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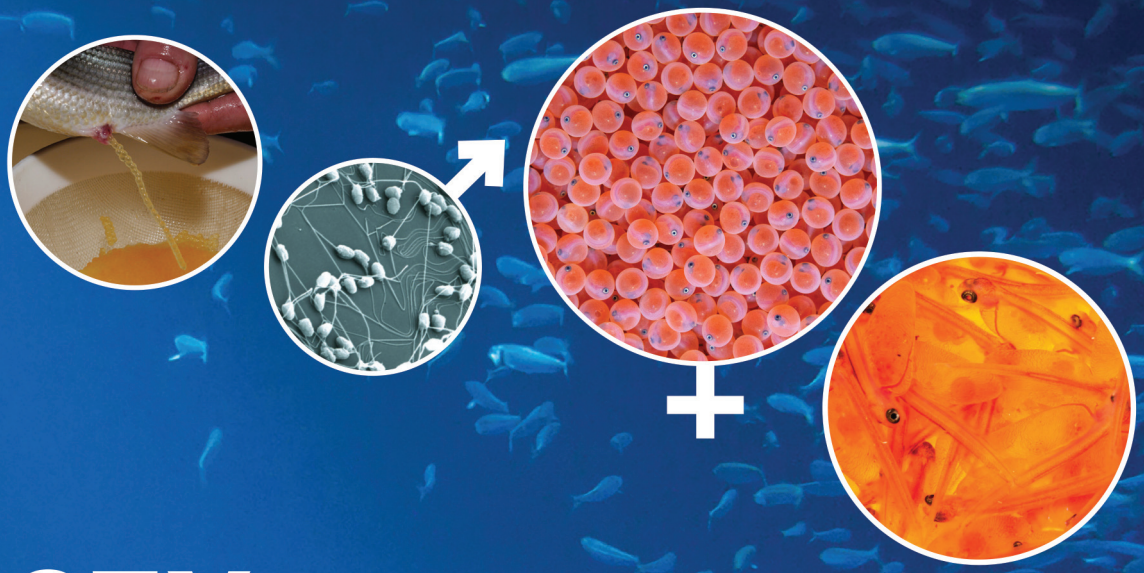

SEX CONTROL IN AQUACULTURE

VOLUME 1

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Sex Control in Aquaculture

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Volume I

Edited by

Han-Ping Wang

Aquaculture Genetics and Breeding Laboratory, The Ohio State University South Centers, Piketon, OH, USA

Francesc Piferrer

Institute of Marine Sciences, Spanish National Research Council (CSIC), Barcelona, Spain

Song-Lin Chen

Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China

Associate Editor

Zhi-Gang Shen

Aquaculture Genetics and Breeding Laboratory,
The Ohio State University South Centers,
Piketon, OH, USA
College of Fisheries, Huazhong Agricultural University,
Wuhan, China

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Editorial Board

Han-Ping Wang
Dr. Han-Ping Wang is a Principal Scientist and the Director of the Aquaculture Research Center and Genetics and Breeding Laboratory at The Ohio State University South Centers. He has provided leadership as the PI for more than 70 research projects, with funding of approximately $10 million. He achieved success in controlled breeding and culture of Reeves shad, and in developing all-male bluegill and all-female yellow perch populations, and superior perch strains. He also completed whole genome sequencing of these two species. Dr. Wang has published more than 100 papers in prestigious international journals and two books, and has two pending patents. He has supervised around 30 PhD students and Post-Doctoral Fellows. Dr. Wang has won six S&T Achievement Awards, 10 Best Paper and other professional awards from national and international agencies.

Francesc Piferrer
Dr. Francesc Piferrer is a Research Professor at the Institute of Marine Sciences (CSIC) in Barcelona. He has studied sex determination and differentiation in Pacific salmon, European sea bass, turbot, and Senegalese sole. He has significantly contributed to demonstrating the importance of estrogens for female sex differentiation in fish. Dr. Piferrer has authored more than one hundred papers in peer-reviewed international journals, has supervised a dozen PhD theses, and has been the PI in many research projects. He has significantly contributed to the development of protocols for sex and maturity control in fish farming, collaborates with private companies, and has developed a patent for the thermal control of sex ratios. In 2013, he was awarded the XII Jacumar Prize for the Best Aquaculture Research.

Song-Lin Chen
Dr. Song-Lin Chen is a Research Professor and the Director of Lab for Aquatic Biotechnology and Genomics in the Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (CAFS). His research interest is involved in genomics, sex control, molecular breeding, cell culture, and sperm and embryo cryopreservation in fish. Dr. Chen has completed the whole genome fine maps of half-smooth tongue sole and Japanese flounder, exploited female-specific AFLP and SSR markers, and found *dmrt1* to be a male-determining gene in half-smooth tongue sole. He has published four books and over 300 research papers, including two papers in Nature Genetics. He has won several State Technological Invention Awards and S&T Progress Award of China.

Zhi-Gang Shen (Associate Editor)
Dr. Zhi-Gang Shen is currently an associate professor at the College of Fisheries, Huazhong Agricultural University. He did his doctoral thesis in aquaculture genetics as a joint PhD student of The Ohio State University (OSU) and Huazhong Agricultural University, and completed his postdoctoral training at the OSU. His research interest has been focused on molecular, physiological and epigenetic mechanisms involved in sex differentiation, sex determination, and sex control in fish. He also studies the sexual growth dimorphism, using experimental biology and bioinformatics.

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.

To Arianna and Núria Piferrer, the most precious all-female progeny that one of the editors of this book has ever produced.

To Youmei Li, Shengqin Xia and Ying Chen, the most important three ladies – mother, wife, and daughter of one of the editors of this book.

Contents

List of Contributors

Katsutoshi Arai
Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hokkaido, Japan

Cristian Araneda
Department of Animal Production, Faculty of Agronomic Sciences, University of Chile, Santiago, Chile

Tulin Arslan
Department of Aquaculture, Mugla Sitki Kocman University, Mugla, Turkey

Jean-François Baroiller
ISEM, Université de Montpellier, CNRS, IRD, EPHE, Montpellier, France; CIRAD, UMR ISEM, Montpellier, France

Sylvain Bertho
French National Institute for Agricultural Research (INRA), Rennes, France

Mercedes Blázquez
Institute of Marine Sciences, Spanish National Research Council (ICM-CSIC), Barcelona, Spain

Helena D'Cotta
ISEM, CNRS, Univ. Montpellier, IRD, EPHE, Montpellier; CIRAD, Montpellier France

Bruce W. Draper
Department of Molecular and Cellular Biology, University of California Davis, CA, USA

Alicia Felip
Institute of Aquaculture Torre de la Sal, Spanish National Research Council (CSIC), Castellón, Spain

Alexis Fostier
French National Institute for Agricultural Research (INRA), Rennes, France

Takafumi Fujimoto
Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hokkaido, Japan

Ze-Xia Gao
College of Fisheries, Huazhong Agricultural University, Wuhan, China

Yann Guiguen
French National Institute for Agricultural Research (INRA), Fish Physiology and Genomics, Rennes, France

Amaury Herpin
French National Institute for Agricultural Research (INRA), Rennes, France

Patricia Iturra
Faculty of Medicine, University of Chile, Santiago, Chile

Natalia Lam
Department of Animal Production, Faculty of Agronomic Sciences, University of Chile, Santiago, Chile

Sang-Go Lee
World Fisheries University Pilot Program, Pukyong National University, Busan, South Korea

James J. Nagler
Department of Biological Sciences and Center for Reproductive Biology, University of Idaho, ID, USA

Nguyen Hong Nguyen
University of the Sunshine Coast, DC, Australia

Paul O'Bryant
The Ohio State University South Centers, Piketon, OH, USA

Francesc Piferrer
Institute of Marine Sciences, Spanish National Research Council (CSIC), Barcelona, Spain

S.M. Rafiquzzaman
Department of Fisheries Biology and Aquatic Environment, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh

M. Aminur Rahman
World Fisheries University Pilot Programme, Pukyong National University (PKNU), Busan, South Korea

Dean Rapp
The Ohio State University South Centers, Piketon, OH, USA

Eric Saillant
Gulf Coast Research Laboratory, School of Ocean Science and Technology, The University of Southern Mississippi, Ocean Springs, MS, USA

Zhi-Gang Shen
Aquaculture Genetics and Breeding Laboratory, The Ohio State University South Centers, Piketon, OH, USA
College of Fisheries, Huazhong Agricultural University, Wuhan, China

Marc Vandeputte
INRA, Jouy-en-Josas, France

Han-Ping Wang
Aquaculture Genetics and Breeding Laboratory, The Ohio State University South Centers, Piketon, OH, USA

Claus Wedekind
Department of Ecology and Evolution, Biophore, University of Lausanne, Lausanne, Switzerland

Hong Yao
The Ohio State University South Centers, Piketon, OH, USA

Fatimah Md. Yusoff
Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

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 broodsrock 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.

May, 2018

Han-Ping Wang,
Francesc Piferrer,
and Song-Lin Chen

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12

Development and Application of Sex-Linked Markers in Salmonidae

Cristian Araneda[1], Natalia Lam[1], and Patricia Iturra[2]

[1] *Department of Animal Production, University of Chile. Santiago, Chile*
[2] *Faculty of Medicine, University of Chile. Santiago, Chile*

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 X_1X_2Y 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-inked 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

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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 cm^2. 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 *Hinf*I 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 *Rsa*I enzyme. Combinations of size and restriction polymorphisms produced three *OmyP9* variants: variant A (894 bp, with two *Rsa*I restriction sites), which generated three fragments (441, 114, and 339 bp); variant B (899 bp, with one *Rsa*I site), which generated two fragments (555 and 344 bp); and variant C (840 bp, with one *Rsa*I 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 F_1 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 (*Eco*RI/*Mse*I, *Pst*I/*Mse*I and *Bam*HI/*Mse*I), 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 *Oncorhychus* 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 Huililco 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: CTGTTGAAGAGCATCA CAGGGTC). 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

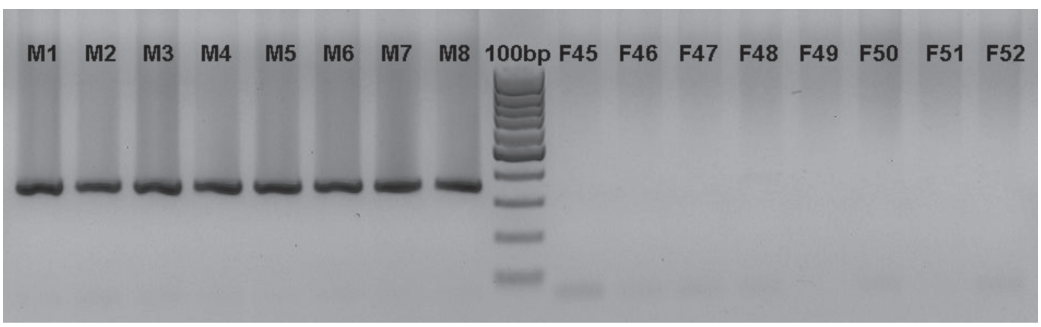

Figure 12.1 Agarose gel electrophoresis, showing the PCR amplification of *sdY* gene (exon 2) in eight males (M1 to M8) and eight females (F45 to F52) from Atlantic salmon. Males shown an amplicon of ≈ 350 bp, which is absent in females.

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 200 bp 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 93 bp 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 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:

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

		Genotype (Molecular Assay)		
		Positive (Male)	**Negative (Female)**	**Total**
Phenotype	Male	*TP*	*FN*	*PM*
	Female	*FP*	*TN*	*PF*
	Total	*GM*	*GF*	

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.

		Marker positive fish								
Gen/Marker	**Assay**	**Male**	**Female**	***Sensibility***	***Specificity***	***PPV***	***NPV***	***LR+***	***DOR***	***ACC***
Atlantic salmon:										
sdY[1]	PCR	542/555	4/384	0.9766	0.9896	0.9894	0.9769	93.75	3961	0.9819
sdY[2]	PCR	64/65	2/65	0.9846	0.9692	0.9697	0.9844	32.00	2016	0.9769
Chinook salmon:										
sdY[3]	TaqMan®	45/45	13/157	1.0000	0.9172	0.9235	1.0000	12.08	974[†]	0.9356
OtY1[4]	PCR	396/396	88/530	1.0000	0.8340	0.8576	1.0000	6.02	3965[†]	0.9050
GH-Ψ[5]	PCR	91/91	0/89	1.0000	1.0000	1.0000	1.0000	179.02[†]	32757[†]	1.0000
OtY3[6]	PCR	143/143	0/127	1.0000	1.0000	1.0000	1.0000	255.11[†]	73185[†]	1.0000
Rainbow trout:										
sdY[7]	PCR	218/218	0/207	1.0000	1.0000	1.0000	1.0000	415.05[†]	181355[†]	1.0000
Omy-163[8]	PCR	386/427	21/480	0.9040	0.9563	0.9538	0.9088	20.66	206	0.9313
OmyP9[9]	PCR	35/47	12/84	0.7447	0.8571	0.8390	0.7705	5.21	18	0.8168
OtY2-WSU[10]	trio-PCR	94/94	0/104	1.0000	1.0000	1.0000	1.0000	208.89[†]	39501[†]	1.0000
OmyY1[6]	PCR	139/144	5/134	0.9653	0.9627	0.9628	0.9652	25.87	717	0.9640
Brown trout:										
sdY[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:										
GH-2[11]	PCR	41/41	0/47	1.0000	1.0000	1.0000	1.0000	94.86[†]	7885[†]	1.0000
Masu salmon:										
GH-Ψ[12]	PCR	63/70	2/61	0.9000	0.9672	0.9649	0.9063	27.45	266	0.9313
Sockeye salmon:										
OtY2-WSU[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 contingence 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.

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