Exxon Valdez Oil Spill
Restoration Project Annual Report

Construction of a Linkage Map for the Pink Salmon Genome

Restoration Project 01190
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, 00190 and 01190. Annual reports entitled <u>Construction of a Linkage Map for the Pink Salmon Genome</u> were submitted in 1997, 1998, 1999, 2000 and 2001. In this annual report we describe Restoration Project 01190. 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 project 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. Undergraduates involved with parts of the project have also presented the results of their work. Joe Meng presented a poster on the rate of mutation in pink salmon at the National Conference of Undergraduate Research at the University of Kentucky in March 2001. In April 2001, Kristin Bott presented the results of her work on a sex-linked DNA marker in pink salmon at the Western Regional Honors Conference at Portland Pacific University, Oregon. 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). A third article will be published this year, Steinberg, E.K., K.R. Lindner, J. Gallea, A. Maxwell, J. Meng, F.W. Allendorf. 2002. Rates and

patterns of microsatellite mutations in pink salmon. Molecular Biology and Evolution. 19(7). 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 have constructed a genetic linkage map for pink salmon (*Oncorhynchus gorbuscha*) and will use it to examine marine survival and fitness in pink salmon in Prince William Sound. We analyzed segregation of 596 DNA fragments in an odd-year female and 94 haploid progeny. Of these markers, 553 were assigned to one of 44 linkage groups. We mapped 319 loci relative to their centromeres using gynogenetic diploid progeny. 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 were marked and released into Resurrection Bay from the Alaska SeaLife Center. In August 1999, we collected 68 adult pink salmon from Likes Creek and produced 68 families. These families were raised at the Alaska SeaLife Center and approximately 24,000 fry were marked and released into Resurrection Bay in May 2000. Only 36 returning adults from the 1998 experimental cohort were collected in August 2000. In August 2001, 259 returning adults from the 1999 cohort were collected. We assigned the adult returns to family using genotype data from 10 loci. Initial analysis has revealed nearly random family survival and heritability of body length.

Project Data: We have two primary sets of data: 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 were tested for nonrandom segregation between all pair-wise combinations of 14 allozyme and three microsatellite loci. The haploid data set consists of 596 polymorphic DNA fragments loci in female A95-103 and 94 of her haploid progeny, and 123 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 319 loci and of genotypes of 54 gynogenetic diploid progeny from female V96-13 at 40 loci. Data for the marine survival and fitness experiment are genotypes at ten PCR-based loci for 50 families (50 parent pairs with 10 embryos each) from the 1998 experimental release. An additional 36-40 embryos from seven of the 1998 families were analyzed at nine microsatellite loci to investigate mutation rates and patterns. The parents for the experimental cohort produced in 1998 were genotyped at 12 additional PCR-based loci and 34 allozyme loci. The 1999 parents have were genotyped at 10 PCR-based loci and 30 allozyme loci. Their progeny, the 259 returning adult fish recovered in August 2001, were genotyped at the same 10 PCR-based loci and this information has been used to assign them to parental family. Four meristic characters, as well as body length, egg mass, and egg number were recorded for both sets of parents as well as the 36 marked adults collected in August 2000. The 259 marked adults collected in August 2001 were

measured for body length, egg mass, and egg number. All data sets are currently recorded in Microsoft Excel spreadsheets. Data will be made available to individuals within the reasonable bounds of sharing unpublished data. For information regarding data contact Joe Tyburczy, Division of Biological Sciences, University of Montana, Missoula, MT 59812. Phone: (406) 243-6749. E-mail: joet@selway.umt.edu.

Citation: Tyburczy, J., K. R Lindner, K. L. Knudsen, P. Spruell, and F. W. Allendorf. 2002. Construction of a linkage map for the pink salmon genome, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 01190), University of Montana, Missoula, Montana.

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EXECUTIVE SUMMARY

The project to construct a genetic linkage map of the pink salmon (*Oncorhynchus gorbuscha*) genome, and to use this map to study the marine survival and fitness in this species, is in its seventh year. The linkage map will allow the evaluation of genetic impacts of the March 1989 *Exxon Valdez* oil spill on pink salmon populations and will help to document the recovery of affected 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 mortality was 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 596 DNA markers segregating in the gynogenetic haploids produced a genetic map comprising 44 linkage groups covering a distance of 4550 centiMorgans. Assuming a minimum distance of 28 centiMorgans for linkage detection and accounting for all the gaps and unlinked markers the minimum distance of the pink salmon genome is 6472 centiMorgans. The haploid pink salmon genome consists of approximately 2.72 million kilobase pairs, thus we estimate approximately 420 kilobase pairs per centiMorgan.

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 609 markers.

The microsatellites and genes of known function added to the linkage map serve as landmarks, or "anchor loci" and will facilitate comparisons between maps. These loci allow comparison of genetic linkage of odd- and even-year pink salmon, estimation of recombination rates of males and females, and incorporation of 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 selective effects of the marine environment on the pink salmon genome.

A complementary even-year map has been constructed. Development of this map, based on the segregation of loci in family V96-13, followed the same design as the odd-year map. This map enabled us to compare odd- and even-year pink salmon as well as add seven new markers to the odd-year map. This even-year map consists of 123 loci, 103 of which have been assigned to one of 33 linkage groups. One locus included on this map is a gene of known function (*MHCBa2*).

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 be required to span the entire genome of pink salmon. We have successfully created a genome map with 44 linkage groups. However despite the number of the markers examined, we were unable to consolidate the map enough to reduce the number of linkage groups to 26, the number of chromosome pairs in pink salmon (2N = 52). Additional markers must be mapped in order to consolidate the map. We have collected gene-centromere distances of 319 loci using gynogenetic diploids. Comparison of the genome maps of odd- and even-year fish has revealed no significant differences between them. We will submit a publication on the results of our mapping efforts.

We are now focusing on the marine survival and fitness portion of the study. Two experimental cohorts have been produced and their returning adult progeny collected. Both cohorts were hatched and released from the Alaska SeaLife Center in Seward. In August 1998, gametes and tissue from 150 pink salmon from Likes Creek were collected and 75 single-cross families were produced. 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-sibling 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.

In August 2000 we planned to collect experimental adult pink salmon as they returned to the fish pass at the Alaska SeaLife Center. Failure of the fish pass to attract any fish forced us to modify these plans. A total of 36 marked pink salmon were collected by seining freshwater streams in upper Resurrection Bay and from recreational fishermen 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.

Though the fish pass at the Alaska SeaLife Center did attract fish 2001, probably due to increased water flow, it still failed to capture them. We collected fish through alternate means including seining nearby rivers, hook-and-line, and an incentive to recreational fishermen. We recovered 259 adult fish from the 1999 experimental cohort.

We genotyped the 36 returning marked pink salmon recovered in August 2000 at nine microsatellite loci and two genes of known function (*MHCα1* and *GH2*) and successfully assigned them back to their family of origin.

The 259 fish from the 1999 cohort have been genotyped at nine microsatellite loci and one gene of known function (*GH2*) which have been used to place them into families. Analysis based on this information has revealed nearly random family returns, and strong heritability of length in both males and females (0.38 and 0.41 respectively).

Several mutations were detected at two of the nine microsatellite loci (*SSA408* and *OGO1c*) in the embryos of the 1998 cohort during the analysis of the transmission of genetic markers used to evaluate parentage. Further analysis revealed a great deal of heterogeneity in microsatellite mutation rates and patterns. Mutation rate estimates ranged from 8.5×10^{-3} and 3.9×10^{-3} mutations per transmission in *SSA408* and *OGO1c* respectively, to 0.0 at the other seven loci. 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. Genetic analysis of the adult progeny collected from the 1999 cohort has revealed similar patterns of mutation. Mutations were detected at both *SSA408* and *OGO1c* at frequencies of 1.9×10^{-3} and 1.2×10^{-2} mutations per transmission. These rates are comparable to those found in the 1998 cohort, and as in that cohort, no mutations were detected at the other seven microsatellite loci. These findings indicate that microsatellite mutation dynamics are complex and likely vary substantially among loci.

The mapping portion of this project is complete. We are also on schedule with the marine survival and fitness experiment. We have released and collected both cohorts of experimental progeny. We have determined the genotypes of the parents used to generate both cohorts, the genotypes of returning adult progeny from both cohorts, as well as genotypes of embryos sampled from the 1998 cohort. We have also collected morphological data on the parents and the returning adults the 1998 cohort.

INTRODUCTION

The project to construct a linkage map for the pink salmon (*Oncorhynchus gorbuscha*) genome and to use this map to study marine survival and fitness of this species is in its seventh 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 mortality was 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, 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), are not conserved across taxa, and therefor cannot 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 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 have similar distributions within 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). Using a total of 609 markers we were able to reduce the number of linkage groups to 44, but could not reduce that number to equal the number of chromosome pairs in pink salmon (1n=26).

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 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)

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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. While our primary map is based on an odd-year female (A95-103), we have also created a complementary map based on an even-year female (V96-13). Having linkage data from both odd- and even-year individuals made it possible to map more markers. Interestingly, linkage relationships are conserved between the reproductively isolated year classes for all markers we examined.

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 have used 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 allowed us to test for sex specific differences in recombination rate and to test for differences in recombination rates based on chromosomal location (centromeric vs. telomeric).

The pink salmon linkage map and the facilities at the Alaska SeaLife Center (ASLC) allow us to test questions that were previously impossible to address in salmonids. For example, it is 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 modified our plans for recovering returning fish from the 1999 cohort to increase the number of fish collected. Alternative collection methods allowed us to recover 259 returning adult salmon in the summer of 2001.

We have determined the genotype for the 259 fish from the 1999 cohort at nine polymorphic microsatellites and one gene of known function and used this data to unambiguously assign the fish to families.

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, and the marine survival and fitness experiment including analysis of the returning fish collected in August 2001. We include results of comparative mapping of odd- and even-year lineages and analysis of recombination rate differences. We also describe preliminary analysis of data from the 2001 returns. Finally, we have attached two published papers, one manuscript that has been accepted for publication describing our mutation analysis, and one manuscript in preparation that describes results of our mapping efforts.

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 whether 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

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(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 macrolesions. 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).

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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*.

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.

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 selected 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 hatchling fry from 67 half-sibling families were pooled. All of the eggs from one of the original 68 families perished before hatching. In April 2000 we marked fry by clipping the adipose fins and subsequently released 24,216 marked individuals into Resurrection Bay.

2001 returns

Through snag-hooking near the fishpass at the ASLC, seining the rivers in upper Resurrection Bay, and a lottery to encourage recreational fishermen to turn in marked fish, we collected 260 marked pink salmon.

Parentage assignment

We analyzed each of the 68 parents from the 1999 cohort at 10 PCR-based loci, including one duplicated locus (*SSA20.19-1,2*) and one gene of known function (*GH2*). 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. The returning adult progeny have been analyzed at all 10 of these loci. After initial scoring at three microsatellite loci (*OTS1*, *RGT6*, and *SSA408*), fish were placed into families. Progeny were then run next to assigned parents for analysis of all subsequent loci. This allowed unambiguous family assignment of fish, and detection of any progeny with alleles whose lengths differ from that of their parents.

Morphometric measurements

We measured length, egg weight, total egg weight, and number of eggs as well as meristic characteristics for the 1999 parents. We measured length, egg weight, total egg weight, and number of eggs for the returning adult progeny. Meristics of the returns will be measured this year.

RESULTS

Genetic Mapping

Linkage Map

Results of the even- and odd-year linkage maps are detailed in Lindner et al. (Appendix 4). We assigned 553 of the 596 DNA markers analyzed for segregation in family A95-103 to one of 44 linkage groups which cover a distance of 4550 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 6472 cM. The haploid pink salmon genome is approximately 2.72 million kilobase pairs (kbp; Johnson et al 1987b); thus, we estimate approximately 420 kbp/cM.

In addition, we have analyzed the segregation pattern of 123 loci in an even-year family V96-13. We have assigned 102 of 123 loci to one of 33 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 319 loci. The data shows 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 41 microsatellite loci and $MHCB\alpha2$ 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

Neither haploid nor gynogenetic diploid analysis 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 4.

Recombination rate differences

Analysis of diploid progeny from the 1998 cohort revealed 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 between sexes 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 16 embryos from this cohort with genotypes at two (*SSA408* and *OGO1c*) of the nine microsatellite loci examined that are best explained as being the result of mutation events. We found 11 mutations in 1,300 transmissions at SSA408, and 5 mutations in 1,278 transmissions of OGO1c, yielding mutation rate estimates of 8.5×10^{-3} and 3.9×10^{-3} mutations per transmission, respectively. A large portion of the novel alleles detected appears to be the result of premeiotic cluster mutations at *SSA408*. Steinberg et al. (Appendix 3) provide more detailed 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 fishermen.

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

Detection of Mutations

We found seven fish with genotypes indicative of a mutation at the same two loci in which mutations were detected in embryos from the 1998 cohort (*SSA408* and *OGO1c*). No mutations were found in the other seven loci (Table 6). One mutation was found in 520 transmissions (260 fish) at *SSA408* for a mutation rate of 2.0×10^{-3} mutations per transmission. Six mutations were found in 520 transmissions of *OGO1c* for a rate of 1.2×10^{-2} mutations per transmission.

Sample collection

We collected 262 marked pink salmon in August and September 2001. Approximately two thirds (183) were collected at the mouth of the ASLC fish pass using snagging gear (Figure 4). A total of 61 marked fish were turned in by recreational fisherman in response to a lottery incentive for a chance to win one of two $500 prizes. The remaining 18 fish were collected in seine nets from several freshwater streams in upper Resurrection Bay. Experimental fish were collected from six different locations (Lowell Creek, Spring Creek, Resurrection River, the ASLC, boat harbor, and culvert; Figure 4). We surveyed other freshwater streams but did not find any of our marked pink salmon at these sites (Tonsina Creek, Spruce Creek, Bear Creek and Salmon Creek).

Sample assignment

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 expectations. Of 262 returning pink salmon collected, 259 were unambiguously assigned to families in the 1999 experimental cohort based on data from nine microsatellite loci and one gene of known function (*GH2*; Table 7). The return of 259 adults constitutes a survival rate of 0.93 percent of the 27,841 pooled fry, and 1.1 percent of the 24,500 fry released. Two fish were eliminated because they had unique alleles or combinations of alleles that could not have been produced by any possible combination of parents. One fish had alleles that indicated it was result of sperm contamination resulting from an unintentional cross between two parents and therefore did not belong to an experimental family. Family returns of the 259 fish was nearly random (Figure 5).

Morphometric measurements

Regression analysis revealed significant heritability of length in both males and females (h^2=0.38, p<0.001 and h^2=0.41, p<0.001, respectively; Figure 6). Egg weight exhibited a positive slope, which, though not significant, is suggestive of some degree of genetic influence (h^2=0.32, p<0.10; Figure 7a). Total egg weight and number of eggs had slightly negative slopes indicating that there is no evidence of genetic influence (Figure 7b,c).

DISCUSSION

Evaluation of Even-year Families for Mapping

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. Further work is necessary in order to place *MHCBα2* on the odd-year map.

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). In addition, this gene is a 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.

Our genetic analysis of the odd and even-year stocks from Likes Creek did not detect 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 supports the incorporation of results from the even-year map onto the more comprehensive odd-year map. Finer resolution mapping with a greater number of loci is necessary to determine the existence and location of any differences between these year classes.

Of the 41 microsatellite loci on the pink salmon linkage map, 27 are included on the rainbow trout map (Sakamoto et al. 2000). Two of these loci included on our map are one of a duplicated pair in pink salmon, but are only known to have a single copy in the rainbow trout map. It is unknown which copies are included on our map. 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; Figure 6). All mutations detected, both in embryos from the 1998 cohort, and returning adults from the 1999 cohort were at two of the nine loci (*OGO1c* and *SSA408*). These two loci are, by far, the most variable examined in this study, both in number of alleles and in length. It seems likely that the high rates of mutation at these loci are responsible for their high levels of genetic variation. These two loci are also the only tetranucleotide repeats; the other seven loci are dinucleotide repeats. All mutant alleles detected differed from the parental allele by four base pairs which is suggestive of addition or deletion of a single repeat unit. The mutation rate estimates at *OGO1c* and *SSA408* 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). Investigation of the adult returns from the 1999 cohort yielded similar estimates of mutation rates for both *OGO1c* and *SSA408*.

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 differentiation (e.g., when conducting stock structure analysis). Loci with a high mutation rates violate the customary assumption that the effect of mutation is negligible, and may be less useful in estimating gene flow and historical patterns of isolation because these signals will be obscured by the accumulation of mutations. 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 detect and be drawn toward 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 correlation between genes and fitness traits.

One problem with the fishpass was that the amount of freshwater the facility was releasing was probably inadequate for the returning adults to detect.

2001 returns

Increased outflow from the fish pass at the ASLC in August and September 2001 was likely responsible for successfully attracting fish. However, it did not actually catch any fish which necessitated other means of retrieving them. The 260 fish recovered is slightly greater than one percent of the released fry. We have only recently completed genetic analysis of these fish at nine of ten loci examined in the 1999 parents. This has enabled us to ambiguously assign the fish into parental families, and observe trends of heritability of length for this cohort.

The near random distribution of returns among families indicates that the influence of selection favoring some families over others is limited. The index of variability (mean family size divided by variance) tends toward 1 under random survival, and increases with deviation from random survival (as families tend to survive or perish as a unit; Crow and Morton, 1955). The index of variability value of 1.82 in this cohort indicates some departure from complete random survival but is much lower than the values of 4.03 and 4.97 found by Geiger et al. (1997) in the two cohorts of pink salmon in which they were able to detect a significant sire effect on survival.

The relatively high heritability of length found in both males and females (0.38 and 0.41; Figure 6) is similar to the values (0.4 and 0.2) found in pink salmon from Auke Creek, AK, released into the wild by Smoker et al. (1994). Heritability of length combined with random family returns suggests that, at least under the oceanic conditions this cohort

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experienced, inherited body length had little effect on marine survival. However, this does not address the effect of length on mating success since these fish were mated in captivity.

Further work will entail completion of allozyme analysis and measurement of meristic characteristics on the adult returns, as well as determining the genotype and family origin of the 500 fry taken from the 1999 cohort at the time of release. When this work is finished, complete analysis incorporating all of these data will be undertaken to investigate correlation between genetic markers and survival and fitness traits.

CONCLUSIONS

We have constructed odd- and even-year linkage maps 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*. Comparisons between odd and even-year maps have not detected any differences in gene order. We have completed two successful years of marking and releasing pink salmon fry into Resurrection Bay. A manuscript has been accepted 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 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. The 259 fish collected in 2001 should be sufficient to allow us to examine relationships between alleles and fitness traits.

ACKNOWLEDGMENTS

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Table 1. Summary of heterozygous loci detected in female V96-13 and percent of loci linked on the even-year map.

	Number of polymorphic loci	Number of markers unlinked	Percent assigned to linkage group
Microsatellites	19	6	68
PINEs	65	17	74
$MHCB^{\alpha}2$	1	0	100
Total	85	23	73

Table 2. Gynogenetic diploid genotypes at nine loci. Chi-square tests for equal numbers of homozygotes (1 d.f.). Chi-square values in the total row tests for significant difference in y between families (* $p < 0.05$).

Locus	Family	Progeny 11	Progeny 12	Progeny 22	Proportion heterozygotes (y)	Chi-square* (df)
OGO1c	A95-20	29	14	28	0.20	0.02
	A95-103	24	16	27	0.24	0.18
	V96-20	14	4	16	0.12	0.13
	V96-13	19	6	17	0.14	0.11
	Total	86	40	88	0.19	2.85 (3)
OMY301	A95-103	7	54	7	0.79	0.00
	V96-13	7	29	6	0.69	0.08
	Total	7	29	6	0.69	1.51 (1)
OMYFGT19	A95-103	25	8	35	0.12	1.67
	V96-13	16	8	19	0.19	0.26
	Total	16	8	19	0.19	1.00 (1)
OMYRGT6	A95-103	27	9	27	0.14	0.00
	V96-13	15	14	25	0.26	2.50
	Total	15	14	25	0.26	2.49 (1)
ONE14	A95-103	1	63	2	0.95	0.33
	V96-13	2	44	2	0.92	0.00
	Total	2	44	2	0.92	0.69 (1)
ONE102	A95-103	10	48	10	0.71	0.00
	V96-13	9	25	19	0.47	3.57
	Total	9	25	19	0.47	6.83 (1)
OTS1	A95-29	14	7	11	0.22	0.36
	A95-103	30	12	26	0.18	0.29
	A95-114	17	17	22	0.30	0.64
	A95-120	15	11	18	0.25	0.27
	V96-13	28	11	14	0.21	4.67
	V96-19	19	30	26	0.40	1.09
	Total	123	88	117	0.27	11.37 (5)

Locus	Family	Progeny			Proportion heterozygotes	
		11	*12*	*22*	*(y)*	Chi-square* (df)
SSA311	A95-103	26	7	35	0.10	1.33
	V96-13	20	13	15	0.27	0.71
	Total	20	13	15	0.27	5.56 (1)
SSA408	A95-103	23	11	29	0.17	0.69
	V96-13	18	10	13	0.24	0.81
	Total	18	10	13	0.24	0.74 (1)

Table 3. Comparison of the recombination fractions (rf) calculated for loci analyzed in families A95-103, and V96-13. Chi-square tests for differences in rate of recombination between families. No comparisons were significant.

Locus	rf		χ^2
	A95-103	V96-13	
SSA408 *OmyFGT19*	0.13	0.25	3.32
ONE102 *SSA293*	0.11	0.05	2.72
OTS101 *OTS1*	0.21	0.27	0.11
OMY301 *SSA197*	0.19	0.19	0.00

Table 4. Comparison of recombination rates between males and females. The first two columns indicate the loci analyzed. The number in parentheses is the gene-centromere distance for each locus (y) based on segregation analysis of gynogenetic diploids in family A95-103 (Lindner et al. 2000a). Sex of informative parent is indicated by F = female and M = male. Recombination rates are averaged for each sex in the total row. Chi-square tests for differences in recombination rates between individuals; the significance level is reported under P.

		Sex	Parental	Recombinants	rf	χ^2	P
SSA408 (0. 17)	*FGT19* (0.12)	F	38	6	0.14		
		F	35	4	0.10		
		F	23	14	0.38	6.73	0.03
		Total	96	24	0.20		
		M	39	5	0.11		
		M	37	2	0.05		
		M	34	1	0.03		
		M	34	1	0.03	3.11	0.37
		Total	144	9	0.06		
ONE102 (0.71)	*ONE18* (0.86)	F	38	4	0.10		
		M	44	0	0.00		
ONE18 (0.86)	*SSA293* (0.97)	F	46	0	0.00		
		M	32	1	0.03		
		M	46	0	0.00	1.29	0.52
		Total	78	1	0.01		

Table 5. Summary of marked adult pink salmon collected in August 2000 from freshwater streams in upper Resurrection Bay. Sex is abreviated, F = female and M = male. Each individual was assigned to its family of origin as reported in the last column.

Individual #	Sex	Date Collected	Location	Length (mm)	Family #
1	F	7 August	Culvert	480	73
2	M	7 August	Culvert	490	46
3	M	15 August	Lowell Cr.	478	11
4	M	15 August	Harbor	478	61
5	F	16 August	Lowell Cr.	441	69
6	M	16 August	Spring Cr.	430	6
7	F	17 August	Culvert	444	27
8	M	17 August	Culvert	473	50
9	M	17 August	Culvert	510	72
10	M	17 August	Harbor	496	23
11	F	18 August	Spring Cr.	457	63
12	F	18 August	Culvert	456	74
13	M	18 August	Spring Cr.	467	28
14	M	18 August	Culvert	435	9
15	M	21 August	Spring Cr.	437	73
16	F	22 August	Culvert	446	49
17	F	22 August	Resurrection R.	484	19
18	F	22 August	Spring Cr.	475	25
19	F	22 August	Resurrection R.	457	38
20	M	22 August	Resurrection R.	418	12
21	F	23 August	Resurrection R.	475	19
22	F	24 August	Culvert	458	15
23	F	24 August	Harbor	483	58
24	M	24 August	Harbor	447	7
25	F	25 August	Resurrection R.	466	75
26	F	25 August	Resurrection R.	470	75
27	M	25 August	Spring Cr.	397	53
28	M	25 August	Culvert	478	64
29	M	27 August	Culvert	508	23
30	F	28 August	Harbor	475	49
31	F	28 August	Resurrection R.	519	45
32	M	28 August	Resurrection R.	473	40
33	M	29 August	Harbor	470	41
34	F	30 August	Spring Cr.	499	45
35	F	30 August	Spring Cr.	470	21
36	F	2 September	Culvert	466	51

Table 6. Variability of nine microsatellite loci in wild-caught parents, including size range (in base pairs), number of alleles, expected heterozygosity (H_e), and mutation rate observed in progeny.

Locus	Cohort	H_e	No. of Alleles	Allele Size Range (bp)	Mutation Rate
OGO1c	1998	0.983	77	275-584	3.9×10^{-3}
	1999	0.975	54	261-440	1.2×10^{-2}
OGO8	1998	0.334	17	88-171	0.0
	1999	0.872	25	88-186	0.0
OMY301	1998	0.856	21	75-113	0.0
	1999	0.846[a]	13	75-113	0.0
ONE3	1998	0.507	3	160-167	0.0
	1999	0.479	2	162-167	0.0
OTS1	1998	0.829	15	218-248	0.0
	1999	0.850	15	216-246	0.0
RGT6	1998	0.922	18	172-255	0.0
	1999	0.934	19	172-258	0.0
SSA20.19-1	1998	0.058	2	77-79	0.0
	1999	0.463	3	77-81	0.0
SSA20.19-2	1998	0.307	3	62-74	0.0
	1999	0.240	3	62-74	0.0
SSA408	1998	0.972	49	300-500	8.5×10^{-3}
	1999	0.968	39	304-528	1.9×10^{-3}

[a] *OMY301* deviates from Hardy-Weinberg proportions (p<0.03) but this difference was driven by a small number of homozygotes at rare alleles.

Table 7. Summary of adult progeny recovered in 2001 from each family and parent, the number of alevins from each parent pooled prior to freshwater rearing, and the percentage of alevins from each parent recovered as adults.

Fam. No.	Progeny per family A	B	Dam	Progeny	%Survival	Alevins	Sire	Progeny	%Survival	Alevins
1	9	3	1	12	0.84	1424	101	18	1.44	1246
2	9	6	2	15	1.14	1321	102	9	0.60	1499
3	2	1	3	3	0.77	391	103	9	1.44	624
4	7	6	4	13	1.57	828	104	7	1.17	596
5	4	2	5	6	0.80	754	105	9	1.13	797
6	5	2	6	7	0.85	821	106	4	0.51	778
7	0	1	7	1	0.22	450	107	6	0.83	721
8	6	9	8	15	1.48	1017	108	10	1.34	746
9	4	2	9	6	1.61	374	109	7	2.40	291
10	3	6	10	9	1.97	456	110	8	1.48	539
11	0	1	11	1	0.17	597	111	8	1.14	702
12	8	3	12	11	1.21	911	112	4	0.50	805
13	2	11	13	13	2.07	628	113	6	1.22	490
14	4	7	14	11	0.96	1149	114	18	1.40	1287
15	2	4	15	6	0.57	1057	115	6	0.65	929
16	4	7	16	11	1.29	854	116	11	1.12	983
17	4	1	17	5	0.57	877	117	12	1.31	918
18	8	5	18	13	1.65	788	118	6	0.80	747
19	3	2	19	5	0.73	687	119	14	1.25	1124
20	11	5	20	16	1.77	905	120	7	1.50	468
21	3	5	21	8	0.76	1058	121	9	0.77	1171
22	6	0	22	6	0.41	1465	122	5	0.37	1351
23	4	2	23	6	0.55	1096	123	8	0.97	827
24	4	3	24	7	0.95	740	124	5	0.50	1010

Table 7. (continued)

Fam. No.	Progeny per family A	B	Dam	Progeny	%Survival	Alevins	Sire	Progeny	%Survival	Alevins
25	6	3	25	9	0.97	925	125	9	0.97	927
26	3	5	26	8	0.89	904	126	8	0.89	901
27	2	2	27	4	0.43	941	127	6	0.58	1041
28	4	3	28	7	0.62	1125	128	5	0.49	1025
29	2	0	29	2	0.59	341	129	7	1.07	653
30	5	0	30	5	1.29	388	130	0	0.00	75
31	1	6	31	7	0.93	753	131	2	0.28	706
32	1	4	32	5	0.49	1017	132	10	0.94	1064
33	1	1	33	2	0.86	231	133	4	1.14	352
34	3	1	34	4	0.71	567	134	2	0.45	447
Total	259			259		27841		259		27841
Avg.	3.81			7.62	0.93	819		7.62	0.93	819

Figure 1. Genetic linkage map of pink salmon based on the inheritance of 85 polymorphic loci in one even-year family (V96-13). Numbers to the left indicate recombination rates (cM). Locus names are to the right.
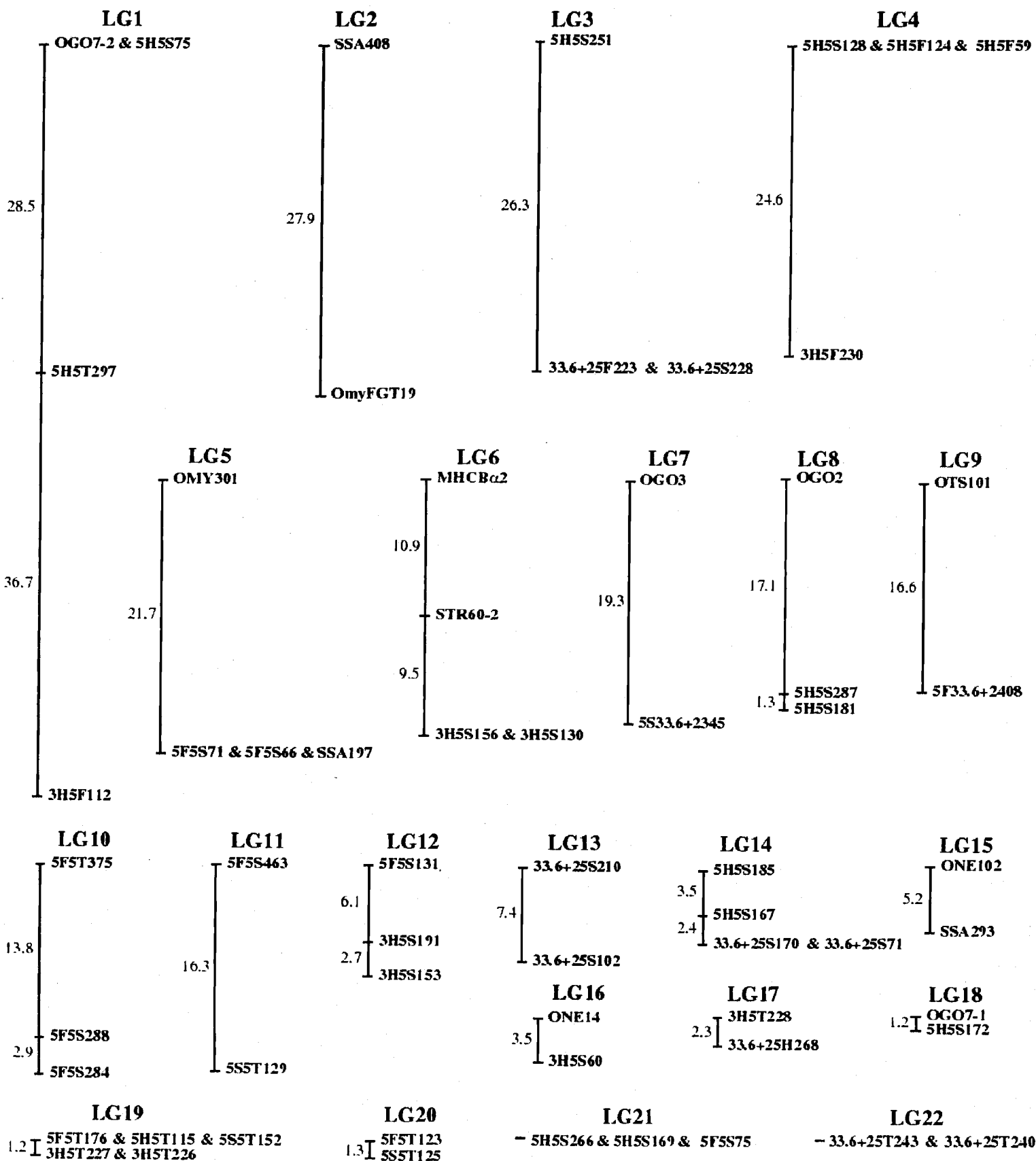
Figure 2. Nautical map of upper Resurrection Bay. Circles indicate where marked pink salmon determined to be part of our study were collected in August and September 2000. Arrow indicates the location of the Alaska SeaLife Center.
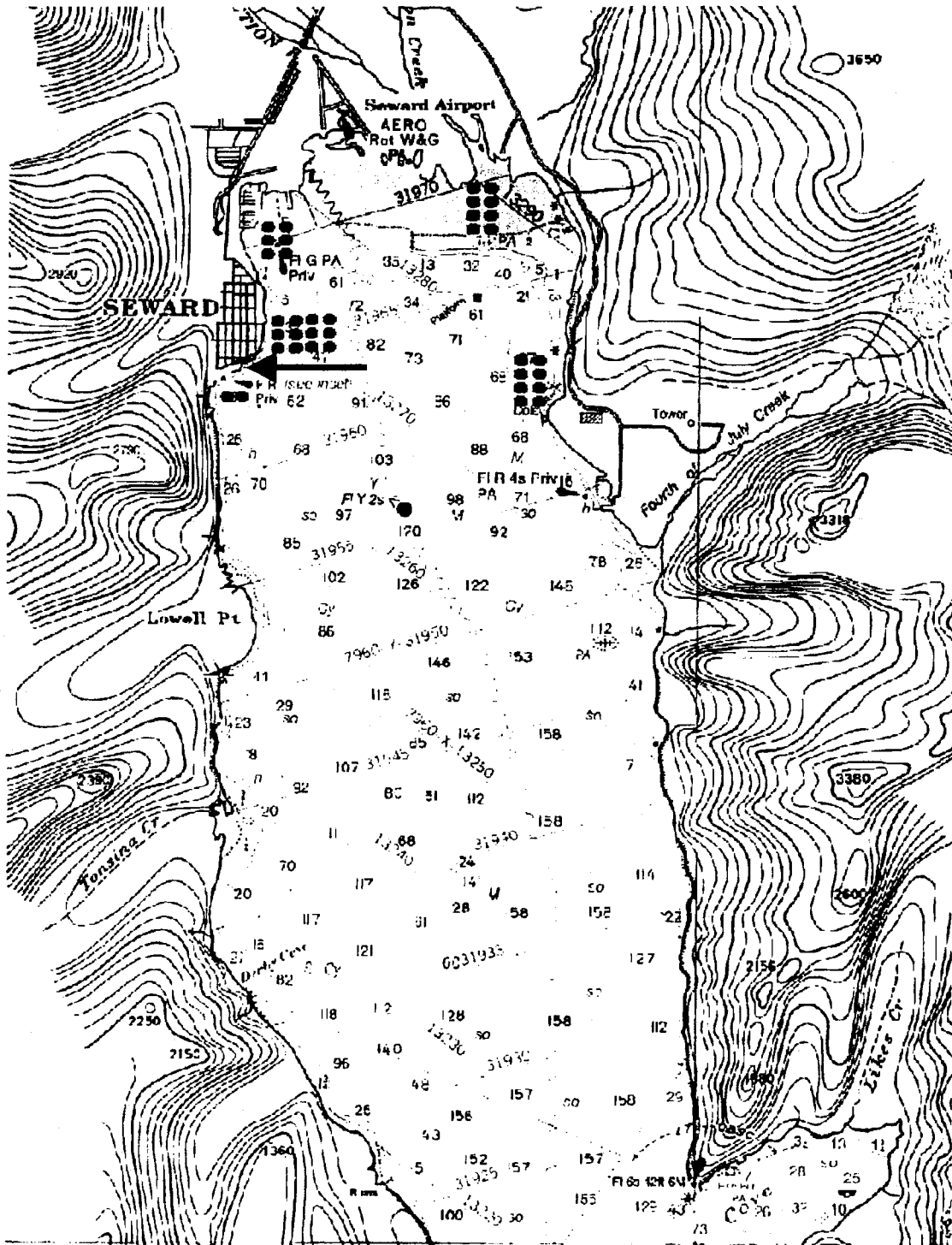
Figure 3. Lengths of marked pink salmon collected from upper Resurrection Bay in August and September 2000.

Females

Males
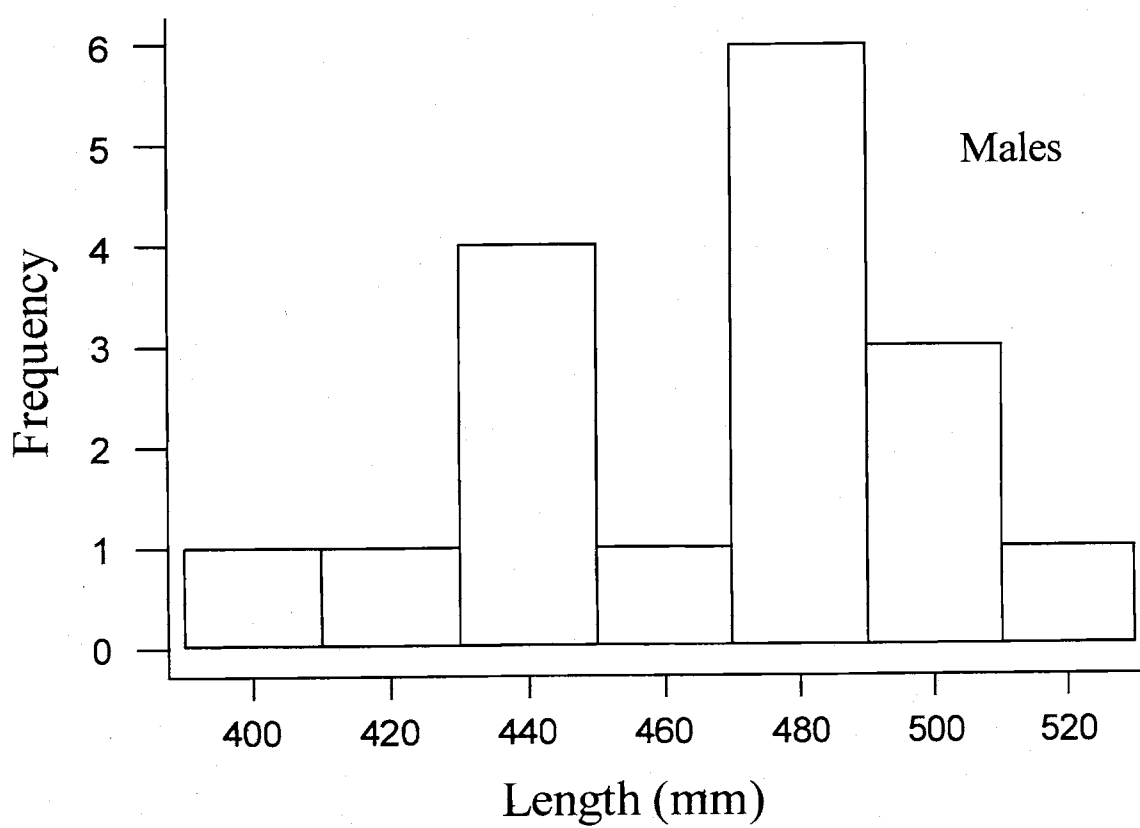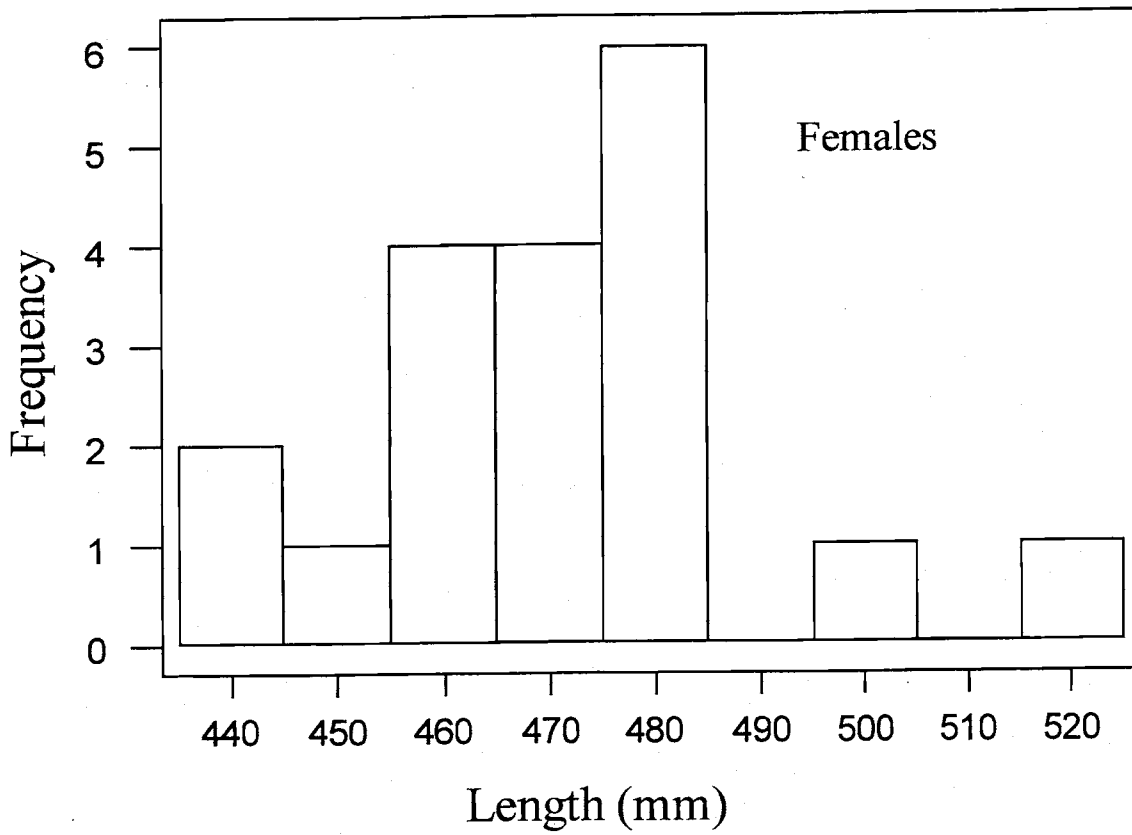
Figure 4. Map of Resurrection Bay. Numbers indicate the location where experimental fish were collected in 2001 as designated below. LC designates Likes Creek, the location where the parents were collected.
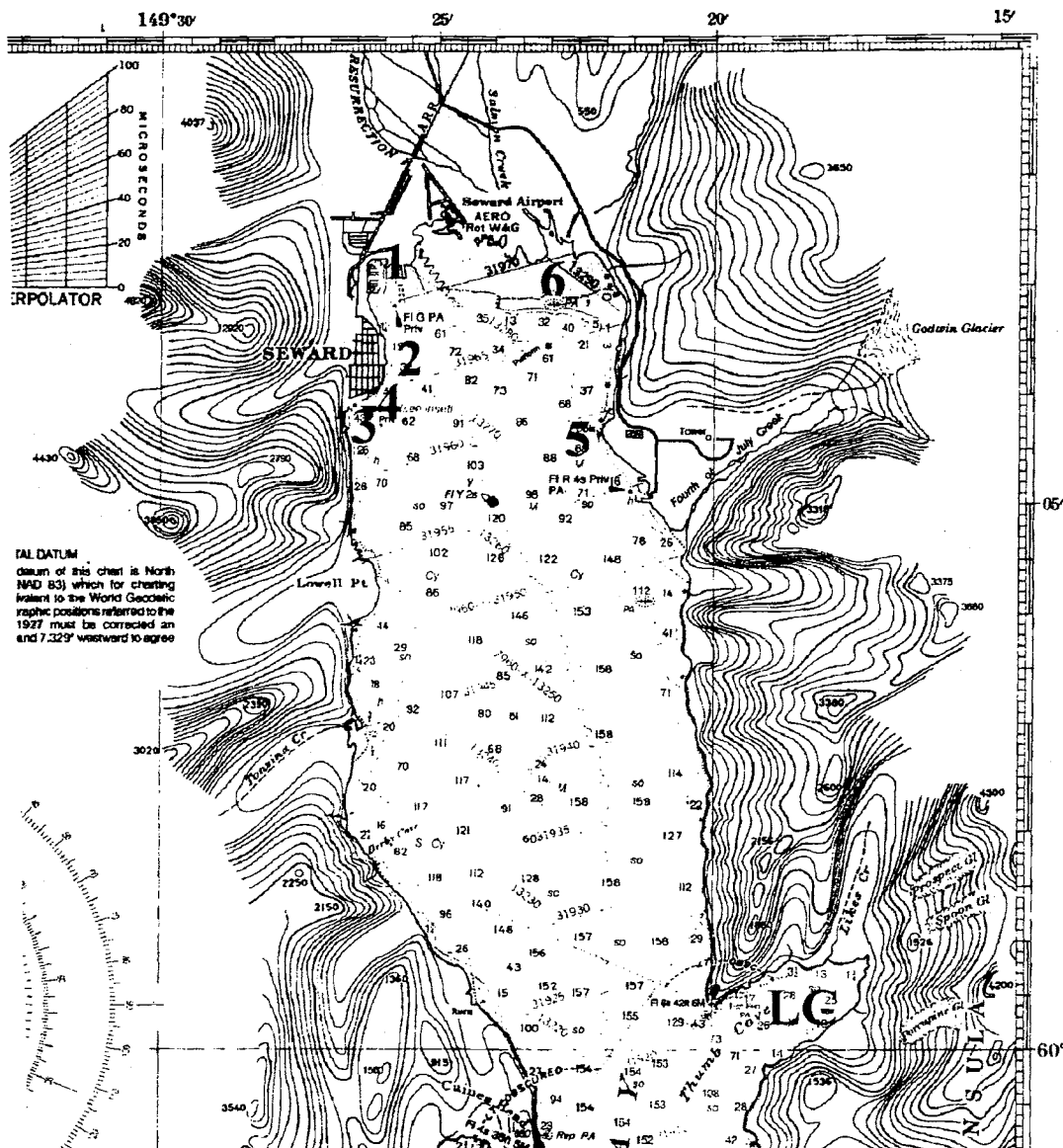
Map Number	Location	Number of fish collected
1	Boat Harbor	2
2	Culvert	31
3	Lowell Cr.	26
4	ASLC	182
5	Spring Cr.	4
6	Resurrection R.	10
	Unknown	4
	Total	259

Figure 5. Number of returns in August 2001 for 67 families.
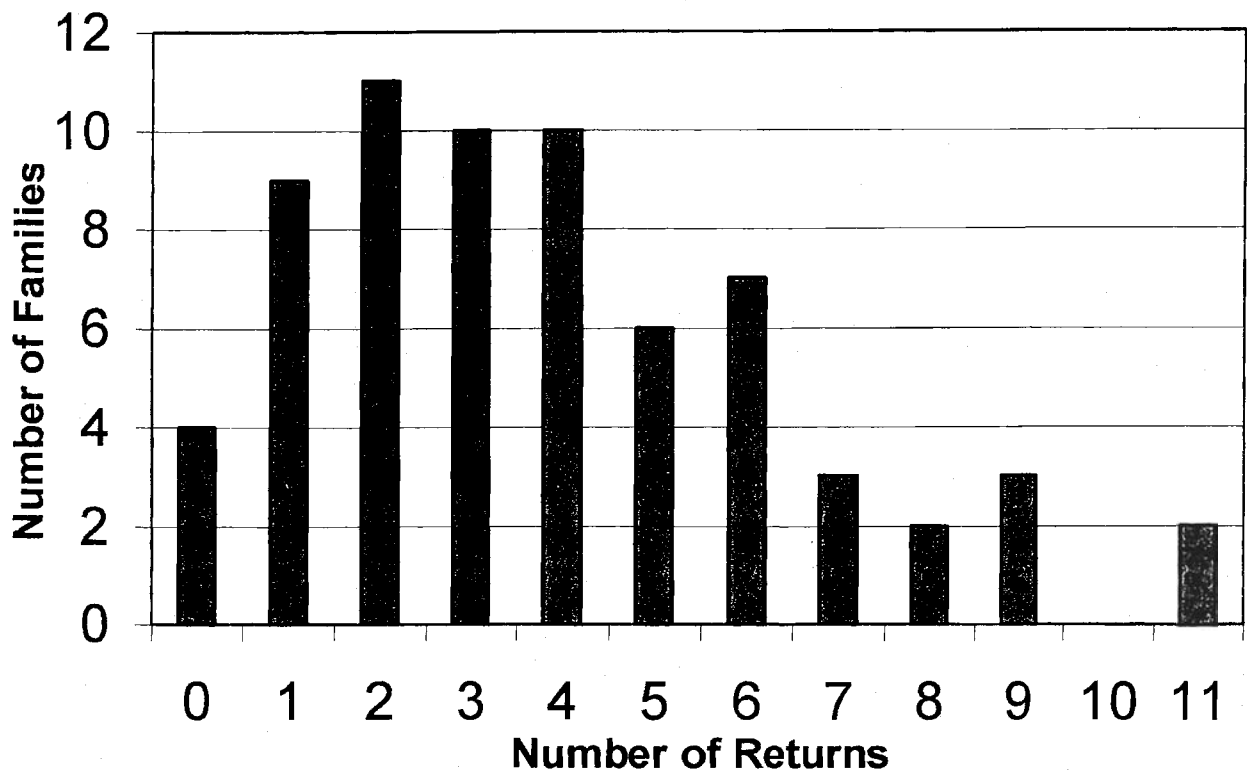
Figure 6. Regression of length of male progeny on mid-parent length (a) and female progeny length on mid-parent length (b) for 1999 cohort.

(a)

$h^2 = 0.38**$
n = 136

Son Length (mm)

Mid-Parent Length (mm)

(b)

$h^2 = 0.41**$
n = 122

Daughter Length (mm)

Mid-Parent Length (mm)

** p < 0.001

46
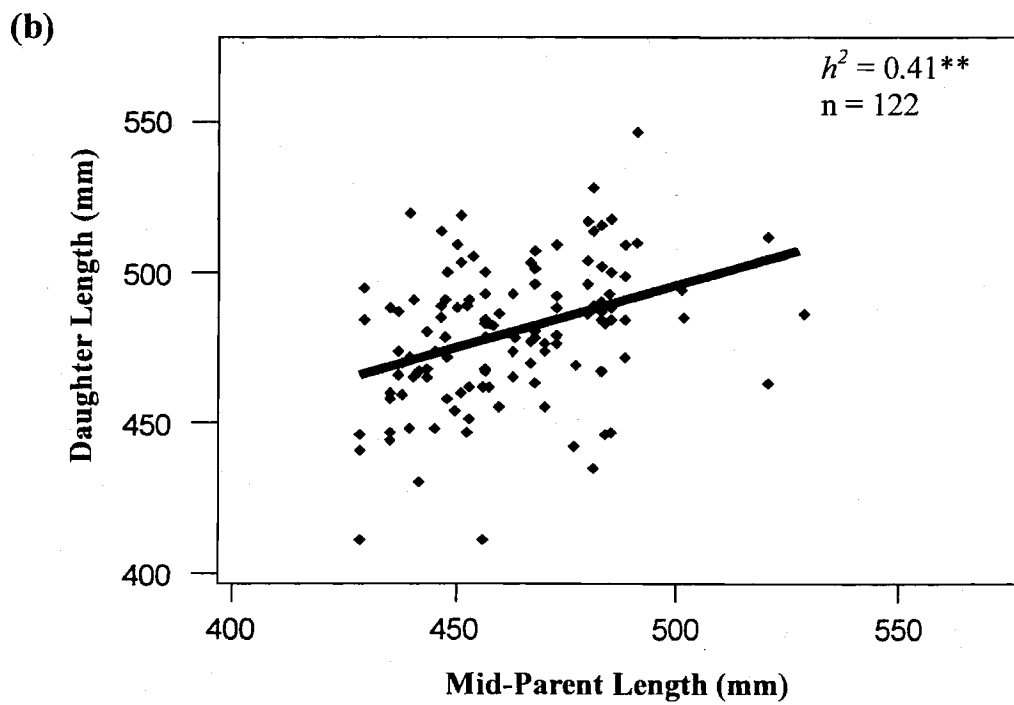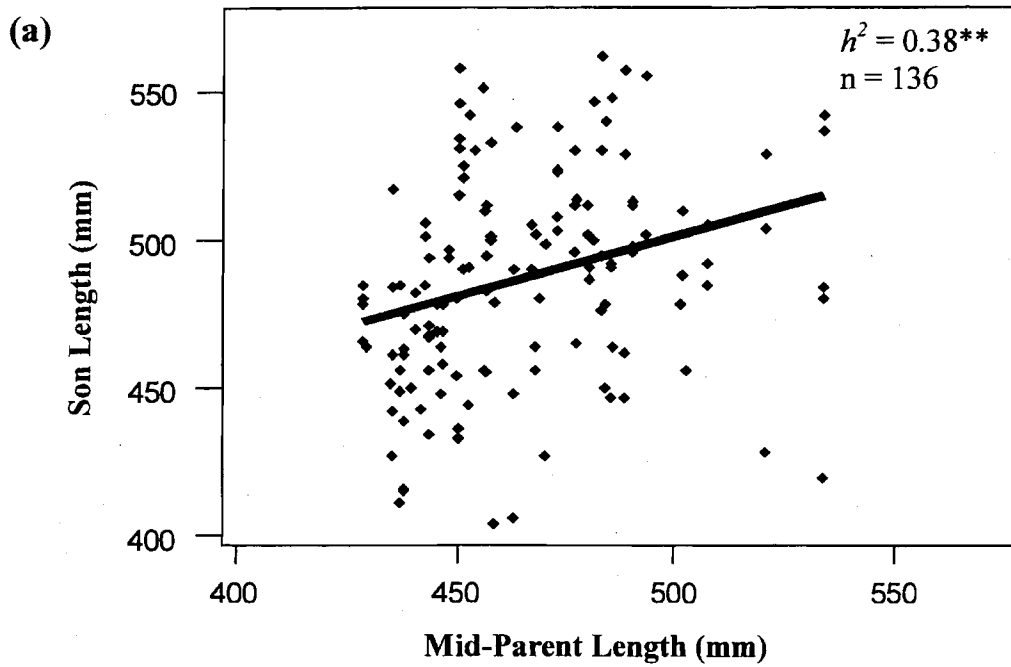
Figure 7. Regression of egg weight (a), total egg weight (b), and number of eggs (c) of daughters on maternal values for 1999 cohort.
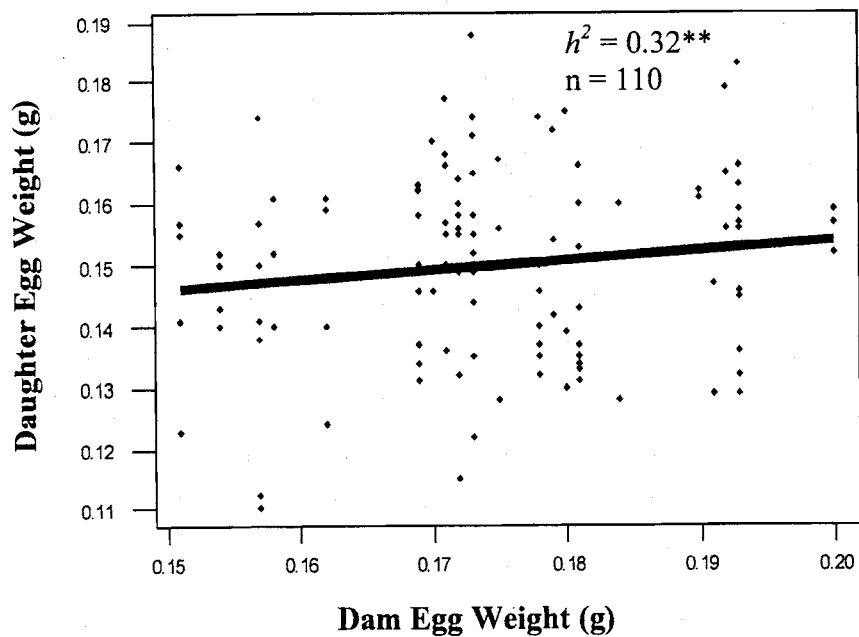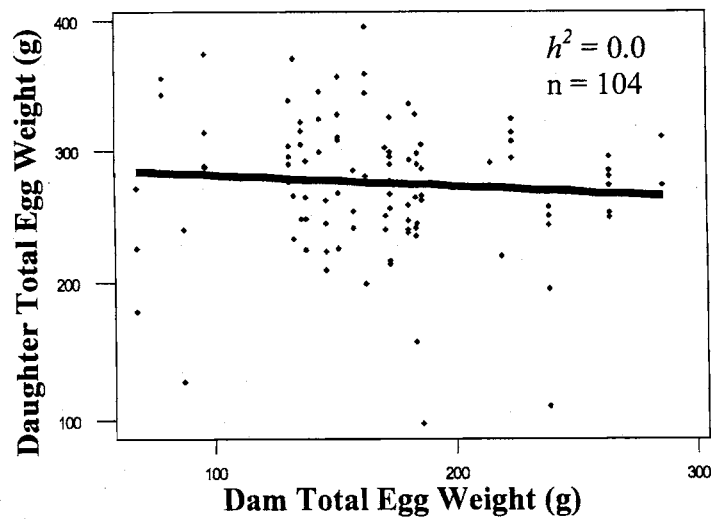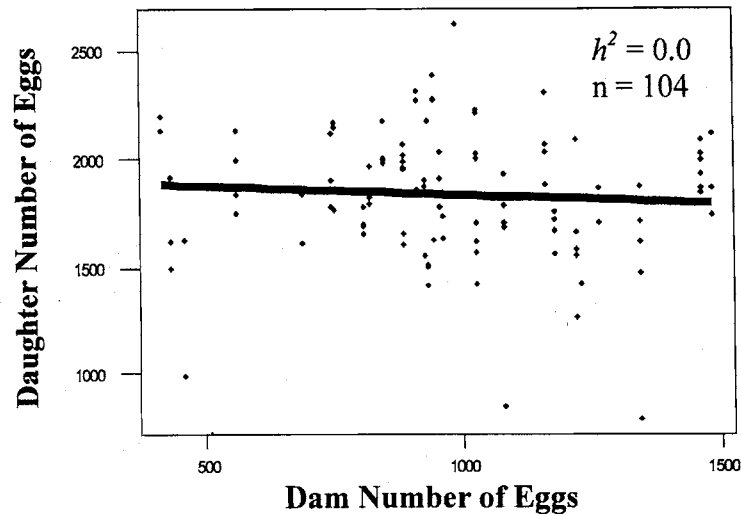
Appendix 1

Inheritance of Nuclear DNA Markers in Gynogenetic Haploid Pink Salmon

P. Spruell, 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

We describe the inheritance of 460 PCR-based loci in the polyploid-derived pink salmon (*Oncorhynchus gorbuscha*) genome using gynogenetic haploid embryos. We detected a length polymorphism in a growth hormone gene (*GH-2*) intron that is caused by an 81 bp insertion homologous to the 3' end of the salmonid short interspersed repetitive element (SINE) *Sma*I. Such insertion polymorphisms within species bring into question the use of SINEs as phylogenetic markers. We confirmed that a microsatellite locus encodes a PCR-null allele that is responsible for an apparent deficit of heterozygotes in a population sample from Prince William Sound. Another set of microsatellite primers amplified alleles of the same molecular weight from both loci of a duplicated pair. In our analysis of several PCR-based multilocus techniques, we failed to detect evidence of comigrating fragments produced by duplicated loci. Segregation analysis of PCR-based markers using gynogenetic haploid embryos ensures that the interpretation of molecular variation is not complicated by heterozygosity, diploidy, or gene duplication. We urge investigators to test the inheritance of polymorphisms in salmonids prior to using them to measure genetic variation.

Fishes of the family Salmonidae comprise a monophyletic group descended from a single tetraploid ancestor (Allendorf and Thorgaard 1984; Behnke 1992). Salmonids have extensive gene duplication at protein loci resulting from this polyploid event (Allendorf and Thorgaard 1984). Studies of DNA sequences have confirmed the presence of many duplicate genes. For example, Agellon et al. (1988) reported duplicated growth hormone genes in rainbow trout (*Oncorhynchus mykiss*), and several other hormones have been found to be encoded by duplicated genes in *Oncorhynchus* species (Hiraoka et al. 1993). In addition, Dautigny et al. (1991) described the sequence divergence between two rainbow trout lysozyme genes.

The polyploid derived genome of salmonids has resulted in complex patterns of segregation and inheritance that have been revealed by the investigation of isozyme loci. Only disomic inheritance has been reported in females. Most loci in males are also inherited disomically. However, some loci show variable patterns of segregation in males, ranging from disomic ratios in some populations to tetrasomic ratios in other populations (Allendorf and Danzmann 1997). The residual

tetrasomic inheritance observed in males apparently results from a two-stage pattern of pairing during male meiosis in which homologous chromosomes pair first followed by homeologous pairing. Disjunction of paired chromosomes occurs so that homologs segregate at the first meiotic division in males. Recombination events between homeologs produce segregation ratios approaching tetrasomic expectations for loci that are distant from their centromere and therefore more likely to be exchanged between homeologs.

The extensive gene duplication in salmonids makes genetic interpretation of molecular variation more difficult than in diploid species. Isoloci (two loci that result from a duplication event and share alleles with identical electrophoretic mobility) are especially problematic and constitute approximately 25% of isozyme markers in rainbow trout (Allendorf and Thorgaard 1984). Individuals have four gene copies at isoloci, and it is difficult to determine how many copies (doses) of a particular allele are present in an individual. In addition, genotypes cannot be determined unambiguously, and there is no way to assign observed variation to a par-

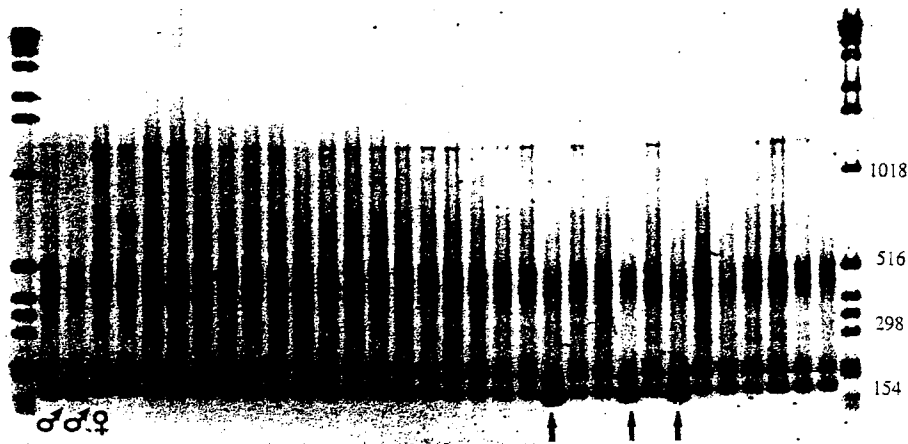**Figure 1.** Growth hormone pseudogene amplification products separated in a 2% agarose gel. Individuals of known sex are indicated by the symbols on the bottom left of the gel. The arrows along the bottom of the gel indicate males that were detected in family 95-103.

ticular locus of the pair without extensive experimental matings (Waples 1988).

There are inherent difficulties in using the polymerase chain reaction (PCR) to study genetic variation. Preferential amplification of alleles at a single locus because of priming site polymorphisms and amplification of multiple paralogous loci are both potentially serious problems. Hare et al. (1996) encountered and discussed these problems in an analysis of anonymous nuclear DNA markers in American oysters (*Crassostrea virginica*). These problems are likely to be even more serious in organisms such as salmonids that, as a result of their polyploid ancestry, have more duplicated loci. PCR primers designed without detailed knowledge of differences between paralogous loci may or may not amplify sequences from both loci. Moreover, even if only one locus is amplified, it will be difficult to ensure that homologous loci are being studied when comparing samples from two populations or two species. The complexities of tetrasomic inheritance and sex-specific recombination in salmonids further confounds these problems.

The complications in interpreting molecular variation in salmonids make it important to test the genetic basis of observed variation with inheritance experiments. Fortunately, external fertilization and well-developed culture systems make salmonids amenable to direct analysis of inheritance. Gametes can be stored and mixed together as desired to produce many full-sib groups from the gametes of a single male or female. In addition, methods of genome manipulation are available to produce large numbers of gynogenetic

diploid and haploid progeny that provide more powerful methods of genetic analysis (Thorgaard and Allen 1987).

Examination of gynogenetic haploids provides an efficient system to test for Mendelian segregation and linkage without the complications associated with diploidy and heterozygosity. For example, the use of haploid embryos avoids the difficulties associated with dominant PCR markers (those in which alleles are expressed as the presence or absence of an amplification product) since recessive alleles are not obscured by their dominant alternatives (Lie et al. 1994). Haploid embryos are not viable; however, they do develop until just prior to hatching (Stanley 1983), providing an embryo from which a sufficient quantity of DNA can be isolated to complete most analyses.

In this article we describe the inheritance of a variety of PCR-based markers in haploid pink salmon (*Oncorhynchus gorbuscha*). These include an intron length polymorphism in a gene-encoding growth hormone, eight microsatellite loci, and over 400 other loci detected by the presence or absence of specific fragments produced by several techniques that amplify multiple fragments from a single set of PCR primers. We also test for the presence of duplicated loci encoding fragments amplified by multilocus PCR-based techniques. The primary objective of this study is to detect and describe hundreds of genetic markers in the pink salmon genome so that we can eventually construct a linkage map that will allow us to better understand the transmission genetics of this polyploid-derived species.

Materials and Methods

Samples and Haploid Gynogenesis

In August 1995, gametes and tissues of 31 pink salmon were collected from the Armin F. Koernig hatchery, Prince William Sound, Alaska. This hatchery stock originated from adult fish collected at several spawning sites in Prince William Sound, Alaska. Seven families of 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 the 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 95-103). Embryos from these families were incubated until just prior to hatching, when they were collected and preserved in ethanol.

Muscle or liver tissue was collected from each parent and embryos were dissected away from the egg chorion and yolk sac. DNA was isolated from these tissues using the Puregene™ DNA isolation kit (Gentra Systems Inc., Minneapolis, MN). The concentration of DNA was determined using a scanning spectrofluorometer. DNA extractions from haploid embryos yielded an average of 45.30 µg of DNA.

Prior to segregation analysis, we screened all putative gynogenetic haploid individuals to eliminate diploids that could be produced by the failure of sperm inactivation. We first used a Y chromosome–specific growth hormone pseudogene (Du et al. 1993, Forbes et al. 1994; primer sequences: 5'-TTTCTCTACGTCTACATTCT-3' and 5'-GTC-TGGCTAGGGTACTCCA-3'; courtesy R. H. Devlin) to identify diploid males. Since haploids were produced by excluding the paternal chromosome complement, any individual containing a Y chromosome must be diploid. Males were identified based on the presence of a 143 bp fragment that is absent in females (Figure 1); males were eliminated from subsequent analyses. Failure of haploid induction could also produce diploid females. To identify diploid females, embryos were screened with six nonduplicated microsatellite loci, described later in this article. Individuals that had more than one allele at any of these loci were excluded from inheritance analysis.

Growth Hormone (GH) Intron

We amplified intron C of *GH-2* using previously described PCR primers and conditions (Forbes et al. 1994). PCR products

were electrophoresed on a 2.0% agarose gel containing ethidium bromide in TAE buffer (Ausubel et al. 1989) and visualized with a Hitachi FMBIO-100 fluorescent imager. PCR amplification products from haploid individuals were purified from agarose gels using the GENECLEAN kit (BIO 101 Inc., La Jolla, CA) and sequenced by direct automated sequencing (Applied Biosystems Inc., Foster City, CA).

Microsatellites

Analysis using seven previously described microsatellite primer sets followed the conditions reported by the original authors with minor modifications. Primers and annealing temperatures are as follows: Fgt-1 and Fgt-4, 51°C (Sakamoto et al. 1994); Oneμ3, 52°C (Scribner et al. 1996); μSat60, 55°C (Estoup et al. 1993); Ots1, 55°C (Hedgecock DE, personal communication); Ssa85 and Ssal97, 57°C (O'Reilly et al. 1996). PCR products were electrophoresed on a 7% denaturing polyacrylamide gel and visualized with a Hitachi FMBIO-100 fluorescent imager.

Randomly Amplified Polymorphic DNA (RAPDs)

RAPD fragments were amplified in a total reaction volume of 10 μl consisting of 10 ng of genomic DNA, 6.7 mM RAPD primer (Operon Technology Inc., Alameda, CA), 4.0 mM MgCl$_2$, 0.2 mM of each dNTP, 1× Stoffel buffer, and 0.25 U Amplitaq DNA polymerase Stoffel fragment (Perkin-Elmer, Norwalk, CT). Thermal cycling was performed in an MJ Research PTC-200 DNA engine. Two cycles of higher stringency PCR were performed with the following thermal profile: denaturation at 96°C for 5 s, annealing at 40°C for 20 s, and extension at 72°C for 30 s. This was followed by 43 cycles with an annealing temperature of 36°C for 20 s and a final extension at 72°C for 2 min. PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized with a Hitachi FMBIO-100 fluorescent imager.

Amplified Fragment Length Polymorphisms (AFLPs)

AFLP restriction/ligation and preselective amplification steps were completed following the Perkin-Elmer/Applied Biosystems AFLP plant mapping protocol, with the modifications outlined below. Thermal cycling was performed in an MJ Research PTC-200 DNA engine. The 10 μl PCR mixture for the selective amplification consisted of 1.5 μl of the preselective ampli-

fication products as DNA template, 0.5 μl EcoRI selective primers, 0.5 μl MseI selective primers, 2 mM MgCl$_2$, 0.1 mM of each dNTP, 2× Amplitaq PCR buffer, and 0.5 U Amplitaq DNA polymerase. The following thermal profile was used for the selective amplification: initial denaturation at 96°C for 2 min followed by a series of 7 cycles with denaturation at 96°C for 1 s, annealing at 65°C for 30 s, extension at 72°C for 2 min. The annealing temperature was decreased by 1°C/cycle for 6 cycles, resulting in a final annealing temperature of 59°C. An additional 30 cycles with an annealing temperature of 59°C for 30 s were also completed. Products were electrophoresed on a 7% denaturing polyacrylamide gel and visualized using a Hitachi FMBIO-100 fluorescent imager.

Paired Interspersed Nuclear Element (PINE) PCR

PCR amplification of anonymous DNA fragments flanked by SINEs (short interspersed elements) and the Tc1 transposon was conducted in a total volume of 10 μl. Primers were designed on the basis of published sequences (Table 1). Each reaction contained approximately 20 ng of genomic DNA, 1 μl 1× Perkin-Elmer PCR buffer, 2.5 mM MgCl$_2$, 0.2 mM of each dNTP, 0.38 pM labeled primer, and 0.5 U Perkin-Elmer AmpliTaq DNA polymerase Stoffel fragment. Reactions were completed in an MJ Research thermocycler using an annealing temperature of 60°C. Products were electrophoresed on a 4.5% denaturing polyacrylamide gel and visualized using a Hitachi FMBIO-100 fluorescent imager.

Nomenclature

A standard method for naming microsatellite loci in salmonids has been informally adopted (see Olsen et al. 1996). Primers are typically named after the species from which they are derived: Ssa (Atlantic salmon, Salmo salar) and Ots (chinook salmon, Oncorhynchus tshawytscha). Some microsatellites were named prior to the advent of this standardized nomenclature. The μSat60 primers were isolated from brown trout (Salmo trutta) and the Fgt1 primers

were isolated from rainbow trout but named fish GT-repeat. Locus names are the primer pair name in uppercase and italics (e.g., OTS1) to make them analogous to the nomenclature for allozyme loci (Shaklee et al. 1990). The nomenclature for duplicated loci follows the format used for duplicated allozyme loci (Shaklee et al. 1990). For example, FGT1-1,2 designates isoloci produced by the Fgt-1 primer set.

We followed nomenclature used for zebrafish (Brachydanio rerio) in naming loci for RAPD markers (Johnson et al. 1996). The name consists of the name of the 10 nucleotide long primer followed by the approximate size of the amplification product. Thus the locus 20A.760 is amplified by primer A20 and results in a 760 bp amplification product.

The nomenclature for AFLPs is consistent with Young et al. (1998) in their description of a rainbow trout linkage map. The names start with the three base selective primer extensions used to produce the loci and end with the length of the fragment measured in base pairs (e.g., AAA/CAT250).

PINE loci are named using a number designating the end of the element from which the primer was derived (3′ or 5′) followed by a one-letter designator for the element from which the primer was derived. If more than one primer was used during the amplification the primers are placed in alphabetical order. The primer designations are followed by the length of the fragment. For example, the locus 5F3H250 amplifies a 250 bp fragment using a primer sequence from the 5′ end of the FokI SINE and the 3′ end of the HpaI SINE.

Alleles are designated as *p for the presence of a product and *a for the absence of a product for multifragment PCR-based techniques (RAPDs, AFLPs, and PINEs). Alleles that differ in length are designated by a number representing their size. For example, 5F3H250*270 designates an allele encoding a 270 bp fragment at a PINE locus at which the common allele encodes a 250 bp fragment.

```
Sockeye     GTAAGTTACCGGGCTGAGACAATCCTCCATGATGCACAATTCCAACATGAATAATAGGGC   60
GH2*C446    .........T..................................................   60
GH2*C527    .........T..................................................   60

Sockeye     ATCTCAAGTTGAACAATCGATACAACTTAGTCATTAGTTATTGGGCAAGCAGATCCCCGA  120
GH2*C446    .......T....................................................  120
GH2*C527    .......T....................................................  120

Sockeye     TTGTCTAAACTCCATGGGTAAATATATACTGTAGATAAGAAGAACCAGCATCATGCATGG  180
GH2*C446    ............................................................  180
GH2*C527    ............G...............................................  180

Sockeye     TAGAAATTAAATCTAGCCATGACAGGGAGTTTTAAATTGTACACTTAAAA-TCGGCAGGA  239
GH2*C446    .G................................................A.........  240
GH2*C527    .G................................................A.........  240

Sockeye     AAATGTTGCTATACCTCAGTGCCTTCAAAAACAACCACATGTCATAGTCCTTGTAAGTAA  299
GH2*C446    .............A........................T.......A.............  300
GH2*C527    .............A........................T.......A.............  300

Sockeye     AACCCATCACTCTCTAATCGGCGGTTTCTCTACGTCTACATTCTCCAGCAATGTGTCATG  359
GH2*C446    ..................T.......................G.........A.....    360
GH2*C527    ..................T.......................AG........A.....    360

Sockeye     TAAA                                                         363
GH2*C446    ....                                                         364
GH2*C527    .... TAATA-TAATAATA-TAATAATATATGCCATTTAGCAGACGCTTTTATCCAAAGC  417
Sma I            TAATAATAATAATAATA-TAATATATGCCATTTAGCAGACGCTTTTATCCAAAGC  105

Sockeye                                  TGATATGGCATCTCAAGCTGTACAATTACAA  394
GH2*C446                                  ..............................  395
GH2*C527    GACTTACAGTCATGTGTGCATACATAAA ..............................  476
Sma I       GACTTACAGTCATGTGTGCATACATTCT

Sockeye     CTCAACTTCATTTTCTAATAATCTGTGGTTTCTCTACATCTACACACACAG          445
GH2*C446    ...................................................          446
GH2*C527    ...................................................          527
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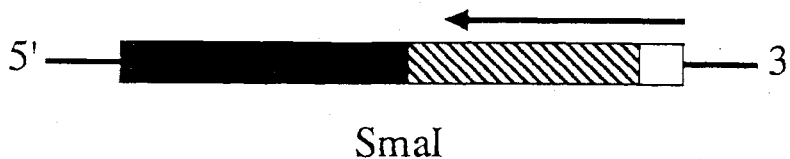
SmaI

Figure 2. Aligned sequences of *GH-2* intron C from sockeye (Devlin 1993) and pink salmon. The 81 bp insert found in *GH2*C527* is indicated by the dark bars in the sequence. The complete *SmaI* element is shown below the sequences. The region that corresponds to the 81 bp insert and its orientation relative to the *GH-2* gene is denoted by the arrow above the element. The solid shaded area corresponds to the tRNA-related region, the hatched region corresponds to the tRNA-unrelated region, and the open region is the AT rich region (Okada 1991).
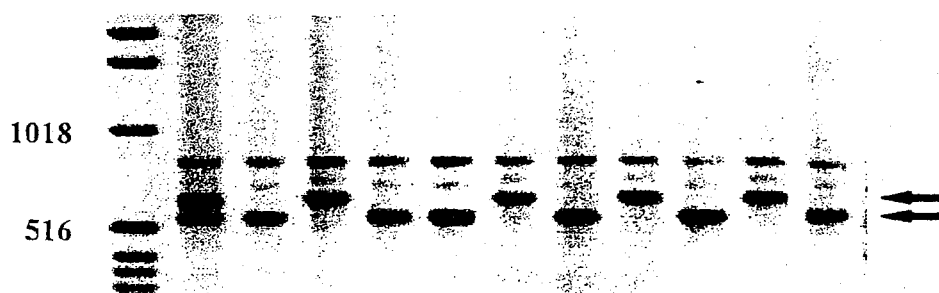
Figure 3. Segregation of *GH-2* polymorphism. The individual denoted by the female symbol is female 95-105, the 10 individuals to the right are haploid offspring from this female segregating for the *GH-2*C446* and *C527* alleles indicated by the arrows.

Results

Growth Hormone

The *GH-2* intron C primers gave products of two different lengths (446 bp and 527 bp; Figures 2 and 3). We designated the two alleles that produce these fragments as *GH-2*C446* and *GH-2*C527* according to the nomenclature guidelines for protein-coding loci in fish (Shaklee et al. 1990). Sequencing revealed that this length difference is caused by an 81 bp insert that is nearly identical to the 3' end of the consensus sequence of the *SmaI* SINE in pink salmon (Kido et al. 1991; Figure 2).

Seven of the 31 adults were heterozygotes at this locus and the remaining 24 were *C446* homozygotes. Two females, 95-105 and 95-115, were heterozygous at this locus; their 72 haploid progeny displayed the expected 1:1 Mendelian segregation for these alleles.

Microsatellites

The seven microsatellite primer sets examined were polymorphic in the 31 adult fish. Four microsatellite loci (*FGT4, ONEμ3, OTS1,* and *μSAT60*) were in Hardy–Weinberg proportions in the adult fish (Table 2) and exhibited expected Mendelian segregation (e.g., Table 3). *SSA85* exhibited expected Mendelian segregation, but was not used in the analysis of adult fish because genotypes were difficult to score. This locus had a minimum of 12 alleles in the adult fish and each allele produced multiple "stutter" bands (Hayashi 1994; Litt and Luty 1989). The overlapping patterns of these additional products makes unambiguous identification of alleles impossible when alleles of similar size are present. The alleles in the heterozygous females were sufficiently different in size so that there was no overlap in the patterns generated in haploids from a single female.

One of the microsatellite primer sets (Fgt1) produced phenotypes that indicated more than two alleles in diploid individuals and more than one allele in haploids. *FGT1-1,2* has previously been described as duplicated isoloci in sockeye salmon (*Oncorhynchus nerka*; Allendorf et al., submitted) and rainbow trout (Young et al. 1998). Inheritance results confirm that *FGT1-1,2* are also isoloci in pink salmon (Table 4). This is seen most clearly in the progeny from female 95-106 who possessed both the *155 and *157 alleles. All 37 of her progeny received both alleles, thus she must have been homozygous at both *FGT1* loci (-1 and -2), as indicated in

1-5

Locus	No. individuals	No. alleles	Heterozygosity		F
			Observed	Expected	
FGT4	31	2	0.290	0.398	0.271
ONEμ3	31	3	0.548	0.505	−0.085
OTS1	31	8	0.806	0.791	−0.019
μSAT60	31	4	0.290	0.414	0.300
SSA197	29	15	0.586	0.912	0.361***
SSA197ª	31	16	0.935	0.885	−0.056

ª Including the null allele.

*** $P < .001$.

F is the fixation index (the proportional excess of heterozygotes).

Table 4. This locus was not scored in the sample of adult fish because of the problems in scoring doses at isoloci (Allendorf et al., submitted).

PCR amplification of SSA197 produced 15 alleles and a highly significant excess of apparent homozygotes in the adult fish (Table 2). In addition, no PCR products were detected in 2 of the 31 fish. The cause of these results became clear in the segregation experiments. All four single-banded females for which we examined haploid progeny were actually heterozygotes for a PCR-null allele (SSA197*a) that produced no amplification product (Table 5). Approximately half of the progeny from each of these females had the same fragment as the mother, while the other half produced no PCR product (Figure 4). PCR products were detected at all other loci in the two adults and all haploid progeny that contained the null, eliminating the possibility that these results were caused by poor-quality DNA samples.

We reanalyzed the genotypes at this locus in the 31 adult fish including the null allele. We assumed that all apparently homozygous fish were heterozygotes for a null allele and the two fish lacking product were null homozygotes (SSA197*a/a). The estimated frequency of SSA197*a under these assumptions is 0.258 and the observed genotypic proportions do not differ from Hardy–Weinberg expectations (Table 2).

Multilocus Primer Sets

We screened 140 RAPD primers or primer pairs in the haploid progeny from female 95-103. Each primer set produced approximately 5–8 fragments from 400 to 1500 bp. We detected 36 repeatable presence/absence polymorphisms amplified by 25 RAPD primers. All of these markers demonstrated Mendelian segregation in 94 haploid progeny from female 95-103.

We screened 77 AFLP primer combinations in the haploid progeny from female 95-103. Each AFLP primer combination produced at least 30 bands ranging from 50 to 600 bp. We selected 43 primer combinations that amplified 284 clear polymorphisms that segregated in 94 progeny from female 95-103. Almost all of the AFLP polymorphisms were presence/absence differences. However, four of the polymorphisms appeared to be caused by a length polymorphism within a fragment. For all of these polymorphisms, individuals had one of two different-size fragments produced by the same primer combination.

We used DNA sequences of salmonid-specific SINEs and the transposon Tc1 as primers to generate multiple DNA fragments from a single PCR. This procedure is similar to the use of the human SINE *Alu1* to identify human chromosomes in somatic cell hybridization experiments (Nelson et al. 1989). Primers identical to one end of the element are oriented such that they initiate DNA synthesis from the end of the element, progressing into the

surrounding genomic DNA. A single primer or pairs of primers may be used to generate multilocus patterns (Greene and Seeb 1997).

A minimum of 30 fragments is amplified by each combination of primers (Figure 5). We have scored 94 haploid offspring from female 95-103 with 16 PINE primer combinations that produce a total of 131 polymorphic loci. In six cases it appears that PINE fragments are segregating as co-dominant alleles that vary in length (Figure 5).

Discussion

The examination of haploid embryos is a powerful tool for segregation analysis (Slettan et al 1997). It allows the unambiguous detection of the transmission of recessive alleles to progeny. Similarly it allows the direct detection of PCR-null alleles at microsatellite loci, such as SSA197*a. Haploid progeny also facilitates the direct sequencing of allelic variants without the problems of heterozygosity (either known or cryptic).

The AFLP technique is particularly well suited for use with haploid embryos. Two properties of AFLPs maximize the information that can be obtained from the limited DNA available. First, many bands are produced per reaction and therefore more polymorphic loci are produced per PCR amplification. Second, the selective amplification step uses a subsample of the PCR products of the preamplification. Up to 133 selective amplifications can be completed from a single preamplification that originally used only 0.5 μg of genomic DNA. This is particularly important when using haploids in which there is little tissue, limiting the amount of DNA available for analysis. Much more genomic DNA is needed to produce fewer bands using other methods such as RAPDs.

Segregation of Recessive Alleles

The occurrence of isoloci makes the interpretation of recessive markers in salmo-

Table 3. Inheritance of OTS1 in gynogenetic haploid progeny.

Female		Progeny phenotype						
Number	Genotype	218	220	222	224	226	228	230
91-101	222/226	—	—	22	—	17	—	—
95-102	218/224	21	—	—	16	—	—	—
95-103	224/230	—	—	—	53	—	—	38
95-104	224/224	—	—	—	39	—	—	—
95-105	220/226	—	20	—	—	15	—	—
95-106	220/224	—	21	—	17	—	—	—
95-115	226/228	—	—	—	—	17	18	—

Table 4. Inheritance of FGT1-1,2 isoloci in gynogenetic haplod progeny.

Female		Progeny phenotype			
Number	Phenotype	155	155/157	157	Female genotype
95-101	155/157	—	20	19	155/157 157/157
95-102	155/157	20	16	—	155/155 155/157
95-103	155/157	—	44	47	155/157 157/157
95-104	155/157	—	24	14	155/157 157/157
95-105	155/157	—	18	18	155/157 157/157
95-106	155/157	—	37	—	155/155 157/157
95-115	155/157	17	19	—	155/155 155/157

1–6

Female Number	Phenotype	Progeny phenotype									Female genotype
		130	142	146	154	162	164	190	196	Null	
95-101	146/162	—	—	21	—	18	—	—	—	—	146/162
95-102	162	—	—	—	—	15	—	—	—	20	162/a
95-103	164	—	—	—	—	—	46	—	—	45	164/a
95-104	196	—	—	—	—	—	—	—	22	17	196/a
95-105	130/154	41	—	—	22	—	—	—	—	—	130/154
95-106	142	—	20	—	—	—	—	—	—	18	142/a
95-115	154/190	—	—	—	24	—	—	12	—	—	154/190

nids problematic. Observed segregation patterns may result from a pair of isoloci (e.g., LOCUS-1,2) that are both heterozygous for alleles associated with the presence or absence of a particular fragment (LOCUS-1*p/a; LOCUS-2*p/a). In this case we expect a 3:1 presence-to-absence ratio of the fragment in haploid progeny (25% p/p:50% p/a:25% a/a). In addition, this same ratio results if a female is heterozygous (*p/a) at two nonhomologous loci that happen to produce fragments of the same size.

It is difficult to distinguish between a 1:1 and 3:1 ratio for an individual fragment except with very large sample sizes. However, the presence of such pairs of loci segregating 3:1 for the presence or absence of a fragment should affect the observed segregation ratios. In the absence of any such cases, we expect our observed segregation ratios to fit a binomial distribution with an expectation of 0.5 (1:1 segregation). The presence of markers segregating 3:1 should result in a "shoulder" in the distribution at a value of 0.75. There is perhaps a slight excess of loci segregating with a value of 0.6 or greater at 451 loci segregating from female 95-103 (Figure 6). We conclude that fragments segregating 3:1 represent at most a small fraction of the total fragments that we have examined.

PINEs

SINEs and transposons occur in high copy number and are believed to be ubiquitously dispersed throughout the genomes of many species (Okada 1991). These characteristics make PINEs potentially valuable tools for genomic mapping efforts. Unlike other multilocus techniques, the primers used to generate PINEs are based on repetitive elements known to exist in the salmonid genome. In addition to generating markers, the inclusion of PINEs in our mapping efforts may also increase our understanding of SINEs and transposons in the salmonid genome.

Others have used the presence or absence of families of SINEs or specific SINEs to make phylogenetic inferences (Kido et al. 1991; Murata et al. 1993, 1996). However, the mechanisms of SINE amplification are not entirely known and evidence is accumulating that the genomic distribution of SINEs may be more complex than previously believed (Spruell and Thorgaard 1996; Takasaki et al. 1997; Young et al. 1998).

The insertion that we have described in intron C of GH-2 corresponds to the 3′ end of the SmaI element (Figure 2). This result is consistent with the observations of Spruell and Thorgaard (1996) and Young et al. (1998) who suggested that the sequences corresponding to some regions of

SINEs may be distributed independently of the remainder of the element. Moreover, this GH-2 insertion is not present in other Oncorhynchus species for which this intron has been sequenced (Figure 1; O. mykiss, Agellon et al. 1988; O. nerka, Devlin 1993; O. kisutch, Forbes et al. 1994; O. keta, Shen et al. 1993). Thus the insertion seen in the GH-2*C446 allele apparently occurred after pink salmon diverged from other Oncorhynchus species.

This pink salmon–specific insertion is unexpected if the amplification of SmaI and SmaI-related sequences occurred in a common ancestor of pink and chum (Oncorhynchus keta) salmon as proposed by Kido et al. (1991). Takasaki et al. (1997) also report a lack of concordance between the presence or absence of specific SmaI elements within pink and chum salmon. They propose several possible explanations for this phenomenon. Among these is the possibility of temporal differences in amplification within lineages. If this hypothesis is correct, the possibility of insertion polymorphisms within species must be addressed before these elements are used as phylogenetic markers. These studies frequently use a single individual to represent an entire taxon, providing no possibility to detect such polymorphisms.

Gene Duplication

Duplicated loci are extremely difficult to use for population genetic analysis. Accurate estimation of allele frequencies at isoloci requires determining the numbers of copies of each allele in individuals (Waples 1988). Isoloci at allozymes are routinely used for population genetic analysis. This is possible because there is a correspondence between band intensity and doses of an allele present in allozymes (Allendorf and Danzmann 1997; Shaklee and Phelps 1992). In addition, the presence of heteromeric isozymes and tissue specificity of many loci aid in estimating doses for enzymes (Waples 1988).

It is difficult to determine how many doses of each allele are present in PCR-based techniques because the amount of amplified product may not accurately reflect allelic doses (Wagner et al. 1994). The many alleles present at most microsatellite loci will also make analysis and allele frequency estimation much more difficult. For a tetrasomic locus with n alleles, there are $(n + 3)!/(n − 1)!4!$ different genotypes (Hartl and Clark 1989, p. 610). Thus there are 330 possible genotypes at OTS1 with eight alleles. May et al. (1997) recently suggested a method for estimating doses

160

140

♀

120
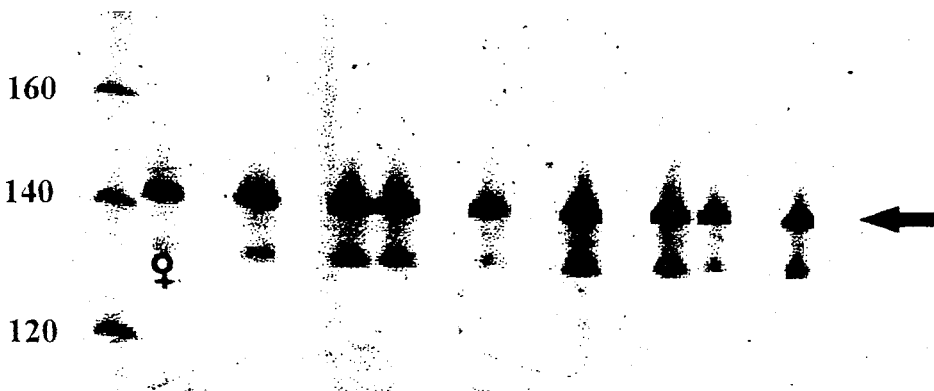
Figure 4. Segregation of a "null" allele (no PCR product is amplified) at SSA197. The individual denoted by the female symbol is female 95-106, the 14 individuals to the right are haploid offspring from this female. The arrow indicates allele SSA197*142.
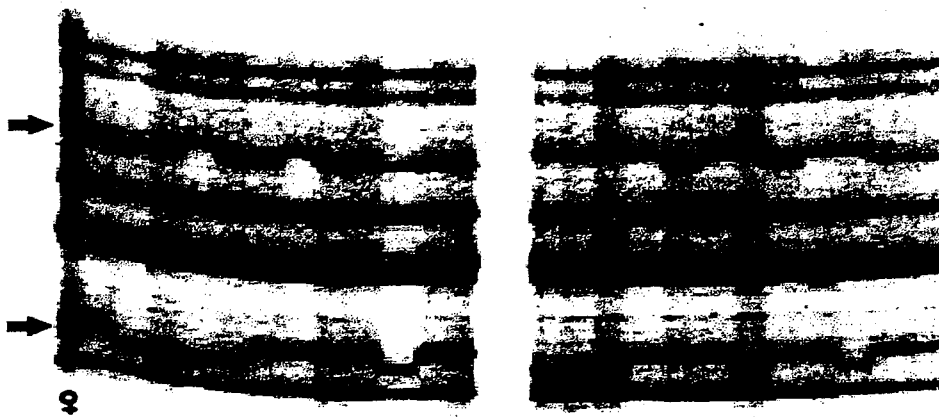
Figure 5. Hpa 3' and Tc1 PINE primer amplification products separated on a 4.5% polyacrylamide gel. Female 95-103 is indicated. The two arrows along the left side of the gel indicate length polymorphisms: top bands 181/182 bp, bottom bands 166/167 bp.

at microsatellite loci that may be helpful when working with duplicated loci.

Perhaps the best way to deal with duplicated microsatellite loci in salmonids is to not use them for population genetic analysis. There are enough microsatellite markers available to obtain a sufficient number of markers without using duplicated microsatellites. Approximately 25% of isozyme markers in rainbow trout are encoded by isoloci (Allendorf and Thorgaard 1984). We would expect the proportion of microsatellites encoded by isoloci to be somewhat less than this since their higher mutation rate will cause more rapid

divergence between alleles at two loci that are no longer undergoing residual tetrasomic inheritance (Allendorf and Danzmann 1997). Nevertheless, the process of diploidization in salmonids is incomplete and we would expect recombination between homeologs to transfer alleles between some microsatellite loci (Allendorf and Danzmann 1997).

Duplicated microsatellite loci in salmonids can be used in many applications (e.g., paternity and kinship analysis). However, it is critical that the inheritance of such loci be tested in the population being investigated because of PCR null alleles
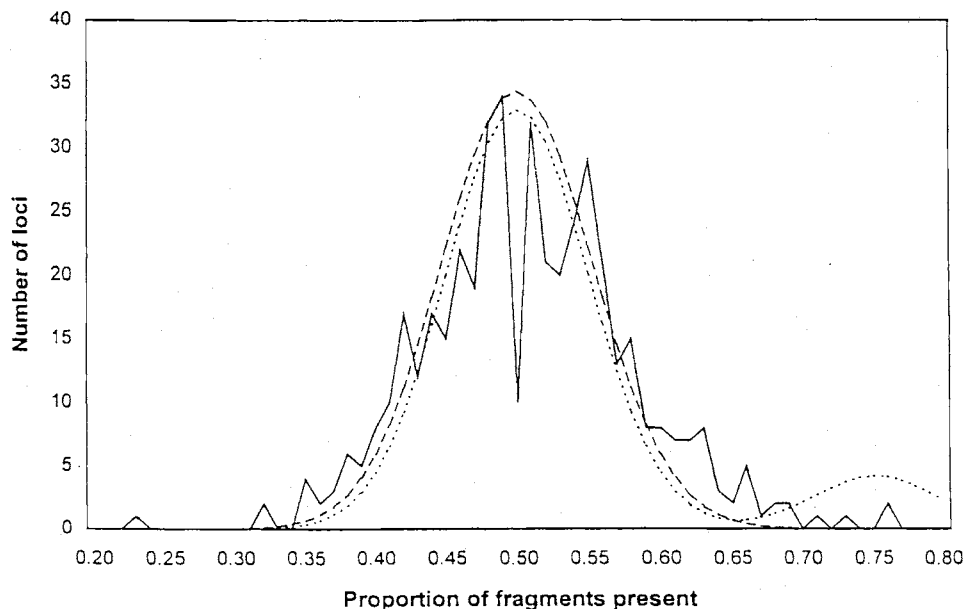
and the possibility of residual tetrasomy in some populations and not others (Allendorf and Danzmann 1997).

The many nuclear DNA markers available offer a wealth of opportunities for greatly improving our understanding of the transmission and population genetics of salmonids. Nevertheless, problems in genetic interpretation are in some ways greater in the direct examination of DNA itself using PCR than in the study of genetic variation in proteins. Allozyme electrophoresis only detects functional genes so that pseudogenes are not a complication. Moreover, the tissue-specific expression of protein loci has been used to identify specific loci within sets of paralogous loci (Ferris and Whitt 1979). For example, only one of the two paralogous duplicates of the vertebrate lactate dehydrogenase B gene (LDH-B) in salmonids is expressed in liver tissue, and the product of the other paralogous locus predominates in heart tissue. This pattern of expression has been conserved over a long period of evolutionary time and is shared among all species of two of the three subfamilies of salmonids: Thymallinae (grayling) and Salmoninae (trout, salmon, and char) (Allendorf and Thorgaard 1984). This consistent pattern of expression makes it easy to identify each of the two paralogous LDH-B loci in salmonids. However, there are an insufficient number of polymorphic allozymes for many applications. PCR-based markers are virtually unlimited in number but are amplified solely on the basis of DNA sequences present, therefore differentiation of pairs of paralogous loci is impossible without inheritance data.

The analysis of gynogenetic haploids is a powerful tool for understanding the transmission of genetic markers in salmonid fishes. Population frequencies of PCR-amplified gel bands alone will not be adequate to understand the genetic basis and significance of observed variation in salmonids. We urge investigators to use inheritance studies to confirm the genetic basis of observed polymorphisms in salmonids whenever possible.

Figure 6. Distribution of segregation ratios for 451 fragments in haploid progeny from female 95-103 (solid line). The dashed line is the expected binomial distribution with an expected value of 0.5 (1:1 segregation). The dotted line is the expected binomial distribution for 90% of the fragments segregating 1:1 and 10% of the fragments segregating 3:1.

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Appendix 2

Gene–centromere mapping of 312 loci in pink salmon by half-tetrad analysis

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Abstract: We estimated recombination rates between 312 loci and their centromeres in gynogenetic diploid pink salmon (*Oncorhynchus gorbuscha*) that we produced by initiating development with irradiated sperm and blocking the maternal second meiotic division. Amplified fragment length polymorphisms (AFLPs) were significantly more centromeric than loci identified by three other techniques (allozymes, microsatellites, and PCR using primer sequences from interspersed nuclear elements). The near absence of AFLPs in distal regions could limit their utility in constructing linkage maps. A large proportion of loci had frequency of second division segregation (y) values approaching 1.0, indicating near complete crossover interference on many chromosome arms. As predicted from models of chromosomal evolution in salmonids based upon results with allozyme loci, all duplicated microsatellite loci that shared alleles (isoloci) had y values of nearly 1.0.

Key words: meiosis, AFLP, microsatellites, isoloci, *Oncorhynchus gorbuscha*.

Résumé : Les auteurs ont mesuré les taux de recombinaison entre 312 loci et leurs centromères respectifs chez un saumon rose (*Oncorhynchus gorbuscha*) diploïde et gynogénétique qui a été produit en initiant le développement à l'aide de sperme irradié et en empêchant la seconde division méiotique maternelle. Les marqueurs AFLP (polymorphisme de longueur de fragments amplifiés) ont montré une localisation beaucoup plus centromérique que ceux identifiés au moyen de trois autres techniques (alloenzymes, microsatellites et amplification PCR avec des amorces spécifiques de séquences nucléaires répétitives dispersées). La quasi absence de marqueurs AFLP dans les régions distales pourrait limiter leur utilité en vue de l'établissement de cartes génétiques. Une forte proportion des loci présentait des valeurs de y s'approchant de 1,0 indiquant par là une interférence presque complète des recombinaisons sur plusieurs bras chromosomiques. Tel que prédit par plusieurs modèles de l'évolution chromosomique chez les salmonidés basés sur les loci alloenzymatiques, tous les loci microsatellites dupliqués qui partageaient des allèles (isoloci) montraient des valeurs de y s'approchant de 1,0.

Mots clés : méiose, AFLP, microsatellites, isoloci, *Oncorhynchus gorbuscha*.

[Traduit par la Rédaction]

Introduction

We are currently investigating the transmission genetics of several hundred PCR-based markers in pink salmon (*Oncorhynchus gorbuscha*) to construct a linkage map and to investigate chromosomal evolution following tetraploidy in salmonids. Half-tetrad analysis is a powerful tool for mapping genes and understanding chromosomal behavior during meiosis. Half-tetrad analysis can be performed if two of the four products from a single meiosis are recovered. Half-tetrads can be produced in many fish species by initiat-

ing development with UV-irradiated sperm and inhibiting the second meiotic division so that the polar body is retained (Thorgaard et al. 1983; Johnson et al. 1996). This results in gynogenetic diploid individuals that receive two chromosome sets from their female parent and none from their male parent (Thorgaard et al. 1983). This procedure allows analysis of meiosis II (MII) half-tetrads as classified by Zhao and Speed (1998).

All MII half-tetrad progeny of a heterozygous female will be homozygous if there are no crossovers between the locus and its centromere. A single crossover between the locus and its centromere will produce heterozygous progeny. The proportion of heterozygous progeny is a measure of the frequency of second division segregation (y) and thus, an estimate of gene–centromere distance. The maximum value of y is 0.67, unless there is chiasma interference inhibiting subsequent crossovers and resulting in y values greater than 0.67. The presence of strong chiasma interference has been reported in salmonids (Thorgaard et al. 1983; Allendorf et al. 1986; May et al. 1989) and other fish species (Naruse et al. 1988; Streisinger et al. 1986).

Amplified fragment length polymorphism (AFLP) markers have become very popular for constructing linkage maps

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in a wide variety of organisms because of their relative technical ease and reproducibility (Mueller and Wolfenbarger 1999). The ability to use different AFLP primer combinations allows a nearly unlimited supply of markers. Nevertheless, recent evidence suggests that in some species AFLPs tend to be clustered in centromeric regions (Keim et al. 1997, Qi et al. 1998; Alonso-Blanco et al. 1998). Young et al. (1998) suggested that AFLPs are centromeric in rainbow trout (*O. mykiss*) because they tended to cluster at the center of linkage groups. Clustering of AFLP markers in centromeric regions would lessen their general utility for constructing linkage maps.

Salmonid fishes diverged from a single tetraploid ancestor approximately 25–50 million years ago (Allendorf and Waples 1996). Residual tetrasomic inheritance still occurs at some loci in males, but only disomic inheritance has been reported in females. The persistence of disomic segregation in females suggests that the tetrasomic ratios in males result from distal recombination between homeologous chromosomes (Wright et al. 1983, Allendorf and Danzmann 1997). This can be explained conceptually by a two-stage model of pairing in which, first, homologous chromosomes pair and recombine in the proximal region of the chromosome. Next, homeologous chromosomes pair and recombine distally. If the crossover nearest the centromere determines the pattern of disjunction (Burnham 1962), then each gamete would receive one copy of each homolog, allowing continued disomic segregation in females.

Extensive gene duplication in salmonids has made genetic interpretations more complex than in species without a polyploid ancestry. Isoloci were originally described as pairs of duplicated allozyme loci whose allelic products have identical electrophoretic mobility (Allendorf and Thorgaard et al. 1984). It has been hypothesized that genetic divergence between isoloci has been prevented by the chromosomal exchanges between homeologs as described in the previous paragraph. It was predicted on the basis of this model that all isoloci should map far from their centromeres (Allendorf and Thorgaard 1984). Gene–centromere mapping of allozyme loci has supported this hypothesis (Allendorf et al. 1986).

Pink salmon have a fixed two-year life cycle (Heard 1991). This is a shorter generation time than most other salmonid species, which is helpful for genetic investigation. In addition, the fixed two-year cycle in pink salmon has resulted in nearly complete reproductive isolation between odd- and even-year fish. Chromosomal differences have been reported between odd- and even-year fish in the same geographical area (Phillips and Kapuscinski 1988). Gharrett et al. (1999) have also demonstrated outbreeding depression in experimental crosses between the two year classes. Based on these results, we would expect to find differences in gene–centromere distances for some loci between odd- and even-year fish.

In this paper, we describe the frequency of second division segregation of 312 loci, including nine allozyme loci, 34 microsatellite loci, 168 AFLPs, and 101 anonymous DNA loci flanked by paired interspersed nuclear elements (PINEs, Spruell et al. 1999). We have previously described the Mendelian inheritance of these loci in gynogenetic haploid pink salmon (Spruell et al. 1999). The primary objectives of this paper are to test for clustering of AFLPs near their centromere, to test for the predicted telomeric location of isoloci detected with microsatellite primers, and to test for differences in gene–centromere distances in odd- and even-year pink salmon.

Materials and methods

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.

Families of gynogenetic diploid individuals were produced by UV irradiation of the sperm and heat shock to block the second meiotic division as described by Thorgaard et al. (1983). Embryos were incubated in separate trays at 4–5°C until 7 months of age, when they were approximately 40 mm in length. Muscle, liver, heart, and eye tissues were sampled and frozen at –80°C until analysis. Enzyme electrophoresis followed Aebersold et al. (1987); tissue and buffer combinations were those described in Seeb et al. (1999). DNA was isolated using the Puregene™ DNA isolation kit (Gentra Systems Inc., Minneapolis, Minn.).

We used a Y-chromosome-specific growth hormone pseudogene (Spruell et al. 1999) to screen putative gynogenetic diploid individuals to eliminate triploids that could have been produced by the failure of sperm inactivation. Only one half of all progeny in which the sperm was not inactivated would be expected to have the Y chromosome. Seven female triploids and diploids that resulted from failure of the UV and heat shock treatment were eliminated because they contained alleles at allozyme or microsatellite loci that were not present in their mother.

Microsatellite loci were amplified as reported by the original authors with minor modifications. Primers and annealing temperatures are listed in Table 1. The countdown PCR profile reduces the annealing temperature by one degree each cycle, in this case from 58°C to 52°C or 54°C to 40°C, finishing with 24 cycles at an annealing temperature of 52°C or 40°C. One primer of most pairs was fluorescently labeled. Products from unlabeled primers were fluorescently tagged by incorporating a TAMRA-labeled dUTP or dCTP (Perkin–Elmer) during PCR. All PCR products were electrophoresed in a 7% denaturing polyacrylamide gel and visualized with a Hitachi FMBIO-100 or ABI 377 fluorescent imager.

AFLPs and PINEs were amplified as described in Spruell et al. (1999). One primer not described in Spruell et al. (1999) was also used to generate PINEs. We created a primer based on the core repeat of Jeffreys' minisatellite, 33.6 (TGGAGGAGGGCTGGA-GGAGGGCGC, Jeffreys et al. 1985). Two bases (GC) were added to the 3′ end of this sequence in an attempt to anchor the primer at the 3′ repeat in the tandem array.

Females and families are designated using the first letter of their origin (A, AFK; V, VFDA), the year of reproduction, and a sequential number. For example, family A95-103 contains the progeny from female 103 collected at Armin F. Koernig (AFK) hatchery in 1995. Nomenclature for allozyme loci follows Shaklee et al. (1990) and Seeb et al. (1999). Genetic nomenclature for microsatellites, AFLPs, and PINEs follows Spruell et al. (1999).

Results

Codominant loci

The genotypes of gynogenetic diploid progeny at 9 codominant allozyme and 31 codominant microsatellite loci are given in Tables 2 and 3. In addition, Table 4 presents the

Table 1. Microsatellite locus names, annealing temperatures, and references.

Locus	Annealing temperature (°C)	Reference
*FGT1-1,2**	51 (60)	Sakamoto et al. (1994)
OCL2	56	Condrey and Bentzen (1998)
*OGO1C**	60 (60)	Olsen et al. (1998)
*OGO2**	(60)	Olsen et al. (1998)
*OGO3**	(60)	Olsen et al. (1998)
*OGO4**	(60)	Olsen et al. (1998)
*OGO5**	64 (60)	Olsen et al. (1998)
OGO7-1,2	60	Olsen et al. (1998)
OGO8	56	Olsen et al. (1998)
OKI3	54–40[†]	Smith et al. (1998)
OMY276-1,2	58–52[†]	Danzmann and Ferguson (personal communication)
OMY301	58–52[†]	Sakamoto et al. (2000)
OMYFGT25	58–52[†]	Sakamoto et al. (2000)
OMYOGT4	58–52[†]	Sakamoto et al. (2000)
OMYRGT1	58–52[†]	Sakamoto et al. (2000)
OMYRGT2	58–52[†]	Sakamoto et al. (2000)
OMYRGT6	58–52[†]	Sakamoto et al. (2000)
OMYRGT13	58–52[†]	Sakamoto et al. (2000)
OMYRGT44	58–52[†]	Danzmann and Sakamoto (personal communication)
*ONEµ3**	(52)	Scribner et al. (1996)
ONEµ14	58–52[†]	Scribner et al. (1996)
ONEµ18	58–52[†]	Scribner et al. (1996)
*OTS1**	55 (52)	Banks et al. (1999)
*OTS101**	56 (52)	Small et al. (1998)
*OTS102**	(52)	Nelson and Beacham (personal communication)
*OTS103**	(58)	Small et al. (1998)
µSAT60-1,2	55 (58)	Estoup et al. (1993)
SSA20.19-1,2	58–52[†]	Sanchez et al. (1996)
*SSA85**	57 (58)	O'Reilly et al. (1996)
*SSA197**	57 (58)	O'Reilly et al. (1996)
SSA293	58–52[†]	McConnell et al. (1995)
SSA311	58–52[†]	Slettan et al. (1996)

*Indicates loci analyzed using an ABI 377 fluorescent imager. Numbers in parentheses indicate annealing temperatures used on samples analyzed with the ABI 377.

[†]Countdown PCR profile as described in text.

half-tetrad genotypes for eight PINE and four AFLP loci that were found to segregate as allelic polymorphisms in the length of the PCR amplicon produced by a single primer pair (Spruell et al. 1999).

There is no evidence of differences in the frequencies of the two homozygous classes (Tables 2–4). Unequal numbers of homozygous classes would be expected if one of the homozygotes had reduced viability because of linkage to a recessive deleterious allele. There must be at least six homozygotes observed before the frequencies of the two homozygous classes can be significantly different ($P < 0.05$) using the exact binomial test; we therefore tested for differences between the number of homozygotes only in families in which there were at least six homozygotes. Only 5 of a total of 58 possible tests had a probability less than 0.05, and none of these are significant when corrected for the 58 independent tests (Rice 1989).

There is little evidence for differences in the proportion of heterozygotes between families (Table 2 and 3). Five loci showed a significant difference between families based on contingency chi-square analysis (*OGO2, OGO5*: $P < 0.01$;

ONEµ3, OTS1, and *SSA85*: $P < 0.05$). However, none of these differences are significant if corrected for the 16 independent simultaneous tests (Rice 1989).

The frequency of heterozygotes is expected to vary according to the frequency of second meiotic division segregation (y). Gene–centromere map distances can be estimated by ($\frac{1}{2}$)y, assuming complete interference. Seven of the 52 codominant loci had y values of 1.00, which occurs only when there is exactly one crossover between the locus and its centromere. This indicates extremely strong chiasma interference on at least some chromosome arms.

Dominant loci

Segregation analysis can be used to estimate the frequency of second division segregation of dominant markers if the female is known to be heterozygous at those loci. Otherwise, the presence of a fragment in all progeny could result either from the female being homozygous or from a high frequency of second division segregation. We identified 164 AFLP and 93 PINE loci at which female A95-103 is heterozygous for the presence or absence of a product based on haploid prog-

Table 2. Half-tetrad genotypes at nine allozyme loci.

Locus	Family	Maternal genotype	Progeny 11	12	22	Proportion heterozygotes (y)	χ^2(d.f.)*
sAAT3	A95-103	100/91	5	47	7	0.80	0.33
	A95-114	100/91	8	43	8	0.73	0.00
	V96-13	100/91	2	7	3	0.58	0.20
	Total		15	97	18	0.75	2.57 (2)
sAAT4	A95-120	210/100	1	46	1	0.96	0.00
	V96-02	290/210	0	27	0	1.00	—
	V96-13	210/100	0	49	3	0.94	3.00
	Total		1	122	4	0.96	1.57 (2)
ADA2	A95-103	100/90	1	52	1	0.96	0.00
CKC2	A95-103	105/100	23	16	18	0.28	0.61
G3PDH1	V96-19	100/60	0	79	0	1.00	—
G3PDH2	V96-19	100/90	29	16	29	0.22	0.00
GDA1	A95-103	108/100	17	23	19	0.39	0.11
	A95-120	118/108	11	26	12	0.53	0.04
	V96-02	108/100	6	14	10	0.47	1.00
	Total		34	63	41	0.46	2.15 (2)
PEPB1	V96-19	138/100	22	4	16	0.10	0.95
PEPD2	A95-103	120/100	3	49	1	0.92	1.00
	A95-114	120/100	3	43	2	0.90	0.20
	A95-120	120/100	0	31	1	0.97	1.00
	V96-13	100/80	3	47	4	0.87	0.14
	V96-19	100/80	2	75	3	0.94	0.20
	Total		11	245	11	0.92	3.45 (4)

*Chi-square test for equal numbers of homozygotes (1 d.f.). Chi-square (d.f.) in the total row is the contingency chi-square value for differences in y between families.

eny (Spruell et al. 1999). Seventy gynogenetic diploid progeny from female A95-103 were examined to estimate the frequency of second division segregation at these loci.

To estimate the proportion of heterozygotes at these markers, we assumed equal numbers of each homozygote class. This assumption is supported by the results from the codominant loci. The frequency of second division segregation (y) can then be estimated by

$$y = \frac{(N_t - 2N_{aa})}{N_t}$$

where N_t is the total number of progeny screened and N_{aa} is the observed number of recessive homozygotes.

Female A95-103 was heterozygous for a null allele at one microsatellite locus (SSA197*164/a, Spruell at al. 1999). Sixty-eight of the 70 gynogenetic diploid progeny had the *164 allele ($y = 0.94$); this is similar to the y values estimated with other females in which this locus could be treated as codominant (Table 3).

Duplicated loci

The PCR products of five microsatellite primer pairs indicated the presence of four gene copies, consistent with the presence of duplicated loci. The loci in three of the five duplicated pairs shared the same alleles and, therefore, are isoloci: OGO7-1,2, OMYFGT276-1,2, and FGT1-1,2. Two primer sets (SSA20.19 and µSAT60) produced patterns consistent with four gene doses. However, the two loci in these

pairs do not share alleles in the pink salmon that we have examined.

Several of the 31 adults from the AFK hatchery had more than two different PCR products amplified by OGO7 primers; consistent with the same alleles occurring at both loci. Female A95-103 had three different PCR products: 182 bp, 200 bp, and 226 bp. All of her haploid progeny had the *200 allele, while the *182 and *226 alleles segregated 1:1 in her haploid progeny (unpublished data). Thus, she was apparently homozygous *200/*200 at one locus (arbitrarily specified OGO7-1) and heterozygous *182/*226 at the other (OGO7-2). All 70 gynogenetic diploid progeny from this female were heterozygous at this locus ($y = 1.00$, Table 2).

Several of the adults also had more than two different PCR products amplified by OMYFGT276 primers; consistent with the same alleles occurring at both loci. Female A95-103 had three different PCR products: 168 bp, 175 bp, and 188 bp. The *168 and *188 alleles segregated 1:1 in her haploid progeny, while approximately one half of her haploid progeny inherited the *175 allele (unpublished data). Thus, she was apparently heterozygous *168/*188 at one locus (arbitrarily designated OMYFGT276-1) and heterozygous for *175 and a null allele (*a) at the other (OMYFGT276-2). Sixty-one of 64 gynogenetic diploid progeny were heterozygous at OMYFGT276-1 ($y = 0.95$). Three of 60 gynogenetic diploid progeny were homozygous for the null allele at OMYFGT276-2. This results in an estimated y of 0.90 using the above equation for dominant markers.

The FGT1 microsatellite primer pair has previously been shown to amplify two loci (FGT1-1,2) in pink salmon

Table 3. Half-tetrad genotypes at 31 microsatellite loci.

Locus	Family	Maternal genotype	Progeny			Proportion heterozygotes (y)	χ^2(d.f.)*
			11	12	22		
OCL2	A95-103	127/135	9	46	13	0.68	0.73
OGO1c	A95-20	292/300	29	14	28	0.20	0.02
	A95-103	280/312	24	16	27	0.24	0.18
	V96-20	332/344	14	4	16	0.12	0.13
	V96-13	444/460	19	6	17	0.14	0.11
	Total		86	40	88	0.19	2.85 (3)
OGO2	A95-20	236/268	15	42	14	0.59	0.03
	V96-02	254/278	2	27	2	0.87	0.00
	V96-13	238/322	8	24	19	0.47	4.48
	V96-19	240/308	16	54	13	0.65	0.31
	V96-20	320/326	3	20	11	0.59	4.57
	Total		44	167	59	0.62	13.82 (4)
OGO3	A95-20	330/340	2	53	1	0.95	0.33
	V96-02	340/350	1	26	0	0.96	1.00
	V96-13	322/372	0	52	0	1.00	—
	V96-20	338/354	1	30	0	0.97	1.00
	Total		4	161	1	0.97	2.72 (3)
OGO4	A95-20	234/250	4	57	1	0.92	1.80
	V96-02	210/226	0	31	0	1.00	—
	V96-13	210/214	3	43	4	0.86	0.14
	V96-19	218/236	4	75	4	0.90	0.00
	Total		11	206	9	0.91	4.77 (3)
OGO5	A95-103	184/188	32	0	36	0.00	0.24
	V96-13	200/208	26	5	22	0.09	0.33
	Total		26	5	22	0.09	6.69 (1)
OGO7-2	A95-103	182/226	0	70	0	1.00	—
OGO8	A95-103	106/144	0	70	0	1.00	—
OKI3	A95-103	296/320	1	63	2	0.95	0.33
OMY301	A95-103	88/90	7	54	7	0.79	0.00
OMYFGT25	A95-103	180/184	14	46	10	0.66	0.67
OMYFGT276-1	A95-103	168/188	1	61	2	0.95	0.33
OMYOGT4	A95-103	400/420	12	45	7	0.70	1.32
OMYRGT1	A95-103	74/76	12	47	11	0.67	0.04
OMYRGT2	A95-103	218/250	27	25	14	0.38	4.12
OMYRGT6	A95-103	188/192	27	9	27	0.14	0.00
OMYRGT13	A95-103	179/183	13	41	13	0.61	0.00
OMYRGT44	A95-103	162/186	11	49	7	0.73	0.89
ONEμ3	A95-29	162/168	0	32	0	1.00	—
	A95-114	162/168	3	54	1	0.93	1.00
	A95-120	162/168	0	44	0	1.00	—
	V96-13	162/168	0	51	2	0.96	2.00
	V96-19	162/168	0	85	0	1.00	—
	Total		3	266	3	0.98	10.15 (4)
ONEμ14	A95-103	212/228	1	63	2	0.95	0.33
ONEμ18	A95-103	262/290	3	59	7	0.86	1.60
OTS1	A95-29	236/246	14	7	11	0.22	0.36
	A95-103		30	12	26	0.18	0.29
	A95-114	220/224	17	17	22	0.30	0.64
	A95-120	224/232	15	11	18	0.25	0.27
	V96-13	228/238	28	11	14	0.21	4.67
	V96-19	224/232	19	30	26	0.40	1.09
	Total		123	88	117	0.27	11.37 (5)
OTS101	A95-20	310/386	23	22	21	0.33	0.09
	A95-103	310/344	25	21	23	0.30	0.08
	V96-02	358/408	8	8	3	0.42	2.27
	Total		56	51	47	0.33	0.92 (2)

Table 3 (*concluded*).

Locus	Family	Maternal genotype	Progeny 11	12	22	Proportion heterozygotes (y)	χ^2(d.f.)*
OTS102	V96-02	*282/298*	0	24	0	1.00	—
	V96-19	*262/290*	1	73	0	0.99	1.00
	Total		1	97	0	0.99	0.33 (1)
OTS103	V96-13	*238/258*	9	35	2	0.76	4.45
µSAT60-1	A95-103	*109/113*	16	37	10	0.59	1.38
	A95-114	*109/113*	12	33	13	0.57	0.04
	Total		28	70	23	0.58	0.04 (1)
SSA20.19-1	A95-103	*77/79*	0	67	0	1.00	—
SSA85	A95-29	*185/194*	16	5	11	0.16	0.93
	A95-103	*155/201*	22	18	30	0.26	1.23
	A95-114	*165/205*	23	7	26	0.13	0.18
	V96-13	*197/213*	19	7·	25	0.14	0.82
	V96-19	*166/209*	29	25	27	0.31	0.07
	Total		109	62	119	0.21	10.15 (4)
SSA197	A95-114	*136/148*	0	57	0	1.00	—
	A95-120	*128/148*	0	44	0	1.00	—
	V96-13	*128/176*	0	52	1	0.98	1.00
	V96-19	*156/160*	0	82	0	1.00	—
	Total		0	235	1	1.00	3.47 (3)
SSA293	A95-103	*178/218*	2	68	0	0.97	2.00
SSA311	A95-103	*170/238*	28	5	35	0.07	0.78

*Chi-square test for equal numbers of homozygotes (1 d.f.). Chi-square value in the total row is contingency chi-square for difference in y between families.

(Spruell et al. 1999) and sockeye salmon (Allendorf and Seeb 2000). Figure 1 shows the electropherograms of individuals having different numbers of doses of the two alleles (155 bp and 157 bp) detected at this locus. We examined gynogenetic diploid progeny in 4 families at *FGT1-1,2* and arbitrarily designated the *155* allele as *1* and the *157* allele as *2* to simplify the notation (Table 5). Females A95-114 (**1112*) and A95-103 (**1222*) had three copies of one allele and one copy of the other, so these females must be homozygous at one locus and heterozygous at the other (Fig. 1a and 1b, respectively). Estimates of the frequency of second division segregation can be made in these families at the single segregating locus as if it were a nonduplicated locus. Both families had y values of 0.90 or greater.

Two females, A95-29 and A95-120, had the duplex genotype (**1122*, Fig. 1c, Allendorf and Danzmann 1997) at *FGT1-1,2*. Duplex individuals can either be homozygous at both loci (**11*, **22*) or heterozygous at both loci (**12*, **12*). Segregation analysis of normal diploid progeny from these two females indicated that they were heterozygous at both loci (J.E. Seeb, unpublished results). Therefore, the genotype of each progeny in these two families is the result of separate meiotic events at *FGT1-1* and *FGT1-2*.

All 42 gynogenetic diploid progeny in family A95-120 had the duplex genotype. The simplest explanation of this result is that all progeny resulted from second division segregation at both loci, thus, $y = 84/84 = 1.00$. Two of the 22 gynogenetic progeny in family A95-29 had the triplex genotype (**1222*), which is expected to result from second division segregation at one of the two loci and first division segregation at the other locus; thus, $y = 42/44 = 0.95$. The average y at this locus in all families is 0.97.

Two distinct loci resulted from the SSA20.19 primer pair. One locus (arbitrarily designated *SSA20.19-1*), was heterozygous *77/79* in female A95-103. All gynogenetic diploid progeny were heterozygous at this locus (Table 3). The other locus (*SSA20.19-2*) was apparently homozygous for the **74* allele in the fish described in this study, but is polymorphic for three alleles (**62*, **72*, and **74*) in a population of pink salmon that we have examined from Likes Creek, Alaska. Similar results were found for the µSAT60 primers. Two females in this study were heterozygous, **109/113*, at *µSAT60-1* (Table 3), and homozygous, **236/236*, at *µSAT60-2*. Pink salmon from Likes Creek are polymorphic for more than 15 alleles (206–270 bp) at the second locus. Approximately 60% of the gynogenetic diploid progeny were heterozygous at *µSAT60-1* (Table 3).

Distribution of markers

Figure 2 shows the distribution of y values for PINEs, AFLPs, and microsatellites. The distribution of y values in PINEs and microsatellites is similar with many y values greater than 0.90. The few allozyme loci have a similar distribution in that four of the nine loci have y values greater than 0.90 (Table 2). AFLP loci are much more proximal than the other classes of loci; the mean y for AFLP loci is 0.40, in comparison to a mean y of 0.69 for both PINEs and microsatellites.

We arbitrarily divided y-values for each class of markers into four regions (0–0.25, 0.26–0.50, 0.51–0.75, and 0.76–1.00) and used chi-square contingency analysis to test for differences. The distribution of AFLPs is significantly different ($P < 0.001$) from the distribution of both PINEs ($\chi^2 = 57.2$, 3 d.f.) and microsatellites ($\chi^2 = 24.6$, 3 d.f.). The distri-

Fig. 1. Electropherograms for individuals with different genotypes at *FGT1-1,2*. The area under the curve at each peak (155 bp and 157 bp) is proportional to the number of doses of each allele (*1 and *2, respectively).

Allendorf et al. (1986) suggested that the absence of evidence for recessive deleterious alleles in gynogenetic diploids may be associated with the redundancy in the salmonid genome because of polyploidy.

There is little indication of differences in y-values within loci between families derived from odd- and even-year females (Tables 2 and 3). There were 14 loci at which we estimated y-values in both odd- and even-year fish. We used Fisher's exact test to test for differences between the proportion of second-division segregation in odd- and even-year fish. Only one locus had a probability less than 0.05 (*OGO5*, $P = 0.014$). This value is not significant if corrected for the 14 independent tests (Rice 1989).

Crossover interference

The presence of a large number of loci with high y values is evidence for the presence of strong interference in the pink salmon genome, as has been found in other fishes (Thorgaard et al. 1983; Allendorf et al. 1986; Streisinger et al. 1986). The maximum value of y with no interference is 0.67. In contrast to this expectation, 12 of the 34 microsatellite loci have y values greater than 0.95. Similarly, 34 of the 101 PINE loci have y values greater than 0.95. The large number of loci with y values near 1.0 is the result of reduced recombination on the telomeric portion of chromosome arms due to interference.

Comparison among species

Numerous allozyme loci in salmonids have been mapped in relation to their centromeres (see review in May and Johnson 1993). Gene–centromere recombination data are available for *sAAT3*, *sAAT*, *4ADA2*, *G3PDH1*, *PEPB1*, and *PEPD2* in pink salmon and other species (Table 6).

Most loci show similar gene–centromere recombination rates in different species. However, *PEPB1* and *sAAT3* do not map to the same location in relation to their centromere in different species. For *PEPB1*, paired comparison between brook trout × Arctic char hybrids (spartic) and pink salmon is significant ($P < 0.01$), as is comparison between sparctic hybrids and rainbow × cutthroat trout hybrids ($P < 0.01$). For *sAAT3*, paired comparison between chum salmon (*O. keta*) and pink salmon is significant ($P < 0.001$), as is comparison between chum salmon and rainbow trout ($P < 0.001$).

Most map distances between the same pair of loci in different salmonid species have been found to be similar (May and Johnson 1993). Thus, a substantial proportion of the numerous chromosomal rearrangements in salmonids apparently took the form of Robertsonian fusions and fissions, where entire chromosome arms were translocated intact, conserving linkage arrangements among species (May and Johnson 1993). However, the lack of conservation of gene-centromere distance among species suggests that other forms of chromosome evolution occurred, potentially disrupting linkage associations on some chromosome arms.

Duplicated microsatellite loci

All three pairs of microsatellite isoloci had y-values greater than 0.90. The telomeric location of these loci supports the previous model for residual tetrasomic inheritance (Allendorf and Thorgaard 1984). All allozyme isoloci are telomeric as well (Allendorf et al. 1986; Johnson et al.

butions of PINEs and microsatellites are not significantly different. The difference between AFLPs and other markers is primarily due to a near absence of AFLP loci with y values greater than 0.9 (Fig. 2). The significance of the differences between AFLPs and other markers disappears if the 0.76–1.00 class is left out of the contingency chi-square analysis (PINEs: $\chi^2 = 0.7$, 2 d.f.; microsatellites: $\chi^2 = 5.2$, 2 d.f.).

Discussion

The lack of evidence for unequal numbers of homozygotes supports our procedure for estimating y at dominant loci by assuming equal numbers of homozygotes. There is no evidence for differential survival of the two homozygous classes in the 58 comparisons made at codominant loci in which enough homozygotes were observed to detect a significant difference. Based on the number of comparisons, it is somewhat surprising that we did not detect deleterious recessive alleles which we expect would reduce the observed number of homozygotes at loci to which they are closely linked. Similar results have been found, however, for allozyme loci that have been examined in gynogenetic diploids in other salmonid species (Thorgaard et al. 1983; Guyomard 1986; Seeb and Seeb 1986; Johnson et al. 1987).

Fig. 2. The distribution of second division segregation (y) for AFLPs, PINEs, microsatellites, and all markers (including allozymes).
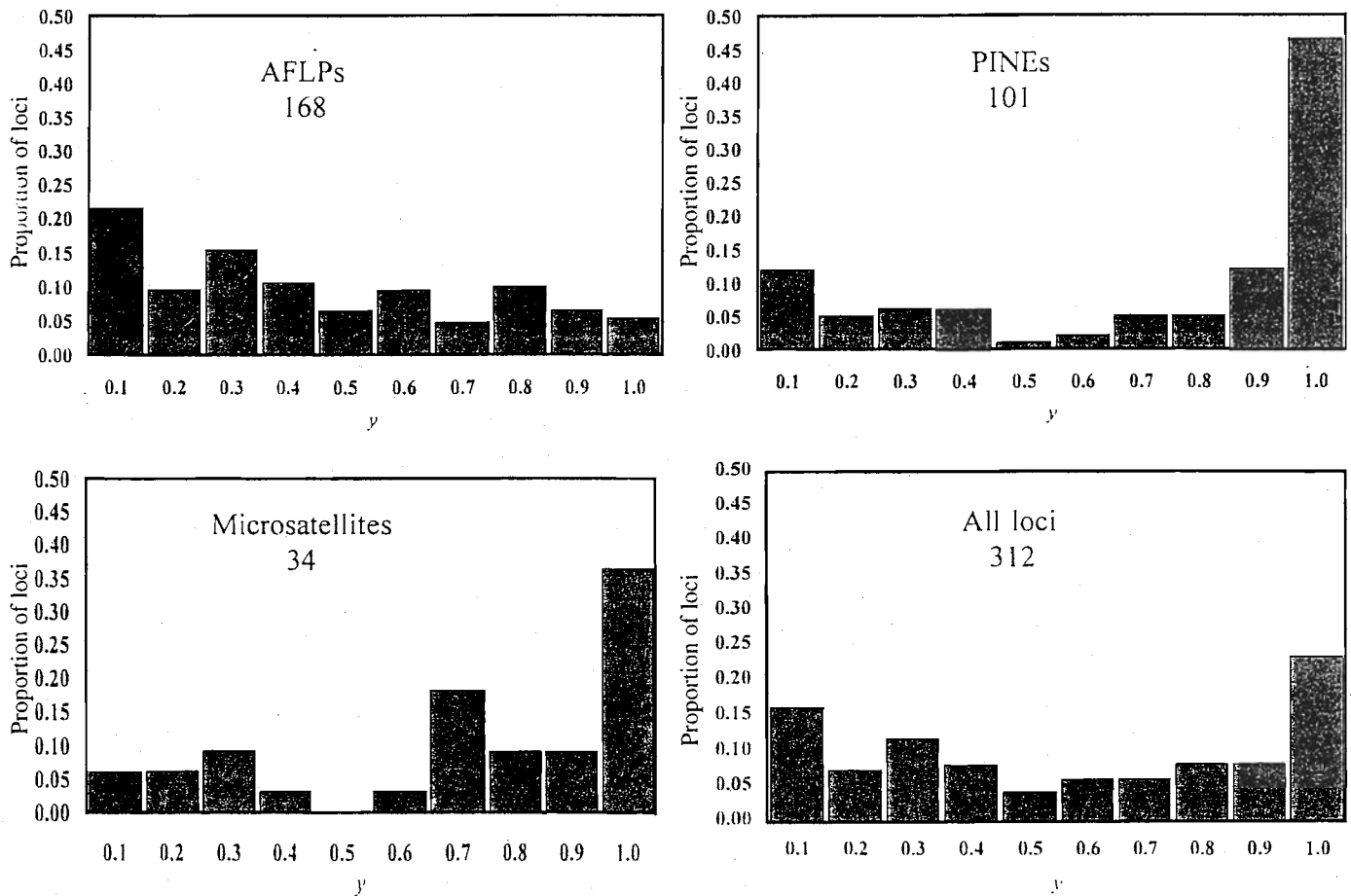
Table 4. Half-tetrad genotypes at eight PINE and four AFLP codominant loci in family A95-103.

| Locus | Maternal genotype | Progeny | | | Proportion heterozygotes (y) | χ^2* |
		11	12	22		
33.6+2/5T384	384/387	2	66	2	0.94	0.00
5F745	745/748	33	0	37	0.00	0.23
3H5T182	182/183	27	15	29	0.21	0.07
5F5T217	217/232	0	70	0	1.00	—
5H5T76	76/77	27	19	22	0.28	0.51
5H5T125	125/128	0	68	0	1.00	0.00
5H5T224	224/249	22	22	24	0.32	0.09
5S5T203	203/207	35	5	30	0.07	0.38
AAC/CGT157	157/158	21	23	18	0.37	0.23
AAT/CTG293	293/295	15	30	20	0.46	0.71
ACC/CAC328	328/367	16	40	10	0.61	1.38
AGA/CTC204	204/205	28	13	26	0.19	0.07

*Chi-square test for equal numbers of homozygotes (1 d.f.).

1987). Homeologous pairing of chromosomes at distal loci apparently causes the exchange of alleles so that the same alleles will be present at both loci.

The gene–centromere distance for pairs of duplicated loci that did not share alleles varied. *SSA20.19-1* had a y-value of 1.00 in one family; we did not map *SSA20.19-2*. *μSAT60-1* had an average y-value of 0.58 in two families; we did not map *μSAT60-2*. Both primer sets for these two pairs of du-

plicated loci were described from species in the genus *Salmo* (*S. salar* and *S. trutta*, respectively) that have not shared a common ancestor with pink salmon or other *Oncorhynchus* species for approximately 4–5 million years (Grewe et al. 1990).

The amplification of two loci with these primer sets reflects slow sequence divergence. Sufficient sequence similarity has been retained at both priming sites resulting in am-

Table 5. Half-tetrad analysis of *FGT1-1,2*. See text for explanation of estimation of *y* (proportion of second division segregation).

Family	Maternal genotype			Progeny					*y*
	-1, -2	*-1*	*-2*	*1111*	*1112*	*1122*	*1222*	*2222*	
A95-29	*1122*	*1/2*	*1/2*	0	0	20	2	0	42/44 = 0.95
A95-103	*1222*	*1/2*	*2/2*	0	0	0	69	1	69/70 = 0.99
A95-114	*1112*	*1/2*	*1/1*	3	35	1	0	0	35/39 = 0.90
A95-120	*1122*	*1/2*	*1/2*	0	0	42	0	0	84/84 = 1.00
									230/237 = 0.97

Table 6. Frequency of second-division segregation (*y*) for six allozyme loci in salmonids.

Locus	Species	*y*	*F*	*N*	Reference
sAAT3	Pink salmon	0.75	3	130	This paper
	Chum salmon	0.98	3	168	Seeb and Seeb (1986)
	Rainbow trout	0.66	2	67	Allendorf et al. (1986)
sAAT4	Pink salmon	0.96	3	127	This paper
	Brown trout	1.00	2	35	Guyomard (1986)
	Sparctic*	0.92	3	188	May and Johnson (1993)
	Splake[§]	0.94	4	119	May and Johnson (1993)
ADA2	Pink salmon	0.96	1	54	This paper
	Cutbow[†]	0.96	7	113	Johnson et al. (1987)
G3PDH1	Pink salmon	1.00	1	79	This paper
	Sparctic*	0.92	3	157	May and Johnson (1993)
	Splake[§]	1.00	2	115	May and Johnson (1993)
	Splake[§]	0.96	1	29	Johnson et al. (1987)
	Rainbow trout	1.00	2	20	Allendorf et al. (1986)
	Char hybrid[‡]	0.87	2	47	Arai et al. (1991)
	Brook trout	1.00	1	7	Arai et al. (1991)
PEPB1	Pink salmon	0.10	1	42	This paper
	Cutbow[†]	0.12	8	152	Johnson et al. (1987)
	Sparctic*	0.26	2	148	May and Johnson (1993)
PEPD2	Pink salmon	0.92	5	267	This paper
	Cutbow[†]	1.00	1	12	Johnson et al. (1987)
	Splake[§]	0.90	2	42	Johnson et al. (1987)

Note: *F* = number of females, *N* = total number of progeny.
*Hybrid between brook trout (*Salvelinus fontinalis*) and Arctic char (*S. alpinus*).
[§]Hybrid between brook trout and lake trout (*S. namaycush*).
[†]Hybrid between cutthroat trout (*Oncorhynchus clarki*) and rainbow trout (*O. mykiss*).
[‡]Hybrid between brook trout and Japanese char (*S. leucomaenis*).

plification of alleles at both loci. However, the divergence in alleles present at these pairs of loci indicates that they are not involved in regular homeologous exchanges in pink salmon. The absence of homeologous exchanges is compatible with the relatively low gene–centromere distance for *μSAT60-1*. Although *SSA20.19-1,2* is telomeric it may be located on a chromosome that is not involved in homeologous exchanges, or the rate of exchange is not sufficient to maintain the same alleles at both loci (Allendorf and Danzmann 1997).

Distribution of different marker types

AFLP loci are distributed differently than the other classes of loci (Fig. 2). The mean *y* for AFLP loci is 0.40, in comparison to a mean *y* of 0.69 for both PINEs and microsatellites. The distribution of *y* at nine allozyme loci is similar to the other non-AFLP markers with a mean *y* of 0.63 (Table 2). This is similar to the distribution of *y* values at 25 allozyme loci in rainbow trout (mean *y* = 0.56, Allendorf et al. 1986).

The differences between markers can be explained in two fundamentally different ways. First, the gene–centromere distances of markers are genetic distances that may not reflect the physical location of markers. For example, there are regions of the chromosome in which recombination is suppressed. Markers will cluster in these regions based on linkage analysis despite being physically distributed uniformly. In addition, all markers beyond 50 cM from the centromere will be assigned a *y* of 1.0 using half-tetrad analysis if there is complete crossover interference; this will cause a clustering of all distal loci.

Alternatively, markers may be physically arranged along the chromosome in clusters. If so, the physical distance would correspond to the spatial distribution estimated by gene–centromere distances. In this case, clustering of markers would reflect that loci tend to occur in particular chromosomal regions. We can compare the results from various marker types and use the results of mapping in other taxa to begin to differentiate between these two hypotheses.

Suppression of recombination in centromeric regions has been well documented. Roberts (1965) first described this phenomenon in *Drosophila* and estimated a reduction in recombination of up to 40% around the centromere. More recently, Tanksley et al. (1992) observed clustering of markers on a linkage map of tomato and concluded that this was due to a 10-fold reduction in recombination that corresponded to centromeric heterochromatin. This conclusion was supported using additional evidence from the physical map of tomato to locate centromeres (Ganal et al. 1989). Based on these results, we might expect an accumulation of markers in centromeric regions.

Our data indicate a near absence of AFLPs in distal regions relative to the other marker types (Fig. 2). Linkage analyses in several other taxa also show a non-uniform distribution of AFLP-based markers. Qi et al. (1998) assigned 51% of the AFLP markers in barley to centromeric clusters. Similarly, Keim et al. (1997) reported a clustering of AFLP markers in soybean. AFLPs were also found in centromeric clusters in *Arabidopsis thaliana* (Alonso-Blanco et al. 1998).

Young et al. (1998) inferred from their haploid linkage map that AFLPs are centromeric in rainbow trout. This is based on the presence of a cluster of tightly linked AFLPs at the center of most of their linkage groups. This clustering includes a much higher proportion of AFLPs than we have observed in pink salmon. However, comparisons between the rainbow trout map and the gene–centromere distances estimated in pink salmon must take into consideration the difference in recombination rate between males and females. Their rainbow trout map was constructed using androgenetically derived homozygous lines and is therefore based upon recombination rates in males. Our gene–centromere data from pink salmon estimates recombination in females. It has been previously reported that the recombination in males is lower than in females in salmonid species (May et al. 1989). Thus, we expect a tighter clustering of markers around the centromere in males than in females.

It is possible that the distribution of AFLPs reflects a bias in the base composition of certain genomic regions. In both our study and that of Young et al. (1998) the restriction enzymes *Eco*RI and *Mse*I were used to generate the AFLP fragments. The recognition sites for these enzymes (GAATTC and TTAA, respectively) are highly biased toward A and T. At least some centromeric regions are also known to be >90% AT.

This base pair composition bias may result in an accumulation of AFLPs near the centromeres. In addition, Young et al. (1998) used an A as the first selective nucleotide on both primers. We also used an A on the *Mse*I primer, but we used a C on the *Eco*RI primer. Thus, if there is a centromeric bias in AFLPs resulting from regional differences in genomic composition, we would expect the AFLPs examined in rainbow trout to be even more biased toward AT-rich sequences.

Young et al. (1999) recently examined the distribution of AFLPs in soybeans and concluded that clustering was associated with heterochromatic regions of reduced recombination rather than a base composition bias. They found no correlation between the percent GC in each selective primer and clustering of AFLPs. In addition, analysis of AFLPs generated using *Eco*RI and *Mse*I differed in distribution from those generated using *Pst*I and *Mse*I. AFLPs produced using *Eco*RI, which is not affected by cytosine methylation, were highly clustered. In contrast, those produced using *Pst*I, in which restriction is inhibited in methylated regions, were not clustered. Thus, it appears in soybeans, that the clustering of *Eco*RI-derived AFLPs can be attributed to regions of highly methylated heterochromatin, such as is found in centromeric regions.

PINEs provide a good fit to our expectations for the distribution of loci (Fig. 2). There is a small cluster of PINE loci in centromeric regions, as expected if there is suppressed recombination in this area. In addition, there is a large cluster of PINEs with y values approaching 1.0, perhaps reflecting the maximum distance from the centromere that can be detected using gene–centromere analysis with strong interference.

We expected PINEs to most closely approximate a random physical location of markers due to the origin of the primers. We have produced PINEs using three different classes of repeats: SINEs (*Hpa*I, *Fok*I, *Sma*I), a transposon (*Tc*1), and a minisatellite (33.6, Jeffreys 1985). Each class of element is inserted into the genome by a different mechanism and is influenced by a different set of evolutionary constraints; most of the PINE fragments that we have mapped were amplified by primers complementary to two different classes of elements.

Greene and Seeb (1997) reported that fragments amplified using primers homologous to *Sma*I and *Tc*1 were nearly all centromeric in pink salmon. However, they were unable to identify markers with large y values because they did not have haploid segregation data from the same females. Thus, they could not distinguish between fragments with y values near 1.0 and fragments for which the female was not segregating, and could not detect the higher peak in the somewhat bimodal distribution of PINEs. Our results are concordant with Greene and Seeb (1997) if the markers with $y > 0.7$ are ignored in our data.

It is important to identify any biases in the distribution of types of markers being used in the construction and consolidation of a linkage map. We have used half-tetrad analysis to demonstrate that using a variety of techniques provides a uniform coverage of the genome. However, other marker characteristics must also be considered when compiling the large number of markers needed for a linkage map. The AFLP technique provides many polymorphic markers and requires less DNA than the other techniques used; an important consideration for the analysis of haploid embryos (Spruell et al. 1999). However, the exceptional distribution of AFLPs appears to be common and may limit the utility of AFLPs for mapping distal regions of chromosomes and identifying quantitative trait loci.

Acknowledgements

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Appendix 3

Rates and Patterns of Microsatellite Mutations in Pink Salmon

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The tendency of microsatellites to be highly polymorphic is a major factor responsible for their popularity as markers for ecological and evolutionary studies. Microsatellite polymorphism is generally attributed to slip-strand mispairing errors, causing the addition or deletion of repeat units during replication (Levinson and Gutman 1987). Valdes, Slatkin, and Freimer (1993) found that human microsatellite evolution appears to follow a stepwise mutation model (SMM) (Ohta and Kimura 1973). Following the SMM, single repeat units are added or deleted with equal and constant probability across all alleles. Several statistical methods to evaluate patterns of microsatellite variability and differentiation that assume variants of the SMM have since been developed (e.g., Goldstein et al. 1995a, 1995b; Slatkin 1995; Rousset 1996) and incorporated into widely used software programs such as GENEPOP (Raymond and Rousset 1995).

Although slippage during replication clearly plays a key role in the overall instability of the microsatellites (reviewed by Eisen 1999), mounting evidence indicates that microsatellite mutation dynamics are more complex than is reflected by the SMM. Numerous examples of mutations that do not constitute single repeat unit changes or those that reflect heterogeneity or bias in the mutational processes of particular loci or alleles have been documented (see reviews by Ellegren 2000a, 2000b and Schlotterer 2000). In particular, the length, type, and number of repeat units have been identified as important factors contributing to the complexity of microsatellite evolution.

Mutation events occurring early in gametogenesis can further complicate mutation dynamics. An underlying assumption of many population genetics models is that mutations occur and enter the gene pool independently. However, Woodruff and Thompson (1992) found that as many as 20% of new mutations detected in large-scale Drosophila screens did not occur as independent events but rather represented clusters of identical mutant alleles sharing a common premeiotic origin. Subsequently, Woodruff, Huai, and Thompson (1996) have shown that the occurrence of premeiotic cluster mutations can not only bias estimates of mutation rates but can also influence basic population genetic processes such as fixation probabilities. Cluster mutations have been documented at microsatellite loci in only two species: pipefishes (*Sygnathus typhle*, Jones et al. 1999) and green turtles (*Chelonia mydas*, FitzSimmons 1998). However, as pointed out by Ellegren (2000a, 2000b),

this may be because of the difficulty in detecting cluster mutations in organisms that produce small numbers of offspring per generation.

As stressed by Chambers and MacAvoy (2000), "a clear knowledge of the process of mutational change at microsatellite loci is imperative for the correct selection of theoretical models upon which statistical methods can be based" however, a knowledge of mutation dynamics requires more information than is typically available. In particular, a lack of inheritance data often precludes direct evaluation of the markers used in population studies. However, this is not the case for many salmonid fishes that are commonly raised in hatcheries and for which many microsatellite markers have been developed (e.g., Scribner, Gust, and Fields 1996; Olsen, Bentzen, and Seeb 1998; Banks et al. 1999). In this paper, we examine the transmission of nine microsatellite loci in 50 families to evaluate the dynamics of microsatellite mutations in pink salmon (*Oncorhyncus gorbuscha*).

We raised families of pink salmon by randomly pairing mature adults collected in Resurrection Bay, Alaska. We collected embryos from each of the families after eye pigment became apparent in the embryo. We extracted DNA from the embryos and fin clips taken from adults using the Puregene™ DNA isolation kit (Gentra Systems Inc., Minneapolis, Minn.). We amplified microsatellites using primers for salmonid fishes developed in other laboratories: *OGO1c* and *OGO8* (*Ogo1c* and *Ogo8*, Olsen, Bentzen, and Seeb 1998); *OMY301* (*Omy301UoG*, R. Danzmann, personal communication); *OMYRGT6-1,2* (*OmyTRG6/I,iiTUF*, N. Okamoto, personal communication); *ONEμ3* (*Oneμ3*, Scribner, Gust, and Fields 1996); *OTS1* (*Ots1*, Banks et al. 1999); *SSA20.19-1,2* (*μ-20.19**, Sanchez et al. 1996); and *SSA408* (*Ssa408*, M. Cairney, personal communication). We used fluorescent primers and followed the PCR conditions recommended by the original authors. We visualized PCR products with a Hitachi FMBIO-100 or FMBIO II fluorescent imager after electrophoresis in 4.5% denaturing polyacrylamide gels. We scored alleles relative to commercial size standards (BioVentures, Inc.).

For the seven loci isolated from species other than pink salmon, we determined the repeat arrays in pink salmon by sequencing at least one allele. If multiple products were produced in the PCR reaction, we isolated bands in 3%–4% agarose gels. We then either purified and sequenced the bands or reamplified, purified, and sequenced them with both the forward and reverse primers. We purified PCR products with Qiaquick™ columns (Qiagen) following the supplier's protocol. Direct sequencing of PCR products was performed by a commercial laboratory. We did not sequence alleles from the two loci that were developed from pink salmon (*OGO1c* and *OGO8*).

We initially genotyped parents and 10 progeny from each of the 50 families. Alleles present in progeny

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Key words: microsatellite, mutation, salmon, *Oncorhyncus gorbuscha*.

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that were not present in either of that individual's parents were considered to be mutants after confirming that correct parentage could be assigned at the other loci. The progenitor of the mutant allele was assumed to be the parental allele that was closest in size to the mutant allele. Because we detected multiple mutations at *SSA408* in progeny from two of the families (98–23 and 98–26) in the initial analysis, we analyzed all the remaining embryos available from these families (38 and 40 embryos, respectively). To increase our sample size, we also genotyped 36 additional progeny from each of the five randomly selected families.

Our sequence analysis of alleles at six of the seven loci originally developed from other salmonids confirmed that these markers comprise similar microsatellites in pink salmon (table 1). The one exception (*OMYRGT6-1,2*) is a duplicated locus that was reported as a CA repeat in rainbow trout (*Oncorhynchus mykiss*, Sakamoto et al. 2000, table 1). In pink salmon, PCR amplifications using *OMYRGT6-1,2* primers consistently produced a smaller band that is present in all individuals as well as larger polymorphic bands that segregated after Mendelian inheritance. The polymorphic *OMYRGT6-1,2* locus in pink salmon contains a CACT repeat array, and the smaller monomorphic locus contains the short interrupted repeat $(CA)_7$-TA-$(CA)_2$ (table 1). The flanking sequences of the rainbow trout CA repeat and the pink salmon CA and CACT repeats can be readily aligned, suggesting that *OMYRGT6-1* and *OMYRGT6-2* have evolved two different microsatellite arrays. In the rainbow trout, the two copies of *OMYRGT6-1,2* map to different linkage groups (Sakamoto et al. 2000). However, whether these loci comprise different microsatellite arrays in the rainbow trout has not been determined (T. Sakamoto, personal communication).

We detected mutations only at the two loci that have the greatest number of alleles in the parental population (*OGO1c* and *SSA408*, table 1). Both these loci are composed of tetranucleotide repeats, whereas the other seven loci comprise a mixture of simple and interrupted dinucleotide repeats (table 1). These data are consistent with the findings of other studies, suggesting that tetranucleotide repeats have higher mutation rates than dinucleotide repeats (Weber and Wong 1993; Lee et al. 1999; Ellegren 2000a, 2000b). However, mutation rates can also depend on allele size (e.g., Schlotterer et al. 1998); the relative importance of repeat motif versus allele size on mutation rates at these nine loci is not known. In total, 24 of the 758 progeny analyzed had genotypes best explained as resulting from mutation events. Five of the mutations were at *OGO1c* and 19 were at *SSA408* (table 2). All these progeny had genotypes at all the other loci that were consistent with their parents. In addition, the second allele at the locus containing the putative mutation was shared with one parent (table 2).

At *SSA408* in the family 98-26, the 320-bp mutant allele was transmitted to 9 of the 50 embryos, and in the family 98-23, the 370-bp mutant allele was transmitted to 4 of the 48 embryos (table 2). These clusters of identical mutations suggest that many novel alleles

Table 1
Characterization of Microsatellite Loci in 50 Pink Salmon Families

Locus[a]	Reported Repeat Sequence[a]	Repeat Sequence in Pink Salmon	Size of Sequenced Allele[b]	Size Range of Alleles[b]	Number of Alleles	Estimated Mutation Rate[c]
OGO1c	$(GTCT)_{26}$-N_{70}-$(AT)_5$-$(GT)_{11}$	$(GTCT)_{26}$-N_{70}-$(AT)_5$-$(GT)_{11}$	Not sequenced	275–584	77	3.9×10^{-3}
OGO8	$(GA)_{22}$	$(GA)_{22}$	Not sequenced	88–171	17	0
OMY301	$(CA)_{20}$	$(CA)_3$-A-$(CA)_{15}$	83	75–113	21	0
OMYRGT6-1,2	$(CA)_{20}$	$(CA)_2$-$(CACT)_{15}$/$(CA)_7$-TA-$(CA)_2$	188/170	172–225/170–170	18/1	0/0
ONEμ3	$(GA)_{18}$	$(GA)_5$-N_7-$(GA)_4$	167	160–167	3	0
OTS1	$(TG)_3$-N_8-$(TG)_3$-N_4-$(TG)_3$-N_2-$(TG)_7$-N_{16}-$(TG)_6$	$(TG)_3$-N_8-$(TG)_3$-N_4-$(TG)_3$-N_2-$(TG)_7$-N_{16}-$(TG)_6$	228	218–248	15	0
SSA20.19-1,2	$(AC)_{13}$-G-$(CA)_4$	$(AC)_8$/$(AC)_4$-AG-$(AC)_5$	79/74	77–79/62–74	2/3	0/0
SSA408	$(GACA)_{27}$	$(GACA)_{39}$	370	300–500	49	8.5×10^{-3}

[a] See text for references. *OMYRGT6-1,2* and *SSA20.19-1,2* are duplicated loci in pink salmon; data for the paralogs at these loci (when available) are separated by "/".

[b] Length of PCR product including both repeat and flanking sequence.

[c] For confidence limits see text; zeros represent loci at which no mutations were detected in approximately 1,300 transmissions.

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Table 2
Microsatellite Mutations Observed in Pink Salmon Families

| Locus | Family | Parental Genotypes[a] | | Progeny Genotypes[b] | | | | | | | Size Change of Mutation[c] | |
		Dam a/b	Sire c/d	a/c	a/d	b/c	b/d	Genotypes	Not Matching	Parents (No.)	No. Increase	No. Decrease
SSA408....	98–15	334/382	322/<u>370</u>	14	9	9	13	*334/**374*** (1)			1 (1)	—
	98–19	<u>338</u>/350	378/404	15	12	8	10	*342/**404*** (1)			1 (1)	—
	98–22	334/404	<u>350</u>/366	4	1	1	2	*334/**354*** (1)			1 (1)	—
	98–23	326/382	<u>366</u>/386	7	18	8	9	*326/**370*** (1)	*382/**370*** (3)	*326/**362*** (2)	4 (1)	2 (1)
	98–26	<u>316</u>/404	312/450	9	12	12	8	*320/**450*** (4)	*320/**312*** (5)		9 (1)	—
	98–34	354/386	370/<u>440</u>	12	10	6	17	*345/**444*** (1)			1 (1)	—
Total											17 (6)	2 (1)
OGO1c....	98–44	342/350	408/<u>474</u>	1	1	3	3	*342/**478*** (1)			1 (1)	—
	98–51	295/366	303/<u>362</u>	1	2	4	2	*295/**366*** (1)			1 (1)	—
	98–71	269/420	346/<u>450</u>	8	16	10	8	*420/**446*** (2)			—	2 (1)
		a/a[c]	c/d								—	—
	98–64	348/348	309/<u>448</u>	5	4	0	0	*348/**444*** (1)			—	1 (1)
Total											2 (2)	3 (2)

[a] The most likely progenitor of the mutant allele (selected on the basis of size similarity) is underlined.

[b] The mutant allele is indicated by bold-face type, and the number of each mutant genotype observed is indicated (*n*).

[c] The number in parenthesis is the number of mutation events inferred, assuming that multiple copies of the same mutant allele within a family resulted from a single mutation event.

that appear in a population may reflect mutations that occurred quite early in development. In sexually reproducing animals, gametes develop from primordial germ cells (PGCs) that differentiate from the somatic cells during the first several divisions in the developing zygote. These cells eventually migrate to the area of gonadial development where the germ cells are produced. The number of PGCs produced varies among organisms (see review by Matova and Cooley 2001).

In zebrafish (*Danio rerio*), researchers combining morphological and mRNA expression studies using germ line markers have recently determined that by the 5-somite (32-cell) stage and until about the 1,000-cell stage, there are four PGCs (Braat et al. 1999). During their migration toward the gonads, the four PGCs give rise to a total of 20–30 cells that populate the gonad and differentiate into germ cells (Braat et al. 1999). If a mutation occurs in one of the original four PGCs (and there is no attrition of cell lines), approximately one out of eight (12.5%) of the progeny should inherit the mutant allele. If gametogenesis is similar in pink salmon, our findings of 9 identical mutant alleles out of the 50 transmitted maternally (18%) in family 98-26 and 4 of the 46 identical mutant alleles (8.7%) transmitted paternally in family 98-23 suggest that each of these mutations likely occurred either in one of the four PGCs or in the subsequent one or two generations of cells that populated the gonad.

The occurrence of clustered mutations results in nonuniform distributions of novel alleles in a population which could influence interpretations of mutation rates and patterns as well as estimates of genetic population structure. For example, Woodruff, Huai, and Thompson (1996) have shown that mutant alleles that are a part of clusters are more likely to persist and be fixed in a population than mutant alleles entering the population independently. In the present study, 15 of the 24 mutant alleles detected at *SSA408* (54%) apparently resulted

from premeiotic mutations. Jones et al. (1999) similarly found that a high proportion (40%) of new mutants observed in pipefish occurred in clusters.

We estimated mutation rates by counting each mutant allele detected as one mutation, regardless of whether the allele appeared to be part of a mutational cluster. However, we only included randomly selected individuals in this analysis (i.e., we eliminated the 78 additional progeny from families 98-23 and 98-26 that we analyzed because we had detected multiple mutations in our initial analysis). The remaining 11 mutations in 1,300 transmissions at *SSA408* and 5 mutations in 1,278 transmissions at *OGO1c* yield mutation rate estimates of 0.0085 (0.0042–0.0151) and 0.0039 (0.0013–0.0091) mutations per gamete, respectively, with the numbers in parentheses being the 95% Poisson confidence intervals. We did not detect mutations at any of the other seven loci; the upper 95% confidence limit using a Poisson distribution for detecting zero mutations in 1,300 transmissions is 0.0028. The proportion of mutations observed varied significantly among the nine loci (contingency chi-square, $P < 0.001$). However, our mutation rate estimates for all the loci are within the range reported for other organisms (see Ellegren 2000*b*, table 1).

All the mutations detected were size changes of four bases (table 2) which is consistent with single-step addition or deletion mutations at both *SSA408* and *OGO1c*. Determining to what degree our data reflect a tendency for mutations to result in size increases or decreases depends on how mutations are counted. If all mutations are treated as single events, 17 mutations at *SSA408* reflected size increases and two reflected size decreases (table 2). Similarly, two mutations detected at *OGO1c* resulted in size increases and three resulted in size decreases (table 2). Alternatively, treating all within-family clusters of the same mutant allele as single mutations that were propagated during gametogenesis

reduces the number of size increases at *SSA408* to six and size decreases to one (table 2). Similarly, because two of the progeny in family 98-71 share the same mutation at *OGO1c*, the number of size decreases would be two rather than three (table 2).

Is the SMM appropriate for pink salmon microsatellites? All 11 of the unique mutations detected in this study were consistent with the addition or deletion of a single repeat unit, which is in accordance with the SMM. Furthermore, our data reflect a high incidence of homoplasy, as 7 of the 11 different mutant alleles detected were alleles already present in other families in this study (E. K. Steinberg et al., unpublished data). Because SMM-based estimators assume that alleles of similar sizes are related, these estimators are expected to be more accurate in the presence of size homoplasy than estimators that assume all mutations are independent and result in novel alleles (Estoup and Angers 1998). These findings suggest that genetic differentiation estimators based on the SMM would be appropriate. However, we also detected a tendency toward size increases in mutant alleles. Estoup and Angers (1998) recommend comparing results from estimators based on different underlying mutational models for concordance because it is not clear how different types of mutational biases affect SMM-based estimators. Given our findings, we agree with this recommendation.

The duplicated locus *OMYRGT6-1,2* apparently comprises two different microsatellite arrays in pink salmon. In rainbow trout, the two copies of this duplicated locus map to different linkage groups (Sakamoto et al. 2000). However, whether these loci comprise different microsatellite arrays in rainbow trout has not been analyzed (T. Sakamoto, personal communication). It would be informative to compare the sequences of *OMYRGT6-1* and *OMYRGT6-2* in rainbow trout, as well as other salmonid fishes, to study the evolution of these two divergent paralogous microsatellite loci. Salmonid fishes have undergone extensive gene duplication compared with other organisms, having diverged from a tetraploid ancestor approximately 25–50 MYA (Allendorf and Thorgaard 1984). Given the prevalence of duplicated loci in salmonid fishes, these organisms may provide an exceptional opportunity to use comparative approaches to study the molecular evolution of microsatellites.

In conclusion, we found evidence for heterogeneity in the rates and patterns of mutation among loci, suggesting that no single model will likely represent the complexity underlying the evolutionary dynamics of microsatellites. Our findings add to the evidence in support of the argument made by Woodruff, Huai, and Thompson (1996) that the occurrence of premeiotic cluster mutations may play an important role in the evolutionary dynamics of microsatellites. Finally, our identification of duplicated microsatellite loci comprising different repeat arrays indicates that the use of comparative analysis to study mutation dynamics (e.g., Amos 1999) could be misleading if the duplication is not detected.

Acknowledgments

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Appendix 4

Estimation of chiasma interference and construction of a linkage map for pink salmon

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ABSTRACT:

We constructed a genetic linkage map for pink salmon (*Oncorhynchus gorbuscha*) consisting of 609 polymorphic loci assigned to one of 44 linkage groups. The map covers 6472 cM with an average resolution of 8 cM. Gene-centromere distance for 319 of these loci were estimated using gynogenetic diploid individuals. The gene-centromere data allow us to locate the centromere on 19 linkage groups and to determine the degree of interference in some linkage groups.

INTRODUCTION:

Salmonid fishes diverged from a single tetraploid ancestor approximately 25-50 million years ago (Allendorf and Waples 1996). Residual tetrasomic inheritance still occurs at some loci in males, but only disomic inheritance has been reported in females. The persistence of disomic segregation in females suggests that the tetrasomic ratios in males result from distal recombination between homeologous chromosomes (Wright et al. 1983, Allendorf and Danzmann 1997). This can be explained conceptually by a two-stage model of pairing in which, first, homologous chromosomes pair and recombine in the proximal region of the chromosome. Next, homeologous chromosomes pair and recombine distally. Yet, if the crossover nearest the centromere determines the pattern of disjunction (Burnham 1962), then each gamete would receive one copy of each homolog, allowing continued disomic segregation in females.

Extensive gene duplication in salmonids has made genetic interpretations more complex than in species without a polyploid ancestry. Isoloci were originally described as pairs of duplicated allozyme loci whose allelic products have identical electrophoretic mobility (Allendorf and Thorgaard et al. 1984). It has been hypothesized that genetic divergence between isoloci has been prevented by the chromosomal exchanges between homeologs as described in the previous paragraph.

Pink salmon have a fixed two-year life cycle (Heard 1991). This is a shorter generation time than most other salmonid species which is helpful for genetic investigation. In addition, the fixed two-year cycle in pink salmon has resulted in nearly complete reproductive isolation between odd- and even-year fish. Chromosomal differences have been reported between odd- and even-year fish in the same geographical area (Phillips and Kapuscinski 1988). Gharrett et al. (1999) have also demonstrated outbreeding depression in experimental crosses between the two year classes. Based on these results, we would expect to find differences in linkage distances for some loci between odd- and even-year fish.

Genetic linkage maps and gene-centromere mapping have become a relatively common tool used to determine where loci are located relative to each other and on each chromosome. Detailed linkage maps exist for several fish species such as, *Oncorhynchus*

mykiss, and *Danio rerio.* Through the comparison of linkage maps we can gain a better understanding of how salmonid genomes have evolved.

In this paper we describe a genetic linage map constructed for pink salmon (*Oncorhynchus gorbuscha*). We expected this map to be large based on the duplicated genome in salmonids and the higher rate of recombination detected in females. Our results support the model that isoloci are more likely located farther out on the chromosome arm.

MATERIALS AND METHODS:

Samples: 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 in August 1995. 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.

Haploid Gynogenesis: Families of gynogenetic haploid embryos were produced by sperm inactivation (Thorgaard et al. 1983). Sperm from four males was pooled for the odd-year crosses, individual males were used for the even-year crosses. UV irradiated sperm was mixed with the eggs from individual females; producing 15 odd-year and 12 even-year families . Embryos from these families were incubated until just prior to hatching when they were collected and preserved in ethanol.

Muscle, liver, heart, and eye tissues were collected from the parents and frozen at −80° C until analysis. Embryos were dissected away from the egg chorion and yolk sack. DNA was isolated using the Puregene(TM) DNA isolation kit (Gentra Systems Inc.). The concentration of DNA was determined using a scanning spectrofluorometer. DNA extractions from haploid embryos yielded an average of 45.30 µg of DNA.

Prior to segregation analysis, all putative gynogenetic haploid individuals were screened to eliminate diploids that could be produced by the failure of sperm inactivation. A Y chromosome-specific growth hormone pseudogene was used to identify diploid males and six single copy microsatellite loci to identify diploid females (Spruell et al. 1998).

Diploid Gynogenesis: Families of gynogenetic diploid embryos were produced as described by Thorgaard et al. (1983). Lindner et al. (2000) describes analysis of gynogenetic diploid progeny.

Markers: 41 microsatellite loci were amplified as reported by the original authors with minor modifications. The annealing temperature and reference for 27 of these loci is reported in Lindner et al. (2000). An additional 14 loci were analyzed (Table 1). Products from unlabeled primers were fluorescently tagged by incorporating a TAMRA labeled dUTP or dCTP (Perkin & Elmer) during PCR. All PCR products were electrophoresed in a 7% denaturing polyacrylamide gel and visualized with a Hitachi FMBIO-100 or ABI 377 fluorescent imager.

Enzyme electrophoresis followed Aebersold et al. (1987); tissue and buffer combinations were those described in Seeb et al. (1999). Amplified fragment length polymorphisms (AFLPs) and Paired Interspersed Nuclear Elements (PINEs) were amplified as described in Spruell et al. (1999).

Nomenclature: Females and families are designated using the first letter of their origin (A=Armin F. Koernig, V=Valdez Fisheries Development Association), the year of reproduction, and a sequential number. For example, family A95-103 contains the progeny from female 103 collected at Armin F. Koernig hatchery in 1995. Nomenclature for allozyme loci follows Shaklee et al. (1990) and Seeb et al. (1998). Genetic nomenclature for microsatellites, AFLPs, and PINEs follow Spruell et al. (1999).

Selection of a Reference Family: 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 an initial screening for polymorphic microsatellite loci. DNA was extracted from 150 putative haploid embryos in this family. Based on microsatellite and Y-linked pseudogene data we detected 29 diploid individuals and eliminated them from subsequent analysis. Ninety-four of the remaining 131 embryos contained sufficient DNA for extensive analysis. Seven of the 77 gynogenetic diploid individuals were eliminated based on the presence of alleles not present in their mother. Gene-centromere mapping studies were conducted on the remaining 70 individuals.

Family V96-13 was chosen 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*. DNA was extracted from 162 putative haploid embryos and tissue of 54 gynogenetic diploid individuals. 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). 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 of female V96-13 is based on 90 haploid and 54 gynogenetic diploid individuals.

Linkage Analysis: We used MapMaker software (Lander et al. 1987) to assign markers segregating in haploid progeny to linkage groups. Grouping of markers used 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 was unknown. Therefore, the segregation pattern of each locus was entered into the program in both possible phases. The Kosambi mapping function was used to calculate the genetic distance. The MapMaker "ripple" function was used to confirm the best order.

Gene-centromere distances were estimated for 319 polymorphic loci using 70 gynogenetic diploid progeny from female A95-103. In addition, gene-centromere distances were estimated for 40 polymorphic loci using 54 gynogenetic diploid progeny from female V96-13. Dominant markers can be used to estimate the frequency of second division segregation if the female is known to be heterozygous through segregation analysis. Without segregation analysis, the presence of a fragment in all progeny could result either from the female being homozygous or from a high frequency of second division segregation.

A program written in FoxPro computer language was developed (Weaver, pers. com.) to determine linkage associations of loci for the gynogenetic diploid segregation

data. The power to determine non-random association between loci is inversely related to their distance from the centromere. Therefore, only loci with a $y \le 0.79$ were included in the analysis. All pairwise comparisons of these loci were evaluated for significant non-random associations using the chi-square test with a cutoff of $\chi^2 = 11.0$ (p<0.005).

Interference analysis: Interference was estimated from gene-centromere data using the analysis described in Zhao and Speed (1999). Segregation data from at least two loci in the same linkage group with y values < 1.0 are required for this analysis.

Interference for the haploid data was estimated using the traditional method of analyzing the number of recombination events across three loci of known order. The *coefficient of coincidence* (cc) was calculated as the ratio of observed double recombinants to the expected number of double recombinants assuming no interference. Interference was calculated as 1-cc.

RESULTS:

A95-103 haploid linkage map: We assigned 553 of the 596 markers analyzed for segregation in family A95-103 to one of 44 linkage groups covering a distance of 4550 cM (Figure 1; Tables 2 and 3). 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 6472 cM. The haploid pink salmon genome is approximately 2.72 billion base pairs or 2.72 million kilobase pairs (kpb; Johnson et al 1987b); thus, we estimate approximately 420 kbp/cM.

Each of the 41 microsatellite loci analyzed for linkage in female A95-103 was assigned to one of the 44 linkage groups. Segregation analysis of four loci *Fgt1-1,2, Ogo7-1,2, OmyFGT276-1,2,* and *Ocl1-1,2* identified these loci as being duplicated (Lindner et al. 2000). *OmyFGT276-1,2* and *Ocl1-1,2* were the only duplicated loci for which female A95-103 was polymorphic at both loci allowing all four loci to be mapped. Each locus of the duplicated pair mapped to different linkage groups, *OmyFGT276-1 & -2* linked to LG18 and LG53; *Ocl1-1 & -2* linked to LG19 and LG40 respectively. In additional loci such as, *μSat60-1,2, Fgt25-1,2, Oki3-1,2, Fgt34-1,2, Omy008-1,2,* and

Ssa20.19-1,2 only one of the duplicated pair segregates in female A95-103. These loci were confirmed to be duplicated through segregation analysis in different pink salmon families (unpublished data). Additional segregation analysis identified a PCR null allele at *Ssa197, One2*, and *OmyFGT276-2*. A null allele was identified when half of the haploid progeny amplified the same allele present in female A95-103 and half did not amplify any product (Spruell et al. 1999).

AFLPs and PINEs amplified the greatest number of reproducible polymorphic loci, 393 and 162 respectively (Table 3). A total of 519 of the AFLP and PINE polymorphisms are presence / absence differences. In addition, ten of the AFLP polymorphisms and eight PINE polymorphisms appeared to be caused by a length polymorphism within a fragment. For all of these polymorphisms, individuals have one of two different sized fragments produced by the same primer combination (Spruell et al 1999).

We analyzed 168 AFLP and 101 PINE loci in gynogenetic diploid progeny at which female A95-103 is heterozygous for the presence or absence of a product based on haploid progeny (Spruell et al. 1999, Lindner et al. 2000). In addition, gene-centromere data was collected for five allozyme loci (*sAAT3, CKC2, ADA2, GDA1,* and *PEPD2*) and 39 microsatellite loci in female A95-103.

A total of 216 out of 319 loci met the gynogenetic diploid linkage analysis criteria discussed above. Only two of the five allozyme loci polymorphic in female A95-103 could be included with a y≤ 0.79 (CKC2 y=0.29 and GDA1 y=0.35). We detected 365 non-random associations between pairs of loci ($\chi^2 \geq 11.0$). Of these pairwise associations, 281 confirmed linkage previously detected with the haploid data. However, 60 non-random associations are between loci from two different linkage groups based on the haploid data. There were two or more significant pairwise associations between ten pairs of linkage groups. Two separate linkage groups based on the haploid data were consolidated to one linkage group, LG40, based on the gynogenetic diploid analysis.

Linkage analysis conducted for allozyme and microsatellite loci in 16 normal diploid pink salmon families resulted in the addition of 11 allozyme loci to the A95-103 haploid map. Five allozyme loci are linked to microsatellite loci already placed on the map using the haploid data (Table 4). One allozyme locus, *CKC2* is linked to two linkage

groups. Based on the gene-centromere data from female A95-103 $CKC2$ is linked to three loci in LG53, $OmyRGT43$ ($\chi^2 = 22.03$), $ACG/CAA240$ ($\chi^2 = 14.54$), and $ACC/CAA106$ ($\chi^2 = 13.88$). Based on the normal diploid data from family A95-103 this locus is linked to $\mu Str60-1,2$ in LG 25 (rf = 0.348, $\chi^2 = 4.26$; Table 4).

V96-14 haploid linkage data: Data from family V96-13 was used to support linkage relationships in the even year map as well as to include additional loci for which female A95-103 did not segregate. We assigned 102 of the 123 loci analyzed for segregation in family V96-13 to one of 33 linkage groups. 25 of the 41 microsatellite loci that segregated in family V96-13 also segregated in family A95-103. Six linkage relationships are conserved between the two families (A95-103 and V96-13). One gene of known function $MHCB\alpha2$ is linked to a microsatellite locus ($Str60-2)$ and two PINE loci.

Interference: The degree of interference was estimated in nine linkage groups based on both half-tetrad (gynogenetic diploid) data as well as haploid data (Table 5). The two methods used to estimate interference agree on the degree of interference in only one of the nine comparisons (LG8; Table 5). In four cases (linkage groups 27, 33, 34, 57) the estimates were very close, either high or complete interference. In three of the four cases (linkage groups 2, 25, and 40) when the two methods do not agree, the half-tetrad analysis resulted in high or complete interference and the haploid data results moderate to no interference. In one comparison (LG5) the half-tetrad data resulted in no interference and the haploid data resulted in moderate interference.

Interference analysis of half-tetrads across six loci in LG2 results in an estimate of high interference. A subset of two loci from this group ($RGT6$ and $5H5T224$) was analyzed using the half-tetrad method, and no interference was found.

Discussion:

Linkage map: A total of 603 polymorphic loci have been analyzed to construct the pink salmon linkage map (Figure 1, Table 3). Yet, the presence of extra linkage groups and unlinked markers indicates that more loci need to be added to consolidate the map. A large number of linkage groups (54%) consist of ten or fewer loci. In addition, 13 linkage groups (33%) are composed of markers with $y \geq 0.46$ indicating they are more

telomeric sections of the chromosomes and need to be linked to the appropriate centromere. A majority of the markers mapped on our linkage map are AFLPs (Table 3) which have been reported to be biased toward the centromeric region (Young et al. 1998, Lindner et al. 2000). This may contribute to our difficulty in linking telomeric linkage groups to their appropriate centromeric region. The gene-centromere data is limited in helping to consolidate these telomeric groups due to the lack of power to assess linkage in markers located far from the centromere. We need to place more intermediate loci on the map in order to consolidate these linkage groups.

Gene-centromere data allowed us to locate the centromere on 19 linkage groups ($y \leq 0.05$). Of these groups the centromeres is metacentric in ten linkage groups (Figure 1). Ref? found that # of the 26 chromosomes in pink salmon are metacentric indicating the need to link chromosome arms to the appropriate centromere. Ten pairs of linkage groups are potentially linked to each other based on multiple non-random associations between pairs of loci in the gene-centromere data. If we relax the LOD to 3.0, one of these pairs of groups (LG34 & LG28) link to each other. However, when the haploid data is reanalyzed for all of these pairs of linkage groups we were unable to consolidate the groups without very large recombination fractions.

The estimated size of the pink salmon genome is consistent with maps constructed in other fishes (Table 6). Due to the polyploid ancestry and the increased recombination rate in females we expect our estimate of genetic size for pink salmon to be approximately twice that of non-polyploid or male based maps.

Markers: The AFLP technique is especially useful when building a map based on haploids for two reasons. First, many bands are produced per reaction and, therefore, more scoreable polymorphic loci are produced per unit effort. Second, less genomic DNA is required; the selective amplification step uses a sub-sample of the PCR products of the preamplification. Up to 133 selective amplifications can be completed from a single pre-amplification that originally used only 0.5 μg of genomic DNA. This technique allows the addition of many loci without the relatively large amount of DNA required for other techniques.

Comparative mapping: Approximately 25-100MYA the salmonids shared a

common ancestor. Through comparative mapping we can start to examine how genomes evolve into different species. Two of the linkage relationships in pink salmon agree with the results of the composite salmonid linkage map constructed by May et al. 1987. *MDHB1-2* and *FH* link to LG13 in the composite salmonid map and both are assigned to LG19 in pink salmon. In addition, *PEPD2* and *GPIB1,2* are assigned to LG4 on the composite map and assigned to LG40 in pink salmon.

Of the 35 microsatellites on the pink salmon linkage map, 25 are included on the rainbow trout map (Sakamoto et al. 2000). Two loci, *Omy301* and *Ssa197* are assigned to one group in both pink salmon (LG27) and rainbow trout (B). In addition, three pairs of loci that are linked in the pink salmon map are pseudo-linked in rainbow trout (Sakamoto et al. 2000). In pink salmon *Ogo7-1,2* and *Fgt25-1,2* are assigned to LG2 but in rainbow trout these loci are assigned to Oi and Oii, *μStr60-1,2* and *Ots100* are assigned to LG25 in pink salmon but to Fii and Fi in rainbow trout, and *Ocl2-1,2* and *OmyRGT1* are assigned to LG57 in pink salmon but to LG15 and LG5 in rainbow trout. LG15 and LG5 are pseudolinked in the composite salmonid map (May and Johnson 1990). These three pairs of loci are all located proximal to the teleomere ($y \geq 0.60$) in pink salmon. In addition, four of the six loci have been identified as duplicated in pink salmon (*Ogo7*, *μStr60-1,2*, *Ocl2*, and *Fgt25*).

One explanation for the difference in linkage relationship between the two species is that these loci are in the process of diploidization after the ancestral chromosome duplication event 25-100MYA. (Allendorf and Thorgaard 1984). In rainbow trout the loci on homologous chromosomes have differentiated enough that the duplicate pair of these loci do not amplify. Yet, the primers amplify each locus *Ogo7* and *Fgt25* on one of the two homeologs. Due to the telomeric location of these loci and tetrasomic inheritance the loci on homeologous chromosomes are pseudolinked in male rainbow trout.

Differences in recombination rate between males and females: We examine recombination rates between linked loci *ADA2-SSA197* as well as *sIDHP2-OTS1* in both male and female pink salmon diploid families. At loci that are farther from the centromere (*SSA197 y* = 0.94) our data agree with Sakamoto et al.; males have a higher rate of recombination (Table 4). However, at loci located close to the centromere (*OTS1 y* =

0.18) recombination rates are once again higher for males (Table 4). This does not agree with Sakamoto et al. 2000. More data needs to be analyzed before we can determine sex specific recombination rates in pink salmon.

Interference: This large data set allows us to take a closer look at interference across large sections of the pink salmon genome in multiple linkage groups. There is evidence of high or complete interference in five of the nine groups analyzed. This is consistent with previous estimates of interference in fish (Allendorf et al. 1986). However, there is evidence that in some linkage groups (LG5) interference may not be complete. In addition, results for LG2 indicate that the interference may vary within a linkage group.

TABLE 1

Microsatellite locus names, annealing temperature, and references.

Locus	Annealing Temperature (^{0}C)	Reference
OMY008	58-52[a]	Holm & Brusgaard 1998
OmyFGT19	58-52[a]	Sakamoto et al. 2000
OmyFGT34	58-52[a]	Sakamoto et al. 2000
OmyRGT43	56	Sakamoto et al. 2000
ONE2	58-52[a]	Scribner et al. 1996
ONE102	58-52[a]	Olsen et al. in press
OTS100	56	Nelson & Beacham 1998
SSA408	60	Cairney & Taggart personal communication

[a] Indicates a countdown PCR.

TABLE 2

A breakdown of linkage groups based on number of markers in each group and the average size in cM.

Number of Markers	Number of groups	Average size (cM)
1-5	10	23.26
6-10	14	53.51
11-15	9	118.50
16-20	6	181.27
21-25	1	189.20
26-30	1	243.70
31-35	2	263.05
36-40	0	--------
41-45	0	--------
46-50	0	--------
over 50	1	457.40

TABLE 3

Summary of polymorphic loci detected by four different techniques.

	Number of polymorphic loci	Percent assigned to linkage group	Percent of loci codominant
AFLP	393	91	2.5
PINE	162	96	4.9
Micro	35	100	91.4
Allozymes	13	100	100
Total	603		

TABLE 4

Summary of linkages in normal diploid families between allozymes and microsatellites.

Loci	Family	Informative Parent	N	r	χ^2 1 df
sAAT3 - FH	A14	F	86	0.337	9.12
sAAT3 - sMDHB1,2	A14	F	89	0.112	53.49
sAAT4 - $^\mu$Str60	A104	F	21	0.238	5.76
ADA2 - PGDH	A120	M	56	0.125	31.50
ADA2 - SSA197	A103	F	42	0.024	38.10
	A120	M	18	0.111	10.89
CKC2 - $^\mu$Str60	A120	F	46	0.348	4.26
FH - MDHB1,2	A14	F	86	0.291	15.07
bGALA - G3PDH1	V2	M	75	0.346	7.05
GDA - PEPD2	A8	M	82	0.012	78.05
	A20	M	95	0.105	59.21
	A29	M	45	0.000	45.00
G3PDH1 - PEPLT	V5	M	75	0.240	20.28
GPIB1,2 - PEPD2	V2	M	75	0.013	71.05
sIDHP2 - Ost1	A29	M	41	0.366	2.95
	A104	F	33	0.303	5.12
PGDH - Ssa197	A120	M	20	0.050	12.20

TABLE 5

Comparison of interference estimates based on half-tetrad and haploid data from nine
linkage groups. The number in parenthesis corresponds to the relative amount of
interference for each method. The half-tetrad interference estimates are grouped into
no(0), low (1-3), moderate (4-6), high (7-9) or in some cases complete interference. The
haploid data range is grouped into no (0), low (0.01-0.33), moderate (0.34-0.66), high
(0.67-0.99), and complete (1.0) interference.

Linkage Group	Estimated degree of Interference	
	Half-tetrad	Haploid
2	High (9)	Moderate (0.46)
5	No (0)	Moderate (0.47)
8	Complete	Complete (1.00)
25	High (9)	No (0.00)
27	Complete	High (0.67)
33	High (8)	Complete (1.00)
34	High (9)	Complete (1.00)
40	High (8)	Low (0.23)
57	?	Complete (1.00)