Genetic Techniques Provide Evidence of Chinook Salmon Feeding on Walleye Pollock Offal

Thaddaeus J. Buser, Nancy D. Davis, Isadora Jiménez-Hidalgo, and Lorenz Hauser

School of Aquatic and Fishery Sciences, University of Washington,
Box 355020, Seattle, WA 98195-5020, USA

Buser, T.J., N.D. Davis, I. Jiménez-Hidalgo, and L. Hauser. 2009. Genetic techniques provide evidence of Chinook salmon feeding on walleye pollock offal. N. Pac. Anadr. Fish Comm. Bull. 5: 225–229.

Abstract: Declining runs of Chinook salmon in western Alaska have focused interest on the ocean condition and food habits of Chinook salmon in the Bering Sea, including potential mortality from bycatch in the pollock fishery. Examination of Chinook salmon stomach contents collected in the eastern Bering Sea by the U.S. North Pacific Groundfish Observer Program (NOAA Fisheries) revealed isolated pieces of skin, bones, and fins (offal) belonging to large-bodied fish which were physically identified as either walleye pollock (*Theragra chalcogramma*) or Pacific cod (*Gadus macrocephalus*). To confirm the species identification of the offal, we matched DNA sequences of these offal samples to known sequences of walleye pollock and Pacific cod. Novel mitochondrial DNA (mtDNA) primers were designed to amplify a 174-base pair (bp)-long section of the cytochrome *c* oxidase subunit I (COI) gene, which was sequenced and compared with sequences downloaded from the GenBank database. Typically, much longer sections (~700 bp) of DNA are used for species identification but due to the state of digestion of the samples, long sequences of DNA were no longer present. The specific design of our primers, however, allowed us to make positive identification and differentiation of walleye pollock and Pacific cod. Of the 15 offal samples, nine yielded usable sequences, all of which were positively identified as walleye pollock. Our results clearly demonstrate the utility of a short COI sequence for species identification of Chinook salmon stomach contents that might otherwise be unidentifiable due to either the state of digestion, or because the salmon consumed isolated body parts (offal) rather than whole fish. These results suggest that walleye pollock offal supplements the diet of Chinook salmon during winter.

Keywords: Chinook salmon, walleye pollock, offal, genetics, food habits

INTRODUCTION

Understanding the ecology of a species is a fundamental component in developing conservation and management plans. Recent declines of Chinook salmon (*Oncorhynchus tshawytscha*) returns to western Alaska have prompted restrictions on commercial fishing (Hayes et al. 2008). Changes in abundance can often be attributed to variability in conditions during the marine life history (Botsford et al. 2002), yet there are large gaps in our understanding of the feeding ecology of Chinook salmon during their time at sea. Food habits studies are basic to gaining insights into salmon marine life history (Beamish and Mahnken 2001; Armstrong et al. 2008).

Stomach content analyses from Chinook salmon gathered in summer and fall in the North Pacific, Gulf of Alaska, and the Bering Sea indicate they feed primarily on fish and gonatid squids, although euphausiids, crab larvae, and other invertebrates can also be found in Chinook salmon diets (e.g., Volkov et al. 1995; Kaeriyama et al. 2004; Davis et al. 2005, 2009a; Volkov et al. 2007; Weitkamp and Sturdevant 2008). However, little is known about the food habits of Chinook salmon at sea during winter, primarily because of the difficulty in conducting winter surveys.

Our samples were obtained from stomach samples collected by U.S. groundfish observers during the winter walleye pollock (*Theragra chalcogramma*) fishery in the eastern Bering Sea. Analysis of these samples revealed the presence of skin, flesh, fins, and bone (Davis et al. 2009b). Visual examination of skin pigmentation, fin and bone morphology, flesh consistency, and myotome structure revealed that among the possible prey species of Chinook salmon, walleye pollock and Pacific cod (*Gadus macrocephalus*) were the only reasonable possibilities. However, due to the condition of the tissues, further identification to the species level was not possible for all samples. Instead, we used genetic techniques to make positive species identifications.

Genetic identification is possible by comparing DNA sequences from unknown samples to those of known taxa. This approach can become quite costly if one must secure, extract, and sequence DNA from all possible candidate taxa. Although DNA sequences are available on public databases (GenBank), they often stem from different genes in different taxa, thus preventing direct comparison for species identifi-

All correspondence should be addressed to T. Buser.
e-mail: tbuser@u.washington.edu

cation. In a recent standardization effort, Hebert et al. (2003) proposed that a single gene sequence was sufficient to differentiate between the majority of species on the planet and suggested using the mitochondrial DNA (mtDNA) gene, cytochrome *c* oxidase subunit I (COI). The COI gene has been termed the "barcode of life" and sequences from different species have been compiled in order to provide a database by which sequences from new or unknown species can be compared (Ratnasingham and Hebert 2007). This effort has been extended to fish (Ward et al. 2005), and COI sequences are now available for a wide variety of species on GenBank (www.ncbi.nlm.nih.gov) and on the Fish Barcode of Life database (www.fishbol.org; Ward et al. 2009).

Genetic tools have been used to determine the identification of prey species after partial digestion by amplifying relatively small (162 bp and 327 bp) sections of mtDNA (Parsons et al. 2005). Short sequences have also helped to identify highly degraded DNA samples using the barcoding gene, COI (Hajibabaei et al. 2006). In this study, our objective was to identify the fish species of offal found in the stomach contents of Chinook salmon. To achieve this objective, we developed novel primers for gene amplification of short DNA fragments, and compared those sequences to reference data from a public database (GenBank) and to positive control DNA samples from known species.

MATERIALS AND METHODS

Laboratory Analysis

Chinook salmon stomach samples were obtained from the winter pollock fishery during January to March, 2007 in the eastern Bering Sea and examined by Davis et al. (2009b). Offal refers to fish body parts (e.g., head, tail, spine, skin) that are discarded after processing. When Chinook salmon stomach contents were identified as fish offal they were collected and frozen at -20°C. In total, 15 samples were selected for genetic analysis (Table 1). Samples were thawed and divided into subsamples, which were then soaked in a 2% bleach solution to reduce contamination. To account for differing degrees of digestion present in each sample and the effect of bleach on our target DNA, we used two different soak times per sample. One subsample was soaked for 1 min and a second subsample for 3 min. After bleach soaking, each subsample was rinsed twice in distilled water and then preserved in a 95% ethanol solution according to the protocol outlined in Mitchell et al. (2007). This procedure reduced DNA contamination from Chinook salmon and other prey items by destroying the DNA in the external layers of the tissue.

A sample of walleye pollock positive control DNA was extracted from fin tissue (collected in the northeast Bering Sea) using the same protocol as that for the offal samples. Two Pacific cod positive control DNA samples were obtained from the study by Cunningham et al. (2009).

Offal DNA was extracted with a Qiagen DNeasy® micro-extraction kit following the manufacturer's protocol (Qiagen Inc., Valencia, CA). Novel primers were designed that amplified DNA from walleye pollock and Pacific cod in order to reduce the likelihood of contamination from other prey sources and from the salmon itself.

Walleye pollock, Pacific cod, and Atlantic cod (*G. morhua*) sequences were downloaded from GenBank and aligned in BioEdit (Ibis Biosciences, Carlsbad, CA). Primers were designed using Primer 3 (Rozen and Skaletsky 2000). The forward (5' – TTGGGATGGACGTAGACACA – 3') and reverse (5' – AGCCCCCAACTGTAAAGAGG – 3') primers amplified a 174-bp-long fragment of the mtDNA COI gene to avoid problems with amplification of large fragments from degraded DNA.

The reaction mixture comprised 20 ng of DNA, 1 X reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, and 0.5 U DNA Taq polymerase. The polymerase chain reaction (PCR) conditions were as follows: preheating at 94°C for two min; 40 cycles of 94°C denaturation for 30 sec, 55°C annealing for 90 sec, and 72°C extension for 90 sec; and a final 72°C extension for three min. The PCR products were examined on 1% agarose gels and directly sequenced in both directions with PCR primers on a high-throughput capillary sequencer at the University of Washington High-Throughput Genomics Unit (Dept. of Genome Science, University of Washington).

Table 1. Offal samples chosen for genetic testing, including month of collection and the tissue type analyzed. All samples were collected in the eastern Bering Sea during January to March, 2007. Offal, in this study, refers to fish body parts (e.g. head, tail, spine, skin) that are discarded after processing.

Sample No.	Collection Month	Tissue Type
8-20	February	Fin
20-9A	February	Bone and Muscle
28-11	February	Skin
43-28D	March	Fin
48-13A	March	Skin
50-7A	March	Fin
51-8A	March	Skin
52-2C	January	Skin
52-3A	January	Bone and Muscle
52-4	January	Skin
52-5A	January	Skin
59-13B	February	Muscle
59-16	February	Muscle
60-19	February	Muscle
84-16	March	Muscle

Data Analysis

The sequence fluorograms were aligned using Sequencher™ (Gene Codes Inc. Ann Arbor, MI). Low-quality base calls at the end of sequences were removed, and sequences were checked for consistency between forward and reverse sequences. Samples with low and/or confounding peaks in the sequence chromatogram were rejected. In addition to the fish offal sequence data, known sequences of walleye pollock (accession numbers AF081699 and DQ174028) and Pacific cod (accession number AF081697) retrieved from the GenBank database were included in the analysis as reference points to compare with our sequences. An Atlantic cod sequence (accession number DQ173997) was also downloaded from GenBank and included in our analysis as a genetic outgroup.

Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007) using the neighbor-joining method (Saitou and Nei 1987) with Kimura two-parameter distances (Kimura 1980) including all three codon positions. In order to evaluate the reliability of the tree, bootstrap values were generated with 1000 iterations and only those values above 50 were reported and indicated at the nodes.

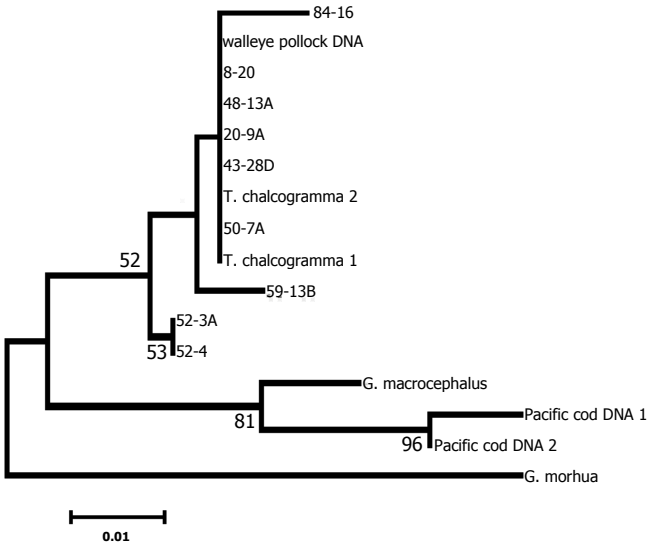

Fig. 1. Neighbor-joining tree showing the evolutionary relationship of nine offal sample haplotypes, three known DNA sample haplotypes and four known haplotypes (from GenBank). The tree is labeled as follows: walleye pollock = *T. chalcogramma* 1 (accession number AF081699) and *T. chalcogramma* 2 (accession number DQ174028); Pacific cod = *G. macrocephalus* (accession number AF081697); and Atlantic cod = *G. morhua* (accession number DQ173997). Offal sample haplotypes are labeled by sample numbers as in Table 2. Walleye pollock positive control DNA sample haplotype is labeled walleye pollock DNA (GenBank accession number GQ302982). Pacific cod positive control DNA sample haplotypes are labeled as Pacific cod DNA 1 (GenBank accession number GQ302983) and Pacific cod DNA 2 (GenBank accession number GQ302984). The tree was generated with Kimura two-parameter distances. Bootstrap values indicated at nodes were generated with 1000 replicates and only values above 50 are reported.

RESULTS

All samples of positive control walleye pollock and Pacific cod DNA amplified with our primers and produced usable haplotypes. Additionally, nine of the 15 offal samples yielded usable haplotypes. Samples 28-11, 51-8A, 52-2C, 52-5A, 59-16, and 60-19 were amplified but rejected due to low and/or confounding peaks. Of those six rejected haplotypes, four were from skin samples (Table 1). However,

Table 2. Biological characteristics of Chinook salmon stomachs containing fish offal identified using genetic techniques. Sequences are available on the GenBank database and can be accessed using the GenBank accession number of each sample. Chinook salmon stomach samples collected by U.S. groundfish observers in the walleye pollock fishery operating in the eastern Bering Sea during January to March, 2007. Chinook salmon age determined from scales, where the number before (after) the period is the number of winters spent in fresh water (ocean). The X indicates that age could not be determined. Chinook salmon biological data and percentage of stomach content weight comprising fish offal from Davis et al. (2009b).

Fish Offal Sample No.	GenBank Accession No.	Chinook Salmon					Fish Offal	
		Sex	Maturity	Fork Length (cm)	Body Weight (kg)	Age	Species Identification	% of Stomach Content Weight
8-20	GQ302973	female	immature	44	0.94	1.2	pollock	100
20-9A	GQ302974	male	immature	52	1.67	1.2	pollock	100
43-28D	GQ302975	male	maturing	77	5.41	1.4	pollock	100
48-13A	GQ302976	female	maturing	82	5.92	1.4	pollock	100
50-7A	GQ302977	female	maturing	62	2.66	1.3	pollock	100
52-3A	GQ302978	female	immature	77	5.74	1.4	pollock	100
52-4	GQ302979	female	immature	47	1.6	X.X	pollock	54
59-13B	GQ302980	male	immature	59	2.34	1.2	pollock	95
84-16	GQ302981	female	immature	67	3.75	1.3	pollock	45

there was no obvious difference in DNA quantity among tissue types. No correlation was detected between the amount of DNA extracted and the duration of soak time in the bleach solution. The length of usable DNA sequences ranged from 108 bp to 152 bp but all sequences were trimmed to a length of 108 bp. Sequences were uploaded to the GenBank database (see Table 2 for offal sample accession numbers; see caption of Fig. 1 for positive control accession numbers). There was a total of 13 variable sites, four of which were diagnostic for differentiating walleye pollock from Pacific cod. Although our primers were designed to amplify DNA from both species, the neighbor-joining tree generated from the sequence data proved to be sufficient for differentiating the two (Fig. 1).

All offal haplotypes showed a closer relationship to the control sample of walleye pollock DNA and the walleye pollock sequences from GenBank than they did to the control samples of Pacific cod DNA and the Pacific cod sequence from GenBank, indicating the offal samples were in fact pieces of walleye pollock (Fig. 1). This relationship was supported by a bootstrap value of 52%. The Pacific cod positive controls were more closely related to each other (96% bootstrap value) and to the Pacific cod sequence (81% bootstrap value) than they were to any other sequence. All pollock samples were more closely related to Pacific cod than they were to the sequence of Atlantic cod from GenBank.

DISCUSSION

Nine of the fish offal samples collected from Chinook salmon stomach contents were identified as originating from walleye pollock (Table 2) due to their genetic similarities with known walleye pollock DNA and sequence data (Fig. 1). Those samples that did amplify but were rejected because of low and/or confounding peaks were likely contaminated by other contents of the stomach from which they were gathered. Four out of six of the rejected sample haplotypes were from skin tissue (Table 1), which was the thinnest tissue type. It is possible that the contamination from other stomach contents completely permeated the tissue. It is also possible that the exclusion of these samples may have introduced a degree of bias in our results, however, because the primary aim of this study was to demonstrate the presence of pollock offal, and not to quantify it, this possible bias is unlikely to have affected our results.

Our results clearly demonstrated the utility of a short COI sequence for species identification of Chinook salmon stomach contents. The specificity of our primer design was possible because morphological characters allowed the identification of offal as either cod or pollock. Further species identification was possible by sequencing the DNA and comparing results with known sequences. Much longer sequences are more typical for identifying species (Ward et al. 2009). The relatively short (108 bp) sequences are likely responsible for the low bootstrap values in the neighbor-joining tree (i.e., 52% for the grouping of all pollock together), but they were sufficient to distinguish two species and, importantly, they could be obtained from degraded DNA (Hajibabaei et al. 2006).

This technique may prove invaluable for identifying fish prey from stomach contents that might be unidentifiable due to the state of digestion, or when isolated body parts are consumed rather than whole fish. Future DNA research will focus on the development of techniques for identification of invertebrate salmon prey, such as cephalopods and cnidarians, which can be difficult to identify in the absence of fresh or intact specimens.

Our results suggest fish offal derived from pollock might supplement the diet of Chinook salmon during winter. The scavenging of commercially discarded fish parts has been well documented in seabirds (Bertellotti 2000; Garthe and Scherp 2003). However, consumption of offal by Chinook salmon has yet to be investigated for the possible changes in feeding strategy and behavior it may elicit in the affected populations. Currently, only direct mortality of Chinook salmon in the pollock fishery has been well documented (Berger 2008). Future research will be needed in order to determine the positive or negative consequences for Chinook salmon survival through the winter and the magnitude of the direct and indirect effects of offal consumption on the total population.

ACKNOWLEDGEMENTS

We thank Jerry Berger, Kerim Aydin, and the U.S. Groundfish Observer Program, Alaska Fisheries Science Center (AFSC), National Oceanic and Atmospheric Administration (NOAA) for their help in obtaining stomach samples. We also thank Michael Canino (AFSC, NOAA) for providing walleye pollock fin tissue. Participation of N. Davis was funded by award #NA04NMF4380162 from NOAA, U.S. Department of Commerce, administered by the Alaska Department of Fish and Game (ADFG) for the Arctic-Yukon-Kuskokwim Sustainable Salmon Initiative (http://www.aykssi.org/). The statements, findings, conclusions, and recommendations are those of the authors and do not necessarily reflect the views of NOAA, the U.S. Dept. of Comm., or ADFG.

REFERENCES

Armstrong, J.L., K.W. Myers, D.A. Beauchamp, N.D. Davis, R.V. Walker, J.L. Boldt, J.J. Piccolo, L.J. Haldorson, and J.H. Moss. 2008. Interannual and spatial feeding patterns of hatchery and wild juvenile pink salmon in the Gulf of Alaska in years of low and high survival. Trans. Am. Fish. Soc. 137: 1299–1316.

Beamish, R.J., and C. Mahnken. 2001. A critical size and period hypothesis to explain natural regulation of salmon abundance and the linkage to climate and climate

change. Prog. Oceanogr. 49: 423–437.

Berger, J.D. 2008. Incidental catches of salmonids by U.S. groundfish fisheries in the Bering Sea/Aleutian Islands and the Gulf of Alaska, 1990–2008. N. Pac. Anadr. Fish Comm. Doc. 1104. 10 pp. (Available at www.npafc.org).

Bertellotti, M. 2000. Utilisation [sic] of fishery waste by Kelp Gulls attending coastal trawl and longline vessels in northern Patagonia, Argentina. Ornis Fenn. 77: 105–115.

Botsford, L.W., C.A. Lawrence, M.F. Hill, A. Hastings, and K.S. McCann. 2002. Dynamic response of California Current populations to environmental variability. Am. Fish. Soc. Symp. 32: 215–226.

Cunningham, K.M., M.F. Canino, I.B. Spies, and L. Hauser. 2009. Genetic isolation by distance and localized fjord population structure in Pacific cod (*Gadus macrocephalus*): limited effective dispersal in the northeastern Pacific Ocean. Can. J. Fish. Aquat. Sci. 66: 153–166.

Davis, N.D., M. Fukuwaka, J.L. Armstrong, and K.W. Myers. 2005. Salmon food habits studies in the Bering Sea, 1960 to present. N. Pac. Anadr. Fish Comm. Tech. Rep. 6: 24–28. (Available at www.npafc.org).

Davis, N.D., A.V. Volkov, A.Ya. Efimkin, N.A. Kuznetsova, J.L. Armstrong, and O. Sakai. 2009a. Review of BASIS salmon food habits studies. N. Pac. Anadr. Fish Comm. Bull. 5: 197–208. (Available at www.npafc.org).

Davis, N.D., K.W. Myers, and W.J. Fournier. 2009b. Winter food habits of Chinook salmon in the eastern Bering Sea. N. Pac. Anadr. Fish Comm. Bull. 5: 243–253. (Available at www.npafc.org).

Garthe, S., and B. Scherp. 2003. Utilization of discards and offal from commercial fisheries by seabirds in the Baltic Sea. ICES J. Mar. Sci. 60: 980–989.

Hajibabaei, M., M.A. Smith, D.H. Janzen, J.J. Rodriguez, J.B. Whitfield, and P.N. Hebert. 2006. A minimalist barcode can identify a specimen whose DNA is degraded. Mol. Ecol. 6: 959–964.

Hayes, S.J., F.J. Bue, B.M. Borba, K.R. Boeck, H.C. Carrol, L. Boeck, E.J. Newland, K.J. Clark, and W.H. Busher. 2008. Annual management report Yukon and northern areas 2002–2004. Alaska Department of Fish and Game Fishery Management Report No. 08-36, Anchorage.

Hebert, P.D.N., A. Cywinska, S.L. Ball, and J.R. DeWaard. 2003. Biological identifications through DNA barcodes. Proc. Roy. Soc. Lond. Series B 270: 313–321.

Kaeriyama, M., M. Nakamura, R. Edpalina, J. Bower, H. Yamaguchi, R. Walker, and K. Myers. 2004. Change in feeding ecology and trophic dynamics of Pacific salmon (*Oncorhynchus* spp.) in the central Gulf of Alaska in relation to climate events. Fish. Oceanogr. 13: 197–207.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111–120.

Mitchell, D., P. McAllister, K. Stick, and L. Hauser. 2007. Sperm contamination in archived and contemporary herring samples. Mol. Ecol. Res. 8: 50–55.

Parsons, K.M., S.B. Pierney, S.J. Middlemass, P.S. Hammond, and J.D. Armstrong. 2005. DNA-based identification of salmonid prey species in seal feces. J. Zool. 266: 275–281.

Ratnasingham, S., and P.D.N. Hebert. 2007. BOLD: The Barcode of Life Data System. Mol. Ecol. Notes 7: 355–364. (Available at www.barcodinglife.org).

Rozen, S., and H. Skaletsky. 2000. Primer 3 on the WWW for general users and for biologist programmers. *In* Bioinformatics methods and protocols: methods in molecular biology. *Edited by* S. Krawetz, and S. Misener. Humana Press, Totowa, NJ. pp. 365–386.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596–1599.

Volkov, A.F., V.I. Chuchukalo, and A.Ya. Efimkin. 1995. Feeding of Chinook and coho salmon in the northwestern Pacific Ocean. N. Pac. Anadr. Fish Comm. Doc. 124. 12 pp. (Available at www.npafc.org).

Volkov, A.F., A.Ya. Efimkin, and N.A. Kuznetsova. 2007. Results of the BASIS studies on Pacific salmon feeding habits in 2002–2006. Izv. TINRO 151: 365–402. (In Russian with English abstract).

Ward, R.D., T.S. Zemlak, B.H. Innes, P.R. Last, and P.D.N. Hebert. 2005. DNA barcoding Australia's fish species. Phil. Trans. Roy. Soc. B 360: 1847–1857.

Ward, R.D., R. Hanner, and P.D.N. Hebert. 2009. The campaign to DNA barcode all fishes, FISH-BOL. J. Fish Biol. 74: 329–356.

Weitkamp, L.A., and M.V. Sturdevant. 2008. Food habits and marine survival of juvenile Chinook and coho salmon from marine waters of Southeast Alaska. Fish. Oceanogr. 17: 380–395.