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RESEARCH ARTICLE

Stocking may increase mitochondrial DNA diversity but fails to halt the decline of endangered Atlantic salmon populations

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Abstract Over the last 50 years, Spanish Atlantic salmon (*Salmo salar*) populations have been in decline. In order to bolster these populations, rivers were stocked with fish of northern European origin during the period 1974–1996, probably also introducing the furunculosis-inducing pathogen, *Aeromonas salmonicida*. Here we assess the relative importance of processes influencing mitochondrial (mt)DNA variability in these populations from 1948 to 2002. Genetic material collected over this period from four rivers in northern Spain (Cantabria) was used to detect variability at the mtDNA ND1 gene. Before stocking, a single haplotype was found at high frequency (0.980). Following stocking, haplotype diversity (h) increased in all rivers (mean h before stocking was 0.041, and 0.245 afterwards). These increases were due principally to the dramatic increase in frequency of a previously very low frequency haplotype, reported at higher frequencies in northern European populations proximate to those used to stock Cantabrian rivers. Genetic structuring increased after stocking: among-river differentiation was low before stocking (1950s/1960s Φ_{ST} = –0.00296–0.00284), increasing considerably at the height of stocking (1980s Φ_{ST} = 0.18932) and decreasing post-stocking (1990s/2002 Φ_{ST} = 0.04934–0.03852). Gene flow from stocked fish therefore seems to have had a substantial role in increasing mtDNA variability. Additionally, we found significant differentiation between individuals that had probably died from infectious disease and apparently healthy, angled fish, suggesting a possible role for pathogen-driven selection of mtDNA variation. Our results suggest that stocking with non-native fish may increase genetic diversity in the short term, but may not reverse population declines.

Keywords *Salmo salar* · Stocking · Mitochondrial DNA · Conservation genetics · Foreign gene introgression

Introduction

The Atlantic salmon (*Salmo salar)* is currently experiencing a global decline, largely due to anthropogenic activities (WWF 2001). In common with other economically important fish species, human interventions to reverse declines have been attempted. Restocking activities are widespread for salmonid populations (Aprahamian et al. 2003), but have also been attempted in other taxa (e.g. Britt et al. 2004). Stocking can be used to supplement small native populations with individuals transplanted from larger stable populations to try to bolster census size. If donor populations are genetically distinct from the recipient population, successful matings between native and non-native individuals may be considered hybridization events and

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can cause genetic introgression (Rhymer and Simberloff 1996). If stocked individuals survive to reproduce, increased gene flow from non-native populations into the stocked population and ultimately increased genetic diversity may result. However, if a substantial and sustained increase in the census size of the breeding population is not achieved through stocking, potentially harmful genetic effects due to increased inbreeding may occur (Wang and Ryman 2001). There are conservation concerns associated with genetic introgression: swamping of indigenous (and distinct) gene pools and the potential for disruption for locally adapted gene complexes (reviewed by Hindar et al. 1991). The stocking rate and effort may be a factor in the level of genetic introgression (Buckmeier et al. 2003). In addition to genetic effects, stocking or translocation of individuals may allow transmission of diseases to naïve populations, which can have devastating consequences for local populations, for example in USA raccoons (Smith et al. 1984).

Most populations of Atlantic salmon are anadromous, making a long-distance marine feeding migration before homing, often with high fidelity, to their natal rivers to reproduce. Atlantic salmon populations are therefore reproductively isolated from others, and studies of genetic structuring among populations using a range of molecular markers have revealed genetic divergences between different populations (Danielsdottir et al. 1997; Nielsen et al. 1996; Nilsson 1997; Verspoor et al. 1999; King et al. 2001), even between tributaries of major river systems (Verspoor, 1997). Reproductive isolation provides the potential for adaptation to local environmental conditions, for which there is some evidence in Atlantic salmon (Verspoor and Jordan 1989; Taylor 1991; Jordan et al. 1997).

In Cantabria, northern Spain, historically Atlantic salmon inhabited seven rivers. In three of these rivers, the species is now extinct. Of the four remaining rivers with extant Atlantic salmon populations, two populations are critically endangered and in serious decline (the Asón and Pas), one is endangered and in decline (Deva) and one is endangered but stable (Nansa), (García de Leániz et al. 2001). These rivers are close to the southernmost limit of the range and are extreme environments for Atlantic salmon mainly due to high water temperatures and droughts. All of these rivers were stocked to a greater or lesser degree during the period 1974–1996 with fertilized ova and juveniles of foreign origin, from farmed and wild source populations. The rivers Asón, Nansa and Deva had the greatest relative stocking effort, whilst the Pas had a much smaller relative stocking effort. Non-native stocking effort peaked during the early 1980s, for example, in 1982 the River Asón received 90,000 eyed ova from Scotland and 200,000 eyed ova from Iceland compared with 120,000 eyed ova from Denmark in 1974 or 20,000 fry and parr from Scotland in 1990 (García de Leániz, unpublished). Non-native stocking of Cantabrian populations is believed to have caused the introduction of at least one bacterial disease, furunculosis (causative agent *Aeromonas salmonicida*) which has resulted in high mortalities especially in the rivers Asón and Pas. Non-native gene introgression has been detected in some southern European salmon populations (Martinez et al. 2001), though in most cases the number of non-native adults has been shown to be very small (García de Leániz et al. 1989; Verspoor and García de Leániz 1997).

In this study we compared mitochondrial (mt)DNA variability in the four extant Cantabrian populations for the latter half of the 20th century. We used the ND1 gene region, which codes for the NADH1 subunit, because previous studies have shown this region to be most variable in Atlantic salmon (Verspoor et al. 1999), other studies on other Atlantic salmon populations have used this region to examine genetic structure (Nielsen et al. 1996; Nilsson 1997, Verspoor et al. 1999; Nilsson et al. 2001, Consuegra et al. 2002; Consuegra et al. 2005a) and the haplotypes have been well described.

The majority of the samples used in this study were derived from single historical fish scales. Historical collections of fish scales and fish otoliths have been made, generally for use in ageing individuals, but in more recent times, protocols have been developed for their use in molecular genetic work (Hutchinson et al. 1999; Nielsen et al. 1999a), for example in the amplification of mtDNA and microsatellites (Nielsen et al. 1999b). Historic collections (especially of fish scales) are particularly useful for the investigation of long-term genetic processes and perturbations to genetic structure. Several previous studies have been able to use such historic collections to examine long-term temporal trends of populations structure (Nielsen et al. 1997) and questions such as immigration of hatchery released fish into wild populations with the effect of genetic homogenization (Vasemagi et al. 2001).

We used a polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) based approach to examine temporal and spatial genetic structure of these populations. Our aim was to examine the influences of random genetic drift, gene flow and selection on these populations over time. With our data we were able to test two main hypotheses. First, we tested whether non-native stocking activities have

affected the variability of ND1 in these populations via gene flow. If northern European mtDNA genes did introgess into Spanish populations during stocking, we would expect to see an increase in haplotype diversity and possibly reduced genetic differentiation due to homogenization through foreign gene flow. Based on previous work, our a priori expectation was that foreign stocking did not leave a genetic signature on populations, and therefore decreased variability was expected to be due to random genetic drift in a declining population.

Second, we tested whether individuals that showed signs of infection with disease were genetically differentiated from those fish that were angled whilst apparently healthy, which may have indicated a role for selection. We would not expect a direct role of mtDNA genes in resistance to infectious disease, and therefore mtDNA genetic differentiation between individuals that succumbed to infectious diseases and those that did not was not expected.

Materials and methods

Samples

Adipose fin tissue was collected in 2002 from adult Atlantic salmon returning to the rivers Asón (n = 30), Deva (n = 32), Nansa (n = 31) and Pas (n = 32) (Table 1) and stored in 95% ethanol at 4°C prior to genetic analysis. Scale collections from adult fish caught by anglers for each river have been made since 1948. Given the limitations of the number of scales available for genetic analysis, samples of individuals representing the time periods 1948–1957, 1960–1963, 1980–1989, 1990–1996 were pooled. No scale samples were available for any river from the 1970s, or for the river Nansa pre-1960. Therefore, 19 samples, stratified by river and decade and comprising at least 24 individuals each, were generated for analysis (Table 1). During the 1990s, there were outbreaks of mortality in the River Asón, likely to be mainly due to furunculosis, and therefore the 1990s Asón sample comprised both individuals who were removed from the river dead due to infectious diesease (n = 26), and individuals who were angled while visibly healthy (n = 45) (for brevity, the terms ''diseased'' and ''healthy'' will be used to describe these two groups).

Table 1 Cantabrian Atlantic salmon samples available for genetic analysis

Sample	Years available/included	*N*
Asón 1950s	48,49,50,53,57	36 (2)
Asón 1960s	60,61,62,63	31 (3)
Asón 1980s	87,88,89	32 (2)
Asón 1990s	90,91,92,93	71 (2)
Asón 2002	02	30 (5)
Deva 1950s	53,57	24 (1)
Deva 1960s	60	30 (2)
Deva 1980s	87,88,89	43 (3)
Deva 1990s	91,92,93,94,95,96	37 (3)
Deva 2002	02	32 (5)
Nansa 1960s	61,62,63	38 (2)
Nansa 1980s	88,89	31 (2)
Nansa 1990s	90,91,92	32 (2)
Nansa 2002	02	31 (7)
Pas 1950s	48,49,50,51,53	35 (3)
Pas 1960s	60,61,62,63	53 (1)
Pas 1980s	87,88,89	60 (2)
Pas 1990s	92,93,94	39 (4)
Pas 2002	02	32 (4)
Total		717 (55)

N gives number of individuals analyzed by PCR/RFLP and numbers in brackets are numbers of individuals analyzed by direct sequencing

DNA extraction and amplification of the ND1 gene region

Total DNA was extracted from all samples using the Promega™ Wizard SV 96 Genomic DNA Purification System. When extracting from modern adipose fin clippings, the manufacturer's protocol was strictly adhered to. All historic scale extractions were carried out in a dedicated historic DNA laboratory, physically separated from PCR procedures. When extracting from the historic scales between 1 and 3 uncleaned scales were used and two modifications to the manufacturer's protocol were made: an increase of the incubation time during the elution steps from 2 min to 5 min and a decreased elution volume of 80–100 μl (reduced from 500 μl). For all sets of scale extractions, a blank control was extracted concurrently and subsequently amplified in PCR reactions. All eluted DNA was stored at –20°C.

The modern tissue-derived DNA samples were amplified using PCR and the primers 5′-acgtgatctgagttgacaacgg-3′ (forward) (Palumbi 1996) and 5′-ggtatgagcccgatagctta-3′ (reverse) (Cronin et al. 1993) to generate a 1409 bp fragment. 4 μl of extracted total DNA was used to amplify this region in a 50 μl reaction volume including 0.3 μM each primer, 2.5 μM dNTPs (Bioline), 0.06 units/μl of *Taq* DNA polymerase and 1× the included ThermoPol Buffer (20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100) (New England Biolabs). The PCR profile involved an initial denaturation step of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension step of 72°C for 6 min.

To amplify all the scale samples, a total of 12 primers generating 6 short overlapping fragments were used (Table 2, Fig. 1). These primers were specifically designed from tissue-derived sequences (and amplification of the tissue sequences using these results did not change either the RFLP typing or the sequence analysis) to generate shorter fragments that captured all the variable sites. The contig of these fragments represent a shortened, 1043 bp fragment of the ND1 gene which contained all observed nucleotide variability in the 1.4 kb fragment amplified from the modern tissues. Two microlitres of extracted DNA was used in a 25 μl PCR as described above except with the addition of 0.2 μM BSA. The thermocycling profile for all PCR reactions consisted of initial denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 45 s and a final extension step of 72°C for 6 min. Following PCR, all products were stored at –20°C.

RFLP digestion of ND1 fragments

Following PCR amplification, variability in the ND1 region was detected through digestion with restriction enzymes. Previous work has shown that variation is detectable in this region of DNA for these Spanish populations with *Hae*III, *Hinf*I, and *Rsa*I restriction enzymes (Consuegra et al. 2002). This study used *Hae*III, *Hinf*I, *Rsa*I, *Dra*I and *Ava*II enzymes to be comparable with studies in other areas of the species range (Verspoor et al. 1999; Nilsson et al. 2001). Each single digestion reaction was conducted using 8 μl of PCR product incubated with 3 units of the relevant restriction enzyme and 1 × buffer (NEB) in a total reaction volume of 10 μl for 18–20 h at 37°C. To stop the digestion reaction, 8 μl of loading buffer was added (50% v/v glycerol, 50 mM EDTA, bromophenol blue).

The digestion products were run on 2% agarose TBE gels stained with ethidium bromide against a commercial size standard at 125 V for approximately 45 min and visualized under UV light using GENESNAP™ software (Syngene).

Haplotypes were designated following Verspoor et al. (1999)

DNA sequencing

A total of 55 individuals, 21 from the tissue samples and 34 from the scale samples, were sequenced, representing at least one individual of each of the different haplotypes found in each of the 19 spatial/temporal samples (Table 1).

Following PCR reactions, and prior to sequencing, PCR products were purified. For the 1.4 kb PCR product amplified from modern tissue, purification was achieved using the QiaQuick™ PCR Purification

Table 2 Primers used in PCR amplification and direct sequencing

Name	Primer sequence 5′–3′	Orientation Forward/Reverse	Start position Hurst et al. (1999)
16sRNA_F	ACG TGA TCT GAG TTG ACA ACG G[c]	F	3593
RsaI_F	CGC TTT CCT CAC CTT ACT CGA ACG[b]	F	3914
RsaI_R	TTT AGG CCG TCC GCG ATA GG[b]	R	4024
471_F	CCA TAC CTT GCC CTT ACG CTT G[b]	F	4087
471_R	CAA GCG TAA GGG CAA GTA TGG[b]	R	4107
679_F	GGC ACA AAC CAT TTC CTA CG[d]	F	4271
687_R	GTT TGT GCC ACT GCT CGT AG[d]	R	4279
HaeIII_F	GTG CAC CCT TGA CCT TAC AGA AGG AG[b]	F	4447
859_R	TGC ACG GTT TGT TTC AGC TA[d]	R	4451
HaeIII_R	TAT TCG GCT ACA AAG AAG AGG GCG[b]	R	4545
1020_F	CCT TCC CCG AAT TAA CAG C[d]	F	4611
1063_R	GGC GGC CTT TGT TAT TAG GT[d]	R	4654
DraI_F	CCC TTG TCC TAT GAC ACC TAG CAC TTC[b]	F	4758
DraIHinfI_F	CTA GCA CTT CCA ACC GCA AT[d]	F	4775
1199_R	CGG TTG GAA GTG CTA GGT GT[d]	R	4790
DraI_R	CAG CCA CGC TAT CAA GGT GGT CC[b]	R	4871
DraIHinFI_R	GTG GTG TAG TGG AAG CAC CA[d]	R	4960
CrND1_R	GGT ATG AGC CCG ATA GCT TA[a]	R	5002

[a] (Cronin et al. 1993)
[b] (Knox et al. 2002)
[c] (Palumbi et al. 1996)
[d] Designed from sequences generated in this study

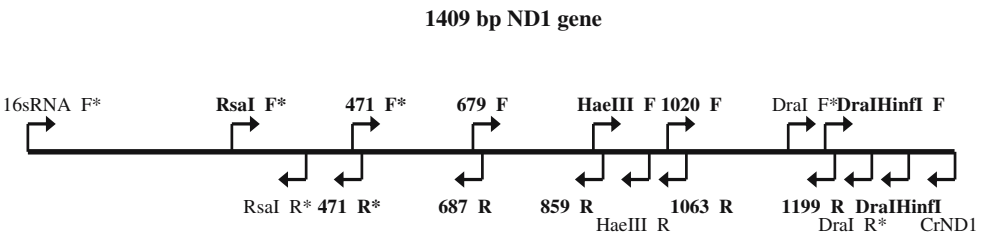

Fig. 1 Schematic of primers used in PCR amplification and direct sequencing. A* indicates use in tissue sequencing reactions, bold indicates use in scale PCR and sequencing reactions

procedure (Qiagen), and purified product was eluted in 50 μl of deionized water. For the smaller PCR fragments derived from historic scale samples, the Microcon YM-50 purification system was used. In this case, the PCR products were eluted in a final volume of 15 μl of deionized water.

For tissue samples, a total of 10 internal primers spanning the ND1 region were used in direct sequencing reactions (Table 2, Fig. 1). For scales, the same forward and reverse primers used to generate each fragment were used in the sequencing reaction (Table 2, Fig. 1). All sequencing reactions were carried out using 3 μl of template DNA, 5 μl of Better Buffer^{TM} (Microzone), 1 μl of BigDye (BigDye V3.1 Sequencing Kit^{TM}, ABI), 0.16 ρmol of primer and deionisied water to a total reaction volume of 15 μl. The thermocyler reaction profile involved an initial denaturation at 96°C for 3 min followed by 25 cycles of 96°C for 15 s, 50°C for 10 s, 60°C for 4 min.

The sequencing reactions were precipitated using the recommended ethanol/EDTA protocol (ABI). Precipitated sequencing reactions were stored at –20°C prior to resuspension in 10 μl of HiDi formamide (ABI) injection buffer. Samples were loaded onto an ABI Prism 3100^{TM} automatic capillary sequencer. Analysis of sequences used Sequencing Analysis 5.1 (ABI) and alignment of the sequence fragments for each individual was conducted using SEQUENCHER^{TM} (Genecodes) with the published *S. salar* mtDNA genome sequence as a reference (Hurst et al. 1999). The sequences were deposited in GenBank (DQ237847–DQ237856).

Analysis of mitochondrial DNA data

Frequencies of RFLP haplotypes were calculated and levels of variation within each sample estimated as haplotype diversity (h) or nucleotide diversity (π) (Nei 1987). A minimum spanning network of haplotypes was constructed based on restriction site presence/absence. Genetic differentiation among samples was assessed using analyses of molecular variance (AMOVA) of RFLP frequencies (Excoffier et al. 1992) or pairwise F_{ST} values. All analyses were conducted using ARLEQUIN version 3 (Excoffier et al. 2005).

ND1 nucleotide sequences were aligned using BIOEDIT (Hall 1999) and checked visually for additional nucleotide polymorphisms not revealed by restriction digests. Pairwise absolute nucleotide differences between all unique sequences were calculated and used to construct a minimum spanning network, using a method implemented in Minspnet (Excoffier and Smouse 1994). We computed, and visualized, genetic distances based on RFLP and sequencing data in the same manner to allow direct comparisons. After translating the sequences, the numbers of synonymous and non-synonymous polymorphisms were calculated. Nucleotide diversity (π) was estimated using DNASP version 4.0 (Rozas et al. 2003).

To explore the possibility of mtDNA differences between healthy and diseased individuals, we calculated haplotype diversities and computed the pairwise F_{ST} values between healthy and diseased fish from the Asón during the period 1990–1993 using ARLEQUIN version 3 (Excoffier et al. 2005). We consider only these samples in this analysis because there was a significant outbreak of furunculosis in the River Asón (the causative pathogen has not yet been detected in the River Nansa) during the early 1990s, and samples were recorded by river bailiffs as presenting with infectious disease symptoms, or else as healthy fish.

RFLP haplotype frequency data from Cantabrian populations were combined with RFLP haplotype frequencies reported for the northern Spanish Narcea population in (Verspoor et al. 1999). The number of net nucleotide substitutions D_A (Nei et al. 1983) between all pairs of samples was calculated for RFLP data using DISPAN (Ota 1993). The resulting matrix of pairwise D_A values was used to construct a UPGMA dendrogram to show relationships among spatial and temporal samples using NEIGHBOUR from the PHYLIP package, version 3.57c (Felsenstein 1994).

Results

Relationship between mtDNA RFLP and sequence diversity

Composite mtDNA ND1 haplotypes were generated by PCR-RFLP for 717 of the 817 (87.8%) Atlantic salmon samples available for analysis from the four Cantabrian rivers that have extant populations. Among the older samples, PCR amplification success was lower (across all scale (pre-2002) samples success rate was 85.5%; for 2002 tissue samples only success rate was 95.4%), probably due to degradation of DNA. The 5 restriction enzymes resolved four variable cut sites producing a total of five composite haplotypes. Estimated sizes of fragments and their restriction digest patterns were as in (Verspoor et al. 1999). Four of the Spanish haplotypes had been reported in Verspoor et al. (1999): AAABA corresponded to haplotype I of Verspoor et al. (1999), AABBA corresponded to haplotype II, DBBBB corresponded to haplotype III and DABBB corresponded to haplotype IV. The Spanish haplotype AAAAA was not reported in Verspoor et al. (1999).

Sequencing of 1043 bases of the ND1 gene for 55 individuals revealed 8 additional polymorphisms that were not detected by the RFLP analysis. However, comparison of minimum spanning networks based on RFLP and sequence data showed that each RFLP haplotype consisted of a single high frequency sequence haplotype, with a variable number (0–4) of low frequency, closely-related sequence haplotypes per RFLP haplotype also detected (Fig. 2). Comparison of nucleotide diversities calculated using RFLP and sequence data on the same individuals (grouped by decade across all rivers) showed that RFLP analysis consistently overestimated the nucleotide diversity (by almost two orders of magnitude) but the temporal pattern was comparable: i.e. the direction of change in nucleotide diversity from decade to decade was generally the same for both data types (Fig. 3). The higher levels of diversity in the RFLP data result from a systematic bias due to the fact that the restriction sites analyzed were chosen because they had previously been shown to be polymorphic.

The ratio of non-synonymous to synonymous polymorphisms in the sequences was 0.71.

MtDNA diversity within populations

Estimates of haplotype diversity (h) varied within and between rivers (from a minimum of 0.00 to a maximum of 0.48) but with a general trend to increase in all rivers immediately post the onset of stocking, for example from 0.056 in the 1950s sample to 0.405 in the 2002 sample of the River Ason (Table 3). However, the increase in haplotype diversity in the 1980s and 1990s, appears to have been reversed in all 2002 samples (Table 3).

One haplotype (AAABA) was predominant and observed with high frequency (0.625–1.000) in each temporal/spatial sample (Table 3). However, in the Asón and Deva a previously undetected haplotype (AABBA) appeared at relatively high frequency (0.375 and 0.140, respectively in samples taken immediately after stocking had commenced (Table 3). The same RFLP haplotype increased in frequency in the post-stocking period in the Nansa (Table 3) and was detected at low frequency in a post-stocking sample from the Pas (Table 3). However, sequencing data revealed that the only individual of RFLP haplotype AABBA detected in a pre-stocking sample (i.e. in the Nansa 1960s: sequence haplotype AABBA-3) differed from the corresponding RFLP haplotypes in all post-stocking samples (sequence haplotypes AABBA-1, AABBA-2 and AABBA-4).

Genetic differentiation within and between rivers

We grouped samples in two ways to resolve potential genetic differentiation. First, we tested whether there was genetic differentiation among rivers in different decades. The AMOVA failed to reject genetic homogeneity of spatial populations during the 1950s and 1960s (Table 4), but did reject genetic homogeneity in all samples after the 1960s. Significant differentiation among rivers was observed in the 1980s, 1990s and 2002 samples, but the strength of differentiation decreased from the 1980s onwards.

The second grouping of samples was to use an AMOVA to test for temporal stability in haplotype frequencies. Significant differences in genetic composition among years were detected for both the Asón and Pas rivers were detected, but not for either the Nansa or Deva (Table 4).

The UPGMA dendrogram (Fig. 4), rooted with another Spanish population, the River Narcea, from Verspoor et al. (1999) grouped all Cantabrian populations into the same cluster but resolved two groups within that cluster. The first group contained populations that had experienced the highest relative stocking effort (post 1970s Asón, Deva and Nansa populations). The second group comprised all of the pre-stocked (pre-1970s) populations for all rivers and all of the post-stocked Pas populations. The pre-stocked group of samples had significantly lower haplotype diversity

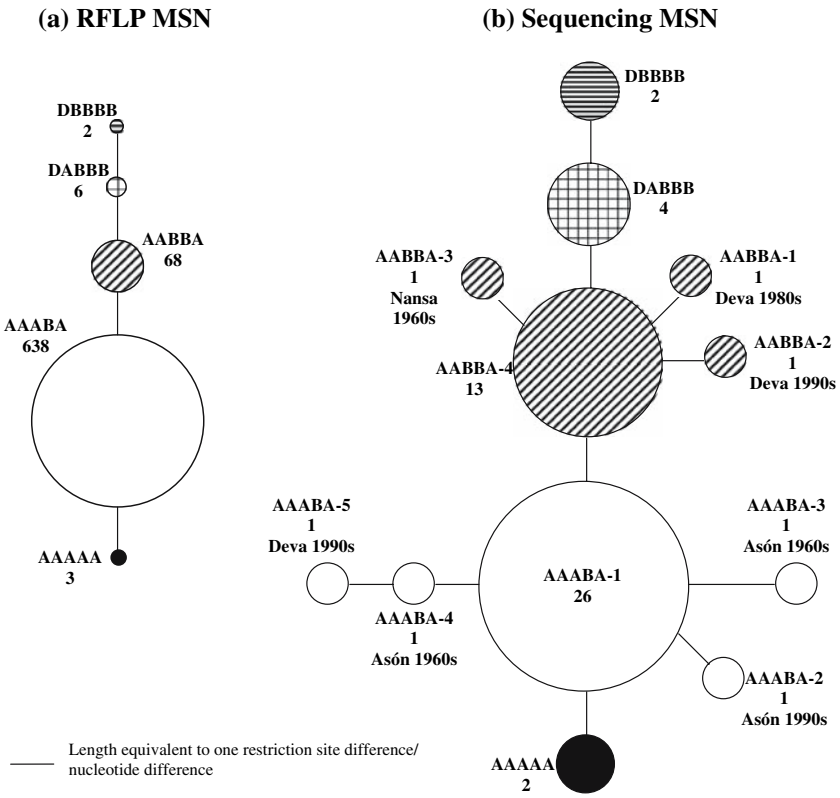

Fig. 2 Minimum spanning networks (MSNs) of haplotypes using (**a**) RFLP sites and (**b**) sequencing polymorphisms. The area of the circle is proportional to number of individuals of that haplotype in the total sample (number given below haplotype). Each branch in the RFLP-based MSN represents a gain/loss of a single restriction site

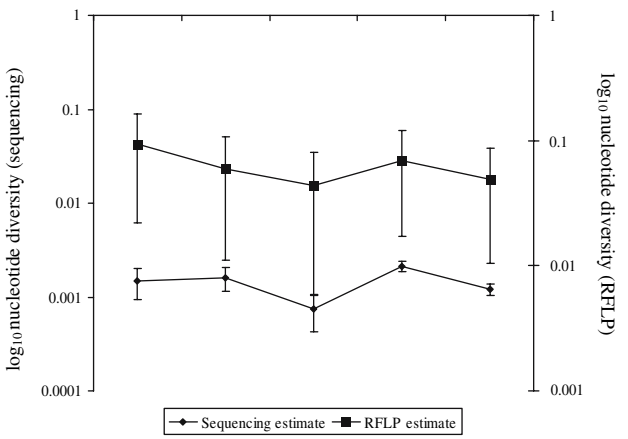

Fig. 3 Graph showing estimates of nucleotide diversity (± standard deviation) estimated using RFLP and sequencing data from the same sample set

than the post-stocked group of samples (Mann–Whitney $U = 8.500$, $P = 0.003$).

Genetic differentiation among diseased and asymptomatic River Asón individuals

Two haplotypes, AAABA and AABBA, dominated the healthy and diseased groups (one individual of the 45 healthy fish had an AAAAA haplotype). Significant genetic differentiation was observed between these two groups ($F_{ST} = 0.154$, $P = 0.01$), with the frequency of the AABBA haplotype higher in the diseased fish (0.500) compared with the healthy fish (0.200). This increase in the frequency of the AABBA haplotype in the diseased group was reflected in a higher level of haplotype diversity in this group ($h = 0.52$, SD = 0.0277) compared to the healthy group ($h = 0.36$, SD = 0.0749).

Discussion

This study demonstrates the utility of the ND1 mtDNA gene in identifying the impact of non-native stocking in Atlantic salmon populations. An alternative to the use of mtDNA markers is the use of nuclear markers (e.g. microsatellites) as they are often more polymorphic than mtDNA, and could resolve population structure to a finer scale than mtDNA. Multilocus microsatellite genotypes can also be used to assign individuals to populations, which could be used to identify specifically which individuals are non-native in a stocked system (Englbrecht et al. 2002). Further, microsatellites are biparentally inherited, and therefore information about male inheritance patterns would be

Table 3 Haplotype frequencies and haplotype diversities for all samples

	N	AAABA	AABBA	AAAAA	DABBB	DBBBB	Haplotype diversity	Standard deviation
Asón 1950s	36	0.972	0.000	0.000	0.000	0.028	0.056	0.052
Asón 1960s	31	1.000	0.000	0.000	0.000	0.000	0.000	0.000
Asón 1980s	32	0.625	0.375	0.000	0.000	0.000	0.484	0.048
Asón 1990s	71	0.676	0.310	0.014	0.000	0.000	0.453	0.044
Asón 2002	30	0.733	0.267	0.000	0.000	0.000	0.405	0.078
Deva 1950s	24	1.000	0.000	0.000	0.000	0.000	0.000	0.000
Deva 1960s	30	0.967	0.000	0.000	0.033	0.000	0.067	0.061
Deva 1980s	43	0.860	0.140	0.000	0.000	0.000	0.246	0.077
Deva 1980s	37	0.838	0.135	0.000	0.027	0.000	0.287	0.088
Deva 2002	32	0.906	0.063	0.000	0.000	0.031	0.179	0.088
Nansa 1960s	38	0.974	0.026	0.000	0.000	0.000	0.053	0.049
Nansa 1980s	31	0.903	0.097	0.000	0.000	0.000	0.181	0.086
Nansa 1990s	32	0.906	0.094	0.000	0.000	0.000	0.175	0.084
Nansa 2002	31	0.871	0.129	0.000	0.000	0.000	0.232	0.090
Pas 1950s	35	0.971	0.000	0.029	0.000	0.000	0.113	0.072
Pas 1960s	53	1.000	0.000	0.000	0.000	0.000	0.000	0.000
Pas 1980s	60	1.000	0.000	0.000	0.000	0.000	0.000	0.000
Pas 1990s	39	0.872	0.026	0.026	0.077	0.000	0.239	0.088
Pas 2002	32	0.969	0.000	0.000	0.031	0.000	0.063	0.058

Table 4 Results of AMOVA of spatial population structure in each decade of sampling and temporal variability within Cantabrian populations

	Φ_{ST}	*P*-value
Decades		
1950s[a]	–0.002	0.606
1960s	0.002	0.227
1980s	0.190	0.000
1990s	0.049	0.007
2002	0.039	0.048
Groups		
River Asón	0.121	0.000
River Deva	0.010	0.222
River Nansa	–0.005	0.420
River Pas	0.035	0.016

[a] Group includes Rivers Asón, Deva and Pas samples

known. However, mtDNA has technical advantages over nuclear DNA for analysis of historic samples in that there are many more copies (in the order of thousands) of mtDNA per cell compared to nuclear DNA (Ballard and Whitlock 2004). Therefore it is more likely to yield useable PCR products, since degradation and low DNA concentration is less likely to affect mtDNA loci compared to nuclear loci. Beyond this technical consideration, there is a certain advantage to using a maternally inherited marker for Atlantic salmon. In anadromous populations of Atlantic salmon, an alternative to anadromy is to become sexually mature at the parr life stage (''precocious maturation''). Precocious parr slow the loss of genetic diversity by increasing the effective population size, so a time lag effect in the loss of genetic diversity might be observed. However, this life history strategy is much more common for males than females (2–100% in males, <1% in females (Fleming 1998)), so the impact of mature parr on the variability of a maternally-inherited marker would be negligible, removing a possible complication to the results of a

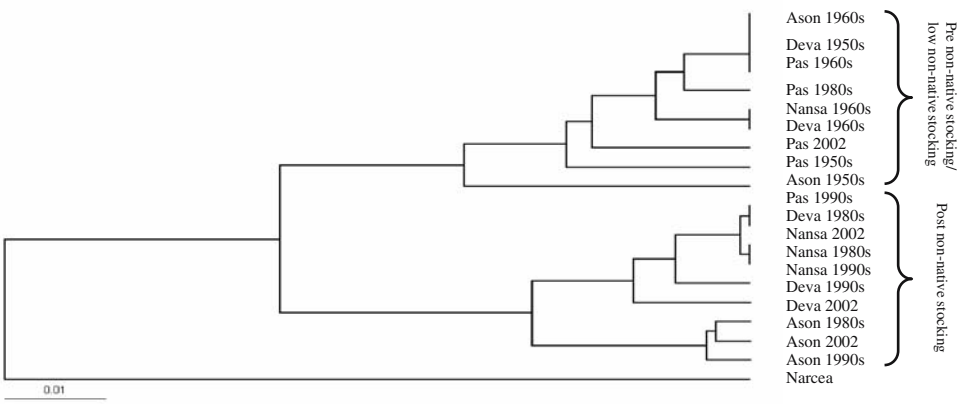

Fig. 4 UPGMA dendrogram based on Nei's D_A distances showing relationships among Cantabrian river samples and other European Atlantic salmon populations, using some data from Verspoor et al. (1999)

study such as this. This study has also shown that, in this case, the ND1 marker is sufficient to differentiate between populations of Atlantic salmon on a small geographic scale.

Our aim in this study was to examine the impact of non-native stocking and disease introduction on variability in the mtDNA ND1 gene in Cantabrian Atlantic salmon populations. We hoped to use this data to infer which genetic processes (drift, gene flow, selection) had most impact on mtDNA variability for these four small Spanish populations. From angling catch figures and catch per unit effort data (García de Leániz et al. 2001), it appears that these populations have suffered sustained census population size declines since the late 1940s (García de Leániz et al. 2001). Our results showed that mtDNA diversity in all populations has increased, mainly due to the large increase of a single haplotype, with small increases in rarer haplotypes. We also showed that after non-native stocking began, these extant Cantabrian populations became more genetically differentiated from one another. In addition, we detected significant genetic differentiation between samples caught in the River Asón in the 1990s which had either died due to infectious disease (most typically furunculosis), or had been angled without external symptoms for infectious disease.

We assigned individuals haplotypes based on RFLP patterns and subsequently sequenced a number of individuals of each haplotype from different samples. A greater number of haplotypes were resolved on the basis of nucleotide sequences than with RFLP analysis. However different sequences which shared the same RFLP haplotype also clustered together in the sequence-based minimum spanning network. Moreover, the estimates of nucleotide diversity produced using the RFLP and sequence data reflected a similar temporal pattern. That the RFLPs gave a higher estimate of nucleotide diversity compared to the sequence data is probably due to the fact that the enzymes we selected were known to give the most polymorphic cut patterns, and that in general, there is little variability in the rest of the nucleotide sequence of the ND1 gene. In summary, the similarity of these patterns indicated that using an RFLP-based analysis, whilst not capturing all of the possible sequence variation, was a valid approach to estimating mtDNA ND1 sequence variability in this instance.

Five RFLP haplotypes were found in the Cantabrian populations, four of which were already described in Verspoor et al. (1999). There were four haplotypes observed across the contemporary Cantabrian populations (two or three per river), which is comparable to the number of haplotypes per river reported for other populations of Atlantic salmon, for example two haplotypes were observed in the Narcea, and four were observed in the North Esk (Verspoor et al. 1999). However, the haplotype diversity in contemporary Cantabria populations was lower than reported for other European populations, for example 0.316 and 0.691 in the rivers Narcea and North Esk, respectively (Verspoor et al. 1999). This may be because one haplotype (AAABA) was extremely common in Cantabrian populations, with other haplotypes being represented by only one or two individuals per sample.

Temporal changes to mtDNA variability in Cantabrian populations

The Cantabrian rivers were historically and are currently characterized by one predominant haplotype (AAABA) which is highly common in northern European populations (Verspoor et al. 1999; Consuegra et al. 2002). However, there was a general trend for haplotype and nucleotide diversity to increase over time, coincident with non-native stocking activities and outbreaks of infectious diseases (mainly furunculosis). Therefore, it seems unlikely that random genetic drift, often associated with population declines, has had a significant impact on these populations during their recent history because we would expect a reduction in magnitude of genetic diversity indices. In fact the opposite trend is observed.

Processes that could serve to increase haplotype diversity over this relatively short period include gene flow and diversifying selection. In this study we have found evidence that non-native stocking has affected mtDNA variability. Before non-native stocking began, the rivers were not genetically differentiated in terms of ND1 haplotype, which is likely to be reflective of low overall levels of mtDNA diversity, their geographic proximity and a small number of migrants per generation. Following stocking, Φ_{ST} among populations increased, and the populations became strongly genetically differentiated. In addition, the number of haplotypes observed in each of the rivers has, in general, increased. The most striking example is in the River Asón, where the AABBA haplotype was not observed in either of the pre-stocked populations, but emerged with a high frequency in the 1980s sample. Similarly, this haplotype was only observed, albeit at a lower frequency, in the River Deva after stocking. It is not possible to determine haplotype frequencies in the original donor populations at the time of stocking, however the AABBA haplotype is common in current northern European populations, geographically close to the original donor populations, and we assume these

to have similar frequencies of this haplotype. The River Pas also had several emergent haplotypes, which occurred at low frequency, after non-native stocking began, which are typical of Scandinavian lineages. The sudden appearances of these haplotypes, common in populations further north (Verspoor et al. 1999), at high frequencies immediately subsequent to the onset of stocking, is supportive of the hypothesis that stocked individuals did impact upon mtDNA diversity.

Furthermore, although Φ_{ST} among the rivers and haplotype diversity within them increased immediately after stocking began, generally both have decreased since the 1980s, which fits with the history of non-native stocking, which peaked during the 1980s, and ceased in 1996. The most likely scenario for these results is that introgression from northern European genes during the stocking period caused the significant changes to haplotype frequencies and haplotype diversity. An alternative scenario is that, over the same period, population bottlenecks within each of the rivers, and reduced migration among them caused increased genetic differentiation. However, that explanation is not consistent with the increases observed in haplotype diversity. After the peak of stocking the input of northern European genes into these populations would have decreased. Moreover, degraded habitat quality, causing more individuals to disperse, may have increased gene flow between the rivers since the 1980s. Consuegra et al. (2005b) used microsatellite and physical tagging data and concluded that there is currently a substantial number of migrants among these rivers. Lack of gene flow from stocked fish and increased gene flow between the Cantabrian rivers seem the most likely reasons for the gradual decreases in mtDNA diversity and genetic structuring in the period after the peak of stocking.

Significant temporal differentiation was detected within the Rivers Asón and Pas - both these rivers have the smallest, most rapidly declining Atlantic salmon populations of the Cantabrian rivers, and poor habitat quality (García de Leániz et al. 2001). The River Asón was also the most heavily stocked of all the rivers (García de Leániz unpublished), and the contemporary population has the highest levels of haplotype diversity of all the rivers. Although significant differences among temporal samples were not detected in the Rivers Deva and Nansa, higher frequencies of 'foreign' haplotypes and increased haplotype diversity was observed after stocking. That significant differences in the genetic composition of temporal populations of the Rivers Deva and Nansa were not observed could be due to the larger and more stable nature of these populations, and the better habitat quality (García de Leániz et al. 2001). Englbrecht et al. (2002) found that stocking had not significantly affected the genetic composition of Alpine populations of Arctic charr in lakes where ecological integrity had been better preserved, however in one polluted lake, the ancestral population had been completely replaced by non-native individuals. Therefore, perhaps some combination of poorer environmental conditions, more severe rate of decline and relative level of stocking effort caused the significant temporal instability in the Rivers Asón and Pas, but not the Deva or Nansa.

Further evidence that adds credibility to the stocking hypothesis is the clear clustering of pre-stocked populations and post-stocked populations in the UPGMA dendrogram and the highly significant difference between the haplotype diversities of the pre-stocked and post-stocked samples. The River Pas post-stocked populations clustered with the pre-stocked populations of the other rivers, despite the emergence of several 'foreign' haplotypes post-stocking. The reasons for this are unclear, but the Pas did receive the smallest stocking effort, and this peaked later than for the other rivers, in the late 1980s (García de Leániz unpublished). This apparent anomaly may be resolved by using more molecular markers.

Contrary to this study, there is little previous evidence for strong genetic signatures of stocking in other comparable systems using mitochondrial and nuclear markers (Verspoor and García de Leániz 1997) (but see Martinez et al. (2001). It may be that original small size and relatively high level of sustained foreign stocking effort of the Cantabrian Atlantic salmon populations made them more susceptible to foreign genetic introgression, at least in the mitochondrial lineage, despite lower survivorship of non-native individuals (Verspoor and García de Leániz 1997).

McGinnity et al. (2004) compared the performance of Atlantic salmon in the Burrishoole river (western Ireland), both native and those from an adjacent river (the Owenmore). The authors found that the non-native individuals did not achieve as high lifetime fitness as native individuals in this system, and we would hypothesis that reduced fitness of non-native individuals is also true for non-native individuals transplanted to Cantabrian rivers. However, given that mtDNA has a smaller effective population size due its inheritance and haploid nature, only a small number of females would be required to survive to reproduce in the rivers to effect changes to mtDNA variability. Indeed, hybrid vigour in F1 individuals has been observed for Atlantic salmon (Einum and Fleming 1997), and this may allow a mechanism for propagation of a non-native haplotype, at least into the F2.

We found a difference in the haplotype composition of two samples taken from the same river (the Asón) during the same period (1990s), one of which was made up fish that had been removed dead from the river and presented with symptoms of infectious dieseases, the second of which were angled and did not present with disease symptoms. These two samples were significantly genetically differentiated, and those fish that had died as a result of this disease had a higher frequency of the AABBA haplotype than the healthy individuals. The ND1 gene codes for the NADH1 subunit, mutations in which have been implicated in some heritable vertebrate diseases (Kirby et al. 2004; Valentino et al. 2004). However, the polymorphic site between the two most common forms of this haplotype in Spanish populations occurs at the third codon position and confers no change to the protein structure. Moreover, the ratio of non-synonymous to synonymous substitutions in the sample of nucleotide sequences did not indicate positive selection at this locus. We therefore find it unlikely that direct selection is acting on ND1 with respect to resistance to infectious disease. However, the mtDNA molecule behaves as a single locus, so it is possible that other genes linked to ND1 haplotypes are somehow involved in resistance to infectious diseases. For example, linkage disequilibrium between mtDNA and nuclear genes (some of which are involved in resistance/susceptibility to infectious diseases) might be maintained through cyto-nuclear incompatibility in crosses between Spanish and non-native fish (Ballard and Rand 2005).

An alternative to a direct genetic role of the mitochondrion in resistance to infectious diseases is that the different haplotypes experience different life histories in terms of date of return to the river. Consuegra et al. (2005a) reported genetic differentiation in early running fish compared with late running fish. In the Asón, early running fish had a higher frequency of AABBA than late running fish (the opposite was seen in the other three rivers), so it might be that these early running fish have had a longer exposure period to infectious diseases and have therefore succumbed to infectious disease at a higher rate. However the fluctuating nature of this pattern among rivers makes the role for indirect selection on ND1 less compelling.

Conservation implications

With the exception of the River Asón, recent historic and contemporary Cantabrian Atlantic salmon populations possess a similar number of haplotypes but have lower haplotype diversity than other European populations (Verspoor et al. 1999; Consuegra et al. 2002). At the last glacial maximum, the Iberian peninsula was a glacial refugium for several species (Jaarola and Searle 2002; Hampe et al. 2003) and acted as one of the main refugia for Atlantic salmon (Consuegra et al. 2002), along with the North Sea and lakes east of the Baltic Sea (Nilsson et al. 2001). That contemporary and historic Cantabrian populations possess less diversity than derivative populations further north may suggest that Cantabrian populations had lost genetic diversity due to random genetic drift as a result of population decline (Parrish et al. 1998), or alternatively that more northern populations had multiple founders (but see Consuegra et al. 2005b). It is also possible that over-exploitation may have resulted in loss of genetic diversity in these southern populations, as anglers appear to exploit distinct population components (Consuegra et al. 2005a).

The important conclusion from our study is that stocking in this instance has succeeded in its proximate goal—to increase genetic diversity—but has not succeeded in its ultimate goal—census population size did not increase as a result. Stocking has resulted in an increase in mitochondrial DNA diversity (as suggested by our estimates of haplotype diversity) probably due to the genetic introgression of ‘foreign’ haplotypes. While, with our current data set it is not possible to conclude with certainty what long term effects this genetic introgression will have on these populations of Atlantic salmon, in the short term it does not seem to have been advantageous.

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