# Genetic variation in restored Atlantic salmon (*Salmo salar* L.) populations in the Ulla and Lérez rivers, Galicia, Spain

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The populations of Atlantic salmon in the Ulla and Lérez rivers, located in Galicia in north-
western Spain, were close to extinction early in the 1990s. A restoration programme involv-
ing supportive breeding has been conducted since 1995, using a mixture of salmon
populations from several Galician rivers. The programme utilizes progeny of adults return-
ing to the rivers and wild parr reared in fresh water until maturity. Five microsatellite loci
were used to compare genetic variability in the restored populations with that in populations
before their collapse in the 1950s. DNA samples were obtained from scale collections (old
samples) and from tissue samples of live fish caught in the rivers (modern samples). Aver-
age heterozygosities and allelic richness are very similar in modern and old samples. Pop-
ulations inhabiting the Ulla and Lérez rivers today are more similar than they were in the
past, possibly because they originated in the same stock mixture.

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## Introduction

The Ulla and Lérez rivers are located at the southern limit
of the European distribution of wild Atlantic salmon, *Salmo
salar* L. (Figure 1). The populations of salmon in both
rivers have been seriously depleted since the late 1970s,
particularly that in the River Lérez, which by 1991, was
close to extinction. These populations are exploited only
by recreational (rod and line) fishing; marine fisheries for
salmon are prohibited. In the River Ulla, the mean annual
catch declined from 264 salmon in the period 1960−1969
to 13 in 1990. In the River Lérez, it has declined from 85
salmon in 1964 to one in 1990. The decline in catches
indicates declining salmon abundance (Consuegra *et al.*,
2005). Supplemental stocking using foreign-origin Atlantic
salmon (mainly from Scotland) was carried out in
1970−1992 in most salmon rivers in Spain, including the
Ulla and the Lérez. Although the number of stocked fish
was high, the number of returning adults derived from
this stocking has been low and the genetic consequences
of the stocking minimal (García de Leaniz *et al.*, 1989;
Blanco *et al.*, 2005; Morán *et al.*, 2005).

A comprehensive restoration programme for Atlantic
salmon in Galician rivers, including the Ulla and the Lérez,
began in 1995 (Caballero, 2002). First, river accessibility
was improved by modifying existing fish passes or
installing new ones, some of which incorporated trapping
facilities to monitor returning adult salmon. Second, restric-
tive regulations were introduced for the recreational fishery,
including closure when necessary. Third, a long-term sup-
portive breeding programme was established in 1995, based
on native juveniles and returning adults. In rivers with
available spawners, stocking used hatchery-reared progeny
of adult salmon obtained from the same river. In addition,
500 juveniles were caught from four Galician rivers (Eo,
Mandeo, Ulla, and Miño) and transferred to the Carballedo
hatchery to produce a stock for reintroduction into rivers for
which adult spawners were not available (e.g. River Lérez).
Since 1999, only broodstock derived from the river
being stocked have been used in the supportive breeding
programme. The juvenile progenies of these salmon are
tagged and released into their parental river. Some kelts
have been reconditioned in the hatchery to be mated the fol-
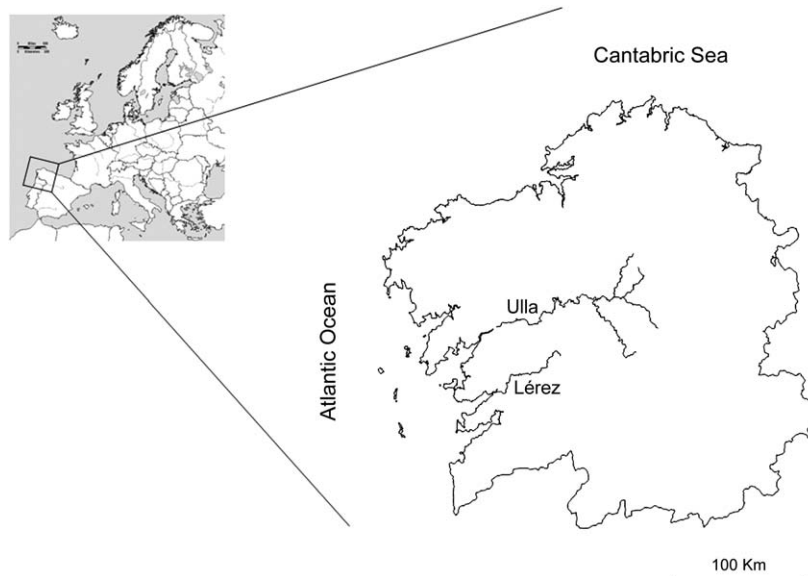lowing year, and some juveniles have been retained in the

Figure 1. Map of Europe and northwestern Spain showing the location of the Ulla and Lérez rivers.

hatchery until maturity (holobiotic rearing). Monitoring has indicated that the number of returning adult salmon derived from fin-clipped, stocked parr has increased since 1999 (Table 1). The descendants of salmon stocked during the supportive breeding programme are now part of the "native" salmon populations in the rivers, which have increased in abundance as the scale of the supportive breeding programme has increased.

The current populations of salmon in the Ulla and Lérez rivers are derived from a small number of natural spawners and salmon stocked during the supportive breeding programme. These artificially founded populations could differ genetically from the populations of the 1950s because of the potential loss of genetic variability, resulting from the

use of a small number of broodstock, and because of stochastic temporal fluctuations in allelic frequency, resulting from genetic drift. To evaluate the effectiveness of the supportive breeding programme in maintaining genetic diversity, we compared the levels of genetic variation for five microsatellite loci in adults returning to the rivers in 1997−2004 with those present in the populations in 1950−1960.

## Material and methods

### Sample collection

Since 1950, regulations have required that scale samples be collected from all salmon caught in the recreational fishery to determine fish age and growth. One or two scales per fish from this historical archive were used to extract DNA from salmon returning to the Ulla and Lérez rivers in 1950−1960 (hereafter referred to as "old samples"). In total, 22 and 44 samples were available from the Ulla and Lérez rivers, respectively. Muscle and fin samples were obtained for DNA extraction from prespawning adults caught in trapping facilities or in the recreational fisheries in the two rivers in 1997−2004 (hereafter referred to as "modern samples"; Table 1). Trapping facilities allow monitoring of 100% and 35% of the adult populations in the Lérez and Ulla rivers, respectively.

### DNA extraction and microsatellite analysis

Chelex-based DNA extraction was performed according to Estoup *et al.* (1996). Five microsatellite loci were screened: *SSOSL311* and *SSOSL85* (Slettan *et al.*, 1995), *Ssa85* and *Ssa202* (O'Reilly *et al.*, 1996), and *Ss4* (Martínez *et al.*,

Table 1. The number of wild and stocked adult salmon monitored at permanent traps or caught in the recreational fisheries in the Ulla and Lérez rivers since the beginning of the restoration programme. The number of salmon analysed in this study is shown in parenthesis.

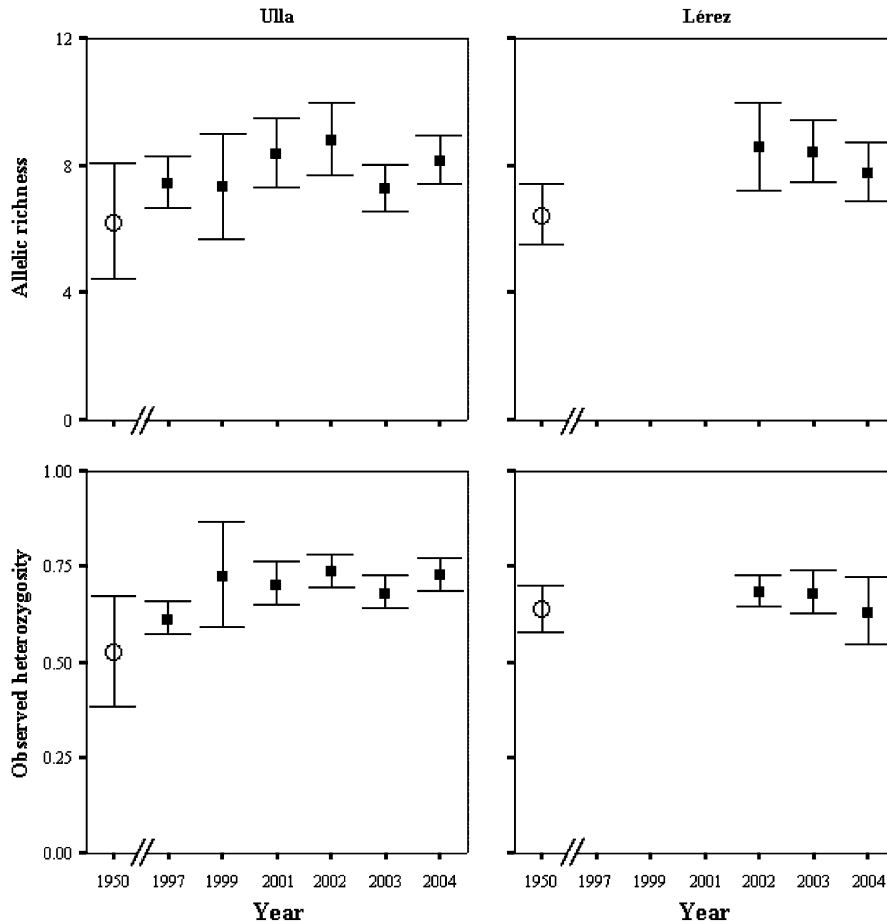| Year | River Ulla | | River Lérez | |
|------|------|------|------|------|
|      | Wild | Stocked | Wild | Stocked |
| 1996 | 25 (0) | 0 | 4 (0) | 0 |
| 1997 | 42 (39) | 0 | 2 (0) | 0 |
| 1998 | 16 (0) | 0 | 0 (0) | 0 |
| 1999 | 41 (29) | 0 | 2 (0) | 2 (0) |
| 2000 | 18 (0) | 6 (0) | 9 (0) | 16 (0) |
| 2001 | 44 (40) | 37 (18) | 10 (0) | 11 (0) |
| 2002 | 66 (49) | 43 (28) | 47 (38) | 17 (8) |
| 2003 | 42 (37) | 6 (5) | 24 (23) | 40 (40) |
| 2004 | 46 (46) | 34 (33) | 23 (19) | 25 (21) |

Figure 2. Allelic richness and observed heterozygosity averaged over loci for the old and modern samples. Vertical bars indicate one standard error. Open circles represent old samples (pooled from 1950 to 1960) and squares represent modern samples.

1999). Polymerase Chain Reactions (PCR) were carried out containing 1 μl of extracted genomic DNA, 2 μl of reaction buffer 10×, 2.5 mM MgCl$_2$, 0.35 μM of each primer (forward primer labelled with FAM, HEX, or NED), 1 U of *Taq* DNA polymerase, and deionized water up to a final volume of 20 μl. Cycling conditions consisted of 5 min denaturation at 95°C, followed by 35 cycles of 20 s denaturation at 95°C, 20 s annealing at 55°C (*Ssa85*, *SSOSL311*, *Ss4*, and *Ssa202* microsatellite loci) and at 53°C (*SSOSL85* microsatellite locus), and 20 s extension at 72°C, followed by a final 7 min extension at 72°C. Amplifications of *SSOSL311*, *Ss4*, and *Ssa202* were carried out in a single multiplex PCR.

PCR products (1–3 μl) of separate reactions were combined with water into a final volume of 10 μl. Two microlitres of this mixture were added to 12 μl of deionized formamide and 0.5 μl of the internal size standard GENESCAN 400HD [ROX] for electrophoresis. This mixture was denatured at 95°C for 5 min and immediately chilled on ice and subjected to capillary electrophoresis on an ABI PRISM Genetic Analyser. Loci were identified according to their size range and

attached fluorescent label. Genotype data were generated using the GENESCAN software (Applied Biosystems).

## Data analysis

Observed and expected heterozygosities were calculated using GENETIX 4.01 (Belkhir *et al.*, 1998). The observed numbers of alleles and private alleles were calculated with GENEPOP 3.3 (Raymond and Rousset, 1995). Deviations from Hardy–Weinberg equilibrium were assessed for each locus and year using GENEPOP 3.3 by exact tests with 1000 iterations; levels of significance were adjusted for multiple tests using the sequential Bonferroni correction (Rice, 1989). Allelic richness was calculated in each group using the FSTAT package (Goudet, 2001), based on the minimum sample size for each corresponding locus. To contrast heterozygosities and allelic richness between modern and old samples for each river, analyses of variance and *post hoc* Student–Newman–Keuls (SNK) tests were performed using the SPSS package. Pairwise $F_{ST}$ (Weir and Cockerham, 1984) values were calculated using GENETIX

Table 2. The observed number of alleles ($A_N$), allelic richness ($A_R$), and observed ($H_O$) and expected ($H_E$) heterozygosities for five microsatellite loci. The number of individuals sampled ($n$) is also shown. All values for modern samples are averages across years. Tests for deviation from Hardy–Weinberg proportions at the 5% level, adjusted according to the sequential Bonferroni procedure, are indicated by the superscript $s/t$, where $s$ is the number of significant tests out of the total number of years analysed, $t$.

| | | Modern Ulla | Old Ulla | Modern Lérez | Old Lérez |
|---|---|---|---|---|---|
| *Ssa85* | $n$ | 309 | 13 | 134 | 28 |
| | $A_N$ | 10.5 | 6.0 | 8.7 | 6.0 |
| | $A_R$ | 6.3 | 6.0 | 6.1 | 4.9 |
| | $H_O^{s/t}$ | $0.55^{4/6}$ | $0.62^{0/1}$ | $0.47^{3/3}$ | $0.75^{0/1}$ |
| | $H_E$ | 0.65 | 0.74 | 0.70 | 0.76 |
| *SSOSL311* | $n$ | 297 | 22 | 138 | 43 |
| | $A_N$ | 13.0 | 6.0 | 14.0 | 10.0 |
| | $A_R$ | 10.8 | 6.0 | 11.9 | 8.7 |
| | $H_O^{s/t}$ | $0.76^{1/6}$ | $0.68^{0/1}$ | $0.71^{2/3}$ | $0.79^{0/1}$ |
| | $H_E$ | 0.83 | 0.76 | 0.86 | 0.72 |
| *SSOSL85* | $n$ | 308 | 14 | 132 | 22 |
| | $A_N$ | 9.5 | 11.0 | 10.7 | 8.0 |
| | $A_R$ | 6.4 | 11.0 | 7.4 | 7.1 |
| | $H_O^{s/t}$ | $0.66^{1/6}$ | $0.71^{1/1}$ | $0.64^{1/3}$ | $0.54^{0/1}$ |
| | $H_E$ | 0.65 | 0.87 | 0.70 | 0.75 |
| *Ss4* | $n$ | 305 | 1 | 140 | 22 |
| | $A_N$ | 11.5 | — | 10.7 | 8.0 |
| | $A_R$ | 9.4 | — | 9.2 | 8.0 |
| | $H_O^{s/t}$ | $0.71^{3/6}$ | — | $0.78^{1/3}$ | $0.65^{0/1}$ |
| | $H_E$ | 0.79 | — | 0.80 | 0.75 |
| *Ssa202* | $n$ | 310 | 22 | 138 | 38 |
| | $A_N$ | 7.2 | 2.0 | 7.7 | 4.0 |
| | $A_R$ | 6.7 | 2.0 | 6.9 | 3.6 |
| | $H_O^{s/t}$ | $0.82^{0/6}$ | $1.00^{1/1}$ | $0.75^{0/3}$ | $0.47^{0/1}$ |
| | $H_E$ | 0.77 | 0.50 | 0.77 | 0.52 |

4.01 (1000 permutations). The pattern of gene frequency differentiation between modern and old samples was represented by a factorial analysis of correspondences (AFC) using GENETIX 4.01. Assignment tests were performed with GENECLASS 2.0 (Piry *et al.*, 2004) using the Bayesian direct approach (Rannala and Mountain, 1997). To test the significance of the correct assignments, Chi-square values were calculated by comparing the observed number of correct assignments with the number of correct classifications expected by chance.

## Results

The total number of alleles per locus ranged from 12 (*Ssa202*) to 29 (*Ss4*). Allelic number, allelic richness, and observed and expected heterozygosities, averaged over years for the modern samples (Figure 2 and Table 2), did not differ significantly in old and modern populations within rivers (allelic richness: SNK test $p = 0.329$ and 0.506 for the Ulla and Lérez rivers, respectively; observed heterozygosity: SNK test $p = 0.516$ and 0.730 for the Ulla and Lérez rivers, respectively). Deviations from Hardy–Weinberg expectations for modern populations were observed for some years (Table 2), generally resulting from a homozygous excess. The number of private alleles ranged from 5 to 16 (out of 10–24 different alleles) in modern Ulla samples, from 5 to 12 (out of 10–17 different alleles) in modern Lérez samples, and from 0 to 1 (out of 2–11 different alleles) in the old samples from both rivers. Note, however, that the size of the modern samples was, on average, approximately 13 times larger than that of the old samples, which explains the substantial difference in the number of private alleles between the sampling periods.

Pairwise $F_{ST}$ values (Table 3) were all significant after sequential Bonferroni correction. Similar results were obtained when only samples from 2004 were used to represent modern populations in the two rivers. A factorial analysis of correspondences revealed that modern populations cluster together, particularly for the first component, which explains 69% of the variation ($p < 0.01$). The old populations were separated more from each other and from the modern populations, suggesting that the genetic composition has changed since the 1950s (Figure 3).

Assignment tests revealed a significant number of correct classifications (an average of 66%) of individuals into the

Table 3. $F_{ST}$ values for pairwise comparisons between old and modern samples.

|  | Modern Lérez | Old Ulla | Old Lérez |
|---|---|---|---|
| Modern Ulla | 0.002* | 0.084*** | 0.126*** |
| Modern Lérez |  | 0.088*** | 0.129*** |
| Old Ulla |  |  | 0.066*** |

*$p = 0.049$, ***$p < 0.001$; all are significant after sequential Bonferroni correction.

four groups considered (Table 4). Old samples showed the highest number of correct assignments and modern Lérez the lowest. When the samples from both rivers were grouped in old and modern samples, the percentage of correctly assigned individuals was close to 95%.

## Discussion

Two main findings have emerged from this study. First, significant genetic differences were observed between old and modern samples, as well as between old samples from both rivers. However, it is necessary to consider the small sample sizes from the old populations, which exhibit fewer private alleles than the modern samples. The modern populations from the Ulla and Lérez rivers were quite similar, and 66 (44%) of 149 salmon from the River Lérez were assigned to the River Ulla. Similarly, 114 (35%) of 324 salmon from the River Ulla were assigned to the River

Table 4. Percentages of correct assignments to the river of origin.

|  | Modern Ulla | Modern Lérez | Old Ulla | Old Lérez | All modern | All old |
|---|---|---|---|---|---|---|
| Modern Ulla | 197 | 66 | 0 | 3 | 458 | 4 |
| Modern Lérez | 114 | 81 | 0 | 1 |  |  |
| Old Ulla | 5 | 2 | 16 | 7 | 15 | 62 |
| Old Lérez | 8 | 0 | 6 | 33 |  |  |
| Sample size | 324 | 149 | 22 | 44 | 473 | 66 |
| % Correctly assigned* | 60.8 | 54.4 | 72.7 | 75.0 | 96.8 | 93.9 |

*All percentages of correct assignment are significant ($p < 0.05$).

Lérez. This is not surprising considering that the Lérez population was close to extinction in the early 1990s and was successfully restored using salmon from the River Ulla and other Galician rivers. The observed difference between both modern populations, although small, can be explained by natural reproduction of salmon and fidelity to natal rivers. These differences can be expected to increase with time, perhaps recovering to the levels of isolation observed in the old populations.

Second, levels of allelic richness and heterozygosity in modern samples are of the same magnitude or even slightly greater, though not significantly so, than those in the old samples (Figure 2), and they are also similar to those in salmon populations inhabiting rivers throughout the range
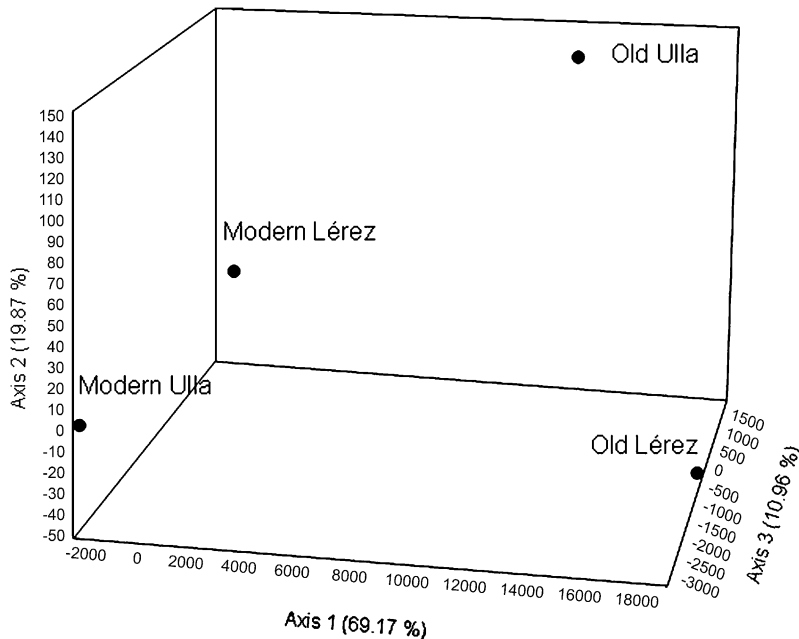
Figure 3. Factorial analysis of correspondences.

of the species (King *et al.*, 2001; Wennevik *et al.*, 2004). The complex life cycle of the Atlantic salmon, with overlapping generations and multiple paternity, may help to minimize or delay the loss of genetic diversity in the face of demographic catastrophes (Waples, 1991). The contribution of mature parr to reproduction may also help increase the effective population size and genetic diversity of salmon populations, especially in southern rivers, where development is fast and maturation rates are high (Martínez *et al.*, 2000). Restoration of the River Lérez has depended on the supportive breeding programme, but remnants of the indigenous salmon population remained in the River Ulla. Therefore, it is possible that, although catches in the recreational fishery in the River Ulla were low, suggesting low population abundance, genetic variability in the remnant population before establishment of the restoration programme was great enough to preserve diversity in the long term. Studies conducted in 1996–2004 found high levels of genetic variability in other small populations of salmon in Spain (Consuegra *et al.*, 2005).

The changes in allelic composition between old and modern populations can be explained by the programme's initial use of a mixture of salmon stocks derived from different Galician rivers. This could have resulted in the introduction of new alleles. The mixture of different stocks, with the additional mixture of cohorts within years, might also explain the homozygous excess observed in some modern samples. Ten years after the programme began, levels of heterozygosity and allelic richness in the restored populations are similar to, or even marginally greater than, those in the ancestral populations, suggesting that the programme successfully maintained the genetic variation present in these populations. However, monitoring the genetic variability and inbreeding in cultured fish used in the programme remains important because a 10% increase in inbreeding can result in a reduction in fitness of about 3–15% (Wang *et al.*, 2002).

Tagging studies have demonstrated that this supportive breeding programme, utilizing local stocks, has increased adult returns (Caballero, 2002) and that it has been more successful than earlier restoration efforts utilizing foreign-origin salmon (Morán *et al.*, 2005). The goal of restoration programmes should be self-sustaining salmon populations (Koljonen *et al.*, 2002). In the meantime, however, as the populations increase, the use of returning adult salmon in the supportive breeding programme should be continued to maintain local adaptations (Hindar *et al.*, 2004).

## Acknowledgements

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