

# DNA species identification of archaeological salmon bone from the Pacific Northwest Coast of North America

Dongya Y. Yang<sup>a\*</sup>, Aubrey Cannon<sup>b</sup>, Shelley R. Saunders<sup>b</sup>

<sup>a</sup>*Ancient DNA Laboratory, Department of Archaeology, Simon Fraser University, 8888 University Drive, Burnaby BC, Canada V5A 1S6*

<sup>b</sup>*Department of Anthropology, McMaster University, Hamilton ON, Canada L8S 4L9*

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## Abstract

This paper reports on the development and application of methods for using DNA analysis for species identification of archaeological salmon bone. Short fragments (less than 300 bp) of mitochondrial DNA from the control region (D-loop) and cytochrome B (CytB) gene were targeted for amplification using the polymerase chain reaction (PCR) technique. The method was used on more than 20 salmon bone samples (dated 7000 to 2000 BP) from the site of Namu on the central coast of British Columbia. Four species: coho, sockeye, pink and chum salmon were identified from the samples. The results are considered valid since systematic contaminations were not detected, multiple species and multiple DNA haplotypes of the same species were identified from the same set of bone samples, and the identified species are consistent with those inferred from other lines of evidence. The results demonstrate the applicability of the ancient DNA technique to species identification of even single salmon vertebrae from archaeological sites in the Pacific Northwest of North America.

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## 1. Introduction

Species level identification of ancient salmon bone is important for a wide range of issues of concern to Pacific Northwest archaeologists, including the season of site occupation [8], the overall intensity and value of fisheries, variability over time due to environmental change [10] or possible resource depression [6], and unequal access to specific fisheries both within [4] and between communities [17]. Accurate and precise salmon species identification, however, is normally impossible due to the lack of specific morphological variation among skeletal remains, particularly the vertebrae, which are the majority of most assemblages [8,13].

Ancient DNA analysis [5,26,33,38] provides an alternative to skeletal species identification of salmon remains [7]. A variety of faunal species have been identified using the ancient DNA technique [3,29–32],

but, to date, there has been only one reported attempt to use this method for the species identification of Pacific salmon [7]. Our study successfully extracted DNA from ancient salmon vertebrae dating to 7000–2000 BP. We developed sensitive PCR methods to target on degraded DNA samples, and applied the technique in the species identification of salmon remains from the site of Namu, on the central coast of British Columbia (Fig. 1).

## 2. Materials and methods

### 2.1. Archaeological salmon bones

We used archaeological salmon bone recovered in excavations conducted at Namu by Roy L. Carlson of Simon Fraser University in 1994 [11,14]. Extensive radiocarbon dating based on these and past excavations indicate Namu was continuously occupied for the past 11,000 years [14]. Analysis of faunal remains which represent the last 7000 years of site occupation shows the salmon fishery was a critical component of the subsistence economy throughout this time [9,14]. An earlier

\* Corresponding author. Tel.: +1-604-291-4651; fax: +1-604-291-5666

E-mail address: donyang@sfu.ca (D.Y. Yang).

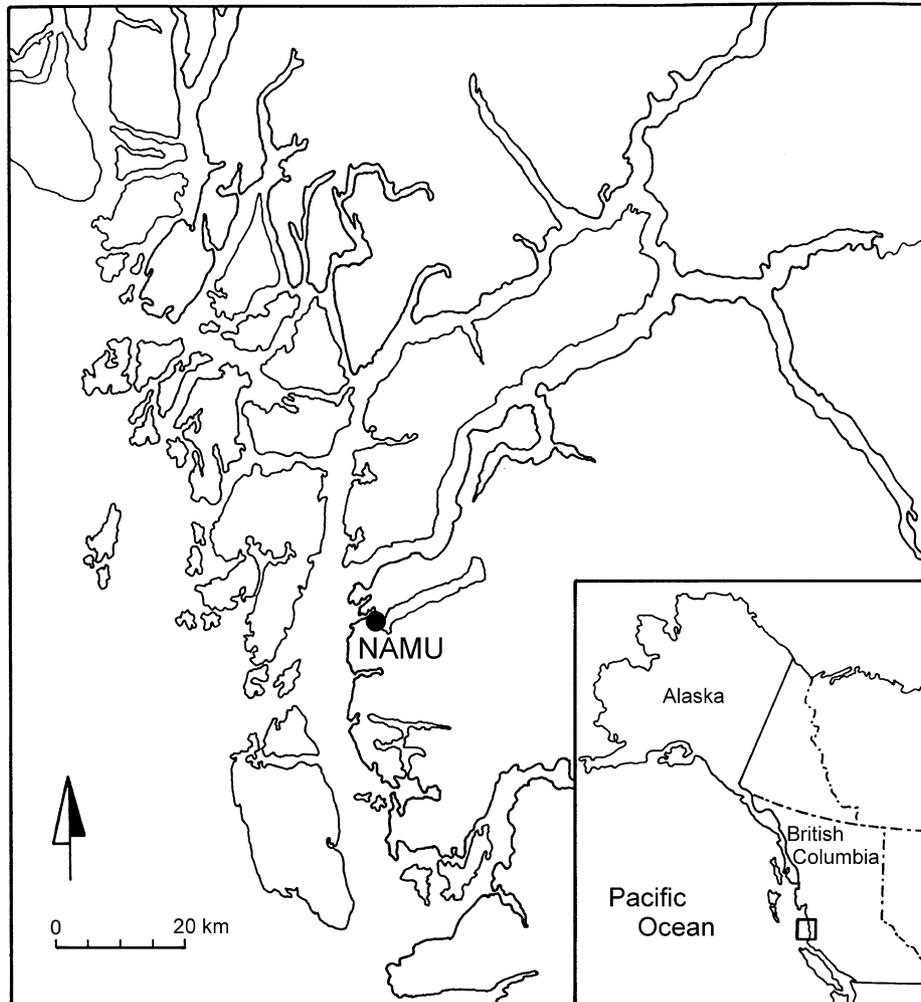


Fig. 1. Location of the Namu site on the central coast of British Columbia.

attempt to establish the probable species composition of the Namu salmon assemblage, based on radiographic analysis, had suggested a range of possible species, but was unable to establish definitively the precise focus of the fishery [8].

For our initial efforts to establish a method for ancient DNA identification, we selected 22 vertebrae from a  $2 \times 2$  m unit excavated in 1994 in the river mouth area of the site [14]. Six vertebrae from deposits 70–80 cm in depth, dating to ca. 2500 BP, were chosen in 2000 and 2001 for initial experimentation in the extraction and amplification of DNA from single (extract 1 and 4) and double vertebrae (extract 2 and 3) (Table 1). The use of two vertebrae was planned to increase the chance for successful retrieval of DNA from salmon remains. These DNA samples were re-amplified along with the DNA extracted in 2002 from a larger set of vertebrae from the same excavation unit, but from a wider range of depths and ages (Table 1). Morphologically, most salmon vertebrae used in this study were in good state of preservation (Fig. 2). A double-blind test

[43] was used in the analysis. Bone samples were made available for the study, but information on possible species identification based on morphological and other archaeological evidence was not initially provided. Some vertebrae were measured to record sizes (Table 1).

Along with salmon bones, a dog bone and a deer bone sample from the same unit and dating to ca. 2500 BP were processed to estimate the state of DNA preservation at the site. Studies have demonstrated that DNA can be well preserved in ancient mammalian bones [22], but less is known about DNA preservation in ancient fish bones. Positive recovery of DNA from the dog or deer bone would indicate the potential for DNA preservation in salmon bone. Approximately 1 g of bone was taken from a dog vertebra and from a cortical portion of a fragmentary deer long bone.

## 2.2. Bone decontamination and DNA extraction

An initial preliminary test conducted to extract DNA from salmon vertebrae showed that adequate DNA

Table 1  
Salmon vertebra samples and DNA species identification

Extract	CytB	Smc1/Smc2	Smc3/Smc4	Smc7/Smc8	Diameter (cm)	Age (y BP)	Deposit level (cm)
1	Sockeye	Sockeye	Sockeye	Sockeye	n/m 1 medi. vert.	4000–2000	70–80
2	Chum	Chum	Chum	Chum	n/m 2 large vert.	4000–2000	70–80
3	<i>Sockeye</i>	<i>Coho/sockeye</i>	<i>Sockeye</i>	<i>Sockeye</i>	n/m 2 small vert.	4000–2000	70–80
4	Chum	Chum	Chum	Chum	n/m 1 large vert.	4000–2000	70–80
5							Blank Extract 2001
6	Chum	Chum	Chum	Chum	1.15	4000–2000	110–120
7	Chum	Chum	Chum	Chum	1.04	4000–2000	110–120
8	Sockeye	Sockeye	Sockeye	Sockeye	0.73	4000–2000	110–120
9	Sockeye	Sockeye	Sockeye	Sockeye	0.73	4000–2000	110–120
10	Chum	Chum	Chum	Chum	1.04	5000–4000	170–180
11	Coho	Coho	Coho	Coho	1.04	5000–4000	170–180
12	Pink	Pink	Pink	Pink	0.83	5000–4000	170–180
13	Pink	Pink	Pink	Pink	0.73	5000–4000	170–180
14	Coho	Coho	Coho	Coho	1.04	7000–6000	320–330
15	Sockeye	Sockeye	Sockeye	Sockeye	1.04	7000–6000	320–330
16	Pink	Pink	Pink	Pink	0.83	7000–6000	320–330
17	Pink	Pink	Pink	Pink	0.83	7000–6000	320–330
18	Chum	Chum	Chum	n/a	1.12	4000–2000	70–80
19	Chum	Chum	n/a	Chum	0.98	4000–2000	70–80
20	Sockeye	Sockeye	Sockeye	Sockeye	0.81	4000–2000	70–80
21	Sockeye	Sockeye	n/a	Sockeye	0.79	4000–2000	70–80
22							Blank Extract 2002
23							Blank Extract 2002

Note: Extract: DNA samples from individual DNA extractions; Code: sample codes for double-blinded test and for internal lab use; n/m: measurement was not made; n/a: sequence was not available; Deposit: depth below the surface; Age: all dates are in calibrated years BP (see [14]:84).

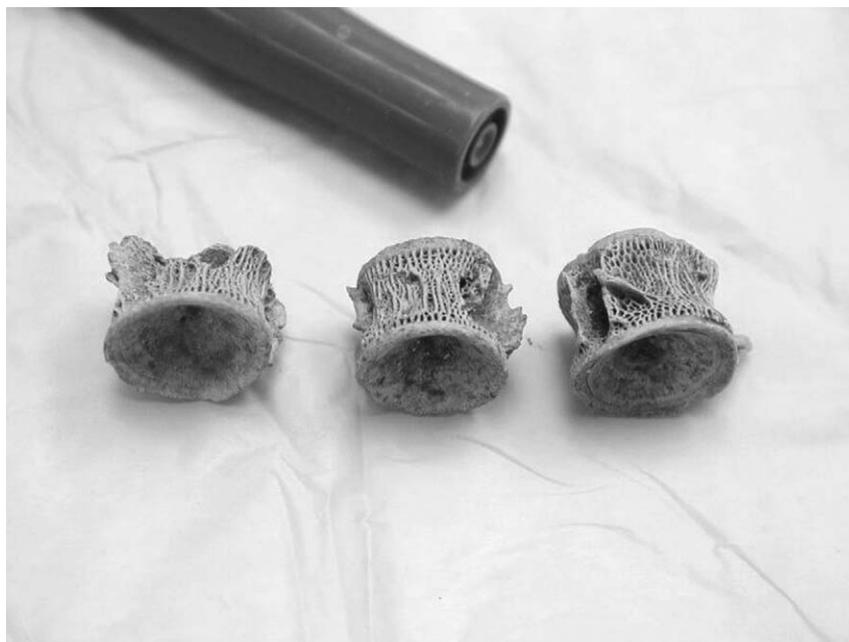


Fig. 2. Salmon vertebrae in good state of preservation.

could be retrieved from a single vertebra. This single vertebra approach was then adopted to ensure that only one species should show up from one DNA sample.

We developed a new protocol to combine various chemical and physical methods for decontamination. Sandpapers were first used to remove possible contaminated surfaces. Due to their complex and porous

Table 2  
Primers for PCR amplifications

Primer	Sequence (5'-3')	bp	Amplicon
Smc1 (F)	AAT GTA GTA AGA ACC GAC CAA C (313–334)	22	Smc1+Smc2 (113 bp)
Smc2 (R)	TAG GAA CCA AAT GCC AGG AAT (425–405)	21	
Smc3 (F)	ACT TGG ATA TCA AGT GCA TAA GGT (680–703)	24	Smc3+Smc4 (135 bp)
Smc4 (R)	CCT GGT TTA GGG GTT TAA CAG G (814–793)	22	
Smc7 (F)	AAC CCC TAA ACC AGG AAG TCT CAA (800–823)	24	Smc7+Smc8 (249 bp)
Smc8 (R)	CGT CTT AAC AGC TTC AGT GTT ATG CT (45–20)	26	
CytB5 (F)	AAA ATC GCT AAT GAC GCA CTA GTC GA (34–59)	26	CytB5+CytB6 (168 bp)
CytB6 (R)	GCA GAC AGA GGA AAA AGC TGT TGA (224–201)	24	

Note: The position was numbered according to the D-loop (tRNA-Phe for Smc8) and the CytB of mitochondrial genome (NC\_001717) of rainbow trout (*O. mykiss*). F is for the forward and R for the reverse primer.

surfaces, however, salmon bone could not be adequately polished, making this method of physical decontamination less effective. Chemical decontaminates were therefore employed for thorough decontamination. Bone samples were first soaked in a 10% bleach solution in a new test tube (15 ml or 50 ml) for 10 min; and then rinsed twice with ultra-pure water to remove any residue of bleach. After the water was poured out, an appropriate amount of 1N HCl (approximately 2 ml for each salmon sample, and more for the dog and deer bone samples) was added into the tube to submerge the sample. Almost immediately, the HCl solution was poured out, and then an appropriate amount of 1N NaOH was added to submerge bone samples again. The NaOH was added to neutralize the residue of HCl that had migrated deep into the bone sample. At the same time NaOH can destroy more contaminant DNA on the surface or within shallow layers of bone. After immediate removal of NaOH, ultra-pure water was used to rinse the sample for two or three times before the sample was put on a clean weighing tray into a crosslinker for UV irradiation for 20 min. A second irradiation was added for the other side of the samples for another 20 min.

A modified silica-spin column method was used to extract DNA from bone samples [42]. Bone samples were ground into fine powders using a liquid nitrogen-grinding mill. The powders were incubated overnight with lysis buffer (0.5 M EDTA pH 8.0, 0.5% SDS and 0.5 mg/ml proteinase K) in a rotating hybridization oven at 50 °C. After centrifugation, 1.5–2.0 ml of supernatant was transferred to a Centricon™ 30 column (Millipore, Billerica, MA, USA) for concentrating DNA samples. Approximately 100–150 µl of supernatant was passed through QIAquick columns (Qiagen, Hilden, Germany) for purifying DNA [42]. Finally, 100–120 µl of DNA was eluted from the column for PCR amplification.

### 2.3. PCR primer design and PCR amplifications

Four sets of primers were designed for amplifying three control region (D-loop) fragments and one

cytochrome B (CytB) fragment. We compiled the D-loop and CytB sequences of the species of the genus *Oncorhynchus* and Atlantic salmon (*Salmo salar*) that were available in GenBank (www.ncbi.nlm.nih.gov) and in published sources [36]. Primers were designed on conserved regions of DNA sequences while allowing variations to exist between the two primer sites. Since there was no report to indicate clearly that DNA could be well preserved in fish bones thousands of years old, a conservative approach was first taken to design primers to target on less than 200 bp fragments (Table 2). Primers Smc7 and Smc8 were later added to amplify for a longer fragment (249 bp) after positive results were obtained from the first sets of primers (Table 2).

Two salmon species were chosen as the source of positive DNA samples for PCR optimization. Samples of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) were obtained from a supermarket in Hamilton, Ontario and both muscle and skin tissues were used to extract DNA using QIAamp™ (Qiagen, Hilden, Germany). The extracted DNA samples were quantified using UV spectrophotometry at 260 nm and a series of diluted DNA samples were then used to optimize PCR conditions. Strong amplifications were achieved at 40 cycles of amplification when templates even dropped to below 10 picograms of DNA samples. Expected specific bands of amplification were observed on both Atlantic salmon and rainbow trout (data not shown), indicating the capability of these primers to amplify other salmon species. The PCR products were sequenced and the results matched those of respective species from GenBank.

Four PCR amplifications were conducted for each sample to obtain DNA sequences of three fragments for the D-loop and one for the CytB (Table 2). PCR amplifications were performed using the GeneAmp™ Thermocycler Model 2400 (Perkin-Elmer, Norwalk CT) in a 50 µl reaction volume containing 50 mM KCl and 10 mM Tris-HCl, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 1.0 mg/ml BSA, 0.3 µM each primer, 5 µl DNA sample and 1.25 U AmpliTaq Gold™. PCR was run at 55–60

cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. To effectively activate the polymerase, an initial denaturing was performed at 95 °C for 12 min. Five µl of PCR product was separated by electrophoresis onto an 8% non-denaturing polyacrylamide gel. PCR products were purified using a QIAquick™ purification kit and were submitted for direct sequencing. The sequencing was carried out on an ABI 3100 at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

The amplification of a mtDNA D-loop fragment of dog was carried out using a pair of published primers MitH52 (forward) 5-TCGAGGCATGGTGATTAAG-3 and MitL63 (reverse) 5-ACCCCTACATTCATATAT TGAAT-3 [25]. The amplified products are 239 bp in length (from np1030 to np1268 according to the D-loop from the dog genome sequence NC\_002008). No DNA was previously extracted from ancient dog bones and no modern dog DNA was handled in the lab. Along with the dog DNA sample, the deer DNA sample was also run for amplifications using the dog primers to determine the specificity of the dog primers and to serve as another blank control for contamination detection.

#### 2.4. Sequence analysis and species identification

All sequencing electropherograms were carefully examined to retrieve reliable sequences. A part or the whole sequence was discarded if signals were too weak or multiple mixed peaks were present. Consequently, DNA was re-amplified and re-sequenced for the discarded whole sequence. The obtained individual DNA sequences were first compared against GenBank through the BLAST search ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)) to examine whether the sequence would match any unexpected sequences or species. Detailed comparisons were then conducted through building a multiple alignment with all reference sequences of related species using ClustalW ([www.ebi.ac.uk/clustalw](http://www.ebi.ac.uk/clustalw)). Reference DNA sequences of all Pacific salmon available in GenBank were collected with the assumption that ancient DNA should come from one of these species or closely related species. For the D-loop, the reference sequences were mainly from the published data [36]. If an ancient DNA sequence showed too much variation, phylogenetic analysis would be conducted using MEGA2 [27].

For each DNA fragment, a tentative species designation could be made if an ancient sequence was found to be identical or very similar to a particular reference sequence. However, final species determination was made only if all available fragments pointed to the same species. It was hoped this approach could overcome the possible problem of random mismatch caused by the shortness of amplified ancient DNA sequences.

#### 2.5. Contamination controls

All DNA extractions and PCR setups were carried out in a dedicated ancient DNA laboratory and all necessary precautions were taken for effective contamination controls [16,39]. Among these, all bone samples and extraction reagents were exposed to UV irradiation in an ultraviolet crosslinker for 20–30 min. Multiple blanks and negative controls were set up along with all ancient DNA samples.

Work on modern DNA samples of Atlantic salmon and rainbow trout was carried out in another DNA laboratory located in a separate building. The modern DNA work was done one year before the ancient DNA samples were extracted. Modern DNA was initially used as positive controls for amplifications along with ancient DNA samples set up at a different room, but was left out when PCR optimization was achieved.

The use of Atlantic salmon and rainbow trout as modern samples for optimization and positive controls also makes any contamination from this source immediately evident. For example, if an Atlantic salmon DNA sequence was obtained from ancient Pacific salmon remains, it must be due to contamination since Pacific salmon and Atlantic salmon DNA are so different from each other.

### 3. Results

#### 3.1. PCR amplifications

Strong PCR amplifications were observed in all salmon samples when PCR was run over 55 cycles while negative controls and blank extracts remained negative, indicating, at this level, no systematic contamination occurred with the DNA extraction and the PCR setup (Table 1, Fig. 4). However, weak or negative PCR amplifications were encountered from the first set of ancient salmon DNA samples during the optimization stage and from the Smc7–Smc8 amplification of longer fragments (for example, a failure of sequencing for extract 18, see Table 1), indicating DNA degradation. The amplification of salmon DNA samples using primer Smc3 and Smc4 showed that sample 12–34, 13–34, 16–34 and 17–34 seemed to have longer PCR amplicons (Fig. 3).

When using the dog-specific primers, only the dog DNA sample yielded a strong PCR amplification, one that was not observed in the deer DNA and blank extract samples (Fig. 4), indicating the effectiveness of contamination controls and adequate specificity of dog-specific primers for PCR amplification.

#### 3.2. Direct sequencing

For most samples, PCR products generated clear sequencing results, though some showed “messy”

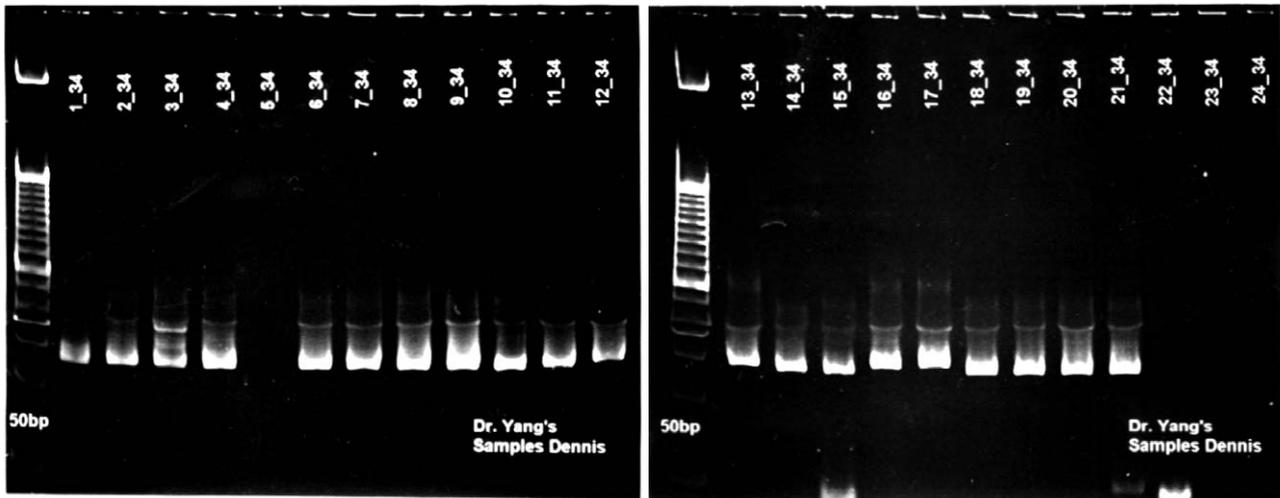


Fig. 3. Electrophoresis of PCR products of salmon DNA samples using primer Smc3 and Smc4 (50 bp ladder; 5–34, 22–34 and 23–34: blank extracts; 24–34: PCR negative control). Samples were named using extract number (Table 1) and primers Smc3/Smc4 (Table 2).



Fig. 4. Electrophoresis of PCR products using dog-specific primers (Marker: 50 bp ladder; N1 and N2: PCR negative controls; Deer: deer DNA; Dog: dog DNA; Blank: blank extract).

sequences at the beginning. This messy start is likely due to incomplete removal of primer dimmer during the purification of PCR products or the presence of non-specific PCR products, resulting in mixed and confusing base callings. All sequences were therefore double-checked on electrophoregrams. Figs. 5–7 show parts of corrected/conformed sequences for the fragments by Smc3/Smc4, CytB5/CytB6 and Smc7/Smc8, respectively. Due to an unexpected “accident” during the purification of PCR products, PCR samples of extracts 19 and 21 were lost and no samples were sent out for direct sequencing of the Smc3–Smc4 fragments (Fig. 5).

It should be noted that among all the specimens, extracts 2 and 3 were extracted from two vertebrae (instead of from one). This is reflected in sequencing electrophoregrams that have mixed base positions (Fig. 5).

### 3.3. BLAST search and multiple sequence alignment

BLAST searches showed that all DNA from the bone samples matched, more or less, those of Pacific salmon. Although a BLAST search of the amplified dog sequence found a great number of dog DNA sequences, none were identical matches, with at least one base pair difference. Obviously, BLAST searches indicated that the DNA sequence was from dog (*Canis familiaris*).

When all the ancient salmon DNA and reference sequences are aligned together, variations can clearly be seen among individual species and specimens (Figs. 5–7). As expected, individual variations were observed in amplified D-loop fragments.

### 3.4. Species identification

Four different salmon species were identified from the ancient DNA samples. Based on the similarity between the ancient DNA and reference sequences, species designations were made, which are better illustrated through multiple alignments (Figs. 5–7). In total, there were seven chum salmon (*O. keta*), four pink salmon (*O. gorbuscha*), two coho salmon (*O. kisutch*) and seven sockeye salmon (*O. nerka*).

Intra-species sequence variations of the ancient DNA samples were observed only in the D-loop fragments (Figs. 5 and 7). For primers Smc1 and Smc2, intra-species variations were only observed in chum, among which there are two sequences that seemed to have one base different from the others. However, due to an unreliable sequence reading at the start of each sequence, it is difficult to determine if any variation was missed for the region (data not shown). However, for primers Smc3 and Smc4, among four pink salmon

	10	20	30	40	50	60	70	80	90		
RainbowT.	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Chum	TATTTCTTC	ACAGATACCT	A-AGATCGCT	CCC-GGCTTT	T-----	--GCGGGTA	AACCCCTTA	CCCCCTAA-	GCTGAAAGAT	CCTTATGTT	85
2-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
4-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
6-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
7-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
10-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
18-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
Sockeye	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
1-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
3-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
8-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
9-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
15-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
20-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	85
Pink	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	88
12-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	96
13-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	96
16-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	96
17-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	96
Coho	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	84
11-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	84
14-34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	84
Chinook	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	84
Atlantic	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	82

Fig. 5. Part of the amplified D-loop sequence by Smc3 and Smc4. All reference sequences were from Shedlock et al. [36] except rainbow trout (NC\_001717) and Atlantic salmon (NC\_001960). Dots indicate identical bases with rainbow trout. Samples were named using extract number (Table 1) and primers Smc3/Smc4 (Table 2).

	10	20	30	40	50	60	70	80	90		
RainbowT.	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Chum	CTTCTAATAT	CTCAGTCTGG	TGAAACTTTG	GCTCACTACT	AGGCCTATGT	TTAGCTACCC	AAATTCTTAC	CGGGCTCTTC	CTAGCCATGC	ACTATACCT	99
2-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
4-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
6-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
7-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
10-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
18-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
19-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
Sockeye	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
1-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
3-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
8-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
9-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
15-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
20-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
21-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
Pink	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
12-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
13-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
16-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
17-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
Coho	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
11-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
14-56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
Chinook	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99
Atlantic	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	99

Fig. 6. Part of the amplified D-loop sequence by CytB5 and CytB6. Reference sequences were retrieved from GenBank: rainbow trout (NC\_001717), chum (AJ314561), coho (AJ314563), sockeye (AJ314568), pink (AJ314562), chinook (AJ314566) and Atlantic salmon (NC\_001960). Dots indicate identical bases with rainbow trout. Samples were named using extract number (Table 1) and primers CytB5/CytB6 (Table 2).

samples, there were two unique DNA sequences with one base pair difference (Fig. 5). For primers Smc7 and Smc8, chum salmon showed three haplotypes of six samples, and pink salmon showed three haplotypes of four sequences (Fig. 7).

A striking variation was observed between the ancient DNA and the reference sequences for pink salmon. A 10 bp insertion (TCCCGGCTTC) appeared in all pink salmon samples (12–34, 13–34, 16–34 and 17–34) (Fig. 5). This insertion should be responsible for the appearance of longer fragments on the electrophoresis (Fig. 3). BLAST searches did not reveal any sequences

that share this unique insertion. As a result, species identity could not be easily made for these Smc3–Smc4 sequences. Even a phylogenetic analysis using MEGA2 failed to reveal their closeness to pink salmon. Fortunately, CytB, Smc1–Smc2 and Smc7–Smc8 sequences clearly pointed to pink salmon. If the insertion was removed from the Smc3–Smc4 sequence, then pink salmon could be easily identified (Fig. 5).

DNA samples extracted from two vertebrae proved to be problematic for species identification. For extract 3, many Ns were observed in the Smc3–Smc4 sequence (Fig. 5) though a sockeye species could still probably

	10	20	30	40	50	60	70	80	90	100					
RainbowT.	AAA-TCAGCA	--ATATTTT	TTTTA-TACA	TTAATAAACT	TTTA-TGCAC	TTT--AGCAT	TTGGCACCGA	CAGCGCTGTA	ATGCGTAC-A	CTTCCATAAA	91				
Chum	.....G T.A.	.....-A	.....G	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	95				
2-78	.....G T.A.	.....-G	.....G	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	95				
4-78	.....G T.A.	.....-A	.....G	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	95				
6-78	.....G T.A.	.....-A	.....G	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	95				
7-78	.....G T.A.	.....-A	.....G	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	95				
10-78	.....G T.A.	.....-A	.....G	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	95				
19-78	.....G T.A.	.....-A	.....G	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	95				
Sockeye	.....A	.....G T.T.	.....-A	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
1-78	.....A	.....G T.T.	.....-A	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
3-78	.....A	.....G T.T.	.....-A	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
8-78	.....A	.....G T.T.	.....-A	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
9-78	.....A	.....G T.T.	.....-A	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
15-78	.....A	.....G T.T.	.....-A	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
20-78	.....A	.....G T.T.	.....-A	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
21-78	.....A	.....G T.T.	.....-A	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
Pink	.....A	.....G T.A.	.....-G	.....G	.....GA	.....AT	.....A	.....T	.....A	.....T	95				
12-78	.....G T.A.	.....-A	.....T	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
13-78	.....G T.A.	.....-A	.....T	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	95				
16-78	.....G T.A.	.....-A	.....T	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	96				
17-78	.....G T.A.	.....-A	.....T	.....GA	.....AT	.....A	.....T	.....A	.....T	.....T	95				
Coho	.....T.G	.....A	.....GG	.....TT	.....A	.....T	.....A	.....T	.....T	.....T	96				
11-78	.....T.G	.....A	.....GG	.....TT	.....A	.....T	.....A	.....T	.....T	.....T	96				
14-78	.....T.G	.....A	.....GG	.....TT	.....A	.....T	.....A	.....T	.....T	.....T	96				
Chinook	.....T.G	.....A	.....G	.....AT	.....C	.....T	.....A	.....T	.....T	.....T	95				
Atlantic	.....G CTA	.....C	.....A	.....TGCCA	.....T	.....AT	.....C	.....TA	.....A	.....C	.....TG	.....C	.....TT	.....T	98

	110	120	130	140	150	160	170	180	190															
RainbowT.	TAAAGTATAC	ATTAATAAAC	TTTTCGATCC	ACTTTG-TAG	CA--CCTAGC	ACCAACAACG	CTGTTATCAA	TGCCATTTC-	-CACGCAC-A	G-CCCGCC	182													
Chum	.....TC	.....CAC	.....TC	.....G	.....A	.....G	.....C	.....T	.....C	.....C	.....T	184												
2-78	.....TC	.....CAC	.....TC	.....G	.....A	.....G	.....C	.....T	.....C	.....C	.....T	185												
4-78	.....TC	.....TAC	.....TC	.....G	.....A	.....G	.....C	.....T	.....C	.....C	.....T	185												
6-78	.....TC	.....TAC	.....TC	.....G	.....A	.....G	.....C	.....T	.....C	.....C	.....T	185												
7-78	.....TC	.....TAC	.....TC	.....G	.....A	.....G	.....C	.....T	.....C	.....C	.....T	185												
10-78	.....TC	.....CAC	.....TC	.....G	.....A	.....G	.....C	.....T	.....C	.....C	.....T	185												
19-78	.....TC	.....CAC	.....TC	.....G	.....A	.....G	.....C	.....T	.....C	.....C	.....T	185												
Sockeye	.....C	.....C	.....T	.....GGCAT	.....C	.....G	.....A	.....C	.....GG	.....CA	.....TA	.....C	.....TG	.....A	.....T	187								
1-78	.....C	.....C	.....T	.....GGCAT	.....C	.....G	.....A	.....C	.....GG	.....CA	.....TA	.....C	.....TG	.....A	.....T	187								
3-78	.....C	.....C	.....T	.....GGCAT	.....C	.....G	.....A	.....C	.....GG	.....CA	.....TA	.....C	.....TG	.....A	.....T	187								
8-78	.....C	.....C	.....T	.....GGCAT	.....C	.....G	.....A	.....C	.....GG	.....CA	.....TA	.....C	.....TG	.....A	.....T	187								
9-78	.....C	.....C	.....T	.....GGCAT	.....C	.....G	.....A	.....C	.....GG	.....CA	.....TA	.....C	.....TG	.....A	.....T	187								
15-78	.....C	.....C	.....T	.....GGCAT	.....C	.....G	.....A	.....C	.....GG	.....CA	.....TA	.....C	.....TG	.....A	.....T	187								
20-78	.....C	.....C	.....T	.....GGCAT	.....C	.....G	.....A	.....C	.....GG	.....CA	.....TA	.....C	.....TG	.....A	.....T	187								
21-78	.....C	.....C	.....T	.....GGCAT	.....C	.....G	.....A	.....C	.....GG	.....CA	.....TA	.....C	.....TG	.....A	.....T	187								
Pink	.....GC	.....T	.....AC	.....C	.....G	.....CG	.....G	.....T	.....C	.....G	.....C	.....T	.....ACT	.....C	.....T	184								
12-78	.....GC	.....T	.....AC	.....C	.....G	.....CG	.....G	.....T	.....C	.....G	.....C	.....T	.....ACT	.....C	.....T	186								
13-78	.....GC	.....T	.....AC	.....C	.....G	.....CG	.....G	.....T	.....C	.....G	.....C	.....T	.....ACT	.....C	.....T	185								
16-78	.....GC	.....T	.....AC	.....C	.....G	.....CG	.....G	.....T	.....C	.....G	.....C	.....T	.....ACT	.....C	.....T	186								
17-78	.....GC	.....T	.....AC	.....C	.....G	.....CG	.....G	.....T	.....C	.....G	.....C	.....T	.....ACT	.....C	.....T	185								
Coho	.....C	.....AGC	.....C	.....ACG	.....GGA	.....G	.....A	.....AG	.....CA	.....T	.....C	.....C	.....GC	.....A	.....T	186								
11-78	.....C	.....AGC	.....C	.....ACG	.....GGA	.....G	.....A	.....AG	.....CA	.....T	.....C	.....C	.....GC	.....A	.....T	187								
14-78	.....C	.....AGC	.....C	.....ACG	.....GGA	.....G	.....A	.....AG	.....CA	.....T	.....C	.....C	.....GC	.....A	.....T	187								
Chinook	.....C	.....C	.....C	.....AGC	.....G	.....T	.....A	.....T	.....C	.....C	.....T	.....A	.....A	.....T	158									
Atlantic	.....G	.....C	.....CTA	.....A	.....A	.....T	.....A	.....A	.....ACTAGT	.....A	.....AG	.....C	.....CG	.....C	.....T	.....G	.....AA	.....AT	.....TA	.....TAA	.....G	.....TA	.....T	188

Fig. 7. Part of the amplified D-loop sequence by Smc7 and Smc8. All reference sequences were from Shedlock et al. [36] except rainbow trout (NC\_001717) and Atlantic salmon (NC\_001960). Dots indicate identical bases with rainbow trout. Samples were named using extract number (Table 1) and primers Smc7/Smc8 (Table 2).

be determined. These were confirmed by the CytB and Smc7–Smc8 sequences (Figs. 6 and 7). Since a more informative segment for sockeye and coho separation is missing for the Smc1–Smc2 sequence, reliable species identification could not be made for extract 3 (data not shown). The confusion must derive from the possibility that the two vertebrae used in this extract represent two individuals or even species, resulting in mixed peaks and an unidentifiable sequence. This example clearly illustrates the importance of extracting DNA from single vertebrae for salmon species identification.

## 4. Discussion

### 4.1. Authenticity of the ancient DNA results

Any possibility of contamination from modern sources must be excluded before amplified DNA can be accepted as authentic ancient DNA, and then used to address any specific research questions [16,18,23,39]. Challenges for such authentication were anticipated in advance in this study, and an appropriate research design was therefore developed to provide the data needed for authentication.

#### 4.1.1. Contamination controls

All necessary precautions were taken to minimize the risk of contamination. A dedicated ancient DNA laboratory was used in this study, and strict contamination controls were exercised throughout all stages of pre-PCR laboratory work. No blank extracts or PCR negative controls show any positive PCR amplifications, indicating the absence of contamination. Modern DNA samples from related species were handled with great care. Physically, the modern DNA work was carried out in another building, and temporally, it was done one year before the ancient DNA work was conducted.

#### 4.1.2. Decontamination measures

This study employed probably the most rigorous decontamination measures developed to date in an attempt to remove all possible contaminant DNA on the salmon remains. For faunal ancient DNA studies, researchers themselves may not be a source of contamination if primers are intentionally designed to avoid homologous sequences with humans [41]. Ancient faunal remains, however, can be contaminated through cross-contamination between modern reference specimens and ancient remains, and/or between ancient remains when archaeologists use comparative specimens in their identifications and analyses. The archaeological specimens used in this study were never brought into contact with modern reference specimens. Although they were potentially in contact with one another, they were randomly selected from bags containing hundreds or thousands of vertebrae, and were unlikely to be systematically con-

taminated as a result. Nevertheless, these factors were not used to “waive” the need for vigorous decontamination. Instead, a seemingly over-destructive protocol was used in this study. It may further damage minute amounts of preserved DNA in ancient remains, but the possible negative impacts obviously did not diminish the potential for extracting adequate ancient DNA templates from the remains, since a high success rate of amplification was achieved.

#### 4.1.3. DNA sequence analysis

As a bonus benefit of working on ancient DNA samples of multiple species, the obtained DNA sequences themselves are indicative of the absence of systematic contamination in this study. Firstly, the sequences made valid sense in terms of phylogenetic relationship since all were found to derive from Pacific salmon. Secondly, four different species were identified from the same set of bone samples, and nucleotide differences were observed in the D-loop fragments from individual samples with the same species identity. If contaminations took place at the species level, contaminant DNA must have come from at least four different sources (i.e. each salmon species); and a higher number of sources would be needed if individual sequences (haplotypes) with D-loop variation were taken into consideration. Thirdly, if contamination took place, there should be more mixed peaks present on the electrophoregrams, as observed in the DNA sample extracted from two vertebrae (data not shown), however, no DNA samples from single vertebrae showed distinctive mixed peaks. Fourthly, the amplifications of three different mtDNA fragments all pointed to the same species for almost all samples. Lastly, no DNA sequences of Atlantic salmon and rainbow trout were observed in ancient salmon bones, demonstrating the absence of contamination from these modern DNA samples.

The low intra-specific variation observed from the ancient salmon bones is consistent with studies of modern salmon DNA [40]. In this study, all four species showed no intra-specific variation for the CytB fragment (Fig. 6). For the D-loop fragments; only chum sequences indicated two possible haplotypes (with one single base difference) for the Smc1–Smc2 fragment (data not shown), and only pink salmon showed two haplotypes (with one single base difference) for the Smc3–Smc4 sequence (Fig. 5). Although a slightly higher variation appeared in the Smc7–Smc8 fragment, only chum and pink salmon showed three haplotypes (with one or two-base difference) for each species (Fig. 7). If the time depth of 5000 years is taken into consideration, the variations are quite low. The low variation may reflect the recent postglacial recolonization of salmon in the Pacific Northwest of North America. Park et al.’s study [34] on the D-loop of modern chum DNA samples from

various geographic locations on the northern Pacific Rim and Smith et al.'s research [37] on modern coho salmon of numerous populations from Alaska to California demonstrate the existence of low intra-specific variation of the D-loop. Smith et al.'s sequence data of about 300 coho individuals show no intra-specific variation for the fragment that we also amplified using Smc1 and Smc2 from the Namu samples. The low variation of the D-loop can also be confirmed by the comparison of different segments of the mitochondrial genome [15]. Churikov et al.'s data on multiple Pacific salmon species show that it is the NADH-dehydrogenase genes instead of the D-loop that are the most variable regions of the mitochondrial genome. After studying inter-specific variation of mtDNA D-loop of Pacific salmon, Shedlock et al. suggest a higher sequence variation exists in the left domain of the D-loop [36], which was amplified through Smc7 and Smc8 in this study. Our results are consistent with this suggestion.

#### 4.1.4. DNA preservation

If the positive amplification and sequencing of dog DNA cannot be used to directly authenticate ancient DNA in this study, it clearly does not invalidate such consideration. DNA seems to be well preserved in ancient salmon and dog remains from this Pacific Northwest Coast site. Generally, this geographic environment cannot be considered ideal for DNA preservation since soil acidity and water saturation can be expected in this coastal region. These factors can accelerate the degradation of ancient DNA [28]. The absence of more severe degradation of these DNA samples could be attributed to the fact that the bone samples were preserved in the shell midden matrix, whose alkalinity neutralized the acidity of surrounding soils, creating an ideal condition for DNA preservation. The year-round relatively low temperature in the region may also have favored DNA preservation. Future studies have been planned to conduct amino acid racemization analysis to investigate this issue further.

#### 4.1.5. Amplifications of both D-loop and CytB as a reproducibility test

Amplifications of both D-loop and CytB can be considered an alternative to the reproducibility test. As discussed extensively in ancient DNA literature, ancient DNA results have to be repeated to be proven authentic [2,35]. The small size and disarticulated state of salmon vertebrae, however, prevent us from extracting DNA from another piece of bone of the same individual. We believe that PCR amplifications of four different mtDNA fragments, if all point to the same species identification, could serve as the reproducibility test.

From methodological and technical points of view, the results strongly suggest the absence of

contamination. The obtained DNA sequences can therefore be considered authentic, though other lines of evidence from archaeological and morphological analysis provide additional crosschecks.

#### 4.2. Species identification by other lines of evidence

One measure of the accuracy of the DNA species identifications is their consistency with other lines of evidence. Paradoxically, however, DNA identification cannot be confirmed or refuted by another reliable and independent method. This was the reason for applying DNA analysis to this problem in the first instance. As noted earlier, morphological variation does not permit species identification from the majority of skeletal elements, and certainly not from the vertebrae. Our only recourse for comparison with independently derived results is therefore the earlier attempt to infer the species composition of the Namu fishery through radiographic analysis and determination of the age profile of the salmon assemblage [8]. Since salmon are caught at the time they return to rivers and streams to spawn, and since different species spawn at characteristically different ages, the age profile allows for the inference of the probable species composition of the fishery.

Radiographic examination of a large sample of Namu vertebrae indicated the presence of 2-year-old fish, which were inferred to be pink salmon, since this is the only Pacific salmon species to spawn almost without exception at this particular age. The majority of the vertebrae, however, were from fish exhibiting two annuli (i.e. in their third year of life), though a sizeable percentage exhibited three annuli (i.e. in their fourth year of life). Unfortunately for the application of this method, there is considerable overlap in the typical spawning ages of chum, coho, and sockeye. Only chinook salmon (*O. tshawytscha*), which typically spawn in their fifth to seventh years of life [20], were considered unlikely to be present on the basis of the age profile. We think it is significant that chinook salmon was also not identified in the ancient DNA analysis. In the radiographic study, fish in their third year of life were reasoned to be either coho or chum, since this is the typical spawning age for coho [19]. The greatest number of chum spawn in their fourth year [24], but substantial numbers spawn in their third year [21]. Unfortunately, though these are typical spawning ages, there is a range of variability in the age of maturity among all species of salmon, with the notable exception of pink salmon [20]. Any age profile therefore can provide only a rough indication of the likely presence of particular species.

Sockeye were considered unlikely to be present in the Namu assemblage based on erroneous reasoning from published reports that the majority of sockeye are confined to major river systems and typically spawn in their fourth or fifth years of life [1]. This error could have

been easily rectified on the basis of local knowledge that the Namu River historically supported a robust sockeye fishery and still supports a major spawning run. Unfortunately, this information was not obtained at the time of Cannon's (1988) study, and the error was perpetuated in the 1991 report on the Namu fauna. Sockeye salmon also mature at a wide range of ages, though they typically exhibit three, four, or five annuli at the time of spawning [20]. Younger mature sockeye therefore also fall, along with coho and chum, well within the age profile of the Namu salmon. This further highlights the inherent ambiguity of using age profiles for species identifications.

The ancient DNA results we have obtained allow us to specify with greater precision the presence of pink, chum, coho, and sockeye, and the absence of chinook. It is particularly gratifying to be able to use this method for more precise identification to rectify Cannon's erroneous conclusion that sockeye were not present or at least not a major component of the Namu salmon fishery [8,9]. In fact, our results show sockeye was a component of the fishery throughout the period 7000–2000 BP.

Although the radiographic study results were far from definitive, we think their consistency with the ancient DNA results with respect to the absence of chinook, the presence of pink, coho, and chum, and the correct identification of sockeye, which was always a likely component of the fishery, allow us a small measure of further confidence in the accuracy of the ancient DNA results. The double-blind test used in this study should also add some "power" to the authentication process [43]. The first author was not aware of the archaeological evidence or interpretations until the ancient DNA work was completed. This may have avoided any possible bias in interpreting the DNA results.

#### 4.3. Archaeological implications

Although this study was directed primarily toward the development and refinement of methods for the consistent extraction of ancient DNA from archaeological specimens of salmon bone, and its use for accurate, precise, and reliable identifications of salmon species, these initial results also have important archaeological implications. They show the presence of a diverse, multi-species salmon fishery at Namu from as early as 7000 BP. The identification of sockeye salmon also extends the seasonal presence at the site to include the middle to late summer, when their spawning run takes place. Along with other salmon species, which spawn throughout the fall, an intensive late winter/early spring herring fishery [12], and neonatal harbor seal, indicating a presence in late spring, this is evidence of virtually year round site occupation.

Ancient DNA analysis is now available as a method for the identification of salmon species in a variety of archaeological contexts throughout the Pacific Northwest. It will enable precise intra- and inter-site and temporal comparisons of the focus of fisheries. These may be used to determine inequality in access to favored species, such as sockeye or coho, over those less rich in fat and therefore less favored, such as pink and chum. This method will also make it possible to monitor changes in the composition of fisheries that might be indicative of changing environmental conditions, technological innovation, or shifting patterns of seasonal settlement. We plan further studies of salmon from Namu and other sites in the vicinity to assess the extent and direction of temporal and spatial variability in the regional fishery.

#### 5. Conclusions

More than 20 salmon vertebrae (dated 7000 to 2000 BP) from Namu, on the central coast of British Columbia, were processed for extracting DNA for species identification. In total, four short fragments (less than 300 bp) from both mtDNA D-loop and CytB were successfully amplified using the PCR technique. Species identifications were made based on DNA sequence analysis.

In summary,

1. DNA was successfully extracted from ancient salmon remains. Our result is consistent with those of others [7,32] that indicate DNA can be well preserved in ancient fish bones. This study also demonstrates that identifiable salmon DNA sequences can be retrieved from a single vertebra as old as 7000 years.
2. Amplifications of both mtDNA D-loop and CytB fragments were accomplished and were used to crosscheck the species identity of salmon DNA samples.
3. Systematic contamination was closely monitored and controlled. The absence of contamination in this study was probably due to vigorous decontamination measures, strict contamination controls, and effective intrinsic contamination detections.
4. Four species (chum, sockeye, pink, and coho salmon) were identified from the remains, which is consistent with other lines of evidence.
5. DNA can be well preserved in remains from the Pacific Northwest Coast of North America, which is most likely due to favorable neutralized pH in shell middens and relatively low year-round temperature.
6. Successful species identification will provide new data to interpret the faunal remains at Namu and provide workable protocols to identify more

vertebra samples from more sites to study fishery activity in the region.

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