

Exxon Valdez Oil Spill
Restoration Project Final Report

Construction of a Linkage Map for the Pink Salmon Genome

Restoration Project 030190
Final Report

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Construction of a Linkage Map for the Pink Salmon Genome

Restoration Project 030190 Final Report

Study History: Project 030190 was initiated in March 1996 to construct a genetic linkage map for pink salmon (*Oncorhynchus gorbuscha*) and to use this map to evaluate effects of natural selection on the genome of this species. Such a map was proposed initially to provide the necessary platform to identify genetic damage in pink salmon inhabiting oiled streams following the March 1989 *Exxon Valdez* oil spill (EVOS). This research was designed to aid recovery efforts with pink salmon, including estimation of straying rates, description of stock structure, and testing if marine survival and other organismal measures of phenotypic variation have a genetic basis. This work was designed to support work with pink salmon under the project *Oil-Related Embryo Mortalities* (Restoration Study \191A). The objective of that project was to identify germline mutations in pink salmon exposed to oil. Genetic damage induced by oil may either be small changes in nucleotide sequence (microlesions) or large-scale changes in chromosome structure (macrolesions). Annual reports were submitted in 1997 through 2002. One thesis has been published: Pilgrim 1999). Four journal articles were published: Spruell et al. (1999), Lindner et al. (2000), Steinberg et al. (2002), and Funk et al. (2005).

Abstract: We have constructed a genetic linkage map for pink salmon (*Oncorhynchus gorbuscha*) and have experimentally investigated marine survival and fitness of pink salmon in Prince William Sound. We analyzed segregation of 596 DNA fragments in odd-year pink salmon. Of these markers, 553 were assigned to one of 44 linkage groups. We estimated gene-centromere distances for 312 loci using gynogenetic diploid progeny. In August 1998, we collected gametes and tissue from 150 pink salmon from Likes Creek and used single-pair mating 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. We found nearly random family survival and high heritability of body length. Morphological traits were all moderately to highly heritable, but egg number and egg weight were not heritable, suggesting that past selection has eliminated additive genetic variation in egg number and egg weight or that there is high environmental variance in these traits. Genetic correlations were similar for nonadjacent morphological traits and adjacent traits. Genetic correlations predicted phenotypic correlations fairly accurately, but some pairs of traits with low genetic correlations had high phenotypic correlations, and vice versa, emphasizing the need to use caution when using phenotypic correlations as indices of genetic correlations.

Key Words: adaptation, fitness, gene-centromere mapping, genetics, linkage map, marine survival, mutation, *Oncorhynchus gorbuscha*, pink salmon.

Project Data: *Description of 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 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. *Format* -- All data were entered as Excel spreadsheets. *Custodian* -- Contact Fred W. Allendorf, Division of Biological Sciences, University of Montana, Missoula, MT 59812.

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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 complete. This work resulted in four peer-reviewed publications in the journals (Appendices B through E) and a published thesis (Appendix A). These journal articles represent the core of this final report.

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 (*MHC*Ba*2*).

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 were 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 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 also collected morphological data on the parents and the returning adults the 1998 cohort.

Understanding the genetic basis of phenotypic variation is essential for predicting the direction and rate of phenotypic evolution. We estimated heritabilities and genetic correlations of morphological (fork length, pectoral and pelvic fin ray counts, and gill arch raker counts) and life history (egg number and individual egg weight) traits of pink salmon from Likes Creek, Alaska, in order to characterize the genetic basis of phenotypic variation in this species. Families were created from wild-caught adults, raised to the fry stage in the lab, released into the wild, and caught as returning adults and assigned to family using microsatellite loci and a growth hormone locus.

Morphological traits were all moderately to highly heritable, but egg number and egg weight were not heritable, suggesting that past selection has eliminated additive genetic variation in egg number and egg weight or that there is high environmental variance in these traits. Genetic correlations were similar for non-adjacent morphological traits and adjacent traits. Genetic correlations predicted phenotypic correlations fairly accurately, but some pairs of traits with low genetic correlations had high phenotypic correlations, and vice versa, emphasizing the need to use caution when using phenotypic correlations as indices of genetic correlations.

INTRODUCTION.

We proposed to construct a genetic linkage map for pink salmon (*Oncorhynchus gorbuscha*) and to use this map to evaluate effects of natural selection on the genome of this species. Such a map was proposed initially to provide the necessary platform to identify genetic damage in pink salmon inhabiting oiled streams following the March 1989 *Exxon Valdez* oil spill (EVOS). We also conducted a series of experiments based at the Alaska SeaLife Center (ASLC) to identify regions of the genome that affect various organismal traits and to test for the effects of natural selection on regions of the genome that include markers used to describe genetic population structure. This research was designed to aid recovery efforts with pink salmon, including estimation of straying rates, description of stock structure, and testing if marine survival and other organismal measures of phenotypic variation have a genetic basis.

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

This work was designed to support work with pink salmon under the project *Oil-Related Embryo Mortalities* (Restoration Study \191A). The objective of that project was to identify germline mutations in pink salmon exposed to oil. Genetic damage induced by oil may either be small changes in nucleotide sequence (microlesions) or large-scale changes in chromosome structure (macrolesions). A detailed genetic map for pink salmon would have been invaluable for interpreting the results of Restoration Study \191A in several ways. First, it would be possible by following the inheritance of any DNA lesions to determine if they are micro- or macro-lesions. Second, these lesions could 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 was not completed.

Molecular genetic techniques have been used extensively to describe population structure of Pacific salmon (Utter et al. 1993; Gharrett and Smoker 1994; Seeb et al. 1998). Genetic divergence among populations has been interpreted as largely reflecting the patterns of exchange of individuals among populations (gene flow) and random changes in frequency of selectively neutral alleles within populations (genetic drift) (Allendorf and Phelps 1981; Waples 1995). This is a useful approach that allows description of the pattern and amount of gene flow among populations.

This approach to describe population structure is based upon the assumption that the pattern and amount of divergence observed is not affected by natural selection or mutation. However, even weak natural selection may have a substantial effect on the pattern of genetic divergence among populations (Allendorf 1983). In addition, different mutation rates at marker loci may also effect the amount of genetic differentiation between populations, in particular if mutation rates at some loci are high (e.g., Jin and Chakraborty 1995). Thus, the high frequency of mutations that we have detected may also have a substantial effect on the amount and pattern of genetic divergence at some loci.

Our work resulted in four peer-reviewed publications in the journals (Appendices B through E) and a published thesis (Appendix A). These journal articles represent the core of this final report.

OBJECTIVES. This project originally had the following overall specific objectives:

1. Develop several hundred variable DNA markers in pink salmon and test them for Mendelian inheritance.
2. Construct a linkage map based upon joint segregation patterns of the DNA polymorphisms detected in previous objective.
3. Map putative lesions identified in Restoration Study \191A.
4. Test for Mendelian inheritance of markers throughout the genome in progeny of fish exposed to oil. Regions that show aberrant segregation ratios in progeny of fish exposed to oil and normal 1:1 ratios in fish not exposed to oil would be candidates for oil-induced lesions.
5. Test for regions of the genome that are associated with traits of adaptive significance (e.g., marine mortality or run-timing).
6. Test if protein markers (allozymes) are under natural selection such that they may not provide accurate information about the genetic structure and amount of gene flow among populations.

We completed Objectives 1 and 2 (Appendices A, B, C, and D). We did not pursue Objective 3 because Restoration Study /191A did not identify any putative lesions for mapping, and Objective 4 because this aspect of Restoration Study \191A was not completed. We completed Objectives 5 and 6 (Appendix E)

METHODS

Gametes for the inheritance studies and linkage map were collected from Prince William Sound in collaboration with the project Oil-Related Embryo

Mortalities (Restoration Study \191A). Embryo incubation took place at the Genetics Lab facilities of ADFG. The laboratory analyses were done at the University of Montana and the ADFG genetics lab in Anchorage.

We began in FY 1998 to use the ASLC Research Facilities at Seward for experiments designed to test for natural selection at loci throughout the genome of pink salmon. Sexually mature pink salmon used in the experimental matings in 1998 and 1999 were collected from Likes Creek in Resurrection Bay. The progeny were marked with an adipose fin clip and released into Resurrection Bay. Due to the failure of the fish pass at the ASLC to attract returning adults we adjusted our plans for recapturing returning adults from the 1999 cohort to include sampling in upper Resurrection Bay.

Our initial map was constructed using gynogenetic haploid and gynogenetic diploid progeny from an odd-year individual female (95-103). This is the same procedure that has been used to build the zebrafish linkage map (Postlethwait et al. 1994). Stanley (1983) reported that haploid embryos of Atlantic salmon will develop until just prior to the stage of hatching if development of the eggs is activated by sperm in which the DNA has been inactivated by UV-radiation. We have used this technique routinely with fishes of the genus *Oncorhynchus* (Forbes et al. 1994; Appendix B). This allows us to follow the segregation and linkage relationships in haploid progeny from females. The use of haploid progeny avoids possible difficulties of dominance with some types of DNA markers because recessive alleles are not obscured by their dominant alternatives in haploids (Lie et al. 1994). Our odd-year map is primarily based on 603 segregating markers in 94 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 placed a number of so-called "anchor" loci on this map.

In addition we completed the construction of a linkage map based on the segregation pattern of 90 haploid individuals in an even-year female (V96-13). 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) 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. Having linkage data from both odd- and even-year individuals will make it possible to map more markers and will allow us to determine whether linkage relationships are conserved between the reproductively isolated year classes.

Marine Survival and Fitness Experiment

This aspect of the research was performed at the ASLC research facilities. Approximately 50,000 and 24,000 marked fish were released in spring of 1999 and 2000 respectively. We collected 36 sexually mature adults in Resurrection Bay from the 1998 cohort and 259 sexually mature adults in Resurrection Bay from the 1999 cohort produced from wild pink salmon collected from Likes Creek. A sample of the fish was collected at release and will be analyzed so that their genetic characteristics prior to the marine phase of the life cycle can be compared to the returning adults. Mousseau et al. (1998) have used a similar approach to estimate heritabilities for weight, length, and age at sexual maturation in chinook salmon.

RESULTS

Gene-Centromere Map

We estimated recombination rates between 312 loci and their centromeres using half-tetrad analysis in a recently published manuscript (Appendix C). We produced the half-tetrads by initiating development with irradiated sperm and blocking the maternal second meiotic division (Appendix B). AFLPs were significantly more centromeric than loci identified by three other techniques (allozymes, microsatellites, and PINES). The near absence of AFLPs in distal regions could limit their utility in constructing linkage maps. A large proportion of loci had y values approaching 1.0, indicating near complete crossover interference on many chromosome arms. As predicted from models of chromosomal evolution in salmonids, all duplicated microsatellite loci that shared alleles (isoloci) had y values of nearly 1.0. This is consistent with previous data from allozyme loci.

Haploid Linkage Map

We assigned 546 of the 590 markers analyzed for segregation in family A95-103 to one of 44 linkage groups covering a distance of 4559 cM (Figure 1; Tables 1-3). 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 6691 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 406 kpb/cM.

Each of the 35 microsatellite loci analyzed for linkage in female A95-103 was assigned to one of the 44 linkage groups. Segregation analysis of three loci *Fgt1-1,2*, *Ogo7-1,2*, and *OmyFGT276-1,2* identified these loci as being duplicated (Lindner et al. 2000). *OmyFGT276-1,2* was the only duplicated locus for which female A95-103 was polymorphic at both loci allowing both loci to be mapped. The two loci map to different linkage groups, LG18 and LG53. Additional loci such as, *μSat60-1,2*, *Ocl2-1,2*, *Fgt25*, and *Ssa20.19-1,2* where only one of the duplicated pair of loci segregates in female A95-103 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*. Half of the haploid progeny amplified the same allele present in female A95-103 and half did not amplify any product (Appendix B).

AFLPs and PINES amplified the greatest number of reproducible polymorphic loci, 393 and 162 respectively (Table 2). 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 (Appendix B).

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 (Appendix B, Lindner et al. 2000). In addition, gene-centromere data was collected for five allozyme loci (*sAAT3*, *CKC2*, *ADA2*, *GDA1*, and *PEPD2*) and 34 microsatellite loci in female A95-103.

A total of 202 out of 304 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 $y \leq 0.79$ (*CKC2* $y=0.29$ and *GDA1* $y=0.35$). We detected 345 non-random associations between pairs of loci. Of these pairwise associations, 293 confirmed linkage previously detected with the haploid data. However, 52 non-random associations are between loci from two different linkage groups based on the haploid data. Ten pairs of linkage groups included two or more significant pairwise associations. 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 haploid map. Five allozyme loci are linked to microsatellite loci already placed on the map using the haploid data (Table 3). 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 $\mu\text{Str}60-1,2$ in LG 25 ($r_f = 0.348$, $\chi^2 = 4.26$; Table 3).

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 4). The two methods used to estimate interference agree on the degree of interference in only one of the nine comparisons (LG8; Table 4). 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. If a subset of only two loci from this group is analyzed using the half tetrad method the interference estimate is no interference.

Comparison of Odd- and Even-Year Linkage Map

We described the segregation of 590 markers in haploid progeny from female A95-103; we also mapped 13 allozyme loci in the same female. We assigned 546 of the 590 DNA markers and all of the allozyme loci to one of 44 linkage groups covering a distance of 4559 cM. Given the haploid number of 26 chromosomes for pink salmon, our mapping efforts produced 18 extra linkage groups. Taking into account the extra linkage groups and 44 unassigned markers as well as the distance to the telomeres we estimate the size of the pink salmon genome to be 6691 cM. The haploid pink salmon genome is approximately 2.72 million kilobase pairs (kpb; Johnson et al 1987b); thus, we estimate approximately 406 kpb/cM. These results are consistent with our expectations when comparing to maps constructed in other fishes.

We completed construction of a linkage map for even-year pink salmon from Prince William Sound. We analyzed the segregation pattern of 85 loci in an even-year family (V96-13) and assigned 63 of 85 loci to one of 22 linkage groups. One gene of known function, *MHCB^{α2}*, is assigned to a linkage group that consists of one microsatellite and two PINE loci.

We estimated gene-centromere distances for 13 additional microsatellite loci in both odd and even year pink salmon (Figure 2). Most loci fall near our expectation for equal *y*'s in both years. One locus, *FGT34*, appears to be telomeric in the even year family and centromeric in the odd year family. *RGT1* displays the opposite pattern, appearing to be proximal in the even year family but distal in the odd year family.

Mutation Analysis

Our results have provided exciting and important information about mutation processes in microsatellites that are accepted for publication in the journal *Molecular Biology and Evolution* (Appendix D). Our experimental design depends upon being able to place returning adults into their correct family on the basis of their multiple-locus genotypes. We tested this by examining inheritance data at 11 loci (nine microsatellites and two genes of known function) for 10 progeny from each of the 50 families that were released in spring of 1999. In the process of analyzing the inheritance data, we detected several mutations at two of the microsatellite loci (*SSA408* and *OGO1c*), indicating that these loci have particularly high mutation rates. Furthermore, at *SSA408* the mutations detected were not distributed randomly among families. Rather, clusters of identical mutant alleles were found in certain families, suggesting they may have resulted from mutation events occurring very early in gametogenesis, prior to meiosis.

Our results have important significance for the use of microsatellite loci in management. Mutations are expected to have a substantial effect on the amount and pattern of genetic divergence among populations if the mutation rate approaches the rate of migration among populations (see discussion in Allendorf and Seeb 2000). Not surprisingly, the number of mutations detected was correlated with the number of alleles in the sample. We detected mutations at the two loci that have the greatest number of alleles in the parental population (*OGO1c* and *SSA408*). The mutation rate estimates at *OGO1c* and *SSA408* (3.7×10^{-3} and 5.4×10^{-3}) are at the high end of the range of 10^{-3} to 10^{-6} reported for other organisms (Dallas 1992, Weber and Wong 1993, Schug et al. 1997). The variability of these two loci makes them powerful tools for assigning parentage. We were able to unambiguously assign parentage to 35 of the 36 returning fish from the 1998 cohort based on these two loci alone. However, given the high probability of mutation at these loci, our results indicate that it is important to use a combination of low and high variability markers for parentage analysis. Our mutation analysis also suggests that *OGO1c* and *SSA408* are inappropriate as markers for analysis of stock structure in pink salmon.

Marine Survival and Fitness Experiment: 1998 cohort

In August 1998, 150 (75 male and 75 female) mature pink salmon were collected from Likes Creek, Resurrection Bay, and transported to the ASLC for controlled matings. We made 75 families of full-sibs by crossing one male and one female. One hundred progeny from each family were collected to test marker inheritance for parentage analysis. We then selected 50 of these families on the basis of egg number and survival during incubation for the release experiment. These families were pooled together into a single tank in March shortly after hatching. In May 1999, approximately 1,500 progeny from each of these 50 single-pair mating families were marked and released from the ASLC facility.

Progeny from this experiment returned in August 2000. We had anticipated a return rate of 2%, for a total of 1,000-2,000 individuals expected to be recovered for genetic and morphological analyses (approximately 30 fish per family). However, no fish returned to the ASLC fish pass, and we captured a total of 36 fish throughout Resurrection Bay. These 36 fish were placed into 30 families on the basis of 10 microsatellite loci. This sample size was too small to answer the questions that we are addressing.

Marine Survival and Fitness Experiment: 1999 cohort

We repeated this experiment with odd-year pink salmon in August 1999. We collected 68 adults (34 females and 34 males) from Likes Creek, and released their marked progeny from the ASLC in May 2000. This cohort returned in the summer of 2001. We used a different experimental mating scheme with these fish to allow a more powerful genetic analysis of the progeny. Each male and each female was crossed with two individuals in a series of 2 x 2 diallel crosses (Figure 3).

We collected 259 sexually mature adults from this cohort that returned to Resurrection Bay in summer 2001 (Figure 4). We identified the parents of these fish based upon genotypes at eight microsatellite loci and a growth hormone locus. The distribution of returning progeny (i.e., reproductive success) of the 34 males and females is close to expected with random reproductive success (Table 5; Figure 5). The only apparent exception is that there are 2 males that each produced an exceptionally large number of returning progeny.

The results from quantitative genetic analysis of these progeny are described in Appendix E.

DISCUSSION

Evaluation of Even-year Families for Mapping

We placed a gene of known function, *MHCB α 2*, 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 *MHCB α 2* 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 ancestral 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 3). 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 (Appendix D). 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 unambiguously 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; Appendix E) 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 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.

CONCLUSIONS

We 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 placed a gene of known function on the even-year map, *MHCBA2*. Comparisons between odd and even-year maps have not detected any differences in gene order.

Understanding the genetic basis of phenotypic variation is essential for predicting the direction and rate of phenotypic evolution. We estimated heritabilities and genetic correlations of morphological (fork length, pectoral and pelvic fin ray counts, and gill arch raker counts) and life history (egg number and individual egg weight) traits of pink salmon from Likes Creek, Alaska, in order to characterize the genetic basis of phenotypic variation in this species. Families were created from wild-caught adults, raised to the fry stage in the lab, released into the wild, and caught as returning adults and assigned to family using microsatellite loci and a growth hormone locus.

Morphological traits were all moderately to highly heritable, but egg number and egg weight were not heritable, suggesting that past selection has eliminated additive genetic variation in egg number and egg weight or that there is high environmental

variance in these traits. Genetic correlations were similar for non-adjacent morphological traits and adjacent traits. Genetic correlations predicted phenotypic correlations fairly accurately, but some pairs of traits with low genetic correlations had high phenotypic correlations, and vice versa, emphasizing the need to use caution when using phenotypic correlations as indices of genetic correlations.

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Table 1. 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 2. 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 3. Summary of linkages in normal diploid families between allozymes and microsatellites.

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

Table 4. 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)

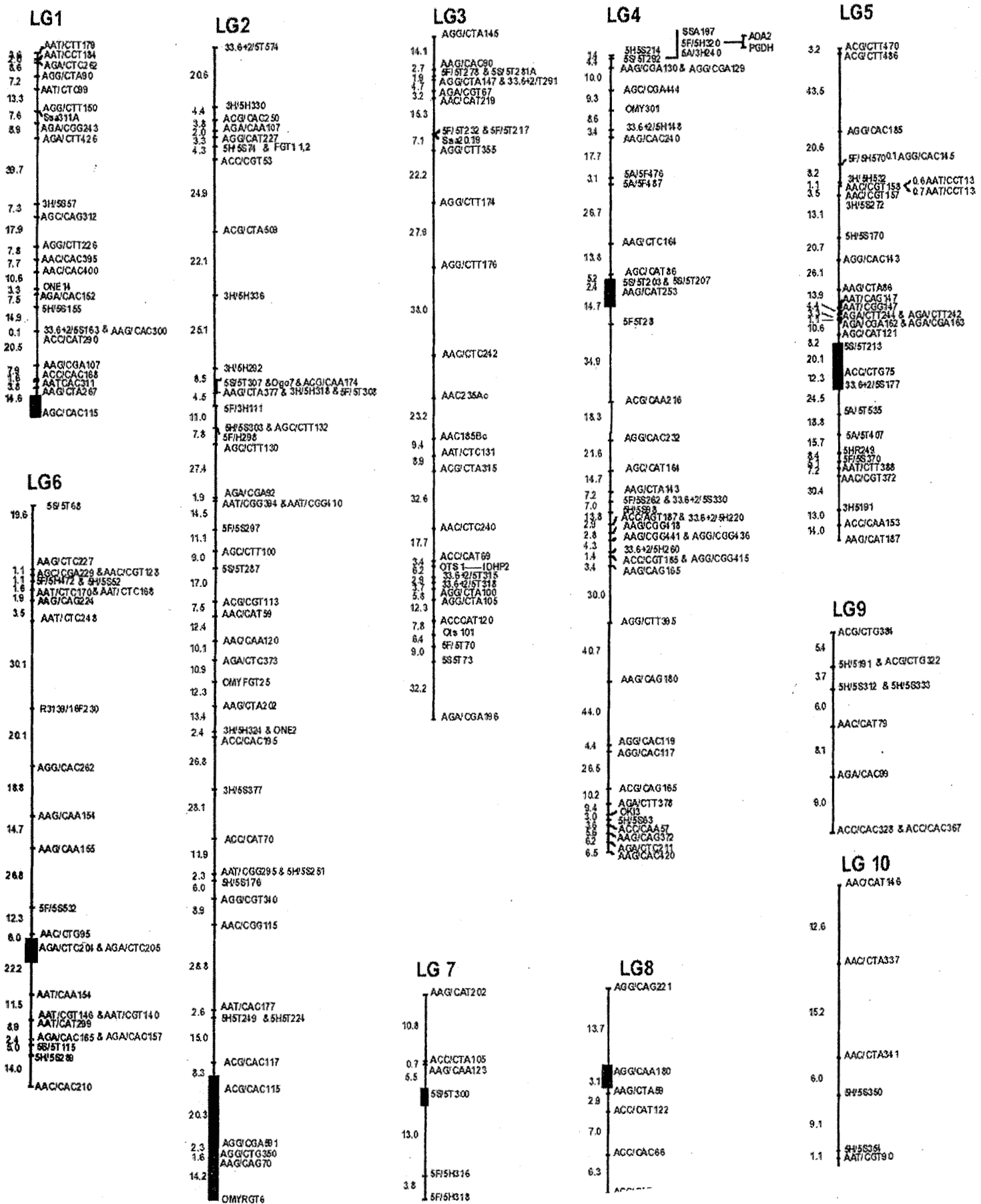
Table 5. Summary of adult progeny recovered from each family and parent, the number of alevins from each parent pooled prior to freshwater rearing, and the percentage of alevins from each parent recovered as adults. See Figure 1 for an explanation of parental cross schemes and family designations.

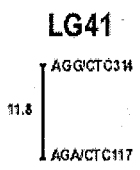
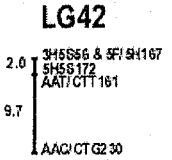
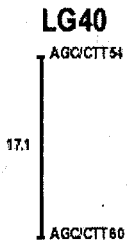
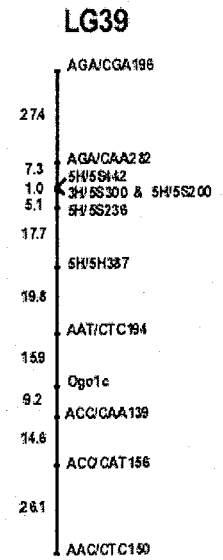
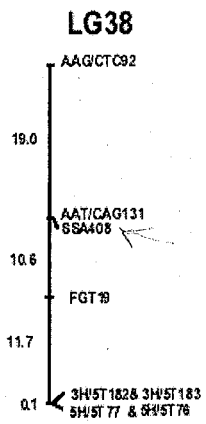
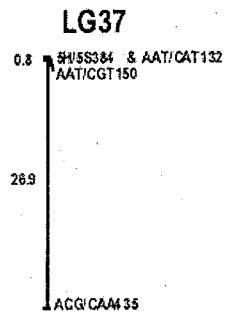
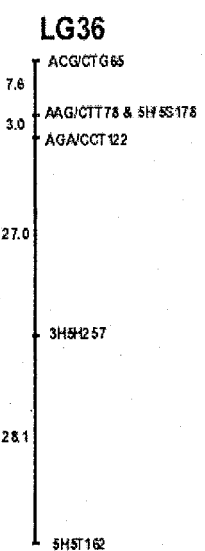
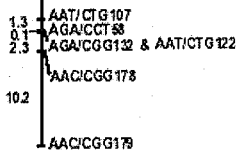
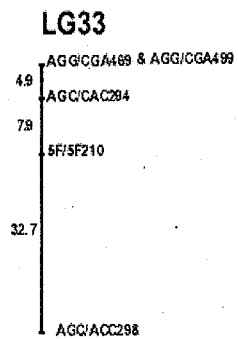
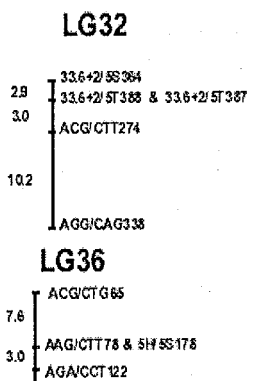
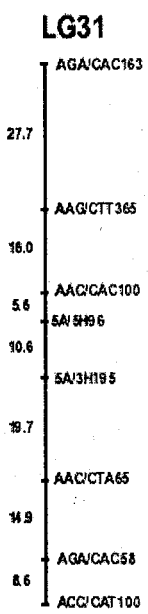
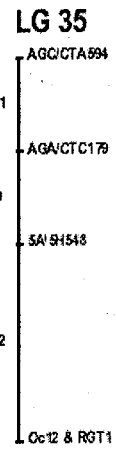
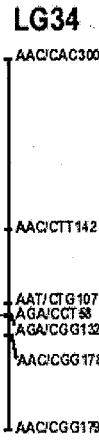
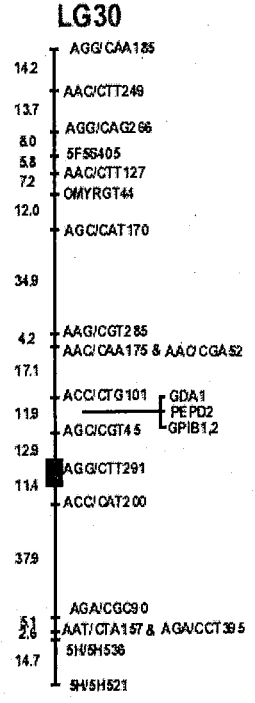
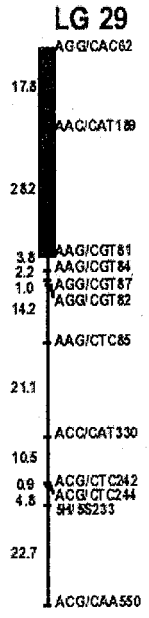
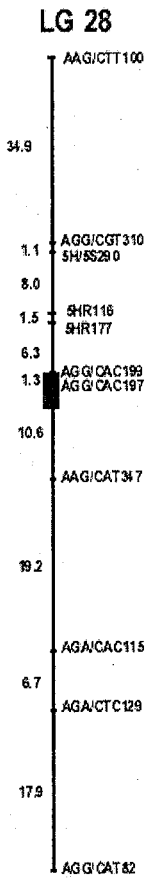
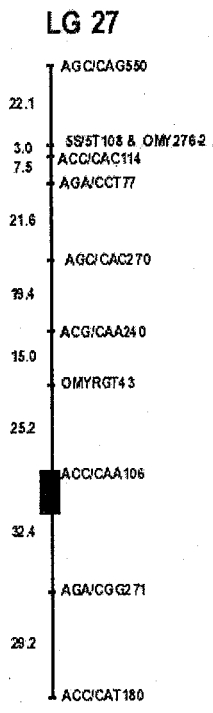
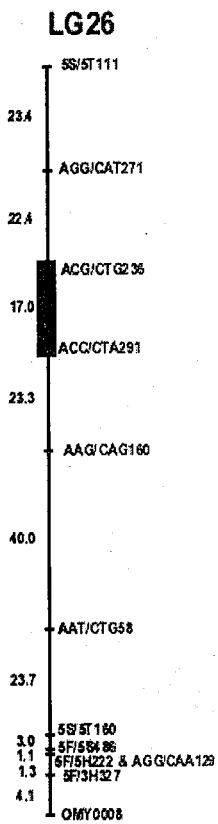
Fam. No.	Progeny per family		Dam	Progeny	%Survival	Alevins	Sire	Progeny	%Survival	Alevins
	A	B								
1	9	3	1	12	0.84	1424	101	18	1.44	1246
2	9	6	2	15	1.14	1321	102	9	0.60	1499
3	2	1	3	3	0.77	391	103	9	1.44	624
4	7	6	4	13	1.57	828	104	7	1.17	596
5	4	2	5	6	0.80	754	105	9	1.13	797
6	5	2	6	7	0.85	821	106	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

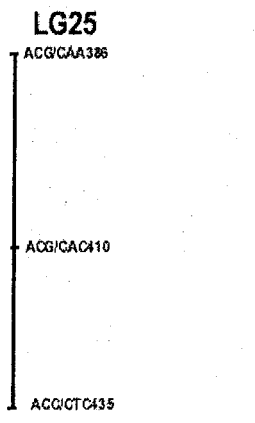
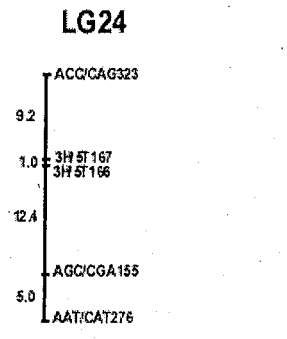
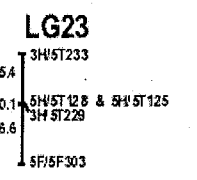
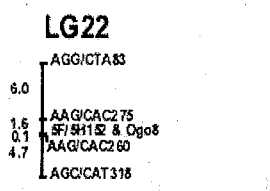
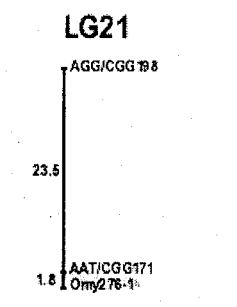
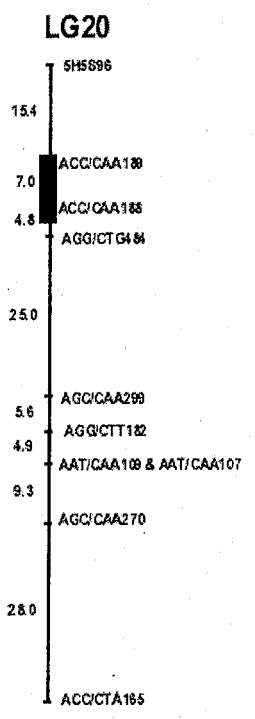
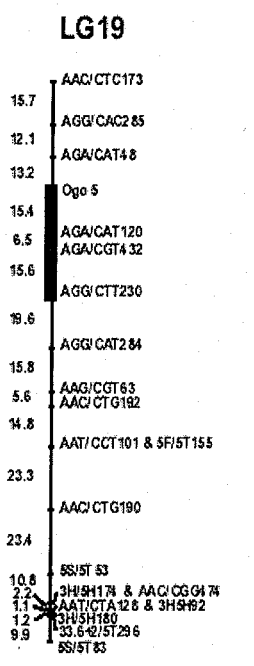
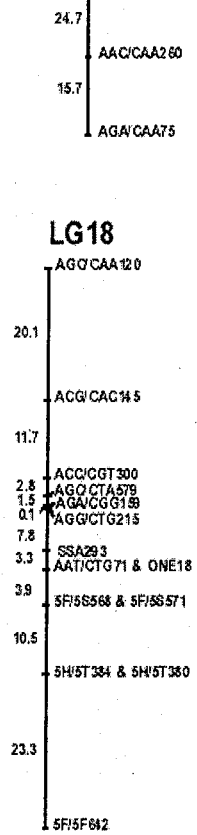
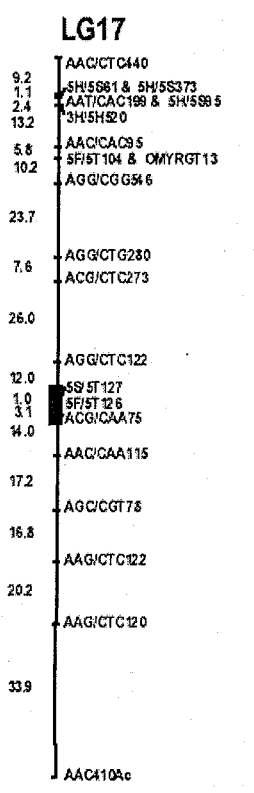
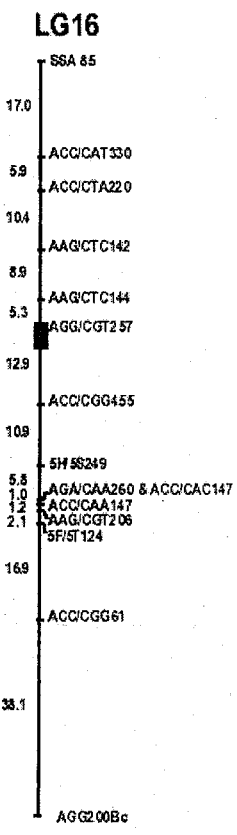
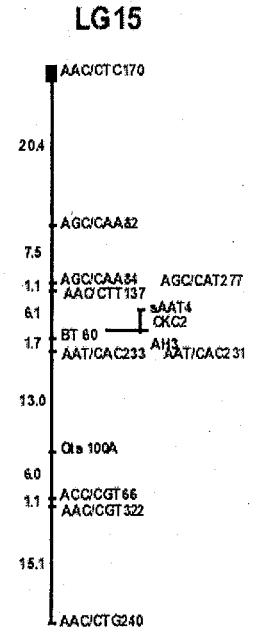
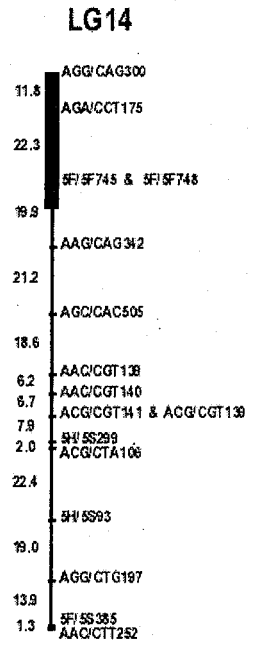
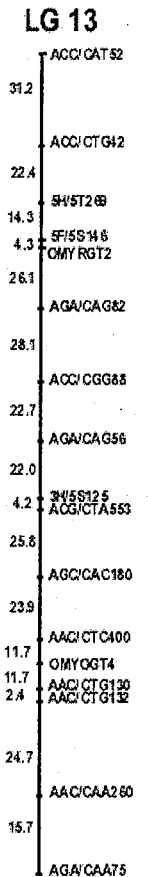
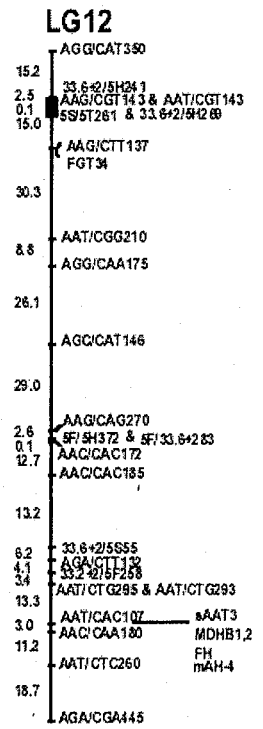
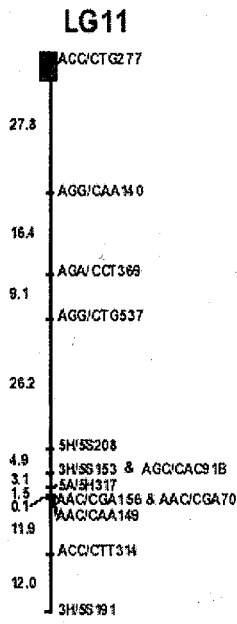
Table 5. (continued)

Fam. No.	Progeny per family		Dam	Progeny	%Survival	Alevins	Sire	Progeny	%Survival	Alevins
	A	B								
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 602 polymorphic loci. Numbers to the left indicate recombination rates (cM). Locus names are to the right. Centromeres are indicated by black rectangles.







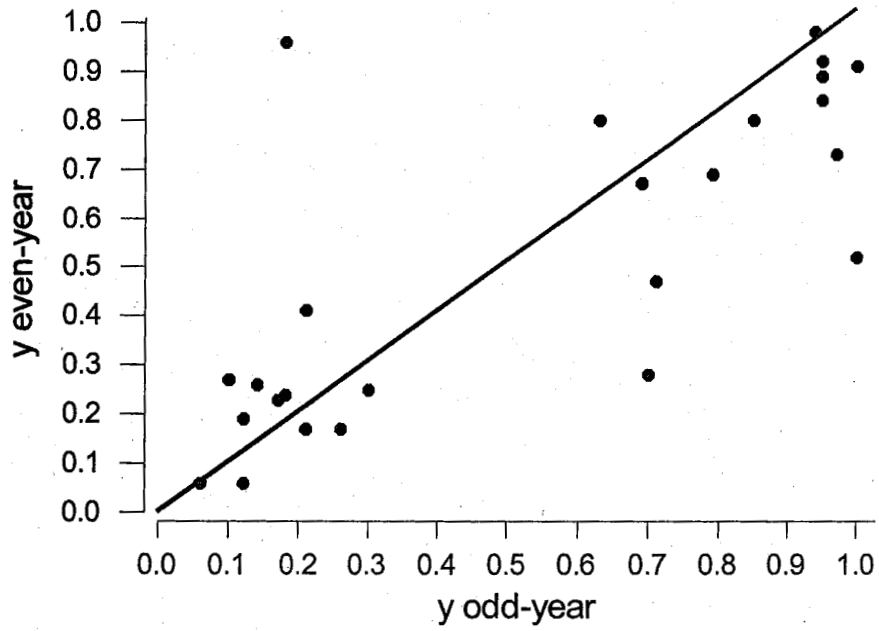
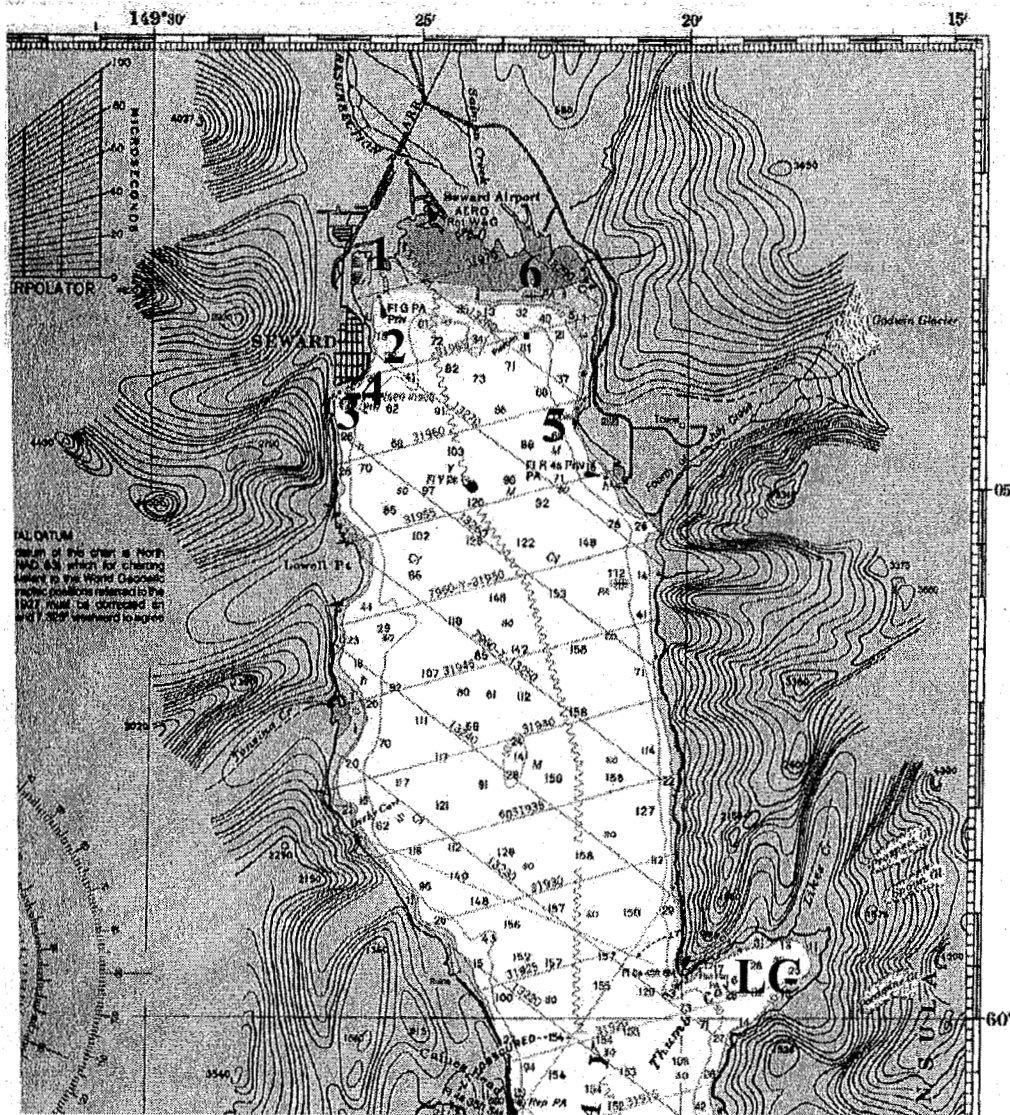


Figure 2. Comparison of gene-centromere distances (y) for odd and even year pink salmon. The gray line indicates the expectation if y values are equivalent in both years.

Figure 3. Diagram of our half-sib family experimental design. Numbers across the top represent females, numbers down the side represent males. The squares contain the family designation and the numbers of individuals used to make each family. The number of the dam is the family number in each half-sibling cross. The letter A or B for each family designates the sire. The parents of family 5A are dam 5 and sire 105, 5B are dam 5 and sire 106, 6A dam 6 and sire 105, and 6B are dam 6 and sire 106, and so forth.

		FEMALES									
		01	02	03	04	05	06	...	33	34	
MALES	101	1A 01x101	2A 02x101								
	102	1B 01x102	2B 02x102								
	103			3A 03x103	4A 04x103						
	104			3B 03x104	4B 04x104						
	105					5A 05x105	6A 06x105				
	106					5B 05x106	6B 06x106				
		133								33A 33x133	34A 34x133
		134								33B 33x134	34B 34x134

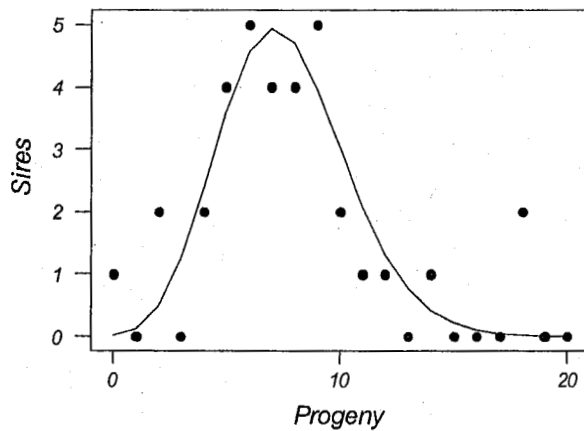
Figure 4. Map of Resurrection Bay. Numbers indicate the location where experimental fish were collected in 2001 as designated below. LC designates Likes Creek, the location where the parents were collected.



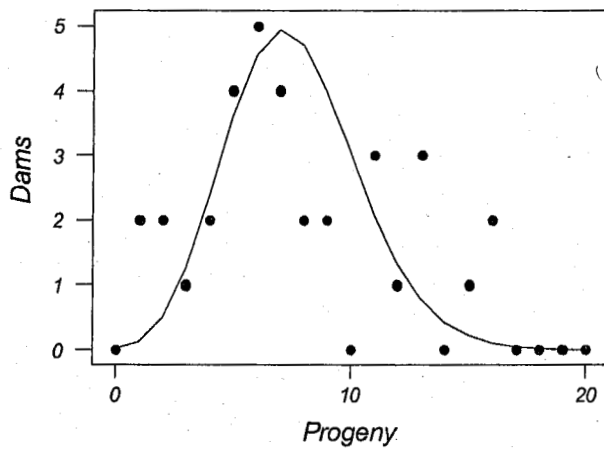
Map Number	Location	Number of fish collected
1	Boat Harbor	2
2	Culvert	31
3	Lowell Cr.	26
4	ASLC	182
5	Spring Cr.	4
6	Resurrection R.	10
	Unknown	4
	Total	259

Figure 5. Scatterplot of the number of progeny returning for sires (a) and dams (b). For example, there were two sires that produced 10 returning progeny. The line is the expected Poisson distribution.

(a)



(b)



A. Pilgrim, K. 1999. Identification of the sex-determining locus in pink salmon. M.A., Organismal Biology and Ecology, University of Montana.