NPAFC
Doc. 539
Rev.

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Submitted to the

NORTH PACIFIC ANADROMOUS FISH COMMISSION

by

JAPAN

October 2001

THIS PAPER MAY BE CITED IN THE FOLLOWING MANNER:
Abe, S., H. Kojima, N. Davis, T. Nomura, and S. Urawa. 2001. Molecular identification of parental species in a salmonid hybrid caught in the Bering Sea. (NPAFC Doc. 539) 11 pages. Laboratory of Animal Cytogenetics, Center for Advanced Science and Technology, Hokkaido University, North 10, West 8, Kita-ku, Sapporo 060-0810, Japan.

MOLECULAR IDENTIFICATION OF PARENTAL SPECIES IN A SALMONID HYBRID CAUGHT IN THE BERING SEA

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ABSTRACT

Polymerase chain reaction (PCR)-based molecular identification was carried out on a salmonid hybrid (age 0.2) that was found among maturing pink salmon (*Oncorhynchus gorbuscha*, age 0.1) caught in the central Bering Sea in July 2000. The fish was larger in body size than co-captured pink salmon, had immature ovaries, and had external and internal characters that pertained most likely to pink and chum salmon (*O. keta*). In this fish, the nucleotide sequence of about 500 bp from a PCR-amplified 5' fragment of mitochondrial DNA control region was identical to that of a previously reported haplotype of chum salmon. In addition, *Dra*I enzyme digestion of the amplified intron C fragment of the nuclear growth hormone 1 (GH1) gene revealed restriction fragment length polymorphisms of pink and chum salmon, indicating GH1 alleles of both species in this fish. These findings showed the fish was a hybrid between a female chum and a male pink salmon, which may have occurred in the wild.

Key words: salmonid hybrid, chum salmon, pink salmon, PCR, mtDNA, GH1 intron C, Bering Sea

INTRODUCTION

In the North Pacific Ocean, occasional capture by Japanese fishermen of a salmon bearing morphological characteristics pertaining to more than one species has been reported for decades (Numachi et al. 1979). These fish were considered hybrids, which seemingly arose from a cross between chum (*Oncorhynchus keta*) and pink salmon (*O. gorbuscha*). However, previous studies to biologically characterize and identify the parental species for such hybrids have been inconclusive (Hunter 1949; Numachi et al. 1979).

In the present study, we are the first to identify a chum-pink salmon hybrid caught in the Bering Sea using polymerase chain reaction (PCR)-based sequencing of the mitochondrial DNA (mtDNA) control region and restriction fragment length polymorphisms (RFLPs) in the intron C of growth hormone 1 (GH1) gene.

MATERIALS AND METHODS

Fish samples

A possible salmonid hybrid was found among 38 fish identified as pink salmon. These fish were caught by gill nets during the research cruise of R/V *Wakatake maru* sponsored by Fishery Agency of Japan in the Bering Sea in July, 2000 (58°30'N, 180°; Fig. 1). The hybrid fish and nine pink salmon were kept in the round at -30 °C and transferred to the National Salmon Resources Center, Sapporo, Japan, for examination.

DNA extraction

Genomic DNA extraction was performed on frozen livers of the hybrid and pink salmon as described previously (Sato et al. 2001). In brief, about 50 µl of liver homogenate were added to 500 µl sodium tris EDTA buffer (0.1 M NaCl, 10 mM Tris-HCl, and 1 mM Na_2-EDTA, pH8.0) containing 500 µg/ml proteinase K and 0.5% SDS, and incubated at 37 °C overnight. DNA was extracted with a mixture of phenol (250 µl) and 24:1 chloroform:isoamylalcohol (250 µl) three times and then twice with 500 µl of 24:1 chloroform:isoamylalcohol alone. DNA in aqueous phase was recovered by ethanol precipitation, and dried in air. DNA samples were dissolved in TE buffer (10 mM Tris-HCl and 1 mM Na_2-EDTA, pH 7.5) and stored at 4°C.

DNA sequencing

Direct nucleotide sequencing of about 500bp from the 5' end of salmonid mtDNA control region was performed as described in Sato et al. 2001. The control region of mtDNA was amplified by PCR in a 100 µl of reaction mixture containing 25-100 ng of template DNA, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 250 nM each dNTPs, 250 nM each of forward and reverse primers, 0.001% gelatin, and 1.25 U of *Taq* DNA polymerase (TaKaRa, Tokyo). The forward and reverse primers were tRNAthr-2, 5'-TCT TGT AAT CCG GAA GTC GGA-3' and tRNAphe-2, 5'-AAC AGC TTC AGT GTT ATG CT-3', respectively (Sato et al. 2001). The method of PCR amplification using a Gene Amp PCR System 9700 was as follows: preheating at 94 °C for 5 min, followed by 35 denaturation cycles at 94 °C for 45 sec, annealing at 58 °C for 45 sec, elongation at 72 °C for 1 min, and extension at 72 °C for 7 min. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) after examining the size and quality with 1.5% agarose-gel electrophoresis and 0.02% ethidium bromide staining.

The sequence reaction was performed with the forward and reverse sequence primers labeled with Texas Red, using a Thermo Sequenase™ pre-mixed cycle sequencing kit (Vistra Systems, Sunnyvale, CA) according to the manufacturer's instruction. The forward and reverse sequencing primers were tRNAthr-3, 5'-GGT TAA AAC CCT CCC TAG TG-3'; and Okdl-H2, 5'-TGG GTA ACG AGC AAT AAG AT-3', respectively, both being internal to the above pair of PCR primers (Sato et al. 2001).

The sequence data from the present study and those reported previously (Schedlock et al. 1992; Sato et al. 2001) were aligned by the GENETIX-WIN version 4.0.1 software (Software Development Co., Ltd, Japan) to examine homology between the sequences of the control region. The phylogenetic relationship among the sequences was estimated by the neighbor-joining method (Saitou and Nei, 1987) based on the genetic distance estimated by Kimura's two-parameter method (Kimura, 1980).

RFLP analysis

GH1 is one of the best studied nuclear genes in salmonid species (Devlin 1993). It is not associated with sex, but has a high interspecific polymorphism of intronic sequences, and therefore is a valuable nuclear DNA marker for identification of species. PCR primers to amplify GH1 intron C were designed referring to the deposited sequences from DDBJ/EMBL/GenBank with accession numbers AF005926 for pink salmon, AF005927 for chum salmon, AF005925 for coho salmon (*O. kisutch*), AF005923 for steelhead trout (*O. mykiss*), and AF005924 for cutthroat trout (*O. clarki*). The primers included GH1CF (forward), 5'-GTA AGT AAC CTG GCT GAG AC-3' and GH1CR (reverse), 5'-TTG TAC AGC TTG AGA TGC CC-3'. The GH1 intron C was amplified in a 10 µl of reaction mixture containing the same components used for amplification of the mtDNA control region, as described above. The PCR cycling profile was preheating at 94 °C for 3 min, followed by 35 denaturation cycles at 94 °C for 30 sec, annealing at 58 °C for 30 sec, elongation at 72 °C for 45 sec, and final extension at 72 °C for 5 min.

After PCR, 8 µl of reaction mixture was incubated with 1 µl each of restriction enzyme and 10x reaction buffer at 37 °C for 1 hour. Five µl of the mixture was electrophoresed on 1.5% agarose gel, followed by staining with 0.02% ethidium bromide to visualize the RFLPs. The most appropriate restriction enzyme was chosen after a search for the restriction sites in the deposited intron C sequences of salmonid GH1 gene as previously described, using GeneWorks version 2.5.1 (Oxford Molecular Grope, Campbell, CA).

RESULTS

Morphological characteristics

Body size of the hybrid was 545 mm fork length and 2,111 g body weight, which was larger than co-captured pink salmon (Fig. 2; Table 1). Examination of fish scales indicated the age of this fish was 0.2 (numbers of winters spent in freshwater, followed by a dot and the number of winters spent at sea) and its ovary was immature (Fig. 3). The number of lateral line scales and other external characters were similar to those of pink salmon, but scale appearance and the number of pyloric caecae most likely pertained to chum salmon (Table 1). Morphological characters of the hybrid showed a mixture of chum and pink salmon characteristics.

mtDNA control region sequences

A fragment of approximately 1200 bp was amplified with a primer pair of tRNAthr-2 and tRNAphe-2 on DNA samples from a hybrid (L6) and four pink salmon (L7, L11 to L13). Direct sequencing on the amplified fragments as templates was successfully performed with nested sequencing primers of tRNAthr-3 and Okdl-H2. Multiple alignment and a neighbor-joining phenogram of the 481bp sequences obtained from these individuals and those of three chum salmon (OK-1, -7 and -9) reported previously (Sato et al. 2001) (DDBJ/EMBL/GenBank accession numbers AB039890, AB039896 and AB039898) revealed that the observed sequence in the hybrid was identical to the sequence of a haplotype found in chum salmon (OKDL-7) (Fig. 4).

RFLP patterns

A computer search for the restriction site in the salmonid GH1 intron C revealed the occurrence of *Dra*I site (5'-TTTAAA-3') due to a base substitution at the 235 nucleotide position from the 5' end in pink salmon (Fig. 5). Digestion with *Dra*I of 719 bp PCR product of intron C will therefore result in 485 bp and 234 bp fragments in pink salmon, but the enzyme will not cleave a 685 bp PCR fragment in chum and other salmon.

Of the two PCR products (about 720 bp and 450 bp) obtained from three pink salmon (L7, L11 and L12), the larger fragment was expected, while the smaller fragment could be a byproduct that occurred from the priming sites highly homologous and internal to GH1CF and GH1CR sequences in the larger fragment (Fig. 6). Three chum salmon (22 to 24), captured during another research cruise in the Bering Sea, showed single PCR product with an expected size of about 690 bp (Fig. 6). *Dra*I digestion did not alter the size of the PCR fragment in all the chum salmon, whereas enzyme digestion of the PCR product yielded two restriction fragments (about 490 bp and 240 bp) in pink salmon L7 and L12 (Fig. 6). Lack of 450 bp byproduct and a broad restriction band of about 240 bp suggested that the byproduct contains an internal sequence with a *Dra*I site. The PCR product in L11 was resistant to *Dra*I because of further base substitution in the restriction site (data not shown). The hybrid (L6) showed three fragments of PCR product after digestion with *Dra*I . Of these fragments, the largest one was the same in size as the 690 bp fragment of chum salmon, and the two others were the same size as the 490 and 240 bp fragments of pink salmon L7 and L12 (Fig. 6). Thus, the hybrid had both pink and chum salmon GH1 alleles .

DISCUSSION

The present molecular genetic study revealed that the hybrid caught in the Bering Sea had a chum salmon mtDNA haplotype and nuclear GH1 gene alleles from chum and pink salmon. MtDNA is maternally inherited, without recombination with nuclear genes (Meyer 1993). Combining the mtDNA and nuclear GH1 findings, we can conclude the hybrid salmon arose from a cross between a female chum and a male pink salmon. Molecular identification of the present chum-pink hybrid raises questions as to whether it was created in the wild, or in a hatchery, its geographic origin, and what might be the biological significance of natural hybrids.

Unlike most interspecific crosses in hatchery trials, chum-pink hybrids have been known to provide fairly good viability (90-99.6%), with a better survival of female chum-male pink crosses than the reverse combination (Simon and Noble 1968). Relying on a good hatching success, release of chum-pink hybrids from hatcheries in Russia, Japan and USA has been conducted to determine the survival of F_1 hybrids in the ocean (Pavlov 1959; Hikita and Yokohira 1964; Simon and Noble 1968). However, the rate of return was inconclusive (Simon and Noble 1968). Experimentally-produced hatchery crosses are likely not to have been produced recently, therefore, it is probable the chum-pink hybrid we examined was naturally produced in the wild.

The OKDL-7 haplotype of chum salmon mtDNA was present in the hybrid. It is the most widely distributed haplotype thus far examined in chum salmon populations from Japan, Russia and North America (Sato et al. 2001 and in preparation). Therefore, our analysis was not sufficient to identify the geographical origin of the hybrid. The summer in the Bering Sea is a highly productive area for salmon feeding, and pink and chum salmon stocks from both Asia and North America are present in this area in summer (Myers et al. 1996; Winans et al. 1998). The use of molecular markers will need further refinements in order for them to be useful for determination of continent of origin of stock mixtures of Pacific salmon on the high seas.

The incidence of chum-pink hybridization in nature is unknown. Natural chum-pink hybrids occur in Alaska (Jack Helle and Anthony Gharrett, personal communication) and British Columbia (Hunter 1949). Though few in number, the occurrence of hybrids is not a rare experience for Japanese fishermen fishing in the ocean (Numachi et al. 1979). As suggested by a preliminary allozyme analysis, the occurrence of .F_1, F_2 and backcross hybrids might be possible in the wild because of a high fertility and faster growth of F_1 hybrids than the parental species (Simon and Noble

1968; Numachi et al. 1979). Infrequent observation of natural hybrids may be associated with either the limited number as compared with abundant "pure" crosses of parental species, or limited by their viability because survival of hybrids decreases with each generation, as they are influenced by parental combination (Simon and Noble 1968). These factors may become a barrier to limit the establishment of a hybrid population in nature.

The question remains open as to whether natural chum- pink hybrids present consequences to wild populations of both species. Fertile hybrids could cause reciprocal genetic introgression in parental population, if such hybrids can participate in natural reproduction of both species. This condition would be confirmed by finding a chum salmon with a pink gene and *vice versa* using more refined molecular markers.

ACKNOWLEDGMENTS

The authors thank Drs. Jack Helle and Bill Heard, Auk Bay National Laboratory, NOAA, and Dr. Anthony Gharrett, Juneau Center, School of Fisheries and Ocean Science, University of Alaska Fairbanks, for their unpublished information on natural chum-pink hybrids.

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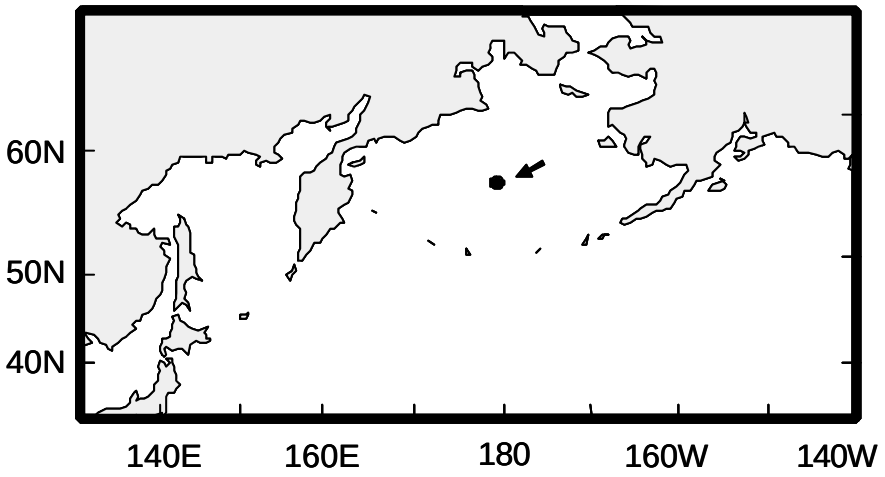


Fig. 1. Location (arrow) in the central Bering Sea where the chum-pink salmon hybrid was captured.

Fig. 2. A chum-pink salmon hybrid (top) and a co-captured pink salmon (bottom) caught in the central Bering Sea in July 2000.

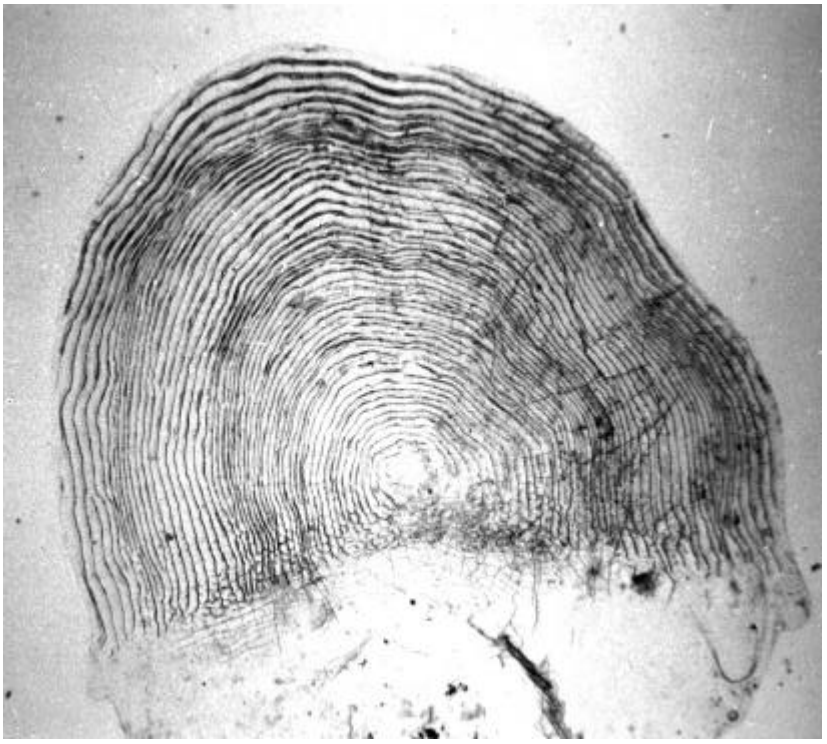


Fig. 3. Scale pattern of an age 0.2 chum-pink salmonid hybrid.

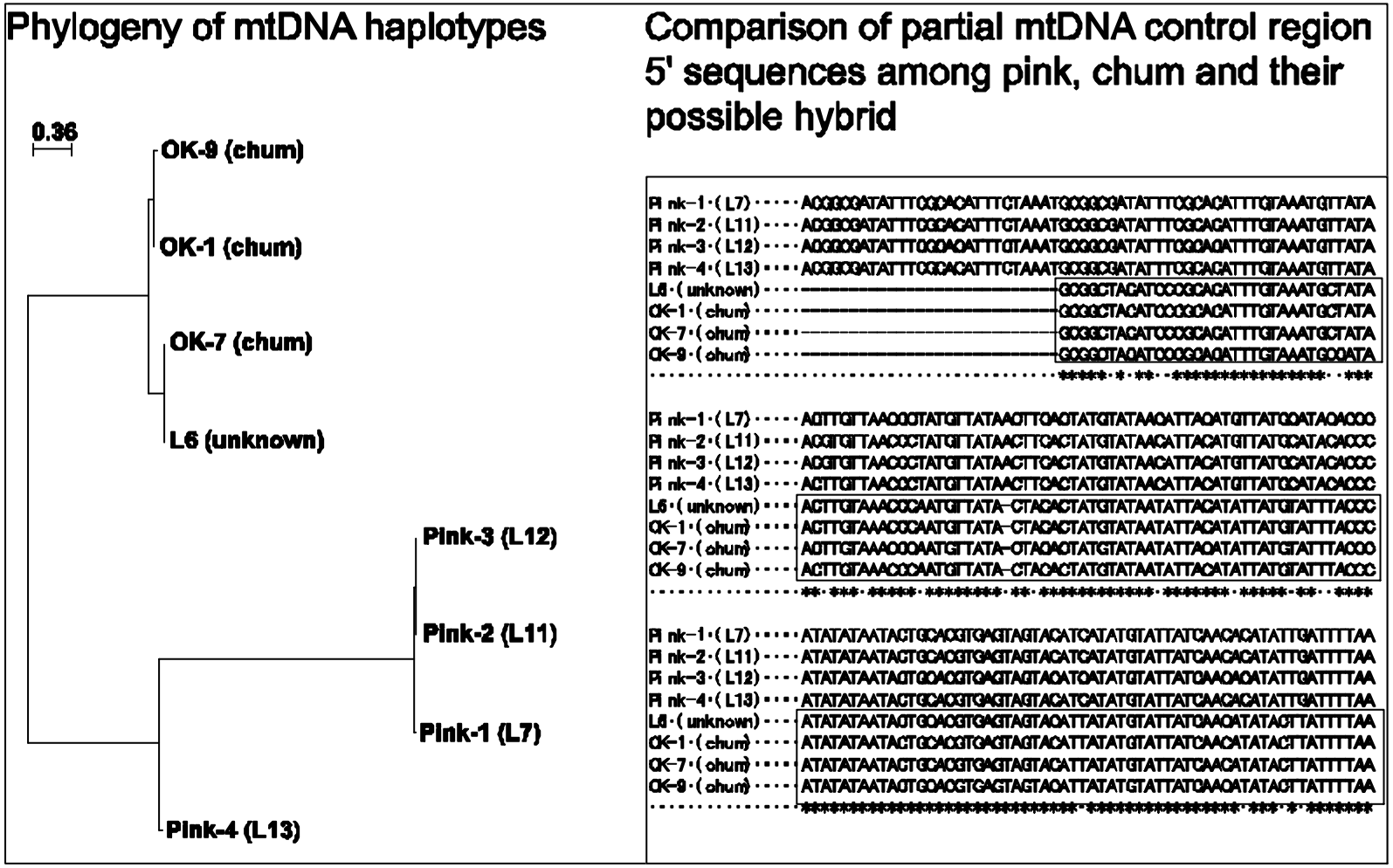


Fig. 4. Alignment of mtDNA control region sequences from pink, chum, and a chum-pink hybrid (right), and neighbor-joining phenogram of the observed sequences (left).

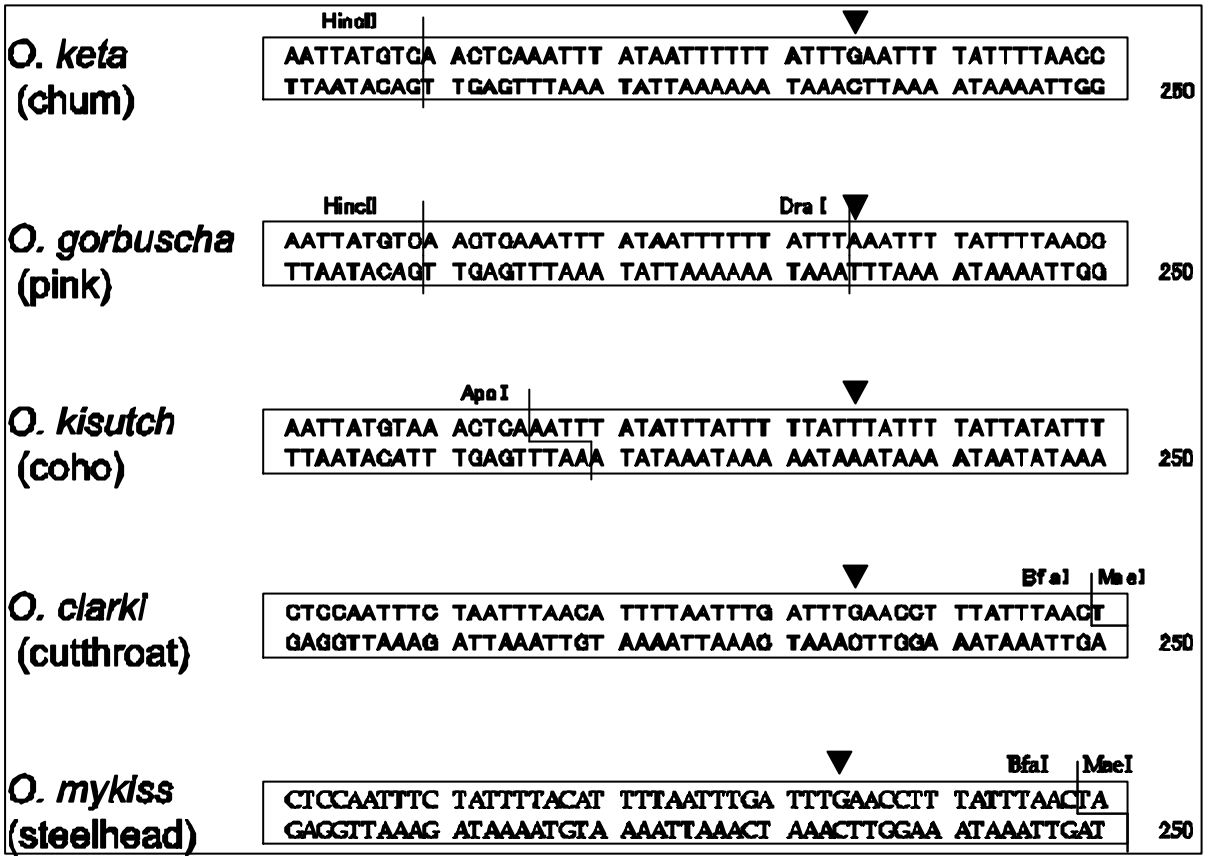
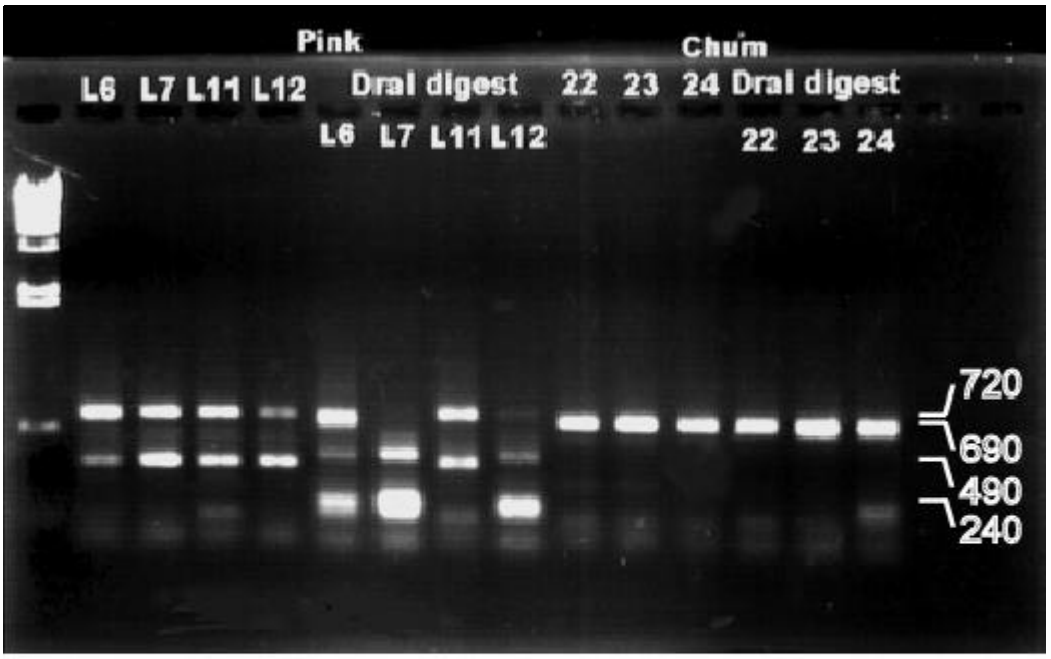


Fig. 5. Restriction sites in partial sequences of salmonid GH1 intron C among salmonid species. Arrowhead points to the 234 or 235 nucleotide position with or without a base substitution.



	Pink	Chum
PCR fragment	719 bp	685 bp
*Dra*I restriction fragment	485 bp 234	685 bp

Fig. 6. *Dra*I RFLP of GH1 intron C in pink (L7, L11 and L12), a chum-pink hybrid (L6), and chum salmon (22 to 24). Expected molecular sizes of GH1 intron C by PCR and *Dra*I digestion are boxed.

Table 1. Morphological data of a chum -pink hybrid and co-captured pink salmon caught in the central Bering Sea, July 2000.

No.		**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L8**	**L9**	**L10**	**L20**
Species		pink	pink	pink	pink	pink	hybrid	pink	pink	pink	pink
Date		3-Jul-00	3-Jul-00	3-Jul-00	3-Jul-00	3-Jul-00	4-Jul-00	4-Jul-00	4-Jul-00	4-Jul-00	7-Jul-00
Latitude		5730	5730	5730	5730	5730	5830	5830	5830	5830	5730
Longitude		18000	18000	18000	18000	18000	18000	18000	18000	18000	17900 W
Gear		115	121	93	93	82	121	93	106	115	93
Sex		female	male	female	female	female	female	female	female	female	female
Maturity		mature	mature	mature	mature	mature	immature	mature	mature	mature	mature
Age		0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1
Fork length (mm)		402	441	401	390	420	545	432	400	420	423
Head length (mm)		81.25	98	86.5	84	87	110	92	82	84	90
Snout length (mm)		21.68	30.8	24.4	20	21.7	31	23.4	19	24	24
Upper jaw length (mm)		40.34	53	42	39	40.5	56.8	44	38	43	44
Body depth (mm)		94.8	109.7	109	91	108	127.1	104	94	92	108
Caudal peduncle depth (mm)		27.24	29.16	30	27	29	38.4	29.2	29	27	30.5
Eye diameter (mm)		10.26	10.2	11.9	9.9	10.1	14.3	11.6	10	11	11
Body weight (g)		903.7	1159.3	1080.7	745	1065.5	2110.9	1168.5	848.1	950.2	1065.5
Gonad weight (g)		80.8	76.2	86.1	75	97.5	5.1	103.5	80.4	71.3	100.7
Liver weight (g)		29	23.8	36.2	30.9	43.3	64.3	38.4	26.6	39.2	30.3
Lateral line scales		178	187	181	188	186	177	190	188	183	185
Dorsal fin rays		12	16	14	15	14	13	12	14	14	15
Pectoral fin rays	L	15	15	16	14	15	15	14	14	15	15
	R	16	14	15	15	15	16	15	15	15	15
Ventral fin rays	L	10	11	10	10	10	11	9	10	10	10
	R	10	10	10	10	10	10	9	10	10	10
Anal fin rays		17	16	17	16	15	17	15	15	16	15
Gill rakers	L	32	30	31	29	UK	29	32	29	29	29
	R	29	29	29	29	UK	29	30	30	30	30
Pyloric caeca		107	100	104	108	110	170	93	110	89	138
Stomach		yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Scales		yes	yes	yes	yes	yes	yes	yes	yes	yes	yes