

# **Sex Determination in Tasmanian Atlantic Salmon**

**by**

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## **Abstract**

Although male heterogamety controls Atlantic salmon sex, hormone treatment can induce sex reversal. In Australia where Atlantic salmon males are unmarketable, sex reversed females (neo-males) are crossed with females to produce all female stock. However, neo-males are indistinguishable from males making early male culling difficult. Therefore, a sex-specific genetic marker was needed to make this distinction. With no such marker available offspring sex was predicted via familial microsatellite analysis. Markers from Chromosome 2 (Ssa02), where the sex locus (*SEX*) previously mapped, predicted test family offspring sex inaccurately. A 64 SNP genome-wide scan suggested Chromosome 6 (Ssa06) housed *SEX* instead. Analysis of 38 male lineages revealed three sex loci on Ssa02, Ssa06 and Ssa03 with 34, 22 and 2 representative families respectively. An exon PCR test for the rainbow trout master sex-determining gene (*sdY*) was consistent with a single sex-determining gene that jumps around the genome in Atlantic salmon.

**Keywords:** Atlantic salmon; sex loci; genetic mapping; *sdY*; jumping gene

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# Table of Contents

Approval.....	ii
Partial Copyright Licence .....	iii
Abstract.....	iv
Acknowledgements.....	v
Table of Contents.....	vi
List of Tables.....	viii
List of Figures .....	viii
<b>1. Introduction .....</b>	<b>1</b>
1.1. Sex determination morphology and implications .....	1
1.2. Sex determination and sex differentiation .....	2
1.3. Sex determination in mammals.....	3
1.4. Environmental sex determination.....	5
1.4.1. Temperature dependent sex determination .....	6
1.4.2. Behavioural sex determination .....	7
1.4.3. Exogenous steroids.....	8
1.4.4. Parthenogenesis .....	8
1.5. Genetic sex determination .....	9
1.5.1. Male heterogamety.....	11
1.5.2. Female heterogamety .....	13
1.5.3. Allelic sex determination.....	14
1.6. Other vertebrate sex-determining systems .....	14
1.7. Genome duplication and sex in fish .....	16
1.8. Sex determination in Salmonidae .....	17
1.9. Sex and Aquaculture.....	18
1.10. Project aims .....	19
<b>2. Methods .....</b>	<b>20</b>
2.1. SALTAS Atlantic salmon population samples.....	20
2.2. Genotyping analyses .....	21
2.3. PCR-based <i>sdY</i> test for Atlantic salmon males .....	25
<b>3. Results .....</b>	<b>27</b>
3.1. Screening Tasmanian Atlantic salmon with microsatellite markers on Ssa02 .....	27
3.2. Mapping of <i>SEX</i> to Ssa06 in Tasmanian Atlantic salmon.....	27
3.3. Screening the SALTAS Atlantic salmon broodstock with Ssa02 and Ssa06 genetic markers .....	28
3.4. Development of a PCR-based test to identify Tasmanian Atlantic salmon males .....	31

<b>4. Discussion.....</b>	<b>33</b>
4.1. Multiple sex loci within a species is rare.....	33
4.2. A single conserved sex-determining gene.....	33
4.3. Mechanisms of <i>sdY</i> movement from one chromosome to another .....	37
4.4. Why these three loci? .....	38
4.5. Are there more than three <i>SEX</i> loci in Atlantic salmon?.....	39
<b>5. Conclusions .....</b>	<b>41</b>
<b>References .....</b>	<b>42</b>
<b>Appendix. Supplementary Tables and Figures .....</b>	<b>51</b>
Supplementary Table S1. Pedigree of the lineages with representation in the 2009 year class .....	51
Supplementary Table S2. The 64 SNPs chosen for the genome wide scan. Detailed linkage groups (LG)s can also be found at <a href="http://www.asalbase.org">www.asalbase.org</a> . Additional SNP information is available online at <a href="http://www.asalbase.org">www.asalbase.org</a> .....	53
Supplementary Table S3. Comprehensive Survey of the 2009 Year class. Microsatellite SNP and <i>sdY</i> raw data is organized by families.....	55
Supplementary Table S4. Microsatellite and SNP analysis of SALTAS broodstock families in which the sex-determining gene resides on Ssa06 (AS-4). Markers are arranged in order corresponding to their placement on the female European Atlantic salmon linkage map (See Phillips et al. 2009 for nomenclature) <a href="http://www.asalbase.org">www.asalbase.org</a> . The seven families originally analyzed are highlighted yellow.....	63
Supplementary Table S5. Microsatellite and SNP analysis of SALTAS broodstock families in which the sex-determining gene housed on Ssa02 (AS-1). Markers are arranged in order corresponding to their placement on the female European Atlantic salmon linkage map ( <a href="http://www.asalbase.org">www.asalbase.org</a> ).....	64
Supplementary Table S6. Microsatellite and SNP analysis of SALTAS broodstock families in which the sex-determining gene housed on Ssa03 (AS-11). Markers are arranged in order corresponding to their placement on the female European Atlantic salmon linkage map. <a href="http://www.asalbase.org">www.asalbase.org</a> .....	65
Supplementary Table S7. Summary of individuals in which <i>sdY</i> call, phenotype call and/ or genotype call do not agree. Discrepancies are highlighted green. ....	66
Supplementary Table S8. Concordance of <i>sdY</i> with genotype and phenotype of the 2009 year class (YC) families.....	68
Supplementary Table S9. Individuals in which only a single exon of <i>sdY</i> (exon 2 or exon 4) was present. A 1 indicates the presence of a particular condition (Fabp6b, No Fabp6b). ....	69

## List of Tables

Table 3.1.	Phenotypic male individuals from the 2009 year class in which SEX may have moved by recombination, translocation or transposition (see Figure 4.1 for details). .....	30
Table 3.2.	Some explanations of inconsistent outcomes between phenotype, genotype and sdY. For a fuller discussion see Figure 4.1. ....	31

## List of Figures

Figure 1.1.	A simplified model highlighting some important factors in male and female sex determination and differentiation pathways. Initially the bipotential gonad is primed to develop into testis or ovaries. Figure taken from Li 2010.....	3
Figure 1.2.	Summary of genetic sex determining mechanisms.....	10
Figure 2.1.	An example of genotype analysis using a single marker linked to the sex locus. ....	21
Figure 2.2.	Lineage containing the four sires of the seven families in the initial pilot study. ....	22
Figure 2.3.	An example of the TaqMan probe marker genotyping result. ....	24
Figure 3.1.	An abstract of the second largest family (2009_32), in which the sex locus mapped Ssa03, to illustrate the comparisons of phenotype to sdY phenotype predictions and haplotype predictions of phenotype. ....	29
Figure 3.2.	sdY triplex PCR test with primers (see methods) from exon 2 (~110 bp) exon 4 (~250 bp) and fabp6b (~450 bp) (Y. Lai personal communication). ....	31
Figure 4.1.	Mechanisms of sdY movement during meiosis of a sire with SEX located on Ssa02.....	36
Figure 4.2.	Predicted structure of the sdY protein (green ribbon) based on sequence similarity to Interferon regulatory factor 3 association domain (white ribbon: 1QWT) and the well conserved DNA binding domain (blue ribbon 1T2K). Note the orientation of the DNA binding domain is not positioned physiologically with respect to the association domain.....	36



# 1. Introduction

Regardless of how or why sexual reproduction arose, vertebrates today consequently exist primarily as male, female (gonochoristic species) or some combination of the two (hermaphroditism) (Devlin and Nagahama 2002). In gonochorists, an individual develops as either male or female and remains male or female for its lifetime. Hermaphroditic individuals can be either sequential or synchronous. As the terms imply sequential hermaphrodites develop first as one sex (male–protoandrous / female-protogynous) and later convert to the opposite sex while synchronous hermaphrodites produce and maintain functional male and female gametes simultaneously throughout their life (Devlin and Nagahama 2002). However, in some synchronous animals like the Poey (*Rivulus marmoratus*) self-fertilization has been taken to an extreme resulting in clonal reproduction of totally homozygous offspring (Kallman and Harrington 1964, Devlin and Nagahama 2002).

## 1.1. Sex determination morphology and implications

In either case the general pathway leading to male or female gonads consists of two main stages: i. development of the urogenital ridge and subsequent bipotential gonad and ii. morphological differentiation of the bipotential gonad to form either male or female gonads (Shoemaker and Crews 2009). The latter of the two stages differs minimally between highly diverse vertebrates suggesting that the underlying pathways of sex differentiation are conserved (Graves and Peichel 2010). However, the mechanisms that determine whether an individual will become male or female are highly diverse including a wide array of environmental and genetic determinants. Although this diversity exists across many different eukaryotes, all these systems can be observed in a single group, fish (Devlin and Nagahama 2002, Volff et al. 2007). Moreover, any one of these systems is labile to environmental influences that may alter sex phenotype even after a particular sex determination pathway has been initiated, particularly if certain factors are not maintained (Devlin and Nagahama 2002). Fish sex determination and

differentiation in particular can be highly influenced by exogenous steroid and pollutants in the environment (Devlin and Nagahama 2002, Hewitt et al. 2008).

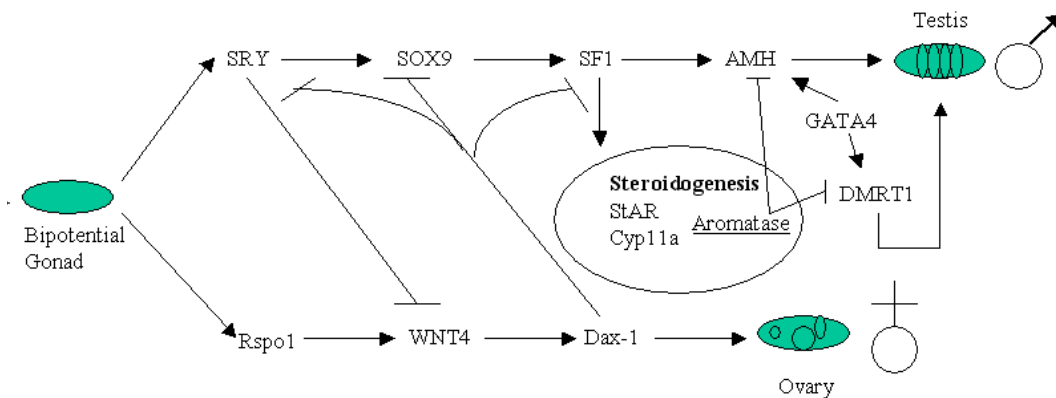
## **1.2. Sex determination and sex differentiation**

Before continuing, it is important to distinguish between sex determination and sex differentiation. Due to the great variety in factors and mechanisms that govern sex determination it is difficult to give a black and white definition for this term. In general sex is considered determined when the bipotential gonad begins to follow a stable pathway leading to morphological development of either female or male gonads. For example, in mammals, a high mobility group (HMG) transcription factor, the sex-determining region Y (*Sry*) gene (Sinclair et al. 1990, Koopman et al. 1990) sets off a cascade that ultimately results in male development (Munger and Capel 2012). *Sry*, similar to other HMG transcription factors alters transcription of target genes by bending bound DNA (Hsiao et al. 2003). In this case sex is considered to be determined upon fusion of gametes because *Sry* is either present or absent in the genome of the resulting individual. Alternatively in many reptiles the incubation temperature during a short window determines the sex of the offspring. In this case it is unknown what the temperature during the window will be until that window period arrives and thus sex is determined in this short window (Shoemaker and Crews 2009). In yet other cases sex determination can be considered to occur multiple times. For example, in sequential hermaphrodites intrinsic or extrinsic factors initiate a male determining pathway, which later may convert to a female pathway due to further environmental factors. This flexibility in sex determination makes the definition of sex determination as well flexible (Devlin and Nagahama 2002). Therefore, the term sex determination here will refer to the presence or absence of any factor that initiates a temporarily stable sexual differentiation pathway. Thus, this allows sex determination to occur multiple times followed by a particular differentiation pathway and the timing of sex determination is therefore also flexible.

### 1.3. Sex determination in mammals

Because the pathways of sex determination and sex differentiation share many conserved components among vertebrates the analysis of the sex-determining pathway in one vertebrate can provide insight to the pathways of many other vertebrates (Devlin and Nagahama 2002, Graves and Peichel 2010). Of course the sex-determining and differentiation pathways are most extensively studied in mammals and thus are the choice for review here.

Today evidence suggests the development of the urogenital ridge and bipotential gonad in mammals is controlled and maintained by a multitude of male and female factors that act antagonistic to one another in a tug a war fashion. Thus the bipotential gonad remains in an undifferentiated state until this scale is tipped (by the presence or absence of *Sry*) in favour of the male or female pathway (Figure 1.1, Munger and Capel 2012).



**Figure 1.1.** A simplified model highlighting some important factors in male and female sex determination and differentiation pathways. Initially the bipotential gonad is primed to develop into testis or ovaries. Figure taken from Li 2010.

In the presence of sex determining region Y (*Sry*) female factors such as Wingless related MMTV integration site 4, (*Wnt4*) are inhibited tipping the scale in favour of the male pathway. *Sry* binds the promoter of sex determining region box Y-box 9 (*Sox9*) which in turn binds its own promoter with higher affinity in addition to inducing expression of many other male promoting genes ultimately leading to testis formation. If *Sry* is absent the female pathway dominates, *Sox9* is not upregulated due to inhibition by female promoting genes such as dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (*dax1*) and ovaries develop. SF1: steroidogenic factor 1; AMH: anti-Mullerian hormone; Aromatase: cytochrome P450, family19; GATA4: GATA binding protein 4.

It is important to remember that even though a gonad is initially undifferentiated the sex of the individual has already been determined by the presence or absence of *Sry*. Several antagonistic factors are expressed in what seems like a gradient fashion where, for instance wingless related MMTV integration site 4 (*Wnt4*), a female promoting gene is predominantly expressed at one end of the gonad while, Fibroblast growth factor 9 (*Fgf9*), a male promoting gene is predominantly expressed at the other (Munger and Capel 2012). Both of these antagonistic proteins can act extracellularly influencing neighbouring cells indirectly via respective pathways initiated by the appropriate receptor (Bernard et al. 2012, Eggers and Sinclair 2012). As a result, the region where the expression of these two genes meets, each of their independent expression levels is decreased and the gonad remains undifferentiated. The mechanisms as to how each cell chooses which side to be on is likely dependent upon several external factors from surrounding cells, however there is also evidence that the tug of war between the male and female genes occurs within cells (Munger and Capel 2012). Moreover the finding that both male and female pathways appear to be primed to go in both XX or XY embryos suggests the gonad is capable of becoming male or female even in the absence of *Sry* (Jameson et al. 2012). In the absence of *Sry* the female pathway becomes dominant after a short window (~1.5 days in mice) and as a result is termed the default pathway (Hiramatsu et al. 2009).

It is not known what exactly controls *Sry* expression but loss of function experiments suggest a number of factors that may be involved (Munger and Capel 2012). Moreover, due to the sweeping pattern of *Sry* expression initiated at the center of the gonad and progressing outward committing cells to a pre-sertoli state, it is likely that up regulation of *Sry* in one cell may influence surrounding cells to adopt a similar fate. However, if *Sry* is expressed within the narrow window, the resulting HMG box transcription factor, *Sry*, up regulates another downstream HMG box transcription factor, *Sox9*. In turn *Sox9* binds the promoters of a multitude of other genes involved in the male determining pathway many of which, including *Fgf9* (Kim et al. 2006) and intracellular prostaglandin D<sub>2</sub> synthase (PGD<sub>2</sub>S) (Wilhelm et al. 2005), function in feed forward loops to further upregulate *Sox9* as well. Both *Fgf9* (mentioned previously) and PGD<sub>2</sub>S exert their actions indirectly. PGD<sub>2</sub>S converts prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) to PGD<sub>2</sub>, which in turn is thought to stimulate *Sox9* indirectly via a cell surface receptor (Wilhelm

et al. 2005). Moreover Sox9 binds its own promoter with higher affinity than *Sry* further upregulating itself (Sekido and Lovell-Badge 2008). It is not currently known whether or not Sox9 inhibits the expression of *Sry* (Munger and Capel 2012).

The feed forward loops initiated by *Sry* tip the tug of war in favour of male development and the potentially antagonistic genes from the female pathway such as *Wnt4*, *Rspo1* (Rspodin homolog 1),  $\beta$ -catenin, and *Foxl2* (forkhead box L2), can no longer keep *Sox9* expression at basal levels to maintain the bipotential gonad (Kim et al. 2006, Uhlénhaut et al. 2009). *Wnt4*, *Rspo1* and  $\beta$ -catenin operate in the same pathway to suppress *Sox9* expression via  $\beta$ -catenin competing antagonistically with Sf1 for the testis specific enhancer (Tesco) (Bernard et al. 2012). For the *Foxl2* transcription factor the target is also Tesco (Eggers and Sinclair 2012). The level of *Sox9* appears to be dose dependent and is critical for determining differentiation of the support cells of the gonad (Morrish and Sinclair 2002). If *Sox9* does not reach a pre-determined threshold, supporting cells will adopt an ovarian state (Munger and Capel 2012). It appears that even after a particular pathway has been chosen that pathway must still be maintained and therefore *Sox9* and other male promoting factors must continually repress ovarian promoting genes and likewise the reverse is true for maintenance of ovaries in females (Uhlénhaut et al. 2009, Matson et al. 2011). It should be noted that not all cells that initially express *Sry* reach critical levels of *Sox9* to adopt a pre-sertoli state and as the short window closes before the ovarian pathway takes over, a minimum number of pre-sertoli cells expressing *Sox9* above threshold level must be reached before the gonad commits to the male pathway. If too few cells are present the female pathway is adopted (Munger and Capel 2012).

## **1.4. Environmental sex determination**

Several physiological factors within the environment are capable of influencing offspring sex. These influencing factors include temperature as seen in many reptiles (Shoemaker and Crews 2009), exogenous chemicals typically affecting fish (Devlin and Nagahama 2002), and social behavioural interactions and cues generally observed in hermaphroditic species (Devlin and Nagahama 2002). In particular, poikilotherms including both reptiles and fish are subject to temperature changes due to the many enzymes involved in sex determination and differentiation pathways. It is well known

that enzyme activity is highly dependant upon temperature (Devlin and Nagahama 2002).

#### **1.4.1. Temperature dependent sex determination**

In many reptiles including turtles, crocodylians, tuataras and some lizards sex is determined by incubation temperature of the developing embryo (Shoemaker and Crews 2009). This phenomenon, termed temperature dependent sex determination, occurs in a narrow window of time for which the embryo is temperature sensitive. The effects of temperature during this sensitive period are unique to each species. For example, during the temperature sensitive period in the red-eared slider turtle (*Trachemys scripta*) an increase in incubation temperature from 26°C to 31°C promotes female development while the American alligator (*Alligator mississippiensis*) exhibits a narrow male promoting temperature range (32.5°C – 33°C) flanked by female promoting temperatures (Shoemaker and Crews 2009). In addition to the different responses to changes in temperature, the timing of the temperature sensitive period differs as well depending on the species chosen.

It is important to note that many of the factors involved in genetic sex-determining mechanisms such as Sf1, Sox9, Sox8, Fgf9, Mis (amh) (see below) are also present in individuals with temperature dependant sex-determining mechanisms; however, their roles are not necessarily the same. For instance, while Sox9 is a key regulator of testis formation in mammals, in reptiles (sea turtle *Lepidochelys olivacea*, as an example), Sox9 is expressed early at both male and female promoting temperatures but at the end of the temperature sensing window is present only in males (Torres-Maldonado et al. 2001, Torres-Maldonado et al. 2002). In addition, shifts from the male promoting temperatures to female promoting temperatures during the sensitive window, resulted in Sox9 expression and protein level down regulation and vice versa for female to male promoting temperature shifts (Torres-Maldonado et al. 2001, Moreno-Mendoza et al. 2001). The same pattern was observed in 3 other reptiles, *T. scripta* (Shoemaker et al. 2007), *Chelydra serpentina* (Rhen et al. 2007) and *Eublepharis macularius* (Valleley et al. 2001), but not the American alligator where Sox9 is absent until the end of the temperature-sensing period (Western et al. 1999). Therefore Sox9 can be considered an important factor involved in commitment to the male pathway, although other

molecules affected by temperature are likely responsible for the early stages of sex determination and directly or indirectly regulate *Sox9* expression.

Of the other genetic factors involved in vertebrate sex determination, *Sf1* shows the greatest potential as a temperature-sensing molecule as it is expressed early in the bipotential gonad of two turtle species (*Trachemys scripta* and *Chrysemys picta*) (Fleming et al. 1999). In these species, *Sf1* expression increased at male promoting temperatures and decreased at female promoting temperatures (Fleming et al. 1999, Valenzuela et al. 2006). However, in the American alligator and other turtle species tested this was not the case, and in fact, *Sf1* levels went up during the female promoting temperature (Shoemaker and Crews 2009). Thus, *Sf1* may be responsible for testis development in the two turtle species in addition to its apparent more conserved role in development of the bipotential gonad discovered in mammals (Luo et al. 1994, Shoemaker and Crews 2009).

These two genes are merely examples. For a full discussion about genes involved in sex-determining pathways and their comparisons between organisms with genetic and temperature dependent sex-determining mechanisms, please see Shoemaker and Crews (2009).

The above examples of temperature sex determination in reptiles are illustrative of gonochoristic species where the individual's sex remains more or less fixed following the temperature sensitive window. However, in fish species temperature influences can also be present in conjunction with other sex-determining mechanisms, such as in genetic sex determination, and will be discussed further later.

#### **1.4.2. Behavioural sex determination**

In hermaphroditic species, sex determination may occur multiple times and sex differentiation may proceed down either a male or female promoting pathway dependent upon environmental cues. Most important is the lack of a sex-determining window, outside of which sex becomes fixed. Consequently, sex determination seems to remain pliable throughout an individual's life and social and behavioural cues related to status within a population determine an individual's sex (Devlin and Nagahama 2002).

Ultimately, an individual's status is controlled by level of dominance with respect to others in the population (Fishelson 1970), size compared to others in the population (Devlin and Nagahama 2002), number of individuals of the same sex in the population and potential pheromones or chemical stimuli present (Devlin and Nagahama 2002). For example, in the protogynous species *Anthias squamipinnis* the presence of a dominant male prevents sex reversal of the larger group of females (Fishelson 1970). When the male is removed from the tank one of the larger females in the group will sex reverse to become male (Fishelson 1970). Similar patterns can be observed for several protogynous and protoandrous species (Devlin and Nagahama 2002). However, other less dominant individuals in a population may sex reverse even in the presence of a dominant individual (Moyer and Zaiser 1984).

#### **1.4.3. Exogenous steroids**

Exogenous steroids are used routinely in aquaculture to manipulate offspring sex creating monosex stock (Davidson et al. 2009). This will be discussed further in later sections. However, there are several examples of sex reversal or cases of intersex individuals in wild populations of fish that have been directly affected by steroid containing pollutants and this has also been extensively reviewed in (Devlin and Nagahama 2002, Hewitt et al. 2008, Söffker and Tyler 2012).

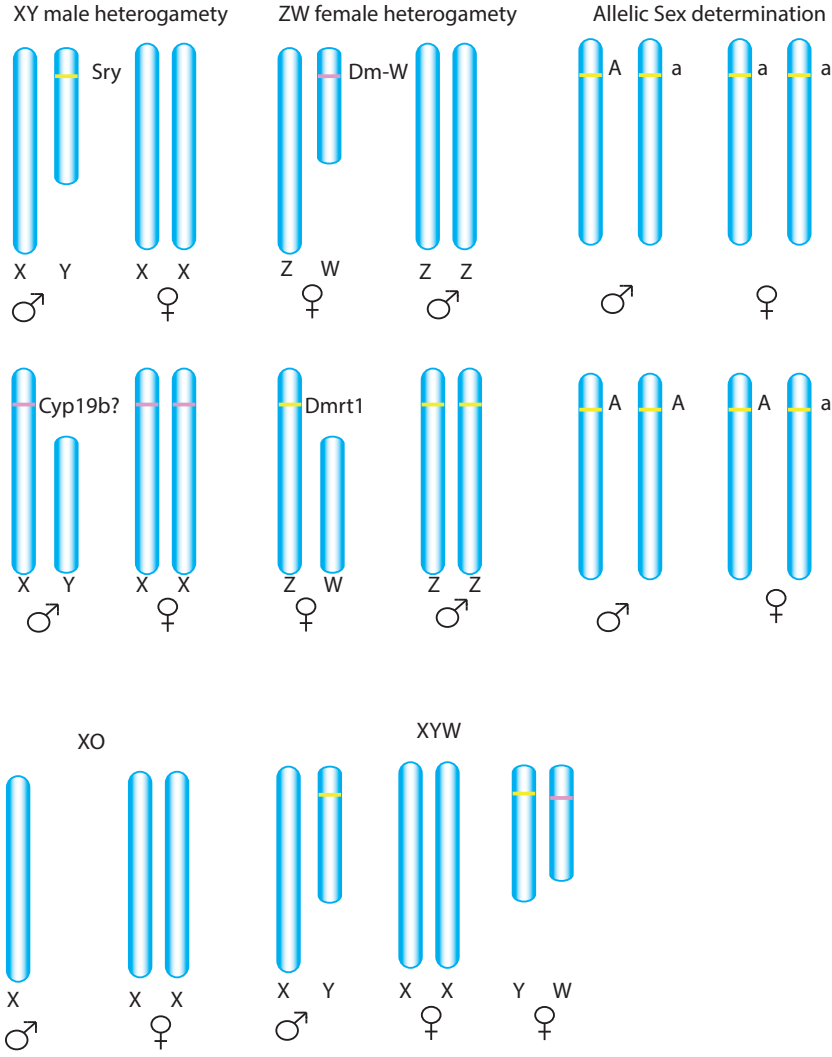
#### **1.4.4. Parthenogenesis**

In some species a female individual may give birth to offspring in the absence of fertilization by male sperm. These instances and variations of unisexual reproduction, including activation by, and exclusion of, male genetic material from the developing embryo are termed parthenogenesis (Hubbs and Hubbs 1932, Neaves and Baumann 2011). The incidence of parthenogenesis was originally thought to be rare among vertebrates but it is becoming clear that this mode of reproduction is much more common than originally thought with incidence observed in about 80 taxa of unisexual taxa and more still observed today (Neaves and Baumann 2011). For a full review of parthenogenesis please see (Devlin and Nagahama 2002, Neaves and Baumann 2011).



## 1.5. Genetic sex determination

Genetic sex determination encompasses male heterogamety, as in mammals, female heterogamety, common to birds, XO systems, observed in the mole-vole (*Ellobius lutescens*), and allele specific mechanisms identified in two fish, *Takifugu rubripes* (Kamiya et al. 2012) and *Oryzias luzonensis* (Myosho et al. 2012). Although many of the components of the downstream pathways initiated by these master sex-determining genes are conserved, the way the components fit together can be unique (Kikuchi and Hamaguchi 2013).



**Figure 1.2. Summary of genetic sex determining mechanisms.**

For both male heterogamety and female heterogamety individual sex is determined by the presence or absence of a single male or female promoting factor (e.g. *Sry* and *Dm-W*) or in a dosage dependent manner (e.g. *Dmrt1* and possibly *Cyp19b*). Alternatively sex can be determined by a single nucleotide change within a gene as observed in allelic sex determination. Other less common mechanisms include an XO system where the Y chromosome has been deleted all together and an XYW system where Y and W compete for dominance. A yellow band denotes a male promoting gene while a pink band denotes a female promoting gene with the exception of allelic sex determination where the yellow band denotes a gene present in both males and females. Although distinct heteromorphic chromosomes are illustrated for male and female heterogamety in this figure, in reality the difference between these two chromosomes only needs to be a single gene. For example, rainbow trout possess a male heterogametic sex determining system, but cryptic differences between the X and Y cannot be seen under the microscope.

### **1.5.1. Male heterogamety**

Of all the vertebrate sex-determining mechanisms known the most thoroughly studied is our own; a genetic male (XY) heterogametic sex-determining system (Figure 1.2). In this case males will always be the heterogametic sex. This mechanism can exist in one of two forms (Figure 1.2): i. A female sex-determining factor on the X chromosome is required in a dosage dependent manner where two copies are required for female gonad development or ii. A male sex-determining factor on the Y initiates the male pathway. Of course we are most familiar with the latter case due to the discovery of *Sry* in mammals (Koopman et al. 1990, Sinclair et al. 1990).

However, the male determining factor, which may be an activator of a male pathway (as *Sry* is in mammals) or an inhibitor of a female pathway or both, is not conserved among vertebrates (Devlin and Nagahama 2002, Kikuchi and Hamaguchi 2013). For example, the master sex-determining gene *Dmy* in medaka (*Oryzias latipes*) is not found in many closely related species that also possess a male heterogametic sex-determining system (Konodo et al. 2003, Myosho et al. 2012). Although the sex-determining genes in one other medaka related species has been identified (Myosho et al. 2012) many more species of the *Oryzias* genus lack a known sex-determining gene even though they have an identified male or female (see below) heterogametic sex-determining system (Takehana et al. 2005, Takehana et al. 2008). Even though several more fish and other animal species are also known to have a male heterogametic sex-determining system, none of them until recently in a few exceptions, have identified sex-determining genes. The Patagonian perjury (*Odontesthes hatcheri*), which possesses a duplicated Y-linked anti-mullerian hormone gene (*amhy*), is one of these few (Hattori et al. 2012). In contrast to *Sry* and *Dmy*, which both code for transcription factors, *amhy* is the first master sex-determining gene to dictate phenotypic sex at the hormone level (Hattori et al. 2012). However, all three of these examples consist of a single male promoting gene linked on the Y chromosome of the respective species and to date no male heterogametic systems are known to possess a female promoting master sex-determining gene that operates in a dosage dependant manner (Figure 1.2, Cutting et al. 2013).

The three-spine stickleback may be a promising candidate to identify such a mechanism. Evidence suggests that the three-spine stickleback possess a male heterogametic genetic sex-determining mechanism (Peichel et al. 2004). Yet this system appears to be labile to exogenous hormone treatment and thus the stickleback can be used as a model to study endocrine disruption caused by synthetic human pollutants (Hahlbeck et al. 2004). In addition, this species serves as an important reference for evolutionary studies of other fish species such as Atlantic salmon and rainbow trout (Danzmann et al. 2008, Lien et al. 2011), which have both economic and societal value (Thorgaard et al. 2002). Despite an abundance of resources including the three-spine stickleback reference genome (Jones et al. 2012), the master sex-determining switch in the three-spine stickleback remains a mystery.

Although much work has been done to identify sex linked markers and sex-determining systems in stickleback (see Urton et al. (2011) for review), no one has yet identified a master sex-determining gene. The complex nature and diversity of sex-determining mechanisms, including both male and female heterogametic systems as well as the influence of environmental factors, may be confounding the identification of a single genetic factor controlling sex determination (Urton et al. 2011). Even within the three-spine stickleback two different male heterogametic systems have been observed (Peichel et al. 2004, Ross and Peichel 2008, Kitano et al. 2009, Urton et al. 2011, Ocalewicz et al. 2011). In addition to the different systems, XXY and XY, within the XY system the genetic map produced by Peichel et al. (2004) does not match the deletion observed by Ross and Peichel (2008). Two markers Stn191 and Stn192, are present in the male map of Peichel et al. (2004) but are deleted from the Y in Ross and Peichel (2008). The differences here may be attributed to the differences in populations chosen for analysis and collectively imply different origins of sex-determining genes and mechanisms among different stickleback populations. This is not unlikely given the wide range of freshwater and saltwater habitats this fish has colonized (Jones et al. 2012). In particular, many of the freshwater habitats formed from receding glaciers, have isolated a number of stickleback populations and it begs the question how similar these populations of three-spine stickleback really are to one another. Although it appears the three-spine stickleback have repeatedly evolved similar adaptations to make the transition from saltwater to fresh water (Jones et al. 2012), the flexibility in the number of

ways in which sex can be determined allows the possibility of novel sex-determining factors to arise in each population.

The best candidate in the marine Pacific three-spine stickleback species is the *Cyp19b* gene, which is absent from the Y chromosome (Ross and Peichel 2008). *Cyp19b* codes for aromatase, the terminal enzyme in the pathway responsible for the conversion of androgens into oestradiol (E2), which promotes female development in many species including stickleback (Hahlbeck et al. 2004, Kroon et al. 2005). Thus, this would lead to a dosage dependent male heterogametic sex-determining mechanism where two copies of aromatase are necessary for female development. Consistent with this hypothesis is evidence that sex reversal of sticklebacks via synthetic oestrogens is dose dependent (Hahlbeck et al. 2004).

### **1.5.2. Female heterogamety**

In contrast to the absence of an identified dosage dependent male heterogametic system, birds display a female heterogametic (ZZ/ZW) system in which a male promoting gene *Dmrt1* does act in a dosage dependent manner where two copies are required for the male phenotype to develop (Figure 1.2, Smith et al. 2007, Smith et al. 2009). Alternatively, a single copy of *Dmrt1* results in female development. It should be noted that *Dmrt1* function has not been tested in birds other than the chicken although its conserved presence on the Z and absence from the W across birds suggest a conserved function across birds (Smith et al. 2009, Chue and Smith 2011). Yet the possibility of other master sex-determining genes in birds should not be excluded despite what seems to be a widespread conservation of the ZZ/ZW female activating system (Smith et al. 2009).

Other examples of female heterogamety are seen in *Xenopus laevis*, which possesses a female promoting master sex-determining gene (*Dm-W*) (Yoshimoto et al. 2008). This case is both analogous and opposite to the examples of male heterogamety observed in mammals (Figure 1.2). Therefore females are heterozygous ZW and males are homozygous ZZ where *Dm-W* is on the W chromosome.

### 1.5.3. Allelic sex determination

Until recently, evidence suggested master sex-determining genes were either single copy genes that operated analogous to *Dm-W* (Yoshimoto et al. 2008) or *Sry* (Koopman et al. 1990, Sinclair et al. 1990), or dosage dependent genes such as *Dmrt1* (Smith et al. 2009). In contrast to this belief two recent publications have shown genetic master sex determinants do not have to be an entire gene but rather can be a single base pair change in a gene (Figure 1.2). The first is an allele of *Amhr2* in the tiger puffer fish (*Takifugu rubripes*) where the presence of a G/C SNP denotes male phenotype while C/C denotes female phenotype (Kamiya et al. 2012). This is the second case, *amhy* in *O. hatcheri* being the first (Hattori et al. 2012), where the anti-Mullerian hormone pathway has been involved in sex determination. However, in this case it is a single amino acid change (His/Asp384) in the target of amh, anti-Mullerian hormone receptor 2, that plays the role in sex determination (Kamiya et al. 2012). Similarly in *Oryzias luzonensis*, a closely related species to medaka described earlier, *Dmy* is absent and instead an allele of *gsdf* (gonadal soma derived growth factor) controls maleness (Myosho et al. 2012). Moreover, interspecific transgenic experiments between *O. luzonensis* and medaka provide the first example for the evolution of a new master sex-determining gene via a mutation in a downstream player which subsequently takes over the downstream pathway in the absence of the original master sex-determining gene (*Dmy* in this case) (Myosho et al. 2012, Kikuchi and Hamaguchi 2013). However, in both cases the master sex-determining genes, *Amrh2* and *gsdf*, of *O. hatcheri* and *O. luzonensis*, respectively are present on both the X and Y chromosome and it is only small changes within these genes that differ between Y and X chromosome.

## 1.6. Other vertebrate sex-determining systems

It is quite remarkable to find that only a single base pair change in an entire genome can dictate the sex of an organism. Yet in some organisms even less is required to become male. For example the mole vole (*Ellobius lutescens*) has lost the Y chromosome all together and thus females are XO while males are X<sup>H-Y</sup>O (Figure 1.2, Yukifumi and Ohno 1977). The process by which this likely evolved suggests the H-Y antigen, which would have been on a previous Y chromosome, jumped to the X

chromosome removing the need for a Y. At the same time there must have been selection to remove individuals with  $XX^{H-Y}$  since these individuals will be sterile (Yukifumi and Ohno 1977). In this case H-Y represents the male determining factor.

Another exception among mammals is the platypus, which like other extant monotremes exhibits multiple sex chromosomes as opposed to just X and Y (Tsend-Ayush et al. 2012). In the case of the platypus there are 5X and 5Y chromosomes and unlike other mammals *Sry* is absent among monotremes (Tsend-Ayush et al. 2012). Instead, it has recently been suggested that *Crspy* and *CrspX* genes may be able to activate the testis-specific enhancer element and thus *Crspy*, which is located on Y chromosome 5, serves as a master sex-determining gene candidate among monotremes (Tsend-Ayush et al. 2012).

The platyfish (*Xiphophorus maculatus*) utilizes an out of the ordinary mode of sex determination by merging both the ordinary female heterogametic ZW and ordinary male heterogametic XY systems (Figure 1.2). In this system X, Y and W chromosomes are all present. All three of these chromosomes are thought to contain male-determining factors but it appears only the allele on the Y is expressed while the alleles on the X and W are suppressed by autosomal influences (Volff and Schartl 2001, Schartl and Volff 2002). As a result males can be XY or YY while XX, XW and WY individuals are female (Volff and Schartl 2001, Schartl and Volff 2002). In this case WY fish are female because the W chromosome has an additional suppressor that is specific for the male determining allele on the Y (Volff and Schartl 2001). This intriguing system allows the observation of the relationship of male heterogamety and female heterogamety and insight to evolutionary transitions between male and female heterogamety (Ezaz et al. 2006). The idea that sex chromosomes systems can flip back and forth is consistent with the observation of both male and female heterogametic systems observed among the species of the previously discussed stickleback and *Oryzias* (Urton et al. 2011, Myosho et al. 2012). To date no master sex-determining gene has been identified in the platyfish and thus the above proposed sex determining system remains as a model; however, this model is consistent with genetic results observed from atypical crosses resulting in spontaneous XX, XW, YW, and WW males and XY and YY females (Volff and Schartl 2001). The recent publication of the platyfish genome (Schartl et al. 2013) will certainly be an asset in the search for a master sex-determining gene in this species.

Although the sex-determining mechanism used by the platyfish is extraordinary, it is not quite so rare as the Cichlid fish, *Metriaclima pyrsonotus*, was found to possess a similar mechanism as well (Ser et al. 2010). In *M. pyrsonotus*, *SEX* (the sex locus) is linked to LG7 in the XY system and LG5 in the ZW system (Ser et al. 2010). Once again it appears the W dominates the Y and WY individuals are female. More interesting in this case is the observation of female skewed families in *M. pyrsonotus* that operate in similar manner to but are absent of the LG5 ZW system thus suggesting a second dominant W locus (Ser et al. 2010). From an evolutionary standpoint the identification of multiple sex loci between and within Cichlid fish species in this case appears to be the result of recruitment of novel sex-determining genes as opposed to a single mobile gene (Ser et al. 2010). This is similar to the case in medaka where closely related species do not share the same master sex-determining gene (Konodo et al. 2003).

## **1.7. Genome duplication and sex in fish**

Since it was first proposed by (Ohno 1970), genome duplication has long been argued as a means for speciation and radiation. In addition to the proposed two rounds of whole genome duplication (WGD) known as the 2R hypothesis (Sidow 1996, Kasahara et al. 2007) a third WGD is thought to have given rise to the teleost lineage (Christoffels et al. 2004, Meyer and Van der Peer 2005). Consistent with these proposed hypotheses is the observation that mammals have four HOX gene clusters while invertebrates have only one (Amores et al. 1998, Naruse et al. 2000). By this logic teleosts should have eight clusters of HOX genes. The identification of only seven clusters in turn implies loss of one of the clusters (Hoegg et al. 2008). An additional so called 4R genome duplication is thought to have occurred approximately 25-100 million years ago in the common ancestor leading to radiation of the salmonids (Allendorf and Thorgaard 1984) and this is well supported by several independent observations. In addition to the identification of approximately twice as many members, compared to teleosts, of fatty acid binding proteins in salmon (Lai et al. 2012), both Atlantic salmon and rainbow trout contain 13 Hox gene clusters, approximately twice that found in zebrafish and medaka (Moghadam et al. 2005a, Moghadam et al. 2005b). Salmonids not only also contain approximately twice as much DNA as found in other teleosts but



twice as many chromosome arms as well (Phillips and Rab 2001, Mank and Avise 2006, Phillips et al. 2009).

It is well established that genome duplication plays some role at least in driving evolution but how does such an event drive this process? It has been proposed that once a whole genome duplication has occurred the resulting paralogous (duplicated) genes may: i. become silenced or lost (nonfunctionalization), ii. acquire a new beneficial function(s) (neofunctionalization), or iii. divide the load each contributing part of the function carried out by the ancestral gene (subfunctionalization) (Innan and Kondrashov 2010). Put simply, the idea is that once the genome has become duplicated since only one gene is necessary for survival the other is free to accumulate mutations and thus face one of the three fates above. However, these affects are slow acting across entire chromosomes and thus much homology exists between paralogous chromosomes as is evident by quadravalent meiotic pairing observed in Atlantic salmon (Wright et al. 1983). In turn this allows one to identify paralogous chromosomes by mapping markers from the chromosomes of one species to an ancestral species. This is the means by which homeologous chromosomes have been identified in Atlantic salmon (Lien et al. 2011).

Following genome duplication one of the major problems facing an organism is sex determination (Davidson et al. 2009). However, the same three fates can face a duplicated master sex-determining gene that face any other duplicated gene in the genome. Currently evidence for neofunctionalization can be observed in medaka where a duplicate of *Dmrt1* (*Dmy*) assumed the sex-determining role. Alternatively, one of the sex-determining genes may be pseudogenized or lost as it now seems in rainbow trout (Yano et al. 2012). Note this hypothesis assumes *sdY* was the master sex-determining gene in the common ancestor of the salmonids.

## **1.8. Sex determination in Salmonidae**

In contrast to mammals in which a male heterogametic sex determination system dominates, sex determination in fish includes all of the various systems described in the previous sections (Devlin and Nagahama 2002). Moreover, due to their lability to surrounding temperature changes, fish sex chromosomes are much more susceptible to turnover, and thus are not only ideal for studying the process by which chromosomes

turnover but also the early stages of sex chromosome evolution (Devlin and Nagahama 2002). Due to their economic and societal value, the Salmonidae (salmon, trout, charr, grayling and freshwater whitefish; Nelson 2006) have received a great deal of attention (Thorgaard et al. 2002, Davidson et al. 2010). Moreover, the Salmonidae are of great interest to the scientific community due to an autotetraploidization event making them ideal candidates for the study of early stages of sex chromosome evolution (Thorgaard et al. 2002, Davidson et al. 2009).

Sex determination operates primarily via a male heterogametic system in several members of the Salmonidae (Davidson et al. 2009). This has been shown via crosses between sex reversed males (XY females) with normal males (XY) males which yield a 3:1 ratio of male to female offspring, while crossing sex reversed females (XX males) with normal females produces all female offspring (Davidson et al. 2009).

Although male heterogamety is conserved among salmonids, there has been great debate as to whether or not a single master sex gene dominates, similar to *Sry* in mammals (Sinclair et al. 1990, Koopman et al. 1990), or if each species of this family possesses their own master sex-determining gene as seen in the genus *Oryzias* (Myosho et al. 2012) and stickleback (Urton et al. 2011). The observation that genetic markers linked to the sex-determining locus (*SEX*) in one species (e.g. Atlantic salmon) map to autosomal chromosomes in brown trout (*Salmo trutta*), rainbow trout and Arctic charr (*Salvelinus alpinus*) by Woram et al. (2003) suggested multiple genes exist possibly as a result of an autotetraploidization event in the common ancestor of the salmonids (Allendorf and Thorgaard 1984). However, it was also argued that a single sex-determining gene exists and has moved about the genome, giving rise to the interspecific sex chromosome polymorphisms observed between members of this family (Phillips et al. 2001, Davidson et al. 2009). The discovery by Yano et al. (2012) of the master sex-determining gene in rainbow trout (*sdY*) and its presence in other salmonid species suggest that the latter hypothesis is correct (Yano et al. 2013).

## **1.9. Sex and Aquaculture**

Sex manipulation is commonly practiced in aquaculture as females are often preferred for production purposes. The Tasmanian Atlantic salmon aquaculture industry

relies on all female production stock because the relatively warm Tasmanian seawater leads to rapid sexual development in males, which gives rise to poorer flesh quality and increased disease susceptibility (Aksnes et al. 1986, Elliott and Kube 2009). The strategy is to treat some females with 17- $\alpha$ -methyl-testosterone at an early age, which makes them develop as phenotypic males. Crossing these sex-reversed females (neo-males) with normal females produces all female offspring (Johnstone and Youngson 1984). Phenotypic differentiation of sex-reversed females from true males is not feasible until the fish become sexually mature, and even then only with destructive sampling. A male-specific genetic marker would overcome this problem and enable the culling of surplus genetic males at an early age, thus reducing the cost associated with tank space and feed.

## 1.10. Project aims

Although sex-specific (i.e., male) genetic markers had been identified in *Onchorhynchus* sp. including:OP-P9/ OmyP9 in rainbow trout (*O. mykiss*) (Iturra et al. 1997); OtY1 (OTY8) and OtY2 in Chinook salmon (*O. tshawytscha*) (Devlin et al. 1991, Devlin et al. 1998, Brunelli and Thorgaard 2004); and a growth hormone pseudogene in Chinook, coho (*O. kisutch*), chum (*O. keta*) and pink (*O. gorbuscha*) salmon (Zhang et al. 2001), none had been identified in Atlantic salmon (*Salmo salar*) (McGowan and Davidson 1998) at the start of this project. Therefore, the aim of this project was to identify a genetic marker that could consistently be used to accurately predict offspring sex in Tasmanian Atlantic salmon. Subsequent aims of this project were:

- i. To generate a comprehensive data set that identifies maintained male lineages within the SALTAS Tasmanian Atlantic salmon selective breeding population as having SEX on either chromosome 2, chromosome 3 or chromosome 6.
- ii. Determine if the rainbow trout master sex-determining gene (*sdY*) is present in all three male lineages identified.
- iii. Determine the relationship between the three identified sex loci and identify individuals in which the proposed master sex-determining gene may have jumped.

## **2. Methods**

### **2.1. SALTAS Atlantic salmon population samples**

All Atlantic salmon samples used in this study come from the SALTAS selective breeding program population. Historically the origins of this population can be traced to the River Philip in Nova Scotia, Canada. The first stocks from this source were initially imported to Garden, New South Wales in 1965 to 1967, for sport fishing, and later transferred to Tasmania between 1984-1986. Until 2004, when the SALTAS selective breeding program was initiated, the population was managed with the primary goal of maintaining genetic diversity. The objectives and desirable traits selected in the program are described in (Elliott and Kube 2009).

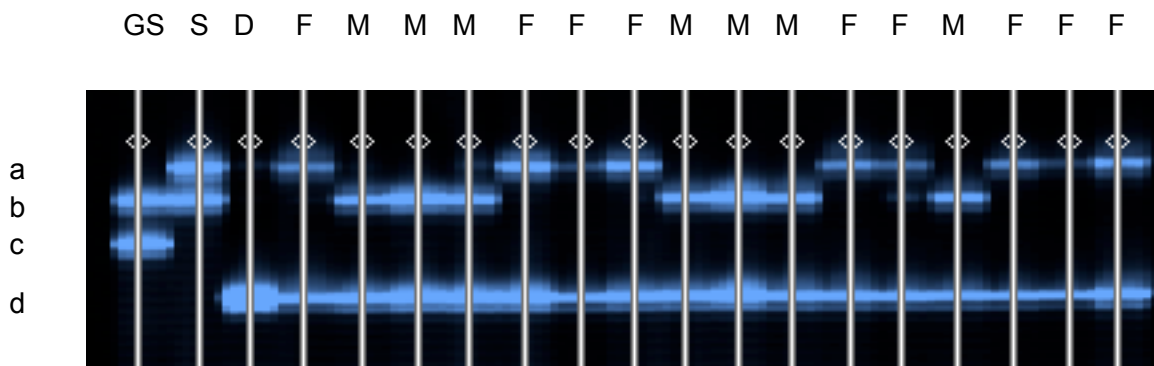
A subset of 47 out of 152 families from the 2009 year class were chosen for analysis in this study based on criteria described later. This subset representing a total 38 of the 50 maintained sire lineages constitutes 1128 individuals of the sixth year class of the selective breeding program. The 47 families and the male lineages to which they belong are shown in Supplementary Table S1. Families chosen were the result of crosses between 44 sires and 36 dams where some parents were used in multiple matings. For instance one sire crossed to two different dams. The sires belong to the 2005, 2006 and 2007 year classes, with 57% one generation from founding stock and 43% two generations from founders. The dams belong to the 2005 and 2006 year classes, where 92% were one generation from founders and 8% two generations from founders.

Families were chosen to maximize the number of sire lineages represented in the data set and confined to a minimum of 15 individuals per family. Individuals were also excluded from the final data set if a confident genotype or phenotype call could not be made. As a result the number of individuals per family in the final data set ranged from 11-54 with between 4-23 males and 4-29 females per family. Sex phenotypes were recorded at either 23 months post fertilization during culling of early maturing individuals

or 29 months post fertilization when final harvest assessment is conducted. All sex phenotypes were assessed by gonad inspection at slaughter.

## 2.2. Genotyping analyses

Microsatellite and SNP (single nucleotide polymorphism) based linkage analyses were used to identify *SEX* in Atlantic salmon. In order for a microsatellite or SNP marker to be useful for sexing offspring (informative), the sire of the family must be heterozygous for the given marker while the dam of the family may be homozygous or heterozygous but cannot have a genotype identical to the sire. These methods are described previously in (Danzmann et al. 2008); however, details will be described here and Figure 2.1 provides an example of how offspring can be sexed via this method.



**Figure 2.1.** *An example of genotype analysis using a single marker linked to the sex locus.*

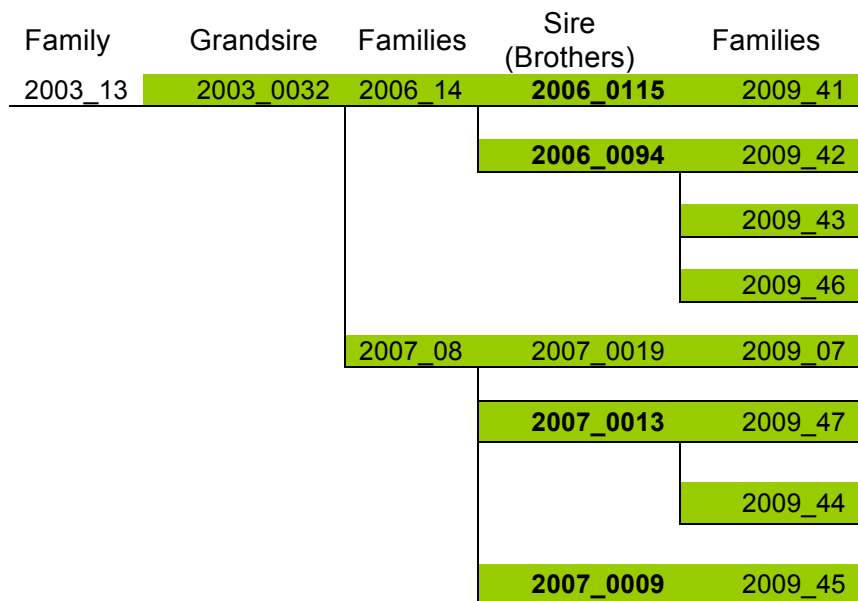
The paternal or male allele b is passed from the Grandsire (GS) to the sire (S) to all male (M) offspring. Predicted males and females are indicated with an M and F while a D denotes D the dam. The alleles are denoted a, b, c and d arbitrarily.

Each 6  $\mu$ l PCR reaction contained 4.05  $\mu$ l nuclease free water (IDT technologies) 0.19  $\mu$ M M13 tailed forward primer, 0.43  $\mu$ M reverse primer and Fam or Hex (M13 tail shown below), 0.18  $\mu$ M dNTP's (Invitrogen), 0.579  $\mu$ l 10X Coral buffer (QIAGEN) (15 mM  $MgCl_2$ ), 0.29 U of Taq (Roche) and 0.5  $\mu$ l of genomic DNA (15 ng/ $\mu$ l).

M13 tail: TGTAACGACGGCCAGT, on individuals where the *sdY* exon based PCR test did not agree with sex phenotype.

All primers used in this project were synthesized by IDT Technologies. PCR reactions were carried out in either a T1, T personal, or T3000 Biometra thermocycler. Thermocycler conditions included an initial denaturation at 94°C (2min) followed by a touchdown involving 10 cycles of 94°C (30s), 60°C-50°C touch down (30s) and 72°C (30s). An additional 24 cycles with annealing temperature 55°C followed.

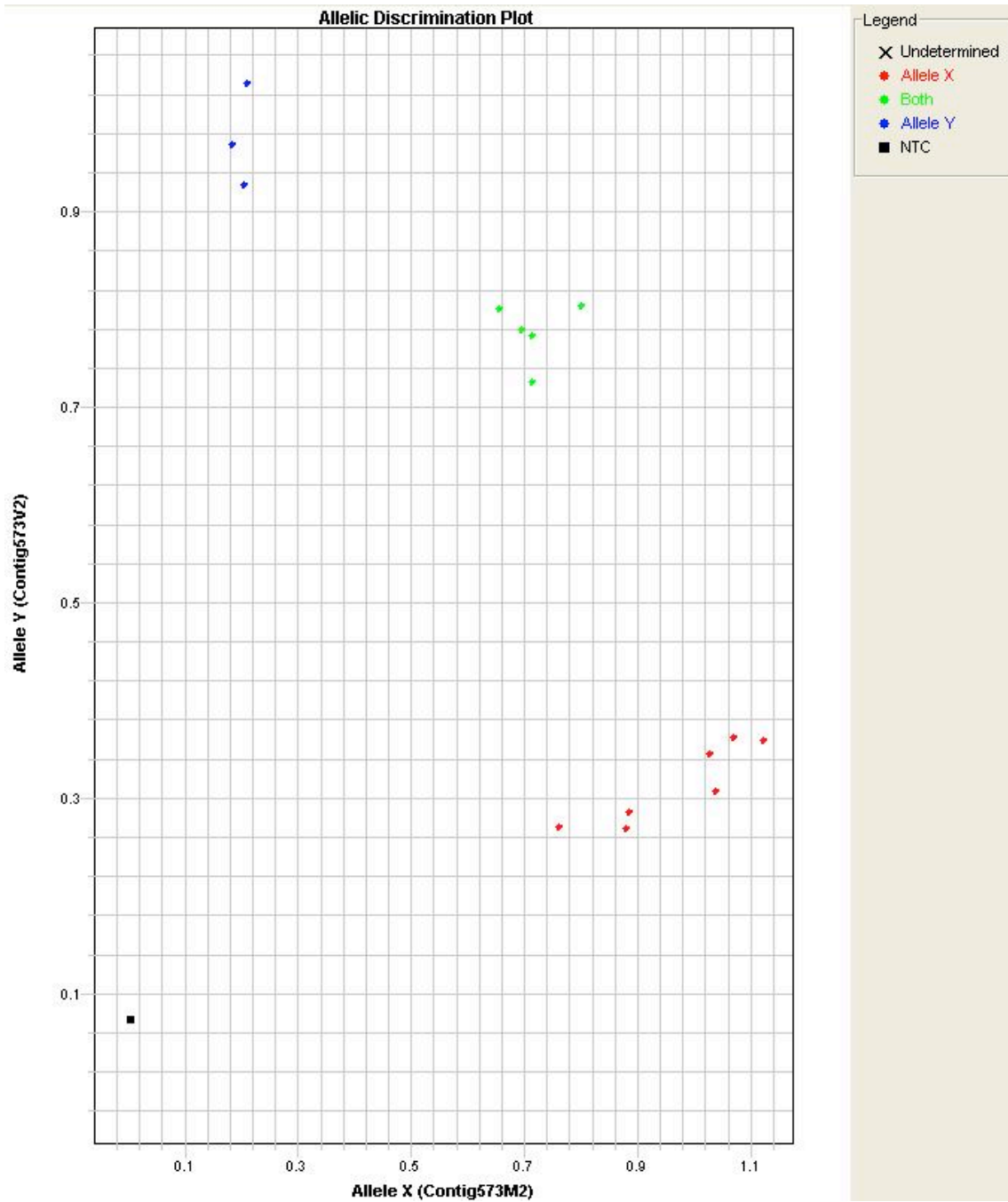
Two µl of formamide loading dye (ABI) was added to 2 µl of each PCR reaction. The mix was denatured at 94°C for 5min in a T1 Biometra thermocycler and subsequently placed on ice before loading on to a 7% urea polyacrylamide gel run on a 377 ABI sequencer. Detailed information for the microsatellite and SNP markers, along with respective integrated linkage maps are available at [www.asabase.org](http://www.asabase.org). For the first seven 2009YC families (Figure 2.2) DNA, extracted from an adipose fin clip, was obtained from Agresearch ([www.agresearch.co.nz](http://www.agresearch.co.nz)). For all other 2009YC families, DNA was provided by Landcatch Natural Selection Ltd. ([www.landcatch.co.uk](http://www.landcatch.co.uk)).



**Figure 2.2. Lineage containing the four sires of the seven families in the initial pilot study.**

All of these families were identified to have the sex-determining gene located on Ssa06. Individuals (males) are shown in the format, Year class (YC),\_, individual ID, (e.g. 2003\_0032; YC 2003, ID 0032). Families are classified in the same way (e.g. 2003\_13; YC 2003, family ID 13). Pedigrees of all 40 lineages can be found in Supplementary Figure S1. The four sires are shown in bold.

A TaqMan 64 SNP genome wide assay (Applied Biosystems) was conducted in 10  $\mu$ l reactions on the 2009YC families. Each 10  $\mu$ l TaqMan PCR reaction contained, 5  $\mu$ l 2x genotyping master mix (Applied Biosystems), 0.25  $\mu$ l of 40x genotyping assay mix (Applied biosystems), 3.75  $\mu$ l Nuclease Free Water (IDT Technologies) and 1  $\mu$ l DNA (15 ng/ $\mu$ l). Initial denaturation at 95<sup>o</sup>C was held for 10minutes followed by 40 cycles of 95<sup>o</sup>C 15 sec and 60<sup>o</sup>C 1 min. Although reactions could be carried out in either a 7900HT Fast Real-Time PCR system (ABI) machine or a Biometra T1 thermocycler, all reactions had to be analyzed on the ABI machine. Figure 2.3 shows an example of an informative family genotyped using this method. The 64 SNP markers are listed in Supplementary Table S2. Additional SNP data related to *SEX* from the 2005YC and 2006YC (Dominik et al. 2010, Lien et al. 2011) were used to predict the sex locus of unknown lineages. Genotypes were compared to male and female phenotypic calls to assess linkage to *SEX*.



**Figure 2.3.** An example of the TaqMan probe marker genotyping result.

Alleles are color coded as indicated in the legend. NTC represents the negative control. “Allele X” and “allele Y” represent homozygous individuals while, “both” represents individuals who are heterozygote or XY.



SALTAS personnel made phenotype calls at harvest, maturation grading and spawning. Fish with questionable phenotypes are indicated in Supplementary Table S3. To determine the chromosomal location of *SEX* in each family, the following criteria were set: (1) at least one marker from Ssa02, Ssa03 or Ssa06 accurately predicts offspring sex; and (2) at least one marker from each of the other loci does not predict offspring sex accurately (negative confirmation). For markers with duplicate loci each informative locus was distinguished by the addition of /1 and /2 to the marker name to indicate locus 1 and 2, respectively (Danzmann et al. 2008). In cases where the grandsire and sire genotype were identical, the male allele was inferred based on the comparison between genotype and phenotype of the offspring.

Logarithm of Odds (LOD) scores (Morton 1962) were calculated individually for each family marker combination in Maple 16 using the following equation.

$$Z = \log_{10} \frac{(1 - \theta)^x \theta^y}{0.5^{(x+y)}}$$

Where the LOD score  $Z$  is given as a function of the number of non recombinant offspring  $x$  and the number of recombinant offspring  $y$  for a given recombination rate  $\theta$ . Cases where the sex phenotype predicted by genotype was 100% accurate were considered to be significant regardless of LOD score.

### **2.3. PCR-based *sdY* test for Atlantic salmon males**

Yano et al. (2012) recently identified a male specific master sex-determining gene, *sdY*, (sexually dimorphic on the Y chromosome) in rainbow trout. Two primer pairs, one specific for exon two and the other specific for exon four of the *sdY* gene, were designed based on an alignment (not shown) of Atlantic salmon *sdY* (Palibroda *et al.*, in preparation) with Danube salmon (*Hucho hucho*), Chinook salmon (OTY-3), rainbow trout Y chromosome genomic sequence, and rainbow trout *sdY*. GenBank accession numbers for the Danube salmon, Chinook salmon, rainbow trout Y and rainbow trout *sdY* sequences are JF951962.1, DQ393568.1, EU081756.1 and AB626896.1, respectively. A triplex PCR reaction was developed to screen the parents and offspring of the tested 2009YC families, with the following primers (IDT Technologies):

Exon 2:

Forward: TGATGGATGGGATCCCCGTCATCTCTCTCCCAAAG

Reverse: TCCCTCATGGAGGGTGGAGTGGTTTTAAGCTCTA

Exon 4:

Forward: AGTTGGAACGCTTCAGCAGAGCAGATGG

Reverse: GGACAAGACTCATCACTCAGTGCACCAATCT

Fabp6b (DNA quality control; Y. Lai, personal communication):

Forward: AATTACGATGAGTTTCTGGAGGCAA

Reverse: CTTTCCGATGGTGAATTTGTTAGTCAA

Each 8.7  $\mu$ l reaction contained 2.25  $\mu$ l nuclease free water (IDT technologies) 1  $\mu$ l of DNA (~50 ng/ $\mu$ l), 0.3  $\mu$ l DMSO (100%) (New England Bio Labs), 1  $\mu$ l dNTPs (Invitrogen) (10 $\mu$ M), 1  $\mu$ l 10X Coral buffer (QIAGEN) (15 mM MgCl<sub>2</sub>), 0.15  $\mu$ l of Taq (Roche) (5 U/ $\mu$ l) and forward and reverse primers of: Exon 2 (0.15  $\mu$ l at 10  $\mu$ M), Exon 4 (1  $\mu$ l at 10  $\mu$ M) and Fabp6b (0.35  $\mu$ l at 10  $\mu$ M). When 15 ng/ $\mu$ l DNA working stocks were used, 4  $\mu$ l of DNA was added to each reaction (total volume 11.7  $\mu$ l). PCR conditions, run on a Biometra T1 Thermocycler machine, were 94°C for 2 min followed by 36 cycles of 94°C for 30 s, 60°C for 30 s, 68°C for 30 s. A final extension of 68°C for 10 min was used. The parents and offspring of the 2009YC families were screened with this multiplex and PCR products were separated on a 1.5% 1 X TBE agarose gel and visualized using ethidium bromide.

Exon 3 was tested, using primers:

Forward: AGTTGGAACGGCTTCAGCAGAGCAGAT and

Reverse: AGATTGGTGCCTGAGTGATGAGTCTTGTC

on individuals where the *sdY* exon based PCR test did not agree with sex phenotype. Here each reaction contained 6.85  $\mu$ l nuclease free water (IDT technologies), 0.5  $\mu$ l forward primer and reverse primer (IDT technologies), 1  $\mu$ l 10X Coral buffer (QIAGEN) (15 mM MgCl<sub>2</sub>), 1  $\mu$ l dNTPs (2  $\mu$ M) 0.15  $\mu$ l of Taq (Roche). Thermocycler conditions were as above except the extension temperature was 70°C.

## **3. Results**

### **3.1. Screening Tasmanian Atlantic salmon with microsatellite markers on Ssa02**

*SEX* was previously mapped to Atlantic salmon linkage group 1 (LG1) (Woram et al. 2003), and Artieri et al. (2006) showed that this corresponds to chromosome 2 (Ssa02). Therefore, to identify a sex-linked genetic marker, which could be used to predict males in the Tasmanian Atlantic salmon population, seven pilot test families from the SALTAS breeding population with microsatellite markers from Ssa02 were screened first ([www.asalbase.org](http://www.asalbase.org); Figure 2.2, Supplementary Table S1 and Table S3). However, *SEX* was not associated with any alleles from these loci, which suggested that *SEX* was not on Ssa02 in these families.

### **3.2. Mapping of *SEX* to Ssa06 in Tasmanian Atlantic salmon**

A screen of these seven Tasmanian Atlantic salmon families using 64 SNP markers, distributed across the genome, indicated that *SEX* resided on Ssa06 in these families (Supplementary Table S1, Table S2 and Table S3). Linkage analysis using microsatellite markers from Ssa06 confirmed this prediction (Supplementary Table S3 and Table S4). An examination of the pedigree records of the SALTAS breeding program revealed that these seven Atlantic salmon families had a common grandsire, and thus represented a single male lineage in the SALTAS population (Figure 2.2). Therefore, some additional 2009YC families from different ancestral male lineages in the SALTAS Atlantic salmon breeding population were screened with microsatellite markers from Ssa06. Although these markers predicted the gender of offspring accurately in some families, in other families there was no association between the genotypic results and offspring sex phenotypes (Supplementary Table S3, Table S4 and Table S5). For non-Ssa06 *SEX* linked families, accurate sex predictions were obtained using genetic markers from Ssa02 (Supplementary Table S5).

### **3.3. Screening the SALTAS Atlantic salmon broodstock with Ssa02 and Ssa06 genetic markers**

With evidence for two sex-determining loci in the SALTAS Atlantic salmon broodstock, a total of 40 families from 34 male lineages with representatives in the 2009YC of the selective breeding program were screened for *SEX* linked to Ssa02 or Ssa06. SNP markers associated with *SEX* from Dominik *et al.* (2010) and Lien *et al.* (2011) predicted a number of male lineages to have *SEX* associated with Ssa02 (data not shown). This increased the number of male lineages with an identified *SEX* locus to 38 (Supplementary Table S1). *SEX* was predominantly associated with Ssa02, being found in 34 of 58 families, which corresponds to 25 of the 38 male lineages. In contrast, *SEX* was associated with Ssa06 in 22 of the 58 families, representing 11 of the 38 identified male lineages (Supplementary Table S4 and Table S5).

Although confident in the assignment of *SEX* to Ssa02 or Ssa06 in 56 of 58 families, a lack of consistency was observed between sex phenotype predictions made by markers mapping to either of these chromosomes for two families (2009\_32 and 2009\_37), containing 35 and 17 offspring, respectively. These results suggested the presence of a third sex-determining locus in Tasmanian Atlantic salmon. Colleagues at CIGENE, Norway recently identified *SEX* on Ssa03 in some European Atlantic salmon (S. Lien, personal communication). Therefore, genetic markers from Ssa03 were used to screen these two Tasmanian families. In both families, 2009\_32 and 2009\_37, there was linkage between sex phenotype and Ssa03 (LOD scores of 5.3 and 3.2, respectively) (Supplementary Table S6).

Although there was evidence for association of sex phenotype with three different chromosomes, there were some instances when sex phenotypes and genotype predictions did not match. For example, in one family (2009\_32) with 35 offspring, in which the sex phenotype was linked to Ssa03, sex predictions using six Ssa03 markers were inconsistent for four individuals (Figure 3.1 and Supplementary Table S3). Of these four individuals, three carried a Y specific allele passed from grandsire to sire (predicted males), but were found to be female at harvest. Two of these three predicted males (phenotypic females) gave PCR product only for exon 3 of the potential master sex gene, *sdY*, by PCR (see below) whereas the other one gave PCR products for exon 3

and exon 4, but not exon 2 (Figure 3.1. and Supplementary Table S3). Exon 3 of *sdY* was tested only on individuals in which the presence or absence of *sdY* did not agree with the phenotype assessment of the fish (Supplementary Table S7). The other individual, which had a female microsatellite haplotype and male phenotype, gave a positive PCR result for exons 2, 3 and 4 of the *sdY* gene (Supplementary Table S3).

2009_32		Ssa03										
parents and <i>sdY</i> agree 100%												
	AS1-2002_0013	AS2	S1-2007_0001	S2	2009_0598	2009_0967	2009_0279	2009_0558	2009_0636	2009_0137	2009_0609	2009_0470
Ssa0649BSFU	2	1	2	1	2	2	2	2	2	1	2	1
Ssa0017BSFU	3	2	3	1	3	3	3	3	3	1	1	1
Ssa0368BSFU	1	3	1	4	1	1	1	1	1	4	4	4
Ssa0516BSFU	3	3	3	4	3	3	3	3	3	4	4	4
OMM5019	2	1	2	1	2	2	2	2	?	1	1	1
OMM1267	1	3	1	2	1	1	?	?	1	2	2	?
SsaD144BSFU	1	3	1	2	1	1	1	1	1	2	2	2
<i>sdY</i>				Exon 2	1	1	0	0	0	0	0	1
				Exon 4	1	0	1	0	0	0	0	1
				<i>Fabp6b</i>	1	0	1	1	1	1	1	1
Sex call from Phenotype				Male	Male	Female	Female	Female	Female	Female	Female	Male

**Figure 3.1. An abstract of the second largest family (2009\_32), in which the sex locus mapped *Ssa03*, to illustrate the comparisons of phenotype to *sdY* phenotype predictions and haplotype predictions of phenotype.**

AS-1 (ancestral sire-1) represents the paternal or Y linked allele housed by the grandsire (2002\_0013) while AS-2 represent the maternal or X linked allele housed by the grandsire. Likewise S-1 (sire-1), inherited from the AS, and S-2, inherited from the ancestral dam (AD) represent the Y linked and X linked alleles housed by the sire (2007\_0001) respectively. Offspring ID's run in the same row where Individual ID's are shown in the format year class\_IDXXXX (e.g. 2009\_0598 is the 2009 Year class ID 0598. Haplotype offspring sex predictions that do not match the sex call from phenotype are highlighted green. The *sdY* predictions are based on the presence of Exon 2 and Exon 4 (predicting male) indicated by a 1 or absence indicated by a 0. *Fabp6b* represents a quality control of DNA where a 1 indicates presence and 0 indicates absence of an amplified product. Full details of this family can be found in Supplementary Table S3.

Seventeen phenotypic males from 10 other families also tested positive for exons 2 and 4 of *sdY* even though they carried the sire's maternally inherited haplotype (Table 3.1; Supplementary Table S3). We also found additional inconsistencies among predicted sex phenotype based on haplotype analysis, sex phenotype observed at harvest and the presence of the *sdY* gene, and these are summarized in Table 3.2 and Supplementary Table S7. Unfortunately none of these fish were used as broodstock, and therefore they have no associated offspring of their own that could be analyzed.

**Table 3.1. Phenotypic male individuals from the 2009 year class in which SEX may have moved by recombination, translocation or transposition (see Figure 4.1 for details).**

<sup>1</sup> Individual ID	Number of markers predicting female haplotype	sdY positive	Chromosome housing (SEX) in family
2009_0756	2	Yes	2
2009_0582	3	Yes	2
2009_0013	1	Yes	2
2009_0252	1	Yes	2
2009_0316	1	Yes	2
2009_0604	1	Yes	2
2009_0953	2	Yes	2
2009_0947	1	Yes	6
2009_0924	1	Yes	6
2009_0192	1	Yes	6
2009_0528	3	Yes	6
2009_0390	1	Yes	6
2009_0641	1	Yes	6
2009_0982	2	Yes	6
2009_0369	2	Yes	6
2009_1080	5	Yes	6
2009_0470	4	Yes	3
2009_0578	4	Yes	3

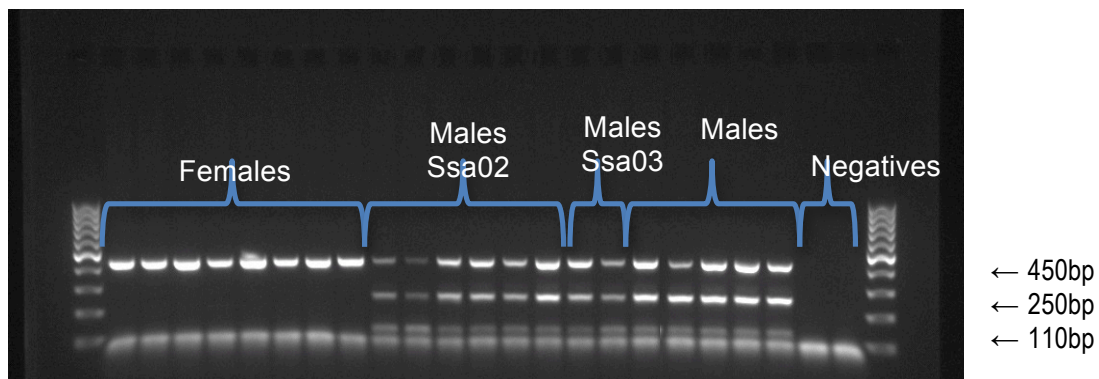
<sup>1</sup>Individual IDs are shown in the format year class\_ individual ID, for example, 2009\_0756 for 2009 year class \_ ID 0756

**Table 3.2. Some explanations of inconsistent outcomes between phenotype, genotype and *sdY*. For a fuller discussion see Figure 4.1.**

Phenotype	Haplotype	<i>sdY</i> test	Total # of individuals	Possible Explanation
Female	Male	Male	1	Phenotype miscall or loss of function of <i>sdY</i>
Female	Female	Male	3	
Female	Male	Female	7	Movement of marker or <i>sdY</i>
Male	Female	Male	20	Movement of marker or <i>sdY</i>
Male	Female	Female	12	Temperature dependent sex reversal or phenotype miscall (King <i>et al.</i> 2012)
Male	Male	Female	4	

### 3.4. Development of a PCR-based test to identify Tasmanian Atlantic salmon males

The recent publication of the male-specific sex-determining gene in rainbow trout, *sdY*, by Yano *et al.* 2013 enabled us to design two pairs of Atlantic salmon specific primers, one from exon 2 and the other from exon 4, to screen the Tasmanian Atlantic salmon for the presence or absence of the *sdY* gene using a PCR based assay (Figure 3.2).



**Figure 3.2. *sdY* triplex PCR test with primers (see methods) from exon 2 (~110 bp) exon 4 (~250 bp) and *fabp6b* (~450 bp) (Y. Lai personal communication).**

Sires are compared to dams from families with *SEX* on Ssa02/ Linkage group 1, Ssa06/ Linkage group 4 and Ssa03/Linkage group 11. The 100 bp ladder is shown in the first and last lanes.

Since both exon 2 and exon 4 should be present in a fully functional *sdY* gene, any individuals in which only one of these two exons amplified were designated as predicted females (e.g. 2009\_0279; Figure 3.1) with the following exception: the absence of the quality control *Fabp6b* amplicon, which is indicative of DNA degradation, but presence of the exon 2 product (e.g. 2009\_0967; Figure 3.1). In this particular case a male designation was assigned. A full list of the 19 individuals with only an exon 2 or exon 4 amplicon present can be found in Supplementary Table S9. All the parents in the tested 2009YC families were screened using these primer pairs for *sdY*, and there was 100% agreement between parent phenotype and the presence or absence of *sdY* PCR products (Figure 3.1; Supplementary Table S3). Expanding the sample set to include all offspring from the 47 families, yielded a concordance between *sdY* and phenotype to 539/567 for males and 405/409 for females (Supplementary Table S3 and Table S8). Of the total 957 offspring *sdY* test results that were consistent with sex phenotype, 27 disagreed with sex predictions based on genotype (Figure 2.2; Supplementary Table S3 and Table S8). For eleven individuals, in which *sdY* predictions did not match phenotype calls, microsatellite haplotype predictions agreed with predictions made by the *sdY* test (Supplementary Table S8). Figure 3.1 illustrates the examples of most of these instances that have been observed within a single family (2009\_32).



## 4. Discussion

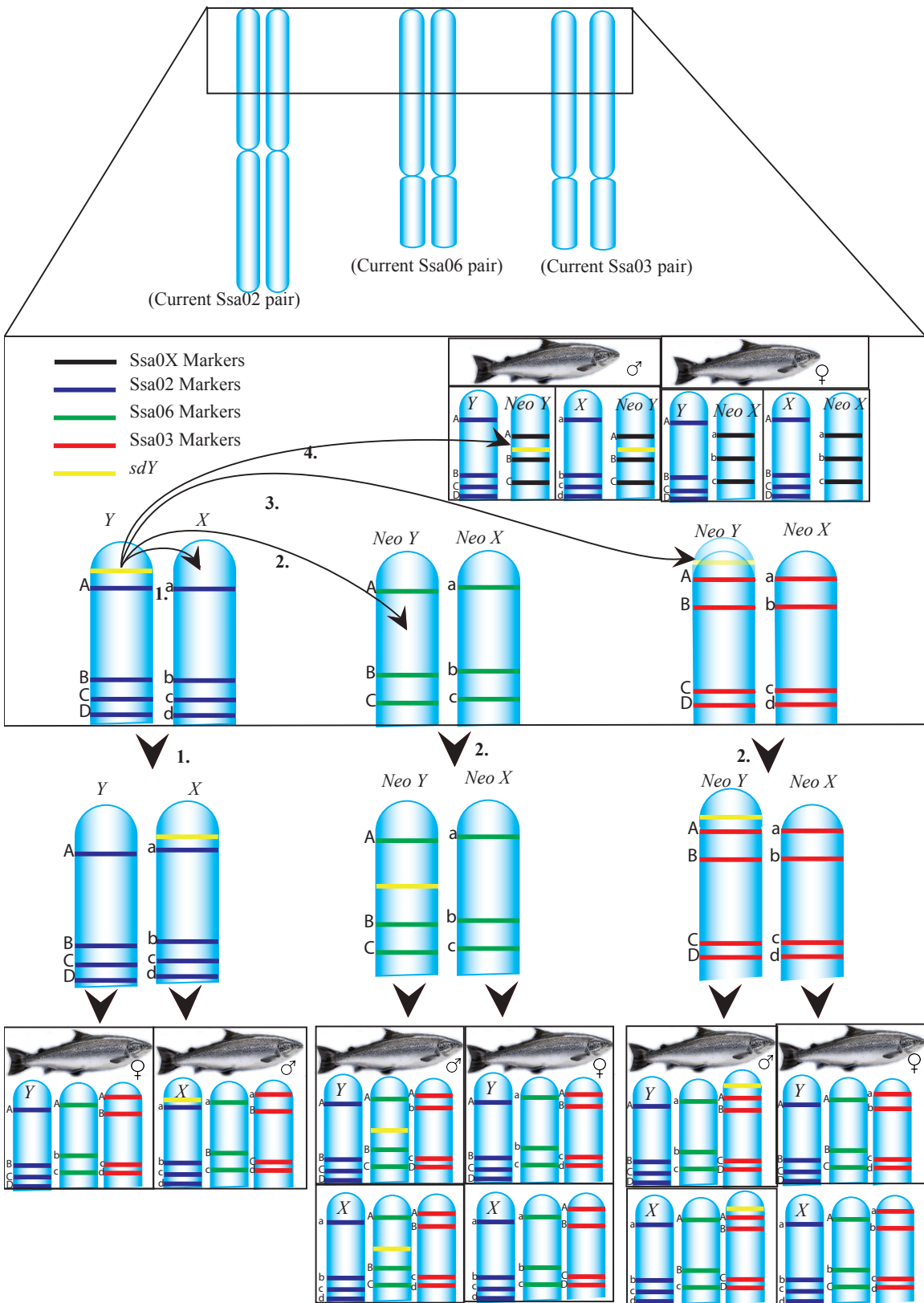
### 4.1. Multiple sex loci within a species is rare

Fish do not share conservation of a master sex-determining gene, and even closely related species can have different sex-determining genes (Konodo et al. 2003, Myosho et al. 2012, Kikuchi and Hamaguchi 2013). However, it is rare to find this phenomenon within orders of other vertebrates such as mammals and birds. The observation of different master sex-determining genes within a species has not been reported. Although rare, multiple sex-determining gene loci, as shown here for Atlantic salmon, have been described previously in Arctic charr (Moghadam et al. 2007, Küttner et al. 2011).

### 4.2. A single conserved sex-determining gene

The occurrence of multiple sex linkage groups in salmonid species led to two competing hypothesis: either a single conserved sex-determining gene that can jump between chromosomes exists; or different sex loci represent unique sex-determining genes (Woram et al. 2003, Phillips et al. 2001, Davidson et al. 2009). The recent reports of Yano et al. (2012, 2013) suggest the former hypothesis is correct. Likewise the PCR based survey here, using primers designed from exon 2 and exon 4 of the *sdY* gene, suggests that *sdY* is present in all three male lineages identified in the Tasmanian Atlantic salmon population. However, in a number of individuals, the inherited microsatellite and SNP marker haplotype, the presence or absence of the *sdY* gene and the sex phenotype did not agree. The single individual (2009\_0838) that was positive for exon 2 and exon 4 of the *sdY* gene and carried a male haplotype but was phenotypically assessed to be female may represent an error in phenotype assignment. Likewise the same explanation is possible for the eleven *sdY* negative individuals that possessed a female haplotype but were assessed to be phenotypically male (Table 3.2 and Supplementary Table S7). Phenotype assessment can be difficult to make due to variation in gonadal tissue and human error. Human error could have also been

introduced through mix up of samples during DNA extraction; however, because genotyping and *sdY* tests were conducted on the same samples, observed inconsistencies between these two data sets are unlikely to have resulted from this type of error. Alternatively, a male phenotype could be observed when both *sdY* and haplotype tests predict female phenotype due to temperature dependent sex reversal, which has been reported in Tasmanian Atlantic salmon (King et al. 2012). Table 3.2 summarizes explanations for the inconsistencies between *sdY* sex predictions and phenotype while Figure 4.1 summarizes the explanations for possible inconsistent outcomes between *sdY* and phenotypic sex predicted by haplotype. The simplest of these explanations to explain inconsistencies between phenotypic sex predicted by haplotype and the *sdY* test is homologous recombination between the Y and X chromosome (Figure 4.1). Although this study was not set up to test this explanation, which is consistent with the lack of morphological differences between the Y and X (Davidson et al. 2009), six instances of a crossover between the Y chromosome and the X chromosome were observed in individuals 2009\_0439, 2009\_0090, 2009\_0784, 2009\_0609, 2009\_1089 and 2009\_1095 (Supplementary Table S3), one of which, 2009\_0609 is also shown in Figure 3.1. Due to the criteria set to assign *SEX* to a particular chromosome (see methods) the depth of coverage of markers from any one linkage group for any one family is too small to assess how often this event actually occurs. However, because recombination is suppressed on the male Y chromosome (Woram et al. 2003, Lien et al. 2011), whether it be chromosome 2, 3 or 6, the rate of crossover is expected to be low. In addition, only a single recombination between the Y and X chromosomes was observed in Family 2009\_32 (Figure 3.1, Supplementary Table S3), the second largest family with a male haplotype comprised of markers mapping from 3.1 cM to 113.5 cM on the female linkage map ([www.asalbase.org](http://www.asalbase.org)). To date, the function of *sdY* is unknown, but the protein product is predicted to contain an association domain while lacking a DNA binding domain (Figure 4.2, Yano et al. 2012).



**Figure 4.1. Mechanisms of sdY movement during meiosis of a sire with SEX located on Ssa02.**

Each major pathway is designated a different number: (1). Homologous recombination including sdY between X and Y; (2). Transposition or (3). Translocation of sdY to Ssa03 or Ssa06; or (4). Translocation or transposition of sdY to another chromosome within the genome (labeled N). Upon transposition to a new chromosome e.g. Ssa06 or Ssa03 the homologous pair becomes a new X and Y (designated neo X and neo Y) after which point segregation can produce different phenotype/sdY/genotype predictions. Note that case 4 could not be observed because individuals in which sex likely jumped were harvested and thus had no offspring that could be analyzed. Following any of these three major events chromosomal segregation further alters the haplotype / sdY / phenotype sex predictions (shown at bottom). Homeologous recombination could also occur between the paralogous chromosomes Ssa06 and Ssa03 (outcomes the same as in 2.) This figure also does not include sex-reversal due to temperature (see Table 3.2).



**Figure 4.2. Predicted structure of the sdY protein (green ribbon) based on sequence similarity to Interferon regulatory factor 3 association domain (white ribbon: 1QWT) and the well conserved DNA binding domain (blue ribbon 1T2K). Note the orientation of the DNA binding domain is not positioned physiologically with respect to the association domain.**

Therefore, because interferon proteins have been shown to inhibit testosterone production, and important factor in male differentiation and development, in leydig cells

it is likely that *sdY* may function as an inhibitor of this action via binding to the interferon regulatory factors (Yano et al. 2012). Since interferon regulatory factors act as a dimer (Chen et al. 2008) an inactive heterodimer may be formed between the *sdY*, lacking a DNA binding domain and the interferon regulatory factor. Alternatively, *sdY* may have a completely new function via interaction with another factor involved in sex determination and this could add another level of complexity to the relationship of the presence of the *sdY* gene, the familial *SEX* genotype and the sex phenotype. Consistent with the idea of a completely novel function is the observation that functionally as a sex determinant *sdY* is lineage specific despite sequence similarity to interferon like proteins in other vertebrates (Yano et al. 2012, 2013). However, the proposed mechanism above is still a novel function compared to the interferon regulatory factors. Along the line of increased complexity in salmonid sex determination, changes in any of several other factors including modifier genes and other key genes in the Atlantic salmon sex-determining pathway could result in naturally observed changes to sex phenotype as is found in some human sex reversal disorders (Eggers and Sinclair 2012). In addition, autosomal influences from what seems to be a weaker sex locus has been observed to sex reverse some rainbow trout females to males in all female populations generated by gynogenesis (Quillet et al. 2002, Valdivia et al. 2013). Therefore, the possibility that *sdY* is not the master sex-determining gene in Atlantic salmon but rather an important downstream player that can alter the sex phenotype should not be ruled out. However, the presence or absence of the *sdY* gene did show 100% concordance with the sex phenotypes of the parents. These individuals have the most accurate sex phenotype calls because progeny have been reared from them.

### **4.3. Mechanisms of *sdY* movement from one chromosome to another**

With a seemingly conserved sex-determining gene (*sdY*) identified in the Tasmanian Atlantic salmon that exhibit three *SEX* loci on different chromosomes, I sought to identify mechanisms by which the *sdY* gene could move about the genome. Given the large regions of homology between the homeologous chromosomes Ssa03 (LG11) and Ssa06 (LG4) in European Atlantic salmon (Lien et al. 2011), recombination between homeologous regions may allow *sdY* movement between Ssa03 and Ssa06. However,

SNP markers did not detect homeology between Ssa02 (LG1) and Ssa03 or Ssa06 (Lien et al. 2011). Rather, Ssa02 shares large homeologous regions with Ssa05 and Ssa12 (Lien et al. 2011). Therefore, it is suggested that the *sdY* gene likely moves by a chromosomal translocation between Ssa02 and Ssa03 or Ssa06. Alternatively, the gene may be able to jump via transposition events (Davidson et al. 2009). At this time it can only be speculated which of these potential mechanisms may have been involved in producing the multiple sex-determining loci observed in Tasmanian Atlantic salmon.

#### **4.4. Why these three loci?**

Although the possibility that more than three sex loci exist in Atlantic salmon, it is interesting to speculate why only three loci were observed. Is it merely a coincidence? Has the gene only had the opportunity to jump once or twice? Is there some ancestral homology between chromosomes 2, 3 and 6? or is there some selective advantage to having *SEX* on one of these three chromosomes? The first two questions will require expansion to larger sample sets including families from Europe and North America and thus cannot be answered here.

The third question poses the hypothesis that chromosome 2 is the ancestral locus while chromosomes 3 and 6 which represent few lineages in the sample set may have arisen more recently. Consistent with this hypothesis is the observed slight homology between the Arctic charr sex linkage group 1/21 and Atlantic salmon linkage group 1/6 (Küttner et al. 2011). Linkage group 1 and Linkage group 6 correspond to chromosome 2 (Ssa02) and chromosome 12 (Ssa12) in Atlantic salmon, respectively. In fact comparative homology with stickleback by Lien et al. (2011) showed Ssa02 and Ssa12 q arms were paralogous. However, the homology observed between Atlantic salmon and Arctic char chromosomes constitutes only a single marker and no homology could be found between Atlantic salmon chromosomes 2 and 3 and 6 by ancestral genome comparison method. Therefore at the present moment no deep homology can be found that would have acted as a means for *SEX* movement from Ssa02 to Ssa03 or Ssa06.

What if not all chromosomes are equally good at being sex chromosomes and some are in fact predisposed to carrying out such a role? Such chromosomes house a

collection of sex-determining and differentiation factors (Brelsford et al. 2013). One gene in particular that appears to have deep homology among vertebrates is *Dmrt1* (Brelsford et al. 2013). This gene and or its duplicates seem to continually show up on vertebrate sex chromosomes studied including birds (Smith et al. 2009), mammals (Matson et al. 2011), medaka (Nanda et al. 2002), *Bufo siculus* (Sicilian green toad), *Hyla arborea* (European tree frog), *Rana temporaria* (common frog) and *X. tropicalis* (Yoshimoto et al. 2008, Brelsford et al. 2013). Could it be possible then that this gene and other important sex-determining or differentiating factors are located on these three chromosomes? FISH analysis using bacterial artificial chromosomes (BACs) containing *Dmrt1* and thirteen other characterized sex determination and differentiation factors did not hybridize to any of the three chromosomes identified here (Li 2010). Therefore it seems unlikely that these three chromosomes were predisposed to becoming sex chromosomes in Atlantic salmon.

In summary, all of these different speculations require further testing with the exception of the last hypothesis, which can likely be ruled out. Characterization of the region surrounding *sdY* in the three male lineages would indeed be most valuable to answer the question if deep homology exists between the three sex chromosomes identified here or if a mobile element constitutes the entire homology between Ssa02 and Ssa06 or Ssa03.

#### **4.5. Are there more than three *SEX* loci in Atlantic salmon?**

It is surprising how little genetic mapping of sex-determining loci has actually been carried out in salmonids, particularly Atlantic salmon. For example, only two SALMAP mapping families with parents from the River Tay, Scotland were used in the identification of LG1 (Ssa02) as the *SEX* containing linkage group in Atlantic salmon (Woram et al. 2003, Danzmann et al. 2008). Subsequent genetic mapping studies involving Atlantic salmon, including those using predominantly SNP markers (Lien et al. 2011, Brenna-Hansen et al. 2012), did not incorporate the sex phenotype as a variable when genetic maps were constructed. In Arctic charr two genetic mapping families were initially used to assign *SEX* to LG4 (Woram et al. 2003), and it was not until mapping studies were conducted on other families of Arctic charr that evidence for additional sex-determining loci in this species was reported (Moghadam et al. 2007, Küttner et al.

2011). This rather unexpected observation, coupled with the results reported here, indicate the importance of using many families when mapping *SEX* in salmonid species.

The Tasmanian Atlantic salmon ancestral population came from the River Phillip, Nova Scotia, Canada. Given the chromosomal differences and sub species status of Scottish (European) and Tasmanian (North American derived) Atlantic salmon it was not so surprising to find that *SEX* mapped to a different locus in each strain (Brenna-Hansen et al. 2012). What was astonishing was that three *SEX* loci were found in the Tasmanian population. This population was founded by three introductions in consecutive years in the 1960s. It begs questions then concerning year class differences in the ancestral population, and how many *SEX* loci there are in Atlantic salmon and how these *SEX* loci are distributed across the species range. Moreover, as mentioned earlier, is it possible to identify the ancestral *SEX* locus in *Salmo salar*, and if so does this provide a clue how the *SEX* locus moves about the genome? Based on linkage analysis in four families, *SEX* was mapped to LG28 in brown trout, the sister species to Atlantic salmon in the genus *Salmo* (Woram et al. 2003). Brown trout LG28 corresponds to LG8 (Ssa15) in Atlantic salmon (Li et al. 2011). Thus, no common ancestral *SEX* locus is obvious for the genus *Salmo*. As with Atlantic salmon, it may be worth mapping the sex-determining gene in families of brown trout from several different geographical populations.



## 5. Conclusions

In summary, three sex loci lineages were identified in the SALTAS Tasmanian Atlantic salmon breeding population, all of which show evidence for the presence of *sdY*. However, at this point it can only be speculated which chromosome houses the ancestral locus and how *sdY* might be moving between these three chromosomes. Characterization of the genomic sequence surrounding the *sdY* gene as well as further linkage analysis to identify individuals in which *sdY* may have jumped are necessary to provide answers for these questions. It still remains unknown how many sex loci there are in Atlantic salmon and if there are any constraints on which chromosomes are appropriate hosts for the sex-determining gene. Thus, further *SEX* linkage analysis across the entire species range of Atlantic salmon should be conducted.

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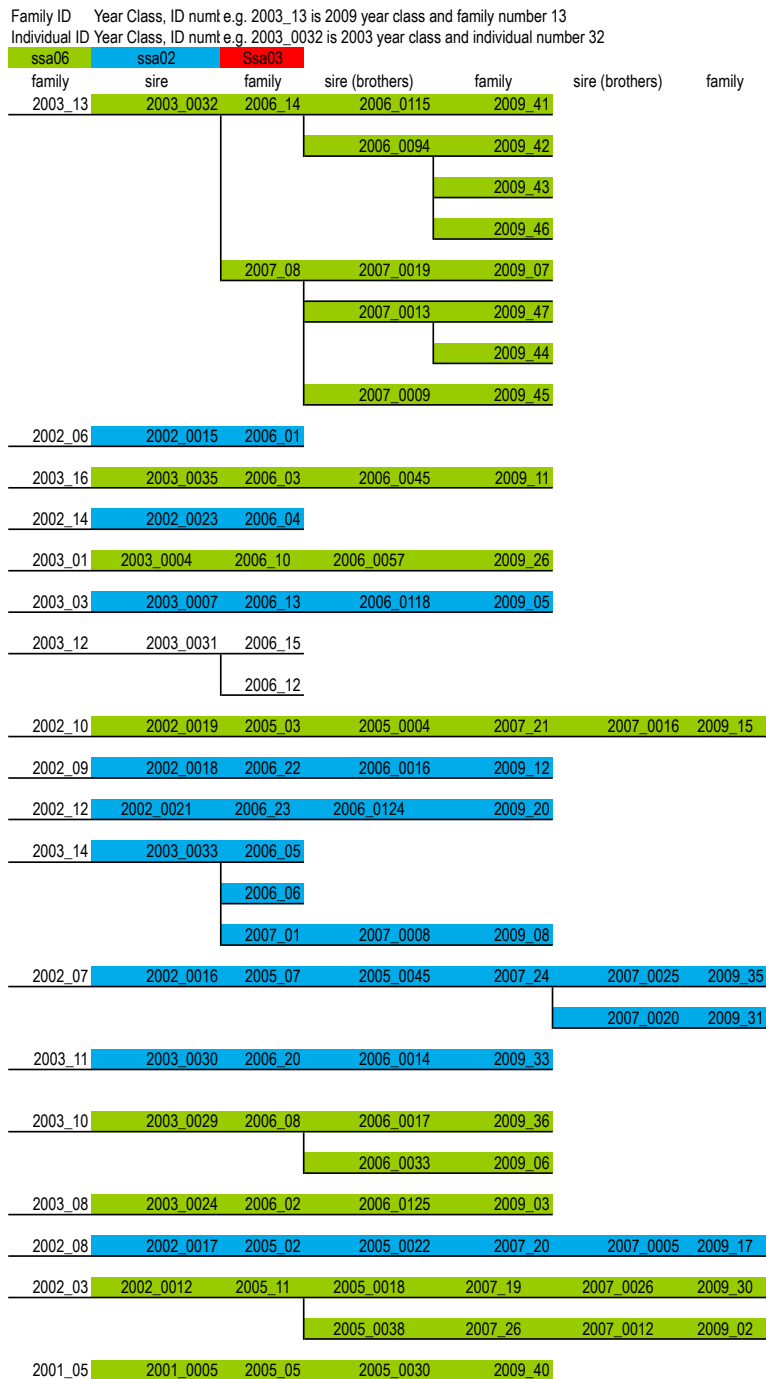
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# Appendix.

## Supplementary Tables and Figures

### Supplementary Table S1.

#### Pedigree of the lineages with representation in the 2009 year class



family	sire	family	sire (brothers)	family	sire (brothers)	family
2002_05	2002_0014	2005_12	2005_0066	2007_17	2007_0011	2009_16
2003_05	2003_0013	2006_11	2006_0067	2009_10		
			2006_0098	2009_24		
2002_11	2002_0020	2005_08	2005_0029	2009_04		
2001_08	2001_0016	2004_05	2004_0002	2007_09	2007_0014	2009_37
2003_11	2003_0030	2006_20	2006_0014	2009_33		
2001_04	2001_0004	2004_08	2004_0007	2007_06	2007_0006	2009_01
2001_03	2001_0003	2004_03	2004_0010	2007_13	2007_0023	2009_39
2001_02	2001_0002	2004_01	2004_0009	2007_11	2007_0022	2009_09
					2007_0024	2009_25
2003_07	2003_0023	2006_07	2006_0069	2009_13		
2001_06	2001_0006	2004_02	2004_0004	2007_03	2007_0004	2009_14
2001_01	2001_0001	2004_04	2004_0006	2007_07	2007_0017	2009_21
			2005_0048	2009_18		
2003_15	2003_0034	2007_02	2007_0003	2009_22		
2003_06	2003_0018	2007_12	2007_0007	2009_23		
2002_02	2002_0011	2005_10	2005_0012	2007_15	2007_0010	2009_27
2001_09	2001_0017	2004_06	2004_0003	2007_04	2007_0021	2009_28
2001_07	2001_0015	2004_07	2004_0001	2007_10	2007_0018	2009_38
				2007_14	2007_0015	2009_29
2002_04	2002_0013	2005_06	2005_0035	2007_23	2007_0001	2009_32
2002_13	2002_0022	2007_05	2007_0002	2009_34		
2003_02	2003_0006	2006_17	2006_0013	2009_19		
2002_01	2002_0010	2005_01	2005_0055	2007_16		
				2007_22		
			2005_0065	2007_18		
				2007_25		
		2005_13	2005_0037	2008_01		
				2008_02		
2003_04	2003_0010	2006_16				
2003_09	2003_0027	2006_18				

**Supplementary Table S2.**

The 64 SNPs chosen for the genome wide scan. Detailed linkage groups (LG)s can also be found at [www.asalbase.org](http://www.asalbase.org). Additional SNP information is available online at [www.asalbase.org](http://www.asalbase.org)

SNPs taken from across the genome.	ID	Alternate ID
LG2	Ssa0193ECIG	16654_489
LG3 only Moen map	Ssa0169aECIG	16265_112
LG4	Ssa0074ECIG	14686_659
LG5	Ssa0131aECIG	15686_402
LG13 only Moen map	Ssa0258ECIG	17495_0157
LG21	Ssa0063cECIG	14392_0452
LG6	Ssa0156ECIG	16129_0239
LG17 only Moen map	Ssa0064ECIG	14468_0119
LG19	Ssa0158ECIG	16147_0613
LG24	Ssa0030ECIG	13579_599
LG28 only Moen map	Ssa0023ECIG	13197_0590
LG33	Ssa0099ECIG	15153_346
LG11	Ssa0137ECIG	15804_0172
LG13 only Moen map	Ssa0142ECIG	15912_0572
LG18	Ssa0082ECIG	14834_0524
LG18	Ssa0052ECIG	14186_163
LG23	Ssa0045ECIG	14053_0820
LG25 only Moen map	Ssa0037ECIG	13907_0121
LG16	Ssa0207ECIG	16791_0446
LG14 only Moen map	Ssa0214ECIG	16856_0321
LG20	Ssa0244ECIG	17230_430
LG31	Ssa0172ECIG	16317_0150
LG15	Ssa0205ECIG	16783_0060
LG7	Ssa0194ECIG	16677_0620
LG23	Ssa154SSFU	Contig925
LG18	Ssa150SSFU	Contig831
LG1 only Moen map	Ssa0182ECIG	16475_1011
LG2	Ssa0078ECIG	14746_388
LG8	Ssa0218ECIG	16903_662
LG9	Ssa0081ECIG	14800_360
LG9	Ssa0028ECIG	Contig13513_0493
LG5	Ssa0170ECIG	Contig16294_0244
LG8	Ssa0217ECIG	Contig16890_1072
LG15	Ssa0248ECIG	Contig17293_0832
LG3	Ssa0250ECIG	Contig17321_0691
LG25	Ssa0147SSFU	Contig 573
LG11	Ssa0062ECIG	Contig14389_0361
LG2	Ssa0072ECIG	Contig14649_80
LG12	Ssa0079bECIG	Contig14757_562
LG20	Ssa0109ECIG	Contig15309_266
LG16	Ssa0119ECIG	Contig15513_384
LG10	Ssa0126aECIG	Contig15610_504

SNPs taken from across the genome.	ID	Alternate ID
LG18	Ssa0157ECIG	Contig16140_0475
LG24	Ssa0167ECIG	Contig16258_0579
LG25	Ssa0168ECIG	Contig16260_0757
LG23	Ssa0004ECIG	Contig11981_0368
LG19	Ssa0091ECIG	Contig15004_0676
LG14	Ssa0257ECIG	Contig17489_0078
LG22	Ssa0005ECIG	Contig12000_0548
LG21	Ssa0019ECIG	Contig12869_0119
LG28	Ssa0044ECIG	Contig14035_0356
LG17	Ssa0055ECIG	Contig14230_0080
LG31	Ssa0127ECIG	Contig15618_92
LG13	Ssa0190ECIG	Contig16609_0285
LG32	Ssa0032bECIG	Contig13615_543
LG6	Ssa0049ECIG	Contig14161_559
LG33	Ssa0087ECIG	Contig14962_456
LG4	Ssa0103aECIG	Contig15190_1379
LG1	Ssa0166ECIG	Contig16240_0204
LG10 only Moen map	Ssa0093ECIG	15042_307
LG2	Ssa1SSFU	Contig94
LG25	Ssa147SSFU	Contig573
LG2	Ssa10SSFU	Contig1167
LG6	Ssa158SSFU	Contig1062











Legend AS-Ancestral sire S1 or S2 is sire D1 or D2 is dam Unknown Known Male Predicted male Known female Predicted female Unknown sex predicted for this family sdy presence and absence indicated by 1 and 0 respectively for Exon 2 Exon 4 Fabp6 (DNA quality control)

Markers Ssa02.LG1 Ssa03.LG1 Ssa04.LG1 Actual known phenotypes are displayed in the 'Sex call from Phenotype' row. Family ID in format Year class\_Family number e.g. 2009\_05 = 2009 Year class\_Family 5. Individual ID in format Year class\_Individual number e.g. 2003\_0007 = 2003 Year class\_Individual 0007

2008\_28 Ssa02 parents and sdy agree 100%

AS1-2007_0017	AS2	S1-2007_0021	S2	2008_0791	2008_0815	2008_0844	2008_0870	2008_0897	2008_0917	2008_0944	2008_0913	2008_0940	2008_0921	2008_0915	2008_0942	2008_0919	2008_0941	2008_0911	2008_0943	2008_0920	2008_0946	2008_0923	2008_0913	2008_0921	2008_0916	2008_0945	2008_0921	2008_0916
Ssa01888SFU	3	4	3	4	3	3	3	3	3	4	3	4	4	4	3	4	4	4	4	4	4	3	3	3	3	4	3	4
Ssa01889SFU	1	3	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssa01890SFU	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

2008\_29 Ssa02 parents and sdy agree 100%

AS1-2007_0015	AS2	S1-2007_0015	S2	2008_0911	2008_0933	2008_0934	2008_0925	2008_0945	2008_0945	2008_0938	2008_0948	2008_0938	2008_0948	2008_0934	2008_0948	2008_0914	2008_0948	2008_0914	2008_0948	2008_0914	2008_0948	2008_0914	2008_0948	2008_0914	2008_0948	2008_0914	2008_0948	2008_0914	2008_0948
Ssa01891SFU	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	
Ssa01892SFU	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
Ssa01893SFU	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	

2008\_31 Ssa03 parents and sdy agree 100%

AS1-2007_0001	AS2	S1-2007_0001	S2	2008_0923	2008_0928	2008_0911	2008_0921	2008_0929	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928
Ssa01894SFU	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2		
Ssa01895SFU	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3		
Ssa01896SFU	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3		

2008\_34 Ssa02 parents and sdy agree 100%

AS1-2007_0022	AS2	S1-2007_0022	S2	2008_0923	2008_0928	2008_0911	2008_0921	2008_0929	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928
Ssa01897SFU	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4		
Ssa01898SFU	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
Ssa01899SFU	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4		

2008\_37 Ssa03 parents and sdy agree 100%

AS1-2007_0016	AS2	S1-2007_0016	S2	2008_0916	2008_0917	2008_0947	2008_0924	2008_0925	2008_0927	2008_0926	2008_0926	2008_0921	2008_0924	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928
Ssa01900SFU	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
Ssa01901SFU	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
Ssa01902SFU	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	

2008\_38 Ssa02 parents and sdy agree 100%

AS1-2007_0016	AS2	S1-2007_0016	S2	2008_0921	2008_0917	2008_0919	2008_0924	2008_0925	2008_0927	2008_0926	2008_0926	2008_0921	2008_0924	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928	2008_0922	2008_0927	2008_0924	2008_0928
Ssa01903SFU	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
Ssa01904SFU	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
Ssa01905SFU	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	

2008\_39 Ssa03 parents and sdy agree 100%

AS1-2007_0023	AS2	S1-2007_0023	S2	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924	2008_0924
Ssa01906SFU	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssa01907SFU	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Ssa01908SFU	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1







**Supplementary Table S4.**

**Microsatellite and SNP analysis of SALTAS broodstock families in which the sex-determining gene resides on Ssa06 (AS-4). Markers are arranged in order corresponding to their placement on the female European Atlantic salmon linkage map (See Phillips et al. 2009 for nomenclature) [www.asalbase.org](http://www.asalbase.org). The seven families originally analyzed are highlighted yellow.**

Marker	21	18	39	40	30	02	15	26	10	24	03	06	07	11	45	44	47	41	42	43	46		
<b>Ssa02</b>																							
Ssa1077BSFU			18/24 (1.4)					6/23 (0)		7/14 (0)													
Ssa0056BSFU	<sup>2</sup> 25/33 (2.0)	9/17 (0)			13/25 (0)	11/18 (0.2)	7/16 (0.1)	15/22 (0.6)		6/16 (0)		11/21 (0)	10/32 (0)					11/16 (0.5)	12/14 (1.7)				
Ssa0163BSFU		17/24 (0.9)	8/18 (0)		8/18 (0)	8/18 (0)							21/32 (0.7)	10/13 (0.9)	4/13 (0.4)	7/11 (0.2)	9/11 (1.0)				5/12 (0.1)	10/12 (1.3)	
Ssa202DU									7/21 (0)														
Ssa0233BSFU																							
Ssa1266BSFU																							
Ssa0162ECIG																							
<b>Ssa06</b>																							
OMM5037	31/33 (6.6)														15/15 (4.5)	10/10 (3.0)	11/11 (3.3)	14/15 (2.9)	13/13 (3.9)	12/12 (3.6)	12/12 (3.6)	118/121	
Ssa0074ECIG					25/25 (7.5)			17/21 (1.9)		14/14 (4.2)	21/21 (6.3)	20/21 (4.6)											30.3
Ssa0376BSFU			23/25 (4.5)	16/16 (4.8)	25/25 (7.5)			21/23 (4.0)	20/21 (4.6)	14/16 (3.2)	14/14 (4.2)		32/32 (9.6)	13/13 (3.9)	15/15 (4.5)	11/11 (3.3)	10/10 (3.0)	14/15 (4.2)	14/14 (3.9)	12/12 (3.6)	12/12 (3.6)	97/102	
Ssa0789BSFU		16/16 (2.9)	23/25 (4.5)				14/16 (3.2)								14/14 (4.2)	10/10 (3.0)	14/16 (4.2)	14/15 (4.2)	14/14 (3.9)	11/11 (3.3)	13/13 (3.9)	269/277	
Ssa0043BSFU															11/13 (1.5)	5/5 (1.5)	10/10 (3.0)	14/16 (3.2)	11/11 (3.3)	13/13 (3.9)	140/145		
Ssa0103aECIG				19/19 (5.4)																		64/68	
Ssa0142BSFU															13/13 (3.9)	11/11 (3.3)	11/11 (3.3)	14/15 (2.9)	14/14 (4.2)	12/12 (3.6)	12/12 (3.6)	19/19	
Ssa0360BSFU							14/16 (3.2)								44 (1.2)	10/11 (3.3)	11/11 (3.3)	13/16 (3.2)	14/14 (4.2)	12/12 (3.6)	12/12 (3.6)	82/83	
Ssa1299BSFU						17/18 (3.7)	21/22 (4.8)															21.1	
Ssa0752BSFU						14/16 (3.2)									10/10 (3.0)	10/10 (3.0)	10/10 (3.0)	10/10 (3.0)	11/11 (3.3)	13/13 (3.9)	14/14 (4.2)	0	
<b>Ssa03</b>																						5.7	
Ssa0646BSFU				6/19 (0.6)																		22.6	
Ssa0343BSFU																						0.01	
OMM1272			11/24 (0)																			21.1	
Ssa0017BSFU								8/22 (0.3)		8/15 (0)					9/15 (0.1)							13.9	
ESTNV_28224_109																						0	
Ssa0366BSFU	18/34 (0)	9/17 (0)					9/16 (0.1)			14/17 (1.7)		10/21 (0)	11/21 (0.1)	17/31 (0.1)					8/14 (0.1)	6/12 (0)	9/12 (0.7)	0	
Ssa0816BSFU																						0.03	
Ssa0212BSFU																						0.03	
Ssa0516BSFU																						0.02	
OMM5019																						0.03	
OMM1267																						0	
Ssa0144																						0.02	
OMM50372																						14.2	

<sup>1</sup>Family (2009 year class where family ID's are given as XX, values below). <sup>2</sup>Chromosome number shown in Ssa0X. <sup>3</sup>Number of offspring for which genotype/phenotype agree / total number of offspring with genotype/phenotype. <sup>4</sup>LOD score corresponding to the ratio of correct genotype/phenotype matches:incorrect genotype/phenotype matches in a given family.

**Supplementary Table S5.**  
**Microsatellite and SNP analysis of SALTAS broodstock families in which the sex-determining gene housed on Ssa02 (AS-1). Markers are arranged in order corresponding to their placement on the female European Atlantic salmon linkage map ([www.asalbase.org](http://www.asalbase.org)).**

Marker	25	9	1	29	38	28	27	16	31	35	17	12	04	20	34	19	05	23	13	33	08	22	14	Total	LOD	T <sub>h</sub> el <sub>h</sub>	Ssa02	
Ssa02 <sup>1</sup>																												
Ssa1077BSFU		21/23 <sup>3</sup> (4.0) <sup>4</sup>							28/29 (6.6)													16/17 (3.4)		65/69	14.1		Ssa1077BSFU 0.06	
Ssa0056BSFU	24/25 (5.7)	20/20 (6.0)	23/23 (6.9)	23/24 (5.4)	23/24 (5.4)	31/31 (9.3)	9/9 (2.7)	23/24 (5.4)	28/29 (6.6)	25/25 (7.5)	27/30 (4.6)	46/50 (9.0)	28/27 (6.2)	12/12 (3.6)	14/14 (4.2)	23/25 (7.7)	26/26 (4.5)	23/23 (6.9)	25/27 (6.3)					428/444	103.7		Ssa0056BSFU 0.04	
Ssa0188BSFU				21/22 (4.9)					23/24 (5.4)	28/29 (6.6)														98/102	23.4		Ssa0188BSFU 0.04	
Ssa202DU											23/23 (6.9)												14/14 (4.2)	14/14	4.2		Ssa202DU 0	
Ssa0233BSFU															17/18 (3.7)									89/90	24.7		Ssa0233BSFU 0.01	
Ssa0182ECIG			23/23 (6.9)																					23/23	6.9		Ssa0182ECIG 0	
Ssa06																												
OMM1937																												
Ssa0074ECIG																												
ESTNV_21223_122																												
Ssa0376BSFU																												
Ssa0789BSFU																												
Ssa0043BSFU																												
Ssa0103aECIG																												
Ssa0142BSFU																												
Ssa0306BSFU																												
Ssa1298BSFU																												
Ssa0752BSFU																												
Ssa0649BSFU																												
Ssa0438BSFU																												
OMM1272																												
Ssa0076BSFU																												
ESTNV_29224_109																												
Ssa0368BSFU																												
Ssa0818BSFU																												
Ssa0212BSFU																												
Ssa0616BSFU																												
OMM1919																												
OMM1267																												
Ssa0144																												

<sup>1</sup>Family [2009] year class where family IDs are given as XX, values below). <sup>2</sup>Chromosome number shown in Ssa0X. <sup>3</sup>Number of offspring for which genotype/phenotype agree / total number of offspring with genotype/phenotype. <sup>4</sup>LOD score corresponding to the ratio of correct genotype/phenotype matches:incorrect genotype/phenotype matches in a given family.



**Supplementary Table S6.**

**Microsatellite and SNP analysis of SALTAS broodstock families in which the sex-determining gene housed on Ssa03 (AS-11). Markers are arranged in order corresponding to their placement on the female European Atlantic salmon linkage map. [www.asalbase.org](http://www.asalbase.org)**

Marker	2009_32 <sup>1</sup>	2009_37	Total	LOD	Theta
<b>Ssa02<sup>2</sup></b>					
Ssa1077BSFU	28/35 <sup>3</sup> (2.9) <sup>4</sup>				
Ssa0055BSFU					
Ssa0183BSFU					
Ssa202DU		13/17 (1.1)			
Ssa0233BSFU					
Ssa1265BSFU					
Ssa0182ECIG					
<b>Ssa06</b>					
OMM5037	16/35 (0)				
Ssa0074ECIG		10/17 (0.1)			
Ssa0376BSFU					
Ssa0789BSFU					
Ssa0043BSFU					
Ssa0103aECIG	12/35 (0)				
Ssa0142BSFU					
Ssa0350BSFU					
Ssa1299BSFU		8/17 (0)			
Ssa0752BSFU					
<b>Ssa03</b>					
Ssa0649BSFU	28/33 (3.8)		28/33	3.8	0.15 Ssa0649BSFU
Ssa0017BSFU	30/34 (4.9)	15/16 (3.2)	45/50	8	0.1 Ssa0017BSFU
Ssa0368BSFU	31/35 (5.1)		31/35	5.1	0.11 Ssa0368BSFU
OMM1267	24/25 (5.7)	14/15 (2.9)	38/40	8.6	0.05 OMM1267
Ssa0516BSFU	30/34 (4.9)	15/16 (3.2)	45/50	8	0.1 Ssa0516BSFU
OMM5019	29/32 (5.3)		29/32	5.3	0.09 OMM5019
SsaD144	30/34 (4.9)				

<sup>1</sup>Family (2009 year class where family ID's are given as XX, values below). <sup>2</sup>Chromosome number shown in Ssa0X.

<sup>3</sup>(Number of offspring for which genotype and phenotype agree) / (total number of offspring with genotype and phenotype). <sup>4</sup>LOD score corresponding to the ratio of correct genotype and phenotype matches:incorrect genotype and phenotype matches in a given family.

**Supplementary Table S7.**

**Summary of individuals in which *sdY* call, phenotype call and/ or genotype call do not agree. Discrepancies are highlighted green.**

<sup>1</sup> Family ID	<sup>2</sup> Individual ID	Phenotype	Phenotypic sex predicted by:		
			Haplotype	<sup>7*</sup> <i>sdY</i> test	Exon 3
2009_32	2009_0558	<sup>4</sup> F	M	<sup>3</sup> F	<sup>5</sup> N
2009_20	2009_0064	F	M	F	<sup>6</sup> Y
2009_20	2009_1027	F	M	F	Y
2009_20	2009_1008	F	M	F	Y
2009_32	2009_0636	F	M	F	N
2009_24	2009_0924	M	F	M	Y
2009_20	2009_0672	M	F	F	Y
2009_08	2009_0373	F	M	F	N
2009_04	2009_0053	M	F	F	N
2009_15	2009_0427	M	F	F	N
2009_38	2009_0261	M	F	F	N
2009_22	2009_0352	M	F	F	N
2009_26	2009_0459	M	F	F	Y
2009_10	2009_0102	M	F	F	Y
2009_23	2009_0559	M	F	F	Y
2009_28	2009_0599	M	F	F	Y
2009_23	2009_0535	M	F	F	N
2009_04	2009_0408	M	M	F	N
2009_05	2009_0048	M	M	F	Y
2009_12	2009_0342	M	M	F	Y
2009_06	2009_0406	M	M	F	Y
2009_41	2009_1080	M	F	M	Y
2009_30	2009_0400	F	F	M	Y
2009_34	2009_1030	F	F	M	Y
2009_26	2009_0988	F	F	M	Y
2009_25	2009_0838	F	M	M	Y
2009_32	2009_0279	F	M	F	N
2009_08	2009_0439	M	F	M	Y
2009_31	2009_0756	M	F	M	Y
2009_35	2009_0582	M	F	M	Y
2009_04	2009_0013	M	F	M	Y
2009_04	2009_0252	M	F	M	Y
2009_02	2009_0192	M	F	M	Y
2009_15	2009_0528	M	F	M	Y

<sup>1</sup> Family ID	<sup>2</sup> Individual ID	Phenotype	Phenotypic sex predicted by:		
			Haplotype	<sup>7*</sup> <i>sdY</i> test	Exon 3
2009_32	2009_0470	M	F	M	Y
2009_01	2009_0316	M	F	M	Y
2009_01	2009_0604	M	F	M	Y
2009_18	2009_0390	M	F	M	Y
2009_03	2009_0940	M	F	M	Y
2009_24	2009_0641	M	F	M	Y
2009_34	2009_0953	M	F	M	Y
2009_37	2009_0578	M	F	M	Y
2009_39	2009_0982	M	F	M	Y
2009_39	2009_0369	M	F	M	Y
2009_36	2009_0947	M	F	M	Y

<sup>1</sup> Family ID in format Year class\_ID e.g. 2009\_01 = 2009 Year class\_family 01

<sup>2</sup> Individual ID in format Year class\_ID e.g. 2009\_0001 = 2009 year class\_individual 01

<sup>3</sup> F= female

<sup>4</sup> M= Male

<sup>5</sup> N= No (Call does not agree with *sdY* call)

<sup>6</sup> Y= yes (call agrees with *sdY* call)

<sup>7</sup> *sdY* call based on multiplex

\* For *sdY* multiplex tests exon 2 and exon 4 must be present for a male call. Note a male call was given if only Exon 2 showed up but the Fabp6b quality control did not amplify (see Supplementary Table S9 for details).

**Supplementary Table S8.**  
**Concordance of sdY with genotype and phenotype of the 2009 year class (YC)**  
**families.**

Family ID	phenotype and genotypes available	Total correct calls		Total miscalls		total with only one representative exon 2 or 4		Phenotype of miscalls sdY		Phenotype miscalls genotype	
		agree with genotype	disagree with genotype	agree with genotype	disagree with genotype	1	0	male	female	male	female
2009_05	14	13	0	0	1	1	0	1	1	0	1
2009_08	27	25	2	0	0	0	0	0	0	0	0
2009_12	21	20	1	0	1	1	0	2	1	0	1
2009_19	12	12	0	0	0	0	0	0	0	0	0
2009_31	24	23	1	0	0	0	0	0	0	0	0
2009_33	23	23	0	0	0	0	0	0	0	0	0
2009_35	29	28	1	0	0	0	0	1	0	0	0
2009_17	25	25	0	0	0	0	0	1	0	0	0
2009_20	50	46	3	1	1	0	0	0	1	1	3
2009_04	29	25	2	1	1	1	0	0	2	3	1
2009_02	17	16	1	0	0	0	0	0	0	0	0
2009_07	32	32	0	0	0	0	0	3	0	0	0
2009_11	13	13	0	0	0	0	0	0	0	0	0
2009_15	16	15	1	1	1	0	0	0	1	0	0
2009_30	23	22	0	0	0	1	0	2	0	1	0
2009_40	15	15	0	0	0	0	0	0	0	0	0
2009_06	20	19	0	0	0	1	0	3	1	0	0
2009_26	23	21	0	0	1	1	0	0	1	1	0
2009_36	21	20	1	0	0	0	0	0	0	0	0
2009_01	22	20	2	0	0	0	0	0	0	0	0
2009_03	14	13	1	0	0	0	0	0	0	0	0
2009_09	21	21	0	0	0	0	0	0	0	0	0
2009_10	21	20	0	0	1	0	0	0	1	0	0
2009_13	26	26	0	0	0	0	0	0	0	0	0
2009_14	14	14	0	0	0	0	0	0	0	0	0
2009_16	8	8	0	0	0	0	0	1	0	0	0
2009_18	16	15	1	0	0	0	0	0	0	0	0
2009_21	33	31	0	0	2	0	0	0	0	0	0
2009_22	17	16	0	1	0	0	0	0	2	2	1
2009_23	24	22	0	2	0	0	0	0	1	1	0
2009_24	15	13	2	0	0	0	0	0	2	2	0
2009_25	24	23	0	1	0	0	0	1	0	0	0
2009_27	28	28	0	0	1	0	0	0	1	1	0
2009_28	22	21	0	0	0	0	0	0	0	0	0
2009_29	23	23	0	0	0	0	0	0	1	1	0
2009_32	33	29	4	0	0	0	0	1	0	0	0
2009_34	26	24	1	0	0	0	0	2	0	1	3
2009_37	15	14	1	0	0	1	0	0	0	1	0
2009_38	24	23	0	1	0	0	0	0	0	0	0
2009_39	24	22	2	0	0	0	0	0	1	1	0
2009_42	14	14	0	0	0	0	0	0	0	0	0
*2009_41	16	15	1	0	0	0	0	0	0	0	0
*2009_47	11	11	0	0	0	0	0	0	0	0	0
*2009_45	15	15	0	0	0	0	0	0	0	0	0
*2009_43	12	12	0	0	0	0	0	0	0	0	0
*2009_46	13	13	0	0	0	0	0	0	0	0	0
*2009_44	11	11	0	0	0	0	0	0	0	0	0
Totals	976	930	27	13	7	20	16	4	31	9	9

Total males: 567  
 Total correct males: 539  
 Total females: 409  
 Total correct females: 405  
 Total correct calls: 957

\*Family ID shown as Year class\_ family number e.g. 2009\_01 = 2009 Year class\_ family 01

**Supplementary Table S9.**

**Individuals in which only a single exon of sdY (exon 2 or exon 4) was present. A 1 indicates the presence of a particular condition (Fabp6b, No Fabp6b).**

Individual	Individuals only carrying exon 4 of sdY		Individuals only carrying exon 2 of sdY		Phenotype
	No Fabp6b	Fabp6b	No Fabp6b	Fabp6b	
2009_0048	1				male
2009_0342	1				male
2009_0725	1				female
2009_0068	1				female
2009_0009			1		male
2009_0911				1	female
2009_0147				1	female
2009_0355		1			female
2009_0404		1			female
2009_0304		1			female
2009_0772		1			female
2009_0227			1		male
2009_0457			1		male
2009_0279		1			female
2009_0967			1		male
2009_0091				1	female
2009_0190		1			female
2009_0537		1			female
2009_0406		1			female
Total:		12	4	3	

<sup>†</sup>Individual ID given as year class\_XXXX e.g. 2009\_0048 is 2009 yearclass individual 0048