

The importance of genetic verification for determination of Atlantic salmon in north Pacific waters

J. L. NIELSEN*, I. WILLIAMS, G. K. SAGE
AND C. E. ZIMMERMAN

*USGS Alaska Science Center, Biological Science Office, 1011 East Tudor Road,
Anchorage, AK 99503, U.S.A.*

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Genetic analyses of two unknown but putative Atlantic salmon *Salmo salar* captured in the Copper River drainage, Alaska, demonstrated the need for validation of morphologically unusual fishes. Mitochondrial DNA sequences (control region and cytochrome b) and data from two nuclear genes [first internal transcribed spacer (ITS-1) sequence and growth hormone (GH1) amplification product] indicated that the fish caught in fresh water on the Martin River was a coho salmon *Oncorhynchus kisutch*, while the other fish caught in the intertidal zone of the Copper River delta near Grass Island was an Atlantic salmon. Determination of unusual or cryptic fish based on limited physical characteristics and expected seasonal spawning run timing will add to the controversy over farmed Atlantic salmon and their potential effects on native Pacific species. It is clear that determination of all putative collections of Atlantic salmon found in Pacific waters requires validation. Due to uncertainty of fish identification in the field using plastic morphometric characters, it is recommended that genetic analyses be part of the validation process.

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Key words: Alaska; DNA; invasive species; molecular identification.

INTRODUCTION

Significant controversy has surrounded the farming of Atlantic salmon *Salmo salar* L. on the Pacific coast of western North America, primarily due to accidental escapement of farm fish from their pens due to storms, tides, marine mammal net-pen damage and losses during transfer or harvest. This controversy feeds on fears of interbreeding, colonization, predation, transfer of diseases and competition by Atlantic salmon on native populations of Pacific salmonids (Naylor *et al.*, 2001). Observation and reports of escapement of Atlantic salmon in Pacific waters, both fresh water and marine, vary by time and region. It has been estimated that 396 522 Atlantic salmon have escaped from farms in British Columbia from 1991 to 2001 (Gaudet, 2002). Over 595 000 fish were accidentally released in three large-scale escapements from Washington State farms, 1996–1998 (Noakes *et al.*, 2000; Gaudet, 2002). Salmonid farming of any species

*Author to whom correspondence should be addressed. Tel.: +1 907 786 3670; fax: +1 907 786 3636; email: jennifer_nielsen@usgs.gov

is not permitted in Alaska; however, farmed Atlantic salmon tend to migrate north after escapements and have been caught in Alaska fisheries. Since 1991, over 600 recoveries from Alaskan waters have been documented (Gaudet, 2002). Primarily these recoveries have occurred in south-east Alaska, but fish have been caught as far north as the Bering Sea (Brodeur & Busby, 1998). Only three recoveries, however, have been documented in fresh water (Mecklenburg *et al.*, 2002).

On 30 April 2001, during a scientific survey for genetic introgression between coastal cutthroat trout *Oncorhynchus clarki* (Richardson) and steelhead *Oncorhynchus mykiss* (Walbaum) in the Martin River (60°23' N; 144°35' W) (Copper River drainage), a salmon was captured whose species of origin could not readily be determined in the field. Although the species was cryptic, its general appearance and several morphometric and meristic characters led the collectors to think it might be an Atlantic salmon. Based on colouration, the fish appeared to have recently migrated from the ocean. The sides of the fish were predominately silvery with greenish shading along the back. 'X-shaped' spots were present along the back and sides above the lateral line with no visible spots on the caudal fin. The head was rounded and the gums at the base of the teeth in the lower jaw were white with no colouration in the margins. There were 11 anal fin rays. This fish was photographed and a fin clip was sent to the laboratory for genetic analyses. A second putative Atlantic salmon was captured in salt water at the tidal edge of the Copper River delta (60°17' N; 145°1' W) off Grass Island on 9 July 2002 and fin tissue was also sent to the laboratory for genetic analyses. Analyses of both mitochondrial and nuclear DNA markers were used to allow rigorous identification of genus and species with the maximum number of diagnostic loci available in the laboratory and to prevent misidentification of hybrids.

MATERIALS AND METHODS

DNA was extracted from fin tissue taken from both unknown samples. Extractions were done two times, independently, using Puregene[®] DNA isolation tissue kits (Gentra Systems, Minneapolis, MN, U.S.A.). Initial genetic analyses included surveys of restriction fragment length polymorphisms (RFLP) of mitochondrial (mtDNA) ND 5/6 region cut with *Dde* I and cytochrome b mtDNA region cut with *Dde* I and *Dpn* II restriction enzymes. Gene regions were amplified with primer sequences given by Nielsen *et al.* (1998). Amplification and visualization followed procedures from Scribner *et al.* (1998), with minor modification. ND 5/6 and cytochrome b RFLP analyses tested species status among sockeye salmon *Oncorhynchus nerka* (Walbaum), coho salmon *Oncorhynchus kisutch* (Walbaum), chum salmon *Oncorhynchus keta* (Walbaum), coastal cutthroat trout and steelhead samples. Atlantic salmon reference samples, two each from four populations: Narraguagus River, Maine, U.S.A., Saint Jean River, Québec, Canada, Ellidaar River, Iceland and Vosso River, Norway, were obtained (USGS, Leetown Science Center, WV, U.S.A.) and analysed.

Sequence analyses of mtDNA (control region and cytochrome b), and nuclear [first internal transcribed spacer (ITS-1)] gene fragments included the two unknown fish, Atlantic salmon from North America and Europe, coho, chum, chinook *Oncorhynchus tshawytscha* (Walbaum) and steelhead samples. Amplification of mtDNA D-loop sequence used M13 reverse and T3 promoter tails (Steffens *et al.*, 1993; Oetting *et al.*, 1995) respectively synthesized onto the 5' ends of the S-phe and P2 primers (Nielsen *et al.*, 1994). Amplification was carried out in a 50 µl reaction consisting of *c.* 50 ng of genomic

DNA, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01% each of gelatin, NP-40 and Triton X-100, 190 µM each dNTP, 50 pmoles of each primer and 1.25 U of DNA polymerase (Promega). An initial 1.5 min, 94°C denaturing cycle was followed by 40 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 1 min. Polymerase chain reaction (PCR) products were visualized on ethidium bromide-stained agarose gels. Sequencing products were purified using Amicon Microcon PCR Centrifugal Filter Devices (Millipore Corporation, Bedford, MA, U.S.A.).

Sequencing protocols followed the procedures outlined in Li-Cor (2000). Sequences were captured using e-Seq software (Li-Cor, Lincoln, NE, U.S.A.). Alignments were adjusted using MEGA software in reference to GENE BANK sequence number U12143 for *S. salar* mtDNA control region, bases 1–1006 (Hurst *et al.*, 1999).

Cytochrome b sequence amplification used primers Cyt(b)L17 (5'-CTACAAGAACC-TAATGGC-3') and Cyt(b)H552 (5'-AAGGCGAAAAATCGTGTTAG-3'), developed in the laboratory from salmonid sequences available on GENE BANK. Primers were designed to align with Atlantic and seven Pacific salmonids (including cutthroat and rainbow trout). M13 forward and M13 reverse tails were respectively synthesized onto the 5' ends of the Cyt(b)L17 and Cyt(b)H552 primers. Amplification reactions and PCR protocols were identical to those for the control region. Sequences were aligned and adjusted as above with reference to GENE BANK sequence number U12143 for *S. salar* mtDNA cytochrome b region, bases 1–1141 (Hurst *et al.*, 1999).

Nuclear ITS-1 primers were designed to amplify and sequence a fragment consisting of c. 150 base pairs of the 18SrDNA gene and 330 base pairs of the ITS-1 of the ribosomal DNA. Primers were designed using ITS-1 sequence from Domanico *et al.* (1997) and data accessioned in GENE BANK. The 18S-F primer (5'-CGTTGATTAAGTCCCTGC-3') was designed to anneal to the 5' end of the 18SrDNA gene and the ITS330R (5'-GGTTCCCAGTGCCGCGCGA-3') primer was designed to anneal to the salmonid ITS-1 gene. This primer pair was tested on the following species: *S. salar*, *O. keta*, *O. kisutch*, *O. nerka* and *O. tshawytscha*. The *O. nerka* sample sequenced poorly, however, and was not included in this study.

The 18S-F and ITS-1(R) primers were modified by the addition of universal M13F and M13R tails, respectively. Product amplification and sequencing followed methods previously described for the mtDNA control region. Species-specific ITS-1 sequences were aligned in reference to *S. salar*, GENE BANK accession number AF201312 (Phillips *et al.*, 2000); *O. kisutch*: GENE BANK accession number AF097563 (Domanico *et al.*, 1997); *O. keta* and *O. tshawytscha* (Domanico *et al.*, 1997).

The nuclear growth hormone gene GH1 (Forbes *et al.*, 1994) was used to amplify and compare the unknown samples with known Atlantic and Pacific salmonids. Amplification followed the procedures of Forbes *et al.* (1994) with minor modification. Amplification products were fractionated in 1.5% agarose gels (120 V for 3 h), stained with ethidium bromide and photographed under UV light. Product sizes were approximated by comparisons with Bio-Rad (Hercules, CA, U.S.A.) EZ Load 100 bp PCR Ruler (100–3000 bp).

RESULTS

Both mtDNA (RFLP and sequence) and nuclear genes indicated that the fish captured in the Martin River was a coho and not an Atlantic salmon. The second sample from the Copper River delta at Grass Island was an Atlantic salmon. Sequence alignment for mtDNA control region showed 80 variable sites in comparisons among Atlantic salmon, coho, chinook and steelhead (Table I). Control region sequence divergence found between Atlantic salmon and the Martin River fish was 25.7%. Within species sequence divergence for Atlantic salmon for this region was only 4.3%. The Martin River fish aligned perfectly with Glacier Bay coho mtDNA sequences at all nucleotide sites. In contrast, the

TABLE I. Mitochondrial DNA control region (D-loop; 987 bp) variable sites and nucleotide changes in Atlantic salmon, coho, chinook, steelhead and the unknown fish from the Copper River area of Alaska. Base numbers follow those given in GENEBANK by Hurst *et al.* (1999). *, insertion or deletion at that site. Deletions in the Atlantic salmon reference sequence are denoted by letters following the previous base number

Species	Sample	Base pair number																																
		817	828	829	831	834	860a	860b	867	871	879	888	889	892	897	900	901	902	906	912	917	934	941	943	944	946	947	948	949	951				
'Unknown' 'Unknown' Atlantic salmon	Martin River, Alaska	C	A	*	G	A	G	A	G	A	G	A	G	A	G	C	A	*	C	T	A	A	T	A	G	A	C	A	C	*	C	T		
	Grass Island, Alaska	C	G	C	A	C	*	*	T	A	C	T	A	A	C	T	G	G	C	T	T	G	T	A	G	A	A	A	A	A	A	T	T	
	GENEBANK-U12143	C	G	C	A	C	*	*	T	A	C	T	A	A	C	T	G	G	C	T	T	G	T	A	G	A	C	A	A	A	A	T	T	
	Narraguagus Maine	C	G	C	A	C	*	*	T	A	C	T	A	A	C	T	G	G	C	T	T	G	T	A	G	A	A	A	A	A	A	A	T	T
	Ellidaar R., Iceland	C	G	C	A	C	*	*	T	A	C	T	A	A	C	T	G	G	C	T	T	G	T	A	G	A	A	A	A	A	A	A	T	T
	Vosso River, Norway	C	G	C	A	C	*	*	T	A	C	T	A	A	C	T	G	G	C	T	T	G	T	A	G	A	A	A	A	A	A	A	T	T
	Saint Jean R., Quebec	C	G	C	A	C	*	*	T	A	C	T	A	A	C	T	G	G	C	T	T	G	T	A	G	A	A	A	A	A	A	A	T	T
	Glacier Bay, Alaska	C	A	*	G	A	G	G	A	G	C	T	T	A	G	A	G	C	A	*	C	T	A	T	A	T	G	A	C	C	*	C	T	T
	Alsek R., Alaska	C	A	*	G	A	G	G	A	G	C	T	T	A	G	A	G	C	A	*	C	T	A	T	A	T	G	A	C	C	*	C	T	T
	Noyo R., California	C	A	*	G	A	G	G	A	G	C	T	T	A	G	A	G	C	A	*	C	T	A	T	A	T	G	A	C	C	*	C	T	T
Chinook salmon	Sacramento R., California	T	A	*	G	A	A	G	C	A	T	A	*	G	A	G	C	A	*	C	T	A	T	A	C	G	A	C	C	C	*	C	T	*
	Sashin Creek, Alaska	C	A	*	*	A	A	G	C	A	T	A	*	G	A	G	C	C	G	*	C	A	A	T	A	C	G	A	C	C	*	C	T	*
	Eel River, California	C	A	*	*	A	A	*	C	*	T	A	G	A	G	A	G	C	C	G	*	C	A	A	T	A	C	G	A	C	C	*	C	T
Species	Sample	952	952a	953	955	958	960	961	962	963	964	964a	964b	964c	964d	964e	965	966	966a	967	967a	967b	967c	967d	967e	968	969a	969b	970	971				
'Unknown' 'Unknown'	Martin River, Alaska	A	G	C	G	C	C	G	G	C	A	C	C	G	*	A	A	G	C	A	*	C	T	G	T	T	*	T	T	*	A	G	A	
	Grass Island, Alaska	A	*	T	G	T	T	A	G	C	C	A	*	A	*	A	A	*	A	*	C	C	*	G	*	T	*	T	*	T	*	G	T	*

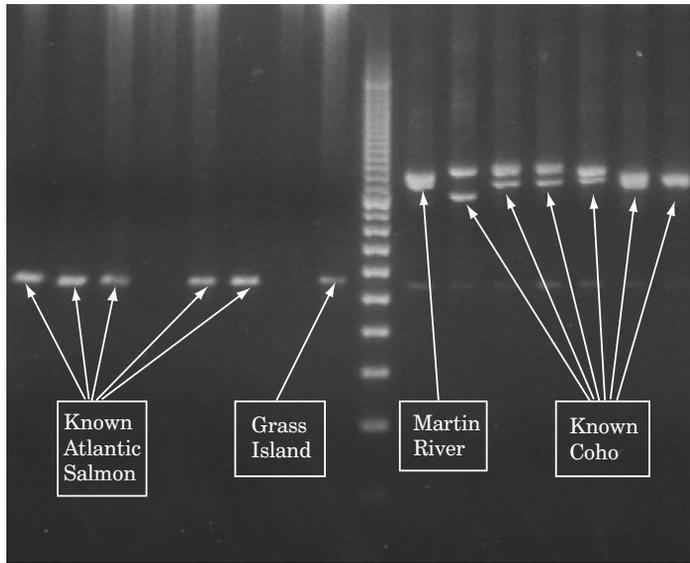


FIG. 1. Growth hormone (GH1) amplification products from known Atlantic salmon, coho salmon and the unknown samples from fish found in the Copper River basin, Alaska, 30 April 2001 (Martin River, fresh water) and 10 May 2002 (Grass Island, intertidal zone).

Grass Island fish aligned perfectly with European strains of Atlantic salmon from Norway and Iceland.

Sequence alignment for cytochrome b revealed 71 variable sites in comparisons among Atlantic and Pacific salmon. Sequence divergence found between Atlantic salmon and the Martin River fish was 9.2%. The Martin River fish aligned perfectly with Glacier Bay coho sequences at all nucleotide sites.

ITS-1 sequence alignment revealed 63 variable sites in comparisons among Atlantic and Pacific salmon. Sequence divergence found between Atlantic salmon and the Martin River fish was 14.3%. Within species sequence divergence for Atlantic salmon for the same region was only 1.0%. In addition, the Martin River fish aligned perfectly with Glacier Bay coho ITS-1 sequence at all nucleotide sites. Finally, nuclear genetic diversity for GH1 showed significant size differences between Pacific coho and Atlantic salmon with the amplification product of the unknown sample from the Martin River sharing fragment size with coho and the Grass Island fish sharing fragment size with Atlantic salmon (Fig. 1).

DISCUSSION

Conventional field-based identification of adult salmon is primarily based on colouration and gross morphology including qualitative and quantitative occurrence of spotting, colouration of tissues such as gums and relative shape of head or caudal peduncle. For example, Mecklenburg *et al.* (2002) list the following as primary characteristics for identification of Atlantic salmon: black spots of which some are X- or Y- shaped on the body dorso-laterally and sometimes on the caudal fin, black spots on operculum, head small and caudal fin slightly indented. Hart (1973) lists sparse X-shaped dark markings, slender caudal

peduncle and teeth on shaft of vomer as characters for recognition of Atlantic salmon.

Anal fin ray counts of 13–19 are diagnostic for *Oncorhynchus* sp. according to Hart (1973) and Mecklenburg *et al.* (2002). The Canada Department of Fisheries and Oceans includes anal fin ray counts of 8–12 as a diagnostic feature for Atlantic salmon (<http://www.pac.dfo-mpo.gc.ca/ops/fm/Salmon/atlantic.htm>). Based on these sources, the Martin River fish, with an anal fin ray count of 11, would not be identified as an *Oncorhynchus* species.

Meristic characters, morphology and colouration are all subject to phenotypic variation (Swain & Foote, 1999). Many of the characters suggested for the identification of Atlantic salmon are probably derived from studies of variation in their native range and from wild fish. Changes in these characteristics resulting from intensive culture, artificial selection and translocation should be examined. Phenotypic variation, ambiguity of qualitative characters and potential difficulty applying characters on live fish in a field setting underscore the importance of genetic identification.

Despite cryptic morphology and meristic evidence to the contrary, the unusual fish collected from the Martin River in April 2001, first suspected and later reported as an Atlantic salmon, was shown to carry coho mtDNA and nuclear genes. The U.S. Forest Service personnel who captured this fish have significant field experience with adult Pacific salmonids from throughout Alaska and the Pacific north-west. Coho salmon are not thought to migrate up the Copper River drainage at the time this fish was captured and no clear morphological or phenotypic evidence that the fish was a coho was found. Clear confirmation of species status using several molecular markers from both maternally inherited mtDNA and bi-parentally inherited nuclear genes eliminated the possibility that recent hybridization contributed to this unusual coho phenotype. Identification of the likely population of origin of the Grass Island Atlantic salmon, *i.e.* European strains from Norway or Iceland, confirmed the power of this technique to investigate the fate of escapees from cage culture of exotic salmonids.

Species determination of unusual fish based solely on limited or conflicting physical characteristics described in the field will clearly add to the controversy over farmed Atlantic salmon and their potential effects on native species in the Pacific Ocean. There has been significant discussion of the need for the retention and verification of specimens examined in scientific studies (Ruedas *et al.*, 2000). This is especially true in cases where animals are moved outside of their natural range, such as farmed Atlantic salmon. It is clear that viable identification of all putative collections of Atlantic salmon found in Pacific waters require validation and genetic conformation is essential. It is highly recommended that genetic analyses be part of any validation programme and that a chain of custody is established for each collection. It is further recommended that all putative Atlantic salmon collections be archived for future examination.

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