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Ancient DNA from Salmon Bone: a Preliminary Study

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In the Pacific Northwest of North America, archaeological salmonid remains have great potential to inform on issues of prehistoric cultural use, salmonid paleobiology and past environments. One hindrance to using prehistoric salmonid remains has been the gross level of taxonomic identification generally achieved. Species diagnostic elements are rarely found in archaeological contexts. Recent breakthroughs in ancient DNA research using the polymerase chain reaction (PCR) suggest a new way to obtain species-level and possibly finer taxonomic information from prehistoric samples. Salmonid-specific primers were developed to target short, taxonomically informative regions of mitochondrial DNA. PCR amplifications of DNA extracted from modern salmonid muscle and bone samples were successful, demonstrating the utility of the primers and the presence of DNA in salmonid bone. PCRs on extracts from archaeological and noncultural surface bone deposits, ranging in age from 9000 to 20 years old, also were successful, although retrievable DNA was more fragmented and degraded than in modern bone and muscle tissue. Direct sequencing of amplified products from one ancient sample confirmed that the product was from *Oncorhynchus* and tentatively suggested that the sample was from *O. nerka*.

Keywords: Ancient fish DNA, Archaeological salmon bone, Mitochondrial DNA, *Oncorhynchus*

INTRODUCTION

Salmonid remains are found in hundreds of archaeological sites from California to Alaska, on the coast and along rivers in the interior, dating to the last 10,000 years. Such remains have great potential to inform on issues of prehistoric cultural use, salmonid paleobiology, and past environments (Schalk, 1977; Butler, 1993; Chatters *et al.*, 1995; Matson and Coupland, 1995). However, a major hindrance to using archaeological salmonid remains to address such issues has been the gross level of taxonomic identifications generally achieved. In western North America, the dominant taxon in the family (in number of species and individuals) is *Oncorhynchus*, which is represented by seven species of salmon and trout. Unfortunately, based on skeletal morphology, only a few cranial elements are species diagnostic (Casteel, 1974; Gorschkov *et al.*, 1979) and these are recovered rarely from archaeological sites (probably because of preservation factors: Butler and Chatters, 1994). Archaeological

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deposits are dominated by postcranial elements, particularly vertebrae, and while these typically can be recognized as *Oncorhynchus* and thus tell us that salmon or trout were being captured and are present in a given drainage and time period, they could clearly tell us more about past human use patterns and environments if species-level identifications were possible.

Recent breakthroughs in ancient DNA research using the polymerase chain reaction (PCR) suggest a new way to obtain species-level and possibly finer taxonomic information from prehistoric samples (e.g. Pääbo, 1989). Given the limited work on ancient DNA in fish bone and the potential value of extracting DNA from prehistoric salmonid bone in particular, we endeavored to establish whether extractable amounts of DNA were present in salmon bony tissues and to what extent this DNA could be obtained from archaeological bone samples. To examine the variation in DNA quantity and quality across environments and time periods, we included samples from several depositional contexts across Pacific Northwest North America, and ages ranging from about 9000 to 20 years old. We report on the preliminary results from this work here.

RESULTS

PCR Amplification

Modern Muscle and Bone

PCR amplifications using the seven salmonid-specific primer pairs (Table I) were successful on modern salmon muscle tissue. Thus the samples served as positive controls during subsequent PCRs on modern and ancient bone. PCR amplifications using these primers also were successful on modern bone samples. Figure 1 shows the successful amplification of modern muscle and bone samples from *Oncorhynchus nerka* (sockeye salmon) and *O. mykiss* (rainbow trout), using six of the primer pairs. Similar results (not depicted) were obtained using bone samples from *O. keta* (chum).

Ancient Bone

PCR amplifications on ancient bone samples (Table II) using primers that targeted sequences between 209 and 231 bp long were not successful, even after removal of contaminants and optimization of reaction conditions. To determine

TABLE I Identification, gene region, targeted primer sequences and expected product size (in bp) for the primer pairs tested (all regions targeted are mitochondrial DNA)

Gene/region	Primer pair	Sequence (5′→3′)	Length (bp)	Reference[a]
ND3		ATGCGGATCCT(T/C)TTGAGCCGAAATCA ACGTGAATTCGTA(T/G)(A/G)(A/C)GTG(A/G)CTTCCAA		1
ND3		CCCTACGAATGTGGATT TCATAGATTAGGCCAAGAGTAAG	209	2
		CCCTATGAGTGCGGATT[b] see above	209	2
		GCAGTACTAGCCACT GATTAGAAAGAAGCGTAAGGAGAA	132	2
$tRNA^{Phe}$		GCTTTAGTTAAGCTACG TGTTAAACCCCTAAACCAG	231	3
Control region		TTCCTGTCAAACCCCTAAACCAGG AAAGTCAGGACCAAGCCTT	280	4
Control region		AATGTAGTAAGAACCGACCAAC TAGGAACCAAATGCCAGGAAT	119	

[a]1. McKay *et al.*, 1996; 2. This paper; 3. Nielsen *et al.*, 1994; 4. Shedlock *et al.*, 1992. [b]Specific to *O. keta*.

whether shorter DNA fragments were present in the bone samples, primers C-1 and C-2 were developed to target a sequence 119 bp in length (Table I) located in the control region. PCR amplifications were conducted using four ancient bone samples (ARC-7, ARC-9, ARC-10, ARC-14); initial PCR products were reamplified twice. PCR products of the same length as those produced by amplifications of modern bone were generated from the ancient samples (Fig. 2). DNA extraction blanks carried through three amplification cycles did not yield any products (Fig. 2). The extra band in ARC-7 is probably an artifact due to the repetitive PCR amplifications.

Although we were able to successfully extract DNA from several of the ancient bone samples, failure to amplify regions of DNA greater than 120 bp suggests that the DNA is somewhat degraded and fragmented.

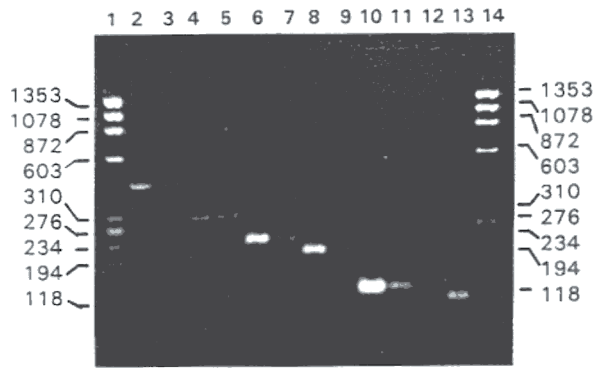

FIGURE 1 PCR amplification of modern salmonid muscle and bone tissues using salmonid-specific primers. Lanes: 1 and 14, ϕX174 digested with *Hae*III (band sizes shown in bp); 2, muscle DNA amplified with the Arg and Gly primers; 3, bone DNA, Arg and Gly; 4, muscle, F+ and 12S1−; 5, bone, F+ and 12S1−; 6, muscle, S-phe and P2; 7, bone, S-phe and P2; 8, muscle, V-1 and N-1; 9, bone, V-1 and N-1; 10, muscle, T-2 and V-3; 11, bone, T-2 and V-3; 12, muscle, C-1 and C-2; 13, bone, C-1 and C-2. Tissues from *Oncorhynchus nerka* (96-12) except sample amplified in lane 2, which is from *O. mykiss* (96-9).

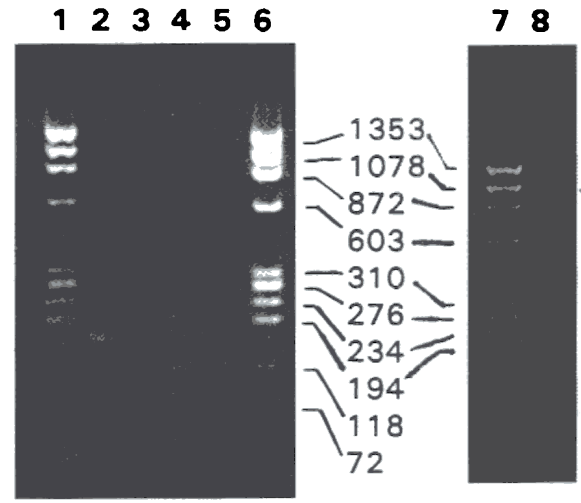

FIGURE 2 PCR amplification of ancient DNA extracts from salmonid remains using the C-1 and C-2 primers. Lanes: 1, 6 and 7, ϕX174 digested with *Hae*III (band sizes shown in bp); 2, ARC-7; 3, ARC-9; 4, ARC-10; 5, ARC-14; 8, DNA extraction blank carried through three amplification cycles showing no product. See Table II for additional information about samples.

TABLE II Description of archaeological salmonid specimens used in study

Catalog number	Weight (g)	Site	Environment	Age (BP)
ARC-2		45CL1	Alluvial	
ARC-3		45CL1	Alluvial	
ARC-4		45CL1	Alluvial	
ARC-5		45CL1	Alluvial	
ARC-6		35CO5	Alluvial	
ARC-7		35CO5	Alluvial	
ARC-8		35WS8	Eolian	
ARC-9		35WS8	Eolian	
ARC-10		Point bar	Surface; alluvial	
ARC-11		45DO372	Eolian and alluvial	
ARC-14		Point bar	Surface; alluvial	

All bony elements used were vertebrae, except ARC-14, which was a dentary. Weights shown are bone powder used for DNA extraction.

DNA Sequencing

The 119 bp region amplified using C-1 and C-2 primers was direct sequenced and approximately 85 bp could be reliably aligned in all of the samples (Fig. 3). Our sequences derived from modern muscle tissue from *O. tshawytscha* and *O. nerka* were identical to previously reported sequences (Shedlock *et al.*, 1992) (Fig. 3).

Results from the direct sequencing of PCR products from the ancient samples were mixed. Background signal tended to be higher than normal, especially in the older samples, resulting in frequent ambiguous base calling. Sequences were obtained from ARC-7, ARC-9 and ARC-10, but only results from ARC-10 could be reliably aligned with the modern sequences (Fig. 3). Importantly, similarity between the ARC-10 sequence and the known sequences from *Oncorhynchus* (Fig. 3) supports the claim that the amplified ARC-10 product is from *Oncorhynchus* and not an exogenous source. Furthermore, comparison of the ARC-10 sample to the *Oncorhynchus* species sequences allows for tentative species identification of the ancient sample as *O. nerka*. As shown in

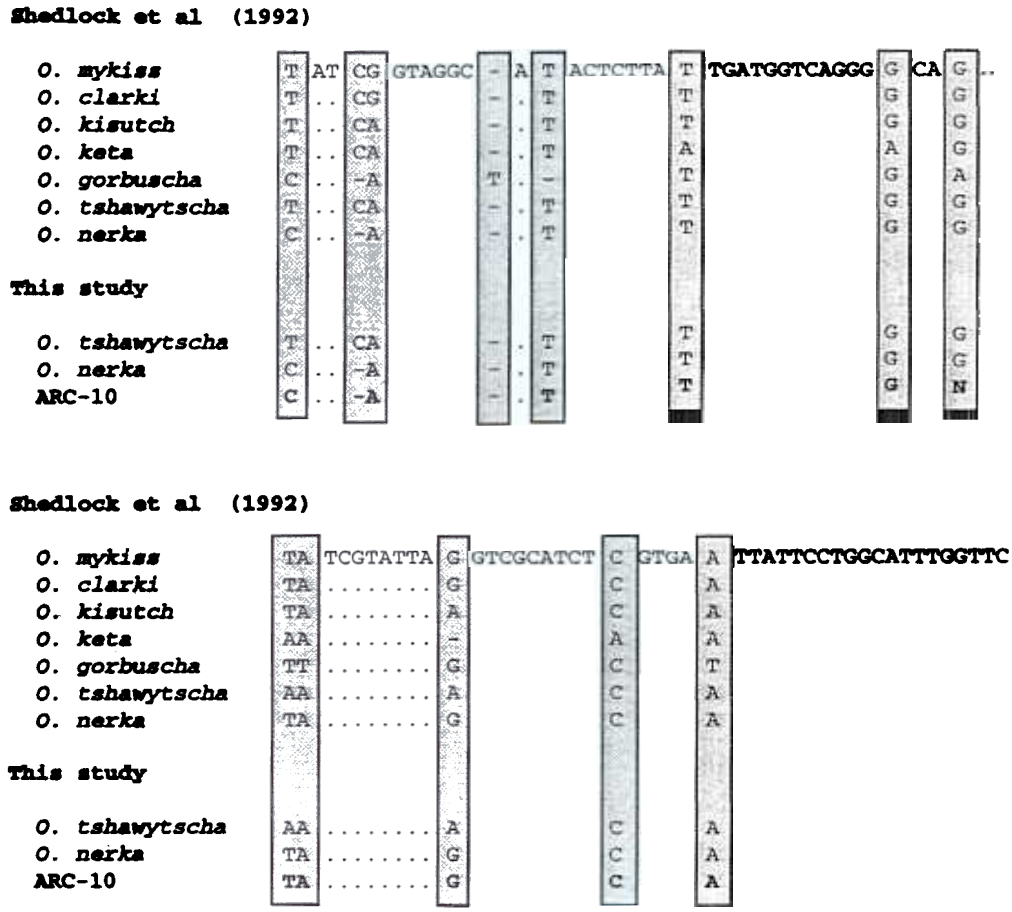

FIGURE 3 Comparison of aligned sequences of the control region of salmonid mtDNAs from modern and ancient samples using C-1 primer. Identity is indicated with dots; gaps in sequence shown by dashes; ambiguity in base assignment is designated with the letter 'N'. Sequences from modern salmonid muscle tissue obtained in this study are from 96-4 (*O. tshawytscha*) and 96-12 (*O. nerka*). Region depicted corresponds to sites 375–462 in the control region obtained by Shedlock *et al.* (1992). Three species included in Shedlock *et al.* (1992) not shown here (*O. masou*, *Salmo salar* and *Thymallus arcticus*).

the shaded areas of Fig. 3, the ARC-10 sequence differs from the *O. mykiss*, *O. clarki* and *O. kisutch* sequences at three locations, from *O. gorbuscha* and *O. tshawytscha* at four locations, and from *O. keta* at seven locations. The ARC-10 sequence is identical to that derived from *O. nerka*, which suggests the ancient sample is from this species.

Contamination

A primary concern of ancient DNA research is controlling for contamination (Austin *et al.*, 1997). As noted elsewhere in this paper, a number of precautions were taken to prevent contamination of the ancient samples. DNA extraction blanks carried through PCR amplifications never yielded any products. We note as well that the lack of amplification of large products from the ancient samples indirectly argues against contamination by modern salmonid DNA. Ancient DNA is typically much more degraded and fragmented than that from modern tissues. Others have noted (e.g. Fox, 1996; Hardy *et al.*, 1997) that longer sequences can be targeted and amplified more frequently in modern samples than in ancient ones. Our study successfully amplified DNA sequences between 200 and 350 bp long from modern muscle and bone tissue. Yet for the ancient sources, only sequences less than 120 bp long were successfully amplified. Therefore, our results are fully consistent with an ancient origin for the DNA from the archaeological and premodern samples. Finally, the overall similarity in the sequences provided by the ancient sample ARC-10 and known sequences from *Oncorhynchus* indicates the amplified products are from a salmonid and not an exogenous source.

DISCUSSION

Compared to other vertebrates, particularly human and nonhuman mammals, ancient DNA in fish bone has been little studied. Indeed, bibliographic searches and direct enquiries yielded no publications demonstrating the presence of ancient DNA in fish bone. Anecdotal evidence profferred by colleagues in biology and paleontology provided doubts about whether fish bone even contained DNA. With these concerns in mind, the primary questions our study posed were simple: do fish bones, specifically from salmonids, contain DNA and are extractable quantities of DNA present in archaeological bone samples? Our studies of modern salmon demonstrate that DNA is present in bony tissue. Our research on pre-modern bone samples also showed that ancient DNA does survive in such samples; degradation of the DNA, however, is much greater than that found in the modern bone samples.

Significantly, the ancient DNA described here was recovered from salmonid bone samples ranging in age from 9000–8000 (ARC-9) to 500 years (ARC-7) to about 20 years old (ARC-10, ARC-14) (Table II). The bone samples came from widely varying depositional environments, including deeply buried eolian sediments from the semi-arid shrub steppe vegetation zone of eastern Oregon (ARC-9), to buried alluvium in the temperate forest zone of western Oregon (ARC-7), to the relatively recent surface collected sample (ARC-10, ARC-14) from the temperate forest of western Washington. While the sample sizes we studied are admittedly small, these results suggest that preservation of ancient salmonid DNA is not contingent on age *per se* or general depositional environment, but may have more to do with very local burial conditions within a site.

Documenting the presence and recovery of DNA in salmonid bones, including very old ones, is important and clearly a prerequisite to establishing a research program that applies techniques of molecular biology to archaeological fish bone samples. However, in order for our results to be useful to studies of salmonid paleobiology or prehistoric human fisheries, species-level or perhaps finer taxonomic identification of ancient DNA samples must be obtained. Species specific

variation exists in the short portion of the control region we amplified; sequence data we obtained from ancient sample ARC-10 is identical to the sequence from *O. nerka*, which suggests the ancient sample is from this species. Definitive species identification, however, will require additional regions of amplified DNA to independently corroborate identifications.

Recent records of species abundance and distribution support this taxonomic assignment. ARC-10 is from a naturally produced surface accumulation of salmon remains on a point bar in the Cedar River; the bones and teeth are from spawned-out salmon carcasses that rafted onto the bar between 12 and 20 years ago (Butler, 1993). The dominant *Oncorhynchus* species in the Cedar River system is *O. nerka*, which, during the 1980s, had spawning populations averaging 282,000 individuals/year and represents the largest run of the species in the continental United States. During the fall of 1985, one of us (V.L.B.) observed *O. nerka* spawning in the river next to the point bar. Only negligible quantities of other salmonids (*O. tshawytscha*, *O. kisutch*, and *O. mykiss*) occupy the river system. Thus, based on modern species distributions and the context of ARC-10, we would expect that the fish bone is from *O. nerka*. This prediction was met with our sequence data, which shows sequences from ARC-10 and *O. nerka* are in complete agreement.

We are currently testing the utility of additional primer pairs directed towards variable regions in the mitochondrial (mt) DNA genome. Krings *et al.* (1997) recently demonstrated the success of targeting multiple small regions of mtDNA and using cloning in conjunction with sequencing to produce longer regions of DNA for phylogenetic purposes; we anticipate these methods will be useful in our future research on ancient salmon DNA.

The archaeological salmonid record has enormous potential to address questions of salmonid paleobiology and prehistoric human subsistence patterns. Unfortunately, given traditional approaches to taxonomic identification of archaeological salmonid remains that rely on skeletal morphology and the scarcity of elements in archaeological contexts that are species diagnostic, that potential has not been realized. Our work, that documents the survival of ancient salmonid DNA from multiple time periods and depositional settings, suggests that molecular approaches to study of archaeological bone may extend our knowledge of prehistoric salmonids much beyond traditional approaches to faunal analysis.

MATERIALS AND METHODS

Because of the recognized pitfalls associated with ancient DNA studies, several protocols were established to minimize the possibility of contamination (based on Hagelberg, 1994; Handt *et al.*, 1994; Taylor, 1996; Austin *et al.*, 1997): (1) modern tissue and ancient samples were processed in separate laboratories; (2) dilute sodium hypochlorite was used to rinse off work areas and clean tools (drill parts) between sample processing; (3) salmon-specific primers were used to target DNA molecules specific to the sample (which would limit the extent to which exogenous DNA is amplified); (4) positive and negative controls were included during the PCR experiments; and (5) aerosol resistant pipette tips were used to aliquot ingredients.

Specimens

The archaeological sites that provided the salmonid specimens represent several depositional environments (alluvial, eolian, deeply buried, surface; moist and semi-arid) and ages (9000 to 20 years) (Table II, Fig. 4). A total of 11 specimens from four archaeological sites and one very recent (20 years and younger) natural deposit of salmon remains was included in the study. Preservation conditions appeared good in the buried deposits, indicated by the abundance of faunal remains and plant tissues and other organics that were recovered. We focused our study on vertebrae

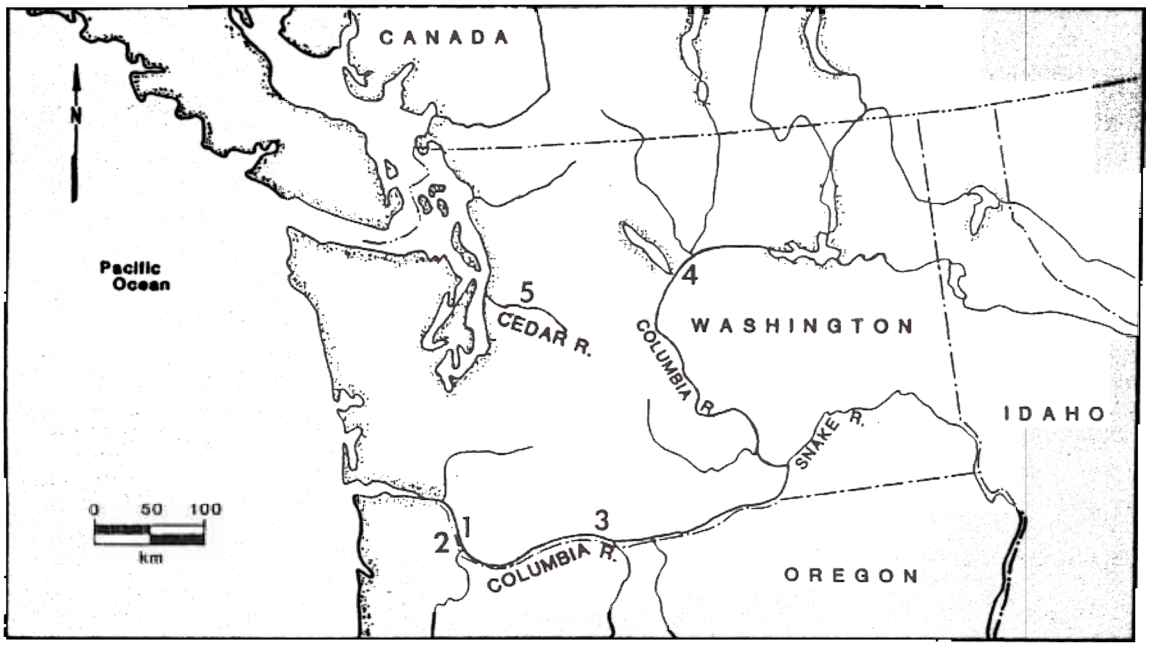

FIGURE 4 Location of sites that provided ancient salmonid DNA samples. 1, 45CL1; 2, 35CO5; 3, 35WS8; 4, 45DO372; 5, point bar.

because of the element's abundance in regional archaeological sites, its relatively large size, and recognition as belonging to a salmonid species. Further, to maximize the amount of bone available for DNA extraction, we selected large vertebrae (>10 mm diameter) from the available assemblages. Because vertebrae at the recent surface accumulation site were quite small, however, we also separately processed a dentary (lower jaw bone with teeth, ARC-14) to ensure that sufficient quantity of bone was available.

Muscle and bone tissues from modern fish carcasses were also included in the study to document the utility of the salmonid-specific primers on control tissues, to establish that the DNA extraction protocols used for mammal bone were suitable for fish, particularly salmon bone, and to provide positive controls during the PCR experiments. Modern tissues of muscle and bone were obtained from salmonid carcasses representing the seven extant species of *Oncorhynchus* that inhabit rivers draining to the Pacific Ocean (Table III). Tissues from selected species (96-4, 96-9, 96-12) were included in this preliminary study.

Bone Processing

Vertebrae from the modern samples were cut away from the fish's trunk and muscle and connective tissue were removed using a scalpel. The partially cleaned vertebrae were boiled for several minutes to loosen residual soft tissue, which was removed with forceps and scalpel. The outer surface of the vertebrae was sanded off using a dremel tool that had been fitted with an aluminium oxide-coated drill bit. The vertebrae were then soaked in a dilute bleach solution (sodium hypochlorite), for 30 min, then put through two rinses of sterilized distilled water. The vertebrae were then placed in a drying oven (80°C) for >1 h.

The outer surface of the archaeological bone samples was removed using a dremel tool with an aluminium oxide-coated drill bit. The vertebrae were then soaked briefly in bleach, then soaked

TABLE III Description of modern salmonid samples included in this study

Species	Catalog number	Collection location (State)
Oncorhynchus kisutch	96-2	Cascade Hatchery, Columbia R. (OR)
O. tshawytscha	96-3	Round Butte Hatchery, Deschutes R. (OR)
O. tshawytscha	96-4	Bonneville Hatchery, Columbia R. (OR)
O. clarki	96-5	Alsea Hatchery, Alsea R. (OR)
O. clarki	96-6	Leaburg Hatchery, MacKenzie R. (OR)
O. mykiss	96-7	Big Creek Hatchery, Columbia R. (OR)
O. mykiss	96-8	Round Butte Hatchery, Deschutes R. (OR)
O. mykiss	96-9	Oak Springs Hatchery, Deschutes R. (OR)
O. gorbuscha	96-10	Auke Bay (AK)
O. keta	96-11	Auke Bay (AK)
O. nerka	96-12	Lake Washington (WA)

and rinsed in sterilized distilled water, covered and air dried. Both the modern and ancient bone samples were reduced to a fine powder using a dremel tool equipped with a diamond-coated drill bit. Typically, the modern and ancient samples provided between 0.3 and 0.4 g of bone powder.

DNA Extraction

Muscle

Muscle tissue from the modern carcasses was removed from the midline of the fish close to the vertebrae. DNA was extracted by initially digesting approximately 250 g of muscle in 500 µl extraction buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA, 10 mM NaCl, 1% SDS, 8 mg ml^{-1} dithiothreitol, and 0.4 mg ml^{-1} proteinase K) at 36°C for 12 h, and then phenol extracted (Hillis *et al.*, 1996). DNA was precipitated by the addition of 2 vols absolute ethanol at 4°C, washed in 70% ethanol, resuspended in 250 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and stored at −20°C.

Ancient and Modern Bone

To initially digest the bone samples, 5 ml 0.5 M EDTA, 250 µl 10% SDS, and 25 µl proteinase K (100 mg ml^{-1}) was added to the bone powder and incubated at 37°C for at least 2 h. The mixture was then centrifuged and DNA was extracted from the supernatant using guanidinium thiocyanate (Elu-Quik Hi-Volume Genomic Kit, Schleicher & Schuell). Extracted DNA from both muscle and bone was stored in 250–500 µl TBE (0.89 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA) at −20°C.

Additional Processing of DNA

Although PCR amplifications utilizing DNA extracted from modern muscle and bone using the above method were successful, initial attempts to amplify the DNA in the ancient samples were not. Contaminants that interfere with PCR are often present in bones, especially archaeological and paleontological samples (Höss and Pääbo, 1993; Hänni *et al.*, 1995) and therefore further manipulation of the extracted DNA was attempted to concentrate and cleanup the DNA. To concentrate the extracted DNA, 0.1 vol. of 3 M sodium acetate and 0.6 vols. of cold absolute ethanol was added to each sample. Samples were mixed thoroughly and left at −20°C overnight. DNA was precipitated by centrifugation, the pellet was washed with 70% ethanol and then resuspended in 50 µl TE.

Several attempts at PCR amplification of these concentrated samples were not successful. Inability to successfully amplify samples containing equal volumes of either modern muscle or bone and ancient bone DNA revealed the presence of contaminants in the ancient bone DNA. Centricon-100 spin columns (Amicon) were used to try to remove the inhibitors, but PCR using

combined modern DNA and ancient bone samples still was not successful. Samples were further treated with hexadecyltrimethyl-ammonium bromide (CTAB), a detergent which has been used successfully to eliminate carbohydrates, phenolics, and other contaminants often present (Fain *et al.*, 1992; Hillis *et al.*, 1996). PCR experiments on the CTAB-treated DNA extracts combined with modern muscle DNA samples were successful, indicating that the CTAB removed the inhibiting agents.

PCR Amplification

PCR amplifications using primer pairs shown in Table I were carried out in 50 µl volumes containing 10 × *Taq* DNA polymerase buffer (Promega: 500 mM KCl, 100 mM Tris-HCl pH 8.8, 15 mM $MgCl_2$, 1% Triton X-100), 50 µg μl^{-1} BSA, 0.2 mM each dNTP, 1.5 units *Taq* polymerase (Promega), 1 mM of each primer and 1–3 µl of DNA template. PCR reactions were conducted in a Thermolyne Amplitron (Dubuque, Iowa) and several variations in both the annealing temperature and the number of cycles were used in an attempt to optimize reaction conditions for specific samples. The standard conditions were: 93°C for 30 s; 45–55°C for 60 s; and 72°C for 90 s for 30–45 cycles. For ancient samples, PCR products from initial reactions were reamplified one or two times to provide sufficient amounts of PCR product.

DNA products were visualized on a 2.5–3.5% agarose gel, using 1 × TAE buffer (40 mM Tris-acetate pH 7.6, 1 mM EDTA) and stained with ethidium bromide; results were viewed under UV irradiation.

We used both published salmonid-specific primers and designed some ourselves based on published gene sequences to amplify segments of the mtDNA genome (Table I). Our work focused on mtDNA because its high copy number favors its survival in decayed tissues. Attempts to amplify the 351 bp ND3 gene segment (McKay *et al.*, 1996), as well as PCRs with three primer pairs that targeted gene segments between 209 and 280 bp long (Table I) using modern bone DNA extracts were successful. PCRs conducted on the ancient bone samples using primers that targeted sequences between 209 and 231 bp long were not successful, even after removing contaminants and optimization of reaction conditions. To determine whether shorter DNA fragments were present in the bone samples, we developed primers (C-1, C-2) to target a 119 bp sequence found in the control region (Table I). These primers were tested on four ancient samples (ARC-7, ARC-9, ARC-10, and ARC-14) and modern muscle and bone tissue.

DNA Sequencing

Direct sequencing of the 119 bp region using the C-1 and C-2 primers was carried out to confirm that the amplified products were in fact from *Oncorhynchus* and to tentatively identify which *Oncorhynchus* species was represented in the ancient samples. Theoretically, species identification using the amplified product was possible because research (Shedlock *et al.*, 1992) has showed that species specific variation exists in the targeted gene fragment.

Prior to sequencing, unincorporated nucleotides and other reaction components were removed from the PCR products using Centricon-50 spin tubes (Amicon). Sequencing was conducted using a Perkin-Elmer ABI 373 automated DNA sequencer and the DyeDeoxy Cycling Sequencing reaction (Perkin-Elmer). Sequences were obtained using both the forward and reverse primers (C-1 and C-2, respectively). DNA amplification products from muscle tissue from two species of *Oncorhynchus* were direct sequenced (*O. tshawytscha* and *O. nerka*) and sequencing was attempted on ancient products ARC-7, ARC-9, and ARC-10. Since amplification of DNA from ARC-7 produced two bands, the PCR product of the appropriate size was purified from the ARC-7 sample using gel purification prior to sequencing. Resulting sequence data were compared to previously published sequences (Shedlock *et al.*, 1992)

for the seven extant species of *Oncorhynchus* in the region.

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