A Detailed Linkage Map of Rainbow Trout Produced Using Doubled Haploids

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ABSTRACT

We report the first detailed genetic linkage map of rainbow trout (*Oncorhynchus mykiss*). The segregation analysis was performed using 76 doubled haploid rainbow trout produced by androgenesis from a hybrid between the "OSU" and "Arlee" androgenetically derived homozygous lines. Four hundred and seventy-six markers segregated into 31 major linkage groups and 11 small groups (<5 markers/group). The minimum genome size is estimated to be 2627.5 cM in length. The sex-determining locus segregated to a distal position on one of the linkage groups. We analyzed the chromosomal distribution of three classes of markers: (1) amplified fragment length polymorphisms, (2) variable number of tandem repeats, and (3) markers obtained using probes homologous to the 5′ or 3′ end of salmonid-specific small interspersed nuclear elements. Many of the first class of markers were clustered in regions that appear to correspond to centromeres. The second class of markers were more telomeric in distribution, and the third class were intermediate. Tetrasomic inheritance, apparently related to the tetraploid ancestry of salmonid fishes, was detected at one simple sequence repeat locus and suggested by the presence of one extremely large linkage group that appeared to consist of two smaller groups linked at their tips. The double haploid rainbow trout lines and linkage map present a foundation for further genomic studies.

GENETIC linkage maps have become powerful research tools in many organisms (Postlethwait *et al.* 1994; Wada *et al.* 1995; Knapik *et al.* 1996; Dib *et al.* 1997; Dietrich *et al.* 1997). A complete linkage map is necessary in order to efficiently carry out molecular-based analyses such as marker-based selection (Cho *et al.* 1994), quantitative trait locus (QTL) analysis (Lander and Botstein 1989) and loss of heterozygosity (LOH) studies in tumorigenesis (Dietrich *et al.* 1994) and for comprehensive investigations of genome evolution between lineages (Morizot *et al.* 1977; Morizot 1983; Lyons *et al.* 1997).

Rainbow trout are one of the most intensively studied fish species because of their importance as a food and sport fish, a model research organism (Wolf and Rumsey 1985) and as a model for genome evolution following tetraploidization (Ohno 1970). A detailed linkage map of the rainbow trout genome would further enhance the potential of this organism for these endeavors. Previous mapping attempts were limited by an inadequate number of polymorphic markers, relying mainly on allozyme polymorphisms (May and Johnson 1990). The development of molecular techniques that identify nucleotide-level DNA sequence polymorphisms between individuals has created an unlimited source of genetic markers which can be used to create detailed linkage maps (Botstein *et al.* 1980; Williams *et al.* 1990; Vos *et al.* 1995).

The rainbow trout genome consists of from 58 to 64 chromosomes with 104 chromosome arms (Thorgaard 1983) and contains approximately 2.4×10^9 base pairs, or 80% that of mammals (Ohno and Atkin 1966). The sex chomosomes are subtelocentric and heteromorphic in most populations (Thorgaard 1977, 1983). Rainbow trout are in the family Salmonidae, which evolved by tetraploidization from a diploid ancestor; the genome is still in the process of rediploidization with many undiverged duplicated loci and some homeologous pairing during meiosis (Allendorf and Thorgaard 1984).

In plants and animals with short generation times, an efficient mapping strategy utilizes homozygous parental strains and recombinant inbred (RI) lines produced by multiple generations of sib mating or self fertilization (Festing 1979; Burr and Burr 1991; Silver 1995). This strategy has many advantages, but is difficult in a species with long generation times such as rainbow trout (2–3 years). However, doubled haploid (DH) individuals can be produced from line hybrids in a single generation using androgenesis (Thorgaard and Allendorf 1988). DH lines are identical to RI lines except that they are the product of only one segre-

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gating meiosis instead of multiple meioses in RI lines. Like RI lines, DH lines constitute a resource that can be used for mapping and research for as long as the lines are perpetuated. Markers and genes can be continually added to the map in future studies and the DNA or lines can be sent to other labs for additional marker and trait analyses (Festing 1979; Burr *et al.* 1988). In contrast, backcross or F_2 progeny are only useful until the DNA supply from an individual is exhausted. RI lines have been used to construct linkage maps in many important agricultural species of plants (Burr and Burr 1991; Tahir and Muehlbauer 1994; Keim *et al.* 1997) and mammalian research models such as mice (Silver 1995).

Highly developed mouse and human maps consist of thousands of simple sequence repeats (SSRs), which appear to be ideal markers (Dib *et al.* 1997; Dietrich *et al.* 1997) because of their high level of heterozygosity, wide dispersal and abundance throughout the genome and transferability across strains or species. The drawback is that extensive resources of time and money are required to develop a set of single-locus SSRs that adequately cover the genome. Uncharacterized species for which limited resources are available such as rainbow trout initially require alternative methods of generating molecular markers to cover the genome (Silver 1995). Two such methods are amplified fragment length polymorphisms (AFLPs) and multilocus DNA fingerprinting. These techniques detect high levels of heterozygosity, are informative in nearly all organisms and do not require extensive DNA manipulations (Jeffreys *et al.* 1985; Schafer *et al.* 1988; Vos *et al.* 1995). The AFLP technique utilizes the polymerase chain reaction (PCR) to amplify restriction fragment length polymorphisms (Vos *et al.* 1995). DNA fingerprinting utilizes probes that hybridize to variable number of tandem repeat (VNTR) sequences in the genome (Jeffreys *et al.* 1985). The high levels of variability detected with these methods enable large numbers of informative loci to be rapidly analyzed even in crosses involving relatively closely related strains.

We report the production of a DH panel of rainbow trout and a detailed genetic linkage map consisting of AFLP, VNTR, small interspersed nuclear element (SINE), SSR and randomly amplified polymorphic DNA (RAPD) markers. The map represents a useful framework of markers in addition to characterizing the recombination patterns and marker distribution for the rainbow trout genome. This represents the most complete linkage map for rainbow trout to date and one of the most detailed genetic maps of any fish species.

MATERIALS AND METHODS

Production of doubled haploid rainbow trout: Homozygous parental lines and doubled haploid rainbow trout were produced by androgenesis using the protocols developed by Parsons and Thorgaard (1985), and Scheerer *et al.* (1991). Successful androgenesis results in a diploid organism that contains two sets of paternal chromosomes. Androgenesis was accomplished by irradiating rainbow trout eggs with 40,000 rads of γ-radiation from a Cobalt-60 source, destroying the maternal nuclear genome. The eggs were then fertilized with normal sperm from a chosen male to initiate development of a haploid zygote. Diploidy was restored by applying a heat shock of 31.5° for 5 min at 210 min after fertilization. This prevents the first cleavage division from occurring but does not prevent chromosome division, thereby doubling the paternal chromosome set and restoring diploidy. Male rainbow trout are the heterogametic sex (Thorgaard 1977), so both male (*YY*) and female (*XX*) homozygote individuals were produced in the first generation of androgenesis. The homozygous parental lines, which were clones of the original first generation homozygote, were produced by androgenesis or gynogenesis (with polar body retention) from the gametes of the first generation homozygous males or females, respectively. The parental lines were confirmed homozygous by DNA fingerprinting (Young *et al.* 1996).

The source populations of the homozygous parental lines used to produce the mapping panel were rainbow trout from the Oregon State University (Corvallis, OR) research strain (OSU) and from the Arlee National Fish Hatchery (Arlee, MT; ARL). DH fish were produced by androgenesis using sperm from an F_1 individual (OSU × ARL) from a cross of the OSU (*XX*) and ARL (*YY*) clonal lines. The OSU × ARL F_1 was an *XY* individual so both male (*YY*) and female (*XX*) DH progeny were produced, which allowed for phenotypic identification and mapping of the sex-determining locus. Doubled haploid fish were grown in recirculating systems until they reached 4–6 inches, then tagged with passive integrated transponder (PIT) tags which allowed each fish to be individually identified.

Molecular markers: Segregation of molecular markers was analyzed in 76 DH individuals. AFLP and multilocus DNA fingerprinting were used to produce the majority of the 475 molecular markers analyzed. Other markers included single locus microsatellites and RAPDs. The numbers of each marker type used in the linkage analysis are listed in Table 1.

DNA was extracted from blood or fin clips. The blood or fin clips were collected from the parental and the DH fish and immediately placed in digestion buffer containing 1 mg/ml protease K, 10 mm EDTA, 10 mm Tris, 1% sodium dodecyl sulfate and 0.01% dithiothreitol. If the fin clips were not going to be extracted immediately they were placed in 95% ethanol to prevent degradation until the tissue could be digested. DNA extraction was performed using standard phenol/chloroform protocols (Sambrook *et al.* 1989).

AFLP markers: AFLP marker analysis was performed as described in Vos *et al.* (1995) and as modified by Travis *et al.* (1996) using the restriction enzymes *Eco*RI and *Mse*I and adenine (A) as the first selective nucleotide. Extracted DNA samples were purified before the restriction digest/ligation step using the Gene Clean kit (Bio101, Inc., Vista, CA), with gentle resuspension. AFLP markers were named so the primer combination that produced the marker and the resulting band size could be identified from the locus name. The first three letters represented the +3 nucleotides for the *Eco*RI primer, the second three letters represented the +3 nucleotides of the *Mse*I primer, the number represented the size in base pairs (bp) of the detected band and the last letter represented the parental line that the band was detected in (o or a) or if it was codominantly inherited (c).

Multilocus DNA fingerprinting and salmonid SINE detection: Restriction enzyme digestion, electrophoresis, Southern transfer, hybridization with alkaline-phosphatase-labeled probes

and detection were carried out as described by Spruell *et al.* (1994). The probes included microsatellite repeats, minisatellite repeats and salmonid-specific SINEs (Spruell and Thorgaard 1996) and were all 5′ end-labeled with alkaline phosphatase for detection. The VNTR oligo probes included the simple repeats ATCC, ATC, ATAG, CTT, GCT and CGC, which were purchased alkaline-phosphatase labeled from FMC (Rockland, ME). The minisatellite probes included Jeffreys 33.6 (Jeffreys *et al.* 1985), M13 (Vassart *et al.* 1987) and Per (Shin *et al.* 1985) which were purchased from Synthetic Genetics, Inc. (San Diego, CA) SINE loci were detected using oligonucleotide probes complementary to the 5′ and 3′ ends of the *Hpa*I element and a probe complementary to the 5′ end of the *Fok*I element (Kido *et al.* 1991; Spruell and Thorgaard 1996). Three restriction enzymes were used for the Southern blot analysis; *Hae*III (h), *Rsa*I (r) and *Dpn*II (d).

Fingerprint markers were named so that the probe, enzyme, band size and parental line that the band was detected in could be determined. For example, the name ATCCh8.6o designated a marker that was detected by the probe ATCC using the restriction enzyme *Hae*III and produced a band that was 8.6 kilobases (kb) in size and detected in the OSU parent.

Alternate alleles at a locus were rarely detected on a DNA fingerprint film (results discussed below). When no alternate alleles were detectable, the presence of a band was scored as one allele and the absence of that band was scored as the alternate allele. This method of allele designation has been successfully used for mapping in mice (Julier *et al.* 1990) and cattle (Georges *et al.* 1990).

Segregation of markers was tested for deviations from Mendelian segregation (divergence from 50:50; chi-square, $P < 0.05$). If a statistically significant deviation was detected at a locus, the marker was closely inspected for possible scoring problems such as co-migrating or unresolvable bands and was not used in the analysis if one of these problems was evident. Additionally, some of the individuals were typed on two separate Southern blots, scored independently and the results compared. This provided an estimate of the scoring error associated with these markers. The error rate detected (<2%) would not significantly influence a map of this resolution (Buetow 1991).

Single locus microsatellites: Five single-locus microsatellite primer pairs were analyzed: FGT-1 (Sakamoto *et al.* 1994), oneu 2 and oneu 6 (Scribner *et al.* 1996) and MS-73 and MS-35 (Estoup *et al.* 1993). The PCR conditions were as follows: 50 ng of DNA was used in a 20 μl PCR reaction containing 1× GIBCO (Grand Island, NY) PCR buffer, 2.0 mM $MgCl_2$, 100 nM of each dNTP, 10 pM of each primer and 1 unit of Taq polymerase. Reaction conditions were an initial denaturation of 94° for 4 min, and then 2 cycles of 94° for 30 sec, 62° for 30 sec and 72° for 1 min; 2 cycles of 94° for 30 sec, 60° for 30 sec and 72° for 1 min; 2 cycles of 94° for 30 sec, 58° for 30 sec and 72° for 1 min and finally 25 cycles of 94° for 30 sec, 55° for 30 sec and 72° for 1 min.

The parental fish were first screened for polymorphisms by electrophoresis in 4% Metaphor agarose (FMC) in Tris-acetate/EDTA buffer and detected with ethidium bromide on a UV light box. If a polymorphism was found that could be resolved by electrophoresis in 4% Metaphor, then DNA samples from the DH progeny were amplified, electrophoresed in 4% Metaphor and detected by ethidium bromide staining. If no polymorphism was observed then the parental DNAs were amplified using 5′ digoxygenin-labeled primers, run on 6% acrylamide in Tris-borate/EDTA buffer, blotted onto nylon membrane (Magnagraph, Micron Separations, Inc., Westborough, MA) and detected by normal protocols as described in the Genius Kit Manual (Boehringer Mannheim, Indianapolis). If a polymorphism was detected using this method then that marker was amplified from DNA of the DH progeny, run on the acrylamide gel and detected as above. For each microsatellite primer pair one of the primers was 5′ end-labeled using Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-Nhydroxy-succinimide Ester (Dig-NHS ester) as described in the Genius Kit Manual (Boehringer Mannheim). Of the five single locus microsatellites analyzed, SSRs oneu 2 and oneu 8 polymorphisms were effectively distinguished on 4% Metaphor agarose (FMC Corp.), FGT-1 polymorphisms were distinguishable on an acrylamide gel and the other two were not polymorphic in this cross.

RAPD amplification: PCR amplification of RAPD loci followed standard protocols described in Williams *et al.* (1990) with slight modifications. For all primers 50 ng of DNA was used in a 25 μl PCR reaction containing 1× GIBCO PCR buffer, 2.5 mm $MgCl_2$, 100 nM of each dNTP, 10 pM of primer and 1 unit of Taq polymerase. The amplification protocol was as follows: 45 cycles of 30 sec at 94°, 30 sec at 36° and 2 min at 72°. Products were electrophoresed in 2.5% agarose in TAE buffer, stained with ethidium bromide and visualized on a UV light box. Approximately 50 primers were screened for polymorphism between the parents. Potential differences were again analyzed and five polymorphisms that were detected with high reproducibility were used in the linkage analysis. Primer sources were either from Operon (Alameda, CA; CS) or University of British Columbia (Vancouver, BC; UBC). The four primers that detected polymorphisms and their sequences were CS32 (CCCACGGATC), CS40 (GACTGCTCGG), CS45 (CACGTCGGAG) and UBC516 (AGCGCCGACG), which detected two polymorphisms. The name used on the map contains the source of the primer (CS or UBC), the molecular weight of the polymorphic band and the parental origin of the band (a, o or c).

Sexing: The two- or three-year-old DH individuals were sexed based on the production of gametes or visual inspection of secondary sexual characteristics in order to avoid sacrificing these valuable individuals. The preferred method of sex identification was the production of gametes. However, for fish which did not produce gametes, sex was determined based on the expression of phenotypic secondary sexual characteristics.

Analysis of segregation: The map was constructed using 76 DH individuals from the OSU × ARL cross. Segregation of the markers was analyzed as a doubled haploid cross using the Macintosh version of Mapmaker 2.0 and the orders checked as an F_2 intercross on Mapmaker/EXP 3.0 using the error detection function (Lander *et al.* 1987; formatted for Macintosh by Dr. Scott Tingley, Dupont Experimental Station, Wilmington, DE). Mapmaker/EXP does not analyze data as a doubled haploid cross but the functions gave nearly identical results. Initial grouping of markers was done using a minimum LOD score of 3.0 and a maximum theta of 0.35. Subsequent analysis of the linkage groups was done with a minimum LOD of 4 and a maximum theta of 0.30 to better detect anomalous linkages. Only a few markers did not maintain linkage with their original groups using the more stringent LOD cutoff. These markers were only placed within the linkage group if their most likely position was supported by >LOD 2 over any other position and significant map expansion did not occur. Scoring errors detected by Mapmaker 3.0 were rechecked and corrected if a typing error was found, and then a final data analysis was performed to produce the current map.

RESULTS

Rainbow trout linkage map: Linkage analysis using 476 markers (475 molecular markers and sex) pro-

duced a genetic map comprising 42 linkage groups and covering a distance of 1997.5 cM. The raw data for our analysis (marker distribution across the 76 individuals analyzed) is available at http://www.wsu.edu:8080/~thorglab/DATA.HTML. The majority of the markers segregated into 31 large linkage groups (>6 markers/group; Figure 1) with the additional markers segregating into one small group of 4 markers, four groups of 3 markers, and 6 marker pairs. There were 9 markers that remained unlinked following the analysis. The majority of the small groups consisted of non-AFLP markers and only one AFLP marker remained unlinked following the analysis. Assuming a minimum distance of 35 cM (minimum theta used in the analysis) to fill in the gaps to the small groups and unlinked markers, the minimum length of the rainbow trout genome is estimated to be 2627.5 cM. Although the mean distance separating markers is 5.6 cM (2627.5/467 linked markers), this averaging is misleading due to marker clustering (see below). The haploid genome size of rainbow trout is approximately 2.4×10^9 bp (Ohno and Atkin 1966) which gives an average distance between crossovers of 913 kilobase pairs (kbp)/cM, which is more similar to the value in humans (Dib *et al.* 1997) than that of other fish species (Postlethwait *et al.* 1994; Wada *et al.* 1995).

Distribution of AFLP and VNTR markers: The AFLP and VNTR markers were not randomly distributed on the map; many AFLPs were clustered at central locations on the linkage groups while the VNTRs were more telomeric in distribution. These clusters consisted of from 5 to 12 AFLPs plus the occasional non-AFLP marker. Interestingly, most of the large linkage groups contained an AFLP cluster, and of those with clusters, all except one (the linkage of groups VI and XI—see below) contained a single cluster.

Relative to the AFLP clusters, other marker types appear to have more distal distributions on the linkage groups. This was analyzed by counting the markers of each type that mapped to the tips of the linkage groups and comparing the observed number of each type to the number expected assuming random distribution using a chi-square test. The AFLPs were significantly underrepresented at the tips of the linkage groups ($\chi^2 = 6.96$, 1 df: $P < 0.01$) and the VNTRs were significantly overrepresented at the tips of linkage groups ($\chi^2 = 17.36$, 1 df: $P < 0.001$). SINEs, the only other marker type with large enough numbers to be analyzed, did not significantly differ from their expected frequencies at the tips of linkage groups (chi-square, $P > 0.05$).

The distal VNTR distribution could also have occurred if the VNTR markers had a significantly higher incidence of scoring error. Misscored markers can have a high LOD score for linkage to a group, but they will not fit into interior positions without significantly affecting the order of adjacent markers and therefore are placed at distal positions to minimize the disruption of correctly scored markers. However, this was likely not a factor in the distribution because the VNTR scoring error estimates were relatively low and the SINE loci which were detected using the same technique as the VNTR markers did not show this telomeric distribution. Additionally, no significant increase in map distance was observed among the marker classes at the most distal interval (*t*-test, $P = 0.325$), which would be expected if one class had a significantly higher error rate. Therefore, the telomeric bias for VNTR markers appears to be a real phenomenon.

Multilocus techniques allow a large number of markers to be detected in a short period of time. The number of each marker type and percent of loci that were codominantly inherited (expressed in both parents) are listed in Table 1. The 332 AFLP markers used in the segregation analysis were produced using 28 primer pairs for an average of 12.1 markers/primer pair. Of these, 45.8% were dominantly inherited bands from the ARL parent, 35.8% were from the OSU parent and 18.4% were codominantly inherited (Table 1). This was slightly higher than the 13% codominantly inherited AFLP markers observed in a linkage analysis of soybean markers (Keim *et al.* 1997). Probes that identified VNTR and SINE markers produce DNA fingerprint-like multilocus patterns containing 20–40 bands per probe-enzyme combination but many could not be scored due to overlapping and co-migrating bands. Probes that detected the fewest bands, in this case ATCC, GATA, ATC and Hpa 3′, gave the most usable markers. It is probable that these simple repeats are less numerous in the genome based on the detection of fewer bands and the greater number of times probes composed of these repeats detected a single locus using two different restriction enzymes. Of these repeats, ATCC and Hpa 3′ loci were each detected using two different restriction enzymes 4 times, and a single ATC locus detected with two enzymes was found once. Only one other repeat locus was detected using two restriction enzymes, a Fok 5′ locus.

Significant deviation from the expected 50:50 Mendelian inheritance pattern was observed for 13.3% of the markers, with all classes giving similar proportions (chi-square, 1 df, $P > 0.05$). These were not randomly distributed on the map but were inherited as blocks of markers that in some cases covered large portions of a linkage group (Figure 1). The segregation of the markers in these regions significantly skewed toward one of the two parents, with regions skewed toward different parents occurring on different linkage groups.

Distribution of SINEs: Markers detected with probes homologous to the 5′ and 3′ end of the *Hpa*I SINE element and the 5′ end of the *Fok*I element were uniformly distributed on the map based on their lack of central clustering and their expected frequency at distal locations. Markers detected with alternate ends of

the *Hpa*I element (5′ and 3′) never mapped to the same position or were even close to one another. Spruell and Thorgaard (1996) demonstrated that a maximum of 50% of the bands detected using the 5′ and 3′ *Hpa*I probes could be homologous based on band mobility. Both results suggest that the large, highly variable bands detected with either one or both of the 5′ and 3′ *Hpa*I probes do not typically contain entire *Hpa*I SINE elements; it appears that the alternate ends of the *Hpa*I SINE element can be distinct from one another and may be similar to minisatellite sequences or make up a yet undescribed class of trout repetitive DNA. Markers detected with the same probe but different restriction enzymes frequently mapped to the same location, indicating detection of the same locus (Julier *et al.* 1990). This occurred more often using the *Hpa* 3′ probe, indicating the *Hpa* 3′ loci detectable by this method were less common in the genome than the *Hpa* 5′ loci.

Tetrasomic inheritance: Two results indicated the presence of tetrasomic inheritance caused by homologous pairing in the generation of this DH family. One single locus microsatellite, FGT-1 (Sakamoto *et al.* 1994), showed an inheritance pattern that could only be explained by tetrasomic inheritance. The locus appeared to be duplicated because of the presence of two distinct bands in both of the homozygous parents and all four bands in the OSU × ARL hybrid. The genotype of OSU was designated *AABB* and that of ARL was *CCDD*. The homozygous progeny also had two bands, with all 6 possible band combinations: *AABB*, *AACC*, *AADD*, *BBCC*, *BBDD* and *CCDD*. Normal disomic inheritance and independent segregation of two loci could result in only four possible combinations of markers, with the alleles at a locus from the same parent never segregating together in the homozygous DH progeny, even with linkage. The presence of individuals with all six possible combinations means that an allele from one parent was segregating with the alternate allele at that locus from the other parent and no alleles were present from the other locus. This could occur if multivalent pairing occurred during meiosis followed by recombination and normal disjunction. Unfortunately it was impossible to map this marker since alleles could not be designated to their respective loci. The multilocus methods used to detect the majority of our mapped markers made it impossible to detect additional duplicated loci, so we cannot estimate the overall proportion of tetrasomically inherited loci.

Although not as definitive as the segregation pattern observed above, the complete map provided additional evidence that homologous recombination occurred in this cross. Linkage groups VI and XI demonstrated linkage with a LOD score of >4, however the end-to-end arrangement of these two groups could not be established based on a significant LOD score for group order. The high LOD score in support of linkage indicated that this was likely true linkage and not an anomaly and therefore may represent another example of homologous pairing and recombination in this cross. These groups do appear to be two separate groups, having a total length significantly greater than any other group and two AFLP clusters (see discussion below). It is unknown if the tetrasomically inherited microsatellite was in this group. It is likely that some of the markers on this group demonstrated tetrasomic inheritance, although widespread homologous recombination would have resulted in the observation of more of these abnormally large groups. Therefore homologous recombination likely did not occur frequently in this cross.

Sex-determining locus: The sex-determining locus was the only phenotypic trait placed on the map and it mapped to a distal position on group I. The sex-determining locus was separated by *ca.* 19 cM from a pair of AFLP markers with no intervening markers. A large amount of recombination occurred between the *X* and *Y* chromosome in this cross which is consistent with the observation of *X-Y* recombination in previous salmonid mapping efforts (May and Johnson 1990; Allendorf *et al.* 1994). Rainbow trout sex chromosomes are heteromorphic in most populations (Thorgaard 1977) and could be distinguished cytologically between the parents of this cross (Carl Ostberg, personal communication).

DISCUSSION

This map represents the most complete linkage analysis in rainbow trout to date and is a first step in producing a highly saturated linkage map of the genome. This map appears to be a good genetic representation of the rainbow trout genome for two reasons. First, there is a good correspondence between the number of large linkage groups and the chromosome number. The haploid chromosome number of the OSU parent was 30 and that of the ARL parent was 32 (Carl Ostberg, personal communication) which reflects the chromosome diversity within the species. The reason for this difference is that the ARL parent contains four acrocentric chromosomes that will be expected to pair normally with two metacentric chromosomes from the OSU parent during meiosis. Therefore, the functional haploid number in this cross was 30. This is very close to the 31 large groups observed from the linkage analysis. The presence of small groups and unlinked markers indicates that there are some gaps that need to be filled. It is likely that these gaps are in telomeric regions, because two of the named groups (group XXX and group XXI) were only short clusters of AFLP markers and additional markers are needed in order to cover the arms of these chromosomes. In addition, the greater proportion of unlinked VNTR markers, which demonstrated a more telomeric distribution, confirm that gaps linking these distally located markers need to be filled. Second, the total map length ob-

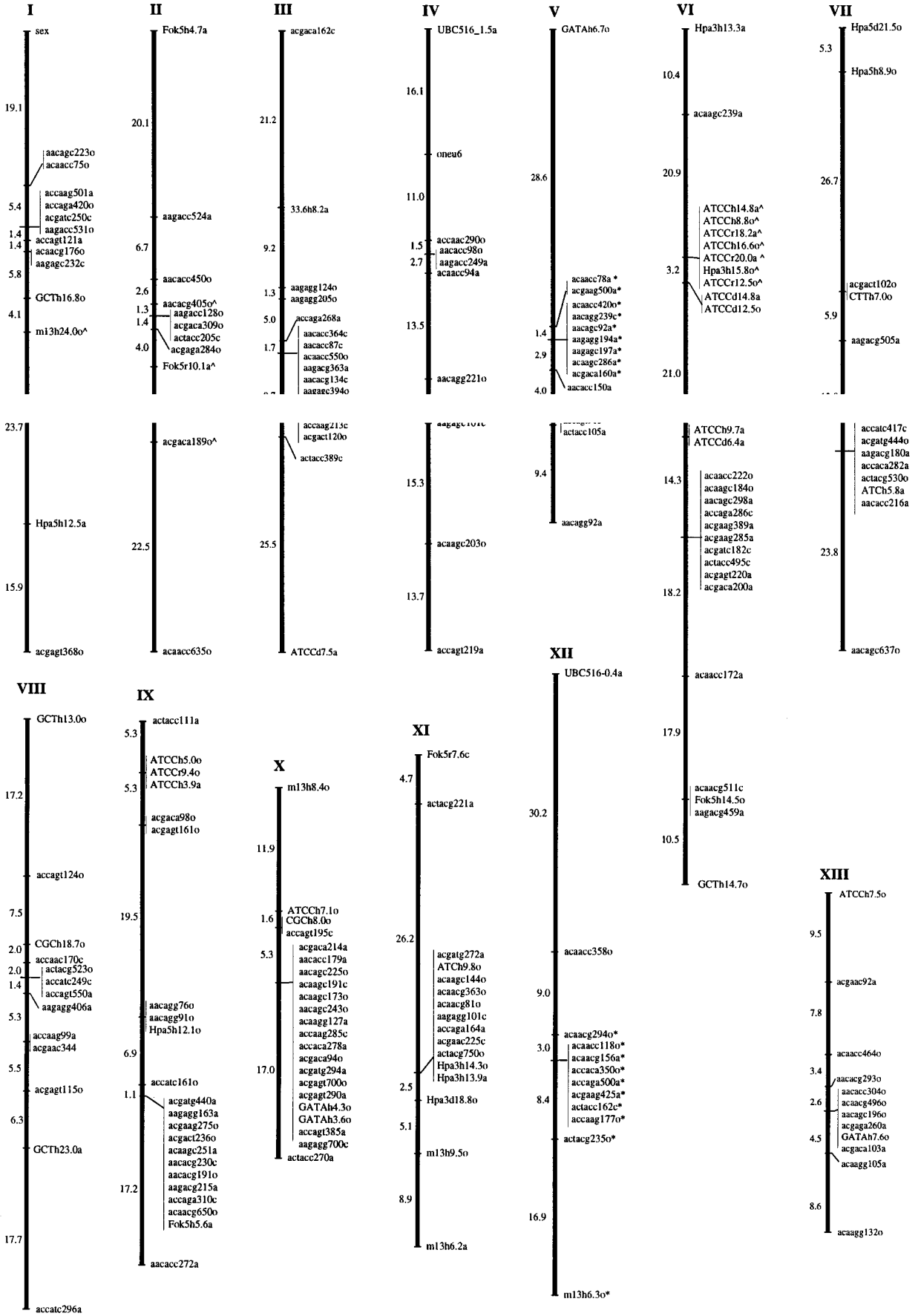
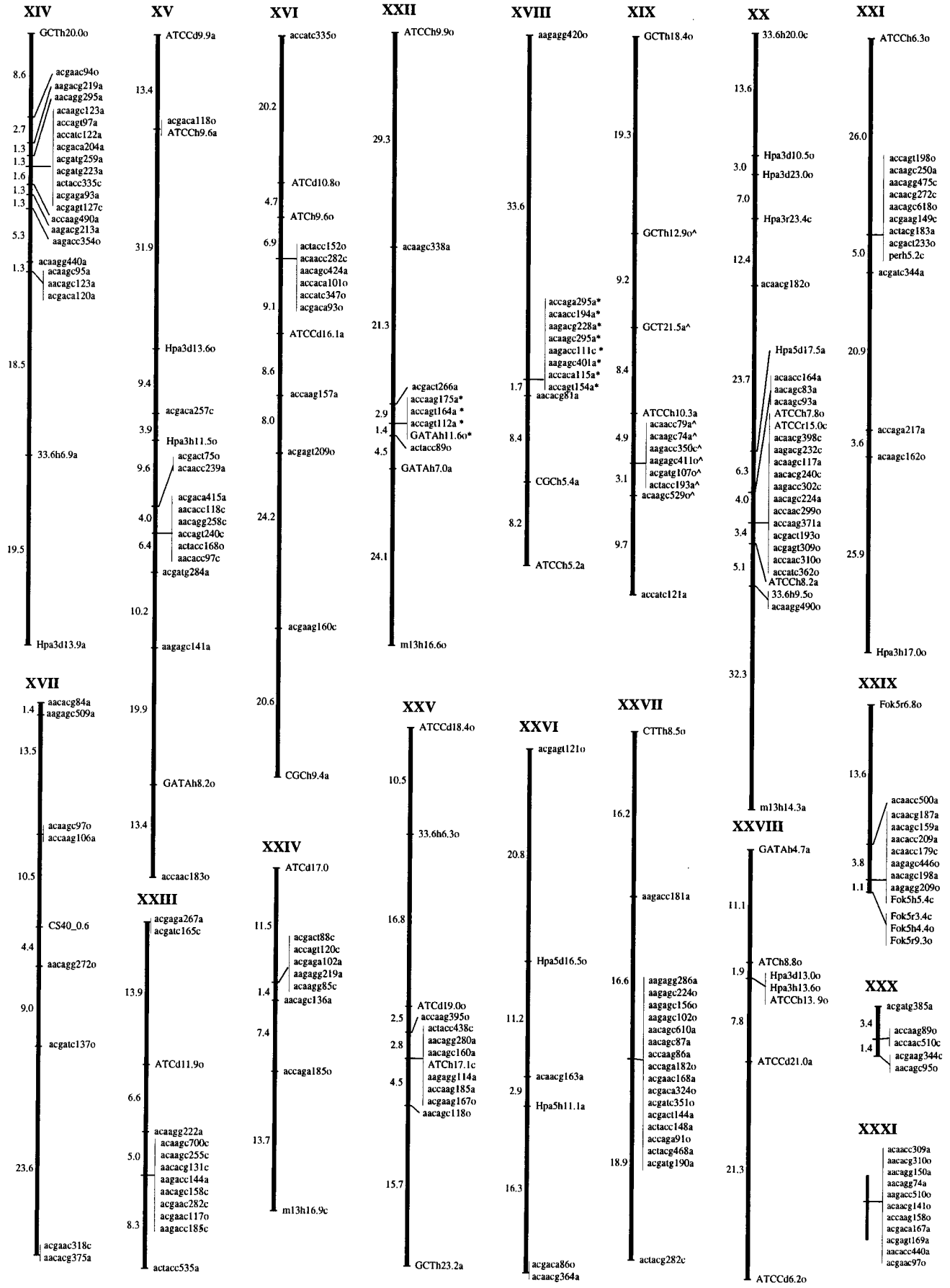


Figure 1.—A genetic map for the rainbow trout genome. Numbers to the left of the linkage groups indicate genetic distance in centiMorgans (Kosombi). Markers followed by symbols demonstrate ratios that significantly deviate from expected Mendelian ratios toward either the male allele (a-*) or the female allele (o-^).


XIV
GCTh20.0o
acgaac94o
aagacg219a
aacagg295a
acaagc123a
accagt97a
accatc122a
acgaca204a
acgatg259a
acgatg223a
actacc335c
acgaga93a
acgagt127c
accaag490a
aagacg213a
aagacc354o
acaagg440a
acaagc95a
aacagc123a
acgaca120a
33.6h6.9a
Hpa3d13.9a
XV
ATCCd9.9a
acgaca118o
ATCCh9.6a
Hpa3d13.6o
acgaca257c
Hpa3h11.5o
acgact75o
acaacc239a
acgaca415a
aacacc118c
aacagg258c
accagt240c
actacc168o
aacacc97c
acgatg284a
aagagc141a
GATAh8.2o
accaac183o
XVI
accatc335o
ATCd10.8o
ATCh9.6o
actacc152o
acaacc282c
aacago424a
accaca101o
accatc347o
acgaca93o
ATCCd16.1a
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acgagt209o
acgaag160c
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XXII
ATCCh9.9o
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acgact266a
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accagt164a *
accagt112a *
GATAh11.6o*
actacc89o
GATAh7.0a
m13h16.6o
XVIII
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accaga295a*
acaacc194a*
aagacg228a*
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Hpa3d10.5o
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aacacg84a
aagagc509a
acaagc97o
accaag106a
CS40_0.6
aacagg272o
acgatc137o
acgaac318c
aacacg375a
XXIII
acgaga267a
acgatc165c
ATCd11.9o
acaagg222a
acaagc700c
acaagc255c
aacacg131c
aagacc144a
aacagc158c
acgaac282c
acgaac117o
aagacc185c
actacc535a
XXIV
ATCd17.0
acgact88c
accagt120c
acgaga102a
aagagg219a
acaagg85c
aacagc136a
accaga185o
m13h16.9c
XXV
ATCCd18.4o
33.6h6.3o
ATCd19.0o
accaag395o
actacc438c
aacagg280a
aacagc160a
ATCh17.1c
aagagg114a
accaag185a
acgaag167o
aacagc118o
GCTh23.2a
XXVI
acgagt121o
Hpa5d16.5o
acaacg163a
Hpa5h11.1a
acgaca86o
acaacg364a
XXVII
CTTh8.5o
aagacc181a
aagagg286a
aagagc224o
aagagc156o
aagagc102o
aacagc610a
aacagc87a
accaag86a
accaga182o
acgaac168a
acgaca324o
acgatc351o
acgact144a
actacc148a
accaga91o
actacg468a
acgatg190a
actacg282c
XXVIII
GATAh4.7a
ATCh8.8o
Hpa3d13.0o
Hpa3h13.6o
ATCCh13. 9o
ATCCd21.0a
ATCCd6.2o
XXIX
Fok5r6.8o
acaacc500a
acaacg187a
aacagc159a
aacacc209a
acaacc179c
aagagc446o
aacagc198a
aagagg209o
Fok5h5.4c
Fok5r3.4c
Fok5h4.4o
Fok5r9.3o
XXX
acgatg385a
accaag89o
accaac510c
acgaag344c
aacagc95o
XXXI
acaacc309a
aacacg310o
aacagg150a
aacagg74a
aagacc510o
acaacg141o
accaag158o
acgaca167a
acgagt169a
aacacc440a
acgaac97o

TABLE 1

Molecular markers analyzed in a doubled haploid population of rainbow trout

Type	Number	% Codominant	Unlinked*
AFLP	332	18.4	1
VNTR	96	3.9	4
SINE	40	7.1	2
RAPD	5	40	1
SSR	2	100	1

* LOD $>$ 3.0, theta $<$ 0.35.

served in this analysis is similar to the expected length based on the physical size and recombination pattern of the chromosomes. Gene-centromere mapping results demonstrate that, in females, a high degree of chiasma interference exists that results in one crossover per chromosome arm (Thorgaard *et al.* 1983; Allendorf *et al.* 1986). One crossover per arm represents *ca.* 50 cM in map length which gives an estimate of 2600 cM (50 cM $\times$ 52 arms) for the length of the genome. This is remarkably close to our estimate of 2627.5 cM.

The total recombination distance of the map was higher than expected based on previous mapping studies which demonstrated a high level of recombination suppression in males compared to females. In some cases the level of recombination between protein markers in males was suppressed by nearly an order of magnitude over that of females (Johnson *et al.* 1987). One major difference between our cross and previous crosses was the evolutionary distance between the parents. Our study was an intraspecific cross of two domestic strains while many of the previous estimates of male recombination suppression were based on the recombination frequencies of interspecific hybrids, which have been shown to have more meiotic abnormalities relating to the ancestral tetraploidization of salmonids. The consequences of a tetraploid ancestry, including multivalent pairing and preferential pairing of homologs, have been shown to be greater in males of interspecific crosses or distant interstrain crosses (Wright *et al.* 1983) and this may explain the higher level of male recombination suppression observed in previous studies. Consequently, recombination estimates from males of interspecific hybrids may not have provided a reliable estimate of the frequencies expected from intraspecific crosses. Linkage analyses using only intraspecific rainbow trout crosses demonstrated that recombination was suppressed in males approximately 40–50% over that of females (May and Johnson 1990; Allendorf *et al.* 1994). Our results are consistent with this level of recombination suppression when compared to the female-based maps of zebrafish and medaka. Both the zebrafish and medaka genomes have approximately one-half the DNA content and chromosome arm number of rainbow trout and map lengths of 2900 cM and 2480 cM, respectively (Postlethwait *et al.* 1994; Wada *et al.* 1995). Assuming similar recombination patterns among these fishes and no male recombination suppression in male rainbow trout, a male-based rainbow trout map should be approximately twice as large as these other species. However, the map length from our analysis is similar to that of these other fishes. This suggests that either recombination suppression occurs in male rainbow trout or a different level of recombination occurs in rainbow trout compared to these other fish species. Additional segregation analyses using female meiosis will be necessary to confirm the presence or degree of recombination suppression in male salmonids.

In the context of DH lines, AFLP, and VNTR markers are extremely useful, being abundant, highly variable and relatively simple to analyze. These markers provide fundamental information about the size and structure of the salmonid genome. Additional efforts will be required to map SSRs, allozymes and other conserved single locus markers to identify intra- and interspecific anchor loci. Given the conservative nature of gene order in fishes of the family Salmonidae observed in previous studies (Johnson *et al.* 1987; May and Johnson 1990), a map with a large number of AFLPs and anchored with single locus SSRs or protein loci will provide a framework for the salmonid genome.

Marker distribution: Clustering of AFLP markers was observed on 27 of the 31 large linkage groups. The AFLP markers may not necessarily be closely spaced physically within the clusters but may appear that way on a recombination-based map because of the lack of recombination in that region (Keim *et al.* 1997). Physical mapping will be necessary to determine the true distribution and order of the AFLP markers within these clusters; however, there is a great deal of evidence suggesting that the AFLP clusters identified the heterochromatic regions associated with centromeres on this map. Although no known centromeric loci were mapped in our analysis, recombination is often reduced in the regions surrounding centromeres and could explain the clustering observed here. In addition, similar AFLP clustering has been observed in other linkage analyses. Clustering of AFLPs has been observed on the chromosomes of corn (Vuylsteke *et al.* 1997) and soybeans (Keim *et al.* 1997) with the clusters segregating to known centromeric positions on the corn linkage groups. The consistent presence of one AFLP cluster per group and the relative position of the AFLP clusters on the linkage groups provided additional support for their identity as centromeric regions. The majority of rainbow trout chromosomes are metacentric (22/30 in this cross), which is consistent with the observation of centrally located AFLP clusters on most of the linkage groups.

Additional evidence that the clusters represent centromeres is the position of the double crossovers on the linkage groups. The position of crossovers can be in-

ferred by examining the raw data of all of the markers in a linkage group in the correct order. Because rainbow trout show an extremely high level of interference, very few double crossovers would be expected on a single chromosome arm (Thorgaard *et al.* 1983). However, interference in other organisms does not affect crossovers on opposite sides of the centromere (Howe 1956; Stadler 1956). Therefore, the only expected double crossovers would occur on opposite sides of the centromere on metacentric chromosomes. Our results indicated that very few double crossovers occurred on the same side of the AFLP cluster. Proof that the AFLP clusters do mark the centromeres will need to be tested using gene-centromere mapping (Thorgaard *et al.* 1983; Streisinger *et al.* 1986) or by mapping known centromere probes. If the AFLP clusters truly mark centromeres, this could provide an important landmark for uniting the karyotype with this genetic linkage map.

Only one other cluster of markers was observed, a group of ATCC markers on linkage group VI (Figure 1). This cluster apparently represents an accumulation of ATCC microsatellites at that position in the genome.

Large regions of some linkage groups contained blocks of markers from one or the other parent that segregated in a non-Mendelian fashion and indicated the nonrandom segregation of the chromosomal regions identified by these markers. Because the parents of this cross were totally homozygous, simple recessive lethal alleles would seem unlikely, but sub-lethal alleles that reduce viability could account for this segregation distortion. The most likely explanation was that individuals possessing chromosomal segments from one parent were less viable than individuals possessing the other parent's segments, causing them to be significantly underrepresented in the population, thus reducing the frequency of alleles from that parent. Similar regions influencing viability as homozygotes have been observed in Arabidopsis (Mitchell-Olds 1995) and in maize, where detailed analyses of these regions demonstrated significant heterosis on yield when heterozygous (Stuber *et al.* 1992). Additional research will be necessary to determine if these regions show heterosis in rainbow trout. If so, mapping and identifying the genes located in these regions could be important to the improvement of commercial aquaculture populations.

Salmonid SINE distribution: Spruell and Thorgaard (1996) first documented the DNA fingerprint-like patterns obtained using oligonucleotide probes from the 5′ or 3′ end of both *Hpa*I and *Fok*I. However, they were unable to explain the high variability of the bands or the discrepancy of patterns obtained using probes homologous to the alternate ends of the *Hpa*I element. In addition, the authors stated that given the proposed number of *Hpa*I loci in the genome, the observation of discrete bands rather than a smear on a Southern blot was puzzling. This was in contradiction to previous results showing that the SINE elements were widely dispersed in stable locations and relatively invariable within species (Kido *et al.* 1991). It was hoped that mapping the loci detected with these probes would provide insight into the distribution of these loci.

Our genetic mapping results demonstrated that the same locus was never detected using probes homologous to alternate ends of the *Hpa*I element. This result combined with that of Spruell and Thorgaard (1996) suggest that these sequences do occur separately in high copy number in the genome and that the structure of this family of repetitive elements is more complex than originally proposed by Kido *et al.* (1991). Most of the bands that were scored were >5 kbp, with many >15 kbp. If these bands contained entire *Hpa*I elements then the 3′ and 5′ probes would detect the same band and it would map to the same locus. We obtained the same result using restriction enzymes that are predicted to cut within the element (*Hae*III and *Rsa*I) or not in the element (*Dpn*II). Takasaki *et al.* (1996) found a partial *Hpa*I element within a Tc1-like transposon, which may indicate a mechanism for relocating partial *Hpa*I sequences. However, this does not explain the high level of variability obtained using these probes, which is very similar to that observed using VNTR probes (Jeffreys *et al.* 1985; Spruell and Thorgaard 1996).

The copy number of loci detected using DNA fingerprinting methods can be estimated by the number of times a probe detected the same locus using different restriction enzymes (Julier *et al.* 1990). In this study the 3′ probe produced fewer bands on the film and detected the same locus using different enzymes more often than the 5′ *Hpa*I probe did. This indicates that there were fewer 3′ *Hpa*I loci than 5′ *Hpa*I loci in the genome. This suggests that *Hpa*I 3′ and 5′ sequences are found distinct of each other in a few highly variable sites in the genome and, given the high variability and fingerprint type pattern, are probably tandemly repeated or associated with VNTRs, which has been observed in sockeye salmon (Cummings *et al.* 1997).

Tetrasomic inheritance: Ohno (1970) proposed the tetraploid ancestry of salmonid fishes based on evidence that salmonids contain nearly twice the DNA and double the chromosome arm number of other closely related fishes. This was confirmed by the observation of duplicate genes, multivalent chromosome pairing during meiosis and homologous inheritance patterns (Wright *et al.* 1983). These meiotic abnormalities occurred only in meiosis of males of interspecific crosses or intraspecific crosses involving distantly related strains. Our observation of tetrasomic inheritance at one microsatellite locus provides direct evidence that at least some residual homologous pairing occurred in this cross.

Because most of the markers used in this study were

detected using multilocus techniques and were mainly dominantly inherited, it was impossible to determine if these loci were duplicated and abnormal inheritance patterns thus could not be detected. However, the completed map provided additional evidence of tetrasomic inheritance. Linkage groups VI and XI (Figure 1) were linked with a LOD score of >4 ($= P > 0.001$) but could not be ordered. This may have been a result of partial homologous pairing. Additional mapping of gene sequences or allozymes will be required to determine if these chromosomes were in fact homologous. Homologous chromosomes should contain copies of gene sequences duplicated at the time of tetraploidy that have not diverged to a great extent because of residual homologous pairing and recombination within this region. The association of such duplicated sequences on the putative homologs will be necessary in order to confirm this result.

Sex determination marker: Previous studies have indicated that the sex-determining locus in rainbow trout is likely to be tightly associated with the centromere on a subtelocentric chromosome (Thorgaard 1977; Allendorf *et al.* 1994). Our results suggest that it is at a distal position on the short arm of that chromosome and that the majority of the length of the *X* and *Y* chromosomes paired and underwent normal recombination, with the nonrecombining portion of the sex chromosomes making up a relatively small portion of the short arm. However, assuming the AFLP clusters represented centromeres, the recombination distance from the sex-determining locus to the centromere was farther than anticipated. It is possible that this discrepancy was due to the misidentification of sex in some DH individuals. Two potential sources for error were misidentified individuals or individuals that were correctly scored but did not exhibit their true genetic sex. Misscoring may have occurred because some of the fish were small and may not have fully exhibited secondary sex characteristics. The most difficult fish to judge would have been immature males which resemble females in appearance. The other source of error comes from the possibility that in homozygous fish, individuals can sometimes spontaneously develop into the opposite sex (Scheerer *et al.* 1991). Either misscoring or developmental switches could have resulted in an apparent increase in recombination frequency between the sex-determining locus and the next closest marker. However, even with these potential scoring problems, a high LOD (>12) score supported the placement of the sex determining locus on this chromosome and we are confident this linkage group represents the sex chromosome.

The relatively limited sex chromosome differentiation in rainbow trout indicates that they are in the early stages of sex chromosome differentiation. A large amount of recombination between the *X* and *Y* chromosome was observed in this cross, confirming previous results (Allendorf *et al.* 1994). The presence of crossovers on the short arm of the chromosome containing the sex-determining locus indicates that the region of differentiation is relatively small and the observed morphological differentiation (Thorgaard 1977) is likely in close proximity to the actual sex determining locus on the short arm. Additional research will be necessary to detail the genetic composition of the sex chromosomes in rainbow trout.

Future uses: The rainbow trout lines developed here represent an extremely valuable resource and will be maintained for future use. Indefinite preservation of the DH lines can be accomplished by cryopreservation of sperm from DH males or sex reversed DH females and the lines can be regenerated in the future by androgenesis. Cryopreservation has been shown to be a reliable method of storing salmonid sperm (Stoss 1983; Wheeler and Thorgaard 1991; Rana 1995) and provides a relatively inexpensive and stable method of preserving the DH panel. These lines are analogous to RI lines used in mapping and genetic analysis of crop plants and vertebrate models such as mice and rats and the powerful techniques developed using these species should be important to the utilization of these rainbow trout lines. Great advances have been made using RI lines in laboratory models such as mice and rats and important agricultural crops, and similar advances made with rainbow trout would greatly benefit the commercial and research potential of the species.

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