Allozyme, mtDNA, and microsatellite variants describe structure of populations of pink and sockeye salmon in Alaska

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Key Words: allozyme, microsatellite, mismatch analysis, mtDNA, *Oncorhynchus gorbuscha*, *Oncorhynchus nerka*, pink salmon, population genetics, sockeye salmon, mixed fishery analysis

5 Jan. 1998

ABSTRACT

We are interested in applying knowledge of population genetics to the sustained management of salmonids of commercial importance. Some populations in Alaska have been adversely affected by climactic or anthropogenic events, and fishery closures to protect these depleted stocks can lead to underutilization of other healthy stocks when both occur in mixed-stock fisheries. A vast array of contemporary gene-detection techniques might identify stocks in a timely manner and thereby improve management. We are currently applying data collected from allozyme electrophoresis and from analysis of restriction fragment length polymorphism of mitochondrial DNA (mtDNA) to solve questions of population structure. We also report pilot studies to collect data on length polymorphisms observed in microsatellite DNA and sequence polymorphisms observed in mtDNA loci and other nuclear DNA (nDNA) loci, testing for site-specific variants that may be assayed using a simple mismatch analysis probe. We focus on results obtained from using these approaches in an array of studies of the structure of commercially important pink salmon (*Oncorhynchus gorbuscha*) and sockeye salmon (*O. nerka*) populations affected by the 41 million liter *Exxon Valdez* oil spill of 1989. In the case of sockeye salmon, the allozyme data was used to guide harvest in-season in order to conserve depleted populations. While we focus our discussion of molecular markers on studies of pink and sockeye salmon in Alaska, similar applications are clearly possible for other salmonids over their entire range.

INTRODUCTION

Describing the genetic structure of populations of Pacific salmon became a central focus of research and management as anthropogenic factors, including overharvest, habitat destruction, and adverse effects on wild populations from hatchery propagation, eroded components of the productivity of the billion-dollar fishery in the North Pacific Ocean (e.g., see reviews in Ryman and Utter 1987; Carvalho and Pitcher 1994). The primary question asked by fisheries managers that seek genetic solutions often is "how can we partition the annual harvest among genetically discrete components of the species in such a way to maximize long-term productivity?" (e.g., Lincoln 1994). Several categories of molecular techniques emerged during the last three decades that may potentially provide answers to this sometimes daunting challenge (see reviews in Park et al. 1994; Ferguson et al. 1995).

Gharrett and Smoker (1994) graph the increasing importance of genetic study as a component of the fish biology literature. Adapting this approach, we demonstrate the relative

increased importance of the four basic types of molecular approaches used in the genetic study of salmonids today (Figure 1A). Advantages of these different approaches include such factors as relative cost and difficulty of field sampling and laboratory analysis (Table 1). One of these molecular approaches, minisatellite analysis, is not pursued by many laboratories because of difficulties in standardizing and interpreting the multi-locus fragment data. Of the remaining categories of molecular markers, allozymes and mitochondrial DNA were used most frequently to define population structure of salmonids. Allozyme analysis remains the preferred approach for study of population genetics for many species of salmonids because of its power to resolve populations of many species in the family by assaying many nuclear loci rapidly and inexpensively (Allendorf 1994). Additional advantages of allozymes include the fact that many laboratories cooperate on inter-institutional examinations of salmonids using allozymes, providing a support structure including a wealth of compatible data for comparison among Pacific Rim populations (e.g., for pink salmon *O. gorbuscha* see Shaklee et al. 1991; White and Shaklee 1991; Shaklee and Varnavskaya 1994; Seeb et al. 1996b).

The utility of mtDNA approaches to study genetic diversity of salmonid populations is controversial because of relatively high cost and slow throughput (Allendorf 1994). Additionally, sometimes mtDNA data reveal less diversity than do allozyme data because mtDNA cannot recombine and is maternally inherited as a single locus so that the variation is absolutely linked (Smouse et al. 1994; contrast the lack of geographic resolution observed for mtDNA data for populations of chum salmon in Park et al. (1993) with the geographic resolution apparent for similar populations in Winans et al. (1994)). One advantage of screening for genetic variation in mtDNA can be that the mitochondrial genome experiences an elevated rate of nucleotide substitution over that of nuclear DNA (nDNA; Lynch 1996). The fidelity of DNA replication in the mitochondria is also reduced, and mtDNA repair systems are less stringent (Alberts et al. 1994). Lynch (1996) not only confirmed that mtDNA accumulates mutations much faster than nDNA, but also showed that even deleterious mutations may become fixed due to the relaxed control over DNA repair and replication. For these reasons, the use of both nDNA and mtDNA approaches sometimes provides complementary information for the resolution of population structure.

In some cases, the disadvantages of allozymes and mtDNA make either technique unsuitable for a particular application. Some species exhibit low or no variation at allozyme loci, making estimates of between population diversity difficult to obtain (e.g., Milner 1993). mtDNA

sometimes provides useful insights into microgeographic structure, but relationships on a macrogeographic scale may become obscured because of convergent evolution (cf., Adams et al. 1994). Thus, additional approaches to genetic analysis focusing upon sequence variants in nDNA emerged in recent years to address these limitations (see Figure 1A, 1B). Of these, the most useful appear to be the techniques that rely on polymerase chain reaction (PCR) amplification of DNA template for further analyses of fragment or sequence variation (e.g., for microsatellites see Estoup et al. 1993; Angers et al. 1995; Morris et al. 1996). Further, the application of a rapid assay to detect site-specific single nucleotide (nt) substitutions for study of fish genetics was promoted by Park and Moran (1994). The use of allele-specific PCR using 3'-primer mismatch analysis (MMA) was shown to be a rapid and sensitive technique for identification of single nt substitutions in rainbow trout (Bailey et al. 1996), and the advent of automated DNA sequencing strategies elevates the opportunity for observing site-specific mutations appropriate for such study.

In this paper we review our studies of genetic variation in pink and sockeye salmon *O. nerka* in order to provide an overview of the utility of allozymes, mtDNA, microsatellites, and potentially MMA for detecting genetic differences that distinguish Pacific salmon populations. These studies were initiated in response to the *Exxon Valdez* oil spill. On March 24, 1989, the supertanker *Exxon Valdez* ran aground on Bligh Reef in Prince William Sound (PWS), Alaska, spilling approximately 41 million liters of crude oil. The oil slick, pushed by winds and currents, moved through western PWS and southern Cook Inlet, depositing toxic polycyclic aromatic hydrocarbons onto spawning gravels and into the salmonid food chain. Subsurface oil remained in some of the beaches for years in spite of the multi-billion dollar clean-up and restoration effort (Wolfe et al. 1994), and populations of some species of salmonids may not be fully recovered (Bue et al. 1996). Of the salmonids inhabiting the spill zone, populations of pink and sockeye salmon were the most adversely impacted.

PINK SALMON STUDIES

Pink salmon is the most abundant North American species of the Pacific salmon (Heard 1991), making it an ecological cornerstone in biological communities of the Pacific Rim and an economic mainstay for many coastal communities. Pink salmon are both anadromous and semelparous: in their natural range, they make long oceanic migrations, home to their natal streams to spawn, and die at age two. Annual catches of pink salmon ranged from 46 to 128 million fish in Alaska alone during the period from 1985-1995.

Many hundreds of streams drain into PWS, and most provide a freshwater home to spawning pink salmon each summer. Little is known about the structure of populations inhabiting PWS or of the genetic relationships of these populations to other populations inhabiting the region. Pink salmon spawn in the intertidal portions of most streams in PWS; some larger rivers host large aggregations of spawners in upstream areas as well. Temporal isolation occurs between aggregations that spawn in mid summer and those that spawn in the fall. Combining this life history evidence with observations of other physiographic and climactic variables in PWS led us to suggest that population substructuring was present. Our objective was to test for both temporal and geographical structuring among even- and odd-year classes by examining genetic differences between early-and late-season spawners, upstream and intertidal spawners, and stream of spawning within each. Additionally, genetic positioning of the local hatchery populations within this structure was of interest because the extensive releases of pink salmon fry in PWS in recent decades may have affected the partitioning of naturally occurring genetic diversity. Important to this study was the fact that even- and odd-year classes have independent population structures because of the rigid two-year life cycle of pink salmon. For example, climactic, tectonic or other such events (such as the major 1964 earthquake (Roys 1971) or the 1989 oil spill) may affect the population structure of one year class, cycle through subsequent generations, and leave the alternate cycle of year-classes relatively unchanged. Therefore, population structure and conservation strategies must be independently assessed for the even- and odd-year classes.

Our primary focus was to screen allozymes and mtDNA for variation that would address the objectives above. Allozymes were selected because extensive baseline data exist for pink salmon (see references above) including a pre-oilspill data set from PWS (Seeb and Wishard 1977). Mitochondrial DNA was selected to add complementary data because we know from other allozyme study that adjacent pink salmon populations can be very closely related (Gharrett et al. 1988).

We also chose to conduct a preliminary screen for microsatellite variation and variation detected at the DNA sequence level. These latter studies were initiated originally to screen for elevated rates of mutation that may have resulted from exposure of individuals to the genotoxic hydrocarbons present due to the oil spill (cf., Bailey et al. 1996). We also wanted to conduct a pilot study to test the viability of these methods to detect genetic variation in pink salmon populations.

Allozyme and Mitochondrial DNA Analyses

To begin the study in 1994, tissues were collected from 92 - 100 individuals from each of 25 spawning aggregations from wild-stock streams and two hatchery collections. PWS was historically divided into subdivisions for management and conservation purposes according to biological, geographical, and geological factors (i.e., see Rugolo 1984), and we distributed sampling effort among the current harvest management zones. Sampling was done to include at least one collection from each of the five major subdivisions (Southeast, East, North, Southwest, Montague; Figure 2A). Although a majority of pink salmon spawning in PWS occurs in areas of tidal influence, some larger tributaries also possess somewhat discrete aggregations that spawn in upstream areas above the influence of tides. Samples were collected from both tidal and upstream sites in five of these creeks.

Our allozyme screen of 77 loci yielded 38 loci that were polymorphic (frequency of common allele less than 0.99; Seeb et al. 1996b). Cavalli-Sforza and Edwards (1967) chord distances were calculated to evaluate genetic relationships and were used as input into a metric multidimensional scaling analysis (MDS; Lessa 1990) using functions in *S-Plus* (Mathsoft, Inc., Seattle, WA).

A subset of 40 individuals from each of the 27 collections was assayed for restriction fragment length polymorphism (RFLP) at sites previously identified in the ND5/ND6 region (Fetzner et al. *in prep.*). After extraction, the ND5/ND6 region was amplified using PCR. Amplified DNA was cut with the six restriction enzymes found to detect haplotype polymorphisms (of the 30 screened in Fetzner et al. (*in prep*.), *Apa I, BstU I, EcoR V, Hinf I, Rsa I, Xba I*) and electrophoresed on agarose gels. Fragments were visualized under UV light, and the restriction sites detected for each enzyme were pooled as composite haplotypes for the statistical analyses.

We tested for genetic structure in three steps. First we used a hierarchical log likelihood analysis (allozymes) and Monte Carlo simulations (mtDNA; Roff and Bentzen 1989) to investigate heterogeneity (1) among wild collections from different elevations (tidal and upstream), (2) among management regions within elevation, and (3) among collections within regions within elevation. Second, we performed pairwise tests within streams where we had both tidal and upstream collections. Third, we performed a gene-diversity analysis to partition variation into hierarchical levels stratified by site, region, and elevation.

Significant differences between overall upstream and tidal collections were detected. Further examination with paired tests revealed that both Lagoon Creek (allozymes) and Koppen

Creek (mtDNA) tidal and upstream collections were significantly different (Table 2). Significant regional heterogeneity was detected within upstream (allozymes and mtDNA) and tidal (allozymes) collections. Pairwise log-likelihood tests between pooled tidal populations within regions were significantly different from the two regions containing the most collections (Southwest and East).

The hierarchical gene-diversity analysis was performed using 30 polymorphic allozyme loci (isoloci were excluded; Table 3). By far the majority of the variation (99.3%) occurred within collections. The remaining heterogeneity was divided among collections within regions (0.5%), among regions within elevation (0.2%), and between elevations (0.1%). A similar analysis of the distribution of molecular variance was made using AMOVA (Excoffier et al. 1992) and utilizing a matrix of Euclidean distances between haplotypes (Table 3). Pairwise Euclidean distances were calculated as the total number of site changes between haplotypes. The AMOVA analysis allows for only a two-level hierarchy, so we were unable to partition regions within elevations as in the preceding analyses; distribution of variation varied little between the two data sets.

An initial MDS was performed with the allozyme data from all collections. This analysis demonstrated the uniqueness of the upstream Lagoon Creek collection. To better visualize the relationships among the other collections, a second MDS was generated excluding the Lagoon Creek upstream collection (Figure 3). Some regional structuring is apparent from the plot. The Southwest collections tend to occupy the left and upper portions of the plot; the East collections occupy a lower area that extends to the extreme right of the plot. Some overlap between the Southwest and East regions occurs. The North collections tend to occupy space across both the Southwest and East regions. The hatchery collections both occur in the central positions of their respective regions, and Armin F. Koernig (AFK) Hatchery is located near the area of overlap between the Southwest and East collections.

The position of the upstream collections is particularly interesting. Upstream collections from Olsen and Koppen Creeks, both in the East region, occupy space within the area bounded by East collections. However, upstream collections from both Mink Creek and Constantine Creek are outliers at the extreme edge of the plot. Interestingly, the tidal collection from Mink Creek is also an outlier and shows affinity to the upstream Mink Creek collection rather than to other tidal collections from the North region. As mentioned earlier, Lagoon Creek upstream was not included in this plot because of its highly distant position.

We also examined the relationship between the hatchery and wild collections. Armin F.

Koernig Hatchery was not different from any of the regions for tidal collections but was significantly different from all upstream collections. Solomon Gulch Hatchery, located in the East region, was different from all regions but East. These hatchery results follow expectations based on the hatchery locations, original brood stock sources, and annual brood stock acquisition methods (e.g., see below). Although both hatcheries cluster into their respective regions in the MDS plot, AFK Hatchery clusters near the area overlapped by the East region collections (Figure 3). Armin F. Koernig Hatchery also clustered closely with Duck River, an eastern PWS site from which gametes were collected to establish the even-year hatchery population in 1976.

Implications for harvest management

Understanding genetic structure of Pacific salmon populations is critical to their management and conservation. Managing on too fine a scale may adversely affect the fishing industry and waste management resources, while managing on too large a scale may result in loss of genetic adaptations and diversity.

Inferences from studies showing genetic homogeneity for allozymes over vast geographic distances (e.g., Shaklee and Varnavskaya 1994) led some to suggest that pink salmon populations within PWS, spanning only 100 kilometers, should be genetically homogenous. In contrast, implications from other allozyme studies (Lane et al. 1990) suggest that pink salmon populations in PWS might be substantially heterogenous. Our objective was to generate molecular genetic data to support or reject these alternatives.

Our analysis of the 1994 collections showed significant substructuring of pink salmon in PWS based upon both allozyme and mtDNA data sets. The heterogeneity analysis, a very conservative analysis because all alleles observed are assumed to exist in all collections thereby inflating the degrees of freedom, showed significant allele frequency differences occurring between stream elevations and among and within regions. Further pairwise analyses indicate that, for tidal spawning aggregates, the East region is distinct from the Southwest region. For upstream spawners, pairwise comparisons show genetic differences occurring among all regions where upstream spawners were sampled.

These data provided insight not only into the structure of the wild fish within PWS, but also into the genetic relationships between hatchery fish and these wild fish. Armin F. Koernig Hatchery could not be distinguished from any of the regions when tidal fish within region were pooled. The even-year lineage for AFK Hatchery was founded originally with more than one stock that included gametes from Duck River, a site across PWS in the East region. Annual

propagation at the hatchery comes from brood stock seined from fish milling in front of the hatchery, and evidence from coded-wire-tag recoveries suggests these milling fish include some wild fish headed for other areas (Sharr et al. 1995). Armin F. Koernig Hatchery is located adjacent to the strait through which most pink salmon enter PWS on their spawning immigration (Templin et al. 1996); consequently hatchery brood stock may include stocks from throughout PWS. Therefore, inability to distinguish the AFK Hatchery brood stock mixture from stocks from other regions is not surprising. Conversely, Solomon Gulch Hatchery is located at the end on the Valdez Arm in eastern PWS. Few pink salmon bound for other regions of PWS are likely to be milling near this hatchery when the brood stock are seined. In addition, the original brood stock for this hatchery was locally obtained. As might be expected, the Solomon Gulch Hatchery collection was not different from other East collections but was different from collections from every other region.

Our preliminary analyses clearly show that significant genetic heterogeneity exists among pink salmon spawning aggregates in both nuclear and mtDNA markers, indicating that pink salmon in PWS do not form a single panmictic unit. We recognize, however, that the data show the even-year lineage to have a shallow genetic structure (in contrast to the structure of sockeye salmon populations from a similar geographic range in Cook Inlet, Alaska; see below), and population differences are not great enough to identify the components of population mixtures during harvest. Yet population structure and barriers to gene flow exist for these fish, and these data confirm that harvest- and hatchery-management decisions made for conservation purposes should best be made on a population-specific rather than species-specific basis. Expansion of this study to include additional odd-year and even-year collections is continuing; the analysis of data from multiple year classes will allow us to better test the appropriateness of current management regions and to test for temporal structuring within year classes.

Microsatellite Variation in Pink Salmon

We conducted a pilot study to test the ability of variation detected at microsatellite loci to discriminate structure of pink salmon populations by looking at three populations in PWS. We selected five prospective loci from those developed in other species based upon criteria reported from an earlier screening of 35 microsatellite primer pairs in five species of Pacific salmon (Olsen et al. 1996): Oneì 3 (developed in sockeye salmon; Scribner et al. 1996), Ots1 (chinook salmon *O. tshawytscha*; Hedgecock et al. *in press*), ì Sat60 (brown trout *Salmo trutta*; Estoup et al. 1993), and Ssa 85 and Ssa197 (Atlantic salmon *Salmo salar*; McConnell et al. 1995, O'Reilly et

al. 1996). For this pilot study, we compared one even-year population collected in 1994 (Koppen Creek late/tidal) and two odd-year populations collected in 1995 (AFK Hatchery, and Koppen Creek late/tidal).

All loci were polymorphic in all populations. Tests to confirm Mendelian inheritance were performed on all loci using full-sib pink salmon families. The results of these tests confirmed Mendelian segregation at all loci and also revealed the presence of a "null" (non-amplifying) allele(s) at locus Ssa197, thus explaining a significant heterozygote deficiency (see below). Rather than attempt a redesign of primers, we chose to exclude Ssa197 from subsequent statistical analysis of these three populations.

The range of detectable variation was considerable (Table 4). Numbers of alleles per locus ranged from 2 (Oneì 3) to 47 (Ssa85), and the allele distribution varied from 12 nucleotides (nt) (Oneì 3) to 116nt (Ssa85). Frequency of the most common allele varied from 9% (Ssa85) to 91% (ì Sat60). Observed heterozygosity ranged from 0.18 to 0.93 for microsatellites Oneì 3, Ots1 and ì Sat60. The mean heterozygosity for all loci (excluding Ssa197) was 0.65 for AFK Hatchery, 0.70 for Koppen Creek (1995) and 0.58 for Koppen Creek (1994). A test of Hardy-Weinberg equilibrium (HWE) revealed no significant differences between observed versus expected heterozygosities within each population at Oneì 3, Ots1 and ì Sat60 ($P>0.0125$). However, observed heterozygosity did differ significantly from expected for microsatellite Ssa197 ($P<0.0125$).

We found significant differences in allele frequencies ($p<0.0011$, $SE<0.0001$) for all populations at microsatellite ì Sat60 and Ssa85 using GENEPOP computer program (Raymond and Rousset 1995) to estimate the probability of independence from an exact Fisher test on multiple RxC contingency tables. Further, a pairwise comparison of all populations showed significant differences (following sequential Bonferroni adjustment using an initial Æ of 0.0125; Rice 1989) in allele frequencies at ì Sat60 and Ssa85 when each odd-year population was compared with the Koppen Creek even-year population. An estimate of the F_{ST} analog (Ł) (Weir and Cockerham 1984) was made for each locus and revealed slight genetic differentiation; values ranged from -0.004 to 0.0350. For all loci combined Ł was estimated at 0.006 with a 95% bootstrap confidence interval of -0.006 to 0.030. This value is of a similar magnitude to those observed for allozymes and mtDNA for the larger group of populations (Table 3).

DNA Sequencing and Site-Specific Mutations

We initially chose to screen tumor suppressor gene *p53* and the mtDNA gene cytochrome *b*

as potential sentinel loci for detecting differences in mutation rates between embryos incubated in oiled and unoiled gravel in the laboratory. It was our hope that we might develop methods to detect site-specific mutations that would provide insight into both genetic damage as a response to genotoxic challenge and into the population structure of damaged populations (Bue et al. 1996).

Tumor suppressor gene *p53* was selected because research establishing a molecular link between pollution and the onset of tumors has been active; much is known about mutations in *p53* and PCR primers are available to amplify it. Mutations within certain 'hot-spot' regions of *p53* are associated with about 50% of all human cancers (Harris 1993). Most mutations are found clustered in exons 5-8 of this gene, allowing efficient screening for DNA sequence alterations. Intense work sequencing this gene from many tumors has now identified more than 5000 cases of nucleotide substitutions (Hollstein et al. 1996). Finally, naturally occurring variation in *p53* exons 7-10 is observed in chinook salmon *O. tshawytscha* populations (Park et al. 1996), making the locus attractive for population screening in pink salmon.

We chose to screen cytochrome *b* for site-specific mutations for two reasons. Cytochrome *b* has been shown to be a diagnostic, mutationally active gene for studies of molecular evolution of salmonids (Shedlock et al. 1992; Palsson and Arnason 1994; Patarnello et al. 1994; Bernatchez and Osinov 1995). Further, Baker et al. (1996) found that the base-pair substitution rates for the cytochrome *b* gene in voles living near the Chernobyl nuclear reactor were hundreds of times greater than is typically found for vertebrates, evidence for a lack of stringent DNA-repair.

A 1066nt region composing exons 7-10 of *p53* was PCR amplified and sequenced (Seeb et al. 1996a). Primers used were:

p53-7F1 5’ CAG GTG GGA TCA GAG TGT ACC 3’ (Park et al. 1996) and

p53-10R1 5’ AGC GTC GGC AAC AGG CAC CAA CTC 3’.

The second primer was developed by selecting a conserved region found through comparison of exon 10 sequence of chinook salmon (Park et al. 1996) with that of rainbow trout (de Fromentel et al. 1992). We developed additional primers to subdivide the 1066nt template by selecting optimal 20mers about 400nt towards the center of the template from the two ends:

p53-8R1 5' CCG ACC CAG GCG CTG CCC 3',

p53-9R1 5' GAG GGG CAG GCA GGG AGG CC 3', and

p53-9F1 5' GGC CTC CCT GCC TGC CCC TC 3'.

We found no evidence of mutations in response to oil and little variation in our initial screen

of the *p53* gene in several dozen individuals originating from two Alaskan populations, including embryos from one population maintained in oiled substrate (Seeb et al. 1996a). However, both populations were polymorphic at the base pair corresponding to nt 1,054 of *p53* in rainbow trout copy DNA (cDNA; de Fromentel et al. 1992), and two heterozygote x homozygote matings demonstrated codominant segregation (data not shown).

A 795nt segment of cytochrome *b* region of mtDNA was PCR amplified and sequenced (Seeb et al. 1996a) using primers:

LGL-765 5’ GAA AAA CCA YCG TTG TWA TTC AAC T 3' (Cronin et al. 1993),

H15498 5’ GGA ATA AGT TAT CTG GGT CTC 3’ (Kocher et al. 1989). Unexpectedly little variation was found in the DNA sequence in our preliminary screen of 20 pink salmon from PWS. Nucleotide 486 in codon 162 was an "A" in all individuals except one female that was a "G".

Both of these polymorphisms in *p53* and cytochrome *b* are candidates for allele-specific PCR assays. At this writing we are optimizing PCR conditions for 24nt primers for cytochrome *b* that contain the polymorphic base at the three prime end (5'-TATGTGGGCGGCGCCCTAGTACA**G**-3', or 5'-23mer-**A**-3'). One can determine the genotype of an individual by using these primers jointly in paired assay by doing the PCR amplification only; no sequence analysis would be required. Similar primers will be developed for the *p53* polymorphism at nt 1,054.

SOCKEYE SALMON STUDIES

Commercial fisheries on sockeye salmon in Cook Inlet have occurred since the late 1800s and represent a significant economic resource to southcentral Alaska. Harvest levels have ranged from 95,000 to 9.5 million fish (Rigby et al. 1991; Ruesch and Fox 1994), and in the last 10 years the total value of the fishery reached $111.1 million (Ruesch and Fox 1994). In July of 1989, however, fishing time in the Cook Inlet area was greatly reduced due to the presence of oil from the *Exxon Valdez* spill.

As a direct result of the reduced exploitation, sockeye salmon spawning in the Kenai River system exceeded optimal escapement goals by three times. Extremely high escapements can produce enough fry to deplete zooplankton prey populations, causing high fry mortality, and can alter the species composition and productivity of prey populations for several years (Schmidt et al. 1995). In response to a potential decline in the fishery, efforts began in 1992 to refine population

identification and management techniques and to increase knowledge of the diversity and abundance of sockeye salmon in Cook Inlet.

Most of the sockeye salmon production in Upper Cook Inlet (UCI) comes from four major river systems. The largest sockeye salmon producer (2.8 million fish annually) is the Kenai River which drains the Kenai Peninsula on the east side of UCI (Figure 2B). Next are the Kasilof and Susitna River systems which each produce approximately 700,000 sockeye salmon annually followed by the Crescent River drainage (200,000 fish). The Kenai, Kasilof and Crescent River systems are characterized by large, central glacial lakes fed by numerous smaller tributaries. The Susitna River system has many smaller lakes, each of which empties into the mainstem through smaller, separate streams. The remainder of the sockeye salmon production in UCI is composed of many minor populations (Ruesch and Fox 1994).

Cook Inlet sockeye salmon have been the focus of a number of population identification studies. Extensive efforts were made to delineate populations through scale pattern analyses (Marshall et al. 1987) and parasites (Waltemeyer et al. 1993). Neither technique proved adequate. Significant temporal and sexual variability within populations exists with scale pattern analyses (Waltemeyer et al. 1996), and it is difficult to obtain population-specific scales on an in-season basis. Similarly, temporal instability eroded the usefulness of parasite data. Grant et al. (1980) found considerable heterogeneity among populations inhabiting the region using allozymes. In evaluations of their resulting mixed-population model, Grant et al. (1980) demonstrated a high degree of success using three loci to classify populations from the Kasilof River and Susitna River drainages, but incomplete baseline data was thought to confound the Kenai River classifications. Additional data from the Russian River, one of the Kenai River drainages, was presented by Wilmot and Burger (1985) who found significant differences between early and late runs from the Russian River. However, no comprehensive genetic survey of Cook Inlet has been undertaken since the 1970s. In this paper, we review the genetic data that were collected to aid in the population identification and restoration of Kenai River sockeye salmon.

Allozyme and Mitochondrial DNA Analyses

Tissue samples from 100 spawning individuals were collected from all major sockeye salmon-producing systems of UCI. Approximately 7,000 individual sockeye salmon were sampled (Figure 2). Mixed-population collections originating from Cook Inlet fisheries (Central District; Figure 2) were collected in a similar manner to that of spawning samples. Samples of muscle, liver, retinal fluid, and heart were dissected from freshly killed individuals. Individual

sample numbers were assigned to uniquely identify all genetic tissues. Allozyme techniques followed those of Aebersold et al. (1987); nomenclature rules followed the American Fisheries Society standard (Shaklee et al. 1990). A total of 68 allozyme loci were resolved (Seeb et al. 1995; Seeb et al. *in press*) .

A subset of 25 populations was surveyed for RFLP variation at mtDNA. Whole DNA was extracted from liver or heart tissue, purified, quantitated, and diluted (100ng/ì l) for PCR amplification. The primers of Cronin et al. (1993) and Park et al. (1993) were used to amplify the mitochondrial NADH dehydrogenase subunits 5 and 6 (ND5/ND6) using PCR. Amplified DNA was cut with six restriction enzymes (*Apa I*, *Hha I*, *Hinf I*, *Kpn I*, *Stu I*, and *Taq I*).

Populations were grouped *a priori* into seven regions: Kenai River, Kasilof River, Susitna River, Yentna River, Northeast Cook Inlet, Knik Arm, and West Cook Inlet. To further describe the subdivision of genetic diversity, a hierarchical gene diversity analysis (Nei 1973; Excoffier et al. 1992) was conducted. The allozyme data was organized to test for the distribution of variability among sites within nursery lakes, among nursery lakes within regions, and among regions. Because fewer samples were analyzed for mtDNA variation, the hierarchy tested for those data were limited to among sites within regions and among regions.

Population contributions to the mixture samples were estimated via maximum likelihood (MLE; Pella and Milner 1987) using a conjugate gradient searching algorithm with square root transformations (Pella et al. 1996) and the computer program SPAM (ADFG 1997); the precision (standard error) of the population composition was estimated by an infinitesimal jackknife procedure (Millar 1987). Individual population estimates were first calculated, then summed into regional groupings (allocate-sum procedure, Wood et al. 1987). Simulated mixtures were used to evaluate the accuracy of the population composition estimates reporting regions. These hypothetical mixtures (n = 400) were generated from the baseline allele frequencies assuming Hardy-Weinberg equilibrium, and the precision of the simulated mixtures was estimated by a parametric bootstrap (Efron and Tibshirani 1986). One hundred bootstrap iterations were performed, and a series of 100% simulations for the seven reporting regions (hypothetical mixtures composed entirely of populations from the individual region). In order to maintain confidence in the estimates, fishery managers desired reporting regions that showed at least 90% allocation to the region of origin.

Heterogeneity within and among regions

A high level of gene diversity revealed by allozymes was found within some regions. Two

lineages of related populations were evident based upon overall similarity: Mainstem Kenai River populations and a second cluster of populations composed of the Tustumena Lake populations from the Kasilof drainage. The Northeastern Cook Inlet and Knik Arm populations also demonstrate regional affinities, but little regional structuring is apparent in the remaining populations. The Russian River, within the Kenai River, was particularly divergent.

A total of 10 composite mtDNA haplotypes were revealed with the six restriction enzymes. Two composite haplotypes representing variation in the *Apa I* enzyme were encountered most frequently (i.e., in 78% of the samples) in Cook Inlet. The distributions of these two haplotypes appear to correlate with geography; haplotype I was present at a high frequency in the Kenai (56.2%) and Kasilof (30.0%) River samples, whereas haplotype II was present at high frequencies in the Kink Arm (55.4%), Susitna River (57.5%), Yentna River (55.4%), Western Cook Inlet (61.9%) samples. The Northeastern Cook Inlet samples were fixed for haplotype II.

A hierarchical gene diversity analysis stratified variation by site, nursery lake, and region. The greatest amount of variation (87.8%) occurred within sites. Relatively little variability was detected among sites within nursery lakes (0.4%; Table 3). However, considerable heterogeneity (7.8%) existed among nursery lakes within regions with the remaining 4.1% of the variability allocated to the among-regions component. An AMOVA analysis similar to the pink salmon analysis was also conducted with the mtDNA; however, only two hierarchical levels, site and region, were analyzed (Seeb et al. 1995).

Mixed-population analyses

Clearly there is far greater among-population diversity in sockeye salmon from Cook Inlet than in pink salmon from PWS (Table 3). This diversity allows for the use of MSA analyses in Cook Inlet, whereas MSA is probably not possible for routine applications for pink salmon in PWS. The performance of the MSA model for Cook Inlet sockeye salmon was investigated through simulations. Fishery managers set an *a priori* goal of 90% accuracy for analysis of simulated mixtures prior to accepting MSA for management purposes. The Kenai River region, the group of greatest concern, showed 91% classification in these simulations; Northeastern Cook Inlet, Kasilof River and Knik Arm also were above or close to the goal (99%, 92% and 88% respectively). Although the Yentna River at 88% was near the goal, the Susitna River misclassified to both the Yentna River and Western Cook Inlet, resulting in a correct classification of only 77%; when the Susitna and Yentna regions were combined, the allocation rose to 87%. Western Cook Inlet, a heterogenous grouping based on geographic proximity, performed at 86%.

Maximum likelihood estimates were calculated for all samples collected from the Central District drift gillnet fisheries. These estimates were then summed by region for use in management. In 1992, 1993, and 1994, few samples were taken, and estimated contributions shed little light on the interactions of regions within the fishery (Figure 4). In 1995 and 1996, five collections were taken from that portion of the season coinciding with the expected presence of Kenai River sockeye salmon (Figure 4). These collections showed an increase through July of Kenai River sockeye salmon in the drift gillnet fishery over the period examined.

Finer scale estimation was also possible for some populations within some river drainages. A 100% simulation was conducted on the Russian River populations above the falls. The simulation result was 99.4% (SD = 0.5%), indicating that the Russian River could be identified in mixtures of Cook Inlet populations with a high degree of accuracy and precision. The proportion of Russian River populations in admixtures within the Kenai River have been estimated during the last several years. The results suggested a pulse of early-run fish, a lull, and then a large pulse of late-run fish.

The mtDNA data was tested for its ability to improve allozyme-based MSA estimates within Cook Inlet. This testing procedure involved running two sets of simulations. The first set of simulations used allozyme data only from the same subset of populations that were analyzed for mtDNA variation. For all simulations, the region being tested composed 100% of the mixture (N = 400), and 100 bootstrap resamplings were conducted. In general, the mtDNA data somewhat improved the accuracy of the estimates. The standard deviations of the mean estimated allocations to the correct regions showed a similar decreasing trend with the addition of mtDNA data, indicating an improvement in precision. However, the small improvements do not warrant the additional costs and substantial additional time required to add mtDNA data into the in-season analysis at this time.

Management implications

The results of the maximum likelihood estimates indicated that Kenai River populations can be identified in mixtures of Cook Inlet sockeye salmon with a level of precision, accuracy, and timeliness useful for fisheries management. The genetics estimates were first incorporated into in-season fishery management in 1995; to date results have been reported for Kenai River/non-Kenai River components only. In future years it is likely that additional reporting groups will be added to the analysis.

Application of genetic data to population identification in fishery management has several advantages over other methods, including stability of allele frequencies over time, ability to process large amount of samples rapidly (allozyme data), and reasonable cost (allozyme data). The accuracy and precision of the estimates can likely be further improved as additional genetic markers become available and processing costs and times of DNA methods decline. These applications are currently underway in Cook Inlet to aid in management and conservation.

Microsatellite Variation in Sockeye Salmon

We also conducted a small-scale pilot study to measure microsatellite variation sockeye salmon by looking at four Cook Inlet populations. Fifteen microsatellite primer pairs were screened to assess quality of amplified PCR product, optimize PCR conditions, and assess their potential for measuring genetic population structure (see Olsen et al. 1996). Of the 15 primer pairs, 5 were chosen for the population screen including Oneì 1, Oneì 2, Oneì 11, and Oneì 14, developed from sockeye salmon (Scribner et al. 1996), and Ssa293, developed from Atlantic salmon (McConnell et al. 1995). The populations surveyed included the Russian River (late run) and Skilak Lake outlet (Kenai River drainage), Moose Creek (Kasilof River drainage), and the Yentna River (Susitna River drainage).

The range of variation was considerable. All loci were polymorphic, and all but Oneì 1 were polymorphic in all populations. Numbers of alleles per locus ranged from 2-10 (Table 5). Observed heterozygosity ranged from 0.00 to 0.90 for microsatellites Oneì 1, Oneì 2, Oneì 11 and Oneì 14. A test of Hardy-Weinberg equilibrium (HWE) revealed no significant differences between observed versus expected heterozygosities within each population at these four loci (P>0.08). However, observed heterozygosity was significantly below that expected for microsatellite Ssa293 (P< 0.001). In fact, some samples failed to exhibit any Ssa293 alleles. We believe the lack of heterozygotes and lack of expression observed at the Ssa293 locus is due to one or more null alleles as observed in the pink salmon data set above. Null alleles have been described previously for human microsatellites (Callen et al. 1993) and are more likely to occur when using primers developed in related species (Forbes et al. 1995). We chose to exclude Ssa293 from subsequent statistical analysis because verification of the null allele hypothesis requires redesigning the primers and conducting inheritance studies-- beyond the scope of this pilot study at this writing.

The mean heterozygosity for the four Oneì loci was approximately 0.50 for Moose Creek, Skilak Lake, and Yentna River. The Russian River exhibited the lowest degree of variability with

a mean heterozygosity of 0.32. Allele frequencies differed among all populations and ranged from 1 to 100% (Figure 5). No clear pattern was evident in the shapes of the allele distributions across populations. For each locus the same allele was most frequent in all populations: Oneì 1(114), Oneì 2(270), Oneì 11(150), Oneì 14(147). At three of the four loci at least one population-unique allele was present. Notably, the 129 and 137 alleles at Oneì 14 had frequencies of about 11% and 8%, respectively, in Moose Creek and Russian River. Additional screening is needed to determine if these alleles are present in other populations.

Significant differences in allele frequencies ($p<0.0001$, $SE<0.0001$) were shown for all populations using the Markov chain algorithm in GENEPOP. Further, a pairwise comparison of all populations using the same algorithm showed significant differences (following sequential Bonferroni adjustment using an initial Æ of 0.0125) in allele frequencies at most loci. The exceptions were: Moose Creek and Skilak Lake at Oneì 1 and Oneì 2, Moose Creek and Russian River at Oneì 11, Russian River and Skilak Lake at Oneì 11, and Skilak Lake and Yentna River at Oneì 14. Finally, an estimate of the F_{ST} analog (Ł) (Weir and Cockerham 1984) was made for each locus and revealed moderate genetic differentiation. Values ranged from 0.042-0.100. For all loci combined Ł was estimated at 0.071, the 95% bootstrap confidence interval being 0.049-0.092. The Ł value for the same group of populations and individuals for 13 allozyme loci was 0.198, greater than that detected through microsatellites.

CONCLUSIONS

Our goals were to use proven genetic techniques to define population structure and thus determine the useful scope of management based upon genetic data; where practicable, collect genetic data on fishery mixtures, in-season, to identify populations intercepted; and explore the utility of new DNA markers for defining population structure for population management.

An array of conservation and restoration alternatives are often proposed for "species" suffering impacts from habitat loss, over fishing, or impacts such as the *Exxon Valdez* oil spill. However, species-based proposals often do not provide the resolution needed to sustain conservation of genetically diverse aggregates of salmon populations; it is essential to manage and restore depleted salmon resources on a population basis in order to conserve between-population diversity and long-term viability.

In this paper we apply allozyme analyses and RFLP analyses of mtDNA to identify Alaskan populations; we further review our development of polymorphism screens using microsatellite

analysis, sequencing of the nuclear gene *p53*, and sequencing the cytochrome *b* region of mtDNA. At this time the allozyme and RFLP analyses appear to provide the most useful results in terms of relative cost, throughput, and discriminating power. The relative ability of these two techniques to delineate populations appears to be a matter of scale. Allozymes often discriminate populations on a large scale where mtDNA data may lack resolving power due to probable convergence of haplotype frequencies (cf., Adams et al. 1994; Fetzner et al. *in prep*.). Mitochondrial DNA may sometimes provide improved resolving power on finer-scale study of population structure. These two approaches provided complementary resolving power on fine-scale study of pink and sockeye salmon structure reported here. Of all of these techniques, in our hands allozymes is the only one that currently provides the resolution and speed necessary to provide in-season fishery estimates.

Yet allozymes do not always provide the resolution necessary to define the relationships of intra-specific populations. Because of this, additional techniques of DNA analysis are being developed (e.g., Figure 1), and we conducted pilot studies of microsatellite and DNA sequence variation.

Of the new techniques, microsatellite analysis has probably received the most recent emphasis (Figure 1B). We found population subdivision using microsatellite analysis, but the between-population diversity detected was not remarkably different from that observed using other techniques. The apparent common occurrence of null alleles may confound the interpretation of variation at some loci. The observed heterozygosity was remarkably high, however, and this in itself may provide advantages to microsatellites in some cases. Our observation is that the slow relative throughput and high relative expense of microsatellite analysis will limit its utility to the study of populations that otherwise cannot be resolved using allozymes or in which lethal sampling is not possible, and we would not expect this approach to have utility for in-season estimates in the near future.

We also report DNA sequence polymorphism detected in our study of potential genetic damage in pink salmon. We are interested in these data for population genetic study in as much as others suggest that allele-specific PCR could provide a rapid-throughput approach for the collection of genetic data. One of the primary reasons that DNA data is not used for stock assessment of harvest mixtures in real-time management is that a comparatively long time is required for data collection. Electrophoretic data from RFLP analyses, let alone DNA sequencing, does not provide data rapidly enough for use in harvest-management decision making. Allele-specific PCR of single nucleotide substations detected by other sequencing or

RFLP assays should provide more rapid results because genotypes are determined directly from the PCR reaction without the need for additional electrophoretic separation of the DNA products.

While the potential for stock assessment using some of the newly developed DNA techniques is great, substantial development and testing remains to be done with many species. Fisheries managers sometimes accuse geneticists of prematurely promoting newly developed but unproven approaches to stock assessment (e.g., see Lincoln 1994). We recommend continued implementation of successful approaches such as allozyme electrophoresis for harvest management; however, we recognize the value of pursuing research into additional genetic technologies that may improve existing assessment capabilities.

ACKNOWLEDGEMENTS

These studies were supported by grants 96255 and 96196 from the *Exxon Valdez* Trustee Council and by the State of Alaska Department of Fish and Game. This paper is contribution number PP97-5002 from the Commercial Fisheries Management and Development Division.

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Table 1. Relative attributes of four molecular techniques for study of population genetics of Pacific salmon.

Technique	Cost	No. Loci	Between-lab Standardization	Throughput	Existing Baseline	Lethal Sampling	Cryo-preservation	Development Needed
Allozymes	low	many	easy	rapid	extensive	usually	yes	very little
mtDNA	med.	one	easy	slow	some	no	no	little
Microsatellite	high	many	easy	slow	little	no	no	new primers
Minisatellite	high	many	hard	slow	little	no	no	low priority

Table 2. Heterogeneity between paired tidal and upstream collections for allozyme and haplotype frequencies. Log-likelihood tests were performed to test homogeneity of allozyme frequencies. Homogeneity of mtDNA was tested using 10,000 Monte Carlo simulations (Roff and Bentzen 1989); probabilities of exceeding the original c^2 by chance alone are given.

	Allozyme			mtDNA	
Stream	Log-likelihood	df	P	$\div^2$	P
Olsen Creek	51.80	47	0.292	2.20	0.905
Mink Creek	58.73	47	0.117	3.94	0.599
Lagoon Creek	115.73	43	0.000*	6.90	0.022
Koppen Creek	56.97	46	0.129	13.56	0.002*
Constantine Creek	63.07	1	0.120	1.17	0.738

*Significant at experimentwise $a = 0.05$ (Rice 1989)

Table 3. Gene diversity analyses. Estimated gene diversity for allozymes were calculated following Nei (1973); estimates for mtDNA were calculated using AMOVA (Excoffier et al. 1992).

Technique	Source	Relative Gene Diversity
PINK SALMON		
Allozyme		
	Within sites	0.993
	Among sites within regions	0.005
	Among regions within elevations	0.002
	Among elevations	0.001
mtDNA	Within sites	0.984
	Among sites within elevations	0.011
	Among elevations	0.006
SOCKEYE SALMON		
Allozyme		
	Within sites	0.878
	Among sites within nurseries	0.004
	Among nurseries within regions	0.078
	Among regions	0.041
mtDNA	Within sites	0.669
	Among sites within regions	0.186
	Among regions	0.148

Table 4. Allelic variability at four microsatellite loci in two odd- and one even-year pink salmon populations from Prince William Sound, Alaska. Samples were from Armin F. Koernig Hatchery (AFK) and from Koppen Creek late intertidal spawners. Sample size (N), number of alleles (A), allele range (R) in nucleotide bases, maximum allele frequency and size of the most frequent allele (M), and observed heterozygosity (H_o) are reported for each population and locus.

Population		Oneì 3	Ots1	ì Sat60	Ssa85	Ssa197[a]	Avg.
AFK	N	52	52	52	52	47	
Hatchery	A	3	17	4	30	17	14
(1995)	R	156 - 168	216 - 268	109 - 121	157-247	128 - 196	
	M	0.61(162)	0.28(232)	0.73(109)	0.11(201)	0.12(160)	
	H_o	0.48	0.85	0.35	0.90	0.55*	0.65
Koppen Creek	N	44	44	44	44	40	
(1995)	A	2	17	5	29	15	13
	R	162 - 168	220 - 260	107 - 117	153-253	132 - 192	
	M	0.61(162)	0.24(232)	0.67(109)	0.09(197)	0.18(144)	
	H_o	0.50	0.93	0.48	0.89	0.60*	0.70
Koppen Creek	N	40	40	40	40	40	
(1994)	A	2	13	2	35	17	13
	R	162 - 168	220 - 248	109 - 113	137-245	124 - 204	
	M	0.51(168)	0.24(228)	0.91(109)	0.09(191)	0.10(144)	
	H_o	0.38	0.90	0.18	0.85	0.85	0.58
All pops.	N	136	136	136	136	127	
	A	3	21	6	47	20	19
	R	156 - 168	216 - 268	107 - 121	137-253	124-204	
	M	0.57(162)	0.25(232)	0.76(109)	0.09(197)	0.12(144)	
	H_o	0.45	0.89	0.34	0.88	0.67	0.64

[a] Ssa197 exhibited 3 alleles of equal frequency in the 1994 sample from Koppen Creek.

Table 5. Sample size (N), number of alleles (A), and observed heterozygosity (H_O) at five microsatellite loci in Cook Inlet sockeye salmon.

Population		Oneì 1	Oneì 2	Oneì 11	Oneì 14	Ssa293	Avg.
Moose Creek	N	50	50	50	50	43	
	A	2	10	3	8	4	5.8
	H_O	0.20	0.76	0.46	0.64	0.28*	0.52
Russian River	N	50	50	50	50	41	
	A	1	6	3	5	5	3.8
	H_O	0.00	0.38	0.36	0.52	0.10*	0.32
Skilak Lake	N	50	48	50	50	40	
	A	3	9	4	8	9	6.0
	H_O	0.24	0.90	0.42	0.54	0.25*	0.52
Yentna River	N	50	50	50	50	41	
	A	3	7	3	5	8	4.5
	H_O	0.38	0.68	0.58	0.38	0.34*	0.51
All pops	N	200	198	200	200	165	
	A	3	13	4	10	10	8.0
	H_O	0.21	0.68	0.46	0.52	0.24*	0.42

[a] Significant differences between observed and expected heterozygostiy were determined using a Bonferroni adjustment (initial Æ = 0.0125) and are indexed with an asterisk (*).