Marine Scotland Science Report

The Scottish Government

Marine Scotland Science Report 22/12

AN OVERVIEW OF POPULATION GENETIC STRUCTURING IN GALLOWAY

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)

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Published by Marine Scotland – Science

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An Overview of Population Genetic Structuring in Galloway

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Executive Summary

This report describes how genetic information from juvenile Atlantic salmon sampled from 14 sites within six Galloway rivers (Figure 1) have been analysed in order to help inform developing fisheries management activities. The key objective for the Galloway Fisheries 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.

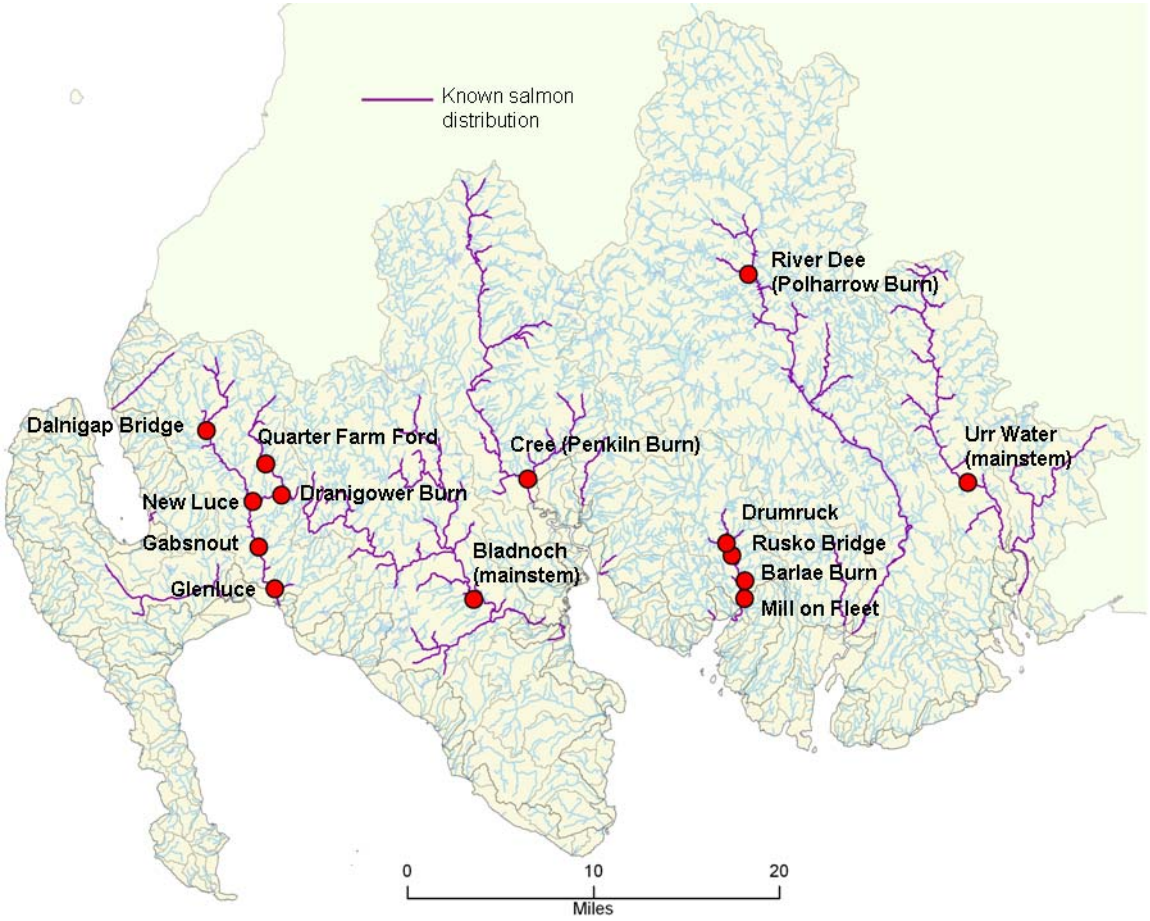

Figure 1. A map of the Galloway Fisheries Trust area, with sample sites that are involved in this report indicated in red with associated site names.

Summary of findings

The analysis showed that, most sites exhibited moderate to no genetic differences from one another with the markers used, indicating low levels of genetic structuring among these sites. The Polharrow Burn (River Dee) site was the most different and was the only location different from all others. The remaining locations showed a mixture of results when compared to each other. There appeared to be a mixture of stable vs. unstable genetic signatures over time, depending on location and method used.

Generally, differences were more pronounced between rivers. The exception was the Bladnoch and Cree rivers, which showed a closer genetic relationship, perhaps reflecting their closer proximity to one another. Only the Water of Fleet and Water of Luce allowed for an assessment of within-river genetic structuring. For the Fleet, none of the sites appeared different from one another, while the results were mixed for the Water of Luce. A more detailed examination of the Water of Luce demonstrated five times more of the genetic variation within the system was attributed to temporal differences within sites as opposed to differences among locations. Overall this suggests moderate among river differences with no or little evidence for stable within-river genetic structuring using the current genetic markers.

The observed degrees of genetic differentiation within the Galloway rivers 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 only 39%, but was on average 66% to river, with four of the six rivers assigning with 80%.

Implications for management

The aim of the current FASMOP project was to identify distinct breeding populations of salmon within six Galloway rivers. The results to date suggest that there are distinct breeding populations, represented by the different rivers. However, currently the distinction of potential populations within a river is limited. 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 moderate to weak 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 within the Galloway rivers were sampled for genetic material by the Galloway Fisheries Trust in order to inform fisheries management following methods outlined by Verspoor and Laughton (2008). Figure 1 shows the locations of the 14 sites that have been included in the genetic analysis. Samples generally consisted of fry and/or parr (n= 25-50, 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 samples sites included two life-history stages (i.e. fry and parr), each of these sub-samples were initially tested for differences 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 20 samples for subsequent analyses.

Data was 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 Galloway Fisheries Trust.

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

Results

Broadly speaking, most samples exhibited weak to moderate genetic differences from one another, indicating varying 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 summarising the main genetic findings in terms of population genetic structuring within and among rivers in Galloway.

Family effects

A total of 669 juvenile salmon were involved in the genetic analysis. All sites were examined for family effects with relatively few samples being removed due to full-sibling relationships, with the exceptions being the Dalnigap Bridge and Drumruck sites (Table 1). The level of family effects differed between samples with the largest family group present in the individual samples ranging from 1 to 7 full-siblings and sample sizes subsequently being reduced by 0-44%. Family effects were controlled for at each site before all further analyses. No individuals were identified as either trout or salmon/trout hybrids at any location.

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
Dalnigap Bridge	1	50	28	36	7	2008
New Luce	2	50	45	59	3	2008
Gabsnout	3	49	44	57	4	2008
Glenluce‡	4	49	48	62	2	2008
Dranigower Burn‡	5	50	43	53	3	2008
Quarter Farm Ford	6	50	47	54	3	2008
Bladnoch‡	7	48	44	64	3	2008
Penkiln Burn‡	8	50	48	57	3	2008
Drumruck	9	50	34	39	7	2008
Rusko Bridge‡	10	50	44	61	3	2009
Barlae Burn	11	25	25	36	1	2009
Mill on Fleet	12	50	44	56	3	2008
Polharrow Burn	13	50	44	47	3	2008
Urr Water‡	14	48	46	61	2	2008

‡ This site contained equal numbers of fry and parr, which were combined as there was no significant difference between them.

Population structuring

All sites contained samples of approximately equal numbers of fry and parr collected in the same year with the exception of the Barlae Burn site, which contained only parr. The CHIFISH analysis showed a mixture of significant and non-significant differences between age classes, depending upon site. Fry and parr were not significantly different from one another within the following sites: Glenluce, Dranigower Burn, Bladnoch, Penkiln Burn, Rusko Bridge and Urr Water. In these six cases, fry and parr from a given location were combined for further analyses. All other locations showed significant differences between age-classes and were therefore kept separate for subsequent analyses. However, the pairwise comparisons based on a genetic differentiation measure (F_{ST}; Appendix 2) between the fry and parr were all non-significant, with the exception of the Polharrow Burn (River Dee) site. The difference between these tests and the CHIFISH analysis likely reflects the difference in power between the two approaches.

The genetic differences among samples show a small to moderate range in the magnitude of genetic differentiation, with 68% (129 out of 190) of the pairwise comparisons being significantly different (Appendix 2). Among these comparisons, the Polharrow Burn (fry and parr) was the only site that was different from all others based on pairwise measures of differentiation (Appendix 2). Most of the other sites show a mixture of significant and non-significant genetic differences to one another. Particularly noticeable from the Jost's *D* measure of differentiation (top half of the matrix, Appendix 2) is a larger degree of differentiation among sites from different rivers (average Jost's $D = 0.081$) compared to sites within the same river from the Water of Luce and Water of Fleet (average Jost's $D = 0.021$). In fact, none of the pairwise comparisons within the Water of Fleet were significantly different from one another, while comparisons among sites within the Water of Luce showed a mixture of significant and non-significant differences. However, these differences were still generally smaller than differences to other rivers. Furthermore, a hierarchical AMOVA conducted on the Water of Luce samples, revealed five times more variation explained by temporal differences (e.g. fry vs. parr) within sites than differences among sites within this system.

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. As can be seen, sites from the Water of Luce (red) tend to group together as do sites within the Water of Fleet (blue), with the Rusko bridge site being the most differentiated in the latter. Furthermore, from Figure 2, it can be seen that fry and parr from a given site are generally quite close together, with the exception of the Polharrow

Burn site, which was the only case where this difference was significant by CHIFISH and the pairwise comparisons (Appendix 2).

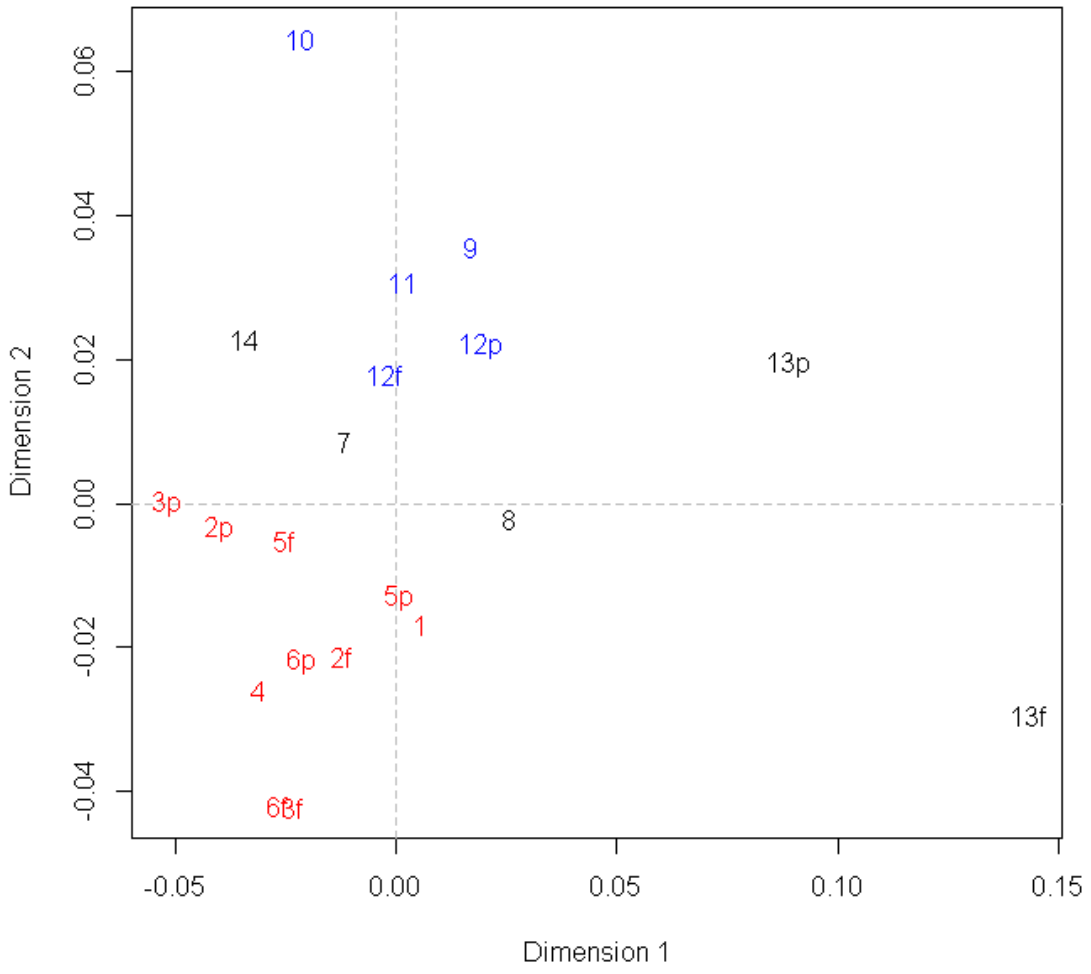

Figure 2. Multi-dimensional scaling (MDS) plot of genetic relationships among all sites based on pairwise estimates of genetic differentiation (Jost's D; see the appendix for details). Points which are closer together on the plot have a more similar genetic makeup while points further apart are more genetically discrete. Samples in red are from the Water of Luce, while samples in blue are from the Water of Fleet. Sample codes as in Table 1 (f = fry, p = parr).

A clustering analysis that explores possible groupings of individuals other than the defined sampling sites was also carried out. This 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. This analysis was done in a hierarchical fashion, as larger

genetic differences among groups may obscure weaker differences at smaller scales. For the sites in Galloway, this analysis determined the most likely number of groups to be two. These two groups corresponded to (1) all six sites within the Water of Luce and (2) all remaining sites. Sites from the Water of Luce were then removed from the analysis and the procedure repeated on the second group 2. The analysis at this second level resulted in the most likely number of groups to be three, corresponding to (1) sites from the Water of Fleet, (2) the River Dee (Polharrow) site, and (3) a group composed of the Bladnoch site, Cree (Penkiln Burn) site, and the Urr Water site. Finally this latter group was re-run in a third round of analysis and defined two groups: (1) the Urr Water site and (2) the Bladnoch and Cree sites. A schematic representing the different levels of clustering and the final groupings is shown in Figure 3.

For both the Water of Luce and Water of Fleet, the clustering analyses were run on these groups separately to see if any within-river groups could be identified. In both cases, the most likely number of groups was determined to be one. This does not necessarily mean, however, that there are not significant genetic differences, but that using this clustering approach, these small differences are more difficult to tease apart and the distinction for splitting individuals into more than one group is less obvious.

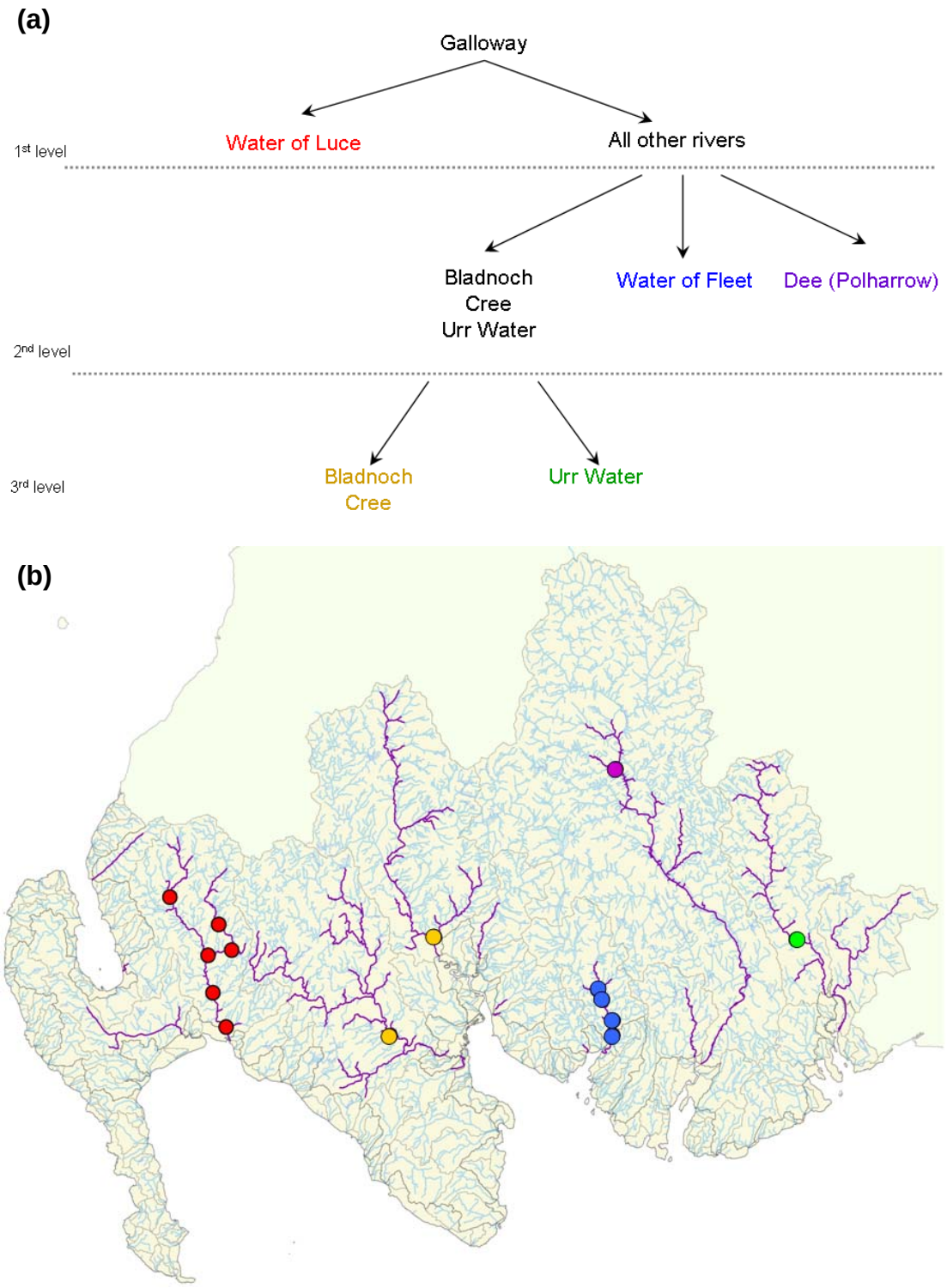

Figure 3 (a) Schematic showing the order in which the different clusters or groupings were resolved according to the hierarchical analysis. (b) Geographic representation of the relationships among sites, following a cluster analysis (STRUCTURE; see appendix). Locations with the same colour are more similar 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 39% of the time (Figure 4a). While this average is greater than would be expected if assignments were purely random (14 sites, random = ~7%), this may reflect the weak 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. A second level of assignment was conducted whereby fish were assigned to a river, rather than a site, as river-level differences may be more pronounced. Average assignment to the six rivers was 66%, which again is higher than expected by random assignment (~17% for six rivers). Assignment was lowest to the Bladnoch and Cree rivers, while the remaining four rivers all showed levels of assignment just below 80%.

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 Galloway rivers, a cut-off does not appear to drastically improve assignments. For example, if we assign only fish that have a minimum of 70% assignment probability, overall correct assignment increases somewhat to site (now 45%) and only marginally to river (now 69%). Applying such a cut-off comes at a potential cost as not all fish in the baseline will be assigned. However the above example for the Galloway rivers (70% cutoff) still resulted in over 75% of fish being assigned.

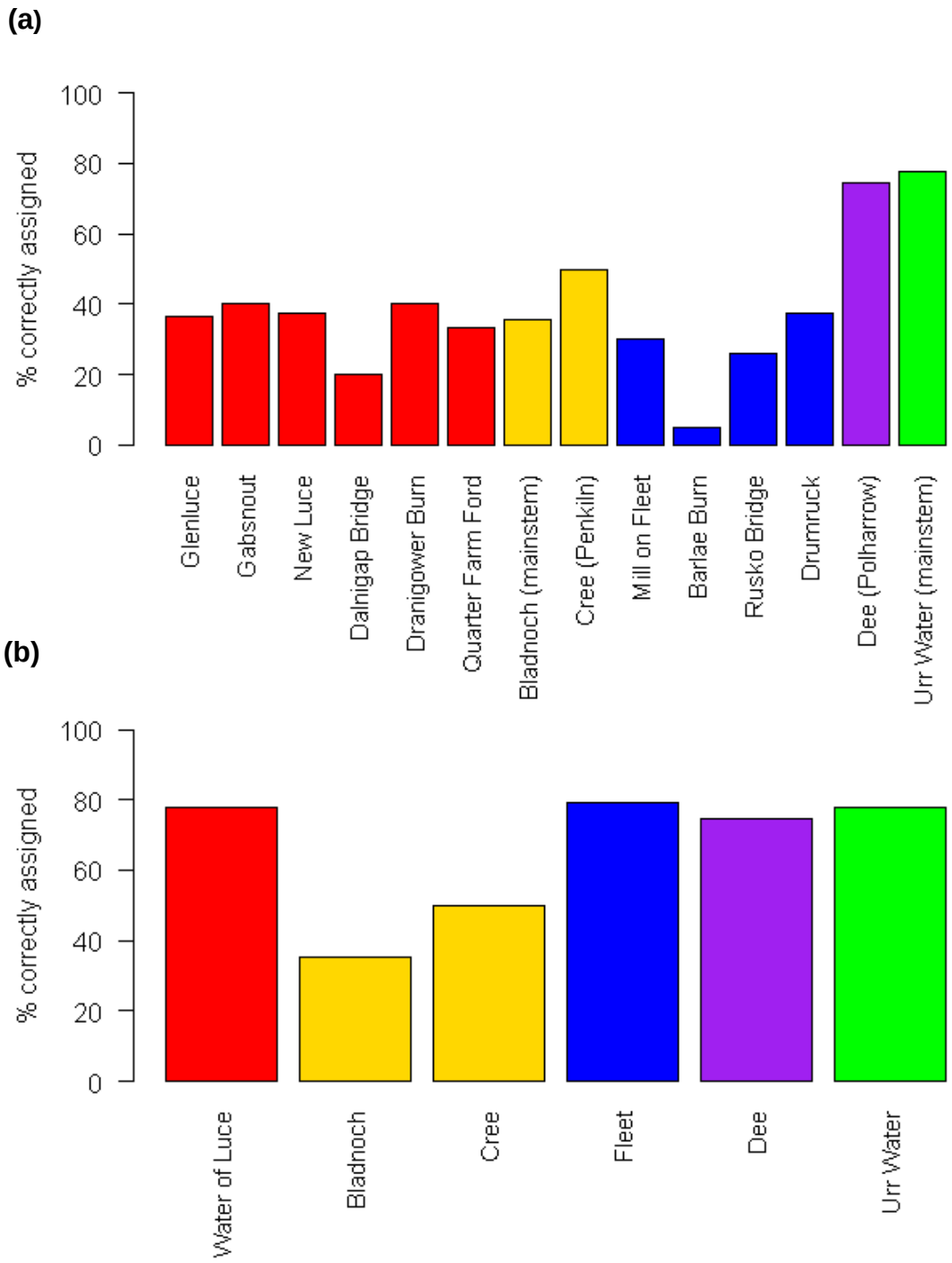

Figure 4. Percentage of fish sampled from each site that correctly assign back to (a) the site where they were sampled or (b) the river where they were sampled.

Discussion

Population structure

The aim of the FASMOP project for the Trust was to identify distinct breeding populations of salmon. The results to date suggest that there are distinct breeding populations within the Galloway rivers. While most sites were significantly different from one another, there was evidence of differences among years within a site (fry vs. parr), suggesting some degree of temporal variation.

A couple of locations had moderate family effects, notably the Dalnigap Bridge and Drumruck sites suggesting fewer numbers of breeders at these sites. Additional sampling at these locations in subsequent years would provide insight as to whether such a result is due to sampling conditions or reflects real biological signatures at these locations. This could be used to assess trends in the numbers of fish breeding at various locations over time.

The clustering analysis demonstrated river-level differences, with the exception of the Bladnoch and Cree rivers. The closer similarity between these latter two rivers may not be surprising given that they share an estuary and are each others closest neighbour. With the exception of the Water of Luce and the Water of Fleet, the four other rivers were represented by only a single sampling site each. Therefore assessment of within-river differences was restricted to the two aforementioned rivers. The clustering analysis in both cases, failed to resolve any within-river differences. While none of the pairwise differences within the Fleet were significant, several comparisons were within the Water of Luce, however the AMOVA analysis demonstrated more temporal variation within sites than among sites for this system. This type of pattern supports the idea of weak meta-population structuring within the system, whereby spatially separated populations are connected by different degrees of interactions or exchange of individuals over time. This is also suggested by the varying results of temporal stability for all systems, depending upon the CHIFISH or F_{ST} (Appendix 2) results. Further sampling, particularly within those rivers represented here by a single sample site, may help to clarify the degree of temporal vs. spatial structuring and may further resolve any differences between the Bladnoch and the Cree rivers.

While a lack or weak level of differentiation, particularly within rivers, may be the result of moderate levels of exchange of spawning adults among sites, caution should still be used in making such an interpretation. This lack of 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.

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 adults to their particular river or 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 4 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%). The average value of correct assignment to site is 39% (Figure 4a), which is somewhat higher than one would expect if there was no genetic structure in the data. While this supports the conclusion that there may be genetic differentiation among some locations, indicative of separate breeding populations, the data does not at present have the power to assign fish of unknown origin (e.g. rod caught adults) to their specific location with higher accuracy. However, assignment to river was generally, much improved with the Luce, Fleet, Dee and Urr Water assigning on average 80% correct (Figure 4b). The site vs. river-level assignments for the Bladnoch, Cree, Dee and Urr Water are the same as they are represented by single sites. However, the assignment to the Water of Luce and Water of Fleet increased from approximately 40% or less to 80%, suggesting that many fish that were misassigned to specific sites were still being assigned to other sites in the same

river. This suggests it may be possible to use such river-level differences in assessment of mixed-stock fisheries or individual assignment to river of origin.

In order to improve assignments for all rivers and possibly gain better distinction for potential breeding populations within rivers, more samples and/or additional genetic markers may be required. 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. This incomplete baseline may explain, in part, the higher misassignment rates between the Bladnoch and Cree. 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 among rivers in Galloway, the level of differentiation with the current marker set is weak and prevents more robust conclusions regarding within system population structuring. Such an outcome is not unique to the Galloway rivers, but is observed in several other systems throughout Scotland. Before it can be concluded that there are little to no genetic differences within these 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. 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 events on the levels of differentiation.

Summary

This analysis demonstrated overall moderate levels of population structuring among most rivers with weak to no structuring observed within rivers, for the two rivers that could be assessed. The results suggest that the different rivers represent distinct breeding populations however, the degree of differences within rivers is not sufficient to allow for robust application to management at this scale presently. For the Water of Luce, the low levels of within-river structuring observed were explained more by temporal variation than that among different sample sites. Clearly more work is needed to clarify the extent of genetic structuring within the Galloway rivers. 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, assist in 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^{o}C for 5 min followed by 32 cycles of 94^{o}C for 30 s, annealing at either 58^{o}C (mix A and C) or 55^{o}C (mix B) for 90 s and extension at 72^{o}C for 60 s. After cycling, a final extension was completed at 60^{o}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.

For the Water of Luce sites a hierarchical AMOVA (Analysis of Molecular Variance) was conducted using the program Arlequin version 3.5.1.2 with 10,000 permutations (Excoffier & Lischer 2010). Given that each of the six sites sampled within the Water of Luce contained two age-classes (fry and parr), this allowed for a comparison of the relative influence of temporal vs. spatial genetic differentiation within this system.

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; f=fry, p=parr). Jost's D above diagonal, F_{ST} below diagonal. Significant pairwise F_{ST} values are indicated in italics and shaded in gray.

	1	2f	2p	3f	3p	4	5f	5p	6f	6p	7	8	9	10	11	12f	12p	13f	13p	14
1	-	0.022	0.027	0.060	0.057	0.032	0.011	0.000	0.025	0.003	0.060	0.032	0.062	0.069	0.068	0.036	0.043	0.121	0.102	0.051
2f	0.006	-	0.043	0.014	0.021	0.003	0.017	0.000	0.031	0.005	0.063	0.051	0.059	0.070	0.054	0.032	0.059	0.136	0.130	0.048
2p	*0.007*	0.009	-	0.036	0.059	0.019	0.048	0.017	0.036	0.028	0.079	0.084	0.061	0.081	0.063	0.051	0.027	0.191	0.125	0.063
3f	*0.013*	0.005	0.011	-	0.023	0.023	0.038	0.018	0.043	0.010	0.068	0.079	0.077	0.100	0.064	0.039	0.081	0.150	0.152	0.103
3p	*0.011*	0.005	*0.013*	0.005	-	0.020	0.026	0.016	0.065	0.002	0.087	0.047	0.065	0.092	0.063	0.039	0.044	0.212	0.122	0.088
4	*0.006*	0.001	*0.007*	*0.009*	*0.006*	-	0.021	0.002	0.054	0.017	0.057	0.054	0.081	0.091	0.062	0.056	0.062	0.162	0.145	0.047
5f	0.002	0.003	0.008	0.008	0.003	0.003	-	0.002	0.023	0.004	0.077	0.058	0.051	0.056	0.046	0.057	0.037	0.165	0.125	0.054
5p	0.001	0.007	0.014	*0.016*	0.008	0.006	-0.002	-	0.021	0.002	0.054	0.020	0.049	0.059	0.028	0.035	0.038	0.123	0.103	0.045
6f	*0.010*	*0.010*	*0.010*	*0.015*	*0.016*	*0.011*	0.006	*0.016*	-	0.011	0.090	0.087	0.092	0.115	0.094	0.072	0.048	0.172	0.131	0.079
6p	0.003	0.002	0.006	0.003	0.002	0.004	-0.002	0.007	0.004	-	0.064	0.053	0.047	0.085	0.083	0.061	0.037	0.161	0.117	0.056
7	*0.011*	*0.014*	*0.017*	*0.016*	*0.017*	*0.011*	*0.012*	*0.016*	*0.018*	*0.011*	-	0.004	0.077	0.076	0.045	0.024	0.045	0.161	0.141	0.085
8	*0.007*	*0.012*	*0.018*	*0.017*	*0.012*	*0.009*	0.009	0.011	*0.019*	*0.011*	0.004	-	0.056	0.092	0.031	0.046	0.017	0.106	0.091	0.078
9	*0.012*	*0.010*	0.010	*0.014*	*0.015*	*0.014*	0.009	0.014	*0.021*	0.009	*0.015*	*0.015*	-	0.013	0.005	0.016	0.024	0.132	0.069	0.074
10	*0.012*	*0.012*	*0.015*	*0.018*	*0.018*	*0.014*	*0.010*	*0.012*	*0.021*	*0.015*	*0.013*	*0.016*	0.003	-	0.000	0.007	0.021	0.182	0.140	0.056
11	*0.013*	*0.013*	*0.015*	*0.016*	*0.016*	*0.012*	0.008	0.011	*0.022*	*0.015*	*0.010*	0.009	0.001	0.002	-	0.000	0.013	0.141	0.121	0.080
12f	*0.008*	0.007	*0.014*	0.010	*0.010*	*0.011*	0.008	0.010	*0.017*	*0.011*	0.005	0.009	0.007	0.003	-0.001	-	0.023	0.145	0.108	0.087
12p	*0.008*	*0.013*	*0.009*	*0.015*	*0.010*	*0.011*	0.008	0.008	*0.018*	*0.010*	*0.010*	0.006	0.006	0.005	0.007	0.007	-	0.114	0.056	0.040
13f	*0.028*	*0.029*	*0.039*	*0.032*	*0.038*	*0.031*	*0.035*	*0.028*	*0.043*	*0.033*	*0.032*	*0.023*	*0.029*	*0.034*	*0.032*	*0.033*	*0.028*	-	0.077	0.190
13p	*0.020*	*0.027*	*0.028*	*0.029*	*0.024*	*0.026*	*0.023*	*0.018*	*0.032*	*0.024*	*0.029*	*0.019*	*0.020*	*0.027*	*0.028*	*0.024*	*0.017*	*0.019*	-	0.136
14	*0.013*	*0.009*	*0.013*	*0.018*	*0.016*	*0.011*	0.011	0.022	*0.018*	0.009	*0.018*	*0.018*	*0.015*	*0.013*	*0.020*	*0.017*	*0.010*	*0.035*	*0.030*	-

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