ICES Journal of Marine Science, 63: 1290–1296 (2006)
doi:10.1016/j.icesjms.2006.03.005

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

María Saura, Pablo Caballero, Armando Caballero, and Paloma Morán

Saura, M., Caballero, P., Caballero, A., and Morán, P. 2006. Genetic variation in restored Atlantic salmon (*Salmo salar* L.) populations in the Ulla and Lérez rivers, Galicia, Spain. – ICES Journal of Marine Science, 63: 1290–1296.

The populations of Atlantic salmon in the Ulla and Lérez rivers, located in Galicia in northwestern Spain, were close to extinction early in the 1990s. A restoration programme involving supportive breeding has been conducted since 1995, using a mixture of salmon populations from several Galician rivers. The programme utilizes progeny of adults returning 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). Average heterozygosities and allelic richness are very similar in modern and old samples. Populations 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.

Keywords: Atlantic salmon, genetic variability, heterozygosity, stocking.

Received 22 September 2005; accepted 17 March 2006.

M. Saura, A. Caballero, and P. Morán: Departamento de Bioquímica, Genética e Inmunología, Facultad de Biología, Universidad de Vigo, Campus Lagoas-Marcosende, 36310 Vigo, Spain. P. Caballero: Centro de Investigaciones Ambientales de Lourizan, Apartado 127, 36080 Pontevedra, Spain. Correspondence to P. Morán: tel: +34 986 813899; fax: +34 986 812556; e-mail: paloma@uvigo.es.

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, restrictive regulations were introduced for the recreational fishery, including closure when necessary. Third, a long-term supportive 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 following year, and some juveniles have been retained in the

1054-3139/$32.00

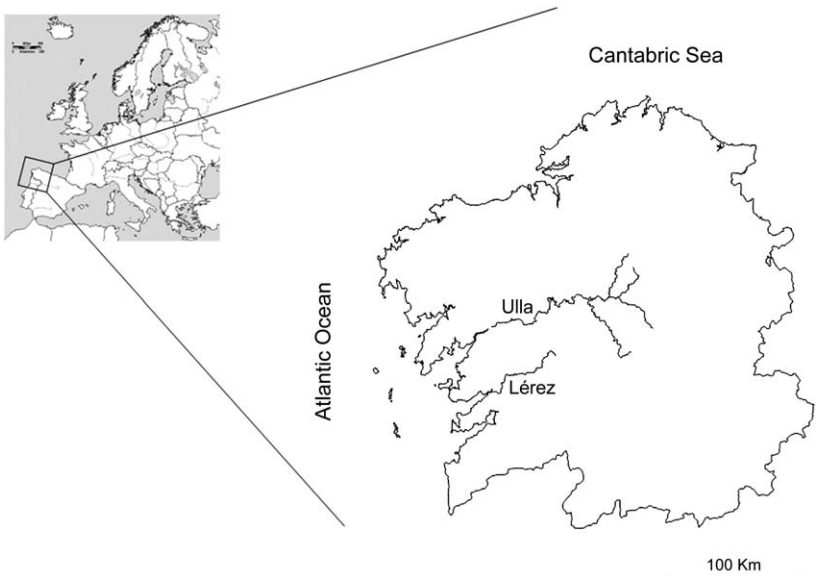

Figure 1. Map of Europe and northwestern Spain showing the location of the Ulla and Lérez rivers.

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.

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.

	River Ulla		River Lérez	
Year	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)

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.*,

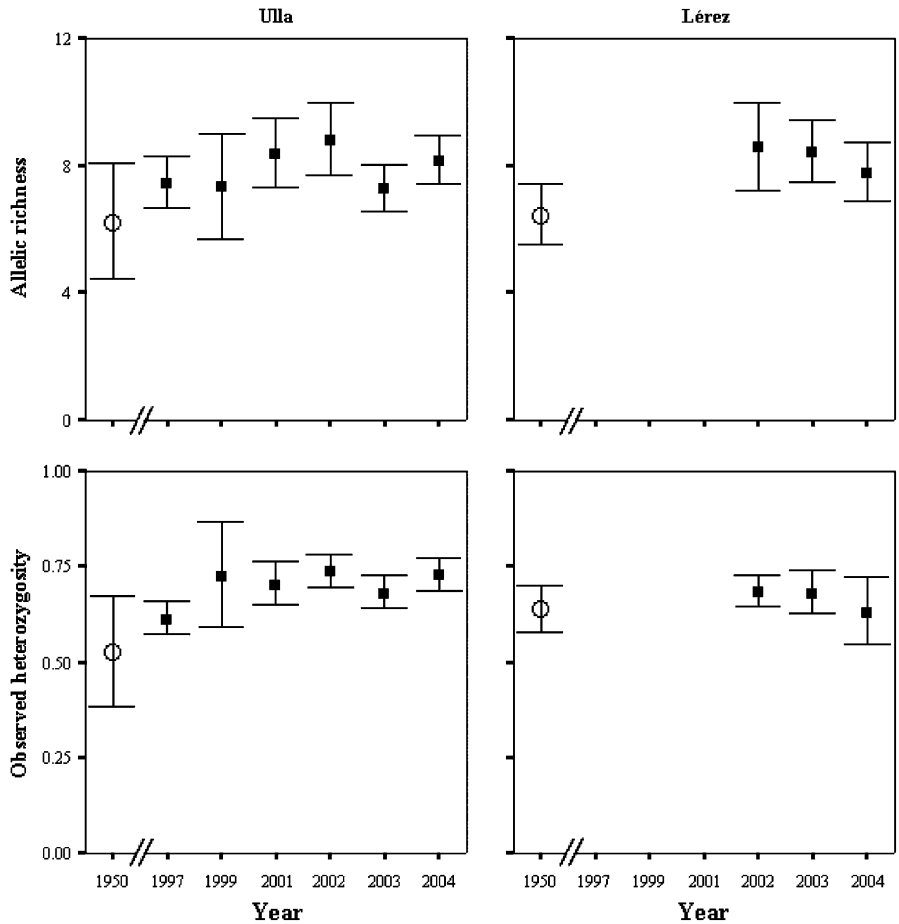

Figure 2. Allelic richness and observed heterozygosity averaged over loci for the old and modern samples. Vertical bars indicate one standard error. Open circles represent old samples (pooled from 1950 to 1960) and squares represent modern samples.

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.

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 ($p < 0.05$).

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 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

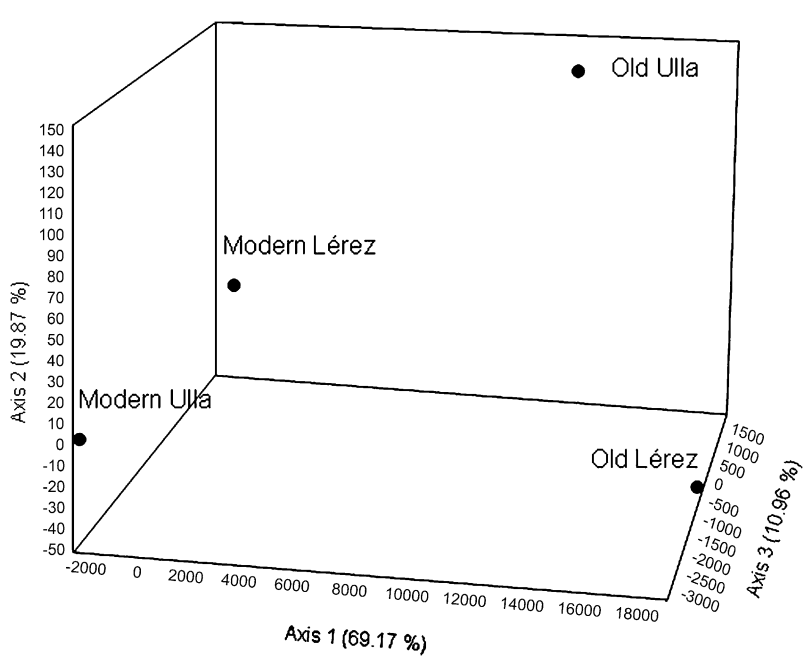

Figure 3. Factorial analysis of correspondences.

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

We are indebted to the staff of Consellería de Medio Ambiente (Xunta de Galicia) for providing samples, and especially to Francisco Hervella Rodríguez. Pilar Alvariño and Nieves Santamaría assisted with technical support. We also thank Peter Hutchinson and two anonymous referees for their corrections to, and comments on, earlier drafts of the manuscript. This work was supported by projects INIA-CPE03-004-C2 and BMC2003-03022 (MEC, Spain), and an FPI grant from MEC to María Saura.

References

Belkhir, K., Borsa, P., Goudet, J., Chikhi, L., and Bonhomme, F. 1998. GENETIX, logiciel sous Windows TM pour la genetique des populations. Laboratoire Genome et Populations. CNRS UPR 9060, Universite de Montpellier II, Montpellier, France.

Blanco, G., Ramos, M. D., Vázquez, E., and Sánchez, J. A. 2005. Assessing temporal and spatial variation in wild populations of Atlantic salmon with particular reference to Asturias (northern Spain) rivers. Journal of Fish Biology, 67(Suppl. A): 169–184.

Caballero, P. 2002. Programas de recuperación del salmón Atlántico (*Salmo salar* L.) en los ríos Ulla, Lérez y Miño. Consellería de Medio Ambiente, II Jornadas del Salmón Atlántico en la Península Ibérica, Xunta de Galicia, pp. 83–116.

Consuegra, S., Verspoor, E., Knox, D., and García de Leaniz, C. 2005. Asymmetric gene flow and evolutionary maintenance of genetic diversity in small, peripheral Atlantic salmon populations. Conservation Genetics, 6: 823–842.

Estoup, A., Largiader, C. R., Perrot, E., and Chourrout, D. 1996. Rapid one-tube extraction for a reliable PCR detection of fish polymorphic markers and transgenes. Molecular Marine Biology and Biotechnology, 5: 295–298.

García de Leaniz, C., Verspoor, E., and Hawkins, A. D. 1989. Genetic determination of the contribution of stocked and wild Atlantic salmon, *Salmo salar* L., to the angling fisheries in two Spanish rivers. Journal of Fish Biology, 35: 261–270.

Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). (Updated from Goudet, J. 1995. FSTAT (version 1.2): a computer program to calculate *F*-statistics. Journal of Heredity, 86: 485–486). Available from. http://www.unil.ch/izea/softwares/fstat.html.

Hindar, K., Tufto, J., Saettem, L. J., and Balstad, T. 2004. Conservation of genetic variation in harvested salmon populations. ICES Journal of Marine Science, 61: 1389–1397.

King, T. L., Kalinowski, S. T., Schill, W. B., Spidle, A. P., and Lubinski, B. A. 2001. Population structure of Atlantic salmon (*Salmo salar* L.): a range-wide perspective from microsatellite DNA variation. Molecular Ecology, 10: 807–821.

Koljonen, M. L., Tähtinen, J., Säisä, M., and Koskiniemi, J. 2002. Maintenance of genetic diversity of Atlantic salmon (*Salmo salar*) by captive-breeding programmes and the geographic distribution of microsatellite variation. Aquaculture, 212: 69–92.

Martínez, J. L., Morán, P., and García-Vázquez, E. 1999. Dinucleotide repeat polymorphism at the *SS4*, *SS6* and *SS11* in Atlantic salmon (*Salmo salar*). Animal Genetics, 30: 464–465.

Martínez, J. L., Morán, P., Pérez, J., de Gaudemar, B., Beall, E., and García-Vázquez, E. 2000. Multiple paternity increases effective sizes of southern Atlantic salmon populations. Molecular Ecology, 9: 293–298.

Morán, P., Pérez, J., and García-Vázquez, E. 2005. Genetic variation in endangered populations of Atlantic salmon (*Salmo salar* L.) from northwestern Spain. Journal of Fish Biology, 67(Suppl. A): 206–212.

O'Reilly, P. T., Hamilton, L. C., McConnell, S. K., and Wright, J. M. 1996. Rapid analysis of Atlantic salmon (*Salmo salar*) by PCR multiplexing of dinucleotide and tetranucleotide microsatellites. Canadian Journal of Fisheries and Aquatic Sciences, 53: 2292–2298.

Piry, S., Alapetite, A., Cornuet, J. M., Paetkau, D., Baudouin, L., and Estoup, A. 2004. GENECLASS 2: a software for genetic assignment and first-generation migrant detection. Journal of Heredity, 95: 536–539.

Rannala, B., and Mountain, J. L. 1997. Detecting immigration by using multilocus genotypes. Proceedings of the National Academy of Sciences of the United States of America, 94: 9197–9201.

Raymond, M., and Rousset, F. 1995. GENEPOP (version 3.3): population genetics software for exact tests and ecumenecism. Journal of Heredity, 86: 248–249.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution, 43: 223–225.

Slettan, A., Olsaker, I., and Lie, Ö. 1995. Atlantic salmon (*Salmo salar*) microsatellites at the *SSOSL 25*, *SSOSL 85*, *SSOSL 311*, *SSOSL 417* loci. Animal Genetics, 26: 281–282.

Wang, S., Hard, J. J., and Utter, F. 2002. Salmonid inbreeding: a review. Reviews in Fisheries Biology and Fisheries, 11: 301–319.

Waples, R. S. 1991. Pacific salmon, *Oncorhyncus* spp, and the definition of "species" under the Endangered Species Act. Acta Marine Fisheries Review, 53: 11–21.

Weir, B. S., and Cockerham, C. C. 1984. Estimating *F*-statistics for the analysis of populations structure. Evolution, 38: 1358–1370.

Wennevik, V., Skaala, O., Titov, S. F., Studyonov, I., and Naevdal, G. 2004. Microsatellite variation in populations of Atlantic salmon from north Europe. Environmental Biology of Fishes, 69: 143–152.