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An efficient and reliable DNA-based sex identification method for archaeological Pacific salmonid *(Oncorhynchus* spp.) remains

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Abstract

Pacific salmonid (*Oncorhynchus* spp.) remains are routinely recovered from archaeological sites in northwestern North America but typically lack sexually dimorphic features, precluding the sex identification of these remains through morphological approaches. Consequently, little is known about the deep history of the sex-selective salmonid fishing strategies practiced by some of the region's Indigenous peoples. Here, we present a DNA-based method for the sex identification of archaeological Pacific salmonid remains that integrates two PCR assays that each co-amplify fragments of the *sexually dimorphic on the Y chromosome* (*sdY*) gene and an internal positive control (*Clock1a* or D-loop). The first assay co-amplifies a 95 bp fragment of *sdY* and a 108 bp fragment of the autosomal *Clock1a* gene, whereas the second assay co-amplifies the same *sdY* fragment and a 249 bp fragment of the mitochondrial D-loop region. This method's reliability, sensitivity, and efficiency, were evaluated by applying it to 72 modern Pacific salmonids from five species and 75 archaeological remains from six Pacific salmonids. The sex identities assigned to each of the modern samples were concordant with their known phenotypic sex, highlighting the method's reliability. Applications of the method to dilutions of modern DNA samples indicate it can correctly identify the sex of samples with as little as ~39 pg of total genomic DNA. The successful sex identification of 70 of the 75 (93%) archaeological samples further demonstrates the method's sensitivity. The method's reliance on two co-amplifications that preferentially amplify *sdY* helps validate the sex identities assigned to samples and reduce erroneous identifications caused by allelic dropout and contamination. Furthermore, by sequencing the D-loop fragment used as a positive control, species-level and sex identifications can be simultaneously assigned to samples. Overall, our results indicate the DNA-

based method reported in this study is a sensitive and reliable sex identification method for ancient salmonid remains.

Introduction

Pacific salmonids (*Oncorhynchus* spp.) were and continue to be an important component of many Indigenous fisheries in northwestern North America [1,2]. Ethnographic records indicate many Indigenous salmonid fisheries in the region likely employed sex-selective fishing strategies [3–10]. Among some groups, such as the Tlingit [7], sex-selective fishing was one of the resource management strategies used to cultivate salmonid stocks [11]. Documenting the deep history of these ethnographically-documented sex-selective salmonid fishing strategies and their use as a resource management strategy requires the accurate sex identification of archaeological salmonid remains. Unfortunately, archaeological salmonid bones are frequently fragmented and typically lack sexually dimorphic features, precluding the sex identification of these remains using conventional morphological approaches. Since sex among many salmonids is believed to be primarily genetically determined [12], ancient DNA (aDNA) analysis can potentially be used to identify the sex of archaeological salmonid bones.

Sex is determined in fish through a variety of behavioural, environmental, and genetic mechanisms [13]. Among salmonids, sex is thought to be primarily determined through a genetic system in which males are the heterogametic sex (XY chromosomal sex-determination system) [12]. For many years, the gene responsible for sex differentiation among salmonids was unknown [12]. However, recent studies suggest the *sexually dimorphic on the Y chromosome* (*sdY*) gene is likely the master sex-determining gene in many salmonids, including Pacific salmonids [14–17]. An early study found that among rainbow/steelhead trout (*Oncorhynchus*

mykiss), the expression of *sdY*, which is limited to the testis and peaks during testis differentiation, is linked to the development of testis [17]. *sdY*'s role in sex determination is further supported by its presence in the vast majority of male Pacific salmonids from all four tested species (cherry [*O. masou*], Chinook [*O. tshawytscha*], sockeye [*O. nerka*], and rainbow/steelhead trout) and absence in most females [14–17]. Since *sdY* is likely the genus's master sex-determining gene, all male Pacific salmonids can be expected to carry the gene, which can be detected through a PCR assay [17]. In such an assay, the absence of *sdY* amplicons is indicative of a female, while the presence of *sdY* amplicons is indicative of a male [17].

In this study, we developed and optimized a DNA-based method for the sex identification of archaeological Pacific salmonid remains that incorporates two PCR assays that co-amplify *sdY* and an internal positive control (IPC). In the first assay (*Clock1a*/*sdY*), a short 95 bp fragment of *sdY* is co-amplified alongside a 108 bp fragment of the autosomal *Clock1a* gene, which serves as an IPC. The second assay (D-loop/*sdY*) co-amplifies the same 95 bp fragment of *sdY* and an IPC consisting of 249 bp fragment of the mitochondrial D-loop region. Based on the results of the two assays, a final consensus sex identity can be assigned to a sample. We evaluated the reliability of this method by comparing the known phenotypic sex of 72 modern Pacific salmonids from five species and the sex identities assigned to them with this method. We subsequently tested the method's sensitivity by applying it to dilutions of modern salmonid DNA and 75 salmonid remains from six species that were recovered from archaeological sites in northwestern North America. Our results indicate the proposed DNA-based method is a highly sensitive and reliable sex identification method for archaeological salmonid remains.

Materials and methods

Development of sex identification method for degraded DNA samples

Using an alignment of published and unpublished *sdY* sequences, we designed ten primer pairs that targeted a ~100 bp fragment of *sdY*. The efficiency and specificity of the primer pairs was evaluated through the software NetPrimer (http://www.premierbiosoft.com/netprimer) and/or by testing them on 6 modern salmonid samples (4 males, 2 females). Based on these results, we selected a single primer pair consisting of primers *sdY*-F19 and *sdY*-R20 to include in two PCR assays (Table 1). These assays were designed to co-amplify the 95 bp fragment of *sdY* targeted by this primer pair and an IPC. The IPC acts as a proxy for the X chromosome, which was not directly targeted due to a lack of data regarding X-linked markers conserved across Pacific salmonids. The IPC is also used to assess whether the failure to amplify *sdY* is due to its biological absence or a lack of amplifiable template DNA [18].

Table 1. Primers included in the PCR assays used in this study.

Locus	Primer	Sequence (5'—3')	Amplicon Size
Clock1a	*Clk1a*-F50 (F)[1]	TAGCCATGTCTGTGTGTTTACTTGC	108 bp
	Clk1a-R60 (R)[1]	GCAGCCAGCTAATTKGATTTG	
D-loop	Smc7 (F)[2]	AACCCCTAAACCAGGAAGTCTCAA	249 bp
	Smc8 (R)[2]	CGTCTTAACAGCTTCAGTGTTATGCT	
sdY	*sdY*-F19 (F)	CCCAACACCCTTCCTATCTCC	95 bp
	sdY-R20 (R)	CCTTCCTCCCTAGAGCTTAAAAC	

[1] F indicates a forward primer and R denotes a reverse primer.
[2] Previously published primers from Yang et al. [19].

In the first assay (*Clock1a*/*sdY*), primers *sdY*-F19 and *sdY*-R20 are included in a co-amplification that amplifies the targeted 95 bp *sdY* fragment alongside a 108 bp fragment of the

autosomal *Clock1a* gene. This *Clock1a* fragment was targeted with primers *Clk1a*-F50 and

Clk1a-R60 (Table 1) and serves as an IPC. The second assay (D-loop/*sdY*), which also includes

primers *sdY*-F19 and *sdY*-R20, co-amplifies the same *sdY* fragment and an IPC consisting of a

249 bp fragment of the mitochondrial D-loop region. This D-loop fragment was amplified with

primers (Smc7 and Smc8) previously published in Yang et al. [19] (Table 1). Following Speller

and Yang [18], we set up the *Clock1a*/*sdY* and D-loop/*sdY* co-amplifications to preferentially

amplify *sdY* by targeting IPCs longer than the *sdY* fragment and weighting the primer ratios in

favour of the *sdY* primers. A primer ratio of 1.5:1 (*sdY*:*Clock1a* primers) was used in the

Clock1a/*sdY* assay while a primer ratio of 6:1 (*sdY*:D-loop primers) was used in D-loop/*sdY*

assay.

Modern salmonid samples

To evaluate the reliability of our proposed sex identification method, we applied it to 72

modern Pacific salmonids of known phenotypic sex. These modern samples consisted of: tissue

samples obtained from pre-deceased salmonids purchased at a public market in Steveston, BC,

from a Fisheries and Oceans Canada (DFO) licensed commercial sockeye salmon fisher

operating in Barkley Sound, BC; muscle and skin tissue collected from carcasses of pre-deceased

spawned-out salmonids washed up on the banks of the Coquitlam River (Port Coquitlam, BC);

archived DNA samples held at Simon Fraser University (Burnaby, BC); archived tissue samples

provided by DFO (West Vancouver, BC); and tissue samples collected from live salmonids

reared by DFO. The live reared salmonids used in this study were reared and collected in

compliance with the Canadian Council for Animal Care guidelines under permit 15-001R1

issued by DFO's Pacific Regional Animal Care Committee. The live salmonids were reared at

DFO's Centre for Aquaculture and Environmental Research (West Vancouver, BC) in a dryland

facility designed to prevent the escape of cultured salmonids. Prior to obtaining tissue samples,

the live reared salmonids were euthanized in a bath of tricaine methanesulfonate (MS-222; 100

mg/L) buffered with sodium bicarbonate (200 mg/L). Tissue samples were obtained from the

sacrificed salmonids after all ventilation activity had ceased. No other permits were required for

this study. The analyzed modern salmonid samples include males and females from five Pacific

salmonid species (Table 2 and S1 Table). DNA was extracted from the modern samples using a

DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocols.

All pre-PCR laboratory work involving modern salmonid samples was conducted at the Centre

for Forensic Research, Simon Fraser University, in a DNA laboratory dedicated to modern

samples.

Table 2. Species and sex distribution of the modern Pacific salmonid samples.

Species	Males	Females	Total
Chinook	10	10	20
Chum	6	5	11
Coho	10	10	20
Pink	7	3	10
Sockeye	6	5	11
Total	39	33	72

Dilution series

The concentration of DNA in a modern female (KCH4) and male (KCH9) Chinook

salmon sample was quantified in triplicate using a NanoDrop 2000c spectrophotometer (Thermo

Fisher Scientific, Waltham, MA). The concentration of total genomic DNA in these two modern

samples was 1,560.1 ± 1.87 ng/μL (KCH4) and 1,575.8 ± 7.96 ng/μL (KCH9) (mean ± SD).

Subsequently, we serially diluted KCH4 and KCH9 10-fold to 1:1,000,000 with distilled H_2O.

To test the sensitivity of our sex identification method, we applied the *Clock1a*/*sdY* and D-loop/*sdY* assays to each of the six dilutions (1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000 dilutions) in the KCH4 and KCH9 dilution series. For both assays, the PCR reaction volumes used for each of the dilutions of KCH4 and KCH9 contained 2.5 µL of DNA solution. Based on their initial concentrations and amount of DNA solution used, the PCR reaction volumes used for the dilutions included approximately 390,025 to 3.9 pg (KCH4) and 393,950 to 3.9 pg (KCH9) of total genomic DNA, respectively.

Archaeological salmonid remains

To further assess its sensitivity, we applied our sex identification method to archaeological Pacific salmonid remains recovered from three archaeological sites located in British Columbia and Oregon (Table 3). Kawumkan Springs Midden (KSM) (35KL9-12) is a residential village located along the Sprague River in the Upper Klamath Basin, Oregon [20,21]. The salmonid remains from Kawumkan Springs Midden we analyzed are approximately 5,300 to 1,200 years old or are of unknown age [20,21]. Keatley Creek (EeRl-7) is a winter pithouse village in the Interior Plateau region of British Columbia and is situated on a river terrace along the east bank of mid-Fraser River [22]. The Keatley Creek salmonid remains examined in this study were recovered from Late Plateau to Early Kamloops Horizon (~1,500 to 1,100 years BP) living floors and storage pits from three residential structures (Housepits 3, 12, and 107) and one specialized structure (Housepit 9) [23]. Say-Umiton (DhHr-18) is a permanent residential site located in a cove along the southwestern shore of Indian Arm, British Columbia [24]. In this study, we included salmonid remains recovered from Late Phase (~1,200 to 250 years BP) activities areas at Say-Umiton [24].

Table 3. Species distribution of the archaeological Pacific salmonid samples.

Site	Site Number	State/ Province	Age of Samples (years BP)	Chinook	Coho	Chum	Pink	Rainbow/ Steelhead Trout	Sockeye	Total
Kawumkan Springs Midden	35KL9-12	OR	5,300-1,200 /Unknown	2	0	0	0	7	0	9
Keatley Creek	EeRl-7	BC	1,500-1,100	8	2	0	0	0	45	55
Say-Umiton	DhHr-18	BC	1,200-250	0	0	9	2	0	0	11
			Total	10	2	9	2	7	45	75

In total, 75 archaeological salmonid remains from these three sites were selected for analysis (Table 3). These archaeological samples were selected for sex identification because of their availability, species diversity, and good mtDNA preservation. Mitochondrial DNA has been amplified from all 75 of these samples during previous projects [20,23,25,26]. DNA was originally extracted from these samples using a modified silica-spin column method [27,28]. All of the samples were previously identified to the species-level through the analysis of cytochrome *b* and/or D-loop fragments [20,23,25,26]. In total, six species of Pacific salmonids are represented among the analyzed assemblages (Table 3 and S2 Table). All pre-PCR laboratory work involving the archaeological samples was conducted in a dedicated ancient DNA laboratory in the Department of Archaeology at Simon Fraser University. To reduce the likelihood of contamination and detect it if it did occur, strict contamination controls, including the analysis of blank extracts, were undertaken [29]. Permission to include the archaeological salmonid samples re-analyzed in this study was granted by the archaeologists who originally provided the samples to the Simon Fraser University Ancient DNA Laboratory.

PCR amplification

PCR amplifications were performed on a Mastercycler Personal or Gradient thermal cycler (Eppendorf, Mississauga, ON, Canada) in a 25 or 30 μL reaction volume. The reaction volume for the *Clock1a*/*sdY* assays contained 1.5× PCR Gold Buffer (Applied Biosystems, Carlsbad, CA, USA), 2 mM MgCl$_2$, 0.2 mM dNTP, 0.45 μM of each *sdY* primer, 0.3 μM of each *Clock1a* primer, BSA (1 mg/mL), 1–5 μL DNA solution, and 0.75–2.25 U AmpliTaq Gold (Applied Biosystems, Carlsbad, CA, USA). The reaction conditions for the D-loop/*sdY* assays were the same as above, except for the primer concentrations, which were as follows: 0.6 μM of each *sdY* primer, 0.1 μM of each D-loop primer. The thermal conditions of the PCRs consisted of an initial denaturation step at 95 ˚C for 12 min followed by 60 cycles at 95 ˚C for 30 s (denaturation), 54 ˚C for 30 s (annealing), and 72 ˚C for 40 s (extension), and a final extension step at 72 ˚C for 7 min. Negative PCR controls were included in each PCR setup to monitor for contamination. The negative controls amplified alongside the dilution series included 2.5 μL of the distilled H$_2$O used to prepare the dilutions.

Sex identification

Five microliters of PCR product were pre-stained with SYBR Green I (Life Technologies, Carlsbad, CA, USA), electrophoresed on a 2% or 3% agarose gel, and visualized with a Dark Reader transilluminator (Clare Chemical Research, Dolores, CO, USA). Due to their similar size (~13 bp difference), the separation of the fragments targeted by the *Clock1a*/*sdY* assays could not be sufficiently resolved using a 2% agarose gel. Consequently, a higher percentage agarose gel (3% agarose) run at 100 v for 99 min was used to separate the fragments amplified by the *Clock1a*/*sdY* assay. PCR products generated by the D-loop/*sdY* assays were typically separated using a 2% agarose gel run at 100 V for 30 to 60 min. The size and intensity of the PCR products generated by the assays was evaluated by visually inspecting the

electrophoresis gels. A sample was confidently identified as a male if *sdY* and the IPC or just *sdY* was amplified with both assays (Table 4). Samples were identified as female if *sdY* failed to amplify and both IPCs were amplified (Table 4). As a quality assurance measure, a sex identity was not assigned to a sample if the assays yielded inconsistent results or did not yield amplified DNA (Table 4).

Table 4. Potential results of the two assays and the final sex identification that would be assigned to a sample in each of these scenarios.

Assay	Marker	Potential Scenarios														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Clock1a/ *sdY*	*Clock1a*	+[1]	-	+	-	+	-	-	+	+	-	-	-	+	+	+
	sdY	+	+	+	+	-	-	-	+	+	+	+	-	-	-	-
D-loop/ *sdY*	D-loop	+	-	-	+	+	-	+	-	+	+	-	-	-	+	-
	sdY	+	+	+	+	-	-	-	-	-	-	-	+	-	+	+
Final Sex ID		♂[2]	♂	♂	♂	♀	N	N	N	N	N	N	N	N	N	N

[1]+ = Amplicon present, - = Amplicon not present
[2]♂ = Male, ♀ = Female, N = No sex identity assigned

Sequence analysis and species identification

To confirm their species identities, we directly sequenced the D-loop fragments amplified from a subset of the archaeological samples with the D-loop/*sdY* assay. Since the archaeological remains have all been previously identified to the species-level through mtDNA analysis, we only sequenced the D-loop amplicons obtained from 12 samples to assess species identification accuracy. These samples included at least one sample from each of the six species and three archaeological sites represented in the set of analyzed archaeological samples. D-loop amplicons

were directly sequenced with the amplification primers in the forward and/or reverse direction at the Eurofins Genomics sequencing facility (Toronto, ON). The obtained sequences were visually edited, truncated to remove the primer sequences, and assembled using ChromasPro (http://www.technelysium.com.au). The resulting edited sequences were compared to reference sequences in GenBank through a BLAST search [30]. In BioEdit [31], the edited sequences were aligned with salmonid reference sequences using ClustalW [32] and trimmed to the same length. Species identifications were then assigned to the samples using the procedure described by Yang et al. [19].

Results

Modern samples

DNA was amplified from all of the modern salmonid samples with both the *sdY*/D-loop and *sdY*/*Clock1a* assays (Fig 1). The sex identifications assigned to each of the modern samples with both assays were concordant, thereby allowing a final consensus sex identity to be assigned to each of the modern samples (Fig 1 and S1 Table). The final consensus sex identities assigned to each of the modern samples matched their known phenotypic sex (S1 Table). None of the negative PCR controls associated with the modern samples yielded PCR products of the expected size.

Fig 1. Negative images of electrophoresis gels showing the (A) *Clock1a*/*sdY* **and (B) D-loop/***sdY*** PCR assay results for modern male and female samples from five Pacific salmonid species.**
The approximate location of the IPC and *sdY* amplicons are indicated by the labelled arrows. The 100 bp ladder used to estimate the size of the amplicons is from Invitrogen (Waltham, MA, USA).

Dilution series

The IPCs were successfully amplified with both assays from the 10-, 100-, 1,000-, 10,000-, and 100,000-fold dilutions of the female Chinook salmon sample (KCH4) (S1 Fig). Similarly, *sdY* and the IPCs were amplified with both assays from the 10- to 100,000-fold dilutions of the male Chinook salmon sample (KCH9) (S1 Fig). No DNA was amplified from the negative PCR controls or the 1,000,000–fold dilution of either KCH4 or KCH9 (S1 Fig), with one exception. The D-loop fragment was weakly amplified from the 1,000,000-fold dilution of KCH9 (S1 Fig). Accordingly, the highest dilution of each sample that could be assigned a sex identity was the 100,000-fold dilution. The concentration of DNA in the 100,000-fold dilutions of KCH4 and KCH9 is estimated to be approximately 15.6 pg/µL (KCH4) and 15.8 pg/µL (KCH9). The reaction volume for the 100,000-fold dilution of KCH4 contained approximately 39.0 pg of DNA, whereas the 100,000-fold dilution of KCH4 contained approximately 39.4 pg of DNA.

Archaeological samples

Among the 75 archaeological samples that were tested, only two samples (SA2 and SD23) consistently failed to yield DNA, and therefore could not be assigned a sex (S2 Table). Of the 73 archaeological samples that yielded DNA, 70 samples yielded sex identification results

that were consistent across both assays and could therefore be assigned a final consensus sex

identity (Fig 2 and S2 Table). At least two samples from each of the species represented among

the archaeological remains were successfully assigned a sex identification (Table 5 and S2

Table). In total, 37 of these samples were identified as male (53%) and 33 were identified as

female (47%) (Table 5 and S2 Table). Table 5 presents the overall sex ratio for each site and the

sex ratios for each of the salmonid species represented at the sites. At each of the sites, both the

overall sex ratio and the sex ratios for each of the identified species were not significantly male

or female biased (Exact binomial test, two-tailed, all p>0.05). Overall sex ratios also did not

significantly differ among sites (Fisher's exact test, two-tailed, p=0.17).

**Fig 2. Negative images of electrophoresis gels showing the (A) *Clock1a*/*sdY* (B) D-loop/*sdY*
assay results for nine of the analyzed archaeological salmonid samples.**
The approximate location of the IPC and *sdY* amplicons are indicated by the labelled arrows. The
100 bp ladder used to estimate the size of the amplicons is from Invitrogen (Waltham, MA,
USA). Note: For SB11, the D-loop/*sdY* assay (B) produced two weak nonspecific bands only
slightly smaller than the predicted size of the *sdY* amplicon, suggesting they might represent *sdY*.
However, the *Clock1a*/*sdY* assay (A) only yielded a fragment of *Clock1a*, confirming the
nonspecific bands likely do not represent *sdY*, verifying SB11's female identity.

**Table 5. Sex ratios (Number of identified males to females) by archaeological site and
species.**

Site	Chinook	Chum	Coho	Pink	Rainbow/Steelhead Trout	Sockeye	Overall
Kawumkam Springs Midden	1:1	—	—	—	6:1	—	7:2
Keatley Creek	3:4	—	1:1	—	—	19:22	23:27
Say-Umiton	—	5:4	—	2:0	—	—	7:4

Three archaeological samples (SD24, SE35, and SE40) yielded DNA but were not

assigned a final consensus sex identity on account of the assays yielding inconsistent results (S2

Table). D-loop was repeatedly amplified from all three of these samples, but the amplification of nuclear DNA was variable. In the case of SE35, *sdY* was amplified once with both the D-loop/*sdY* and *Clock1a*/*sdY* assay, but could not be re-amplified with either assay. Similarly, Clock1a was amplified from SD24 and SE40, but failed to amplify at least two other times. These inconsistent results may reflect allelic dropout related to DNA degradation or rare sequence variations in the *Clock1a* and *sdY* genes. Amplicons approximating the expected size of the targeted products were not amplified from any of the negative PCR controls or the blank extraction controls.

Species identification

D-loop sequences were successfully obtained from the 12 samples that underwent sequencing. BLAST searches indicated each of the samples' D-loop sequences most closely resembled reference sequences from Pacific salmonid species. Through multiple alignment and phylogenetic analysis, we were able to assign a species-level identification to each of the sequenced samples (S2 Table). The species identities assigned to each of the samples matched their previously assigned species identities (S2 Table) [23,25,26].

Discussion

Authenticity of the ancient DNA results

Multiple lines of evidence indicate the sex identification results obtained for the archaeological salmonid samples are authentic and not the result of contamination. First, all pre-PCR laboratory work involving the archaeological samples was conducted in a dedicated aDNA

laboratory that is physically separated from the modern DNA and post-PCR laboratories. Second, the archaeological samples were previously rigorously decontaminated using a combination of chemical washes and UV irradiation [23,25,26]. Third, no amplicons of the expected sized were amplified from any of the negative PCR or blank extraction controls in this or previous studies [23,25,26]. Fourth, the species identities assigned to all 12 of the samples whose D-loop amplicons were sequenced matched the species identities assigned to them in previous studies (S2 Table) [23,25,26].

Sensitivity and cross-species applicability

In this study, we successfully assigned sex identities to 93% of the archaeological salmonid samples we analyzed with our method. The high proportion of samples that were successfully sexed highlights the high sensitivity of our method. However, as remains that did not previously yield mtDNA were not tested, our results likely overestimate the method's sensitivity. Nonetheless, the sexing of nearly all of the remains previously identified to the species-level through mtDNA analysis, suggests our method's sensitivity is comparable to the mtDNA-based assays [19,33] used to identify the remains. Furthermore, our successful sexing of 100,000-fold dilutions of modern male and female Chinook salmon samples, with estimated DNA concentrations of 15.6 and 15.8 pg/µl, further supports the sensitivity of our method. Assuming the C-value of the Chinook salmon genome is 2.45 pg [34], the successful sexing of these dilutions using only ~39 pg of total genomic DNA indicates our method works readily with only ~8 nuclear DNA templates.

Our results also indicate our sex identification method can be used to sex individuals from a number of Pacific salmonid species. The method's cross-species applicability is

demonstrated by our successful sex identification of modern samples from five salmon species, and archaeological samples from six species. We expect that the method can also be applied to other Pacific salmonids, such as cutthroat trout (*O. clarkii*), but additional tests are needed to confirm this possibility. The sensitivity and cross-species applicability exhibited by our sex identification method suggests it is an efficient means for sexing archaeological Pacific salmonid remains from a range of species.

Reliability

In addition to being sensitive and having cross-species applicability, our proposed sex identification method has also proven to be able to produce reliable sex identities. The agreement between the sex we assigned to each of modern samples with our sex identification method, and their known phenotypic sex, highlights our method's reliability. Our method's reliability is partly due to its reliance on assays that screen for the presence of *sdY*, rather than other Y-linked markers (e.g., *GH-Y*) not as strongly associated with phenotypic sex [35,36]. Due to *sdY*'s critical role in controlling sex differentiation in Pacific salmonids, its presence or absence is a reliable proxy for phenotypic sex [17]. However, previous studies have identified modern Pacific salmonids with *sdY* genotypes inconsistent with their phenotypic sex [14,15,17], indicating our method may not always yield accurate sex identifications. Both *sdY*-positive females and *sdY*-negative males have been previously documented among modern Pacific salmonid populations [14,15,17]. Mutations and environmental factors, such as temperature and exposure to certain contaminants, may trigger sex reversals that result in individuals with discordant genotypic and phenotypic sexes [14]. Nevertheless, unless past conditions were more conducive to sex reversals, erroneous sex identifications caused by sex reversals will likely be minimal as less

than 7% of contemporary Pacific salmonids have incongruent genotypic and phenotypic sexes [14–17].

The reliability of the sex identities assigned to archaeological samples with our method is enhanced by its reliance on two PCR assays, rather than a single assay, to sex samples. By using two assays, erroneous sex identifications or no calls caused by the dropout of *sdY* or the IPC due to DNA degradation can be detected [37]. As evidenced by the inconsistent sex identification results obtained for three of the archaeological samples (SD24, SE35, SE40), the dropout of single-copy nuclear markers, such as *sdY*, does occur when dealing with specimens with degraded DNA. The dropout of Y-linked markers is more common when the IPC used in a PCR assay outnumbers the Y-linked marker, as its higher copy number results in it outcompeting the Y-linked marker [38]. Our method is potentially susceptible to *sdY* dropout related to this issue as the IPCs in both the *Clock1a*/*sdY* and D-loop/*sdY* assays have higher copy numbers than *sdY*. However, we reduced the potential for *sdY* dropout and erroneous sex identifications caused by the IPCs outnumbering and outcompeting *sdY* by designing both assays to favor the amplification of *sdY* [18]. Both assays were designed to preferentially amplify *sdY* by skewing the primer ratio in favour of the *sdY* primers and targeting an *sdY* fragment shorter than the IPCs [18]. Among the male samples, the stronger amplifications obtained for *sdY* relative to those obtained for the IPC, particularly D-loop, indicates *sdY* was indeed preferentially amplified (Figs 1 and 2 and S1 Fig). In addition to the above factors, primer-template mismatches can also result in *sdY* dropout and erroneous sex identifications [38]. Although not addressed here, the likelihood of *sdY* dropout caused by primer-template mismatches could be lessened by using alternative *sdY* primers in one of the assays.

Although they can potentially contribute to *sdY* dropout and erroneous sex identifications, the IPCs included in both assays play a critical role in validating the sex identities assigned to archaeological samples. Among samples that did not yield *sdY* amplicons, the amplification of both IPCs indicates their lack of *sdY* likely reflects their female sex rather than degradation or inhibition [18]. Without the inclusion of an IPC, reliably identifying samples as female would be difficult as inhibited, degraded, and female samples would produce identical results: no amplicons.

Detection of contamination and species identification

As archaeological samples are susceptible to contamination, embedding means of detecting contamination within aDNA analyses is of critical importance [29]. Our method's use of two assays fulfills this requirement as the assays act as independent PCR replications that can aid in the detection of contamination and authentication of sex identification results. Furthermore, sequencing the various fragments amplified by the two assays provides an additional means for detecting contamination. The generation of conflicting taxonomic identities through the sequence analysis of different fragments from the same sample is suggestive of contamination [19,33]. Moreover, by sequencing the D-loop fragment targeted by the D-loop/*sdY* assay, a sample's species identity can also be determined [19]. Through the sequence analysis of this D-loop fragment, we successfully assigned species-level identifications to 12 archaeological remains, a task that is typically not possible though morphological analyses [39]. Consequently, this method will allow researchers to simultaneously determine past salmonid fisheries' species and sex preferences.

Archaeological implications

The analyzed salmonid remains from each of the sites examined in this study were not meant to be representative samples, which limits our data's interpretive potential. Nonetheless, our data allows some hypotheses to be drawn about the sex-selectivity of the pre-Contact salmonid fisheries in northwestern North America. The lack of a biased sex ratio among the relatively large sample of sexed salmonid remains from Keatley Creek suggests sex-selective salmon fishing was not a pervasive practice among the site's inhabitants. Conversely, little can be said about the sex-selectivity of the KSM's and Say-Umiton'ssalmonid fisheries given the likely unrepresentativeness of the small number of sexed salmonid remains (n=9 and n=11, respectively) from these sites. Furthermore, the curatorial history of the KSM assemblage, specifically the loss of an unknown number of remains between excavation and aDNA analysis, also makes our KSM sample's representativeness questionable [21]. Consequently, establishing the sex-selectivity of pre-Contact salmonid fisheries in the Upper Klamath Basin will require examining remains from other sites in that region. In the case of Say-Umiton, establishing the sex-selectivity of its salmonid fishery will require analyzing additional remains from the site.

Conclusion

In this study, we developed and optimized a highly-sensitive DNA-based method for the sex identification of archaeological Pacific salmonid remains. This method integrates two PCR assays that co-amplify an IPC (*Clock1a* or D-loop) and this genus' master sex-determining gene: *sdY*. In summary,

1) Using this method, we successfully sexed 70 of the 75 (93%) mtDNA-identified archaeological Pacific salmonid samples we analyzed. This suggests the method has a

high sensitivity comparable to that of mtDNA-based species identification assays, making it an efficient sex identification method for archaeological Pacific salmonid remains.

2) The sex identities assigned with this method to all 72 of the analyzed modern Pacific salmonid samples matched their known phenotypic sex, highlighting the method's reliability. Reflecting the method's sensitivity, dilutions of DNA samples from modern Chinook salmon could be assigned to the correct sex using as little as ~39 pg of total genomic DNA.

3) As evidenced by the successful sex identification of samples from six Pacific salmonid species (Chinook, chum, coho, pink, sockeye, and rainbow/steelhead trout), the method is applicable to remains from multiple Pacific salmonid species.

4) By sequencing the D-loop fragment used as an IPC in the D-loop/*sdY* assay, species-level identifications can be assigned to samples. This will enable the sex and species preferences of past salmonid fisheries to be determined in tandem.

Although we focused on salmonids from a single genus, our findings highlight the potential of using *sdY*-based assays to sex archaeological remains from other salmonids, such as Atlantic salmonids (*Salmo* spp.) and char (*Salvelinus* spp.), that share this master sex-determining gene [17]. More broadly, our results highlight the potential of using aDNA analysis to assign sex identities to archaeological fish remains from species whose sex is genetically determined. By enabling the sex identification of fish remains, aDNA analysis can shed light on the sex-selective fishing strategies employed by past peoples.

Acknowledgements

References

1. Butler VL, Campbell SK. Resource intensification and resource depression in the Pacific Northwest of North America: a zooarchaeological review. J World Prehis. 2004;18: 327–405.

2. McKechnie I, Moss ML. Meta-analysis in zooarchaeology expands perspectives on Indigenous fisheries of the Northwest Coast of North America. J Archaeol Sci Rep. 2016;8: 470-485.

3.   Barnett HG. The Coast Salish of British Columbia. Westport: Greenwood Press; 1975.

4.   Curtis ES. The North American Indian: being a series of volumes picturing and describing the Indians of the United States, the Dominion of Canada, and Alaska, volume 13. Norwood: Plimpton Press; 1924.

5.   Dale C, Natcher DC. What is old is new again: the reintroduction of Indigenous fishing technologies in British Columbia. Local Environ. 2014;20: 1309–1321.

6.   Kennedy DID, Bouchard R. *Stl'átl'imx* (Fraser River Lillooet) salmon fishing. In: Hayden B, editor. A complex culture of the British Columbia Plateau: traditional *Stl'átl'imx* resource use. Vancouver: University of British Columbia Press; 1992. pp. 266–354.

7.   Langdon SJ. Traditional knowledge and harvesting of salmon by *Huna* and *Hinyaa* Tlingit. Anchorage: U.S. Fish and Wildlife Service, Office of Subsistence Management, Fisheries Resource Monitoring Program; 2006.

8.   Ratner NC, Brown P, Rowan J, Yates D, Smith M, Dizard JA, et al. Local Knowledge, customary practices, and harvest of sockeye salmon from the Klawock and Sarkar Rivers, Prince of Wales Island, Alaska. Juneau: Alaska Department of Fish and Game, Division of Subsistence; 2006.

9.   Ritchie M, Springer C. Harrison River chum fishery: the ethnographic and archaeological perspective. Report on file, Agassiz: Sts'ailes Band; 2010.

10.   Simeone WE, Valentine EM. Ahtna knowledge of long-term changes in salmon runs in the Upper Copper River drainage, Alaska. Juneau: Alaska Department of Fish and Game, Division of Subsistence; 2007.

11.   Thornton T, Deur D, Kitka H. Cultivation of salmon and other marine resources on the Northwest Coast of North America. Hum Ecol Interdiscip J. 2015;43: 189–199.

12.   Davidson WS, Huang K, Fujiki T, von Schalburg KR, Koop BF. The sex determining loci and sex chromosomes in the family Salmonidae. Sex Dev. 2009;3: 78–87.

13.   Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture. 2002;208: 191–364.

14.   Cavileer TD, Hunter SS, Olsen J, Wenburg J, Nagler JJ. A sex-determining gene (*sdY*) assay shows discordance between phenotypic and genotypic sex in wild populations of Chinook salmon. Trans Am Fish Soc. 2015;144: 423–430.

15.   Larson WA, McKinney GJ, Seeb JE, Seeb LW. Identification and characterization of sex-associated loci in sockeye salmon using genotyping-by-sequencing and comparison with a sex-determining assay based on the sdY gene. J Hered. 2016;107: 559–566.

16.   Yano A, Guyomard R, Nicol B, Jouanno E, Quillet E, Klopp C, et al. An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*. Curr Biol. 2012;22: 1423–1428.

17.   Yano A, Nicol B, Jouanno E, Quillet E, Fostier A, Guyomard R, et al. The sexually dimorphic on the Y-chromosome gene (*sdY*) is a conserved male-specific Y-chromosome sequence in many salmonids. Evol Appl. 2013;6: 486–496.

18.   Speller CF, Yang DY. Identifying the sex of archaeological turkey remains using ancient DNA techniques. J Archaeol Sci Rep. 2016;10: 520-525.

19.   Yang DY, Cannon A, Saunders SR. DNA species identification of archaeological salmon bone from the Pacific Northwest Coast of North America. J Archaeol Sci. 2004;31: 619–631.

20.   Stevenson AE. Using archaeological fish remains to determine the native status of anadromous salmonids in the Upper Klamath Basin (Oregon, USA) through mtDNA and geochemical analysis. M.Sc. Thesis, Portland State University. 2011. Available from: http://pdxscholar.library.pdx.edu/open_access_etds/444

21.   Stevenson AE, Butler VL. The Holocene history of fish and fisheries of the Upper Klamath Basin, Oregon. J Calif Gt Basin Anthropol. 2015;35: 169–188.

22.   Hayden B. Observations on the prehistoric social and economic structure of the North American Plateau. World Archaeol. 1997;29: 242–261.

23.   Speller CF, Yang DY, Hayden B. Ancient DNA investigation of prehistoric salmon resource utilization at Keatley Creek, British Columbia, Canada. J Archaeol Sci. 2005;32: 1378–1389.

24.   Lepofsky D, Trost T, Morin J. Coast Salish interaction: a view from the inlets. Can J Archaeol. 2007;31: 190–223.

25.   Speller C. Ancient DNA analysis of archaeological salmonid remains from Klamath Basin, Oregon. Report on file, Burnaby: Ancient DNA Laboratory, Department of Archaeology, Simon Fraser University; 2010.

26.   Speller C, Yang D. Species identification of archaeological salmon remains using ancient DNA techniques. Report on file, Burnaby: Ancient DNA Laboratory, Department of Archaeology, Simon Fraser University; 2004.

27.   Yang DY, Eng B, Waye JS, Dudar JC, Saunders SR. Technical note: improved DNA extraction from ancient bones using silica-based spin columns. Am J Phys Anthropol. 1998;105: 539–543.

28.   Yang DY, Liu L, Chen X, Speller CF. Wild or domesticated: DNA analysis of ancient water buffalo remains from north China. J Archaeol Sci. 2008;35: 2778–2785.

29.   Yang DY, Watt K. Contamination controls when preparing archaeological remains for ancient DNA analysis. J Archaeol Sci. 2005;32: 331–336

30. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215: 403–410.

31. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41: 95–98.

32. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22: 4673–4680.

33. Yang DY, Speller CF. Co-amplification of cytochrome b and D-loop mtDNA fragments for the identification of degraded DNA samples. Mol Ecol Notes. 2006;6: 605–608.

34. Hardie DC, Hebert PDN. The nucleotypic effects of cellular DNA content in cartilaginous and ray-finned fishes. Genome. 2003;46: 683–706.

35. Devlin RH, Park L, Sakhrani D, Baker JD, Marshall AR, LaHood E, et al. Variation of Y-chromosome DNA markers in Chinook salmon (*Oncorhynchus tshawytscha*) populations. Can J Fish Aquat Sci. 2005;62: 1386–1399.

36. Muttray AF, Sakhrani D, Smith JL, Nakayama I, Davidson WS, Park L, et al. Deletion and copy number variation of Y-chromosomal regions in coho salmon, chum salmon, and pink salmon populations. Trans Am Fish Soc. 2017;146: 240–251.

37. Quéméré E, Perrier C, Besnard A-L, Evanno G, Baglinière J-L, Guiguen Y, et al. An improved PCR-based method for faster sex determination in brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). Conserv Genet Resour. 2014;6: 825–827.

38. Sinding MHS, Tervo OM, Grønnow B, Gulløv HC, Toft PA, Bachmann L, et al. Sex determination of baleen whale artefacts: implications for ancient DNA use in zooarchaeology. J Archaeol Sci Rep. 2016;10: 345–349.

39. Cannon DY. Marine fish osteology: a manual for archaeologists. Burnaby: Archaeology Press, Simon Fraser University; 1987.

Supporting information

S1 Table. Species and phenotypic sex information and sex identification results for the modern Pacific salmonid samples analyzed in this study.

S2 Table. Sex and species identification results for the archaeological Pacific salmonid samples analyzed in this study.

S1 Fig. Negative images of electrophoresis gels showing the (A) *Clock1a/sdY* (B) D-loop/*sdY* PCR assay results for dilutions of a modern female (KCH4) and male (KCH9) Chinook salmon sample. The approximate location of the IPC and *sdY* amplicons are indicated by the labelled arrows. The 100 bp ladder used to estimate the size of the amplicons is from Invitrogen (Waltham, MA, USA).
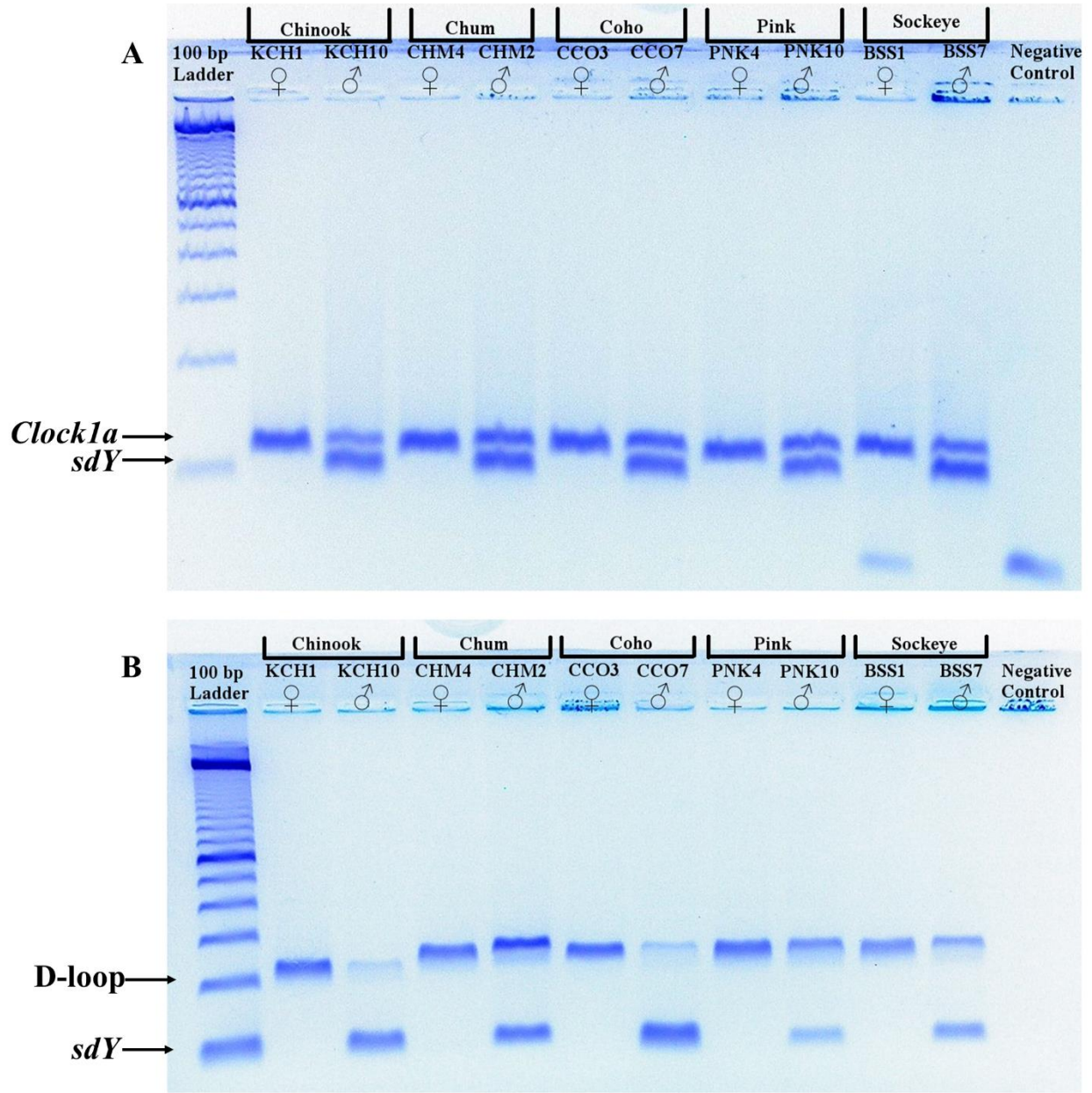
Figure 1.
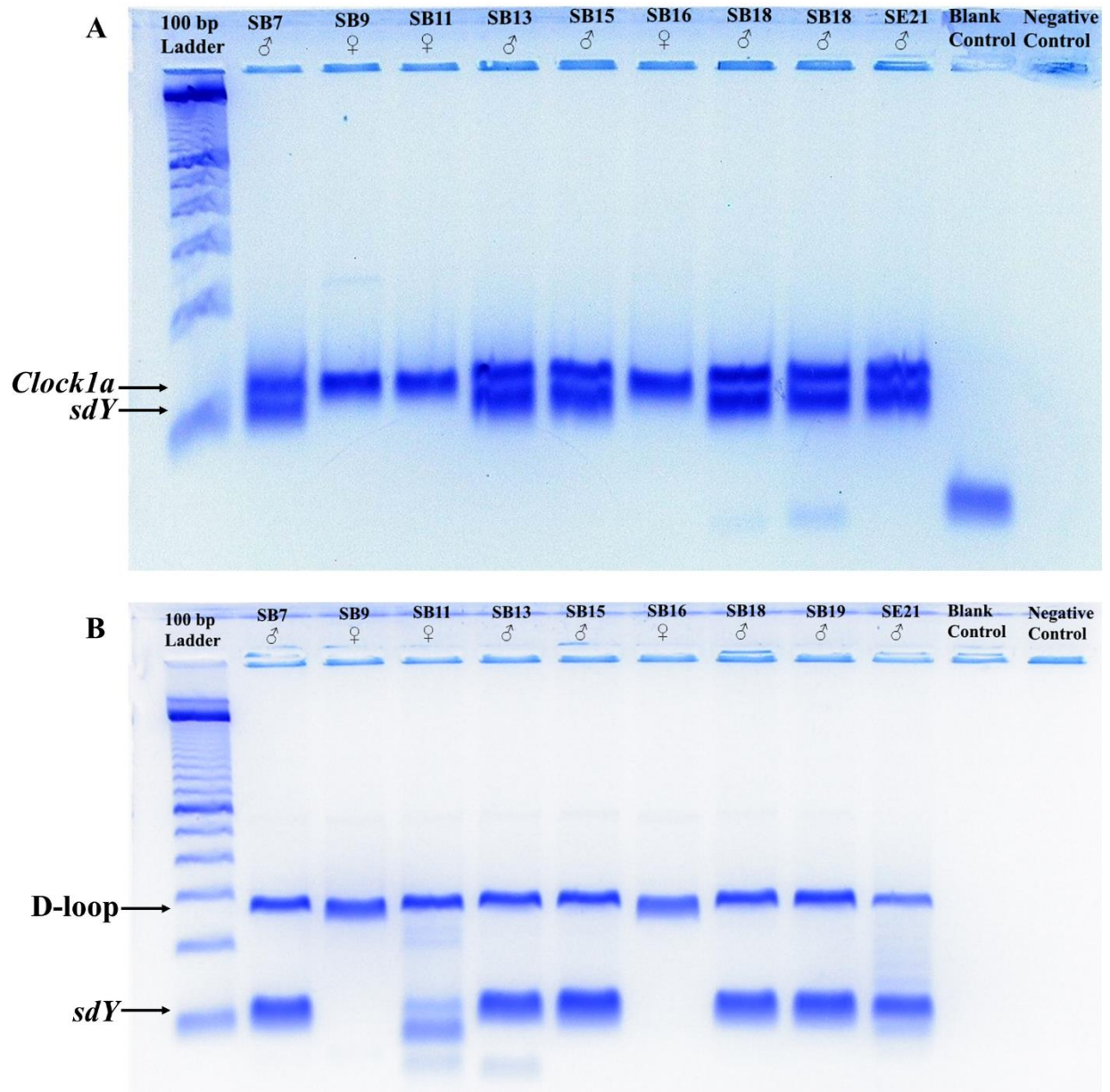
Figure 2.

S1 Table. Species and phenotypic sex information and sex identification results for the modern Pacific salmonid samples analyzed in this study.

Sample ID	Species	Phenotypic Sex	*Clock1a*/*sdY* Assay Sex ID	D-loop/*sdY* Assay Sex ID	Consensus Sex ID
BKS1	Sockeye	♀[1]	♀	♀	♀
BKS2	Sockeye	♀	♀	♀	♀
BKS3	Sockeye	♂	♂	♂	♂
BKS4	Sockeye	♂	♂	♂	♂
BSS1	Sockeye	♀	♀	♀	♀
BSS2	Sockeye	♂	♂	♂	♂
BSS3	Sockeye	♀	♀	♀	♀
BSS4	Sockeye	♂	♂	♂	♂
BSS5	Sockeye	♀	♀	♀	♀
BSS6	Sockeye	♂	♂	♂	♂
BSS7	Sockeye	♂	♂	♂	♂
CCO1	Coho	♀	♀	♀	♀
CCO2	Coho	♀	♀	♀	♀
CCO3	Coho	♀	♀	♀	♀
CCO4	Coho	♀	♀	♀	♀
CCO5	Coho	♀	♀	♀	♀
CCO6	Coho	♂	♂	♂	♂
CCO7	Coho	♂	♂	♂	♂
CCO8	Coho	♂	♂	♂	♂
CCO9	Coho	♂	♂	♂	♂
CCO10	Coho	♂	♂	♂	♂
CHC1	Chinook	♀	♀	♀	♀
CHC2	Chinook	♀	♀	♀	♀
CHC3	Chinook	♀	♀	♀	♀
CHC4	Chinook	♀	♀	♀	♀
CHC5	Chinook	♀	♀	♀	♀
CHC6	Chinook	♂	♂	♂	♂
CHC7	Chinook	♂	♂	♂	♂
CHC8	Chinook	♂	♂	♂	♂
CHC9	Chinook	♂	♂	♂	♂
CHC10	Chinook	♂	♂	♂	♂
CHM1	Chum	♀	♀	♀	♀

CHM2	Chum	♂	♂	♂	♂
CHM3	Chum	♂	♂	♂	♂
CHM4	Chum	♀	♀	♀	♀
CHM5	Chum	♂	♂	♂	♂
CRC1	Chum	♀	♀	♀	♀
CRC2	Chum	♀	♀	♀	♀
CRC3	Chum	♂	♂	♂	♂
CRC4	Chum	♂	♂	♂	♂
CRC5	Chum	♀	♀	♀	♀
CRC6	Chum	♂	♂	♂	♂
DCO1	Coho	♀	♀	♀	♀
DCO2	Coho	♀	♀	♀	♀
DCO3	Coho	♀	♀	♀	♀
DCO4	Coho	♀	♀	♀	♀
DCO5	Coho	♀	♀	♀	♀
DCO6	Coho	♂	♂	♂	♂
DCO7	Coho	♂	♂	♂	♂
DCO8	Coho	♂	♂	♂	♂
DCO9	Coho	♂	♂	♂	♂
DCO10	Coho	♂	♂	♂	♂
KCH1	Chinook	♀	♀	♀	♀
KCH2	Chinook	♀	♀	♀	♀
KCH3	Chinook	♀	♀	♀	♀
KCH4	Chinook	♀	♀	♀	♀
KCH5	Chinook	♀	♀	♀	♀
KCH6	Chinook	♂	♂	♂	♂
KCH7	Chinook	♂	♂	♂	♂
KCH8	Chinook	♂	♂	♂	♂
KCH9	Chinook	♂	♂	♂	♂
KCH10	Chinook	♂	♂	♂	♂
PNK1	Pink	♂	♂	♂	♂
PNK2	Pink	♂	♂	♂	♂
PNK3	Pink	♂	♂	♂	♂
PNK4	Pink	♀	♀	♀	♀
PNK5	Pink	♂	♂	♂	♂
PNK6	Pink	♂	♂	♂	♂

PNK7	Pink	♂	♂	♂	♂
PNK8	Pink	♀	♀	♀	♀
PNK9	Pink	♀	♀	♀	♀
PNK10	Pink	♂	♂	♂	♂

[1]♂ = Male, ♀ = Female

S2 Table. Sex and species identification results for the archaeological Pacific salmonid samples analyzed in this study.
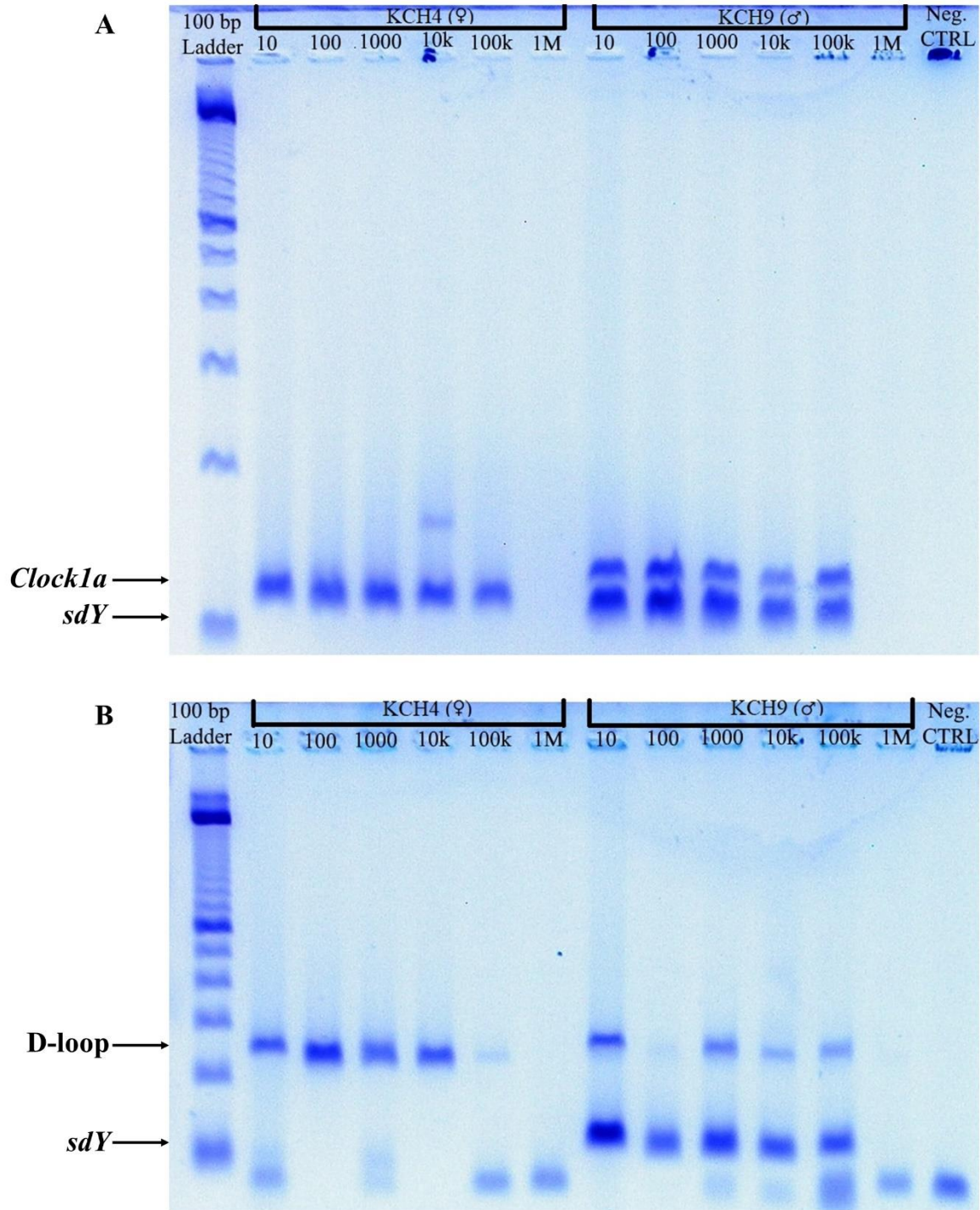
Sample ID	Site	Previous Species ID	Repeat Species ID	*Clock1a/sdY* Assay Sex ID	D-loop/*sdY* Assay Sex ID	Consensus Sex ID
SA2	KC[1]	Chinook	—	PCR Failure	PCR Failure	N[2]
SA4	KC	Sockeye	Sockeye	♂	♂	♂
SA5	KC	Sockeye	—	♀	♀	♀
SA6	KC	Sockeye	—	♂	♂	♂
SA7	KC	Sockeye	Sockeye	♀	♀	♀
SA8	KC	Sockeye	—	♀	♀	♀
SA9	KC	Chinook	—	♂	♂	♂
SA10	KC	Chinook	—	♂	♂	♂
SA11	KC	Sockeye	—	♂	♂	♂
SA12	KC	Chinook	—	♀	♀	♀
SB1	KC	Chinook	Chinook	♂	♂	♂
SB3	KC	Sockeye	—	♀	♀	♀
SB5	KC	Sockeye	—	♀	♀	♀
SB7	KC	Sockeye	—	♂	♂	♂
SB9	KC	Sockeye	—	♀	♀	♀
SB11	KC	Sockeye	—	♀	♀	♀
SB13	KC	Sockeye	—	♂	♂	♂
SB15	KC	Sockeye	—	♂	♂	♂
SB16	KC	Sockeye	—	♀	♀	♀
SB18	KC	Sockeye	—	♂	♂	♂
SB19	KC	Sockeye	—	♂	♂	♂
SBC29	KSM	Rainbow/ Steelhead Trout	—	♂	♂	♂
SBC30	KSM	Rainbow/ Steelhead Trout	Rainbow/ Steelhead Trout	♀	♀	♀
SBC31	KSM	Rainbow/ Steelhead Trout	Rainbow/ Steelhead Trout	♂	♂	♂
SBC32	KSM	Rainbow/ Steelhead Trout	—	♂	♂	♂
SBC33	KSM	Chinook	—	♀	♀	♀

SBC35	KSM	Rainbow/ Steelhead Trout	—	♂	♂	♂
SBC36	KSM	Chinook	—	♂	♂	♂
SBC54	KSM	Rainbow/ Steelhead Trout	—	♂	♂	♂
SBC55	KSM	Rainbow/ Steelhead Trout	—	♂	♂	♂
SD6	KC	Sockeye	—	♂	♂	♂
SD9	KC	Sockeye	—	♀	♀	♀
SD13	KC	Sockeye	—	♂	♂	♂
SD17	KC	Sockeye	—	♀	♀	♀
SD20	KC	Sockeye	—	♂	♂	♂
SD22	KC	Sockeye	—	♂	♂	♂
SD23	KC	Sockeye	—	PCR Failure	PCR Failure	N
SD24	KC	Sockeye	—	PCR Failure/♀	♀	N
SD25	KC	Sockeye	—	♂	♂	♂
SD32	KC	Sockeye	—	♂	♂	♂
SD57	KC	Sockeye	—	♀	♀	♀
SD66	KC	Chinook	—	♀	♀	♀
SD68	KC	Chinook	—	♀	♀	♀
SD70	KC	Sockeye	—	♂	♂	♂
SD76	KC	Sockeye	—	♀	♀	♀
SD77	KC	Sockeye	—	♀	♀	♀
SD78	KC	Sockeye	—	♀	♀	♀
SD79	KC	Sockeye	—	♀	♀	♀
SD80	KC	Sockeye	—	♂	♂	♂
SE1	KC	Sockeye	—	♀	♀	♀
SE9	KC	Sockeye	—	♂	♂	♂
SE15	KC	Sockeye	—	♀	♀	♀
SE21	KC	Sockeye	—	♂	♂	♂
SE23	KC	Sockeye	—	♀	♀	♀
SE25	KC	Sockeye	—	♀	♀	♀
SE35	KC	Sockeye	—	PCR Failure/♂	♀/♂	N
SE40	KC	Sockeye	—	PCR Failure/♀	♀	N
SE45	KC	Sockeye	—	♂	♂	♂

SE47	KC	Sockeye	—	♀	♀	♀
SE48	KC	Chinook	Chinook	♀	♀	♀
SE49	KC	Coho	Coho	♂	♂	♂
SE50	KC	Sockeye	—	♀	♀	♀
SE51	KC	Sockeye	—	♀	♀	♀
SE52	KC	Coho	Coho	♀	♀	♀
ST4	SU	Chum	—	♂	♂	♂
ST7	SU	Chum	Chum	♀	♀	♀
ST10	SU	Chum	—	♂	♂	♂
ST18	SU	Chum	—	♀	♀	♀
ST24	SU	Chum	—	♂	♂	♂
ST28	SU	Chum	—	♂	♂	♂
ST106	SU	Chum	—	♂	♂	♂
ST244	SU	Pink	Pink	♂	♂	♂
ST291	SU	Chum	—	♀	♀	♀
ST531	SU	Pink	—	♂	♂	♂
ST560	SU	Chum	Chum	♀	♀	♀

[1]KC = Keatley Creek (EeRl-7), KSM = Kawumkan Springs Midden (35KL9-12), SU = Say-Umiton (DhHr-18)

[2]♂ = Male, ♀ = Female, N = No sex identity assigned

S1 Fig. Negative images of electrophoresis gels showing the (A) *Clock1a*/*sdY* **(B) D-loop/***sdY***
PCR assay results for dilutions of a modern female (KCH4) and male (KCH9) Chinook
salmon sample.**
The approximate location of the IPC and *sdY* amplicons are indicated by the labelled arrows. The
100 bp ladder used to estimate the size of the amplicons is from Invitrogen (Waltham, MA,
USA).