Approval

Name: Song Lin
Degree: Doctor of Philosophy
Title: Cytogenetic Evolution of Genus Salmo
Examiining Committee: Chair: Tim Audas
                                      Assistant Professor
                                      William Davidson
                                      Senior Supervisor
                                      Professor
                                      Nancy Hawkins
                                      Supervisor
                                      Associate Professor
                                      Nicholas Harden
                                      Supervisor
                                      Professor
                                      Ben Koop
                                      Internal Examiner
                                      Professor
                                      Biology
                                      University of Victoria
                                      Kerry Naish
                                      External Examiner
                                      Professor
                                      School of Aquatic and Fishery Sciences
                                      University of Washington

Date Defended/Approved: February 21, 2019
Abstract

The ancestral genome duplication that occurred around 80-100 MYA in the common ancestor of extant salmonids induced a stressed auto-tetraploid genome. This was followed by expansion of transposable elements which is believed to facilitate large-scale chromosomal rearrangements. The species radiation did not occur until around 40-50 million years later when earth’s climate was going into a cooling stage. As the result of the large-scale genome reorganization, present-day salmonids share large syntenic collinear blocks in a species-specific order. In the present study, our first aim was to visualize these rearrangements by taking advantage of genus Salmo (including two sole members, namely, Atlantic salmon (S. salar) and brown trout (S. trutta)) where species not only exhibit distinct karyotypes, but also intra-species chromosomal polymorphisms. Moreover, another aim of present study was to provide cytogenetic support for confirming the identity of sex determining gene, sdY, in Atlantic salmon by revealing its physical locations in both European and North American derived Tasmanian Atlantic salmon. In the genus of Salmo, large scale rearrangements including fusions, translocations, fissions and possible chromosome arm loss were evident in the present study, giving rise to the extant karyotypes of S. salar and S. trutta. The fact that no polymorphic karyotype of the S. trutta was observed in our study and other publications suggests a relative stable genomic background. In contrast, polymorphic karyotypes due to Robertsonian rearrangements of three pairs of chromosomes were found in Canadian subpopulations of S. salar. The fixation of the homozygous translocation in most Canadian populations indicates possible fixation of advantageous mutations and suggests a mild to near-neutral underdominance of this rearrangement in its ancestral heterozygous state. On the other hand, the observation of potentially deleterious Robertsonian tandem fusions in all Canadian populations indicate the initial rearrangement likely took place in a small effective population with subsequent spreading into other river populations through colonization. In our effort to visualize the physical locations of sdY, a single-copy gene, the gene was indeed found residing on the sub-telocentric region of Ssa02q in European Atlantic salmon, consistent with results from previous SEX mapping studies. In comparison, multiple locations including Ssa02q, Ssa03p and Ssa06p were shown to be locations of sdY in a North American derived Tasmanian population, supporting a previous study suggesting a jumping gene theory.
Keywords: sex determination; sex differentiation; salmonids; cytogenetics
Acknowledgements

To my beloved wife Xiating Huang and my lovely daughter Juliet Lin: because I owe it all to you. Many Thanks!

I am grateful to my father and mother Jiantong Lin and Jianmei Kong, who have provided me through moral and emotional support in my life. I am also grateful to my other family members and friends who have supported me along the way.

A very special gratitude and respect goes out to my senior supervisor, Dr. William S. Davidson for his patient guidance and tremendous trust and support. I sincerely wish him a forever happy retirement life.

I am also grateful to my supervisor committee members: Dr. Nancy Hawkins and Dr. Nicholas Harden. Nick not only taught me the fundamental knowledge related to molecular biology as the instructor for MBB331, but also provides valuable ideas and supports along the way. Nancy has been providing brilliant ideas and guidance whenever it is needed. I truly grateful to all their valuable assistance throughout the PhD research.

And finally, last but by no means least, also to all members of Davidson Lab: Evelyn, Alejandra, Will, Michelle, Rico … it was great sharing laboratory with all of you.

Thanks for all your encouragement!
# Table of Contents

Approval.............................................................................................................. ii
Abstract............................................................................................................ iii
Acknowledgements............................................................................................ v
Table of Contents............................................................................................... vi
List of Tables...................................................................................................... vii
List of Figures..................................................................................................... viii

Chapter 1. **Introduction**.................................................................................. 1
1.1. Whole genome duplication and how it relates to the evolution of Salmonidae ...... 1
1.2. The cytogenetic evolution in *Salmonidae*.................................................. 6
1.3. Sex determination in animals....................................................................... 10
    1.3.1. Sex determination in mammals............................................................ 11
    1.3.2. Sex determination in *Drosophila* ........................................................ 13
    1.3.3. Sex determination in *Caenorhabditis elegans* ....................................... 14
    1.3.4. Sex determination in fish ................................................................... 15
1.4. The importance of conducting molecular cytogenetic research in the genus *Salmo*
    and general procedures used in fluorescence in-situ hybridization (FISH)......... 19
1.5. Objectives of this study.............................................................................. 21
1.6. References.................................................................................................. 22

Chapter 2. **Cytogenetic variation among populations of Canadian Atlantic salmon (Salmo salar)**................................................................. 35
2.1. Abstract...................................................................................................... 36
2.2. Introduction................................................................................................. 37
2.3. Material and methods ................................................................................ 39
    2.3.1. Blood collection and lymphocyte culture.............................................. 39
    2.3.2. BAC probe preparation and FISH ......................................................... 40
    2.3.3. Genotyping with mtDNA and nuclear DNA markers.......................... 42
2.4. Results ...................................................................................................... 42
2.5. Discussion.................................................................................................. 50
2.6. Reference................................................................................................... 53

Chapter 3. **Cytogenetic relationship between members of genus Salmo** ............ 56
3.1. Abstract...................................................................................................... 57
3.2. Introduction................................................................................................. 58
3.3. Material and methods ................................................................................ 60
    3.3.1. Fish blood collection ............................................................................ 60
    3.3.2. C-banding techniques .......................................................................... 61
    3.3.3. BAC marker assignment and fluorescence in-situ hybridization ........ 61
3.4. Results....................................................................................................... 63
3.5. Discussion.................................................................................................. 72
3.6. References.................................................................................................. 75
List of Tables

Table 2-1 List of SNP markers used to genotype Newfoundland samples. Nuclear SNPs are from Pritchard et al. (2016) [21] and genetic position of mtDNA markers can be found in Atlantic salmon mtDNA genome at NCBI (accession number: NC_001960).......................................................... 45

Table 2-2 Results of genotyping with mtDNA SNPs. ......................................................... 45

Table 2-3. Historical data of chromosome counts in North American Atlantic salmon (Roberts 1968, Roberts 1970).......................................................... 45

Table 2-4. Chromosomal rearrangements among strains of Canadian Atlantic salmon. Homozygous (HO); heterozygous (HE); not observed (NO); New Brunswick (NB); Nova Scotia (NS); Quebec (QC); Newfoundland (NL). Each line represents one fish sample.............................................................. 46

Table 3-1 The nomenclature of brown trout chromosome complement with corresponding chromosomes of Atlantic salmon. Genetic mapping information from two previous reports are also included. Gharbi (Gharbi et al. 2006) [15]; Leitwein (Leitwein et al. 2017) [14], N/A (data not found); Missing (Missing counterpart)................................................................. 72
List of Figures

Figure 2-1 Seven samples locations and the number of corresponding samples...........44
Figure 2-2. Ideogram of three Robertsonian rearrangements visualized in the survey of 7 river strains of Canadian Atlantic salmon. Top: homozygous translocation between Ssa01 and Ssa23. This rearrangement is fixed in all populations except samples from Newfoundland; Middle: homozygous or heterozygous fusion between Ssa08 and Ssa29. The resulting dicentric chromosomes will potential interfere with normal meiotic segregation; Bottom: homozygous or heterozygous fusion between Ssa26 and Ssa28. Both fusions appears to independent among all samples surveyed. The red circle represents centromere..............................................47
Figure 2-3. FISH-images of Ss01p/23q translocation with arrows pointing at BAC probes. Metaphase spreads of an European Atlantic salmon (left, [Brenna-Hansen et al., 2012]) and a Canadian Atlantic salmon (right) sampled from River Stewiacke, NS with a 2N=56. Red label with red arrow: S0198E23 on Ssa01p, red label with yellow arrow: S0088O23 on Ssa01q, green label: S0102N22 on Ssa23q..............................................48
Figure 2-4. FISH-images of Ssa08q/29q fusion (left) and Ssa26q/28q fusion (right) with arrows pointing at BAC probes. Metaphase spreads of a Canadian Atlantic salmon sampled from River Stewiacke, NS with a 2N=56. No fusion is occurred in Ssa08q/29q fusion, whereas homozygous fusion of Ssa26q/28q is observed. Red label with red arrow: S0214J02 on Ssa08q (left); S0059P02 on Ssa26q (right), green label with Green arrow: S0016D16 on Ssa29q (left); S0091M08 on Ssa28q (right). ....................49
Figure 3-1. C-banding of European Atlantic salmon. Ssa: Atlantic salmon chromosome. The chromosome complement of Atlantic salmon is comprised of 8 pairs of meta/acro centric chromosomes and 21 pairs of telocentric chromosomes. LG: linkage group..................................................65
Figure 3-2. C-banding of brown trout. Str: brown trout chromosome. The chromosome complement of brown trout is comprised of 11 pairs of meta/acro centric chromosomes and 29 pairs of telocentric chromosomes.........................66
Figure 3-3-A. Ideogram of the cytogenetic relationship between Atlantic salmon and brown trout. Markers on the chromosomes are the BAC probes from CHORI-214 library. Ssa: Atlantic salmon; Str: brown trout.........................67
Figure 4-1. sdY chromosomal locations of European Atlantic salmon revealed by FISH using BAC probe, S0524M13, containing sdY sequences as part of insert. S0524M13 red fluorescence. Green arrows show S0033O17 (Ssa03 marker), S0332O19 (Ssa06 marker) and S0227A12 (Ssa02 marker) in A, B and C, respectively. .........................................................86
Figure 4-2. sdY chromosomal locations of both European and Tasmanian Atlantic salmon revealed by FISH using plasmid probe, containing sdY sequences as the only insert. Green arrow: sdY plasmid; Red arrow: S0227A12 (Ssa02 marker), S0227A12 (Ssa02 marker), S0033O17 (Ssa03 marker) and S0332O19 (Ssa06 marker) in A, B, C and D, respectively.................87
Figure 4-3 Multiple alignment of sdY in Tasmanian families and European Atlantic salmon. From top to bottom, Ss06 Tasmanian sdY, Ssa03 Tasmanian
sdY, Ssa02 Tasmanian sdY, European sdY (contig 292). The sdY genes in Atlantic salmon from different populations have similar sizes (European Atlantic salmon Ssa02, 4190 bp; Tasmanian Ssa06, 4281 bp; Tasmanian Ssa02, 4292 bp; Tasmanian Ssa03, 4294 bp). The differences in length of the Atlantic salmon sdY genes come from different numbers of a CT dinucleotide repeat in Intron I. Please see Figure S1 in Lubieniecki et al. (2015) for the detailed nucleotide comparison [24]. At the protein level the sdY sequences are identical in terms of length.

Figure 4-4 Sexually dimorphic sex chromosomes in Nauyak Lake Arctic charr (The top two are males, the lower two are females). The sdY containing BAC, 524M13 (red signals) was used to visualize the locations of sdY. Because of the similar genetic environment shared in multiple chromosomes, the BAC hybridizes to multiple locations. Since AC04 is the sex chromosomes, marked by BAC S0175J24 in green signal, the sex chromosomes (Y) is the one bearing both red and green signals. From the female images, both X and Y are morphological indistinguishable whereas it is heteromorphic in male images, indicating a fusion with an autosome.
Chapter 1.

Introduction

1.1. Whole genome duplication and how it relates to the evolution of Salmonidae

Salmonidae, collectively known as salmonids, is a family of at least 70 species of cold-water fish found naturally in both saltwater (Pacific and Atlantic Ocean) and freshwater. The earliest fossil record of Salmonidae was found at Driftwood Creek, central British Columbia and dated back to the middle Eocene, around 40-60 million years ago (MYA) [1]. The extant family of Salmonidae consists of three subfamilies including Salmoninae (trout, salmon, and char), Coregoninae (whitefish and cisco) and Thymallinae (grayling). Around 90% of salmonids such as Atlantic salmon and Pacific salmons have developed an anadromous life cycle where the spawning takes place in freshwater systems after returning from a grow-out stage spent in saltwater [2]. In comparison, there are species, such as grayling, which remain strictly as a freshwater habitant. Aside from the diverse life cycle and social, economic and ecological importance of many species of this family, Salmonidae have fascinating genomes owing to the ancestral whole genome duplication.

Following the teleost (ray-finned fish) whole genome duplication (3R WGD) [3], the common ancestor of extant salmonids experienced one additional Salmonid-specific WGD (SsWGD, 4R) at approximately 88 million years ago (MYA) [2]. The 4R SsWGD was historically believed to be the driving force responsible for the species diversification based on a couple of observations. First, two rounds of genome duplication were coincident with the origin of all vertebrates; Second, additional lineage-specific WGDs gave rise to species-rich groups of organisms such as fish with more than 25,000 species and flowering plants with more than 350,000 species. However, this causative relationship is challenged by a 50 million-year gap found between the timing of SsWGD and species diversification in a study with advanced phylogenetic analysis using related molecular clock methods on gene paralogues retained after SsWGD [2]. Intriguingly, the occurrence of anadromy, a major transition in life history that is believed to increase species radiation, did not coincide with SsWGD either [4]. Therefore, the exact role of
WGD in salmonid species diversification and trait innovation is unclear. Perhaps, knowledge obtained from other organisms with similar genomic background (tetraploids) could provide some clues regarding both the origin and evolutionary significance of ancestral genome duplication.

Tetraploids with duplicated genome can arise from either autotetraploidization or allotetraploidization with involvement of unreduced gametes (2N) due to meiotic aberrations [5]. These two processes differ in the involving parents. The former process arose from within populations of individual species. The latter is the result of interspecific hybridization. The former process can occur in two scenarios; the simple one-step process, yet relatively rare, is the union of two unreduced gametes of the same species, resulting from meiotic non-disjunction [5]. For example, one out of around 4000 progeny of an open-pollinated diploid apple species was found to be an autotetraploid [6]. The same phenomenon was also evident in other taxa of plants with low frequency [7]–[9]. The second scenario involves a process known as triploid bridge [5]. Naturally occurring triploid animal and plants are known to produce aneuploid gametes (1N and 3N) [10]–[12] and diploid gametes (2N), and the union of 3N gametes with 1N gametes or two of 2Ns can then give rise to autotetraploids [13]–[15]. Given the fact that naturally occurring triploids are common in many fish including extant salmonids [16], it is tantalizing to assume this pathway as the origin of the salmonid ancestor. Similar to the formation of autotetraploids by triploid bridge, allotetraploid progeny can be encountered in backcrossing of the F1 or F2 of interspecific hybrids due to the improper pairing and segregation caused by the divergent sets of genomes [5]. For example, one triploid progeny was found in a crossing between plants of Galeopsis pubescens and Galeopsis speciose, the subsequent backcrossing with a diploid G. pubescens yield a viable allotetraploid seed [17]. Alternatively, allotetraploids from the union of two unreduced gametes (2N) are frequently observed in F2 generation of interspecific hybrids. For example, crossing between Digitalis ambigua and D. purpurea yielded tetraploids in 90% of the F2 progeny [18]. Moreover, tetraploids were observed in 50% of the F2 hybrid progeny of Allium cepa and A. fistulosum [19]. Compared to autopolyploids, allopolyploids are relatively common and account for the origin of 60-70% of flowering plants [20]–[22]. The different rate of occurrence is possibly due to the fact that hybrids can yield unreduced gamete in an average frequency of 50 times higher than non-hybrids [5], [23].
To explain to the ancestral tetraploidization, there are four major unique genetic and karyotypic features historically identified in salmonids: (1) extant salmonids have twice the size of genome (around 2.5 x 10^9 bp) per diploid cell as most teleosts; (2) their karyotypes comprise approximately 100 chromosomes arms, which is about twice as many as most fish; (3) quadrivalent pairing between homologous and homeologous chromosomes is observed in some males during meiosis; and (4) residual tetrasomic inheritance is seen at some duplicated loci [24]. The last two features also indicate the nature of the SsWGD event being an auto-tetraploidization rather than an allo-tetraploidization (hybridization), which is observed in most polyploid plants and some teleost families such as catostomids [25] and Cyprinidae [26]. Additionally, recent results from mapping studies and whole genome sequencing of Atlantic salmon (Salmo salar) have shown more supporting evidence for the occurrence of the ancestral salmonid auto-tetraploidization [27]–[30].

So, what is the relationship between WGDs and speciation? In a broader context, what is the evolutionary significance of WGDs? The fact that all vertebrates share only two rounds of ancestral genome duplication, and most WGDs in other organisms including angiosperms, moss, fungi and ciliates took place relatively recently rather than at deeper branches [31], has been suggested that organisms with newly duplicated genomes find it harder to survive unless the event happens at some specific time points when certain conditions would grant and even favor their survival [31]. Since the WGDs repetitively gave rise to many species-rich groups such as fish (contains half the species of vertebrates) and plants, it appears the extra raw genetic materials provided by WGDs can significantly increase the potential for species radiation [32]–[35] once the survival condition is met. However, it should be noted that it is still unclear if the link between WGDs and species radiation is simply correlation or causal [31].

In the short term, genome duplication can cause disadvantageous or deleterious changes and individuals with a duplicated genome can be outcompeted by diploid progenitors [33], [34], [36]. However, genome duplication has been suggested to reduce the risk of extinction by providing mutational robustness through functional redundancy [37]; higher evolutionary and adaptational rate [38]. Strong supportive evidence comes from several families of flowering plants where independent WGDs occurred at around the same time as K-T boundary where large-scale mass extinction of animal and plant species was caused by markedly changed environment due to catastrophic events [39].
such as volcano eruption and asteroid impact. Similarly, massive extinction was found close to the periods of 2R vertebrate duplication and 3R teleost duplication [40]–[45]. Moreover, allopolyploid plants are found to tolerate a wider range of environmental factors in comparison to their diploid related species, indicating heterotic effects, also known as hybrid vigor [46], [47]. In vertebrates, unlike its diploid relative *Xenopus tropicalis*, *Xenopus laevis* with a relatively recent allotetraploidization (18MYA), is extremely invasive and can cope with some extreme conditions such as high salt, severe drought, cold and starvation [48], [49]. Therefore, WGDs do seem to enable survival of polyploids through escaping extinction or explore new environments that were not tolerable to diploid progenitors.

In the long run, as first proposed by Ohno [50], morphological innovations such as enhanced nervous, endocrine and circulatory systems can arise after genome duplication through various mechanisms including reciprocal gene loss (RGL) [51], sub-functionalization (differential expression between duplicates in different tissues or developmental stages) [52], neo-functionalization (one of the duplicates gaining a novel function) [53]. In cyprinids fish including carps and gold fish, the 4R allotetraploidization that occurred 8 MYA is the most recent genome duplication among vertebrates. Similar to allotetraploid plants, immediate expression changes of paralogs were observed. RGL is relatively slow (7.8% of paralogs) in comparison to fast expression changes (25% of paralogs), suggesting neo-functionalization and sub-functionalization are more common following allotetraploidization [54]. In the cases where the above mechanisms occur in duplicated loci harboring essential genes, reproductive isolation can be induced and lead towards speciation [55]. Direct evidence was provided in artificial crossings of different accessions (samples collected from the same wild population in different time points) of *Arabidopsis thaliana*, RGL on a duplicated essential gene lowered the fitness of F1 and F2, causing reproductive isolation in further generations [56]. In other cases where these mechanisms occur in duplicated loci harboring regulator genes, an increase in organism complexity can be expected [57]. In plants, WGDs induce the expansion of some important developmental regulators, such as the auxin response regulators and certain MADS-box transcription factor subfamilies [58]–[60]. Similarly, the number of homeobox (Hox) clusters and insulin receptors in vertebrates were expanded mainly through 1R and 2R genome duplication [61], [62]. However, species radiation did not occur right after ancestral genome duplications. A gap of 150 million years is seen between teleost
3R duplication and species radiation [31]; a smaller gap of 40 million years was also found in the SsWGD [2]. Therefore, genome duplications merely provide raw genetic materials for increasing complexity and speciation rate but cannot direct or promote the results [31].

Looking back in the case of salmonids, the species radiation finally took place 40 million years after the duplication during the Eocene–Oligocene transition when the global temperature was dropping significantly. It is arguable that this change in the environment was involved in triggering the species radiation. Moreover, trait innovation such as anadromy could potentially encourage the speciation by providing sufficient food in the ocean and reproductive isolation in the natal rivers. Indeed, higher speciation rates are seen in lineages that developed an anadromous life cycle [2]. However, without increased gene regulators from WGD and potential functional divergence of duplicated paralogues, it would seem to be difficult to develop such a complex life cycle [31]. In rainbow trout, around half of duplicated genes from SsWGD are retained and show signs of pseudogenization rather than complete loss of one copy [63]. A similar number and fate of duplicate retention was also reported in Atlantic salmon [30]. Among the duplicated genes in rainbow trout, a group of genes, miRNA display nearly completed retention, suggesting that an expression controlling mechanism is needed post WGD [63]. Moreover, neo-functionalization that was more common than sub-functionalization for duplicates in Atlantic salmon was not evident in rainbow trout [30], [63]. Since large scale genome rearrangements that was observed post SsWGD in Atlantic salmon was not found in rainbow trout, is it possible the difference on chromosomal rearrangements would affect the fates of duplicated genes? The evolution of duplicate genes relies on the sequence divergency. By breakage and rejoining unrelated double strand DNA, chromosomal rearrangements including deletions, duplications, inversions and translocation could potentially increase the sequence divergency through mechanisms such as suppressed recombination between paralogs [64]. However, the answer is unclear from the literature. In allotetraploid carp, although neo-functionalization is more common than sub-functionalization, no large-scale genome reorganizations were observed. It is worth noting that mutations do occur in a low frequency, therefore, neo or sub-functionalization can still evolve on duplicates without the presence of chromosomal rearrangements.
1.2. The cytogenetic evolution in *Salmonidae*

Polypliodization such as WGD provides raw genetic material, the fate of which can be followed by significant alternations at the cellular and phenotypic level [65] resulting from genetic variation caused by mutation, drift and selection. Ultimately, new adaption and species diversification can be seen during the subsequent evolutionary processes [31], but how does genetic variation accumulate in salmonids? Moreover, newly duplicated genomes will have detrimental effects in meiosis due to improper segregation caused by multivalent pairing. Therefore, it is important for tetraploid genome to block such multivalent pairing and ultimately re-achieve disomic inheritance. This process of returning from an unstable tetraploid genome towards a more stable diploid form is known as re-diploidization [50].

Chromosomal rearrangements that could re-shape the morphology of duplicated chromosomes (also known as homeologous chromosomes) and subsequently prevent quadrivalent pairing between homologs and homeologs were evident post SsWGD in Atlantic salmon, allowing the accumulation of genetic variation without the recombination [24]. Moreover, unlike imbalanced rearrangements which can cause detrimental results such as changes in gene disruption, generation of fused genes and interference of gene regulation [66], special balanced rearrangements called Robertsonian rearrangements that involves the breakage and rejoining at heterochromatic regions such as centromere and telomere are frequently reported in salmonids [24]. These chromosomal movements involving whole chromosome arms (fusion, translocation and fission) are named after William Robertsonian, who first discovered such rearrangements in 1961. Please see Figure 2-2-1 in chapter two for examples of such movements. Since the residual tetrasomic inheritance that is caused by pairing and cross-over between homologous and homeologous chromosomes is still seen across salmonids, the current state of the salmonid genome is therefore considered pseudo-tetraploids and the re-diploidization is still an on-going process [67].

In a comparative mapping study using several species of salmonids, Kodama et al. [68] shown the rate of re-diploidization is “prevented or retard” by the formation of metacentric chromosomes through Robertsonian rearrangements. This argument is based on the observation that homeologous pairings will always involve at least one metacentric chromosome [68]. However, this theory does not necessarily apply to other
salmonid species such as Atlantic salmon where the quadrivalent pairing can be involved with only acrocentric chromosomes [30].

The principle of Robertsonian rearrangements requires centromeric breakage of double-strand DNA, and one might wonder how often such event can happen? The answers might come from one of the intriguing findings from the Atlantic salmon genome assembly where up to 60% of entire genome consists of transposable elements (TEs) [30]. TEs are DNA sequences that can change their genomic locations and often result in duplications of the genetic material and therefore even capable of altering the genome sizes [69]. The first discovery of TEs was Ac/Ds elements in maize by Barbara McClintock in 1950 [70]. TEs can make up to a large fraction of eukaryotic genomes (90% of maize genome [71] and 44% of human genome [72]) and can generally be divided into two groups depending on the mechanism of transposition [73]. The Class I TEs are known as retrotransposons which mimic the retrovirus pathway of moving with RNA intermediates in a manner of “copy and paste”. The Class I TEs can be further subcategorized into three groups, retrotransposons with long terminal repeats; long interspersed nuclear elements (LINEs) that lack of long terminal repeats, but encode reverse transcriptase; short interspersed nuclear elements (SINEs) that lack of both long terminal repeats and genes coding for reverse transcriptase. Since the RNA intermediates require the reverse transcriptase to synthesis the double-stranded DNA for insertion, LINEs and retrotransposons are considered autonomous, while the transpositions of SINEs are non-autonomous and therefore require the reverse transcriptase from either of the other two types of elements. The most “famous” of examples for Class I TEs is the Alu elements in human where it makes up 10.7% of the genome [74]. Alu elements belong to the subclass of SINEs and capture LINE-1 protein’s reverse transcriptase for transposition. The Class II element is called DNA transposons which move by “cut and paste” manner without RNA intermediates. The transposition of DNA transposons relies on the transposase which can target either specific or non-specific insertion sites. Similar to Class I TEs, DNA transposons can be either autonomous or non-autonomous depending on whether or not they contain genes coding for transposase. Despite the “cut and paste” manner, DAN transposons can still be duplicated if the transposition takes place during S phase of the cell cycle when the jumping and landing occurs after and ahead of the replication fork, respectively. The well-known examples of DNA transposons include Ac/Ds in maize and P elements in
Drosophila [75]; color variegation on corn kernels is caused by Ac/Ds transposition where Ac is autonomous element and Ds is non-autonomous and requires the help of Ac to jump [70]; P elements invaded the genome of the laboratory strain of Drosophila around 60 years ago and its autonomous transposition is responsible for the hybrid dysgenesis [76]. Both Class I and Class II TEs create a staggered cut in the target site and therefore result in direct repeats on both sides of flanking region once the transposition is completed. This signature together with other iconic features such as inverted terminal repeats have been used to identify and evaluate the timing of possible transposition events [77].

Active TEs reduce genome stability and are normally considered detrimental. TEs are known to be mutagenic, frequently inserting to protein-coding genes, and also inducing genome rearrangements by chromosome breakage, illegitimate recombination [77]. TEs can also affect splicing and polyadenylation patterns of neighboring genes, or by acting as their enhancers or promoters. In fact, TE-related rearrangements have contributed to 25 human genetic disease [78] and over-expression of retrotransposons are seen in many cancers [79]–[81]. Therefore, active TEs are normally concentrated in heterochromatin regions of chromosomes and are often under strong suppression mechanism such as chromatin remodeling and RNAi silencing [77]. However, certain genetic and environmental stress including UV exposure, temperature shifting and polyploidization are seen to cause epigenetic release of TEs silencing [82]. For example, the transposition rate of some TE containing heat-shock like promoters will increase when the cells are subjected to stress [83].

Without surprise, both categories of TEs are found in salmonids with evidence suggesting horizontal transfer of DNA transposons through a parasite Schistosoma japonicum [84]–[86]. One thing worthy noting is that this particular parasite does not currently use fish as host, but rather snails and humans. However, its related Diplostomum species do use fish as host, suggesting host switching that is known to occur in schistosome evolution [84]. As the salmonid species with the best whole genome assembly, Atlantic salmon is shown to harbor the highest percentage of TEs in vertebrates. This is believed to be the result of TE expansion post SsWGD as a response to genome stress caused by WGD, suggesting ancestral regulatory failure in suppressing the spread of TEs [30]. Moreover, the occurrence of TE expansion at the initiation of rediploidization, is likely prior to the ancestral fusions, strongly suggesting a
role of TEs in the following chromosomal movements [30]. With the largest class of TEs (Tc1-mariner DNA transposon, around 13% of the entire genome) in Atlantic salmon residing in centromeric regions, it is tempting to suggest the commonly seen Robertsonian rearrangements are the result of double-strain breakage cause by DNA transposons. Intriguingly, active transposition can still be expected in Atlantic salmon since an open reading frame in two salmonid specific DNA transposons were reported. Therefore, further salmonid genomic re-organization could still be triggered by active TEs once the suppression mechanism is no longer effective.

From surveying other teleosts that did not experience additional 4R duplication, the ancestral karyotype of salmonids prior to SsWGD consists of 48 acrocentric chromosomes with NF (number of chromosome arms, also known as fundamental number) of 48, and subsequent SsWGD gave rise to karyotype of 96 acrocentric chromosomes with NF of 96 [87]. Through the following evolution, extant salmonid chromosome complements can be classified into 4 major categories [88]-[89]. Type A’ and A” complements both have 2n = 80 with NF of 110-120 and ≥140, respectively; Type B and B’ complements both have 2n = 60 with NF = 104 and NF < 80, respectively. There are two modes of chromosome evolution found in the extant salmonids. In Coregoninae and the Salmoninae, chromosomes have evolved mainly by Robertsonian fusions and translocations, whereas inversions are predominant in Thymallinae. As a result, a decreased chromosome number, with a consistent number of chromosome arms (Type B and B’), are seen in the former two sub-families, while an increased number of chromosome arms and similar number of chromosomes (Type A and A’) as the tetraploid ancestor are evident in Thymallinae. Compared to other families of tetraploid teleosts, salmonids appear to have various rates of chromosomal divergence, possibly due to the two identical copies of genome through autotetraploidization [24]. In addition, the extant salmonids have chromosome numbers ranging from around 52 to 100, whereas the majority of species in the allotetraploid Cyprinidae have 48-52 chromosomes [90] and the allotetraploid Catostomidae apparently have 96-100 chromosomes [91].

The observation that most species of salmonids with an anadromous life history have the type B and B’ complement has made some scientists wonder if there is a relationship between the mode of chromosomal evolution and natural selection. A theory proposed by Qumsiyeh (1994) suggested that there is a link between life history strategy
and chromosomal evolution. The theory states that a species with a higher diploid number and/or number of chromosome arms is expected to have increased genetic recombination and genetic variation, producing novel traits to battle for survival in a less stable environment (fluctuation on temperature, pH and food availability) such as freshwater lakes and lagoons. On the other hand, species with lesser number of chromosomes and/or chromosome arms caused by Robertsonian fusions and translocations have less recombination and higher retention of specialized trait to allows adaptation to a specialized and more constant environment. For example, fishes with an anadromous life history spend a great deal of their life in a stable ocean environment (due to the large body size of oceans comparing to freshwater habitats, oceans have relatively stable temperature, pH and mineral composition) before returning to a specific freshwater breeding ground and can therefore afford a decreased genetic recombination and favor retention of highly specialized trait such as smoltification and precise homing ability [92]. This theory of Qumsiyeh is to be tested when more evidence is collected from functional studies.

In conclusion, the cytogenetic evolution of extant salmonids uses the process of re-diploidization where divergence between duplicated regions are promoted by Robertsonian rearrangements with the help of TEs expansion. Together with natural selection working on the genetic variation, many specialized traits can then arise.

1.3. Sex determination in animals

Sex determination is a vital and complex process to ensure proper gametogenesis where oocytes and sperms are produced from ovary and testis, respectively, of the same individual or separate individuals with the opposite sex. The field of sex determination has been a fascinating area of research in countless organisms including mammals, insects, nematodes, plants, reptile, fish, etc. The emerging knowledge from such research has shown low degree of conservation among groups of animals due to the fact that sex determining mechanisms and involving factors have independently evolved multiple times in different groups. Despite being “endless” fields of research, information obtained from model species such as mice, Drosophila and Caenorhabditis elegans have provided significant understanding of how such a complex process is carried out in each case. Therefore, let us review some key
knowledge learned from such groups before diving into the field of fish sex determination.

1.3.1. Sex determination in mammals

Mammalian sex determination is best studied in therian mammals where both sex determining mechanisms and sex determining genes are confirmed [93]. Male therian mammals normally contain one pair of sexually dimorphic sex chromosomes with XY cytotype while females have XX. In comparison, monotreme mammals such as platypus have 5 pairs of sex chromosomes without confirmed sex determining genes [94]. There are two main milestones in the discovery of mammalian sex determination; 1. Human patient studies in 1959 with Turner syndrome where missing Y chromosomes results in females (XO females) and Klinefelter syndrome where additional X chromosomes does not result in females (XXY males) led to the suggestion that the sex determining gene is located on the Y chromosome [95], [96]; 2. The sex determining gene, Sry (also known as testis-determining factor, TDF), was discovered three decades later from four human XX male patients with translocated Y DNA [97]. The role of the master sex determination of Sry was further confirmed by successful sex reversal on XX mice through inserting human genomic fragments containing Sry [98].

Sry has been a difficult gene to study its evolution and regulation due to the highly degraded nature of Y chromosome and lack of conservation in any group except therian mammals [93]. In fact, incomplete knowledge regarding its upstream regulatory sequences are believed responsible for 30-40% of XY human patients suffering from a sex development disorder [93]. Sry belongs to the protein family of Sox that is found throughout the animal kingdom with function related to embryogenesis, typically acting as cell differentiation switches [99]. Sry may have arisen from duplication of Sox3 on the X chromosome and evolved a neo-function as a sex determining gene [100]. Since there is a dramatic difference on the gene content between X and Y chromosomes, random X inactivation is used as dosage compensation to prevent over expression in females [101]. Despite being the master sex determining gene in most mammals, the only conserved region of Sry is the high mobility group (HMG) domain (please refer to Figure 3 in Kashimada and Koopman (2010)) [93]. Both functions of binding and bending of such domain are important for upregulating Sox9 (please see details below) [102]. The rest of regions on Sry contains species-specific sequence and important for gene
function. Transgenic mice lacking such a region fail to induce male development [103],
and transgenic XX mice with human and goat Sry are able to develop to males [104], [105].

Detailed knowledge related to Sry expression, regulation and how it directs the molecular pathway to successfully trigger the formation of testis are mainly provided in studies on mice [93]. Please see Figure 1 in Kashimada and Koopman (2010) for a detailed overview of sex determination in mice. The expression of Sry is first observed in somatic gonadal cells between 10-10.5 days post coitum (dpc) [106]. Using its HMG DNA-binding domain, Sry together with steroidogenic factor 1 (SF1) binds to (A/T) ACAA (T/A) in the minor groove of promotor sequence of Sox9 gene, resulting in a 60-85° bend and causes upregulation of Sox9 by which proper Sertoli cells are differentiated [102], and they in turn stimulate the development of Leydig cells, spermatagonium, testis vascular cells and other interstitial cell types. In comparison, female-specific genes including Wnt4 and Foxl2 start to express at 11.5-12.5 dpc in female mice and upregulate other downstream players to ensure proper development of granulosa cells, theca cells and primary oocytes [93].

Once triggered by Sry, Sox9 is then transcribed under a positive feedback loop where Sox9 protein together with SF1 bind back to an enhancer region known as TESCO (testis-specific enhancer of Sox9 core) of the upstream sequence of Sox9 to ensure a continued expression ((please refer to Figure 5 in Kashimada and Koopman (2010)) [107]. This feedback loop is important not only to capture the signals for developing Sertoli cells, but also explain the reason why expression of Sry only occurs early and is not maintained in mouse sex determination [108]. Such transient expression of Sry is not observed in any other therian mammals with currently unknown reasons. In comparison to the lack of conservation of Sry among animal species, Sox9 is shared and involved in the gonadal differentiation pathway of many other groups of animals such as chicken, red-eared slider turtles and alligators [93]. In fact, over-expression of Sox9 successful induce male development in XX mice, suggesting Sox9 itself is sufficient for male development and Sry merely acts as a trigger [109].
1.3.2. Sex determination in Drosophila

The sex determination of Drosophila was first discovered by Calvin Bridges in 1916. Based on the observation that flies with XXY genotype developed as females and XO flies were sterile males, he concluded that the ratio between the number of X chromosome versus the number of autosomes (X:A) determines the phenotypic sex and the chromosome Y is only required for male fertility, fundamentally different from mammalian sex determination [110]. Sixty years later, sex-lethal gene (Sxl) was identified as the interpreter of the X:A ratio [111]. The sex-specific expression of Sxl is activated by proteins of four X-linked genes, also known as X-linked signal elements (XSE) including scute, sisA, runt and unpaired, working on the SxlPe promotor of Sxl of somatic cells [112]–[114]. Since the window of opportunity for SxlPe activation is limited and ends abruptly at cellular blastoderm, female flies carrying two X chromosomes are able to produce enough amount of XSE protein to trigger the activation of SxlPe. In contrast, XSE proteins won’t reach the threshold amount in time in males with one X chromosome and consequently no SXL will be produced [115]. Close to the end of cellular blastoderm, a second and later promotor of Sxl, SxlPm is activated in both females and males [116]. The activation of SxlPm ensures the maintenance of Sxl in females whereas the male specific pre-mRNA of SXL from SxlPm will include a stop codon in exon 3. In female flies, such inclusion of exon 3 is prevented by alternative splicing caused by SXL transcribed from SxlPe (reviewed in Salz and Erickson (2010) [115]). Please see Figure 3 in Salz and Erickson (2010) for detailed modes of SxlPe and SxlPm activation.

The functional Sxl in female flies can then facilitate the alternative splicing of downstream factors such as transformer gene (tra) and male-specific-lethal-2 gene (msl-2) [117], [118]. The female specific isoform of TRA in turn cause female specific splicing of doublesex gene (dsx), responsible for cellular development of somatic ovaries [117]. Msl-2 is the key protein for the dosage compensation mechanism used in male flies to double the expression of X chromosomes [118]. In female flies, msl-2 is repressed through intron retention caused by functional SXL. The exact number of downstream factors that SXL might target are still unknown. In Drosophila, the communication between somatic cells and germ cells to shape sexual fate of germ cells is not well understood. The Jak/Stat pathway is believed to be in charge of spermatogenesis. No signal is identified from female somatic cells [119]. Moreover, the autonomous fate
determination by Sxl and other genes such as Ovo and Otu is reported in XX germ cell [120].

1.3.3. Sex determination in Caenorhabditis elegans

C. elegans share a similar sex determination system used in Drosophila, which is based on the ratio between X chromosome and autosomes [121]. An X:A ratio of 0.67 results in male development, while a ratio higher than 0.75 leads to females [122]. Most intriguingly, the Y chromosome is missing in C. elegans where animals are generally females that is protoandric hermaphrodite with XX genotype, and occasionally males that carry just one X chromosomes (XO) would arise from gametes missing an X chromosome due to non-disjunction during meiosis [123]. To cope with the expression difference because of the double copy of X in females, dosage compensation is used by females to reduce the expression of X chromosomes in half [124]. Since females are capable of producing and store functional sperms in the early developmental stage (up to L4 stage), self-fertilization is achieved later when sex reversal produces mature oocytes.

In male C. elegans, xol-1 is the master sex determining gene and its activation is triggered by the low dosage of X chromosomes [124]. In contrast, X linked determinants such as sex-1 and fox-1 would facilitate the inactivation of xol-1 in females [125], [126]. Further downstream, SDC proteins (sdc-1, sdc-2 and sdc-3) expression is activated in the absence of xol-1 in females and results in the repression of her-1 that is otherwise activated by xol-1 in males [127]–[130]. Besides the sex determining role, SDC proteins are also responsible for the dosage compensation in females [131]. The sex reversal in XX females relies on the post transcriptional battle between downstream factor tra-2 and fem-3 (reviewed in [132]). The detailed diagram showing the pathway to either gametogenesis of XX females is shown in Figure 3.1 in Zanetti and Puoti (2013). In brief, GLD-1 and FOG-2 that are required for spermatogenesis is repressed by TRA-2 in the absence of HER-1; FBF and MOG proteins repress FEM-3 to allow TRA-2 to activate TRA-1 that drives oogenesis and prevent spermatogenesis by inhibiting fog-1 and fog-3.
1.3.4. Sex determination in fish

Research that has been done in human and model species such as *Drosophila* reveals a generally consistent genetic sex determination system (GSD) with sexually dimorphic chromosomes and a highly degenerated Y. In human, there are around 1100 genes on the X chromosomes, whereas the Y chromosome harbors only a few dozen genes with half of the chromosome composed of heterochromatin [134], [135]. Similarly, the *D. melanogaster* Y chromosome contains only 15 genes with the entire chromosome being heterochromatic and consisting of highly repetitive and transposable element (TE)-derived DNA [136]. Such highly evolved sex determination system and sex chromosomes with clearly suppressed recombination are in fact the result of 50-300 million years of evolution [137]. With highly degenerated sex chromosomes of these groups, important evolutionary questions are more difficult to answer. For example, were the repetitive sequences of the Y chromosomes in human and *Drosophila* added after the degeneracy or actually participating the process? Perhaps, information gathered from other organisms with varies type of sex determination system and less evolved chromosomes such as fish could shed light on this important area of evolution.

Based on gonadal differentiation, fish sex determination can generally be divided into two categories including gonochoristic and hermaphrodites. Gonochoristic species includes many teleosts when individuals that can only develop into either functional males or females without later reversal to the other sex throughout their life. Hermaphrodites such as many reef fish are the species where individuals are capable of producing both functional gametes during their lifespan [16], [138], [139]. Depending on the direction of sex reversal, there are three groups of hermaphrodites called protoandrous (male to female), protogynous (female to male) and simultaneous hermaphrodites (producing both functional gametes at the same time). The development of functional gonads involves interaction between multiple cell types such as somatic cells and germ cells through endocrine control involving pituitary-derived gonadotropins and sex steroids. [16], [140], [141]. However, due to the complexity of sex determination and later gonadal sex differentiation, as summarized by Devlin and Nagahama [16], it is still unclear if somatic cell or the primordial germ cells are the initial place for triggering the entire process of sex differentiation.
In comparison to mammals where gonadal differentiation is directed by genetic sex determining system, gonadal differentiation in fish can be influenced by both intrinsic factors such as sex determining gene(s) and extrinsic factors such as temperature, pH and social interaction with conspecifics [16], [138], [139]. Therefore, many fish are known to employ various sex determining mechanisms including environmental sex determination (ESD), genetic sex determination (GSD) and social structure dependent sex determination. In some interesting cases such as the tongue sole (Cynoglossus semilaevis) where the phenotypic sex is normally determined by GSD, genetic females can also become phenotypic males when the water temperature is above certain threshold, indicating a gradient of GSD and ESD can exist in the same species [142].

Polygenic and monogenic GSD are both evident in fish. Polygenic GSD relies on cumulative influence of sex factors scattering around the genome and is indicated by varies progeny sex ratio with no apparent extrinsic factors. Moreover, polygenic is considered to be ancestral to monogenic GSD where sex chromosomes finally arise from one single gene gaining enough influence to direct the gonadal differentiation [16]. However, in some populations of guppy and tilapia, monogenic GSD is affected by an autosomal modifier, emphasizing the fluid state of GSD in fish [143]–[145]. Through hormonal manipulation and subsequent progeny testing to observe the sex ratio, the monogenic GSD mechanism used by gonochoristic fish can either be female heterogametic (ZW/ZZ) or male heterogametic (XX/XY). Intriguingly, male heterogametic was hypothesized to be evolved because of positive correlation between fitness and male size and vice versa for female heterogametic [146]. Moreover, both GSD systems can be deployed by different species of fish in the same genus such as tilapia [147]. In the case of salmonids, male heterogametic was adopted in all species examined [148]. Even though the salmonids “traditionally” follow the principle of GSD, rare cases such as temperature influenced sex reversal was reported in farmed Atlantic salmon of Tasmania where the fish were introduced around 60 years ago, emphasizing the potential plasticity of sex determination in this fish family [149]. In fact, the labile sex differentiation has been seen in many other fish and is often used in monosex aquaculture for sex manipulation through administration of exogenous sex steroids, mainly 17α-methyltestosterone for all-male and 17β-estradiol for all-female production [16]. In order to achieve a stable sex reversal, exogenous sex steroids have to be applied in a critical period during the early stage of sex differentiation, suggesting intrinsic sex determining
factors only work in the early stages of sex differentiation and can therefore be overridden by exogenous steroids.

Unlike the highly degenerated and dimorphic sex chromosomes in mammals and *Drosophila*, around 90% of examined fish with GSD system exhibit morphological indistinguishable sex chromosomes. Therefore, the constraints affecting mammalian viability when losing X chromosomes carrying many structure genes or gaining one additional X chromosomes would not necessarily be applicable in most of fish species [50]. In triploid rainbow trout (*Oncorhynchus mykiss*) with an XXY constitution, fish will develop as functional males, suggesting an operative Y sex determination is not upset by the polyploidy [150]. In fact, a dosage compensation mechanism was shown in an allo-triploid hybrid *Cyprinidae* species to cope with an increase in ploidy [151]. Despite salmonids generally exhibiting morphological indistinguishable sex chromosomes, one exception was found in sockeye salmon where the existing Y chromosomes is the result of Robertsonian fusion between the ancestral Y with an ancestral autosome, therefore, an X1X2Y system (females 2N = 58; males 2N = 57) with sexually dimorphic chromosomes [152]. Such a neo-Y sex determination system could encourage the cumulation of novel traits on the ancestral X chromosomes due to lack of recombination in males and potentially induce speciation, as seen in stickleback fish [153].

Moreover, cytogenetically indistinguishable sex chromosomes also suggest the rise of sex determining genes and sex chromosomes is a recent evolutionary event and might not be well conserved across different families of fish [16]. Unlike the well conserved sex determining gene, *Sry*, in mammals [154], fish exhibit a range of sex determining genes even within the same genus such as *dmrt1Y* in *Oryzias latipes* and *gsdfY* in *Oryzias luzonensis* [155]. Therefore, it has been suggested that the existing sex determining factors on the Y or W chromosome may be replaced by sex factors on the autosomes, and the new sex-determining genes can regularly be generated as soon as the existing sex chromosome carries a lethal mutation [16]. As poikilothermic animals, this rapid replacement of sex determining genes should actually be expected in fish where embryonic development is exposed to the external physical environment where large temperature changes can influence the production of sex steroids, interrupting the regular direction of sex differentiation [16].
Despite the tremendous attention researchers have put into revealing the identity of sex determining genes, only a hand full of genes have been discovered in vertebrates, suggesting the limited options in becoming sex determining gene. There are two possible mechanisms giving birth to sex determining genes in fish, which includes simple allelic diversification and complex process of gene duplication followed by neo- or sub-functionalization [156], [157]. For example, allelic diversification gave rise to sole specific dmrt1 gene [156]; the amhY in Patagonian pejerrey (Odontesthes hatcheri) arose from the amh gene duplication [158]. Despite the observation of species specific sex determining genes, nearly all of the genes are conserved as part of the sex determination pathway, often related to production of sex steroids [157]. The relatively recent discovery of sdY in rainbow trout was shown to be an exception [159]. Unlike others that were known to play roles in the sex determination pathway, sdY is the truncated version of an immune related gene, interferon regulatory factor 9 (irf9) which lacks the N terminus DNA binding domain. Please see Figure 1 in Yano et al. (2012) for a sequence comparison between sdY and irf9 of rainbow trout. With both paralogs of irf9 genes found elsewhere in the genome, sdY is suggested to be the result of a transposition mechanism, which was to be expected in a species that is pseudo-tetraploids and still going through the re-diploidization process. In terms of its function, it was recently found that sdY gains its master sex determining role by forming complexes with foxl2 to prevent the production of estrogen through interfering the activation of aromatase, emphasizing the initial sex differentiation took place in somatic cells over primordial germ cells in the case of rainbow trout [160]. In effort to investigate if sex determining gene is conserved in salmonid, two scenarios were proposed from numerous mapping studies. A comparative mapping study indicated the sex chromosome of brown trout (Salmo trutta) together with another acrocentric chromosome correspond to an autosome in Atlantic salmon, suggesting the sex determining gene might differ or able to jump around the genome in these two closely related salmonids [161], [162]. Moreover, intraspecific sex linkage group polymorphism was reported in Atlantic salmon [163] and Arctic charr (Salvelinus alpinus) [164], also suggesting a jumping sex determining gene or different sex determining genes used even within the same species. If sdY is indeed conserved in salmonids as the master sex determining gene, possible jumping mechanism such as transposition and chromosomal rearrangement ought to be investigated.
In conclusion, sex determining genes in salmonid is still in the initial stage of evolution, indicative by the lack of sexually dimorphic chromosomes. With the effects from SsWGD and the broad range of sex determining factors seen in fish species, a large pool of genes involved in or beyond the sex differentiation pathways ought to be expected in salmonids species to be the ultimate sex determination factor if indeed no environmental or social influences are observed. More importantly, research focusing on salmonid species with sexually dimorphic chromosomes would possibly reveal not only the evolutionary process of sex chromosomes after the ancestral auto-tetraploidization, but also the speciation in general.

1.4. The importance of conducting molecular cytogenetic research in the genus Salmo and general procedures used in fluorescence in-situ hybridization (FISH)

The genus Salmo is comprised of two sole members, namely brown trout and Atlantic salmon. The former species carrying a Type A cytogenetic complement and a mainly freshwater life cycle, the latter one on the other hand exhibits a Type B cytogenetic complement with intra-species variation together with an anadromous life cycle. Therefore, Salmo is unique in salmonids and has advantageous for scientists to understand the apparent parallel direction of the cytogenetic evolution following an ancestral WGD. With more available molecular information such as the whole genome assembly of Atlantic salmon and higher resolution methods such as chromosome painting (in-situ hybridization), molecular cytogenetic studies can be used to provide more definitive answers in comparative genome studies with the objective of better understanding the process of re-diploidization. Before getting into the detailed research in the following chapters, some confusions regarding the cytogenetic terminology shall be made aware of here. By “traditional” cytogenetic terminology, chromosome with equal sizes of two arms are named metacentric chromosomes; chromosomes with unequal sizes of two arms are named submetacentric chromosomes with the smaller arm called p arm and larger arm called q arm; chromosomes with two arms where one of them is really small are named acrocentric chromosomes; chromosomes with one single arm are named telocentric chromosomes. This terminology regarding morphology of chromosomes is often modified in the cytogenetic study of salmonids due to the difficulty to distinguish small telocentric chromosomes and acrocentric chromosomes. Therefore,
often “acrocentric chromosome” is the term for both telocentric and acrocentric chromosomes.

Unlike traditional cytogenetic studies in mammals, banding pattern with higher resolution such as the G-banding technique do not perform well in salmonids due to the lack of distributions of G/C or A/T rich regions. Moreover, banding technique does not provide enough resolution for visualization of specific DNA sequence in the genome. Consequently, fluorescence in-situ hybridization (FISH) with higher resolution is adapted in studies of salmonid molecular cytogenetics.

FISH is molecular cytogenetic technique that was invented in 1982 by researchers to visualize gene location in Drosophila [165]. It uses fluorescent probes with a high degree of sequence complementarity that bind to only targeted parts of the chromosome. Therefore, FISH is often used for detecting and localizing DNA for use in genetic counseling, medicine, and species identification [166]. Similar to DNA probes, FISH with RNA probes was later developed to visualize gene expression. The variety of FISH methods mainly differ in ways of labelling method and probe size. Proper size of probes is important to incorporate enough signal while not too bulky to prevent hybridization. The general FISH procedure involves the labelling of probes, the hybridization with target, washing unspecific binding and visualization under fluorescence microscopes. Depending on the labelling methods, indirect labelling with modified nucleotides coupled with digoxienin or biotin requires further hybridization with antibodies conjugated to fluorochromes; direct labelling simply uses modified nucleotides that is already coupled with fluorochromes. Through serious signal amplification through hybridization with antibodies, indirect labelling is generally used when signal was too weak to be detected by direct labelling, however, it is apparently more time consuming and labor intensive [167].

There are generally three critical points to ensure a successful FISH experiment. Firstly, the size of probes should be large enough to visualize the signal while it should not be too big to induce unspecific binding. Since the modified nucleotides can slow down and even stop the probe synthesis by polymerase, large size probes tend to have lower incorporation rate and therefore a series of testing ought to be performed. For example, the ratio between fluorescent dUTP and dTTP should be manipulated in order to achieve enough amounts of probes. Secondly, the hybridization environment such as
the incubation temperature, concentration of denaturing reagent and incubation duration can affect the hybridization result. The last but not the least, how to wash the slides post hybridization can yield significantly different outcomes. For example, the concentration of detergent used, and the necessity of agitation should all be taken into consideration to produce good hybridization with little background noise. For details on how the FISH and banding pattern is performed, please see the project chapter where banding pattern and FISH with various modification are used for each specific project.

1.5. Objectives of this study

Following SsWGD, large-scale genome reorganization is involved in the process of rediploidization. As a result, present-day salmonids share large syntenic collinear blocks in a species-specific order. In the present study, our first aim was to visualize these rearrangements by taking advantage of genus Salmo (including two sole members, namely, Atlantic salmon (S. salar) and brown trout (S. trutta)) where sister species not only exhibit distinct karyotypes, but also intra-species chromosomal polymorphisms. In Chapter three, the homologous chromosomes between sister species of genus Salmo were revealed by FISH, demonstrating how cytogenetic evolution can be influenced by chromosomal rearrangements. Moreover, individual chromosomal morphology that is often undeterminable in mapping studies will be collected in this study to enable us to first draw a nomenclature of the brown trout karyotype with its corresponding relationship with Atlantic salmon genome. In Chapter two, a cytogenetic survey was conducted to visualize the geographical distribution of two fusions and one translocation in Canadian populations of Atlantic salmon, revealing the population ancestry of Canadian Atlantic salmon. Another aim of present study (Chapter four) was to provide cytogenetic support for confirming the identity of sex determining gene, sdY, in Atlantic salmon by revealing its physical locations in both European and a North American derived Tasmanian Atlantic salmon.
1.6. References


[67] “AllendorfThorgaard84.pdf.”


Chapter 2.

Cytogenetic variation among populations of Canadian Atlantic salmon (*Salmo salar*)

Publication (manuscript is written and ready to submit): Cytogenetic variation among populations of Canadian populations of Atlantic salmon, 2019. Cytogenetic and Genome Research.

Authors: Lin S¹, Li J¹, Davidson WS¹

¹ Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, V5A 1S6 Canada.

Author contributions: LS completed all the FISH related experiments. LJ provided the technique support and preliminary experiments. LS and DWS wrote the manuscript.
2.1. Abstract

Following the ancestral salmonid specific genome duplication (SsWGD), the ancestor of extant salmonids carried 48 pairs of acrocentric chromosomes. In the following process of salmonid re-diploidization, Robertsonian rearrangements are the large-structural chromosomal movements that promote the gene divergence and lead towards speciation. As a result, intra-species karyotypic variation is often seen in extant salmonids. As the most derived salmonid species based on the chromosomal morphology (European Atlantic salmon carries 29 pairs of chromosomes with more than half of them showing signatures of ancestral fusions or translocations). The Canadian subpopulation of Atlantic salmon was chosen in this study to exam if the three previously reported rearrangements can explain the survey observations of chromosomal variations from seven rivers across Eastern Canada. Our results obtained by fluorescent in situ hybridization (FISH) revealed the homozygous translocation between Ssa01 and Ssa23 is fixed in six strains of Canadian Atlantic salmon originating from New Brunswick, Quebec and Nova Scotia, while such an event was not observed in European salmon as well as samples from Newfoundland (NF). Moreover, two independent fusions between Ssa26 and Ssa28, Ssa08 and Ssa29, were observed within and among the Canadian strains, giving rise to various number of chromosome and chromosome arms of 2n= 54-57, NF=72-74, respectively. Our results indicate the shared ancestry of Canadian Atlantic salmon is different from the European subpopulation. Moreover, founder effects are thought to explain the populations of Eastern Canada, except Newfoundland. Intriguingly, meiotic drive working on the segregation after the cross-over might be responsible for the absence of fish with 2n=58 with the presence of fish with 2n=57. Considering the potential environmental adaptation, the functional divergence among homeologs residing on the rearranged chromosomes should be a focus for future functional genomic studies. Moreover, future comparative genomic studies focusing on centromeric regions involved in the rearrangements between European and Canadian subpopulation shall be conducted to elucidate the potential triggering mechanism with involvement of transposable elements.
2.2. Introduction

Compared to most fish species which did not experience the 4th round of whole genome duplication, the auto-tetraploids of ancestral salmonids provide redundant genetic material for gene evolution leading to species radiation during the process of re-diploidization where chromosome structures are reassembled to promote divergence among homeologs [1], [2]. Following the ancestral duplication, large and small scale chromosomal rearrangements including Robertsonian fusions, fissions and translocations; pericentric inversions and deletions are the primary means to achieve the re-diploidization that is still an on-going process [1], [3]. As a result, many studies have encountered salmonid species with intra-species cytogenetic variation (For a detailed review, please see [3], [4]). Such intra-species variation caused by Robertsonian rearrangements are also commonly seen in other teleosts [5], [6] and mammals such as the European house mouse [7], [8].

Over several decades of cytogenetic studies, one species of salmonid in particular, Atlantic salmon, has drawn great scientific attention due to its two unique features: first, Atlantic salmon exhibits not only intra-species, but also intra-population cytogenetic variation [9], [10]; secondly, the species possesses the least number of chromosome and chromosome arm (2n=54-58, NF=72-74) within Salmonidae with a clear signature of ancestral fusions or translocations on more than half of the pairs (please see figure 3.1 in chapter three for a clear karyotype of European Atlantic salmon). Consequently, Atlantic salmon is well accepted as the most derived species based on its chromosomal morphology and a great candidate for studying the roles of chromosomal rearrangement in the evolutionary process of re-diploidization. The mega-population of Atlantic salmon can be generally divided into two major populations based on geographical distribution [11]. The European subpopulation resides from northern Portugal to eastern Barents Seas in Russia including Iceland and some freshwater river systems of the Baltic Sea. The North American subpopulation can be found from Labrador in Canada to Virginia in the US. Most of the populations have evolved to have an anadromous life cycle, but a certain amount of landlocked populations do exist owing to the postglacial geographical barriers formed during the retreat of the continental ice. Besides the different locations of natural habitats, these two subpopulations are also differentiated in many aspects of molecular genetic composition such as allozymes [12],
mini- and microsatellites [11], [13], rDNA [14] and mtDNA [15], [16]. Moreover, cytogenetic variation is commonly seen in North American populations, while hardly encountered in European populations [9]. Atlantic salmon from North America were previously been shown to possess chromosome complements varying among 2n= 54-57 and NF=72-74 [10], whereas European strains generally have a stable karyotype composition of 58 chromosomes with NF of 74. The karyotypic differences, together with the blocks of repetitive DNA seen in metacentric and large telocentric chromosomes, has led scientists to believe structural changes such as Robertsonian rearrangements are responsible for the sub-speciation of this mega-population [3], [17].

Unlike traditional cytogenetic studies in mammals, banding patterns with higher resolution such as the G-banding technique do not perform well in salmonids due to the lack of distributions of G/C or A/T rich regions [4]. Consequently, studies such as comparative genetic mapping and chromosome painting are often used to elucidate the chromosomal relationship. Two separate attempts were made to visualize the chromosomal differences between the two subpopulations of Atlantic salmon. In the earlier study with less number of microsatellite markers, Lubieniecki et al. (2010) identified two possible Robertsonian fusions between acrocentric Ssa26q and acrocentric Ssa28q; either p or q arm of metacentric Ssa06 and acrocentric Ssa22q; [18]. The results were partially supported by a later attempt with an additional rearrangement proposed. In addition, Brenna-Hansen et al. (2012) revealed the intra-population variation of chromosome number in one Canadian river strain (Saint John River, New Brunswick) is due to one homozygous Robertsonian translocation between metacentric Ssa01p and acrocentric Ssa23q, and two heterozygous Robertsonian fusions between acrocentric Ssa 26q and acrocentric Ssa 28q; metacentric Ssa08p and acrocentric Ssa29q [19]. In the latter study, chromosome painting by FISH was included to confirm the results from computational analysis and therefore provide a convincing theory to explain the chromosomal variations. Please see Figure 2-2 for a diagram showing these three rearrangements. Please note the number of chromosomes remains the same after either a homozygous or heterozygous translocation, whereas the number of chromosomes will be reduced by one and two after a heterozygous and homozygous fusion, respectively. Moreover, the fundamental number (number of chromosome arm) can also be potentially affected by fusions depending on the orientation of events. For example, a centric-centric fusion will preserve the number of chromosome arms,
whereas telo-centric will reduce the arm number. Therefore, in this study, our first aim is to test the hypothesis that these three rearrangements are indeed responsible for the karyotype variation of Canadian Atlantic salmon through surveying seven river populations across eastern Canada. Considering Atlantic salmon is a species with anadromous life cycle and accurate homing ability, the second aim of this study is to visualize any potential region or population-specific rearrangements. The information gathered in this study aimed to not only provide information for understanding the structural changes involved in the process of re-diploidization in Atlantic salmon, but also point out the population ancestry of Canadian Atlantic salmon.

2.3. Material and methods

2.3.1. Blood collection and lymphocyte culture

The blood of European Atlantic salmon (Mowi strain of Norway) was collected at the facility of Department of Fisheries and Oceans Canada located at Centre for Aquaculture & Environmental Research in West Vancouver, British Columbia. This particular Norwegian strain had been domesticated for a couple of decades before being imported commercially to British Columbia between 1991-1995 [20]. The blood of seven Canadian strains of Atlantic salmon was collected from local rivers with support from local fishery authorities. Due to the endangered status of wild Atlantic salmon, a very low number of fish were allowed to be sampled. Unfortunately, Atlantic salmon from River Philip, Nova Scotia, is unavailable for sampling due to closure of the local hatchery. Therefore, the farmed stock in Tasmania that was imported from River Philip to Australia around 1970 was assumed to represent this population. Please see Figure 2-1 for a map with sampling locations and its corresponding number of samples.

Up to 2 ml of blood was aseptically drawn from the caudal vein of the fish using a sterile syringe inserted near the anal fin. The blood was collected into a Vacutainer tube containing heparin, gently mixed, and transported to the lab at 4 °C. 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 min. The diluted blood was then centrifuged at 1200 rpm for 5 min at room temperature. After centrifugation, the buffy coat (containing lymphocytes)
above the red blood cells was floated in plasma by gentle stirring with a 1 ml pipette. The lymphocyte-enriched plasma was then collected in a new 15 ml sterile plastic tube. The lymphocyte-enriched plasma was centrifuged at 1500 rpm for 5 min, and the resulting cell pellet was suspended in 5 ml of complete media L-15 containing 10% fetal bovine serum (FBS), 60 mg/ml of kanamycin sulfate, 1· antibiotic/antimycotic solution (100 units/ml of penicillin, 100 mg/ml of streptomycin, and 250 ng of amphotericin B), 25 mM of 2-mercaptoethanol, 18 mg/ml of phytohemagglutinin (PHA-W), and 100 mg/ml of lipopolysaccharide (LPS). The cells were cultured at 18 °C in a culture tube slanting to an angle of approximately 30° with gentle daily mixing for 6 day. Approximately 90 min before cell harvest, the lymphocyte culture was supplemented with 500 ng/ml colcemid. The cells were collected by centrifugation at 1500 rpm for 5 min, and the supernatant was discarded. The cell pellet was suspended in 2 ml of 0.075 M KCl hypotonic solution for 20 min at 20 °C. The hypotonic solution was slowly added to a volume of 2 ml. Then, 2 ml of fresh Carnoy’s fixative (3 methanol:1 acetic acid) was added slowly. After centrifugation at 1500 rpm for 5 min, the supernatant was discarded. The fixed cells were gently suspended in 3 ml of Carnoy’s fixative. The fixation step was repeated two more times, and then the cells were suspended in 1–2 ml of Carnoy’s fixative. A microscope slide was exposed to hot water vapor at 73.5 °C for 30 sec. The cell suspension was immediately dropped onto the slide from a height of around 20-30 cm. After the slide surface became “grainy” the slide was immediately exposed again to the hot water vapor at 73.5 °C for 30 sec. The slide was then quickly dried on a hot surface, which provided good chromosome spreading.

2.3.2. BAC probe preparation and FISH

To represent the chromosome arms involved in the three rearrangements, we used the same BAC probes from the same CHORI-214 BAC library as used in the study of Brenna-Hansen et al. (2012) [19]. Namely S0198E23 for Ssa01p; S088O23 for Ssa23q; S0214J02 for Ssa08p; S0016D16 for Ssa29q; S0059P02 for Ssa26q; S0091M08 for Ssa28q. The BAC library was prepared using genomic material from European Atlantic salmon. The BAC probes were first tested by FISH on European Atlantic salmon to confirm their correct location before proceeding to Canadian strains. BAC DNA, prepared using a large construct kit (QIagen), was labeled with either SpectrumOrange (Vysis) or SpectrumGreen (Vysis); 500 ng of extracted BAC DNA was
mixed with 1.25 ml of SpectrumOrange or SpectrumGreen, 2.5 ml of 0.1 mM dTTP, 5 ml 0.1 mM dNTP mix, 2.5 ml 10 X nick translation buffer, and 2.5 ml of nick translation enzyme in 25 ml. The reaction mixture was briefly mixed and centrifuged, and then incubated in a PCR thermocycler at 15 °C for 8 h, followed by 70 °C for 10 min, and then paused at 4 °C; 5 ml of the nick translation reaction mixture of the BAC and 5 ml of the nick translation reaction mixture of a chromosome-specific BAC were combined with 2 mg of Atlantic salmon Cot-1 DNA and 2 mg of human placental DNA in a microcentrifuge tube; 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% EtOH were added to precipitate the DNA. The mixture was incubated at -80 °C for 60 min and then centrifuged at 12,000 rpm for 30 min at 4 °C to pellet the DNA. The supernatant was removed, and the pellet was dried for 15 min at room temperature. The pellet was then suspended in 3 ml of distilled water and 7 ml of hybridization buffer by shaking at 250 rpm for 30 min at 37 °C. The probe was denatured by heating at 80 °C for 5 min and then chilled on ice for 1 min. Then, the probe was incubated at 37 °C from 30 to 60 min prior to hybridization.

Hybridization of the fluorescent-labeled DNA probe with chromosome spreads was performed as suggested by the manufacturer (Vysis). The freshly made metaphase-containing slides were treated with 2X SSC for 30 min at 37 °C, and then serial dehydrated in 70% EtOH, 85% EtOH, and 100% EtOH, with each treatment lasting 2 min. The hybridization area was marked using a tipped scribe. The slide was denatured in 70% formamide in 2X SSC, pH 7.0–8.0, at 73 °C for 3 min. Then, the slide was serial dehydrated in -20 °C 70% EtOH, 5% EtOH, and 100% EtOH, with each treatment being 2 min, and then air-dried; 10 ml 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 37 °C for 16 hr. The coverslip was removed together with the rubber cement seal, and the slide was immediately placed into a 0.4X SSC/0.3% NP-40 wash solution at 73 °C for 4 min, with several agitations per minute. The slide was then treated with the 2X SSC/0.1% NP-40 wash solution at room temperature for 2 min. The slide was dried in the dark, and then 10 mL of DAPI antifade solution (Invitrogen) was applied to the slide. A multiphoton confocal microscope A1R MP (Nikon) was used to check the metaphase spreads. Laser 405 was used to detect the DAPI stain, laser 488 was used for the green dUTP labeling, and laser 560 was used for the orange dUTP labeling. For
each slide, eight images were taken from eight separate spreads to evaluate proper signals and accurate chromosome number of the individual fish.

2.3.3. Genotyping with mtDNA and nuclear DNA markers

Since NL samples share the cytogenetic features of both European (lack of translocation) and Canadian salmon (independent fusions), we suspected that they are the result of hybridization between these two subpopulations. To test the hybridization hypothesis, Taqman assay was used to perform the genotyping with 10 SNP markers (5 in mtDNA (Table 2-1, personal communication with Dr. Willie Davidson at Simon Fraser University) and 5 in nuclear DNA [21] (Table 2-1) ) that are known to be distinguishable between European and North American subpopulations. In the mtDNA genotyping, eight DNA samples from fish with known origin (6 European individuals and 2 Canadian individuals) were used together with our two NL samples. In the nuclear DNA genotyping, our two samples from Newfoundland were incorporated into a population study with a large date set where fish samples were collected from both European and Canadian (unpublished study).

2.4. Results

Agreed with previous data on the cytogenetic complements of Atlantic salmon populations in North America (Table 2-3), the chromosome number of Canadian Atlantic salmon sampled in the present study from seven river populations varied from 2N = 54 to 57 (Table 2-4). Interestingly, fish with 2N = 54 and 55 chromosomes were only found in samples from Tasmania, Australia, representing the population of River Philip in Nova Scotia. Without further sampling from wild stocks of River Philip, it is hard to verify if it is indeed fish with 2N = 54 and 55 chromosomes are only present in this population. Alternatively, a bigger sampling size on other river populations might be able to address this issue. Similar to the study where the three rearrangements were found, fish with heterozygous fusion of both possible fusions were present in our samples. Moreover, unique to our study, we found homozygous fusion of both possible fusions among all populations. Since no apparent population specific pattern of fusions was evident, both fusions appear to be independent (can either be homozygous or heterozygous). Intriguingly, the translocation that is fixed in the homozygous state in all samples was not
found in the population from Newfoundland, indicating a clear population-specific chromosomal rearrangement. Since there is only one population sampled from the entire Newfoundland region, whether this population-specific rearrangement can in fact existing in other nearby rivers is unknown. Therefore, future samplings on other populations in the nearby region is necessary to visualize the boundary of this interesting rearrangement.

Compared to the unaffected number of chromosomes from either homozygous or heterozygous translocation, fusions on the other hand can result in reduction of chromosome and chromosome arm number, providing an explanation of various chromosome numbers seen in the samples (Figure 2-2). Using one particular fish with 2N = 56 from river Stewiacke as an example (Figure 2-3 & 2-4), no fusion was found between Ssa08p and Ssa29q, homozygous fusions between Ssa26q and Ssa28q gave rise to a pair of brand new metacentric chromosomes and reduced the total number of chromosomes by 2. Moreover, an additional brand new pair of metacentric chromosomes were produced from the homozygous translocation, resulting in the reduction of arm number by 2. Taken together, these three rearrangements are indeed able to explain the variation of chromosome number among all samples.

To our surprise, genotyping with 5 nuclear DNA SNPs all indicated homozygous Canadian on both NL samples, and 5 mtDNA SNPs all showed Canadian haplotype for both samples (Table 2-1 & 2-2). Therefore, there is no evidence of hybridization from SNP analysis. Alternatively, the hybridization signals might be located in other genomic locations and more SNPs are required to detect such signatures.
Figure 2-1 Seven samples locations and the number of corresponding samples.
Table 2-1 List of SNP markers used to genotype Newfoundland samples. Nuclear SNPs are from Pritchard et al. (2016) [21] and genetic position of mtDNA markers can be found in Atlantic salmon mtDNA genome at NCBI (accession number: NC_001960).

<table>
<thead>
<tr>
<th>Name of nuclear DNA makers</th>
<th>Position of mtDNA makers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESTV_17201_545</td>
<td>10126</td>
</tr>
<tr>
<td>ESTNV_22249_490</td>
<td>13514</td>
</tr>
<tr>
<td>ESTNV_26441_1376</td>
<td>1441</td>
</tr>
<tr>
<td>ESTNV_35650_2037</td>
<td>3444</td>
</tr>
<tr>
<td>ESTNV_30824_114</td>
<td>8642</td>
</tr>
</tbody>
</table>

Table 2-2 Results of genotyping with mtDNA SNPs.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Scotland_1</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>Scotland_2</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>New Brunswick_1</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td>New Brunswick_2</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td>Sweden_1</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>Sweden_2</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>Norway_1</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>Norway_2</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>Newfoundland_1</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td>Newfoundland_2</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
</tbody>
</table>

Table 2-3. Historical data of chromosome counts in North American Atlantic salmon (Roberts 1968, Roberts 1970)

<table>
<thead>
<tr>
<th>River origin</th>
<th>Chromosome counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Grand Lake, Maine</td>
<td>57</td>
</tr>
<tr>
<td>Lake Memphremagog, Maine</td>
<td>56</td>
</tr>
<tr>
<td>Machias River, Maine</td>
<td>54, 55</td>
</tr>
<tr>
<td>Narraguagus River, Maine</td>
<td>54, 55, 56</td>
</tr>
<tr>
<td>Miramichi River, NB</td>
<td>54, 55, 56</td>
</tr>
</tbody>
</table>
Table 2-4. Chromosomal rearrangements among strains of Canadian Atlantic salmon. Homozygous (HO); heterozygous (HE); not observed (NO); New Brunswick (NB); Nova Scotia (NS); Quebec (QC); Newfoundland (NL). Each line represents one fish sample.

<table>
<thead>
<tr>
<th>River Name</th>
<th>2N</th>
<th>Translocation</th>
<th>Fusion 1</th>
<th>Fusion 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ssa1 and Ssa23</td>
<td>Ssa26 and Ssa28</td>
<td>Ssa8 and Ssa29</td>
</tr>
<tr>
<td>Saint. John, NB</td>
<td>57</td>
<td>HO</td>
<td>HE</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>HE</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>HE</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>NO</td>
<td>HO</td>
</tr>
<tr>
<td>Stewiacke, NS</td>
<td>56</td>
<td>HO</td>
<td>HE</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>HO</td>
<td>HE</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>HO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Gasperau, NS</td>
<td>56</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>HE</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>HE</td>
<td>HE</td>
</tr>
<tr>
<td>River Philips, NS</td>
<td>54</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td>(Tasmania, Austria)</td>
<td>55</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>HO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Jaques Cartier, QC</td>
<td>56</td>
<td>HO</td>
<td>HE</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>HO</td>
<td>HE</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>HE</td>
<td>HE</td>
</tr>
<tr>
<td>Trinity, QC</td>
<td>57</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>HO</td>
<td>HE</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>HO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td>Rocky River, NL</td>
<td>57</td>
<td>NO</td>
<td>NO</td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>NO</td>
<td>NO</td>
<td>HE</td>
</tr>
</tbody>
</table>
Figure 2-2. Ideogram of three Robertsonian rearrangements visualized in the survey of 7 river strains of Canadian Atlantic salmon. Top: homozygous translocation between Ssa01 and Ssa23. This rearrangement is fixed in all populations except samples from Newfoundland; Middle: homozygous or heterozygous fusion between Ssa08 and Ssa29. The resulting dicentric chromosomes will potential interfere with normal meiotic segregation; Bottom: homozygous or heterozygous fusion between Ssa26 and Ssa28. Both fusions appears to independent among all samples surveyed. The red circle represents centromere.
Figure 2-3. FISH-images of Ss01p/23q translocation with arrows pointing at BAC probes. Metaphase spreads of an European Atlantic salmon (left, [Brenna-Hansen et al., 2012]) and a Canadian Atlantic salmon (right) sampled from River Stewiacke, NS with a 2N=56. Red label with red arrow: S0198E23 on Ssa01p, red label with yellow arrow: S0088O23 on Ssa01q, green label: S0102N22 on Ssa23q.
Figure 2-4. FISH-images of Ssa08q/29q fusion (left) and Ssa26q/28q fusion (right) with arrows pointing at BAC probes. Metaphase spreads of a Canadian Atlantic salmon sampled from River Stewiacke, NS with a 2N=56. No fusion is occurred in Ssa08q/29q fusion, whereas homozygous fusion of Ssa26q/28q is observed. Red label with red arrow: S0214J02 on Ssa08q (left); S0059P02 on Ssa26q (right), green label with Green arrow: S0016D16 on Ssa29q (left); S0091M08 on Ssa28q (right).
2.5. Discussion

From the historical data (Table 2-3), the chromosome number of North American subpopulation generally varies from 54 to 57 [10]. However, one single study did report fish with $2n = 58$ in Chaleur-Bay district of Quebec, Canada [22]. In the present study through surveying seven Canadian populations, the observed chromosome complements ($2n = 54-57$) are largely consistent with historical data. Interestingly, samples showing $2n = 54$ and $2n = 55$ are only found in Tasmanian salmon. This is either an indication of small sampling size in our samples from other populations or potential geographic influence after the introduction of salmon to Tasmania about 35 years ago. A definitive conclusion could be drawn upon expanding sampling sizes of other populations and also comparing wild stocks from River Phillip and Tasmanian salmon. However, access to wild populations of Atlantic salmon is difficult due to currently protected status of Canadian Atlantic salmon.

In the present study, the homozygous translocation was found to be fixed in all populations except fish from Newfoundland, indicating a region/population-specific pattern of rearrangement does exist in Eastern Canada. Since the lack of translocation is consistent with European Atlantic salmon, this potentially provides the first cytogenetic evidence to support previous studies suggesting Atlantic salmon originating from Newfoundland being hybrids between European and Canadian strains [16], [23], [24]. However, this conclusion was not supported by our attempt with genotyping using 10 SNP markers known to distinguish between European and North American populations. All 10 markers showed the genotype of Canadian ancestry. A more definitive answers shall be provided when more markers are used. Indeed, results from a recent comparative study using dense SNP maps did show introgression of European markers on Ssa01 and Ssa23 in NL populations [24]. In terms of two possible fusions between Ssa08p and Ssa29, and Ssa26q and Ssa28q, not only did we find both fusions in heterozygous states, same as described previously [19], homozygous fusions for both were observed in all seven populations. These two fusions appear to be lack of specific patterns within and among populations and therefore considered independent.

Considering the highly unlikely possibility of evolving the same translocation and fusion in various river across large area, the fixation of homozygous translocation in all samples except NL and two shared independent fusions suggests a shared origin for all
Canadian salmon. Since Newfoundland has been shown to be a hybrids zone [16], [23], [24], our results suggest the initial post-glacial colonization of Eastern Canada was in Newfoundland where both fusions took place and the following homozygous translocation occurred in a single or very small number of river populations right after further expansion to the rest of Eastern Canada. Moreover, the translocation between Ssa01p and Ssa23q appears to be quickly fixed in the homozygous state while both fusions that occur before the translocation has not reached the fixation and still remain either homozygous or heterozygous, suggesting positive selection and mild selection on the translocation and fusions, respectively. Therefore, future study with functional analysis on genes present on chromosomes involved in the three rearrangements could reveal some key players during the evolution of Canadian Atlantic salmon. Indeed, serval quantitative trait loci (QTL) that are related to early smoltification and ecological related traits were found on Ssa01 [25], [26]. Moreover, SNPs associated with thermal regimes were also found on Ssa01, Ssa08, Ssa29 [27].

Intriguingly, despite the presence of fish with 2n = 57 in all sampled populations, no fish with 2n = 58 was observed. Fish with 2n = 57 ought to produce gametes with either n = 28 or n = 29. The lack of fish with 2n = 58 suggests the possibility of fatal effects when fertilization of both gametes of n = 29 takes place. Considering the homozygous translocation (Ssa01p/23q) does not affect the number of chromosomes, the fish with 2n = 57 is the result of a single heterozygous fusion between Ssa08p and Ssa23q or Ssa26q and Ssa28q (Figure 2-2). Therefore, each gamete of n = 29 should be perfectly normal and carry two unarranged chromosomes, similar to the regular gametes from European salmon. So why don’t we encounter any fish with 2n = 58? One explanation is that the n = 29 gametes would always carry one fused chromosomes and one unarranged chromosome, suggesting meiotic drive is working on the segregation after the cross-over [28]. Alternatively, the gametogenesis can simply be aborted in fish with 2n = 57 due to either dicentric nature of rearranged chromosomes or pairing issues between rearranged and unarranged chromosomes [29], [30]. Breeding experiments will provide a more definitive answer for this interesting finding.

Even though the chromosomal variations seen in Atlantic salmon has been known for decades and can now be explained by the three rearrangements, the ultimate question regarding the triggering mechanism has not been able to be postulated until the recent genome assembly of European Atlantic salmon. Among all known sequenced
genome of vertebrates, the Atlantic salmon genome contains the highest amount of repeat sequences including transposable elements of both DNA transposons and retrotransposons [31]. Despite all SINEs and LINEs appearing as non-functional remnants [32], [33], a couple of salmon specific DNA transposon families including DTSsa1 and DTSsa2 remain active [34]. A large burst of transposons was found before the ancestral Robertsonian fusion leading to the formation of metacentric chromosomes [31]. With the cryptic and non-autonomous TEs known to accumulate in the centromeric region where heterochromatin is, epigenetic influences turning on the TEs could potentially facilitate the centromeric breakage and non-homologous recombination required for these rearrangements [35], [36]. Comparative genomic studies between the European and Canadian subpopulations focusing on these centromeric regions should be conducted to test this hypothesis; however, the great difficulty of sequencing through the highly repetitive centromeric region will make this difficult.

In conclusion, these three rearrangements are indeed responsible for the karyotype variation that results in large scale chromosomal structural changes involved in the re-diploidization of Canadian Atlantic salmon. Since the environmental differences do exist between European and North America subpopulation, functional studies of chromosomes involved in the rearrangement with regard to environmental adaption shall be a focus of future landscape genomics [37] to better understand duplicated genome evolution. As a species with important economic value, our finding is significant for the salmon industry which uses Canadian Atlantic salmon as broodstocks for selective breeding program. Specific crosses among fish with different karyotypes should be made to reveal any special meiosis segregation and potential functional influences. Moreover, the founder effect shown by the fixation of homozygous translocation suggests a different genetic background of Canadian Atlantic salmon in comparison to European subpopulation. Therefore, from conservation aspect, we would not recommend the introduction of European Atlantic salmon for the local salmon aquaculture.
2.6. Reference


Chapter 3. Cytogenetic relationship between members of genus *Salmo*.

**Publication (manuscript is written and ready to submit):** Cytogenetic relationship between members of genus *Salmo*, 2019. Cytogenetic and Genome Research.

**Authors:** Lin S¹, Li J¹, Davidson WS¹

¹ Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, V5A 1S6 Canada.

**Author contributions:** LS completed 80% of the FISH related experiments. LJ provided the technique support and 20% of the FISH related experiments. LS and DWS wrote the manuscript.
3.1. Abstract

Following the ancestral SsWGD around 80-100 million years ago, chromosomal rearrangements including Robertsonian fusions, fissions and translocations are believed to be the primary structural changes to achieve the re-diploidization in present-day salmonid genomes. As a result, the ancestral genome was rearranged into species-specific orders. Within salmonids, the two sole members of genus *Salmo*, namely Atlantic salmon and brown trout, represent the most derived and relatively ancestral karyotype, respectively, providing excellent sample materials to study the possible outcome of rearrangements. In the present study, our aim is to visualize the large-scale structural movements of ancestral homologous chromosomes through comparative cytogenetic analysis of both members of genus *Salmo*. Since the chromosomal variations was detected in North American populations of Atlantic salmon, the European subspecies with a stable karyotype was chosen for the analysis. Through C-banding, the karyotype complement of both Atlantic salmon and brown trout is consistent with previous literature [1]. The European Atlantic salmon comprises of 58 chromosomes (8 pairs of (sub) metacentric and 21 pairs of telocentric chromosomes) with the number of chromosome arms (NF) of 74, whereas brown trout has 80 chromosomes (11 pairs of (sub) metacentric and 29 pairs of telocentric chromosomes) with NF of 100. Of the total of 29 pairs of chromosomes in Atlantic salmon, 12 pairs corresponded to either two or three pairs of chromosomes in brown trout. Three arms of brown trout chromosomes were lacking corresponding counterparts in Atlantic salmon, suggesting the possibility of chromosome arm losses, which might not be unexpected in an autopolyplloid species with a very high percentage of transposons. Moreover, multi-chromosome localization of rDNA in brown trout is consistent with previous reports. The physical morphology and identity of chromosomes collected in this study allows us to propose a nomenclature of the brown trout karyotype with additional information regarding corresponding relationship with Atlantic salmon.
3.2. Introduction

In salmonids, Robertsonian rearrangements including translocations, fusions and fissions are the large-scale chromosomal rearrangements used in the process of re-diploidization where the ancestral duplicated genome differentiates in order to restore disomy status [1], [2]. Compared with imbalanced rearrangements such as deletions, and duplications where there is either net gain or loss of genetic materials, Robertsonian rearrangements through whole-arm movements often results in no apparent gain or loss of genetic material. However, these rearrangements might induce negative effects. Dicentric chromosomes resulted from tandem fusion (telo-centro) caused pairing issue during meiosis [3]; recombination rate was reduced because of translocations [4]. Moreover, gene expression can also be affected. For example, multiple studies reported that the expression of genes that are located in regions of highly conserved arrangement was altered due to Robertsonian translocations [5], [6]. Robertsonian rearrangements can sometime cause loss of genetic material. Alteration of rRNA expression was caused by loss of two nucleolus organization regions (NORs) in a uniparental disomy (UPD) patient suffering from intellectual disability due to a Robertsonian translocation [7]. In the case of salmonids, the formation of metacentric chromosomes by Robertsonian translocation is shown to provide stability of quadrivalent pairing in meiosis I and consequently prevent or retard the re-diploidization in the genus of Oncorhynchus [8], while the same theory is not necessary applicable to Atlantic salmon [9] since the quadrivalent pairing can occur without metacentric chromosomes.

By comparing the karyotypes of present-day salmonids to the ancestral karyotype post SsWGD that is 96 chromosomes with 96 chromosome arms, the two sole members of genus *Salmo*, namely Atlantic salmon and brown trout, represents the most derived and relative ancestral karyotype, respectively [1]. Brown trout populations are mainly freshwater inhabitants and show remarkable karyotype uniformity with a diploid complement of \(2n = 80\) and \(NF = 102-104\) [10]. Although the origin of brown trout is in Europe, this species has been successful introduced to many places across the globe. In contrast, the metapopulation of Atlantic salmon is known to be mainly anadromous with two subpopulations found in North America and Europe, separated by significant divergence of genomic structures. Atlantic salmon from North America including Northeastern USA and Canada, are reported to have chromosome number varying from
54-57 [11], whereas European strains generally have a stable karyotype composition of 58 chromosomes with NF of 74 [12]. Moreover, one of the distinctive features of Atlantic salmon chromosomes are the blocks of heavily DAPI staining and repetitive DNA, which is believed to be the results of ancestral fusion events [13]. In a effort to elucidate the chromosomal relationship between two members of genus *Salmo*, two earlier studies comparing linkage maps of brown trout and Atlantic salmon have shown clear evidence of fusions and translocations involved in the speciation of this genus [14], [15]. Interestingly, by using brook trout (*Salvelinus fontinalis*) as an outgroup, fusions or fissions were only found in Atlantic salmon after the *S. salar* and *S. trutta* speciation, suggesting the karyotype of brown trout is closer to ancestral state [14]. Comparative genomic studies using genetic maps of multiples species is an efficient way to achieve high resolution comparison and can potentially identify not only large-scale rearrangements but also small changes such as paracentric inversions. However, some technical difficulties do exist in such studies. For example, the physical morphology (metacentric or telocentric) of chromosomes can be difficult to determine if the centromere placement is an issue [14], [15]. Moreover, unmatched numbers of linkage groups and the actual numbers of chromosome could occur in studies with low density of genetic markers [15]. Also, it is always difficult to visualize the orientation of rearrangements with additional morphological information. Most importantly, as pointed out by Allendorf et al., (2015), most duplicated markers are not used in building genetic maps of salmonids, therefore observing chromosomal rearrangements by comparative mapping studies will tend to underrepresent the duplicated regions [16].

In this study, our aim is to reveal the large-scale genomic rearrangements during re-diploidization of genus *Salmo* by conducting a comparative cytogenetic analysis between the two sole members, Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*). To avoid complications from chromosomal variations detected in Canadian populations, the European subspecies of Atlantic salmon was chosen for the analysis. The biological question being addressed here concerns how homologous chromosomes are rearranged from the predicted ancestral salmonid karyotype of 2n = 96, NF = 96 to produce the karyotypes seen in extant Atlantic salmon and brown trout [10], [17], [18]. It should be noted that there could also be chromosomal loss or gain during the species divergence. The information gathered in this study will enable speculation not only on the cytogenetic evolution in the genus *Salmo*, but also how chromosomal
rearrangements have shaped salmonid genomes in general. Moreover, individual chromosomal morphology that is often undeterminable in mapping studies will be collected in this study to enable us to first draw a nomenclature of the brown trout karyotype with its corresponding relationship with Atlantic salmon genome.

3.3. Material and methods

3.3.1. Fish blood collection

The blood of the European strain of Atlantic salmon was collected at the facility of Department of Fisheries and Oceans Canada located at the Centre for Aquaculture & Environmental Research in West Vancouver, British Columbia. Brown trout samples were collected from the Aquaculture Centre of Dalhousie University. The procedures for blood collection and lymphocyte culture are the same as that in Chapter Two.

Up to 2 ml of blood was aseptically drawn from the caudal vein of the fish using a sterile syringe inserted near the anal fin. The blood was collected into a Vacutainer tube containing heparin, gently mixed, and transported to the lab at 4 °C. 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 min. The diluted blood was then centrifuged at 1200 rpm for 5 min at room temperature. After centrifugation, the buffy coat (containing lymphocytes) above the red blood cells was floated in plasma by gentle stirring with a 1 ml pipette. The lymphocyte-enriched plasma was then collected in a new 15 ml sterile plastic tube. The lymphocyte-enriched plasma was centrifuged at 1500 rpm for 5 min, and the resulting cell pellet was suspended in 5 ml of complete media L-15 containing 10% fetal bovine serum (FBS), 60 mg/ml of kanamycin sulfate, 1· antibiotic/antimycotic solution (100 units/ml of penicillin, 100 mg/ml of streptomycin, and 250 ng of amphotericin B), 25 mM of 2-mercaptoethanol, 18 mg/ml of phytohemagglutinin (PHA-W), and 100 mg/ml of lipopolysaccharide (LPS). The cells were cultured at 18 °C in a culture tube slanting to an angle of approximately 30° with gentle daily mixing for 6 d. Approximately 90 min before cell harvest, the lymphocyte culture was supplemented with 500 ng/ml colcemid. The cells were collected by centrifugation at 1500 rpm for 5 min, and the supernatant was discarded. The cell pellet was suspended in 2 ml of 0.075 M KCl hypotonic solution
for 20 min at 20 °C. The hypotonic solution was slowly added to a volume of 2 ml. Then, 2 ml of fresh Carnoy’s fixative (3 methanol:1 acetic acid) was added slowly. After centrifugation at 1500 rpm for 5 min, the supernatant was discarded. The fixed cells were gently suspended in 3 ml of Carnoy’s fixative. The fixation step was repeated two more times, and then the cells were suspended in 1–2 ml of Carnoy’s fixative. A microscope slide was exposed to hot water vapor at 73.5 °C for 30 sec. The cell suspension was immediately dropped onto the slide from a height of around 20-30 cm. After the slide surface became “grainy” the slide was immediately exposed again to the hot water vapor at 73.5 °C for 30 sec. The slide was then quickly dried on a hot surface, which provided good chromosome spreading.

3.3.2. C-band techniques

Once the chromosome spreads had been prepared on the slides, the C-banding technique was carried out to examine the karyotype of both European Atlantic salmon and brown trout. The procedure used was a modification based on the classic method by Sumner (1972) [19]. In brief, chromosomes spread were obtained by dropping the fixed cells onto glass slides from a height of 15-20 cms. The slides with chromosome spreads attached were first incubated in 0.2N HCl for 45 minutes at room temperature. Then the slides were washed briefly in distilled water prior to incubation for 8 minutes in 5% Ba(OH)₂ 8H₂O at 35 °C. The slides were then washed three times in distilled water acidulated with acetic acid. Slide incubation for 45 minutes then took place in 2X SSC at 60 °C. After a series of washing with distilled water, the slides were stained with propidium iodide (15 ul/slide) and sealed by attaching the coverslip with rubber cement. The visualization of chromosome spreads was done on a WaveFX Spinning Disc Confocal system from Quorum Technologies (Guelph, Canada). For each slide, eight images were taken from eight separate spreads to evaluate proper banding patterns.

3.3.3. BAC marker assignment and fluorescence in-situ hybridization

European Atlantic salmon was chosen as the reference for this project to eliminate the interference of intra-specific chromosomal rearrangements seen in North American Atlantic salmon. The entire chromosome complement of Atlantic salmon is broken into different sections based on the following rules; each chromosome was first broken into two arms for metacentric chromosomes and further broken down to small
sections based on blocks of repetitive DNA, if there is any. For example, Ssa01 is divided into three sections, namely Ssa01p, Ssa01qa and Ssa01qb. The bacterial artificial chromosome (BAC) probes representing different sections of Atlantic salmon chromosomes were chosen based on the presence of microsatellite markers previously assigned to the locations [13]. All the IDs of BACs are shown in Figure 3-3-A, B and C. After confirmation for their location on salmon chromosomes by fluorescence in situ hybridization (FISH), probes with correct locations were hybridized to brown trout chromosome spreads. The BAC FISH procedure is the same as that used in Chapter two.

The BAC library was prepared using genomic material from European Atlantic salmon. The BAC probes were first tested by FISH on European Atlantic salmon to confirm their correct location before proceeding on brown trout. BAC DNA, prepared using a large construct kit (QIAGen), was labeled with either SpectrumOrange (Vysis) or SpectrumGreen (Vysis); 500 ng of extracted BAC DNA was mixed with 1.25 ml of SpectrumOrange or SpectrumGreen, 2.5 ml of 0.1 mM dTTP, 5 ml 0.1 mM dNTP mix, 2.5 ml 10⁻ nick translation buffer, and 2.5 ml of nick translation enzyme in 25 ml. The reaction mixture was briefly mixed and centrifuged, and then incubated in a PCR thermocycler at 15 °C for 8 h, followed by 70 °C for 10 min, and then paused at 4 °C; 5 ml of the nick translation reaction mixture of the BAC and 5 ml of the nick translation reaction mixture of a chromosome-specific BAC were combined with 2 mg of Atlantic salmon Cot-1 DNA and 2 mg of human placental DNA in a microcentrifuge tube; 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% EtOH were added to precipitate the DNA. The mixture was incubated at -80 °C for 60 min and then centrifuged at 12,000 rpm for 30 min at 4 °C to pellet the DNA. The supernatant was removed, and the pellet was dried for 15 min at room temperature. The pellet was then suspended in 3 ml of distilled water and 7 ml of hybridization buffer by shaking at 250 rpm for 30 min at 37 °C. The probe was denatured by heating at 80 °C for 5 min and then chilled on ice for 1 min. Then, the probe was incubated at 37 °C from 30 to 60 min prior to hybridization.

Hybridization of the fluorescent-labeled DNA probe with chromosome spreads was performed as suggested by the manufacture (Vysis). The freshly made metaphase-containing slides were treated with 2 X SSC for 30 min at 37 °C, and then serial dehydrated in 70% EtOH, 85% EtOH, and 100% EtOH, with each treatment lasting 2 min. The hybridization area was marked using a tipped scribe. The slide was denatured
in 70% formamide in 2 X SSC, pH 7.0–8.0, at 73 °C for 3 min. Then, the slide was serial dehydrated in -20 °C 70% EtOH, 5% EtOH, and 100% EtOH, with each treatment being 2 min, and then air-dried; 10 ml 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 37 °C for 16 hr. The coverslip was removed together with the rubber cement seal, and the slide was immediately placed into a 0.4 X SSC/0.3% NP-40 wash solution at 73 °C for 4 min, with several agitations per minute. The slide was then treated with the 2 X SSC/0.1% NP-40 wash solution at room temperature for 2 min. The slide was dried in the dark, and then 10 mL of DAPI antifade solution (Invitrogen) was applied to the slide. A multiphoton confocal microscope A1R MP (Nikon) was used to check the metaphase spreads. Laser 405 was used to detect the DAPI stain, laser 488 was used for the green dUTP labeling, and laser 560 was used for the orange dUTP labeling. For each slide, eight images were taken from eight separate spreads to evaluate proper signals and accurate chromosome number of the individual fish.

3.4. Results

The karyotype of European Atlantic salmon and brown trout from the current work is consistent with the previous literatures [1]. For Atlantic salmon, the complement is comprised of 8 pairs of (sub) metacentric chromosomes and 21 pairs of telocentric chromosomes with NF of 74 (Figure 3-1). For brown trout, it is comprised of 11 pairs of (sub) metacentric chromosomes and 29 pairs of telocentric chromosomes with NF of 100 (Figure 3-2). Detailed FISH images with various BAC markers on both Atlantic salmon and brown trout chromosomes are available in supplementary material. The FISH results are compiled into the simple ideogram in Figure 3-3-A, B and C.

There are twelve chromosomes of Atlantic salmon corresponding to either 2 or 3 chromosomes in brown trout, indicating the lesser number of chromosomes in Atlantic salmon is caused by a greater number of ancestral Robertsonian fusions or fusion followed by inversions. For example, Ssa01 is the results of two fusions including a likely centro-centro fusion between Str12 and Str01, and a telo-centro fusion between Str01 and Str13. Ssa09 on the other hand is the results of telo-telo fusion between Str19 and Str03 or one telo-centro fusion followed by pericentric inversion. Moreover, a pericentric inversion was clearly evident between Ssa18 and Str02. Most strikingly, one ancestral fission of a metacentric chromosome was followed by a Robertsonian fusion, giving rise
to the acrocentric Ssa16 and Ssa29 with unchanged number of chromosomes (Figure 3-3-B). Moreover, three chromosomes arms of brown trout, namely Str15q, Str23q and Str27p are missing their counterpart in Atlantic salmon, while Ssa8q of Atlantic salmon (heterochromatic arm with rDNAs and NORs, please note that there is an error in Phillips et al. (2009) where Ssa08p was thought to be the arm with rDNA [13]. In fact, it should be the q arm) corresponds to sections on nearly half of brown trout chromosomes with a relative major location on the Str11 (the largest telocentric chromosomes) (Figure 3-4).

In summary, results from present study showed the corresponding relationship between homologous chromosomes in two sole species of genus Salmo. Robertsonian translocations, inversions, fusions and fissions were all evident in the genomic reorganization of the genus. The information collected in this study on the physical morphology of brown trout chromosomes together with its homologous counterparts in Atlantic salmon is then merged into a nomenclature for brown trout karyotype (Table 3-1).
Figure 3-1. C-banding of European Atlantic salmon. Ssa: Atlantic salmon chromosome. The chromosome complement of Atlantic salmon is comprised of 8 pairs of meta/acro centric chromosomes and 21 pairs of telocentric chromosomes. LG: linkage group.
Figure 3-2. C-banding of brown trout. Str: brown trout chromosome. The chromosome complement of brown trout is comprised of 11 pairs of meta/acro centric chromosomes and 29 pairs of telocentric chromosomes.
Figure 3.3-A. Ideogram of the cytogenetic relationship between Atlantic salmon and brown trout. Markers on the chromosomes are the BAC probes from CHORI-214 library. Ssa: Atlantic salmon; Str: brown trout.
Figure 3-3-B. Ideogram of the cytogenetic relationship between Atlantic salmon and brown trout. Markers on the chromosomes are the BAC probes from CHORI-214 library. Ssa: Atlantic salmon; Str: brown trout.
Figure 3-3-C. Ideogram of the cytogenetic relationship between Atlantic salmon and brown trout. Markers on the chromosomes are the BAC probes from CHORI-214 library. Ssa: Atlantic salmon; Str: brown trout.
Figure 3-4. FISH-images of a BAC probe, S0258F18, representing Ssa08q in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right).
<table>
<thead>
<tr>
<th>S. trutta Chromosome ID</th>
<th>Morphology</th>
<th>S. salar Chromosome ID</th>
<th>Linkage ID (Gharbi)</th>
<th>Linkage ID (Leitwein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Telocentric</td>
<td>1qa</td>
<td>BT23</td>
<td>BT30</td>
</tr>
<tr>
<td>2p</td>
<td>Telocentric</td>
<td>18qb</td>
<td>BT23</td>
<td>BT30</td>
</tr>
<tr>
<td>2q</td>
<td>Telocentric</td>
<td>18qa</td>
<td>BT23</td>
<td>BT30</td>
</tr>
<tr>
<td>3p</td>
<td>(Sub)metacentric</td>
<td>9qb</td>
<td>BT02</td>
<td>BT23b</td>
</tr>
<tr>
<td>3q</td>
<td>(Sub)metacentric</td>
<td>9qc</td>
<td>BT02</td>
<td>BT23a</td>
</tr>
<tr>
<td>4p</td>
<td>(Sub)metacentric</td>
<td>4p</td>
<td>BT04</td>
<td>BT15</td>
</tr>
<tr>
<td>4q</td>
<td>(Sub)metacentric</td>
<td>23</td>
<td>BT04</td>
<td>BT15</td>
</tr>
<tr>
<td>5p</td>
<td>(Sub)metacentric</td>
<td>5p</td>
<td>BT12</td>
<td>BT11a</td>
</tr>
<tr>
<td>5q</td>
<td>(Sub)metacentric</td>
<td>5q</td>
<td>BT12</td>
<td>BT11b</td>
</tr>
<tr>
<td>6p</td>
<td>(Sub)metacentric</td>
<td>6p</td>
<td>BT35</td>
<td>BT05a</td>
</tr>
<tr>
<td>6q</td>
<td>(Sub)metacentric</td>
<td>6q</td>
<td>BT06</td>
<td>BT05b</td>
</tr>
<tr>
<td>7p</td>
<td>(Sub)metacentric</td>
<td>7p</td>
<td>BT15</td>
<td>BT02b</td>
</tr>
<tr>
<td>7q</td>
<td>(Sub)metacentric</td>
<td>7q</td>
<td>BT05</td>
<td>BT02a</td>
</tr>
<tr>
<td>8</td>
<td>Telocentric</td>
<td>8q</td>
<td>BT41</td>
<td>BT33</td>
</tr>
<tr>
<td>9qa</td>
<td>Telocentric</td>
<td>11qa</td>
<td>BT13</td>
<td>BT36a</td>
</tr>
<tr>
<td>9qb</td>
<td>Telocentric</td>
<td>11qb</td>
<td>BT13</td>
<td>BT36b</td>
</tr>
<tr>
<td>10p</td>
<td>(Sub)metacentric</td>
<td>12qa</td>
<td>BT27</td>
<td>BT06a</td>
</tr>
<tr>
<td>10q</td>
<td>(Sub)metacentric</td>
<td>12qb</td>
<td>BT27</td>
<td>BT06b</td>
</tr>
<tr>
<td>11qa</td>
<td>Telocentric</td>
<td>19qa</td>
<td>BT16</td>
<td>BT40b</td>
</tr>
<tr>
<td>11qb</td>
<td>Telocentric</td>
<td>19qb</td>
<td>BT16</td>
<td>BT40b</td>
</tr>
<tr>
<td>12</td>
<td>Telocentric</td>
<td>1p</td>
<td>BT13</td>
<td>BT09</td>
</tr>
<tr>
<td>13</td>
<td>Telocentric</td>
<td>1qb</td>
<td>N/A</td>
<td>BT16</td>
</tr>
<tr>
<td>14</td>
<td>Telocentric</td>
<td>2p</td>
<td>N/A</td>
<td>BT12</td>
</tr>
<tr>
<td>15p</td>
<td>(Sub)metacentric</td>
<td>2q</td>
<td>N/A</td>
<td>BT20</td>
</tr>
<tr>
<td>15q</td>
<td>(Sub)metacentric</td>
<td>Missing</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>16</td>
<td>Telocentric</td>
<td>3p</td>
<td>BT16</td>
<td>BT18</td>
</tr>
<tr>
<td>17</td>
<td>Telocentric</td>
<td>3q</td>
<td>BT16 &amp; 24</td>
<td>BT03</td>
</tr>
<tr>
<td>18</td>
<td>Telocentric</td>
<td>4q</td>
<td>BT02</td>
<td>BT31</td>
</tr>
<tr>
<td>19</td>
<td>Telocentric</td>
<td>10qb</td>
<td>BT12 &amp; 35</td>
<td>BT35</td>
</tr>
<tr>
<td>20</td>
<td>Telocentric</td>
<td>21</td>
<td>BT31</td>
<td>BT24</td>
</tr>
<tr>
<td>21</td>
<td>Telocentric</td>
<td>22</td>
<td>BT33</td>
<td>BT26</td>
</tr>
<tr>
<td>22</td>
<td>Telocentric</td>
<td>23p</td>
<td>BT07</td>
<td>BT25</td>
</tr>
<tr>
<td>23p</td>
<td>(Sub)metacentric</td>
<td>Missing</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>23q</td>
<td>(Sub)metacentric</td>
<td>Missing</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>24</td>
<td>Telocentric</td>
<td>24</td>
<td>BT22</td>
<td>BT19</td>
</tr>
<tr>
<td>25</td>
<td>Telocentric</td>
<td>25</td>
<td>BT09</td>
<td>BT07</td>
</tr>
<tr>
<td>26</td>
<td>Telocentric</td>
<td>26</td>
<td>N/A</td>
<td>BT13</td>
</tr>
<tr>
<td>27p</td>
<td>(Sub)metacentric</td>
<td>Missing</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>27q</td>
<td>(Sub)metacentric</td>
<td>27</td>
<td>BT29</td>
<td>BT29</td>
</tr>
<tr>
<td>S. trutta Chromosome ID</td>
<td>Morphology</td>
<td>S. salar Chromosome ID</td>
<td>Linkage ID (Gharbi)</td>
<td>Linkage ID (Leitwein)</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------------------------</td>
<td>-----------------------</td>
<td>---------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>28</td>
<td>Telocentric</td>
<td>28</td>
<td>BT36</td>
<td>BT14</td>
</tr>
<tr>
<td>29p</td>
<td>(Sub)metacentric</td>
<td>16qb</td>
<td>BT10</td>
<td>BT01a</td>
</tr>
<tr>
<td>29q</td>
<td></td>
<td>29</td>
<td>BT06</td>
<td>BT01b</td>
</tr>
<tr>
<td>30</td>
<td>Telocentric</td>
<td>13qa</td>
<td>BT26</td>
<td>BT27</td>
</tr>
<tr>
<td>31</td>
<td>Telocentric</td>
<td>13qb</td>
<td>BT27</td>
<td>BT10</td>
</tr>
<tr>
<td>32</td>
<td>Telocentric</td>
<td>14qa</td>
<td>BT22</td>
<td>BT32</td>
</tr>
<tr>
<td>33</td>
<td>Telocentric</td>
<td>14qb</td>
<td>BT34</td>
<td>BT37</td>
</tr>
<tr>
<td>34</td>
<td>Telocentric</td>
<td>15qa</td>
<td>BT28</td>
<td>BT39</td>
</tr>
<tr>
<td>35</td>
<td>Telocentric</td>
<td>15qb</td>
<td>BT28</td>
<td>BT17</td>
</tr>
<tr>
<td>36</td>
<td>Telocentric</td>
<td>17qa</td>
<td>N/A</td>
<td>BT08</td>
</tr>
<tr>
<td>37</td>
<td>Telocentric</td>
<td>17qb</td>
<td>BT15</td>
<td>BT38</td>
</tr>
<tr>
<td>38</td>
<td>Telocentric</td>
<td>20qa</td>
<td>BT32</td>
<td>BT04</td>
</tr>
<tr>
<td>39</td>
<td>Telocentric</td>
<td>20qb</td>
<td>BT14</td>
<td>BT34</td>
</tr>
<tr>
<td>40</td>
<td>Telocentric</td>
<td>16qa</td>
<td>BT25</td>
<td>BT21</td>
</tr>
</tbody>
</table>

Table 3-1 The nomenclature of brown trout chromosome complement with corresponding chromosomes of Atlantic salmon. Genetic mapping information from two previous reports are also included. Gharbi (Gharbi et al. 2006) [15]; Leitwein (Leitwein et al. 2017) [14], N/A (data not found); Missing (Missing counterpart).

3.5. Discussion

Metacentric and large acrocentric chromosomes bearing blocks of repetitive DNA in Atlantic salmon indeed correspond to multiple small acrocentric chromosomes in brown trout, showing the existence of ancestral Robertsonian fusions (Figure 3-3, 3-4 and 3-5.) and explaining the lower number of chromosomes and chromosome arms seen in Atlantic salmon. The metacentric chromosomes in brown trout, namely Str4 and Str29, both have two corresponding chromosomes in Atlantic salmon (Figure 3-3 and 3-4), indicating the species-specific direction of Robertsonian rearrangements are involved in the speciation of this genus. Our results on the actual cytogenetic relationship between Atlantic salmon and brown trout is largely consistent with earlier findings from comparing high-density linkage maps (Table 3-1). The disagreement lies on the orientation of chromosome arms. In addition, we did not observe the intra-chromosomal inversion between Ssa06 and Str06, as described in Leitwein et al. (2017). Instead, such an inversion was found between Ssa18 and Str02. Considering the physical evidence
collected through banding patterns and FISH in this study, our results are therefore more credible.

There are two intriguing findings in this study. First, there are three brown trout chromosome arms missing corresponding counterparts in Atlantic salmon. Such a finding can be explained by chromosomal arms lost during the speciation of *S. salar*. Considering a burst of transposon took place prior to the Robertsonian fusions in the ancestral Atlantic salmon genome [8], the whole-arm lost is possible by mechanisms such as breakage-fusion-bridge cycle resulted from fusion between sister chromatids following double strand breakages due to transpositions [16]. However, future studies with higher resolution such as whole genome sequencing of brown trout shall be carried to provide definitive confirmation. If indeed the arms are not conserved in Atlantic salmon, functional studies on the genes present in those locations of brown trout can then help provide insight into the speciation of Atlantic salmon. The second intriguing finding is regarding the position of rDNA, which appears to be strictly located on Ssa08p in Atlantic salmon while dispersed on to nearly half of the entire chromosomes complement in brown trout with a relative major location on the largest sub-telocentric chromosomes (Figure 3-6). This finding is largely consistent with previous cytogenetic work with AgNOR/CM-A3 staining on both species [17]. Similar multi-chromosomal locations of rDNA with one pair of chromosomes being the major bearer were also evident in four other salmonid species including rainbow trout (*Oncorhynchus mykiss*), masu salmon (*Oncorhynchus masou*), brook trout and Japanese huchen (*Sakhalin taimen*), suggesting a bidirectional evolution of rDNA is common in salmonids, possibly caused by random recombination [18] and gene duplication or unequal cross-over [17]. One question raised from this observation is if the increased location of rDNA positively correlates with its copy number and expression? Considering all minor locations of rDNA in brown trout is either centromeric or telomeric regions which is consisted of mainly constitutive heterochromatin, the expression of rDNA is therefore likely to be suppressed in these regions. With this in mind, the negative influences such as neuro-degradation from changing the expression of rDNA (in some cases, only certain subunits of rDNA) as seen in human patients [19], [20] might not necessary occur in salmonids.

The differences seen in the cytogenetic complements of brown trout and Atlantic salmon appears to lend support towards the suppressed-recombination models of chromosomal speciation [21], [22], considering their distribution and life cycles. Brown
trout populations are known to occupy much wider distribution than Atlantic salmon with natural and transplanted populations specialized in lacustrine, fluvial and anadromous life histories. In comparison, Atlantic salmon is found to be mainly anadromous and rarely succeed for transplantation. The principle for the different adaption was suggested to be related to Qumsiyeh’s theory (1994) based on mammalian chromosome evolution [22]. In addition, higher recombination is positively correlated to higher chromosome number and therefore encourage the retention of specialized traits required for specific environments [23]. Indeed, a lower recombination rate was observed in chromosomes involved in Robertsonian rearrangements in mice [4].

In conclusion, the cytogenetic evolution of the genus Salmo appears to follow the general trend of re-diploidization with the involvements of varies types of Robertsonian rearrangements and possible arms lost. A nomenclature of brown trout karyotype complement is now generated with physical morphology and corresponding relationship with sister species of the same genus. In comparison to brown trout, more rounds of centric-centric and telo-centric fusions gave rise to a more derived karyotype seen in Atlantic salmon. Since such rearrangements are believed to be facilitated by the burst of transposon elements (TEs) in Atlantic salmon, indicating a loss of control of TEs spread [8], [24]–[26], it is therefore interesting in future research to see how mechanisms governing TEs relate to different evolutionary strategies used in brown trout.
3.6. References


Chapter 4. Turnover of sex chromosomes in Tasmanian Atlantic salmon (*Salmo. salar*)

**Publication:** Genomic instability of the sex determining locus in Atlantic salmon (*Salmo salar*), 2015. G3: Genes, Genomes, Genetics, vol. 5, 2513-2522. Licensed under CC BY 4.0 International.

**Authors:** Lubieniecki KP¹, Lin S¹, Cabana EI¹, Li J¹, Lai YY¹, Davidson WS¹

¹ Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, V5A 1S6 Canada.

**Author contributions:** LS completed all FISH related experiments and sdY sequencing study on Tasmanian salmon and brown trout. LKP, LYY and CEI conducted the sequence assembly of sdY BACs.
4.1. Abstract

Atlantic salmon like other members of the subfamily *Salmoninae*, are gonochoristic with male heterogamety (XX/XY). The SEX locus was mapped to Ssa02 in a European subpopulation whereas fish derived from North America subpopulation were known to harbor SEX on three chromosomes, namely Ssa02, Ssa03 and Ssa06. The sex determining gene, *sdY*, first discovered in rainbow trout, has shown male-specific presence in 13 other species including Atlantic salmon. In the present study, our primary aim is to provide cytogenetic support to confirm the role of *sdY* as the sex determining gene through visualizing its physical locations in both the genome of European and North America derived salmon. FISH analysis using a BAC containing *sdY* reveals a similar genomic environment on these three chromosomes. FISH analysis using plasmid probe containing *sdY*-only insert showed that the signal of *sdY* not only localized to the mapped locations on the chromosomes in both European and North America derived salmon, but also to only one of the sex chromosomes, Y chromosome. From analyzing the *sdY* sequences in both European and North America derived salmon, the gene is well conserved (100%) at the protein level, despite different lengths due to intronic repeats. Together, our study provides the first cytogenetic evident to support *sdY* being the sex determining gene. The similar genomic environment among these three chromosomes may contribute to its translocation.

4.2. Introduction

Teleosts represent a group of fish with around 30,000 species residing in countless aquatic habitats with their own specific environments across the globe. The sex determination systems utilized by teleosts are known to harbor a wide range of mechanisms with numerous factors involved [1]–[3]. Despite the accumulated knowledge gained through a tremendous amount of research, there are only 1700 species (around 6%) with cytogenetic data and several species with known sex determining genes [1], [2]. With more advanced techniques available nowadays such as next generation sequencing (NGS) and high-density single nucleotide polymorphism (SNP) maps, more information shall soon be revealed for this fascinating system.

The sex determination system in teleost can generally be divided into two categories including genetic sex determination (GSD) and environmental sex
determination (ESD) with many species exhibiting overlapping systems. In GSD, both monofactorial male heterogametic (XX/XY) and female heterogametic (ZW/ZZ) are observed. In ESD, factors such as temperature, water pH and population social structures are known to be the determinant of direction towards either ovaries or testes [1]–[3]. In many species such as half smooth tongue sole [4] and Atlantic salmon [5], where GSD is utilized, phenotypically sex reversal is evident when the water temperature is shifted during a certain stage of sex differentiation. This phenomenon is not so surprising considering the poikilotherm’s lack of regulation of body temperature is known to affect many biochemical pathways. [1]

Among all teleost examined cytogenetically, only 10% of them carry heteromorphic sex chromosomes, suggesting a fast turnover in the evolution of sex chromosomes in teleosts [2]. In GSD system, a new sex chromosomes can be generated from an autosome carrying a neo sex determining factor that is powerful enough to independently override the sex differentiation pathway trigged by the old sex determining gene [1]. This scenario was displayed in the emerging of gsdfY in O. luzonensis where gsdf can be independently up-regulated without the signal from dmrt1Y [6]. Alternatively, the same sex determining gene can translocate or transpose into an autosome. Such scenarios involving a Robertsonian fusion was observed in two subpopulation of threespine stickleback (Gasterosteus aculeatus) resulting in a multiple sex chromosome system (X1X1X2X2/X1X2Y) [7]. The mechanism for the fusion is believed to be related to sexually antagonistic traits commonly governed by genes related to mating. In fact, a gene involving sexually dimorphic pigmentation is shown to be closely linked to the female determining locus in Lake Malawi cichlid (WZ/ZZ), giving rise to a new sex chromosomes [8]. In salmonids, sockeye salmon is by far the only species reported for carrying sexually dimorphic chromosomes where males carry a Y chromosome that arose from a fusion between ancestral Y and an autosome, like the stickleback fish. However, there is not yet a link found between such rearrangements and functional traits [9].

Unlike Sry that is conserved in all placental mammals and marsupials [10], sex determining genes are not well conserved in teleost adopting GSD [1], [11], even in close related species. For example, dmrt1Y is used as the sex determining gene in Japanese medaka (Oryzias latipes) [12], whereas gsdfY, a downstream gene trigged by dmrt1Y has emerged as new sex determining gene in the closely related O. luzonensis.
The switch of sex determining gene is caused by mutations in the upstream cis regulatory elements during evolution in the past 5 million years [6], [13]. Interestingly, the original dmrt1Y is no longer present in the genome, probably due to the lack of recombination as a sex determining gene. Prior to the identification of sex determining gene, sdY, in rainbow trout, all the identified sex determining genes in teleost belong to two protein families including DMRT and TGF-β. The former is also conserved in chicken as the sex determining gene. Male (ZZ) chicken is the result of double dosage of DMRT located on chromosome Z [14]. The latter includes several amh related sex determining genes and gsdf related sex determining gene [6], [11], [15], [16]. In salmonids, the lack of conserved sex linkage group from comparative genetic mapping studies indicated the sex determining gene could be species-specific or there is a conserved gene that is able to transpose or translocate into multiple linkages [17], [18]. The latter suggestions was shown to be the case since the sdY, sex determining gene found in rainbow trout, also shown to be male-specific in PCR amplification in 13 other salmonid species [19] [20]. Unlike the other confirmed sex determining genes being known to play direct roles in the sex differentiation pathway, sdY is a duplicated copy of the immune related gene, irf-9 and it determine the maleness by forming a complex with foxl2 to block the production estrogen [21]. It is worth noting that sdY is present in both male and female genome of two relatively young species of subfamily Coregoninae [20]. Therefore, existence of a neo sex determining gene or a different determination system should not be excluded in salmonids.

Within the metapopulation of Atlantic salmon, the sex determining locus was mapped to the sub-telocentric region of Ssa02q in European subpopulations [22], whereas North American derived populations is known to harbor the SEX locus on Ssa02, Ssa03 and Ssa06 depending on the linage. The North American derived populations where multiple SEX locus was found is the farm stocks used in Tasmanian, Australia. These fish were originally transplanted from the River Phillip, Nova Scotia, Canada, back in 1970s [23]. Since the sdY is shown to be male-specific by PCR amplification for the European Atlantic salmon populations [20] and three male lineages of Tasmanian salmon [23], our first aim in the present study was to visualize its surrounding genetic environment by FISH with a BAC probe where sdY is part of the genomic insert. The second aim was to provide cytogenetic support to confirm the role of sdY as the sex determining gene by visualizing its true cytogenetic location among.
European and three lineages of North America derived Tasmanian Atlantic salmon by FISH with a plasmid containing sdY-only insert. Plasmid FISH can also reveal if there were any large-scale chromosomal movements involved in the translocation or jumping. Moreover, nucleotide sequencing of sdY from each of three lineages was carried out to reveal any lineage specific nucleotide changes. Please note this research was a part of published study where flanking regions of sdY were investigated to elucidate any possible mechanism responsible for the multiple locations of the same gene [24]. Interestingly, one recent study with SNPs from whole genome sequencing of these three lineages suggested Ssa02 being the ancestral location of sdY, and translocation to Ssa03 and Ssa06 is a recent event [25]. The information gathered from this present study can be used to widen our understanding of evolution of a newly discovered sex determining gene in teleosts.

4.3. Material and methods

4.3.1. Blood collection and lymphocyte culture

The blood of three European Atlantic salmon (Mowi strain) was collected at the facility of Department of Fisheries and Oceans Canada located at Centre for Aquaculture & Environmental Research in West Vancouver, British Columbia. The blood of one male and one female fish from each of three North America derived Tasmanian lineages (these three lineages had SEX mapped to one of three possible locations including Ssa02, Ssa03 and Ssa06) of Atlantic salmon was collected with support from Commonwealth Scientific and Industrial Research Organization (CSIRO) in Hobart, Tasmania. The same procedure used in Chapter two was adopted for blood collection and lymphocyte culture.

4.3.2. BAC and plasmid probe preparation and FISH

Three marker BACs with known location (www.asalbase.org) were chosen to signal each of three possible chromosomes bearing sdY, respectively. Namely, S0227A12 for Ssa02, S0033O17 for Ssa03, and S0332O19 for Ssa06. The same BAC containing sdY, S0524M13, used in Chapter two was adopted in this chapter. The FISH procedure with BAC markers were the same as described in Chapter two.
FISH analyses using a plasmid containing the Atlantic salmon sdY gene (PCR amplification product obtained using primers from contig 2: Contig 2 FISH 858F and Contig 2 FISH 5976R, Table S1) as the probe required a more elaborate procedure than that for BAC DNA. The plasmid was first labeled using a DIG-nick translation kit (Roche) at 15 °C for 70 min to obtain labeled fragments ranging in size from 200 to 400 bp. The probe mixture for visualizing the location of the sdY gene contained 50 ng of DIG-nick translated plasmid probe and 100 ng of a SpectrumOrange (Vysis) labeled BAC for a particular chromosome (Phillips et al. 2009). Ethanol precipitation of the probe mixture and slide preparation were performed as described for BAC FISH with the modification of air-drying slides for 72 hr at 55 °C before denaturing the chromosomes. The precipitated probe mixture was then dissolved in 10 ml of a solution containing 50% deionized formamide, 2 X SSC, 10% dextran sulfate, 50 mM sodium phosphate, pH 7, before hybridization with chromosome spreads overnight. The coverslip was removed together with the rubber cement seal and the slide was immediately placed in a 2 X SSC wash solution containing 50% formamide at 45 °C for 2 min without agitation. The second wash was performed with immersion in a TNT buffer containing 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 for 2 min. The slide was then incubated with 100 ml of TNB buffer containing 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% blocking reagent for 30 min at 37 °C with a coverslip. The coverslip was then removed by immersion of the slide in TNT buffer for 30 sec. A series of antibody reactions was then performed by incubating 100 ml of each specific antibody for 30 min at 37 °C with a washing step for 3 min in TNT buffer between each of the reactions. The first antibody was mouse monoclonal anti-DIG, 0.5 mg/ml in TNB (Roche Diagnostics, Laval, QC), followed by the second antibody, DIG-conjugated sheep anti-mouse IgG (Millipore (Canada) Ltd., Etobicoke, ON), 2 mg/ml in TNB, and the third antibody was fluorescein conjugated sheep anti-DIG (Roche Diagnostics, Laval, QC), 2 mg/ml in TNB. The antibody attached slide was washed by TNT buffer for 2 min and then subjected to a series of dehydration steps of 70%, 90%, and 100% ethanol for 2 min each. The air-dried slide was finally counterstained with 10 ml of DAPI antifade solution (Life Technologies Inc., Burlington, ON) and sealed with a coverslip using rubber cement. Laser 405 was used to detect the DAPI stain, laser 488 was used for the DIG labeled plasmid probe, and laser 560 was used for the SpectrumOrange labeled BAC probe.
4.3.3. Sequencing of the sdY gene from Tasmanian Atlantic salmon

Various sections of the sdY gene region of Tasmanian Atlantic salmon were first amplified through PCR using TopTaq DNA polymerase (Qiagen) and primers stated in Lubieniecki et al. (2015) [24] to achieve overlapping sequences. Purified PCR products were cloned using either the pSTBlue-1 Acceptor vector kit or pJET 1.2 vector kit. DNA was extracted from 5 ml LB culture of five single colonies picked from each transformation. Restriction digestion was used to confirm the existence of positive inserts in the transformed bacteria prior to the recombinant plasmids being sent for sequencing by Genewiz.

4.4. Results

4.4.1. FISH analyses with BAC and plasmid probes containing sdY

We were curious to determine if S0524M13, the BAC that contains the full sequence of the sdY gene, would hybridize to chromosome 2 (Ssa02) in European Atlantic salmon, as would be predicted from the results of Artieri et al. (2006). Therefore, we performed dual FISH analyses with S0524M13 in combination with other BACs from the set that was used to define the Atlantic salmon chromosomes and integrate them with the linkage groups in the genetic map [26]. S0524M13 hybridized to three pairs of European Atlantic salmon chromosomes, which were identified as Ssa02, Ssa03, and Ssa06 (Figure 4.1). These are the same chromosomes that Eisbrenner et al. (2014) mapped SEX to in Tasmanian Atlantic salmon male lineages [23]. These results suggest that it was not by chance that SEX mapped to these particular chromosomes; rather, there are similar genomic regions in these chromosomes that may facilitate the jumping of the sex-determining gene around the genome. We emphasize that any given male Atlantic salmon is likely to have a single sdY gene, but the BAC containing the sdY gene contains flanking regions that must occur in both male and female salmon and have paralogs that are located in more than one chromosome.

To determine if the sdY gene is located on these chromosomes in the Tasmanian male lineages, we cloned the sdY gene from Atlantic salmon into a plasmid vector (Please see Lubieniecki et al. (2015) for detailed primer information) [24]. This plasmid contains an insert of 5118 bp that did not contain any putative repetitive mobile elements.
on either side of the sdY gene, but the possibility that these elements are located within intronic regions could not be excluded. When this sdY-containing plasmid was used as a probe in dual FISH analyses with BACs known to hybridize to Ssa02, Ssa03, or Ssa06, the results revealed that the sdY gene was indeed on the chromosomes to which SEX had been mapped in these male lineages (Figure 4-2). The plasmid probe hybridized to the ends of the p arm in Ssa03 and Ssa06 and to the end of q arm in Ssa02. Moreover, the sdY-containing plasmid probe hybridized only to one of the chromosomes, presumably the Y chromosome. Also, no large-scale chromosomal movements were evident since the pair of sex chromosomes carries indistinguishable morphological differences. These results supported that sdY is the sex determining gene in both European and North American derived Tasmanian salmon. Moreover, the gene had indeed jumped around the genome in a mechanism similar to transposition rather than chromosomal rearrangements in Tasmanian salmon.

4.4.2. Comparisons of sdY sequences in Atlantic salmon populations

We wanted to determine if the sdY gene sequences were highly conserved in Tasmanian male lineages, which had SEX mapped to Ssa02, Ssa03, or Ssa06 [23]. Our rationale for doing so was that if these sequences were identical, then it would suggest a recent jumping of the sdY gene around the genome. In contrast, if there were several changes, then it would suggest that some time had elapsed since the sdY gene jumped. Using primers [24] we amplified and sequenced the sdY genes from three Tasmanian Atlantic salmon males, which had SEX mapped to Ssa02, Ssa03, or Ssa06. These sequences were compared with the European Atlantic salmon sdY gene sequence from contig 2 (Figure 4-3). The sdY gene consists of four exons (exon 1, 28 bp; exon 2, 360–363 bp; exon 3, 101 bp; and exon 4, 90 bp). The sdY genes in Atlantic salmon from different populations have similar sizes (European Atlantic salmon Ssa02, 4190 bp; Tasmanian Ssa06, 4281 bp; Tasmanian Ssa02, 4292 bp; Tasmanian Ssa03, 4294 bp). The differences in length of the Atlantic salmon sdY genes come from different numbers of a CT dinucleotide repeat in Intron I. Please see Figure S1 in Lubieniecki et al. (2015) for the detailed nucleotide comparison [24]. At the protein level the sdY sequences are identical in terms of length (Figure 4-3). The sdY protein of all Atlantic salmon is 193 amino acids in length. There is 100% protein sequence identity among the sdYs from
three lineages of Tasmanian Atlantic salmon with a synonymous substitution in Ssa02 and Ssa03 male lineages.

Figure 4-1. sdY chromosomal locations of European Atlantic salmon revealed by FISH using BAC probe, S0524M13, containing sdY sequences as part of insert. S0524M13 red fluorescence. Green arrows show S0033O17 (Ssa03 marker), S0332O19 (Ssa06 marker) and S0227A12 (Ssa02 marker) in A, B and C, respectively.
Figure 4-2. sdY chromosomal locations of both European and Tasmanian Atlantic salmon revealed by FISH using plasmid probe, containing sdY sequences as the only insert. Green arrow: sdY plasmid; Red arrow: S0227A12 (Ssa02 marker), S0227A12 (Ssa02 marker), S0033O17 (Ssa03 marker) and S0332O19 (Ssa06 marker) in A, B, C and D, respectively.
Figure 4-3 Multiple alignment of sdY in Tasmanian families and European Atlantic salmon. From top to bottom, Ssa06 Tasmanian sdY, Ssa03 Tasmanian sdY, Ssa02 Tasmanian sdY, European sdY (contig 292). The sdY genes in Atlantic salmon from different populations have similar sizes (European Atlantic salmon Ssa02, 4190 bp; Tasmanian Ssa06, 4281 bp; Tasmanian Ssa02, 4292 bp; Tasmanian Ssa03, 4294 bp). The differences in length of the Atlantic salmon sdY genes come from different numbers of a CT dinucleotide repeat in Intron I. Please see Figure S1 in Lubieniecki et al. (2015) for the detailed nucleotide comparison [24]. At the protein level the sdY sequences are identical in terms of length.
Figure 4-4 Sexually dimorphic sex chromosomes in Nauyak Lake Arctic charr (The top two are males, the lower two are females). The $sdY$ containing BAC, 524M13 (red signals) was used to visualize the locations of $sdY$. Because of the similar genetic environment shared in multiple chromosomes, the BAC hybridizes to multiple locations. Since AC04 is the sex chromosomes, marked by BAC S0175J24 in green signal, the sex chromosomes (Y) is the one bearing both red and green signals. From the female images, both X and Y are morphological indistinguishable whereas it is heteromorphic in male images, indicating a fusion with an autosome.
4.5. Discussion

In the present study, our aim to visualize genetic environments of sdY was accomplished by BAC FISH where a similar genetic environment was found in all three chromosomes known to harbor sdY. The second aim to provide cytogenetic support for sdY being the sex determining gene was achieved by plasmid FISH where sdY signal was only present in Y chromosomes. Moreover, no large-scale genomic movements were linked to the translocation, indicating transposition may be responsible for the multi-chromosome locations of sdY in North American derived Tasmanian salmon. From analyzing the sdY nucleotide sequences among three Tasmanian lineages, the gene is well conserved (100%) at the protein level despite of different length due to intronic repeats located in intron1.

Although the SEX has been mapped to three chromosomes, namely Ssa02, Ssa03 and Ssa06 in Tasmanian Atlantic salmon, the identity of the sex determining gene residing SEX locus was unclear [23]. Cytogenetic dual FISH analyses on European Atlantic salmon showed that a BAC containing the sdY gene hybridized not only to its expected location on European Atlantic salmon chromosomes (i.e., Ssa02) [22] but also to Ssa03 and Ssa06 (Figure 4-1), where SEX had been mapped in Tasmanian families [23]. These results suggested that there are similar sequences in these regions of the genome and that this may facilitate the movement of the sdY gene cassette. The use of a plasmid probe that contains the sdY gene without flanking genomic sequences in dual FISH analyses showed that the sdY gene did indeed localize to the chromosomes where SEX had been mapped in different Tasmanian Atlantic salmon families. Moreover, the probe hybridized primarily to one of the sex chromosomes (Figure 4-2), as would be expected of a male specific gene in a monofactorial GSD.

Since there are not cytogenetical changes in the morphology of the three pairs of chromosomes in all three lineages, chromosomal translocation of large fragments is unlikely to facilitate the sdY jumping. Instead, hybridization of the plasmid sdY gene probe to the telomeric ends of the short arms of Ssa03 and Ssa06 and, as had been predicted previously [22], to the end of the long arm of Ssa02, supports the hypothesis of a male-specific gene (sdY) having been transposed to the ends of different chromosomes not only in different species but also within a species [17], [18]. More supporting evidence favoring transposition came from annotation of flanking region of
The downstream region of sdY where the breakpoint between male and female specific region was found to contain predicted domains of members of the RT_like (reverse transcriptase–like) superfamily, EEP (exonuclease-endonucleasephosphatase) superfamily, and L1-EN (endonuclease of the non-LTR retrotransposon LINE-1) superfamily. Moreover, the upstream region of around 1 kb from 5’ sdY was prone to rearrangement and therefore considered unstable [24].

We searched the literature for other examples of the transposition of a sex-determining gene within a species. Although there is considerable evidence for turnover of sex chromosomes in closely related species of fish [7], [13], [27], [28] in many instances the actual sex-determining gene(s) in the different species is/are not known. These observations rely on genetic mapping of SEX and comparing the locations of sex-linked markers and autosomal markers in different species. We do note, however, that this phenomenon of a jumping sex determining factor has been documented in the fly Megaselia scalaris [29]. Since Tasmanian salmon is the only subpopulation of North American Atlantic salmon showing sdY transposition and all three chromosomes harboring sdY are cytogenetically homomorphic, the transposition may be considered a recent event if it was not found in the original wild stock. Interestingly, a very recent study lends supports to the recent translocation since no divergence was found between Ssa03 and Ssa06 of Tasmanian salmon [25]. Therefore, future research on the wild stock is urgently needed to understand potential unique transposition triggered by potential mechanism such as epigenetic influences on activating cryptic transposons flanking the gene.

Despite our results clearly showing that the transposition of a sex-determining gene, sdY, has occurred in Atlantic salmon, other mechanisms contributing to turn-over of salmonid sex chromosomes can still be in the play. In our preliminary study of Arctic charr sex chromosomes, the sex chromosome (Y) is fused with an acrocentric autosome in one population (Nauyak Lake, NL) while this was not observed in the other population (Tree river, TR). As the result, male NL charr have a diploid number of 77, one chromosome less than female (Figure 4-4). Similar fusion has been previously reported in Japanese stickleback fish and responsible for the reproductive isolation and possible speciation due to the mutations cumulated on the neo-X chromosome [30]. Together with the fact that sdY does not appear to be male specific in subfamily of Coregoninae,
future studies of sex determining gene in salmonid fish will certainly broaden our evolutionary understanding of sex chromosomes and sex determining genes in general.
4.6. References


Chapter 5. Conclusion

Chromosomal rearrangements are observed in a wide range of taxa including insects, teleost and mammals. [1]–[3]. Such large scale genome reorganization has been more frequently reported in recent years due to the advancement of molecular cytogenetic techniques such as comparative genetic mapping and in situ hybridization [4]. However, the outcomes of chromosomal rearrangements are not clear and often vary between species, indicating interference from genomic background [5], [6]. In broad terms, chromosomal rearrangements can be broken into two forms; balanced and unbalanced, depending on whether there is a net gain or loss of genetic materials. Even though both forms can result in deleterious outcomes because of resulting gene disruption, generation of fused genes and interference of gene regulation etc., the balanced form is considered relatively less harmful and therefore conferred with the possibility of transmission into both daughter cells and gametes. The resulting relocations of chromosomal segments are used to categorize these rearrangements into five major classes, namely inversion (paracentric and pericentric), translocation, fusion and fission. In a special case of rearrangements involving whole arm movement, they are often referred to as Robertsonian rearrangements in honor of William Robertson, the first reporter of such genome reorganization [7].

Chromosomal rearrangements have been empirically considered underdominant due to the observation of heterozygotes with reduced fertility and in some cases, infertile [4]. Such outcomes are normally due to mitotic or/and meiotic segregation problems associated with aneuploidy. With the accumulating data available in recent years, a closer examination of possible evolutionary outcomes of each of various rearrangements is possible. Among all five classes of rearrangements, tandem fusion (centromere-telomere and telomere-telomere) and fission are rarely encountered due to severely deleterious outcomes such as dicentric chromosomes arising from the former, resulting in 50% reduced production of balanced gametes [8], [9], and difficulty to reassemble functional telomeres and centromeres in the latter [10]. In comparison to such strong underdominance, translocations (including Robertsonian) and centric fusions (Robertsonian) are often reported and therefore considered mild underdominant [4], [5].
During the years of cytogenetic studies of various chromosomal rearrangements, multiple theoretical models of chromosomal speciation have been proposed [5]. Despite a lack of universal models to explain all observations in different taxa, reduced gene flow due to suppressed recombination has been generally accepted as a common outcome of such a large-scale genome reorganizations [4], [5], although supporting evidence are mostly from inversions and Robertsonian fusions in plants and animals [11]. The reduced recombination is shown to provide protection of co-adapted alleles and consequently confers adaptive advantage and consequent speciation. Numerous examples of unbalanced gametes are often produced from meiotic recombination and non-disjunction in inversion and Robertsonian fusions/translocation, respectively, heterologous synapsis was found to avoid deletion from loop formation in rodents and primates with inversions [12], [13], and no meiotic non-disjunction was reported in dogs with Robertsonian fusions [14]. Therefore, reproductive impairments due to chromosomal rearrangements requires consideration of more involving factors such as size of rearranged segments and genomic background.

Besides providing protection for mutation from suppressed recombination, chromosomal rearrangements are well known triggers for modified gene expression even in cases of balanced rearrangements, especially for the region nearby the break point [6]. Classic examples of modified expression include fused a gene due to the translocation into existing gene sequence; down regulation due to formation of cryptic promoters [15]; gene silencing due to translocation of actively expressed genes into heterochromatin regions [16] and genome-wide effects on expression due to changed nuclear reorganization [17]. Therefore, mild to near-neutral underdominance on reproduction impairment and generation of advantageous mutation or changes on gene expression would synergize the transmission of chromosomal rearrangements [4]. Moreover, recent data has suggested meiotic drive due to centromere strength being involved in the maternal selection of chromosomes resulting from Robertsonian fusion [18]. In the case of strong underdominance such as tandem fusions, drift can be achieved by dramatic changes in demographic conditions, such as a small effective population (population bottleneck) [19].

The accumulating fact that intra-species chromosomal polymorphism can be observed in one species, while closely related species exhibit stable karyotype suggests the occurrence of rearrangements are not random. So what are the possible triggering
mechanisms? Numerous studies have provided possible triggers with evidence related to repeat elements [20], segmental duplication (low-copy repeats with size spanning 150 kb) [21] and genome fragile sites [22]. All three aspects of evidence are contributing to the initial double strand break required for the rearrangements. Once the initial rearrangement took place (for examples, failures on suppressing TEs), the persistence of those in mild underdominance should be longer than those in strong underdominance. Over time, if advantageous mutations occur in the heterozygous karyotype due to the suppressed recombination, selection can then promote the transient from heterozygote into homozygotes rearrangements without the help of meiotic drive [5].

Keeping both theoretical information and empirical data mentioned above in mind, we can then try to interpret our observation in the chromosomal evolution of the genus *Salmo*. From the genome assembly of Atlantic salmon and previous studies of inter-species comparative mapping, a plausible scenario of genome evolution was proposed. The ancestral genome duplication that occurred around 80 MYA in the common ancestor of extant salmonids gave rise to a stressed duplicated genome followed with species radiation during the climate cooling stage around 60 MYA triggered by reactivation of transposable elements, explaining such high content of repeat elements (58-60%) found in the genome [23], [24]. The subsequent large scale genome reorganization gave rise to extant species with large collinear blocks in a species specific orders [2]. In the genus of *Salmo*, large scale rearrangements including tandem fusions, centric fusions and possible chromosome arm loss were evident in the present research to give rise to the extant karyotypes of *S. salar* and *S. trutta*. The fact that no polymorphic karyotype of the *S. trutta* has been observed in present research and other publications suggests a relatively stable genomic background. In contrast, polymorphic karyotypes due to Robertsonian rearrangements of three pairs of chromosomes were found in Canadian subpopulations of *S. salar*. The fixation of the homozygous translocation in most Canadian populations suggests that: 1. the ancestral heterozygous state of translocation was mild to near-neutral underdominance and allowed the transition to a homozygous rearrangement 2. the fixation of homozygous translocation indicates possible fixation of advantageous mutations. On the other hand, the observation of potentially deleterious Robertsonian tandem fusions (Ssa08 and Ssa29, centro-telo fusion produce dicentric chromosomes) in all Canadian populations indicate the initial rearrangement likely took place in a small effective population and
subsequent spreading into other river population through colonization. Besides largescale genomic rearrangements, genic movement was evident in this research where the sex determining gene was found with three possible locations in a population of S. salar originating from a Canadian river, while no such evidence has been reported in European populations. Evidence from the flanking region of sdY suggests a jumping mechanism by a retrotransposon, which is not surprising to see in a genome with 58-60% of repeat content. Therefore, future studies are urged to address the mechanism behind the apparent overall instability of genome of Canadian S. salar. Future research focusing on epigenetic, synapsis analysis, functional studies are necessary to understand any selective force and the precise mechanism facilitating such polymorphic karyotype of Canadian S. salar. For example, a comparative methylation study around sdY region between Tasmanian population and the original Canadian river population could provide a possible epigenetic signature that might facilitate such jumping event; comparative expression studies could potential identify expression changes related to the three rearrangements and synapsis analysis can be carried out to exam any meiotic drive on the rearranged chromosomes.
References


Appendix A. Supplementary images for Chapter two

Supplementary figure A-1: FISH-images of 3 BAC probes representing Ssa01 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa01p (Str12): S0198E23 in red; Ssa01qa (Str01): S0196K13 in green, Ssa01qb (Str13): S0088O23 in red.

Supplementary figure A-2: FISH-images of 2 BAC probes representing Ssa02 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa02p (Str14): S0227A22 in green; Ssa02q (Str15): S0002E22 in red.
Supplementary figure A-3A: FISH-images of 2 BAC probes representing Ssa04 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa04p (Str04p): S0022M13 in green; Ssa04q (Str18): S0045H04 in red.

Supplementary figure A-3B: FISH-images of 2 BAC probes representing Ssa04p and Ssa23 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa04p (Str04p): S0022M13 in green; Ssa23 (Str04q): S0102N22 in red.
Supplementary figure A-4: FISH-images of 2 BAC probes representing Ssa10 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa10qa (Str20): S0439A22 in green; Ssa10qb (Str23): S0143A06 in red.

Supplementary figure A-5: FISH-images of 2 BAC probes representing Ssa03 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa03p (Str16): S0033O17 in green; Ssa03q (Str17): S0492D06 in red.
Supplementary figure A-6: FISH-images of 3 BAC probes representing Ssa09 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa09qa (Str19): S0048N03 in green; Ssa09qb (Str3p): S0109O02 in red; Ssa09qc (Str3q): S0175J24 in green.

Supplementary figure A-7: FISH-images of 2 BAC probes representing Ssa13 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa13qa (Str30): S0061B01 in green; Ssa13qb (Str31): S0229A14 in red.
Supplementary figure A-8: FISH-images of 2 BAC probes representing Ssa14 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa14qa (Str32): S0249L01 in green; Ssa14qb (Str33): S0205B09 in red.

Supplementary figure A-9: FISH-images of 2 BAC probes representing Ssa15 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa15qa (Str34): S0092M14 in green; Ssa15qb (Str35): S0121A09 in red.
Supplementary figure A-10: FISH-images of 2 BAC probes representing Ssa17 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa17qa (Str36): S0148O07 in green; Ssa17qb (Str37): S0031H11 in red.

Supplementary figure A-11: FISH-images of 2 BAC probes representing Ssa20 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa20qa (Str38): S0067N05 in green; Ssa20qb (Str39): S0118K06 in red.
Supplementary figure A-12: FISH-images of 2 BAC probes representing Ssa16 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa16qa (Str40): S0216J09 in green; Ssa16qb (Str29p): S0097E23 in red.

Supplementary figure A-13: FISH-images of 2 BAC probes representing Ssa05 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa05p (Str05p): S0026A22 in green; Ssa05q (Str05q): S0236C16 in red.
Supplementary figure A-14: FISH-images of 2 BAC probes representing Ssa06 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa06p (Str05p): S0322O19 in green; Ssa06q (Str06q): S0003A07 in red.

Supplementary figure 1-15: FISH-images of 2 BAC probes representing Ssa07 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa07p (Str07p): S0225J21 in green; Ssa07q (Str07q): S0222K14 in red.
Supplementary figure A-16: FISH-images of 1 BAC probe representing Ssa08 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa08q (Str08): S00214J02 in red.

Supplementary figure A-17: FISH-images of 2 BAC probes representing Ssa11 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa11qa (Str09qa): S0056B24 in green; Ssa11qb (Str09qb): S0123C06 in red.
Supplementary figure 1-18: FISH-images of 2 BAC probes representing Ssa12 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa12qa (Str10p): S0056C01 in green; Ssa11qb (Str10q): S0457I14 in red.

Supplementary figure A-19: FISH-images of 2 BAC probes representing Ssa19 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa19qa (Str11qa): S0145N16 in green; Ssa11qb (Str09qb): S0006P09 in red.
Supplementary figure A-20: FISH-images of 2 BAC probes representing Ssa21 and Ssa22, respectively, in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa21 (Str21): S0245I09 in green; Ssa22 (Str22): S0018L24 in red.

Supplementary figure A-21: FISH-images of 2 BAC probes representing Ssa24 and Ssa25, respectively, in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa24 (Str24): S0068A03 in green; Ssa25 (Str25): S0105M10 in red.
Supplementary figure A-22: FISH-images of 2 BAC probes representing Ssa27 and Ssa29, respectively, in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa27 (Str27): S0166I14 in green; Ssa29 (Str29): S0016D16 in red.

Supplementary figure A-23: FISH-images of 2 BAC probes representing Ssa26 and Ssa28, respectively, in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa26 (Str26): S0059P02 in green; Ssa28 (Str28): S0091M08 in red.
Supplementary figure A-24: FISH-images of 2 BAC probes representing Ssa29 and Ssa08, respectively, in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa29 (Str29): S0016D16 in green; Ssa08q (Str08): S0214J02 in red.

Supplementary figure A-25: FISH-images of 3 BAC probes representing Ssa01 and Ssa23, respectively, in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa01p (Str12): S0198E23 in red; Ssa01qb (Str01): S0088O23 in red; Ssa23 (Str4q): 102N22 in green. Atlantic salmon photo was previously published in [Breonna-Hansen et al., 2012]
Supplementary figure A-26: FISH-images of 2 BAC probes representing Ssa18 in Atlantic salmon (Left) and corresponding chromosomes in brown trout (Right). Ssa18qa (Str12q): S0214J02 in green; Ssa18qb (Str12p): S0113M17 in green.