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SEX CONTROL IN AQUACULTURE

VOLUME 1

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Sex Control in Aquaculture

Sex Control in Aquaculture

Volume I

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To 97-year old Zhennan Wang and 90-year-old Dusheng Peng, Hong Yao, Alan and Eileen Wang – superior parents, lovely wife, and fast-growing male and female offspring that one of the editors of the book is lucky to have.

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Preface

This book was motivated by an increasing, strong need for the control of sex ratios and monosex production knowledge and technology by the rapid growing global aquaculture industry. Currently, aquaculture – the fastest growing food-producing sector – contributes about 50% of the world's food fish, based on the Food and Agriculture Organization (FAO) latest reports. Sex control in aquaculture serves different purposes.

First and foremost, a wide spectrum of aquacultured species show sexual dimorphism in growth and ultimate size, whereby one sex grows faster than the other or attains a larger size. Thus, there are important benefits in rearing only the fastest-growing sex or monosex production. Second, in some species, precocious maturation and uncontrolled reproduction need to be prevented. Third, some negative impacts of reproduction on product quality or disease resistance need to be prevented in some species. Fourth, in sex-changing hermaphrodites, sex ratio control can benefit broodstock management. Finally, there are some species where the gonads or gametes of females have special economic value, e.g., caviar.

Therefore, sex control for the production of monosex or sterile stocks is extremely important for aquaculture professionals and industries to improve production or to increase revenue, reduce energy consumption for reproduction, and eliminate a series of problems caused by mixed-sex rearing or sexual maturation. Incidentally, the same principles used for sex control in aquaculture can be used in population control to eliminate

undesired invasive species – an aspect that is also dealt with in this book.

The two volumes of “*Sex Control in Aquaculture*” together is composed of 11 parts and a total of 41 chapters, which have been written by leading experts in the field. Volume I consists of Parts I to V (Chapters 1–19), while the remaining Parts VI to XI (Chapters 20–41) make up Volume II.

With eight chapters, Part I is concerned with the theoretical and practical basis of sex determination/differentiation and sex control in aquaculture. These chapters provide the concepts and rationale for sex control in aquaculture, and present our current knowledge on basic aspects of the genetic, endocrine, and environmental mechanisms for sex determination and sex differentiation, including epigenetic regulation. Readers will find a detailed, most up-to-date description of the underlying mechanisms responsible for the establishment of the sexes and, hence, the sex ratios. Several chapters also provide information on chromosome set manipulation techniques, hybridization and new gene knockout, and the application of these different approaches to aquaculture. There is also a chapter on the application of sex ratio manipulation for population control (e.g., for the management of invasive species).

Parts II to XI, or Chapters 9 to 41, contain detailed protocols and key summarizing information for the sex control practice of 35 major aquaculture species or groups with sexual size dimorphism, monosex, or polyploidy culture advantages. These major

aquaculture species include Nile tilapia, blue tilapia, Mozambique tilapia, black-chin tilapia, salmonids, European sea bass, bluegill, largemouth bass, crappies, yellow perch, Eurasian perch, channel catfish, yellow catfish, southern catfish, half-smooth tongue sole, turbot, southern flounder, summer flounder, Japanese flounder, Atlantic halibut, Pacific halibut, spotted halibut, sturgeon, shrimp, prawn, Atlantic cod, malabar grouper, honeycomb grouper, large yellow croaker, rice field eel, the Japanese eel, the European eel, the American eel, and common carp.

All chapters are arranged in the same structure and format for easier reading and the extraction of useful information, but each chapter has its own unique story. Therefore, the two volumes of the book can be read cover to cover, or you can pick any chapter, depending on your interests. However, we suggest that all readers start with Chapters 1 through 8 (Part I), in order to get a comprehensive background before moving to a particular species or group of species.

In summary, the use of sex control in aquaculture is becoming one of the most important topics for both aquaculture research and the aquaculture production industry. This book synthesizes relevant and recent information on sexual development principles and sex control practice, and emphasizes

their applications for use in the aquaculture industry. It bridges the gap between theory and practice in sex control of farmed species, including new developments and methodologies used in sex determination, differentiation, monosex, and polyploidy production for aquaculture.

Thus, the book will appeal to a large audience: Scientists working directly in aquaculture research or food production will find relevant information on the principle and practical aspects of sex control in aquaculture; and scientists working with basic aspects of fish/shrimp biology, reproductive endocrinology, genetics, and evolutionary biology will find abundant information regarding sex in related species. Likewise, biologists working in the farming industry, hatchery management, fisheries, as well as related administrators, will benefit from clear and practical information on how to apply sex control in aquatic animals. Finally, young researchers and graduate students will learn about a field – the establishment of sex in fish/crustaceans and its control – with both basic and applied connotations.

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12

Development and Application of Sex-Linked Markers in Salmonidae

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12.1 Introduction

Most salmonid fish have an XY sex determination system, usually with no morphologically differentiated putative sex chromosomes [1] (see Box 12.1). Sockeye salmon (*Oncorhynchus nerka*) is an exception, with an X₁X₂Y sex determination system, in which females have one more chromosome (2n=58) than males (2n=57) [1–3]. Accurate sexing of salmonids provides many commercial benefits, motivating research to identify sex-linked markers for aquacultured fish. Sexual maturity affects growth, and increases male aggressive and competitive behaviors. Maturing fish may also stop feeding, show decreased vitality due to skin infections or other diseases, and produce lower quality meat (including fillets with altered color or flavor).

Due to the many maturity-related changes relevant to commercial salmonid production, aquaculturists seek to limit pre-harvest sexual maturation, producing sterile males and females by inducing triploidy (see Chapter 13), or monosex specimens, using gynogenesis or androgenesis (see Chapter 13). Given that the XY system is common to most salmonids, the research has focused on finding male-specific sex-linked molecular markers. Markers present in the male (putatively in the Y chromosome, called Y-linked markers) and absent in

females (or the X chromosome) have been detected using various molecular techniques that have evolved from the 1980s to the present day.

In the 1970s and 1980s, allozymes (biochemical markers) were used extensively to assess genetic variation in natural populations and were the first sex-linked markers identified in salmonids. Given their historical importance, we will dedicate a few lines to allozymes, keeping in mind that the polymorphisms underlying these biochemical markers have a genetic basis in the coding sequence of the enzyme. These polymorphisms are expressed in the phenotype, and may have adaptive implications. In rainbow trout (*Oncorhynchus mykiss*), the allozymic loci *bGLUA-2** (formerly *HEX-2*) and *sSOD-1** show linkage with the Y chromosome [14–16] and loci *Ldh-1**, *Aat-5**, and *Gpi-3** in the *Salvelinus* species [17]. Application of these markers for salmonid sexing has been very limited.

The development of polymerase chain reaction (PCR), molecular cloning, and automated Sanger sequencing, have made it possible to perform amplifications from small quantities of genetic material. As a result, small DNA segments are sufficient for performing genetic analyses, determining nucleotide sequences, and comparing

Box 12.1 Sex determination systems in salmonids

Sex determination systems are diverse among vertebrates. Genetic and environmental factors guide the process of determining whether the primordial gonad in the embryo becomes an ovary or testicle. When the gonads begin to function, the respective male or female sexual phenotype emerges.

Fish exemplify the diversity of sex determination systems. Various species have XX/XY, ZZ/ZW, or multiple chromosome systems and, in some species, sex is determined, or strongly influenced, by the environment [4]. Salmonids have separate sexes, and the sex determination is under genetic control. Experimental sex reversal experiments have confirmed that the male is the heterogametic sex. Crossing an XY female (sex-reversed male) with a normal male (XY) yields a 3 : 1 proportion of phenotypic

males and females, and crossing an XX male (sex-reversed female) with a normal female (XX) produces 100% phenotypic female progeny [5–7].

In some salmonids, such as rainbow trout (*Oncorhynchus mykiss*) and various *Salvelinus* species, chromosomal sex (XX/XY) is distinguishable by morphology [8], while other salmonids do not exhibit marked sex-linked morphology [1]. In the latter case, sex chromosomes have been identified using chromosome-banding techniques, such as fluorescence *in situ* hybridization (FISH), involving probes that carry sex-linked markers. Linkage studies and comparative analyses among species have characterized most of the sex chromosomes in this group of fishes [9–13].

findings with results from public databases to identify homologous sequences. Since the 1990s, these techniques have been used to develop PCR-based markers, such as RAPDs (random amplified polymorphic DNA [18, 19]), AFLPs (amplified fragment length polymorphisms [20]), SCARs (sequence-characterized amplified regions [21]), and microsatellites [22], to amplify partial sequences of genes and pseudogenes, and to evaluate associations between these markers and phenotypic sex.

Development of next-generation sequencing methods in the 2000s permitted massive sequencing of RNA from specific tissues (a technology called RNA sequencing). This technology was used to compare the genes transcribed in male and female gonadal tissues, shedding light on a potential salmonid master determining sex gene. This section will review the development of male-specific markers, through the 2012 discovery of the *sdY* gene and their applications, to 2017. The most relevant markers are described below, but various markers developed as an academic exercise with no practical utility are not listed. Only a few markers have been

applied massively to salmonid sexing and, to our knowledge, even these markers are not used routinely in commercial fish farming. Probably, when all these technologies become more cost-effective than echography, they will be routinely used by the industry – but now this is not the case.

12.2 Development of Sex-Linked Markers in Salmonids

Biological samples are required to evaluate genomic DNA for the presence of any of the markers discussed in this chapter. In alevins, the entire adipose fin is often removed. Because the fin may be difficult to cut in adult fish, a small sample called a fin clip is often used instead. This technique requires removing a small piece of dorsal fin – no more than 0.5 cm². Samples can be dried and then stored in paper or in a tube with 95–100% ethanol until DNA extraction. There are many protocols for extracting DNA, including commercial kits (available from many biotech suppliers worldwide),

rapid protocols using Chelex resin [23], and elaborated protocols using phenol and chloroform [24]. Regardless of the protocol, high-quality DNA is necessary for genotyping any molecular marker.

12.2.1 *OtY1/OtY8*

One of the first male-specific salmonid markers identified was the Y-chromosomal DNA probe *OtY1* in Chinook salmon (*Oncorhynchus tshawytscha*), by Devlin *et al.* [25]. This probe was initially developed using the subtractive hybridization method, to produce an enriched fraction of male-specific sequences for cloning. Eighteen clones were subjected to southern blotting, using a radioactive probe. A single 250 bp probe hybridized with an 8 kb fragment in all 30 males, but none of the 29 females were analyzed [25]. Segregation analysis of one family showed *OtY1* was inherited by male progeny from the sire. Because the blotting method was time-consuming and difficult to apply in commercial aquaculture, a rapid PCR-based test for *OtY1* was developed, producing a male-specific 209 bp amplicon [26].

The *OtY1* marker was explored in other salmonids, but found to be male-specific in the Chinook only. In rainbow trout, *OtY1* was not Y-linked, nor did it map in the linkage group bearing the sex determining locus [27, 28]. Furthermore, the above studies detected no recombination between the *OtY1* marker and the sex determining locus [25, 29]. Females positive for *OtY1* have been detected in some wild and hatchery populations (ranging from 4–84% of the female population), indicating a possible recombination event; however, this pattern may be attributable to environmental sex reversion mediated by temperature or estrogen pollution [30, 31].

In a subsequent analysis, the 8 kb fragment detected with the *OtY1* probe was cloned and subjected to southern blotting and PCR analyses, to characterize the genomic organization of the new marker, *OtY8*. As with *OtY1*, this clone was found to be Y-linked,

segregating from the male parent to male progeny [32]. Studies in eight other *Oncorhynchus* species (*O. keta*, *O. nerka*, *O. gorbuscha*, *O. kisutch*, *O. mykiss*, *O. masou*, and *O. clarki*) and Atlantic salmon revealed that *OtY8* is Y-linked only in Chinook salmon [28, 32].

12.2.2 *GH-Ψ/GH-2* Genes

Growth hormones (GH) play an important role in fish growth. Because the growth rate of captive fish has been (and still is) a primary target in fish breeding, there are ongoing efforts to clone, sequence, and characterize the genes associated with this process in salmonids [33, 34]. Salmonids have two expressed growth hormone genes (*GH-1* and *GH-2*), one of which has been identified as a sex-linked marker in Pacific salmon [35]. For example, in coho (*Oncorhynchus kisutch*) and Chinook salmon, two alleles (*a* and *b*) were identified in intron C of the *GH-2* gene. These alleles differ in size (434 and 455 bp, respectively) and *HinfI* enzyme restriction sites [36]. In both species, segregation analyses have shown that allele *b* is male-specific and located in the Y-chromosome, while allele *a* is located in the X-chromosome. Therefore, all males are heterozygous for this allele (genotype *ab*), and females are homozygous for the *a* allele. This type of segregation is absent in rainbow trout, in which the *GH-2* gene does not show a sex-linked pattern [36].

In addition to the sex-linked polymorphism in the *GH-2* gene, a non-functional Y-linked growth hormone pseudogene (*GH-Ψ*) has been described in five Pacific salmon species: Chinook, coho, masu (*O. masou*), chum (*O. keta*), and pink salmon (*O. gorbuscha*) [29, 33, 35, 37]. In all male Chinook and coho salmon, a 290 bp fragment from *GH-Ψ* is amplified by PCR primers *GH5/6*, designed for intron E [33, 34]. In chum and pink salmon, the Y-linked specific fragments are amplified by primers *GH28/GH30*, designed for intron C, resulting in 160 bp and 175 bp amplicons [29]. In masu salmon, the male-specific fragment is 280 bp.

The inheritance pattern indicates some degree of recombination between Y and X chromosomes, and 97.5% and 24.3% of the male fragment is present in phenotypic males and females, respectively [35, 38]. It is likely that some recombination also occurs in Chinook salmon [29], as the estimated distance between *GH-Ψ* and the sex determining gene is approximately 10 centimorgan (cM) in this species. However, no study to date has detected a recombination event with the sex determining locus.

12.2.3 *OmyP9*

In rainbow trout, the first male-specific marker was identified by Iturra *et al.* [39] with bulked segregant analysis (BSA) and RAPD (random amplified polymorphic DNA) screening. These researchers used pooled samples from 12 males and 12 females from the Mount Lassen strain. An RAPD assay with 900 primers identified two sex-associated RAPD fragments (650 and 390 bp), amplified by the primers OP-A11 and OP-P9, respectively. The 390 bp fragment amplified by RAPD primer OP-P9 was present in all 12 males, and absent in all 12 females. When this polymorphism was tested in the Scottish strain, it amplified in all males, but also in 38% of females. The 650 bp fragment amplified by RAPD primer OP-A11 always amplified in a percentage of males, but never in females. Finally, only the fragment amplified by primer OP-P9 was converted to a SCAR (sequence-characterized amplified region) marker, designated *OmyP9*, enlarging the RAPD fragment to 899 bp [40].

A more detailed analysis of *OmyP9* identified three size polymorphisms (899, 894, and 840 bp) and one restriction polymorphism when digested with the *RsaI* enzyme. Combinations of size and restriction polymorphisms produced three *OmyP9* variants: variant A (894 bp, with two *RsaI* restriction sites), which generated three fragments (441, 114, and 339 bp); variant B (899 bp, with one *RsaI* site), which generated two fragments (555 and 344 bp); and variant C (840 bp, with

one *RsaI* restriction site), which generated two restriction fragments (501 and 339 bp). Segregation analyses, in 93 males and 93 females from six different strains of rainbow trout, showed that males are never homozygous for the C variant. However, none of the three variants are strictly associated with male or female phenotypes, indicating that *OmyP9* is not a fully Y-linked locus, and that some recombination between X and Y chromosomes can occur in the region bearing this marker.

In crosses with known parental genotypes, determining the progeny's sex is straightforward. For example, in ten experimental crosses, the male parent always passed his variant A to male progeny and never to female progeny [40]. A similar pattern was observed by Lopez and Araneda [41] in crosses used to evaluate the performance of *OmyP9* in identifying the sex of rainbow trout.

12.2.4 *Omy-163*

This marker was also developed in rainbow trout to identify the Y-chromosome, using amplified fragment length polymorphism (AFLP) screening in pooled samples obtained from crosses between outbred females and F₁ males, derived from crosses between XX individuals from the OSU (Oregon State University) female clonal line, with YY individuals from four different male clonal lines (SW, Swanson; ARL, Arlee; CW, Clearwater; and HC, Hot Creek) [42]. AFLP screening was performed with 486 primer combinations and three pairs of restriction enzymes (*EcoRI/MseI*, *PstI/MseI* and *BamHI/MseI*), resulting in 4374 polymorphic fragments. Fifteen sex-linked AFLP markers were converted to SCAR markers, but only the *Omy-163* marker produced distinctive male vs. female fragment patterns in the trout – that is, a sex-linked amplification pattern [41, 43].

Omy-163 has been tested for genotyping in several strains of rainbow trout, but has not always shown a Y-chromosome

association [43]. In cases where a Y-linked pattern was identified, some recombination between the putative *SEX* determining locus and the SCAR was observed. For example, in the global analysis performed by Felip *et al.* [42], 29 of 380 males were negative for the male pattern, and nine of 396 females were positive for the male pattern. In Lopez and Araneda [41], 16 of 47 males were negative for the male pattern, and 8 of 84 females were positive for the male pattern. Linkage studies show that *Omy-163* is located near the *SEX* locus, separated by a distance ranging from 0.0 to 42.2 cM (average 7.2 cM), making recombination plausible [42, 43].

12.2.5 *OtY2/OtY3/OmyY1*

OtY2-WSU is another marker with a Y-linked inheritance pattern, developed for Chinook salmon and later detected in coho, chum, and sockeye salmon [44]. *OtY2*-WSU shows autosomal inheritance in rainbow trout. A small number of coho ($n=48$) and chum ($n=30$) salmon were also screened; in sockeye salmon, the segregation pattern detected in 119 samples was not fully Y-linked, as 12 phenotypic males were negative and three phenotypic females were positive for the marker. *OtY2*-WSU was detected using AFLP screening for sex-specific fragments in pools of androgenetic diploid Chinook salmon (males and females). It is thought that these androgenetic individuals typically carry two copies of the paternal X-chromosome (in females) or Y-chromosome (in males), facilitating the identification of Y-specific markers [44]. *OtY2*-WSU genotyping was performed using trio PCR, with two pairs of male-specific primers and a primer for the glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*) as an internal control [44].

OtY2-WSU was the basis for developing two other Y-linked molecular markers, one for Chinook salmon (*OtY3*) and the other for rainbow trout (*OmyY1*) [45]. Both markers were studied using PCR screening in 12.5 kb and 21 kb genomic regions flanking *OtY2*-WSU in

Chinook salmon and rainbow trout, respectively. Approximately 10 kb of the sequences were found to be similar between the species. Extensive characterization of these genomic regions indicated that, in Chinook salmon, this region contains an inactive retrotransposon and a minisatellite. These were used to develop a PCR assay to amplify the fully Y-linked marker *OtY3*, which shows two male-specific alleles (725 and 500 bp) [45].

In rainbow trout, the marker contains a region that shows sequence homology with 18S ribosomal RNA and internal transcribed spacer 1 (ITS), the major histocompatibility complex (MHC) class IB intronic region, a LINE-1 type reverse transcriptase, and the *OmyY1* Y-linked marker (in the genomic region homologous with Chinook salmon). However, the retrotransposable element detected in Chinook salmon is absent in rainbow trout. The Y-specific marker *OmyY1* amplifies a 792 bp fragment at a high frequency in males (96.5%) and a low frequency in females (3.7%). This finding may indicate either some degree of recombination with the sex determining region (note that some evidence of mobile elements has been provided for this region) or, as has been argued for other Y-linked markers, may be attributable to environmental sex reversion of some individuals [45].

Several single-nucleotide polymorphism (SNPs) have been identified in a 1058 bp region, including the *OmyY1* Y-specific marker in various male lineages [45]. This male-specific region is not believed to undergo recombination. A Y-haplotype phylogeographic analysis of 333 male rainbow trout obtained from 57 locations in western North America and Russia was recently performed, but no information regarding the inconsistencies between phenotypic sex and *OmyY1* was reported [46].

12.2.6 Microsatellite Markers

With the development of salmonid genetic maps that include phenotypic sex, a number of microsatellite markers have been mapped

near the putative sex determining locus (*SEX*) in a named sex- or Y chromosome-linked group. The first comparative analysis of the *SEX* locus was performed for Arctic char, brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), and rainbow trout, indicating that the microsatellites linked to the *SEX* locus are different in every species [47].

The first microsatellite map for rainbow trout identified the locus *OmyFGT19TUF*, located 1.15 cM from the putatively sex determining locus in males [48]. Advances in rainbow trout genetic maps have confirmed this finding. Other microsatellites detected in this sex-linked group (RT-1) and used to assign sex in rainbow trout include *Ots517NWFSC*, *OMM1026*, and *OMM1372* [27, 42, 43, 47, 49–52]. Finally, the RT-1 linkage group was identified as the sex chromosome (OmySex) in later genetic maps for this species [9].

In Atlantic salmon (*Salmo salar*), the first sex-linked microsatellite reported was *Ssa202DU*, followed by other markers in the linkage group AS1 [47, 53]. This finding was confirmed when the physical map was integrated with the genetic map, anchoring the *SEX* locus between *Ssa202DU* and a large heterochromatin region [55] in the Ssa02 chromosome. Interestingly, the *SEX* locus in this species has also been mapped in two other chromosomes, Ssa06 and Ssa03, depending on mapping families [56].

There are obstacles to using microsatellite loci for sexing salmonids. For one, microsatellite loci are not the sex determining loci. For another, some degree of recombination between the microsatellites and the *SEX* locus is always possible. For example, in Tasmanian Atlantic salmon, the prediction of a phenotypic male, based on a Y-specific haplotype for seven microsatellites inherited from grandsire to sire, fails about 11.4% of the time, probably due to recombination among these markers and the *SEX* locus [56]. Another drawback of microsatellites is that it is necessary to know the paternal and maternal haplotypes to genotype the progeny.

12.2.7 *sdY* Gene

2012 marked the discovery of the *sdY* gene (sexually dimorphic on the Y chromosome), the master sex determining gene in rainbow trout by Yano *et al.* [57]. This gene was discovered by comparing the gonadal transcriptomics of true males and females at the onset of molecular sexual differentiation. The presence of *sdY* was evaluated in 425 trout, and all 218 males were positive for the gene, while all 207 females were negative [57]. *sdY* encodes for a putative protein of 192 amino acids, has four exons, and shares homology with the rainbow trout sex-specific marker *OmyY1* [45] and interferon regulatory factor 9 (*Irf9*). The rainbow trout linkage map containing *sdY* confirmed full linkage with the *SEX* locus in the chromosome OmySex (RT-01 linkage group).

After this revolutionary discovery, screening for the *sdY* gene was performed in other salmonid species, yielding generally similar results to those found in rainbow trout. Species evaluated included graylings (*Thymallus thymallus*), masu salmon, Chinook salmon, Dolly Varden trout (*Salvelinus malma malma*), Arctic charr, brook trout, lake char (*Salvelinus namaycush*), Atlantic salmon, brown trout (*S. trutta*), huchen (*Hucho hucho*), and sakhalin taimen (*Parahucho perryi*) [58]. In all of these species, *sdY* is present in males and absent in females, with few deviations from this pattern.

However, another study carried out in Asian populations from five species of *Oncorhynchus* genus showed high rate of incongruences between presence/absence of *sdY* and phenotypic sex: Chinook salmon (41.2%), chum salmon (18%), sockeye salmon (44%), masu salmon (31%). Only pink salmon presented a 4% on incongruences [59]. These high rates of females positive to *sdY*, and males negative to *sdY*, indicate a possible instability of this sex determining locus in Pacific salmon [59].

More extensive screening for *sdY* has been performed in cultivated Atlantic and wild Chinook salmon. In Chinook salmon, *sdY* is likely the sex determining gene, but some discrepancies have been found

between phenotypic sex and the presence of *sdY*. For example, Yano *et al.* [58] found one female positive for *sdY* among 41 females tested from a wild Alaskan population (USA). Cavileer *et al.* [60] found 13 phenotypic females positive for *sdY* among 107 females tested. In this latter work, four *sdY* coding regions were examined in the *sdY* positive females. Seven females were negative for the *sdY* promoter region and exon 1, but the other six seemed to have the complete coding region, despite a female phenotype. The most probable explanation for females bearing the whole *sdY* gene is that expression was somehow disabled, possibly due to environmental factors (temperature or estrogen contamination), during early development [60].

In Tasmanian Atlantic salmon, there is strong evidence for association among regions bearing the *sdY* gene and phenotypic sex, but there are also some discrepancies [56]. For example, six individuals, evaluated using two sets of *sdY*-specific primers (exon 2 and exon 4), were positive for this gene but phenotypically female, and two phenotypic males were also negative for *sdY* [56].

Similarly, our laboratory tested for the *sdY* gene in Atlantic salmon (mowi strain) breeders from the Huillico aquaculture reproduction program in southern Chile (Figure 12.1). Two phenotypic females were found to be positive for *sdY* among 45 females, and one phenotypic male

was negative for *sdY* among 45 males. Our laboratory used a set of primers published by Yano *et al.* [58] for exon 2 (sdY-E2S1: CCCAGCACTGTTTTCTTGTCTC and sdY-E2AS2: CTGTTGAAGAGCATCACAGGGTC). Interestingly, in Tasmanian Atlantic salmon, *sdY* was found in three different chromosomes, depending on the male lineage of the family. For example, in 58.6% of the 58 families analyzed, this gene was in chromosome Ssa02, but mapped to chromosomes Ssa06 and Ssa03 in 37.9% and 3.5% of families, respectively [56]. Therefore, in this species, the *sdY*-bearing chromosome region and *SEX* locus can suffer recombination with other chromosomes.

Current evidence supports a strong consensus that the *sdY* gene is likely the master sex determining gene in rainbow trout, Chinook salmon, and Atlantic salmon, and probably other salmonid species. The inconsistencies between female phenotypic sex and the presence of the complete *sdY* gene (excluding genotyping or phenotype assignment error) in Chinook and Atlantic salmon may be attributable to temperature-dependent sex reversal [56], contamination with estrogens during early development [60], or an as yet undiscovered factor that must interact with *sdY* gene to produce sex differentiation.

Due to its high rate of success in identifying phenotypic sex, several tests have been developed using the *sdY* gene. For example, a

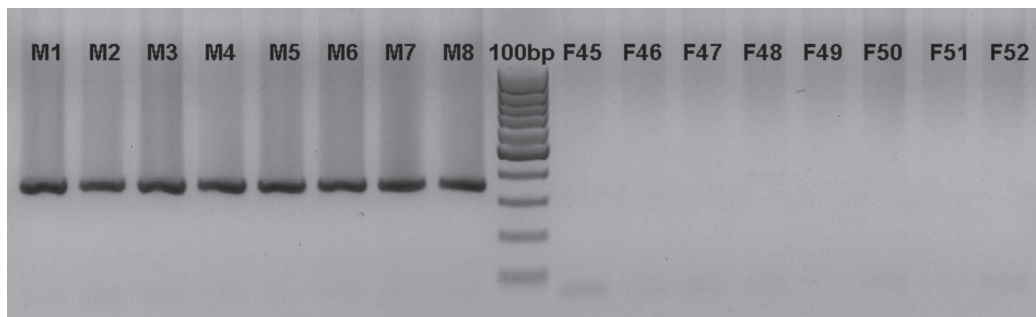


Figure 12.1 Agarose gel electrophoresis, showing the PCR amplification of *sdY* gene (exon 2) in eight males (M1 to M8) and eight females (F45 to F52) from Atlantic salmon. Males shown an amplicon of ≈ 350 bp, which is absent in females.

rapid test, based on high resolution melting analysis (HRM), simultaneously discriminates the sex and species of Atlantic salmon, brown trout, and their hybrids [61], using the two primer pairs published for co-amplification of *sdY* and 18S ribosomal RNA by Yano *et al.* [57]. The test has not been applied in many samples to date. However, it is an interesting, cost-effective, and quick method for sexing, as well as for species and hybrid identification, with potential applications in conservation biology and the food industry.

In the genus *Salmo*, a second assay, based on the amplification of a small section of 200bp of the *sdY* gene, was developed to be multiplexed with microsatellite markers [62]. The method was tested on 65 marine trout (*Salmo trutta*), with a mismatch of 3.2% [62]. Unfortunately, the authors did not provide raw data for a quantitative evaluation of their results using diagnostic tests.

A third quick method for sexing Atlantic salmon with *sdY* gene uses a TaqMan assay, based in the amplification of a fragment of 93bp from the 4th exon of the gene [63]. This method was tested on 2583 individuals, detecting only one female among the 1257 salmons positive to *sdY* (false positive rate=0.08%), however the false negative rate (males negative to *sdY*) was not evaluated [64].

12.3 Evaluation of Sex Marker Applications in Salmonids

As described above, many sex-linked markers have been identified in salmonids, but only a few have been used extensively. To evaluate potential applicability to salmonid sexing, the approach described by Lopez and Araneda [41] is used here to estimate diagnostic statistics for each molecular assay: sensitivity, specificity, positive predictive value (*PPV*), negative predictive value (*NPV*), likelihood ratio of a positive test result (*LR+*), accuracy (*ACC*), and diagnostic odds ratio (*DOR*). A basic description of all of these diagnostic tests can be found in Glas *et al.* [64]. Successful

Table 12.1 Contingency table for sex phenotyping and classification using a molecular assay.

| | | Genotype (Molecular Assay) | | |
|-----------|--------|----------------------------|-------------------|-----------|
| | | Positive (Male) | Negative (Female) | Total |
| Phenotype | Male | <i>TP</i> | <i>FN</i> | <i>PM</i> |
| | Female | <i>FP</i> | <i>TN</i> | <i>PF</i> |
| | Total | <i>GM</i> | <i>GF</i> | |

performance was defined as correct identification of the male fish (XY individual), given that all of the molecular assays tested detect Y-chromosome gene or markers. In this type of analysis, individuals are classified in a 2 × 2 contingency table (Table 12.1), as follows:

TP, *FP*, *FN*, and *TN* denote the number of true positive, false positive, false negative, and true negative results, respectively. *PM* and *PF* are phenotypic males and females, respectively, identified through direct observation of gamete emission or gonads, and *GM* and *GF* are genotypic males and females, respectively, identified through genotyping with the molecular assay (Table 12.2).

The computational formulae for the tests are as follows:

Sensitivity (true positive rate) is the proportion of true (phenotypic) males correctly identified by the molecular assay.

$$Sensitivity = \frac{P(PM \cap GM)}{P(PM)} = \frac{TP}{(TP + FN)}$$

Specificity (true negative rate) is the proportion of true females correctly identified by the assay.

$$Specificity = \frac{P(PF \cap GF)}{P(PF)} = \frac{TN}{(TN + FP)}$$

To evaluate the probability that these molecular assays provide the correct gender identification, positive predictive value (*PPV*, i.e., the proportion of males with positive test results correctly sexed as male) and negative

Table 12.2 Performance of various molecular assays developed for salmonid sexing.

| Gen/Marker | Assay | Marker positive fish | | | | | | | | | |
|-------------------------------|---------------------|----------------------|--------|-------------|-------------|--------|--------|---------------------|---------------------|--------|--|
| | | Male | Female | Sensitivity | Specificity | PPV | NPV | LR+ | DOR | ACC | |
| Atlantic salmon: | | | | | | | | | | | |
| <i>sdY</i> ¹ | PCR | 542/555 | 4/384 | 0.9766 | 0.9896 | 0.9894 | 0.9769 | 93.75 | 3961 | 0.9819 | |
| <i>sdY</i> ² | PCR | 64/65 | 2/65 | 0.9846 | 0.9692 | 0.9697 | 0.9844 | 32.00 | 2016 | 0.9769 | |
| Chinook salmon: | | | | | | | | | | | |
| <i>sdY</i> ³ | TaqMan [®] | 45/45 | 13/157 | 1.0000 | 0.9172 | 0.9235 | 1.0000 | 12.08 | 974 [†] | 0.9356 | |
| <i>OtYI</i> ⁴ | PCR | 396/396 | 88/530 | 1.0000 | 0.8340 | 0.8576 | 1.0000 | 6.02 | 3965 [†] | 0.9050 | |
| <i>GH-ψ</i> ⁵ | PCR | 91/91 | 0/89 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 179.02 [†] | 32757 [†] | 1.0000 | |
| <i>OtY3</i> ⁶ | PCR | 143/143 | 0/127 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 255.11 [†] | 73185 [†] | 1.0000 | |
| Rainbow trout: | | | | | | | | | | | |
| <i>sdY</i> ⁷ | PCR | 218/218 | 0/207 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 415.05 [†] | 181355 [†] | 1.0000 | |
| <i>Omy-163</i> ⁸ | PCR | 386/427 | 21/480 | 0.9040 | 0.9563 | 0.9538 | 0.9088 | 20.66 | 206 | 0.9313 | |
| <i>OmyP9</i> ⁹ | PCR | 35/47 | 12/84 | 0.7447 | 0.8571 | 0.8390 | 0.7705 | 5.21 | 18 | 0.8168 | |
| <i>OtY2-WSU</i> ¹⁰ | trio-PCR | 94/94 | 0/104 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 208.89 [†] | 39501 [†] | 1.0000 | |
| <i>OmyYI</i> ⁶ | PCR | 139/144 | 5/134 | 0.9653 | 0.9627 | 0.9628 | 0.9652 | 25.87 | 717 | 0.9640 | |
| Brown trout: | | | | | | | | | | | |
| <i>sdY</i> ⁷ | PCR | 73/73 | 76/76 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 152.96 [†] | 22491 [†] | 1.0000 | |

(Continued)

Table 12.2 (Continued)

| Marker positive fish | | | | | | | | | | |
|---|-----------|-------|--------|-------------|-------------|--------|--------|--------------------|-------------------|--------|
| Gen/Marker | Assay | Male | Female | Sensibility | Specificity | PPV | NPV | LR+ | DOR | ACC |
| Coho salmon: | | | | | | | | | | |
| <i>GH-2</i> ¹¹ | PCR | 41/41 | 0/47 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 94.86 [†] | 7885 [†] | 1.0000 |
| Masu salmon: | | | | | | | | | | |
| <i>GH-γ</i> ¹² | PCR | 63/70 | 2/61 | 0.9000 | 0.9672 | 0.9649 | 0.9063 | 27.45 | 266 | 0.9313 |
| Sockeye salmon: | | | | | | | | | | |
| <i>OtY2-WSU</i> ¹⁰ | Trio PCR* | 49/61 | 3/58 | 0.8033 | 0.9483 | 0.9395 | 0.8282 | 15.53 | 75 | 0.8739 |

¹ Eisbrenner *et al.* [56].

² Combined data from Yano *et al.* [58] and Araneda (unpublished).

³ Cavileer *et al.* [60].

⁴ Combined data from Devlin *et al.* [25, 29], Nagler *et al.* [30] and Williamson and May [31].

⁵ Combined data from Du *et al.* [33] and Devlin *et al.* [29].

⁶ Brunelli *et al.* [45].

⁷ Yano *et al.* [57].

⁸ Combined data from Felip *et al.* [42] and López and Araneda [41].

⁹ López and Araneda [41].

¹⁰ Brunelli and Thorgaard [44].

¹¹ Forbes *et al.* [36].

¹² Zhang *et al.* [35] and Yamamoto and Kitanishi [38].

[†] Estimated adding 0.5 to all counts due to LR+, and DOR are undefined if the 2 × 2 contingency table contains zeroes.

predictive value (*NPV*, i.e., the proportion of females with negative results correctly sexed as female) were estimated with the equation from Altman and Bland [65]. In the next two equations, *Prevalence* was assumed to be 0.5, as this is the expected proportion of males in a normal population [41].

$$PPV = \frac{Sensitivity \cdot Prevalence}{Sensitivity \cdot Prevalence + (1 - Specificity) \cdot (1 - Prevalence)}$$

$$NPV = \frac{Sensitivity \cdot (1 - Prevalence)}{(1 - Sensitivity) \cdot Prevalence + Specificity \cdot (1 - Prevalence)}$$

The likelihood ratio of a positive test result (*LR+*) was estimated to evaluate the usefulness of molecular assays in identification of males. This statistic is the ratio of a positive “male” test result among phenotypic males to the same positive result among phenotypic females. Larger values of *LR+* indicate better performance.

$$LR = \frac{Sensitivity}{(1 - Specificity)}$$

Accuracy (*ACC*), that is, the proportion of correctly-identified subjects, was estimated as follows:

$$ACC = \frac{(TP + TN)}{(TP + TN + FP + FN)}$$

Finally, the diagnostic odds ratio (*DOR*) of a test is the ratio of the odds of a positive result among phenotypic males relative to the odds a positive result among phenotypic females.

$$DOR = \frac{\left(\frac{TP}{FP}\right)}{\left(\frac{FN}{TN}\right)} = \frac{\left(\frac{Sensitivity}{(1 - Sensitivity)}\right)}{\left(\frac{1 - Specificity}{(Specificity)}\right)} = \frac{\left(\frac{PPV}{1 - PPV}\right)}{\left(\frac{1 - NPV}{NPV}\right)}$$

Higher values of *DOR* indicate better discriminatory test performance, and values close to 1 indicate that the genetic test does not discriminate between the sexes. The *DOR* is highest when sensitivity and specificity are close to 1.0 [64].

The genotypic and phenotypic sex data published for each assay in each salmonid species were used for these estimations.

The only restriction was that the analyzed samples must include at least more than 40 individuals per sex (Table 12.2).

In general, nearly all of the markers developed for sexing salmonids showed high sensitivity and specificity for detecting a true male individual, with a *DOR* value above one (Table 12.2). The performance of various assays developed for different species shows that, in general, markers developed for the *sdY* gene performed better than other markers when enough data were available for analysis.

For Atlantic salmon, the assay developed by Eisbrenner *et al.* [56] showed the best performance. In Chinook salmon, an assay based on the *OtY3* marker [45] showed the best performance among four markers evaluated. In rainbow trout, a comparison of five different markers indicated that the best sexing test was based on the *sdY* gene developed by Yano *et al.* [57]. For brown trout, coho, masu, and sockeye salmon, only one marker was evaluated in each species, based on the *sdY* gene [58], *GH-2* gene [36], *GH-Ψ* [35], and *OtY2-WSU* [44], respectively.

On the other hand, Podlesnykh *et al.* [59] have shown congruence in genotyping between the *sdY* gene and other Y-linked molecular markers in some Pacific salmon. For example, in Chinook salmon and sockeye salmon, sexing performance was similar, with *sdY* and with *OtY2-WSU* marker. Similarly, in masu salmon, sexing performance was also similar between *sdY* and *GH-Ψ* marker. These findings indicate that it is possible to use *sdY* instead of other Y-linked molecular markers in these species. However, considering the small samples used by species (29–50), these results should be considered preliminary.

It is highly probable that the application of the primer sets developed by Yano *et al.* [58], Eysturskarð *et al.* [63] or Quéméré *et al.* [62] in more individuals of other salmonid species would reveal that *sdY*-based tests show the best performance for salmonid sexing if *sdY* is truly the sex determining master gene for all salmonids. However, molecular assay for salmonid sexing must be more cost effective, faster, and validated with international standards such ISO 17025, before they will be extended to the industry.

References

- 1 Davidson W, Huang T-K, Fujiki K, *et al.* (2009). The sex determining loci and sex chromosomes in the family Salmonidae. *Sexual Development* **3** (2–3), 78–87.
- 2 Thorgaard GH. (1978). Sex chromosomes in the sockeye salmon: a Y-autosome fusion. *Canadian Journal of Genetics and Cytology* **20** (3), 349–54.
- 3 Faber-Hammond J, Phillips R, Park L. (2012). The sockeye salmon neo-Y chromosome is a fusion between linkage groups orthologous to the coho Y chromosome and the long arm of rainbow trout chromosome 2. *Cytogenet Genome Research* **136** (1), 69–74.
- 4 Devlin RH and Nagahama Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* **208** (3–4), 191–364.
- 5 Johnstone R, Simpson T, Youngson A, Whitehead C. (1979). Sex reversal in salmonid culture: Part II. The progeny of sex-reversed rainbow trout. *Aquaculture* **18** (1), 13–9.
- 6 Hunter GA, Donaldson EM, Goetz FW, Edgell PR. (1982). Production of all-female and sterile coho salmon, and experimental evidence for male heterogamety. *Transactions of the American Fisheries Society* **111** (3), 367–72.
- 7 Hunter GA, Donaldson EM, Stoss J, Baker I. (1983). Production of monosex female groups of chinook salmon (*Oncorhynchus tshawytscha*) by the fertilization of normal ova with sperm from sex-reversed females. *Aquaculture* **33** (1–4), 355–64.
- 8 Thorgaard GH. (1977). Heterogametic sex chromosomes in male rainbow trout. *Science* **196** (4292), 900–902.
- 9 Phillips RB, Nichols KM, DeKoning JJ, *et al.* (2006). Assignment of rainbow trout linkage groups to specific chromosomes. *Genetics* **174** (3), 1661–1670.
- 10 Phillips RB, DeKoning J, Morasch M, *et al.* (2007). Identification of the sex chromosome pair in chum salmon (*Oncorhynchus keta*) and pink salmon (*Oncorhynchus gorbuscha*). *Cytogenetic Genome Research* **116** (4), 298–304.
- 11 Phillips RB, Keatley K, Morasch M, *et al.* (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 Genetics* **10**, 46.
- 12 Li J, Phillips RB, Harwood A, *et al.* (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 Research* **133** (1), 25–33.
- 13 Phillips RB, Park LK, Naish KA. (2013). Assignment of Chinook salmon (*Oncorhynchus tshawytscha*) linkage groups to specific chromosomes reveals a karyotype with multiple rearrangements of the chromosome arms of rainbow trout (*Oncorhynchus mykiss*). *G3: Genes, Genomes, Genetics* **3** (12): 2289–95.
- 14 Allendorf FW, Seeb JE, Knudsen KL, Thorgaard GH. (1986). Gene-centromere mapping of 25 loci in rainbow trout. *Journal of Heredity* **77** (5), 307–312.
- 15 Allendorf FW, Gellman WA, Thorgaard GH. (1994). Sex-linkage of two enzymes loci in *Oncorhynchus mykiss* (rainbow trout). *Heredity* **72** (5), 498–507.
- 16 Gellman WA, Allendorf FW, Thorgaard GH. (1987). Hexosaminidase is sex linked in rainbow trout. *Isozyme Bulletin* **20**, 14.
- 17 May B, Johnson KR, Wright Jr JE. (1989). Sex linkage in salmonids: evidence from a hybridized genome of brook trout and Arctic charr. *Biochemical Genetics* **27** (5–6), 291–301.
- 18 Williams JG, Kubelik AR, Livak KJ, *et al.* (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18** (22), 6531–6535.

- 19 Welsh J and McClelland M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18** (24), 7213–7218.
- 20 Vos P, Hogers R, Bleeker M, *et al.* (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23** (21), 4407–4414.
- 21 Paran I and Michelmore RW. (1993). Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theoretical and Applied Genetics* **85** (8), 985–993.
- 22 Ellegren H. (2004). Microsatellites: simple sequences with complex evolution. *Nature Reviews Genetics* **5** (6), 435–445.
- 23 Yue GH and Orban L. (2005). A simple and affordable method for high-throughput DNA extraction from animal tissues for polymerase chain reaction. *Electrophoresis* **26** (16), 3081–3083.
- 24 Taggart JB, Hynes RA, Prodóuhl PA, Ferguson A. (1992). A simplified protocol for routine total DNA isolation from salmonid fishes. *Journal of Fish Biology* **40** (6), 963–965.
- 25 Devlin RH, McNeil BK, Groves TD, Donaldson EM. (1991). Isolation of a Y-chromosomal DNA probe capable of determining genetic sex in chinook salmon (*Oncorhynchus tshawytscha*). *Canadian Journal of Fisheries and Aquatic Sciences* **48** (9), 1606–1612.
- 26 Devlin RH, McNeil BK, Solar II, Donaldson EM. (1994). A rapid PCR-based test for Y-chromosomal DNA allows simple production of all-female strains of chinook salmon. *Aquaculture* **128** (3), 211–220.
- 27 Nichols KM, Young WP, Danzmann RG, *et al.* (2003). A consolidated linkage map for rainbow trout (*Oncorhynchus mykiss*). *Animal Genetics* **34** (2), 102–115.
- 28 Noakes MA and Phillips RB (2003). OtY1 is a Y-linked marker in chinook salmon but not in rainbow trout. *Animal Genetics* **34** (2), 156–157.
- 29 Devlin R, Biagi C, Smailus D. (2001). Genetic mapping of Y-chromosomal DNA markers in Pacific salmon. *Genetica* **111** (1–3), 43–58.
- 30 Nagler JJ, Bouma J, Thorgaard GH, Dauble DD. (2001). High incidence of a male-specific genetic marker in phenotypic female chinook salmon from the Columbia River. *Environmental Health Perspectives* **109** (1), 67–69.
- 31 Williamson KS and May B. (2002). Incidence of phenotypic female chinook salmon positive for the male Y-chromosome-specific marker OtY1 in the Central Valley, California. *Journal of Aquatic Animal Health* **14** (3): 176–183.
- 32 Devlin RH, Stone GW, Smailus DE. (1998). Extensive direct-tandem organization of a long repeat DNA sequence on the Y chromosome of chinook salmon (*Oncorhynchus tshawytscha*). *Journal of Molecular Evolution* **46** (3), 277–287.
- 33 Du SJ, Devlin RH, Hew CL. (1993). Genomic structure of growth hormone genes in chinook salmon (*Oncorhynchus tshawytscha*): presence of two functional genes, GH-I and GH-II, and a male specific pseudogene, GH-psi. *DNA and Cell Biology* **12** (8), 739–751.
- 34 Devlin RH. (1993). Sequence of sockeye salmon type 1 and 2 growth hormone genes and the relationship of rainbow trout with Atlantic and Pacific salmon. *Canadian Journal of Fisheries and Aquatic Sciences* **50** (8), 1738–1748.
- 35 Zhang Q, Nakayama I, Fujiwara A, *et al.* (2001). Sex identification by male-specific growth hormone pseudogene (GH-ψ) in *Oncorhynchus masou* complex and a related hybrid. *Genetica* **111** (1–3), 111–118.
- 36 Forbes SH, Knudsen KL, North TW, Allendorf FW. (1994). One of two growth hormone genes in coho salmon is sex-linked. *Proceedings of the National Academy of Sciences* **91** (5), 1628–1631.
- 37 Nakayama I, Biagi C, Koide N, Devlin R. (1999). Identification of a sex-linked GH pseudogene in one of two species of Japanese salmon (*Oncorhynchus masou* and *O. rhodurus*). *Aquaculture* **173** (1), 65–72.

- 38 Yamamoto T and Kitanishi S. (2012). Variable incidences and morphological characteristics of female masu salmon *Oncorhynchus masou* with growth hormone pseudogene. *Journal of Fish Biology* **80** (2), 378–386.
- 39 Iturra P, Medrano J, Bagley M, *et al.* (1997). Identification of sex chromosome molecular markers using RAPDs and fluorescent in situ hybridization in rainbow trout. *Genetica* **101** (3), 209–213.
- 40 Iturra P, Bagley M, Vergara N, *et al.* (2001). Development and characterization of DNA sequence OmyP9 associated with the sex chromosomes in rainbow trout. *Heredity* **86** (4), 412–419.
- 41 Lopez ME and Araneda C. (2012). An evaluation of a diagnostic test to identify the sex of farmed rainbow trout, using sex-specific molecular markers. *Latin American Journal of Aquatic Research* **40** (4), 1085–1089.
- 42 Felip A, Fujiwara A, Young WP, *et al.* (2004). Polymorphism and differentiation of rainbow trout Y chromosomes. *Genome* **47** (6), 1105–1113.
- 43 Felip A, Young WP, Wheeler PA, Thorgaard GH. (2005). An AFLP-based approach for the identification of sex-linked markers in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **247** (1–4), 35–43.
- 44 Brunelli JP and Thorgaard GH. (2004). A New Y-chromosome-specific marker for Pacific salmon. *Transactions of the American Fisheries Society* **133** (5), 1247–1253.
- 45 Brunelli JP, Wertzler KJ, Sundin K, Thorgaard GH. (2008). Y-specific sequences and polymorphisms in rainbow trout and chinook salmon. *Genome* **51** (9), 739–748.
- 46 Brunelli JP, Steele CA, Thorgaard GH. (2010). Deep divergence and apparent sex-biased dispersal revealed by a Y-linked marker in rainbow trout. *Molecular Phylogenetics and Evolution* **56** (3), 983–990.
- 47 Woram RA, Gharbi K, Sakamoto T, *et al.* (2003). Comparative genome analysis of the primary sex-determining locus in salmonid fishes. *Genome Research* **13** (2), 272–280.
- 48 Sakamoto T, Danzmann RG, Gharbi K, *et al.* (2000). A microsatellite linkage map of rainbow trout (*Oncorhynchus mykiss*), characterized by large sex-specific differences in recombination rates. *Genetics* **155** (3), 1331–1345.
- 49 Palti Y, Danzmann RG, Rexroad CE. (2003). Characterization and mapping of 19 polymorphic microsatellite markers for rainbow trout (*Oncorhynchus mykiss*). *Animal Genetics* **34** (2), 153–156.
- 50 Guyomard R, Boussaha M, Krieg F, *et al.* (2012). A synthetic rainbow trout linkage map provides new insights into the salmonid whole genome duplication and the conservation of synteny among teleosts. *BMC Genetics* **13**, 15.
- 51 Guyomard R, Mauger S, Tabet-Canale K, *et al.* (2006). A Type I and Type II microsatellite linkage map of rainbow trout (*Oncorhynchus mykiss*) with presumptive coverage of all chromosome arms. *BMC Genomics* **7**, 302.
- 52 Palti Y, Genet C, Luo M-C, *et al.* (2011). A first generation integrated map of the rainbow trout genome. *BMC Genomics* **12**, 180.
- 53 Phillips RB, Nichols KM, DeKoning JJ, *et al.* (2006). Assignment of rainbow trout linkage groups to specific chromosomes. *Genetics* **174**, 1661–1670.
- 54 Gilbey J, Verspoor E, McLay A, Houlihan D. (2004). A microsatellite linkage map for Atlantic salmon (*Salmo salar*). *Animal Genetics* **35** (2), 98–105.
- 55 Artieri CG, Mitchell LA, Ng SHS, *et al.* (2006). Identification of the sex-determining locus of Atlantic salmon (*Salmo salar*) on chromosome 2. *Cytogenetic and Genome Research* **112** (1–2), 152–159.
- 56 Eisbrenner W, Botwright N, Cook M, *et al.* (2014). Evidence for multiple sex-determining loci in Tasmanian Atlantic salmon (*Salmo salar*). *Heredity*, **113** (1), 86–92.

- 57 Yano A, Guyomard R, Nicol B, *et al.* (2012). An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*. *Current Biology* **22** (15), 1423–1428.
- 58 Yano A, Nicol B, Jouanno E, *et al.* (2013). The sexually dimorphic on the Y-chromosome gene (sdY) is a conserved male-specific Y-chromosome sequence in many salmonids. *Evolutionary Applications* **6** (3), 486–496.
- 59 Podlesnykh AV, Brykov VA, Kukhlevsky AD. (2017). Unstable linkage of molecular markers with sex determination gene in pacific salmon (*Oncorhynchus spp.*). *Journal of Heredity* **108** (3), 328–33.
- 60 Cavileer TD, Hunter SS, Olsen J, *et al.* (2015). A sex-determining gene (sdY) assay shows discordance between phenotypic and genotypic sex in wild populations of chinook salmon. *Transactions of the American Fisheries Society* **144** (2), 423–430.
- 61 Anglès d'Auriac MB, Urke HA, Kristensen T. (2014). A rapid qPCR method for genetic sex identification of *Salmo salar* and *Salmo trutta* including simultaneous elucidation of interspecies hybrid paternity by high-resolution melt analysis. *Journal of Fish Biology* **84** (6), 1971–1977.
- 62 Quéméré E, Perrier C, Besnard A-L, *et al.* (2014). An improved PCR-based method for faster sex determination in brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). *Conservation Genetics Resources* **6** (4), 825–827.
- 63 Eysturskarð J, Dam M, í Kongsstovu SK, *et al.* (2017). Rapid sex identification of Atlantic salmon (*Salmo salar* L.) by real-time PCR. *Aquaculture Research* **48** (5), 2618–2620.
- 64 Glas AS, Lijmer JG, Prins MH, *et al.* (2003). The diagnostic odds ratio: a single indicator of test performance. *Journal of Clinical Epidemiology* **56** (11), 1129–1135.
- 65 Altman DG and Bland JM. (1994). Statistics Notes: Diagnostic tests 1: sensitivity and specificity. *British Medical Journal* **308**, 2552.

