CHARACTERIZING THE DIET AND POPULATION STRUCTURE OF LAMPREYS *LETHENTERON* SPP. USING MOLECULAR TECHNIQUES

By

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Abstract

Lampreys contribute to the health of aquatic ecosystems and are targeted in both subsistence and commercial fisheries. Despite their ecological and commercial importance, the management and conservation of native lampreys have been largely overlooked. The goal of this study was to close current knowledge gaps of lamprey biology through the examination of *Lethenteron* spp. in Alaska. This study applied two molecular techniques, DNA metabarcoding and microsatellite genotyping, to (1) characterize the diet of marine-phase Arctic lamprey *Lethenteron camtschaticum* (N = 250) in the eastern Bering Sea and (2) investigate the population structure of larval lampreys *Lethenteron* spp. (N = 120) within and among three Yukon River tributaries. A combination of visual observations and DNA metabarcoding revealed the presence of diagnostic structures/tissues (i.e., eggs, fin[s], internal organs, otoliths, and vertebrae) and detected DNA sequences of ten ray-finned fishes in the diets of *L. camtschaticum*. The most frequent prey taxa were Pacific sand lance *Ammodytes hexapterus*, Pacific herring *Clupea pallasii*, gadids, and capelin *Mallotus villosus*. Five of the ten taxa identified in this study were reported for the first time as prey for *L. camtschaticum*. To investigate the genetic diversity of larval lampreys, a recognized knowledge gap for populations in Alaska, a total of 81 larval lampreys were successfully genotyped at all loci. Global F_{ST} of larvae was 0.074 (95% CI: 0.042 – 0.110), while pairwise F_{ST} values among the three localities examined ranged from 0.066 – 0.081. Hierarchical model-based Bayesian clustering analyses detected three genetic clusters (K = 3) among all larval lampreys and two genetic clusters (K = 2) among Chena River larvae; no further genetic clustering was identified within the remaining two tributaries. Estimates of contemporary gene flow indicated reciprocal migration among sites. The diet analyses indicated anadromous *L. camtschaticum* function as flesh-feeding predators that prey upon pelagic fishes

in the eastern Bering Sea, while genetic analyses suggested that larval lamprey aggregations within three Yukon River tributaries exhibited higher levels of genetic diversity than are typically found among broad-ranging populations of anadromous lamprey species. Ultimately, this study highlighted the value of molecular techniques to improve our understanding of the biology of a poorly studied fish species in Alaska.

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General Introduction

The taxon Cyclostomata includes two surviving groups of agnathan (jawless) fishes, lampreys and hagfishes. Referred to as 'living fossils', lampreys (Petromyzontiformes) are an order of ancient vertebrate fishes with fossil records dating back to 360 million years before present (Renaud 2011). The life cycle of lampreys includes distinct larval, macrophthalmia (e.g., juvenile), and adult phases. All lampreys begin their life cycle as larvae burrowed in fine silt substrates, filter feeding on diatoms, organic detritus, and bacteria in freshwater tributaries (Hardisty and Potter 1971; Sutton and Bowen 1994, 2009). This larval period is estimated to last from two to seven years in duration, and appears to vary within and among species (Manzon et al. 2015). The metamorphic transition between the larval and macrophthalmia stages is characterized by radical morphological and physiological changes that occur over a period of up to four months (Manzon et al. 2015). During metamorphosis: 1) the larval buccal funnel (e.g., oral hood) develops into an adult oral suctorial disc; 2) eye spots undergo transformation into functional eyes; 3) fins differentiate and enlarge; 4) exterior pigmentation changes; 5) and the shape and structure of the branchial region surrounding gill pouches are modified (Hardisty and Potter 1971; Manzon et al. 2015). Although morphologically similar in their larval form, different lamprey species often exhibit discernable differences in both body size and feeding mechanisms as juveniles (Zanandrea 1959; Vladykov and Kott 1979).

Metamorphosed juvenile lamprey exhibit either parasitic or nonparasitic life-history strategies, depending on the species (Hardisty and Potter 1971; Renaud 2011; Manzon et al. 2015). Parasitic lampreys migrate to the ocean or remain in freshwater lakes or rivers (landlocked) during a maturing growth phase. Nonparasitic forms (referred to as brook lampreys) remain in freshwater throughout their lifecycle and do not feed after metamorphosis upon which

they reach sexual maturity. Parasitic lampreys suction and feed on flesh and/or blood of teleost host fishes (Potter and Hilliard 1987; Renaud et al. 2009). Although rare, lampreys attached to sharks and large marine mammals (e.g., whales) have also been reported (Gallant et al. 2006; Nichols et al. 2011; Samarra et al. 2012). Anadromous juveniles are estimated to remain at sea for up to four years, but a portion of small-bodied individuals (i.e., praecox form) are documented returning to freshwater after only one year at sea upon reaching maturation (Kucheryavyi et al. 2007; Renaud 2011; Orlov et al. 2014). Previous research indicates that sea lamprey *Petromyzon marinus* do not exhibit natal homing during spawning migrations in part due to extensive migrations for marine feeding and involuntary host movements (Bergstedt and Seelye 1995; Waldman et al. 2008; Hatch and Whiteaker 2009). Instead, spawning migrations of sexually maturing, adult lampreys are guided to suitable habitat by pheromone odors (i.e., bile salts) from stream-resident larvae (Moore and Schleen 1980; Sorensen et al. 2003; Vrieze et al. 2011). Although limited research has been conducted on lamprey homing, this behavior is believed to be similar among different lamprey species (Moser et al. 2015).

The functional role of lampreys in aquatic ecosystems should not be underestimated. Characterized as 'ecosystem engineers', lampreys provide an important function by maintaining the health of aquatic ecosystems throughout their life cycle (Shirakawa et al. 2013; Hogg et al. 2014; Boeker et al. 2016). For example, larval lamprey burrowing and feeding mechanisms aerate substrates and contribute to nutrient cycling in freshwater ecosystems (Shirakawa et al. 2013; Boeker et al. 2016). Spawning adults alter stream habitats during redd building and likely contribute marine-derived nutrients from spawned-out carcasses (Hogg et al. 2014; Maitland et al. 2015). Lampreys serve an important function as forage prey in freshwater and marine ecosystems. Fishes in freshwater tributaries prey upon eggs during lamprey spawning as well as

upon emergent and burrowed larvae (Renaud 1997; Cochran 2009). Juvenile lampreys are also susceptible to predation by predatory fishes (e.g., burbot *Lota lota*, inconnu *Stenodus nelma*, northern pike *Esox lucius*) and birds (e.g., gulls *Larus* spp., terns *Sterna* spp., herons *Ardea* spp.) during their out-migration to the marine environment (Renaud 1997; Close et al. 2002; T. Sutton, UAF, personal communication). Although the extent to which predation on lampreys occurs in marine ecosystems remains unquantified, they are recognized as an important component of the diets of pinnipeds, seabirds, and various marine fishes (Roffe and Mate 1984; Close et al. 2002; Cochran 2009).

Current lamprey taxonomy recognizes life-history variants as distinct species (Mecklenburg et al. 2002; Renaud 2011; Potter et al. 2015). However, debate is still ongoing as to the degree of speciation between parasitic and nonparasitic forms (reviewed in Docker 2009). Analyses of mitochondrial DNA (mtDNA) markers suggest a lack of reciprocal monophyly through multiple independent formations of nonparasitic populations from parasitic species (Espanhol et al. 2007; Boguski 2009). Measures of fine-scale genetic variation using nuclear markers further supports gene flow between parasitic and nonparasitic forms (Yamazaki et al. 2006; Docker et al. 2012; Rougemont et al. 2015; Bracken et al. 2015); however, the level of gene flow has been shown to be dependent on the degree of geographic connectivity (e.g., allopatric versus parapatric versus sympatric) among populations (Yamazaki et al. 2011; Taylor et al. 2012; Mateus et al. 2016; Rougemont et al. 2015; Rougemont et al. 2016). A study using a novel reduced representation sequencing approach was the first to report fixed allelic differences between parasitic and nonparasitic forms of *Lampetra*, but that study examined individuals from only a single tributary in Portugal (Mateus et al. 2013). Using a more robust dataset, a separate study similarly reported a subset of diagnostic alleles that could be used to unambiguously

discriminate between parasitic and nonparasitic *Lampetra* regardless of geographic connectivity (Rougemont et al. 2016). These results failed to support the hypothesis of genotypic plasticity between *Lampetra* species pairs; however, a broader application of research is needed to examine the degree of differentiation for the other lamprey genera.

Five lamprey species (Pacific lamprey *Entosphenus tridentatus*, Arctic lamprey *Lethenteron camtschaticum*, river lamprey *Lampetra ayresii*, Alaskan brook lamprey *L. alaskense*, and western brook lamprey *L. richardsoni*) are found in Alaskan tributaries (Mecklenburg et al. 2002; Renaud 2011). Arctic lamprey is the most common species in Alaska, with known spawning aggregations throughout the Yukon, Kuskokwim, and Susitna river drainages (Morrow 1980; Mecklenburg et al. 2002). With a semi-circumpolar distribution ranging from eastern Eurasia to the coast of northwestern North America, Arctic lamprey have a more northerly distribution than any other lamprey species (Potter et al. 2015). Both landlocked and anadromous forms of parasitic Arctic lamprey have a semi-circumpolar distribution (Heard 1966; Kucheryavyi et al. 2007; Yamazaki et al. 2011). Although four brook lamprey species are congeneric with Arctic lamprey (Alaskan brook lamprey *Lethenteron alaskense*, Siberian brook lamprey *L. kessleri*, American brook lamprey *L. appendix*, and the far eastern brook lamprey *L. reissneri*), Alaskan brook lamprey are the only brook lamprey species indigenous to Alaska and Canada (Mecklenburg et al. 2002; Renaud 2011).

Subsistence harvests of Arctic lamprey within the Yukon River drainage date back to the late 1800s (Renaud 2011). Village residents harvested lampreys with dip nets or 'eel sticks' during large, concentrated migrations in rivers that occurred under the ice from late October to early December (Brown et al. 2005). These harvests have not only been important to supplement summer and fall salmon harvests for human consumption and sled dog food, but lamprey skins

and oil have functional uses such as small bags for holding fish and conditioning for animal skin boots (Brown et al. 2005). A test commercial fishery targeting anadromous Arctic lampreys was started in 2003, and currently remains in operation with annual harvest limits set at 20,000 kg (Hayes and Salomone 2004). The purpose of the test fishery was to assess the economic demand for lampreys, test collection gear types, and gain additional information on lamprey distribution and abundance (Hayes and Salomone 2004). Commercially harvested lampreys have been primarily sold to Asian pharmaceutical markets, European and Asian food markets, and research institutions (Renaud 1997, 2011). Despite the commercial and subsistence harvest of this species, the status of lamprey populations in Alaska and ancestral genetic relationships among aggregations in different drainages within the state are currently unknown (ADF&G 2006; Thorsteinson and Love 2016).

The overarching goal of this thesis was to increase our knowledge of lamprey biology through the utilization of molecular genetic techniques. Several research areas have been identified as priorities for the conservation of native lamprey species including (1) the role of anadromous lampreys as predators in marine ecosystems and (2) a further understanding of genetic diversity and fine-scale population structure (Mesa and Copeland 2009). Chapter one of this thesis utilized a novel high-throughput sequencing approach to investigate the diet of marine-phase Arctic lamprey in the eastern Bering Sea. Knowledge about lamprey-host interactions in the marine environment comes primarily from observations of lamprey scars on hosts. For Arctic lamprey specifically, many these observations are limited to Russian estuaries and rivers (Birman 1950; Nikol'skii 1956; Gritsenko 1968). Arctic lamprey are believed to be particularly abundant in the Bering Sea off the coast of Alaska, making this region an ideal system to examine trophic interactions in a different area of their distribution.

Chapter two characterizes the genetic diversity among larval populations of *Lethenteron* spp. within the Yukon River drainage, Alaska, using microsatellite markers. Although studies focused on the population structure of *Lethenteron* spp. have been investigated in Eurasia, little is known about populations in Alaska, despite their abundance (Yamazaki et al. 2006, 2011, 2014; Sutton 2017). The Yukon River is the largest river drainage in Alaska with known spawning aggregations of anadromous Arctic lamprey. In addition, both the commercial and subsistence fisheries are located along the lower Yukon River, making this drainage an important area to conduct initial investigations into the genetic population structure of *Lethenteron* spp. Overall, this research highlights the value of genetics as a research tool to investigate ecological and evolutionary knowledge gaps of *Lethenteron* spp., a largely overlooked Arctic fish species.

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Chapter 1: Utilizing DNA metabarcoding to characterize the diet of marine-phase Arctic lamprey (*Lethenteron camtschaticum*) in the eastern Bering Sea[1]

Abstract

To understand the marine feeding ecology of Arctic lamprey (*Lethenteron camtschaticum*) in the eastern Bering Sea, visual observations and DNA metabarcoding of gut contents were utilized to characterize diet composition (N = 250 lampreys) in 2014 and 2015. Differences among individual diets were evaluated by collection year, capture site, and fish size. Hard structures and tissues were observed during visual examinations of gut contents, and 10 ray-finned fish taxa were identified by DNA metabarcoding. The most frequent taxa included Pacific sand lance (*Ammodytes hexapterus*), Pacific herring (*Clupea pallasii*), gadids, and capelin (*Mallotus villosus*). Five taxa were reported for the first time as prey for Arctic lamprey. Individual diets differed between collection years, among capture sites, and among size classes; however, both collection year and size explained only a small portion of diet variability (R^2 = 0.02 and 0.04, respectively) relative to station site (R^2 = 0.49). These study results indicate that Arctic lamprey are opportunistic flesh eaters, and highlighted the value of DNA metabarcoding to characterize the diet of a poorly understood lamprey species.

[1] Shink, K.G., Sutton, T.M., Murphy, J.M., and López J.A. Utilizing DNA metabarcoding to characterize the diet of marine-phase Arctic lamprey (*Lethenteron camtschaticum*) in the eastern Bering Sea. Prepared for submission to Canadian Journal of Fisheries and Aquatic Sciences.

Introduction

Characterizing marine-phase lamprey diets pose a special challenge to researchers. Occurrences of lamprey wounds on teleost fishes and hard structures (e.g., scales and/or fins) recovered from lamprey intestinal contents have been routinely used to identify prey species (Beamish 1980; Maitland et al. 1984; Novomodnyy and Belyaev 2002; Renaud et al. 2009). While occurrences of lamprey wounds provide insights into lamprey-host marine interactions, identified hosts may be biased toward highly valued and frequently encountered commercial fishes (Hardisty and Potter 1971). Hard structures can be taxonomically informative, but they often have variable recovery and digestion rates and may not be consistently ingested during predation, which can lead to biased or misleading dietary inferences (Cottrell et al. 1996; Cottrell and Trites 2002). In addition, digested blood and/or chunks of flesh recovered from lamprey intestinal tracts generally yield limited details on prey composition and are not taxonomically informative. As a result, marine trophic interactions of anadromous lamprey species remain poorly understood (Mesa and Copeland 2009).

The food habits of closely related species of lamprey can vary from blood to the flesh of their prey (Potter and Hilliard 1987; Renaud et al. 2009). Flesh-feeding species are generally characterized with having smaller buccal glands, a smaller oral disc with fewer teeth, and having an enlarged 'tongue-like piston' (Potter and Hilliard 1987; Renaud et al. 2009). Lampreys that exhibit flesh-feeding food habits target smaller-bodied fishes and inflict severe damage that often results in the death of the prey (Roos et al. 1973; Beamish 1980; Maitland et al. 1984; Renaud et al. 2009). Flesh-feeding species are known to ingest large pieces of flesh and, in some instances, have been shown to penetrate the prey's body cavity to consume their internal organs (Beamish and Williams 1976; Beamish 1980; Maitland et al. 1984). In contrast, blood-feeding lampreys

primarily target larger fish species that are less susceptible to damage because wounds from blood feeders are characterized by a single hole or slide where blood can continuously be extracted (Potter and Hilliard 1987; Renaud et al. 2009; Patrick et al. 2009). Previous research has identified the feeding mode of different lamprey species based on morphological characteristics of the oral disc and dentition (Potter and Hilliard 1987; Renaud et al. 2009).

Both European river lamprey (*Lampetra fluviatilis)* and Western river lamprey (*Lampetra ayresii*) are known to feed on flesh (Beamish and Williams 1976; Beamish 1980; Maitland et al. 1984). Arctic lamprey (*Lethenteron camtschaticum*) was inferred to be a flesh-feeding lamprey species, but this conclusion was based on morphological similarities in dentition to European and Western river lampreys, not explicit diet evaluations (Potter and Hilliard 1987; Renaud et al. 2009). Visual examination of the intestinal contents of European river lamprey revealed the presence of large chunks of flesh, spines, scales, eggs, and internal organs (reviewed in Hardisty and Potter 1971; Maitland et al. 1984; Renaud et al. 2009). Similarly, fins, scales, skin, bones, muscle, and internal organs have been reported in the intestinal contents of Western river lamprey (Beamish and Williams 1976; Beamish 1980). Unidentifiable remains were reported in 0 – 56% of examined lampreys (Beamish 1980; Maitland et al. 1984; Beamish and Neville 1995). To date, no studies have examined the intestinal contents of Arctic lamprey, and the potential occurrence and frequency of these structures in the diet is currently unknown.

Much of what is known about the diet of Arctic lamprey has originated from visual observations of lamprey wounds on teleost fishes captured within Russian estuaries (Nikol'skii 1956; Gritzenko 1968; Heard 1966; Novomodnyy and Belyaev 2002; Shevlyakov and Parensky 2010). Arctic lampreys are believed to be prey upon pelagic schooling fishes (e.g., Osmeridae, Clupeidae) and juvenile Pacific salmon (*Oncorhynchus* spp.; Nikol'skii 1956; Gritzenko 1968;

Novomodnyy and Belyaev 2002; Shevlyakov and Parensky 2010; Siwicke 2014). General marine distributions of Arctic lamprey indicate high concentrations of these predators along the eastern Bring sea shelf, but information on lamprey trophic interactions in this region are scarce (Orlov et al. 2014). Overlapping patterns of abundance inferred from catch-per-unit effort (CPUE) data in the Bering Sea have shown co-occurrence between Arctic lamprey and Pacific herring (*Clupea pallasii)* and juvenile Pacific salmon (Siwicke 2014). In the Laurentian Great Lakes, the distribution of sea lamprey (*Petromyzon marinus)* appears to be influenced by the distribution of prey fish availability (Johnson and Anderson 1980; Bence et al. 2003; Harvey et al. 2008). If this relationship is an important driver of Arctic lamprey distribution in the Bering Sea, research suggests Pacific herring and Pacific salmon are important hosts for this species of lamprey (Siwicke 2014).

The application of molecular techniques to characterize prey species in predator diets improves detection and taxonomic resolution of prey relative to traditional morphological methods (Deagle et al. 2005; Braley et al. 2010; Carreon-Martinez et al. 2011; Moran *et a*l. 2016). Continued development and refinement of 'DNA metabarcoding' approaches for accurate species identification has made it possible to characterize diet components that lack taxonomic characters without *a priori* information on predator diets (Valentini et al. 2009; Pompanon et al. 2012; Taberlet et al. 2012). Prey DNA can be isolated from fecal or gastrointestinal tract samples and used for targeted sequencing of taxonomically informative genome regions (reviewed in Pompanon et al. 2012). However, DNA metabarcoding has only been used in a limited number of studies involving predatory fish diet evaluations (Leray et al. 2013, Berry et al. 2015; Leray et al. 2015; Harms-Tuohy et al. 2016).

The objective of this study was to characterize the diet of marine-phase Arctic lamprey in

the eastern Bering Sea through a combination of visual observations of intestinal contents and DNA metabarcoding. The specific objectives of this study were to: (i) assess if diets of Arctic lamprey change as a function of capture year, capture site, and/or size class; and (ii) evaluate the relative performance of diet composition inferred from previous reports of visual observations of lamprey scars to that revealed by DNA metabarcoding.

Methods

Lamprey Collections

Marine-phase Arctic lamprey (N = 250) were collected using surface rope trawls (Baker 2011) during the U.S. Bering-Aleutian Salmon International Survey (BASIS) on the eastern Bering Sea shelf between September 04 and September 22 in 2014 (n = 122), and September 01 and September 16 in 2015 (n = 128; Figure 1.1). Lamprey were captured at 30 of 57 stations in 2014 and 26 of 37 stations in 2015, and immediately frozen whole. Specimens were shipped to the University of Alaska Museum of the North where they were stored at -20°C until further processing.

Measurements and Processing

In the laboratory, whole Arctic lamprey were thawed and measured for total length (L_T; nearest 1 mm) and total weight (W_T; nearest 0.01 g) prior to dissection. Whole intestinal tracts were removed from each specimen, and contents were designated as anterior or posterior based on its location in the intestine. Contents within the first half of the intestine were identified as anterior, while contents within the second half were identified as posterior. Intestinal contents were separated using a metal forcep and scraped into separate sterile petri dishes and weighed to the nearest 0.01 g. Intestinal contents were examined using a Leica M125C stereoscope (Leica

Microsystems, Wetzlar, Germany) at a magnification of 8 – 100x for the presence of diagnostic hard structures (e.g., scales, otoliths, bone fragments). Observed hard structures were removed and preserved in 96% molecular-grade ethanol. To act as a validation measure for DNA metabarcoding sequences, a subset (n = 61) of diagnostic tissues (e.g., sizable flesh pieces, internal organs) were removed and preserved in 96% molecular-grade ethanol to be used in targeted Sanger sequencing.

Upon completion of visual observations, anterior and posterior intestinal contents were placed in separate 15 mL vials and frozen at -20°C. Between processing each lamprey, all laboratory surfaces were wiped with a 10% bleach solution and covered with a fresh VWR® table protector with leak-proof moisture barrier to minimize the possibility of cross-specimen contamination. In addition, all dissecting tools were submerged in 20% bleach solution for five minutes and thoroughly rinsed with nuclease-free water.

DNA Extractions

Total genomic DNA (gDNA) was isolated from sub-sampled tissues using the Gentra Puregene Tissue Kit (Qiagen, California, USA). During the final elution step, DNA was eluted and suspended in 50 μL nuclease-free water for subsequent molecular analyses, which varied from the manufacturer's recommendations.

To maximize the quality of isolated DNA, anterior intestinal contents were thawed and mechanically homogenized to reduce intra-sample variability and facilitate DNA isolation. The tip of the tissue homogenizer was submerged in a 10% bleach solution, rinsed with nuclease-free water, and wiped dry between samples. Total gDNA was extracted from four 200 mg sub-samples of homogenized anterior content using the DNeasy® *mericon* food kit (Qiagen,

California, USA) following the manufacturer's short fragment recovery protocol. When a total of 800 mg could not be recovered from the anterior content, a combination of anterior and posterior content was used. Extraction negative controls were systematically incorporated during extractions. Final DNA concentrations were determined by fluorometry on a Qubit instrument using reagents from the double stranded broad range DNA fluorometry assay kit (Invitrogen, CA, USA).

PCR – Sanger Sequencing

Polymerase chain reactions (PCRs) for Sanger sequencing of sub-sampled tissues were conducted using universal DNA barcode primers. Primers FishF1 – 5' TCAACCAACCACAAAGACATTGGCAC 3' and Fish R1 – 5' TAGACTTCTGGGTGGCCAAAGAATCG 3' were used to target a 655 basepair (bp) region of the cytochrome c oxidase subunit I (COI) in fishes (Ward et al. 2005). The COI PCR was conducted in 25 μL reaction volumes with 1 μL template DNA and the following reagent concentrations: 1X Go Taq polymerase buffer, 0.4 μM of each primer, 0.8 mM dNTP's, 2.0 mM Mg^{2+}, and 0.025 U/μL of Go Taq polymerase. Temperature cycling conditions for PCRs were an initial denaturation at 94°C for two minutes followed by 32 cycles of 94°C for 45 seconds, 51°C for 30 seconds, and 72°C for 45 seconds, followed by a final extension at 72°C for seven minutes and 30 seconds then held at 4°C. A PCR negative control was included in all amplifications. The PCR products were visualized on 1.5% agarose electrophoresis gels to verify target amplification lengths and lack of external contamination. Sanger sequencing was conducted on an ABI 3730XL at Eurofins MWG Operon (Louisville, KY, USA). Sequences were visually inspected and analyzed with the CodonCode Aligner software (Dedham, MA, USA) and compared to

publically available DNA sequences in GenBank using the basic local alignment search tool (BLAST; Altschul et al. 1990).

PCR – DNA Metabarcoding

A vertebrate-specific primer set targeting a 106 basepair (bp) segment of the mitochondrial genome coding the 12s ribosomal RNA gene was used for DNA metabarcoding (Riaz et al. 2011). Eight forward and 12 reverse primers with internal sequence tags (Appendix A) were generated following the approach described in Glenn et al. (2016) to preserve the ability to assign sequence reads back to individual lamprey specimens. The PCR reactions were conducted in 25 μL reaction volumes with 5 μL template DNA and the following reagent concentrations: 1X Go Taq polymerase buffer, 0.4 μM of each primer, 0.8 mM dNTP's, 2 mM Mg^{2+}, 10 ug/mL of bovine serum albumin (BSA), and 0.025 U/μL of Go Taq polymerase. The PCR conditions were an initial denaturation at 95°C for five minutes followed by 28 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. A PCR negative control was included in all amplifications. Fragment size and absence of contamination was confirmed by visualization of PCR products on 2.5% agarose electrophoresis gels.

Indexed PCR products were combined into four pools. Each pool contained samples with a unique combination of indexes, one randomly selected DNA extraction negative control, and one amplified PCR negative control. Additional library preparation steps were conducted at GeneWiz (South Plainfield, NJ, USA). Pooled libraries were multiplexed and 150PE sequenced on an Illumina MiSeq. The run included 10% PhiX DNA spike-in control to improve the data quality of low-diversity samples.

Bioinformatics

Initial performance of the MiSeq run was evaluated with FastQC v.0.11.5 (Andrews 2010). Individual sequencing reads were demultiplexed using BBDuk within BBTools package (J. Bushnell, Joint Genome Institute, unpublished data) and a modified PERL script by Eric Collins (University of Alaska Fairbanks, https://github.com/rec3141/demult/blob/master/run-bbduk.sh) allowing no mismatches per barcode. Primers were trimmed from demultiplexed reads using CUTADAPT v.1.12 (Martin 2011). Sequencing reads that contained no primer, contained greater than 10% error rates (> 1 primer mismatch), or fell outside of the target read length (96 – 116 bp) were discarded. Paired-end reads were merged with a minimum overlap of 30 bp using PEAR v.0.9.6 (Zhang et al. 2014). Trimmed and merged reads were then run through a VESEARCH v.2.4.0 (Rognes et al. 2016) pipeline that included (1) *de novo* chimera checking, (2) dereplicating 100% identical sequence, and (3) clustering sequences at a ≥ 96% similarity threshold into operational taxonomic units (OTUs). To exclude sequencing noise and/or artifacts, dereplicated sequences assigned to individual samples were removed from downstream analyses using VSEARCH. Dereplicated sequences were classified as noise and/or artifacts when a sequence occurred a fewer number of times in an individual sample when compared to the frequency with which it occurred in DNA and PCR negative controls.

A custom BLAST database of complete mitochondrial fish genomes was generated for this study using the downloaded fish genome files that were compiled in the Mitochondrial Genome Database of Fish (MitoFish; Iwasaki et al. 2013). The database was created using the 'makeblastdb' option within BLAST+ v.2.6.0 (Camacho et al. 2009) and contained 2,148 unique fish mitochondrial genome sequences.

Final OTU sequences were queried against the custom BLAST database using the command-line tool 'blastn' within BLAST+. Search parameters specified an e-value threshold of 10^{-5}, 90% or greater sequence identity, and a maximum retention of 10 sequence alignments. BLAST files were imported into MEGAN (Huson et al. 2016) to visualize taxonomic assignments using customized least common ancestor (LCA) parameters (min score = 100, top percent = 8, min support = 1) and the LCA algorithm weighted at 80%. A sequence similarity of $\geq$ 98% was considered to be a species level match. Otherwise, OTUs were assigned to the highest taxonomic classification that encompassed all significant matches. The final taxonomic incidence (e.g., presence/absence) table contained all individual samples and was exported from MEGAN for subsequent analyses.

Statistical Analysis

Rarefaction analysis and the Chao2 species richness estimator was calculated to assess the effect of sample size on the number of detected host species and estimate the number of additional samples needed to fully describe the diet components of Arctic lamprey. Sample-based estimates and 95% confidence intervals were calculated in EstimateS v.9.1.0 (Coldwell 2013) using 1,000 sample-order randomizations.

Multivariate statistical analyses were conducted using the VEGAN package (Oksanen et al. 2013) in R (R Core Team 2013). The incidence table was used to generate distance matrices among samples using the Jaccard distance measure. Permutational Multivariate Analysis of Variance using distance matrices (PERMANOVA) was run with 999 permutations using the R-VEGAN function ADONIS to examine the statistical significance and percentage of dietary variation that could be explained by collection year (e.g., 2014 and 2015), station sites where

Arctic lamprey were captured (e.g., 39), and total length (e.g., L_T). For L_T analyses, lampreys were grouped into 12 size-class intervals of 25 mm, which corresponded to 1-inch length measurements. The 12 size-class intervals encompassed the smallest and largest individual lampreys (187 – 464 mm, respectively). Nonmetric multidimensional scaling (NMDS) plots were used to visually investigate patterns in the diets of individuals for each of the above factors.

Results

Intestinal contents from were recovered from all Arctic lamprey (N = 250). The L_T of examined specimens ranged from 187 – 465 mm, while W_T ranged from 8.0 – 192.1 g (Figure 1.2). Recovered diagnostic structures included eggs, fins and/or fin rays, internal organs, otoliths, scales, vertebrae and uncategorized bone fragments (Figure 1.3). Hard structures were recovered from 103 (84%) and 112 (88%) intestinal tracts in 2014 and 2015, respectively (Figure 1.4). Fins and/or fin rays were the most abundant structure in both years, while otoliths were the most infrequent structures.

Sanger Sequencing

Genomic DNA from 28 of the 61 (46%) tissue samples were successfully amplified by PCR. Of those successful amplifications, 27 sequences were taxonomically identified to species based on the criteria of ≥ 98% sequence similarity to publicly available sequences in the NCBI database. Seven species were detected in 2014 and 2015, respectively (Table 1.1). Two species, Chinook salmon and yellowfin sole (*Limanda aspera)*, were detected only from samples collected in 2014. Two other species, pink salmon (*Oncorhynchus gorbuscha*) and Pacific sand lance (*Ammodytes hexapterus*), were detected only in 2015 samples. The remaining five species

[walleye pollock (*Gadus chalcogrammus*), Pacific sand lance (*Ammodytes hexapterus*), capelin (*Mallotus villosus*), Pacific herring (*Clupea pallasii*), and saffron cod (*Eleginus gracilis*)] were detected in the samples from both study years.

DNA Metabarcoding

The high throughput sequencing run produced 21,590,316 raw reads of which 18,862,344 were assigned back to unique index tags. A small proportion of the reads (0.1%) were assigned to one of the eight negative control samples; however, no sequencing reads remained in the negative control samples after the filtering process. A total of 7,557,159 high quality reads (Phred score ≥ Q38) were partitioned among 221 (88%) samples and used in downstream analyses. The OTU clustering approach implemented in VSEARCH delineated 261 OTUs in the intestinal contents. All OTUs were identified to the level of taxonomic family, genus, or species. The sample-based rarefication curve appeared to reach a plateau, which indicated that the number of sampled individuals provided an adequate representation of species in the diet of Arctic lamprey in the eastern Bering Sea. However, the Chao2 estimator suggested that the dietary extent of Arctic lamprey had not been fully described (Figure 1.5).

A total of 10 ray-finned fish taxa were detected. These taxa were comprised of eight orders, with four taxa taxonomically identified to family, one identified to genera, and five identified to species (Table 1.2). Pacific sand lance, Pacific herring, Gadidae, and capelin occurred most frequently in the diet of Arctic lamprey (Table 1.2). Capelin and Pacific herring were the dominant taxa for 2014 and 2015, respectively. The number of taxa detected within individual Arctic lamprey intestinal contents ranged from one (66%) to four (0.5%), but two and three taxa were also observed within individual gut contents (27% and 6%, respectively).

Only 27 of 61 (44%) tissue samples yielded high quality sequences. All species that were identified by sequencing a 655 bp region of COI from sub-sampled tissues were represented in the high-throughput final sequence library, but there were three taxonomic groups (i.e., daubed shanny (*Leptoclinus maculatus*), sculpins (Cottidae), and sticklebacks (Gasterosteidae)) were only detected in the high-throughput dataset (Table 1.1). Identical taxa were detected by both methods in 20 of 27 (74%) samples. The DNA metabarcoding approach detected more than one taxonomic group in 6 of 27 (22%) individual gut contents when compared to taxon identified by sub-sampled tissues.

Statistical analysis

The diets of Arctic lamprey were significantly different between collection years (ADONIS: $R^2 = 0.011$, $P = 0.009$) and among the 12 size classes (ADONIS, $R^2 = 0.037$; $P = 0.020$), but each factor accounted for only a small proportion of diet variability. Diets of individual Arctic lamprey were also significantly different among station sites and accounted for a moderate proportion of diet variability (ADONIS: $R^2 = 0.487$, $P = 0.001$). Although NMD scaling produced clustering in a two-dimensional plot and provided a good representation of the data (Kruskal's stress value = 0.04), visual inspections of each plot did not reveal obvious patterns or clusters solely represented by individuals belonging to different temporal, spatial, or biological groups.

Discussion

This study is the first to implement gene-based identification of lamprey diet composition. Specifically, this study characterized the diet of marine-phase Arctic lamprey using

a combination of intestinal content observations and DNA metabarcoding. Metabarcoding analysis detected Pacific sand lance, Pacific herring, gadids, and capelin as the most frequent taxa in the gut contents of Arctic lamprey. Reports of lamprey wounds on clupeids and osmerids are common among flesh-feeding species (Nikol'skii 1956; Maitland et al. 1984; Beamish and Williams 1976; Beamish 1980; Beamish and Neville 1995). Identified diet components largely supported previous reports of Arctic lamprey wounds on species such as saffron cod, rainbow smelt, Pacific herring, and Pacific salmon (Nikol'skii 1956; Gritsenko 1968; Novomodnyy and Belyaev 2002; Shevlyakov and Parensky 2010). Attacks on juvenile Pacific salmon have also been widely reported in estuaries (Beamish 1980; Beamish and Neville 1995; Novomodnyy and Belyaev 2002; Shevlyakov and Parensky 2010). Surprisingly, Pacific salmon were detected in only eleven Arctic lampreys in the current study, which may be attributed to limited sampling of nearshore estuarine habitats or greater abundances of other prey species (Maitland et al. 1984; Siwicke 2014).

The current study was also the first to document three species (i.e., walleye pollock, Pacific sand lance, and daubed shanny) and two families (i.e., Cottidae and Pleuronectidae) as diet components of Arctic lamprey. Although these taxa were reported in lamprey diets for the first time, only Pacific sand lance and walleye pollock were detected in more than one individual. This suggests that the importance of Cottidae, Pleuronectidae, and daubed shanny as prey in this region may be relatively low. These results highlighted the ability of DNA metabarcoding to reveal rare and previously unreported dietary components of a poorly studied lamprey species in the eastern Bering Sea.

The presence of diagnostic hard structures and observations of tissue masses within Arctic lamprey intestinal tracts are indicative of a flesh-feeding approach. Morphological

structure, dentition, and size of the oral disc determine the feeding mode of parasitic lampreys (Potter and Hilliard 1987; Renaud et al. 2009). Previous studies that examined these morphological differences classified Arctic lamprey as a flesh-feeding species, but this conclusion was inferred by morphological similarities to known flesh-feeding species and not visual examinations of intestinal contents (Potter and Hilliard 1987; Renaud et al. 2009). Although the occurrence of hard structures and internal organs in intestinal tracts have been widely documented for other flesh-feeding lamprey species (reviewed in Hardisty and Potter 1971; Maitland et al. 1984; Beamish and Williams 1976; Beamish 1980; Renaud et al. 2009), these results are the first to visually confirm flesh, internal organs, and hard structures in the intestinal tracts of Arctic lamprey. Ultimately, the study results supported the hypothesis that Arctic lamprey are a flesh-feeding species; however, without prior knowledge or direct observations, the feeding modality of marine-phase Arctic lamprey remains unknown.

Flesh-feeding Arctic lamprey may be predators, scavengers, or exhibit a combination of these foraging behaviors. The frequency and type of diagnostic hard structures (i.e., fins, vertebrae, etc.) observed within the gut contents of Arctic lamprey offer limited insight into the specific feeding behavior(s) of this species because skeletal structures may be ingested after predation events or during scavenging. It is presumed that flesh-feeding lampreys actively pursue live prey and use their oral disc as a suction mechanism to facilitate feeding until they dislodge or the prey sustains life-ending injuries. The occurrence of Arctic lamprey attached to live prey and the presence of healed oral disc wounds suggest a predatory behavior (Birman 1950; Nikol'skii 1956; Heard 1966; Shevlyakov and Parensky 2010). Without direct visual observations, it is unknown if Arctic lamprey continues to feed after the death of its prey. However, this behavioral feeding modality has been observed in flesh-feeding Western river

lamprey and the Miller Lake lamprey (*Entosphenus minimus*; Bond and Kan 1973; Beamish 1980). This behavior directly contrasts with that of blood-feeding sea lamprey and Pacific lamprey (*Entosphenus tridentatus*), which are thought to abandon prey that are dead or dying (Hardisty and Potter 1971). Only one species of lamprey, the Caspian lamprey (*Caspiomyzon wagneri*), are thought to be scavengers, but this conclusion was based on indirect evidence (Renaud 2011).

If Arctic lamprey predominantly exhibits predatory behavior, the frequency with which vital skeletal structures and organs were observed suggests that prey attacked in the eastern Bering Sea sustain high mortality rates. Lamprey feeding mode (i.e., blood versus flesh) and prey body size are factors related to the survival probability of prey (Beamish and Williams 1976; Cochran 1984; Potter and Hilliard 1987; Renaud et al. 2009). Flesh-feeding wounds are typically larger in size than localized wounds characteristic of blood-feeding species (Beamish and Williams 1976; Potter and Hilliard 1987). Larger fishes are more likely to recover from a lamprey attack than smaller-bodied prey (Cochran and Jenkins 1994; Swink 2003; Patrick et al. 2009). Flesh-feeding lampreys are thought to prefer smaller adult or juvenile fishes as prey even though some species grow to a larger body size (Birman 1950; Gritsenko 1968; Hardisty and Potter 1971; Roos et al. 1973; Maitland et al. 1984; Cochran and Jenkins 1994; Renaud et al. 2009). Laboratory studies have demonstrated that flesh-feeding Western river lamprey can kill and consume a clupeid host within an hour; these attacks may expose the vertebral column as up to three quarters of the body can be consumed (Beamish and Williams 1976). Although the presence of skeletal structures and musculature suggests a predatory approach, scavenging of dead prey may also account for the presence of hard structures and tissues in intestinal contents.

It is possible that the feeding behavior of lampreys varies with food availability. Although live prey was shown to elicit an immediate feeding response in Western river lamprey and Pacific lamprey, these species were also observed scavenging on previously deceased fishes and carrion (Beamish and Williams 1976; Beamish 1980). A combination of predatory and scavenging behaviors was similarly proposed for flesh-feeding Miller Lake lamprey (Bond and Kan 1973). Likewise, Arctic lamprey may exhibit scavenging behavior during opportunistic and concentrated abundances of carrion (i.e., mass die-offs of semelparous capelin after spawning) in the eastern Bering Sea. Incidental deaths of individual shoaling fishes caused by larger predators may provide further scavenging opportunities. Because of the relatively poor swimming performance of lampreys relative to teleost fishes (Beamish 1974; Dauble et al. 2006), scavenging appears to a plausible scenario under which Arctic lamprey could more easily consume highly mobile fishes (i.e., Pacific sand lance). However, the possibility of 'secondary predation' (i.e., detecting the consumed prey of the primary prey in lamprey intestinal contents) may have influenced the study results.

The observation of entire digestive tracts within the intestinal tracts of Arctic lamprey increased the probability of detecting signals of secondary predation in this study. Secondary predation has been documented in other dietary studies, and is recognized as a limitation of DNA metabarcoding (Deagle et al. 2009; O'Rorke et al. 2012; Bowser et al. 2013; De Barba et al. 2014; Pinol et al. 2014). The eastern Bering Sea is a highly connected and complex marine ecosystem (Aydin and Mueter 2007). Within this region, Pacific herring and gadids feed on larval and juvenile capelin (Hjermann et al. 2004; Godiksen et al. 2006), while Pacific sand lance are important prey for Pacific herring, capelin, rainbow smelt, and Pacific salmon (Robards and Piatt 1999). Pacific sand lance may also feed on Pacific herring eggs and larvae (Sturdevant et al.

2000). Finally, both chum salmon (*Oncorhynchus keta*) and Chinook salmon (*O. tshawytscha*) consume juvenile walleye pollock in addition to Pacific herring and capelin (Davis et al. 2009). Due to the multifaceted roles of detected taxa as both predator and prey, it is not possible to definitively determine the extent of secondary predation in this study. The most frequent prey taxa were detected both individually and with multiple taxa within individual intestinal tracts. Ultimately, the ambiguity of Arctic lamprey feeding behavior and food-web interconnectivities in the eastern Bering Sea highlights a limitation of DNA metabarcoding. Without *a priori* knowledge or direct visual observations of feeding behaviors under natural conditions, key aspects of the feeding ecology of Arctic lamprey remain speculative. The use of DNA barcoding in combination with visual observations and stable-isotope techniques may circumvent the limitations of any one technique and provide additional insight into the trophic position of Arctic lampreys relative to other fish species in the Bering sea ecosystem.

Predator sample sizes were large enough to examine potential spatial (e.g., sample site), temporal (e.g., collection year), and biological (e.g., size class) variability in Arctic lamprey diet composition. Although both biological and temporal variables were significant, they explained only a small portion of the differences between diets (R^2 = 0.011 and 0.037, respectively). Lamprey diet composition does not appear to be driven by inter-annual variation or body size in the eastern Bering Sea ecosystem; however, the significant relationship between lamprey size and consumed prey may support previous observations of larger lampreys consuming larger prey (Maitland et al. 1984; Swink 1990, 2003). Of the four dominant taxa identified in this study, Pacific sand lance were not detected in of large lampreys (> 425 mm). Herring and capelin were detected in gut contents belonging to lampreys of the largest size classes (i.e., 425 – 449 and 450 – 475 mm). Gadids were detected in mid-range size classes (300 – 424 mm), and capelin was

detected in all but one size class (250 – 274 mm). Herring was the only prey taxa that was detected in all size classes. This may reflect a forced transition to larger-bodied prey as lamprey body size and buccal funnel diameter increases, in comparison to lampreys of smaller body size, which may retain flexibility to prey upon fish species of varying body sizes.

Although it is recognized that different lamprey species exhibit specialized feeding mechanisms, Arctic lampreys may be generalist predators that opportunistically consume prey driven by regional species abundances in the eastern Bering Sea. Of the factors examined, spatial variability was significant and explained the greatest portion of differences between individual dietary components. This may be explained by the varying abundances of different species at station sites where lampreys were captured. Abundances of Arctic lamprey and potential hosts (mainly Pacific herring and Pacific salmon) were shown to be highly correlated in the eastern Bering Sea and were hypothesized to be driven by predator-prey interactions (Siwicke 2014). This trend has similarly been observed in regions of the Laurentian Great Lakes where sea lamprey wounds were most frequently observed on fish species with the highest seasonal abundance (Johnson and Anderson 1980; Bence et al. 2003; Harvey et al. 2008). Because flesh-feeding lamprey species are generally of smaller body size, it would be metabolically more efficient to feed on fish species of greater abundance as opposed to seeking out specific rarer prey species. The overlapping distribution of lampreys and pelagic schooling fishes may be primarily driven by habitat preferences along the eastern Bering Sea shelf, and these distributions exhibit temporal and spatial variations (Orlov et al. 2014). Further evaluations are necessary to assess dietary variation of Arctic lampreys that may be driven by seasonal fish assemblages.

Both gene-based identification techniques produced similar dietary results, however, there were discrepancies and biases associated with each approach. Species identification using

DNA metabarcoding and the COI gene fragment detected identical taxonomic groups for 78% of individuals. Additional taxa were detected with DNA metabarcoding in 25% of individuals, suggesting that the tissue samples that could be analyzed with the COI gene fragment did not fully capture the dietary variability within individual lampreys. This also proved to be a limitation of DNA metabarcoding. Samples where different taxonomic groups were identified with different methods indicated that all taxa within individual intestinal tracts were not always detected, which may be an unintended result of isolating DNA from only the anterior portion of intestinal contents. The taxonomic resolution of the DNA metabarcoding primer set was unable to discriminate among sequences from fishes in the families Cottidae, Gadidae, Gasterosteidae, and Pleuronectidae and the genus *Oncorhynchus*. However, the fine-scale taxonomic resolution of the COI gene fragment provided additional trophic insight. Sequenced tissue samples identified one species within the family Gadidae, walleye pollock and pink and Chinook salmon. While it cannot be confirmed that the remaining Gadidae and Pacific salmon sequences were those specific species, it provided additional taxonomic resolution to the trophic dataset. Ultimately, the large sample size and little taxonomic variability observed in this study characterized the dietary variability of Arctic lampreys in the eastern Bering Sea.

We endeavored to improve our knowledge of the food habits of Arctic lamprey. Indeed, visual observations of intestinal contents confirmed the flesh-feeding approach of this species (Potter and Hilliard 1987; Renaud et al. 2009), while the DNA metabarcoding approach identified the importance of pelagic schooling fishes in the diet of Arctic lamprey. However, additional study is still needed to investigate if diet varies by season as well as throughout their geographic distribution. While this approach cannot explicitly ascertain the feeding behavior modality or 'secondary predation', it will provide insight into the food-web dynamics in the

eastern Bering Sea and the need for additional observations of lamprey feeding behavior under both environmental and laboratory conditions. Finally, the term 'parasitic' has been used to describe fishes that consume tissue and/or internal fluids of a host species without killing their host (Elliott et al. 2002). While this description may apply to some lamprey species, the results from this study suggest that the term parasitic should not be used as a generalization to describe the feeding ecology of all lampreys.

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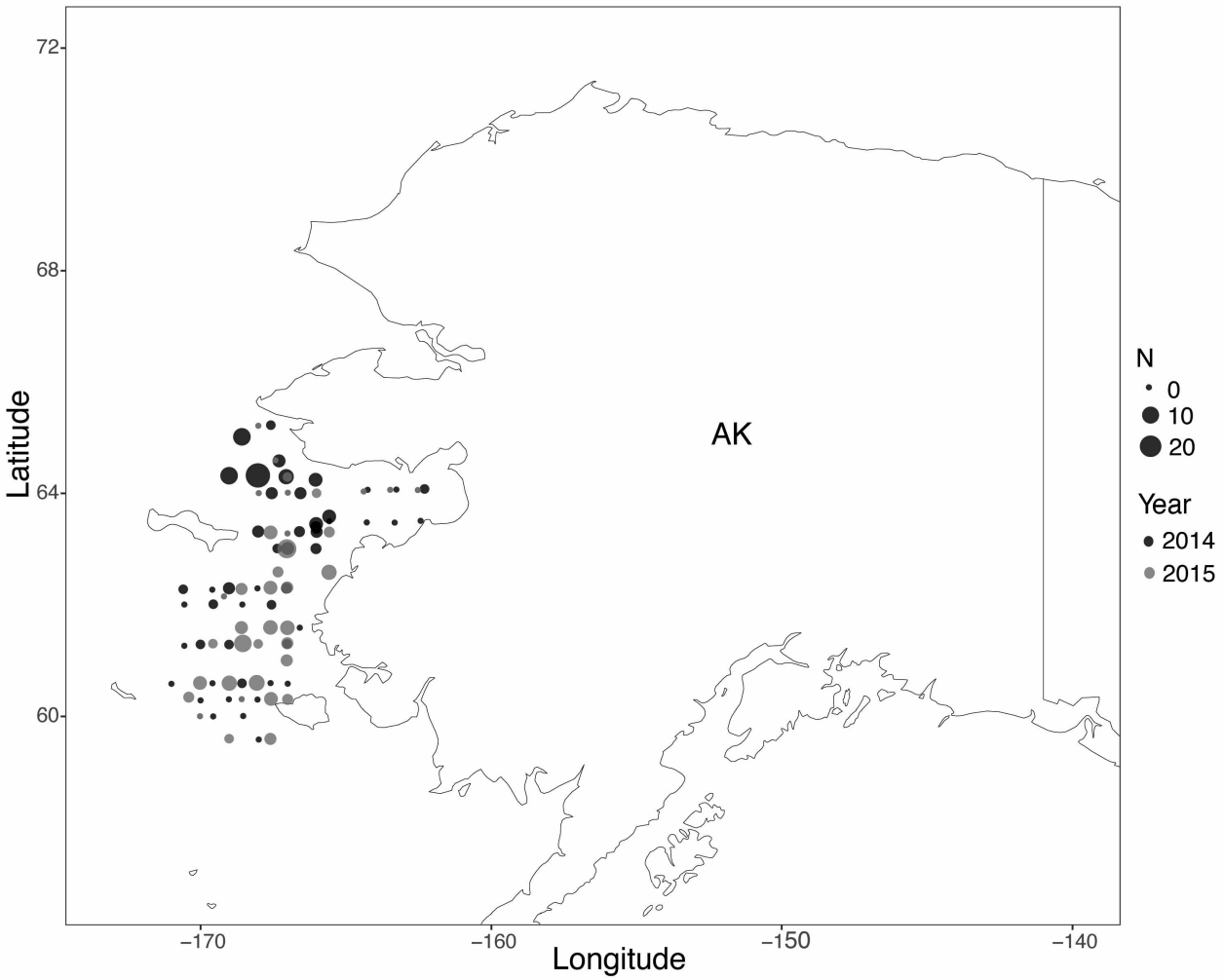

Figure 1.1. Map of the sample sites in the eastern Bering Sea in 2014 (black) and 2015 (gray). Each circle denotes a station where surface trawls were conducted. The diameter of each circle represents the number of lampreys captured at each locality.

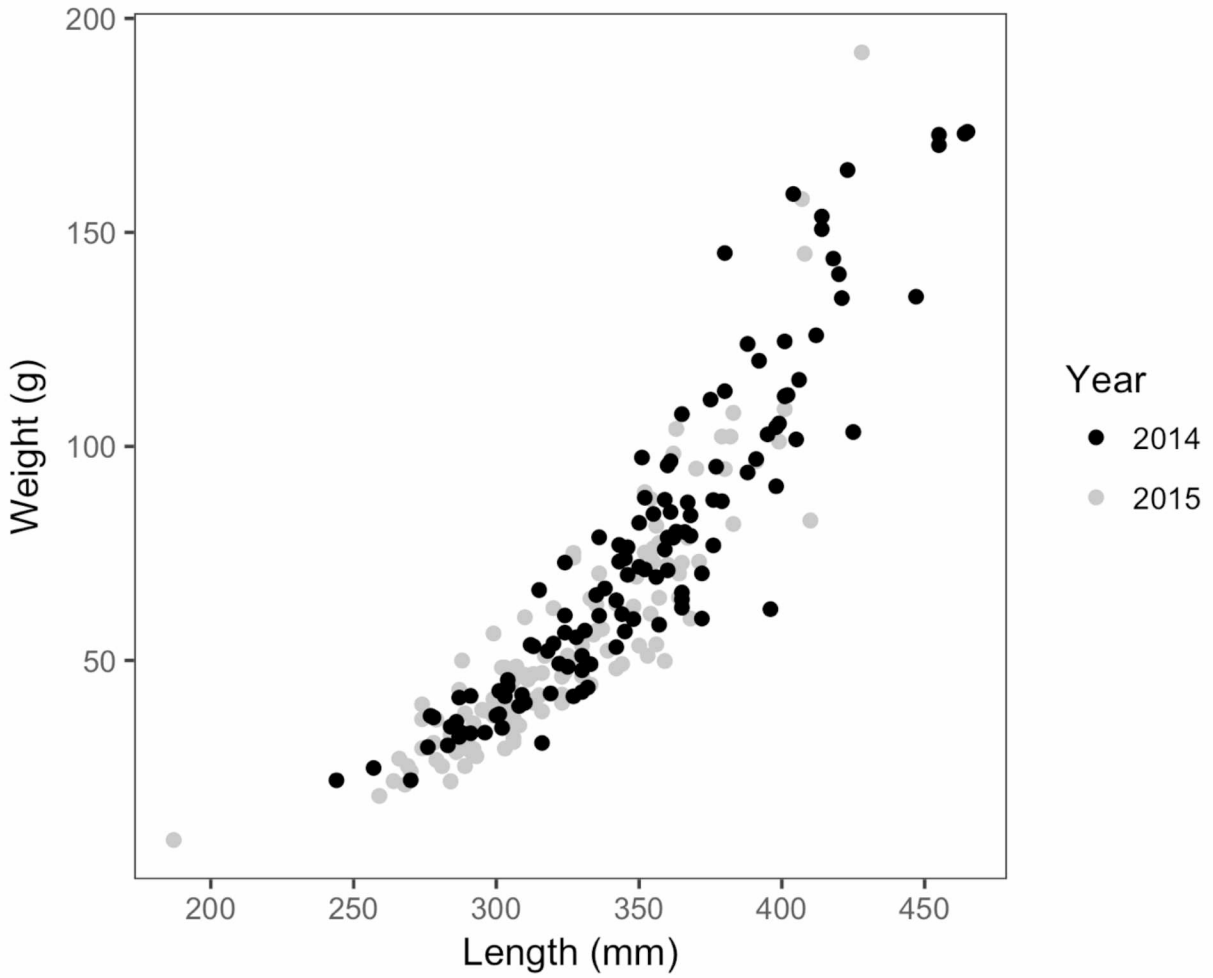

Figure 1.2. Weight versus total length of Arctic lamprey collected from the eastern Bering Sea in 2014 and 2015.

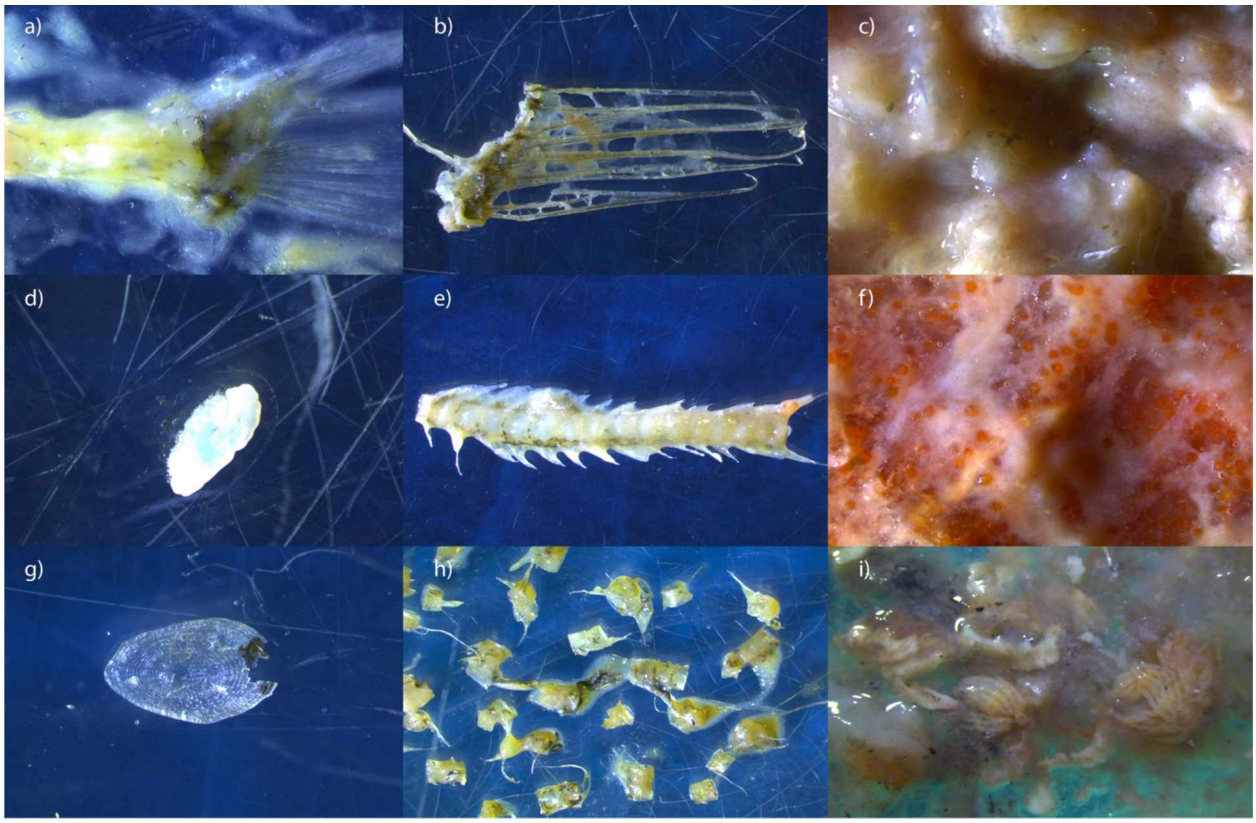

Figure 1.3. Diagnostic hard structures and tissues recovered from intestinal tracts of Arctic lamprey: (a) caudal fin attached to vertebrae; (b) fin; (c) scales embedded within tissue masses; (d) otolith; (e) vertebral column; (f) eggs; (g) scale; (h) individual vertebrae; and (i) internal organs.

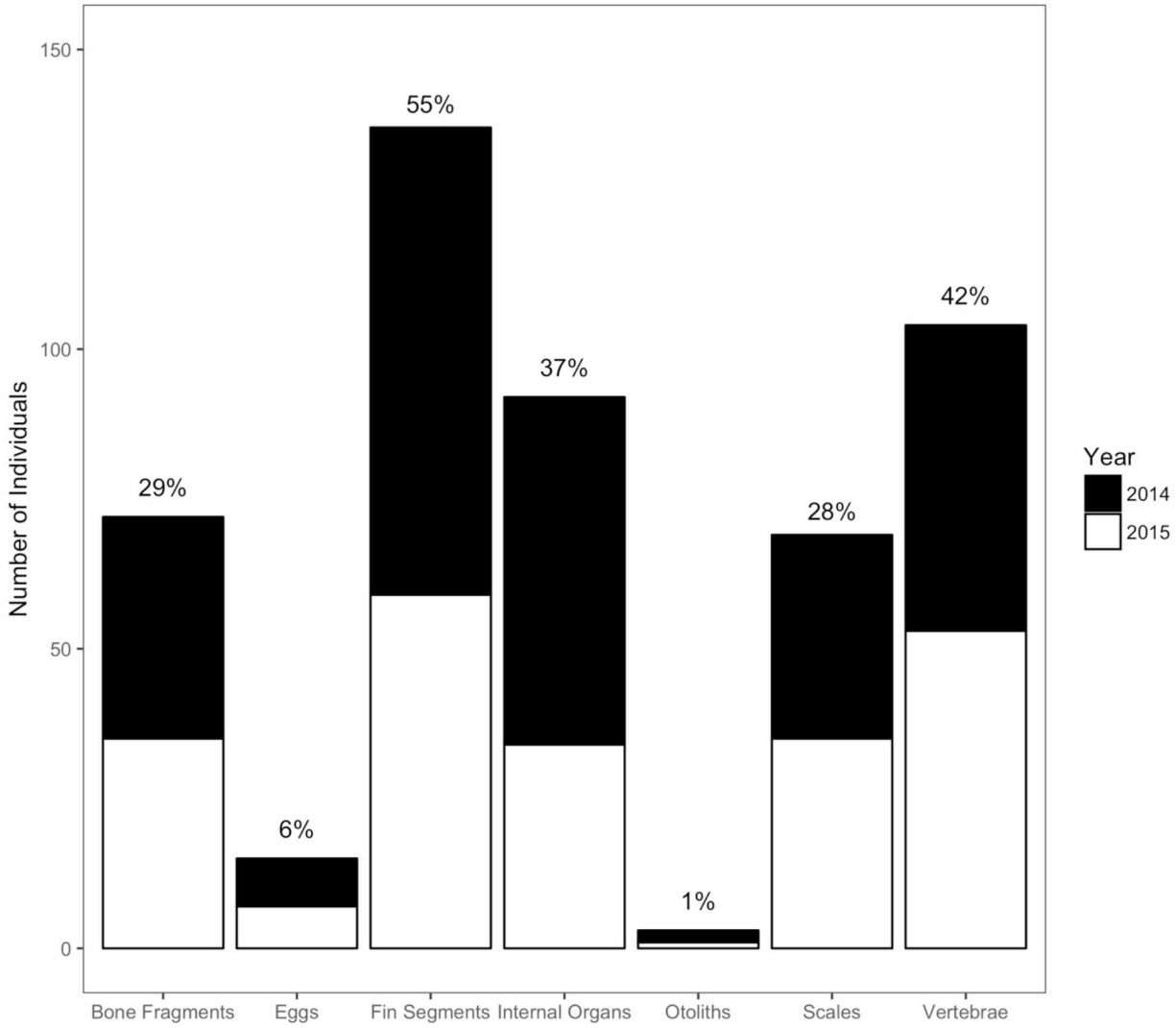

Figure 1.4. Number of individuals with diagnostic hard structures and tissues within intestinal tracts of Arctic lamprey. The percentages above each column are the frequency of occurrence (e.g., presence/absence) relative to the number of individual lamprey for each collection year.

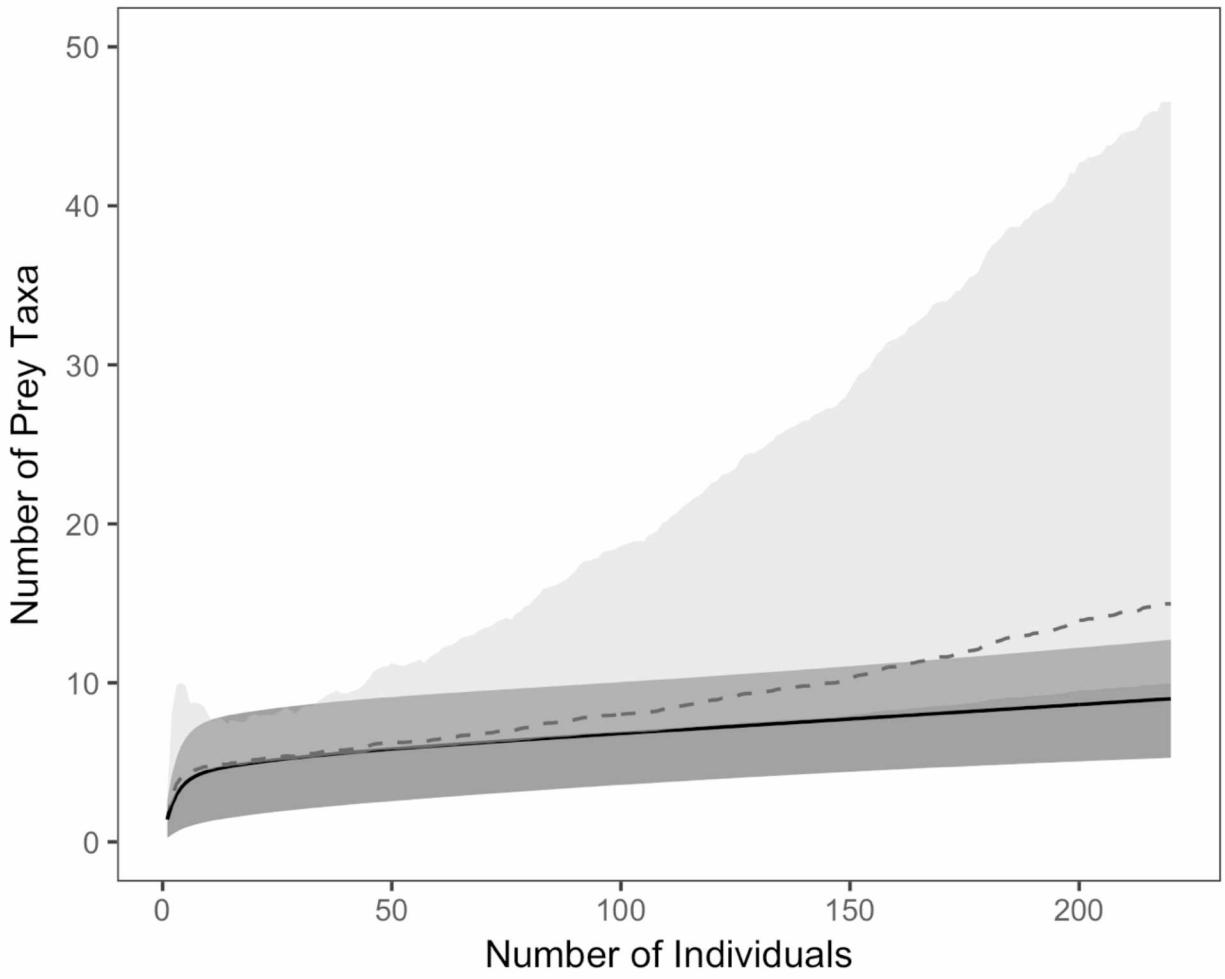

Figure 1.5. Rarefaction curve (solid line) and Chao2 species richness estimator (dotted line). The shaded areas represent 95% confidence limits for each estimate.

Table 1.1. Comparison of taxonomic assignments from tissue samples (Sanger sequencing) and gut contents (DNA metabarcoding). Bold taxa correspond to agreement in the taxonomic assignments. *n* = number of individuals that contained identical taxa identified by sequenced tissue samples.

Year	Tissue type	*n*	Sanger Sequencing	DNA metabarcoding	
2014	flesh	5	***Gadus chalcogramma***	**Gadidae**	
	flesh		***Gadus chalcogramma***	**Gadidae**	
	flesh		***Gadus chalcogramma***	**Gadidae**	
	organ		***Gadus chalcogramma***	**Gadidae**	
	pyloric caeca		*Gadus chalcogramma*	*Clupea pallasii*	
	flesh	1	***Limanda aspera***	**Pleuronectidae**	*Clupea pallasii*
	flesh	1	*Oncorhynchus tshawytscha*	*Clupea pallasii*	
	flesh	1	***Mallotus villosus***	***Mallotus villosus***	
	GI tract	1	*Eleginus gracilis*	*Ammodytes hexapterus*	
2015	flesh	7	*Gadus chalcogramma*	*Mallotus villosus*	
	flesh		***Gadus chalcogramma***	**Gadidae**	
	GI tract		***Gadus chalcogramma***	**Gadidae**	
	organ		***Gadus chalcogramma***	**Gadidae**	
	pyloric caeca		***Gadus chalcogramma***	**Gadidae**	
	pyloric caeca		***Gadus chalcogramma***	**Gadidae**	
	pyloric caeca		***Gadus chalcogramma***	**Gadidae**	
	flesh	5	***Ammodytes hexapterus***	***Ammodytes hexapterus***	*Clupea pallasii*
	flesh		***Ammodytes hexapterus***	***Ammodytes hexapterus***	*Clupea pallasii*
	flesh		***Ammodytes hexapterus***	***Ammodytes hexapterus***	*Clupea pallasii*
	flesh		***Ammodytes hexapterus***	***Ammodytes hexapterus***	*Clupea pallasii*
	organ		***Ammodytes hexapterus***	***Ammodytes hexapterus***	*Clupea pallasii*
	flesh	3	*O. gorbuscha*	*Clupea pallasii*	
	GI tract		*O. gorbuscha*	-	
	GI tract		*O. gorbuscha*	Gadidae	
	organ	2	***Mallotus villosus***	***Mallotus villosus***	Gadidae
	organ		***Mallotus villosus***	***Mallotus villosus***	
	flesh	1	***Eleginus gracilis***	**Gadidae**	
		27			

Table 1.2. Taxonomic assignment of prey items found in Arctic lamprey intestinal contents. Species-level assignments were based on criteria of ≥ 98% sequence similarity to sequences in the reference database. Taxa in bold occurred in over 10% of the gut contents.

Order	Family	Genus	Species	Frequency of occurrence (%)	Total number of samples (*n*)
Clupeiformes	**Clupeidae**	***Clupea***	***pallasii***	**25.3**	**79**
Gadiformes	**Gadidae**			**16.3**	**51**
Gasterosteiformes	Gasterosteidae			0.3	1
Osmeriformes	**Osmeridae**	*Osmerus*	*mordax*	1.6	5
		Mallotus	***villosus***	**31.1**	**97**
Perciformes	**Ammodytidae**	***Ammodytes***	***hexapterus***	**20.8**	**65**
	Stichaeidae	*Leptoclinus*	*maculatus*	0.3	1
Pleuronectiformes	Pleuronectidae			0.3	1
Salmoniformes	Salmonidae	*Oncorhynchus*	spp.	3.5	11
Scorpaeniformes	Cottidae			0.3	1

Chapter 2: Characterizing the genetic variation among larval populations of *Lethenteron* spp. within the Yukon River drainage, Alaska, using microsatellite markers[2]

Abstract

Information regarding the genetic population structure among *Lethenteron* spp. populations within Alaskan rivers is limited. The objective of this study was to investigate the genetic diversity among larval populations of *Lethenteron* spp. from three tributaries within the Yukon River drainage, Alaska, using microsatellite genotyping. A total of 81 larval lampreys were genotyped at eight microsatellite loci. Global F_{ST} was 0.074 (95% CI: 0.042 – 0.110), while pairwise F_{ST} values ranged from 0.066 – 0.081. Model-based Bayesian clustering analyses without sampling locality priors identified one ancestral population typical of panmixia, while the model with sample locality priors detected three genetic clusters (K = 3). Within-river clustering analyses of population structure indicated panmixia within the East Fork of the Andreafsky and Gisasa rivers, but detected reduced levels of admixture within the Chena River. Estimates of contemporary gene flow indicated reduced but reciprocal migration among sites. These results suggest life-history variants of *Lethenteron* spp. may contribute to the moderate degree of genetic differentiation and reduced levels of gene flow among sample sites than typically found among anadromous *L. camtschaticum* populations.

[2] Shink, K. G., Sutton, T. M., Murphy, J. M., and López, J. A. Characterizing the genetic variation among larval populations of *Lethenteron* spp. within the Yukon River drainage, Alaska using microsatellite markers. Submitted to the Journal of Fish Biology.

INTRODUCTION

Arctic lamprey *Lethenteron camtschaticum* (Tilesius 1811) are an agnathan fish widely distributed at mid to high latitudes in northwestern North America and eastern Asia (Mecklenburg et al. 2002; Renaud 2011). Six nonparasitic species are congeneric with *L. camtschaticum*, but the Alaskan brook lamprey *L. alaskense* is the only nonparasitic species endemic to Alaska rivers and streams (Vladykov and Kott 1978; Mecklenburg et al. 2002; Renaud 2011). Although morphologically similar in their larval form, parasitic and nonparasitic species diverge radically in feeding behavior and body size through successive life-history stages (Zanandrea 1959; Vladykov and Kott 1979; Docker 2009; Sutton 2017). Anadromous *L. camtschaticum* feeds on the blood and/or flesh of host fishes in marine environments before returning to freshwater tributaries to spawn, while *L. alaskense* is a fluvial, nonparasitic species that resides in freshwater tributaries for the duration of their lifecycle and do not feed as adults (Mecklenburg et al. 2002; Renaud et al. 2009a, 2011). Current taxonomics rely on morphology and life history to distinguish among species, despite uncertain phylogenetic relationships between parasitic and nonparasitic species pairs (Docker 2009; Renaud 2009b; Potter et al. 2015).

Morphological and molecular studies have sought to resolve taxonomic relationships among parasitic and nonparasitic *Lethenteron* spp. Morphological and meristic evaluations have previously failed to identify diagnostic taxonomic characteristics to distinguish sympatric parasitic and nonparasitic forms within river systems (Kucheryavyi et al. 2007; Nazarov et al. 2011; Sutton 2017). Molecular analyses suggest limited genetic divergence in sequenced regions of the mitochondrial genome among paired *Lethenteron* species (Yamazaki et al. 2006; Artamonova et al. 2011; Balakirev et al. 2014; Artamonova et al. 2015). Investigations of species

differences between *L. camtschaticum* and *L. alaskense* revealed that these two life-history variants were expressed in populations that shared the same mitochondrial gene pool (Lang et al. 2009; April et al. 2011). These results bring into question the taxonomic status of parasitic and nonparasitic species within the genus *Lethenteron*.

Fine-scale population genetic data for *Lethenteron* spp. are restricted in geographical scope to eastern Eurasia. Yamazaki et al. (2011) reported low levels of genetic differentiation and ongoing gene flow between sympatric parasitic and nonparasitic populations of *L. camtschaticum*. Results from microsatellite analyses among anadromous *L. camtschaticum* indicated heterogeneous populations despite broad distributions (Yamazaki et al. 2014). This trend has been observed in other anadromous lamprey species and was attributed, in part, to a lack of natal homing (Bryan et al. 2005; Goodman et al. 2008; Spice et al. 2012). Similarly, Yamazaki et al. (2014) hypothesized that a lack of natal homing contributed to the levels of panmixia among examined *L. camtschaticum* populations; however, the exact biological and/or environmental mechanisms facilitating high levels of gene flow among populations remain speculative. To date, no studies have characterized the genetic structure among populations of lamprey in Alaska rivers (ADF&G 2006; Thorsteinsen and Love 2016). This emphasizes the need of additional data to document relatedness among *Lethenteron* populations in the eastern part of their distribution and compare patterns in population genetic structure across their geographic range.

The objective of this study was to investigate the genetic diversity and levels of gene flow among three larval populations of *Lethenteron* spp. within the Yukon River drainage using microsatellite genotyping. Here, we use the term *Lethenteron* spp. to refer to sampled lamprey populations because a recent field study in the Chena River, Alaska, determined that

morphological and meristic characteristics could not be used to differentiate between *L. camtschaticum* and *L. alaskense* during their larval stage (Sutton 2017). Because there are no physical barriers limiting dispersal and ongoing gene flow has been observed within the genus *Lethenteron*, we hypothesized that there would be low levels of genetic differentiation and high levels of gene flow among sampled sites.

MATERIALS AND METHODS

SAMPLE COLLECTION

Larval lampreys were collected from three tributaries in the Yukon River drainage, Alaska (Figure 2.1; Table 2.1). Larvae from the East Fork of the Andreafsky and Gisasa rivers were collected using a modified bottom sampler (Lasne et al. 2010). Fin tissue samples were removed from 40 individuals within each tributary that were released alive near their collection site. Larvae from the Chena River were collected using a Model ABP-2 backpack electrofishing unit (ETS Electrofishing LLC, Wisconsin, USA), and a maximum of ten individuals were collected at each collection site (Sutton 2017). These larvae were euthanized using tricaine methanesulfonate (MS-222) and frozen for subsequent molecular analyses. All tissue samples were preserved in 96% molecular-grade ethanol in the field and placed in cold storage (-20 °C) for long-term preservation.

MICROSATELLITE GENOTYPING

Total genomic DNA from individual tissue samples were isolated through tissue lysis followed by salt and alcohol precipitation using the Gentra Puregene Tissue Kit (Qiagen, California, USA) following the manufacturer's protocol. Eight microsatellite loci previously identified and developed from a brook lamprey *Lethenteron* spp. N from Japan were targeted for genotyping

(Takeshima et al. 2005). To generate allele amplicons for the targeted loci, a three-primer Polymerase Chain Reaction (PCR) approach was implemented (Schuelke 2000). The primer combination included: (1) a locus specific primer with the standard M13 sequence 5' tail; (2) a locus specific complementary primer; and (3) a fluorescently labeled oligonucleotide corresponding to the M13 segment of the locus specific primer. The standard M13 sequence 5' tail was incorporated on the locus specific forward primer for all loci except Lspn088.

Polymerase Chain Reactions (PCRs) were conducted in a 25 μL reaction volume with 0.5 μL variable concentration genomic DNA template, 1x *GoTaq*® buffer (Promega), 0.05 mM locus specific primer with M13 tail, 0.25 mM locus specific complementary primer, 0.25 mM fluorescently labeled M13 primer (FAM or HEX), 0.6 mM dNTP's, 25 μg/mL BSA, 1.5 - 2 μM Mg^{2+}, and 0.025 U/μL of *GoTaq*® polymerase. The Mg^{2+} concentrations in the reaction mix varied by locus (Table 2.2). Thermal cycler conditions were identical to those reported in Takeshima et al. (2005). The PCR products were run on 2.5% agarose gels with a negative control to confirm amplification length and lack of template contamination. These products were analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, California, USA) and allele sizes were determined using GeneMapper v 3.7 (Applied Biosystems, California, USA).

Potential genotyping errors resulting from null alleles and/or large allelic drop out were evaluated with the software program Micro-Checker (van Oosterhout et al. 2004). Deviations from genotype frequencies expected under Hardy-Weinberg equilibrium (HWE), and tests for linkage disequilibrium (LD) between pairs of loci in each population were evaluated with exact probability tests implemented in GENEPOP v 4.0.10 (10^4 dememorization, 10^3 batches, 10^4 iterations per batch; Raymond and Rousset 1995).

MICROSATELLITE GENETIC DIVERSITY

Statistics summarizing allelic frequencies, mean number of alleles (N_A), expected heterozygosity (H_E), and observed heterozygosity (H_O) were calculated with the program ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010). Estimates of allelic richness (A_R) and inbreeding coefficients (F_{IS}) were calculated with the program FSTAT 2.9.3.2 (Goudet 2001).

The probability of identity (PI) estimate was conducted in GENALEX 6.5 (Peakall and Smouse 2012) to test the probability of identifying two independent samples with identical genotypes at specific combinations of loci. Values within the range of 0.01 – 0.0001 are recommended to increase confidence that the number of genotyped loci are sufficient to identify individuals (Waits et al. 2001). The PI among individuals genotyped at seven loci was well within the suggested range (0.0034 – 0.0001; Figure 2.2).

Because all tissue samples collected for this study came from larval lampreys, we investigated the likelihood that collected samples were from a small number of families. The probability of sampled full-sibling pairs was estimated using the full-pedigree likelihood (FL) approach in COLONY v. 2.0.6.2 (Jones and Wang 2010) under a female and male polygamy mating system, known allele frequencies, no sibship prior, and a long-run length with five independent runs. One randomly selected individual from each full-sibling pair within each tributary was removed from the dataset and estimates of F_{ST} were recalculated (Appendix C). Reported values of F_{ST} without full siblings decreased but remained highly significant, so all individuals were included in modeling analyses.

GENETIC DIFFERENTIATION

To assess the degree of genetic differentiation between populations, Weir and Cockerham's (1984) F_{ST} was estimated by the program GENEPOP. Fisher's exact probability tests for genetic

differentiation for each population pair across all loci was conducted in GENEPOP to evaluate the statistical significance of the observed F_{ST} values. Global F_{ST} and 95% confidence intervals were estimated in FSTAT with 20,000 permutations.

Sampling locations were grouped into two geographical regions (lower and upper Yukon area, respectively) depending on their location upstream or downstream of the village Shagluk within Alaska management areas for the Yukon River (Bue et al. 2009). The East Fork of the Andreafsky and Gisasa Rivers are located within the lower Yukon River area, while the Chena River falls within the upper Yukon River management area for Alaska. The percentage of variation within populations, among populations within geographical regions, and within geographical regions was calculated using a locus-by-locus analysis of molecular variance (AMOVA) test in ARLEQUIN with 20,000 permutations.

Genetic isolation-by-distance (IBD) was evaluated to test for population structure influenced by geographic distance. River distances previously reported by Templin et al. (2005) were used to perform a test for IBD using Isolation by Distance Web Service v 3.21 (Jensen et al. 2005). Pairwise river distances were evaluated by the distance in river kilometers (rkm) between the confluences of sampled tributaries.

GENETIC STRUCTURE

The model-based Bayesian clustering analysis within STRUCTURE v 2.3.4 (Pritchard et al. 2000) was used to identify genetic structure and estimate individual membership probabilities to genetic clusters. Evaluated genetic clusters (K) ranged from 1 to 7, with 20 independent replicates conducted for each K value. In each replicate analysis, Markov chain Monte Carlo (MCMC) simulations consisted of 750,000 iterations using an admixture model with correlated allele frequencies (Falush et al. 2003) discarding samples from the first 250,000 iterations as

burn-in. Analyses of genetic structure were conducted among all individuals with and without prior information on the locality of samples (LOCPRIOR; Hubisz et al. 2009). Population-level analyses were conducted independently to further investigate genetic structure within each tributary. For population-level analyses, tested values of K ranged from 1 to 5 with identical parameters to those described above. The prior model parameter LOCPRIOR was not used for these analyses.

For all STRUCTURE models, the most likely number of clusters was identified with the estimated log likelihood lnPr(X|K) reported in STRUCTURE output and the ΔK method (Evanno et al. 2005) implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2012). The software program CLUMPP v 1.2 (Jakobsson and Rosenberg 2007) was used to identify the optimal alignment of estimated cluster membership matrices over the 20 runs for the chosen k. Plots were visualized using DISTRUCT v 1.1 (Rosenberg 2004).

Contemporary migration rates (m) were estimated using a Bayesian assignment method in the program BAYESASS 3.0.1 (Wilson and Rannala 2003) employing a MCMC procedure that does not assume HWE. Resultant estimates of m were used to estimate proportions of non-migrant individuals within each population and evaluate directionality of migration among population pairs within the last two generations. A total of 21^6 MCMC iterations and 5^6 burn-in were used to estimate m and produce convergent trace outputs for five independent runs with varied seed numbers. Delta values for migration rates, inbreeding coefficient, and allele frequencies were adjusted to attain acceptance rates between 40 and 60% of the total iterations. The convergence of the MCMC algorithm was assessed using the software TRACER 1.6 (Rambaut et al. 2014) by visually plotting posterior parameter estimates.

RESULTS

MICROSATELLITE GENETIC DIVERSITY

A total of 81 larval lampreys were successfully genotyped at eight loci. One locus (Lspn002-2) was monomorphic in all genotyped individuals and was excluded from downstream analyses. Micro-Checker indicated the occurrence of a null allele at locus Lspn019c in the Chena River population and at locus Lspn050 in all three populations (Table 2.3). Significant deviations from HWE were detected at locus Lspn019c and Lspn050 after Bonferroni correction (critical value: P = 0.003). No linkage disequilibrium (LD) was observed between pairs of loci. The number of observed alleles per locus varied from 2 – 6, and allelic richness per locus among all populations ranged from 2.000 – 3.995 (locus Lspn094 and Lspn088, respectively). Additional summary statistics including expected and observed heterozygosity and inbreeding coefficients (F_{IS}) are provided in Table 2.4.

GENETIC DIFFERENTAITION

Measures of F_{ST} for all population pairs ranged from 0.066 – 0.081, with a global F_{ST} of 0.074 (95% CI: 0.042 – 0.110; Table 2.5). Tests of population differentiation remained highly significant among all pairs of populations after Bonferroni correction (critical value: P = 0.0166). The AMOVA results partitioned 92.91% of observed variation within populations (P = 0.000), 9.34% among populations within geographical regions (P = 0.000), and -2.25% among geographical regions (P = 0.724). The trend of IBD among *Lethenteron* populations was negatively correlated but not statistically significant (r = -0.9876, P = 0.1663).

GENETIC STRUCTURE

Results for the STRUCTURE model without prior information on the locality of samples reported the highest log-likelihood for $K = 1$ with ΔK selecting K=2 (Figure 2.2a; 2.6). Average

individual membership coefficients ranged from 0.481 to 0.519, suggesting almost equal proportions of global ancestry between genetic clusters. The STRUCTURE output with the LOCPRIOR parameter identified $K = 3$ as the optimal number of genetic clusters, which was supported by both the highest log-likelihood and ΔK method (Figure 2.2b). The highest average individual membership coefficient within each cluster was 0.849, 0.875, and 0.875 for the East Fork of the Andreafsky, Gisasa, and Chena rivers, respectively.

Models without prior parameters were run in STRUCTURE to investigate potential genetic structure within each tributary. The highest log-likelihood supported $K = 1$ for both the East Fork of the Andreafsky and Gisasa rivers; however, ΔK identified $K = 2$ and $K = 4$, respectively, as the best partition of genetic structure (Figure 2.2c). Average individual membership coefficients were divided equally between two (0.500) and four (0.250) clusters for each system implying genetic structure could not be detected. Within the Chena River, both the highest log-likelihood and ΔK method supported $K = 2$ as the optimal number of clusters. The highest individual membership coefficient (q_i) identified in each cluster was 0.797 and 0.789.

The proportion of non-migrant individuals ranged from 0.940 in the East Fork of the Andreafsky River to 0.799 in the Chena River (Figure 2.3). The highest proportion of migrants ($m = 0.143$) was detected from the Chena River into the Gisasa River. Estimated migration among populations was symmetrical, with the highest estimated migration rates occurring from upstream to downstream among all three localities.

DISCUSSION

These results represent the first examination of *Lethenteron* spp. genetic diversity and population structure within the Yukon River drainage. Both global and pairwise F_{ST} values observed among

sampled tributaries were indicative of moderate genetic differentiation and reduced levels of gene flow. The moderate levels of genetic structure observed in this study were distinctive from the levels of panmixia reported among *Lethenteron* spp. populations throughout the western part of their distribution (Yamazaki et al. 2011, 2014; Artamonova et al. 2015).

Bayesian models with and without the LOCPRIOR model parameter produced contradictory results. The model without prior information on the locality of samples assigned all individuals with almost equal probability to two clusters. These results suggested: (1) all individuals belong to a single ancestral population; or (2) the genetic signal was too weak to be detected without prior parameters. The inability of STRUCTURE to detect genetic clusters without prior parameters can be influenced by numerous factors, including scored loci, sampling intensity and sample size, or recent divergence (Evanno et al. 2005; Hubisz et al. 2009; Fogelqvist et al. 2010; Kalinowski 2011). Integrating informative priors such as sample localities can improve the ability of Bayesian models to detect genetic structure without false positives and may be useful when working with a limited number of individuals and/or loci (Corander et al. 2003; Corander and Marttinen 2006; Hubisz et al. 2009).

Results from the model that incorporated the LOCPRIOR model parameter identified three genetic population clusters supported by both the highest log-likelihood and ΔK methods. Average individual membership coefficients and reduced levels of admixture produced by this model appeared to reflect the observed levels of heterozygosity and positive F_{IS} values within each river as well as the moderate levels of F_{ST} among population pairs. While we believe these results best depict the data, contrasting model outputs highlight the importance of additional sampling efforts across space and time to better inform and evaluate genetic relationships among populations of *Lethenteron* spp. within the Yukon River drainage.

The high F_{ST} values observed in this study were unexpected given the knowledge of *L. camtschaticum* genetic structure in the western part of their geographic distribution and its inferred implications for migration ecology. The global F_{ST} value observed among sampled *Lethenteron* populations was higher than values observed among chum salmon *Oncorhynchus keta* (F_{ST} = 0.018) and Chinook salmon *O. tshawytscha* (F_{ST} = 0.038) within the Yukon River drainage (Beacham et al. 2006, 2009). Natal homing in *Oncorhynchus* spp. largely influences geographic clustering and genetic population structure (Dittman and Quinn 1996; Sato et al. 2004); however, previous research indicated that sea lamprey *Petromyzon marinus* and Pacific lamprey *Entosphenus tridentatus* do not exhibit natal homing (Bergstedt and Seelye 1995; Waldman et al. 2008; Hatch and Whiteaker 2009). Instead, pheromone odors from stream-resident larvae are one of the primary mechanisms that guide adult *P. marinus* to optimal spawning habitat independent of their natal streams (Moore and Schleen 1980; Sorensen et al. 2003; Vrieze et al. 2011). This spawning strategy, referred to as the "suitable river strategy" by Waldman et al. (2008), is believed to contribute to the levels of panmixia among anadromous *P. marinus* and *E. tridentatus* (Bryan et al. 2005; Goodman et al. 2008; Spice et al. 2012). No studies have verified the role of larval pheromones on migratory behavior in *L. camtschaticum*, but it is suspected to be similar among lamprey genera (Moser et al. 2015). Previous genetic studies support this inference; Yamazaki et al. (2014) reported continuous reciprocal gene flow among *L. camtschaticum* populations not influenced by geographical distance. While our study also found the effects of IBD insignificant, we can only speculate as to the mechanisms restricting gene flow among sampled *Lethenteron* spp. populations within the Yukon River drainage.

Our data suggested that nonparasitic *L. alaskense* and/or resident *L. camtschaticum* life-history variants contributed to the moderate values of F_{ST} reported in this study. Significant measures of genetic differentiation have been reported among nonparasitic resident lamprey populations of the same species, despite high levels of gene flow between parasitic and nonparasitic forms (Espanhol et al. 2007; Blank et al. 2008; Mateus et al. 2011; Boguski et al. 2012; Docker et al. 2012; Bracken et al. 2015; Rougemont et al. 2015). Although contemporary estimates of migration were symmetric among sites, estimated proportions of non-migrants were greater in both the East Fork of the Andreafsky and Gisasa rivers. When evaluated with the reduced levels of heterozygosity and admixture detected within these rivers, analyses suggest reduced dispersal capabilities characteristics of resident forms. It appears that gene flow, while ongoing among sampled sites, occurs at reduced levels preventing the formation of a panmictic population.

Within-river analyses of population structure suggest the presence of both parasitic and nonparasitic forms in our samples. Bayesian clustering analyses raised the possibility of genetically diverging *Lethenteron* spp. populations (presumably representing parasitic and nonparasitic forms) occurring in sympatry within the Chena River; however, the small spatial scale of sampling efforts within the East Fork of the Andreafsky and Gisasa rivers (3.5 rkm) compared to efforts in the Chena River (145 rkm) and small sample sizes, may explain the genetic variability (or lack thereof) detected within each tributary. Although the scope of this study did not include an explicit examination of genetic differentiation between *L. camtschaticum* and *L. alaskense* within the Yukon River drainage, adult parasitic and nonparasitic forms are known to inhabit the Chena River (Sutton 2017; A. Gryska, Alaska Department of Fish and Game, personal communication). This further highlights the need to

sample adult *Lethenteron* spp. to provide a comprehensive analysis of gene flow and address questions of divergence between this species pair.

Results of our study suggested that (1) sampled populations exhibited moderate levels of genetic structure and reduced levels of gene flow and (2) the possibility of diverging populations occurring in sympatry within the Chena River. The reported levels of genetic structure among the rivers examined in this study emphasized the need for additional sampling efforts within the Yukon River drainage and other major Alaska river drainages where *Lethenteron* spp. have been observed. In addition, future studies in Alaska should focus on parasitic *L. camtschaticum* and nonparasitic *L. alaskense* to provide a comprehensive evaluation of potential species divergence between these two forms. Continued efforts to address the connectivity of *Lethenteron* populations would inform the management of commercial and subsistence lamprey fisheries along the lower Yukon River drainage and provide further insight into the biology and migration ecology of this species throughout their geographic distribution.

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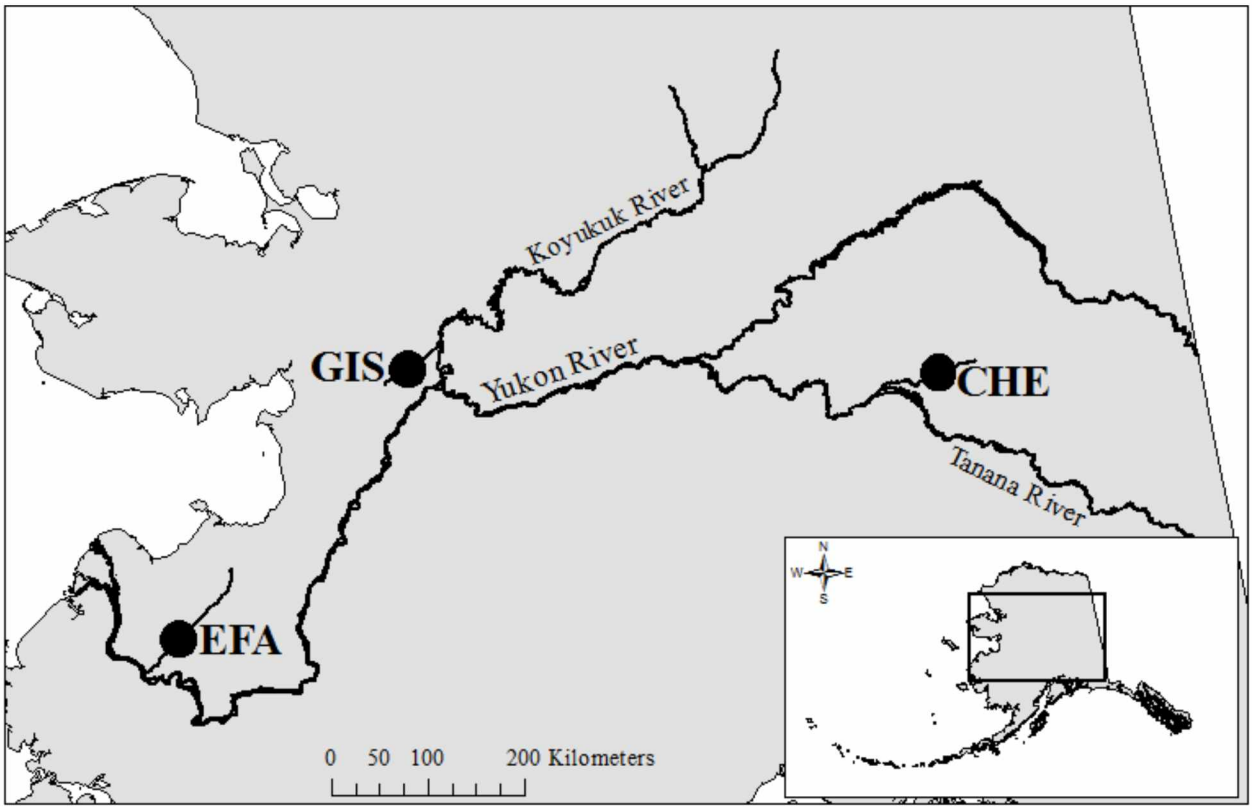

Figure 2.1. Map of sampled tributaries within the Yukon River drainage, Alaska. EFA – East Fork of the Andreafsky River. GIS – Gisasa River. CHE – Chena River.

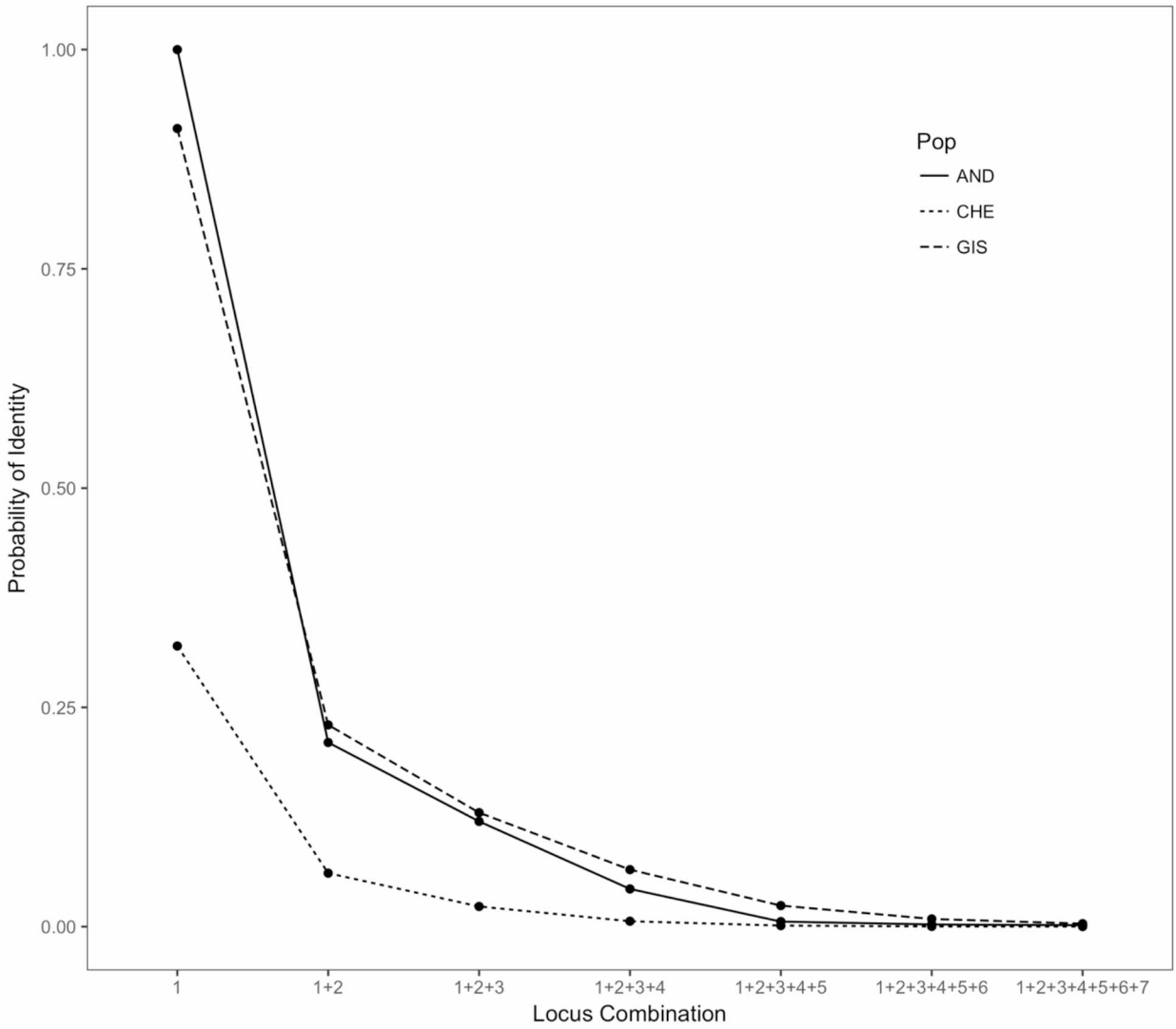

Figure 2.2. Observed probability of identity (P_{ID}) values for sampled populations at increasing combinations of microsatellite loci.

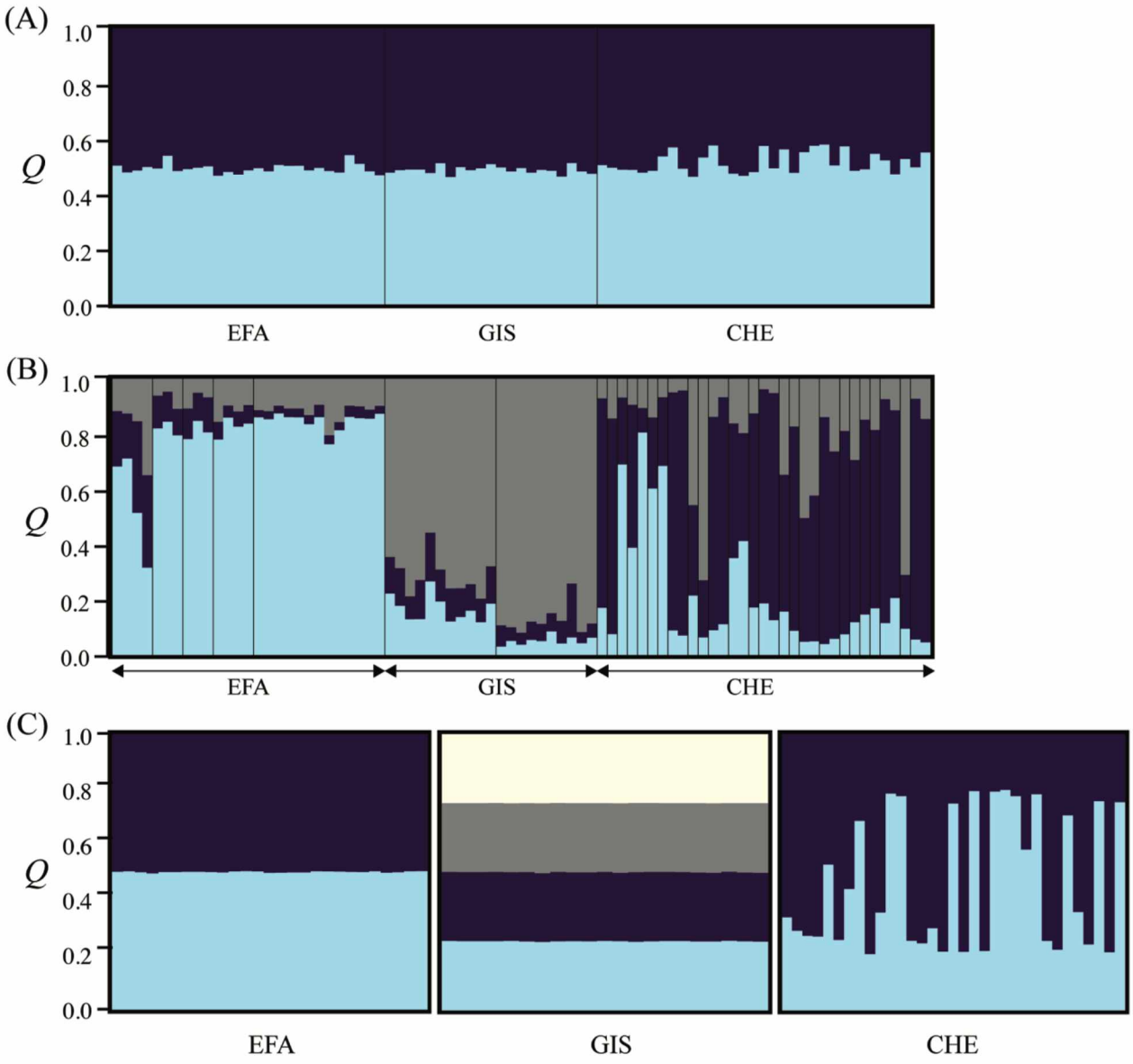

Figure 2.3. STRUCTURE bar plots generated from microsatellite data. (A) global analysis of population structure without prior information on the locality of samples (K = 2); (B) global analysis of population structure with prior information on the locality of samples (LOCPRIOR; K = 3); (C) within-river analyses of population structure without prior information (K = 2, K = 4, and K = 2).

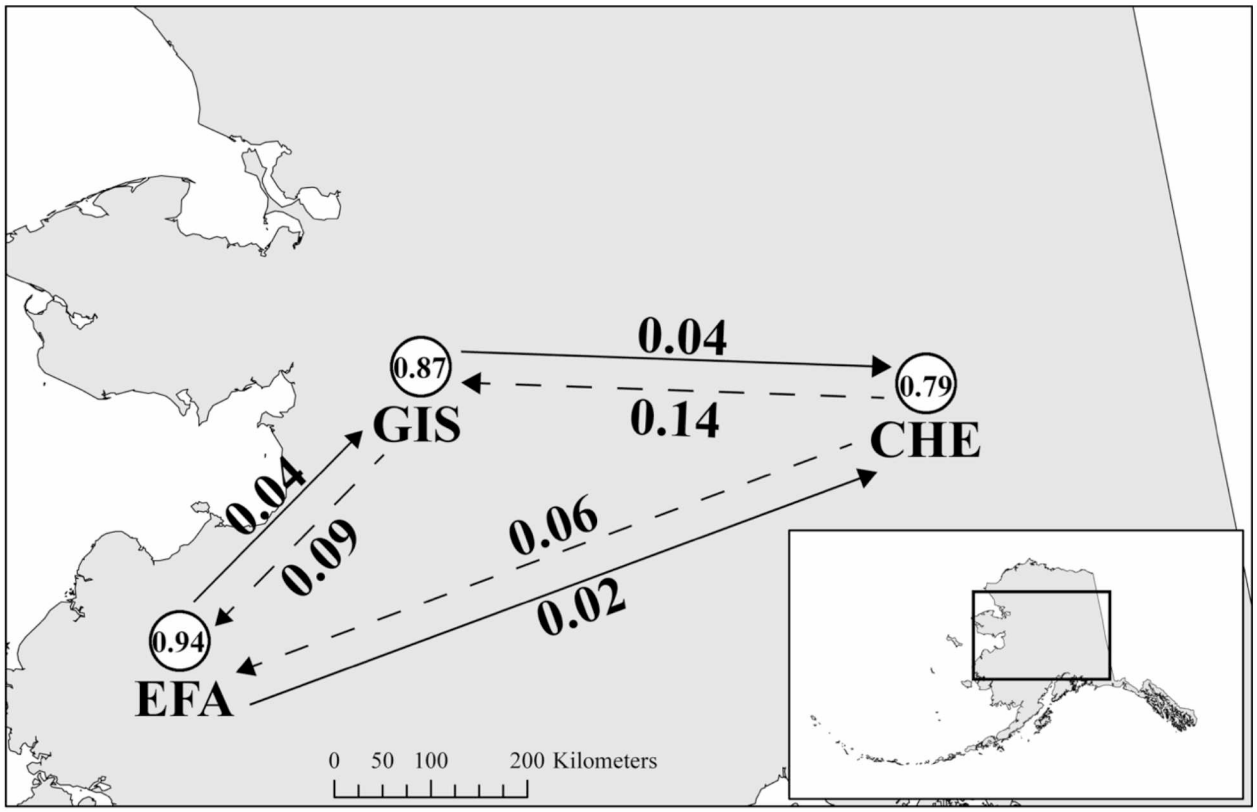

Figure 2.4. Contemporary migration rates (*m*) among populations estimated using BAYESASS. Numbers within circles denote the proportion of non-migrants within populations. Arrows indicate the direction of gene flow and the corresponding *m* values.

Table 2.1. Details for sampled rivers within the Yukon River drainage, Alaska. The number of sampled sites within each river, tissue sample size (N), and collection year is given for each locality.

Abb.	River	Sampled Sites	*N*	Year
EFA	East Fork of the Andreafsky	5	40	2014
GIS	Gisasa	2	40	2014
CHE	Chena	24	40	2011

Table 2.2. Characteristics of eight microsatellite loci selected for this study. Information includes repeat motif, forward and reverse primer sequences, magnesium concentration (Mg^{2+}), and fluorescent label used during PCRs.

Locus	Repeat motif	Primer Sequences 5' - 3'	Mg^{2+}	Fluorescent Label
Lspn019c	$(CA)_5AA(CA)_5$	F: TCTGCCGATATTTGATTCTC§ R: GATCTTTAGTCTTTGCTGTTGT	2.0	HEX
Lspn010-2	$(CA)_5$	F: CAGGACCGGTTCAAATAATCA§ R: TGACCTTTGCTGACAGATGG	1.5	FAM
Lspn050	imperfect (CA)	F: GCTCCGGTTATGAAATGGAA§ R: ATGCATTATATTCGTCCGCC	1.5	FAM
Lspn088	$(CA)_4CG(CA)_6$	F: GGATAATCGTCAGCAGTGTT R: TCCATCTCTCTCGTTACCAT§	2.0	HEX
Lspn044	imperfect (CA)	F: ACGGTTCAGATAATCGTCAC§ R: GACGGTAATTTAATTGCGAA	1.5	HEX
Lspn013	$(CA)_5$	F: GCAGACTTTGCTTTAGGAGA§ R: ATTTGGTATAGCCCTGTGAG	1.5	FAM
Lspn094	imperfect (CA)	F: GGTGTTGACTGAATCGAACT§ R: GTTCTCTAGAGCTGTCGCAC	2.0	FAM
Lspn002-2	$(GA)_9$	F: TCGTGCCAACTCGTCATCTA§ R: GGATACGAACAGCTCCTGCT	1.5	FAM

§M13 tail

Table 2.3. Summary of observed allele frequencies. P values in bold are significant deviations from HWE after Bonferroni correction (P = 0.003). Asterisks indicate the presence of a null allele.

		Sampled Rivers		
Locus	Allele	EFA	GIS	CHE
Lspn019c	150	0.000	0.000	0.136
	152	0.000	0.024	0.182
	158	1.000	0.976	0.682
	P	-	-	**0.000**
	null allele	-	-	*
Lspn010-2	224	0.444	0.357	0.409
	226	0.370	0.524	0.318
	228	0.185	0.119	0.273
	P	0.201	0.463	0.118
	null allele	-	-	-
Lspn050	314	0.037	0.167	0.288
	354	0.111	0.000	0.030
	356	0.852	0.833	0.682
	P	**0.000**	**0.002**	**0.002**
	null allele	*	*	*
Lspn088	163	0.000	0.000	0.015
	169	0.130	0.024	0.045
	181	0.722	0.810	0.545
	183	0.148	0.167	0.364
	185	0.000	0.000	0.015
	187	0.000	0.000	0.015

Table 2.3 cont.

	P	0.646	0.559	0.831
	null allele	-	-	-
Lspn044	218	0.370	0.071	0.318
	220	0.315	0.714	0.409
	226	0.185	0.000	0.000
	228	0.130	0.214	0.273
	P	0.349	0.156	0.348
	null allele	-	-	-
Lspn013	343	0.130	0.214	0.273
	345	0.778	0.714	0.439
	347	0.093	0.071	0.288
	P	1.000	0.804	0.939
	null allele	-	-	-
Lspn094	283	0.111	0.429	0.182
	287	0.889	0.571	0.818
	P	0.267	0.663	1.000
	null allele	-	-	-

Table 2.4. Summary statistics for *Lethenteron* spp. N = number of individuals genotyped at all seven loci, N_A = mean number of alleles, H_E = expected heterozygosity, H_O = observed heterozygosity, and F_{IS} = inbreeding coefficient.

	N	N_A	H_E	H_O	F_{IS}
EFA	27	2.714	0.443	0.389	0.125
GIS	21	2.571	0.379	0.320	0.160
CHE	33	3.286	0.546	0.459	0.162

Table 2.5. Pairwise F_{ST} values for sampled population pairs. F_{ST} values shaded in grey are significant tests of population differentiation after Bonferroni correction (P = 0.0166).

	EFA	GIS	CHE
EFA	-		
GIS	0.080	-	
CHE	0.066	0.081	-

Table 2.6. Comparisons between estimated log-likelihood (lnP(K)) STRUCTURE output and the statistic ΔK evaluated in STRUCTURE HARVESTER over 20 runs for tested values of k. Values in bold indicate the lowest estimated log-likelihood, while values shaded in grey indicate the optimal value of k identified by ΔK.

STANDARD

	LnP(K)		
K	Mean	StDev	ΔK
1	**-943.020**	**0.207**	**-**
2	-1038.465	26.222	1.75
3	-1088.030	95.633	0.159
4	-1152.755	62.480	0.651
5	-1258.180	215.721	0.699
6	-1212.830	162.987	0.045
7	-1174.890	49.730	-

LOCPRIOR

	LnP(K)		
K	Mean	StDev	ΔK
1	-943.125	0.415	-
2	-921.540	7.015	0.928
3	**-893.445**	**4.743**	**17.321**
4	-947.500	37.803	1.125
5	-959.010	26.476	0.104
6	-967.760	35.350	0.350
7	-964.125	34.548	-

EFA

	LnP(K)		
K	Mean	StDev	ΔK
1	**-260.535**	**0.341**	**-**
2	-261.345	0.438	1.996
3	-261.280	0.516	0.087
4	-261.170	0.895	0.078

Table 2.6 cont.

5	-261.130	0.807	-

GIS

	LnP(K)		
K	Mean	StDev	ΔK
1	**-187.970**	**0.264**	**-**
2	-188.380	0.577	0.035
3	-188.770	0.622	0.556
4	-188.820	0.839	0.703
5	-189.460	2.826	-

CHE

	LnP(K)		
K	Mean	StDev	ΔK
1	-428.510	0.412	-
2	**-407.830**	**2.642**	**26.887**
3	-458.175	45.364	0.404
4	-526.865	44.582	0.569
5	-570.180	39.939	-

General Conclusions

The results of this study were two-fold. First, results using a combination of visual observations of intestinal contents and high throughput sequencing reinforced the assumption of *L. camtschaticum* as a flesh-feeding predator with pelagic schooling fishes as their prey in the eastern Bering Sea. The frequency with which diagnostic hard structures and tissue masses were observed within the intestines of *L. camtschaticum* suggested a predatory, flesh-feeding approach. These diagnostic structures and tissues included eggs, fins and fin rays, internal organs, otoliths, scales, and vertebrae. It is likely that *L. camtschaticum* predation on prey fishes causes high mortality rates. The utilization of high-throughput sequencing highlighted the application of 'DNA metabarcoding' to characterize the diet of a marine predator that is difficult to observe under natural conditions. This study was the first to report three taxa (i.e., Cottidae, Gadidae, and Pleuronectidae) within the diets of *L. camtschaticum*. In addition, this method detected rare food items, as a portion of taxa identified in this study were associated with only a few number of individuals. High degrees of interspecies predation among taxa detected in this study proved to be a limitation of using DNA metabarcoding as a dietary technique in a highly connected marine system. Because of the detection sensitivity of high-throughput sequencing, this study was unable to dismiss the possibility of secondary predation that may ultimately affect interpretation of our results. Ultimately, these results not only provided additional insight into the complexity of food web dynamics in the eastern Bering Sea, but improved our understanding of the importance of pelagic schooling fishes as prey for *L. camtschaticum*.

An investigation into the genetic structure of larval *Lethenteron* spp. within the Yukon River drainage suggested moderate levels of genetic structure among examined populations. Hierarchical analyses of genetic clustering without sampling locality priors identified one

ancestral population among all individuals, indicating a panmictic population. Further analyses that examined genetic clustering among individuals within rivers identified two ancestral populations within the Chena River, while no genetic divergence was detected among individuals within the East Fork of the Andreafsky and Gisasa rivers. These results suggest that two diverging populations are present within the Chena River that may be attributed to reduced levels of gene flow between nonparasitic and parasitic forms. Estimates of contemporary migration rates among populations indicated reciprocal migration among sites that may attribute to the levels of admixture identified in this study. Ultimately, these results indicate that the moderate degree of genetic differentiation and reduced levels of gene flow among sample sites may be attributed to life-history variants of *Lethenteron* spp. While this investigative study increased our understanding of the genetic diversity among larval *Lethenteron* spp. within the Yukon River drainage, it also emphasized areas where additional research effort is needed to further understand the degree of genetic divergence and levels of gene flow among nonparasitic and parasitic forms.

This research provided baseline genetic and dietary data on a poorly studied fish species, highlighting the importance and need for additional studies of *Lethenteron* spp. not only in Alaska but throughout their geographic distribution. Recommendations for future research include collecting marine-phase *L. camtschaticum* during different feeding seasons (e.g., winter, spring, etc.) to determine possible seasonality in their diet. Specimens were collected over two years, but collection days were restricted to one feeding season (August – September). Sampling over all seasons would deepen our understanding of *L. camtschaticum* trophic interactions and potential seasonal dietary variability in the eastern Bering Sea. In addition, employing reduced representation sequencing techniques (e.g., RADseq, ddRAD, GBS) to investigate potential

genome-wide divergence between nonparasitic *L. alaskense* and parasitic *L. camtschaticum* is recommended. Because larval *Lethenteron* spp. have no diagnostic morphological characteristics to differentiate between the putative species (Sutton 2017), sampling efforts should target spawning adults when each form can be morphologically distinguished. Reduced representation sequencing provides a method to sample hundreds if not thousands of shared molecular markers among individuals, which is orders of magnitude higher than the small number (i.e., 8) of microsatellites used in this study. Powerful demographic analyses can be conducted with high-throughput sequencing data and then compared to previous conclusions determined using microsatellite genotyping. This is recommended because distribution and abundance estimates of *Lethenteron* spp. within Alaska are limited (ADF&G 2006).

Appendix A. iTru Fusion Primers with custom tags.

Primer name	Primer Sequences : 5' - 3'
iTru_A_12S_V5_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTACTAGAACAGGCTCCTCTAG
iTru_B_12S_V5_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcAACACTAGAACAGGCTCCTCTAG
iTru_C_12S_V5_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTatCGGTTTAGAACAGGCTCCTCTAG
iTru_D_12S_V5_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtcgGTCAATAGAACAGGCTCCTCTAG
iTru_E_12S_V5_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGCGTAGAACAGGCTCCTCTAG
iTru_F_12S_V5_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgCCACATAGAACAGGCTCCTCTAG
iTru_G_12S_V5_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTctGGATGTAGAACAGGCTCCTCTAG
iTru_H_12S_V5_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtgaTTGACTAGAACAGGCTCCTCTAG
iTru_1_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGAATTAGATACCCCACTATGC
iTru_2_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgAGTGGTTAGATACCCCACTATGC
iTru_3_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccACGTCTTAGATACCCCACTATGC
iTru_4_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTttcTCAGCTTAGATACCCCACTATGC
iTru_5_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTAGGTTAGATACCCCACTATGC
iTru_6_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtGCTTATTAGATACCCCACTATGC
iTru_7_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgcGAAGTTTAGATACCCCACTATGC
iTru_8_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaatCCTATTTAGATACCCCACTATGC
iTru_9_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATCTGTTAGATACCCCACTATGC
iTru_10_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgAGACTTTAGATACCCCACTATGC
iTru_11_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcgATTCCTTAGATACCCCACTATGC
iTru_12_12S_V5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtctCAATCTTAGATACCCCACTATGC

Appendix B. Measures of pairwise F_{ST} and exact tests of Arctic lamprey population differentiation were recalculated after one individual from each full-sibling pair identified by COLONY was removed. Adjusted sample sizes for each Yukon River tributary are reported along the diagonal, while pairwise F_{ST} values are reported below diagonal. F_{ST} values shaded in grey are significant after Bonferroni correction (P = 0.0166).

	AND	GIS	CHE
AND	20		
GIS	0.050	16	
CHE	0.054	0.048	26