0+ aged fry (hatched in the same year) were caught by electrofishing in August and September 2021, 2022 and 2023, at the sample sites shown in Figure 1 and Table 1. Each fish was anaesthetised, a sample of approximately 2 x 2 mm was taken from the caudal fin, the fish was allowed to recover and returned to its capture location. Tissue samples were preserved in pre-labelled tubes containing 70% ethanol and these were sent to the IBFC laboratory for genetic analysis.

DNA extraction and genotyping-by-sequencing

All samples were processed in 96-well plates with three 'blank' control wells (containing no salmon tissue) on each plate. DNA was extracted from approximately 2mm^2 of each fin clip using HotSHOT alkaline lysis (Truett *et al*. 2000). DNA concentration was measured by spectrophotometry using the QiaExpert system and diluted with 10mM Tris to a standard concentration of 10ug/µl using a QIAgility liquid handling robot. Each sample was genotyped for a panel of genetic markers: 88 short tandem repeats ('microsatellites'), one genetic sequence on the male sex determining locus, and two single nucleotide polymorphisms (SNPs), each linked to a known functional gene in Atlantic salmon (*six6* and *vgll3*). Markers were amplified in two separate multiplex PCR reactions containing the following: 3µl 2x Qiagen Type-IT multiplex master mix, 0.3µl primer multiplex mix (45 or 46 primer pairs at a mean concentration of 1µM per primer), 2.7µl diluted DNA. Thermocycling conditions were: 95°C for 15min, 25x [94°C 30s, 57°C 3min, 72°C 30s], 72°C for 10min. The two sets of PCR products were pooled for each sample and diluted 40x with water. Six to eight 96-well plates were combined for each DNA sequencing run. Sample-specific forward and reverse index combinations and Illumina sequencing tags were added to each sample (including blanks) in 5µl PCR reactions using the following protocol: PCR mix - 2.35µl H_2O, 0.5µl 10x buffer, 0.25U Taq DNA polymerase, 0.1µl dNTPs (10µM each), 1µl forward and reverse index mix (1µM per index); 1µl diluted multiplex PCR product; thermocycler conditions - 98°C for 2 min, 20x [98°C 10s, 62°C 30s, 72°C 15s], 7C for 10 min. Product for all samples was pooled into a single library, and purification and fragment size selection was performed using Agencourt AmPure XP beads. The concentration of the pooled library was measured using a KAPA library quantification kit on the Agilent AriaMX RT-PCR system and standardized. Each pooled library was single-end sequenced on an Illumina MiSeq using Illumina V3 sequencing chemistry (150 cycles), with sequence reads demultiplexed to individual samples on the basis of their sample-specific indices and output in fastq format.

Statistical analysis

Microsatellite genotypes were called from DNA sequence reads using MEGASAT (Zhan *et al.* 2017), using an IBFC standard pipeline. For sexing and SNP calling, sequence reads were trimmed using cutadapt and aligned to the Atlantic salmon reference genome (ICSASG_v2) using BWA-mem (Li *et al.* 2013), with variants called at the target site using SAMtools mpileup (Li *et al.* 2009). Genetic sex was assigned according to the ratio of sequence read counts from the male determining locus (present in males only) to sequence read counts across the two SNPs (present in both sexes; thresholds: < 0.03 = female; > 0.25 = male; otherwise inconclusive).

Brown trout or first-generation trout-salmon hybrids in the dataset were identified from a known combination of non-amplification of certain microsatellite loci with brown-trout specific alleles at other loci. The package rubias (Moran & Anderson 2018) was used in R 4.0.3 (R Core Team 2020) to check for the presence of genetically identical samples (i.e those taken from the same individual fish). Finally, any remaining fish with > 25% missing data and any genetic marker with >10% missing data was removed from the analysis.

The software COLONY 2.0.6.6 (Jones & Wang 2010) was used to infer family structure among the genotyped juveniles and so infer the number of breeders that produced them. COLONY uses a maximum likelihood approach to infer sibling relationships from shared genetic variation, taking into account possible genotyping error. As the default assumption was that each juvenile was truly 0+ and therefore the product of a spawning event in the previous year we first reconstructed pedigrees separately for 2021, 2022 and 2023. The following parameters were applied: probability of allele drop out 0.001 and other errors 0.001 for all loci; allele frequency not updated; diecious parents; polygamy for both sexes; full sibship scaled; medium sibship prior with an average maternal and paternal sibship size of 2; unknown population allele frequency; combined pairwise likelihood and full likelihood (FLPS) algorithm with medium run length and medium precision. We performed three replicate runs with independent random seeds, and accepted the replicate which returned the largest number of independent parents. Subsequently, we used the same approach to reconstruct a pedigree for the entire three-year juvenile dataset.

We also used COLONY to estimate effective number of breeders ($N\text{b}_{\text{sib}}$). This is a standardized measure of the number of adults contributing to the sampled juvenile cohort which can be used as an approximation of effective population size (N_e) that is directly linked to the 'genetic health' of the population. It is generally lower than the actual number of breeders.

To investigate how number of inferred parents was influenced by number of juveniles sampled, we also used COLONY to reconstruct parentage from random sub-samples of all juveniles collected in 2021, 2022, 2023. We subsampled 100, 150, 200, 250 and 300 fish and inferred family structure using the same parameters as above. Two replicate sets of subsamples were analysed for each year.

The presence of population genetic substructure within the Farrar – for example that which could emerge as a result of fine-scale homing to specific spawning sites within a river, was assessed using the programme STRUCTURE (Pritchard *et al.* 2000). As the presence of sibling groups in a dataset can generate a false signal of genetic substructure, we retained only one offspring from each inferred parent. We combined these juveniles from the three sampling years (n=380) and ran STRUCTURE allowing 1 - 10 possible genetic clusters with the following parameters: admixture model with correlated allele frequencies, no prior population information, 50,000 burn-in followed by 100,000 MCMC reps; all other parameters default. We determined the most likely number of clusters from the joint values of log likelihood and delta k (Pritchard *et al.* 2000; Evanno *et al.* 2005)

The gene *vgll3* has been shown to strongly influence age-at-maturity (i.e. age and size of salmon returning to freshwater) across a wide range of European Atlantic salmon populations in the Atlantic, and for many populations its influence varies by sex (Barson *et al.* 2015, Ayllon *et al.* 2015, Miettinen *et al*. 2024). Similarly, the gene *six6* is strongly associated with the seasonal timing of the adult return migration in both Scottish rivers and further afield (Cauwelier *et al.* 2017, Pritchard *et al.* 2018), as well as interacting with *vgll3* in a complex manner to also influence age-at-maturity (Besnier *et al*. 2022). We examined allelic variation at the two SNPs closely linked to each of these genes to assess the potential of the Farrar population to produce salmon exhibiting a range of genetic maturity and/or migration timing.

Figure 1. Juvenile sampling sites along the River Farrar. Black bars show the location of hydropower dams and light blue bars the location of natural barriers to migration. FAR_01 comprised two sampling locations in the same tributary.

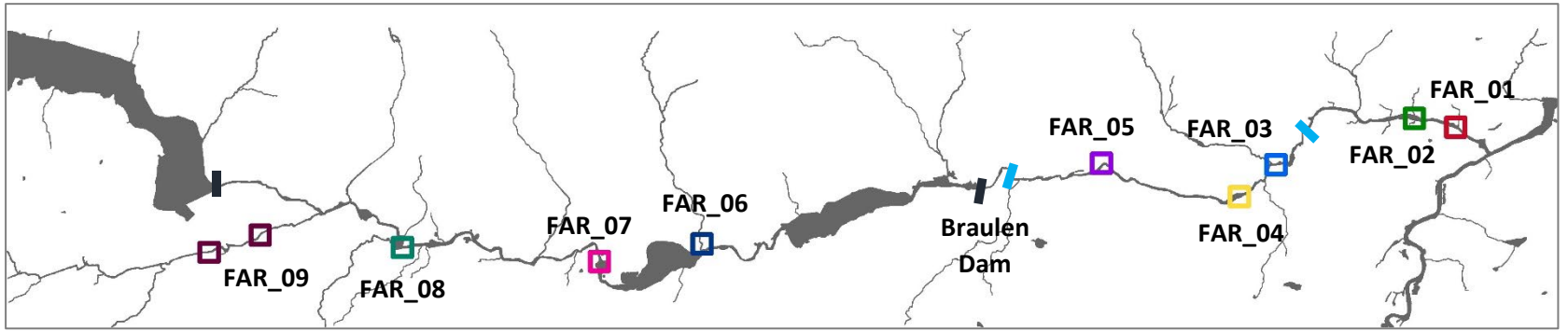


Table 1. Samples collected and samples retained following different quality control steps.

Year	Site	Site Description	Easting	Northing	#Sampled	#Trout	#Failed QC	#Analysed
2021	FAR_01	DS Struey_Bridge	240230	840345	26	0	0	26
2021	FAR_02	NEPS21_0293	239569	840491	20	0	0	20
2021	FAR_03	NEPS21_0289	237349	839719	20	0	0	20
2021	FAR_04	NEPS21_0273	236760	839185	20	0	0	20
2021	FAR_05	FAR2 historic site	234549	839743	45	0	0	45
2021	FAR_06	Allt coire Mhuillidh	228147	838397	51	13	0	38
2021	FAR_07a	Above Loch a'Mhullidh	226501	838104	45	4	1	40
2021	FAR_07b	Above Loch a'Mhullidh	226583	838095				
2021	FAR_08	NEPS21_0290	223340	838337	50	0	1	49
2021	FAR_09a	Uisge Misgeach	220238	838257	60	10	0	50
2021	FAR_09b	Uisge Misgeach	221060	838545				
2021 TOTAL					**337**	**27**	**2**	**308**
2022	FAR_01	DS Struey_Bridge	240227	840362	25	0	0	25
2022	FAR_02	NEPS21_0293	239567	840495	25	0	0	25
2022	FAR_03	NEPS21_0289	237349	839719	21	0	0	21
2022	FAR_04	NEPS21_0273	236760	839185	20	0	0	20
2022	FAR_05	FAR2 historic site	234570	839720	45	0	0	45
2022	FAR_06	Allt coire Mhuillidh	228142	838375	50	10	2	38
2022	FAR_07a	Above Loch a'Mhullidh	226501	838104		0	3	50
2022	FAR_07b	Above Loch a'Mhullidh	226583	838095	53			
2022	FAR_08	NEPS21_0290	223340	838337	50	0	0	50
2022	FAR_09a	Uisge Misgeach	220223	838255	61	1	0	60
2022	FAR_09	Uisge Misgeach	220269	838263				
2022	FAR_09b	Uisge Misgeach	221060	838545				
2022 TOTAL					**350**	**11**	**5**	**334**
2023	FAR_01	DS Struey_Bridge	240227	840362	26	0	1	25
2023	FAR_02	NEPS21_0293	239567	840495	30	0	1	29
2023	FAR_03	NEPS21_0289	237402	839716	20	0	0	20
2023	FAR_04	NEPS21_0273	236760	839185	21	0	0	21
2023	FAR_05	FAR2 historic site	234570	839720	45	0	0	45
2023	FAR_06	Allt coire Mhuillidh	228142	838375	24	1	0	23
2023	FAR_07a	Above Loch a'Mhullidh	226501	838104				
2023	FAR_07b	Above Loch a'Mhullidh	226583	838090	38	0	1	37
2023	FAR_08	NEPS21_0290	223340	838337	50	0	1	49
2023	FAR_09a	Uisge Misgeach	220223	838255				
2023	FAR_09	Uisge Misgeach	220269	838263				
2023	FAR_09b	Uisge Misgeach	221060	838545	60	1	0	59
2023 TOTAL					**314**	**2**	**4**	**308**

Results

A total of 1,001 juvenile salmonids were sampled from eleven electrofishing sites in the Farrar (Figure 1; Table 1) across the three sampling years. Two pairs or trios of geographically close sampling sites (in Uisge Misgeach and above Loch a'Mhullidh) were combined and treated as single sites for data analysis. All samples were put through the genotyping process. Two pairs of genetic duplicates were observed (monozgotic twins or repeat sampling of the same fish - both collected in 2022), and forty samples corresponded genetically to brown trout. Duplicates and trout were removed from the dataset. Nine additional samples failed genotyping quality control, leaving 308 (from 2021), 334 (2022) and 308 (2023) juvenile Atlantic salmon for analysis.

The inferred sexes and familial relationships among the genotyped juvenile salmon, assuming that all were correctly aged as 0+ fish, are shown in Figures 2a, 2b and 2c. Based on the observed genotypes, replicate COLONY runs estimated that between 243 and 248 parents spawned in 2020 to produce the juveniles sampled in 2021, 270-272 parents produced the juveniles sampled in 2022, and 255-260 parents produced the juveniles sampled in 2023.

Across all years, most inferred parents produced juveniles at a single sampling site (72.8%, Table 2) or two neighbouring sampling sites (not including sites separated by Braulen Dam, 11%). However, 10.9% of inferred parents produced juveniles sampled both above and below the Dam. Each year slightly fewer parents were inferred from the juveniles sampled above the Braulen Dam than from juveniles sampled below (2021: 40% vs 48%; 2022: 46% vs 47%; 2023: 37% vs. 48%).

The effective number of breeders ($N_{b(sib)}$) was estimated by COLONY to be 173 in 2021 (95% confidence interval 140-213), 186 in 2022 (95% CI: 150-228), and 196 in 2023 (95% CI: 158-244)

When the juveniles from all sampling years were combined, COLONY inferred that they were produced by 766 separate parents. Of these, 141 parents (18.4%) were linked to juveniles sampled in >1 year. For 58 (41%) of these parents, their inferred offspring were caught at the same or adjacent sites in all years; for the remaining 83 (69%), their offspring were caught at sites further apart. Thirty-nine parents were linked to juveniles caught above the dam in one year, but below the dam in another year. Estimated ($N_{b(sib)}$) across the three years was 560 (95% CI: 500-633).

The STRUCTURE analysis based on the 380 non-sibling fish from 2021-23 found the most likely number of genetic clusters to be one. Thus. there was no evidence for long-term genetic sub-structuring associated with different parts of the river.

COLONY parentage reconstruction using random subsamples of the juveniles from each year showed that inferred number of parents increased linearly as the number of sampled juveniles also increased, with no suggestion of an asymptote (Figure 3). Thus, we expect the number of parents above to be an underestimate of the actual number of salmon that are successfully producing juveniles in the Farrar.

We observed genetic variation within the juveniles for both *vgll3* and *six6* linked SNPs (Table 4). While there was no pattern in allele frequencies at the *vgll3* marker across the sampling sites, we observed an increase in frequency of early-migration associated *six6* allele from the downstream to the upstream sites.

Figure 2a. Reconstructed pedigrees of juveniles sampled along the Farrar in 2021. Coloured points represent sampled juveniles, with colour indicating collection site as on the Figure 1 map; they are connected by lines to their inferred parents. The shape of the point indicates the genetic sex of each sampled juvenile, inferred from the presence or absence of the male sex determining locus. We cannot reconstruct the sexes of the parents, however parents represented by different shades of grey within each mating group are assumed to be different sexes.

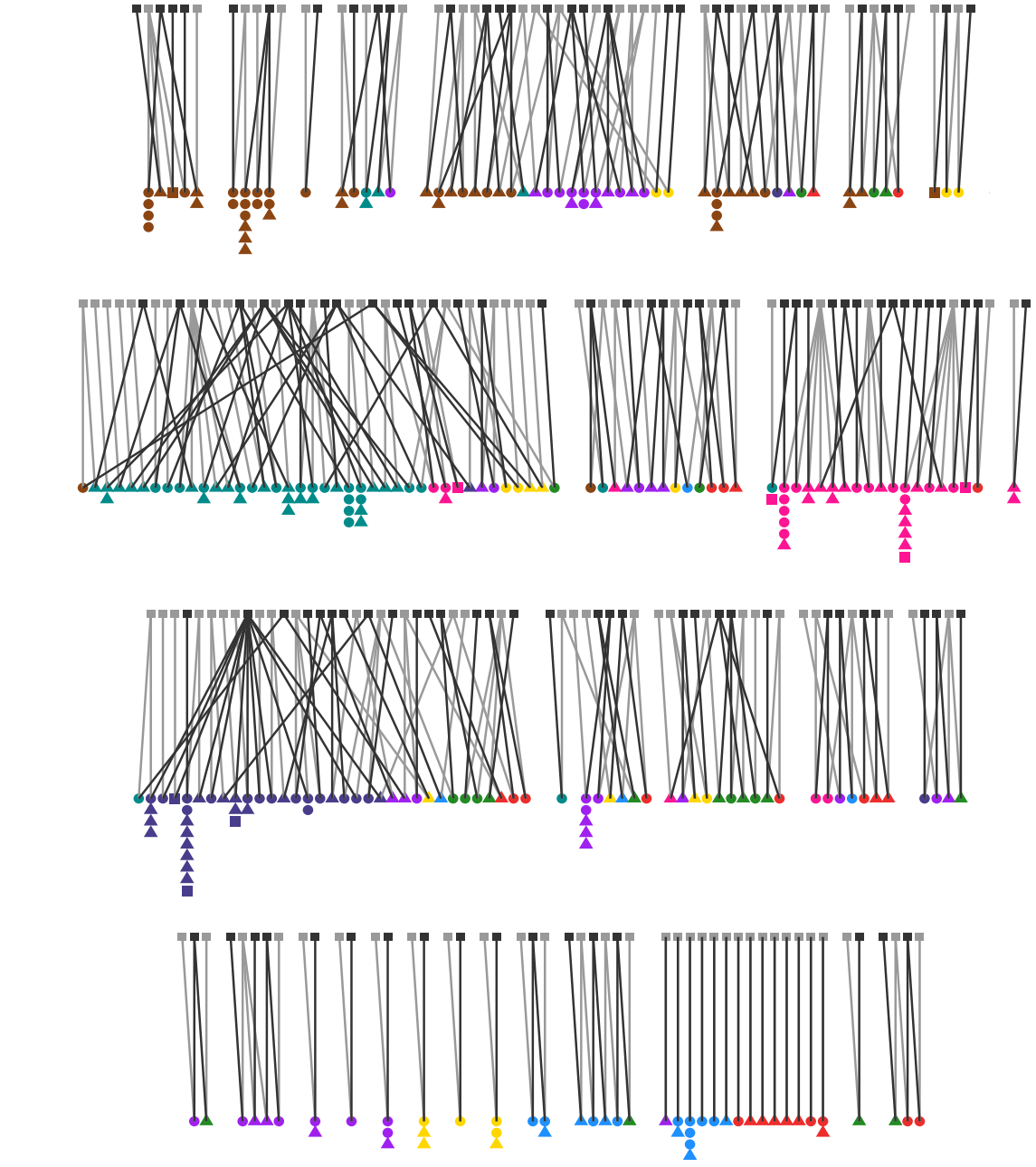
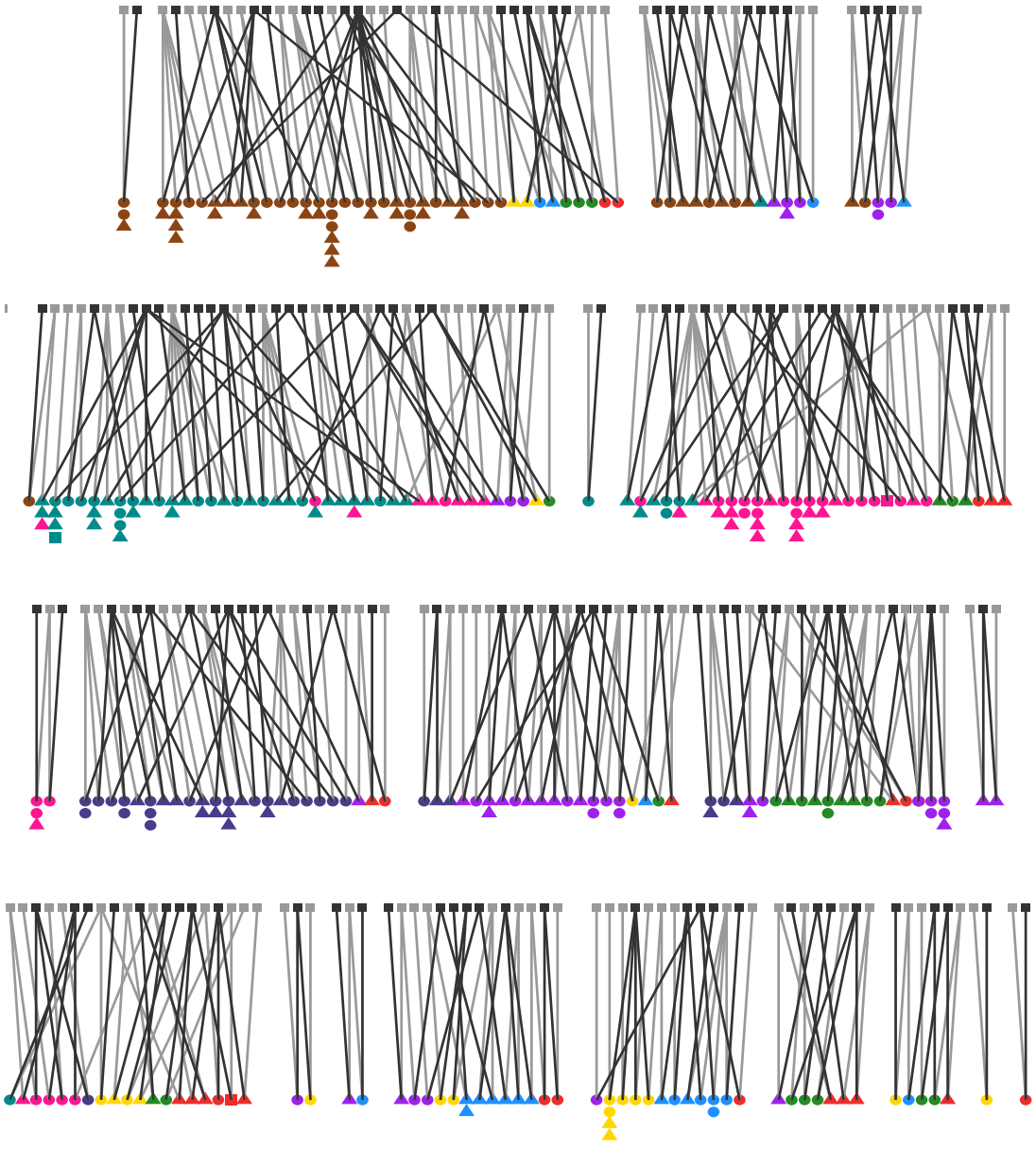


Figure 2b. Reconstructed pedigrees of juveniles sampled along the Farrar in 2022. See Figure 2a for further explanation and Figure 2c for figure legend.



Sampling sites above Braulen Dam

FAR_09 FAR_08 FAR_07 FAR_06

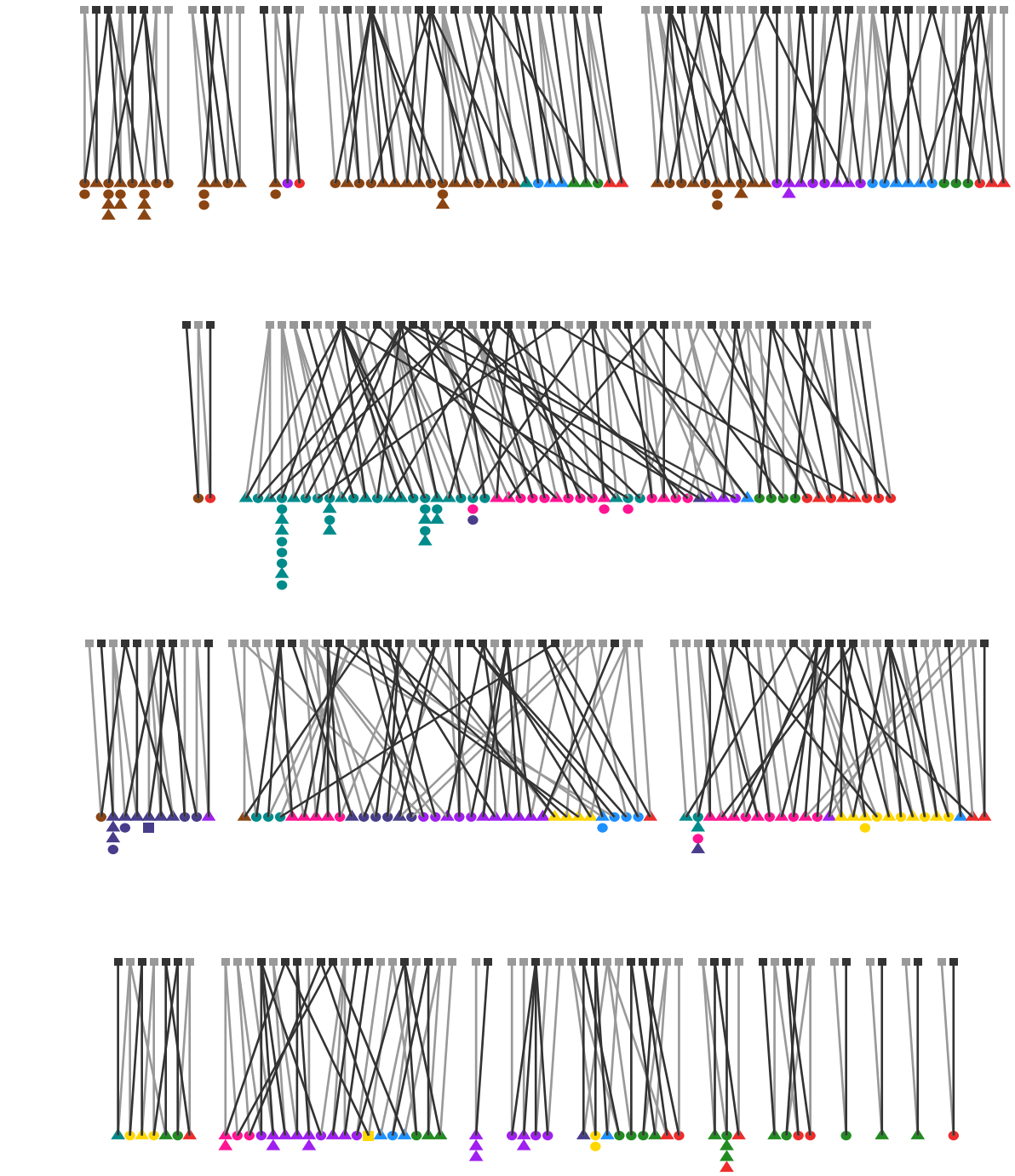
Sampling sites below Braulen Dam

FAR_05 FAR_04 FAR_03 FAR_02 FAR_01

Genetic sex of offspring

Female Male Unknown

Figure 2c. Reconstructed pedigrees of juveniles sampled along the Farrar in 2023. See Figure 2a for further explanation.



Discussion

The results of this study suggest that a relatively healthy Atlantic salmon population is present in the Farrar River, with a minimum of 248-272 breeding adults per year inferred from sibship reconstruction among the 0+ sampled juveniles. The estimated effective number of breeders per year, a proxy for 'effective population size' ranged from 173-196. As a 'rule of thumb' a closed population with an effective size >50 but <500 is considered at low risk from problems associated with inbreeding (Frankham *et al.* 2014) but without migrants from other areas it is expected to lose genetic variation and therefore adaptive potential over time as a result of 'genetic drift'. Note that, for species such as salmon where juveniles spawned in one year return to breed in many different years, the relationship of Nb_{sib} to the true effective population size is not straightforward (Waples, 2024).

We expect the true number of breeders and Nb_{sib} of the Farrar population to be higher than these estimates, as the number of distinct parents identified is limited by the number of juveniles sampled. In practice, in species such as salmon where most adults reproduce only once, it is impossible to discover the true adult population size from reconstruction of juvenile sibships (Waples & Feutry 2021). Our ability to obtain a reliable approximation is also limited by the practicality of sampling and genotyping large numbers of juveniles and the non-random distribution of families along the river. A more accurate picture of the number of breeding adults might be obtained through 'parent-offspring close-kin mark-recapture' (Waples & Feutry 2021), whereby genetic samples (e.g. scales, small fin clips or mucus swabs) are taken from captured adults in one year and their possible 0+ offspring in the following year. The breeding adult population size is then estimated by comparing the number of adults that are 'recaptured' – identified as parents of the juveniles to the total number of parents inferred from the juvenile analysis.

Although most individual salmon reproduce in a single spawning location, we found some juveniles that were sampled at different sites – sometimes a substantial distance apart - but shared a parent. This may be attributable to three factors: i) movement of juveniles between hatching and sampling; ii) repeated spawning of some returning adults at multiple spawning locations, or iii) incorrect family reconstruction by the COLONY algorithm. Although we cannot completely discount (iii), we consider it likely that some 0+ juveniles may have moved among adjacent sampling sites between hatching and sampling, and also that a few individuals may stop off to spawn in lower river sections on their way upstream. Surprisingly many parents were also linked to juveniles sampled in two or more different years. Although incorrect family reconstruction may once again be a factor, this may also be due to a combination of repeat spawners (both returning adults and precocious male parr) and inclusion of some 1+ or even 2+ fish in the sample of putative 0+ juveniles.

Despite the strong clustering of families within different sampling areas in the Farrar, we found no overall evidence for long-term, fine-scale genetic sub-structuring such as could arise if generations of salmon return to spawn to their exact natal location. This is supported by our observation that some parents are linked to juveniles caught in very different parts of the river, both within and across years.

The Farrar Atlantic salmon population is genetically variable at two loci with known large effects on salmon life history in Scotland and/or continental Europe – *vgll3* and *six6*. Both of these are associated with age-at-maturity and adult migration timing, with *six6* being particularly strongly associated with early vs. late run timing in Scotland. *Vgll3* has a sex-specific effect in many populations meaning that males carrying one version of each allele type return as grilse while females with the same genotype return as multi-seawinter salmon. As expected in a population dominated by grilse, the older-age-at-maturity *vgll3* allele is at relatively rare in the Farrar; nevertheless, it is present, meaning that the population retains its genetic capacity to produce later-maturing, larger fish. Similarly, both the 'earlier-migration' and 'later-migration' alleles are present at *six6*, with the 'earlier migration' allele tending to be more frequent in the upper parts of the Farrar which were previously known to have a Spring salmon run. We caution that salmon age-at-maturity and migration timing are also affected by numerous other genes with smaller effects, as well as the freshwater and marine

environment. Thus, these *vgll3* and *six6* genotypes should not be considered absolutely predictive of the eventual adult size and migration timing of the sampled juveniles.

Figure 2: Number of parents inferred from random subsamples of juveniles.

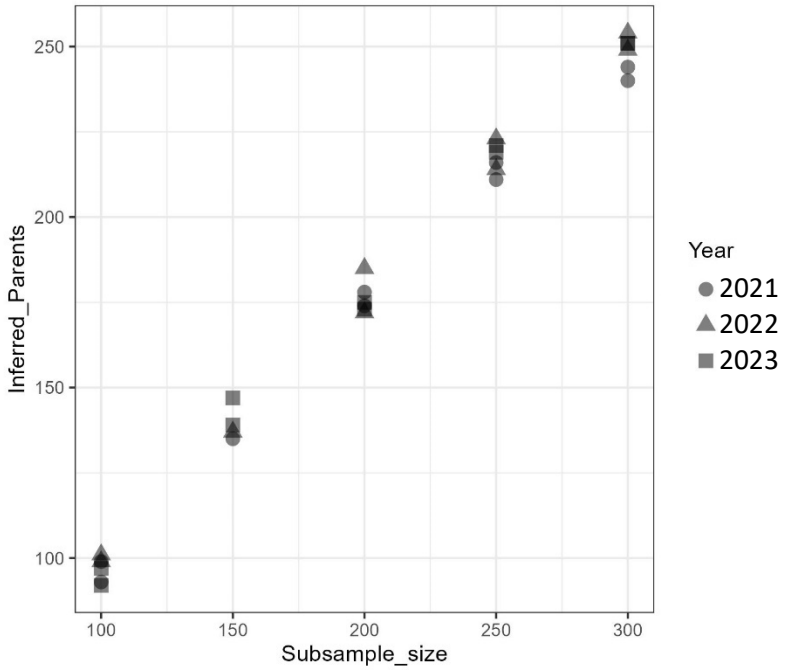


Table 2: Distribution of the inferred parents across the sampling sites.

Offspring location	2021		2022		2023	
Below dam - single location	100	40.3%	91	33.5%	94	36.2%
Below dam - adjacent locations	10	4.0%	21	7.7%	22	8.5%
Below dam - non-adjacent locations	10	4.0%	17	6.3%	10	3.8%
Above dam - single location	92	37.1%	108	39.7%	83	31.9%
Above dam - adjacent locations	6	2.4%	16	5.9%	11	4.2%
Above dam - non-adjacent locations	2	0.8%	0	0.0%	2	0.8%
Above & below dam	28	11.3%	19	7.0%	38	14.6%
Total number of inferred parents	**248**		**272**		**260**	

Table 3: Observed frequencies of the *six6* allele linked to earlier adult return migration timing and the *vgll3* allele associated with older age-at-maturity (i.e. grilse vs. salmon) across sites and years.

	FAR01	FAR02	FAR03	FAR04	FAR05	FAR06	FAR07	FAR08	FAR09
***Six6* earlier 2021**	0.66	0.68	0.58	0.78	0.74	0.72	0.79	0.67	0.76
***Six6* earlier 2022**	0.66	0.72	0.69	0.55	0.7	0.71	0.67	0.74	0.89
***Six6* earlier 2023**	0.56	0.63	0.68	0.83	0.74	0.77	0.69	0.78	0.78
***Vgll3* older 2021**	0.18	0.15	0.08	0.23	0.09	0.08	0.11	0.10	0.07
***Vgll3* older 2022**	0.14	0.06	0.12	0.20	0.10	0.14	0.08	0.21	0.16
***Vgll3* older 2023**	0.07	0.05	0.05	0.08	0.10	0.03	0.04	0.14	0.16

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