Molecular methods for the genetic identification of salmonid prey from Pacific harbor seal (*Phoca vitulina richardsi*) scat

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Twenty-six stocks of Pacific salmon and trout (*Oncorhynchus* spp.), representing evolutionary significant units (ESU), are listed as threatened or endangered under the Endangered Species Act (ESA) and six more stocks are currently being evaluated for listing.[1] The ecological and economic consequences of these listings are large; therefore considerable effort has been made to understand and respond to these declining populations. Until recently, Pacific harbor seals (*Phoca vitulina richardsi*) on the west coast increased an average of 5% to 7% per year as a result of the Marine Mammal Protection Act of 1972 (Brown and Kohlman[2]). Pacific salmon are seasonally important prey for harbor seals (Roffe and Mate, 1984; Olesiuk, 1993); therefore quantifying and understanding the interaction between these two protected species is important for biologically sound management strategies. Because some Pacific salmonid species in a given area may be threatened or endangered, while others are relatively abundant, it is important to distinguish the species of salmonid upon which the harbor seals are preying. This study takes the first step in understanding these interactions by using molecular genetic tools for species-level identification of salmonid skeletal remains recovered from Pacific harbor seal scats.

Most studies of harbor seal food habits rely on morphological identification of indigestible parts (e.g. otoliths and bones) from scat. Otoliths can be used to identify fish species (Ochoa-Acuna and Francis, 1995) but are not always present in scats, which can result in an underestimate of the number of species and the number of fish consumed (Harvey, 1989). Skeletal remains in scat are much more common and generally bones can be identified to the species level (Cottrell et al., 1996). Morphological identification is possible to the family level only with Pacific salmonid bones; however, genetic markers have the ability to discriminate between species, and the feasibility of extracting DNA from bones has been clearly demonstrated (Hochmeister et al., 1991).

Mitochondrial DNA (mtDNA) has been widely employed in systematic studies (reviewed by Avise, 1994) making it ideal for animal species identification. In this study, we explored three regions of the mitochondrial genome that have been previously characterized in Pacific salmonids (Shedlock et al., 1992; Domanico and Phillips, 1995; Parker and Kornfield, 1996). DNA sequencing of these regions provided an unambiguous way to determine species identity. Because high throughput sequencing can be prohibitively expensive for laboratories with limited facilities, restriction fragment length polymorphism (RFLP) analysis was also explored as an alternative for species identification. A previous study had established a species-specific polymerase chain reaction (PCR) test for Pacific Northwest salmon and coastal trout species (McKay et al., 1997). The PCR test is based on the initial amplification of an approximately 1000-bp fragment of the nuclear growth hormone 2 gene. The degraded state of the DNA isolated from bones recovered from scat has generally limited successful PCR to amplicons of 300 bp or less (data not shown). Furthermore, the amount of DNA isolated from bone fragments can be quite small; mtDNA is present in higher copy number per cell than is nuclear DNA. Thus, we considered mtDNA it to be a more

[1] http://www.nwr.noaa.gov/1salmon/salmesa/specprof.htm. [Accessed June 17, 2003.]

[2] Brown, R. F., and S. G. Kohlman. 1998. Trends in abundance and current status of the Pacific harbor seal (*Phoca vitulina richardsi*) in Oregon: 1977–1998. ODFW (Oregon Department of Fish and Wildlife) Wildlife Diversity Program Technical Report, 98-6-01, 16 p. [Available from ODFW, 7118 NE Vandenberg Ave. Corvallis, OR 97333.]

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appropriate target for our assay. We chose to explore smaller regions of the mitochondrial genome, including the d-loop (Shedlock et al., 1992), a portion of the 16s ribosomal gene (Parker and Kornfield, 1996), and a region spanning the cytochrome oxidase III, t-RNA glycine, and ND3 genes (hereafter, referred to as COIII/ND3) (Domanico and Phillips, 1995). Significant interspecific variation but not intraspecific variation was observed in the COIII/ND3 region among salmonid species in previous studies, making it a particularly good candidate region for the development of diagnostic markers (Domanico and Phillips, 1995).

In the first phase of the study, we developed and validated the genetic tools for species identification by using frozen or ethanol-preserved tissues collected from known species and populations. In the second phase, we applied these tools to the identification of bone remains from harbor seal scats collected at the Umpqua River (Oregon). A number of Pacific salmonid species are present in the Umpqua River but of particular concern were the searun cutthroat (*Oncorhynchus clarki*) that were listed as endangered under the ESA during 1996 (Johnson et al., 1999). Here we report the method associated with these two phases of the project. The salmonid bones that were identified genetically were incorporated into a larger study of the harbor seal diet and are reported in a companion paper (Orr et al., 2004).

Materials and methods

Salmonid tissue samples of known species have been collected over the past decade by geneticists from the Conservation Biology Molecular Genetics Laboratory (NOAA/NMFS/NWFSC) or generously donated by others (see "Acknowledgments" section) and maintained either frozen at –80°C or preserved in 95% ethanol. Reference populations were chosen to represent the geographic range of chinook salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), sockeye salmon (*O. nerka*), pink salmon (*O. gorbuscha*), chum salmon (*O. keta*), steelhead (*O. mykiss*), coastal cutthroat trout (*O. clarki clarki*), and Yellowstone cutthroat trout (*O. clarki bouvieri*) (collection information is listed in Table 1). Tissues were extracted with either a standard phenol and chloroform extraction (Sambrook et al., 1989) or by using the DNAeasy 96-well tissue kit (Qiagen, Valencia, CA), following the manufacturer's instruction for tissue preparations. PCR primers were either taken directly from the published studies or designed from the reported sequences (Table 2). All primers were cycled with 2.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.04 μM primers, 0.25 units of *Taq* DNA polymerase (Promega, Madison, WI), 20–40 ng of DNA, and cresol red loading buffer (final concentration 2% sucrose and 0.005% cresol red) for 35–45 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute.

A single individual of each salmonid species listed in Table 1 was sequenced for both the 16s rRNA and COIII/ND3 regions. For DNA sequencing, the PCR products were purified with an Ultrafree MC column (Millipore, Beverly, MA) and resuspended in 20 μL of sterile water. The purified product (1–10 μL depending on band intensity) was manually sequenced by using the USB ThermoSequenase cycle sequencing kit (Cleveland, OH), following the manufacturer's instructions. MACDNASIS (Miraibio Inc., Alameda, CA) and SEQUENCHER (Gene Codes Corp., Ann Arbor, MI) were used for sequence alignment and identification of diagnostic restriction enzyme cut sites.

RFLP analysis of the unpurified COIII/ND3 PCR product was performed in the presence of a cresol red loading buffer. Restriction digests were incubated for 6 to 12 hours at 37°C for *Dpn* II, *Sau* 96I, *Fok* I, *Ase* I, at 50° for *Apo* I, and at 60°C for *Bst* NI with the supplied buffers (NEB, Beverly, MA) and 1–5 units of enzyme. Restricted products were electrophoresed in a 4% 3:1 high-resolution and medium-resolution agarose gel (Continental Laboratory Products, San Diego, CA). DNA bands on the agarose gels were visualized with SYBR Gold, following the manufacturer's instructions (Molecular Probes, Eugene, OR).

Personnel from the National Marine Mammal Laboratory (NMML) collected and processed harbor seal scat samples from the Umpqua River (Orr et al., 2004). NMML researchers identified bone remains to either family or species level by using morphological characteristics of skeletal remains (Orr et al., 2004). From 39 harbor seal scats, 116 bones were identified morphologically to the genus *Oncorhynchus* and subjected to DNA analysis for species identification. For a positive DNA extraction control, we simulated digestion by treating coastal cutthroat bones (collected from Cowlitz Trout Hatchery, Winlock, WA) in a mixture of laboratory-grade trypsin (a digestive enzyme), baking soda, and water for 1 to 2 days. These trypsin-treated bones from a coastal cutthroat trout were used as positive DNA extraction and amplification control.

To prepare samples for DNA extraction, bones were soaked in 10% sodium hypochlorite for 10 minutes to destroy any contaminating DNA that may have adhered to the outside of the bone and were rinsed twice in sterile water. Bones ranged in weight from 0.1 to 105.6 mg and included teeth, vertebrae, gillrakers, radials, and bone fragments (hereafter, all bony parts and teeth will be referred to as "bone"). The bones were decalcified overnight in 0.5M EDTA solution (Hochmeister et al., 1991); fragile or small fragments were not decalcified. The EDTA was removed and the decalcified samples were extracted with the QIAamp tissue extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with the following modifications: 1) samples were proteinase K digested overnight or until completely digested; 2) 10 mg/μL yeast t-RNA carrier was added to the extractant before placement on the QIAQuick column; and 3) DNA was eluted in a reduced volume (50–100 μL) of buffer AE. Negative controls containing no tissue were simultaneously processed to verify that the extraction was free of contaminating DNA. The trypsin-treated coastal cutthroat bones were used as positive extraction and PCR controls.

Five to ten microliters of the extracted DNA were used in each amplification reaction. Amplification success was determined by electrophoresis through a 2% agarose gel followed by staining with ethidium bromide or the more sensitive SYBR Gold (Molecular Probes). Species identifi-

Table 1

Species, locations, and sample sizes (*n*) examined for RFLP analysis.

Species	Population	Location	*n*
Chinook	Walker Creek	Upper Frasier River, British Columbia	10
	Grovers Creek Hatchery	Puget Sound, Washington	12
	Lookingglass Hatchery	Snake River, Oregon	12
	Carson Hatchery	Columbia River, Washington	12
	Abernathy Hatchery.	Columbia River, Washington	11
	Upper Sacramento Mainstem	Sacramento River, California	10
Coho	Edison Creek	Oregon Coast	13
	Sandy River	Columbia River, Oregon	15
	North Fork Moclips River	Washington Coast	15
	Minter Creek Hatchery	Puget Sound, Washington	15
	Yakoun River	Queen Charlotte Island, British Columbia	7
Sockeye	Nehalem Ponds	Oregon Coast	4
	Redfish Lake	Snake River, Idaho	4
	Alturas Lake	Snake River, Idaho	2
	Ozette Lake	Washington Coast	14
	Lake Wenatchee	North Cascades,Washington	10
	Babine Lake	Central British Columbia	2
	Kamchatka River	Kamchatka Peninsula, Russia	9
Chum	Hamma Hamma River	Hood Canal, Washington	11
	Frosty Creek	Alaskan Peninsula	12
	Utka River	Chucotka Peninsula, Russia	9
	Miomote River	West Honshu, Japan	11
Pink	Nisqually River	South Puget Sound, Washington	6
	Snohomish River Even Year	North Puget Sound, Washington	12
	Skagit River	North Puget Sound, Washington	7
	Hood Canal Hatchery	Hood Canal, Washington	9
Steelhead	Gaviota Creek	South California Coast	4
	Coquille River	Oregon Coast	8
	Upper Tucannon River	Snake River, Washington	12
	Finney Creek	Puget Sound, Washington	12
	Quinault Hatchery	Washington Coast	12
	Tigil River	Kamchatka Peninsula, Russia	12
Cutthroat[1]	Alsea River	Oregon Coast	2
	Alsea Hatchery	Oregon Coast	3
	Duwamish River	Puget Sound Washington	12
	Yellowstone River	Yellowstone River, Montana	5

[1] Cutthroat trout from the Yellowstone River are a different subspecies (*O. clarki bouvieri*) from the Washington and Oregon coastal cutthroat trout (*O. clarki clarki*).

cation was accomplished by sequencing of either the d-loop or the COIII/ND3 region. RFLP analysis was performed as described above with the following modifications: *Bst* NI was excluded because it is redundant with *Dpn* II, the enzyme amount was reduced to 0.4–1.0 units per reaction, and incubation time did not exceed 2 hours. The COIII/ND3 primers are specific to the family Salmonidae. To test the possibility that the failure to obtain amplification with the COIII/ND3 primers was due to morphological misidentification of an *Oncorhynchus* species we used the 16s primers that are conserved across a broad set of taxa from Platyhelminthes through Chordata (Parker and Kornfield, 1996).

Results

The COIII/ND3 and 16s sequences were confirmed for all seven salmonid naturally present in the Pacific Northwest (Figs. 1 and 2) and deposited in Genbank (COIII/ND3: AF294827-AF294833; 16S: AF296341-AF296347). Two chinook salmon were sequenced representing two *Dpn* II

Table 2

Primer sequences, size of amplified product in base pairs, and references for mitochondrial loci used in this study.

Locus	Primer sequences (5′ to 3′)	Product size	Reference
d-loop	P2: tgt taa acc cct aaa cca g P4: gcc gaa tgt aaa gca tct ggt	230	Shedlock et al., 1992
COIII/ ND3	F: tta caa tcg ctg acg gcg R: gaa aga gat agt ggc tag tac tg	368	Domanico and Phillips, 1995
16sV	F: tac ata aca cga gaa gac c R: gtg att gcg ctg tta tcc	260	Parker and Kornfield, 1997

Table 3

Restriction fragment length polymorphisms of the cytochrome oxidase III and ND3 region digested with six restriction enzymes. The "A" haplotype does not cut with the enzyme, "B" cuts with the enzyme, and "C" cuts with the enzyme but at a different site than "B."

Species	*Dpn* II	*Sau* 96I	*Fok* I	*Ase* I	*Apo* I	*Bst* NI
Chinook	A/B[1]	B	B	A	A	A
Coho	A	A	B	A	A	A
Sockeye	A	A	A	A	C	B
Chum	A	A	A	B	C	A
Pink	C	A	A	B	C	C
Steelhead	A	A	A	B	B	A
Cutthroat	A	A	A	A	A	A

[1] Spring-running chinook from the Columbia and Snake Rivers were polymorphic for the *Dpn* II cut site. Spring chinook from Carson Hatchery (derived from the upper Columbia River spring-running ESU [evolutionary significant unit]) had the "A" haplotype at a frequency of 0.91 (n=12) and spring chinook from Lookingglass Hatchery (Snake River spring-summer-running ESU) had the "A" haplotype at a frequency of 0.83 (n=12). All other chinook samples from Table 1 were invariant for the "B" haplotype.

haplotypes (A and B) and their sequences are presented in Figure 1; the chinook salmon individuals were from the Upper Columbia River summer and fall ESU (Methow River, WA). A second intraspecific polymorphism in chinook salmon was observed at position 341 between our ND3 sequence and the published sequence (Domanico and Phillips, 1995) (Fig.1). Sufficient nucleotide variation exists in the d-loop (Shedlock et al., 1992) and in the COIII/ND3 region (Fig. 1) to distinguish among the salmon species by sequencing; both regions were used for bone identification.

Six restriction enzymes were selected from the COIII/ND3 sequence that appeared to distinguish among all the species (*Dpn* II, *Sau* 96I, *Fok* I, *Ase* I, *Apo* I, and *Bst* NI) (Fig. 1). The *Dpn* II and *Bst* NI cut patterns are redundant in that only one of these enzymes is required for species identification when used in conjunction with the other four enzymes (however, only *Dpn* II exhibits the intraspecific chinook polymorphism, see below). Haplotype patterns for all species are listed in Table 3. The haplotypes were scored with a simple alphabetic system: "A" was uncut (368 base-pair (bp) band) and "B" was cut (the size differed depending on enzyme). A few of the enzymes had an alternative cut site, and the resulting haplotype we labeled "C." The "B" haplotype produced by *Apo* I occurs in steelhead and the bands migrate at 300 and 68 bp, whereas the bands of the "C" haplotype in sockeye, chum, and pink salmon migrate at 250 and 118 bp. The enzyme *Bst* NI also has two cut patterns: the sockeye salmon "B" haplotype bands migrate at 282 and 87 bp and the "C" haplotype bands in pink salmon migrate at 271 and 98 bp. The *Dpn* II "B" haplotype in chinook salmon creates two fragments, 290 and 80 bp; the "C" haplotype in pink salmon creates three fragments, 292, 53, and 24 bp.

To confirm that the restriction enzyme polymorphisms were diagnostic within each species, we surveyed all seven Pacific salmon species representing multiple populations spanning a large geographic range (Table 1). No intraspecific polymorphisms were detected among populations with the exception of chinook salmon (Tables 1 and 3). A single intraspecific polymorphism was found with the *Dpn* II enzyme in chinook salmon lineages in the Columbia and Snake River basins (Tables 1 and 3). Chinook salmon from the Snake River spring-summer run (Lookingglass

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                              20    DpnII         40                  60
                                    ****
Chinook A : TTACAATCGCTGACGGCGTGTACGGCTCTACTTTCTTTGTCGCCACCGGATTCCATGGCC
Chinook B : ............................................................
Coho      : ...................A..........................A.............
Sockeye   : ...................A....................T.....G............T
Chum      : ...................A........C.................A........C....
Pink      : ...................A.....A..C...........A.....A.............
Steelhead : ...................A.......................T..A.............
Cutthroat : ...........................................T..A..T..........

                        DpnII 80     BstNI       100      ApoI/ Sau96I
                           ****      *****                  ************
Chinook A : TACACGTGATTATTGGCTCAACCTTTCTAGCCGTTTGCCTTCTGCGACAGGTCCAATACC
Chinook B : ................A...........................................
Coho      : .......A.....C.............................A.....A..T.......
Sockeye   : .......A.................C..G..............A.....AA.T.......
Chum      : .......A..C..C..............G..T...........A.....AA.T.......
Pink      : .......A..C..C............T.G.....C........A.....AA.T.......
Steelhead : .......A....................G..............A.....A..T.....T.
Cutthroat : ....T..A....................G..............A.....A..T.......

                  FokI       140                 160                 180
            **********
Chinook A : ACTTTACATCCGAACATCATTTTGGCTTTGAAGCTGCTGCTTGATATTGACACTTTGTAG
Chinook B : ............................................................
Coho      : ............................................................
Sockeye   : ..........T.......................C.........................
Chum      : .T..C.....T.......................C.........................
Pink      : ....C.....T..G....................C.........................
Steelhead : ..........T.............................C...................
Cutthroat : ..........T.................................................

                             200                 220 start tRNA glycine
                                                          -->
Chinook A : ACGTTGTGTGACTCTTCCTATACGTCTCTATTTACTGATGAGGCTCATAATCTTTCTAGT
Chinook B : ............................................................
Coho      : .......A....................................................
Sockeye   : ......................................................C.....
Chum      : .........................T..................................
Pink      : ............................................................
Steelhead : .......A..G.................................................
Cutthroat : .......A....................................................

            AseI             260        BSTNI    280           Start ND3
            ******                      *****                        -->
Chinook A : ATTAACACGTATAAGTGACTTCCAATCACCCGGTCTTGGTTAAAATCCAAGGAAAGATAA
Chinook B : ............................................................
Coho      : ......G.....................................................
Sockeye   : ..C..TGA....................................................
Chum      : .....TTA....................................................
Pink      : .....TTA...CG.................T.............................
Steelhead : .....T........................C.............................
Cutthroat : ............................................................

             ApoI           DpnII                 340                 360
             ******         ****
Chinook A : TGAACTTAATTACAACAATCATCACTATTACCATCACATTRTCCGCAGTACTAGCCACTA
Chinook B : ............................................................
Coho      : ............................C.........C.G..T...............G
Sockeye   : ........G.............................C.G...................
Chum      : ......................T.....C...........G...C...............
Pink      : .....C..........G.....T...............C.G...................
Steelhead : ....T.....C.............................A...................
Cutthroat : ............................C...........G...................


Chinook A : TTTCTTTC
Chinook B : ........
Coho      : ........
Sockeye   : ........
Chum      : .C......
Pink      : .C......
Steelhead : ........
Cutthroat : ........
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Figure 1

Aligned sequences of the 3′ region of the cytochrome oxidase III gene (COIII), the tRNA glycine gene, and the 5′ region of the ND3 gene for seven species of the genus *Oncorhynchus*. The cutthroat trout sequence is represented by the coastal cutthroat subspecies (*O. clarki clarki*). Chinook "A" refers to the "A" *Dpn* II haplotype; chinook "B" refers to the "B" *Dpn* II haplotype. Sequence identity relative to the chinook salmon "A" sequence is denoted by dots; nucleotide substitutions are indicated. The arrow at basepair (bp) 230 is the start of the tRNA glycine gene and the arrow at bp 300 is the start of the ND3 gene. Stars above the sequence correspond to restriction enzyme cut sites used in this study. At position 341 in chinook, the R represents an A or G.

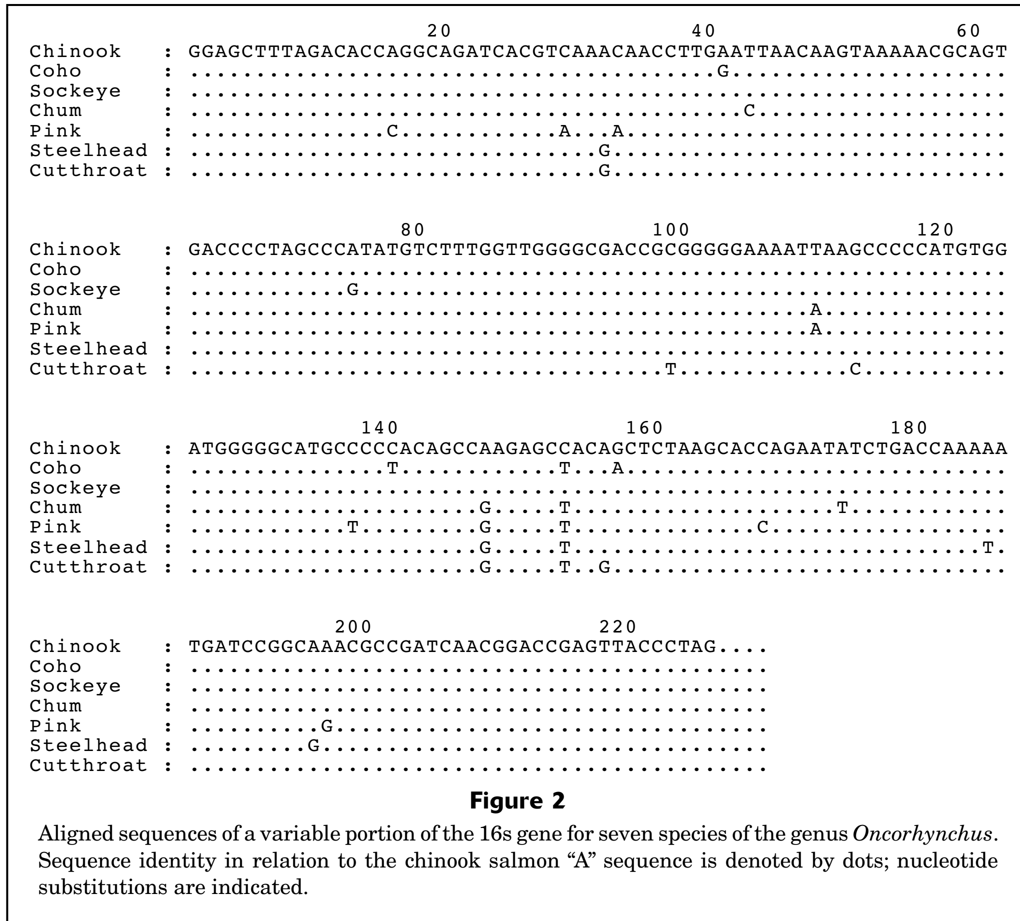

Figure 2

Aligned sequences of a variable portion of the 16s gene for seven species of the genus *Oncorhynchus*. Sequence identity in relation to the chinook salmon "A" sequence is denoted by dots; nucleotide substitutions are indicated.

Hatchery) and hatchery stocks descended from the Upper Columbia River spring run (Carson Hatchery) had the "A" (uncut) haplotype at a frequency of 83% and 91%, respectively, whereas those from the Lower Columbia River ESU were invariant for the "B" (cut) haplotype. The "B" haplotype was also invariant in the other lineages examined (Sacramento River, CA; Puget Sound, WA; and the Fraser River, BC). Despite this *Dpn* II polymorphism, the haplotype patterns were still chinook-specific.

Extractions from the trypsin-treated cutthroat trout bones, used as positive controls, were amplified consistently, but of the 116 salmonid bones from harbor seal scats, only 78 (67%) were amplified. Failed samples were repeated several times with all possible primer sets. Because each scat contained multiple bones, we were able to amplify bones representing 35 of the 39 scats (90%). The smallest bone we successfully amplified was a 0.2-mg tooth and the largest was a 21.8-mg vertebra. There did not appear to be a relationship between bone size and DNA extraction success; no significant difference in mean bone size was detected between 32 bones that either amplified or failed (P=0.280; unpaired t-test; SYSTAT 8.0 [Chicago, IL]). The bone samples that failed to amplify repeatedly were also tested by using the evolutionarily conserved 16s primers. Some samples were still refractory to PCR, indicating that the overall DNA quality or quantity was insufficient for this assay; however, those samples that did amplify were identified by sequencing as salmon. In an unrelated study using river otter bones (data not presented), one bone sample morphologically identified as salmonid yielded a sequence with 100% identity to the published 16s sequence available for Northern squawfish (*Ptychocheilus oregonensis*) (Simons and Mayden, 1998).

After verifying the specificity of the RFLP analysis for differentiating the Pacific salmon species, the assay was applied to the bone samples. Restriction enzyme digestion required some modification when applied to bone. On occasion, the restriction enzyme protocol developed for the fresh tissue resulted in degradation of the amplified bone PCR product. Enzyme amount and digestion times were scaled back for the analysis of the bone samples. The *Fok* I enzyme proved the most difficult for the bone samples, which was likely due to nonspecific restriction that occurs when the enzyme is present at a high concentration in relation to its target or if the reaction is allowed to digest for more than two hours. In some cases, only very weak amplification was achieved with the bone samples and it was difficult to get digestion without degradation. Although sequencing was the main technique used for bone identification, 23 bones in this study were identified by using the RFLP technique. Fourteen of these 23 bones were additionally confirmed by sequencing and the two techniques gave matching results.

Discussion

This study focused on the development of tools for the genetic identification of Pacific salmon skeletal remains recovered from harbor seal scats. These tools help to determine the diet of marine mammals and can also be used to address direct management questions regarding interspecific interactions in rivers such as the Umpqua River where salmonid species of concern (cutthroat trout) occur with protected marine mammal species. The harbor seal diet in the Umpqua River consisted of nonsalmonid fish and chinook, coho, and steelhead; no cutthroat trout were observed in the scat samples (Orr et al., 2004). The majority of salmonid species identifications were possible only by using genetic methods because very few otoliths were recovered in the Umpqua River scats. A number of other sites exist were this technology may also be applicable. In Hood Canal (WA) the summer chum salmon run is listed as threatened under the ESA. A report of seal diets in Hood Canal determined that 27% of the fish consumed by harbor seals were salmonids (Jeffries et al.[3]). The study used both bones and otoliths, but only 25% of the samples contained otoliths that allowed species-level identification. In the Alsea River (OR), coho salmon are listed as threatened. A report by Riemer et al.[4] indicated that 6% of fish consumed by pinnipeds in the Alsea River are salmonids; none of the salmonid remains were morphologically identifiable to species.

Extraction of DNA from bones can be done with a commercially available kit with minor modifications. In our study, only 67% of the bone DNA extracts could be amplified by PCR. PCR failure could be due to DNA degradation during the digestive process or to environmental exposure after defecation. However, multiple bones are often present in scats and we were able to amplify DNA from at least one bone representative from 35 out of the 39 scats examined. Sequencing or RFLP analyses of the COIII/ND3 locus are both viable methods of identifying the seven common *Oncorhynchus* species. This study used manual sequencing with radioactivity and we did have better results using this method compared to the RFLP method. A recently published study also identified restriction enzymes in the cytochrome B gene that distinguish among the salmonid species (Russell et al., 2000). The study reported diagnostic RFLP differences among these species but did not confirm the lack of intraspecific variation in a wide geographic survey of each species. The goal of the cytochrome B RFLP assay designed by Russell et al. (2000) was to identify salmon species found in processed food products but the primers may also prove useful in species identification of bone remains. The 16s primer set is also valuable for bones that are morphologically unidentifiable. However for salmonid species identification, the 16s region contains fewer diagnostic nucleotide substitutions in relation to the d-loop and the COIII/ND3 region. Overall, the techniques established here would be useful for further study of marine mammal diets and may have the potential for forensic application.

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