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Papers from
" Pollock Stock Structure and Identification Workshop"

Yokohama, Japan
7-9 September, 1999

Edited by
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Introduction

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This book contains papers presented at the "Pollock Stock Structure and Identification Workshop", Yokohama, September 7-9, 1999. This workshop was proposed at the annual meeting of the Convention for the Conservation of Pollock resources in the Central Bering Sea, and was coordinated by Dr. R. Marasco of Alaska Fisheries Science Center (AFSC), and Dr. T. Kobayashi of Hokkaido National Fisheries Research Institute (HNF).

The workshop was held at National Research Institute of Fisheries Science (NRIFS) in Yokohama. Over 50 representatives from Korea, Poland, Russia, United States, and Japan took part in the workshop to review and discuss about stock structure of walleye pollock and other marine species in the North Pacific region. The workshop was opened by Dr. Hatanaka (Director of NRIFS), and Dr. Low (AFSC) presented a keynote address on the general information about walleye pollock stock in the North Pacific region. Overall, 15 oral presentations contributed to this workshop. All contributors were invited to submit papers for the HNF Technical Report.

All of the papers were submitted by the summer 2000. However, publication of this book was delayed than our initial expectation for some confusion in the editorial work. In some papers, information may become older. But it is not the author's responsibility, and it should be attributed to the delay of editorial work.

As editor, I would like to thank all those who contributed to the proceeding of this workshop, and I appreciate their patience. And I believe that this book involve precious information for our future stock study of walleye Pollock and other marine species. I hope this book will contribute to the future stock identification study, and will lead to the future international cooperative research activities.

The Workshop and this publication were supported financially by the Fisheries Agency of Japan.

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Pollock Stock Structure and Identification Workshop

September 7-9, 1999
National Research Institute of Fisheries Science
Yokohama, Japan

Agenda

September 7

Opening remarks:	H. Hatanaka. (NRIFS)
Keynote Address:	Loh-Lee Low(NMFS)
	Joint paper by U.S. Japan, Russia, China, Korea, and Poland

Review of Pollock Stock Studies

Japan:	A. Nishimura & T. Yanagimoto (HNF)
Korea:	S. Kim (NFRDI)
Poland:	J. Janusz (SFI)
Russia:	A. Glubokov (VNIRO)
United States:	S. Grant (NMFS)

September 8

Stock Identification Technology

Morphology:	J. Ianelli (NMFS)
Otolith Chemistry:	K. Severin (UAF)
Otolith Chemistry:	S. Thorrold (ODU)
Biochemical Genetics:	J. Seeb (ADFG)
Biochemical Genetics:	S. Chow (NRIFSF)
Biochemical Genetics:	M. Sekino (NFRFE)
Mitochondrial DNA:	O. Katugin (TINRO)
Microsatellite Analysis:	P. O'Reilly & M. Canino (UW)
Microsatellite Analysis:	H. Abe & M. Goto (ICR)

September 9

Workshop to Develop Research Action Plan

(Co-charis: Marasco and Kobayashi)

- Data needs/ Field Sampling/ Sample Processing
- Training and Cooperative Research Opportunities
- Action Plan Development
- Concluding remarks
- Closing remarks

Pollock Stock Structure and Identification Workshop Rapporteur's Report

September 7-9, 1999
National Research Institute of Fisheries Science
Yokohama, Japan

Dr. H. Hatanaka (NRIFS) opened the conference with comments on the need for developing active research programs for stock structure problems facing Bering Sea pollock.

Dr. L. Low (AFSC) presented a review of pollock resources and issues for Bering Sea pollock fisheries management. This was followed by a review of studies occurring in each of the participating countries.

REVIEW OF POLLOCK STOCK STRUCTURE STUDIES

Chair Doug Eggers

In this session, each national section provided a review of pollock stock structure studies.

Japanese studies

Akira Nishimura provided the review of stock structure studies based on phenotypic characteristics. These studies found differences in morphology and in otolith characteristics between areas (western Bering Sea, Eastern Bering Sea, Aleutian Basin, and Northern Japan Sea). Recent studies have focused on otolith characteristics of pollock in northern eastern Bering Sea, southern eastern Bering Sea, and Chukchi Sea and suggest a strong relation between Aleutian Basin and southern eastern Bering Sea. However, biological characteristics have changed over time with declining abundance and increasing growth rates. Takashi Yanagimoto provided the review of genetic studies PCR-RFLP, RAPD-PCR, and AFLP. Early studies of pollock using the allozyme marker SOD suggest some isolation of eastern and western Bering Pollock and suggest these populations are isolated. In future studies, it is important to collect significant numbers of adult pollock from various spawning grounds.

Korean Studies

Soon-Song Kim provided the review of studies based on morphology and genetics. Meristic counts of pollock population through the western north Pacific and Bering Sea were examined. Significant differences were found due mostly to differences between two most southern populations (eastern Korea and Pacific side of Hokkaido) from the more northerly populations. Genetic characteristics of the populations were also examined.

Polish studies

Jerzy Janusz reviewed the age and length characteristics of fishery catches in the northern Bering Sea area, 1995 -1998. Age and length frequency distribution, as well as von Bertalanffy growth curves were presented.

Russian Studies

Alexander Glubokov reviewed the current status of walleye pollock in the northwestern Bering Sea. These populations were monitored using fishery catches and number of surveys. In the northwestern Bering Sea area, the 1990's were marked by decreasing abundance of pollock. Areas of pollock concentration clearly delineated. Stock characteristics of populations in the eastern Koryak shelf, western Koryak shelf and Navarin area were examined using morphological, physiological, and genetic indicators. Differences in physiological morphological indicators were found between the areas.

United States studies

Stewart Grant provided a review of genetic methods for defining stocks of marine fishes. Population segments may be defined with phenetic variability that reflects demographic or life-history responses to spatial or temporal changes. Population segments can also be distinguished by molecular genetics that measure inherited differences among populations as a result of geographic isolation. The phenetic and genetic methods reflect population changes that occur on vastly different time scales and may or may not coincide. There are limits to the molecular genetic methods in populations, such as walleye pollock, with high gene flow between population segments. The rigorous analysis of rapidly evolving genes, such as microsatellite loci and elemental analysis may hold the best promise for identifying population units on spatial and temporal used by managers. Other genetic and non-genetic methods should also play a role in stock identification.

STOCK IDENTIFICATION TECHNOLOGY, PART I.

Chair A. Nishimura

J. Ianelli (AFSC) presented problems in stock structure study using morphometric methods. Length-age data from catches suggested significant differences of pollock growth, and the possibility of the existence of multiple stocks was suggested in the EBS. He then presented alternative hypotheses based on size-related movement and showed that the same differences can occur even from a single stock. The distribution of length-frequency data by area over time showed patterns favoring a single stock. This was due to following the geographic changes in the abundance of strong year-classes.

K. Severin (Univ. of Alaska Fairbanks) and S. Thorrold (Old Dominion Univ.) made presentations about methods used for analyses of otolith chemistry. Technical and/or methodological discussions were made, and a pilot study of juvenile pollock was presented. EPMA is thought to be a useful method for stock identification study for the Bering Sea pollock. Thorrold introduced new method of otolith chemistry with using LA-ICP-MS. Otolith elemental information is thought to be useful for the natural tag of the stocks. The factors affecting otolith elemental characteristics were discussed with specially considering environmental and natal homing mechanism of the fish.

J. Seeb (Alaska Dept. Fish. and Game) introduced his genetic study conducted for the area from Gulf of Alaska to Bogoslof area in the Bering Sea. In his presentation, SOD analysis was suggested to be the useful method for the pollock stock study in the Alaskan waters. S. Chow (NRIFSF) introduced his genetic study for the tuna and swordfish in the global ocean scale. His genetic technique is thought to be fundamental to identify stock structure for those fish group.

STOCK IDENTIFICATION TECHNOLOGY, PART II.

Chair V.I. Radchenko

Four presentations were made during this session. Key points are presented below.

1. M. Sekino. Molecular cloning of microsatellite DNA from marine organisms.

(a) A new technique for microsatellite DNA cloning using sonication has been worked out. It resulted in construction of highly enriched genomic library.
(b) This method has been used to construct enriched libraries for Japanese rockfish and bastard halibut.
(c) This method could have some potential applications to walleye pollock stock structure studies, though it seems to be applicable primarily for isolating microsatellites with low frequency in genomes.

2. O. Katugin. Biochemical genetic variation and population structure study in walleye pollock (*Theragra chalcogramma*) from the Bering Sea.

(a) There exists an overall subdivision of the Bering Sea pollock gene pool into two parts: eastern and western, which had been confirmed by other studies with application of different techniques.
(b) Polymorphic genetic markers, which were used in this study, had not been applied to walleye pollock by other researchers. Nevertheless, the results appeared to be consistent with those derived from other sources of information, including proteins and mtDNA.

3. P. O'Reilly. High-resolution analysis of walleye pollock stock structure using microsatellite DNA markers.

(a) Highly polymorphic microsatellite loci were first used in the analysis of stock differentiation in walleye pollock.
(b) Applicability of the technique has been tested on a large geographic scale from Japan to Puget Sound. Although levels of population structuring were low, there was rather clear differentiation of geographically separated population units.
(c) It was shown that temporal stability of intra-specific differences even at a single locus could indicate existence of stock structure in a migratory fish species.

4. H. Abe. The application of microsatellite DNA for determining population structure of Minke whales.

(a) Population genetic analysis has been successfully applied to Minke whales which have several biologically units.
(b) Several genetic methods, including microsatellites and mtDNA, refined existing knowledge of Minke whale stock differentiation between the Sea of Japan and the western North Pacific.
(c) It was shown that samples for stock structure analysis of highly migratory species should be collected from the breeding areas.

General conclusions

Most presentations made at the afternoon session focused on stock structure analyses techniques and approaches rather than directed pollock research. Applicability of these techniques to the problem of stock structure analysis of pollock will take time, and the power of each method should be tested.

There is a need to optimize pollock stock structure analysis and stock identification methodologies. Calibration is needed between different methods. This implies that initially tests should be performed to determine if available methods can—and to what extent—discriminate between different populations or stocks.

DEVELOPMENT OF AN ACTION PLAN

Co-chairs: R. Marasco and T. Kobayashi

Dr. Marasco suggested that the following questions be used as guideposts in the development of an action plan:

- What methods should be used for stock structure studies?
- What data are required for these methods?
- How should samples be collected?
- How should they be analyzed?

The co-chairs opened discussions on these topics and the development of a summary statement for consideration by the S and T at its November meeting.

Stock ID methods

The following four methods were proposed as the core methodologies for stock ID work. These do not preclude developing and/or using other methods.

Genetic techniques

- Sequence analyses of mtDNA
- Allozymes
- Microsatellite loci analysis

Phenetic methods

- Otolith chemistry

Other methods were identified as follows

- Chromosomal DNA (rapids)
- Meristics and morphometrics
- Parasite fauna
- Tag-recapture programs
- Gonad-somatic studies
- Organ/tissue studies
- Analysis of non-coding DNA (Intron)
- MHC
- Sine

The necessity was discussed of having a central source for lab processing of collected samples. It was agreed that there should be sharing of raw data and coordination of data analyses.

Sampling locations

The following areas were identified for sampling locations:

- Bering Sea
  - Bogoslof Island
  - Eastern Bering Sea NW & SE of Pribilofs
  - Aleutian-Commander Islands
  - Donut hole area
  - Northern Bering sea
  - W. Bering Sea (Karaginski area)
- Other N. Pacific areas (for comparison)
  - Sea of Okhotsk, Peter the Great Bay, Sakhalin Coast, Hokkaido coast, Shelikof, Prince William Sound, and Korean Peninsula.

Timing of sampling

The sampling efforts should be collected over multiple years on spawning grounds for evaluating stationarity in stock ID methods. Samples also should be collected over different periods of the spawning events.

It was recognized that the cooperation of respective neighboring countries is necessary to help in sample collections.

Sample collect

It was recognized that consideration of sample size is extremely important for stock structure studies. It was concluded that sampling protocols be developed within each lab. Drs. Seeb & Grant agreed to develop and circulate draft protocols in time for the November S&T meeting. The information will be made available on the Internet.

Comments on sampling materials were as follows

- Tissue samples from spawning pollock
  - Heart, muscles & gonads
  - By size groups?
- Otolith samples of adults and juveniles

For otoliths, scales, & other hard structures

- Preservation in alcohol?
- Dry in envelopes?
- Single versus multiple samples?

For Tissue Samples

- Frozen —what temp and handling procedures?
- Tissue samples dissolved or preserved in preservative solutions?

Coordinate of data and samples collection

- From ships of opportunity?
- From special sampling schemes by research vessels?
- During spawning conditions only?
- Collection by Russia & U.S. in respective EEZs
- Help from fishing nations
- Designate coordinators by country

Pollock Stocks in the North Pacific and Importance of Stock Structure and Identification Research

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WON-SEOK YANG[4], JERZY JANUSZ[5], AND TANG QISHENG[6]

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2 Russian Federal Research Institute of Fisheries and Oceanography, Moscow, Russia
3 Hokkaido National Fisheries Research Institute, Kushiro, Japan
4 National Fisheries Research and Development Institute, Pusan, Korea
5 Sea Fisheries Institute, Gdynia, Poland
6 Yellow Sea Fisheries Research Institute, Qindao, China

ABSTRACT: The walleye pollock (*Theragra chalcogramma*) resource is presently the largest single species resource in the North Pacific Ocean. Its distribution stretches across the wide expanse of the Pacific Ocean from the western shores of Canada, up through the Gulf of Alaska, into the entire Bering Sea, across the Aleutian Basin to the shores of Siberia, down and across the Kamchatka Peninsula, into the Sea of Okhotsk, the waters of northen Japan, and along the Korean Peninsula. The resource is important to the region from two important aspects—to the ecology of the marine ecosystem and to support a fishing industry. The pollock stocks have a combined biomass of 15 to 30 million metric tons (mmt). They support a massive fishing industry that takes 3-7 million metric tons of fish per year for the past 30 years. Pollock occupies significant roles throughout its life stages in the food web of the ocean and sustains life for all tropic levels of the ecosystem. The paper will review the importance of pollock stock structure and identification for the Bering Sea to the Convention for the Conservation of Pollock resources in the Central Bering Sea. The pollock resource in the Bering Sea is composed of at least 5 major stocks which are transboundary and inter-mixes at various stages of their life history. Knowledge on stock structure, migration, and identification have generally been poor and has not been improved for almost 20 years. New research must be planned to advance their knowledge.

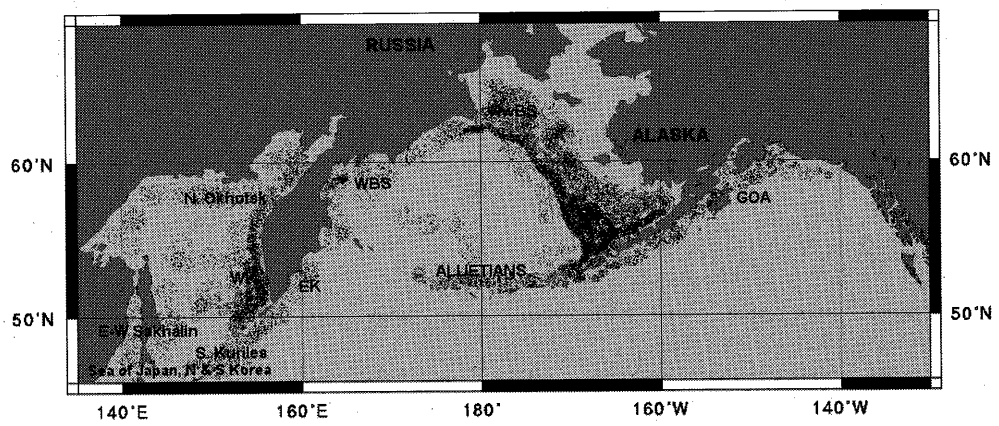

Fig. 1. General distribution of pollock in the North Pacific.

INTRODUCTION

The walleye pollock (*Theragra chalcogramma*) resource is presently the largest single species fisheries resource in the North Pacific Ocean. Its distribution stretches across the wide expanse of the Pacific Ocean from the western shores of Canada, up through the Gulf of Alaska, into the entire Bering Sea, across the Aleutian Basin to the shores of Siberia, down and across the Kamchatka Peninsula, into the Sea of Okhotsk, the waters of northen Japan, and along the Korean Peninsula (Fig. 1). The resource is important to the North Pacific Ocean and its adjacent seas from two important aspects -- to the ecology of the marine ecosystem and to support a fishing industry.

The pollock stocks from the various regions have a combined biomass of 15 - 30 mmt. They support a massive fishing industry that takes 3-7 mmt of fish per year for the past 30 years. Even though pollock fisheries are harvested locally in certain Asian areas in historic times, they are essentially modern day fisheries developed some 30 years ago after the Japanese fishing industry pioneered large-scale at-sea fishing and surimi-processing capabilities. The resource now supports important fishing industries from the Gulf of Alaska to the Korean Peninsula.

The individual fish itself is not particularly special. Its marketable size generally ranges from 25-60 cm in length and weighs 0.3 - 1.4 kg. As a group, however, the species plays an especially significant role in the fisheries and ecology of the North Pacific Ocean. It is the most abundant species in most parts of the region. It occupies significant roles throughout its life stages in the food web of the ocean and sustains life for all trophic levels in the ecosystem. This paper will review the importance of the pollock resource to countries that border the North Pacific Ocean and its adjacent seas and to other nations that have historically fished pollock. It will touch on the importance of pollock stock structure and their identification to the Convention for the Conservation of Pollock resources in the Central Bering Sea. This Convention has only been in force since 1995 to conserve and manage pollock stocks in the Central Bering Sea for the Parties of the Convention—The People's Republic of China, Japan, Republic of Korea, the Republic of Poland, the Russian Federation, and the United States.

MAJOR FISHING AREAS

The major fishing areas may be identified from historical catch statistics (Fig. 2) as:

(1) the Gulf of Alaska, including Shelikof Straits,
(2) the eastern Bering Sea — virtually all areas from the shelf to the continental slope regions,
(3) the Navarin Basin of the northern Bering Sea,
(4) the Olyotorski area of the western Bering Sea,
(5) the Aleutian Islands region,
(6) the central Bering Sea outside of the U.S. and Russian 200-mile exclusive economic zones, otherwise known as the "Donut Hole area",
(7) waters off Kamchatka and the island chain between northern Japan and the southern tip of Kamchatka,
(8) the Sea of Okhotsk,
(9) northern Japan, and
(10) the Korean Peninsula.

The two main centers of abundance are the Sea of Okhotsk and the eastern Bering Sea.

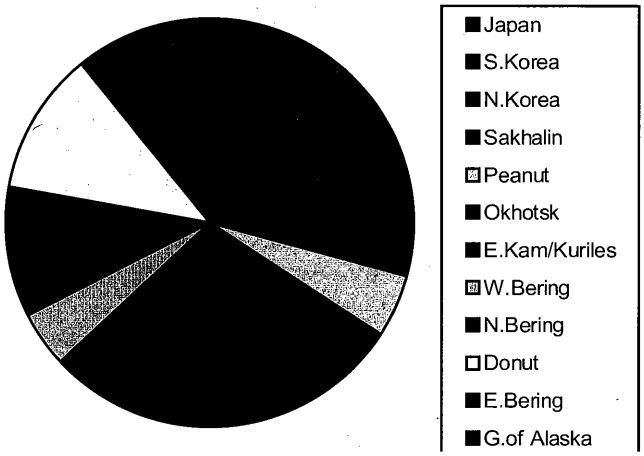

Fig. 2. Percentage distribution of average (1981-1995) catch of pollock by twelve North Pacific areas.

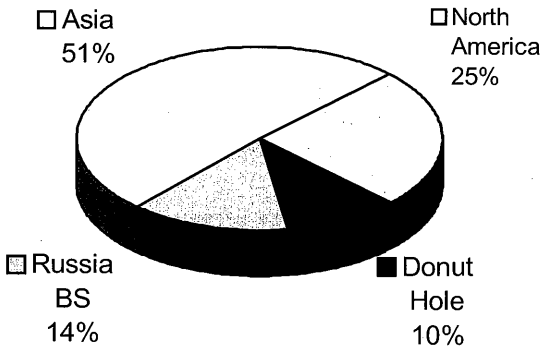

Fig. 3. Percentage distribution of average (1981-95) catch of pollock by four major North Pacific areas.

CATCH HISTORY AND ECONOMIC SIGNIFICANCE

The 15-year average catch for 1981-1995 was 2.9 mmt from the Asia region and 2.8 mmt from the North American region (that includes Russian catches from the Bering Sea) (Table 1, Fig. 3). At $500/t of product value (Joe Terry, personal communications), the recent average annual catch of 5.7 mmt would be worth $2.85 billion. With a simple assumed 2-times multiplier effect for processing, post-processing, transhipment, and marketing, the pollock fisheries would easily account for almost $6 billion worth of economic activity for the region.

Table 1. Walleye pollock catch (in metric tons) in the North Pacific Region

Year	Gulf of Alaska	Eastern Bering Sea	Bogoslof	Donut Hole	Western Bering Sea (a)	Navarin Basin (b)	Eastern North Pacific	Asiatic North Pacific ©	Total
1981	139,200	973,500			279,000	900,000		3,799,800	6,091,500
1982	168,700	956,000			356,000	804,000		3,259,700	5,544,400
1983	215,600	981,500			353,000	722,000		2,817,200	5,089,300
1984	306,700	1,092,100		181,200	376,000	503,000		3,617,400	6,076,400
1985	284,900	1,139,700		363,400	278,000	488,000		3,396,200	5,950,200
1986	93,600	1,142,000		1,039,800	271,000	570,000		3,393,100	6,509,500
1987	69,500	859,400	377,000	1,326,300	300,000	463,000		3,083,700	6,478,900
1988	65,600	1,228,700	87,800	1,396,700	324,000	852,000		2,778,200	6,733,000
1989	78,200	1,229,600	36,100	1,447,600	309,000	684,000		2,692,000	6,476,500
1990	90,500	1,455,200	151,700	917,400	383,000	232,000		2,720,500	5,950,300
1991	107,500	1,217,300	264,800	293,400	309,000	178,000		2,757,100	5,127,100
1992	93,900	1,164,400	200	10,700	281,000	316,000		2,860,100	4,726,300
1993	108,200	1,326,600	900	1,200	363,000	389,000		2,053,000	4,241,900
1994	111,200	1,363,500	600	0	210,000	178,000		1,861,900	3,725,200
1995	67,000	1,262,800	300	0	86,000	320,000		2,072,000	3,808,100
Average	133,353	1,159,487	102,156	581,475	298,533	506,600	2,781,604	2,877,460	5,659,064

(a) Russian EEZ, west of 176 degrees E Longitude
(b) Russian EEZ, east of 176 degrees E Longitude
© Catch data from the Asiatic North Pacific was compiled by Vidar Wespestad as part of the records of the Science Group meetings of the Convention for the Conservation of Pollock Resources in the Central Bering Sea.

In social terms, however, pollock fisheries employ, nurture, and sustain the livelihood of untold numbers of people and their communities. Pollock products provide food to supplement the nutrition of millions of people. The true importance of pollock to the people of our countries cannot be overstated.

The region of particular attention of this Workshop is, however, the Bering Sea region, since it encompasses the "Donut Hole area" of the Convention (Fig 4). The 15-year (1981-95) Bering Sea average catch totaled 2.6 mmt (Table 1, Fig. 5) and were distributed as follows: Eastern Bering Sea/Aleutians (44%), Bogoslof Island area (4%), western Bering Sea (11%), Navarin Basin (19%), and the Donut Hole area (22%). An average of 750,000 t of pollock were taken during the 9-year period (1985-93) when the Donut pollock fisheries were actively fishing (Fig. 6). Since 1993, however, the Donut Hole pollock fishery has been closed as a result of agreements made by the Parties to the Donut Convention. It is the hope of the Parties that the Donut Hole pollock stock would recover soon to support a sizeable fishery into the near future.

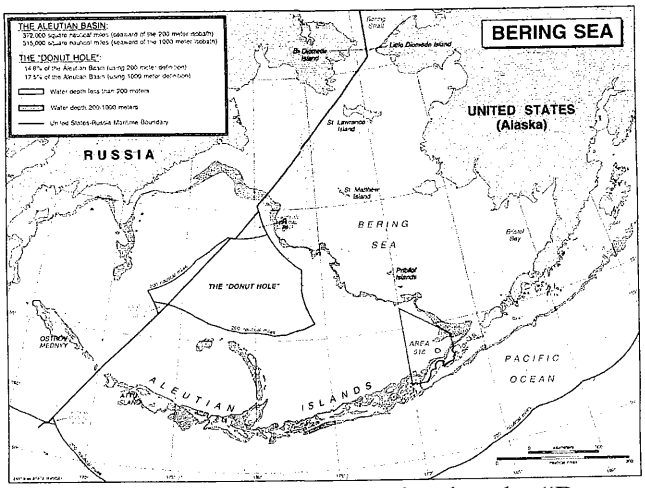

Fig. 4. Map of the Bering Sea showing the "Donut Hole" area.

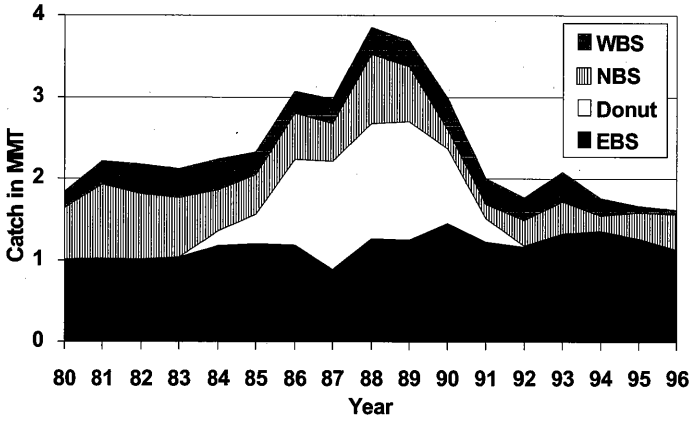

Fig. 5. Catch trend of pollock by region in the Bering Sea, 1980-96.

Table 2. Walleye pollock catch (in metric tons) in the Donut Hole Area of the Bering Sea.

Year	China	Japan	Korea	Poland	Russia	U.S.	Donut Total
1985	1,600	163,510	82,440	115,870	0	0	363,420
1986	3,200	705,620	155,720	163,250	12,000	0	1,039,790
1987	16,530	803,550	241,870	230,320	34,000	0	1,326,270
1988	18,420	749,980	268,600	298,710	61,000	0	1,396,710
1989	31,140	654,910	342,300	268,570	150,700	0	1,447,620
1990	27,830	417,020	244,270	223,450	4,800	0	917,370
1991	16,650	140,450	77,960	54,870	3,470	0	293,400
1992	3,970	2,700	4,050	0	0	0	10,720
1993	0	100	480	600	0	0	1,180
1994	0	0	0	0	0	0	0
1995	0	0	0	0	0	0	0
1996	0	0	0	0	0	0	0
1997	0	0	0	0	0	0	0
Average (1985-93)	13,082	404,204	157,521	150,627	29,552	0	755,164

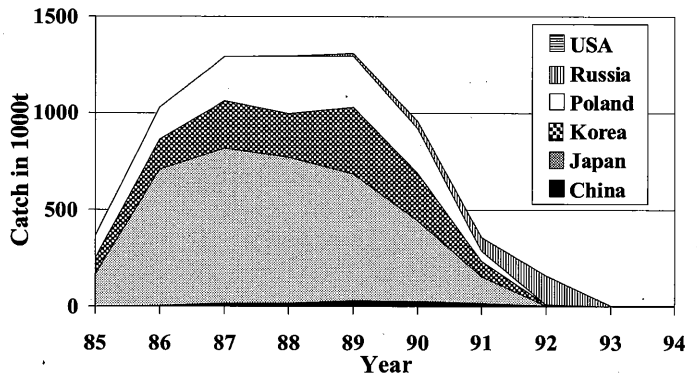

Fig. 6. Catch trend of pollock by country in the Donut Hole area, 1985-94.

SIGNIFICANCE OF POLLOCK IN ECOSYSTEM

Pollock is a focal component of the North Pacific marine food-web. It is dominant in numbers and biomass and contributes important ecological linkages to many marine organisms. Pollock feeds on a broad variety of prey species (Fig. 7) and in turn is a major food source for other fishes, marine mammals, seabirds, and man (Smith *et. al.* 1984).

At larval stages, pollock feeds on calanoid copepod eggs and nauplii and euphausiid larvae. Juvenile and young adult pollock feed on fish larvae, mysids, *Thysanoessa inermis*, *Parathemisto pacifica*, and pandalid shrimps. Feeding on plentiful zooplankton and young fishes, pollock is a major trophic level concentrator of energy to pass on to higher trophic levels. Pollock are in turn eaten by the following major groups (Fig. 8):

Groundfish Species — pollock feeds upon its own species. This is cannibalism of the smaller fish by the larger fish. Many flatfish species in the Bering Sea shelf depends heavily on pollock for food — Greenland turbot, arrowtooth flounder, Pacific cod, Pacific halibut, flathead sole, yellowfin sole, and rock sole. Other less abundance species that consume pollock are: Alaska skate, sablefish, Pacific sandlance, various sculpins, and atka mackerel.

Marine mammals — The major species of marine mammals that prey on pollock are: northern fur seal, northern sea lions, spotted seals, and ribbon seals (Kajimura and Fowler 1984). Other predators of pollock are harbor seal, fin whale, Dalls porpoise, minke whales, and humpback whales.

Seabirds — The main piscivorous birds that consume pollock are: common murre, thick-billed murre, black-legged kittiwakes, red-legged kittiwakes tufted murre, and horned puffin.

Thus, pollock, from egg stage to adult forms, is intricately linked ecologically and play important roles throughout its life history in the ecology of the North Pacific Ocean. It sustains life and biological productivity of the marine ecosystem.

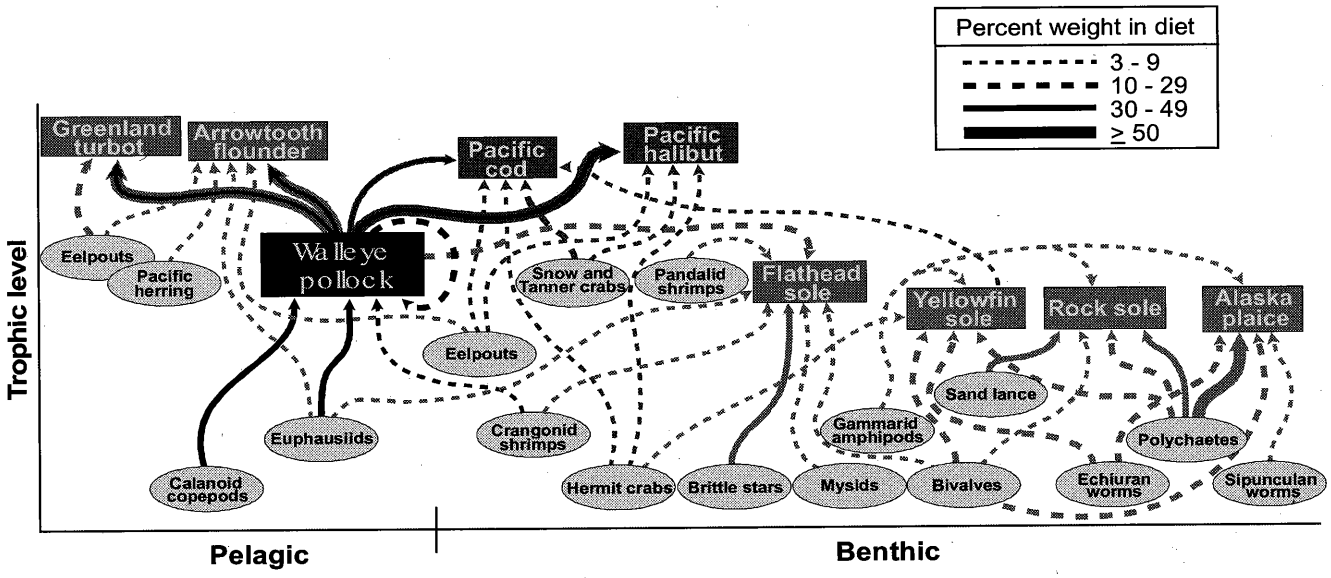

Fig. 7. Diagram showing the role of pollock in the marine food web of the Bering Sea.

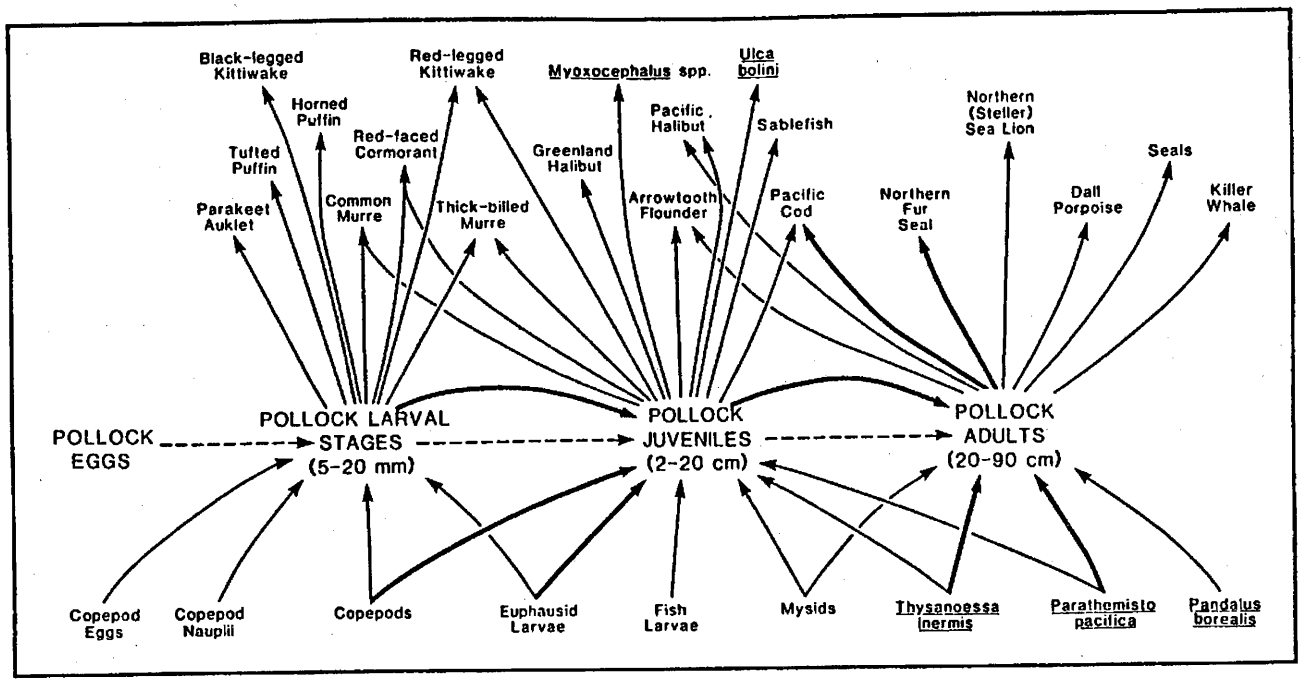

Fig. 8. Diagram showing the role of pollock life stages in the marine food web of the Bering Sea.

POPULATION SIZE

Resource abundance has undergone huge changes in its short recent history. Pollock was not known to be a particularly abundant species in the Bering Sea until the mid 1960's; at least not as a dominant species as it is now. The first comprehensive survey of the Bering Sea that was conducted by Russian scientists from 1958-1961 provided little evidence that pollock resources were dominant in the Bering Sea in the 1950's

Table 3. Estimated biomass of walleye pollock (in metric tons) in Bering Sea region.

Year	Bogoslof Area (a)	Eastern Bering Sea (b)	Western Bering Sea (c)
1964		1,037,000	
1965		1,227,000	
1966		1,096,000	
1967		2,095,000	
1968		2,510,000	
1969		3,810,000	
1970		5,083,000	1,130,000
1971		5,813,000	980,000
1972		5,648,000	830,000
1973		3,922,000	1,390,000
1974		2,342,000	1,640,000
1975		3,014,000	2,850,000
1976		3,008,000	3,180,000
1977		2,894,000	3,380,000
1978		2,867,000	5,060,000
1979		2,933,000	7,990,000
1980		4,294,000	7,450,000
1981		8,569,000	4,480,000
1982		9,778,000	8,920,000
1983		10,705,000	5,760,000
1984		10,179,000	3,830,000
1985		11,919,000	7,090,000
1986		10,913,000	3,270,000
1987		11,116,000	4,970,000
1988	2,400,000	10,274,000	4,720,000
1989	2,130,000	8,546,000	4,340,000
1990		6,659,000	3,810,000
1991	1,290,000	5,180,000	
1992	940,000	8,294,000	
1993	640,000	10,279,000	
1994	490,000	8,917,000	
1995	1,100,000	8,680,000	
1996	680,000	6,811,000	
1997	390,000	5,307,000	
1998	490,000	5,133,000	

(a) From Honkalehto and Williamson (1998). Hydroacoustic survey biomass by NOAA R/V *Miller Freeman*

(b) From Ianelli et al. 1998. Estimated Age 3+ Biomass from Age-structured model.

(c) From Balykin 1996. Estimated commercial stock biomass from Age-structured model.

as they are in the 1980s-90s even though Serobaba (1970) reported that pollock concentrations were "distributed irregularly over a vast range" and noted that fisheries were promising.

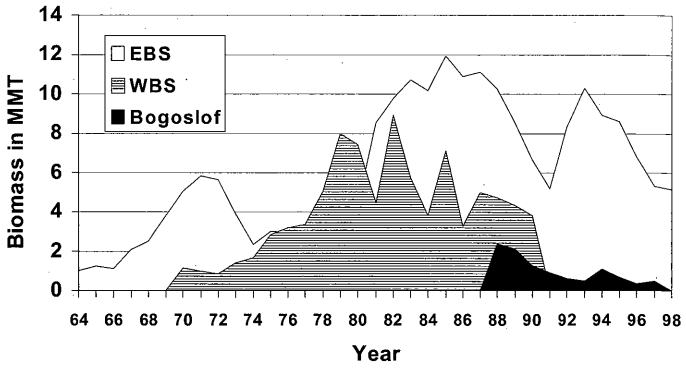

Fig. 9. Estimated pollock biomass of the pollock stock in the eastern Bering Sea, western Bering Sea, and Bogoslof area.

Flatfish populations, particularly yellowfin sole, were the dominant species group in the eastern Bering Sea then (Moiseev P.A. 1963). Herring was also noted to be commercially abundant (Dudnik, Y.I and E.A. Usol'tsev, 1964) and rockfish species were harvested commercially be Russians.

How has pollock abundance changed in various areas of the Bering Sea? Information of such changes are available only for three areas: eastern Bering Sea, western Bering Sea (that includes the Navarin Basin), and Bogoslof area.

Eastern Bering Sea Stock — The latest series of biomass estimates has been made by Ianelli (1998) and shown in Fig. 9 (Table 3). Adult biomass (Age 3+) showed 3 production cycles: 1964-74, 1974-91, and 1991-98. Peak abundance was 5.8 mmt in 1971 for the 1st cycle, 11.9 mmt in 1985 for the 2nd cycle, and 10.3 million t in 1993 for the latest cycle. We are near the bottom of the 3rd cycle and the 1998 abundance is back to the 1971 level at about 5.1 million t.

Western Bering Sea Stocks — Balykin (1996) reported a time series of estimated biomass for the entire western Bering Sea which, presumably, included the Olyotorski and Navarin stocks. The time series was for 1970-90 only (Table 3, Fig. 9). It appears that there was one major abundance cycle that peaked at 8.9 mmt in 1983. Since Balykin's estimates were made, VNIRO has taken the lead in estimating biomass of the Navarin Basin stock by using data from trawl and hydroacoustic surveys. Preliminary estimates reported by VNIRO (by Dr. Kotenev) indicated that total biomass of the Navarin Basin/Northern Bering Sea stock within the Russian EEZ ranged from 1.5 to 2.5 mmt in recent years (1995-1998).

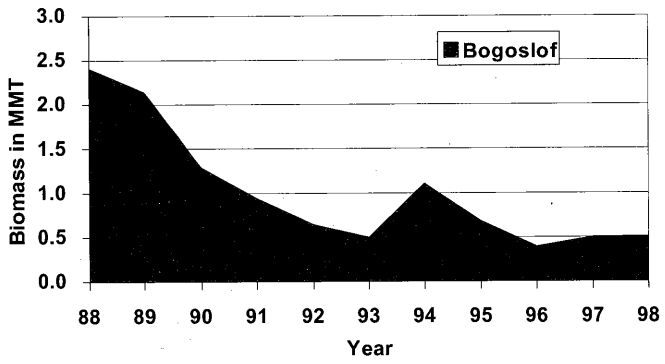

Fig. 10. Estimated pollock biomass in Bogoslof area.

Bogoslof/Aleutian Basin Stock — Hydroacoustic surveys conducted by the U.S. research vessel, *Miller Freeman*, have been used to estimate the spawning biomass of pollock in the Bogoslof area (Honkalehto and Williamson, 1998). This area is used as a reference area and its spawning pollock biomass is used as a proxy of the pollock biomass for the entire Aleutian Basin. The *Miller Freeman* estimates indicated that biomass has decreased substantially since the 2.4 mmt estimated 1988 (Fig 10, Table 3). A recent peak biomass of 1.1 mmt was estimated in 1995 but abundance has again declined to about 0.49 mmt in 1998. In 1999, the *Miller Freeman* did not conduct its Bogoslof survey. Instead, the Japanese R/V *Kaiyo maru*, took over the survey and the preliminary estimate for 1999 is similar to the R/V *Miller Freeman* estimate in 1998.

What does variations and cyclic abundance of pollock mean for the Bering Sea? To begin with, they affect fisheries. Total allowable catches of pollock fisheries are set according to the level of pollock biomass estimated from year to year. In addition to the direct impact on pollock TACs, wide variations in pollock abundance must have significant ecological impacts to other living marine resources in the Bering Sea ecosystem.

STOCK STRUCTURE AND MIX

Knowledge of pollock stock structure and mixing is still largely incomplete for most pollock stocks in the North Pacific region. For the Bering Sea, the definition of a stock and its structure can still be debated, although the major features of stock structures are generally observed by the scientists. For practical management purposes, a stock has generally been defined as a definable management unit. This definition has generally been used by the Parties of the Donut Convention. Biologically, however, a stock would be defined as a genetically distinguishable self sustaining biological unit. Presumable, a self sustaining biological unit can be traced down to an aggregated spawning unit. Such a fine distinction, however, may not be easy to define as pollock may spawn over a large geographical space and time period. Thus, the biological definition of a pollock stock may be defined to its "Ecological significant unit" that may include several spawning aggregations in a broad geographical area. Defined as such, one composite figure of stock mixing is shown in Fig. 11.

Five or more major stocks may be identified:

(i) A large eastern Bering Sea shelf stock that largely spawns over a broad geographical space and time in the U.S. EEZ of the EBS shelf. In reality, spawning concentrations are numerous and discretely distributed throughout the Southeastern Bering Sea, particularly around the Pribilof Islands and extending southwards and northwards of Pribilof Islands along the edge of the continental shelf and slope. Is this one massive spawning concentration of one stock with different time of spawning or spawning by different discrete stocks? The answer to the number of stocks would depend on how one defines a stock. For management purposes of stock utilization, the U.S. has considered the eastern Bering Sea pollock stock as one manageable unit stock that are made up of many ecologically significant spawning units.

(ii) The U.S. has also considered another pollock stock in the Aleutian region where spawning of various pollock schools could be located among the Aleutian chain of islands.

(iii) To the northern Bering Sea-Navarin Basin, Russian scientists have reported several spawning units. They have also reported the possibility that some pollock units may spawn under ice cover. Just like in the case of the eastern Bering Sea stock, the northern Bering Sea area can be considered to have one manageable stock unit.

(iv) On the western side of the Bering Sea, there is another manageable pollock stock that spawns in the Cape Olyutorsk-Bay of Karaginsky area (Balykin 1996).

(v) Then finally, there is the Donut Hole area. Where does the fish come from? Is it a discrete unit or is it made up of fish from the neighboring areas? These are questions yet to be adequately answered.

Throughout the negotiations that led up to the agreement for the Convention on the Conservation of pollock resources in the central Bering Sea, the principal arguments of the Parties was that the Donut area is made up of the Aleutian Basin stock which in turn is an independent stock that is separate from those stocks found in U.S. and Russian waters. The primary spawning area of the Aleutian Basin stock is believed to be the Bogoslof Island area that is located within the U.S. EEZ. There is no question now that the Bogoslof Island area is an important spawning area where 0.4 - 2.4 mmt tons of pollock have been measured to come to spawn each February-March period since 1988 (Table 3). What is less known is the interrelationships of the progeny of these Bogoslof spawners with those pollock that spawns on the southeastern Bering Sea shelf-slope areas.

Where do the progeny of the Bogoslof spawners drift and migrate as they develop in life stages? Based upon the oceanography and water circulation of the Bogoslof area and the SE Bering Sea, they probably intermix with the progeny of spawners from the SE Bering Sea. Since the biomass of the SE Bering Sea pollock resource is many times larger than that in the Bogoslof area, the eastern Bering Sea shelf and slopes would still be dominated by progeny from the SE Bering Sea. As the progeny of the Bogoslof spawners develop into juvenile and adult life, how do they migrate back into the Aleutian Basin, if at all? Did some of the pollock found in the Donut Hole area during past fishing seasons come from the western Bering Sea and from the Navarin Basin as well? These and other questions of stock identification and mixing are still waiting to be answered.

STOCK IDENTIFICATION RESEARCH

Since this is a Workshop of the Parties of the Donut Convention that specifically focuses attention on the central Bering Sea and its immediate neighboring areas, we should review some needs of pollock stock structure and stock identification research as they relate to:

— stock abundance
— user needs (of Korea, Japan, China, Poland, Russia, U.S)
— transboundary interactions and confounding of life stages
— interception, sharing, and mitigation
— Ecosystem health and trophic linkages

Pollock forms the foundation of large scale trawl fisheries in the Bering Sea. As such, the stocks must be healthy and abundant to sustain such commercial fishing enterprises. The resource in the Aleutian Basin has already been too low to allow any fishing in the Donut Hole area since 1993. Fishermen from many countries — Japan, Korea, China, Poland, and Russia — eagerly awaits the rebuilding of the Aleutians Basin pollock resources so that fishing activities can resume.

The pollock stock in the western Bering Sea has already been reduced to such low levels that they no longer sustain large catches. The stocks in the northern Bering Sea, the eastern Bering Sea, and the Aleutian Islands region, while still abundant, are on declining trends. The U.S. is beginning to experience lower catch quotas in the eastern Bering Sea. This and other declining stocks should be rebuilt.

Pollock resources that originate in various spawning areas of the Bering Sea undertake migrations. A general representation of such migration was reviewed by Balykin (1996). Bogoslof area eastern Bering Sea origin fish have been postulated to migrate into the northern Bering Sea and possibly into the Aleutian Basin-Donut Hole area as adults to intermix with stocks of other origin. Likewise, western Bering Sea-Navarin Basin origin fish have also been postulated to migrate into the Aleutian Basin-Donut Hole area and into northerly parts of the U.S. EEZ.

The issues are further complicated by differential migration at the various stages of the pollock life history. There is a general lack of knowledge for quantifying the degree of stock mix and how fisheries in any specific area would affect the health and abundance of stocks in another area at a later point in time. This is why stock identification research would be useful! There is a need to understand the differential impact of fishing on mixed stocks, and at different life history stages, so that proper management measures can be implemented for the various stocks and areas. Eventually, the issue of shared equitable utilization and coordinated management of the various pollock stocks will have to be worked out among the Parties. This applies to the Parties of the Donut Hole Convention and especially so for the United States and Russia that have large pollock resources and fisheries in their respective EEZs.

Beyond direct fishing activities, the health of the pollock resources is important to the balance of the Bering Sea ecosystem. Pollock plays crucial roles throughout all its life history stages on the dynamics and stability of the marine ecosystem. The health of pollock resources have been postulated to affect the welfare of many species of seabirds, marine mammals (especially sea lions), and untold numbers of fish and invertebrate species. But are the issues that simple? We think not. We have rather rudimentary knowledge of how all the marine organisms interact and affect each other.

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Review of Pollock Stock Structure Studies in the Japanese Institutes

1. Phenotypic Characteristic and Otolith Application Studies

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Summary: Walleye pollock (*Theragra chalcogramma*) is an important commercial target species for the Japanese groundfish fisheries in the North Pacific. Japanese commercial vessels operated pollock fisheries throughout the Bering Sea to the western North Pacific, and Okhotsk Sea and Japan Sea around northern Japan. The stock structure of pollock has been a major topic of investigation in the Japanese Institutes. Here, I summarize the pollock stock structure studies in Japan, with focusing their phenotypic characteristic and otolith studies.

POLLOCK STOCK STUDIES BEFORE 1980

Pollock stock structures studies in the Japanese institutes can be divided into two phases. There was a large pollock fishery in broad area in the North Pacific before 1980. Knowledge on the characteristics of the pollock stocks from the broad area was very important to manage those resources. Before 1980, the most of the attentions were focused to the stock in the waters adjacent of the Hokkaido, northern part of Japan. In this phase, pollock stocks in the Okhotsk Sea and Bering Sea were also used as for the comparative data. During this phase, some of the phenotype characteristics were studied, and tagging studies were also conducted in the broad area around Japan. The basic knowledge about pollock stock structure was obtained during this phase. Even in nowadays, pollock stock management around northern Japan is conducted on the basis of the knowledge from this phase.

Ogata (1959) and Iwata and Hamai (1972) studied the geographical variation of the vertebral counts around Japan. In these studies, significant difference was observed between stocks, and the existence of the sub-population or local form was suggested. Koyachi and Hashimoto (1977) also studied variation of the meristic characters of pollock from the adjacent waters to Japan, around Kamchatka and the Bering Sea. Pectoral and dorsal fin rays, gill rakers and the vertebrae were examined. Vertebral counts appeared especially useful for identifying sub-populations. The eastern Bering Sea (EBS) sub-population was recognized but at the same time the existence of the western Bering Sea (WBS) sub-population was pointed out for the future problems.

Hashimoto and Koyachi (1977) studied the geographical variation of relative growth of pollock. Using allometric and morphometric criteria, there appears to be 7 sub-population in the waters adjacent to Japan. Other sub-populations are suggested to be distributed in the waters Kamchatka, EBS, GOA and Canada.

The primary object of Ishida (1954) was to observe the otolith features for age determination. In this study, considerable differences were observed in the growth and the otolith-somatic relationship between areas. Kyushin *et al.*. (1961) observed the two types of otolith from western Pacific, and Ishida (1954, 1957) observed the geographical difference in growth.

Tagging studies were also conducted by scientists in Hokkaido area (Yoshida,1982). These results show us very interesting information about pollock stock structure. The results indicate broad movement of individuals across the areas. However, seasons of mark or recapture are not identified here, and these results do not indicate whether these movements are part of a larger seasonal migration pattern.

POLLOCK STOCK STUDIES AFTER 1980

After mid-1980s, Japanese trawl vessels lose their fishing ground in the eastern Bering Sea, and they concentrated their fishing effort in the international waters (Donut Hole) of the Bering Sea, outside of the US and Russian EEZs. This area is entirely in deep waters of the Aleutian Basin, and the bottom depth is generally deeper than 2000 m. Walleye pollock distributed around 250-500 m deep below the surface. Mid-water trawl fishery has been developed rapidly in this phase, and the international water was the last fishing ground in the Bering Sea for the Japanese Trawler. In order to establish the fishery management in this area and the related area, the knowledge about the stock structure was very important.

In this second phase (1980-1990s), research effort

was focused to the relationship between Aleutian Basin pelagic pollock and eastern/western shelf demersal pollock. In the Bering Sea area, Japanese institutes frequently conducted Echo Integration Mid-water Trawl (EIMWT) survey to obtain the information about the distribution and abundance, and to know the biological characteristics of pelagic pollock. With using samples from these surveys, some phenotypic and biochemical characteristics were studied.

Morphological difference

Morphological difference was studied using Truss Network Measurement method (Wians, 1984). Samples were collected from EBS, WBS, Aleutian Basin and Northern Japan Sea in the spawning season (Fig. 1).

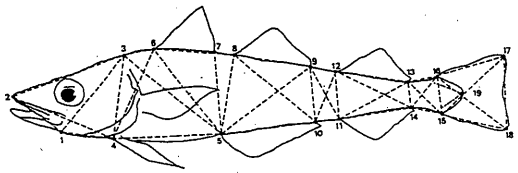

Fig. 1. Location of 19 landmarks for a truss network data set (Nitta and Nishimura, 1991).

	Predicted (%)			
	G1	G2	G3	G4
G1	96.4	0	1.8	1.8
G2	2	93.9	4.1	0
G3	2.7	1.2	94.3	1.8
G4	5.2	0	0	94.8

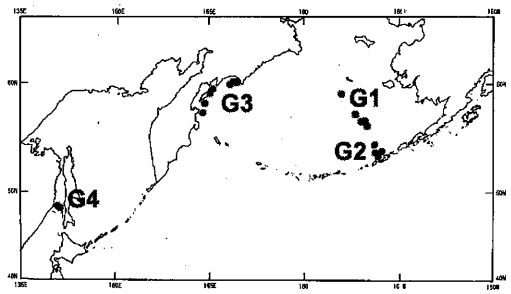

Fig. 2. Results of discriminate analysis of TNM data set (Nitta, 1994).

TNM is thought to be useful method to describe phenotypic characteristics of pollock. Results of the discriminate analyses showed that geographical differences were observed in the morphological characteristics of adult pollock. Higher than 90% of the fish were correctly classified to the each area group (Fig. 2). The preliminary results of this study are reported by Nitta and Nishimura (1991).

Otolith components

In late 1980s to early 1990s, otoliths of the fish from each area group were dissolved in hydro chloride solution, and the major element contents were measured by the atomic absorption method. At the same time, amino acid contents were measured by amino acid automatic analyzer (Nakano *et al.*, 1991).

In these analyses, Ca, Mg, Na and Sr were detected as for the elements, and isoleusin and tyrosine were selected to examine.

These results suggested that the element and amino acid contents might be useful for the stock identification. However, the methodological problems were pointed out for using dissolved whole otolith. The data represents its whole life stage. To get the information about migration and the origin of the fish, the information from early life history is important. But this method does not allow us to get such data.

Electro Probe Micro-analyzer (EPMA)

Naturally acquired tags such as elemental composition of otoliths might indicate the origin of the fish. Information about chemical components around the otolith focus could be utilized to assign fish to their nursery ground in the early life stage.

In the recent year, otolith element study is conducted by using Electro Probe Micro-analyzer (EPMA). Age 1 fish were collected from southern, northern EBS, and Chukchi Sea in the summer, and the central part of these otoliths was examined. P, Na, K, and Sr contents were measured (probe width 3μm; measured step 3μm). Na and K concentrations showed that the age 1 pollock in the Chukchi Sea has a significantly different from those in the Bering Sea (Fig. 1). Within Bering Sea, no obvious difference was observed in Na, K, and P contents. Time-series change of Sr concentration showed different pattern between northern and southern EBS, and it became higher in smaller size range in the northern EBS. Sr content is thought to be affected by the water temperature in the environment.

Table 1. Result of discriminate analysis for otolith components data (Nakano *et al.*., 1994).

Elemental components	EBS	A.Basin	Bogoslof	DH	WBS(west 170E)	WBS(east 170E)	Percent correct
EBS	21	0	1	2	4	10	55.3
A.Basin	2	14	1	4	1	3	56.0
Bogoslof	1	2	5	1	0	1	50.0
DH	0	7	5	8	4	1	32.0
WBS(w. 170E)	2	0	0	1	3	0	50.0
WBS(e. 170E)	6	1	2	0	2	15	57.7

Amino Acid contents	EBS	A.Basin	Bogoslof	DH	WBS(west 170E)	WBS(east 170E)	Percent correct
EBS	20	1	0	1	0	2	83.3
A.Basin	0	14	0	3	1	1	87.5
Bogoslof	0	0	10	2	0	1	76.9
DH	0	3	0	13	2	4	59.1
WBS(w. 170E)	1	2	0	0	8	0	72.7
WBS(e. 170E)	4	0	0	0	0	6	60.0

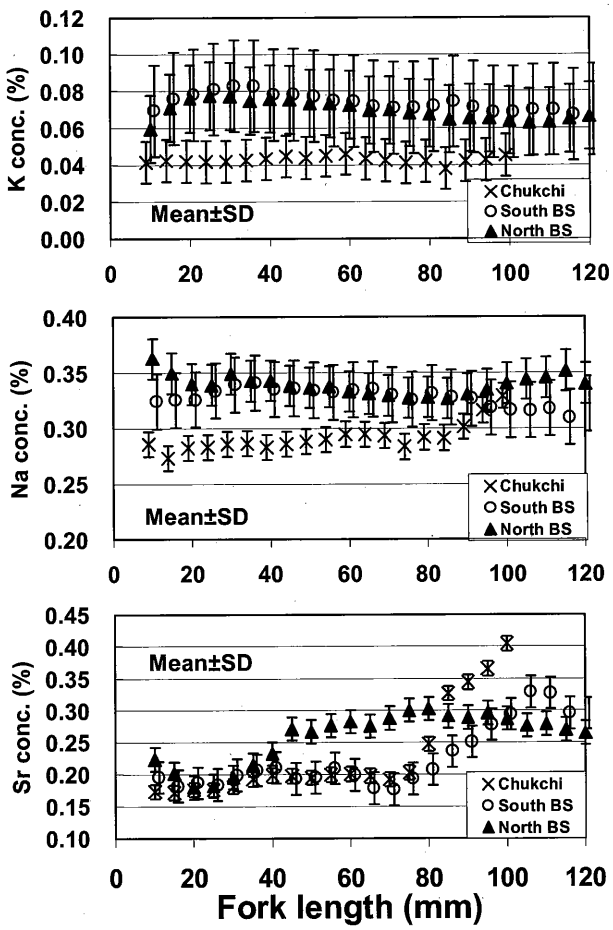

Fig. 3. Transects of otolith microelements concentration measured with a EPMA (Age1 pollock). The horizontal axis indicates fork length (mm) converted from the otolith diameter (n=12 for each area).

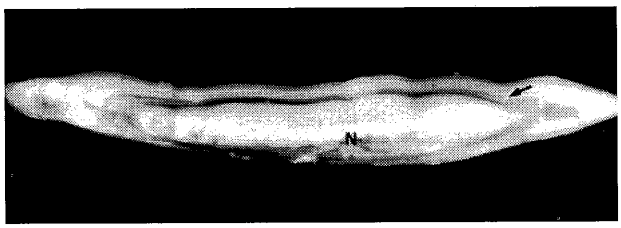

Fig. 4. Otolith of age 1 walleye pollock, showing the 1st annulus (arrow).

Growth analysis

The same kind of the difference was also observed in the 1st annulus formation (Fig. 4). The initiation and the width of the 1st annulus formation were different between age 1 fish from the northern and southern EBS. With using these parameters, 90% of age 1 fish were correctly classified to the northern and southern EBS group (Nishimura, 1998). Those parameters were also obtained from adult basin pollock, and the discriminate function from age 1 results was adopted to

classify. The results suggested that the characteristics of the 1st annulus of the basin pollock were classified to the southern EBS group (Fig.5). This suggests the strong relationship between basin adult pollock and age 1 pollock in the southern EBS. However at this moment, interannual variability is ignored and the data is so limited. Further work will be required with specially focused to the interannual variability in growth and environmental variability.

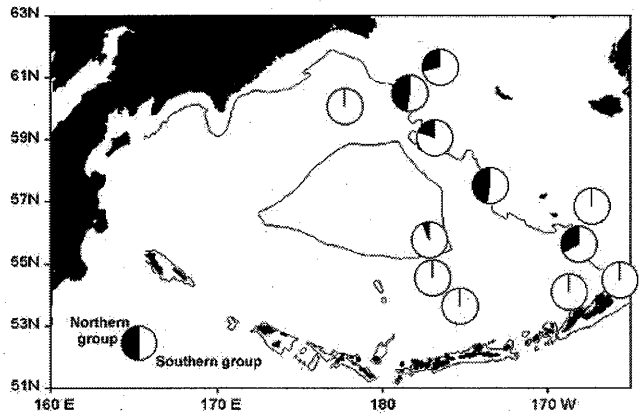

Fig. 5. Results of discriminate analysis for the parameters of the first annulus in the otoliths. Discriminate function was cited from Nishimura (1998).

FOR THE FUTURE STUDY

Briefly, phenotypic characteristics indicate the existence of separate population structure in the northern North Pacific. However, most of these characteristics do not give us the information of their origin. Only vertebra counts and otolith information might have relation to their origin, if these numbers or composition were affected by water temperature in the developing stage.

In the recent year, the abundance of the basin pollock is decreasing and the distribution area is shrinking. At the same time, some biological characteristics such as spawning, growth, and year class are dramatically changing. Is the basin pollock still having the same stock structure as those in 1980's? We hope to get some advanced information with new genetic technology in our future study.

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Review of Pollock Stock Structure Studies in the Japanese Institutes 2. Genetic Analysis

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SUMMARY: The Japanese scientists have performed the genetic analysis to elucidate population structure of walleye pollock in the Bering Sea since 1988. Allozyme, mitochondrial DNA analysis, PCR-RFLP analysis, RAPD analysis, and AFLP analysis were carried out. There were differences between western and eastern area in the Pacific Ocean for allozyme and PCR-RFLP analysis. However the other analysis could not detect genetic variations among sampling area.

KEY WORDS: Walleye pollock, Allozyme, mtDNA, PCR-RFLP analysis, RAPD analysis, AFLP analysis

INTRODUCTION

To elucidate population structure of walleye pollock, the Japanese scientists have examined *Theragra chalcogramma,* in the Bering Sea, morphological characters, metallic elements and amino acid contents in the otolith, and genetic analysis of walleye pollock since 1989. In this report, the results of genetic analysis are summarized.

Allozyme

Wada and Yokawa[1)] and Okazaki[2-5)] examined enzyme electrophoresis analyses. Tissue samples of pyloric appendage, liver, heart, brain, and muscle of walleye pollock were used. Horizontal starch gel electrophoresis was used to detect protein variants using the methods describe by Utter et al.[6)]. Gels were made using 13% starch. Forty-three enzymes were used and 54 loci were detected from 31 enzyme strains. *SOD** was the most efficient enzyme to examine population structure as reported by Iwata[7,8)] and Grant and Utter[9)](Table 1). Allelic frequencies were examined using pollock collected from various areas during 1992-1994. Judging from these results, population of west Bering Sea was discriminated from that of the Donut Hole and the east Bering Sea in boundary of the cape Navarin (Fig. 1). The variance of year and migration was not considered in these reports, but it suggested that the migrations of west and east population were restricted to each area. It was observed that the frequencies of *SOD*50* gradually increased from the cape Navarin through the Hokkaido (Fig. 1.).

mtDNA analysis

Numachi and Kobayashi[10)] analyzed restriction fragment length polymorphism (RFLP) of mtDNA using the usual method, but it was so breakables that they could not get the good results. They developed new method combined southern hybridization method and non-radioactive probe labeled digoxigenin of mtDNA for salmon.[11)] mtDNA analysis was conducted using a grain of sample.[12-14)] Sampling location is shown in Fig. 2. Pattern of restriction fragment length polymorphism in the mtDNA was shown in Fig. 3. Twenty-four haplotypes were detected from 7 endonuclease (Table 2). The significant of geographic homogeneity in haplotype distributions were tested using the chi-square randomization method of Roff and Bentzen[15)] (Monte Carlo Technique). It was found to be significantly different among west continental area, northern east continental area, and southern east continental area of the Bering Sea. But there were no difference between Donut Hole and others ($p<5\%$). Mulligan and Chapman[16)] report that 65 haplotypes were detected from pollock from Gulf of Alaska, Donut Hole, Bogoslof Island, and Adak Island, and significant differences among these areas were observed. The differences between the results of Numachi and Kobayashi[14)] and Mulligan and Chapman[16)] were considered to be dependent on 2 enzymes that did not use in these analyses. In the following study, sample size and enzymes are thought to be important factor to obtain good results.

PCR-RFLP analysis

Numachi and Kobayashi[13,14)] and Okazaki[17)] were amplified Control region (D-Loop region) of mtDNA, and were examined geographical differences using RFLP

Table 1. Names, number, and abbreviations of used enzymes and proteins.[1,2)]

Enzyme or protein name	abbreviation	number	No. of loci	Wada and Yokawa (1990)	Okazaki (1991)
Aconitate hydratase	ACON	4.2.1.3	1	*	*
Adenosine deaminase	ADA	3.5.4.4	2	*	
Alcohol dehydrogenase	ADH	1.1.1.1	1	*	
Adenylate kinase	ADK	2.7.4.3	3?	*	*
Aldolase	ALD	4.1.2.13	1	*	
Glycerophosphatase	ALP	3.1.3.1	1	*	
Creatine kinase	CK	2.7.3.2	2	*	
	DIA	1.6.4.3	2	*	
Esterase	EST	3.1.1.1	4	*	
Fumarate hydrase	FUM	4.2.1.2	1	*	
Glyceraldehyde-3-phosphate dehydrogenase	GA3PD	1.2.1.12	2	*	*
Alphaglycerophosphate dehydrogenase	aGDH	1.1.1.8	2	*	*
Glutamate dehydrogenase	GLUDH	1.4.1.3	1	*	
Aspartate aminotransferase	GOT	2.6.1.1	1	*	*
Glucose-6-phosphate dehydrogenase	G6PD	1.1.1.49	1	*	
Glucose-6-phosphate isomerase	GPI	5.3.1.9	2	*	
Isocitrate dehydrogenase	ICD	1.1.1.42	2	*	*
Lactate dehydrogenase	LDH	1.1.1.27	4	*	
Malate dehydrogenase	MDH	1.1.1.37	3	*	*
Malic enzyme	ME	1.1.1.40	2	*	*
mannose-6-phosphate isomerase	MPI	5.3.1.8	1	*	
Purine-nucleoside phosphorylase	NP	2.4.2.1	2	*	
AminopeptidaseA	PEPA	3.4.3.2	2	*	
AminopeptidaseB	PEPB	3.4.1.3	1	*	
AminopeptidaseD	PEPD	3.4.--	1	*	*
6-Phosphoglucose isomerase	6PGD	1.1.1.44	1	*	*
Phosphoglucomutase	PGM	2.7.5.1	1	*	
Pyruvate kinase	PK	2.7.1.40	4?	*	
Superoxide dismutase	SOD	1.15.1.1	1?	*	*
Triose-phosphate isomerase	TPI	5.3.1.1	1?	*	
Xanthine oxidase	XOD	1.2.3.2	1	*	
Aldehyde dehydrogenase	NAD	1.2.1.3	ND	*	
Fructose-bisphosphatase	FDP	3.1.3.11	ND	*	
Glucose dehydrogenase	GDH	1.1.1.47	ND	*	
Glycerate dehydrogenase		1.1.1.29	ND	*	
3-Hydroxybutyrate dehydrogenase		1.1.1.30	ND	*	
Hexokinase		2.7.1.1	ND	*	
		3.2.2.2	ND	*	
		1.1.1.-	ND	*	
6-Phosphofructokinase		2.7.1.11	ND	*	
Sorbitol dehydrogenase		1.1.1.14	ND	*	
		1.1.1.22	ND	*	
		1.1.1.10	ND	*	

analysis. Okazaki[18,19)] amplified about 2.0Kbp region (NADH5-6) containing the NADH5 and NADH6 genes of the NADH de hydrogenase complex and about 2.0Kbp region (NADH1) coding for the NADH1 and 16sRNA. Sampling location was shown in Fig. 4. Control region and NADH5-6 region were digested with 16 endonuclease, and restricted types were obtained from 5 and 4 endonuclease, respectively (Table 3). Characteristic restricted type of area could not detect in both regions, but frequencies of restricted type had differences among areas (No significance test). They used 10 samples per one area in both regions, and the sample size was limited. NADH1 region was digested with 14 endonuclease, and restricted types were obtained from 10 endonuclease. Twenty-two haplotypes were obtained from NADH1 region (Table 4). But we could not distinguish pollock sub-population within the collected area. We are needed to analyze with combined various regions.

On the other hand, Yanagimoto[20)] amplified

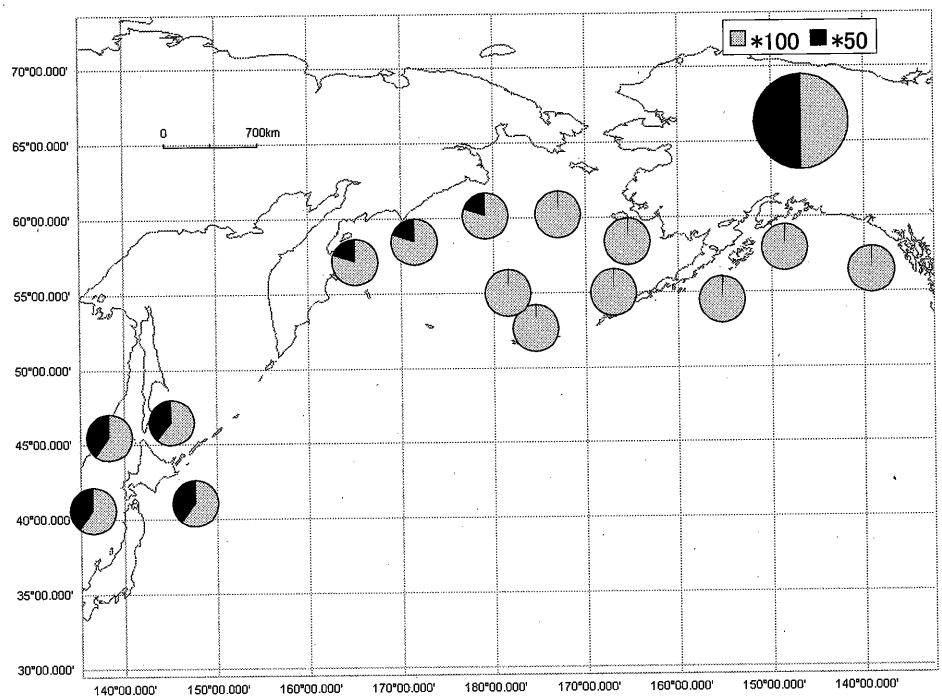


Fig 1. The compositions of allelic frequencies of SOD* of walleye pollock.
The results of Iwata[8] and Grant and Utter[9] and Okazaki[5] were combined.

Table 2. mitochondrial DNA haplotype observed in walleye pollock, *Theragra chalcogramma*.[14] Numerals indicate number of individuals composing each haplotype. Letter sequences from left to right refer to the digestion profiles for the six variable endonuclease: (1) *Apa*I, (2) *Bgl*I, (3) *Dra*I, (4) *Eco*RI, (5) *Hind*III, (6) *Pst*I, (7) *Sac*II.

	Haplotype	Donut Hole	Continental shelf			West Bering Sea			Gulf of Alaska	Sea of Okhotsk	
		SB	SA	SF	SL	SG	SH	SI	SM	SN	SO
1	1111111	1	0	0	0	0	0	0	0	0	1
2	1121111	1	0	0	0	0	0	0	0	0	0
3	2111111	9	10	8	1	3	5	4	1	7	3
4	2111141	0	0	0	1	0	0	0	0	0	0
5	2121111	3	5	8	4	5	1	1	2	2	2
6	2121121	0	0	1	0	0	0	0	0	0	0
7	2121141	1	0	0	0	0	0	1	0	0	0
8	2141111	1	0	0	0	0	0	0	0	0	0
9	2151111	0	0	0	0	0	0	1	0	0	0
10	2161111	0	0	0	0	0	0	0	0	0	1
11	2211111	0	0	0	1	2	0	0	2	2	0
12	2311111	1	1	1	0	0	1	3	0	0	1
13	2311121	0	0	0	0	0	0	1	0	0	0
14	2331111	0	1	0	1	0	0	0	0	0	0
15	2411111	0	0	1	2	0	0	0	0	0	0
16	2421111	0	0	0	0	0	0	1	0	0	0
17	2531111	0	0	0	0	0	1	0	0	0	0
18	2611111	0	0	0	0	0	0	1	1	0	0
19	3311111	1	0	0	0	0	0	0	0	0	0
20	4111111	0	0	0	1	0	0	0	0	0	0
21	5111111	2	0	0	0	0	0	0	0	1	0
22	5111141	0	1	0	0	0	0	0	0	0	0
23	5121111	2	0	0	0	0	0	0	0	0	0
24	6111111	0	0	0	1	0	0	0	0	0	0
	Total	22	18	19	12	10	8	13	6	12	8

Control region and NADH5-6 region of pollock collected from Bogoslof, North Bering Sea, East Bering Sea, Okhotsk Sea, east and west area of Hokkaido, and conducted RFLP analysis. Patterns of restriction fragment length polymorphism were shown in Fig. 5. Characteristic restricted type of area could not detect in both regions, but frequencies of restricted type by *Hinf* I in Control region and by *Msp* I in NADH5-6 region had differences among areas (Fig. 6). The c ompositions of restriction pattern were different with east and west area, and these results were similar to Allozyme results.

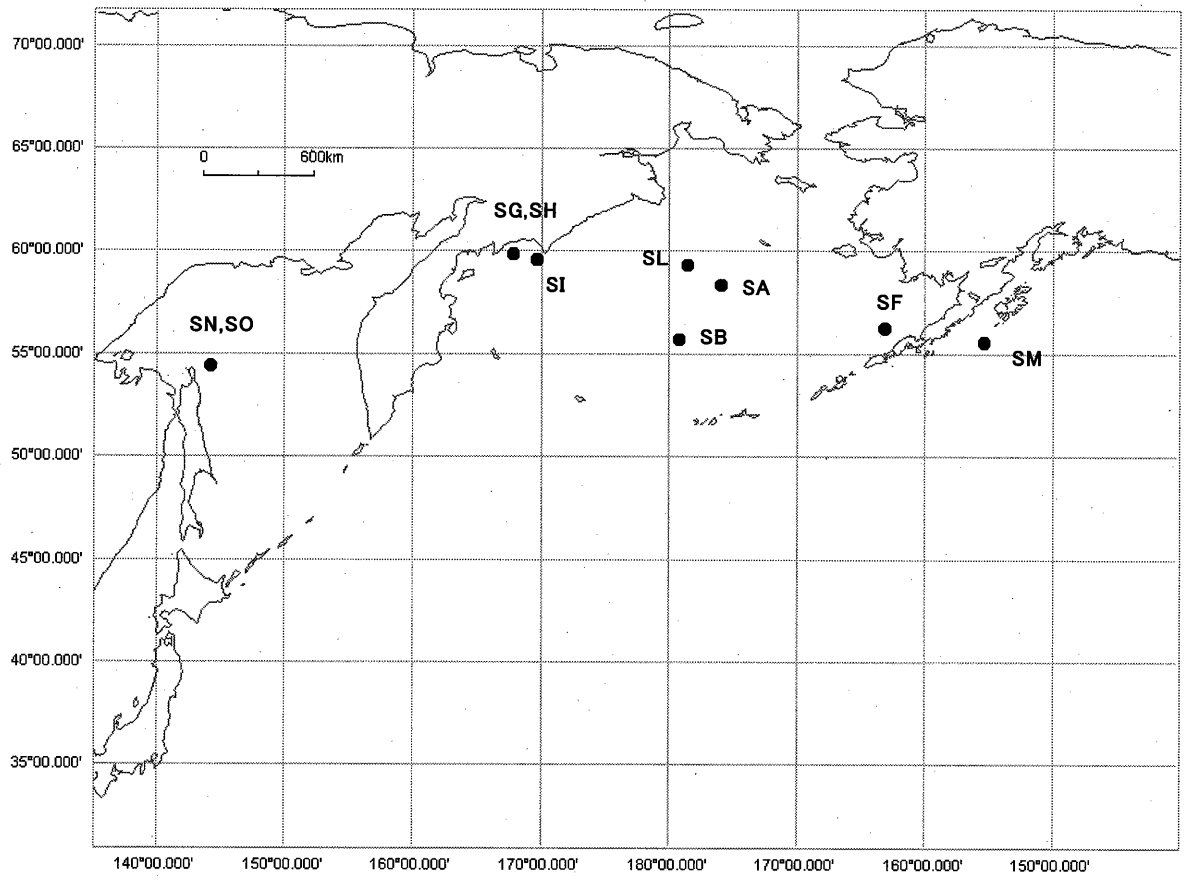


Fig. 2. The sampling location for mtDNA analysis of walleye Pollock (Numachi and Kobayashi)[14].

Table 3. Restriction type compositions in control and NADH 5-6 region.[17,18]

Region	Endonuclease	Restricted type	Sampling Location		
			East	Central	West
Control region	*Hae* Ⅲ	A	6	6	8
		B	4	3	2
		C	0	1	0
	Hinf Ⅰ	A	8	7	10
		B	2	3	0
NADH5–6 region	*Aci* Ⅰ	A	8	7	10
		B	2	3	0
	Alu Ⅰ	A	10	8	10
		B	0	2	0
	Bfa Ⅰ	A	10	10	8
		B	0	0	2
	Msp Ⅰ	A	6	6	9
		B	4	3	1
		C	0	1	0

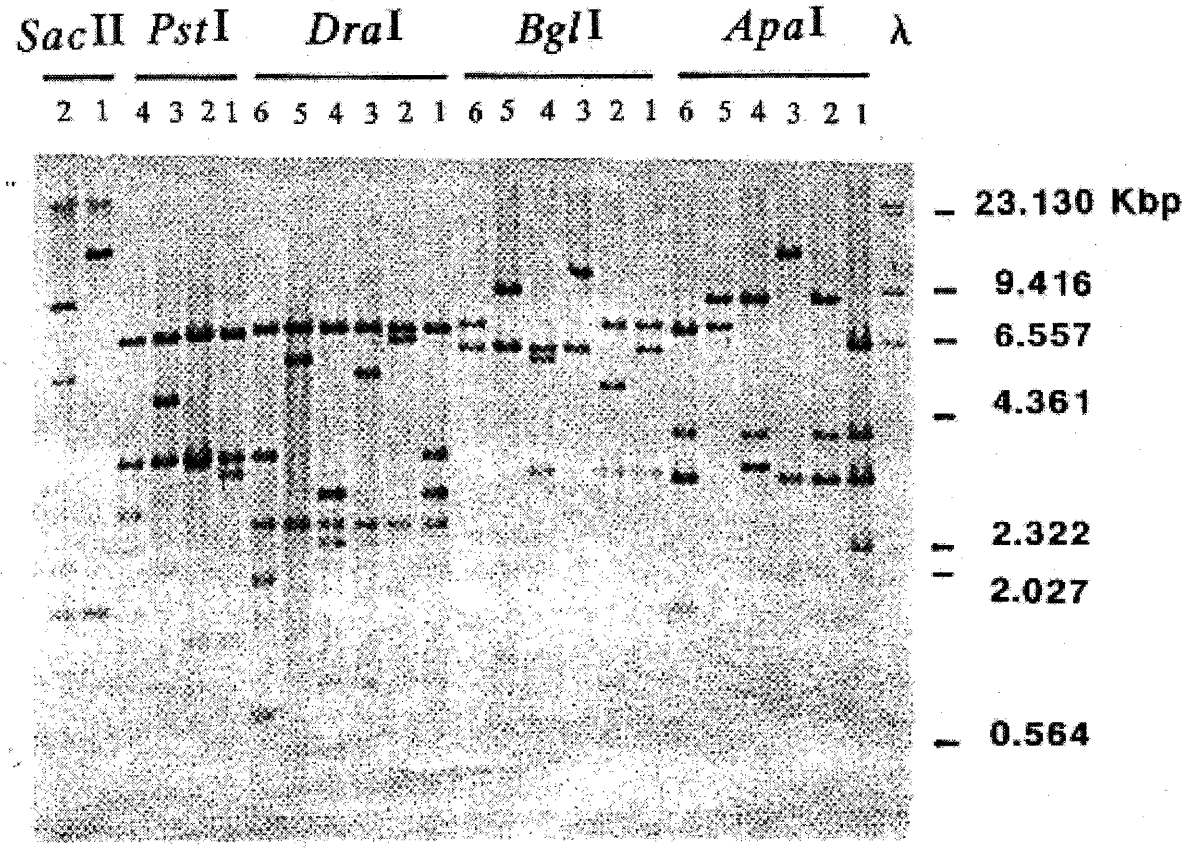


Fig. 3. Pattern of restriction fragment length polymorphism (RFLP) in the mitochondrial DNA of walleye pollock.[14] Righ lane indicate size marker.

Table 4. Haplotype compositions in NADH 1 region of walleye pollock by endonuclease.[19] Numerals indicate number of individuals composing each haplotype. Letter sequences from left to right refer to the digestion profiles for the fourteen endonuclease: (1)*Aci*I, (2)*Alu*I, (3)*Bfa*I, (4)*BstU*I, *Dde*I, (5)*Hae*III, (6)*Hha*I, (7)*Hinf*I, (8)*Msp*I, (9)*Nla*III, (10)*ScrF*I, (11)*Sau96*I, (12)*Taq*I. *; EC: eastern continental, BI: Bogoslof Island, SB: soutern Basin, DH:Donut hole, WC: western contiental area, NB: north Basin.

	Haplotype	Location*					
		EC	BI	SB	DH	WC	NB
1	AAAAAAAAAAAAA	5	4	1	10	6	6
2	AAAAAAAAABAAAA	19	8	8	8	8	7
3	AAAAAACAABAAAA	2	4	3	1	0	0
4	CAAABAAAABAAAA	0	2	0	0	0	0
5	AAAAAAEAABAAAD	1	2	0	0	0	0
6	AAAAAAAAAABAA	0	0	2	1	0	0
7	AAAAAAAAABDAAA	0	1	0	0	0	0
8	DAAAAAAACAAAAA	0	0	1	0	0	0
9	AAAAAAEAAAAACB	0	0	2	0	0	0
10	AAAAAAAAABBAAA	0	0	1	0	0	0
11	AAAAAAAAABAAAC	0	0	1	0	2	0
12	DAAAAAAAAAAAA	0	0	1	0	0	0
13	AACAAAAAAAAAA	0	0	2	0	0	0
14	ABAAAAAAAAAAA	0	0	0	1	0	0
15	ADAAAAAAAAAAA	0	0	0	2	0	0
16	BAAAAAAAAAAAA	0	0	0	0	1	0
17	ABAAAAAAAAAAA	0	2	0	0	1	2
18	AABAAAAABAAAA	0	0	0	0	1	0
19	ACAAAAAAAAAAA	0	0	0	0	0	1
20	ABAAAABAABAABA	0	0	0	0	0	2
21	AAAAAAEAABAACB	0	0	0	0	0	1
22	AAAAAAAAABCAAA	0	0	0	0	0	1
	Total	27	23	22	23	19	20

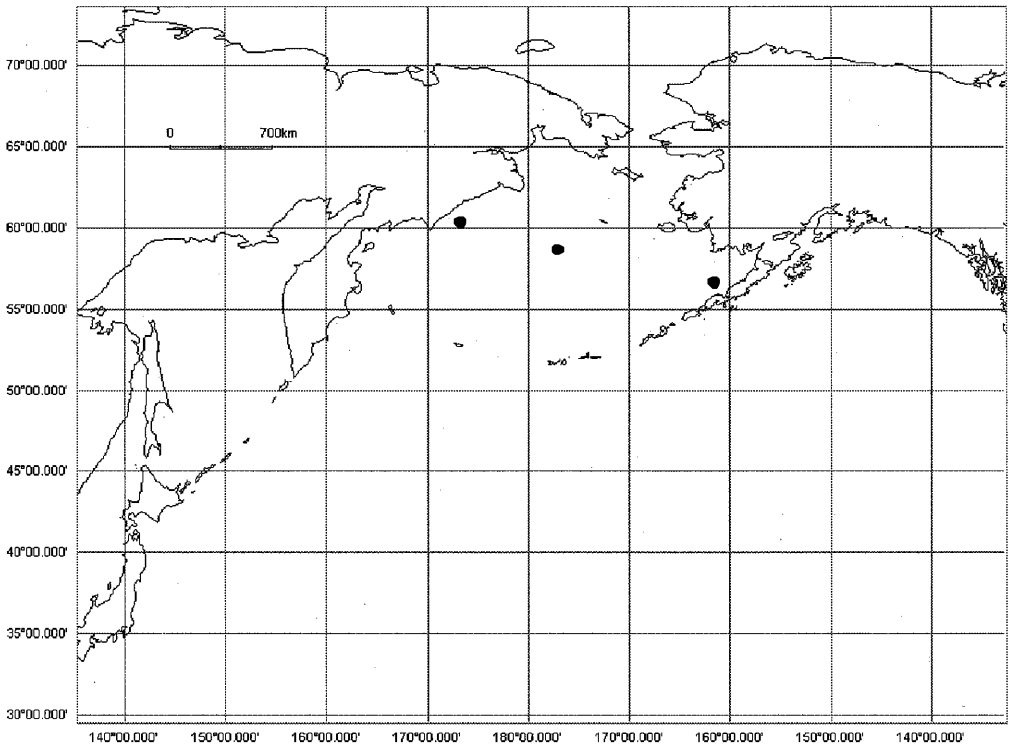


Fig. 4. The sampling location of PCR-RFLP analysis of walleye pollock.[17,18)]

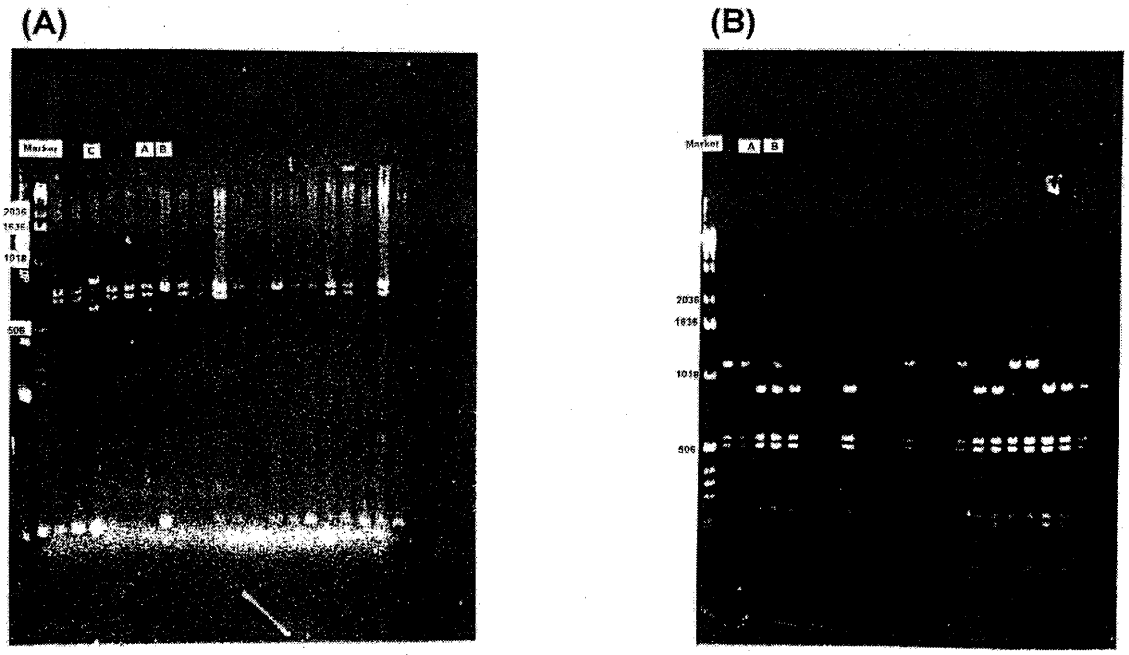
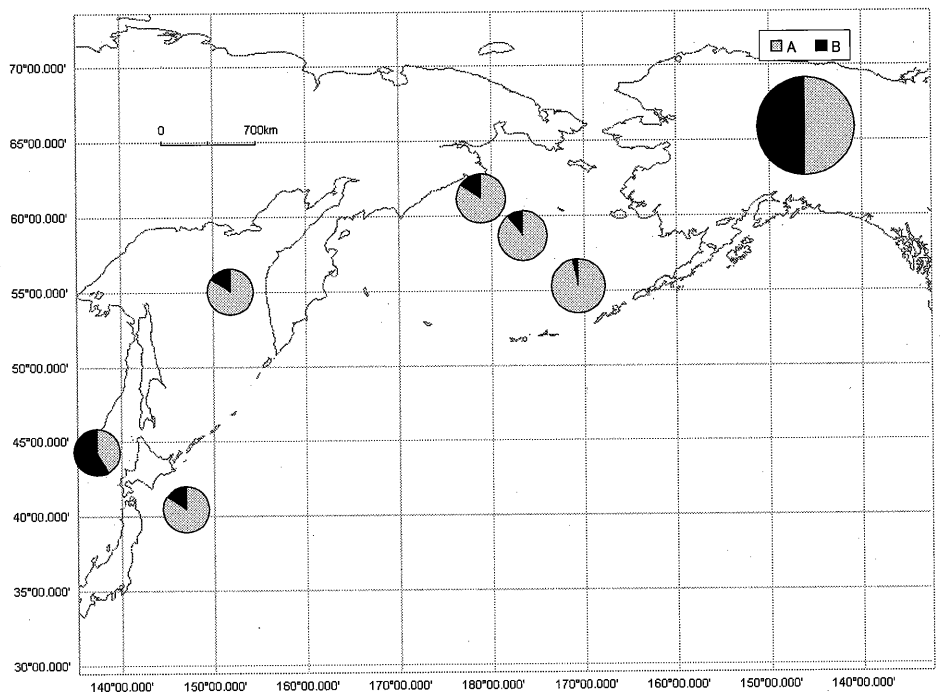


Fig. 5. Fragment pattern in control and NADH5-6 region of walleye pollock.[20)] (A): Control region digested by *Hinf*I. (B): NADH5-6 region digested by *Msp*I.

(A)



(B)

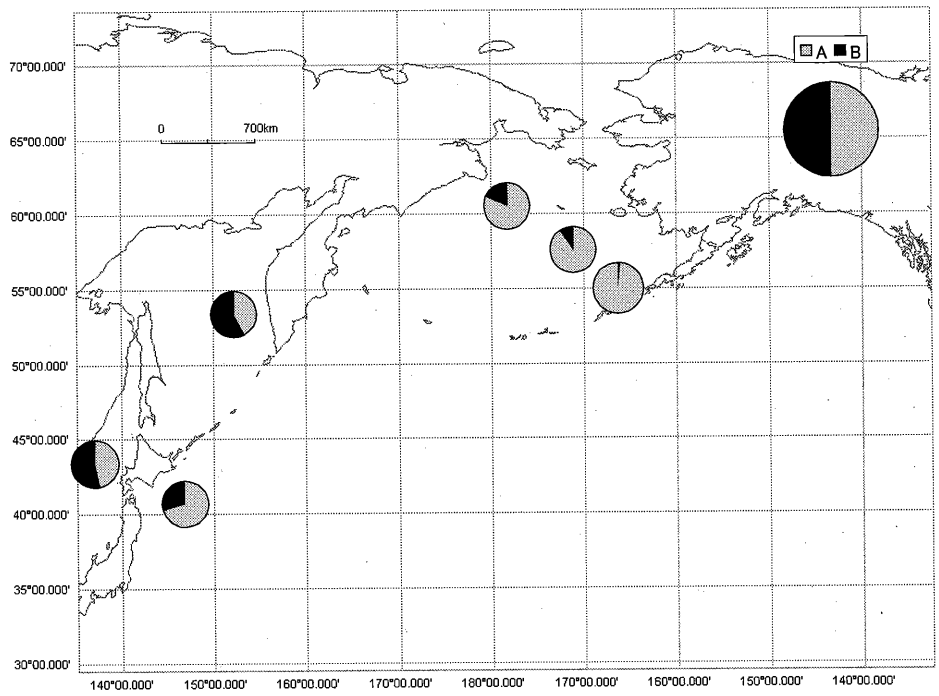


Fig. 6. Fragement pattern compositions of control and NADH5-6 region.[20] (A): Control region digested by *Hin*fI. (B): NADH5-6 region digested by *Msp*I.

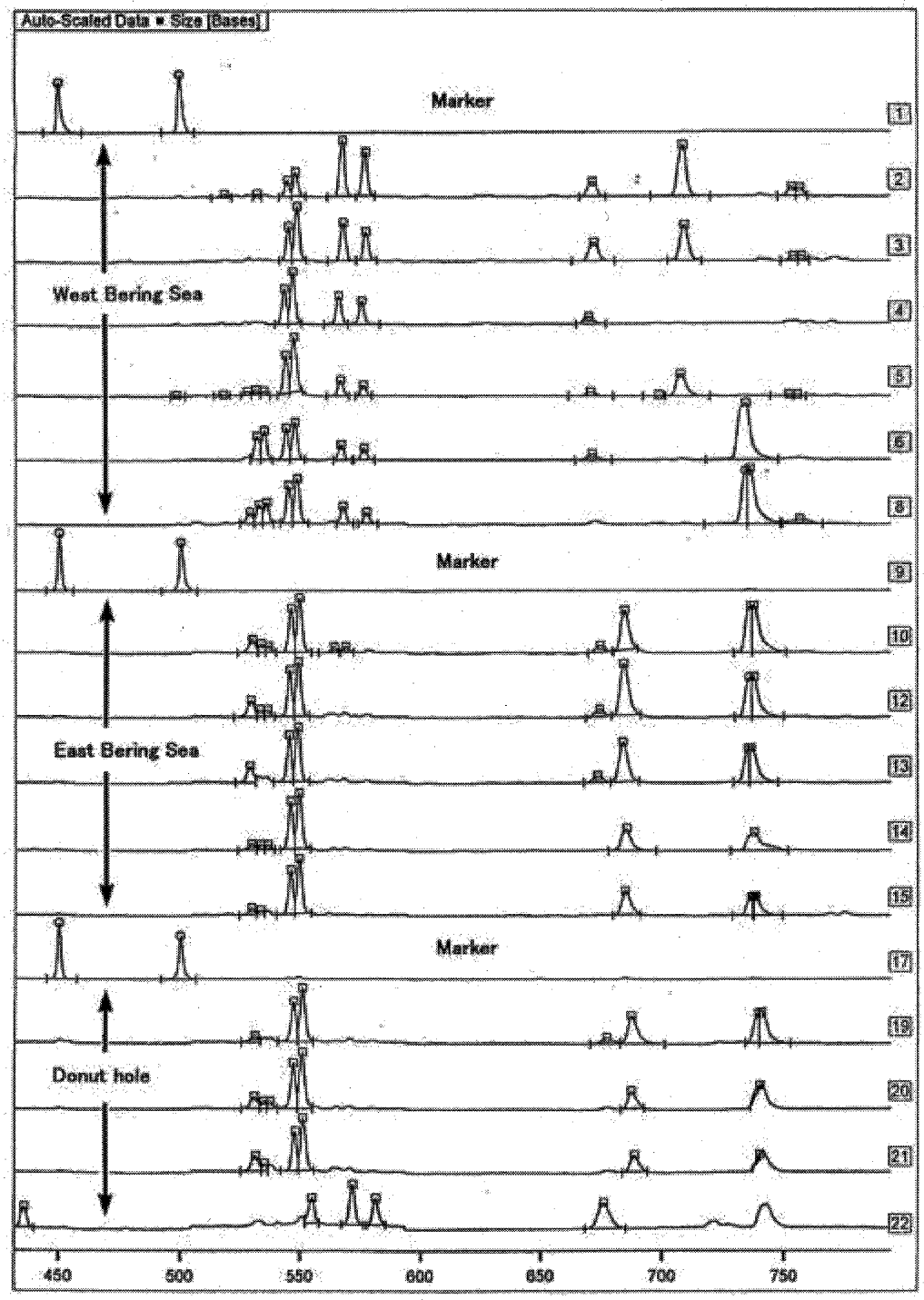


Fig. 7. Patterns obtained by fluoro RAPD-PCR.[24)]

But these compositions were different with east and west area of Hokkaido. In the following studies, we will conduct PCR-RFLP analysis for pollock collected around Hokkaido.

RAPD-PCR analysis

Random amplified polymorphic DNAs (RAPD) PCR analysis was examined to apply to the pollock sub-population study.[21] RAPD-PCR analysis has problem for reappearance, and reaction conditions were tested changing primer length, template density, and *Taq* polymerase and so on. The results indicated that the template density affected reappearance, and decamer primer was suggested to be the best primer length for RAPD-PCR. But we could not find effective primer to distinguish pollock sub-population. Tandem repeat primerd (TREP) PCR analysis was also examined,[22] but we could not obtain good results.

Fluoro RAPD-PCR using automate DNA sequencer was examined.[23,24] Random primer set A (Operon Technologies) for RAPD were used in this method. After primer was labeled Cy5, RAPD-PCR was conducted. Amplified fragments were detected using atuomate DNA sequencer. In this reaction condition, the reappearance was good. The detected range became 50-1000bp using Fluoro RAPD-PCR, but small fragments could detect and many bands could find from one band observed in usual method using ethidium bromide. Judging from these results, it was suggested that Fluoro RAPD-PCR is convenient and sensitive method, and it was also suggested the possibility to detect genetic homology with an objective way.

Fluoro RAPD-PCR was conducted using OPA12 labeled fluorescence for pollock, and characteristic fragments peak of west Bering Sea was found. But we could not find peak that can distinguish between pollock of open sea and east Bering Sea (Fig. 7). We need to examine various genetic markers in the future study.

AFLP analysis

Amplified fragment length polymorphism (AFLP) analysis is able to detect many genetic markers from one analysis, and is chiefly applied plant taxonomy. In this method, after template DNA was digested by two endonucleases, PCR for digested fragments was conducted using same endonucleases adding specific base. Amplified fragments could change by number and species of adding specific base. This method can use automate DNA sequencer by endonuclease labeled fluorescence. But we could not specify that amplified fragments originate from anywhere of DNA, and the reappearance of the results is problem. AFLP analysis was applied for walleye pollock, but appearance fragments changed by analysis.[25] In future study, we must find good reaction condition at first. And we need use many primers to apply AFLP analysis for walleye pollock.

FUTURE WORKS

In the future studies, it is important to collect adult pollock in various spawning grounds with significant number of samples, in the spawning season. Some applied genetic study for North Pacific pollock stocks will be continued at Hokkaido Institute.

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Mitochondrial DNA Preliminary Analysis and Meristic Characters Comparison for Stock Identification of Walleye Pollock, *Theragra chalcogramma*, from the North Pacific

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ABSTRACT: Mitochondrial DNA (mtDNA) variation was examined by restriction endonuclease digestion in 100 walleye pollock from five areas (Central area and Bogoslof area of the Bering Sea, Kuril Islands, Pacific coast of the Hokkaido and east coast of Korea) in the North Pacific Ocean. Six endonucleases produced variant restriction patterns among the populations. The composite mtDNA digestion patterns revealed twenty genotypes. Genetic difference among the populations of the area will be conducted by the RFLPs analysis for stock identification. Meristic variation was also examined by the counts of the seven meristic characters (numbers of vertebrae, numbers of ray of the three dorsalfins, two analfins and one pectloralfin) in 570 walleye pollock from seven areas (the same as above mtDNA sample area, western Bering Sea and Okhotsk Sea). ANOV F-test on these meristic counts except for numbers of the first and second analfin ray showed significant differences among the seven areas.

INTRODUCTION

The walleye pollock, *Theragra chalcogramma*, is the major semi-demersal fish resource in the North Pacific Ocean. For several years pollock has supported one of the world's largest single species fishery with catches ranging from 4.5 to 5.0 million metric tons annually in 1992-1996. The bulk of this catch has come from Asian waters. It is distributed from east coast of Korea to southern Oregon coast throughout off Hokkaido, Okhotsk Sea, Bering Sea and Gulf of Alaska. Within this range a number of spawning grounds isolated are recognized. It has been assumed that these spawning groups are isolated genetically.

A stock is commonly defined as a population of fish that is reproductively isolated from other populations. Morphology are often used to distinguish stocks of fish (Ibssen *et al.* 1981). The morphology of a fish has both genetic and environmental components. There are several limitations to using these measures to separate populations, since the meristic characteristics being measured originate from both genetic and environmental influences. But the morphology methods are useful in identifying stocks where a stock is defined as a "population of fish that behave as a cohesive unit whose members exhibit common responses to environmental conditions within its geographic boundaries."

Electrophoretic studies allowed genetic testing of this discrete stock concept. In the past few years techniques have become available for more direct examination of variability in DNA. The maternally inherited mitochondrial DNA (mtDNA), which appears to evolve more rapidly than nuclear DNA, shows great potential as a marker in examining the population-genetic structure in marine organisms. Recently, restriction endonuclease analysis of mtDNA has been used successfully to identify the stock structure of several important fishes. The technique is thought to be more sensitive than allozyme studies and thus may expose additional differences among populations.

This study has important implications, as it is to examine the genetic and meristic relationships between walleye pollock stock from the Korean waters, off Hokkaido, Kuril Islands, Okhotsk Sea and Bering Sea.

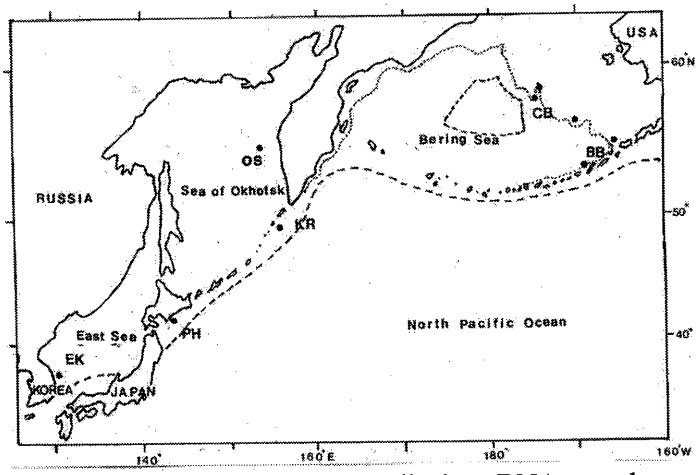

Fig. 1. Locations of walleye pollock mtDNA sample collections in the North Pacific from June 1997 to June 1999. Solid circles designate the collection sites. EK: East coast of Korea, PH: Pacific coast of Hokkaido, KR: Kuril Islands, OS: Sea of Okhotsk, CB: Central Bering Sea, BB: Bogoslof area of Bering Sea.

MATERIALS AND MEUIODS

1. mtDNA

The restriction fragment length polymorphisms (RFLPs) analysis is used here to identify walleye pollock stocks in the North Pacific. Walleye pollock were collected by trawlers during spring and winter from 1997 to 1999. Sampling was conducted in the Bering Sea in the vicinity of Bogoslof Island (BB) and the continental slope of central area (CB). Additional samples were taken in the Sea of Okhotsk (OS), Pacific coast of Kuril Islands (KR), Pacific coast of the Hokkaido (PH) and east coast of Korea (EK). A total of 170 fishes ranged from 31.2 cm FL to 62.0 cm FL were collected for the RFLPs analysis (Table 1, Figure 1).

Mature ovaries were removed from each fish and frozen at –60°C for later use. 5g of each ovarian tissue sample was homogenized at 4°C in 30 ml of STEK buffer (0.25M Sucrose, 50mM Tris, 10mM EDTA, 1.5% KCL, pH 7.5). An alkaline method (Park and Kim, 1995), with modifications described by Chapman and Power (1984), was used to isolate mtDNA. mtDNA samples were digested with the eight six-base recognition endonucleases (*Apa* I , *Bgl* I , *Dra* I, *Eco* RV, *Hind* III, *Ksp* I , *Pst* I and *Xba* I).

Table 1. Collection records of walleye pollock mtDNA samples in the six areas of the North Pacific.

Area	Date	Location	No. of Individual	Size range (FL : cm)
East coast of Korea	15 Dec. '97	38° 20′ N, 128° 45′ E	30	36.0~53.6
Pacific coast of Hokkaido	15 Nov. '97	42° 12′ N, 143° 38′ E	20	39.6~53.2
Pacific coast of Kuril Islands	28 Oct. '97	49° 10′ N, 155° 01′ E	20	42.0~54.8
Sea of Okhotsk	11 Mar. '99	54° 50′ N 153° 00′ E	20	31.2~45.7
Central Bering Sea (Continental slope)	2 June '97	56° 08′ N, 169° 38′ W	10	44.1~56.9
	7 June '97	58° 03′ N, 175° 33′ W	10	41.2~54.2
	11 June '99	58° 47′ N, 174° 43′ W	20	32.5~48.6
Bogoslof area of Bering Sea	7 Mar. '98	53° 13′ N, 169° 04′ W	20	49.0~62.0
	24 May '99	55° 13′ N, 165° 49′ W	20	32.4~62.0
	June '97~ June '99		170	31.2~62.0

The fragments were separated by horizontal electrophoresis on 0.7% agarose gels. λDNA was used as a molecular size marker. The gels were stained for approximately 30 min with ethidium bromide and photographed with polaroid camera.

Haplotype diversity of mtDNA was calculated according to Nei and Tajima (1981), and nucleotide sequence divergence between populations was calculated according to Nei and Li (1979). The unweighted pairgroup method using arithmetic averages (UPGMA) was used to cluster genetic distances (Sneath and Sokai, 1973). Statistical confidence in these cluster was tested using heterogeneity chi-square decomposition.

2. Meristic characters

Walleye pollock samples were collected from three areas of Bogoslof (BB), central Bering Sea (CB) and western Bering Sea (WB) in the Bering Sea and one area (OS) in the Okhotsk Sea, two areas of Kuril Islands (KR) and Hokkaido (PH) in the North Pacific Ocean and one area (EK) in the east coast of Korea from 1997 through 1999. A total of 570 fishes ranged from 22.4 cm FL to 56.8 cm FL were collected for meristic counts in this study (Table 2, Figure 2).

The number of vertebrae (NVe) including urostyle, the numbers of rays of the three dorsalfins (ND_1, ND_2, ND_3), two analfins (NA_1, NA_2) and pectoralfin (NP) were counted through the film used by Hitex X-rayer.

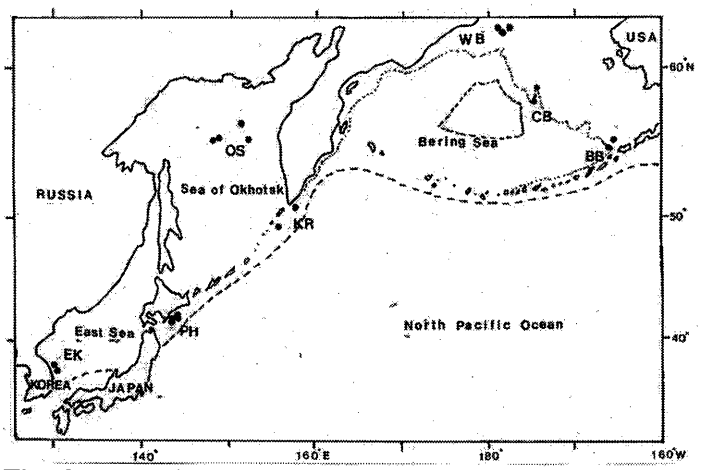

Fig. 2. Locations of walleye pollock meristic sample collections in the North Pacific from May 1997 to June 1999. Solid circles designate the collection sites. EK: East coast of Korea, PH: Pacific coast of Hokkaido, KR: Kuril Islands, OS: Sea of Okhotsk, WB: Western Bering Sea, CB: Central Bering Sea, BB: Bogoslof area of Bering Sea.

Table 2. Collection records of walleye pollock meristic samples in the seven areas of the North Pacific.

Area	Date	Location	No. of Individual	Size range (FL : cm)
East Sea of Korea	4 Dec. '97	38° 15′ N, 128° 40′ E	30	31.5~42.2
	15 Dec. '97	38° 20′ N, 128° 45′ E	30	36.0~53.6
Pacific coast of Hokkaido	28 Dec. '97	42° 12′ N, 143° 38′ E	30	29.7~38.3
	3 Jan. '98	42° 27′ N, 143° 52′ E	20	24.4~34.4
	3 Feb. '98	41° 59′ N, 143° 39′ E	60	24.0~43.6
	28 Mar. '98	42° 25′ N, 143° 51′ E	30	26.8~46.5
Pacific coast of Kuril Islands	10 Sep. '97	50° 46′ N, 157° 37′ E	20	44.4~50.6
	28 Oct. '97	49° 10′ N, 155° 01′ E	20	42.0~54.8
Sea of Okhotsk	8 Dec. '97	56° 56′ N, 151° 14′ E	60	24.4~42.5
	23 Jan. '98	55° 48′ N, 147° 49′ E	30	29.4~56.8
	14 Mar. '98	55° 12′ N, 152° 54′ E	30	33.5~44.3
	3 Apr. '98	56° 03′ N, 148° 48′ E	30	33.4~57.4
Western Bering Sea	3 Aug. '97	62° 17′ N, 177° 18′ W	20	31.9~42.4
	30 Aug. '97	62° 36′ N, 178° 33′ W	20	27.5~35.0
	18 Oct. '97	62° 29′ N, 178° 16′ W	20	36.7~44.9
Central Bering Sea (Continental slope)	7 June '97	58° 03′ N, 175° 33′ W	30	38.7~54.2
	11 June '99	58° 47′ N, 174° 43′ W	30	32.5~48.6
Bogoslof area of Bering Sea	20 May '97	54° 47′ N, 166° 25′ W	30	40.5~55.8
	24 May '99	55° 13′ N, 165° 49′ W	30	32.4~55.4
Total	May '97~ June '99		570	24.4~56.8

Table 3. Molecular weight estimates for restriction fragments of walleye pollock mtDNA produced by the eight endonucleases.

Apa I						*Bgl* I			
A	B	C	D	E	F	A	B	C	D
8.5	8.5	8.5	13.7	6.3	8.5	7.2	7.2	10.5	6.3
3.8	6.9	3.4	3.1	3.8	3.8	6.3	5.1	6.3	5.9
3.1	1.1	3.1		3.1	2.7	3.3	3.3		3.3
1.1	0.3	1.1		2.2	1.1		1.1		1.3
0.3		0.4		1.1	0.7				
		0.3		0.3					
16.8	16.8	16.8	16.8	16.8	16.8	16.8	16.7	16.8	16.8

Dra I				*Eco* RV			*Hind* III
A	B	C	D	A	B	C	A
6.8	6.8	6.8	6.8	8.0	8.0	15.1	5.6
3.4	6.2	3.4	3.4	7.1	7.1	1.7	5.4
2.8	2.5	2.5	3.1	1.5	1.7		2.9
2.5	0.7	1.4	2.8	0.2			1.8
0.7	0.6	1.3	0.7				1.1
0.6		0.7					
		0.6					
16.8	16.8	16.7	16.8	16.8	16.8	16.8	16.8

Ksp I	*Pst* I				*Xba* I	
A	A	B	C	D	A	B
15.1	6.6	6.6	6.6	6.6	7.5	7.8
1.7	3.8	3.8	5.0	4.3	4.7	4.7
	3.5	2.8	3.8	3.8	2.2	2.2
	1.5	1.5	0.9	0.9	2.1	2.1
	0.9	0.9	0.5	0.7	0.3	
	0.5	0.7		0.5		
		0.5				
16.8	16.8	16.8	16.8	16.8	16.8	16.8

Genotypes: A, B, C, D, E, F

ANOV F-test for analysis of valiance and multiple comparison Scheffes test (Zar, 1974) were applied to compare significant differences in means of meristic counts.

PRELIMINARY RESULTS

From the eight restriction endonuclease digestions of mtDNA for 100 walleye pollock, six endonucleases produced variant restriction patterns among populations. *Hind* III and *Ksp* I digest yielded one profile, while *Apa* I generated the most profile with six genotypes. Molecular weight estimates for these fragments are presented in Table 3. The estimated molecular weight for the entire mtDNA molecule was 16.7~16.8 kb. The composite mtDNA digestion patterns revealed twenty genotypes.

The means and standard deviation of the meristic count in each character are shown in Table 4. The mean number of vertebrae from the areas north of 50° N including OS, KR and WB showed greater than those from the areas south of 45° N including EK and PH. In the north area group the mean number of vertebrae was about 52 and in the south area group it was about 50. The results of data on the number of vertebrae is similar from that of Serobaba (1975). Hashimoto and Koyachi (1977) noted that the number of vertebrae increased in northly direction, which our data showed the same results.

ANOV test on 7 meristic counts showed significant differences among the seven geographic areas except for the number of the first and second analfin rays (Table 5).

FUTURE PLAN

From the genotypes revealed by mtDNA digestion patters, composition of the genotypes by area population and estimates of percent nucleotide divergence between populations will be calculated. Genetic distance between populations and log-likelihood G-test for heterogeneity among the populations will be analyzed for stock identification.

Scheffes test for significant differences of meristic character counts among the seven areas population will be analyzed.

Table 4. Means and standard deviation on meristic count of walleye pollock in the areas of the North Pacific from August 1997 to June 1999.

Meristic character	EK	PH	KR	OS	WB	CB	BB
No. of vertebrae	50.15	50.27	51.94	51.48	51.80	52.15	51.77
	(0.79)	(0.71)	(0.83)	(0.81)	(0.89)	(0.73)	(0.72)
No. of the 1st dorsalfin ray	12.79	13.22	13.67	12.92	13.11	13.56	13.60
	(0.78)	(0.87)	(0.72)	(0.92)	(0.88)	(0.90)	(0.89)
No. of the 2nd dorsalfin ray	16.96	17.06	17.00	16.44	16.73	16.49	16.48
	(1.49)	(1.36)	(1.60)	(1.32)	(1.16)	(1.10)	(1.39)
No. of the 3rd dorsalfin ray	20.74	20.87	20.83	19.95	20.38	20.58	20.58
	(1.16)	(1.28)	(1.38)	(1.20)	(1.34)	(1.03)	(1.39)
No. of the 1st analfin ray	22.00	22.15	22.73	21.70	21.91	22.19	22.28
	(1.25)	(1.12)	(1.53)	(1.30)	(1.42)	(1.41)	(1.45)
No. of the 2nd analfin	21.66	21.55	21.92	21.45	21.49	21.50	21.49
	(1.48)	(1.29)	(1.38)	(1.16)	(1.23)	(1.00)	(1.09)
No. of pectoralfin ray	20.46	19.83	-	20.33	-	20.15	20.33
	(0.68)	(1.03)	-	(0.86)	-	(0.68)	(0.73)
No. of sample	47	138	36	149	59	55	60

Figures in parenthesis denote the standard deviation.
EK: East coast of Korea, PH: Pacific coast of Hokkaido,
KR: Pacific coast of Kuril Is., OS: Sea of Okhotsk, WB: Western Bering Sea,
CB: Central Bering Sea, BB: Bogoslof of Bering Sea.

Table 5. ANOV F test of meristic count of walleye pollock from the seven areas of the North Pacific from August 1997 to June 1999.

Meristic characters	ANOV F value	DF (Numerator, Denominator)	$F_{(\alpha,\infty)}$ (p=0.01)	Conclusion
No. of vertebrae	81.19	6, 528	2.84	S
No. of the 1st dorsalfin ray	7.62	6, 516	2.84	S
No. of the 2nd dorsalfin ray	4.07	6, 514	2.84	S
No. of the 3rd dorsalfin ray	11.03	6, 531	2.84	S
No. of the 1st analfin ray	2.63	6, 516	2.84	NS
No. of the 2nd analfin ray	0.93	6, 530	2.84	NS
No. of pectoralfin ray	7.38	4, 376	3.37	S

S : Significant ($p < 0.01$), NS : Nonsignificant ($p > 0.01$).
DF: Degrees of freedom.
Data of pectoralfin ray counts were from four areas excluding Kuril Is. and western Bering Sea.

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Length and Age Structure of the Navarin Pollock Stock (Bering Sea) in 1995-1998 on the Basis of Polish Commercial Catches

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Materials for biological studies were collected on board of Polish commercial vessels fishing for pollock in Navarin area (Russian EEZ) in the periods of 1995-1998. The length structure was determined by measuring fish (fork length), chosen randomly and divided according to their sex at an accuracy 1 cm "below". During biological analysis individual pollock were measured and sex, body weight, gonads maturity (according to 8-grade Maier scale) were determined. Otoliths were removed for age determination. The time and place of collected samples were similar during the four years of investigations (Fig. 1). Taking this into account von Bertalanffy's growth curves for males and females were calculated on the basis of mean length at age from four years of investigation (1995-1998). The length-weight relationship was also calculated.

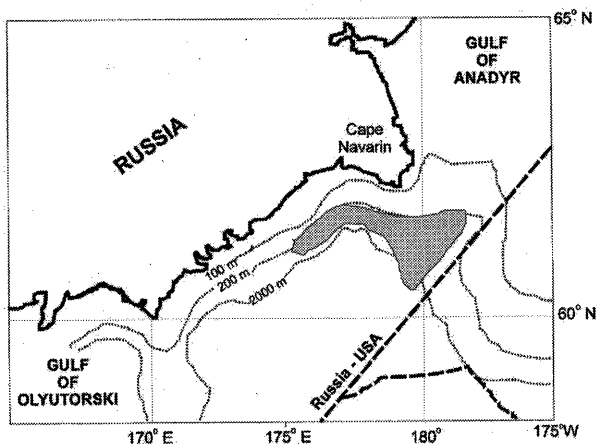

Fig. 1. Area of polish catches (hatched) of pollock in Navarin waters during the time of biological observations in August-October 1995-1998.

Length frequency in length classes is presented in Fig. 2. Length of pollock in commercial catches ranged from 24 to 60 cm. Fish with length over 60 cm were very scarce, their share in catches during four years was only 0.1%. Fish in lengths below 30 cm constituted only 2.0 %. Fish with length 35-45 cm predominated in the catches (63.8%). The mean length of pollock during 1996-1998 years was very stable – 40.4 cm, and only in 1995 it was equal 39.0 cm. The curves of length frequency in the catches differed considerably in shape. They are double-peaked in the years 1995, 1996, 1998 and single-peaked in 1997. The length distribution for males were generally similar to those for females but the curves for females were shifted towards greater lengths. As a result the mean length of females was greater than of males by about 1.0 cm.

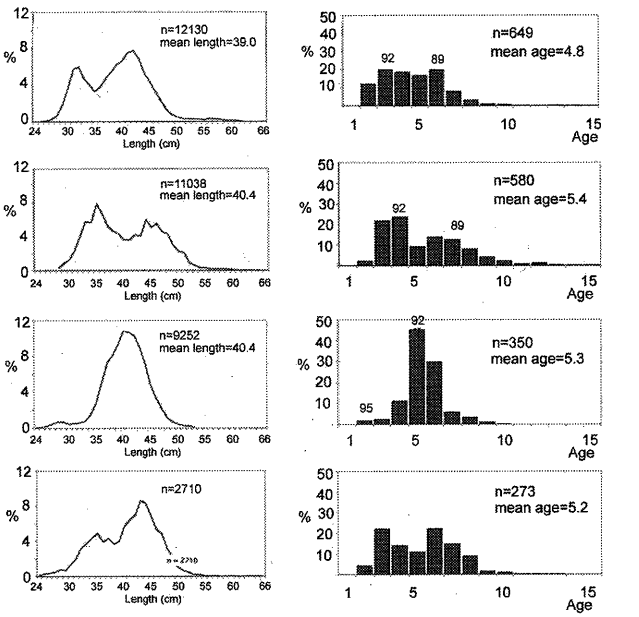

Fig. 2. Size and age composition of walleye pollock in Polish catches in Navarin area 1995-1998.

Age composition of pollock in Polish catches is shown in Fig. 2. Fish aged 2-21 years occurred with fish 3-7 years old dominating. Fish older than 14 years were very scarce. The 1992 year-class was the most abundant during four years of catches. In 1995 and 1996 the year class of 1989 was abundant in catches. The mean age of pollock was very stable during 1995-1998 (about 5.3) with the exception of 1995 when mean age of pollock was lower – 4.8 years.

Mean length at age indicated slightly faster growth of males during the first 4 years of fish life and faster growth of females at older age. The asymptotic length of females is about 7.5 cm higher than for males. The

parameters of von Bertalanffy's growth model for males and females are as follow:

Parameter	Males	Females
L_{inf}(cm)	54.46	62.12
K	0.205	0.156
t_o	- 1.641	- 1.886

Fig.3 shows the theoretical growth curve and observed mean length at age for the four years of catches.

The weight of pollock ranged from 100 to 2750 g. Mean weights at length classes are very similar during the four years of catches. The calculated values of the parameters for the length-weight relationship are as follows:

Parameter	Males	Females
k	0. 0072	0. 0082
n	3.008	2.969

This relationship are presented in graphic form in Fig. 4.

During four years of Polish catches in Navarin area any biological differences in exploited stock of pollock between years were noticed. The differences in length and age composition of catches were the results of strength of most abundant year-classes.

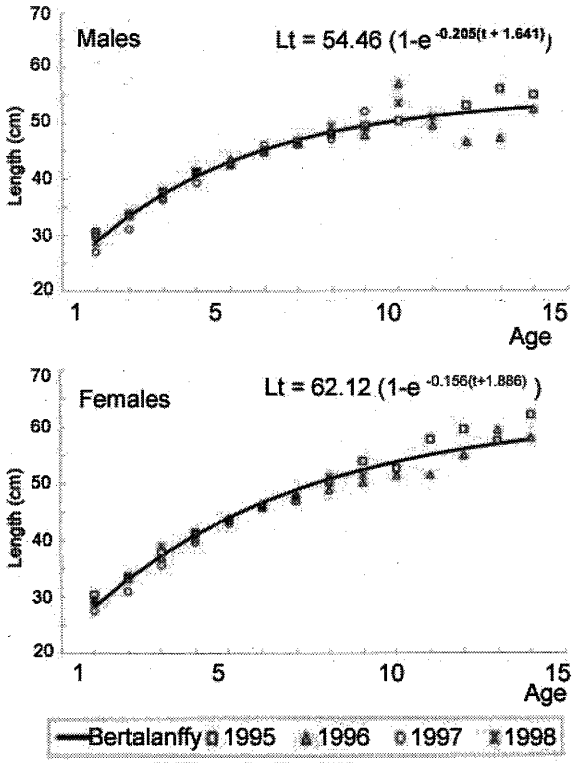

Fig. 3. Mean length at age and von Bertalanffy growth curve for pollock in Polish catches, 1995-1998, in Navarin area.

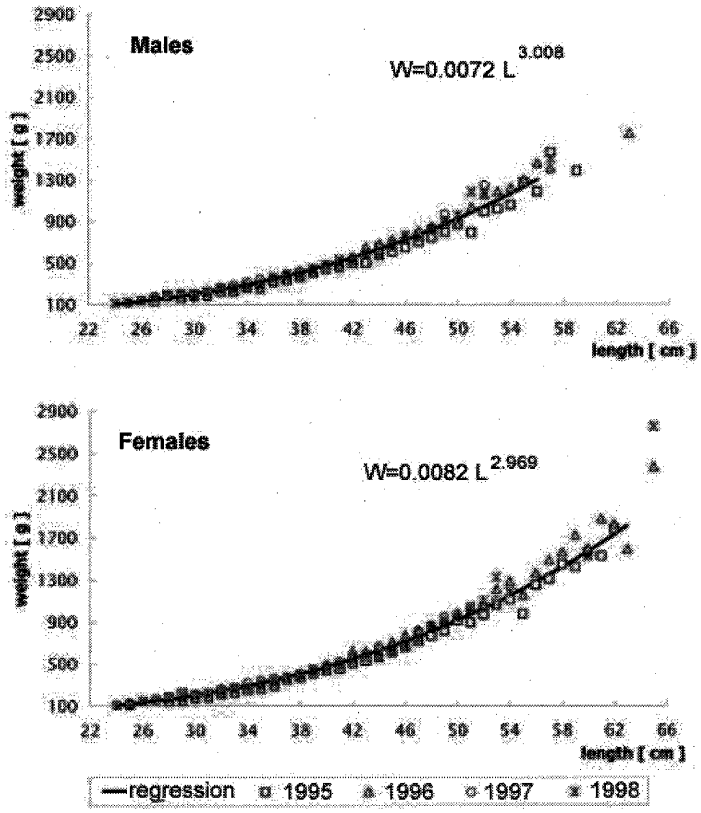

Fig. 4. Length-weight relationship of pollock in Polish catches, 1995-1998, in Navarin area.

Current Status of Walleye Pollock Stock in the North-Western Bering Sea

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Drastic changes in climate and hydrological regime that occurred in the Bering Sea during the last decade caused considerable transformation of the basin ichthyocenosises, fishes abundance and population structure.

A monitoring of commercial fishes stocks in the Bering Sea is being carrying out during last years by Russian Fishery Research Institutes: VNIRO, TINRO, KamchatNIRO. This monitoring includes regular bottom and mid-water surveys combined with echo-integration surveys, fry surveys, ichthyoplancton, zooplankton and hydrological surveys. These surveys provided a wide set of data that allowed us to assess the current state of pollock stocks in the north-western Bering Sea.

The 1990s were marked by a sharp decrease in the pollock abundance throughout its entire distribution area. This decrease changed the spatial distribution pattern of pollock in the north-western Bering Sea. The distribution became patched. The areas of pollock concentrations are clearly separated; the concentrations are composed of fishes of all age groups, from early juveniles to adults. This could be illustrated by the data obtained off Navarin Cape in June 1999 (Fig. 1-3). Dense pollock concentrations were found in areas of specific hydrological conditions—in quasi-stationary eddies (Fig. 4).

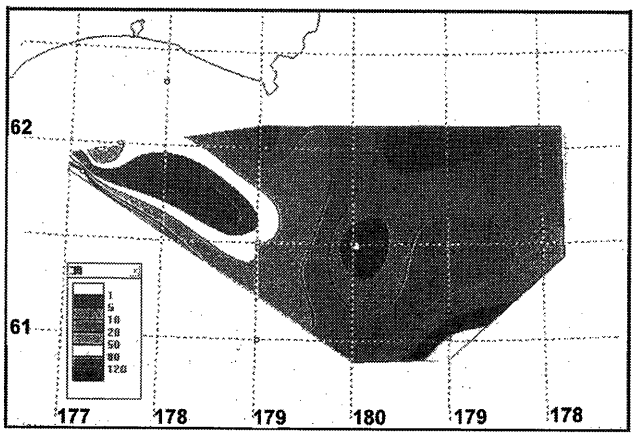

Fig. 1. Distribution of Navarin pollock, June 1999, bottom trawl survey, t/miles^2.

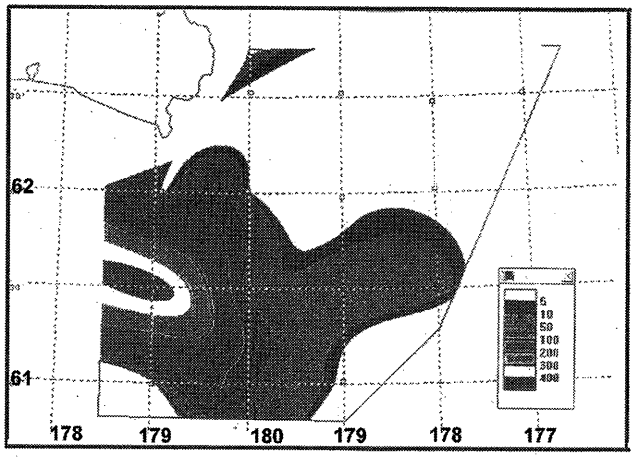

Fig. 2. Distribution of Navarin pollock age 3+ (1996yc), June 1999, fly survey, specimen/catch.

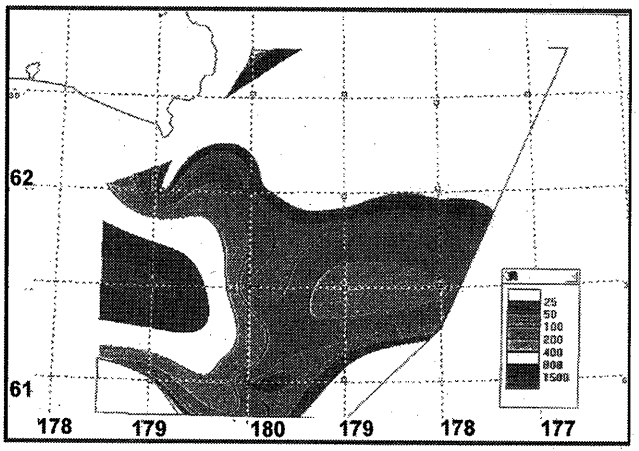

Fig. 3. Distribution of Navarin pollock age 2+ (1997yc), June 1999, fly survey, specimen/catch..

According to plankton surveys, these eddies create favorable conditions for development of phyto- and zooplancton. High abundance of primary producers and low trophic levels consumers in eddies attracts here predators of higher trophic levels, including pollock. Position of eddies in north-western Bering Sea is rather stable throughout the year. Due to low abundance of pollock, there is almost no competition between fishes for space and food, and high concentrations of pollock

remain only within quasi-stationary eddies. These eddies also create favorable conditions for pollock spawning as the egg and larvae are confined by the circulation and are not carried out to the deep water. This results in a patchy pattern of pollock distribution and decline of its migration activity as compared with periods of its high abundance. Fishery data from the north-western Bering Sea prove that there are no sharp changes in position and density of pollock concentrations during entire fishing season.

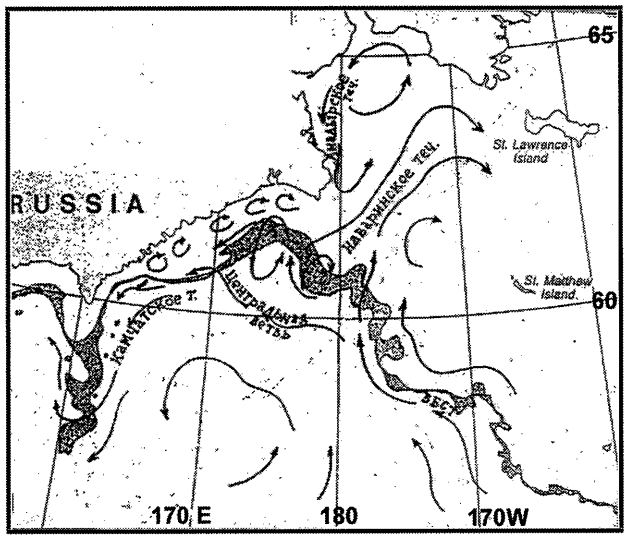

Fig. 4

A lifelong residence of pollock in the north-western Bering Sea to certain areas of quasi-stationary eddies suggests development of variations among fishes from different groups. For example, let us consider pollock shoals in the area from 173°E longitude up to the US-Russia Convention line. The most slender (thin) fishes were registered on the eastern Koryak shelf whereas to the west and to the east from this region there were found better fed individuals (Fig. 5).

Morphometric studies showed that the relative antherodorsal distance of pollock from the western Koryak shelf was significantly different from that of Navarin waters. From the other hand, the pollock from the eastern Koryak shelf shows clear difference from its western and eastern neighbors in relative antheanal distance and the altitude of the first dorsal fin (Fig. 6).

Variations in temperature, hydrological regimes, feeding conditions, and other parameters cause differences in gonadogenesis, energy metabolism, and dynamics of physiological processes. For example, in cold waters off Cape Navarin, the spawning of pollock occurs later than in southern parts of the Bering Sea. The onset of post-spawning feeding period is also delayed as compared with pollock groups from warmer south-western regions. As a result, in the beginning of summer, the hepatsomatic and cubic condition indices of pollock from Navarin region are lowest in the north-western Bering Sea (Fig. 7), while gonadsomatic index of males which finishing the spawning later then females is the highest (Fig. 8). The gonads and liver condition of pollock from different locations testify indirectly that its spawning and feeding take place in the same area. If pollock have migrated to the cold-water feeding grounds from some warmer-water areas, its gonadsomatic and hepatosomatic indices would be similar with those of specimens from warmer areas. However, such similarity is actually absent.

Specific oceanographic conditions in the areas of pollock concentrations cause differences in annual cycles of generative and plastic metabolisms in the specimens from different concentrations. As it was already mentioned, in Russian Exclusive Economic

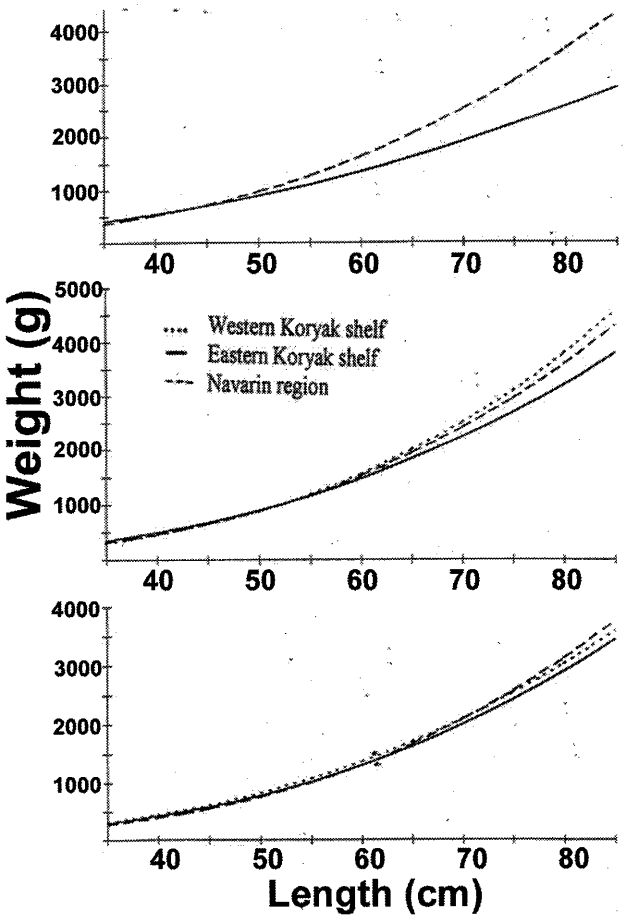

Fig. 5. Length-weight relationships for North-Western Bering Sea pollock, July-August.

Zone of the Bering Sea, the pollock from the north-eastern parts inhabit more colder waters than that from southern parts. Consequently, in the north-eastern survey areas its feeding season is shorter than in the western ones. Therefore, pollock from the north-eastern Koryak shelf and waters off Cape Navarin reveal higher rate of nutrients accumulation than the specimens caught in the Olyutor Bay and over the Shirshov Ridge. However, sharp increase of average gonadsomatic index in pollock from north-eastern Koryak shelf in July-September indicates that in this region the pollock spend energy primarily to the generative metabolism (Fig. 8). The specimens caught off Cape Navarin primarily accumulate energy in the liver (Fig. 7).

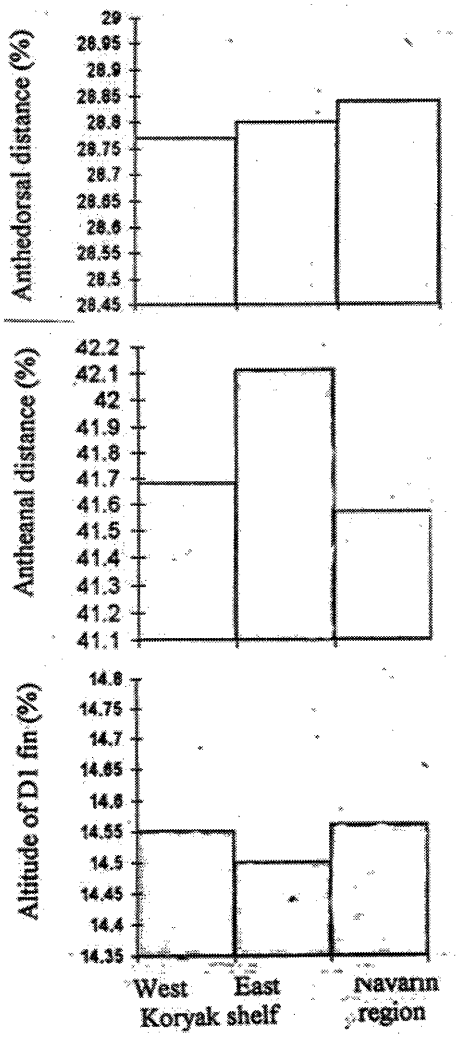

Fig. 6. Morphometric characteristic of North-Western Bering Sea pollock.

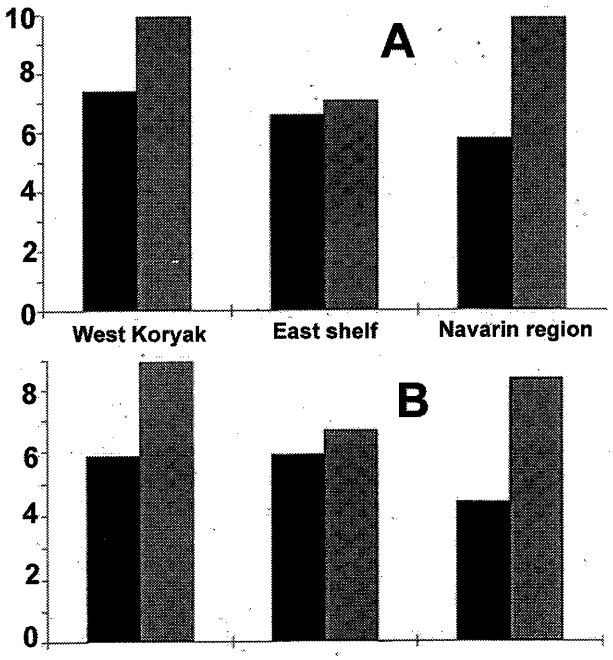

Fig. 7. Seasonal changes in hepatsomatic index of North-Western Bering Sea pollock (A) for females, (B) for males, %. Black-July, grey-September.

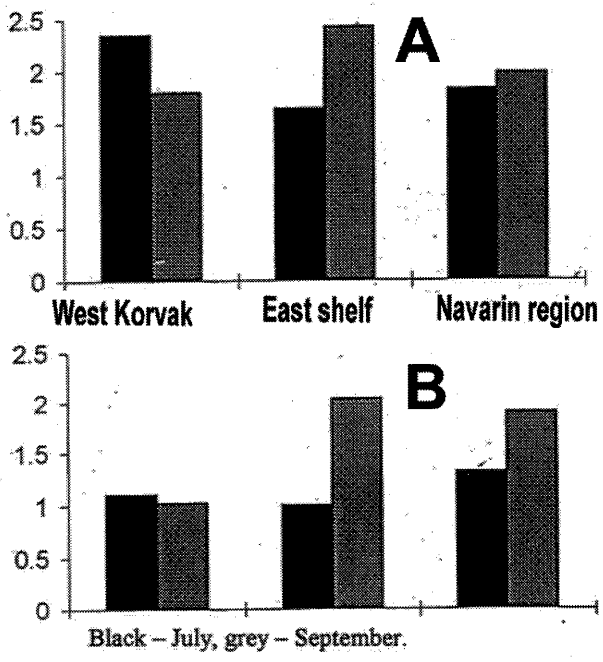

Fig. 8. Seasonal changes in gonad-somatic index of North-Western Bering Sea pollock (A) for females, (B) for males, %.

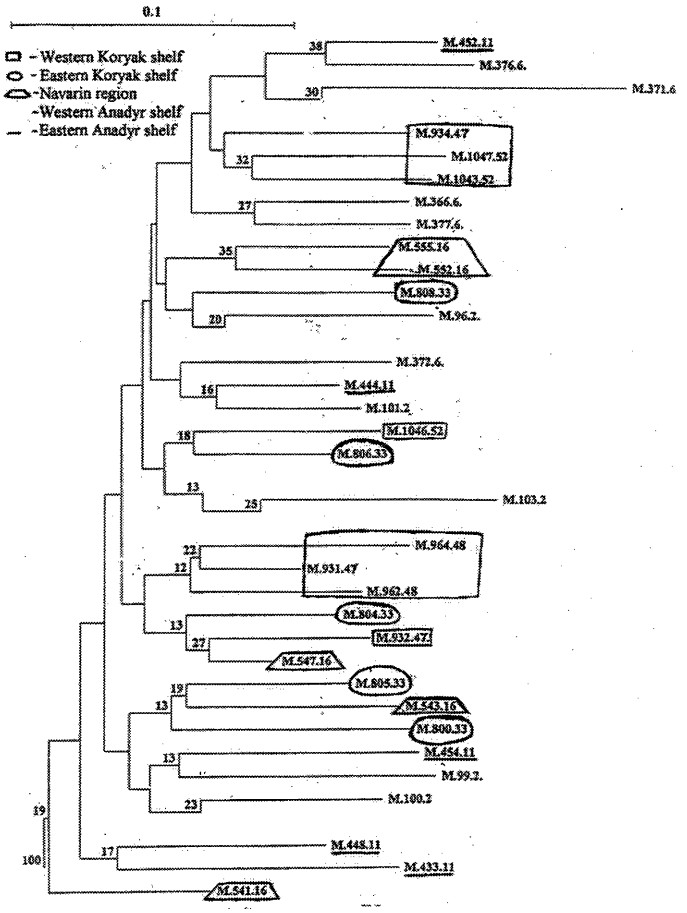

Fig. 9. Genetic tree on the base of differences between DNA molecules of North-Western Bering Sea mature pollock males.

To assess the genetic status of the variations, found in pollock in the Bering Sea, VNIRO scientists turned to Drs. Shubina and Mednikov, from the Biology School of the Moscow University, who analyzed molecules of chromosome DNA of spawner males by the method of arbitary primers-randomly amplified polymorphic DNA using a matrix of pairwise combination of primers (Welsh and McClelland) followed by amplificate electrophoretic fractionating. Reconstruction of the tree based on genetic distances between the objects revealed absence of variations of the population rank of pollock caught in the area from the western Koryak shelf up to the Anadyr shelf and pollock taken off Navarin Cape (Fig. 9). Dr. Katugin will discuss absence of polymorphism of esterases and transferrines in local pollock. Dr. M. Iwata also failed to find difference in polymorphism of tetrazolium oxidase within Asian pollock population (Iwata, 1975). Therefore, we could assume that presently there is no genetically significant difference between individuals caught in the area from Olyutor Bay up to Navarin Cape. Nevertheless, the existence of prominent morphological and physiological peculiarities of pollock from different groups in the given area and a low migration activity of the specimens allow us to consider large groups, for example those off Cape Navarin, as separate sub-units of the stock.

Summarizing the discussion, we could make the following conclusions: 1. Quasi-stationary eddies of the north-western Bering Sea are habitats of individual pollock groups which differ in some biological characteristics. During periods of low abundance of pollock, the reproductive isolation occurs between areas of pollock concentrations. 2. Analyses of chromosome DNA showed no genetic variations between individual groups. This could be associated with a possible exchange of specimen during periods of a larger abundance of pollock when location borders grew less prominent. There is no time for genetic difference to develop because periods of high abundance alternate with periods of low abundance and the interval could be no more than several decades.

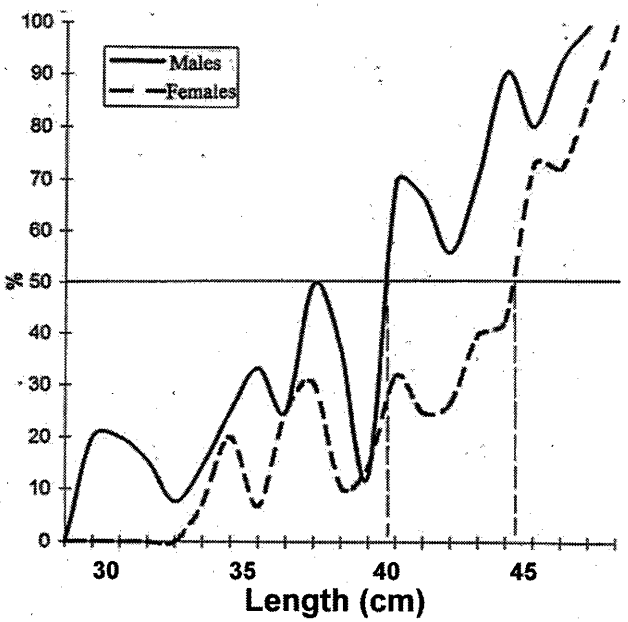

Fig. 10. Maturation curve of North-Western Bering Sea pollock, June 1999.

What are forecasts of further changes in the status of pollock stocks in the north-western Bering Sea? Among reasons of a sharp decline of the pollock number in the early 1990s we could name weak-year classes of 1993 and 1994. Then there was a certain stabilization of the stock abundance supported by three strong-year classes of 1995-1997. The 1999 fry survey showed that

the 1998 year-class was not strong in the north-western Bering Sea. Moreover, sexual maturation of the local pollock is late at the present moment. In 1996-1997 the first mature females were 24-25 cm long and males made 23 cm. Majority of females maturate being less than 40 cm long and mature males were below 35 cm. In 1999 half of females maturated being 45-46 cm long and mature males are 40-41 cm long (Fig. 10). Early sexual maturation of previous years was one of the reason of growing biomass of spawning population and production of strong-year classes. According to estimations by Dr. Babayan, from VNIRO, spawning pollock attained its maximum of 81.2% of the total commercial stocks in 1997. Shifting of maturation to a later age could cause reverse processes and lead to production of several weak generations in the nearest future. Therefore, we could not expect an increase of pollock biomass in the north-western Bering Sea.

However, the current status of ichthyocenosises in the Bering Sea is no unique. In the 1970s, the levels of abundance as well as biology of pollock and other fish species such as cod, one-finned greenling and so on were comparable with the pattern of the 1990's. The VNIRO results and Dr. Andriyashev's data on distribution of commercial fish species indicate that the status of modem ecosystems of the Bering Sea and relevant ichthyocenosises are similar to the pattern of the late 1920s and early 1930S. Consequently, next decade we can possibly see another boom of pollock abundance after the present depression.

Limits of Genetic Methods for Defining Stocks of Walleye Pollock

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ABSTRACT: The identification of demographically independent population units in a harvested species is essential for devising sound management strategies that will ensure the perpetuation of harvested stocks. Researchers in the United States have applied several methods to defining distinct population segments. Population segments can be defined with phenetic variability that reflects demographic or life-history responses to spatial or temporal environmental changes. Some aspects of population structure can also be discerned by molecular genetic methods. These methods measure inherited differences among populations that arise as a result of geographical or ecological isolation. Genetically defined stocks, however, may or may not coincide with stocks defined by demography and life history variability. Different aspects of stock structure are detected by different methods because each method is sensitive to population processes that operate on various spatial and temporal scales. Distinct population morphologies or elemental profiles, for example, can arise on short time scales in response to temperature, salinity, and food availability. In contrast, genetic differences for some phenotypic traits may appear only after many more generations, especially for large populations. The useful time scales for molecular genetic methods are varied, depending upon the mutation rate of the kind of genetic material being surveyed and the amount of random genetic drift in a population. The analysis of slow evolving genes through protein electrophoresis or DNA sequencing can be used to detect population differences arising on scales of thousands to millions of years. More rapidly evolving non-coding DNA, such as microsatellite loci or pseudogenes, can potentially detect differences among populations arising over shorter periods of time. A problem in fisheries management has been to recognize the limitations of some methods in defining independent population units and to match the time scales of phenetic and molecular genetic methods to management requirements

INTRODUCTION

The prevailing paradigm in fishery management is to ensure the life of a stock, while maximizing its harvest. Central to this concept is the identification of groups of fish whose recruitments and mortalities, and hence abundances, are largely independent of other groups of fish. These groups are then managed as separate entities. Defining the geographical extent of a stock can greatly influence the accuracy of estimating abundance trends and of anticipating the effects of harvest. For example, if abundance estimates for a geographical stock are too large, quotas may be set too high, and the stock may be over fished; if quotas are too low, potential harvests may be lost. For depleted, but isolated stocks, the amount of migration into areas of overharvest may not be sufficient to replenish the stock on time scales useful to management. Another problem may be that stocks isolated from one another on spawning grounds may mix at other times. In these cases, less abundant stocks may be inadvertently overharvested, if fishing quotas are based on overall abundance or on the abundance of the larger stock.

Molecular genetic methods have proved to be highly successful for identifying distinct population segments of freshwater and anadromous fishes, which usually consist of semi-isolated populations. However, the usefulness of these methods to marine populations has been limited. Marine fishes differ from freshwater and anadromous species by having a greater potential for movement between populations, because of fewer physical barriers to migration in marine waters. The problem of stock identification in marine fishes is therefore more difficult to address because high levels of interbreeding between populations (gene flow) tend to prevent large differences from arising between populations. Since the genetic signal indicating stock structure commonly is weak, sample errors may dwarf the indications of stock structure, or some techniques may not be powerful enough to detect small differences between stocks.

The use of some population genetic models to interpret the results of population studies and to estimate levels of gene flow between populations also can be problematic. These models often assume equilibrium patterns in gene flow and constant population size. In nature, dispersal and the geographical boundaries of a stock often change over short periods of time in response to environmentl changes, so that gene flow estimates may be inaccurate and misleading. For example, decadal climatic cycles, such as the North Atlantic Oscillation (NAO) and the Pacific Decadal Oscillation (PDO), can produce shifts in migration patterns and

geographic range. Another, often unappreciated, fact is that genetic population structure, as measured by molecular methods, represents the cumulative effects of past events, so that estimates of gene flow may not reflect the present-day dynamics of migration.

The goal of this paper is to explore the limitations of various methods to infer stock structures of marine fishes, and of walleye pollock in particular. Numerous reviews on the use of genetic methods to identify stocks have been written recently, and important conclusions from these reviews will be highlighted here (Sinclair and Solemdal 1988; Smith *et al.*. 1990; Carvalho and Hauser 1994, 1998; Ward and Grewe 1994; Pawson and Jennings 1996; Hauser and Ward 1998; Shaklee and Bentzen 1998; Waples 1998; Grant *et al.*. 1999). The level of stock differentiation in walleye pollock is expected to be low, because pollock appear to be characterized by high levels of gene flow between populations (Bailey *et al.*. 1999). Detecting these low levels of differentiation presents a challenge in choosing appropriate methods and in applying statistically powerful procedures to analyze the data.

MECHANISMS PRODUCING STOCK STRUCTURE

To devise a method of detecting stock structure, we must first understand the mechanisms producing demographic and genetic differences between stocks that are important to management. Although population biologists often use idealized populations to simplify the analysis of data and to build models, it must be understood that few natural populations exhibit all the characteristics of these idealized populations. One concept in population genetics is the hypothetical panmictic population, in which each individual has the same chance of mating with other individuals of the opposite sex (Fig. 1A). In reality, individuals are often isolated from potential mates by life history traits that limit migration. In these circumstances, demographic and phenetic differences can arise between groups through isolation by distance (Fig. 1B). Current and temperature shifts, and behavior may produce permeable barriers between stocks and allow stocks to respond independently to regional differences in food availability or temperature (Fig. 1C). Most open-ocean marine fishes are thought to consist of partially isolated stocks showing low levels of divergence, and it is these very low levels of divergence that managers attempt to detect.

As isolation continues, demographic, morphological, and genetic differences between stocks become greater, and "stock" differences may be marked by morphological traits or recognizable as subspecies differentiation (Fig. 1D). If the cause of isolation, for example a physical barrier to migration, is relaxed, the differentiated stocks may reassociate and interbreed (Fig. 1E). Other stocks in secondary contact may not interbreed, if strong genetic differences have accumulated over long periods of time. Clines in character measurements or gene frequencies, or abrupt genetic or morphological discontinuities may form, depending on the genetic compatibility of the differentiated stocks and recency of contact between the previously isolated stocks. Stages 1B, 1C, and 1E appear to be present in walleye pollock.

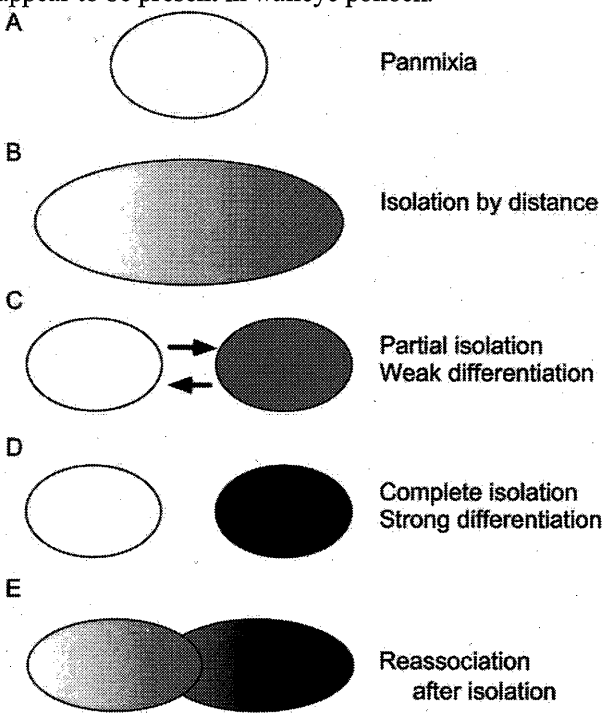

Figure 1. Models of population structure and differentiation.

Table 1. Factors influencing genetic divergence between populations.

Factor	Chief determinant
Random genetic drift	Effective population size (N_e)
Gene flow	Success matings of individuals from non-natal populations
Natural selection	Environmental variables, such as temperature, salinity, etc.
Mutation	Flawed DNA replication; mutagenic compounds
Length of isolation	Long periods of isolation lead to greater genetic change than do short periods of isolation

Several factors can bring about phenetic and genetic changes in an isolated stock (Table 1). Natural selection is most important in influencing adaptive life-history, and morphological traits. Important selective agents in the marine environment include temperature, salinity, food availability, predators, and pollutants, among many others. Other than the elimination of deleterious mutations by purifying selection, natural selection appears to be unimportant in explaining the geographical distributions of most molecular genetic markers. Instead, the distributions of molecular genetic population markers appear to be most influenced by random genetic drift, especially in small populations (or in populations in which a few parents contribute large numbers of offspring). Gene flow, or the movement of individuals that effectively breed in non-natal populations, counters genetic drift and, in some cases, can swamp the effects of natural selection. High levels of mutation can also produce differences between populations and may provide the resolving power for highly polymorphic microsatellite loci. In addition to these fundamental processes, the duration of isolation influences the extent of differentiation between populations, because of increased divergence reflecting the accumulated effects of population events.

METHODS USED TO DEFINE STOCKS OF WALLEYE POLLOCK

The methods used to distinguish between stocks fall into two major groups: 1) phenetic methods, which survey traits that are strongly influenced by environmental factors, and 2) genetic methods, which survey inherited traits influenced indirectly by population variability. The strengths and limitations of these two general methods are discussed briefly in the following sections.

Phenetic Methods

Phenetic methods of stock identification include the analysis of such things as life history variability (*e.g.*, spawning timing), growth rates, morphological variability (*e.g.*, morphometric and meristic variability), and demography (*e.g.*, fecundity, age structure, mortality), among others (Table 2). Although some of these traits may have a broad genetic basis, they are generally strongly influenced by environmental factors over the life time of an individual or over a few generations. Phenetic differences can arise among populations in response to environmental variability among areas and can sometimes be used to infer the degree of independence among stocks. These differences are often not detectable with neutral genetic markers (*e.g.*, Atlantic herring; Ryman *et al.*. 1984).

Table 2. Methods of stock identification.

Trait	Characteristics
Phenetic	Both genetic and environmental influences possible
Life history variability	Genetic and environmental influences possible
Migration patterns	
Timing of spawning	
Growth rate	
Age at maturity	
Fecundity	
Recruitment	
Mortality	
Morphology	Genetic and environmental influences possible
Morphometric variability	
Meristic variability	
Physical tags	No genetic influence
Parasites	Chiefly environmental, possible genetic influence
Elemental profiles	Chiefly environmental, possible genetic influence
Molecular genetic	Predictable genetic inheritance
Protein electrophoresis (allozymes)	Biparental genetic inheritance
	Recombination during reproduction
Nuclear DNA	Biparental inheritance
Coding regions (genes)	Recombination during reproduction
Non-coding regions	
Microsatellite DNA	
Pseudogenes	
Introns	
Organellar DNA	
Mitochondrial DNA	Maternal inheritance
	No recombination during reproduction

Phenetic methods also include the analysis of acquired or natural tags, which lack a genetic basis. These include physical tags that are attached to a fish and later recovered (*e.g.*, Kimura *et al.*. 1998; Metcalfe and Arnold 1997), parasites that are characteristic of specific regions because of differential resistance or occurrence (*e.g.*, Konovalov 1995; Speare 1994; Urawa *et al.*. 1998), and elemental profiles (*e.g.*, Edmonds *et al.*. 1991; Fowler *et al.*. 1995; Thorrold *et al.*. 1998) that reflect local environmental conditions or diets. These tags provide evidence of movement of individuals from one place to another, but not necessarily of gene flow and population connectivity. Since these kinds of population markers are not inherited, they must be

applied each generation or must arise anew each generation.

Mulligan *et al.*. (1989) analyzed the elemental composition of the juvenile portion of otoliths from pollock collected in the eastern Bering Sea. They found elemental profiles that indicated differences between three areas: southeastern shelf, northwestern shelf and the Aleutians. Fish could be assigned to their capture locations based on the juvenile "fingerprint" with an accuracy of 70 to 85%. These results indicate limited dispersal of adults among these three broad areas in the Southeastern Bering Sea. In a more recent study of elemental profiles in otoliths, Severin *et al.*. (1995) examined samples from five localities in the Southeastern Bering Sea and the Western Gulf of Alaska. They found that after correcting for age/length measurable differences existed between areas and that fish could be reclassified to capture locality with 6- 80% accuracy.

Molecular Genetic Methods

Molecular genetic population markers are similar to applied tags, except that every individual in the population possesses tags that are inherited in a predictable fashion from one generation to the next (Table 2). Genetic tags are assumed to remain unchanged over the lifetime of an individual. Unlike physical tags, however, individual genetic tags are generally not unique to individuals or to populations so they are not absolute markers of population origin. Populations, therefore, must be characterized by a suite of gene frequencies that can be used to test for population differentiation. These frequencies are usually stable from one year to the next. Various statistical procedures are used to test hypotheses of stock structure, but the power of these procedures to detect biologically meaningful units depends on several factors including sample size, the distribution of samples, and levels of polymorphism of the genetic marker.

Several molecular genetic markers have been used to estimate stock structure. Some of these markers are more suited to addressing particular questions than are other markers. Variability in genes or DNA located in the nucleus can be assayed indirectly by the analysis of proteins encoded in DNA. One technique, protein electrophoresis, has been the mainstay of stock identification for several years, especially for freshwater and anadromous species which generally have low levels of gene flow, and hence moderate to high levels of genetic differentiation between populations. Marine fishes, in contrast, tend to have higher levels of gene flow, which leads to less differentiation among populations. Differences between these groups are reflected in estimates of F_{st}, a relative measure of allele-frequency differentiation that ranges from 0.0 (no difference) to 1.0 (fixed allele-frequency differences). The median F_{st} in a large collection of published studies is 0.14 for freshwater fishes, 0.081 for anadromous salmonids, but only 0.02 for marine fishes (Ward *et al.*. 1994; Waples 1998) (Table 3).

Protein electrophoresis has limited value in detecting local stocks of many marine fishes. One exception is for fish populations that are subdivided by coast line topography or strong oceanographic gradients. Examples of this are adjoining populations of

Table 3. The proportion of the total gene diversity (H_T) within a species that is due to subdivision among population (F_{st}). Estimates of H_T and F_{st} are based on variability for proteins detected with electrophoresis. (Summarized from Ward *et al.*. 1994 and Waples 1998).

	Number of species	Average number of populations	H_T	F_{ST} Mean	F_{ST} Median
Freshwater	49	5.9	0.062	0.222	0.144
Anadromous	7	13.1	0.057	0.108	0.081
Marine	57	6.4	0.064	0.062	0.020

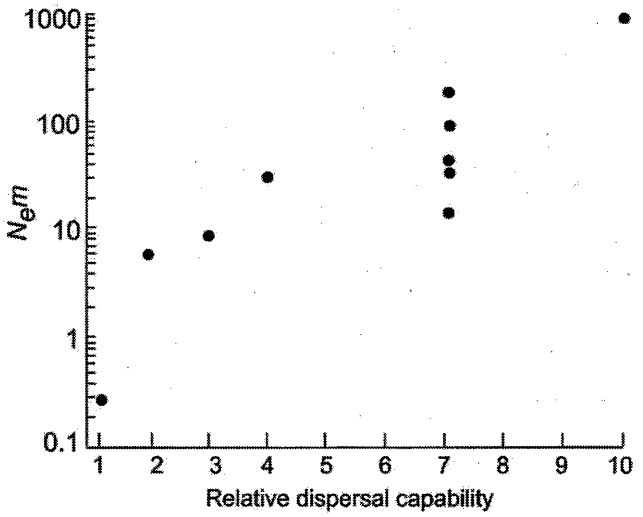

Figure 2. Relationship between dispersal capability and genetic estimates of gene flow from F_{ST} in near shore and reef fishes. N_em is the number of migrants that breed in each population. Data from Waples (1987).

Mediterranean anchovies in the Adriatic Sea that are apparently isolated by strong oceanic gradients (Bembo *et al.*. 1996) and rockfish populations that are isolated by onshore currents (Rocha-Olivares and Vetter 1999). Another exception is for fish which have limited larval and adult dispersal so that local populations are at least partially isolated from other populations, but without strong barriers to dispersal. For example, Waples (1987) found an inverse relationship between F_{st} and the extent of adult and larval dispersal in reef and near-shore fishes (Fig. 2).

On very broad spatial scales across the North Pacific, protein electrophoretic studies of several species detected population differences that apparently resulted from isolation in the distant past. An example of this ocean wide subdivision can be found in Pacific herring (Fig. 3)

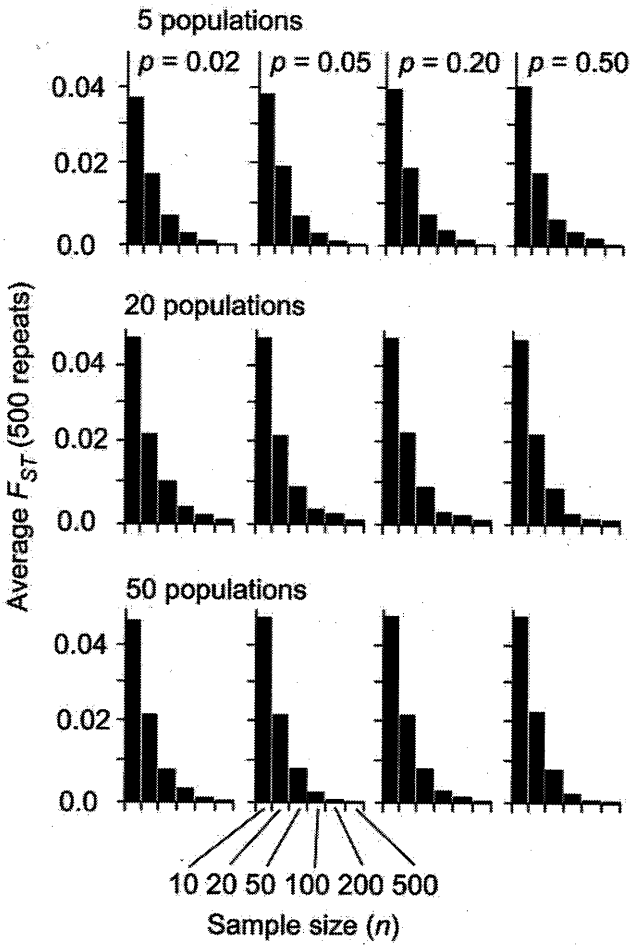

Figure 3. Sources of error ("noise") in estimating F_{st} in natural populations. Simulations incorporated allele frequency (p), the number of populations sampled, and sample size (n). Each estimate of error was based on 500 resamplings of a hypothetical set of populations with no gene frequency differences among them.

(Grant and Utter 1984) and in several other marine species (Grant *et al.*. 1983, 1984, 1987). Few allozyme differences, however, have been detected among oceanic populations in each of these groups. A similar North Pacific Ocean discontinuity in gene frequency has been observed for the enzyme superoxide dismutase (Sod) in walleye pollock (Iwata 1973, 197Sa, b; Grant and Utter 1980). In this case, the demarcation between the two oceanic groups appears to be located on the Asian side of the Bering Sea or in the Okhotsk Sea. These ocean-wide differences indicate that fish generally do not disperse over large distances across the North Pacific. If they did, gene frequency differences across the North Pacific would disappear. The lack of mixing also implies that partially isolated stocks may exist on a smaller geographical scale. The presence of ocean-wide gene frequency differences may provide the basis for identifying stocks and dispersal pathways between stocks in areas of mixing between the two major groups.

Mitochondrial DNA

One widely used method of population analysis is the use of sequence or RFLP (restriction fragment length polymorphism) data for mitochondrial DNA (mtDNA), which codes for several genes that are not found in the cell nucleus. mtDNA differs from nuclear DNA (nDNA) in two important ways. One way is that recombination is lacking in mtDNA, so that gene combinations (haplotypes) are passed unaltered from one generation to the next, except for new mutations. A second way is that mtDNA is inherited from only the maternal parent in most fishes, so that gene phylogenies correspond to female lineages. A greater amount of random genetic drift among populations is expected for mtDNA genes, because the effective population size for mtDNA is about one fourth of that for nuclear genes. These characteristics permit phylogeographical analyses of mtDNA haplotypes, which can potentially indicate dispersal pathways for females and the extent of gene flow between populations (Avise *et al.*. 1987).

mtDNA has been used to study walleye pollock, but with little success in detecting population groupings. Mulligan *et al.*. (1992) sampled four localities: 1) Gulf of Alaska, 2) the "donut hole" in mid Bering Sea, 3) Bogoslof Island in the southeastern Bering Sea, and 4) Adak Island in the Aleutian Archipelago. Tests of haplotypic frequencies showed significant differences between the Adak Island sample and the three other samples. Nevertheless, the overall level of differentiation between these samples was small (F_{st} = 0.019) and was similar to the level detected with allozymes (Grant and Utter 1980). The apparent lack of stock structure in the Bering Sea may lie with the failure to sample populations during spawning, when stock

separation is expected to be largest.

A second study of mtDNA variability in the Bering Sea populations showed another pattern of differentiation among samples. Shields and Gust (1995) sampled walleye pollock from six areas: 1) western Bering Sea, 2) northwestern Bering Sea, 3) the "donut hole", 4) Aleutian Islands, 5) southeastern Bering Sea, and 6) Gulf of Alaska. None of these samples differed from each other in pairwise tests. However, the comparison between samples 1-2 combined (western Bering Sea) and samples 5-6 (eastern Bering Sea-Gulf of Alaska) was significant. These results indicate at least some east-west differentiation across the Bering Sea. The samples for this study also appear to have been collected out of the spawning season when stocks may have been mixed.

Microsatellite DNA

Microsatellite DNA markers can potentially detect stock structure on finer spatial and temporal scales than can other DNA or protein markers, because of higher levels of polymorphism found in microsatellite DNA (reflecting a high mutation rate). When populations are at least partially isolated, genetic markers at loci with high mutation rates may accumulate more rapidly in some areas than in others. In Atlantic cod, the analysis of microsatellite loci resolved fine scale genetic differences between stocks that were not isolated by any apparent barriers to gene flow (Bentzen *et al.*. 1996). Early studies of microsatellite variability in pollock showed variable results, possibly because of technical difficulties in the DNA analysis itself or because of the sampling of mixed populations outside spawning areas. More recent studies of pollock populations with microsatellites are based on improved technologies and on spawning- area samples (O'Reilly *et al.*. this volume; Seeb *et al.*. this volume).

METAPOPULATION STRUCTURE

Even in abundant species, local populations may not be stable through time. Populations inevitably grow and contract in response to regional variability in ocean-climate cycles, shifts in currents, disease, predation, and food availability. Populations in some areas may become extinct or may expand into previously unoccupied habitats, especially during periods of population growth and high abundances. Populations within a metapopulation are usually connected by dispersal and gene flow that serves to limit genetic divergence between populations and to aid in the colonization of unused habitats. Bailey *et al.*. (1999) suggest that walleye pollock also consist of an ocean-wide metapopulation, in which several major populations isolated from each other by distance, geographical barriers to dispersal, currents, and possibly by the timing of spawning. For example, strong genetic differences between western North Pacific populations and eastern Bering Sea populations indicate little connectivity between these population groups. Within these major groups, however, only weak genetic differences have been detected among areas (*e.g.*, Mulligan *et al.*. 1992; Shields and Gust 1995). Nevertheless, the occurrence of temporal allozyme frequency differences in some areas (Seeb *et al.*. this volume), and the occurrence of distinct trace elemental profiles of fish in different areas (Mulligan *et al.*. 1989) indicates the lack of panmixia in some relatively small areas, at least on short time scales.

SUMMARY OF PHENETIC AND GENETIC STUDIES OF POLLOCK

These studies together lead to four important conclusions. One is the presence of broad-scale differences across the North Pacific Ocean that most likely resulted from isolation by ice age cooling and sea level fluctuation. A second observation is that small-scale genetic population structure appears to exist within and between the Bering Sea and Gulf of Alaska, but the exact nature of this structure has not been well understood. If demographically independent stocks of pollock exist in the Bering Sea, they may not have been isolated from one another for a sufficient length of time to show detectable levels of genetic differentiation from each other. The sample designs of these studies have been weak, because samples were not always collected during the spawning season when the greatest amount of stock separation would be expected. Sample sizes may also have been too small to detect the low levels of differentiation expected in a high gene flow species. A third observation is that molecular genetic techniques that assay portions of DNA with high mutation rates may be required to resolve the fine scale population structure of pollock. Alternatively, high levels of gene flow may prevent the appearance of genetic stock structure. Lastly, if walleye pollock consist of subpopulations with constantly changing abundances, patterns of dispersal, and geographical boundaries, a complex sampling scheme over several years may be required to understand the genetic and demographical dynamics of walleye pollock in the Bering Sea.

DETECTING STOCK STRUCTURE IN HIGH GENE-FLOW SPECIES

Apart from choosing appropriate gene markers, biologists are faced with several analytical problems in

attempting to detect stock structure in high gene flow species. These problems can be illustrated by examining potential sources of error in the statistic F_{st}, which is commonly used to measure the magnitude of differentiation among populations. This statistic or its analogue is applied to allele-frequency data generated by any of several methods, including protein electrophoresis and the analyses of nuclear DNA (*e.g.* microsatellite loci, pseudogenes, and introns), and to mtDNA haplotype frequencies. F_{st} is defined as the ratio of the observed variance in gene frequencies to the maximum possible variance given fixation of alleles in different populations in the same proportion as the gene frequencies.

$$F_{st} = \sigma^2 / (pq) \qquad \text{Equation (1)}$$

Where σ^2 is the observed variance, pq is the maximum theoretical variance, and $q = 1 - p$. When frequency data for several polymorphic loci are available, F_{st} is usually based on the average over loci. Values greater than 0.05 represent substantial divergence among populations. Nevertheless, values less than 0.05 may reflect biologically significant differences among populations. Since F_{st} is calculated from gene frequencies, the factors that affect the accuracy of estimating gene frequencies in populations and random differences among loci also affect the accuracy and precision of F_{st} estimates. These include sample size, the number and level of polymorphism of the loci, and number of populations sampled.

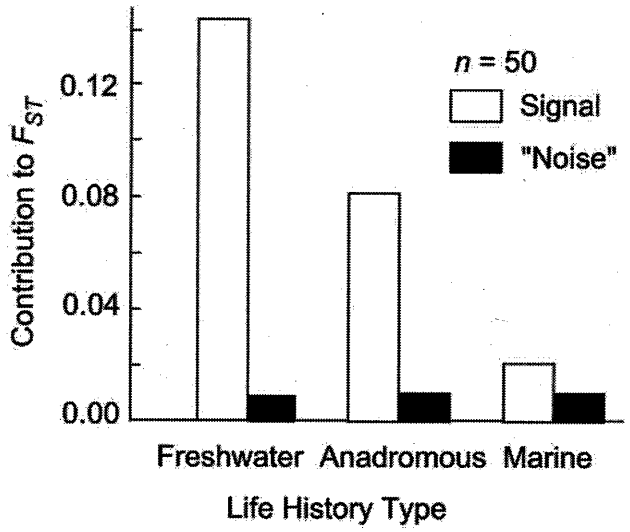

Figure 4. The signal to "noise" ratios in estimating F_{st} in species of fishes with different life-history patterns. F_{st} values represent the median value in studies of natural populations. "Noise" was estimated by $1/2n$, the expected error for a given sample size. F_{st} values for the three life-history types were taken from Ward *et al.*. (1994), and Waples (1998).

The relative contributions of some of these errors can be seen in the results of simulations of hypothetical populations with identical gene frequencies (Fig. 3). One result is that various levels of polymorphism appear to introduce about the same level of error in estimating F_{st}. The use of low frequency alleles (p = 0.02) introduces about the same level of error as does the use of alleles with intermediate frequencies (p = 0.50). Another result is that the level of error in estimating F_{st} increases slightly as the number of populations sampled increases. The largest source of error, however, is from estimating gene frequencies with small samples. For freshwater and anadromous species the error or "noise" relative to the signal of stock structure is small, but for high gene flow marine species, the error in F_{st} can be about the order of magnitude as the level of differentiation between stocks, when small sample sizes are used (Fig. 4).

ESTIMATING DISPERSAL FROM GENE FLOW

For most commercially important species of marine fishes, estimating levels of gene flow and dispersal are important for devising management and conservation strategies. If a stock is depleted by overharvest, how soon will it be replenished by dispersal from other populations? Even if harvesting is not a concern, some populations may not be self supporting because of the loss of recruits from the population and the reliance on recruits from other source populations Physical tags can indicate dispersal patterns, but tag data alone do not indicate how much gene flow occurs between populations.

One common approach to estimating gene flow is to determine the level of divergence between populations from a set of molecular genetic data with F_{st}, and then estimate the number of genetically effective migrants ($N_e m$) with an approximation of the island model of migration (Wright 1943),

$$F_{st} \approx 1 / (1 + 4N_e m) \quad , \qquad \text{Equation (2)}$$

where N_e is the effective population size and m is the proportion of breeding individuals in each population that are migrants each generation. If assumptions of the model are met, the model conveniently provides estimates of the number of migrants. Some of these assumptions are that

1) populations are in drift-migration equilibrium, 2) the number of subpopulations are infinite, 3) N_e is the same in each subpopulation and is unchanged over time, 4) m is also the same between populations and is unchanging, 5) m is small, 6) alleles are selectively neutral, and 7) mutation is absent. Waples (1998) explored the use of F_{st} and the island model of migration to estimate gene

flow and concluded that some of the violations of the model can greatly limit the accuracy of gene flow estimates. For example, in populations where stocks have only recently become isolated from each other, an insufficient amount of time may have elapsed for genetic differences to appear. In other instances, migration may not be consistent over time. F_{st} can also be applied to other models including one of complete isolation between populations in which

$$F_{st} = 1 - e^{(-t(2Ne))} \quad , \qquad \text{Equation (3)}$$

Here N_e is as before and t is the duration of the isolation in generations. Unfortunately, any value of F_{st} used to estimate migration is also consistent with this model of complete isolation given the right combination of N_e and t.

Another important consideration in estimating gene flow with equation (2) is that the shape of the relationship between F_{st} and N_em is concave and changes rapidly for small values of F_{st}. Even a small confidence interval around estimates of F_{st} for high gene flow species can result in a large range of N_em values. For example, a typical confidence interval for an estimate of $F_{st} = 0.020$ (the median value for marine fishes) based on sample sizes commonly used in DNA studies would include estimates of N_e ranging from a few to hundreds of individuals (Fig. 5). Such a wide range of gene flow estimates would be difficult to incorporate into a management plan for a marine fish.

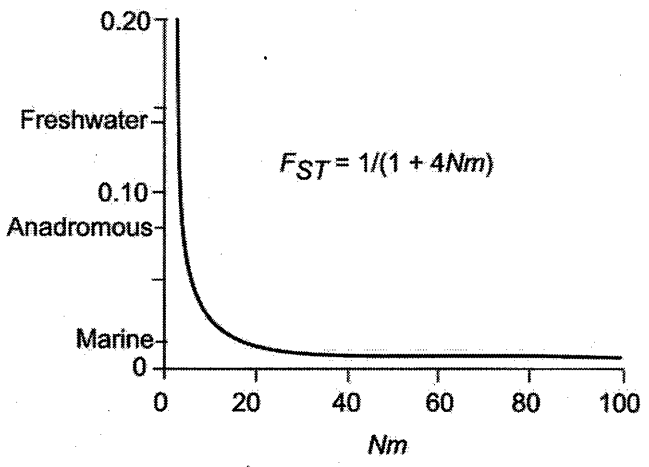

Figure 5. Relationship between F_{st} and gene flow (N_e) in the island model of migration (Wright 1943), in which population sizes are equal and constant, and the proportion of non-natal individuals breeding in each population is the same for all populations.

CONCLUSIONS

Several factors influence the application of phenetic and molecular genetic stock methods to high gene flow species such as walleye pollock. One is that populations are dynamic and are best viewed as constantly changing facets of a mosaic that grow, diminish, and sometimes disappear (metapopulations). Abundances of walleye pollock appear to be driven, in part, by large year classes, which appear sporadically and which may influence population structure by promoting dispersal into less dense areas. Evidence for the metapopulation nature of pollock stock structure can be found in sharp increases in abundance in areas of previous low abundance and in variable allele frequencies over years in the same area (Seeb *et al.*. this volume). In such metapopulations, stock structure can only be understood by sampling over a period of years.

Another factor is that differences between populations accumulate on various time scales, depending on the nature of the phenetic or genetic variability being examined. On short time scales, some morphological, developmental, or life history traits can be influenced by environmental events over the life time of an individual. On intermediate time scales of decades or centuries, gene frequencies of rapidly evolving gene markers can change in response to short-term dispersals, sweepstakes recruitment, range contractions and expansions, and mutation. On time scales of thousands of years, major differences in gene frequencies can accumulate in response to isolations brought about by major climatic and oceanic events. These factors must be considered in the choice of a particular method and in the interpretation of the results of a study Because of the need to delineate demographically independent population units that sometimes exist only on short time scales, many molecular genetic markers may have limited application to the management of high gene flow species.

Another important factor in detecting stock structure in high gene flow species is sample design. In species where fish migrate to spawning areas, but mix at other times, the timing and location of sample collections will have an overriding influence on the ability to detect stock structure. Another consideration is the recognition that only small genetic or phenetic differences between stocks are expected in high gene flow species. A rule of thumb is that sample sizes of 100 or more individuals are required to detect such small, but real, differences between stocks (Fig 6). Sample size requirements for tests of gene-frequency differences, when the expected differences are small, are quite stringent. For example, sample sizes range from 237 to 802 individuals, depending on the frequencies of the

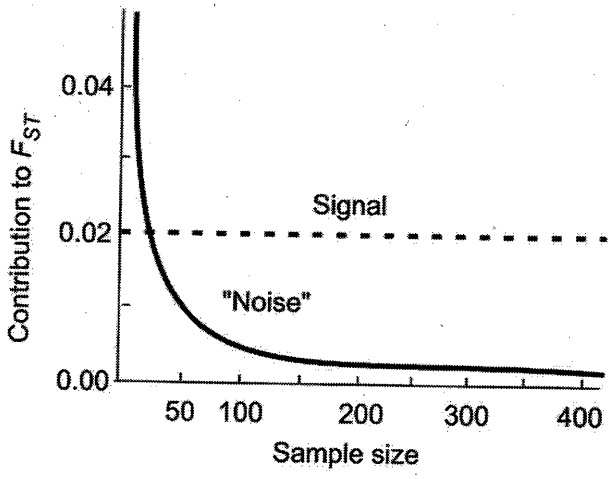

Figure 6. Relationship between error in estimating F_{st} and sample size. F_{st} equal to 0.02 (broken line) is the median value for marine fish species.

Table 4. Samples size required to detect a real gene frequency difference between populations for a given amount of certainty. Sample sizes computed according to Sokal and Rohlf (1981).

		Frequency in sample 2					
Frequency in sample 1	Power (1 - β)	0.10	0.20	0.30	0.40	0.50	0.60
0.05	0.6	156	30	15	10	7	5
	0.8	237	44	21	13	9	7
0.20	0.6			102	31	16	10
	0.8			157	46	23	14
0.45	0.6					513	61
	0.8					802	93

alleles, to ensure an 80% chance detecting a 0.05 frequency difference between two populations (Table 4).

The purpose of this article has been to point out the sources of limitations on the use of molecular genetic methods to identify stocks of high gene flow species, such as walleye pollock. Molecular genetic methods have been applied with great success to some species, particularly species with limited amounts of gene flow which allows genetic differences to arise on relatively short time scales. These methods, however, may not always be successful in delineating stocks of high gene flow marine species for use in stock assessment. The rigorous analysis of rapidly evolving genes, such as microsatellite loci, holds the best promise for identifying population units that are on the same spatial and temporal scales used by managers to estimate abundance trends. Non-genetic methods, such as the analysis of morphological and demographic variability, the analysis of trace elements, and the use of tag-and-recapture information should also play prominent roles in the identification of pollock stocks, because these methods can track the effects of environmental events that occur on short time scales.

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Bering Sea Walleye Pollock Stock Structure Using Morphometric Methods

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ABSTRACT: The assessments of Eastern Bering Sea (EBS) walleye pollock presented to managers imply that the stock areas are adequately defined for conservation assessment and management purposes. If these assumptions are incorrect, then effective management may be compromised. Stock structure research is therefore an important area of research. We evaluate the use of morphometric methods for stock identification purposes using the most recently available fisheries length and age data. Significant differences were found between areas within Eastern Bering Sea suggesting, at first, that multiple "stocks" may play important roles in contributing to the fishery production. However, we demonstrate that with relatively small migration patterns driven partly by body size, the differences can be easily reproduced with only one "true" stock. Other contributions to the observed differences may also include local food supply and oceanographic conditions. We conclude that the such simulation models are useful for examining alternative stock structure hypotheses.

INTRODUCTION

Stock delineation or identification is needed for effective fisheries management. Understanding the rates of exchange between populations or sub-stocks that may compromise effective single-stock management is crucial. Most stock identification methods are designed to highlight the *potential* for interaction between stocks. Few studies involve the direct estimation of rates of exchange. Knowing the potential is an important first step, especially in cases where the stock structure is poorly understood (e.g., Ianelli *et al.* 1998).

For walleye pollock (*Theregra chalcogramma*) in the North Pacific, a variety of methods have been applied for stock identification purposes (e.g., Serobaba 1977, Grant and Utter 1980, Lynde *et al.* 1986, Dawson 1989, Gong *et al.* 1990, Nitta and Sasaki 1990, Severin 1995). Bailey *et al.* (1999) provides an excellent review of stock identification techniques and hypotheses around stock structure issues for pollock. Among these methods, the use of morphometrics and meristics to differentiate stocks continues to advance. For example, Kim (this volume) presents analyses of counts and measurements from a large number of samples collected using X-ray photography. Since morphometrics and meristics can represent phenetic expressions due to environmental conditions in addition to genetic factors, they are regarded as being useful for fisheries management. For example, a common difference may be a manifestation of growth variability—a quantity of direct impact to fisheries management since quotas and harvest rates can change substantially for different growth attributes. Another advantage of morphometric and meristic methods is that the data can be relatively easy to collect and inexpensive to process. This can lead to a long time series of data which is useful for testing the persistence of stock structure patterns.

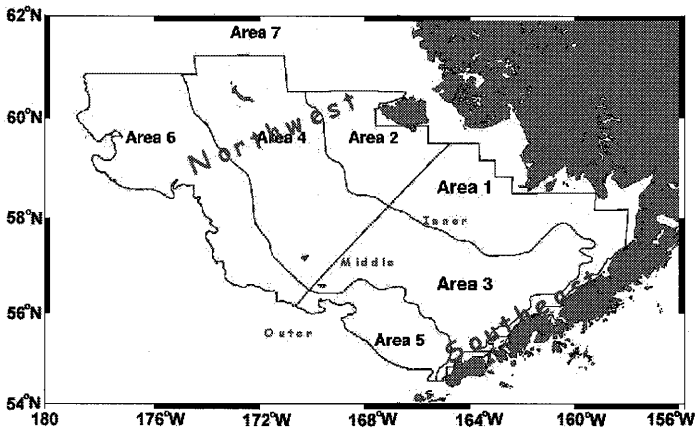

Figure 1. Strata definitions used for this study. These are based on the NMFS bottom-trawl survey strata as applied to the commercial fishery data.

Table 1. Length frequency sample sizes by area, 1991-1998.

Year	SE Inner	SE Middle	NW Middle	SE Outer	NW Outer
1991	241	99526	12831	175984	242808
1992	179	136105	25758	178332	204449
1993	45	146889	10156	191468	56780
1994	459	213653	10055	168499	43798
1995	10	200869	1382	147902	23349
1996	376	251876	266	174371	24042
1997	100	131139	424	133519	108849
1998	212	227126	942	175499	62575

Table 2. Number of fish aged by area and year, 1991-1998.

Year	SE Middle	NW Middle	SE Outer	NW Outer
1991	135	47	266	253
1992	121	41	213	113
1993	187	58	155	117
1994	172	37	128	97
1995	162		143	88
1996	346		94	
1997	171		93	137
1998	69		56	
Total	1806	348	1595	1477

Unfortunately, there are some concerns with using such apparent phenetic marks for stock classifications. For example geographic patterns in morphometric types may also reflect a fundamental characteristic in the ability of fish to move or migrate. In this paper we present an approach to show how, through a simple growth mechanism, a single population may appear to have different, geographically distinct, growth patterns. This is due to a general ontogenetic migration pattern where larger fish-at-age tend to move further. This can affect observed "synoptic" growth rate differences. We present some new data based on fishery observations to illustrate the types of differences commonly observed.

METHODS

Data collected by observers during the foreign and joint venture fisheries (1979-1989) were combined with the domestic observer data collections (1989-1998). Commercial fishery data were re-stratified by months and depth-areas corresponding to the NW and SE eastern Bering Sea shelf (Fig. 1). We only used samples from observed hauls because distinct haul location data are not available at ports-of-landing. We constructed catch length frequencies by area and time for comparisons (Table 1) and also size-at-age data for comparisons (Table 2).

The conceptual simulation model has population numbers-at-size that grow according to a probability distribution and move, also according to a probability distribution (Fig. 2). We evaluated scenarios operating from an assumed single dispersed stock but with simulated observations collected within each area stratum for compiling length-at-age data. Currently, implementation of the conceptual model is incomplete. Further refinements to the approach are unlikely to change the general nature of the simulation patterns discussed here.

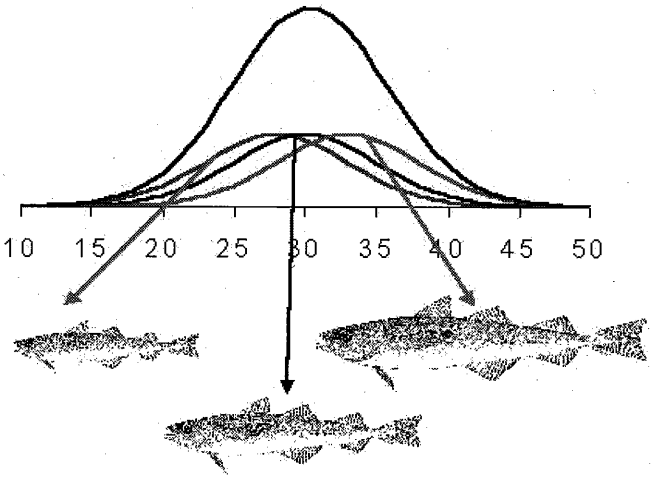

Fig. 2. Simulation scenario illustrating the process of a "single stock" segregation by size to different areas (where the fish images represent different areas).

RESULTS

The data show a distinct pattern of movement around the Eastern Bering Sea by age (Fig. 3). Computing an overall average length-frequency for the years 1991-1998, then differencing area-specific length frequencies (each normalized to sum to one) provides a way of summarizing characteristics of fish in each area (Fig. 4).

Relative to the mean length frequency pattern, the northwest outer shelf area (Area 6) had high levels of smaller fish and low levels of large fish. The southeast area (Area 3) generally lacked small fish and had the highest proportion in the 40-50 cm range.

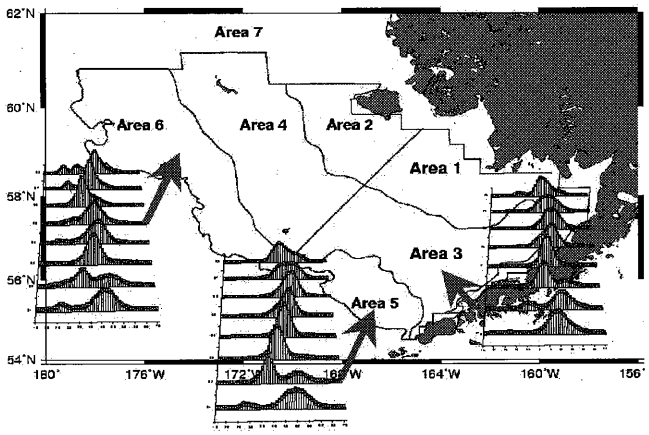

Figure 3. Patterns in fishery length-frequency data by region from 1991-1998.

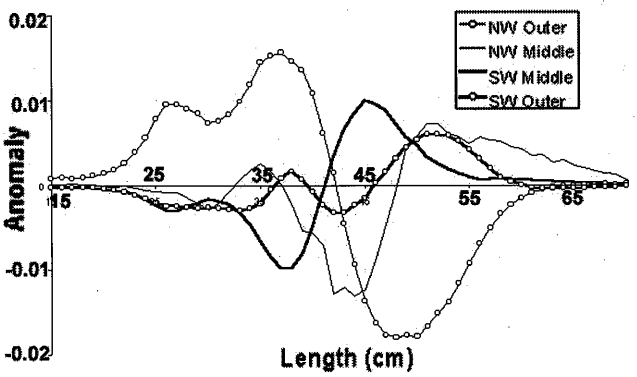

Figure 4. Pollock length frequency anomaly by area based on all eastern Bering Sea fishery data from 1991-1998. The values were computed by differencing the individual area length frequencies from the overall mean (all areas combined). All length frequencies were normalized to sum to one.

This area also had higher levels of fish greater than 60 cm. The southwest portion of the eastern Bering Sea (Area 5) had the lowest levels of frequency anomalies. The highest positive anomaly for this area occurred in the 50-55 cm range. This pattern is consistent with a single-stock hypothesis with a general ontogenetic movement southward and east along the shelf as they approach maturity. At around 5-7 years age (45-55 cm) they congregate in the southeast portion of the shelf, eventually moving toward the western part of the eastern Bering Sea where spawning occurs.

Examination of growth rates by area showed several significant differences. For the outer-shelf areas, the northwest area was consistently smaller for a given age than fish caught in the southeast area (Fig. 5). The middle shelf area showed very similar sizes-at-age except for the youngest ages when comparing the southeast region with the northwest (Fig. 6).

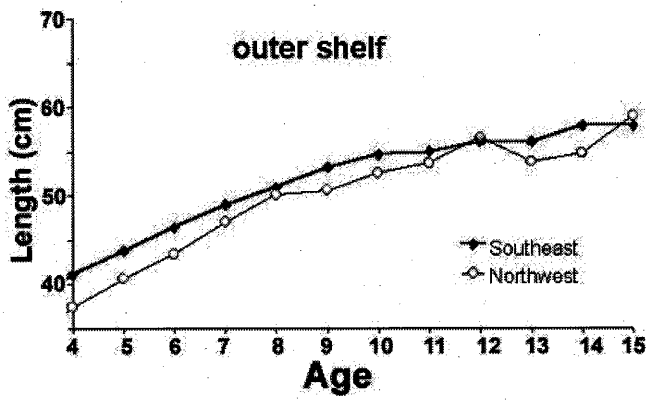

Figure 5. Average length-at-age comparison between the southeast and northwest zones of the EBS for the outer part of the shelf.

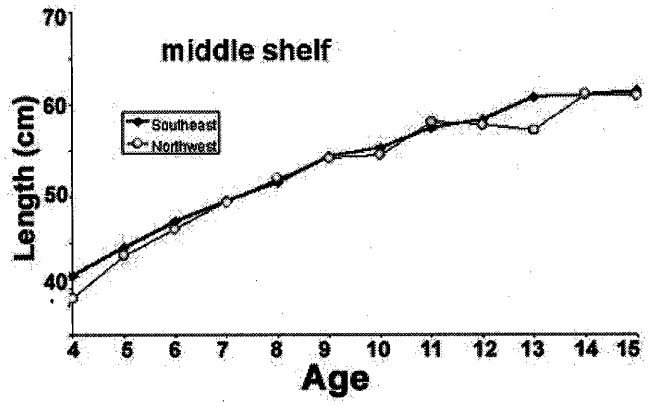

Figure 6. Average length-at age comparison between the southeast and northwest zones of the EBS for the middle part of the shelf.

Comparing the aggregate across-slope mean length-at-age for the southeast region showed that the larger fish of a given age were found on the inner part of the shelf, followed by the middle and outer shelf (Fig. 7). For the northwest region, the pattern was the same but with even larger differences, especially between the middle and outer shelf areas (Fig. 8).

Comparing pair wise differences at a single age (here chosen to be age 5 since this represents an age that is generally well sampled in all areas) provides a summary of inter-annual variability and of the general differences (Fig. 9). This figure shows that the northwest outer region was consistently smaller than the other areas while the most similar regions were the southeast outer and northwest middle regions.

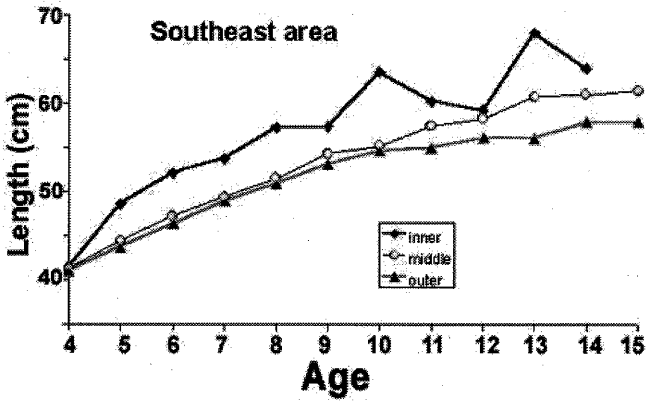

Figure 7. Average length-at-age comparison between the shelf regions in the southeast zone of the EBS.

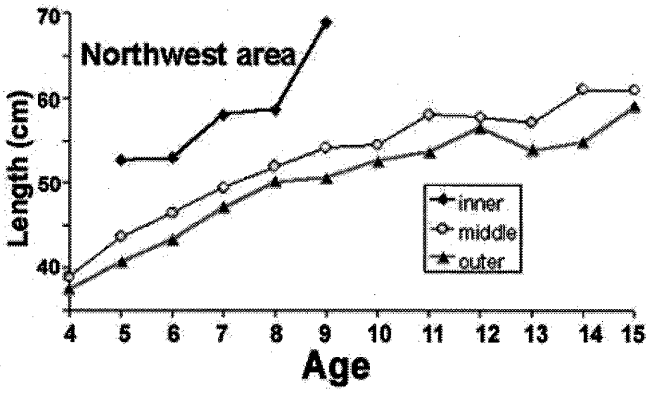

Figure 8. Average length-at-age comparison between the shelf regions in the northwest zone of the EBS.

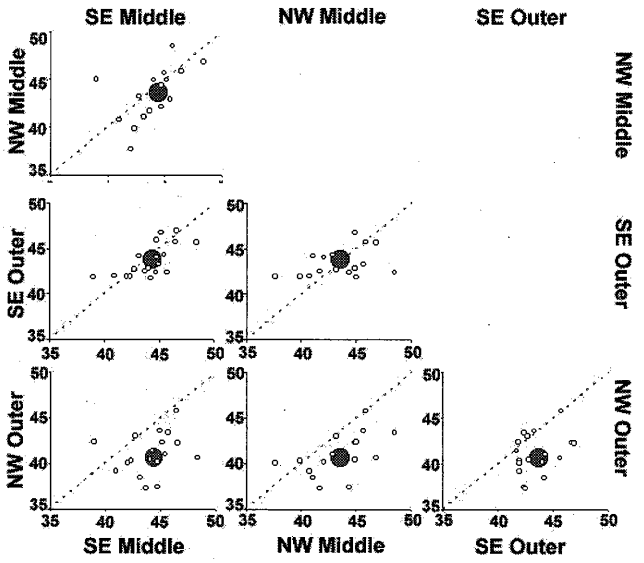

Figure 9. Average length-at-age 5 years by zones of the EBS. The small dots represent annual mean values while the large circle represents the overall averages for all years.

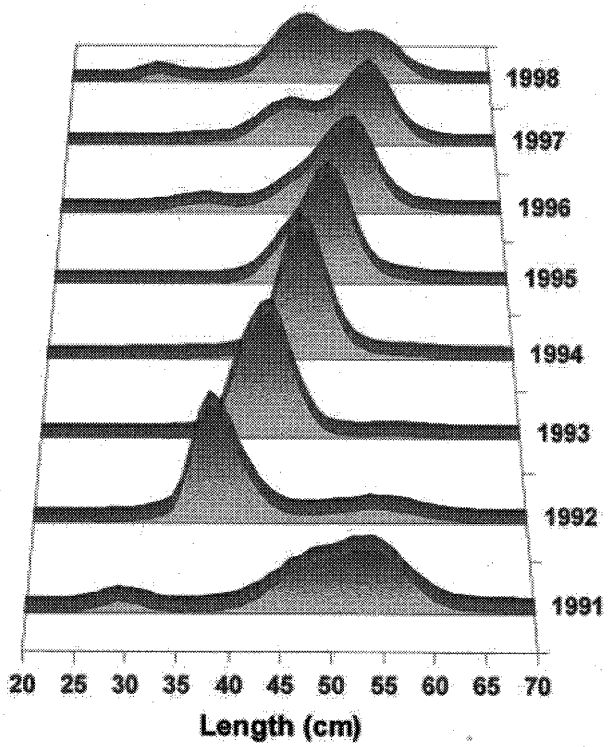

Figure 10. Fishery length frequency for late summer EBS pollock season, 1991-1998.

DISCUSSION

The idea that fish of different sizes may exhibit differential movement is not new. Lynde *et al.* (1986) explained that the differences in size-at-age they found might be due to processes related to greater movement of larger fish within the same cohorts. If pollock exhibit such ontogenetic movements as the data suggest they do, then this seems plausible. The alternative that areas with distinct size-at-age differences represent discrete reproductive stocks is inconsistent with the known changes in the overall abundance changes that occur seasonally. For example, in the late winter and early spring the concentration of pollock is much higher in the southeast portion of the Bering Sea than during the summer months when the stock appears to be highly dispersed.

The effects of year-class variability will continue to hamper strong conclusions about stock structure (e.g., Fig. 10). Recruitment fluctuates widely and can represent a large fraction of the annual stock size variability. Stepanenko (1997) discussed these expansions and contractions as having a large effect on the distribution of stock(s). If spawning site fidelity is not extremely high for pollock, and if transfer between putative populations does occur, it is likely that the transfer rate varies considerably from year to year due to the high year-class variability.

Stock structure questions remain difficult to resolve. The work of Fadeeyev (1990) suggests that migrations play an important role in the population dynamics of pollock, particularly for the eastern Bering Sea. The data we presented here is consistent with the migration patterns proposed by Fadeeyev and additionally proposes an explanation for some of the growth differences observed by different areas of the eastern Bering Sea.

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Electron Probe Micro Analysis of Otoliths

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ABSTRACT: Otoliths are aragonite (a form of calcium carbonate) and proteinaceous structures in a fish's head. They function in hearing and balance. Material precipitates on their surface throughout the life of the fish, and is only occasionally resorbed. Otoliths contain optical bands that correspond to various growth increments and also contain varying amounts of trace elements that are incorporated into the aragonite. The abundance of these elements is controlled by both ecological and biological factors. Otoliths contain a complete chemical history of the fish's life. Early studies of otolith composition were made by analyzing whole otoliths. While these studies were useful, they could not detect changes that occurred as the fish grew. For this, micro analytical techniques capable of sampling small areas were needed. Electron Probe Micro Analysis (EPMA) is one of the more widely used techniques for doing this. Other methods in use include Laser Ablation - Inductively Coupled Mass Spectrometry (LA-ICPMS) and Proton Induced X-ray Emission (PIXE). EPMA has been used examine otoliths for the past twenty years or so. EPMA is done by bombarding a sample with a fine beam of electrons and observing the X-rays that are generated in the sample. The energies of the X-rays are associated with the kind of elements present in the sample. The number of X-rays of a given energy is proportional to the abundance of a particular element in the sample. Otoliths are prepared for EPMA by making thin transverse sections in much the same way as they are prepared for examination by optical methods. EMPA can then quantitatively analyze areas that are approximately 10 microns across, and qualitatively examine areas a micron or so wide. This limitation is caused because the aragonite degrades under the electron beam, making it difficult to maintain sample stability and generate enough X-rays for statistically reliable quantitation with smaller diameter beams. Quantitative analyses are typically made along transects from the nucleus to the margin of the otolith, giving an elemental life history of the fish. While the spatial resolution remains constant across the transect, time resolution decreases toward the margin as otolith growth slows as the fish ages. Qualitative work generally takes the form of X-ray maps, which give information about the abundance of various elements across the entire surface of the otolith. This can be valuable if the otolith has grown abnormally. Some nine elements can be detected in otoliths using EPMA. Of them, strontium (Sr) has been the most widely studied. It appears as though Sr concentrations are controlled by salinity as well as temperature and diet, and are correlated with biological factors such as growth rate and stress. The other elements have not been associated directly with any physical or biological parameters, but do vary through a fish's life. The general thought is that the concentrations of these elements are controlled by a combination of physical (e.g. temperature, salinity, various oceanographic parameters) and biological (*e.g.*. growth rate, stress, diet) parameters. If this is the case then fish taken from the same area would have the same elemental signatures. A pilot study of 57 juvenile pollock otoliths taken from five areas in the Gulf of Alaska and the Bering Sea showed that it was possible to assign some 60-80% to the correct capture locality based on the otolith composition as measured at the otolith margin. Further studies are being undertaken to determine how accurately pollock migrations can be traced using otolith chemistry.

INTRODUCTION

Otoliths are mineral structures that lie within endolymphatic hollows associated with the semi-circular canal network near the brain in teleost fish (Platt and Popper 1981). They are composed primarily of calcium carbonate amid a proteinaceous matrix (Degens *et al.* 1969). They grow throughout a fish's life as material precipitates on the outer surface. In many species, optical and physical growth increments, similar to tree rings, are apparent both on daily intervals for juvenile fish (Campana and Neilson 1985), and on annual intervals for adults (McFarlane and Beamish 1995). Otoliths generally contain an uninterrupted record of life history information within their growth increments (Gauldie and Nelson 1990) as they are only rarely resorbed, although resorption during times of high stress has been documented (Mugiya and Uchimura 1989).

As an otolith grows various trace elements are incorporated into it. The concentration of some of these elements is related to the salinity of the water the fish inhabits (Tzeng *et al.* 1997). A variety of other forces,

both intrinsic (growth rate, stress, breeding potential) and extrinsic (temperature, sea water chemistry) have also been suggested as controlling the concentration of these trace elements in otolith aragonite. Several studies have shown that it is possible to distinguish fish taken from different localities based on the chemistry of their otoliths (*e.g.*. Nishimura, this volume, Thorrold, this volume, Edmonds *et al* 1989, Edmonds *et al.* 1991, Severin *et al.* 1995).

In this paper I will describe how otoliths are prepared for EPMA, the basic theory behind EPMA analysis, some ways in which EPMA can be used to analyze otoliths, and present data on a preliminary study on walleye pollock from the Gulf of Alaska and the South-Eastern Bering Sea.

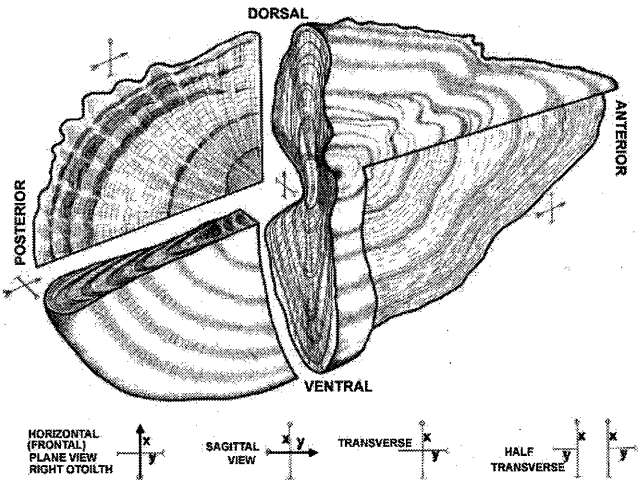


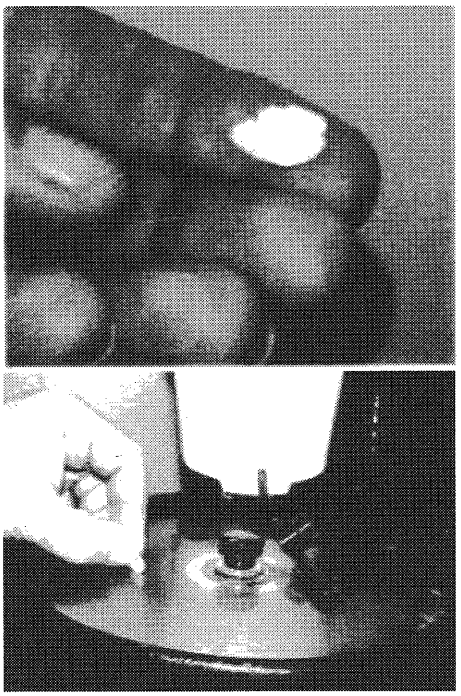
Figure from Pannella, 1980.
Most otoliths are sectioned transversely.

SAMPLE PREPARATION

Proctor and Thresher (1998) found that the concentration of some elements detectable by EPMA was affected by manner in which the fish was stored (*e.g.*. extracted immediately after capture versus extracted after the fish had been stored in ethanol) before the otolith was extracted. They recommended that all fish used in a study be treated in the same manner and that comparisons among otoliths processed in different ways be made with caution if at all. They found that otoliths from fish stored frozen to be the most similar to those extracted immediately after collection.

After the otolith is extracted from the fish most researchers clean it in whatever clean water is available. It is then air dried and stored dry in a vial or paper envelope.

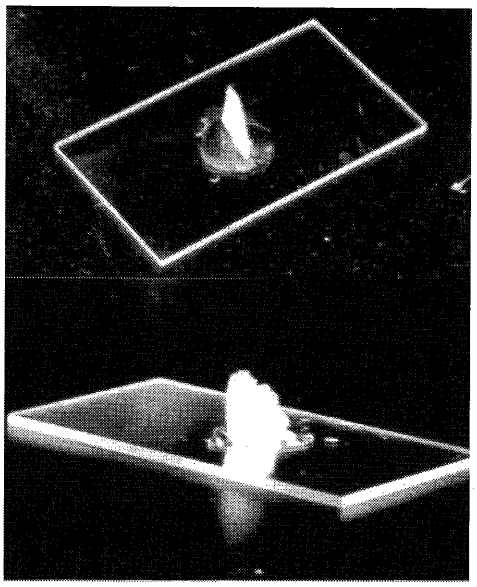
There are as many ways to prepare otoliths for EPMA as there are researchers, and no method is in and of itself superior to another — the goal is to create a sample that has a perfectly flat exposed surface and includes the areas of interest. Samples are generally transverse sections taken through the core of the otolith so that the earliest portion of the otolith can be examined. Preparation is much the same as for examination for optical methods: Sections are cut from the otolith, mounted in a manner appropriate for the particular microprobe, and then ground and polished flat with a series of increasingly fine powders.

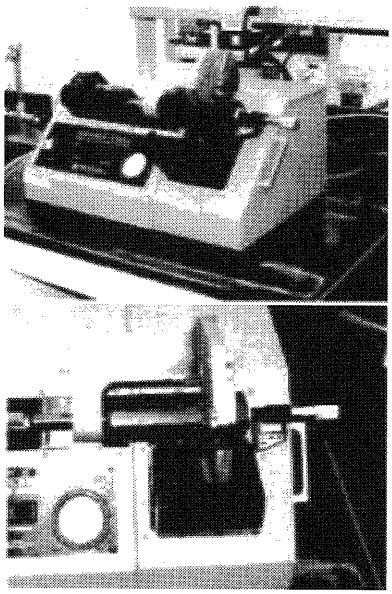


Otoliths large enough to be manipulated by hand can be sectioned with a diamond saw or, alternatively, can be held in the fingers and either anterior or posterior end removed on a fairly coarse (typically 250 to 600 grit) abrasive surface. I prefer to use a diamond impregnated rotating wheel but carborundum or aluminum oxide paper or grit on glass also can be used. In either case, the area of interest should not be exposed. The section is then mounted on a glass slide. Most electron microprobes are designed to analyze geologic samples which are typically mounted on 25mm x 50mm (petrographic) glass slides. While many electron microprobes can accommodate the standard 25mm x 75mm glass slides used in biological work, some cannot, so it is wise to check with the microprobe laboratory before selecting a mounting method. Petrographic slides are also thicker than biological slides and can better resist the stresses of grinding and polishing. The other common sample format is circular, typically between 25mm and 50mm. While sample thickness does not mater for the purposes of EPMA, it is

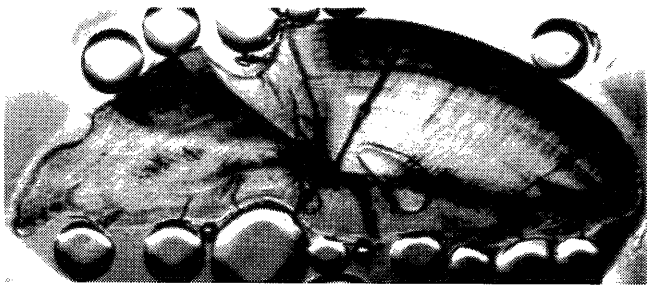
convenient to have a sample thin enough for optical examination to aid in orientation.

Thermoplastic glue such as Crystalbond 509 is quick and convenient and allows the specimen to be readily removed from the slide. It is not as strong as a thermosetting epoxy. In any case it is important to test the glue as a source of contamination of the sample. This can be done with X-ray mapping which will be discussed below.





Next the sample is ground to the desired surface with a successively finer series of grits. It is essential to remove all the coarse grit before using the next finer grit to prevent scratching the sample. This is done in an ultrasonic cleaner. I prefer to use a geologic thin section machine (such as Buehler's "Petrothin"), where the sample is ground against a diamond-impregnated wheel, because there is no need to change grinding compound. The sample is ready for polishing when removed from the machine. The machine also allows precise control of sample thickness and insures that the surface of the sample is exactly parallel with the mounting slide, something that can be difficult to achieve when grinding by hand. After grinding the sample with 1200 grit the sample can be polished. I have found that polishing with a water-based slurry of 3 micron alumina powder on glass greatly reduces the amount of time needed for final polishing. Final polishing generally takes 5-15 minutes and is done on rotating wheels using either 1 micron oil based diamond compound on coarse paper or 0.05 micron alumina and water on silk. The final polish is monitored using a reflected light petrographic microscope and will appear clear when it is completely flat.



After the sample is polished it is cleaned. I have found that ammonia-based household cleaning solutions work the best and leave no residue. After cleaning the sample is coated with a 250-300 Angstrom layer of carbon to make its surface electrically conductive. It is then ready for examination by EPMA.

Otoliths that are too small to be readily manipulated by hand are embedded in plastic blocks (usually a thermosetting epoxy) in a manner similar to the way specimens are prepared for sectioning for Transmission Electron Microscopy. After the block is cured it can be ground and polished using the methods described above. The block can be attached to a slide, but if there is no need for optical examination, the block can be of the proper size and shape for direct placement in the electron microprobe.

THEORY OF ANALYSIS

A complete understanding of the theory behind X-ray generation and the analysis of X-ray spectra is not necessary to use EPMA, nor does the user need to understand the complete operation of the instruments used in EPMA. However, the user of any analytical technique should have at least a rudimentary understanding of the theory that allows that kind of analysis. Complete descriptions of EPMA can be found in Williams (1987), Goldstein *et al.* (1992), Reed (1993), or Scott *et al.* (1995).

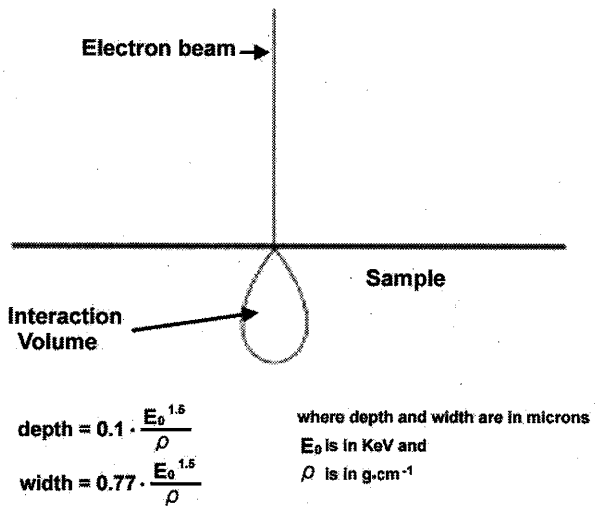
EPMA can be done because of the X-rays that are produced from atoms when they are bombarded by high energy electrons. A beam of electrons is accelerated through a potential of several tens of kilovolts and focused to a fine point on the sample where they interact with the atoms that comprise it. The energy of the beam is generally measured in kilo electron volts (keV) and is equal to the accelerating voltage. The number of electrons in the beam is known as the beam current and is typically in the range of one to ten nanoamperes (One nanoampere is $1x10^{-9}$ coulomb•second^{-1}; a coulomb is the charge on 6.24 x 10^{18} electrons). This means that some $6x10^{9}$ to $60x10^{9}$ electrons are hitting the sample each second. Each one of these electrons has a chance of producing an X-ray when it interacts with the sample. Clearly increasing the beam current will increase the number of X-rays generated in the sample. Unfortunately there are limits to the amount of beam current that can be used when analyzing otoliths.

The electron beam does not just interact with the sample surface, it penetrates the sample. In addition to penetrating the sample it also spreads horizontally. The depth (z) and width (y) of penetration in microns can be approximated by

$$y = 0.077 \cdot \frac{E_0^{1.5}}{\rho} \quad \text{and} \quad z = 0.1 \cdot \frac{E_0^{1.5}}{\rho}$$

where E_o is the energy of the electron beam in kilo electron volts and ρ is the density of the sample in gm·cm^{-3}. (Equations modified from Potts (1987). See Gunn *et al.* (1992) for other approximations.). For a typical otolith this means that the smallest possible volume that can be analyzed, even with a beam that is infinitely narrow, is 1.5 microns wide and 2.0 microns deep given a density of 2.92 gm·cm^{-1} for aragonite and using a 15 keV beam.

Electron beam-Specimen Interaction Volume



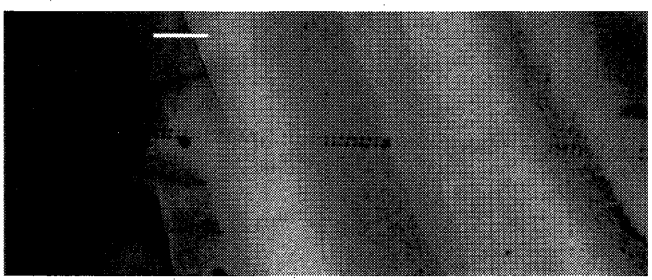
In reality the volume is quite a bit bigger because typically 10 micron or so diameter beams are used. Larger beams are used because some of the energy of the

electron beam is transferred to the sample as heat. Aragonite is heat sensitive and will decompose into CaO and CO_2 if it is exposed to large beam currents for a long period of time. The CO_2 gets pulled into the vacuum system of the electron microprobe and the concentration of the remaining elements appears increased. Gunn *et al.* (1992) defined beam power density as

$$BPD = \frac{E_0 \times I}{A}$$

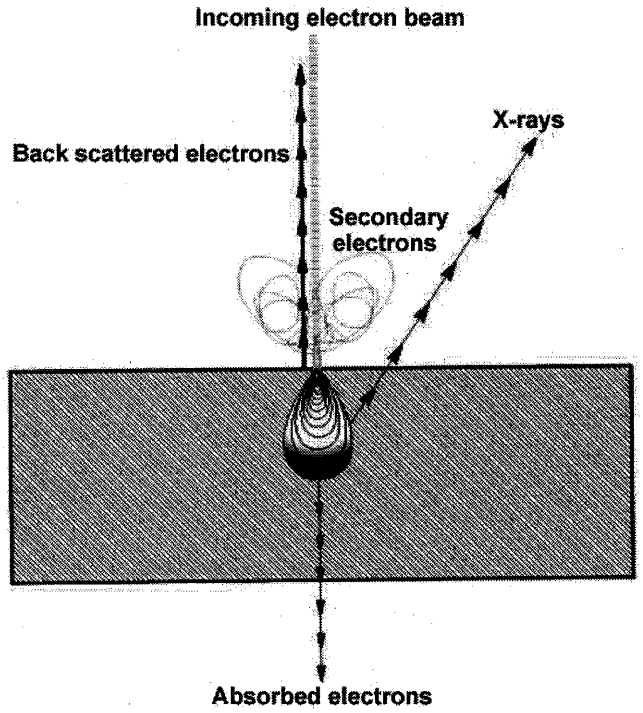
where E_0 is the accelerating voltage, I is the beam current, and A is the area over which the beam is spread. They suggested that the beam power density be kept below 3 $\mu W\ \mu m^{-2}$ for analysis of otoliths. This is a beam power density that is approximately an order of magnitude less than what is typically used in EPMA for most material science work and is something that the investigator should ask the electron probe operator to check, particularly if the operator is not experienced in analyzing otoliths. Looking for the change in the production of X-rays as the beam is on the sample is a practical method for determining if the sample is being affected adversely by the beam. It is also worth noting that defocusing the beam does not increase the depth of penetration into the sample.

This is a scanning electron micrograph of beam damage on an otolith. The scale bar is 100 microns.



The beam damage marks can be useful if the specimen is viewed in transmitted light as the analyzed positions can readily be located relative to optical bands. The scale bar is 100 microns.

There are four types of interactions between the electron beam and the sample that are of interest to the typical EPMA user. A brief review of the structure of an atom makes these interactions easier to understand. The nucleus of the atom is a tightly bound package of neutrons and protons in the center of the atom. The number of protons determines the kind of element and is known as the atomic number of the atom. For a neutral atom the number of electrons surrounding the nucleus is equal to the number of protons in the nucleus. These electrons are in shells, with the inner shell electrons being more tightly bound to the atom than the outer shell electrons. Each shell can each contain a specific number of electrons, and each shell is at a specific energy. The energy level of each shell is determined by the number protons in the nucleus, i.e. the energy levels vary according to element. If an electron is removed to cause a vacancy in a particular shell, certain other electrons can move (or transition) from a lower energy shell to fill it. Quantum mechanics dictates that only certain transitions are allowable.



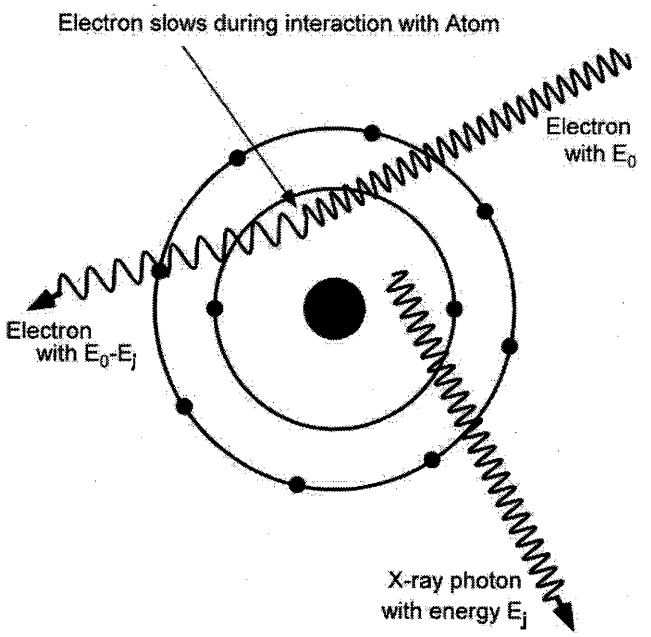
Some of the electrons in the electron beam undergo elastic collisions with the atoms and are reflected directly back out of the sample. These are called backscattered or Rutherford scattered electrons. The number of backscattered electrons is highly dependent on the average atomic number of the sample. Backscattered electrons can be used to make qualitative estimates of the relative composition across a sample.

Other electrons interact with the loosely bound outer shell electrons of the atoms in the sample and dislodge them at low energies. These are known as secondary electrons. The number of secondary electrons is related to the angle between the sample and the electron beam, thus they give information about the shape of the sample. They are what are commonly imaged in Scanning Electron Microscopes.

The rest of the electrons interact with the atoms in the specimen to generate X-rays and are known as the electrons that form the absorbed current.

Some of these electrons undergo inelastic collisions with atoms in the sample and loose energy. This energy is emitted from the sample in the form of a photon. This photon can have any energy between 0 and the energy of the original electron beam, with the most common energy being half that of the beam. Photons in this energy range are classified as X-rays, and these particular X-rays carry little information of use for most EPMA work. They do, however, contribute to the total X-ray spectrum and complicate its interpretation. These X-rays are known as continuum X-rays or Bremsstrahlung.

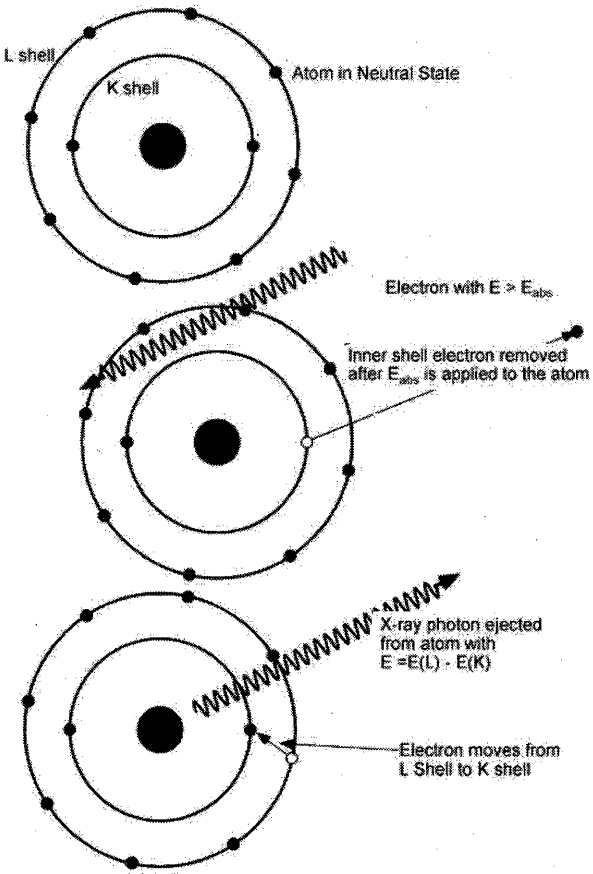
Background X-ray Generation
(Bremsstrahlung, or Braking Radiation)



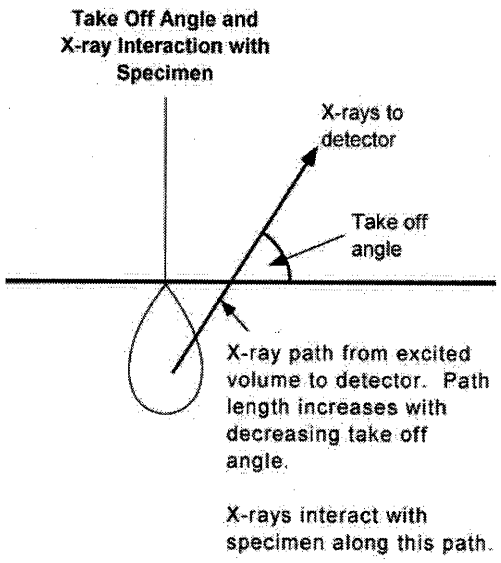
If an electron has an energy greater than the binding energy of an inner shell electron of an atom in the sample then it may cause that electron to be ejected from the atom, leaving the atom ionized. An electron from an outer shell then falls to the inner shell to fill the vacancy and stabilize the atom. When this happens the energy that is equivalent to the difference of the energy of the two shells is released from the atom. At this point two things can happen. The energy can be reabsorbed by the atom and an electron, called an Auger electron, is released, leaving the atom lacking two electrons. The energy can also be released as a photon. This photon is in the X-ray range, and because it was formed by a transition of an electron between two energy levels that are specific to a particular kind of element, it will have an energy that is specific to that element. These X-rays are known as characteristic X-rays. They are what are used to determine both the type of element that is present in the sample by their energy and how much of that element is present by the number of X-rays of that particular energy.

There is generally more than one possible transition that can fill an inner shell vacancy. This means that there is more than one possible energy for a characteristic X-ray. For higher atomic number atoms the characteristic X-ray spectrum can become quite complex. In some cases the characteristic X-rays of one element can overlap with the characteristic X-rays of another element, but by examining the complete characteristic X-ray spectrum it is possible to identify the element positively.

Characteristic X-ray Generation



Beam penetration of the sample limits the spatial resolution achievable by EMPA and also complicates the calculations needed to turn counts of characteristic X-rays into elemental concentrations. Most of the interaction between the electron beam and the atoms in the sample takes place beneath the sample surface, and most of the characteristic X-rays are generated there. These X-rays must pass through the sample before they can get to the X-ray detector. While they are traveling through the sample there is a chance that they will be absorbed by some of the atoms in the sample (absorption) and generate other X-rays (fluorescence). The amount of absorption and fluorescence is fairly easy to calculate if the composition of the material that the X-rays are traveling through is known. The sample, however, is of unknown composition, which is the very reason it is being examined! Well-developed iterative techniques are used to estimate the amount of absorption and fluorescence. These techniques rely on the X-rays passing through a known length of sample. The relationship between the beam and the detector is fixed by the design of the instrument, and if the geometry of the sample is known then the path length may readily be calculated. In almost all cases the calculations are designed for a sample that is flat, perpendicular to the beam, and located at a particular position in the instrument. It is clear that a sample with surface irregularities will complicate quantification calculations not only because the beam will excite an irregular volume within the sample but also because the path length for the X-rays leaving the sample will be difficult to quantify. It is not important for the general EPMA user to know the details of these calculations, but it is important to know that they are based on specimens with a well-characterized geometry"



Compositional calculations for EPMA are performed by comparing the number of characteristic X-rays of a given element measured on the sample with those measured on a well characterized standard. Different standards can be used for different elements, and to some extent the choice of standards depends on which method of compositional calculation is used. Characteristic X-ray counts must be corrected for continuum radiation —the X-ray detector cannot differentiate between them as there is no difference between a photon of a given energy that was produce by an inner shell ionization and one produced by a beam electron slowing as it passes and atom. This correction is made by measuring the continuum X-ray intensity at an energy close to that of the characteristic X-ray energy, taking care that the energy is not that of some other characteristic peak. The measurement of continuum X-ray intensity is particularly important when the concentration of the element of interest is low. In one case (Campana *et al.*, 1997) an unexpected change in the continuum spectrum made measurements of low levels of strontium extremely inaccurate. Correcting the background measurements made the results quite accurate.

Precision in EPMA measurements is limited by the number of X-rays counted for each element as well as the number of X-rays counted for the continuum correction for each element. X-ray production follows a Poisson distribution, which can be approximated by a Gaussian distribution with a mean μ and a standard deviation $\sqrt{\mu}$. In other words, if 10,000 X-rays of some particular energy were observed in a given amount of time, then a repeated series of measurements would have a standard deviation of 100 counts. This is true whether measuring characteristic or continuum X-rays. To obtain the number of characteristic X-rays, the continuum X-rays much be subtracted from it, and according to the rules of error propagation the variance (the square of the standard deviation) associated with the counts must be added. Assuming equal counting times on the characteristic and continuum energies, the relative error associated with a particular characteristic X-ray measurement is

$$\frac{\sqrt{CR_{char+cont} + C_{cont}}}{C_{char+cont} - C_{cont}}$$

Where $C_{char+cont}$ is the number of X-rays counted at the characteristic X-ray energy and C_{cont} is the number of X-rays counted in a properly selected energy close to that of the characteristic X-rays. The upper term is the standard deviation of the difference between the measure peak at the characteristic X-ray energy (which consists of characteristic and continuum X-rays) and the continuum X-rays. The lower term is the estimate of the number of characteristic X-rays.

If counting times are not equal the equation becomes

$$\frac{\sqrt{\frac{CR_{char+cont}}{t_{char+cont}} + \frac{CR_{cont}}{t_{cont}}}}{CR_{char+cont} - CR_{cont}}$$

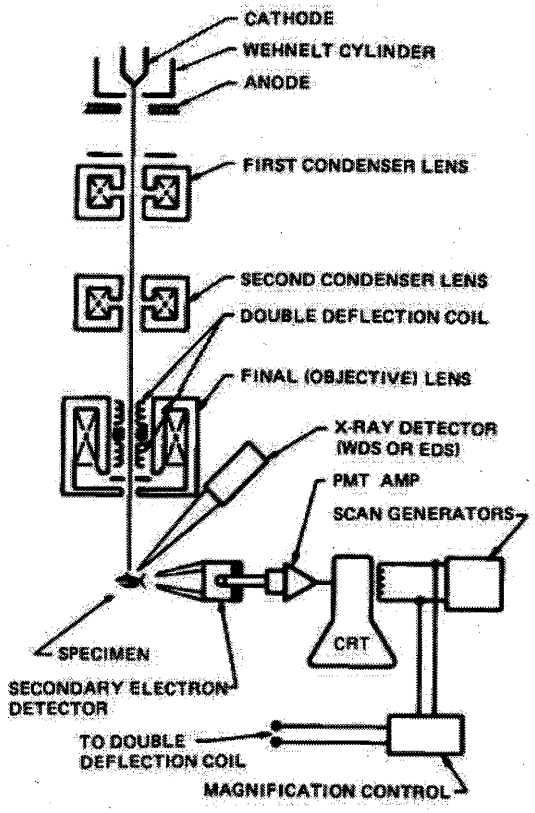
where $CR_{char+cont}$ is the count rate of the X-rays at the characteristic X-ray energy, CR_{cont} is the count rate of the X-rays counted in a properly selected energy close to that of the characteristic X-rays and $t_{char+cont}$ and t_{cont} are the counting times at each energy. Notice that the relative error decreases as the count rates increase and also as the counting time increases. Thus more precise analyses will be obtained with high count rates and long counting times. Unfortunately high count rates demand the use of large beam currents, which are destructive to aragonite. Similarly, long counting times can also affect the aragonite in addition to adding to the expense of analysis. The investigator must decide on the precision required for the study in mind and decide on analytical conditions accordingly.

INSTRUMENTATION

An EPMA instrument may be thought of as two systems, the electron beam generation and control system and the X-ray detection system. The electron beam is typically emitted from a tungsten filament, accelerated through an anode held at the desired potential, and then focused through a series of electromagnetic lenses. The instrument typically also includes scanning coils so that the beam can be moved across the specimen in a raster pattern. The instrument also typically includes detectors for secondary and backscattered electrons and could be used as a Scanning Electron Microscope. This is typically not a routine use for an electron microprobe because of the stage design: As was noted above, the calculations used to convert observed X-ray counts into weight fractions are based on measurements being taken from a flat sample that is oriented perpendicular to the beam. Stages on most electron microprobes are usually designed so that the flat surface of the sample must be held perpendicular to the beam —there are rarely options for tilting the stage as there are in SEMs. Another major difference between dedicated EPMA instruments and SEMs is in the beam current control. Because X-ray intensity is proportional to beam current it is important to have a very stable and well characterized beam for EPMA. SEMs typically lack the elaborate beam control and measuring circuitry that is an integral part of an electron microprobe.

The X-ray detection part of the system consists of one or more X-ray spectrometers. Two kinds of spectrometers are in common use: Energy Dispersive Spectrometers (EDS) and Wavelength Dispersive Spectrometers (WDS).

The heart of an EDS detector is a crystal, usually silicon but sometimes germanium. When an X-ray interacts with the crystal it creates electron-hole pairs. The number of pairs is directly proportional to the energy of the X-ray. The number of pairs created during the interaction is measured and though a conversion process is displayed as a count of an X-ray of a particular energy. An EDS system can detect and display all energies of X-rays simultaneously. EDS systems have an energy resolution of approximately 130- 150 electron volts.

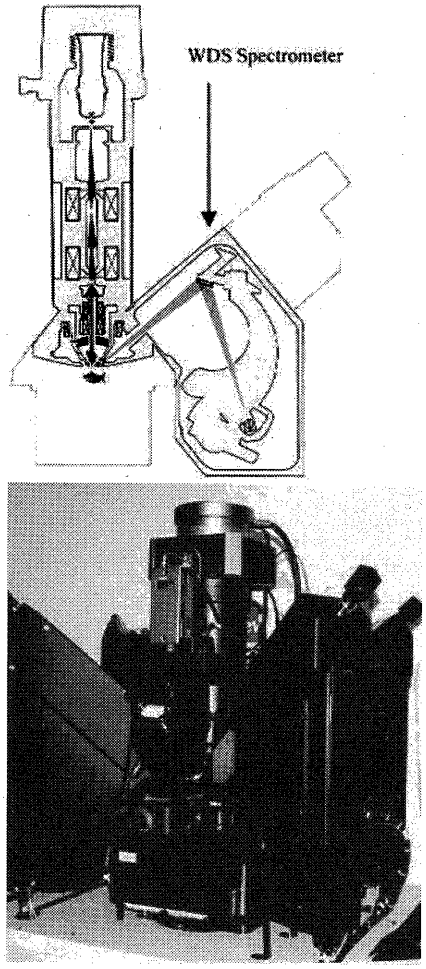


WDS spectrometers work by diffracting X-rays of a crystal according to Bragg's Law (Bragg's Law states that there will be constructive interference when

$$n\lambda = 2d \cdot \sin\theta$$

where n is an integer, λ is the wavelength of the diffracted wave, d is the latice spacing in the crystal, and θ is the angle between the wave and the crystal. The angle of the spectrometer is adjusted to the wavelength of interest.). They can detect only a single energy of wavelength at one time, hence most EPMA instruments are fitted with multiple WDS spectrometers. The diffracted X-rays are detected in a flow proportional gas detector, which is similar to the detector used in a Geiger counter. They typically have an energy resolution of

about 1 ev. The increased energy resolution of a WDS spectrometer makes it by far the spectrometer of choice for doing EPMA analysis of otoliths.

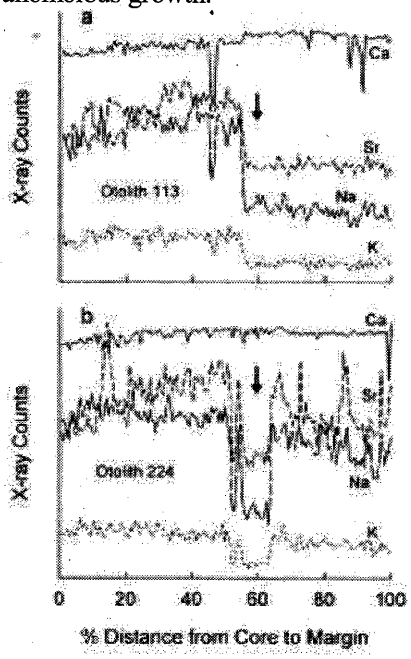


A modern electron microprobe

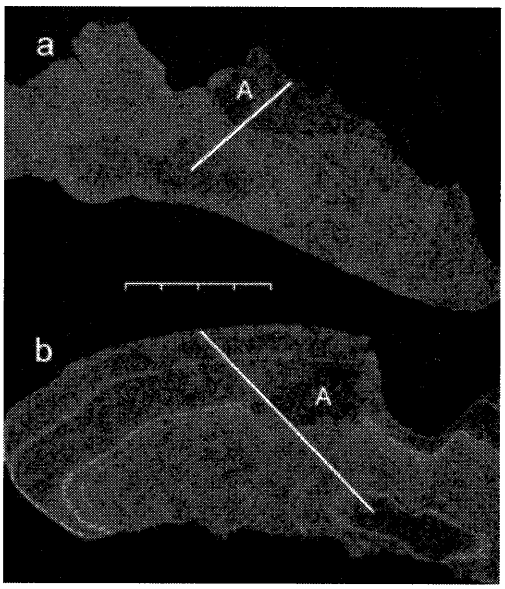
APPLICATIONS

As mentioned above, the number of X-rays of a given energy is roughly proportional to the quantity of a particular element in the sample. The number of X-rays of a given energy from the sample can be compared to a standard, proper calculations can be made and an exact composition of the sample can be made. This is probably the most typical EPMA application. However, on occasion, it is just as useful to record simply the number of X-rays of a particular energy that were collected at a given location (Brown and Severin, in press). This is completely adequate when the investigator is looking for major changes in chemistry.

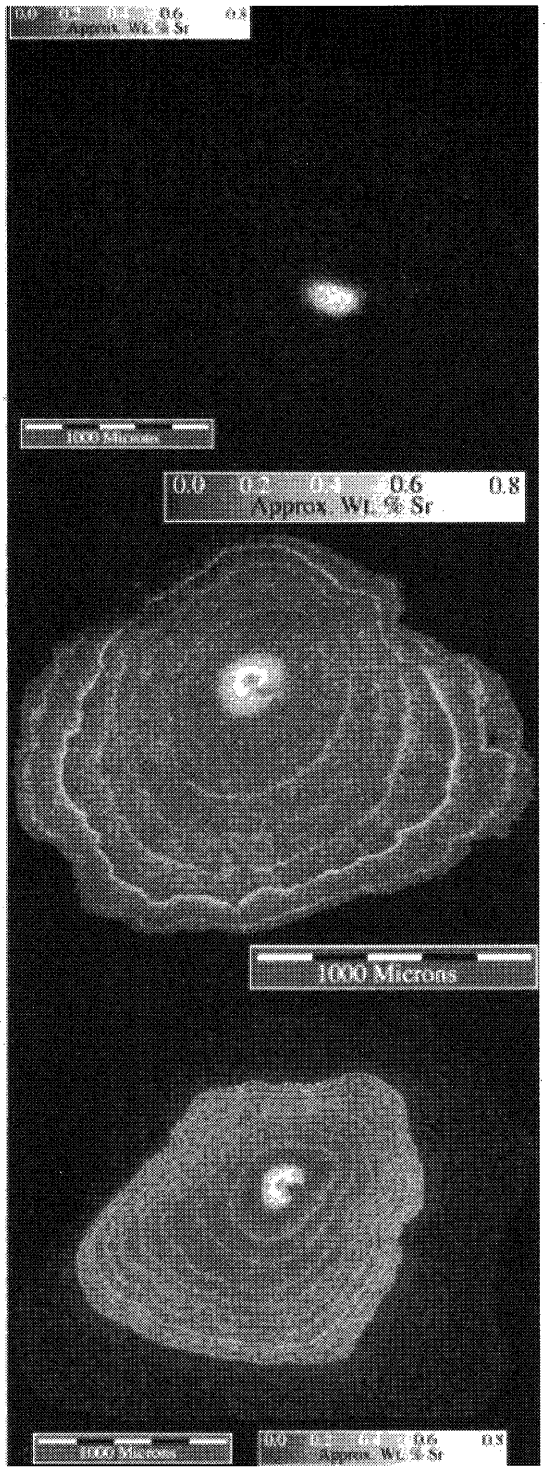
In some cases X-ray maps are appropriate. X-ray maps essentially map composition across a two dimensional area. While it is possible to quantify them, generally they are used as indicators of relative abundance. It is possible to easily detect similarities in overall patterns using X-ray maps, as well as to detect areas of anomolous growth.



Transects across inconnu (*Stenodus leucichthys*) otoliths. The anomalous areas marked with arrows were investigated further with x-ray maps.



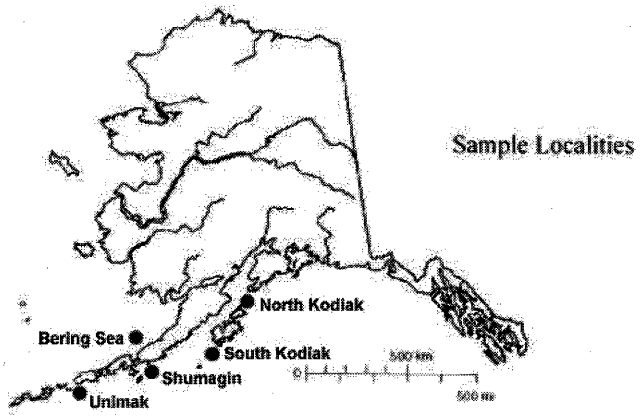
X-ray maps of the inconnu otoliths. The brighter areas are areas of higher strontium abundance. Lines show transects from previous figure. Scale bar is 1 mm. Anomalous areas are marked with"A". These maps confirmed that the otolithshad grown abnormally.



These X-ray maps of eel otoliths clearly show that the fish were exposed to different factors that affect the uptake of strontium. The authors (Tzeng *et al.*, 1997) attributed the differences to the salinity of the water that the fish were living in. The X-ray maps were used to visualize dramatic differences in life history.

AN APPLICATION OF EPMA TO POLLOCK

This is a summary of Severin *et al.* 1995. EPMA was used to measure Na, Mg, P, S, Cl, K, Ca, and Sr concentrations on the outer margins of 57 juvenile walleye pollock otoliths from five locations in the Gulf of Alaska and Bering Sea. Discriminant analyses that used various combinations of Na, P, K, Sr, and fish-standard length and/or age showed that 60 to 80% of the samples could be assigned to the correct capture locality. While concentrations of some of the measured elements correlated with standard length or age of the fish, there were measurable differences among localities when concentrations are length or age corrected, mainly due to differences in Na and K concentrations.

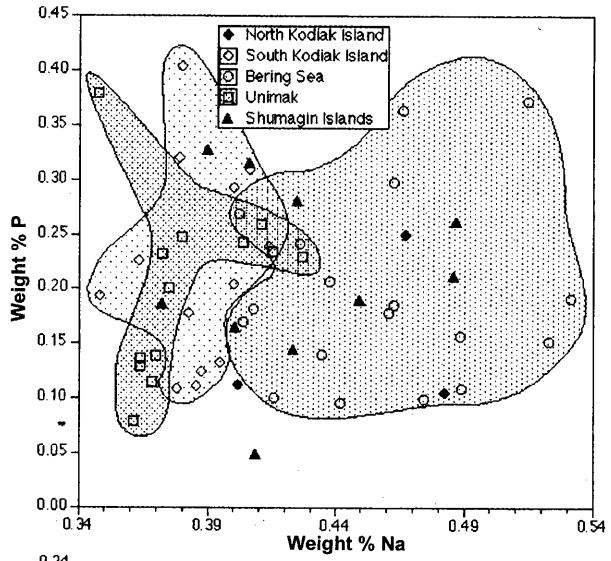


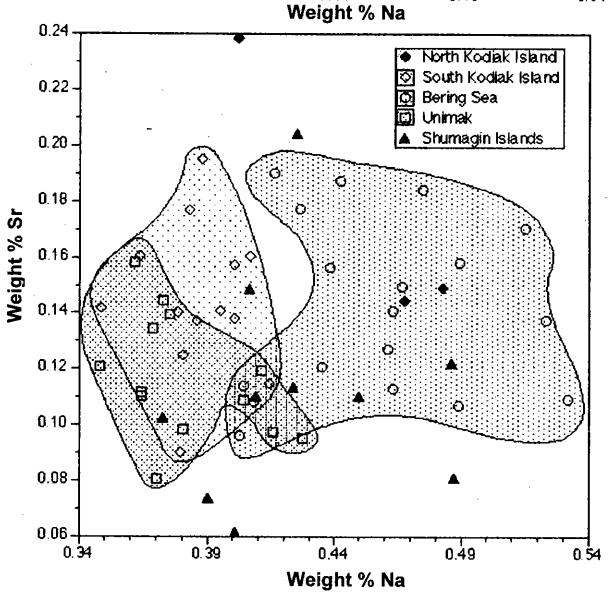
Beam Conditions: 15kV, 6nA, 20 micron diameter beam

Specimens	Unimak 13	Shumagin 10	Bering 18	S.Kodiak 13	N.Kodiak 3		
	Mean Concentrations (Weight %)					3 sigma detection limit	Counting Times (sec)
Na	0.381	0.425	0.458	0.386	0.450	0.043	80
* Mg	0.008	0.007	0.012	0.007	0.006	0.035	80
P	0.201	0.213	0.194	0.218	0.155	0.057	60
* S	0.031	0.034	0.044	0.033	0.031	0.047	80
* Cl	0.028	0.026	0.035	0.024	0.018	0.044	60
K	0.063	0.062	0.093	0.079	0.085	0.036	80
Ca	38.670	38.391	38.689	38.610	38.840	N/A	40
Sr	0.116	0.112	0.141	0.144	0.177	0.089	80

* indicates elements that were overall at or below the 3 sigma detection limit, although they were present in some analyses.

Summary of elemental concentrations measured at otolith margins.

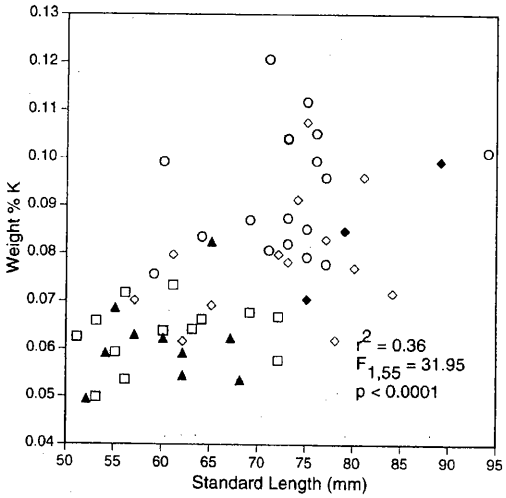




Simple plots of elemental abundance at otolith margins suggested that the various areas could be distinguished based on otolith chemistry. Discriminant analysis showed that the elements could discriminate the areas with a 77% correct accuracy. Na, K, and Sr were the elements most important in the analysis.

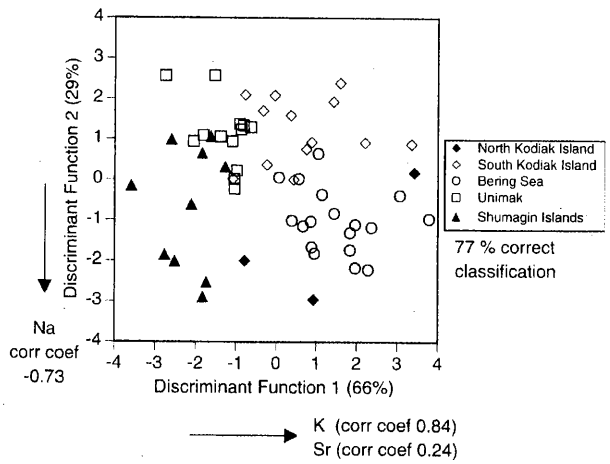
Further investigation of the data revealed that simply using standard length of the fish could also distinguish most the capture localities. Concentrations of K, Na, and Sr were found to be significantly correlated with standard length as well.

K Concentrations Measured at Otolith Margins



	SL (mm)	Age (days)
◆ North Kodiak Island	81.0	-
◇ South Kodiak Island	72.2	114.2
○ Bering Sea	62.8	-
□ Unimak	60.4	108.6
▲ Shumagin Islands	60.2	116.8

Discriminanant Analysis Based on Elemental Concentrations



A discriminant analysis based on elemental concentration and standard length was able to distinguish the localities with a 79% correct classification. While standard length was an important discriminator, elemental abundance of K and Na was also important in discriminating among the localities.

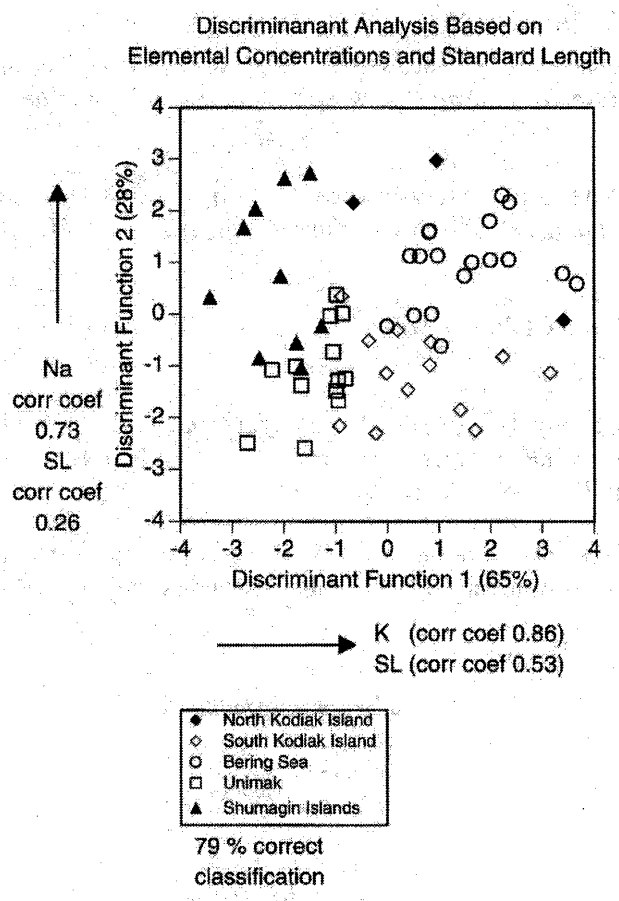
CONCLUSION

Na, Mg, P, S, Cl, K, Ca, and Sr (as measured with an electron microprobe) are present in the otoliths of juvenile walleye pollock from Alaskan waters.

These elements, particularly Na, K, and Sr, can be used to distinguish some geographic areas with 70-80% correct classification.

Na, K, and Sr are correlated with standard length and, presumably, age. The elemental differences seen in the

otoliths from the areas studied here may be the result of sampling fish of different ages, rather than environmental factors. Life history profiles of these elements are needed before concluding that the variability seen here is the result of geochemical variation.



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Stock Delineation in Marine Fishes through Analyses of Otolith Chemistry: Potential and Likely Pitfalls

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INTRODUCTION

Fisheries ecologists continue to search for natural markers of population structure. Most workers have concentrated on developing genetic tags of population structure. However, this approach has proved problematic, as significant genetic exchange among presumed populations by larval dispersal, adult vagrancy and deliberate stock transfers has hampered this approach in a number of anadromous and marine fishes (*e.g.* Graves *et al.*, 1992; Epifanio *et al.*, 1995). The difficulty with genetic data is not so much when significant differences among populations are found, but rather when the null hypothesis of no difference among presumed populations cannot be rejected. In this instance, it is impossible to determine if there is considerable genetic exchange among populations, or if the genetic data is reflecting historical rather than present-day gene flow (Gold *et al.*, 1997). A further problem pertains to the extremely low rates of exchange needed to prevent differentiation between populations — perhaps on the order of 20 females per generation (Smith *et al.*, 1996). In statistical terms, this means there is a high chance of failing to reject the null hypothesis (no differentiation among populations) when, over ecologically-relevant time scales, the populations are disjunct.

Recently, several studies have suggested that variations in the trace element chemistry of fish otoliths reflect population associations, often in the absence of concomitant genetic variations (Campana *et al.*, 1995; Thresher *et al.*, 1994). Otoliths, or ear stones, are accretionary structures located within the inner ear of teleost fish, and are composed primarily of aragonite deposited on a proteinaceous matrix. The utility of otolith chemistry as a retrospective history of water mass residency relies upon three properties of otoliths. First, the deposition time of otolith material can be estimated by reference to concentric rings in otoliths that are routinely used in fish aging studies (Beamish and McFarlane, 1987). Second, the metabolically inert nature of otoliths ensures that the aragonite mineralogy remains unaltered after deposition (Campana and Neilson, 1985). Third, the calcium carbonate and trace elements that make up greater than 90% of the otolith appear to be derived mainly from water, as modified by ambient temperature (Fowler *et al.*, 1995; Farrell and Campana, 1996). The elemental composition of the otolith will, therefore, reflect to some degree the environmental characteristics of the water in which the fish lives (Thorrold *et al.*, 1997). Since physical and chemical composition characteristics of water vary spatially, otolith microchemistry should record the water mass characteristics specific to a particular natal area.

INSTRUMENTATION

Although a thorough discussion of the instrumentation used in the analysis of otolith chemistry is beyond the scope of this paper (see Campana *et al.*, 1997), a brief description of techniques is warranted. Instrumentation can be divided into bulk or solution based approaches, including atomic absorption spectroscopy (AAS), neutron activation analysis (INNA), and inductively coupled plasma atomic emission spectroscopy (ICP-AES), and probe-based techniques electron probe microanalysis (EPMA), proton induced x-ray emission (PIXE), synchroton radiation x-ray fluorescence spectroscopy (SRXFS), and the ion microprobe. This distinction is, however, becoming increasingly blurred by the development of probe interfaces for techniques that have traditionally been considered bulk methods, such as for INAA and inductively coupled plasma mass spectrometry (ICP-MS).

The most widely used tool for routine analysis of otolith chemistry has been the electron microprobe (Gunn *et al.*, 1992). The instrument is available at many universities and research institutions, which may explain at least some of its popularity. Although spatial resolution of the instrument is excellent, with beam diameters of < 10 μm, sensitivity limits quantification to no more than 6 elements present in the otolith at minor to percent levels (Na, S, Cl, K, Ca, and Sr), as detection limits for most elements are typically around 100 $ug.g^{-1}$ (Figure 1). Unfortunately, this list includes only one element, Sr, whose concentration in the otolith is likely to be reflective of environmental availability. Further, EPMA analysis is generally not considered sufficiently precise for use in Sr thermometry, despite several applications for that purpose in the literature.

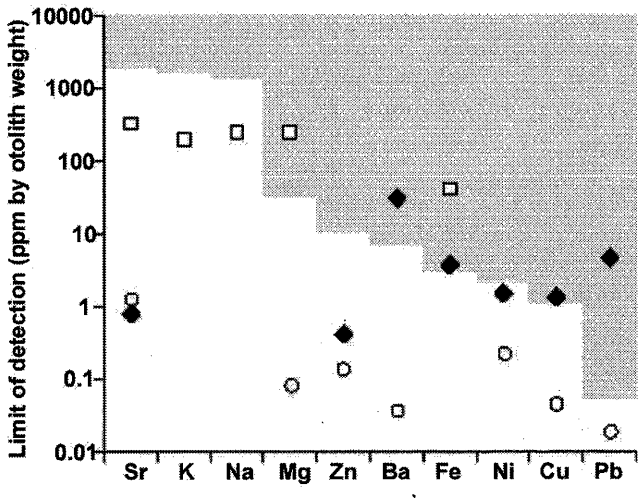

Figure 1. Limits of detection for the wave-length dispersive electron microprobe (□), proton-induced x-ray emission (♦), and laser ablation ICP-MS (○) in otoliths. Limits of detection indicated by symbols within the shaded area are higher than likely concentrations in otoliths from marine fish, and therefore are unable to be quantified by the instrument in question. Data from Campana *et al.* (1997).

Otolith chemistry applications that depend upon relatively large differences among water mass chemistries, such as tracing movements of fish from oceanic spawning grounds to low salinity estuarine nursery areas through differences in Sr/Ca profiles, can be adequately addressed using EPMA.

A second technique, related to EMPA, is proton-induced x-ray emission (PIXE). Sie and Thresher (1992) provide an introduction to the technique, as applied to otolith chemistry. In essence, PIXE uses a beam of protons, as opposed to a beam of electrons in EPMA, to generate characteristic x-rays from the sample surface which are then detected by energy dispersive spectrometry. The instrument is one to two orders of magnitude more sensitive than EPMA, with limits of detection approaching 1 $\mu g \cdot g^{-1}$. Although this is obviously a significant improvement over EPMA, concentrations of the majority of trace elements in otoliths still remain near or below detection limits of the instrument (Sie and Thresher 1992). Indeed, given that the trace-metal. clean techniques were not been routinely used in otolith studies until quite recently, it is likely that actual levels in otoliths were even lower than those recorded by Sie and Thresher (1992). An examination of otolith chemistry studies that have used PIXE suggests that only two elements, Zn and Ba, are detected with any regularity that were below the detection limits of EPMA (Campana *et al.*, 1997). Measurement precision is similar to that of EPMA (Sie and Thresher 1992), and hence the technique is not suited to high precision Sr thermometry applications.

Recently, a number of studies of otolith chemistry have been published based en assays using ICP-MS (see Campana 1999 for a review). The technique is very sensitive, with limits of detection in solution-based mode of around 1–10 $ng.g^{-1}$ otolith weight. This, in turn, means that it is possible to assay a number of elements in otoliths that cannot be quantified using EPMA or PIXE, including Li, B, Mg, Mn, Ni, Cu, Cd, and Pb. By coupling the ICP-MS with a laser ablation system, probe-based analyses of sectioned otoliths can be performed with a spatial resolution that is now, with the use of excimer lasers, approaching that of EPMA (Sinclair *et al.,* 1998). The instrumentation is less than two decades old, and development of the technique is still proceeding rapidly. For instance, most ICP-MS sold to date have been single collector instruments based on quadrupole mass analyzers. However, new generation ICP-MS instruments have recently become commercially that utilize sector field mass analyzers. Single collector sector field instruments have several advantages over quadrupoles based instruments, including the ability to operate in high mass resolution. This, in turn, allows molecular interferences on isotopes of interest to be resolved and eliminated. Molecular interferences are particularly common in high Ca matrices like otoliths, particularly on transition-row metals such as Ni and Cu (Figure 2). These interferences make quantification of these elements using quadrupole ICP-MS difficult, if not impossible. Sector field ICP-MS instruments can also assay inter-element ratios, such as Sr/Ca, with precision (0.05% RSD) approaching that of thermal ionization mass spectrometry (Rosenthal *et al.,* 1999, Latkoczy and Thorrold unpubl. data).

APPLICATIONS

Through funding from the National Science Foundation, we are examining the movement of Atlantic croaker (*Micropogonias undulatus*) from offshore spawning locations to estuarine nursery areas. We hypothesized that the trace element and isotopic composition of the water in which larvae are spawned will be recorded in the otolith chemistry. Life-history scans across the otoliths of juvenile croaker, using laser ablation sector field ICP- MS, revealed variation in Mn/Ca, Sr/Ca and Ba/Ca ratios across individual otoliths, and among otoliths from juveniles collected in different locations (Figure 3).

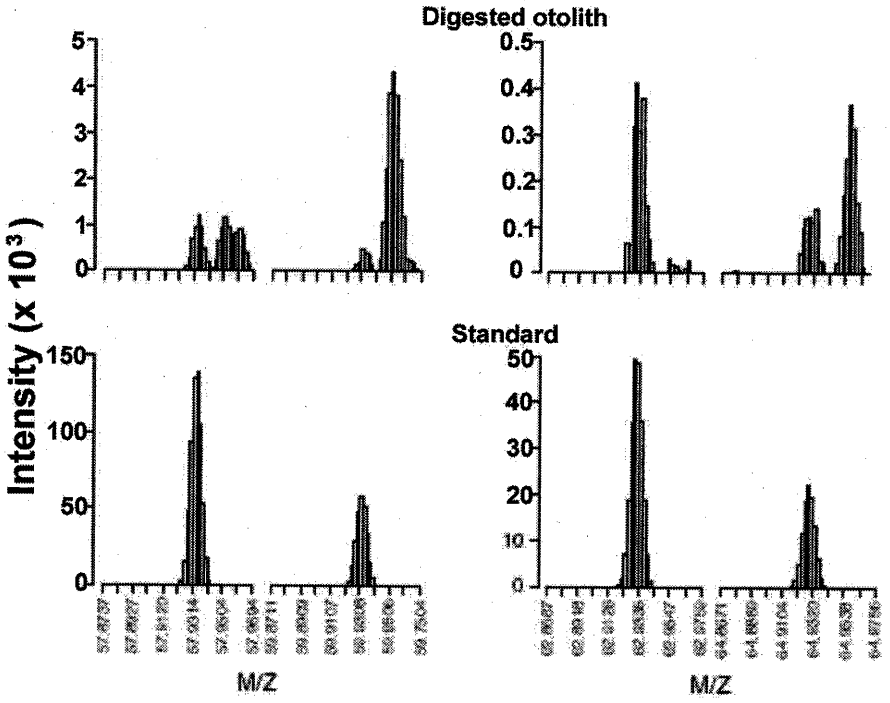

Figure 2. Results of high resolution ICP-MS (r=3000) analysis of Ni and Cu isotopes from an unseparated dissolved weakfish otolith (top panel) and Ni and Cu standard (bottom panel).

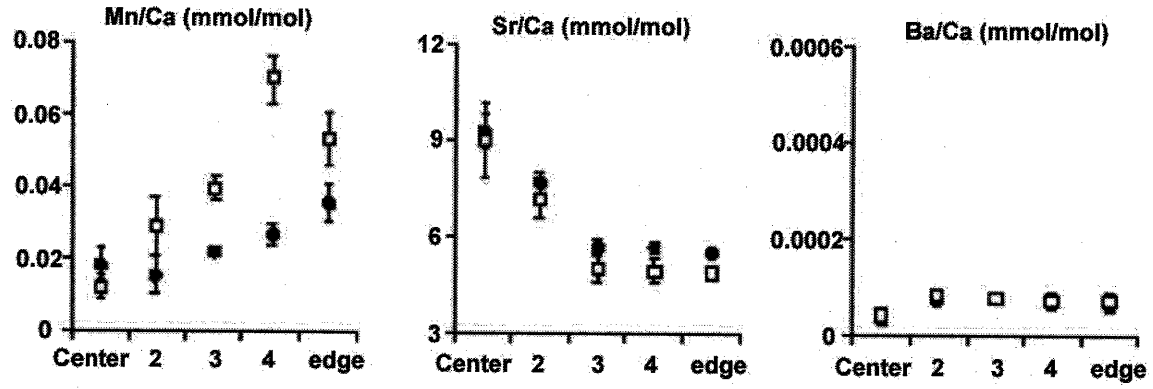

Figure 3. Results from laser ablation sector field ICP-MS analysis of juvenile Atlantic croaker (*Micropogonias undulatus*) otoliths from Pamlico Sound, North Carolina. Analyses were conducted at 5 sites from the center to the edge of each otolith. Results are means (+/- SE) of three otoliths from the Pamlico River (●) and the Neuse River (□).

The most obvious feature in the Mn:Ca profiles was the extremely high ratios seen towards the edge of otoliths collected from the Neuse River. The pattern was not, however, seen in Pamlico River otoliths. If Mn/Ca ratios in otoliths are reflecting Mn:Ca ratios in the water column, then dissolved Mn in the Neuse River must have been comparatively high in March and April of 1997. We suspect that high Mn levels may be due to the presence of hypoxic bottom waters in the Neuse River (Thorrold and Shuttleworth in press). If this hypothesis can be validated, Mn:Ca levels in otoliths may be a useful proxy for hypoxia in aquatic environments. Otolith Sr:Ca profiles from both the Neuse and Pamlico River samples declined sharply from the center of the otoliths to the middle position, and remained constant thereafter out to the edge (Figure 3), These results indicate movement from offshore waters with comparatively high Sr/Ca values to estuarine systems with low Sr/Ca values. These data are consistent with a number of earlier studies that have found that Sr/Ca ratios can change dramatically as fish move from freshwater to the coastal ocean or vice versa. Ba:Ca

ratios from Pamlico Sound fish were surprisingly constant among replicate fish from the same river, and within otoliths from individual fish. Although these was a slight increase in Ba/Ca ratios from the center to the second position on the otoliths, the difference was insignificant. This is in contrast to life history scans from juvenile croaker collected in Chesapeake Bay (Thorrold *et al.*, 1997, Thorrold and Shuttleworth in press), which we suspect is due to a lack of freshwater input in Pamlico Sound compared to Chesapeake Bay.

Stock structure of weakfish (*Cynoscion regalis*)

We are also investigating stock structure and age-specific migration patterns of weakfish (*Cynoscion regalis*) along the Atlantic coast of the United States. Efforts to manage this fishery are presently handicapped by an inability to determine the spatial extent of weakfish stocks. Previous studies based on mark-recapture, morphology and life history differences have suggested there may be multiple stocks of weakfish along the mid-Atlantic coast (reviewed by Graves *et a.*, 1992). However, allozyme and mtDNA studies failed to support a multi-stock hypothesis. Weakfish spawn in estuarine and near-coastal waters, and larvae are believed to use selective tidal stream transport to remain within the estuary in which they were spawned (Rowe and Epifanio 1994). If larval drift is insignificant, and if spawning fish home to their natal area, weakfish should show evidence of stock separation along a geographic range that spans the entire U.S. Atlantic coast from Florida to Maine. However the power of the genetic studies to test the null hypothesis of a single homogeneous stock was limited due to the low amount of overall genetic variation that was revealed. The mean nucleotide sequence diversity (the average amount of nucleotide differences found among individuals of a population) in the mtDNA analysis averaged 0.15%, one of the lowest values reported for any vertebrate (Graves *et al.* 1992). There is no way to determine, at this stage, if there is sufficient larval drift, or adult vagrancy, to prevent the formation of stock structure, or if the genetic techniques are not powerful enough to reveal what may be subtle genetic differences among adjacent populations.

We investigated the ability of geochemical signatures in otoliths to record the nursery areas of juvenile weakfish, *Cynoscion regalis*. Juvenile *C. regalis* were collected from multiple sites in Doboy Sound (Georgia), Pamlico Sound (North Carolina), Chesapeake Bay (Virginia), Delaware Bay (Delaware) and Peconic Bay (New York) from July to September 1996. One sagittal otolith from each specimen was assayed for Mg/Ca, Mn/Ca, Sr/Ca and Ba/Ca ratios using inductively coupled plasma mass spectrometry (ICP-MS), while $\delta^{13}C$ and $\delta^{18}O$ values from the other sagittal otolith in the pair were determined using isotope ratio mass spectrometry (IR-MS). On the basis of multivariate differences in these chemical signatures (Figure 4), linear discriminant function analysis and artificial neural network models were able to successfully classify individual fish to their natal estuary with overall error rates of less than 10%. Further, even better results were obtained from samples in 1997. However, on a more cautious note, we were not able to classify 1997 fish using models parameterized by 1996 juveniles. We will be able to use chemical signatures from the juvenile portion of adult *C. regalis* otoliths to accurately classify these fish to their natal estuary. We anticipate that we will not only be able to follow migration in young fish, but will also be able to determine age-specific mixing rates and thus, the extent of philopatry in these estuarine-dependent fishes.

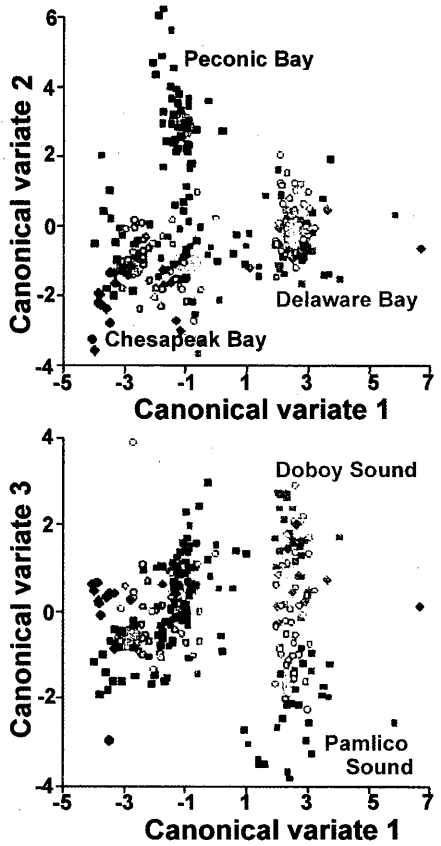

Figure 4. Canonical variates 1 and 2 (top), and canonical variates 1 and 3 (bottom), summarizing variations in chemical signatures in *Cynoscion regalis*, otoliths, collected from live locations along the Atlantic coast of the U.S. Symbol colors map to locations, while symbol shapes indicate different sites within a location. Shaded areas represent bootstrapped 95% confidence ellipses around location means for each variate.

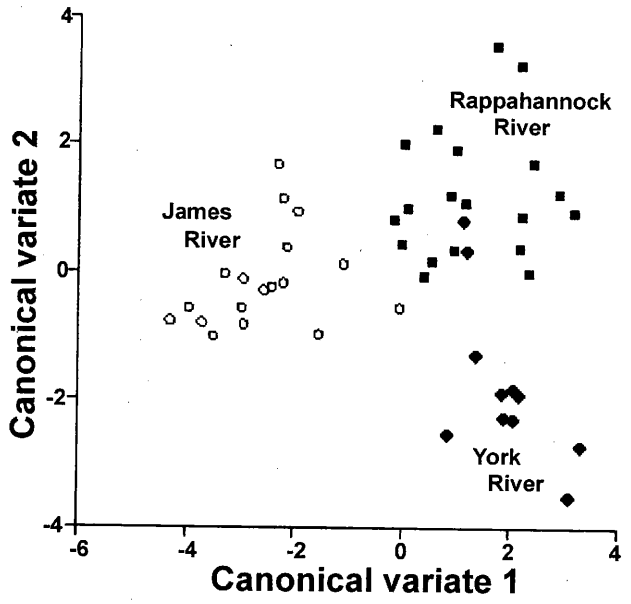

Figure 5. Plot of first 2 canonical variates summarizing variations in chemical signatures in otoliths among juvenile weakfish from the Rappahannock River, and James River in Chesapeak Bay.

The highly significant differences in chemical signatures among sites at some locations suggested that otolith chemistry varied over smaller spatial scales as well as among estuaries. To examine this in more detail, we used samples collected from 3 rivers that flow into the western shore of Chesapeake Bay- the James, York and Rappahannock Rivers. While sample sizes were smaller than the among location comparisons, CDA plots still showed that there was considerable separation among the rivers using the combined elemental and isotopic data (Figure 5). Indeed, only two fish from the Rappahannock River overlapped in discriminant space with fish from the York River; the remaining 52 samples separated clearly into the specific river in which they were captured.

FUTURE DIRECTIONS

We anticipate that instrumentation will continue to be developed and that these advances will lead to further refinement in our techniques in analyzing chemical signatures in otoliths. One potential problem, as we start quantifying elements in otoliths that may be toxic to the fish, is that physiological regulation across the branchial membranes may de-couple the chemical composition of the water from that of the endolymphatic fluid. In such cases, absolute levels in the otolith may tell us little about environmental exposure to a metal. However, these problems can largely be avoided by using isotopic tracers, which are unlikely to fractionate during transport and deposition from the water to the otolith. Isotopic ratios of Li, Ca, Cu, Sr, and Pb, for instance, vary in natural waters, and may prove to be useful tracers of water mass residency and pollution exposure (*e.g.* Kennedy *et a.*, 1997). Although precise measurements of isotope ratios have traditionally been on thermal ionization mass spectrometry, the recent development of multi-collector ICP-MS has provided an alternative that may be particularly useful in otolith research (Halliday *et al.*, 1998). The ability of multi-collector ICP-MS to generate extremely precise isotopic ratios, with a minimum of sample preparation and the option of sample introduction via laser ablation, leads us to believe that this instrument will be become a routine, but powerful, tool in the arsenal of otolith chemistry researchers.

Although further technique development is ongoing, we are encouraged by our first successes. Because we can now isolate a day or two of growth in the otolith with new laser-probe techniques, we will be able to match the scale of biological events to physical events. Future studies will permit us to couple the timing of larval advection with meso-scale physical oceanographic processes and meterological forcing events. This knowledge is fundamental in predicting recruitment of estuarine-dependent marine fishes, so far an elusive goal in assessing the health of fish populations. However, the promise of otolith chemistry is greater than the application to recruitment that we have presented in this paper. For the first time, scientists will be able to place the origins and movements of fish in space and time. The chemical signatures in fish otoliths can be used to determine the extent of stock mixing across jurisdictional boundaries to provide evidence in resolving transboundary disputes as is already being attempted for Atlantic bluefin tuna and walleye pollock. The ability to determine origins in mixed-stock fisheries will permit managers to tailor regulations to maximize the persistence of vulnerable stocks.

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Population Structure of Walleye Pollock Inferred from Fluctuating Allele Frequencies at the Allozyme Locus *SOD*

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SUMMARY: Exceptional genetic markers with unique discriminating power have been identified in both nuclear DNA and allozymes applied to stock identification problems in marine species. In this study we focus on the distribution of *SOD* variation, as this allozyme locus appears to be uniquely valuable for describing walleye pollock *Theragra chalcoglamma* populations. Expanding on the sampling of Iwata (1975) we find that *SOD* allele frequencies distinguish Eastern Bering Sea and Gulf of Alaska collections from each other and from our one collection from the Kamchatka area. Interestingly, the sample of spawning fish taken from Prince William Sound, Alaska, in 1998 differ significantly (P = 0.001) from those taken in 1997. This latter result suggests that the spawners in Prince William Sound may be a sink population originating from genetically discrete sources in the Gulf of Alaska.

INTRODUCTION

The pollock fishery off the coast of North America is highly valued, and an understanding of stock identification can provide information key to sustained management. Management issues that would benefit from a clearer understanding of population structure include (1) response to the depletion and potential U.S. Endangered Species Act (ESA) listing of stocks off the coast of Washington, (2) prey/predator interactions and the fishing restrictions invoked because of existing ESA protection of the Stellar sea lion, and (3) meta-population structure and interaction of big basin spawners with fjord spawners such as found in Prince William Sound, Alaska. Genetic markers have yet to provide clear insight into high-seas issues such as the origin of the fish intercepted in Donut-Hole fisheries. Many hope that development of new markers may provide such insights as well as identify larvae to population of origin to aid forecasting efforts.

The population structure of pollock is not well understood. Large aggregations are contained in the big basins of the Bering Sea and Sea of Okhotsk. Smaller stocks spawn in fjords and valleys of the continental shelf ranging from Japan to Puget Sound, Washington, USA. In 1995 a new fishery emerged on spawners in Prince William Sound, Alaska, managed by the State of Alaska. State regulation of this fishery may confound the inter-jurisdictional management allocations now in place to conserve the Gulf of Alaska stocks. Knowledge of the demographic and genetic relationships of the Prince William Sound spawners to those inhabiting the greater Gulf of Alaska would help managers to better administer the quotas of the Prince William Sound fishery.

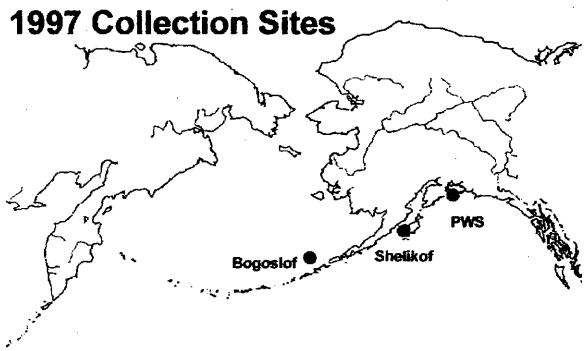
We have three primary objectives:
(1) *Re-examine an array of different genetic markers for stock-discriminating capability.* Different classes of gene markers have shown limited resolving power in historical studies of pollock; some of these studies were hampered by the fact that data were collected from samples of fish collected in the summer. Pollock segregate into spawning groups in winter and early spring, and summer collections would potentially be from mixtures not representing the discrete stocks of interest. We propose to test an array of gene markers, including those previously examined, from spawning aggregations to maximize our chance to detect any stock differences.
(2) *Use promising gene markers to test for stock structure among Prince William Sound spawners and other spawning aggregations in the Gulf of Alaska.*
(3) *Apply promising markers to study the stock structure of pollock inhabiting the North Pacific Ocean and Bering Sea.* We are expanding our examination to include other collections from the N. Pacific and Bering Sea to address broader questions such as stock distribution and migration, origin of Donut Hole stocks, or identification of larvae to stock of origin.

In this oral communication we focus primarily on our findings of the distribution of variation at the allozyme locus *SOD*, as this locus appears uniquely valuable for describing pollock populations.

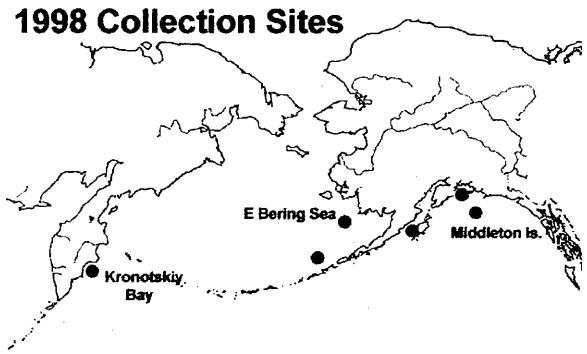
MATERIALS AND METHODS

We started this project by screening for variation at (1) restriction fragment length polymorphism at several mtDNA genes, (2) seven microsatellite loci first described as polymorphic in Atlantic cod (*Gmos*, Brooker *et al.,* 1994), and (3) forty allozyme loci including those described by Iwata (1975) and Grant and Utter (1980). After the initial screening we chose cytochrome *b*, cytochrome oxidase, and ND 5/6 regions of mtDNA where we ultimately detected 62 haplotypes. Four of the seven *Gmo* loci appeared to perform reliably at first. Ultimately we chose to only use *Gmo1* and *Gmo2* for the population surveys because null alleles confused the scoring of the other loci. We identified 29 allozyme loci that we could reliably score to address project objectives two and three.

In 1997 we collected muscle, liver, heart, and eye tissue from spawners in Prince William Sound (N=100), and the US National Marine Fisheries Service provided tissues from spawners from Shelikof Strait (N = 100) and Bogoslof Island (N=80) (Slide 1).



Slide 1. Collection sites in 1997 including Bogoslof Island, Shelikof Strait, and Prince William Sound.



Slide 2. Collections sites in 1998, in addition to repeats of 1997, also included Middleton Island in the Gulf of Alaska, Eastern Bering Sea, and Kronotskiy Bay.

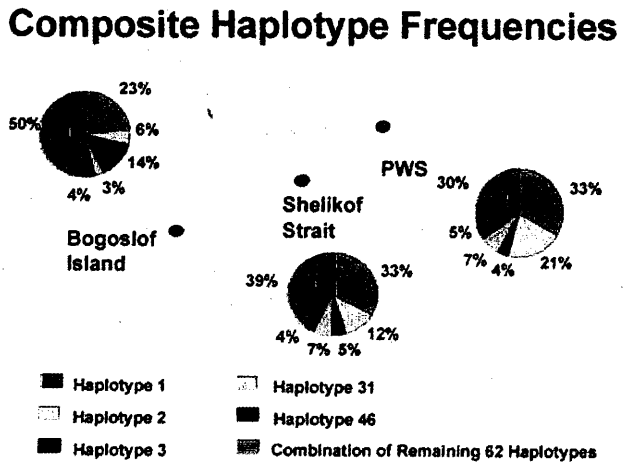
In 1998 we obtained tissues from 100 spawners from each of the three 1997 collection sites. We also collected tissues from 100 spawners from a newly detected stock spawning near Middleton Island in the Gulf of Alaska, and National Marine Fisheries Service provided an additional collection of 40 spawners from the Eastern Bering Sea (Slide 2). Kamchatka Research Institute of Fisheries and Oceanography provided 100 samples from Kronotskiy Bay (southeast Kamchatka).

RESULTS AND DISCUSSLON

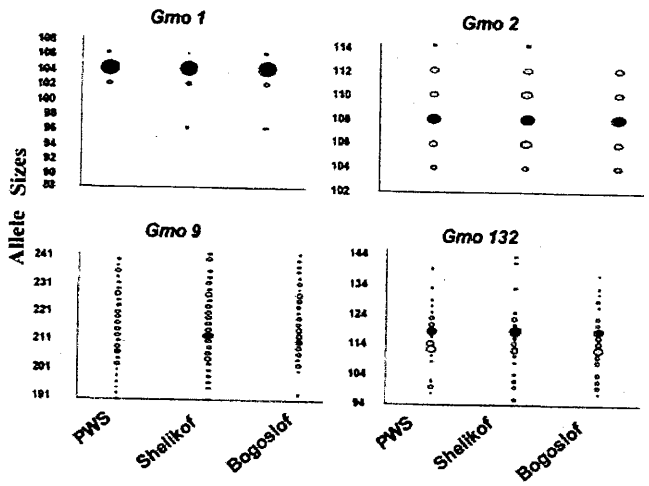
We have completed the mtDNA and microsatellite analyses of the 1997 collections and allozyme analysis of the 1997 and 1998 collections.

Briefly, we found no haplotype differences between the Gulf of Alaska collections from Prince William Sound and Shelikof Strait. The Bogoslof Island collection was genetically discrete, distinguished by frequencies of haplotypes 1, 2, and 3 (Slide 3).

No differences among the three 1997 collections were observed for the *Gmo* loci (Slide 4). Single-pair matings suggested that *Gmo1* and *Gmo2* were segregating in a Mendelian fashion and confirmed that *Gmo9* and *Gmo132* were expressing null alleles. *Gmo9* and *Gmo132* were dropped from further analyses.

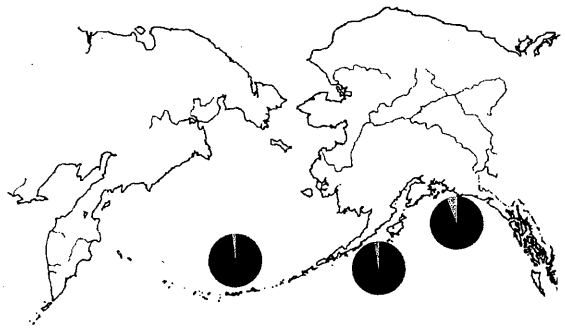


Slide 3. Composite haplotype frequencies. The Bogoslof Island collection is genetically discrete from Prince William Sound (*P=0.0001*) and Shelikof Strait (*P=0.0005*). Prince William Sound and Shelikof Strait collections are not genetically discrete (*P=0.8668*).



Slide 4. Bubble-graph representation of allele frequencies for the *Gmo* microsatellites for the 1997 collections. Allele size (base pairs) on the y-axis; population on the x-axis. Allele frequency is proportional to size of bubble. *Gmo* loci do not differentiate the 1997 collections from Prince William Sound, Shelikof Strait, and Bogoslof Island.

SOD variation in pollock, 1997

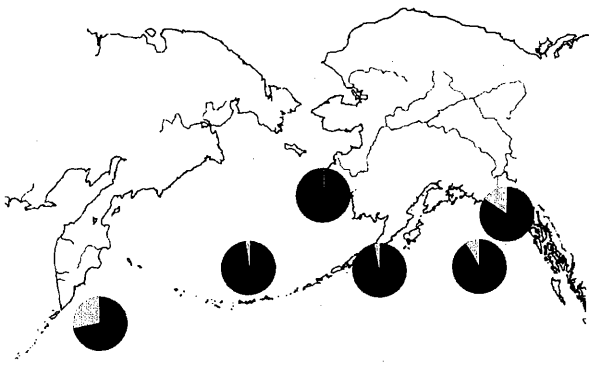


Slide 5. We observed a low frequency of a fast migrating *SOD* allele in our Gulf of Alaska and Bogoslof collections in 1997. The frequencies were not significantly different among the collections.

With one exceptions, allozyme loci showed no striking differences among collections. Expanding on the sampling of Iwata (1995), we found that *SOD* allele frequencies distinguish Eastern Bering Sea and Gulf of Alaska collections from each other and from our one collection from the Kamchatka area (Slide 5,6,7).

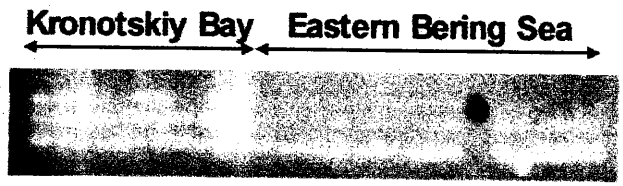
Interestingly, the sample of spawners from Prince William Sound, Alaska, in 1998 differ significantly (P = 0.001) from those taken in 1997. This latter result suggests that the spawners in Prince William Sound may be a sink population originating from genetically discrete sources in the Gulf of Alaska (Slide 8).

SOD variation in pollock, 1998

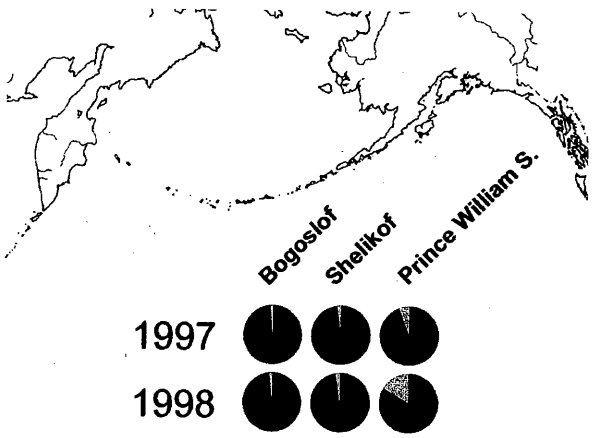


Slide 6. We observed elevated frequencies of the fast-migrating allele in the 1998 collections from Prince William Sound, Middleton Island, and Kronotskiy Bay.

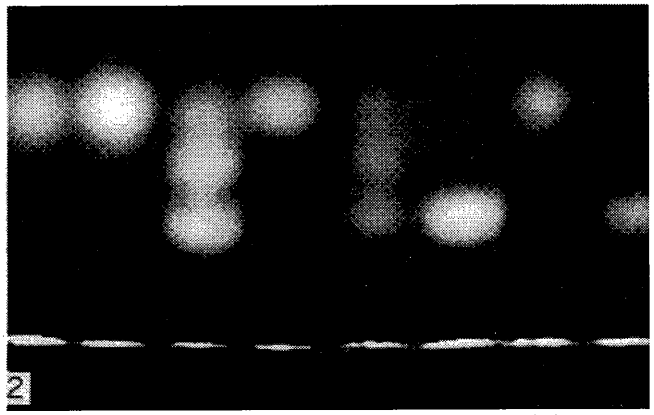
SOD Phenotypes
Observed 1997-1999



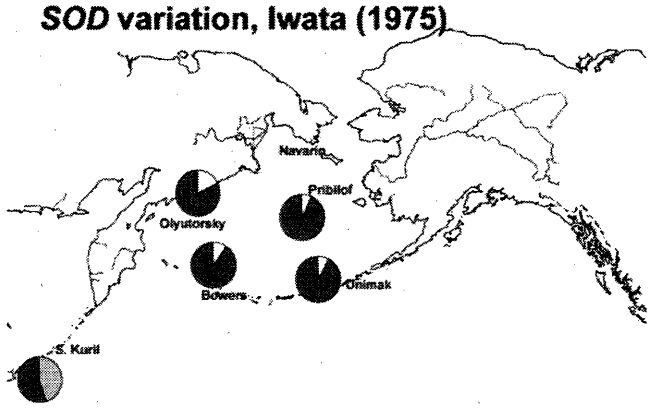
Slide 7. Allozyme phenotypes observed in our study.



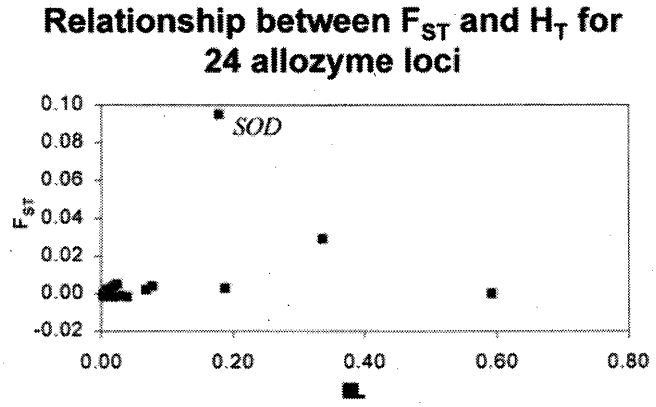
Slide 8. Temporal variation of allete frequencies at *SOD* between 1997 and 1998 for three populations. The 1998 collection from Prince William Sound differed significantly from the 1997 collection (P=0.001).



Slide 9. *SOD* allozyme variation described in Iwata (1975).



Slide 10. Distribution of *SOD* allele frequencies among collections of spawning pollock.



Slide 11. *SOD* demonstrates elevated between-population variability (Fst) for a given total heterozygosite (H_T) compared to other allozyme loci. Such a relationship suggests that directional selection may be driving *SOD* allozyme frequencies to differing endopoints, enhancing the discriminating power of this locus to detect discrete populations.

We are especially interested in the fluctuating population structure observed in Prince William Sound. Pollock populations are driven by strong year classes, so we want to collect matched otolith and tissue samples to examine possible year-class effects on gene frequencies.

Of the historical studies of genetic variation in pollock, none report more discriminating power than that reported by Iwata (1975) for the allozyme locus *SOD* (Slide 9). Iwata (1975) report demographic variables that describe various stocks in the Eastern Pacific Ocean and Eastern Bering Sea; his *SOD* alleles were expressed in a clinal pattern along a SW-NE axis (Slide 10).

Alleles of *SOD* genes have been shown to be under strong directional selection in other species. The elevated F_{st} for *SOD* among pollock populations suggests that *SOD* alleles are under the influence of such directional selection (Slide 11). We believe that this property may make the locus *SOD* especially valuable for identifying discrete stocks of pollock.

In recent years hope for the development of new DNA markers with increased resolving power led to the near abandonment of allozymes as a tool for studying the genetics of pollock populations. Yet, to date none of the modern DNA studies show the resolving power demonstrated by *SOD* allozymes (e.g., see Mulligan *et al.* 1992, Shields and Gust 1995, see also O'Reiley *et al.* this symposium). Exceptional markers with unique discriminating power have been identified in both nuclear DNA and allozymes applied to stock identification problems in other marine species. Also, mitochondrial DNA markers may reflect evolutionary signatures different from those observed with nuclear markers. We advocate the use of several marker classes to produce the best understanding of the population genetics of a species by taking advantage of exceptional markers as well as diversity of evolutionary processes. In the case of pollock, we believe that the collection of samples from spawners over multiple years is essential to clarify the genetic relationships among various populations.

ACKNOWLEDGEMENTS

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Recent Advances in Genetic Stock Study for Tunas and Billfishes

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SUMMARY: Tunas and billfishes are highly migratory large pelagic fish distributed in a very wide area through tropical to temperate occasionally in cold waters. Their reproductive activity is widely extending east and west in an ocean basin but confined in tropical and sub-tropical waters. These aspects have given us an impression that the population may be genetically homogeneous. Understanding the stock structure may improve fishery management strategies. If multiple stocks occupy the same area, they should be managed separately and we have to determine the mixing ratio of individuals in the catch. Fish population geneticists have sought for gene markers with higher variation. Because highly polymorphic genetic markers may be potentially capable of detecting subtle signals of population subdivision on small spatial scales and over short periods of time. The inherent large variance of the highly polymorphic genetic markers, however, may sometimes mask existing genetic difference between target populations. Here, I summarize several genetic stock studies for tuna and billfish species, in which low to high variable genetic markers were applied, and also briefly introduce an attempt to develop a rapid detection system on nucleotide sequence differences between tuna species.

Northern bluefin tuna (*Thunnus tynnus*)

Diagnostic difference in nucleotide sequences of coding regions in mtDNA (cytochrome *b* gene and a segment flanking ATPase and COIII genes designated *ATCO*) were found between the Atlantic and Pacific populations to the extent that single restriction enzyme digestion could nearly identify the origin of an individual (Chow and Inoue, 1993; Chow and Kishino, 1995). This mtDNA marker was nearly monomorphic in each ocean basin sample, meaning no capability to investigate genetic stock study in each ocean. Broughton and Gold (1997) and Takagi *et al.* (1999) isolated five and four microsatellite loci, respectively, finding all were hyper variable. Significant differences in allele frequencies at these loci were observed between the Atlantic and Pacific samples, but none of these loci were comparable with the simple and diagnostic mtDNA marker for separating the Atlantic and Pacific individuals. Concerning the putative eastern and western stocks of Atlantic northern bluefin tuna, Broughton and Gold (1997) and Takagi *et al.* (1999) compared samples from northwestern Atlantic and Mediterranean Sea. Nucleotide sequence and RFLP analyses in the hyper variable mtDNA *D-loop* region and several nuclear gene introns have been performed using larval samples from the Gulf of Mexico and Mediterranean Sea (Chow, unpublished). But none of the genetic analyses have so far detected any signal of genetic differentiation between eastern and western stocks.

Swordfish (*Xiphias gladius*)

Kotoulas *et al.* (1995) first found heterogeneous haplotype frequencies of mtDNA between swordfish samples collected in the Mediterranean Sea and tropical Atlantic Ocean. Nucleotide sequence analysis of hyper variable control region (*D-loop*) of mtDNA revealed highly significant difference in the haplotype frequencies between samples from the Mediterranean Sea, Atlantic and Indo-Pacific (Rosel and Block, 1995; Alvarado-Bremer *et al.* 1996). Many rare genotypes produced by sequence analysis, however, did not increase resolving power or even have obscured existing differences between stocks. Restriction assay naturally detected less polymorphism in this hyper variable region, but Chow *et al.* (1997) demonstrated RFLP analysis to delineate better profile on the population structure than sequence analysis. A single restriction enzyme digestion detected no variation in the Mediterranean sample but high variation in the other samples, indicating that no exogenous swordfish enters the Mediterranean Sea. Chow and Takeyama (in press) performed nucleotide sequence and restriction analyses on calmodulin gene intron locus (*CaM*) isolated by Chow (1998) and revealed low variation, in which only two alleles (*A* and *B*) were observed. Yet, substantial differences in the allele frequencies were observed between Mediterranean-Northwest Atlantic samples (CaM^A=34.7 to 49.3%) and those from tropical Atlantic and Indo-Pacific (CaM^A=84.0 to 100%). Chow and Takeyama (in press) further attempted combined analysis of these *CaM* and *D-loop* loci and suggested that there are at least four breeding units: the Mediterranean Sea, western Atlantic (possibly the Gulf of Mexico-Caribbean), tropical-east to South Atlantic

(possibly the Gulf of Guinea), and Indo-Pacific.

Bigeye tuna (*Thunnus obesus*)

Alvarado-Bremer *et al.* (1998) using nucleotide sequencing analysis of a short segment of mtDNA control region (*D-loop*) found that mtDNA genotypes of bigeye tuna fell into two highly divergent clades (I and II) which were unequally distributed between the Atlantic and Indo-Pacific. Chow *et al.* (in press) also found two genotypes (designated α and β) in a segment flanking the ATPase and COIII genes (*ATCO* segment), which were simply identified by a single restriction digestion. Type α was predominated in the Atlantic, where 178 out of 244 individuals examined were type α. In contrast, only one out of 195 individuals collected in the Indo-Pacific was type α, indicating that gene flow and fish migration between the Atlantic and Indo-Pacific are severely restricted. They also observed that the variation in the other segment containing *D-loop* region was much higher and that the genotype distributions were highly heterogeneous between the Atlantic and Indo-Pacific. However, former less polymorphic marker was more diagnostic because of its much smaller error. Frequency of type α considerably varied from 0 to 80 % among seven samples collected off Cape of Good Hope, indicating that fishes from these two distinct stocks (Atlantic and Indo-Pacific) are mixing around South Africa. This simple and diagnostic genetic marker appears to be useful for estimating mixing ratio around South Africa.

As shown above, highly polymorphic genetic markers are not always better than less polymorphic one. In management point of view, simple and diagnostic genetic markers to separate stocks or to assess mixing ratio between stocks appear to be suited for practical use. Matsunaga *et al.* (1999) have designed tuna species-specific DNA probes based on the nucleotide sequences in the *ATCO* segment and constructed rapid species identification system using DNA chip. Higher fluorescence intensity was observed in the hybridization of PCR products with its specific probe in DNA chip than with the probes for the other tuna species. In this system, single or two nucleotide differences within a short DNA segment (20 to 30bp) were enough to identify tuna species. If stock-specific DNA sequences are detected, this high-throughput detection system may be applied for stock identification study and/or estimating mixing ratio in the catch not only in the laboratory but also in the field.

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Molecular Cloning of Microsatellite DNA from Marine Organisms

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PROCEEDINGS

Microsatellite DNA sequences are tandem arrays of short nucleotide motifs, such as (dC-dA)n·(dG-dT)$_n$ repeats (Weber, 1990). Microsatellite DNA markers have proven to be powerful tools for various genetic studies, for instance, studies of population structure, evaluation in breeding, and construction of genetic linkage maps, because of their high informativeness of repeat length polymorphisms. However, as a problem, cloning microsatellites using the conventional method is difficult in some cases.

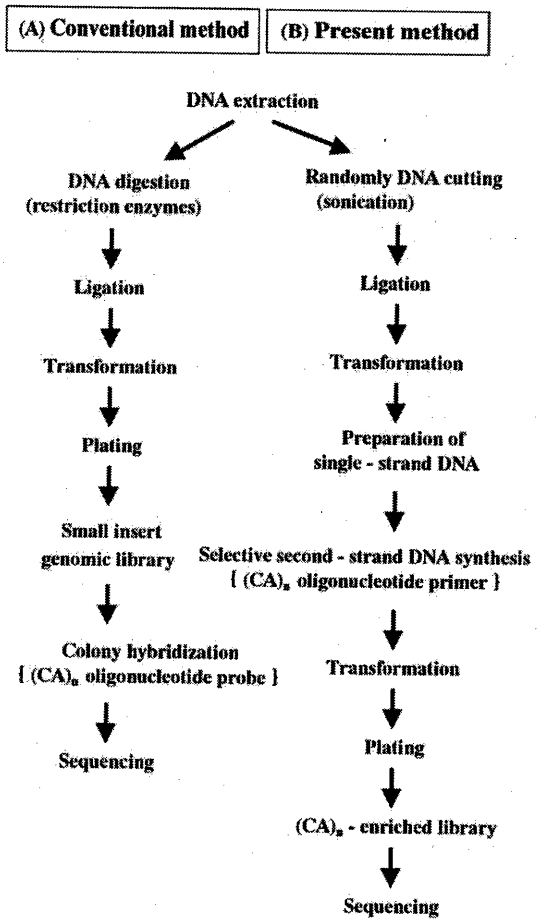

Fig. 1. Experimental procedures in the conventional colony hybridization method (A) and in present method (B). The conventional method was reffered to Brooker et al., (1994).

Fig. 1A shows a conventional experimental procedures that commonly used for cloning microsatellites. We thought the problems in the conventional method would be summarized into two points as follows: 1) A severe bias of DNA fragments obtained by restriction enzymes digestion; This is caused by a property that restriction enzymes can digest genomic DNA at only a specific DNA sequences. Therefore, use of DNA fragments digested by restriction enzymes at ligation step leads to a number of duplicated clones in a small insert genomic library. To minimize appearance of duplicated clones, we used sonicated DNA fragments (physical cutting of genomic DNA at random sites). 2) Low frequency of clones possessing microsatellite repeats in the library; This is a serious problem especially in several species which have small number of microsatellite copies in their genomes. In these species, a large number of Petri-plates must be screened for isolating a number of microsatellites. The complicated screening steps are labor and much time and costs consuming. To resolve this problem, we constructed a marker selected library. In the conventional method transformed cells are directly plated on Petri-plate medium, and these are referred to small insert genomic library. Meanwhile, in present method single strand DNA was prepared after transformation, and selective second strand DNA synthesis *in vitro* was employed using CA-repeat oligonucleotide primer. Resulant double strand DNA was transformed into competent cells again, and plated on. Yielding transformants were referred to a (TG/CA)$_n$ enriched library. Fig. 1B shows an experimental procedure in our method.

We tried to isolate CA-repeat microsatellites from Pacific abalone *Haliotis discus discus* genome using our method. First, we constructed a conventional small insert genomic library to estimate the frequency of microsatellite copies per haploid genome. As the results, four out of 2,400 clones (0.2±0.1%) were CA-positive (Fig. 2). Based on some assumptions, the results indicate that every 100-290 kbp contains CA-repeat microsatellites in the genome. Our data suggests that the frequency of CA-repeat microsatellites in Pacific abalone is quite low in comparison with teleost fishes (Brooker *et al.*, 1994, McConnell *et al.*, 1995) and

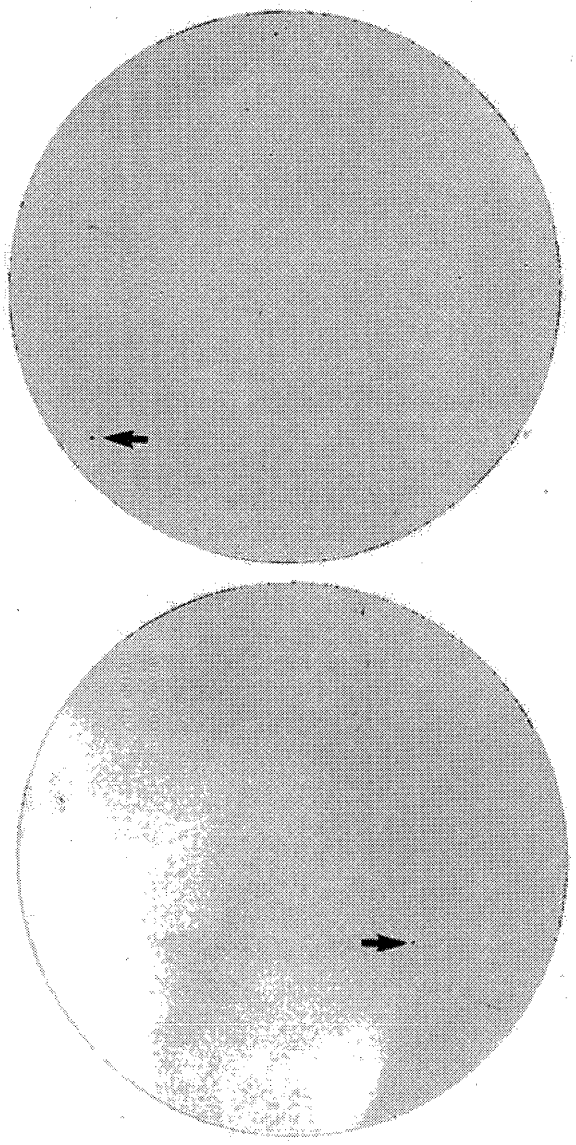

Fig. 2. The results of colony hybridization screening in a conventional small insert genomic library. Two sheets of membranes are shown. Arrows indicate (CA)-positive clones.

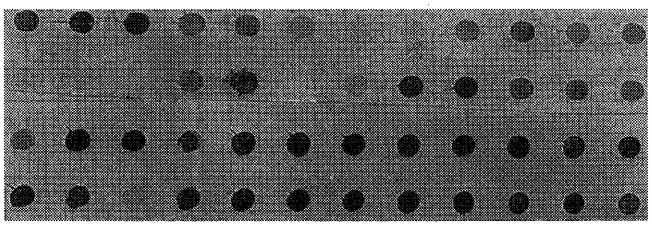

Fig. 3. The results of dot-blot hybridization screening of randomly selected clones from a $(CA)_n$-enriched library.

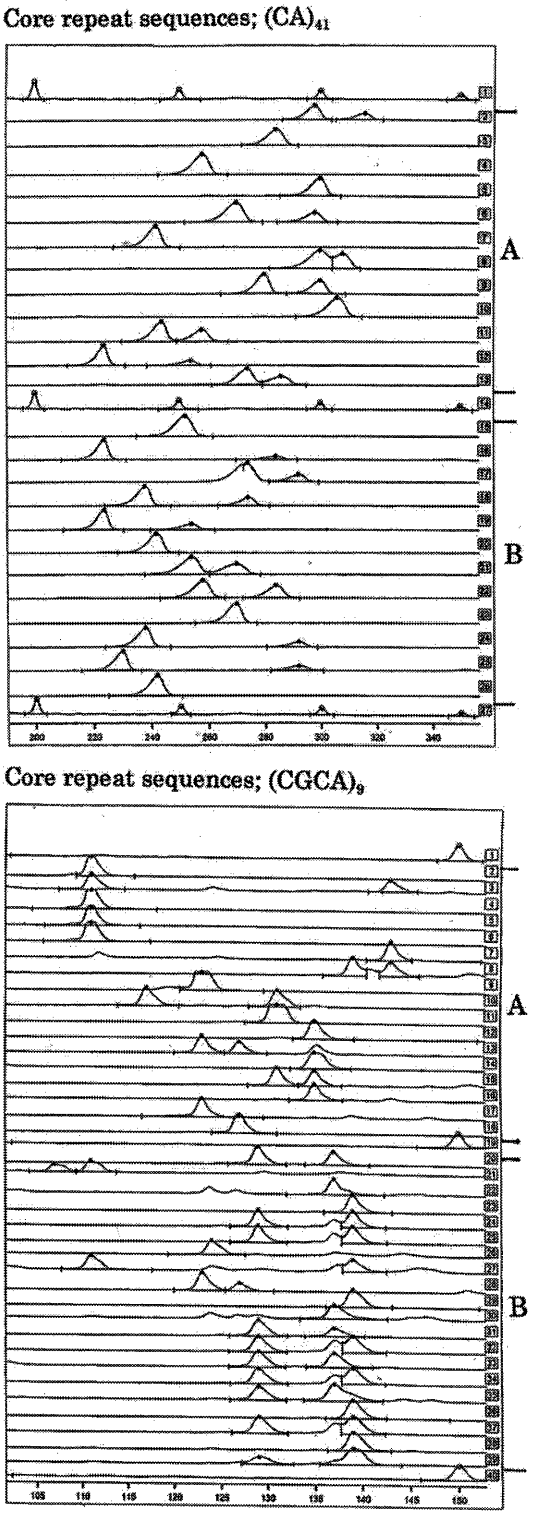

Fig. 4. CA- and CGCA-repeat length polymorphisms in Pacific abalone *Haliotis discus discus* (A) and *H. discus hannai* (B). Lane 1, 14, and 27 in the left one, and Lane 1, 19, and 40 in the right one are size marker every 50 bp. *Scale is bases.

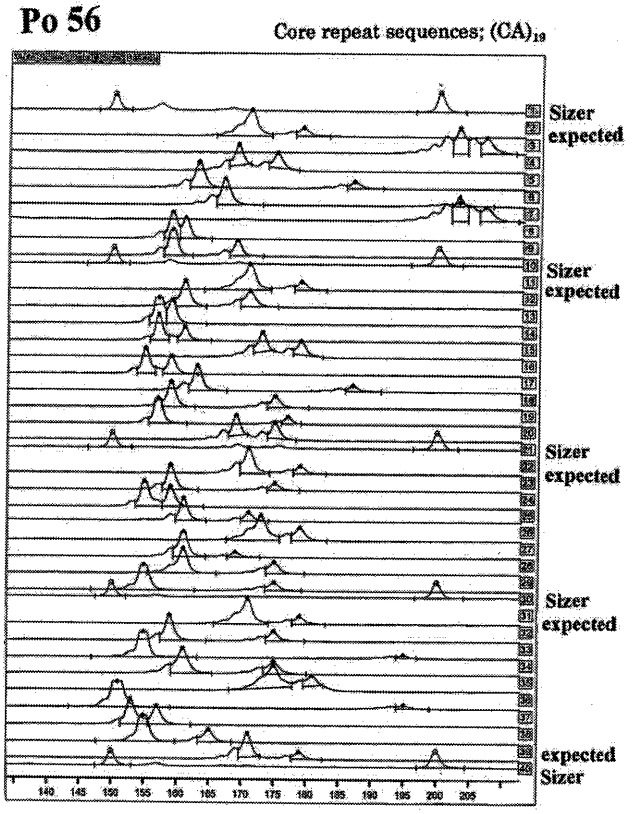

Fig. 5. CA-repeat length polymorphisms in Japanese flounder *Paralichthys olivaceus*. Lane 1, 10, 21, 30, and 40 are size marker every 50bp. Lane 2, 11, 22, 31, and 39 are PCR products from genomic DNA defined as a criterion. *Scale is bases.

mammals (Hamada *et al.*, 1982), being similar level to the European flat oyster (Naciri *et al.*, 1995). A $(TG/CA)_n$-enriched library was constructed using our method. As the results, 41 out of 48 clones (85.4±5.1%) gave CA-positive signals (Fig. 3). In spite of quite low frequency of CA-repeat microsatellites estimated in Pacific abalone genome, our method could construct about 400 fold more $(CA)_n$-enriched library in comparison with the conventional method. Thirty five out of 39 clones which were randomly chosen from the library contained independent 44 microsatellites (Table 1). A problem that Takahashi *et al.* (1996) pointed out, i.e., a half of clones in their library was duplicated, was minimized since the 35 clones contained different sequences each other. Twenty six out of the 35 clones had enough sequence information to design PCR primer sets flanking microsatellite sequences. This meant that about 60% of the clones which was randomly selected in the library could take into PCR primer designs. Although we have not yet examined all primer sets, highly polymorphisms were detected using several primer sets designed (Fig. 4).

Table 1. The Pacific abalone microsatellite repeat sequences from a $(CA)_n$-enriched library.

Clone No.	Sequence (5' → 3')	Primer design
1.	$(CA)_7(CGCA)_2CGA_2(CGCA)_2A_2(CA)_2(CG)_2$	No
2.	$(TG)_2(CA)_8C(TG)_2$, $(TG)_2(CA)_6AT$, $TA(CA)_6AT$, $TG(CA)_8CT$, $A_4(CA)_6AG$	No
3.	$(CA)_{41}$	Yes
4.	$(GACT)_2(CTCA)_7(CA)_2CT(CA)_9$	Yes
5.	$AC(GT)_9AG$, $CAC_2(CA)_{12}TA(CA)_8$	Yes
6.	$CAC_8(CA)_9C_4$	No
7.	$(CA)_6(GA)_2$	No
8.	$(CA)_{20}$	No
9.	$CA_2C(CA)_7$	Yes
10.	$CA_2T(CA)_{25}$	No
11.	$(CA)_{16}$	Yes
12.	$(GA)_2CAGA(CA)_5$	Yes
13.	$C_3A_2(AC)_6$	Yes
14.	$(CGCA)_9$	Yes
15.	$(CT)_3(CAA)_2(CA)_8$	Yes
16.	$CA_2G(CA)_{25}$, $(CACCT)_6$	Yes
17.	$CACT(CA)_{16}TACA$	Yes
18.	$(CA)_{30}$	Yes
19.	$CAG(CA)_6TACA$	No
20.	$CA_2GCA_2C(CA)_{25}$	Yes
21.	$A(CA)_2CT(CA)_{13}(CGCA)_{11}(CA)_6$	Yes
22.	$(CA)_8(CG)_4$	Yes
23.	$(CA)_6(CG)_4$	Yes
24.	$G_3A_2(CA)_5$	Yes
25.	$(CA)_{28}$	Yes
26.	$(GTT)_2(CA)_6$, $TGTA(CA)_8$, $TA(CA)_{12}TC$	Yes
27.	$(CA)_2CA_2(CA)_6$	Yes
28.	$(CAAA)_3(CA)_4CA_2(CA)_{12}G_2CA(CGG)_3$, $(CAAA)_5$	No
29.	$(CA)_2AC(CA)_2AC(CAC)_2(CA)_5$	Yes
30.	$(CA)_8(TGCA)_2$	Yes
31.	$(CA)_{34}$	Yes
32.	$TA(CA)_5CTAC$	No
33.	$(CA)_2CGCACG(CA)_{21}CGCA$	Yes
34.	$(CA)_6(CGCA)_4(CA)_5GA(CA)_{12}GA(CA)_6GA(CA)_9(CT)_2$	Yes
35.	$(CA)_2GACACG(CA)_6$	Yes

Besides the high efficiency for cloning microsatellites, our method could be more widely applicable. Actually, we have succeeded in cloning microsatellites from some teleost fishes and bivalve (Fig 5). Our method could overcome the difficulties to isolate microsatellites from a species which has low frequency of microsatellites in the genomes, and will contribute to the pollock population genetics leading to optimal fisheries resource management.

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Biochemical Genetic Variation and Population Structure Study in Walleye Pollock (*Theragra chalcogramma*) from the Bering Sea

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ABSTRACT: Genetic variation in walleye pollock (*Theragra chalcogramma*) from the Bering Sea have been examined by two basic approaches: starch gel electrophoresis of water soluble proteins, and mitochondrial DNA (mtDNA) analysis with the use of PCR technology. Three genetic loci, coding for muscle esterases (locus *mEst*), blood serum esterases (locus *sEst*), and a zone of serum general proteins, presumably transferrins (locus *Trf*), were sufficiently polymorphic to be used in the analysis of walleye pollock differences on the geographic scale. The use of all the three polymorphic loci revealed that eastern Bering Sea pollock differs significantly from northern Bering Sea and Gulf of Alaska pollock. The analysis of sEst allele frequencies showed that there is a major differentiation of the Bering Sea walleye pollock into two genetically distinct groups: western, or Asian, and eastern, or American. A technique for amplification of three fragments of mtDNA from the pollock's heart tissue have been worked out. These fragments cover mostly variable regions of fish mtDNA. Of eight restriction enzymes three revealed restriction sites polymorphism, which could be used in population structure study of walleye pollock.

INTRODUCTION

The use of genetic markers is one of several possible major approaches towards a problem of intraspecific differentiation and stock structure of walleye pollock. At least two techniques proved to be highly valuable in population genetic analysis of fish. One is the electrophoretic analysis of proteins, mostly enzymes, with subsequent genetic interpretation of different protein variants. Another is the analysis of mitochondrial DNA (mtDNA) using polymerase chain reaction (PCR), and revealing restriction sites variation in amplified mtDNA fragments. The results of applying these two basic techniques to the study of population differentiation in the Bering Sea walleye pollock are presented herein.

Pollock collections for multilocus protein electrophoresis have been made during several years in different regions of the Bering Sea and adjacent areas: Gulf of Alaska and east Aleutian Islands from the east, and Kamchatka Bay from the west (Figure 1). Pollock sampling have been made during spring time and occasionally in the beginning of summer period, assuming that sampled fish represented locally spawning populations. We also used one sample from the western Bering Sea, and one sample from Sea of Okhotsk to reveal variation in PCR amplified mtDNA regions. Total number of fish, subjected to genetic analysis, amounted almost 1000.

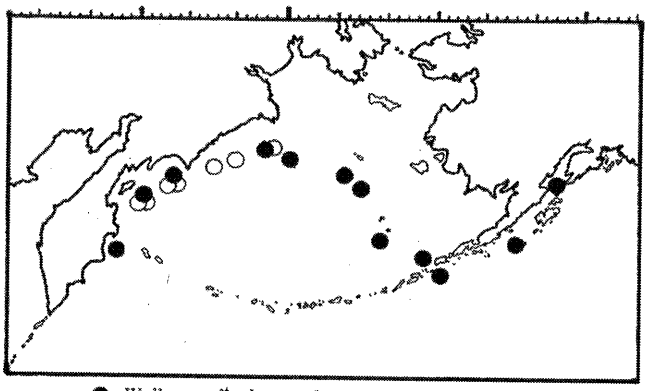

Figure 1. Map showing locations of the samples of walleye pollock used in the biochemical genetic population study.

PROTEIN VARIATION AND DIFFERENTIATION OF WALLEYE POLLOCK

Two sources of data were used to analyze geographic variation in allelic frequencies of polymorphic genetic loci in walleye pollock. One included the results of research, conducted by TINRO scientists across the entire Bering Sea in 1980 and 1986. Another part of database was obtained from joint TINRO and IBM (Institute of Marine Biology, Far Eastern Branch of Russian Academy of Sciences) investigations in 1990, following the research cruise to the western Bering Sea.

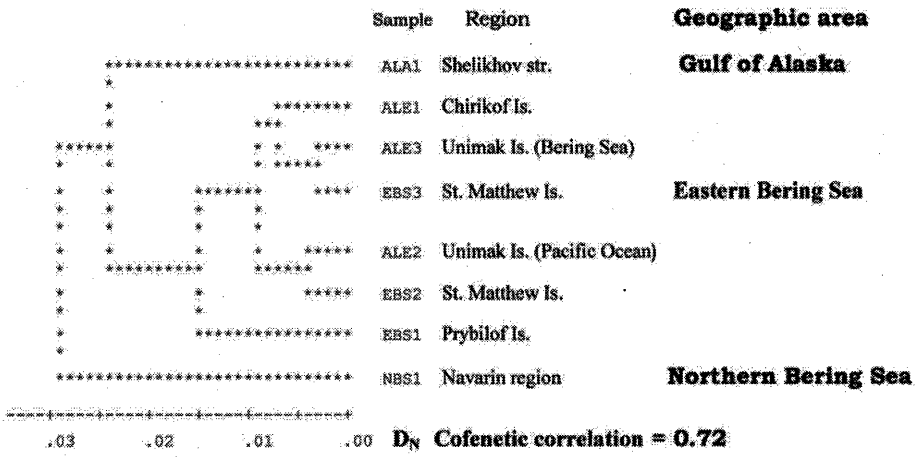

Figure 2. UPGMA Nei's genetic distance (D_N) dendrogramm showing genetic relationships between samples of walleye pollock from the eastern Bering Sea and adjacent areas (data from allele frequencies of three polymorphic loci *mEst, s Est u Trf*).

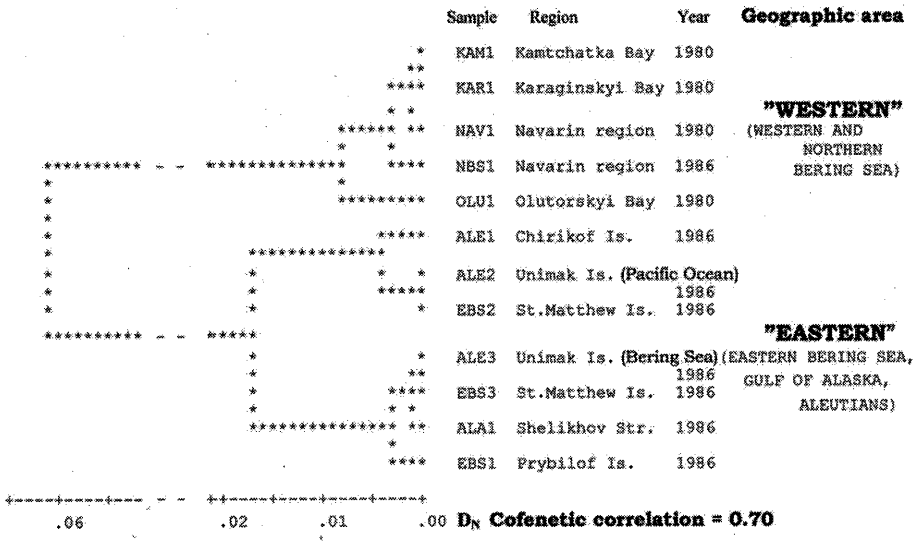

Figure 3. UPGMA Nei's genetic distance (D_N) dendrogramm showing genetic relationships between samples of walleye pollock from the Bering Sea and adjacent areas (data from allele frequencies of polymorphic locus *sEst*).

Three highly polymorphic protein systems were used for comparisons between samples, and those protein zones were first revealed and described by TINRO scientists (Flusova, Bogdanov, 1986; Flusova, 1987). They suggested genetic basis for the following variable proteins of pollock: muscle esterases (locus *mEst*), blood serum esterases (locus *sEst*), and blood serum general protein zone, presumably corresponding to transferrins (locus *Trf*). Two electrophoretically detectable codominant alleles were revealed at *sEst*, three—at *mEst*, and five—at *Trf*. No significant violations of Hardy-Weinberg principle were detected at any of the three genetic loci, confirming the hypothesis of inheritance of protein variants, and suggesting a random mating pattern for fish from sampled populations.

Of 42 enzyme-coding loci, mentioned in the electrophoretic survey of walleye pollock's genetic variability, 14 loci were polymorphic under a 0.99 criterion (Mikhailova, Efremov, 1993). Allelic frequencies of those 14 loci were used (under permission of Dr. V. V. Efremov) in this study to analyze genetic differences between samples of pollock from the northwestern Bering Sea.

The analysis of walleye pollock from the spawning sites in the northern and eastern Bering Sea (from the region off Cape Navarin down to Unimak Island), and from the Gulf of Alaska (Shelikhov Strait and south of Chirikov Island), revealed considerable genetic differences between samples. UPGMA cluster analysis of Nei's standard genetic distances (Nei, 1972), showed that all samples were grouped in accordance with a geographic scale (Figure 2). Those pollock samples, which were taken in the eastern Bering Sea, are clustered together, while sample from Shelikhov Strait (ALA1), as well as sample from Navarin region (NBS1) outbranch on the graph, and thus differ from clustered samples and between each other.

Table 1. Genetic variation between samples
Contingency chi-square tests: 1986, three genetic loci.

Locus	Aleutians	Eastern Bering Sea	Aleutians/ Eastern Bering Sea	All samples
sEst	n.s.	n.s.	n.s.	**P<0.001**
mEst	n.s.	n.s.	n.s.	**n.s.**
Trf	n.s.	n.s.	n.s.	**P<0.01**
Total	n.s.	n.s.	n.s.	**P<0.001**

Hierarchical F-statistics

Comparison X Y	F_{XY} Groups: Gulf of Alaska, Aleutians, eastern and northern Bering Sea	F_{XY} Groups: northern Bering Sea and the rest of samples together
sample - group	0.004	0.003
sample - total	0.009	0.009
group - total	**0.004 (50%)**	**0.006 (66%)**

Table 2. Genetic variation between samples
Contingency chi-square tests: 1980 and 1986,locus *mEst*.

Western bering Sea	Eastern Bering sea, Aleutians, Gulf of Alaska	All samples
n.s.	n.s.	**P<0.0001**

Hierarchical F-statistics

Comparison X Y	F_{XY} Groups: western and eastern Bering Sea (including adjacent areas: Gulf of Alaska and Aleutians)
sample - group	0.008
sample - total	0.052
group - total	**0.045 (86%)**

Table 3. Genetic variation between samples
Contingency chi-square tests: 1990, 14 loci.

Locus	Western Bering sea (west of 176° E)	Northern Bering Sea (east of 1976° E)	All samples
Pgm-1	n.s.	n.s.	**P<0.05**
13 others	n.s.	n.s.	**n.s.**
Total	n.s.	n.s.	**n.s**

Hierarchical F-statistics

Comparison X Y	F_{XY} Groups: western and northern Bering Sea
sample - group	0.002
sample - total	0.000
group - total	- 0.001

Contingency chi-square analysis revealed no significant heterogeneity between samples taken in the eastern Bering Sea and off eastern Aleutians (Table 1). Total genetic heterogeneity is highly significant when samples of Alaskan and Navarin pollock are included in the analysis. Regional heterogeneity have been observed at two of three loci: *sEst* and *Trf*. Partitioning observed allelic variability using hierarchical F-statistics (Wright, 1978) revealed that half of total variance in the "Eastern" group of samples was due to differences between Alaskan and eastern Bering Sea pollock. The share of variance, attributed to between-group differences was somewhat higher, being almost two-thirds of total variance between samples, when Navarin pollock was included into the comparison.

Large-scale comparison of inherited protein variation of walleye pollock from the entire upper Bering Sea rim, was conducted using allelic frequencies of highly polymorphic locus *sEst*. Twelve samples were clustered into two genetically distinct geographical groups on the UPGMA dendrogram of Nei's (1972) genetic distances (Figure 3). One cluster combined all Asian pollock samples from the western and northern parts of the Bering Sea, including sample from adjacent Kamchatskyi Bay. We named this group of the Bering Sea walleye pollock "Western", or "Asian". Another cluster consists of the eastern Bering Sea pollock samples, including those from adjacent Aleutian and Gulf of Alaska regions. We named this second group of pollock "Eastern", or "American".

The contingency chi-square analysis of genetic variation between samples revealed that there was no sufficient heterogeneity between samples within each of two geographical groups: "Asian" and "American" (Table 2). At the same time, there was highly significant total heterogeneity, attributable to allelic differences between pollock from eastern and western sites of the surveyed area. The appliance of hierarchical F-statistics showed that 86% of all between-sample variance in allelic frequencies was due to differences between "Western" and "Eastern" major geographic groups of pollock. The rest 14% was attributed to differences between samples within each group. We can assume from the branching pattern on UPGMA graph of genetic distances that greater part of within-group genetic variability could be explained by differences between the "Eastern" samples, while those from the "Western" group look less genetically separated, and thus more compact in terms of tree topology.

No total genetic heterogeneity have been observed among walleye pollock samples from the Asian populations basing on data from fourteen enzyme-coding polymorphic loci (Table 3). Only one locus, *Pgm-* 1, yielded significant heterogeneity between samples.

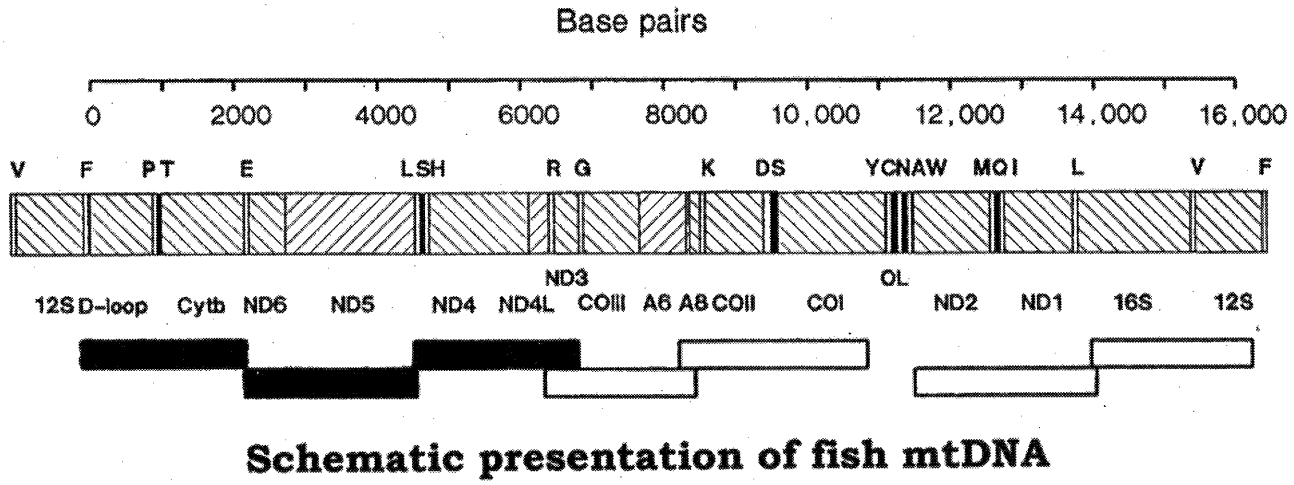

Fig. 4. Schematic presentation of fish mtDNA
-mtDNA regions of pollock amplified with PCR technology.

Application of hierarchical analysis of genetic variation with partitioning sampled populations into two minor geographic groups: "western" (west from 176°E), and "northern" (east from 176°E), revealed no spatial pattern in between-sample variance of allelic frequencies. It suggested that there are no geographically dependent genetic differences between pollock from the western (Karaginskyi and Olutorskyi Bays) and northern (sites adjacent to Cape Navarin) Bering Sea areas.

Summing it up, we observed significant genetic differentiation of mature walleye pollock from the spawning sites in the Bering Sea and adjacent areas, and this pattern of intraspecific variation has a strong geographic component in it. Basing on our own data, we assume that there exists a general subdivision of the Bering Sea pollock's gene pool into two distinct parts according to two geographic areas: "Eastern" ("Asian"), and "Western" ("American"). Stocks of walleye pollock from the Kamchatskyi, Karaginskyi, and Olutorskyi Bays, and those from Navarin region constitute the "Western" population group, while fish from the eastern Bering Sea shelf together with those eastern Aleutians belong to the "Eastern" population group. Walleye pollock from the Shelikhov Starit spawning ground differs from the Bering Sea fish, and represents another separate population unit. Those results should be considered to a greater part preliminary. We think that there is a strong need in new polymorphic genetic markers with a high degree of variation between samples and between regions, and with an increased possibility to discriminate between population units of walleye pollock.

MITOCHONDRIAL DNA ANALYSIS

During the last years, a restriction analysis of entire mtDNA or its fragments have been successfully applied to infer intraspecific variation and population structure of various zoological species. A pioneer research into mtDNA variation of the eastern Bering Sea and Gulf of Alaska walleye pollock revealed that restriction sites variation analysis provides good resolution between geographically separate population samples of the species (Mulligan *et al.*, 1992). For the eastern Bering Sea pollock, this approach appeared to be more sensitive, than analysis of the species population samples from the same regions using protein electrophoresis of a large number of loci (Grant and Utter, 1980).

In a joint TINRO-Centre-IBM research, there have been developed a technique for PCR amplification of mtDNA fragments, 2100-2200 bp each (Figure 4). Taking into account the fact that size of entire pollock's mtDNA is about 16,800 bp (Mulligan *et al.*, 1992), almost 40% of the whole mtDNA molecule was subjected to the analysis. The research is conducted in IBM under supervision of Dr. V. A. Brykov, who kindly consented to present first optimistic results for this report. We used primers, which had been developed on the basis mtDNA primary sequence of mykiss (Zardoya *et al.*, 1995, Carney *et al.*, 1997). First fragment incorporates genes for two NAD-oxydehydrogenase subunits NAD3 and NAD4, second—NAD5 and NAD6, and third—cytochrome B and D-loop. Ten restriction endonucleases were used in the digestion analysis of the first fragment: *Bgl* I, *Pst* I, *Hinf* I, *Ama87* I, *Pvu* II, *Hpa* I, *Sty* I, *AccB* I, *Sau96* I, and *Dra* I. Endonuclease digestions of mtDNA for 50 fish from two localities

revealed restriction sites polymorphism. Variable patterns were observed for *Bgl* I, *Pst* I, *Sau96* I, and *Hinf* I. Restriction digests for walleye pollock from the western Bering Sea (Ozernoi Bay) and northern Okhotsk Sea were compared. All four endonucleases yielded variant profiles in the Bering Sea pollock sample, while Okhotsk Sea sample appeared to be invariant. At present, we are processing the other two PCR amplified products of walleye pollock's mtDNA, and are going to expand the area of our research.

CONCLUSION

All our data combined and considered, we can see rather good and premising future perspectives in the field of population genetic structure research of the Bering Sea walleye pollock, especially when alternative molecular approaches, *e.g.* protein electrophoresis and PCR analysis of mtDNA, are applied to the problem simultaneously. And the most appropriate and fruitful should undoubtedly be a complex way of research using not only genetic baseline data analyses, but also analysis of morphological variation, *e.g.* external traits, otoliths, etc., and basing on a fundamental knowledge of the species ecology, reproductive biology and distribution patterns.

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High Resolution Analysis of Walleye Pollock Stock Structure Using Microsatellite DNA Markers

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ABSTRACT: Previous published investigations of genetic structuring of walleye Pollock (*Theragra chalcogramma*) populations in the North Pacific Ocean and Bering Sea have been based on either multiple low variability allozyme loci, or a single high variability mtDNA or microsatellite locus. Here, we report the development of a large suite of primarily tetranucleotide microsatellite markers that exhibit minimal PCR induced stutter for population analyses of walleye pollock. We also discuss preliminary findings of genetic heterogeneity among populations from the western Pacific (Funka Bay, Japan), the Gulf of Alaska (Prince William Sound), Port Townsend (Washington State), and the northwest and southeast Bering Sea.

Overall, multi-locus pair-wise estimates of population structuring (F_{ST}, R_{ST}) and genetic distance (D_{LR}) were small (equal to or less than 0.01, 0.04, and 0.80, respectively), though similar to what is commonly observed for mobile marine species. The largest multi-locus estimates of genetic differentiation, and the largest number of significant single-locus differences in allele frequencies, were observed between northwest and northeast Pacific populations. Comparatively moderate multi-locus estimates of genetic differentiation, and an intermediate number of single locus differences were observed between Port Townsend and Prince William Sound.

Small multi-locus estimates of differentiation were observed between two populations in the Bering Sea and within a single putative population in the Bering Sea (Unimak) that was sampled at two different times. One or two single-locus significant differences were observed between the northwest Bering Sea and Unimak populations and within the Unimak population. The biological significance of these results is currently being evaluated through analyses of additional loci and replicate samples from within regions.

INTRODUCTION

During the last half century, walleye pollock has supported an increasingly important commercial fishery in the North Pacific and Bering Sea, and now accounts for approximately 40% of U.S. fisheries production. Although the North American catch has remained stable during the last 20 or so years (~1.3 millions tons, Wespestad 1998), downward trends in numbers have been noted in several regions, including the Okhotsk Sea, west and central Bering Sea, the Gulf of Alaska, and Puget Sound (Bailey 1998; Wespestad 1988).

Typically, marine fisheries are managed assuming little population structuring across broad geographic expanses. However, undetected fine-scale structuring within larger management units can lead to over-exploitation of more accessible populations (Waples 1998), and thus long-term reductions in overall regional catches, and possible biological or commercial extinction of local stocks. Walleye pollock stocks from the Gulf of Alaska are managed separately from those from the Bering Sea, and west Bering Sea pollock are managed separately from east Bering Sea pollock (Bailey *et al.* 1998). Given the mobility of adults, and the pelagic nature of pollock larvae, management on such a broad scale would seem appropriate. However, hypothesized larvae retention mechanisms, and possible philopatry or natal homing of dispersed adults could result in more fine-scale population structuring within these regions.

Numerous attempts using phenotypic and acquired characteristics have been made to identify patterns and levels of population structuring in walleye pollock (reviewed in Bailey 1998). Many of these studies have indicated broad and fine-scale structuring of walleye pollock populations in the North Pacific Ocean and Bering Sea, although there is not a consensus on the details of the patterns of structuring observed. Furthermore, plastic phenotypic traits and acquired may not reveal genetic population structuring owing to strong natal homing of widely dispersed adults.

Genetic character states of individuals are not influenced by ontogenetic histories, and thus offer another perspective of population structuring. Studies using traditional molecular genetic markers (allozyme and mtDNA) to date have all identified significant and often comparatively large differences between Asian and

North American pollock populations. However, the degree of more fine-scale structuring observed has varied among reports, even between studies encompassing the same regions. Johnson (1977) and Grant and Utter (1980) reported little or no structuring within the Bering Sea or Gulf of Alaska. Mulligan *et al.* (1992) found significant structuring of mtDNA restriction fragment length polymorphisms (RFLP) within the Bering Sea, and between the Bering Sea and Gulf of Alaska. Shields and Gust (1995) reported a lack of overall heterogeneity at two mtDNA sequences analyzed within the Bering Sea and Gulf of Alaska, but tentative evidence of differences between the west and east Bering Sea. Powers (1998) found significant differences between "American" (Gulf of Alaska and eastern Bering Sea) and "Asian" (Sea of Japan and western Bering Sea) populations, and differences "within each group". The apparent increased resolution observed using mtDNA markers over allozyme loci may be due to (1) smaller effective population size of mtDNA (N) versus allozyme (nuclear) (4N) markers, and hence the greater potential for drift in small or bottlenecked populations, or (2) higher mutation rates at compared to nuclear allozyme loci. However, sample sizes were sometimes very limited in these earlier studies (fewer than 25 individuals were assayed in many of the populations surveyed) suggesting the need for caution in the interpretation of some of these findings.

Microsatellite molecular genetic markers have only recently been applied to analyses of stock structure in marine fishes. Attributes that make microsatellites highly useful genetic markers include their moderate to high variability, their abundance and dispersed distribution in genomes, and their relative ease of assay using the polymerase chain reaction (PCR) (O'Reilly and Wright, 1995). In addition, because each individual carries two copies (at least for autosomal loci), sample sizes are larger than would be for comparable mtDNA studies. The advantages of microsatellites as genetic markers are countered to some extent by several technical problems that can make it difficult to efficiently and precisely determine allele sizes in all individuals assayed. First is the failure of PCR to produce enough copies of some alleles to permit detection, often because of mutations in primer sequence (Callen *et al.* 1993; Ede and Crawford 1995; Paetkau and Strobeck 1995), but also due to difficulties amplifying very long alleles, consisting of many repeats. This second contributing factor has been termed short allele dominance (Wattier *et al.* 1998; E. Zouros, pers. comm.), and may involve "competition" between short and long alleles. In such cases, the genotype is interpreted as homozygous for the single visible product, hence, shorter alleles are over-represented, and long alleles under-represented.

A second technical problem is that individual alleles often do not appear as a single discrete product, but rather as a series of bands descending in size and intensity from the first main product. These additional bands are referred to as stutter or shadow bands, and are widely thought to occur during PCR due to replication slippage (Schlotterer and Tautz 1992; Hauge and Litt 1993). Stutter bands can make it difficult to distinguish between homozygous states and heterozygous states where alleles differ by a single repeat unit (O'Reilly *et al.* 1996), and can hamper precise determination of allelic states.

The effect of stutter bands on the efficiency and accuracy of allele assignment has caught the attention of several researchers, and has precipitated a range of analytical and laboratory "fixes". Perlin *et al.* (1995) have developed a deconvolution method to mathematically remove PCR stutter bands. Miller and Yuan (1997) devised a model that predicts the shape of stutter patterns, and used a least-squares technique to correctly resolve overlapping allelic patterns. Methods such as these, however, are based on assumptions involving the gain and loss of repeat units during PCR. Little is known about the effects of such factors as template quality, the presence of impurities in the PCR reaction, array sequence, flanking sequence, array length etc., on these assumptions, and hence the general applicability of these methods. Another approach taken has been to consider bands that coincide with stutter positions as either possible artifact bands or alleles (Sparkes and Buckleton 1998).

Laboratory based methods involve minimizing the intensity of stutter bands. Meldgaard and Morling (1997) found that for some loci, denaturing immediately prior to gel loading, use of sample loading buffer with a minimum of 50% formamide, and maintaining a minimum temperature of denaturing gels of 43°C, minimized secondary peaks (stutter bands) in electropherograms. Other procedures devised specifically to ameliorate stutter bands include use of formamide plus urea in the preparation of denaturing gels, and blotting fragments onto nylon membranes and probing with oligonucleotides complementary to the array motif involved (Litt *et al.* 1993). In addition to being cumbersome and time consuming, many of these methods resulted in only moderate reductions in the intensity of stutter bands (as would be expected if the major source was slipped strand mispairing during PCR), and their efficacy varied among loci tested.

It is well known that microsatellite loci vary greatly in the amount of stuttering exhibited. Two strong correlates of stutter intensity are repeat unit length and the number of repeats within arrays (henceforth referred to as array size). In general, stuttering increases as the

repeat unit length decreases; arrays with 4 base pair (bp) repeats tend to stutter less than arrays with 3 bp repeats, which stutter less than arrays with 2 bp repeats (Edwards *et al.* 1991). The intensity of stutter bands relative to the "correct" PCR product has also been shown to increase with increasing array size (Walsh *et al.* 1996). Both correlates are of concern in studies of microsatellite polymorphism in fishes. First, the great majority of microsatellites isolated from fishes to date are dinucleotide loci. Second, microsatellite arrays tend to be substantially larger in fishes than in mammals (Slettan *et al.* 1993; Brooker *et al.* 1994; McConnell *et al.* 1995, see also Table 1), and it is worth noting that heterozygote deficiency is very prevalent in population studies of fishes (reviewed in O'Connell and Wright 1997). This departure from Hardy-Weinberg equilibrium may be due to population admixture (Wahlund) effects, but may also reflect longer array lengths in fishes, and increased incidence of both short allele dominance and stutter related allele scoring errors.

Here we report the development of 20 primarily tetranucleotide microsatellite markers from the commercially important gadid fish, walleye pollock *(Theragra chalcogramma)*, isolated using magnetic bead hybridization selection methods. These loci were chosen from an initial pool of 112 microsatellite-bearing clones for properties such as short array length, and thus minimal expected short allele dominance and PCR induced stutter; moderate to high levels of variability; and robust amplification. We also discuss the results of tests confirming the Mendelian inheritance of these loci, and the conservation of these loci in other gadoid fishes. Preliminary results of a survey of genetic variation at ten microsatellite loci among six pollock populations from the North Pacific Ocean and Bering Sea is presented.

Table 1. Examples of allele sizes for dinucleotide and tetranucleotide microsatellites in several mammakian and fish species.

Species	Average range (bp)*	Number of loci surveyed	Number of individuals sampled	Reference
Dinucleotide *loci*				
Humans	11.5	4	58	Huang *et al.* 1992
Pigs	21.7	357	104	Rohrer *et al.* 1994
Cattle	12	1	426	Machugh *et al.* 1994
Atlantic salmon	45	3	162	McConnell *et al.*1995ab
Rainbow Trout	40	2	614	Morris *et al.* 1996
Atlantic Cod	71	6	127	Brooker *et al.* 1994
Tetranucleotide loci				
Humans	27	3	300	Edwards *et al.* 1992
Humans	36	7	150	Urquhart *et al.* 1995
Atlantic salmon	54	3	109	O'Reilly *et al.* 1996

modified from O'Reilly et al. 1996.

METHODS

Microsatellite Development

Most of the loci described here were derived from a library enriched for tetranucleotide loci, following Kijas *et al.* (1994), but with some modifications. Approximately 2g of ethanol-preserved pollock tissue was minced into 1-2 mm^3 pieces, and extracted using standard proteinase K/phenol-chloroform methods (Sambrook *et al.* 1989). DNA digested to completion with the restriction enzyme *Sau*3A (Pharmacia), was size fractionated in 1.5% agarose-TAE gels. The 300-800 bp DNA was excised, and recovered from the gel by centrifugation through filter paper-slurry columns according to Chuang and Blattner (1994), phenol extracted once, chloroform extracted once, and precipitated following Sambrook *et al.* (1989), then resuspended in 20 µl TE (10mM Tris-HCl ph 8.0, 1mM EDTA). Purified, size selected DNA was then ligated into *Bam*Hl-dephosphorylated pUC18 (Pharmacia), using T4 DNA ligase (Gibco BRL). The ligated DNA was purified by a single phenol extraction followed by a single chloroform extraction.

Insert DNA was amplified via PCR, using M13 forward (GTA AAA CGA CGG CCA GT) and M13 reverse (GGA AAC AGC TAT GAC CAT G) primers. Amplified DNA was ethanol precipitated, and approximately 50 ng of resuspended PCR product used to seed a second, single strand PCR using M13 reverse primer only. Following PCR, the reaction was diluted to 65 µl with distilled water, and heated to 95°C for 5 minutes. Immediately following denaturation, single stranded genomic DNA was combined with 35 µl of $(GATA)_7$ oligonucleotides bound to streptavidin coated para-magnetic beads (Dynal Inc.) suspended in 10X SSC. The hybridization was carried out for 20 minutes at room temperature, with occasional agitation to resuspend the beads. The 1.5 ml tube was then placed in the magnetic particle concentrator (MPC)(Dynal) for 30 seconds, after which, material not bound to the beads was removed by aspiration. To further purify the enriched fraction, 100 µl of low stringency wash solution (2XSSC; 0.5 ng/µl M13 forward and reverse primers) was added, and the tube was removed from the MPC. The solution was

then vortexed to disperse the beads, and returned to the MPC for 30 seconds. Solution not bound to the beads was again aspirated off. This low stringency wash procedure was repeated four times. An additional five high stringency washes (2XSSC; 0.5 ng/µl M13 forward and reverse) were carried out using the same procedure outlined above for low stringency washes. Following the final wash, microsatellite-enriched DNA was released by re-suspending the beads in a 20 µl of 0.15M NaOH for 20 minutes. The mixture was returned to the MPC for 30 seconds, and the enriched fraction no longer bound to the beads was drawn off and retained. The enriched DNA was neutralized by adding 100mM Tris-HCl pH 8.0 until the pH was below 9.0. The recovered DNA was amplified and made double stranded by symmetric PCR using M13 forward and M13 reverse primers, as described previously. Double stranded microsatellite enriched DNA was ethanol precipitated, digested with *Sau* 3A and ligated to the dephosphorylated *Bam* H1 site of pUC18 (Pharmacia). The ligant was diluted 5X with distilled water, and 1 µl used to transform 50 µl aliquots of DH5 alpha library efficiency competent cells (Gibco/BRL), as per the manufacturer's specifications. Following the 60-minute recovery incubation, cells were transferred to LB agar plates containing 50 mg ml^{-1} ampicillin (Sambrook *et al.* 1989). Clones were initially screened for insert size by PCR amplification directly off the colonies using M13 forward and M13 reverse primers (Kilger and Schmid 1994), followed by size fractionation in 1.0 % agarose-TAE gels. Colonies containing insert sizes of 150 bp or less were not considered further, because of the high probability of containing insufficient flank to develop primers from both sides of the array. All clones with inserts in excess of 150 bp were sequenced using standard PCR cycle sequencing methods.

Two microsatellite loci, Tch3 and Tch4, were obtained using conventional cloning and screening techniques described by Wimberger *et al.* (1999).

Table 2. Microsatellite designation, array sequence, array type, size range (in base pairs) and number of alleles, for all 20 microsatellites optimized to data.

Locus	*Array sequence*	Anneal. temp.	Array type	Size range	# diff. alleles
Tch3	$(GT)_{14}$	58	D	77-110	10
Tch4	$(GT)_{10}CT(GT)_7$	58	D	135-149	8
Tch5	$(GATA)_{14}$	42	T	176-296	32
Tch6	$GTCT((GTCT)(GCCT))_4GCCT(GTCT)_7GTTT(GTCT)_8$	54	T	130-330	9
Tch7	$(GTCT)_{14}GTCGCTCT(GTCT)_8CTCGCTCT(GTCT)_{18}$	47	T	186-226	8
Tch8	$(GATA)_{16}GACA(GATA)_{19}$	42	T	125-229	48
Tch9	$((GATA)(GATG))_4GATG(GATA)_3$	54	T	98-146	5
Tch10	$(GGCT)_6CTCT(GTCT)_2$	54	T	127-247	32
Tch11	$(GATA)_{22}$	54	T	117-209	24
Tch12	$(GGTT)_{22}$	54	T	118-162	12
Tch13	$(GT)_9$	54	D	74-106	17
Tch14	$(GAAA)_{31}$	54	T	116-244	34
Tch15	$(GA)_3(CA)_2GACA(GA)_5CAGATA(GA)_8$	54	D	77-111	18
Tch16	$(GTCT)_4$	54	T	86-98	5
Tch17	$(GTCT)_{27}$	54	T	165-245	11
Tch18	$(GT)_{15}$	54	D	67-115	25
Tch19	$(GTCT)_{15}$	54	T	70-174	27
Tch20	$(GA)_6(GGAA)_3GGAT(GGAA)_2GGAAT(GAAA)_{10}GAAG(GAAA)_5$	54	T	142-300	56
Tch21	$(GT)_{17}$	54	D	86-98	5
Tch22	$(GACA)_8$	54	T	76-132	15

Laboratory analyses of population samples

Small fin clips (200-500 mg) were placed in 2 ml screw cap vials filled with absolute ethanol. After several days, when possible, the original ethanol was replaced with fresh ethanol. Samples were stored for between one and eight months prior to extraction.

Approximately 50 mg of preserved tissue was placed in a 1.5 ml tube containing 1 ml of distilled water, and incubated for 15-30 mins to remove most of the ethanol. After aspirating off the water, 150 µl of digestion buffer (10 mM Tris-HCl pH8.3, 50 mM KCl, 0.8% Tween 20, NaCl, 100 ug/ml Proteinase K) was added. Samples were incubated at 65°C for three to four hours with intermittent vortexing. Samples were phenol extracted three times, and chloroform extracted once to remove impurities following Sambrook *et al.* (1989). DNA was concentrated and salts removed using standard ethanol precipitation procedures.

Amplification reactions were carried out in a 10 µl volume containing 10-100 ng of template, 10mM Tris-HCl (pH 8.3), 50mM KCL, 2.0 mM $MgCl_2$, 200 µM each dNTP, 0.50 µM of forward and reverse primers, and 0.5 U *Taq* Polymerase. Thermal cycling conditions were as follows: (94°C for 3 min) X 1; (94°C for 1 min, Y°C for 30 sec) X 5; (90°C for 3 min; Y°C for 30 sec; 72°C for 30 sec) X 20, where Y = the locus specific annealing temperatures given in Table 2. PCR amplifications were carried out on a Techne Genius 384 well thermal cycler. Following PCR, 2 µl of a 6 X-glycerol based loading buffer (see Sambrook *et al.* 1989) was added to each 10 µl sample. Percent acrylamide and allele size had a marked effect on the

resolution of bands. Loci with alleles in excess of 150 bp were analyzed on 6% native acrylamide gels, and sample volume was constrained to 1.5 µl or less (increased % acrylamide and increased sample loading volumes caused bands to smear, making allele size determinations difficult). Loci with alleles below 150 bp were analyzed on 8% acrylamide gels, and much greater sample volumes (up to 4 µl for loci below 100 bp) could be accommodated. Acrylamide gels were run at 200 V for 3 to 8 hours, depending on allele sizes expected, which varied considerable amongst loci (Table 2). A combination of allelic ladder standards and 20/100 bp ladders used to size unknown fragments. Gels were stained with SYBR® Green I (Molecular Probes) following Rodzen *et al.* (1998), and visualized using a Fluorlmager 575 (Molecular Dynamics).

Statistical Analyses of Allele Frequency Information

Conformance to Hardy-Weinberg equilibrium was evaluated using the Option 3 (probability test) of Genepop3.ld (Raymond and Rousset 1995). Single locus tests of allele frequency differences between pairs of populations were performed using the Genic option of Genepop3.ld (Fisher's Exact test of Independence). Pairwise F_{ST} estimates were calculated using Genepop, and R_{ST} estimates and associated p values calculated using R_{ST} CALC (Goodman, 1997). Pairwise D_{LR} (Likelihood Ratio distances) were estimated using the program *Assignment calculator*, written J. Brzustowski, University of Alberta, following Paetkau *et al.* (1997). Significance of multiple tests were evaluated using sequential Bonferroni methods (Rice, 1989). Global significance across loci was tested according to Chapman *et al.* (1999).

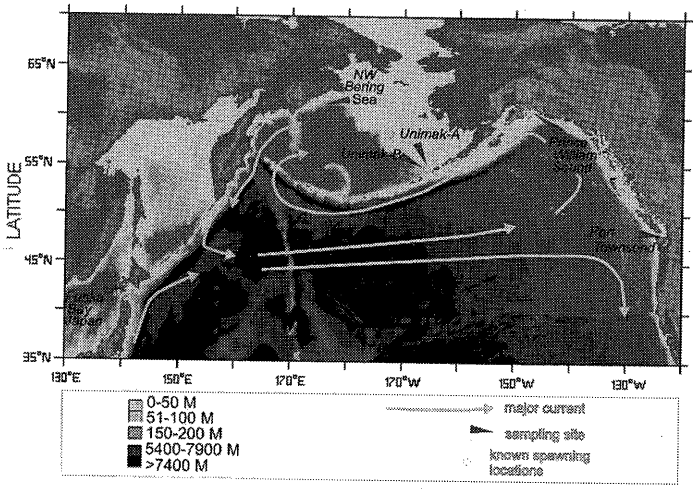

Figure 1. Sample collection sites from the North Pacific and Bering Sea.

Table 3. Sample information from six collection sites from the North Pacific Bering Sea.

	Unimak A (SE Bering Sea)	Unimak B (SE Bering Sea)	NW Bering Sea	PWS (G of A)	Funka Bay (Japan)	Port T (WA STATE)
Date	March 1997	April 1997	October 1997	March 1997	March 1998	June 1998
Sample Size (# Indiv.)	85-99 x=96	80-91 X=88.7	59-65 X=62.4	77-90 X=86.2	75-81 X=78.8	55-82 X=82
Life History Stage	ADULT	ADULT	ADULT	ADULT	ADULT	JUV.
Spawning Aggregation	YES	YES	*NO*	YES	YES	*NO*
Temporal Replicates Planned	YES		?	?	?	?

Sample Collections

Samples were obtained from Funka Bay, Japan, the northwest Bering Sea, Prince William Sound (Gulf of Alaska), Port Townsend (WA state), and the southeastern Bering Sea (Unimak) (Figure 1). Sampling dates, sample size, life history stage, and other details are given in Table 3.

RESULTS AND DISCUSSION

Library enrichment

The microsatellite-enriched library yielded approximately 1000 colonies, of which 685, contained insert sizes of 150 bp or less. Because of the low probability of such clones containing tetranucleotide microsatellite arrays, with sufficient flank to permit the development of primers, these clones were not considered further. All of the remaining approximately 300 clones were sequenced. Approximately 40% (112) contained one or more microsatellite arrays. Eighty eight (~30%) consisted of primarily tetranucleotide repeats, (GATA)n, but also (GACA)n, (GAAA)n, (GGTT)n, and (GGTC)n motifs. The remaining loci consisted mostly of dinucleotide repeats, (GA) and (GT), though several trinucleotide loci consisting of either (ATT), and (GAC) motifs were also found.

Many of the 88 tetranucleotide microsatellite loci occurred multiple times in the library, exhibiting occasional base substitutions in flanking regions and/or differences in numbers of repeats comprising the arrays. Due to the speculated occurrence of these loci in satellite or other repeat elements, seen in microsatellite libraries from other taxa (Beckmann and Weber 1992; Kaukinen and Varvio 1992; Buchanan *et al.* 1993; Wilke *et al.*

1994; Coltman and Wright 1994), these loci were not considered further. Several other loci exhibited insufficient flank from which to develop primers. Many loci were also excluded from further analyses because of excessive numbers of tetranucleotide repeats (>50). Alleles with multiple repeats may not be amplified via PCR sufficiently to permit detection, especially in the presence of markedly shorter alleles (Wattier *et al.* 1988).

As has been documented for other fish species (Slettan *et al.* 1993; Brooker *et al.* 1994; McConnell *et al.* 1995), the majority of dinucleotide arrays were quite long, containing 20 or more repeats. Given the greater likelihood of (1) increased stutter with increased numbers of repeats at dinucleotide loci (Walsh *et al.* 1996), (2) greater potential for short allele dominance, and the negative effects of both on the precision of allele size determinations (He *et al.* 1996; O'Reilly *et al.* 1996), these loci were not considered further.

Primers were developed and tested for 30 microsatellite loci: no detectable product was observed at six microsatellite loci, two were invariant in 10 or more individuals from two populations, and four loci did not amplify consistently in different samples. Locus names, array sequences and array type are given in Table 2.

Locus characteristics-variability and PCR stutter

Most of the 20 microsatellite loci developed here were very variable, exhibiting high levels of heterozygosity, number of alleles, and wide allele size ranges (Table 2). Heterozygosity levels of approximately 1/4 of the loci tested were above 90%; in contrast, approximately 1% of the 814 loci assayed in humans were equally variable (see Weissenbach *et al.* 1992). Number of alleles and allele size range of many of the loci were also very large, up to ~50 alleles spanning 150 bp. Reports of similar levels of variability are rare in the literature, but comparable values have been found in other marine fishes, including Atlantic cod (Brooker *et al.* 1994), Pacific herring (O'Connell *et al.* 1998) and Pacific halibut (Bentzen, unpublished data). These heightened levels of variability may be due to increased mutation rates resulting from greater levels of replication slippage and/or lower rates of correction; reduced constraints on increased array lengths; large historical population sizes, and reduced pruning of extreme alleles by fixation; or other unknown factors. Eleven loci exhibited moderate levels of heterozygosity (53-80 %), and many fewer alleles.

The wide range of allele sizes seen at a large portion of loci in pollock and cod suggest that array size and stutter may be problematic for PCR based assays of dinucleotide microsatellites in gadids.

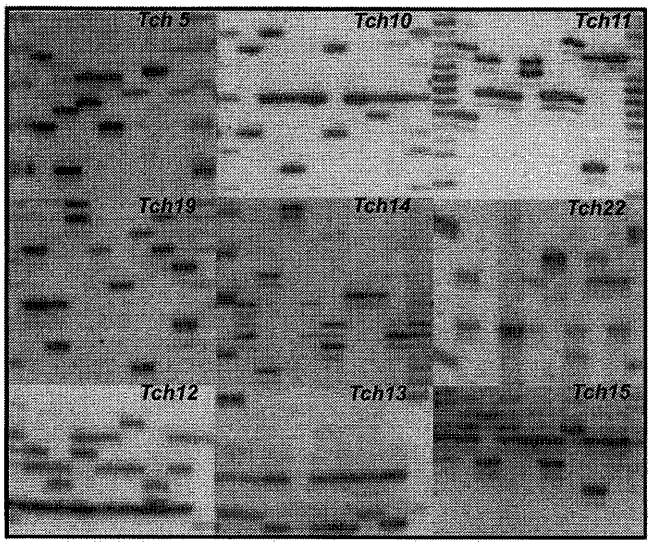

Figure 2. Di-and tetranucleotide microsatellites developed from walleye pollock (*Theragra chalcogramma*). Following PCR amplification, the 10 microsatellites chosen for the first phase of this study were size fractionated in 6% or 8% native acrylamide gels, stained with SYBR green and visualized using the Molecular Dynamics Fluorlmager 575. In each of the nine panels, allelic ladder standards, 20/100 bp ladders, or both, flank every set of seven or eight walleye pollock samples. PCR induced stutter bands are, in each case, much reduced compared to the primary "correct" product. A single non-stutter related product occurs at 120 bp at TCH 12. However, allelesat this locus occur at 118 and 122 bp, and are clearly distinguishable from this artifact band.

All 20 microsatellites developed here exhibited little or no stuttering, as seen in this sample of nine loci PCR amplified from walleye pollock (Figure 2). Heterozygozotes with alleles one repeat unit apart are clearly distinguishable from homozogyotes (see Tch10, Tch11 , Tchl9, Tch22, Tch12, and Tch 15, Figure 2).

Preliminary Analyses of population structuring

Number of alleles, range of allele size observed, and effective heterozygosity values are given for each population in Table 4. The fewest number of alleles, and the lowest levels of heterozygosity are often observed in the northwest Bering Sea sample, though we have not yet tested the statistical significance of these trends. The reduced number of alleles could simply reflect smaller sample size (N=59-65) compared to other populations. No other cross-population trend is readily apparent.

Table 4. Locus specific sample size, number of alleles observed, and effective heterozygosity for all six populations surveyed.

	Variable	Unimak A (SE Bering)	Unimak B (SE Bering)	NW Bering	PWS (G of A)	*Funka Bay* (Japan)	Port T (WA State)
Tch11	N	94	91	64	88	80	74
	#alleles	20	18	18	20	18	19
	He	0.92*	0.90*	0.91	0.92*	0.91*	0.92*
Tch10	N	98	90	64	88	80	82
	#alleles	21	23	15	20	20	20
	He	0.79	0.82	0.78	0.85	0.79	0.80
Tch14	N	85	84	61	84	81	80
	#alleles	22	25	25	22	24	22
	He	0.94	0.93	0.94	0.93	0.93	0.93
Tch15	N	99	91	55	88	80	80
	#alleles	14	11	12	12	12	11
	He	0.62+	0.67	0.61	0.71+	0.68+	0.72
Tch12	N	99	90	65	89	80	84
	#alleles	8	9	8	10	10	9
	He	0.78	0.76	0.78	0.79	0.83	0.80
Tch13	N	99	91	64	88	76	78
	#alleles	7	7	7	7	8	9
	He	0.68	0.68	0.65	0.67	0.83	0.80
Tch14	N	90	80	59	77	76	55
	#alleles	24	27	19	23	23	21
	He	0.94*	0.93*	0.93*	0.94*	0.94*	0.92*
Tch22	N	98	90	65	89	80	80
	#alleles	9	9	7	10	9	6
	He	0.70*	0.68	0.68	0.75	0.72	0.72
Tch19	N	99	89	65	81	75	78
	#alleles	20	20	18	19	19	20
	He	0.93*	0.93*	0.93*	0.93*	0.93*	0.92*
Tch18	N	99	91	62	90	80	81
	#alleles	17	16	13	14	16	18
	He	0.78	0.77	0.77	0.77	0.82	0.86

*significant deficit of heterozygotes at 0.05 after sequential-table wide corrections for multiple tests

+ significant excess of heterozygotes at 0.05 after sequential-table wide corrections for multiple tests

Significant departures from Hardy-Weinberg equilibrium, in the direction of a deficit of heterozygotes, occur much more frequently at high variability loci (loci with effective heterozygosity above 80% and with allele size range in excess of 50 bp)(Table 4). Despite efforts taken during the microsatellite development phase of this research to avoid the longest arrays, this effect may be due to short allele dominance and the difficulty detecting PCR amplified alleles consisting of many repeats. Recent re-analyses of several homozygous individuals at Tch19 using different electrophoresis and detection technology has revealed large faint alleles previously undetected. Given that the observed heterozygosity at these variable loci is quite high (>75%, unpublished data), the number of missing alleles is relatively small (approximately 1/2 of the difference between the observed and expected homozygosity). Therefore, the effect on the following analyses is expected to be

Table 5. Locus specific exact tests of differentiation between pairs of populations, at $p<0.05$.

	Unimak A (SE Bering Sea)	Unimak B (SE Bering Sea)	NW Bering Sea	PWS (G of A)	Funka Bay (Japan)
Unimak A (E Bering)	--------				
Unimak B (E Bering)	3,*9*	--------			
NW Bering Sea	*10*	*5,8*	--------		
PWS (G of A)	2,5,6,*9,10**	1,2,*7,8,9,10**	1,2,4,5,6,*8**	--------	
Funka Bay	1,2,3,6,*7,9,** ***10***	1,2,3,5,6,*8,** *9,10*	1,3,4*	1,2,3,6,*9,** *10*	--------
Port T (WA)	3,*8,10**	2,3,*7,8,9,** *10*	3,*8,9,10**	2,5,*8,10**	3,4,6,*8,9,** ***10***

NW Bering Sea- northwest Bering Sea, PWS- Prince William Sound, Port T- Port Townsend

1) Tch5: 2) Tch10; 3) Tch12; 4) Tch13; 5) Tch18;) Tch22; 7) Tch11; 8) Tch15; 9) Tch14; 10) Tch19

Exact test performed using the Genic option of Genepop 3.1b (Raymond and Rousset, 1995)

Bold type indicates significance at $p<0.05$ following table wide (sequential Bonferroni) adjustments for multiple tests.

* indicates global significance across loci following Chapman et al. (1999)

minimal. Regardless, we are currently retyping "suspect" homozygous individuals at these longer-array loci, and regard these findings to be preliminary.

Significant single-locus differences were found between all population pairs, with the greatest number observed between Japan and east Pacific populations (up to 8 of 10 loci, Table 5). Numerous differences were also observed between both Port Townsend and Prince William Sound, and other east Pacific populations (up to 6 of 10 loci). One or two single locus differences were observed between the northwest and southeast Bering Sea populations and within the southeast Bering Sea population sampled in March and April, 1997. Many of the single locus differences reported above are not significant after sequential Bonferroni adjustments (Rice 1989) for multiple tests (Table 5). However, the emerging consensus is that this correction may be overly conservative. Chapman *et al.* (1999) have developed a maximum likelihood approach for determining global significance from multiple single locus tests of differentiation. Based on their method, global significance was observed between Japan and all populations; between Port Townsend and all populations; and between Prince William Sound and all other populations (Table 5).

Several additional indices of population differentiation were also estimated, including traditional F_{ST} and R_{ST} statistics, indicators of levels of population structuring,

Table 6. Estimates of F_{ST} between pairs of walleye pollock population for all 10 loci combined.

	Unimak A (SE Bering Sea)	Unimak B SE Bering Sea)	NW Bering Sea	PWS (G of A)	Funka Bay (Japan)
Unimak A (SE Bering)	---------				
Unimak B (SE Bering)	0.002	---------			
NW Bering	0.000	0.003	---------		
PWS (G of A)	0.003	0.003	0.004	---------	
Funka Bay (Japan)	0.007	0.010	0.007	0.005	---------
Port T. (WA State)	0.006	0.007	0.006	0.003	0.007

All estimtes calculated using Genepop version 3.1b (Raymond and Rousset, 1995)

Table 7. Estimated R_{ST} values for pairwise population comparisons below diagonal, significance of departure from zero above diagonal.

	Unimak A (SE Bering)	Unimak B (SE Bering))	NW Bering Sea	PWS (G of A)	Funka Bay (Japan)	Port T (WA State)
Unimak A	------	0.690	0.060	0.070	**0.000**	0.040
Unimak B	-0.001	------	0.220	0.030	**0.000**	**0.000**
NW Bering	0.007	0.002	-------	0.040	**0.000**	0.030
PWS	0.003	0.007	0.009	------	**0.000**	**0.000**
Funka Bay	0.027	0.035	0.039	0.020	------	**0.000**
Port T	0.005	0.011	0.009	0.004	0.031	------

p values calculated using bootstrapping techniques.
All calculations performed using R_{ST} CALC (Goodman, 1997)
Bolded p values indicate significance at $p < 0.05$ after sequential Bonferroni adjustment for multiple tests.

and Likelihood Ratio distances (D_{LR}), developed for highly variable microsatellite genetic markers (Paetkau *et al.* 1997). Pairwise F_{ST} values ranged from 0 to 0.01 (Table 6), small compared to that reported for mammals and freshwater fishes over similar geographic scales, but comparable to values typically reported for mobile marine fishes (O'Connell and Wright, 1997). It should also be noted that this statistic is affected by the variability of loci used in its estimation. The maximum F_{ST} value for pairs of populations (assuming a sample

Table 8. D_{LR} genetic distance estimates are given below the diagonal, above the diagonal area the results of a test of the null hypothesis of panmixia between population pairs. The value given is the likelihood of finding fewer cross assignments than observed, and was based on 1000 bootstrap replicates of individuals across populations.

	Unimak A (SE Bering Sea)	Unimak B SE Bering Sea)	NW Bering Sea	PWS (G of A)	Funka Bay (Japan)	Port T (WA State)
Unimak A	-------	0.7515	0.591	0.5123	0.130	0.735
Unimak B	0.189	-------	0.859	0.100	0.088	0.192
NW Bering	0.143	0.146	-------	0.276	0.654	0.064
PWS	0.116	0.344	0.287	-------	0.219	0.250
Funka Bay	0.460	0.760	0.402	0.224	-------	0.021
Port T	0.297	0.657	0.742	0.317	0.800	-------

Estimates calculated using programs written by John Brzustowski, University of Alberta, following Paetkau *et al.* 1997.

size of 25 or greater) is 1/2 the homozygosity of the locus. For example, the maximum possible F_{ST} value for populations with completely non-overlapping allele frequency distributions is 0.025 for loci with heterozygosity levels of 95% or higher. R_{ST} values are similarly constrained by high locus variability, but rather than being based solely on allele frequencies, they also take into account allele size differences. R_{ST} estimates ranged from 0 to 0.039, with the largest values observed between Funka Bay and east Pacific populations (Table 7).

D_{LR} pairwise distance measures ranged from 0.116 to 0.8, and were greatest between Japan and east Pacific populations (Table 8). The largest values given here are similar to the minimal values reported by Paetkau *et al.* (1997) for black bears across the North American continent. Comparisons with marine or freshwater fishes could not be made, as no such estimates were found in the literature, perhaps reflecting the recent development of D_{LR}. In a comparison with five other distance measures, Paetkau *et al.* (1997) found the greatest correlation between genetic distance and geographic distance using D_{LR} estimates.

An unrooted Neighbor joining tree of D_{LR} distance estimates shows genetic relationships among populations (Figure 3). Population pairs surveyed here appear to follow an isolation by distance model, where distance is

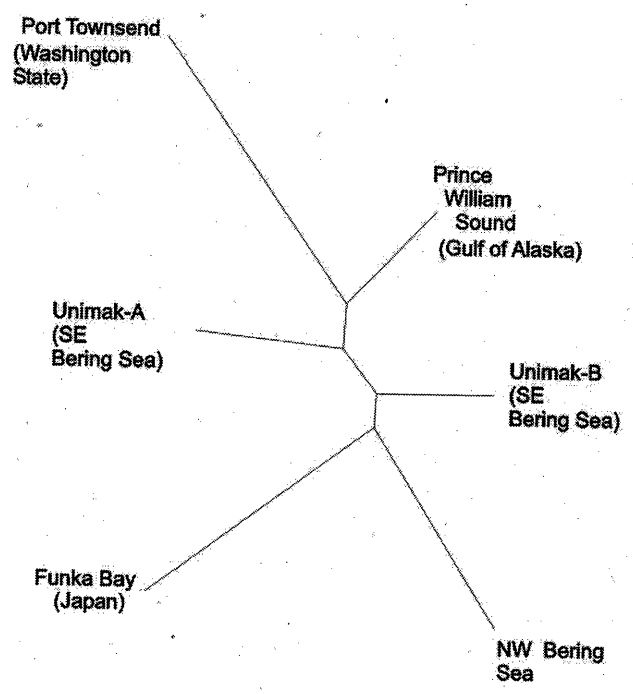

Figure 3. Neighbor joining tree of D_{LR} genetic distance estimates.

measured along the continental shelf of Asia, across the Bering Sea, and down along the continental shelf of North America. The Funka Bay population is most closely related to northwest Bering Sea pollock, then to the southeast Bering population (Unimak), followed by Prince William Sound, and finally Port Townsend.

CONCLUSIONS

Based on several indices, genetic structuring of walleye pollock is clearly evident on a Pacific basin wide scale, with populations from Funka Bay Japan very distinct from east Pacific pollock, consistent with previous reports using allozyme and mtDNA genetic markers. The magnitude of differentiation (based on F_{ST}, R_{ST} and D_{LR} measures) within and between populations from the Bering Sea and the northeast Pacific is comparatively moderate. However, global significance of single locus tests of differentiation were observed between walleye pollock from Port Townsend and Prince William Sound, suggesting genetic structuring within the northeast Pacific. Within the Bering Sea, and between the Bering Sea and Prince William Sound, F_{ST}, R_{ST} and D_{LR} estimates were small. However, this may reflect more shallow levels of population structuring, rather than the absence of genetic differentiation. Significant differences were found at a single locus between northwest and southeast Bering Sea populations, and two-single locus significant differences were observed between two samples from the southeast Bering Sea population (Unimak). To test whether these differences reflect genetic structuring within these regions, as opposed to sampling error (non-representative sampling of the true putative populations), additional analyses of these sites from one or more years, and analyses of samples collected from Shelikof Strait during 1997 and 1998, are currently underway.

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The Application of Microsatellite DNA for Determining Population Structure of the Minke Whale

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ABSTRACT: In order to try to define biologically meaningful stock units for use in management situation, we examined the intraspecific population structure within the North Pacific and Antarctic minke whales using 5~9 microsatellite primer sets that had been reported in previous studies. In the analysis of western North Pacific minke whales, significant differences were detected in the levels of allelic diversity and expected heterozygosity between groups from Sea of Japan (sub-area 6) and Pacific Ocean (sub-area 7, 9). However, no significant difference was found between coastal (sub-area 7) and offshore (sub-area 9) samples by genotypic differentiation test. These results were consistent with mtDNA results, which clearly showed the genetic heterogeneity between western and eastern side of Japan. Furthermore, the population structure in the Antarctic minke whale was also examined. Despite these samples were obtained from widely separated locations, little differences were found in their allele frequencies. Significant deviations from Hardy-Weinberg equilibrium were detected in the total sample (overall estimation) and eastern part of Area III using three different statistic approaches, suggesting some degree of stock structure in the Antarctic Ocean.

INTRODUCTION

Microsatellite loci are regions of genomic DNA that contain short (2~6 bp) tandemly repeated sequences. They are rapidly becoming the dominant source of nuclear genetic markers for a wide range of application such as population genetics, migratory movement, forensic testing, individual recognition in wild animals and abundance estimation. In cetacean studies, more than 30 microsatellite loci have been isolated both from mysticeti and odontoceti genome, and some of these loci showed highly polymorphic enough to be applicable to population genetics studies in the North Pacific and Antarctic minke whale (*Balaenoptera acutorostrata*).

In the western North Pacific, the existence of two distinct minke whale stocks — J stock (the Sea of Japan, Yellow Sea and East China Sea) and O stock (the Pacific coast of Japan and Sea of Okhotsk) were illustrated by genetic approaches (Goto and Pastene, 1997; Wada, 1984) as well as morphological studies (Ohsumi, 1983; Kato *et al.*, 1992). Furthermore, a Working Group on North Pacific Minke Whale Management Trials proposed to introduce the possibility of an additional new stock, W stock (West Pacific) in the off shore of Pacific side of Japan. The purpose of this study was to examine the current understanding of population structures around Japan and to clarify whether hypothetical W stock actually exists in the West Pacific.

As regard to the Antarctic minke whale, several attempts have been made to reveal stock structure using various molecular tools (Van Pijlen *et al.*, 1991 ; Hoelzel and Dover, 1991; Wada *et al.*, 1991; Pastene *et al.*, 1993, 1996). These approaches could clarify the genetic divergence between the minke whale of the Southern Hemisphere and those of the Northern Hemisphere, nevertheless it was quite difficult to detect stock structures probably due to low genetic differences among this region. Our final goal is to define the stock boundaries in the North Pacific and Antarctic for the effective management of this species.

When considering the application of microsatellite markers to resolve questions of stock structure and identification, both biological and technical issues must be carefully examined. For example, biological issues relevant to population criteria include yearly migration, environmental factors, reproduction. On the other hand, technical issues include the number of microsatellite loci, type of repeated units (di-, tri- or tetranucleotides), statistical methods.

Table 1. Summary of microsatellite loci used in this study.

Loci	Source	Simple sequence	North Pacific (alleles)	Antarctic (alleles)	Ref.
EV1 *Pm*	Sperm whale	(AC)13(TC)8	25	18	Valsecchi & Amos (1996)
EV37 *Mn*	Humpback whale	(AC)24	10	Null alleles	Valsecchi & Amos (1996)
EV104 *Mn*	Humpback whale	(AC)14(GCAC)2	4	17	Valsecchi & Amos (1996)
GATA 417	Humpback whale	(GATA)14	11	Microvariant	Palsbøll *et al* . (1997)
GATA 028	Humpback whale	Unknown	14	Microvariant	Palsbøll *et al* . (1997)
GATA 098	Humpback whale	(GATA)15	6	10	Palsbøll *et al* . (1997)
GT 023	Humpback whale	Unknown	15	20	Palsbøll (Unpublished)
GT 211	Humpback whale	Unknown	16	24	Palsbøll (Unpublished)
GT 509	Humpback whale	Unknown	13	ambigous	Palsbøll (Unpublished)

MATERIALS AND METHODS

Samples and DNA extraction

Minke whale samples were collected both from JARPN (Japanese Whale Research Program under Special Permit in the North Pacific) and JARPA (Japanese Whale Research Program under Special Permit in the Antarctic) surveys. In addition, we used samples which were obtained from coastal Korea (sub-area 6 in the Sea of Japan) by Korean small-type whaling operations. Sampling localities and the number of specimen in each study area was shown in Fig.1and Fig.2. Genomic DNA was extracted from several sorts of tissues (muscle, liver, heart) by means of standard proteinase K, phenol-chloroform procedure (Sambrook *et al.*, 1989).

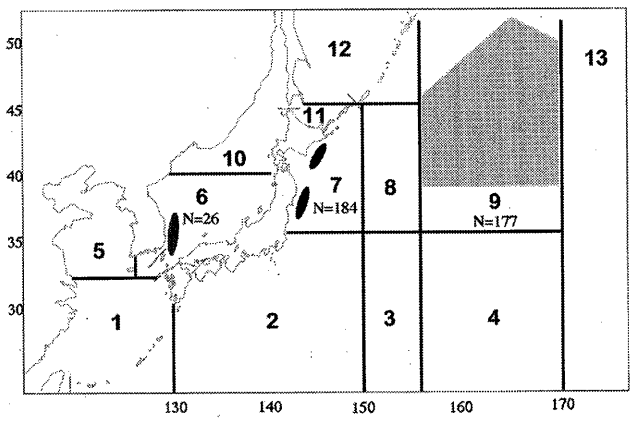

Fig. 1. Geographic origin of samples. The numbers in bold denote the sub-areas defined by the Working Group on North Pacific Minke Whale Management Trials (IWC, 1994). Samples from sub-area 6, 7, and 9 were used in this study.

STR Genotyping

Nearly 30 primers sets, which were obtained from personal communication (Dr. Palsbøll, University of Wales-Bangor, Wales, UK) and previous studies (Valsecchi and Amos, 1996 ; Palsbøll *et al.*, 1997), were tested for PCR amplification on the North Pacific and Antarctic minke whales under several conditions. By this screening procedures, 9 loci turned out to be highly polymorphic and appropriate for population analysis on the North Pacific minke whales. However, some of them could not be used for population study of the Antarctic minke whale because they produced microvariant and null alleles (Table 1). Detail of the reaction mixture and conditions for PCR amplification were described in our previous paper (Abe *et al.*, 1997).

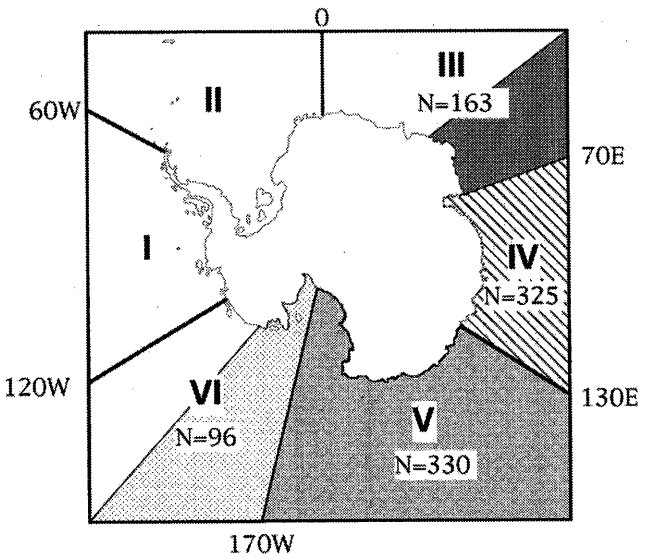

Fig.2. Geographic localities examined for micro- satellite analysis in the Antarctic minke whales.

Statistical analyses

Deviation from Hardy-Weinberg (HW) proportions were tested for all areas both in the North Pacific and Antarctic. The heterogeneity test was conducted using an unbiased estimate of the G-based test which was performed for all pair of populations for all loci using the computer program GENEPOP version 3.1 (Raymond and Rousset, 1995).

RESULTS

Western North Pacific

The chi-square based test for HW equilibrium in the North Pacific populations detected significant deviations at two loci (EV1 *Pm* and GATA417) in sub-area 7 (Table 2). Genotypic differentiation test clearly showed genetic heterogeneities between the Sea of Japan (sub-area 6) and Pacific side of Japan (sub-area 7, 9) populations. However, we did not detect significant difference between coastal (sub-area 7) and offshore (sub-area 9) area of the western North Pacific (Table 3).

Table 2. Chi-square test for differences in allele frequencies of six microsatellite loci.

	χ^2 test		
	6	7	9
GT 023	ns	ns	ns
GATA 028	ns	ns	ns
GATA 098	ns	ns	ns
GATA417	ns	P<0.05	ns
EV1 *Pm*	ns	P<0.05	ns
EV37 *Mn*	ns	ns	ns

Table 3. Results of genotypic differentiation test between North Pacific populations.

Sub-areas	6 vs 7	6 vs 9	7 vs 9
GT 023	<0.01	<0.01	0.5382
GATA 028	<0.01	<0.01	0.3532
GATA 098	<0.01	<0.01	0.1701
GATA 417	<0.01	<0.01	0.5570
EV1 *Pm*	<0.01	<0.01	0.8164
EV37 *Mn*	<0.01	<0.01	0.2054

Table 4. Results of three tests for Hardy-Weinberg equilibrium.

	AREA III	AREA IV	AREA V	AREA VI	OVERALL
LIKLI.test	0.0275	0.8157	0.4258	0.0466	0.0020
EXACT test	0.0102	0.8331	0.4837	0.0962	0.0026
HOMOZ. test	0.1522	0.2826	0.0908	0.6589	0.0014
Combined	0.0026	0.7704	0.2412	0.0703	<0.001

LIKLI.= Likelihood ratio HOMOZ.= Homozygosity

Antarctic

Departures from HW equilibrium were examined by three statistical methods for each area and microsatellite locus (Table 4). We could find significant deviations in Area III and Area VI. Given that whole samples were combined together as single data set, clear decline of P-values ($P<0.01$) were observed in all three tests (overall estimation). No significant deviations were detected in Area IV and Area V at 5% level.

DISCUSSION

Western North Pacific

The results of the microsatellite analysis of the western North Pacific minke whales presented here were consistent with the genetic data derived from mtDNA and allozyme analyses (Goto and Pastene, 1997; Wada, 1983; 1984). All these results indicate a clear genetic differentiation between the coastal Korea and Pacific side of Japan. Furthermore, we observed a significant low level of genetic variations in the Korean samples as compared with that of Pacific side of Japan. This definitive distinction between two groups may be explained as the consequence of founder effect in the Sea of Japan. From a paleoenvironmental point of view, it should be noted that the Sea of Japan had experience of being completely isolated from the North Pacific Ocean by Japanese land mass at the middle of Pleistocene (Riss II stadial; Fujii, 1990). It is plausible that invasion of a limited number of minke whales into this area occurred as the rise of sea level have severely affected their genetic variation mainly due to founder effect.

We detected significant deviation for HW equilibrium in sub-area 7 at two loci. This might suggest that some J stock animals penetrated into this sub-area through the Okhotsk Sea, and partial mixing of J and O stock could affect the results of H-W test. Our hypothesis is analogous to the *scenario* derived from mtDNA and allozyme analyses (Pastene *et al.*, 1997; Wada, 1991).

Antarctic

Heterozygosity values in the Antarctic minke whale were higher than those observed in the North Pacific minke whale in our previous study (Abe *et al.*, 1998). Allele frequencies of the five loci were very similar among four Areas. One of the main factor influencing the stock structure is level of gene flow between adjacent populations. Mutation rates of most nuclear loci are so low that new mutations have not had sufficient time to appear and became fixed. In this study, we could not observe major difference in allelic distributions among the Antarctic Areas, and the range of genetic distance estimated by F-statistics was quite narrow ($Fst<0.02$). These are evidences that gene flow could have a strong influence in homogenizing genetic composition in the Antarctic minke whale across biological boundaries that surely existed for a certain period. Although the gene flow event could have prevented the Antarctic minke whales from gathering diagnostic characters specific for each putative stock, statistical tests for heterogeneity could partially reveal hierarchical structure in the Antarctic. In overall estimation, significant deviations from HW equilibrium were detected by homozygosity test even though no departure from HW proportions were found in each Area (Table 4). This is the typical case of Wahlund effect which is well characterized by heterozygote deficiency suggesting the existence of more than one stock.

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