

Introgression and genetic structure in northern Spanish Atlantic salmon (*Salmo salar* L.) populations according to mtDNA data

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Abstract We have used PCR-RFLP analysis to screen ancestral and modern genetic variation of the mitochondrial ND1 region at eight Atlantic salmon rivers in northern Spain. Using old scales, we show that genetic variation in NW Spanish salmon populations has not changed significantly during the past 50 years. Comparing our data with that from the literature we can conclude that, in general, modern salmon populations from NW Spain are distinct from putative northern European donor populations. Taken together, these observations suggest that, despite the non-native introductions carried out during 1970–1993, introgression of northern European mitochondrial genomes in NW Spanish salmon populations has been generally low. Different results were obtained for populations in NE Spain and southern France, where rivers Nansa, Asón, Bidasoa and Nive presented a high frequency of haplotypes characteristic of northern European rivers that reflect introgression. We show that the levels of introgression are correlated with the foreign stocking intensity. Thus, the rivers that were more intensely stocked are more similar to northern European salmon rivers. We also observed an East–West cline in the levels of foreign introductions and introgression. In addition, we provide the first description of the overall genetic structure of Atlantic salmon populations in southern Europe. We have observed evidences of genetic differentiation among populations and isolation by

distance as a consequence of salmon homing behaviour. The results obtained do not call for changes in introduction policies in Spain, currently based on the use of native stocks.

Keywords Atlantic salmon · Stocking · mtDNA · Population differentiation · Introgression

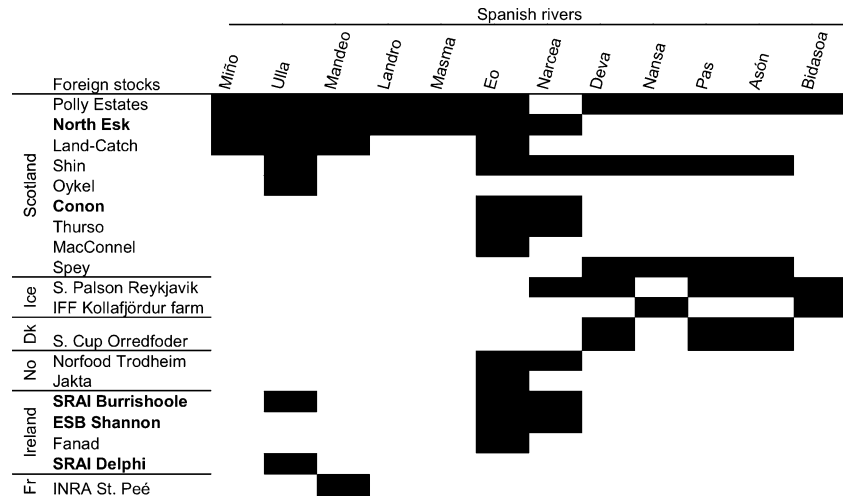
Introduction

Spanish salmon populations suffered a severe demographic decline in the 1970s, becoming extinct in many rivers. Only four Spanish rivers are currently not considered vulnerable (Sella, Narcea, Deva and Bidasoa) (WWF 2001). To overcome the effects of this decline, Spanish salmon populations were supported with stocks from northern Europe (British Isles, Iceland and Scandinavia) during the 1970's–1990's (Consellería de Medio Ambiente de Galicia; Consejería de Medio Ambiente de Asturias; Dirección General de Montes y Conservación de la Naturaleza de Cantabria; Dirección General de Medio Ambiente de Navarra) (Fig. 1). The number of released fish varied considerably across years and rivers (Table 1). The same stock was often used to supplement different rivers, and most rivers were stocked with salmon from several parts of Europe (see Fig. 1). However, and according to allozyme studies, the overall success of these foreign introductions was very low (García de Leániz et al. 1989; Vázquez et al. 1993; Morán et al. 1994; Verspoor and García de Leániz 1997; Blanco et al. 2005; Morán et al. 2005a; Morán et al. 2005b), except in some rivers like Navia and Nivelles (the latter in France) (Morán et al. 2005a). However, mitochondrial DNA (Consuegra et al. 2002) and microsatellites (Martínez et al. 2001; Ayllón et al. 2006) suggest that

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Fig. 1 Introductions of foreign stocks in Spanish rivers. Black boxes indicate the foreign stocks used to supplement each river. In bold are foreign stocks previously screened for the ND1. Abbreviations are as follows: Iceland (Ice), Denmark (Dk), Norway (No) and France (Fr). Data not available for rivers Lérez and Nive



some introgression took place in rivers Asón, Nivelle, Eo, Esva, Narcea, Sella, Cares or Nive. Indeed, allozymes might underestimate levels of introgression because of their limited power to detect genetic variation (Lewontin 1974). Most allozymes are diallelic, and they can be under the effect of selection, like MEP-2*, where variation is determined by temperature-mediated natural selection (Verspoor and Jordan 1989). Moreover, archival scales cannot be used to compare current and past allozyme variation. We therefore expect that assessing mitochondrial DNA variation should provide better estimates of the levels of introgression of non-native strains in natural populations. Importantly, mitochondrial variation has not been assessed in rivers from NW Spain (Galicia region), where allozyme studies suggest low levels of foreign introgression (Morán et al. 2005b). A suitable mitochondrial marker to carry out this task is ND1, where considerable differentiation has been reported among European salmon populations (Verspoor et al. 1999; Nilsson et al. 2001; Consuegra et al. 2002; Asplund et al. 2004). In addition, it is often possible to compare the genetic variation at ND1 before and after introductions in order to detect potential changes when old scale collections are available (Nielsen et al. 1999; Martínez et al. 2001; Hansen 2002; Heath et al. 2002; Ostergaard et al. 2003).

Salmon populations show a considerable reproductive isolation that has allowed the development of local adaptations. Indeed, this species presents many life trait differences among populations (Taylor 1991), and genetic studies using different genetic markers support population differentiation not only between North America and Europe but also within regions and even among rivers within these regions (Taggart et al. 1995; Verspoor et al. 1999; King et al. 2001; Nilsson et al. 2001; Asplund et al. 2004; Tonteri et al. 2005; Verspoor et al. 2005). In contrast, genetic structure is generally low in southern European rivers,

according to allozyme (Sánchez et al. 1996; Blanco et al. 2005; Morán et al. 2005a; Morán et al. 2005b) and microsatellite data (Ayllón et al. 2006). Indeed, it has been suggested that gene flow can be very high between some of these rivers (Consuegra et al. 2005). Still, allozyme studies indicate that some differentiation can occur (Blanco et al. 2005). Furthermore, there is genetic differentiation for the ND1 locus in several Spanish populations (Ayllón 2005). Importantly, studies in southern Europe have focused so far on particular areas within this region, and an overall comparison of southern European salmon populations is still lacking.

The main objective of this study is to provide a better characterization of the levels of introgression of northern European stocks in Spanish salmon rivers. Secondly, we want to describe the temporal and spatial patterns of mitochondrial genetic variation in southern European salmon populations. To accomplish these objectives we contrast the mitochondrial variation at the ND1 locus between old and recent temporal samples from NW Spain. We also combine our data with available data from previously screened populations to offer a broad perspective of the effects of introgression of foreign translocations in southern Europe, providing at the same time the first description of the overall genetic structure of Atlantic salmon in this region.

Material and methods

Sample collection

We screened mtDNA variation of salmon populations in eight rivers in northern Spain (Fig. 2). We analysed 1,290 individuals (Table 2). A total of 1,195 samples were collected from rivers Miño, Lérez, Ulla, Mandeo, Landro,

Table 1 Management data of all the studied rivers during the period of foreign salmon introductions in southern Europe (1981–1994)

River	Year	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	Mean	SE	TOTAL
Miño	TC	113	43	36	106	63	105	22	62	20	57	27	37	3	18	50.86	9.47	712
	FS	0	0	0	0	0	0	0	8	21	20	25	0	0	0	5.286	2.5	74
	FS/TC	0	0	0	0	0	0	0	0.13	1.05	0.35	0.93	0	0	0	0.175	0.1	0.10
Ulla	TC	256	104	104	180	175	265	56	62	13	12	33	23	0	4	91.93	24.7	1287
	FS	0	0	0	0	0	0	24.3	30	46	49.6	47.5	30	44.8	–	20.94	5.74	272.2
	FS/TC	0	0	0	0	0	0	0.43	0.48	3.54	4.13	1.44	1.3	–	–	0.944	0.39	0.21
Mandeo	TC	15	0	0	10	11	33	14	15	0	15	11	9	3	12	10.57	2.31	148
	FS	6	2.5	0	0	0	0	10	25	12	25	0	20.7	0	0	7.229	2.6	101.2
	FS/TC	0.4	–	–	0	0	0	0.71	1.67	–	1.67	0	2.3	0	0	0.613	0.23	0.68
Landro	TC	7	2	10	15	12	18	22	1	B	B	B	3	11	7	9.818	1.79	108
	FS	0	0	0	0	0	0	7.5	32.2	18	0	0	0	0	0	4.121	2.55	57.7
	FS/TC	0	0	0	0	0	0	0.34	32.2	–	–	–	0	0	0	2.958	2.59	0.53
Masma	TC	27	19	12	35	41	105	48	40	28	25	84	32	24	23	38.79	6.87	543
	FS	0	0	0	0	0	0	30.8	38.2	69	50	0	0	0	0	13.43	6.26	188
	FS/TC	0	0	0	0	0	0	0.64	0.96	2.46	2	0	0	0	0	0.433	0.22	0.35
Eo	TC	835	187	489	343	286	702	300	193	24	10	94	40	27	19	253.5	70.4	3549
	FS	0	0	0	0	10	25	25	177	155	91	113	91.4	0	0	49.1	17	687.4
	FS/TC	0	0	0	0	0.03	0.04	0.08	0.92	6.46	9.1	1.2	2.29	0	0	1.437	0.75	0.19
Narcea	TC	756	198	851	956	201	534	781	1181	282	523	766	430	917	1036	672.3	84.2	9412
	FS	1.5	100	21	7.1	15	63.5	150	100	97	131	60	9	0	0	53.94	14.2	755.1
	FS/TC	0	0.51	0.02	0.01	0.07	0.12	0.19	0.08	0.34	0.25	0.08	0.02	0	0	0.122	0.04	0.08
Deva	TC	776	302	775	451	360	760	527	731	323	234	452	987	515	445	545.6	59.8	7638
	FS	3	175	95	228	4	47.2	200	75	90	117	154	0	0	0	84.84	21.5	1187.7
	FS/TC	0	0.58	0.12	0.5	0.01	0.06	0.38	0.1	0.28	0.5	0.34	0	0	0	0.206	0.06	0.15
Nansa	TC	65	30	41	25	27	49	63	52	39	94	65	57	44	35	49	5.03	686
	FS	0	100	100	100	100	100	100	0	0	35	30.8	0	0	0	47.56	12.9	665.8
	FS/TC	0	3.33	2.44	4	3.7	2.04	1.59	0	0	0.37	0.47	0	0	0	1.282	0.41	0.97
Pas	TC	214	185	428	186	150	250	200	98	45	352	453	86	16	77	195.7	36.4	2740
	FS	0	98.6	50	80	0	140	0	0	0	67	73	0	0	0	36.33	12.7	508.6
	FS/TC	0	0.53	0.12	0.43	0	0.56	0	0	0	0.19	0.16	0	0	0	0.142	0.06	0.19
Asón	TC	54	3	70	94	262	145	51	238	101	85	101	226	75	76	112.9	20.6	1581
	FS	0	290	200	375	100	100	292	300	0	21	30.2	0	0	0	122	37.3	1708.2
	FS/TC	0	96.7	2.86	3.99	0.38	0.69	5.73	1.26	0	0.25	0.3	0	0	0	8.008	6.84	1.08

Table 1 continued

River	Year	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	Mean	SE	TOTAL
Bidasoa	TC	17	0	35	16	8	39	48	42	16	27	22	59	59	61	32.07	5.33	449
	FS	6	18	23	43.2	27.5	62.8	100	50	38.4	149	56.3	12.5	0	0	41.93	15.2	587,067
	FS/TC	0.35	-	0.66	2.7	3.44	1.61	2.08	1.19	2.4	5.53	2.56	0.21	0	0	1.749	0.58	1.31

TC: total catches; FS: thousands of foreign individuals released; FS/TC: average stocking effort, calculated as the ratio between FS and TC. We provide also the mean and standard error of TC, FS and FS/TC. Note that we calculated the average stocking effort in two different ways: (1) as the mean of the 14 FS/TC (SE1) estimated per river shown in the column "Mean" (2) as the ratio of the cumulative values (from 1981–1994) of TC and FS (SE2) shown in the column "TOTAL".

B: banned period

–: data not available or FS/TC could not be calculated

Masma and Bidasoa in 1992–2004. Most of these samples were returning adults obtained from angling catches during the fishing season (March–August/September). We also analysed 95 archival scales from rivers Miño, Lérez, Ulla and Eo from 1950–1960.

Given the absence of reliable salmon counts for these rivers, we used angling catches as a proxy of population size that reflects stock abundance (Chadwick 1985; Ryan 1986; Nicieza et al. 1990). Among these eight salmon rivers and based on the official number of captures (Table 1), rivers Eo and Bidasoa should harbour the largest salmon populations, whereas in rivers Lérez, Mandeo and Landro the reduced number of captures suggests that these populations are much smaller. The remaining rivers (Miño, Ulla and Masma) should present intermediate population sizes. All of these rivers are sustained since 1992 by supportive breeding using native stocks (Official records of the corresponding local authorities).

Laboratory analyses

Total genomic DNA was isolated following the protocol described by Taggart et al. (1992). PCR amplification of the polymorphic sites of the ND1 mtDNA was carried out using primers described by Knox et al. (2002). We amplified by PCR two segments of the ND1 region, the first using primers 4D and 7R amplified mitochondrial region 4447–4959 following Hurst et al. (1999). This 4D-7R fragment included polymorphic positions 4517, 4821 and 4910 that can be detected after the digestion with *Hae*III and *Ava*II for position 4517 and *Dra*I and *Hinf*I for the other two positions, respectively. The second PCR was made using primers 5F and 5R to amplify region 3914–4024. This 111 bp fragment included the polymorphic position 3971 that can be detected with *Hae*III and *Ava*II and position 3989 detected after the digestion with *Rsa*I. Historical scales were amplified using sets of primers 4, 5, 6 and 7 described by Knox et al. (2002), allowing the amplification of fragments around 100 bp that included all the polymorphic sites mentioned above. PCR reactions were made in a total volume of 20 µl that included: 2 µl of 10× PCR Buffer (10 mM Tris–HCl pH 8.8, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton×-100), 1 µl of dNTPs (10 mM each), 1 µl of each primer (20 µM) and 0.5 units of Dynazyme II (Finnzymes Oy). PCRs were performed in an ABI 9700 thermocycler for 35 cycles. A first step of denaturation for 5 min was made and then 35 cycles of denaturation at 95°C for 20 s, annealing at 42°C for 20 s and extension at 72°C for 20 s.

In order to explore other polymorphisms apart from the restriction sites in this region, we screened all modern samples ($N = 430$) for both ND1 fragments using single strand conformation polymorphisms (SSCPs). Eight µl of

Fig. 2 Rivers screened for the ND1 in this study (in bold). We also include rivers previously described for the ND1 region (Verspoor et al. 1999; Consuegra et al. 2002). Pie diagrams show the distribution of haplotype frequencies after pooling temporal samples of the same river together. In Asón we show the haplotype frequencies of the period 1980–1990

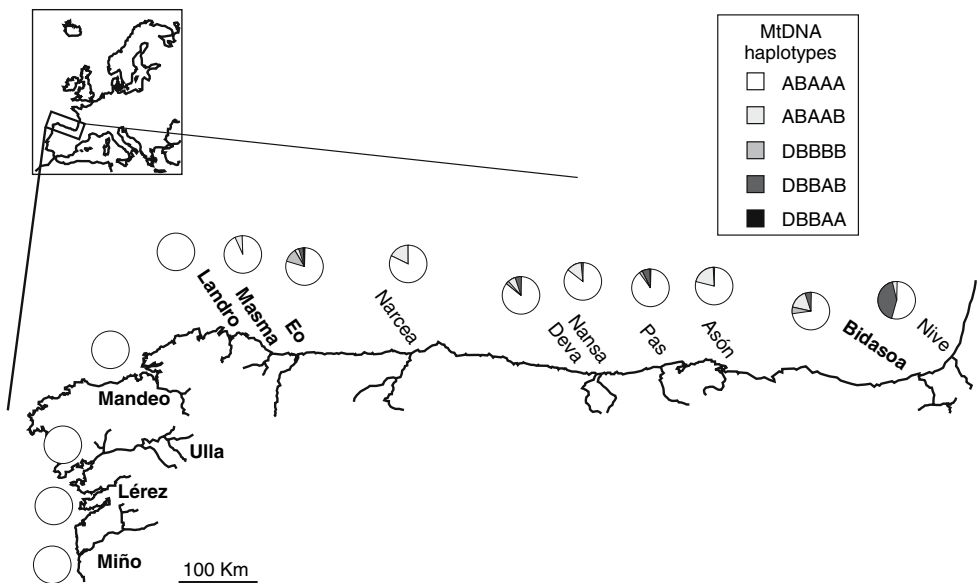


Table 2 Haplotype frequencies for ND1 and haplotypic (*h*) and nucleotide (π) diversities of the samples of this study (Galician-Bidasoa dataset)

	N	ND1 haplotype (<i>Hae</i> III, <i>Dra</i> I, <i>Ava</i> II, <i>Hinf</i> I, <i>Rsa</i> I)					<i>h</i>	π
		ABAAA	ABAAB	DBBBB	DBBAB	DBBAA		
Miño 50–60	17	1	0	0	0	0	0	0
Miño 93–94	16	1	0	0	0	0	0	0
Miño 03–04	16	1	0	0	0	0	0	0
Lérez 52–60	5	1	0	0	0	0	0	0
Lérez 2003	20	1	0	0	0	0	0	0
Ulla 50–60	25	1	0	0	0	0	0	0
Ulla 92–94	100	1	0	0	0	0	0	0
Ulla 97–00	150	1	0	0	0	0	0	0
Mandeo 92–94	25	1	0	0	0	0	0	0
Landro 93–94	10	1	0	0	0	0	0	0
Masma 93	12	0.917	0.083	0	0	0	0.167	0.0003
Masma 94	13	0.923	0.077	0	0	0	0.154	0.0003
Masma 03–04	20	1	0	0	0	0	0	0
Eo 92–93	3	1	0	0	0	0	0	0
Eo 1994	15	0.867	0.067	0.067	0	0	0.216	0.0007
Eo 1997	10	0.800	0.100	0.100	0	0	0.378	0.0013
Eo 1998	10	0.900	0	0.100	0	0	0.200	0.0010
Eo 1999	8	0.875	0	0.125	0	0	0.250	0.0013
Eo 2000	2	1	0	0	0	0	0	0
Eo 50–60	48	0.729	0.021	0.187	0.042	0.021	0.440	0.0019
Bidasoa 2002	459	0.754	0.163	0.044	0.039	0	0.403	0.0011
Bidasoa 2003	190	0.695	0.211	0.047	0.047	0	0.471	0.0012
Bidasoa 2004	116	0.655	0.164	0.069	0.112	0	0.531	0.0015

For each sample we provide the name of the river and sampling period. N: sample size

each PCR were separated in non-denaturing polyacrylamide gels (50:1) at 10% and ran at 4°C for 90 min at 30 W. Gels were silver stained and bands were analyzed by

visual inspection. Different SSCP patterns were cleaned using GFX PCR DNA purification kits (Amersham Biosciences) and sequenced, at least twice per SSCP pattern,

using a dRhodamine terminator cycle sequencing kit and an automated sequencer ABI PRISM 310. As we did not observe other polymorphisms than those present at the restriction sites (see results), we screened the remaining samples using RFLP-PCR.

Digestions with the restriction enzymes were made following manufacture recommendations (Roche). Fragments were run in polyacrylamide gels together with a 100 bp molecular marker and visualized after silver staining. Haplotype designation followed Verspoor et al. (1999). This nomenclature has also been used in other studies of Atlantic salmon (King et al. 2001; Consuegra et al. 2002).

Population genetic analyses

Three different datasets were assembled to perform the different analyses:

- (1) Galician-Bidasoa dataset: this includes the eight rivers that we have screened for the ND1 in this study (rivers Miño, Léz, Ulla, Mandeo, Landro, Masma, Eo and Bidasoa).
- (2) Southern European dataset: this includes samples from Galician rivers and Bidasoa, plus data from other southern European rivers (Narcea, Deva, Nansa, Pas, Asón and Nive) previously screened for the ND1 by Verspoor et al. (1999) and Consuegra et al. (2002).
- (3) Global dataset: this includes datasets 1 and 2 plus data from possible donor populations of northern Europe, i.e. rivers screened for the ND1 in British Isles (Ireland and UK), Scandinavia (Norway, Denmark and the Atlantic coast of Sweden) and Iceland (see Nielsen et al. 1996; Verspoor et al. 1999; Nilsson et al. 2001).

All the statistical tests were performed with the program ARLEQUIN version 2.0 (Excoffier et al. 1992), unless indicated in the text. We calculated haplotype frequencies, haplotype (h) and nucleotide (π) diversities (Nei 1987) within populations for all three datasets.

Temporal homogeneity

For the Galician-Bidasoa dataset, we tested for temporal homogeneity within rivers performing exact tests of differentiation among temporal samples. We carried out the same analysis for the samples of river Asón (see Consuegra et al. 2002). We also performed an AMOVA with the haplotype frequencies to test for significant variation among three periods: 1950–1960, 1993–1994 and 1997–2004. Because we did not detect temporal heterogeneity (except in river Asón), in the subsequent analyses all temporal samples from the same river were pooled.

Population structure and phylogeography

For the southern European dataset we calculated pairwise F_{ST} and associated P -values (10,000 permutations) to describe the current population structure. To represent the Asón river we used the 1980–1990 samples (see Consuegra et al. 2002). A sequential Bonferroni correction (Hochberg 1988) was performed to correct for multiple tests. We performed an AMOVA with the haplotype frequencies considering four geographical regions: Atlantic (rivers Miño, Léz, Ulla and Mandeo), Western Cantabric (rivers Landro, Masma, Eo and Narcea), Eastern Cantabric (rivers Deva, Nansa, Pas and Asón), and Basque region (rivers Bidasoa and Nive). We performed a Mantel test comparing geographical distances and F_{ST} values between populations. To test whether isolation by distance was an artefact of effects of different levels of foreign stocking across rivers we performed a partial mantel test (Smouse et al. 1986) correcting for stocking level. The indicator matrix for this test consisted of the pairwise difference of number of foreign released individuals (FS) between rivers. If foreign introductions had contributed significantly to the genetic differences across populations we would expect that pairs of rivers with a higher difference in stocking would present also higher F_{ST} and vice-versa. This correlation was performed with only 12 rivers. We did not include rivers Léz and Nive because the former's salmon population became extinct (Saura et al. 2006) and for the latter we could not obtain detailed stocking information. To perform this analysis we used the IBDWS v3.03 software (Jensen et al. 2005).

In addition, we implemented a nested clade phylogeographic analysis (NCPA, Templeton et al. 1995), using the programs TCS 1.18 (Clement et al. 2000) and GeoDis 2.0 (Posada et al. 2000). The NCPA is based on the biological interpretation of clade distances (D_c), nested clade distances (D_n) and interior-tip clade distances (IT_c and IT_n). D_c measures how geographically widespread are the individuals that bear haplotypes from a specific given clade. D_n measures how far the individuals bearing haplotypes from a given clade are from all other individuals that bear haplotypes included in the immediate higher step clade. IT_c and IT_n measure the average difference in clade and nested clade distances (respectively) between interior and tip clades in the network. Statistical significance was estimated by 10,000 random permutations under the null hypothesis of no geographic association of the genetic distribution. Phylogeographical interpretations for significantly small and large statistics were achieved using an updated version of the inference key in Templeton et al. (1995) available at <http://darwin.uvigo.es>.

To assess the relationships among populations, using the global dataset, we constructed a neighbour-joining

(NJ) tree based on Cavalli-Sforza's genetic distances (D_{CS}) using PHYLIP 3.6 (Felsenstein 1993). We included in the NJ tree all the southern European rivers and all rivers from the three possible donor European regions that have been screened for the ND1 (see Nielsen et al. 1996; Verspoor et al. 1999; Nilsson et al. 2001). Bootstrap values (Felsenstein 1985) were not calculated for these trees because our data includes only one locus. Note that resampling individuals does not result in a direct evaluation of the confidence on the population tree nodes.

Admixture analysis

We performed an admixture analysis using the Bayesian method of Pella and Masuda (2001) implemented in the software BAYES. For each river, we considered the possible donor stocks that have been described for the ND1 contributing to the admixture (see Fig. 1), and one resident stock (the ancestral sample from the corresponding river). Therefore we only analyzed rivers from which we had old scales (Miño, Ulla and Eo). We excluded river Lérez because of its low sample size (only five individuals were available from 1950–1960). We ran several chains with different values for population proportions: one chain was begun for each stock, with that stock composing 0.95 of the mixture and the remaining three stocks composing equal proportions. To monitor the convergence of the chains to the target posterior distribution, the shrink factor was computed (Gelman and Rubin 1992). Chains were combined after discarding the first half of each chain as burn-in. We report the mean and the standard deviation from the resulting posterior distribution.

Population management analysis

To check whether the intensity of foreign introductions could explain the different observed levels of introgression, we analysed the available data for twelve Spanish rivers from the period 1981–1994, when foreign introductions were carried out in Spain. The management data include total angling catches (TC) per river and year, thousands of foreign individuals released (FS) and stocking effort ($StE_1 = FS/TC$) (see Table 1). To test for significant differences in stocking among southern European rivers we carried out the non-parametric Kruskal-Wallis test using FS and StE_1 as variables to be contrasted (with 14 replicates per river) and rivers as grouping factor.

We also performed three different linear regressions to test whether there was a significant correlation between foreign stocking and introgression of allochthonous stocks in these rivers. As a measure of introgression we calculated

the genetic similarity (using Cavalli-Sforza's genetic distance, D_{CS}) between each Spanish river and the northern European group. The latter was obtained by pooling all the ND1 data available of all the rivers of the regions where foreign stocks originated (i.e. British Isles, Scandinavia and Iceland). To measure the intensity of foreign stocking at each river we used three different parameters: (1) the cumulative values of FS (FS_T) (from 1981–1994); (2) the mean of the 14 FS/TC estimated per river (StE_1) and (3) the ratio of the cumulative values of TC and FS (StE_2) from the 1981–1994 period. Therefore, we calculated the linear regression and correlation of FS_T , StE_1 and StE_2 with D_{CS} .

To check whether there was an East–West cline in the intensity of foreign introductions we performed three different linear regressions using the river's mouth longitude as the independent variable and FS_T , StE_1 and StE_2 as dependent variables. Also, to test whether there was a similar cline in the level of introgression we performed a linear regression between longitude (independent variable) and D_{CS} (dependent variable).

Results

Genetic variation

SSCPs analysis performed in salmon adults did not reveal other polymorphic positions than those previously described by Verspoor et al. (1999). Therefore, our haplotype designations followed these authors. Moreover, the different sequenced patterns were identical to those reported previously by Nilsson et al. (2001). We found five haplotypes based on restriction enzyme digestions (Tables 2 and 3). The most frequent haplotype was ABAAA. The Miño, Ulla, Lérez, Mandeo and Landro rivers did not exhibit any variation in any of the temporal samples studied and all populations were fixed for ABAAA. Samples from river Bidasoa and Eo 1950–1960 were the most diverse.

Temporal homogeneity

For the Galician-Bidasoa dataset, pairwise F_{ST} and exact tests were not significant among temporal samples within rivers, after Bonferroni correction. The AMOVA also indicated that the distribution of genetic variation in these rivers is not significantly different among temporal periods (4.27%; $P = 0.14$). According to the results, temporal samples from the same river were pooled for subsequent analyses (Table 3). For the river Asón we detected heterogeneity before and after the 1960–1980 period, and samples were pooled accordingly into Asón 1950–1960 and Asón 1980–1990.

Table 3 Haplotype frequencies for ND1 and haplotypic (h) and nucleotide (π) diversities for each southern European river, after pooling homogenous temporal samples of the same river

	N	ND1 haplotype (<i>HaeIII</i> , <i>DraI</i> , <i>AvaII</i> , <i>HinfI</i> , <i>RsaI</i>)						h	π
		ABAAA	ABAAB	DBBBB	DBBAB	DBBAA	SBBAB		
Southern Europe									
Miño	49	1	0	0	0	0	0	0	0
Lérez	25	1	0	0	0	0	0	0	0
Ulla	275	1	0	0	0	0	0	0	0
Mandeo	25	1	0	0	0	0	0	0	0
Landro	10	1	0	0	0	0	0	0	0
Masma	45	0.956	0.044	0	0	0	0	0.087	0.0002
Eo	96	0.802	0.031	0.135	0.021	0.011	0	0.340	0.0014
Narcea ^a	42	0.818	0	0.182	0	0	0	0.304	0.0016
Deva ^b	166	0.928	0.036	0.012	0.024	0	0	0.138	0.0006
Nansa ^b	240	0.863	0.117	0	0.020	0	0	0.250	0.0007
Pas ^b	245	0.914	0.033	0	0.049	0.004	0	0.162	0.0006
Asón 50–60 ^b	61	0.967	0	0.016	0.017	0	0	0.065	0.0003
Asón 80–90 ^b	237	0.765	0.227	0	0.009	0	0	0.366	0.0007
Bidasoa	765	0.724	0.175	0.049	0.052	0	0	0.440	0.0012
Nive ^a	32	0.563	0.031	0	0.406	0	0	0.534	0.0017
European regions									
Southern Europe	2252	0.826	0.105	0.027	0.035	0.001	0	0.292	0.0008
British Isles ^{acd}	420	0.477	0.287	0.115	0.121	0	0	0.680	0.0019
Scandinavia ^c	587	0.560	0.109	0.070	0.240	0	0.020	0.612	0.0019
Iceland ^a	49	0.224	0	0.143	0.633	0	0	0.540	0.0017

We also include variation reported in donor European regions (see Nielsen et al. 1996; Verspoor et al. 1999; Nilsson et al. 2001; Consuegra et al. 2002)

^a Verspoor et al. (1999)

^b Consuegra et al. (2002)

^c Nilsson et al. (2001)

^d Nielsen et al. (1996)

Population structure

Many pairwise F_{ST} and exact tests comparisons between rivers in the Galician-Bidasoa dataset were significant (Table 4). The AMOVA indicated that the distribution of genetic variation in these rivers is significant among populations within periods (6.62%; $P < 0.001$).

For the southern European dataset, the AMOVA was not significant among regions (4.78%; $P = 0.10$), but it was significant within regions (4.58%; $P < 0.0001$). The Mantel test indicated a significant pattern of isolation by distance in this area ($R = 0.42$; $P = 0.001$) (Fig. 3). The partial mantel test for 12 rivers (after excluding Lérez and Nive) indicated that the correlation between genetic and geographic distances was still significant after correcting for the intensity of foreign stocking ($R = 0.32$; $P = 0.035$). The NCPA revealed a significant nonrandom association of clades and sampling locations (Fig. 4). There was no geographical association of haplotypes within clade 1–1.

The inference chain for clade 1–2 suggested a history of contiguous range expansion. For the whole network we inferred a pattern of restricted gene flow with isolation by distance. When we performed the NCA excluding rivers Nansa, Asón, Bidasoa and Nive (the rivers that showed evidence of introgression, see discussion) we obtained the same result.

In the NJ tree depicted in Fig. 5, most Spanish rivers grouped together and separately from northern European rivers. However, there were exceptions: rivers Asón 80–90, Nansa, Bidasoa and Nive grouped with northern European rivers. Interestingly, river Shannon (Ireland) grouped within the Spanish clade.

Admixture analysis

The estimated contributions of the ancestral samples to the modern populations were very high in all rivers (>0.90) (Table 5). Northern European stocks contribution was very

Table 4 Pairwise F_{ST} and exact tests of population differentiation for all southern European salmon rivers

	Miño	Lérez	Ulla	Mandeo	Landro	Masma	Eo	Narcea	Deva	Nansa	Pas	Asón 50–60	Asón 80–90	Bidasoa
Lérez	0													
Ulla	0	0												
Mandeo	0	0	0											
Landro	0	0	0	0										
Masma	0.026	0.004	0.132	0.004	-0.035									
Eo	0.103	0.077	0.248*	0.077	0.038	0.062								
Narcea	0.172	0.121	0.441*	0.121	0.065	0.104	-0.011							
Deva	0.020	0.007	0.061*	0.007	-0.026	-0.008	0.061*	0.099*						
Nansa	0.065	0.050	0.124*	0.050	0.018	0.019	0.045*	0.077*	0.022					
Pas	0.027	0.015	0.063*	0.015	-0.018	0.001	0.064*	0.101*	-0.002	0.022				
Asón 50–60	0.004	-0.011	0.056	-0.011	-0.046	-0.001	0.072	0.116	0.001	0.047	0.008			
Asón 80–90	0.141*	0.123	0.239*	0.123	0.091	0.088	0.069*	0.099*	0.101*	0.031	0.103*	0.125*		
Bidasoa	0.110*	0.099	0.145*	0.099	0.072	0.072	0.034*	0.054*	0.072*	0.029*	0.071*	0.093*	0.007	
Nive	0.453*	0.358*	0.766*	0.358*	0.268*	0.360*	0.212*	0.233*	0.404*	0.299*	0.375*	0.405*	0.225*	0.153*

Significant results at the 5% level after Bonferroni correction for F_{ST} are shown in bold. Significant exact tests are identified with an asterisk (*)

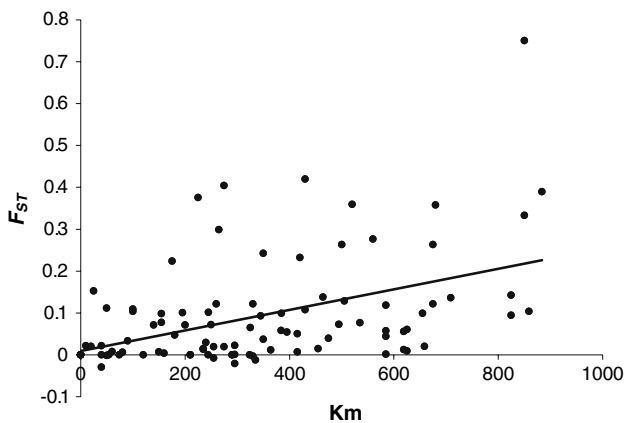


Fig. 3 Correlation between F_{ST} and geographic distance (Km). The line represents the regression slope ($R = 0.42$; $P = 0.001$)

low (<7%) except for the contribution of the Shannon to the river Eo (66%).

Data management analysis

The Kruskal-Wallis test was significant for both FS ($P = 0.000$) and StE_1 ($P = 0.014$), indicating that stocking intensity has been significantly different across rivers. Moreover, we observed a negative and significant correlation between FS and genetic distance with northern European salmon populations ($R = -0.61$; $P = 0.034$). In other words, Spanish populations that were more heavily stocked were more similar to northern European salmon

rivers and vice versa. However, none of the stocking effort measures (StE_1 and StE_2) were significantly correlated with genetic similarity ($R = -0.20$; $P = 0.6$ and $R = -0.45$; $P = 0.39$, respectively).

We also observed a significant correlation between FS and StE_2 with longitude ($R = -0.67$; $P = 0.019$ and $R = -0.60$; $P = 0.040$, respectively), suggesting that stocking was more intense in the East. However, we did not detect a significant correlation between StE_1 and longitude ($R = -0.34$, $P = 0.28$). The correlation between introgression and longitude was also significant ($R = 0.88$, $P = 0.000$).

Discussion

Our results indicate that (matrilineal) introgression of foreign salmon stocks in Northern Spain has been different among rivers. In general, it has been much lower in northwestern than in northeastern Spain (except for rivers Deva and Pas). We did not observe genetic similarity between the western rivers and the northern European populations. Moreover, we did not detect significant temporal variation in the Galician rivers where old scales were available (Miño, Lérez, Ulla and Eo). Moreover, the admixture stock analysis in rivers Miño, Ulla and Eo suggests that in most cases the estimated contribution of foreign rivers was very small. Indeed, some similarity with foreign rivers can just arise by haplotype sharing between northern and southern European populations, and this might be the case for rivers Shannon and Eo. The Shannon

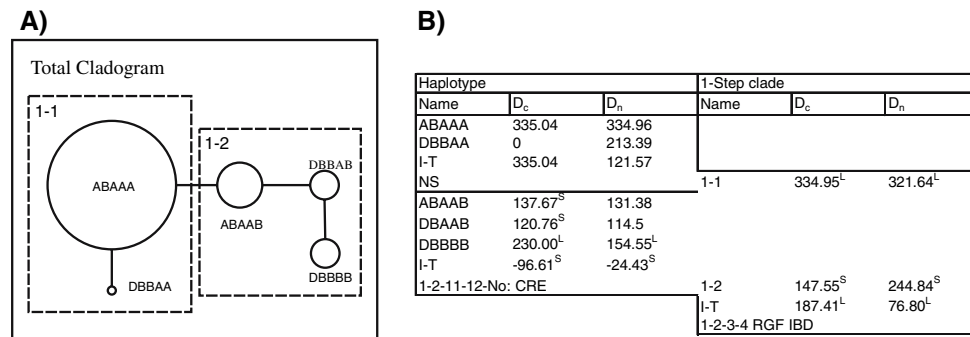


Fig. 4 (A) TCS network and nested design for the five mitochondrial ND1 haplotypes reported in Atlantic salmon populations in southern European rivers. The size of the circle represents the frequency of each haplotype. Dashed boxes represent the one-step clades. (B) Results of the nested clade analysis. Column “Name” is the name of the clade, D_c is the clade distance and D_n the nested clade distance at each one of the levels of the analysis (haplotype and one-step levels).

The row *I-T* indicates the average difference between interior and tip clades. Superscript S means that the statistic was significantly small and superscript L that the statistic was significantly large (at the 5% level). The lines in bold describe the steps followed in the inference key and the conclusion reached: NS (not significant), CRE (Contiguous range expansion) and RGF IBD (Restricted gene flow with isolation by distance)

population is almost fixed for the most common haplotype (ABAAA), similarly to the NW Spanish populations. This population is of hatchery origin, which could help to explain the low variability present in this river. Indeed, it is known that hatchery stocks can deviate significantly from wild source populations (Verspoor 1988).

The pattern of isolation by distance observed also suggests that introductions with similar foreign stocks have not completely homogenised the genetic structure in this region. The observed East-West cline in the intensity of stocking and the level of introgression explain just in part the genetic differentiation, which is also a consequence of restricted gene flow.

The effects of foreign introductions have been more important in the Eastern rivers. Nansa, Bidasoa, Asón and Nive showed evidence of introgression. Presence of foreign stocks has been previously detected in rivers Nansa and Asón using allozymes, although returns of allochthonous salmon was lower than returns for wild fish (Verspoor and García de Leániz 1997). Moreover, temporal differences between samples before and after the introductions in Asón suggest the occurrence of introgression in this river (Consuegra et al. 2002). Similarly, we observed that river Bidasoa was related to foreign rivers, a fact that has not been reported in allozyme studies (Blanco et al. 2005). This could be due to a difference of statistical power between these two types of markers, but also due to the occurrence of introgression only for the mitochondrial genome.

Rivers Nansa, Asón and Bidasoa show a high frequency of haplotype ABAAB, which is common in the British Isles, Norway and in the Swedish Atlantic coast. At least in river Asón, this haplotype was absent before foreign introductions (Consuegra et al. 2002), suggesting that the presence of this haplotype is a consequence of foreign

translocations. For the other two rivers introgression is more difficult to confirm due to the lack of pre-stocking temporal samples. In river Nive, the high frequency of haplotype DBBAB suggest strong introgression levels (Verspoor et al. 1999). Also in river Nivelle, close to Nive, introgression has been high (Martínez et al. 2001).

Several factors could explain the heterogeneity in the success of foreign salmon populations in southern Europe. Most likely, the intensity of introductions, that has been significantly different among rivers, has been crucial. Most heavily stocked rivers (based on *FS*) are more similar to populations in Northern Europe. However, although we observed a similar trend, the correlation between stocking effort and genetic distance with Northern Europe was not significant. Certainly, using the number of catches as a proxy for population size will have some associated error, especially because catches depend on the duration of the fishing season and fishing quotas that differ among rivers. Morán et al. (2005a) demonstrated previously that there was a clear association between stocking effort and the genetic pattern observed in south European Atlantic salmon populations. Indeed, there are also other factors that should also influence the success of foreign introductions, like particular characteristics of the river including both environmental and biological factors (Morán et al. 2005a). For instance, the accessible area of the river and therefore its carrying capacity could influence the differential success of foreign stocks among rivers (see Verspoor and García de Leániz 1997). Also, the occurrence of droughts could determine the success of non-native salmon (see Morán et al. 2005a). As biological factors we can mention the differential predation of foreign and native stocks. In a common garden experiment carried out in river Eo using salmon populations from Ireland, Scotland, Norway, native river Eo and inter-stock hybrids Eo-Norway, different

Fig. 5 Neighbour-joining population tree of all southern and northern European rivers screened for the ND1. Abbreviations are as follows: Iceland (Ice), Sweden (Sw), Denmark (Den), Norway (No), Ireland (Ir), Scotland (Sc), France (Fr) and Spain (Sp). In bold are southern European samples



predation rates by wild otters on each stock were observed. The results showed that predation was higher in non native stocks (García de Leániz et al. 1994).

Studies carried out so far concerning the success of foreign introductions support that they have been in general ineffective (García de Leániz et al. 1989; Vázquez et al. 1993; Morán et al. 1994; Verspoor and García de Leániz 1997; Blanco et al. 2005; Morán et al. 2005a; Morán et al. 2005b). This study confirms this general trend in NW Spain but not in the NE rivers. However, it is important to remark that introgression can occur via mature male parr (Morán et al. 1994) and we cannot detect this using mtDNA. Southern and northern European salmon populations show genetic, physiological and ecological differences (García de Leániz et al. 1989), and there are evidences that foreign

stocks are less adapted to Iberian rivers than wild stocks (García de Leániz et al. 1994). Our results do not call for changes in introduction policies, currently based on the use of native stocks. Moreover, the occurrence of introgression in some southern European rivers (like Nansa, Asón, Bidasoa or Nive) reinforces the idea that native stocks should be used to avoid disrupting locally adapted populations.

Our results do suggest that southern European populations are generally differentiated, as in northern Europe (Nilsson et al. 2001; Asplund et al. 2004). This structure occurs mainly among rivers within regions rather than among geographic areas. Previous studies using allozymes and microsatellites suggested that the genetic structure of this area was low (Blanco et al. 2005; Morán et al. 2005a; Morán et al. 2005b; Ayllón et al. 2006), although

Table 5 Parameters of the posterior distribution for admixture proportions

Stock	Mean	SD
Eo		
Burrishoole	0.077	0.108
Shannon	0.660	0.260
Conon	0.029	0.049
North-Esk	0.031	0.061
Eo 1950–1960	0.203	0.276
Ulla		
Burrishoole	0.002	0.004
Delphi	0.001	0.003
North-Esk	0.002	0.004
Ulla 1950–1960	0.995	0.006
Miño		
North-Esk	0.032	0.044
Miño 1950–1960	0.968	0.044

differentiation was already observed for mtDNA (Consuegra et al. 2002; Ayllón 2005). These opposite results might be related to the distinct nature of the molecular markers used, like patterns of inheritance and substitution rates, but also to the different geographical scales considered. Mitochondrial DNA is maternally inherited, and it has a smaller effective size than nuclear genes. For instance, Ayllón (2005) found different patterns of genetic isolation in several Spanish salmon rivers using nuclear and mitochondrial genes. Similar differences were also observed in the Baltic Sea (Nilsson 1997). Contrary to most genetic studies of this area, we have considered a large number of populations that represent the whole range of variation across southern Europe.

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