Genetica **101:** 209–213, 1998.
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Identification of sex chromosome molecular markers using RAPDs and fluorescent *in situ* hybridization in rainbow trout

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Received 16 June 1997 Accepted 4 January 1998

Key words: rainbow trout, sex chromosome markers, RAPD, FISH

Abstract

The goal of this work is to identify molecular markers associated with the sex chromosomes in rainbow trout to study the mode of sex determination mechanisms in this species. Using the RAPD assay and bulked segregant analysis, two markers were identified that generated polymorphic bands amplifying preferentially in males of the Mount Lassen and Scottish strains of rainbow trout. Chromosomal localization using fluorescent *in situ* hybridization of a 900 bp probe developed from one of these markers revealed a brightly defined signal on a chromosome that could morphologically be classified as the Y chromosome.

Introduction

Several studies have shown the existence of an XX/XY sex chromosome system in rainbow trout (*Oncorhynchus mykiss,* Walbaum). The cytogenetic evidence, including surveys in different populations of this species, chromosome banding, and the characterization of the meiotic synapsis during male gametogenesis, indicates that the X and Y chromosomes are morphologically similar and in their initial stages of differentiation (Thorgaard, 1977; 1983; Hartley, 1987; Oliveira et al., 1995). In contrast to what has been observed in other salmon species such as *O. tschawytscha*, *O. kisutch*, and *Salvelinus namaycush* (Devlin et al., 1991; Forbes et al., 1994; Reed, Bohlander & Phillips, 1995), the search for DNA molecular markers for identifying the heterogametic sex in trout has not been successful (Ferreiro, Medrano & Gall, 1989; Nakayama et al., 1994).

Molecular genetic techniques could be important tools in the study of these chromosomes, especially in the characterization of specific markers, to clarify the differentiation mechanisms of sexual chromosomes and the mode of sex determination in this species. Moreover, the identification of a specific sex-linked marker can be useful for sex diagnosis in early stages of development.

The RAPD assay (Random Amplified Polymorphic DNA) described by Williams et al. (1990) and Welsh and McClelland (1990) has become a widely used method to identify DNA polymorphisms for genetic analysis in plants and in animals (Cushwa & Medrano, 1996). The application of the RAPD assay together with bulked segregant analysis (Giovanonni et al., 1991; Michelmore, Paran & Kisseli, 1991) has created an efficient approach to identify genetic markers associated with specific regions of the genome (Horvat & Medrano, 1996). The method of bulked segregant analysis uses DNA pools of segregant individuals for a particular trait. The two bulks are screened with RAPD primers to detect polymorphisms, which can be associated to the character or chromosome region of interest. Furthermore, RAPD markers of interest can be converted into sequence-characterized amplified regions, or SCARs, to be used as PCR-based genetic markers with potential applications in physical mapping (Paran & Michelmore, 1993; Cushwa et al., 1996).

In insects (Traut, 1994), birds (Levin, Crittenden & Dodgson, 1993; Griffiths & Tiwari, 1993), mammals (Wardell et al., 1993; Cushwa & Medrano, 1996;

Gutierrez et al., 1997), and plants (Hormaza, Dollo & Polito, 1994) sex-specific markers using RAPDs have been identified. The goal of the work discussed herein was to utilize the RAPD assay and bulked segregant analysis to search for molecular markers associated with the sex chromosomes in a random sample of two cultivated populations of rainbow trout. Our screening allowed the identification of RAPD markers that preferably amplify in the male genome of the two strains. One of these RAPD markers was also localized to a chromosome using fluorescent *in situ* hybridization.

Materials and methods

Adult and sexually mature specimens of rainbow trout were used from a strain from Mount Lassen Trout Farm, California and from the Scottish strain maintained at the Rio Blanco-UCV Hatchery, V Region, Chile.

From each previously anesthetized specimen, a blood sample was collected from the lateral vein in 5-ml EDTA-vacutainer tubes. DNA sample-pools of each sex were made by mixing an aliquot of 30 μl of blood from 12 males and 12 females of the Mount Lassen strain of rainbow trout. DNA was extracted from red blood cells by digestion with proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Several experiments were made to optimize the reproducibility of the RAPD assay, following very closely the protocol of Horvat and Medrano (1996). Amplification reactions (15 μl) contained: 1x Stoffel PCR buffer, 100 μM of each dNTP, 4 mM $MgCl_2$, 0.4 μM of primer (random decamers), 1.5 units of the Amplitaq DNA polymerase-Stoffel fragment (Perkin Elmer Cetus, Norwalk, Conn.), 25 ng of template DNA, and a mineral oil overlay of 50 μl. Reamplification reactions were carried out using 0.5 U of Taq DNA polymerase (Promega). PCR reactions were performed using MJ Research PTC-100 96-V thermal cyclers. The amplification profile was 94 °C (2 min), 3 cycles at 94 °C (1 min), 35 °C (1 min), 72 °C (2 min), followed by 32 cycles at 94 °C (10 s), 35 °C (30 s), and 72 °C (1 min; last cycle 5 min). PCR products of both DNA pools were electrophoresed in adjacent lanes in 1.8% agarose gels stained with ethidium bromide and visualized under UV light. Two pools of male and female rainbow trout DNA were screened with Operon (OP, Operon Tech., Alameda, CA) and University of British Columbia (UBC, Vancouver, British Columbia) RAPD primers. To determine the degree and specificity of the observed polymorphism, primers amplifying bands in only one DNA pool were examined in all individual males and females that made up the pools.

Fluorescent *in situ* hybridization (FISH) was carried out in the Scottish strain chromosome preparations obtained from peripheral blood lymphocyte cultures (Colihueque et al., 1992). A 898 bp SCAR fragment, designated as P9All, was used as a probe. P9All was developed from a band that amplified only in males of the Mount Lassen rainbow trout strain using RAPD primer OP-P9. The probe was biotinylated using a PCR Nonradioactive Labeling System kit Gibco (BRL) precipitated with ethanol and dissolved in hybridization solution (50% formamide, 2 × SSC, and 10% dextran sulfate). Chromosome preparations aged for more than two days were dehydrated in a series of ethanol gradient solutions. Chromosomes and probe were denaturated simultaneously in a flat plate block at temperatures between 70 °C and 80 °C for 5 min. Slides were flooded with 11 μl of hybridization mixture and incubated overnight at 37 °C. Hybridization signals were detected with avidin-fluorescein and two rounds of antiavidin-biotin amplification using the Oncor Chromosome In situ Kit (Oncor, Inc.). As a counterstain, propidium iodide was used. Observations and photographs were made using an Optiphot Nikon Microscope with the appropriate filters.

Results and discussion

Screening of the male and female DNA pools of the Mount Lassen strain with 900 RAPD primers identified 8 potential markers that produced polymorphisms between the pools. However, after analyzing the individuals in the DNA pools, only two primers (OP-A11 5′-CAATCGCCGT-3′ and OP-P9 5′-GTGGTCCGCA-3′) were found to generate a predominantly-male polymorphism. Primer OP-A11 generated a marker band of approximately 650 bp, which was present in 9 of the 12 males in the DNA pool and absent in all the females (Figure 1). A similar result was observed in rainbow trouts of the Scottish strain, where 13 out of 20 males and none of 14 females examined showed the OP-A11 polymorphism. Primer OP-P9 revealed a polymorphism of approximately 390 bp in the Mount Lassen strain that was present in all males and absent in all the females (Figure 2). However, in the Scottish strain, the OP-P9 390 bp polymor-

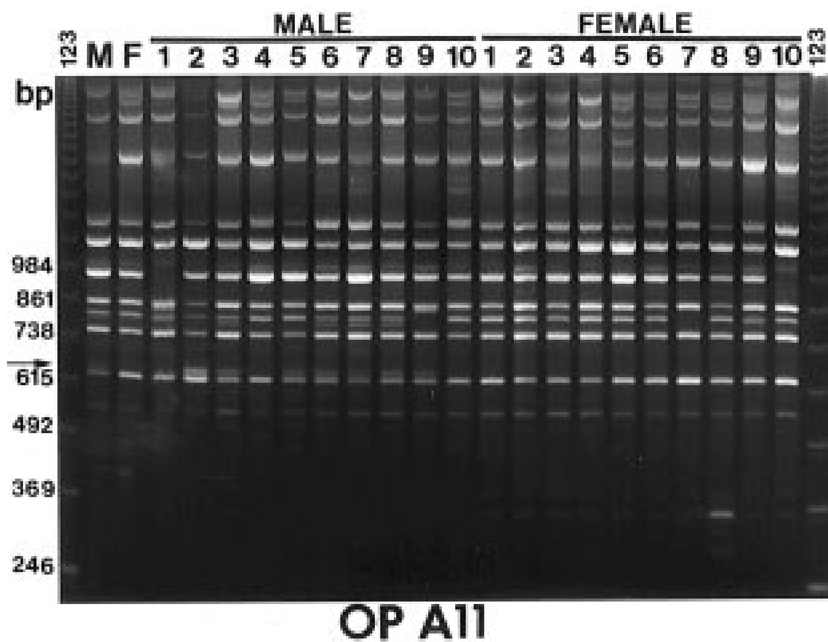

Figure 1. Agarose gel (1.8%) of RAPD PCR amplification products using primer OP-A11. Lane 1, 123 bp ladder size marker; M and F, combined DNA pools of 12 male and 12 female Mount Lassen rainbow trout; Male 1–10 and Female 1–10 represent 10 of the individual males and females included in the respective DNA pools. Arrow indicates the 650 bp band that amplified in 75% of the males and none of the females.

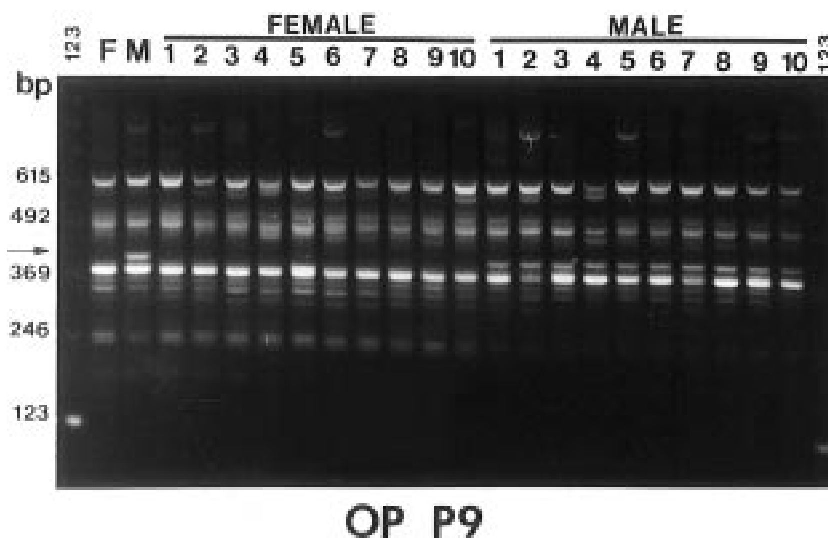

Figure 2. Agarose gel (1.8%) of RAPD PCR amplification products using primer OP-P9. Lane 1, 123 bp ladder size marker; M and F, combined DNA pools of 12 male and 12 female Mount Lassen rainbow trout; Male 1-10 and Female 1-10 represent 10 of the individual males and females included in the respective DNA pools. Arrow indicates the 390 bp band that amplified in all of the males and none of the females.

phic band appeared in 38% of the females, although it consistently amplified in all males.

The observed differences in RAPD marker polymorphisms between the Mount Lassen and the Scottish strains are not surprising given that there is a high degree of differentiation among cultured populations of rainbow trout. Several RAPD primers amplify specific Y chromosome markers in birds and mammals (Levin, Crittenden & Dodgson, 1993; Griffiths & Tiwari, 1993; Wardell et al., 1993). In contrast, we found only two

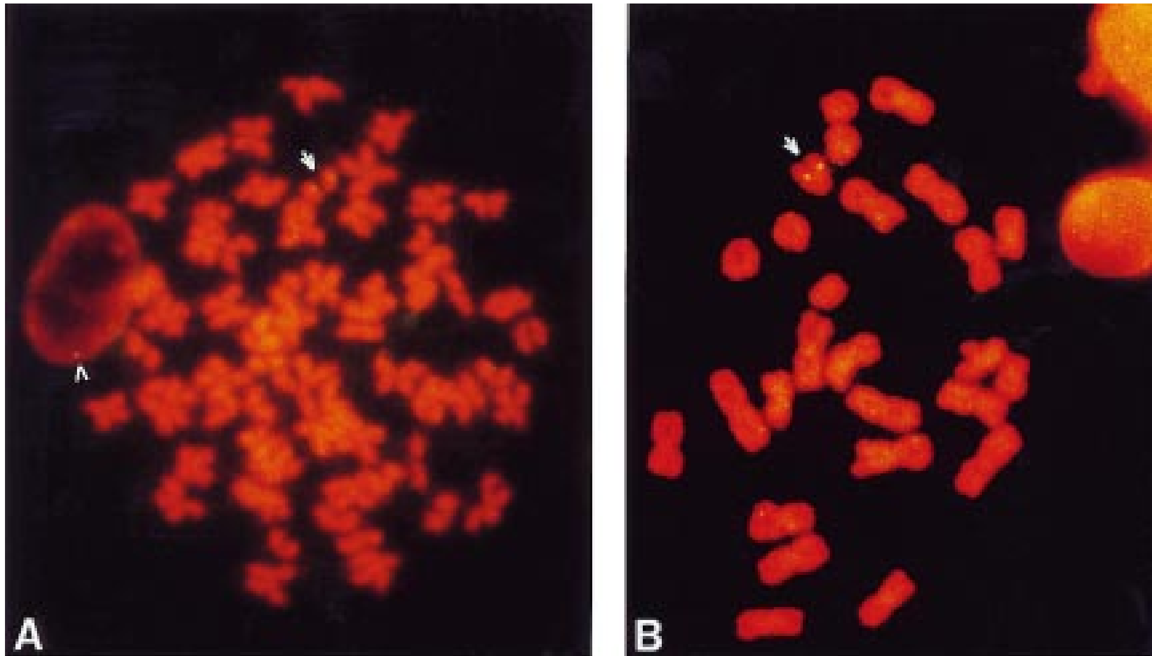

Figure 3. Fluorescence *in situ* hybridization of biotinylated SCAR probe P9All detected with FITC in male rainbow trout chromosomes (A) metaphase plate, (B) partial metaphase plate. Arrows indicate fluorescent spot in the putative Y chromosome. Arrowhead shows a single spot in the interphase nucleus.

RAPD sex-associated markers in rainbow trout. This may be explained because in this species the X and Y chromosomes are morphologically and genetically similar (Thorgaard, 1977, 1983; Allendorf, Gellman & Thorgaard, 1994). Therefore, the identification of sex-associated markers, like OP-A11 and OP-P9, can be considered as a molecular entry point to study sex chromosome differences in this species. The present markers could be used for sexing rainbow trout in specific strains, like Mount Lassen, but further development is necessary, such as studying the nature and variability of the DNA sequences of these markers in a larger sample of the population. The development of markers that could be physically mapped by *in situ* hybridization will be important in understanding the characteristics of the sex-determining mechanisms in trout. Work is in progress to examine some of these aspects.

FISH results are shown in Figure 3A, B. In the karyotype of rainbow trout males, hybridization signals were observed in both cromatides of a uniarmed chromosome. The morphological characteristics of this chromosome suggest that it could correspond to the rainbow trout Y chromosome (Thorgaard, 1977, 1983). By increasing the amplification rounds we also observed weak signals on the X chromosome, which can be morphologically identified. The increase in amplification rounds also increases the background, making it difficult to discard the possibility that target sequences also exist on autosomes. The difference in the intensity of the signals between the X and the putative Y chromosome could be explained by differences in copy number of the target sequences and/or organization of these sequences as a result of the process of Y chromosome differentiation in this species (Devlin et al., 1991). It is possible that in some strains of rainbow trout, copies of the highly repetitive Y chromosome P9 sequences appeared by recombination with the X chromosome. These results confirm that RAPD markers can be converted into probes that can be physically mapped to specific chromosomes using *in situ* hybridization techniques (Cushwa & Medrano, 1996; Cushwa et al. 1996).

Acknowledgements

This work was supported in part by grant Fondecyt 1940371. We thank Alison Van Eenennaam and Alma Islas for their assistance in the preparation of the RAPD

gel and picture. We also thank Mount Lassen Trout Farm, Redbluff, California for providing rainbow trout samples and Río Blanco-UCV Fish Hatchery, Chile.

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