

## ORIGINAL ARTICLE

# Evidence for multiple sex-determining loci in Tasmanian Atlantic salmon (*Salmo salar*)

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Phenotypic sex in salmonids is determined primarily by a genetic male heterogametic system; yet, sex reversal can be accomplished via hormonal treatment. In Tasmanian Atlantic salmon aquaculture, to overcome problems associated with early sexual maturation in males, sex-reversed females are crossed with normal females to produce all female stock. However, phenotypic distinction of sex-reversed females (neo-males) from true males is problematic. We set out to identify genetic markers that could make this distinction. Microsatellite markers from chromosome 2 (Ssa02), to which the sex-determining locus (*SEX*) has been mapped in two Scottish Atlantic salmon families, did not predict sex in a pilot study of seven families. A TaqMan 64 SNP genome-wide scan suggested *SEX* was on Ssa06 in these families, and this was confirmed by microsatellite markers. A survey of 58 families in total representing 38 male lineages in the SALTAS breeding program found that 34 of the families had *SEX* on Ssa02, in 22 of the families *SEX* was on Ssa06, and two of the families had a third *SEX* locus, on Ssa03. A PCR test using primers designed from the recently published *sdY* gene is consistent with Tasmanian Atlantic salmon having a single sex-determining gene that may be located on at least three linkage groups.

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## INTRODUCTION

Sexual reproduction is a biological feature of most vertebrates. Yet, the mechanisms of sex determination, which is the production of individuals with male or female phenotypes, are diverse, and involve both genetic and environmental factors (Graves and Peichel, 2010). This is particularly true for fish in which male heterogamety, as found in mammals, female heterogamety, commonly associated with birds, parthogenesis, and behavioral/age sex reversal and environmental sex determination (for example, temperature) are observed (Devlin and Nagahama, 2002; Volff *et al.*, 2007). The conservation of morphology in gonadal development among species suggests that the pathways responsible for producing male and female phenotypes are also conserved (Graves and Peichel, 2010). In contrast, several different vertebrate sex-determining master switches have been identified: *Sry* in most mammals (Koopman *et al.*, 1990; Sinclair *et al.*, 1990); *Dmy* (*Dmrt1bY*) in the medaka (*Oryzias latipes*) (Matsuda *et al.*, 2002; Nanda *et al.*, 2002); *Dm-W* in *Xenopus laevis* (Yoshimoto *et al.*, 2008); *Dmrt1* in chicken (Smith *et al.*, 2009); a Y-linked anti-Mullerian hormone gene duplicate in the Patagonian perjury (*Odontesthes hatcheri*) (Hattori *et al.*, 2012); an allele of *Amhr2* in *Takifugu rubripes* (Kamiya *et al.*, 2012); and an allele of *gsdf* in *Oryzias luzonensis* (Myosho *et al.*, 2012). Moreover, sex-determining master genes and sex-linkage groups are not conserved in closely related species in the genus *Oryzias* (Myosho *et al.*, 2012).

The Salmonidae (salmon, trout, charr, grayling and freshwater whitefish; Nelson, 2006) have received a great deal of attention due to

their economic and societal value (Thorgaard *et al.*, 2002; Davidson *et al.*, 2010). Sex determination in several members of this family involves male heterogamety (Davidson *et al.*, 2009). Although sex-specific (that is, male) genetic markers have 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, 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 have been identified in Atlantic salmon (*Salmo salar*) (McGowan and Davidson, 1998). Indeed, the observation that genetic markers linked to the sex-determining locus (*SEX*) are different in Atlantic salmon, brown trout (*Salmo trutta*), rainbow trout and Arctic charr (*Salvelinus alpinus*) (Woram *et al.*, 2003) has led to two hypotheses: (1) suggesting that there were multiple sex-determining loci in the salmonids, perhaps as a result of the autotetraploidization in their common ancestor (Allendorf and Thorgaard, 1984), and (2) suggesting that there is a single sex-determining gene that jumps around the genome (Phillips *et al.*, 2001; Davidson *et al.*, 2009). The discovery by Yano *et al.* (2012a) of the master sex-determining gene in rainbow trout (*sdY*) and its presence in other salmonid species suggests that the latter hypothesis is correct (Yano *et al.*, 2012b).

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

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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 (Davidson *et al.*, 2009). 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. As no male-specific marker has been described for Atlantic salmon, we set out to search for genetic markers that could distinguish male and female Atlantic salmon in the Tasmanian aquaculture broodstock. Here we present evidence that there are multiple sex-determining loci in the Tasmanian Atlantic salmon population, one of which corresponds to the previously mapped *SEX* on chromosome 2 (Ssa02) (Artieri *et al.*, 2006). PCR primers that amplify exons of the *sdY* gene (Yano *et al.*, 2012a,b) yield male-dominant amplification products in families representing all three identified sex-determining loci, suggesting that there is a single sex-determining gene in Atlantic salmon, which has the ability to move around the genome.

## MATERIALS AND METHODS

### SALTAS Atlantic salmon population samples

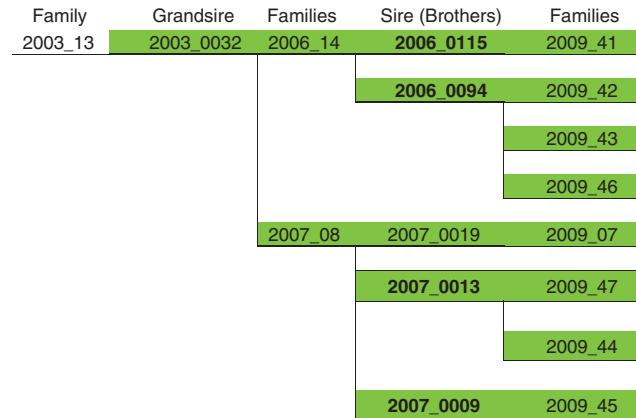
The Atlantic salmon samples used in this study are from the SALTAS selective breeding population. This population is derived from the Tasmanian landrace, which originates from the River Philip in Nova Scotia, Canada. Stocks were imported first to Gaden, New South Wales, during 1965–1967, and then transferred to Tasmania during 1984–1986. They were managed, essentially to maintain genetic diversity, until 2004 when the SALTAS selective breeding program started. The breeding objectives and selection traits used in the program are described in Elliott and Kube (2009).

The samples used in this study represent a subset from the 2009 year class, which is the sixth year class of the selective breeding program. Samples were taken from 1128 individuals, representing 47 families from a total of 152 families for that year class (Supplementary Table S1). Sampled families were the result of matings between 44 sires and 36 dams, with some parents being used in multiple matings. Sires were from the 2005, 2006 and 2007 year classes, with 57% being one generation from founding stock and 43% being two generations from founders. Dams were from the 2005 and 2006 year classes, with 92% being one generation from founders and 8% being two generations from founders. The sampled families represented 38 founding sire lineages from a total of 50 maintained sire lineages.

Sampled families were chosen to maximize the representation of sire lineages and were limited to those with at least 15 individuals per family. Individuals for which a confident phenotype or genotype could not be made were excluded. In the final data set, the number of individuals per family ranged from 11 to 54, the number of males per family was between 4 and 23, and the number of females was between 4 and 29. All sex phenotypes were recorded at slaughter by gonad inspection. This was done at either 23 months from fertilization as part of a grading and culling of early maturing individuals, or at 29 months as part of the final harvest assessment.

### Genotyping analyses

Microsatellite and SNP-based linkage analyses were used to identify the *SEX* in Atlantic salmon. Detailed information for the microsatellite and SNP markers, along with respective integrated linkage maps are available at [www.asalbase.org](http://www.asalbase.org) or available upon request. For the first seven 2009YC families (Figure 1) 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)). Informative



**Figure 1** Lineage containing the four sires of the seven families in the initial pilot study. All of these families were identified to have *SEX* located on Ssa06. Individuals (males) are shown in the format, Year class (YC), \_, individual ID, (for example, 2003\_0032; YC 2003, ID 0032). Families are classified in the same way (for example, 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.

microsatellite markers (see Phillips *et al.* (2009) for nomenclature) were genotyped by methods previously described (Danzmann *et al.*, 2008).

A TaqMan 64 SNP genome-wide assay (Life Technologies, Carlsbad, CA, USA) was conducted in 10- $\mu$ l reactions on the 2009YC families. The 64 SNP markers are listed in Supplementary Table S2. Additional SNP data related to *SEX* from the 2005YC and 2006YC families (Dominik *et al.*, 2010; Lien *et al.*, 2011) were used to predict the sex locus of unknown lineages. Genotypes were compared with male and female phenotypic calls to assess linkage to *SEX*.

SALTAS personnel assessed phenotypic 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, we set the following criteria: (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 were calculated individually for each family marker combination. We considered cases where the sex phenotype predicted by genotype was 100% accurate to be significant regardless of LOD score.

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

Yano *et al.* (2012a) 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 2 and the other specific for exon 4 of the *sdY* gene, were designed based on an alignment (not shown) of Atlantic salmon *sdY* (E Palibroda, personal communication) 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, Coralville, IA, USA):

Exon 2:

Forward: 5'-TGATGGATGGGATCCCCGTCATCTCTCTCCCAAAG-3'

Reverse: 5'-TCCCCTATGGAGGGTGGAGTGGTTTAAAGCTCTA-3'

Exon 4:

Forward: 5'-AGTTGGAACGCTTCAGCAGAGCAGATGG-3'

Reverse: 5'-GGACAAGACTCATCACTCAGTGCCACCAATCT-3'  
Fabp6b (DNA quality control; Y Lai, personal communication):  
Forward: 5'-AATTACGATGAGTTTCTGGAGGCAA-3'  
Reverse: 5'-CTTCCGATGGTGAATTTGTTAGTCAA-3'

Each 8.7-µl reaction contained 2.25 µl nuclease-free water (IDT technologies), 1 µl of DNA (~50 ng µl<sup>-1</sup>), 0.3 µl DMSO (100%) (New England Bio Labs), 1 µl dNTPs (Life Technologies) (10 µM), 1 µl 10 × Coral buffer (QIAGEN, Valencia, CA, USA) (15 mM MgCl<sub>2</sub>), 0.15 µl of Taq (Roche, Mississauga, ON, Canada) (5 U µl<sup>-1</sup>), and forward and reverse primers of: Exon 2 (0.15 µl at 10 µM), Exon 4 (1 µl at 10 µM) and Fabp6b (0.35 µl at 10 µM). When we used 15 ng µl<sup>-1</sup> DNA working stocks, 4 µl of DNA was added to each reaction (total volume 11.7 µl). PCR conditions, run on a Biometra T1 Thermocycler machine (Montreal Biotech Inc., Kirkland, PQ, Canada), were 94 °C for 2 min followed by 36 cycles of 94 °C for 30 s, 60 °C for 30 s and 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 × TBE agarose gel, and visualized using ethidium bromide.

**RESULTS**

**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, we first screened seven families from the SALTAS breeding population with microsatellite markers from Ssa02 (www.asalbase.org; Figure 1, Supplementary Tables S1 and S3). However, SEX was not associated with any alleles from these loci, which suggested that SEX was not on Ssa02 in these families.

**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 Tables S1, S2 and S3). Linkage analysis using microsatellite markers from Ssa06 confirmed this prediction (Supplementary Tables S3 and 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 1). Therefore, we screened some additional 2009YC families from different ancestral male lineages in the SALTAS Atlantic salmon breeding population 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 Tables S3, S4 and S5). For non-Ssa06 SEX-linked families, accurate sex predictions were obtained using genetic markers from Ssa02 (Supplementary Table S5).

**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 from Dominik *et al.* (2010) and Lien *et al.* (2011) allowed us to predict a number of male lineages in which SEX was 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 Tables S4 and S5).

Although confident in our assignment of SEX to Ssa02 or Ssa06 in 56 of 58 families, we observed a lack of consistency 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. Our colleagues at CIGENE, Norway, recently identified SEX on Ssa03 in some European Atlantic salmon (S Lien, personal communication). Therefore, we used genetic markers from Ssa03 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 2 and Supplementary Table S3). Of these four individuals, three carried a Y-specific haplotype passed from grandsire to sire (predicted males), but were found to be female at harvest. Two of these three predicted males (phenotypic females)

2009_32		Ssa03											
parents and sdY 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	2	1	2	1
Ssa0017BSFU	3	2	3	1	3	3	3	3	3	3	1	1	1
Ssa0368BSFU	1	3	1	4	1	1	1	1	1	1	4	4	4
Ssa0516BSFU	3	3	3	4	3	3	3	3	3	3	4	4	4
OMM5019	2	1	2	1	2	2	2	2	2	?	1	1	1
OIM1267	1	3	1	2	1	1	?	?	?	?	2	2	?
Ssa0144BSFU	1	3	1	2	1	1	1	1	1	1	2	2	2
			sdY		Exon 2	1	1	0	0	0	0	0	1
					Exon 4	1	0	1	0	0	0	0	1
					Fabp6b	1	0	1	1	1	1	1	1
					Sex call from Phenotype	Male	Male	Female	Female	Female	Female	Female	Male

**Figure 2** An abstract of the second largest family (2009\_32), with 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 (for example, 2009\_0598 is the 2009 Year class ID 0598). Haplotype offspring sex predictions that do not match the sex call from phenotype are highlighted in 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.

**Table 1 Phenotypic male individuals from the 2009 year class in which *SEX* may have moved by recombination, translocation or transposition (see Figure 2 for details)**

Individual ID <sup>a</sup>	Number of markers predicting female haplotype	<i>sdY</i> positive	Chromosome housing ( <i>SEX</i> ) 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_0470	4	Yes	3
2009_0578	4	Yes	3

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

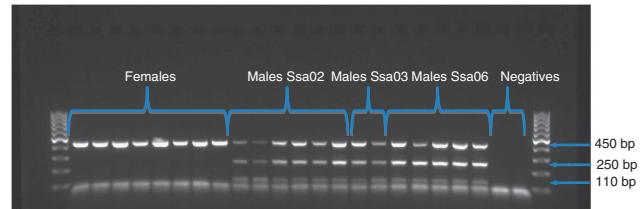
**Table 2 Some explanations of inconsistent outcomes between phenotype, genotype and *sdY***

Phenotype	Phenotype predicted by		Total no. of individuals	Possible explanation
	Haplotype	<i>sdY</i> test		
Female	Male	Male	1	
Female	Female	Male	5	Phenotype miscall or loss of function of <i>sdY</i>
Female	Male	Female	6	Movement of marker or <i>sdY</i>
Male	Female	Male	18	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	1	

For a fuller discussion see Figure 3.

did not amplify a product for exon 2, 3 or 4 of the *sdY* gene by PCR (see below), whereas the other one gave PCR products for exon 3 (primer sequences available upon request) and exon 4, but not exon 2 (Figure 2 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).

Seventeen phenotypic males from ten other families also tested positive for exons 2 and 4 of *sdY*, even though they carried the sire's maternally inherited haplotype (Table 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 2 and Supplementary Table S7. Unfortunately, none of these fish were used



**Figure 3** *sdY* triplex PCR test with primers (see Materials and Methods) from exon 2 (~110 bp), exon 4 (~250 bp) and *fabp6b* (~450 bp) (Y. Lai personal communication) comparing sires with 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.

as broodstock, and therefore they have no associated offspring of their own that could be analyzed.

### 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.* (2012a) enabled us to design two pairs of Atlantic salmon specific primers (E Palibroda, personal communication), 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). As 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 (for example, 2009\_0279; Figure 2) 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 (for example, 2009\_0967; Figure 2). In this particular case a male designation was assigned. A full list of all 19 individuals with only an exon 2 or exon 4 amplicon present can be found in Supplementary Table S9. We screened all the parents in the tested 2009YC families using these primer pairs for *sdY*, and found 100% agreement between parent phenotype and the presence or absence of *sdY* PCR products (Figure 3; Supplementary Table S3). Expanding the sample set to include all offspring from the 47 families, yielded a concordance between *sdY* and phenotype to 542/555 for males and 380/384 for females (Supplementary Tables S3 and S8). Of the total 922 offspring *sdY* test results that were consistent with sex phenotype, 27 disagreed with sex predictions based on genotype (Figure 2; Supplementary Tables S3 and S8). For 11 individuals, in which *sdY* predictions did not match phenotype calls, genotypic calls agreed with predictions made by the *sdY* test (Supplementary Table S8). Figure 2 illustrates the examples of most of these instances that were observed within a single family (2009\_32).

## DISCUSSION

### 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 (Myosho *et al.*, 2012). 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, to our knowledge, has not been reported. Although rare, multiple sex-determining gene loci, as we show here for Atlantic salmon, have been described previously in Arctic charr (Moghadam *et al.*, 2007; Küttner *et al.*, 2011).

### A single conserved sex-determining gene

The occurrence of multiple sex-linkage groups in salmonid species led to two competing hypotheses: either a single conserved sex-determining gene that can jump between chromosomes exists, or different sex loci represent unique sex-determining genes (Phillips *et al.*, 2001; Woram *et al.*, 2003; Davidson *et al.*, 2009). The recent reports of Yano *et al.* (2012a,b) suggest the former hypothesis is correct. Likewise our PCR-based survey, 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 that 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 12 *sdY*-negative individuals that possessed a female haplotype but were assessed to be phenotypically male (Table 2 and Supplementary Table S7). Phenotype assessment can be difficult to perform 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, as 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 2 summarizes explanations for the inconsistencies between *sdY* sex predictions and phenotype, while Figure 4 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). 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), we did observe six instances of a crossover between the Y chromosome and the X chromosome 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 2. Due to the criteria we set to assign *SEX* to a particular chromosome (See Materials and 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, as 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 2, Supplementary Table S3), the second largest family with a male haplotype comprised of markers mapping from 3.1 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 (Yano *et al.*, 2012a). Thus, it is likely that another sex-determining factor is necessary for *sdY* to function as the sex-determining gene, 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. Along this line, 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 found in some human sex-reversal disorders (Eggers and Sinclair, 2012). 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, as progeny have been reared from them.

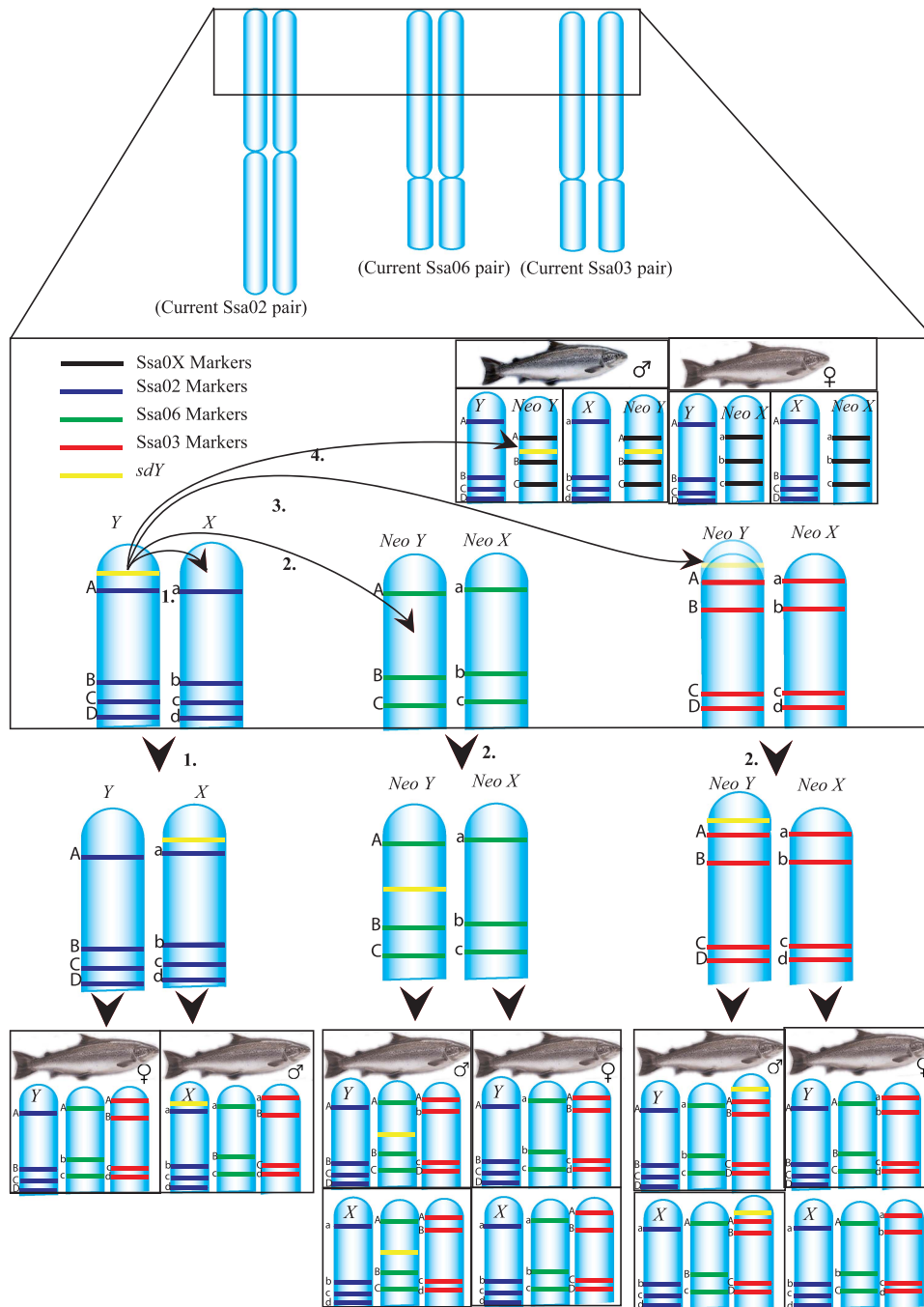
### Mechanisms of *sdY* movement from one chromosome to another

With a seemingly conserved sex-determining gene (*sdY*) identified in the Tasmanian Atlantic salmon that exhibit three *SEX* loci on different chromosomes, we 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 the 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, we suggest 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, we can only speculate which of these potential mechanisms may have been involved in producing the multiple sex-determining loci we observe in Tasmanian Atlantic salmon.

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

It is remarkable how little genetic mapping of sex-determining loci has actually been carried out in salmonids, particularly Atlantic salmon. For example, the identification of LG1 (Ssa02) as the *SEX* containing linkage group in Atlantic salmon was based solely on the analysis of the two SALMAP mapping families, whose parents came from the River Tay, Scotland (Woram *et al.*, 2003; Danzmann *et al.*, 2008). Other genetic mapping projects on 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. The assignment of *SEX* to LG4 in Arctic charr initially came from genetic mapping in two families (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, indicates the importance of using many families when mapping *SEX* in salmonid species.

The Tasmanian Atlantic salmon ancestral population is from the River Phillip, Nova Scotia, Canada. It may not have been a great surprise that *SEX* mapped to a different locus in the Tasmanian (North American derived) Atlantic salmon from that reported in the Scottish (European) Atlantic salmon given the chromosomal differences and subspecies status that have been assigned to North American and European Atlantic salmon (Brenna-Hansen *et al.*, 2012). What was astonishing, however, was that three *SEX* loci were found in the Tasmanian population. This population was founded from 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, is it possible to identify the ancestral *SEX* locus in *Salmo salar*, and if



**Figure 4** 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 for example, 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). Homologous 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 2).

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.

In summary, we have identified three *SEX* loci in the SALTAS Tasmanian Atlantic salmon breeding population, all of which show evidence for the presence of *sdY*. However, at this point we can only

speculate 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, is 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.

## DATA ARCHIVING

The Atlantic salmon linkage maps, genetic markers and primers used for genotyping are available at [www.asalbase.org](http://www.asalbase.org). All genotype data are included in the Supplementary Information.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Aksnes A, Gjerde B, Roald SO (1986). Biological, chemical and organoleptic changes during maturation of farmed Atlantic salmon, *Salmo salar* *Aquaculture* **53**: 7–20.

Allendorf FW, Thorgaard GH (1984). Tetraploidy and the evolution of salmonid fishes. In: Turner BJ (ed) *Evolutionary genetics of fishes*. Plenum Press: New York, NY, USA, pp 1–46.

Artieri CG, Mitchell LA, Ng SHS, Parisotto SE, Danzmann RG, Hoyheim B *et al.* (2006). Identification of the sex-determining locus of Atlantic salmon (*Salmo salar*) on chromosome 2. *Cytogenet Genome Res* **112**: 152–159.

Brenna-Hansen S, Li J, Kent MP, Boulding EG, Dominik S, Davidson WS *et al.* (2012). Chromosomal differences between European and North American Atlantic salmon discovered by linkage mapping and supported by fluorescence in situ hybridization analysis. *BMC Genomics* **13**: 432.

Brunelli JP, Thorgaard GH (2004). A new Y-chromosome-specific marker for Pacific salmon. *Trans Am Fish Soc* **133**: 1247–1253.

Danzmann RG, Davidson EA, Ferguson MM, Gharbi K, Koop BF, Hoyheim B *et al.* (2008). Distribution of ancestral proto-Actinopterygian chromosome arms within the genomes of 4R-derivative salmonid fishes (Rainbow trout and Atlantic salmon). *BMC Genomics* **9**: 557.

Davidson WS, Huang T, Fujiki K, von Schalburg KR, Koop BF (2009). The sex determining loci and sex chromosomes in the family Salmonidae. *Sex Dev* **3**: 78–87.

Davidson WS, Koop BF, Jones SJM, Iturra P, Vidal R, Maass A *et al.* (2010). Sequencing the genome of the Atlantic salmon (*Salmo salar*). *Genome Biol* **11**: 403.

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

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

Devlin RH, Stone GW, Smail DE (1998). Extensive direct tandem organization of a long repeat DNA sequence on the Y-chromosome of chinook salmon (*Oncorhynchus tshawytscha*). *J Mol Evol* **46**: 277–287.

Dominik S, Henshall JM, Kube PD, King H, Lien S, Kent MP (2010). Evaluation of an Atlantic salmon SNP chip as a genomic tool for the application in a Tasmanian Atlantic salmon (*Salmo salar*) breeding population. *Aquaculture* **308**: S56–S61.

Eggers S, Sinclair A (2012). Mammalian sex determination –insights from humans and mice. *Chromosome Res* **20**: 215–238.

Elliott N, Kube P (2009). Development and early results of the Tasmanian Atlantic salmon breeding program. *Proc Assoc Advmt Anim Breed Genet* **18**: 362–365.

Graves JAM, Peichel C (2010). Are homologies in vertebrate sex determination due to shared ancestry or to limited options? *Genome Biol* **11**: 205.

Hattori RS, Murai Y, Oura M, Masuda S, Majhi SK, Sakamoto T *et al.* (2012). A Y-linked anti-Mullerian hormone duplication takes over a critical role in sex determination. *Proc Natl Acad Sci USA* **109**: 2955–2059.

Iturra P, Medrano JF, Bagley M, Lam N, Vergara N, Marin JC (1997). Identification of sex chromosome molecular markers using RAPDs and fluorescent in situ hybridization in rainbow trout. *Genetica* **101**: 209–213.

Kamiya T, Kai W, Tasumi S, Oka A, Matsunaga T, Mizuno N *et al.* (2012). A trans-species missense SNP in Amhr2 is associated with sex determination in the tiger pufferfish, *Takifugu rubripes* (fugu). *PLoS Genet* **8**: e1002798.

King H, Ruff N, Evans B, Elliott N (2012). Evidence that sex differentiation in Atlantic salmon is thermolabile. *ICISB, Oslo* P-018.

Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R (1990). Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature* **348**: 450–452.

Küttner E, Nilsson J, Skúlason S, Gunnarsson S, Ferguson MM, Danzmann RG (2011). Sex chromosome polymorphisms in arctic charr and their evolutionary origins. *Genome* **54**: 852–861.

Li J, Phillips RB, Harwood AS, Koop BF, Davidson WS (2011). Identification of the sex chromosomes of brown trout (*Salmo trutta*) and their comparison with the corresponding chromosomes in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Cytogenet Genome Res* **133**: 25–33.

Lien S, Gidskehaug L, Moen T, Hayes BJ, Berg PR, Davidson WS *et al.* (2011). A dense SNP-based linkage map for Atlantic salmon (*Salmo salar*) reveals extended chromosome homeologies and striking differences in sex-specific recombination patterns. *BMC Genomics* **12**: 615.

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T *et al.* (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature* **417**: 559–562.

McGowan C, Davidson WS (1998). The RAPD technique fails to detect a male-specific genetic marker in Atlantic salmon. *J Fish Biol* **53**: 1134–1136.

Moghadam HK, Ferguson MM, Danzmann RG (2007). Linkage variation at the sex-determining locus within Fraser strain Arctic charr *Salvelinus alpinus*. *J Fish Biol* **71**: 294–301.

Myosho T, Otake H, Masuyama H, Matsuda M, Kuroki Y, Fujiyama A *et al.* (2012). Tracing the emergence of a novel sex-determining gene in Medaka, *Oryzias latipes*. *Genetics* **191**: 163–170.

Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A *et al.* (2002). A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA* **99**: 11778–11783.

Nelson JS (2006). *Fishes of the World*, 4th edn. John Wiley and Sons: New York, NY, USA.

Phillips RB, Keatley KA, Morasch MR, Ventura AB, Lubieniecki KP, Koop BF (2009). Assignment of Atlantic salmon (*Salmo salar*) linkage groups to specific chromosomes: Conservation of large syntenic blocks corresponding to whole chromosome arms in rainbow trout (*Oncorhynchus mykiss*). *BMC Genomics* **10**: 46.

Phillips RB, Konkol NR, Reed KM, Stein JD (2001). Chromosome painting supports lack of homology among sex chromosomes in *Oncorhynchus*, *Salmo*, and *Salvelinus* (Salmonidae). *Genetica* **111**: 119–123.

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ *et al.* (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346**: 240–244.

Smith CA, Roesler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ *et al.* (2009). The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature* **461**: 267–271.

Thorgaard GH, Bailey GS, Williams D, Buhler DR, Kaattan SL, Ristow SS *et al.* (2002). Status and opportunities for genomics research with rainbow trout. *Comp Biochem Physiol B Biochem Mol Biol* **133**: 609–646.

Volf JN, Nanda I, Schmid M, Scharl M (2007). Governing sex determination in fish: regulatory putesches and ephemeral dictators. *Sex Dev* **1**: 85–99.

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

Yano A, Guyomard R, Nicol B, Jouanno E, Quillet E, Klopp C *et al.* (2012a). An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*. *Curr Biol* **22**: 1–6.

Yano A, Nicol B, Jouanno E, Quillet E, Fostier A, Guyomard R *et al.* (2012b). The sexually dimorphic on the Y-chromosome gene (*sdY*) is a conserved male-specific Y-chromosome sequence in many salmonids. *Evolutionary Applications* **6**: 486–496.

Yoshimoto S, Okada E, Umemoto H, Tamura K, Uno Y, Nishida-Umehara C *et al.* (2008). A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. *Proc Natl Acad Sci USA* **105**: 2469–2474.

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



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