Exxon Valdez Oil Spill
Restoration Project Annual Report

Construction of a Linkage Map for the Pink Salmon Genome

Restoration Project 00190
Annual Report

This annual report has been prepared for peer review as part of the *Exxon Valdez* Oil Spill Trustee Council restoration program for the purpose of assessing project progress. Peer review comments have not been addressed in this annual report.

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Study History: This project was initiated under Restoration Project 96190 in March 1996 and was continued under Restoration Projects 97190, 98190, 99190, and 00190. Annual reports entitled Construction of a Linkage Map for the Pink Salmon Genome were submitted in 1997, 1998, 1999 and 2000. In this annual report we describe Restoration Project 00190. Twelve oral presentations reporting the results of this project have been given at professional meetings or university seminar series. Dr. Fred Allendorf gave an overview of the project at the national meeting of the American Fisheries Society in Dearborn, Michigan, August 1996, at the Arctic Division of the American Association for the Advancement of Science annual meeting in Valdez, AK, September 1997 and to the general public at the ASLC, November 1998. In addition, Dr. Allendorf presented an overview of this study at the Swedish Agricultural University, Umeå, Sweden in January 1998 and Aarhus University, Aarhus, Denmark, in February 1998. Kate Lindner presented an update at the semi-annual Coast-wide Salmonid Genetics meeting in Seattle, March 1997 as well as at the Alaska Department of Fish and Game in Anchorage Alaska, September 1997. Ms. Lindner also presented at the SALMAP meeting in Toronto Canada, September 1998. In addition, Ms. Lindner presented an overview of the project to several Elder Hostel groups at the ASLC in August 2000. Dr. Paul Spruell represented our lab at United States Department of Agriculture (USDA) panels on aquaculture in 1997, 1998, and 1999. Dr. Spruell and Ms. Lindner each presented mapping results at the Plant and Animal Genome Mapping Meeting in San Diego, January 1999. Ms. Lindner was invited to present our findings to the USDA panel on mapping in aquaculture species that is held in conjunction with the mapping meeting each year. Dr. Spruell was invited to participate in a workshop sponsored by Hitachi Software and used our pink salmon linkage data to illustrate the advantages of fluorescent technologies to produce linkage maps. In March 1999, Kathy Knudsen presented an overview of the project to the Kenai High School freshman biology class. In May 1999, Eleanor Steinberg was awarded a National Science Foundation postdoctoral fellowship in Biological Informatics to work in the Allendorf laboratory on the pink salmon project. Two journal articles are published; Spruell, P., K. L. Pilgrim, B. A. Greene, C. Habicht, K. L. Knudsen, K. R. Lindner, J. B. Olsen, G. K. Sage, J. E. Seeb, and F. W. Allendorf. 1999. Inheritance of nuclear DNA markers in gynogenetic haploid pink salmon. Journal of Heredity (90:289-296), and Lindner, K. R., J. E. Seeb, C. Habicht, E. Kretschmer, D. J. Reedy, P. Spruell, and F. W. Allendorf. 2000. Gene-centromere mapping of 312 loci in pink salmon by half-tetrad analysis. Genome (43:538-549). In addition a manuscript (Steinberg, E. K., K. R. Lindner, J. Gallea, J. Meng, A. E. Maxwell, and F. W. Allendorf; Rates and patterns of microsatellite mutations in pink salmon) has been submitted to Molecular Biology and Evolution. Another manuscript (Lindner, K. R., P. Spruell, C. Habicht, K. L. Knudsen, J. E. Seeb, H. Zhao, and F. W. Allendorf; Estimation of chiasma interference and construction of a linkage map for pink salmon) is currently being prepared for submission to Genetics.

Abstract: We are characterizing genetic markers and constructing a genomic linkage map for pink salmon (Oncorhynchus gorbuscha) for use in recovery efforts for pink salmon in Prince William Sound. In August 1998, we collected gametes and tissue from 150 pink salmon from Likes Creek and performed single-pair matings to produce 75 families. In May 1999, approximately 48,000 individuals from 49 of these families were marked and released into Resurrection Bay from the Alaska SeaLife Center (ASLC). In August 1999, we collected 68 adult pink salmon from Likes Creek and produced 68 half-sibling families. These families were raised at ASLC and approximately 24,000 fry were marked and released into Resurrection Bay in May 2000. Due to the failure of the fishpass at the ASLC only 36 returning adults from the 1998 experimental cohort were collected in August 2000. We are currently developing a plan to increase the number of returning marked adults collected in August and September 2001. We plan to compare genotypes in the released fry and returning adults from the 1999 experimental cohort to test for regions of the genome associated with marine survival and other life history traits (e.g. body size, egg number, and egg size).

Project Data: Two primary sets of data are being developed; one for the linkage map and one for the marine survival and fitness experiment. Data for the linkage map are the inheritance of DNA fragments in the haploid and gynogenetic diploid progeny of two pink salmon females (A95-103 and V96-13). Sixteen additional diploid families have been tested for nonrandom segregation between all pair-wise combinations of 14 allozyme and three microsatellite loci. The haploid data set consists of 590 polymorphic DNA fragments in female A95-103 and 94 of her haploid progeny, and 85 polymorphic DNA fragments in female V96-13 and 90 of her haploid progeny. The diploid data set consists of genotypes of 70 gynogenetic diploid progeny from female A95-103 at 312 loci and of genotypes of 54 gynogenetic diploid progeny from female V96-13 at 19 loci. Data for the marine survival and fitness experiment are genotypes at ten PCR-based loci for 50 families (50 parent pairs and 10 progeny from each) produced for the experimental release in 1998. The parents for the experimental cohort produced in 1998 have been genotyped at 12 additional PCR-based loci and 34 allozyme loci. The 1999 parents have been genotyped at 11 PCR-based loci and 30 allozyme loci. An additional 36-40 embryos from seven of the 1998 families were analyzed at nine microsatellite loci to investigate mutation rates and patterns. Four meristic characters, body length, egg size, and egg number have been recorded for both cohorts of adults used in experimental crosses as well as the 36 marked adults collected in August 2000. All data sets are currently recorded in Microsoft Excel spread-sheets. Data will be made available to individuals within the reasonable bounds of sharing unpublished data. For information regarding data contact Kate Lindner, Division of Biological Sciences, University of Montana, Missoula, MT 59812. Phone: (406) 243-5503. E-mail: klindner@selway.umt.edu.

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EXECUTIVE SUMMARY

The project to construct a genetic linkage map for the pink salmon (*Oncorhynchus gorbuscha*) genome and to use this map to study the evolutionary biology of this species is in its sixth year. The linkage map will allow the characterization of the genetic impacts of the March 1989 *Exxon Valdez* oil spill on pink salmon populations and will help to document the recovery of effected populations in Prince William Sound. A linkage map will be essential for detecting and understanding causes of reduced egg and embryo survival in oiled areas. In addition, the markers that are mapped and characterized in detail will aid other recovery efforts with pink salmon, including estimation of straying rates, description of stock structure, and testing for a genetic basis of marine survival.

Elevated embryo mortalities were detected in populations of pink salmon inhabiting oiled streams following the spill. These increased rates of mortality persisted through the 1993 field season, three generations after the spill. This suggests that genetic damage may have occurred as a result of exposure to oil during early developmental life-stages.

The genetic linkage map will provide the platform to address the genetic impact of the oil spill. The initial framework of the map used haploid progeny to avoid the difficulties associated with dominant markers that obscure recessive alternatives in diploids. Gynogenetic diploids from the same family were also examined to locate centromeres of chromosomes and facilitate the consolidation of the map.

Gametes and tissues of pink salmon were collected from the Armin F. Koernig hatchery in August of 1995 as well as the Solomon Gulch hatchery in August 1996. Families of gynogenetic haploid and diploid embryos were produced in cooperation with the Alaska Department of Fish and Game by mixing irradiated sperm with eggs from individual females. One family (A95-103) was chosen to be the primary reference family upon which initial mapping efforts were focused.

Linkage analysis of 590 markers segregating in the gynogenetic haploids produced a genetic map comprising 44 linkage groups covering a distance of 4559 centiMorgans (cM). Assuming a minimum distance of 30 cM for linkage detection and accounting for all the gaps and unlinked markers the minimum distance of the pink salmon genome is 6691 cM. The haploid pink salmon genome consists of approximately 2.72×10^6 kilobase pairs (kbp), thus we estimate approximately 391 kbp/cM.

Thirteen allozyme loci have been added to the map using gynogenetic diploid and normal diploid data. Five allozyme loci are polymorphic in female A95-103 and thus could be tested for nonrandom segregation using the gynogenetic diploid data. The other eight loci were placed on the map through classic linkage analysis of diploid pink salmon families. With the addition of these markers the linkage map consists of a total of 603 markers.

We are also adding microsatellites and genes of known function to the linkage map. These markers will serve as landmarks, or "anchor loci" that facilitate comparisons between maps. These loci will allow us to compare genetic linkage of odd and even-year pink salmon, estimate recombination rates of males and females, and incorporate data from other salmonid linkage maps. The known genes will be of particular interest during the second phase of this project in which we examine the effects of the marine environment on the pink salmon genome.

Work continues on the construction of a complementary even-year map. Development of this map, based on the segregation of loci in family V96-13, follows the same design as the odd-year map. This map will enable us to compare odd- and even-year pink salmon well as add new markers to the odd-year map. Currently, this even-year map consists of 85 loci, 62 of which have been assigned to one of 22 linkage groups. One gene of known function (*MHCBa2*) is included on this map.

We have generated a large number of markers distributed throughout the genome using haploid embryos and multilocus techniques. Due to their polyploid ancestry, salmonid genomes are large, therefore many markers will likely be required to span the entire genome of pink salmon. We are attempting to "consolidate" the pink salmon map by reducing the number of linkage groups to 26, the number of chromosome pairs in pink salmon (2N = 52). We have collected gene-centromere distances of 312 loci using gynogenetic diploids. Ultimately, these data will allow us to identify the centromere of each chromosome and assign the linkage groups identified using the haploids to a specific chromosome. The even-year map will also help to consolidate the odd-year map by increasing the chance of detecting linkage between groups currently unlinked on the odd-year map.

We are now focusing on the marine survival and fitness portion of the study. Two experimental cohorts have been produced. In August 1998, gametes and tissue from 150 pink salmon from Likes Creek were collected and 75 single-cross families were produced. These families were raised at the Alaska SeaLife Center in Seward. Ten embryos from each family were analyzed to evaluate inheritance of genetic markers. A total of 48,329 individuals from 49 of these families were marked and released into Resurrection Bay in May, 1999. At the time of release, 1000 fry from the experimental families were randomly sampled for genetic analysis. In August 1999, gametes and tissue from 68 pink salmon from Likes Creek were collected and 68 half-sib families were produced. A total of 24,216 fry from all 68 families were marked and released into Resurrection Bay in April 2000. A sample of 500 fry was collected at the time of release. We will measure body size and egg size, count egg number, and collect gametes and tissue from returning marked fish to examine the association of genetic markers with fitness traits such as marine survival and fecundity.

In August 2000 we planned to collect experimental adult pink salmon as they returned to the fish pass at the ASLC. Due to a complete failure of the fish pass to attract any fish we were forced to modify these plans. A total of 36 marked pink salmon were collected by

seining freshwater streams in upper Resurrection Bay and from recreational fisherman responding to an incentive. Based on the collection effort and the number of fish collected it is our belief that a significant number of marked pink salmon returned to Resurrection Bay, but due to our limited resources we were unable to collect more returning adults.

We have genotyped all 36 returning marked pink salmon at nine microsatellite loci and two genes of known function (*MHCα1* and *GH2*). Based on the initial genetic analysis using a subset of four loci (*OGO1c, SSA408, SSA20.19-1* and *SSA20.19-2*) we were able to assign each fish back to the family from which it originated.

Several mutations at two microsatellite loci (*OGO1c* and *SSA408*) were detected during the analysis of the transmission genetics of markers used to evaluate parentage. Expanding on this analysis revealed a great deal of heterogeneity in microsatellite mutation rates and patterns. Mutation rate estimates for the nine loci ranged from 0.0 to 8.5×10^{-3} mutations per generation. The detection of large clusters of identical mutations at one locus, *SSA408*, indicated that the majority of mutant alleles identified reflected mutational events that occurred early in the differentiation of the germline. Evidence for a hypermutable allele at *SSA408* was also detected. These findings indicate that microsatellite mutation dynamics are complex and likely vary substantially among loci.

We are on schedule with the mapping portion of this project. More anchor loci will be included on the map. This will both aid in consolidation and provide more markers to detect regions of the genome associated with fitness traits. Even-year mapping has succeeded in linking one gene of known function (*MHCBα2*) to a microsatellite locus in one of the 22 linkage groups. We are also on schedule with the marine survival and fitness experiment. We have released both cohorts of experimental progeny and have collected returning adults from the 1998 cohort. We have determined the genotypes of the parents used to generate both cohorts and from embryos sampled from the first cohort. We have also collected morphological data on the parents and the returning adults. In addition, we have developed a sample collection plan that will not rely on the fish pass at the ASLC. We will collect as many marked pink salmon as possible in August and September 2001.

INTRODUCTION

The project to construct a linkage map for the pink salmon (*Oncorhynchus gorbuscha*) genome and to use this map to study the evolutionary biology of this species is in its sixth year. This map will provide the framework to evaluate potential impacts of the March 1989 *Exxon Valdez* oil spill on pink salmon. In addition, the mapping effort will produce valuable tools to improve our understanding of the fundamental population biology and genetics of pink salmon. Genetic markers that are mapped can be used to identify regions of the genome that are associated with important fitness traits, as well as to track population dynamics. This information will be critical for effective monitoring of recovery efforts of pink salmon in Prince William Sound.

Following the 1989 oil spill in Prince William Sound, elevated embryo mortalities were detected in populations of pink salmon inhabiting oiled streams. These increased rates of mortality persisted through the 1993 field season, three generations after the spill, suggesting that genetic damage may have occurred as a result of exposure to oil during early developmental life-stages (Bue et al. 1998). The consequences of the putative genetic damage include impaired physiological function of individuals and reduced reproductive capacity of pink salmon populations.

Documenting possible effects of the spill on pink salmon requires a detailed understanding of this species' genome. Since the rediscovery of Mendel's principles early in this century genetic linkage maps have provided important information for understanding genetic variation in species. A genetic map plays a similar role for a geneticist that a geographical map plays for the explorer of new territories. For many years, genetic maps could only be constructed in a very few model species that were suitable for extensive genetic manipulation (e.g., Drosophila and mice). Recent advances in molecular genetics now make it possible to uncover enough genetic markers to construct a detailed genetic linkage map in almost any species (Postlethwait et al. 1994).

A genetic linkage map is generated by analyzing segregation patterns of polymorphic genetic markers, typically within a single lineage (e.g., a mother and her progeny). By analyzing many polymorphic markers spread throughout the genome, correlations in segregation patterns of markers occurring in the same chromosomal region can be detected. Ultimately, if a sufficient number of linked markers are analyzed, the number of linkage groups identified will equal the number of chromosomes; at this point the map is considered to be "consolidated". This is ideal because markers can then be assigned to chromosomes. However, a genetic map that is not completely consolidated can be used to address many basic questions concerning genomic and organismal evolution.

The degree to which linkage groups are conserved over evolutionary time can be evaluated by comparing linkage maps from different taxa. Alternatively, if linkage relationships are known to be conserved, the resolution of a linkage map can be improved by incorporating information from linkage maps from closely related taxa. In fishes, linkage relationships are reported to persist after 300 million years of evolution (Graf

1989), thus many loci should occupy similar chromosomal positions in closely related taxa. However, it is important to note that many of the dominant multilocus genetic markers that are widely used in map construction, such as amplified fragment length polymorphisms (AFLPs), can not be used for comparisons between maps. Comparative mapping can be accomplished using dominant markers to determine linkage relationships of co-dominant markers that are conserved across taxa. These conserved markers can be used as reference points ("anchor loci") for map comparisons. Anchor loci are typically single locus markers such as allozymes, microsatellites, or genes of known function. Genetic linkage maps that contain many anchor loci are especially useful for evolutionary studies.

Genes of known function are important genetic markers to include on a linkage map because they serve as anchor loci, and they also can be used to study interactions between genes and the environment. For example, the major histocompatability complex, *MHC*, has been extensively studied in vertebrates for a variety of reasons including its role in disease resistance or susceptibility. Various class I and class II *MHC* alleles have been characterized in Pacific and Atlantic salmon (Katagiri et al. 1996, Miller et al. 1996, Miller et al. 1997). The addition of the *MHCB2α1* locus to the even-year linkage map is the first step to understanding how *MHC* is organized in pink salmon. In addition, we will be able to test for correlation's between fitness traits of returning adults and this gene of known function.

Many interesting and important questions about genome organization can be explored using a linkage map that includes various types of genetic markers. For example, linkage relationships can be evaluated to determine whether functional genes tend to cluster together relative to other markers. In addition, linkage assignments derived from genetic maps can be assessed to determine whether different types of markers are distributed similarly across a genome (e.g., Spruell et al. 1999; Appendix 1). Genetic linkage maps also allow the possibility of identifying the sex-determining region, which can provide important information for population level studies.

We have constructed a pink salmon linkage map using gynogenetic haploid and diploid progeny from an individual female (see Spruell et al. 1999; Appendix 1). This is the same procedure used to construct the zebrafish linkage map (Postlethwait et al. 1994). Our linkage map is based on segregating markers in haploid progeny from a single pink salmon female (A95-103) that returned to Armin F. Koernig hatchery in Prince William Sound in August 1995. We used the gene-centromere mapping approach (Johnson et al. 1996) to link markers to centromeres (see Lindner et al. 2000, Appendix 2). We are continuing to add markers to our map to reduce the number of linkage groups to match the number of chromosome pairs in pink salmon (26). In addition, we continue segregation analyses in other individuals to map markers that are not polymorphic in female A95-103 and, more specifically, to include particular genes of known function.

Odd- and even-year pink salmon are reproductively isolated due to the fixed two-year life cycle of this species (Aspinwall 1974). Beacham et al. (1988) report substantial allozyme

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and morphological evidence for differentiation of alternate brood years. In addition, Phillips and Kapuscinski (1988) and Phillips et al. (1999) detected chromosomal rearrangements between odd- and even-year populations that occur in the same geographical area. Furthermore, in a recent experimental study, Gharrett et al. (1999) demonstrated outbreeding depression in crosses between the two year classes. Together, these findings suggest that the alternate brood years are reproductively isolated and genetically distinct. Our primary map is based on an odd-year female (A95-103) therefore, we are now focusing our efforts on an even-year female (V96-13). Having linkage data from both odd- and even-year individuals makes it possible to map more markers and will allow us to determine whether linkage relationships are conserved between the reproductively isolated year classes.

A high resolution linkage map will also allow us to re-evaluate the validity of assumptions about meiotic processes in salmonids. Thorgaard et al. (1983) and Allendorf et al. (1986) concluded that there was a high level of chiasma interference in salmonids. However, this conclusion was based on the limited number of polymorphic allozyme loci available. It is possible that high levels of interference are not uniform and that some regions of the genome may violate the assumption of complete interference. The odd-year map we have generated is of much finer resolution, which allows us to test for interference along the chromosome arm. We are currently using our map to test the assumption of uniform interference (see Lindner et al., Appendix 4).

Previous studies in salmonids have detected differences in recombination rates among individuals (Sakamoto et al. 2000 and May et al. 1990). We are able to test for these differences in pink salmon through the analysis of markers we have already characterized in pink salmon. The 75 families produced for the marine survival and fitness study provide us with the samples necessary to test for sex specific differences in recombination rate and to test for differences in recombination rates based on chromosomal location (centromeric vs. telomeric).

A completed linkage map for pink salmon and the facilities at Alaska SeaLife Center (ASLC) will allow us to test questions that were previously impossible to address in salmonids. For example, it is has been notoriously difficult to detect and measure the effects of natural selection in natural populations (Lewontin 1991). The most powerful method to detect natural selection is to compare genotype distributions in a single cohort sampled at different life history stages (Lynch and Walsh 2000). We have used the facilities at ASLC to produce the families necessary for such an experimental design. Two cohorts (1998 and 1999) have been produced, marked, and released into Resurrection Bay. Over 48,000 progeny from 49 full-sibling families were released in May 1999 and over 24,000 progeny from 68 half-sibling families were released in April 2000. Unfortunately, due to unexpected failure of the fish pass at the ASLC, collecting the returning 1998 cohort adults in the summer of 2000 was exceptionally difficult and only 36 individuals were recovered. As this sample size is too small to test for natural selection, we are modifying our plans for recovering returning fish from the 1999 cohort to increase the number of fish collected.

We tested our ability to place returning adults to their correct families by examining inheritance of nine polymorphic microsatellite markers in embryos from the 1998 cohort. Microsatellite markers tend to be highly polymorphic relative to other markers, making them a good choice for use in parentage analysis based on multilocus genotypes. The results of our testing confirmed that we could unambiguously assign returning adults to a family with four microsatellite loci.

Interestingly, we detected several mutations at two of the microsatellite loci in our inheritance analysis. Building on this dataset has allowed us to empirically evaluate the rate and pattern of mutations at nine microsatellite loci in pink salmon (see Steinberg et al., Appendix 3). Knowledge of the underlying evolutionary dynamics of markers used in population studies is important for correct interpretation of patterns of variability detected with these markers. Our inheritance dataset revealed a great deal of heterogeneity in mutation rates and patterns among the nine microsatellite loci. This finding has direct relevance to management applications of these markers.

In this report we update our progress on the construction of the pink salmon linkage map, highlighting mapping results for an even-year family (V96-13). We include preliminary results of comparative mapping of odd- and even-year lineages and analysis of recombination rate differences. We also provide an overview of progress on the marine survival and fitness experiment. Finally, we have attached two published papers, one manuscript in preparation that describes results of our mapping efforts, and one manuscript that has been submitted for publication describing our mutation analysis.

OBJECTIVES

1. Develop several hundred variable DNA markers in pink salmon and test them for Mendelian inheritance.

2. Construct a linkage map based upon joint segregation patterns of the DNA polymorphisms detected in previous objective.

3. Map putative lesions identified in Restoration Study \191A.

4. Test for Mendelian inheritance of markers throughout the genome in progeny of fish exposed to oil. Regions that show aberrant segregation ratios in progeny of fish exposed to oil and normal 1:1 ratios in fish not exposed to oil would be candidates for oil-induced lesions.

5. Test for regions of the genome that are associated with traits of adaptive significance (e.g., marine mortality or run timing).

6. Test if protein markers (allozymes) are under natural selection such that they may not provide accurate information about the genetic structure and amount of gene flow among populations.

The linkage map research was originally designed to support work with pink salmon under the project *Oil-Related Embryo Mortalities* (Restoration Study \191A). The objective of that project was to identify germline mutations in pink salmon exposed to oil. Genetic damage induced by oil may either be small changes in nucleotide sequence (microlesions) or large-scale changes in chromosome structure (macrolesions). A detailed genetic map for pink salmon would be invaluable for interpreting the results of studies such as Restoration Study \191A in several ways. First, it will be possible by following the inheritance of any DNA lesions to determine if they are micro- or macro-lesions. Second, these lesions can be mapped to determine if they are randomly spread throughout the genome or if they occur at mutational "hot spots" that are susceptible to oil induced damage. However, Restoration Study \191A is no longer ongoing, and thus our work concentrates on objectives 1 - 2 and 5 - 6.

METHODS

Production of Progeny for Mapping

In August 1995, gametes and tissues of 31 pink salmon were collected from the Prince William Sound Aquaculture Corporation's Armin F. Koernig (AFK) hatchery, Prince William Sound, Alaska. Gametes and tissues of 22 pink salmon were collected from the Valdez Fisheries Development Association's (VFDA) Solomon Gulch Hatchery, near Valdez, Alaska in August 1996. Both of these hatchery stocks originated from adult fish collected at several spawning sites in Prince William Sound, Alaska. We designated families using the first letter of their place of origin (A=AFK, V=VFDA), the year of reproduction, and a sequential number corresponding to the maternal parent. For example, family A95-103 contains the progeny from female 103 collected at Armin F. Koernig hatchery in 1995.

Gynogenetic Haploids

Gynogenetic haploid embryos were produced by sperm inactivation as described by Thorgaard et al. (1983). Sperm from four males was pooled prior to UV irradiation, then mixed with eggs from individual females. Females were numbered and their progeny were designated by year class and the number assigned to that female (e.g., family A95-103). Embryos from these families were incubated until just prior to hatching when they were collected and preserved in 95% ethanol. DNA extraction was completed on the haploids as previously described (Spruell et al. 1999; Appendix 1).

Gynogenetic Diploids

Gynogenetic diploid progeny were produced using gametes from the same parents used to produce the haploid progeny. Eggs were fertilized with sperm that had been UV irradiated. Diploidy was restored by applying a heat shock that causes the retention of the second polar body (Thorgaard et al. 1983). These diploid embryos are viable and were raised until they reached sufficient size (approximately 40 mm, total length) for allozyme analysis. Individuals were sacrificed and tissue samples collected and frozen at -80°C for allozyme analysis. In addition, the caudal peduncle and fin were collected for DNA extraction (Spruell et al. 1999; Appendix 1).

Selection of Reference Families

Family A95-103 was chosen as the reference family to generate an odd-year pink salmon linkage map based on the number of embryos produced and on results from initial screening of microsatellites (see Allendorf et al. 1997 and Appendix 4).

Family V96-13 was chosen as a candidate to generate a complementary even-year linkage map based on an acceptable number of embryos produced in this family and because

preliminary screening showed the mother to be heterozygous at a gene of known function, *MHCBα2*.

Analysis of V96-13 progeny

DNA was extracted from 162 putative haploid embryos and tissue from 54 gynogenetic diploids. For the convenience of using 96 well microtiter plates, DNA is being analyzed for a subset of 95 haploid embryos and the mother. Gynogenetic origin was confirmed as described in Spruell et al. (1999; Appendix 1). We detected evidence of non-maternal alleles (i.e. due to incomplete sperm inactivation) in 4 of the 95 putative haploid embryos and none of the 54 putative gynogenetic diploids. One additional haploid embryo has been excluded from further analysis based on degraded DNA resulting in the production of poor PCR products. Currently mapping analysis is based on 90 haploid and 54 gynogenetic diploid individuals.

Evaluation of Genetic Markers for Mapping

Genetic markers were included on the haploid linkage map based on two criteria. First, fragments from loci polymorphic in the mother had to segregate in the progeny. Second, the segregation of each fragment in the progeny had to be 1:1 as expected under simple Mendelian genetic models.

Map Construction

Linkage Analysis Software

We used the UNIX version of MapMaker software (Lander et al. 1987) to assign markers segregating in haploid progeny to linkage groups. Grouping of markers was conducted using a minimum LOD score of 4.0 and a maximum recombination fraction (θ) of 0.28 (P<0.001). The linkage phase of the markers is unknown. Therefore, we entered the segregation pattern of each locus into the program in both possible phases. The Kosambi mapping function was used to calculate the genetic distance.

Gene-Centromere Distances

We estimated how far markers are located from their centromere (the gene-centromere distance) using diploid gynogens produced from females A95-103 and V96-13 (Thorgaard et al. 1983; Allendorf et al. 1986). The details of these calculations are provided in Appendix 2.

Evaluation of Recombination rates between sexes

DNA was extracted from a total of 46 embryos from six families produced from the 1998 adults collected from Likes Creek. Recombination rates were estimated for linked loci using families in which at least one parent is heterozygous at both loci.

Mutation Analysis

In Lindner et al. (2000b) we reported genotypes that appeared to result from mutation events in the 1998 cohort embryos used to evaluate transmission genetics of microsatellite loci. We have examined this possibility in more detail by analyzing additional embryos and additional loci. The details of this analysis are provided in Appendix 3.

Marine Survival and Fitness Experiment: 1998 Cohort

2000 returns

Experimental adult pink salmon were expected to return to the fish pass at the ASLC. As a result of the failure of the fish pass to attract any fish, the experimental pink salmon had to be collected from freshwater steams in upper Resurrection Bay in seine nets. In addition, 20 experimental pink salmon were turned in by recreational fisherman in response to a $1,000 lottery that was conducted as an incentive.

Parentage assignment

All experimental pink salmon collected were initially analyzed at four microsatellite loci (*SSA408, OGO1c, SSA20.19-1* and *SSA20.19-2*). PCR products were electrophoresed in 4.5% polyacrylamide gels and products were visualized on a Hitachi FMBIO fluorescent scanner. Allele size ladders for *OGO1c* and *SSA408* consisting of between six and eight evenly spaced alleles were developed using template DNA from select 1998 parents. Allele sizes for the return samples were determined relative to these allele ladders as well as a commercial size ladder (MapMarker). Comparing the sample genotype with the 1998 parental genotypes at these four loci we were able to determine if the sample was actually part of our experimental population. To confirm the family assignment, PCR products from the return sample and the expected parents at the two most variable loci, *OGO1c* and *SSA408,* were electrophoresed side by side.

We analyzed nine microsatellite loci and two genes of known function, *GH2* and *MHCα1,* in all 36 collected returns. PCR conditions and annealing temperature for these loci are reported in Lindner et al. (2000a).

Meristics and Morphological measurements

Length was measured from the mid-eye to fork. Meristic counts were completed for four paired characters, pectoral fin, pelvic fin, upper 1st gill arch, and lower 1st gill arch.

Marine Survival and Fitness Experiment: 1999 Cohort

Release families

In February 2000 approximately 24,500 fry from 68 half sibling families were pooled. In April 2000 we marked fry by clipping the adipose fins and subsequently released 24,216 marked individuals into Resurrection Bay.

Genetic analysis of parents

We analyzed each of the 68 parents from the 1999 cohort at 10 PCR-based loci, including one duplicated locus (*SSA20.19-1,2*). We used GenePop (Raymond and Rousset 1995) to calculate the number of alleles per locus and to assess departures from expected Hardy-Weinberg proportions of genotype frequencies.

RESULTS

Genetic Mapping

Linkage Map

Results of the odd-year linkage map are detailed in Lindner et al. (Appendix 4). We assigned 546 of the 590 markers analyzed for segregation in family A95-103 to one of 44 linkage groups which cover a distance of 4559 cM. Given the haploid number of 26 chromosomes for pink salmon, our mapping efforts produced 18 extra linkage groups. Taking into account the extra linkage groups and 43 unassigned markers as well as the distance to the telomeres we estimate the size of the pink salmon genome to be 6691 cM. The haploid pink salmon genome is approximately 2.72 million kilobase pairs (kpb; Johnson et al 1987b); thus, we estimate approximately 406 kbp/cM.

In addition, we have analyzed the segregation pattern of 85 loci in an even-year family V96-13. We have assigned 62 of 85 loci to one of 22 linkage groups (Table 1, Figure 1). One gene of known function, *MHCBα2* is assigned to a linkage group that consists of one microsatellite and two PINE loci (Figure 1).

Gene-centromere Analysis

Lindner et al. (2000a; Appendix 2) reports the estimated gene-centromere distance for 312 loci. The data showes that amplified fragment length polymorphisms (AFLPs) are significantly more centromeric than loci identified by three other techniques (allozymes, microsatellites, and PINEs). Gene-centromere distances were also estimated for all 19 microsatellite loci and *MHCBα2* in 54 gynogenetic diploid progeny from family V96-13.

The genotypes of progeny from V96-13 at ten loci are given in Table 2; all other families were previously analyzed and are included for comparison. No significant differences in the frequencies of the two homozygote classes were found, indicating that lethal alleles were not influencing our results. The exact binomial test requires detection of six homozygotes for the frequencies of the two homozygous classes to be considered significantly different ($P<0.05$). Only two out of a total of 26 tests had a probability less than 0.05, these are not significant when corrected for the number of independent tests (Rice 1989).

Comparative mapping

Initial results from both haploid and gynogenetic diploid analysis have not identified any significant differences between odd (A95-103) and even (V96-13) year stocks of pink salmon (Table 2 & 3). Comparisons between the odd-year pink salmon map and other teleost maps are included in Appendix 3.

Recombination rate differences

We detected a significant difference in recombination rate between females at *SSA408* and *OmyFGT19* ($P<0.03$; Table 4). All other comparisons between individuals of the same sex were not significant.

Females tend to have a higher recombination rate for loci located close to the centromere *(SSA408/FGT19)*. However, we found no significant difference in recombination rate for loci located farther from the centromere (*ONE102/ONE18* and *ONE18/SSA293*; Table 4).

Marine Survival and Fitness Experiment: 1998 Cohort

Detection of Mutations

We have observed a total of 24 individuals with genotypes at two (*SSA408* and *OGO1c*) of the nine microsatellite loci examined that are best explained as being the result of mutation events. A high frequency of the novel alleles detected appear to be the result of premeiotic cluster mutations at *SSA408*. Steinberg et al. (Appendix 3) provide the results of this analysis.

Sample collection

A total of 41 marked fish were collected during August and September 2000. Of the 41 fish collected, 16 were collected live from Resurrection river and Spring creek using a seine net and 25 were turned in dead by recreational fisherman.

Sample assignment

Each of the 41 marked fish collected was initially genotyped at four polymorphic microsatellite loci (*OGO1c, SSA408, SSA20.19-1,* and *SSA20.19-2*). Two of the samples were identified as juvenile chinook salmon marked with an adipose fin clip (presumably from a hatchery population). Three samples collected with degraded adipose fins had alleles at *OGO1c* and *SSA408* that were not present in the 1998 parents and therefore were not part of our experimental population.

All but one of the 36 marked pink salmon could be assigned unambiguously to a single family based on the analysis of four loci. The remaining individual could be placed in either of two families. After further analysis with three additional loci (*OmyRGT6, MHCα1,* and *OGO8*), we were able to place this individual in one family, LCP98-73. Based on these results we were able to assign 36 individuals to one of the 49 release families (Table 5, Figure 2). All 36 marked pink salmon identified as part of our experimental population were analyzed next to their assigned parents at a total of 11 PCR based loci (9 microsatellites, *MHCα1,* and *GH2*). All initial family assignments were confirmed.

Morphometric measurements

We measured length and estimated meristic variation for the 36 adult pink salmon determined to be part of our experimental population. Males are slightly smaller and significantly more variable in length than females (P<0.025, Figure 3). The average number of asymmetric traits (FA) is 1.39 at the four bilateral traits. The average number of asymmetric characters for males (1.35) and females (1.43) is not significantly different.

Marine Survival and Fitness Experiment: 1999 Cohort

Genetic Analysis

Parents of the 1999 cohort have been analyzed at nine microsatellite loci and one gene of known function (*GH2*). None of the loci deviate from Hardy-Weinberg expections.

DISCUSSION

Evaluation of Even-year Families for Mapping

One of our goals is to add genes of known function to our linkage map. We have placed a gene of known function, *MHCBα2,* on the even-year linkage map. This gene is currently linked to two PINE loci and one of a duplicated microsatellite locus (*STR60-2*). Unfortunately, *STR60-2* is not mapped on our more comprehensive odd-year map. With the addition of more microsatellite loci that are linked on both the odd and even-year map we hope to be able to link *MHCBα2* to loci already on the odd-year map.

Placing *MHCBα2* on a map will allow us to start examining gene locations relative to other loci in pink salmon. There are two classes of *MHC* genes, class I and class II. Class I *MHC* is involved with the ability of the body to recognize altered "self" cells and *MHC* class II is involved in recognizing foreign invaders. Studies of the organization of MHC suggest that the class I and II regions are not linked in bony fishes (Sato et al. 2000). We will examine if this is true for pink salmon by focusing on mapping other MHC loci. In addition, this gene is a perfect candidate for analysis in the marine survival and fitness experiment.

Comparative mapping

Pink salmon are unique in that they exhibit a rigid two-year life cycle that has resulted in two reproductively isolated odd- and even-year lineages (Aspinwell 1974). Beacham et al. (1988) found substantial allozyme and morphological evidence for differentiation of alternate brood years. In addition, Phillips and Kapuscinski (1988) and Phillips et al. (1999) detected chromosomal rearrangements between odd- and even-year populations that occur in the same geographical area. Furthermore, in a recent experimental study, Gharrett et al. (1999) demonstrated outbreeding depression in crosses between the two year classes. Together, these findings suggest that the alternate brood years are reproductively isolated and genetically distinct.

Based on our preliminary genetic analysis of the odd and even-year stocks from Likes Creek we have not detected any differences in recombination fraction at linked loci between year class. The comparison of both haploid linkage data and gene-centromere distances between odd and even-year classes support findings that gene order is highly conserved (Graf 1989). The similarity in gene order between these two year classes also support the incorporation of results from the even-year map onto the more comprehensive

odd-year map. We need to compare more loci to determine where differences exist between these year classes.

Of the 35 microsatellites on the pink salmon linkage map, 25 are included on the rainbow trout map (Sakamoto et al. 2000). A comparison of the odd year pink salmon linkage map and the rainbow trout map (Sakamoto et al. 2000) is discussed in Appendix 4.

Differences in recombination rates

The analysis of recombination rates in pink salmon detected large differences between individuals. Sakamoto et al. (2000) suggest that this might be a result of anscestral tetrasomic inheritance and pseudolinkage. When homeologous chromosomes pair and exchange material, the resulting homologous chromosomes are less similar to each other than when homologous pairing occurs (Allendorf and Danzmann 1997). Presumably this makes it more difficult in subsequent generations for pairing and exchanges to occur resulting in a lower rate of recombination in those individuals produced from parents in which multivalent pairing occurred.

Previous studies in salmonids have detected differences in recombination rates between males and females (Wright et al. 1983, and Sakamoto et al. 2000). Due to large differences detected between individuals within each sex we compared the average recombination rate of females to that of males at each locus. Initial results agree with Sakamoto et al. (2000); females have a higher recombination rate at loci located close to the centromere ($y < 0.17$; Table 4). Due to our small data set we are unable to draw conclusions for loci that are farther from the centromere ($y > 0.71$).

In tetraploid species such as pink salmon, it has been suggested that the difference in recombination rate between sexes is due to constraints imposed on crossing-over during multivalent pairing (Sakamoto et al. 2000). Multivalent pairing has only been reported in males and generally occurs in the telomeric region (Wright et al. 1983; Allendorf and Danzmann 1997). It has been suggested that multivalent pairing in males explains the tendency for males to have a higher rate of recombination than females in telomeric regions. Recombination in the telomeric regions of males can occur between homologous and homeologous chromosomes increasing the chance for exchange in that region.

Mutation Analysis

Our inheritance dataset revealed a great deal of heterogeneity in mutation rates and patterns among the nine microsatellite loci analyzed (Steinberg et al., Appendix 3). All 24 mutations detected were at two of the nine loci (*OGO1c* and *SSA408*). The mutation rate estimates at *OGO1c* and *SSA408* (3.7×10^{-3} and 5.4×10^{-3}) are at the high end of the range of 10^{-3} to 10^{-6} reported for other organisms (Dallas 1992, Weber and Wong 1993, Schug et al. 1997).

The variability of these two loci makes them powerful tools for assigning parentage. We were able to unambiguously assign parentage to 35 of the 36 returning fish from the 1998 cohort based on these two loci alone. However, given the high probability of mutation at these loci, our results indicate that it is important to use a combination of low and high variability markers for parentage analysis.

Mutations at *SSA408* were not distributed randomly among families, but rather tended to be clustered within families. This pattern of inheritance suggests that a high proportion of novel alleles resulted from mutations occurring early in gametogenesis. Clustering of mutations within single families has been shown to bias estimates of mutation rates and to influence basic population genetic processes such as fixation probabilities (Woodruff et al. 1996). Another potential source of bias we detected at *SSA408* was the tendency for mutations to increase allele size and for particular alleles to be hypermutable. The variability of mutations within and among loci and among families suggests that mutation should not be ignored when interpreting patterns of genetic differention (e.g., when conducting stock structure analysis). Certainly, if data from both highly polymorphic and less polymorphic loci are being combined, the possibility for locus-specific effects should be evaluated.

2000 returns

In August and September 2000 no fish returned to the ALSC fish pass. We expected most of our returning population to "home" to the freshwater signal at the ASLC. However, due to the failure of the fish pass we were forced to survey freshwater streams in upper Resurrection Bay for marked pink salmon using seine nets. We also relied on recreational fisherman to turn in marked pink salmon. Though we were able to collect 36 marked pink salmon this sample is too small to test for correlations between genes and fitness traits.

One problem with the fishpass is the amount of freshwater the facility is able to release is inadequate for the returning adults to detect. At this stage there is no way to remedy this problem. We are currently developing a plan for the 2001 field season that will enable us to collect more returning fish from the freshwater streams in upper Resurrection Bay.

CONCLUSIONS

We have constructed an odd-year linkage map that can be used to test for effects of regions of the genome on traits that are important to the recovery of pink salmon (e.g., growth and survival) and to evaluate stock structure. We have placed a gene of known function on the even year map, *MHCBα2*. Initial comparisons between odd and even-year maps have not detected any differences in gene order. We will continue to use the even-year map to add anchor loci to the odd-year map. We have completed two successful years of marking and releasing pink salmon fry into Resurrection Bay. A manuscript has been submitted for publication describing microsatellite mutation patterns and rates based on genotyping data from the 1998 experimental families. The experimental families from 1998 and 1999 as well as the linkage map have allowed us to begin to examine sex specific recombination differences and the role of gene location. Due to the failure of the fish pass we were able to collect only 36 returning adults from the 1998 cohort. Plans are being developed to improve the number of returning adults collected in August and September 2001.

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