Ancient DNA Analysis of Archaeological Fish Remains: Methods and Applications

by

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Declaration of Committee

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Abstract

Despite their cultural importance, relatively few ancient DNA (aDNA) studies have focused on fish. Consequently, the methods available for the aDNA analysis of fish remains are underdeveloped relative to those available for other fauna, particularly mammals. This thesis addresses this methodological gap through a series of three projects focused on developing and applying new DNA-based methods for analysis of archaeological fish remains.

The first project presents a DNA-based method for the sex identification of archaeological Pacific salmonid (*Oncorhynchus* spp.) remains. In this method, two PCR assays that each co-amplify fragments of the Y-linked *sexually dimorphic on the Y chromosome* (*sdY*) gene and an internal positive control (*clock1a* or D-loop) are used to assign sex identities to samples. This method's reliability, sensitivity, and efficiency was evaluated by applying it to 72 modern Pacific salmonids from five species and 75 archaeological remains from six species. The results of these tests indicate this method is a reliable and efficient method for the sex identification of Pacific salmonid remains.

Building on the first project, the second project modified the sex identification method developed for Pacific salmonids to make it applicable to archaeological Atlantic salmonid (*Salmo* spp.) and char (*Salvelinus* spp.) remains. This method was subsequently applied to 61 Atlantic salmon (*Salmo salar*) and lake trout (*Salvelinus namaycush*) remains from the 13th century CE Antrex site (AjGv-38) in southern Ontario, Canada. Using this method, we successfully assigned sex identities to 51 of these remains (83.61% success rate), highlighting the method's sensitivity and efficacy.

In the third project, a new two-tiered approach to the DNA-based species identification of archaeological fish remains was developed. In this approach, novel universal primers are first used to amplify a short fragment of the mitochondrial *cytochrome oxidase I* DNA barcode region, which is used to assign an initial taxonomic identification to samples. This initial identification is then used to guide the selection of taxon-specific primers targeting a secondary marker capable of refining the initial identification to the species-level. Application of this method whole or in part to 33 modern fish samples and 89 archaeological fish remains suggests it is an efficient species identification method.

Keywords: Ancient DNA; DNA Barcoding; Environmental Archaeology; Ichthyoarchaeology; Sex Identification; Species Identification

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Chapter 1. Introduction

Fish are a paraphyletic group of vertebrates that includes extant and extinct members of Acanthodii (spiny sharks), Agnatha (jawless fish), Chondrichthyes (cartilaginous fishes), Placodermi (armoured fish), and Osteichthyes (bony fish) (Helfman et al. 2009:3; Nelson et al. 2016). Conventionally, the term fish is used to refer members of these groups that are ectothermic (cold-blooded) aquatic vertebrates with gills and fins (Helfman et al. 2009:3). Since the first putative fish appeared in the Cambrian Period (Benton 2015:46), fishes have diversified into over 34,000 living species, and presently constitute the largest group of vertebrates (Eschmeyer et al. 2018; Nelson et al. 2016:1). Today, fishes can be found around the world in a range of freshwater and marine habitats, as well as almost every habitat in between (Cohen 1970; Helfman et al. 2009:329–354; Nelson et al. 2016:9–11). The species and ecological diversity of fishes has allowed for the global proliferation of fishing among past and present human societies. Despite their cross-cultural importance, few studies have applied ancient DNA (aDNA) analysis to archaeological fish remains (Grealy et al. 2016; Oosting et al. 2019). Consequently, the methods available for the aDNA analysis of fish remains are underdeveloped relative to those available for the aDNA analysis of mammals, and other more ‘charismatic’ fauna. In this thesis, I sought to address this methodological gap by developing and applying new DNA-based methods for the species and sex identification of archaeological fish remains.

1.1. The History and Dietary Importance of Fishing

The consumption of fish by humans is not a recent phenomenon but has a deep history. Small numbers of fish remains have been recovered from early *Homo habilis* and *Homo erectus* sites in East Africa, suggesting humans may have been exploiting fish sporadically since the emergence of *Homo* (e.g., Braun et al. 2010; Stewart 1994). However, the association between *Homo habilis* and *Homo erectus* and fish remains is often tenuous (Erlandson 2001). While earlier hominins possibly opportunistically consumed some fish, more regular harvesting of fish did not occur until the Middle Palaeolithic and emergence of modern humans (*Homo sapiens sapiens*) (Erlandson 2001). Relative to earlier sites, the modern human-associated Blombos Cave on the

coast of South Africa has yielded a moderate amount of fish remains, suggesting fish were being consumed (Henshilwood et al. 2001). Fish remains, some of which were likely accumulated by modern humans, have also been recovered from similarly aged deposits at the coastal Klasies River site located to the west (von den Driesch 2004). As evidenced by the recovery of ~90 kya catfish bones associated with bone harpoons from the inland Katanda locality, Congo-Kinshasa, fish were also being consumed by modern humans in the continent's interior during the Middle Palaeolithic (Yellen et al. 1995). Outside of Africa, stable sulfur, and nitrogen isotope analyses of Tianyuan 1, a 42 to 39 cal thousand-year-old modern human from northern China, indicates this individual ate large amounts of freshwater fish (Hu et al. 2009). In Asia, artifactual and zooarchaeological evidence, suggest that marine-adapted cultures focused on the exploitation of aquatic resource were established in East Timor and Okinawa by the 30,000 years ago (Fujita et al. 2016; O'Connor et al. 2011). The consumption of fish was not limited to modern humans during the Middle Paleolithic. Faunal remains from Figueira Brava, Portugal, indicate the site's Neandertal occupants were routinely consuming substantial quantities of various fish species and other aquatic resources between 86 and 106 kya (Zilhão et al. 2020). Fish remains were similarly important to anatomically modern humans in Europe during the Upper Paleolithic. Recent Bayesian mixing models of stable isotope data from modern humans, for instance, indicate that at Kendrick's Cave —an Upper Palaeolithic site in Wales —20 ± 13% to 27 ± 15% of individual's caloric intake was freshwater fish, whilst 5± 5% to 6±5% of calories were derived from marine fish (Pickard and Bonsall 2020).

While post-dating the Middle Palaeolithic due its later settlement, fishing also has a deep history in the Americas, with some of the earliest people on the continent systematically exploiting fish. Zooarchaeological and chemical analyses of hearth sediment indicate people were regularly consuming both freshwater fish and anadromous salmon, including chum salmon, at the inland Upward Sun River site, Alaska, ca. ∼13,200–11,500 cal BP (Choy et al. 2016; Halffman et al. 2015). Bayesian mixing models of stable isotopic data from human remains from Upward Sun River have confirmed that, while terrestrial protein made up the bulk of individuals' protein (62 ± 8%), salmon were also an important protein source (32 ± 6%) (Halffman, Potter, Mckinney, et al. 2020). Early use of fish by the early inhabitants of the Americas has also been documented within coastal regions of North America. On the Channel Islands off

the coast of California, 78 marine bony fish remains, including remains from rockfish, sculpin, flatfish, greenling, clupeids, and surfperch, have been recovered from Late Pleistocene (ca. 12,000–11,350 cal BP) deposits at CA-SRI-512 (Erlandson et al. 2011). Similarly, in neighbouring Baja California, Mexico, late Pleistocene and early Holocene deposits at the Richard's Ridge (PAIC-49) and Cerro Pedregoso (PAIC-44) sites have yielded abundant remains (*n*=1,495) from a diverse range of fish taxa (*n*=25) (Des Lauriers et al. 2017). The taxa recovered inhabiting a range of habitats including the intertidal, neritic, and oceanic zones, indicating exploitation of a range of fish habitats (Des Lauriers et al. 2017). Moreover, the presence of 11,165–8875 cal BP shell fishhooks at these sites indicates that their inhabitants, even at these early times had specialized fishing technology, attesting to the importance of fish (Des Lauriers et al. 2017). Excavations along South America's western coast have similarly produced mounting evidence that fishing was an important subsistence activity during the Late Pleistocene (Sandweiss 2008). For instance, at Huaca Pretia, Peru, the remains of shark, primarily requiem shark (Carcharhinus sp.), as well as various other animals inhabiting wetlands or the nearshore, but few terrestrial animal remains, have been recovered from Late Pleistocene layers (Dillehay et al. 2017). Similarly, fish predominate late Pleistocene and early Holocene assemblages from the Quebrada Jaguay and the Ring sites in Peru (Reitz et al. 2016).

In both hemispheres, fishing continued to play an important dietary and economic resource in more recent times. In the North America's Pacific Northwest, stable isotope analyses of human remains (Chisholm et al. 1982; Lovell et al. 1986; Schwarcz et al. 2014) and zooarchaeological data (Butler and Campbell 2004) indicate marine fish were a significant protein source for the region's Indigenous peoples throughout the Holocene. Likewise, an abundance of marine fish and other marine taxa and general lack of terrestrial fauna at early through middle Holocene (10,000–3.750 BP) coastal Peruvian sites, reflects the continuing dietary importance of fish (Reitz 2001). Fish were similarly important among groups living in more inland locales in the Americas, where rivers and lakes provided sources for fish. For example, in southern Ontario, stable isotope and zooarchaeological analyses (Feranec and Hart 2019; Hawkins et al. 2019), indicate that fish, particularly large piscivores, composed a significant portion of protein consumed by Late Woodland (ca. 950–200 cal BP) Iroquoian peoples. In South America's Lake Titicaca basin, fish by weight compose 23% to 46% of the faunal assemblages from

Formative Period (3449–450 cal BP CE) sites examined by Capriles et al. (2014), attesting to their dietary significance. In northern Europe, the so-called ca. 1000 CE Fish Event Horizon, characterized by marked increase in the abundance of marine fish remains, particularly gadids, at archaeological site attest to the growing importance of marine fishing during the Medieval Period (Barrett et al. 2004; Orton et al. 2014; Oueslati 2019). On Madagascar, a variety of marine fish predominate (52.46–73.67% *NISP*) assemblages from late Holocene (1400 BP–Present) sites in the southwest portion of the island, highlighting their dietary importance (Douglass et al. 2018). Today, fish continue to be economically important commodity and a staple across the globe. In 2018 alone it its estimated 179 million tonnes of fish were harvested (Food and Agriculture Organization of the United Nations [FAO] 2020:2). In addition, fish and seafood represented $\gtrsim 20\%$ of the animal protein consumed by ~3.3 billion people (FAO 2020:5)

However, the universality of the dietary importance of fish should not be overstated. Among some groups, such as the Hopi of the American Southwest, the Maasai of East Africa, the Tibetans of East Asia, and Tasmanian Aborigines, a dietary aversion to fish has been documented (Simoons 1994). Aversion from the consumption of fish was also likely present in the distant past. Dobney and Ervynyck (2007) suggest that the general absence of fish in Iron Age sites in England and Belgium reflect an aversion to fish consumption among peoples in these areas during this period. While such absence of fish remains at sites from this period could reflect taphonomic biases, they argue recovery of remains from other small-bodied taxa suggest this might not be the case (Dobney and Ervynyck 2007). Even among societies where fish consumption is or was the norm, some sub-groups or sub-cultures may refrain from fish consumption. For instance, priests of the Greco-Roman god Poseidon (Neptune) refrained from consuming fish and other seafoods, despite fish being regularly consumed in Greco-Roman society (Simoons 1994:276). Conversely, in cultures where fish avoidance was commonplace, some individuals, often the poor, did consume fish (Simoons 1994). Often, fish were avoided because they were viewed as unclean or similar to other animals perceived as unclean or because they or the waters they inhabited were seen as sacred (Simoons 1994). In areas of Asia where Buddhism or Jainism were influential, the religious dictate to minimize suffering was instrumental in driving abstinence from fish and many other animal products (Simoons 1994).

1.2. The Non-Dietary Importance of Fish

Like other food sources and subsistence activities, fish and fishing are often conceptualized by archaeologists solely in terms of their dietary significance. However, the cultural importance of fish and fishing extends well beyond their caloric value. Every culture's relationship with fish is embedded within a socio-ecological system through a series of dialectical relationships (Berkes 2011). Through these dialectical links, fishing, fish populations, and other facets of cultural systems all influence each other (Berkes 2011; Cannon 1998; Moss 2012). Consequently, fish and fishing have played a role in shaping many societies' technology, social structure, gender relations, symbolic systems, and relationships with other groups.

1.2.1. Technology

Cross-culturally, the need to increase the efficiency of both the harvesting and processing of fish, as well as minimize risk has stimulated the development of new technologies (Acheson 1981). Notably, it has been hypothesized that the need to efficiently process increasing harvests of aquatic resource, including fish, stimulated the development of pottery in many regions (Haaland 2015). Fishing's role in the development of pottery is supported by the association of early ceramics with fish remains and sites near aquatic environments (Haaland 2015). For example, the San Jacinto 1 site in Colombia, which has yielded some of the earliest pottery in the Americas, contains the remains of various aquatic resources, and is on a floodplain (Oyuela-Caycedo 1995; Stahl and Oyuela-Caycedo 2007). The results of recent residue analyses lend further credence to notion that fishing played a role in the development of ceramics. Residue analyses of early ceramics from many regions in Eurasia, including areas of Northern Europe (e.g., Demirci et al. 2020; Oras et al. 2017), Japan (e.g., Craig et al. 2013; Lucquin et al. 2018), Korea (e.g., Shoda et al. 2017), and the Russian Far East (e.g., Gibbs et al. 2017; Shoda et al. 2020), have identified lipids from aquatic animals, likely fish. In the Americas, residue analyses of early ceramics from the Arctic (e.g., Anderson et al. 2017) and Eastern Woodlands (e.g., Taché and Craig 2015; Taché et al. 2019) indicate they were also used to process aquatic resources.

1.2.2. Social Structure

In many societies, elites owned or controlled various aspects of the chain of production for fish products, which played a role in promoting and maintaining social differentiation. Depending on the society, elite ownership or control variously extended to aquaculture facilities (e.g., Kikuchi 1976; Thompson et al. 2020), fishing grounds (e.g., Kalland 1995:146; Romanoff 1992), fish stocks (e.g., Wilson 2003:35), equipment (e.g., Norr and Norr 1975; Norr and Norr 1978), and trade routes (e.g., Kusimba and Kusimba 2010; Vésteinsson 2016). For instance, in England, sturgeon have been owned by the monarch since the Middle Ages (Wilson 2003:35), with the *Prærogativa Regis* promulgated by King Edward II in 1322 stating the monarch has rights over all "Whales and great Sturgeons taken in the Sea or elsewhere within the Realm, except in certain Places privileged by the King (Parliament of England 1322)". Elites also indirectly asserted control over fish production by financing others' fishing efforts. As a result of the amount of capital required to obtain fishing gear, fishers often received loans from elites or other individuals to purchase equipment (e.g., Walker 2001). In return for loans and/or the provision of equipment, fishers ceded a degree of economic control to their financiers and/or the owners of fishing equipment (Norr and Norr 1975; Norr and Norr 1978; Walker 2001). For example, among the Fanti of Ghana, fishtraders who provided loans to fishers were in a "position of dictating, or at least influencing, the fishing practices of the crews that are in service or debt to them (Walker 2001:164)". Elites' direct or indirect ownerships of aspect of fish production and fishers' cessation of some of their economic self-determination often effectively transformed fishers into a "sea-going proletariat" (Acheson 1981:292).

Through direct or indirect control of all or parts of the chain of production for elites, elites were able to accumulate resources. For instance, in both industrial and pre-industrial fisheries, owners and financiers of fishing gear and equipment were entitled to a portion of the catch, providing them with a pool of surplus fish (Norr and Norr 1975; Norr and Norr 1978). Under the *mafungu* system practiced by the Swahili, one third of the returns less expenses generated by a catch and three shares of the remaining two thirds of the net returns divided among the crew was reserved for the gear owner (Prins 1965:166–167). Such elite-controlled resource pools can promote social complexity as they can be used by in elites in ways that generate and/or maintained their power and status (Roscoe 2008:79–86). Elites, for example, could attract subservient followers by

using the resource pools they control to reward loyal followers with gifts of surplus fish or fishing privileges (cf. Hayden 1990:36; Roscoe 2008:81). For example, in Hawaii the royal-controlled fishponds provided fish that the monarch redistributed to court (Kikuchi 1976). However, such gifts simultaneously socially indebt followers to elites due to expectation of reciprocity , providing elites with a degree of power over the gift recipients (Hayden 1990; Hayden and Gargett 1990). For instance, in return for being provided access to fishing grounds by their feudal lord, Japanese villagers during the Tokugawa shogunate (AD 1600–1868) were forced to perform *corvée* labour or pay taxes in the form of a portion of their catch (Kalland 1995:146). Surplus fish could also be mobilized by elites in displays of conspicuous consumption that symbolically reinforced their status by signalling to others their power and wealth (cf. van der Veen 2003). In the case of the Tsimshian of coastal British Columbia, eulachon, a fish whose runs were controlled by households (Patton et al. 2019), were used in a form of conspicuous consumption that entailed elites burning their oil at feasts (MacDonald and Cove 1987:182–183). Elites, such as those in late Medieval and early modern Iceland (Perdikaris and McGovern 2007; Vésteinsson 2016), also sought to symbolically maintain their position by using the fish resources they controlled to obtain prestige goods.

Certain fish have in various societies being regarded as prestige goods that are emblematic of one's status. For instance, according to the 16th century Dutch fish trader Adriaen Coenen, six different social classes (Poor fisherman, poor people, farmers and labourers, common people, rich and wealthy, skipper and their family and friends) in the Netherlands could be distinguished by the fish and seafood they consumed (Bennema and Rijnsdorp 2015). In Hungary, it has been hypothesized the frequent occurrence of sturgeon at Medieval high-status sites and its absence from peasant sites reflects its status as a prestige good (Bartosiewicz and Bonsall 2008). At Kahikinui, Maui, Hawaii, remains from large fish taxa, specifically groupers (*Epinephelus* spp.), jacks (Carangidae), and sharks (Carcharhinidae), are more common in pre-contact elite households relative to commoner households, suggesting they were luxury foods (Kirch and Jones O'Day 2003). What fishes were regarded as prestigious varied between societies. Previous studies have identified a variety of traits, including size, rarity, degree of associated danger, expense, spiritual significance, taste, and visually attractiveness, as influencing the relative prestige attached to fish species (Bartosiewicz et al. 2008; Fabinyi and Liu 2014; Gault et al. 2008; Ten Eyck 2000). By partaking in fish taxa seen

as luxury goods, elites signal their wealth and power to others, which symbolically reinforce their elevated status and may also attract followers (Hayden 1998:10–11). For example, Živaljević et al. (2019) hypothesizes the presence of sturgeon remains at the royal-founded medieval Studenica Monastery, Serbia, reflects its monks reasserting their high status vis-à-vis their connection to royalty by consuming prestigious sturgeon at feasts with the monarch.

1.2.3. Gender Roles

In fishing societies, the sexual division of labour is often structured by a gendered sea-land dichotomy heavily centred around fishing related activities (Acheson 1981; Schwerdtner Máñez and Pauwelussen 2016; Thompson 1985). Within this dichotomy, the sea is typically viewed as the province of males, with the result being fishing is across cultures typically performed by men (Acheson 1981; Schwerdtner Máñez and Pauwelussen 2016; Thompson 1985). Conversely, land is seen as female space with shoreside activities, such as fish processing, largely being performed by women (Harper et al. 2013; Schwerdtner Máñez and Pauwelussen 2016; Thompson 1985). For instance, prior to World War II, British women referred to as herring girls or lassies, followed male operated herring fleets as they traversed the coast and worked in crews of three to process the catch (Thompson 1985). Other shoreside tasks were likewise frequently performed by women. Among the Fante of West Africa, the fish trade was largely traditionally operated by women who set the price for fish, which they purchased from fisherman on beaches and then processed and resold to middleman (Christensen 1977; Overå 1993; Walker 2002). This pattern continues today with women currently marketing approximately 85–90% of the sardinella (*Sardinella* spp.) landed by Liberian Fante fisherman (Jueseah et al. 2020). The Fante were not alone in this regard, as the fish trade cross-culturally was traditionally a female activity (Acheson 1981; Harper et al. 2013; Schwerdtner Máñez and Pauwelussen 2016). Women in fishing societies also often exercise a high degree of control over household finances and activities due to the prolonged absence of men from the community (Acheson 1981; Harper et al. 2013; Thompson 1985). Take the case of 20th century rural Newfoundland, where a survey by Sinclair and Felt (1992) found that 59.4% of female participants and only 15.3% of male participants reported being responsible for household banking. Similarly, Overå (1993:123) notes that the wives of Fante fisherman were “the bank of the household”.

Control over domestic affairs also means that within fishing societies women are typically the primary caregiver for children and therefore play a critical roles in the social reproduction of society (Ram-Bidesi 2015; Thompson 1985).

Although fishing is often portrayed as solely a male activity, it is important to stress that around the globe women do frequently fish (Chapman 1987; Harper et al. 2013; Harper et al. 2020; Kleiber et al. 2015). Globally, it is estimated 11% ± 4% of present-day small-scaler fishers are women, with the exact proportion regionally varying between 45% ± 15% in Melanesia and 2% ± 1% Western Asia, Northern Africa, and Eastern Europe (Harper et al. 2020). However, the fishing activities undertaken by men and women tend to differ. Cross-culturally, fishing in offshore environments tends be restricted to men, whilst fisheries carried out by women tend to be restricted to nearshore environments (Kleiber et al. 2015). For instance, on Ngazidja Island, Comoros, most of the fishing carried out by women occurs in the reef flats located close to shore (Hauzer et al. 2013). Locating fishing activities within the nearshore allows women to continue to meet other household obligations (Kleiber et al. 2015). As a result of fishing in different environments, men and women also having divergent ecological knowledge bases, with each more familiar with the habitat they exploit (Chapman 1987). Despite having divergent but complementary ecological knowledge, women have historically been widely excluded from the fisheries management decision making processes (e.g., Matsue et al. 2014; Vunisea 2008). In addition to exploiting different habitats, the purpose of male and female fishing activities often differs. The harvests of female-lead fisheries are often retained for household consumption, providing an important source of nutrition (Harper et al. 2013). On Ngazidja Island, for example, 40–100% of the fish landed by female fishers, is consumed by members of their household (Hauzer et al. 2013). Conversely, fish landed by males are typically sold or traded (Harper et al. 2013).

The sea-land sexual division of labour commonly observed in fishing communities is maintained through several mechanisms including the physical separation of the genders, child socialization practices, and belief systems (Acheson 1981; Thompson 1985; Yodanis 2000). However, this sexual division of labour is not immutable as changes in the dynamics of fisheries can prompt a renegotiation of gender roles (e.g., Davis 1993; Godden 2013; Hamilton and Butler 2001; Gopal et al. 2014; Hapke 2001). In Newfoundland, the collapse of the cod fishery in the 1980s and 1990s

has resulted in the societal ideal of maleness no longer being centered around fishing (Davis 1993). Consequently, males on the island are increasingly rejecting fishing as career, preferring instead a service economy job, and women now prefer not to marry individuals working in the fishing economy (Davis 1993). Moreover, with men no longer being at sea for long periods of time fishing, they are increasingly involved in managing the household, a traditionally female domain (Davis 1993). Changes in the dynamics of fisheries have similarly challenged female gender roles in certain regions, such as Kerala, India. Here, the shift of landing sites from beaches in local fishing villages to harbour prompted by fishing mechanization (e.g., installation of outboard motors on boats) has reduced female participation in the fish trade (Gopal et al. 2014; Hapke 2001). Many are unable to travel the long-distances into the city where fish are landed and those who do are limited to trading in small non-wholesale markets, as large wholesale markets are male spaces (Gopal et al. 2014; Hapke 2001).

1.2.4. Symbolic Systems

Unsurprising, given their dietary and socio-economic importance, spiritual and symbolic importance was placed on fish by many past and present cultures. In many cultures, ceremonies or ritual centered around fishing or fish are practiced, highlighting the spiritual and symbolic significance of fish. Examples of such ceremonies include the First Salmon (Gunther 1926) and Sucker (Hunn 1990:55,58; Post 1938:18–19; Spier 1930:148–149). Ceremonies practiced by Indigenous peoples in Northwestern North American, the Argungu Fishing Festival in northern Nigeria (Shyllon 2007), the *mattanaza* in Sicily (van Ginkel 2010), and the making of the *vanua* practiced by fishers in the Batanes Island, Philippines (Mangahas 2010). Fish also figure prominently in the beliefs, stories, and imagery of many religious traditions (Hooke 1961). Within the folk religion of the Tanka people of Hong Kong, various fish, such as sturgeon (*Acipenser sihensis*) and sawfish (*Pristis* spp.), are regard as sacred fish (Anderson 1969). In Christian traditions, canonical and non-canonicals texts are replete with stories and references related to fishing, and fish, many of which likely had deeper symbolic meanings rooted in Judeo-Roman society (Batten 2017; Hanson 1997). For example, there is the story of Jesus feeding a crowd of 5000 with five loaves and two fish (Matthew 14:13–21; Mark 6:34–44; Luke 9:10–17; John 6:1–15) or the use of the phrase “fishers of men” as metaphor for the disciples’ job (Matthew 4:19) (Confraternity of

Christian Doctrine 2011). Furthermore, the ichthys (Figure 1A),a stylized fish with 2nd century CE antecedents (Rasimus 2012), continues to be used in the present day by individuals to identify themselves as Christian. More recently, individuals have appropriated the ichthys and transformed it into the Darwin fish (Figure 1B), which is used to signal a rejection of religion and adherence to scientism (Lessl 2007).

Figure 1. (A) Ichthys symbol used by individuals to identify themselves as Christians. (B) A modified ichthys symbol referred to as a Darwin fish that is used by individuals to signal their belief in scientism and rejection of religion. Modified from figure courtesy of Reaper35 and Wikimedia Commons. Original figure licensed under CC0 1.0.

Archaeological data also attests to the spiritual importance of fish. The symbolic importance of fish to past peoples is exemplified by the inclusion of fish remains or fishing implements in burial and non-mortuary ritual contexts. Such deposits have been identified in Africa (e.g., Brunton et al. 2013), Eurasia (e.g., Brinker et al. 2020; Connor et al. 2017; Molodin et al. 2012; Molodin et al. 2015), and the Americas (e.g., Betts et al.

2012; Klokler 2020; Maxwell 2000). At the Jabuticabeira II shell midden, for instance, Kloker (2020) identified seven 2880 to 1400 year old burials containing over 100 otoliths, which they note is a pattern seen at other Brazilian coastal sites. Artistic depictions of fish further attest to the symbolic important of fish to past peoples (e.g., Bicho et al. 2007; Capriles et al. 2014; Conway 2010:24; Rita and Mainfort 2012). In some instances, archaeological data indicate that the spiritual significance of fish and fishing has a very deep history. In Indonesia, fishhooks fashioned from bivalves have been recovered from a burial on Alor Island dating to the Terminal Pleistocene (ca. 12,000 to 10,000 cal BP), reflecting fishing's long-standing spiritual importance in the region (Connor et al. 2017).

As they are imbued with symbolism and reflective of broader socio-economic systems, fish and fishing in many societies are deeply entangled with and emblematic of social identity. This linkage between fish and identity is highlighted by the etymology of some groups' endonyms being rooted in fish or fishing terminology. For instance, the translation of *Lhuuge Lęą,* the endonym for the Dane-zaa living near Charlie Lake, British Columbia, is 'suckerfish' people (Ridington and Ridington 2013:3). Likewise, *Gitanmaax* , which refers to a Gitx̱san people residing near, Hazelton, British Columbia, means the 'people who fish by torchlight'(Muckle 1998:96). Groups may also use piscine-related terms to refer to other groups, as demonstrated by the use of the term ichthyophagi by ancient Greeks to refer to several groups (Simoons 1994:261). Oftentimes, the use of such terms simultaneously defines both the 'other' and themselves by contrasting their real or imagined relationships with fish (Fischler 1988). Within groups, fish and fishing may also be used as markers for certain sub-groups, such as kin groups. Amongst, the Ojibway there were five clans named after fish: Catfish (*Mizi*), Pike (*Kinozhae*), Sturgeon (*Numae*), Sucker (*Numaebin*), and Whitefish (*Addikmeg*) (Johnston 1976:60).

Since food preferences are culturally informed, fish consumption practices are also symbolic of social identity and play a role in identity formation (Morales-Muñiz and Llorente-Rodriguez 2018; Crabtree 1990; Hesse 1986; Fischler 1988). Through the consumption of fish in prescribed manner informed by a group's foodways, individuals, consciously or unconsciously, assert their belonging to that group (Fischler 1988). This is the case among the Vezo of Madagascar, where a distinction is made between the *Vezo vatane* (true Vezo) and *Vezom-potake* (Vezo of mud) based on the degree to

which individuals rely on fishing versus agriculture (Marikandia 2001). Within the archaeological record, differing social identities may be reflected in variation of the species represented in ichthyofaunal assemblages and their relative importance, as well as differing butchering patterns (Crabtree 1990; Hesse 1986; Ijzereef 1989). Izereef (1989), for example, hypothesized 17th to 18th century AD households in Amsterdam's Waterlooplein with comparably low numbers of fish remains and an absence of eels remains were inhabited by Jews. The absence of eel remains within this household is inferred to reflect individuals adhering to kashrut dietary prohibitions that prohibit the consumption of fish without scales (Ijzereef 1989). Similarly, the differences between the ichthyofaunal assemblage recovered from the Calvert site, home of Maryland's former governor, and nearby site has been suggested to differing social identities (Yentsch 1992). Yentsch (1992) posits the relative high abundance of fish remains and diversity species represented at site reflects the continuation of West African fishing traditions by its slave population. Variation in other dietary indicators that are indicative of fish consumption, such as stable nitrogen and carbon isotope analyses of human remains (e.g., Barrett et al. 2001), likewise may reflect differing social identities.

1.2.5. Intergroup Relations

Fish and fisheries can also play an influential role in shaping in the relationships between groups. Particularly when they are scarce, fish can be a *casus belli* for disputes and conflicts (Pomeroy et al. 2016). In the 20th and 21st century, various conflicts between state actors, such as the Turbot War (1995) between Canada and Spain (Schaefer 1996), the multiple Cod Wars (1952–1956, 1958–1961, 1972–1973, and 1975–1976) between the United Kingdom and Iceland (Steinsson 2016), and the Migingo Island Dispute between Kenya and Uganda (2004–Present) (Rossi 2017), have been triggered by fisheries disputes. These so-called 'fish wars' are not a new phenomenon. Cannon's (1992) analysis of turn of the 20th century ethnographic data from British Columbia's Interior Plateau found intergroup conflict in the region was historically structured by spatial variation in salmon abundance. This analysis indicated Indigenous peoples located further up the Fraser River system, with less access to salmon, raided downstream groups in order to gain access to salmon or slaves that could be traded for salmon (Cannon 1992). The defensibility of Late Period (ca. 3500–200 BP) village sites in the salmon rich Mid-Fraser suggests this pattern of conflict has

pre-Contact origins (Morin et al. 2008; Sakaguchi et al. 2010). As others have noted, fisheries disputes can rapidly destabilize socio-political structures, leading to broader societal transformations (Pomeroy et al. 2016; Sumaila and Bawumia 2014). While fisheries can be a source of intergroup conflict, they can also foster cooperation and collaboration between groups. For instance, in the Interior Plateau extensive trade networks developed as a means of exchanging salmon among other goods, which fostered the development of a regional interaction sphere with shared cultural traits (Cannon 1992; Hayden and Schulting 1997; Rousseau 2004) .

Within colonial societies, fisheries also played an important role in shaping social structures by being an arena throughout which power relations between colonizers and Indigenous peoples are contested. Through laws that limited Indigenous people's fishing rights, colonizers sought to assimilate Indigenous peoples into the colonial economy and society as well as assert sovereignty over both Indigenous peoples and their lands (Harris 2001; Ichikawa 2001). Such laws were commonplace in Canada, where various laws, such as the 1868 *Fisheries Act*, banned the use of traditional fishing methods (Harris 2001:40). Canadian laws also sought to restrict the types of fisheries Indigenous peoples could conduct. Notably, in British Columbia, 19th and 20th century discriminatory laws that explicitly or implicitly prevented Indigenous people from obtaining the licenses required to independently participate in commercial fisheries (Harris 2001). These laws both increased settler access to fisheries by eliminating competition and transformed Indigenous peoples into a labour pool that could be exploited by commercial fisheries and canneries (Harris 2001). In Ghana, legal rulings by the colonial British judges sought to overturn traditional marine tenure systems and implement the European notion that the sea was common property (Walker 2002). Legal regimes that disenfranchised Indigenous peoples from their traditional fishing rights were also presents in other colonial states, such as Japan (Ichikawa 2001), and Norway (Ahrén 1999).

Fisheries have also been a medium through which Indigenous peoples have exerted sovereignty, challenging colonial dominance. Indigenous communities, such as the Paulatuuqmiut in the Northwest Territories (Todd 2014), have resisted government efforts to commercialize their resources for external profit by barring some commercial fisheries in their territories. The revival of traditional fishing practices has also been used to protest colonial control. For instance, in 2008, the Cowichan erected a fish weir—gear outlawed by the Canadian government—on the Cowichan River without federal approval

as a visual testament to their sovereignty over the river's salmon fishery (Dale and Natcher 2015). Furthermore, Indigenous people have contested state control and gained legal recognition of their resource rights through legal cases, such as *R v Marshall* in Canada, initiated over fisheries disputes (Davis and Jentoft 2001). As Davis and Jentoft (2001:224), note court cases recognizing fishing rights are symbolically important to Indigenous peoples as they are "an affirmation of their unique political status within the nation-state."

1.3. Ichthyoarchaeology

Due to their multi-dimensional and cross-cultural importance, a whole subdiscipline of zooarchaeology focused on the analysis of archaeological fish remains has developed. Ichthyoarchaeology is a subdiscipline of zooarchaeology that seeks to explore the dialectical relationships between people, fish, and the environment through the recovery and analysis of archaeological fish remains (Morales-Muñiz and Llorente-Rodriguez 2018). The earliest studies of archaeological fish remains were conducted in the 19th century by scholars such as Rütimeyer (1861) and Sauvage (1875) in Europe and Wyman (1875) in the Americas (See Casteel (1976) for a detailed review of the early history of ichthyoarchaeology.). However, ichthyoarchaeological studies, did not become routine until the second half of the 20th century (Morales-Muñiz and Llorente-Rodriguez 2018). This coincided with Post-war growth in zooarchaeological studies spurred by interpretative frameworks, such as conjunctive and processual archaeology, that sought to move beyond culture history and address questions about lifeways (Reitz and Wing 2008:18–20). Morales-Muñiz and Llorente-Rodriguez (2018) contend that ichthyoarchaeology did not become a full-fledged subdiscipline until the start of the Fish Remains Working Group meetings. These biennial meetings, which began in 1981 in Copenhagen, have grown from 16 participants at first meeting to 62 participants at the latest meeting held in 2019 in Portland (Bartosiewicz and Butler 2020; Morales Muñiz 1996). The geographic origins of scholars attending these meetings has also diversified, with the number of countries represented growing from a few European countries and Israel at the first meeting to 18 countries from across the globe at the most recent edition (Bartosiewicz and Butler 2020; Morales Muñiz 1996) (Figure 2). Morales-Muñiz and Llorente-Rodriguez (2018) credit the Fish Remains Working Group meetings with the

internationalization and formalization of the analysis of archaeological fish remains that has allowed ichthyoarchaeology to emerge a distinct subdiscipline.

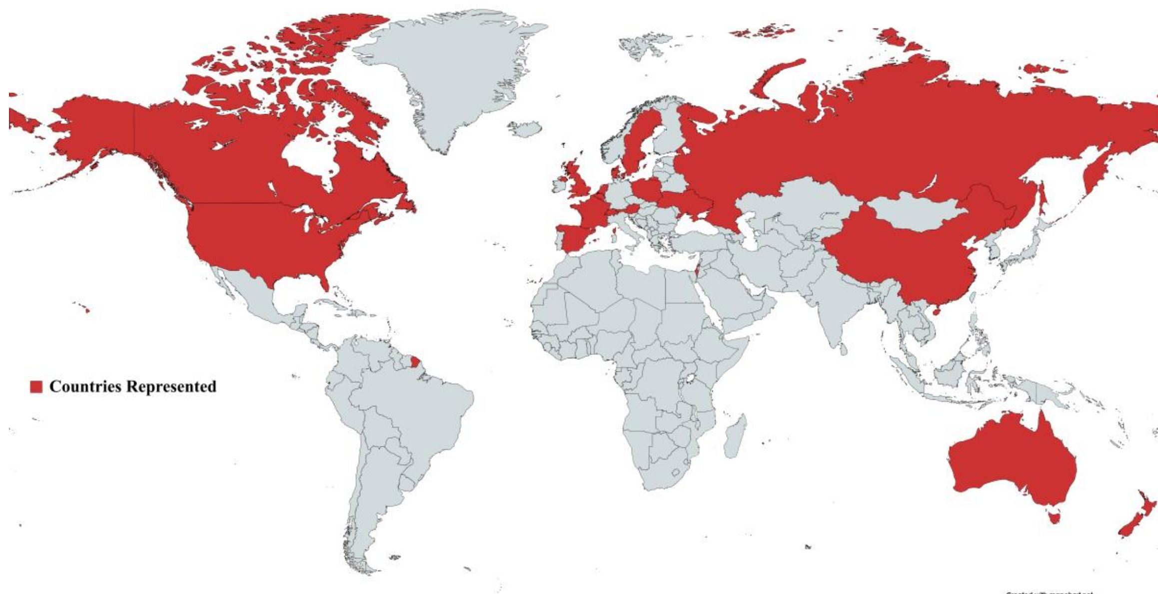

Figure 2. Countries with participants at the 20th Meeting of the Fish Remains Working Group held from August 26th–30th, 2019, in Portland, Oregon, USA. Data compiled from Butler et al. (2019). Figure created with MapChart (http://www.mapchart.net) and licensed under CC BY-SA 4.0.

1.4. Ancient DNA and Ichthyoarchaeology

For much of its history, morphological and metric analyses of fish remains have been the foundation of most ichthyoarchaeological studies (See Casteel (1976) ;and Wheeler and Jones (1989) for overviews of the morphological and metric methods conventionally used to analyze fish remains). Today, however, ichthyoarchaeological studies are also increasingly drawing upon data generated through the analysis of biomolecules recovered from archaeological fish remains (Morales-Muñiz and Llorente-Rodriguez 2018). Biomolecules are organic molecules synthesized by living organisms, with most biomolecules found in organisms belonging to one of four classes: carbohydrates, lipids, nucleic acids, and proteins (Brown and Brown 2011:4–5). In recent years, various biomolecular analyses, including aDNA analysis (See Oosting et al. (2019) for review), peptide mass fingerprinting or zooarchaeology by mass spectrometry (ZooMS) (e.g., Harvey et al. 2018; Guiry, Buckley, Orchard, Hawkins, et al. 2020; Richter et al. 2011; Korzow Richter et al. 2020), and stable isotope analyses of bone

collagen (e.g., Barrett et al. 2008; Braje et al. 2017; Fuller et al. 2012; Guiry, Buckley, Orchard, Hawkins, et al. 2020; Guiry, Royle, Matson, et al. 2020; Guiry, Royle, Orchard, et al. 2020; Häberle et al. 2016), have been used to study archaeological fish remains. Interest in the aDNA analysis of ichthyoarchaeological remains, in particular, has seen marked growth since its earliest application to archaeological materials in the late 1990s and early 2000s (e.g., Butler and Bowers 1998; Ciesielski et al. 2002). Following this pioneering work, both the number of studies involving the aDNA analysis of fish remains and the range of questions being address through such analyses has increased. Through aDNA analysis, researchers have been able to identify the species, population affinity, and sex of individual fish remains, and assess the genetic and phenotypic diversity of past fish populations.

1.4.1. Preservation of DNA in Ancient Fish Remains

Within mammals, the preservation of DNA in bone has been observed to often correlate with bone density, with dense bones, such as the petrous pyramid, exhibiting the best DNA preservation (e.g., Alberti et al. 2018; Gamba et al. 2014; Leney 2006). As Star (2017) notes, based on this relationship observed in mammals, some have presumed, on account of their low bone density, that DNA preservation in fish bones is poor. Morales-Muñiz and Llorente-Rodriguez (2018), for instance, note that DNA preservation in fish bones is poor due to "the porous nature of fish bones that facilitates "drainage" of molecules into the sediment." However, multiple studies have demonstrated that archaeological fish remains often exhibit exceptional DNA preservation, making them an ideal sample-type for aDNA analysis. Studies using PCR to amplify mitochondrial DNA markers routinely achieve amplification success rates greater than 70%, with success rates above 90% not uncommon (e.g., Nicholls et al. 2003; Rodrigues et al. 2018; Yang et al. 2004; Cannon and Yang 2006). Despite having a low copy number, high success rates for the amplification of nuclear DNA markers have also been routinely achieved for archaeological fish bones (e.g., Hutchinson et al. 2015; Royle et al. 2018; Royle et al. 2020; Speller et al. 2012). These high amplification success rates may reflect the fact that fish remains appear to often contain relatively high levels of endogenous DNA (e.g., Boessenkool et al. 2017; Ferrari et al. 2021; Star et al. 2017). For instance, a whole-genome study of Viking Age (800–1066 CE) cod remains by Star et al. (2017) found that endogenous DNA accounted for 15% to 46% of

the DNA extracted from 15 of the 19 analyzed samples. Ferrari et al. (2021) hypothesize that the lack of bone remodelling among most extant bony fish may contribute to the well-preserved DNA frequently observed in archaeological fish remains. Unlike mammals, recent high-throughput sequencing of a large-sample of archaeological Atlantic cod remains (*n*=204) from 38 sites in Europe suggests DNA preservation in fish remains does not significantly vary between elements (Ferrari et al. 2021).

Although they often exhibit good DNA preservation, fish remains, like all biological remains, undergo DNA degradation processes that reduce the length, quantity, and quality of their DNA molecules. The primary process responsible for DNA fragmentation is depurination (Dabney et al. 2013). Depurination results in the formation of abasic sites through the hydrolytic cleavage of the glycosidic bond between a purine (adenine or guanine) and the sugar-phosphate backbone of DNA (Dabney et al. 2013; Lindahl 1993). These abasic sites then undergo β-elimination, which causes chain breaks that fragment DNA molecules (Dabney et al. 2013; Lindahl 1993). Consequently, most of the DNA molecules recovered from ancient remains are less than 500 bp long (Dabney et al. 2013). Ultimately, such fragmentation will result in few or no DNA molecules being present in ancient fish remains, leading to low analytical success rates or complete failure. This is particularly true in warm regions, such as the tropics (e.g., Hlinka et al. 2002; Kemp and Huynen 2014), since depurination and chain breakages rates are positively correlated with temperature (Lindahl and Nyberg 1972; Lindahl and Andersson 1972). For instance, a fragment of the *16S rRNA* gene could only be amplified from 1 of the 51 archaeological fish remains from late Holocene contexts in subtropical southeast Queensland, Australia, examined by Hlinka et al. (2002). As depurination is also acid-catalyzed (Lindahl and Nyberg 1972), fish remains from archaeological contexts, such as shell middens, with alkaline or neutral soils conditions often exhibit good DNA preservation (e.g., Cannon and Yang 2006; Ferrari et al. 2021; Puncher et al. 2019; Yang et al. 2004). Like depurination, blocking lesions formed through oxidative damage and cross-linking that prevent polymerase extension will also reduce the length of amplifiable templates (Dabney et al. 2013). In addition to processes the reduce the amount and length of DNA molecules, ancient remains are also subject to degradation processes that alter their nucleotide sequence (Dabney et al. 2013). This is predominately the results of miscoding lesions caused by cytosine deamination, which

results in cytosine to thymine and guanine to adenine transitions by converting cytosine to uracil (Dabney et al. 2013; Lindahl 1993).

1.4.2. Applications of aDNA Analyses of Fish Remains

Species Identification

The species identification of fish remains is foundational to ichthyoarchaeological studies. However, for various reasons, identifying fish remains to the species-level using traditional morphology-based approaches is often difficult (Wheeler 1978). Since the morphology of many skeletal elements does not vary between closely related species, it is not possible to assign species identifications to many elements through morphological analysis (Wheeler 1978). Even when *inter*specific morphological differences exist, *intra*specific variation can obscure the magnitude of these differences, complicating the delineation of species through skeletal morphology (Gobalet et al. 2004; Thieren et al. 2016). The fragmentation of fish remains further complicates morphology-based species identification as it often results in the loss of taxonomically informative features (Colley 1990). Due to their relatively weak and labile collagen ultrastructure, fish remains are particularly susceptible to such damage (Szpak 2011). The species identification of archaeological fish remains through morphological analyses can also be hindered by factors extrinsic to the remains. These factors include the existence of undescribed species, time limitations on analyses, lack of access to comprehensive comparative collections, high fish biodiversity in the vicinity of an archaeological site, and gaps in individual researchers' ichthyological knowledge (Colley 1990; Cooke and Jiménez 2004; Gobalet 2001; Wake 2004; Wheeler 1978).

Due to these barriers to morphology-based species identification, researchers are increasingly using aDNA analysis to identify fish remains (Table 1**)**. The identification of fish remains through aDNA analyses is made possible by the fact that genetic variation exists between fish species. Interspecific genetic variation exists regardless of whether an element exhibits taxonomically informative traits. Consequently, unlike conventional zooarchaeological analysis, DNA-based approaches can be used to identify heavily fragmented fish remains and elements lacking taxon-specific morphological traits. For example, Yang and colleagues (e.g., Cannon and Yang 2006; Cannon et al. 2011; Ewonus et al. 2011; Guiry, Royle, Matson, et al. 2020; Speller et al.

2005; Yang et al. 2004) have used aDNA analyses to assign species identifications to Pacific salmon (*Oncorhynchus* spp.) vertebrae, which lack species-specific morphological features. In DNA-based species identification approaches, taxonomic identifications are assigned to samples by comparing their sequence and/or profile for a genetic marker or markers to that of reference specimens of known taxonomy. A sample is assigned to the species its sequence(s) and/or profile(s) for the analyzed marker(s) match or most closely resemble. While aDNA analysis can and has been used to identify fish remains from a variety of taxonomic groups, there has been substantial focus on identifying salmonid and sturgeon remains (Table 1).

A variety of genetic markers have been used to assign ancient fish remains to species (Table 1). However, as mitochondrial DNA (mtDNA) has a higher copy number per cell than nuclear DNA (nuDNA) (1000s of copies vs 2 copies) and thus more likely to preserve (Brown and Brown 2011:16,117), most studies have analyzed mtDNA markers (Table 1). Nonetheless, a handful of studies have assigned species-level identifications through the analysis of nuDNA markers in conjunction with mtDNA markers (Table 1). Since DNA degradation generally precludes the amplification of large fragments from ancient remains, the markers used to identify fish remains typically consists of fragments of DNA less than 300 bp long (Brown and Brown 2011:118). However, high-throughput sequencing (HTS) or next-generation sequencing approaches now enable the entire genome and mitochondrial genome of samples to be sequenced (Hofreiter et al. 2015), which can facilitate species identification. For example, de Flamingh et al. (2018) were able to obtain complete mitogenome sequences for four Pacific salmon remains recovered from the 49-KEN-147, Alaska. By analyzing these mitochondrial genome sequences, they were able to confirm the species identifications assigned to them through the analysis of short fragments of the mitochondrial *cytochrome b* gene and D-loop (de Flamingh et al. 2018).

Table 1. Published studies that have used aDNA analysis to identify ancient fish remains. The marker(s) used by a study to identify the remains is indicated with a checkmark.

Study	Focal Family/Group	Country	Mitochondrial DNA								Nuclear DNA			
			COI[1]	*Cytb*	D-loop	*NADH1*	*NADH5*	*12S*	*16S*	Whole mitogenome	*ATPase*	*ITS1*	Microsatellites	Sequence Flanking Microsatellite
Brosse et al. (2009)	Acipenseridae	France		✓										
Chassaing et al. (2013)	Acipenseridae	France		✓	✓								✓	
Grindle et al. (2021)	Acipenseridae	USA		✓										
Fopp-Bayat (2005)	Acipenseridae	Poland		✓										
Ludwig et al. (2008)	Acipenseridae	Germany			✓									✓
Ludwig, Makowiecki, et al (2009)	Acipenseridae	Poland			✓									
Ludwig, Ardnt, et al. (2009)	Acipenseridae	Portugal/Spain			✓									
Macheridis et al. (2020)	Acipenseridae	Denmark	✓											
Nikulina and Schmölcke (2016a)	Acipenseridae	Germany		✓										
Nikulina and Schmölcke (2016b)	Acipenseridae	Germany		✓										
Pagès et al. (2008)	Acipenseridae	France		✓										
Popović et al. (2014)	Acipenseridae	Poland		✓	✓		✓	✓	✓				✓	
Robles et al. (2010)	Acipenseridae	Spain						✓						
Thieren et al. (2016)	Acipenseridae	Belgium/Netherlands/UK		✓									✓	
Shirak et al. (2013)	Cichlidae	Israel	✓											
Shepherd and Campbell (2021)	Chondrichthyes	New Zealand	✓											
Živaljević et al. (2017)	Cyprindae	Serbia		✓							✓			
Bas et al. (2020)	Merlucciidae	Argentina		✓										
Palmer et al. (2018)	Osmeridae	USA						✓						
Brzuzan et al. (2004)	Salmonidae	Poland			✓	✓								
Butler and Bowers (1998)	Salmonidae	USA			✓									
Cannon and Yang (2006)	Salmonidae	Canada		✓	✓									
Cannon et al. (2011)	Salmonidae	Canada	**Details Not Reported**											
Ciesielski and Brzuzan (2003)	Salmonidae	Poland			✓									
de Flamingh et al. (2018)	Salmonidae	USA		✓	✓					✓				
Ewonus et al. (2011)	Salmonidae	Canada		✓	✓									
Ewonus et al. (2020)	Salmonidae	Canada		✓	✓									
Grier et al. (2013)	Salmonidae	Canada						✓						
Guiry, Royle, Orchard, et al. (2020)	Salmonidae	USA		✓	✓									
Guiry, Royle, Matson, et al. (2020)	Salmonidae	Canada		✓	✓									

Study	Focal Family/Group	Country	Mitochondrial DNA								Nuclear DNA			
			COI[1]	***Cytb***	**D-loop**	***NADH1***	***NADH5***	***12S***	***16S***	**Whole mitogenome**	***ATPase***	***ITS1***	**Microsatellites**	**Sequence Flanking Microsatellite**
Halffman et al. (2015)	Salmonidae	USA	✓	✓	✓			✓						
Halffman et al. (2020)	Salmonidae	USA						✓						
Johnson et al. (2018)	Salmonidae	USA						✓						
Kemp et al. (2014)	Salmonidae	Canada						✓						
Kemp et al. (2020)	Salmonidae	USA						✓						
Korzow Richter et al. (2020)	Salmonidae	USA		✓	✓									
Lanman et al. (2021)	Salmonidae	USA						✓						
Lepofsky et al. (2007)	Salmonidae	Canada	**Details Not Reported**											
Monroe et al. (2013)	Salmonidae	Canada						✓						
Morin et al. (2021)	Salmonidae	Canada		✓	✓									
Moss et al. (2014)	Salmonidae	USA						✓						
Royle et al. (2018)	Salmonidae	Canada/USA			✓									
Royle et al. (2020)	Salmonidae	Canada		✓	✓									
Speller et al. (2005)	Salmonidae	Canada		✓	✓									
Splendiani et al. (2016)	Salmonidae	Italy			✓									
Stevenson and Butler (2015)	Salmonidae	USA	**Details Not Reported**											
Thompson et al. (2019)	Salmonidae	USA		✓	✓									
Yang et al. (2004)	Salmonidae	Canada		✓	✓									
Yang and Speller (2006)	Salmonidae	Canada		✓	✓									
Puncher et al. (2019)	Scombridae	Spain/Turkey	✓		✓							✓		
Rodrigues et al. (2018)	Sebastidae	Canada			✓				✓					
Nicholls et al. (2003)	Serranidae	Cook Islands							✓					
Douglass et al. (2018)	Multiple	Madagascar						✓						
Grealy et al. (2016)	Multiple	Madagascar						✓						
Hlinka et al. (2002)	Multiple	Australia							✓					
Seersholm et al. (2018)	Multiple	New Zealand						✓	✓					

[1]*COI=Cytochrome oxidase I, Cytb=Cytochrome b, NADH1=NADH dehydrogenase subunit 1, NADH5=NADH dehydrogenase subunit 5, 12S=12S rRNA, 16s=16s rRNA, ATPase=ATP synthase beta subunit, ITS1=Internal transcribed spacer 1*

HTS has also revolutionized the DNA-based species identification of fish remains by enabling bulk-bone metabarcoding (BBM) (e.g., Grealy et al. 2016; Seersholm et al. 2018). Whereas conventional genetic approaches involve extracting and analyzing DNA from each sample individually, BBM entails the processing and analysis of multiple samples in tandem (Murray et al. 2013). In BBM, a fragment of DNA is PCR amplified from a mixed DNA sample consisting of DNA co-extracted from a pool of samples and then sequenced through HTS (Murray et al. 2013). By analyzing multiple samples in tandem, BBM allows researchers to rapidly identify the range of fish species represented in an assemblage (e.g., Grealy et al. 2016; Seersholm et al. 2018). Moreover, pooling samples together also reduces overall analytical costs (Murray et al. 2013). Using this approach, Seersholm et al. (2018) was able to identify the fish species represented at 650 to 150 year-old Māori sites from across New Zealand, enabling an examination of geographic variability in fishing practices. Their analysis found that the species composition of Māori fisheries varied with latitude, with pronounced species composition differences existing between fisheries on the country's North and South Island (Seersholm et al. 2018). In Madagascar, Grealy et al. (2016) were able to identify the presence of 23 families of fishes in a 100- to 300-year-old midden.

Although it is a cost-effective and powerful DNA-based species identification method, BBM does have some limitations (see Grealy et al. (2015) for an in-depth discussion). First, as it involves pooling multiple fish remains, species identification cannot be connected back to a particular sample, erasing the ability to cross-reference other data (e.g., stable isotope data, size reconstruction) and species identity. Second, BBM only provides information about the presence or absence of fish species in assemblage, and not their relative abundance (Grealy et al. 2015). Finally, PCR biases can result in differential amplification between taxa, impeding the detection of some taxa through BBM (Grealy et al. 2015). However, this limitation can be overcome by using multiple primers sets for PCR-based BBM or relying on shotgun sequencing (Grealy et al. 2015).

Through the DNA-based species identification of archaeological fish remains, archaeologists can address a range of questions regarding past peoples. First and foremost, the genetic assignment of species-level identifications to fish remains provide information about the range of species harvested by past fisheries, and if a non-BBM approach is used, their relative importance. By applying aDNA analysis to smelt

(Osmeridae) remains from five late pre-contact and protohistoric sites in northern California, Palmer et al. (2018), for instance, determined that surf smelt (*Hypomesus pretiosus*) was the focus of each site's smelt fishery. Conversely, the aDNA analysis of Pacific salmon remains from a series of sites on British Columbia's Central Coast found that the composition and focus of fisheries varied between sites (Cannon et al. 2011). When the biology of the identified species is considered, DNA-based species identifications can also shed light on other aspects of fishing strategies, such as the seasonality (e.g., Ewonus et al. 2011; Grier et al. 2013) and catchment area of fisheries (e.g., Nicholls et al. 2003). For example, as the various Pacific salmon (*Oncorhynchus* sp.) species run at different times, the genetic species identifications of salmon remains has been used to infer the seasonality of fisheries and by association sites (e.g., Ewonus et al. 2011; Grier et al. 2013). On the Cook Islands, aDNA analysis of serranid remains found that most were inshore species, suggesting fishing took place primarily within the inner rather than outer-reef or deep-water (Nicholls et al. 2003). Reflecting the dialectical relationship between fishing and other aspects of society (see above), the species identification of fish remains can also shed light on broader social structures. At Keatley Creek, British Columbia, Speller et al. (2005) investigated differential access to fish resources through the DNA-based species identification of Pacific salmon remains from housepits of different statuses.

The DNA-based species identification of archaeological fish remains can also provide insights into aspects of the historical ecology of fish species, notably their past range (e.g., Brosse et al. 2009; Chassaing et al. 2013; Ludwig et al. 2008; Ludwig, Arndt, et al. 2009; Ludwig, Makowiecki, et al. 2009; Nikulina and Schmölcke 2016a; Nikulina and Schmölcke 2016b; Popović et al. 2014; Robles et al. 2010; Živaljević et al. 2017). Such studies have important implications for modern conservation efforts as they establish baseline of what species were present in area before anthropogenic impacts (e.g., Brosse et al. 2009). For instance, while Atlantic sturgeon was historically thought to be confined to the east coast of North America, aDNA analysis of archaeological and museum sturgeon specimens indicate it historically had much wider range (Chassaing et al. 2013; Ludwig et al. 2002; Ludwig, Makowiecki, et al. 2009; Nikulina and Schmölcke 2016a; Nikulina and Schmölcke 2016b; Popović et al. 2014). These analyses found that prior to the 20th century it also inhabited river systems draining into the French Atlantic coast as well as the Baltic, and North Sea (Chassaing et al. 2013; Ludwig et al. 2002;

Ludwig, Makowiecki, et al. 2009; Nikulina and Schmölcke 2016a; Nikulina and Schmölcke 2016b; Popović et al. 2014). Similarly, aDNA analysis of cyprinid remains from the Danube Gorges demonstrated that range of vyrezub (*Rutilus Frisii*) extended into the Middle and Lower Danube during Mesolithic and Neolithic, whereas it is presently only found in its upper reaches (Živaljević et al. 2017). The species identification of fish remains also opens up to the door to additional aDNA (e.g., Thompson et al. 2019) and stable isotopes analyses (e.g., Bas et al. 2020; Guiry, Royle, Orchard, et al. 2020) that provide additional details about species' historical ecology. By conducting stable carbon and nitrogen analysis on genetically identified Atlantic salmon (*Salmo salar*) remains from New York, Guiry et al. (2020) demonstrated that the now extirpated Lake Ontario Atlantic salmon spawning in the state were potamodromous. In Argentina, Bas et al. (2020) documented temporal changes in the diet breath of Argentine hake (*Merluccius hubbsi*) by conducting stable isotope analysis on genetically identified remains.

In recent years, an emerging tend in ichthyoarchaeological studies has been validating new species identification methods through aDNA analysis. Recently, Korzow Richter et al. (2020) demonstrated the reliability of ZooMS as species identification method for Pacific salmon by confirming their ZooMS-based species identification through aDNA analysis. Conversely, discrepancies between the species identities assigned to Pacific salmon remains through a morphometric method (Huber et al. 2011) and aDNA analysis, indicated this morphometric method was unreliable (Moss et al. 2014). aDNA analysis can also be used as a quality control method for conventional morphology-based identification approaches (Wolverton 2013). For instance, by using aDNA analysis, Thieren et al. (2016) found that 13% of the sturgeon scutes they morphologically identified as Atlantic or European sturgeon, respectively, were the other species. This suggests that the morphological trait—surface patterning— used to distinguish scutes from these species is not diagnostic (Thieren et al. 2016).

Ancient DNA analysis is undeniably a powerful tool for determining the species identity of fish remains. However, it is not without limitations. Genetic species identification approaches are dependent on the existence of significant DNA sequence variation between species. However, some closely related species do not exhibit significant interspecific genetic variation within some markers, making the unambiguous identification of such taxa through aDNA analysis difficult (cf. Ward et al. 2009). For

example, a fragment of mitochondrial *12S RNA* gene frequently used for the species identification of ichthyofaunal remains (e.g., Grier et al. 2013; Johnson et al. 2018; Moss et al. 2014; Palmer et al. 2018) cannot differentiate pink (*Oncorhynchus gorbuscha*) and some masu salmon (*Oncorhynchus masou*) due to haplotype sharing (Jordan et al. 2010). A lack of significant genetic differentiation between species can be the result of a multitude factors. In instances where species have recently diverged (i.e., following the Last Glacial Maximum), there may have been insufficient time for the accumulation of inter-specific mutational differences and lineage sorting (Ward et al. 2009). In addition, interspecific hybridization, which is not uncommon among fishes (Montanari et al. 2016; Scribner et al. 2000), can result in the introgression of DNA from one species into another, complicating the genetic delineation of species (Ward et al. 2009). Such hybridization is particularly problematic for DNA-based species identification using mtDNA markers. As mtDNA is maternally inherited, hybrids cannot be detected with mtDNA markers and individuals with hybrid ancestry will be identified as the maternal species (Ward et al. 2009). To detect hybrids, the analysis of nuDNA markers is required (Ward et al. 2009) but are less likely to be amplifiable due to nuDNA's low copy number. Nonetheless, nuDNA markers have been successfully used to identify archaeological fish remains as hybrids. For instance, to identity archaeological sturgeon remains with hybrid ancestry, researchers frequently analyze nuclear microsatellites or the sequence of the region flanking a microsatellite in conjunction with mtDNA markers (e.g., Chassaing et al. 2013; Ludwig et al. 2008; Popović et al. 2014). Using this approach, Popović et al. found that (2014) 52% of the pre-Roman and 34% of the Medieval samples from the Baltic they identified as Atlantic sturgeon through the analysis of mtDNA had European sturgeon (*Acipenser sturio*) ancestry. Hybridization can also in some instances results in hybrid speciation, with the new species having markers similar to those of the parent species (Unmack et al. 2014).

Population Affinity

In the context of ichthyoarchaeology, geographic provenancing refers to the identification of the location where the fish represented at an archaeological site were caught (cf. Lubinski and Partlow 2012). Such geographic provenance data allows archaeologists to document the long-distance transport or trade of fish (e.g., Van Neer et al. 2004) and identify fisheries' catchment areas (e.g., Lepofsky et al. 2007). Information regarding the catch location of fish can also be used to establish the historical presence

of now extirpated species in specific regions (e.g., Miller et al. 2011; Stevenson 2011). To ascertain the geographic origins of archaeological fish remains, archaeologists have traditionally used a variety of methods (See Lubinski and Partlow 2012 for review.). The presence of exotic taxa at archaeological sites has historically been a key indicator for the use of non-local fish (Lubinski and Partlow 2012; Van Neer et al. 2004). However, the range of many taxa have shifted over time, which can hinder the applicability of to species' whose past distribution is not known (Lubinski and Partlow 2012; Van Neer et al. 2004). Moreover, the importation of locally available taxa from non-local sources, such as the import of Newfoundland Atlantic cod into Britain (e.g., Hutchinson et al. 2015), is invisible with this method. The relative abundance of different skeletal elements has also been used by archaeologists to infer whether a particular species was harvested locally or transported to the site (Lubinski and Partlow 2012). As fish are often decapitated prior to transport, it has often been suggested that taxa whose post-cranial elements are overrepresented in assemblage, represent fish that were transported from non-local sources (Lubinski and Partlow 2012). However, processes other than preparation for transport can also result in an underrepresentation of cranial elements and overrepresentation of post-cranial elements. Notably, if a taxon's cranial bones are less dense than their vertebrae, density-mediated destructive taphonomic process may also reduce the relative abundance of cranial elements (Butler and Chatters 1994). The skeletal-part abundance approach is also hindered by the fact that some fish were transported whole, resulting in their cranial bones being underrepresented despite being transported (Lubinski and Partlow 2012).

More recently, zooarchaeologists have used isotope and elemental analyses to provenance fish remains (e.g., Barrett et al. 2008; Barrett et al. 2011; Disspain et al. 2012; Dufour et al. 2007; Hutchinson et al. 2015; Miller et al. 2011; Nehlich et al. 2013; Orton et al. 2011). As the chemical composition of fish tissues reflects the chemistry of their aquatic environments, fish remains can be provenanced through isotope and elemental analyses (Disspain et al. 2015; Guiry 2019; Lubinski and Partlow 2012). For instance, through the stable carbon and nitrogen analysis of bone collagen, Barrett and colleagues (e.g., Barrett et al. 2008; Barrett et al. 2011; Hutchinson et al. 2015; Orton et al. 2011) have identified the geographic source of Atlantic cod remains at European archaeological sites, providing insights into the history of the cod trade. However, diagenetic processes that alter the chemical composition of remains (e.g., Andrus and

Crowe 2002; Miller et al. 2011; Proctor and Thresher 1998) and overlaps in the water chemistry of different areas (Guiry 2019; Fuller et al. 2020) can impede geographic provenancing through chemical analyses.

In light of the limitations of other geographic provenancing approaches, there has been interested in using DNA-based methods to help provenance fish remains. Due to the existence of inter-population genetic variation (e.g., Hartman et al. 2019; Lewallen et al. 2016; Yi et al. 2019; Winters et al. 2010), it is possible to assign individual fish to population by ascertaining the population with which it exhibits the highest genetic affinity (Hansen et al. 2001; Ogden 2008). Assessing the relationship of individual specimens to populations can be accomplished through various methods or combinations of method, including assignment tests (e.g., Ludwig et al. 2008), principal component analysis (e.g., Star et al. 2017), and the construction of phylogenetic trees (e.g., Živaljević et al. 2017) and networks (e.g., Wooller et al. 2015). Genetic approaches have been widely used in modern contexts to assign fish to populations or stocks (Hansen et al. 2001). Although widely used in modern contexts, only a handful of studies have used DNA-based approaches to provenance fish remains (e.g., Arndt et al. 2003; Hutchinson et al. 2015; Star et al. 2017). The first such study was conducted by Arndt et al. (2003), who used aDNA analysis to provenance African sharptooth catfish (*Clarias gariepinus*) from the Roman and Byzantine layers at Sagalassos, Turkey. Through the phylogenetic analysis of a fragment of the mitochondrial control region, Arndt et al. (2003) sourced these remains to the Lower Nile. More recently, researchers have used aDNA analysis to identify the origins of cod remains in order to trace the emergence of the historic cod trade. By genotyping samples for 28 nuclear SNPs, Hutchinson et al. (2015) were able to source cod remains from the wreck of the 16th century AD Mary Rose. These data indicated the specimens were from non-local sources in the northern North Sea, Barents Sea/Iceland, and Newfoundland. By combining the genetic data with stable carbon and nitrogen isotope data obtained from the same samples, it was possible to confirm and in some instances further refine their geographic origin (Hutchinson et al. 2015). More recently, Star et al. (2017) determined the geographic origin of Viking Age and Medieval cod remains from sites in northern Europe, by genotyping individuals for a large number of SNPs and inversion loci.

Documenting the population affinities of ancient specimens has also been used to document the historical phylogeography of fish populations. In Europe, multiple

studies have investigated historical relationships of sturgeon populations through the aDNA analysis of archaeological and archival samples (e.g., Chassaing et al. 2013; Ludwig et al. 2002; Ludwig et al. 2008; Ludwig, Makowiecki, et al. 2009; Nikulina and Schmölcke 2016a; Popović et al. 2014). For instance, by analyzing microsatellites and mtDNA control region sequences, researchers have been able to determine the relationships between the now extinct Baltic population of Atlantic sturgeon and extant conspecific populations (Ludwig et al. 2002; Ludwig et al. 2008; Popović et al. 2014). These results indicate this extinct population is most closely related to present-day populations in eastern Canada, suggesting they are the founding source of the historic Atlantic sturgeon population in the Baltic Sea (Ludwig et al. 2002; Ludwig et al. 2008; Popović et al. 2014). Genetic analysis of archaeological fish remains have similarly been used to document the genetic affinities of the vyrezub population that occupied the Middle and Lower Danube during the Neolithic and Mesolithic (Živaljević et al. 2017). Through phylogenetic analyses of short *cytochrome b* and *ATPase* sequences obtained from archaeological specimens, Živaljević et al. (2017) determined that this extirpated vyrezub population was most closely related to present-day populations in the Upper Danube. In Alaska, Wooller et al. (2015) conducted aDNA analysis on a 8820 cal year old northern pike in order to determine its phylogenetic relationships and investigate the species' post-glacial dispersal. Phylogenetic analysis of control region and *cytochrome b* sequences obtained from this individual indicated they shared a haplotype with modern populations located south of the Wisconsin ice sheet (Wooller et al. 2015). Wooller et al. (2015) suggest this indicates that following deglaciation northern pike dispersed southward from Beringia. Understanding the historical phylogeography of fish populations has important implications for modern fish conservation efforts. Such data can aid in conservation programs by facilitating the identification of population closely related to the extirpated population that may act as sources for reintroductions (Hartman et al. 2019).

Successfully identifying the geographic origins or population affinities of fish remains through aDNA analysis is dependent on the availability of reference genetic data from a broad range of populations (Hansen et al. 2001; Ogden 2008). However, such reference data are often unavailable, particularly for non-commercially harvested or lesser-known taxa. Even in instances where such data are available, they often consist of genetic markers that cannot be readily analyzed among ancient specimens. Due to

their high mutation rate, microsatellites, a nuclear marker, have historically been the marker of choice of population-level studies of modern fish (Cuéllar-Pinzón et al. 2016). However, obtaining comparable microsatellite data from ancient ichthyofaunal specimens is often difficult due to DNA degradation. As a result of DNA degradation, allelic dropout is very common when attempting to amplify microsatellites from ancient remains (e.g., Speller et al. 2012), leading to genotyping errors. Fortunately, due to advances in high-throughput sequencing, nuclear single nucleotide polymorphisms (SNPs) are rapidly displacing microsatellites as the marker of choice for modern fish population genetic studies (Cuéllar-Pinzón et al. 2016). Unlike microsatellites, ancient fish remains can be reliably genotyped for SNPs through PCR-based approaches (e.g., Hutchinson et al. 2015; Speller et al. 2012). Nonetheless, allelic dropout still often occurs when attempting to use PCR approaches to genotype ancient fish remains for SNPs (e.g., Hutchinson et al. 2015; Speller et al. 2012). However, high-throughput sequencing techniques have greatly improved researchers' ability to obtain SNP data from ancient fish specimens. For instance, Star et al. (2017) used high-throughput sequence to genotype ancient Atlantic cod specimens for SNPS at 156,695 positions. Such genomic studies allow researchers to consider variation across the genome rather than at a limited number of loci when trying to assess a specimen's population affinities.

Diachronic changes in the population structure can also potentially confound attempts to use aDNA analysis to identity geographic origins or population affinities of ancient fish remains. By altering geographic barriers and the ecological conditions of habitats, anthropogenic and natural processes can alter the genetic structure of fish populations (Bull and Maron 2016; Crispo et al. 2011). In some instances, recent natural and anthropogenic processes have promoted genetic divergence among populations, creating genetic structure where it may not have been present before (e.g., Haponski et al. 2007; Heggenes and Røed 2006; Meldgaard et al. 2003; Roberts et al. 2013; Zhao et al. 2016). For example, Meldgaard et al. (2003) found evidence for fish weirs altering the genetic structure of Danish grayling (*Thymallus thymallus*) populations. Amongst the Danish grayling (*Thymallus thymallus*) populations examined, there was a positive correlation between genetic differentiation and the number of weirs separating populations (Meldgaard et al. 2003). On the other hand, recent processes have reduced genetic differentiation among some fish populations or species (e.g., Behm et al. 2010; Bhat et al. 2014; Taylor and Piercey 2018; Taylor et al. 2006; Faulks and Östman 2016).

In the case of European whitefish (*Coregonus lavaretus*) in Lake Skrukkebukta, Finland, an erosion of phenotypic and genetic differences between benthic and pelagic ecotypes of this species has occurred as result of the introduction of vendace (*Coregonus albula*) (Bhat et al. 2014). Through competitive exclusion, vendace has limited the pelagic ecotype's access to its preferred pelagic feeding zone, promoting gene flow between these two ecotypes of European whitefish (Bhat et al. 2014). Due to such changes in the population structure of fish species, caution needs to be exercised when using modern genetic data to infer the geographic origin or population affinities of ancient fish remains (Hutchinson et al. 2015).

To an extent, the issues posed by changes in populations' genetic compositions can be mitigated by analyzing ancient control samples to confirm past population structure. Control samples should consist of elements, such as cranial elements, that are unlikely to have been transported long distance and were likely deposited at archaeological sites close to where the fish they derive from was caught (Hutchinson et al. 2015). Ideally, control samples should be obtained for all potential source population (Hutchinson et al. 2015). The use of control samples has been used to evaluate past population structure of cod, prior to the DNA-based geographic provenancing of archaeological samples from the Mary Rose (Hutchinson et al. 2015).

Sex Identification

To date, the sex composition of ichthyofaunal assemblages has not been widely considered (see Matsui (2005) and Robson et al. (2013) for notable exceptions). This is likely partially due to the difficulties surrounding the sex identification of fish remains through conventional zooarchaeological approaches. Many, if not most, sexually dimorphic traits exhibited by fish do not archaeologically preserve (e.g., colour) and few skeletal elements exhibit sex-based size or shape differences (e.g., Hilton and Cox Fernandes 2006), hindering sex identification. Some taxa do exhibit sexual size dimorphism or have elements whose morphology varies between sexes that potentially allow sex to be inferred through size estimation (e.g., European eel [*Anguilla Anguilla*]) (Robson et al. 2013) or morphological analysis (e.g., premaxilla in medaka [*Oryzias latipes*]; endopterygoid in *Apteronotus bonapartii*) (Yabumoto and Uyeno 1984; Hilton and Cox Fernandes 2006), respectively. For instance, Robson et al. (2013) reconstructed the length of 139 eels represented in Neolithic Danish assemblages and

identified 21 of them as female due to their length exceeding maximum 55 cm length of males. In Japan, Matsui (2005) identified two Pacific salmon teeth from the 8th to 9th century AD Matoba site as male on account of their morphological similarity to modern male salmon teeth. Nonetheless, even among such taxa, the sex identification of fish skeletal elements remains difficult. Among taxa that exhibit sexual size dimorphism, the size ranges of males and females often overlap, precluding the sex identification of individual in this overlap through size estimations. In the case of the eels examined by Robson et al. (2013), 118 of their 139 samples (84.89%) could not be assigned to a sex as their length fell within the size overlap between males and females. Moreover, elements that exhibit inter-sex morphological variation may not preserve. For example, while the dentary of most male salmonids elongates to form a kype during the breeding season (Witten and Hall 2003), salmonid cranial elements rarely preserve in archaeological contexts due to their low density (Lubinski 1996; Butler and Chatters 1994; Hawkins et al. 2019).

In situations where fish remains cannot be assigned to a sex through conventional zooarchaeological approaches, aDNA analysis can in some instances be used for sex identification. Sex determination pathways in fish are extremely varied. Depending on the taxa, sex may be determined by behavioural, genetic, or environmental mechanisms (See Devlin and Nagahama (2002) for a detailed review of sex determination pathways amongst fish.). If archaeological remains are from a species whose sex is genetically determined, aDNA analysis can potentially be used to assign them to a sex. For example, Royle and colleagues (2018; 2020) have used PCR assays to assign sex identities to archaeological Pacific salmonid, Atlantic salmon, and lake trout remains. These assays screen for the presence of the male-specific *sexually-dimorphic on the Y-chromosome* gene, which is the master sex determining gene in most salmonids (Bertho et al. 2018; Yano et al. 2012; Yano et al. 2013). Application of these assays to modern individuals of known phenotypic or genotypic sex indicates they are reliable (Royle et al. 2018; Royle et al. 2020). While genetic techniques to date have only been applied to archaeological salmonid remains, similar approaches can be used to sex fish from taxa with genetic sex determination systems. Genetic markers potentially useful for sex identification have been identified in various other culturally important taxa such as sablefish (*Anoplopoma fimbria*) (Rondeau et al. 2013), gadids

(Kirubakaran et al. 2019), and northern pike (*Esox lucius*) (Pan et al. 2019; Pan et al. 2021).

Though its application has been limited, the accurate sex identification of archaeological fish remains through aDNA analysis has been used to address some archaeologically relevant questions. Among some fish species, such as northern pike (Casselman 1974) and Atlantic salmon (Harvey et al. 2017), the sex ratio of individuals accessible to fishers changes seasonally, making it possible to infer a fishery's seasonality from an ichthyofaunal assemblage's sex composition. This approach has been used been Royle et al. (2020) to infer the seasonality of the Atlantic salmon fishery at the 13th century AD Antrex site in Ontario. Royle et al. (2020) hypothesize that the prevalence of Atlantic salmon sample genetically identified as female at Antrex indicates its inhabitants were harvesting salmon during the early part of spawning runs, which are female dominated. Documenting the proportion of males and female fish in an assemblage may also shed light on whether past peoples were managing fisheries through sex-selective fishing. In situations where taxa display a biased operational sex ratio, the preferential harvesting to surplus sex can contribute to the sustainability of a fishery (Mathisen 1962; Reed 1982). In northwestern North America, ethnographic data indicate Indigenous people used such a strategy to manage Pacific salmonids. Here, groups such as the Ahtna (Simeone and Valentine 2007:13), Cowichan (Dale and Natcher 2014), Tla'min (Barnett 1955:88), Tlingit (Langdon 2006; Ratner et al. 2006), Shasta (Curtis 1924:113).and the Sts'ailes (Ritchie and Springer 2010), preferentially harvested male Pacific salmonids, which are the surplus sex (Mathisen 1962; Reed 1982). Through DNA-based sex identifications of salmonid remains, Royle et al. (2018; 2020) have investigated whether Indigenous peoples in northwestern North America and Ontario used similar sex-selective fishing strategies to manage salmonids in the deep past.

Genetic Diversity

Around the world, numerous marine and freshwater fish taxa are being threatened by a variety of factors, including overfishing, climate change, habitat alteration, and the introduction of invasive species (Arthington et al. 2016; Hilborn et al. 2003; Jackson et al. 2001). The FAO (2020:47), for instance, estimates that as of 2017 34.2% of harvested marine fish stocks are overfished. As a result of these threats, many

fish populations have experience marked declines since the Industrial Revolution (Arthington et al. 2016; Hilborn et al. 2003; Jackson et al. 2001). For example, since 1980, approximately 80% of steelhead trout populations in British Columbia, Oregon, and Washington have declined (Kendall et al. 2017). In the Laurentian Great Lakes Basin of North America, 82 fish species are now endangered in at least one of the basin's watershed as a results of having undergone significant population declines (Mandrak and Cudmore 2010). The populations declines have resulted in the extirpation of some species from parts of their range, and in some instances the global extinction of a taxon. For example, the Chinese paddlefish (*Psephurus gladius*), a large fish (7 m max length) which inhabited the Yangtze River, was recently declared globally extinct after not having been sighted since 2003 (Zhang et al. 2020).

Population declines can have a significant effect on the genetic structure of fish populations. Notably, population bottlenecks are often, but not always, associated with reductions in genetic diversity (Bouzat 2010). Reductions in genetic diversity may compound threats to taxa by limiting their adaptability to new and changing pressures. Without genetic diversity, populations may not retain alleles that are beneficial and selected for in novel environments, limiting their adaptability (Barrett and Schluter 2008). Due to this link between genetic diversity and species resilience, there has been much interest in quantifying the effect of population bottlenecks on the genetic diversity of fish populations. Many studies have sought to accomplish this comparing the genetic diversity of fish collected in the early and mid 20th century, with that of modern fish populations (e.g., Guinand et al. 2003; Haponski and Stepien 2014; Price et al. 2019; Riccioni et al. 2010). However, using early 20th century specimens is potentially problematic as these fish may have been collected after impacts had already occurred. In many ecosystems, significant environmental changes and fishing pressures that may have impacted fish populations occurred well before the 20th century. For example, stable isotope analyses of archaeological salmonid bone from the Lake Ontario watershed indicate deforestation had caused shifts in the lake's nitrogen cycle had by the mid-19th century (Guiry, Buckley, Orchard, Hawkins, et al. 2020). Consequently, the genetic diversity observed among 20th century samples may represent a shifted baseline not representative of pre-industrial or pre-bottleneck conditions (Pauly 1995).

Fortunately, the aDNA analysis of fish remains provides researchers with a way to overcome the issue posed by the fact that recent historical samples may represent a

shifted baseline. Through the analysis of ancient fish remains, the genetic diversity of fish populations that lived well before recent impacts can be determined, providing an understanding of their genetic variability over the *longue durée* (Hofman et al. 2015). By comparing the genetic diversity of ancient and modern populations, temporal shifts in genetic diversity can be identified (Hofman et al. 2015). In recent years, a few studies have used this approach to evaluate the effects of various anthropogenic activities on the genetic diversity of fish populations. For example, Johnson et al. (2018) investigated the effects of recent human activities (e.g., damming, overfishing, habitat destruction, introduction of hatchery fish) on the genetic diversity of Columbia River Chinook salmon. An analysis of a fragment of the mitochondrial control region indicated that relative to ancient Columbia River Chinook salmon populations, modern ones are characterized by lower haplotype and nucleotide diversity (Johnson et al. 2018). These data indicate the recent human activities have had significant impact on the genetic diversity of Chinook salmon in the Columbia basin (Johnson et al. 2018). Similarly, Speller and colleagues (Speller et al. 2012; Moss et al. 2016) have examined the possibility of documenting temporal changes in the genetic diversity of Pacific herring through the aDNA analysis of archaeological herring bones. Within the Northeast Pacific, based on analyses of mitochondrial DNA and nuclear SNPs, Speller and colleagues (Speller et al. 2012; Moss et al. 2016) found that despite intense harvest pressures from Euro-North American fisheries the genetic diversity of ancient and modern herring are comparable. However, analyses of microsatellites indicates significant differentiation between ancient and modern populations, but they hypothesize this likely reflects allelic drop-out (Speller et al. 2012).

Analyses of the genetic diversity of fish populations over long time periods can also highlight the response of fish populations to past climate change. In Italy, comparison of mitochondrial control region haplotypes obtained from brown trout from successive layers at Grotta del Santuario della Madonna that span the late Pleistocene to early Holocene revealed temporal turnover in haplotypes (Splendiani et al. 2016). These haplotype turnovers corresponded with climatic shifts, suggest climate change drove population replacements (Splendiani et al. 2016). However, the number of analyzed samples (*n*=12) was low limiting inferences about past population dynamics. Analyses of *cytochrome b* sequences obtained from Icelandic archaeological cod vertebrae dating between AD 1550 and 1910 found a decrease in genetic diversity over

time. This reduction in genetic diversity appears to have been the result of a population bottleneck, with Bayesian Skyride and coalescence modeling indicating a marked population contraction during the 15th or 16th century AD. Olafsdottir et al. (2014) hypothesizes that this bottleneck may be the result of habitat and environmental changes caused by the Little Ice Age, whose onset occurred during this timeframe. Understanding the response of fish populations to past climatic shifts has relevance to modern day fish conservation efforts. By understanding how fish populations responded to past climate change, we can predict how they might respond to future climate change. For instance, the case of the brown trout *(Salmo trutta*) from Grotta del Santuario della Madonna, suggests that future climate change might drive similar population replacements (Splendiani et al. 2016).

Phenotype Determination

Within aDNA analysis, there has been growing interest using genotype data obtained from ancient specimens to reconstruct or estimate their phenotype (see Fortes et al. (2013) for review.). By identifying which alleles an ancient specimen exhibit for a gene(s) coding for a particular trait, its phenotype can potentially be determined (Fortes et al. 2013). Understanding how the phenotypes represented in a species or population changed over time can shed light on how they adapted or were affected by environmental changes and/or human activities (Fortes et al. 2013). In the case of fish, such data can be used to document past instances of fisheries-induced evolution (see Kuparinen and Merilä (2007) for review.) or establish a baseline for identifying modern cases. While most studies seeking to reconstruct the phenotypes of ancient specimens have focused on mammals, particularly humans (see Fortes et al. (2013) for review.), there has been increased interest in applying similar approach to fish remains. For example, Thompson et al. (2019) determined the migration timing of Chinook salmon recovered from late Holocene archaeological sites in Klamath Basin, Oregon, by genotyping them for two SNPs located within the greb1l gene at positions 640,165 and 670,329. Individuals' genotype for these SNPs is strongly associated with migration timing: spring-run individuals are homozygous for the T/A allele, fall-run individuals being homozygous for the A/T allele, and heterozygotes having an intermediary migration time (Thompson et al. 2019). Comparisons of the phenotypes exhibited by their ancient specimens with modern individuals demonstrated that 20th century dam construction in the Klamath Basin drove a reduction in the abundance of spring-run Chinook salmon

(Thompson et al. 2019). Such studies are dependent on the genetic architecture underlying a phenotypic trait being well understood. In the case of Thompson et al. (2019), they identified SNPs associated with migration timing by using capture baits and high-throughput sequencing to sequence *greb1l* in modern Chinook salmon with known migration times. To further validate their association with migration timing, they genotyped modern Chinook salmon from the Klamath Basin for these SNPs. Ongoing reductions in the cost of HTS and microarray genotyping will facilitate genome wide association studies of modern fish that can be used to identify SNPs that are useful for phenotype determination (e.g., Barría et al. 2018; Tsai et al. 2015).

1.5. Research Objectives and Thesis Organization

Despite the cultural importance of fish and its proven potential, the aDNA analysis of archaeological fish remains is—relative to other taxonomic groups—uncommon (Grealy et al. 2016; Oosting et al. 2019). Based on a literature review of aDNA studies, Grealy et al. (2016:83) estimates only 2.5% of aDNA studies have focused on the analysis of fish remains. In addition to assumptions about poor DNA preservation, Morales-Muñiz and Llorente-Rodriguez (2018) attributes the dearth of fish-centric aDNA studies to the small size of fishbones impeding DNA extraction as well as a lack of genetic reference data, methods tailored to fish, and communication between aDNA researchers and ichthyoarchaeologists. Due to the paucity of aDNA analyses of fish remains, the methods available for such studies are underdeveloped relative to those available for other fauna, particularly mammals. This methodological gap is exemplified by the delayed incorporation of high-throughput sequencing methods into aDNA analyses of fish remains. Although the first application of high-throughput sequencing to an ancient sample—a mammoth—occurred in 2006 (Poinar et al. 2006), the first application of these methods to fish did not occur until 2016 (Grealy et al. 2016). The methodological underdevelopment of aDNA analyses of fish remains has limited researchers' ability to generate potentially informative genetic data from ichthyofaunal materials. For instance, due to the delayed application of HTS to fish remains, the first ancient fish genome sequences (Star et al. 2017) were not published until nearly ten years after the sequencing of the first ancient genome (Miller et al. 2008).

In this thesis, I sought to address the lack of an extensive methodological toolkit for the aDNA analysis of fish remains through a series of three projects. In these

projects, I developed and applied new DNA-based methods for the sex (Chapter 2 and Chapter 3) and species identification (Chapter 4) of archaeological fish remains. In the first project (Chapter 2), I developed a PCR-based method for the sex identification for ancient Pacific salmon remains and applied it to materials from three archaeological sites in British Columbia and Oregon. The second project (Chapter 3) adapted this sex identification method for Pacific salmon to make it applicable to Atlantic salmonids (*Salmo* spp.) and char (*Salvelinus* spp.) remains. By applying this method to Atlantic salmon and lake trout remains from the Antrex site in Ontario, I investigated whether Middle Ontario Iroquoians were managing salmonid through male-selective fishing. Versions of Chapter 2 and Chapter 3 have been previously published as articles in *PLOS One* (Royle et al. 2018) and the *Journal of Archaeological Science: Reports* (Royle et al. 2020), respectively. The final project (Chapter 4) focused on developing a new universal genetic species identification method for archaeological fish remains, which was applied to middle and late Holocene fish remains from EeRb-144, British Columbia. The final chapter summarizes and synthesis the results of these studies, highlights their limitations, and discuss future research avenues that could be pursued with these methods.

Chapter 2. An Efficient and Reliable DNA-Based Sex Identification Method for Archaeological Pacific Salmonid (*Oncorhynchus* spp.) Remains

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2.1. 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.

2.2. Introduction

Pacific salmonids (*Oncorhynchus* spp.) were and continue to be an important component of many Indigenous fisheries in northwestern North America (Butler and Campbell 2004; McKechnie and Moss 2016). Ethnographic records indicate many Indigenous salmonid fisheries in the region likely employed sex-selective fishing strategies (Barnett 1975; Curtis 1924; Dale and Natcher 2015; Kennedy and Bouchard 1992; Langdon 2006; Ratner et al. 2006; Ritchie and Springer 2010; Simeone and Valentine 2007). Among some groups, such as the Tlingit (Langdon 2006), sex-selective fishing was one of the resource management strategies used to cultivate salmonid stocks (Thornton et al. 2015). 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 (Davidson et al. 2009), 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 (Devlin and Nagahama 2002). 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) (Davidson et al. 2009). For many years, the gene responsible for sex differentiation among salmonids was unknown (Davidson et al. 2009). 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 (Cavileer et al. 2015; Larson et al. 2016; Yano et al. 2012; Yano et al. 2013). 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 (Yano et al. 2012). *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 (Cavileer et al. 2015; Larson et al. 2016; Yano et al. 2012; Yano et al. 2013). 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 (Yano et al. 2013). 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 (Yano et al. 2013).

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.

2.3. Materials and Methods

2.3.1. 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 2). 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 (Speller and Yang 2016).

Table 2. 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. (2004).

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 2) 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. (2004) (Table 2). Following Speller and Yang (2016), 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.

2.3.2. 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 methane sulfonate (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 3 and Table A1). 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 3. 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

2.3.3. 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.

2.3.4. 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 4). Kawumkan Springs Midden (KSM) (35KL9-12) is a residential village located along the Sprague River in the Upper Klamath Basin, Oregon (Stevenson 2011; Stevenson and Butler 2015). The salmonid remains from Kawumkan Springs Midden we analyzed are approximately 5,300 to 1,200 years old or are of unknown age (Stevenson 2011; Stevenson and Butler 2015). 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 (Hayden 1997). 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) (Speller et al. 2005). Say-Umiton (DhHr-18) is a permanent residential site located in a cove along the southwestern shore of Indian Arm, British Columbia (Lepofsky et al. 2007). In this study, we included salmonid remains recovered from Late Phase (~1,200 to 250 years BP) activities areas at Say-Umiton (Lepofsky et al. 2007).

In total, 75 archaeological salmonid remains from these three sites were selected for analysis (Table 4). 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 (Stevenson 2011; Speller et al. 2005; Speller 2010; Speller and Yang 2004). DNA was originally extracted from these samples using a modified silica-spin column method (Yang et al. 1998; Yang et al. 2008). All of the samples were previously identified to the species-level through the analysis of *cytochrome* b and/or D-loop fragments (Stevenson 2011; Speller et al. 2005; Speller 2010; Speller and Yang 2004). In total, six species of Pacific salmonids are represented among the analyzed assemblages (Table 4 and Table A2). 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 (Yang and Watt 2005). 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.

Table 4. 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

2.3.5. 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 (Table 2), 0.3 µM of each *clock1a* primer (Table 2), 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 (Table 2), 0.1 µM of each D-loop primer (Table 2). 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_2O used to prepare the dilutions.

2.3.6. 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 5). Samples were identified as female if *sdY* failed to amplify and both IPCs were amplified (Table 5). 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 5).

Table 5. 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

2.3.7. 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, Canada). 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 (Altschul et al. 1990). In BioEdit (Hall 1999), the edited sequences were aligned with salmonid reference sequences using ClustalW (Thompson et al. 1994) and trimmed to the same length. Species identifications were then assigned to the samples using the procedure described by Yang et al. (2004).

2.4. Results

2.4.1. Modern Samples

DNA was amplified from all of the modern salmonid samples with both the *sdY*/D-loop and *sdY*/*clock1a* assays (Figure 3). 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 (Figure 3 and Table A1). The final consensus sex identities assigned to each of the modern samples matched their known phenotypic sex (Table A1). None of the negative PCR controls associated with the modern samples yielded PCR products of the expected size.

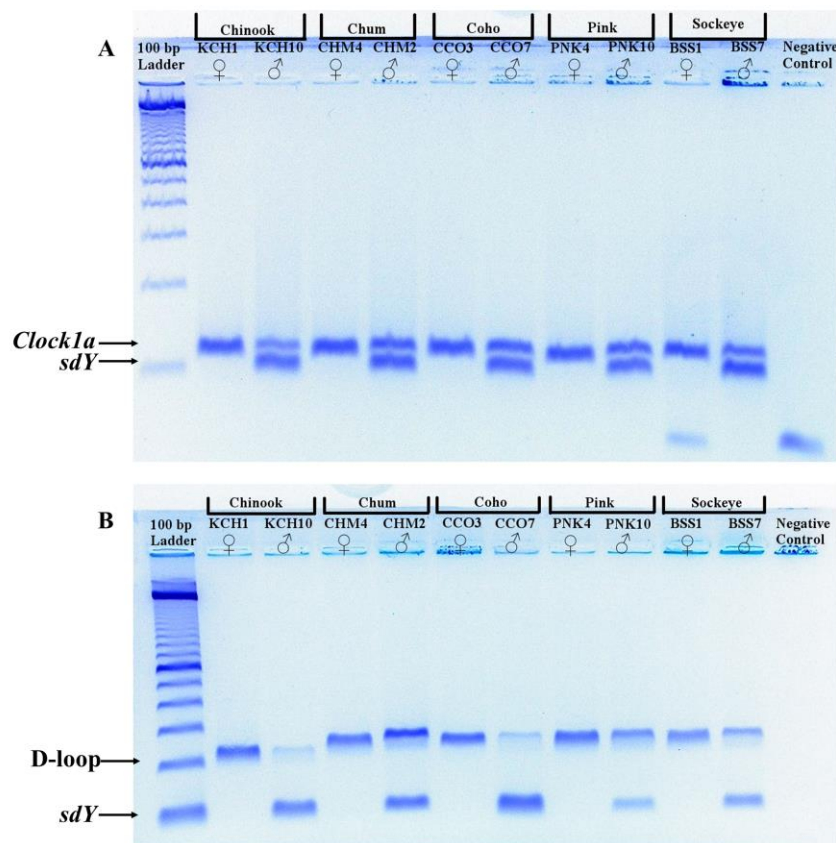

Figure 3 Negative images of electrophoresis gels showing the (A) *clock1a*/*sdY* and (B) D-loop/*sdY* PCR assay results for modern male and female samples from five Pacific salmonid species. The approximate location of the IPC and *sdY* amplicons are indicated by the labelled arrows. The 100 bp ladder used to estimate the size of the amplicons is from Invitrogen (Waltham, MA, USA).

2.4.2. 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) (Figure A1). 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) (Figure A1). No DNA was amplified from the negative PCR controls or the 1,000,000–fold dilution of either KCH4 or KCH9 (Figure A1), with one exception. The D-loop fragment was weakly amplified from the 1,000,000-fold dilution of KCH9 (Figure A1). 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 KCH9 contained approximately 39.4 pg of DNA.

2.4.3. 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 (Table A2). 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 (Figure 4; Table A2). At least two samples from each of the species represented among the archaeological remains were successfully assigned a sex identification (Table 6; Table A2). In total, 37 of these samples were identified as male (53%) and 33 were identified as female (47%) (Table 6; Table A2). Table 6 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, $p=0.17$).

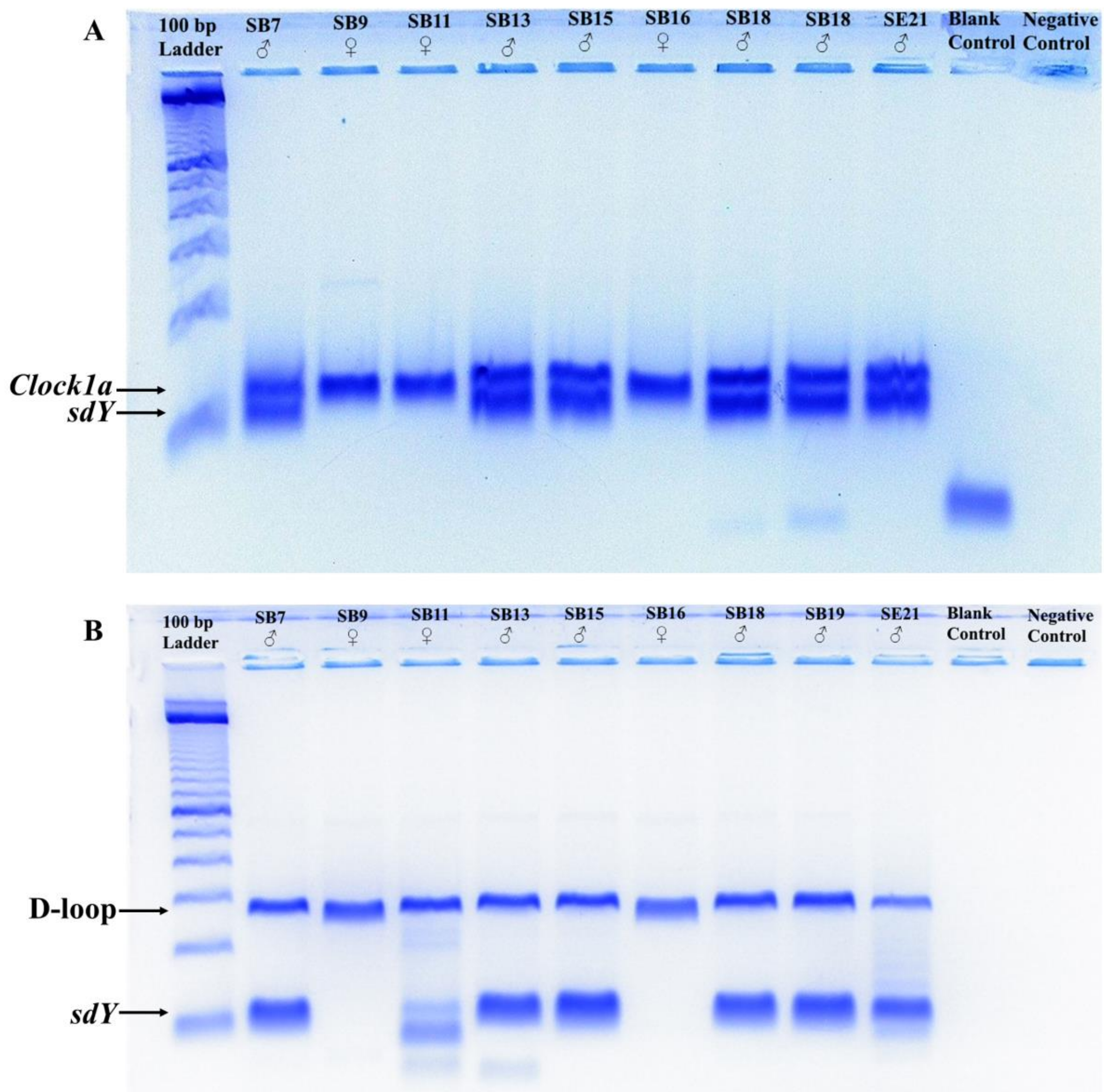

Figure 4. Negative images of electrophoresis gels showing the (A) *clock1a*/*sdY* (B) D-loop/*sdY* assay results for nine of the analyzed archaeological salmonid samples. The approximate location of the IPC and *sdY* amplicons are indicated by the labelled arrows. The 100 bp ladder used to estimate the 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 6. 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 (Table A2). 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.

2.4.4. 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 (Table A2). The species identities assigned to each of the samples matched their previously assigned species identities (Table A2) (Speller et al. 2005; Speller 2010; Speller and Yang 2004).

2.5. Discussion

2.5.1. 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 (Speller et al. 2005; Speller 2010; Speller and Yang 2004). Third, no amplicons of the expected sizes were amplified from any of the negative PCR or blank extraction controls in this or previous studies (Speller et al. 2005; Speller 2010; Speller and Yang 2004). 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 (Table A2) (Speller et al. 2005; Speller 2010; Speller and Yang 2004).

2.5.2. 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 (Yang et al. 2004; Yang and Speller 2006) 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 (Hardie and Hebert 2003), 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.

2.5.3. 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 (Devlin et al. 2005; Muttray et al. 2017). Due to *sdY*'s critical role in controlling sex differentiation in Pacific salmonids, its presence or absence is a reliable proxy for phenotypic sex (Yano et al. 2013). However, previous studies have identified modern Pacific salmonids with *sdY* genotypes inconsistent with their phenotypic sex (Cavileer et al. 2015; Larson et al. 2016; Yano et al. 2013), 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 (Cavileer et al. 2015; Larson et al. 2016; Yano et al. 2013). 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 (Cavileer et al. 2015). 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 (Cavileer et al. 2015; Larson et al. 2016; Yano et al. 2012; Yano et al. 2013).

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 (Quéméré et al.

2014). 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 (Sinding et al. 2016). 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* (Sinding et al. 2016)]. 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 (Sinding et al. 2016). 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 (Figure 3; Figure 4; Figure A1). In addition to the above factors, primer-template mismatches can also result in *sdY* dropout and erroneous sex identifications (Sinding et al. 2016). 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 (Speller and Yang 2016). 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.

2.5.4. 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 (Yang and Watt 2005). 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 (Yang et al. 2004; Yang and Speller 2006). Moreover, by sequencing the D-loop fragment targeted by the D-loop/*sdY* assay, a sample's species identity can also be determined (Yang et al. 2004). 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 (Cannon 1987). Consequently, this method will allow researchers to simultaneously determine past salmonid fisheries' species and sex preferences.

2.5.5. 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's salmonid 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 (Stevenson and Butler 2015). 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.

2.6. 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 (Yano et al. 2013). 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.

Chapter 3. Investigating the Sex-Selectivity of a Middle Ontario Iroquoian Atlantic salmon (*Salmo salar)* and Lake Trout (*Salvelinus namaycush*) Fishery through Ancient DNA Analysis

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3.1. Abstract

Prior to European settlement, Indigenous peoples sustainably harvested Atlantic salmon (*Salmo salar*) and lake trout (*Salvelinus namaycush*) from Lake Ontario for centuries. Previous studies have suggested Indigenous peoples were able to maintain the productivity of Atlantic salmon and lake trout fisheries in the Great Lakes region through the use of resource management strategies. Since males tend to be the surplus sex among salmonids, one way in which Indigenous peoples could have managed Atlantic salmon and lake trout stocks was through the preferential harvesting of males. Here, we sought to investigate whether Indigenous peoples traditionally used sex-selective fishing to manage Lake Ontario Atlantic salmon and lake trout stocks. To address this question, we modified a DNA-based sex identification method developed for ancient Pacific salmonid (*Oncorhynchus* spp.) remains to make it applicable to archaeological Atlantic salmonid (*Salmo* spp.) and char (*Salvelinus* spp.) remains. This

method assigns sex identities to samples through two PCR assays that co-amplify a fragment of the Y-specific salmonid master sex-determining gene (*sexually dimorphic on the Y-chromosome* gene) and an internal positive control, consisting of a fragment of the mitochondrial D-loop or nuclear *clock1b* gene. We applied this method to 61 Atlantic salmon and lake trout remains from the Antrex site (AjGv-38), a Middle Ontario Iroquoian (ca. 1250 to 1300 CE) village located in the Lake Ontario watershed. Using this method, we successfully assigned sex identities to 51 of these remains (83.61% success rate), highlighting our method's sensitivity and efficacy. Statistical analyses indicate neither the aggregate sex ratio nor the sex ratios obtained for the individual species were male-biased. This suggests Antrex's Middle Ontario Iroquoian inhabitants probably did not practice male-selective fishing for Atlantic salmon or lake trout.

3.2. Introduction

Lake Ontario, in northeastern North America, was historically renowned for its substantial populations of lake trout (*Salvelinus namaycush*) and potamodromous Atlantic salmon (*Salmo salar*) (Guiry et al. 2016; Parson 1973; Smith 1995; Dymond et al. 2019). During the 19th century, these populations supported large-scale Euro–North American commercial fisheries, as well as subsistence and recreational fisheries (Elrod et al. 1995; Tiro 2016; Bogue 2000). However, by the mid-nineteenth century, Euro–North American–driven overfishing, habitat alteration, pollution, and species introductions, had caused Atlantic salmon and lake trout stocks in Lake Ontario to collapse (Elrod et al. 1995; Parson 1973; Ketola et al. 2000; Smith 1995; Dymond et al. 2019). As a result, Atlantic salmon were extirpated from Lake Ontario by 1900, with the last sighting occurring in 1899 (Parson 1973; Dymond et al. 2019). Although lake trout continued to be commercially harvested into the 20th century, this taxon, too, became locally extinct, by the end of the 1950s (Elrod et al. 1995).

Prior to their extirpation, Atlantic salmon and lake trout were harvested from Lake Ontario by Indigenous peoples for centuries (Hawkins et al. 2019). It has been hypothesized that Indigenous peoples maintained the productivity of salmonid fisheries in the Great Lakes through the use of resource management strategies (Recht 1997; Tiro 2016; Thoms 2004). Ethnohistoric and ethnographic data indicate these resource management strategies included restricted fishing seasons (Tiro 2016), tenure systems that regulated access to fisheries (Thoms 2004), and the use of selective fishing

technologies, such as weirs (Recht 1997). These fisheries management strategies were underpinned by what the Wendat historian Georges E. Sioui (1999) terms a fishing theology. This fishing theology consisted of a series of rituals and beliefs that cultivated a reciprocal and respectful relationship between humans and fish (Recht 1997; Sioui 1999; but see Tiro 2016; Thoms 2004). Understanding the repertoire of strategies that Indigenous peoples traditionally used to manage Lake Ontario's Atlantic salmon and lake trout stocks can inform present-day restoration efforts focused on these taxa (Morales et al. 2017).

One way in which Atlantic salmon and lake trout stocks can be managed is through sex-selective fishing. As males tend to be the surplus sex among salmonids, preferentially harvesting males can enhance the sustainability of salmonid fisheries (Mathisen 1962; Reed 1982; Fleming and Einum 2011). In another salmonid-bearing region of the Americas, northwestern North America, such preferential harvesting of male salmonids, specifically Pacific salmonids (*Oncorhynchus* spp.), was widely practiced by a variety of Indigenous peoples, including the Ahtna (Simeone and Valentine 2007), Cowichan (Dale and Natcher 2015), Shasta (Curtis 1924), Sts′ailes (Ritchie and Springer 2010), Tla'amin (Barnett 1975) ,and Tlingit (Langdon 2006; Ratner et al. 2006). Within this region, male-selective Pacific salmonid fishing was commonly achieved through the use of weirs and traps, which enabled the release of female fish (Dale and Natcher 2015; Langdon 2006; Ratner et al. 2006; Ritchie and Springer 2010). Alternatively, in river pools with clear water and light-coloured substrates it was possible to visually discern male Pacific salmonids and preferentially harvest them with spears or gaffs (Curtis 1924; Langdon 2006; Ratner et al. 2006). Ethnographic accounts indicate that in many instances male-selective fishing was purposefully done by Indigenous peoples in order to maintain the productivity of local Pacific salmonid stocks (Langdon 2006; Ritchie and Springer 2010; Barnett 1975; Dale and Natcher 2015). However, it is important to note that male Pacific salmonids were also preferentially harvested for reasons unrelated to management. For instance, males were targeted by some individuals on account of their larger size or were incidentally harvested in higher numbers due to fluctuations in the relative abundance of the sexes (Langdon 2006; Simeone and Valentine 2007; Ritchie and Springer 2010). As similar fishing technologies were also used by Indigenous peoples in the Great Lakes region (Cleland 1982; Recht

1997), it is feasible that they also managed Lake Ontario Atlantic salmon and lake trout stocks through similar male-selective fishing strategies.

Here, we sought to investigate whether male-selective fishing was one of the strategies Indigenous peoples used to manage Lake Ontario's Atlantic salmon and lake trout stocks. To address this question, we developed a DNA-based sex identification method for archaeological Atlantic salmonid (*Salmo* spp.) and char (*Salvelinus* spp.) remains by adapting a method developed for ancient Pacific salmonid remains (Royle et al. 2018). Following Royle et al. (2018), this method uses two PCR assays that co-amplify a fragment of the Y-specific salmonid master sex-determining gene (*sexually dimorphic on the Y-chromosome* gene) (Yano et al. 2012; Yano et al. 2013), and an internal positive control consisting of a fragment of the mitochondrial D-loop or nuclear *clock1b* gene. Royle et al. (2018) have demonstrated that this DNA-based approach is an efficient sex identification method for archaeological Pacific salmonid remains, but it has yet to be applied to remains from other salmonids. To investigate the sex-selectivity of Indigenous Atlantic salmon and lake trout fisheries in the Lake Ontario basin, we applied our modified DNA-based sex identification method to 28 Atlantic salmon and lake trout remains from the Middle Ontario Iroquoian (ca. 1250–1300 CE) Antrex site (AjGv-38). Our results indicate that our modified method is an efficient sex identification method for archaeological Atlantic salmonid and char remains and suggest Antrex's inhabitants likely did not practice male-selective fishing for these species.

3.3. Archaeological Context

Antrex is an Ontario Iroquoian village located near the north shore of Lake Ontario, in present-day Mississauga, Ontario, Canada (Figure 5). The site is bounded by a tributary of Cooksville Creek and is also situated near the Credit River (Archaeological Services Inc. 2010). During the 19th century, the Credit River supported a substantial Atlantic salmon run harvested by Anishinaabeg and Euro–Canadians (Tiro 2016; Parson 1973; Thoms 2004). The combined results of excavations and surveys conducted by Archaeological Services Inc. (Archaeological Services Inc. 2010; Archaeological Services Inc. 1991); the Erindale College (now University of Toronto Mississauga) Field School (Smith 1993); and Mayer, Poulton, and Associates Inc. (Mayer Poulton and Associates Inc. 1991; Mayer Heritage Consultants Inc. 1998) indicate Antrex was a partially palisaded, 0.65 ha village composed of 8 longhouses, some of which were

contemporaneous. Analyses of the site's ceramic assemblage indicate it was inhabited during the Middle Ontario Iroquoian period, with multiple radiocarbon dates suggesting a ca. 1250 to 1300 CE occupation (Archaeological Services Inc. 2010; Mayer Heritage Consultants Inc. 1998; Mayer Poulton and Associates Inc. 1991). Within this timeframe, Antrex, like other Middle Ontario Iroquoian villages (Warrick 1988), was likely only occupied for approximately 20 years before being abandoned (Robertson and Williamson, 2002).

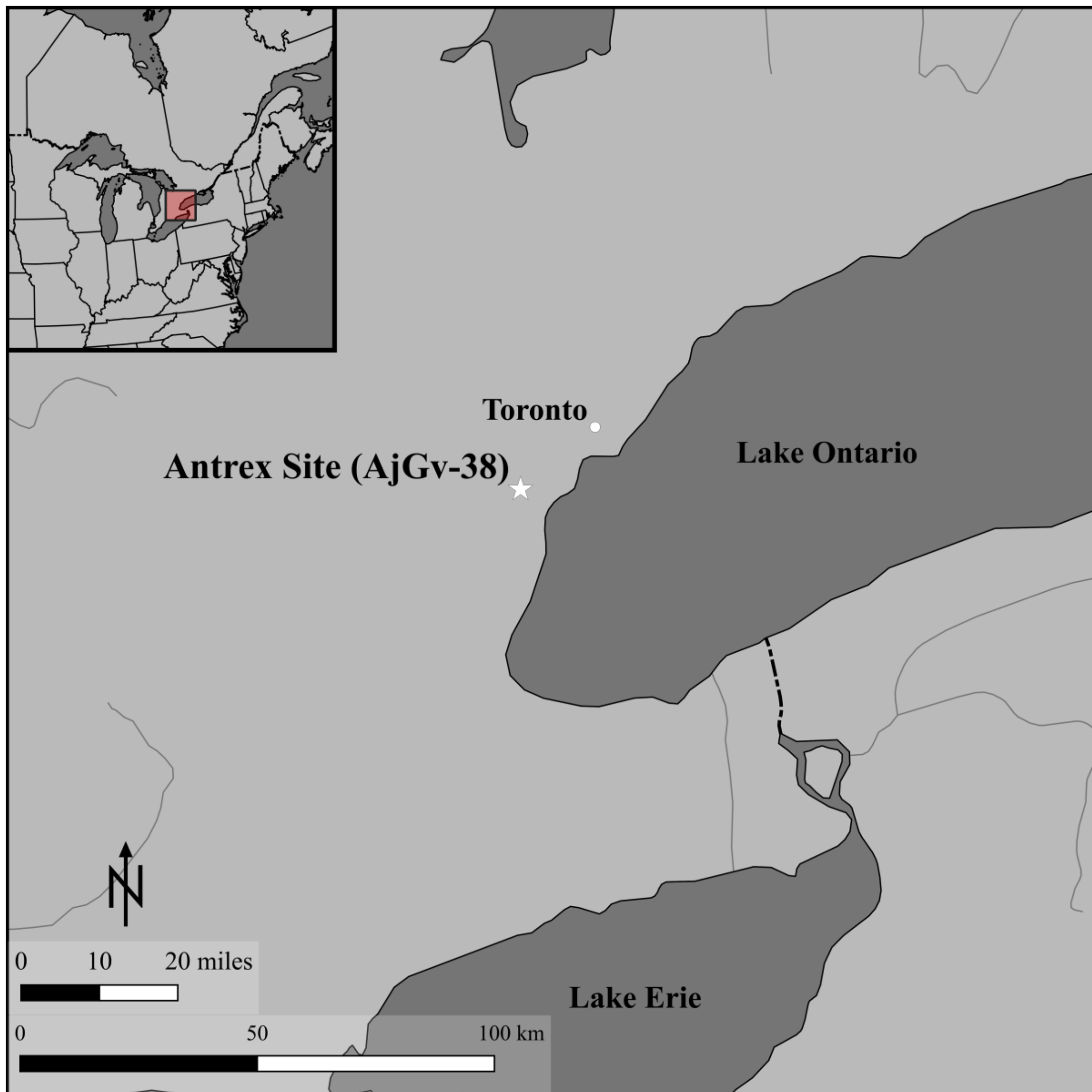

Figure 5. Location of the Antrex (AjGv-38) site.

During the Middle Ontario Iroquoian period, subsistence patterns were characterized by an increased dependence on cultigens, most notably maize (*Zea mays*

ssp. *mays*) (Dodd et al. 1990). Although maize and other crops were important foodstuffs, stable isotope analyses of Middle Ontario Iroquoian human remains indicate that fish, particularly large piscivorous species, were significant sources of protein (Pfeiffer et al. 2014; Pfeiffer et al. 2016; van der Merwe et al. 2003; Feranec and Hart 2019). The abundance of fish remains at many archaeological sites dating to this period further reflects the dietary importance of fish at this time (Pfeiffer et al. 2014; Hawkins et al. 2019). The results of a preliminary zooarchaeological analysis, namely an assignment of vertebrate remains to taxonomic class, indicates that fish constitute 33.78% (*NISP*=4,724) of Antrex's inventoried faunal assemblage (*NISP*=13,986) (Balmer 2010). This suggests that, proportionately, fish were a similarly important subsistence item at Antrex. As a more detailed, below-class, analysis of the Antrex faunal assemblage has yet to be completed, the relative abundance of Atlantic salmon, lake trout, and other individual fish species at the site is unknown.

3.4. Materials and Methods

3.4.1. Sampling and Zooarchaeological Analysis

A total of 61 salmonid vertebral elements recovered from Antrex were selected for ancient DNA (aDNA) analysis. We sampled vertebrae rather than unique cranial elements in order to maximize our sample size. In contrast to salmonid vertebrae, which are often archaeologically abundant, salmonid cranial elements are typically rare due to their low bone density relative to vertebrae, which increases their susceptibility to destructive taphonomic processes (Butler and Chatters 1994; Lubinski 1996; Hawkins et al. 2019). However, since vertebral elements other than the atlas, penultimate, and ultimate vertebra, are repetitive elements, sampling vertebrae can potentially result in sampling an individual fish multiple times, which would bias our results. Following Cannon and Yang (2006), we sought to mitigate the potential for repeated sampling of individual fish by selecting vertebral elements recovered from different units, features, and layers. Detailed provenience information for each of the analyzed samples is provided in Table B1. As Antrex was likely only occupied for about 20 years, all of the samples, despite coming from different contexts, are roughly contemporaneous.

Taxonomic identifications were assigned to the selected samples by Orchard through comparisons with reference specimens held in the Deborah J. Berg Faunal

Collection at the Department of Anthropology, University of Toronto Mississauga (Mississauga, ON, Canada). Uncertain taxonomic identifications were double-checked and confirmed by Needs-Howarth using reference specimens from the Howard G. Savage Faunal Archaeo-Osteology Collection at the Department of Anthropology, University of Toronto (Toronto, ON, Canada). Of the 61 salmonid vertebrae selected for analysis, 35 (Samples LOS1–LOS35) were identified as Atlantic salmon and 26 (Samples LON1–LON26) were identified as lake trout or likely lake trout (*Salvelinus* cf. *namaycush*) (Table B1).

Prior to aDNA analysis, a portion of some of the samples (Samples LOS1–LOS17 and LON1–LON11) was removed and subjected to stable carbon and nitrogen isotope analysis (Table B1) and, in some instances (Samples LOS4, LOS14, LON7, LON9, LON10, and LON11), zooarchaeology by mass spectrometry (ZooMS) (Table B1) (Guiry, Buckley, Orchard, Hawkins, et al. 2020). ZooMS confirmed the zooarchaeological taxonomic identifications assigned to four of the six analyzed samples (LOS4, LOS14, LON10, and LON11) (Guiry, Buckley, Orchard, Hawkins, et al. 2020). The remaining two samples (LON7 and LON9) could not be assigned a species identification through ZooMS (Guiry, Buckley, Orchard, Hawkins, et al. 2020).

3.4.2. Decontamination and DNA Extraction

Decontamination, DNA extractions, and PCR setups were all conducted in a dedicated aDNA laboratory in the Department of Archaeology, Simon Fraser University (Burnaby, BC, Canada) and followed strict contamination controls (Yang and Watt 2005). In instances where samples were sufficiently large, only a portion of the individual bone was used for DNA extraction. All of the samples were decontaminated prior to DNA extraction using a previously published protocol (Speller et al. 2012). To decontaminate the samples, each sample was, in brief, immersed in a 100% commercial bleach solution (~5% w/v NaOCl) for ≈6–8 mins; rinsed in distilled water for 30 sec–1 min; rinsed again in distilled water for ≈6–11 mins; and UV irradiated for 15–30 mins on two sides. Subsequently, the decontaminated samples were incubated overnight at 50 ˚C in 2.8–5 mL of lysis buffer (0.5 M EDTA [pH 8.0], 0.125–0.25% SDS, and 0.5 mg/mL proteinase K) in a rotating hybridization oven. Following incubation, DNA was extracted from the digested samples using a modified silica-spin column method (Yang et al. 1998; Yang et al. 2008). DNA extraction was repeated for three of the Atlantic salmon samples (LOS7,

LOS9, and LOS15) using the remaining bone. Repeat DNA extractions were conducted by an independent analyst within the same laboratory as the initial extractions. To monitor for contamination, blank extraction controls were included in each DNA extraction procedure and subjected to amplification with each combination of primers.

3.4.3. Development of DNA-based Sex Identification Method

Across the family Salmonidae, sex is principally determined through an XY genotypic sex-determination system wherein males are the heterogametic sex (Davidson et al. 2009). Among most salmonids, including Atlantic salmonids and char, the master sex-determining gene responsible for sex differentiation is hypothesized to be *sdY (sexually dimorphic on the Y-chromosome* gene), a male-specific gene located on the Y-chromosome (Yano et al. 2013). The results of recent studies suggest the expression of *sdY* in developing gonads triggers male differentiation by preventing estrogen synthesis, which promotes testis development (Yano et al. 2013; Yano et al. 2012; Bertho et al. 2018). Recently, Royle et al. (2018) have demonstrated that archaeological Pacific salmonid remains can be assigned accurate sex identities using two PCR assays that screen for the presence of *sdY* and an internal positive control (IPC). However, not all the primers in these assays are conserved in Atlantic salmonids and chars, necessitating the modification of this method to make it applicable to our samples.

Atlantic salmonid and char *sdY* sequences obtained from GenBank (Sayers et al. 2019) were aligned with ClustalW (Thompson et al. 1994) through BioEdit v7.2.5 (Hall 1999). Through a visual examination of this alignment in BioEdit, we designed several primer pairs that targeted small fragments (<100 bp) of *sdY*. NetPrimer (http://www.premierbiosoft.com/netprimer) and Primer-BLAST (Ye et al. 2012) were used to assess the potential efficiency and specificity of these potential primer pairs. We subsequently included these primers in various potential PCR sex identification assays that, following Royle et al. (2018), co-amplify *sdY* alongside an IPC consisting of a fragment of mitochondrial or nuclear DNA. We evaluated the efficiency of these potential assays by testing them on modern Atlantic salmon (3 males, 1 female) and Arctic char (*Salvelinus alpinus*) (1 male) samples whose genotypic sex was known and a subset of our archaeological samples. The genotypic sex of the modern samples was determined using the *18S rRNA* gene/*sdY* co-amplification PCR sex identification assay described

by Yano et al. (2013). Reaction conditions for the assays were optimized by applying them with varying PCR conditions to subsets of our modern and ancient samples.

Based on the results of these tests, we selected two PCR sex identification assays to apply to the entire set of Atlantic salmon and lake trout samples from Antrex. Following Royle et al. (2018), the first assay co-amplifies a 98 bp fragment of *sdY* alongside an IPC consisting of a 255 bp fragment of the mitochondrial D-loop. The *sdY* fragment targeted in this assay is amplified with primers *sdY*-F100 and *sdY*-R101, whilst the D-loop fragment was amplified with previously published primers Smc7 and Smc8 (Yang et al. 2004) (Table 7). In the second assay, primers *sdY*-F102 and *sdY*-103 were used to amplify a 98 bp fragment of *sdY*, which was amplified in tandem with a 116 bp fragment of the nuclear *clock1b* (*clk1b*) gene (Table 7). This *clock1b* fragment serves as the IPC in this assay and was amplified with primers *clk1b*-F106 and *clk1b*-R107 (Table 7). Since the X-chromosome is not conserved between or within salmonid species due to *sdY* being a transposable element (Eisbrenner et al. 2014; Lubieniecki et al. 2015; Faber-Hammond et al. 2015), primers directly targeting it were not included in either assay. In both assays, the co-amplification of the IPC functions as a surrogate for the presence of the X-chromosome. Application of these assays to our small sample of genotypically-sexed modern Atlantic salmon (3 males, 1 female) and Arctic char (*Salvelinus alpinus*) (1 male) produced sex identification results concordant with their known genotypic sex (Table B2). All pre-PCR laboratory work involving the modern samples was conducted in a laboratory in the Centre for Forensic Research, Simon Fraser University (Burnaby, BC), that is dedicated to the analysis of modern DNA samples and physically separated from the aDNA laboratory.

Table 7. Primer pairs used in this study.

Locus	Primer[1]	Sequence (5'–3')	Amplicon Size[2]	Source
cytochrome b	CytB5 (F)	AAAATCGCTAATGACGCACTAGTCGA	168 bp	Yang et al. (2004)
	CytB6 (R)	GCAGACAGAGGAAAAAGCTGTTGA		Yang et al. (2004)
clock1b	*clk1b*-F106 (F)	CTGGTGCAGATGTTCCTCCAAC	116 bp	This study
	clk1b-R107 (R)	ACCACCTGGCCCTGCATGTTGAGAGC		This study
D-loop	Smc7 (F)	AACCCCTAAACCAGGAAGTCTCAA	255 bp	Yang et al. (2004)
	Smc8 (R)	CGTCTTAACAGCTTCAGTGTTATGCT		Yang et al. (2004)
sdY	*sdY*-F100 (F)	ATCTCTCTCCCAAAGCCCCC	98 bp	This study
	sdY-R101 (R)	CTTAAAACCACTCCACCCTCCAT		This study

sdY	*sdY*-F102 (F)	GGGGAGTGATGTCAGAATTGC	98 bp	This study
	sdY-R103 (R)	AGATGGGAATGGTGTCGGG		This study

[1]F denotes a forward primer and R denotes a reverse primer.

[2]Predicted size of mitochondrial DNA, *clock1b*, and *sdY* amplicons is based on the position of their corresponding primer pair within Atlantic salmon mitochondrion genome (Genbank accession number: NC001960) (Hurst et al. 1999) , *clock1b* (Genbank accession number: GU228525) (Paibomesai et al. 2010), *sdY* (Genbank accession number: KP898412) (Lubieniecki et al. 2015) reference sequences, respectively.

3.4.4. PCR Amplification and Sex Identification

PCR amplifications and post-PCR procedures were conducted in a dedicated post-PCR laboratory physically separated from the aDNA laboratory. PCR amplifications for the sex identification assays were performed on a Mastercycler Personal or Gradient thermal cycler (Eppendorf, Mississauga, ON, Canada) in a 30 µL reaction volume that contained 1.5× PCR Gold Buffer (Applied Biosystems, Carlsbad, CA, USA), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6 µM of each *sdY* primer (Table 7), 0.1 µM of each D-loop (D-loop/*sdY* assay) or *clock1b* (*clock1b*/*sdY* assay) primer (Table 7), BSA (1 mg/mL), 1–4 µL of DNA solution, and 0.75–1.25 U/µL AmpliTaq Gold (Applied Biosystems, Carlsbad, CA, USA). The thermal program for 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), 58 °C (D-loop/*sdY* assay) or 56 °C (*clock1b*/*sdY* assay) for 30 s (annealing), and 70 °C for 40 s (extension), and a final extension step at 72 °C for 7 min. To identify instances of allelic drop-out, a multi-tube procedure was used for both sex identification assays (Taberlet et al. 1996). Both sex identification assays were applied to each of the samples between two and five times (Sugimoto et al. 2006). Negative PCR controls were included in each PCR run.

Five microlitres of PCR product was pre-stained with SYBR Green I (Life Technologies, Carlsbad, CA, USA), electrophoresed on a 2% (D-loop/*sdY* assay) or 3% (*clock1b*/*sdY* assay) agarose gel, and visualized with a Dark Reader transilluminator (Clare Chemical Research, Dolores, CO, USA). Sex identities were assigned to the samples with each of the assays through a visual analysis of the electrophoresis gels of the generated PCR products using a modified version of the criteria outlined by Sugimoto et al. (2006). For both assays, a sample was identified as male if *sdY* or both *sdY* and the IPC were amplified at least twice (Sugimoto et al. 2006). Samples were identified as female if the IPC was amplified at least three times with an individual assay (Sugimoto et al. 2006) and *sdY* was not amplified by any of the five PCR replicates

carried out for potential females (Janečka et al. 2008). A sex identity was not assigned to a sample with an individual assay if neither of these criteria were met. Following Royle et al. (2018), a final consensus sex identity was assigned to the samples based on the sex identities assigned with the individual assays. A final consensus sex identity was assigned to a sample if it was successfully identified as the same sex by both the D-loop/*sdY* and *clock1b/sdY* assay. A sample was not assigned a sex identity if the assays yielded inconsistent results or if a sex identity could not be assigned to the sample with one or both of the assays.

3.4.5. Statistical Analyses of Sex Identification Results

Statistical analyses of the sex identification results were performed in R v3.5.1 (R Core Team 2018) through RStudio v1.1.456 (RStudio Team 2015). Two-tailed exact binomial tests were used to assess whether the aggregate sex ratio or the sex ratios obtained for each of the species was significantly male or female biased (McDonald 2014). The significance of inter-specific sex ratio differences was evaluated through a two-tailed Fisher's exact test of independence (McDonald 2014). *P*-values less than or equal to 0.05 were considered significant.

3.4.6. Species Identification

To confirm the samples' species identities, we sequenced and analysed the D-loop fragment co-amplified by the D-loop/*sdY* assay (Royle et al. 2018). In instances where this D-loop fragment was only weakly amplified by this assay or failed to amplify, we attempted to amplify this fragment with the same D-loop primers in a singleplex PCR. Following Yang et al. (2004), we sought to confirm the D-loop–based species identifications though the analysis of a 168 bp fragment of *cytochrome b* amplified in a singleplex PCR with primers CytB5 and CytB6 (Table 7). The conditions for the singleplex PCRs targeting these D-loop and *cytochrome b* fragments were the same as above, with the exception of their primer concentrations, polymerase concentrations, and annealing temperatures which were as follows: 0.3 μM of each D-loop or *cytochrome b* primers, 1–1.5 U AmpliTaq Gold, and 54 °C, respectively. Negative PCR controls were included in each of the singleplex PCR runs. The PCR products generated by the singleplex PCRs were separated on a 2% agarose gel and visualized in the same manner as described above. Unpurified D-loop and *cytochrome b* amplicons were

directly sequenced in the reverse and/or forward direction with their respective amplification primers at Eurofins Genomics (Toronto, ON, Canada).

The sequences obtained from the Antrex samples were visually edited, truncated to remove the primer sequences, and assembled using ChromasPro v2.1.8 (http://www.technelysium.com.au). To determine their closest taxonomic match, the edited sequences were compared against reference sequences accessioned in GenBank through a BLASTn search (Altschul et al. 1990). Multiple alignments of the edited sequences, reference sequences from all Atlantic salmonid and char species (Atlantic salmon, brook trout [*Salvelinus fontinalis*], and lake trout) native to southern Ontario (Holm et al. 2009), and a huchen (*Hucho hucho*) reference sequence to serve as an outgroup in the phylogenetic analyses, were performed for each marker using ClustalW (Thompson et al. 1994) through BioEdit v7.2.5 (Hall 1999). Maximum-likelihood phylogenetic trees were constructed for each of the aligned datasets using PhyML v3.1 (Guindon et al. 2010) with 1000 bootstrap replicates. Each phylogenetic analysis was performed with the best–fit substitution model determined by PhyML's automated Smart Model Selection (SMS) method (Lefort et al. 2017) using the Akaike Information Criterion. SMS selected HKY85 as the best-fit substitution model for the D-loop sequences and HKY85+G as the best-fit substitution model for the *cytochrome b* sequences. Both of the resulting phylogenetic trees were visualized and annotated with iTOL v4.4.1 (Letunic and Bork 2019). Species-level identifications were assigned to samples if all the sequences obtained from a given sample matched or closely resembled sequences from a single species and differed from other, closely related species.

3.5. Results

3.5.1. Sex Identification

DNA was successfully amplified with the sex identification assays from 60 of the 61 samples (Table B3; See Figure 6 and Figure 7 for exemplar electrophoresis gels). The results of the individual PCR replicates carried out for each sample with both sex identification assays are provided in Table B3, whilst Table 8 presents a summary of the sex identification results for each of the samples. Of the 60 samples that yielded amplicons, the D-loop/*sdY* and *Clock1b*/*sdY* assays generated concordant sex identities

for 51 samples, enabling a sex identification to be assigned to these samples (83.6% success rate) (Table 8; See Figure 6 and Figure 7 for exemplar electrophoresis gels). The sex identification results obtained for the repeat DNA extractions of samples LOS7, LOS9, and LOS15 matched the sex identities generated from the initial extractions (Table B3). Of the 51 remains that were successfully sexed, 29 were Atlantic salmon and 22 were lake trout. The remaining ten samples could not be assigned a sex identity using the outlined criteria (Table 8). Likely owing to DNA degradation, one of these samples (LOS16) could not be assigned a sex identity as a result of the failure to amplify DNA with either assay (Table B3). Stable isotope analyses of LOS16 indicate it has poorly preserved collagen (Table B1) (Guiry, Buckley, Orchard, Hawkins, et al. 2020), suggesting that overall biomolecular preservation in this sample was poor. DNA was amplified at least once with both assays from the remaining nine samples, but these could not be assigned to a sex due to the replicates of one or both assays yielding inconsistent results (Table 2; Table B3). The failure to obtain consistent results for these samples potentially reflects allelic drop-out due to degradation, inhibition, amplification competition with the IPC in the case of males, or a combination thereof. No DNA was amplified from any of the blank extraction or negative PCR controls with either the sex identification assays or singleplex PCRs.

Table 8. Sex and species identification results for the analyzed samples. ZooMS species identifications are from Guiry, Buckley, Orchard, Hawkins, et al. (2020).

Sample	Zooarchaeological Species ID	ZooMS Species ID	D-loop Species ID	*Cytb* Species ID	Consensus Genetic Species ID	D-loop/*sdY* Sex ID	*clock1b*/*sdY* Sex ID	Consensus Sex ID
LOS1	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS2	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS3	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Male	Male
LOS4	Atlantic salmon	Atlantic salmon	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Male	Male
LOS5	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Male	Male
LOS6	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Indeterminate	Indeterminate	Indeterminate
LOS7	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS8	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS9	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Male	Male
LOS10	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS11	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS12	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS13	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Male	Male
LOS14	Atlantic salmon	Atlantic salmon	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS15	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS16	Atlantic salmon	-	Indeterminate	Indeterminate	Indeterminate	Indeterminate	Indeterminate	Indeterminate
LOS17	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS18	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Indeterminate	Indeterminate
LOS19	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS20	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Indeterminate	Indeterminate	Indeterminate
LOS21	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS22	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Indeterminate	Indeterminate
LOS23	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female

Sample	Zooarchaeological Species ID	ZooMS Species ID	D-loop Species ID	*Cytb* Species ID	Consensus Genetic Species ID	D-loop/*sdY* Sex ID	*clock1b*/*sdY* Sex ID	Consensus Sex ID
LOS24	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS25	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Male	Male
LOS26	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Indeterminate	Indeterminate
LOS27	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Male	Male
LOS28	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS29	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Male	Male
LOS30	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS31	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS32	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS33	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Male	Male	Male
LOS34	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LOS35	Atlantic salmon	-	Atlantic salmon	Atlantic salmon	Atlantic salmon	Female	Female	Female
LON1	Lake trout	-	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON2	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON3	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON4	Lake trout	-	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON5	Lake trout	-	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON6	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON7	Lake trout	Indeterminate	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON8	Lake trout		Lake trout	Lake trout	Lake trout	Male	Male	Male
LON9	Lake trout cf.	Indeterminate	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON10	Lake trout cf.	Lake trout	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON11	Lake trout cf.	Lake trout	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON12	Lake trout cf.	-	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON13	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Indeterminate	Indeterminate

Sample	Zooarchaeological Species ID	ZooMS Species ID	D-loop Species ID	*Cytb* Species ID	Consensus Genetic Species ID	D-loop/*sdY* Sex ID	*clock1b*/*sdY* Sex ID	Consensus Sex ID
LON14	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON15	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON16	Lake trout	-	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON17	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON18	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON19	Lake trout cf.	-	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON20	Lake trout cf.	-	Lake trout	Lake trout	Lake trout	Male	Indeterminate	Indeterminate
LON21	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON22	Lake trout	-	Lake trout	Lake trout	Lake trout	Male	Male	Male
LON23	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Indeterminate	Indeterminate
LON24	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON25	Lake trout	-	Lake trout	Lake trout	Lake trout	Female	Female	Female
LON26	Lake trout	-	Lake trout	Lake trout	Lake trout	Male	Indeterminate	Indeterminate

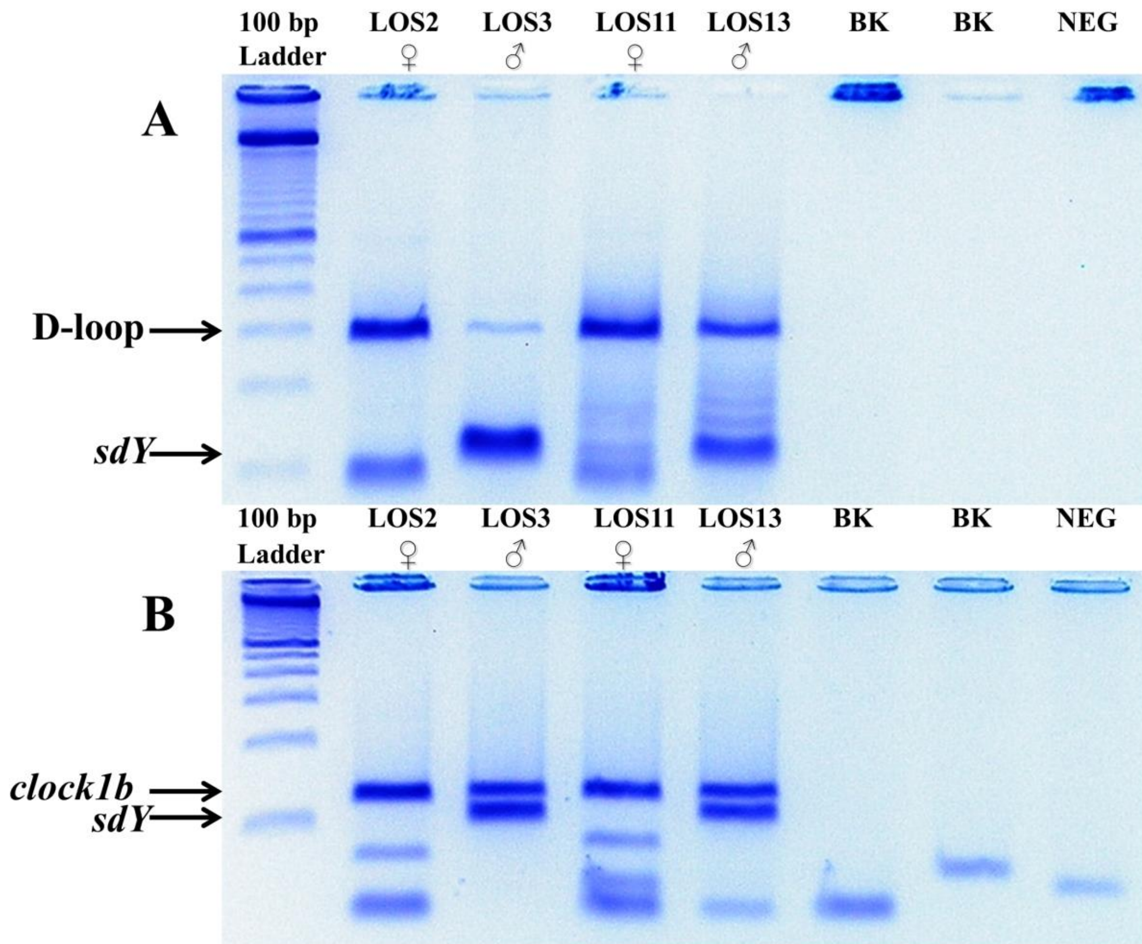

Figure 6. Negative images of electrophoresis gels showing the (A) D-loop/*sdY* PCR and (B) *clock1*b/*sdY* assay results for four of the analyzed Atlantic salmon (*Salmo salar*) samples (LOS#). The Mars (♂) and Venus (♀) symbols beneath the sample names denote samples identified as male and female, respectively . The approximate positions of the internal positive control (D-loop and *clock1b*) and *sdY* amplicons generated by the assays are indicated by the labelled arrows. BK denotes the blank extraction controls processed alongside the samples. NEG indicates negative PCR controls. The 100 bp ladder is from Invitrogen (Waltham, MA, USA).

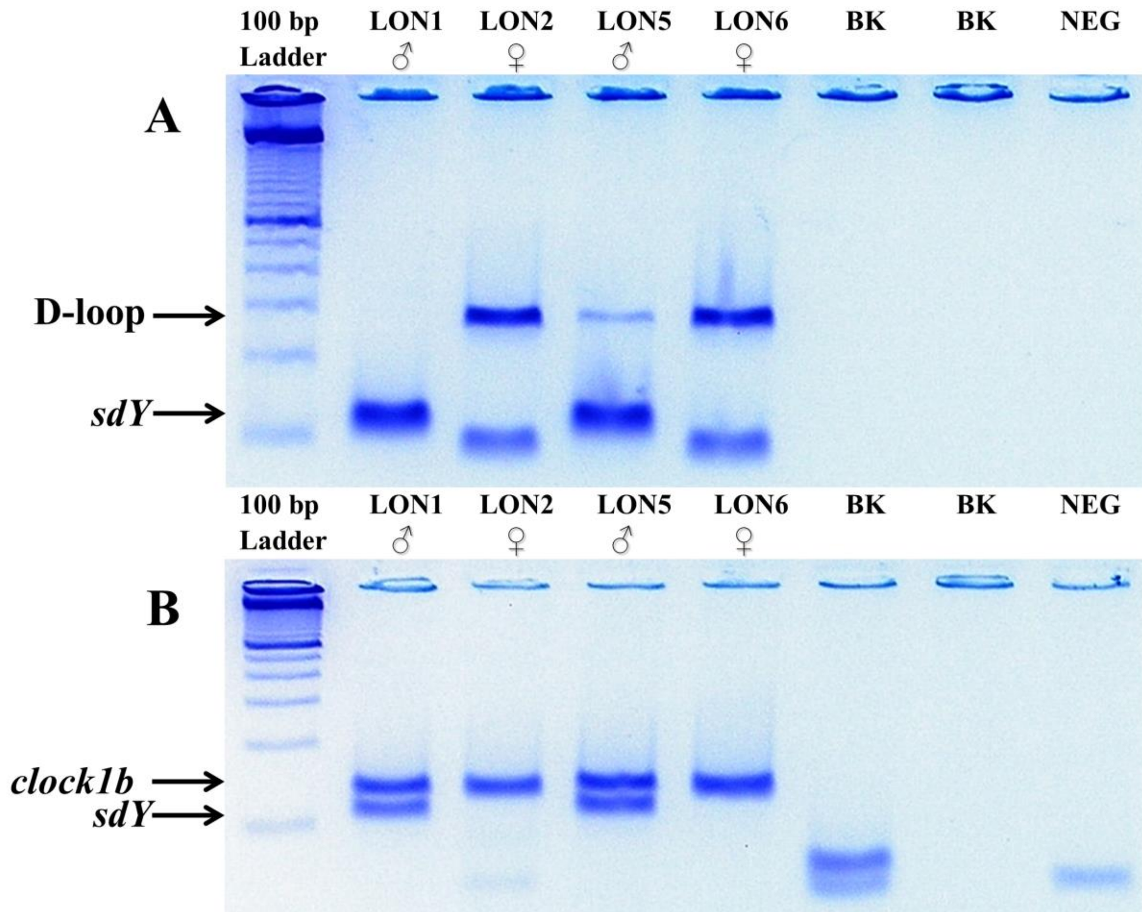

Figure 7. Negative images of electrophoresis gels showing the (A) D-loop/*sdY* PCR and (B) *clock1b*/*sdY* assay results for four of the analyzed lake trout (*Salvelinus namaycush*) samples (LON#). The Mars (♂) and Venus (♀) symbols beneath the sample names denote samples identified as male and female, respectively. The approximate positions of the internal positive control (D-loop and *clock1b*) and *sdY* amplicons generated by the assays are indicated by the labelled arrows. BK denotes the blank extraction controls processed alongside the samples. NEG indicates negative PCR controls. The 100 bp ladder is from Invitrogen (Waltham, MA, USA).

When all 51 of the sexed samples are considered as a whole, irrespective of species, no sex bias is evident. Although females were more abundant than males (Table 9), no significant difference from a 1:1 sex ratio was observed (Exact binomial test, two-tailed, p=0.2624). Amongst the Atlantic salmon samples assigned a sex, females were more than twice as abundant as males (Table 9). However, the sex ratio obtained for the Atlantic salmon samples is not significantly sex-biased (Exact binomial test, two-tailed, p=0.06143). No sex bias was observed in the sample of sexed lake trout

(Exact binomial test, two-tailed, *p*=0.8318), with male and female lake trout being roughly equally abundant (Table 9). The sex ratios obtained for each species did not significantly differ from each other (Fisher's exact test, two-tailed, *p*=0.1504).

Table 9. Number of identified females and males by species.

Species	Females	Males	Sex Indeterminate	Total Analyzed
Atlantic salmon	20	9	6	35
Lake trout	10	12	4	26
Aggregate	30	21	10	61

3.5.2. Species Identification

Both D-loop and *cytochrome b* were successfully amplified from 60 of the 61 samples, with LOS16 being the only sample to not yield any mitochondrial DNA. The D-loop and *cytochrome b* sequences obtained from the repeat DNA extractions of samples LOS7, LOS9, and LOS15 matched those obtained from the initial extractions. The results of the BLASTn searches indicate the D-loop and cytochrome *b* sequences obtained from each sample matched or closely resembled Atlantic salmon or lake trout reference sequences. Each sample's D-loop and cytochrome *b* sequences matched reference sequences from the species to which it was identified using zooarchaeological methods and differed from closely related taxa. The phylogenetic analyses yielded similar results and (Figure 8 and Figure 9). All the sequences obtained from samples zooarchaeologically identified as lake trout formed a group with lake trout reference sequences, whilst those from samples zooarchaeologically identified as Atlantic salmon clustered with references sequences from that species (Figure 8 and Figure 9). Based on these data, species-level identifications could be confidently assigned to each of the 60 samples that yielded mitochondrial DNA (Table 8). The DNA-based species identities assigned to the samples agreed with the species identities assigned to them through zooarchaeological methods and, in the case of four samples, ZooMS (Table 8).

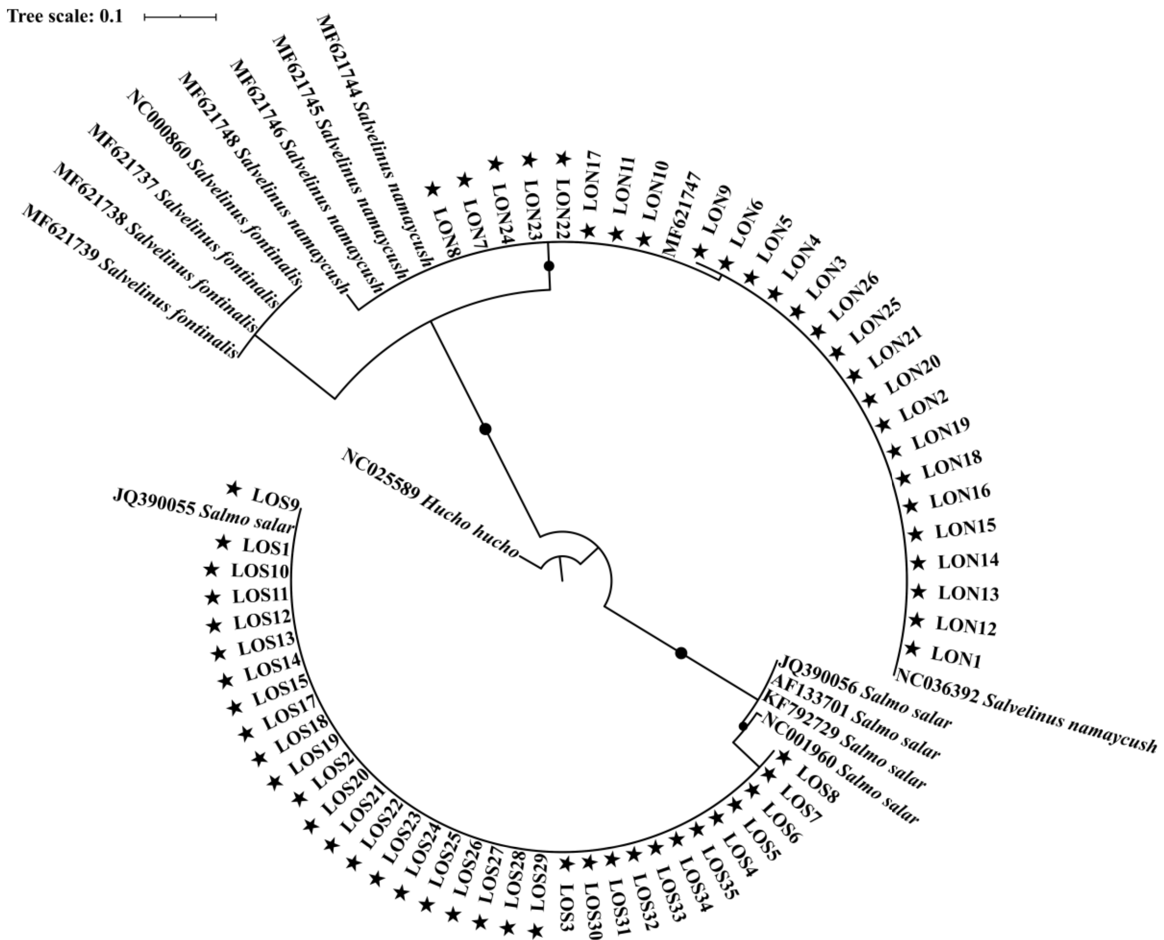

Figure 8. Maximum-likelihood phylogenetic trees displaying the relationship between the D-loop sequences obtained from the Antrex samples (denoted with stars) and references sequences (GenBank accession number shown) from all Atlantic salmonid (*Salmo* spp.) and char (*Salvelinus* spp.) species native to southern Ontario. The tree was rooted using a huchen (*Hucho hucho*) reference sequence as an outgroup. LOS# indicates samples from Antrex identified through conventional zooarchaeological methods or ZooMS as Atlantic salmon (*Salmo salar*), whereas LON# denotes samples identified as lake trout (*Salvelinus namaycush*). The circles indicate nodes with bootstrap support values greater than 50% after 1,000 replications. The scale bar represents the number of nucleotide substitutions per site.

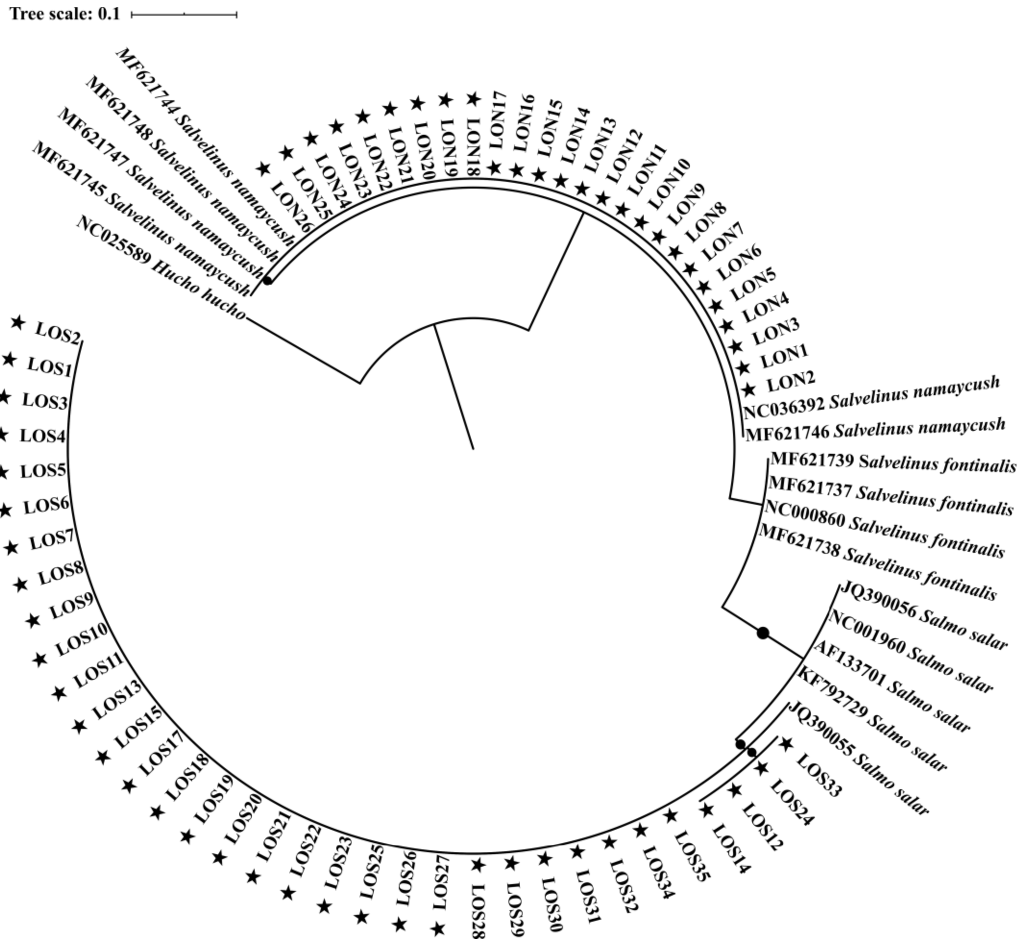

Figure 9. Maximum-likelihood phylogenetic trees displaying the relationship between the *cytochrome b* sequences obtained from the Antrex samples (denoted with stars) and references sequences (GenBank accession number shown) from all Atlantic salmonid (*Salmo* spp.) and char (*Salvelinus* spp.) species native to southern Ontario. The tree was rooted using a huchen (*Hucho hucho*) reference sequence as an outgroup. LOS# indicates samples from Antrex identified through conventional zooarchaeological methods or ZooMS as Atlantic salmon (*Salmo salar*), whereas LON# denotes samples identified as lake trout (*Salvelinus namaycush*). The circles indicate nodes with bootstrap support values greater than 50% after 1,000 replications. The scale bar represents the number of nucleotide substitutions per site.

3.6. Discussion

3.6.1. Authenticity of Ancient DNA Data

Although archaeological fish remains often exhibit exceptional DNA preservation (Oosting et al. 2019), they, like all ancient skeletal remains, are highly susceptible to

contamination from exogenous sources of modern DNA (Yang and Watt 2005). However, various lines of evidence suggest our aDNA data are authentic rather than the result of systematic contamination. First, all pre-PCR laboratory work was conducted in a dedicated aDNA laboratory that is physically separated from modern DNA and post-PCR laboratories (Cooper and Poinar 2000). Second, prior to DNA extraction, the samples were decontaminated using both bleach and UV irradiation (Yang and Watt 2005). Third, no DNA was amplified from any of the blank extraction or negative PCR controls, indicating a lack of systematic contamination (Cooper and Poinar 2000). Fourth, the sex identities assigned to samples were successfully reproduced with two independent PCR assays and two to five replicates of each assay (Cooper and Poinar 2000). Fifth, analysis of the amplified D-loop and *cytochrome b* fragments yielded identical species identifications for each of the 60 samples that yielded mitochondrial DNA (Yang et al. 2004). Sixth, the DNA-based species identities assigned matched those assigned to them through conventional zooarchaeological methods and ZooMS in the case of four samples, providing independent support for the aDNA data (Yang et al. 2004). Seventh, all repeat DNA extractions produced sex identities as well as D-loop and *cytochrome b* sequences that matched those obtained from the initial extractions (Cooper and Poinar 2000). Eighth, the successful amplification of DNA from associated passenger pigeon (*Ectopistes migratorius*) remains from Antrex (Guiry, Orchard, Royle, Cheung, et al. 2020), provides supporting evidence for the preservation of DNA in fish remains from the site (Cooper and Poinar 2000). Finally, with the exception of LOS16, all of the samples (n=27) that underwent stable isotope analysis had well-preserved collagen (Table B1) (Guiry, Buckley, Orchard, Hawkins, et al. 2020), indicating that the samples exhibit good overall biomolecular preservation (Cooper and Poinar 2000).

3.6.2. Efficacy of Sex Identification Method

In order to be an efficient sex identification method for archaeological or palaeontological remains, PCR-based sex identification methods must be both sensitive and accurate. The high proportion of samples to which we successfully assigned sex identities (83.61%) with our method indicates it is highly sensitive. In this study, we did not assess our method's accuracy by applying it to a large sample of Atlantic salmonids and char of known phenotypic sex. However, the congruence between the sex identifications we assigned to a small sample of modern Atlantic salmon and Arctic char

with our method and the validated method described by Yano et al. (2013), suggests our method is reliable. The results of previous studies provide further support for the reliability of our method. Previous studies have demonstrated a strong relationship between Atlantic salmonids' *sdY* genotype and their phenotypic sex (Eisbrenner et al. 2014; Yano et al. 2013; Quéméré et al. 2014; King and Stevens 2020). For instance, amongst the Atlantic salmon analyzed by Eisbrenner et al. (2014), *sdY* was present in 97.66% of analyzed males (*n*=555) and absent in 98.96% of analyzed females (*n*=384). Although *sdY* in char has not been as extensively studied, Yano et al. (2013) found a similar strong relationship between *sdY* genotype and phenotypic sex amongst char species, including lake trout. This correspondence between *sdY* genotype and phenotypic sex observed among Atlantic salmonids and char indicates *sdY* is an accurate sex identification marker for these taxa, suggesting our method is reliable. However, males lacking *sdY* and females possessing *sdY* have been documented among char and Atlantic salmonids (e.g., Eisbrenner et al. 2014; Yano et al. 2013), indicating our method is not foolproof.

Several other design aspects of our sex identification method also contribute to its reliability. Critical to our method's reliability is the use of two PCR assays to assign sex identities to samples. By facilitating the detection of Y-chromosome dropout due to degradation (Royle et al. 2018; Taberlet et al. 1996; Quéméré et al. 2014), a common issue in aDNA studies (Kim et al. 2013), the use of two assays reduces false female identifications. False female identifications are further reduced in our method through the co-amplification of an IPC in both assays. The co-amplification of these IPCs provides for ascertaining whether the failure to amplify *sdY* is indeed due to the sample being female or due to a lack of amplifiable DNA as result of inhibition or degradation. However, the co-amplification of the IPCs in the assays can, by outcompeting *sdY*, lead to *sdY* drop-out, resulting in the erroneous identification of males as females (Sinding et al. 2016). Following Royle et al. (2018) and Speller and Yang (2016), our method reduces the probability of the IPCs outcompeting *sdY* by designing the assays to preferentially amplify *sdY*. Both assays promote the preferential amplification of *sdY* by targeting *sdY* fragments shorter than the IPCs fragment and by using a higher concentration of *sdY* primers relative to the IPC primers (Royle et al. 2018; Speller and Yang 2016). Although these measures promoted the preferential amplification of *sdY*, our data indicates that the amplification of the IPC, but not *sdY*, from male samples did

still occur. For example, one of the D-loop/*sdY* and two of the Clock1a/*sdY* PCR replicates performed for LON7, which was identified as male, failed to amplify *sdY* but amplified the IPC (Table B3). However, the performance of PCR replicates for both assays enabled the identification of instances of *sdY*, and in the case of females, IPC dropout, that could influence the sex identification results, minimizing their effect. In addition to the above factors, the drop-out of *sdY* and subsequent misclassification of male salmonid samples as females can also occur as a result of primer–template mismatches (King and Stevens, in press). To an extent, by using different *sdY* primers in each assay, our method mitigates the potential for such misidentification related to primer–template mismatches (Royle et al. 2018; Szpak et al. 2020).

On top of being an efficient sex identification method for Atlantic salmonid and chars remains the method described in this study is also useful for species identification. Through the sequence analysis of the D-loop fragment co-amplified as an IPC in the D-loop/*sdY* assay, we were able to assign species-level identifications to 60 of the 61 samples. As this fragment exhibits intra-specific variation amongst both Atlantic salmon and lake trout, analysis of this fragment may also shed light on the historic genetic diversity of these taxa. Here, we confirmed the D-loop species identities assigned to these 60 samples through the amplification and analysis of a fragment of *cytochrome b.* Although not needed for species identification, the amplification and analysis of *cytochrome b* functions as an internal reproducibility test useful for detecting contamination (Yang et al. 2004). Any discrepancies between the species identities indicated by these D-loop and *cytochrome b* fragments might be indicative of contamination (Yang et al. 2004).

3.6.3. Sex-Selectivity of Antrex's Atlantic Salmon and Lake Trout Fisheries

At Antrex, neither the aggregate sex ratio nor the sex ratio obtained for the individual species were significantly male-biased. In the case of lake trout, the Antrex fishery appears to have targeted males and females relatively equally, whilst female fish appear to have been to some extent preferentially harvested by the site's Atlantic salmon fishery. This suggests the site's Middle Ontario Iroquoian inhabitants did not preferentially target male Atlantic salmon and lake trout, and, by inference, did not manage these salmonids through male-selective fishing. The lack of evidence at Antrex

for the management of Atlantic salmon and lake trout through male-selective fishing is potentially the product of a myriad of factors. These include the fishing technologies used by the site's inhabitants, a lack of pronounced sexual dimorphism amongst lake trout, natural biases in Atlantic salmon sex ratios, and the local abundance of both species.

Fishing Technology

Traditionally, gillnets were commonly used by the Wendat and other Indigenous peoples in the Great Lakes region to harvest salmonids, particularly chars and whitefish (*Coregonus* spp.) (Tooker 1964; Cleland 1982). Gillnets are nets suspended in the water column that passively ensnare fish that fall within a narrow size range, with the size range of ensnared fish being determined by the net's mesh gauge (Hubert et al. 2012). Fish that are substantially larger than a gillnet's mesh gauge are unable to breach the net, whilst small fish are able to slip through the net without being ensnared (Hubert et al. 2012). However, as male and female Atlantic salmon and lake trout often overlap considerably in size (e.g., Halttunen et al. 2013; Miller and Kennedy 1948), it is potentially difficult to preferentially target individuals from these taxa belonging to a specific sex with gillnets. Nonetheless, through regular monitoring of gillnets and the release of salmonids belonging to an undesired sex, gillnets could potentially be operated in a sex-selective manner. Historic Wendat fishers, however, often left gillnets in place for extended periods of time (Tooker 1964), which reduces the potential for the release of undesired individuals by increasing mortality among ensnared fish (Buchanan et al. 2004). The unbiased lake trout sex ratio at Antrex might reflect its inhabitants' reliance on similar gillnetting strategies with limited sex-selective capabilities to harvest this species. However, there is scant direct evidence for the use of gillnets by Antrex's inhabitants. Nonetheless, the presence of bone netting needles at the site indicates fishing nets—potentially gillnets—were manufactured and/or mended, and hence used, by its inhabitants (Cooper 2010).

Degree of Sexual Dimorphism

Due to their greater accessibility and predictability during their spring to fall spawning run, Ontario Iroquoians likely harvested Atlantic salmon as they migrated upstream from Lake Ontario (Hawkins et al. 2019; Holm et al. 2009). Similarly, lake trout were likely harvested during the fall, when they aggregate on shoals in Lake Ontario in

order to spawn (Needs-Howarth and Thomas 1998; Holm et al. 2009; Martin and Olver 1980). During this spawning period, lake trout, unlike many other salmonids, exhibit relatively muted sexual dimorphism (Martin and Olver 1980). Notably, spawning male lake trout do not typically develop the prominent kype seen among spawning males belonging to other salmonid species (Royce 1951). Male lake trout do develop dark bands during the spawning season that set them apart from females, but only for a very brief period (Martin and Olver 1980; Royce 1951). In addition, whilst breeding tubercles are a male-specific trait in some lake trout populations, this trait is not universally male-specific, with females also developing breeding tubercles in some populations (Martin and Olver 1980). By impeding the ready sex identification of individual lake trout, this lack of pronounced, sustained, and consistent, sexual dimorphism may have hampered Middle Ontario Iroquoians' ability to fish sex-selectively for this species. As spawning Atlantic salmon exhibit pronounced sexual dimorphism (Fleming and Einum 2011), this hypothesis likely does not account for the lack of a male-selective Atlantic salmon fishery at Antrex.

Naturally Biased Sex Ratios

Amongst some modern Atlantic salmon populations, the sex ratio of spawning runs has been observed to temporally vary (Harvey et al. 2017; Pérez et al. 2005). Reflecting females' earlier migration timing, Atlantic salmon spawning runs in some populations have been observed to be female dominated during the early portion of the spawning season (Dahl et al. 2005; Harvey et al. 2017; Pérez et al. 2005; Sparholt et al. 2018). As the spawning season progresses, and males begin to migrate in larger numbers, spawning runs become less female-biased (Harvey et al. 2017; Pérez et al. 2005). During the peak of the spawning run, the sex ratio may be relatively unbiased, yet it may become male-biased following this peak, with some males persisting in spawning creeks throughout the winter (Harvey et al. 2017; Holm et al. 2009).

In modern recreational fisheries targeting Atlantic salmon spawning runs, the sex demographics of harvested salmon often mirror those of the spawning run at their time of harvest (Pérez et al. 2005; Harvey et al. 2017). Consequently, assuming they were harvested during their spawning runs in a non-sex-selective manner, the sex ratios of archaeological Atlantic salmon assemblages may provide insights into when they were harvested. Although not statistically more abundant, the predominance of female rather

than male Atlantic salmon at Antrex might reflect the harvesting of salmon early in their spring to fall spawning run when females may have been more prevalent. Support for such targeting of early-run Credit River salmon by Indigenous fisheries can be found in Euro-Canadian historic records. In a diary entry dated June 16th, 1796, Elizabeth Simcoe (Robertson, 1911:328) reported that Indigenous people congregated along the Credit River “at this season to fish for salmon.” While likely referring to Mississauga rather than Iroquoians related to Antrex’s inhabitants, Simcoe’s statement does indicate Indigenous peoples did harvest the early-run salmon that migrated up the Credit River during the spring. Alternatively, the predominance of female Atlantic salmon at Antrex could also reflect females being incidentally harvested in larger numbers due to the Credit River run, similar to some modern populations (Fleming 1998), having a female-biased sex ratio, regardless of season.

Historically, the condition of Atlantic salmon running up the Credit River and other nearby Lake Ontario tributaries appear to have seasonally varied, which may have influenced the timing of Antrex’s Atlantic salmon fishery. During the early nineteenth-century, Thomas W. Magrath (1833:299) described salmon taken from the Credit River during the spring as being in "fine” condition and “firm and full of curd”. Similarly, Samuel Wilmot (1872:79) later in the century remarked that spring running salmon in the nearby Humber River were “rich and fat in flesh, in prime condition” while fall running salmon were “lean and lank, out of condition.” The early-run timing potentially suggested by Antrex’s female-dominated Atlantic salmon sex ratio, might reflect a strategy to maximize access to prime condition salmon. However, we stress additional samples from Antrex need to be analyzed in order to confirm the female-bias of the site’s Atlantic salmon fishery.

Local Abundance

Historical records suggest Atlantic salmon and lake trout were potentially abundant in the vicinity of the Antrex site during its occupation. For instance, the nearby Credit River was historically described as supporting one of the largest Atlantic salmon runs on the north shore of Lake Ontario (Parson 1973; Dymond et al. 2019). Although lake trout were likely less abundant than Atlantic salmon, they are hypothesized to have been quite abundant in the lake (Smith 1995; Elrod et al. 1995). As Antrex was likely only occupied by approximately 400 people for roughly 20 years (Robertson and

Williamson 2002), the fishing pressure exerted by the site's inhabitants may have been insufficient to depress these locally abundant Atlantic salmon and lake trout stocks. Since only 11,000 Iroquoians are estimated to have occupied south-central Ontario during the early Middle Ontario Iroquoian period, when Antrex was occupied (Warrick 2008), regional fishing pressures may have also been relatively minimal. Without resource depression of the locally abundant Atlantic salmon and lake trout stocks, there may have been little incentive for Antrex's inhabitants to manage them through male-selective fishing (Alvard and Kuznar 2004). Alternatively, other management strategies, such as the ethnographically documented tenure systems (Thoms 2004), seasonal closures (Tiro 2016), and a fishing theology (Sioui 1999), may have also been sufficient to maintain the productivity of local Atlantic salmon and lake trout stocks.

3.7. Conclusion

Here, we reported on a DNA-based sex identification method for archaeological Atlantic salmonid and char remains that is adapted from a method developed for ancient Pacific salmonid remains. This method assigns sex identities to samples through two PCR assays that screen for the presence of the Y-linked *sdY* gene and IPCs consisting of D-loop or *clock1b* fragments. Reflecting this method's efficiency and sensitivity, we were able to assign sex identities to 51 of the 61 analyzed Atlantic salmon and lake trout remains from the Antrex site. By sequencing the D-loop fragment co-amplified as IPC and additional *cytochrome b* fragment, this method also allowed for species identifications to be assigned to 60 of the remains. Although only applied to remains from a single site in Ontario, this DNA-based sex identification method likely has applicability to Atlantic salmonid and char assemblages from sites across their global range. Moreover, the high proportion of salmonid remains assigned sex identities in this study and that of Royle et al. (2018) highlights the potential for PCR-based sex identification methods for other fish taxa represented in zooarchaeological assemblages. Similar PCR-based sex identification methods can potentially be developed for the remains of other fish taxa, such as Atlantic cod (*Gadus morhua*) (Kirubakaran et al. 2019), and sablefish (*Anoplopoma fimbria*) (Rondeau et al. 2013), whose putative master sex-determining genes have also been identified.

The sex identification data generated in this study suggests Antrex's Middle Ontario Iroquoian inhabitants did not manage Atlantic salmon and lake trout fisheries

through male-selective fishing. As Ontario Iroquoian fishing strategies geographically varied due to differing local environmental conditions (Hawkins et al. 2019), this lack of male-selective fishing may not have been universal among Middle Ontario Iroquoians. Likewise, mirroring temporal changes in other aspects of Ontario Iroquoian fishing strategies (Hawkins et al. 2019), the sex-selectivity of fisheries may have also temporally varied in response to changing cultural and environmental conditions. Documenting such potential geographic and temporal variation in the sex-selectivity of Ontario Iroquoian fisheries will require the analysis of remains from sites from other regions and time periods. Conducting such studies will provide insights into the factors that influenced the sex-selectivity of Ontario Iroquoian Atlantic salmon and lake trout fisheries.

Chapter 4. A Two-Tiered Approach to the DNA-Based Species Identification of Archaeological Bony Fish (Osteichthyes) Remains

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4.1. Abstract

In this study, we propose a new two-tiered approach to the DNA-based identification of archaeological bony fish (Osteichthyes) remains. In this approach, novel universal primers are first used to amplify a ~166 bp fragment of the *cytochrome c oxidase I* (*COI*) DNA barcode region, which is used to assign preliminary identifications to samples. Taxon-specific primers are then selected based on this initial identification and used to amplify a secondary marker (e.g., *cytochrome b*, D-loop) that can refine these *COI*-based identifications to the species-level. To evaluate efficacy of this approach, we applied it whole or in part to 33 modern and 89 archaeological fish specimens. A fragment of *COI* was successfully amplified from 32 (96.97%) of the modern and 53 (58.43%) of the archaeological samples. The successfully amplified samples represent at least 37 bony fish species from 28 genera, 18 families, and 10 orders, highlighting these primers' universality. Through the analysis of the amplified *COI* fragment, all of the samples as well as contaminant fish DNA amplified from one of the modern samples could be identified to the family- (*n*=1), genus- (*n*=51), or species-level (*n*=34). The reliability of these identifications is supported by the concordance observed between the species identities assigned to the modern samples that yielded endogenous DNA and their known species identities. Using taxon-specific primers, we successfully amplified a secondary marker from 43 of the 53 (81.13%) archaeological remains that yielded a fragment of *COI*. Analysis of the secondary markers enabled the identifications assigned to 36 of the archaeological remains to be refined to the species-level and

confirmed the *COI*-based species identifications assigned to 7 remains. These data demonstrate our proposed two-tiered approach to DNA-based species identification is an efficient and broadly applicable means for maximizing the number of fish remains with species-level identifications.

4.2. Introduction

The taxonomic identification of fish remains plays a foundational role in ichthyoarchaeological studies. Through the identification of archaeological fish remains, researchers can identify the range of taxa harvested by past fisheries and their relative importance (e.g., Abhayan et al. 2020; Béarez et al. 2016; Douglass et al. 2018; Hopt and Grier 2018; Moss 2011; Needs-Howarth and Thomas 1998; Ono and Intoh 2011; Rodrigues et al. 2018; Speller et al. 2005; Zabilska-Kunek 2019). By considering the ecology, ethology, size, genetic profiles, and/or stable isotope values of the taxa represented at sites, it is possible to reconstruct past fisheries' seasonality (e.g., Moss 2011; Needs-Howarth and Thomas 1998; Zabilska-Kunek 2019), harvest methods (e.g., Béarez et al. 2016; Butler 1994; Greenspan 1998), and fishing grounds (e.g., Abhayan et al. 2020; Douglass et al. 2018; Hutchinson et al. 2015). As fishing is embedded within a socio-ecological system (Berkes 2011), taxonomic identification can also provide insights into aspects of cultural systems beyond what, how, when, and where fish were caught. Variation in the abundance of particular taxa between and within sites, for instance, has been used to investigate resource ownership (e.g., Bartosiewicz and Bonsall 2008; Moss 2011; Speller et al. 2005). By identifying the fish remains in ritual deposits, researchers have also examined the spiritual and symbolic importance of particular taxa (e.g., Betts et al. 2012; Maxwell 2000). Documenting the fish taxa represented at archaeological sites can also be used to address a range of questions regarding palaeoenvironments, including the historical abundance (e.g., Braje et al. 2017; McKechnie et al. 2014) and range of taxa (e.g., Dombrosky et al. 2016; Pagès et al. 2008), Such data can also be used to characterize palaeoenvironments' biotic and abiotic factors (e.g., Lambrides and Weisler 2018; Zangrando et al. 2016). The identification of taxa also opens the door to further analyses that may provide additional insights into palaeoecological questions. Conducting stable isotope analyses on identified remains, for example, has provided insights into the migratory (e.g., Guiry et al. 2016; Guiry, Royle, Matson, et al. 2020; Guiry, Royle, Orchard, et al. 2020; Halffman et

al. 2015) and feeding behavior (e.g., Bas et al. 2020; Braje et al. 2017) of taxa, as well as anthropogenic or natural shifts in aquatic nutrient cycles (e.g., Guiry, Buckley, Orchard, Hawkins, et al. 2020; Häberle et al. 2016). Moreover, by conducting aDNA analysis on remains identified to the species-level, both the historic genetic and phenotypic diversity (e.g., Johnson et al. 2018; Ólafsdóttir et al. 2014; Thompson et al. 2019; Speller et al. 2012) and population affinity of fish populations can be investigated (e.g., Ludwig et al. 2008; Živaljević et al. 2017).

Ideally, fish remains should be identified to the species-level. The species identification of fish remains allows for more fine-scale archaeological and palaeoenvironmental interpretations than is possible with identifications to higher taxonomic ranks (Colley 1990; Giovas et al. 2017). However, as many skeletal elements lack interspecific morphological variation, identifying ancient fish remains to the species-level using conventional morphology-based zooarchaeological approaches is often difficult (Morales-Muñiz and Llorente-Rodriguez 2018; Wheeler 1978). Even when elements exhibit *inter*specific morphological differences, *intra*specific variation can reduce the magnitude of these differences, complicating the delineation of species through their skeletal morphology (e.g., Gobalet et al. 2004; Thieren et al. 2016). Moreover, archaeological fish remains are often fragmented which further hinders morphology-based species identification as fragmentation frequently results in the loss of taxonomically informative features (Colley 1990). Due to their relatively weak and labile collagen ultrastructure, fish remains are particularly susceptible to such fragmentation (Szpak 2011). Factors extrinsic to fish remains, including the existence of cryptic and undescribed species, time constraints, lack of access to comprehensive reference collections, high fish biodiversity in the vicinity of a site, and gaps in researchers' ichthyological knowledge, can also impede morphology-based species identification (Colley 1990; Cooke and Jiménez 2004; Gobalet 2001; Wake 2004; Wheeler 1978; Yeomans and Beech 2021). Due to these factors, archaeological fish remains are often identified to the genus-level or above (e.g., Ono and Intoh 2011; Stevenson and Butler 2015; Zabilska-Kunek 2019) and on occasion are assigned divergent identifications by different researchers (e.g., Giovas et al. 2017; Gobalet 2001) or misidentified (e.g., Harvey et al. 2018; Moss et al. 2016; Rodrigues et al. 2018). The difficulties surrounding the identification of fish remains may also bias the taxonomic composition of ichthyofaunal assemblages in favour of taxa with many robust and

morphologically distinctive remains, complicating interpretations (Colley 1990; Ewonus 2011; Wheeler and Jones 1989).

Researchers attempting to morphologically identify modern fish specimens to the species-level often face similar obstacles (Ward et al. 2009). Consequently, researchers now routinely rely on DNA barcoding to identify modern fish specimens to the species-level (Hebert et al. 2003; Ward et al. 2009). In DNA barcoding, specimens are identified by comparing their DNA barcode, which consists of the sequence of a standardized fragment of DNA, to that of specimens of known identity (Hebert et al. 2003; Ward et al. 2009). In the case of fish and other animals, the DNA barcode commonly used for species identification consists of a ~650 bp fragment of 5' end of the mitochondrial *cytochrome c oxidase I* (*COI*) gene (Folmer et al. 1994; Hebert et al. 2003; Ward et al. 2009). Through the analysis of this fragment, it is possible to discriminate approximately 99% of fish species (Ward et al. 2009).

Although useful for identifying modern fish specimens, conventional *COI*-based DNA barcoding cannot be used to identify archaeological fish remains. Since ancient samples have been subjected to taphonomic processes that degrade DNA, long DNA fragments (>300 bp), including *COI* DNA barcodes, cannot generally be amplified from ancient samples (Hajibabaei et al. 2006; Pääbo et al. 2004). However, short fragments (<300 bp) of the *COI* DNA barcode, so-called mini-barcodes, can often be amplified from samples with degraded DNA (Bhattacharjee and Ghosh 2014; Hajibabaei et al. 2006; Meusnier et al. 2008; Shokralla et al. 2015). Despite their diminutive size, *COI* mini-barcodes often retain enough sequence variation to separate many fish species (Bhattacharjee and Ghosh 2014; Hajibabaei et al. 2006; Meusnier et al. 2008; Shokralla et al. 2015). This mini-barcoding approach has been previously successfully used to identify processed fish products (Shokralla et al. 2015) and archival fish tissues (Bhattacharjee and Ghosh 2014) with degraded DNA.

Despite its potential, only a handful of studies have attempted to identify archaeological fish remains through the analysis of *COI* mini-barcodes (e.g., Halffman et al. 2015; Macheridis et al. 2020; Puncher et al. 2019; Shirak et al. 2013). The few studies that have used fragments of the *COI* barcode region to identify fish remains have typically relied on primer systems developed for specific taxa (e.g., Macheridis et al. 2020; Puncher et al. 2019; Shirak et al. 2013). The efficient application of such taxon-

specific primers require *a priori* knowledge about the taxonomic identity of fish remains, which hinders their applicability to ichthyofauna remains only identified as fish (Speller et al. 2016). Such fish remains comprise in many archaeological contexts a significant portion of the recovered ichthyoarchaeological assemblage (e.g., Abhayan et al. 2020; Douglass et al. 2018; Stevenson and Butler 2015; Zabilska-Kunek 2019). Identifying the numerous remains categorized simply as fish through mini-barcoding requires universal primers capable of amplifying a fragment of *COI* from an array of taxa. Unfortunately, few universal mini-barcoding primers are available for fish, with those developed often targeting long fragments (>200 bp) of *COI* unlikely to preserve in many fish remains (e.g, Shokralla et al. 2015). Moreover, universal eukaryotic primers that amplify a 130 bp of the *COI* barcode (Meusnier et al. 2008) cannot be efficiently applied to archaeological fish remains as they can readily amplify contaminant DNA.

In this study, we develop new universal *COI* mini-barcoding primers useful for identifying archaeological samples of bony fish (Osteichthyes), a superclass that includes more than 33,000 species (Fricke et al. 2020). We propose incorporating these universal primers within a two-tiered approach to DNA-based species identification that sequentially refines through the analysis of multiple markers (Figure 10). In this approach, our novel universal mini-barcoding primers are first used to amplify a *COI* mini-barcode, which is used to assign an initial family-, genus-, or species-level identification to a sample (Figure 10). Since no single mini-barcode can identity all fish taxa to the species-level (Shokralla et al. 2015), we propose utilizing this initial identification to guide the selection of taxon-specific primers that target a marker capable of refining these initial identifications to the species-level (Figure 10). Depending on the availability of reference sequences and the results of population genetic studies, this secondary marker may be another *COI* mini-barcode, *cytochrome b* (*Cytb*), D-loop, or any other segment of mitochondrial DNA (mtDNA). To evaluate the efficacy of this approach, we first tested our universal mini-barcoding primers on 33 modern fish specimens and 89 archaeological fish remains. We subsequently used taxon-specific primers to amplify a secondary marker from the archaeological remains. Our results demonstrate that our proposed two-tiered DNA-based approach to species identification is an efficient and broadly applicable method for assigning species-level identifications to fish remains.

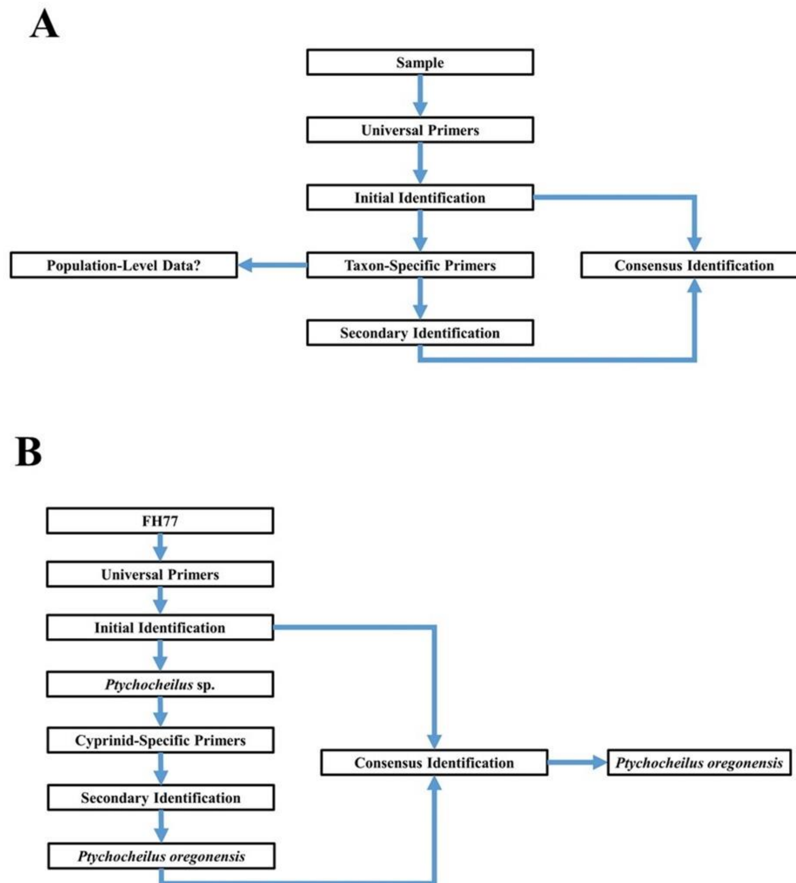

Figure 10. (A) Idealized workflow for the proposed two-tiered approach to the DNA-based species identification of archaeological bony fish remains. (B) Workflow for an archaeological fish specimen from EeRb-144 identified as a northern pikeminnow (*Ptychocheilus oregonensis*) through our two-tiered DNA-based species identification approach.

4.3. Materials and Methods

4.3.1. Development of Universal *COI* Mini-Barcoding Primers

We assembled a database of all the bony fish species with complete mitochondrial genome sequences accessioned in MitoFish version 2.96 (Iwasaki et al. 2013). Subsequently, we used a random number generator to select at least one species belonging to each of the families represented in the database. In total, this sample consisted of 306 species belonging to 288 families, and 45 orders (Table C1). Complete COI sequences from each of these taxa were retrieved GenBank (Sayers et al. 2020) and then aligned with Clustal Omega through the EMBL-EBI server (Goujon et

al. 2010; Sievers et al. 2011). We subsequently examined the alignment in Bioedit version 7.2.5 (Hall 1999) to design multiple primer pairs that flank a variable region within the DNA barcode region and amplify a fragment less than 200 bp long. The potential efficiency of these primers was then evaluated by determining their melting temperatures and potential to form secondary structures through NetPrimer (http://www.premierbiosoft.com/netprimer) and/or Oligoanalyzer version 3.1 (Owczarzy et al. 2008). We further evaluated the specificity and efficiency of several of the primer pairs by applying them to a small set of modern fish specimens. To optimize the reaction conditions for the primers, we tested them on modern specimens with varying PCR conditions. Based on these analyses, we selected a single pair of universal primers (F134 and R294) to apply to a sample of archaeological fish remains and a larger set of modern fish specimens (Table 10). These primers flank a 118 bp mini-barcode located near the 5' end of the COI DNA barcode region and amplify a fragment that is approximately 166 bp long.

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Table 10. Primer pairs used in this study.

Primers	Taxon	Locus	Sequence (5'——3')	Amplicon Size (bp)	T_a (°C)	Source
F134(F)[1]	Osteichthyes	*COI*	CTCTATCTAGTATTTGGNGCYTG	166	50	This study
R294 (R)			ATTGGCATTACTATAAAGAARATYAT			This study
F600 (F)	Cyprinidae	*Cytb*	TCAACCGCATTTCRTCVG	172	52	This study
R720 (R)			CACCAATATTTCAGGTYTCYTTA			This study
F647 (F)	Catostomidae	*COI*	CACCAATATTTCAGGTYTCYTTA	233	52	This study
R647 (R)			CCTGCCAGGTGAAGAGAAAAGAT			This study
Smc7 (F)	Salmonidae	D-loop	AACCCCTAAACCAGGAAGTCTCAA	249	52	Yang et al. (2004)
Smc8 (R)			CTTAACAGCTTCAGTGTTATGCT			Yang et al. (2004)

[1] Forward primers are denoted with an (F) and reverse primers are denoted with an (R).

4.3.2. Specimens

Modern Specimens

To further evaluate their efficacy as well as the utility of the amplified mini-barcode for species identification, we applied F134 and R294 to 33 modern fish specimens whose species identity was known. This set of specimens includes 33 species from 26 genera, 17 families, and 9 orders (Table 11). Whole genome DNA was extracted from the modern samples using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocols. DNA extractions and PCR setups involving the modern specimens were conducted in a laboratory in the Centre for Forensic Research, Simon Fraser University (Burnaby, BC), that is dedicated to analysis of modern materials.

Table 11. Taxonomy of the modern fish specimens analyzed in this study.

Sample Number	Order	Family	Species
MF15	Beloniformes	Scomberesocidae	*Cololabis saira*
CM26	Clupeiformes	Clupeidae	*Clupea pallasii*
MF20	Clupeiformes	Clupeidae	*Konosirus punctatus*
MF10	Clupeiformes	Clupeidae	*Sardinops melanostictus*
MF18	Gadiformes	Gadidae	*Gadus chalcogrammus*
MF9	Gadiformes	Gadidae	*Gadus macrocephalus*
MF27	Gadiformes	Gadidae	*Micromesistius australis*
MF24	Perciformes	Carangidae	*Trachinotus ovatus*
MF29	Perciformes	Carangidae	*Decapterus macrosoma*
MF12	Perciformes	Centropomidae	*Lates calarifer*
MF8	Perciformes	Percidae	*Perca flavescens*
MF11	Perciformes	Percidae	*Sander vitreus*
MF16	Perciformes	Sciaenidae	*Larimichthys crocea*
MF19	Perciformes	Scombridae	*Scomber scombrus*
MF7	Perciformes	Scombridae	*Thunnus albacares*
MF28	Perciformes	Trichiuridae	*Trichiurus lepturus*
MF25	Pleuronectiformes	Paralichthyidae	*Paralichthys olivaceus*
MF22	Pleuronectiformes	Pleuronectidae	*Hippoglossus stenolepis*
MF26	Pleuronectiformes	Pleuronectidae	*Parophrys vetulus*
MF14	Osmeriformes	Osmeridae	*Mallotus villosus*
PNK5	Salmoniformes	Salmonidae	*Oncorhynchus gorbuscha*
CHM4	Salmoniformes	Salmonidae	*Oncorhynchus keta*
MF4	Salmoniformes	Salmonidae	*Oncorhynchus kisutch*
MF1	Salmoniformes	Salmonidae	*Oncorhynchus mykiss*

Sample Number	Order	Family	Species
MF5	Salmoniformes	Salmonidae	*Oncorhynchus nerka*
MF3	Salmoniformes	Salmonidae	*Oncorhynchus tshawytscha*
MF13	Salmoniformes	Salmonidae	*Salmo salar*
MF2	Salmoniformes	Salmonidae	*Salvelinus alpinus*
MF23	Scorpaeniformes	Anoplopomatidae	*Anoplopoma fimbria*
MF6	Scorpaeniformes	Hexagrammidae	*Ophiodon elongatus*
RFM1	Scorpaeniformes	Scorpaenidae	*Sebastes nebulosus*
RFM2	Scorpaeniformes	Scorpaenidae	*Sebastes pinniger*
MF17	Siluriformes	Pangasiidae	*Pangasius hypophthalmus*

Archaeological Specimens

In total, 89 archaeological fishbones were analyzed as part of this study. All of the analyzed specimens are from EeRb-144, British Columbia, and date to the middle and late Holocene (Nicholas et al. 2012). Decontamination, DNA extraction, and PCR setup procedures involving the ancient samples were conducted in a dedicated ancient DNA laboratory in the Department of Archaeology, Simon Fraser University (Burnaby, BC). Following standard contamination control protocols (Poinar 2003; Yang and Watt 2005), this laboratory is positively pressured, equipped with a UV-HEPA air ventilation system, and is physically separated from both the modern DNA and post-PCR laboratories. Additional strict contamination controls, including the use of protective clothing and the regular cleaning of bench surfaces with bleach, were adhered to throughout the analyses (Yang and Watt 2005).

Prior to DNA extraction, all of the samples were decontaminated using one of two protocols. The majority of the samples (*n*=84) were decontaminated using a modified version of the protocol described by Speller et al. (2012). In brief, samples were soaked in a 100% commercial bleach solution (≈5% w/v NaOCl) for 5–7 min, submerged twice in distilled water for ≈30 s and 5–12 min, and then UV irradiated in a crosslinker for 15 min on two sides. The remaining samples (*n*=5; FH1–FH5) were decontaminated using a modified version of the protocol outlined by Yang et al. (2004). This decontamination protocol mirrored the protocol described above except for the inclusion of additional rinses in 1 M HCl for 30–60 s and 1 M NaOH for 30–60 s prior to the second submersion of the samples in distilled water. However, this treatment was discontinued after the processing of these samples as the HCl appeared to be rapidly dissolving thin elements.

Following decontamination, the samples were incubated overnight at 50 ˚C in 2–5 ml of lysis buffer (0.5 M EDTA [pH 8.0], 0.5% SDS and 0.5 mg/mL proteinase K) in a rotating hybridization oven. DNA was extracted from the lysed samples using a modified silica-spin column method (Yang et al. 1998; Yang et al. 2008). To monitor for contamination, blank extraction controls were processed alongside the ancient samples and subjected to amplification.

4.3.3. PCR Amplification and DNA Sequencing

All PCR amplifications and post-PCR procedures were conducted in a dedicated post-PCR laboratory in the Centre for Forensic Research, Simon Fraser University (Burnaby, BC). PCR amplifications were performed with a Mastercycler Personal or Gradient thermocycler (Eppendorf, Mississauga, ON) in a 25–50 µL reaction volume containing 1.5× PCR Gold Buffer (Applied Biosystems, Carlsbad, CA), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 µM of primers F134 and R294, BSA (1 mg/mL), 1–6 µL DNA extract, and 0.75–3.45 U AmpliTaq Gold (Applied Biosystems, Carlsbad, CA). The reaction volumes for the PCRs carried out for the archaeological fish remains also included BSA (1 mg/mL). The thermal conditions for the PCRs consisted of an initial denaturation step at 95 ˚C for 12 min followed by 60 cycles at 95 ˚C for 30 (denaturation) s, 50 ˚C for 30 s (annealing), and 70 ˚C for 40 s (extension) and a final extension step at 72 ˚C for 7 min. The conditions for the PCR amplifications of the secondary markers were the same as above but used appropriate taxon-specific primers and annealing temperatures (Table 10). A negative control was included in each PCR run in order to detect instances of contamination.

Following amplification, 5–10 µL of PCR product was pre-stained with SYBR Green I (Life Technologies, Carlsbad, CA), electrophoresed on a 2% agarose gel, and visualized with a Dark Reader transilluminator (Clare Chemical Research, Dolores, CO). Prior to sequencing, some of the amplicons were cleaned with ExoSAP-IT (USB Corporation, Santa Clara, CA) (Bell 2008). Amplicons were directly sequenced in the forward and/or reverse direction at Eurofins Genomics (Louisville, KY and Toronto, ON) with the amplification primers. The sequences obtained from each specimen were visually edited, truncated to remove the primer sequences, and assembled using ChromasPro version 1.7.6 (http://www.technelysium.com.au) or SeqTrace version 0.9.0 (Stucky 2012).

4.3.4. Taxonomic Identification

The edited sequences were compared to reference sequences in the BOLD Public Record Database using the BOLD Identification Engine (Ratnasingham and Hebert 2007) and/or GenBank through a BLAST search (Altschul et al. 1990). A sample was assigned a species-level identification with a marker if its sequence matched or very closely resembled reference sequences from a single species and differed significantly from those of other closely related species (Yang et al. 2004; Rodrigues et al. 2018). If its sequence matched or was equally similar to reference sequences from multiple species, a specimen was assigned to the lowest taxonomic rank shared by these species, which was identified using the Integrated Taxonomic Information System (http://www.itis.gov). If its sequence did not closely resemble any reference sequences, the specimen was regarded as unidentified. Consensus identifications were assigned to specimens by considering the identifications assigned to them through the analysis of both markers. No consensus identification was assigned to a specimen if the analysis of different markers suggested discordant taxonomic identities. In such instances, specimens were considered unidentified.

4.4. Results

4.4.1. Modern Samples

A fragment of *COI* was successfully amplified with the universal primers from all of the modern samples. No DNA was amplified from any of the negative PCR controls amplified in tandem with the modern samples. BOLD Identification Engine and BLAST searches indicated that the mini-barcode sequences obtained from the modern samples all closely resembled bony fish references sequences. Using the criteria outlined above, 24 samples were identified to the species-level, 8 to the genus-level, and 1 to the family-level through the analysis of the amplified mini-barcode (Table 12). The identifications assigned to 32 of the 33 modern specimens were concordant with their known species identities (Table 12).

The only sample that was assigned an identification inconsistent with its known identity was MF25. This specimen was an olive flounder (Paralichthys olivaceus; Paralichthyidae), but was identified as a sparid (Sparidae). This discrepancy is likely the

result of two factors. First, multiple bases in middle of the R294 primer sequence are not conserved in olive flounder, which may have impeded the efficient amplification of endogenous DNA from this specimen. Second, as it was purchased from a supermarket where multiple species are processed and sold, MF25 was likely contaminated with sparid DNA. In the absence of a suitable endogenous template, our use of a high cycle number (n=60) and a high efficiency DNA polymerase (Amplitaq Gold) likely enabled the amplification of this trace contaminant sparid DNA from MF25. Although purchased from a Canadian supermarket, it is unlikely MF25 represents seafood substitution, a widespread phenomenon in Canada (Wong and Hanner 2008). A nearly complete COI DNA barcode obtained from this sample with primers (FishF2 and FishR2) designed by Ward (2005) matched olive flounder barcode reference sequences.

Table 12. Identifications assigned to the modern samples through the analysis of the fragment of *COI* amplified with the universal primers.

Sample Number	Known Species Identity	Assigned Taxonomic Identity	Known and Assigned Identities Match?	Level of Identification
CHM4	*Oncorhynchus keta*	*Oncorhynchus keta*	✓	Species
CM26	*Clupea pallasii*	*Clupea pallasii*	✓	Species
MF1	*Oncorhynchus mykiss*	*Oncorhynchus* sp.	✓	Genus
MF2	*Salvelinus alpinus*	*Salvelinus* sp.	✓	Genus
MF3	*Oncorhynchus tshawytscha*	*Oncorhynchus tshawytscha*	✓	Species
MF4	*Oncorhynchus kisutch*	*Oncorhynchus kisutch*	✓	Species
MF5	*Oncorhynchus nerka*	*Oncorhynchus nerka*	✓	Species
MF6	*Ophiodon elongatus*	*Ophiodon elongatus*	✓	Species
MF7	*Thunnus albacares*	*Thunnus* sp.	✓	Genus
MF8	*Perca flavescens*	*Perca flavescens*	✓	Species
MF9	*Gadus macrocephalus*	*Gadus* sp.	✓	Genus
MF10	*Sardinops melanostictus*	*Sardinops melanostictus*	✓	Species
MF11	*Sander vitreus*	*Sander vitreus*	✓	Species
MF12	*Lates calcarifer*	*Lates calcarifer*	✓	Species

Sample Number	Known Species Identity	Assigned Taxonomic Identity	Known and Assigned Identities Match?	Level of Identification
MF13	*Salmo salar*	*Salmo salar*	✓	Species
MF14	*Mallotus villosus*	*Mallotus villosus*	✓	Species
MF15	*Cololabis saira*	*Cololabis saira*	✓	Species
MF16	*Larimichthys crocea*	*Larimichthys crocea*	✓	Species
MF17	*Pangasius hypophthalmus*	*Pangasius* sp.	✓	Genus
MF18	*Gadus chalcogrammus*	*Gadus* sp.	✓	Genus
MF19	*Scomber scombrus*	*Scomber scombrus*	✓	Species
MF20	*Konosirus punctatus*	*Konosirus punctatus*	✓	Species
MF22	*Hippoglossus stenolepis*	*Hippoglossus stenolepis*	✓	Species
MF23	*Anoplopoma fimbria*	*Anoplopoma fimbria*	✓	Species
MF24	*Trichiurus lepturus*	*Trichiurus lepturus*	✓	Species
MF25	*Paralichthys olivaceus*	Sparid	✗	Family
MF26	*Parophrys vetulus*	*Parophrys vetulus*	✓	Species
MF27	*Micromesistius australis*	*Micromesistius australis*	✓	Species
MF28	*Trachinotus ovatus*	*Trachinotus ovatus*	✓	Species
MF29	*Decapterus macrosoma*	*Decapterus macrosoma*	✓	Species
PNK5	*Oncorhynchus gorbuscha*	*Oncorhynchus gorbuscha*	✓	Species
RFM1	*Sebastes nebulosus*	*Sebastes* sp.	✓	Species
RFM2	*Sebastes pinniger*	*Sebastes* sp.	✓	Species

4.4.2. Ancient Samples

DNA was successfully amplified from 55 of the 89 (61.80%) archaeological fish remains with our universal primers. However, two samples (FH40 and FH41) yielded *COI* sequences that did not resemble any bony fish reference sequences in BOLD or GenBank, reducing the true amplification success rate to 58.43%. The sequences

obtained from these specimens most closely resembled reference sequences from domestic cattle (*Bos taurus*) (FH40) and bacteria (FH41). A *COI* sequence resembling that of cattle was also obtained from FH87. However, subsequent amplification and sequencing attempts yielded a sequence from FH87 that matched reference sequences from bony fish. The *COI* sequences obtained from the remaining 51 archaeological fish remains that yielded DNA matched or most closely resembled piscine reference sequences.

The success rate for the amplifications carried out with the taxon-specific primers had a slightly lower success rate than those performed with the universal mini-barcoding primers. A secondary marker was successfully amplified with appropriate taxon-specific primers from 43 of the 53 (81.13%) fish remains that yielded a fragment of *COI*. In cases where the amplification of a secondary marker failed, the taxon-specific primers targeted a fragment longer than the one targeted with the universal primers (>166 bp) and thus less likely to be preserved. The secondary marker sequences obtained from the 43 successfully amplified samples matched or closely resembled reference sequences from bony fish species. No DNA was amplified with any of the primers from any of the blank extraction or negative PCR controls

Table 13. Number of identified specimens (*NISP*) of the different taxa identified among the anlayzed archaeological remains through the analysis of the *COI* mini-barcode in isolation and in conjunction with a secondary marker.

Order	Family	Species	*COI* NISP	*COI* + Secondary Marker NISP	Secondary Marker
Cypriniformes	Catostomidae	*Catostomus catostomus*	2	2	*COI*
Cypriniformes	Catostomidae	*Catostomus* sp.	31	7	*COI*
Cypriniformes	Catostomidae	*Catostomus* macrocheilus	0	24	*COI*
Cypriniformes	Cyprinidae	*Mylocheilus caurinus*	7	7	*Cytb*
Cypriniformes	Cyprinidae	*Ptychocheilus* sp.	11	0	*Cytb*
Cypriniformes	Cyprinidae	*Ptychocheilus oregonensis*	0	11	*Cytb*
Salmoniformes	Salmonidae	*Oncorhynchus* sp.	1	0	D-loop
Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	0	1	D-loop
Salmoniformes	Salmonidae	*Oncorhynchus tshawytscha*	1	1	D-loop
Genus-Level Identifications			43	7	
Species-Level Identifications			10	46	
Total			53	53	

Taxonomic identifications were successfully assigned to all of the archaeological samples that yielded endogenous DNA. Through the analysis of the targeted *COI* mini-barcode, 43 of the archaeological remains (81.13%) were identified to the genus-level and 10 (18.87%) were identified the species-level (Table 13; Table C2). Sequence analysis of a secondary marker enabled the genus-level identifications assigned to an additional 36 samples to be refined to the species-level, increasing the proportion of samples with species-level identifications to 86.79% (Table 13; Table C2). The genus-level identities assigned to the remaining seven samples (*Catostomus* sp.) could not be further refined as a secondary marker consisting of a second fragment of *COI* could not be amplified from them. With the exception of three samples where the amplification of a secondary marker failed, analysis of the secondary marker confirmed the identities of those samples (*n*=8) successfully identified to the species-level through the analysis of the *COI* mini-barcode (Table C2). The taxonomic identities assigned to each of the samples through the analysis of each marker and the consensus identifications assigned to them are presented in Table C2.

4.5. Discussion

4.5.1. Contamination and Authenticity of Ancient DNA Data

Due to PCR's hypersensitivity and DNA degradation, ancient fish remains are susceptible to contamination with exogenous modern DNA from various sources, including DNA from modern specimens, environmental DNA, and PCR products (Yang and Watt 2005). The amplification of bacterial or cattle DNA from three of the archaeological fish remains indicates that such contamination did occur in this study. The amplification of contaminant cattle DNA from two specimens may reflect the previously documented presence of contaminant DNA from domestic animals, including cattle, in reagents (Leonard et al. 2007). On the other hand, the bacterial sequence retrieved from FH41 possibly represents the amplification of DNA from environmental microbes, which typically constitute a large proportion of the DNA extracted from ancient fish remains (e.g., Boessenkool et al. 2017). While the amplification of contaminant DNA resulted in the universal primers generating false positive amplifications, it should not result in erroneous identifications provided the contamination originates from non-fish taxa. The contaminant bacterial and mammalian DNA sequences we obtained from

samples could be readily identified as contamination and discarded. Nonetheless, the amplification of contaminant cattle and bacterial DNA demonstrates the need to use strict contamination controls when conducting genetic analyses on archaeological, archival, and palaeontological fish remains.

Although contamination with exogenous mammalian and bacterial DNA occurred, multiple lines of evidence suggest the fish sequences obtained from the archaeological remains are authentic. First, all pre-PCR procedures involving the archaeological remains were conducted in a dedicated aDNA laboratory that is physically separated from the modern DNA and post-PCR laboratories used in this study (Poinar 2003). Second, prior to DNA extraction, the archaeological fish remains were decontaminated using a combination of chemical washes and UV irradiation (Yang et al. 2004). Third, DNA was not amplified from any of the negative PCR or blank extraction controls (Poinar 2003). Fourth, multiple species were identified among the archaeological remains, reducing the parsimony of contamination as an explanation for the aDNA results as it would have to originate from multiple sources (Yang et al. 2004). Fifth, the inverse correlation between amplification success and amplicon length that characterizes aDNA was observed (Poinar 2003). Finally, in instances where *COI* mini-barcode and secondary marker sequences were both available for a sample, sequence analyses of the two markers yielded concordant results (Table C2) (Yang et al. 2004; Yang and Speller 2006).

4.5.2. Universality of *COI* Mini-Barcoding Primers

Between the ancient and modern specimens, we successfully amplified a fragment of *COI* with our newly designed universal primers from at least 37 bony fish species from 28 genera, 18 families, and 10 orders. If the contaminant sparid DNA amplified from MF25 is considered, an additional species, genus, and family can be added to these totals. Many of the taxon from which we successfully amplified DNA have been diverging for hundreds of millions of years (Hurley et al. 2007; Peng et al. 2009). The amplification of DNA from a range of taxa belonging in many instances to long divergent lineages suggests our mini-barcoding primers exhibit a high degree of universality. Despite their broad applicability among bony fish, we failed to amplify human DNA with our universal primers suggesting they are specific enough to exclude the amplification of this ever-present contaminant (Yang and Watt 2005). However, the

successful amplification of cattle and bacterial DNA with these primers demonstrates they are not specific enough to prevent the amplification of all contaminant DNA. Nonetheless, their universality and limited ability to amplify some common contaminants suggests our universal primers are an efficient system for amplifying DNA from faunal remains simply identified as bony fish.

Although they can efficiently amplify DNA from a range of taxa, our universal *COI* mini-barcoding primers are not truly universal. Our failure to amplify endogenous DNA from a modern olive flounder specimen (MF26)—possibly due to primer-template mismatches—demonstrates they will not be able to amplify fragments of *COI* from every fishbone. In future studies, the potential for PCR failures related to primer-templates mismatches can be assessed by examining whether the primers are conserved among the taxa likely to be encountered within an archaeological assemblage. In situations where primer-template mismatches are likely, alternative universal primers (e.g, Grealy et al. 2016; Jordan et al. 2010; Seersholm et al. 2018) may need to be used to assign initial identifications to fish remains.

4.5.3. Efficacy for Species Identification

In our proposed two-tiered approach to DNA-based species identification, preliminary identifications are assigned to fish remains by analyzing a fragment of the *COI* barcode region amplified with our universal primers. Our results demonstrate that the targeted *COI* fragment is indeed a useful marker for assigning these preliminary identifications to fish remains. Through the analysis of this *COI* mini-barcode, we were able to assign all the of the modern and archaeological specimens that yielded endogenous DNA and the contaminant sparid DNA to at least the family-level. In most instances, it was possible to assign species- or the genus-level identifications to samples. The concordance observed between the *COI*-based species identities assigned to 32 of the 33 modern samples and their known species identities indicates these identifications are not only precise, but also reliable.

In practice, previously designed universal primers for fish that target short fragments of the mitochondrial *12S* (e.g., Jordan et al. 2010; Grealy et al. 2016) and *16S rRNA* (e.g., Seersholm et al. 2018) genes could also be used to assign preliminary identifications to ichthyoarchaeological remains. However, the use of a *COI* mini-

barcode to assign initial identifications to fish remains provides a key advantage. Namely, a relatively large number of *COI* reference sequences are available in public databases. Currently, there are more than 173,000 *COI* reference sequences from over 15,000 bony fish species available in BOLD. In some regions, *COI* reference sequences are available for almost every species. For instance, *COI* reference sequences are available for over 95% of Canadian freshwater fish species, with some taxonomic groups (e.g., Salmonidae) having complete coverage (Hubert et al. 2008). In comparison, less than 40,000 *12S* or *16S rRNA* reference sequences are available in GenBank. Without a taxonomically comprehensive database of reference sequences, specimens may be misidentified or remain unidentified (Hubert et al. 2015; Yang et al. 2005).

The numerous modern and archaeological samples that were not assigned a species-level identification through the analysis of targeted *COI* mini-barcode indicates a secondary marker is indeed often needed to assign species-level identifications. By using the *COI*-based identifications as a guide, it was possible to select taxon-specific primers to target more informative secondary markers that allowed for the discrimination of closely related species sharing *COI* sequences. For instance, while the *COI* sequence obtained from the pikeminnow specimens (*n*=11) matched sequences from both northern (*Ptychocheilus oregonensis*) and Umpqua (*Ptychocheilus umpquae*) pikeminnow, the analysis of an additional *Cytb* allowed for these species to be discerned. As such, by relying on multiple rather than a single marker, our two-tiered approach to DNA-based identifications maximizes the number of remains assigned species-level identifications. However, in instances where a secondary marker cannot be amplified, honing down identifications to the species-level may not be possible, as was the case for seven suckers (*Catostomus* sp.) specimens analyzed in this study. In the case of these sucker specimens, the targeted secondary marker was considerably larger than the fragment amplified with the universal primers (233 bp vs. 166 bp), and thus less likely to be preserved on account of DNA degradation. Consequently, to maximize the number of species-level identifications, the secondary marker should ideally consist of a DNA fragment that is shorter than or of comparable size to the fragment amplified with the universal primers.

The analysis of a secondary marker offers additional benefits beyond facilitating species identification. First, targeting a secondary marker that exhibits intra-specific variation may provide genetic data useful for populations studies. Second, it aids in

authenticating the aDNA results. As Yang and colleagues note (Yang et al. 2004; Yang and Speller 2006), significant discrepancies between the identities assigned to samples through the analysis of different markers may be indicative of contamination. Since repeat DNA extractions can often not be performed on fish remains on account of their small size, embedding other reproducibility tests within aDNA analyses of fish remains is often necessary in order to authenticate the results.

In this study, the various secondary markers we used to confirm or refine the initial *COI*-based identifications are all located within the mitochondrial genome. Such a reliance on mtDNA markers for both tiers of identification can limit the success of our species identification approach. Since the mitochondrial genome is maternally inherited, our approach cannot identify hybrids if both markers consist of mtDNA fragments (Ward et al. 2009). Moreover, in instances where introgressive hybridization, hybrid speciation, and recent divergence times have resulted in taxa sharing the same or similar mtDNA haplotypes, it may not be possible to identify remains with out approach if both markers are mitochondrial (Ward et al. 2009). In some cases, the use of a biparental nuclear DNA (nuDNA) marker as the secondary marker may enable the identification of hybrids and members of taxonomic groups with complex evolutionary histories (Ward et al. 2009). Such a pairing of nuDNA and mtDNA markers has been previously used to identify Atlantic (*Acipenser oxyrinchus*) and European (*Acipenser sturio*) sturgeon hybrids (e.g., Chassaing et al. 2013; Ludwig et al. 2008; Popović et al. 2014) and tuna (*Thunnus* spp.) species (Puncher et al. 2019) that have experienced episodes of introgressive hybridization within archaeological assemblages. Consequently, if taxa with complex evolutionary histories or high hybridization rates are identified through the analysis of the *COI* fragment, we recommend using a nuDNA fragment for the secondary marker.

4.6. Conclusion

In this study, we proposed and tested a new two-tiered approach to the DNA-based species identification of archaeological bony fish remains. By examining *COI* reference sequences from a large and diverse set of bony fish species, we developed novel universal primers for bony fish that target a short fragment of the *COI* DNA barcode region. Reflecting their universality, fragments of *COI* were successfully amplified from an array of long divergent fish taxa were with these primers. When

analyzed in isolation the *COI* mini-barcodes generated with these primers can be used to identify fish remains to at least the family-level. However, it was possible to refine these initial *COI*-based identifications to the species-level by using them to select taxon-specific primers that target a more variable a secondary marker. Moreover, the analysis of a secondary marker may provide genetic data useful for population studies and can serve as a reproducibility test. Due to the universality of our mini-barcoding primers, our two-tiered approach to DNA-based species identification should be applicable to a range of archaeological fish remains, including those simply identified as bony fish. By maximizing the number of fish remains with species-level identifications, researchers will be able to better address questions regarding human-fish interactions, palaeoenvironments, and the past ecology of fish species.

Chapter 5. Conclusion

Although dealing with fish remains from different regions, time periods, and taxa, the three studies presented in this thesis are linked by a shared overarching research objective. Each study sought to promote the incorporation of aDNA analysis into ichthyoarchaeology by developing and applying new genetic methods for the analysis of fish remains. In the first study, a DNA-based method for the sex identification of archaeological Pacific salmonid remains was developed. Building on the results of this first study, the second study modified this method to make it applicable to archaeological Atlantic salmonid and char remains. The final and third study (Chapter 3) developed a DNA-based species identification method that is applicable to the remains of a broad range of taxa. In this concluding chapter, I summarize and discuss the results and significance of these studies and provide future directions for research.

5.1. Summary and Discussion of Key Findings

5.1.1. DNA-Based Sex Identification of Archaeological Salmonid Remains

The first two studies presented in this thesis focused on developing and applying DNA-based methods for the sex identification of ancient Pacific salmonid (Chapter 2), Atlantic salmonid, and char (Chapter 3) remains. Both methods assign sex identities to samples using two PCR assays that co-amplify the Y-linked salmonid master-sex determining gene (*sdY*) alongside a positive control consisting of a fragment of the mitochondrial D-loop or one of the autosomal *clock* genes. The sex identities assigned with these methods to modern specimens of known phenotypic (Chapter 2) or genotypic (Chapter 3) sex were concordant, highlighting their reliability. In addition to being reliable, high success rates were obtained when they were applied to archaeological salmonid remains indicating both methods are highly sensitivity. In the case of the method developed for Pacific salmonids, application of the method to dilutions of modern Chinook salmon DNA indicated only a few DNA templates are needed for positive results. Their reliability and sensitivity make these methods highly efficient sex identification methods for archaeological salmonids remains. Although this thesis focused on applying them to materials from North American archaeological sites, these

methods have much broader applicability. All the genera examined in Chapter 2 and Chapter 3 have geographic ranges that extend beyond North America, meaning they are applicable to materials from other regions.

The use of aDNA analysis to assign sex identities to ancient human and animal specimens is not new. Genetic techniques have been used for nearly 30 years to determine the sex of archaeological and paleontological remains (See Hummel and Herrmann 1991 for an early application.). Previous studies, however, have largely concentrated on using aDNA analysis to assign sex identifications to human remains (e.g., Faerman et al. 1995; Faerman et al. 1998; Hummel and Herrmann 1991; Kennett et al. 2017; Matheson and Loy 2001; Mays and Faerman 2001; Skoglund et al. 2013; Stone et al. 1996) as well as specimens of various non-humans mammals (e.g., Nistelberger et al. 2019; Pagès et al. 2009; Szpak et al. 2020; Svensson et al. 2008; Svensson et al. 2012) and birds (e.g., Allentoft et al. 2010; Bunce et al. 2003; Speller and Yang 2016). Little attention has been given to the genetic sex identification of remains belonging to other taxonomic groups. In the case of fish, the DNA-based sex identification methods for salmonid remains presented in this thesis are the first such genetic methods available for ancient fish remains. The efficacy of these sex identification methods for salmonid remains, however demonstrate that despite this lack of attention, aDNA analysis is a viable method for the sex identification of archaeological fish remains. Methods analogous to ones developed for salmonids in this study could be applied to other fishes with similar genetic determination systems. Although these results highlight the potential for the DNA-based sex identification of fish remains, there are some caveats that limit the applicability of such approaches. If fish remains are from one of the many species whose sex is not directed by genetics but by environmental or behavioral mechanisms (Devlin and Nagahama 2002), aDNA analysis cannot be used to identify their sex. Moreover, the methods developed in this study for salmonids cannot be directly applied to other species due to complexities of fish genetic sex determination systems.

Relative to other taxonomic groups, fish genetic sex determination systems are incredibly diverse as result of having evolved independently multiple times (Devlin and Nagahama 2002; Kikuchi and Hamaguchi 2013; Mank and Avise 2009). Among fish, both polygenetic sex determination systems where sex is controlled by multiple gene and monogenic systems in which sex is controlled by a single gene have been observed

(Devlin and Nagahama 2002; Kikuchi and Hamaguchi 2013; Mank and Avise 2009). In addition to variability in the number of genes controlling phenotypic sex, the exact genes that play a role in sex determination also vary (Kikuchi and Hamaguchi 2013). For instance, among taxa with monogenetic sex determination systems, the master-sex determining gene can vary. This is true within the superorder Protacanthopterygii where *sdY* is the master-sex determining gene shared by most salmonids (Yano et al. 2012; Yano et al. 2013), while *amhby* (*Y-chromosome-specific anti-Müllerian hormone paralog b*) dictates sex in many esocids (Pan et al. 2019; Pan et al. 2021). Even among closely related species the genes responsible for sex determination may vary. For example, while the male-specific *dmrt1bY* d*oublesex and mab-3 related transcription factor 1b on the Y-chromosome)* gene is the master sex determining gene in medaka (*Oryzias latipes*), the $gsdf^{Y}$ (*gonadal soma derived growth factor on the Y chromosome*) gene fulfills this role in the congeneric *Oryzias luzonensis* (Myosho et al. 2012). In extreme instances, sex determination genes may also even vary between different populations of some species. In the case of northern pike (*Esox lucius*), population studies have found that *amhby* is the master sex-determining gene among Eurasian populations and North America populations west of the Continental Divide but has been lost within other North American populations (Pan et al. 2021; Johnson et al. 2020). The variability of the genetic sex-determination systems exhibited by fish complicates the sex identification of ichthyofaunal remains through aDNA analysis as it means bespoke methods and markers have to developed for different taxa. Developing such tailored methods requires knowledge of the genes responsible for sex determination and/or other sex-linked markers within the taxa being analyzed. In many instances, such information is not known, precluding the genetic sex identification of remains belonging to such species. The growth in genomic analyses, however, is facilitating the identification of master-sex determining genes and other sex-linked markers in various fish taxa. Genomic studies have recently identified potential master-sex determining genes in gadids (*zinc knuckle on the Y chromosome* gene [*zkY*]) (Kirubakaran et al. 2019), and sablefish (*Anoplopoma fimbria; gene gonadal-soma derived factor gene* [*gsdf*]) (Rondeau et al. 2013).

The application of these DNA-based sex identification method to archaeological materials also provided insights into the sex-selectivity of salmonid fisheries in the Pacific Northwest (Chapter 2) and Great Lakes (Chapter 3). At Keatley Creek, British Columbia, where a large number of salmonid remains were successfully assigned a sex

identity, no evidence for sex-selective fishing was observed. The other sites from the Pacific Northwest that were examined in Chapter 2 did not have sufficiently large samples of sexed remains to address questions regarding the sex-selectivity of their salmonid fisheries. Likewise, no evidence for the preferential harvesting of male Atlantic salmon and lake trout was observed at Antrex, Ontario. However, female Atlantic salmon may have been preferentially targeted by Antrex's inhabitants, which was hypothesized to potentially reflect the harvesting of salmon during the spring when runs are female dominated. These data suggest that at both Antrex and Keatley Creek male-selective salmonid fishing strategies similar to those ethnographically documented in the Pacific Northwest (e.g., Barnett 1975; Curtis 1924; Dale and Natcher 2015; Langdon 2006; Ratner et al. 2006; Ritchie and Springer 2010; Simeone and Valentine 2007) were not used as a resource management strategy.

The sex-selectivity patterns (or lack thereof) exhibited by the salmonid fisheries at Antrex and Keatley Creek may not be reflective of the fisheries at other sites in the Great Lakes or Pacific Northwest. In both regions, meta-analyses of the species composition of late Holocene faunal assemblages indicate Indigenous fishing strategies varied spatially (Hawkins et al. 2019; McKechnie and Moss 2016). This reflects the fact that fishing practices are not performed in isolation from their environmental and cultural contexts but are informed by their socio-ecological milieu (Berkes 2011; Cannon 1998; Moss 2012). As they too were possibly informed by local social and ecological factors, the sex-selectivity of salmonid fisheries in these regions also were potentially geographically variable. In areas with low salmonid productivity, for instance, there may have been more of an incentive to manage salmonids through male-selective fishing than at Antrex and Keatley Creek, which are in areas with a hyperabundance of salmonids. It is also possible that the sex-selectivity of salmonid fisheries varied in response to changing cultural and environmental conditions. For example, in southern Ontario, a late 14th century population explosion and village expansion following Antrex's occupation may have put additional stress on local resources (Birch 2015; Warrick 2008), necessitating more intensive management. Collapses in local salmonid populations , such as the ca. 4000 to 1000 cal BP pink salmon collapse documented at Namu, British Columbia (Cannon and Yang 2006; Cannon and Densmore 2008), may have similarly incentivized the management of salmonids through sex-selective fishing. Documenting how the sex-selectivity of salmonid fisheries in these regions

geographically and temporally varied in response to environmental and cultural factors will require the analysis of remains from additional sites separated in time and space.

5.1.2. DNA-Based Species Identification of Archaeological Fish Remains

The third study included in this thesis detailed the development of a two-tiered DNA-based approach useful for identifying fish remains from a broad range of taxa to the species-level. In this method, universal primers are first used to amplify and sequence a fragment of the mitochondrial *COI* DNA barcode region or mini-barcode. The successful amplification of this fragment with these primers from a diverse array of fish taxa indicate they exhibit a high-degree of cross-species applicability. The cross-species applicability of these primers makes this method useful for identifying remains from a broad range of taxa. Through the sequence analysis of the *COI* fragment amplified with these universal primers, a preliminary identification is assigned to a sample. By using a fragment of *COI* to generate an initial identification, this method leverages the large number of *COI* reference sequences available for fish taxa to generate accurate species identifications. As the *COI* barcode region has become the gold-standard for the DNA-based species identification of modern fish (Ward et al. 2009; Becker et al. 2011), the number of reference sequences available for this marker will continue to expand. Ultimately, this will increase the reliability of the identification assigned through the analysis of the *COI* fragment amplified with our universal primers. The preliminary identifications assigned to samples are then confirmed or refined through the analysis of a second fragment of DNA amplified with taxon-specific primers. Using this method, species identifications were successfully assigned to middle through late Holocene fish remains from EeRb-144, British Columbia. A future publication will discuss the archaeological implications of the species composition of the identified samples.

While the results of this study highlighted the usefulness of our two-tiered DNA-based approach identification method, it also highlighted its limitations. As a result of the poor DNA preservation exhibited by the analyzed fish remains from EeRb-144 (see Royle (2014) for a detailed discussion), the success rate of our approach was relatively low. In addition, as presented in Chapter 4, this approach cannot identify hybrids. All of the markers used in this study are located within the maternally inherited mitochondrial

genome. As such, they only provide information about a specimen's maternal ancestry and therefore cannot be used to identify individuals with a father belonging to a different species (Ward et al. 2009). If hybridization is common amongst the taxonomic groups identified in an assemblage through the analysis of a fragment of *COI*, a fragment of nuDNA could be analyzed to confirm the identical identifications and identify hybrids. The analysis of a nuDNA marker as the secondary marker may also facilitate the differentiation of species with complex evolutionary histories, characterized by processes such as recent divergence times, hybrid speciation, and introgressive hybridization (Ward et al. 2009).

Although not their primary purpose, both sex identification methods can also be used to genetically identify salmonid remains to the species-level. By sequencing the D-loop fragment co-amplified as a positive control in one of the assays used in both sex identifications methods, species-level identities were successfully assigned to samples. In the case of the Atlantic salmon and lake trout samples examined, the identifications assigned to samples with this D-loop fragment were confirmed through the analysis of a fragment of *cytochrome b* amplified through a singleplex PCR. While not used confirm to the D-loop based species identities assigned to the Pacific salmonid remains examined in Chapter 2, this *cytochrome b* fragment could be similarly used to corroborate these identifications (e.g., Guiry, Royle, Matson, et al. 2020).

Though not explored here, sequencing the nuDNA fragments co-amplified by the sex identification assays could in some instances also aid in the taxonomic identification of salmonid remains. BLAST searches indicate the sequence of the *sdY* fragment co-amplified by the D-loop/*sdY* assay developed for Atlantic salmonids and chars exhibits inter-specific variation making it potentially useful for species identification. Within this fragment, Atlantic salmon differs by a C→T transition from the closely related brown trout (*Salmo trutta*) as well as the char species (Arctic char and brook trout) for which sequence data is available. The fragment of *clock1a* amplified by the *clock1a*/*sdY* developed for Pacific salmonids, similarly, varies between some species within this genus. Due to this variability, the remains of some salmonid species could be identified through the analysis of these nuDNA fragments. As such, the analysis of this fragment may provide additional support for species identification assigned through the analysis of mtDNA fragments.

Perhaps more importantly, sequencing fragments of nuDNA co-amplified by some of the PCR sex identification assays can aid in the identification of salmonid hybrids. Unlike mtDNA, which is maternally inherited, *sdY* is paternally inherited whilst autosomal genes, such as *clock1a*, are biparentally inherited. Due to their differing inheritance patterns, discrepancies between the species identifications assigned to a sample with mtDNA markers like D-loop *cytochrome b* and those assigned with *clock1a* or *sdY* may be indicative of hybrids. As hybrids occur naturally between many salmonids (Baxter et al. 1997; Hartley 1996), the ability to distinguish individuals with hybrid ancestry—something not possible with conventional mtDNA markers—is important when dealing with salmonids. However, as the fragments do not vary between every species, not all species or hybrids can be identified through the analysis of these fragments.

5.2. Methodological Synergies

By providing detailed information about the species and sex composition of ichthyofaunal assemblages, the DNA-based species and sex identification method presented here can, on their own, be used to address various questions. However, methodological synergies exist between these methods and other genetic, biomolecular, and conventional zooarchaeological methods. Pairing these species and sex identification with these complementary methods substantially increases their utility and power.

In recent years, there has been a growing interest in the application of high-throughput sequencing to fish remains (Oosting et al. 2019). Application of the DNA-based species and sex identification to samples may facilitate such high-throughput sequencing. At the most basic-level, these methods can be used to assess the mitochondrial and — in the case of the sex identification method — nuDNA preservation exhibited by samples before high-throughput sequencing. Identifying remains to the species-level with the methods described here can further facilitate high-throughput sequencing approaching by enabling the selection of taxonomically appropriate baits for hybridization enrichment. By increasing the proportion of endogenous DNA in libraries, hybridization enrichment can enable the generation of high-quality mitochondrial or whole genomes (Hofreiter et al. 2015). Due to their universality, the primers targeting *COI* developed in Chapter 4 could also potentially be used for bulk-bone metabarcoding of assemblages of fish remains (e.g., Grealy et al. 2016; Seersholm et al. 2018). These

methods may also enable other conventional genetic analyses. In the case of conventional PCR and Sanger sequencing, the species identification of remains can be used to guide the selection of taxonomic-specific primers that can be used to amplify and sequence variable regions. Analyses of whole or mitochondrial genome sequences and sequences of variable regions can provide insights into the trade of fish (e.g., Arndt et al. 2003; Star et al. 2017), demographic responses of species to natural and anthropogenic factors (e.g., Johnson et al. 2018; Ólafsdóttir et al. 2014), and aid in phenotype reconstruction (e.g., Thompson et al. 2019).

A more nuanced understanding of the dynamics of past fisheries can also be obtained by applying the sex and species identification methods described here in tandem with other non-genetic methods. For instance, as Korzow Richter et al. (2020) note, DNA-based sex identification could be applied to salmonid remains identified to species through ZooMS to reconstruct the sex and species composition of past fisheries. Combining the DNA-based sex and species identification approaches described in this study with stable isotope analysis may be a particularly fruitful effort for future research into past fisheries. In the case of the sex identification method developed for Pacific salmonid remains (Chapter 2), applying it in tandem with stable carbon and nitrogen analysis of collage has already proven successful. A recent study has successfully applied both this sex identification method and stable nitrogen and carbon isotope analyses to late Holocene Pacific salmonid bones (Guiry, Royle, Matson, et al. 2020). The pairing of these methods has provided a wealth a data about the salmon harvested by late Holocene Plateau Pithouse Tradition and ancestral Tsilhqot'in in the Chilcotin Plateau. Using the method described in Chapter 2, the species and sex of the harvested Pacific salmonids were determined, while stable isotope analysis was able to identify their migratory behaviour (anadromous versus freshwater resident) (Guiry, Royle, Matson, et al. 2020). Pairing the DNA-based species identification methods with stable isotope analysis may also enhance our understanding other aspects of past fisheries. Within a fish species the $\delta^{13}C$ and $\delta^{15}N$ of individuals and the variation exhibited by individuals, can vary depending on their habitat and how they were harvested (Guiry 2019). Consequently, documenting the $\delta^{13}C$ and $\delta^{15}N$ of fish remains genetically identified to the species-level can provide insights into harvest methods and locations (Guiry 2019). The genetic identification of fish remains to the species-level can be used to guide the selection of appropriate formulas for reconstructing the size of individuals

from measurements taken before DNA extraction. By documenting the size range of individuals belonging to a given species within an assemblage, it is possible to infer harvest methods (e.g., Greenspan 1998).

Combining the methods described in this study with other biomolecular and non-biomolecular methods can also enhance our understanding of the past ecology of fish species. By applying both stable isotope analysis and DNA-based sex identification to salmonid remains, exploring sex-based variation within the ecology of past salmonid populations becomes possible. Such an approach has previously been employed to documented sex-based differences in migration and marriage patterns among the Norse (Krzewińska et al. 2018) and the feeding ecology of 19th century beluga whales in Nunvaut (Szpak et al. 2020). Comparing the $\delta^{13}C$ and $\delta^{15}N$ of male and female Lake Ontario Atlantic salmon from the Antrex site (Table B1), for instance, indicates males exhibit more isotopic variability despite fewer samples being identified as male (Figure 11). This suggests sex-based differences in the feeding ecology may have existed in this extinct population, but additional analyses are needed due to the small sample size. Moreover, comparing size reconstructions of salmonids who have been genetically sexed could be used to document the degree of sexual dimorphism exhibited by past salmonid populations (cf. Bunce et al. 2003). By comparing the size difference between males and females over time, it is possible to examine whether reductions in sexual size dimorphism associated with overharvesting of large individuals have occurred.

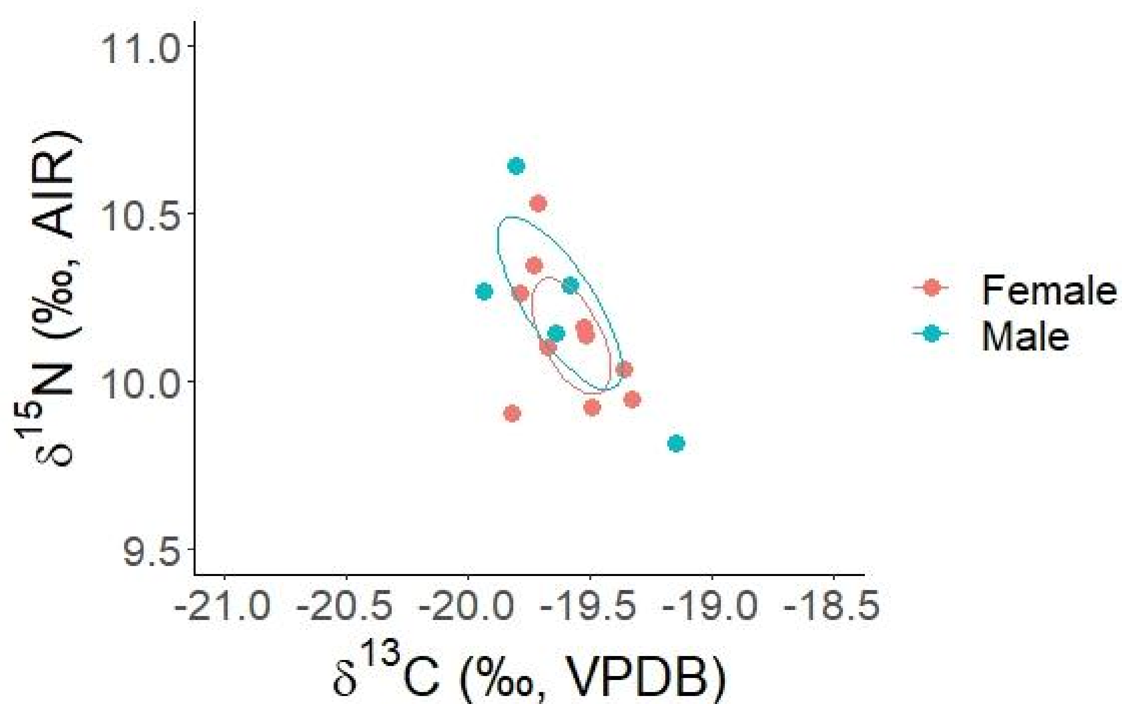

Figure 11. Bivariate plot of stable carbon and nitrogen isotope compositions for bone collagen from genetically sexed Atlantic salmon (*Salmo salar*) remains from the 13th century CE Antrex site (AjGV-38), Ontario. Standard ellipses were created with R version 4.0.3 (R Core Team 2018) as implemented in RStudio version version 1.1.456 (RStudio Team 2015) using the package ggplot2 (Wickham 2016) and a *p*-value set at 40%.

Pairing the DNA-based sex and species identification methods described here with other biomolecular methods can alleviate some of the ethical issues surrounding their application to ancient faunal remains. Although minimally destructive protocols exist (e.g., McGrath et al. 2019; Sirak et al. 2017), many commonly employed methods for biomolecular approaches, including aDNA analysis, stable isotope analysis, and ZooMS, are destructive, raising ethical issues (Kaestle and Horsburgh 2002; Pálsdóttir et al. 2019; Prendergast and Sawchuk 2018). Their destructiveness places these methods at odds with the professional codes of conduct by various archaeological organizations, such as the Society for American Archaeology (Society for American Archaeology 2016) and International Council for Archaeozoology (Reitz et al. 2009), that implore archaeologists to preserve collections. Most discussions surrounding the ethics of destructive sampling for biomolecular methods have focused on human materials (e.g., Hublin et al. 2008; Prendergast and Sawchuk 2018; Wagner et al. 2020). However,

archaeologists are also increasingly cognizant of the ethical concerns surrounding sampling faunal material (e.g., Pálsdóttir et al. 2019; Reitz et al. 2009; Guiry, Royle, Matson, et al. 2020). At the same time, it is recognized that while destructive, biomolecular analysis can provide information otherwise unobtainable through non-destructive methods (Pálsdóttir et al. 2019; Reitz et al. 2009). To an extent, conducting DNA-based sex and species identification methods presented in this study in tandem with other biomolecular approaches helps balance these conflicting imperatives. First, it contributes to the sustainability of collections by curtailing the need to destroy different samples for separate biomolecular analysis (Pálsdóttir et al. 2019; Guiry, Royle, Matson, et al. 2020). Second, by applying these methods to fish remains on which ZooMS or stable isotope analyses of collagen has already been conducted, it reduces the likelihood of destroying samples unlikely to yield DNA. As the preservation of DNA is correlated with collagen preservation, ZooMS or collagen-based stable isotope analysis can be used to flag samples more likely to yield positive DNA-based sex and species identification results (Götherström et al. 2002).

5.3. Concluding Remarks

With the first aDNA studies conducted in the early 1980s (e.g., Higuchi et al. 1984; Pääbo 1984; Pääbo 1985a; Pääbo 1985b) , aDNA analysis now has an over 40-year-long history in archaeology and paleontology. Over the course of this more than 40-year-long history, aDNA has had a transformative effect on our understanding of the past by providing unparalleled insights into a variety of topics ranging from human evolution and population histories to domestication processes to the effects of climate change and human activities on animal populations (see Ermini et al. 2015 for review.). Due to its transformative effect on archaeology, aDNA analysis, alongside other natural science techniques, has been heralded as being part of the 'third science revolution' in archaeology (Kirstiansen 2014). This revolution, however, has been slow to take hold in the field of ichthyoarchaeology as the number of aDNA studies of fish remains have been few and far between. The relative dearth of aDNA studies focused on fish remains has been attributed to their suspected poor DNA preservation and the lack of method available for genetic analyses of fish remains (Morales-Muñiz and Llorente-Rodriguez 2018). By developing DNA-based sex and species identification methods, this thesis sought to rectify this by providing researchers with a genetic toolkit they can use to study

fish remains. As the methods described in this thesis rely on conventional PCR and Sanger sequencing, these methods should be accessible to most laboratories. The growing interest in applying high-throughput sequencing technologies to fish remains as well as the lowering costs of these techniques will further expand this toolkit by making genomic approaches accessible (Oosting et al. 2019). The generally high amplification success rates obtained in this study also contribute to the growing body of evidence that indicates that — contrary to expectations (Morales-Muñiz and Llorente-Rodriguez 2018) — DNA is generally well-preserved in fish remains (e.g., Ferrari et al. 2021; Star et al. 2017). With an expanding genetic toolkit available for fish remains and knowledge of their good DNA preservation, aDNA analysis is poised, just as it has done in other subdisciplines, to radically transform ichthyoarchaeology.

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Appendix A. Supplementary Information for Chapter 2

Table A1. 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	♂	♂	♂	♂

Sample ID	Species	Phenotypic Sex	*clock1a*/*sdY* Assay Sex ID	D-loop/*sdY* Assay Sex ID	Consensus Sex ID
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	♂	♂	♂	♂

Sample ID	Species	Phenotypic Sex	*clock1a*/*sdY* Assay Sex ID	D-loop/*sdY* Assay Sex ID	Consensus Sex ID
PNK1	Pink	♂	♂	♂	♂
PNK2	Pink	♂	♂	♂	♂
PNK3	Pink	♂	♂	♂	♂
PNK4	Pink	♀	♀	♀	♀
PNK5	Pink	♂	♂	♂	♂
PNK6	Pink	♂	♂	♂	♂
PNK7	Pink	♂	♂	♂	♂
PNK8	Pink	♀	♀	♀	♀
PNK9	Pink	♀	♀	♀	♀
PNK10	Pink	♂	♂	♂	♂

[1]♂ = Male, ♀ = Female

Table A2. 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	—	♂	♂	♂

Sample ID	Site	Previous Species ID	Repeat Species ID	*clock1a*/ *sdY* Assay Sex ID	D-loop/*sdY* Assay Sex ID	Consensus Sex ID
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	—	♂	♂	♂

Sample ID	Site	Previous Species ID	Repeat Species ID	*clock1a*/ *sdY* Assay Sex ID	D-loop/*sdY* Assay Sex ID	Consensus Sex ID
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

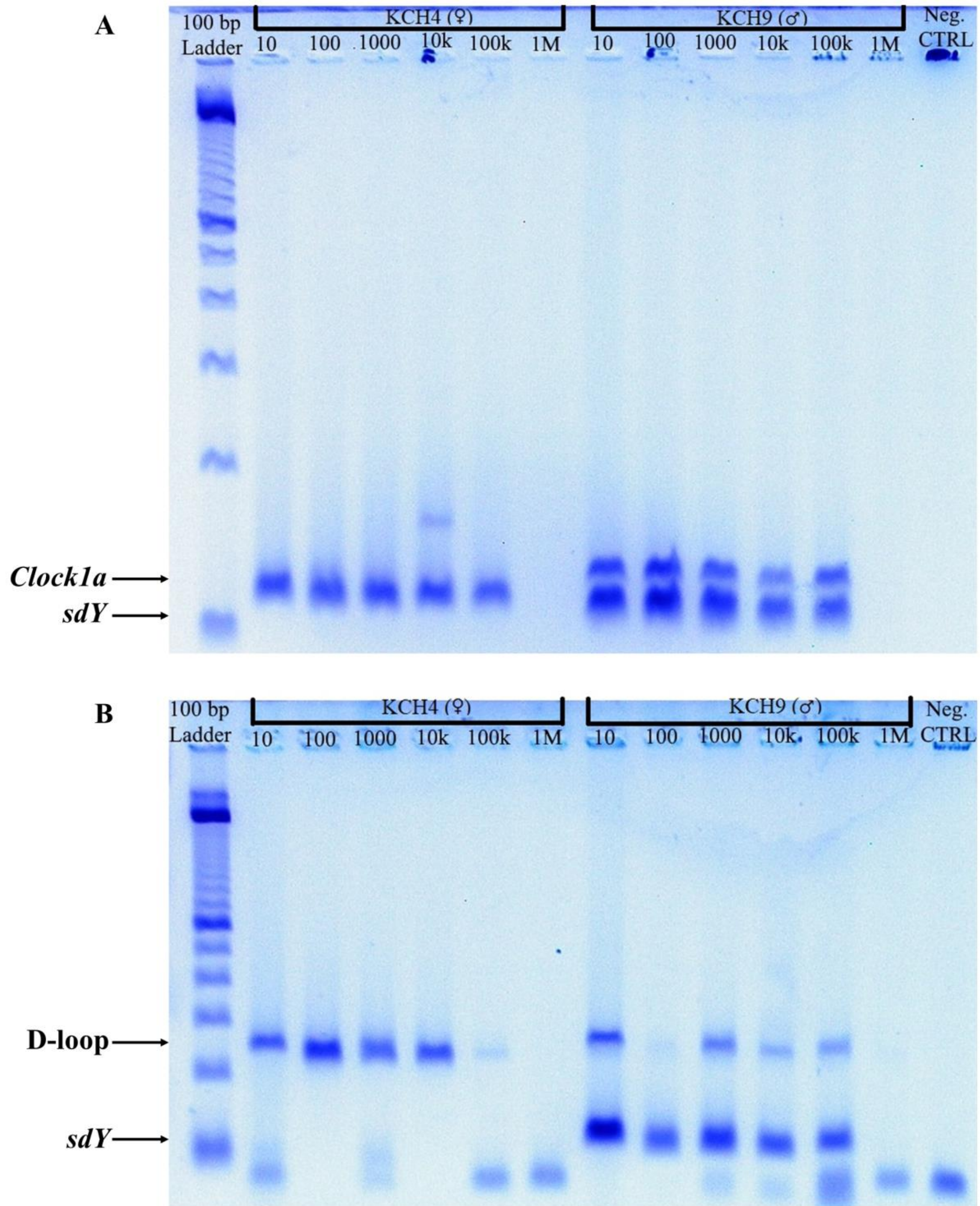

Figure A1. Negative images of electrophoresis gels showing the (A) *clock1a*/*sdY* (B) D-loop/*sdY* PCR assay results for dilutions of a modern female (KCH4) and male (KCH9) Chinook salmon sample. The approximate location of the IPC and *sdY* amplicons are indicated by the labelled arrows. The 100 bp ladder used to estimate the size of the amplicons is from Invitrogen (Waltham, MA, USA).

Appendix B. Supplementary Information for Chapter 3

Table B1. Provenience information and stable isotope data for the analyzed Atlantic salmon and lake trout samples from Antrex. The stable isotope data and ZooMS species identifications are from Guiry Guiry, Buckley, Orchard, Hawkins, et al. (2020).

aDNA Lab Code	UTM[1] Catalogue Number	Isotope Lab Code	Site	Borden Number	Element	Zooarchaeological Species ID	ZooMS Species ID	Genetic Species ID	Genetic Sex ID	Unit	Feature	Quad	Level	Collagen Yield (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%C	%N	C:N
LOS1	64	IUBC727	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N448-E183	-	-	A Horizon	4.1	-19.5	10.2	36.4	13.0	3.3
LOS2	47	IUBC719	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N462-E215	Midden 2	-	Level 2	10.5	-19.5	10.1	39.7	14.7	3.2
LOS3	14	IUBC708	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Male	N469-E208	Midden 3	-	-	10.3	-19.6	10.3	39.7	14.5	3.2
LOS4	89	IUBC737	Antrex	AjGv-38	Vertebra	*Salmo salar*	*Salmo salar*	*Salmo salar*	Male	N517-E184	-	-	-	5.0	-19.8	10.6	39.7	14.4	3.2
LOS5	114	IUBC746	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Male	N502-E195	Midden 1	-	All Levels	7.4	-19.6	10.1	39.0	14.3	3.2
LOS6	91	IUBC738	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Indeterminate	N483-E178	-	-	Topsoil	0.5	-19.7	9.9	34.7	12.1	3.3
LOS7	27	IUBC715	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N463-E214	Midden 2	-	-	5.0	-19.8	10.3	40.6	14.7	3.2
LOS8	70	IUBC730	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N517-E189	-	-	-	10.7	-19.3	9.9	40.0	14.6	3.2
LOS9	3	IUBC705	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Male	N485-E195	Feature 143	3	Living Floor	10.1	-19.2	9.8	22.6	8.1	3.3
LOS10	75	IUBC731	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N514-E178	-	-	-	7.9	-19.7	10.5	39.5	14.4	3.2
LOS11	51	IUBC720	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N465-E209	Midden 2	-	-	10.4	-19.5	9.9	39.2	14.6	3.1
LOS12	62	IUBC726	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N464-E209	Midden 2	-	10-20 cm	12.2	-19.7	10.3	39.8	14.5	3.2
LOS13	109	IUBC743	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Male	N503-E195	Midden 1	-	0-10 cm	2.8	-19.9	10.3	38.4	13.7	3.3
LOS14	59	IUBC723	Antrex	AjGv-38	Vertebra	*Salmo salar*	*Salmo salar*	*Salmo salar*	Female	N461-E213	Midden 2	-	Level 1	1.4	-19.4	10.0	39.5	13.8	3.3
LOS15	15	IUBC709	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N470-E209	Midden 3	-	Topsoil	9.8	-19.8	9.9	40.6	14.7	3.2

aDNA Lab Code	UTM[1] Catalogue Number	Isotope Lab Code	Site	Borden Number	Element	Zooarchaeological Species ID	ZooMS Species ID	Genetic Species ID	Genetic Sex ID	Unit	Feature	Quad	Level	Collagen Yield (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%C	%N	C:N
LOS16	110	IUBC744	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	Indeterminate	Indeterminate	N503-E197	Midden 1	-	10-20 cm	0.1	Insufficient Collagen				
LOS17	111	IUBC745	Antrex	AjGv-38	Atlas	*Salmo salar*	-	*Salmo salar*	Female	N504-E197	Midden 1	-	21-30 cm	7.1	-19.7	10.1	39.7	14.3	3.2
LOS18	402	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Indeterminate	N472-E186	-	-	A Horizon	-	-	-	-	-	-
LOS19	404	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N468-E186	-	-	Topsoil	-	-	-	-	-	-
LOS20	406	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Indeterminate	N471-E187	-	-	A Horizon	-	-	-	-	-	-
LOS21	407	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N471-E194	-	-	-	-	-	-	-	-	-
LOS22	409	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Indeterminate	N469-E188	-	-	Topsoil	-	-	-	-	-	-
LOS23	410	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N510-E175	-	-	Topsoil	-	-	-	-	-	-
LOS24	411	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N513-E175	-	-	A Horizon	-	-	-	-	-	-
LOS25	412	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Male	N452-E178	-	-	-	-	-	-	-	-	-
LOS26	414	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Indeterminate	N453-E195	-	-	-	-	-	-	-	-	-
LOS27	416	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Male	N494-E200	-	-	-	-	-	-	-	-	-
LOS28	418	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N501-E194	Midden 1	-	1-10 cm	-	-	-	-	-	-
LOS29	421	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Male	N506-E194	Midden 1	-	11-20 cm	-	-	-	-	-	-
LOS30	423	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N503-E198	Midden 1	-	0-10 cm	-	-	-	-	-	-
LOS31	425	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N504-E194	Midden 1	-	11-20 cm	-	-	-	-	-	-
LOS32	428	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N467-E209	Midden 2	-	-	-	-	-	-	-	-
LOS33	429	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Male	N464-E212	Midden 2	-	-	-	-	-	-	-	-

aDNA Lab Code	UTM[1] Catalogue Number	Isotope Lab Code	Site	Borden Number	Element	Zooarchaeological Species ID	ZooMS Species ID	Genetic Species ID	Genetic Sex ID	Unit	Feature	Quad	Level	Collagen Yield (%)	δ13C (‰)	δ15N (‰)	%C	%N	C:N
LOS34	431	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N461-E212	Midden 2	-	0-10 cm	-	-	-	-	-	-
LOS35	433	-	Antrex	AjGv-38	Vertebra	*Salmo salar*	-	*Salmo salar*	Female	N466-E191	-	-	A Horizon	-	-	-	-	-	-
LON1	52	IUBC721	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Male	N465-E209	Midden 2	-	-	10.9	-20.1	11.9	40.1	14.6	3.2
LON2	58	IUBC722	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N461-E213	Midden 2	-	Level 1	6.3	-19.4	12.1	40.2	14.7	3.2
LON3	61	IUBC725	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N464-E209	Midden 2	-	10-20 cm	11.0	-20.1	12.1	38.8	14.1	3.2
LON4	65	IUBC728	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Male	N448-E183	-	-	A horizon	5.9	-19.8	11.8	36.2	13.0	3.2
LON5	24	IUBC712	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Male	N469-E207	Feature 147; Midden 3	-	Topsoil	4.8	-20.4	12.0	40.8	14.3	3.3
LON6	35	IUBC717	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N464-E209	Midden 2	-	0-10 cm	11.0	-20.1	12.0	40.7	15.0	3.2
LON7	88	IUBC736	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	Indetermina te	*Salvelinus namaycush*	Male	N517-E184	-	-	-	11.3	-23.7	7.9	40.1	14.4	3.3
LON8	97	IUBC741	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Male	N460-E200	Feature 56	-	-	3.6	-19.4	12.2	39.7	14.6	3.2
LON9	44	IUBC900	Antrex	AjGv-38	Vertebra	*Salvelinus* cf. *namaycush*	Indetermina te	*Salvelinus namaycush*	Male	N465-E208	Midden 2	-	-	10.1	-19.1	11.8	40.0	14.4	3.2
LON10	112	IUBC909	Antrex	AjGv-38	Vertebra	*Salvelinus* cf. *namaycush*	*Salvelinus namaycush*	*Salvelinus namaycush*	Male	N504-E197	Midden 1	-	21-30 cm	1.2	-20.0	12.3	37.1	12.8	3.4

aDNA Lab Code	UTM[1] Catalogue Number	Isotope Lab Code	Site	Borden Number	Element	Zooarchaeological Species ID	ZooMS Species ID	Genetic Species ID	Genetic Sex ID	Unit	Feature	Quad	Level	Collagen Yield (%)	δ13C (‰)	δ15N (‰)	%C	%N	C:N
LON11	113	IUBC910	Antrex	AjGv-38	Vertebra	*Salvelinus* cf. *namaycush*	*Salvelinus namaycush*	*Salvelinus namaycush*	Male	N502-E195	Midden 1	-	All Levels	6.1	-20.3	12.0	39.8	14.1	3.3
LON12	401	-	Antrex	AjGv-38	Vertebra	*Salvelinus* cf. *namaycush*	-	*Salvelinus namaycush*	Male	N470-E208	-	-	-	-	-	-	-	-	-
LON13	403	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Indeterminate	N472-E195	-	-	Topsoil	-	-	-	-	-	-
LON14	405	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N468-E186	-	-	Topsoil	-	-	-	-	-	-
LON15	408	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N469-E188	-	-	Topsoil	-	-	-	-	-	-
LON16	413	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Male	N452-E183	-	-	-	-	-	-	-	-	-
LON17	415	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N453-E195	-	-	-	-	-	-	-	-	-
LON18	417	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N494-E200	-	-	-	-	-	-	-	-	-
LON19	419	-	Antrex	AjGv-38	Vertebra	*Salvelinus* cf. *namaycush*	-	*Salvelinus namaycush*	Male	N501-E194	Midden 1	-	1-10 cm	-	-	-	-	-	-
LON20	420	-	Antrex	AjGv-38	Vertebra	*Salvelinus* cf. *namaycush*	-	*Salvelinus namaycush*	Indeterminate	N506-E194	Midden 1	-	1-10 cm	-	-	-	-	-	-
LON21	422	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N503-E198	Midden 1	-	0-10 cm	-	-	-	-	-	-

aDNA Lab Code	UTM[1] Catalogue Number	Isotope Lab Code	Site	Borden Number	Element	Zooarchaeological Species ID	ZooMS Species ID	Genetic Species ID	Genetic Sex ID	Unit	Feature	Quad	Level	Collagen Yield (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%C	%N	C:N
LON22	424	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Male	N504-E194	Midden 1	-	11-20 cm	-	-	-	-	-	-
LON23	426	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Indeterminate	N449-E181	-	-	A Horizon	-	-	-	-	-	-
LON24	427	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N462-E211	Midden 2	-	-	-	-	-	-	-	-
LON25	430	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Female	N459-E215	Midden 2	-	-	-	-	-	-	-	-
LON26	432	-	Antrex	AjGv-38	Vertebra	*Salvelinus namaycush*	-	*Salvelinus namaycush*	Indeterminate	N466-E191	-	-	A Horizon	-	-	-	-	-	-

[1]UTM = Department of Anthropology, University of Toronto Mississauga

Table B2. Results of the PCRs performed on the modern Atlantic salmon and Arctic char samples with the D-loop/*sdY* and *clock1b*/*sdY* co-amplification sex identification assays designed in this study as well as the *18S rRNA* gene/*sdY* co-amplification sex identification assay designed by Yano et al. (2013).

Sample	Species	D-loop/*sdY* Sex Identification Assay			*clock1b*/*sdY* Sex Identification Assay			*18S*/*sdY* Sex Identification Assay		
		D-loop	*sdY*	D-loop/*sdY* Sex ID	*clock1b*	*sdY*	*clock1b*/*sdY* Sex ID	*18S*	*sdY*	*18S*/*sdY* Sex ID
ATL1	*Salmo salar*	P[1]	P	Male	P	P	Male	P	P	Male
ATL2	*Salmo salar*	P	P	Male	P	P	Male	P	P	Male
ATL3	*Salmo salar*	P	P	Male	P	P	Male	P	P	Male
ATL4	*Salmo salar*	P	N	Female	P	N	Female	P	N	Female
MF2	*Salvelinus alpinus*	N	P	Male	P	P	Male	P	P	Male

[1]P = Amplified, N = Not amplif

Table B3. Results of the PCRs performed on the Atlantic salmon and lake trout samples from Antrex with the D-loop/*sdY* and clock1b/*sdY* co-amplification sex identification assays.

Sample	D-loop/*sdY* Sex Identification Assay											*clock1b*/*sdY* Sex Identification Assay											Consensus Sex ID
	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		D-loop/*sdY* Sex ID	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		*clock1b*/*sdY* Sex ID	
	D-loop	*sdY*	D-loop	*sdY*	D-loop	*sdY*	D-loop	*sdY*	D-loop	*sdY*		*clock1b*	*sdY*	*clock1b*	*sdY*	*clock1b*	*sdY*	*clock1b*	*sdY*	*clock1b*	*sdY*		
LOS1[1]	N[2]	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS2	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS3	P	P	P	P	P	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LOS4	P	P	N	P	-	-	-	-	-	-	Male	P	P	P	P	-	-	-	-	-	-	Male	Male
LOS5	N	P	N	P	N	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LOS6	P	N	N	N	P	N	P	N	N	P	Indeterminate	N	N	N	P	N	N	N	N	N	N	Indeterminate	Indeterminate
LOS7	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	A	N	Female	Female
LOS7 R[3]	P	N	P	N	P	N	P	N	P	N	Female	A	N	P	N	P	N	P	N	P	N	Female	Female
LOS8	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS9	P	P	P	P	-	-	-	-	-	-	Male	P	P	P	P	-	-	-	-	-	-	Male	Male
LOS9 R	P	P	P	P	-	-	-	-	-	-	Male	P	P	P	P	-	-	-	-	-	-	Male	Male
LOS10	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS11	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS12	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	A	N	Female	Female
LOS13	P	P	P	P	P	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LOS14	P	N	P	N	P	N	P	N	P	N	Female	N	N	P	N	N	N	P	N	P	N	Female	Female
LOS15	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS15 R	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS16	N	N	N	N	N	N	N	N	N	N	Indeterminate	N	N	N	N	N	N	N	N	N	N	Indeterminate	Indeterminate
LOS17	P	N	N	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS18	P	N	P	N	P	N	P	N	P	N	Female	N	P	N	N	N	N	N	N	N	N	Indeterminate	Indeterminate
LOS19	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS20	P	N	P	N	P	N	P	P	P	N	Indeterminate	N	N	N	N	P	N	P	P	P	N	Indeterminate	Indeterminate
LOS21	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS22	P	P	P	N	P	N	P	N	P	P	Male	P	N	N	N	P	N	P	N	P	P	Indeterminate	Indeterminate
LOS23	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	N	N	P	N	P	N	Female	Female
LOS24	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS25	P	N	P	P	P	N	P	P	P	N	Male	N	N	N	N	P	N	N	P	P	P	Male	Male
LOS26	P	N	P	N	P	N	P	N	P	N	Female	N	P	N	N	P	N	P	N	P	N	Indeterminate	Indeterminate
LOS27	P	P	P	P	P	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LOS28	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS29	P	P	P	P	P	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LOS30	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female

Sample	D-loop/*sdY* Sex Identification Assay											*clock1b*/*sdY* Sex Identification Assay											Consensus Sex ID
	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		D-loop/*sdY* Sex ID	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		*clock1b*/*sdY* Sex ID	
	D-loop	*sdY*	D-loop	*sdY*	D-loop	*sdY*	D-loop	*sdY*	D-loop	*sdY*		*clock1b*	*sdY*	*clock1b*	*sdY*	*clock1b*	*sdY*	*clock1b*	*sdY*	*clock1b*	*sdY*		
LOS31	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS32	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS33	P	N	P	N	P	N	P	P	P	P	Male	P	N	N	P	P	N	P	P	P	P	Male	Male
LOS34	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LOS35	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON1	N	P	N	P	N	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LON2	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON3	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON4	P	P	P	P	P	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LON5	P	P	P	P	P	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LON6	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON7	N	P	N	N	N	P	P	N	N	P	Male	N	N	P	N	N	P	P	P	P	N	Male	Male
LON8	P	P	P	P	P	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LON9	P	P	P	P	-	-	-	-	-	-	Male	P	P	P	P	-	-	-	-	-	-	Male	Male
LON10	P	P	P	P	-	-	-	-	-	-	Male	P	P	P	P	-	-	-	-	-	-	Male	Male
LON11	P	P	P	P	P	P	-	-	-	-	Male	P	P	P	P	P	P	-	-	-	-	Male	Male
LON12	N	P	P	P	-	-	-	-	-	-	Male	P	P	P	P	-	-	-	-	-	-	Male	Male
LON13	N	N	P	N	P	N	P	N	P	N	Female	N	N	N	N	N	N	P	N	P	N	Indeterminate	Indeterminate
LON14	P	N	P	N	N	N	N	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON15	N	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON16	N	P	P	P	P	P	P	P	-	-	Male	P	P	P	P	P	P	P	P	-	-	Male	Male
LON17	P	N	P	N	N	N	N	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON18	N	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON19	N	P	P	P	P	P	-	-	-	-	Male	P	P	P	N	P	P	-	-	-	-	Male	Male
LON20	N	P	P	N	P	P	P	P	P	P	Male	P	N	A	N	N	N	N	P	P	N	Indeterminate	Indeterminate
LON21	P	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON22	N	P	P	P	-	-	-	-	-	-	Male	P	P	P	P	-	-	-	-	-	-	Male	Male
LON23	P	N	P	N	P	N	P	N	P	N	Female	N	N	N	N	N	N	N	P	N	N	Indeterminate	Indeterminate
LON24	N	N	P	N	P	N	P	N	P	N	Female	P	N	A	N	P	N	P	N	P	N	Female	Female
LON25	N	N	P	N	P	N	P	N	P	N	Female	P	N	P	N	P	N	P	N	P	N	Female	Female
LON26	N	P	P	N	P	N	P	P	P	P	Male	P	N	P	N	P	N	P	P	P	N	Indeterminate	Indeterminate

[1]LOS# = Atlantic salmon samples, LON# = Lake trout samples
[2]P = Amplified, N = Not amplified, A = Ambiguous
[3]R = Repeat DNA extraction

Appendix C. Supplementary Information for Chapter 4

Table C1. Taxonomy of reference sequences used to design universal mini-barcoding primers for bony fish.

Order	Family	Species	Genbank Accession Number
Acipenseriformes	Acipenseridae	*Acipenser stellatus*	NC005795
Acipenseriformes	Polyodontidae	*Polyodon spathula*	NC004419
Albuliformes	Albulidae	*Pterothrissus gissu*	NC005796
Albuliformes	Halosauridae	*Aldrovandia affinis*	NC005801
Albuliformes	Notacanthidae	*Notacanthus chemnitzii*	NC005144
Amiiformes	Amiidae	*Amia calva*	NC004742
Anguilliformes	Anguillidae	*Anguilla japonica*	NC002707
Anguilliformes	Chlopsidae	*Kaupichthys hyoproroides*	NC013607
Anguilliformes	Congridae	*Heteroconger hassi*	NC013629
Anguilliformes	Derichthyidae	*Derichthys serpentinus*	NC013611
Anguilliformes	Heterenchelyidae	*Pythonichthys microphthalmus*	NC013601
Anguilliformes	Moringuidae	*Moringua edwardsi*	NC013622
Anguilliformes	Muraenesocidae	*Cynoponticus ferox*	NC013617
Anguilliformes	Muraenidae	*Rhinomuraena quaesita*	NC013610
Anguilliformes	Myrocongridae	*Myroconger compressus*	NC013631
Anguilliformes	Nemichthyidae	*Avocettina infans*	NC013624
Anguilliformes	Nettastomatidae	*Hoplunnis punctata*	NC013623
Anguilliformes	Ophichthidae	*Myrichthys maculosus*	NC013635
Anguilliformes	Serrivomeridae	*Stemonidium hypomelas*	NC013628
Anguilliformes	Synaphobranchidae	*Simenchelys parasitica*	NC013605
Ateleopodiformes	Ateleopodidae	*Ateleopus japonicus*	NC003178
Atheriniformes	Atherinidae	*Hypoatherina tsurugae*	NC004386
Atheriniformes	Atherinopsidae	*Menidia menidia*	NC011174
Atheriniformes	Melanotaeniidae	*Melanotaenia lacustris*	NC004385
Atheriniformes	Notocheiridae	*Iso hawaiiensis*	NC011178
Aulopiformes	Aulopidae	*Aulopus japonicus*	NC002674
Aulopiformes	Chlorophthalmidae	*Chlorophthalmus agassizi*	NC003160
Aulopiformes	Synodontidae	*Harpadon microchir*	NC003161
Batrachoidiformes	Batrachoididae	*Porichthys myriaster*	NC006920
Beloniformes	Adrianichthyidae	*Oryzias latipes*	NC004387
Beloniformes	Belonidae	*Ablennes hians*	NC011180
Beloniformes	Exocoetidae	*Cypselurus hiraii*	NC007403
Beloniformes	Hemiramphidae	*Hyporhamphus sajori*	NC011173
Beloniformes	Scomberesocidae	*Cololabis saira*	NC003183

Order	Family	Species	Genbank Accession Number
Beryciformes	Anomalopidae	*Anomalops katoptron*	NC008128
Beryciformes	Anoplogastridae	*Anoplogaster cornuta*	NC004391
Beryciformes	Berycidae	*Beryx decadactylus*	NC004393
Beryciformes	Diretmidae	*Diretmoides veriginae*	NC008126
Beryciformes	Diretmidae	*Diretmus argenteus*	NC008127
Beryciformes	Holocentridae	*Ostichthys japonicus*	NC004394
Beryciformes	Monocentridae	*Monocentris japonica*	NC004392
Beryciformes	Trachichthyidae	*Hoplostethus japonicus*	NC003187
Ceratodontiformes	Ceratodontidae	*Neoceratodus forsteri*	NC003127
Characiformes	Alestiidae	*Phenacogrammus interruptus*	NC004699
Characiformes	Characidae	*Chalceus macrolepidotus*	NC004700
Characiformes	Characidae	*Hasemania nana*	NC022724
Characiformes	Citharinidae	*Citharinus congicus*	NC015805
Characiformes	Citharinidae	*Distichodus sexfasciatus*	NC015836
Characiformes	Hemiodontidae	*Apareiodon affinis*	NC015834
Characiformes	Hemiodontidae	*Hemiodus gracilis*	NC015816
Characiformes	Lebiasinidae	*Piabucina astrigata*	NC015745
Clupeiformes	Chirocentridae	*Chirocentrus dorab*	NC006913
Clupeiformes	Clupeidae	*Clupea pallasii*	NC009578
Clupeiformes	Clupeidae	*Etrumeus teres*	NC009583
Clupeiformes	Denticipitidae	*Denticeps clupeoides*	NC007889
Clupeiformes	Engraulidae	*Coilia nasus*	NC019625
Clupeiformes	Pristigasteridae	*Ilisha africana*	NC009584
Clupeiformes	Sundasalangidae	*Sundasalanx praecox*	NC016725
Coelacanthiformes	Coelacanthidae	*Latimeria menadoensis*	NC006921
Cypriniformes	Balitoridae	*Schistura balteata*	NC008679
Cypriniformes	Balitoridae	*Sinogastromyzon puliensis*	NC011922
Cypriniformes	Balitoridae	*Vaillantella maassi*	NC008680
Cypriniformes	Catostomidae	*Ictiobus bubalus*	NC013071
Cypriniformes	Cobitidae	*Misgurnus anguillicaudatus*	NC011209
Cypriniformes	Cyprinidae	*Danio rerio*	NC002333
Cypriniformes	Gyrinocheilidae	*Gyrinocheilus aymonieri*	NC008672
Cypriniformes	Psilorhynchidae	*Psilorhynchus homaloptera*	NC011210
Cyprinodontiformes	Aplocheilidae	*Aplocheilus panchax*	NC011176
Cyprinodontiformes	Aplocheilidae	*Nothobranchius furzeri*	NC011814
Cyprinodontiformes	Aplocheilidae	*Rivulus marmoratus*	NC003290
Cyprinodontiformes	Cyprinodontidae	*Jordanella floridae*	NC011387
Cyprinodontiformes	Fundulidae	*Fundulus heteroclitus*	NC012312
Cyprinodontiformes	Goodeidae	*Xenotoca eiseni*	NC011381
Cyprinodontiformes	Poeciliidae	*Xiphophorus maculatus*	NC011379

Order	Family	Species	Genbank Accession Number
Elopiformes	Elopidae	*Elops hawaiensis*	NC005798
Elopiformes	Megalopidae	*Megalops atlanticus*	NC005804
Esociformes	Esocidae	*Esox lucius*	NC004593
Esociformes	Umbridae	*Novumbra hubbsi*	NC022455
Gadiformes	Bregmacerotidae	*Bregmaceros nectabanus*	NC008124
Gadiformes	Gadidae	*Boreogadus saida*	NC010121
Gadiformes	Gadidae	*Lota lota*	NC004379
Gadiformes	Macrouridae	*Squalogadus modificatus*	NC008223
Gadiformes	Macrouridae	*Ventrifossa garmani*	NC008225
Gadiformes	Merlucciidae	*Merluccius merluccius*	NC015120
Gadiformes	Moridae	*Physiculus japonicus*	NC004377
Gasterosteiformes	Aulorhynchidae	*Aulichthys japonicus*	NC011569
Gasterosteiformes	Aulostomidae	*Aulostomus chinensis*	NC010269
Gasterosteiformes	Centriscidae	*Aeoliscus strigatus*	NC010270
Gasterosteiformes	Fistulariidae	*Fistularia petimba*	NC024190
Gasterosteiformes	Gasterosteidae	*Pungitius pungitius*	NC011571
Gasterosteiformes	Hypoptychidae	*Hypoptychus dybowskii*	NC004400
Gasterosteiformes	Indostomidae	*Indostomus paradoxus*	NC004401
Gasterosteiformes	Pegasidae	*Pegasus volitans*	NC010271
Gasterosteiformes	Solenostomidae	*Solenostomus cyanopterus*	NC010267
Gasterosteiformes	Syngnathidae	*Hippocampus erectus*	NC022722
Gonorynchiformes	Chanidae	*Chanos chanos*	NC004693
Gonorynchiformes	Gonorynchidae	*Gonorynchus greyi*	NC004702
Gonorynchiformes	Kneriidae	*Parakneria cameronensis*	NC007891
Gonorynchiformes	Phractolaemidae	*Phractolaemus ansorgii*	NC007892
Gymnotiformes	Apteronotidae	*Apteronotus albifrons*	NC004692
Gymnotiformes	Hypopomidae	*Brachyhypopomus occidentalis*	NC015078
Lampridiformes	Lamprididae	*Lampris guttatus*	NC003165
Lampridiformes	Stylephoridae	*Stylephorus chordatus*	NC009948
Lampridiformes	Trachipteridae	*Zu cristatus*	NC003167
Lepidosireniformes	Lepidosirenidae	*Lepidosiren paradoxa*	NC003342
Lepidosireniformes	Protopteridae	*Protopterus aethiopicus*	NC014764
Lophiiformes	Antennariidae	*Tetrabrachium ocellatum*	NC013879
Lophiiformes	Caulophrynidae	*Caulophryne pelagica*	NC016020
Lophiiformes	Ceratiidae	*Cryptopsaras couesii*	NC013880
Lophiiformes	Chaunacidae	*Chaunax tosaensis*	NC004382
Lophiiformes	Diceratiidae	*Bufoceratias thele*	NC013869
Lophiiformes	Gigantactinidae	*Gigantactis vanhoeffeni*	NC013885
Lophiiformes	Himantolophidae	*Himantolophus groenlandicus*	NC013868
Lophiiformes	Linophrynidae	*Haplophryne mollis*	NC013865

Order	Family	Species	Genbank Accession Number
Lophiiformes	Lophiidae	*Lophiodes caulinaris*	NC013872
Lophiiformes	Melanocetidae	*Melanocetus johnsonii*	NC013866
Lophiiformes	Neoceratiidae	*Neoceratias spinifer*	NC013864
Lophiiformes	Ogcocephalidae	*Coelophrys brevicaudata*	NC013886
Lophiiformes	Oneirodidae	*Oneirodes thompsoni*	NC013871
Lophiiformes	Thaumatichthyidae	*Thaumatichthys pagidostomus*	NC013875
Mugiliformes	Mugilidae	*Myxus capensis*	NC017892
Myctophiformes	Myctophidae	*Lampadena urophaos*	NC020151
Myctophiformes	Neoscopelidae	*Scopelengys tristis*	NC020149
Ophidiiformes	Bythitidae	*Cataetyx rubrirostris*	NC004375
Ophidiiformes	Carapidae	*Carapus bermudensis*	NC004373
Ophidiiformes	Ophidiidae	*Lamprogrammus niger*	NC004378
Osmeriformes	Alepocephalidae	*Bathytroctes breviceps*	NC013574
Osmeriformes	Argentinidae	*Glossanodon semifasciatus*	NC004595
Osmeriformes	Bathylagidae	*Lipolagus ochotensis*	NC004591
Osmeriformes	Galaxiidae	*Galaxias gollumoides*	NC015239
Osmeriformes	Leptochilichthyidae	*Leptochilichthys agassizii*	NC011006
Osmeriformes	Microstomatidae	*Nansenia ardesiaca*	NC004596
Osmeriformes	Opisthoproctidae	*Opisthoproctus soleatus*	NC004600
Osmeriformes	Osmeridae	*Mallotus villosus*	NC015244
Osmeriformes	Osmeridae	*Plecoglossus altivelis*	NC002734
Osmeriformes	Platytroctidae	*Sagamichthys abei*	NC011010
Osmeriformes	Retropinnidae	*Retropinna retropinna*	NC004598
Osmeriformes	Salangidae	*Salangichthys microdon*	NC004599
Osteoglossiformes	Gymnarchidae	*Gymnarchus niloticus*	NC012707
Osteoglossiformes	Hiodontidae	*Hiodon tergisus*	NC015082
Osteoglossiformes	Mormyridae	*Paramormyrops gabonensis*	NC015107
Osteoglossiformes	Notopteridae	*Papyrocranus congoensis*	NC012714
Osteoglossiformes	Osteoglossidae	*Arapaima gigas*	NC010570
Osteoglossiformes	Osteoglossidae	*Osteoglossum bicirrhosum*	NC003095
Osteoglossiformes	Pantodontidae	*Pantodon buchholzi*	NC003096
Perciformes	Acanthuridae	*Acanthurus leucosternon*	NC009830
Perciformes	Acropomatidae	*Doederleinia berycoides*	NC009867
Perciformes	Ammodytidae	*Ammodytes personatus*	NC021374
Perciformes	Anabantidae	*Anabas testudineus*	NC024752
Perciformes	Anarhichadidae	*Anarhichas lupus*	NC009773
Perciformes	Apogonidae	*Apogon semilineatus*	NC022510
Perciformes	Ariommatidae	*Ariomma lurida*	NC022497
Perciformes	Arripidae	*Arripis trutta*	NC015787
Perciformes	Blenniidae	*Petroscirtes breviceps*	NC004411

Order	Family	Species	Genbank Accession Number
Perciformes	Bramidae	*Pteraclis aesticola*	NC022487
Perciformes	Caesionidae	*Pterocaesio tile*	NC004408
Perciformes	Callionymidae	*Synchiropus splendidus*	NC024195
Perciformes	Carangidae	*Trachurus japonicus*	NC002813
Perciformes	Centracanthidae	*Spicara maena*	NC009854
Perciformes	Centrarchidae	*Lepomis cyanellus*	NC020359
Perciformes	Centrolophidae	*Schedophilus velaini*	NC021759
Perciformes	Centropomidae	*Lates calcarifer*	NC007439
Perciformes	Chaetodontidae	*Chaetodon auripes*	NC009870
Perciformes	Channichthyidae	*Chionodraco myersi*	NC010689
Perciformes	Channidae	*Channa marulius*	NC022713
Perciformes	Chiasmodontidae	*Dysalotus alcocki*	NC022482
Perciformes	Cichlidae	*Ptychochromoides katria*	NC011169
Perciformes	Cynoglossidae	*Cynoglossus bilineatus*	NC023226
Perciformes	Draconettidae	*Draconetta xenica*	NC024185
Perciformes	Echeneidae	*Echeneis naucrates*	NC022508
Perciformes	Elassomatidae	*Elassoma zonatum*	NC011388
Perciformes	Eleotridae	*Eleotris acanthopoma*	NC004415
Perciformes	Embiotocidae	*Ditrema temminckii*	NC009060
Perciformes	Emmelichthyidae	*Emmelichthys struhsakeri*	NC004407
Perciformes	Enoplosidae	*Enoplosus armatus*	NC013181
Perciformes	Gempylidae	*Thyrsitoides marleyi*	NC022492
Perciformes	Gobiesocidae	*Aspasma minima*	NC008130
Perciformes	Gobiidae	*Oxyurichthys formosanus*	NC020345
Perciformes	Haemulidae	*Diagramma pictum*	NC009856
Perciformes	Haemulidae	*Hapalogenys analis*	NC019646
Perciformes	Helostomatidae	*Helostoma temminkii*	NC022728
Perciformes	Istiophoridae	*Kajikia audax*	NC012678
Perciformes	Kuhliidae	*Kuhlia mugil*	NC013142
Perciformes	Kurtidae	*Kurtus gulliveri*	NC022477
Perciformes	Kyphosidae	*Girella punctata*	NC013137
Perciformes	Labridae	*Pteragogus flagellifer*	NC010205
Perciformes	Lateolabracidae	*Lateolabrax japonicus*	NC018045
Perciformes	Lethrinidae	*Lethrinus obsoletus*	NC009855
Perciformes	Lutjanidae	*Lutjanus johnii*	NC024572
Perciformes	Luvaridae	*Luvarus imperialis*	NC009851
Perciformes	Malacanthidae	*Branchiostegus albus*	NC012905
Perciformes	Monodactylidae	*Monodactylus argenteus*	NC009858
Perciformes	Moronidae	*Morone saxatilis*	NC014353
Perciformes	Mullidae	*Parupeneus multifasciatus*	NC024192

Order	Family	Species	Genbank Accession Number
Perciformes	Nemipteridae	*Nemipterus japonicus*	NC023972
Perciformes	Nomeidae	*Cubiceps pauciradiatus*	NC013150
Perciformes	Nototheniidae	*Notothenia coriiceps*	NC015653
Perciformes	Odacidae	*Odax cyanomelas*	NC009061
Perciformes	Odontobutidae	*Perccottus glenii*	NC020350
Perciformes	Opistognathidae	*Opistognathus jacksoniensis*	NC017895
Perciformes	Oplegnathidae	*Oplegnathus fasciatus*	NC010968
Perciformes	Osphronemidae	*Macropodus ocellatus*	NC024753
Perciformes	Pentacerotidae	*Pentaceros japonicus*	NC021758
Perciformes	Percichthyidae	*Gadopsis marmoratus*	NC024436
Perciformes	Percidae	*Etheostoma radiosum*	NC005254
Perciformes	Pholidae	*Pholis crassispina*	NC004410
Perciformes	Platax teira	*Platax teira*	NC024580
Perciformes	Plesiopidae	*Trachinops taeniatus*	NC017900
Perciformes	Polynemidae	*Eleutheronema tetradactylum*	NC021620
Perciformes	Pomacanthidae	*Centropyge loricula*	NC009872
Perciformes	Pseudochromidae	*Labracinus cyclophthalmus*	NC009054
Perciformes	Rachycentridae	*Rachycentron canadum*	NC011219
Perciformes	Rhyacichthyidae	*Rhyacichthys aspro*	NC004414
Perciformes	Scaridae	*Chlorurus sordidus*	NC006355
Perciformes	Scatophagidae	*Scatophagus argus*	NC021968
Perciformes	Sciaenidae	*Chrysochir aureus*	NC016987
Perciformes	Scombridae	*Thunnus albacares*	NC014061
Perciformes	Serranidae	*Epinephelus coioides*	NC011111
Perciformes	Siganidae	*Siganus guttatus*	NC024088
Perciformes	Sillaginidae	*Sillago sihama*	NC016672
Perciformes	Sparidae	*Acanthopagrus latus*	NC010977
Perciformes	Sparidae	*Sparus aurata*	NC024236
Perciformes	Sphyraenidae	*Sphyraena japonica*	NC022489
Perciformes	Stromateidae	*Pampus chinensis*	NC024044
Perciformes	Terapontidae	*Rhynchopelates oxyrhynchus*	NC013141
Perciformes	Tetragonuridae	*Tetragonurus cuvieri*	NC022499
Perciformes	Toxotidae	*Toxotes chatareus*	NC013151
Perciformes	Trichiuridae	*Aphanopus carbo*	NC022506
Perciformes	Trichodontidae	*Arctoscopus japonicus*	NC002812
Perciformes	Xiphiidae	*Xiphias gladius*	NC012677
Perciformes	Zanclidae	*Zanclus cornutus*	NC009852
Perciformes	Zoarcidae	*Lycodes toyamensis*	NC004409
Percopsiformes	Aphredoderidae	*Aphredoderus sayanus*	NC004372
Percopsiformes	Percopsidae	*Percopsis transmontana*	NC003168

Order	Family	Species	Genbank Accession Number
Pleuronectiformes	Achiridae	*Achirus lineatus*	NC023768
Pleuronectiformes	Bothidae	*Crossorhombus azureus*	NC022446
Pleuronectiformes	Paralichthyidae	*Paralichthys olivaceus*	NC002386
Pleuronectiformes	Pleuronectidae	*Verasper moseri*	NC008461
Pleuronectiformes	Psettodidae	*Psettodes erumei*	NC020032
Pleuronectiformes	Samaridae	*Samariscus latus*	NC024263
Pleuronectiformes	Scophthalmidae	*Psetta maxima*	NC013183
Pleuronectiformes	Soleidae	*Solea senegalensis*	NC008327
Polymixiiformes	Polymixiidae	*Polymixia japonica*	NC002648
Polypteriformes	Polypteridae	*Polypterus weeksii*	NC020655
Saccopharyngiformes	Cyematidae	*Cyema atrum*	NC013609
Saccopharyngiformes	Eurypharyngidae	*Eurypharynx pelecanoides*	NC005299
Saccopharyngiformes	Monognathidae	*Monognathus jesperseni*	NC013612
Saccopharyngiformes	Saccopharyngidae	*Saccopharynx lavenbergi*	NC005298
Salmoniformes	Salmonidae	*Hucho taimen*	NC016426
Scorpaeniformes	Anoplopomatidae	*Anoplopoma fimbria*	NC018119
Scorpaeniformes	Cottidae	*Clinocottus analis*	NC013828
Scorpaeniformes	Cyclopteridae	*Aptocyclus ventricosus*	NC008129
Scorpaeniformes	Dactylopteridae	*Dactyloptena peterseni*	NC003194
Scorpaeniformes	Hexagrammidae	*Pleurogrammus monopterygius*	NC023475
Scorpaeniformes	Platycephalidae	*Platycephalus indicus*	NC022481
Scorpaeniformes	Scorpaenidae	*Pterois miles*	NC024746
Scorpaeniformes	Scorpaenidae	*Sebastes schlegelii*	NC005450
Scorpaeniformes	Triglidae	*Satyrichthys amiscus*	NC004403
Semionotiformes	Lepisosteidae	*Lepisosteus oculatus*	NC004744
Siluriformes	Amblycipitidae	*Liobagrus marginatoides*	NC021122
Siluriformes	Aspredinidae	*Bunocephalus coracoideus*	NC015811
Siluriformes	Auchenipteridae	*Tetranematichthys quadrifilis*	NC015743
Siluriformes	Bagridae	*Leiocassis longirostris*	NC014586
Siluriformes	Callichthyidae	*Corydoras rabauti*	NC004698
Siluriformes	Clariidae	*Clarias batrachus*	NC023923
Siluriformes	Cranoglanididae	*Cranoglanis bouderius*	NC008280
Siluriformes	Diplomystidae	*Diplomystes nahuelbutaensis*	NC015823
Siluriformes	Doradidae	*Amblydoras gonzalezi*	NC015745
Siluriformes	Heteropneustidae	*Heteropneustes fossilis*	NC015827
Siluriformes	Ictaluridae	*Ictalurus punctatus*	NC003489
Siluriformes	Loricariidae	*Pterygoplichthys disjunctivus*	NC015745
Siluriformes	Malapteruridae	*Malapterurus electricus*	NC015833
Siluriformes	Mochokidae	*Synodontis schoutedeni*	NC015808
Siluriformes	Pangasiidae	*Pangasius larnaudii*	NC015839

Order	Family	Species	Genbank Accession Number
Siluriformes	Percichthyidae	*Siniperca scherzeri*	NC015815
Siluriformes	Pimelodidae	*Pimelodus pictus*	NC015797
Siluriformes	Schilbeidae	*Pareutropius debauwi*	NC015837
Siluriformes	Siluridae	*Silurus glanis*	NC014261
Siluriformes	Sisoridae	*Pareuchiloglanis sinensis*	NC024434
Stephanoberyciformes	Barbourisiidae	*Barbourisia rufa*	NC012046
Stephanoberyciformes	Cetomimidae	*Cetostoma regani*	NC004389
Stephanoberyciformes	Melamphaidae	*Scopelogadus mizolepis*	NC003171
Stephanoberyciformes	Rondeletiidae	*Rondeletia loricata*	NC003186
Stomiiformes	Gonostomatidae	*Diplophos taenia*	NC002647
Stomiiformes	Gonostomatidae	*Sigmops gracilis*	NC002574
Stomiiformes	Stomiidae	*Chauliodus sloani*	NC003159
Synbranchiformes	Mastacembelidae	*Mastacembelus favus*	NC003193
Synbranchiformes	Synbranchidae	*Monopterus albus*	NC003192
Tetraodontiformes	Balistidae	*Balistes vetula*	NC011948
Tetraodontiformes	Diodontidae	*Chilomycterus reticulatus*	NC011331
Tetraodontiformes	Molidae	*Mola mola*	NC005836
Tetraodontiformes	Monacanthidae	*Nelusetta ayraud*	NC011921
Tetraodontiformes	Ostraciidae	*Kentrocapros aculeatus*	NC009864
Tetraodontiformes	Ostraciidae	*Lactoria diaphana*	NC011330
Tetraodontiformes	Tetraodontidae	*Chelonodon pleurospilus*	NC015369
Tetraodontiformes	Triacanthidae	*Triacanthus biaculeatus*	NC009863
Tetraodontiformes	Triacanthodidae	*Triacanthodes anomalus*	NC009861
Tetraodontiformes	Triodontidae	*Triodon macropterus*	NC009859
Zeiformes	Caproidae	*Antigonia capros*	NC003191
Zeiformes	Oreosomatidae	*Neocyttus rhomboidalis*	NC004399
Zeiformes	Parazenidae	*Parazen pacificus*	NC004396
Zeiformes	Zeidae	*Zenopsis nebulosa*	NC003173
Zeiformes	Zeidae	*Zeus faber*	NC003190
Zeiformes	Zeniontidae	*Zenion japonicum*	NC004397

Table C2. Species identifications assigned to the analyzed specimens.

Sample	*COI* Species ID	Secondary Marker Species ID	Consensus Species ID
FH1	PCR Failure	N/A	N/A
FH2	PCR Failure	N/A	N/A
FH3	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH4	PCR Failure	N/A	N/A
FH5	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH6	PCR Failure	N/A	N/A
FH7	PCR Failure	N/A	N/A
FH8	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH9	PCR Failure	N/A	N/A
FH10	PCR Failure	N/A	N/A
FH11	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH12	PCR Failure	N/A	N/A
FH13	PCR Failure	N/A	N/A
FH14	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH15	PCR Failure	N/A	N/A
FH16	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH17	*Mylocheilus caurinus*	*Mylocheilus caurinus*	*Mylocheilus caurinus*
FH18	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH19	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH20	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH21	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH22	PCR Failure	N/A	N/A
FH23	PCR Failure	N/A	N/A
FH24	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH25	*Catostomus catostomus*	*Catostomus catostomus*	*Catostomus catostomus*
FH26	PCR Failure	N/A	N/A
FH27	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH28	*Mylocheilus caurinus*	PCR Failure	*Mylocheilus caurinus*
FH29	*Mylocheilus caurinus*	*Mylocheilus caurinus*	*Mylocheilus caurinus*
FH30	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH31	PCR Failure	N/A	N/A
FH32	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH33	*Mylocheilus caurinus*	*Mylocheilus caurinus*	*Mylocheilus caurinus*
FH34	PCR Failure	N/A	N/A
FH35	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH36	PCR Failure	N/A	N/A
FH37	*Oncorhynchus* sp.	*Oncorhynchus mykiss*	*Oncorhynchus mykiss*

Sample	*COI* Species ID	Secondary Marker Species ID	Consensus Species ID
FH38	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH39	PCR Failure	N/A	N/A
FH40	PCR Failure	N/A	N/A
FH41	PCR Failure	N/A	N/A
FH42	PCR Failure	N/A	N/A
FH43	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH44	*Mylocheilus caurinus*	PCR Failure	*Mylocheilus caurinus*
FH45	PCR Failure	N/A	N/A
FH46	PCR Failure	N/A	N/A
FH47	PCR Failure	N/A	N/A
FH48	PCR Failure	N/A	N/A
FH49	PCR Failure	N/A	N/A
FH50	PCR Failure	N/A	N/A
FH51	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH52	PCR Failure	N/A	N/A
FH53	PCR Failure	N/A	N/A
FH54	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH55	PCR Failure	N/A	N/A
FH56	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH57	PCR Failure	N/A	N/A
FH58	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH59	*Catostomus* sp.	*Catostomus* sp.	*Catostomus* sp.
FH60	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH61	*Catostomus catostomus*	*Catostomus catostomus*	*Catostomus catostomus*
FH62	PCR Failure	N/A	N/A
FH63	*Mylocheilus caurinus*	*Mylocheilus caurinus*	*Mylocheilus caurinus*
FH64	*Catostomus* sp.	PCR Failure	*Catostomus* sp.
FH65	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH66	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH67	PCR Failure	N/A	N/A
FH68	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH69	*Oncorhynchus tshawytscha*	PCR Failure	*Oncorhynchus tshawytscha*
FH70	*Catostomus* sp.	PCR Failure	*Catostomus* sp.
FH71	*Catostomus* sp.	PCR Failure	*Catostomus* sp.
FH72	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH73	PCR Failure	N/A	N/A
FH74	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH75	*Catostomus* sp.	PCR Failure	*Catostomus* sp.
FH76	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*

Sample	*COI* Species ID	Secondary Marker Species ID	Consensus Species ID
FH77	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH78	*Ptychocheilus* sp.	*Ptychocheilus oregonensis*	*Ptychocheilus oregonensis*
FH79	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH80	*Mylocheilus caurinus*	*Mylocheilus caurinus*	*Mylocheilus caurinus*
FH81	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH82	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH83	PCR Failure	N/A	N/A
FH84	PCR Failure	N/A	N/A
FH85	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*
FH86	PCR Failure	N/A	N/A
FH87	*Catostomus* sp.	PCR Failure	*Catostomus* sp.
FH88	*Catostomus* sp.	PCR Failure	*Catostomus* sp.
FH89	*Catostomus* sp.	*Catostomus macrocheilus*	*Catostomus macrocheilus*