SEX DETERMINATION IN THE GENUS SALMO

by

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Abstract

Salmonids share a strictly genetic mechanism for sex determination with males being the heterogametic sex. FISH analysis using BACs containing sex-linked microsatellite markers in Atlantic salmon identified chromosome 2 as its sex chromosome. The same sized chromosome 2 pairs in both sexes suggesting that the sex chromosome in Atlantic salmon is not highly differentiated. Combining information from sex determination pathways in different phyla, I created a list of 14 candidate sex determining genes. Candidate gene mapping identified all gene locations. However, their positions rule them out as the master regulatory gene. Comparative genomic analysis shows that the closest related species brown trout sex linkage group (LG) is syntenic to Atlantic salmon autosomal LG 8. FISH analysis revealed the brown trout sex chromosomes as a pair of small submetacentric chromosomes. Although they are closely related, I predict that Atlantic salmon and brown trout are likely to have different sex determining genes.

Keywords: Atlantic salmon; brown trout; sex determination; sex determining gene candidates; gene mapping; comparative genomic analysis; FISH analysis.

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Table of Contents

List of Figures

List of Tables

1: Introduction

It is well known that sexual reproduction has tremendous advantages over asexual reproduction. Sexual reproduction provides genetic recombination and linkage disequilibrium that allows one species to quickly adapt to the changing environment rather than going to extinction due to the accumulation of deleterious mutations. Sex determination is a fundamental process for sexual reproduction that leads to the development of two sexes, male and female, for almost all animal species. Although mechanisms for sex determination vary from purely environmental influences to strict genetic regulation, the sex-determining pathways are surprisingly conserved within vertebrates from mammals to teleosts. For those species which are under strict genetic regulation for proper sex determination and development, interestingly, the genetic triggers (sex-determining master gene) to initiate the sex-determining pathways are versatile. SRY is the sex-determining trigger gene in human, but it is restricted to mammals (Ferguson-Smith, 2007). The DM-domain containing gene Doublesex and mab-3 related transcription factor 1 (DMRT1) plays a sex-determining role in both vertebrates and invertebrates. The DMRT1 homolog DMRT1bY (DMY) was recently found to be the sex-determining master gene in medaka (*Oryzias latipes*) (Nanda *et al.,*2002; Matsuda *et al.,* 2002). However, the closely related species (*Oryzias curvinotus*, *Oryzias celebensis*) lack this gene (Koopman and Loffler, 2003). Salmonids share a strictly genetic mechanism for sex

determination. Fluorescent *in situ* hybridization (FISH) analysis using bacterial artificial chromosomes (BACs) containing sex-linked microsatellite markers in Atlantic salmon (*Salmo salar*) identified chromosome 2 as its sex chromosome (Artieri *et al.,* 2006). The chromosome 2 pairs are the same size in both females and males, which suggests that the sex chromosome in Atlantic salmon is not highly differentiated. For decades, it has been of great interest to find what gene is the trigger in Atlantic salmon for sex determination, and if its closely related species share the same sex-determining gene.

1.1 Sex-determining mechanisms

The mechanisms for sex determination can be divided into two broad categories: environmental mechanisms and genetic mechanisms.

1.1.1 Environmental mechanisms

Species using environmental mechanisms to determine phenotypic sex require external stimuli. It is quite common in reptile and fish species. Temperature or salinity at which the eggs are incubated determines the phenotypic sex of embryos for many of those species (Merchant-Larios *et al.,* 2010; Lee *et al.,* 2009). Species utilizing environmental sex-determining mechanisms can also alter their phenotypic sex after becoming sexually mature. For example, population dynamics is a common environmental factor that can affect an individual's sex. When dominant individuals of a certain sex become scarce in a population, an individual of the opposite sex may undergo sex reversal to balance the ratio of two sexes within the population (Larson *et al.,*

2003). There is growing evidence that anthropogenic environmental changes can also lead to sex reversal of fish (Nagler *et al.,* 2001; Scholz and Klüver, 2009). For species using this type of sex-determining mechanism, one's genotype has little influence on its final phenotypic sex. However, the action of genes is still required for sex determination and proper sex development. It is just that the gene product that acts as a trigger for sex determination is controlled by environmental factors.

1.1.2 Genetic Mechanisms

Genetic sex-determining mechanisms involve inheritance of master gene(s) on sex chromosomes that act(s) as a switch to trigger the sex-specific development of the embryonic gonad into either a testis or an ovary. The two major types of genetic sex-determining mechanism are male heterogamety (XY system) and female heterogamety (ZW system).

For the male heterogamety, all males have X and Y sex chromosomes and all females have two X sex chromosomes. All zygotes can either have X and Y chromosomes to become a male or two X chromosomes to become a female. Female heterogamety is the reverse of male heterogamety, and thus all females have Z and W sex chromosomes and all males have two Z sex chromosomes. All zygotes either have Z and W chromosomes to become a female or two Z chromosomes to become a male. There are other genetic sex-determining mechanisms such as a polyfactorial system, in which the sex of the embryo can be determined by genetic factors inherited from the male and female parents

(Bull, 1983); or haplo-diploidy, in which males arise from unfertilized eggs and females arise from fertilized eggs (Freeman and Herron, 2007).

1.2 Sex-Determining Pathways

1.2.1 Description of sex-determining pathways

The sex-determining pathways can be described as a complex series of activation of signals that ultimately lead to sex differentiation and sex determination. Comparison among vertebrates reveals the shared genetic pathway of sex determination although various sex-determining mechanisms are present. Many genes involved in the common sex-determining genetic pathway are known to activate testis differentiation in all vertebrates (Marshall Graves, 2008; von Hofsten and Olsson, 2005). The known interaction among genes involved in the sex-determining pathway in mammals is illustrated in Figure 1.1.

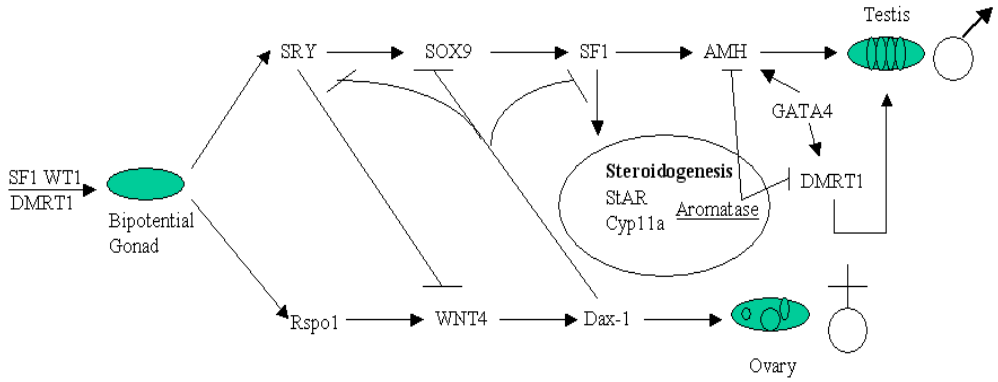


Figure 1.1 Important genes involved in general mammalian gonad differentiation pathways.

The gonad is initially bipotential, and expression of SRY leads to testis differentiation. If no testis forms, the bipotential gonad will become an ovary by the influence of genes such as Dax-1. (SRY: Sex-determining Region Y; SOX9: SRY (sex-determining region Y)-box 9; SF1: steroidogenic factor 1; AMH: anti-Mullerian hormone; Aromatase: cytochrome P450, family19; DAX-1: dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; GATA4: GATA binding protein 4).

It is surprising, however, how versatile triggers can initiate the highly conserved strategy to determine the sex. The location of the trigger gene defines the sex chromosome (Marshall Graves, 2008).

1.2.2 Sex determination in placental mammals

In all placental mammals, the gonad arises from a ridge of cells around the embryonic kidney. It is initially undifferentiated and is bipotential. During later stages of embryogenesis the bipotential gonad can develop into testis in males or ovary in females. Although the complete pathway is still unknown, studying sex-reversal patients discovers many genetic pathway steps involved in sex determination in humans, such as the involvement of SOX9 in XY sex reversal syndrome or SRY involvement of Swyer syndrome (Wilhelm and Koopman, 2006). The SRY protein is a transcription factor containing the High Mobility Group (HMG) domain that can bind specifically to a six base consensus DNA sequence. Some researchers suggest that the binding of SRY to a specific DNA sequence will introduce a bend which leads to activation of downstream genes (Graves, 1995; Waters *et al*., 2007). Its intronless gene structure also becomes a criterion for finding sex-determining master genes in other species. Although the function of SRY is crucial, it is poorly conserved even within the HMG box region (Marshall Graves, 2008). SOX9, which is closely related to SRY, is another important gene that is upregulated in testis development. Too little of the SOX9 expression leads to male-to-female sex reversal, and too much of the gene product will cause the opposite result (Marshall Graves, 2008). Upregulation of other genes such as Dax-1 favours female development (Sinisi *et al.*, 2003).

Genes such as SOX9 or DMRT1 that encode a protein with a zinc-finger DNA binding domain (DM domain) involved in the pathway show dosage dependency (Jameson *et al.,* 2003; Raymond *et al.,* 1999).

1.2.3 Sex determination in marsupials

Marsupials diverged from placental mammals approximately 180 million years ago (Mya) (Koina *et al*., 2006). Compared to mammals, marsupials have a smaller X chromosome. The marsupial X chromosome corresponds to the long arm and pericentric region of human X chromosome (Graves, 1995). Only four genes on the marsupial Y chromosome are also present on the human Y chromosome. The ZFY gene, which was the first gene isolated from the human Y chromosome, was thought to be the testis-determining gene for a long time. This hypothesis was later rejected partly because of its autosomal location in marsupials (Marshall Graves, 2008). The SRY gene was then found to be the sex-determining master gene for almost all placental mammals. An SRY ortholog is also present on the marsupial Y chromosome, and appears to play a sex-determining role in all therian mammals (Foster *et al.,* 1992). However, without further experiments, the question if SRY is also the sex-determining master gene in marsupials cannot be answered. The sex-reversing ATRY gene is another strong candidate sex-determining gene in marsupials, because it also presents only on the Y chromosome of marsupials (Marshall Graves, 2008). The presence of SRY on the marsupial Y chromosome suggests the gene is at least 180 My old (Marshall Graves, 2008).

1.2.4 Sex determination in birds

Birds and mammals diverged approximately 310 Mya (Taylor and Berbee, 2006). Birds use a ZZ/ZW sex-determining system. Females represent the heterogametic sex, and the W chromosome is only found in female birds. The Z chromosome is as large and gene rich as mammalian X chromosomes and the W chromosome is much smaller and relatively gene poor like the mammalian Y chromosome. There are several hypotheses for the modes of sex determination in birds, such as a dominant female determinant SRY-like gene on the W chromosome, or a dosage sensitive male determinant gene on the Z chromosome so that two copies of this gene product are required for male development (Marshall Graves, 2008). The dosage sex-determining modes have been found in *Drosophila melanogaster* (Burtis and Baker, 1989) and *Caenorhabditis elegans* (Stothard and Pilgrim, 2003; Pires-daSilva, 2007). The female determining master gene on the W chromosome has not been found yet. The gene DMRT1 is located on the Z chromosome for all the bird species studied so far (Ezaz *et al*., 2006). DMRT1 is orthologous to doublesex (*dsx*) in *D. melanogaster* and male abnormal-3 (*mab*-3) in *C. elegans*. These orthologs play important roles in sex determination in both organisms (Baker *et al.,* 1989; Zarkower, 2002). Its position together with its conserved role involved in sex determination makes it a strong sex-determining candidate in birds and it supports the hypothesis of a dosage dependent male determining sex determination mode. The DMRT1 containing region on the chicken Z chromosome shares an extensive conservation of synteny with human chromosome 9 (Nanda *et al.,* 2000). The fact that DMRT1 in human is dosage

sensitive in the sex-determining pathway (deletion of the DMRT1 region causes XY sex reversal (Raymond *et al.,* 1999) further supports the dosage sensitive hypothesis.

1.2.5 Sex determination in fishes

Ray-finned fishes (Actinopterygii) represent the extant common ancestor of mammals. They diverged from lobe-finned fishes (Sarcopterygii) approximately 450 Mya (Kumar and Hedges, 1998). Teleosts are a monophyletic group in ray-finned fishes. There are about twenty four thousand teleost fish species worldwide (Christoffels *et al.,* 2004), and almost every sex-determining mechanism and sex-determining mode established so far can be found in one fish species or another. Thus, fish species provide tremendous resources for the study of sex determination and the evolution of sex chromosomes. Most fish species utilizing genetic mechanisms for sex determination have homomorphic sex chromosomes, and most of the YY males are viable in species such as Tilapia (Kobayashi and Nagahama, 2009). In addition, YY males of many species are fertile. This indicates that the Y-chromosomes in fish still contain functional autosomal genes and are not as degenerated as in human. Thus, for fish species that utilize genetic sex-determining mechanisms, the sex chromosomes might still be in the early stage of evolution. Only about ten percent of the cytogenetically characterized fish species have distinct sex chromosomes (for review, see Devlin and Nagahama, 2002). For those which carry homomorphic sex chromosomes, environmental influences often play a large role in sex determination. This might be due to the sex chromosome not having had

sufficient time to undergo degeneration to gain a dominant influence on sex determination. Devlin and Nagahama (2002) have hypothesized that an autosomal gene could cause the replacement of the original sex chromosome once a new mutation occurred which could override the effect of the existing sex-determining gene.

Medaka is an example that carries homomorphic sex chromosomes. Medaka utilizes an XX/XY sex-determining system as in human. Oogenesis of medaka begins early in female gonads, and the number of germ cells in females is much greater than in males only 8 to 10 days post fertilization. Spermatogenesis, on the other hand, does not begin until 5 to 6 weeks post fertilization (Saito and Tanaka, 2009). One approach to identify the sex-determining master gene in medaka was to find candidate genes that are known to be involved in sex determination and differentiation in other vertebrates, and check if any candidate was located on the sex linkage group. An alternative approach was positional cloning by chromosome walking from sex-linked markers towards the end of the sex chromosome. Both approaches identified DMY as the sex-determining master gene in medaka. When DMY is mutated, it can cause XY fish to become females (Matsuda *et al*., 2002; Otake *et al*., 2006). DMY, which is a DMRT1 ortholog, is the only gene that has been found as a sex-determining gene in fish species. It is believed that DMY was transposed on to chromosome 1 after a gene duplication event and gained the new function of being the sex determination switch (Marshall Graves, 2008). The environment also plays a role in medaka sex determination, as raising the temperature to

32℃ can cause a forty percent increase in the male proportion of the population (Hattori *et al.,* 2007). As mentioned previously, many closely related species of medaka lack this DMY gene, suggesting the duplication of DMRT1 in *O. latipes* was a very recent event. In addition, some closely related species to *O. latipes* such as *O. javanicus* and *O. hubbsi* utilize a ZZ/ZW system (Takehana *et al*., 2008), which suggests that new sex determination mechanism can evolve rapidly among closely related fish species.

Cichlid fish (tilapias) sex-determining mechanisms vary from major genetic factors on the sex chromosomes in both an XX/XY system and a ZZ/ZW system (Cnaani *et al.,* 2008), parental minor genetic factors to environmental influences such as temperature change in a critical period of development (Baroiller *et al.,* 2009). Nile tilapia (*Oreochromis niloticus*) has been a great model for gonadal sex differentiation with the XX/XY male heterogametic system or ZZ/ZW female heterogametic system and also sex chromosome evolution in vertebrates (Cnaani *et al.,* 2008). By FISH analysis and meiotic chromosome paring, the sex chromosomes were found to be the largest chromosome pair, and they are still at an early stage of differentiation (Ezaz *et al.,* 2004; Ocalewicz *et al*., 2009). However, it has been found that sex-reversal XX females and YY males can be induced easily by sex hormones, and there is evidence for the existence of XX males in natural populations (Kobayashi and Nagahama, 2009; Baroiller *et al.,* 2009). So far the sex-determining gene has not been discovered in Nile tilapia. Several genes have been found to be involved in gonadal differentiation by studying their expression profiles. Foxl2 expression is more dominant in the

female gonad than the male gonad in early development. Co-localization of Foxl2 with Ftz-f1 and aromatase in stromal cells in XX gonads and also in the ovary of XY females sex reversed by estrogens suggests the importance of Foxl2 in the regulation of aromatase for proper sex development (Kobayashi *et al*., 2003; Wang *et al*., 2007). The DMRT1 expression is specific to the XY gonad, and it can be detected even before the appearance of any morphological change in the gonad. Sox9 is expressed in germ cell surrounding cells in both sexes before gonadal differentiation and later is only expressed in those cells in the male gonad. The AMH expression pattern is similar to Sox9 (Kobayashi and Nagahama, 2009).

Zebrafish (*Danio rerio*) is another well-studied fish model. No sex chromosome has been found, and some studies suggest that sex chromosomes do not exist in zebrafish (Wallace and Wallace, 2003). Both XX/XY and ZZ/ZW sex-determining systems have been suggested, and these inconsistencies suggest that the sex-determining system is not stable in zebrafish (von Hofsten and Olsson, 2005). Unlike what happens in medaka, female ovary development is the default pathway. Oogenesis occurs in all individuals, regardless of their sex, and gonad differentiation happens afterwards so that oogenesis is completed in females and testis development is initiated in males with ovarian degeneration (Saito and Tanaka, 2009; von Hofsten and Olsson, 2005). Aromatase (Cyp19a) inhibition can lead to a sex reversal female (XX male) in zebrafish (Uchida *et al*., 2004), which indicates its role in female sex differentiation. AMH is expressed in Sertoli cell in testis and the follicular layer in

ovaries (Rodriguez-Mari *et al*., 2005). It might be an Ftz-f1 homolog ff1d target, as it is co-expressed with AMH in these two locations (von Hofsten and Olsson, 2005). In mammals, Ftz-f1 regulates AMH and AMH in turn inhibits aromatase during gonadal development (di Clemente *et al*., 1992). Other genes such as SOX9 or WT1 might also play important roles in zebrafish sex determination as suggested by their expression patterns and possible regulatory roles.

1.2.6 Evolutionary conservation in sex-determining pathways

Recent work regarding sex determination tends to find that more and more genes involved in the sex-determining pathway are conserved among different taxa. DM domain containing genes are on the top of the list because of their involvement in sex determination in both vertebrates such as mammals and medaka and invertebrates such as *Drosophila* and *C. elegans* (Raymond *et al*., 1998). This might suggest either some aspect of a common evolutionary origin of sex determination or convergent evolution (Raymond *et al*., 1998) because sexual reproduction is so beneficial, whenever it is acquired during evolution it will remain in that lineage. The DMY gene is the upstream switch of the medaka sex-determining pathway. Its orthologs in *Drosophila* and *C. elegans*, however, act as downstream regulators for sex determination. The trend is that the conserved genes found to be involved in sex-determining pathways among different taxa are more likely to be in the downstream element of the pathway, and upstream regulators tend to be different, even in closely related species. This leads to the theory of retrograde evolution of sex-determining pathways, in which the downstream segment of the pathway is the most ancient, and the

upstream regulatory element was added later independently in different lineages (Wilkins, 1995). The occurrence of whole genome duplications (WGD) could provide sources of the upstream regulatory elements for sex determination that can be recruited later. This can also explain why DMRT1 and its orthologs can act both upstream and downstream in sex-determining pathways in different organisms. The identification of the primary sex-determining master gene is fundamental to the characterization of the sex-determining pathway in a species. Genes that act as the sex-determining switch have only been found in a few organisms. For many organisms being studied, this switch remains elusive. Nevertheless, knowing the conserved sex-determining pathway may provide clues about what the switch gene should look like.

1.3 Salmonid Fish

1.3.1 Family Salmonidae

The family Salmonidae consists of three sub-families: Coregoninae, Thymallinae, and Salmoninae (Nelson, 1994). The sub-family Salmoninae includes trout, salmon and charr (Figure 1.2).

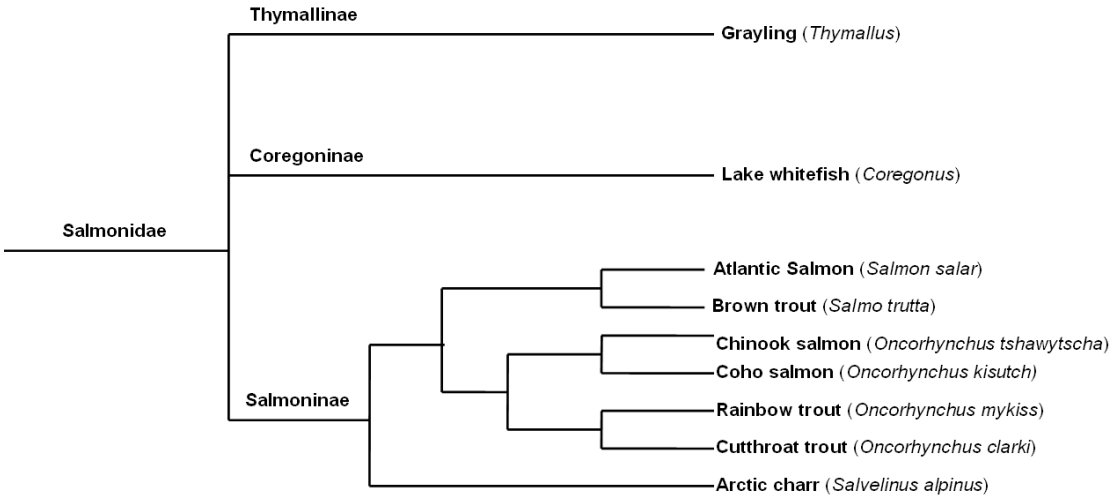


Figure 1.2 Phylogenetic tree of Salmonidae fishes.
(This tree is based on Davidson *et al.,* 2009.)

There is great interest in studying members of the Salmoninae because of their importance in wild fisheries and aquaculture industries, especially Atlantic salmon (*Salmo salar*), Pacific salmon (*Oncorhynchus* spp.), rainbow trout (*Oncorhynchus mykiss*), Arctic charr (*Salvelinus alpinus*) and brown trout (*Salmo trutta*), which is the closest species to Atlantic salmon.

It is believed that three rounds of whole genome duplications (WGD) happened before the emergence of teleost fishes. The first round of WGD occurred before the emergence of vertebrates. The second round led to the appearance of jawed vertebrates, and the third round took place in the lineage that led to the development of teleost fishes (Ohno, 1970; Sidow, 1996; Kasahara, 2007; Christoffels *et al.,* 2004). Allendorf and Thorgaard (1984) suggested a fourth WGD occurred 25 to 100 Mya in the common ancestor of salmonids. The strongest evidence for this comes from studies of *HOX* gene clusters. Invertebrates only have one *HOX* gene cluster, mammals have four gene clusters, zebrafish and medaka have seven gene clusters, and both Atlantic salmon and rainbow trout have thirteen gene clusters (Amores *et al*., 1998; Naruse *et al*., 2000; Moghadam *et al*., 2005a, 2005b). Karyotype studies also show the chromsome arm number (NF) in many salmonids is between 96-104, (in Atlantic salmon NF is 74) which is almost twice that seen in most other teleost fishes (Phllips and Rab, 2001; Mank and Avise 2006). Tetrasomic inheritance patterns at male meiosis segregation are observed in salmonids which suggest that salmon genomes are still undergoing a rediploidization process (Allendorf and Danzmann, 1997; Wolfe, 2001). The recently duplicated salmonid genomes

provide us with tremendous resources to study what can happen during the rediploidization process, especially how an organism can overcome the problem of sex determination after the WGD (Davidson *et al.,* 2009).

1.3.2 Atlantic salmon

In order to study the Atlantic salmon genome, a linkage map was generated based on microsatellite markers (Gilbey *et al.,*2004) and a another linkage map was generated based on both microsatellite and AFLP markers (Moen *et al.,* 2004). The female recombination rate can be up to 8.26 times higher than males (Moen *et al.,* 2004). Although a higher female recombination rate is a common phenomenon in many XY sex-determining species (Petkov *et al.,* 2007), such a large difference between female and male recombination rates is rare. Some have hypothesized that this is due to the inhibition of recombination in males (Allendorf and Thorgaard, 1984; Sakamoto *et al.,* 2000; Nichols *et al.,* 2003). Fifteen LGs were built initially by Gilbey *et al.* (2004). Moen *et al.* (2004) generated a female linkage map with 33 LGs and a male linkage map with 31 LGs, with 25 LGs in common between the two maps. The LG numbers were assigned randomly. Approximately 1,600 microsatellite markers have been mapped on to different LGs (Danzmann *et al.,* 2008).

To understand the Atlantic salmon genome better, the Genome Research on Atlantic Salmon Project (GRASP) was initiated, involving many universities and research institutions. A large-insert BAC genomic library was constructed as part of the GRASP project. This CHORI-214 Atlantic salmon BAC library was constructed using three segments of partial *Eco*RI and a fourth segment of partial

*Sau3*AI digested genomic DNA from a single Norwegian male strain of Atlantic salmon. The library provides at least 20 fold of coverage of the Atlantic salmon genome. BAC library filters, each representing more than 18,000 distinct BAC clones in duplicate, are also available for hybridization screening (Thorsen *et al*., 2005). *Hin*dIII fingerprinting was performed on the BAC library clones, which enabled the generation of a physical map for Atlantic salmon genome consisting of approximately 4,400 contigs (Ng *et al.,* 2005). Each contig containing a BAC with an informative maker within the BAC end sequence could be directly integrated to the corresponding position of the marker in the linkage map (Danzmann *et al.,* 2008). The linkage map has also been integrated with the karyotype using FISH analysis (Phillips *et al.,* 2009).

1.4 Sex determination in Salmonids

In salmonids, sex reversed XX phenotypic males crossed with normal females produce all female progeny (Johnstone *et al*., 1979; Hunter *et al*., 1982, 1983; Johnstone and Youngson, 1984). This evidence not only leads us to believe that salmonids utilize a strictly genetic sex-determining mechanism, but also indicates a male heterogametic system. However, morphologically distinct sex chromosomes are not common in salmonids. Nevertheless, for some species such as least cisco (*Coregonus sardinella*), rainbow trout (*O. mykiss*), sockeye salmon (*O. nerka*), and lake trout (*Salvelinus namaycush*), heteromophic sex chromosomes are observed (Phillips and Rab, 2001). Interestingly, some rainbow trout populations do not have heteromorphic sex chromosomes; while some rainbow trout hatchery populations have different sized sex chromosomes,

which indicates that the sex chromosomes in rainbow trout are still in the early stages of differentiation (Thorgaard, 1977, 1983). Microsatellite linkage maps were constructed for a number of salmonid species, including Arctic charr, brown trout, Atlantic salmon, and rainbow trout (Woram *et al.,* 2003). The sex linkage groups were defined as the linkage groups that contain the phenotypic male sex marker (*SEX)*. *SEX* was mapped on to the end of sex linkage groups of Atlantic salmon, brown trout and Arctic charr. Comparative genetics among these species using microsatellite markers on the sex linkage group of these species revealed that there is no sex linkage group conservation among these species (Woram *et al*., 2003). This evidence could support the hypothesis of Phillips *et al*. (2001), which suggests that a common sex-determining master gene has been transposed within the genomes of some salmonids, which causes the non-conservation of their sex linkage groups.

1.4.1 Sex determination in Atlantic salmon

Inter-individual as well as intra-individual chromosome polymorphisms in Atlantic salmon populations are observed, probably due to Robertsonian translocations. Although the chromosome number varies from 54 to 58 in the same species, NF is usually 74 (Hartley and Horne, 1984). This number is less than what is seen in brown trout (NF=102) or rainbow trout (NF=104). The C-banding pattern also shows that Atlantic salmon has more heterochromatin than the other two species (Hartley and Horne, 1984). This observation suggests that salmonids have undergone independent rediploidization processes after the

fourth WGD event, and Atlantic salmon has undergone more extensive genome rearrangement than other closely related salmonids.

Atlantic salmon utilizes a male heterogametic sex-determining system (Johnstone *et al.*, 1979). Previous studies have shown that LG1 is associated with *SEX* (Woram *et al.*, 2003; Artieri *et al.*, 2006). Because the recombination rate in females is approximately ten times greater than in male salmonids, the female linkage map was used for the integration with the physical map. FISH analysis was done for six BACs each containing a LG1 microsatellite marker (OmyFGF8TUF, One18ASC, BHMS447, One102ADFG, BHMS150 and Ssa202DU), and all the BACs hybridized to chromosome 2 – the sex chromosome in Atlantic salmon. The positions the BACs hybridized to the chromosome 2 correlate with the order of the markers on LG1. The Y chromosome cannot be identified at this stage because the sex chromosome pair is homomorphic, and all the BACs gave positive signals for both chromosomes. Both cytogenetic analysis and linkage group analysis point *SEX* towards the region between microsatellite marker Ssa202DU and the heterochromatin region near the end of the q arm on chromosome 2 (Artieri *et al.,* 2006).

Hybridization of probes designed from microsatellite markers on LG1 on to the BAC library filters allowed an initial identification of 12 contigs that contain these markers. These resources enabled the initiation of building a physical map for chromosome 2 based on microsatellite marker locations on LG1. The current map of LG1 contains 38 microsatellite markers, 5 single nucleotide polymorphisms (SNPs) and 6 amplified fragment length polymorphisms (AFLPs).

Extensive chromosome walking and minimum tiling path construction have built the physical map of chromosome 2 with 22 contigs, including 5 super-contigs (Huang, 2008). The sex-determining master gene may be within one of those contigs. The repetitive nature of the Atlantic salmon genome has prevented the joining of all the contigs. From all the contigs that were confirmed on chromosome 2, three contigs (contig 783, contig 818 and contig 2705) on the q arm of chromosome 2 were chosen for sequencing and annotation. Contig 2705 is close to *SEX,* and thus the annotation of the sequence is a good resource for sex-determining gene candidates. The BAC sequence annotation for contig 2705 shows that the contig contains three interesting genes: zinc finger matrin-type 4 (Zmat4), zinc finger FYVE domain containing 27 (ZFYVE27) and testis-specific gene 118 (TSG118). All three genes were expressed in the ovary of an Atlantic salmon smolt (Fujiki, unpublished data).

1.5 Sex-Determining Candidate Genes in Atlantic salmon

As mentioned previously, the *SEX* locus of Atlantic salmon is believed to be at the end of the q arm on chromosome 2. The repetitive nature of the Atlantic salmon genome makes chromosome walking in chromosome 2 extremely difficult near the end of its q arm. The genetic mechanism for sex determination in Atlantic salmon could be either a dominant male determining gene on the Y chromosome that leads to male development, or a dosage sensitive sex-determining gene on the X chromosomes that leads to proper sex development. Based on the hypothesis of retrograde evolution of the sex-determining master gene and the evidence that Atlantic salmon is still in its rediploidization stage,

any genes that have a conserved role involved in the sex-determining pathway in other species could be a sex-determining gene candidate in Atlantic salmon. Several genes caught my attention because of their potential roles in sex determination in other fish species, or if they have a conserved sex-determining role across taxa. These genes are listed in Table 1.1. The rationale for considering each gene as a candidate for the sex-determining gene of Atlantic salmon is given in Chapter 3.

Table 1.5.1 Fourteen sex-determining gene candidates for Atlantic salmon.

Gene Candidates	Description
Aromatase	Cytochrome P450, family 19, subfamily A, polypeptide 1
Ftz-F1	Steroid hormone receptor Ad4BP
Dax1	Nuclear receptor subfamily 0, group B, member 1
Sox9	SRY (sex determining region Y)-box 9
Sox9 α2	SRY (sex determining region Y)-box 9 α2
WT1	Wilms tumor 1
DMRT1	Doublesex and mab-3 related transcription factor 1
AMH	Anti-Mullerian hormone
Foxl2 ortholog	Forkhead box L2 ortholog
Foxl2 paralog	Forkhead box L2 diverged paralog
AR α	Androgen receptor α
AR β	Androgen receptor β
Figla	Folliculogenesis specific basic helix-loop-helix
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2

The relative dosage of gene expression is very sensitive in a sex-determining pathway and battles between the antagonists finally lead to the proper sex development. Nevertheless, there are many possibilities for how exactly sex is determined in Atlantic salmon. The genes listed here only subset a small group of genes that should be investigated.

1.6 Comparative Genome Analysis

1.6.1 Comparing Genomes from Different Species

Comparative genome analysis allows us to compare the gene contents between different organisms, and can help us predict how genomes evolve in different lineages. With the increasing number of genomes sequenced recently, it also becomes very useful for gene location prediction by comparing genomes between closely related species. One can achieve this by finding syntenic regions of DNA markers or gene homologs among genomes. Determining the syntenic regions among different genomes not only allows one to hypothesize the evolutionary relationships, but also allows one to reconstruct the vertebrate ancestor genomes. In recent studies, the genomes of human, pufferfish, zebrafish and medaka have been compared in a pairwise manner, and these studies confirm the 3R duplication event that led to the teleost lineage (Kohn *et al.*, 2006; Jaillon *et al.*, 2004; Mulley and Holland, 2004; Naruse *et al.*, 2000, 2004). Comparative genome analysis has also been carried out for Atlantic salmon, rainbow trout, zebrafish and medaka (Danzmann *et al.*, 2008). The result showed that the two homeologous chromosomes in zebrafish and medaka (due to the 3R duplication) contain conserved regions that correspond to four

chromosome arms (partial or whole) in salmonids, which also confirmed that the common ancestor of salmonids had undergone an additional independent WGD event. In addition, comparisons of conserved syntenic regions can allow us to predict how genomes have become rearranged after the WGD. It is also possible to discover sex-determining gene candidates in Atlantic salmon by searching for syntenic regions between Atlantic salmon LG1 and the four fish genomes (pufferfish, stickleback, zebrafish and medaka) available at the Ensembl website (http://www.ensembl.org). To search for the sytenic regions and gene orthologs between Atlantic salmon LG1 and other fish genomes, a separate tBLASTx was performed for all BAC-end sequences from the contigs assigned to LG1 and each of the fish proteomes. These results are given on the AsalBase website (http://www.AsalBase.org). A previous study reconstructed four Atlantic salmon LG1 maps based on rearrangements of the orthologous chromosomes from pufferfish, stickleback, zebrafish and medaka (Huang. 2008). The comparative analysis revealed that there are significant inter- and intra-chromosome rearrangements between Atlantic salmon and other fishes, whose genomes have been sequenced. The gene ZFYVE27 was found at the telocentric end of LG1, the sex-determining region of Atlantic salmon, on maps reconstructed based on stickleback, zebrafish and medaka orthologous chromosomes. This evidence makes the gene ZFYVE27 a strong candidate for the sex-determining switch in Atlantic salmon.

1.6.2 Comparing Genomes within Salmonidae

Comparative genome analysis has also been carried out in salmonids. For example, recent studies indicate both Atlantic salmon and rainbow trout share conserved syntenic regions in their genomes. As mentioned above, comparing locations of sex-linked markers among different salmonids revealed that the sex-linked markers in one species are on one or more autosomes of other species. Thus, the fish species within the salmonid group share different sex linkage groups as determined by comparative genomic analysis. For example, the sex linkage group microsatellite markers of brown trout, the closest species to Atlantic salmon, are found on LG8 of Atlantic salmon (Woram *et al.,* 2003). To predict the sex-determining gene in brown trout, we can use the Atlantic salmon LG8 physical map integration as a reference, and then search for candidate genes via a syntenic search in AsalBase (http://www.AsalBase.org).

1.7 Aim of the Thesis

The overall aim of my MSc thesis was to study and better understand the sex determination mechanisms in Atlantic salmon, and possibly gain insight into sex determination in its closely related species.

There were three major objectives of my MSc thesis:

(1) To investigate sex-determining candidate genes for Atlantic salmon, by looking at their physical locations in the Atlantic salmon genome. If a candidate is found to be located at the q arm of chromosome 2, or at the end of LG1, it will be a strong candidate for sex determination in Atlantic

salmon. If not, it will not likely be the sex-determining master gene. There were two main approaches to achieve this objective. The first was to screen the Atlantic salmon BAC libraries for all the candidate genes listed above, find the contig each gene belongs to, and check if there is an informative marker within the contig, and on which LG the marker is mapped on. The second approach was to use FISH analysis for the candidates whose locations cannot be identified by the first approach.

(2) To identify where sex-linked markers in other salmonids are located in the Atlantic salmon genome. This will provide information and perhaps indicate if salmonids share the same sex-determining master gene. If they have the same sex-determining gene, this evidence will support the hypothesis that the same sex determination master gene rearranged on to the end of different linkage groups in different species independently during evolution by genome rearrangements. If they do not share the same sex-determining gene, the evidence will support the hypothesis of retrograde evolution, in which the sex-determining master gene is independently recruited in each species during evolution. This could also explain why a sex-determining master gene in one species is not sex determining in other closely related species. The main approach was to use comparative genomics and linkage mapping.

(3) To identify the sex chromosome in brown trout, and possibly to find the sex-determining gene in brown trout. The approach was to use FISH analysis and comparative genomics.

2: Material and Methods

2.1 Candidate gene screening

2.1.1 Oligonucleotide hybridization probe and primer design

The sequences used to design the hybridization probes and primers for the 14 sex-determining master gene candidates were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/) or from the cGRASP EST database (http://web.uvic.ca/grasp/). The sequences obtained from EST databases were compared to the nt/nr database on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the information regarding the EST was correct in the database. The sequences were then compared with other fish genome sequences in Ensembl (http://uswest.ensembl.org/index.html) (medaka, zebrafish, stickleback, fugu, and tetraodon) to find conserved exon intron boundaries. Sequences within a single exon for each gene were used as the input to design oligonucleotide probes (40-mers) and corresponding complementary primers (20-mers) using Primer3 online software (http://frodo.wi.mit.edu/primer3/). The oligonucleotides were designed such that they had a GC content of 50% or higher, with a Tm of the probe of at least 55^{o}C.

2.1.2 BAC library screening

The CHORI-214 Atlantic salmon BAC library filters (Thorsen *et al.,* 2005) were screened with the 40-mer probes designed from the 14 candidate genes.

The 40-mers were end-labeled with ^{32}PγATP using T4 polynucleotide kinase and hybridized to six BAC filters at a time as described by Johnstone *et al.* (2008). The pre-hybridization step was carried out in 5 x saline-sodium citrate buffer (SSC), 0.5% sodium dodecyl sulfate (SDS) and 5 x Denhardt's solution at 65℃ for 2 hours, and then the ^{32}P-labeled probes were added and incubated overnight for at least 16 hours. The filters were washed three times, each for 1 hour, at 50℃, in 1 x SSC and 0.1% SDS. The filters were then wrapped in Saran^{TM} wrap and exposed to phosphor screens that were subsequently scanned using the Typhoon Imaging System and visualized using ImageQuant software, giving an image of the ^{32}P-labeled hybridization-positive BACs containing the microsatellite markers.

2.1.3 PCR confirmation for the true positives

The hybridization-positive BAC clones were picked from the library, cultured on a LB agar plate containing chloramphenicol (50 µg/mL) overnight at 37ºC overnight for a PCR check. The PCR reaction mixture contained the following: 1 µL of 10 x PCR buffer containing $MgCl_2$ (QIAGEN), 1 µL of 2 mM dNTPs, 0.5 µL of 10 µM 40-mer probe, 0.5 µL of 10 µM complementary primer, 0.15 µL of Taq DNA polymerase (QIAGEN) and 6.8 µL of dH_2O. A small amount of BAC clone on the agar plate was added into the PCR reaction mix as template. The PCR protocol was: 95ºC for 5 min followed by, 35 cycles of 95ºC for 45 sec, 65ºC for 45 sec and 72ºC for 2 minutes, and then 72ºC for 10 min. PCR products were separated by electrophoresis through a 1.3% agarose gel with 1 x TBE, stained with ethidium bromide and visualized using a UV trans-

illuminator (Alpha Innotech). The hybridization positive BACs that give a PCR product of the correct size were considered to be true positives.

2.1.4 Contig and LG identification

Hybridization and PCR-positive BACs for the candidate genes were matched to fingerprint contigs within the Atlantic salmon physical map (Ng *et al.,* 2005 as shown on www.AsalBase.org). The linkage groups (LG) information is also available for contigs with microsatellite markers that have been mapped in the SALMAP Atlantic salmon mapping families (Danzmann *et al.,* 2008).

2.1.5 Microsatellite marker genotyping analysis

The microsatellite markers all have the 21M13 sequences added to the 5' end of the forward primer. 21M13 sequence is 5' TGTAAAACGACGGCCAGT 3'. The PCR reaction mixture used for microsatellite mapping contained the following: 1 µL of 10 x PCR buffer containing $MgCl_2$ (QIAGEN), 0.2 µL of 10 mM dNTPs, 0.2 µL of 10 uM forward primer, 0.5 µL of 10 uM reverse primer, 0.5 µL of 10 µM 21M13 labelled primer (either FAM or HEX labelled), 0.1 µL of Taq DNA polymerase (QIAGEN), 25 ng of genomic DNA and 7 µL of dH_2O. The PCR protocol was touch down: 94ºC for 2 min followed by, 21 cycles of 94ºC for 30 sec, 60ºC for 30 sec, -0.5ºC/cyle and 72ºC for 30 sec, and then an additional 14 cycles of 94ºC for 30 sec, 50ºC for 30 sec, 72ºC for 30 sec, and then 72ºC for 10 min. 2.5 µL of the loading dye (formamide) was added into 2 µL of the PCR reaction mixture, heated at 94ºC for 4 minutes and then immediately put on ice. 2 µL of the cold loading mixtures were applied to a polyacrylamide gel made with

following recipe: 11.6 g of UREA, 13.2 mL of H_2O, 3.85 mL of Long Range Buffer, and 2.75 mL of 10 X TBE with 150 µL of 10% ammonium persulphate (APS) and 25 µL of TEMED. The gel was run for 2 hours on an ABI 377 automated sequencer.

2.1.6 Intron region SNP searching

The exon intron boundaries for each of the sex-determining gene candidates were found as described in section 2.1.1. Primers were designed to flank a single intron in this case instead of within a single exon. The sequences with exon/intron boundaries in the middle were used as the input to design forward primers and corresponding reverse primers using Primer3 online software (http://frodo.wi.mit.edu/primer3/). Both forward and reverse primers were designed about 20 nucleotides in length. The oligonucleotides were designed such that they had a GC content of 50% or higher, with a Tm of the probe of at least 55^{o}C.

The single intron flanking primer sets were used to amplify the genomic DNA of the Br5 male, Br5 female, Br6 male, and Br6 female (the parents in the SALMAP mapping families). The PCR products were obtained from the SYBR stained agarose gel after electrophoresis by cutting out from the gel, and purified using an Ultrafree-DA column (Millipore).

The PCR products were then used as templates for direct sequencing reactions. The sequencing reaction mixture contained the following: 2 µL of Amersham DYEnamic ET terminator cycle sequencing kit master mix, 0.5 µL of

10 uM forward or reverse primer, and 2.5 µL of purified PCR product. The PCR protocol is as follows: 96ºC for 1 minutes, followed by 40 cycles of 96ºC for 10 sec, 50ºC for 5 sec, and 60ºC for 2 minutes. The sequencing reaction clean up protocol was: add 1 µL of sodium acetate/ EDTA buffer and 80 µL of 95% EtOH into the sequencing reaction mixture, vortex and centrifuge at 13,000 rpm for 20 minutes. Remove the supernatant, and add 200 µL of 70% EtOH and centrifuge at 13,000 rpm for 10 minutes. Remove the supernatant again and let air dry for 20 minutes, then add 2 µL of formamide loading dye to dissolve the DNA pellet, and load on to the sequencing gel in an ABI 377 DNA sequencer (Applied Biosystems). The sequencing gel recipe is as follows: 18 g of urea, 5 mL of Long Ranger Gel solution, 5 mL of 10 X TBE, and 20 mL of H_2O, with 250 µL of 10% APS and 30 µL of TEMED.

2.1.7 FISH on Atlantic salmon chromosomes

Atlantic salmon of 2-5 kg body weight was anaesthetized using MS222. The fish skin was cleaned with 70% EtOH and Kimwipes^{Tm}. Up to 2 mL of blood was aseptically taken from the caudal vein of the fish by a sterile syringe near the anal fin with heparinised Vacutainers^{Tm}, as shown in Figure 2.1. Some of the blood samples were taken by Carlo Biagi, who showed me how to do this.

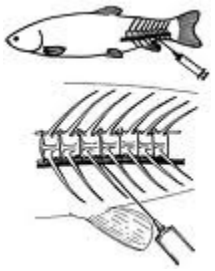


Figure 2.1 How to take blood from caudal vein.

This figure was from FAO corporate document repository on line at http://www.fao.org/docrep/field/003/ac160e/ac160e09.htm.

The heparinized blood was thoroughly mixed with 5 mL of media L-15 (Gibco) in a 15 mL sterile plastic tube and placed on ice for 5 minutes. The diluted blood was then centrifuged at 1,200 rpm for 5 minutes at room temperature. After centrifugation, the buffy coat (containing lymphocytes) above the red blood cells was floated in plasma by a gentle stirring with a 1 mL pipette. The lymphocyte enriched plasma was then collected in a new 15 mL sterile plastic tube. The buffy coat may be hard to observe depending on fish physiological conditions. The process is illustrated in Figure 2.2.

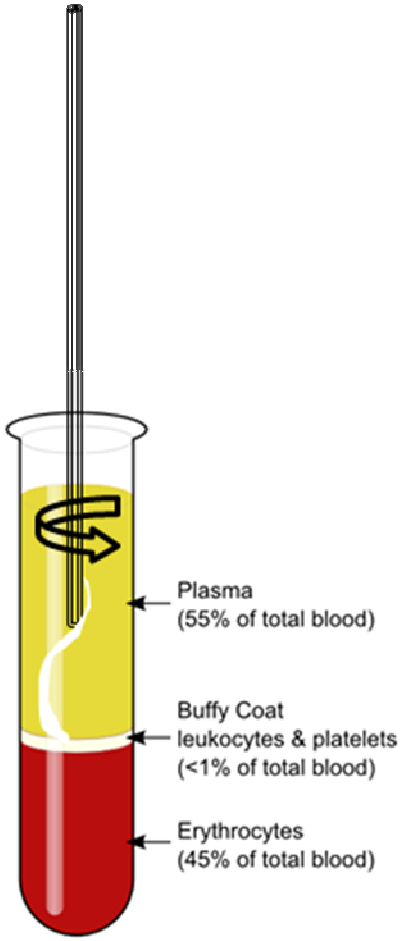


Figure 2.2 Illustration of getting the lymphocyte enriched plasma.

This figure is modified based on File:Blood-centrifugation-scheme.png on Wikipedia at http://en.wikipedia.org/wiki/File:Blood-centrifugation-scheme.png.

The plasma was centrifuged at 1,500 rpm for 5 minutes, and the resulting cell pellet was resuspended in 5 mL of complete media L-15 containing 10% fetal bovine serum (FBS), 60 µg/mL of kanamycin sulfate, 1 x antibiotic-antimycotic solution (100 U/mL of penicillin, 100 µg/mL of streptomycin and 250 ng of amphotericin B), 25 µM of 2-mercaptoethanol and 18 µg/mL of phytohemagglutinin (PHA-W) and 100 µg/mL of lipopolysaccharide (LPS). The last two reagents are mitogens. The cells were cultured at 20ºC in a culture tube slanting to an angle of about 30º with gentle daily mixing for 6 days. About 2.5 hours before cell harvest, the lymphocyte culture was supplemented with 500 ng/mL colcemid. The cells were collected by centrifugation at 1,500 rpm for 5 minutes, and the supernatant was discarded. The cell pellet was resuspended in 2 mL of 0.075 M KCl hypotonic solution for 20 minutes at 20ºC. The hypotonic solution was slowly added to 2 mL. Then, 2 mL of fresh Carnoy's fixative (3 methanol: 1 acetic acid) was added slowly. Centrifuge at 1,500 rpm for 5 minutes, and the supernatant was discarded. The fixed cells were gently resuspended in 3 mL of Carnoy's fixative. Repeat the fixation step two more times, and then add 1-2 mL of Carnoy's fixative. The cell suspension was now ready for chromosome spreading. The slide was exposed to hot water vapor for 30 seconds. The cell suspension was immediately dropped on to the slide. After the slide surface becomes grainy, the slide was immediately exposed again to the hot water vapor for 30 seconds. The slide was then quickly dried on a hot surface, which provided good chromosome spreading.

The nick translation reaction mixture used for generating the fluorescent probe for FISH contained the following: 500 ng of extracted BAC DNA, specific amount of water to make up the total volume of 25 µL of reaction, 1.25 µL of SpectrumGreen for the BAC containing gene of interest (or 1.25 µL of SpectrumOrange for the marker BAC as the reference), 2.5 µL of 0.1 mM dTTP, 5 µL 0.1 mM dNTP mix, 2.5 µL 10 X nick translation buffer and 2.5 µL of nick translation enzyme. The reaction mixture was briefly mixed and centrifuged, and incubated at 15ºC for 16 hours. The reaction was stopped by incubating at 70ºC for 10 minutes, and then chilled on ice. The probe size was determined by running a 1.3% agrose gel containing EtBr at 140 volts for 45 minutes together with the 100 bp ladder.

Cot-1 DNA is used as a blocking agent for FISH analysis. It is prepared as follows: About 90 µg of genomic DNA was obtained and adjusted to the volume of 300 µL, and then 0.1 X volume of 3M NaCl was added. Aliquot into 5 tubes, and sonicate the tube for 6 minutes between power 4-5 using a sonicator (Heat System Ultrasonics). 2 µL of each tube was taken out and run on an agarose gel to check for correct sonication size (100 bp up to 1 kb). The reassociation time was calculated by the formula: time (minutes) = 5.92/DNA concentration (mg/mL). The sonicated samples were chilled on ice for a few seconds and allowed to reassociate for the calculated time at 65ºC. One unit of S1 nuclease was used for every µg of starting DNA. Add 5 X S1 nuclease buffer and let sit for 30 minutes at 37ºC. Then add 0.12 X volume of 3M NaoAC (pH = 5.2), and add 2.5 X volume of 95 % EtOH, place in -80ºC freezer for 30 minutes, gently vortex

and centrifuge at 13,000 rpm for 15 minutes at 4ºC. The supernatant was removed to dry the pellet for 10 minutes, and the pellet was resuspended in 20 µL TE buffer.

Human placental DNA was also used as a blocking agent. One unit of human placental DNA (Invitrogen) was dissolved in 167 µL of TE overnight at room temperature. The dissolved DNA was transferred into 3 different tubes and sonicated for 7 minutes between power 4-5 using a sonicator (Heat System Ultrasonics). 2 µL of the sonicated DNA from each tube was taken out and run on an agarose gel to check for correct sonication size (500 bp up to 1 kb).

Five µL of the nick translation reaction mixture of the BAC for the gene candidate and 5 µL of the nick translation reaction mixture of the BAC containing the sex reference marker were mixed together with 2 µg of Atlantic salmon Cot-1 DNA and 2 µg of human placental DNA in a microcentrifuge tube. 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% EtOH were added to precipitate the DNA. The mixture was incubated at -80ºC for 30 minutes and then centrifuged at 12,000 rpm for 30 minutes at 4ºC to pellet the DNA. The supernatant was removed and the pellet was dried for 15 minutes at room temperature. The pellet was then resuspended in 3 µL of dH_2O and 7 µL of hybridization buffer by shaking at 250 rpm for 30 minutes at 37ºC. The probe was denatured by heating at 73ºC for 10 minutes and then chilled on ice for 5 minutes. Then the probe was incubated at 37ºC from 30 to 60 minutes for pre-hybridization.

The freshly made metaphase containing slides were treated with 2X SSC for 30 minutes at 37ºC, and then serial dehydrated in 70% EtOH, 85% EtOH and 100% EtOH, each treatment for 2 minutes. The hybridization area was marked by a tipped scribe. The slide was denatured in 70% formamide in 2X SSC, pH 7.0-8.0, at 73ºC for 3 minutes. Then the slide was serial dehydrated in -20ºC 70% EtOH, 85% EtOH and 100% EtOH, each treatment for 2 minutes, and then air dried. 10 µL denatured probe was added to the slide, and a coverslip was immediately applied and sealed with rubber cement. The slide was put in a sealed humidified box at 37ºC room for 16 hours.

The coverslip was removed together with the rubber cement seal, and the slide was immediately placed into the 73ºC 0.4 X SSC/0.3% NP-40 wash solution in a hot water bath for 4 minutes, with several agitations every minute. The slide was then treated with 2 X SSC/0.1% NP-40 wash solution at room temperature for 2 minutes. The slide was dried in the dark, and then 10 µL of DAPI antifade solution (Invitrogen) was applied to the slide. The multiphoton confocal microscope A1R MP (Nikon) was used to check the metaphase spreads. Laser 405 was used for the DAPI stain, laser 488 was used for the green dUTP labelling and laser 560 was used for the orange dUTP labelling.

2.2 Genotyping and linkage analysis of Atlantic salmon with sex-linked markers in closely related species

The procedure for genotyping and linkage analysis was exactly the same as described in section 2.1.5.

2.3 Physical location of brown trout sex-linked markers in Atlantic salmon

2.3.1 Brown trout sex-linked markers probe design

The brown trout sex-linked markers were used as primers to amplify Atlantic salmon genomic DNA using the same protocol described in section 2.1.3. The PCR products were separated by electrophoresis through a 1.3% agarose gel with 1 x TBE, and stained with SYBR Safe (Invitrogen). Then the PCR products were cut out from the gel, and each PCR product was purified using an Ultrafree-DA column (Millipore). The purified PCR product was then used for sub-cloning and sequencing (as described in sections 2.3.2-2.3.4). The PCR product sequences were uploaded on Primer3 online primer design software (http://frodo.wi.mit.edu/primer3/), and probes and corresponding reverse primers were chosen as described as section 2.1.1.

2.3.2 Sub-cloning the PCR product

Two µL of the PCR product was mixed with 2.5 µL of 2 X Ligation Premix and 0.5 µL of AccepTor Vector (50 ng/µL) from Novagen AccepTor Vector Kit in a 14 mL Polypropylene Round-Bottom Tube on ice. The ligation mixture was left

at 16ºC for 2 hours, and the kept at 4ºC until use. 25 µL of the Novablue Singles Competent Cells (Novagen) were added to the ligation mixture, and incubated on ice for 30 minutes. The mixture was heat shocked at 42ºC in a water bath for exactly 45 seconds then immediately put back in ice for 5 minutes. 250 µL of SOC medium was added into the cell mixture and incubated for 30 minutes at 37ºC with shaking at 250 RPM. The cell culture was then spread on the ampicillin/X-gal/IPTG agar plate (diameter of 90mm) (50 µL of 4% X-gal, 25 µL of 0.1 M IPTG and 50 µg/mL ampicillin) gently, and incubated at 37ºC overnight.

2.3.3 Insert check by colony PCR

Several white colonies were picked up from the plate and transferred on to a new 50 µg/mL ampicillin agar plate as the “master plate”. The PCR mixture was as follows: 1 µL of 10 x PCR buffer containing $MgCl_2$ (QIAGEN), 1 µL of 2 mM dNTPs, 0.5 µL of 10 uM T7/Sp6 universal primers, 0.15 µL of Taq DNA polymerase (QIAGEN) and 6.8 µL of dH_2O. The toothpick with the leftover of the colony after transfer on to the “master plate” was dipped into the PCR mixture as template. The PCR protocols, PCR product separation and visualization were exactly the same as mentioned in section 2.1.3. The white colonies with insert confirmed by PCR were transferred from the master plate to 5 mL of LB containing 100 µg/mL of ampicillin, and shaken at 250 RPM at 37ºC overnight. The plasmid DNA was isolated using a QIAprep Spin Miniprep kit (QIAGEN) and saved for sequencing.

2.3.4 Plasmid DNA Sequencing

The sequencing reaction mixture contained the following: 2 µL of Amersham DYEnamic ET terminator cycle sequencing kit master mix, 0.5 µL of 10 µM primer (either T7 or Sp6), and 2.5 µL of the PCR product. The PCR protocol is as follows: 96ºC for 1 minute, followed by 40 cycles of 96ºC for 10 sec, 50ºC for 5 sec, and 60ºC for 2 minutes. The sequencing reaction clean up and sequencing was as described in section 2.1.6.

2.3.5 BAC library screening

The procedure for screening the BAC library for brown trout sex-linked microsatellites was the same as described in section 2.1.2.

2.3.6 PCR confirmation for the true positives

The procedure for screening the BAC library for true positives for probes was the same as described in section 2.1.3.

2.3.7 Contig and linkage group identification

The procedure for searching the available information for the true positive BACs through the online database was the same as described in section 2.1.4.

2.3.8 Comparative Genomics

The AsalBase online database (http://dev.AsalBase.org) has the option for comparative genome analysis. The genomes of medaka, zebrafish, stickleback and tetraodon are available for comparison with different contigs of the Atlantic salmon physical map. A separate BLASTx was performed between all available

BAC-end sequences from an inquiry contig and each of the proteomes of medaka, zebrafish, stickleback and tetraodon. The syntenic region of the contig of these fish genomes show up as a result page, with the annotated BAC end sequences at the bottom of the page. It is possible to extend the syntenic region and this allows one to search for possible adjacent contigs, using BLASTx hits between the annotated genes from the extended syntenic region and all available BAC end sequences from all the contigs in the database.

2.3.9 Chromosome walking

Prior to probe and reverse primer design, the available T7 and Sp6 BAC end sequences from a contig were downloaded from dev.AsalBase.org. The Repeat Masker program on the Unviersity of Victoria cGRASP website (http://lucy.ceh.uvic.ca/repeatmasker/cbr_repeatmasker.py) was used to mask the repetitive DNA sequences. The masked sequences were used as input to design probes and reverse primers as described in section 2.1.1. The minimum tiling path of the BACs along a contig was generated by a series of PCR reactions (reaction mixture and PCR protocols as described in section 2.1.3.) using both T7 and Sp6 primer pairs for a single BAC and its adjacent BACs (according to the contig map) as templates to see which pair was able to amplify BACs from the left side of the BAC and which pair was able to amplify the adjacent BACs on the right side. Finally the minimum tiling path was generated. The probes and reverse primers from the BAC end sequences pointing outward from the BACs that were tested to be the outmost were used to screen the library

to find the next adjacent contig. The screening and PCR confirmation procedures were as described in section 2.1.2 and section 2.1.3.

2.3.10 Searching for male/female specific SNPs

PCR reactions were done using two male and two female brown trout genomic DNAs as templates with the BHLH-B4 primer set. The reaction mixture and PCR protocol were as described in section 2.1.3. The PCR products were sub-cloned and sequenced exactly the same way as described in sections 2.3.2-2.3.4.

3: Results

3.1 The genomic locations of Atlantic salmon sex-determining candidate genes

The flow chart in Figure 3.1 illustrates the approaches taken to find the genomic locations for each of the Atlantic salmon sex-determining gene candidates.

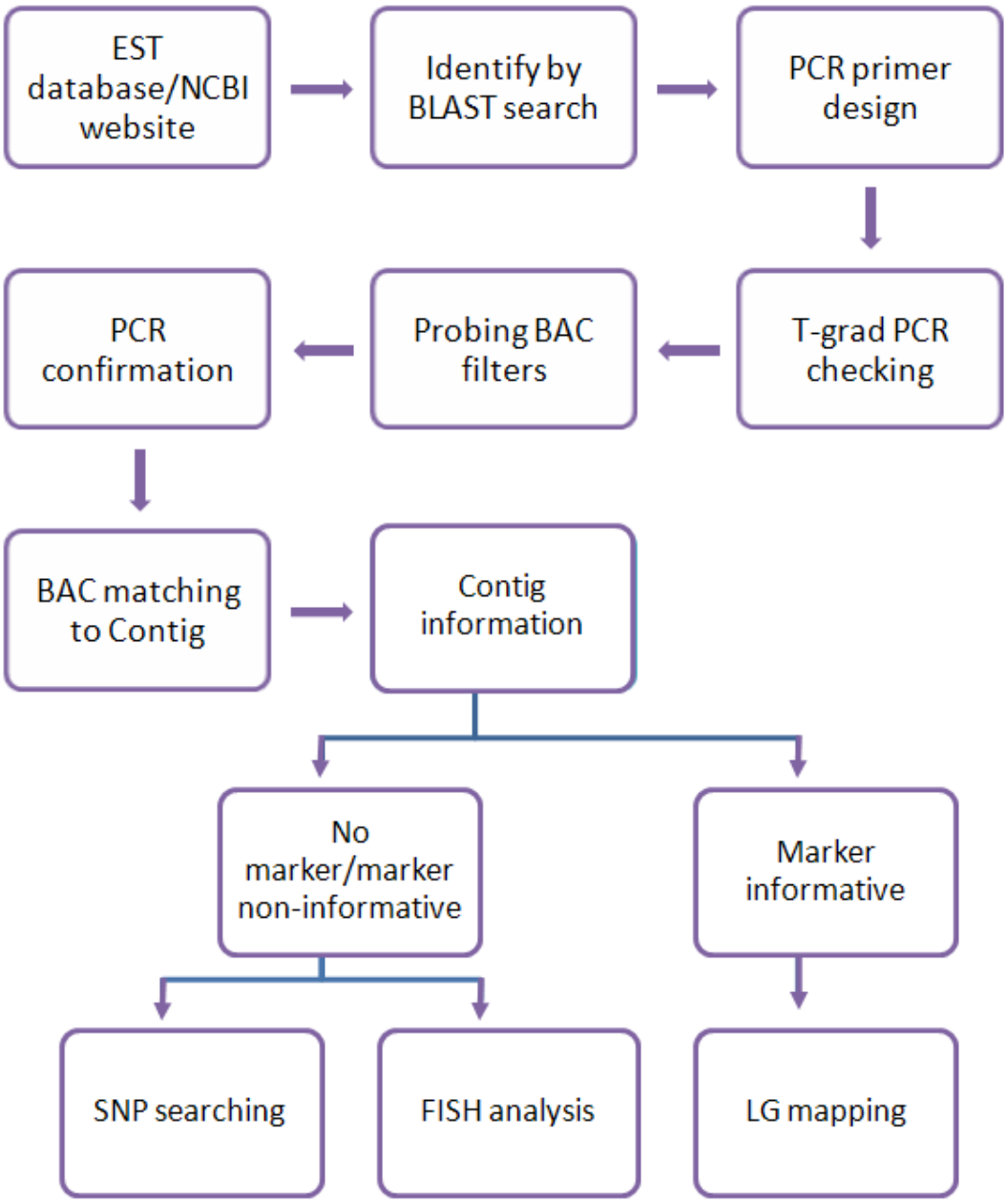


Figure 3.1 The flow chart for finding the genomic locations for sex-determining candidate genes in Atlantic salmon.

3.1.1 Finding the BAC contigs that contain the sex-determining gene candidates

Probes were designed for 14 sex-determining gene candidates in Atlantic salmon. The probes were designed based on an Atlantic salmon EST if possible. Otherwise, probes were designed based on ESTs of closely related species, such as rainbow trout. The probe sequences are approximately 40 nucleotides in length, and they also can act as forward primers for PCR. Exon boundaries for sex-determining gene candidates were identified by comparisons of the exon boundaries among five fish species whose genomes have been annotated in the Ensembl genome browser: fugu, medaka, stickleback, tetraodon and zebrafish. These fish species are quite divergent from one another, thus sometimes they have different exon numbers for the same gene. The rationale is that if all five species share an exon boundary somewhere in the same gene, then it is more likely that the situation will be the same in Atlantic salmon. The probes and corresponding reverse primers were designed within a single exon or across a single intron for PCR checking purposes. The reverse primers were approximately 20 nucleotides in length. The probes were used to screen Atlantic salmon CHORI-214 BAC library filters. Before the probing experiment, the probes and their corresponding reverse primers were checked for optimal annealing temperature and specificity by T-grad PCR for every sex-determining gene candidate using Atlantic salmon genomic DNA as the template. A good indication for optimal annealing temperature and specificity is a very clean, clear yet strong bright band for the PCR product. After hybridization, the positive BACs

of probes from the filters were selected from the Atlantic salmon CHORI-214 BAC library. PCR reactions were carried out again using the corresponding probe/reverse primer pairs and BAC DNAs as templates. The genomic DNA was also used as template for a positive control, and water was added as a mock template for negative control to check for possible contamination. The true positive BACs were those whose PCR product sizes were the same as that of genomic DNA. The probe and reverse primer sequences corresponding to each of the sex-determining gene candidates are listed in Table 3.1.

Table 3.1.1 The sequences of probe and reverse primers for each of the sex-determining gene candidates.

[1]RT = rainbow trout, [2]CI = cross intron, [3]R = reverse primer, [4]AS = Atlantic salmon, [5]EST = EST database sequence.

Probe/Reverse primers	Sequences
DMRT1-Probe RT[1] (CI[2])	ACCAAGCTGCTGGAGTGTGCCGGTCCCCCGTCCGCGTCTC
DMRT1-R[3] RT (CI)	TACAGAGCCCCATCTCCT
DMRT1 AS[4] probe-2	AGACTGTCAGTGCCAGAAATGCAAACTGATCGCCGAGCGG
DMRT1 AS R-2	CTAAAGTTAGCCAGGTCCAC
FTZ-F1 RT Probe	GTGGGGGCAGGAATAAGTTTGGCCCCATGTACAAGCGGGA
FTZ-F1RT R	TGGGATGGAGGGCATAAG
SOX9 AS EST[5] Probe	AGTCACTACAGTGAGCAGCAGGGCTCCCCTCCCCAGCATG
SOX9 AS EST R	GGCTCATATAGCTGCTGAAG
SOX9 AS α2 Probe	GTCTCCTACACCGGTACCTACGGCATCAGCAGCTCTGTGG
SOX9 AS α2 R	CTCTGCTGATCGTTGTAGTG
AR α RT probe 2	TAATAGGCATAATGAACTGCCGGAACCGTCATGGGACTTTCAGTA
AR α RT R2	CTGTCACCAACCTCATTCTT
AR β RT probe	ATGACAACACACAATATGGACCGAGGCAGGGTATGAATCC
AR β RT R	TGGCATCTGTGTAAGAGACA
Aromatase RT EST probe	CGGAGAGGAGACATTCATACTCAGCAGTTCCTCTGCAGTG
Aromatase RT EST R	CGAGACACACACGTCTACTG
Dax-1 AS EST probe	CTGCAAAGCCGCATCTACGGTCCTCATGAAGACTCTGCGA
Dax-1 AS EST R	CAGGATCCGCTGCAACAT
wt1 AS Probe-1	CAGACGTACGTGACCTCAACGCCCTGCTACCCCCGGTGCC
wt1 AS R-1	GTGAACTGGCCAGAGAAGT
Foxl2 paralog RT probe	AGAAGCCCCCATACTCTTACGTCGCCCTCATTGCCATGGC
Foxl2 paralog RT R	GGAACTGATGGTTGTGGTAG
Foxl2 ortholog RT probe	GACACTTACCAAAACCCAGAGGATGACGCAATGGCCCTGA
Foxl2 ortholog RT R	TCTGACAGGAGGTGTAGGAC
CITED2 AS Probe	CGTCCAGAGAAAGACTGAACCACAAACTAGACTAGCCACA
CITED2 AS R	AGGTAACGAGGAAGAAATCC
Figla AS EST probe	GGGTAACATTTTGGTGAGTGAAACAGGTGAGCCCTTGCTC
Figla AS EST R	TTTACTGGGTTTACGGTCAC
AMH AS Probe	CTGGAGAAATACCTGCTGTCTCCTCCTGAGGCCACCATCT
AMH AS R	CAGATATTGGTTCCATGCTC

Each of the probes and its corresponding reverse primer was checked for specificity using T-grad PCR. A T-grad gel image result for AMH is given as an example and is shown in Figure 3.2.

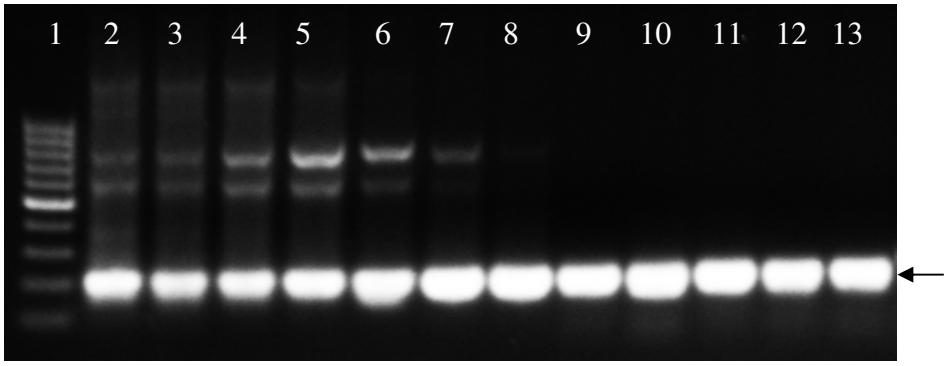


Figure 3.2 The gel picture for T-grad PCR result of AMH.

Lane 1: 100 bp ladder; lane 2: PCR at 45°C; lane 3: PCR at 45.5°C; lane 4: PCR at 46.9°C; lane 5: PCR at 49°C; lane 6: PCR at 51.4°C; lane 7: PCR at 53.8°C; lane 8: PCR at 56.2°C; lane 9: PCR at 58.5°C, lane 10: PCR at 60.9°C; lane 11: PCR at 63.1°C; lane 12: PCR at 64.5°C; lane 13: PCR at 65°C. All PCR reactions used Atlantic salmon genomic DNA as template. The arrow indicates expected size of amplification product.

Each of the probes for the sex-determining gene candidates was hybridized to Segment 1 (filters 1-6) of the Atlantic salmon BAC library CHORI-214. Segment 4 (filters 18-20) and Segment 2 (filters 7-12) were used in cases where the probing result of Segment 1 was not informative. All 14 sex-determining gene candidates gave positive BACs by hybridization. An example of the result for filter 5 hybridized with the AMH probe is shown in Figure 3.3.

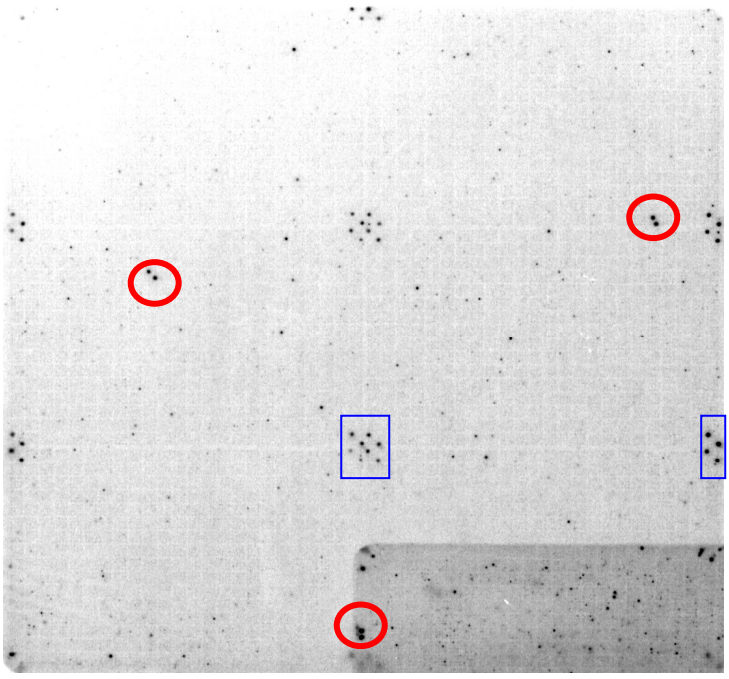


Figure 3.3 CHORI-214 Atlantic salmon BAC library filter #5 hybridized with the probe for AMH.

The red circles are the positive BACs that were picked for PCR confirmation. The blue rectangles are the reference points that help to identify the correct BACs from the filters.

PCR reactions were performed to screen for true positive BACs containing the sex-determining gene candidates. AMH is used again as an example to illustrate a PCR result in Figure 3.4.

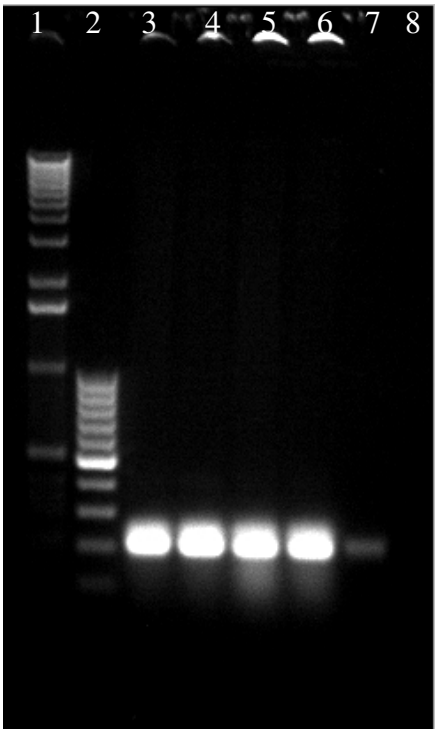


Figure 3.4 The BACs that were hybridization positive for AMH probe were picked for PCR confirmation.

The agarose gel was made of 1.3%, and genomic DNA was used as a positive control and water was used as a negative control for all PCR reactions. Lane 1: 1 kb ladder, lane 2: 100 bp ladder, lane 3: S0112A06, lane 4: S0212A05, lane 5: S0244J24, lane 6: S0255B24, lane 7: Atlantic salmon genomic DNA, lane 8: water.

The true positive BACs may belong to a contig that has been assembled and included in the AsalBase database. This can be checked by the bac2contig in-house tool in the developmental AsalBase website. The PCR confirmed hybridization positive BACs for AMH that are part of contig 1652 are shown in yellow in Figure 3.5, which is a screen shot from the developmental Asalbase website.

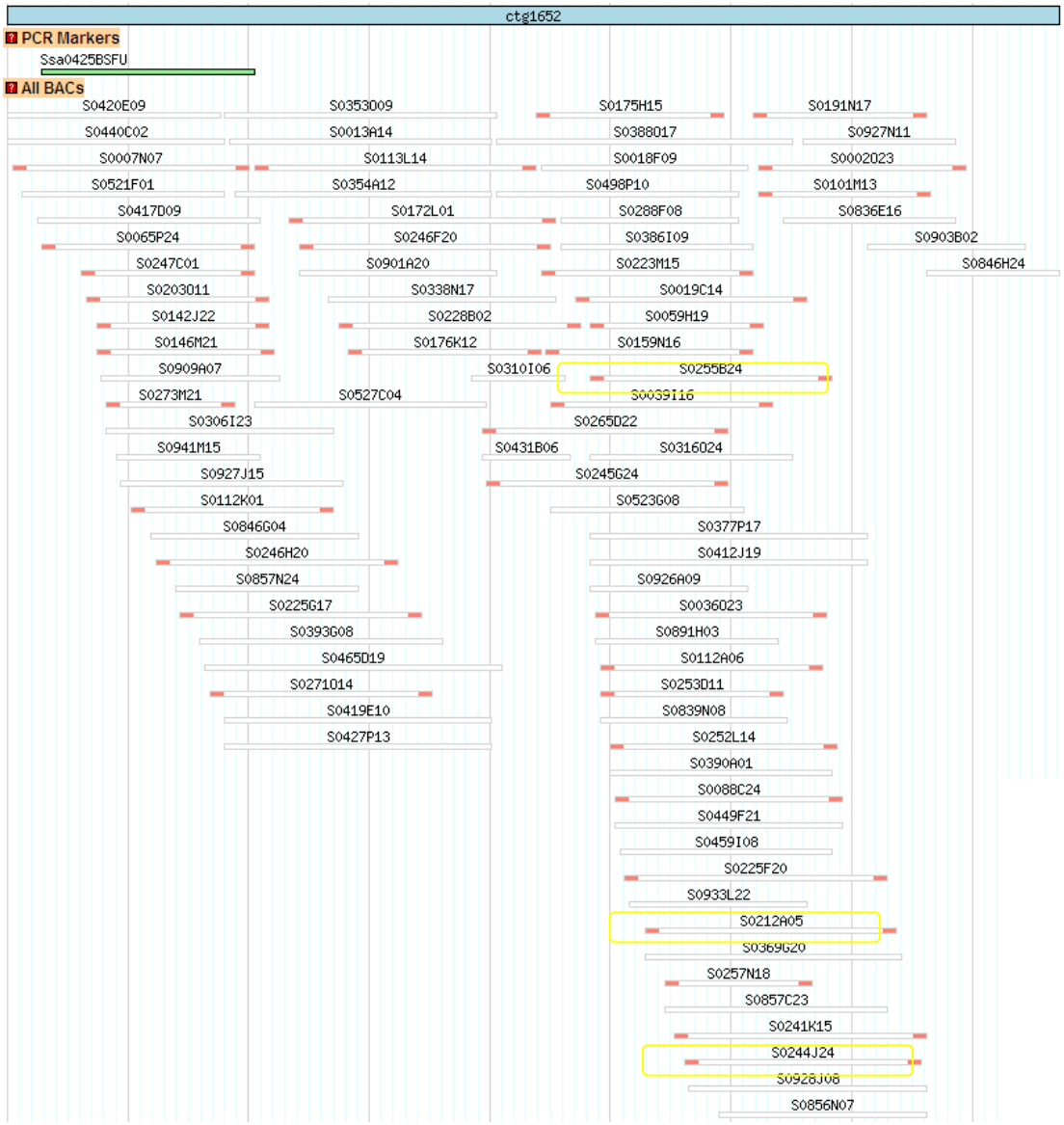
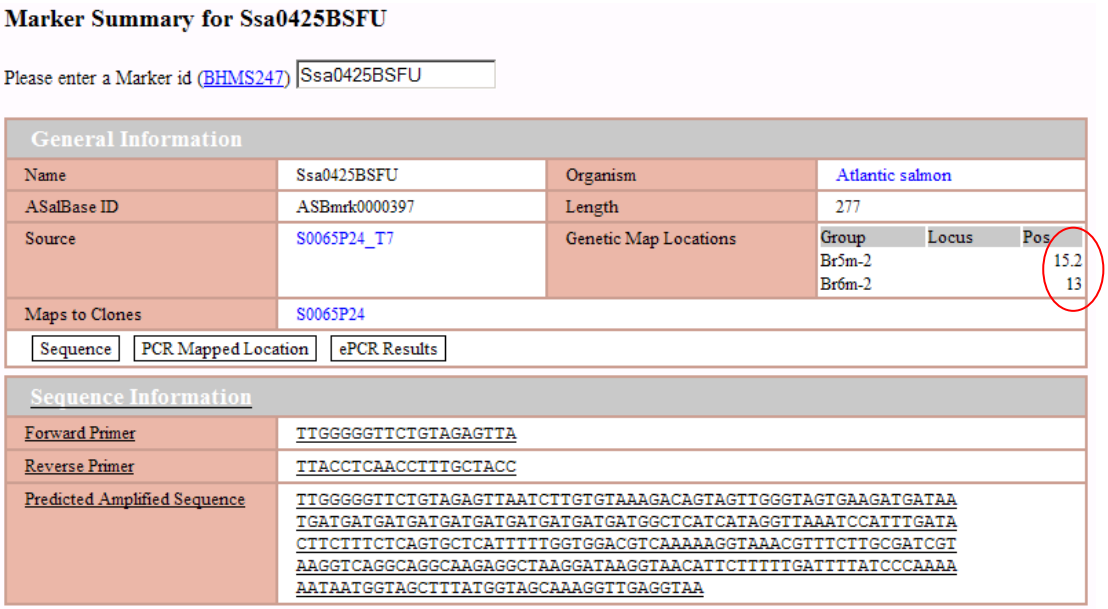


Figure 3.5 Developmental AsalBase view of Contig1652.
AMH positive clones are highlighted in yellow.

3.1.2 Mapping the sex-determining gene candidates

After identification of the contigs to which each of the sex-determining gene candidate belongs, the next step was to check if linkage groups (LG)s have been assigned to those contigs. Each contig contains many BACs with available BAC end sequences, and if any BAC end sequences contain possible microsatellite markers, the primers flanking microsatellite markers were designed by an automated pipeline. The microsatellites were then checked for variation in the parents of the two Atlantic salmon mapping families, Br5 and Br6. If a microsatellite was variable in one of the parents of the families, it was informative for mapping and was used for LG analysis. Vice versa, if the microsatellite was not variable in any of the parents of the families, it was considered not informative, and could not be used for linkage analysis. The LG analysis software LINKMFEX (Danzmann, 2006) was used for LG analysis. The LOD score for linkage was set at 4 because of the Atlantic salmon recently duplicated genome. Many microsatellites were checked for polymorphism, and LGs were assigned to each of the markers tested that were informative. The LG information was updated on the AsalBase database (http://www.AsalBase.org) for contigs.

By clicking on the PCR marker Ssa0425BSFU as shown in Figure 3.5, the marker summary page will show up with all available marker information. Figure 3.6. shows how the marker summary looks using marker Ssa0425BSFU as an example.



Marker Summary for Ssa0425BSFU

Please enter a Marker id (BHMS247) Ssa0425BSFU

General Information

Name	Ssa0425BSFU	Organism	Atlantic salmon
ASalBase ID	ASBmrk0000397	Length	277
Source	S0065P24_T7	Genetic Map Locations	Group / Locus / Pos Br5m-2 15.2 Br6m-2 13
Maps to Clones	S0065P24		

Sequence | PCR Mapped Location | ePCR Results

Sequence Information

Forward Primer	TTGGGGGTTCTGTAGAGTTA
Reverse Primer	TTACCTCAACCTTTGCTACC
Predicted Amplified Sequence	TTGGGGGTTCTGTAGAGTTAATCTTGTGTAAAGACAGTAGTTGGGTAGTGAAGATGATAA TGATGATGATGATGATGATGATGATGATGGCTCATCATAGGTTAAATCCATTTGATA CTTCTTTCTCAGTGCTCATTTTTGGTGGACGTCAAAAAGGTAAACGTTTCTTGCGATCGT AAGGTCAGGCAGGCAAGAGGCTAAGGATAAGGTAACATTCTTTTTGATTTTATCCCAAAA AATAATGGTAGCTTTATGGTAGCAAAGGTTGAGGTAA

Figure 3.6 Summary page for marker Ssa0425BSFU from developmental AsalBase.

The red-circled region gives the genetic map locations of this marker. It was mapped to LG2 for Br5 male and Br6 male.

Some of the microsatellites were not checked by the time the contigs had been identified. In these cases, the microsatellite primers were picked from the stocks and tested for informativeness. For example, Figure 3.7 shows the microsatellite gel image for the marker Ssa1746BSFU from the SP6 end sequence of BAC S0240D02 from contig 1378.

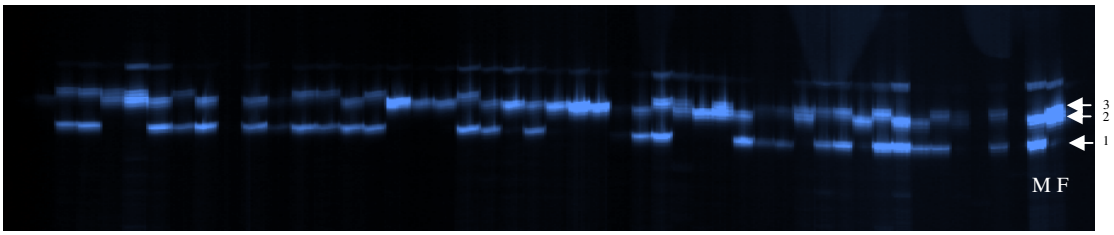


Figure 3.7 Microsatellite gel image for marker Ssa1746BSFU.

The blue bands on the gel are each individual's genotype in the Br5 mapping family. The M indicates the male parent, whose genotype was scored as 1/2, and the F indicates the female parent, whose genotype was scored as 2/3. The bands on the left are the offspring genotypes; their genotypes can either be 1/2, 1/3, 2/3 or 2/2.

In cases where no marker is present or markers are not informative in the contig to which a sex-determining gene candidate belongs, an alternative approach is to try to identify SNPs at a restriction site for certain enzymes in the sex-determining gene candidate. In this way, our mapping families (Br5 and Br6) can be used for LG analysis using restriction site fragment length polymorphism (RFLP) markers.

Forward and corresponding reverse primers for Ftz-f1, Foxl2 ortholog, Sox9 and Sox9α2 were designed about 20 nucleotides in length, and to cross a single intron using the conserved exon-intron boundaries found in other fish species as described in section 3.1.1. After T-grad PCR checking, the primer sets were used to amplify genomic DNA for the Br5 and Br6 mapping family parents. The PCR products were isolated, cleaned and ligated into the plasmid vector pSTBlue-1 and used to transform *E. coli*. Eight colonies were selected for each parent and the plasmid inserts were sequenced. Sox9α2 is given as an example in Figure 3.8.

```
                                10         20         30         40         50
                         ....|....|  ....|....|  ....|....| ....|....| ....|....|
Br5 male Sox9α2 5-1t     -AGATT-GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT  48
Br5 male Sox9α2 5-2t     GTGATTTGTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAATCGCTCT  50
Br5 male Sox9α2 5-3t     GTGAT--GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAATCGCTCT  48
Br5 male Sox9α2 5-6t     GTGATTTGTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT  50
Br5 male Sox9α2 5-7t     GTGATTTGTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT  50
Br5 male Sox9α2 5-8t     GTGAT--GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT  48
Br5 male Sox9α2 5-9t     GTGAT--GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT  48
Br5 female Sox9α2 6-3t   GTGATTTGTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT  50
Br5 female Sox9α2 6-5t   GTGATT-GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT  49
Br5 female Sox9α2 6-6t   -AGATTTGTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT  49
Br5 female Sox9α2 6-7t   GTGATT-GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAATCGCTCT  49
Br5 female Sox9α2 6-8t   GTGATT-GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT  49

                               60         70         80         90        100
                         ....|....|  ....|....|  ....|....|  ....|....| ....|....|
Br5 male Sox9α2 5-1t     GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT  98
Br5 male Sox9α2 5-2t     GCCTCCTCCA CGAACGGACG CTTCTCACCC TCATTGAGTA GCCTGTGCGT  100
Br5 male Sox9α2 5-3t     GCCTCCTCCA CGAACGGACG CTTCTCACCC TCATTGAGTA GCCTGTGCGT  98
Br5 male Sox9α2 5-6t     GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT  100
Br5 male Sox9α2 5-7t     GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT  100
Br5 male Sox9α2 5-8t     GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGC  98
Br5 male Sox9α2 5-9t     GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT  98
Br5 female Sox9α2 6-3t   GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT  100
Br5 female Sox9α2 6-5t   GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT  99
Br5 female Sox9α2 6-6t   GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GTCTGTGCGT  99
Br5 female Sox9α2 6-7t   GCCTCCTCCA CGAACGGACG CTTCTCACCC TCATTGAGTA GCCTGTGCGT  99
Br5 female Sox9α2 6-8t   GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT  99

                              110        120        130        140        150
                         ....|....| ....|....| ....|....| ....|....| ....|....|
Br5 male Sox9α2 5-1t     GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA  148
Br5 male Sox9α2 5-2t     AAAAGGCAAA TGGATACATG AGTAAATAAA ATATTCAATT GACAGCAAAA  150
Br5 male Sox9α2 5-3t     AAAAGGCAAA TGGATACATG AGTAAATAAA ATATTCAATT GACAGCAAAA  148
Br5 male Sox9α2 5-6t     GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA  150
Br5 male Sox9α2 5-7t     GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA  150
Br5 male Sox9α2 5-8t     GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA  148
Br5 male Sox9α2 5-9t     GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA  148
Br5 female Sox9α2 6-3t   GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA  150
Br5 female Sox9α2 6-5t   GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA  149
Br5 female Sox9α2 6-6t   GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA  149
Br5 female Sox9α2 6-7t   AAAAGGCAAA TGGATACATG AGTAAATAAA ATATTCAATT GACAGCAAAA  149
Br5 female Sox9α2 6-8t   GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA  149
                              160        170        180        190        200
                         ....|....| ....|....| ....|....| ....|....| ....|....|
Br5 male Sox9α2 5-1t     TAATTTACCA ---------- ---------- ---------- -CAAAGGTGA  167
Br5 male Sox9α2 5-2t     TAGTTTCCTA TTTTAATGCA GCATAACATA CCTTTTTATT TCAAATGTTC  200
Br5 male Sox9α2 5-3t     TAGTTTCCTA TTTTAATGCA GCATAACATA CCTTTTTATT TCAAATGTTC  198
Br5 male Sox9α2 5-6t     TAATTTACCA ---------- ---------- ---------- -CAAAGGTGA  169
Br5 male Sox9α2 5-7t     TAATTTACCA ---------- ---------- ---------- -CAAAGGTGA  169
Br5 male Sox9α2 5-8t     TAATTTACCA ---------- ---------- ---------- -CAAAGGTGA  167
Br5 male Sox9α2 5-9t     TAATTTACCA ---------- ---------- ---------- -CAAAGGTGA  167
Br5 female Sox9α2 6-3t   TAATTTACCA ---------- ---------- ---------- -CAAAGGTGA  169
Br5 female Sox9α2 6-5t   TAATTTACCA ---------- ---------- ---------- -CAAAGGTGA  168
Br5 female Sox9α2 6-6t   TAATTTACCA ---------- ---------- ---------- -CAAAGGTGA  168
Br5 female Sox9α2 6-7t   TAGTTTCCTA TTTTAATGCA GCATAACATA CCTTTTTATT TCAAATGTTC  199
Br5 female Sox9α2 6-8t   TAATTTACCA ---------- ---------- ---------- -CAAAGGTGA  168
```

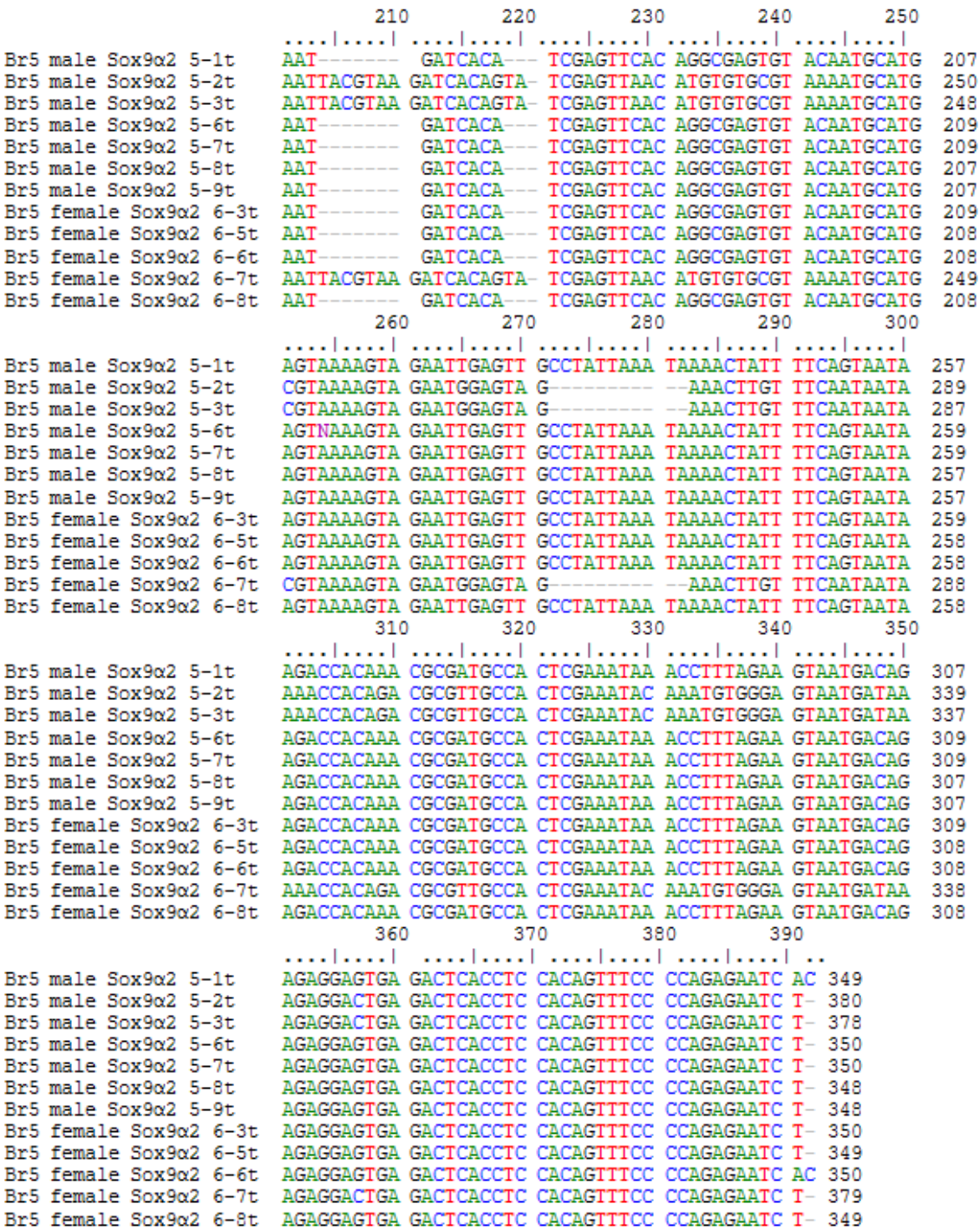


Figure 3.8 The ClustalW alignment for Br5 male and Br5 female Sox9α2 cross-intron sequences obtained from different colonies.

Although the overall sequences are very similar, there are two different sequences for both Br5 male and Br5 female, which can easily be distinguished by the gaps. Thus the sequences listed in Figure 3.8 were separated into two groups and realigned as shown in Figure 3.9 and Figure 3.10.

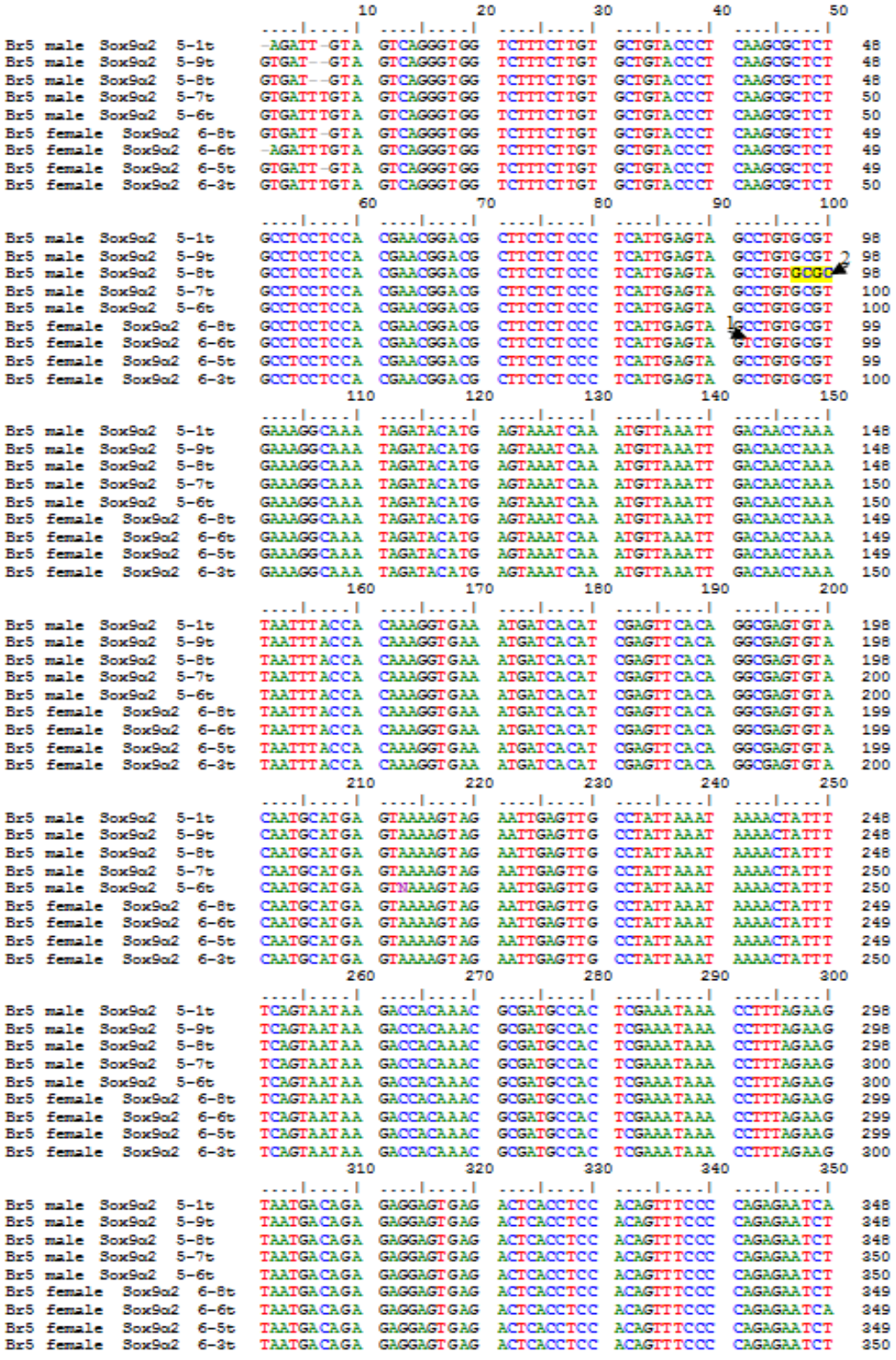

10 20 30 40 50
Br5 male Sox9a2 5-1t -AGATT-GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT 48
Br5 male Sox9a2 5-9t GTGAT--GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT 48
Br5 male Sox9a2 5-8t GTGAT--GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT 48
Br5 male Sox9a2 5-7t GTGATTTGTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT 50
Br5 male Sox9a2 5-6t GTGATTTGTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT 50
Br5 female Sox9a2 6-8t GTGATT-GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT 49
Br5 female Sox9a2 6-6t -AGATTTGTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT 49
Br5 female Sox9a2 6-5t GTGATT-GTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT 49
Br5 female Sox9a2 6-3t GTGATTTGTA GTCAGGGTGG TCTTTCTTGT GCTGTACCCT CAAGCGCTCT 50
60 70 80 90 100
Br5 male Sox9a2 5-1t GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT 98
Br5 male Sox9a2 5-9t GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT 98
Br5 male Sox9a2 5-8t GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGC 98
Br5 male Sox9a2 5-7t GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT 100
Br5 male Sox9a2 5-6t GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT 100
Br5 female Sox9a2 6-8t GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT 99
Br5 female Sox9a2 6-6t GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT 99
Br5 female Sox9a2 6-5t GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT 99
Br5 female Sox9a2 6-3t GCCTCCTCCA CGAACGGACG CTTCTCTCCC TCATTGAGTA GCCTGTGCGT 100
110 120 130 140 150
Br5 male Sox9a2 5-1t GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA 148
Br5 male Sox9a2 5-9t GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA 148
Br5 male Sox9a2 5-8t GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA 148
Br5 male Sox9a2 5-7t GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA 150
Br5 male Sox9a2 5-6t GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA 150
Br5 female Sox9a2 6-8t GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA 149
Br5 female Sox9a2 6-6t GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA 149
Br5 female Sox9a2 6-5t GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA 149
Br5 female Sox9a2 6-3t GAAAGGCAAA TAGATACATG AGTAAATCAA ATGTTAAATT GACAACCAAA 150
160 170 180 190 200
Br5 male Sox9a2 5-1t TAATTTACCA CAAAGGTGAA ATGATCACAT CGAGTTCACA GGCGAGTGTA 198
Br5 male Sox9a2 5-9t TAATTTACCA CAAAGGTGAA ATGATCACAT CGAGTTCACA GGCGAGTGTA 198
Br5 male Sox9a2 5-8t TAATTTACCA CAAAGGTGAA ATGATCACAT CGAGTTCACA GGCGAGTGTA 198
Br5 male Sox9a2 5-7t TAATTTACCA CAAAGGTGAA ATGATCACAT CGAGTTCACA GGCGAGTGTA 200
Br5 male Sox9a2 5-6t TAATTTACCA CAAAGGTGAA ATGATCACAT CGAGTTCACA GGCGAGTGTA 200
Br5 female Sox9a2 6-8t TAATTTACCA CAAAGGTGAA ATGATCACAT CGAGTTCACA GGCGAGTGTA 199
Br5 female Sox9a2 6-6t TAATTTACCA CAAAGGTGAA ATGATCACAT CGAGTTCACA GGCGAGTGTA 199
Br5 female Sox9a2 6-5t TAATTTACCA CAAAGGTGAA ATGATCACAT CGAGTTCACA GGCGAGTGTA 199
Br5 female Sox9a2 6-3t TAATTTACCA CAAAGGTGAA ATGATCACAT CGAGTTCACA GGCGAGTGTA 200
210 220 230 240 250
Br5 male Sox9a2 5-1t CAATGCATGA GTAAAAGTAG AATTGAGTTG CCTATTAAAT AAAACTATTT 248
Br5 male Sox9a2 5-9t CAATGCATGA GTAAAAGTAG AATTGAGTTG CCTATTAAAT AAAACTATTT 248
Br5 male Sox9a2 5-8t CAATGCATGA GTAAAAGTAG AATTGAGTTG CCTATTAAAT AAAACTATTT 248
Br5 male Sox9a2 5-7t CAATGCATGA GTAAAAGTAG AATTGAGTTG CCTATTAAAT AAAACTATTT 250
Br5 male Sox9a2 5-6t CAATGCATGA GTNAAAGTAG AATTGAGTTG CCTATTAAAT AAAACTATTT 250
Br5 female Sox9a2 6-8t CAATGCATGA GTAAAAGTAG AATTGAGTTG CCTATTAAAT AAAACTATTT 249
Br5 female Sox9a2 6-6t CAATGCATGA GTAAAAGTAG AATTGAGTTG CCTATTAAAT AAAACTATTT 249
Br5 female Sox9a2 6-5t CAATGCATGA GTAAAAGTAG AATTGAGTTG CCTATTAAAT AAAACTATTT 249
Br5 female Sox9a2 6-3t CAATGCATGA GTAAAAGTAG AATTGAGTTG CCTATTAAAT AAAACTATTT 250
260 270 280 290 300
Br5 male Sox9a2 5-1t TCAGTAATAA GACCACAAAC GCGATGCCAC TCGAAATAAA CCTTTAGAAG 298
Br5 male Sox9a2 5-9t TCAGTAATAA GACCACAAAC GCGATGCCAC TCGAAATAAA CCTTTAGAAG 298
Br5 male Sox9a2 5-8t TCAGTAATAA GACCACAAAC GCGATGCCAC TCGAAATAAA CCTTTAGAAG 298
Br5 male Sox9a2 5-7t TCAGTAATAA GACCACAAAC GCGATGCCAC TCGAAATAAA CCTTTAGAAG 300
Br5 male Sox9a2 5-6t TCAGTAATAA GACCACAAAC GCGATGCCAC TCGAAATAAA CCTTTAGAAG 300
Br5 female Sox9a2 6-8t TCAGTAATAA GACCACAAAC GCGATGCCAC TCGAAATAAA CCTTTAGAAG 299
Br5 female Sox9a2 6-6t TCAGTAATAA GACCACAAAC GCGATGCCAC TCGAAATAAA CCTTTAGAAG 299
Br5 female Sox9a2 6-5t TCAGTAATAA GACCACAAAC GCGATGCCAC TCGAAATAAA CCTTTAGAAG 299
Br5 female Sox9a2 6-3t TCAGTAATAA GACCACAAAC GCGATGCCAC TCGAAATAAA CCTTTAGAAG 300
310 320 330 340 350
Br5 male Sox9a2 5-1t TAATGACAGA GAGGAGTGAG ACTCACCTCC ACAGTTTCCC CAGAGAATCA 348
Br5 male Sox9a2 5-9t TAATGACAGA GAGGAGTGAG ACTCACCTCC ACAGTTTCCC CAGAGAATCT 348
Br5 male Sox9a2 5-8t TAATGACAGA GAGGAGTGAG ACTCACCTCC ACAGTTTCCC CAGAGAATCT 348
Br5 male Sox9a2 5-7t TAATGACAGA GAGGAGTGAG ACTCACCTCC ACAGTTTCCC CAGAGAATCT 350
Br5 male Sox9a2 5-6t TAATGACAGA GAGGAGTGAG ACTCACCTCC ACAGTTTCCC CAGAGAATCT 350
Br5 female Sox9a2 6-8t TAATGACAGA GAGGAGTGAG ACTCACCTCC ACAGTTTCCC CAGAGAATCT 349
Br5 female Sox9a2 6-6t TAATGACAGA GAGGAGTGAG ACTCACCTCC ACAGTTTCCC CAGAGAATCA 349
Br5 female Sox9a2 6-5t TAATGACAGA GAGGAGTGAG ACTCACCTCC ACAGTTTCCC CAGAGAATCT 349
Br5 female Sox9a2 6-3t TAATGACAGA GAGGAGTGAG ACTCACCTCC ACAGTTTCCC CAGAGAATCT 350

Figure 3.9 ClustalW alignment for Br5 male and Br5 female Sox9α2 cross-intron sequences 1.
The arrow 1 pointed T within a putative C/T SNP could not be confirmed because there was no restriction site around this SNP region. The arrow 2 pointed C within a putative C/T SNP could be tested using the enzyme HhaI because its restriction site is G_CG'C.

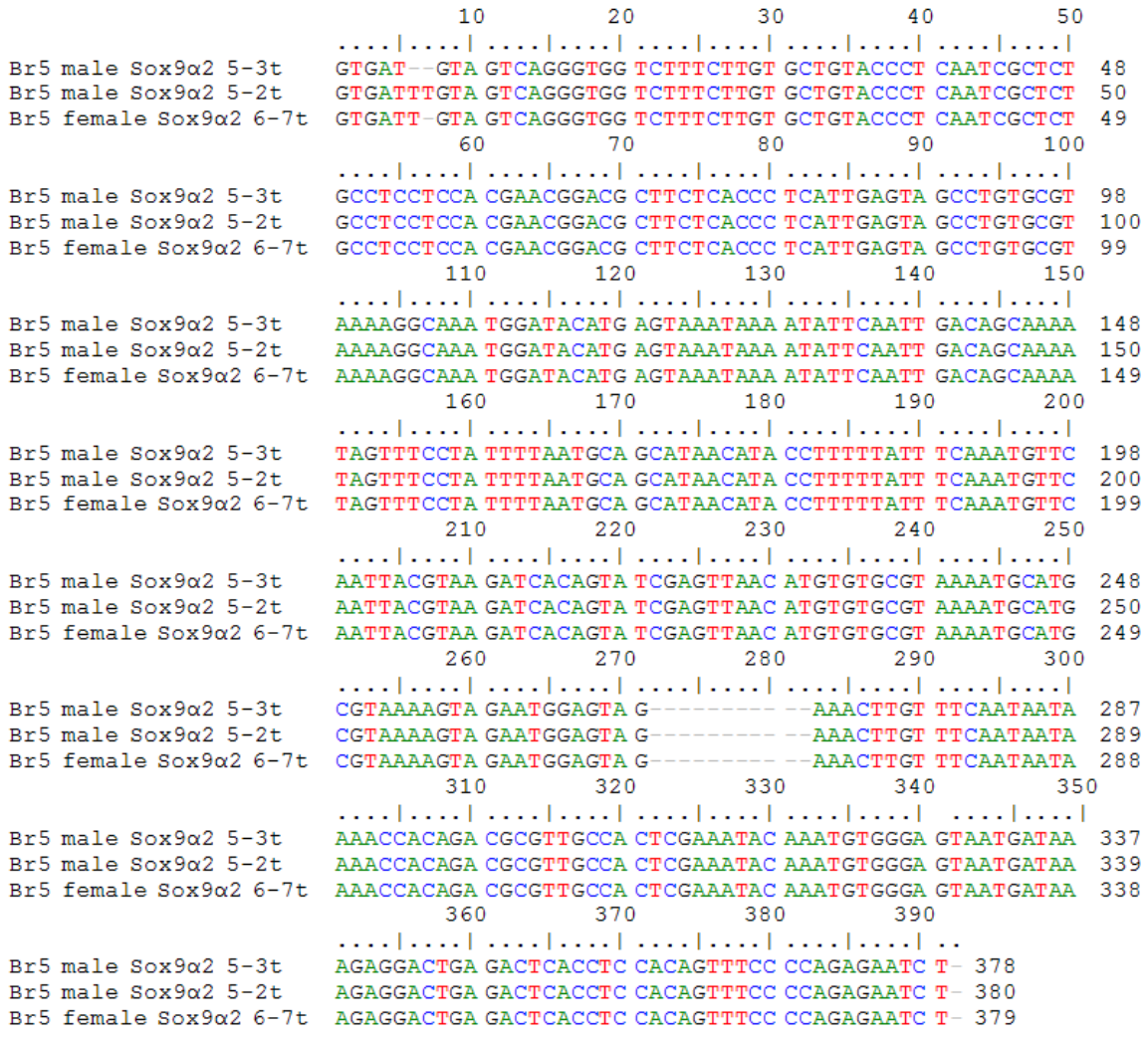


Figure 3.10 ClustalW alignment for Br5 male and Br5 female SOX9 α2 cross intron sequences 2.

These sequences of Br5 male and Br5 female are identical.

PCR reaction was done again for Br5 male and Br5 female using the cross intron primers for Sox9α2, and the PCR products were subjected to digestion with HhaI. The result is shown in Figure 3.11.

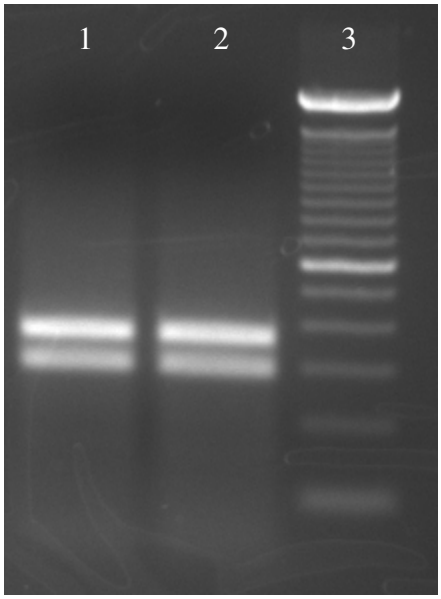


Figure 3.11 Gel image for HhaI digestion for both Br5 male and Br5 female Sox9α2 cross intron PCR product.

Lane 1: Br5 male; lane 2: Br5 female; lane3: 100 bp ladder. The expect PCR product size is 380-390 bp. The HalI should cut the PCR product at position 93, leaving a product of 93 bp and another product of 300 bp.

Both parent HhaI digestion patterns were the same, which is unexpected. This can be explained by the number of colonies picked for sequencing not being enough to get the SNP for Br5 female, or because there is a duplicated locus for Sox9α2 so the primer set actually amplifies 2 different sequences during the PCR reaction, and there were a SNP at the second locus that contains the HhaI restriction site in the Br5 female. A similar situation is found with the Foxl2 ortholog.

Two different yet very similar cross intron sequences for Sox9α2 also occur in the Br6 parents. A similar situation was also found for Sox9 in the Br5 and Br6 mapping families. This may indicate that actually there are duplicated loci for those genes.

Direct sequencing was also performed for Sox9, Sox9α2, Ftz-f1, CITED2, and Dax1 from both ends using PCR products from parents of both mapping families. After contig assembly by Sequencher 4.1 (Gene Codes Coperation), no obvious SNPs were identified.

3.1.3 Locating sex candidate genes on Atlantic salmon chromosomes

Dual-FISH analysis was applied for the each of the nine genes Sox9, Sox9α2, ARα, ARβ, Foxl2 ortholog, Figla Ftz-f1, CITED2, and Dax, for which the linkage groups are not available together with marker Ssa233BSFU. The DNA of the BACs containing the nine gene candidates is shown in Figure 3.12(a), and the DNA of the BAC containing the marker Ssa233BSFU is shown in Figure 3.12(b).

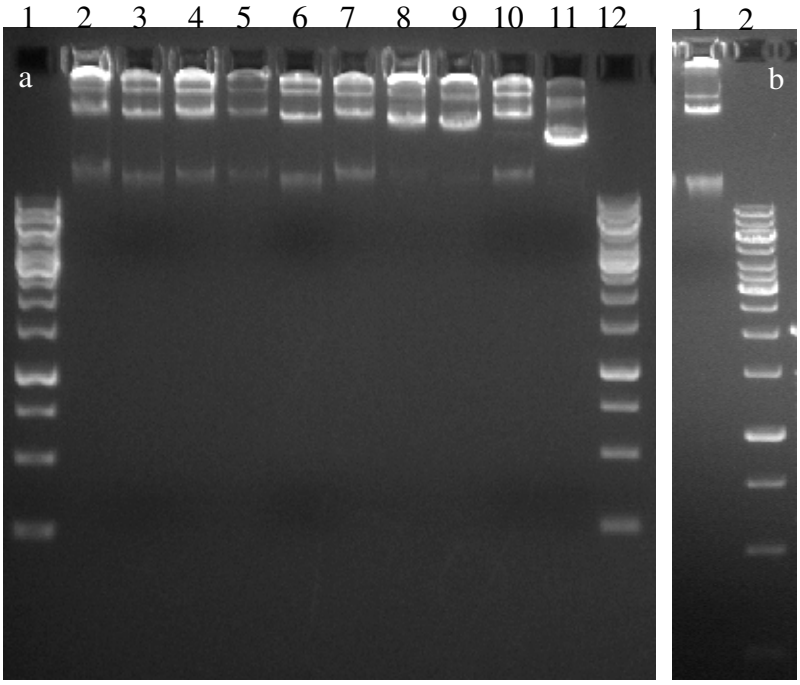


Figure 3.12 (a) Gel image for BAC DNA of the 9 gene candidates that will be used for nick translation of the FISH analysis.

Lane 1: 1 kb ladder; lane 2: S0016F17; lane 3: S0029O27; lane 4: S0062H08; lane 5: S0140M02; lane6: S0224M18; lane 7: S0088L03; lane 8: S0163F11; lane 9: S0473K20; lane 10: S0263M10; lane 11: S0612P18; lane 12: 1 kb ladder. (b) Gel image for the reference BAC DNA that will be used in FISH analysis. Lane 1: S0002E22; lane 2: 1 kb ladder.

The BACs were nick translated by the nick translation kit (Vysis). The products of the nick translocation reaction are shown in Figure 3.13. Note the smear of DNA from 200 to 400 bp.

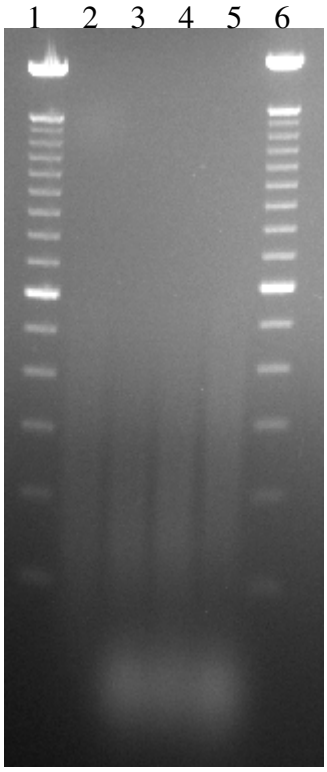


Figure 3.13 Gel image for the nick translation of 4 different BACs to be used in hybridization of the FISH analysis.

Lane 1: 100 bp ladder; lane 2: S0002E22; lane 3: S0473K20; lane 4: S0263M10; lane 5: S0163F11; lane 6: 100 bp ladder.

The nick translation products for each gene together with the marker nick translation product were prepared for hybridization on to the metaphase chromosome containing slide. The results of the dual-FISH experiments are shown in Figure 3.14.

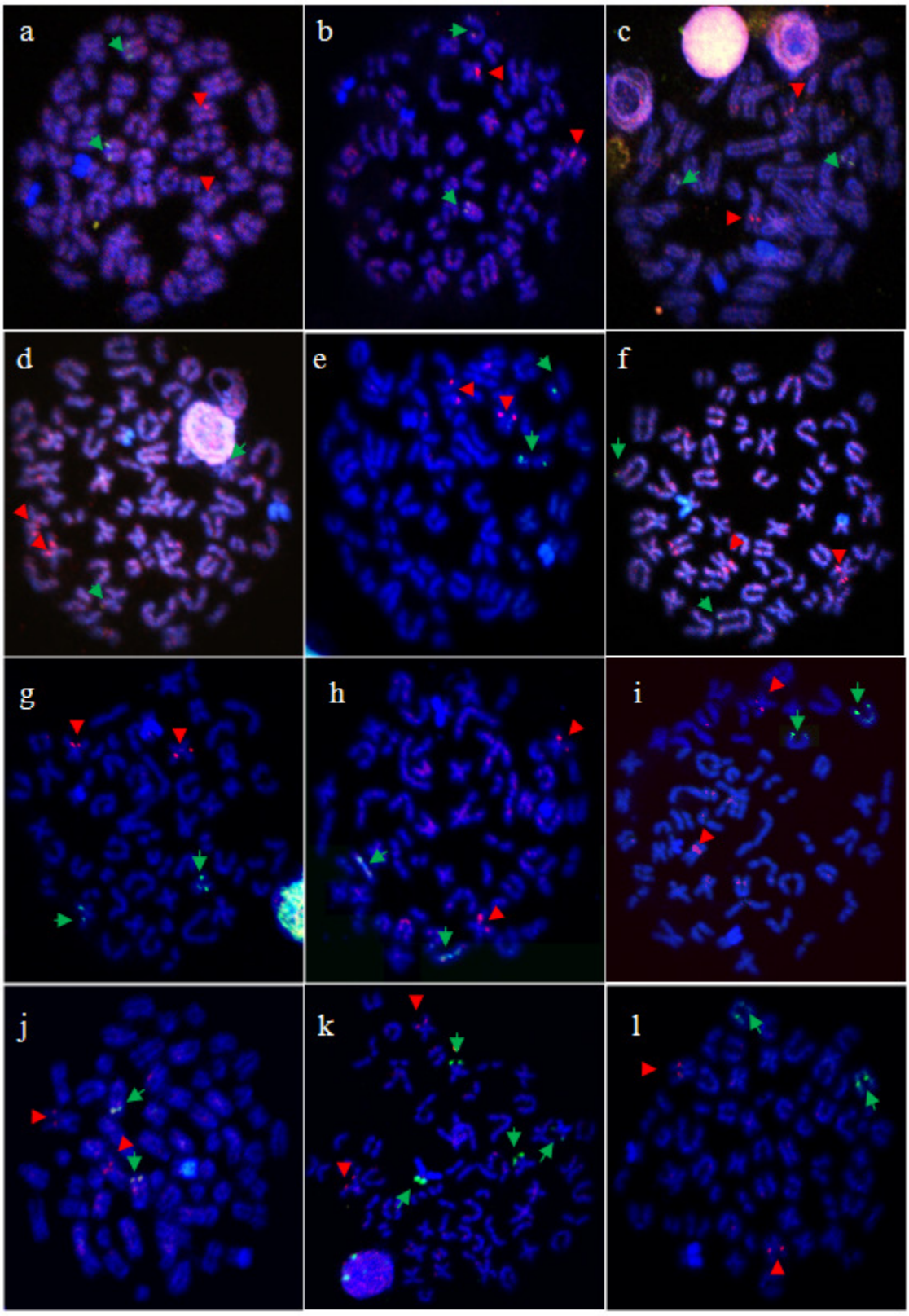

a
b
c
d
e
f
g
h
i
j
k
l

Figure 3.14 Dual-FISH result for the 9 BACs (green labelled) that contain the gene candidate together with reference BAC S0002E22 (red labelled) that contains marker Ssa0233BSFU that is very close to *SEX*.
a: S0088L03 and S0002E22; b: S0140M02 and S0002E22; c: S0224M18 and S0002E22; d: S0473K20 and S0002E22; e: S0263M10 and S0002E22; f: S0062H08 and S0002E22; g: S0163F11 and S0002E22; h: S0029O27 and S0002E22; i: S0016F17 and S0002E22; j: S0066D20 and S0002E22; k: S0082B16 and S0002E22; l: S0893A20 and S0002E22. The green arrow indicates which chromosome pair the gene candidate containing BAC hybridized to, and the red arrow head indicates which chromosome the reference BAC hybridized to.

3.1.4 DMRT1 as a sex-determining gene candidate

DMRT1 is of interest because of its important role in sex determination in all organisms studied. The DMRT1 homologs, *dsx* in *Drosophila* and *mab-3* in *C. elegans* are required for male specific development (Baker and Ridge, 1980; Shen and Hodgkin, 1988; Yi *et al.*, 2000). This suggests that some sexual development mechanisms are shared between nematodes and arthropods (Raymond *et al.*, 1998). DMRT1 in birds is located on the Z chromosome, which suggests a dosage dependent sex-determining role (Nanda et al., 2000). As mentioned previously, a DMRT1 paralog, DMY, is the sex-determining gene in medaka (Nanda *et al.,* 2002; Matsuda *et al.,* 2002). In all vertebrates studied, DMRT1 is expressed in the male genital ridge during embryo development and in testis after sexual maturation (Raymond *et al.,* 2000). Thus, it is a strong sex-determining gene candidate for Atlantic salmon. A DMRT1 probe and reverse primer were designed based on the rainbow trout DMRT1 mRNA sequence from the NCBI website (NM_001124269). The primer set was designed to be within a single exon according to other fish DMRT1 exon boundaries. However, the T-grad result showed a few thousand basepair amplification product, which indicates that the primer set actually crosses an intron. Subcloning and sequencing of the PCR product revealed that in Atlantic salmon, this exon is split into two exons by an additional intron. From the subcloned PCR sequence, an Atlantic salmon specific DMRT1 primer set was redesigned, in order to probe the BAC library. BACs from contig 169 and contig 4652 were PCR confirmed positive

for DMRT1. The recently duplicated Atlantic salmon genome is likely to be still in the stage of rediploidization, and many genes involved in sex determination are known to be dosage sensitive. Thus it is quite possible that some of the sex-determining candidates can be located in two different places in the Atlantic salmon genome, and the varying dosage effects of these genes could also play an important role in sex determination (see also in aromatase and WT1 later in this section). However, neither of these contigs contains any informative microsatellite markers. FISH for BAC S0082B16 from contig 169 showed that it hybridized to two pairs of chromosomes. It hybridized near the end of the q arm of a pair of submetacentric chromosomes, which is chromosome 8, LG 19, based on the DAPI staining pattern of the rRNA genes. It also hybridized to a different metacentric chromosome pair for which the LG information cannot be identified. FISH for BAC S0893A20 from contig 4652 revealed that it hybridized to a telocentric chromosome pair. Interestingly, a microsatellite was found in the cross intron PCR product sequence. This microsatellite was informative in the SALMAP families, and linkage group analysis revealed its location in LG7. LG7 corresponds to a telocentric chromosome pair, which could be the chromosome pair that BAC S0893A20 hybridized to, but without banding patterns, I cannot tell if this is the case or not. Nevertheless, this information rules out DMRT1, the sex-determining switch in medaka, as the sex-determining master gene in Atlantic salmon.

3.1.5 Ftz-f1 as a sex-determining gene candidate

Ftz-f1 (fushi tarazu factor-1) was first found to be a key regulator of *fushi tarazu* (ftz), a homeobox segmentation gene in *Drosophila* (Lavorgna *et al*., 1991). Its homologs have many different names in different species. They are now grouped as NR5A (Nuclear Receptors Nomenclature Committee, 1999). In mammals, Ftz-f1 has two homologs, NR5A1 and NR5A2. NR5A1 is a key regulator of steroidogenesis and is involved in the male specific sex-determining pathway (Ikeda *et al.,* 1993; Sadovsky *et al.,* 1995). Differential expression of Ftz-f1 in the two sexes during sexual gonadal histogenesis in Tilapia also suggests its involvement in sex determination (Wang *et al*., 2007). A Ftz-f1 EST sequence (contig # 4011644) from the Atlantic salmon EST database on the UVIC cGRASP website (http://web.uvic.ca/grasp/) was confirmed by BLAST searching using the EST sequence as the query, and the probe and corresponding reverse primer set was designed based on the EST sequence within a single exon. However, there is only one Ftz-f1 EST sequence in the database, which may not represent all Ftz-f1 genes in the Atlantic salmon genome. Yet the Ftz-f1 present in the EST database is the only clue we have at present. The PCR confirmed BACs that are positive for the Ftz-f1 probe are all within contig 1195. The contig contains a microsatellite marker Ssa1643BSFU which had not been tested for informativeness when the contig was identified. This marker was then tested, but it is not informative in our mapping families. Thus, this approach did not identify the location of Ftz-f1 in the Atlantic salmon genome by linkage group analysis. SNP searching also found no obvious SNPs that can be used for RFLP. FISH analysis reveals that it resides on a pair of

telocentric chromosomes. The fact that it locates on a different chromosome pair than the sex chromosome pair rules out the possibility of this copy of Ftz-f1 as the master gene switch for the sex determination gene in Atlantic salmon.

3.1.6 Sox9 as a sex-determining gene candidate

Although teleosts lack the testis determining gene SRY of mammals, several HMG box containing genes such as SOX genes do exist. **SOX9** is downstream of SRY in the sex-determining pathway in mammals. It is of interest because it shows a conserved sex-determining pathway involvement from mammals to teleosts. Many fish contain two SOX9 genes, named either SOX9a and Sox9b (Jonas and Per Erik, 2005) or Sox9α and Sox9α2 (Kobayashi and Nagahama, 2009). Expression studies for Sox9 genes in several fish species show that the type of Sox9 that is involved with sex determination differs from one species to another. Studies in Nile tilapia show that Sox9 is initially expressed in the cells surrounding the gonad of both sexes before sex differentiation and later only in XY gonadal surrounding cells (Kobayashi and Nagahama, 2009). In rainbow trout, Sox9 is expressed predominantly in testis and brain (Takamatsu *et al.,* 1997), and a temporal gene expression pattern study during gonadal differentiation and early gametogenesis suggests that it is an early expressed gene involved in trout testicular differentiation (Baron *et al.,* 2005). In medaka, Sox9b and Ftz-f1 are expressed in the gonadal somatic mesoderm, and later the Sox9b expressing cells differentiate into Sertoli and granulose cells (Kondo *et al*., 2009). Sox9 in zebrafish can bind an HMG-box containing element in the promoter region of Ftz-f1 *in vitro*, indicating that there is

a conserved pathway between Sox9 and Ftz-f1 in zebrafish. In rainbow trout, two copies of Sox9 are present: Sox9(α) and Sox9α2. Because information for Sox9 in Atlantic salmon was not available at the beginning of this study, it was hypothesized that Atlantic salmon would also have two copies of Sox9, Sox9(α) and Sox9α2, as in its closely related species rainbow trout. Either one of them or both of them could be involved in sex determination. Rainbow trout Sox9(α) (AB006448) and Sox9α2 (AB209872) mRNA sequences were used to design probes and corresponding reverse primers within a single exon. BACs that were confirmed positive for the Sox9(α) probe all belong to contig 1241 and BACs positive for the Sox9α2 probe all belong to contig 5893. However, neither contig contains any microsatellite markers. Thus, linkage group analysis was not available to check the physical locations of these two genes. SNP searching for cross intron sequences for Sox9(α) and Sox9α2 also could not find any obvious SNPs for RFLP analysis. FISH shows that Sox9(α) went to a metacentric chromosome pair, and Sox9 α2 went to a pair of telocentric chromosomes. Neither of them resides on the sex chromosomes, which rules out their possibility as the sex-determine gene in Atlantic salmon.

3.1.7 Androgen receptor as a sex-determining gene candidate

Androgen receptor (AR) is also believed to be involved in the sex-determining pathway in mammals, because mutations in AR cause Androgen Insensitivity Syndrome, and the phenotype can vary from nearly normal male to nearly normal female (Wilhelm and Koopman, 2006). The androgen receptor gene duplicated into two genes, ARα and ARβ, after the teleost lineage split from

Acipenseriformes, probably due to a WGD event (Douard *et al.,* 2008). The ARα and ARβ were very similar when the teleost fish diverged, but ARβ has diverged significantly as the teleost fish species evolved (Douard *et al.,* 2008). Gene comparisons have been done between mutations present in the diverged ARβ in fish and AR in human. The comparison reveals the mutations in ARβ are related to mutations seen in human Androgen Insensitivity Syndrome, and this might imply a potential function of the two ARs in sex determination in fish (Douard *et al*., 2008). ARα and ARβ in Atlantic salmon might also be involved in sex determination because steroid hormone treatment can cause sex reversal (Sower *et al.,* 1984). The information for Atlantic salmon ARα and ARβ was not available when the study commenced. Thus, ARα (AB012095) and ARβ (AB012096) mRNA sequences from its closely related species rainbow trout were used to design the probes and corresponding reverse primers. Because the rainbow trout ARα and ARβ mRNA sequences are very similar to each other, (percent identity= 90% by Megablast from NCBI), the probes and corresponding reverse primers were designed from positions where the two sequences have most mismatches in order to distinguish ARα and ARβ one from another. BACs that were confirmed positive for the ARβ probe belong to two contigs: contig 1437 and contig 690. BACs that were positive for the ARα probe all belong to contig 690. Sequencing the PCR product for BACs that are positive for ARβ only and positive for both ARα and ARβ revealed that BACs from contig1437 contain ARβ and BACs from contig 690 contain ARα. The ARβ probe hybridized to both ARα and ARβ containing BACs because the two gene sequences are very

similar. Contig 1437 does not contain any microsatellite markers; thus, linkage analysis is not available for ARβ. Although contig 690 contains two markers, Ssa0857BSFU and Ssa1366BSFU, neither of them is variable in the SALMAP families. This makes linkage analysis unavailable for ARα. Nevertheless, this indicates the two genes are not overlapping in the genome as the BACs from the two contigs are not overlapping. The FISH results show that the gene containing BAC for both genes hybridize to telocentric chromosome pairs other than the sex chromosomes, which means they cannot be the sex-determining gene in Atlantic salmon.

3.1.8 AMH as a sex-determining gene candidate

AMH (Anti-Müllerian hormone) has another common name MIS (Müllerian-inhibiting substance). It inhibits the formation of Müllerian ducts in the human male embryo (Behringer, 1994). In addition, it is also an important regulator of gonadal steroidogenesis as it inhibits aromatase in developing gonads (di Clemente *et al.,* 1992). In several fish species the expression of AMH is similar to Sox9 (von Hofsten and Olsson, 2005; Tobayashi and Nagahama, 2009). Thus, AMH is also thought to be involved in sex determination in Atlantic salmon. An Atlantic salmon AMH mRNA sequence (FJ609190) is available on the NCBI website, and thus the probe and corresponding reverse primers were designed based on this mRNA sequence. BACs that are positive for the AMH probe all belong to contig 1652, which contains an informative marker Ssa0425BSFU. However, this marker was mapped on to LG2, which rules out AMH as the sex-determining gene in Atlantic salmon.

3.1.9 Aromatase as a sex-determining gene candidate

Aromatase (Cyp19) controls the balance between the terminal sex hormone androgens and estrogens. This balance ensures the development of the proper sex. Many fish species contain two copies of aromatase, Cyp19a and Cyp19b. Cyp19a is usually involved in the sex-determining process, while Cyp19b is expressed in the brain (von Hofsten and Olsson, 2005). The temperature at which eggs are incubated influences the expression level of aromatase in many reptiles, which ultimately determines the sex of the developing embryo (Jeyasuria and Place, 1998; Wibbels *et al.,* 1998; Bogart, 1987). Inhibitors of aromatase can also lead to sex reversal of females in many fish such as zebrafish (Fenske *et al.,* 2004) and bird species, which illustrates the conserved importance of aromatase in sex-determining pathways. Recent studies suggest that Foxl2 is an upstream regulator of aromatase in the sex-determining pathway for ovarian differentiation in mammals and fishes (Kondo, *et al*., 2009; Baron *et al*., 2004; Pannetier *et al*., 2006). The aromatase probe and corresponding reverse primer were designed based on rainbow trout EST (contig # Omyk-BX083177) from the UVIC cGRASP EST database. The BACs that were confirmed positive for the aromatase probe belong to two non-overlapping contigs: contig 1378 and contig 6832. Contig 1378 contains a marker Ssa1746BSFU and contig 6832 contains another marker Ssa2761BSFU. Both markers were informative: marker Ssa1746BSFU maps to LG10 and Ssa2761BSFU maps to LG28. Thus, there are two aromatase genes which reside on two different LGs. This might explain its dosage dependent role in sex determination in Atlantic salmon. Yet based on the fact that neither gene maps to

LG1, it is quiet unlikely that either is the sex-determining master switch in Atlantic salmon.

3.1.10 Foxl2 as a sex-determining gene candidate

In snapping turtle (*Chelydra serpentine*) which utilizes temperature dependent sex determination, the **Foxl2** level is higher in developing gonads at temperatures that favor female development. In rainbow trout, there are at least two copies of Foxl2, named Foxl2 ortholog and Foxl2 diverged paralog, or Foxl2a and Foxl2b. Foxl2b is much diverged and few similarities can be found between Foxl2a and Foxl2b (Baron *et al*., 2004). This indicates a fast evolutionary rate of Foxl2b, which in turn may mean it can acquire new functions. In rainbow trout both Foxl2a and Foxl2b are expressed in female gonad with independent expression profiles, and aromatase expression pattern is highly correlated with Foxl2a (Baron *et al*., 2004). Thus, it is reasonable to propose that Foxl2a and Foxl2b are also involved in sex determination in its closely related species, Atlantic salmon. Foxl2a and Foxl2b probes and corresponding reverse primers were designed based on rainbow trout Foxl2a (AY507927) and Foxl2b (AY507926) mRNA sequences due to the lack of information in Atlantic salmon. BACs that were confirmed positive for the Foxl2b probe all belong to contig 377, and the microsatellite maker Ssa0679BSFU was mapped LG5, which rules out Foxl2b as the sex-determining gene in Atlantic salmon. BACs that were confirmed positive for the Foxl2a probe all belong to contig1077, but contig1077 does not contain any microsatellite markers. Thus, it was not possible to use linkage group analysis to identify the physical locations of Foxl2a in the Atlantic

salmon genome. The FISH analysis reveals that the Foxl2a containing BAC hybridized to a pair of telocentric chromosomes, which rules out its possibility as the sex-determining gene in Atlantic salmon.

3.1.11 Dax1 as a sex-determining gene candidate

Dax1 is usually considered to be the female sex-determining gene in mammals, because of its involvement in female sex development. In Atlantic salmon, the default sex is proposed to be female as in zebrafish. So far the sex-determining gene has not been found in zebrafish. It has been proposed that zebrafish might not have sex chromosomes. However, in Atlantic salmon, the evidence is strong that it utilizes a genetic mechanism for sex determination, and the sex chromosomes have been identified. Thus, it is possible that a female determining factor such as DAX-1 could lead to sex determination in fishes whose default sex is female, and they could utilize a dosage sensitive sex-determining system, such that females have two active copies of Dax-1, whereas males only have one. The Dax1 EST sequence (DW181004) is available on the UVIC cGRASP Atlantic salmon EST database, and thus this EST sequence was used to design the probe and corresponding reverse primer. The probe result showed that the true positive BACs all belong to contig 4837, which does not contain any informative genetic markers. Thus, it was not possible to determine the location of Dax1 in the Atlantic salmon genome by LG analysis. The FISH result shows that the Dax1 gene is located on a pair of telocentric chromosomes, which rules out its possibility as the sex-determining gene in Atlantic salmon.

3.1.12 WT1 as a sex-determining gene candidate

WT1 is a Wilms tumor suppressor gene, which has four zinc-finger motifs at the C-terminus. Specific mutations in the zinc-finger region can lead to abnormal urogenital development in Denys-Drash syndrome and Frasier syndrome in human (Wilhelm and Koopman, 2006). Mouse mutant studies reveal that WT1 is essential for some mesoderm-derived tissue, including kidney and gonad (see review by Hohenstein and Hastie, 2006). Ftz-f1 in human is a direct target of WT1 (Buaas *et al*., 2009). In zebrafish WT1 is involved in steroidogenic interrenal development together with ftz-f1 (von Hofsten and Olsson, 2005). Thus, it might also be involved in sex determination in Atlantic salmon. An Atlantic salmon WT1 mRNA sequence on NCBI website (BT059480) was used to design a probe and corresponding reverse primer. The true positive BACs belong to two contigs: contig 2265 and contig 88. Contig 2265 contains marker Ssa10009BSFU and Ssa10036BSFU. However, they are not informative in our mapping families. Thus, it was not possible to identify the physical location using this approach. Contig 88 contains a marker Ssa0679BSFU, and it had been mapped to LG23. Thus, although these two contigs are not overlapping, we could not tell if these two contigs reside on one linkage group or different linkage groups. BAC S0066D20 from contig 2265 was used for FISH analysis. It hybridized to a large telocentric chromosome pair near the telomere, which rules out its possibility as the sex determining gene in Atlantic salmon.

3.1.13 Figla as a sex-determining gene candidate

Figla (folliculogenesis specific basic helix-loop-helix) is a key regulator of multiple oocyte specific genes that are important for early development in mammals (Joshi *et al.,* 2007). Figla was also found to be important in medaka for XX individual gonad development (Matsuda, 2003). Figla is highly expressed during the gonad differentiating to ovary period in zebrafish, which suggests a conserved role of Figla in female sex determination. Thus, Figla is also on the candidate gene list for sex determination in Atlantic salmon. An Atlantic salmon Figla EST in the UVIC cGRASP EST database (contig # CK891846) was used to design the probe and corresponding reverse primer. The positive BACs all belong to contig 851. The contig does not contain any microsatellite markers, which again precludes the identification of its location in the Atlantic salmon genetic map. The FISH analysis reveals that Figla is located on a pair of telocentric chromosomes, which again rules out its function as the sex-determining gene in Atlantic salmon.

3.1.14 CITED2 as a sex-determining gene candidate

CITED2 (Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2) is a non-DNA binding protein that can act as a transcription co-regulator (Braganca *et al.,* 2003; Bhattacharya *et al.,* 1999). It regulates Ftz-f1 expression levels by interacting with WT1 during early adrenal development in mammals (Val *et al.,* 2007). It is the first co-factor that has been found for mammalian sex determination involved in the WT1/Ftz-f1 pathway (Buaas *et al.,* 2009). Thus, it could also be a candidate for the Atlantic salmon sex

determination factor. According to CITED2 from other fishes, it is a single exon gene. The probe and reverse primer were designed based on an Atlantic salmon CITED2 mRNA sequences from NCBI (NM_001141711). The CITED2 probe only hybridized to a few BACs, and only two are true positive BACs (S0163F11 and S0896E03). Both of them are singletons, and the BAC end sequences did not contain obvious microsatellite markers for LG analysis. This makes CITED2 the only gene that the true positive BACs did not assemble into a contig. This also makes CITED2 very interesting as we know that the Atlantic salmon sex-determining master gene was hypothesized to be located in a region that is very close to a heterochromatin region near the end of q arm on chromosome 2. The highly repetitive nature of heterochromatin makes the BACs containing the heterochromatin region hard to assemble into a contig. Thus CITED2 is likely in or very near to a heterochromatin region, which was speculated to be the same heterochromatin region where the sex-determining gene resides. However, the FISH result shows that it is located on a different submetacentric chromosome pair than the sex chromosomes, which rules out its possibility as the sex-determining gene in Atlantic salmon.

3.1.15 Summary

Ten out of the 14 gene candidates have verified positive BACs that belong to a single contig: Sox9 (contig 1241), Sox9 α2 (contig 5893), Ftz-f1 (contig 1195), AR α(contig 1437), ARβ(contig 690), AMH (contig 1652), DAX-1 (contig 4837), foxl2 paralog (contig 377), foxl2 ortholog (contig 1077), and Figla (contig 851). Another three gene candidates have verified positive BACs that belong to

two different, non-overlapping contigs: DMRT1 (contig 169 and contig 4652), aromatase (contig 1378 and contig 6832), WT1 (contig 2265 and contig 88). Of the 16 identified contigs for the sex candidates, only three of them had available linkage group information: AMH (contig 1652, LG2), WT1 (contig 88, LG23) and foxl2 paralog (contig 377, LG5). Another two contigs contained informative microsatellite makers and linkage groups were assigned: aromatase (contig 1378, LG10), aromatase (contig 6832, LG28). The available information regarding the gene candidates is listed in Table 3.2.

For the nine genes remaining, I tried to design cross intron primers to amplify Br5 and Br6 parental genomic DNA, and search for SNPs by sequencing. However, no obvious SNPs were found, which is unexpected. One explanation could be that the intron regions still contain essential regulatory elements. Nevertheless, linkage group analysis is not the only way to identify the gene locations on the Atlantic salmon genome. Dual-FISH analysis revealed the locations of the remaining nine gene candidates on different types of chromosome pairs relative to the sex chromosomes. None of them reside on the same chromosome pair as the sex-linked marker Ssa0233BSFU. The FISH results of the nine gene candidates are listed in Table 3.3.

Table 3.1.2 Positive BACs, contigs to which the BACs belongs, available markers in the contigs, LG analysis and SNP search result for each of the 14 sex-determining candidates.

Bold LGs were identified by LG analysis carried out by me.

Candidate genes	**Positive BACs**	**Contigs**	**Markers**	**LG analysis**
DMRT1	S0082B16	ctg169	Ssa0539BSFU&Ssa1174BSFU	N/A
	S0893A20	ctg4652	no	N/A
			dmrt1 specific	**LG7**
Ftz-f1	S0088L03	ctg1195	Ssa1643BSFU	N/A
Sox9(α)	S0473K20	ctg1241	no	N/A
Sox9α2	S0263M10	ctg5893	no	N/A
AR β	S0016F17	ctg1437	no	N/A
AR α	S00140M02	ctg690	Ssa0857BSFU&Ssa1366BSFU	N/A
AMH	S0112A06	ctg1652	Ssa0425BSFU	LG2
aromatase	S0243A19	ctg1378	Ssa1746BSFU	**LG10**
	S0212G18	ctg6832	Ssa2761BSFU	**LG28**
foxl2 paralog	S0009J17	ctg377	Ssa0679BSFU	LG5
foxl2 ortholog	S0062H08	ctg1077	no	N/A
DAX-1	S0224M18	ctg4837	no	N/A
WT1	S0066D20	ctg2265	Ssa10009BSFU&Ssa10036BSFU	N/A
WT1	S0120C04	ctg88	Ssa0489BSFU	LG23
Figla	S0029O27	ctg851	no	N/A
CITED2	S0163F11	no	no	N/A

Table 3.1.3 Chromosome locations for the nine gene candidates that the LG information is not available.

Gene candidates	BACs	Chromosomes	position
Ftz-f1	S0088L03	Telocentric	Close to the centromere
Sox9(α)	S0473K20	Metacentric	Close to the centromere
Sox9α2	S0263M10	Telocentric	Close to telomere
AR β	S0016F17	Telocentric	Close to telomere
AR α	S0014M02	Telocentric	Close to telomere
foxl2 ortholog	S0062H08	Telocentric	Close to telomere
Dax-1	S0224M18	Telocentric	Close to telomere
DMRT1	S0082B16	Submetacentric	Close to telomere of q arm
		Metacentric	Close to telomere of one chromosome arm
DMRT1	S0893A20	Telocentric	In the middle
WT1	S0066D20	Telocentric	Close to telomere
Figla	S0029O27	Telocentric	Close to the centromere
CITED2	S0163F11	Submetacentric	Close to the centromere

3.2 Sex-linked markers in Atlantic salmon and its closely related species

The flow chart of the approaches to map the sex-linked markers in closely related species to Atlantic salmon on to the Atlantic salmon linkage map is shown in Figure 3.15.

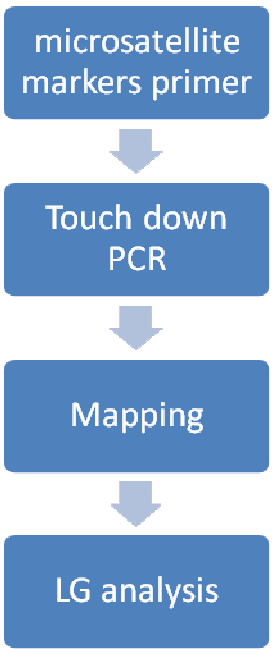


Figure 3.15 The flow chart of finding the locations of sex-linked markers in closely related species to Atlantic salmon on to the Atlantic salmon linkage map.

3.2.1 Finding where the sex-linked markers in closely related species are located in the Atlantic salmon genome

Twelve *SEX*-linked microsatellite markers from closely related species were tested for linkage analysis in Atlantic salmon: OmyRT5TUF, OmyFGT27TUF, OmyFGT2TUF, Omy10INRA, Omy7INRA, and Omy325UoG from brown trout (Woram *et al.,* 2003; Davidson *et al.,* 2009), and Omm1295, Omm1265, Omm1318, Ots514NWFSC, Ots553NWFSC and Ots212OSU from Coho salmon (McClelland and Naish, 2008; Davidson *et al*., 2009). These sex-linked markers of closely related species were chosen to map in the Atlantic salmon genome for comparative genomic studies, which will allow us to determine if they share the same or different sex linkage group, and also potentially provide the answer to the ultimate question if they share the same sex determining gene or not. The Ots212OSU primer set did not give a PCR product using Atlantic salmon genomic DNA as template, which may suggest that the Atlantic salmon genome and Coho salmon genome differ in the region flanking this marker; or a genome rearragement in Atlantic salmon happened so that there was a break somewhere within the marker. Another seven markers mentioned above were not informative in the Atlantic salmon Br5 and Br6 SALMAP mapping families: OmyFGT27TUF, OmyFGT2TUF, Omy7INRA, Omm1295, Omm1265, Omm1318, and Ots553NWFSC. Ots514NWFSC was informative in the Br5 family, and it mapped to LG14 in the male parent at 104.3 cM, which is at the end of LG14. This was the first time a Coho salmon sex-linked marker was mapped on to the Atlantic salmon linkage map. Perhaps a

genome rearrangement in Atlantic salmon caused the relocation of the Ots514NWFSC marker and its surrounding regions, which makes the sex linkage groups different between Coho salmon and Atlantic salmon. As other Coho salmon sex-linked markers were not informative, it is not possible to say if any of these markers would also map to LG14 in Atlantic salmon, or to other LGs. Omy10INRA was informative in both the Br5 and Br6 families. It was mapped on LG8 in both families. The merged female linkage map distance was 61.8 cM. Omy325UoG was informative in both parents of the Br6 family, and it mapped to LG8. The mapped distance was 8.6 cM in the Br6 male linkage map and was 76.9 cM in Br6 the female linkage group. OmyRT5TUF, the closest marker to the brown trout *SEX*, was informative in Br6 family, and it mapped to LG8 in the male linkage map at 8.6 cM. All the brown trout sex-linked markers (Omy10INRA, OmyRT5TUF and Omy325UoG) that were informative in Br5 or Br6 families mapped to LG8 in Atlantic salmon. Due to the cross-over rate in Atlantic salmon males being about tenfold less than in females, the linkage map of male usually does not contain as much information as the female linkage map. Many markers are clustered together in the male map, and in female linkage maps the markers are more dispersed. It is generally assumed that the order of markers is the same between male and female, thus the more informative merged female linkage map of Br5 and Br6 is used if possible. The Atlantic salmon LG8 merged female map and Br6 male map, together with brown trout sex linkage group 28, are shown in Figure 3.16. From the merged LG8 map we can tell that all the markers that were mapped on brown trout sex linkage group, LG28, were located

at the bottom half of the linkage group. Although the marker OmyRT5TUF was not shown in the female merged map, it can be seen with Omy325UoG and Omy10INRA in the Br6 male map. It is reasonable to assume that OmyRT5TUF would be somewhere above Omy301UoG in the Atlantic salmon female merged map for LG8.

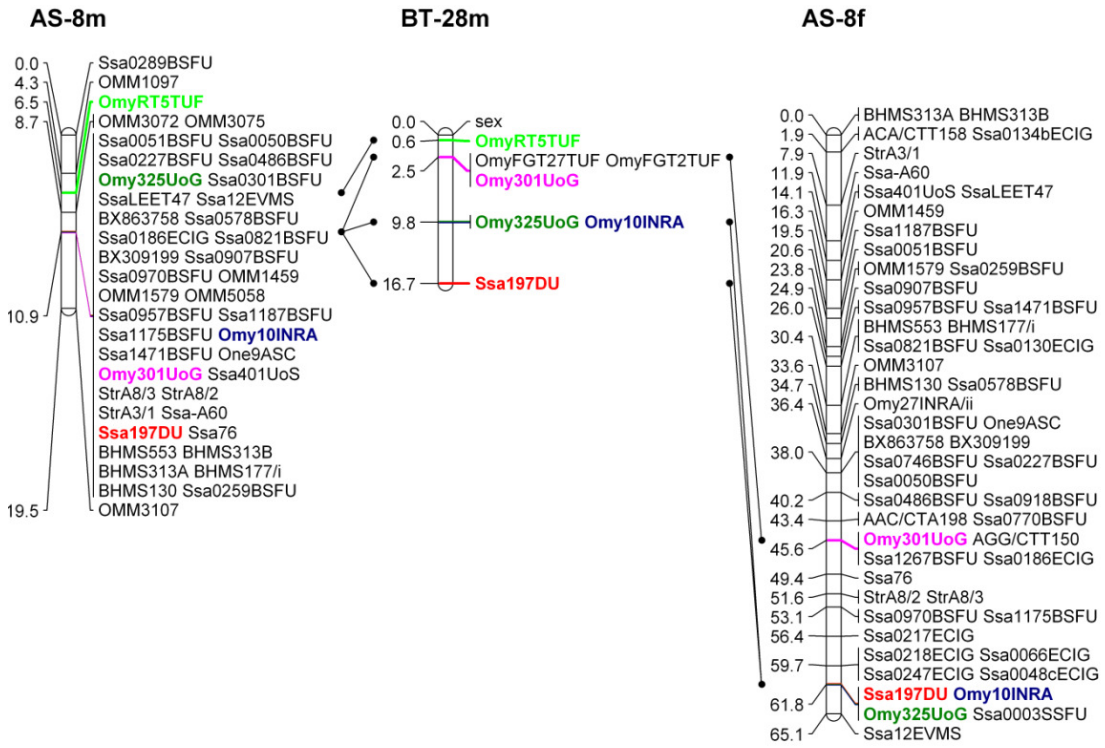


Figure 3.16 Atlantic salmon Br6 male LG8, Atlantic salmon Br5 and Br6 female merged LG8 and brown trout sex linkage LG28 maps.

The colored markers are the markers mentioned in the text, the straight lines are to show the correlation of marker positions between Atlantic salmon LG8 and brown trout LG28.The black dots refer to the bars which represent the marker locations in the linkage map.

The top half of Atlantic salmon LG8 corresponds to brown trout LG19, which is shown in Figure 3.17. This suggests that there was a chromosome fusion as part of the Atlantic salmon genome rearrangement after the split between *S. salar* and *S. trutta*.

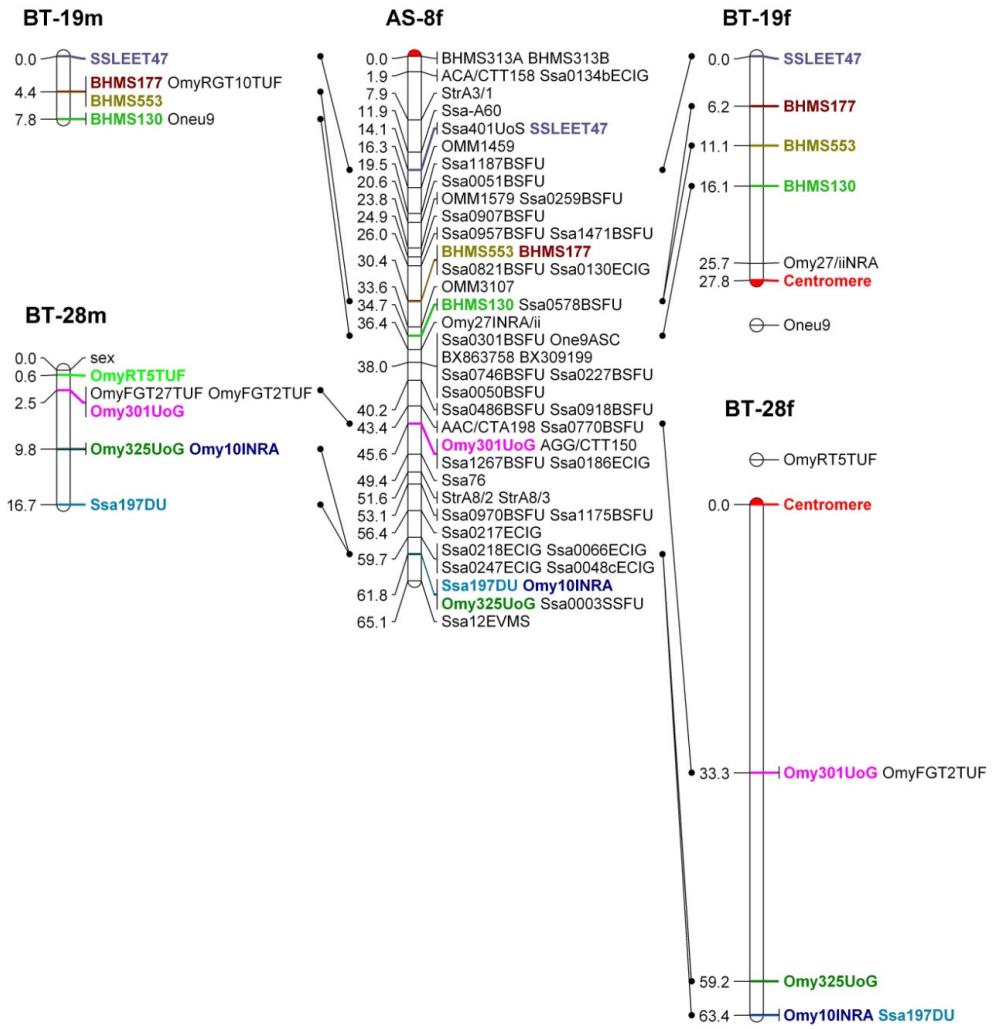


Figure 3.17 Comparison of Atlantic salmon linkage group AS-8f with brown trout linkage groups, BT-19 and BT-28, that share colinear conserved syntenic blocks of genetic markers.

The locations of the centromeres, shown in red, were determined by centromere mapping in the case of BT-19f and BT-28f (Gharbi *et al.*, 2006) and inferred from cytogenetic analysis in the case of AS-8f (Phillips *et al.*, 2009). The black dots refer to the bars which represent the marker locations in the linkage map.

The fusion of two chromosomes would probably cause at least one gene disruption; gene silencing or gene deletions by loss of a bit of one or both chromosomes. Figure 3.17 can explain why the sex linkage groups are different between Atlantic salmon and brown trout, and it would support the hypothesis that Atlantic salmon and brown trout utilize different sex-determining master genes.

3.3 Finding the sex chromosome and sex-determining gene candidate of Atlantic salmon's closest related species brown trout

The flow chart of the approach to identify the sex chromosome and sex-determining gene candidate in brown trout is shown in Figure 3.18.

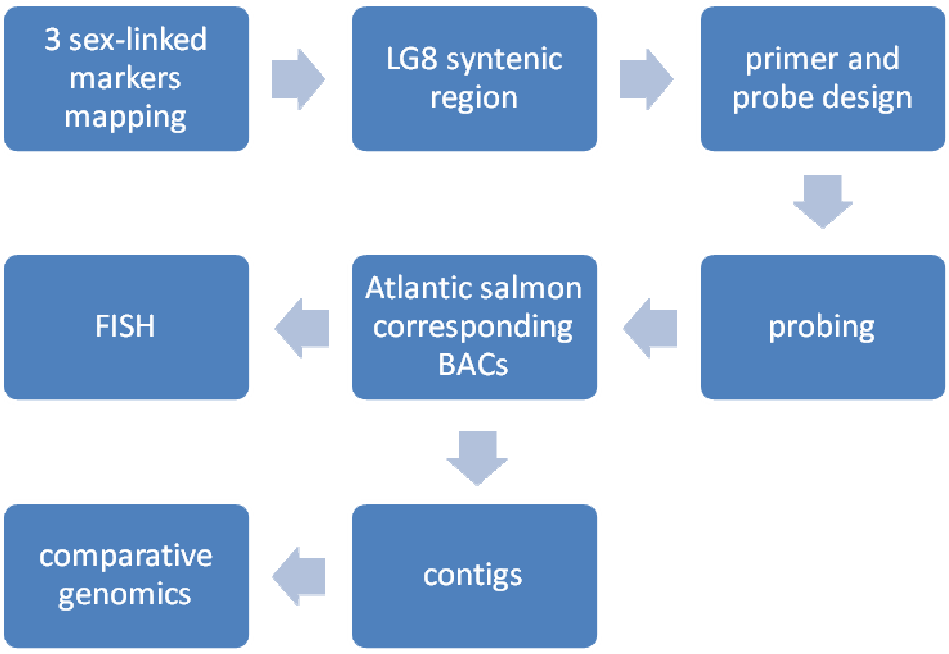


Figure 3.18 The flow chart for identifying brown trout sex chromosomes and a sex-determining gene candidate.

Brown trout sex-linked microsatellite marker primers for Omy10INRA, Omy325UoG, Omy301UoG, and OmyRT5TUF were used to amplify Atlantic salmon genomic DNA, and the PCR products were sub cloned and sequenced. Four different sized PCR product bands were seen on a 1.3% agarose gel for the OmyRT5TUF microsatellite primers. The gel picture is shown in Figure 3.19.

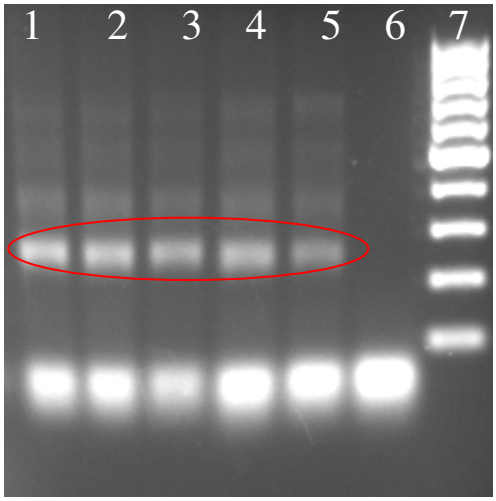


Figure 3.19 The gel image of amplification products of Atlantic salmon genomic DNA using the OmyRT5TUF primer set.

Lanes 1-5: Atlantic salmon genomic DNA, Lane 6: no template control, Lane7: 100 bp ladder. The bottom bands circled in red were the PCR products length about 200 bp. This piece of the gel was cut out for cloning the PCR product because the microsatellite length is usually about 200 bp.

Probes (~40 nucleotides) and reverse primers (~20 nucleotides) were designed based on corresponding PCR product sequences for Omy10INRA, Omy325UoG and OmyRT5TUF for screening the Atlantic salmon CHORI-214 BAC library filters. The microsatellite reverse primer for Omy301UoG was used as a probe to screen Atlantic salmon CHORI-214 BAC library filters directly because the primers were designed too close to the marker, and a probe of 40 nucleotides cannot be designed from the PCR sequence. The hybridization temperature was set to be 55^{o}C instead of 65^{o}C because a lower stringency was needed for the 20-mer reverse primer probe. True positive BACs were selected the same way as described in section 3.1.1. BAC S0164H12 was identified as a true positive for Omy301UoG and BAC S0133F23 was identified as a true positive for OmyRT5TUF. Marker Ssa197DU at the end of LG8 already had been identified on BAC S0121A09 (Phillips *et al.,* 2009). The DNA from these three BACs was isolated using a large construct kit and sent to R. Phillips for FISH analysis. The rationale is that if all three BACs, which correspond to OmyRT5TUF on the top, Omy301UoG in the middle and Ssa197DU at the bottom of the brown trout sex LG hybridize to the same chromosome, then the sex chromosome for brown trout can be identified by FISH analysis. The FISH results for the three BACs on the brown trout chromosomes are shown in Figure 3.20. The BACs that were true positives to corresponding microsatellite markers for FISH analysis are listed in Table 3.4.

Table 3.3.1 The true positive BACs and their corresponding microsatellites.

Microsatellite marker	**Positive BACs**
Omy301UoG	S0164H12
OmyRT5TUF	S0133F23
Ssa197DU	S0121A09

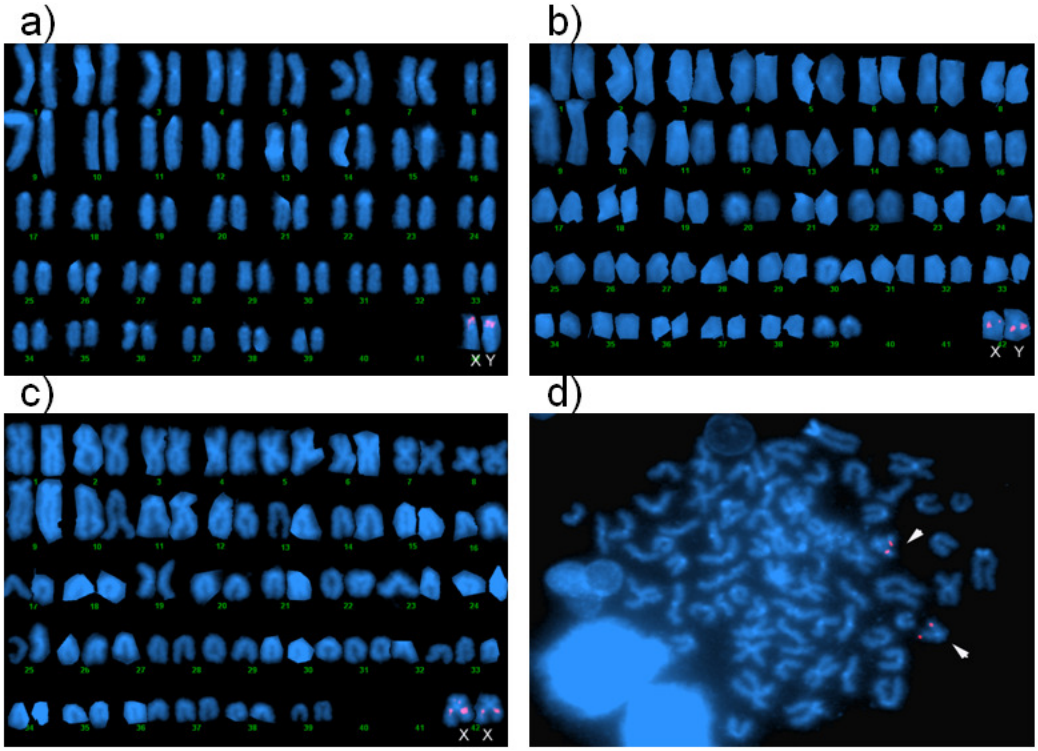


Figure 3.20 The FISH results for the three BACs S0164H12 (Omy301UoG), S0133F13 (OmyRT5TUF) and S0121A09 (Ssa197DU).

a) is the hybridization of BAC S0133F13 on to male brown trout metaphase chromosomes, b) is the hybridization of BAC S0164H12 on to male brown trout metaphase chromosomes, c) is the hybridization of BAC S0164H12 on to female brown trout metaphase chromosomes, and d) is the hybridization of BAC S0121A09 on to male brown trout metaphase chromosomes.

The three BACs did indeed hybridize to the same chromosome pair, revealing the sex chromosomes of brown trout. The sex chromosomes are homomorphic and correspond to a submetacentric chromosome pair.

3.3.1 Finding the sex-determining master gene of Atlantic salmon's closest related species, brown trout

BACs that were true positives for marker OmyRT5TUF were checked in AsalBase, and they were all at one end of fingerprint contig67. Comparative genomic analysis with the medaka genome suggested that contig1967 and contig67 are in close proximity to one another, as shown in Figure 3.21.

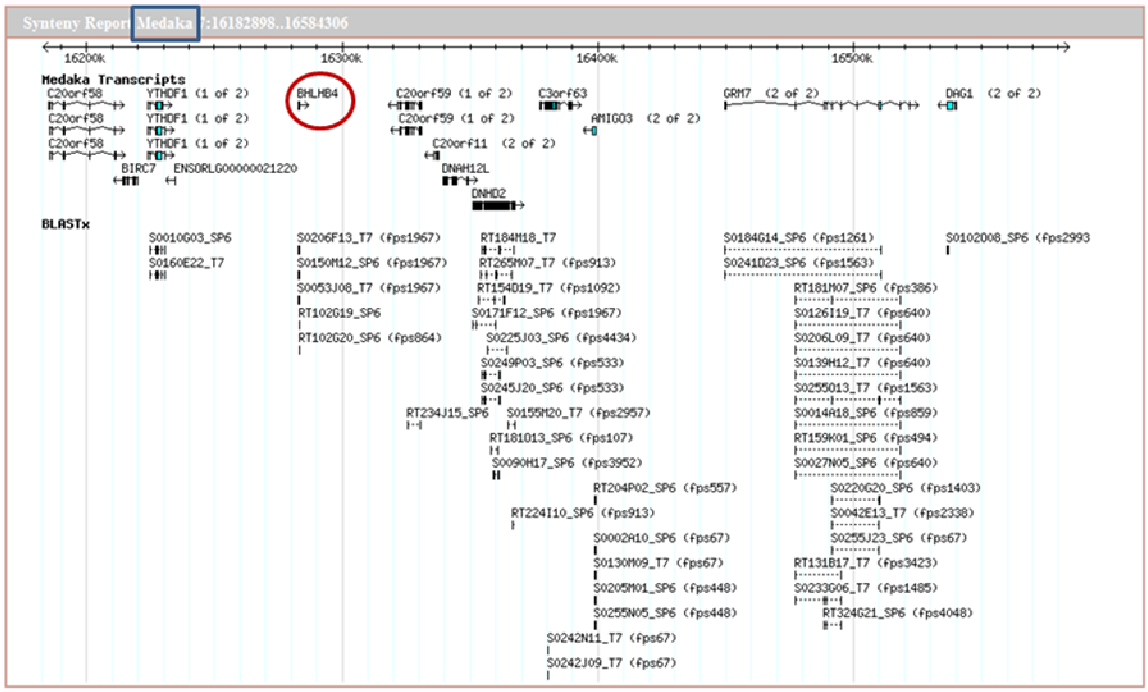


Figure 3.21 The snapshot for part of the summary page of a synteny analysis on the developmental AsalBase based on the medaka genome.

The red-circled gene is BHLH-B4 in contig1967.

BAC S0150M12 within contig1967 contains a very interesting gene, BHLH-B4. The predicted helix loop helix structure of the gene product as well as its single exon character make it a strong candidate for the brown trout sex determination gene. BAC S0007P15 within contig1967 contained a microsatellite. Primers were designed for this microsatellite and it was found to be informative in the Br5 family. However, the linkage analysis result revealed its location in LG7 rather than LG8. It was reasonable to hypothesize that the genomic region in BAC S0150M12 still corresponds to the brown trout sex chromosome. Therefore, BAC S0150M12 and BAC S0007P15 were selected for FISH analysis as described in section 3.2.2.

The probing result showed that BAC150M12 hybridized to a different chromosome from the putative sex chromosome in brown trout (R. Phillips, personal communication). PCR primers were designed for BHLH-B4 and two male and two female brown trout genomic DNA as templates were used for PCR amplification. The PCR products were sequenced and examined for sex specific differences. Unfortunately, no such difference was found. This evidence appears to eliminate BHLH-B4 as a sex-determining master gene candidate for brown trout. Thus, I conclude that the brown trout sex-determining master gene is not BHLH-B4.

Minimum tiling path were generated for contig67 using BAC end sequences to design primers. Figure 3.22 shows the minimum tiling path for contig67.

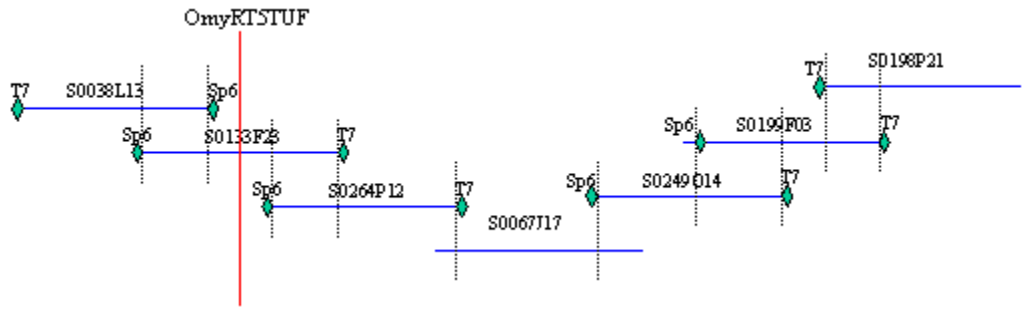


Figure 3.22 Minimum tiling path for contig67.

Probes cannot be designed at the end where OmyRT5TUF was located for chromosome walking because of its repetitive nature. It is reasonable to assume that this end is towards the centromere as centromeres contain highly repetitive sequences. As it is known that the putative sex chromosome of brown trout should be a submetacentric chromosome with the closest marker to *SEX* OmyRT5TUF on the tiny p arm on the top, the sex-determining master gene would be somewhere on top of OmyRT5TUF away from the centromere. Thus, probe and reverse primers were also designed for chromosome walking at the other end of contig67 based on BAC S0199F03. The PCR confirmation check showed that BACs from five different contigs produce a single PCR product band. Figure 3.23 shows the gel image of the PCR confirmation check for true positives of BAC S0199F03.

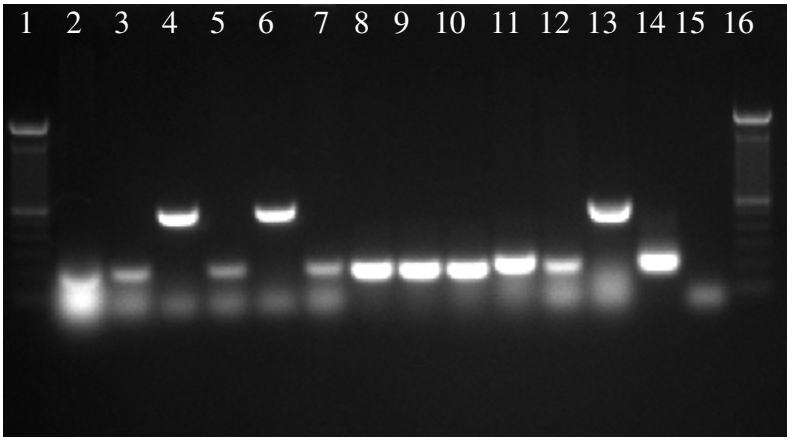


Figure 3.23 Gel image of PCR result for checking the true positive BACs of S0199F03.

Lane1: 100 bp ladder; lane2: S0054D12, lane3: S0059K09, lane4: S0062E06, lane5: S0062M14, lane6: S0068F10, lane7: S0075C12, lane8: S0192O17, lane9: S0243J18, lane10: S0250B04, lane11: S0259D06, lane12: S0261C01, lane13: S0283K02, lane14: Atlantic salmon genomic DNA, lane15: no template control, lane16: 100 bp ladder.

BACs from three different contigs yielded a clean PCR amplification product of the expected size: lane8: BAC S0192O17 from contig289, lane9: BAC S0243J18, lane10: BAC S0250B04 and lane12: BAC S0261C01 from contig2368 and lane11: BAC S0259D06 from contig580. Contig289 contains a marker from Atlantic salmon LG4 and contig580 contains a marker from LG17 according to available information on AsalBase. This result showed that BAC S0199F03 also contains repetitive elements, probably from the WGD. This might suggest the master gene for sex determination of brown trout is actually within contig67. When the Atlantic salmon genome sequence becomes available, it will be possible to apply resequencing techniques to the brown trout genome to check for other candidate genes in this region.

4: Discussion

There is good evidence to support sex determination being a strictly genetic mechanism in Atlantic salmon, with the male as the heterogametic sex (Johnstone *et al*., 1979; Hunter *et al*., 1982, 1983; Johnstone and Youngson, 1984). The sex chromosome of Atlantic salmon was found to be chromosome 2 based on its correspondence to linkage group (LG) 1, which contains male-linked alleles in pedigree analysis (Artieri *et al.,* 2006). The sex-determining region *SEX* is close to the end of the q arm of chromosome 2. A previous study tried chromosome walking along the whole of chromosome 2 (Huang, 2008), but this encountered great difficulties because of the repetitive nature of the Atlantic salmon genome (de Boer *et al.,* 2007), especially at the q end of chromosome 2 where *SEX* is thought to be very close to a heterochromatic region with many repeat elements. As both positional cloning and a candidate gene search identified DMRT1 as the sex-determining gene in medaka (Matsuda *et al*., 2002; Nanda *et al*., 2002), a candidate gene mapping became an alternative approach for a sex determination study in Atlantic salmon. Fourteen candidate genes were selected based on the fact that they are all involved in sex determination pathways among different phyla, as illustrated in Figure 1.1. By trying to find where they are located in the Atlantic salmon genome I hoped to predict if any one among them would be a strong candidate as a sex-determining gene in Atlantic salmon. Specifically, if any of them is located on the q arm of the

chromosome 2, or maps to LG1, then it will become a strong candidate for sex determination in Atlantic salmon. Using a combination of LG analysis and FISH analysis, I was able to pinpoint genomic locations for the fourteen gene candidates. Based on these locations all fourteen genes were excluded as candidates for sex determination in Atlantic salmon. However, there is every reason to believe that the fourteen gene candidates are involved somehow in sex determination in Atlantic salmon and other salmonids. Now that the BACs containing these genes have been identified, it will be possible to sequence the entire genes and their potential controlling regions. This information might provide insight into, or even reveal, common controlling elements in these genes that interact with a common transcription factor that is the sex-determining gene product. Thus, It may be possible to use the sequence information to fish out the transcription factor using a gel shift assay.

The big question remains concerning what the sex-determining gene could be and if other candidate genes could be predicted? I am now very confident that DMRT1, which is the sex-determining gene in medaka, is not the sex-determining gene in Atlantic salmon. Most genes involved in the sex determination pathway are transcription factors, and this gives a clue that it might be some transcription factors that operates upstream of DMRT1. The non-cell type specific (i.e., general) transcription factors usually gain cell specificity through interaction with transcriptional co-activators or co-repressors that are cell type specific (Hobert, 2004). Moreover, during many developmental processes the activation of certain genes is by a double negative reaction, in which the

activation is led by repression of a transcription repressor (Gray and Levine. 1996). This leads to another interesting family of gene regulatory elements, namely microRNAs, which could potentially be involved in controlling the sex-determining pathway in Atlantic salmon. MicroRNAs mostly regulate gene expression by repression, which is an important mechanism for defining cell types (Hobert *et al.,* 2008). The fact that a transcription factor can be regulated by a microRNA, and microRNAs and transcription factors are linked in the gene regulatory networks make microRNAs more interesting. This would also explain the inability to identify sex-specific markers in Atlantic salmon (McGowan and Davidson, 1988) while these have been identified in several Pacific salmon species (Devlin *et al.,* 2001). Although some microRNAs have been identified from rainbow trout (Ramachandra *et al.,* 2008; Salem *et al.,* 2010), none are known for Atlantic salmon. This may be a fruitful area of research. The sequencing of the Atlantic salmon genome through the sex-determining region would give us a list of strong gene candidates based on their genomic location and potential function. However, it is the genome of “Sally the salmon” that is being sequenced. “Sally the salmon” is a female double haploid Atlantic salmon (Davidson *et al.,* 2010), which means it is unlikely to have the sex-determining gene unless it is a dosage dependent sex-determining mechanism. Resequencing of a male double haploid Atlantic salmon, available from Unni Grimholt in Oslo, may solve this problem, but that is a few years away at this time.

It is known that even closely related species of medaka utilize a different sex-determining gene other than DMRT1. Thus, another question that must be

addressed is if the sex-determining gene in salmonids, or at least in the genus *Salmo*, is the same. There are two popular hypotheses for salmonid sex determination. One suggests that salmonids utilize the same sex-determining gene that is jumping from the end of one chromosome to the end of a different chromosome mediated either by transposons or by homeologous recombination during meiosis (Phillips *et al.,* 2001). The other suggestion is that different species among the salmonids have different sex-determining genes that evolved independently and may be related to speciation events (Davidson *et al.,* 2009). Comparative genomic analysis showed that microsatellite markers from the sex linkage groups in closely related species such as brown trout and Coho salmon mapped to different autosomal linkage groups rather than the sex linkage group in Atlantic salmon. This was also observed for Arctic charr and rainbow trout as well as chinook salmon and cutthroat trout (Woram *et al.,* 2003; Davidson *et al.,* 2009). These results suggest that species closely related to Atlantic salmon have evolved different sex linkage groups. The brown trout sex linkage group actually mapped to the bottom half of a big acrocentric chromosome in Atlantic salmon, in which the top half corresponds to another linkage group in brown trout. This suggests that many independent genome wide rearrangements have occurred during the rediplodization of the ancestral salmonid genome. However, the question if the sex determination genes in salmonids are the same or different still cannot be resolved.

In order to get more information about sex determination in the genus *Salmo*, I set out to identify the brown trout sex chromosome(s). A comparative

genomic study and FISH analysis showed that the sex chromosomes of brown trout correspond to a small acrocentric chromosome pair. Linkage analysis indicates that *SEX* is located at the very top of the acrocentric pair. Comparative genetic analysis suggested BHLHB4 as a gene candidate for sex determination in brown trout. However, the location of the BHLHB4 gene was later found to be on a chromosome pair other than the sex chromosomes, which excluded its possibility as the sex-determining gene in brown trout. Although I was not able to identify the sex-determining gene in brown trout, the sex chromosomes in brown trout were found to be an acrocentric pair and they are different from those in Atlantic salmon.

5: Conclusion

More questions remain to be answered after the investigation of sex determination in Atlantic salmon and its closely related species. Do the salmonids share the same sex-determining gene that jumps around among different chromosome ends? Did salmonids obtain different sex-determining genes independently during their evolution? Is Atlantic salmon really utilizing a single sex-determining gene like SRY in human? Is the gene a transcription factor or a microRNA? Does Atlantic salmon utilize a dosage dependent sex-determining mechanism, with two copies defining the female sex? Some of these questions can be answered by sequencing a double haploid Atlantic salmon male, and trying to find the sequence differences between the male fish and "Sally the salmon" chosen for the Atlantic salmon reference genome sequence. The genome of "Sally the salmon" is being produced using Sanger sequencing, which gives the longest reads and is the most reliable method for species like Atlantic salmon, which contain too many repeats due to the 4R WGD. However, it is expensive and time consuming. Luckily the next generation sequencing is becoming more reliable and is becoming cheaper. With the availability of a reference Atlantic salmon genome, resequencing a male double haploid salmon by next generation sequencing will be quite feasible and relatively inexpensive. This is probably the best hope for identifying not only the sex-determining factor

in Atlantic salmon but also the sex-determining gene/factor for other salmonids such as brown trout.

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