Marine Scotland Science Report

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Marine Scotland Science Report 18/12

AN OVERVIEW OF POPULATION GENETIC STRUCTURING IN AYRSHIRE'S RIVERS FOR AYRSHIRE RIVERS 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)

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An Overview of Population Genetic Structuring in the Ayrshire Rivers Trust

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Executive Summary

This report describes how genetic information from juvenile Atlantic salmon sampled from 28 sites within the Ayrshire Rivers Trust area (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.

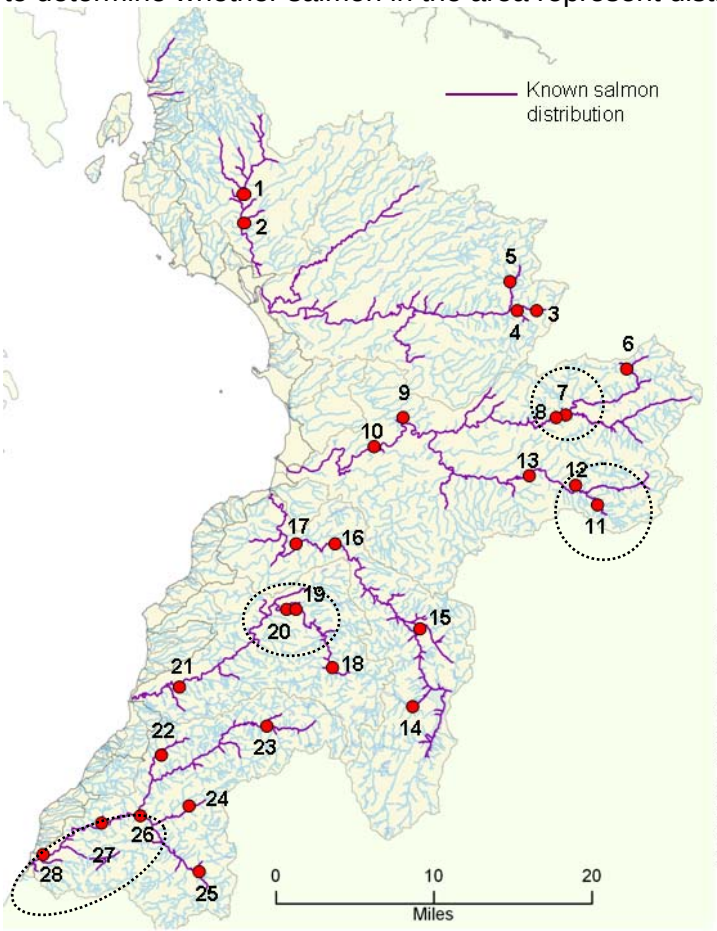

Figure 1. A map of the Ayrshire Rivers Trust area, with sample sites included in this report indicated in red with associated site names. Sites enclosed in ellipses were grouped for analysis (see main text).

Summary of findings

The analysis showed that, most sites exhibited weak to no genetic differences from one another with the markers used, indicating low levels of genetic structuring among these sites. The Glenoul and Garpel Burn sites were the most different and several sites were severely affected by the presence of small numbers of breeders. The remaining locations showed smaller levels of differentiation, with a tendency for the River Ayr in particular to be more distinct than the others. For two locations, which were sampled in different years, there appeared to be stable genetic signatures over time. Overall this suggests a stable, but very weak level of population genetic structuring within and among rivers in the Ayrshire Rivers Trust area using the current genetic markers.

This weak degree of genetic differentiation observed among sites 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 25% (or 47% to river), 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 is not large enough to assign fish with higher accuracy

Implications for management

The aim of the current FASMOP project was to identify distinct breeding populations of salmon within the Ayrshire rivers that were sampled. The results to date suggest that there *may* be distinct breeding populations. However, currently the distinction of these potential populations 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 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 throughout Ayrshire 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 28 sites that have

been included in the genetic analysis. Samples generally consisted of fry and/or parr (n= 5-54, 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 sites sampled close together and/or nearby sites with low sample sizes, 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 23 samples for subsequent analyses (see Table 1 and Figure 1).

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 no significant genetic differences from one another, indicating 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 Ayrshire Rivers Trust.

Family effects

A total of 714 juvenile salmon from the six Ayrshire River catchments sampled 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 exception of a few sites [e.g. Garpel Burn & Muck Water (Stinchar); Table 1]. The level of family effects differed between samples with the largest family group present in the individual samples ranging from 0 to 18 full-siblings and sample sizes subsequently being reduced by 0-71%. Family effects were controlled for at each site before all further analyses. There was a single trout and one salmon/trout hybrid sample identified across all locations (Table 1).

Population structuring

Several sites were sampled in close proximity and in some cases, had small sample sizes and/or were sampled in different years. These sites were tested for differences in the frequencies of genetic variants prior to analysis (using the program CHIFISH). The CHIFISH analysis showed no significant differences between the Limmerhaugh (2009) samples and the Ayr midreaches (2010) sample and so were combined for subsequent analyses. The same was true for three other groups of sites: (1) the Glenmuir (2003) and Guelt Waters (2003), (2) Blairquhan (2005) and the mainstem Girvan (2008) and (3) the three sites on the Stinchar mainstem (all sampled in 2003). These four groupings are reflected in Table 1 and Figure 1. On the other hand, the three sites on the upper Irvine (all sampled 2003) showed significant differences from one another.

The genetic differences among sites show a small range in magnitude of genetic differentiation, with 67% (170 out of 253) of the pairwise comparisons being significantly different (Appendix 2). Most sites show a close genetic relationship with a mixture of significant and non-significant comparisons to all other sites (Appendix 2). 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.

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	Code for analysis	Original sample size	Sample size analysed (sibs removed)	Number of breeders contributing to sample	Largest single family	Year sampled
Garnock (mainstem)	1	20	20	36	1	2003
Dusk Water	2	16	16	26	1	2003
R. Irvine (mainstem)*	3	5	n/a	n/a	n/a	2003
R. Glenoul	4	19	15	26	2	2003
Glen Water	5	16	16	25	1	2003
Greenock Water[1]	6	30	21	31	5	2002
Upper Ayr† (Limmerhaugh/ midreaches)	7	50/42	47/36	62/51	3/7	2009/2010
Stairaid/Barskimming	8	42	38	55	4	2010
Lower Ayr (Stair dam)[2]	9	49	45	64	3	2009
Glenmuir & Guelt Waters‡	10	40	40	69	1	2003
Lugar Water	11	41	40	53	2	2010
Garpel Burn	12	31	9	12	17	2008
Muck Water (Doon)	13	27	22	32	3	2005
R. Doon (Skeldon Mills)	14	29	29	49	1	2005
R. Doon (Holms)	15	13	13	25	1	2008
R. Girvan (Balbeg)	16	31	28	48	2	2005
R. Girvan (mainstem)§	17	48	47	77	2	2003/ 2005
R. Girvan (Cairnhill)	18	31	31	49	1	2004
Lower Water of Assel	19	24	19	30	3	2004/ 2005
Stinchar (Balloch)	20	31	29	46	2	2004
Muck Water (Stinchar)	21	30	9	13	18	2003
Duisk River	22	37	32	47	3	2005
Stinchar¶	23	54	52	71	2	2003

1. One sample from this location was identified as a trout.
2. One sample from this location was identified as a salmon/trout hybrid.

* Due to such low sample size, this location was not analyzed for family structure.

† Samples from Limmerhaugh and midreaches were combined for analysis.

‡ Samples from Glenmuir & Guelt Waters were combined for analysis.

§ Samples from Blairquhan and the River Girvan (mainstem) were combined for analysis.

¶ Samples from the lower, middle & upper Stinchar mainstem were combined for analysis.

Note: Sample codes in the above table refer to the codes in Figure 2 and Appendix 2 based upon the analysis and *not* Figure 1 showing sampling locations.

The largest differences are seen to the Garpel Burn (Doon) and the River Glenoul (Irvine) as both of these are plotted furthest apart from the central cluster of the remaining sites (Figure 2). The central cluster represents all remaining sites from all six river catchments within the Ayrshire Rivers Trust area. As can be seen in the plot, there is overlap among rivers, for example, the two Garnock sites are not closest together but instead are each plotted closer to sites within the Girvan, Doon or Stinchar. On the other hand, there is a tendency for sites of some rivers to group closer together, such as the six sites within the River Ayr (all to the right and above the origin of the plot). This is also somewhat true for the Stinchar.

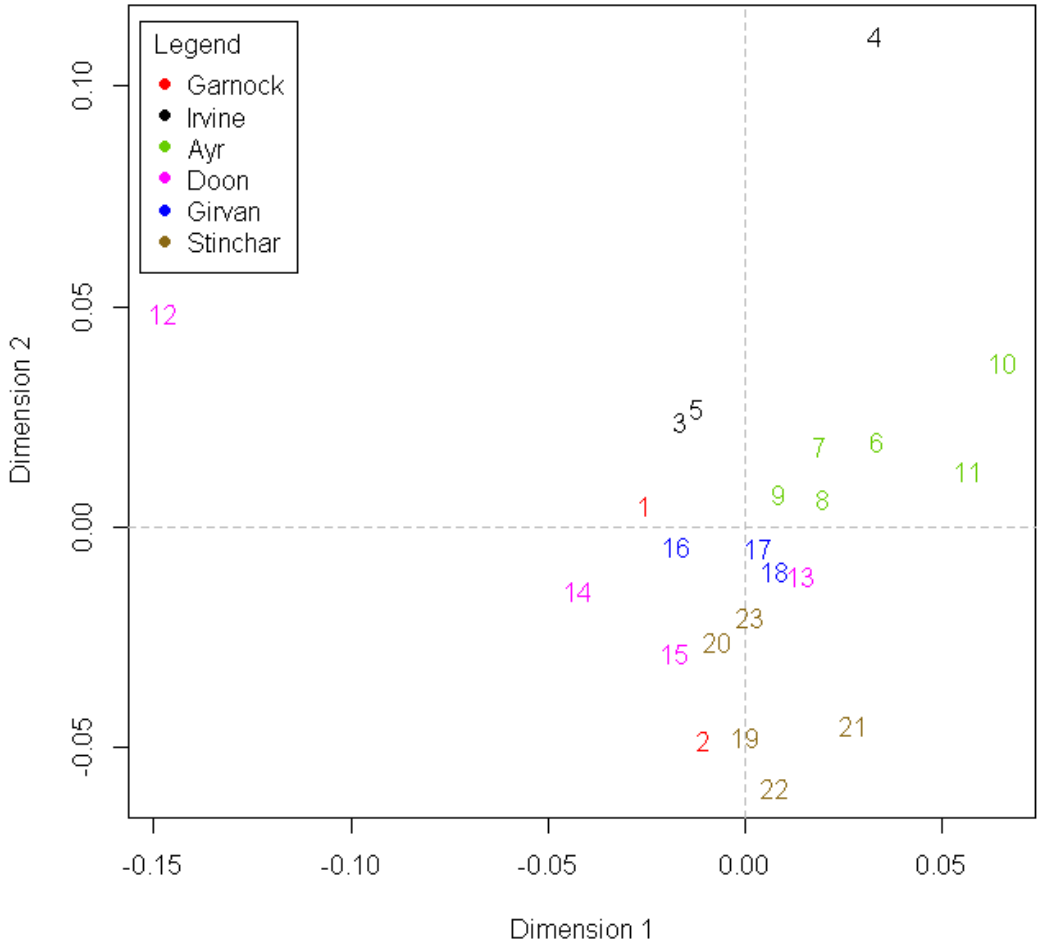

Figure 2. Multi-dimensional scaling (MDS) plot of genetic relationships among all sites based on pairwise estimates of genetic differentiation (Jost's D; see the appendix for details). Points which are closer together on the plot have a more similar genetic makeup while points further apart are more genetically discrete. Sample sites are colour-coded by river to assist visualization of river-level groups. [**Note:** the sample IDs presented here refer to the codes in Table 1 and *not* the site numbers in Figure 1.]

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. For the current sites, this analysis determined the most likely number of groups to be two. These two groups (Figure 3, orange vs. green) generally corresponded to 1) the Irvine and Ayr rivers and 2) the Garnock, Doon, Girvan and Stinchar. Several of the sites were an even split between the two groups and was due to a lack of ability to assign those sites to one group or the other (represented by a mixture of the two colours in Figure 3).

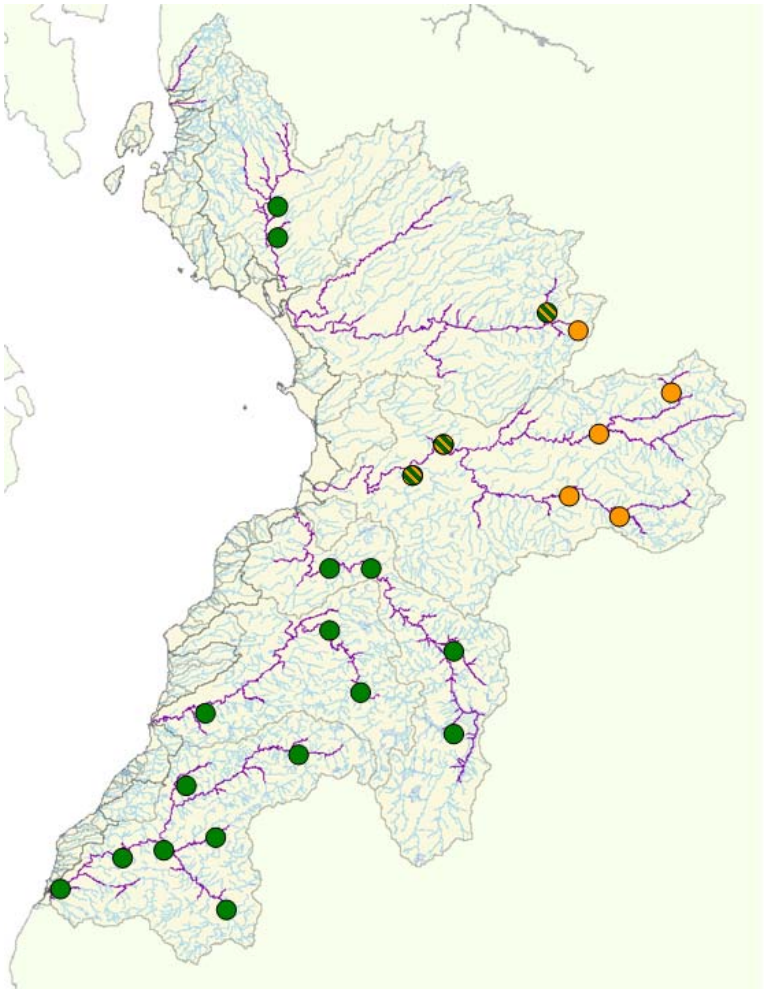

Figure 3. Geographic representation of the relationships among sites, following a cluster analysis (STRUCTURE; see appendix). Locations with the same colour are more similar to one another and belong in the same cluster. Locations with a mixture of the two colours are due to a lack of ability to assign that site to one cluster or the other.

A second round of clustering was performed on either the ‘orange’ group separately or the ‘green’ group separately to determine if further structure within either group could be resolved using this approach. 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 below this level, but that using this clustering approach, these small differences we observe here are more difficult to tease apart and the distinction for splitting individuals into more than one group is less obvious.

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. 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 25% of the time (Figure 4a). While this average is greater than would be expected if assignments were purely random (23 sites, random = ~4%), 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. Furthermore, several individual sites do in fact show an assignment success close to the random level or no assignments back to that site at all (e.g. Garnock (main), Glen Water (Irvine), Irvine (main) and Assel; Figure 4a).

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. In other words, while an individual may not assign back to the particular site from which they were sampled, they may still be assigning to other sites within the same river. Within the Ayrshire Rivers Trust sampled areas, average assignment to the six rivers was 47% (Figure 4b), which again is higher than expected by random assignment (16% for six rivers). Correct river-level assignment is highest to the River Ayr (~80%) and lowest for the River Irvine (~12%) with the remaining rivers assigning between ~15%-50% (Figure 4b).

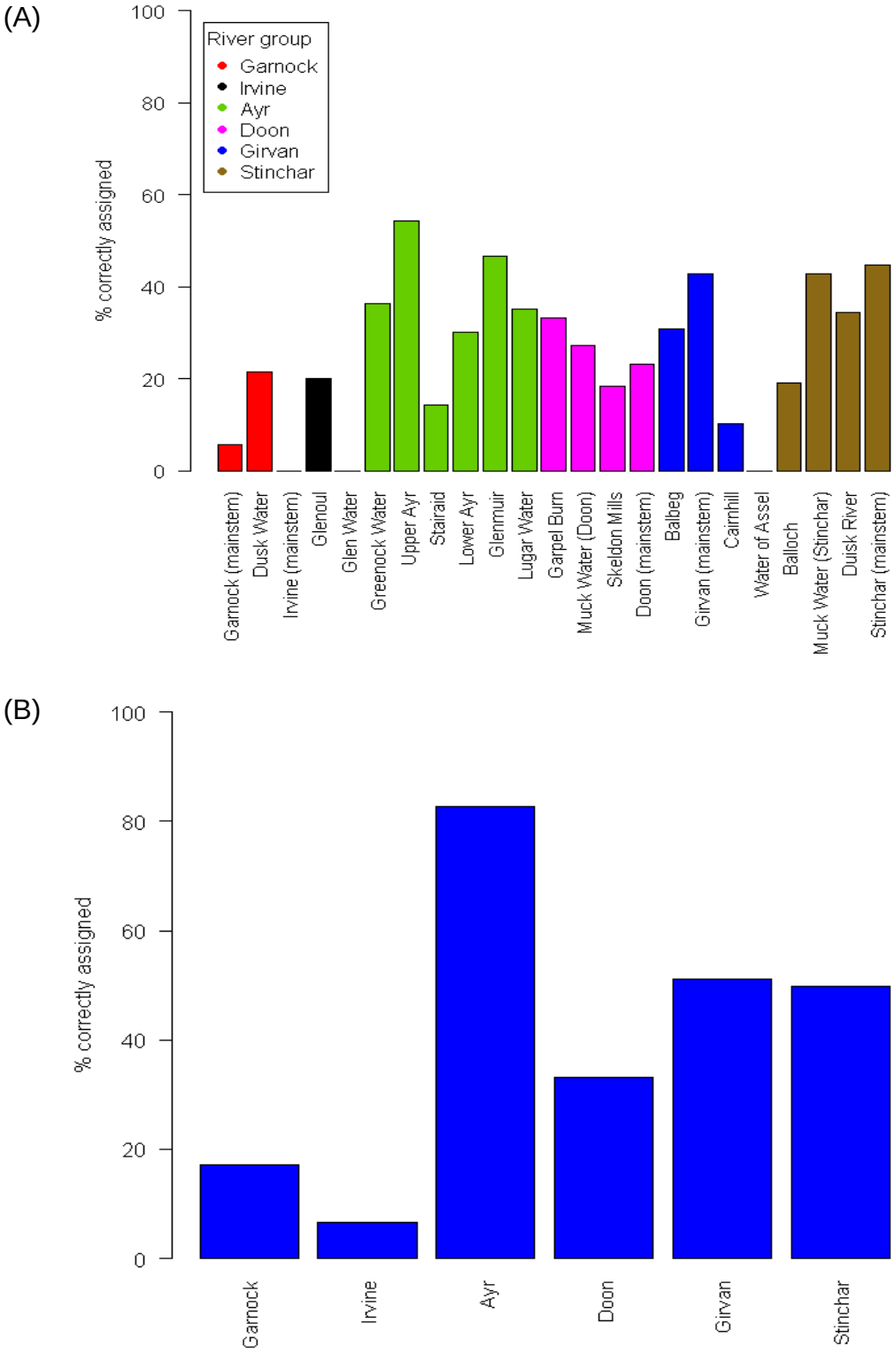

Figure 4. Percentage of fish sampled from each site that correctly assign back to that site.

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 Ayrshire rivers sampled, 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 improves only slightly (32% versus 25% previously). 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 Ayrshire rivers (70% cutoff) still resulted in 69% of fish being assigned. This suggests that many fish are being assigned, with high probability, to sites other than those at which they were sampled. Furthermore, the 70% cutoff did not change the overall average assignment to river level.

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 *may be* distinct breeding populations within the Ayrshire rivers included in this study. However, using our current set of genetic markers the distinction of these potential breeding populations is not easily defined. This is due to the low magnitude of differences observed among sites, and is reflected in part by the low accuracy of trying to assign individuals to the location from which they were originally sampled.

The Glenoul (Irvine) and Garpel burn (Doon) sites were the most distinct sites based on pairwise comparisons (Figure 2; Appendix 2). The Glenoul site was plotted far apart from the other two sites on the Irvine, however all had relatively small sample sizes and pairwise measures of differentiation (based on F_{ST}; Appendix 2) were not significantly different. This was in contrast to the CHIFISH results and likely to be associated with the different statistical power of the two tests. The Garpel burn site was severely affected by full-siblings (Table 1). This finding was not surprising as a survey of the whole stream was necessary to collect the sample (B. Shaw, personal communication). The Muck Water site (Stinchar) was also severely affected by full-siblings and while field observations of this outcome were less obvious than the Garpel burn site, the sample was taken within approximately one kilometre of the top of the system. Interestingly, however, the Muck Water site did not separate out on the MDS plot (Figure 2) and grouped quite closely with the remaining sites on the Stinchar.

The remaining sites tended to group toward the centre of the MDS plot (Figure 2). There was, however, a tendency for sites on the River Ayr to group together and to a lesser

extent, the sites on the Stinchar. Sites from the Garnock, Doon and Girvan all plotted close together and not always with sites from the same river system.

Two locations allowed for an assessment of the temporal stability of the genetic signature at that particular location. The Upper Ayr samples (midreaches & Limmerhaugh) were sampled very close together in different years (2009 & 2010), as were the Blairquhan and mainstem sites on the River Girvan (2003 & 2005). In both cases the two timepoints were not significantly different from one another, suggesting temporal stability on small spatial scales, at least where this was investigated. More temporal samples would need to be screened to determine if such a pattern of temporal stability is widespread throughout the systems. This suggests that there is a stronger signal of differentiation among sites (albeit still quite weak) compared to a given site over time. 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.

The clustering analysis (Figure 3) was consistent with little to weak structuring across sites, however it identified the most likely number of groups to be two. This does not necessarily mean, however, that there are not significant genetic differences below this level, but that using this approach any further genetic differences among sites are more difficult to tease apart. One of the groups involved sites from the Ayr and Irvine rivers. Among the Ayr sites, it was primarily the most upstream sites that were in this grouping with sites further downstream indicating a more mixed membership between the two groups. This distinctiveness of the River Ayr in particular, is consistent with the high level of assignment seen to this river (see below). The Upper Ayr was largely blocked to fish passaged until the 1980s, at which point the Ayr side of the catchment was heavily stocked with salmon fry from the Lugar side of the catchment. It may be the case that founder effects could be contributing to the more distinct genetic signature of the Upper Ayr catchment.

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%). The average value of correct assignment to site is 25% or to river is 47% (Figure 4), which is somewhat higher than one would expect if there were 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 do not at present have the power to assign fish of unknown origin (e.g. rod caught adults) to their location with high accuracy. Assignment to the River Ayr is quite high (~80%) reflecting the grouping of sites from this river on the MDS plot (Figure 2) and with the clustering analysis (Figure 3). This is likely a result, at least in part, of the widespread coverage for this system, compared to others as well as relatively larger sample sizes. More robust coverage of other systems with increased sample sizes may help to improve assignments to those rivers. However, it is likely that additional genetic markers will also need to be utilized.

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. The assignment of individuals in the analysis was only to sites represented in the baseline. If the ‘true’ site has not been sampled, fish from these missing sites will be assigned incorrectly to some site that is in the baseline. For the most accurate and complete picture of assignments within a system, detailed knowledge of *all* breeding groups defined by the set of markers used is required, so these would need to be resolved first. 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 suggestion of genetic structuring within and among the Ayrshire rivers, the level of differentiation with the current marker set is weak and in prevents more robust conclusions. Such an outcome is not unique to Ayrshire, but is observed in several other systems throughout Scotland. However, 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 levels of differentiation.

Summary

This analysis demonstrated overall weak levels of population structuring within and among Ayrshire rivers. The results suggest that there *may* be distinct breeding populations however the degree of these differences is not sufficient to allow for robust application to management at present. For several of the sites where there was temporal sampling, the analysis suggests that there is stability of the genetic make-up at these sites as they were less differentiated across years than they were to other sites. Clearly more work is needed to clarify the extent of genetic structuring within and among Ayrshire 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, 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^{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.

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	15	16	17	18	19	20	21	22	23
1	-	0.019	-0.003	0.083	0.020	0.075	0.046	0.019	0.001	0.095	0.082	0.107	0.006	0.001	0.020	0.043	0.025	0.011	0.011	0.000	0.086	0.035	0.019
2	0.007	-	0.001	0.180	0.065	0.079	0.051	0.077	0.019	0.102	0.060	0.155	0.053	0.049	0.045	0.044	0.048	0.055	0.031	0.042	0.077	0.057	0.058
3	-0.005	0.011	-	0.007	-0.005	0.011	0.000	0.000	-0.012	0.007	0.017	0.073	0.001	-0.009	0.001	-0.001	-0.001	0.000	0.000	-0.001	0.012	0.003	-0.002
4	*0.038*	*0.056*	0.033	-	0.052	0.087	0.050	0.076	0.066	0.073	0.071	0.176	0.097	0.133	0.143	0.111	0.104	0.095	0.157	0.134	0.148	0.167	0.124
5	0.013	*0.024*	0.006	0.022	-	0.060	0.014	0.021	0.032	0.084	0.090	0.121	0.040	0.033	0.082	0.050	0.072	0.051	0.049	0.075	0.073	0.075	0.056
6	*0.022*	*0.022*	0.014	*0.037*	*0.019*	-	0.036	0.011	0.036	0.065	0.049	0.186	0.092	0.081	0.053	0.051	0.074	0.076	0.052	0.080	0.117	0.102	0.082
7	*0.009*	*0.011*	0.004	*0.033*	*0.009*	*0.009*	-	0.009	0.012	0.043	0.030	0.160	0.063	0.036	0.041	0.039	0.043	0.039	0.048	0.027	0.084	0.067	0.053
8	0.008	*0.013*	0.002	*0.031*	*0.015*	0.005	0.003	-	0.000	0.056	0.063	0.168	0.040	0.021	0.025	0.032	0.034	0.039	0.031	0.019	0.080	0.049	0.024
9	0.003	0.006	-0.005	*0.034*	*0.014*	*0.013*	0.003	0.001	-	0.080	0.046	0.156	0.037	0.016	0.009	0.007	0.016	0.023	0.021	0.012	0.098	0.056	0.026
10	*0.017*	*0.021*	0.012	*0.041*	*0.027*	*0.016*	*0.010*	*0.012*	*0.015*	-	0.005	0.210	0.076	0.100	0.134	0.088	0.065	0.086	0.116	0.085	0.114	0.114	0.098
11	*0.015*	*0.013*	0.013	*0.036*	*0.023*	*0.011*	*0.007*	*0.012*	*0.011*	0.003	-	0.202	0.042	0.098	0.046	0.078	0.047	0.062	0.084	0.055	0.099	0.095	0.065
12	*0.034*	*0.039*	0.030	*0.063*	*0.039*	*0.048*	*0.041*	*0.040*	*0.038*	*0.052*	*0.047*	-	0.171	0.077	0.139	0.120	0.151	0.157	0.170	0.140	0.209	0.188	0.159
13	0.005	*0.015*	0.009	*0.043*	*0.019*	*0.019*	*0.012*	*0.010*	*0.009*	*0.019*	*0.013*	*0.036*	-	0.017	0.024	0.048	0.039	0.013	0.007	0.002	0.090	0.018	0.022
14	0.003	*0.014*	-0.008	*0.043*	*0.014*	*0.020*	*0.009*	*0.007*	*0.005*	*0.021*	*0.018*	*0.023*	0.005	-	0.010	0.029	0.024	0.006	0.009	0.001	0.049	0.030	0.008
15	0.012	0.012	0.009	*0.050*	*0.023*	*0.016*	*0.014*	0.010	*0.010*	*0.029*	*0.014*	*0.034*	0.010	0.006	-	0.019	0.037	0.070	0.011	0.009	0.081	0.046	0.064
16	*0.010*	*0.015*	0.000	*0.039*	*0.021*	*0.016*	*0.009*	0.006	0.004	*0.019*	*0.015*	*0.031*	*0.011*	0.005	0.008	-	0.001	0.010	0.010	0.016	0.078	0.046	0.032
17	*0.008*	*0.011*	0.002	*0.040*	*0.024*	*0.021*	*0.009*	*0.006*	*0.007*	*0.017*	*0.014*	*0.032*	*0.009*	*0.007*	*0.016*	0.003	-	0.015	0.008	0.008	0.097	0.048	0.010
18	0.004	*0.011*	-0.003	*0.039*	*0.018*	*0.018*	*0.007*	*0.008*	0.004	*0.016*	*0.011*	*0.039*	*0.008*	0.004	*0.015*	0.003	0.005	-	0.013	0.015	0.031	0.027	0.011
19	0.009	0.011	0.011	*0.053*	*0.019*	*0.015*	*0.011*	*0.008*	*0.008*	*0.023*	*0.016*	*0.033*	0.008	0.004	0.008	0.004	0.004	0.006	-	0.000	0.061	0.010	0.004
20	0.002	0.007	-0.001	*0.040*	*0.019*	*0.015*	*0.005*	0.004	0.004	*0.016*	*0.011*	*0.030*	0.001	0.001	0.005	0.003	0.003	0.003	0.002	-	0.048	0.010	0.003
21	*0.018*	*0.018*	0.005	*0.048*	0.021	*0.028*	*0.018*	*0.019*	*0.017*	*0.025*	*0.020*	*0.045*	*0.021*	*0.014*	*0.025*	*0.019*	*0.016*	0.010	0.014	*0.015*	-	0.070	0.078
22	*0.011*	*0.016*	0.003	*0.051*	*0.019*	*0.022*	*0.013*	*0.010*	*0.011*	*0.023*	*0.020*	*0.034*	0.005	0.005	0.009	*0.009*	*0.012*	*0.009*	0.007	0.003	*0.021*	-	0.028
23	0.006	*0.013*	0.001	*0.041*	*0.017*	*0.017*	*0.009*	*0.005*	*0.007*	*0.018*	*0.015*	*0.032*	0.005	0.003	*0.015*	*0.008*	*0.003*	*0.005*	0.002	0.001	*0.015*	*0.006*	-

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