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Microsatellite screening in Pacific halibut (*Hippoglossus stenolepis*) and a preliminary examination of population structure based on observed DNA variation

by

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Contents

Abstract

A genetic study was initiated in 2002 with the aim of more thoroughly examining the population structure of the eastern Pacifc halibut (*Hippoglossus stenolepis*) stock. Here, we detail the initial steps of the study: optimization of a suite of dinucleotide microsatellites and application of those markers in a preliminary analysis of gene frequencies from 3 sites generally representing the edges of the geographic range of the stock. Tissue samples were collected from the southern end of the range at Newport, Oregon, and at northern and western sites of St. Paul and Adak Island in the Bering Sea. A total of sixteen microsatellite loci previously developed for Atlantic halibut (*Hippoglossus hippoglossus*) were screened in Pacific halibut. In addition, we attempted to amplify one marker under selection (pantophysin).

Fourteen microsatellite loci were successfully optimized, of which ten appeared sufficiently variable to be used for population studies. Pantophysin was not successfully amplified. Microsatellite analysis of 236 Pacific halibut revealed relatively high genetic variability, with about 40 alleles per locus for the whole dataset (range 19-59), and an average heterozygosity of about 90% (range 73-97%). Three out of the ten variable loci showed genotype frequencies that were out of Hardy-Weinberg equilibrium. There was no significant genetic differentiation between the three sites using most of the commonly applied measures and tests. However, permutation tests yielded significant F_{ST} results at the 10% significance level. In this test, the Adak sample was found to be significantly different from the other two sites. Similar results were obtained whether the three loci with deviations from Hardy Weinberg equilibrium (HhiD34, HhiJ42, Hhi59) were included or not. Possible biological mechanisms for the apparent genetic differentiation are discussed.

This is the first report to demonstrate that these microsatellite markers can be used in Pacific as well as Atlantic halibut. The other results are preliminary and will be used to guide future research, which is to include analysis of fish sampled on their breeding grounds during the winter spawning season, more thorough sampling of summer feeding grounds, and examination of temporal stability in allele frequencies.

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Introduction

The idea that fisheries management should be based on local self-sustaining population rather than the typological species can be traced back to the turn of the 19th century (Heincke 1898; Hjort 1914). Fisheries models developed since then are based on fish 'stocks', which are the unit for the estimation of parameters such as the number of spawning individuals and recruitment (Hilborn and Walters 1992). Despite this long history of the stock concept (Sinclair 1988; Carvalho and Hauser 1994), our knowledge of the population structure of many marine species is still very limited, not only because of the apparent lack of environmental barriers to dispersal, but also because of ecology and life history of many species, which seems to promote large scale dispersal (Hauser and Ward 1998). Furthermore, early molecular studies based on protein variability often revealed little genetic differentiation, suggesting the existence of large panoceanic populations. However, recently the combination of improved sampling design and more sensitive genetic markers has provided evidence for population structure on a surprisingly small scale (e.g. cod, Hutchinson et al. 2001; Ruzzante et al. 2000), and thus has allowed the identification of stocks relevant to fisheries management.

An example for a species with uncertain population structure is the Pacific halibut (*Hippoglossus stenolepis*), which are distributed in the North Pacific from southern California through the northern Sea of Japan, and within the Bering Sea north to Norton Sound and the Gulf of Anadyr (Hart 1988). The species has long represented an important fishery resource in western North America, being fished by the indigenous peoples of the US and Canada for hundreds of years, and by a commercial fleet since the late 1880s (IPHC 1998). Over the last decade, commercial catches in the US and Canada have fluctuated around an annual mean of ~50 million pounds (IPHC 2001). The value of this resource is recognized throughout the region, as evidenced by the establishment of the International Pacific Halibut Commission (IPHC) in 1923 by the governments of the US and Canada to jointly manage and study the eastern Pacific population(s).

Despite the importance of the resource and its recognition by the relevant agencies, little is known about the population structure of the species. Presently, the eastern Pacific halibut resource is managed under the assumption that a single panmictic population (i.e., a fully mixed population in which members from all geographic regions regularly interbreed) exists from California through the eastern Bering Sea. This assumption rest largely upon a long history of tagging studies (see review in Kaimmer 2000) and analyses of larval distribution (Skud 1977;

St. Pierre 1989) indicating northwesterly larval drift throughout the Gulf of Alaska and into the Bering Sea, balanced by southeasterly migration of juveniles and adults over broad geographic expanses. In particular, individuals tagged in the southeast Bering Sea have been recovered as far south as California, and maximum annual movements of over 1500 km have been observed (Skud 1977). Thus, Pacific halibut in the eastern Pacific Ocean are treated as a single unit stock with regard to reproduction and recruitment. However, with respect to harvest guidelines, management is conducted within a series of regulatory areas based on observed subtleties of population dynamics. The Gulf of Alaska fishery has traditionally been separated into two broad regulatory regions located east and west of Cape Spencer, Alaska. This division was originally based on differences in size- and age-structure within the stock as well as tagging studies that suggested adult fish move more freely within regulatory areas than between them (Thompson and Herrington 1930; VanCleve and Seymour 1953). Recent analyses indicate that population dynamics differ between the two regions, as well (Clark and Hare 2002). The Bering Sea fishery is effectively treated as an extension of the Gulf of Alaska. Bering Sea harvest limits are based upon population abundance estimated for the Gulf of Alaska, scaled to the proportion of suitable benthic habitat that is available for adults in the Bering Sea relative to the Gulf (Clark and Hare 2000).

While the management scenario rests upon the best available information regarding movements and spatial population structure, there is reason to believe that actual structure could be more complex than presently understood. Conclusions drawn from tagging studies are sensitive to patterns of fishing effort, tag loss and reporting (Hilborn et al. 1995). Patterns observed in size structure and abundance between regions can be caused by factors other than reproductive isolation, such as regional differences in mortality or responses to environmental conditions. In light of this, attempts have been made to identify reproductive units using a variety of genetic techniques. Use of allozyme electrophoresis (Tsuyuki et al. 1969; Grant et al. 1984) has generally not demonstrated significant genetic variation within the eastern north Pacific, though significant genetic separation between the eastern and western Pacific has been identified (Grant et al. 1984). More recent research using nuclear DNA microsatellites supported the hypothesis that eastern and western Pacific stocks are genetically separate, but also suggested that the eastern Pacific population may "be structured in distinct reproductive groups" (Bentzen et al. 1998). In particular, the true nature of the relationship between the Bering Sea and Gulf of Alaska sub-populations remains elusive; the results of existing genetic analyses are difficult to interpret due to a number of study limitations.

Here, we report the results of laboratory work conducted on Pacific halibut to isolate microsatellite markers that were originally developed for Atlantic halibut (*Hippoglossus hippoglossus*). Our primary objective was to determine whether Atlantic halibut markers could be optimized for Pacific halibut, and be of use in future genetic studies. In order to asses the utility of the markers that were successfully optimized, we conducted a preliminary analysis of population structure using fish sampled at 3 locations representing the edges of the range of the eastern Pacific stock.

Microsatellites consist of 1-5 base pair (bp) repeats that form tandem arrays up to 300 bp in length, and exhibit high levels of allelic variation in repeat number. Polymorphism exhibited by specific microsatellites is readily detected by amplification of the microsatellite through the use of oligonucleotide primers specific to the non-repetitive regions that flank the repeat array, in combination with the polymerase chain reaction (PCR). Allelic variation is scored by gel electrophoresis of the PCR products, most commonly on high throughput automated systems allowing the analysis of up to 300 genotypes in an hour. However, species-specific primers usually have to be isolated, incurring considerable costs and labor, unless primers for closely related species are already available. The first aim of the study was to determine the applicability of Atlantic halibut markers for Pacific halibut, and to estimate their variability and power for

population analysis. In the second phase of the study we genotyped samples from three Pacific halibut collection sites (St. Paul, AK; Adak, AK; and Newport, OR) using successful loci from the first phase.

In addition, we carried out preliminary experiments on DNA regions under selection, which have been shown to be more sensitive markers of population structure (Beacham et al. 2001). Selected markers have not yet been applied to Pacific halibut and may need considerable development effort for optimization. Among the variety of potential candidate genes, we concentrated our initial efforts on the pantophysin locus (*Pan I*). This locus encodes for an integral membrane protein that has been localized in small cytoplasmic vesicles in the cell, though its exact functions in microvesicle trafficking and exocytotic pathways are still poorly understood (Windoffer et al. 1999; Brooks 2000). Although the agent of selection is thus unknown, population studies of *Pan I* variation in Atlantic cod have shown striking levels of differentiation (Pogson et al. 1995, 2001; Pogson and Fevolden 2003; Karlson and Mork 2003). Contrasting two marker classes, one neutral (microsatellites) and the other influenced by selection (*Pan I*), can provide significant insight into the relative strengths of the evolutionary forces responsible for population structuring in Pacific halibut. In an applied context, the availability of markers under diversifying selection may allow the identification of essentially self-recruiting populations, which are demographically independent but may have sufficient gene flow with neighboring populations to prevent differentiation at neutral molecular markers.

Materials and methods

Sample collection

Halibut samples were collected from three geographic regions between June and July of 2002 (Fig. 1; Table 1). Samples consisted of fin tissue preserved in 100% ethanol. Fin clips were taken during IPHC port sampling activities from halibut captured in local commercial fisheries. At each site, the otoliths were removed from a random subsample of fish and the ages of those individuals determined via enumeration of internal growth annuli. All ages were determined first by surface reading (Forsberg, 2001), and all fish estimated to be greater than 15 years old were re-aged using break-and-burn techniques (Blood, 2003).

DNA extraction

DNA was extracted using Qiagen (Valencia, CA) DNeasy 96-well silica membrane based kits, following manufacturer's instructions. The DNA was diluted to approximately 12-50 ng/µl with low concentration TE buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Table 1. Pacific Halibut samples used in this study: "N" denotes the total number of samples used in genetic analyses, "L" the number of fish for which lengths were determined, and "A" the number of fish for which ages were determined at each sampling location.

Sampling Location	Date Collected	N	L	A
Adak, AK	June 2002	96	100	52
Newport, OR	July 2002	96	98	41
St. Paul, AK	June 2002	44	44	21

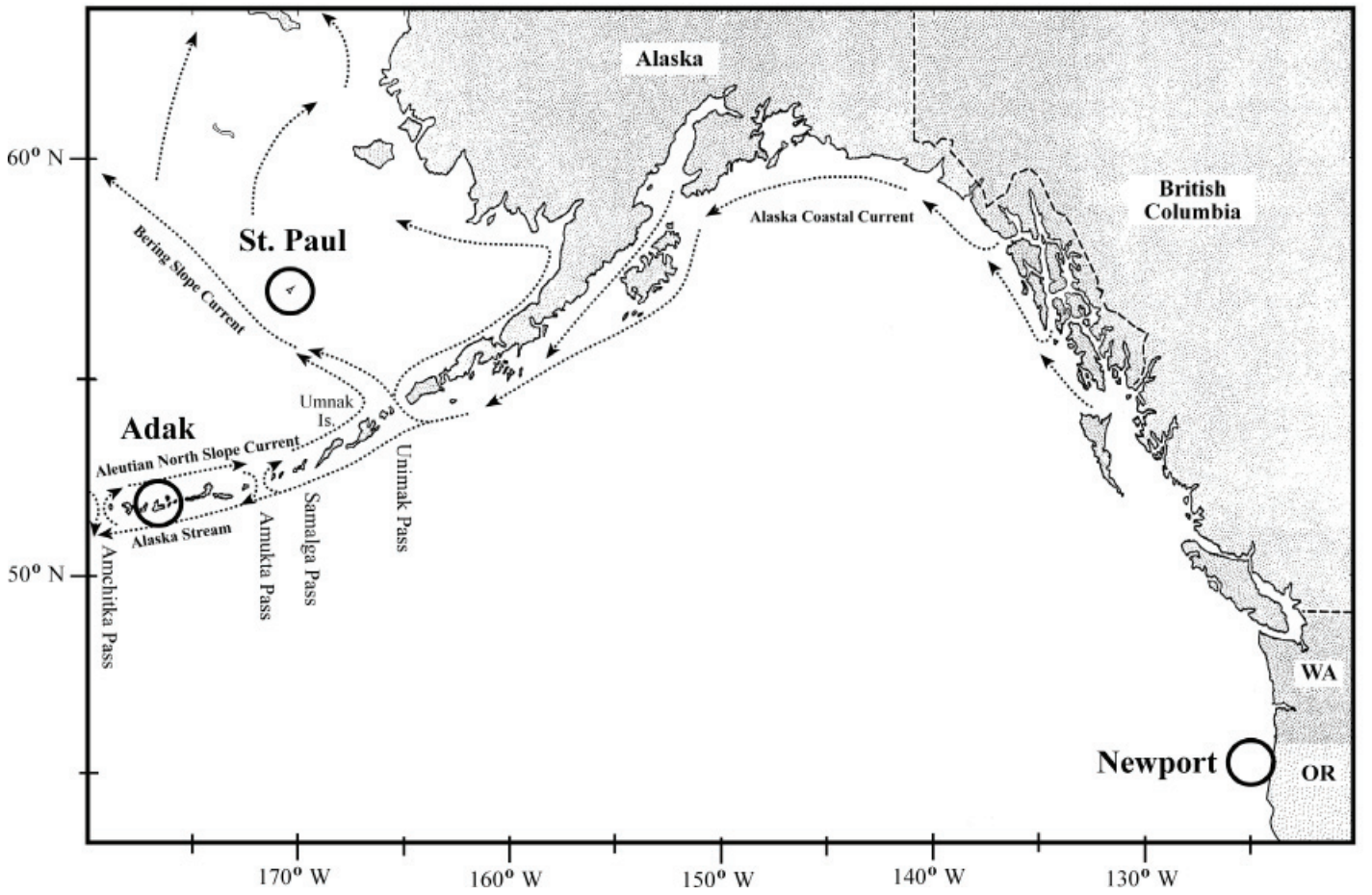

Figure 1. Locations at which tissue samples were collected (large circles), geography of the Aleutian Islands, and major circulation patterns in the northern Gulf of Alaska and southeast Bering Sea. Dashed lines depict long-term mean flow (adapted from Kinder and Schumacher 1981, Stabeno et al. 1999).

Screening of microsatellite loci

Sixteen microsatellite loci previously developed for Atlantic halibut (McGowan and Reith 1999, Coughlan et al., 2000) were screened in Pacific halibut (Table 2). Loci were initially screened for amplification using the polymerase chain reaction (PCR) carried out in 10µl volumes comprised of 10 mM Tris-HCl pH 8.3, 50mM KCl, and varying concentrations of $MgCl_2$, dNTP's, primer, and *Taq* (GeneChoice, Frederick, MD). Each sample was amplified at three different annealing temperatures (55°C, 56°C, and 57°C), two different *Taq* concentrations (0.05 and 0.1 u/µl), two $MgCl_2$ concentrations (1.5 and 2.0 mM), two dNTP concentrations (200 and 250 µM each dNTP), and two primer concentrations (0.1 and 0.5 µM). The amplification profile comprised the following: one cycle of 95C (2 min) and 25 cycles of 95C (30 sec) + T_M (30 sec) + 72C (30 sec), followed by 72C (40 min).

Table 2. Loci tested in the present study, primer sequences, GenBank accession number and amplification success and variability in Pacific halibut.

Locus	Primer sequence (5'-3')	Repeat motif (Atlantic halibut)	Reference	Accession number	Amplification, Variability (Pacific halibut)
HhiA44	**F:**CAACTGTGGG-TATGTGCCTG **R:**GTGTCAGCACT-GTGCTTAAACC		McGowan et al. 1999	AF133243	Yes, Variable
HhiC17	**F:**TTAGGTCTGAT-CACCGCTATG **R:**GTTTA-CAAAGGTTTCT-GATGGC	(CA)23	McGowan et al. 1999	AF133244	Yes, Variable
HhiD34	**F:**GCCTGGTCT-CATTGTGTTCC **R:**AGGTTAAAT-GATTTCCT-GAAGCTG	CACT(CA)13	McGowan et al. 1999	AF133245	Yes Variable
HhiI29	**F:**GCTTCGGTTA-CACCTTTGC **R:**AGGACAGT-GAGGATGTCCG	(GT)27(GA)3	McGowan et al. 1999	AF133246	No
HhiJ42	**F:**CACAAACTCAA-GATGTTGCG **R:**AAGCTCACTG-GAAAATAATACCC	(CA)33(TA)3GCA (GA)4	McGowan et al. 1999	AF133247	Yes Variable
Hhi-1	**F:**GGAATA-	$(GT)_4GA(GT)_6$ $GA(GT)_5(GA)_2$ $(GT)_7(GA)_2$	Coughlan *et al*. 2000	AJ270779	Yes Not variable
Hhi-3	**F:**TCAGACAG-GAAGGAAGTTT-GG **R:**CCTCTCGGAAT-CACACACAG	$(CA)_{32}$	Coughlan *et al*. 2000	AJ270780	Yes Variable

Table 2. continued

Hhi-51	**F:**TTGAGCCAGT-TACAGAGAAGC **R:**ACTG-TATCCTCTGTTTA-CATCCA	$(TG)_8$ AG($TG)_5$	Coughlan *et al*. 2000	AJ270781	Yes Not variable
Hhi-52	**F:**ATTGAGA-AAGCAAATGTAC-GACC **R:**GTTCTTTTTAT-GTGAGCGACT-GTG	$(CTGTAACATA-CAACAA)_3$ $(CTG-TAACATACAA)_2$	Coughlan *et al*. 2000	AJ270782	Yes Variable
Hhi-53	**F:**ACCAA-CAGTGACA-CATAGCTCCT **R:**ATGCTAAT-GGGCTCTAAAATC	$(CA)_{29}$	Coughlan *et al*. 2000	AJ270783	Yes Variable
Hhi-55	**F:**CTTTTTCCT-GAGACGCTTG **R:**TAACC-GTTCCTCCACTGC	$(GT)_7$ $TT(GT)_8$ $(GA)_4$	Coughlan *et al*. 2000	AJ270784	No
Hhi-56	**F:**CACCAAAGA-CAGATGAAGCA **R:**CTACACTAT-CAGCAGCCCAG	$(GT)_2$ $AT(GT)_{12}$	Coughlan *et al*. 2000	AJ270785	Yes Variable
Hhi-57	**F:**GATTGCTGCT-GTTGCCTC **R:**TCCGCT-GCTCCCTCTA	$(CA)_2$ $CT(CA)_2$ $CT(CA)_3$ CT $(CA)_5$ CTCACG $(CA)_5$ $GA(CA)_5$ $GA(CA)_{11}$ GA $(CA)_5$ $GA(CA)_4$	Coughlan *et al*. 2000	AJ270786	Yes Variable
Hhi-59	**F:**GAGTGAGAGA-AACCAAAAGGC **R:**GC-GAGGGAAGAGA-GGAACAAC	$(CT)_2$($GT)_{12}$	Coughlan *et al*. 2000	AJ270787	Yes Variable
Hhi-60	**F:**CAGA-CAAAAACTCACA-CACGCTC **R:**	$(CA)_{12}$	Coughlan *et al*. 2000	AJ270788	Yes Not variable
Hhi-63	**F:**TCTCTATGTTT-GCCTGCCACCTTC **R:**TCGAC-CATCGTTT-GAATCTTTTG	$(CA)_{28}$ $G(CA)_2$ $(CGCA)_9$	Coughlan *et al*. 2000	AJ270789	Yes Variable

PCR products were visualized by electrophoresis of 5 µl of PCR product and 1µl of 6x glycerol loading buffer (Sambrook and Russell 2000) on a 6% native mini Bio-Rad polyacrylamide gel (Hercules, CA). Gels were run at 200V for 30 min, stained with Sybr Green II, and visualized on an FMbioII (MiraiBio, Alameda, CA). Following the initial screening, suitable loci were chosen depending on amplification success and variability (Table 3).

Population survey

Microsatellite fragment sizes were determined using a MegaBACE DNA genotyper/ sequencer (Amersham/Molecular Dynamics). Loci were grouped into panels, or "MegaBACE multiplexes" (Table 3) in order to maximize the number of loci screened at a time. Allele sizes were quantified by comparison with a 900 base pair (bp) molecular weight internal size standard and Genetic Profiler version 1.1 (http://www.mdyn.com). Genotypes for each locus were then exported into other statistical software for further analysis.

The Microsatellite Toolkit (Add-In for Microsoft Excel, Park 2001) was used to check data for errors and to convert data into Genepop (Raymond and Rousset 1995) and FSTAT format (Goudet 1995). The number of alleles, allelic range, and both observed and expected heterozygosity (H_O and H_E) were calculated for each population using GENETIX v. 4.01 (Belkhir et al. 2000). Test for departures from Hardy-Weinberg equilibrium (HWE) were performed for each locus and population using probability tests available in Genepop v3.3 (Raymond and Rousset 1995). Tests for genotypic and genic differentiation were carried out using the same program. F_{ST} values per locus and overall was calculated using FSTAT (Goudet 1995). The significance of overall and pairwise F_{ST} values was calculated in GENETIX v 4.01 using 1000 random permutations of the data. We decided not to calculate and test R_{ST}, because it assumes a stringent mutation model that is not supported in most empirical evaluations (e.g. Weetman et al. in press).

Table 3. PCR conditions, allelic range, and MegaBACE panels for multiplexing for the 10 loci used in this study.

Locus	Annealing Temp (*C)	[$MgCl_2$]	[dNTPs]	Panel	Range
HhiA44	**55**	0.8 mM	200 µM	B	137-263
HhiC17	**55**	0.8 mM	250 µM	B	118-207
HhiD34	**56**	0.8 mM	250 µM	C	196-288
HhiJ42	**55**	0.8 mM	250 µM	B	98-241
Hhi-3	**57**	0.8 mM	250 µM	C	165-238
Hhi-52	**57**	0.8 mM	250 µM	A	115-250
Hhi-53	**55**	0.8 mM	250 µM	A	205-332
Hhi-57	**55**	0.8 mM	250 µM	A	101-225
Hhi-59	**57**	0.8 mM	250 µM	A	135-164
Hhi-63	**56**	0.8 mM	200 µM	C	192-304

Selected markers - Pantophysin

Pantophysin was amplified in four halibut and two walleye pollock samples, using the *Pan* I primers and PCR protocol used in Canino & Bentzen (2004). The PCR product was subjected to electrophoresis on an agarose gel, excised out of the gel, and resuspended in 10 µl of low TE buffer. One µl of the purified PCR product was cloned into competent cells with the Topo TA cloning kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. DNA was purified using a Qiagen Miniprep kit (Quiagen, Valencia, CA). Inserts of four clones from each

sample were amplified using a PCR with M13 primers. An agarose gel showed that the insert had approximately the expected size of the *Pan I* locus (985 basepairs, Canino & Bentzen 2004). Positive clones containing inserts were grown up overnight in LB broth (Sambrook and Russell 2001) in a shaking incubator (37ºC and 220 rpm). Approximately 200ng of purified DNA or 300ng of PCR product was used in a sequencing reaction with the Amersham cycle sequencing kit (Amersham Biosciences) and 35 cycles with an annealing temperature of 57ºC. Cloning and sequencing was repeated as described above, with 16 halibut samples. Sequences were submitted to BLAST (GenBank) to confirm the identity of the sequenced fragment.

Results

Size and age structure

The sex composition of the sample populations was unknown because halibut are eviscerated prior to landing. Fish were of legal commercial size only (Fig. 2). Mean forklength was 119.5 ± 1.76 cm at Adak (range = 84-164 cm), 107.7 ± 2.22 cm at St. Paul (range = 74-177 cm), and 107.7 ± 1.76 cm at Newport (range = 83-126). Age in sampled fish (Fig. 3) averaged 17.6 ± 0.70 yr at Adak (range = 10-32 yr), 14.3 ± 0.47 yr at Newport (range = 10-21 yr), and 11.7 ± 0.69 yr at St. Paul (range = 8-17 yr). Nonparametric tests were used to examine differences between sample populations due to non-normality of age and size distributions. Kruskal-Wallis rank sum tests (Hollander and Wolfe, 1999) indicated significant difference among sites with respect to both fish length and age. However, pairwise comparisons based on Bonferroni inequalities (Gibbons, 1993) suggested only that median age of the Adak subsample was significantly greater than St. Paul, at the 0.05 level (Table 4).

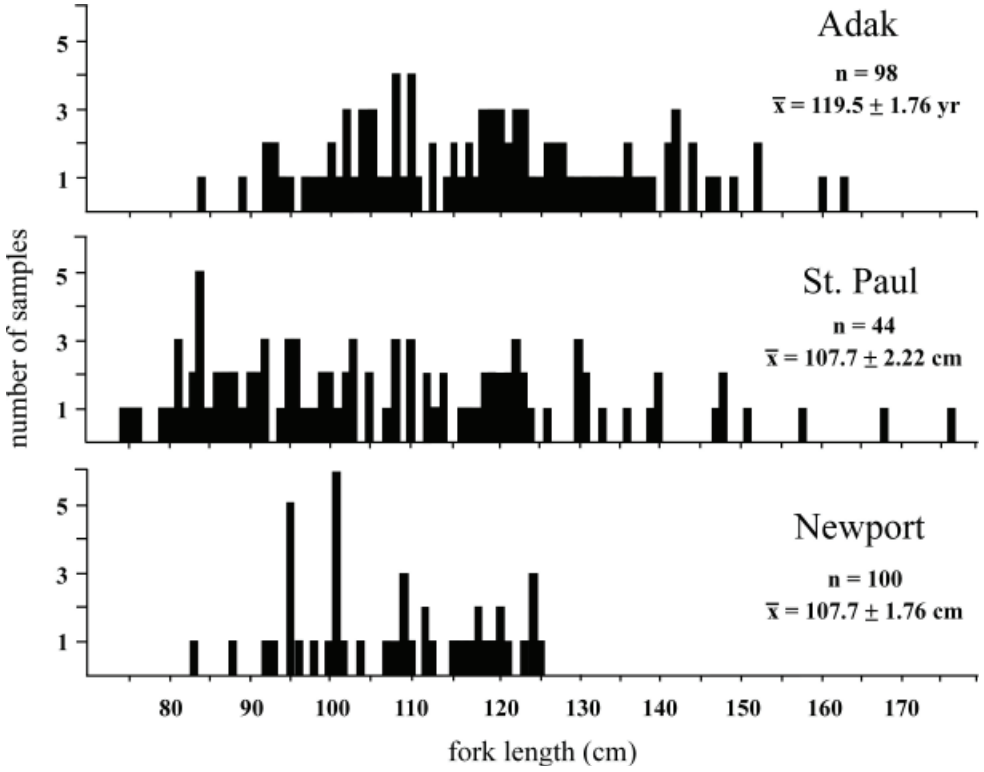

Figure 2. Length frequency distribution of fish sampled at each site.

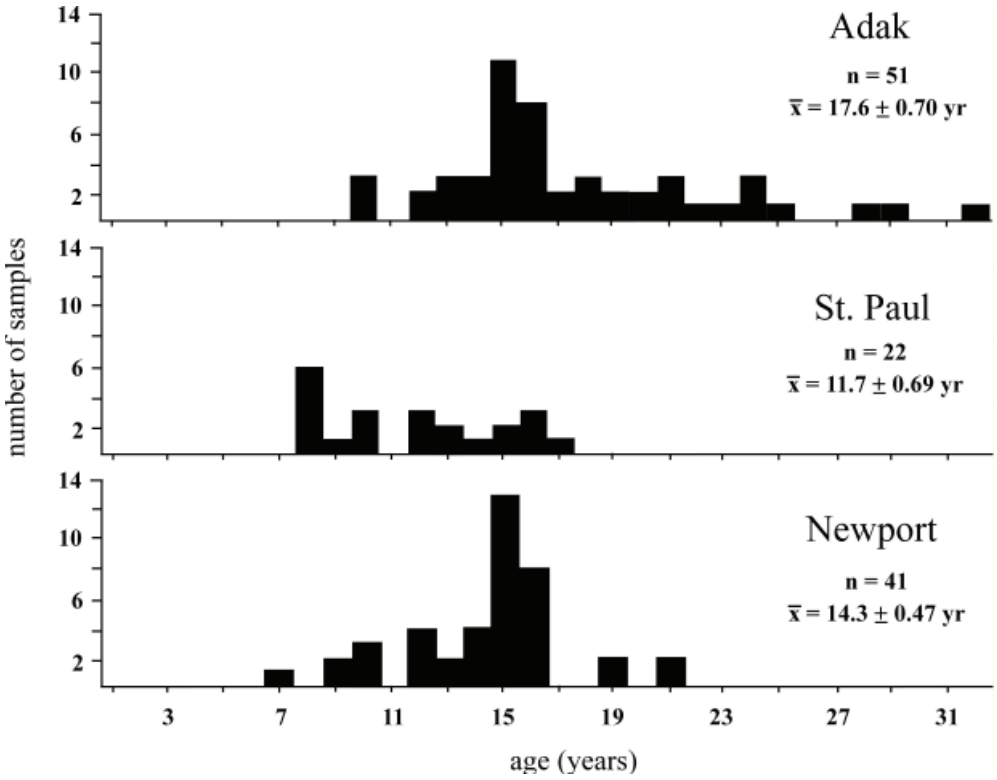

Figure 3. Age composition of fish sampled at each site.

Microsatellites

All but two of the loci (HhiI29, Hhi55) could be successfully optimized for Pacific halibut. Of those fourteen loci, ten appeared variable, and were used in the second phase of the study (Table 3). Analysis of 236 Pacific halibut revealed relatively high genetic variability, with about 40 alleles per locus for the whole dataset (range 19-59), and an average heterozygosity of about 90% (range 73-97%).

Three out of the ten variable loci showed genotype frequencies that were out of Hardy-Weinberg equilibrium (Table 5). Most of these deviations were due to deficiency of heterozygotes, most likely due to non-amplifying null alleles (loci HhiD34, HhiJ42). One locus showed a highly significant deficiency of heterozygotes in one sample, and a highly significant excess in the other two samples (Hhi59). This result may have been due to scoring problems, though the magnitude of the deviation (F_{IS}) was relatively small.

There was no significant genetic differentiation between the three samples using most of the commonly applied measures and tests (Table 6). Neither tests for genic nor genotypic differentiation provided evidence for genetic differences among populations. Similar results were obtained whether the three loci with deviations from Hardy Weinberg equilibrium (HhiD34, HhiJ42, Hhi59) were included or not. However, the permutation tests in GENETIX, which randomizes genotypes among samples and calculates F_{ST} values from the randomized datasets for comparison with the real value, yielded significant results at the 10% significance level (F_{ST}=0.001, P=0.077, after exclusion of loci out of Hardy-Weinberg equilibrium: F_{ST}=0.001, P=0.070). Subsequent pairwise analyses between samples using the same test provided evidence for differentiation of the Adak sample from the other two samples (Table 7).

Table 4. Results of Kruskal-Wallis rank sum tests (Hollander and Wolfe, 1999) and pairwise comparisons based on Bonferroni inequalities (Gibbons, 1993) comparing length and age of the aged subsample of fish at each site.

Length analysis:			
	Mean length	**N**	
Adak	119.5 ± 1.76	98	
St. Paul	107.7 ± 2.22	44	
Newport	107.7 ± 1.76	100	
K-W test statistics:	**H'**	**DF**	**p**
	26.73	2	<0.001
Pairwise comparisons:	**Critical adjusted rank diff**	**Observed mean rank diff**	**p**
Adak v. Newport	73.40	42.58	>0.30
Adak v. St. Paul	57.49	46.43	>0.30
St. Paul v. Newport	73.17	3.85	>0.30
Age analysis:			
	Mean age	**N**	
Adak	17.6 + 0.70	51	
St. Paul	14.3 + 0.47	22	
Newport	11.7 + 0.69	41	
K-W test statistics:	**H'**	**DF**	**p**
	24.15	2	<0.001

Table 5. Sample sizes (N), Number of alleles (A), expected and observed heterozygosity (H_E and H_O), and F_{IS} (Weir and Cockerham, 1984) for each sample at each locus. Positive F_{IS} values indicate a deficiency of heterozygotes, negative values an excess. The P-value (p) is the probability that the genotype frequencies are in Hardy-Weinberg equilibrium (Genepop analysis). The total column present mean sample size (N), total number of alleles in the dataset (A), and weighted mean expected (H_E) and observed heterozygosity (H_O).

		Adak, AK	Newport, OR	St. Paul, AK	Total / average	Reported from Atlantic halibut
Hhi52	N	93	42	94	229	20
	A	18	12	18	22	9
	H_E	0.7701	0.6774	0.7183	0.732	0.78
	H_O	0.7419	0.7857	0.7553	0.755	0.70
	F_{IS}	+0.042	-0.148	-0.046		
	p	0.2770	0.7325	0.0548		
Hhi59	N	96	43	92	231	20
	A	17	10	13	19	4
	H_E	0.7979	0.8202	0.8071	0.806	0.30
	H_O	0.8542	0.8140	0.9348	0.879	0.35
	F_{IS}	-0.065	+0.019	-0.153		
	p	0.0000	0.0000	0.0000		
HhiA44	N	74	44	72	190	55
	A	33	32	39	53	18
	H_E	0.9491	0.9403	0.9519	0.948	0.86
	H_O	0.8514	0.9318	0.9167	0.895	0.87
	F_{IS}	+0.110	+0.021	+0.044		
	p	0.0368	0.3771	0.4577		
HhiC17	N	94	43	95	232	55
	A	37	33	37	40	22
	H_E	0.9633	0.9608	0.9608	0.962	0.95
	H_O	0.9681	1.0000	0.9474	0.966	0.89
	F_{IS}	+0.000	-0.029	+0.019		
	p	0.6951	0.7352	0.8318		
HhiJ42	N	62	38	66	166	55
	A	38	32	41	51	13
	H_E	0.9585	0.9522	0.9622	0.959	0.79
	H_O	0.5968	0.7368	0.7121	0.675	0.67
	F_{IS}	+0.401	+0.266	+0.267		
	p	0.0000	0.0057	0.0099		
Hhi53	N	88	44	84	216	20
	A	47	36	52	59	14
	H_E	0.9728	0.9628	0.9724	0.971	0.94
	H_O	0.9432	0.9545	0.9524	0.949	0.90
	F_{IS}	+0.036	+0.020	+0.027		
	p	0.1886	0.2538	0.1267		
Hhi57	N	93	44	95	232	20
	A	42	30	43	55	12
	H_E	0.9237	0.9233	0.9141	0.920	0.79
	H_O	0.9032	0.8864	0.9474	0.918	0.70
	F_{IS}	+0.028	+0.051	-0.031		
	p	0.1108	0.1847	0.7882		

Table 5. continued.

HhiD34	*N*	93	43	93	229	55
	A	25	19	28	33	9
	H_E	0.9205	0.8899	0.9083	0.910	0.73
	H_O	0.6129	0.6047	0.6774	0.638	0.80
	F_{IS}	+0.339	+0.331	+0.259		
	p	0.0000	0.0000	0.0000		
Hhi63	*N*	78	44	90	212	20
	A	41	37	45	50	14
	H_E	0.9589	0.9535	0.9639	0.960	0.87
	H_O	0.9231	0.8864	0.9222	0.915	0.75
	F_{IS}	+0.044	+0.082	+0.049		
	p	0.1056	0.0606	0.0473		
Hhi3	*N*	76	43	74	193	20
	A	31	30	31	35	16
	H_E	0.9559	0.9511	0.9551	0.955	0.88
	H_O	0.9474	0.9070	0.9730	0.948	0.95
	F_{IS}	+0.016	+0.058	-0.012		
	p	0.3693	0.1117	0.8248		
Average	**N**					
		84.7	42.8	85.5	213	34
	A	32.9	27.1	34.7	41.7	13.1
	H_E	0.91707	0.90315	0.91141	0.912	0.789
	H_O	0.83422	0.85073	0.87387	0.853	0.758

Table 6. Tests for genetic differentiation from GENEPOP and GENETIX. P F_{ST} shows the probability of F_{ST} being random. The final row (Total-3) indicates values obtained when the three loci that were out of Hardy Weinberg equilibrium (HhiD34, HhiJ42, Hhi59) were excluded from the analyses.

	GENEPOP		*GENETIX*	
Locus	genotypic	genic	F_{ST}	P F_{ST}
HhiA44	0.121	0.000		
HhiC17	0.891	0.851	-0.002	
HhiD34	0.741	0.516	-0.003	
HhiJ42	0.494	0.027	0.000	
Hhi-3	0.950	0.925	-0.002	
Hhi-52	0.443	0.411	0.015	
Hhi-53	0.127	0.189	0.000	
Hhi-57	0.317	0.105	-0.001	
Hhi-59	0.572	0.429	0.005	
Hhi-63	0.906	0.782	-0.000	
Total	0.767	0.203	0.001	0.077
Total - 3	0.601	0.459	0.001	0.070

Table 7. Pairwise F_{ST} values calculated in Genetix, and compared to 1000 random permutations, used as the significance level (percentage of random permutations greater than those achieved by the data).

	All loci		Without HhiD34, HhiJ42, Hhi59	
Comparison	***F_{ST}***	***P - value***	***F_{ST}***	***P - value***
Adak vs. Oregon	0.015			
Adak vs. St. Paul	0.0011	0.083	0.00134	0.047
Oregon vs. St. Paul	-0.0018	0.966	-0.00180	0.970

Pantophysin

Amplification of the *Pan* I locus appeared to be successful; it produced a fragment of the expected size, about 1kb, in both walleye pollock and halibut. Cloning was equally successful, and after PCR amplification of inserts, the inserts had the expected size as well. However, the sequences we obtained from the first cloning exercise did not align with pantophysin sequences from cod in GenBank, and were thus probably not pantophysin.

The second round of cloning produced inserts of about the correct size, but ranging from 850 bp to 1100 bp. We sequenced eight of these clones; again a BLAST search using these sequences did not yield pantophysin as closest match. Attempts to increase the stringency (accuracy) of the PCR by increasing annealing temperatures were not successful, nor were application of alternative primers provided by Mike Canino (NOAA Fisheries, 7600 Sand Point Way NE. Seattle, WA 98115, pers. comm.).

Discussion

Microsatellite amplification

Our microsatellite study demonstrated clearly that the primers isolated from Atlantic halibut are applicable to Pacific halibut. Such cross-species amplification of microsatellites has been reported before: especially closely related groups of species, such as Pacific salmon (Scribner et al. 1996), cichlids (Noack et al. 2000) and Pacific rockfishes (Gomez-Uchida et al. 2003) are known to allow the use of microsatellites used in other species of the same group. Similar species in Atlantic and Pacific also often allow successful cross-amplification, for example, herring (e.g. O'Connell et al. 1998), cod (M. Canino, NOAA Fisheries. 7600 Sand Point Way NE. Seattle, WA 98115, pers. comm.), and now halibut.

The success of the application of heterologous primers (primers from other species) is not only dependent on the phylogenetic relationships among species, but also on the specific locus to be amplified. Very conserved regions (for example, those present in mitochondrial DNA (mtDNA)) allow amplification of some DNA regions across entire classes or even phyla of animals and plants (e.g. Kocher et al. 1989). While such conserved regions can be specifically targeted in the well-studied mtDNA, microsatellite flanking regions are usually anonymous, that is, their region of origin in the genome is not known. Therefore, there is considerable variability in the application of heterologous microsatellite primers – indeed, some loci seem to be conserved across almost all fish species (Rico et al. 1996; Zardoya et al. 1996). However, such loci are rare, and usually the number of successfully amplified loci drops rapidly with increasing phylogenetic divergence from the species from which primers were isolated (the focal species) (Scribner and Pearce 2000).

Reduced amplification success is not the only complication with heterologous primers. Null alleles, alleles that do not PCR amplify because of mutations in the priming sites flanking the microsatellite, often cause a bias in the estimation of allele frequencies (Estoup and Angers 1998).

Such mutations are more likely to occur if the species in question is more distantly related to the focal species. The main effect of such null alleles is that heterozygotes are scored as homozygotes, because one of the two alleles was not amplified, resulting in a heterozygote deficit compared to Hardy Weinberg expectations. One way to correct for null alleles is to redesign primers to avoid the mutated site (Paetkau and Strobeck 1995), though they can also simply be excluded from the analysis. In the present study, only two loci showed consistent deficiency of heterozygotes – as we had a sufficient number of loci, these could be excluded without much effect on results. Interestingly, one of the two loci (HhiJ42: McGowan and Reith 1999) previously showed some indication for null alleles in Atlantic halibut, the species from which it was isolated.

One commonly observed feature of heterologous primers is their shorter length and lower variability compared to that described in the focal species. This observation has led to discussions whether differences in microsatellite length between species are mainly due to ascertainment bias or indeed reflect genuine genomic differences (e.g. Amos et al. 2003). Once again, variability is expected to decrease with increasing phylogenetic distance from the focal species (Scribner and Pearce 2000). In the present case, variability of seven out of ten microsatellite loci was actually higher in Pacific halibut than in Atlantic halibut, a result which may be due to demographic factors such as a larger population size.

The variability of the loci tested here is relatively high, as commonly expected for marine species with large population sizes (DeWoody and Avise 2000). Extremely high variability may be problematic for population comparisons, especially if the distribution of allele frequencies is very skewed (Hauser et al. 2001). Recent modeling, however, suggests that high variability in microsatellites, on the order reported here, may be more powerful for the estimation of genetic distances (Kalinowski 2002). Our set of loci should therefore allow a fairly powerful assessment of the population structure of Pacific halibut, in particular in association with three other loci for Pacific halibut previously isolated by Bentzen et al. (1998).

Microsatellite population differentiation

Although tests for population differentiation showed few significant differences among the three collection sites, these preliminary data were interesting and suggested some degree of population structure. Tests for genic and genotypic differentiation carried out in GENEPOP (Raymond and Rousset 1995) were generally not significant (Table 6), though tests for the significant difference of F_{ST} from random expectations, as carried out in GENETIX (Belkhir et a*l.* 2000) were marginally significant (F_{ST}=0.1%, P=0.077, Table 6), and showed a significant difference between the sample of Adak from the other two samples (Table 7). This apparently surprising discrepancy between GENEPOP and GENETIX can be explained by the difference in tests used by the programs. GENEPOP carries out Fisher's exact tests on allele numbers (genic differentiation) or on allele numbers derived from genotype frequencies (genotypic differentiation), while GENETIX calculates an F_{ST} value from the data, and compares it with F_{ST} values derived from 1000 permutations of genotypes in the original data set. While the latter approach aims to measure correlations of genes within and among populations, the former follows classic tests of contingency tables (Excoffier 2001). The relative power of the two approaches is not well known, though it could be argued that in species with high intra-population variability permutation tests on genetic correlations may be more powerful than contingency tests based on many alleles and genotypes of low frequency. In any case, the significant test result produced by the permutation tests warrants further investigation.

Perhaps surprisingly, pairwise permutation tests suggested that the sample from Adak was different from the other two samples. If true, this implies that gene flow is greater between the Pribilof Islands and Oregon than it is between the Pribilofs and Adak. Initially, this appears counterintuitive. St. Paul and Adak are both within the southeast Bering Sea, separated by ~800 km, while Newport lies over 3000 km southward in a different ocean basin. One explanation is

that the apparent genetic differences could be sampling artifacts. The aged subsamples indicate significant differences in average fish age among sites. If genetic signatures do not remain stable over time, differences in age composition among sites could generate spurious site-specific genetic differences. However, while Adak appeared to be genetically distinct from both St. Paul and Newport (Table 7), it was different only from St. Paul with respect to age distribution (Table 4). Thus, it is difficult to support the hypothesis that differences in age composition alone could be responsible for all of the observed genetic separation between Adak and the other sites. Furthermore, the genetic results obtained here are not inconsistent with earlier research that found no difference between central Gulf of Alaska individuals and those just north of Unimak Pass, but found individuals sampled near Hokkaido to be significantly different from both of the eastern Pacific regions (Grant et al. 1984). If the eastern and western Pacific actually support different populations, one can conclude that segregation must exist somewhere along either the Kuril or Aleutian Ridges. This segregation might functionally occur during at least two phases of halibut life history: perhaps adult halibut do not migrate across deep Aleutian passes, or larvae are not advected along the Aleutian chain.

The continental shelf, defined as water depths <200m (Stabeno et al. 1999) is contiguous throughout the Gulf of Alaska and into the southeast Bering Sea, extending westward into the Aleutian Chain to Unimak Island (~169° W lon; refer to Fig. 1). However, the shelf does not extend uninterrupted along the Aleutians as far westward as Adak. Rather, shelf habitat west of Umnak Island is confined to small segments that surround groups of volcanic islands, with shelf segments separated from each other by deep oceanic passes. The Adak shelf segment is separated from the southeast Bering Sea and western Gulf of Alaska shelf by four deepwater passes, the deepest of which are Samalaga and Amukta (Fig. 1). Catch per unit effort in annual IPHC setline surveys in the Bering Sea and Aleutian Islands is typically very low at depths greater than about 450m (S. Hare, IPHC, P.O. Box 95009, Seattle, WA 98145, unpub. data), and the IPHC uses this 450 m as the lower limit defining halibut habitat for the region. Both Samalga and Amukta Pass approach this depth at their shallowest, potentially producing constrictions to halibut movement. Fish moving along isobaths at shallow depths near passes might be guided around the islands and tend to remain on each shelf segment as opposed to moving freely amongst them.

There is also evidence for fragmentation of current patterns and oceanographic features along the Aleutian Chain that could affect larval dispersal (Fig. 1; for a detailed description of Bering Sea current patterns, please refer to Stabeno et al. 1999). The Gulf of Alaska is characterized by a large cyclonic gyre with westward-flowing currents along its northern boundary. In relatively shallow water, the Alaska Coastal Current (ACC) transports terrestrial runoff from at least southeast Alaska to Unimak Pass, while the Alaska Stream (AS) represents geostrophic flow along the shelf-edge. At Unimak Pass portions of the ACC and AS turn northward along the shelf edge to join the Bering Sea Slope Current (BSC), and also flow along the coast into Bristol Bay. From a larval transport perspective, it is easy to imagine that larvae spawned in the western Gulf, particularly along the southern Alaska Peninsula, are likely to be advected through Unimak Pass and delivered to the vast nursery grounds of Bristol Bay. If those individuals do not undergo ontogenetic migration back to the Gulf prior to maturity, they may eventually contribute to spawning groups located in the southeast Bering Sea.

South of the Aleutian Chain the AS continues westward as far as Near Pass (~172° E lon) where much of it enters the Bering Sea. A considerable proportion of the water entering the Bering Sea at Near Pass continues moving to the north and west, but a portion of the AS also turns back to the east to form the Aleutian North Slope Current (ANSC). The ANSC flows eastward along the north side of the Aleutians, generally countering the AS. However, unlike the relatively unidirectional flow of the ACC and BSC, flow along the Aleutians is more complex and often fragmented by the deep Aleutian passes. That is, flow through a number of the passes tends to be northward along the eastern side of the pass, and south along the western side of

the pass. In particular, this has been observed at both Amchitka and Amukta Passes (Fig. 1). Thus, the ANSC and AS combine with through-pass flows to generate a series of oval nearshore gyres that surround each Aleutian shelf region and enclose groups of islands in their own coastal current system. The resulting currents may be well suited for larval retention within the central and western Aleutians. For example, larvae spawned north of Adak Island may be transported eastward by the ANSC, advected south through Amukta Pass and into the AS, travel westward to Amchitka Pass, then be advected northward back into the ANSC and eventually delivered back to Adak. Local larval retention may result in gene flow during the larval phase that is lower between the Aleutians and southeastern Bering Sea than it is between the western Gulf and southeastern Bering Sea.

The low differentiation among populations observed here may at least in part also be due to inappropriate sampling. The samples collected for this study were collected opportunistically from fishing vessels, and consisted mainly of non-spawning individuals. Studies suggest that halibut can carry out extensive migrations between shallow water summer feeding and winter spawning grounds located near the shelf edge. Such migrations have long been documented based on tagging studies that date to the 1920s and supported by seasonal changes in the overall distribution of mature fish (Skud 1977; St. Pierre 1984). Analysis of historical tagging data indicates that halibut in the Gulf of Alaska tend to aggregate in the northern Gulf during winter months, and that individuals found in the western Gulf and British Columbia during the summer may undergo considerable migration northward (Leaman et al. 2002). In one study conducted in 1979, halibut tagged on a single spawning ground off southeast Alaska and recaptured in the commercial fishery dispersed over at least 750 km of coastline within two years (Hoag et al. 1983). Recent work using pop-up satellite-transmitting archival tags in the Gulf of Alaska has suggested that average seasonal migration distances may be on the order of ~350 km and the maximum observed migration distance was over 1100 km for one individual (T. Loher, IPHC, P.O. Box 95009, Seattle, WA 98145, unpub. data). Seasonal migration of up to 500 km has been observed in the southeast Bering Sea using satellite tags (A. Seitz, University of Alaska, Fairbanks, Institute of Marine Science, P.O. Box 757220, Fairbanks, AK 99775, unpub. data). Notably, not all halibut appear to undergo extensive movements. The satellite tagging indicates that while some halibut travel large distances, others tagged in close proximity show relatively little displacement between summer and winter. Aggregations of non-spawning fish may consist of a mixture of both local and distant spawning populations that only segregate during the spawning season. Such population mixtures are difficult to identify, unless differentiation among spawning populations is large (unlikely in a marine species like halibut) or data from contributing populations are available. It will therefore be crucially important in a future research project to concentrate sampling on spawning fish on their spawning grounds.

It is important to remember here that molecular genetic markers provide a very conservative estimate of stock structure relevant to management: although populations with very small levels of exchange (few individuals / generation) can be identified, populations with a demographic exchange of 1-5% per generation may appear genetically homogenous (Carvalho and Hauser 1994). Therefore, low F_{ST} values as presented here are no proof, or even an indication, of the existence of a single panmictic population. The significant difference of the F_{ST} value from random expectation, on the other hand, warrants the further investigation of the population structure of halibut. Given the promising initial results of the current study, we have expanded our sampling program to include spawning fish, as well as a much broader and evenly spaced distribution of summer collections that should help us refine conclusions regarding geographic variation. These samples will be analyzed in future work and comprise future report(s). Additionally, the inclusion of a temporal aspect seems warranted in future work to demonstrate the stability of any observed geographic patterns.

Pantophysin

DNA regions under selection potentially offer a powerful method to identify essentially self-recruiting populations. In marine species, neutral markers, which differentiate only by genetic drift and mutation, may take many generations to accumulate genetic differences, and very low levels of gene flow are sufficient to homogenize populations. Therefore, even populations which are essentially self-recruiting, may be indistinguishable genetically (Carvalho and Hauser 1994; Hauser and Ward 1998). Selected markers, on the other hand, may show genetic differentiation much sooner after population separation, and may maintain such differences even under considerable gene flow. The use of these markers may therefore provide an additional way to identify self-recruiting populations, though the combination of selected sequences with neutral markers and extensive temporal analyses are advisable to gauge the selection differential between areas, and to avoid the identification of 'nursery stocks' (Smith et al. 1990), populations that show genetic differences because of fast selection during the lifetime of individual cohorts, but recruit from a common gene pool.

In the present project, we attempted to apply the pantophysin locus *Pan I*, which has been shown in many gadoids to be under selection and show significant differentiation at small geographic scales. Although we were successful of amplifying a fragment of the expected size of *Pan I*, sequencing data could not confirm the identity of the fragment. Indeed, the sequence did not align with published gadoid *Pan I* sequences, and therefore our fragment may have been another region of the genome. As we tried two different cycles of cloning of these fragments and analyzed over 20 fish, this result is unlikely to be due to methodological problems. It is more likely that gadoid primers do not work on halibut due to mutations in the priming site, suggesting that considerably more development effort is required to analyze *Pan I*.

Because of these complications with the analysis of the *Pan I* locus, we did not attempt to analyze any more selected markers. However, a wide range of coding DNA regions is available for analysis and could be tested. In addition, it may be possible to use AFLP (amplified fragment length polymorphisms, Vos et al. 1995) to identify DNA regions distinguishing among populations and to isolate SNPs (single nucleotide polymorphisms, Breen et al. 1999) from these regions (Nicod and Largiader 2003). The combined use of neutral and selected markers is the most powerful approach for the analysis of marine species with presumably high levels of gene flow.

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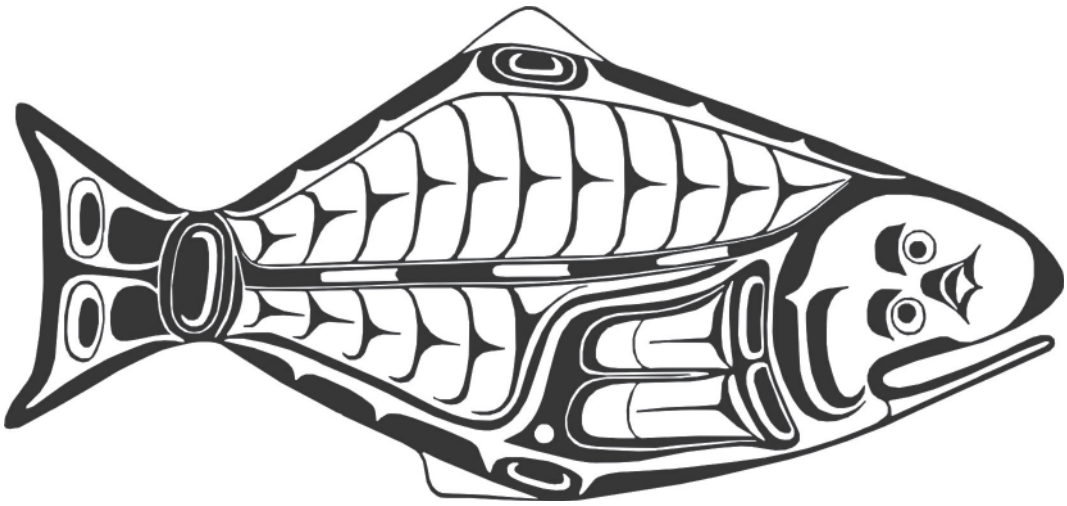

Halibut Crest - adapted from designs used by Tlingit, Tsimshian and Haida Indians