# Test cross explores if *sdY* is the sole genetic requirement for maleness in Atlantic Salmon

*J. Kijas[1], M. Menzies[1], S. McWilliam[1],H. King[2] & B. Evans[3]*

*[1] CSIRO Agriculture & Food, 4067 Brisbane, Australia*
*James.Kijas@csiro.au (Corresponding Author)*
*[2] CSIRO Agriculture & Food, 7004 Hobart, Australia*
*[3] Tassal Operations Pty Ltd, 7001, Hobart, Australia*

## Summary

The ability to diagnose the sex of immature animals is important in salmon breeding. The *sdY* gene has been proposed as the master regulator of sex determination, as it is located in the male specific region of multiple salmonid species. Diagnostic PCR assays directed against *sdY* predict sex, however we observe phenotypic males in the Tasmanian Selective Breeding Program that test negative for the presence of the gene. This experiment sought to determine if *sdY* negative males are capable to generating viable progeny of both sexes, and to explore if alternative genetic pathways in Atlantic salmon can confer maleness. We constructed test crosses between 10 *sdY* negative males and 5 females, to evaluate the sex ratio of their progeny and their genetic composition. Progeny were raised as a single management group until 14 months post fertilization, when 204 fish were examined by dissection to assign sex. This revealed 11 male and 191 female progeny (sex of 2 fish could not be determined). The presence of anatomic males prompted PCR testing, revealing all were *sdY* negative. We considered the possibility these fish have *sdY* sequence variability that confounded our DNA test, or that alternative sequence elements located in the male specific region can confer maleness in the absence of *sdY*. In response, a PCR method was developed to establish the presence or absence of the entire male specific region. Application to the test-cross male progeny revealed they appear to lack the male specific region. This excluded the possibility that *sdY* testing error is the basis for our observations, as each animal lacked not only *sdY* but the entire male specific region. Our findings leave open the possibility that an alternative to *sdY* may exist to trigger the development of male Atlantic salmon, and we have generated a test-cross population which can be used for its genetic mapping.

*Keywords: salmon, sex determination, sdY, genomics.*

## Introduction

The genetic control of sex determination in livestock is rigidly controlled by the *SRY* gene. Teleost fish, however, exhibit a remarkable array of sex determination systems that may include strict genetic control, total environmental control or a combination of the two. For those species under genetic control, at least five master sex genes have been identified (Martinez et al., 2014). Sex determination in salmonids involves the *sdY* gene (sexually dimorphic y chromosome), and it's presence is strongly associated with maleness (Yano et al., 2013). The ability to assign the sex of immature salmon is important in the context of selective breeding programs, and juvenile fish are tested for *sdY* in the Tasmanian Selective Breeding Program (SBP) before confirmatory phenotypic assessment is performed prior to

spawning. This has identified multiple examples of phenotypic male fish that lack the *sdY* gene (Eisbrenner et al 2014). This raises the possibility that an alternative genetic mechanism is in operation in some fish to confer maleness, or that a technical artefact disrupts the DNA diagnostic in a proportion of fish. The objective of the current study was to evaluate these possibilities, and to conclusively determine if *sdY* is the sole master regulator gene conferring maleness in Atlantic salmon.

## Results

### Test Crossing *sdY* Negative Male Salmon

Test crosses were constructed using 10 'running' male Atlantic salmon that tested negative for the presence of *sdY* exon 2 and 4 (data not shown). Milt was collected and used to fertilise eggs from five 2013 year class breeding females (Figure 1). Test cross progeny were raised as a single management group until the age of 14 months post fertilization when they were assigned phenotypic sex by internal examination. A total of 204 animals were assessed, of which 11 (or 5.4 %) were confirmed to be phenotypic males. In the absence of pedigree, it is currently unclear if the male offspring were generated by a single sire at high frequency or by multiple sires, each at low frequency. The presence of male progeny demonstrates the ability of *sdY* negative fish to generate offspring of both sexes.

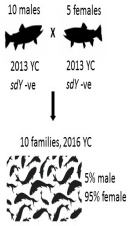


*Figure 1. Test cross design. Each female was mated with two sdY negative males to generate a total of 10 families. Test cross progeny were managed as a single cohort until the age of 14 months, when were assessed to assign their phenotypic sex.*

**Sequence Characterisation of the Male Specific Region**

To evaluate the genomic status of male offspring from the test cross, we first sought to characterise the genomic architecture of male and female fish where *sdY* correctly predicted sex. Twenty SBP animals (11 male and 9 female) were used for whole genome sequencing to 30X coverage, before reads were aligned to the reference genome assembly ICSASG_v2 (accession GCA_000233375.4). The reference assembly was constructed using a female individual (Lien et al., 2016), and is therefore not expected to contain either *sdY* or the male specific region (MSR) in which it resides. In response, we also mapped raw reads against an available male fish BAC contig containing *sdY* (Lubieniecki et al., 2015). Figure 2 shows mapped reads from three representative fish, across a 1.5 Kb region of the *sdY* BAC contig. Female fish lack reads up to position 14,320 bp, whereas male fish contain mapped reads for the same region. This identifies the MSR which by definition is only present in males. Both female and male fish have reads mapped to the contig at bp positions higher than 14,320. This defines the male – female common region (MFCR). Importantly, the analysis identified the precise junction point that separates the MSR from the MFCR at base pair position 14,320 (Figure 2). This sequence level characterization forms the basis with which to study animals where *sdY* fails to correctly assign phenotypic sex.

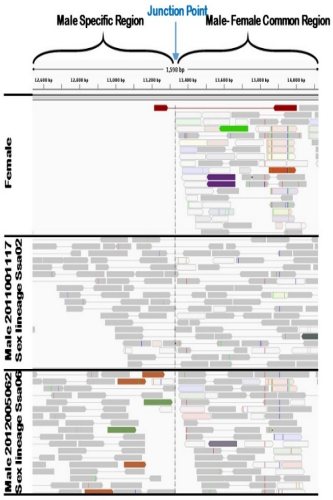


*Figure 2. Alignment of sequence reads compared between male and female fish. Reads were aligned to a 20 Kb sdY gene BAC contig constructed from a male fish and visualised using the Integrated Genome Viewer. A 1.5 Kb region is shown, to illustrate the male specific region, the male – female common region and junction point that separates them.*

## Junction Point Test Design and Results

The *sdY* test for predicting sex relies on sequence homology to the coding exons of the gene (Eisbrenner et al., 2014). If a subset of SBP fish carry substantial sequence variability in these sequences, or if sequence elements other than *sdY* in the male specific region can confer maleness, fish may test negative using the *sdY* diagnostics that are in fact genetically male. To evaluate these possibilities, PCR tests were developed to establish the presence or absence of the entire male specific region (Figure 3). The tests exploit the junction point, and are designed to generate PCR products only in animals that carry the MSR. Complexity arises from the finding the MSR is located on chromosome 2, 3 or 6 (Eisbrenner et al., 2014). This defines individual males as belonging to one of three sex lineages (SL-02, SL-03 or SL-06). Sequence differences between the MSR insertion site necessitated development of dedicated junction point (JP) assays for males from different sex lineages (Figure 3). Evaluation using DNA from 20 SBP animals with available genome sequence confirmed the JP1 and JP2 tests successfully distinguished males from females, as well as between males from different sex lineages (Figure 3C, D). After confirming the tests are diagnostic, we applied them to evaluate DNA from male test cross progeny. This revealed they i) do not contain a copy of *sdY* and ii) returned negative results for both the JP1 and JP2 tests. This strongly suggests they lack the MSR.

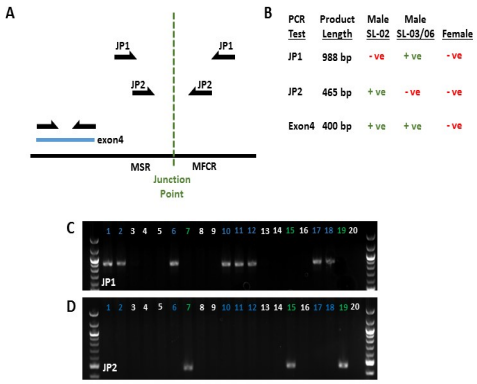


*Figure 3. Testing for the presence of the male specific region. (**A**) The approximate location of three PCR primer pairs are shown in relation to the junction point (vertical line) identified in Figure 2. The location of sdY exon 4 is given in blue. (**B**) PCR primers were designed to amplify three different products, and the name and expected length (in base pairs) is given for each. Based on whole genome sequencing data, junction point test 1 (JP1) should amplify a product only in male fish from sex lineage 03 or 06. Similarly, JP2 has been designed to be specific for male fish from sex lineage 02. Gel images showing the results of JP1 (**C**) and JP2 (**D**) testing of 20 SBP fish used for whole genome sequencing. Lane numbers are color coded to indicate females (white), males from lineage 03/06 (blue) and males from lineage 02 (green).*

**Conclusion**

The test cross described here confirmed *sdY* negative males are capable of generating viable offspring, and that a proportion are anatomically male. PCR testing for *sdY* and the male specific region confirmed these male progeny lacked both genomic features. This strongly suggests that in this subset of animals either i) a separate genetic determinant has substituted for the action of *sd*Y to trigger maleness, ii) an environment cue (eg temperature) has acted alone to trigger maleness or iii) a combination of the two is in action whereby a latent genetic mechanism that is inactive in the presence of *sd*Y moves to an active state in response to an environmental trigger to direct maleness.

## List of References

Eisbrenner WD, Botwright N, Cook M, Davidson EA, Dominik S, Elliott NG, et al. 2014. Evidence for multiple sex-determining loci in Tasmanian Atlantic salmon (*Salmo salar*). Heredity 113(1): 86-92.

Lien S, Koop BF, Sandve SR, Miller JR, Kent MP, Nome T, et al. 2016. The Atlantic salmon genome provides insights into rediploidization. Nature 533(7602): 200-205.

Lubieniecki KP, Lin S, Cabana EI, Li J, Lai YY, Davidson WS. 2015. Genomic Instability of the Sex-Determining Locus in Atlantic Salmon (*Salmo salar*). G3 5(11): 2513-2522.

Yano A, Nicol B, Jouanno E, Quillet E, Fostier A, Guyomard R, et al. 2013. The sexually dimorphic on the Y-chromosome gene (*sdY*) is a conserved male-specific Y-chromosome sequence in many salmonids. Evol Appl. 6(3): 486-496.