



Characterization of sex chromosomes in rainbow trout and coho salmon using fluorescence *in situ* hybridization (FISH)

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Abstract

With the aim of characterizing the sex chromosomes of rainbow trout (*Oncorhynchus mykiss*) and to identify the sex chromosomes of coho salmon (*O. kisutch*), we used molecular markers OmyP9, 5S rDNA, and a growth hormone gene fragment (*GH2*), as FISH probes. Metaphase chromosomes were obtained from lymphocyte cultures from farm specimens of rainbow trout and coho salmon. Rainbow trout sex marker OmyP9 hybridizes on the sex chromosomes of rainbow trout, while in coho salmon, fluorescent signals were localized in the medial region of the long arm of one submetacentric chromosome pair. This hybridization pattern together with the hybridization of a *GH2* intron probe on a chromosome pair having the same morphology, suggests that a submetacentric pair could be the sex chromosomes in this species. We confirm that in rainbow trout, one of the two loci for 5S rDNA genes is on the X chromosome. In males of this species that lack a heteromorphic sex pair (XX males), the 5S rDNA probe hybridized to both submetacentrics. This finding is discussed in relation to the hypothesis of intraspecific polymorphism of sex chromosomes in rainbow trout.

Introduction

Unlike mammals and birds, there are certain fish groups that are characterized by sex chromosomes (XY or ZW) that are morphologically indistinguishable or only slightly differentiated in the karyotype, even when different chromosome banding methods are used. In many cases, the heterogametic sex has been identified by experimental crosses with sex-reversal specimens (Yamazaki, 1983). Among these fish groups the salmonid species have heterochromosomes with differing stages of morphological and genetic differentiation. Heteromorphic chromosomes have only been described in three species. In lake trout, (*Salvelinus namaycush*) that differ in the heterochromatin of the sex pair, the X chromosome has a C-band that is Q bright on the short arm, but absent in the Y chromosome (Phillips & Ihssen, 1985; Phillips & Hartley, 1987). A multiple sex chro-

mosome system $X_1X_2Y-X_1X_1X_2X_2$ characterizes the sockeye salmon (*Oncorhynchus nerka*) which has a metacentric Y chromosome, possibly as a result of a Robertsonian translocation (Thorgaard, 1978). The sex pair in rainbow trout (*O. mykiss*) is characterized by a small difference in the length of the short arm between submetacentric X and Y-chromosomes (Thorgaard, 1977; Thorgaard, 1983). Utilizing banding techniques, it has been demonstrated that the X chromosome has a pericentromeric heterochromatin band and that the region shows a brilliant band, whether stained with DAPI or Hoescht33258/Actinomycin D (Colihueque et al., 1992; Moran et al., 1996). Moreover, one of the 5S ribosomal DNA gene loci has been localized on the X-chromosome by fluorescent *in situ* hybridization (FISH) (Moran et al., 1996; Fujiwara et al., 1998). In spite of these differences, the X and Y chromosomes pair homologously along their whole length during meiosis, suggesting a close homology

between both chromosomes (Oliveira et al., 1995). Genetic conservation between these chromosomes has also been suggested because of the viability shown by androgenetic YY specimens (Parson & Thorgaard, 1985). The X and Y-chromosomes of rainbow trout appear to be in an initial stage of differentiation. This hypothesis is supported by the finding that some male specimens from different natural and cultured populations show absence of heteromorphism between X and Y chromosome (Thorgaard, 1977, 1983; Colihueque et al., 2001).

In coho salmon (*O. kisutch*) which has a karyotype of 60 chromosomes and NF110, heteromorphic sex chromosomes have not been revealed even using chromosome banding methods (Hartley, 1987; Lozano et al., 1991; Colihueque, 1998). Genetic evidence indicates, however, that in this species an XY sex determination system is operating (Hartley, 1987).

The search for specific sex chromosome DNA markers in diverse fish species has permitted the isolation of repetitive sequences associated with these chromosomes (Devlin et al., 1991; Nanda et al., 1993; Nakayama et al., 1994; Reed & Phillips, 1995; Devlin et al., 1998). Knowledge of sex-linked genes in salmonids is still scarce, though some anonymous DNA sex-linked markers have been identified in rainbow trout as a result of recently developed genetic linkage maps (Young et al., 1998; Sakamoto et al., 2000). In the genome of various salmonid species the presence of duplicated growth hormone genes and of sex linked sequences of pseudogenes has been demonstrated (Du, Devlin & Hew, 1993; McKay, Devlin & Smith, 1996). Through linkage studies in progenies using restriction enzyme polymorphisms, Forbes et al. (1994) established that one of the *GH* genes is found in the sex chromosomes of coho salmon. Similar results have been recently obtained for the *O. masou* species complex (Nakayama et al., 1998). In previous studies, we have characterized the molecular marker OmyP9, and using genetic analysis in crosses we have shown that this marker is localized in the sex chromosomes of rainbow trout. OmyP9 is possibly a repeat sequence with differing degrees of representation in the sex chromosomes, as shown by FISH in male chromosomes of this species (Iturra et al., 1998).

In the present study, we use molecular markers OmyP9, 5S rDNA and a *GH2* gene fragment as probes for FISH with the aim of characterizing the sex chromosomes in rainbow trout and to identify the sex chromosomes in coho salmon.

Materials and methods

Metaphase plates were obtained by culturing peripheral blood lymphocytes from sexually mature adult specimens of rainbow trout, Cofradex, Americana and Escocesa strains and cultured coho salmon from Piscícola Huililco Ltda., Pucón, Region IX, Chile (Colihueque et al., 2001). To prepare the FISH probes, the same specimens were used to extract genomic DNA from red blood cells by conventional methods described previously (Iturra et al., 2001).

Probes and FISH

OmyP9

This 898 base pair (bp) sequence (GenBank Accession no. AF323613) originated from a sex-linked RAPD marker isolated from the rainbow trout genome of this species. This marker has variants A, B and C identified by a *RsaI* restriction polymorphism. We demonstrated that variant A is localized on the Y chromosome for the males studied (Iturra et al., 2001). Using OmyP9-specific primers a sequence in coho salmon was amplified that was 84% identical to OmyP9 of rainbow trout. The OmyP9 FISH probe was made following the procedure outlined by Iturra et al. (1998). Two males and four females of rainbow trout and three males and four females of coho salmon were studied.

5S rDNA

Primers A; 5'-TACGCCCCGATCTCGTCCGATC-3' and B; 5'-GCTTACGGCCATACCAGCCTG-3' (Pendas et al., 1994) were used in genomic DNA to PCR amplify a fragment corresponding to the coding region +NTS of the 5S rDNA genes of rainbow trout. The PCR reaction mix and amplification conditions used were as described by Moran et al. (1996). The PCR product was subjected to electrophoresis and a band of approximately 300 bp was eluted from the gel and used as a template for reamplification using biotin-dCTP (Gibco) to obtain a FISH probe.

GH2

Primers GH48; 5'-CAATACCATTTGTGGT-3' and GH53; 5'-ACAGAGAGAGATCGATGG-3' were used in genomic DNA to PCR amplify a 900 bp intron sequence of the *GH* gene (McKay et al., 1996). The amplified fragment was labeled with biotin and used as a hybridization probe. This fragment was purified from agarose gel using a QIAquick Gel extraction kit (Qiagen) and sequenced in an automated ABI 377

sequencer to confirm it corresponded to the *GH2-D* intron. Three males and three females of coho salmon were studied.

FISH was performed following the protocol previously described (Iturra et al., 1998). Briefly, dehydrated chromosomes and the respective probe were simultaneously denatured in a flat plate block at 80–85°C for 5 min. Hybridization signals were detected using the Oncor Chromosome In Situ kit (Oncor, Inc.). In some protocols, one to four rounds of avidin-fluorescein amplification were performed. Chromosomes were stained with propidium iodide and the slides were mounted using the Vectashield (Vector Laboratories Inc.) antifade solution. In some slides, chromosomes were also sequentially stained with DAPI (4',6-diamidino-2-phenylindole) to identify the X chromosomes (Colihueque et al., 1992). Metaphase plates were evaluated and photographed in a Nikon Optiphot microscope equipped with the appropriate fluorescent filters. PhotoshopTM version 5.0 (Adobe) computer software was used for processing the images.

Results

Figure 1(A) shows hybridization signals with OmyP9 in the karyotype of a female rainbow trout. The signals can be observed in the medial region of the long arm of both chromatids of one of the two members of the X chromosome pair. Successive rounds of amplification revealed a fluorescent mark in both X-chromosomes, although non-specific signals in metaphase plates increased. The different response to the probe detection reaction between both X chromosomes possibly reflects differences in the copy number of the OmyP9 target sequence. The X-chromosomes were identified by the characteristic pericentromeric brilliant DAPI band (Figure 1(B)).

OmyP9 hybridization in coho salmon chromosomes is shown in Figure 2. Of 116 metaphases analyzed, signs of hybridization were consistently observed in a pair of subtelocentric chromosomes corresponding to pair 25 or 26 in accordance with Colihueque (1998). The hybridization site was localized to the medial region of the long arm of the chromosomes. In some metaphase plates, hybridization signals were observed on one of the chromatids (Figure 2(A)). This result is not uncommon as has already been observed in other FISH studies from different species (Benabdelmouna et al., 1999). In our study, signal detections

on the respective sister chromatids were obtained with successive rounds of amplification (Figure 2(B)).

The probe constructed from the *GH2-D* intron sequence showed a pattern of fluorescent signals mainly associated with a pair of subtelocentric chromosomes. The hybridization sites were in close proximity to the telomere in males and females (Figure 3). Weak signal was also observed on the short arm of a single subtelocentric chromosome in a position close to the centromere in both sexes. This chromosome is different from the subtelocentrics with telomeric signals. All these chromosomes have similar morphology to that identified by hybridization with the OmyP9 probe.

The 5S rDNA probe was identified on the pericentromeric region of the X chromosome in both male and female rainbow trout, showing a much more intense signal than that observed in the short arm of the NOR bearing chromosomes. Among the five specimens studied, two were males with morphologically indistinguishable X and Y chromosomes with two similar subtelocentric chromosomes (XX males). One of these individuals was of the Scottish strain with $2n = 58$ and the other one, of Cofradex strain showed $2n = 60$, both with NF104. In these specimens, 5S rDNA hybridization was detected on the pericentromeric region of both chromosomes (Figure 4(A)). Different intensity of the hybridization signs among the two subtelocentric chromosomes was observed in all the metaphase plates examined. These slides were then stained with DAPI, showing that both subtelocentric chromosomes had a pericentromeric brilliant band (Figure 4(B)).

Discussion

The use of DNA markers as probes for FISH contributes to the study of fish sex chromosomes, not only because the method provides greater knowledge of the structure of these chromosomes, but also because it makes it possible to compare the genomes of different species. Our results show that the OmyP9 probe is informative as a comparative approach to studying the sex chromosomes in both related species of rainbow trout and coho salmon. OmyP9 is localized in the X chromosomes in rainbow trout, which is consistent with our previous FISH results in males of this species where we also found hybridization signals on the Y chromosome. OmyP9 was also found in the coho salmon genome localized to the medial region of a subtelocentric pair in this species. It is

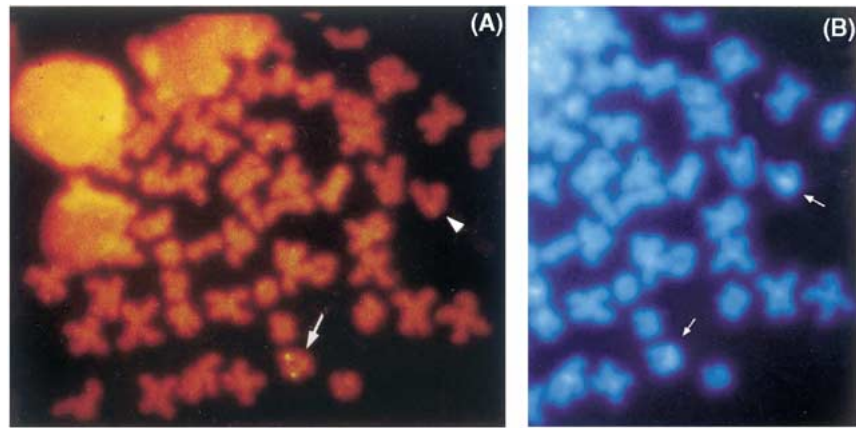


Figure 1. (A) FISH detection with biotin-labeled OmyP9 marker in female rainbow trout chromosomes. Arrow indicates a single X chromosome with fluorescent signal. Arrowhead shows the other X chromosome. (B) DAPI staining of the same female metaphase plate. Arrows show the X chromosomes with their typically brilliant DAPI bands.

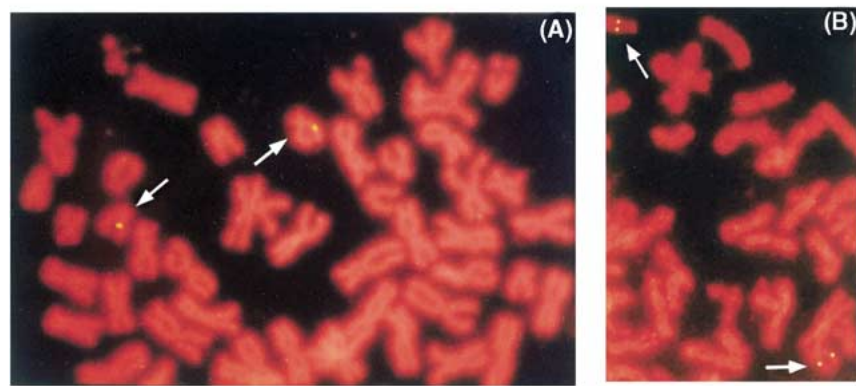


Figure 2. FISH detection with biotin-labeled OmyP9 to coho salmon chromosomes. (A) Arrows indicate fluorescent spots on the medial region of long arm in one of the chromatids of subtelocentric chromosomes. (B) Arrows show signals detection on both chromatids of subtelocentric chromosomes after successive rounds of amplification.

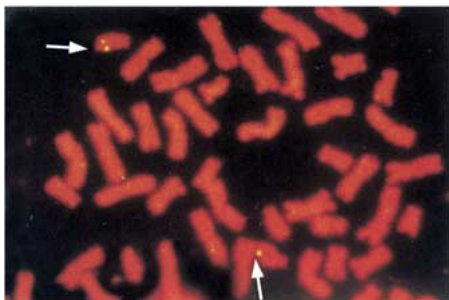


Figure 3. FISH detection with biotin-labeled *GH2*-intron D fragment to coho salmon chromosomes. Arrows indicate hybridization spots on the distal region of the long arm of a subtelocentric chromosome pair.

interesting to note that this hybridization pattern is in accordance with that observed in rainbow trout, where OmyP9 is located in a similar position in the

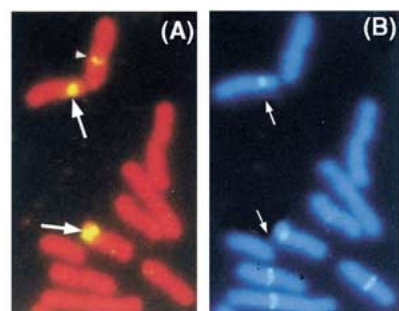


Figure 4. FISH of 5S rDNA genes in 'XX' male rainbow trout chromosomes. This male has not heteromorphism between X and Y chromosomes. (A) The arrows show the location of 5S rDNA hybridization signals on two subtelocentric chromosomes. Arrowhead show signal location on one of the NOR bearing pair. (B) DAPI staining of the same metaphase plate. Arrows show brilliant DAPI bands on the same 5S rDNA bearing subtelocentric chromosomes.

long arm of the X and Y chromosomes. As in rainbow trout, the sex chromosomes in sockeye salmon are also subtelocentric or uniarmed (Thorgaard, 1978). This chromosome comparison suggests conserved uniarmed morphology of sex chromosomes among these species. Given the evidence of the localization of the rainbow trout sex marker OmyP9, we suggest that this subtelocentric pair could be the sex chromosomes in coho salmon based as well as the morphological similarity of this pair with heterochromosomes of related species. Although this hypothesis needs further confirmation, if correct, our results would indicate that OmyP9 is a conserved sequence in the sex chromosomes of rainbow trout and coho salmon. In a manner similar to that observed in the rainbow trout, the increase of amplification rounds showed no specific weak signals in other chromosomes in coho salmon. As in rainbow trout, it is difficult to discard the possibility that OmyP9 or related sequences are present in other regions of the genome (Iturra et al., 1998).

For the differentiation process of sex chromosomes (X and Y) structural and/or DNA changes occur that are very important to the partial or complete suppression of crossing-over between the two primitive homomorphic chromosomes in the heterogametic sex (Ohno, 1967; Charlesworth, 1978). Accumulation of repetitive sequences, pseudogenes and transposable elements, has been detected in the sex chromosomes of fish species. This has suggested their participation in the early stages of differentiation of heteromorphic chromosomes and/or that their sole presence is a consequence of suppression of recombination between heteromorphic sex chromosomes (Nanda et al., 2000). Among salmonids, lake trout and chinook salmon, tandem repeat sequences characteristic of the sex chromosomes of each species have been isolated (Devlin et al., 1991; Reed & Phillips, 1995; Devlin, Stone & Smailus, 1998). OmyP9 is a repetitive sequence in rainbow trout located on the X and Y chromosome in a non recombinant chromosome region (Iturra et al., 2001). Although this sequence is present in the genome of coho salmon, and possibly on the sex chromosomes of this species, it could exhibit a different organization and different number of repeats in relation to rainbow trout (Devlin, Stone & Smailus, 1998). The hybridization of each marker on both members of a chromosome pair, presumably X and Y, supports the idea that they are at an early stage of differentiation in coho salmon.

The identification of subtelocentric chromosomes as possible sex chromosomes in coho salmon is, in

part, reinforced by the hybridization pattern of the *GH2-D* intron probe in chromosomes having the same morphology. It is expected that the *GH2-D* probe may hybridize in more than one chromosome pair, as more than one locus exists for the growth hormone gene (Du, Devlin & Hew, 1993). Just now, our results do not allow us accurately to establish which ever of the subtelocentric chromosomes with *GH2-D* signs of hybridization could correspond to the sex pair, since we need to demonstrate its chromosomal colocalization with OmyP9 probe. Studies of the distribution pattern of the 5S rDNA genes in the genome of salmonids indicates that these genes can occupy one or more loci (Pendás et al., 1994; Moran et al., 1996; Pardo et al., 2000). Our FISH analysis with the 5S ribosomal DNA probe in diverse rainbow trout strains confirms results described by Morán et al. (1996). As expected, fluorescent signals are localized in the NOR bearing chromosome pair and in males with heteromorphic sex chromosomes on one subtelocentric chromosome. In females they are localized in two of these chromosomes, which are recognized as the X chromosome in this species with DAPI banding. We agree with Moran et al. (1996), that with the FISH technique, it is not possible to rule out the presence of a minor cluster of the 5S ribosomal genes in the Y chromosome. To clarify this point we would need to identify polymorphisms in this marker in order to perform genetic analyzes in the progeny. On the other hand, in rainbow trout males with no morphological differences among X and Y chromosomes (XX males) the hybridization of the 5S rDNA marker in two subtelocentric chromosomes, which also show a bright DAPI band, indicates that both subtelocentrics share a common chromosomal domain that includes heterochromatin and one of the 5S ribosomal gene loci. The second subtelocentric chromosome may correspond to an extra X chromosome in the karyotype of these males, given the XXY condition in this species. This explanation does not seem plausible since both individuals present NF104 as has been discussed by Colihueque et al. (2001). We cannot rule out that in different rainbow trout populations a structural chromosome rearrangement could have occurred as a non-reciprocal translocations among an X chromosome and an autosome, or an X chromosome and the Y chromosome. However, Thorgaard (1977, 1983) suggests an intraspecific polymorphism in the sex chromosome pair in rainbow trout where some populations retained an undifferentiated Y chromosome. The 'extra X' chro-

mosome present in the karyotype of the males studied could thus correspond to an ancestral Y chromosome, which remains conserved with respect to the X chromosome in both its heterochromatin constitution as well as the presence of 5S rDNA genes. In this case, structural changes such as deletions could partly accompany sex chromosome differentiation in rainbow trout. In addition, in our ongoing studies of the coho salmon karyotype (three males and three females), we have found that females have one locus for 5S rDNA genes, whereas the males show three hybridization signals, one of those being localized in only one subtelocentric chromosome in a similar position to that observed for the rainbow trout X chromosome. This may thus correspond to the coho salmon Y chromosome (Lam et al., in preparation). Further studies on the genomic distribution of 5S rDNA and new sex chromosome markers in *Oncorhynchus* species will help to understand the genome reorganization mechanisms and processes involved in the sex chromosome differentiation in this species.

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