Exxon Valdez Oil Spill
Restoration Project Annual Report

Construction of a Linkage Map for the Pink Salmon Genome

Restoration Project 98190
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.

Kate R. Lindner
Paul Spruell
Kathy L. Knudsen
Ben Sneed
Fred W. Allendorf

Division of Biological Sciences
University of Montana
Missoula, MT 59812

for:

Alaska Department of Fish and Game
Habitat and Restoration Division
333 Raspberry Road
Anchorage, Alaska 99518

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Study History: This project began in FY 96. However, we did not receive authorization to proceed until 5 March 1996. This project was initially focused on the construction of a genetic linkage map for pink salmon (*Oncorhynchus gorbuscha*). We are currently working on consolidating the linkage map as well as including genes of known function. In August 1998 we initiated the second part of this project, testing for the association between fitness traits and markers distributed throughout the genome. We collected gametes from 150 adult pink salmon from Likes Creek, Resurrection Bay. We produced 75 single-pair families and raised them in the Alaska SeaLife Center (ASLC), Seward AK. We have pooled 49 of these families in one saltwater tank and will release them into Resurrection Bay in late May. 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. In addition Ms. Lindner presented an update at the Alaska Department of Fish and Game in Anchorage Alaska, September 1997. Dr. Paul Spruell has represented our lab at the United States Department of Agriculture (USDA) panel on aquaculture for the past three years. 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. This panel may result in additional funding for salmonid mapping efforts. 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. 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, Journal of Heredity (90:289-296) was published. We have prepared 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 302 loci in pink salmon by half-tetrad analysis.) for submission to Genome. Another 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.

Abstract: We are constructing a detailed genomic linkage map for pink salmon (*Oncorhynchus gorbuscha*) that will assist pink salmon recovery efforts in Prince William Sound (PWS). In August 1995, families of gynogenetic haploid, gynogenetic diploid and normal diploid pink salmon were produced using gametes collected at the Koernig hatchery in PWS. We selected one reference family and analyzed the segregation pattern of 585 DNA fragments in the maternal parent and 94 of her haploid progeny; 559 have been assigned to one of 42 linkage groups. We mapped 303 loci in relation to their centromeres using gynogenetic diploid progeny. Currently we are working on reducing the number of linkage groups to 26, the number of chromosomes present in the female pink salmon genome. In August 1998, gametes and tissue from 75 female and 75 male pink salmon from Likes Creek were collected. Single-pair matings produced 75 families that are currently being raised at the Alaska SeaLife Center (Seward). Individuals from 49 of these families will be marked and released into Resurrection Bay this spring. Upon their return in 2000, we will compare genotypes in released fry and returning adults to test for genetic differences in marine survival and other life history traits.

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

Project Data: Data are the inheritance of DNA fragments in the haploid and gynogenetic diploid progeny of a single pink salmon female (95-103). Sixteen additional diploid families were tested for nonrandom segregation between all pair-wise combinations of 14 allozyme and 3 microsatellite loci. The haploid data set consists of 585 polymorphic DNA fragments in female 95-103 and 94 of her haploid progeny. The diploid data consist of genotypes of 70 gynogenetic diploid progeny from female 95-103 at 301 loci. The Likes Creek pink salmon data set consists of genotypes for 150 individuals at 44 loci. All data sets are recorded in Microsoft Excel spread sheets. Data will be made available to individuals within the reasonable bounds of sharing unpublished data. For information regarding data contact Kate Lindner, Division of Biological Sciences, University of Montana, Missoula, MT 59812. Phone: (406) 243-5503. E-mail: klindner@selway.umt.edu.

Citation:

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EXECUTIVE SUMMARY

The construction of a genetic linkage map for the pink salmon (*Oncorhynchus gorbuscha*) genome is in its fourth year. This linkage map will allow the characterization of the genetic impacts of the March 1989 *Exxon Valdez* oil spill on pink salmon populations and help to document the recovery of effected populations in Prince William Sound. A genetic linkage map will be essential for detecting and understanding causes of reduced egg and embryo survival in oiled areas. In addition, a detailed genetic map 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 is being constructed using haploid progeny to avoid the difficulties associated with dominant markers that obscure recessive alternatives in diploids. Gynogenetic diploids from the same family are also being examined to locate the centromere of each chromosome 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 95-103) was chosen to be the reference family upon which our initial mapping efforts were based. Embryos were confirmed to be of gynogenetic origin using a sex-specific pseudogene locus and several microsatellites. After removal of individuals not of gynogenetic origin, family 95-103 consists of 94 haploid embryos, and 70 gynogenetic diploid progeny from female 95-103.

Linkage analysis of 585 markers segregating in the gynogenetic haploids produced a genetic map comprised of 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 2.72×10^6 kilobase pairs (kbp) thus, we estimate a physical recombination rate of approximately 391 kbp/cM.

In addition 13 allozyme loci have been added to the map using gynogenetic diploid and normal diploid data. Five allozyme loci are polymorphic in female 95-103 and 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 598 markers.

We have generated a large number of markers throughout the genome using haploid embryos and multilocus techniques. Due to polyploid ancestry of salmonids their genome size is large and will require many markers to cover the genome. Our next goal is to "consolidate" the map and reduce the number of linkage groups to 26, the number of chromosomes in pink salmon. In order to achieve this goal, we have collected gene-centromere distances of 311 loci using gynogenetic diploids. 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. The microsatellites will serve as land marks, 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 also serve as anchor loci and will be of great interest during the next phase of this project in which we examine the effects of the marine environment on the pink salmon genome.

We have initiated the second portion of the study at the Alaska SeaLife Center. In August 1998 gametes and tissue from 150 pink salmon, 75 females and 75 males, from Likes Creek were collected. The linkage map was used to identify loci in pink salmon that are distributed across the entire genome. These markers are used to collect genotypes from the 150 adult pink salmon. Eggs from each female were mixed with sperm from one male resulting in 75 single cross families. These families are currently being raised at the ASLC Center in Seward. A total of 72,833 individuals from 49 of these 75 families will be marked and released from the ASLC into Resurrection Bay this May. In the fall of the year 2000 we will collect gametes and tissue from all returning marked fish to examine the association of genetic markers with fitness traits such as marine survival and fecundity.

We are on schedule with the mapping portion of this project. More anchor loci will be included on the map which will both consolidate the map and provide more markers to detect regions of the genome of the genome associated with fitness traits. The second portion of this project is underway. We have collected genotypes from the adult pink salmon and the fry are growing at the ASLC in preparation for a late May release.

INTRODUCTION

The construction of a genetic linkage map for the pink salmon (*Oncorhynchus gorbuscha*) genome is in its fourth year. This map will provide a more thorough understanding of the genetic impacts of the March 1989 *Exxon Valdez* oil spill on pink salmon populations. In addition the map will increase our understanding of the genetic characteristics of pink salmon and help document the recovery of effected populations in Prince William Sound.

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, 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.

A detailed genetic map of pink salmon will be invaluable for documenting the effects the spill may have had on pink salmon. Such a map will also aid other pink salmon recovery efforts, including estimating straying rates, describing stock structure, and testing for a genetic basis of marine survival.

The construction of a linkage map requires analyzing the genetic transmission of hundreds of DNA polymorphisms. These markers must meet several criteria to be useful for mapping purposes. The techniques used must generate reproducible markers and it is preferred that they detect multiple loci from a single polymerase chain reaction (PCR). These multilocus techniques more efficiently generate the large number of loci necessary to serve as the framework of the map. The fragments detected by each technique should also be dispersed throughout the genome to avoid exclusion of specific chromosomal regions. Finally, the markers must be present in the maternal parent and segregate in a ratio expected in simple Mendelian inheritance models.

Many of the multilocus techniques commonly used for linkage map construction result in variation that is measured by the presence or absence of a given PCR product. Fragments that segregate in this "dominant" (present) versus "recessive" (absent) manner are problematic in that it is impossible to distinguish individuals that are homozygous dominant (two alleles that produce the DNA fragment) from those that are heterozygous (one allele produces the fragment, the other does not). We are avoiding the difficulties of dominance with these markers by using haploid progeny in which recessive alleles are not obscured by their dominant alternatives (Lie et al. 1994). Although these embryos are not viable, development progresses until just prior to hatching (Stanley 1983), providing an embryo from which we obtain sufficient DNA for PCR-based analyses.

We have analyzed many such markers however, we have not yet reduced the number of markers to the number of chromosomes in pink salmon (26). This process, known as "consolidation of the map" is our next goal.

Identification of the centromeres of each chromosome would assist in consolidation. The analysis of gynogenetic diploids is an effective method to identify the centromeres of chromosomes (Thorgaard 1983) and will aid in the consolidation of the map in two ways. First, gynogenetic diploids allow the analysis of recombination rates of individual markers based on the frequency of heterozygotes. These recombination rates are a function of the distance the marker is located from the centromere, the gene-centromere distance. Once markers tightly linked to centromeres have been identified, markers linked to the centromeric markers can be assigned to a specific chromosome (Johnson et al. 1996). Second, the analysis of co-segregation between markers used in gene-centromere mapping will identify groups that are linked, thereby consolidating the map.

Dominant markers, such as amplified fragment length polymorphisms (AFLPs) and paired interspersed nuclear element PCR (PINEs) are very useful to construct linkage maps but do not facilitate comparison between maps. However, comparative mapping can be accomplished by linking the dominant markers to markers that are conserved across taxa thus placing the anchor loci on the map. 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 known genes.

Comparisons between the pink salmon linkage map and other teleost linkage maps is currently difficult due to the lack of shared anchor loci. Linkage relationships in fishes persist after 300 million years of evolution (Graf 1989), suggesting that many anchor loci should occupy a similar chromosomal position in closely related taxa. As linkage maps of fish become more common, it is important to be able to incorporate information from other linkage maps onto the pink salmon linkage map. Therefore it is necessary to include more anchor loci on the pink salmon linkage map.

We currently are adding numerous, allozymes, microsatellites, and known genes to the map that will serve as anchor loci. PCR primers for many salmonid microsatellite loci are published and many of these should amplify homologous loci in pink salmon. Given the high level of polymorphism typical for these markers, screening microsatellite loci is probably the most efficient method to add anchor loci to the map.

Genes of known function can also serve as anchor loci and are particularly interesting in the study of 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, including pink 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. The presence or absence of a six base pair insertion differentiates these allele classes. Female 95-103 does not show a length polymorphism at this locus. However, other techniques to detect sequence polymorphisms should allow us to add this and other genes to the linkage map.

We will also use the map to investigate questions of salmonid evolution and genome structure. We are using various types of markers to construct the map. We can use the linkage assignments derived from the map to test for a uniform distribution of each marker type. In addition, as we place genes of know function on the map we will be able to document any clustering or bias in chromosomal position for functional genes.

A high resolution linkage map will also allow us to re-examine 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. We have generated a map with much finer resolution. It is possible that high levels of interference are not uniform and some regions of the genome map violate this assumption. We can now test this assumption using our map.

Another benefit to generating a linkage map is the ability to identify the sex-determining region in pink salmon. Sex-linked genetic markers are important for the study of population genetics. Several such markers have been described and developed for a variety of species (i.e., mouse, human, chicken and fruit fly) yet few are known in salmonids (Devlin et al. 1991, Du et al. 1993, Allendorf et al. 1994, Forbes et al. 1994, Moran et al. 1996). Sex-linked markers are useful for the gender identification of immature fish, for investigating the genealogy and phylogeny of species, for comparative gene mapping, and for detecting geographic population structures. Sex-linked markers can also help compare rates of recombination between the X and Y chromosomes in males, the X chromosomes in females, and aid in the determination of the size of the non-recombining region on the Y-chromosome.

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 the distributions of genotypes in a single cohort at different life history stages (p. 303, Lynch and Walsh, in preparation). We have used the facilities at ASLC to produce the families necessary for such an experimental design. We have genotyped the adults used to make these families. We therefore know the expected genetic profile of each family. We will have samples of these families at three time points during their life cycle (1) the time of eye pigment deposition, (2) the time of release into the marine environment, and (3) when they return as mature adults. We will characterize these samples using genetic markers known to be distributed throughout the genome and identify genomic regions associated with specific fitness traits at each life history stage.

In this report, we update our progress on the construction of the pink salmon linkage map and describe the experimental families we will use in conjunction with the map to address questions of pink salmon survival and fitness. We describe the segregation analysis of 585 loci in the haploid gynogens, 311 loci in the diploid gynogens, and 13 allozyme loci

in normal diploids. We are using these data to construct the linkage map. We have used the gynogenetic diploid data to confirm linkages suggested by the haploid data and to calculate gene centromere distances for those 311 loci. We also demonstrate how gene centromere distances allow us to compare the genomic distribution of loci amplified by AFLPs, PINEs, and microsatellites. In addition, we begin to examine the assumption of complete interference in salmonid fish. Finally, we describe 75 families of pink salmon produced at the ASLC that we will use in combination with the linkage map to test for associations between genomic regions and traits of adaptive significance.

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 Experimental Progeny

Gynogenetic Haploid Production

In August 1995, gametes and tissues of 31 pink salmon were collected from the Armin F. Koernig (AFK) hatchery, Prince William Sound, Alaska. This hatchery stock originated from adult fish collected at several spawning sites in Prince William Sound. 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 95-103). Embryos from these families were incubated until just prior to hatching when they were collected and preserved in ethanol. DNA extraction was completed on the haploids as previously described (Appendix 1).

Gynogenetic Diploid Production

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 (Appendix 1).

Biparental Diploid Production

We produced 16 "normal" (biparental) diploid families by mixing eggs and sperm from individuals collected at the AFK hatchery. These progeny were raised at the Alaska Department of Fish and Game (ADFG) facility in Anchorage in separate Heath trays at 4 °C. When fry reached approximately 40mm total length they were sampled and kept frozen at -80°C until allozyme analysis could be completed.

Selection of a Reference Family

A single family (95-103) was chosen as the reference family to generate the linkage map. DNA was extracted from 150 putative haploid embryos in this family. Confirmation of gynogenetic origin was completed on the haploids (Appendix 1). Ninety-four of the remaining 131 haploid embryos contained sufficient DNA for extensive analysis.

DNA was extracted from 77 putative gynogenetic diploids in family 95-103. Individuals were confirmed to be gynogenetic diploid progeny of female 95-103 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.

Genetic Markers

Genetic markers are included on the haploid linkage map based on two criteria. First, polymorphic fragments in the haploids had to be present in female 95-103. Second, the segregation of each fragment in the progeny had to be 1:1 as expected under simple Mendelian genetic models. We identified 620 fragments that met these criteria and included them in the analysis of joint segregation ratios. 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 have been eliminated from the linkage analysis.

Thirty microsatellite loci were amplified as reported by the original authors with minor modifications. Primers and annealing temperatures are listed in Table 2.

Genotypes of the 70 gynogenetic diploid offspring of female 95-103 were scored at 168 AFLP loci, 101 PINE loci, 23 microsatellite loci and five allozyme loci. The PCR-based techniques for each marker type are identical to those used for amplification of markers in the haploid embryos (Appendix 1).

Tissues were analyzed using enzyme electrophoresis after storage at -80 °C following Aebersold et al. (1987) and Seeb et al. (1996).

Map Construction

Gene-Centromere Distances

We used half tetrad analysis to estimate how far loci are from the centromere (the gene-centromere distance) for each locus we have analyzed in the diploid gynogens. (Thorgaard et al. 1983; Allendorf et al. 1986). The details of these calculations are provided in Appendix 2.

Linkage Analysis Software

We used MapMaker software (Lander et al. 1987) to assign markers segregating in haploid progeny to linkage groups. Grouping of markers was completed using a minimum LOD score of 3.0 and a maximum recombination fraction (θ) of 0.30 ($P<0.001$). The linkage phase of the markers is unknown. Therefore, we entered the segregation pattern of each locus into the program in both possible phases. This resulted in a data set that was too large to use the MapMaker "group" command on the entire set. The markers present in each group were assigned to chromosomes and all additional markers were analyzed using the "assign" command. The Kosambi mapping function was used to calculate the genetic distance. When analysis of the entire data set was

required Mark Daly at the Whitehead Institute analyzed the entire set using a UNIX-based computer system.

A computer program was written for the gynogenetic diploid data to analyze all pair-wise combinations of loci for non-random segregation (Noel Weaver). Loci with a proportion of heterozygotes (y) ≤ 0.80 were analyzed using this program.

An additional 16 diploid families were analyzed for nonrandom segregation between all pair-wise combinations of 14 allozyme and three microsatellite loci.

Estimates of Interference

We have initiated a collaboration 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 will compare our findings in the haploid data with the results from the gynogenetic diploid data.

Likes Creek Experiment

Families

In August of 1998, we collected gametes from 75 female and 75 male pink salmon from the mouth of Likes Creek, Resurrection Bay, Alaska. We assigned each individual a unique number and measured it's length from the middle of the eye to the fork of the caudal fin. We also collected liver, eye, heart, and muscle tissue from each individual for subsequent genetic analysis. We then removed the head of each fish and froze them for future otolith removal and meristic counts.

We dissected otoliths from the adult pink salmon collected from Likes Creek. Alaska Department of Fish and Game personnel analyzed otoliths for presence of temperature rings used to mark hatchery stocks. They found no indication that any of the adults collected from Likes Creek originated from a hatchery stock.

We used the gametes collected from Likes Creek to produce 75 single-pair families (one female x one male) to be reared at the ASLC. Eggs and sperm from a single male and female were mixed together. After 2 minutes the eggs were rinsed with fresh water and treated with a dilute betadine solution to reduce infection and fungus growth. Each family was placed in a separate Heath tray and raised in fresh water at $5^{O}C$.

We visually checked eggs for developing embryos in November 1998. We weighed a subsample 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 100 embryos from each family in 100% ethanol for subsequent DNA analysis.

We selected 50 families on the basis of egg number and survival during incubation for the release experiment. These families were pooled together into a single tank in March shortly after hatching; one of these families was inadvertently not included in the pool. In May 1999, approximately 1,500 progeny from each of these 49 single-pair mating families will be released from the ASLC facility.

Genetic Analysis

We examined each of the 150 parents using 11 PCR based markers and 63 allozyme loci following the methodology described above. We detected thirty-four polymorphic allozyme loci in this population. The number of alleles per locus was calculated using GenePop (Raymond and Rousset 1995). Genotypic frequencies were also tested for departures from expected Hardy-Weinberg proportions using GenePop. We calculated the observed (H_o) and expected heterozygosity (H_e) directly from the genotypes. We examined males and females separately to test for differences between the sexes.

Fluctuating Asymmetry

Five bilaterally paired meristic traits, pectoral fins, pelvic fins, upper and lower gill arches, and the mandibular pores were examined for fluctuating asymetry (FA) in all 150 adult pink salmon. We quantified asymmetry as the number of traits that were asymmetric in each individual. We subsequently removed the mandibular pore count data due to scrapes on the lower jaw which made scoring this trait difficult.

RESULTS

Linkage Map

Linkage analysis of 585 markers (Table 1) segregating in the gynogenetic haploids produced a genetic map comprised of 42 linkage groups, covering a distance of 5371 centimorgans (cM) (Figure 1). Twelve groups include five markers or less and the largest group contains 52 markers (Table 3). Twenty-six markers remain unlinked. Anchor loci have been assigned to 17 linkage groups, 11 linkage groups include more than one anchor locus.

Assuming a minimum distance of 30 cM for linkage detection and accounting for all the gaps and unlinked markers, the estimated minimum size of the pink salmon genome is 6872 cM. The haploid pink salmon genome consists of approximately 2.72×10^6 kilobase pairs (kbp) thus, we estimated that each centiMorgan corresponds to a physical distance of approximately 391 kbp.

Gynogenetic Diploids

Gene-centromere distances (Appendix 2, Figure 2) were estimated by the proportion of gynogenetic diploid progeny that were heterozygous (y). Since gynogenetic progeny are the result of second polar body retention, heterozygotes can only be produced if there is a crossover between the centromere and that marker (Thorgaard et al. 1983). These recombination rates are a function of the gene-centromere distance.

Markers with very low gene-centromere distances must be located in centromeric regions. We have identified markers near the centromere ($y \leq 0.10$) on 21 linkage groups. Seven of these centromeric regions are located at the end of the linkage group.

Contingency tests of linkage on all pair-wise combinations of loci in the gynogenetic diploids supports most of the haploid linkage groups. Gene-centromere data for most markers linked in the haploid map have significant contingency values ($P<0.025$). Three out of the five allozyme loci, that segregate in female 95-103, *GDA1, CKC2,* and *sAAT3*, were included in the test for non-random segregation. Two of these loci, *GDA1* and *sAAT3*, non-randomly segregate ($P<0.001$) with loci already placed on the linkage map. In addition two pairs of loci in two separate linkage groups had significant contingency chi-square values ($P<0.001$) suggesting that these two groups were actually linked. These two groups have been combined into one larger group (linkage group 30).

We have used the data obtained from the gynogenetic diploids to examine the distribution of each marker type along the length of the chromosome. These data are described in Appendix 2.

Biparental Diploids

We have added 11 allozyme loci to the map using data from bi-parental diploid families. Five allozyme loci are linked to microsatellite loci already included on the linkage map (Table 5). *IDHP2* is linked to *OTS1*. Two loci, *ADA2* and *PGDH,* are linked to the same microsatellite, *SSA197*, and linked to each other. However *sAAT4* and *CKC2* are linked to the same microsatellite, *STR60*, but do not link to each other in the families tested. Four allozymes could be added to the map because they are linked to each other as well as loci already on the map; *sMDHB1,2* and *FH*, are linked to *sAAT3* in addition *GBIB1,2* and *PEPD2* are linked to *GDA1* (Table 5). Both sAAT3 and GDA1 have been included on the map through the gynogenetic diploid analysis. Two more allozyme loci can be included based on other linkage studies conducted in pink salmon (Makoto 1998); *MAH-4* is linked to *sAAT3* and *AH3* is linked to *sAAT4*.

With the addition of these 13 allozyme loci the linkage map consists of a total of 598 loci.

Likes Creek Experiment

Families

Over 95,000 eggs produced in the initial crosses survived through hatching. In March 1999 we pooled 49 of the largest families in an outdoor saltwater tank. Families contained an average of 1486 fry. Over 73,000 fry will be released into Resurrection Bay in May 1999. Of the remaining families two consisted of less than 200 eggs and were eliminated, eight families are being raised in separate tanks for future inbreeding studies, and the remaining 16 families are being raised for use by the ASLC.

Genetic Analysis

We detected polymorphisms at 34 of 63 allozyme loci analyzed. We eliminated *PEPD1* from subsequent analysis due to a large amount of missing data. In addition, genotypes have been collected at 9 polymorphic microsatellite loci, growth hormone 2 (*GH2*), and the α 1 region of *MHC*.

Observed and expected heterozygosity for each polymorphic locus are presented in Table 4. No deviations from expected Hardy-Weinberg proportions were detected (Table 4). However, when males and females were examined separately, we observed a large difference in allele frequencies at *MHCα1* (Table 6).

Assignment of Progeny to Family

We estimated our power to genetically assign progeny to the correct family using the computer program ProbMax (Danzmann et al. 1997). We used the empirically determined genotypic profile of the experimental parents and generated 100 hypothetical offspring from each family. Offspring genotypes were determined by randomly assigning each individual one of the possible genotypes at each of 33 allozyme loci and 11 PCR-based loci. We used these simulated offspring to test how reliably we could determine the family to which each belonged. We were able to assign all but one of the 49,000 simulated progeny back to the correct family.

Morphology

We measured all 150 adult pink salmon collected from Likes Creek. Males have significantly greater variance in length then females ($P<0.01$, Figure 2) and they tend to be smaller ($P<0.06$).

The population average FA for the four meristic traits is 1.52. The average number of asymmetric characters for females (1.16) is significantly lower then the average for males (1.54, $P<0.02$, Figure 3). The variance in the number of asymmetric characters for females ($\sigma^2 = 0.66$) was also significantly lower than the variance for males ($\sigma^2 = 1.04$, $P<0.001$; Figure 3)

DISCUSSION

Consolidation of the Linkage Map

We have collected a large amount of data from both haploid and diploid progeny. However, we have yet to reduce the number of linkage groups to 26, the number of chromosomes in the pink salmon genome. We will continue to add markers to the linkage map and to reduce the number of linkage groups using data from family studies and other salmonid mapping efforts.

We expected the pink salmon map to be large because of the polyploid ancestry of salmonids. We also expected the pink salmon map to be larger than the rainbow map based on different recombination rates in males and females. Young et al. (1998) estimated the rainbow trout (*Oncorhynchus mykiss*) linkage map to be 2628 cM based upon recombination rates in males. However, the pink salmon linkage map is based on recombination in females. We expect female-based maps to be longer than male-based maps because of the reduced recombination rate in male salmonids (Johnson et al. 1987a).

We initially anticipated that it would be necessary to map over 500 markers to ensure that new markers can be assigned to an existing linkage group with high probability (Van der Beek and Van Arendonk 1993). We based this estimate on data from zebrafish in which 99% of all loci were estimated to be within 20 cM of a marker on the map based upon an earlier report using 414 markers (Postlethwait et al. 1994). We expected the pink salmon map to require more markers than the zebrafish map based on the relative genome sizes but had no way to know the exact number of loci necessary.

Our goal is to produce a linkage map with 26 linkage groups each corresponding to a pink salmon chromosome and we will continue to work toward that goal. We have exceeded our original estimate of 500 markers and have yet to consolidate the map. However, several of the linkage groups are small and should join other groups with the addition of markers. Centromeric regions are located close to the ends of several of the linkage groups. Pink salmon chromosomes are predominately metacentric or submetacentric (Gorshkov et al. 1981) which is inconsistent with the centromeric location suggested by the linkage map. We suspect that several of our linkage groups represent the different arms of the same chromosome and will eventually be joined when centromeric markers for each chromosome have been identified. We believe that although the number of linkage groups has not yet been reduced to the number of chromosomes, the map is useful in its current state.

Application of the Linkage Map

Pink Salmon Management

The existing genome map for pink salmon allows us to address important genetic issues related to other components of the Pink Salmon Restoration Program and fundamental questions of salmonid evolution. The numerous genetic markers identified in the course of this study will provide greatly increased power and resolution to identify stocks of pink salmon on a very fine scale (Stock Separation and Management). The genetic map also allows us to test for the presence of genes having major effects on phenotypes of importance for the management of pink salmon, and to test for phenotypes associated with specific combinations of multilocus genotypes (Lander and Schork 1994). These genetic markers will be of great value in genetically identifying fish from supplementation programs and detecting their ecological and genetic interactions with wild fish (Supplementation).

Salmonid Evolution

The map has also provided new insight into the genomic structure and evolution in pink salmon. We have analyzed gene-centromere data and concluded that markers produced by various techniques are not uniformly distributed throughout the genome. These results are detailed in Appendix 2.

In addition, analysis of the combined haploid and diploid data sets has suggested that the level of chiasma interference is not uniform throughout the genome. We have constructed the map assuming complete interference in all regions based on previously available data (Thorgaard et al. 1983). However, if there is less interference in some areas our assessment of linkage and linkage distances may be incorrect. We have just initiated a collaboration with Dr. Zhao to analyze these data. Further analysis will help us better understand genomic processes in salmonids and test the impacts of these findings on the construction of linkage maps.

Estimation of Fitness

We are entering the next phase of the linkage map project. We have almost completed the assembly of the map itself. We are now ready to begin the application of the map to questions of pink salmon ecology and evolution. We have produced full-sib families that will be used in conjunction with the map to test for regions of the genome associated with survival and fitness. The linkage map provides us with a detailed guide to the pink salmon genome. The Likes Creek families and the facilities at the ASLC provide the opportunity to apply this map to a group of pink salmon families reared in a natural environment.

We have examined the adult fish used to produce these families for length, egg number, and meristic counts for four bilateral traits to measure developmental stability (Leary et al. 1992). The males have significantly greater variance in length than females ($P<0.01$,

Figure 2). Beacham et al. (1988) described a similar result in pink salmon from throughout British Columbia. In addition, males were significantly more asymmetric for the four bilateral traits ($P<0.02$). This is a surprising result because our work with rainbow trout showed that these traits are determined long before sexual maturity. The difference in FA between males and females must then either result from an early effect of gender or from a sex mediated differential survival in the marine environment of fish with different amounts of FA. Many studies have found a negative relationship between fluctuation asymmetry and heterozygosity (Leary et al. 1984). To date none of these studies have looked at differences in this relationship between sexes. We will investigate this relationship within the population and determine when these differences arise.

We will compare our results of the parents genotypes to a sample of 1,000 progeny taken at the time of release into the marine environment. We will test these progeny for a relationship between multiple locus heterozygosity, length and condition factor. We can sex the smolts at release using a sex-linked PCR marker (Appendix 1). This will allow us to determine if morphological differences in length and fluctuating asymmetry are present at this early stage.

In addition to parents and immature offspring, we will also be able to do similar analyses on adults returning in 2000. We will clip the adipose fin of each individual to confirm that our results are not confounded by strays entering the fishway at ASLC. We used single-pair matings and structured our sampling to produce a replicated system in which fitness traits can be measured for both the freshwater and marine phases of the pink salmon life cycle. We have also identified enough polymorphic genetic markers to identify the family of origin for each individual. We can therefore pool individuals into a single tank, eliminating possible tank effects during freshwater rearing. Assigning progeny to families using genotypes also excludes the possibility of differential mortality due to various tagging regimes that might have been necessary to physically identify each fish back to a family.

This is an extremely powerful experimental design that will allow us to measure a multitude of parameters for the first time in pink salmon, salmonid fishes, or any vertebrate in some cases. The most powerful aspect of this experiment will be the capability of measuring fitness for loci spread throughout the genome at multiple points during the life cycle of pink salmon. In the case of males, fitness will be estimated by survivorship (viability) from egg to return at sexual maturity. In the case of females, we will use both survivorship and the number of eggs produced so that we can take into account both viability and fecundity. We will also be able to estimate the heritabilities of a variety of traits (e.g., size at sexual maturity) by parent-offspring regression (Leary et al. 1985).

Perhaps the most significant aspect of the proposed research is the power to detect the effects of natural selection on loci spread throughout the genome. Comparison of genotypes in thousands of fry during the freshwater phase of their life cycle and in this same cohort when they return as adults will allow a powerful test for regions of the genome that affect survival. The failure to detect differential survival would provide

strong evidence for the assumption of selective neutrality of genetic markers used to describe population structure.

We believe that the most likely result is that there will be some regions of the genome associated with differential survival. This experimental design will allow us to determine what proportion of the genome is affected and how strong effect is. We will also be able to test if this differential survival is associated with regions of the genome marked by allozyme loci.

CONCLUSIONS

We have made significant progress toward the goal of consolidating the map with the addition of 31 anchor loci and gene-centromere analysis. The combined data sets from the haploid and diploid gynogens provides a great deal of information on the structure of the pink salmon genome despite the fact that the number of linkage groups exceeds the number of chromosomes. We will continue to add anchor loci on the map to allow comparisons with other maps. In so doing, we expect the number of linkage groups to be further reduced. Despite the fact that the map has not been consolidated it is already useful for assessing other projects such as the description of stock structure in pink salmon.

Our first year of gamete collection and fish rearing at ASLC has been highly successful. We produced over 70,000 fry that we will release in Spring of 1999. Assuming a 4% return we should obtain enough returning adults in August 2000 to begin the analysis of the first generation of this study.

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Personal Communications

Mark Daly, Whitehead Institute, 9 Cambridge Center, Cambridge MA 02142.

Roy Danzmann, Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1 Canada.

Chris Habicht, Alaska Department of Fish and Game, Genetics Program, 333 Raspberry Road, Anchorage AK 99518.

Dennis Hedgecock, University of California, Davis, Bodega Marine Laboratory, Bodega Bay, CA 94923.

Jeffrey B. Olsen, Marine Molecular Biotechnology Laboratory, University of Washington, 3707 Brooklyn Ave. NE, Seattle, WA 98105-6715.

James E. Seeb, Alaska Department of Fish and Game, Genetics Program, 333 Raspberry Road, Anchorage AK 99518.

Noel Weaver, Craighead Institute, Upper Miller Creek Rd. Missoula, MT 59801.

Hongyu Zhao, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06520.

Table 1. Summary of primer pairs screened in haploid and diploid individuals 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
Microsatell	30	100	90[α]
Allozymes	13	100	100
Total	598	96	

[α] *SSA197*, *OGO7*, and *OMY276* could not be scored as a codominant marker due the presence of a null allele in female 95-103 (Appendix 1).

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

Locus	Annealing Temperature (°C)	Reference
*μSAT60**	55(58)	Estoup et al. 1993
*FGT1-1,2**	51(60)	Sakamoto et al. 1994
GH2	70	Forbes et al. 1994
MHC α 1	55	Katagiri et al. 1996
OCl2	56	Condrey et al. 1998
*OGO1C**	60(60)	Olsen 1998
*OGO3**	60(60)	Olsen 1998
*OGO5**	(60)	Olsen 1998
OGO7-1,2	60	Olsen 1998
OGO8	56	Olsen 1998
OMY276-1,2	58-52 †	Danzmann & Ferguson personal communication
OMY301	58-52 †	Danzmann & Ferguson personal communication
OMYFGT25	58-52 †	Danzmann & Sakamoto personal communication
OMYOGT 4	58-52 †	Danzmann & Sakamoto personal communication
OMYRGT1	58-52 †	Danzmann & Sakamoto personal communication
OMYRGT13	58-52 †	Danzmann & Sakamoto personal communication
OMYRGT2	58-52 †	Danzmann & Sakamoto personal communication
OMYRGT43	58-52 †	Danzmann & Sakamoto personal communication
OMYRGT44	58-52 †	Danzmann & Sakamoto personal communication
OMYRGT6	58-52 †	Danzmann & 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. in press
*OTS101**	56(52)	Small et al. 1998
*OTS102**	55(52)	Nelson & Beacham
*OTS103**	55(58)	Small et al. 1998
*SSA197**	57(58)	O'Reilly et al. 1996
SSA20.19	58-52 †	Powell personal communication
SSA293	58-52 †	McConnell et al. 1995a
SSA311	58-52 †	Slettan et al. 1995a
*SSA85**	57(58)	O'Reilly et al. 1996

*Indicates loci analyzed using an ABI 377. Numbers in parentheses indicate annealing temperatures used on samples analyzed with the ABI 377.
†Countdown PCR profile as described in Appendix 2.

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	50.3
11-15	8	122.5
16-20	4	204.7
21-25	4	213.2
26-30	0	-----
31-35	1	354.1
36-40	0	-----
41-45	2	532.8
>50	1	470.4

Table 4. Polymorphic loci, number of alleles present at each locus, the observed heterozygosity (H_o) and the expected heterozygosity (H_e) for 150 adult pink salmon collected from Likes Creek, Resurrection Bay, Alaska.

Locus	Number of Alleles	H_o	H_e	Locus	Number of Alleles	H_o	H_e
ADA2	3	0.120	0.121	*OGO8*	22	0.367	0.368
AK	3	0.027	0.026	*OMY301*	23	0.897	0.843
bGALA	3	0.184	0.180	*ONE3*	3	0.553	0.504
CKA2	3	0.020	0.020	*OTS1*	16	0.913	0.856
CKC1	2	0.014	0.014	*PEPB1*	3	0.240	0.246
FDHG	2	0.020	0.020	*PEPD2*	2	0.673	0.617
G3PDH1	2	0.253	0.270	*PEPLT*	3	0.253	0.261
G3PDH2	3	0.280	0.282	*PGDH*	4	0.490	0.441
G3PDH3	2	0.013	0.013	*PGK2*	2	0.007	0.007
GAPDH2	3	0.020	0.020	*PGM2*	2	0.007	0.007
GH2	2	0.233	0.217	*sAAT3*	2	0.322	0.306
GPIB1,2	2	0.013	0.026	*sAAT4*	3	0.473	0.511
IDDH1	2	0.067	0.065	*sIDHP1*	2	0.007	0.077
LDHB2	2	0.020	0.020	*sIDHP2*	3	0.367	0.453
mAAT1	2	0.007	0.007	*sMDHA1,2*	3	0.033	0.033
mAAT2	2	0.033	0.033	*sMDHB1,2*	3	0.033	0.033
mAH1	2	0.007	0.007	*SSA14*	5	0.087	0.096
mAH4	2	0.067	0.065	*SSA20.19-1*	2	0.053	0.052
MHC	2	0.523	0.497	*SSA20.19-2*	3	0.347	0.316
mMEP1	2	0.480	0.466	*STR60-2*	5	0.277	0.257
mSOD	3	0.013	0.013	*TPI2*	2	0.013	0.013
OCL2	6	0.367	0.380	*TPI4*	2	0.013	0.013

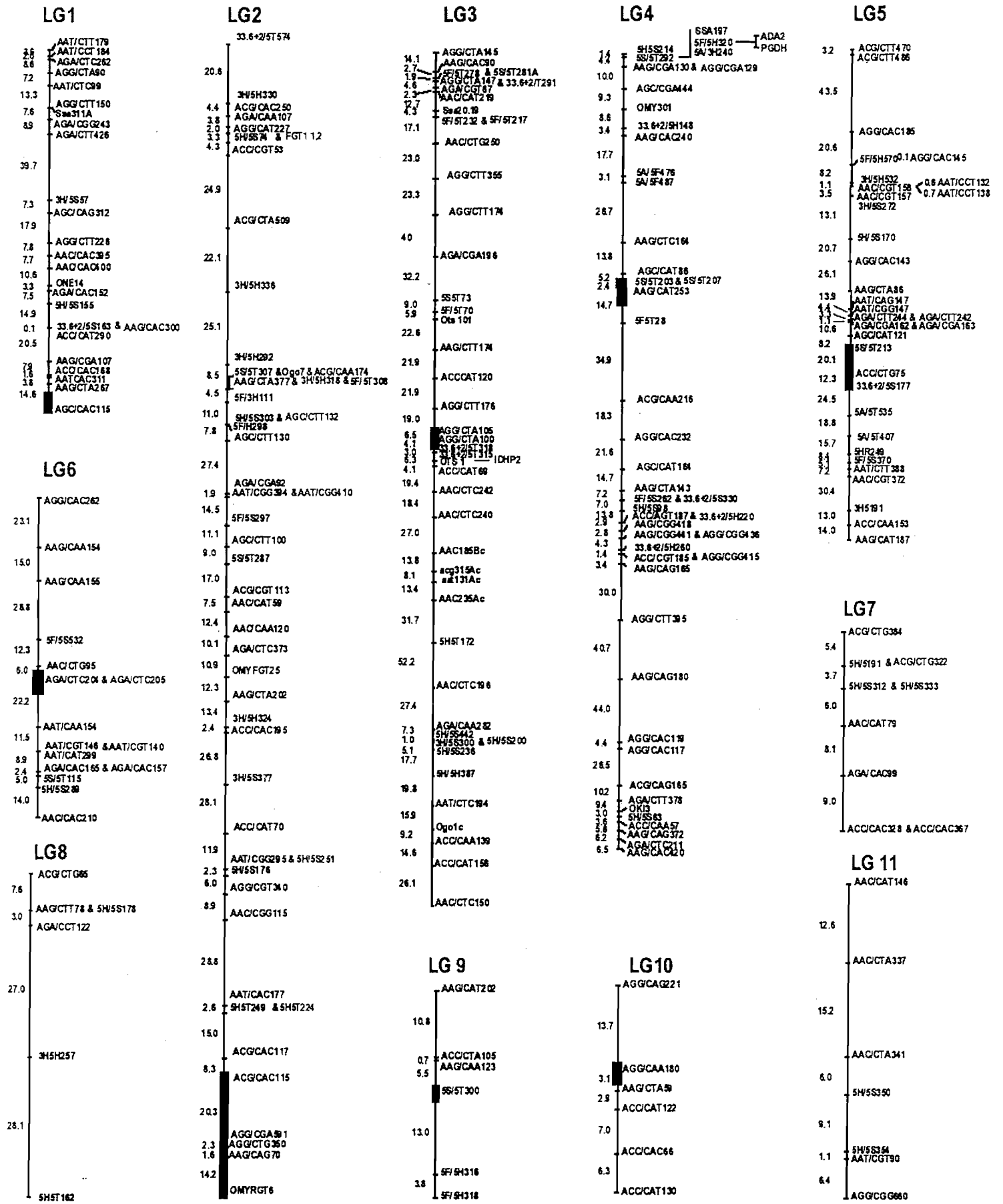
Table 5. Summary of linkages in normal diploid families for allozymes and microsatellites.

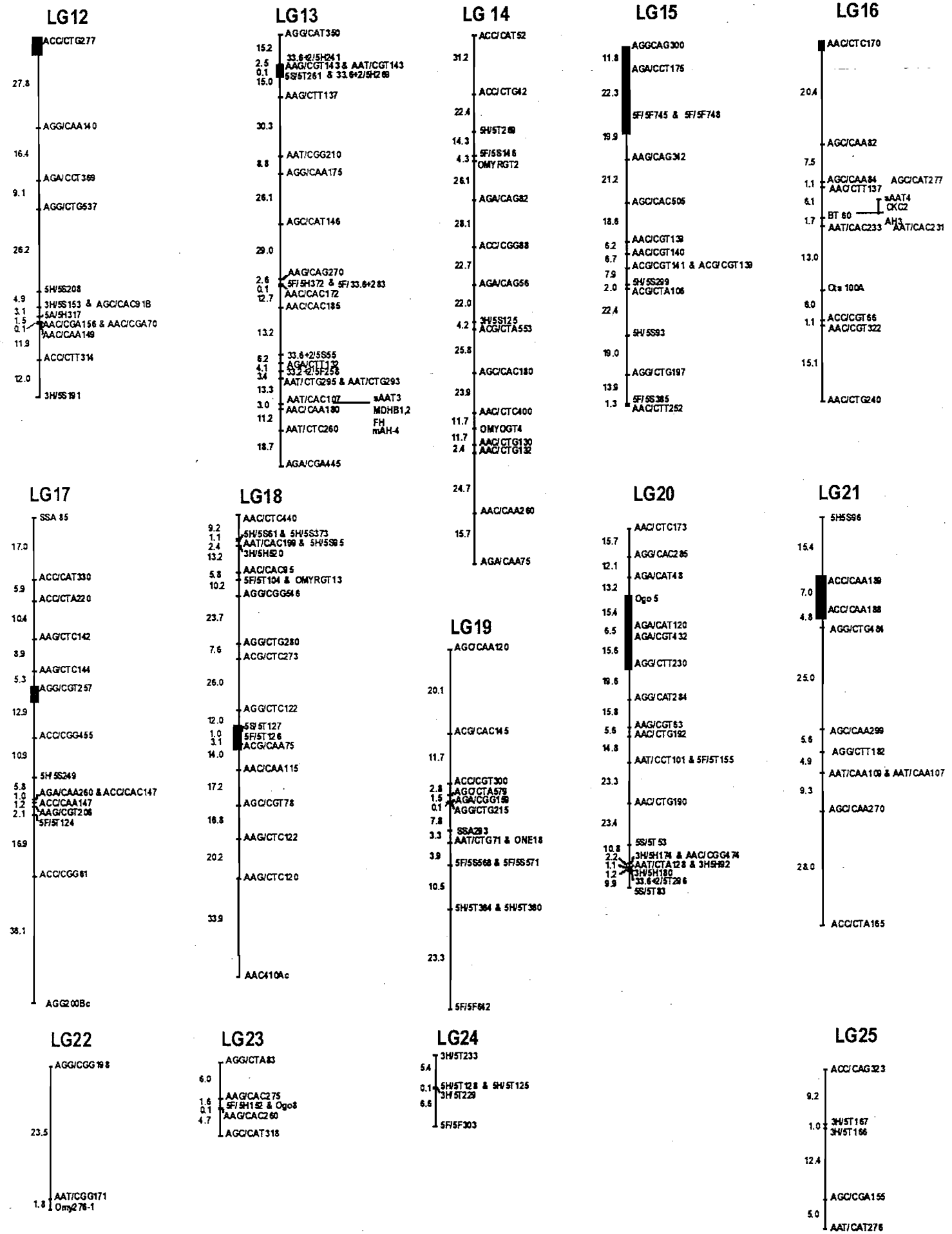
Loci	Fam	Inform. Parent	N	r	Chi-square (1 df)
sAAT3 - FH	A14	Fem	86	0.337	9.12
sAAT3 - sMDHB1,2	A14	Fem	89	0.112	53.49
sAAT4 - STR60	A104	Fem	21	0.238	5.76
ADA2 - PGDH	A120	Mal	56	0.125	31.50
ADA2 - SSA197	A103	Fem	42	0.024	38.10
	A120	Mal	18	0.111	10.89
CKC2 - STR60	A103	Fem	46	0.348	4.26
FH - MDHB1,2	A14	Fem	86	0.291	15.07
bGALA - G3PDH1	V2	Mal	75	0.346	7.05
GDA1 - PEPD2	A8	Mal	82	0.012	78.05
	A20	Mal	95	0.105	59.21
	A29	Mal	45	0.000	45.00
G3PDH1 - PEPLT	V5	Mal	75	0.240	20.28
GPIB1,2 - PEPD2	V2	Mal	75	0.013	71.05
sIDHP2 - OTS1	A29	Mal	41	0.366	2.95
	A104	Fem	33	0.303	5.12
PGDH - SSA197	A120	Mal	20	0.050	16.20

Table 6. Genotypes for *MHCα1* in adult pink salmon from Likes Creek.

	MHCα1 Parental Genotypes				
	264/264	*264/270*	*270/270*	Freq(**264*)	F_{IS}
Females	21	27	27	0.46	+ 0.28*
Males	7	51	16	0.44	- 0.39*
Total	28	78	43	0.45	- 0.05

Figure 1. Genetic linkage map of pink salmon based on the inheritance of 598 polymorphic loci. Numbers to the left indicate recombination rates (cM). Locus names are to the right. Centromeres are indicated by black rectangles.




LG12
ACC/CTG277
27.8
AGG/CAA140
16.4
AGA/CCT369
9.1
AGG/CTG537
26.2
5H/5S208
4.9
3H/5S153 & AGC/CAC91B
3.1
5A/5H317
1.5
AAC/CGA156 & AAC/CGA70
0.1
AAC/CAA149
11.9
ACC/CTT314
12.0
3H/5S191
LG13
AGG/CAT350
15.2
33.6+2/5H241
2.5
AAG/CGT143 & AAT/CGT143
0.1
5S/5T261 & 33.6+2/5H269
15.0
AAG/CTT137
30.3
AAT/CGG210
8.8
AGG/CAA175
26.1
AGC/CAT146
29.0
AAG/CAG270
2.6
5F/5H372 & 5F/33.6+283
0.1
AAC/CAC172
12.7
AAC/CAC185
13.2
33.6+2/5S55
6.2
AGA/CTT132
4.1
33.2+2/5F258
3.4
AAT/CTG295 & AAT/CTG293
13.3
AAT/CAC107
sAAT3
3.0
AAC/CAA180
MDHB1,2
11.2
AAT/CTC260
FH
mAH-4
18.7
AGA/CGA445
LG 14
ACC/CAT52
31.2
ACC/CTG42
22.4
5H/5T289
14.3
5F/5S146
4.3
OMYRGT2
26.1
AGA/CAG82
28.1
ACC/CGG88
22.7
AGA/CAG56
22.0
3H/5S125
4.2
ACG/CTA553
25.8
AGC/CAC180
23.9
AAC/CTC400
11.7
OMYOGT4
11.7
AAC/CTG130
2.4
AAC/CTG132
24.7
AAC/CAA260
15.7
AGA/CAA75
LG15
AGGCAG300
11.8
AGA/CCT175
22.3
5F/5F745 & 5F/5F748
19.9
AAG/CAG342
21.2
AGC/CAC505
18.6
AAC/CGT139
6.2
AAC/CGT140
6.7
ACG/CGT141 & ACG/CGT139
7.9
5H/5S299
2.0
ACG/CTA106
22.4
5H/5S93
19.0
AGG/CTG197
13.9
5F/5S385
1.3
AAC/CTT252
LG16
AAC/CTC170
20.4
AGC/CAA82
7.5
AGC/CAA84
AGC/CAT277
1.1
AAC/CTT137
6.1
sAAT4
CKC2
BT 60
1.7
AAT/CAC233
AH3
AAT/CAC231
13.0
Ots 100A
8.0
ACC/CGT66
1.1
AAC/CGT322
15.1
AAC/CTG240
LG17
SSA 85
17.0
ACC/CAT330
5.9
ACC/CTA220
10.4
AAG/CTC142
8.9
AAG/CTC144
5.3
AGG/CGT257
12.9
ACC/CGG455
10.9
5H/5S249
5.8
AGA/CAA260 & ACC/CAC147
1.0
ACC/CAA147
1.2
AAG/CGT206
2.1
5F/5T124
16.9
ACC/CGG61
38.1
AGG200Bc
LG18
AAC/CTC440
9.2
5H/5S61 & 5H/5S373
1.1
AAT/CAC199 & 5H/5S85
2.4
3H/5H520
13.2
AAC/CAC95
5.8
5F/5T104 & OMYRGT13
10.2
AGG/CGG546
23.7
AGG/CTG280
7.6
ACG/CTC273
26.0
AGG/CTC122
12.0
5S/5T127
1.0
5F/5T126
3.1
ACG/CAA75
14.0
AAC/CAA115
17.2
AGC/CGT78
16.8
AAG/CTC122
20.2
AAG/CTC120
33.9
AAC410Ac
LG19
AGC/CAA120
20.1
ACG/CAC145
11.7
ACC/CGT300
2.8
AGC/CTA578
1.5
AGA/CGG159
0.1
AGG/CTG215
7.8
SSA293
3.3
AAT/CTG71 & ONE18
3.9
5F/5S568 & 5F/5S571
10.5
5H/5T384 & 5H/5T380
23.3
5F/5F642
LG20
AAC/CTC173
15.7
AGG/CAC285
12.1
AGA/CAT48
13.2
Ogo 5
15.4
AGA/CAT120
6.5
AGA/CGT432
15.6
AGG/CTT230
19.6
AGG/CAT284
15.8
AAG/CGT63
5.6
AAC/CTG192
14.8
AAT/CCT101 & 5F/5T155
23.3
AAC/CTG190
23.4
5S/5T53
10.8
3H/5H174 & AAC/CGG474
2.2
AAT/CTA128 & 3H/5H92
1.1
3H/5H180
1.2
33.6+2/5T296
9.9
5S/5T83
LG21
5H5S96
15.4
ACC/CAA189
7.0
ACC/CAA188
4.8
AGG/CTG486
25.0
AGC/CAA299
5.6
AGG/CTT182
4.9
AAT/CAA109 & AAT/CAA107
9.3
AGC/CAA270
28.0
ACC/CTA165
LG22
AGG/CGG198
23.5
AAT/CGG171
1.8
Omy276-1
LG23
AGG/CTA83
6.0
AAG/CAC275
1.6
5F/5H152 & Ogo8
0.1
AAG/CAC260
4.7
AGC/CAT318
LG24
3H/5T233
5.4
5H/5T128 & 5H/5T125
0.1
3H/5T229
6.6
5F/5F303
LG25
ACC/CAG323
9.2
3H/5T167
1.0
3H/5T166
12.4
AGC/CGA155
5.0
AAT/CAT276

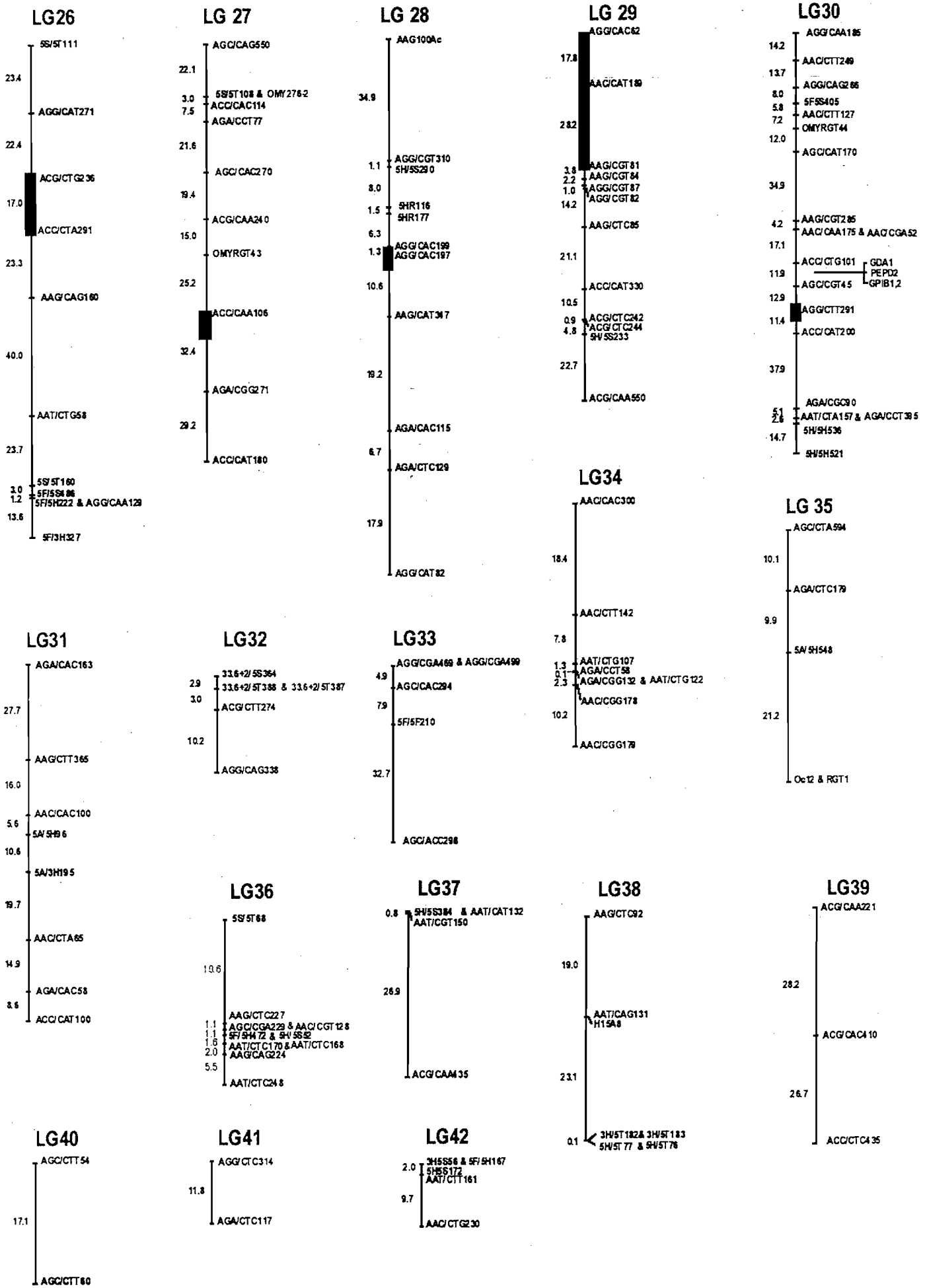

LG26
5S/5T111
23.4
AGG/CAT271
22.4
ACG/CTG236
17.0
ACC/CTA291
23.3
AAG/CAG160
40.0
AAT/CTG58
23.7
5S/5T160
3.0
5F/5S486
1.2
5F/5H222 & AGG/CAA129
13.6
5F/3H327
LG 27
AGC/CAG550
22.1
5S/5T108 & OMY2762
3.0
ACC/CAC114
7.5
AGA/CCT77
21.6
AGC/CAC270
19.4
ACG/CAA240
15.0
OMYRGT43
25.2
ACC/CAA106
32.4
AGA/CGG271
29.2
ACC/CAT180
LG 28
AAG100Ac
34.9
AGG/CGT310
1.1
5H/5S290
8.0
5HR116
1.5
5HR177
6.3
AGG/CAC199
1.3
AGG/CAC197
10.6
AAG/CAT317
19.2
AGA/CAC115
6.7
AGA/CTC129
17.9
AGG/CAT82
LG 29
AGG/CAC62
17.8
AAC/CAT189
28.2
AAG/CGT81
3.8
AAG/CGT84
2.2
AGG/CGT87
1.0
AGG/CGT82
14.2
AAG/CTC85
21.1
ACC/CAT330
10.5
ACG/CTC242
0.9
ACG/CTC244
4.8
5H/5S233
22.7
ACG/CAA550
LG30
AGG/CAA185
14.2
AAC/CTT249
13.7
AGG/CAG266
8.0
5F5S405
5.8
AAC/CTT127
7.2
OMYRGT44
12.0
AGC/CAT170
34.9
AAG/CGT285
4.2
AAC/CAA175 & AAC/CGA52
17.1
ACC/CTG101
11.9
GDA1
PEP02
GPIB1,2
AGC/CGT45
12.9
AGG/CTT291
11.4
ACC/CAT200
37.9
AGA/CGC90
5.1
AAT/CTA157 & AGA/CCT385
2.6
5H/5H536
14.7
5H/5H521
LG34
AAC/CAC300
18.4
AAC/CTT142
7.8
AAT/CTG107
1.3
AGA/CCT58
0.1
AGA/CGG132 & AAT/CTG122
2.3
AAC/CGG178
10.2
AAC/CGG179
LG 35
AGC/CTA594
10.1
AGA/CTC179
9.9
5A/5H548
21.2
Oc12 & RGT1
LG31
AGA/CAC163
27.7
AAG/CTT365
16.0
AAC/CAC100
5.6
5A/5H96
10.6
5A/3H195
19.7
AAC/CTA65
14.9
AGA/CAC58
8.6
ACC/CAT100
LG32
33.6+2/5S364
2.9
33.6+2/5T388 & 33.6+2/5T387
3.0
ACG/CTT274
10.2
AGG/CAG338
LG33
AGG/CGA469 & AGG/CGA499
4.9
AGC/CAC294
7.9
5F/5F210
32.7
AGC/ACC298
LG36
5S/5T68
10.6
AAG/CTC227
1.1
AGC/CGA229 & AAC/CGT128
1.1
5F/5H472 & 5H/5S52
1.6
AAT/CTC170 & AAT/CTC168
2.0
AAG/CAG224
5.5
AAT/CTC248
LG37
5H/5S384 & AAT/CAT132
0.8
AAT/CGT150
26.9
ACG/CAA435
LG38
AAG/CTC92
19.0
AAT/CAG131
H15A8
23.1
3H/5T182& 3H/5T183
0.1
5H/5T77 & 5H/5T76
LG39
ACG/CAA221
28.2
ACG/CAC410
26.7
ACC/CTC435
LG40
AGC/CTT54
17.1
AGC/CTT60
LG41
AGG/CTC314
11.8
AGA/CTC117
LG42
3H5S56 & 5F/5H167
2.0
5H5S172
AAT/CTT161
9.7
AAC/CTG230

Figure 2. Length of pink salmon collected from Likes Creek.

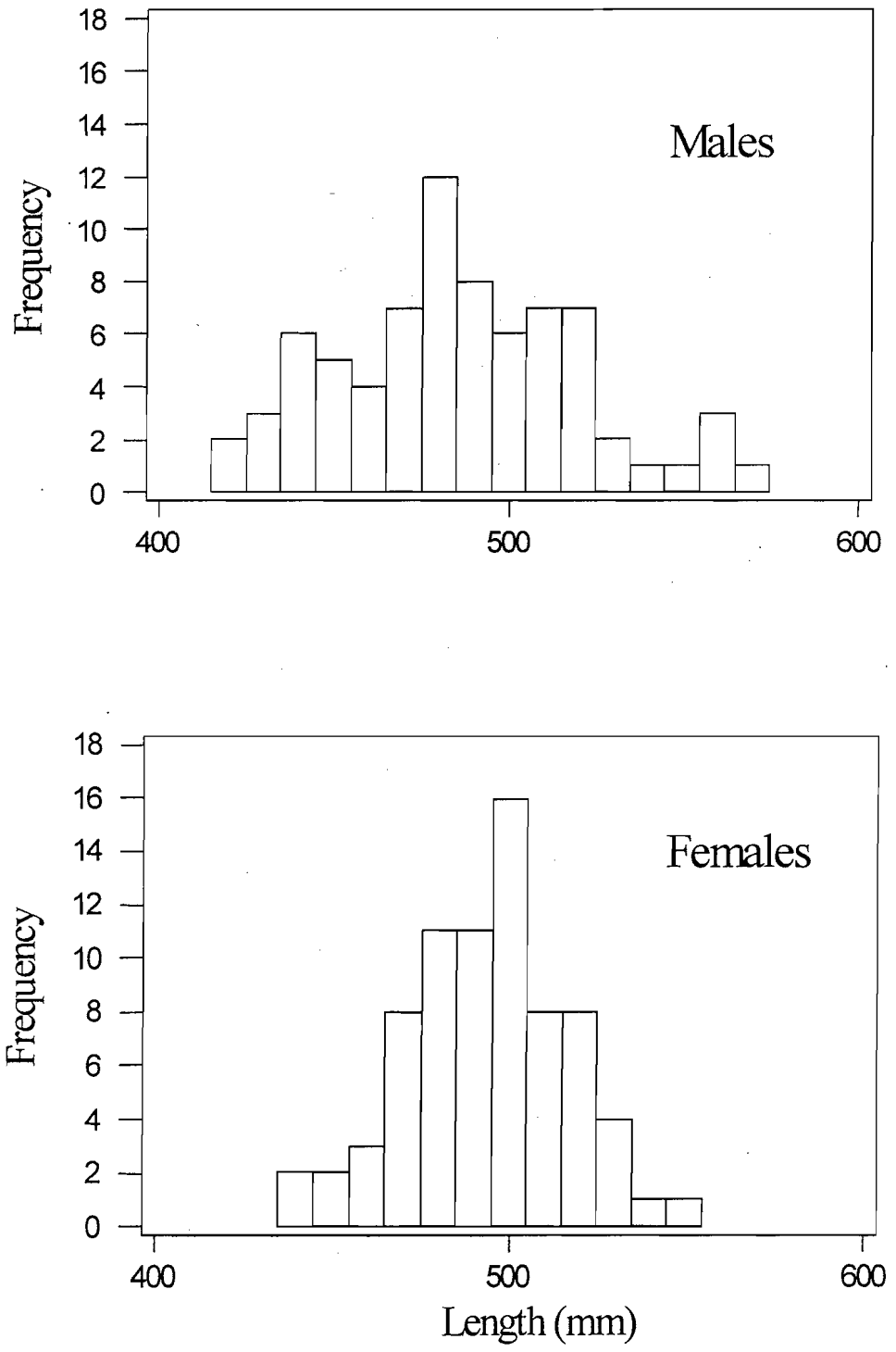
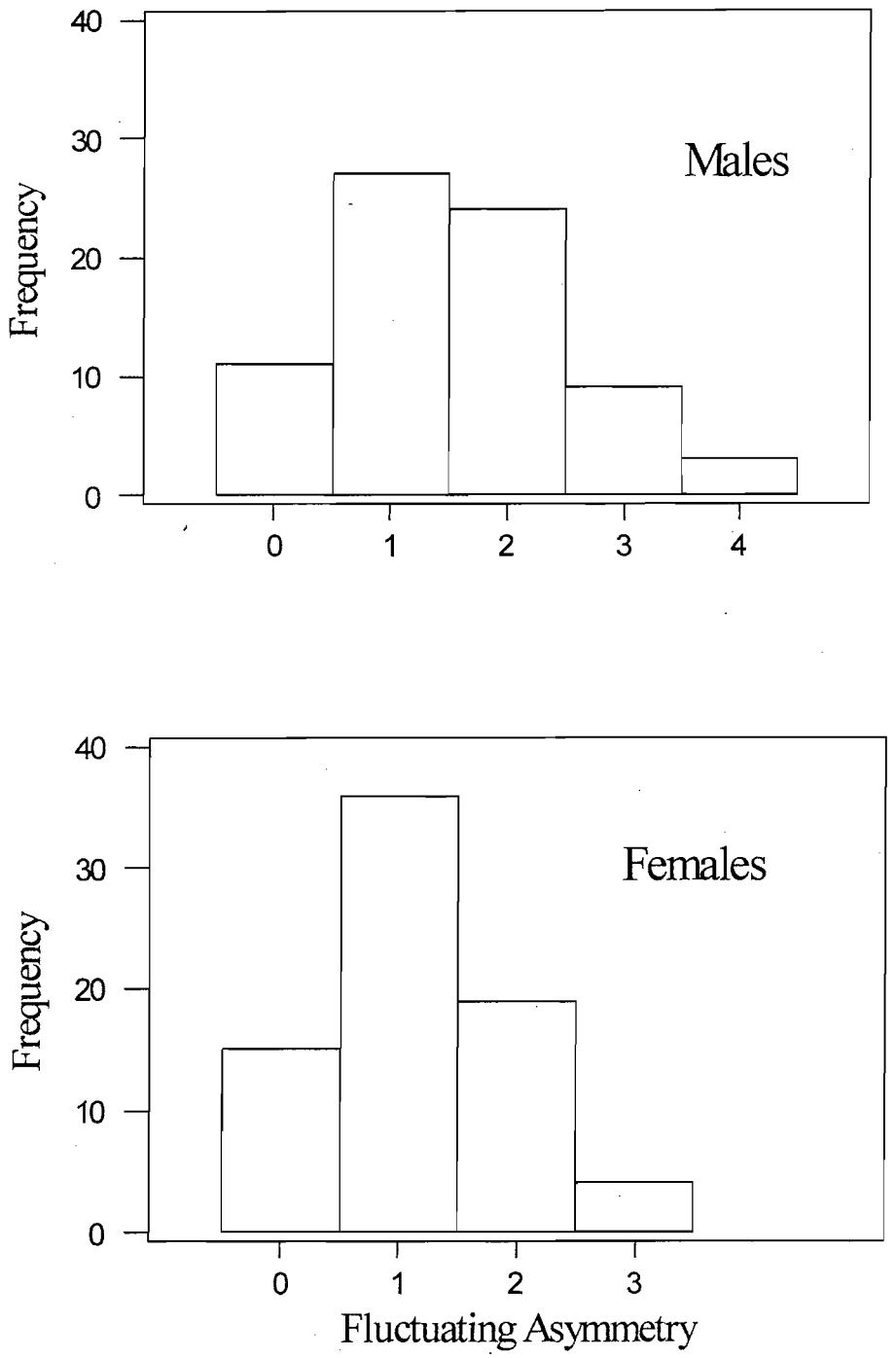


Figure 3. Number of asymmetric characters in pink salmon collected from Likes Creek.



APPENDIX 1

Inheritance of Nuclear DNA Markers in Gynogenetic Haploid Pink Salmon

P. Spruell, K. L. Pilgrim, B. A. Greene, C. Habicht, K. L. Knudsen, K. R. Lindner, J. B. Olsen, G. K. Sage, J. E. Seeb, and F. W. Allendorf

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

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

The polyploid derived genome of salmonids has resulted in complex patterns of segregation and inheritance that have been revealed by the investigation of isozyme loci. Only disomic inheritance has been reported in females. Most loci in males are also inherited disomically. However, some loci show variable patterns of segregation in males, ranging from disomic ratios in some populations to tetrasomic ratios in other populations (Allendorf and Danzmann 1997). The residual tetrasomic inheritance observed in males apparently results from a two-stage pattern of pairing during male meiosis in which homologous chromosomes pair first followed by homeologous pairing. Disjunction of paired chromosomes occurs so that homologs segregate at the first meiotic division in males. Recombination events between homeologs produce segregation ratios approaching tetrasomic expectations for loci that are distant from their centromere and therefore more likely to be exchanged between homeologs.

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

From the Division of Biological Sciences, University of Montana, Missoula, MT 59812 (Spruell, Pilgrim, Knudsen, Lindner, Sage, and Allendorf), Alaska Department of Fish and Game, Anchorage, Alaska (Greene, Habicht, and Seeb), and School of Fisheries, University of Washington, Seattle, Washington (Olsen). B. A. Greene and G. K. Sage are currently at the United States Geological Survey, Anchorage, Alaska. Address correspondence to Paul Spruell at the address above or e-mail: spruell@seiway.umt.edu. This research was supported by the *Exxon Valdez* Oil Spill Trustee Council (96160) and the National Science Foundation (DEB-9300135). We thank Robert H. Devlin for helpful suggestions on the use of the growth hormone pseudogene primers and generously sharing his unpublished primer sequences, Joan Strange for her assistance in sequence analysis, David Arsenault for technical assistance, and the Prince William Sound Aquaculture Corporation and the Valdez Fisheries Development Association for providing adult fish and gametes.

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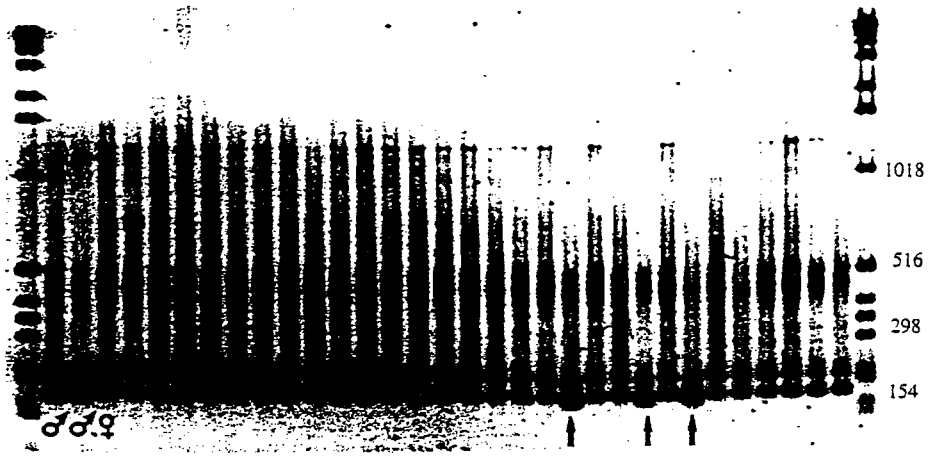

Figure 1. Growth hormone pseudogene amplification products separated in a 2% agarose gel. Individuals of known sex are indicated by the symbols on the bottom left of the gel. The arrows along the bottom of the gel indicate males that were detected in family 95-103.

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

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

Microsatellites

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

Randomly Amplified Polymorphic DNA (RAPDs)

RAPD fragments were amplified in a total reaction volume of 10 μl consisting of 10 ng of genomic DNA, 6.7 mM RAPD primer (Operon Technology Inc., Alameda, CA), 4.0 mM $MgCl_2$, 0.2 mM of each dNTP, 1× Stoffel buffer, and 0.25 U Amplitaq DNA polymerase Stoffel fragment (Perkin-Elmer, Norwalk, CT). Thermal cycling was performed in an MJ Research PTC-200 DNA engine. Two cycles of higher stringency PCR were performed with the following thermal profile: denaturation at 96°C for 5 s, annealing at 40°C for 20 s, and extension at 72°C for 30 s. This was followed by 43 cycles with an annealing temperature of 36°C for 20 s and a final extension at 72°C for 2 min. PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized with a Hitachi FMBIO-100 fluorescent imager.

Amplified Fragment Length Polymorphisms (AFLPs)

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

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

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
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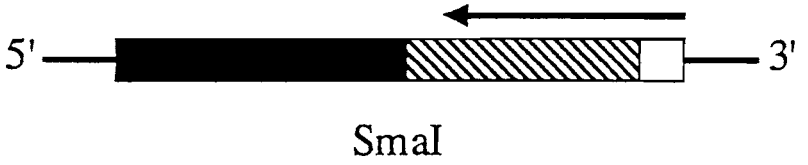

Figure 2. Aligned sequences of *GH-2* intron C from sockeye (Devlin 1993) and pink salmon. The 81 bp insert found in *GH2*C527* is indicated by the dark bars in the sequence. The complete *SmaI* element is shown below the sequences. The region that corresponds to the 81 bp insert and its orientation 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).

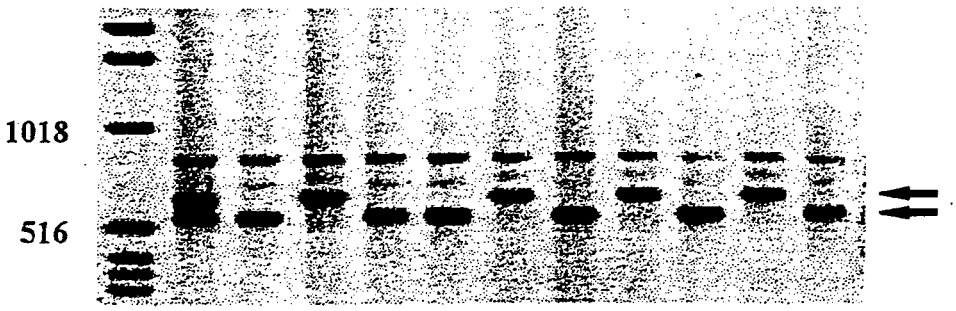

Figure 3. Segregation of *GH-2* polymorphism. The individual denoted by the female symbol is female 95-105, the 10 individuals to the right are haploid offspring from this female segregating for the *GH-2*C446* and **C527* alleles indicated by the arrows.

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

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

Locus	No. individuals	No. alleles	Heterozygosity		
			Observed	Expected	*F*
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

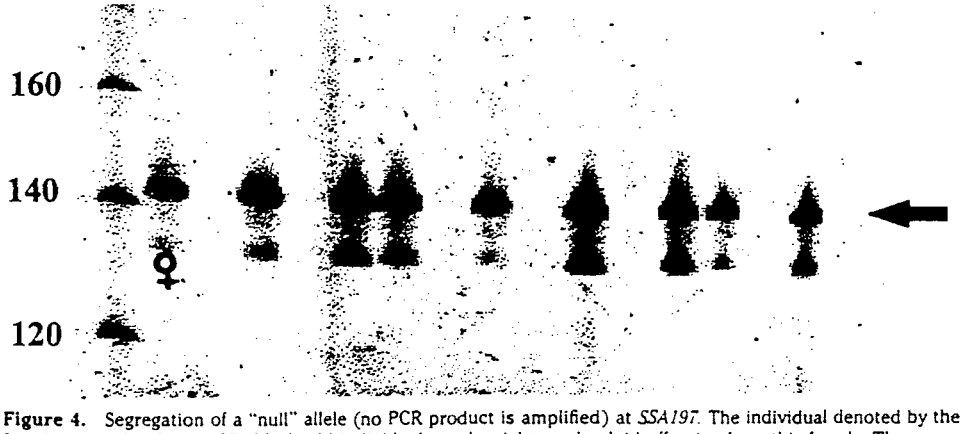

Figure 4. Segregation of a "null" allele (no PCR product is amplified) at *SSA197*. The individual denoted by the female symbol is female 95-106, the 14 individuals to the right are haploid offspring from this female. The arrow indicates allele *SSA197*142*.

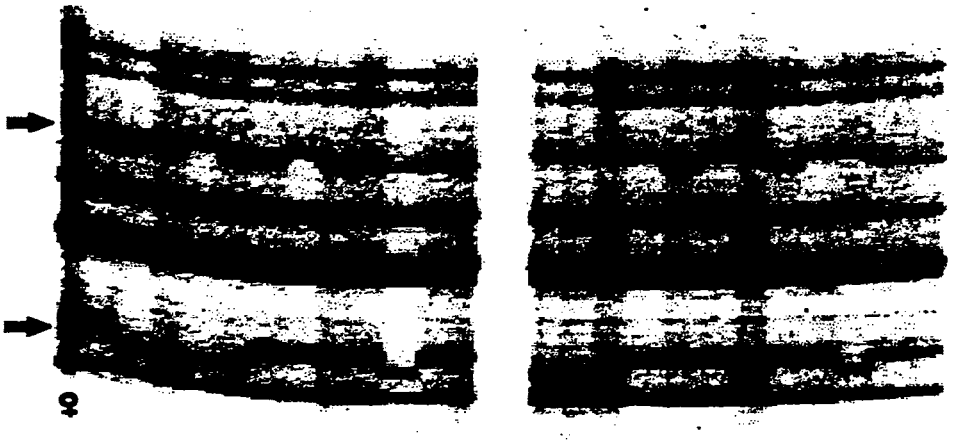

Figure 5. Hpa 3' and Tc1 PINE primer amplification products separated on a 4.5% polyacrylamide gel. Female 95-103 is indicated. The two arrows along the left side of the gel indicate length polymorphisms: top bands 181/182 bp, bottom bands 166/167 bp.

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.

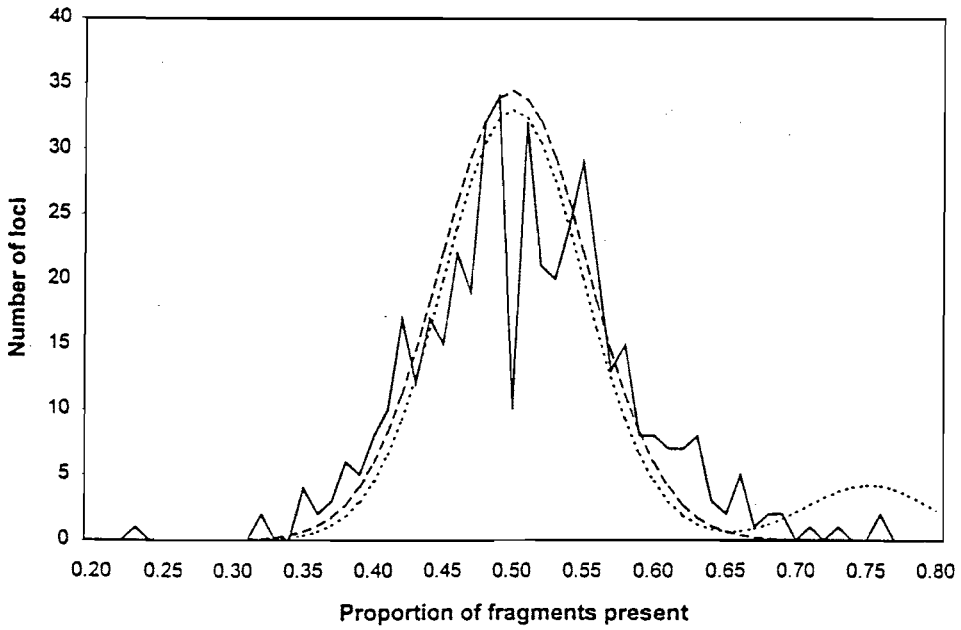

Figure 6. Distribution of segregation ratios for 451 fragments in haploid progeny from female 95-103 (solid line). The dashed line is the expected binomial distribution with an expected value of 0.5 (1:1 segregation). The dotted line is the expected binomial distribution for 90% of the fragments segregating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

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APPENDIX 2

Submitted

Genome

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

K. R. Lindner*, J. E. Seeb†, C. Habicht†, K. L. Knudsen*, E. Kretschmer†, D. J. Reedy*, P. Spruell*, and F. W. Allendorf*[1]

*Division of Biological Sciences, University of Montana, Missoula, MT 59812, and †Alaska Department of Fish and Game, Anchorage, AK 99518

[1] Author to whom all correspondence should be addressed (e-mail:darwin@selway.umt.edu).

Phone (406) 243-5503; fax (406) 243-4184.

Results

Codominant loci

The genotypes of gynogenetic diploid progeny at nine codominant allozyme and 31 codominant microsatellite loci are given in Tables 2 and 3, respectively. In addition, Table 4 presents the 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 (Table 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 detected before the frequencies of the two homozygous classes can be significantly different ($P<0.05$) using the exact binomial test. 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 no evidence for differences in the proportion of heterozygotes between families. Only one locus (*OGO2*) showed a significant difference between families based on contingency chi-square analysis. However, this difference is not significant if corrected for the 16 independent simultaneous tests (Rice 1989).

The frequency of heterozygotes is expected to vary according to the frequency of second meiotic division segregation (y). Gene-centromere map distances can be estimated by $(\frac{1}{2})y$, assuming complete interference. Seven of the 52 codominant loci had y values of 1.00 which will occur only when there is exactly one crossover between the

Abstract: We estimated recombination rates between 312 loci and their centromeres in gynogenetic diploid pink salmon (*Oncorhynchus gorbuscha*). Gynogenetic diploids are produced by initiating development with irradiated sperm and blocking the maternal second meiotic division. The proportion of heterozygous gynogenetic diploid progeny is a measure of the frequency of second division segregation (y) and thus, an estimate of the gene-centromere distance. A large proportion of loci had y values approaching 1.0, indicating near complete crossover interference on many chromosome arms. We compared the chromosomal distribution of loci produced using four techniques. Amplified fragment length polymorphisms (AFLPs) were significantly more centromeric than loci identified by the other techniques. 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. In addition, we compared the gene-centromere distances in reproductively isolated odd- and even-year pink salmon and found no differences.

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

Introduction

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 0.66, unless there is chiasma interference inhibiting subsequent crossovers and resulting in y values greater than 0.66. The presence of strong chiasma interference has been reported in salmonids (Thorgaard et al. 1983; Allendorf et al. 1986; May & Johnson 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 in a wide variety of organisms because of their relative technical ease and reproducibility. The ability to use different AFLP primer

combinations allows a nearly unlimited supply of markers. Nevertheless, recent evidence suggests that 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 (*Oncorhynchus 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. Yet, if the crossover nearest the centromere determines the pattern of disjunction (Burnham 1962), then each gamete would receive one copy of each homolog, allowing continued disomic segregation in females.

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

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. 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 and Smoker (1991) 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 amplified fragment length polymorphisms (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, Alaska.

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. Enzyme electrophoresis followed Aebersold et al. (1987); tissues were stored at -80 °C until analysis. DNA was isolated using the Puregene(TM) DNA isolation kit (Gentra Systems Inc.).

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. Female triploids and diploids resulting 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.

Amplified fragment length polymorphisms (AFLPs) and paired interspersed nuclear elements (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 (TGG AGG AGG GCT GGA GGA GGG CGC, 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 hatchery in 1995. Nomenclature for allozyme loci follows Shaklee et al. (1990) and Seeb et al. (1998). Genetic nomenclature for microsatellites, AFLPs, and PINEs follows Spruell et al. (1999).

locus and its centromere (i.e., no double-crossovers). 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 have 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 progeny (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.

The distribution of y for 164 AFLP loci and 93 PINE loci is presented in Figure 1. This figure also includes the four AFLP and eight PINE loci that are codominant as discussed in the previous section. In addition, 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 at this locus with other females (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 adults had more than two different PCR products consistent with the same alleles occurring at both loci using the OGO7 primers. 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. 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 consistent with the same alleles occurring at both loci using the OMYFGT276 primers. 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. 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 (Spruell et al. 1999) and sockeye salmon (Allendorf and Seeb, in press). Figure 2 shows the electropherograms of individuals having different number 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 3). 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. 2a and 2b, 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. 2c; 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 (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 (206bp - 270 bp) at the second locus. Approximately 60% of the gynogenetic diploid progeny were heterozygous at *μSAT60-1* (Table 3).

Distribution of markers

Figure 1 shows the distribution of y values for PINEs, AFLPs, and microsatellites. The y values in PINEs and microsatellites have the same distribution with a peak greater than

0.90. The few allozyme loci appear to 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 centromeric 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 the distribution of 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 from the distribution of both PINEs and microsatellites ($P<0.001$). None of the other comparisons between marker types are significantly different.

Discussion

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 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). 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 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 0.66. 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.

Duplicated loci

All three pairs of isoloci had y-values that were greater than 0.90. The telomeric location of these loci supports the previous model for residual tetrasomic inheritance (Allendorf and Thorgaard 1984). Homeologous pairing of chromosomes at distal loci apparently enables 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*. *uSAT60-1* had an average *y* value of 0.58 in two families; we did not map *uSAT60-2*. 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 amplification 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 *uSAT60-1*. 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

The distribution of proportions of heterozygotes in gynogenetic diploid progeny (y) for all loci indicates that markers are distributed along the length of the chromosomes. However, marker types are distributed differently. AFLP loci are much more centromeric than the other classes of loci (Fig. 1). 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 clustering of 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 50cM from the centromere will be assigned a y of 1.0 using half-tetrad analysis if there is complete crossover interference; this will cause a clustering of all distal loci.

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

Suppression of recombination in centromeric regions has been well documented. Roberts (1965) first described this phenomenon in *Drosophila* and estimated a reduction in recombination of up to 40% around the centromere. More recently, Tanksley et al. (1992) observed clustering of markers on a linkage map of tomato and concluded that this was due to a ten-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 the clustering of AFLPs in centromeric regions relative to the other marker types (Figure 1). Linkage analysis 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 and Johnson 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. (in press) 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 (Figure 1). 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 *Tc1* 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 a y greater than 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 centromeric clustering of *Eco*RI-based AFLPs appears to be common and may limit the utility of these markers for mapping distal regions of chromosomes. Centromeric clustering of AFLPs may also reduce the likelihood of identifying quantitative trait loci using this technique. However, based on the results of Young et al.

(in press), other restriction enzymes could be used to generate AFLPs that are not clustered and may be preferentially located in gene-rich regions.

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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 et al. 1998
*OGO1C**	60(60)	Olsen 1998
*OGO2**	(60)	Olsen 1998
*OGO3**	(60)	Olsen 1998
*OGO4**	(60)	Olsen 1998
*OGO5**	64(60)	Olsen 1998
OGO7-1,2	60	Olsen 1998
OGO8	56	Olsen 1998
OKI3	54-40 †	Smith et al. 1998
OMY276-1,2	58-52 †	Danzmann & Ferguson personal communication
OMY301	58-52 †	Sakamoto et al. submitted #
OMYFGT25	58-52 †	Sakamoto et al. submitted #
OMYOGT 4	58-52 †	Sakamoto et al. submitted #
OMYRGT1	58-52 †	Sakamoto et al. submitted #
OMYRGT2	58-52 †	Sakamoto et al. submitted #
OMYRGT6	58-52 †	Sakamoto et al. submitted #
OMYRGT13	58-52 †	Sakamoto et al. submitted #
OMYRGT44	58-52 †	Danzmann & 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 & 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. 1995

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

† Countdown PCR profile as described in text.

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

Locus	Family	Maternal Genotype	Progeny *11*	Progeny *12*	Progeny *22*	Proportion heterozygotes *(y)*	Chi-square* (df)
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 (4)
PEPB1	V96-13	*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 (8)
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 (4)
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 (4)

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

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

Locus	Family	Maternal Genotype	Progeny			Proportion heterozygotes	Chi-square* (df)
			11	*12*	*22*	*(y)*	
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 (6)
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 (8)
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 (6)
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 (6)
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 (2)

Locus	Family	Maternal Genotype	Progeny			Proportion heterozygotes	Chi-square* (df)
			11	*12*	*22*	*(y)*	
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
ONEu3	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 (8)
ONEu14	A95-103	*212/228*	1	63	2	0.95	0.33
ONEu18	A95-103	*262/290*	3	59	7	0.86	1.60

Locus	Family	Maternal Genotype	Progeny			Proportion heterozygotes	Chi-square* (df)
			11	*12*	*22*	*(y)*	
OTS1	A95-29	*236/246*	14	7	11	0.22	0.36
	A95-103	*232/246*	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 (10)
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 (4)
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 (2)
OTS103	V96-13	*238/258*	9	35	2	0.76	4.45
uSAT60-1	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 (2)
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 (8)

Locus	Family	Maternal Genotype	Progeny			Proportion heterozygotes	
			11	*12*	*22*	*(y)*	Chi-square* (df)
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 (6)
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 df). Chi-square value in the total row is contingency chi-square for difference in y between families.

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

Locus	Maternal Genotype	Progeny			Proportion heterozygotes	Chi-square*
		11	*12*	*22*	*(y)*	
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

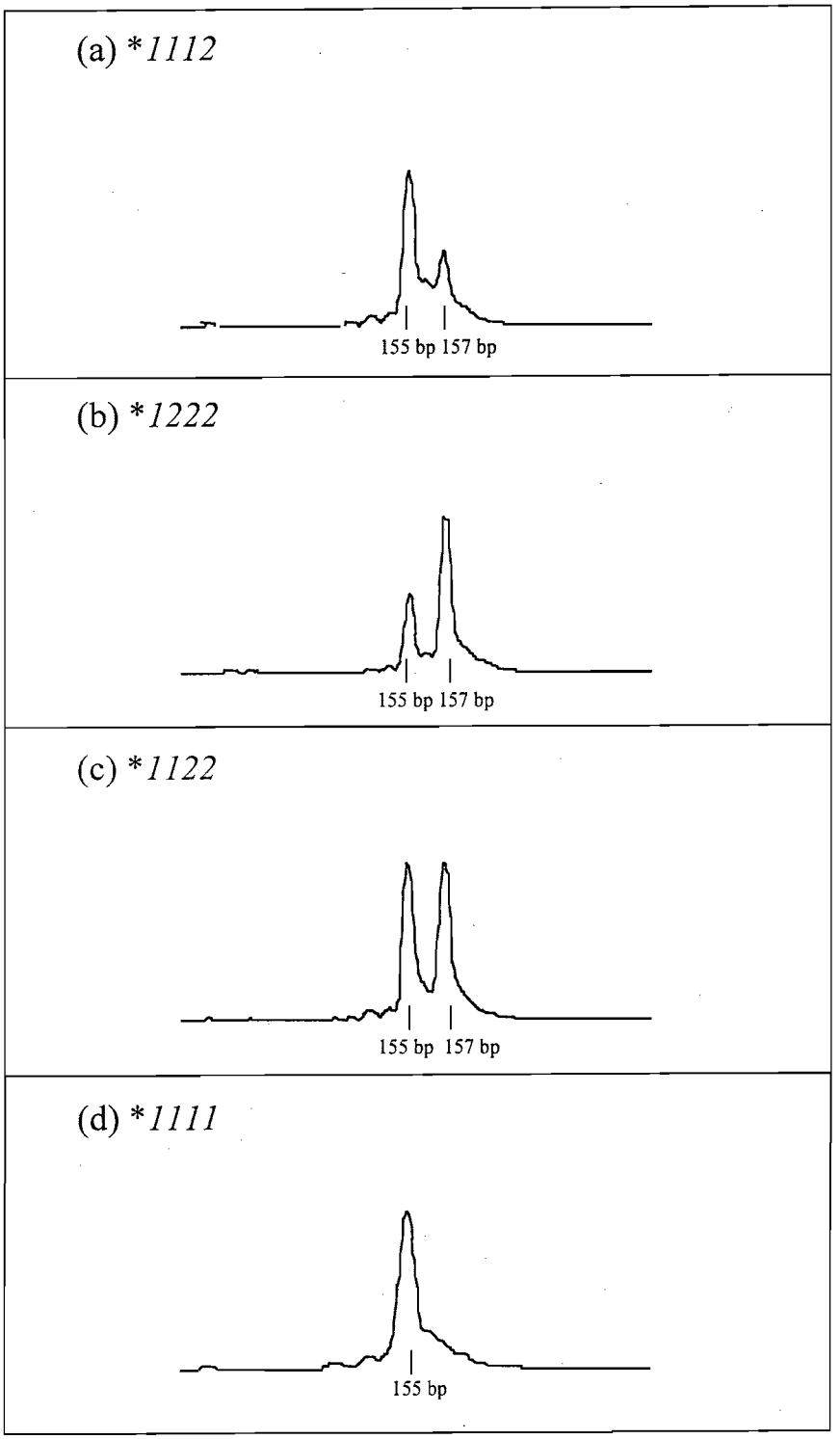
*Chi-square test for equal numbers of homozygotes (1 df).

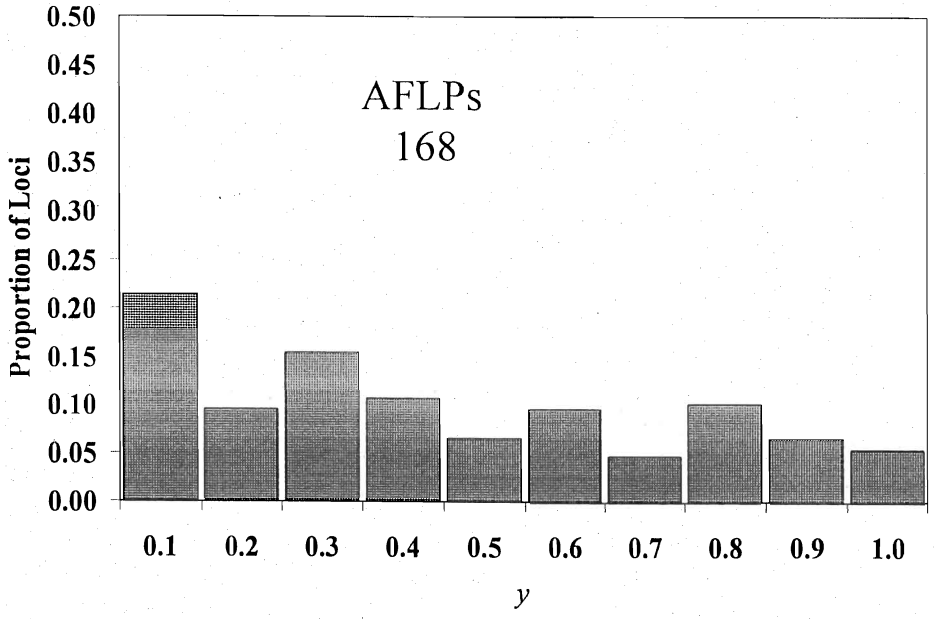
Table 5. Half-tetrad analysis of *FGT1-1,2*. See text for explantaion of estimation of *y* (proportion of second division segregation).

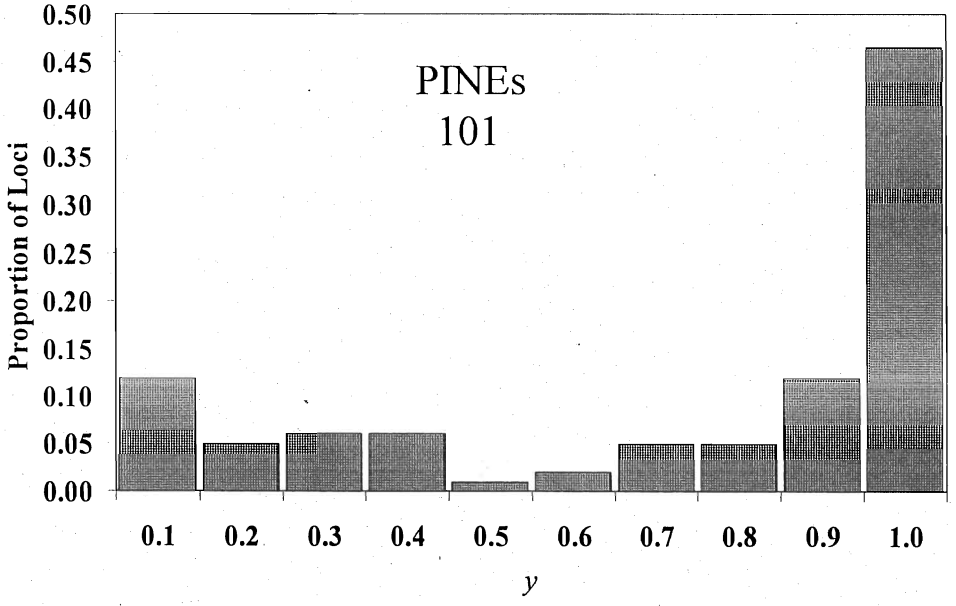
	Maternal Genotype			Progeny					
Family	*-1,2*	*-1*	*-2*	*1111*	*1112*	*1122*	*1222*	*2222*	*y*
A95-29	*1122*	*1/2*	*1/2*	0	0	20	2	0	42/44=0.95
A95-103	*1222*	*1/2*	*2/2*	0	0	0	69	1	69/70=0.99
A95-114	*1112*	*1/2*	*1/1*	3	35	1	0	0	35/39=0.90
A95-120	*1122*	*1/2*	*1/2*	0	0	42	0	0	84/84=1.00
									230/237=0.97

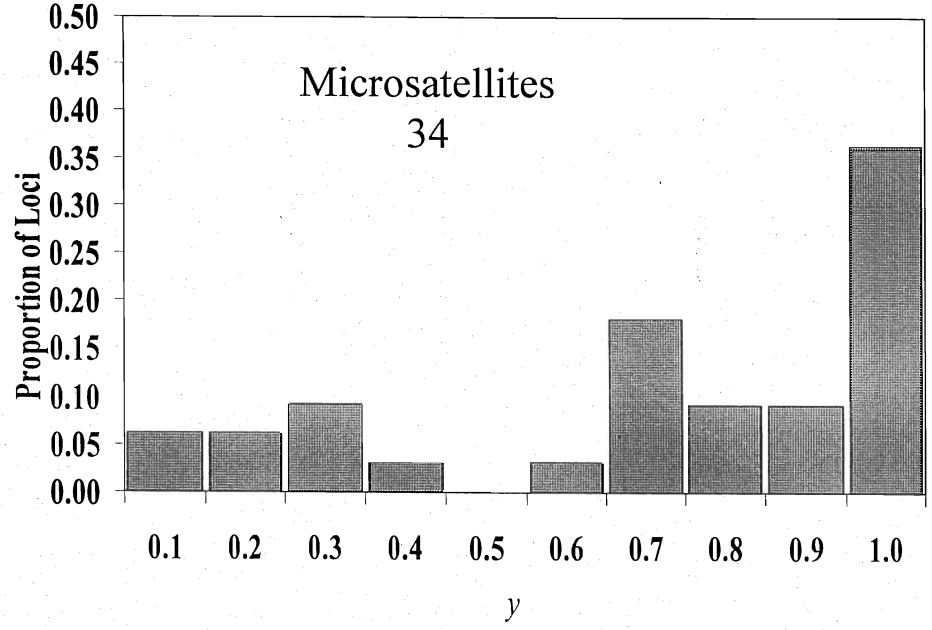
Fig. 1. The distribution of second division segregation (y) for AFLPs, PINEs, microsatellites, and all markers (including allozymes).

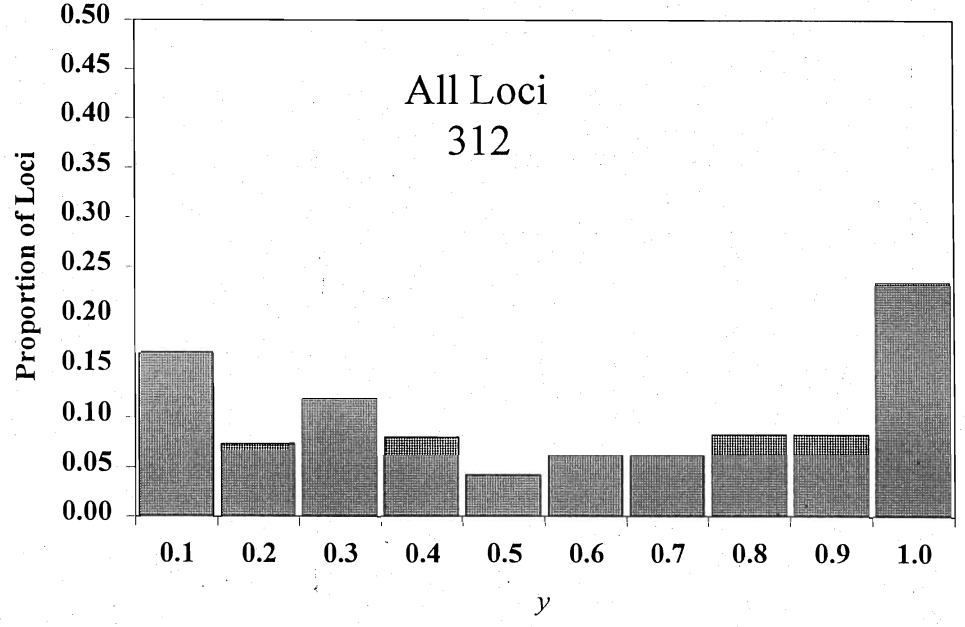
Fig. 2. Electropherograms for individuals with different genotypes at *FGT1-1,2.* The area under the curve at each peak (155 bp and 157 bp) is proportional to the number of doses of each allele (*1 and *2 respectively).

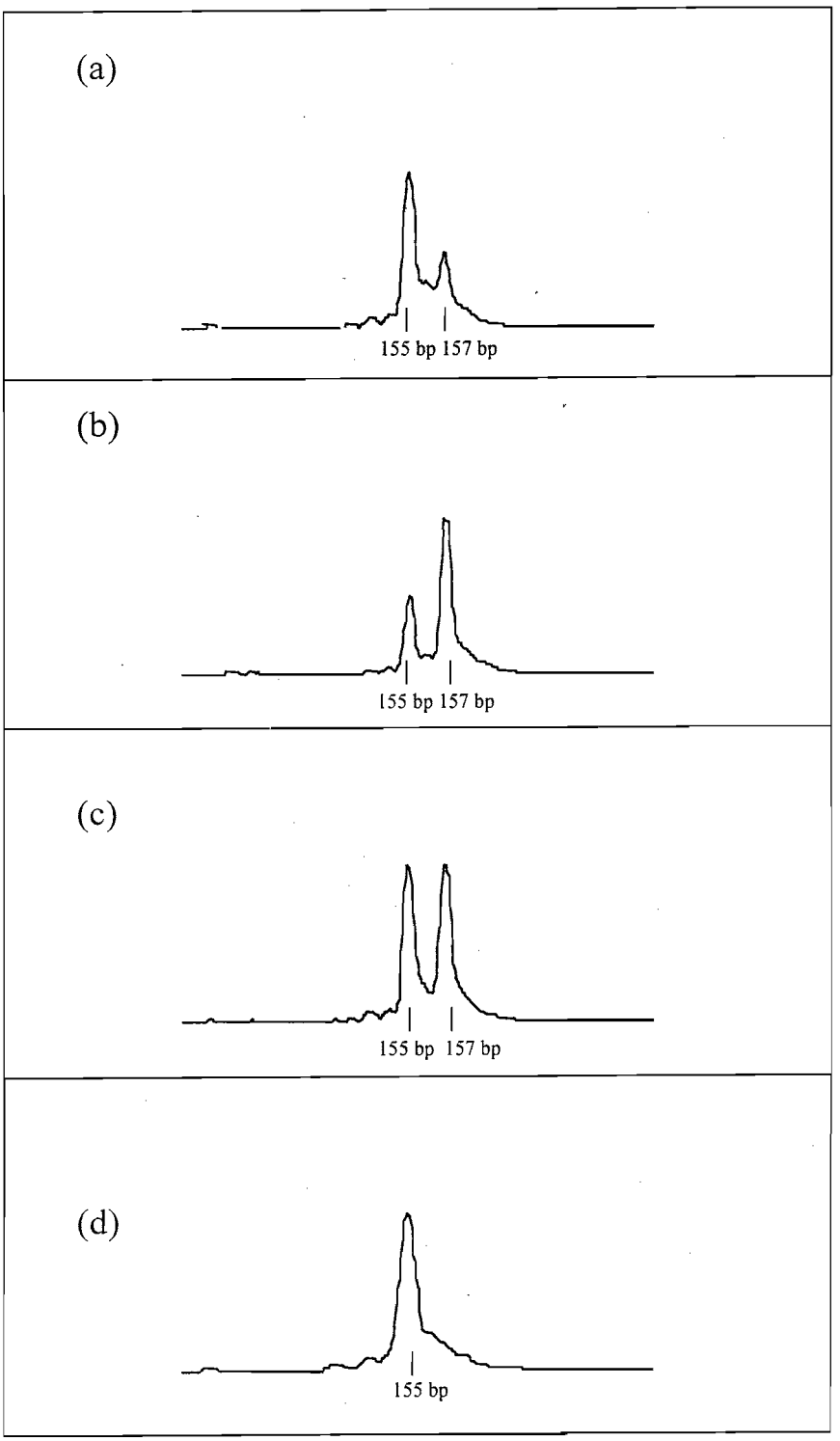

(a) *1112
155 bp 157 bp
(b) *1222
155 bp 157 bp
(c) *1122
155 bp 157 bp
(d) *1111
155 bp


AFLPs
168
Proportion of Loci
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
y


PINEs
101
Proportion of Loci
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
y


Microsatellites
34
Proportion of Loci
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
y


All Loci
312
Proportion of Loci
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
y


(a)
155 bp 157 bp
(b)
155 bp 157 bp
(c)
155 bp 157 bp
(d)
155 bp