Exxon Valdez Oil Spill
Restoration Project Annual Report

Construction of a Linkage Map for the Pink Salmon Genome

Restoration Project 99190
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, and 99190. Annual reports entitled Construction of a Linkage Map for the Pink Salmon Genome were submitted in 1997, 1998, and 1999. In this annual report we describe Restoration Project 99190. Ten 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 at the semi-annual Coast-wide Salmonid Genetics meeting in Seattle, March 1997 as well as at the SALMAP meeting in Toronto Canada, September 1998. Ms. Lindner also presented an update at the Alaska Department of Fish and Game in Anchorage Alaska, September 1997. Dr. Paul Spruell represented our lab at United States Department of Agriculture (USDA) panels on aquaculture in 1997, 1998, and 1999. Dr. Spruell and Ms. Lindner each presented mapping results at the Plant and Animal Genome Mapping Meeting in San Diego, January 1999. Ms. Lindner was invited to present our findings to the USDA panel on mapping in aquaculture species that is held in conjunction with the mapping meeting each year. Dr. Spruell was invited to participate in a workshop sponsored by Hitachi Software and used our pink salmon linkage data to illustrate the advantages of fluorescent technologies to produce linkage maps. In March 1999, Kathy Knudsen presented an overview of the project to the Kenai High School freshman biology class. In May 1999, Eleanor Steinberg was awarded a National Science Foundation postdoctoral fellowship in Biological Informatics to work in the Allendorf laboratory on the pink salmon project. She is currently developing a Microsoft Access database to manage and analyze the large amount of data being generated. A journal article (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; Inheritance of nuclear DNA markers in gynogenetic haploid pink salmon) is published in the Journal of Heredity (90:289-296). In addition a manuscript (Lindner, K. R., J. E. Seeb, C. Habicht, E. Kretschmer, D. J. Reedy, P. Spruell, and F. W. Allendorf; Gene-centromere mapping of 312 loci in pink salmon by half-tetrad analysis) is published in the journal Genome (43:538-549). A manuscript (Lindner, K. R., P. Spruell, C. Habicht, K. L. Knudsen, J. E. Seeb, H. Zhao, and F. W. Allendorf; A linkage map for pink salmon based on gynogenetic haploids and half tetrads) is currently being prepared for submission to Genetics. Another manuscript (Steinberg, E. K., K. R. Lindner, A. E. Maxwell, and F. W. Allendorf; Pattern and rate of mutation at microsatellite loci in pink salmon) is being prepared for submission to Molecular Ecology.

Abstract: We are developing genetic markers and constructing a genomic linkage map for pink salmon (*Oncorhynchus gorbuscha*) for use in recovery efforts for pink salmon in Prince William Sound. We have analyzed segregation of 589 DNA fragments in an odd-year female and 94 of her haploid progeny. Of these fragments, 563 have been assigned to one of 42 linkage groups. We have mapped 312 loci in relation to their centromeres using gynogenetic diploid progeny. We continue to add markers to the map to reduce the number of linkage groups to 26, the haploid number of chromosomes present in female pink salmon (25 autosomes and an X-chromosome). We have initiated work on a complementary even-year linkage map to incorporate additional markers on the map and for use in comparative studies. We have also made substantial progress on experiments that will allow us to apply mapped genetic markers to study marine survival and fitness in pink salmon. In August 1998, we collected gametes and tissue from 150 pink salmon from Likes Creek and performed single-pair matings to produce 75 families. In May 1999, approximately 48,000 individuals from 49 of these families were marked and released into Resurrection Bay from the Alaska SeaLife Center.

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

Project Data: Two primary sets of data are being developed; one for the linkage map and one for the marine survival and fitness experiment. Data for the linkage map are the inheritance of DNA fragments in the haploid and gynogenetic diploid progeny of two pink salmon females (A95-103 and V96-02). Sixteen additional diploid families have been tested for nonrandom segregation between all pair-wise combinations of 14 allozyme and three microsatellite loci. The haploid data set consists of 589 polymorphic DNA fragments in female A95-103 and 94 of her haploid progeny, and five polymorphic DNA fragments in female V96-02 and 95 of her haploid progeny. The diploid data set consists of genotypes of 70 gynogenetic diploid progeny from female A95-103 at 312 loci and of genotypes of 43 gynogenetic diploid progeny from female V96-02 at five loci. Data for the marine survival and fitness experiment are genotypes at ten PCR-based loci for 50 families (50 parent pairs and 10 progeny from each) produced for the experimental release in 1998. The parents for the experimental cohort produced in 1998 have been genotyped at 12 additional PCR-based loci and 34 allozyme loci and the 1999 parents have been genotyped at 30 allozyme loci. Four meristic characters, body length, egg size, and egg number have been recorded for both cohorts of adults used in experimental crosses. All data sets are currently recorded in Microsoft Excel spread sheets. Data will be made available to individuals within the reasonable bounds of sharing unpublished data. Contact Kate Lindner, Division of Biological Sciences, University of Montana, Missoula, MT 59812. Phone: (406) 243-5503. E-mail: klindner@selway.umt.edu.

Citation:

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2. 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 43:1-12. 2-1

EXECUTIVE SUMMARY

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

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

The genetic linkage map we are constructing will provide the platform to address the genetic impact of the oil spill. The initial framework of the map was constructed using 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. 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 (number A95-103) was chosen to be the primary reference family upon which initial mapping efforts were focused.

Linkage analysis of 589 markers segregating in the gynogenetic haploids produced a genetic map comprising 42 linkage groups covering a distance of 5352 centiMorgans (cM). Assuming a minimum distance of 30 cM for linkage detection and accounting for all the gaps and unlinked markers the minimum distance of the pink salmon genome is 6872 cM. The haploid pink salmon genome consists of approximately 2.72 billion base pairs or 2.72 x 10^6 kilobase pairs (kbp) thus, we estimate approximately 391 kbp/cM.

Thirteen allozyme loci have been added to the map using gynogenetic diploid and normal diploid data. Five allozyme loci are polymorphic in female A95-103 and thus could be tested for nonrandom segregation in 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 602 markers.

We have generated a large number of markers distributed throughout the genome using haploid embryos and multilocus techniques. Due to polyploid ancestry, salmonid

genomes are large, therefore many markers will likely be required to span the entire genome of pink salmon. We are attempting to "consolidate" the pink salmon map by reducing the number of linkage groups to 26, the number of chromosome pairs in pink salmon (2N = 52). We have collected gene-centromere distances of 312 loci using gynogenetic diploids. Ultimately, these data will allow us to identify the centromere of each chromosome and assign the linkage groups identified using the haploids to a specific chromosome.

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

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

To date, parents and embryos collected from each of the 1998 release families have been genotyped using ten markers identified in the course of this study. Based on these data, we have selected a subset of markers that can be used to accurately place returning fish to families. These data have also allowed us to perform preliminary estimates of mutation rates and to ascertain the presence of null alleles. We have detected mutations at two microsatellite loci, and null alleles at one gene of known function (*MHC*). These results are particularly important for the application of genetic markers to questions concerning population biology (e.g., stock structure). For example, loci with high mutation rates or null alleles could bias estimates of gene flow and effective population size.

We are on schedule with the mapping portion of this project. More anchor loci will be included on the map which will both aid in consolidation and provide more markers to detect regions of the genome associated with fitness traits. Even-year mapping is underway. We are also on schedule with the marine survival and fitness experiment. We have released one cohort of experimental progeny and have raised a second cohort. We have determined the genotypes of the parents used to generate both cohorts and from

embryos sampled from the first cohort. We have also collected morphological data on the parents. We are prepared to assign returning fish to families which will be critical both for determining which individuals to use in the next round of crosses as well as for estimating fitness.

INTRODUCTION

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

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

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

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

The degree to which linkage groups are conserved over evolutionary time can be evaluated by comparing linkage maps from different taxa. Alternatively, if linkage relationships are known to be conserved, the resolution of a linkage map can be improved by incorporating information from linkage maps from closely related taxa. In fishes, linkage relationships are reported to persist after 300 million years of evolution (Graf 1989), thus many loci should occupy similar chromosomal positions in closely related

taxa. However, it is important to note that many of the dominant multilocus genetic markers that are widely used in map construction, such as amplified fragment length polymorphisms (AFLPs), can not be used for comparisons between maps. Comparative mapping can be accomplished by finding links between the dominant markers and 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). Katagiri et al. (1996) sequenced the entire MHC class I cDNA in pink salmon and described the presence of two main allele types in the alpha 1 domain. Because it is known to be variable in pink salmon, this locus should be a particularly good candidate for mapping.

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

We are constructing the pink salmon linkage map using gynogenetic haploid and diploid progeny from an individual female (see Spreull 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 Appendix 2). Analysis of segregation patterns of markers linked to the same centromere enabled us to consolidate the map to 42 linkage groups. We are continuing to add markers to our map to reduce the number of linkage groups to match the number of chromosome pairs in pink salmon (26). In addition, we have initiated segregation analyses in other individuals to map markers that are not polymorphic in female A95-103 and, more specifically, to include particular genes of known function.

Odd- and even-year pink salmon are reproductively isolated due to the fixed two-year life cycle of this species (Aspinwall 1974). Beacham et al. (1988) report substantial allozyme 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 finding suggest that the alternate brood years are reproductively isolated and genetically distinct. Our map is based on an odd-year female (A95-103); therefore, as we evaluate additional potential mapping females we are focusing our efforts on even-year families. 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.

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 map we have generated is of much finer resolution, which allows us to test for interference along the chromosome arm. We are currently using our map to test the assumption of uniform interference.

Finally, a completed linkage map for pink salmon and the facilities at Alaska SeaLife Center (ASLC) will allow us to test questions that were previously impossible to address in salmonids. For example, it is has been notoriously difficult to detect and measure the effects of natural selection in natural populations (Lewontin 1991). The most powerful method to detect natural selection is to compare genotype distributions in a single cohort sampled at different life history stages (Lynch and Walsh, in preparation, p. 303). 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. Over 48,000 marked fish from 49 families of the 1998 cohort were released in May 1999; surviving individuals will be collected when they return to the facility at sexual maturity. The 1999 cohort is currently being reared at the ASLC for release in May, 2000.

In this report we update our progress on the construction of the pink salmon linkage map, including descriptions of the addition of new markers and initial results on mapping *MHC-α1* in an even-year family. We also describe progress on the marine survival and fitness experiment. Specifically, we have released nearly 48,000 marked fry into Resurrection Bay and have genotyped parents and progeny from the 1998 experimental cohort at ten loci. We summarize the genotyping data and describe our findings with respect to mutation rates and detection of null alleles. In addition, we explain how we use these data to select markers which will allow us to place returning fish back to family groups. Finally, we describe the production and initial genetic analysis of additional families that we will release in May 2000.

OBJECTIVES

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

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

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

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

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

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

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

METHODS

Production of Progeny for Mapping

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

Gynogenetic Haploids

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

Gynogenetic Diploids

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

Selection of Reference Families

Family A95-103 was chosen as the reference family to generate a pink salmon linkage map based on the number of embryos produced and on initial screening of microsatellites (see Allendorf et al. 1997). DNA was extracted from 150 putative haploid embryos in this family. Confirmation of gynogenetic origin was completed on the haploids (Spreull et al. 1999; Appendix 1). Ninety-four of the remaining 131 haploid embryos contained sufficient DNA for extensive analysis. In addition, DNA was extracted from 77 putative gynogenetic diploids from family A95-103. Individuals were confirmed to be gynogenetic diploid progeny of female A95-103 as described in Spruell et al. (1999;

Appendix 1). The seven individuals that were not gynogenetic diploids may have resulted from incomplete inactivation of sperm (Thorgaard et al. 1983) or may have been individuals from another treatment. These seven individuals were removed from subsequent analyses.

Family V96-02 was chosen as a candidate to generate a complementary even-year linkage map because an acceptable number of embryos were produced in this family and because particular loci were polymorphic (see below). DNA was extracted from 95 putative haploid embryos and tissue from 43 gynogenetic diploids. Gynogenetic origin was completed as described in Spruell et al. (1999; Appendix 1). We did not detect evidence of non-maternal alleles (i.e. due to incomplete sperm inactivation) in any of the 95 putative haploid embryos or the 43 putative gynogenetic diploids.

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. We identified 602 fragments that met these criteria and included them in the analysis of joint segregation ratios for family A95-103. Specifically, we amplified 35 RAPD loci, 393 AFLP loci, and 168 PINE loci as described in Spruell et al. (1999, Appendix 1, Table 1). However, due to consistent problems determining linkage relationships of RAPD markers, all RAPD loci were eliminated from the linkage analysis. In addition, thirty-four microsatellite loci were amplified with minor modifications to published specifications (Table 2).

Three genes of known function (*GH-2,* Forbes et al. 1994; *MHC-α1*, Katagiri et al. 1996; and *p53,* Bhaskaran 1999) could not be included in our mapping efforts because we did not detect variation in female A95-103. However, the standard electrophoresis methods we used only identify length differences among fragments. To detect possible sequence polymorphisms in these three loci, we tested an alternative screening method which takes advantage of the differences in electrophoretic mobility of folded conformations of single-stranded DNA fragments comprising different nucleotide sequences. This 'single-strand conformation polymorphism' (SSCP) approach was developed to detect point mutations (i.e. single base substitutions) for human biomedical applications (Orita et al. 1989). We used SSCP analysis to screen *GH-2, MHC-α1*, and *p53* in multiple odd- and even-year individuals, including our odd-year mapping female (A95-103). PCR products were electrophoresed according to the protocol provided with the Mutant Detection Enhancement (MDE™) gel solution (FMC Corporation) with the following exceptions; the gel stock included 10% glycerol and gels were run for 12-14 hours at six watts constant power in a 4° C cold room.

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 V96-02 (Thorgaard et al. 1983; Allendorf et al. 1986). The details of these calculations are provided in Appendix 2.

Estimates of Interference

We are continuing work with Dr. Hongyu Zhao from Yale Universty School of Medicine who has developed a statistical analysis for half tetrad data that estimates the amount of chiasma interference present in each linkage group (Zhao and Speed 1998). Interference can be estimated by measuring the number of crossovers that occur between chromatids. The linkage map provides information about the linkage phase and distance between markers. We can use this information to estimate a coefficient of interference through the analysis of multiple loci in one linkage group. We are comparing our findings in the haploid data with the results from the gynogenetic diploid data.

Marine Survival and Fitness Experiment: 1998 Cohort

Release Families

In March 1999 approximately 73,000 fry from 49 families of the 1998 cohort were pooled. In May 1999 we marked fry by clipping the adipose fin and released 48,329 marked individuals into Resurrection Bay. In addition, we randomly sampled 1,000 progeny at the time of release to test for relationships between multiple locus heterozygosity, length, and condition factor in fry both within and between families. This sample of fry will also be used to compare genotypes and relationships between genotypes and phenotypes of released and returning fish. We will also analyze these fry using a sex-linked PCR-marker originally described by Forbes et al. (1994) to test if the length differences we previously detected in adults (Lindner et al. 1999) are present at this life-history stage.

Genetic Analysis

We analyzed each of the 150 parents from the 1998 cohort at 22 PCR-based loci, including two duplicated loci (*SSA20.19-1,2* and *uSAT60)*. Based on this screening, we selected ten loci that could be readily and unambiguously scored for parentage analysis. 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 frequences. We calculated the expected heterozygosity (H_e) from the genotype data. We examined males and females separately to test for differences between the sexes. In addition, we analyzed ten embryos from each of the 49 release families as well as one family (98-04) that was not released at the ten loci to check for Mendelian inheritance.

Mutation Analysis

Analysis of mutations was performed using 1998 cohort genotype data by comparing alleles present in progeny (i.e. embryos) to those in their parents. An allele amplified in progeny but not present in either parent is considered a putative length mutation (see Figure 1). To allow a more detailed assessment of the putative mutation events, additional 1998 release families were selected for further analysis. To date, additional progeny from three families have been analyzed at locus *SSA408* .

Parentage Analysis Software

We evaluated our ability to genetically assign progeny to the correct family by analyzing genotypes from the 1998 cohort and their parents using the computer program ProbMax2 (Danzmann et al. 1997). We initially tested the entire data set. In subsequent runs, we eliminated loci with null alleles (i.e. *MHC-α1*) and individuals for which we were missing data. We also tested many different groups of loci to assess whether a subset of the ten markers analyzed could be used to rapidly and accurately assign returning adults to families.

Marine Survival and Fitness Experiment: 1999 Cohort

Production of Families

In August of 1999, we collected gametes from 34 female and 34 male pink salmon from the mouth of Likes Creek, Resurrection Bay, Alaska. We assigned each individual a unique number and measured its length from the middle of the eye to the fork of the caudal fin. We dissected otoliths which will be analyzed by Alaska Department of Fish and Game personnel for presence of temperature rings used to mark hatchery stocks. We also collected liver, eye, heart, muscle, and fin tissue from each individual as well as sperm from each male for genetic analysis.

We used gametes collected from Likes Creek to produce 68 half-sibling families for rearing at the ASLC. Eggs collected from each female were divided into two equal groups, and each group was fertilized with sperm from one male. Each male was used to

fertilize the eggs of two females, producing the block-design depicted in Figure 2. We raised embryos following the same procedures we used for the 1998 cohort (see Lindner et al. 2000). We visually checked eggs for developing embryos in November 1999. We weighed a sub-sample of 100 eggs from each female. We used the weight of these 100 eggs to calculate an average weight per egg for each female and then weighed all eggs in each family to estimate total egg number. We preserved 20 embryos from each family in 100% ethanol for DNA analysis.

Progeny from all 68 families were pooled together into a single tank in March 2000, shortly after hatching. In May 2000, approximately 400 fry from each of these 68 half-sibling families will be marked and released from the ASLC facility into Resurrection Bay.

Genetic Analysis

Allozyme loci were analyzed using enzyme electrophoresis for each of the 68 parents from tissues stored at-40 °C following Aebersold et al. (1987) and Seeb et al. (1996).

Morphology and Fluctuating Asymmetry

We measured body size (mid-eye to fork length) of all 68 adult pink salmon collected in August 1999. In addition, we examined four bilaterally paired meristic traits (pectoral fins, pelvic fins, and upper and lower gill arches) for fluctuating asymmetry (FA). Traits were considered asymmetric if the right and left sides were not equivalent. We quantified FA as the number of traits that were asymmetric in each individual.

RESULTS

Genetic Mapping in Reference Family A95-103

Linkage Map

We have described the segregation of 602 markers in haploid progeny from female A95-103; we have also mapped 13 allozyme loci using gynogenetic-diploid progeny and classic linkage analysis in normal families. We have assigned 563 of the 602 markers to one of 42 linkage groups covering a distance of 5352 centiMorgans (cM) (Figure 3; Tables 1 and 3). Only 26 markers remain unlinked. The estimated size of the pink salmon linkage map based on these data is 6872 cM. This includes 5352 cM mapped in Figure 3, an estimated 260 cM to account for the distance from the end markers to their adjacent telomeres, and an estimated 1260 cM in unfilled gaps in the map. The haploid pink salmon genome consists of approximately 2.72 x 10^6 kilobase pairs (kbp) thus, we estimated that each cM corresponds to a physical distance of approximately 391 kbp. Anchor loci have been assigned to 18 linkage groups including 11 linkage groups with more than one anchor locus.

Gene-centromere Analysis

Results of gene-centromere distance analyses are detailed in Lindner et al. (2000; Appendix 2).

Evaluation of Families for Even-year Mapping

SSCP Analysis

SSCP was used to analyze *GH-2, MHC-αl*, and *p53* PCR products from five odd- and five even-year females. No differences were detected among the PCR products from the ten females at *GH-2* or *p53*. However, two fragments were amplified in nine individuals and four fragments were amplified in one individual (V96-02) at *MHC-αl* indicating the potential for a sequence polymorphism. Segregation analysis of 95 haploid progeny confirmed that a sequence polymorphism is present at *MHC-αl* in family V96-02. This analysis also allowed the association between pairs of fragments and alleles, arbitrarily designated *270a* and *270b*, to be determined. The four fragments present in female V96-02 represent each of the four single strands of DNA that have folded in different conformations based on their nucleotide sequence (see Figure 4). The different conformation results in a different migration rate through the MDE gel. The two fragments present in each of the haploid progeny represent the forward and reverse strands of DNA for each allele (*270a and 270b*).

Gene-centromere Distances

The genotypes of the 43 gynogenetic diploid progeny of V96-02 at ten loci are given in Table 4; all other families we have analyzed at these loci 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 used requires detection of six homozygotes for the frequencies of the two homozygous classes to be considered significantly different ($P<0.05$). Only one out of a total of 21 tests had a probability less than 0.05, which is not significant when corrected for the number of independent tests (Rice 1989).

Marine Survival and Fitness Experiment: 1998 Cohort

Genotype Analysis

The parents of the 1998 cohort have been analyzed using a wide array of genetic markers. In addition to these data, we have now genotyped at least ten progeny (embryos collected in 1998) from each of 50 families (including the 49 families released) at ten DNA loci, (8 microsatellites, *GH-2* and *MHC-αl*). Table 5 lists the data compiled for the 1998 cohort and summarizes the variability detected in these markers. This table suggests that loci closer to the centromere may be more variable.

Null alleles at MHC-α1

In the 150 adults analyzed in Lindner et al. (2000, Table 6), we found that genotype frequencies at *MHC-αl* of both males and females differed significantly from expected Hardy-Weinberg proportions, and that males included a greater frequency of heterozygotes than females. We analyzed the segregation of *MHC-αl* alleles in 500 progeny and determined that a null allele (i.e. an allele that is not amplified by our primers) was present at this locus.

Detection of Mutations

We have observed 28 progeny that have genotypes that are best explained as being the results of mutation events at one of two microsatellite loci (Table 6). These 28 progeny all have genotypes at eight of nine loci that are compatible with their parents; in addition, one allele at the locus containing the putative mutation in each of these progeny is compatible with alleles from either their mother or father. Thus, all of these individuals appear to possess *de novo* mutations at these loci. One of the families included in Table 6 that was used for screening additional individuals (98-26) was chosen because it had a high mutation rate in our initial screen. Therefore, our estimates of mutation rates are based upon our initial screening of 10 individuals per family. We initially detected 14 mutations at *SSA408* in a total of 467 individuals (Table 6). Each individual inherits two gene copies of each gene. Therefore, our estimate of mutation rate at *SSA408* is 14/(467 x 2)=0.015. Similarly, we detected 8 mutations in 488 individuals at *OGO1c* for an estimated mutation rate of 0.008.

Assignment of Progeny to Family

We used the computer program ProbMax2 (Danzmann et al. 1997) to evaluate our ability to genetically assign progeny to the correct family. We analyzed the genotypes of the 50 sets of parents and their 500 progeny from the 1998 cohort at the loci described. We excluded *MHC-αl* from this analysis because the presence of null alleles (see above) confounds parentage analysis. Using the remaining nine loci, 484 individuals were assigned to their correct parents, three individuals to two possible sets of parents, and 13 individuals could not be assigned. The four individuals assigned to multiple parents had not been successfully genotyped at several loci. New DNA extracts will be prepared and analyzed for these individuals. All 12 individuals for whom no possible parents were identified could be accounted for by mutations in *OGO1C* or *SSA408* (see below).

Selection of Loci for Parentage Analysis of Returning Progeny

ProbMax2 was also used to determine whether a subset of loci could be analyzed to efficiently assign progeny to the proper family. This will be necessary for us to be able to quickly determine family relationships of returning individuals so that we can select appropriate parents for the next round of crosses. After testing a series of marker combinations on the 496 individuals for which we had genotype data at most loci, we found that 472 (95%) could be correctly assigned to parents with data from a subset of

four loci (*OGO1C*, *SSA20.19-1*, *SSA20.19-2*, and *SSA408*). By repeating the parentage analysis using subsets of these four loci; first excluding *OGO1C*, then including *OGO1C* but excluding *SSA408*, we found that we could correctly determine parentage for all unassigned individuals.

Marine Survival and Fitness Experiment: 1999 Cohort

Genetic Analysis

Table 7 presents the number of alleles and observed and expected heterozygosities for the 19 allozyme loci we have analyzed to date. We have detected variation at 13 of the 19 loci.

Morphology and Fluctuating Asymmetry

We measured length and estimated meristic variation for the 68 adult pink salmon collected from Likes Creek in August 1999. We found that males are more variable in length than females ($P<0.0025$, Figure 5) and that males also tend to be smaller ($P<0.02$). The population average FA for the four meristic traits is 1.38. The average number of asymmetric characters for males (1.42) versus females (1.33) was not significantly different (Figure 6).

DISCUSSION

Evaluation of Even-year Families for Mapping

We initially selected female V96-02 as the focus of our even-year mapping efforts because a large number of her gynogenetic progeny had been produced and because our SSCP assays detected polymorphism at the locus *MHC-α1* in this individual. Preliminary analysis of gynogenetic diploid progeny at four microsatellite loci (*OGO1c*, *RGT6*, *SSA311*, and *SSA408*) revealed a greater proportion of heterozygotes (y) in V96-02 than in A95-103 at all of these loci (see Table 4). The differences were statistically significant for all loci except *RGT6* ($p<0.05$). Based on these results we compared the proportion of heterozygotes in V96-02 to all other females for which we had generated data at ten loci (Table 4). Two microsatellite loci (*OGO3* and *OTS102*) had y values greater than 0.95 in all families tested, limiting our power to test for differences; therefore, we excluded these loci from comparative analyses. We found that family V96-02 had the greatest proportion of heterozygotes at each of the remaining eight loci (Table 4).

To assess whether the differences in heterozygosities of gynogenetic diploid progeny detected in V96-02 could potentially be attributed to year-class, we compared the proportion of heterozygotes at four of the eight loci described above (*OGO1c*, *OGO2*, *OGO4*, and *OTS101*) for which we had data for more than two families. Significant among-family differences were found at *OGO1c* and at *OGO2* ($p<0.05$); when family

V96-02 was excluded from the analysis the differences were no longer statistically significant. Comparisons of V96-02 to the other families combined at these two loci indicated that the differences detected among families could be attributed to V96-02 ($p<0.05$). It thus appears that recombination in family V96-02, not in even-year families in general, is unusually high. This is an interesting result that warrants further investigation; however, given that V96-02 appears to be unusual, we need to identify a different even-year reference family for comparisons of odd- and even-year maps.

Genetic Analysis of 1998 Cohort

Null alleles at MHC-α1

Based on comparisons between the genotypes of 1998 cohort embryos and those of their parents, we determined that there is at least one null allele at *MHC-α1*. However, our data do not allow us to determine whether there is a single or multiple null alleles. Furthermore, our data suggest that whether or not certain alleles are amplified may depend on the type of sample (i.e. fin clips vs. embryos). Based on the assumption that some alleles are not amplifying due to mutations in the priming site, we plan to try two strategies for optimizing conditions for this locus. First, we will try reamplifying samples using a lower annealing temperature. If we continue to see evidence of missing alleles, we will then try redesigning our primers using degenerate bases at the 3' end (e.g., Palumbi 1996).

Microsatellite Mutations

Our results have provided exciting and important information about the mutation process in microsatellites. We have observed two different types of germline mutations: singletons and clustered mutations (Woodruff et al. 1996). Singletons are mutations that occur in late meiosis and are transmitted to a single gamete (Figure 7). Clustered mutations occur during premeiotic replication of the germline and thus are expected to be transmitted to approximately one-half of all progeny. Although it is commonly believed that new mutants result from unique, independent mutation events, recent studies in Drosophila suggest that clustered mutations may account for 20-50% of all new mutant alleles (Woodruff et al. 1996). Jones et al. (1999) have reported cluster mutations at microsatellite loci in the pipefish (*Syngnathus typhle*); to our knowledge this is the only published account of cluster mutations in fish.

We have observed a total of 18 singleton mutations at *SSA408* and *OGO1c* in nine different families (Tables 6 and 9). Both of these loci are tetranucleotide repeats, and all of the putative mutant alleles differ by just one repeat-unit (4 base pairs) from an allele in the parent transmitting the mutant allele. In addition, 17 of the 18 putative mutant alleles detected appear to be one repeat-unit longer than their likely progenitors, whereas one mutant allele was one repeat-unit shorter (Table 8). Similarly, Banks et al. (1999) found a single mutation in their study of the inheritance of microsatellites in chinook salmon that was a gain of a single repeat-unit. The single-step change with a bias towards

increasing the number of repeats is consistent with the theory that microsatellite mutations result from DNA polymerase strand slippage (Levinson and Gutman 1987, Weber and Wong 1993). The mutations we detected appear to be randomly distributed among families with a single exception. Nine of the singleton mutations are in family 98-26 at *SSA408*. Eight of these nine mutations appear to be a one repeat-unit gain at the same allele (*316*). Thus, this appears to be a so-called "hypermutable" allele such as has been reported in other species (Schlötterer et al. 1998).

Clustered mutations were observed in family 98-65 at *SSA408* and in 98-40 at *OGO1c* (Table 8). In both cases, it appears that an allele in the father mutated before meiosis so that a mutant allele was transmitted to approximately one-half of all progeny. Both of these mutations increased allele size by multiple repeat-units. The mutant allele at *SSA408* in 98-65 appears to be a gain of 3 repeat-units, and the mutant allele at *OGO1c* in 98-40 appears to be a gain of 15 repeat-units. Thus all of the singleton mutations were single repeat-unit changes while both clustered mutations were larger.

Garza et al. (1995) have suggested that mutations producing changes greater than a single repeat-unit may be due to a different mutational mechanism than DNA polymerase slippage, such as unequal crossover. Our linkage map would allow us to evaluate such differences by looking for recombination events coupled with the production of mutations in specific regions. Furthermore, Table 5 suggests there may be a correlation between chromosomal position (i.e. distance to the centromere) and variability (alleles and heterozygosity) which may reflect a positional bias in mutation rate. Examining additional mapped loci will allow us to explore this possibility in more detail.

These results have important significance for the use of microsatellite loci in analyzing genetic population structure. Most of the models applied in population genetics virtually ignore mutation, assuming that mutation events are rare and thus unimportant in comparison to other factors, such as migration and genetic drift. In theory, if the probability of mutation approaches that of migration, mutation will have a substantial effect on the amount and pattern of genetic divergence among populations (see discussion in Allendorf and Seeb 2000). Furthermore, it is generally assumed that mutant alleles arise independently and not as clusters within lineages, such as we have detected. Woodruff et al. (1996) have argued that clustered mutations "may cause us to reconsider many of the fundamental relationships on which population genetic theory is based". The fact that cluster mutations account for 45% (10/22) of the new mutations that we detected in our initial screen of 10 individuals from each family at both loci (Table 6) indicates that the common practice of ignoring mutation processes may produce misleading interpretations when microsatellite markers are used to evaluate genetic population structure.

We have begun writing a paper for submission to Molecular Ecology that describes the rate and pattern of mutations that we have observed to date. We will obtain more data on mutations when we genotype and place returning fish into their families of origin.

Assignment of Progeny to Family

Our analysis of ten DNA loci in the 1998 brood year families used in the experimental release for the marine survival and fitness experiment indicates that we have selected loci that encompass a broad range of variability (see Table 5). For the purpose of parentage assignment, highly variable loci are particularly useful in that they provide greater discriminatory power than less variable loci. However, our data suggest that they are also more mutable, which can seriously confound parentage analysis. In addition, we have found that the highly variable loci tend to be more difficult to type accurately. Thus, we expected that using a combination of high and low variability markers would be optimal for parentage assignment. We found that by using the combination of *OGO1c*, *SSA20.19-1*, *SSA20.19-2*, and *SSA408*, we could accurately assign parentage to all of the progeny for which we had genotypes, if we took mutations into account.

This is an important finding because it suggests that we will be able to rapidly genotype and assign to families individuals returning to the ALSC in August. We hope to mate full-sibs from the 1998 cohort to test for effects of inbreeding on marine survival and other life history traits (e.g., survival and body size during early life history stages). The genotyping will be critical for the selection of appropriate parents to use for our experimental crosses which must be performed within a few days of the adults returning to spawn. Hitachi Genetic Systems has agreed to set up a fluorescent scanner at the ASLC for our use during the period of the 1998 cohort return. Parentage will be determined on the basis of these genotypes and experimental crosses will be made within two or three days of sampling the adults.

Morphological Differences Between Even- and Odd-year Pink Salmon

In our 1998 samples of adults from Likes Creek, we found that even-year females were larger than even-year males, whereas adult females sampled in 1999 were significantly smaller than males (Table 9). In both years, females were less variable in length than males. In a multi-year study of pink salmon populations in British Columbia, Beacham et al. (1988) similarly found that even-year adult females were larger than males produced the same year, and that the females were less variable in length in both year classes. However, in contrast to our results, they found no significant difference in length between sexes in odd-year populations. These data support the idea that different selective forces may act on females and males. Importantly, our experimental design will allow us to assess at which life history stage the apparent morphological differences arise by measuring the size of fry before release in our experimental families. In addition, we are raising families at the ASLC which have not been released. We can use these fish to test whether the same morphological differences arise in these fish which have been maintained in captivity.

CONCLUSIONS

We have constructed an odd-year linkage map that can be used to test for effects of regions of the genome on traits that are important to the recovery of pink salmon (e.g., growth and survival) and to evaluate stock structure. Identification of a family for even-year mapping is now underway. We now have completed two successful years of gamete collection and rearing of fry at the ASLC. We released approximately 48,000 marked fish in 1999 and raised another cohort for release in May 2000. We have identified genetic markers that can be used to accurately and efficiently assign returning fish to families. Our initial analysis of genotyping data from embryos collected from the 1998 experimental families has uncovered interesting findings with respect to microsatellite mutations, which we are continuing to analyze with hopes of producing a manuscript by the end of the year.

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Table 1. Summary of primer pairs screened and polymorphic loci detected by four different techniques. The second column is the number of polymorphic loci detected by each technique. The percentage of those markers assigned to a linkage group is given in the third column. The fourth column is the percent of the loci that are inherited in a co-dominant manner.

	Number of Polymorphic Loci	Percent Assigned to a Linkage Group	Percent of Markers Codominant
AFLPs	393	95	1
PINEs	162	97	5
Microsatellites	34	100	91
Allozymes	13	100	100
Total	602	96	

Table 2. Microsatellite locus names, annealing temperatures, and references.

Locus	Annealing Temperature (°C)	Reference
FGT1-1,2	51	Sakamoto et al. 1994
FGT19	58-52 †	Sakamoto et al. in press
OCL2	56	Condrey et al. 1998
OGO1C	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	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 & Ferguson personal communication
OMY301	58-52 †	Sakamoto et al. in press
OMYFGT25	58-52 †	Sakamoto et al. in press
OMYFGT34	58-52 †	Sakamoto et al. in press
OMYOGT 4	58-52 †	Sakamoto et al. in press
OMY0008	58-52 †	Holm & Brusgaard 1998
OMYRGT1	58-52 †	Sakamoto et al. in press
OMYRGT2	58-52 †	Sakamoto et al. in press
OMYRGT6	58-52 †	Sakamoto et al. in press
OMYRGT13	58-52 †	Sakamoto et al. in press
OMYRGT43	58-52 †	Sakamoto et al. in press
OMYRGT44	58-52 †	Danzmann & Sakamoto personal communication
ONEμ2	58-52 †	Scribner et al. 1996
ONEμ14	58-52 †	Scribner et al. 1996
ONEμ18	58-52 †	Scribner et al. 1996
OTS1	55	Banks et al. 1999
OTS100	56	Nelson & Beacham 1998
OTS101	56	Small et al. 1998
OTS102	52	Nelson & Beacham, personal communication
μSAT60-1,2	55	Estoup et al. 1993
SSA20.19-1,2	58-52 †	Sanchez et al. 1996
SSA85	57	O'Reilly et al. 1996
SSA197	57	O'Reilly et al. 1996
SSA293	58-52 †	McConnell et al. 1995
SSA311	58-52 †	Slettan et al. 1995
SSA408	58-52 †	Cairney & Taggart personal communication

† Countdown PCR profile.

Table 3. Summary of linkage groups in the pink salmon genome map based on inheritance in 94 haploid progeny of a single female (95-103).

Number of markers in linkage group	Number of linkage groups	Average Size (cM)
2-5	12	27.2
6-10	10	50.32
11-15	8	122.5
16-20	4	204.7
21-25	4	213.2
31-35	1	354.1
41-45	2	532.85
50+	1	470.4

Table 4. Gynogenetic diploid genotypes at ten loci. Data for additional families at *OGO1c, OGO2, OGO3, OGO4, OTS101,* and *OTS102* are from Lindner et al. 2000. Chi-square tests for equal numbers of homozygotes (1d.f.). Chi-square value in the total row is the contingency chi-square for difference in *y* between families; *(P<0.05).

Locus	Family	Maternal Genotype *a/b*	Progeny Genotypes *aa*	*ab*	*bb*	Proportion Heterozygotes *(y)*	Chi-square (df)
MHC-α1	**V96-02**	***270a/270b***	**3**	**21**	**12**	**0.58**	**5.40**
	A95-114	*264/270*	19	17	21	0.30	0.10
	Total		22	38	33	0.41	7.42 (1)*
OGO1c	**V96-02**	***284/354***	**6**	**17**	**8**	**0.55**	**0.29**
	V96-20	*332/344*	14	4	16	0.12	0.13
	V96-13	*444/460*	19	6	17	0.14	0.11
	A95-20	*292/300*	29	14	28	0.20	0.02
	A95-103	*280/312*	24	16	27	0.24	0.18
	Total		39	27	41	0.25	22.24 (4)*
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)

Locus	Family	Maternal Genotype *a/b*	Progeny Genotypes *aa*	*ab*	*bb*	Proportion Heterozygotes (*y*)	Chi-square (df)
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)
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)
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)
RGT6	**V96-02**	***204/216***	**9**	**9**	**13**	**0.29**	**0.73**
	A95-103	*188/192*	27	9	27	0.14	0.00
	Total		36	18	40	0.19	2.92 (1)
SSA311	**V96-02**	***168/195***	**8**	**24**	**9**	**0.59**	**0.06**
	A95-103	*170/238*	28	5	35	0.07	0.78
	Total		36	29	44	0.27	34.32 (1)*
SSA408	**V96-02**	***375/402***	**5**	**18**	**7**	**0.60**	**0.33**
	A95-103	*336/350*	23	11	29	0.17	0.69
	Total		28	29	36	0.31	17.14 (1)*

Table 5. Loci amplified in 50 sets of parents analyzed for the marine survival and fitness experiment. The first two loci are genes of known function. The remaining 11 loci are microsatellites. Number of alleles and expected heterozygosizy (H_e) are reported. The first eight microsatellite loci were amplified in the parental pairs and at least 10 of their progeny. See text for mutation rate (μ) calculation. The last three loci were amplified only in the parents. Gene-centromere distances (y) were calculated using data from gynogenetic diploids (Lindner et al. 2000). Missing data are represented by ---.

Locus	# of Alleles	H_e	μ	y
GH-2	2	0.270	0	---
MHC-α1	2 (+null)	0.497	0	0.43
OGO1c	77	0.983	0.008	0.19
OGO8	17	0.334	0	1.00
OMY301	21	0.856	0	0.79
ONEμ3	3	0.507	0	0.98
OTS1	15	0.829	0	0.27
SSA20.19-1	2	0.058	0	1.00
SSA20.19-2	3	0.307	0	---
SSA408	49	0.972	0.015	0.17
OCL2	6	0.380	---	0.68
SSA14	5	0.096	---	---
μSAT60-2	5	0.257	---	0.58

Table 6. Mutations observed at two microsatellite loci. Numbers in parentheses represent individuals analyzed and mutations detected in addition to the original screening.

	SSA408		*OGO1c*	
Family	#Individuals	#Mutations	#Individuals	#Mutations
98-1	8	0	10	0
98-4	8	0	10	0
98-5	9	0	10	0
98-6	9	0	10	0
98-7	8	0	10	0
98-9	9	0	10	0
98-10	10	0	10	0
98-11	10	0	10	0
98-12	10	0	10	0
98-13	9	0	10	0
98-14	10	0	10	0
98-15	10 (33)	0 (1)	10	0
98-19	10 (35)	1 (0)	10	0
98-20	10	0	10	0
98-21	10	0	10	0
98-22	9	1	10	0
98-23	10	2	10	0
98-25	10	0	10	0
98-26	10 (39)	4 (5)	10	0
98-27	9	0	10	0
98-28	10	0	9	0
98-32	10	0	9	0
98-34	9	0	10	1
98-35	9	0	10	0
98-38	9	0	10	0
98-39	10	0	9	0
98-40	10	0	10	4
98-41	10	0	10	0
98-43	9	0	10	0
98-44	9	0	9	1
98-45	10	0	10	0
98-46	8	0	9	0
98-49	9	0	9	0
98-50	8	0	10	0
98-51	10	0	10	1
98-53	9	0	10	0
98-58	9	0	10	0
98-60	10	0	10	0
98-61	10	0	10	0
98-63	9	0	10	0
98-64	8	0	10	1
98-65	10	6	10	0
98-67	9	0	10	0
98-68	10	0	9	0
98-69	9	0	10	0
98-71	9	0	10	0
98-72	10	0	10	0
98-73	9	0	10	0
98-74	10	0	6	0
98-75	8	0	9	0
Total	467 (107)	14 (6)	488	8

Table 7. Allozyme loci, number of alleles present at each locus, the expected heterozygosity (H_e), and the observed heterozygosity (H_o) for 68 adult pink salmon collected in August 1999 from Likes Creek, Resurrection Bay, Alaska.

Locus	Number of Alleles	H_e	H_o
6PDGH	2	0.09	0.1
AAT1	3	0.5	0.67
AAT2	2	0.03	0.03
ACO	1	----	----
AK	1	----	----
CK	2	0.04	0.04
G3PDH	3	0.18	0.19
HEX	3	0.41	0.53
IDDH	1	----	----
IDH	2	0.23	0.02
LDH1	1	----	----
LDH2	3	0.17	0.18
MDH1	1	----	----
MDH2	3	0.13	0.13
MEP1	3	0.11	0.09
MEP2	2	0.03	0.03
PEPB	2	0.03	0.03
PGM	2	0.11	0.12
sSOD	1	----	----

Table 8. Pattern of mutations observed in 1998 cohort families. Parent genotypes are reported with the underlined allele as the most likely progenitor of the mutant allele. Progeny genotypes are summarized with the putative mutant allele in bold followed by the number observed (n).

Locus	Family	Dam	Sire	Progeny Genotypes					Total
SSA408		*a/b*	*c/d*	*a/c*	*a/d*	*b/c*	*b/d*	genotypes not matching parents (#)	
	98-15	334/382	322/370	12	9	9	12	334/**374** (1)	43
	98-19	338/350	378/404	15	12	8	9	**342**/404 (1)	45
	98-22	334/404	350/366	4	1	1	2	334/**354** (1)	9
	98-23	326/382	366/386	3	2	1	2	326/**370** (1) 382/**370** (1)	10
	98-26	316/404	312/450	9	12	12	7	**320**/450 (3) **320**/312 (5) 316/**454** (1)	49
	98-65	330/354	330/464	2	0	2	0	330/**476**(1) 354/**476**(5)	10
OGO1c									
	98-34	350/450	309/346	3	1	3	2	**354**/346 (1)	10
	98-40	277/360	360/400	3	0	3	0	277/**460**(1) 360/**460**(3)	10
	98-44	342/350	408/474	1	1	3	3	342/**478** (1)	9
	98-51	295/366	303/362	1	2	4	2	295/**366** (1)	10
		a/a	c/d						
	98-64	348/348	309/448	5	4	0	0	348/**444** (1)	10

Table 9. Average length of adult pink salmon collected from Likes Creek in 1998 and 1999.

	N	Length	Standard Deviation
1998			
Male	75	483.72	34.60
Female	75	492.79	22.52
1999			
Male	34	474.44	38.71
Female	34	456.03	23.31

Figure 1. PCR products from *SSA408* in family 98-23 electrophoresed in a 7% polyacrylamide gel. The first two lanes on the left show products from the mother and father. The next ten lanes show products amplified from their progeny. Numbers at the right represent the size of the parental alleles in base pairs (bp). Arrows indicate individuals (7 and 9) with a mutant allele (370 bp). Size ladder is shown at the far right.

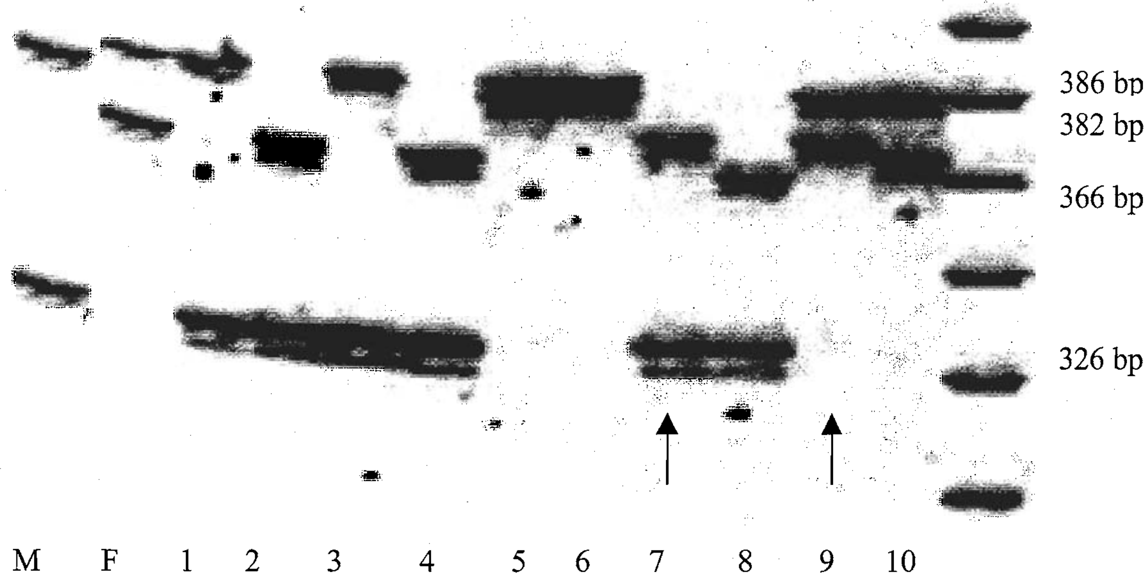
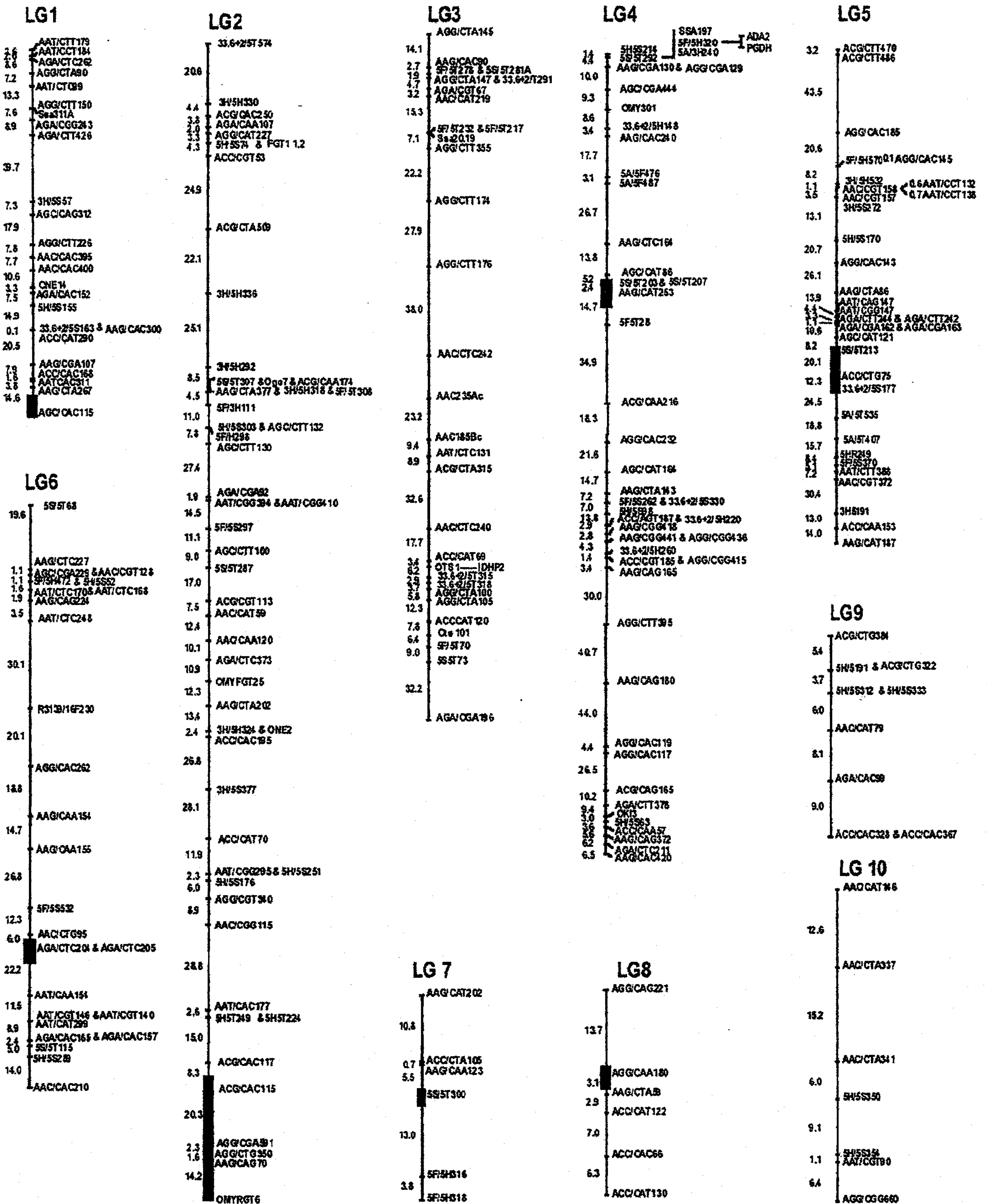


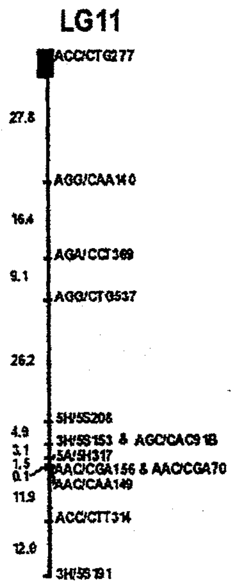
Figure 2. Diagram of our half-sib family experimental design. Numbers across the top represent females, numbers down the side represent males. The squares represent individuals used to make each family.

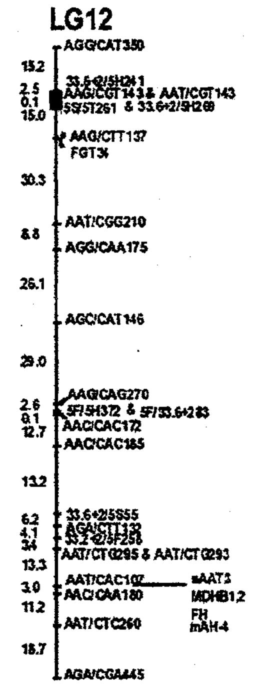
FEMALES

MALES	01	02	03	04	05	06	...	33	34
101	01x101	02x101							
102	01x102	02x102							
103			03x103	04x103					
104			03x104	04x104					
105					05x105	06x105			
106					05x106	06x106			
.							.		
.							.		
.							.		
133								33x133	34x133
134								33x134	34x134

Figure 3. 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.




LG11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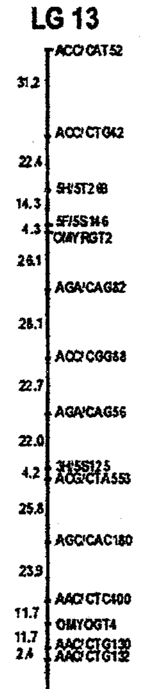
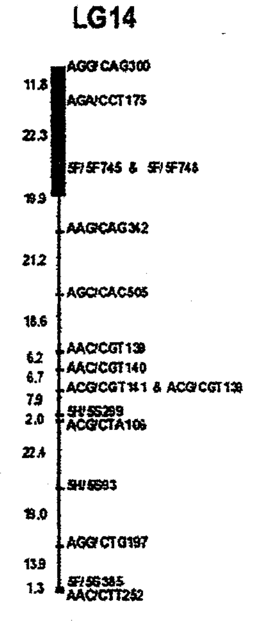

LG 13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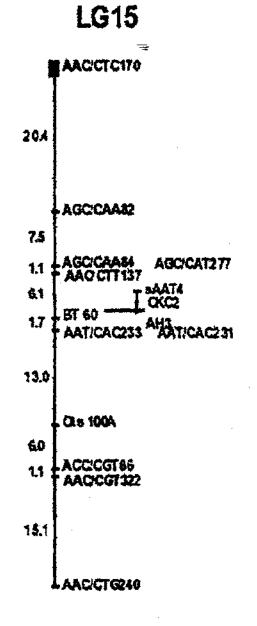
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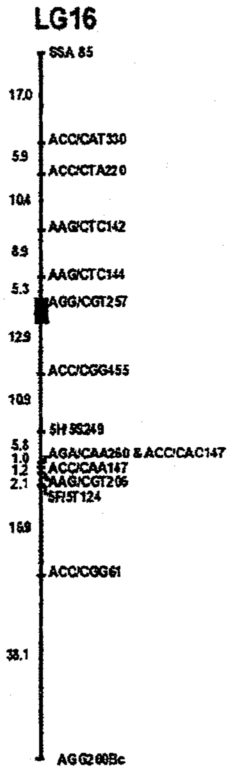
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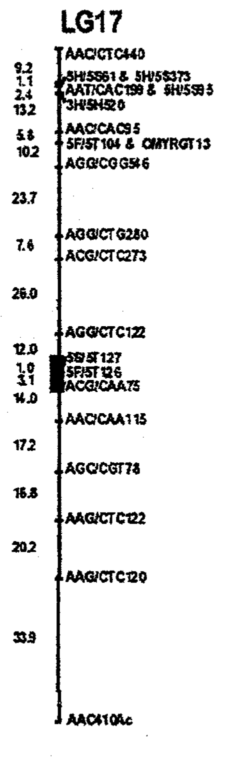
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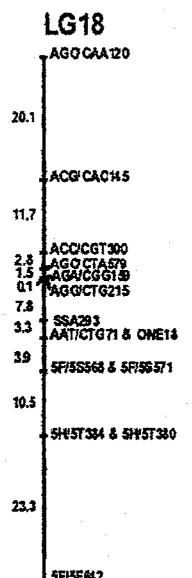
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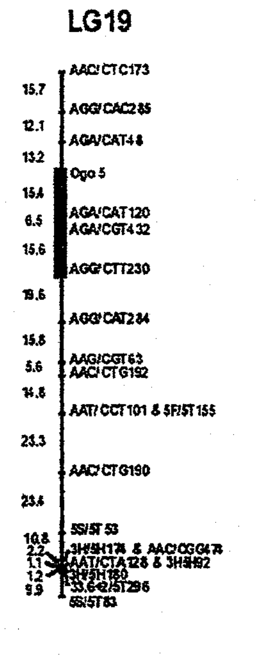
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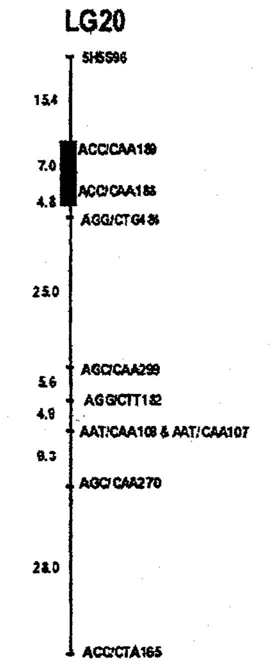
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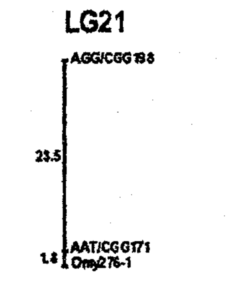
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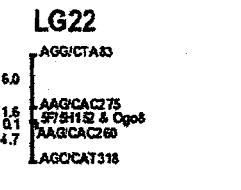
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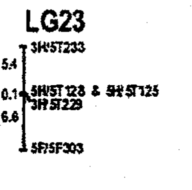
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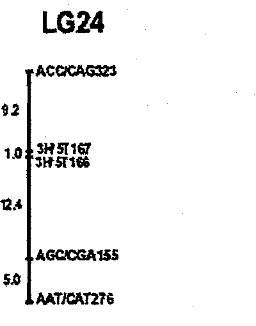
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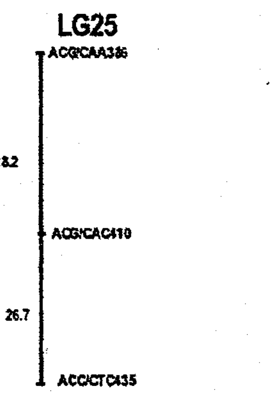

LG25

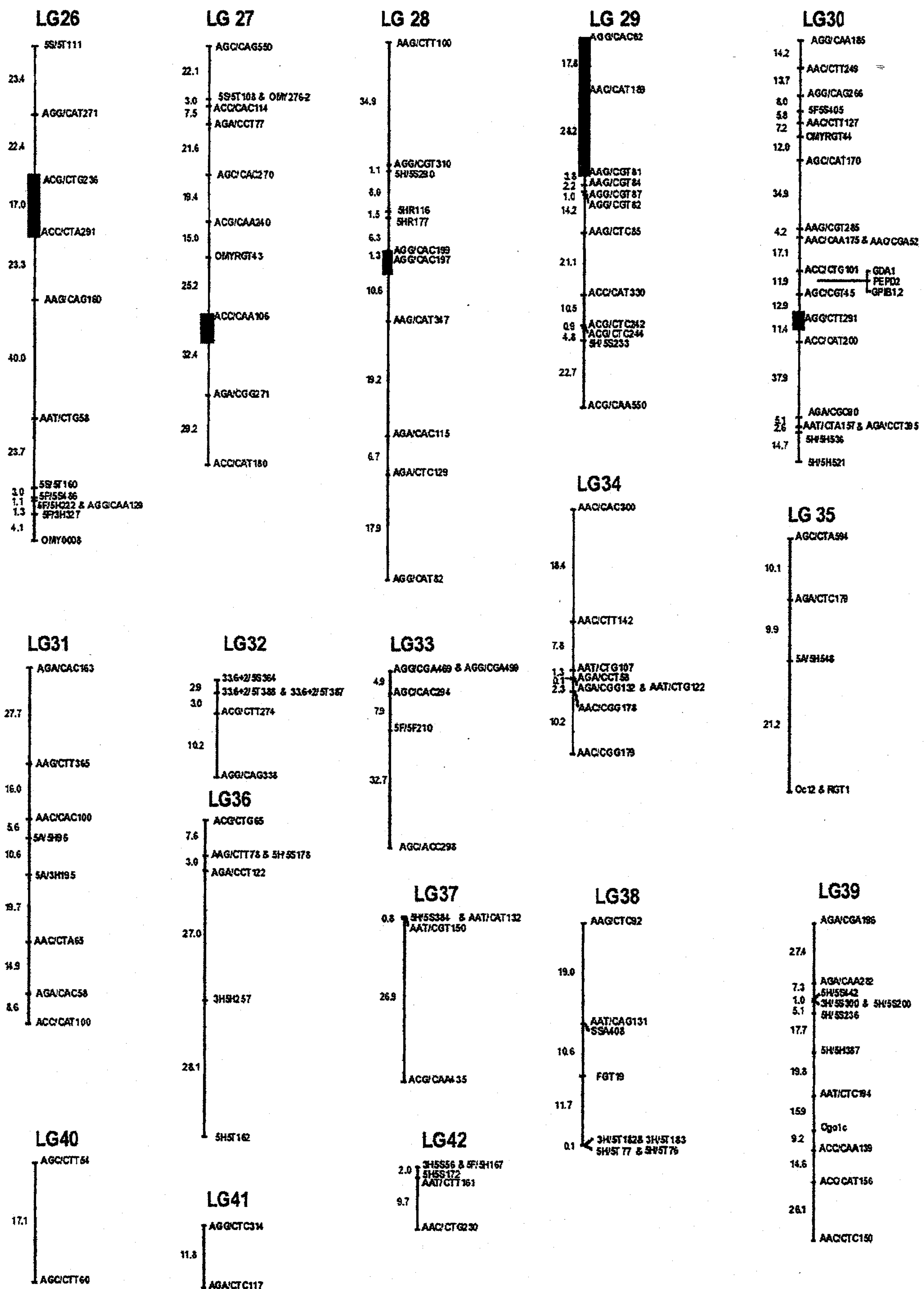

LG26
5S/5T111
AGG/CAT271
ACG/CTG236
ACC/CTA291
AAG/CAG160
AAT/CTG58
OMY0008
LG 27
AGC/CAG550
ACC/CAC114
AGA/CCT77
AGC/CAC270
ACG/CAA240
OMYRGT43
ACC/CAA106
AGA/CGG271
ACC/CAT180
LG 28
AAG/CTT100
AGG/CGT310
5HR116
5HR177
AGG/CAC199
AGG/CAC197
AAG/CAT347
AGA/CAC115
AGA/CTC129
AGG/CAT82
LG 29
AGG/CAC62
AAC/CAT189
AAG/CGT81
AAG/CGT84
AAG/CGT87
AGG/CGT82
AAG/CTC85
ACC/CAT330
ACG/CTC242
ACG/CTC244
ACG/CAA550
LG30
AGG/CAA185
AAC/CTT249
AGG/CAG266
AAC/CTT127
OMYRGT44
AGC/CAT170
AAG/CGT285
AAC/CAA175 & AAG/CGA52
ACC/CTG101
GDA1
PEPD2
GPIB1,2
AGC/CGT45
AGG/CTT291
ACC/CAT200
AGA/CGG80
AAT/CTA157 & AGA/CCT395
LG31
AGA/CAC163
AAG/CTT365
AAC/CAC100
AAC/CTA65
AGA/CAC58
ACC/CAT100
LG32
ACG/CTT274
AGG/CAG338
LG33
AGG/CGA469 & AGG/CGA499
AGG/CAC294
5F/5F210
AGC/ACC298
LG34
AAC/CAC300
AAC/CTT142
AAT/CTG107
AGA/CCT58
AGA/CGG132 & AAT/CTG122
AAC/CGG178
AAC/CGG179
LG 35
AGC/CTA594
AGA/CTC179
Oc12 & RGT1
LG36
ACG/CTG65
AGA/CCT122
LG37
AAT/CGT150
ACG/CAA435
LG38
AAG/CTC92
AAT/CAG131
SSA408
FGT19
LG39
AGA/CGA196
AGA/CAA282
AAT/CTC194
ACC/CAA139
ACC/CAT156
AAC/CTC150
LG40
AGC/CTT54
AGC/CTT60
LG41
AGG/CTC314
AGA/CTC117
LG42
AAT/CTT161
AAC/CTG230

Figure 4. PCR products from *MHC-αl* in haploid family V96-02 analyzed using the SSCP technique. Female V96-02 is indicated by the arrow. Alleles segregate in the haploid progeny based on a conformational polymorphism effecting the mobility of single stranded DNA. Products from alleles *270a* and *270b* are labeled on the left.

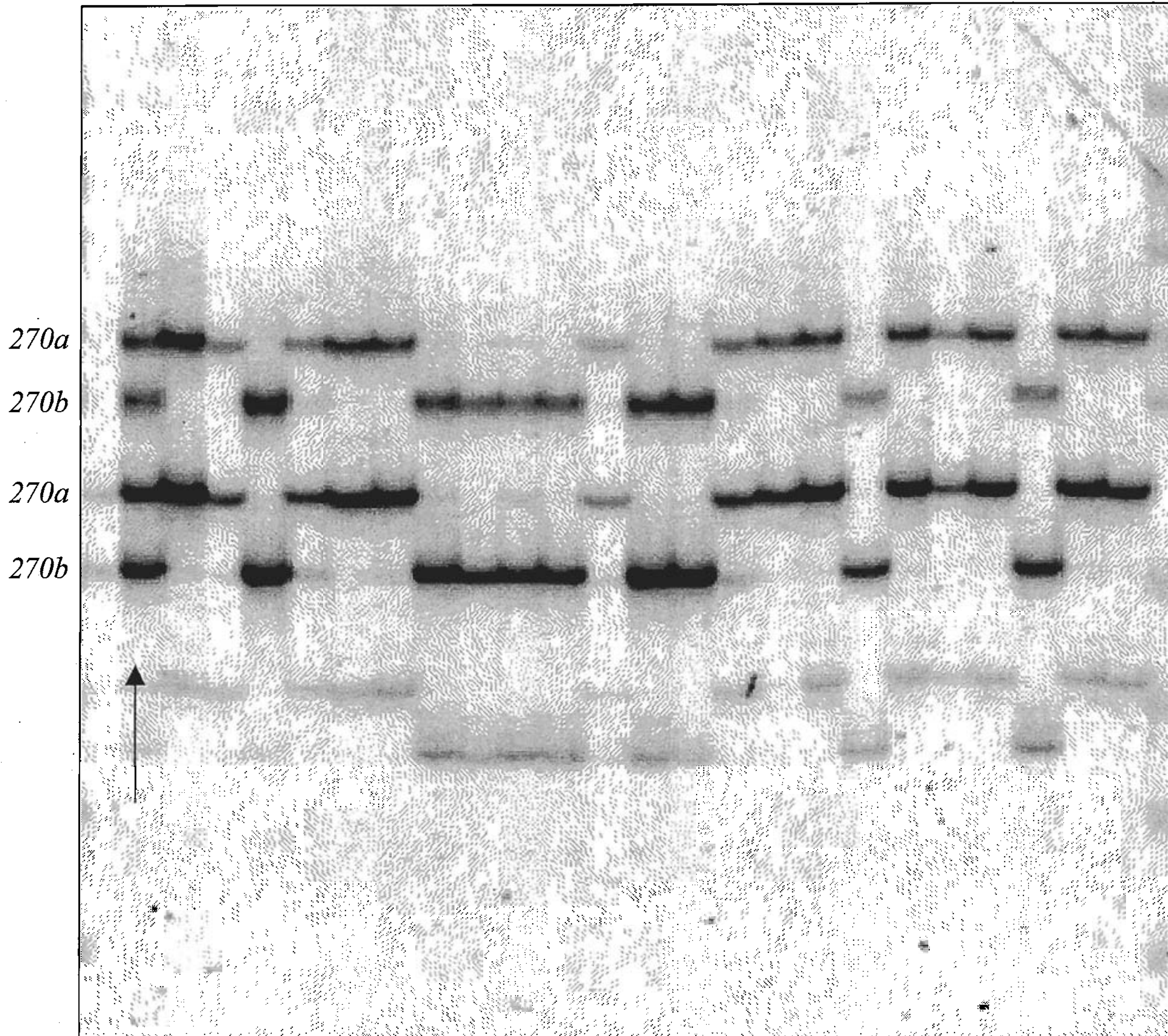
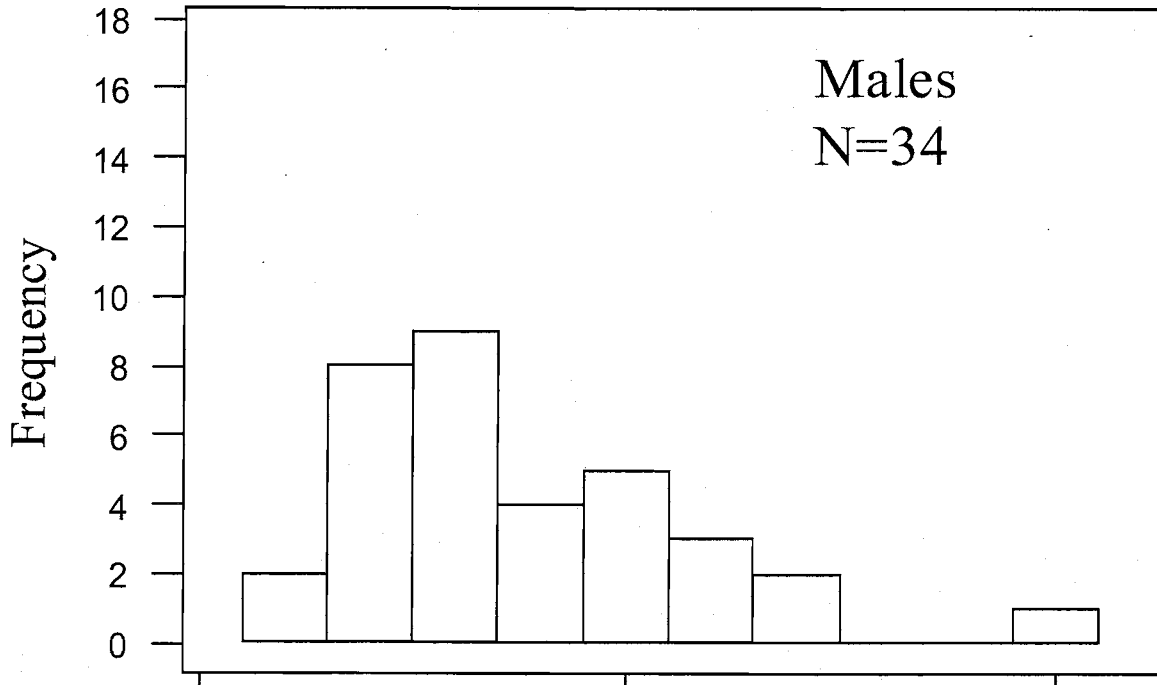


Figure 5. Lengths of adult pink salmon collected in August 1999 from Likes Creek.



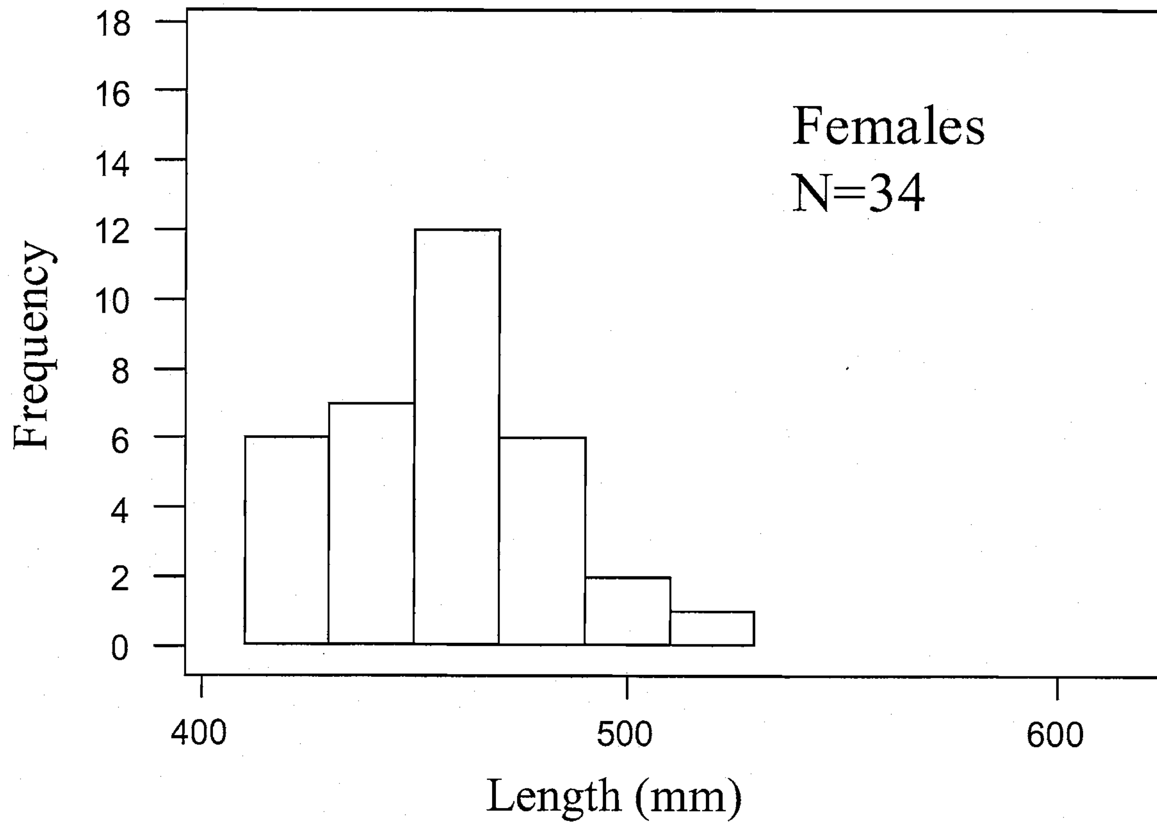


Figure 6. Number of asymmetric characters in pink salmon collected from Likes Creek.

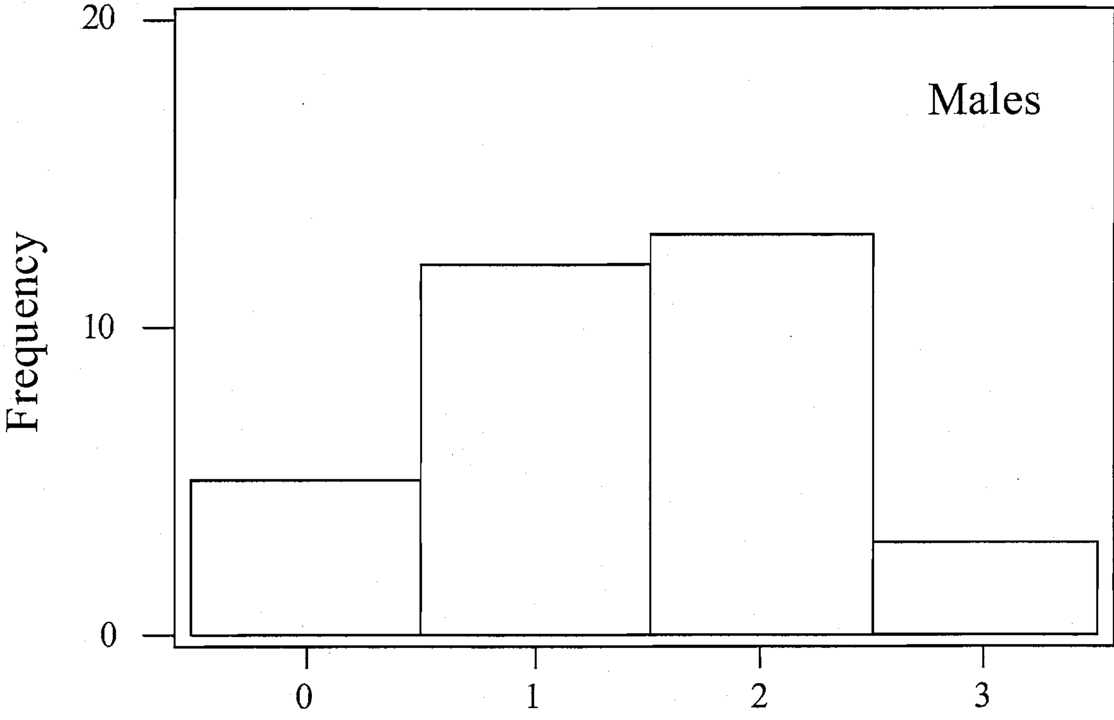
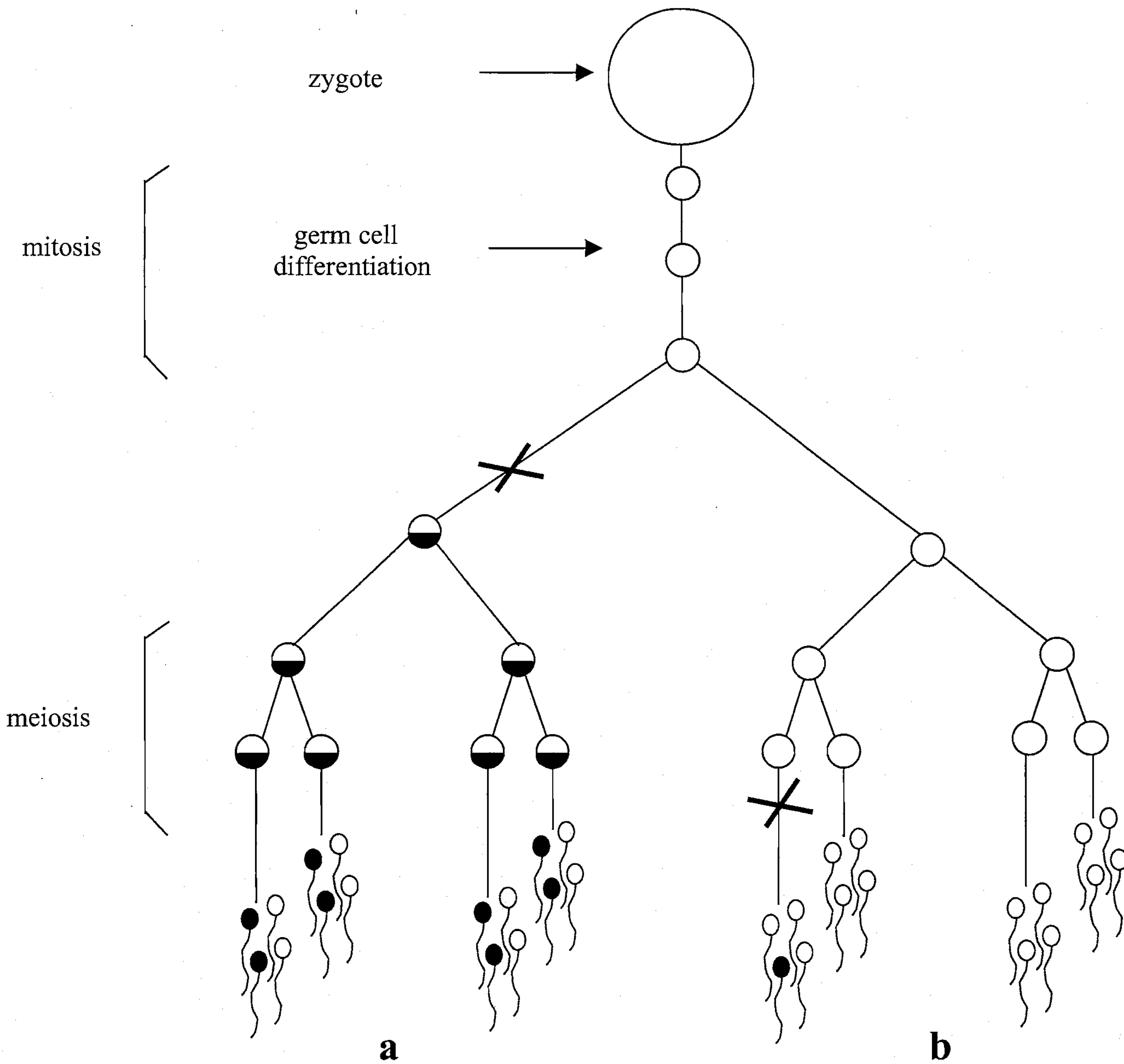


Figure 7. Schematic of cell lineages during ontogeny (modified from Woodruff et al. 1996). Mutations are denoted by X. (a) A mutation arises before meiosis and is replicated into a cluster of copies in multiple gametes; (b) a mutation arises late in meiosis in a single gametic cell.



Appendix 1

Inheritance of Nuclear DNA Markers in Gynogenetic Haploid Pink Salmon

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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) *SmaI*. 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-

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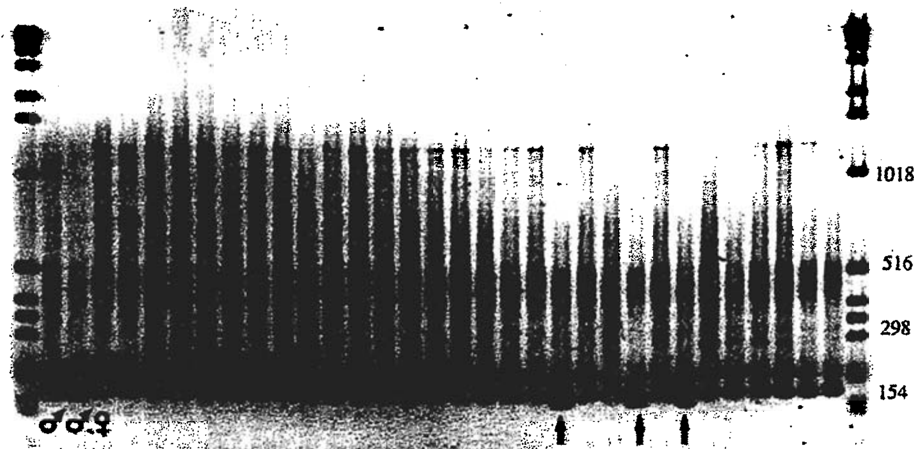

Figure 1. Growth hormone pseudogene amplification products separated in a 2% agarose gel. Individuals of known sex are indicated by the symbols on the bottom left of the gel. The arrows along the bottom of the gel indicate males that were detected in family 95-103.

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′-GTCTGGCTAGGGTACTCCA-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

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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).

Table 1. Primer sequences used for paired interspersed nuclear element (PINE) PCR and references.

Primer name	Sequence (5′-3′)	Reference
*Hpa*I 5′	AACCACTAGGCTACCCTGCC	Kido et al. 1991
*Hpa*I 3″	ACAGGCAGTTAACCCACTGTTCC	Kido et al. 1991
*Fok*I 5′	CTACCAACTGAGCCACACG	Kido et al. 1991
*Sma*I 5′	AACTGAGCTACAGAAGGACC	Kido et al. 1991
Tc1 5′	GTATGTAAACTTCTGACCCACTGG	Greene and Seeb 1997

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 Ssa197, 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 amplification products as DNA template, 0.5 μl *Eco*RI selective primers, 0.5 μl *Mse*I 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 *Fok*I SINE and the 3′ end of the *Hpa*I 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
```

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).

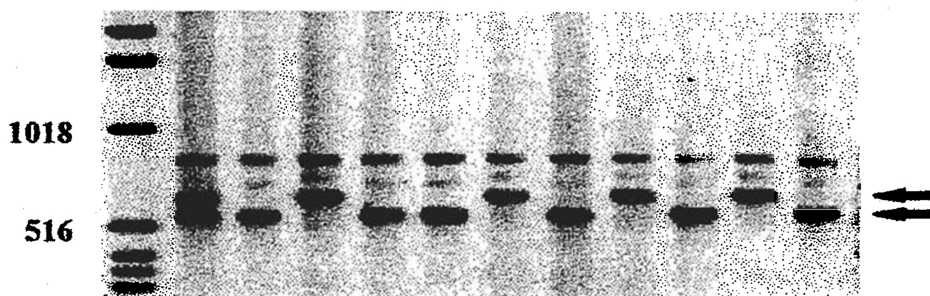

Figure 3. Segregation of *GH-2* polymorphism. The individual denoted by the female symbol is female 95-105, the 10 individuals to the right are haploid offspring from this female segregating for the *GH-2*C446* and **C527* alleles indicated by the arrows.

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

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Table 2. Summary of genetic variation at five microsatellite loci in adult pink salmon from Prince William Sound.

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***
SSA197[a]	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 *AluI* 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 codominant 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*

Table 5. Inheritance of *SSA197* in gynogenetic haploid progeny.

Female		Progeny phenotype									Female
Number	Phenotype	130	142	146	154	162	164	190	196	Null	genotype
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

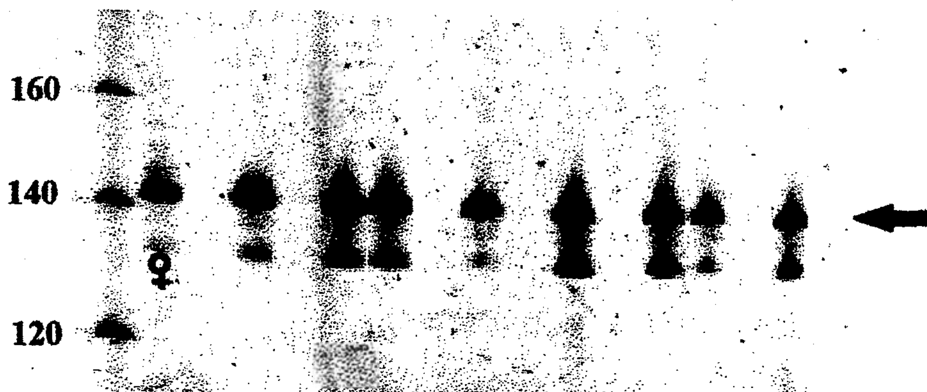

Figure 4. Segregation of a "null" allele (no PCR product is amplified) at *SSA197*. The individual denoted by the female symbol is female 95-106, the 14 individuals to the right are haploid offspring from this female. The arrow indicates allele *SSA197*142*.

1–6

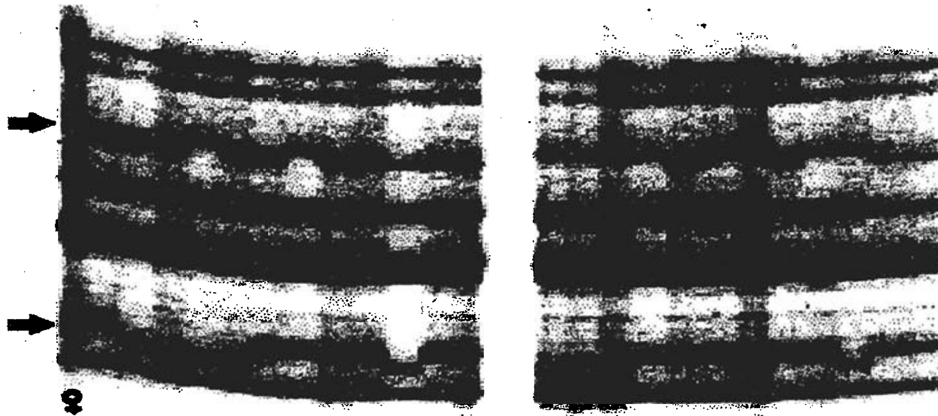

Figure 5. Hpa 3' and Tc1 PINE primer amplification products separated on a 4.5% polyacrylamide gel. Female 95-103 is indicated. The two arrows along the left side of the gel indicate length polymorphisms: top bands 181/182 bp, bottom bands 166/167 bp.

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.

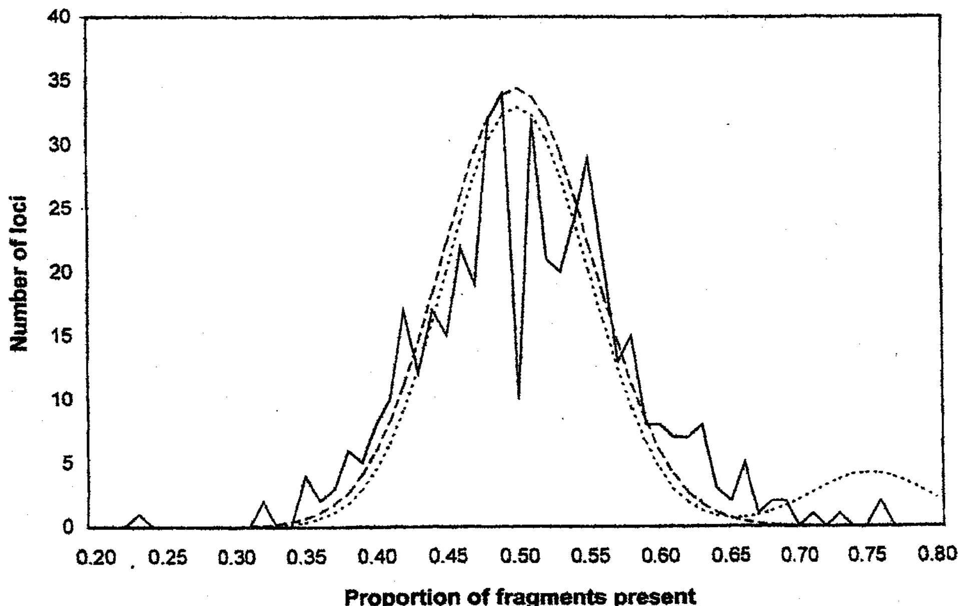

Figure 6. Distribution of segregation ratios for 451 fragments in haploid progeny from female 95-103 (solid line). The dashed line is the expected binomial distribution with an expected value of 0.5 (1:1 segregation). The dotted line is the expected binomial distribution for 90% of the fragments segregating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

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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 (½)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-

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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

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

Locus	Family	Maternal genotype	Progeny 11	12	22	Proportion heterozygotes (y)	χ^2(d.f.)*
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)

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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. 1*a* and 1*b*, 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. 1*c*, 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 (χ^2 = 57.2, 3 d.f.) and microsatellites (χ^2= 24.6, 3 d.f.). The distri-

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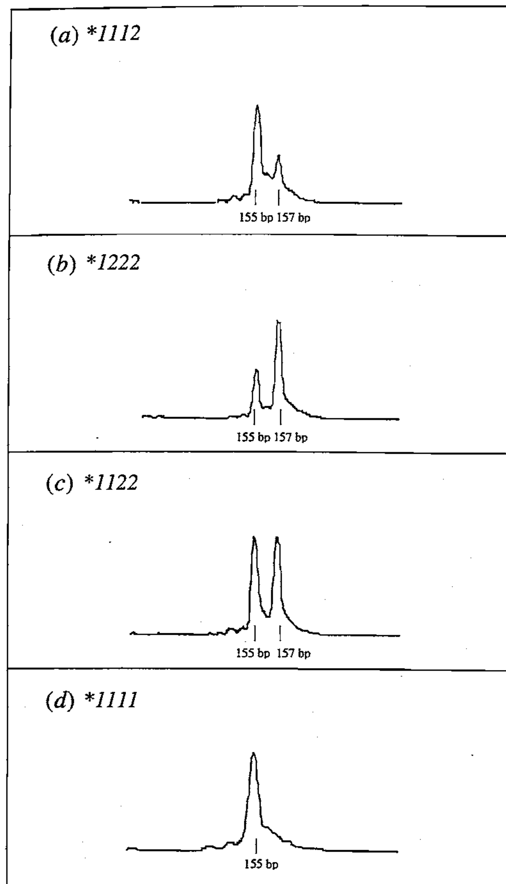

Fig. 1. Electropherograms for individuals with different genotypes at *FGT1-1,2*. The area under the curve at each peak (155 bp and 157 bp) is proportional to the number of doses of each allele (**1* and **2*, respectively).

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). 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.

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Fig. 2. The distribution of second division segregation (y) for AFLPs, PINEs, microsatellites, and all markers (including allozymes).

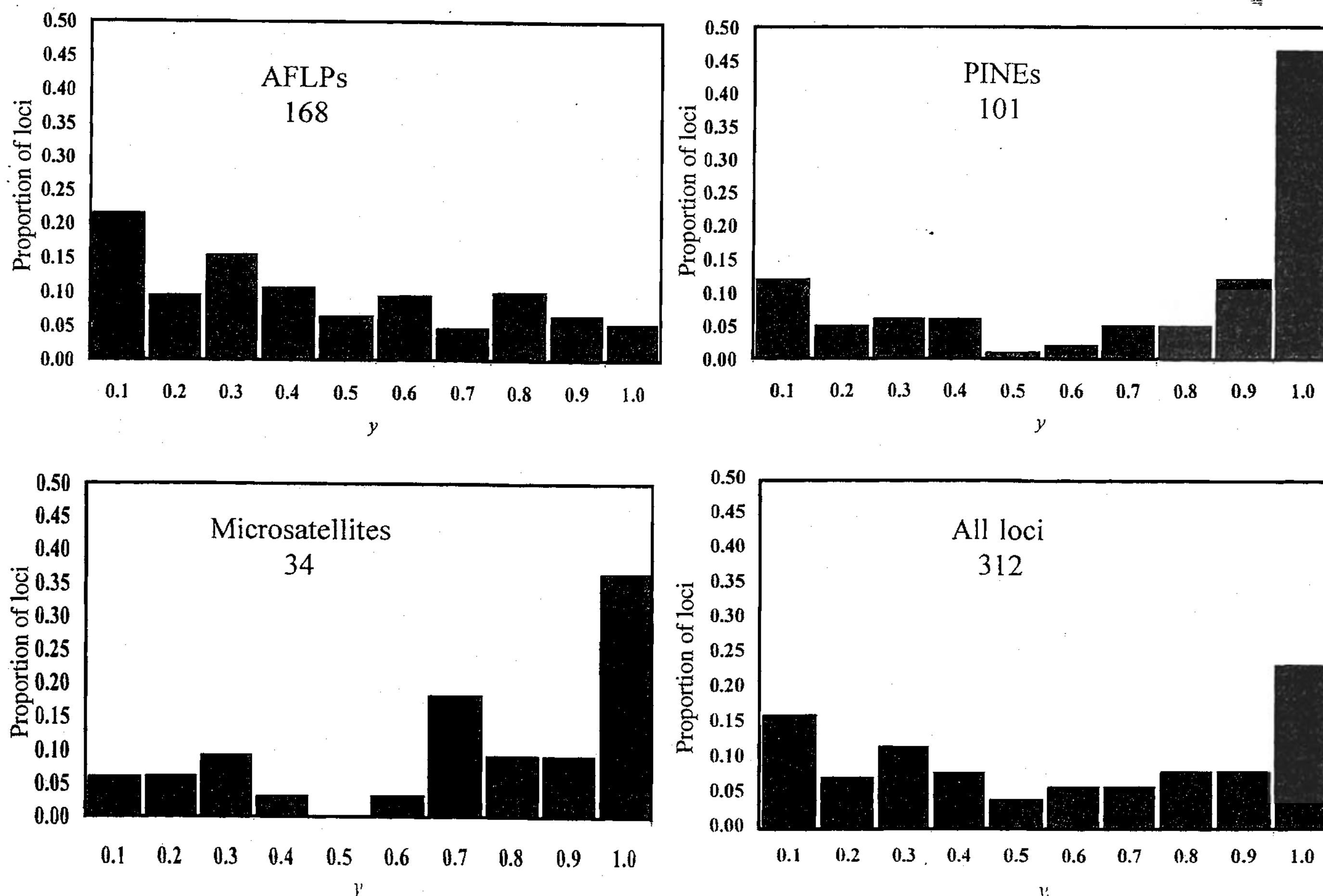

Table 4. Half-tetrad genotypes at eight PINE and four AFLP codominant loci in family A95-103.

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 duplicated 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-

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

	Maternal genotype			Progeny					
Family	*-1, -2*	*-1*	*-2*	*1111*	*1112*	*1122*	*1222*	*2222*	*y*
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.

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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.

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