



## **K-*ras* oncogene DNA Sequences in Pink Salmon in Streams Impacted by the *Exxon Valdez* Oil Spill: No Evidence of Oil-induced Heritable Mutations**

MATTHEW A. CRONIN,<sup>1\*</sup> JEFFREY K. WICKLIFFE,<sup>2</sup> YELENA DUNINA<sup>2</sup> AND  
ROBERT J. BAKER<sup>2</sup>

<sup>1</sup>*LGL Alaska Research Associates, 1101 East 76th Avenue, Anchorage, AK 99518, USA*

<sup>2</sup>*Department of Biological Sciences, Texas Tech University, Lubbock TX 79409, USA*

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**Abstract.** It was hypothesized in previous studies that the *Exxon Valdez* oil spill in Prince William Sound, Alaska, induced heritable mutations and resulted in mortality of pink salmon (*Oncorhynchus gorbuscha*) embryos. In one of these studies, laboratory exposure of pink salmon embryos to crude oil resulted in apparent mutation-induction in exon 1 and exon 2 of the *K-ras* oncogene, but no fish from the area impacted by the oil spill were analyzed. We assessed *K-ras* exon 1 and exon 2 DNA sequences in pink salmon from five streams that were oiled and five streams that were not oiled by the *Exxon Valdez* oil spill in Prince William Sound, and two streams with natural oil seeps and one stream without seeps on the Alaska Peninsula. Of the 79 fish analyzed for exon 1 and the 89 fish analyzed for exon 2, none had the nucleotide substitutions representing the mutations induced in the laboratory study. Other variable nucleotides occurred in similar proportions in oiled and non-oiled streams and probably represent natural allelic variation. These data do not support the hypothesis that heritable mutations in the *K-ras* gene were induced by the *Exxon Valdez* oil spill or oil seeps.

**Keywords:** *Exxon Valdez* oil spill; *K-ras* gene DNA sequences; mutations; pink salmon

### **Introduction**

It was hypothesized that exposure to crude oil following the *Exxon Valdez* oil spill in Prince William Sound, Alaska in 1989 resulted in mortality of pink salmon (*Oncorhynchus gorbuscha*) embryos (Bue et al., 1996, 1998). A pattern of greater embryo mortality in oiled streams than in non-oiled streams over two generations led Bue et al. (1998) to hypothesize that the impact was trans-generational,

and possibly due to oil-induced germ line genetic mutations. They expressed concern that such mutations could affect the viability of populations for generations after a pollution event.

A possible mechanism of mutation-induced mortality was suggested by laboratory study of pink salmon embryos experimentally exposed to crude oil (Roy et al., 1999). In this study, three putative mutations, at codon 12 nucleotide position 35 and codon 13 nucleotide position 37 in exon 1 and at codon 61 nucleotide position 70 in exon 2 of the *K-ras* oncogene, were detected in pink salmon embryos exposed to crude oil but not in control embryos. Mutations at the same three *K-ras* codons

\*To whom correspondence should be addressed: Tel.: (907) 562-3339; Fax: (907) 562-7223; E-mail: mcronin@lgl.com.

are often found in tumors in humans and animals, including salmonid fish (Fong et al., 1993; Hendricks et al., 1994; Bailey et al., 1996; Roy et al., 1999 and references therein). Assessments of *Exxon Valdez* oil impacts on other genetic markers (including the *P53* gene, the mitochondrial DNA cytochrome *b* gene, multiple microsatellite loci, and DNA content assessed with flow cytometry) in experimental fish populations failed to detect mutations or genetic damage (Roy et al., 1999; Miller et al., 1994; Seeb et al., 1996).

Roy et al. (1999) concluded that crude oil may induce *K-ras* mutations in pink salmon, but they cautioned that this does not necessarily mean that either heritable or somatic mutations were induced in fish in the areas impacted by the *Exxon Valdez* oil spill. Roy et al. (1999) explicitly recognized the need to assess whether oil actually induced *K-ras* mutations in Prince William Sound pink salmon populations. In this paper, we provide such an assessment. Specifically, we analyzed *K-ras* DNA sequences in pink salmon from streams that were oiled by the *Exxon Valdez* oil spill and non-oiled streams in southwest Prince William Sound. We also included samples from streams with and without natural oil seeps on the Alaska Peninsula for comparison of fish from the oil spill area with fish from chronically oiled sites and a different geographic area. Our primary objective was to determine if the oil-induced nucleotide substitutions (i.e., mutations) observed by Roy et al. (1999) occur in pink salmon in the streams in Prince William Sound impacted by the *Exxon Valdez* oil spill. Another objective was to compare the patterns of *K-ras* DNA sequence variation between oiled and non-oiled streams in Prince William Sound and in areas on the Alaska Peninsula with natural oil seeps. Our samples were from adults collected in 1997 and 1999, four to five generations after the oil spill that occurred in 1989. Pink salmon have a two-year life history in which all adults die at two years of age after spawning. Our samples therefore were not from fish directly exposed to the oil spill and our analysis is focused on persistent heritable mutations, not somatic mutations directly induced by exposure to oil.

## Methods

Tissues were obtained from spawning pink salmon from ten streams in Prince William Sound in

September 1997 and 1999 (Fig. 1), and three streams in an area with natural oil seeps on the Alaska Peninsula in September 1999 (Fig. 2). This includes five streams that were oiled and five streams that were not oiled by the *Exxon Valdez* oil spill in southwestern Prince William Sound, and two streams with oil seeps and one stream without oil seeps on the Alaska Peninsula (Table 1). The Prince William Sound streams are identified in Table 1 and Fig. 1 by name and reference number, and are described by Brannon et al. (1995) and Maki et al. (1995). The streams on the Alaska Peninsula (Oil Creek, Teresa Creek, and Portage Creek) shown in Fig. 2 are described by Blasko (1976) and Becker and Manen (1989). Oil Creek and Teresa Creek are reported to have oil seeps, although we observed seeps only at Oil Creek. Portage Creek is not known to have oil seeps. Fish were collected in a dip net or by hand from the stream channel and killed, and liver tissue was collected and put on ice for 2–8 h and then kept frozen until analysis.

Each sample was given a unique identification number and all samples were randomized so that laboratory personnel did not know if the samples came from an oiled or non-oiled stream. Therefore, all of the procedures outlined below were conducted in a blind fashion to avoid bias in characterizing the *K-ras* nucleotide sequences.

Genomic DNA was isolated from liver tissue (~0.1 g) with a standard cell lysis, protein digestion, and organic extraction procedure followed by ethanol precipitation. *K-ras* exon 1 and exon 2 were amplified in separate polymerase chain reactions (PCR) using the primers described by Roy et al. (1999):

- Exon 1: Forward primer HO1: 5'-ATGACGGAA-TACAAGCTG-3'  
Reverse primer C37: 5'-CTCGATGGTG-GGGTCATATT-3'
- Exon 2: Forward primer H38: 5'-GACTCGTA-CAGGAAGCAGGT-3'  
Reverse primer C79: 5'-CATGGCGCTG-TACTCCTCCT-3'.

Amplification reactions were performed in 25  $\mu$ l volumes consisting of approximately 100 ng of genomic DNA, 0.4  $\mu$ M each primer (Integrated DNA Technologies; Coralville, IA), 0.2 mM dNTPs (GeneAmp<sup>®</sup>, Applied Biosystems (ABI); Foster City CA), 1.5 mM MgCl<sub>2</sub> (Promega Corporation;

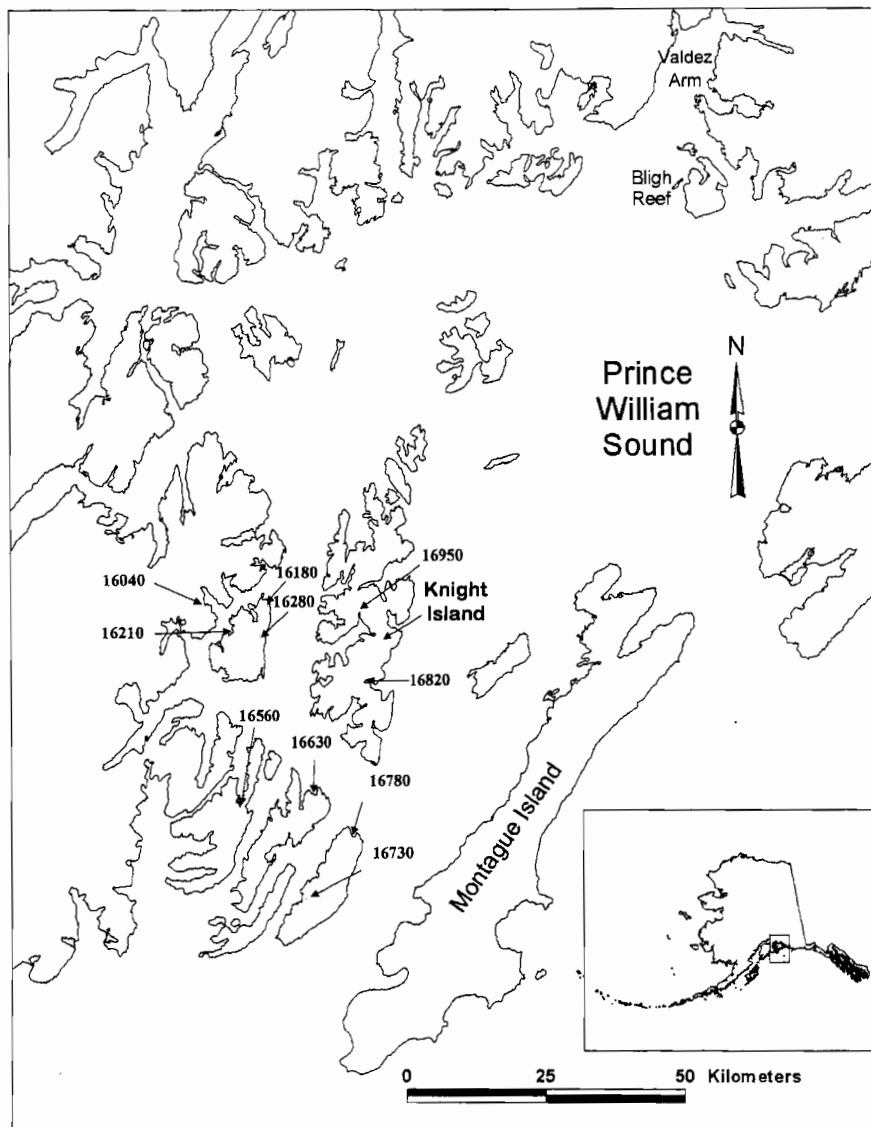


Figure 1. Map of western Prince William Sound, Alaska with the streams sampled for this study identified by number.

Madison, WI),  $1\times$  *Taq* buffer (Promega Corp.), and 2U *Taq* polymerase (Promega Corp.). Amplicons were subjected to agarose (1.0%) gel electrophoresis, stained with ethidium bromide, and visualized using an Eagle Eye<sup>®</sup> II Still Video System (Stratagene Corporation; LaJolla, CA). Amplicons were then purified according to the manufacturer's recommendations using the QIAquick PCR Purification Kit (QIAGEN Inc.; Valencia, CA). Purified amplicons were then cycle-sequenced with the PCR primers in both directions using the ABI PRISM<sup>®</sup> dRhodamine or

ABI PRISM<sup>®</sup> BigDye<sup>™</sup> dye-terminator chemistry following the manufacturer's recommendations (ABI). DNA sequences were generated using capillary electrophoresis and a fast sequencing protocol on an ABI PRISM<sup>®</sup> 310 Genetic Analyzer according to the manufacturer's recommendations (ABI). Chromatograms and sequences were then proofed and aligned using the Sequencer ver. 3.1 software for the Macintosh (Gene Codes Corp.; Ann Arbor, MI). Computer-assigned nucleotide calls were verified with visual inspection of the chromatograms.

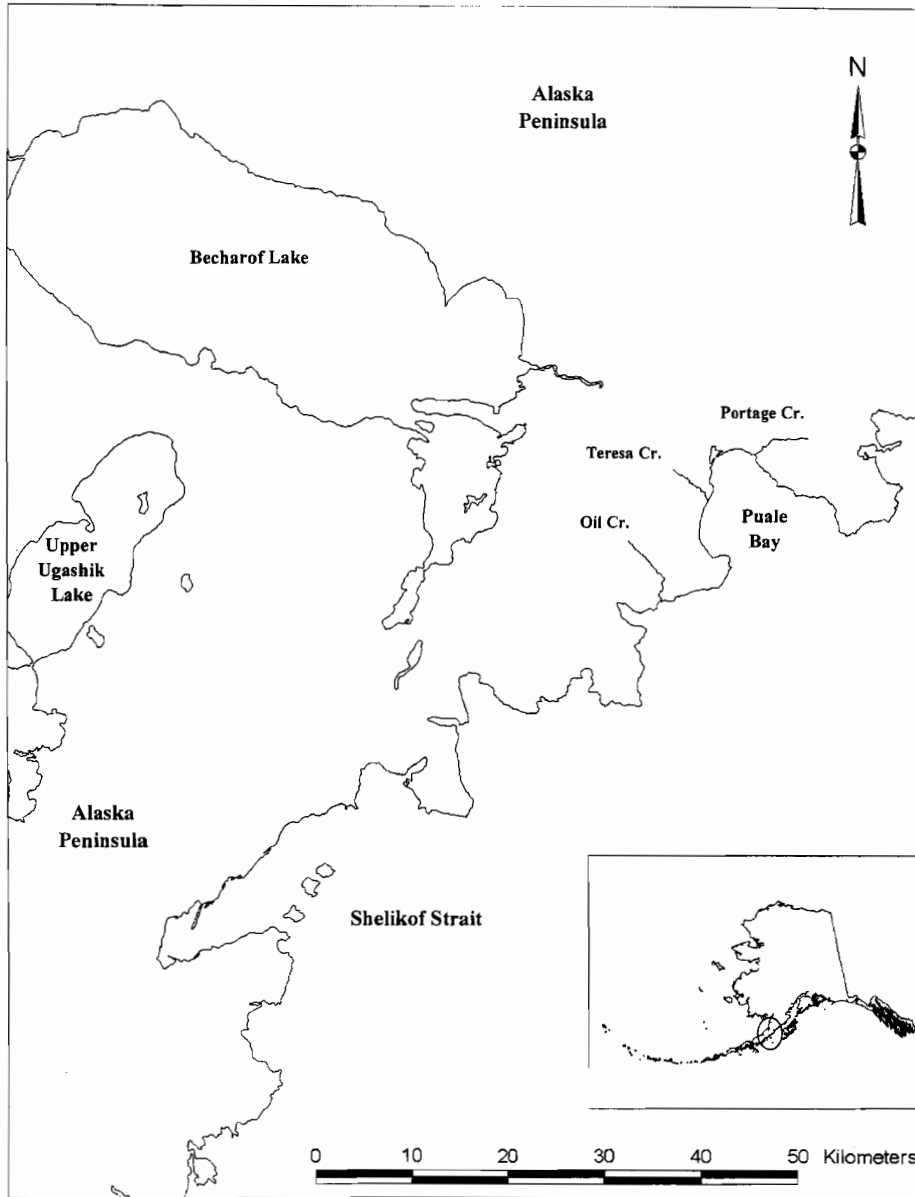


Figure 2. Map of the Puale Bay area on the Alaska Peninsula with the streams sampled for this study identified.

We first assessed potential variable nucleotides at the sites at which mutations were identified by Roy et al. (1999): codon 12 nucleotide position 35 and codon 13 nucleotide position 37 in exon 1 and at codon 61 nucleotide position 70 in exon 2. We also assessed variable nucleotides (i.e. substitutions) at other sites within and between samples. We considered the non-mutant sequence of Roy et al. (1999) as the standard sequence, and nucleotides that varied from this

sequence as substitutions. In some individual fish samples there were two nucleotides at single nucleotide positions. These could reflect heterozygotes or mosaic somatic genotypes. The BigDye chemistry that we employed was developed to yield uniform chromatogram peak heights and allows detection of heterozygotes and mosaic DNA sequences within individuals. In practice, however, peak heights are not uniform within or between sequences and a ratio

Table 1. Streams and numbers of pink salmon for which K-ras DNA sequences were determined

Stream	Exon 1			Exon 2		
	1997	1999	Total	1997	1999	Total
<i>Oiled streams</i>						
Prince William Sound						
16180 North Chenega	6	0	6	7	0	7
16280 East Chenega	8	9	17	5	8	13
16630 North Evans	2	1	3	1	4	5
16780 Sleepy Bay	7	2	9	6	2	8
16820 Snug Harbor	0	2	2	1	4	5
Alaska Peninsula						
Oil Creek <sup>a</sup>	0	5	5	0	8	8
Teresa Creek <sup>a</sup>	0	4	4	0	4	4
<i>Non-oiled streams</i>						
Prince William Sound						
16040 Ewan	3	7	10	1	7	8
16210 Totemoff	10	2	12	10	5	15
16560 Bainbridge	3	1	4	4	1	5
16730 Horseshoe	1	0	1	3	0	3
16950 Drier	0	4	4	1	2	3
Alaska Peninsula						
Portage Creek <sup>a</sup>	0	2	2	0	5	5

<sup>a</sup>Alaska Peninsula, only sampled in 1999.

of 70:30 (wildtype:mutant) is a threshold below which mutant sequences cannot be reliably detected (ABI, Application Note-DOC # 100640). We therefore used a conservative approach and only identified two nucleotides at a given position for a sample if the two peaks on the chromatogram were above the baseline signal in the sequences in both directions.

Following identification of sequence variation, we assessed the numbers and proportions of fish with variable nucleotides from the oiled and non-oiled streams. We used the MEGA 2 computer program (Kumar et al., 2001) to assess nucleotide variation between sequences, including the average nucleotide substitutions per nucleotide site, synonymous substitutions per synonymous site, and non-synonymous substitutions per non-synonymous site with the Jukes and Cantor (1969) method.

## Results

### Exon 1

We obtained sequence for the first 102 of the 111 nucleotide positions for exon 1 of the K-ras gene

reported by Roy et al. (1999). Some of our exon 1 sequences were identical to those reported by Roy et al. (1999) for non-mutant pink salmon. Other exon 1 sequences varied from the Roy et al. (1999) non-mutant sequence by 1, 2, or 3 nucleotides (Fig. 3). The nucleotide substitutions in exon 1 included three synonymous and four nonsynonymous substitutions (Table 2). The exon 1 sequences were submitted to GenBank (Accession numbers AF467659 and AF465429–AF465436).

We obtained exon 1 sequences for 68 fish from Prince William Sound, including 37 fish from oiled streams and 31 fish from non-oiled streams. We also obtained exon 1 sequences for 11 fish from the Alaska Peninsula oil seep area, including 9 fish from streams with seeps and two fish from a stream without seeps. None of our sequences had either of the G–A mutations at codon 12 nucleotide position 35 or codon 13 nucleotide position 37 for exon 1 reported by Roy et al. (1999). There were also no mutations observed in the other nucleotide positions in codons 12 and 13.

The numbers and proportions of fish with other nucleotide substitutions in our exon 1 sequences are shown in Table 2. There were synonymous transitions at codon 10 nucleotide position 30 (GGG–GGA) and codon 24 nucleotide position 72 (ATT–ATC) that occurred in similar proportions of the fish from both oiled and non-oiled streams in Prince William Sound and the Alaska Peninsula streams with oil seeps. The substitution at codon 10 position 30 was observed in 65% of the fish from oiled streams and 71% of the non-oiled streams in Prince William Sound, and in 78% of the fish from streams with oil seeps on the Alaska Peninsula. The substitution at codon 24 position 72 was in 38% of the fish from oiled streams and 32% of the fish from non-oiled streams in Prince William Sound, and in 44% of the fish from the streams with oil seeps on the Alaska Peninsula (Table 2). There was also a synonymous GCA–GCC transversion at codon 11 nucleotide position 33 in one fish from an oiled stream in Prince William Sound. There were four nonsynonymous substitutions in exon 1 in one fish each (3%) from the non-oiled streams. These included a GGG–CGG (Gly–Arg) transversion at codon 10 nucleotide position 28, an ATC–ATG (Ile–Met) transversion at codon 21 nucleotide position 63, a CAG–CAT (Gln–His) transversion at codon 22 nucleotide position 66, and

EXON 1

				111	111	111	122	222	222	223	333	333	333	444	444	444	455	555	555	556	666	666	666	777	777	777
Roy et al.	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678
Roy et al. mut	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
PWS1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
PWS2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
PWS3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
PWS4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
PWS5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
PWS6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
PWS7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
PWS8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
PWS9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

								111	111	111	111
Roy et al.	788	888	888	889	999	999	999	000	000	000	011
Roy et al. mut	...	...	...	...	...	...	...	...	...	...	...
PWS1	...	...	...	...	...	...	...	...	...	...	...
PWS2	...	...	...	...	...	...	...	...	...	...	...
PWS3	...	...	...	...	...	...	...	...	...	...	...
PWS4	...	...	...	...	...	...	...	...	...	...	...
PWS5	...	...	...	...	...	...	...	...	...	...	...
PWS6	...	...	...	...	...	...	...	...	...	...	...
PWS7	...	...	...	...	...	...	...	...	...	...	...
PWS8	...	...	...	...	...	...	...	...	...	...	...
PWS9	...	...	...	...	...	...	...	...	...	...	...

Figure 3. DNA Nucleotide sequences for K-ras exon 1 in pink salmon. The Roy et al. and Roy et al. mut sequences represent the non-mutant and mutant sequences from Roy et al. (1999), respectively. The sequences identified in this study are identified by PWS designations. Dots represent identity with the Roy et al. sequence in the top row. Dashes indicate sequences not resolved in our analysis. Nucleotide positions are indicated by the numbers along the top of the sequences.

a GAA–GGA (Glu–Gly) transition at codon 31 nucleotide position 92.

For the exon 1 sequences in Fig. 3 there was an average of 0.022 (SE 0.008) substitutions per site using the Jukes–Cantor distance method (Jukes and Cantor, 1969). There was an average of 0.046 (SE 0.036) synonymous substitutions per synonymous site and 0.016 (SE 0.006) non-synonymous substitutions per non-synonymous site. The synonymous and non-synonymous substitution rates were not significantly different ( $Z = 0.971$ ,  $P = 0.334$ ).

Exon 2

The exon 2 sequences included the 90 nucleotide positions described by Roy et al. (1999). Some of our exon 2 sequences were identical to those reported by Roy et al. (1999) for non-mutant pink salmon. Other exon 2 sequences varied from the Roy et al. (1999) non-mutant sequence by 1, 2 or 3 nucleotides (Fig. 4). The nucleotide substitutions in exon 2 included three synonymous substitutions and two non-synonymous substitutions (Table 2). The sequences for exon 2

were submitted to GenBank (Accession numbers AF 467658 and AF465420–AF465428).

We obtained exon 2 sequences for 72 fish from Prince William Sound. This included 38 fish from oiled streams and 34 fish from non-oiled streams. We also obtained exon 2 sequences for 17 fish from the Alaska Peninsula oil seep area, including 12 fish from streams with oil seeps, and 5 fish from one stream without seeps. None of our sequences had the C–A mutation at codon 61 nucleotide position 70 for exon 2 reported by Roy et al. (1999). There were also no mutations observed in the other nucleotide positions in codon 61.

The numbers and proportions of fish with other nucleotide substitutions in exon 2 sequences are shown in Table 2. There were three common synonymous substitutions in exon 2 in both the oiled and non-oiled streams in Prince William Sound and the Alaska Peninsula oil seep area. Each of these substitutions was found in similar proportions of the sampled fish in the oiled and non-oiled streams in Prince William Sound. These included a GAT–GAC transition at codon 47 nucleotide position 30, a

Table 2. Numbers and proportions of pink salmon with the nucleotide substitutions in K-ras exon 1 and exon 2 observed in this study. The first letters of each nucleotide position are those in the Roy et al. (1999) non-mutant sequence. The second letters are substitutions observed in this study

	Nucleotide Position								
	Synonymous substitutions			Non-synonymous substitutions					
Exon 1 (Number of fish)	30 G-A	33 A-C	72 T-C	28 G-C	63 C-G	66 G-T	92 A-G	35 <sup>a</sup> G-A	37 <sup>a</sup> G-A
Prince William Sound									
Oiled streams (37)	24	1	14	0	0	0	0	0	0
Proportion of fish	0.649	0.027	0.378	0	0	0	0	0	0
Non-oiled streams (31)	22	0	10	1	1	1	1	0	0
Proportion of fish	0.710	0	0.323	0.032	0.032	0.032	0.032	0	0
Alaska Peninsula									
Oil seeps (9)	7	0	4	0	0	0	0	0	0
Proportion of fish	0.778	0	0.444	0	0	0	0	0	0
No oil seeps (2)	0	0	0	0	0	0	0	0	0
Proportion of fish	0	0	0	0	0	0	0	0	0
Exon 2 (Number of fish)	30 T-C	33 G-A	63 A-T	5 C-T	62 C-T	70 <sup>a</sup> C-A			
Prince William Sound									
Oiled streams (38)	34	11	37	0	0	0			
Proportion of fish	0.895	0.289	0.974	0	0	0			
Non-oiled streams (34)	32	6	33	1	1	0			
Proportion of fish	0.941	0.176	0.971	0.029	0.029	0			
Alaska Peninsula									
Oil seeps (12)	11	5	012	0	0	0			
Proportion of fish	0.917	0.417	1.000	0	0	0			
No oil seeps (5)	5	4	5	0	0	0			
Proportion of fish	1.000	0.800	1.000	0	0	0			

<sup>a</sup>Substitutions detected in experimentally-oiled pink salmon by Roy et al. (1999).

GGG-GGA transition at codon 48 nucleotide position 33, and an ACA-ACT transversion at codon 58 nucleotide position 63. These substitutions ranged in frequency from 18% to 100% of the fish from the different sampling areas (Table 2). There were two nonsynonymous substitutions in exon 2, and each was observed in one fish (3%) from non-oiled streams in Prince William Sound. These included a TCG-TTG (Ser-Leu) transition at codon 39 nucleotide position 5 and an ACT-ATT (Thr-Ile) transition at codon 58 nucleotide position 62.

For the sequences in Fig. 4 there was an average of 0.024 (SE 0.011) substitutions per site using the Jukes-Cantor distance method (Jukes and Cantor, 1969). There was an average of 0.075 (SE 0.047) synonymous substitutions per synonymous site and 0.009 (SE 0.006) non-synonymous substitutions

per non-synonymous site. The synonymous and non-synonymous substitution rates were not significantly different ( $Z = 1.504$ ,  $P = 0.135$ ).

## Discussion

The K-ras exon 1 and exon 2 mutations experimentally induced in pink salmon by Roy et al. (1999) did not occur in fish collected from streams in Prince William Sound that were impacted by the *Exxon Valdez* oil spill. These mutations also did not occur in fish collected from streams in Prince William Sound that were not impacted by the oil spill, and in streams on the Alaska Peninsula with and without natural oil seeps. These data do not support the hypothesis that heritable mutations in the K-ras gene were induced

EXON 2		111	111	111	122	222	222	223	333	333	333	444	444	444	455	555	555	556	666	666	666	777	777	777			
Roy et al.		123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678
Roy et al. mut		GAC	TCG	TAC	AGG	AAG	CAG	GTG	GTG	ATT	GAT	GGG	GAG	ACG	TGT	CTG	CTG	GAC	ATC	CTG	GAC	ACA	GCA	GGT	CAG	GAG	GAG
PWS1		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS2		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS3		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS4		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS5		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS6		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS7		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS8		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS9		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS10		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
		788	888	888	889																						
		901	234	567	890																						
Roy et al.		TAC	AGC	GCC	ATG																						
Roy et al. mut		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS1		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS2		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS3		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS4		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS5		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS6		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS7		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS8		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS9		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
PWS10		.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

Figure 4. DNA Nucleotide sequences for K-ras exon 2 in pink salmon. The Roy et al. and Roy et al. mut sequences represent the non-mutant and mutant sequences from Roy et al. (1999), respectively. The sequences identified in this study are identified by PWS designations. Dots represent identity with the Roy et al. sequence in the top row. Nucleotide positions are indicated by the numbers along the top of the sequences.

by the oil spill or natural oil seeps. However, it is possible that heritable mutations were induced but have been removed by selection (Cronin and Bickham, 1998), or that mutations were induced in low frequency, and we did not detect them in our samples. In any event, it is unlikely that oil-induced mutations had a measurable impact on the populations because temporal patterns of mortality in oiled streams are not consistent with those expected if heritable mutations were responsible (Cronin and Bickham, 1998).

When assessing the potential for mutation-induction resulting from the *Exxon Valdez* oil spill, it is important to consider the degree of exposure to oil. The high frequency of K-ras mutations observed by Roy et al. (1999) followed laboratory exposure to relatively high concentrations of oil (5.7 g oil/kg gravel) from the *Exxon Valdez*. Polycyclic aromatic hydrocarbons (PAH) comprise about 2% of Alaska North Slope crude oil (A. E. Bence, personal communication) so this exposure equates to 0.114 g PAH/kg gravel. In comparison, the mean PAH concentrations in pink salmon spawning substrate in streams impacted by the *Exxon Valdez* oil spill in 1989–1991 were orders of magnitude lower. The

maximum PAH concentration in the spawning substrate was 0.0028 g/kg (range  $5 \times 10^{-7}$ – $2.8 \times 10^{-3}$  g/kg, Brannon et al., 1995; Maki et al., 1995; Brannon and Maki, 1996). The oil concentrations in pink salmon spawning areas following the oil spill may have been below that necessary for mutation-induction.

Roy et al. (1999) did not report any nucleotide variation except for the mutations induced in codons 12, 13 and 61. Their study included 40 fish from a hatchery, and offspring of single-pair matings of these fish. We observed several variable nucleotide sites among our samples that were not detected by Roy et al. (1999) probably because we sampled larger numbers of fish from several spawning streams and/or because we had better resolution of DNA sequence variation. In any event, there are no substantive differences in distribution of the substitutions in oiled and non-oiled streams that suggest heritable mutation-induction. In fact, all of the nonsynonymous substitutions were in fish from non-oiled streams. Unlike the mutations experimentally induced by Roy et al. (1999), the nucleotide variation we observed probably represents natural allelic variation at the K-ras locus.

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