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

Final Project Report, Years 1-4.

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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 provide a complementary measure of minimum spawner abundance to that which can be obtained using fish counters or catch data. It also enables estimation of 'effective number of breeders' (N_b) per year, an important parameter linked to per-generation effective population size (N_e) and 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 hydropower-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 spawners producing sampled juveniles in each location, and the effective number of breeders per year.
- 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.

Sampling

Tissue samples were collected from juvenile Atlantic salmon in the Farrar by the Beauly District Fishery Board and volunteers, following a previously developed sampling plan. All electrofishing and tissue collection was performed following standard protocols under required licences and permissions. Briefly, juveniles of sizes corresponding to 0+ aged fry (hatched in the same year) were caught by electrofishing in August and September 2021 -2024, 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 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: 87 short tandem repeats ('microsatellites'), nine single nucleotide polymorphisms (SNPs) – two of which are linked to a known functional gene in Atlantic salmon (*six6* and *vgll3*) - and one genetic sequence on the male sex determining locus. 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 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 nine SNPs.

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 >15% 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. Previously, we have inferred family structure separately for juveniles collected in different years. However, for this final report we performed a single family reconstruction for all genotyped individuals. This enabled the algorithm to use all genetic information available while accounting for possible repeat spawning events and misidentification of parr as fry. We also slightly adjusted COLONY parameters from previous years. Specifically, guided by an analysis of individuals with known parentage that indicated that COLONY can underestimate the true number of parents, and that assuming a higher average sibship size can make this underestimation worse, we changed our prior for maternal and paternal sibship sizes from 2.0 to 1.0. 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; weak sibship prior with an average maternal and paternal sibship size of 1; unknown population allele frequency; combined pairwise likelihood and full likelihood (FLPS) algorithm with medium run length and medium precision.

We also used COLONY to estimate effective number of breeders per year ($N\mathrm{b}_{sib}$). This is a standardized measure of the number of adults contributing to the sampled juvenile cohort. It is directly related to effective population size per generation (N_e), an important genetic parameter which can be thought of as an indicator of the 'genetic health' of a population. The smaller the N_e of a population, the faster genetic diversity is expected to be lost over time and the more likely inbreeding is to occur.

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 four sampling years (n=348) 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. To avoid large full-sibling families influencing the results, all but one fish from each full-sibling group was removed before allele frequencies were estimated.

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_07 and FAR_09a combine two closely adjacent sampling locations.

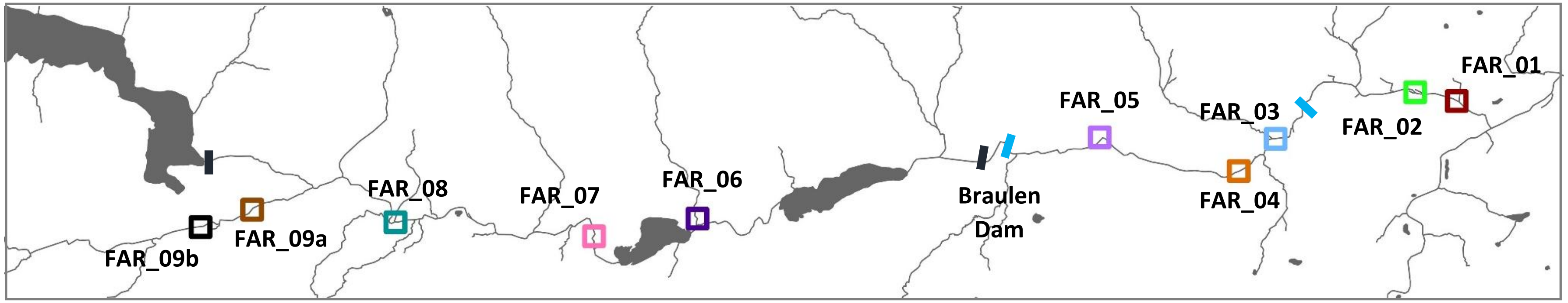


Table 1. Samples collected and samples retained following different quality control steps.

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		226583	838095				
2021	FAR_08	NEPS21_0290	223340	838337	50	0	1	49
2021	FAR_09a	Uisge Misgeach	221060	838545	22	5	0	17
2021	FAR_09b	Uisge Misgeach Top	220238	838257	38	5	0	33
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	1	39
2022	FAR_07a	Above Loch a'Mhullidh	226501	838104	53	0	2	51
2022	FAR_07b		226583	838095				
2022	FAR_08	NEPS21_0290	223340	838337	50	0	0	50
2022	FAR_09a	Uisge Misgeach	221060	838545	20	1	0	19
2022	FAR_09b	Uisge Misgeach Top	220223	838255	41	0	0	41
2022			220269	838263				
2022	**TOTAL**				**350**	**11**	**3**	**336**
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	38	0	1	37
2023	FAR_07b		226583	838090				
2023	FAR_08	NEPS21_0290	223340	838337	50	0	1	49
2023	FAR_09a	Uisge Misgeach	221060	838545	20	1	0	19
2023	FAR_09b	Uisge Misgeach Top	220223	838255	40	0	0	40
2023			220269	838263				
2023	**TOTAL**				**314**	**2**	**4**	**308**
2024	FAR_01	DS Struey_Bridge	240227	840362	31	0	0	31
2024	FAR_02	NEPS21_0293	239567	840495	30	0	0	30
2024	FAR_03	NEPS21_0289	237402	839716	32	0	0	32
2024	FAR_04	NEPS21_0273	236760	839185	27	0	0	27
2024	FAR_05	FAR2 historic site	234570	839720	50	0	2	48
2024	FAR_06	Allt coire Mhuillidh	228142	838375	25	0	0	25
2024	FAR_07a	Above Loch a'Mhullidh	226501	838104	65	0	2	63
2024	FAR_07b		226583	838090				
2024	FAR_08	NEPS21_0290	223340	838337	55	0	6	49
2024	FAR_09a	Uisge Misgeach	221060	838545	30	0	0	30
2024	FAR_09b	Uisge Misgeach Top	220223	838255	15	0	1	14
2024			220269	838263				
2023	**TOTAL**				**360**	**0**	**11**	**349**

Results

A total of 1,361 juvenile salmonids were collected from ten electrofishing sites in the Farrar (Figure 1; Table 1) across the four sampling years. Two pairs of geographically close sampling locations (in Uisge Misgeach: FAR_09a and above Loch a'Mhullidh: FAR_07) 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, trout, and 18 additional samples with >25% missing genotypes were removed from the dataset. Thirteen microsatellite markers were also removed due to high levels of missing data. This left 308 (from 2021), 336 (2022), 308 (2023) and 349 (2024) juvenile Atlantic salmon, genotyped at 74 microsatellites and 9 SNPs, for analysis. Across years, 47% of juveniles were genotypically male, 49% genotypically female and 4% could not be genotypically sexed.

The reconstructed familial relationships among the genotyped juvenile salmon, split by year of collection, are shown in Figures 2a-2d. Based on the observed genotypes, COLONY estimated that 311 parents spawned in 2020 to produce the juveniles sampled in 2021, 332 parents produced the juveniles sampled in 2022, 329 parents produced the juveniles sampled in 2023, and 337 parents produced the juveniles sampled in 2024. One hundred and fifty-six (13.8%) inferred parents were linked to juveniles sampled in two different years and 12 (1.1%) were linked to juveniles sampled in three years. Thus, 1,129 total parents were inferred to have produced the 1,301 genotyped offspring. Figure 3 shows the reconstructed familial relationships for the entire four-year juvenile sample.

Within a year, the majority (91.5% - 95.8%) of reconstructed parents were linked to juveniles sampled at a single collection site or two adjacent sites (excluding sites above and below the Braulen Dam). Despite larger numbers of juveniles being collected above the dam each year, more parents were always inferred for the below-dam samples (Table 2).

Of the 168 parents that were inferred to have offspring in multiple years, 50% were linked to juveniles sampled one year apart, 36% were linked to juveniles sampled two years apart, and 14% were linked to juveniles sampled three years apart. Forty-four percent of the juveniles that were sampled in different years but linked to the same parent were captured in the same or immediately neighbouring sampling sites. To address the possibility that juveniles linked to the same parent but sampled one year apart originated from the same spawning event - i.e. they were 0+ fry in the first year but 1+ parr in the second year - we examined the length distribution of these fish (Figure 4). Only in half of the cases were fish sampled in the second year larger than those sampled in the first year.

The effective number of breeders (Nb_{sib}) was estimated by COLONY to be 176 in 2021 (95% confidence interval 142-223), 206 in 2022 (95% CI: 169-256), 199 in 2023 (95% CI: 162-244) and 178 (95% CI:145-223) in 2024. If we make the (likely inaccurate) assumptions that each salmon breeds only once, that mean generation time is 3.5 years and that there is no gene flow from other parts of the Beauly, then this would translate to a generational N_e of 661 (95% CI: 538-825) (Waples, 2002, N_e = harmonic mean (N_b) * generation time).

The STRUCTURE analysis based on the 348 non-sibling fish from 2021-24 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.

We observed genetic variation within the juveniles for both *vgll3* and *six6* linked SNPs (Figure 5). While there was no pattern in allele frequencies at the *vgll3* marker across the sampling sites, we observed a general increase in frequency of the early-migration associated *six6* allele from the downstream to the upstream sites.

Table 2. Results of parentage reconstruction based on juvenile genotypes.

Year	Inferred # parents	# Parents with offspring at only one site	# Parents with offspring at adjacent sites	# Parents with offspring above dam	Parent per offspring above dam	# Parents with offspring below dam	Parent per offspring below dam	# Parents with offspring above & below dam
2021	311	277 (89.1%)	18 (5.8%)	131	0.74	171	1.30	9
2022	332	293 (88.3%)	25 (7.5%)	152	0.76	176	1.30	4
2023	329	283 (86.0%)	18 (5.5%)	139	0.83	177	1.27	13
2024	337	286 (84.9%)	37 (11.0%)	139	0.77	194	1.15	4

Discussion

The results of this study suggest that a relatively healthy Atlantic salmon population is present in the Farrar River, with >300 successful spawners per year inferred from sibship reconstruction among the 0+ sampled juveniles. These breeders are expected to include both adult returners from the sea and precocious male parr, which can make up 30% or more of the parents (Saura *et al.* 2008). Despite slightly more juveniles being sampled above Braulen Dam in each year, more parents were always inferred below the dam, suggesting approximately 2x as many spawners in this lower part of the river. The estimated effective number of breeders (N_b) per year ranged from 178-206, potentially translating to effective population size per generation (N_e) > 600. As a 'rule of thumb' an isolated population with N_e > 500 is considered at low risk from problems associated with inbreeding or long-term loss of adaptive genetic variation due to 'genetic drift' (Frankham *et al.* 2014), and in the case of the Farrar N_e will be further increased by gene flow from other parts of the Beauly system.

Our results provide only a minimum estimate of the true number of breeders in the population, as the number of distinct parents identified is limited by the number of juveniles sampled. In practice it is impossible to discover the true adult population size from reconstruction of juvenile sibships (Waples & Feutry 2021). If desired, a more accurate picture of the number of adults (returners plus precocious parr) could be obtained through 'parent-offspring close-kin mark-recapture' 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 adult population size is then estimated by examining the proportion of adults that are 'recaptured' by being identified as parents of the juveniles (Waples & Feutry 2021).

Although most individual salmon reproduced in a single spawning location, we found some juveniles that were sampled at different sites – sometimes a substantial distance apart - but shared a parent. There are several possible explanations for this: 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 adults may stop off to spawn in lower river sections on their way upstream.

Slightly more surprisingly, many parents were also linked to juveniles sampled in two or more different years. Although we might expect some of this to be caused by 1+ parr being mistakenly identified as 0+ fry, comparison of lengths among years did not reveal any strong trend for the juveniles collected in the second year to be larger. The repeated identification of the same parent across years likely reflects a combination of some mis-aged juveniles, actual repeat spawning of individuals as precocious parr and/or returning adults, and the COLONY algorithm conflating close relationships so that, for example, some juveniles identified as siblings are actually parent-offspring pairs or first cousins.

Our identification of closely related juveniles at different sampling sites, both within and across years, suggests that there is no strong long-term population genetic substructure within the Farrar associated with spawning sites. This is supported by the results from our STRUCTURE analysis.

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 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. Vertically stacked points indicate full siblings. 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.

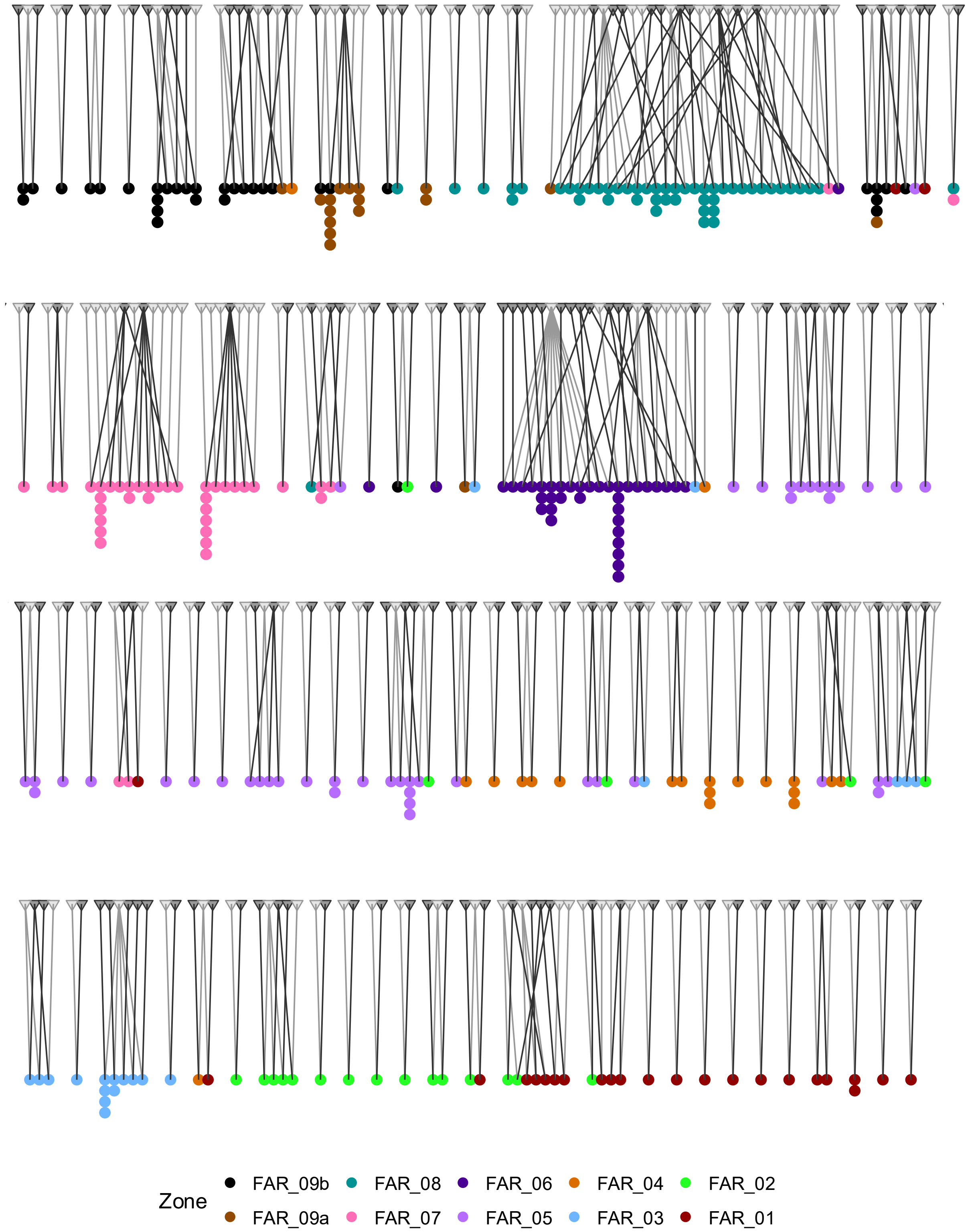


Figure 2b. Reconstructed pedigrees of juveniles sampled along the Farrar in 2022. See Figure 2a for further explanation.

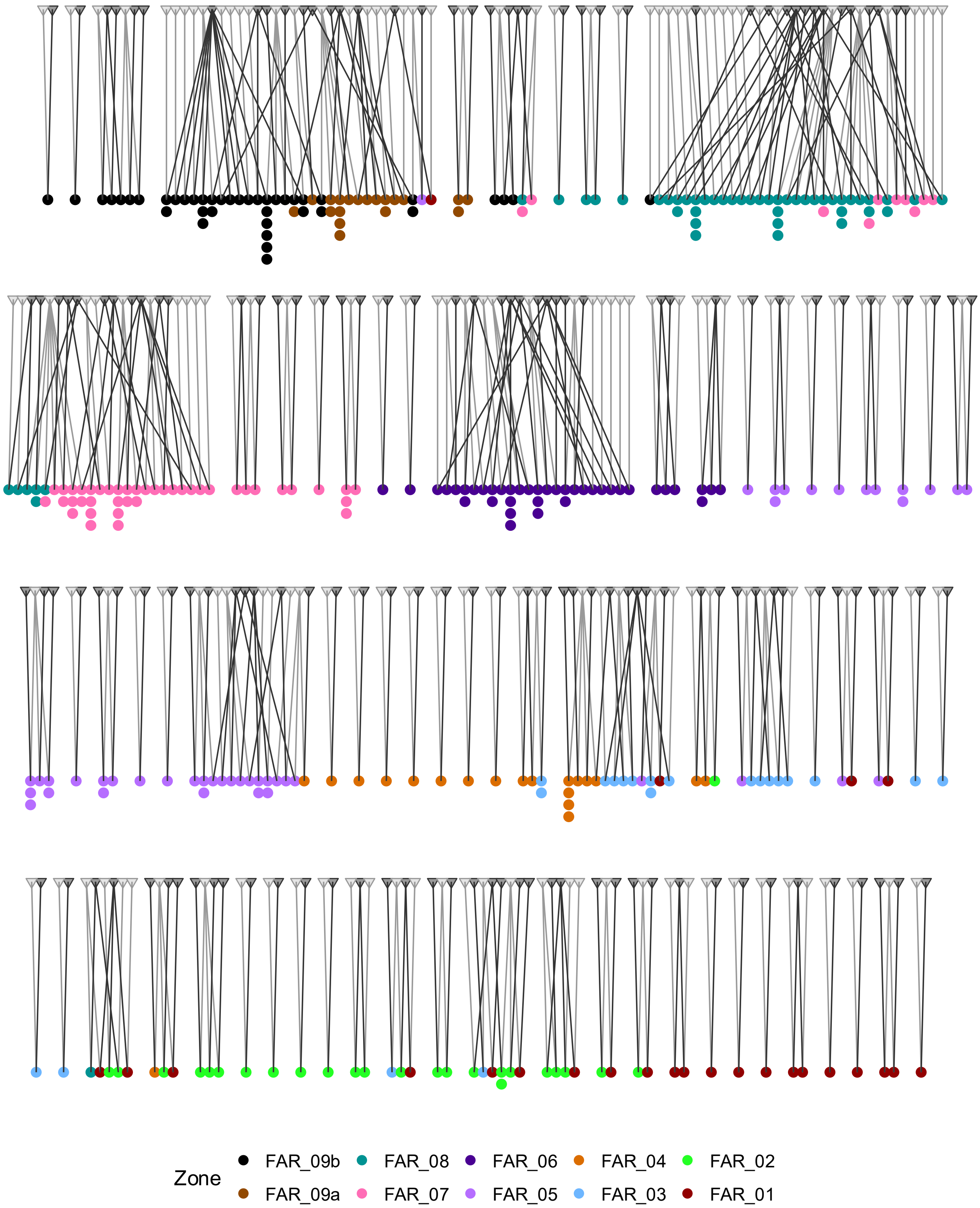


Figure 2c. Reconstructed pedigrees of juveniles sampled along the Farrar in 2023. See Figure 2a for further explanation.

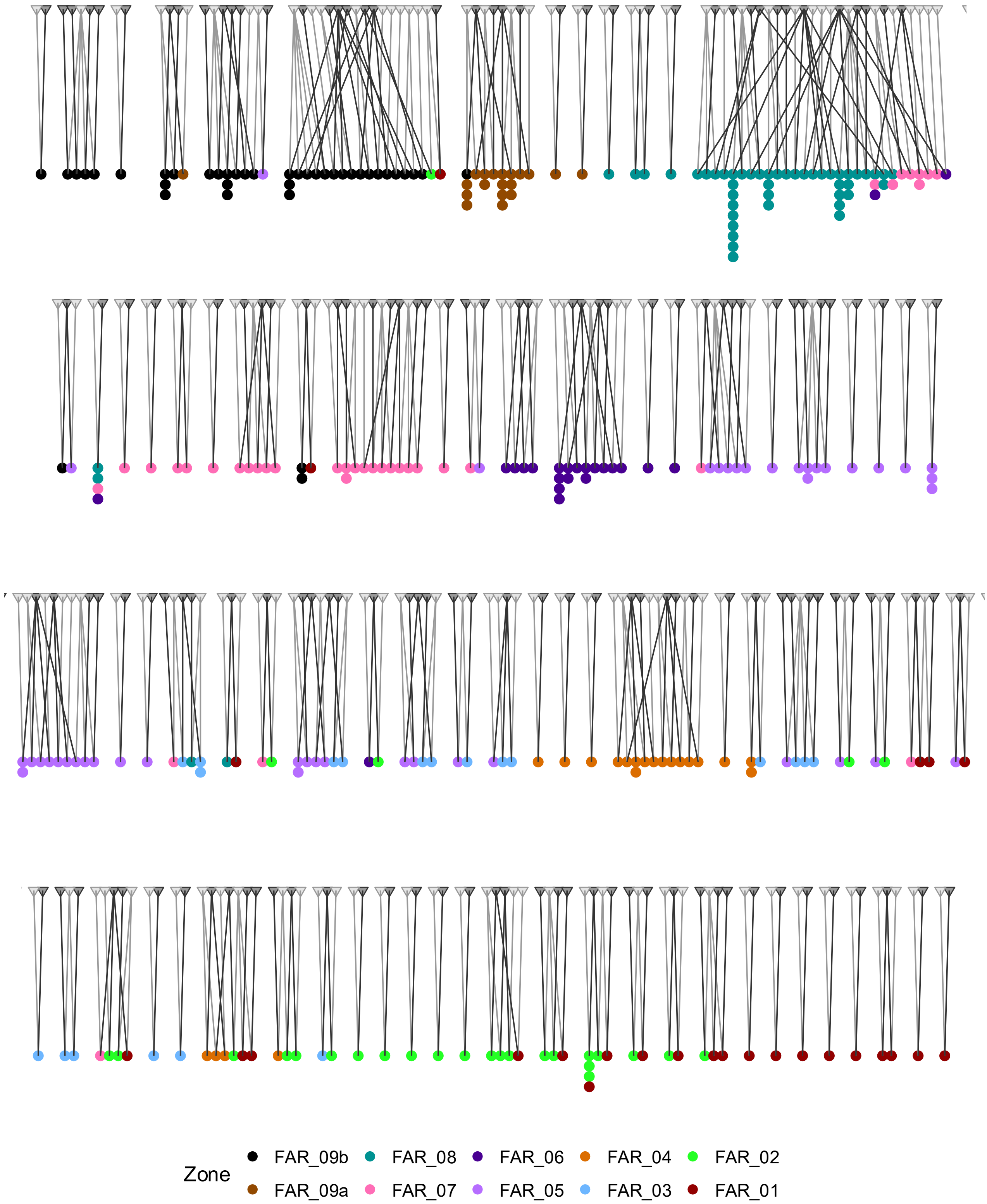


Figure 2d. Reconstructed pedigrees of juveniles sampled along the Farrar in 2024. See Figure 2a for further explanation.

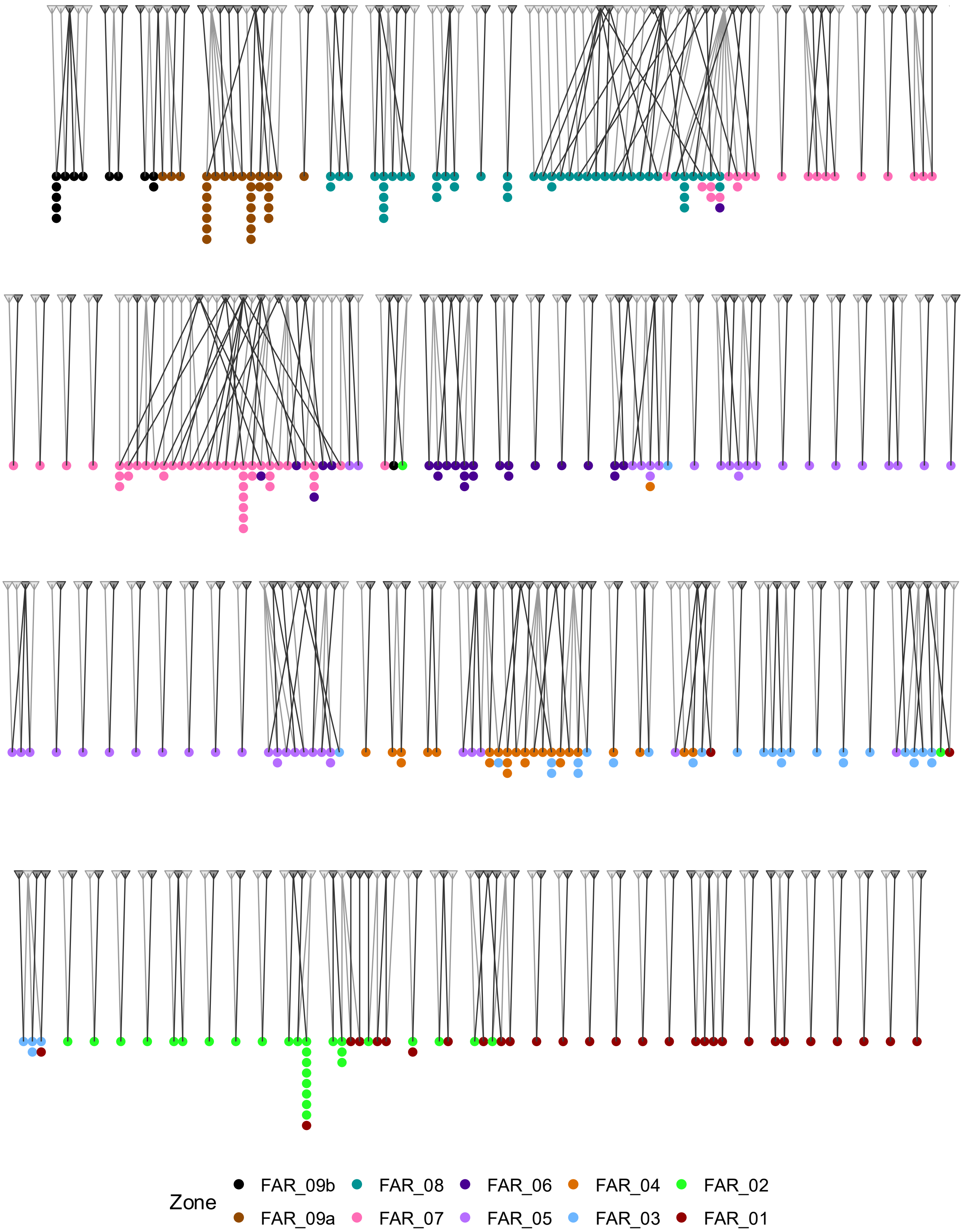


Figure 3. Inferred pedigree for juveniles sampled in all years. Colour indicates sampling site and shape indicates sampling year.

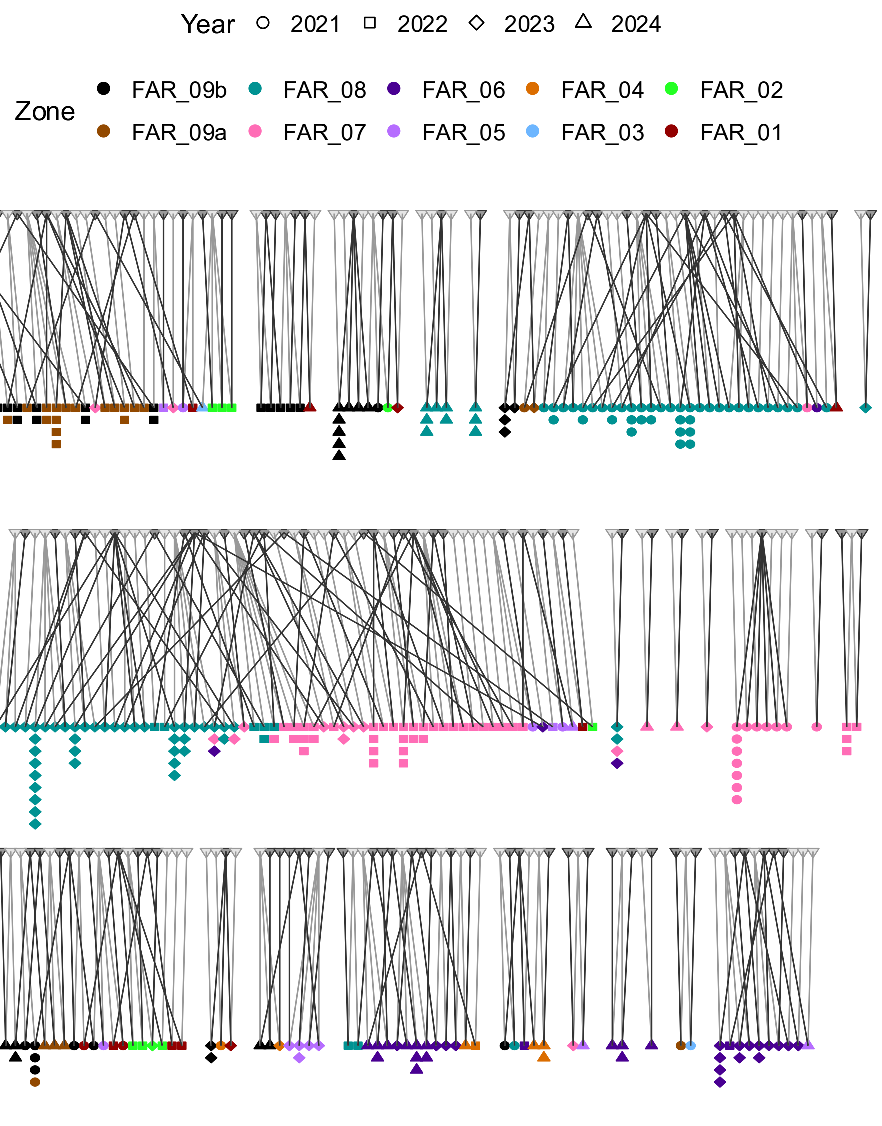


Figure 3. continued

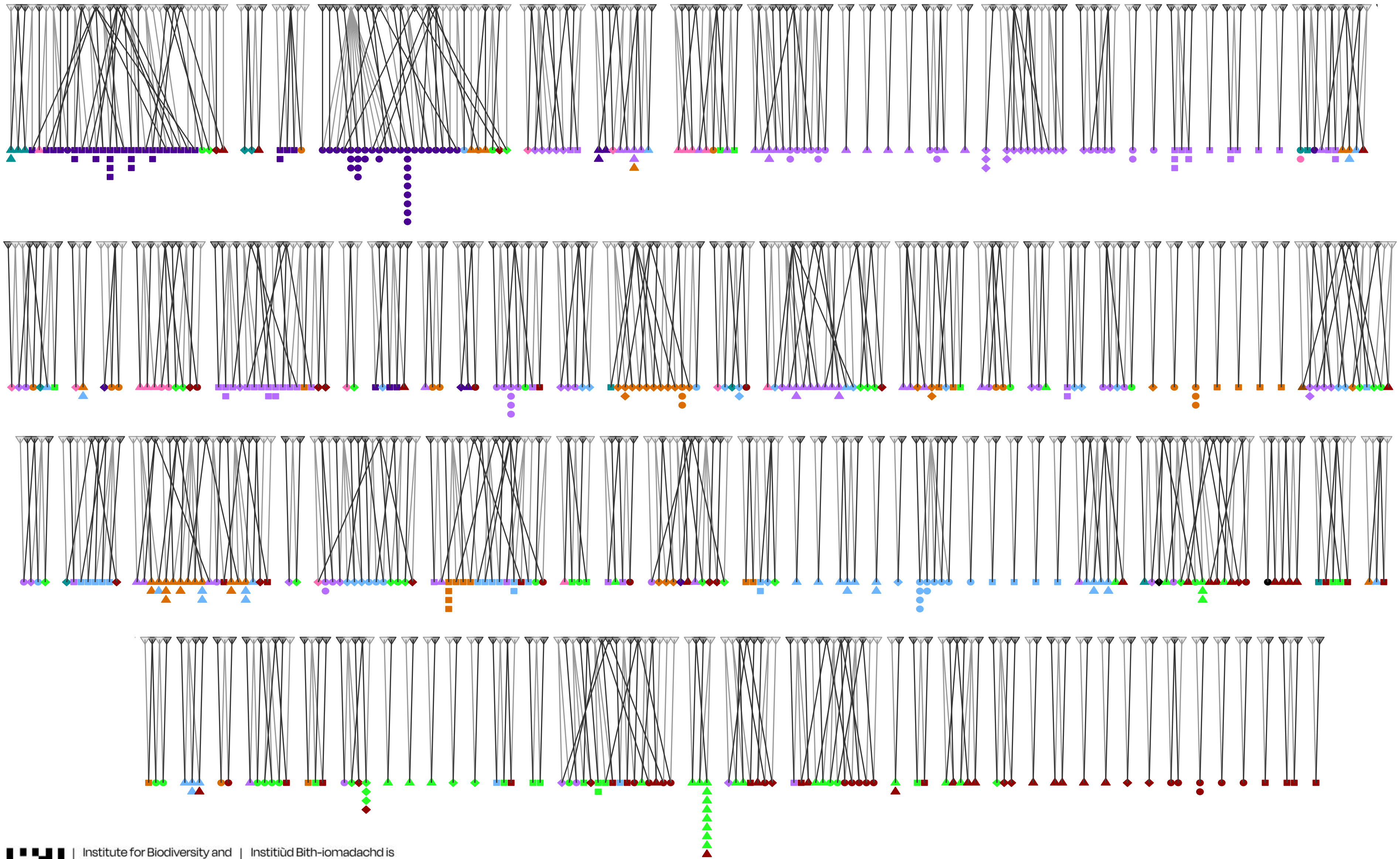


Figure 4. Mean lengths of juveniles sampled one year apart but linked to the same parent. Measurements were not available for all juveniles.

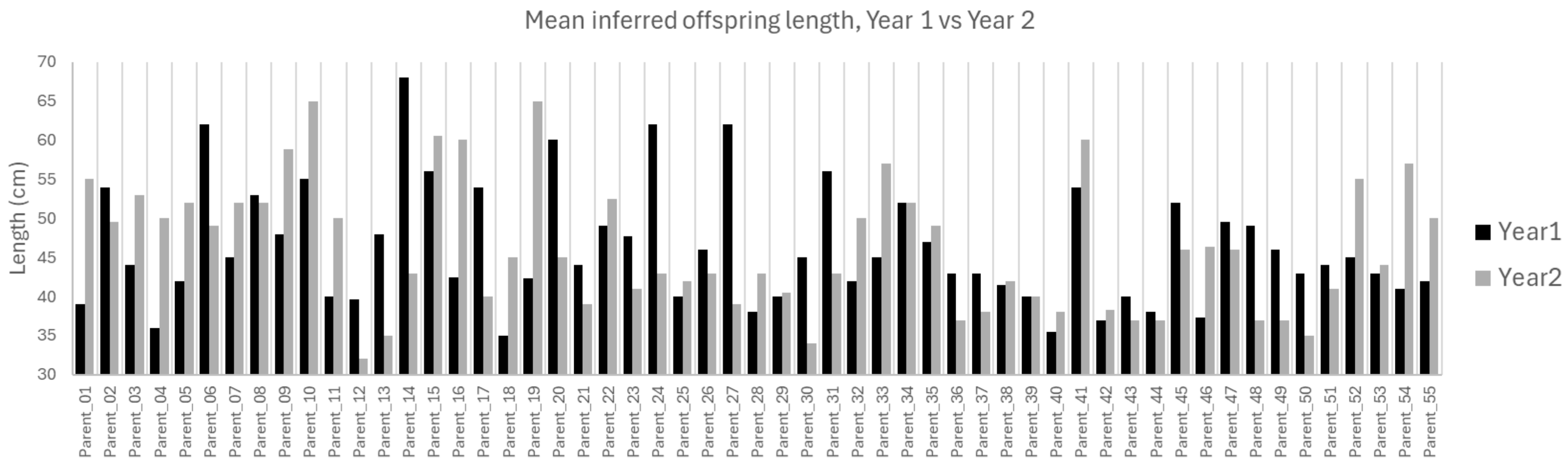
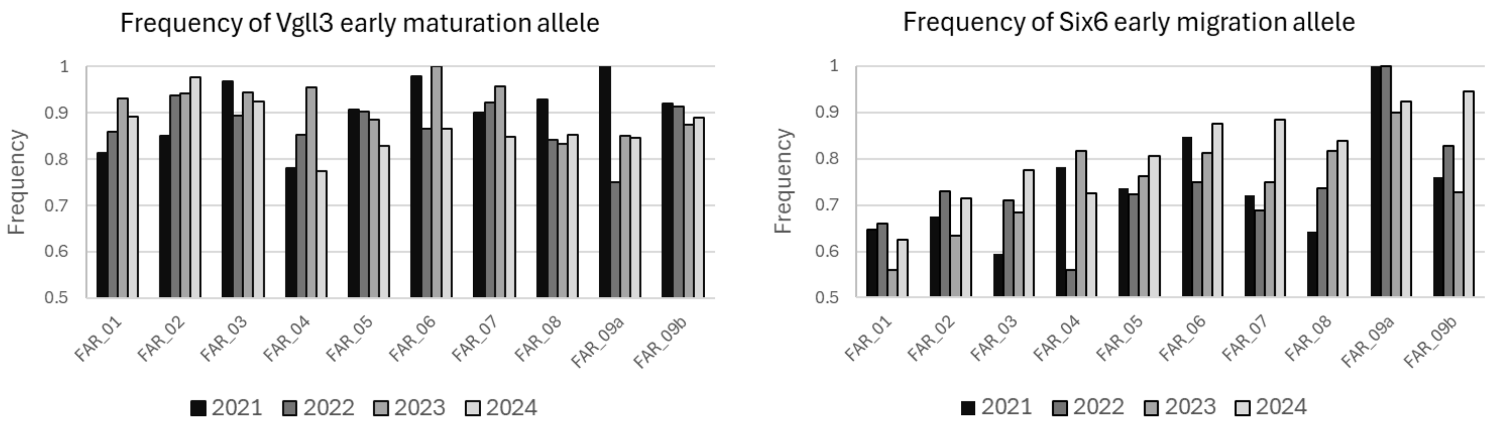


Figure 5. Observed frequencies of the *six6* allele linked to earlier adult return migration timing and the *vgll3* allele associated with younger age-at-maturity (i.e. grilse vs. salmon) across sites and years.



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