MAPPING AND EVOLUTION OF CANDIDATE SEX DETERMINING LOCI, SEX CHROMOSOMES, AND SEX LINKED SEQUENCES IN RAINBOW AND CUTTHROAT TROUT

By

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To the Faculty of Washington State University:

The members of the committee appointed to examine the dissertation of MAHMOUD AHMAD ALFAQIH find it satisfactory and recommend that it be accepted.

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Abstract

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Fish provide an intriguing model for the study of how sex determination mechanisms evolve. Salmonids have an XX-XY mechanism for sex determination where males are the heterogametic sex. Sex chromosomes of most salmonid species can not be cytogenetically distinguished suggesting that they are at an early stage of evolution. Salmonids have also evolved from a common tetraploid ancestor 25-100 million years ago. The role of genome duplications in shaping the karyotype of vertebrates is well established. The presence of a genome duplication event in salmonids' recent evolutionary history makes them a good model for understanding the structuring of genomes following polyploidization. This dissertation examines the evolution of sex determination mechanisms, and the evolution of duplicated loci following polyploidization using the two model salmonid species rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout (*Oncorhynchus clarki*). In this dissertation, we first show that unlike other Pacific salmon and trout species examined, rainbow and cutthroat trout have their sex locus on the same linkage group which appears to be largely homologous. We then use a BAC clone

containing sequences of one of the markers used to establish synteny between the sex chromosomes of the two species, to identify the Y chromosome of cutthroat trout. We also test the linkage of a number of candidate sex-determining genes to the Y chromosome of rainbow trout. We exclude the role of all of the tested genes as candidates for sex-determination. We also describe duplication of one of the candidate genes, *Sox6* and discuss its inheritance in our mapping families. *Sox9*, a candidate sex-determining gene is part of a chromosomal block shown to be conserved among different species. Recent evidence indicates conservation of the block in rainbow trout. Two *Sox9* gene copies were isolated from rainbow trout, *Sox9* and *Sox9α2*. Previous evaluations of their evolutionary history gave ambiguous results. We hypothesize that mapping of *Sox9* and *Sox9α2* would allow us to more properly evaluate their evolutionary relationship. The map position of *Sox9* and *Sox9α2* genes indicates that *Sox9* belongs to the *Sox9b* clade while *Sox9α2* belongs to the *Sox9a* clade.

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CHAPTER ONE

GENERAL INTRODUCTION

Polyploidy has long been proposed to have played a major role in structuring the vertebrate genome (Ohno 1970). Two rounds of genome duplication are hypothesized to have occurred before divergence of all vertebrates (Hughes and Friedman 2003; Sidow 1996).With over 24,000 species, teleost fishes encompass about half the number of this phylum (Nelson 1994). The astounding morphological diversity seen among teleost species is speculated to have been promoted by an additional genome duplication event that preceded teleost radiation. This hypothesis is strongly supported by the presence of additional genes and genomic clusters in a number of teleosts (Amores et al. 1998; Christoffels et al. 2004; Naruse et al. 2004). However, despite the established importance of polyploidy in driving vertebrate genome evolution, our understanding of the molecular organization of genomes following polyploidy is still in its early stages. A better understanding of the mechanisms involved in the structural differentiation of duplicated homologous chromosomes into two distinct homeologues, and the fate of duplicated loci within these chromosomes is essential for this process.

Salmonidae is a monophyletic group of teleost fish containing twelve genera and some 72 species (Nelson 1994). All species of this single lineage descended from a common ancestor which underwent an auto-tetraploidization event 25-100 million years ago (Allendorf and Danzmann 1997; Allendorf and Thorgaard 1984). Salmonids have twice as many chromosome arms as other teleosts (Phillips and Rab 2001). In addition to chromosome number counts, the tetraploid ancestry of salmonids is supported by genome size estimates (Ohno et al. 1968) and patterns of gene duplication(Allendorf et al. 1975). Diploidization across salmonid genomes is

still not complete (Allendorf and Danzmann 1997). Tetrasomic segregation ratios of duplicated loci which occurs in males indicate that some chromosomes can still exchange genetic material with their ancestral duplicates (Johnson et al. 1987).

Salmonids appear to have undergone extensive chromosomal variation following the genome duplication event (Allendorf and Thorgaard 1984; Phillips and Rab 2001). The diploid chromosome number of salmonid species ranges from 52 to 102 chromosomes (Hartley 1987). However, most salmonid species share a fairly similar number of chromosome arms (NF~100). This fact strongly supports that centric fusions were the predominant form of genetic rearrangement that followed the genome duplication event (Allendorf and Thorgaard 1984). More recently (Phillips and Rab 2001), salmonid species that have unusual number of chromosome arms were documented; Lenoks (NF=110-116), huchens (NF=112-116) and graylings (NF=146-170). Since Robertsonian translocations generally do not lead to changes in chromosome arm numbers, other types of rearrangements must have been involved in shaping the karyotype of the aforementioned taxa. Possible rearrangements may have involved tandem fusions and/or pericentric inversions.

In conclusion, salmonids are one of the better models for studying polyploidization and genome evolution. A comprehensive understanding of the evolutionary forces that shape vertebrate genome following polyploidization requires the isolation and comparative mapping of genes and gene families across different salmonid species; such an analysis would allow us to identify conserved and distinctive evolutionary patterns pertaining to genome duplication events.

Fishes are also one of the most interesting research models for the exploration of how sex determining mechanisms evolve (Devlin and Nagahama 2002). Because of their tremendous

diversity (Nelson 1994), fishes not only provide unique examples of sex determination mechanisms, they also posses sufficient diversity to share sex determination processes and pathways of other vertebrates. Cumulative research indicates that fishes possess a wide variety of evolutionarily labile sex determination mechanisms which have evolved repeatedly among different lineages (Devlin and Nagahama 2002).

Salmonids have an XX-XY genetic mechanism for sex determination where males are the heterogametic sex (Allendorf and Thorgaard 1984; Devlin and Nagahama 2002; Donaldson and Hunter 1982). The sex-determining mechanism is believed to function via a 'dominant' factor located on the Y chromosome as evidenced by the production of XXY triploid rainbow trout (*Oncorhynchus mykiss*) males (Thorgaard and Gall 1979). Male heterogamety is also supported by the analysis of male to female ratios in the progeny of crosses that involve hormonally sex reversed individuals; rainbow trout females XX, which are sex reversed to males, produce all-female progeny when crossed to normal XX females (Johnstone et al. 1978). This is further supported by the identification of a variety of allozyme, AFLP and microsatellite markers that map close to the sex locus in a number of salmonid species (Allendorf et al. 1994; Du et al. 1993; Felip et al. 2004; Nichols et al. 2003; Sakamoto et al. 2000).

The sex chromosomes of all salmonids identified to date have small non-recombining regions and large pseudo-autosomal regions (Artieri et al. 2006; Phillips 2002; Phillips et al. 2007; Phillips et al. 2005; Phillips and Ihssen 1985; Stein et al. 2001; Thorgaard 1977). Small morphological differences between the X and Y chromosomes have been identified in lake trout (*Salvelinus namaycush*) (Phillips and Ihssen 1985), brook trout (*Salvelinus fontinalis*) (Phillips 2002), and rainbow trout (Thorgaard 1977). However, some rainbow trout populations have

cytogenetically indistinguishable sex chromosomes suggestive of differences in the level of their differentiation (Felip et al. 2004; Thorgaard 1983). This finding indicates the presence of intraspecific polymorphisms in salmonid sex chromosomes. The aforementioned observations indicate that the sex chromosomes of salmonids are at an early evolutionary stage where genetic rearrangements differentiating the sex chromosomes are still in the process of fixation. This makes them an intriguing model for the study of sex chromosome evolution.

Accordingly, studying the sex differentiation pathway in salmonids is pivotal to understanding the early stages of sex chromosome evolution. If the loss of genetic material from the mammalian Y chromosome continues at the same rate, it might eventually disappear in 5-10 million years (Marshall Graves 2002). Consequently, deciphering the mechanism by which the sex chromosomes evolve in salmonids might provide us with candidate scenarios of how the sex-determining mechanism of mammals would re-evolve after the eventual fall of *Sry* (Marshall Graves 2002).

Comparative analysis of the Y chromosome linkage map of four salmonid species (Arctic char, *Salvelinus alpinus*; Atlantic salmon, *Salmo salar*; Brown trout, *Salmo trutta*; and rainbow trout) showed that the sex locus of each of the species is located on a different linkage group although the positioning of markers proximal to the sex locus is conserved among homologous autosomes (Woram et al. 2003). In fact, the only two salmonid species demonstrated to date to have the sex locus on a homologous shared chromosome are lake trout and brook trout, species that form viable hybrids and are completely inter-fertile (Phillips et al., 2002). One explanation for the lack of conservation of the sex chromosomes of different salmonid species could be the presence of a small DNA motif involved in the rearrangement of

the sex determining region across different species (Woram et al. 2003). This relocation might involve the presence of the sex-determining gene within a region that has transposed to a different linkage group in each species (Woram et al. 2003). Another explanation for the lack of sex chromosome homology could be the separate evolution of sex-determining mechanisms in almost every salmonid species (Woram et al. 2003).

The current view of the sex determination cascade describes it as a gene hierarchy (Wilkins 1995). Genes that reside on top of this hierarchy have been recruited only relatively recently (Marin and Baker 1998) and are only found in specific branches of the phylogenetic tree. However, downstream genes are conserved between the different phyla, regardless of the sex determination mechanism, and thus seem to have been present for a much longer time. The recent discovery that the sex determining gene of medaka is a homologue of a downstream gene of the pathway (Matsuda et al. 2002) led researchers to propose that downstream genes are recruited to the top of the hierarchy by gene duplication (Schartl 2004).

Three papers are presented in this dissertation in the form of manuscripts. In the first paper we show that rainbow trout and, its closest relative, cutthroat trout have their sex locus on the same linkage group. We also use a BAC clone, containing the sequence of one of the markers that genetically mapped close to the sex locus of rainbow and cutthroat trout, as a probe to identify the Y chromosome of cutthroat trout.

In the second paper, we test five loci of four candidate genes for their linkage to the Y chromosome of rainbow trout. We show that none of the loci tested could function as the "master switch" of the sex determination cascade. However, we report a salmonid-specific

duplication of one of the candidate genes and discuss inheritance of the duplicates in our mapping families.

In the third paper, we hypothesize that ambiguity in tracking the evolutionary history of *Sox9* and *Sox9α2* in rainbow trout can be resolved by mapping these loci. *Sox9* is part of a chromosomal block which has been shown to be conserved among a number of different species of different phyla, and recent evidence indicates conservation of the same block in rainbow trout. Our mapping result indicates that *Sox9* is duplicated in rainbow trout and belongs to the *Sox9b* clade, while *Sox9α2* belongs to the *Sox9a* clade. We finally propose that previous phylogenetic reconstructions failed to identify correct evolutionary relationships of the genes because of gene conversion between the two *Sox9* paralogs.

References cited

Allendorf FW, Danzmann RG (1997) Secondary tetrasomic segregation of MDH-B and preferential pairing of homeologues in rainbow trout. Genetics 145:1083-92

Allendorf FW, Gellman WA, Thorgaard GH (1994) Sex-linkage of two enzyme loci in *Oncorhyncus mykiss* (rainbow trout). Heredity 72 (Pt 5):498-507

Allendorf FW, Thorgaard GH (1984) Tetraploidy and the evolution of salmonid fishes The Evolutionary Genetics of Fishes. Plenum publishing, New York

Allendorf FW, Utter FM, May BP (1975) Gene duplication within the family Salmonidae: detection and determination of the genetic control of duplicate loci through inheritance

studies and examination of populations. In: Markert CL (ed) Isozymes. Academic Press, New York, p 415–431

Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH (1998) Zebrafish hox clusters and vertebrate genome evolution. Science 282:1711-4

Artieri CG, Mitchell LA, Ng SH, Parisotto SE, Danzmann RG, Hoyheim B, Phillips RB, Morasch M, Koop BF, Davidson WS (2006) Identification of the sex-determining locus of Atlantic salmon (*Salmo salar*) on chromosome 2. Cytogenet Genome Res 112:152-9

Christoffels A, Koh EG, Chia JM, Brenner S, Aparicio S, Venkatesh B (2004) Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes. Mol Biol Evol 21:1146-51

Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208:191-364

Donaldson EM, Hunter GA (1982) Sex control in fish with particular reference to salmonids. Canadian Journal of Fisheries and Aquatic Sciences 39:99-110.

Du SSJ, Devlin RRH, Hew CCL (1993) Genomic structure of growth hormone genes in chinook salmon (*Oncorhynchus tshawytscha*): presence of two functional genes, GH-I and GH-II, and a male-specific pseudogene, GH-psi. DNA and cell biology 12:739-51

Felip A, Fujiwara A, Young WP, Wheeler PA, Noakes M, Phillips RB, Thorgaard GH (2004) Polymorphism and differentiation of rainbow trout Y chromosomes. Genome 47:1105-13

Hartley SE (1987) The chromosomes of salmonid fishes. Biological Reviews 62:197-214

Hughes AL, Friedman R (2003) 2R or not 2R: Testing hypotheses of genome duplication in early vertebrates. Journal of Structural and Functional Genomics 3:85-93

Johnson KR, Wright JE, Jr., May B (1987) Linkage relationships reflecting ancestral tetraploidy in salmonid fish. Genetics 116:579-591

Johnstone R, Simpson TH, Youngson AF (1978) Sex reversal in salmonid culture. Aquaculture 13:115-134

Marin I, Baker BS (1998) The evolutionary dynamics of sex determination. Science 281:1990-4

Marshall Graves JA (2002) The rise and fall of SRY. Trends Genet 18:259-64

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417:559-63

Naruse K, Tanaka M, Mita K, Shima A, Postlethwait J, Mitani H (2004) A medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. Genome Res 14:820-8

Nelson JS (1994) Fishes of the World

Nichols KM, Young WP, Danzmann RG, Robison BD, Rexroad C, Noakes M, Phillips RB, Bentzen P, Spies I, Knudsen K, Allendorf FW, Cunningham BM, Brunelli J, Zhang H, Ristow S, Drew R, Brown KH, Wheeler PA, Thorgaard GH (2003) A consolidated linkage map for rainbow trout (*Oncorhynchus mykiss*). Anim Genet 34:102-15

Ohno S (1970) Evolution by Gene Duplication

Ohno S, Wolf U, Atkin NB (1968) Evolution from fish to mammals by gene duplication. Hereditas 59:169–187

Phillips R, Rab P (2001) Chromosome evolution in the Salmonidae (Pisces): an update. Biological Reviews 76:1-25

Phillips RB (2002) Characterization of Charr Chromosomes Using Fluorescence in Situ Hybridization. Environmental biology of fishes 64:223

Phillips RB, DeKoning J, Morasch MR, Park LK, Devlin RH (2007) Identification of the sex chromosome pair in chum salmon (*Oncorhynchus keta*) and pink salmon (*Oncorhynchus gorbuscha*). Cytogenet Genome Res 116:298-304

Phillips RB, Morasch MR, Park LK, Naish KA, Devlin RH (2005) Identification of the sex chromosome pair in coho salmon (*Oncorhynchus kisutch*): lack of conservation of the sex linkage group with chinook salmon (*Oncorhynchus tshawytscha*). Cytogenet Genome Res 111:166-70

Phillips RRB, Ihssen PPE (1985) Identification of sex chromosomes in lake trout (*Salvelinus namaycush*). Cytogenetics and cell genetics 39:14-8

Sakamoto T, Danzmann RG, Gharbi K, Howard P, Ozaki A, Khoo SK, Woram RA, Okamoto N, Ferguson MM, Holm LE, Guyomard R, Hoyheim B (2000) A microsatellite linkage map of rainbow trout (*Oncorhynchus mykiss*) characterized by large sex-specific differences in recombination rates. Genetics 155:1331-45

Schartl M (2004) Sex chromosome evolution in non-mammalian vertebrates. Curr Opin Genet Dev 14:634-41

Sidow A (1996) Gen(om)e duplications in the evolution of early vertebrates. Current Opinion in Genetics & Development 6:715-722

Stein J, Phillips RB, Devlin RH (2001) Identification of the Y chromosome in chinook salmon (*Oncorhynchus tshawytscha*). Cytogenet Cell Genet 92:108-10

Thorgaard GH (1977) Heteromorphic sex chromosomes in male rainbow trout. Science 196:900-2

Thorgaard GH (1983) Chromosomal differences among rainbow trout populations. Copeia 3:650-662

Thorgaard GH, Gall GAE (1979) Adults triploids in a rainbow trout family. Genetics 93:961-973

Wilkins AS (1995) Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. Bioessays 17:71-7

Woram RA, Gharbi K, Sakamoto T, Hoyheim B, Holm LE, Naish K, McGowan C, Ferguson MM, Phillips RB, Stein J, Guyomard R, Cairney M, Taggart JB, Powell R, Davidson W, Danzmann RG (2003) Comparative genome analysis of the primary sex-determining locus in salmonid fishes. Genome Res 13:272-80

CHAPTER TWO

The cutthroat trout Y chromosome is conserved with that in rainbow trout

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Abstract

Five genetic markers previously shown to be located on the sex chromosomes of rainbow trout (*Oncorhynchus mykiss*) were tested for their linkage with the sex locus of Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri*) in a genetic cross created from a rainbow X cutthroat male hybrid. We show that the sex locus of both rainbow and cutthroat trout is on the same homologous linkage group. Fluorescence in situ hybridization (FISH) using a probe to the microsatellite marker Omm1665, which maps close to the sex locus of Yellowstone cutthroat trout, was used to identify the Y chromosome of cutthroat trout in the hybrid. The Y chromosome of cutthroat trout is subtelocentric and lacks a DAPI band found on the short arm of the Y chromosome of some rainbow trout males. The physical location of the Omm1665 probe is different between the rainbow and cutthroat strains used for making the hybrid. The potential uses of this difference for the stock identification and conservation programs of rainbow and cutthroat populations and for the identification of species hybrids are discussed.

1. Introduction

Wide varieties of evolutionarily labile sex determination mechanisms, which have evolved repeatedly among different lineages, make fish one of the most interesting models for the exploration of how sex determination mechanisms evolve (Devlin and Nagahama, 2002). With more than 27,000 species (Nelson, 2006), fishes provide unique examples of sex determination mechanisms and possess sufficient diversity to share sex determination processes and pathways with other vertebrates.

Trout, salmon, and char of the subfamily Salmoninae all have an XX-XY genetic mechanism for sex determination where males are the heterogametic sex (Donaldson and Hunter, 1982; Allendorf and Thorgaard, 1984; Devlin and Nagahama, 2002). The sex chromosomes of all salmonids identified to date have small non-recombining regions and large pseudo-autosomal regions. This is supported by pairing of the X and Y chromosomes of rainbow trout *(Oncorhynchus mykiss)* during meiosis (Oliviera et al., 1995) and by genetic mapping studies of rainbow trout (Nichols et al., 2003), brown trout (*Salmo trutta*) (Gharbi et al., 2006) and Arctic char (*Salvelinus alpinus*) (Woram et al., 2004). Small morphological differences between the sex chromosome pair have been identified in lake trout (*Salvelinus namaycush*) (Phillips and Ihssen, 1985), brook trout (Phillips et al., 2002) sockeye salmon (*Oncorhynchus nerka*) (Thorgaard, 1978), and rainbow trout (Thorgaard, 1977). However, in some rainbow trout populations the sex chromosomes are homomorphic and cannot be microscopically distinguished (Thorgaard, 1977; Thorgaard, 1983). This indicates the presence of intraspecific polymorphisms in salmonid sex chromosomes. The aforementioned observations indicate that the sex chromosomes of salmonids are at an early evolutionary stage, making them an intriguing model for the study of sex chromosome evolution.

Comparative analysis of the Y chromosome linkage map of four salmonid species (Arctic char; Atlantic salmon, *Salmo salar*; brown trout; and rainbow trout), members of the three major genera within the subfamily Salmoninae, showed that the sex locus of each of the species is located on a different linkage group although the positioning of markers proximal to the sex locus is conserved among homologous autosomes (Woram et al., 2003). The sex chromosomes of seven other salmonid species have also been identified: lake trout, brook trout, sockeye salmon, chinook salmon, coho salmon, chum salmon and pink salmon (Phillips and Ihssen, 1985; Phillips et al., 2002; Phillips et al., 2005; Phillips et al., 2007). The latter five species are all members of the genus *Oncorhynchus* (Pacific salmon and trout), which includes in addition to the five aforementioned species; rainbow trout, cutthroat trout, masu salmon and amago salmon. The only two salmonid species demonstrated to date to have the sex locus on a homologous shared chromosome are lake trout and brook trout, species that form viable hybrids and are completely inter-fertile (Phillips et al., 2002). One explanation for the lack of conservation of the sex chromosomes of different salmonid species could be the presence of a small DNA motif involved in the rearrangement of the sex determining region across different species (Woram et al., 2003). This relocation might involve the presence of the sex-determining gene within a region that has transposed to a different linkage group in each species (Woram et al., 2003). Another explanation for the lack of sex chromosome homology could be the separate evolution of sex determining mechanisms in almost every salmonid species (Woram et al., 2003; Phillips et al., 2005; Phillips et al., 2007).

A variety of sex-linked markers have been isolated from salmonid species. OtY1 (Devlin

et al., 1991) is a male specific repetitive marker found on the Y chromosome only in chinook salmon. A growth hormone pseudogene, termed GH-Y, has been shown to be linked to the Y chromosome of all Pacific salmon species except sockeye salmon and rainbow trout (Du at al., 1993; Nagler at al., 2004). OtY2 is yet another male-linked marker shared by most Pacific salmon species (Brunelli and Thorgaard, 2004) except for rainbow trout.

The sex chromosomes of rainbow trout are the largest subtelocentric pair of chromosomes in the karyotype (Thorgaard, 1977). Allendorf et al. (1994) reported the first sex-linked loci in this species. Segregation data from their mapping families showed that HEX-2 and sSOD-1 allozyme markers are both located in the recombining region of rainbow trout sex chromosomes at a distance of 8.1 and 34.9 cM from the sex locus respectively. This finding was confirmed in the linkage map published by Nichols et al. (2003), who mapped the sex locus to linkage group 1, and later by Felip et al. (2004). A variety of microsatellite and AFLP markers also map close to the sex locus (Young et al., 1998; Sakamoto et al., 2000; Nichols et al., 2003; Felip et al., 2004).

Cutthroat trout (*Oncorhynchus clarki*) is a sister species of rainbow trout. Both species are believed to have evolved from a common ancestor about 2 million years ago (Behnke, 1992). However, since the species evolved in allopatry, they typically lack reproductive isolation mechanisms. Consequently, hybrids of the two species are viable and are completely inter-fertile (Behnke, 1992). Hybridization that results from secondary contact between introduced and native forms of these species represent a major concern from a conservation standpoint. This practice is believed to be directly involved in the extinction of at least one species, the Alvord cutthroat trout (Leary et al., 1984; Gyllensten et al., 1985; Bartley and Gall, 1991). Accordingly, the identification of genetic markers that are distinctively different between the two species is of

great value for the identification of species hybrids and for assessing the level of introgression between species populations.

In this study, we tested five loci shown by different studies to be located on the sex chromosomes of rainbow trout (Allendorf et al., 1994; Nichols et al., 2003; Guyomard et al., 2006) for their linkage to the Y chromosome of Yellowstone cutthroat trout. We show that the sex locus in both species is located on the same linkage group which appears to be largely homologous. Using chromosomes of the same rainbow X cutthroat trout hybrid used for generating the mapping family, we also used a Bacterial Artificial Chromosome (BAC) clone containing one of the loci mapped by linkage analysis as a probe for in situ hybridization to identify the Y chromosome of Yellowstone cutthroat trout.

2. Materials and methods

2.1. Mapping family

The mapping family was created by crossing outbred rainbow trout eggs (XX), supplied by Troutlodge Inc (Summer, WA), with an F1 rainbow X cutthroat hybrid male (XY). The hybrid was derived by crossing a rainbow trout from the Oregon State University (OSU) female clonal line (XX) with a Yellowstone cutthroat male (XY). Males of the mapping family inherited the sex-determining locus from the Yellowstone cutthroat male. OSU is one of ten homozygous clonal lines of rainbow trout at Washington State University derived by the chromosome set manipulation techniques of androgenesis and gynogenesis (Parsons and Thorgaard, 1985; Young et al., 1996; Robison et al., 1999). Fish of the mapping family (N=54)

were reared until they became mature enough for their phenotypic sex to be determined by examining their gonadal morphology.

2.2. Marker analysis

Phenotypic sex was used as a marker for sex in linkage analysis. Four microsatellite markers and one known gene marker previously shown to be located on the sex chromosomes of rainbow trout (Table 1) were examined for linkage with the sex locus as determined by phenotypic sex. Genomic DNA was extracted from fin clips of the mapping family, the OSU clonal line, and the OSU X cutthroat male hybrid parent using the Puregene DNA Isolation Kit (Gentra systems, Minneapolis, MN).

2.2.1. Microsatellites

All the primer sequences, PCR reaction parameters and optimum annealing temperatures of the different microsatellites can be found in references listed in Table 1. Size differences between OSU and cutthroat alleles for all four microsatellites were large enough to be typed in a 2.5% agarose gel stained by ethidium bromide and viewed under UV light.

2.2.2. Cu-Zn superoxide dismutase 1 (*Sod-1*)

Gene specific primers were designed to flank the first intron of the gene. Exon-intron boundaries of *Sod-1* were identified by comparing cDNA sequence of the gene (GenBank

accession number: AF469663) to relevant genomic sequences of *Takifugu rubripes* and *Danio rerio.* The following primer combination;

5' GTGTTGTCTCCATAAGCATGGACGTG '3 and

5'AGGACGTGAAGAGATGGCAATGAAGG '3 was used to amplify the first intron of *Sod-1* from OSU and from the OSU X cutthroat male hybrid. One strong amplicon from OSU and two strong amplicons, of differing size, from the OSU X cutthroat hybrid were cloned into pGEM-T Easy plasmid system (Promega, Madison, WI) and sequenced. Sequencing was performed at the Washington State University Laboratory for Biotechnology and Bioanalysis. The sequence of all three amplicons was confirmed to be *Sod-1* using the BLASTn algorithm of the NCBI website. Sequence alignment revealed that the size difference between the OSU and the cutthroat alleles is due to an insertion/deletion in their respective introns. The size difference was utilized to score fish of the mapping family using the same primer combination used for cloning and then running the PCR products in a 2% agarose gel.

2.3. Construction of the Y chromosome linkage map

Construction of the Y chromosome linkage map was performed using Map Maker 3.0/EXP. Grouping of markers was performed using the Kosambi map function with a minimum LOD score of 3.0 and a maximum theta of 0.45.

2.4. In situ Hybridization and Karyotyping

Chromosome preparations were obtained from blood of a hybrid between the rainbow trout OSU strain (2N=60) (Ristow et al., 1998) and Yellowstone cutthroat (2N=64) (Loudenslager and Thorgaard, 1979) by methods described previously (Reed and Phillips, 1995). The Omm1665 BAC clone was isolated from the Swanson (YY) BAC library (Palti et al., 2004) by screening PCR pools made from this library and BAC DNA was extracted using a standard kit (Quiagen Inc.). The DNA was labeled with spectrum orange (Vysis, Inc.) and hybridizations and washes done according to methods recommended by the manufacturer with minor modifications (Phillips et al., 2006). Slides were examined using an Olympus BX60 microscope and photographed with a Sensys 1400 digital camera. Images were captured with Cytovision software (Applied Imaging Inc.) and selected karyotypes were prepared using Genus software (Applied Imaging Inc.). Chromosome pairs were identified using relative size, centromere staining, chromosome arm ratios, and centromere probes. Chromosomes were sorted according to size using the software described above and adjustments made by hand to conform to the standard chromosome arm ratios and DAPI staining of centromeres.

3. Results

3.1. The sex locus of rainbow and cutthroat trout is on the same homologous linkage group

All five markers previously shown to map to rainbow trout sex chromosomes mapped on the same linkage group along with the sex locus as determined by phenotypic sex (Fig. 1a). Since

the genetic cross used in this study was designed so that all male fish of the mapping family would inherit their male-determining sex locus from the cutthroat trout, it can be concluded that the sex locus of both rainbow and cutthroat trout is on the same linkage group.

In salmonids, it is generally accepted that large sex- specific differences exist in the rate and localization of recombination (Sakamoto et al., 2000; Gharbi et al., 2006). Females have a much higher recombination rate with crossovers localized to centromeric regions of linkage groups, while male crossovers are more distal in location (Sakamoto et al., 2000). Accordingly, if the linkage map constructed from this cross (Fig. 1a) is compared with the female-based linkage map of rainbow trout X chromosome (Fig. 1b) adapted from Guyomard et al., (2006), it can be concluded that the order of markers is conserved, also supporting their overall homology.

3.2. Localization of OMM 1665 in rainbow trout and identification of the Y chromosome of Yellowstone cutthroat trout

Clear hybridization signals can be seen in rainbow trout for the Omm1665 probe (red probe, Fig. 2) where it is found on the long arm just below the centromere of the sex chromosome pair. The X chromosome of rainbow trout can be easily distinguished in the karyotype as the largest subtelocentric chromosome with a bright DAPI band on the short arm. The Y chromosome lacks this bright band in many strains including the one shown in Figure 2.

After determining by linkage analysis that the microsatellite marker Omm1665 maps close to the sex locus of Yellowstone cutthroat trout, we used a BAC clone containing Omm1665

as a probe on chromosome preparations of the OSU X cutthroat male hybrid to identify the Y chromosome of Yellowstone cutthroat trout and determine the physical location of that marker on the chromosome. Two clear hybridization signals for the Omm1665 probe are noticeable in the metaphase spread of the OSU X cutthroat hybrid (Fig. 3). One signal is located on the long arm, just below the centromere, of a large subtelocentric chromosome (Fig. 4). The short arm of the same chromosome has a bright DAPI band. The localization of the Omm1665 signal and the bright DAPI band indicate that this chromosome represents the X chromosome of OSU (rainbow trout). The other signal is located above the centromere of another subtelocentric chromosome which lacks the DAPI band. This chromosome was identified as the Y chromosome of Yellowstone cutthroat trout. Intriguingly, this result demonstrates that the physical location of the Omm1665 probe is different between the rainbow and the cutthroat strain used for making the hybrid.

4. Discussion

We have shown that the sex locus of both rainbow and cutthroat trout is located on the same linkage group. We later used one of the markers that maps close to the sex locus in our cross as a probe to identify the Y chromosome of cutthroat trout. This brings the number of salmonids in which sex chromosomes and or sex linkage groups have been identified to eleven species. This is only the second case, in addition to lake trout and brook trout, in which two salmonid species have been found to share the same sex chromosome pair. Interestingly, in both cases the two species can form viable hybrids and are completely interfertile. This might indicate

that, in salmonids, species that have the sex locus on the same linkage group will produce more fertile hybrids than those that have different sex chromosomes.

The identification of cutthroat trout Y chromosome leaves masu and amago salmon as the only two species of the genus Oncorhynchus in which the sex chromosome and or sex linkage group have not yet been identified. These two species are closely related and might also have the same sex chromosome and sex linkage group. The linkage of GH-Y to the Y chromosome of masu salmon has been documented (Zhang et al., 2001) and GH-Y could be used as a probe for the identification of the sex chromosomes in these species as it was in coho salmon, chum salmon and pink salmon (Phillips et al., 2005; Phillips et al., 2007). Once this is done, then we will know whether cutthroat and rainbow trout are the only species pair in the genus Oncorhynchus that have the same sex chromosome. The fact that most species of this genus lack conserved sex-linkage groups but do share markers proximal to the sex locus (Devlin et al., 2001; Zhang et al., 2001; Brunelli and Thorgaard, 2004; Phillips et al., 2005; Phillips et al., 2007) supports the hypothesis that the sex-determining gene is part of a small male-specific region that has repeatedly transposed to a different linkage group throughout species evolution.

Both microsatellite markers, Omm1665 and Ssa408uos, that mapped close to the sex locus of cutthroat trout Y chromosome mapped on top of each other 11.3 centimorgans away from the sex locus. This is analogous to the situation in rainbow trout in which many sex-linked markers map at the same location on the male map (Nichols et al., 2003; Felip et al., 2004). This lack of meiotic recombination appears to be present in the rainbow X cutthroat male hybrid. This can be inferred by comparing the location of the two sex-linked microsatellites, Omm1665 and Ssa408uos, in this study and in the female- based genetic map of rainbow trout adapted from

Guyomard et al., (2006). These two markers map on top of each other in this study (Fig 1a). However, they map 26.9 centimorgans away from each other in the rainbow trout female map (Fig. 1b). In rainbow trout, Ots517NWFSC and Omm1372 are sex-linked microsatellite markers that map on top of each other in a rainbow trout doubled haploid linkage map produced from male hybrid clonal lines (Fig.1c) (Felip et al., 2004). However, these two markers are 24.3 centimorgans apart in a linkage map produced by gynogenesis from a female hybrid (Fig. 1b) (Guyomard et al., 2006). Another example is SNP-B4 which physically maps proximal to the centromere of the rainbow trout sex chromosome pair. However, genetic mapping in a malebased map places this marker on top of Omm1372 (Felip et al., 2004). Omm1372 maps 41.9 cM away from the centromere in a female-based linkage map (Fig. 1b) (Guyomard et al., 2006). Omm1372 wasn't mapped in this study but is very closely linked to OMM5136 which was mapped in cutthroat.

Because recombination near the sex locus appears to be reduced in male trout, we believe that failure to identify markers that exhibit complete linkage to the sex locus in genetic crosses is the product of spontaneous sex reversal (Felip et al., 2004). During the construction of the linkage map presented here and the published rainbow trout linkage maps (Young et al., 1998; Nichols et al., 2003; Felip et al., 2004) genotypic females may have been scored as phenotypic males as a result of female to male sex reversal. Male to female sex reversal may also have occurred. Sex reversal accompanied by a low rate of meiotic recombination in that chromosomal region would result in sex-linked markers mapping on top of each other at a distance away from phenotypic sex. This has so far prevented investigators from identifying the exact, accurate location of the sex locus in rainbow trout and will have the same effect in cutthroat trout.

Clear hybridization signals of the Omm1665 probe are visible on both the X and Y chromosomes of rainbow trout and on the Y chromosome of cutthroat trout. The presence of this marker on the X chromosome indicates that this marker is not part of the male determining region of rainbow trout and is most likely located in the pseudoautosomal regions of the sex chromosome pair. This is presumably true for cutthroat sex chromosomes as well. However, since the FISH experiment with the Omm1665 probe was not performed on pure cutthroat samples, but rather on a rainbow X cutthroat hybrid, more data is necessary to confirm this finding.

Interestingly, the physical location of the Omm1665 probe was different between the sex chromosome pair of rainbow trout, where it mapped on the long arm of the chromosomes just below the centromere on both the X and the Y, and the Y chromosome of cutthroat trout where the same probe mapped on the short arm just above the centromere. Two possible scenarios can explain this finding; (1) Omm1665 is part of an inversion that is specific to the Y chromosome of all cutthroat trout. It was previously proposed in theories of sex chromosome evolution (Ohno, 1967) that once a sex determining locus has been established on one of the sex chromosomes, the next task would be to shield that locus from recombination to maintain its status as 'sex determining'. An attractive postulated mechanism was to surround the incipient locus with a large inversion (Vallender and Lahn, 2004; Schartl, 2004). (2) This represents an intraspecific polymorphism in the morphology of the sex chromosomes that is specific to the source population of the cutthroat male used for making the OSU X cutthroat hybrid and may not be typical of all cutthroat trout subspecies. Intraspecific polymorphism in the morphology of the Y chromosome is a character of evolutionarily young sex chromosomes and has been documented

in rainbow trout populations (Thorgaard 1977, 1983; Iturra et al., 2001(a); Iturra et al., 2001(b); Felip et al., 2004).

In order to distinguish between these scenarios we need to characterize the sex chromosomes and associated male-specific regions of cutthroat strains from various populations of the species range. The order of markers in that area of the chromosome also requires further evaluation in female-based maps in view of a higher rate of recombination in areas that are proximal to the centromere in females.

References cited

1. Allendorf FW, Gellman WA, Thorgaard GH: Sex-linkage of two enzyme loci in *Oncorhynchus mykiss* (rainbow trout). Heredity 72:498-507 (1994).

2. Allendorf FW, Thorgaard GH: Tetraploidy and the evolution of salmonid fishes, in Turner BJ (ed): The Evolutionary Genetics of Fishes, pp 1-53 (Plenum Publishing Company, New York 1984).

3. Bartley DM, Gall GAE: Genetic identification of native cutthroat trout (*Oncorhynchus clarki*) and introgressive hybridization with introduced rainbow trout (*Oncorhynchus mykiss*) in streams associated with the Alvord basin, Oregon and Nevada. Copeia 3:854– 859 (1991).

4. Behnke RJ: Native trout of Western North America (American Fisheries Society Monograph 6, Bethesda 1992).

5. Brunelli JP, Thorgaard GH: A new Y chromosome-specific marker for Pacific salmon. Transactions of the American Fisheries Society 133:1247-1253 (2004).

6. Devlin RH, McNeil BK, Groves TDD, Donaldson EM: Isolation of a Y-chromosomal DNA probe capable of determining genetic sex in chinook salmon (*Oncorhynchus tshawytscha*). Can J Fish Aquat Sci 48:1606-1612 (1991).

7. Devlin RH, Biagi CA, Smailus DE: Genetic mapping of Y-chromosomal DNA markers in Pacific salmon. Genetica 111:43-58 (2001).

8. Devlin RH, Nagahama Y: Sex determination and sex differentiation in fish: an overview of genetic physiological and environmental influences. Aquaculture 208:191-364 (2002).

9. Donaldson EM, Hunter GA: Sex control in fish with particular reference to salmonids. Canadian Journal of Fisheries and Aquatic Sciences 39:99-110 (1982).

10. Du SJ, Devlin RH, Hew CL: Genomic structure of growth hormone genes in chinook salmon (*Oncorhynchus tshawytscha*): presence of two functional genes, GH-I and GH-II, and a male-specific pseudogene, GH-psi. DNA Cell Biol 12:739-751 (1993).

11. Felip A, Fujiwara A, Young WP, Wheeler PA, Noakes M, Phillips RB, Thorgaard GH: Polymorphism and differentiation of rainbow trout Y chromosomes. Genome. 47:1105- 13 (2004).

12. Gharbi K, Gautier A, Danzmann RG, Gharbi S, Sakamoto T, Høyheim B, et al: A linkage map for brown trout (*Salmo trutta*): chromosome homeologies and comparative genome organization with other salmonid fish. Genetics 172:2405-19 (2006).

13. Guyomard R, Mauger S, Tabet-Canale K, Martineau S, Genet C, Krieg F, et al: A type I and type II microsatellite linkage map of rainbow trout (*Oncorhynchus mykiss*) with presumptive coverage of all chromosome arms. BMC Genomics 7:302-315 (2006).

14. Gyllensten U, Leary RF, Allendorf FW, Wilson AC: Introgression between two cutthroat trout subspecies with substantial karyotypic, nuclear and mitochondrial genomic divergence. Genetics 111:905–915 (1985).

15. Iturra P, Bagley M, Vergara N, Imbert P, Medrano JF: Development and characterization of DNA sequence *OmyP9* associated with the sex chromosomes in rainbow trout. Heredity 86:412-419 (2001a).

16. Iturra P, lam N, de la Fuente M, Vergara, N, Medrano JF: Characterization of sex chromosomes in rainbow trout and coho salmon using fluorescence *in situ* hybridization (FISH). Genetica 111:125-131 (2001b).

17. Leary RF, Allendorf FW, Phelps SR, Knudsen KL: Introgression between west-slope cutthroat and rainbow trout in the Clark Fork drainage, Montana. Proceedings of the Montana Academy of Sciences 43:1–18 (1984).

18. Loudenslager EJ, Thorgaard GH: Karyotypic and evolutionary relationships of the Yellowstone (*Salmo clarki bouvieri*) and west-slope cutthroat trout (*Salmo clarki lewisi*). J Fish Res Board Can 36:630-635 (1979).

19. Nagler JJ, Cavileer T, Steinhorst K, Devlin RH: Determination of genetic sex in chinook salmon (*Oncorhynchus tshawytscha*) using the male-linked growth hormone pseudogene by real-time PCR. Mar Biotechnol 6:186-91 (2004).

20. Nelson JS: Fishes of the World (Wiley, New York 2006).

21. Nichols KM, Young WP, Danzmann RG, Robison BD, Rexroad C, Noakes M, et al: A consolidated linkage map for rainbow trout (*Oncorhynchus mykiss*). Animal Genetics 34:102-15 (2003).

22. Ohno S: Sex Chromosomes and Sex-Linked Genes (Springer, Berlin 1967).

23. Oliveira C, Foresti F, Rigolino M G, Tabata YA: Synaptonemal complex analysis in spermatocytes and oocytes of rainbow trout *Oncorhynchus mykiss* (Pisces,Salmonidae): the process of autosome and sex chromosome synapsis. Chromosome Res 3:182-190 (1995).

24. Parsons JE, Thorgaard GH: Production of androgenetic diploid rainbow trout. J. Hered 76:177-181 (1985).

25. Palti Y, Gahr SA, Hansen JD, Rexroad CE: Characterization of a new BAC library for rainbow trout: evidence for multi-locus duplication. Animal Genetics 35:130-133 (2004).

26. Phillips RB, Ihssen PE: Identification of sex chromosomes in lake trout (*Salvelinus namaycush*). Cytogenet Cell Genet 39:14-18 (1985).

27. Phillips RB, Matouka MP, Reed KM: Characterization of charr chromosomes using Fluorescence in Situ Hybridization. Environ Biol Fish 64:223-228 (2002).

28. Phillips RB, Morasch MR, Park LK, Naish KA, Devlin RH: Identification of the sex chromosome pair in coho salmon (*Oncorhynchus kisutch*): lack of conservation of the sex linkage group with chinook salmon (*Oncorhynchus tshawytscha).* Cytogenet Genome Res 111:166-70 (2005).

29. Phillips RB, DeKoning J, Morasch MR, Park LK, Devlin RH: Identification of the sex chromosome pair in chum salmon (*Oncorhynchus keta*) and pink salmon (*Oncorhynchus gorbuscha*). Cytogenet Genome Res 116:298-304 (2007).

30. Phillips RB, KM Nichols, DeKoning JJ, Morasch MR, Keatley KA, Rexroad C, et al: Assignment of rainbow trout linkage groups to specific chromosomes. Genetics 174:1661–1670 (2006).

31. Reed KM, Phillips RB: Molecular cytogenetic analysis of the double CMA3 chromosome in lake trout, *Salvelinus namaycush.* Cytogenetics and Cell Genetics 70:104-107 (1995).

32. Ristow SS, Grabowski LD, Ostberg C, Robison B, Thorgaard GH: Development of Long-Term Cell Lines from Homozygous Clones of Rainbow Trout. Journal of Aquatic Animal Health 10:75-82 (1998).

33. Robison BD, Wheeler PA, Throgaard GH: Variation in development rate among clonal lines of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 173:131-141 (1999).

34. Sakamoto T, Danzmann RG, Gharbi K, Howard P, Ozaki A, Khoo SK, et al: A microsatellite linkage map of rainbow trout (*Oncorhynchus mykiss*) characterized by large sex-specific differences in recombination rates. Genetics 155:1331-1345 (2000).

35. Schartl M: Sex chromosome evolution in non-mammalian vertebrates. Curr Opin Genet Dev 14:634-41 (2004).

36. Thorgaard GH: Heteromorphic sex chromosomes in male rainbow trout. Science 196:900-902 (1977).

37. Thorgaard GH: Sex chromosomes in the sockeye salmon: a Y-autosome fusion. Can J Genet Cytol 20:349-54 (1978).

38. Thorgaard GH: Chromosomal differences among rainbow trout populations. Copeia 3:650-662 (1983).

39. Vallender EJ, Lahn BT: How mammalian sex chromosomes acquired their peculiar gene content. Bioessays 26:159-69 (2004).

40. Woram RA, Gharbi K, Sakamoto T, Hoyheim B, Holm LE, Naish K, et al: Comparative genome analysis of the primary sex-determining locus in salmonid fishes. Genome Res 13:272-280 (2003).

41. Woram RA, McGowan C, Stout JA, Gharbi K, Ferguson MM, Hoyheim B, et al: A genetic linkage map for Arctic char (*Salvelinus alpinus*): evidence for higher recombination rates and segregation distortion in hybrid versus pure strain mapping parents. Genome 47:304-15 (2004).

42. Young WP, Wheeler PA, Fields RD, Thorgaard GH: DNA fingerprinting confirms isogenecity of androgenetically derived rainbow trout lines. J Hered 87:77-81 (1996).

43. Young WP, Wheeler PA, Coryell VH, Keim P, Thorgaard GH: A detailed linkage map of rainbow trout produced using doubled haploids. Genetics 148:839-850 (1998).

44. Zhang Q, Nakayama I, Fujiwara A, Kobayashi T, Oohara I, Masaoka T: Sex identification by male-specific growth hormone pseudogene (GH-Ψ) in *Oncorhynchus masou* complex and a related hybrid. Genetica 111:111–118 (2001).

Table 1. Y linked loci used in this study and references demonstrating their linkage to rainbow trout sex chromosomes.

Marker	Type	Reference
Omm1026	Microsatellite	Nichols et al (2003), Felip et al (2004), Guyomard et al. (2006).
Omm5136	Microsatellite	Guyomard et al. (2006)
Omm1665	Microsatellite	Guyomard et al. (2006)
Ssa408uos	Microsatellite	Guyomard et al. (2006)
Sod	Gene/ Cu-Zn superoxide dismutase 1	Allendorf et al. (1994), Nichols et al. (2003), Felip et al (2004).

Figure legends

Figure 1. Genetic maps of Y-linked loci in (a) A male-based genetic map of a female rainbow trout (OSU) crossed with a Yellowstone cutthroat male which was constructed in this study (b) A female-based genetic map of rainbow trout adapted from Guyomard et al. (2006) (c) A male-based genetic map of rainbow trout of a cross involving OSU with HC (male clonal line of rainbow trout) adapted from Felip et al. (2004). Numbers to the left of each linkage group indicate genetic distances in centimorgans (Kosambi).

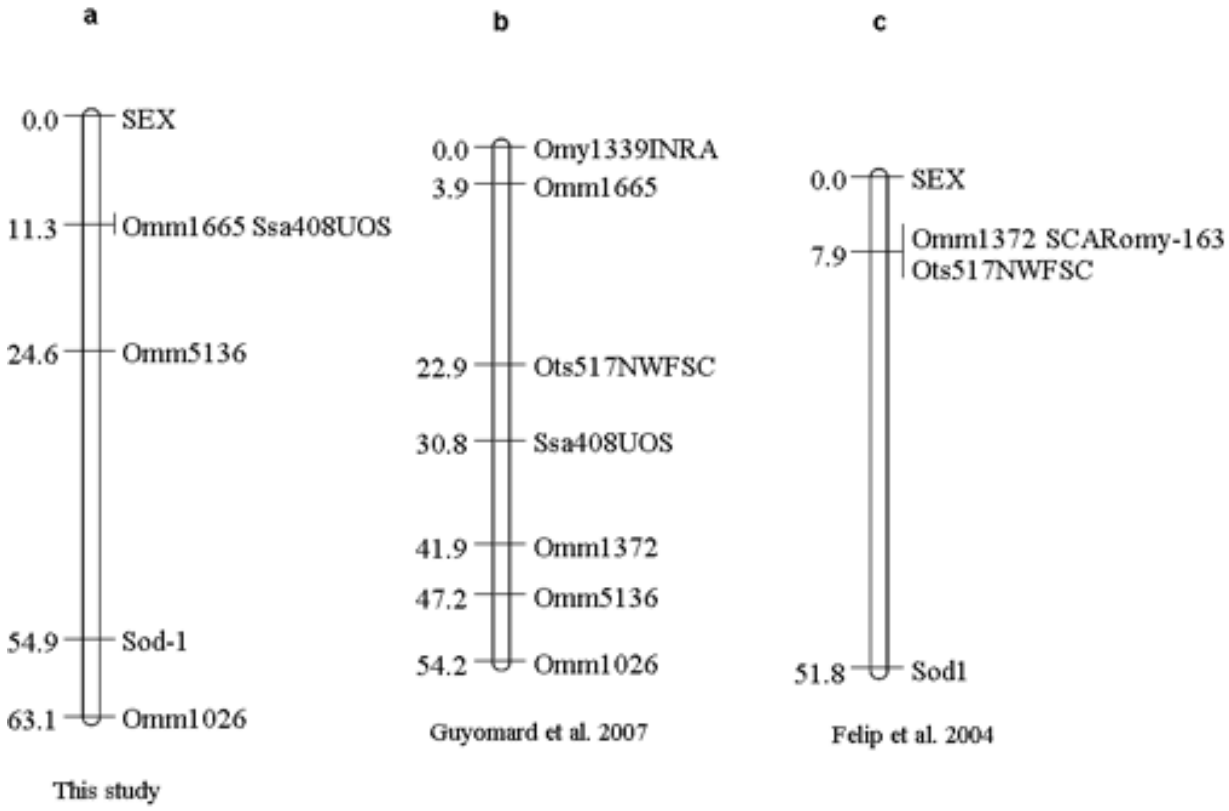
Figure 2. Male karyotype of rainbow trout showing the location of Omm1665 (red probe) on the long arm of the sex chromosomes just below the centromere. The sex chromosomes (pair # 30) are the largest subtelocentric pair in the karyotype and the X chromosome has a bright DAPI band on the short arm.

Figure 3. Metaphase spread of the OSU X cutthroat male hybrid, used for making the mapping family, hybridized with the Omm1665 probe (labeled in red). Signals are detected on two subtelocentric chromosomes indicated by arrows in the figure. The upper chromosome has a bright DAPI band on its short arm and is identified as the X chromosome of OSU. The lower chromosome lacks the bright DAPI band and is identified as the Y chromosome of cutthroat trout.

Figure 4. Partial karyotype of OSU X cutthroat male hybrid showing a subtelocentric pair of chromosomes with one of the chromosomes in the pair having a bright DAPI band on its short

arm. These chromosomes are from metaphase spread of OSU X cutthroat male hybrid (see Figure 3.) hybridized with the Omm1665 probe (labeled in red).

Figure 1.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11.3 Omm1665 Ssa408UOS
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22.9 Ots517NWFSC
30.8 Ssa408UOS
41.9 Omm1372
47.2 Omm5136
54.2 Omm1026
Guyomard et al. 2007
c
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Ots517NWFSC
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Felip et al. 2004

Figure 2.

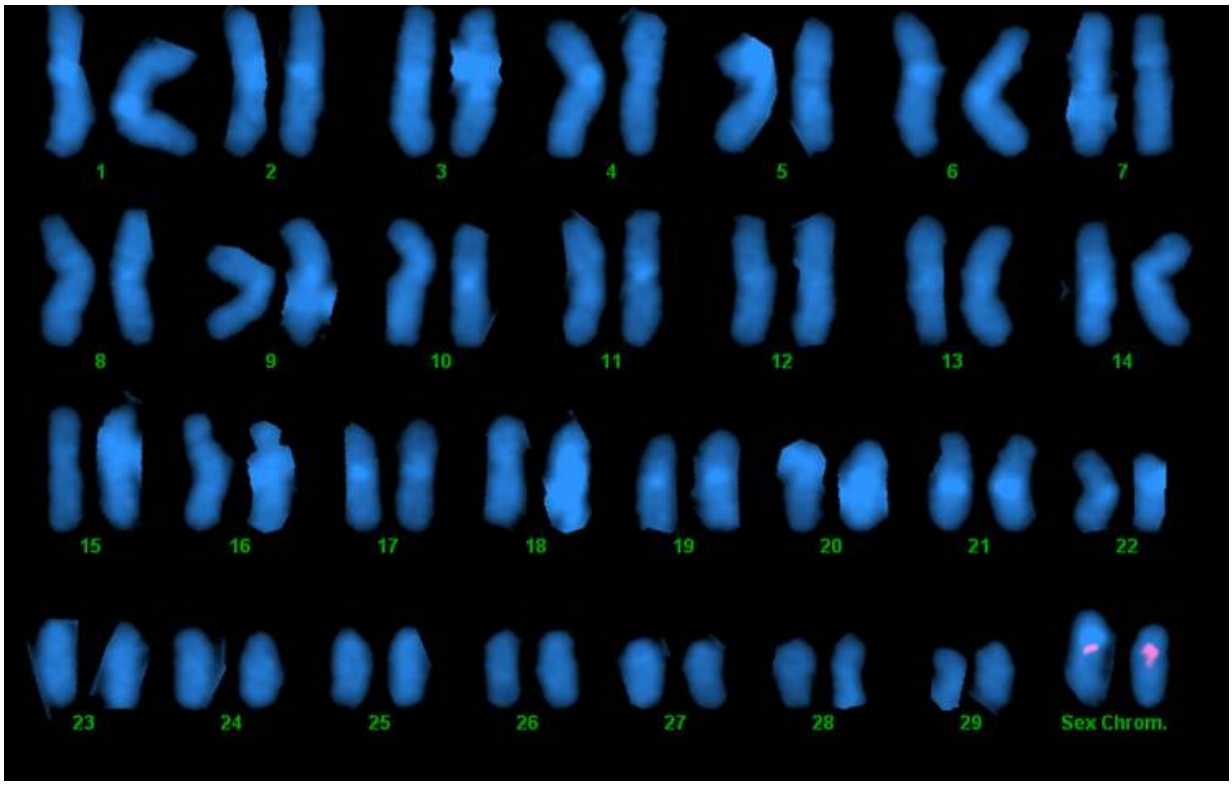
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Figure 3.

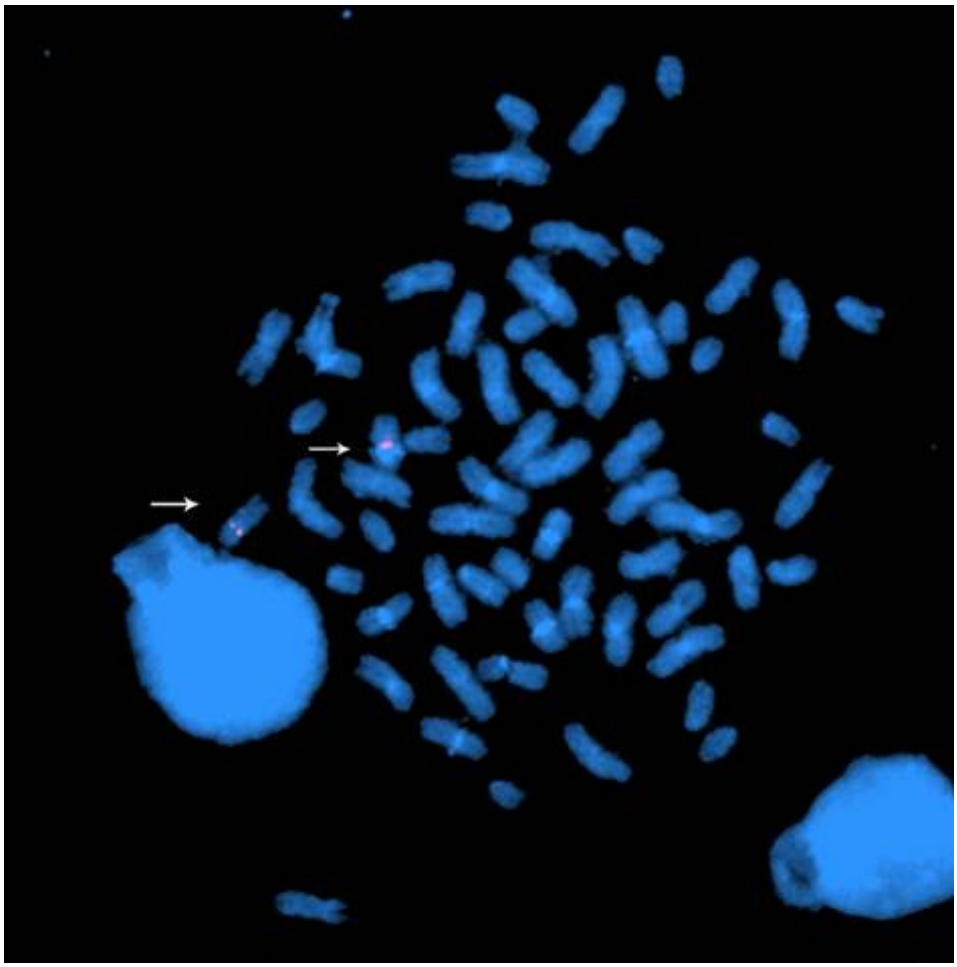
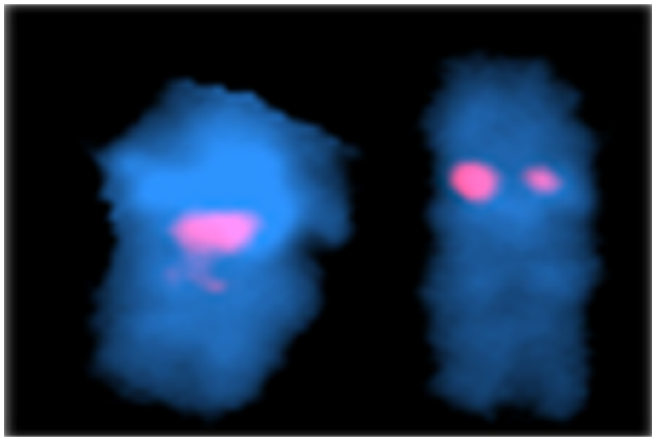


Figure 4.



CHAPTER THREE

Mapping of candidate sex determining genes in rainbow trout (*Oncorhynchus mykiss*)

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and

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Abstract

Rainbow trout have an XX/XY genetic mechanism for sex determination where males are the heterogamtic sex. Rainbow trout evolved from a tetraploid ancestor and thus have a high repertoire of duplicated genes. Recent studies in medaka have identified the sex determining gene as a homologue of *Dmrt1*. The origin of the sex-determining gene in medaka suggested that downstream genes of the sex-determining pathway can assume the function of the master switch of the cascade following their duplication We tested five loci of four candidate genes, for which there is evidence for their involvement in sex determination, for linkage to the Y chromosome of rainbow trout in genetic crosses: *Amh*, *Dax1*, *Dmrt1* and *Sox6*. None of the genes tested proved to be Y-linked. *Amh* and *Dax1* mapped to linkage groups 8 and 5 respectively. *Dmrt1* was not assigned to any linkage group. However, a chi-square test did not support its linkage to the Y chromosome. *Sox6* is duplicated in rainbow trout. *Sox6(1)* and *Sox6(2)* mapped to homeologous linkage groups 10 and 18 respectively. Genotyping doubled haploids of the OSU X Arlee mapping family for the different *Sox6(1)* and *Sox6(2)* alleles indicated that they are tetrasomically inherited. No evidence of tetrasomic inheritance of *Sox6(1)* and *Sox6(2)* alleles was observed in the OSU X HC mapping family. The map position of *Sox6(1)* and *Sox6(2)* and their pattern of inheritance indicated that duplication of the *Sox6* locus is recent and specific to the salmonid lineage.

1. Introduction

With about 24,000 species, teleosts represent the most diverse and species-rich group of vertebrates. A whole genome duplication event that preceded the radiation of teleosts is speculated to have provided the genetic material that promoted both adaptation and rapid radiation of fishes (AMORES *et al.* 1998). A more recent genome duplication event (25 – 100 million years ago) is believed to have taken place before the radiation of teleosts of the family Salmonidae (ALLENDORF and THORGAARD 1984). Species of that family have diploid karyotypes rangeing between 54-92 with 100 chromosome arms in most species while the most frequent diploid karyotype in teloests is 48-50 acrocentric chromosomes (PHILLIPS and RAB 2001). In addition to chromosome counts, the tetraploid ancestry of salmonids is supported by estimates of genome size (OHNO *et al.* 1968) and patterns of gene duplication (ALLENDORF *et al.* 1975). Diploidization has been restored across most of the genome of salmonids (ALLENDORF and THORGAARD 1984). However, inheritance patterns of duplicated loci indicate that some chromosomes can still form multivalents and exchange segments with their ancestral duplicates.

Rainbow trout (*Oncorhynchus mykiss*) ranks as one of the most extensively studied salmonid species. Research areas which have been particularly well-studied include comparative immunology and physiology, carcinogenesis, and evolutionary genetics (THORGAARD *et al.* 2002). The nature of the genes controlling sex determination in lower vertebrates can be studied using the rainbow trout model. Rainbow trout offers advantages over some other fish models in ease of conducting controlled breeding experiments and the ability to manipulate ample quantities of gametes in controlled in vitro fertilization procedures (BROMAGE *et al.* 1992). Other advantages include a slow rate of embryonic development which facilitates a thorough study of embryonic changes, and a stable XX-XY genetic mechanism for sex determination (FELIP *et al.*

2004) as opposed to zebrafish, where the mode of sex determination is currently unknown (BRUNNER *et al.* 2001) despite a high-resolution genetic map.

In rainbow trout the general consensus is that sex is determined by an XY system, where males are the heterogametic sex (ALLENDORF and THORGAARD 1984; DONALDSON and HUNTER 1982). Rainbow trout have a pair of subtelocentric sex chromosomes. The sex chromosomes are heteromorphic in most populations (THORGAARD 1977; THORGAARD 1983) with the X chromosome having a slightly longer short arm. However, some populations have cytogenetically indistinguishable sex chromosomes suggestive of differences in the level of their differentiation (FELIP *et al.* 2004; THORGAARD 1983). Genetic mapping studies indicate that the differential region on the Y chromosome is small, with most of the X and Y chromosomes consisting of pseudo-autosomal regions (SAKAMOTO *et al.* 2000; YOUNG *et al.* 1998). This can be further concluded from the viability of YY males (PARSONS and THORGAARD 1985) as well as from pairing of the X and Y chromosomes during spermatogenesis (OLIVEIRA 1995). Intraspecific polymorphisms among different populations and a small NRY suggest that rainbow trout sex chromosomes are primitive and have a low level of differentiation.

Accordingly, studying the sex-differentiation pathway in rainbow trout is pivotal to understanding the early stages of sex chromosome evolution. Moreover, identifying the sex-determining gene could facilitate the production of all-female stocks of fish. This is of enormous importance in aquaculture in view of a higher commercial value of later-maturing females (BYE and LINCOLN 1986).

The sex-determining gene of medaka has been identified (MATSUDA *et al.* 2002). Interestingly, rainbow trout have several parallels to medaka. Both have a stable XX-XY genetic

sex determination system with the male being the heterogametic sex (DONALDSON and HUNTER 1982; NANDA *et al.* 2002; THORGAARD 1977). Both species have a number of duplicated loci (ALLENDORF and THORGAARD 1984; NARUSE *et al.* 2004) and both species have sex chromosomes believed to be at an early stage of evolution (FELIP *et al.* 2004; ITURRA *et al.* 2001; NANDA *et al.* 2002; THORGAARD 1977; THORGAARD 1983). In medaka, the sex-determining gene, *Dmy*, is part of a chromosomal fragment that was duplicated from autosomal linkage group 9 and inserted into what became the Y chromosome (NANDA *et al.* 2002). *Sry*, the sex determining gene of mammals, is believed to have evolved from *Sox3* on the X chromosome (GRAVES and FOSTER 1994). Expression profiles of the genes involved in the cascade of sex differentiation in mice, chickens and alligators indicate conservation of the genes *per se*. However, pronounced variation exists between the timing of expression of the genes relative to the critical period for sex-determination across the different species (MORRISH and SINCLAIR 2002).

In conclusion, the sex differentiation cascade in vertebrates appears to be governed by the same molecular players. However, different branches of the phyla vary in the pattern of interaction of the molecular players and most importantly the starting trigger of the cascade (SCHARTL 2004). Based on the above observations, a model was proposed for evolution of the sex-determining gene cascade (SCHARTL 2004). According to this model, the sex-determining gene of the cascade is originally a downstream gene whose expression is dependent on the expression of the gene directly upstream in the cascade. Gene duplication allows that gene to be over-expressed above a certain threshold level. Over-expression liberates the gene from the upstream signal controlling its expression and with additional functional divergence allows it to assume the function of the ‘master switch’ of the cascade. Thus, in a tetraploid organism like

rainbow trout (ALLENDORF and THORGAARD 1984) which has a high repertoire of duplicated genes, it is legitimate to anticipate that any gene in the sex differentiation cascade could potentially function as the sex determining gene. Based on what we currently know from *Sry* in mammals and *Dmy*, in the medaka, the sex-determining gene has an upstream position in the cascade, shows stronger expression in male as compared to female gonads, is expressed before the time sex differentiation of the gonad starts and is linked to the Y chromosome. In a recent study in the tilapia model (*Oreochromis* spp) the authors were able to identify two candidate genes, *Amh* and *Dmrta2*, which map within quantitative trait locus, QTL, regions for sex determination in that species (SHIRAK *et al.* 2006). We are investigating several candidate genes for sex determination in the rainbow trout model.

Orthologous sequences of the major genes of the mammalian sex determination pathway have been reported in the rainbow trout. Examples of those genes include *Wt1*, *Sox9*, *Dmrt1*, *Amh* and *Dax1*. Three *Wt1* loci, and three *Sox9* loci (ALFAQIH *et al*., In prep) have been previously mapped in our lab (BRUNELLI *et al.* 2001) and none of the loci showed linkage to the Y chromosome. In this paper we tested for linkage of the candidate genes *Sox6*, *Dmrt1, Dax1 and Amh* to the Y chromosome of rainbow trout. Although our results clearly indicate that none of the candidate loci tested can potentially act as the primary sex-determining gene in the rainbow trout, we report a salmonid lineage specific duplication of *Sox6* and discuss the inheritance of the duplicated locus in our mapping families. We also report the complete genomic sequence of *Dax1* and promoter subsequence analysis of *Dax1* and *Dmrt1*.

2. Materials and methods

2.1. Mapping families and Linkage maps

Two doubled haploid mapping families and their respective linkage maps have been used for this study. The first family was produced by androgenesis from a hybrid of two clonal lines. OSU, an XX clonal line, and HC**,** an YY clonal line. The clonal lines were produced by androgenesis and gynogenesis (PARSONS and THORGAARD 1985; SCHEERER *et al.* 1991). Each clonal line is homozygous at all loci and its isogenecity has been confirmed by multilocus DNA fingerprinting (YOUNG *et al.* 1996). The original OSU X HC map was constructed by genotyping 106 doubled haploid fish at 484 polymorphic AFLP markers, 36 microsatellites and phenotypic sex, and has been originally used for identifying a single major QTL that affects natural killer cell-like activity in the rainbow trout model (ZIMMERMAN *et al.* 2004). DNA originating from 85 doubled haploid fish of the original mapping family was used for genotyping polymorphisms in non-coding regions of the candidate genes *Sox6*, *Dmrt1* and *Dax1*.

The second mapping family was produced by androgenesis from a cross between the OSU (XX) and Arlee (YY) clonal lines. The original OSU X Arlee map was constructed by genotyping 76 doubled haploid fish at 476 markers and the sex phenotype (YOUNG *et al.* 1998) and was later updated by genotyping the family at an additional 883 markers thus bringing the total number of markers to 1359 (NICHOLS *et al.* 2003). Genomic DNA originating from the 76 doubled haploid fish used for the construction of the original map was used for genotyping polymorphisms in non-coding regions of the candidate genes *Sox6* and *Amh.* Synteny between the linkage groups of the OSU X HC and OSU X Arlee maps used in this study has been previously established (ZIMMERMAN *et al.* 2004).

2.2. PCR reactions

All PCR reactions were performed using a Thermolyne Amplitron thermocycler II. PCR reaction conditions begin with a 94°C 2 minutes denaturation step, followed by 30 cycles of 94°C for 45 seconds, 60 °C for 45 seconds and 72 °C for 45 seconds. The reaction ends with a final extension step of 72 °C for 2 minutes. All reactions were 20 µls in volume containing: 1 µl of 50 ηg/ µl DNA template, 2 µls of 10X PCR buffer (Invitrogen, Carlsbad, CA), 1 µl of 50 mM $MgCl_2$, 1 µl of 10 pmoles of each primer, 0.5 ul of 10 mM dNTPs (Invitrogen, Carlsbad, CA) and 1.5 U of Taq DNA Polymerase (Promega, Madison, WI).

2.3. Primer design

All the primers were designed to amplify genomic DNA fragments of non-coding regions of candidate genes. The primers were designed to anneal to exon sequences flanking tentative introns. To predict exon-intron boundaries of the candidate genes *Sox6*, *Dmrt1* and *Amh*, the cDNA sequence of the aforementioned genes was compared to the relevant genomic sequences of *Takifugu rubripes* and *Danio rerio.*

2.4. Cloning and sequencing of non-coding regions of candidate genes

Genomic DNA was extracted from OSU, HC and Arlee fin clips using Gentra kit (Puregene, MN) according to the manufacturers' instructions. Non-coding regions of candidate genes were amplified by PCR. PCR amplification products were resolved in a 2% agarose gel

stained with ethidium bromide. Desired DNA fragments were gel-purified using Gene Clean (Bio 101) and ligated into pGEM-T Easy (Promega) plasmid system. Recombinant plasmids were transformed into sub-cloning efficiency chemically competent DH5α E. *coli* (Invitrogen) and selected upon LB ampicillin plates. Colony PCR was performed on white colonies using T7 and Sp6 as forward and reverse primers respectively. For OSU, HC and Arlee**,** 8 to 10 PCR amplicons, which had sizes similar to the size of the ligated PCR product (excluding the size of the multiple cloning site), were selected for sequencing using either T7 or SP6 as the sequencing primer. Sequencing was performed at the Washington State University Laboratory for Biotechnology and Bioanalysis. The sequence was verified to be that of the target gene using the BLASTn algorithm of the NCBI website. Alignment of the recovered sequences to look for sequence polymorphisms between parental clonal lines or nucleotide substitutions between duplicates of *Sox6* of the same clonal line was performed using Sequencher 3.11 software. Once more sequence information was obtained, tentative polymorphisms / substitutions were confirmed by direct amplification of candidate gene fragments from genomic DNA and sequencing with a nested primer.

2.5. Isolation of *Dax1* and *Dmrt1* genomic sequences

A PCR-based approach was used to isolate a lambda clone of *Dax1* and *Dmrt1* from a lambda Zap II (Stratagene, La Jolla, CA) genomic library created from an OSU X HC clonal hybrid. Following sequencing of the *Dax1* and *Dmrt1* lambda library clones by PCR walking, the structure of the *Dax1* gene was deduced based on sequence alignment with the relevant sequences of *Dicentarchus labrux* and *Oreochromis noliticus.* Intron and promoter sequences of

Dmrt1 were identified based on alignment of the recovered genomic clone with rainbow trout *Dmrt1* published cDNA sequence (Genbank Accession no. AF209095). To identify the map position of *Dax1* and *Dmrt1*, genomic DNA fragments of *Dax1* promoter and *Dmrt1* first intron were amplified by PCR using OSU and HC DNA. The fragments were cloned, sequenced, and the sequences aligned to look for polymorphisms as for the other candidate genes.

2.6. Genotyping by PCR-RFLP

Genomic sequences of the candidate genes *Sox6, Dmrt1 and Amh* have sequence substitutions between parental clonal lines and/or among gene duplicates which alter recognition sequences of restriction enzymes. To score doubled haploids of the mapping family for having either allele (presence or absence of the restriction site), a genomic DNA fragment containing the polymorphic restriction site was amplified using genomic DNA from parental clonal lines and doubled haploids of the respective mapping family. Four µls of the PCR reaction were run in a 2% agarose gel to check for amplification of the correct size product. The remaining DNA was ethanol-precipitated and the DNA pellet was re-suspended in 10 µls of distilled water. The re-suspended DNA was digested with the appropriate restriction enzyme (New England Bio-labs) in 20 µl volume reactions according to the manufacturer's directions. The products of the digestion reaction were fractionated in 1.5% agarose gels stained with ethidium bromide. The RFLP pattern was visualized under UV light and the doubled haploids were scored accordingly.

2.7. Genotyping by Taqman assay

A single nucleotide polymorphism (A/G) was detected between OSU and HC in the deduced promoter region of *Dax1*. A Taqman assay (ABI) was used to score doubled haploids of the OSU X HC mapping family for having either OSU or HC alleles.

2.8. Genotyping by size polymorphisms

A microstaellite repeat found nested in the *Sox6* fourth intron was amplified by PCR using DNA from OSU, HC, Arlee and doubled haploids of each respective family. PCR products were run in a 2.5% agarose gel stained with ethidium bromide and visualized under UV light. A size difference in the amplified microsatellite between *Sox6* of parental clonal lines and/or between *Sox6* duplicates was used to score doubled haploids of each family for having different *Sox6* alleles.

2.9. Gene mapping

Genotyping data of candidate genes was incorporated into the pre-constructed OSU x HC and OSU x Arlee linkage maps using Map Maker 3.0/EXP. Grouping of markers was performed using the Kosambi map function with a minimum LOD score of 3.0 and a maximum theta of 0.45.

2.10. Promoter sequence analysis

A 2.2 Kb region upstream of *Dax1* and 0.6 kb region upstream of *Dmrt1* coding regions were sequenced from the isolated lambda clones. The promoter sequences were analyzed for the presence of putative regulatory elements of other genes known to be involved in sex differentiation using Matinspector software (Genomatix Inc., www.genomatix.de).

3. Results

3.1. Genomic organization and map position of *Dax1* gene

Only a partial cDNA sequence of rainbow trout *Dax1* is found in nucleotide databases (Genbank accession number BX298095). Using primers that amplify a 221 bp region of the first exon of the reported sequence, we isolated and sequenced a complete genomic clone of the *Dax1* gene. Tentative locations of the start codon, stop codon and exon-intron boundaries were inferred based on sequence alignment with the reported sequences of *Dax1* from *Dicentarchus labrux* (AJ633646) and *Oreochromis noliticus* (AY135397). Our analysis indicates that the coding region of *Dax1* contains an ORF of 909 bp. The gene is organized into two exons 660 and 240 bp in size respectively. The exons are separated by one intron 563 bp in size. A 302 amino acid polypeptide was deduced from the inferred cDNA sequence (Figure 1). The intron is located between amino acid positions 222-223.

A 1.1 kb genomic fragment of the deduced 5' UTR and promoter region of *Dax1* was amplified and sequenced from OSU and HC clonal lines to look for sequence polymorphisms. A single A/G nucleotide polymorphism was found in the sequenced region (Table 2). Doubled

haploids of the OSU X HC mapping family were genotyped for either allele. The results of genotyping mapped *Dax1* to linkage group 5 (Figure 2).

3.2. Isolation of *Dmrt1* genomic sequences

We isolated and sequenced a 3.37 Kb lambda clone of *Dmrt1* genomic sequences from a genomic library created from an OSU X HC clonal hybrid. Sequence alignment with the reported cDNA sequence of *Dmrt1* indicated that the clone contained 649bp of sequence from the promoter region of *Dmrt1*, the entire 239 bp of the first exon, the first intron of the gene 1.757 kb in size and a 724 bp partial sequence of the second exon.

3.3. Linkage analysis of *Dmrt1* in the OSU X HC mapping family

We amplified, cloned and sequenced a 1.89 Kb genomic fragment which contains the first intron of *Dmrt1* from OSU and HC clonal lines. Sequence analysis revealed the presence of a single nucleotide polymorphism (SNP) between OSU and HC that leads to the loss of an *RsaI* site in the HC type (Table 2). PCR-RFLP using *Rsa1* restriction was used to score doubled haploids of the OSU X HC mapping family for the *Dmrt1* types (Figure 3(B)). Genotyping doubled haploids of the OSU X HC mapping family did not assign *Dmrt1* to any linkage group. However, chi-square analysis did not support linkage of the HC (YY) *Dmrt1* type with the Y chromosome of rainbow trout as indicated by phenotypic sex.

3.4. Promoter subsequence analysis of *Dax1* and *Dmrt1*

Subsequence analysis of the upstream 2.2 Kb promoter sequence of *Dax1* and the 649 bp promoter sequence of *Dmrt1* identified a number of transcription factor binding sites or *cis* regulatory elements (Table 3, Table 4). Intriguingly, some of these elements are binding sites for products of genes known to be involved in the sex differentiation cascade of mammals. These elements include SF1 (steroidegenic Factor 1), WT1 (wilms' tumor suppressor), COUP-TF (chicken ovalbumin upstream promoter-transcription factor), HMGA (high mobility group A) and PAX (paired domain box). Interestingly, previous experiments in mammalian systems have demonstrated the interaction of the *Dax1* gene product with SF1, WT1, and COUP-TF (BURRIS *et al.* 1995; KIM *et al.* 1999; YU *et al.* 1998).

3.5. Genomic organization and map position of *Amh*

Comparison of *Amh* cDNA sequence from rainbow trout with zebrafish (*Danio rerio*) orthologous genomic sequences indicated the presence of four tentative introns in *Amh* of rainbow trout. We amplified, cloned and sequenced a 1.35 kb genomic fragment that contains the 2nd, 3rd and 4th introns of *Amh* from OSU and Arlee clonal lines. Intron sizes were 337 bp, 106 bp and 93 bp respectively. Comparison of OSU and Arlee intron sequences indicated the presence of a SNP in the 4th intron of OSU that changes the recognition sequence of *Tsp509I* restriction enzyme (Table 2). PCR-RFLP with *Tsp509I* was used to score doubled haploids of the OSU X Arlee mapping family for receiving either allele (Figure 3(A)). The results of genotyping mapped *Amh* to linkage group eight (Figure 2).

3.6. *Sox6* is duplicated in rainbow trout

Exon-intron boundaries of eight tentative introns were identified in *Sox6* of rainbow trout by comparing the cDNA sequence of *Sox6* from rainbow trout to relevant genomic sequences of zebrafish (*Danio rerio*) and fugu (*Takifugu rubripes*). PCR amplification of the fourth intron of *Sox6* from OSU and HC produced two products of differing size. The OSU products were 1067 and 984 bp in size, while the sizes of the amplified products in HC were 1097 bp and 984 bp respectively (Table 5). All four products were cloned and sequenced. The sequence of all four products was verified to be that of *Sox6*. The larger-sized OSU and HC types were named *Sox6(1)* while the smaller-sized types were named *Sox6(2)*. Sequence analysis of the four different *Sox6* alleles in OSU and HC reveals the presence of a microsatellite repeat nested in the 4^{th} intron of *Sox6* (data not shown). The size difference between *Sox6(1)* and *Sox6(2)* is due to an expansion in the size of the microsatellite repeat. The size difference between *Sox6(1)* of HC and its counterpart in OSU is due to a greater expansion in the same microsatellite repeat in HC. The presence of two *Sox6* products in each of the homozygous clonal lines OSU and HC indicated that Sox6 is duplicated in the rainbow trout. Intriguingly, PCR amplification of the same intron from Arlee produced only one product 984 bp in size. To look for small insertions, deletions or single nucleotide substitutions between the *Sox6* duplicates of Arlee that cannot be resolved in agarose gels, the Arlee product was cloned and sequenced. However, sequence information did not reveal any nucleotide substitutions in the amplified region between *Sox6* duplicates of Arlee.

3.7. Inheritance of *Sox6* alleles in the OSU X Arlee mapping family

In addition to the size difference between the *Sox6(1)* and *Sox6(2)* loci of OSU, sequence comparison between the different *Sox6* duplicates of OSU and Arlee indicated the presence of a substitution in *Sox6(2)* of OSU that changes the recognition sequence of *AvaII* restriction enzyme (Table 5 and Figure 4(A)). PCR-RFLP with *AvaII* was used to genotype doubled haploids of the OSU X Arlee mapping family for the different *Sox6* alleles. Double reduction division, which can occur in polyploid organisms as a result of multivalent formation during meiosis (BURNHAM 1962), is described as the formation of gametes that carry two copies of the same allele on sister chromatids (ALLENDORF and DANZMANN 1997). Genotype analysis of the OSU X Arlee mapping family showed that 9 out of 76 doubled haploids have only received the OSU *Sox6(2)* allele (Genotype 4 in Figure 4(A) and (B)). This result can be explained by the occurrence of double reduction divisions in the OSU X Arlee male hybrid used for constructing the mapping family. This might also be explained by failure of the *AvaII* restriction assay in samples of all nine doubled haploids that displayed evidence of double reduction division. However, the genotype of the aforementioned doubled haploids was confirmed by digestion with *NlaIII* which also produces a distinctive polymorphic restriction profile for each of the four genotypes (data not shown).

3.8. Inheritance of *Sox6* alleles in the OSU X HC mapping family

Double reduction division in an OSU X HC male hybrid would lead to the formation of doubled haploids that have two alleles of the same type (i.e : *Sox6(1)* or *Sox6(2)*) from the same

parent. Utilizing size differences between the different *Sox6* alleles in OSU and HC, doubled haploids of the OSU X HC mapping family were genotyped for the different *Sox6* alleles to evaluate the occurrence of double reduction divisions in the OSU X HC male hybrid used for making the family (Figure 5). Genotyping 85 doubled haploids of the OSU X HC mapping family provided no evidence of double reduction division of *Sox6* loci in that cross.

3.9. Map position of *Sox6(1)* and *Sox6(2)*

The results of genotyping doubled haploids of the OSU X Arlee mapping family for the different *Sox6* alleles mapped *Sox6(1)* to linkage group 18 and *Sox6(2*) to linkage group 10 (Figure 2). Homeologous linkage groups are linkage groups or segments of linkage groups that arose from a common ancestor following a proposed whole genome duplication event that preceeded salmonid radiation and thus are involved in multivalent formation and might exchange chromatid segments during meiosis (ALLENDORF and DANZMANN 1997). A homeologous relationship between linkage groups 10 and 18 has been suggested in a number of studies (GUYOMARD *et al.* 2006; NICHOLS *et al.* 2003; PHILLIPS *et al.* 2006) and is supported in this study.

Sequence information recovered from the 4th intron of *Sox6(2)* of OSU and HC did not show any sequence substitutions between the two lines and thus we were not able to map it in the OSU X HC cross. However, in view of the clear size difference in *Sox6(1)* between OSU and HC, we were able to genotype doubled haploids of the mapping family for that *Sox6* allele and it

mapped to linkage group 18, which is concordant with the result obtained upon mapping the same locus in the OSU X Arlee cross.

4. Discussion

We tested five loci for their linkage to the Y chromosome of rainbow trout in this study. Three *Sox9* loci (ALFAQIH *et al*. In prep) and three *WT1* loci (BRUNELLI *et al.* 2001) have also been mapped in our genetic crosses. This brings the number of loci of candidate genes of rainbow trout sex determination tested for linkage to the Y chromosome to eleven. All of the loci tested were chosen based on broad agreement of their role in mammalian sex determination and/or based on expression studies that indicate over-expression of the candidate genes during testicular differentiation of rainbow trout (BARON *et al.* 2005). However, none of the loci tested could potentially act as the primary sex-determining gene in rainbow trout because none of the loci showed linkage to the Y chromosome.

A number of candidate genes can still be tested for their linkage to the sex locus of rainbow trout. Ten out of the 30 genes discovered so far in the *Sox* gene family have been shown to be involved in sex determination or expressed in embryonic testes or ovaries (BOWLES *et al.* 2000; TAKADA and KOOPMAN 2003). Rainbow trout orthologs of five of those genes are described in either Genbank or the TIGR rainbow trout gene index. Genes of the DM domain family are also strong candidates for sex determination in rainbow trout. There are six DM domain genes in fish, most of them with unknown functions (VOLFF *et al.* 2003). In addition to *Dmrt1*, orthologous sequences of *Dmrt2* and *Dmrt4* have been isolated from rainbow trout

(MARCHAND *et al.* 2000). Studying the gene expression profile of 102 genes involved in gonadal differentiation of rainbow trout. BARON *et al.* (2005) identified *Pax2a* as an early expressed gene displaying a testis specific expression profile. This study identified potential binding sites of *Pax2* in the promoter sequences of *Dmrt1* and *Dax1*. Accordingly, members of this gene family are also strong candidates for sex determination in rainbow trout.

Despite the presence of a clear polymorphism that differentiates between the parental *Dmrt1* types of the OSU X HC mapping family, we were not able to assign that gene to any linkage group. However, chi-square analysis excluded linkage between HC type of *Dmrt1* and the sex locus of rainbow trout. Failure to genetically map *Dmrt1* to any linkage group might be explained by the absence of closely-linked markers co-segregating with *Dmrt1*. It might also be explained by cross-amplification of *Dmrt1* with a paralogous gene or a gene duplicate of the same size. In such a case, doubled haploids of the mapping family will not be genotyped correctly for receiving the corresponding allele of each gene. However, a chi square test (data not shown) did not support significant deviation of the marker distribution from expected Mendelian segregation ratios which might occur in the event of cross amplifying a paralogous gene.

All salmonids, including rainbow trout, are believed to have evolved from a single tetraploid ancestor 25-100 million years ago (ALLENDORF and THORGAARD 1984). Multivalent formation between homologous chromosomes and their ancestral duplicates results in tetrasomic segregation ratios of duplicated loci. In salmonids, meiotic multivalents and tetrasomic inheritance are restricted to males. Restoration of disomic inheritance and diplodization of the genome occurs gradually via the structural organization of duplicated chromosome sets into two distinct homeologues (WAINES 1976). An estimated 20% of rainbow trout loci are isoloci which

are still recombining in males (ALLENDORF and DANZMANN 1997; ALLENDORF and THORGAARD 1984; PHILLIPS *et al.* 2006) . This indicates that diplodization of the rainbow trout genome is still not complete. This finding is supported by genetic maps of allozyme loci (MAY and JOHNSON 1993) and by physical mapping of BAC clones containing sequences of duplicated loci (PHILLIPS *et al.* 2006). Consequently, the inheritance pattern of *Sox6*(1) and *Sox6*(2) duplicated loci in the OSU X Arlee mapping family (i.e.: formation of double reduction gametes) indicates that duplication of the *Sox6* locus is recent and is the result of the proposed whole genome duplication event that preceded salmonid radiation. This also indicates that linkage groups 10 and 18 of rainbow trout are still pairing and recombining in meiosis in this genetic cross. It is more difficult for proximal areas of homeologous chromosomes to recombine because of the structural constraint imposed by multivalent formation on recombination. Therefore, the telomeric location of *Sox6(1)* and *Sox6(2)* on linkage groups 10 and 18 respectively further supports recent duplication of the *Sox6* locus.

Differences in pairing affinity between homeologous chromosomes (ALLENDORF and DANZMANN 1997) explain why tetrasomic inheritance is more frequent in rainbow trout crosses that involve distantly-related parental lines (PHILLIPS *et al.* 2006). The HC clonal research strain is more closely related to OSU strain than Arlee. This relationship is supported by comparing the chromosome numbers of each of the aforementioned strains (OSU and HC have 60 chromsomes while Arlee has 64); (RISTOW *et al.* 1998) and is further supported by an AFLP similarity tree that compares distances of the different clonal research strains propagated in the lab (Nichols, unpublished data) based on the number of AFLP bands they share. This relationship might explain the absence of evidence of tetrasomic inheritance of the *Sox6(1)* and *Sox6(2)* loci in the OSU X HC mapping family.

A number of models were proposed to explain tetrasomic segregation ratios of duplicated loci in male salmonids (ALLENDORF and DANZMANN 1997; ALLENDORF and THORGAARD 1984; WRIGHT *et al.* 1983). However, we currently lack direct chromosomal evidence supporting any of these models. We have genetically shown in this study that linkage groups 10 and 18 are involved in tetrasomic inheritance of duplicated loci. Using BAC clones that hybridize to these linkage groups (PHILLIPS *et al.* 2006) would allow us to more properly model the steps involved in tetrasomic inheritance. This would involve performing *in situ* hybridization experiments on meiotic preparation of rainbow trout males to attempt to visualize formation of multivalents and track their resolution.

In conclusion, the origin of the sex determining gene in medaka (MATSUDA *et al.* 2002) suggested that downstream genes of the sex determining pathway can assume the function of the master key regulator of the cascade following their duplication (SCHARTL 2004). Using linkage analysis in genetic crosses, we excluded the role of five candidate loci as sex-determining genes in rainbow trout. We also identified a recent duplication of the *Sox6* locus specific to the salmonid lineage and added four known genes to its genomic map. Enrichment of the rainbow trout genomic map with type I markers allows for the identification of conserved syntenic blocks between rainbow trout and other vertebrate organisms and also allows for comparative genome analyses.

References cited

ALLENDORF, F. W., and R. G. DANZMANN, 1997 Secondary tetrasomic segregation of MDH-B and preferential pairing of homeologues in rainbow trout. Genetics **145:** 1083-1092.

ALLENDORF, F. W., and G. H. THORGAARD, 1984 Tetraploidy and the evolution of salmonid fishes in *The Evolutionary Genetics of Fishes*. Plenum publishing, New York.

ALLENDORF, F. W., F. M. UTTER and B. P. MAY, 1975 Gene duplication within the family Salmonidae: detection and determination of the genetic control of duplicate loci through inheritance studies and examination of populations, pp. 415–431 in *Isozymes*, edited by C. L. MARKERT. Academic Press, New York.

AMORES, A., A. FORCE, Y. L. YAN, L. JOLY, C. AMEMIYA *et al.*, 1998 Zebrafish hox clusters and vertebrate genome evolution. Science **282:** 1711-1714.

BARON, D., R. HOULGATTE, A. FOSTIER and Y. GUIGUEN, 2005 Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod **73:** 959-966.

BOWLES, J., G. SCHEPERS and P. KOOPMAN, 2000 Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. Dev Biol **227:** 239-255.

BROMAGE, N., J. JONES, C. RANDALL, M. THRUSH, B. DAVIES *et al.*, 1992 Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout (Oncorhynchus mykiss). Aquaculture **100:** 141-166.

BRUNELLI, J. P., B. D. ROBISON and G. H. THORGAARD, 2001 Ancient and recent duplications of the rainbow trout Wilms' tumor gene. Genome **44:** 455-462.

BRUNNER, B., U. HORNUNG, Z. SHAN, I. NANDA, M. KONDO *et al.*, 2001 Genomic organization and expression of the doublesex-related gene cluster in vertebrates and detection of putative regulatory regions for DMRT1. Genomics **77:** 8-17.

BURNHAM, C. R., 1962 *Discussions in cytogenetics*. Burgess Publ. Co, St. Paul, MN.

BURRIS, T. P., W. W. GUO, T. LE and E. R. B. MCCABE, 1995 Identification of a Putative Steroidogenic Factor-1 Response Element in the DAX-1 Promoter. Biochemical and Biophysical Research Communications **214:** 576-581.

BYE, V. J., and R. F. LINCOLN, 1986 Commercial methods for the control of sexual maturation in rainbow trout (Salmo gairdneri R.). Aquaculture **57:** 299-309.

DONALDSON, E. M., and G. A. HUNTER, 1982 Sex control in fish with particular reference to salmonids. Canadian Journal of Fisheries and Aquatic Sciences **39:** 99-110.

FELIP, A., A. FUJIWARA, W. P. YOUNG, P. A. WHEELER, M. NOAKES *et al.*, 2004 Polymorphism and differentiation of rainbow trout Y chromosomes. Genome **47:** 1105-1113.

GRAVES, J. A., and J. W. FOSTER, 1994 Evolution of mammalian sex chromosomes and sex-determining genes. Int Rev Cytol **154:** 191-259.

GUYOMARD, R., S. MAUGER, K. TABET-CANALE, S. MARTINEAU, C. GENET *et al.*, 2006 A type I and type II microsatellite linkage map of rainbow trout (Oncorhynchus mykiss) with presumptive coverage of all chromosome arms. BMC Genomics **7:** 302.

ITURRA, P., M. BAGLEY, N. VERGARA, P. IMBERT and J. F. MEDRANO, 2001 Development and characterization of DNA sequence OmyP9 associated with the sex chromosomes in rainbow trout. Heredity **86:** 412-419.

KIM, J., D. PRAWITT, N. BARDEESY, E. TORBAN, C. VICANER *et al.*, 1999 The Wilms' Tumor Suppressor Gene (wt1) Product Regulates Dax-1 Gene Expression during Gonadal Differentiation. Mol. Cell. Biol. **19:** 2289-2299.

MARCHAND, O., M. GOVOROUN, H. D'COTTA, O. MCMEEL, J. LAREYRE *et al.*, 2000 DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, Oncorhynchus mykiss. Biochim Biophys Acta **1493:** 180-187.

MATSUDA, M., Y. NAGAHAMA, A. SHINOMIYA, T. SATO, C. MATSUDA *et al.*, 2002 DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature **417:** 559-563.

MAY, B., and K. R. JOHNSON, 1993 Composite linkage maps of salmonid fishes (*Salvelinus*, *Salmo*, *Oncorhynchus*), pp. 309-317 in *Genetic maps: Locus map of complex genomes*, edited by S. J. O' BRIEN. Cold Spring Harbor Laboratory press, Woodbury, NY.

Table legends

Table1. F and R, forward and reverse primers used for amplifying genomic fragments that were later cloned and sequenced; LF and LR, forward and reverse primers used for library screening; MF and MR, forward and reverse primers used for mapping each respective fragment; SF and SR; forward and reverse primers used for genotyping by size polymorphisms.

Table1. Primer sequences.

Gene	Cloning/Library screening primers	Marker position	Mapping primers
Sox6	F: TTCACAGGCAGCAAGACCAG R: AACAGCGCTGTGGAGTTCAG	4th intron	MF: TTCACAGGCAGCAAGACCAG MR: AACAGCGCTGTGGAGTTCAG SF: CTAGAGCTGCGGATGTTGTAAC SR: TGAGCCCACTGGCAGGTGTTC
Amh	F: CATCACTTTCACCAGTCACTC R: TCGGTACTGCGTCTCACTG	4th intron	MF: CATCACTTTCACCAGTCACTC MR: TCGGTACTGCGTCTCACTG
*D*ax1	LF: CTCCGGTCACCGCAGGTTAC LR: AGGATCCGTTGCAACATGC F: ACCTACAGCACCGAATATCAC R: GTTGTTGCCTTAGCTCAAGC	5' UTR	Taqman assay
Dmrt1	F: AGGAACCACGGCTACGTGT R: CAACCTCCTGACTGGACAG	1st intron	MF: CAGAAATGCAAACTGATCGC MR: CAACCTCCTGACTGGACAG

Table 2. Typing of *Amh, Dax1* and *Dmrt1* genes and alleles by sequence polymorphisms.

Gene	Accession numbers	Parent	Site of polymorphism	Polymorphism	Method of genotyping
Amh		Arlee	79-82 of fourth intron	**(A)**ATT	PCR-RFLP *Tsp509I*
		OSU	79-82 of fourth intron	**(G)**ATT	
Dax1		HC	-10 of 5' UTR	**G**	Taqman assay
		OSU	-10 of 5' UTR	**A**	
Dmrt1		HC	744 of first intron	GTA**(C)**	PCR-RFLP *Rsa1*
		OSU	744 of first intron	GTA**(A)**	

Table 3. Typing of *Sox6* genes and alleles by sequence polymorphisms.

Gene	Accession numbers	parent	Site of polymorphism	Polymorphism	Method of genotyping	Size of marker
Sox6		Arlee	725-729 of fourth intron	G(**G**)ACC	PCR-RFLP *Ava II*	984 bp
Sox6(1)		OSU	806-810 of fourth intron	G(**G**)ACC		1067 bp
Sox6(2)		OSU	725 of fourth intron	G(**A**)ACC		984 bp
Sox6(1)		HC	Size of fourth intron	30 bp expansion	Size polymorphism	1097 bp
Sox6(2)		HC	No polymorphism with *Sox6(2)* of OSU			984 bp

Table 4. Promoter sequence analysis of *Dax1*.

Element	Sequence	Location (bp)	Strand
PAX5	CTGTAGCACTGAAATGCAGTGCCTTAGAC	-1096/-1124	+
SF1	ATCTCAAGGCCAT	-816/-828	+
COUP-TF	CCGAGTGGGGCAGCGGTCTAAGGCA	-1082/-1105	-
HMGA	ACAAATTCACAACTCCC	-523/-539	-
TATA box	TAATATAAATGTGCTGC	-123/-139	+
WT1	TTGCGCGGGGGTATCAG	-104/-119	+
PAX 2/5/8	CTTTGAAGAGTTG	-52/-63	-
SOX-5	TTCTGACAATGCAGTAA	-488/-504	-

Table 5. Promoter sequence analysis of *Dmrt1*.

Element	Sequence	Location (bp)	Strand
SF1	CCTACAAGGTTAC	-176/-188	-
SF1	TTTGCAAGGCCAC	-438/-450	+
PAX2	CAACTTCTCGCGACATTAAACAC	-138/-160	+
PAX3	ACTGTCCCACGTGTACTCT	-187/-205	-
PAX6	ATTAAACACTTCAATAAGA	-128/-146	+

Figure legends

Figure 1. Inferred amino acid sequence of *Dax1*. Nucleotide and amino acid positions are numbered. Exon-intron junction is indicated by a triangle.

Figure 2. Genetic map of the rainbow trout (*Oncorhynchus mykiss*) candidate sex determining loci. Numbers on the left indicate distances in centimorgans. Marker names are indicated on the right. The linkage group number and the mapping family used for linkage analysis are indicated on top of each linkage group. Candidate sex determining loci are boxed.

Figure 3. *Amh* and *Dmrt1* are not linked to the Y chromosome. (A) Segregation of *Amh* alleles in OSU X Arlee doubled haploids for linkage analysis of the *Amh* locus. (B) Segregation of *Dmrt1* alleles in OSU X HC doubled haploids for linkage analysis of the *Dmrt1* locus. A genomic DNA fragment containing a polymorphic *Tsp509I* site, for *Amh* linkage analysis, and a polymorphic *RsaI* site, for *Dmrt1* Linkage analysis, was amplified by PCR. Following digestion with the appropriate restriction enzyme, the PCR products were fractionated in a 1.5% agarose gel and viewed under UV light. The image is inverted for better resolution. Samples of the parental OSU, HC and Arlee lines are labeled. Males of the doubled haploid family are labeled with the letter (M) while females are labeled with the letter (F). The results of genotyping only six individuals of the mapping family are presented in each figure.

Figure 4. Inheritance of *Sox6* alleles in the OSU X Arlee mapping family. (A) A schematic diagram showing the different *Sox6* alleles in OSU and Arlee parental lines and the different genotype classes that were observed in doubled haploids of the OSU X Arlee mapping family. The OSU types are shown in solid lines while the Arlee types are shown in dashed lines.

Restriction sites of *AvaII* enzyme are indicated by an inverted triangle on each respective *Sox6* type. The distance of each restriction site away from the 5' end of each respective type is indicated above the triangle. Sizes of the different *Sox6* types before their digestion with *AvaII* and the sizes observed in the mapping family following *AvaII* digestion are indicated with arrows. (B) A 2% agarose gel image of the different *Sox6* alleles in OSU and Arlee parental lines and the different genotype classes observed in doubled haploids created by androgenesis from an OSU X Arlee hybrid. Look at part (A) for details.

Figure 5. Inheritance of *Sox6* alleles in the OSU X HC mapping family. The size of a microsatellite repeat nested in the fourth intron of *Sox6(1)* is different between OSU and HC lines. No differences between the two lines exist in the size of the same microsatellite in the fourth intron of *Sox6(2)*. Markers of *Sox6(1)* and *Sox6(2)* loci were amplified simultaneously using primers that flank the microsatellite repeat in OSU, HC and fish of the OSU X HC mapping family. All individuals of the mapping family received markers of both *Sox6(1)* and *Sox6(2)*. The gel image is inverted for better resolution. Samples of the parental OSU and HC lines are labeled. Males of the doubled haploid family are labeled with the letter (M) while females are labeled with the letter (F). The results of genotyping only six individuals of the mapping family are presented in the figure.

Figure 2.

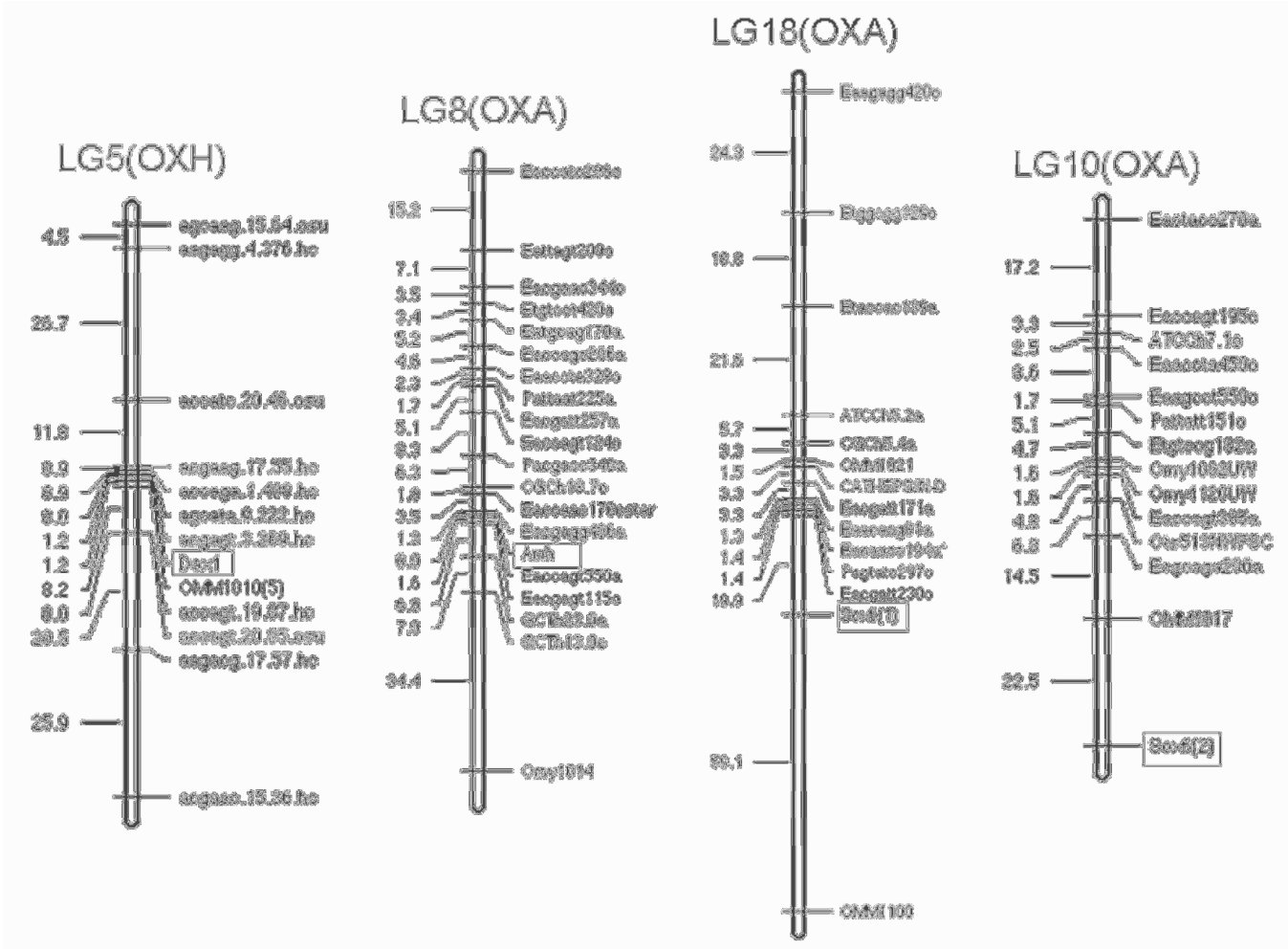


Figure 3.

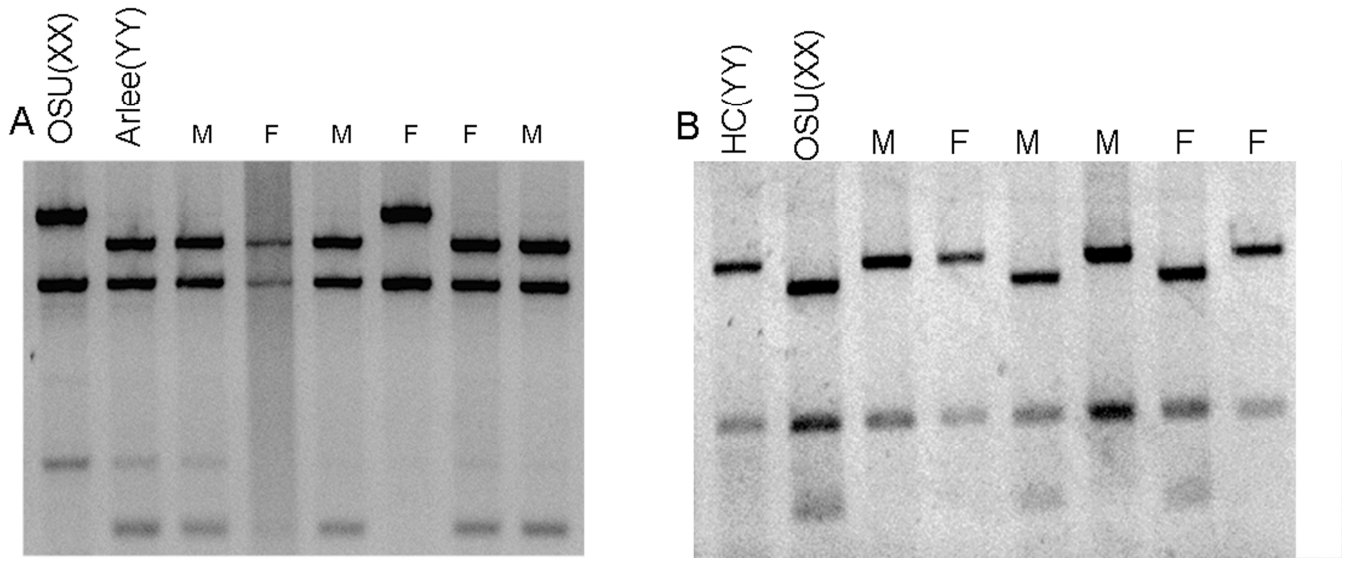


Figure 4.

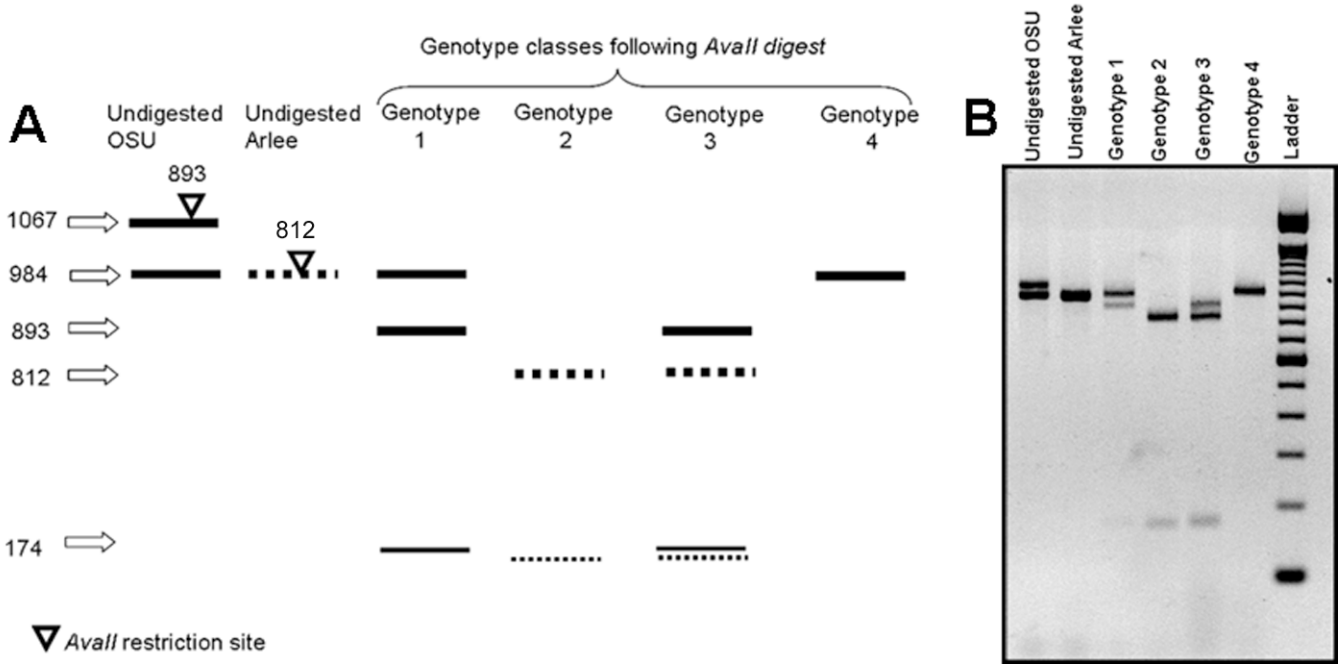
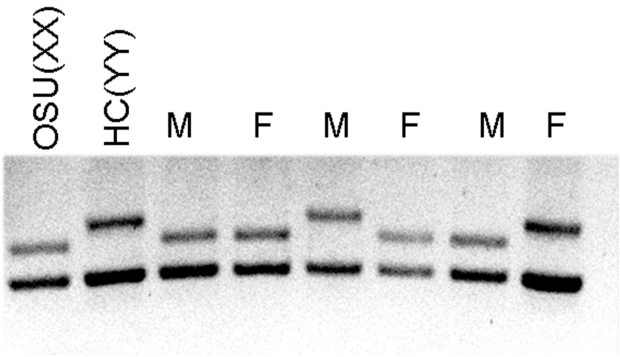

A
Undigested OSU
Undigested Arlee
Genotype classes following AvaII digest
Genotype 1
Genotype 2
Genotype 3
Genotype 4
893
812
1067
984
893
812
174
AvaII restriction site
B
Undigested OSU
Undigested Arlee
Genotype 1
Genotype 2
Genotype 3
Genotype 4
Ladder

Figure 5



CHAPTER FOUR

Comparative genome mapping reveals evidence of gene conversion between *Sox9* paralogous sequences of rainbow trout

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Abstract

Teleost fishes are the most diverse group of vertebrates. A whole genome duplication event that preceded their divergence spurred their rapid radiation. A more recent genome duplication event is believed to have taken place before the radiation of teleosts of the family Salmonidae. Two *Sox9* genes have been isolated from a number of teleost species. The first paralogous *Sox9* genes were isolated from zebrafish and are called *Sox9a* and *Sox9b*. *Sox9* orthologs were later isolated from a number of teleosts including rainbow trout. The *Sox9* copies of rainbow trout are called *Sox9* and *Sox9α2*. Previous evaluations of the evolutionary history of *Sox9* gene copies in teleosts using phylogenetic reconstructions gave ambiguous results. For example, zebrafish *Sox9a* and *Sox9b* did not clearly fall within their respective clades. In the same phylogeny the coding sequences of *Sox9* and *Sox9α2* of rainbow trout clustered together and fell within the *Sox9b* clade. However, this relationship was only weakly supported as evidenced by a bootstrap value of 66. We hypothesized that genetic mapping of *Sox9* and *Sox9α2* of rainbow trout would allow us to more properly evaluate the evolutionary relationship between *Sox9* gene copies in rainbow trout. Specifically, we believed that additional insights were likely because *Sox9* is part of a chromosomal block which has been shown to be conserved among a number of different species of different phyla, and recent evidence indicates conservation of the same block in rainbow trout. We show that *Sox9* locus is duplicated in rainbow trout and that the two copies map to homeologous linkage groups 2 and 9. Genotype frequencies of doubled haploids of the mapping family, for the duplicated *Sox9* copies, suggest that the *Sox9* copies are pseudo-linked. The map position of the *Sox9* gene copies indicates that they belong to the *Sox9b* clade. We also genetically map *Sox9α2* to linkage group 22. The map position of *Sox9α2* indicates that it belongs to the *Sox9a* clade. Phylogenetic reconstruction of teleost *Sox9* gene

copies using their 3' UTR regions supports the result obtained with comparative genome mapping. The mosaic evolutionary history of *Sox9* gene copies in rainbow trout suggests the presence of recombination / gene conversion events between *Sox9* and *Sox9α2*. This hypothesis is supported by a number of methods that detected potential gene conversions between the two paralogous sequences.

1. Introduction

Polyploidy has long been recognized to have played a major role in the evolution of vertebrates (Ohno 1970). Two rounds of genome duplication are hypothesized to have been pivotal in structuring the vertebrate genome (Hughes and Friedman 2003; Sidow 1996). With 24,000 species, teleosts, an infra-class of ray finned fish, are considered to be the most species rich group of vertebrates (Nelson 1994). The observation of extra genes and genomic clusters in this group compared to other vertebrate species has led researchers to propose the presence of an additional genome duplication event before teleost radiation. This genome duplication event is hypothesized to have taken place after divergence of ray-finned fish (Actinopterygii) and lobe-finned fish lineages (Sarcopterygii) (Amores et al. 1998; Naruse et al. 2004; Postlethwait et al. 2000) but before teleost radiation, and is also suggested to have provided the major driving force that promoted the extensive diversity seen among teleost species (Amores et al. 1998; Meyer and Schartl 1999).

Salmonids, a family of teleost fishes containing some 72 species and twelve genera (Nelson 1994) have all diverged from a common tetraploid ancestor 25-100 million years ago (Allendorf and Danzmann 1997; Allendorf and Thorgaard 1984). The polyploid ancestry of salmonids is supported by genome size estimates (Ohno et al. 1968), chromosome number and arm number counts (Phillips and Rab 2001) and the observation of multivalent formation during meiosis (Johnson et al. 1987). Tetrasomic segregation ratios of duplicated loci indicate that salmonids are still in the process of diploidization which would eventually restore disomic inheritance (Allendorf and Danzmann 1997; Allendorf and Thorgaard 1984; Moghadam et al. 2005). This makes salmonids one of the best vertebrate models for understanding how genomes

are organized following polyploidization and how homologous chromosomes that result from genome duplication diverge into two distinct homeologues. A more comprehensive understanding of the consequences of polyploidy within fishes and vertebrates in general requires comparative genome analysis of conserved gene families among closely related species and among diverse taxa.

Gene conversion is increasingly becoming recognized as an important genetic mechanism that shapes the evolution of multigene families. Well-studied examples of gene families where gene conversion is established to have occurred include; the globin gene family in primates (Scott et al. 1984), heat shock loci in *Drosophila melanogaster* (Leigh Brown and Ish-Horowicz 1981) and the ribosomal genes in hominoids (Arnheim et al. 1980). Gene conversion acts on members of gene families and maintains their sequence homogeneity. Because gene conversion does not necessarily affect entire sequences of genes it may lead to different regions of genes having different evolutionary histories (Posada and Crandall 2002). In that event, using phylogenetic analysis to evaluate the evolutionary history of members of gene families where gene conversion has occurred may not reflect the true history of the family (Posada et al. 2002; Posada and Crandall 2002). Phylogenetic trees with different topologies may result depending on the region used to reconstruct the phylogeny (Posada and Crandall 2002).

SOX9 is a member of a family of transcription factors that share the HMG box of SRY (Gubbay et al. 1990). Haploinsuffeciency of *Sox9* in humans results in campomelic dysplasia, a disease characterized by skeletal malformations and male to female sex reversal (Foster et al. 1994; Wagner et al. 1994). The symptoms of this disease illustrate the functional roles of *Sox9* during development; *Sox9* plays an important role in the regulation of cartilage formation and

male gonad development. *Sox9* has been shown to be required for chondrogenesis of all vertebrate species examined to date, in addition to teleost fish (Bi et al. 1999; Chiang et al. 2001; Foster et al. 1994; Yan et al. 2002). Interestingly, a conserved role for *Sox9* in male gonad development has also been demonstrated in all vertebrates examined to date, regardless of the switching mechanism involved in sex determination (Kent et al. 1996); da Silva et al. 1996; Western et al. 1999).

Two *Sox9* genes have been isolated from a number of teleost species. Chronologically, the first two teleost *Sox9* genes were isolated from zebrafish (Chiang et al. 2001). The two genes were considered to be co-orthologous of tetrapod *Sox9* and were believed to have arisen as a result of the genome-wide duplication proposed to have occurred before the divergence of teleosts. The two *Sox9* genes were accordingly called *Sox9a* and *Sox9b* (Chiang et al. 2001).

Two *Sox9* genes have been found in rainbow trout, *Sox9* (Takamatsu et al. 1997) and *Sox9α2* (Baron et al. 2005). *Sox9* was first considered to belong to the *Sox9a* clade because it branched with *Sox9a* of zebrafish in a phylogenetic analysis of a number of *Sox9* gene copies (Zhou et al. 2003). The aforementioned analysis did not include *Sox9α2* because it was not yet isolated. Following the isolation of additional *Sox9* orthologous from medaka (Kluver et al. 2005), fugu (Koopman et al. 2004) and stickleback (Cresko et al. 2003), zebrafish *Sox9a* and *Sox9b* turned out to be more divergent than other teleost *Sox9* genes and did not clearly fall within *Sox9a* or *Sox9b* clades (Kluver et al. 2005). This more recent phylogenetic analysis of *Sox9* gene copies from teleost species shows that *Sox9* and *Sox9α2* of rainbow trout cluster together and fall within the *Sox9b* clade alongside *Sox9b* of medaka, stickleback and rice field eel. However, this relationship is only weakly supported as indicated by a bootstrap value of 66.

In that same phylogeny, *Sox9a* of zebrafish falls within *Sox9b* clade while *Sox9b* of zebrafish falls as an outgroup to the other teleost *Sox9* genes.

Extensive chromosomal rearrangements have occurred during the evolution of teleost species. However, comparative genome analyses of the two teleost model organisms, zebrafish medaka and humans have shown that despite those rearrangements large syntenic chromosomal regions still exist among their genomes (Naruse et al. 2004; Postlethwait et al. 2000). A large region in human chromosome 17 is an example of one of those syntenic blocks. This chromosomal region has been shown to be conserved across zebrafish, medaka, mouse and pufferfish (Naruse et al. 2004; Postlethwait et al. 2000; Vandepoele et al. 2004). Recently, a syntenic relationship of the same chromosomal region has also been proposed for the rainbow trout genome (Moghadam et al. 2005). Genetic mapping of the *HoxBaii* cluster (Moghadam et al. 2005) and of the growth hormone gene type 1 (Nichols et al. 2003) in rainbow trout to linkage group 9 indicate that this linkage group, or part of it, is syntenic with human chromosome 17. Two big blocks of linkage groups 3 and 12 of zebrafish are hypothesized to be co-orthologs of human chromosome 17 which were derived by chromosome duplication (Postlethwait et al. 2000). Interestingly, *Sox9* of humans maps to chromosome 17 while *Sox9b* and *Sox9a* of zebrafish map to linkage groups 12 and 3 respectively (Chiang et al. 2001). In view of the presence of a conserved syntenic block between linkage group 9 of rainbow trout and human chromosome 17, the co-orthologous relationship between human chromosome 17 and linkage groups 3 and 12 of zebrafish and the fact that human *Sox9* maps to chromosome 17, we hypothesized that genetic mapping of *Sox9* and *Sox9α2* of rainbow trout would allow us to more properly evaluate the evolutionary relationship between *Sox9* gene copies in rainbow trout and

overcome the ambiguity in clade assignment based on evaluations that solely depend on phylogenetic reconstructions of *Sox9* gene copies in teleost species.

In this study, we show that the *Sox9* locus is duplicated in rainbow trout. Using a doubled haploid mapping family we genetically map the two copies to homeologous linkage groups 2 and 9 respectively. Genotype frequencies for the duplicated *Sox9* copies in the mapping family suggest that the *Sox9* copies are pseudo-linked. Linkage of *Sox9* copies with growth hormone gene type 1 and type 2 respectively supports that they are co-orthologs of *Sox9b*. We also genetically map *Sox9α2* to linkage group 22. Linkage of *Sox9α2* to *HoxB5bii* suggests that it belongs to the *Sox9a* clade. We finally show that phlyogenetic analysis of several teleost *Sox9* gene copies using their 3' untranslated regions supports the result obtained with comparative genome mapping. The mosaic evolutionary history of Sox9 gene copies in rainbow trout suggests the presence of recombination / gene conversion events between *Sox9* and *Sox9α2*. This hypothesis is supported by a number of methods that detected potential gene conversions between the two paralogs.

2. Materials and methods

2.1. Mapping families

A doubled haploid mapping family of rainbow trout (n=85) provided the source material for this study. The mapping family was created by androgenesis from an OSU X HC hybrid and was originally used to detect quantitative trait loci (QTL) for natural killer cell- like activity (Zimmerman et al. 2004). Details for the background of the family are provided in Zimmerman

et al. (2004). DNA originating from 85 doubled haploid fish of the original mapping family was used for genotyping polymorphisms in non-coding regions of *Sox9* and *Sox9α2*.

2.2. Primer design, PCR amplification, cloning, sequencing and identification of polymorphisms

All the primers were designed to amplify genomic DNA fragments of non-coding regions of genes. The exon-intron boundaries of *Sox9* were previously reported (Takamatsu et al. 1997). To infer the position of introns in *Sox9α2,* the cDNA sequence of *Sox9α2* of rainbow trout was aligned with that of *Sox9* using ClustalW and the primers were designed based on the sequence alignment and the potential conservation of intron positions. All PCR reactions were performed using a Thermolyne Amplitron thermocycler II. PCR reaction conditions begin with a 94°C 2 minutes denaturation step, followed by 30 cycles of 94°C for 45 seconds, 60 °C for 45 seconds and 72 °C for 45 seconds. The reaction ends with a final extension step of 72 °C for 2 minutes. All reactions were 20 µls in volume containing: 1 µl of 50 ηg/ µl DNA template, 2 µls of 10X PCR buffer (Invitrogen, Carlsbad, CA), 1 µl of 50 mM MgCl2, 1 µl of 10 pmoles of each primer, 0.5 ul of 10 mM dNTPs (Invitrogen, Carlsbad, CA) and 1.5 U of Taq DNA Polymerase (Promega, Madison, WI). PCR amplification products were resolved in a 2% agarose gel stained with ethidium bromide. Desired DNA fragments were gel purified using Gene Clean (Bio 101) and ligated into pGEM-T Easy (Promega) plasmid system. Recombinant plasmids were transformed into sub-cloning efficiency chemically competent DH5α E. *coli* (Invitrogen) and selected upon LB ampicillin plates. Colony PCR was performed on white colonies using T7 and Sp6 as forward and reverse primers respectively. Eight to 10 PCR amplicons which had sizes similar to the size of the ligated PCR product, excluding the size of the multiple cloning site,

were selected for sequencing using either T7 or SP6 as the sequencing primer. Sequencing was performed at the Washington State University Laboratory for Biotechnology and Bioanalysis. The sequence was verified to be that of the target gene using the BLASTn algorithm of the NCBI website. Alignment of the recovered sequences to look for sequence polymorphisms between parental clonal lines or nucleotide substitutions between duplicates of *Sox9* of the same clonal line was performed using Sequencher 3.11 software. Once more sequence information was obtained tentative polymorphisms / substitutions were confirmed by direct amplification of the gene fragments from genomic DNA and sequencing with a nested primer.

2.3. Genotyping by PCR-RFLP

Genomic sequences of *Sox9* and *Sox9α2* have sequence substitutions between parental clonal lines and/or among gene duplicates which alter recognition sequences of restriction enzymes. To score doubled haploids of the mapping family for having either allele (presence or absence of the restriction site), a genomic DNA fragment containing the polymorphic restriction site was amplified using genomic DNA from parental clonal lines and doubled haploid fish of the mapping family. Four µls of the PCR reaction were run in a 2% agarose gel to check for amplification of the correct size product. The remaining DNA was ethanol-precipitated and the DNA pellet was re-suspended in 10 µl of distilled water. The re-suspended DNA was digested with the appropriate restriction enzyme (New England Bio-labs) in 20 µl volume reactions according to the manufacturer's directions. The products of the digestion reaction were fractionated in 1.5% agarose gel stained with ethidium bromide. The RFLP pattern was visualized under UV light and the doubled haploids were scored accordingly.

2.4. Genotyping by direct sequencing

A nucleotide substitution (T/G) was detected between *Sox9* duplicates of HC. A genomic DNA fragment containing the site of the substitution was amplified by PCR using DNA from OSU, HC and doubled haploid fish of the mapping family. The PCR reaction was treated with Exo-SAP (USB) and the amplified product was sequenced with a nested primer to score doubled haploids for having that *Sox9* allele.

2.5. Gene mapping

Genotyping data of *Sox9* and *Sox9 α2* was incorporated into the pre-constructed OSU X HC linkage maps using Map Maker 3.0/EXP. Grouping of markers was performed using the Kosambi map function with a minimum LOD score of 3.0 and a maximum theta of 0.45

2.6. Phylogenetic analysis

Sequence data from the 3' UTR region was used to examine evolutionary relationships among gene copies. Sequences were obtained for several teleost species from GenBank for *Sox9a* (*Gasterosteus aculeatus*, AY351914.1; *Takifugu rubripes*, AY277965.1) and *Sox9b* (*Gasterosteus aculeatus*, AY351915.1; *Takifugu rubripes*, AY277964.1; *Cyprinus carpio*, AY956415.1; *Odontesthes bonariensis*, AY319415.2; *Danio rerio*, AY029578.1). Sequences were aligned in ClustalW with Genbank sequences for the *Sox9* (AB006448.1) and *Sox9α2*

sequences from rainbow trout (AF209872.1). Homologous regions that could be aligned were retained and used for phylogenetic analysis. Non-homologous regions that could not be aligned, including large insertions or deletions, were removed and coded as missing data for the analysis. Phylogenetic history of the *Sox9* gene was estimated from a Bayesian analysis conducted in MRBAYES 3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). A Bayesian analysis was run for 1 x 10^6 generations and sampled every 100th generation. Two independent runs were performed simultaneously and the average standard deviation of split frequencies at completion was 0.00294, suggesting convergence of the two runs on a stationary distribution. Examination of the distribution of posterior probabilities suggested stationarity was reached within 1000 generations, but the first 25% of samples (2.5 x 105 generations) were discarded as 'burn in' to ensure sampling from a stationarity posterior distribution.

2.7. Recombination (gene conversion) analysis

Detection of putative recombination events was carried out on the cDNA alignment of *Sox9* sequences from a number of teleost species using the software package RDP3 (Martin et al. 2005b) (http://darwin.uvigo.es/rdp/rdp.html). The package implements the following eight methods; RDP(Martin and Rybicki 2000), GENECONV (Padidam et al. 1999), BOOTSCAN (Martin et al. 2005a), MAXCHI (Smith 1992), CHIMAERA (Posada and Crandall 2001), SISCAN (Gibbs et al. 2000), LARD (Holmes et al. 1999) and 3Seq (Boni et al. 2007). Each analysis was run using default settings and only events detected between paralogs in the same genome by at least half of the methods (to control for false positives) were reported based on a Bonferroni corrected P-value cutoff of 0.05.

3. Results

3.1. *Sox9* is duplicated in rainbow trout

PCR amplification of the second intron of *Sox9* from OSU and HC produced a 0.8 kb fragment (data not shown). The fragment was cloned, sequenced and the sequence was verified to be that of *Sox9*. Sequence alignment revealed three areas of nucleotide substitutions between the recovered sequences (Table 2). Two of the substitutions were within sequences recovered from the HC clonal line. HC is a homozygous clonal line propagated in our lab through the process of androgenesis. The homozygosity of this clonal line has been confirmed through the process of multilocus DNA fingerprinting (Young et al. 1996). This result indicated duplication of the *Sox9* locus.

One of the substitutions in one of the HC types changed the recognition sequence of *Dra1* restriction enzyme (Table 2). To confirm duplication of the *Sox9* locus, a genomic DNA fragment containing the polymorphic *Dra1* restriction site plus another *Dra1* site common between all OSU and HC types was amplified from OSU, HC and doubled haploids of the mapping family and digested with *DraI*. The resulting restriction profile (Figure 1) confirmed duplication of the *Sox9* locus and provided an assay to score doubled haploids of the mapping family for receiving that *Sox9* allele. Furthermore, the first intron of *Sox9* from HC was amplified, cloned and sequenced and sequence analysis confirmed the presence of two types in HC (data not shown). The HC *Sox9* type that contains the additional *DraI* restriction site was called *Sox9(1)* while the other HC type was called *Sox9(2)*. Interestingly, sequence analysis of the first and second intron of *Sox9* from OSU did not reveal any sequence polymorphisms between the duplicated loci.

3.2. *Sox9(1)* and *Sox9(2)* map to homeologous linkage groups 2 and 9 respectively

Homeologous linkage groups are linkage groups or segments of linkage groups that arose from a common ancestor following a proposed whole genome duplication event that proceeded salmonid radiation (Nichols et al. 2003) and are accordingly involved in multivalent formation and might exchange chromatid segments during meiosis (Allendorf and Danzmann 1997). *Sox9(1)* and *Sox9(2)* mapped to linkage groups 2 and 9 respectively. The homeology of these two linkage groups has been suggested by a number of studies (Guyomard et al. 2006; Moghadam et al. 2005; Nichols et al. 2003; Phillips et al. 2006) and is further confirmed in this study.

3.3. *Sox9(1)* and *Sox9(2)* loci are pseudo-linked

Genotyping of the OSU X HC doubled haploids for the different sequence substitutions between the *Sox9(1)* and *Sox9(2)* types revealed the presence of four different genotype classes; two parental genotypes and two non-parental recombinants. Assuming independent assortment of the different *Sox9* types, the number of doubled haploids in each class should be equal. However, our analysis shows the presence of an excess number of non-parental recombinants (Figure 2). This is in agreement with a pseudo-linkage model in which homologous chromosomes pair with their homeologues forming a quadrivalent during meiosis. Alternate segregation of the quadrivalent is believed to cause the excess number of non-parental recombinants seen among pseudo-linked markers (Johnson et al. 1987; Wright et al. 1983).

Without prior knowledge of the phase of the alleles, pseudo-linked markers will appear in linkage disequilibrium (Gharbi et al. 2006) and thus the usage of the term pseudo-linkage.

3.4. *Sox9α2* maps to linkage group 22

Based on potential intron conservation between *Sox9* and *Sox9α2,* we amplified both introns of *Sox9α2* from the OSU and HC clonal lines and sequenced them to look for sequence polymorphisms between the two lines. Sequence analysis revealed the presence of a polymorphism in the first intron which changes the recognition sequence of *DraI* restriction enzyme in HC (Table 2). PCR-RFLP with *DraI* was used to score doubled haploids of the OSU X HC mapping family for having either the OSU or HC alleles (Figure 3). The results of genotyping mapped *Sox9α2* to linkage group 22.

3.5. Phylogenetic analysis of teleost *Sox9* gene copies based on their 3' UTR

Sox9 is up-regulated in the testis of all vertebrates examined to date regardless of the mechanism employed for sex determination (Morrish and Sinclair 2002). This indicates that the *Sox9* sequence has been under strong selection throughout evolution. Accordingly, we chose to estimate the evolutionary relationship of rainbow trout *Sox9* genes to each other and to those of other teleost fishes based on the more neutrally evolving 3' UTR sequences. Our results place *Sox9* of rainbow trout within the *Sox9b* clade and *Sox9α2* within the *Sox9a* clade (Figure 4).

3.6. Gene recombination analysis detects gene conversion between *Sox9* and *Sox9α2* of rainbow trout

Five out of the eight methods implemented by RDP3 software to detect gene recombination (P-value of 0.05) indicate that such an event has occurred between *Sox9* and *Sox9*α2 of rainbow trout (Table 3). RDP3 defined the putative recombinant daughter sequence as *Sox9α2* and the parent sequence as *Sox9* of rainbow trout.

A phylogeny constructed from the *Sox9* coding region of rainbow trout and other teleost fishes shows that the *Sox9* genes from rainbow trout group together in the same clade (Kluver et al. 2005). The fact that ectopic recombination was detected between these genes suggests that a gene conversion event may have occurred between these paralogs thus would explain the observed tree topology since gene conversions tend to homogenize sequences, making them more similar to each other.

4. Discussion

Comparative genome mapping between zebrafish and humans revealed the presence of a large chromosomal block that is syntenic between human chromosome 17 (HSA 17) and zebrafish linkage group 3 (LG 3) (Postlethwait et al. 2000). The same chromosomal block is also found on LG 12 of zebrafish, which suggested that LG 3 and LG 12 of zebrafish are duplicates of each other and are both co-orthologous of HSA 17. Mapping of *Hoxbaii* and growth hormone gene type 1 (*GH1*) to LG 9 of rainbow trout suggested that the same chromosomal block is also syntenic with rainbow trout LG 9 (Moghadam et al. 2005). The presence of the paralogous

sequences *GH2* and *Hoxbai* on LG 2 of rainbow trout suggested that parts of LG 2 and LG 9 are co-orthologous of LG 3 of zebrafish and are perhaps the result of the proposed whole genome duplication event specific to the salmonid lineage. Our study supported these relationships. We genetically map another locus, *Sox9*, localized within the same syntenic chromosomal block in humans to LG 9 of rainbow trout. We also show that *Sox9* locus is duplicated in rainbow trout and we map the duplicated copy to LG 2. The inheritance pattern of the duplicated *Sox9* copies in our mapping family (pseudo-linkage) supports previously suggested homeology between LG 2 and LG 9 and indicates that these two linkage groups can still pair up during meiosis, which further indicates that they resulted from a recent genome duplication event. The map position of *Sox9b* of zebrafish to LG 3 indicates that *Sox9(1)* and *Sox9(2)* of rainbow trout belong to the *Sox9b* clade which is similar to the result obtained with previous phylogenetic reconstructions of evolutionary history based on cDNA sequences of teloest *Sox9* gene copies (Kluver et al., 2005) and with a phylogenetic reconstruction based on 3' UTR sequences presented in this study.

Further, we suggest a possible conserved synteny between LG 22 of rainbow trout and LG 12 of zebrafish. This relationship is suggested by linkage of *Sox9α2*, mapped in this study, with *Hoxb5bii*, mapped by Moghadam et al. (2005). Synteny between these two linkage groups indicates that *Sox9α2* belongs to the *Sox9a* clade. Although this result is not supported by phylogenetic reconstructions based on cDNA sequences of teleost *Sox9* gene copies (Kluver et al. 2005), it is similar to the result obtained with phlyogenetic reconstructions presented in this study where evolutionary history of Sox9 gene copies is evaluated using their 3' UTR sequences.

The Mosaic evolutionary history of *Sox9α2* of rainbow trout, where phylogenetic analysis of its cDNA sequence infers a different relationship with *Sox9* than the relationship inferred with

reconstructions based on 3' UTR sequences, implied that gene conversion between *Sox9* and *Sox9α2* might be responsible for the difference seen in the topology of the two phylogenetic trees. Such a discrepancy in evaluating evolutionary history based on the region of the gene used for phylogenetic analysis have previously uncovered three gene conversion events between members of the major histocompatibility complex multigene family (Hughes et al. 1993). Gene conversion between *Sox9* and *Sox9α2* of rainbow trout was further supported by five methods commonly used to detect gene conversion between paralogous sequences. Such an event would have the effect of homogenizing the sequence of *Sox9α2* with *Sox9* and would explain their grouping in phylogenetic reconstructions based on their cDNA sequences. Escape of the 3'UTR sequence of *Sox9α2* from gene conversion might indicate that the border between the translated and untranslated regions of the gene represents a recombination breakpoint.

In conclusion, the data presented in this study provide further support for the presence of a conserved syntenic block between humans and salmonids which indicates linkage of the loci that fall on this block in the last common ancestor of the ray finned and lobe finned fish lineages, taxa that separated 400 million years ago. It also supports homeology between LG 2 and LG 9 of rainbow trout and suggests that these two linkage groups, or parts of them, have evolved by genome duplication from an ancestral linkage group orthologous to LG 3 of zebrafish. It also supports the proposed tetraploidization event in the lineage leading to salmonids. Furthermore, it shows that evaluation of evolutionary history of gene families based solely on phylogenetic reconstructions can sometimes give wrong inferences because of recombination between members of the gene family.

References cited

Allendorf FW, Danzmann RG (1997) Secondary tetrasomic segregation of MDH-B and preferential pairing of homeologues in rainbow trout. Genetics 145:1083-92

Allendorf FW, Thorgaard GH (1984) Tetraploidy and the evolution of salmonid fishes The Evolutionary Genetics of Fishes. Plenum publishing, New York

Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH (1998) Zebrafish hox clusters and vertebrate genome evolution. Science 282:1711-4

Arnheim NN, Krystal MM, Schmickel RR, Wilson GG, Ryder OO, Zimmer EE (1980) Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and apes. Proceedings of the National Academy of Sciences of the United States of America 77:7323-7

Baron D, Houlgatte R, Fostier A, Guiguen Y (2005) Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 73:959-66

Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B (1999) *Sox9* is required for cartilage formation. Nat Genet 22:85-89

Boni MF, Posada D, Feldman MW (2007) An Exact Nonparametric Method for Inferring Mosaic Structure in Sequence Triplets. Genetics 176:1035-1047

Chiang EF, Pai CI, Wyatt M, Yan YL, Postlethwait J, Chung B (2001) Two sox9 genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. Dev Biol 231:149-63

Cresko WA, Yan YL, Baltrus DA, Amores A, Singer A, Rodriguez-Mari A, Postlethwait JH (2003) Genome duplication, subfunction partitioning, and lineage divergence: Sox9 in stickleback and zebrafish. Dev Dyn 228:480-9

da Silva SM, Hacker A, Harley V, Goodfellow P, Swain A, Lovell-Badge R (1996) Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat Genet 14:62-68

Foster JW, Dominguez-Steglich MA, Guioli S, Kowk G, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, et al. (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 372:525-30

Gharbi K, Gautier A, Danzmann RG, Gharbi S, Sakamoto T, Hoyheim B, Taggart JB, Cairney M, Powell R, Krieg F, Okamoto N, Ferguson MM, Holm LE, Guyomard R (2006) A linkage map for brown trout (Salmo trutta): chromosome homeologies and comparative genome organization with other salmonid fish. Genetics 172:2405-19

Gibbs MMJ, Armstrong JJS, Gibbs AAJ (2000) Sister-scanning: a Monte Carlo procedure for assessing signals in recombinant sequences. Bioinformatics 16:573-82

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, Lovell-Badge R (1990) A gene mapping to the sex-determining region of the

mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature 346:245-50

Guyomard R, Mauger S, Tabet-Canale K, Martineau S, Genet C, Krieg F, Quillet E (2006) A type I and type II microsatellite linkage map of rainbow trout (Oncorhynchus mykiss) with presumptive coverage of all chromosome arms. BMC Genomics 7:302

Holmes EEC, Worobey MM, Rambaut AA (1999) Phylogenetic evidence for recombination in dengue virus. Molecular Biology and Evolution 16:405-9

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755

Hughes AL, Friedman R (2003) 2R or not 2R: Testing hypotheses of genome duplication in early vertebrates. Journal of Structural and Functional Genomics 3:85-93

Johnson KR, Wright JE, Jr., May B (1987) Linkage Relationships Reflecting Ancestral Tetraploidy in Salmonid Fish. Genetics 116:579-591

Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P (1996) A male-specific role for SOX9 in vertebrate sex determination. Development 122:2813-22

Kluver N, Kondo M, Herpin A, Mitani H, Schartl M (2005) Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. Dev Genes Evol 215:297-305

Koopman P, Schepers G, Brenner S, Venkatesh B (2004) Origin and diversity of the Sox transcription factor gene family: genome-wide analysis in Fugu rubripes. Gene 328:177-186

Leigh Brown AAJ, Ish-Horowicz DD (1981) Evolution of the 87A and 87C heat-shock loci in Drosophila. Nature 290:677-82

Martin D, Rybicki E (2000) RDP: detection of recombination amongst aligned sequences. Bioinformatics 16:562-563

Martin DDP, Posada DD, Crandall KKA, Williamson CC (2005a) A modified bootscan algorithm for automated identification of recombinant sequences and recombination breakpoints. AIDS research and human retroviruses 21:98-102

Martin DP, Williamson C, Posada D (2005b) RDP2: recombination detection and analysis from sequence alignments. Bioinformatics 21:260-262

Meyer A, Schartl M (1999) Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. Curr Opin Cell Biol 11:699-704

Moghadam HK, Ferguson MM, Danzmann RG (2005) Evidence for Hox gene duplication in rainbow trout (Oncorhynchus mykiss): a tetraploid model species. J Mol Evol 61:804-18

Morrish BC, Sinclair AH (2002) Vertebrate sex determination: many means to an end. Reproduction 124:447-57

Naruse K, Tanaka M, Mita K, Shima A, Postlethwait J, Mitani H (2004) A medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. Genome Res 14:820-8

Nelson JS (1994) Fishes of the World

Nichols KM, Young WP, Danzmann RG, Robison BD, Rexroad C, Noakes M, Phillips RB, Bentzen P, Spies I, Knudsen K, Allendorf FW, Cunningham BM, Brunelli J, Zhang H, Ristow S, Drew R, Brown KH, Wheeler PA, Thorgaard GH (2003) A consolidated linkage map for rainbow trout (Oncorhynchus mykiss). Anim Genet 34:102-15

Ohno S (1970) Evolution by Gene Duplication

Ohno S, Wolf U, Atkin NB (1968) Evolution from fish to mammals by gene duplication. Hereditas 59:169–187

Padidam M, Sawyer S, Fauquet CM (1999) Possible Emergence of New Geminiviruses by Frequent Recombination. Virology 265:218-225

Phillips R, Rab P (2001) Chromosome evolution in the Salmonidae (Pisces): an update. Biological Reviews 76:1-25

Phillips RB, Nichols KM, DeKoning JJ, Morasch MR, Keatley KA, Rexroad C, 3rd, Gahr SA, Danzmann RG, Drew RE, Thorgaard GH (2006) Assignment of rainbow trout linkage groups to specific chromosomes. Genetics 174:1661-70

Posada D, Crandall KA (2001) Evaluation of methods for detecting recombination from DNA sequences: Computer simulations. Proceedings of the National Academy of Sciences 98:13757-13762

Posada D, Crandall KA, Holmes EC (2002) Recombination in evolutionary genomics. Annual Review of Genetics 36:75-97

Posada DD, Crandall KAKA (2002) The effect of recombination on the accuracy of phylogeny estimation. Journal of molecular evolution 54:396-402

Postlethwait JH, Woods IG, Ngo-Hazelett P, Yan YL, Kelly PD, Chu F, Huang H, Hill-Force A, Talbot WS (2000) Zebrafish comparative genomics and the origins of vertebrate chromosomes. Genome Res 10:1890-902

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574

Scott AF, Heath P, Trusko S, Boyer SH, Prass W, Goodman M, Czelusniak J, Chang LY, Slightom JL (1984) The sequence of the gorilla fetal globin genes: evidence for multiple gene conversions in human evolution. Mol Biol Evol 1:371-389

Sidow A (1996) Gen(om)e duplications in the evolution of early vertebrates. Current Opinion in Genetics & Development 6:715-722

Smith JM (1992) Analyzing the mosaic structure of genes. Journal of Molecular Evolution 34:126-129

Takamatsu N, Kanda H, Ito M, Yamashita A, Yamashita S, Shiba T (1997) Rainbow trout SOX9: cDNA cloning, gene structure and expression. Gene 202:167-70

Vandepoele K, De Vos W, Taylor JS, Meyer A, Van de Peer Y (2004) Major events in the genome evolution of vertebrates: paranome age and size differ considerably between ray-finned fishes and land vertebrates. Proc Natl Acad Sci U S A 101:1638-43

Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E, et al. (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 79:1111-20

Western PS, Harry JL, Graves JA, Sinclair AH (1999) Temperature-dependent sex determination in the American alligator: AMH precedes SOX9 expression. Dev Dyn 216:411-9

Wright JE, Jr., Johnson K, Hollister A, May B (1983) Meiotic models to explain classical linkage, pseudolinkage, and chromosome pairing in tetraploid derivative salmonid genomes. Isozymes Curr Top Biol Med Res 10:239-60

Yan YL, Miller CT, Nissen RM, Singer A, Liu D, Kirn A, Draper B, Willoughby J, Morcos PA, Amsterdam A, Chung BC, Westerfield M, Haffter P, Hopkins N, Kimmel C, Postlethwait JH (2002) A zebrafish sox9 gene required for cartilage morphogenesis. Development 129:5065-79

Young WP, Wheleer PA, Fields RD, Thorgaard GH (1996) DNA fingerprinting confirms isogenecity of androgenetically derived rainbow trout lines. J. Hered 87:77-81.

Zhou R, Liu L, Guo Y, Yu H, Cheng H, Huang X, Tiersch TR, Berta P (2003) Similar gene structure of two Sox9a genes and their expression patterns during gonadal differentiation in a teleost fish, rice field eel (Monopterus albus). Mol Reprod Dev 66:211-7

Zimmerman AM, Evenhuis JP, Thorgaard GH, Ristow SS (2004) A single major chromosomal region controls natural killer cell-like activity in rainbow trout. Immunogenetics 55:825-35

Table 1. Primer sequences.

Gene	Cloning primers	Marker position	Mapping primers
Sox9	F: CACATCTCTTCCGGTGACATC	2nd intron	MF: AAGTCGATGTTGAGCTGGC
	R: AAGTACTGGTCGAACTCATGGA		MR: GAAGTGCGTTCTCCCAGTG
			NP: TAGGGCTCTCATGAACTAACAC
Sox9	F: TCAAGAGACCCATGAACGCGT	1st intron	Cloned to confirm gene duplication
	R:GATGTCACCGGAAGAGATGTG		
Sox9α2	F: CATCTCCATAACGCTGAGCTG	1st intron	MF: CATCTCCATAACGCTGAGCTG
	R: TGCGCGTTTTTCATGGACTTC		MR: TGCGCGTTTTTCATGGACTTC

F and R, forward and reverse primers used for amplifying genomic fragments that were later cloned and sequenced; MF and MR, forward and reverse primers used for mapping each respective fragment; NP; nested primer used for genotyping polymorphisms by direct sequencing.

Table 2. Typing of *Sox9* genes and alleles by sequence polymorphisms

Gene	Accession numbers	Parent	Site of substitution	Polymorphism	Method of genotyping
.First substitution					PCR-RFLP *Dra1*
Sox9(1)		HC	101-106 of second intron	TTTAAA	
Sox9(2)		HC	99-104 of second intron	AATCAT	
Sox9		OSU	99-104 of second intron	AATCAT	
.Second substitution					Direct sequencing
Sox9(1)		HC	481 of second intron	T	
Sox9(2)		HC	479 of second intron	T	
Sox9		OSU	481 of second intron	C	
.Third substitution					Direct sequencing
Sox9(1)		HC	980 of cDNA	G	
Sox9(2)		HC	980 of cDNA	T	
Sox9		HC	980 of cDNA	G	
Sox9α2		HC	161-166 of first intron	TT(**G**)AAA	PCR-RFLP *Dra1*
Sox9α2		OSU	161-166 of first intron	TT(**T**)AAA	

Table 3. Results of eight different recombination tests implemented in RDP3

Method	P-value
RDP	3.993×10^{-04}
GENECONV	1.889×10^{-02}
BootScan	2.144×10^{-05}
MaxChi	5.349×10^{-04}
Chimaera	3.225×10^{-01}
SiScan	1.043×10^{-09}
LARD	1.107×10^{-09}
3Seq	4.612×10^{-02}

Figure legends

Figure 1. *Sox9* is duplicated in rainbow trout. (A) Sequence analysis of *Sox9* types in OSU and HC revealed the presence of a *Dra1* restriction site common between all four types and another polymorphic site found only in one of the HC types. (B) A genomic DNA fragment containing both *Dra1* sites was amplified from OSU, HC in addition to males and females of the mapping family. The fragment was later digested with *DraI* and fractionated in a 2% agarose gel. The presence of four bands in HC restriction profile and only two in OSU confirmed duplication of the *Sox9* locus. Samples of the parental HC and OSU lines are labeled. Males of the doubled haploid family are labeled with the letter (M) while females are labeled with the letter (F).

Figure 2. Pseudo-linkage of *Sox9* types in OSU X HC mapping family. OSU types are shown in solid lines and HC types are shown in spotted dash lines. The genotypes of the doubled haploids produced from the OSU X HC hybrid are shown in circles. The observed number of each class of doubled haploids is shown beneath each respective genotype (circle). If we assume normal disomic inheritance of *Sox9*, where the duplicated copies of *Sox9* assort independently, the expected number of doubled haploids of each class will be equal (n=21). However, there is an excess of non-parental recombinants supporting pseudo-linkage between the two *Sox9* types.

Figure 3. Segregation of *Sox9α2* alleles in OSU X HC doubled haploids for linkage analysis of the *Sox9α2* locus. A 0.4 Kb genomic DNA fragment containing a polymorphic *DraI* site was amplified by PCR. Following digestion with *DraI*, the PCR products were fractionated in a 2% agarose gel and viewed under UV light. The image is inverted for better resolution. Samples of the parental HC and OSU lines are labeled. Doubled haploid fish of the mapping family that

received the OSU allele are labeled with the letter (O) while those that received the HC allele are labeled with the letter (H).

Figure 4. Phylogenetic reconstructions of *Sox9* gene copies in teleosts using their 3' UTR sequences. Results indicate that *Sox9* of rainbow trout belongs to the *Sox9b* clade while *Sox9α2* belongs to the *Sox9a* clade.

Figure 1

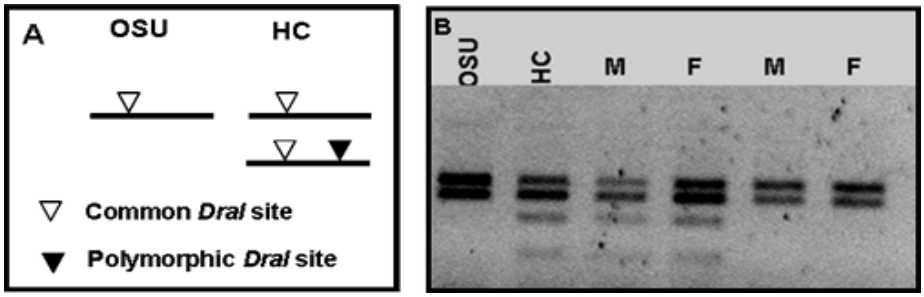
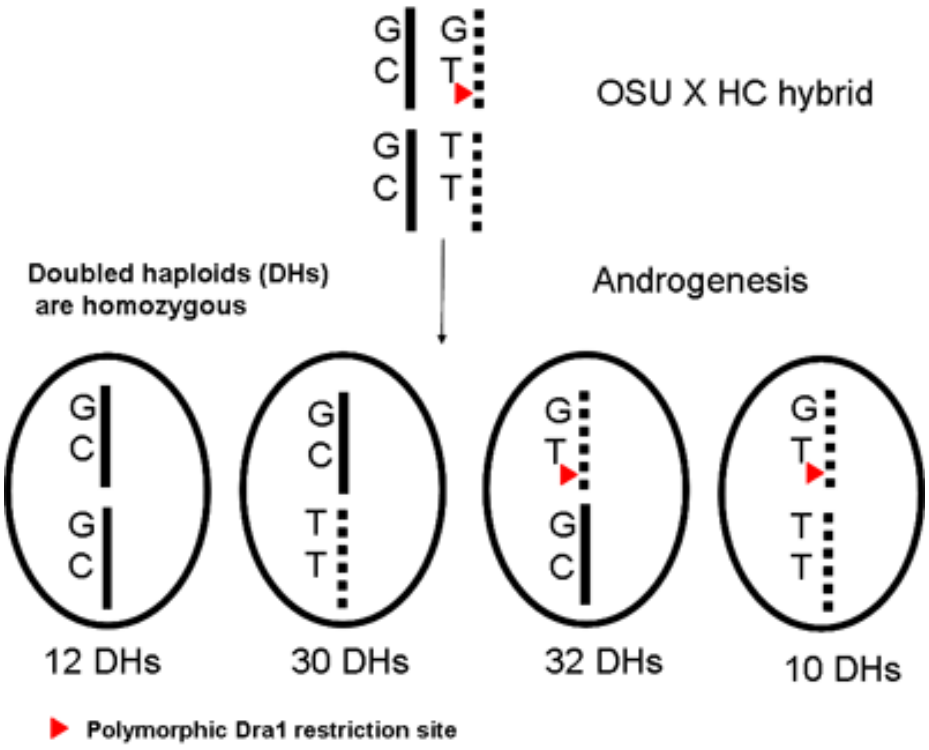


Figure 2


G
C
G
T
OSU X HC hybrid
G
C
T
T
Doubled haploids (DHs)
are homozygous
Androgenesis
G
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T
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12 DHs
30 DHs
32 DHs
10 DHs
Polymorphic Dra1 restriction site

Figure 3

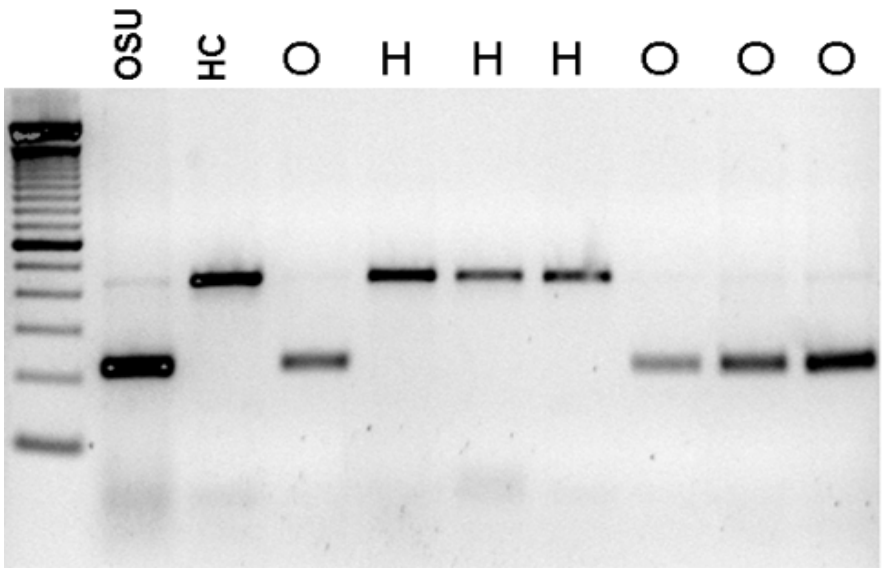
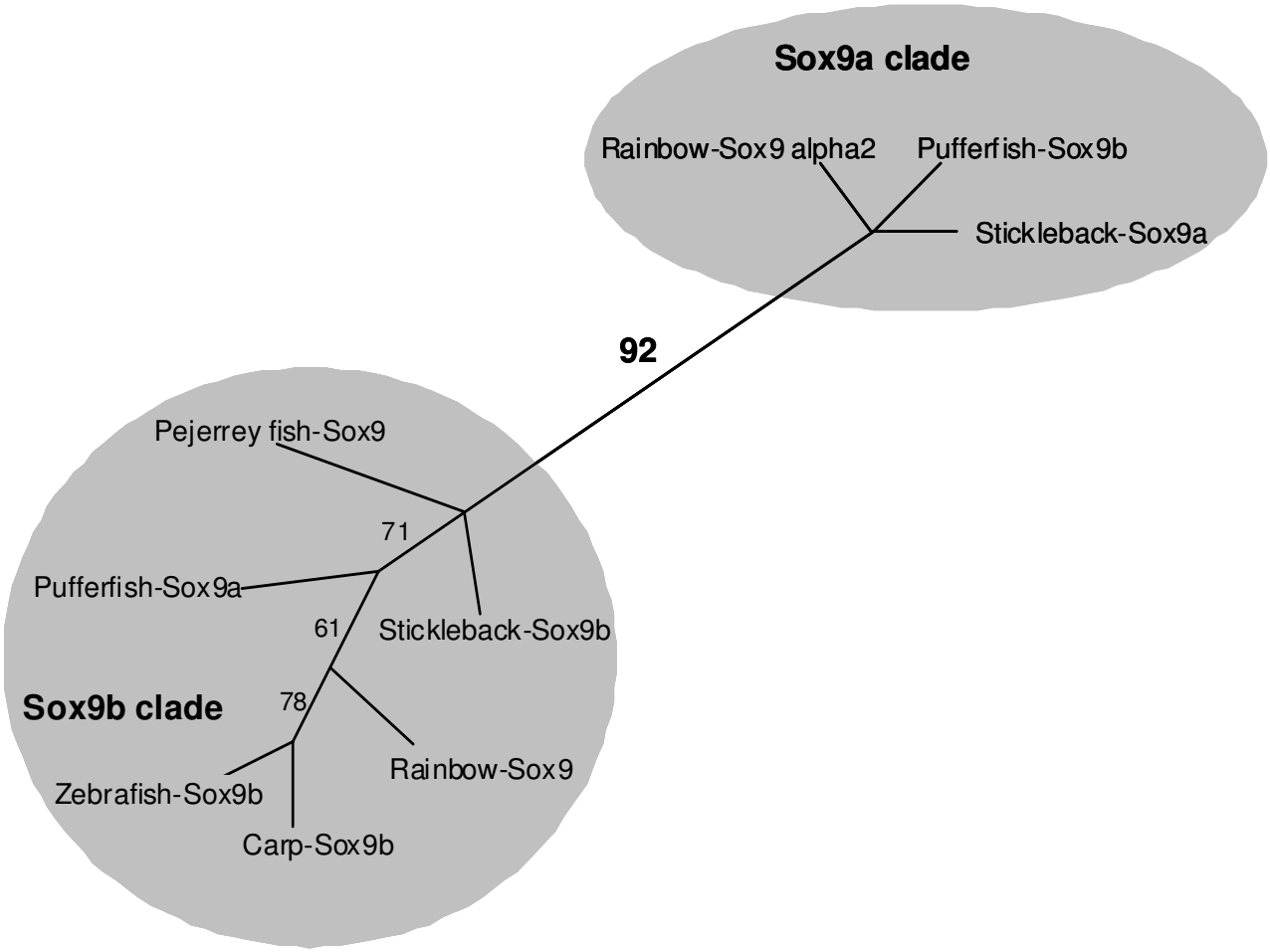


Figure 4


Sox9a clade
Rainbow-Sox9 alpha2
Pufferfish-Sox9b
Stickleback-Sox9a
92
Pejerrey fish-Sox9
71
Pufferfish-Sox9a
61
Stickleback-Sox9b
Sox9b clade
78
Rainbow-Sox9
Zebrafish-Sox9b
Carp-Sox9b

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

1. Conclusions

The studies presented in this dissertation only represent one step forward toward a better understanding of two big themes in Biology: sex determination and genome duplication (polyploidization). Although we were never able to identify the sex-determining gene in rainbow trout, one goal of this study, many other notable findings were made and numerous opportunities for future research were created by these findings.

We were able to demonstrate that, unlike other *Oncorhynchus* species, rainbow trout and cutthroat trout share the same sex chromosome pair. We also show that rainbow and cutthroat trout sex chromosomes appear to be largely homologous as indicated by comparative genetic mapping of a number of loci, previously shown to be located on rainbow trout sex chromosomes, with cutthroat trout. However, despite their overall homology, inferred by genetic mapping, the physical location of the BAC clone containing the microsatellite marker Omm1665 is different between cutthroat trout Y chromosome and rainbow trout sex chromosome pair (X and Y). This marker maps just below the centromere of rainbow trout sex chromosomes. However, it maps above the centromere of cutthroat trout Y chromosome. This difference can be utilized for stock identification of rainbow and cutthroat trout populations and for assessing species introgression and hybridization.

We also identify two pairs of homeologous linkage groups (2 and 9, 10 and 18) which appear to be involved in multivalent pairing as indicated by the inheritance pattern of two

duplicated loci that genetically mapped on the above linkage groups. Despite the presence of a number of models that attempt to explain the unusual segregation ratio of duplicated loci observed in tetraploid derivative species of the salmonid lineage (Allendorf and Danzmann 1997; Allendorf and Thorgaard 1984), there is no direct physical evidence supporting or refuting any of the models. Numerous BAC clones which contain loci that physically map on each of the above linkage groups, shown to be involved in tetrasomic inheritance, have been identified (Phillips et al. 2006). Accordingly, FISH experiments on meiotic preparations of rainbow trout males using these BAC clones can help us better understand the mechanics of tetrasomic inheritance and asses the validity of the previous models.

Comparative genome analysis within and between salmonids is essential for understanding the structural differentiation of genomes following their polyploidization. Salmonid species provide a "natural laboratory" for studying this process because they represent a single lineage for which there is cumulative evidence of a whole genome duplication event in its recent evolutionary history. We have added six known gene markers to the rainbow trout map. These markers would definitely be beneficial for such comparisons.

2. Future research

Studies on the molecular factors involved in sex determination across divergent species have revealed that downstream genes of the cascade are conserved. However, genes that trigger the pathway are only present in specific branches of the phylogenetic tree (Marin and Baker 1998). This has led researchers to propose that the sex-determination cascade evolves from the

bottom upward, where genes located at the top of the hierarchy have become involved in triggering the pathway only relatively recently (Marin and Baker 1998). Furthermore, it was proposed that the "master switch" of the sex determination cascade evolves from downstream genes of the pathway by gene duplication (Schartl 2004).

Depending on the above hypothesis we tested a number of genes, for which there is evidence in the scientific literature for their involvement in sex determination, for linkage to the Y chromosome of rainbow trout. Unfortunately, despite our intensive efforts we did not find any gene that can potentially act as the sex-determining gene of rainbow trout. Although our results do not undermine the validity of our approach, two approaches are presented here with the ultimate goal of finding the sex-determining gene.

The first approach presented discusses a strategy that can be applied to find new candidate genes for sex determination in Pacific salmon and trout. The strategy is based on results which indicate that the sex-determining gene of medaka is part of a fragment originating from one of the austosomal chromosomes which was duplicated and inserted into the Y chromosome. We strongly believe that a similar scenario might be involved in evolution of the sex-determining mechanism of Pacific salmon and trout.

The second approach presented discusses a strategy which can be applied to generate a finer linkage map of rainbow trout sex chromosomes. This map can be later used as a framework to find the sex-determining gene by chromosome walking using bacterial artificial chromosome (BAC) genomic clones.

2.1. Candidate gene approach

Medaka (*Oryzias latipes*) have an XX-XY mechanism for sex determination where males are the heterogametic sex (Matsuda et al. 1998). The sex chromosomes are homomorphic and cannot be cytogenetically distinguished. A gene responsible for male sexual development, *Dmy*, has been recently isolated from medaka (Matsuda et al. 2002; Nanda et al. 2002). This gene product belongs to a family of transcription factors that share a highly conserved DNA binding domain known as the DM domain. The prototype member of this family of proteins, DMRT1, is involved in sex determination in a variety of phyla, ranging from nematodes to mammals (Burtis and Baker 1989; Raymond et al. 1999; Zhu et al. 2000). *Dmy* appears to have evolved from an autosomal homologue of *Dmrt1* (Kondo et al. 2006; Matsuda et al. 2002). Sequence analysis of the Y specific region in medaka indicates that *Dmy* is part of an autosomal fragment that was duplicated and inserted into what became the Y chromosome (Kondo et al. 2006; Matsuda et al. 2002). *Dmy* is the only functional gene within the duplicated fragment, all the other co-duplicated genes degenerated (Kondo et al., 2006). Interestingly, *Dmrt1* is part of a genomic cluster formed with two additional *Dmrt* genes. The organization of this cluster is highly conserved across different phyla, from fish to mammals (Brunner et al. 2001).

A similar scenario might be involved in the evolution of the sex-determining factor of some salmonid species of the genus *Oncorhynchus*. A growth hormone pseudogene (*GH-Y*) is linked to the Y chromosome of the four salmon species; chum, pink, chinook and coho (Devlin et al. 2001). Fluorescence in situ hybridization (FISH) experiments with a probe containing *GH-Y* sequence indicate that this marker is part of the Y-specific region of all aforementioned species and is not part of the pseudo-autosomal region shared with the X chromosome (Phillips et al.

2007; Phillips et al. 2005). In fact, the above probe was used to identify the Y chromosome of chinook, coho, chum and pink salmon. Phylogenetic analysis indicates that *GH-Y* evolved from growth hormone gene type 2 (*GH2*). Intriguingly, recent evidence (Moghadam et al. 2005) suggests that *GH2* is part of a chromosomal segment that is conserved across rainbow trout, zebrafish, fugu, mouse and humans.

Based on the above observations, we hypothesize that analogous to the situation in medaka, *GH-Y* is part of a chromosomal fragment that was duplicated from an autosomal linkage group and inserted into the Y chromosome of some *Oncorhynchus* species. The sex-determining gene would be the only gene on that fragment that remained functional. All the other co-duplicated genes, including *GH2*, became degenerate (pseudo-genes). Accordingly, the sex determining gene might be a paralogue of a gene linked to *GH2*.

None of the salmonid species have their complete genome sequenced. All of the currently published maps do not have a high density of known gene markers. However, *GH2* is part of chromosomal fragment that appears to be syntenic with zebrafish. Accordingly, candidate genes linked to the growth hormone gene of zebrafish can be identified from zebrafish genomic databases. Duplicates of the candidate genes can be isolated and later tested for their linkage to the Y chromosome of salmonid species.

2.2. A better linkage map of rainbow trout sex chromosomes

Despite the publication of a number of rainbow trout sex chromosome linkage maps (Felip et al. 2004; Guyomard et al. 2006; Nichols et al. 2003; Sakamoto et al. 2000; Young et al.

1998), the true location of the sex determining locus remains ambiguous. This is largely attributed to the fact that all markers in that area of the chromosome cluster on top of each other some distance away from phenotypic sex (Felip et al. 2004; Nichols et al. 2003). This feature of male-based linkage maps, is explained by (1) the lack of meiotic recombination in that area of the chromosome (Felip et al. 2004; Sakamoto et al. 2000; Zhang et al. 2001), which explains the clustering of genetic markers. (2) sex reversal, which explains the lack of makers that map on top of phenotypic sex.

In rainbow trout, as in other trout and salmon species, large sex-specific differences exist in the rate and localization of recombination (Gharbi et al. 2006; Sakamoto et al. 2000). Females have a much higher recombination rate (ratio F: M, 3.25:1) with crossovers localized to centromeric regions of linkage groups, while male crossovers are more distal in location (Sakamoto et al. 2000). OmyY1 is a sex linked marker in all pacific salmon species tested except for pink salmon (Brunelli, personal communication). The fact that the sex locus of most *Oncorhynchus s*pecies is on a different linkage group except for those that can form viable hybrids (Phillips 2002; Phillips et al. 2007; Phillips et al. 2005) suggest that OmyY1 is part of the male determining region of all *Oncorhynchus* species, which appears to be transposing to a different linkage group during species evolution.

The following is an outline for the development of a rainbow trout doubled haploid mapping family. The development of such a family would allow us to benefit from the higher female recombination rate and at the same time produce a mapping family that segregates for the sex locus: (1) Oregon State University, OSU, XX female line is crossed with one of the YY male lines to create a clonal male hybrid. (2) the aforementioned male hybrid is sex reversed into a

phenotypic female via the administration of 17 β estradiol (Simpson et al. 1976; Johnstone et al. 1978). (3) A doubled haploid mapping family is generated from the sex reversed female by the process of gynogenesis.

Guyomard et al. (2006) published an updated microsatellite linkage map of rainbow trout with comprehensive coverage of all chromosome arms. Twenty five of those microsatellites mapped to linkage group 1 of rainbow trout which represents the sex chromosome pair. Individuals of the mapping family can be genotyped for the 25 microsatellites. To overcome the problem of sex reversal, OmyY1 can be used as a marker for phenotypic sex. This approach would likely give us the closest approximation of the true location of the sex locus.

The Y chromosome linkage map produced from this study would likely have a higher resolution than any of the previously published maps. This approach would allow us to identify markers on both sides of rainbow trout sex locus which would set up the stage for a BAC walking experiment that follows the linkage mapping and eventually lead to the identification of the sex determining-gene of the species.

3. Final remark

Despite the established role of rainbow trout and other salmonids as a suitable research model in a wide range of applications including carcinogenesis, toxicology, nutrition, disease and immunology (Thorgaard et al. 2002), and the documented suitability of the clonal lines as powerful tools for investigations pertaining to these research applications. Research in rainbow trout is still far from achieving its fullest potential. This unfortunate reality is largely attributed

for the lack of a complete sequence of its genome, despite the obvious benefit of such a step (Thorgaard et al. 2002). Being directly involved in salmonid genetic research over the past four years I can understand the disappointment associated with the lack of such a project and the immediate necessity to have such a step initiated.

References cited

Allendorf FW, Danzmann RG (1997) Secondary tetrasomic segregation of MDH-B and preferential pairing of homeologues in rainbow trout. Genetics 145:1083-92

Allendorf FW, Thorgaard GH (1984) Tetraploidy and the evolution of salmonid fishes The Evolutionary Genetics of Fishes. Plenum publishing, New York

Brunelli JP, Thorgaard GH (2004) A New Y-Chromosome-Specific Marker for Pacific Salmon. Transactions of the American Fisheries Society 133:1247-1253

Brunner B, Hornung U, Shan Z, Nanda I, Kondo M, Zend-Ajusch E, Haaf T, Ropers HH, Shima A, Schmid M, Kalscheuer VM, Schartl M (2001) Genomic organization and expression of the doublesex-related gene cluster in vertebrates and detection of putative regulatory regions for DMRT1. Genomics 77:8-17

Burtis KC, Baker BS (1989) Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell 56:997-1010

Devlin RH, Biagi CA, Smailus DE (2001) Genetic mapping of Y-chromosomal DNA markers in Pacific salmon. Genetica 111:43-58

Felip A, Fujiwara A, Young WP, Wheeler PA, Noakes M, Phillips RB, Thorgaard GH (2004) Polymorphism and differentiation of rainbow trout Y chromosomes. Genome 47:1105-13

Gharbi K, Gautier A, Danzmann RG, Gharbi S, Sakamoto T, Hoyheim B, Taggart JB, Cairney M, Powell R, Krieg F, Okamoto N, Ferguson MM, Holm LE, Guyomard R (2006) A linkage map for brown trout (Salmo trutta): chromosome homeologies and comparative genome organization with other salmonid fish. Genetics 172:2405-19

Guyomard R, Mauger S, Tabet-Canale K, Martineau S, Genet C, Krieg F, Quillet E (2006) A type I and type II microsatellite linkage map of rainbow trout (Oncorhynchus mykiss) with presumptive coverage of all chromosome arms. BMC Genomics 7:302

Kondo M, Hornung U, Nanda I, Imai S, Sasaki T, Shimizu A, Asakawa S, Hori H, Schmid M, Shimizu N, Schartl M (2006) Genomic organization of the sex-determining and adjacent regions of the sex chromosomes of medaka. Genome Res 16:815-26

Marin I, Baker BS (1998) The evolutionary dynamics of sex determination. Science 281:1990-4

Matsuda M, Matsuda C, Hamaguchi S, Sakaizumi M (1998) Identification of the sex chromosomes of the medaka, Oryzias latipes, by fluorescence in situ hybridization. Cytogenet Cell Genet 82:257-62

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417:559-63

Moghadam HK, Ferguson MM, Danzmann RG (2005) Evidence for Hox gene duplication in rainbow trout (Oncorhynchus mykiss): a tetraploid model species. J Mol Evol 61:804-18

Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Schartl M (2002) A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. Proc Natl Acad Sci U S A 99:11778-83

Nichols KM, Young WP, Danzmann RG, Robison BD, Rexroad C, Noakes M, Phillips RB, Bentzen P, Spies I, Knudsen K, Allendorf FW, Cunningham BM, Brunelli J, Zhang H, Ristow S, Drew R, Brown KH, Wheeler PA, Thorgaard GH (2003) A consolidated linkage map for rainbow trout (Oncorhynchus mykiss). Anim Genet 34:102-15

Phillips RB (2002) Characterization of Charr Chromosomes Using Fluorescence in Situ Hybridization. Environmental biology of fishes 64:223

Phillips RB, DeKoning J, Morasch MR, Park LK, Devlin RH (2007) Identification of the sex chromosome pair in chum salmon (Oncorhynchus keta) and pink salmon (Oncorhynchus gorbuscha). Cytogenet Genome Res 116:298-304

Phillips RB, Morasch MR, Park LK, Naish KA, Devlin RH (2005) Identification of the sex chromosome pair in coho salmon (Oncorhynchus kisutch): lack of conservation of the sex linkage group with chinook salmon (Oncorhynchus tshawytscha). Cytogenet Genome Res 111:166-70

Phillips RB, Nichols KM, DeKoning JJ, Morasch MR, Keatley KA, Rexroad C, 3rd, Gahr SA, Danzmann RG, Drew RE, Thorgaard GH (2006) Assignment of rainbow trout linkage groups to specific chromosomes. Genetics 174:1661-70

Raymond CS, Kettlewell JR, Hirsch B, Bardwell VJ, Zarkower D (1999) Expression of Dmrt1 in the Genital Ridge of Mouse and Chicken Embryos Suggests a Role in Vertebrate Sexual Development. Developmental Biology 215:208-220

Sakamoto T, Danzmann RG, Gharbi K, Howard P, Ozaki A, Khoo SK, Woram RA, Okamoto N, Ferguson MM, Holm LE, Guyomard R, Hoyheim B (2000) A microsatellite linkage map of rainbow trout (Oncorhynchus mykiss) characterized by large sex-specific differences in recombination rates. Genetics 155:1331-45

Schartl M (2004) Sex chromosome evolution in non-mammalian vertebrates. Curr Opin Genet Dev 14:634-41

Thorgaard GH, Bailey GS, Williams D, Buhler DR, Kaattari SL, Ristow SS, Hansen JD, Winton JR, Bartholomew JL, Nagler JJ, Walsh PJ, Vijayan MM, Devlin RH, Hardy RW, Overturf KE, Young WP, Robison BD, Rexroad C, Palti Y (2002) Status and opportunities for genomics research with rainbow trout. Comp Biochem Physiol B Biochem Mol Biol 133:609-46

Young WP, Wheeler PA, Coryell VH, Keim P, Thorgaard GH (1998) A detailed linkage map of rainbow trout produced using doubled haploids. Genetics 148:839-50

Zhang QQ, Nakayama II, Fujiwara AA, Kobayashi TT, Oohara TT, Masaoka SS, Kitamura RRH, Devlin (2001) Sex identification by male-specific growth hormone pseudogene (GH-psi) in Oncorhynchus masou complex and a related hybrid. Genetica 111:111-8

Zhu L, Wilken J, Phillips NB, Narendra U, Chan G, Stratton SM, Kent SB, Weiss MA (2000) Sexual dimorphism in diverse metazoans is regulated by a novel class of intertwined zinc fingers. Genes Dev. 14:1750-1764