Marine Scotland Science Report

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AN OVERVIEW OF POPULATION GENETIC STRUCTURING IN THE OUTER HEBRIDES FISHERIES TRUST

Prepared as part of the Focusing Atlantic Salmon Management on Populations (FASMOP) Project

Delivered in partnership with the Rivers and Fisheries Trusts of Scotland (RAFTS)

M W Coulson, L M I Webster, D Kelly, A Armstrong,

E Cauwelier, L Stradmeyer, J Gilbey, C Sinclair & E Verspoor

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An Overview of Population Genetic Structuring in the Outer Hebrides Fisheries Trust

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Executive Summary

This report describes how genetic information from juvenile Atlantic salmon sampled from 15 sites within the Outer Hebrides Fisheries Trust (Figure 1) have been analysed in order to help inform developing fisheries management activities. The key objective for the Trust was to define the genetic structure of the locations under investigation, in order to determine whether salmon in the area represent distinct breeding populations.

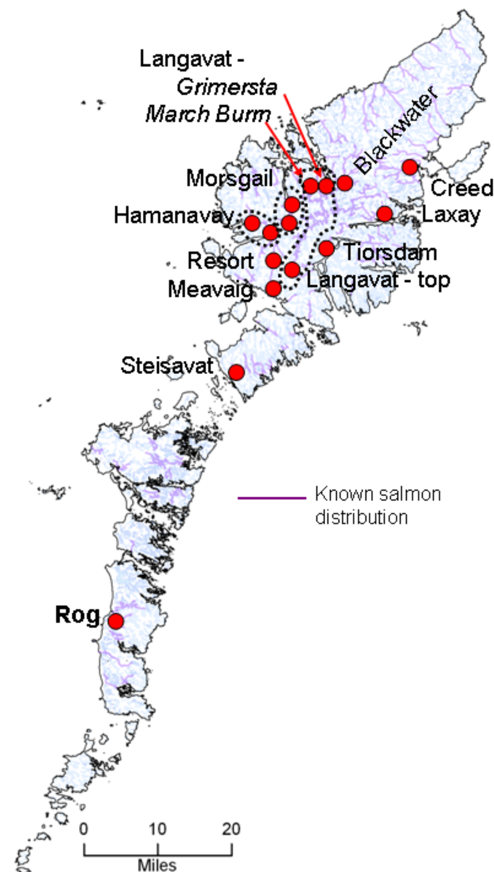


Figure 1. A map of the Outer Hebrides, with sample sites that are involved in this report indicated in red with associated site names. Sites enclosed within a circle were grouped for analysis (see main text).

Summary of findings

The analysis showed that, most sites exhibited significant genetic differences from one another with the markers used, indicating low to moderate levels of genetic structuring among these sites. Several sites were different from all others including: the upper Langavat site, March Burn, the upper Hamanavay, Creed, Rog, Laxay and Steisavat sites. The Rog site was by far the most distinct, given its more remote location from all others.

The degree of genetic differentiation observed here is largely reflected by the ability to predict where a sample is from using only genetic information (genetic assignment); where genetic signatures are strongly related to location, individuals are more likely to be assigned to the location from which they were originally sampled. The average value of correct assignment to site was 46%, which is higher than one would expect if there was no genetic structure in the data. However, the magnitude of differences observed with the current markers varied, depending upon location, as is not large enough to assign fish with higher accuracy to all sites. Assignment was highest to Rog (85% accuracy).

Implications for management

The aim of the current FASMOP project was to identify distinct breeding populations of salmon within the Outer Hebrides. The results to date suggest that there are distinct breeding populations. Most systems were genetically different from all other systems and there was evidence for within-river genetic structuring for both the Langavat and Hamanavay systems. Overall this suggests that there are distinct breeding populations of salmon within the Outer Hebrides. Temporal sampling at these sites would allow for an assessment of the stability of the observed patterns and levels of genetic differences. For a number of locations the distinction of these potential populations is limited and do not show any sort of obvious geographical pattern.

There are two possible reasons for the observed low levels of genetic structuring seen:

- There is reproductive mixing of individuals between the different parts of the system. This could include possible stocking events in the past.
- The microsatellites in the study do not give the resolution required to adequately describe population structuring within the river.

The current genetic markers show overall weak to moderate genetic differentiation. However, this observation cannot be used to rule out the possibility of locally adapted traits being present within the system. This may be further clarified with the development and application of newer, more targeted, genetic markers. To determine if it is possible

to improve assignments and gain better distinction for potential breeding populations, larger sample sizes and/or newer genetic markers will be required and possibly a more complete baseline of potential populations sampled.

Introduction

Atlantic salmon (*Salmo salar* L.) are one of the world's most widely recognized and prized fish species. However, declines in numbers across much of the species' range have been cause for concern and the focus of intense management schemes and research efforts. Central to these efforts is the recognition of how the species is structured spatially across both broad and fine scales. Given the large native range encompassed by the species and their well-known ability to home to natal rivers, it is expected that Atlantic salmon will demonstrate a considerable degree of population structuring, representing discrete breeding units that are reproductively separated to varying degrees (Webb et al. 2007; King et al. 2007). This reproductive separation underlies the basis for locally adapted traits to establish across a widely variable environment. Indeed, ample evidence exists in favour of local adaptation in many salmonid species (Taylor 1991; Garcia de Leaniz et al. 2007; Fraser et al. 2011).

A principal tool used to resolve population structuring is the use of genetic markers. Such approaches have been used for decades, with most of the contributions towards salmonids occurring within the past 20 years (Verspoor, 2007). Initially, this work focused on range-wide patterns of differentiation (e.g. King et al. 2001; Verspoor et al. 2005) or documenting the expected structuring existing between different river systems (e.g. O'Reilly et al. 1996; Fontaine et al. 1997; McConnell et al. 1997; Spidle et al. 2003; Castric and Bernatchez 2004; Dillane et al. 2007). However, not as much is known about structuring within river systems and few examples exist for Scottish rivers (but see for example, Verspoor et al., 1991; Jordan et al., 2005).

Ecological studies have shown that different tributaries within a river may exhibit differences in traits such as run-timing (Stewart et al. 2002, 2006), variation in age at smolting (Englund et al. 1999) and sea-age at maturity (Niemela 2006), among others. When such differences are shown to have a genetic basis (e.g. Stewart et al. 2002, 2006), then salmon breeding in separate locations, for instance, above and below waterfalls or other natural features may often be heritably different in ways that affect their behaviour, survival and reproductive success. This may be true of neighbouring tributary populations and, since intermixing of these populations runs a risk of unknown magnitude, may not be desirable. Indeed, at its worst, mixing in vulnerable populations could have long term negative effects on population viability by reducing survival (McGinnity et al., 2003). Recent genetic analyses of Atlantic salmon have indicated that

rivers may be structured on fine scales into multiple distinct breeding populations. Such studies have used both neutral genetic markers (Garant et al. 2000; Vaha et al. 2007; Dionne et al. 2008; Dillane et al. 2008) as well as markers for which there is an underlying basis for natural selection (Landry and Bernatchez 2001).

The suite of genetic markers used in the current survey are assumed to be "neutral" (meaning they are not known to be linked to heritable characteristics that may differ among locations such as run timing, growth rate, etc.). They will therefore largely reflect the shared ancestry of salmon among sites rather than make a direct assessment of the heritable trait characteristics that differ among them. Differences at such markers may *imply* that groups are sufficiently distinct for heritable trait differences to have evolved, however, where no difference is observed, we cannot rule out the possibility that these sites differ in heritable traits.

Given the recognition of the 'population' as a focal unit for management, it follows that knowledge of the genetic structuring among sites is required for certain management and conservation schemes. The potential for multiple, distinct populations to become established within a single river means that detailed knowledge needs to be gained regarding the scale at which such populations occur. As a first step in this process, a baseline for systems needs to be constructed and built upon as more information becomes available.

In 2009, a partnership between the Rivers and Fisheries Trusts of Scotland (RAFTS), Marine Scotland Science (MSS), and the participating individual Fisheries Trusts and Boards was established. It set out to undertake a Scotland-wide survey of genetic structuring within all Scotland's major salmon-producing rivers. This project, entitled **F**ocusing **A**tlantic **S**almon **M**anagement **O**n **P**opulations (FASMOP), had as its central aim to undertake a program of genetic sampling of Atlantic salmon stocks in river systems across Scotland. The purpose of this sampling scheme was to define the genetic structure among locations in order to determine whether salmon within and among the various systems in a given area represent distinct breeding populations This work, alongside the EU SALSEA-MERGE and other MSS projects, is creating a genetic map of salmon populations across Scotland, to help inform management and conservation efforts.

Summary of Methods

Juvenile salmon from various locations throughout the Outer Hebrides were sampled for genetic material by the Trust in order to inform fisheries management following methods outlined by Verspoor and Laughton (2008). Figure 1 shows the locations of the 15 sites that have been included in the genetic analysis for the Trust. Samples generally

consisted of fry and/or parr (n= 28-50, depending on site) and for each individual, data from 17 genetic markers (microsatellites) were collected. The results from the microsatellite marker SsaF43 allowed us to identify any trout or trout/salmon hybrids that may be present among samples. These individuals were then removed prior to analysis.

It is possible that samples are more reflective of families rather than populations, given the life-history stage(s) targeted by sampling and the potentially fine-scale geographic coverage (Hansen et al. 1997). This occurrence can alter the genetic signature of the sample and obscure population level differences. Therefore, prior to population level analyses, each site was screened for the presence of full-siblings, representing family groups and when identified, all but one individual of a full-sib family were removed. Additionally, this analysis can estimate how many breeders contributed to producing each sample, which may include contributions from precocious parr. Initial sample sizes as well as sample sizes after full-siblings were removed are presented in Table 1.

When two sites were sampled close together (<5 km), these sub-samples were initially tested for differences between each other using the program CHIFISH (Ryman 2006). Where no significant differences were found, data from these sub-samples were combined; otherwise they were left separate for all further analyses. This resulted in 14 samples for subsequent analyses.

Data were then analysed using standard population genetic methods to evaluate the genetic relationships and groupings among the sample sites in order to obtain a general overview of population structure and address the objectives of the Trust.

A detailed methods and analysis section can be found in Appendix 1.

Results

Broadly speaking, most sites exhibited weak or moderate genetic differences from one another, indicating overall low levels of genetic structuring among these sites with the current set of markers. The interpretation of the pattern and degree of differences in terms of the relationships among populations, combined with the known history and geographical proximity of sites can be useful to inform fisheries management decisions. Here we discuss the results of the FASMOP project summarizing the main genetic findings in terms of population genetic structuring within the Outer Hebrides Fisheries Trust.

Family effects

A total of 582 juvenile salmon from the Outer Hebrides were involved in the genetic analysis. All sites were examined for family effects with relatively few samples being removed due to full-sibling relationships, although there were a few exceptions (Table 1). The level of family effects differed between samples with the largest family group present in the individual samples ranging from 2 to 9 full-siblings and sample sizes subsequently being reduced by 4-41%. Family effects were controlled for at each site before all further analyses. There were three samples identified as salmon/trout hybrids across all locations (Table 1).

Table 1

Details of samples used for this analysis, including original sample size, and details of each site following COLONY analysis to re-construct family relationships.

Site	Site ID	Original sample size	Sample size analysed (sibs removed)	Number of breeders contributing to sample	Largest single family	Year sampled
Langavat – top	1	48	43	64	2	2009
Langavat – March Burn	2	41	28	37	6	2005
Langavat - Grimersta	3	32	30	48	2	2005
Tiorsdam[1]	4	28	20	27	5	2005
Hamanavay – upper[2]	5	43	32	42	5	2008
Hamanavay – mid	6	50	45	52	2	2008
Blackwater	7	34	20	30	9	2005
Creed	8	34	30	41	3	2005
Rog	9	44	42	58	2	2008
Laxay	10	50	48	58	2	2010
Meavaig	11	45	36	48	5	2005
Morsgail[2,†]	12	36	27	44	9	2008
Resort[2]	13	47	42	58	2	2010
Steisavat	14	50	44	60	2	2009

1. Two samples from this site were found to be trout/salmon hybrids.
2. One sample from this site was identified as a trout/salmon hybrid.

† Upper and lower Morsgail showed significant differences from one another, however these two sites were combined for analysis due to small sample sizes (18 & 9, respectively)

Population structuring

Several sites within a system were sampled close together (<5 km) and in each case the two sites were tested for differences using the program CHIFISH (Appendix 1). Two sites on the Hamanavay (upper and mid) showed significant genetic differences between them ($P < 0.05$) and were therefore kept separate for the remainder of the analyses. Significant differences ($P < 0.05$) were also found between the two sites on the Morsgail. However, these sites contained only 18 and 9 individuals and under conditions of small sample sizes, the tests as implemented in CHIFISH may be unreliable (Ryman, 2006). Pooling of small (<25) samples has been shown to produce more informative analyses of genetic structuring (Gomez-Uchida & Banks, 2005) and the Morsgail sites were therefore combined for subsequent analysis. Finally, the three sites sampled within the Langavat were all significantly different from one another.

Most sites were genetically different from one another, with 85% (77 out of 91) of the pairwise comparisons being significantly different (Appendix 2). Among these comparisons, several sites were different from all other sites. These included the top Langavat site, March Burn (Langavat), the upper Hamanavay site, Creed, Rog, Laxay and Steisavat sites (Appendix 2). The remaining sites all show a mixture of significant and non-significant levels of differentiation among them. A visual representation of these relationships among locations can be found in Figure 2, which uses multi-dimensional scaling to represent pairwise estimates of genetic differentiation among sites (Appendix 2). Points which are closer together on the plot have a more similar genetic makeup while points further apart are more genetically discrete.

The most differentiated site is the Rog as it is plotted furthest apart from the remaining sites (Figure 2). The next most differentiated site is the upper Hamanavay site, which interestingly, is plotted quite far apart from the mid Hamanavay site, which is more similar to sites from other systems. The remaining sites are mostly still significantly different from one another, but are plotted closer together.

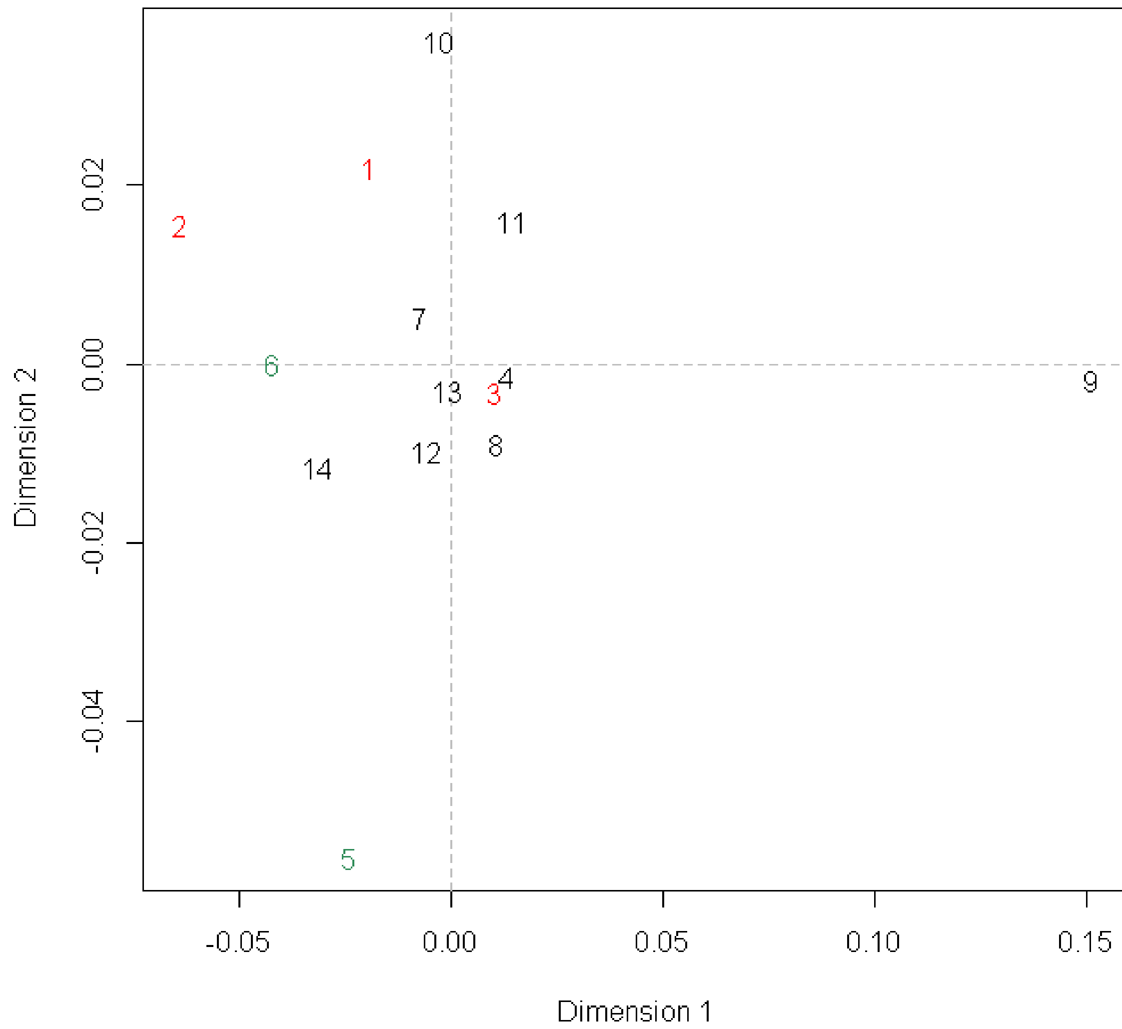
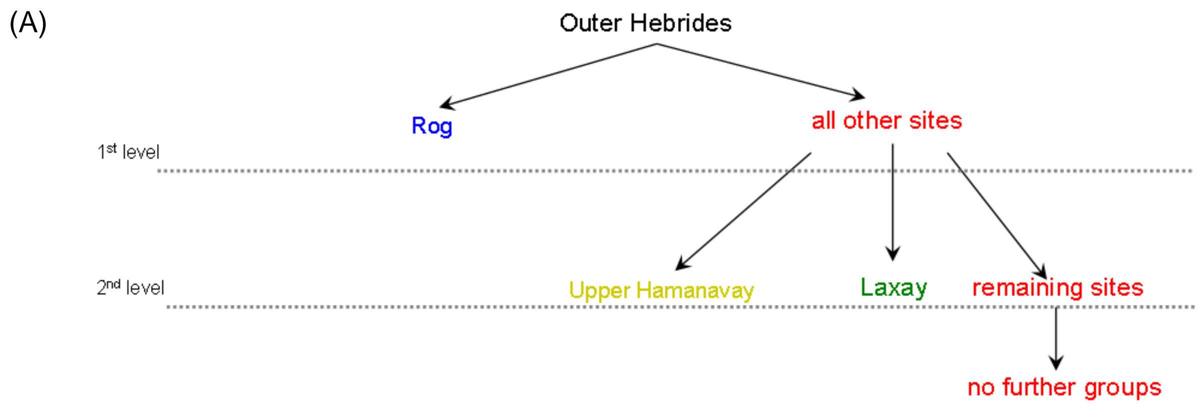


Figure 2. Multi-dimensional scaling (MDS) plot of genetic relationships among all sites based on pairwise estimates of genetic differentiation (Jost's D; see the appendix for details). Points which are closer together on the plot have a more similar genetic makeup while points further apart are more genetically discrete.

A clustering analysis that explores possible groupings of individuals other than the defined sampling sites was also carried out. This analysis was done in a hierarchical fashion, as larger genetic differences among groups may obscure weaker differences at smaller spatial scales. For each level, the analysis aims to determine from a given number of samples, the most likely number of groups and the membership of each individual into those groups. Each of the groups identified by the first round, are then separately analyzed in a second round and this process is repeated until further identification of groups is not possible. For the Outer Hebrides sites, at the broadest level, this analysis determined the most likely number of groups to be two. These two groups corresponded to 1) Rog and 2) all remaining sites. The distinction of Rog based upon this approach is consistent with its placement in the MDS plot (Fig. 2). Among the remaining sites, the analysis was re-run and the most likely number of groups was determined to be three. These were 1) the upper Hamanavay site, 2) the Laxay, and 3) all remaining sites. This third group was re-run and the most likely number of groups was determined to be one. This does not necessarily mean, however, that there are not significant genetic differences below this level, but that using this clustering approach, these smaller differences are more difficult to tease apart and the distinction for splitting individuals into more than one group is less obvious.



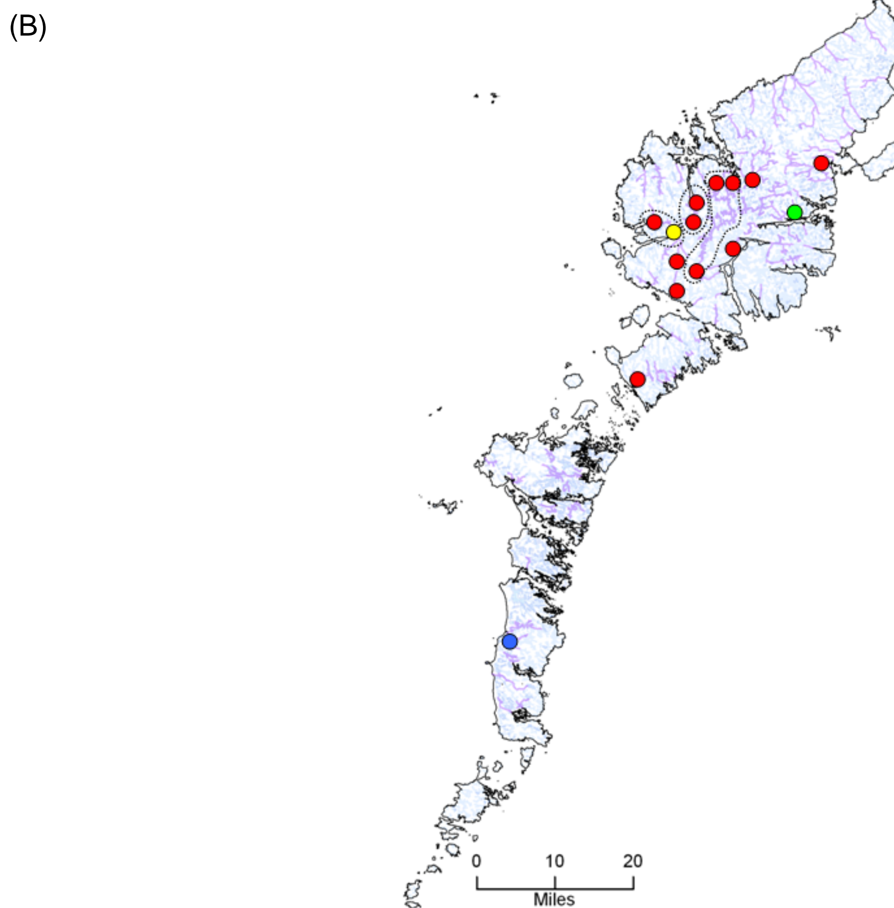


Figure 3. (A) Schematic showing the order in which the different clusters or groupings were resolved according to the hierarchical analysis. (B) Geographic representation of the relationships among sites, following a cluster analysis (STRUCTURE; see appendix). Locations with the same colour are more similar to one another and belong in the same cluster.

Genetic assignment of individuals

The assignment analysis shows how useful this baseline genetic information is to identify which of the sampled sites a fish of unknown origin is from (Figure 4). Each individual fish is taken in turn and it is assessed from which of the sampling locations provided in the baseline, that individual is most likely to have originated. Assignment of fish back to their specific site of collection was, on average, correct 46% of the time. While this average is much greater than would be expected if assignments were purely random (14 sites, random = ~7%), this may reflect the weak to modest population genetic structure underlying the data, but the magnitude of differences observed with the current markers among sites is not large enough to assign fish to location of sampling with higher accuracy. Assignment is highest to the Rog site (85%), reflecting the distinctiveness of this site in both the MDS plot (Figure 2) and the clustering analysis (Figure 3). A number of other sites assign around 60% with several sites noticeably lower (as low as 10% for the Tiorsdam).

It may be possible to improve accuracy by implementing a cut-off rule for the probability that an individual gets assigned or by allowing individuals to not be assigned to any of the sites in the baseline (i.e. came from an unsampled population). However, for the Outer Hebrides, a cut-off does not appear to improve assignments. For example, if we assign only fish that have a minimum of 70% assignment probability, overall correct assignment changes only marginally to 51% (compared to 46% above) and individual-site assignment increases at most ~16%. Applying such a cut-off comes at a potential coast as not all fish in the baseline will be assigned. However the above example for the Outer Hebrides (70% cutoff) still resulted in over 76% of fish being assigned. This suggests that many fish are being assigned, with high probability, to sites other than those they were sampled.

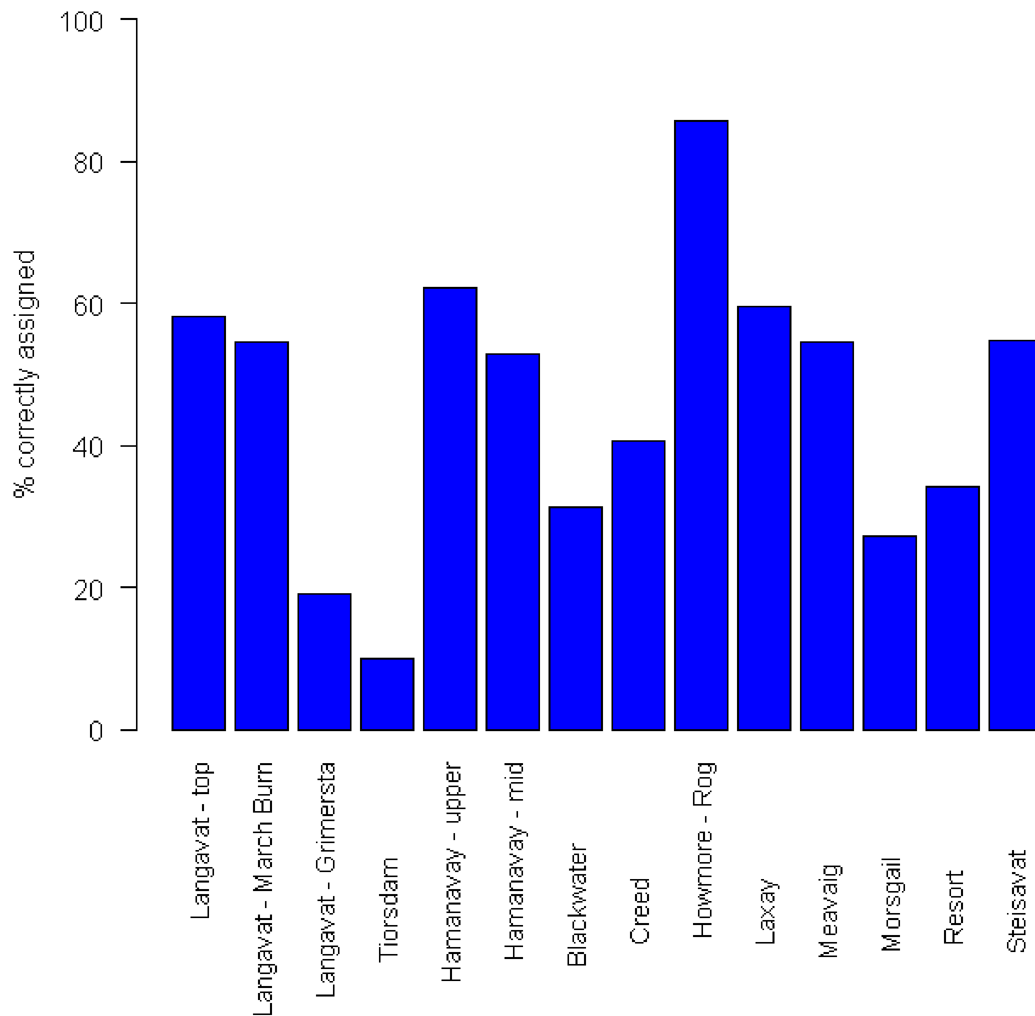


Figure 4. Percentage of fish sampled from each site that correctly assign back to that site.

Discussion

Population structure

The aim of the FASMOP project for the Trust was to identify distinct breeding populations of salmon within and among systems. The results to date suggest that there are distinct breeding populations in the Outer Hebrides. Some of these populations are quite distinct while for other the distinction of potential breeding populations is not as easily defined using the current set of genetic markers.

There are various reasons why one might expect some sites to be more distinct than others. Here, the most distinct site was from Rog. Given the geographic distance between this site and the others (Figure 1), and the known homing ability of salmon, it is

perhaps not surprising that this sample should be so distinct. This undoubtedly represents a distinct breeding population from the other sites. Relationships among all the other sites are less distinct and the magnitudes of genetic differences among most sites are weak to modest, but still statistically significant, suggesting they *may* still represent distinct breeding populations (Appendix 2). The patterns of these differences are not easily interpreted by geographic proximity, with some closer sites having large genetic differences than with more distance locations (Figure 2).

Three systems allowed for assessment of within-system genetic structuring (Morsgail, Hamanavay and Lanagavat). All three systems showed evidence of genetic structuring within them, however, sample sizes for the Morsgail were small and likely influenced the outcome for this particular system. The Langavat and Hamanavay systems both supported within-river genetic structuring. The Langavat system is quite a complicated and dendritic system which may contribute to the evolution and maintenance of distinct breeding populations. One of the sites (Top Langavat) was sampled in 2009 while the other two (March Burn & Grimersta) were sampled in 2005. Some of the difference between the former and latter two sites may be due to temporal variation, however, this would not be the case for the two sites samples in 2005. Furthermore, the upper Langavat site is quite far up the system and located far apart from the remaining sites. The two sites on the Hamanavay are more different from one another than to other systems. As these two samples were collected in the same year, this suggests that the pattern is not due to temporal variation and family effects were relatively small and accounted for. However, these two sites are separated by a couple of lochs. Presence of lochs on other systems throughout Scotland has been associated with within-river genetic structuring and therefore may represent at least a partial barrier. Furthermore, there is no known history of stocking on this system (D. Kelly, personal communication), but this cannot definitively be ruled out. This does, however, suggest the existence of more than one breeding population within the Hamanavay as well as the Langavat systems. Sampling of sites in subsequent years would help to further establish whether certain sites represent distinct breeding populations by assessing if the observed genetic differences and associated patterns are stable over time. This could lend further support for the definition of such separated populations.

While a lack or weak level of differentiation among some sites may be the result of moderate levels of exchange of spawning adults among sites, caution should still be used in making such an interpretation. Weak genetic differences may be due, at least in part, to the current set of genetic markers. For instance, adaptive differences may be present (e.g. for run timing behaviour) which our neutral genetic markers could not detect. Therefore, other types of markers, which may be associated with adaptive traits, may help to further address the degree to which these locations represent distinct breeding populations.

Therefore, when there is clear evidence of distinct breeding populations, then a continued caution with respect to sourcing brood stock would be desirable in respect of stocking programmes. However, even with weak to little observed differentiation, the same caution should be exercised. As mentioned above, a lack of genetic differences with a given set of markers may not necessarily imply a single breeding population. Locations may still differ with respect to adaptive traits and until such issues can be addressed, then locally sourced brood stock should reduce the risk of disrupting any local adaptations that lead to increased survival.

Genetic assignment

The power to assign fish of unknown origin to their location of origin with high accuracy is possible where candidate locations show strong genetic differentiation. Such an approach is useful for discriminating the composition of mixed-stock fisheries. This could be useful, for instance, in assigning rod caught adults to their particular stock component. For example, it may be possible to use genetic assignments to determine whether salmon returning to a river at different time points are destined for different parts of the catchment if there is well defined structuring between these components and with genetic markers which may be associated with that particular trait. Genetic assignment allows one to calculate the probability that a given fish originated from a particular location. Then the location with the highest probability is taken as the site from where that individual originated. This is done for each individual and Figure 3 shows the proportion of individuals from a given site, which was assigned back to that site based on their genetic profile. If each location exhibits large differences from everywhere else, one would expect the accuracy of assigning individuals to the location from which they were sampled to be high (e.g. 90-100%). Assignment is highest to the Rog (Figure 4), which is not surprising given the much higher level of differentiation observed at that site (Appendix 2). Several other locations assign with moderate levels of accuracy (~60%; Figure 4), however, the average value of correct assignment to site is 46% (Figure 4). This average is higher than one would expect if there was no genetic structure in the data. While this supports the conclusion that there are genetic differences among some locations, indicative of separate breeding populations, the data do not at present have the power to assign fish of unknown origin (e.g. rod caught adults) to their location with higher accuracy.

In order to improve assignments and gain better distinction for potential breeding populations, larger sample sizes and/or additional genetic markers may be required as well as a more complete baseline of potential populations sampled. As many assignments will try to assign individuals to sites represented in the baseline, if the 'true' site has not been sampled, fish from these missing sites will be forced to be incorrectly

assigned. At present, these assignments represent our best estimates since all fish assigned were known to originate from sites in the baseline. For the most accurate and complete picture of assignments within a system, detailed knowledge of *all* breeding populations is required. It should be noted, however, that at certain geographical scales or for certain systems, assignment may not be possible with high accuracy, regardless of the samples and markers employed. If there is exchange of even modest amounts of spawning individuals over time between sites, then the genetic make-up of these sites will look relatively similar and prevent assignment to defined groups with high accuracy.

Future work

While there is evidence of genetic structuring within among sites in the Outer Hebrides, the level of differentiation with the current marker set is moderate to weak depending upon location and prevents more robust conclusions across all systems. Such an outcome is not unique to the Outer Hebrides, but is observed in several other systems throughout Scotland. Before a complete picture can be formed regarding the extent of population structuring within and among systems, a much more detailed survey is warranted. Currently, the development and application of a different class of genetic marker (**S**ingle **N**ucleotide **P**olymorphisms, or SNPs) is underway in Scotland to address the resolution of population structuring in more detail and provide a more robust assessment. This approach offers at least two distinct advantages over the current suite of markers in that (1) the number of markers screened for SNPs is much larger than that for microsatellites (100s - 1000s vs. 10s, respectively) and (2) that while microsatellites are selectively “neutral”, SNP markers should be associated with both “neutral” as well as actual traits, the latter of which some may be adaptive. The use of SNPs, either as an alternative to or in conjunction with microsatellites, has been shown to be promising for resolving different stock components with respect to fisheries management for various salmonid species (e.g. Narum et al. 2008, Glover et al. 2010, Beacham et al. 2010). Trying to target the underlying genetic differences that are associated with known biological (e.g. run-timing) or habitat (e.g. pH, elevation) differences will help to shed light on different stock components. For instance, finding a genetic marker associated with run-timing would allow for direct application toward the identification of spring vs. late-running stock components. This would allow for a more diagnostic application rather than using a set of random, ‘neutral’ genetic markers.

A number of factors may underlie population genetic structuring. At least one of these, not addressed here, is the potential impact played by stocking practices. Stocking in many areas has been common for Atlantic salmon both within and between systems. Such practices may influence why certain locations appear very distinct if they were sourced from a different location compared to the surrounding stock components. Alternatively, if stocking was widespread in an area, this could result in a more similar

genetic make-up among stock components than would otherwise be the case. For instance, the Grimersta has been used as a source for stocking among other, the Morsgail. However, in order to address the degree, if any, to which stocking plays a role in genetic structuring, detailed knowledge of the stocking history and records are essential. Furthermore, including genetic samples from possible donor sources would provide an insight into whether those donors had made a lasting impact on the local stock. Additionally, the availability of historical samples that pre-date the stocking history of an area would be of particular value in addressing the impact as it would offer a comparison of the genetic make-up pre- and post-stocking levels of differentiation.

A second unexplored contribution to the patterns of population structure observed may be aquaculture escapes. Intermixing of farm fish with native strains may profoundly affect the genetic structuring of wild populations (Ferguson et al, 2007). It may also potentially cause reduced differentiation among wild strains where there is significant introgression into wild populations from the same farm strains. However, it is important to remember that there will be a lot of genetic variation among Scottish fish farm strains, which underscores the importance of a broad sample of baseline farm strains for comparison with wild populations. The collection of such a farm baseline as well as new markers that have been developed specifically to address such issues (Karlsson et al. 2011) will potentially allow for a comprehensive survey of the potential impacts of farm escapes on wild populations. It would seem sensible to consider this aspect when planning future work for the Outer Hebrides Fisheries Trust, given the long history of fish farming in the area.

Summary

This analysis demonstrated overall weak to moderate levels of population structuring among sites within the Outer Hebrides. The results suggest that there are distinct breeding populations between most rivers but as well as evidence for within-river structuring. However, the number of distinct populations and degree of these differences is not sufficient to allow for robust application to management at present, except perhaps for some locations (e.g. Rog). Clearly more work is needed to clarify the extent of genetic structuring within the Outer Hebrides. This will likely involve the use of newer genetic tools and a more targeted approach to contribute to our overall understanding of the underlying salmon population structure and in turn, assisting the efficient management and conservation of this valuable resource.

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Appendix 1

Laboratory Procedures

DNA was extracted from individual fin clips using a standard proteinase K digestion (Fisher Scientific UK). The crude DNA extract was diluted (1 in 10) in 1xTE (Tris-EDTA) buffer for all further work. Seventeen microsatellite markers that have previously been developed for Atlantic salmon were amplified from each DNA extract by polymerase chain reaction (PCR) using fluorescently-labelled primers. The microsatellite markers used were: SP2201, Sp2210, SPG7, SP1605, SP1608, SP2216, SP3016 (Paterson et al., 2004), SsaD144, SsaD157, SsaD48, SsaD71 (King et al., 2005), Ssa14, Ssa289 (McConnell et al., 1995), Ssa202, Ssa171, Ssa197 (O'Reilly et al., 1996), SsaF43 (Sánchez et al., 1996). These 17 markers were amplified in three multiplex reactions according to the mixtures in Table 1 of this appendix. PCR reactions were conducted using the Type-it Microsatellite PCR kit (Qiagen). Cycle conditions were as follows : an initial denaturation at 95°C for 5 min followed by 32 cycles of 94°C for 30 s, annealing at either 58°C (mix A and C) or 55°C (mix B) for 90 s and extension at 72°C for 60 s. After cycling, a final extension was completed at 60°C for 30 min.

PCR products were run on a MegaBACE capillary sequencer (Amersham Biosciences) and compared against a size standard of Et Rox 550 (GE Healthcare) run along with each sample. Fragment sizes were scored with Fragment Profiler version 1.2 software (GE Healthcare). For data quality control, all results were independently checked by two people and in addition one in five results were scored “double-blind” and the results compared to calculate error rates.

Data Analysis

The results from the microsatellite marker SsaF43 allowed us to identify any trout/salmon hybrids that may be present among samples, and also any mis-identified trout. The genetic information from these individuals was then removed from further analysis.

In order to remove bias in the data due to over-representation of family groups, an analysis of family relationships was performed using the software COLONY (Wang & Santure, 2009, Jones & Wang 2010) to identify full-sibling individuals. Furthermore, this analysis allowed for a prediction as to the number of breeders that contributed to each sample. For each location sampled, all but one member of a full-sibling group were removed from analysis.

Table 1

List of microsatellites used in the genetic survey with primer sequences, multiplex mixture, final primer concentration in the PCR and the reference reporting the microsatellite locus.

Microsatellite marker	Sequence forward primers 5'-3'	Sequence reverse primers 5'-3'	Multiplex mixture	Final primer concentration (μM)	reference
Sp2201	TTTAGATGGTGGGATACTGGGAGGC	CGGGAGCCCCATAACCCTACTAATAAC	A	0.02	Paterson et al., 2004
Sp2210	AAGTATTCATGCACACACATTCACTGC	CAAGACCCTTTTTCCAATGGGATTC	A	0.02	Paterson et al., 2004
SPG7	CTTGGTCCCGTTCTTACGACAACC	TGCACGCTGCTTGGTCCTTG	A	0.02	Paterson et al., 2004
Ssa 202	CTTGGAATATCTAGAATATGGC	TTCATGTGTTAATGTTGCGTG	A	0.02	O'Reilly et al., 1996
SsaD144	TTGTGAAGGGGCTGACTAAC	TCAATTGTTGGGTGCACATAG	A	0.03	King et al., 2005
SsaD157	ATCGAAATGGAACTTTTGAATG	GCTTAGGGCTGAGAGAGGATTAC	A	0.03	King et al., 2005
Sp1605	CGCAATGGAAGTCAGTGGACTGG	CTGATTTAGCTTTTTAGTGCCCAATGC	B	0.015	Paterson et al., 2004
Sp1608	AGCACACTCATCATCTTACCTAGAG	ATGGACAGAAAGATAATGAGGG	B	0.015	Paterson et al., 2004
Sp2216	GGCCCAGACAGATAAACAAACACGC	GCCAACAGCAGCATCTACACCCAG	B	0.015	Paterson et al., 2004
Ssa171	TTATTATCCAAAGGGGTCAAAA	GAGGTCGCTGGGGTTTACTAT	B	0.015	O'Reilly et al., 1996
Ssa14	CCTTTTGACAGATTTAGGATTTC	CAAACCAAACATACCTAAAGCC	B	0.02	McConnell et al., 1995
Ssa289	GTTTCTTTACAAATAGACAGACT	TCATACAGTCACTATCATC	B	0.02	McConnell et al., 1995
Sp3016	GACAGGGCTAAGTCAGGTCA	GATTCTTATATACTCTTATCCCCAT	C	0.02	Paterson et al., 2004
Ssa197	GGGTTGAGTAGGGAGGCTTG	TGGCAGGGATTTGACATAAC	C	0.02	O'Reilly et al., 1996
SsaF43	AGCGGCATAACGTGCTGTGT	GAGTCACTCAAAGTGAGGCC	C	0.02	Sánchez et al., 1996
SsaD48	GAGCCTGTTCAGAGAAATGAG	CAGAGGTGTTGAGTCAGAGAAG	C	0.03	King et al., 2005
SsaD71	AACGTGAAACATAAATCGATGG	TTAAGAATGGGTTGCCTATGAG	C	0.03	King et al., 2005

Where there was more than one site sampled within a 5-km distance, two life-history stages (i.e. fry and parr) sampled at the same site and/or a site was sampled in different years, the data were initially tested for differences using the program CHIFISH (Ryman 2006). Where no significant differences were found, data from these sites or time points were combined; otherwise they were left separate for all further analyses.

The program MICROCHECKER (Van Oosterhout et al. 2004) was used to screen for genotyping errors and non-amplifying variants (null alleles) in the raw data. In addition markers were checked for conformity to linkage equilibrium (probability test) and Hardy-Weinberg equilibrium (exact test), as implemented by GENEPOP version 4 (Rousset, 2008). In all cases, inference of significance was corrected for multiple-testing using the false discovery rate (FDR) method (Narum, 2006). Allelic richness is an estimate of the number of genetic variants found in a sample after controlling for sample size. This was calculated using the program HP-Rare (Kalinowski, 2005), and allows an assessment of differences in genetic diversity among samples standardized to a common sample size.

The genetic structure between groups was examined using two measures of genetic differentiation - pairwise F_{ST} (calculated as θ; Weir & Cockerham 1984) calculated in the program GENETIX (Belkhir et al. 2004) and assessed for significance with permutation tests using 500 randomizations. The second measure of differentiation, pairwise Jost's D (Jost, 2008) was calculated with the program SMOGD (Crawford 2010). A pairwise matrix of both distance measures is presented in Table 2 of this appendix. A multi-dimensional scaling (MDS) plot was drawn to illustrate the relationships among sites using the Jost's D measure of differentiation.

Clustering of individuals among potential distinct groups was undertaken with STRUCTURE 2.3.3, using the admixture model with correlated alleles (Pritchard et al., 2000). Briefly, this method assumes the number of distinct groups (*K*) in turn to be from 1 to some defined upper limit (i.e. the number of sites sampled). The analysis then determines which *K* is most consistent with the observed data,and assigns each individual to one of the defined groups. Furthermore, prior information on sampling sites was used to initiate the analysis with the LOCPRIOR option available in STRUCTURE 2.3.3 (Hubisz et al. 2009). A burn-in phase of 150,000 iterations was followed by a run phase of 250,000, using a minimum of five independent runs for each number of groups (*K*) being tested. Both the log-likelihood probabilities and the delta *K* method (Evanno et al., 2005) were examined to find the most likely *K*.

The utility of the data to assign fish of unknown origin to sample site was examined by running individual assignment tests using the program ONCOR (Kalinowski et al. 2007). Assignments were conducted using the method of Rannala & Mountain (1997). Location of assignment was taken as the site with the highest probability. Only individuals with a

complete multi-locus genotype (i.e. all 17 microsatellites) were chosen for assignment as comparing criterion values for individuals with differing number of markers typed is difficult (Piry et al 2004). Caution should be used when interpreting these results as the locations used may not represent the full spread of genetic diversity or populations present within the catchment, as well as differences in sample size, may affect the results.

Appendix 2

Pairwise estimates of genetic differentiation among groups as defined in Table 1 (main text). Jost's D above diagonal, F_{ST} below diagonal. Significant pairwise F_{ST} values are indicated in italics and shaded in gray.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	-	0.079	0.030	0.056	0.077	0.039	0.032	0.066	0.173	0.032	0.031	0.065	0.027	0.033
2	*0.013*	-	0.064	0.078	0.103	0.054	0.065	0.089	0.219	0.103	0.070	0.069	0.046	0.072
3	*0.009*	*0.010*	-	0.009	0.046	0.014	0.025	0.045	0.127	0.034	0.018	0.012	0.000	0.033
4	*0.017*	*0.013*	0.002	-	0.066	0.052	0.018	0.054	0.132	0.053	0.022	0.025	0.013	0.054
5	*0.015*	*0.017*	*0.010*	*0.015*	-	0.059	0.065	0.085	0.184	0.095	0.080	0.047	0.041	0.054
6	*0.010*	*0.011*	0.002	*0.010*	*0.010*	-	0.064	0.074	0.194	0.063	0.030	0.017	0.018	0.043
7	*0.010*	*0.015*	0.007	0.011	*0.015*	*0.014*	-	0.066	0.158	0.054	0.050	0.047	0.038	0.048
8	*0.013*	*0.016*	*0.009*	*0.012*	*0.015*	*0.012*	*0.012*	-	0.145	0.089	0.037	0.054	0.055	0.061
9	*0.032*	*0.041*	*0.027*	*0.029*	*0.035*	*0.032*	*0.031*	*0.030*	-	0.164	0.126	0.154	0.141	0.185
10	*0.009*	*0.018*	*0.007*	*0.012*	*0.016*	*0.010*	*0.012*	*0.015*	*0.031*	-	0.031	0.039	0.041	0.074
11	*0.010*	*0.012*	0.004	0.006	*0.015*	*0.007*	*0.010*	*0.006*	*0.023*	*0.008*	-	0.016	0.002	0.026
12	*0.013*	*0.012*	0.003	*0.008*	*0.010*	0.002	*0.011*	*0.011*	*0.031*	*0.009*	0.004	-	0.024	0.033
13	*0.008*	*0.010*	0.001	0.005	*0.009*	0.004	*0.008*	*0.010*	*0.025*	*0.007*	0.001	0.006	-	0.015
14	*0.010*	*0.013*	*0.006*	*0.012*	*0.011*	*0.008*	*0.009*	*0.010*	*0.032*	*0.012*	*0.005*	*0.008*	*0.004*	-

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