

**Genome Mapping
and Genomics
in Animals**

Chittaranjan Kole

Series Editor

Thomas D. Kocher
Chittaranjan Kole
Editors

Genome Mapping and Genomics in Fishes and Aquatic Animals



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Genome Mapping and Genomics in Animals
Volume 2

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Thomas D. Kocher, Chittaranjan Kole
(Editors)

Genome Mapping and Genomics in Fishes and Aquatic Animals

With 29 Illustrations, 12 in Color



THOMAS D. KOCHER
PROFESSOR
Department of Biology
1210 Biology/Psychology Building
University of Maryland
College Park
MD 20742
USA

e-mail: tdk@umd.edu

CHITTARANJAN KOLE
Department of Genetics & Biochemistry
Clemson University
111 Jordan Hall
Clemson, SC 29634
USA

e-mail: ckole@clemson.edu

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Preface to the Series

The deciphering of the sequence of a gene for the first time, the gene for bacteriophage MS2 coat protein to be specific, by Walter Fiers and his coworkers in 1972 marked the beginning of a new era in genetics, popularly known as the genomics era. This was followed by the complete nucleotide sequence of the same bacteriophage in 1976 by the same group; DNA sequencing of another bacteriophage (Φ -X174) in 1977 by Fred Sanger, Walter Gilbert, and Allan Maxam, working independently; and first use of any DNA marker in gene mapping in 1980 for the human system by David Botstein. These landmark discoveries were immediately embraced by the life science community and were followed by an array of elegant experiments leading to the development of several novel concepts, tools, and strategies for elucidation of genes and genomes of living organisms of academic and economic interests to mankind.

The last two decades of the twentieth century witnessed the invention of the polymerase chain reaction; several types of molecular markers; techniques of cloning large DNA segments in artificial chromosomes; approaches to isolate and characterize genes; and tools for high-throughput sequencing, to name just a few. Another noteworthy development had been the formulation of different computer software to analyze the huge amount of data generated by genome mapping experiments, and above all deployment of information technology to store, search, and utilize enormous amounts of data particularly of cloned genes, transcripts, ESTs, proteins, and metabolites. This sweet and swift marriage of biology and information technology gave birth to bioinformatics and the new “omics” disciplines such as genomics, transcriptomics, proteomics, and metabolomics.

The tide of genome mapping and genomics flooded all phyla of the animal kingdom and all taxa of the plant kingdom and most obviously the prokaryotes. In the animal systems, we already had the gene sequence for the CFTR protein in humans in 1989; genome sequence of the model organism *Caenorhabditis elegans* in 1998; genetic maps of many higher animals with map positions of genes and gene-clusters during the nineties. We also happily witnessed the beginning of genome sequencing projects of three domestic animals (cow, dog, and horse) and poultry in 1993. All these achievements and endeavors culminated in the whole-genome sequence of the fruit-fly *Drosophila*, the garden pea of the animal system, in 2000 declaring a successful and pleasant ending of the genome science efforts of the twentieth century. The new millennium in 2001 started with the publication of the draft sequence of the human genome on February 15th by The International Human Genome Mapping Consortium and on February 16th by The Celera Genomics Sequencing Team.

A flurry of new concepts and tools in the first few years of the first decade of the twenty-first century has enriched the subject of genomics and the field has broadened to include the young and fast-growing disciplines of structural genomics, functional genomics, comparative genomics, evolutionary genomics, and neutraceutical genomics, to name just a few. We now have more, faster, cheaper, and cleverer mapping and sequencing strategies, association mapping and the

454 for example; several tools, such as microarrays and cDNA-AFLP to isolate hundreds of known and unknown genes within a short period, elegantly assisted by transcript-profiling and metabolic-profiling; identifying new genes from the knowledge-base of homologous genomes; and precise depiction of the road map of evolution of human and other members of the animal kingdom and their phylogenetic relationships with members of other species or genera. Within less than a decade of the deciphering of the first complete genome sequence for an animal species in 1998, we have complete sequences of some seventeen species of the animal kingdom including nematodes (2), arthropods (4), domestic animals and poultry (2), marsupial (1), wild animals (2), aquatic animals (4), human (1), and non-human primate (1). Many more genome mapping projects are progressing rapidly and their results are expected to be published soon.

The list of achievements in the fields of genome mapping and genomics in human and other members of the animal kingdom is enormous. It is also true that in today's world, in the global village of the new millennium, we have access to almost all information regarding the initiation, progress, and completion of all endeavors of animal genome sciences and can enrich our knowledge of the concepts, strategies, tools, and outcomes of the efforts being made in animal genome mapping and genomics. However, all this information is dispersed over the pages of periodicals, reviews on particular types of animals or their specific groups in hard copy versions, and also in electronic sources at innumerable links of web pages for research articles, reports, and databases. But we believe that there should be a single compilation, in both hard copy and electronic versions, embodying the information on the work already done and to be done in the fields of genome mapping and genomics of all members of the animal kingdom that are of diverse interests to mankind: academic, health, company, or environment.

We, therefore, planned for this series on Genome Mapping and Genomics in Animals with five book volumes dedicated to Arthropods; Fishes and Aquatic Animals; Domestic Animals; Laboratory Animals; and Human and Non-Human Primates. We have included chapters on the species for which substantial results have been obtained so far. Genomes of many of these species have been sequenced or are awaiting completion of sequencing soon. Overview on the contents of these volumes will be presented in the prefaces of the individual volumes.

It is an amazingly interesting and perplexing truth that only four nucleotides producing only twenty amino acids in their triplet combination could create anywhere between five to thirty million species of living organisms on the earth. An estimated number of about a half million vertebrate animal species have been described so far! Genomes of the few animal species from this enormous list that we know today are also too diverse to elucidate. It is therefore daring to edit a series on depiction of the diverse genomes we are presenting in over sixty chapters in the five volumes. Seven globally celebrated scientists with knowledge and expertise on different groups of animal systems, and human and non-human primates provided me with the inspiration and encouragement to undertake the job of the series editor. Noelle (Noelle E. Cockett), Paul (Paul Denny), Wayne (Wayne B. Hunter), Tom (Thomas D. Kocher), Ravi (Ravindranath Duggirala), Tony (Anthony G. Comuzzie), and Sarah (Sarah Williams-Blangero) were always available for consultations and clarifications on any aspect while editing the manuscripts of this series. While working on this series, I have been a student first, a scientist second, and an editor third and last, with the mission to present a comprehensive

compilation of animal genome mapping and genomics to the students, scientists, and industries currently involved and to be involved in the study and practice of animal genome sciences. I express my thanks and gratitude as a humble science worker to these seven volume editors for giving me an opportunity to have an enriching and pleasant view of the wide canvas of animal genome mapping and genomics. I also extend my thanks and gratitude to all the scientists who have generously collaborated with their elegant and lucid reviews on the rationale, concepts, methodologies, achievements, and future prospects of the particular systems they are working on, and for the subtle touches of their own experiences and philosophies.

As expected, the editing jobs of this series comprised communication with the volume editors, authors, and publishers; maintenance of the files in hard and soft copies; regular internet searches for verification of facts and databases; and above all maintenance of an environment to practice and enjoy science. My wife Phullara, our son Sourav, and our daughter Devleena were always with me on my travels as a small science worker on a long road of "miles to go before I sleep," not only for the successful completion of this series but also in all my efforts for teaching, research, and extension wherever I worked and stayed in my life.

We have already completed a seven-volume series on Genome Mapping and Molecular Breeding in Plants with Springer that has been very popular among students, scientists, and industries. We are also working on a series on Genome Mapping and Genomics in Microbes with Springer. It was, is, and will be enriching and entertaining to work with the experienced and wonderful people involved in the production of this series, including Sabine (Dr. S. Schwarz), Jutta (Dr. J. Lindenborn), and Cornelia (Mrs. C. Gründer), among many from the Springer family. I record my thanks and gratitude to them, here (and also submit in the databanks for future retrieval) for all their timely co-operation and advice when publishing these volumes.

I trust and believe that we must have missed deliberations on many significant animal species and left many mistakes on the pages of these volumes. All these lapses are surely mine, and all the credits must go to the volume editors, the authors, and the publisher. In the future these errors will be rectified on receipt of suggestions from the readers, and also there will be further improvement of the contents and general set-up of the volumes of this series.

Clemson
January 10, 2008

Chittaranjan Kole

Preface to the Volume

Efforts to bring modern genetic tools to the study of aquaculture species began in earnest with an international workshop in Dartmouth, Massachusetts, in 1997. In the years that followed, researchers, many newly recruited to the field of aquaculture, developed genetic maps of DNA polymorphisms, contributed thousands of expressed sequence tags to the public databases, and began to construct physical maps by fingerprinting large insert clone libraries. This work largely followed similar work in livestock species like cows, pigs, and chickens and has provided a solid foundation for future research.

Ten years ago, the idea that the genomes of these aquaculture species might be completely sequenced seemed hopelessly optimistic. The cost of sequencing was high, and funding priorities favored traditional biomedical models. Since then, costs have continued to drop, and several aquaculture species have been recognized as useful biomedical models. It is encouraging that the tilapia genome is scheduled for sequencing at the Broad Institute in 2008. We expect that many more aquaculture species will be sequenced over the next few years.

This volume summarizes the first era of genomic studies of aquaculture species, in which the tools and resources necessary to support whole-genome sequencing were developed. We now look forward to the application of a new generation of sequencing and genotyping technologies that will transform the selective breeding of these species. We hope that improved strains of these aquaculture species, with higher growth rates and improved resistance to disease, will contribute to a “blue revolution” that will help feed an overcrowded planet.

The eight chapters included in this volume have been contributed by 30 scientists from 13 countries. We express our thanks and gratitude to them for their cooperation and patience in bringing this project to a successful conclusion.

College Park
Clemson
January 10, 2008

Thomas D. Kocher
Chittaranjan Kole

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Contributors

- Cristian Araneda
Departamento de Producción Animal
Facultad de Ciencias Agronómicas
Universidad de Chile
P.O.B 1004, Santiago, Chile
craraned@uchile.cl
- C. Batargias
Nireus Chios Aquaculture S.A.
Kardamila, GR-82 300 Chios
Greece
and
Present address:
Faculty of Agricultural Technology
Department of Aquaculture
and Fisheries
Technological Education Institute
of Messolonghi, Nea Ktiria
GR-30 200 Messolonghi
Greece
- Robert A. Bullis
Advanced BioNutrition Corporation
7155 Columbia Gateway Drive
Columbia, MD 1046, USA
- A. Canario
Centro de Ciencias do Mar
Universidade do Algarve
Campus de Gambelas, P-8005-139 Faro
Portugal
- D. Chatziplis
Animal Genetics and Breeding
Department of Animal Production
Faculty of Agriculture
Aristotle University of Thessaloniki
GR-54 006 Thessaloniki, Greece
and
Present address:
Animal Breeding and Genetics
Department of Animal Production
School of Agricultural Technology
- Alexander Technological Educational
Institute of Thessaloniki
P.O. Box 141, GR-57 400 Sindos
Thessaloniki, Greece
- D. Chistiakov
Department of Molecular Diagnostics
National Research Center
GosNIIgenetika
1st Dorozhny Proezd 1
113545 Moscow, Russia
- Avner Cnaani
Department of Poultry and Aquaculture Sciences
Institute of Animal Sciences
Agricultural research Organization
Volcani Center
Bet Dagan 50250, Israel
avnerc@volcani.agri.gov.il
- M.R.M. Coimbra
Faculty of Marine Science
Tokyo University of Marine Science
and Technology
Konan 4, Minato, Tokyo 108-8477
Japan
and
Departamento de Pesca e Aquicultura
Universidade Federal Rural
de Pernambuco
Dois Irmãos, 52171-900 Recife-PE
Brazil
- Arun K. Dhar
Advanced BioNutrition Corporation
7155 Columbia Gateway Drive
Columbia, MD 21046, USA
adhar@abn-corp.com
- K. Fuji
Faculty of Marine Science
Tokyo University of Marine Science

and Technology
Konan 4, Minato, Tokyo 108-8477
Japan

Ximing Guo
Haskin Shellfish Research Laboratory
Institute of Marine
and Coastal Sciences
Rutgers University
6959 Miller Avenue, Port Norris,
NJ 08349, USA
xguo@hsrl.rutgers.edu

C. Haley
Roslin Institute
Division of Genetics and Genomics
Roslin, Midlothian EH25 9PS, UK

Gideon Hulata
Department of Poultry
and Aquaculture Sciences
Institute of Animal Science
Agricultural Research Organization
Volcani Center, Bet Dagan 50250
Israel
vlaqua@volcani.agri.gov.il

Patricia Iturra
Programa de Genética Humana
Instituto de Ciencias Biomédicas
Facultad de Medicina
Universidad de Chile
P.O.B 70061-7, Santiago, Chile

K. Kobayashi
Faculty of Marine Science
Tokyo University of Marine Science
and Technology
Konan 4, Minato, Tokyo 108-8477
Japan

Natalia Lam
Programa de Genética Humana
Instituto de Ciencias Biomédicas
Facultad de Medicina
Universidad de Chile
P.O.B 70061-7, Santiago, Chile

Jeong-Ho Lee
Haskin Shellfish Research Laboratory
Institute of Marine
and Coastal Sciences
Rutgers University
6959 Miller Avenue, Port Norris,
NJ 08349, USA

A. Libertini
CNR-ISMAR
Istituto di Scienze Marine –
Biologia del Mare
Riva 7 Martiri, 1364/A
I-30122 Venezia, Italy

Zhanjiang Liu
The Fish Molecular Genetics
and Biotechnology Laboratory
Department of Fisheries
and Allied Aquacultures
and Program of Cell and Molecular
Biosciences, Aquatic Genomics Unit
Auburn University
Auburn, AL 36849, USA
zliu@acesag.auburn.edu

Roberto Neira
Departamento de Producción Animal
Facultad de Ciencias Agronómicas
Universidad de Chile
P.O.B 1004, Santiago, Chile

N. Okamoto
Faculty of Marine Science
Tokyo University of Marine Science
and Technology,
Konan 4, Minato, Tokyo 108-8477
Japan
nokamoto@kaiyodai.ac.jp

Laszlo Orban
Reproductive Genomics Group
Temasek Life Sciences Laboratory
Singapore
laszlo@tll.org.sg
and
Department of Biological Sciences
National University of Singapore
Singapore

Betsy Read College of Arts and Sciences California State University San Marcos, CA 92096, USA	F.A.M. Volckaert Laboratory of Animal Diversity and Systematics Katholieke Universiteit Leuven Ch. Deberiotstraat 32 B-3000 Leuven, Belgium filip.volckaert@bio.kuleuven.be
C. Castaño Sánchez Faculty of Marine Science Tokyo University of Marine Science and Technology Konan 4, Minato, Tokyo 108-8477 Japan	Lingling Wang Haskin Shellfish Research Laboratory Institute of Marine and Coastal Sciences Rutgers University 6959 Miller Avenue, Port Norris, NJ 08349, USA
T. Sakamoto Faculty of Marine Science Tokyo University of Marine Science and Technology Konan 4, Minato, Tokyo 108-8477 Japan	Yongping Wang Haskin Shellfish Research Laboratory Institute of Marine and Coastal Sciences Rutgers University 6959 Miller Avenue, Port Norris, NJ 08349, USA
C. Tsigenopoulos Hellenic Centre of Marine Research Institute of Marine Biology and Genetics Department of Genetics and Molecular Biotechnology P.O. Box 2214, Gournes Pediados GR-71 500 Heraklion, Crete Greece	Qingjiang Wu Institute of Hydrobiology Chinese Academy of Sciences Wuhan People's Republic of China

Abbreviations

AFLP	Amplified fragment length polymorphism
AFR	Anal fin ray counts
AIMS	Australian Institute of Marine Science
ARL	Arlee National Fish Hatchery, Montana, USA
ARS	Agricultural Research Service
BAC	Bacterial artificial chromosome
BC	Backcross
BES	BAC-end sequencing
BLUP	Best linear unbiased predictor
BM	Body mass
bp	Base pair
CATS	Comparative anchor-tagged sequences
CCD	Charge-coupled device
cDNA	Complementary DNA
cGRASP	Consortium for Genomics Research on All Salmonids Project
CIM	Composite interval mapping
cM	Centimorgan
CV	haploid C-value; the measure of genome size
CW	Dworshak National Fish Hatchery on the Clearwater River, Idaho, USA
<i>cyt b</i>	Cytochrome b (gene)
DH	Doubled haploid
D-loop	Mitochondrial control region
EDR	Embryonic development rate
EL	Embryonic length
ESC	Enteric septicemia of catfish
eSNPs	Expressed single nucleotide polymorphisms
EST	Expressed sequence tag
ETL	Economic trait loci
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed conversion ratio
FISH	Fluorescence in situ hybridization
FL	Fork length
fluoMEP	Fluorescent motif enhanced polymorphism
FPC	Fingerprinted contig
GAS	Gene-assisted selection
Gb	Gigabase (one billion base pairs)
GCHV	Grass carp hemorrhagic virus
HC	Hot Creek Hatchery, California, USA
IHHNV	Infectious hypodermal and hematopoietic necrosis virus
IHN	Infectious hematopoietic necrosis disease
IHNR	Infectious hematopoietic necrosis resistance
IHNV	Infectious hematopoietic necrosis virus
Indel	Insertion/deletion polymorphism

IPN	Infectious pancreatic necrosis disease
IPNR	Infectious pancreatic necrosis resistance
IPNV	Infectious pancreatic necrosis virus
ISA	Infectious salmon anemia disease
ISSR	Inter-simple sequence repeat
IUPAC	International Union of Pure and Applied Chemistry
JOD	Juvenile oyster disease
K	Fulton's condition factor
kb	Kilobase (one thousand base pairs)
LDV	Lymphocystis disease virus
LG	Linkage group
LOD	Logarithm of the odds
MAS	Marker-assisted selection
Mb	Megabase (one million base pairs)
MHC	Major histocompatibility complex
MIM	Multiple interval mapping
mRNA	Messenger ribonucleic acid
MS	Microsatellites
MSX	M multinucleated sphere X
MT	Metric tons
mtDNA	Mitochondrial DNA
mya	Million years ago
<i>n</i>	Haploid chromosome number
N50	Defines the size of contig "A", which splits the whole size range of contigs in such a way that half of the total contig size is contained in contigs larger than A
NAGRP	National Animal Genome Research Program
NCBI	National Center for Biotechnology Information
NCC	Nonspecific cytotoxic cell
NCCCWA	National Center for Cool and Cold Water Aquaculture
NF	Number of fundamental chromosome arms
NK	Natural killer cell
NOR	Nucleolar organizer region
NRSP-8	National Research Support Project-8
NTS	Non-transcribed spacers
ORF	Open reading frame
OSU	Oregon State University, USA
PAC	P1 artificial chromosome
PB2	Polar body 2
PCN	Pyloric caeca number
PCR	Polymerase chain reaction
PCR-RFLP	Restriction fragment length polymorphism, applied to fragments amplified by polymerase chain reaction
PEG	Polyethylene glycol
pg	Picogram
PINE	Paired interspersed nuclear elements
PRINS	Primed in situ labeling
QTL	Quantitative trait locus
QX	Queensland unknown

<i>rag 2</i>	Nuclear recombination activating gene 2
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RH	Whole-genome radiation hybrid
SALL	Scales above lateral line
SCAR	Sequence characterized amplified region
SINE	Short interspersed elements
SLP	Specifically listed pathogen
SNP	Single nucleotide polymorphism
SP	Spawning date
SPF	Specific pathogen free
SPR	Specific pathogen resistant
SSCP	Single-strand conformation polymorphism
SSH	Suppression subtractive hybridization
SSR	Simple sequence repeat (microsatellite)
STC	Sequence-tagged connector
TDT	Transmission disequilibrium test
TSV	Taura syndrome virus
US	United States
USDA	United States Department of Agriculture
USMSFP	US Marine Shrimp Farming Program
UTT	Upper temperature tolerance
VN	Vertebrate number
VNTR	Variable number tandem repeats
WGS	Whole genome shotgun (sequencing)
WSSV	White spot syndrome virus
YAC	Yeast artificial chromosome
YHV	Yellowhead virus
ZFIN	Zebrafish Information Network
ZMAP	Integrated map of the zebrafish genome
Zv6	Zebrafish genome assembly (version 6)

1 Salmonids

Cristian Araneda¹, Roberto Neira¹, Natalia Lam², and Patricia Iturra²

¹ Departamento de Producción Animal, Facultad de Ciencias Agronómicas, Universidad de Chile, P.O.B 1004, Santiago, Chile
e-mail: craraned@uchile.cl

² Programa de Genética Humana, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, P.O.B 70061-7, Santiago, Chile

1.1 Introduction

The modern salmon industry has had an explosive growth rate since the 1980s (Dunham et al. 2001), especially in Norway and the United Kingdom. Salmon and trout production requires cold waters with high oxygen content and low levels of pollutants, with a preference for protected coastal sectors far from big urban centers. So, even though cultured salmonid species originated in the Northern Hemisphere, close to 40% of the salmon produced in captivity today comes from the Southern Hemisphere, where they have been successfully introduced and cultivated.

1.2 History of Salmon Culture

1.2.1 Early History of Salmon Culture

The first attempts to artificially reproduce these species began in the middle of the fourteenth century in France. The French monk Dom Pinchon incubated trout eggs that he collected from the rivers where the fish bred. However, most authors attribute the development of artificial fertilization of trout and salmon eggs to Prussian Stephan Ludwing Jacobi (1711–1784) who published his experiences in 1763 in the “Hannoverschem magazinn” (Huet 1972). These findings were used in 1842 by Remy and Gehin in Vosges, France. They fertilized rainbow trout eggs

and developed fry and fingerling production in ponds to restock streams in the Moselle River basin. In 1848, the French Academy sent a scientific commission to corroborate the findings, and eventually professor Coste, a specialist in embryology, obtained support of the French government for the construction of a hatchery in Huningue (Alsace) in 1851 (Blanco 1995). In the United States, the first hatchery was inaugurated in Maine in 1871. A second US hatchery was constructed in 1872 on the McCloud River, a tributary of the Sacramento River in California. The first artificial incubation of salmonids in Japan began with a lot of 17,000 eggs obtained from the Nakagawa River in 1876. Experiments in artificial incubation and release into different rivers of the main Japanese island, Honshu, continued until 1888 but without great success.

France, Germany, England, the United States, and Japan led the efforts to establish artificial reproduction of salmonids. In addition, these countries made great efforts to transplant salmonids to other places and latitudes. First was England, which distributed salmonids to its colonies in New Zealand and Australia. Although salmonids did not naturally exist in the Southern Hemisphere, the English government, with the support of the United States, sent eggs of Pacific salmon to be released into the southern English possessions. Their persistence finally resulted in the introduction of salmons in a wild state in New Zealand, now recognized as the first successful introduction of salmon in the Southern Hemisphere. Beginning in 1870, and continuing for more than 60 years, the United States led efforts to introduce eggs of Pacific salmon to different countries in Europe and the Southern Hemisphere, including Chile and New

Zealand. These eggs were obtained from the McCloud River hatchery.

1.2.2

From 1890 to 1975

In 1890, Danish trout farmers began the development of trout culture in a system of earthen ponds, with freshwater flux through each fishpond. This system radically improved fish yield and reduced disease. This breakthrough led to the beginning of the commercial trout farming industry. Norway tried to implement the Danish system, but was not successful, due to the low temperatures of freshwaters in Norwegian winters. The seas around Norway are warmed by the influence of the Gulf Stream, which is an advantage for faster fish growth (Sedgwick 1988). In 1912, the Norwegians made the first attempts to cultivate rainbow trout in the sea. But it was not until the middle of the 1950s that the culture of salmon and rainbow trout in the sea began to grow. The industry became profitable and reached a production level near 500 MT in 1965 and 2,200 MT in 1974. The Norwegian system using floating cages to culture salmonids in sea water was adopted around the world (Willoughby 1999).

1.2.3

From 1975 to the Present Time

With the decline of capture fisheries for wild salmon and trout in the Northern Hemisphere, aquaculture of salmonid species became increasingly important around the world. The development of culture systems that reproduced the complete life cycle in captivity, and the incorporation of artificial dry pellet diets in 1964 (Halver 1972), allowed the culture of salmonid species to become industrialized. Chile and Norway now produce 76% of the world's aquaculture salmon and trout. Other relevant producers are the UK, Canada, Turkey, Denmark and the US (Table 1). From 1980 to 1991, world production of farmed salmon grew from 7,149 MT to almost 325,563 MT, an increase of 4,600% (FAO 2005). At present, the world salmon and trout aquaculture production is more than two million MT per year, having tripled with respect to production at the beginning of the 1980s (Fig. 1). Farmed salmon, which is recognized in the market for its homogenous quality and constant supply, represents more than two

Table 1 Main salmonid producing countries in the world
(Source: FAO global databases, Aquaculture production 2005a)

Country	Production 2005 (Metric Tons)
Norway	641,174
Chile	598,251
United Kingdom	142,613
Canada	103,164
Turkey	49,282
Denmark	37,001
United States of America	36,905
France	35,001
Iran (Islamic Rep. of)	34,760
Italy	30,564
Spain	26,132
Japan	24,461
Faeroe Islands	23,455
Germany	19,343
Australia	16,317
Poland	15,700
Ireland	15,378
China	14,507
Finland	13,713
Russian Federation	8,800
Other	64,057
Total World Production	1,886,521

thirds of the total. Atlantic salmon (*Salmo salar*) is the most important farmed species, followed by rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*) (Fig. 2).

1.3

Taxonomic Status and Distribution

Salmonids have a Holarctic distribution throughout Eurasia and North America (Scott and Crossman 1973) and are probably of freshwater origin, based on the evidence that all salmonids spawn in fresh water. Tchernavin (1939) suggested that ancestral salmonids were "small brightly colored fishes living in cool streams and lakes of the northern hemisphere. Using fresh water routes, they spread over a wide area. The environmental diversity of regions over which they spread favored the formation of numerous species" (Neave 1958). This suggests that a trout-like fish was ancestral to a salmon. Pacific salmon prob-

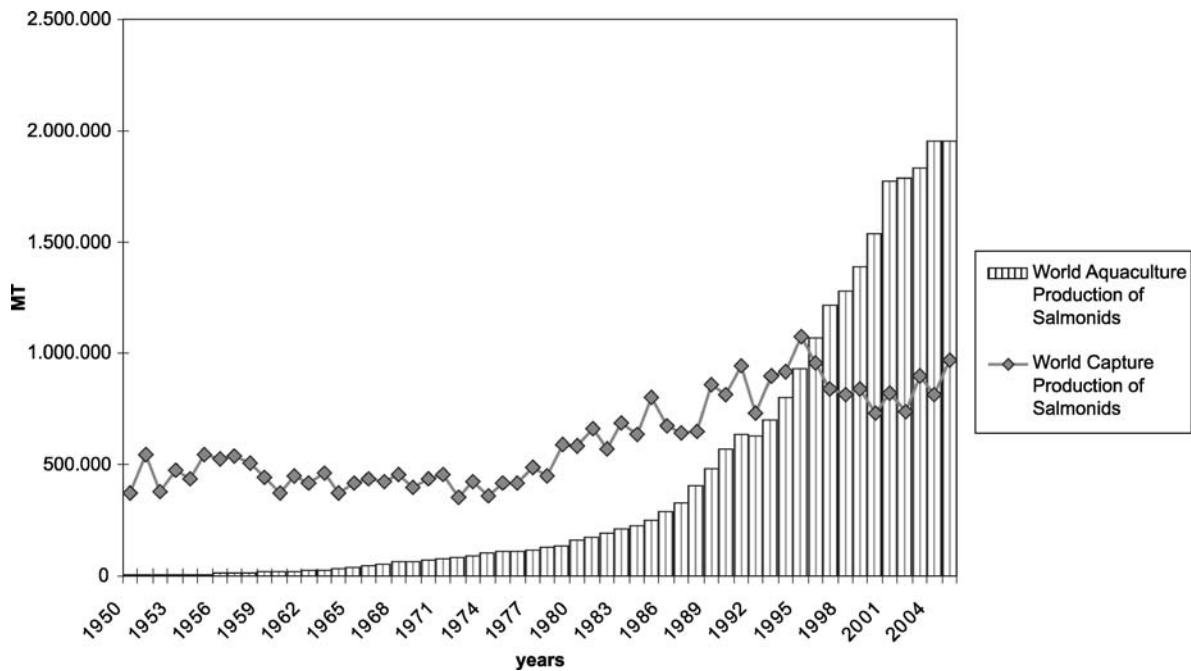


Fig. 1 A comparison of the world capture production and world aquaculture production of salmonids. (Source: FAO global databases 2005a, 2005b; Aquaculture production: Quantities 1950–2005 and Capture production 1950–2005)

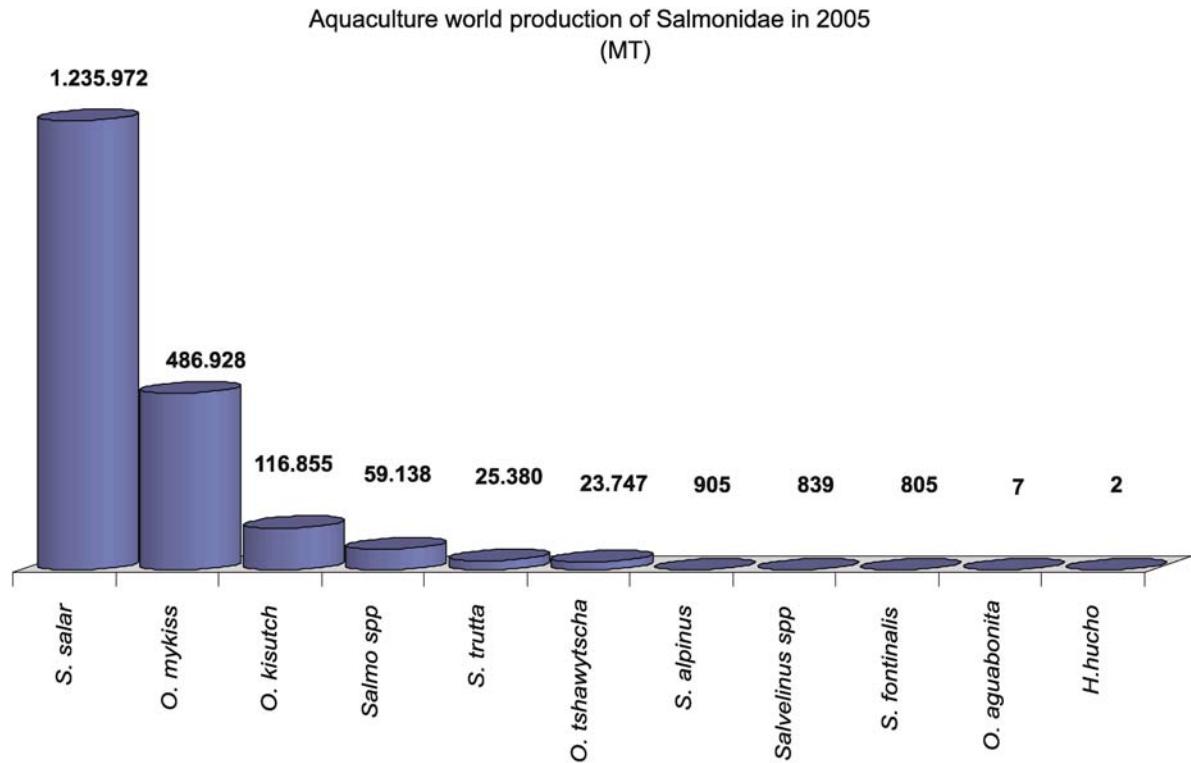


Fig. 2 The main species of farmed salmon and trout in the world in metric tons (MT). (Source: FAO global databases: Aquaculture production 2005a)

ably descend from a *Salmo* ancestor (Neave 1958). It is thought that separate Atlantic and Pacific salmon species arose after the closure of the Arctic link between the North Atlantic and the North Pacific oceans, around one million of years ago.

There are three subfamilies of salmonid fishes: *Coregoninae* (whitefishes and ciscoes), *Thymallinae* (graylings), and *Salmoninae* (lenoks, huchen, trouts, charrs, and salmons). The taxonomic classification of salmon is shown below:

Phylum: Chordata

Subphylum: Vertebrata

Superclass: Osteichthyes

Class: Actinopterygii

Order: Salmoniformes

Suborder: Salmonoidea

Family: Salmonidae

Subfamily: Coregoninae

Subfamily: Thymallinae

Subfamily: Salmoninae

Phylogenetic relationships among salmonid species are complex and sometimes difficult to resolve. Phillips and Oakley (1997) suggest the following reasons: (1) these fishes underwent a rapid adaptive radiation following tetraploidization around 50–100 million years ago (Allendorf and Thorgaard 1984), (2) hybridization and introgression have been common in this group (Utter and Allendorf 1994), and (3) recolonization of lakes released from glaciations within the past 10,000 years has resulted in assemblages of sympatric morphotypes or ecotypes with different degrees of reproductive isolation in Northern Hemisphere lakes.

The subfamily *Salmoninae* includes six genera of trout, salmon, and charr: *Brachymystax*, *Hucho*, *Salmothymus*, *Salvelinus*, *Salmo*, and *Oncorhynchus*. In this section, we describe the most important characteristics of cultivated salmonids in their natural environment.

1.3.1

Genus *Salmo*

This group doesn't necessarily die after spawning, and therefore repeated spawnings can be obtained from the same broodstock over several years. Good care of farmed broodstock can lead to high survival rates.

Atlantic Salmon (*S. salar*)

Natural Range: Atlantic salmon are found from north of the Hudson River to southern Greenland and Iceland on the western side of the Atlantic. On the eastern Atlantic, they are found from northern Portugal to the Kara Sea in Russia, including the Baltic Sea. **Life Characteristics:** Atlantic salmon are migrant, returning to freshwater during the 12 months preceding spawning, usually between October and December in the Northern Hemisphere. The female excavates a nest in silt-free gravel, usually in a tributary close to the salt water. On hatching, fry remain in the gravel using their remaining yolk sac. Fingerlings are carnivorous. After one or two years as freshwater “parr,” the surviving fish undergo physiological and behavioral changes (smoltification) and migrate to begin the seawater phase of their life cycle. Salmon may return to their native rivers, after spending one winter and a few months at sea, but most return to freshwater after two or more winters at sea. Female salmon usually weigh between 3 and 6 kg at maturity and can produce around 1,200 and 2,000 eggs/kg of body weight.

Brown Trout (*S. trutta*)

Natural Range: Brown trout originate in the mountain waters of Central and Western Europe. **Life Characteristics:** The brown trout is a very polymorphic fish, with several subspecies that constitute simple geographic races or populations of different behavior. There are subspecies of brown trout that remain in rivers (*Salmo trutta fario*) and one less-abundant subspecies (*Salmo trutta trutta*) that migrates to the sea. Brown trout are a stenothermal coldwater fish that need freshwater and do not withstand high temperature variations. They spawn in the autumn or at the beginning of winter, leaving their normal habitat (seas, lakes, streams, or brooks) to swim upstream and spawn on gravel beds. They are an important sport fish around the world.

1.3.2

Genus *Oncorhynchus*

The main characteristic of this genus is that they generally die after spawning.

Pink Salmon (*O. gorbuscha*)

Common Names: pink, humpback (USA and Canada), karafutomaru (Japan), gorbuscha (Russia). **Native Range:** Pink salmon live in large rivers in North America and Asia. **Life Characteristics:** This is the most common Pacific salmon and also the most cold tolerant. Females produce around 2,000 relatively small eggs. Seaward migration takes place a few days after hatching. The young fish may spend 3–5 months in estuaries and coastal waters. They return to home rivers after 16–20 months, averaging 1.5 kg.

Chum Salmon (*O. keta*)

Common Names: chum, dog (USA and Canada), sake (Japan), and keta (Russia). **Native Range:** This species has a wide geographical distribution, from Oregon to the Arctic coast of Alaska in the eastern Pacific, and from Japan to the Arctic coast of Siberia in the western Pacific. **Life Characteristics:** Chum salmon are the second-most abundant Pacific salmon and also very cold tolerant. Spawning usually takes place in the lower reaches of rivers, within 150 km of sea. Eggs are laid between October and July on the Northern Hemisphere, depending on the latitude of the river. Females lay around 3,000 eggs. Fry migrate to sea within 1–3 months after emerging from the gravel. They spend up to 4 years in sea and may complete two or three ocean circuits.

Sockeye Salmon (*O. nerka*)

Common Names: sockeye (Canada and USA), red (Alaska), blueback (Columbia River), Benimasu (Japan), nerka (Russia). **Native Range:** Sockeye salmon are found from the Klamath River in California to the Yukon River in Alaska. In the western Pacific they range from the northern Bering Sea to the northern shore of the Okhosk Sea (Russia). **Life Characteristics:** The average spawning weight is 3.5 kg, and the female lays 3,500–4,000 small eggs. Fry can migrate to the sea, but usually migrate first to freshwater lakes where they grow slowly, feeding on planktonic crustaceans for 1–3 years. They eventually spend about 3 years at sea before returning to spawn.

Coho Salmon (*O. kisutch*)

Common Names: coho (Canada and Alaska), blueback (Canada), silver (USA), ginmaru (Japan),

kizhuch (Russia). **Native Range:** This species is found in coastal streams from California to Norton Sound in Alaska, and from northern Hokkaido (Japan) to the Anadyr River (Russia). **Life Characteristics:** Similar to that of Atlantic salmon. In the wild, coho salmon stay in freshwater for 1–2 years. Most coho salmon spend 2 years at sea and grow fast during the second year. Spawning takes place in the autumn or early winter. Females produce between 1,000 and 2,000 eggs/kg of body weight.

Chinook Salmon (*O. tshawytscha*)

Common Names: king, (USA), spring (Canada), masunosuka (Japan), chavycha (Russia). **Native Range:** This species is found from the Ventura River in southern California to Point Hope in Alaska on the eastern rim and from Hokkaido to the Anadyr River (Russia) on the Asian side. **Life Characteristics:** This is the least abundant of all the Pacific salmon. Females lay about 3,000–12,000 eggs. Fingerlings migrate to sea after about 120 days and spend between 1 and 5 years in sea, mostly near shore or in inshore waters. The average weight at the end of marine life is about 10 kg.

Rainbow Trout (*O. mykiss*)

Common Names: steelhead (USA). **Native Range:** The natural range for rainbow trout extends from Alaska to Mexico and includes British Columbia, Washington, Oregon, California, Idaho, and Nevada. **Life Characteristics:** Trouts are found in cold, clear waters of creeks, rivers, and lakes (steelhead may be found in estuaries or oceans) with complex structures, such as riffles, submerged wood or boulders, and aquatic vegetation. Steelhead trout is the same species as rainbow trout, but steelhead is a migratory form, and rainbow is a landlocked form. During the course of artificial cultivation, this species has been widely cross-bred. Because of crossbreeding, there is an impressive variety of types or strains of *O. mykiss*, where the main difference is the spawning season. Most populations spawn between January and May, but some strains have been obtained that start spawning in December or earlier in the Northern Hemisphere, and even, there are twice annually spawning strains with two spawns in one year (spring and autumn).

1.3.3

Genus *Salvelinus*

Arctic Charr (*S. alpinus*)

Common Names: omble chevalier (France), seesaibling (German), salvelino (Spanish), goylets (Russia), and arupusuiwana (Japan). **Native Range:** This species lives in the cold lakes of the northern slopes of Europe, Asia, and North America. **Life Characteristics:** Charr bear resemblance to salmon, but with a longer and more colorful body. They can weigh more than 4 kg and spawn in the autumn in the same way as other European salmonids.

Brook Trout (*S. fontinalis*)

Native Range: The brook trout is native to eastern and central North America, especially the Great Lakes and upper Mississippi River basin. **Life Characteristics:** Spawning generally occurs in the months of October and November. Females can produce between 100 and 400 eggs, depending upon the size and age of the individual. Depending upon water temperatures, the eggs hatch after 2–3 months of development. Brook trout take 2–3 years to mature and usually do not live longer than 6 years.

1.3.4

Genus *Coregonus*

Lake Whitefish (*C. clupeaformis*)

Native Range: Lake whitefish are widely distributed in North American freshwaters from the Atlantic coast across Canada and the northern United States to British Columbia, the Yukon Territory, and Alaska. **Life Characteristics:** This species inhabits lakes and large rivers, including brackish water. Whitefish spawn in early winter, and fry hatch the following spring. Some ecotypes of lake whitefish can reach a size of more than 9 kg and an age of more than 25 years. Other species of this genus occur across the northern land mass of North America, Asia, and Europe. Across this vast geographical range, this species exists in numerous forms or ecotypes, which has resulted in great nomenclatural confusion.

1.3.5

Genus *Hucho*

Danube Salmon (*H. hucho*)

Native Range: This is a continental salmon originally from the Danube basin. **Life Characteristics:** The Danube salmon live in fast-running water and migrate over short distances upstream at the spawning season. They can grow to more than 1 mt in length.

1.4

Genetic Improvement

Genetic improvement of aquaculture species did not begin until sophisticated varieties of *koi* carps were developed in Japan during the 1800s (Dunham et al. 2001). Fish improvement programs became more important in the 1900s with the rediscovery of Mendelian genetics and eventually grew into modern selection programs in the 1960s. Intensive genetic improvement programs began in 1970 coincident with the introduction of advanced methodologies and concepts of animal production. The continuing development of molecular genetics has led to the application of diverse biotechnologies and techniques to improve cultured fish stocks. Selection on quantitative characters of economic interest generally has produced much higher gains in aquatic species than those registered for domestic animals. For growth rate, for example, average genetic gains per generation of 15% for tilapia, of 14% for Atlantic salmon, 9–10% for coho salmon, and 14–20% for channel catfish have been reported (Bondari 1983; Gjerde 1986; Durham 1987; Hershberger et al. 1990; Gjerde and Korsvoll 1999; Rye and Eknath 1999; Neira et al. 2006a). These large gains can be explained by the enormous genetic variation and comparatively high fecundity of aquatic organisms, allowing high selection intensities (Gjedrem 1997). In contrast, for dairy cattle, the maximum expected genetic gain is about 2% per generation, with generation intervals of 6 years, while in pigs and birds, the genetic gains per generation can reach 2.5–4.5%. The generation interval for salmon in the Northern Hemisphere is about 3 years for Pacific salmon and rainbow trout and 4 years for Atlantic salmon. In Chile, a 2-year cycle has been achieved for coho salmon, while 3 and 4 years is the norm for rainbow trout and Atlantic

salmon, respectively. Shorter generation intervals are possible in Chile because of the availability of water with a higher temperature that allows the production of smolts within one year. This shorter generation interval is a significant advantage and translates into rates of growth gain of 4–5% per year in coho salmon.

1.4.1

Genetic Improvement Programs in Salmon Aquaculture

There are a limited number of experimental or commercial genetic improvement programs of aquatic species. In salmonids, there are results published on Atlantic salmon (Gjerde 1986; Gjerde and Korsvoll 1999), coho salmon (Hershberger et al. 1990; Gall and Neira 2004; Neira et al. 2006a), and rainbow trout (Kinkaid 1983; Gjerde 1986; Kause et al. 2003). Other breeding programs have been developed commercially in Scotland, Canada, Chile, the United States, the Netherlands, Iceland, Finland, Norway, and New Zealand.

Most of the established genetic improvement programs focus on salmonids. Data obtained from the First Fish Breeders Round Table meeting at the Akvaforsk Institute of Aquaculture Research, in Norway, indicated that there are ten genetic programs for Atlantic salmon (four in Norway, three in Chile, and one each in Canada, Ireland, Iceland, and Scotland), three for coho salmon (two in Chile and one in Canada), and five for rainbow trout (two in Norway and Chile and one in Finland) (T. Gjedrem unpublished).

Growth rate traditionally has been included as selection criteria in all salmonid programs, with very good results. Other characteristics of interest have been to improve carcass quality traits, such as flesh color, fat content, and texture of flesh in rainbow trout, Atlantic salmon, and coho salmon (Gjerde and Gjedrem 1984; Rye and Refstie 1995; Neira et al. 2004); early sexual maturity in Atlantic salmon (Gjerde 1986; Gjerde and Korsvoll 1999); skin color in rainbow trout (Kause et al. 2003); and early spawn date in coho salmon and rainbow trout (Siitonen and Gall 1989; Sadler et al. 1992; Su et al. 1999; Neira et al. 2006b). In general, there is great interest in the genetics of disease resistance, and promising results on infectious diseases such as furunculosis, infectious pancreatic

necrosis virus (IPNV), and parasitic infection have been obtained for Atlantic salmon and rainbow trout (Gjedrem 1979; Gjedrem et al. 1991; Okamoto et al. 1993; Kolstad et al. 2005).

Despite these successes, there is still a lot of opportunity to improve the efficiency of the methods in salmonid selection programs. Several of these programs are applying methodologies of genetic evaluation designed for terrestrial animals, such as the BLUP system (best linear unbiased predictor) for breeding value prediction (Gall et al. 1993; Gall and Bakar 2002; Neira et al. 2006a). Also, even when a series of DNA markers and specific genes associated with productive traits have been published, fish breeding program have not incorporated marker-assisted selection (MAS) or gene-assisted selection (GAS) in their routine genetic evaluation systems.

Limitations of traditional selection are evident when the trait under selection is hard to measure, the individuals need to be sacrificed to measure the trait, the trait is sex-limited, or the heritability is low. In all these cases, the use of molecular markers linked or associated with the trait and accounting for a significant proportion of the variation (~10% or more) are good candidates for MAS (Dekkers and Hospital 2002). The practical applicability of MAS or GAS involves an additional cost of genotyping at least 500 individuals every generation. Actually, the few molecular applications published in salmon breeding are limited to parental assignment or analysis (Norris et al. 2000) or to early sex identification using SSR (simple sequence repeat or microsatellite) markers in experimental populations (Reid et al. 2005).

1.5

Genetic Mapping in Salmonids

Genetic maps of salmonid species are still under development. The first blueprint of a salmonid map was published in 1990. It was a composite map for “all salmonid fishes,” based on combined recombination rates obtained from different species and studies, with a small number of 54 polymorphic allozyme loci marking 22 chromosomal arms (May and Johnson 1990). There are four published genetic maps for rainbow trout, three partial maps for Atlantic salmon,

one for Arctic charr, one for lake whitefish and one for brown trout. A preliminary linkage map for pink salmon (*Oncorhynchus gorbuscha*) was presented at a genome conference in San Diego, California (Linder et al. 1999; Spruell et al. 1999), but these data have not been formally published in a map. So, currently there is published map information for four genera of salmonid fishes (*Oncorhynchus*, *Salmo*, *Salvelinus*, and *Coregonus*), and two of these (*Oncorhynchus* and *Salmo*) are commercially important. In addition to these more complete maps, there are also partial maps of specific linkage groups developed as a byproduct of quantitative trait loci (QTL) mapping for different traits in different species, principally rainbow trout.

It is interesting to note that linkage mapping in salmonid fishes is more similar to mapping in plants than mapping in other vertebrates. For example, family sizes are large, like in plants, and therefore only a small number of families are necessary for mapping. In addition, endogamic strains, isogenics, and doubled haploid (DH) lines can be constructed for

mapping. As in plants, anonymous molecular markers, such as amplified fragment length polymorphism (AFLP, Vos et al. 1995), have been extensively used in fish (Moen et al. 2004a). In general, the standard nomenclature for AFLP markers is to use a code of six letters and a number representing the fragment size in base pairs, where the first three letters correspond to +3 nucleotides for the first restriction-enzyme-specific primer and the second three letters represent +3 nucleotides for the second restriction-enzyme-specific primer (Young et al. 1998). For SSR (simple sequence repeat) or microsatellite markers, a standard locus identification used in salmonid studies was proposed by Jackson et al. (1998). The designation begins with a three-letter acronym for the salmon species the SSR was isolated from (*Omy* = *O. mykiss*, *One* = *O. nerka*, *Ssa* = *S. salar*, *Str* = *S. trutta*, etc.) followed by a laboratory-specific designation for each marker and a suffix acronym describing the lab of the origin of the primers (Table 2). Duplicated loci amplified by one primer set are identified by an “/i” or “/ii”

Table 2 Source of the SSR primers used for mapping in salmonid fish*

Laboratory suffix acronym	Complete Name of Laboratory
ASC	Alaska Science Center, USA.
BFRO	Biotechnical Faculty, University of Ljubljana, Groblje Slovenia.
BML	Bodega Marine Laboratory, University of California, Davis USA.
CNRS	Centre National de la Recherche Scientifique, Chantal Poteaux France.
DIAS	Danish Institute for Animal Science, Tjele Denmark.
DU	Dalhousie University, Halifax, Nova Scotia Canada.
INRA	Institut National de la Recherche Agronomique, Jouy-en-Josas France.
LEE	National Fish Health Research Laboratory, Leetown, West Virginia USA.
Lav	Université Laval, Québec Canada.
OSL	Norwegian College of Veterinary Medicine, Oslo Norway.
NUIG	National University of Ireland, Galway Ireland.
NVH	National Veterinary Hospital, Norway.
OVIE	Universidad de Oviedo, Oviedo Spain.
SSBI	SeaStar Biotech Incorporated, Victoria, British Columbia Canada.
TUF	Tokyo University of Fisheries, Tokyo Japan.
UCH	Universidad de Chile, Santiago Chile.
UoG	University of Guelph, Ontario Canada.
UoS	University of Stirling, Scotland.
UoV	University of Victoria, British Columbia Canada.
UoP	University of Porto, Portugal.
UW	University of Washington, USA.

* Adapted from Sakamoto et al. (2000) and Gharbi (2001)

suffix within the marker name (Woram et al. 2004). In some cases, several SSR markers were published before this standard nomenclature was used, and for this, original names of the markers from the publications are adopted; for example, the designation SSOSL corresponds to a marker developed by Slettan et al. (1997) for Atlantic salmon.

1.5.1

First Generation Map

Rainbow Trout (*Oncorhynchus mykiss*)

The construction of genetic maps in salmonids is difficult due to a long generation interval in these fishes, normally 2–4 years depending on the species and culture conditions. However, in these fishes, it is possible to produce doubled haploid (DH) lines in a single generation using androgenesis (Pearson and Thorgaard 1985), and this approach was used to develop the first genetic map in rainbow trout. Today, this is the salmonid species with the best characterized genetic map and the most QTLs mapped (Table 6). The first genetic map was published by Young and colleagues in 1998. It included 332 AFLP markers and a few codominant markers (Table 3). Doubled haploid fishes were produced by the team of Gary Thorgaard at Washington State University (Young et al. 1996). The DH lines were produced from the F₁ hybrids of a cross between isogenic lines from Oregon State University (OSU) and Arlee (ARL) National Fish Hatchery (Montana, USA). Linkage groups identified in this hybrid used the acronym OA, due to the parental cross origin (OSU × ARL), and to distinguish them from linkage groups derived from crosses among other rainbow trout strains. AFLP markers were used instead of microsatellites because they took less time to develop and were cheaper to score. Two years later, the first map with predominantly codominant markers (191 SSR polymorphic loci; Sakamoto et al. 2000; Table 3) by the team of Roy Danzmann at the University of Guelph (Ontario, Canada) was published. The strategy used for mapping was to use the three backcross families initially used for QTL mapping for upper temperature tolerance (UTT) by Jakson et al. (1998). These families were obtained from crosses between fish with high and low tolerance to high temperature, from trout strains produced at Maple (Ontario, Canada) by the Ontario Ministry of Natural Resources. Finally, both groups produced a consolidated map with a length

of 4,590 cM (Fig. 3), principally combining markers used in previous studies (Nichols et al. 2003a). This updated map was developed using the DH rainbow trout OA and AFLP used by Young et al. (1996), adding codominant markers (SSR) from Sakamoto et al. (2000) and some allozymes from May and Johnson (1990). Other interesting loci anchored in this map are 29 type I polymorphisms detected using single-strand conformation polymorphism (SSCP), indel, restriction fragment length polymorphism (RFLP), or single nucleotide polymorphism (SNP) screening. These loci were mapped to 20 different linkage groups. The 33 genes and allozyme loci mapped are listed in Tables 4 and 5, and from this, *SOD*, *bGLUA** and *CBR1* were mapped to the sex chromosome of this species. The allozymic locus *bGLUA**, formerly *HEX*, had been previously mapped to the sex chromosome by Allendorf et al. (1994) in this same species.

A fourth map for rainbow trout has been published recently by a French team lead by René Guyomard. The primary objective was to cover the overall 52 chromosome arms from the base karyotype of this species (Guyomard et al. 2006). The mapping panel consisted of two DH families, and the map includes 901 SSR loci, with an important fraction (389 markers) developed from expressed sequence tag (EST) libraries, and two SNP markers (Table 3). Also in this work, 369 transcript EST sequences of rainbow trout together with blastn were used to identify 49 ESTs syntenic with ESTs from zebrafish (*Danio rerio*) in 20 different linkage groups of this model fish.

Atlantic Salmon (*Salmo salar*)

For this species, there were three draft maps published in 2004 by two different laboratories. One map, developed principally by a team from Scotland, is based on 64 codominant loci with 50 SSRs effectively anchored in 15 linkage groups (Table 3), with 11 SSRs and 3 allozyme loci remaining unlinked (Gilbey et al. 2004). The approach used for mapping in this study was a backcross design using two families obtained from a cross between an F₁ hybrid male and a female from River Don (Aberdeenshire, Scotland). The parents of the F₁ male were a non-anadromous male Atlantic salmon obtained from Bristol Cove River (Newfoundland, Canada) and a female salmon from River Don (Scotland). An interesting finding of this study was the identification in silico (using the BlastX algorithm) that flanking sequences of marker SS11 (GenBank AJ133370) anchored in linkage group 10 showed 91%

Table 3 Comparative information on salmonid genetics maps available until 2006

Reference	Map Size ¹ (cM)	Linkage Groups	Number and type of molecular markers anchored in the map	AFLP	VNTR ²	SINE	Allozymes	Genes ³	Other ⁴	SEX mapped to Linkage group ⁵
<i>Oncorhynchus mykiss</i>										
Young et al. 1988	2627.5	31	2	332	5	96	40			1
Sakamoto et al. 2000	1134	29	191		3					18
Nichols et al. 2003	4590	40	226	973	5	84	38	4	29	OA-I (18)
Guyomard et al. 2006	2750	31	901					2		RT-1
<i>Oncorhynchus gorbuscha</i>										
Linder et al. 1999	Not informed	55	28	393	35			164		Not mapped
<i>Salmo salar</i>										
Gilbey et al. 2004	630.5	15	50							Group 1
Moen et al. 2004	103–901	31–33	54	473						Not mapped
<i>Salmo trutta</i>										
Gharbi et al. 2006	346.4–912.5	35	288							BT28
<i>Salvelinus alpinus</i>										
Woram et al. 2004	390–992	46	184	129				5		Not mapped
<i>Coregonus clupeaformis</i>										
Rogers et al. 2001	1462	29		119						Not mapped

¹ When two values are reported, first value corresponds to male map and second value to female map.² Includes VNTR detected as multilocus by probe hybridization and minisatellites amplified by PCR.³ Genes identified using SNP, SSCP, or SSR located in its flanking regions or introns.⁴ Includes Paired Interspersed Nuclear Elements (PINEs) and Comparative Anchor Tagged Sequences (CATS).⁵ Name of the linkage group that contained SEX locus obtained from original references.

Table 4 Allozyme loci mapped in different salmonid genomes

Locus	Allozyme name	IUPAC Enzyme Commission	Location in Linkage Groups on map of Rainbow Trout ¹		Location in Linkage Groups on map of Brown Trout ²
<i>bGLUA*</i>	b-N-acetylhexosaminidase	3.2.1.52	OA-I		
<i>sSOD-1*</i>	Superoxide dismutase 1	1.15.1.1	OA-I		
<i>sMDH-B1,2*</i>	Malato dehydrogenase 1	1.1.1.37	OA-VI		BT-23
<i>sLDH-B2*</i>	L-lactate dehydrogenase 2	1.1.1.27	OA-XV		
<i>sIDHP-3*</i>	Isocitrate dehydrogenase 3	1.1.1.42	OA-XVI		
<i>mMEP-2*</i>	Malic enzyme	1.1.1.40	OA-XVI		
<i>PGK-2*</i>	Phosphoglycerate kinase	2.7.2.3	OA-XX		
<i>mIDHP-2*</i>	Isocitrate dehydrogenase 2	1.1.1.42	OA-XXVII		BT-31
<i>sG3PDH-1*</i>	Glyceraldehyde-3-phosphate dehydrogenase 1	1.1.1.8	OA-XXIX		BT-1
<i>sAAT-1,2*</i>	Aspartate aminotransferase	2.6.1.1			BT-1
<i>sMDH-A2*</i>	Malato dehydrogenase 2	1.1.1.37			BT-8
<i>sIDHP-1*</i>	Isocitrate dehydrogenase 1	1.1.1.42			BT-9
<i>PGDH*</i>	Phosphogluconate dehydrogenase	1.1.1.44			BT-28
<i>EST-1*</i>	Esterase 1	3.1.1.-			BT-7
<i>FH-1*</i>	Fumarate hydratase 1	4.2.1.2			BT-23
<i>FH-2*</i>	Fumarate hydratase 2	4.2.1.2			BT-35
<i>MPI-2*</i>	Mannose-6-phosphate isomerase	5.3.1.8			BT-7
<i>TF-1*</i>	Transferrine	Not enzymatic protein			BT-16

¹ Combined data from Sakamoto et al. (2000) and Nichols et al. (2003)

² Data from Gharbi (2001)

of sequence similarity with Aralar 1 protein (calcium-binding mitochondrial carrier protein) from several species, including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, and *Rattus norvegicus*.

A second team of Norwegian researchers developed two more complete sex-specific maps using both SSR and AFLP markers (Table 3). The female map included 33 linkage groups and 230 markers, while the male map included 31 linkage groups with 251 markers. There were 22 linkage groups in common between sexes (Moen et al. 2004a). These maps were developed using one parental half-sib family from an AquaGen commercial breeding population, class year 1999. This family was subdivided into two groups of 69 and 67 offspring, and not all markers were genotyped in all 136 offspring. This study detected the greatest difference of recombination frequencies between sexes reported for any vertebrate: 8.26 in favor of female. This difference was translated in a difference in map size between male and female, 103 cM vs. 901 cM, respectively.

An examination of Atlantic salmon maps shows some shared markers that could be used to develop

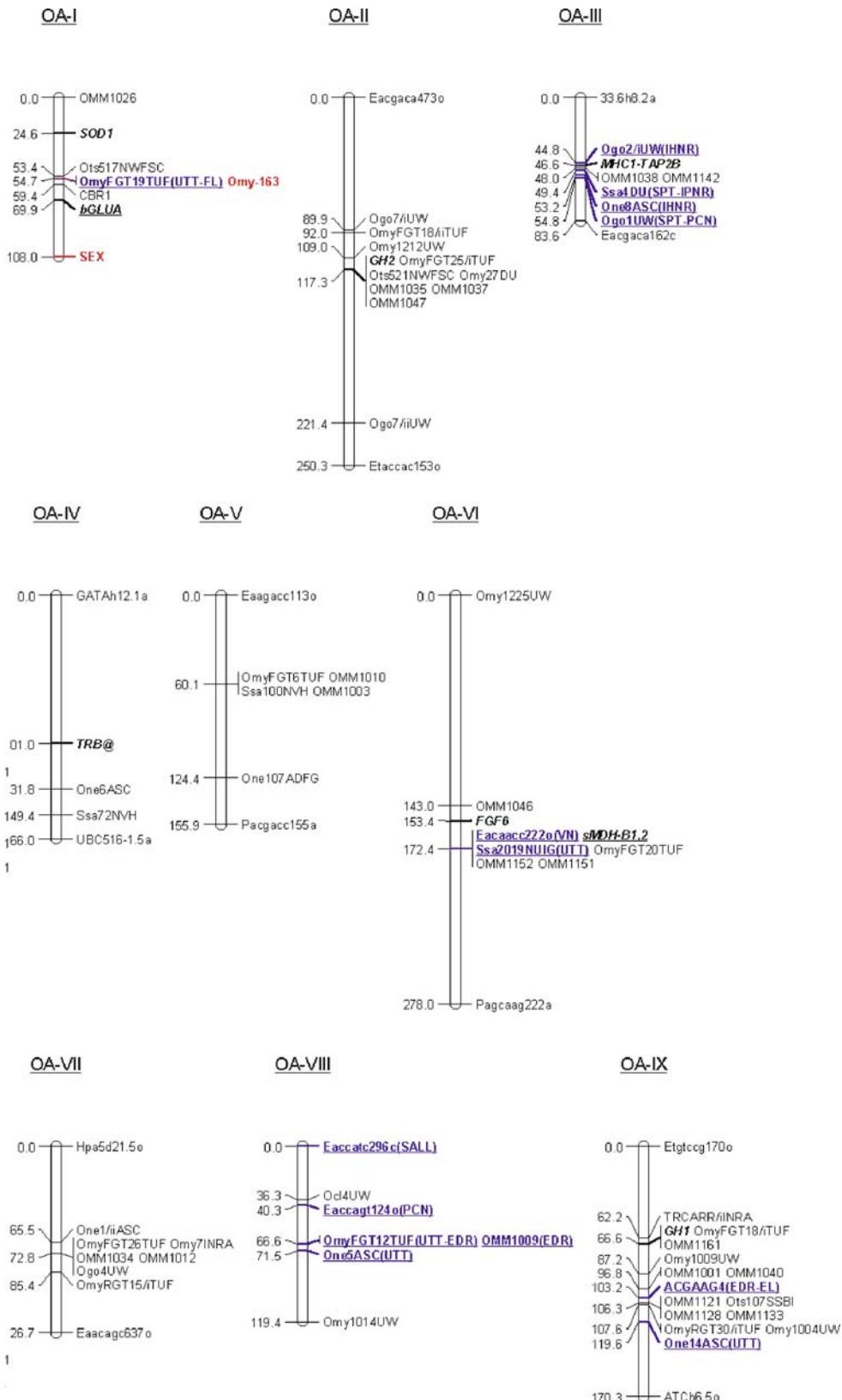
a consolidated map for this species. For example, Moen et al. (2004a) mapped the locus *Ssa197* in the linkage group 1, and Gilbey et al. (2004) mapped this marker in the linkage group 12. Similar patterns occurred for the locus *SSOSL438* that mapped in the linkage groups 9 and 8 in the respective maps (Fig. 4). In both cases, the maps have different markers on the homologous linkage groups (Fig. 4). It is, therefore, possible to combine the marker information and increase the map saturation. These maps for Atlantic salmon are two good approximations to produce a complete map for the species; however, more research will be necessary to produce a complete map. It is possible that the true map length should be similar to the rainbow trout map, because these species share a common ancestor and similar haploid chromosome number (Table 9). A consolidated map will be possible in a near future due to the cGRASP initiative to obtain the complete sequence of the Atlantic salmon genome and to develop more codominant markers (SNPs and SSRs) for mapping in this species (Davison et al. unpublished).

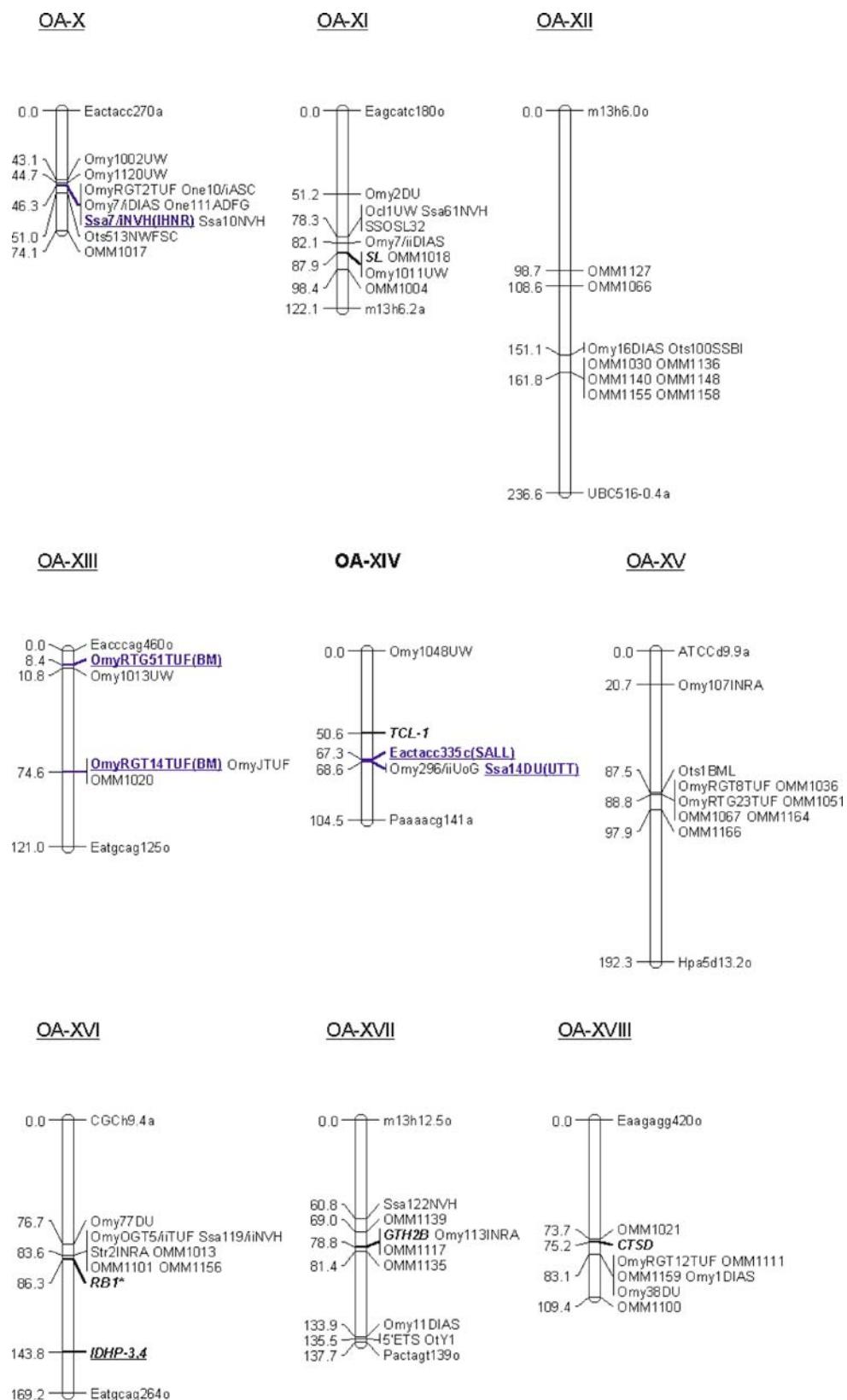
Table 5 Genes mapped in different salmonid genomes

Locus	Gene name	Location in Linkage Groups on map of Rainbow Trout ¹ Brown Trout ² Arctic Char ³		
<i>SOD1</i>	Cu/Zn-superoxide dismutase 1	OA-I		
<i>CBR1</i>	Carbonyl reductase	OA-I		
<i>GH2</i>	Growth hormone 2	OA-II		AC-20
<i>MHC1-TAP2B</i>	MHC I transport and activating protein 2B	OA-II		
<i>TRB@</i>	T-cell receptor beta	OA-IV		
<i>FGF6</i>	Fibroblast growth factor 6	OA-VI		
<i>WT1-b</i>	Wilms tumor – type 1a	OA-VI		
<i>GH1</i>	Growth hormone 1	OA-IX		
<i>hsc70</i>	71 kD heat shock cognate protein	OA-IX		
<i>CYP1A2</i>	Cytochrome P50 subfamily I polypeptide 2	OA-X		
<i>TRCARR<i>i/ii</i></i>	Trout red cell arrestin	OA-XI & OA-XX	BT-27 & BT-30	AC-20
<i>TCL-1</i>	C-type lectin	OA-XIV		
<i>RB1</i>	Retinoblastoma	OA-XVI		
<i>5'ETS</i>	External transcribed spacer for 18/S28S rDNA	OA-XVII		
<i>GTH2B</i>	Gonadotropin hormone II β subunit	OA-XVII		
<i>CTSD</i>	Cathepsin D	OA-XVIII		
<i>MYC</i>	C-myc proto-oncogene	OA-XIX		
<i>VIM</i>	Vimentin	OA-XIX		
<i>TP53</i>	p53 proto-oncogene	OA-XX		
<i>TCL-2</i>	ITIM-bearing C-type lectin	OA-XXI		
<i>ID1</i>	Inhibitor of DNA binding/differentiation 1	OA-XXII		
<i>PRL</i>	Prolactin	OA-XXIV		
<i>HRAS</i>	Ras-1 oncogene	OA-XXVII		
<i>MT1B</i>	Metallothionein B	OA-XXVII		
<i>RAG1</i>	Recombination activation gene-3' UTR	OA-XXVII		
<i>WT1-a</i>	Wilms tumour – type 1b	OA-XXVII		
<i>NRAMP-α</i>	Natural resistance associated macrophage protein α	OA-XXIX		
<i>UQCRCI</i>	Ubiquinol-cytochrome c reductase core I protein	OA-XXIX		
<i>ZNFN1A1</i>	Zinc finger protein, subfactor 1 A	OA-XXX		
<i>SL</i>	Somatolactin	OA-XI	BT-14	AC-22
<i>PPAR-γ</i>	Peroxisomal proliferator activator receptor			AC-7
<i>SOX-9</i>	SRY-related high mobility group – box protein 9			AC-20
<i>MYOD<i>i/ii</i></i>	Myogenic differentiation antigen 1		BT-13 & BT-21	
<i>TF<i>i/ii</i></i>	Transferrin		BT-16	

¹Combined data from Sakamoto et al. (2000) and Nichols et al. (2003)²Data from Gharbi (2001)³Data from Woram et al. (2004)

Fig. 3 Consolidated map of rainbow trout (OSU \times ARL). Only codominant markers are represented along with the most distal SSR or AFLP markers on every linkage group. *Markers* linked to QTLs are indicated in blue and underlined on corresponding linkage groups. *UUT* upper temperature tolerance, *SP* spawning date, *EDR* embryonic development rate, *EL* embryonic length, *FL* fork length, *BM* body mass, *PCN* pyloric caeca number, *VN* vertebrate number, *SALL* scales above lateral line, *AFR* anal fin ray counts, *IPNV* resistance to IPNV, *IHNR* resistance to IHNR, *NK* NK-like cytotoxicity. (Modified from Nichols et al. 2003; QTLs references in Table 6; Graphics made using MapChart, Voorrips et al. 2002)



**Fig. 3** (continued)

**Fig. 3** (continued)

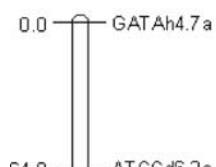
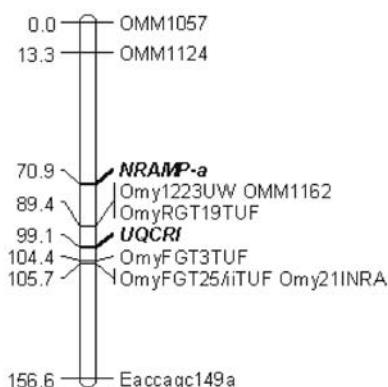
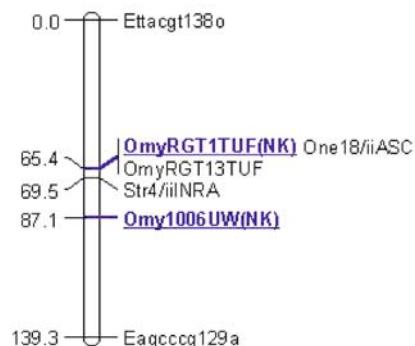
OA-XXVIIIOA-XXIXOA-XXXOA-XXXI

Fig. 3 (continued)

Arctic Charr (*Salvelinus alpinus*)

The first detailed linkage map for this species was published by a team from the University of Guelph, Ontario, Canada (Woram et al. 2004). This map has 46 linkage groups with 322 markers including SSRs, AFLPs, and SNPs (Table 3). It was developed using two commercial but not genetically improved strains of Arctic char, Nauyuk and Fraser. These strains were collected in Canada from Nauyuk Lake (Nunavut) in 1978 and the Fraser River (Labrador) from 1980 to 1984, from a reduced number of wild parents (Woram et al. 2004). For map construction, two backcross families were obtained from crossing a Fraser male with an F₁ hybrid (Fraser × Nauyuk) female and the reciprocal cross, an F₁ hybrid (Fraser × Nauyuk) male with Fraser female.

From each family, 48 progeny were genotyped for mapping.

In this map, it was possible to anchor genes (type I markers) using SNP polymorphisms (detected by heteroduplex analysis) and SSR markers located within intron regions of trout red cell arresting (TRCARR), somatolactin (SL), growth hormone 2 (GH2), and peroxisome proliferator-activated receptor (PPAR- γ) genes. SOX9 was mapped by a cluster of markers located in the introns 1 and 2 of this gene (Table 5). Three of these genes (SOX9, TRCARR, and GH2) were mapped to the same linkage group AC-20, and it is interesting that these same genes are located in a single chromosome in the mouse, suggesting conserved orthologous synteny (Woram et al. 2004).

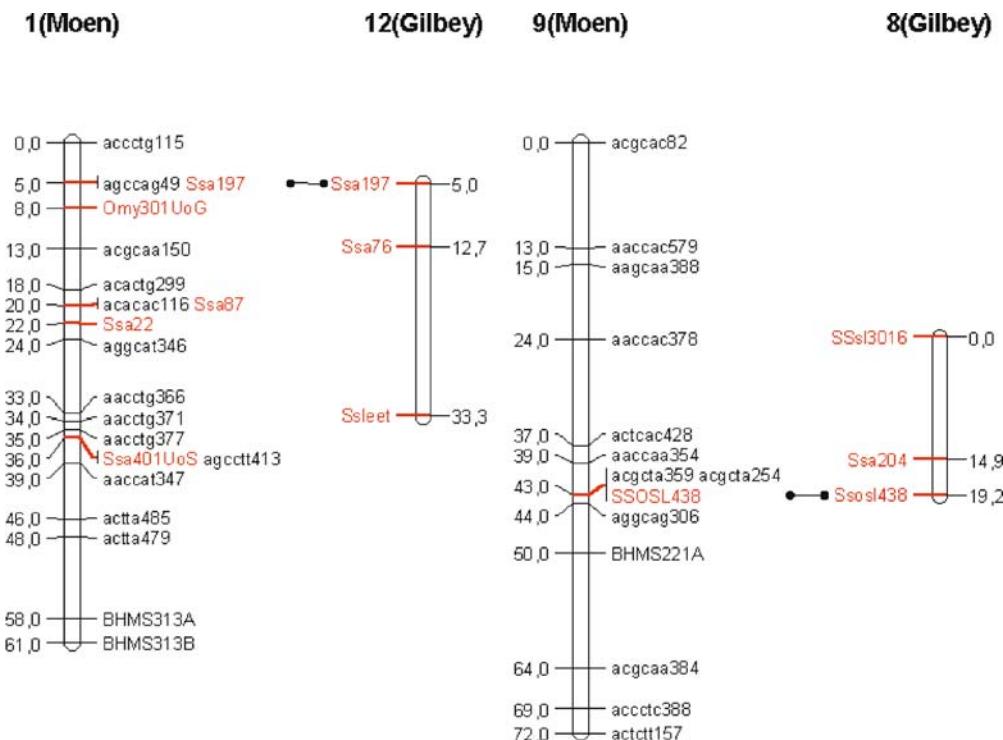


Fig. 4 Comparison of homologous linkage groups from Atlantic salmon maps from Moen et al. (2004a) and Gilbey et al. (2004) with the same loci mapped (*Ssa197* and *SSOSL438*). (Graphics made using MapChart, Voorrips et al. 2002)

Brown Trout (*Salmo trutta*)

The partial map for this species was developed by Karim Gharbi in his Ph.D. thesis work at the Institut National Agronomique Paris-Grignon, France (Gharbi 2001; Gharbi et al. 2005, 2006). This map has 302 codominant SSR and allozyme markers in 37 linkage groups (Table 3). The four mapping families used to develop this map were raised by a classical backcross design, where parental fish from different phylogenetic groups of brown trout (i.e., Atlantic, Mediterranean, or *marmoratus*) were used to produce an F₁ male that was mated with females from pure Atlantic origin. Finally, 45 or 48 progeny from each family (186 progenies in all) were genotyped for different numbers of different types of markers (fluctuating between 42 and 279) in the mapping experiment. Sex-specific maps were estimated to cover 346.4 and 912.5 cM of the male and female genomes, respectively.

Lake Whitefish (*Coregonus clupeaformis*)

For this salmonid species, there is a partial genetic map developed based on 119 AFLP markers by Rogers

et al. (2001) as part of research on the genetic architecture and population divergence in this fish due to ecological shifts which occurred as a consequence of Pleistocene glaciation. Five divergent lineages in the *Coregonus* genus arose during this glaciation, and at present there is a secondary contact zone where different ecotypes cohabit lakes located from southeastern Québec (Canada) to northern Maine (USA). The mapping effort in *Coregonus* has been made by crossing two ecotypes, dwarf (limnetic) and normal (benthic), where the main differences are the age at sexual maturity and adult size. Dwarf fishes generally mature at the first year of age and seldom exceed a body size of 20 cm and weight of 100 g, while normal fishes mature between two and five years of age and frequently exceed 40 cm and 1,000 g (Rogers and Bernatchez 2005).

The map for this species was developed using a classical backcross design, where the parental fishes were dwarf whitefish from Témiscouata Lake (Québec, Canada) and normal whitefish from Aylmer Lake (Northwest Territories, Canada) collected in

1996. From these stocks, an F₁ hybrid progeny dwarf/normal was produced using a complete diallele cross with 20 males and 20 females (Lu and Bernatchez 1998). The mapping family consisted of 60 progenies derived from a cross between an F₁ hybrid female with a pure dwarf male, and it produced a map of 1,462 cM and 29 sex-specific linkage groups (Table 3), where the average size of linkage groups was 50.5 cM and the number of markers per linkage groups ranged from 2 to 17. Future mapping efforts in this species should use codominant markers to increase the coverage of the genome (Rogers et al. 2001).

Pink Salmon (*Oncorhynchus gorbuscha*)

The construction of the genetic map in this species was made using 620 molecular markers of different types: AFLP, paired interspersed nuclear elements (PINE), randomly amplified polymorphic DNA (RAPD), and comparative anchor-tagged sequences (CATS) (Table 3). Preliminary results of mapping in this species were presented at the Plant & Animal Genome Conference in San Diego (California, USA) by Linder et al. (1999) and Spruell et al. (1999). However, a partial or complete map has not been formally published. In this preliminary map, 563 markers were anchored to 55 linkage groups, more than twice the haploid number of this species. A set of 310 loci were mapped in relation to their centromeres using gynogenetic progeny.

1.5.2

Second Generation Map

The construction of an integrated map with high resolution and saturation is a pending task in salmonids. However, the integration of maps will be a natural consequence of the gene mapping efforts in all salmonid species, principally because these fish evolved from a common ancestor 25–100 million years ago (Allendorf and Thorgaard 1984). There is mounting evidence from QTL mapping of conserved synteny of chromosomal regions, in spite of the differences in chromosome number and chromosome arm heterogeneity between and within species. For example, three QTLs for body weight have been detected in homologous chromosomal regions of rainbow trout and Arctic charr (Reid et al. 2005), and in our laboratory,

we have suggestive evidence that three SSR loci linked to QTL for spawning date in rainbow trout to show allelic heterogeneity between coho salmons (*O. kisutch*) selected for early and late spawning date, as indicative of association with spawning time QTL (Diaz et al. unpublished). The mapping of the sex-determining locus also shows some evidence of SSR loci conserved between the Y chromosomes of salmonids (Woram et al. 2003), although the linkage of markers to the Y chromosome in rainbow trout has been highly variable and strain specific (Iturra et al. 1998), indicating that the differentiation of sex chromosomes is a more complex phenomenon in salmonid fishes. At the nucleotide sequence level, in general, it is possible to amplify SSR loci in different species of salmon, indicating the conservation of priming sites in these genomes. This feature is favorable to identification of homologous chromosomes between salmonid species. For example, in the Sakamoto et al. (2000) map for rainbow trout, SSR markers obtained from eight other species of salmon were used with cross-amplification with *Oncorhynchus mykiss*: cutthroat trout (*O. clarki*), pink salmon (*O. gorbuscha*), chum salmon (*O. keta*), sockeye salmon (*O. nerka*), chinook salmon (*O. tshawytscha*), marble trout (*Salmo marmoratus*), Atlantic salmon (*S. salar*), and brown trout (*S. trutta*). This phenomenon of cross-amplification has made it possible to map SSR loci from different species in all salmonid genetic maps that have incorporated these types of markers (Table 3). Additionally, Rexroad et al. (2005) showed cross-species amplification in all 89 polymorphic SSR markers developed from the rainbow trout genome. This cross-amplification included species of *Salvelinus*: Arctic charr (*S. alpinus*) and brook trout (*S. fontinalis*).

Until 2005, the main type of markers used for mapping in salmon were anonymous type II markers. However, based on EST sequences principally obtained from a normalized cDNA library (NCC-CWA 1RT) developed in the USDA National Center for Cool and Cold Water Aquaculture in West Virginia, it has been possible to develop and map new SSR markers in rainbow trout (Rexroad et al. 2005). These SSR markers are particularly useful as type I makers for comparative mapping. It is noteworthy that a fraction of these markers could be mapped to chromosomes of freshwater puffer fish (*Tetraodon nigroviridis*), mice, and humans. Other ESTs containing

SSRs were also identified in the genome databases of fugu (*Takifugu rubripes*) and zebrafish (*Danio rerio*) but without any mapping information (Rexroad et al. 2005). This and other cDNA libraries are being used to develop SSR and SNP type I markers for mapping in rainbow trout and Atlantic salmon (Davison et al. unpublished).

Allozyme markers have been mapped in only two species of salmon (rainbow trout and brown trout, Table 4), and polymorphisms in type I markers have been mapped in only three salmon species (rainbow trout, brown trout, and Arctic charr, Table 5). For both types of markers, only six loci have been mapped in more than one species, and only trout red cell arrestin (*TRCARR*) and somatolactin (*SL*) genes have been mapped in three species of salmons (Table 5). This lack of information about type I markers has so far prevented the development of integrated maps in salmonids.

1.5.3

Mapping Consequences of Ancestral Tetraploidy of Salmonids

Another phenomenon that complicates the map construction in salmonid fishes is that fishes of the *Salmonidae* family descend from a presumed autotetraploid ancestor (Allendorf and Thorgaard 1984), and from this event, the genomes of salmonids have been returning to diploid state. This event of tetraploidization and the restoration of diploidy have consequences in the map construction, because it involves the evolution from four homologous chromosomes in the ancestral fish to two new pairs of homologous and homeologous chromosomes. It is believed that this process of diploidization is not complete in males, because some chromosomes in the male fish still pair with their homeologous counterparts by multivalent formation during meiosis. From segregation analysis of duplicate allozymic locus *MDH-B1,2** in more than 8,000 rainbow trout progeny, it was evident that females always produce diploid gametes. Males, on the other hand, showed variable levels of tetrasomic segregation with a minimum of 25% (Allendorf and Danzmann 1997). The separation of multivalents and recombination may produce “pseudolinkage” in males. That is to say, it produces an aberrant

pattern of nonrandom assortment, where an excess of nonparental type of progeny is observed when the phase of alleles is known (Wright et al. 1983). As a consequence, males have a lower recombination rate in comparison with females, as has been seen in all salmonid genetic recombination maps. Differences in recombination rates between sexes are well documented and have been explained by lower frequencies of crossing over in the heterogametic sex. Apparently, in salmonid fishes, this is increased by the formation of multivalents only in males. Evidence from rainbow and brown trout shows that in males, large chromosomal regions proximal to the centromere have recombination frequencies close to zero, in comparison with telomeric regions that exhibit higher recombination rates (Sakamoto et al. 2000; Gharbi et al. 2006). In rainbow trout, the recombination rate is 10:1 in favor of females close to the centromere and 0.14:1 in favor of males close to the telomere (Sakamoto et al. 2000). In Atlantic salmon, the difference in the recombination rate between the sexes is 3.92 (Gilbey et al. 2004) and 8.26 (Moen et al. 2004a) in favor of females. In the brown trout map, this difference is 6.4 in favor of females (Gharbi et al. 2006), while in the Arctic charr map, it is only 1.69, the lowest published in a salmon (Woram et al. 2004).

As a consequence of residual tetraploidy, some salmonid loci exhibit a tetrasomic inheritance pattern. Evidence of this phenomenon involving the allozyme loci has been reported in several studies since Bailey et al. (1970). For example, in rainbow trout, cytosolic isocitrate dehydrogenase loci (*sIDHP-3,4**, formerly *Idh-3* and *Idh-4*) share alleles. A similar phenomenon occurs for hepatic malate dehydrogenase (MDH) loci (*sMDH-1,2**, formerly *Mdh-1* and *Mdh-2*), and for the skeletal muscle form of MDH (*sMDH-3,4**, formerly *Mdh-3* and *Mdh-4*). These results are indicative of tetrasomic inheritance (Thorgaard et al. 1983). At the DNA marker level, duplicate loci have been detected for *Ogo2UW* SSR that detected loci in linkage groups OA-III and OA-XXV of a rainbow trout genetic map (Sakamoto et al. 2000). We also have evidence that this locus shows tetrasomic inheritance in coho salmon (C. Araneda unpublished data). A similar pattern was detected for SSR loci *OmyFGT-1TUF* during the construction of a rainbow trout map by Young et al. (1998). The phenomenon is rather general because O’Malley et al. (2002) detected homeolo-

gous regions among thirteen pairs of linkage groups, with 26 SSR loci being duplicated. In the brown trout map, 23 duplicated chromosomal regions were identified on 35 linkage groups, with more than 50 duplicated markers (Gharbi 2001; Gharbi et al. 2005, 2006).

1.6

Detection of Quantitative Trait Loci

Several QTLs have been mapped to different linkage maps on the salmon genome. The mapping strategies used have been composite interval mapping (CIM) to scan an AFLP-based map (Robison et al. 2001), and single marker analysis, which separately tests every segregating maternal or paternal SSR allele (Jackson et al. 1998). The QTLs for several normally distributed quantitative traits are furnished in Tables 6 and 7. Single markers associated with monogenic or polygenic traits without mapping data will be discussed in the next part of this chapter (Table 8).

1.6.1

Upper Temperature Tolerance

The first QTL described for any salmon was published by Jackson and colleagues (1998) for upper temperature tolerance (UTT) in rainbow trout based on the segregation of 24 loci in three backcross families (Table 6). This trait corresponds to the length of time that a fish is able to survive at 25.7 °C. The families used were obtained by crossing F₁ males with high-temperature-tolerant (two families) and intolerant parental females (one family). Hybrid F₁ progenies were obtained by crossing a high-temperature-tolerant female with an intolerant male. This study could detect two main chromosomal regions explaining 13% and 9% of the within-family variance of UTT, linked to *Omy325UoG* and *Ssa14DU*, respectively. These markers were anchored to linkage groups B and D on the Sakamoto et al. (2000) map (Table 6). It is interesting to note that these two linkage groups have some homology, because these SSR loci had been mapped to linkage groups OA-XXI and OA-XIV, respectively, in the consolidated map. However, the group OA-XIV carries a combination of markers formerly mapped to groups B and

D in Sakamoto's map. Later studies in this species using the same BC design (three families) showed that the SSR locus *Ssa20.19NIUG* maps near a QTL with a stronger effect on UTT than those reported previously by Jackson et al. (1998), and the other locus *One14ASC* shows a weaker effect (Danzmann et al. 1999, Table 6).

Further evidence of an association of *Ssa20.19NIUG* with UTT QTL was obtained using a diallel panel derived from a cross of two outbred rainbow trout strains unselected for temperature tolerance. This study confirmed the association of the locus *Ssa20.19NIUG* with UTT, explaining 7.5% of the phenotypic variance of this trait in these outbred crosses (Perry et al. 2001). However, it was not possible to detect any effect on temperature tolerance on chromosomal regions close to *Omy325UoG* and *Ssa14DU* loci. The authors attributed this lack of association to the different population designs, where the overall phenotypic variance in the outbreed study is considerably less than in the backcross design. Because of this, only the QTLs with a stronger effect will be detected in outbred populations (Perry et al. 2001).

In addition to these autosomal QTLs, evidence was recently obtained for a sex-linked QTL close to the locus *OmyFGT19TUF*, accounting for 9.6% of phenotypic variance of UTT, and also having a pleiotropic effect on fork length (FL) in rainbow trout (Perry et al. 2005). This work was performed using two designs, a backcross with three half-sib families and an outbred diallel panel with 40 full-sib families (Perry et al. 2005). Recent evidence shows that the duplicate uncoupling protein genes (*UCP2A* and *UCP2B*) map close to the SSR linked to UTT QTLs in both linkage groups in rainbow trout and are candidate genes to explain this temperature tolerance phenotype (Coulibaly et al. 2006). In mammals, the *UPC2* gene is involved in multiple functions, including energy balance. The protein encoded by this gene is located in the inner mitochondrial membrane (Coulibaly et al. 2006).

Arctic charr (*Salvelinus alpinus*) has been used to test UTT QTL effects in homologous chromosomal regions of rainbow trout on the basis of genetic maps available for both species (Somorjai et al. 2003). This study used a backcross family obtained from crossing of two strains characterized by different thermal selection regimens. One strain was obtained

Table 6 QTLs mapped on rainbow trout (*Oncorhynchus mykiss*) genome

Trait	Markers detected as linked to QTLs and its respective linkage group in brackets	References
Upper temperature tolerance	<i>OmyFGT19TUF</i> (OA-I*); <i>Ssa2019NTUG</i> (OA-VI*); <i>One14ASC</i> (OA-IX*); <i>Ssa14DU</i> (OA-XIV*);	Jackson et al. 1998, Danzmann et al. 1999, Fishback et al. 2000, Perry et al. 2001
Spawning date	<i>Omy325UoG</i> , <i>OmyFGT27TUF</i> (OA-XXI*); <i>Ssa4DU</i> , <i>OmyRGT41TUF</i> , <i>Ogo1UW</i> (OA-III*); <i>OmyFGT12TUF</i> , <i>Ssa311NCVM</i> , <i>One5ASC</i> (OA VIII*); <i>OmyFGT34TUF</i> , <i>Ssa103NVH</i> (OA-XIX*); <i>Ssa289DU</i> , <i>Ssa439NCVM</i> (OA-XXII*); <i>One8ASC</i> , <i>One19ASC</i> , <i>Ots4BML</i> , <i>Ssa85DU</i> , <i>OmyRGT36TUF</i> (OA-XXIV*); <i>OmyRGT7TUF</i> (OA-XXXV*)	Sakamoto et al. 1999, Fishback et al. 2000, O'Malley et al. 2002
Embryonic development rate	<i>ACGAGA5</i> (R6**); <i>OmyFTG12TUF</i> , <i>Omy18INRA</i> , <i>OMM1009</i> (OA-VIII*); <i>ACGAAAG4</i> (OA-IX*)	Robison et al. 2001, Sundin et al. 2005
Embryonic length	<i>AGCCTC1</i> (R6**); <i>ACGAAG4</i> (OA-IX*)	Robison et al. 2001
Embryonic weight	<i>ACGAGA5</i> (R6**); <i>AGCGCG4</i> (R11**)	Robison et al. 2001
Fork length	<i>OmyFGT19TUF</i> (OA-I*)	Perry et al. 2001
Body mass	<i>OmyRTG51TUF</i> , <i>OmyRTG47TUF</i> , <i>OmyRTG14TUF</i> (OA-XII*); <i>OmyRTG11TUF</i> (OA-XXXI*)	Martyniuk et al. 2003
Pyloric ceaca number	<i>AGCATA4.355.1hc</i> , <i>Ogo1UW</i> , <i>AGCATA4.355.hc</i> (OA-III*); <i>EACCAGT124o</i> , <i>ACCACA12.62.osu</i> (OA-VIII*); <i>AGCGAGA15.235.osu</i> , <i>ACCATC12.249.hc</i> (OA-XXXII*)	Zimmerman et al. 2005
Vertebrate number	<i>ACGATC1</i> , <i>ACGAAG15</i> (OA-VI*)	Nichols et al. 2004
Scales above lateral line	<i>ACGAAAG12</i> , <i>AGCAGC16</i> (OA-VIII*); <i>ACGAGA93ac</i> , <i>ACGATG7c</i> (OA-XIV*)	Nichols et al. 2004
Anal fin ray counts	<i>AGCACG150o</i> , <i>AGCACA8</i> (OA-XXII*)	Nichols et al. 2004
IPNV Resistance/susceptibility	<i>OmyRGT41TUF</i> , <i>Ssa4DU</i> (OA-III*); <i>OmyOGT41TUF</i> , <i>OmyRGT6/iiTUF</i> (OA-XXII*)	Nichols et al. 2004
IHNV Resistance	<i>GCTCTC178</i> , <i>GATCAC291</i> , <i>One8ASC</i> , <i>OMM1054</i> , <i>Ogo2Uj/W</i> (OA-III*); <i>Ssa7NVH</i> , <i>OtsG78</i> (OA-X*)	Ozaki et al. 2001
NK-like cytotoxicity	<i>GGTCCCC60</i> , <i>GATCC510</i> , <i>OmyFGT-1TUF</i> , <i>Ssa94NVH</i> , <i>OtsG3</i> (OA-XXVII*)	Rodriguez et al. 2004
Ceratomyxosis resistance	<i>Agaag-17.120.osu</i> , <i>Eacgaaac97o</i> , <i>Omy1006UW</i> , <i>OmyRGT1TUF</i> , <i>accagt.11.274.hc</i> (OA-XXXI*)	Zimmerman et al. 2004
	<i>Acgact12</i> (OC17*****)	Nichols et al. 2003b

* Current linkage groups identification after Nichols et al. (2003), consolidated rainbow trout OSU × ARL map.

** Original linkage group nomenclature from Robison et al. (2001).

*** Original linkage group nomenclature from Rodriguez et al. (2004).

**** Original linkage group nomenclature from Nichols et al. (2003b).

Table 7 QTLs mapped on different salmonids genomes (excluding rainbow trout)

Species	Trait	Markers detected as linked to QTLs and its respective linkage group in brackets	References
<i>Salvelinus alpinus</i>	Upper temperature tolerance	<i>Ssa14DU</i> (AC-12); <i>Ssa189NVH</i> , <i>Ssa85DU</i> , <i>Ssa185 NV</i> <i>Ssa119NVH</i> (AC-13); <i>SsaF43NUIG</i> , <i>One10ASC</i> (AC-26).	Somorjai et al. 2003
<i>Salmo salar</i>	Body weight	<i>Ssa401UoS</i> (AS-8); <i>Ssa417UoS</i> , <i>OmyRGT32 TUF</i> , <i>BHMS211</i> (S-11).	Reid et al. 2005
	Fulton's condition factor	<i>BHMS159</i> (AS-2); <i>Str58CNRS</i> (AS-5); <i>BHMS211</i> (AS-11); <i>OtsG249UCD</i> (AS-14).	Reid et al. 2005
	ISA resistance	aaccac74, agccta290 (7f)	Moen et al. 2004b
<i>Coregonus clupeaformis</i>	Absolute growth rate	GGTG199, GGT060n, GGTG104, GGTG107 (Lg1-m); ACTA135, CTTC220 (Lg6-f); AGAC126, AGAC175 (Lg12-m)	Rogers and Bernatchez 2005

from Nauyuk Lake (Northwest Territories, Canada) and is adapted to cooler temperatures with less tolerance to upper temperatures. The other strain originates from Fraser Lake (Labrador, Canada) and is considered more tolerant of high temperature. Significant associations with UTT were detected close to loci *Ssa189NVH* and *SsaF43NUIG*, and suggestive associations were detected for SSR loci *Ssa85DU*, *Ssa185NVH*, *One10ASC*, *Ssa14DU*, and *Ssa119NVH* (Somorjai et al. 2003; Table 7). Unfortunately, the authors did not report the contribution of each QTL to the overall phenotypic variance for UTT. In relation to QTLs reported for this trait in rainbow trout, the marker *Ssa14DU* shows association in both species. Also, the marker *Ssa119NVH* maps syntetically to *Omy77DU* in a region that in rainbow trout shows physiological epistasis with *Ssa2019NIUG* and *Ssa14DU* (Danzmann et al. 1999).

In summary, evidence from different populations, different experimental designs, and different species of salmonids confirms at least three QTLs for UTT that map close to the loci *Ssa2019NIUG*, *OmyFGT19TUF*, and *Ssa14DU*. This evidence, obtained from different sources, argues strongly in favor of the idea that true QTLs for UTT have been detected in salmonid fishes (Complex Trait Consortium 2003). These studies of UTT QTLs have been made by Roy Danzmann's team using a compendium of visual basic programs (LINKMFEX) developed by him and designed to perform linkage analysis in outcrossing mapping panels (Danzmann 2005).

1.6.2 Spawning Date

In salmonids, spawning date (ovulation) is an important life-history trait determining the time of fertilization, date of progeny emergence, and even the probability of survival and the growth rate of the fry (Quinn et al. 2002). In salmon farming, extending the reproductive period allows for better management of fish production according to season, as well as increasing the period during which eggs are available on the market (Gall and Neira 2004).

The spawning date of rainbow trout is a trait with high additive genetic variation ($0.53 \leq h^2 \leq 0.65$; Siitonen and Gall 1989; Su et al. 1999). There are strains which spawn in spring (February to April, in the North Hemisphere), and strains which spawn in fall (September to December). The first evidence of chromosomal regions affecting spawning time was presented by Sakamoto et al. (1999), who used 54 SSR markers in a backcross family of 45 female progeny for mapping. This family was produced by crossing an F₁ hybrid male fish with a fall spawning female. The F₁ hybrid was obtained from mating of spring spawning male with a fall spawning female. Eleven SSR loci on five linkage groups showed significant association with spawning date QTLs: *OmyFGT12TUF*, *Ssa311NCVM*, and *One5ASC* on linkage group OA-VIII; *One2ASC*, *One19ASC*, *Ots4BML*, and *Ssa85DU* on group OA-XXIV; *Ssa4DU* on group OA-III; *Ssa289DU* and *Ssa439NCVM* on group OA-XXII; and *OmyFGT34TUF* on group

OA-XIX (Table 6). The effects on phenotypic variance of these QTLs were not reported in this study, but the approximate extent of the QTL regions on each linkage group was 4.6, 0, 17.8, 13, and 21 cM, respectively (Sakamoto et al. 1999). Later evidence obtained from a commercial trout farm where fish were spawning in different seasons (spring and fall) shows that females maturing at different times showed heterogeneity in the distribution of allelic frequencies for loci linked with spawning date QTLs *OmyFTG12TUF*, *OmyRGT41TUF* (linked to *Ssa4DU*), and *SSOSL439* (formerly *Ssa439NCVM* in Sakamoto et al. 1999; Table 6), compared to other loci not linked to QTL (Fishback et al. 2000). Fish spawning at the same time of year but in different years showed little heterogeneity in allele frequencies on QTL-linked loci (Fishback et al. 2000).

Another study performed by the same laboratory detected evidence of six QTL regions in rainbow trout, confirming the presence of four strong spawning date QTLs on four different linkage groups. This work used 201 SSR markers and 90 progeny of a backcross family made between two outbred strains of rainbow trout spawning in fall and spring, where a hybrid F₁ male was crossed with a fall spawning female (O'Malley et al. 2002). This study, like Sakamoto et al. (1999), confirmed the presence of one spawning date QTL on linkage group OA-XIX, in the interval between *OmyFTG34TUF* and *Ssa103NVH*, and a second QTL on linkage groups OA-VIII near the centromere and spanning the region from *OmyFTG12TUF* to *One5ASC*. A third region, also identified in previous studies, was detected on linkage group OA-XXIV in the interval flanked by *OmyRGT36TUF* and *Omy2ASC*. Another QTL region was confirmed on linkage group OA-III, close to the locus *Ogo1UW* and linked to *OmyRGT41TUF* and *Ssa4DU*. Finally, there was marginal evidence for two more QTLs, near marker *Ssa298DU* on linkage group OA-XXII (also detected by Sakamoto's work) and OA-XXV (marker *OmyRGT7TUF*; O'Malley et al. 2002).

In coho salmon (*Oncorhynchus kisutch*), three loci linked to QTL in rainbow trout (*One2ASC*, *One19ASC*, *OmyFTG34TUF*), show strong differences between female fish divergently selected for early and late spawning date for three generations (N. Diaz et al. unpublished data). This evidence supports the presence of QTLs close to these loci and is indirect evidence of

conservation of chromosomal segment with QTLs for spawning time on salmonid genomes, although due to the lack of a linkage map for coho salmon, it is not possible to confirm the synteny of coho salmon and rainbow trout linkage groups.

In general, these studies indicate that spawning date is a highly polygenic trait in salmonid genomes, with at least four QTLs confirmed (Table 6). Again, mapping of QTLs for spawning time was performed using LINKMFEX software for single marker analysis (Danzmann 2005) and MultiQTL (version 2.1.2) for the interval mapping study by O'Malley et al. (2002).

1.6.3

Embryonic Development Rate

The embryonic development rate, evaluated principally as the time from fertilization to hatching, plays a significant role in the life history of wild and cultured salmon stocks. In nature, this trait determines the time of fry emergence, resulting in reduced predation rates, optimal availability of exogenous food, and appropriate conditions for fry migration (Robison et al. 1999). Mapping of QTLs linked to this trait has been performed using two homozygous clonal (DH) lines of rainbow trout with divergent hatching times, using composite interval mapping (CIM) and principally AFLP markers (Robison et al. 1999, 2001). The F₁ fishes used in this study were obtained by crossing homozygous clonal lines of all-male (YY) rainbow trout from the Swanson River (Alaska, USA) and a domesticated all-female (XX) stock from Oregon State University. These two lines have been shown to differ in hatching times (Robison et al. 1999). The F₁ all-male progeny (XY) were used to produce two DH mapping families. Ultimately, 170 progeny were genotyped with 219 AFLPs, two SSRs (*One2ASC* and *One19ASC*), and an *AluI* polymorphism in the 3' UTR of the *P53* gene. The DH design is known to increase the power to detect QTLs in experimental populations (Martinez et al. 2001). The marker genotypes were used to construct a genetic linkage map with MAPMAKER/EXP version 3.1 and MAPMAKER version 2.0 (Lander et al. 1987). A standardized nomenclature for the linkage groups relative to the consolidated map was not fully realized because only seven markers shared with those of the map of

Nichols et al. (2003a). Therefore, the linkage groups were named using the letter R followed by a number, as in the original paper (Table 6; Robison et al. 2001).

CIM (Zeng 1993, 1994), implemented in QTL Cartographer software, was used to detect QTLs for embryonic development rate, embryonic length, and weight at swim-up. Three QTLs influencing time to hatch were detected on linkage groups R13 (OA-IX), R6, and R9, accounting for 14.7%, 5.3%, and 4.6% of the phenotypic variance for this trait, respectively. On the other hand, a QTL influencing embryonic length was detected on linkage group R13 (OA-IX) at the same position of a QTL for time to hatch. A second QTL was detected on linkage group R6, but 40 cM away from the QTL for time to hatch anchored at the same linkage group. The proportion of variance explained by these two QTLs was 13.1% and 9.5%, respectively. QTLs for embryonic weight were detected on linkage group R11 (accounting for 10.9% of phenotypic variance), and on linkage group R6 at the same position as the QTL for time to hatch (accounting for 15.3% of the trait variance). An interesting finding of this study was the probable detection of two QTLs on linkage groups R13 (OA-IX) and R6, with pleiotropic effects on time to hatch and embryonic length, and time to hatch and weight at swim-up, respectively (Table 6; Robison et al. 2001).

Further fine-mapping for time to hatch in rainbow trout was performed by the same laboratory using a congenic line carrying a major development rate QTL developed at Oregon State University through advanced backcross QTL analysis and marker-assisted selection (Sundin et al. 2005). With this approach, one QTL was identified for embryonic development rate on linkage group OA-VIII, linked to SSR loci *OmyFGT12TUF*, *OmyI8INRA*, and *OMM1009* (Table 6), and explaining between 26% and 28% of variation in time to hatch in two different mapping backcross families (Sundin et al. 2005). This QTL may influence early sexual maturity (gamete production) as well as development rate and is interesting because the locus *OmyFGT12TUF* is also linked to the QTL for spawning time in this species (Table 6). In summary, four chromosomal regions influencing embryonic development rate (and other embryonic traits) have been identified in rainbow trout. Confirmation of these QTL in additional crosses is still needed.

1.6.4

Morphological and Meristic Traits

QTLs for several morphological traits, including fork length, body weight, absolute growth rate, and Fulton's condition factor (K, measured as $100 \times \text{body weight} \cdot \text{fork length}^{-3}$), and meristic traits (counts of fin rays, lateral line scales, vertebrae, and pyloric caeca) have been identified recently on salmonid genomes (Table 6).

In the study of Perry et al. (2005) on upper temperature tolerance in rainbow trout, morphological traits that showed phenotypic correlation with UTT were evaluated, such as fork length and Fulton's condition factor (Jackson et al. 1998). The QTL mapping population and strategies were similar to those used for UTT (Perry et al. 2005). A QTL affecting fork length was identified close to the sex-linked SSR locus *OmyFTG19TUF* on linkage group OA-I. Therefore, there is evidence for two QTLs close to *OmyFTG19TUF*: one for UTT and another for fork length, accounting for 9.7% and 9.6% of the variance in these traits, respectively. An alternative hypothesis is that there is only one QTL with pleiotropic effects on both these traits (Perry et al. 2005). This study did not detect any association with Fulton's condition factor in rainbow trout using 2,001 SSR markers.

In Atlantic salmon, four QTLs for condition factor and two QTLs for body weight have been detected in comparative studies with rainbow trout and Arctic charr (Table 7; Reid et al. 2005). In this case, several QTLs influencing these two traits altogether had been mapped using 91 SSR loci and 46 progenies from each of three full-sib families. The parental fishes were obtained from the Atlantic Salmon Broodstock Development Program, in Chamcook (New Brunswick, Canada) and from the St. John River. One strong QTL explaining 20.1% of variation in body weight was detected on linkage group AS-8 (locus *Ssa401UoS*). Another QTL with a relatively strong effect was found on group AS-11 (locus *Ssa417UoS*) and accounted for approximately 12% of phenotypic variation. A previous study by this research group in rainbow trout showed marginal evidence that several SSR loci previously associated with spawning time (for example *One2ASC*, *One19ASC*, *OmyRGT36TUF*) also showed association with body mass, but with modest phenotypic effects (variation explained between 0.7% and 11.3%). The strongest association for locus *OmyRGT1TUF* ex-

plained only 1.8% of the phenotypic variation (Martyniuk et al. 2003). Two other QTLs accounting for between 0.7% and 5.0% of total variance were found. One mapped close to the telomeric loci *OmyRTG51TUF* and *OmyRTG47TUF*, and the other mapped near *OmyRTG14TUF* on linkage group OA-XIII (Table 6).

Significant QTLs for condition factor have been detected on linkage groups AS-14 (locus *OtsG249UCD*), AS-11 (locus *BHMS211*), AS-5 (locus *Str58CNRS*), and AS-2 (locus *BHMS159*), accounting for 24.9%, 16.9%, 17.6%, and 17.6% of variation, respectively (Table 7). This result suggests that some portion of quantitative variation in body weight and condition factor in Atlantic salmon is under the control of a few QTLs with relatively large effect (Reid et al. 2005). Many of the morphological growth-related traits show phenotypic and genetic correlations, and there is evidence for QTLs with pleiotropic effects (Martyniuk et al. 2003; Reid et al. 2005). However, due to the low density of markers on existing salmonid linkage maps, it is still difficult to distinguish between single QTLs and overlapping QTLs (Reid et al. 2005). As has been usual for Danzmann's laboratory, QTLs mapping was performed using LINKMFEX software for single marker analysis (Danzmann 2005) and multiple interval mapping (MIM) implemented in MultiQTL version 2.1.2 (O'Malley et al. 2002).

QTLs for absolute growth rate have been mapped in lake whitefish in the same mapping population used for the construction of a recombination map based on AFLP markers (Rogers et al. 2001). Growth rate was estimated on the basis of monthly weight measurements performed for four months on a sample of 50 adult fish (more than 2 years old) belonging to a back-cross family derived from mating between normal and dwarf parental ecotypes (Rogers and Bernatchez 2005). The objective of this study was to identify markers flanking potential QTLs among intervals rather than analyze the effect of single QTLs. This analysis revealed significant associations with growth on 11 linkage groups with LOD scores ranging from 3.4 to 8.7. In linkage groups Lg4-f, Lg5-f, Lg7-f, Lg8-f, Lg11-f, Lg13-m, Lg24-f, and Lg26-m, potential QTLs span over all 30 markers anchored in the map published by Rogers and colleagues (2001). The abundance of QTLs on a larger chromosomal interval seems consistent with the general approach used by these researchers, which could produce occasional type I er-

ror in the QTL identification. Only in the larger linkage group Lg1-m are there two more delimited regions carrying presumptive QTLs for absolute growth spanning 18.5 cM (between telomeric loci *GGTG199* and *GGT060*) and 6.7 cM (between more centromeric loci *GGTG104* and *GGTG107*; Rogers and Bernatchez 2005). Two other linkage groups also had relatively well-defined QTL regions, spanning 21.8 cM on Lg6-f (between loci *ACTA135* and *CTTC220*) and 28.3 cM on Lg12-m (between *AGAC126* and *AGAC175*). The map used in this study had relatively few markers, but if there is linkage between markers, such limited sampling should be sufficient to detect QTLs with strong effects. At this time, the chromosomal regions with potential QTLs are candidates for a subsequent fine-scan with codominant markers and marker types that are more transferable across species (Rogers and Bernatchez 2005). The QTL analyses in this study were performed using an algorithm of maximum likelihood interval analysis implemented in MAP-MAKER/QTL version 1.0 (Lander and Botstein 1989).

Meristic or discontinuous quantitative traits in fish have in general higher heritability ($h^2 \geq 0.5$) and are used for fish identification and taxonomy (Kirpichnikov 1981). One interesting meristic trait with high heritability ($h^2 = 0.43$; Bergot et al. 1981), and for which QTLs have been identified, is the number of pyloric caeca in rainbow trout, a trait involved in nutrient absorption and therefore possibly indirectly involved in growth rate. Pyloric caeca are sac-shaped anatomical structures, distal to the pylorus in the upper zone of the small intestine, and histologically similar to the small intestine (Rust 2002). The inner lumens of pyloric caeca are extensively folded with large numbers of microvilli and secretory cells, producing a variety of digestive enzymes. These enzymes facilitate the enzymatic breakdown of proteins, lipid hydrolysis, and the absorption of amino acids, fats, water, sodium, carotenoids, and vitamins. In adult rainbow trout, the numbers of pyloric caeca range between 31 and 147, depending on the strain, and this number appears to become fixed in alevins when fish reach 4 cm in length (Zimmerman et al. 2005).

In the QTL mapping strategy, clonal lines of rainbow trout from an experimental population at Oregon State University (OSU) and from a cultured population from the state hatchery in Hot Creek (HC), California (USA) were used. The strains differ by approximately 34 pyloric caeca. An OSU female (XX)

fish was crossed with an HC male (YY) to produce a hybrid F₁ male, which was used to produce a doubled haploid population (OSU × HC) of 54 individuals by androgenesis. This population was genotyped with 330 AFLP and 39 SSR markers (Zimmerman et al. 2005). A genetic map was developed using MAPMAKER/EXP version 3.1 and MAPMAKER version 2.0 (Lander et al. 1987), and QTL mapping was performed by CIM (Zeng 1994) using model 6 of the QTL Cartographer software. Three QTLs for pyloric caeca number were identified (Table 6). The QTL explaining the highest percentage of phenotypic variance (19.2%) was located on linkage group OA-III in a chromosomal interval spanning 21.8 cM between AFLP markers *agcata.4.355.hc* and *aagacg.6.350.hc* and containing the SSR loci *Ogo1UW*. A second QTL accounting for 18.6% of trait variation was mapped on linkage group OA-VIII between markers AFLP *Eaccagt124o* and *accaca.12.62.osu*, spanning 38.2 cM. The SSR locus *OmyFGT12TUF* mapped in the same linkage group but was not associated with pyloric caeca number. The distance between the more proximal AFLP marker (*accaca.12.62.osu*) and *OmyFGT12TUF* was about 17.3 cM. The third QTL mapped in the linkage group OA-XIII in a region flanked by AFLP markers *agcaga.15.235.osu* and *accatc.12.249.hc*. The interval spanned 29.6 cM and explained 13.5% of phenotypic variance. No SSR locus mapped in the interval, but the locus *OMM1109* was 70.1 cM away from AFLP marker *agcaga.15.235.osu* (Table 6; Zimmerman et al. 2005). It is interesting that QTLs for spawning time and shorter time of hatch have been identified on linkage group OA-VIII, close to the loci *OmyFGT12TUF*, and on linkage group OA-III, near the locus *Ogo1UW*. This suggests a possible underlying basis for these three traits in rainbow trout, or as an alternative hypothesis, a bias toward the same chromosomal intervals in the QTL mapping in salmonids due to the reduced pool of markers for mapping.

QTLs have been reported for several other meristic traits, including the number of vertebrae, scales above the lateral line, and anal fin rays (Nichols et al. 2004). In this case, the QTL mapping was performed using 238 AFLP markers in DH progeny derived by androgenesis from an F₁ hybrid male. The parental fish were clonal lines from Oregon State University (OSU) and the Dworshak National Fish Hatchery on Clearwater River (CW) at Ashakha (Idaho, USA). Composite interval mapping (Zeng 1994), implemented in the

QTL Cartographer software, was used for QTL detection, and MAPMAKER/EXP version 3.0 was used to assemble markers into linkage groups. Synteny with the consolidated rainbow trout map was determined using some shared AFLP markers. This analysis detected a QTL explaining 23% of the variation in vertebrae number within a 6 cM interval between markers *ACGATC1* and *ACGAAG15* on linkage group OA-VI. Two QTLs were detected for count of scales above the lateral line on linkage groups OA-VIII and OA-XIV, accounting for 27.5% and 15.9% of phenotypic variation, respectively (Table 6). One QTL was detected for anal fin ray counts on linkage group OA-XXII (linked marker *agcacg150o*), explaining 13.4% of variation in this trait.

In general, meristic variation has been negatively correlated with differences in embryonic developmental rate (time to hatch). Fish reared at higher temperatures show accelerated growth and fewer elements in meristic traits (Nichols et al. 2004). In this study, in spite of a strong negative association between time to hatch and counts of meristic traits, only one QTL (for scales above the lateral line) was mapped to the same linkage group (OA-VIII) bearing a QTL for developmental rate. The genetic distance between these QTLs is more than 50 cM, indicating independent segregation of these QTLs (Fig. 3) and suggesting that developmental rate and number of meristic traits are not tightly coupled in rainbow trout (Nichols et al. 2004). However, it is interesting to note that the two QTLs for meristic traits mapped close to the QTL controlling upper temperature tolerance. These QTL are number of scales above the lateral line on linkage group OA-XIV and vertebrae number on the OA-VI chromosome (Fig. 3), possibly indicating a common genetic mechanism for these traits.

1.6.5

Resistance to Disease and Immunologic Traits

Breeding for disease resistance (or tolerance) is an important area in livestock and aquaculture production, due to the multiple effects of epidemic events such as production loss, zoonotic problems, barriers for international trade, and animal welfare (Bishop 2002). Polymorphisms in major histocompatibility complex (MHC) or related disease response genes have been associated with disease resistance in several livestock

species. However, today it is clear that there is no gene, combination of genes, or level of genetic variation (heterozygosity) that confers a kind of “general resistance effect.” Therefore, searching for individual QTLs affecting resistance/susceptibility to specific bacterial, viral, or parasitic diseases, along with identification of QTLs for immunological responses to pathogens, is a challenge that is relevant in salmonid fish. So far, QTLs for resistance to infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV), and enzootic myxozoan parasite *Ceratomyxa shasta* have been identified (Table 6).

IPNV is a highly contagious and virulent disease with great impact in hatcheries. Differences in resistance have been found among cultivated varieties of rainbow trout (Hill 1982). This variation has allowed the selection of rainbow trout strains resistant and susceptible to IPNV at the Yoshida Research and Training Station of Tokyo (Okamoto et al. 1993). The average mortality evaluated in challenge tests in the period from 1979 to 1989 on the resistant and susceptible strains used for QTL mapping varied between 4.42% and 96.13%, respectively (Ozaki et al. 2001). These strains were crossed to produce an F₁ hybrid family, and an F₁ male was crossed with a susceptible female to produce a backcross family of 152 progeny. A linkage map using 51 SSR segregating loci was constructed. Interval mapping and calculation of likelihood ratio statistics was performed using Map Manager QT28 software (Manly and Olson 1999). Two putative QTLs were detected. One QTL was close to loci *OmyRGT41TUF* and *Ssa4DU* on linkage group OA-III. A second QTL was located close to *OmyOGT4TUF* and *OmyRGT6/iiTUF* on linkage group OA-XXII. Each QTL explained about 17% of phenotypic variance (Ozaki et al. 2001; Table 6).

Another viral disease for which QTLs have been mapped is IHNV, which affects almost all stages of salmonid production. Under intensive culture conditions, losses can approach 100% in alevins, principally in rainbow trout aquaculture (Palti et al. 1999). Several observations suggest that interspecific hybrids of rainbow trout and cutthroat trout (*Oncorhynchus clarkii*), and interstrain hybrids of steelhead and rainbow trout, have significantly reduced susceptibility to IHNV relative to commercial strains of rainbow trout (Palti et al. 1999; Rodriguez et al. 2004). QTL mapping associated with resistance to IHNV was performed by genotyping 185 AFLP and 72 SSR markers

in an interstrain hybrid. Crosses between steelhead (resistant) from the Idaho Department of Fish and Game (USA) and rainbow trout (susceptible) from Clear Spring Food (Buhl, Idaho, USA) were used to produce several hybrid F₁ families. Members of these families were backcrossed to rainbow trout. Challenge tests with the IHNV virus were performed on approximately 200 individuals of the F₁ and BC generations, and mortalities were recorded during the three weeks after the challenge.

Sex-specific linkage maps were produced with MAPMAKER/EXP version 3.0 (Lander et al. 1987), and eight putative QTLs for IHNV resistance were identified. Three were anchored on linkage groups of the male map, and five were anchored on the female map. Assignment of the QTLs to rainbow trout linkage groups was difficult, because several markers had not been mapped before. This was especially true for presumptive QTLs mapped on the female map. However, for the male map, there are syntenic markers that permit the identification of these QTL regions on the standard rainbow trout map (Fig. 3). The first QTL region on the male map was detected on linkage group OA-X (loci *Ssa7i/NVH* and *OtsG78*). A second QTL was mapped to linkage group OA-XXVII, where six AFLP markers (between *GGTCCC60* and *GATCC510*) and three SSR loci (*OmyFGT-1TUF*, *Ssa94NVH*, and *OtsG3*) showed significant association. A third QTL region was anchored in linkage group OA-III close to eight AFLP markers (from *GCTCTC178* to *GATCAG291*) and three SSR loci (*One8ASC*, *OMM1054*, and *Ogo2Ui/W*; Table 6). These three regions bearing presumptive QTLs on the male-based map have been confirmed in a separate analysis of three other families (Rodriguez et al. 2004). Interestingly, the locus *Ogo2Ui/W* has been mapped 1.8 cM from a gene of the major histocompatibility complex (Nichols et al. 2003; Fig. 3). *MHC1-TAP2B* encodes an ATP-binding transporter involved in peptide translocation for MHC class 1 molecules from the cytosol to the endoplasmic reticulum. *Ogo2Ui/W* maps close to locus *Ssa4DU*, which has been linked to a QTL for resistance to the IPN virus (Ozaki et al. 2001; Fig. 3). This is indirect evidence supporting a possible association of this chromosomal region with immunologic functions or resistance/susceptibility to disease. On the other hand, three loci linked with IHNV resistance QTLs (one by each linkage group bearing QTLs) were detected to be duplicated on a previous map, i.e.,

OmyFGT-1TUF (Sakamoto et al. 2000), *Ssa7i/NVH*, and *Ogo2Ui/W* (Nichols et al. 2003a). Also, the QTL mapping was performed on the basis of a male map, which has a reduced recombination rate in comparison with a female map (Sakamoto et al. 2000). Therefore, it is not possible to eliminate the probability of pseudolinkage, an artifact in which markers appear to be linked to QTLs, when in reality they are on different chromosomes. Therefore, the evidence on resistance/susceptibility for IHN virus QTLs must be analyzed carefully and confirmed by other studies.

Only one immunological trait has been mapped on the salmonid genome, and it concerns the activity of nonspecific cytotoxic cells (NCC), analogous to mammalian natural killer cells (NK), in rainbow trout (Zimmerman et al. 2004). In fish, as in mammals, NK-like cells are involved in protection against bacterial and protozoan parasites and the elimination of tumor cells. The mapping strategy was to use 106 DH fish progeny of a cross between clonal lines of the OSU and HC strains of rainbow trout. The fish were screened with 330 AFLP and 39 SSR markers. NK-like activity was evaluated on NCC cells obtained from a blood sample and incubated with ^{51}Cr -label YAC-1 target cells to determine the percentage of cytotoxicity (Ristow et al. 2000). The QTL mapping was performed using CIM implemented in QTL Cartographer software (Zeng 1994). The genetic map was generated using MAPMAKER/EXP v3.0. This study revealed a unique chromosomal region affecting the NK-like cytotoxicity on linkage group OA-XXXI, spanning 25.7 cM between the AFLP markers *saagaag.17.120.osu* and *accagt.11.274.hc*, and accounting for 63.4% of phenotypic variation in the trait (Zimmerman et al. 2004). In this wide region, two SSR loci are also anchored, *Omy1006UW* and *OmyRGT1TUF* (Table 6; Fig. 3). All evidence shows that this QTL is unlinked with other QTL regions affecting disease resistance or immunological-related genes (Zimmerman et al. 2004), although the locus *OmyRGT1TUF* was previously associated with body mass (Martyniuk et al. 2003).

Evidence for multiple QTLs for resistance to a parasitic disease caused by *Ceratomyxa shasta* has been detected using clonal lines of rainbow trout (Nichols et al. 2003b). *Ceratomyxa shasta* is an enzootic myxozoan parasite that requires two hosts to complete its life cycle. Actinospores in the water column infect the digestive tract of salmon and cause intestinal

inflammation, tissue degradation, and posterior mortality. Salmonids that migrate through or are reared where ceratomyxisis is endemic are more resistant or tolerant than fish outside these areas (Nichols et al. 2003b). Clonal hybrid fish were obtained by crossing females of OSU clonal rainbow trout susceptible to parasites with males of ARL or CW clonal lines, and 45 DH progeny were produced. After exposure to parasites, the mortality (live or dead) and days to death of every fish were recorded. Single marker associations for both traits were evaluated using a panel of 343 AFLP markers. Markers that showed evidence of association were tested in multiple regression models (linear and logistic) to determine the number of loci influencing these traits. A linkage map was constructed using MAPMAKER/EXP v3.0 to group and order these markers together (Lander et al. 1987). Interval mapping by regression was used to ascertain the position of binary trait QTLs (Haley and Knott 1992) implemented in r/QTL (Broman et al. 2003). For the metric trait (days to death), a Cox proportional hazard model was used to test differences between survival curves and AFLP genotypes in each marker. The analyses consistently identified three AFLP markers associated with QTL, explaining 41% of total variance. Two of these markers (*acgaga9* and *agcaga6*) were unlinked in the genetic map. The last marker (*acgact12*) was anchored in linkage group OC-17, but synteny with the published genetic map for rainbow trout has not been established (Table 6).

Infectious salmon anemia (ISA) is a viral disease of Atlantic salmon that causes severe losses in the aquaculture of this species (Thorud and Djupvik 1988). Challenge tests for ISA were carried out on two full-sib family and 79 progenies using intraperitoneal injection of infectious material. The mortality was monitored until 50% of the progenies were dead and the cause of death was verified by bacteriological test (Moen et al. 2004b). QTLs for resistance were identified using survival analysis and the transmission disequilibrium test (TDT), two statistically robust methods traditionally used in human genetics to detect associations between single nucleotide polymorphism (SNP) markers and disease (Lin et al. 2004). TDT is a nonparametric association-segregation-based test that compares the number of times that an allele is transmitted or not transmitted from a heterozygous parent to an affected progeny (Spielman et al. 1993).

This test is usually used only in cases where there is previous evidence of a close association between a marker and a candidate gene. However, the test has been successfully applied for the initial detection of associations between markers and QTLs. Survival analysis has been widely used in human medicine for testing medical treatments, and it is expected to be more powerful than TDT, since the variable used is continuous (time of survival of individuals), which is more informative than the binary variable (susceptible/resistant) used in TDT (Moen et al. 2004b). QTLs for resistance to ISA were mapped using 340 AFLP markers. Two AFLP markers (*aaccac74* and *agccta290*) were significant, producing a reduction of approximately 25% of mortality among fish inheriting the band-present allele in both markers. Both markers were anchored to the same linkage group 12f on the Moen et al. (2004a) map, tagging a presumptive QTL for ISA resistance on this chromosome spanning 7 cM. In this same linkage group, the SSR loci *BHMS394*, 17 cM away from *aaccac74*, was mapped (Moen et al. 2004a).

As it has been described in this section, QTL mapping in salmonid is an ongoing activity, and many putative QTLs must be confirmed with more extensive studies. It is noteworthy that in rainbow trout, QTLs for a number of different traits have been mapped to similar chromosomal positions. For example, the linkage group OA-VIII carries QTLs for scales above the lateral line, number of pyloric caeca, spawning date, and time to hatch. Linkage group OA-III carries QTLs for number of pyloric caeca, spawning date, and resistance to IPNV and IHNV (Table 6; Fig. 3). However, other large linkage groups with similar marker saturation do not carry any QTL, suggesting that there is no bias restricting QTL identification to larger or marker-saturated chromosomes. It is possible that these QTLs have pleiotropic effects on several traits, but this is unlikely due to the very different nature of the traits on each linkage group. An alternative scenario is that QTLs for different traits are located in a cluster, but with present map saturation, it is impossible to separate their individual effects. High-resolution QTL mapping, along with molecular marker mapping, physical mapping, positional cloning, and analysis of candidate genes and gene expression will be necessary for a precise identification of the genes determining the phenotypic expression of traits under study.

1.7 Association of Traits with Specific Molecular Markers

The low density of markers on many salmonid genetic maps, and the complete absence of maps for several species, has driven many researchers to look for associations of anonymous markers, generally RAPD or AFLP dominant markers, with monogenic and polygenic traits. The approach most often used is bulked segregant analysis (BSA; Michelmore et al. 1991) or selective genotyping (Darvasi and Soller 1992). Once a polymorphism segregating with the trait of interest is identified, the polymorphic fragment is cut from the gel (agarose or polyacrylamide), purified, cloned, and sequenced to design specific primers for PCR amplification. These primers are tested on genomic DNA, and the amplicons obtained tested for association with the trait of interest. To accelerate the search, the marker screening is usually performed on pools of DNA obtained from five to twenty individuals of the phenotypic extremes (Darvasi and Soller 1994).

1.7.1 Bulked Segregant Analysis

In BSA, the co-segregation of many markers is followed in a segregating population obtained by crossing a single pair of parental fish with different phenotypic expressions for the discrete trait under study. A number of segregating individuals are used in each pool, with the objective of minimizing variation not associated with the trait of interest (Michelmore et al. 1991). This approach was used to identify sex-specific sequence characterized amplified region (SCAR) markers *Omy-163* and *OmyP9* linked to the Y chromosome in rainbow trout using AFLP screening (Felip et al. 2005) or RAPD screening (Iturra et al. 2001a). In salmon aquaculture, the sex phenotype is an important commercial trait. Growers prefer to stock all-female fingerlings because they mature later than males (Table 8; Brunelli and Thorgaard 2004).

BSA was also used to identify markers associated with the dominant albino phenotype in a Japanese strain of rainbow trout (Table 8; Nakamura et al. 2001).

Table 8 Primers sequences and PCR conditions of molecular markers identified as associated with several traits in different salmon species

Marker	Type	Reference	Primers	Trait	Species
<i>OmyP9</i>	SCAR	Iturra et al. (2001a)	F 5'-TGCAATTCCACTTCTCTCGTT-3' R 5'-GACGTCAAAGGTGTCGGTT-3'	Sex	Rainbow trout
<i>Omy-163</i>	SCAR	Felip et al. 2005	F 5'-CTTCTGTCTACCAAAATC-3' R 5'-CATCAAGTCACATGACTAAC-3'	Sex	Rainbow trout
<i>OmyD-AlbnTUF</i>	SSR	Nakamura et al. 2001	F 5'-GAATTACGGAGTGGAGTG-3' R 5'-TCATGATGCCACGGATTCT-3' F 5'-ATGGACAAGTAAGGCCT-3'	Albinism	Rainbow trout
<i>GHI</i>	RFLP(<i>TaqI</i>)-PCR	Gross and Nilsson 1999	R 5'-CTTGAGGTGCTGAGCTC-3' F 5'-ATCGTGACGCCAATCGACAAGCAC-3'	Body Weight	Atlantic salmon
<i>GH2</i>	RFLP(<i>HinfI</i>)-PCR	Forbes et al. 1994	F 5'-GGTACTCCAGGATTCAATCGGA-3' R 5'-GAGGACGTCCACCTTACAATCC-3'	Sex	Coho salmon
<i>Okiz06</i>	SCAR	Araneda et al. 2005	F 5'-GAGGACGTCCACCTTACAATCC-3' R 5'-GAGGACGTCCACCTTACAATCC-3'	Flesh color	Chinook salmon Coho salmon
<i>OrY1</i>	PCR based	Noakes and Phillips 2003	F 5'-GAITGCTGGATTGG-3 R 5'-CCAGCGATGGTTGGTAG-3	Sex	Chinook salmon ¹
<i>OtY2</i>	SCAR	Devlin et al. 1994	F 5'-CTGGTTCGAGCCTAACGTAG-3' R 5'-GATGCAGTAGGAGCAGATG-3'	Sex	Pacific Salmons ²
<i>Omy207</i>	SSR	Heath et al. 2002	F 5'-ACCCTAGTCAGTCAGTCAGG-3' R 5'-GATCACTGTGATAACATCG-3'	Reproductive female traits	Chinook salmon
<i>GHRH/PACAP2</i>	SNP A/G	Tao and Bouling 2003	F 5'-TAAATAAAGCCTACAGGAAAGCGC-3' R 5'-GGCGTCCTTGYTTCTATATCTCT-3'	Early juvenile growth	Arctic charr

NI Not informed.

¹ With exception of sockeye salmon (*O. nerka*) and rainbow trout.² With exception of rainbow trout and not tested in pink salmon (*O. gorbuscha*).

In this case, the AFLP marker could be converted into a microsatellite marker *OmyD-AlbnTUF* due to the presence of pentanucleotide repeats (GGAGT) in the AFLP sequence. This discovery is not infrequent, and we have found variable number tandem repeats (VNTR) motifs, and micro- and minisatellites inside RAPD markers. For example, the dinucleotide microsatellite marker *OkiCAT229UCH* associated with spawning time (GenBank accession number AY926352) was isolated from a salmon coho RAPD marker during SCAR construction (C. Araneda et al. unpublished data). BSA has also been used to identify three RFLP markers associated with resistance to the IHN virus in hybrid fish between rainbow trout females and cutthroat trout males (*O. clarki*) from the facilities of the Department of Fish and Game at Hayspur and Henry's Lake (Idaho, USA), respectively (Palti et al. 1999). However, these three RFLP fragments associated with IHN resistance/susceptibility have not been characterized by cloning and sequencing.

1.7.2 Selective Genotyping

Selective genotyping is similar to BSA with the difference that the polymorphism is scored in only the most extreme phenotypes from the population. The hypothesis that underlies this approach is that the individuals on the ends of distribution for a quantitative trait have a higher probability of sharing more alleles that increase or diminish the trait value, and therefore they are more informative than the individuals in the middle of the distribution (Lander and Botstein 1989). Using a similar approach, but on the basis of breeding values instead of phenotypic values, a dominant SCAR marker (*Oki206*) was associated with variation in flesh color of coho salmon (Table 8; Araneda et al. 2005).

Markers such as *OmyD-AlbnTUF* and *Omy-163* have the advantage that they have been anchored in a recombination map (Fig. 3). The SCAR *OmyP9* has been assigned physically by means of fluorescent in situ hybridization (FISH) to a submetacentric chromosome pair in rainbow trout (Iturra et al. 2001b), but *Oki206* has not been assigned a chromosomal position, because there is not yet a genetic map for coho salmon.

1.7.3

Other Single Locus Markers

Several single markers have been developed for sex discrimination in salmonid fishes. The first step toward this objective was the identification of a growth hormone pseudogene (*GHψ*) present only in males of chinook salmon and other Pacific salmon (Du et al. 1993), with the exception of sockeye salmon (*O. nerka*) and rainbow trout (Devlin et al. 2001). This discovery allowed the development of a male-specific, PCR-based marker (*OtY1*) for chinook salmon (Devlin et al. 1994). A *HinfI* RFLP in intron C of growth hormone gene *GH2* has also been described as sex linked in coho and chinook salmon (Table 8; Forbes et al. 1994). And, as mentioned previously, two allozymes (*bGLUA** and *sSOD-1**) are sex linked in rainbow trout (Allendorf et al. 1994).

Local liver expression of PGM-1 allozyme alleles coded by the *PGM-1r** regulatory locus are associated with an increase of embryonic developmental rate (time to hatch) and age at first reproduction in rainbow trout, possibly due to an accelerated metabolism on the glycolysis pathway in fish embryos (Allendorf et al. 1983). However, a syntenic locus in Atlantic salmon does not show a similar phenotypic effect (Verspoor and Moyes 2005).

Evidence of association of a PCR RFLP (*TaqI*) in the growth hormone (*GH1*) gene with body weight has been reported in Atlantic salmon. Different haplotypes at this marker show differences in size (Table 8; Groos and Nilsson 1999). An A/G polymorphism in the growth hormone-releasing hormone and pituitary adenylate cyclase-activating polypeptide genes (*GHRH/PACAP2*) accounts for 9.4% of the phenotypic variance in juvenile growth rate of Arctic charr. The heterozygous genotype A/G is associated with faster growth rate, and the homozygous genotype G/G shows suggestive association with early gonadal development, which may help to predict early maturation in males (Table 8; Tao and Boulding 2003).

Finally, a population analysis of correlations between heterozygosity and reproductive fitness in chinook salmon shows that one SSR locus (*Omy207*) is significantly associated with female reproductive traits, such as relative fecundity, egg size, and egg survival (Table 8; Heath et al. 2002). This locus has been proposed as a good candidate for further QTL analysis of reproductive traits in this species.

1.8

Physical Mapping

Physical maps can be constructed by a variety of different approaches and can be classified into two general types. Cytogenetic maps describe the order and spacing of markers on a DNA molecule based on microscopic analysis. Cytogenetic maps show the specific physical locations of genes and/or molecular markers on each chromosome. The second type of physical map consists of a set of cloned fragments of DNA that represent a complete chromosome or chromosomal segment, together with information about the order of the cloned sequences. Both types of physical maps are being developed in salmonids.

The complexity of salmonid genomes derives from the existence of duplicate loci arising from an ancestral tetraploid event. This introduces an additional difficulty in the construction of genetic maps in these species. Furthermore, it is known that differences in genetic recombination among the sexes are due to differences in the meiotic behavior of the chromosomes between males and females. Additional chromosome variation has arisen from Robertsonian rearrangements, inversions, and tandem translocations. So, the assembly of genetic and physical maps is of particular importance in these species.

1.8.1

Karyotypes

The cytological characteristics of salmon genomes have been widely described and are summarized in Table 9. The diploid number ($2n$) ranges from 52 to 84 chromosomes with a DNA content (C-value) ranging from 2.23 to 3.70 pg (Ojima et al. 1963; Johnson et al. 1987; Phillips and Ráb 2001; Hardie and Hebert 2004).

The chromosome evolution of salmonid species has been reviewed by Hartley (1987) and, more recently, by Phillips and Ráb (2001). These authors have proposed two categories of karyotypes in this species: Type A and Type B based on chromosome numbers, and two new subcategories, A' and B', according to the variation of the chromosome arm number (NF). As is shown in Table 9, cultured species of the genus *Salvelinus* and *S. trutta* belong to the Type A karyotype with

diploid chromosome numbers between $2n = 78 - 84$ and NF = 98–102. All *Oncorhynchus* species show the Type B karyotype with $2n$ around 60 chromosomes. *S. salar* belongs to Type B' karyotype with an NF = 72–74, lower than the typical salmonid NF, which is around 100 (Phillips and Ráb 2001).

Intraspecific polymorphism as a consequence of Robertsonian rearrangements is well documented in wild and farmed populations of rainbow trout, brown trout, and Atlantic salmon (Thorgaard 1983; Colihueque et al. 2001; Phillips and Ráb 2001). Different salmonid species also present structural chromosomal polymorphisms, including changes in the position of the nucleolar organizer region (NOR; Porto-Foresti et al. 2002), variation in the number and size of this chromosome region (Phillips and Hartley 1988), and in the distribution patterns of the constitutive heterochromatin (Phillips and Ráb 2001).

The application of fluorescence in situ hybridization (FISH) is one of the most powerful tools for the construction of cytogenetic maps in fishes. This method allows the localization of DNA sequences in the metaphase and meiotic chromosomes and in the nucleus. The DNA sequences used as probes can correspond to individual genes, specific regions of chromosomes, complete chromosomes, and even complete genomes (Phillips and Reed 1996). FISH probes are labeled with modified nucleotide precursors tagged with biotin or digoxigenin by enzymatic methods or PCR. They are then hybridized to denatured chromosomes and detected at the site of hybridization using proteins linked to fluorescent dyes. The chromosomes are observed by epifluorescence microscopy equipped with digital imaging devices, such as cooled charge-coupled device (CCD) cameras. The sensitivity of these cameras has permitted the development of FISH probes with modified nucleotides directly conjugated to a fluorescent dye.

During the last few years, bacterial artificial chromosomes (BAC) libraries (Katagiri et al. 2001; Palti et al. 2004) and a considerable database of expressed sequence tags (ESTs) (Rexroad et al. 2003, 2005) have become available for several species of salmonids. These resources represent important progress toward the creation of high-resolution genetic maps for these species. BAC and cDNA libraries for several salmonid species are also a valuable resource for preparing FISH probes. Identification of polymorphic genetic

Table 9 DNA content (pg), chromosome diploid number ($2n$), arm chromosome number (NF) and karyotype categories (follows Phillips and Rab 2001) of cultured salmonid species

Species	C-value (pg)	$2n$	NF	Type
Oncorhynchus				
<i>O. mykiss</i>	2.4 ¹	58–64	104	B
<i>O. kisutch</i>	2.6 ²	60	100	B
<i>O. masou</i>	2.07–3.29 ³	66	100	B
<i>O. tshawytscha</i>	2.45 ¹	68	100	B
<i>O. gorbuscha</i>	2.57 ²	52–54	100	B
<i>O. nerka</i>	3.04 ³	57♂–58♀	100	B
<i>O. keta</i>	2.97 ³	74	100	B
Salmo				
<i>S. salar</i>	3.27 ¹	54–58	72–74	B'
<i>S. trutta</i>	2.94 ³	78–84	98–102	A
Salvelinus				
<i>S. alpinus</i>	3.70 ¹	78	98	A
<i>S. fontinalis</i>	3.27 ¹	84	100	A
<i>S. namaycush</i>	3.04 ¹	84	100	A
Coregonus				
<i>C. clupeaformis</i>	2.44 ¹	80	94	A

¹ Hardie and Hebert 2004

² Johnson et al. 1987

³ Ojima et al. 1963

markers in these clones provides an excellent tool for the integration of physical and linkage genetic maps. Moreover, it will enrich the comparative studies among the salmonid species and contribute to an understanding of the genomic architecture of these species.

1.8.2

Physical Mapping of Centromeric Repeats

Centromeric repeated sequences show very little evolutionary conservation among vertebrates, even among closely related species. This is also true of the centromeric sequences that have been isolated from the genome of some salmonid species. Three families of centromeric repeated sequences have been isolated in *Salvelinus* species by Hartley and Davidson (1994) and Reed et al. (1995). Physical mapping, using the sequences *AluI/RsaI*-Type A, *AluI/RsaI*-Type B, and

DraI/EcoRI as FISH probes on the chromosomes of *S. alpinus*, *S. fontinalis*, and *S. namaycush*, showed that these sequences are located mainly in the centromere of several metacentric chromosomes as well as in some acrocentric chromosomes in these species. Experiments using dual-FISH probes with combinations of these sequences show that the centromere of some chromosomes is characterized by more than one type of sequence. These studies have demonstrated that for each *Salvelinus* species, there is a unique pattern of hybridization of these centromeric sequences (Phillips et al. 2002). Recently, Viñas et al. (2004) have isolated a highly repeated *HpaI* DNA family from the *Salmo salar* genome. This sequence, conserved in *S. trutta*, is absent in the genome of *Oncorhynchus* but shows certain similarity with *AluI/RsaI* in *Salvelinus*. FISH using *HpaI* sequences shows hybridization in the centromere of three pairs of acrocentric chromosomes of the 29 pairs that constitute the karyotype of *S. salar*.

1.8.3

Physical Mapping of Telomeric Repeated Sequences

In vertebrates, telomeres constitute the physical end of the chromosomes, and the subjacent DNA corresponds to highly conserved sequences organized in tandemly repeated, G-rich sequences like $(TTAGGG)_n$. Probes of telomeric sequences constitute a valuable tool in the characterization of chromosomal rearrangements, like tandem fusions, that are implied in the intraspecific chromosomal variation in diverse species of salmonids (Hartley 1987; Phillips and Ráb 2001). Two strategies have been used for mapping the telomeric sequences using FISH. Perez et al. (1999) isolated and cloned a DNA sequence containing telomeric sequences of *S. salar*. They used a clone containing the sequence $(TTAGGG)_a$ as a FISH probe, and it showed hybridization only in the terminal ends of all the Atlantic salmon chromosomes. Abuin et al. (1996) described the distribution of telomeric sequences using $(TTAGCG)_n$ oligonucleotides as a probe to hybridize on chromosomes of *S. salar*, *S. trutta*, *O. kisutch*, and *O. mykiss*. They obtained similar results, but in addition detected interstitial hybridization in a long chromosome pair in *S. salar*. The detection of interstitial telomere sites in chromosomes of brook trout and androgenetic individuals of rainbow trout (*Oncorhynchus mykiss*) using primed in situ labeling (PRINS) has been reported by Ocalewicz et al. (2004a) and Ocalewicz et al. (2004b).

1.8.4

Physical Mapping of Multicopy Genes: Ribosomal Genes

Vertebrates have two units that code for ribosomal RNA genes (rDNA). The major unit, containing genes for 5.8S, 18S, and 28S rRNA, is repeated in tandem, separated by intergenic spacers, and located in one or several specific chromosomal regions called nucleolar organizer regions (NORs). In metaphase chromosomes, the NOR-bearing chromosomes are identified by means of AgNOR staining, because they have been transcriptionally active in the preceding interphase. In fishes, it is also possible to identify the NOR-bearing chromosomes using the fluorescent

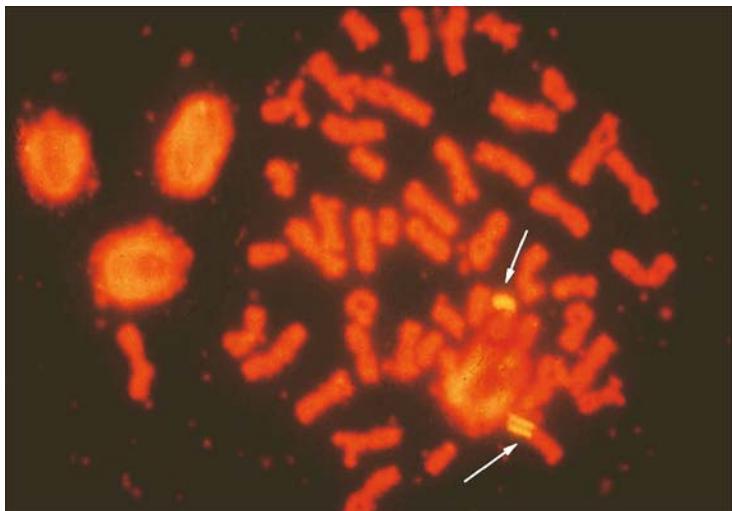
stain chromomycin A3. This fluorochrome has a great affinity to CG-rich regions of the 18S + 28S genes. However, FISH has been the most informative tool for mapping the ribosomal genes in salmonid karyotypes. It has been established that the coding sequences of the 18S + 28S rDNA genes in vertebrates are evolutionarily conserved, which makes it possible to map these genes using heterologous FISH probes.

The minor units contain the 5S rDNA genes and consist of tandem repeats of a highly conserved coding sequence of 120 bp, flanked by non-transcribed spacers (NTS). FISH studies of the 5S rDNA genes in salmonid genomes indicate that these genes are localized to one or several loci (Pendas et al. 1994; Moran et al. 1996; Fujiwara et al. 1998; Pardo et al. 2000; Iturra et al. 2001a; Stein et al. 2001).

FISH studies have revealed that in rainbow trout, the ribosomal 18S + 28S genes are located in a single medium size submetacentric chromosome pair, which agrees with the results obtained with silver staining and chromomycin A3 (Fujiwara et al. 1998; Porto-Foresti 2002). Nevertheless, variation in the position of 18S + 28S genes in the NOR-bearing chromosomes has been detected in Brazilian farmed stocks of rainbow trout as the result of pericentric inversions (Porto-Foresti et al. 2002). A single chromosome pair bearing the 18S + 28S genes has been demonstrated in *Oncorhynchus masou* (Fujiwara et al. 1998) and in *O. kisutch* (Fig. 5). Different results were observed in *Salmo* and *Salvelinus*. *S. trutta* presents brilliant signals of hybridization in one pair of chromosomes and dispersed weak signals in eight chromosomes, while in Atlantic salmon, the ribosomal genes map to a single chromosomal pair (Pendas et al. 1993a, b). In Arctic charr, lake trout, and brook trout, these genes are dispersed over several chromosomes (Reed and Phillips 1995; Phillips 1996; Fujiwara et al. 1998).

The 5S rDNA probes show a diverse distribution pattern of these genes in the karyotypes of salmonids. In *Salvelinus*, the chromosomal distribution of the 5S rDNA sequences varies from a single metacentric pair in *S. fontinalis*, to three pairs in *S. namaycush*, and 3–4 acrocentric pairs in *S. alpinus* (Phillips et al. 2002). One locus of 5S rDNA genes in rainbow trout and Atlantic salmon is located adjacent to the cluster of 18S + 28S ribosomal genes (Moran et al. 1996; Fujiwara et al. 1998; Iturra et al. 2001a). This relationship among NORs and 5S rDNA genes is also observed in Brazilian rainbow trout stocks in which these clus-

Fig. 5 Chromosomal mapping of 18S + 28S rDNA genes labeled with biotin on metaphase chromosomes of *O. kisutch*. Arrows show the NOR bearing pair with a bright hybridization signal along the long arm. (Source: Lam and Iturra unpublished)



ters are located in a different position in the chromosome (Porto-Foresti et al. 2002). In several rainbow trout strains, the second locus of the 5S ribosomal genes is located on the X chromosome (Moran et al. 1996; Iturra et al. 2001a; Ocialewicz 2002; Phillips et al. 2004). In chinook salmon, the 5S rDNA genes map on the Y chromosome, as well as on several other acrocentric chromosomes (Stein et al. 2001). Our results of physical mapping of 5S ribosomal genes in coho salmon indicate that these gene clusters are located in a medium size metacentric pair in males and females (N Lam et al. unpublished). Furthermore, we observed brilliant signals in the short arm of a single acrocentric chromosome in the males, which could correspond to a Y chromosome in this species (Iturra et al. 2001a; Lam 2002). The singular distribution of the 5S ribosomal genes in the genome of male and females of these species suggests the existence of differential regulation of gene expression among the sexes (Martins and Galletti 2001; Phillips et al. 2004).

1.8.5

Physical Mapping of Coding Genes

Physical maps of coding genes are at an early stage of development in salmonid species. Nevertheless, the currently ongoing genomics projects will make advances possible through the availability of resources, like BAC clones and ESTs, from which FISH probes can be prepared. An example is the elegant physical and genetic mapping of the major histocom-

patibility complex (MHC) in rainbow trout (Hansen et al. 1999; Phillips et al. 2003). Hansen et al. (1999) demonstrated by segregation analysis that rainbow trout MHC class I and II regions are located on different linkage groups. Recently, these authors used FISH probes of different BAC clones containing the MHC class II gene (*DAB*) class Ia and Ib loci and *ABCB2* gene respectively to demonstrate that the rainbow trout MHC regions are located on at least four different chromosomes, 17p, 3p, 18q, and 14q (Phillips et al. 2003).

Unique anonymous markers have been found within BAC clones, making it possible to link these probes to specific linkage groups in rainbow trout (Nichols et al. 2003a). A good example is the chromosome localization of the rainbow trout vitellogenin gene (*Vtg*) cluster. The *Vtg* genes form a large and complex family in the rainbow trout genome located on the short arm of an acrocentric pair, as demonstrated using FISH by Trichet et al. (2000). The multigene families for each of the five histone genes were physically mapped in rainbow trout, Atlantic salmon, and brown trout karyotypes (Pendas et al. 1994). They found hybridization signals of the histone DNA probes on a single submetacentric chromosome pair in the three species, suggesting that the histone cluster maps to a single locus (Pendas et al. 1994). Perez et al. (2000) isolated and studied the physical location of the methionine tDNA in the genome of Atlantic salmon and brown trout. They found evidence for a single locus in the chromosomes of both species.

1.8.6

Physical Mapping of Sex Markers

Fishes are characterized by the diversity of their sex determination systems. The most common genetic mechanism of sex determination among vertebrates is the existence of cytological differentiated sex chromosomes in one of the sexes. This situation is rather uncommon in the fish species studied until now. In many cases, the heterogametic sex has been identified by experimental crosses with sex reversal specimens (Yamazaki 1983). Male heterogamety has been identified in salmonid species, and this has been confirmed by cytogenetic evidence in lake trout, brook trout, rainbow trout, and sockeye salmon (Thorgaard 1977, 1978, 1983; Phillips and Ihssen 1985; Phillips and Hartley 1988). More recently, sex-linked molecular markers and FISH probes have given support to an XY sex determination system in salmonid species, but the sex chromosomes are morphologically indistinguishable or only slightly differentiated in the karyotype (Forbes et al. 1994; Devlin et al. 1994, 1998; Iturra et al. 2001a, b; Zhang et al. 2001; Stein et al. 2002; Phillips et al. 2001, 2002; Woram et al. 2003; Brunelli and Thorgaard 2004).

In *S. namaycush* and *S. fontinalis*, the sex chromosome pair corresponds to the largest submetacentric pair, which shows a heterochromatic band only on the short arm of the X chromosome (Phillips et al. 2001). Sex chromosome paint probes for FISH obtained by microdissection of the Yp of *S. namaycush* and a Yq-specific probe prepared from the largest acrocentric chromosome pair in rainbow trout have been used to investigate the sex chromosomes in North American charr species and the possible homology among sex chromosomes in rainbow trout and chinook salmon, and Atlantic salmon and brown trout (Phillips et al. 2001, 2002). The probes hybridized to the largest submetacentric chromosome pair in all of the studied charr species. However, the authors suggest that linkage studies are necessary to determine if this chromosome pair is conserved in all *Salvelinus* species (Phillips et al. 2002). On the other hand, the hybridization pattern with these probes in rainbow trout, chinook salmon, brown trout, and Atlantic salmon suggests that the sex chromosomes of these species would not show homology, but that they have evolved independently (Phillips et al. 2002).

Sequences related to the sex chromosomes have been mapped by FISH in chinook salmon, rainbow trout, and coho salmon. *Oty1* is a male-specific repetitive DNA sequence isolated from the chinook salmon genome by Devlin et al. (1991) and used as a male sex marker to identify the genetic sex by PCR in this species (Table 8; Devlin et al. 1994). This Y-chromosome-specific sequence is located in a distal position of the short arm of a medium size acrocentric chromosome identified as the Y chromosome in this species by Stein et al. (2001). The sex pair in rainbow trout is characterized by small differences in the length of the short arm among subtelocentric X and Y chromosomes (Thorgaard 1977, 1983). The hypothesis that the X and Y chromosomes of rainbow trout appear to be in an initial stage of differentiation has been supported by the finding that some male specimens from different natural and cultured populations do not show heteromorphism between X and Y chromosomes (Thorgaard 1977, 1983; Colihueque et al. 2001). Three sex molecular markers have been physically located on the sex chromosomes of rainbow trout. *OmyP9* (Table 8) is a polymorphic SCAR marker that shows FISH-hybridization to the sex chromosomes in rainbow trout (Iturra et al. 1998, 2001a, b; Lam et al. 2003). The *OmyP9* probe hybridizes in the medial region of the long arm of the acrocentric/subtelocentric Y chromosome. These results, together with the *OmyP9* pattern of inheritance in several Chilean-farmed rainbow trout strains, allows us to suggest the proximity of *OmyP9* to the sex determination locus in this species (Iturra et al. 2001b).

Recently, two new sex linked markers, SCAR *Omy-163* (Table 8) and SNP-*B4*, have been physically and genetically mapped on the Y chromosome of rainbow trout (Felip et al. 2004, 2005; Phillips et al. 2004). Both probes hybridize on the long arm of the sex chromosome pair but in different chromosomal positions. SNP-*B4* is localized close to the centromere, and SCAR *Omy-163* hybridizes distal to the first one, in the same region on the long arm as *OmyP9*. The Y chromosome location of all of these three markers supports the hypothesis that the sex determination locus is localized on the long arm of the sex chromosomes in rainbow trout (Iturra et al. 1998, 2001a; Felip et al. 2004, 2005; Phillips et al. 2004). Other molecular markers related to sex in this species correspond to AFLPs and microsatellites that have been used in the

construction of genetic maps of the sex-linked group in several rainbow trout strains, including clonal lines (Young et al. 1998; Sakamoto et al. 2000; Nichols et al. 2003a; Woram et al. 2003; Palti et al. 2004; Felip et al. 2005). Iturra et al. (2001b) used *OmyP9* to identify the sex chromosomes in coho salmon. This probe shows fluorescent signals in the medial region of the long arm of a subtelocentric pair with similar morphology as rainbow trout sex chromosomes, being identified as the sex pair in salmon coho (Lam 2002). Recently, Phillips et al. (2007) have identified the sex chromosomes of *O. gorbuscha* and *O. keta* using a probe to the male-specific GH-Y (growth hormone pseudogene).

Different approaches have been used to construct genetic linkage maps to identify syntenic markers which might help identify the primary sex-determining locus in salmonids. Recently, comparative maps of the sex-determining region among salmonid species, using mainly microsatellite and AFLP markers, have been published (Woram et al. 2003; Marshall et al. 2004; Felip et al. 2005). These studies confirm the sex linkage of markers among several species of salmonid. An example is the male-specific pseudogene *Ghy*, which is linked to the Y chromosome in *Oncorhynchus*, but not in rainbow trout, sockeye salmon, or *Salmo salar* (Devlin et al. 2001). Also, the Y-linked marker *Oty1* in chinook salmon is not sex-linked in rainbow trout (Noakes and Phillips 2003). A new Y-specific chromosome marker derived from an AFLP polymorphism has been described by Brunelli and Thorgaard (2004), which is useful in chinook and coho salmons, but not in rainbow trout, among other salmonid species. The availability of the cloned probes for FISH studies will be useful in the construction of sex chromosome maps for these species.

1.9 Integration of Genetic and Cytogenetic Maps

The full utilization of genetic maps requires knowledge of the correspondence between the genetic and cytogenetic maps. Several methods have been used to correlate genetic and cytogenetic maps (Stephens et al. 2004). FISH provides the most direct way of

physically mapping DNA sequences on chromosomes. However, FISH mapping of fish genomes depends heavily on the development of recombinant DNA BAC libraries.

Recently, thanks to the information generated by genomics projects in rainbow trout, it has been possible to assign linkage groups to specific chromosomes of rainbow trout by means of FISH (Phillips et al. 2006). In this case, BAC clones containing markers previously identified from each linkage group were used as fluorescent probes.

The genetic and cytogenetic maps will be helpful for the QTL mapping in salmonids for breeding applications and for conducting genome comparisons between these species. Moreover, cytogenetic maps can quickly sort out duplicate loci, which help in building contigs, to determine linkage groups for duplicated loci from FISH experiments, and to determine which linkage groups are fused in other strains and species. SNPs mined from BACs could provide haplotypes of linked markers for population studies (Phillips et al. 2006).

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2 Cyprinids

Laszlo Orban^{1,2} and Qingjiang Wu³

¹ Reproductive Genomics Group, Temasek Life Sciences Laboratory, Singapore

e-mail: laszlo@tll.org.sg

² Department of Biological Sciences, National University of Singapore, Singapore

³ Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, People's Republic of China

2.1 Introduction

Cyprinids are arguably the most important group of freshwater teleosts. Their most popular representative, the common carp, is the most extensively cultivated fish species and perhaps the best-known teleost in the world. A number of detailed reviews have been published about the genetics and aquaculture of common carp (e.g., Balon 1995; Gomelsky 2003; Horvath and Orban 1995; Vandepitte 2003). Some of them discussed these topics in wider context by including results on related cyprinids as well (e.g., Hulata 1995b, 2001; Penman 2005).

In this chapter, we aim to review the history and current status of cyprinid genetics and genomics. Throughout the whole chapter, we use every opportunity to broaden the topic with comparative aspects by including information about other cyprinids and occasionally other teleost species important for research or aquaculture.

2.2 The Biology and History of Farmed Cyprinids

2.2.1 The Taxonomy and Physiology of Farmed Cyprinids

Cyprinids are ray-finned teleosts (Class *Actinopterygii*) from the infraclass *Teleosteii* and the order *Cyprinidae*.

According to FishBase¹ the family *Cyprinidae* contains 210 genera and over 2,000 species (Froese and Pauly 2005).

The typical features of the cyprinid body are the following: toothless jaw, pharyngeal teeth in the throat (ideal for characterization), the presence of the Weberian organ at the beginning of the vertebrate column, and the complete lack of spiny rays on the dorsal fin. Cyprinid species naturally occur in wide-ranging habitats in the northern temperate zone and the tropical regions as well. Most of them are confined to freshwater bodies; only a few species are found at estuaries. For a review on morphological and taxonomic analysis of cyprinid subfamilies and their geographical distribution, see Howes (1991).

Cyprinids exhibit a wide variety of spawning behaviors (Mills 1991). Some species, such as common carp and goldfish, lay their adhesive eggs onto the vegetation or the bottom. Others drop their pelagic eggs in the stream (grass carp and silver carp) or shed their eggs in gravelly bottoms (zebrafish). Due to their wide geographical range and excellent adaptational abilities, cyprinids play an important role in the food chain of natural freshwater bodies and in the ponds of commercial fisheries as well.

The selected set of most important cyprinid species discussed in this chapter is shown in Table 1. In addition to common carp, the so-called "Chinese carp" species (i.e., bighead carp, grass carp, and silver carp) are the most important for aquaculture. Additional cyprinids often used in culture (and/or research) are the following: goldfish, ginbuna, Prussian

¹ For a list of important and useful websites related to this topic, see the end of the chapter.

Table 1 The most important cyprinid species and subspecies for research and aquaculture

Common name	Latin name*	Haploid Chr. No	Chr. sets	Genome size	Haploid CV*** (pg)
Bighead carp	<i>Aristichthys nobilis</i> (Richardson, 1845)	24	2	Small	1.04 (0.01)
Grass carp	<i>Ctenopharyngodon idella</i> (Valenciennes, 1844)	24	2	Small	1.02 (0.03)
Silver carp	<i>Hypophthalmichthys molitrix</i> (Valenciennes, 1844)	24	2	Small	1.01 (0.02)
Zebrafish	<i>Danio rerio</i> (Hamilton, 1822)	25	2	Medium	1.78 (0.25)
Common carp	<i>Cyprinus carpio</i> (Linnaeus, 1758)	50	2	Medium	1.79 (0.10)
Crucian carp	<i>Carassius carassius</i> (Linnaeus, 1758)	50	2	Medium	1.99 (0.21)
Goldfish	<i>Carassius auratus auratus</i> (Linnaeus, 1758)	50	2	Medium	1.78 (0.14)
Ginbuna	<i>Carassius auratus langsdorffii</i> (Temminck and Schlegel, 1846)	U**	3	Large	2.55
Prussian carp	<i>Carassius gibelio</i> (Bloch, 1782)	50	3	Large	2.92 (0.21)

* – According to FishBase (Froese and Pauly 2005), and to Global Biodiversity Information Facility (GBIF; accessed through www.gbif.net, 2007.04.01).

** – Unknown

*** – According to Animal Genome Size Database (Gregory 2005a). For species with multiple measurements the median value (+/- S.D.) is displayed here.

carp (silver crucian carp or gibel carp), and crucian carp. The list is completed by the striped pet fish commonly known as zebrafish. This species, which has become one of the major vertebrate research models during the past three decades, also belongs to the family *Cyprinidae*. Research on this species provides useful data, especially for the comparative aspects. Additional cyprinid species, such as the black carp (*Mylopharyngodon piceus*; Richardson 1846), the so-called “Indian major carps,” such as the catla (*Catla catla*; Hamilton 1822), the mrigal (*Cirrhinus cirrhus*; Bloch 1795), and the rohu (*Labeo rohita*; Hamilton 1822; Jhingran and Pullin 1988; Singh and Mittal 1990) or the tench (*Tinca tinca*; Linnaeus 1758; Buchtova et al. 2003, Flajshans et al. 1993a), are not being discussed in detail here, and they are only referred to occasionally.

2.2.2 Classical Studies on the Taxonomy and Origin of Farmed Cyprinids

Most experts agree that common carp originated from East Asia and apparently split from other members of the *Cyprininae* subfamily in the middle of the Miocene (Zardoya and Doadrio 1999). There are, however, several different hypotheses on the phylogeography of

the various phenotypic forms of common carp that exist today. According to Berg (1964), carp originally inhabited a continuous range in Eurasia, which was divided later into eastern and western populations during the Pleistocene. Balon’s opinion was entirely different: he postulated that common carp originated from the area bordered by the drainages of the Black, Caspian, and Aral seas and later dispersed to the east and west (Balon 1995).

On the basis of morphological differences, four different subspecies of common carp have been proposed by Kirpichnikov (1967): European-Transcaucasian carp (*Cyprinus carpio carpio*), Central Asian carp (*C.c. aralensis*), East Asian carp (*C.c. haematopterus*) and South Asian carp (*C.c. viridiviolaceus*). Wu (1977) accepted only three subspecies with different zoogeographical ranges: *C.c. carpio*, *C.c. haematopterus*, and *C.c. rubrofuscus*². *C.c. carpio* had the widest range from the Danube River to the Volga River, with additional populations in the Xinjiang Uygur Autonomous Region of China; *C.c. haematopterus* originated from north of the Nanning Mountains in China, whereas *C.c. rubrofuscus*

² *Cyprinus carpio rubrofuscus* is also mentioned in the literature as *C.c. viridiviolaceus* and *C.c. nigroauratus*. FishBase classifies this taxon on the basis of morphological data as a separate species (*C. rubrofuscus*); however, on the basis of molecular data, we refer to it as a subspecies in this chapter.

originated from south of the Nanling Mountains. Balon (Balon 1995) listed only two subspecies: *C.c. carpio* and *C.c. haematopterus*, which show distinctly different morphological characteristics as described earlier by Svetovidov (1933). He questioned the existence of the other subspecies.

There is no data on the exact origin of carassids in the peer-reviewed literature. The ancestors of this group are thought to have originated from the Far East. There are two different species from the *Carassius* genus discussed in the chapter, namely *C. carassius* and *C. auratus*. The second species is divided into three subspecies: the goldfish (*C.a. auratus*), the Prussian carp or silver crucian carp (*C.a. gibelio*³), and the ginbuna (*C.a. langsdorffii*). The goldfish has more phenotypic forms than any other ornamental aquarium species (Smartt and Bundell 1996).

The Prussian carp and ginbuna differ from the rest of the members of the group as they are both triploid and gynogenetic. The former is widely distributed in China, Russia, and large parts of Europe, whereas the latter is found only in Japan.

According to FishBase (Froese and Pauly 2005), zebrafish is native to several South Asian countries. On the basis of original collection data, the geographic range of the species has been reconstructed as follows: "from Pakistan in the west to Myanmar (Burma) in the east and from Nepal in the north to the Indian state of Karnataka in the south" (Engeszer et al. 2007).

2.2.3

The History of Cyprinid Aquaculture

The Aquaculture of Common Carp

Ancient Chinese documents show that cultivation of common carp started around 4,000 years ago. Pond fish culture in China began in the Shang Dynasty, twelfth century BC, with common carp as the main cultivated species. Common carp has been reared on a large scale in China since the Zhou Dynasty, fifth to second century BC. The book of Fan Li from 460 BC (*Fish Culture Classic*) is the earliest written document about fish cultivation. It stimulated prosperous development of freshwater fish cultivation in China at the end of the Zhou Dynasty and had a positive influence

on common carp culture of South Asia and later on that of Europe as well.

During the Han Dynasty (from 206 BC to 220 AD), common carp cultivation reached its prosperous period. During that period, common carp was cultured as the primary species, not only in ponds, but in open water bodies as well. Records from the age of the Han Dynasty, such as *History Record* by Shima, show that common carp was cultured in waters spanning hundreds of hectares. During the Tang Dynasty (618–907 AD), common carp culture was banned because the Chinese name of common carp ("Li") was same as the emperor's family name, thus common carp was regarded as a symbol of their family. The legislation banned people from sacrificing and selling common carp, and captured individuals had to be returned to the river.

It is unknown when exactly common carp was first cultured Japan, but according to Chiba and colleagues (1966), a pond was built in Kawachi Province in 31 BC. Although a Chinese book of the Western Jin Dynasty (fourth century) mentioned carp with various colors, systematic koi carp breeding is thought to have begun only around the seventeenth century in Japan. Monks were probably responsible for some of the early carp introductions to Southeast Asia (Steffens 1999).

According to the prevalent view (e.g., Chiba et al. 1966; Günther 1868; Vooren 1972), the ancestor of European domestic carp was the Asian common carp, which was transported from Asia to Europe during the ancient Greek and Roman times. The oldest written European document is apparently from the sixth century AD: "Among the older writers Cassiodorus, of the sixth century, terms the *Carpa* of the Danube a costly fish of delicate flavor and supplied to the table of princes," as cited in Day (1880). Thus it appears likely that Europeans began to captivate carp for consumption before the sixth century. From the thirteenth century AD onwards, German and other European pisciculturists had begun keeping Danube wild carp in their ponds, mainly at monasteries. Carp breeding was mentioned at that time in documents from Austria and later spread to other countries (e.g., France and Denmark). There are indications that carp cultivation was practiced in the British Isles as early as the fourteenth century (Lever 1996). From around the fifteenth century AD, meals made from common carp became very popular at Christmas dinners in several Eastern European nations, such as Czechs, Hungar-

³ FishBase refers to it as a separate species (*C. gibelio*).

ians, and Poles. By the end of the sixteenth century, domesticated Danube carp had spread throughout Europe, and the mountain areas of Bohemia became the center of pond culture.

Unfortunately, no accurate records are available concerning the origin of pond-based carp culture in Russia. It is believed that Danube carp was transferred into the European part of Russia in the Middle Ages; nevertheless, the Amur River wild carp might have been found earlier in the ponds of eastern Russia.

Common carp was introduced to America from France in 1831 (Lachner et al. 1970) and later from Asia and Europe to California in 1872 and 1877 (Moyle 1976). The precise date for the introduction into Australia is unknown. According to estimates, it happened between the 1850s and 1870s (Brown 2005). Farming of common carp in Israel was initiated in 1939 through a number of introductions, mainly from Europe (Hulata 1995a).

solution to reduce egg stickiness during egg incubation (Woynarovich 1961) revolutionized carp culture by making hatchery-induced breeding possible (for review, see Horvath et al. 1984). Cryopreservation of the sperm has further extended the possibilities of carp aquaculture (Cognie et al. 1989; Horvath et al. 2003; Lubzens et al. 1997; Magary et al. 1996).

Live strain banks of common carp (and to a lesser extent of other cyprinid species, such as Chinese carp and tench) have been established at Szarvas (Hungary; Csizmadia et al. 1995; Gorda et al. 1995), Vodnany (Czech Republic; Flajshans et al. 1999), and Golysz (Poland; Rakus et al. 2003). Besides acting as centers for preserving the genetic diversity of the species, they have also served as focal points of breeding and selection work. Activities at these strain banks have yielded several valuable man-made hybrid varieties, such as the Szarvas 215 and the Szarvas P.31 three-line hybrids (Bakos and Gorda 1995), for the benefit of aquaculture production all over the world.

Man-Made Common Carp Varieties and Their Propagation

The various selection programs used over time had yielded different varieties in pond-cultured common carp populations. More than a dozen different common carp varieties can be found in China. Among these Chinese common carp varieties, some are distinguished and are widely cultured: Xinguo red carp, purse red carp, and Qingtian color carp originated from *C.c. haematopterus*, whereas big belly originated from *C.c. rubrofucus*.

During the nineteenth century, many distinctive varieties of common carp were developed in Germany, Bohemia, and Slovakia, such as Aischgründer and Galician mirror carp, leather carp, Bohemian, etc. Several distinct varieties of the carp and commercial hybrids have been generated – mostly in the twentieth century – in Russia, among them the so-called Ukrainian breed, the Ropsha breed (produced by long-term selection of hybrids between Galician mirror carp and Amur wild carp), and the Krasnodar breed, developed for increased resistance against dropsy (Kirpichnikov 1967, 1999; Kirpichnikov et al. 1993). Some varieties or inbred strains (e.g., Blue-grey, Gold, and Dor-70) were developed in Israel from local carp by crossing between replicate groups within selected lines.

The development of various technologies, such as hormonal induction of brooders and salt-carbamide

The Aquaculture of Other Cyprinid Species

When common carp culture was banned under the Tang Dynasty (618–907 AD; see above), Chinese fish farmers realized that other cyprinid teleosts, such as silver carp, bighead carp, grass carp, and black carp, were also quite suitable for freshwater cultivation. They started to collect their fry from the Changjiang and Pearl rivers during spawning season. This practice continued later – even after the ban on the culture of common carp was lifted – and lasted more than a thousand years. During that period, these four species – together with common carp – became essential for the traditional Chinese polyculture.

In the polyculture system, silver carp, bighead carp, grass carp, black carp, and common carp are co-stocked in the same pond. The usual stocking ratio is more than 60% silver carp and bighead carp; 20–30% grass carp; and 10–20% black carp, common carp, or crucian carp. The different cyprinid species co-stocked into the same pond must be complementary not only in their nutritional requirements but in feeding habits as well. Artificial propagation of bighead and silver carp was achieved first in 1958, followed soon by that of grass carp and black carp (for review, see Liu and He 1992). These achievements have made the collection of Chinese carp fry from natural waters unnecessary.

The goldfish is thought to have originated from a red-colored mutant crucian carp found in the wild over 2,000 years ago in southern China. During the Song Dynasty (tenth to thirteenth century), goldfishes were cultured in ponds of temples. They were brought to Japan from China at the beginning of the sixteenth century and to Europe in the seventeenth century. By the middle of the nineteenth century, they became affordable and popular pets of the general public. Today, there are more than a hundred varieties of goldfish, mostly used for ornamental purposes (Smartt and Bundell 1996).

In order to complete the history of cyprinid culture, Prussian carp also needs to be mentioned. Similarly to common carp, the culture of Prussian carp was also initiated in China. The earliest activity can be traced back to the East Han Dynasty (25–189 AD). Production at that time was limited to a rather small scale. The aquaculture of this species had been restricted to China and Japan until the mid-1960s and has gradually expanded to several other countries and regions since then. The major producer of Prussian carp has always been China, whose production has expanded from less than 2,000 metric tons in 1950 to nearly 1.7 million metric tons in 2002 (99.6% of the world's total). The species became really important for freshwater aquaculture in the 1960s, when it was introduced into central and south China from the Heilongjiang River (northeast China). The most common grow-out technology for crucian carp is based on polyculture in ponds and rice paddies.

2.3 Classical Genetics

2.3.1 Phenotypic Markers

The different intensities of selection had their impact on the genetic structure of carp populations, resulting in the emergence of various genotypic and phenotypic traits in domesticated varieties of common carp.

The scale pattern has been a well-known phenotypic trait in different varieties of common carp. According to Kirpichnikov (1967, 1999), the scale pattern is determined by two unlinked genes, *S* and *N*. Based upon the distribution and number of scales, four different phenotypes are distinguished: scaled

carp (scales completely cover the body; genotype: $SSnn$ or $Ssnn$); linear carp (a straight row of enlarged scales along the lateral line; $SSNn$ or $SsNn$); mirror carp (enlarged, irregularly placed scales; $ssnn$); and nude or leather carp (very few or no scales; $ssNn$). It was proposed that all the *NN* genotypes should be lethal, independently from the type of the alleles present for the other gene (Kirpichnikov 1967, 1999). Recent data (Bercsenyi M, Czuczka P, Harris M, and Rohner N, pers. comm.) indicate that the genetic regulation of scale formation in cyprinids might be more complex than indicated by the Kirpichnikov model.

The coloration of common carp can vary among types and is determined by the relative amount of melanophores, iridophores, xanthophores, and guanophores (for a review on pigments, see Kelsh 2004). Red or orange individuals are deficient in melanophores, whereas white ones are deficient in both melanophores and xanthophores. The absence of guanophores yields a semitransparent individual. Red Xingguo common carp was crossed with mirror carp (wild coloration), and their offspring generated by backcross were used to study color inheritance. The results demonstrated that, similar to scale pattern, red coloration is also controlled by two genes (*B* and *R*). Individuals with homozygous recessive genotype for both genes ($bb;rr$) showed red coloration (Wu et al. 1979). Additional color varieties described include: German blue, Polish blue, grey, steel, and patterned (Kirpichnikov 1967, 1999). Recently, classical and molecular genetic analyses have been performed on three varieties of koi carp to learn more about the genetic basis of their color inheritance (David et al. 2004).

Body shape is another phenotypic variable among common carp breeds. Generally, the wild common carp has a longer, torpedo-like body shape, whereas most domesticated varieties have a deeper body. The genetics of this trait are still unknown. Other phenotypic traits include dwarfism, missing or additional fins, and distorted (dolphin-like) head (Kirpichnikov 1967, 1999).

2.3.2 Biochemical Markers

The allelic frequency of several polymorphic proteins is different between subspecies and races of com-

mon carp. Therefore, studies on biochemical polymorphisms of the common carp, including wild carp and cultured varieties, have provided valuable genetic information about the evolution and origin of varieties for several decades. These investigations have also yielded potential markers for selective breeding. A few cases are described below, exemplifying the usefulness of these markers during the 1970s and early 1980s.

The polymorphisms of myogen (Truveller et al. 1973), transferrin (Valenta et al. 1976), esterases (Paaver 1983), and lactate dehydrogenase (Kirkpichnikov 1967, 1999; Paaver 1983) were used to identify the subspecies and strains of common carp. Transferrin and esterase alleles have also been used as markers for stocks selected for resistance against dropsy (Shart and Iljasov 1979). Kohlmann and Kersten (1999) have compared the isoenzyme patterns of eight enzymatic systems in eleven German and five Asian common carp lines and found distinct differences between the two groups.

Although the phenotypic and biochemical markers have been very useful both for fish research and production during the 1970s and 1980s, their limitations in terms of the relatively low allele numbers and total number of polymorphic markers became obvious. From the mid-1980s, DNA technology started to provide a new generation of tools, which led to improved datasets and a better understanding of basic genetic problems. From the accumulation of results of medium- to high-throughput genetic methods, a new area of science, called genomics, was born. Genomics is the structural and functional analysis of genomes, chromosomes, and genes, or in other words, genetics on a large scale.

2.4

Cyprinid Chromosome Sets and Their Manipulation

2.4.1

Chromosome Sets

Cyprinid chromosome sets have been studied extensively by cytogenetic methods (for reviews, see Buth et al. 1991; Klinkhardt et al. 1995; Rab and Collares-Pereira 1995; Sola and Gornung 2001; Yu et al. 1987).

The total chromosome number in cyprinids varies from 42 to more than 200 (Buth et al. 1991). However all cyprinids, including the eight species and subspecies described in this chapter, contain either 24–25 chromosomes or a duplicate of that, as the haploid set (see Table 1 for details).

The chromosome number of grass carp, bighead, and silver carp is the same, $2n = 48$. At the same time, their karyotypes show the following slight differences: grass carp has 18 metacentric, 24 submetacentric, and 6 subtelocentric chromosomes (the number of fundamental chromosome arms, or NF = 90), whereas both bighead carp and silver carp have fewer submetacentric and more subtelocentric ones (18m + 22sm + 8st; NF = 88) (Yu et al. 1987).

The karyotype of common carp is 22m + 34sm + 22st + 22t (NF = 156) (Rab and Collares-Pereira 1995). There are, however, variants with different chromosome numbers (e.g., koi carp varieties with 101 and 102 chromosomes; Ojima and Takai 1981). The haploid chromosome number of goldfish equals that of the common carp ($2n = 100$), and their karyotype also seems identical (Wu and Gui 1999) (Fig. 1A).

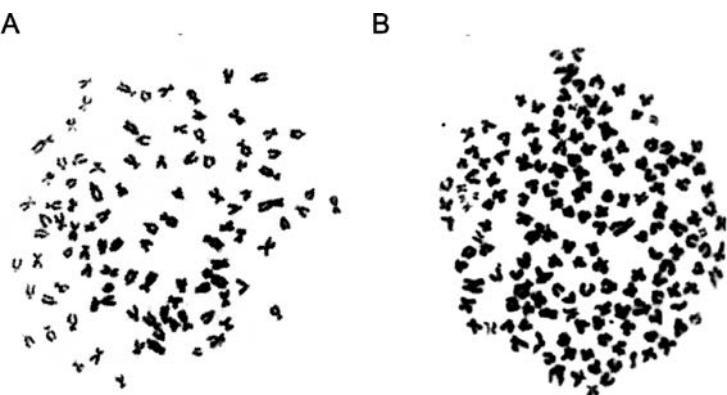
The karyotype of zebrafish presents an interesting picture: its haploid chromosome number of 25 nearly equals that of grass carp, bighead, and silver carp, but on the other hand, its genome size is considerably higher, close to those of the re-duplicated common carp and goldfish genomes. The zebrafish karyotype is: 4m + 16sm + 30st (NF = 70) (Sola and Gornung 2001).

Most individuals of Prussian carp and ginbuna populations are triploids⁴, meaning they contain an additional chromosome set and approximately 50% higher haploid C-values⁵ in comparison to common carp, crucian carp, and goldfish. Triploid Prussian carp and ginbuna reproduce by natural gynogenesis, producing clones (Dong et al. 1996, Umino et al. 1997). The karyotype of Prussian carp contains 162 ($3n$) chromosomes (34m + 58sm + 42st + 28t; NF = 254) (Wu and Gui 1999) (Fig. 1B). Ginbuna populations in Japan include triploid and occasionally tetraploid individuals. The chromosome number for

⁴ To follow the convention, all cyprinids discussed in the chapter are referred to as diploids, with the exception of Prussian carp and ginbuna, which are called triploids.

⁵ The haploid C-value, or CV (in picograms), is the measure of genome size.

Fig. 1 Mitotic metaphase chromosome set from A goldfish and B Prussian carp



the former is 156, whereas that for the latter is 206 (Kobayashi et al. 1977).

2.4.2 Gynogenesis

Genome manipulations are very important tools, not only for basic research, but also for practical fish breeding (for reviews, see Horvath and Orban 1995; Pandian and Koteeswaran 1998; Purdom 1969). Fish embryos normally inherit two chromosome sets, one from the maternal and another from the paternal source. Induced subtraction of a chromosome set can be achieved by preventing a full haploid chromosome set either from the sperm cell (gynogenesis) or from the egg (androgenesis) to be transmitted into the embryo. This process yields haploids that can be diploidized by inhibiting the cell duplication during the first division of the embryo.

In gynogenesis, the egg develops without genetic contribution from the paternal nucleus. Natural gynogenesis occurs when the sperm penetrates the oocyte and activates it, but the paternal genome fails to incorporate into the zygote. In these cases, the second polar body is usually retained and completes the chromosome set of the offspring to diploid level. The process was first described in the guppy (*Poecilia formosa*; Girard 1859), then in several additional species, among them Prussian carp and ginbuna (see below).

In order to achieve artificial gynogenesis, two “issues” had to be solved: the genetic inactivation of the sperm cell and the need to bring the resulting genome to diploid level. The first evidence for successful application of the technique was reported by Opperman (1913) in trout (*Salmo trutta*). The begin-

ning of systematic studies in fishes was laid down by experiments on loach (Neyfakh 1956, Romashov et al. 1960) and later on common carp (Cherfas 1975, 1977; Romashov et al. 1960).

The genetic inactivation of the paternal chromosome set can be achieved by exposing the sperm cells to chemical mutagens or, more frequently, high doses of radiation (e.g., X-rays, γ -rays, or UV). The vast majority of gynogenetic embryos developing from eggs fertilized with inactivated sperm are haploid and inviable due to developmental problems. Although the frequency of spontaneous diploidization in artificial gynogenesis is very low, about 0.2% in the common carp, the availability of a large number of induced eggs permits the generation of a few diploid individuals for experimental purposes (Wu et al. 1991).

Diploid gynogenetic offspring can be produced artificially by inhibiting extrusion of the second polar body or by inhibiting the first mitotic division of the gynogenetic embryos. This can be achieved by treatment with high or low temperature or chemical agents (e.g., colchicine). The initiation, duration, and intensity of treatment are critical for successful production of meiotic (primary or polar body) (Wu et al. 1981) and mitotic (Hollebecq et al. 1986; Komen 1990; Nagy 1987; Nagy et al. 1978) gynogenetic common carp.

Meiotic gynogenesis through polar body retention results in increased homozygosity, although some heterozygous loci are retained because of crossing over between homologous chromosome segments. Nevertheless, the resulting individuals can be used for rapid inbreeding within several generations. For common carp, in the first meiotic gynogenetic generation, the inbreeding coefficient is equal to about 60% (Cherfas 1977).

Gynogenesis through mitotic interference produces truly homozygous diploids. Although the percentage of gynogenetic diploids induced by temperature shock may be as high as 38%, up to 80% of these individuals are sterile because of chromosome deletion or breakage (Streisinger et al. 1981). On the other hand, no sterile female has ever been found among gynogenetic individuals resulting from spontaneous diploidization.

The haploids produced by andro- or gynogenesis (without subsequent duplication) are favorable materials for karyotype analysis and construction of genetic linkage maps. Nonetheless, the most attractive use of artificial gynogenesis is apparently the production of inbred lines. Since gynogenesis in common carp produces exclusively female offspring, the procedure had to be first combined with hormonal sex reversal (Wu et al. 1981). Streisinger and his colleagues (1981) generated the first clonal cyprinid lines in zebrafish by successfully combining induced mitotic gynogenesis with hormonal masculinization. A few years later, a Hungarian team successfully used the same approach in the common carp to produce near-isogenic lines (Nagy 1987; Nagy and Csanyi 1984). A new inbred line, called red common carp 8305, was generated subsequently in China based on the same principles, and it was utilized in crosses to generate hybrid vigour (Wu et al. 1991).

2.4.3 Androgenesis

Androgenesis is the “inverse” of gynogenesis: the maternal chromosome set is destroyed by treating the egg, resulting in offspring with chromosomes of exclusively paternal origin (for review, see Gomelsky 2003; Horvath and Orban 1995; Kirankumar and Pandian 2004b; Pandian et al. 1999). The procedure has been applied successfully to a number of teleost species, including common carp and zebrafish.

The first androgenetic cyprinids were produced by inactivating common carp eggs by X-ray, fertilizing them with normal sperm, and restoring the diploidy by heat shock (Grunina et al. 1990). Komen’s team in Wageningen (The Netherlands) exposed common carp eggs immersed in a synthetic ovarian fluid to UV irradiation to produce haploid androgenetic embryos (Bongers et al. 1994). Diploidy was then re-

stored by heat shock. The procedure was then applied repeatedly in several successive generations to produce near-isogenic all-male (YY) lines (Bongers et al. 1999).

The technique was successfully adapted to zebrafish as well. Androgenetic diploid embryos were generated by irradiating eggs with X-rays and heat-shocking them following fertilization with untreated sperm (Corley-Smith et al. 1999; Corley-Smith et al. 1996). However, the yield of viable individuals was quite low (around 2%).

Androgenesis can also be performed using sperm and egg from different species. The first such experiment in cyprinids was reported by Grunina and her co-workers (1991), who produced “diploid androgenetic hybrids” by fertilizing inactivated common carp eggs with normal (untreated) crucian carp sperm. Bercsenyi and colleagues (1998) performed interspecific androgenesis by fertilizing γ -ray-irradiated carp eggs with goldfish sperm followed by heat shock-based genome duplication. According to the comparative phenotypic and molecular analysis of the “parents” and offspring, the resulting diploid androgenic individuals did not contain carp-derived traits or sequences. Pandian’s lab produced androgenic diploid individuals by fertilizing inactivated eggs of the grey Sumatra barb (*Puntius tetrazona*; Bleeker 1855) with sperm of the golden rosy barb (*Puntius conchonius*; Hamilton, 1822; Kirankumar and Pandian 2004b). Polyethylene glycol (PEG 4000) was used to enhance the frequency of dispermia⁶, allowing for the production of non-homozygous diploids without heat-shock treatment. Later, the same laboratory achieved interspecific androgenetic restoration of rosy barb using cadaveric sperm – retrieved from a deceased male individual – to fertilize inactivated tiger barb eggs, followed by heat shock (Kirankumar and Pandian 2004a). This is the first successful restoration experiment in the history of fish genome manipulation, raising hopes for successful future applications of similar technologies on endangered species (Corley-Smith and Brandhorst 1999).

Androgenetic progeny can also be generated accidentally during interspecific hybridization. When grass carp eggs were fertilized by common carp sperm, 43 diploid androgenetic progeny were pro-

⁶ Dispermia occurs when two different sperm cells fertilize the same egg.

duced from 18,000 fertilized eggs without any thermal or chemical shock (Stanley 1976).

2.4.4 Induced Triploidy

Individuals harboring more than two chromosome sets in the nucleus of their cells are called polyploids. Polyploidy may either arise spontaneously, or it can be induced experimentally by disrupting the metaphase spindle in either somatic or germ cells, thus preventing the replicated chromosome sets from separating into two daughter cells. Allopolyploids originate from a zygote containing different genomes (e.g., from hybridization of two species), whereas autopolyploids have only homologous genomes in their nuclei (for review, see Van de Peer and Meyer 2005).

Triploids contain three chromosome sets. They can be generated by the manipulation of meiotic process (for reviews, see Gomelsky 2003; Horvath and Orban 1995; Ihssen et al. 1990; Pandian and Koteeswaran 1998). The induction of triploidy is analogous to meiotic gynogenesis, except that normal (i.e., untreated) spermatozoa are used for fertilization, and the triploid is produced by polar body retention. Another possibility for the production of triploids is crossing tetraploid brooders with diploid ones. This technique was used successfully in rainbow trout (Chourrout et al. 1986, Diter et al. 1988); however, it has only been applied occasionally in common carp, primarily due to the very low survival rate of induced tetraploids (Cherfas et al. 1993).

Induced triploidy has been performed in common carp by a number of investigators who inhibited the second meiotic division of eggs fertilized with untreated (i.e., normal) spermatozoa either by cold shock (Cherfas et al. 1990; Gervai et al. 1980; Ojima and Makino 1978) or heat shock (Cherfas et al. 1994; Recoubratsky et al. 1992). The success of such treatment can be evaluated and the polyploidy verified by karyotyping or nuclear analysis of red blood cells (Wu et al. 1986). Most induced triploids are sterile, since they are aneuploid or carry chromosomes with deletion and/or breakage. In theory, the few fertile individuals can be used to propagate the line by means of natural or induced gynogenesis.

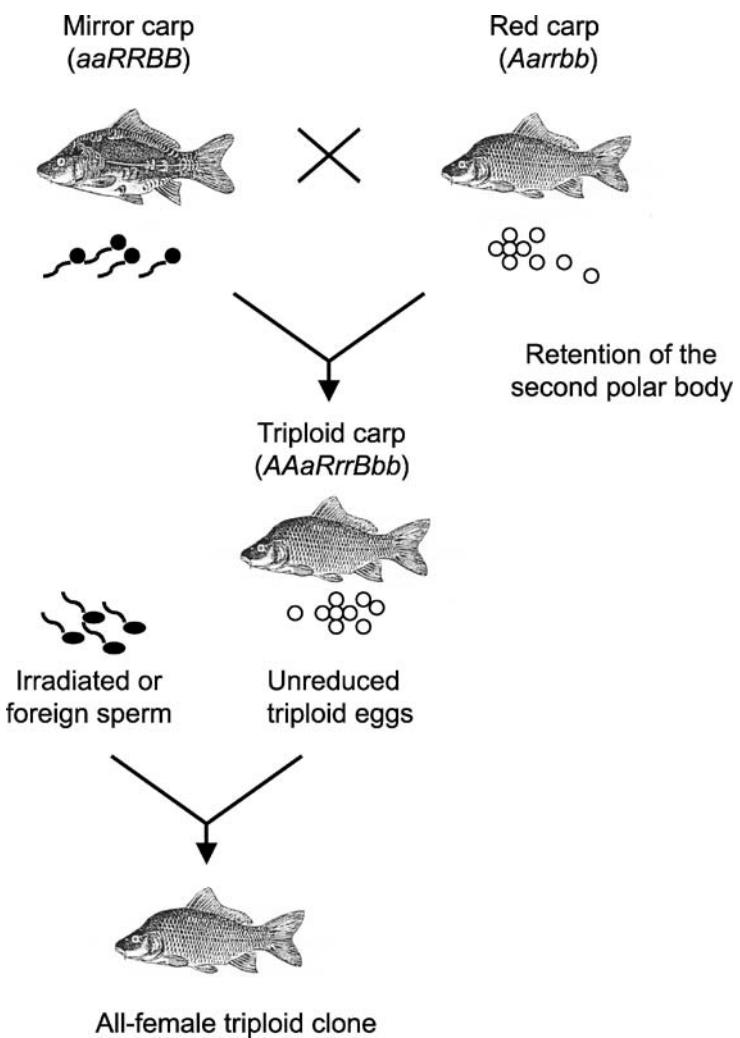
The diploid hybrid females produced by crossing common carp and crucian carp turned out to

be fertile. Their mature eggs activated by irradiated sperm developed normally, and a chromosome count revealed that their karyotype was $2n = 100$. This indicated that the female pronucleus of the diploid hybrid mature egg maintained both chromosome sets. When these eggs were fertilized with normal (non-irradiated) sperm of mirror carp, all resulting offspring individuals were triploid ($3n = 150$; Wu et al. 1993), suggesting that the female pronucleus of the diploid hybrid fused with the male pronucleus and formed an allotriploid individual with two genomes from common carp and one from crucian carp. The testes of a three-year-old male allotriploid individual were fully developed; however, normal spermatozoa have never been detected in them. Allotriploid ovaries of some individuals were filled with a large amount of mature oocytes at stage IV, whereas oocytes at stages I–III were observed in other individuals (Wu et al. 2003b).

The mature eggs of these allotriploids developed normally and passed the hatching stage after being activated by inactive sperm of related species. The chromosome count of these gynogenetic embryonic cells revealed that they contained not only triploid ($3n = 150$), but also of haploid ($1n = 50$), diploid ($2n = 100$), and aneuploid ($1.5n = 75$) cells (Wu et al. 1993, 2003b). When non-irradiated sperm of related species, such as Wuchang bream (*Megalobrama amblycephala*; Yih 1955; $2n = 48$), stone moroko (*Pseudorasbora parva*; Temminck and Schlegel 1846; $2n = 50$) and sharpbelly (*Hemiculter leucisculus*; Basilewsky 1855; $2n = 48$), were used to activate mature eggs of these allotriploids, the viable offspring were all allotriploids ($3n = 150$), and their morphological traits were identical with those of their mother (Wu et al. 2003b). This suggested that the eggs maintained their three chromosome sets and that their pronucleus did not fuse with the penetrating male pronucleus, but instead they developed gynogenetically following activation. After seven successive generations of gynogenetic reproduction, an allotriploid gynogenetic common carp clone was established (Wu et al. 1993, 2003b) (Fig. 2).

In addition to common carp, induced triploids were generated from several other farmed cyprinids, including grass carp (Rodriguez-Gutierrez 1995; Rothbard et al. 2000; Thompson et al. 1987), bighead carp (Aldridge et al. 1990), black carp (Rothbard et al. 1997), and tench (Flajshans et al. 1993a,b).

Fig. 2 A scheme for maintaining vigor by means of induced triploidy. AA or Aa scaled, aa mirror, $rrbb$ red color, $Rrbb$ or $rrBb$ grey color



Triploid grass carps were tested as potential means of controlling vegetation in lakes in the US (Bonar et al. 2002; Pine et al. 1990; Venter and Schoonbee 1991).

Polyploidy has numerous applications in genetic studies and breeding programs. Induced triploids have good potential as food fishes with enhanced growth, as well as for reproduction control due to the fact that they are generally sterile. On the other hand, the fact that a small portion of the triploid individuals retain their reproductive ability calls for caution (e.g., van Eenennaam et al. 1990). These individuals would be able to reproduce through bisexual means with each other or with related species. The latter might threaten the gene pool of endemic populations when hybrids are released into open water. A possible approach for preventing genetic segregation from

the triploid offspring, by generating a triploid from a hybrid, has been proposed. The assumption is that such a triploid hybrid would only reproduce by natural gynogenesis (Wu et al. 1979).

2.4.5 Induced Tetraploidy

Individuals with four chromosome sets in their nuclei are called tetraploids. Those tetraploids that contain four homologous chromosome sets are autotetraploids, whereas the rest with one or more heterologous sets of the four are allotetraploids (for reviews on artificial tetraploid teleosts – and among them cyprinids – see Horvath and Orban 1995; Ihssen et al. 1990; Pandian and Koteeswaran 1998).

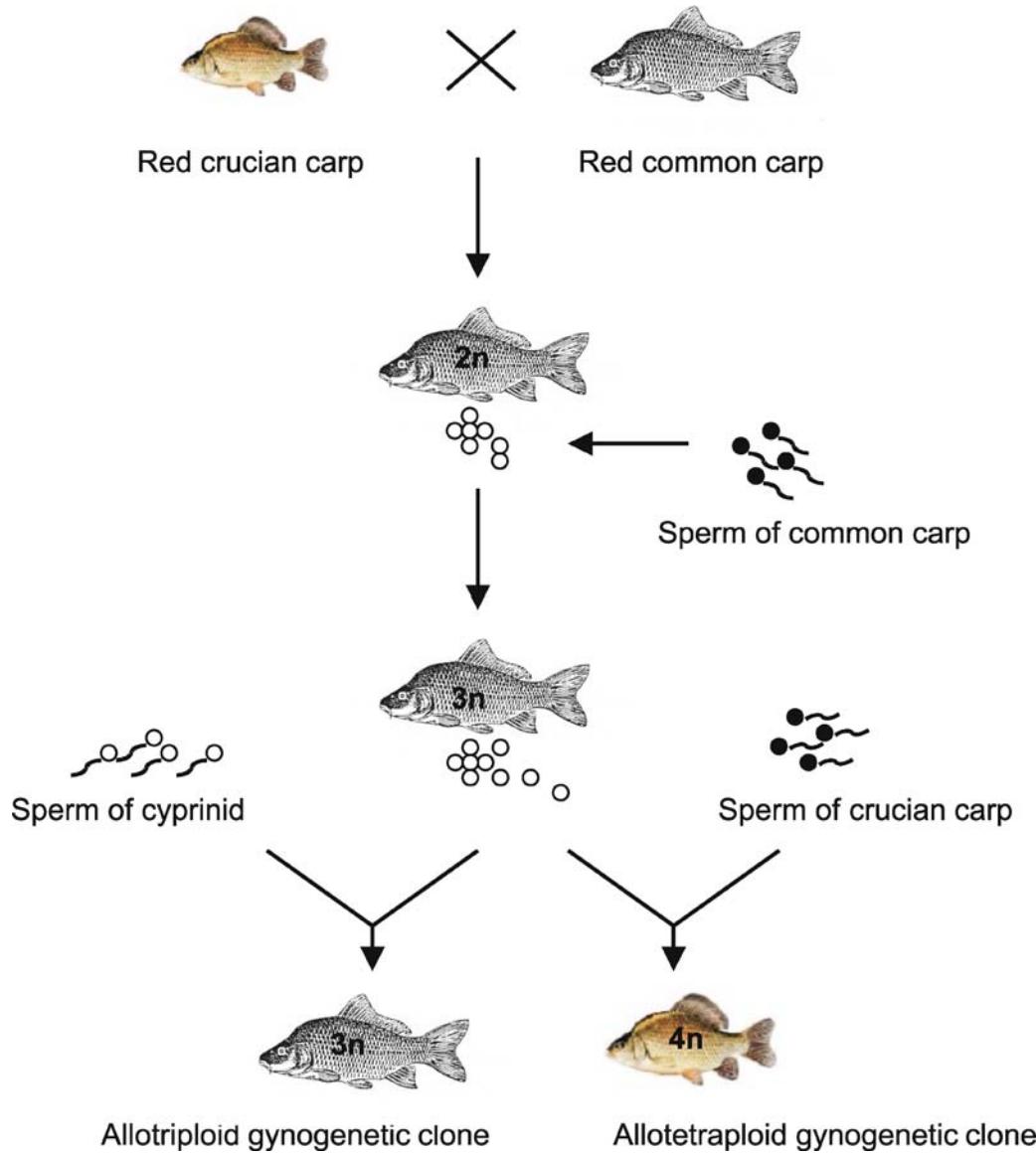


Fig. 3 A diagram illustrating the establishment of unisexual clones of allotriploid and allotetraploid lines

When normal (i.e. untreated) haploid ova are fertilized with normal haploid spermatozoa, and the first mitotic division is interrupted, tetraploid carp individuals can be produced. Only a few reports can be found in the literature about induced tetraploidy in common carp, indicating the difficulty of the procedure (Cherfas et al. 1993; Taniguchi et al. 1986). Induced tetraploids have also been produced from grass carp (Cassani et al. 1990), bighead (Aldridge et al. 1990), crucian carp (Gui et al. 1991), and tench (Flajshans et al. 1993b).

Tetraploidy can also be generated in cyprinids by interspecific hybridization (Chen et al. 1987, 1997). The female pronucleus of rare individuals of allotriploid carp (with two common carp genomes and one crucian carp genome) can fuse with the penetrating male pronucleus and produce allotetraploid offspring (Wu et al. 2003b). The genome of these allotetraploid individuals contains three maternal genomes and a paternal one. All the survivors are females, and they are fertile. Comparative analysis of allotetraploid carp females with their offspring – involving the study

of morphological traits, chromosomes, and molecular markers – indicated that the mode of reproduction of this allotetraploid carp was natural gynogenesis (i.e., allotetraploid carps produced allotetraploid clones). Therefore, a tetraploid ($4n$) line can be produced and maintained (Wu et al. 2003b; Ye et al. 2002) (Fig. 3).

The third way to form allotetraploid stocks from diploid hybrids of crucian carp and common carp was reported recently (Liu et al. 2001). In this system, allotetraploidy occurred from the ability of diploid hybrids to produce unreduced diploid ova and diploid spermatozoa. By fertilizing unreduced diploid ova with unreduced diploid sperm (both from diploid hybrids) allotetraploid individuals were obtained. These allotetraploids were fertile, and they produced diploid gametes. By crossing allotetraploid females with allotetraploid males through several successive generations, an allotetraploid stock was established (Liu et al. 2001, 2004).

2.4.6 Nuclear Transfer

The first report on nuclear transfer in vertebrates was published by Briggs and King (1952). Later, Gurdon (1962) managed to generate adult frogs from the nucleus of a single somatic cell. Successful nuclear transplantation in goldfish and bitterling (*Rhodeus sericeus*; Pallas 1776) using embryonic cells as donor nuclei was reported one year later (Tung et al. 1963). The detailed history of nuclear transplantation experiments performed on fish in China, and some theoretical studies on nucleo-cytoplasmic interactions, were published later in retrospect (Lu and Chen 1993, Yan 1998). In the latter source, it was proposed that the character variety of nucleo-cytoplasmic hybrids resulted from the interaction between the heterogeneous nucleus and cytoplasm (Yan 1998). During the subsequent years, the technology has been successfully used on several different fish species, including the zebrafish (Ju et al. 2004a, b).

The development of somatic cell engineering for fish breeding was first established by Chen and colleagues (1986). Somatic nuclear transplantation in crucian carp was obtained by using primary cultures of kidney cells from an adult crucian carp as nuclear donor. The transplanted crucian fish developed normally and reached maturation (Chen et al. 1986). Since

then, extensive work has been performed with the aim of generating virus-resistant fish lines by using this technology. Several cell lines resistant to viral diseases were produced from grass carp (Li and Mao 1990; Wang et al. 1993).

Microinjection of donor cells into mature, enucleated eggs has been the most commonly used method to produce transplants in fish. However, the technique requires specific skills, extensive experience, and the success rate is still low. An electrofusion method called polar injection that was developed in the late eighties (Liu et al. 1988) and used later to produce transplants in loach (Fu and Wu 2001) may offer an alternative approach.

2.5 Genetic and Genomic Resources for Cyprinid Research

2.5.1 DNA Markers and Their Applications

Minisatellites

Minisatellites are variable number tandem repeat (VNTR)⁷ sequences containing a core unit (10–15 base pairs) flanked by restriction sites and often tandemly repeated up to 1,000 times (Jeffreys et al. 1985a). Most minisatellites are polymorphic. The polymorphism can be detected by digesting genomic DNA with various restriction endonucleases and hybridizing by labeled “multilocus probes” that contain a concatamer of the core sequence of minisatellites occurring at a high copy number in the genome to be tested (Jeffreys et al. 1985b). The method was named restriction fragment length polymorphism (RFLP); however, it is often referred to simply as “DNA fingerprinting” due to the fact that the resulting multilocus profiles were the first DNA-based tools for the identification of individuals.

RFLP has been applied widely for the identification of human individuals in various areas including

⁷ Variable number tandem repeats (VNTRs) are short nucleotide sequences that are 12–100 bp long. They are usually repeated in tandem with units of 4–40. There are two kinds of VNTRs: the longer minisatellites (10–15 bp core) and the shorter microsatellites (2–4 bp core).

forensics, immigration, and paternity cases (for review, see Jeffreys 2005). It has also been used in various areas of fish research. Its uses in cyprinid studies include confirmation of homozygosity in common carp lines (Ben-Dom et al. 2001; Wiegertjes et al. 1996), confirmation of tri- and tetraploidy in ginbuna (Dong et al. 1997), phylogenetics in the genus *Carassius* (Murakami and Fujitani 1997), and confirmation of inadvertent hybridization among Indian major carps (Padhi and Mandal 1997).

Random Amplified Polymorphic DNA

The random amplified polymorphic DNA (RAPD) assay (Welsh and McClelland 1990; Williams et al. 1990) is a method based on the polymerase chain reaction (PCR), which uses commercial 10-mer primers for “blind” screening of genomes. The product of a RAPD assay is a “molecular barcode”, which usually contains up to a dozen potential markers when separated on agarose gels and detected by ethidium bromide staining (and about 10 times more if visualized by more laborious methods with higher sensitivity, e.g., silver staining of band patterns separated on polyacrylamide gels).

The RAPD assay is very easy to set up and needs no prior information about the template. Therefore, it is an ideal tool for quick studies on lesser-known or even completely unknown genomes (Table 2). Its disadvantages are that most RAPD markers are dominant/recessive, they usually do not work in every strain/population, and the assay is highly sensitive to changes in reaction conditions, thus requiring careful optimization when being set up in a new laboratory (Benter et al. 1995).

The first applications of this technique in cyprinid research were the identification of markers showing high levels of polymorphism among zebrafish strains (Johnson et al. 1994) and then creation of a genetic linkage map based entirely on RAPD markers for the zebrafish (Postlethwait et al. 1994). Studies on farmed cyprinids soon followed. The first few applications were diverse, including a study on natural gynogenesis in Prussian carp (Chen et al. 1987) and ginbuna (Dong and Taniguchi 1996), analysis of heterosis in common carp (Dong and Zhou 1998), and a search for genetic differences among koi carps with different color patterns (Jackson et al. 2000). Later, this technique was used to verify the success of androgenesis in zebrafish

(Corley-Smith et al. 1996) and goldfish (Bercsenyi et al. 1998).

RAPD markers have also been used for the analysis of genetic diversity of farmed and natural populations of common carp (Bartfai et al. 2003), crucian carp (Luo et al. 1999; Yoon and Park 2002) and Prussian carp (Zhou et al. 2000). Although RAPD markers are potentially useful to complement genetic maps based primarily on microsatellites (Horvat and Medrano 1996), the primary application of the technology on cyprinids is expected to be for the analysis of individuals produced by artificial or natural genome manipulation (Bercsenyi et al. 1998; Yan et al. 2005) or hybridization (Chrisanfova et al. 2004; Toth et al. 2005).

Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP; Vos et al. 1995) is a PCR-based amplification technique similar to RAPD which is ideally suited for the analysis of unknown genomes. The major difference between the two methods is that in AFLP, the complexity of the genomic template is reduced during the amplification by using anchored primers (Table 2).

AFLP has primarily been used for the study of prokaryotes and plants, and reports from animal studies are fewer (Bensch and Akesson 2005). The use of AFLP has also been limited in cyprinid research: it has only been utilized for mapping mutations in zebrafish (Ransom and Zon 1999), and for the characterization of various koi and common carp strains (David et al. 2001).

Fluorescent Motif Enhanced Polymorphism

Fluorescent motif enhanced polymorphism (flu-oMEP; Chang et al. 2007) is a new genotyping method combining the advantages of RAPD and AFLP. The link between the two methods is provided by a fluorescently labeled “common primer” that is used in a two-primer PCR together with an unlabeled, short (10-mer) RAPD primer (Chang et al. 2007). All the products originating from the common primer are labeled and can be separated by capillary gel electrophoresis using commercial sequencing machines. Thousands of RAPD primers can be used for co-amplification with one or more common primers tapping into various areas of the genomes. The common primer can be designed to bind frequently occurring motifs distributed in

Table 2 Comparing the amplification conditions of genotyping PCR reactions with different markers with that of a general PCR reaction

	RAPD	AFLP	fluoMEP	Microsatellite	PCR
Known template	no	no	no	yes	yes
Number of primers	1	2	2	2	2
Type of primers	short	anchored	common+short	flanking	flanking
Annealing temp	low	high	low	high	high
Number of products	0–10*	10–100**	10–100	2	1
Product	unknown	unknown	unknown	known	known

* – if separated on agarose gel and detected by ethidium bromide

** – if separated on a polyacrylamide gel and detected by silver staining

the genome in a non-random manner, providing theoretical opportunity for targeting.

Microsatellites

Microsatellites (simple sequence repeats or short tandem repeats) are short repetitive sequences containing a minimum of 6 repeat units (for review, see Ellegren 2004). They are classified according to the size of repeat unit as mono- (e.g., A), di- (e.g., CA), tri- (e.g., GAG), or tetranucleotide (e.g., GATA) types. Microsatellites can easily be amplified by PCR using primer pairs designed to anneal to their flanking regions (Table 2). They are ideal markers for population genetics as well as genomics due to their co-dominant nature, high polymorphism, and heterozygosity. Microsatellites are abundant in all eukaryotic genomes and thus provide universal and relatively dense coverage for the whole genome.

Microsatellite markers were introduced to cyprinid research and aquaculture with substantial delay in comparison to the area of farm animal husbandry. Like RAPD markers, the first set of cyprinid microsatellites were described from zebrafish (Goff et al. 1992), and later, vastly extended collections were used for setting up a genetic linkage map (Knapik et al. 1998; Shimoda et al. 1999). To date, polymorphic microsatellites have been isolated from the following cyprinid species: common carp (e.g., Aliah et al. 1999; Crooijmans et al. 1997; David et al. 2001; Yue et al. 2004), Prussian carp (Yue and Orban 2002), ginbuna (Ohara et al. 1999), goldfish (Yue and Orban 2004; Zheng et al. 1995), and silver carp (Mia et al. 2005).

Many of these markers have been shown to work in cross-species amplifications performed in related species (e.g., Tong et al. 2002, 2005; Yue et al. 2004; Yue and Orban 2002), thus opening up new possibilities for the study of other cyprinid species lacking characterized microsatellites (see Sect. 2.7.1 for more details). Amplification products from such cross-species applications should always be sequenced and compared to those in the host species, as some products might lack similarity to the original microsatellite locus, especially in case of distantly related species pairs as detected in plants (Chen et al. 2002) and teleosts (Yue GH, Kovacs B, and Orban L, unpublished data) as well.

The primary applications for microsatellites in cyprinid research (as in other areas of animal husbandry) are the analysis of genetic relatedness in natural and farmed populations (e.g., Bartfai et al. 2003; Desvignes et al. 2001; Kohlmann et al. 2003; Lehoczky et al. 2005) and the generation of genetic linkage maps (Sun and Liang 2004). Additional uses in cyprinids include the detection of interspecific hybrids (Mia et al. 2005) and estimating the age of ancient genome duplications (David et al. 2003).

Mitochondrial DNA-derived Markers

The mitochondrial DNA (mtDNA) of vertebrates is typically a 16–20 kb long, circular DNA molecule, found in as many as several thousand copies per cell (Burger et al. 2003; Meyer 1993). Due to its matrilineal, clonal inheritance, mtDNA is an essential tool for evolutionary and phylogeographic studies (Avise 2000; Lang et al. 1999).

The sequencing of complete mitochondrial genomes of cyprinids started with the following three species: ginbuna (Murakami et al. 1998), zebrafish (Broughton et al. 2001) and common carp (Chapman et al. 2004; Mabuchi et al. 2006). Later Saitoh and colleagues added the fully sequenced mitogenomes of an additional 29 species from the *Cyprinidae* family (Saitoh et al. 2003, Saitoh et al. 2006). In addition to these, unpublished complete mtDNA sequences are also available for goldfish (NC_006580) and crucian carp (NC_006291) from GenBank (Benson et al. 2005), bringing the total number to 34.

The most important use of mtDNA-derived markers is for phylogenetic analysis of the family *Cyprinidae* (see below for details), as well as genetic analysis of various farmed stocks and natural populations of common carp (Kohlmann et al. 2003; Zhou et al. 2003b).

Expressed Sequence Tags

Expressed Sequence Tags (ESTs) are short (500–800 bp) single-pass sequences produced typically from ends of cDNA clones (for review, see Slater 1998). Although prone to errors (Hillier et al. 1996), they are nonetheless extremely useful for collecting “snapshot” information about expressed gene sets and providing help for gene prediction during genome annotation (Poustka et al. 2003).

The dbEST database (Boguski et al. 1993) contains EST sequences from only four cyprinid species (dbEST release 060107, accessed on June 12, 2007). Not surprisingly, the vast majority of cyprinid ESTs are from the zebrafish (around 1.35 million ESTs). The three other members of the family with ESTs listed in the database with fewer sequences are the fat-head minnow (*Pimephales promelas*; Rafinesque 1820; around 250,000 ESTs); common carp (nearly 20,000 ESTs), and grass carp (531 ESTs).

Single Nucleotide Polymorphism

Single nucleotide polymorphisms (SNPs) are variations between individuals of the same species affecting the genome at one particular position (i.e., a single base pair) (for review, see Vignal et al. 2002). Although theoretically, a nucleotide can mutate to any of the three other types, SNPs are usually bi-allelic with the less frequent allele having more than 1% frequency.

The two main sources of SNP discovery are large-scale sequencing of genomic DNA (Haga et al. 2002

and “mining” of EST databases (Picoult-Newberg et al. 1999). According to the data provided by these tools, their estimated average frequencies in vertebrate genomes are highly variable, ranging from one SNP per 4.1 kb (*Salmonidae*; Smith et al. 2005a) to one SNP per 225 bp (broiler and layer lines of chicken; Schmid et al. 2000), making them the markers with the highest density in eukaryotes.

The primary applications of SNPs are the saturation of a particular region of a genetic linkage map with markers to confirm a candidate gene or a QTL (fine-mapping). The only cyprinid sequences in the dbSNP database are 2,111 SNPs from zebrafish, additional teleost markers are from Chinook salmon (*Oncorhynchus tshawytscha*; Walbaum 1792; 395 SNPs) and from rainbow trout (*Oncorhynchus mykiss*; Walbaum 1792; 139 SNPs). Bradley and colleagues (2007) have screened through 39% of the Zv6 and identified nearly 550,000 potential SNPs. Subsequent verification on a subset indicated that over 70% of them are likely to be valid polymorphisms, resulting in nearly 390,000 estimated SNPs (Bradley et al. 2007). This number is expected to triple once the Zebrafish Genome Project is completed. SNPs have not been described yet for the other cyprinid species discussed in this chapter. Applications of SNPs in teleost research have been largely restricted to the *Salmonidae* family, using conserved sites to extend the validity of SNPs across species (Smith et al. 2005a) and estimating the origin of wild caught salmon (Smith et al. 2005b).

Cyprinid Evolution as Revealed by DNA Markers

Comparison of a detailed genetic linkage map of zebrafish with sequenced genomes of *Tetraodon* and human has revealed large regions of conserved synteny among the three genomes, pointing toward an ancestral teleost haploid chromosome set of 12–13 (Woods et al. 2005). Prior to the radiation of the ray-finned teleosts (*Actinopterygii*), their common ancestor apparently went through a whole-genome duplication (Postlethwait et al. 1998) either at ca. 320–350 million years ago (mya) (Christoffels et al. 2004; Taylor et al. 2003; Vandepoele et al. 2004) or at 226–316 mya (Hurley et al. 2007). This duplication yielded the 24–25 chromosomes (haploid set; *n*) commonly found today in many cyprinids and other ray-finned fishes (Cataudella et al. 1977; Postlethwait 2004; Yu et al. 1987) (Table 1).

The doubled haploid chromosome number ($n = 50$) and increased genome size of common carp, goldfish, and crucian carp can be explained by an additional, more recent duplication event in their lineage. Genetic analysis of various genes and/or their protein products in common carp have indeed pointed toward an additional genome duplication in its ancestor (Ferris and Witt 1977; Larhammar and Risinger 1994; Wang et al. 2005; Zhang et al. 1995); however, the estimates for the timing were widely different, ranging between 16–58 mya. Detailed analysis of duplicated DNA markers in common carp estimated the time of that duplication to be 12 mya (David et al. 2003). According to the currently accepted theory, this extra whole-genome duplication – which happened after the separation of the ancestors of today's "small genome" and "medium/large genome" cyprinids (Table 1) – was due to the hybridization of two closely related but different species resulting in allotetraploidy (for review, see Gregory and Mable 2005). The genome of the affected successors then dealt with the consequences of the duplication in different ways during their evolution, yielding a range of different genome sizes and chromosome numbers observable today (Animal Genome Size Database; Gregory 2005a). In addition to whole-genome duplication, partial (segmental) duplications seem to have taken place in the common carp genome between 2.3 and 6.8 mya (David et al. 2003) and in the zebrafish genome as well (Taylor et al. 2001) (Fig. 1).

An interesting alternative explanation for the presence of "duplicated" and "non-duplicated" genomes within the *Cyprinidae* family was proposed by Collares-Pereira's laboratory in the 1990s. According to their theory, ancestral cyprinids had a haploid set with 50 chromosomes ($2n = 100$), and during the evolution, some successor species have lost half of them, ending up with only 25 ($2n = 50$; Alves et al. 2001; Collares-Pereira and Coelho 1989; Collares-Pereira and Da Costa 1999).

Molecular Phylogenetics of Cyprinids and Molecular Phylogeography of Common Carp Subspecies

Polymorphic DNA markers have helped researchers to collect new data on the phylogenetics and phylogeography of the family *Cyprinidae*. Studies based on the analysis of the mitochondrial cytochrome b (*cyt b*) gene (Briolay et al. 1998; Durand et al. 2002;

Gilles et al. 1998; Zardoya and Doadrio 1998, 1999; Zardoya et al. 1999), partial 16S RNA (Gilles et al. 1998), and the mitochondrial control region (Gilles et al. 2001) in European, Mid-Eastern, Asian, and American cyprinids have yielded somewhat conflicting results. While all have supported the traditional subdivision of *Cyprinidae* into the *Cyprininae* and *Leuciscinae* subfamilies, some of them suggested additional subfamilies, such as *Rasborinae*, *Gobioninae*, and *Tincinae* (Gilles et al. 2001). The exact phylogenetic position of some of the latter clades was still under dispute.

Recently, Saitoh and colleagues (2006) have generated a phylogenetic tree of the *Cyprinidae* family based on 53 fully sequenced mitogenomes. Out of the eight main species targeted in this chapter, only three (common carp, ginbuna and zebrafish) were included. The phylogenetic tree contained a major cyprinid and leuciscin clade divided into a total of 12 subfamilies, out of which 2 (*Barbinae* and *Rasborinae*) were not monophyletic. Wang and colleagues (2007) have sequenced the nuclear recombination activating gene 2 (*rag 2*) from over 100 East Asian cyprinid species. According to their data, the *Cyprinidae* family likely originated around 46–49 mya, as opposed to earlier estimates of 39 mya, based on *cyt b* analysis (Zardoya and Doadrio 1999). The new data also indicate that the most basal cyprinid subfamily is *Danioninae* (estimated divergence: 31 mya), whereas the *Cyprininae*–*Leuciscinae* split occurred at about 26 mya (Wang et al. 2007). Six of the eight species discussed in this chapter have been analyzed in this study: common carp and goldfish were classified into the *Ciprinini* clade; grass carp, silver carp, and bighead carp into the monophyletic *Xenocipridini* clade containing all the endemic taxa from East Asia; whereas zebrafish fell in the proposed *Danioninae* subfamily (Wang et al. 2007).

The introduction of polymorphic DNA markers has also allowed researchers to re-visit the issue of the origin of common carp and its subspecies. Aimed with the advanced tools of genotyping (primarily based on mtDNA and microsatellites) new sets of data have been collected.

Kohlmann's team has used PCR–RFLP analysis of two mitochondrial genes to analyze five populations of European and three populations of East Asian subspecies and found them distinctly different (Gross et al. 2002). Later, they extended their studies to 23 populations and additional markers, including al-

lozymes and microsatellites (Kohlmann et al. 2003, 2005). The results of these recent studies confirm the taxonomic status of the European (*C.c. carpio*) and East Asian (*C.c. haematopterus*) subspecies, reject the separate subspecies status of the Central Asian samples (*C.c. aralensis*) suggested earlier by Kirpichnikov (1967), and leave open the possibility of a third subspecies (*C.c. rubrofuscus* or *C.c. viridiviolaceus*) (Kohlmann et al. 2005).

A research team from Wuhan (China) found that the three subspecies of common carp (*Cyprinus carpio carpio*, *C.c. haematopterus*, and *C.c. rubrofuscus*) can be distinguished using mitochondrial (Zhou et al. 2003a, b; 2004b) and microsatellite markers (Zhou et al. 2004a). PCR-RFLP study of the ND5/6 region showed the presence of a single haplotype within each subspecies, but the pattern of each subspecies pair could be clearly separated from each other by at least two enzymes (Zhou et al. 2003a) (Fig. 4). Phylogenetic analysis based on the sequencing of the mitochondrial control region (D-loop) and cytochrome b gene indicated that common carp could indeed be subdivided into three subspecies (Zhou et al. 2003b, 2004b). On the basis of these two mitochondrial sequence regions, any common carp individual can be easily classified (see Fig. 5 as an example). Similar comparisons performed on European carp varieties showed that the ancestor of German domestic carp is most likely Danube wild carp (Zhou et al. 2003a).

The mtDNA of wild common carp from Lake Biwa (Japan) was compared with those of the Eurasian wild and domesticated forms (Mabuchi et al. 2005). The data indicate that the Lake Biwa form is more ancient than the Eurasian one (split ca. 1.7–2.5 mya), pointing toward the East Asian origin of European wild common carp, as proposed earlier by Froufe and colleagues (2002).

David and his colleagues (2007) studied the genetic relationship of common carp strains cultured for food and ornamental purposes using AFLPs and microsatellites. They found marked genetic differences between the two groups, despite their recent divergence. Microsatellite and RAPD analysis of two phenotypically distinctly different Hungarian common carp farmed varieties has indicated that the genetic differences in such man-made stocks might be less than expected (Bartfai et al. 2003), confirming earlier data obtained from German stocks by allozymes (Kohlmann and Kersten 1999).

2.5.2

Genetic Maps, Map-Based Cloning, and QTLs

Genetic linkage maps are ordered, linear sets of genes and genetic markers depicting their relative locations on the chromosomes (for reviews, see Liu 1998; Primrose 1998). The order and relative distance of these genes/markers is estimated on the basis of their recombinational frequency during meiosis, as detected in the F₂ generation.

The first genetic linkage map for zebrafish was based on a mapping panel containing 94 haploid offspring from a heterozygous female produced by crossing individuals from two different strains (Postlethwait et al. 1994). The map was based on 401 RAPD markers, together with 9 mapped mutations. A reference DNA panel from an F₂ intercross (Knapik et al. 1996) was used to produce the first microsatellite-based linkage map with 705 markers (Knapik et al. 1998), which was extended subsequently to a high-density map containing 2,000 markers (Shimoda et al. 1999). Another mapping panel, called the HS panel, containing homozygous diploid F₂ progeny from two F₁ hybrid zebrafish females (Kelly et al. 2000) was used to map 4,073 polymorphic markers, including 3,417 of them from expressed sequences (Woods et al. 2000, 2005).

Whole-genome radiation hybrids (RH) are produced by fusing irradiated cells of the target species with normal diploid cells of the other species (Walter et al. 1994). The resulting chimeric RH cells contain chromosomal fragments from the target species, and their panel can be used for quick physical mapping of sequences. Two RH maps have been produced for zebrafish (Geisler et al. 1999; Hukriede et al. 2001), and used successfully to map thousands of sequences with strong contributions from the zebrafish community.

The Zebrafish Information Network (ZFIN) website lists mapping information from four different meiotic panels and the two radiation hybrid panels described above. The data from the HS meiotic panel and the RH panels has been consolidated into an integrated map of the zebrafish genome (ZMAP) containing 33,266 markers (accessed on June 15, 2007).

Physical maps identify the location (bp) of different loci on the chromosomes. The physical map with lowest resolution is the banding pattern of chromosomes, the status of which for zebrafish was summarized first by Amores and Postlethwait (1999)

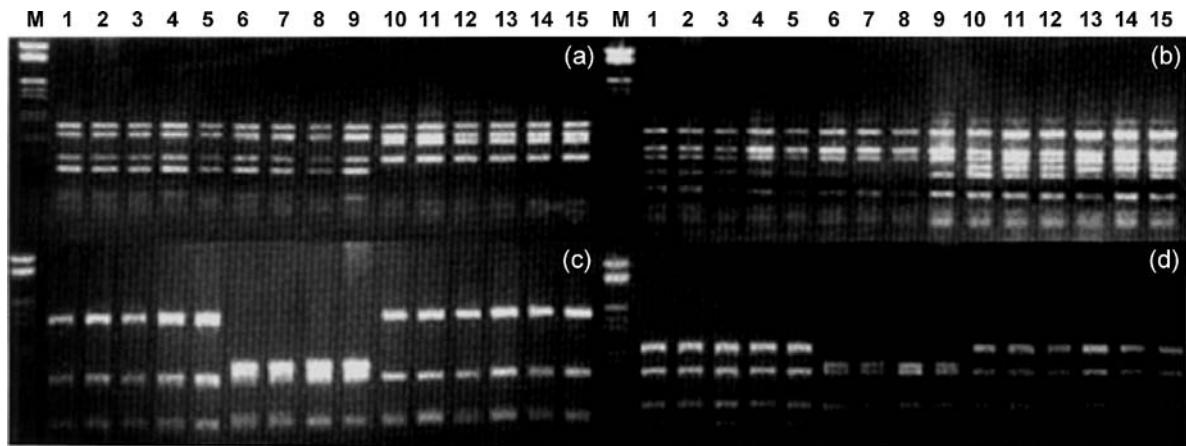


Fig. 4 PCR-RFLP patterns of the ND5/6 sequence amplified from the three subspecies of common carp and digested by *Hae* III (a), *Dde* I (b), *Mbo* I (c), and *Taq* I (d). Samples: *M* molecular weight marker (λ DN A /EcoRI+HindIII), 1–5 *Cyprinus carpio haematopterus*, 6–9 *Cyprinus carpio rubrofuscus*, 10–15 *Cyprinus carpio carpio*. (Reproduced from Zhou et al. 2003a with permission)

RCC	ttacgcattt	tttcttatcg	-aaattcgg	gaggctgtat	ctacggcg-g	acgta
<i>C.c.c.</i>	GMC1t.cc	c.ct....g.	t...gggt.t	tga.t...t.	tacggcgcta .t...
<i>C.c.c.</i>	GMC2t.cc	c.ct....g.	t...gggt.t	tga.t.....	tacggcgcta .-...
<i>C.c.c.</i>	VWC1t.cc	c.ct....ga	t...gggt.t	tgaat.ag..	tacggcgcta .-...
<i>C.c.c.</i>	VWC2t.cc	c.ct....g.	t...gggt.t	tga.t.....	tacggcgcta .-...
		c-	g-		tg-	t-
<i>C.c.h.</i>	YWC1	..gtat.c.c	c.c....ct.a	t...gggt.t	..aat.....	tacggcgcca .-...
<i>C.c.h.</i>	YWC2	ccg.at.c.c	c.c.....ta	t...aggt.t	..aat.....	tacggcgcca .-...
<i>C.c.h.</i>	RMC1	..g.at.c.c	c.c.....ta	t...gggt.t	..aat.....	tacggcgcca .-...
<i>C.c.h.</i>	RMC2	..g.at.c.c	c.c.....ta	t...gggt.t	..aat.....	tacggcgcca .-...
<i>C.c.h.</i>	XRC1	..g.at.c.c	c.c.....ta	t...gggt.t	..aat.....	tacggcgcca .-...
<i>C.c.h.</i>	XRC2	..g.at.c.c	c.c.....ta	t...gggt.t	..aat.....	tacggcgcca .-...
<i>C.c.h.</i>	JKC1	..g.at.c.c	c.c.....ta	t...gggt.t	..aat.....	tacggcgcca .-...
<i>C.c.h.</i>	JKC2	..g.at.c.c	c.c.....ta	t...gggt.t	..aat.....	tacggcgcca .-...
<i>C.c.h.</i>	QTC1	..g.at.c.c	c.c.....ta	t...gggt.t	..aat.....	tacggcgcca c...
<i>C.c.h.</i>	QTC2	..g.at.c.c	c.c.....ta	t...gggt.t	..aat.....	tacggcgcca .-...
		g-a-			a-	
<i>C.c.r.</i>	PRC1	.c....t.c.c	cc....g.t.a	tg...ggg..t	..a.t.....	tacggcgcca .-c.g
<i>C.c.r.</i>	PRC2	.c....t.c.c	cc.....ta	tg...ggg..t	..a.t.....	tacggcgcca .-c.g
<i>C.c.r.</i>	BBC1tg..c	c..t....ta	t...gggg..t	..a.tc...c	tacggcgcca .-cg
<i>C.c.r.</i>	BBC2tg..c	c..t....ta	t...ggg..t	..a.tc....	tacggcgcca .-cg
<i>C.c.r.</i>	SWC1t....c	cc.....ta	cgg...ggg..t	..a.t.....	tacggcgcca .-c.g
<i>C.c.r.</i>	SWC2t....c	c..tc....a	c...ggg.att.....	tacggcgcca .-...g
						g

Fig. 5 Sequences of D-loop region of mitochondrial DNA from different varieties of the common carp. *Bold bases* below every group label genotypes specific to the subspecies. *C.c.c.* *Cyprinus carpio carpio*, *C.c.h.* *Cyprinus carpio haematopterus*, *C.c.r.* *Cyprinus carpio rubrofuscus*, *RCC* reference sequence obtained from GenBank (ID: X61010; dots indicate agreement with reference sequence), *GMC* German mirror carp, *VWC* Volga River wild common carp, *YWC* Yangtze River wild common carp, *RMC* Russian mirror common carp, *XRC* Xinguo red common carp, *JKC* Japanese koi carp, *QTC* Qingtian common carp, *PRC* purse red common carp, *BBC* big belly common carp, *SWC* Yuanjiang River wild common carp

and later by Sola and Gornung (2001). The current state-of-the-art technology for zebrafish is an advanced cytogenetic map with the first generation bacterial artificial chromosome (BAC)⁸ probe panel mapped to individual chromosomes. The panel contains 75 chromosomally mapped BAC probes (three for each chromosome), which hybridize to near-telomeric or near-centromeric positions (Lee and Smith 2004).

The present status of genetic maps for farmed cyprinids is rather preliminary. The only map generated to date is a low-density genetic linkage map for common carp (Sun and Liang 2004). The mapping cross was between common carp and Boshi carp (*Cyprinus pellegrini pellegrini*; Tchang 1933), and the mapping panel contained 46 haploid F₂ embryos from an interspecific F₁ hybrid female. The genetic linkage map covers 4,111 cM, and it contains 268 markers in 50 linkage groups (LGs), equaling the haploid chromosome number. The markers are the following type: 26 common carp microsatellites, 19 crucian carp microsatellites, and 65 zebrafish microsatellites, as well as 105 genes and 57 RAPD markers. No radiation hybrid map or cytogenetic map has been reported for any of the farmed cyprinids to date.

According to the peer-reviewed literature, the only search performed in cyprinids for the identification of quantitative trait loci⁹ (QTLs; Liu 1998) was made on the common carp linkage map described above. Four RAPD markers putatively associated with cold tolerance were identified (Sun and Liang 2004). One of the markers (5N1451c) was successfully mapped to LG 5, whereas the other three remain unmapped.

The map-based positional cloning¹⁰ approach (Bahary et al. 2004; Talbot and Schier 1999) has been used successfully to identify zebrafish genes with important roles in development and disease (e.g.,

Brownlie et al. 1998; Nechiporuk et al. 2003; Zhang et al. 1998). Due to the absence of proper maps, no such feat has been attempted in any of the farmed cyprinids yet.

2.5.3

Libraries, cDNAs and Proteins

Clone libraries are DNA fragment collections archived in suitable vectors that allow for the amplification and long-term storage of the inserted fragments. The libraries are classified according to the origin of DNA (e.g., genomic or cDNA) and the type of vector (e.g., phage or plasmid) (for details, see Sambrook and Russell 2001).

Three decades of research on zebrafish genetics have yielded a large number of libraries, which provide essential resources for those working on the species and other teleosts. Large-insert genomic libraries are available in various different vectors, including P1 artificial chromosomes (PACs)¹¹, yeast artificial chromosomes (YACs),¹² and BACs (Amemiya et al. 1999; Amemiya and Zon 1999; Koch et al. 2004; Zhong et al. 1998). Hundreds of cDNA libraries have also been generated by research labs and commercial companies. An oligonucleotide-fingerprinted¹³ and EST-characterized set with 25,102 clones is available to the community in membrane-printed form (Clark et al. 2001).

There are very few peer-reviewed publications about libraries from farmed cyprinid species. A BAC library was described both from common carp (Kata-giri et al. 2001) and from goldfish (Luo et al. 2006). A commercial lambda phage-based genomic DNA library for common carp is available from Stratagene (La Jolla, CA, USA). The first cDNA library from a farmed teleost was produced from goldfish

⁸ Bacterial artificial chromosomes, or BACs, are cloning vectors derived from the F-plasmid naturally occurring in *Escherichia coli* bacteria. BAC vectors are able to accept large, 100–300 kb inserts.

⁹ A QTL is polymorphic locus with alleles having different effects on a phenotypic trait and showing continuous distribution.

¹⁰ Map-based positional cloning is a method for the identification of a gene based on the chromosomal location of its locus by utilizing a high-density genetic linkage map. No functional information about the gene is necessary for positional cloning.

¹¹ P1 artificial chromosomes (PACs) are vectors for propagating large DNA inserts in *Escherichia coli*. PACs are derived from the genome of the P1 bacteriophage.

¹² Yeast artificial chromosomes (YACs) are plasmid-derived chimeric vectors containing a centromere, two telomeres, and a replication origo from yeast. They are able to accept DNA inserts of up to 400 kb.

¹³ During this procedure, the redundancy of the cDNA library is reduced by hybridizing a number of labeled oligonucleotides against the clones and selecting a single one from a group of clones with identical hybridization profile.

and was used to isolate prolactin genes (Chan et al. 1996). Seven tissue-derived common carp cDNA libraries normalized by subtraction were generated by Cossins' team at Liverpool University (UK) (Gracey et al. 2004). A testis-derived common carp cDNA collection, containing over 3,000 unique clones, has been produced by "in silico" normalization recently (Christoffels et al. 2006).

The GenBank database (Benson et al. 2005) contains 55,011 and 661 mRNA sequences as well as 65,595 and 2,004 protein sequences for zebrafish and common carp, respectively (all four sets being redundant).

2.5.4

The Zebrafish Genome Project

The Zebrafish Genome Project was initiated by the Wellcome Trust Sanger Institute in 2001 (Butler 2000). Two strategies were applied in parallel: (1) a whole-genome shotgun (WGS)¹⁴ sequencing approach, followed by automated annotation and (2) sequencing of physically mapped, overlapping clones¹⁵ from BAC libraries, followed by manual annotation (Jekosch 2004).

The DNA template used for the shotgun sequencing was isolated from ~1,000 embryos of the Tubingen strain. Later reads from a library produced from a double-haploid Tubingen individual were also included in the Zv6 assembly (see below) to ease the task of the assemblers. For the traditional "clone mapping and sequencing" approach, clones from six different libraries (four BAC libraries, one PAC, and one fosmid library) were used as templates. In the framework of the Zebrafish Genome Fingerprinting Project, a physical map was produced by first identifying clones containing markers for a selected region, then clustering them by high-throughput fingerprinting, and finally

selecting the optimal ones for sequencing on the basis of their position in the contig (Marra et al. 1997).

The results for the first approach are displayed on the Ensembl browser under the species name (WGS data for zebrafish), whereas those for the second can be found on the Vega Zebrafish browser. Both of these browsers are accessible through the homepage of the Sanger Institute.

According to the above sources, the status of the Zebrafish Genome Project at the time of finalizing this chapter (July 2007) was the following: The WGS approach based on nearly 19 million reads produced 14.2 Gb of sequence (6–7.5× coverage¹⁶), yielding nearly 106,000 supercontigs (N50 size¹⁷ = 687 kb). The traditional approach yielded nearly 214,000 finger-printed clones sequenced with 21× coverage, equaling nearly 0.4× coverage of the 1.69 Gb zebrafish genome assembled into 1,775 reference sequence contigs. The combined assembly – called Zv6, which integrates WGS data with those of the clones – contains more than 1.63 Gb of assembled sequence arranged into 6,653 scaffolds. The biggest scaffold is more than 9.1 Mb long, and the N50 size is nearly 1.25 Gb ($n = 327$, Zv6 assembly). The assembly contains information on 32,143 transcripts in the form of mRNA, EST, or protein sequences.

At the time of finalizing this chapter, the website of Sanger Institute indicated the end of 2008 as the projected date for providing a completely finished and manually annotated genome sequence in Vega for zebrafish.

2.5.5

cDNA Arrays and Oligonucleotide Chips

cDNA and oligonucleotide arrays (or DNA chips) are dense, organized collections of probes (transcripts or their tags) fixed to the surface of carriers (Schena 1996). They allow for parallel assessment of the expression level of thousands of genes in two different situations on the basis of their differential hybridization (for reviews, see Bowtell 1999; Burnside 1999; Stears et al. 2003; Watson et al. 1998).

¹⁴ A whole-gene shotgun refers to mass sequencing by fragmenting genomic DNA into relatively small fragments and assembling them mostly on the basis of their overlaps and not on their location in larger genomic fragments (i.e., BAC clones).

¹⁵ This is the traditional approach, which is based on sequencing clones generated from a single BAC clone at a time. It is slower than the shotgun approach, but it is expected to provide a more precise assembly, especially in the case of complex genomes.

¹⁶ On average, every base pair is read 6–7 times.

¹⁷ The N50 size for a contig collection defines the size of contig "A," which splits the whole size range of contigs in such a way that half of the total contig size is contained in contigs larger than "A".

The first generation of the arrays were the membrane-based macroarrays (e.g., Rockett et al. 2001) with low probe density. Macroarrays are now seldom-used tools, because they were made obsolete by high-density microarrays containing up to 10^5 – 10^6 probes per slide. Probes used on the microarrays are either directly synthesized or printed onto glass slides (for review, see Granjeaud et al. 1999).

The first zebrafish array was a printed microarray containing more than 4,500 unique cDNAs from heart and skeletal muscle (Ton et al. 2002). Since then, more than half a dozen “general microarrays” (based mostly on genes expressed in embryos/larvae) have been generated, and most of them are available for use by the community (Table 3). Additional probe sets derived from a single organ have also been described (Coimbra et al. 2002; Li et al. 2004). The latter was later converted into a microarray (Sreenivasan et al. 2008). They will be useful to extend our knowledge about tissue- and organ-specific transcriptomes.

Applications for these arrays in zebrafish research range from gene expression profiling during development (Linney et al. 2004; Mathavan et al. 2005; Ton et al. 2002) and organ regeneration (Cameron et al. 2005; Lien et al. 2006), the analysis of organ transcriptomes (Sreenivasan et al. 2008), detecting the effect of environmental changes (Handley-Goldstone et al. 2005; Ton et al. 2003), and drug testing (Tamaru et al. 2005; van der Ven et al. 2006; Voelker et al. 2007), to functional analysis of various loss-of-function situations (Leung et al. 2005; Qian et al. 2005; Sumanas et al. 2005).

So far, the three non-zebrafish cyprinid arrays described in the peer-reviewed literature are two printed cDNA microarrays from common carp (Glacey et al. 2004; Moens et al. 2006; Reynders et al. 2006) and a brain-derived cDNA array from goldfish (Martyniuk et al. 2006). The biggest of these arrays contains more than 13,000 probes from subtracted common carp cDNA libraries derived from seven somatic tissues,

Table 3 Cyprinid microarrays

Name of array	Species	Tissue/ stage	Type	Probes	Source	Available*
Zebrafish Muscle	<i>D. rerio</i>	Muscle	cDNA	ca. 4.5 K	(Ton et al. 2002)	?
2ndG Zebrafish Microarray	<i>D. rerio</i>	Mixed	cDNA (norm.)	ca. 15 K	(Lo et al. 2003)	?
Zebrafish 14 K Oligo array	<i>D. rerio</i>	Mixed	Long Oligos	ca. 14 K	MWG	no
Zebrafish OligoLibrary	<i>D. rerio</i>	Mixed	Long oligos (65mer)	16,399	Sigma-GenoSys	yes
Zebrafish Oligo Array	<i>D. rerio</i>	Mixed	Long oligos (60mer)	ca. 22 K	Agilent	yes
Array-ready Oligo set	<i>D. rerio</i>	Mixed	Long oligos (70mer)	3,479	Operon	yes**
Zebrafish 14 K OciChip	<i>D. rerio</i>	Mixed	Long oligos (50mer)	14,067	Ocimum Biosolutions	yes
Zebrafish Whole Genome Expression Microarray	<i>D. rerio</i>	Mixed	Long oligos (60mer)	32,899	NimbleGen Systems Inc.	yes
GeneChip Zebrafish Genome Array	<i>D. rerio</i>	Mixed	Short oligos	14,900	Affymetrix	yes
Zebrafish Gonad Uniclonal	<i>D. rerio</i>	Gonads	cDNA (i.s.n.***)	6,370	(Sreenivasan et al. 2008)	yes
Common carp Microarray	<i>C. carpio</i>	7 tissues	cDNA	13,349	(Glacey et al. 2004)	yes
Common carp Microarray	<i>C. carpio</i>	unknown	cDNA	960	(Moens et al. 2006)	?
Common carp Gonad	<i>C. carpio</i>	Gonads	cDNA (i.s.n.)	ca. 3 K	(Christoffels et al. 2006)	yes****
Goldfish brain	<i>C. auratus</i>	Brain	cDNA	ca. 1.2 K	(Martyniuk et al. 2006)	yes****
Cyprinid Microarray	<i>C. carpio</i>	10 tissues	cDNA	ca. 24 K	(Williams et al. 2008)	
	<i>C. auratus</i>					

* Availability for other researchers on commercial or collaborative basis in the form of printed slides.

** Not as a slide, only in the form of printable probes as a set.

*** i.s.n. – in silico normalized. (During in silico normalization randomly picked cDNA clones are sequenced, clustered and the longest representative from each cluster is selected for printing to reduce the redundancy of the clone set.)

**** As part of the Cyprinid Microarray.

and it was used to analyze transcriptional changes in poikilotherm organisms as a response to cold shock (Gracey et al. 2004). This array has now been extended to contain 24,000 probes (Williams et al. 2008), including the gonad-derived sequences described by the Orban lab (Christoffels et al. 2006) and goldfish brain derived sequences isolated by the Trudeau lab (Martyniuk et al., 2006).

2.5.6 Induced Mutants and Transgenic Lines

Zebrafish mutants have been generated by three large-scale forward genetic screens¹⁸ (Driever et al. 1996; Haffter et al. 1996) and several smaller ones (e.g., Torres-Vazquez et al. 2003; Wingert et al. 2003; Xiao et al. 2005). At the beginning, all screens were performed by chemical mutagenesis.¹⁹ Later zebrafish mutant sets have also been successfully generated by a pseudotyped retrovirus,²⁰ by the *tol2* transposon isolated from the Japanese medaka (*Oryzias latipes*; Temminck and Schlegel 1846; Kawakami and Shima 1999; Kawakami et al. 2004; Parinov et al. 2004), and even by the plant-derived transposon *Ac/Ds* (Emelyanov et al. 2006). The advantage of the latter three methods over the chemical one is the easier identification of the position of mutation due to the presence of vector sequences. In the case of the transposons, the possibility of hitting neighboring regions of the original insertion by re-mobilized transposons might provide an additional bonus. Recently, the targeted mutagenesis procedure called TILLING – originally developed for plant studies – has been successfully adapted for use in zebrafish (Wienholds et al. 2002, 2003).

The community-wide zebrafish mutant collection, which contains several thousand lines, has been used to understand the role of genes in development (for review, see Grunwald and Eisen 2002) and disease (for review, see Dodd et al. 2000; Dooley and Zon 2000).

¹⁸ Forward genetics generates mutations in genes by random methods and uses the resulting phenotype to identify the mutated gene.

¹⁹ Ethyl-nitrozo-urea treatment of males was used to induce mutations in the germ line.

²⁰ This engineered retrovirus had a genome based on the Moloney murine leukemia virus packaged into the envelope protein of the vesicular stomatitis virus.

Although natural mutations and hybrids are available for the farmed cyprinids, especially goldfish (Smartt and Bundell 1996) and common carp (e.g., Bongers et al. 1997; Kirpichnikov 1967, 1999) we know of no mutant line that has been produced by random or targeted mutagenesis from these species to date.

There are hundreds of stable transgenic lines generated from zebrafish, primarily with the purpose of understanding the role of genes in essential developmental and cellular processes (for reviews, see Lele and Krone 1996; Tomasiewicz et al. 2002; Udvadia and Linney 2003). Stable transgenic lines have only been generated in three farmed cyprinids: common carp, goldfish, and grass carp. Transgenic goldfish lines were used primarily for testing the expression and inheritance of reporter genes from various recombinant constructs (e.g., Yoon et al. 1990; Zhu et al. 1985). Two common carp lines transgenic for growth hormone genes were used to test the possibility of growth enhancement (for review, see Horvath and Orban 1995; Wu et al. 2003a). A grass carp line transgenic to human lactoferrin was developed recently in China in order to increase the resistance of the species against hemorrhage induced by the grass carp hemorrhagic virus (GCHV; Zhong et al. 2002).

2.6 Speculation on Sequencing the Genome of a Farmed Cyprinid Species

Although the importance of genetic linkage maps, radiation hybrid maps and cytogenetic maps in genetic and genomic research is undeniable, they typically provide a “low-resolution view” of the genetic material. Whole-genome sequences offer the ultimate physical map, fully uncovering the genetic information encoded in the chromosomes of a species. Having one or more additional cyprinid genomes sequenced would provide researchers much-needed insight into the evolution of this very interesting group, which contains diploid, tetraploid, and even hexaploid species. It would also allow for further improvements in the commercial selection process by more effective identification of QTLs.

What is the chance for having the genome of a farmed cyprinid sequenced in the near future?

Despite the continuously decreasing costs of sequencing reactions due to technological improvements, whole-genome sequencing with 10–12× coverage and high-quality assembly remains a rather expensive business. Funding for the sequencing of new species by international or national agencies is allocated by considering a number of criteria, among which expected scientific benefits, commercial importance, and genome size seem to be the most important (Gregory 2005b, Reid 2002).

According to the websites of the National Human Genome Research Institute and Ensembl, the genomes of at least 60 vertebrate species have been selected for whole-genome sequencing, and the list is growing continuously. While about four dozen of these species are mammals, interestingly, ten of the remaining non-mammalian species are teleosts. The genomes of Japanese fugu (*Takifugu rubripes*; Temminck and Schlegel 1850; Aparicio et al. 2002) and green spotted puffer fish (*Tetraodon nigroviridis*; de Proce 1822; Jaillon et al. 2004) are available in the form of a draft assembly, whereas work on the genomes of zebrafish (Jekosch 2004), Japanese medaka (Naruse and Takeda 2006), three-spined stickleback (*Gasterosteus aculeatus*; Linnaeus 1758; Kingsley et al. 2004), elephant shark (*Callorhinchus milii*; de Saint-Vincent 1823; Venkatesh et al. 2007), and coelacanth (*Latimeria chalumnae*; Smith 1939; Noonan et al. 2004) is in progress. The sequencing of the following fish genomes has also been approved: Nile tilapia (*Oreochromis niloticus*; Linnaeus 1758), spotted gar (*Lepisosteus oculatus*; Winchell 1864) and skate (*Raja erinacea*; Mitchell 1825). Once the “in progress” genomes are completed, the ten assembled fish genome drafts will offer a goldmine for researchers working on the comparative genomics of teleosts, especially those with a limited amount of sequence information.

On the other hand, the relatively high representation of fish among sequenced non-mammalian vertebrates seems to work against the selection of additional teleost species, even if their commercial importance is obvious, their genome is of reasonable size, and their status is fully ready for such an exercise. None of the farmed cyprinid species discussed in this chapter fulfills these three criteria. Their commercial importance is based primarily on their Asian production quantity. Moreover, some of them are considered to be pests in North America and Australia (Roberts and Tilzey 1997). Therefore,

it does not seem likely that the major American or European sequencing centers will add them to their list soon. The best chance for these species could be offered by the quickly growing sequencing capacity of China, which “flexed its muscles” recently by delivering the draft sequence for the genome of rice (Yu et al. 2002) and the silkworm (Xia et al. 2004).

Which species would be the best candidate for the first farmed cyprinid genome project? The answer is not easy. From the viewpoint of the readily available resources and classical (cyto)genetic data, common carp appears to be the best option for the short term. At Wageningen University (The Netherlands), there are (near)-isogenic lines – generated by successive rounds of androgenesis by Komen’s team – available from various different strains of common carp (Bongers et al. 1997, 1998; Tanck et al. 2002). In the case of complex genomes with a lot of duplicated regions, such individuals provide excellent sequencing templates essential for a good quality assembly. If necessary, these (near)-isogenic lines could also be crossed to generate hybrid individuals to maximize the number of single nucleotide polymorphisms (SNPs) to be found. When compared to the zebrafish, the common carp genome could provide valuable information on the consequences of a recent whole-genome duplication as the first such comparison between two closely related vertebrate species (similar comparisons have been performed between tetraploid and diploid plant species; e.g., Brubaker et al. 1999; Rong et al. 2005).

On the other hand, from the technological and financial point of view, the much smaller genome of the three “truly diploid” farmed cyprinids (grass carp, bighead, and silver carp) appear to be better choices for a sequencing project aiming for a high-quality assembly. The problem with the latter species is the limited knowledge of genetic diversity in farmed stocks and natural populations, as well as a near-complete lack of genomic resources. Should these become available for any of the truly diploid cyprinids in the near future, that species would become a strong contender in the genome sequencing race, especially if financial resources available for such a purpose were limited.

Taking all of the above into account, a two-step approach appears to be the most suitable. A draft sequence with 1–2× coverage could be produced first for common carp. Such a sequence, when complemented with a strong EST program, could

provide the community of cyprinid biologists with an extremely valuable resource. In the meanwhile, the production of the resources essential for sequencing (e.g., double-haploid lines) for grass carp, bighead, or silver carp should be expedited to set up the stage for the second cyprinid genome project, aiming for a high-quality draft assembly.

2.7

The Urgent Need for Comparative Cyprinid Genomics

As described in detail throughout the previous sections, the amount of genetic and genomic information available is highly skewed among cyprinid species. Whereas the zebrafish offers researchers a near-complete genome project (see Sect. 2.5.4), different kinds of high-density maps, and more than 1.3 million ESTs, the farmed fish species with the highest ranking on this cyprinid list is common carp, with a low-density genetic linkage map (<300 markers) and 60 times fewer ESTs than those of zebrafish. The gap between common carp and the rest of the farmed cyprinids is even bigger: altogether, fewer direct resources are available for the genomic research of the remaining six commercial cyprinid species discussed in this chapter than are available for common carp alone.

Therefore, it would make sense for researchers working on the genomics of farmed cyprinids to orchestrate their efforts to create toolsets ideal for comparative genomic approaches. Such resources would allow them to take advantage of the existing sequence and mapping information from zebrafish, the three other teleosts, and the rest of the vertebrates with deeply studied genomes, without the need of extraordinary investments.

This section reviews the possibilities for enhancing the available genomic resources in farmed cyprinids in such a way that they would allow for more efficient data exchange and comparisons between their genomes and that of zebrafish and other vertebrate models. The recommendations concentrate primarily on those areas that are either essential for such an approach or offer the best potential for fast improvements in the field of comparative genomics of cyprinids. Several of the issues analyzed

here were raised earlier during a workshop on the worldwide genomic resources for non-model fish species organized by Andrew Cossins at Cranage Hall, Cheshire, UK, and were discussed in general by two reviews, with topics tightly connected to the workshop (Clark 2003, Cossins and Crawford 2005). Our chapter narrows down the focus from the teleosts to cyprinids and provides recommendations to advance the comparative genomics of the most important members of this family with occasional outlook to other species.

2.7.1

Genome Maps Need to Be Established in Farmed Cyprinids

The starting point to establish comparative genomic studies is the generation of genome maps at all available levels. Genetic linkage maps would be useful to obtain information about the phylogeny of the *Cyprinidae* family, as they would allow researchers to track how syntenic regions and chromosome segments originating from the common ancestor have evolved in the member species. As currently there is only a single, low-density genetic linkage map available for common carp (Sun and Liang 2004), there is an urgent need to generate such a resource for most (all?) species described in this chapter. If this is not possible, then the minimal target should be to generate a genetic linkage map for at least one representative from the three different groups with small (~1 Gb), medium (~1.7–2.0 Gb), and high (~2.7 Gb) genome sizes (see Table 1). The authors' recommendations for the latter case are the following species: grass carp, common carp (further extension of the existing map), and Prussian carp.

These genetic linkage maps should be based primarily on an overlapping set of (conserved) microsatellites, but they should also contain a sufficient number of conserved intragenic repeats to ensure maximal level of cross-compatibility among them. There is a large set of these markers available from the dbSTS database and from the Zebrafish Genome Project as well. Data from several studies indicate that many cyprinid microsatellites could be used for cross-species amplification in other cyprinid species (e.g., Tong et al. 2002; Tong et al. 2005; Yue et al. 2004; Yue and Orban 2002).

Radiation hybrid maps should also be developed to complement genetic linkage maps. The former would allow researchers to complement their maps with non-polymorphic genetic sequences based on zebrafish loci, allowing an increased level of “cross-talk” across the group. Chromosome-specific BAC probe sets selected for zebrafish cytogenetics (Lee and Smith 2004) should be tested in farmed cyprinids to reveal relationships between chromosomes from related species and to gain a better understanding about their evolution. If necessary, such probe sets should also be developed for some of the farmed cyprinids as well.

2.7.2

More Genomic and cDNA Libraries Are Needed

There is an urgent need to produce libraries with genomic and cDNA inserts from farmed cyprinids. Large-insert genomic (BAC and YAC) libraries are essential for the analysis of chromosomal regions and for positional cloning of genes. Ideally, these libraries should be normalized by fingerprinting and provided to the community in the form of high-density filters, as is done for the zebrafish. End-sequenced BAC and YAC libraries are essential for the sequencing of complex genomes, as they allow for more efficient assembly of sequence information obtained by the whole-genome shotgun approach.

High quality, full-length cDNA libraries should be produced from the major organs of all important farmed cyprinid species. End sequencing of their inserts could quickly improve the current imbalance of EST sets. This would be an ideal community project, as the cost of cDNA/EST work is relatively low. EST clusters would yield intragenic SNPs, which could be mapped into the genetic linkage maps to enhance their resolution in the coding regions, as was done for the human map (Miller et al. 2005). Comparative analysis of clustered EST sets could provide valuable information about the composition of transcribed gene sets and the relative quantity of particular messages in various tissues and about the fate of gene orthologs in various cyprinid species.

Mapping of ESTs and full-length cDNAs is expected to help with comparative genomics of closely related species. Our preliminary data seem to indicate that they might be useful for improving the gene

prediction and gene identification of quite distantly related species as well (Christoffels A, Bartfai R and Orban L, unpublished data).

2.7.3

Comparative Transcriptomics of Cyprinids – A Treasure Trove Waiting to Be Opened

One of the indirect benefits of the EST databases and cDNA sequence collections is that the normalized sequence sets derived from them allow for the production of microarrays. Currently, such tools are only available for the zebrafish and common carp (see Sect. 2.5.5 for details), and they should be developed for additional cyprinids as well.

However, researchers working on “sequence-poor” species do not necessarily need to wait for years to study gene expression in their favorite research object. There is a growing body of evidence in the peer-reviewed literature that cDNA microarrays developed for a given vertebrate species can be used to analyze differential gene expression in related species (Adjaye et al. 2004). Similar observations have been made among teleosts as well (Renn et al. 2004; von Schalburg et al. 2005). An array produced from brain cDNA of *Astatotilapia burtoni* (Gunther 1894) showed detectable signals for the majority of 4,500 cichlid-derived cDNA probes when hybridized with labeled targets from the distantly related zebrafish (Renn et al. 2004). Preliminary observations from our laboratory show that zebrafish cDNA microarrays work well with labeled targets from goldfish (Sreenivasan R and Orban L unpublished data). It seems highly likely that the applicability of these arrays can easily be extended to every cyprinid species discussed in the present chapter.

Similar analysis for cross-species applicability has never been performed for oligonucleotide arrays in cyprinids. These arrays contain long (60–70 mer) or short (20 mer) oligonucleotides, which are designed to be highly specific to a particular transcript. They are capable of differentiating even between alternative transcripts produced from the same locus or among the mRNAs transcribed from gene paralogs. This specificity is expected to make these arrays less efficient in cross-species studies than cDNAs, although data from several intervertebrate comparisons seem

to indicate that in the absence of the latter, they might still be applicable (Chalmers et al. 2005; Chan et al. 2005; Grigoryev et al. 2004; Ji et al. 2004).

Harmonized use of the same microarrays across several farmed cyprinid species could generate a large dataset on the expression level of thousands of genes at different developmental stages, in various tissues, and at various environmental conditions. Such a cross-cyprinid dataset would be immensely valuable to understanding the developmental biology and physiology of this important group of species.

2.7.4

A Call for Cross-compatible Cyprinid and Teleost Databases

One of the biggest problems of comparative genomics is that most data are deposited in databases that are not linked to each other. Since user-friendly protocols of data transfer and data comparison are not available for most of the cases, researchers without immense knowledge in bioinformatics are unable to move easily among datasets of different species – a basic necessity for comparative genomics.

The possibility of cross-comparing large datasets is available for most sequenced teleost genomes. Since data from several teleost genome projects – (Taki)fugu, medaka, stickleback, *Tetraodon* – is available on the same server at Ensembl, a pairwise comparative analysis can be performed. However, a parallel analysis of a larger number of genes in more than two species at the same time is not facilitated.

The HomoloGene project at the National Center for Biotechnology Information allows the three-way comparison of sequenced eukaryotic genomes and identifies the homologs. However, it is not prepared to accept a large number of sequences from un-annotated genomes. The software package offered by VISTA Tools (Frazer et al. 2004; Mayor et al. 2000) offers the possibility of alignments of uploaded sequences; however, the size limit is 300 kb, and none of the sequenced fish genomes are offered as base sequences.

Researchers working on farmed cyprinids should develop their own tools for automated comparative analysis of their sequences on a small to medium scale. We would like to propose here that a website allowing for cross-cyprinid comparisons should be set up in

the near future. The example to follow was generated by the Hubbard Center of Genomic Studies of the University of New Hampshire (USA) in an effort championed by Thomas Kocher. Their Comparative Genome Database was created primarily to facilitate comparative mapping among their main vertebrate model group, the cichlids (the genome of some of which is mapped, but not sequenced). However, the software allows comparisons with the genomes of the puffer fish, zebrafish, and medaka. Moreover, their system is open to the possibility of including new species in the future.

The comparative cyprinid database could start on a similar platform and should be linked to all existing and future teleost genomics databases to allow for easy surfing among the various datasets.

2.8

Synopsis

The family of *Cyprinidae* is the most important taxonomic group of freshwater teleosts both for basic biology and aquaculture production. Although an extensive set of methods have been developed and thoroughly tested on the classical genetic analysis and genome manipulation of farmed cyprinids, the amount of data obtained from these species with modern genetic and genomic methods is negligible.

There is an urgent need to collect more data about the genomes, chromosomes, and genes of farmed cyprinids in order to take advantage of the opportunities offered by the various areas of molecular genetics and genomics. A comparative approach is recommended for these studies as the number of species is high and so is the size of some of their genomes. Wisely selected toolsets and approaches would allow the cyprinid community to shuttle information across the whole range of species discussed in the chapter, leading to more efficient allocation of resources and to an intense crosstalk among all researchers involved (and even those outside of cyprinid circles).

The fact that the prime teleost model of developmental biology, the zebrafish, belongs to this group makes the task of data collection easier since researchers working on the farmed representatives can take advantage of the tremendous amount of infor-

mation accumulated on this small, striped, pet fish species over the past 30 years or so.

The presence of large-bodied farm fish and small pet fish within the same family also offers a possibility of an efficient “division of labor” when experiments are designed as suggested by some of the leading zebrafish researchers in a recent article (Cheng 2004). Common carp, goldfish, and the other larger-sized species would be more ideal objects for physiological, anatomical, and cell culture experiments than zebrafish. On the other hand, functional analysis of genes discovered in those species could be performed in the zebrafish using the vast array of tools (e.g., morpholinos, transgenics, microarrays, etc.) optimized for that species (Cheng 2004).

The prospects for cyprinid genetics and genomics are bright. Researchers should work together to understand more about this interesting teleost group for the purposes of basic research and for improved aquaculture production.

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Important Databases and Websites

*Animal Genome Size Database:*²¹

<http://www.genomesize.com/summary.htm>

ArkDB:

<http://www.thearkdb.org/>

Comparative Mapping Database:

<http://hgcs.unh.edu/comp/>

dbEST:

<http://www.ncbi.nlm.nih.gov/dbest/>

dbSNP:

<http://www.ncbi.nlm.nih.gov/snp/>

²¹ Those mentioned in the text are labeled with bold and italics.

<i>dbSTS:</i> http://www.ncbi.nlm.nih.gov/dbSTS/	<i>National Human Genome Research Institute:</i> http://www.genome.gov/10002154
<i>Ensembl:</i> http://www.ensembl.org/	<i>Nile Tilapia page:</i> www.cichlidgenome.org
Entrez Genome: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome	Norwegian Microarray Consortium: http://microarray.no/
<i>FishBase:</i> http://www.fishbase.org/	Salmon Genome Project: http://www.salmongenome.no/cgi-bin/sgp.cgi
<i>GenBank:</i> http://www.ncbi.nlm.nih.gov/Genbank/	<i>Sanger Institute:</i> http://www.sanger.ac.uk/
Genomic Resources for Zebrafish (at ZFIN): http://zfin.org/zf_info/catch/catch.html	Stanford Genome Evolution Center: http://cegs.stanford.edu/index.jsp
<i>Global Biodiversity Information Facility (GBIF):</i> http://www.secretariat.gbif.net/portal/index.jsp	The Institute of Genome Research (TIGR): http://www.tigr.org/
GRASP: http://web.uvic.ca/cbr/grasp/	<i>VISTA Tools:</i> http://genome.lbl.gov/vista/index.shtml
<i>HomoloGene:</i> http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=homologene	<i>WGS data for zebrafish:</i> http://www.ensembl.org/Danio_reario/index.html
<i>ZMAP (an integrated map of the zebrafish genome):</i> http://zfin.org/cgi-bin/webdriver?M!val=aa-crossview.apg&OID=ZDB-REFCROSS-010114-1	UCSC: http://www.genome.ucsc.edu/
Global Invasive Species Database: http://www.issg.org/database/welcome/	Zebrafish Genome Fingerprinting Project: http://www.sanger.ac.uk/Projects/D_reario/WebFPC/zebrafish/small.shtml
<i>Medakafish Homepage</i> http://biol1.bio.nagoya-u.ac.jp:8000/	<i>Zebrafish Genome Project:</i> http://www.sanger.ac.uk/Projects/D_reario/
Michael Smith's Genome Sciences Centre: http://www.bcgsc.ca/	<i>Zebrafish Information Network (ZFIN):</i> http://zfin.org/
MitoFish – Fish Mitochondrial Genome database: http://mitofish.ori.u-tokyo.ac.jp/	<i>Vega Zebrafish browser:</i> http://vega.sanger.ac.uk/Danio_reario/
<i>NCBI:</i> http://www.ncbi.nlm.nih.gov/	<i>Zv6 assembly:</i> http://www.sanger.ac.uk/Projects/D_reario/Zv6_assembly_information.shtml

3 Catfish

Zhanjiang Liu

The Fish Molecular Genetics and Biotechnology Laboratory, Department of Fisheries and Allied Aquacultures and Program of Cell and Molecular Biosciences, Aquatic Genomics Unit, Auburn University, Auburn, AL 36849, USA
 e-mail: zliu@acesag.auburn.edu

3.1 Introduction

Catfish is the major aquaculture species in the United States, accounting for more than 60% of all US aquaculture production. Its global importance is increasing as several countries in Asia, such as China and Vietnam, are now heavily involved in catfish aquaculture. Of the cultured catfish in the US, channel catfish (*Ictalurus punctatus*) is the major cultured species. However, a closely related species, blue catfish (*I. furcatus*) is also important because of its ability to produce hybrid catfish with channel catfish. In addition, a number of characteristics of blue catfish make it a highly useful model for genome research.

Channel catfish belongs to the phylum Craniata, class Actinopterygii, order Siluriformes, family Ictaluridae, and genus *Ictalurus*. It belongs to a general group referred to as catfish, i.e., a group of fishes with smooth skin, large flat heads, and long barbels near the mouth. Like the carps, this group of fishes is hardy such that they are more adaptable for artificial spawning, handling, and culture. They have all the characteristics necessary for aquaculture, e.g., easy to produce seeds, ability to manipulate spawning (including artificial), easy to culture, high tolerance to low dissolved oxygen, and efficient feed conversion. Catfish is particularly adaptable for western lifestyles because it lacks small bones and can therefore be processed into fillets on an industrial scale.

Catfish culture consisted of only sporadic activities before the 1970s in the United States. Starting in the 1960s, a group of scientists at Auburn University, led by Homer Scott Swingle, started pond culture of channel catfish. Research conducted at Auburn Uni-

versity and elsewhere optimized pond design, aeration, nutrition, feeding strategies, hatchery production, and disease management, allowing catfish to become a significant national aquaculture industry in the United States. The industry took off in the early 1980s and has been steadily growing since then (Fig. 1). In 2006, catfish production reached 700 million pounds, becoming one of the fastest growing sectors in American agribusiness.

The major catfish cultured in the US is channel catfish. However, the closely related blue catfish is quite important, not only for research, but also for production. Channel catfish and blue catfish exhibit different phenotypes for important production and performance traits. Channel catfish is superior to blue catfish in growth rate, resistance to columnaris disease, and perhaps feed conversion efficiency since that has been found to correlate with growth rate. Blue catfish is more resistant than channel catfish to the most serious bacterial disease, enteric septicemia

Major US Aquaculture Production

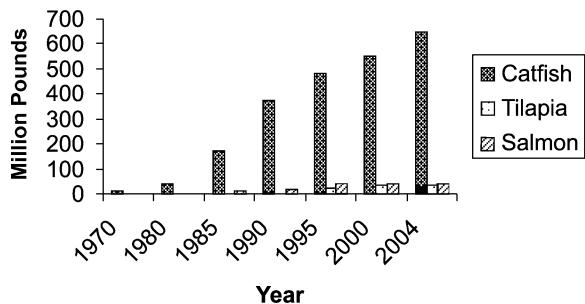


Fig. 1 Catfish is the major aquaculture species in the United States

of catfish (ESC) caused by *Edwardsiella ictaluri*. The bacterial pathogen can infect blue catfish in nature, but the incidence rate is much lower. Blue catfish also has a smaller head and a more uniform body shape, making it more adaptable to processing machines to provide a greater fillet yield than channel catfish. Blue catfish stays higher in the water column, making it easier to harvest by seining. One way of exploiting these desirable traits from blue catfish is through the use of an interspecific hybrid catfish. The other way is to introgress the beneficial genes into channel catfish to develop synthetic breeds.

Channel catfish female × blue catfish male interspecific hybrid catfish are superior to either parent in most production and performance traits. Experiments conducted at Auburn University using various combinations of hybrid catfish production involving channel catfish, blue catfish, white catfish, and flat-head catfish demonstrated that only the female channel catfish × blue catfish male hybrid results in heterosis or hybrid vigor. All other hybrids produced by crossing channel catfish with any other tested ictalurid catfishes, or combinations of any other mating scheme, including the reciprocal hybrid of blue catfish female × channel catfish male produced hybrids that performed worse than channel catfish and, therefore, do not have any application values. The channel catfish female × blue catfish male interspecific hybrid catfish, however, is better in almost all important traits. It grows faster, has a more aggressive feeding behavior, exhibits a greater feed conversion efficiency, is more resistant to major bacterial diseases, is easier to harvest, and provides a greater fillet yield. Wide commercial application of the hybrid catfish could revolutionize the catfish industry. However, mass production of the hybrid seeds is still difficult. Due to reproductive isolation, artificial spawning must be conducted to produce the hybrid catfish fingerlings. In addition, the fertilization rate and hatching rate of the hybrid catfish is significantly lower than the natural spawning of channel catfish. Research is needed to figure out ways to mass produce the hybrid catfish fingerlings to provide sufficient sources of seed stocks for the catfish industry.

Domestic channel and blue catfish exhibit significant phenotypic and genetic variation for economic traits such as disease resistance, growth rate, feed conversion efficiency (found highly correlated with growth), environmental stress tolerance, carcass

yield, seinability, and reproduction (Dunham et al. 1982, 1983, 1984, 1985, 1987a, b, 1990, 1992, 1993a, 1993b; Dunham and Smitherman 1983a, b, 1984, 1987; Bondari 1984; Hallerman et al. 1986; Cadieu 1993; Wolters and Johnson 1994; Dunham 1996; Wolters et al. 1996). Auburn University established an ongoing catfish genetics research program in 1969 to evaluate traditional selective breeding and molecular genetics for improving these quantitative traits. Growth rate and feed conversion efficiency have been improved by as much as 50% through selection (Bondari 1983; Dunham and Smitherman 1983a, 1987; Rezk 1993; Padi 1995), intraspecific crossbreeding (Bondari 1983, 1984; Dunham and Smitherman 1983b), interspecific hybridization (Dunham et al. 1990; Ramboux 1990; Dunham 1996), and genetic engineering (Dunham et al. 1992; Dunham 1996). Disease resistance has been improved primarily through interspecific hybridization (Plumb and Chappell 1978; Dunham et al. 1990, 2000; Dunham 1996; Wolters et al. 1996; Argue et al. 2003), intraspecific crossbreeding (Plumb et al. 1975), and strain selection (Dunham and Smitherman 1984; Wolters and Johnson 1994). Tolerance to low oxygen was improved primarily by interspecific hybridization (Dunham et al. 1983). Seinability can be improved by interspecific hybridization (Dunham 1996) and strain selection (Chappell 1979), and carcass yield by strain selection (Dunham et al. 1984), hybridization (Dunham 1996; Dunham et al. 2000; Argue et al. 2003), and indirect selection (Dunham et al. 1985; Rezk 1993). Heritabilities and genetic correlations have been calculated (Patino 1986; Cadieu 1993; Dunham et al. 2000; Argue et al. 2003).

It is widely recognized that aquaculture must grow rapidly to become an alternative seafood source to the world's collapsing fisheries. It is also clear that improved brood stocks must be developed to overcome the major biological and production hurdles blocking the development of an intensive, reliable, cost-effective, and sustainable aquaculture industry. The greatest obstacle to the catfish industry is the disease problems related to intensive aquaculture. In fact, a recent survey performed by the Auburn University Extension System indicated that all the participating catfish farmers listed disease problems as their top concern (John Jensen unpublished). "To keep my fish alive" is the greatest wish of catfish farmers. Diseases are the primary cause of losses of all operations (NAHMS 1997). The catfish industry suffers an annual

loss of more than \$100 million due to diseases. Other hurdles include low profit margins to catfish producers due to high levels of spending on feeds and the reduction of environmental stresses.

Genetic improvement of catfish is a proven method of addressing these problems. Previous research in selective breeding and molecular genetics has resulted in genetically improved catfish (Dunham and Smitherman 1983a, b, 1987; Dunham et al. 1990, 1992; Dunham 1996) and four releases of genetically improved catfish to the industry. The catfish industry has reached a new milestone based upon research with the establishment of the first few viable breeding companies in the industry within the past 10 years, as well as the increased use of genetically improved catfish and breeding principles by catfish farmers over the past 20 years. An understanding of the chromosomal location of economic trait loci (ETL) is required for further improvement in disease resistance, growth, and carcass yields and other traits using marker-assisted selection (MAS), genetic engineering, or introgression of genes from both channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*). In many cases, the selected traits could be counterproductive to one another. For instance, a recent release of a line referred to as the USDA103, a genetically improved catfish line with enhanced growth rate and feeding behavior by USDA-ARS, has resulted in severe disease occurrence in the catfish industry, demonstrating that it is important to not just concentrate on a single trait, but rather to consider most, if not all, important traits.

In spite of the progress made by traditional selection programs, further progress is limited by lack of genome information. The rationale for creating genetic maps of catfish is to increase the efficiency of selection. Breeders wish to find molecular markers correlated with genetic loci controlling economic traits and use these markers to select superior brood stocks (Waldbieser et al. 2001). Traits such as growth rate are relatively easy to measure and select using phenotypic information alone. A genetic map will be more useful to select fish for traits for which measurement is difficult or expensive (e.g., disease resistance) or lethal to brood stocks (e.g., carcass composition). A genetic map will also be beneficial for introgression of alleles into channel catfish from other species with which hybrid production is feasible, such as blue catfish. In addition, a gene map would provide a guide to selection on multiple traits. For instance, fast growth is often

correlated with low reproductive capacity. A gene map would allow both traits to be mapped such that brood stocks can be obtained to harbor superior traits on both growth and reproduction traits.

Catfish genome studies started in the 1980s. However, due to limitations in technology, very limited genome characterization was conducted. Channel and blue catfish both have 29 pairs of chromosomes and genomic sizes of approximately 1.0×10^9 bp per haploid genome (LeGrande et al. 1984; Tiersch et al. 1990, Tiersch and Goudie 1993). The recombination genomic size is still not well known but is estimated to be 3,000–4,000 cM. Several studies on linkage analysis were conducted in early stages of catfish genome research. Hallerman et al. (1986) found that 9 of 13 polymorphic allozyme loci changed their frequency in response to selection for growth rate, indicating these loci were linked with growth trait. Three electrophoretic studies (Dunham and Smitherman 1984; Hallerman et al. 1986; Carmichael et al. 1992) have documented extensive genetic variability within and between blue and channel catfish at 70 isozyme loci. This genetic variability was first used in gene mapping to estimate gene-centromere distances for six loci in gynogenetic channel catfish (Liu et al. 1992) and for additional polymorphic loci in blue–channel hybrids. The first *Ictalurus* linkage group, comprised of loci coding for glutathione reductase and phosphoglucomutase (Morizot et al. 1994), was identified by studies of segregation in intraspecific channel catfish crosses and interspecific F₁ (channel × blue) × channel catfish backcrosses. Genetic segregation analysis using allozyme markers demonstrated that the marker inheritance from the interspecific hybrid backcrossing was all normal (Liu et al. 1992; Morizot et al. 1994), as indicated by karyotype studies (LeGrande et al. 1984). This early genome-related research paved the way for large-scale catfish genome research. Here in this chapter, I will summarize the recent progress made in catfish genome research and also briefly introduce the National Aquaculture Genome Project in the United States.

3.2 Construction of Genetic Maps

The US National Aquaculture Genome Project is a part of the National Animal Genome Research

Program (NAGRP). Details of the NAGRP can be found at http://www.csrees.usda.gov/nea/animals/in_focus/an_breeding_if_nagrp.html. The Aquaculture Genome Project was launched officially in 1997 when the first Aquaculture Genome Workshop was held in Dartmouth, Massachusetts. The workshop, organized by Acacia Warren of Tufts University, marked the start of large-scale aquaculture genomic research. The US aquaculture genome project was initially established as a regional project (NE-186), and in 2003 joined the National Animal Genome Project (NRSP-8). Under the umbrella of the US National Aquaculture Genome Project, six species groups are listed: catfish, salmonids, tilapia, shrimps, oysters, and striped bass. For more information on the US Aquaculture Genome Project, visit <http://www.animalgenome.org/aquaculture/>. Below, I will summarize research progress in catfish genomics.

3.2.1 Development and Evaluation of Molecular Markers

Facing a genome about which there was little information available, our first task was to develop molecular markers for marking the catfish genome. The initial effort in catfish genomics was devoted to the development of polymorphic markers and evaluation of their applications in catfish. Because we did not have any previous knowledge, we first evaluated marker systems which required no prior molecular information. Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers do not require any specific probes or sequence information. They are applicable to species where there is no known information. Therefore, they were adoptable to catfish. RAPD polymorphisms were abundant using the channel catfish × blue catfish hybrid system but were relatively infrequent among channel catfish (Liu et al. 1998a). A total of 142 primers were tested for their application in genetic studies of catfish (Liu et al. 1999a). RAPD markers were highly useful for hybrid identification in catfish but were not most suitable to genome mapping because of their relatively low reproducibility.

AFLP is similar to RAPD in its inheritance as dominant markers (Liu et al. 1998b). However, several fea-

tures of AFLP make it one of the most preferred markers in catfish. First, it is a highly robust marker system, allowing multi-locus analysis to be conducted in a single analysis. Second, polymorphism rates are high, especially for analyses using the channel catfish × blue catfish hybrid resource families. In contrast to the low reproducibility of RAPD, AFLP is highly reliable. More than 3,000 polymorphic AFLPs were identified using 64 primer combinations (Liu et al. 1999b). AFLP markers were not only very useful for genome mapping in catfish (Liu et al. 2003), but also highly useful for population studies (Mickett et al. 2003; Simmons et al. 2006).

As progress was made in catfish genomics, microsatellite markers were demanded. Microsatellites are very useful markers in catfish. Their major strengths lie in their high polymorphism, codominant inheritance, high abundance, even distribution in the genome, and small locus sizes facilitating genotyping using PCR. Their major drawback is the cost and effort involved in the development of the markers. Like RAPD and AFLP, most microsatellites are type II markers that prohibit information communication among different species through the evolutionary spectrum. Several hundred microsatellite markers were developed in catfish through microsatellite-enriched libraries and analysis of expressed sequence tags (ESTs) (Waldbieser and Bosworth 1997; Liu et al. 1999c, 2001a; Tan et al. 1999; Karsi et al. 2002a; Kocabas et al. 2002b). Very recently, several thousands of microsatellites have been identified from BAC end sequences (Xu et al. 2006).

3.2.2 Development of Type I Markers

One of the lessons learned from the initial efforts of catfish genomics is that not enough attention was paid to the development of type I markers. Type I markers are associated with known genes. Like type II markers, type I markers are useful for genetic linkage and QTL mapping. However, additional benefits of being able to conduct comparative genome mapping, to study genome evolution, to allow interspecies information exchange, and to enhance interlaboratory communications can only be offered by type I markers. Three approaches have been taken to develop type I markers in catfish. The first approach

was to identify microsatellites within cDNAs through bioinformatic mining of microsatellites from EST sequences (Serapion et al. 2004a). While the coding regions of these genes allow us to identify the sequences, microsatellites offer high polymorphism to the sequences. Polymorphic microsatellites within genes of known functions make highly informative type I markers. It appears that catfish ESTs are rich in microsatellites. About 9% of ESTs deposited in GenBank contain microsatellites, twice the rate of zebrafish and seven times the rate in mammals (Liu 2003).

The second approach was to identify single nucleotide polymorphisms among expressed sequences for expressed single nucleotide polymorphisms (eSNPs). In this effort, we have taken advantage of the channel catfish \times blue catfish interspecific hybrid system. Comparative analysis of expressed sequence tags (ESTs) has proven to be a very effective way for development of type I SNPs. He et al. (2004) analyzed a total of 86,603 bases from 159 genes, of which 63,537 bp were analyzed from 131 known genes. Among the 131 known genes, a total of 840 eSNPs were identified, i.e., 1.32 eSNP per 100 bp of known genes. The vast majority of the genes harbor at least one SNP between channel catfish and blue catfish.

The third approach is to identify microsatellites within introns. Catfish introns are rich in microsatellite sequences. PCR primers were designed from adjacent exons of selected genes. The intron sequences were amplified and sequenced for the existence of microsatellites (Serapion et al. 2004b) or length polymorphism. It appears that this approach is also effective for the development of type I markers in catfish. Among the three approaches, the largest effort to date is being devoted to the first approach. A recent EST project sequenced more than 20,000 more channel catfish ESTs and more than 10,000 blue catfish ESTs (Li et al. 2007). Bioinformatic mining of microsatellites from these ESTs allowed the identification of several thousands of EST-derived microsatellites.

In spite of the importance of type I microsatellites, it is clear that SNPs will soon serve as the predominant marker type because of their high abundance. With the recent EST project conducted at the Joint Genome Institute, 400,000 channel catfish ESTs will be produced, and 200,000 blue catfish ESTs will be produced. These resources will allow the identification of a large number of SNPs. Obviously, once the whole genome sequence is produced, SNP markers

will play an even greater role in genome studies of catfish.

Recent progress in sequencing the ends of bacterial artificial chromosome (BAC) clones (Xu et al. 2006) has also generated many microsatellite markers that can be used not only for genome mapping of catfish, but also for integration of the genetic linkage maps based on meiosis and the BAC-based physical map (Xu et al. 2007). By assigning a common set of markers to both BACs and to the meiotic map, the two maps are merged together, providing greater resolution.

3.2.3

Resource Families and Linkage Maps

Linkage maps of catfish were constructed using both interspecific hybrid resource families (Liu et al. 2003) and intraspecific channel catfish resource families (Waldbieser et al. 2001). Each of the two mapping populations has its own advantages. By using the channel catfish intraspecific resource families, recombination frequency is more natural, and thus the genetic distances between markers are not distorted. Practical objectives of the map may be toward fine-mapping of performance traits showing variation among various strains/lines of channel catfish. A genetic linkage map has been constructed using the channel catfish resource families. To date, some 270 microsatellites have been mapped in 32 linkage groups (Waldbieser et al. 2001). In contrast, the use of the interspecific hybrid resource families allows exploitation of an experimental system where maximum polymorphism can be created for markers of various kinds. In this regard, most markers should be species markers, and thus data is most likely transferable among different interspecific resource families.

Because of the high polymorphism between the channel catfish and blue catfish, the hybrid system should allow mapping of various markers, such as RAPD, AFLP, microsatellites, and SNP markers. The practical objectives of using the interspecific hybrid system are to construct synthetic catfish breeds through introgression. As mentioned above, while channel catfish are different from and superior to blue catfish in growth rate, feed conversion efficiency, and resistance to columnaris disease (caused by *Flavobacterium columnare*, the most common bacterial disease

in catfish), blue catfish are different from and superior to channel catfish in resistance to enteric septicemia of catfish (ESC, the most severe bacterial disease in catfish), harvestability, and processing yield. This interspecific system, therefore, provides a model system for analysis of major QTLs involved in disease resistance and disease defenses.

3.2.4

A Summary of the Linkage Map

A genetic linkage map has been constructed using the interspecific hybrid resource families. A total of 418 AFLP markers have been mapped to 44 linkage groups (Liu et al. 2003). Another genetic linkage map was constructed using microsatellite markers (Waldbieser et al. 2001) using the intraspecific resource families. In this linkage map, a total of 293 microsatellites were mapped in 35 linkage groups.

A key demand of catfish genetic mapping is the construction of a gene-based linkage map. To that end, more than 300 type-I microsatellite markers have been genotyped in the interspecific resource family. This gene-based linkage map will serve as the base for comparative genome analysis.

A second demand of catfish genetic mapping is the integration of the various genetic maps. Genotyping the same markers across multiple resource families should allow integration of the maps and provide higher map resolution. Besides, it should be interesting to compare maps constructed using different resource families.

3.3

QTL Mapping and Identification of Candidate Genes

Catfish offer unique advantages for analysis of QTLs. Thousands of individuals can be produced per spawn. The use of large full-sib families for analysis of quantitative traits should minimize any variation due to the use of different families. However, phenotypic evaluations of aquatic animals can be challenging. Marking fish is often difficult and intrusive and causes wounding or stress of fish that interferes with phenotypic evaluations and measurements. For instance, labeling

fish, no matter whether it is heat branding or PIT tagging, often leads to wounding that may stress the fish and result in infections by bacterial pathogens. Traits important for aquaculture include growth rate, feed conversion efficiency, disease resistance, body conformation and processing yield, meat quality, stress response, tolerance to low dissolved oxygen, and tolerance to low water quality. Feed conversion efficiency is very important, because feed accounts for more than 50% of the variable production costs. Diseases can cause up to 30% of annual losses. Under intensive aquaculture conditions, disease problems have been one of the top concerns of catfish producers. Faster growth means shortening of culture period, thereby reducing cost of production.

Selective genotyping is a very effective approach for initial QTL analysis in catfish. Because families are large, phenotypic extremes can be selected for genotyping. For certain markers such as SNPs, selective genotyping coupled with pooling of DNA samples has proven to be very efficient for initial identification of markers linked to performance traits. To date, three markers have been tentatively linked to feed conversion efficiency, and several markers are being evaluated for their linkage with resistance to ESC (e.g., Fig. 2). QTL projects are ongoing at Auburn University. Our most important objective is to identify DNA markers linked to disease resistance. The interspecific hybrid system is currently being used for the assessment of major genes affecting resistance to ESC disease.

3.4

Landscape of the Catfish Genome

An understanding of the basic genomic landscape is very important for decision making related to marker development, linkage mapping, physical mapping, and whole-genome sequence assembly. In this regard, we have given major attention to identifying repetitive elements in the catfish genome. Our recent BAC-end sequencing experiments suggest that about 10% of sequences in the catfish genome are highly repetitive. By using the Repeatmasker web server (<http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker>) and the zebrafish repeat database, about 8% of the catfish sequences were

Fig. 2 Putative microsatellite markers associated with feed conversion efficiency. Shown on the *left* are the best performers and on the *right* are worst performers of feed conversion. Note the great differences in allele usages. Marker names are marked on the *left margin*

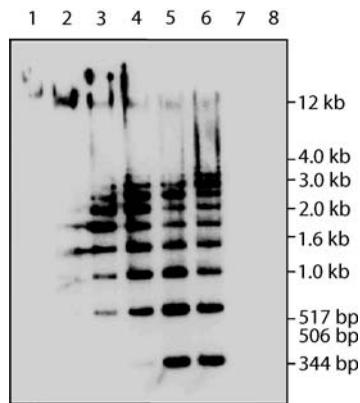
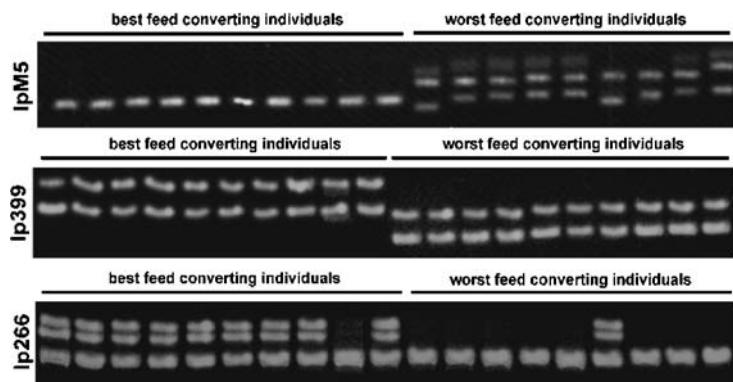


Fig. 3 Southern blot analysis of the *Xba* elements revealed its head-to-tail tandem arrangements. *Lanes 1–6*, catfish genomic DNA was digested with incremental amount of *Xba*I endonuclease, generating a ladder of monomer, dimer, trimer, etc. *Lane 7*, negative control, and *lane 8*, molecular weight marker

masked. Additional use of Fugu repeat databases allowed masking of another 2% of the catfish sequences. The largest group of the repeats contains transposons accounting for approximately 4% of the catfish genome. Simple sequence repeats also account for a significant amount of the catfish repeats in the genome (Xu et al. 2006).

Several repetitive elements have been identified and characterized in catfish. The *Xba* elements are highly repetitive, accounting for about 5% of the catfish genome. This element was initially identified by the observation of prominent bands after catfish genomic DNA was digested with restriction endonuclease *Xba*I. Sequencing analysis revealed that it is about 330 bp in size and highly A/T-rich. Further analysis using Southern blot hybridization indicated

that the *Xba* element was arranged in head-to-tail arrays because incremental amounts of the restriction enzyme led to the generation of a ladder-like pattern representing monomers, dimers, trimers, etc. (Fig. 3). These elements appeared to be specific only for channel catfish and blue catfish but were not in the genomes of several other ictalurid catfishes (Liu et al. 1998c). Fluorescent in situ hybridization (FISH) experiments suggest that these elements are located in the centromeric regions of the catfish chromosomes (Quiniou et al. 2005).

The second major class of repetitive elements identified from catfish was the Tc1-like transposons. Several families of Tc1-like transposon elements have been identified by PCR using a single primer designed from the inverted repeats. Three of these families have been characterized. The largest Tc1-like element, referred to as *Tip1*, is 1.6 kb in size, representing the full-length Tc1-like elements. They are highly similar to those identified from zebrafish and other teleosts (Radice et al. 1994; Izsvák et al. 1995; Liu and Li 2003). The second family of Tc1-like elements identified from catfish, referred to as *Tip2*, was 1.0 kb in size. They represent the deleted forms of Tc1-like elements. Sequence comparison of the *Tip2* with known Tc1-like elements from various organisms indicated that they are more similar to Tc1-like elements from invertebrates than to those from vertebrates, especially when the functional domains were considered. The third family of Tc1-like elements is non-autonomous Tc1-like elements, referred to as *Tipnon* (Liu et al. 1999e). They include inverted repeats that share a sequence identity with the Tc1-like elements but do not have any sequences homologous to the transposase gene. They are very small with a size of about 530 bp. However, *Tipnon* is highly abundant with about 32,000 copies

accounting for about 1.6% of the catfish genome. Most of the Tc1 elements appear to exist as short remnants of the transposon. Further analysis through large-scale EST sequencing suggested that a significant fraction (0.6%) of the entire transcriptome contains Tc1-related sequences in both the natural and antisense orientations (Nandi et al. 2007).

In addition to the *Xba* elements and the Tc1-like elements, the *Mermaid* and *Merman* short interspersed elements (SINE) were also identified and characterized in catfish. About 9,000 copies of *Mermaid* and 1,200 copies of *Merman* exist in the channel catfish genome. They were so named because of their coexistence (Kim et al. 2000).

3.5 BAC Libraries and Physical Mapping

Development of genomic resources and technology in catfish has been a major focus in the last few years. To date, one genomic λ -DNA library has been made (Kim et al. 2000; Kocabas et al. 2002a). Two large insert BAC libraries have been made (Quiniou et al. 2003; Wang et al. 2007). The two libraries were constructed using different restriction enzymes EcoR1 and Hind III, and should be useful to complement each other for gap filling.

BAC contig-based physical maps have been constructed in catfish (Xu et al. 2007; Quiniou et al. 2007). One physical map was constructed using BAC library CHORI 212, and the other using CCBL1 BAC library. With the CHORI 212 BAC library, fingerprints of 40,000 BAC clones ($6.5 \times$ genome coverage) were processed, generating 34,580 BAC clones ($5.6 \times$ genome coverage) for the fingerprinted contig (FPC) assembly of the BAC contigs. A total of 3,307 contigs were assembled. Each contig contains an average of 9.25 clones with an average size of 292 kb. The combined contig size for all contigs was 0.965 Gb, approximately the genome size of the channel catfish. The reliability of the contig assembly was assessed by both hybridization of gene probes to BAC clones contained in the fingerprinted assembly and validation of randomly selected contigs using overgo probes designed from BAC end sequences (Xu et al. 2007). With the CCBL1 BAC library, 46,548 BAC clones were fingerprinted and assembled into 1,782 contigs and covered an estimated

physical length of 0.93 Gb (Quiniou et al. 2007). The use of gynogenetic catfish DNA for the construction of the CCBL1 BAC library allowed cloning of nearly completely homozygous DNA into the CCBL1 library, providing advantages for physical mapping. These physical maps should greatly enhance genome research in the catfish, particularly aiding in the identification of genomic regions containing genes underlying important performance traits. These genome resources lay solid ground for whole genome sequencing in catfish.

The successful sequencing of the human and mouse genomes stirred up a wave of excitement in genome biology. As a result, and partly also due to advances in sequencing technology, sequencing the entire genomes of vertebrate species is no longer an overwhelming challenge. Currently, whole-genome sequencing has been completed or is being completed for a number of vertebrate animals, including cattle, pigs, chickens, and several species of fish, such as the zebrafish, *Danio rerio*, *Takifugu rubripes*, *Tetraodon nigroviridis*, and medaka. In addition, the NIH announced the whole genome sequencing for tilapia – the first aquaculture species. The availability of these genomic sequences will make it possible to trace genomic differences related to functional differences and evolution and fundamentally understand the genetic elements determining expression and function.

For the most efficient whole-genome sequencing, the ideal situation is to avoid repeated sequencing of overlapping regions, but also to cover all gaps such that the sequence of the entire genome can be assembled. Three paradigms have been used for selecting minimally overlapping clones for sequencing. The first is a map-based approach in which fingerprints of clone pairs that appear to have minimum levels of overlapping are picked. This approach was used by the *C. elegans* project and on human chromosomes 1, 6, 20, 22, and X. This approach works well, except that the average overlaps were found to be significant (47.5 kb). If the fingerprinting is conducted by four-color fluorescence-based labeling, this situation probably could be improved, but such a prediction is yet to be proved.

The second approach is based on sequence-tagged connectors using BAC-end sequences (Venter et al. 1996). This approach alleviated the problem of large overlapping regions, but the risk of false positives is high. The third approach is a hybrid of the first two approaches and has been used in various sequencing

projects such as *Arabidopsis* and *Drosophila*. By examining both the overlapping fragments and the overlapping sequences, this approach has been both efficient and reliable. However, many genome resources must be developed to prepare a species for effective whole-genome sequencing. Among many resources, large-insert BAC libraries are crucially important because of their capacity to serve as the basis for physical mapping as well as for the standard clone sets for high-throughput genomic sequencing (Tomkins et al. 2001; Osoegawa et al. 2000, 2003). The BAC-end sequencing (BES), or sequence-tagged connector (STC), approach has proven to be an effective strategy for large-scale sequencing (Venter et al. 1996). This approach involves sequencing the ends of BAC inserts to scatter sequence tags randomly across the genome (Mahairas et al. 1999). Once any BAC or other large segment of DNA is sequenced to completion by conventional shotgun approaches, the STCs can be used to identify a minimum tiling path of BAC clones overlapping the nucleation sequence for sequence extension (Siegel et al. 1999; Chen et al. 2004), thereby greatly reducing redundant sequencing. BES is also useful for confirming genome assembly and for obtaining a non-biased sample of the genome for the purpose of analysis for gene contents, status of repetitive elements, and simple sequence repeats (Larkin et al. 2003; Winter et al. 2003). Thus, production of a BAC-based physical map and BAC-end sequences in channel catfish should lay the scientific foundation for sequencing the whole genome of this species. Although other methods have been used to generate contigs for targeted comparative sequencing, large-scale BAC-end sequencing is currently the most efficient strategy for building whole-genome comparatively anchored physical maps in map-poor species (Larkin et al. 2003) such as catfish.

Recently, we have sequenced 25,000 BAC ends, generating almost 50,000 BESs. This project produced more than 27 million base pairs of genomic sequences, representing approximately 3% of the catfish genome, allowing, for the first time in catfish, a look at gene content, repeat structure and arrangements, and conserved syntenies. The BAC-end sequences are equivalent to one BES every 20 kb, providing a valuable resource toward whole-genome sequencing of catfish. Furthermore, many genes and microsatellites were assigned to BACs, allowing them to be used for in-

tegration of the physical map with linkage maps. We also confirmed conserved syntenies using overgo hybridization, demonstrating the value of the draft zebrafish genome sequence to catfish research. Once BAC-based contigs are produced, this BES resource will allow integration of the maps, development of regional markers for fine QTL mapping, and large-scale comparative genome analysis. Such resources, therefore, are important for genetic analysis of performance traits important to aquaculture, whether or not the catfish genome will be sequenced in the near future.

3.6 EST Analysis and Transcriptome Analysis

Twenty-one cDNA libraries have been made from channel catfish, including 15 cDNA libraries made from various channel catfish tissues and six cDNA libraries made from cultured cell lines. Tissues used for construction of the cDNA libraries include head kidney (anterior kidney), spleen, skin, liver, brain, stomach, intestine, ovary, gill, muscle, testis, pituitary, olfactory tissue, and trunk kidney (posterior kidney). Fourteen of the 15 cDNA libraries were made in the pSport-1 vector (Life Technologies, MD), and one was made in the lambda Unizap cloning vector (Stratagene, CA). In consideration of their uses for the identification of SNPs, tissues from 15 fishes were used; as genomic resources to include potentially most, if not all, transcripts for the study of disease-related genes, tissues were collected from both healthy and infected fishes at various times after infection (Li et al. 2007). The six cDNA libraries from the cultured cell lines of channel catfish were from the catfish autonomous (immortal) B cell line, the catfish autonomous (immortal) T cell line, one-week-old catfish mixed leukocyte culture, the catfish autonomous (immortal) macrophage cell line, and the catfish nonautonomous (mortal) cytotoxic T cell lines (<http://morag.umsmed.edu/libraries/index.html>).

Six cDNA libraries were also constructed from blue catfish using tissues of head kidney, spleen, liver, gill, skin, and heart. These cDNA libraries were used to conduct comparative analysis of ESTs between the channel catfish and blue catfish for the identification

of eSNPs. EST analysis has proven to be one of the most efficient ways for gene identification, gene expression profiling, and cataloguing. It also produces resources for the development of cDNA microarrays. To date, about 43,000 ESTs from channel catfish and 10,000 from blue catfish have been produced and deposited in GenBank. The Institute of Genome Research (TIGR) has constructed a gene index that includes more than 23,000 unique sequences (<http://www.tigr.org>). This number will increase, since several more thousands of ESTs sequenced recently have not been deposited in GenBank yet.

The majority of cDNA libraries have only been sequenced at a low depth (Fig. 4). In order to acquire a greater coverage of the catfish transcriptome, more ESTs must be sequenced. In our previous sequencing experience, the gene discovery rate was quite high. Our initial sequencing efforts were limited to non-normalized libraries, including libraries made from brain (Ju et al. 2000), head kidney (Cao et al. 2001), skin (Karsi et al. 2002a), and spleen (Kocabas et al. 2002b). On top of these efforts, a recent EST sequencing project generated 30,000 additional ESTs (Li et al. 2007). In this project, overgo probe hybridization subtraction was used to enhance the gene discovery rate. Overall, the sequencing redundancy rate was below 30%, an excellent rate. In a recent effort attempting to characterize the entire catfish transcriptome, the author of this chapter took a lead along with 80 scientists and obtained approval for a large EST sequencing project by the Joint Genome Institute of the Department of Energy. In the project, 300,000 catfish EST clones will be sequenced from both ends to generate 600,000 ESTs. I believe that through this project, we should be able to capture the largest proportion of the catfish transcriptome, and this project will soon be completed.

One of the major uses of ESTs is to provide technological resources for the development of microarrays. The first catfish microarray was a 660 gene channel catfish (*Ictalurus punctatus*) cDNA array printed on nylon and used to identify channel catfish brain genes responsive to cold acclimation at four time points (0, 2, 24, and 48 h) after a shift from 24 °C to 12 °C (Ju et al. 2002). Cultured catfish in the US must be able to tolerate wide ranges in water temperatures throughout the year. The findings of this study (e.g., transient induction of chaperone and signal transduction pathway genes) provided insight into the genes and molecular

pathways altered in the catfish brain during exposure to a type of environmental stress that is relevant to the aquaculture industry (decreased ambient temperature) (Ju et al. 2002). A comprehensive understanding of the genes involved in cold acclimation may reveal suitable targets (e.g., expression biomarkers or SNPs) for marker-assisted selection (MAS) of cold-resistant catfish broodstock. A 19 K oligo microarray platform was developed to identify catfish spleen genes responsive to LPS (Li and Waldbieser, 2006). This study confirmed that many immune-relevant genes (e.g., TLR5, interferon regulatory factor 1, a chemokine receptor) responded in LPS-stimulated catfish spleen.

Infectious diseases are among the most serious threats to the global aquaculture industry. Therefore, one of the main applications of fish genomic resources and techniques is to identify genes that may be suitable targets for MAS of disease-resistant broodstock. Channel catfish is the most economically important aquaculture finfish species in the US, and the immune system and responses of this species are well-characterized. While channel catfish are relatively susceptible to *E. ictaluri* infection, blue catfish are relatively resistant. A 28 K catfish in situ oligo microarray platform was recently developed, with features representing all discrete transcripts currently known in *I. punctatus* (21 K genes) and *I. furcatus* (7 K genes). This is an outstanding genomics platform for comparative transcriptomic studies aimed at identifying candidate genes or heritable expression biomarkers of natural resistance to *E. ictaluri*. Two studies using the 28 K catfish microarray provide a comprehensive look at the hepatic gene expression responses of channel catfish and blue catfish to challenge with *E. ictaluri* (Peatman et al. 2007, 2008). A comparison of the results of these two microarray studies identifies a suite of 58 genes that responded to the pathogen in blue catfish but not in channel catfish. This differentially responsive suite included immune-relevant genes such as CC chemokine SCYA106, MHC class I alpha chain, and matrix metalloproteinase 13 (MMP13) (Peatman et al. 2007b). These studies identified numerous genes that potentially play roles in the differential sensitivity of channel and blue catfish to *E. ictaluri*, forming a solid foundation for future functional characterization, genetic mapping, and QTL analysis of immunity-related genes from catfish (Peatman et al. 2008).

EST analysis allows a brief assessment of tissue expression of genes. It also allows identification of alter-

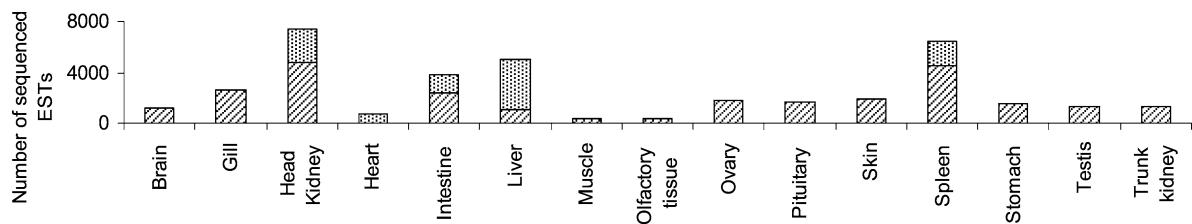


Fig. 4 Catfish libraries and EST sequences produced to date. Sketched areas indicate channel catfish libraries, and dotted areas indicate blue catfish libraries

native splicing and alternative polyadenylation. Most importantly, it is probably the most efficient way to identify polymorphic markers such as EST-associated microsatellites. In a recent analysis of 43,000 ESTs, we have identified more than 4,000 EST-associated microsatellites. Though additional work is still required for these microsatellites to be used as markers, it is the first step to the mapping of a large number of type-I markers.

ESTs also provide the material basis for the study of gene duplication. Gene duplication is a widespread phenomenon in teleost fishes. The most popular theory currently states that teleost fish went through a stage of whole-genome duplication, making them tetraploid organisms, followed by rapid random gene losses in various lineages. Such high levels of gene duplications present many difficulties for aquaculture genomicists. The availability of ESTs from closely related species of channel catfish and blue catfish provide phylogenetic approaches to differentiate if the genes are orthologous or not. For instance, if a channel catfish EST is more similar to a blue catfish EST than another channel catfish EST, it would suggest the presence of two copies of the related genes in channel catfish.

3.7 Systematic Characterization of Genes and/or Full-Length cDNAs

Systematic analysis of complete cDNAs and/or genes using a genomic approach is part of our genome program. While understanding the genome on a large scale is important, detailed analysis of genes and their expression is mandatory for the understanding of gene structure, gene evolution, gene families, or-

thologs versus paralogs, and gene expression in relation to functions. Additional information concerning transcript processing can also be obtained regarding alternative splicing and alternative polyadenylation. All such information should facilitate comparative functional genomics.

A set of transcripts involved in a specific metabolic pathway or a specific process can be obtained systematically during large-scale EST analysis without screening for specific cDNAs one by one. After the initial identification of ESTs representing genes of interest, complete cDNA sequences can be easily obtained and their expression analyzed. Using such an approach, we have characterized a complete set of all 32 small ribosomal protein cDNAs and a complete set of all 47 large ribosomal protein cDNAs from channel catfish (Karsi et al. 2002b; Patterson et al. 2002). Other genes we have analyzed include myostatin (Kocabas et al. 2002a), gonadotropin (GnRH) alpha subunit (Liu et al. 1997), GnRH beta subunits 1 and 2 (Liu et al. 2001b), alpha actin gene (Kim et al. 2000), creatine kinase (Liu et al. 2001c), a large number of CC (He et al. 2004; Bao et al. 2006; Peatman et al. 2005, 2006) and CXC chemokine genes (Baoprasertkul et al. 2004, 2005; Chen et al. 2005), hepcidin (Bao et al. 2005), LEAP-2 antimicrobial peptide (Bao et al. 2006), bacterial permeability increasing protein (Xu et al. 2005), three NK-lysin genes (Wang et al. 2005, 2006), a number of cytokine genes, a number of complement genes, and a large number of cytochrome P450 genes (Z Liu unpublished).

The significance of characterizing complete coding sequences has been realized. NIH started the mammalian Full-Length cDNA Initiative (<http://grants.nih.gov/grants/guide/rfa-files/RFA-CA-99-005.html>) in 1999 for the purpose of functional and comparative genomics. Full-length cDNA databases of human (<http://www.ornl.gov/meetings/wccs/helix>.

htm) and mouse (<http://www.jsbi.org/journal/GIW99/GIW99P34.pdf>) cDNAs have also been established in Japan. While our efforts and resources for the analysis of full-length cDNAs are limited, our high-quality cDNA libraries should be a valuable resource for such purposes.

3.8 Future Perspectives

Efforts in catfish genome research should be enhanced. Specifically, I believe the following areas need to be addressed in the very near future. First, genetic linkage mapping should be continued. Denser genetic maps must be constructed for practical usefulness in breeding programs. QTL mapping efforts should be increased. With the initial identification of performance traits-linked markers, genome regional markers should be developed for fine-mapping the putative QTLs. Candidate gene identification using microarray and other approaches should be conducted in order to pin down the potential genes involved in important QTLs, especially for disease resistance. Integration of the linkage and physical maps is very important for the identification of genes underlining production and performance traits. More importantly, regional markers can then be developed from adjacent BAC clones for fine-mapping of QTLs and for the eventual cloning of economically important genes. In the long term, the BAC contigs should be useful as the guide for entire or partial genome sequencing in catfish. Mapping of common markers on the physical map and the linkage maps will also allow integration of various maps that will greatly increase the resolution of catfish maps. Comparative mapping efforts should be increased. Much information can be obtained by the comparative mapping.

Benefits of great investment into basic studies using model species can only be realized by comparative genomics. This includes the “transfer” of information from map-rich species to catfish and also comparative studies among several aquaculture fish species such as tilapia and salmonids. A large number of type-I markers are being mapped in catfish. Upon completion, comparison of the map location of these type-I markers can be directly compared to those of the human, cattle, swine, and zebrafish. Coordination and

mapping of the same set of type I markers in other aquaculture fish species will also allow development of comparative maps in several other aquaculture species. In addition, hybridization of a common set of type-I marker probes to catfish, tilapia, and salmonids has been planned (TD Kocher, pers. comm.). A direct comparison of their location on the physical map is also possible using BAC contigs. Gene expression in relation to function should be studied in a comparative way through evolution. While many genetic mechanisms may have been evolutionarily conserved, specific mechanisms discovered from catfish should fill the gap it represents as an important aquaculture species among the lower teleost fish.

Whole-genome sequencing must be considered for catfish in the near future. This is mandated by several realities. Several funding agencies are now starting to limit their funding to species with entire genome sequences. This would mean a double penalty on aquaculture species if we do not push for genome sequencing. First, because of the lack of genome sequences, we cannot do *in silico* cloning, *in silico* Southern blot, and many other functions available to species with genome sequences; and second, the lack of genome sequences certainly does not disqualify anyone for applying for grants to do functional genome research. Also, the genome research of catfish has come to a stage where further progress is limited without the genomic sequences, and advances in sequencing technology make it feasible now to carry out the entire genome sequencing of catfish. At present, it would take about \$10 million to sequence and assemble the catfish genome. Whole-genome sequencing was not previously even a possibility at this price.

In spite of funding limitations, catfish genome research has made major advances in recent years. We expect that as the genome research progresses, genome information will be applied to selective breeding programs, allowing enhanced broodstock development. Along with research from many other fields, genome research will prove to be the fundamental basis for a sustainable aquaculture industry.

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4 Tilapias

Avner Cnaani and Gideon Hulata

Hubbard Center for Genome Studies, University of New Hampshire, Gregg Hall, 35 Colovos Road, Durham, NH 03824, USA
e-mail: vlaqua@volcani.agri.gov.il

4.1 Introduction

4.1.1

Brief History: Center of Origin, Domestication, Dissemination

Fishes of the family Cichlidae, to which tilapias belong, are abundant in tropical and subtropical regions. Originating from Africa, where they dominate freshwater lakes, they are native to South and Central America, the Indian subcontinent, and the Middle East. Later introductions, throughout the 20th century, mostly for commercial purposes, spread the family all over Asia, southern Europe, and the southern USA (Keenleyside 1991a). The natural distribution of tilapias is limited to Africa and the Middle East. Tilapias have often been transferred from their native habitats to new locations within Africa, and from Africa and the Middle East to other continents for culture purposes (Philippart and Ruwet 1982; Moreau 1983). Some of these transfers and introductions may have resulted in negative impacts on native fish stocks, including the loss or disturbance of natural genetic resources for tilapias.

The genera *Oreochromis*, *Sarotherodon*, and *Tilapia* of the family Cichlidae (commonly referred to as tilapias; Trewavas 1983) consist of some 100 species; however, only a handful are considered of importance in aquaculture (Pullin 1991). Among those are the blue (or Jordan) tilapia *O. aureus* Steindachner, the Nile tilapia *O. niloticus* L., and to a lesser extent *O. spilurus* spilurus Gunther, *O. andersonii* Castelnau, *S. melanotheron* Ruppell, *T. rendalli* Boulenger, and red tilapias (a diverse group of single species – *O. niloticus* and *O. mossambicus*

Peters – or more often hybrids involving the above two species with *O. aureus* and/or *O. urolepis hornorum* Trewavas).

Like most aquatic cultured species, and in contrast to many other agricultural species, tilapias are at a very early stage of domestication. Farmers often rely on broodstock that have only recently been removed from wild populations. The need, and opportunity, for genetic improvement of these species is great. Quantitative performance of cultured fish is strongly affected by genetic determinants. Therefore, a better understanding of the genes regulating economically important traits should benefit selective breeding of aquatic species, and such breeding would improve the quality and performance of these animals in commercial aquaculture (Hulata 2001).

4.1.2

Zoological Descriptions

Tilapiine fishes (Trewavas 1983) belong to the family Cichlidae, order Perciformes, within the Teleostei. Tilapias are sturdy fish, well suited to aquaculture under a broad range of conditions, and they have been introduced into most tropical regions. Due to their tropical origin, their distribution is limited by climatic regimes. Tilapias are mostly cultured in inland, fresh, or brackish water, but due to their salinity tolerance, several species are cultured in full-strength seawater, mainly in cage farming (Watanabe et al. 1989, 2002; Costa Pierce and Hadikusumah 1990; Cruz and Ridha 1990, 1991; Suresh and Lin 1992). Increasingly, tilapias are being raised at high density in intensive systems with formulated feeds, but most tilapias are still cultured in semi-intensive pond systems. The ability of these fish to feed low in the food chain, as either a col-

umn filter feeder or benthic (bottom feeding) omnivore, explains much of their appeal as cultured fish. Due to their mild flavor, culinary versatility, and ease in rearing, tilapias have been dubbed the “aquatic chicken” (ICLARM 1984; Coward and Little 2001).

Tilapias (like all cichlids) are bony, perch-like, bilaterally-compressed fishes. Their body is covered with scales, except for the head, which is incompletely covered. The lateral line on both sides of the trunk is interrupted into two parts. The single dorsal fin is well developed, with spiny rays in its anterior part and soft rays in the posterior part. The pelvic fins are located in the anterior part of the trunk and are equipped with a spiny ray in the front. The anal fin is also equipped with a few spiny rays. Like all cichlids, they have a single nostril on each side of the head. The jaws in the mouth can be protracted for feeding purposes. They are lined with teeth of variable number and shape, in accordance with the species’ specialized feeding habits. Deep in their mouth, the throat is equipped with a pair of bones covered with teeth. The lower pharyngeal bone is shaped like a triangle while the upper one is shaped like a plate. Together they form the “pharyngeal apparatus,” which plays a significant role in chopping the food while feeding; again, the number and shape of the teeth, and the shape of the triangular bone, reflect feeding specializations.

The most important feeding characteristic of tilapias is their feeding low in the food web. For aquaculture, this means they can be fed cheaper feeds (largely based on plants or plant-derived components) than those used to feed carnivorous species (which require large quantities of animal protein and fat). They can be grown in a wide range of culture systems, from extensive and semi-intensive systems where they feed on the components of the natural food web developing in the ponds, to highly intensive systems where they are fed protein-rich diets in a pelleted form. They can be cultured in monoculture, or in polyculture with other fish species.

Tilapias (like all cichlids) are unique in their mating systems and parental care of their young (Barlow 1991; Keenleyside 1991b). For a period of a couple of weeks after spawning, the progeny are protected by one or both parents until they become independent. Tilapias can be divided into two subgroups based on their breeding behavior. Substrate

spawners (guarders) are monogamous, forming pair bonds for long periods of time, and their eggs are adhesive and guarded in a nest until becoming free swimming. Mouth brooders are polygamous; mating is brief and females depart from the males’ territories after collecting the eggs deposited in a nest into their mouth. In some rare cases, both parents or only the males collect and brood the eggs and larvae in the mouth. In fact, the systematic classification introduced by Trewavas (1983) is based largely on breeding patterns – *Oreochromis* being maternal mouth brooders, *Sarotherodon* being paternal or bi-parental mouth brooders, and *Tilapia* being substrate brooders.

Tilapias are easily bred in captivity, and in the tropics they can breed all year round. In subtropical regions, the breeding season is limited by water temperature and may range from 5 to 7 months. Fry production can be practiced in a wide range of facilities (earthen, concrete, and plastic-lined ponds; fine-mesh cages; aquaria; and tanks). Due to their investment in parental care, fecundity is relatively low compared to most fishes and ranges from several hundred to several thousand per spawn, depending on the species and size of the breeding female. The eggs, however, are quite large. The larvae start feeding as soon as they finish absorbing the yolk sac, and they easily accept dry or live food. Females can breed every 3–4 weeks during the breeding season. Tilapias attain sexual maturity when rather young (4–6 months) while still being smaller than marketable size.

Together, these characteristics cause a major management problem to tilapia culturists, namely uncontrolled reproduction in grow-out ponds that results in over-crowding and slow growth of stocked fish. To avoid this, many tilapia farmers are using monosex instead of mixed-sex populations, although in Third World countries, mixed-sex is still widely used. Various methods are used to establish monosex populations, including genetic manipulations. During the 1960s–1970s, production of all-male F₁ hybrids was the focus of much of the genetic research efforts devoted to tilapias (Wohlfarth and Hulata 1983) and triggered research into understanding the mechanism controlling sexual differentiation in tilapias. Results suggested the existence of two genetic systems primarily controlling sexual differentiation among *Oreochromis*

species – XX/XY and WZ/ZZ. Efforts to uncover the genetic basis of the sex determination in tilapias continue using molecular tools and genomics. Chromosomal regions strongly associated with sex in tilapia have been identified recently (Lee et al. 2003, 2004).

The primary domesticated Cichlidae species of the genus *Oreochromis* have $2n = 44$ chromosomes (Korff et al. 1979; Majumdar and McAndrew 1986); a few species have exceptional chromosome numbers ranging from $2n = 38$ to $2n = 48$ (Harvey et al. 2002a, b). The genome contains one pair of large chromosomes, the rest being medium-sized to small chromosomes (Harvey et al. 2002a, b; Martins et al. 2004). The genome size of tilapias has been estimated to 1.1 pg, and therefore it is predicted that the tilapia haploid genome contains about 1.06×10^9 bp (Majumdar and McAndrew 1986). The latest tilapia linkage map spans a total of 1,311 cM (Lee et al. 2005).

4.1.3 Economic Importance

Tilapias are among the most important food fishes cultured in tropical and subtropical countries (Beveridge and McAndrew 2000). Worldwide aquaculture production of tilapias (and other cichlids) exceeded 1.8 million tons per year in 2007 (FAO), making them one of the most important cultured aquatic species (4% of total world aquatic animal production), and they are ranked eighth on the Food and Agriculture Organization of the United Nations's (FAO) 2004 major cultured species list. The main producers (aquaculture and captive fisheries together) were: China (~900,000 tons), Egypt (~200,000 tons), the Philippines (~145,000 tons), Indonesia (~140,000 tons), Thailand (~100,000 tons), Taiwan (~90,000 tons), and Brazil (~70,000 tons).

The flesh of tilapias is white and rather delicate. It has a sweet flavor and tender flakes when cooked and is considerably leaner than farmed salmon. It only contains 1 gram of fat (0.4 grams of saturated fats) in a 100 gram portion. Fillet yield is about 40% of the whole fish body weight. The edible part of the carcass of male and female tilapia consists of ~53% and ~51%, respectively (Dikel and Celik 1998). Body moisture ranges from 69.2–74.6%; protein,

lipid, and ash range from 49.3–52.5%, 34.3–37.1%, and 11.0–12.5%, respectively (El-Sayed et al. 2003). According to the American Tilapia Association (<http://ag.arizona.edu/azaqua/ista/nutrition.htm>), the US Food and Drug Administration (FDA) approved nutritional characteristics of tilapia are: 1 g total fat (0.5 g saturated fat), 21 g protein, 40 mg sodium, 55 mg cholesterol, 90 mg omega-3 fatty acids, and 93 calories (9 from fat) per 113 g (4 oz) fillet portion.

4.1.4

Breeding Objectives and Classical Breeding Achievements

Selective breeding for improved growth rate has been performed seriously on only one species, the Nile tilapia (*O. niloticus*), resulting in an improved breed known as the GIFT strain that has been disseminated to various countries in Southeast Asia (Eknath et al. 1993, 1998; Gupta and Acosta 2004). Other populations of Nile tilapia, and various other cultured species, have had little or no genetic selection initiated for performance traits, such as growth or disease resistance (Brzeski and Doyle 1995; Sanchez et al. 1995; Basiao and Doyle 1999).

Hybridization between tilapia species, to produce all-male progeny, was considered a promising method to prevent the uncontrolled reproduction that leads to over-population of culture ponds. Hybridization between some species of tilapias such as Nile tilapia, *O. niloticus*, and the blue tilapia, *O. aureus*, results in the production of near all-male offspring. This hybrid combines the advantageous characteristics of both species, being more cold tolerant than *O. niloticus* and burrowing in the mud less than *O. aureus*. It also has good salinity tolerance. Other tilapia crosses producing predominantly male offspring include Nile tilapia \times *O. urolepis hornorum* or *O. macrochir* Boulenger, and *O. mossambicus* \times *O. urolepis hornorum* (Wohlfarth and Hulata 1983; Penman and McAndrew 2000). Culture of (nearly) all-male hybrid tilapias is most common in Israel and Taiwan. The potential of tilapia hybrids for culture is underexploited, due mainly to management problems (Wohlfarth 1994). The major reason for this failure is the instability of the production of all-male hybrids. All-male progeny are produced only by cross-

ing pure species. Without careful broodstock management, the system breaks down, and females start to appear in what was previously an all-male hybrid. "It appears that this breakdown is largely due to the infiltration of parental broodstock by individuals of a different genotype, predominantly hybrids between the two species involved, which are difficult to distinguish from their parents" (Wohlfarth 1994).

Maintaining the purity of broodstock for hybridization is a difficult and tedious task for a well-managed tilapia hatchery, let alone fingerling production facilities in less-developed countries. Hybridization of tilapia has been largely replaced by hormonal sex inversion and more recently by breeding sex-inversed neo-males (see below) as alternative methods of producing all-male tilapias. It should be noted, however, that maintaining purity of broodstocks for the latter suffers from exactly the same problems. YY male genotypes of Nile tilapia proved to be as viable and as fertile as normal XY males, and to sire progeny that are nearly 100% males (GC Mair 2000, pers. comm.). Their all-male XY progeny, known as genetically male tilapia (GMT), are mass produced on a commercial scale, providing a reliable solution to the problem of early sexual maturation and uncontrolled reproduction leading to overpopulation in Nile tilapia culture (Mair et al. 1995, 1997; Tuan et al. 1998, 1999; Abucay et al. 1999; Beardmore et al. 2001). Results from on-station trials indicate that GMT have considerable benefits under culture, significantly increasing yields compared to mixed-sex tilapia of the same strain or sex-reversed male tilapia (Mair et al. 1995). The YY male line has been further selected for growth rate and for combining ability for GMT sex ratio, using intensive within-family selection in a synthetic female line (Abucay and Mair 2004). Culture of all-male Nile tilapia, based on the YY-GMT, has been widely practiced in the Philippines since 1995, in Thailand since 1997, and to a lesser extent in a number of other countries, including China, Fiji, Vietnam, Central American countries, and the USA (GC Mair 2000, pers. comm.). A similar approach was also applied to *O. aureus*. By breeding sex-inverted *O. aureus* males (which became ZZ neo-females) with normal (ZZ) males, all-male progeny are produced (Lahav 1993; Rosenstein and Hulata 1994).

4.1.5

Classical Marker Development and Application

Classical taxonomy of tilapiine species used various morphological and meristic traits, such as count of gill rakers, size and shape of teeth on jaws and pharyngeal bone, counts of anal and dorsal fin spines, as well as various body measurements. Other visual markers include color patterns, but species-specific visual markers are rare; a notable exception is the typical dark vertical striation on the caudal fin of *O. niloticus* (Trewavas 1983).

Protein variation has been investigated and used mainly for species and hybrid identification. The need for species purity identification arose from some failures in implementing interspecific hybridization for production of monosex (all-male) populations as a means of reproduction control. General proteins, hemoglobin (Macaranas et al. 1996), and enzyme loci (Chen and Tsuyuki 1970; Avtalion and Wajdani 1971; Basasibwaki 1975; Avtalion et al. 1975, 1976; Herzberg 1978; Kornfield et al. 1979; Avtalion 1982; Wu and Wu 1983; Basiao and Taniguchi 1984; Galman et al. 1988) were used in early studies. Later development of enzyme-specific staining methods increased the number of gene loci detected (McAndrew and Majumdar 1983; Brummett et al. 1988; Sodsuk and McAndrew 1991; Macaranas et al. 1995; Sodsuk et al. 1995; Falk et al. 1996; Appleyard et al. 2001). Penman and McAndrew (2000) list a series of diagnostic loci having fixed allele differences that enable discrimination between the major cultured or hybridized species – *O. aureus*, *O. niloticus*, *O. mossambicus*, and *O. urolepis hornorum*. Using electrophoretic markers, several cases of species impurity due to introgressive hybridization have been detected (Avtalion 1982; Taniguchi et al. 1985; Macaranas et al. 1986).

4.1.6

Development of DNA Markers

New techniques for developing and analyzing DNA markers were rapidly integrated into studies in several fish species, including tilapia. The first DNA markers to be used in tilapia were the human minisatellite probes 33.1 and 33.6 (Carter et al. 1991; Har-

ris et al. 1991; Harris and Wright 1995; Naish et al. 1995). RAPD (random amplified polymorphic DNA) markers were used for species and strains identification (Bardacki and Skibinski 1994, 1999; Naish et al. 1995).

Tilapia specific microsatellite primers were first introduced in the mid 1990s. Lee and Kocher (1996) developed 140 markers (133 dinucleotide repeats and 7 trinucleotide repeats) from *O. niloticus*. Ambali (1996) developed eight markers (dinucleotide repeats) from *O. shiranus* Boulenger. As part of the construction of tilapia genomic resources, additional markers were needed. Carleton et al. (2002) developed 165 microsatellites and a private company (Genomar ASA, Oslo, Norway) developed an additional 1,319 microsatellites for *O. niloticus* (Lee et al. 2005). Six more microsatellites were developed for *O. shiranus* (Ambali et al. 2000), and six microsatellites were found inside gene sequences (Yue and Orban 2002).

AFLPs (amplified fragment length polymorphisms) were developed as part of the genetic linkage mapping process and were used to construct the linkage groups where there were not enough microsatellites (Kocher et al. 1998; Agresti et al. 2000).

4.1.7 Limitations of Conventional Genetics and Breeding Approaches and the Utility of Molecular Mapping

Protein electrophoresis has limited discrimination power among populations or subspecies, due to low levels of genetic variation. The development of various molecular techniques enabled more powerful applications for that purpose, e.g., mtDNA RFLP (Seyoum and Kornfield 1992), PCR-based RAPD markers (Bardacki and Skibinski 1994, 1999; Naish et al. 1995; Dinesh et al. 1996), and more recently PCR-based microsatellite loci (Lee and Kocher 1996; Moreira et al. 2000; Appleyard et al. 2001; Rutten et al. 2004; Wilson et al. 2004). Microsatellite markers were also used for genetic identification of cultured tilapia stocks and for studying the status of introduced tilapiine fishes in the wild and in captivity (Costa-Pierce and Doyle 1997; Costa-Pierce 2003).

4.2 Genetic Mapping in Tilapia

4.2.1 First-Generation Maps

Several linkage maps of DNA markers were constructed for tilapia in recent years. The first comprehensive attempt to map the tilapia genome (Kocher et al. 1998) documented the segregation of 62 microsatellite and 112 AFLP markers in 41 haploid embryos derived from a single *O. niloticus* female. The map consisted of 30 linkage groups spanning 704 cM and estimated a total map length of ~1,200 cM. Agresti et al. (2000) used a sample of the above microsatellites, together with AFLP markers, to create genome maps for each of the parents in an [*O. mossambicus* × (*O. aureus* × red *O. niloticus*)] 63 full-sib family. The female *O. mossambicus* parent had a total of 78 segregating markers (17 microsatellites, 61 AFLPs). Of these, 62 (13 microsatellites, 49 AFLPs) were linked to 14 linkage groups covering a total length of 514 cM. The first generation (F_1) hybrid male parent had a total of 229 segregating markers (62 microsatellites, 167 AFLPs), of which 214 (60 microsatellites, 154 AFLPs) were linked to 24 linkage groups covering a total length of 1,632 cM. McConnell et al. (2000) used 49 offspring of a backcross (male *O. niloticus* × *O. aureus* that was crossed with *O. niloticus* female). A partial genetic linkage map was constructed. The *O. aureus* male linkage map comprised 28 markers and 10 linkage groups, covering 213 cM, and a smaller genetic linkage map of the *O. niloticus* female comprised nine markers and four linkage groups, covering 41 cM. All three studies used the same set of microsatellite DNA markers that were developed by Lee and Kocher (1996), and comparison of the different maps showed a remarkable conservation among linkage maps of the different species and hybrids.

4.2.2 Second-Generation Map

The recent development of hundreds of microsatellite markers enabled construction of a second-generation linkage map of tilapia from the F_2 progeny of an interspecific cross between *O. aureus* and *O. niloticus*.

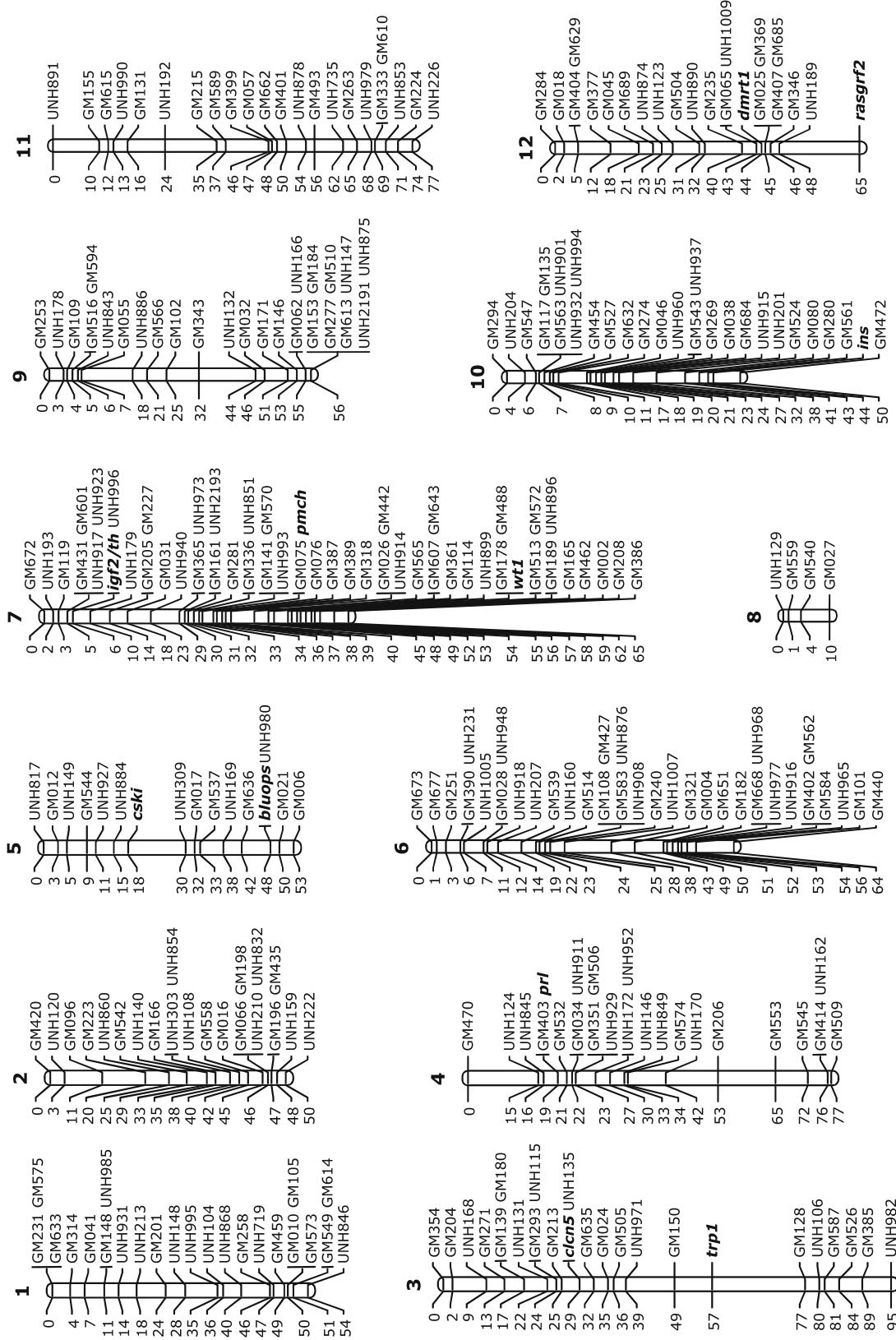


Fig. 1 Second-generation linkage map of the tilapia genome (From Lee et al. 2005)

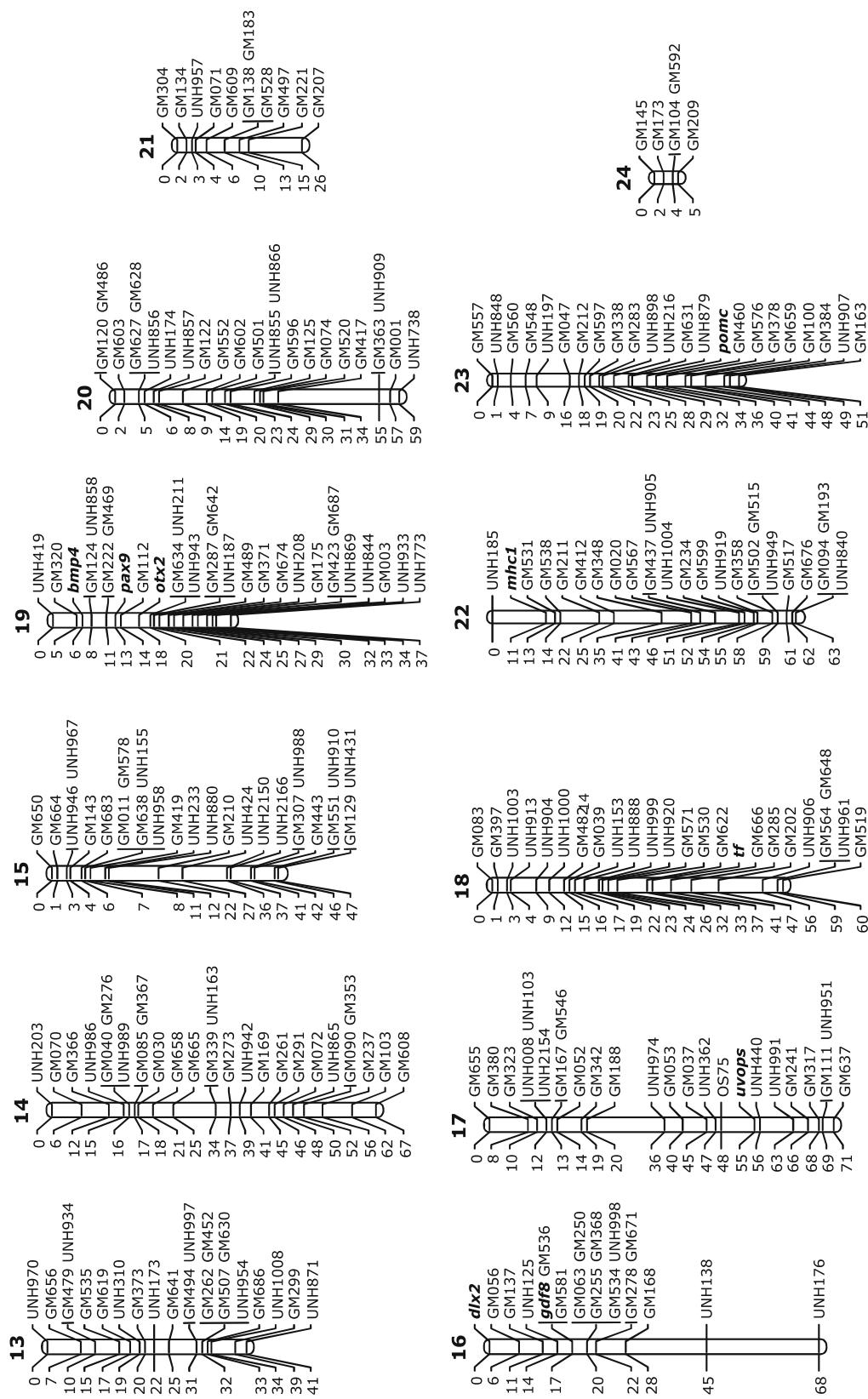


Fig. 1 (continued)

(Fig. 1). This map contains 525 microsatellite and 21 gene-based markers. It spans 1,311 cM, for an average marker spacing of 2.4 cM. The markers are linked in 24 linkage groups, 22 large and two small ones. The number of markers per linkage group ranges from four to 51. Marker density varies among linkage groups, from a high of 1 cM/marker on LG24 to a low of 3.96 cM/marker on LG3 (Lee et al. 2005). The linkages and order of markers in this map are largely congruent with the previous linkage maps of tilapia (Kocher et al. 1998; Agresti et al. 2000; McConnell et al. 2000). As this map is based largely on sequenced microsatellite markers, it is easy to make comparisons of the maps constructed for different strains and species, and the expected position of another 13 microsatellites can be determined by comparison with the previous linkage map, bringing the total number of mapped markers to 559. Furthermore, these microsatellite markers are highly polymorphic and therefore informative in most crosses. The several linkage maps of tilapia can be viewed and compared using the comparative genome viewer at the website www.cichlidgenome.org. The distribution of recombination along linkage groups can be very different between males and females, suggesting that tilapia have a sex-specific pattern of recombination. Although the overall levels of recombination are nearly identical, internal linkages are often larger in females, while terminal linkages are larger in males (Lee et al. 2005).

This comprehensive map of the tilapia genome is one of the most extensive linkage maps available for fishes. This map provides the infrastructure for systematic genome scans for detection of quantitative trait loci (QTL) in tilapias, as already demonstrated in several studies (Lee et al. 2003, 2004, 2005; Cnaani et al. 2004, 2008).

4.2.3 Physical Mapping

Four bacterial artificial chromosome (BAC) libraries have been constructed from the sperm of a single male *O. niloticus*. The libraries represent 6–64× coverage of the tilapia genome, and the average insert sizes are 65 kb, 105 kb, 145 kb, and 194 kb (Katagiri et al. 2001).

A genome-wide physical map of the tilapia genome was constructed by restriction fingerprinting 35,245 BAC clones from the two libraries with the

largest average insert size, representing approximately 5.6 fold coverage of the tilapia genome. The map consists of 3,621 contigs and is estimated to span 1.752 Gb in physical length. The contigs contain an average of nine clones each and have an average length of 390 kb (Katagiri et al. 2005).

4.3 Gene Mapping

The first-generation tilapia linkage maps were based on microsatellite and AFLP markers and did not contain known genes. Only a few genes were mapped into the second-generation linkage map that contains more than 500 microsatellites.

Polymorphisms in known genes were subsequently identified by one of the two general approaches. Eight genes were already deposited in GenBank and contained simple-sequence motifs, which were polymorphic in the mapping cross (*Blue opsin*, *Csks*, *Clcn5*, *Igf2/th*, *Ins*, *Prl*, *Rasgrf2*, and *UVopsin*). For 26 other genes (*Amh*, *Bmp4*, *Cyp19a1*, *Dax1*, *Dlx2*, *Dmo*, *Dmrt1*, *Dmrt2*, *Dmrt2a*, *Fhl3l*, *Foxl2*, *Ixl*, *Lhx9*, *Mhc-1*, *Otx2*, *Pax9*, *Pmch*, *Pomc*, *Sfl*, *Sox2*, *Sox8*, *Sox14*, *Tf*, *Trp1*, *Wt1_1*, and *Wt1b*), degenerate primers were used to amplify genomic DNA or cDNA. Resulting PCR products were cloned, sequenced, and verified by BLAST. Gene-specific primers were designed, and polymorphic sequences, distinguishing *O. niloticus* and *O. aureus*, were identified and used to genotype the F₂ mapping family (Lee and Kocher 1998; Cnaani et al. 2002b, 2003b, 2007; Lee et al. 2005; Lee and Kocher 2007a; Shirak et al. 2006).

By using the flanking sequences of the short tandem repeat in tilapia microsatellites as a query in a BLAST search, 16 genes were identified (Cnaani et al. 2002a). With the expected addition of numerous sequences from other fish species (*Fugu rubripes*, *Tetraodon nigroviridis*, *Danio rerio*, and *Oryzias latipes*) and EST libraries of cichlid fishes (<http://www.tigr.org/tdb/tgi/>), we expect that more genes will be predicted from the tilapia microsatellite sequences.

In the process of QTL positional cloning, a comparative mapping approach is used in order to add markers to the QTL interval. These are usually type

I markers, which are based on gene polymorphisms (Lee and Kocher 2007b). Additional genes were already cloned and mapped in QTL studies but have not yet been published (B-Y Lee, AE Howe, A Cnaani, A Shirak and TD Kocher, unpublished results).

4.4 **QTL in Tilapias**

4.4.1 **Detection of QTL**

The ability of tilapiine fishes to create viable interspecific hybrids makes them an ideal organism for genetic studies, using backcrosses or F₂ intercrosses as a segregating population, as suggested by Poompuang and Hallerman (1997). Indeed, most of the QTL studies so far have used populations of interspecific hybrids (Streelman and Kocher 2002; Cnaani et al. 2003a, 2004; Howe 2004; Lee et al. 2005).

So far, only a few QTL studies have been conducted in tilapia. The traits of interests have been sex determination, stress response, body color, and fish size. In the following sections, we review the results of these studies by studied traits, and therefore some of the studies will be mentioned several times as they aimed for detection of QTL for multiple traits. The QTL found in tilapias are summarized in Table 1.

Sex Determination

Monosex all-male culture is preferred in the commercial production of tilapias. Males have a higher growth rate than females, and the all-male culture prevents an uncontrolled reproduction that detracts the management of the ponds due to uneven growth, unknown biomass, and inefficient feeding. Sex determination is predominantly determined by the existence of a major locus but is also influenced by minor genetic and environmental factors. Repeated studies since the early 1960s have shown that different tilapia species have different sex determination mechanisms. In *O. niloticus* and *O. mossambicus*, the male is heterogametic (XX-XY), while in *O. aureus* and *O. urolepis hornorum*, the female is heterogametic (WZ-ZZ). In recent years, several studies were conducted in order to detect the major loci of sex determination in these species and to characterize the sex chromosomes.

A gynogenetic line of *O. aureus* was used in order to reduce the population heterozygosity and isolate loci where heterozygosity is essential for fish development, such as the sex determination loci in females (which are the heterogametic) (Palti et al. 2002; Shirak et al. 2002). Deleterious effects were observed during embryonic development for allele combinations of three unlinked microsatellites (*UNH159* residing in LG2, *UNH231* in LG6, and *UNH216* in LG23) (Palti et al. 2002). Allele combinations of these markers were also associated with sex determination (Shirak et al. 2002).

Lee et al. (2005) constructed the tilapia linkage map from a family of *O. aureus* × *O. niloticus* F₂ hybrids (156 offspring). In this family, association was found between sex and microsatellites on LG3. Genotyping families from the pure species revealed association of markers in LG1 (*UNH104*, *UNH995*, and *GM201*) with sex determination in *O. niloticus*, and the locus was mapped into a 10 cM interval (Lee et al. 2003). Three families of *O. niloticus* were used in this study, and in one of them, markers on LG1 did not show an association with sex. However, this family was relatively small (only 26 fish) compared to the other two (46 and 47 fish). In one family of *O. aureus*, four markers (*GM354*, *UNH168*, *GM271*, and *UNH131*) in a 20 cM region within LG3 had a strong association with sex determination, with 95% of the females having the same haplotype in these markers. Markers in LG1 (*UNH104* and *GM201*) were also associated with sex determination in this family and had epistatic interaction with the markers in LG3 (Lee et al. 2004). The results of these studies were the basis for a comprehensive characterization of the sex chromosomes in six tilapiine species: *O. aureus*, *O. niloticus*, *O. mossambicus*, *O. karongae*, *T. zillii*, and *T. mariae*. Linkage and physical mapping demonstrated that there are two distinct sex chromosomes in these species, with a male heterogametic system on LG1, a female heterogametic system on LG3, and some cases of loci interactions between and within chromosomes that are affecting the sex determination in tilapia (Cnaani et al. 2008).

Gynogenesis has been used to create XX and YY families in *O. niloticus*. Genotyping of AFLP markers was performed on bulked segregants (males vs. females) at the family level, as well as on individuals. The screening identified three Y-linked and one X-linked marker (Ezaz et al. 2004). The X-linked marker (*OniX420*) and one of the Y-linked markers (*OniY425*)

Table 1 Summary of tilapia's QTL, with their location in the tilapia linkage map¹

Linkage Group ¹	Trait	Species ²	Reference ³
1	Stress response	Om × Oa	2
	Blood biochemical parameters	Om × Oa	2
	Sex	On, Oa, Om × Oa	4, 5, 2
	Size	Om × Oa	2
3	Stress response	Om × Oa	2
	Sex	Oa, Om × Oa, Oa × On	5, 2, 6
	Size	Om × Oa	
	Red color	Oa × On, Om × On	3, 6
4	Stress response	Om × Oa	2
	Growth in high salinity	Om × On	9
11	Stress response	Om × Oa	2
16	Blood biochemical parameters	Om × Oa	2
19	Stress response	Om × Oa	2
20	Blood biochemical parameters	Om × Oa	2
22	Stress response	Om × Oa	2
23	Cold tolerance	Om × Oa, 4WC	1, 7
	Stress response	Om × Oa	2
	Sex	Om × Oa, Oa	1, 2, 8
	Size	Om × Oa, 4WC	1, 2, 7

¹ Linkage groups numbers as assigned in Lee et al. 2005.

² Species abbreviations: Om – *O. mossambicus*, Oa – *O. aureus*, On – *O. niloticus*, 4WC – Four-way cross.

³ The numbers in this column correspond to the following entries in the reference list: (1) Cnaani et al. 2003a; (2) Cnaani et al. 2004; (3) Howe 2004; (4) Lee et al. 2003; (5) Lee et al. 2004; (6) Lee et al. 2005; (7) Moen et al. 2004; (8) Shirak et al. 2002; (9) Streelman and Kocher 2002.

were shown to be allelic. The linkage of these markers with sex differs between families, and recombination rates varied from zero to 20%. Two BAC clones containing the markers *OniY227* and *OniY382* were isolated and physically mapped by fluorescence in situ hybridization (FISH) to the long arm of the large tilapia chromosome. The results of this study were consistent with an earlier karyotypic work suggesting that the sex determination region is present on the largest chromosome pair of this species (Carrasco et al. 1999).

O. mossambicus × *O. aureus* F₂ hybrids families were used for QTL studies of several traits. Markers associated with sex were detected in a genome scan that covered ~80% of the tilapia genome. Markers in LG1 (*UNH868*) and LG3 (*UNH925*) had a strong effect,

and markers in LG19 (*UNH858*) and LG23 (*UNH848*) had a weaker effect on sex (Cnaani et al. 2004). In a similar hybrid family that was genotyped only with markers in LG23, one marker (*UNH879*) was associated with sex (Cnaani et al. 2003a).

Karayucel et al. (2004) studied gynogenetic and inbred clonal lines of *O. niloticus* and found two unlinked loci that are affecting sex determination, in addition to the major locus on the sex chromosome. They were called “sex reversal” loci, and it seems that they are involved in sex reversal at both directions – male to female and female to male. Both loci seem to be autosomal, and one of them is linked to red body color. These loci may also be correlated with the phenomenon of temperature-dependent sex determination in tilapia.

Stress Response

Cultured fish, especially under intensive culture conditions, are under prolonged stress, and their physiological responses under these circumstances affect energy-dependent processes like growth, maturation, and disease resistance. Studies in different species and interspecific hybrids have shown genetic variance in several stress-related traits. QTL studies were aimed at detecting loci associated with traits related to common stresses in the commercial tilapia culture, such as low temperature and exposure to air.

Linkage analysis between microsatellite markers and cold tolerance was conducted in *O. mossambicus* × *O. aureus* F₂ hybrids, as these two species were found to differ significantly for this trait. In a preliminary experiment, a single family was genotyped for 20 randomly selected polymorphic markers. After finding a putative QTL in LG23 (marker UNH180), six markers in this linkage group were screened in a larger family. The marker UNH879 was associated with cold tolerance (Cnaani et al. 2003a). A genome scan with 54 microsatellites and 23 AFLP markers, in a four-way tilapia cross (two different families from a cross between *O. niloticus* × *S. galilaeus* L. male and *O. mossambicus* × *O. aureus* female) was also conducted to identify QTL for cold tolerance. The results of this study support the existence of the QTL that was previously detected in LG23 (Moen et al. 2004).

A genome scan with 42 markers was conducted in another family of the same F₂ hybrid (*O. mossambicus* × *O. aureus*), searching for QTL for innate immunity response to stress and for blood biochemical parameters which are indicators of fish health (Cnaani et al. 2004). QTL were found in one family, and several of them were confirmed in a second family. QTL for stress response traits were detected in LG1 (UNH868), LG3 (GM180 and UNH925), LG4 (GM553 and UNH952) and LG23 (GM47, UNH848, and UNH898). QTL for blood biochemical components were detected in LG16 (UNH998) and LG20 (GM552).

Size

Fish size is a highly important trait in any cultured species, and previous studies have demonstrated that there is a large genetic variation for growth rate in tilapias. However, only a few studies aimed at QTL

detection for this trait, and in most of them, it was a secondary trait and not the main objective.

In a genome scan conducted in a full-sib family of *O. mossambicus* × *O. aureus* F₂ hybrids, markers associated with body weight and length were located in LG1 (UNH868), LG3 (GM180 and UNH925), LG10 (UNH912), LG13 (UNH934), LG18 (UNH1003) and LG23 (GM47, UNH848, and UNH898). The markers in LG1, LG3, and one of the markers in LG23 are also sex-linked markers, and therefore the size differences probably reflect the different growth rate between males and females. One marker in LG23 (UNH130) was associated with fish size in two separate studies: in a similar F₂ hybrid family (Cnaani et al. 2003a) and in a four-species cross (Moen et al. 2004).

Appleyard et al. (2001) found a population-wide association between growth and heterozygosity of the marker UNH146 in a commercial strain of *O. niloticus*. However, when they tested a closely linked marker, it was not correlated with growth, and therefore they concluded that the original linkage was a false positive result.

Color

There are several commercial strains of tilapia with red body color, a trait that can add to the market price of the fish. Cultured strains of red tilapia were isolated from *O. niloticus*, *O. mossambicus*, and their hybrids. Several studies, using classical breeding experiments, determined that red body color is controlled by a single gene, with different modes of action among strains.

The tilapia family that was used for creating the linkage map was an *O. aureus* × *O. niloticus* F₂ hybrid. Since the grand-sire *O. niloticus* was a red mutant (homozygous for a dominant red allele), the F₂ hybrids segregated for this trait. Linkage analysis of the red color with a genome-wide set of microsatellite markers revealed a single QTL, residing on LG3. Interval analysis showed the strongest likelihood in the 24 cM region between markers GM150 and GM128 (Howe 2004; Lee et al. 2005). Association of the same microsatellites with the red body phenotype was found also in an F₂ family of *O. niloticus* × *O. mossambicus* cross that was segregating for this trait, and the QTL was located in the same region of LG3 (Howe 2004). Fine-mapping of this QTL was done by developing new markers through comparative mapping with *Fugu* and medaka, and by sequencing AFLP markers tightly

linked to the QTL. By coupling these two approaches, the QTL interval was reduced to 200 kb, within two BACs (Howe 2004; AE Howe and TD Kocher, unpublished data).

4.4.2

Gene-Trait Association

Fewer than 40 genes have been mapped so far in tilapia. Most of these genes were partly sequenced and mapped because of their known role in sex determination, pigmentation, or immune response pathways. However, associations between gene polymorphism and quantitative traits have been found for only a few of them.

Streelman and Kocher (2002) found an association between a polymorphism of a microsatellite within the prolactin 1 (*prl1*) promoter and differences in the gene expression. It was also correlated with growth rate in high salinity challenge. Fish that were homozygous for the longer allele had higher expression of prolactin and slower growth in 16 ppt salinity.

Polymorphisms in two genes, the MHC class I and the IgM light chain, were associated with stress-related traits (Cnaani et al. 2004). However, these genes were not studied beyond genotype-trait association, and it is not known whether the polymorphisms in these genes are influencing these traits or if they are just linked to a QTL.

Several genes known for their role in the sex determination pathway were mapped in tilapia. Five of these genes were found to be closely located to sex determination loci (*Cyp19a1* and *Wt1b* on LG1, *Amh*, *Dmrt2* and *Sox14* on LG23); however, they were excluded from being the major sex determination genes in tilapia (Shirak et al. 2006; Cnaani et al. 2007; Lee and Kocher 2007a).

4.5

Future Scope and Related Tilapia Genomic Research

The main goal in tilapia genomic research is the use of molecular information for breeding improvement. Therefore, we expect that future research will be fo-

cused on QTL positional cloning and the development of the infrastructure for this work. Combining linkage and physical maps will be a powerful tool for the fine-mapping of QTL and for comparative mapping approaches. The development of EST libraries will open more opportunities for gene expression studies and analyses of candidate genes.

The Joint Genome Institute (JGI) of the US Department of Energy (DOE) recently completed the partial sequencing of five species of fish of the family Cichlidae from Lake Malawi in East Africa. Genomic sequence of these closely related species will be a high value addition to the available genomic resources as there is nearly complete colinearity between the tilapia linkage map and the Lake Malawi cichlid map and similarities of more than 95% in coding sequences within the family Cichlidae.

Finally, the NHGRI of the National Institutes of Health has committed funds to produce a draft assembly of the Nile tilapia genome. It is expected that sequencing and assembly of this genome will be completed in 2009, opening a new era for genetic research in tilapia and related cichlid fishes.

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5 European Sea Bass

F.A.M. Volckaert¹, C. Batargias^{2,9}, A. Canario³, D. Chatziplis^{4,10}, D. Chistiakov⁵, C. Haley⁶, A. Libertini⁷, and C. Tsigenopoulos⁸

¹ Laboratory of Animal Diversity and Systematics, Katholieke Universiteit Leuven, Ch. Deberiotstraat 32, B-3000 Leuven, Belgium, e-mail: filip.volckaert@bio.kuleuven.be

² Nireus Chios Aquaculture S.A., Kardamila, GR-82 300 Chios, Greece

³ Centro de Ciencias do Mar, Universidade do Algarve, Campus de Gambelas, P-8005-139 Faro, Portugal

⁴ Animal Genetics and Breeding, Department of Animal Production, Faculty of Agriculture, Aristotle University of Thessaloniki, GR-540 06 Thessaloniki, Greece

⁵ Department of Molecular Diagnostics, National Research Center GosNIIgenetika, 1st Dorozhny Proezd 1, 113545 Moscow, Russia

⁶ Roslin Institute, Division of Genetics and Genomics, Roslin, Midlothian EH25 9PS, UK

⁷ CNR-ISMAR, Istituto di Scienze Marine – Biologia del Mare, Riva 7 Martiri, 1364/A, I-30122 Venezia, Italy

⁸ Hellenic Centre of Marine Research, Institute of Marine Biology and Genetics, Department of Genetics and Molecular Biotechnology, P.O. Box 2214, Gournes Pediados, GR-715 00 Heraklion, Crete Greece

⁹ Present address: Faculty of Agricultural Technology, Department of Aquaculture and Fisheries, Technological Education Institute of Messolonghi, Nea Ktiria, GR-30200 Messolonghi, Greece

¹⁰ Present address: Animal Breeding and Genetics, Department of Animal Production, School of Agricultural Technology, Alexander Technological Educational Institute of Thessaloniki, P.O. Box 141, GR-57 400 Sindos, Thessaloniki Greece

5.1 Introduction

The European sea bass (*Dicentrarchus labrax* L.) is well known to the fishermen and inhabitants of the Mediterranean Sea. The oldest references date as far back as the classical texts (Caius Plinius Secundus 23–79 AD) in *Naturalis historia (IX liber)*. Sea bass has been a highly appreciated food item throughout history, but without any indication of intensive aquaculture until recently. Traditional extensive culture consists of the polyculture of juvenile mullet, European eel, sea bass and/or sea bream in coastal lagoons. After fattening, they are captured as they migrate to the sea through narrow channels (often specially modified for this purpose). Extensive culture has been a tradition throughout the Mediterranean (e.g., valliculture in Italy).

5.1.1 History

The first documented record of intensive aquaculture dates back to the early 1970s, when G Barnabé (Montpellier, France) closed the reproductive cycle through the description of the life cycle and experiments on controlled spawning on the French Mediterranean coast (Barnabé 1972, 1974; Barnabé and Rene 1972). Similar interests were pursued in Italy (Lumare and Villani 1973). The extensive lagoons of Thau, Mauguio, and Pérols, and the Biological Station of Sète represented a suitable environment for the capture of adult wild sea basses and experiments in aquaculture. At that time, the demand for wild captured fish by Mediterranean customers was high. The second half of the 1970s saw a focus on improved husbandry and nutrition at sites across the western Mediterranean basin (Barnabé 1986) and on the Atlantic

coast (Barahona-Fernandes et al. 1977). The initiation of commercial aquaculture dates back to the period 1980–1985, a time when major progress was made on improving larval survival (Haffray et al. 2004). The first genetic studies date from 1973, when the karyotype of European sea bass was prepared (Cataudella et al. 1973). The commercial scaling up of aquaculture production, and the steady increase in tonnage, occurred throughout the 1990s and continues today. Expectations are that production will level off due to space and market constraints before reaching the massive production figures of Atlantic salmon or Nile tilapia.

5.1.2

Zoological Descriptions

The presently accepted scientific name for the European sea bass (or European seabass) is *Dicentrarchus labrax*. Previous synonyms are amongst others *Morone labrax*, *Roccus labrax*, *D. elongatus*, *D. lupus*, *Labrax lupus*, and *Perca labrax* (Pickett and Pawson 1994; Froese and Pauly 2006). The generally accepted taxonomic hierarchy for the sea bass is reported in Table 1.

The family Moronidae is phylogenetically closely related to the hermaphroditic Serranidae. Only two genera pertain to the Moronidae: the genus *Morone*, with four described species (all inhabiting the western Atlantic Ocean), and the genus *Dicentrarchus* with two species: *D. labrax* and the spotted sea bass *D. punctatus* (Bloch 1792), all inhabiting the eastern Atlantic Ocean (Pickett and Pawson 1994).

Table 1 Taxonomic hierarchy of the European sea bass

Phylum	Chordata
Subphylum	Vertebrata
Superclass	Osteichthyes
Class	Actinopterygii
Subclass	Neopterygii
Infraclass	Teleostei
Superorder	Acanthopterygii
Order	Perciformes
Suborder	Percoidei
Family	Moronidae
Genus	<i>Dicentrarchus</i> Gill, 1860
Species	<i>Dicentrarchus labrax</i> (Linnaeus, 1758)

The European sea bass shows demersal behavior, inhabiting coastal waters down to 100 m, but it is more common in shallow waters. It lives in the littoral zone on various kinds of bottoms in estuaries, lagoons, and occasionally rivers (Lloris 2002). It is distributed along the coasts of the Mediterranean Sea, the Black Sea, the eastern Atlantic Ocean from Norway to Morocco, the Canary Islands, and Senegal (Froese and Pauly 2006), and has been reported near Iceland (Jonsson 1992). It has been introduced for culture purposes in Israel (Red Sea), and more recently in Oman and the United Arab Emirates.

Morphology and General Biology

The body shape is rather elongated and approaches a “typical” fish shape (Fig. 1). Reported maximal sizes are 1.03 m in length and 12 kg in weight. The opercle has two flat spines, and the preopercle has large, forward-directed spines on its lower margin. The mouth is terminal, moderately protractile and endowed by vomerine teeth. On the back, there are two separate dorsal fins, the first with spiny rays, the second with soft rays. Pelvic fins are situated well forward of the belly, slightly behind the opercle. The caudal fin is moderately forked and normally has 17 branched rays. The body is covered with regular scales, and its color varies considerably from silvery grey to bluish on the back, silvery on the sides. The belly is sometimes tinged with yellow. The young fish sometimes have dark spots on the upper part of the body. In adults, a diffuse dark spot characterizes the edge of the opercle (Froese and Pauly 2006). Sexual dimorphism in length and weight has been observed in *D. labrax* even before sexual maturity (Gorshkov et al. 1999).

D. labrax is a eurythermal fish (8–24 °C, or even up to 2–32 °C) with high tolerance to salinity changes. It enters coastal waters and river mouths in summer but migrates offshore in colder weather and occurs in deep water during winter. Larvae are located preferentially within the depth range 1–20 m. The growing larvae drift from the open sea inshore, and eventually into creeks, backwaters, and estuaries. These sheltered habitats are used by juvenile sea bass for the next 4–5 years, before they mature and adopt the migratory movements of adults (Pickett and Pawson 1994). Adults disperse mainly during the winter period, while dispersal during summer is quite limited (Fritsch 2005). The (sub)adult European sea bass is

Fig. 1 European sea bass (*Dicentrarchus labrax* L.) (photo: A. Libertini)

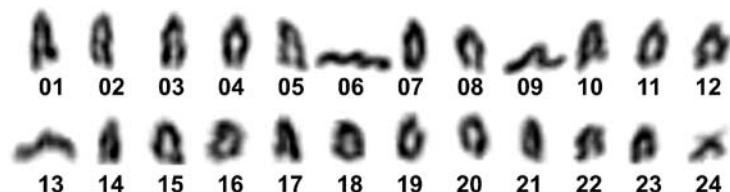


Fig. 2 Haploid karyotype of the European sea bass from a cultured haploid embryo cell. On the heteroplastic shorter arm of chromosome 24 lies the NOR; this arm usually shows size polymorphism. Reproduced from Chistiakov et al. (2005) with permission of the Genetics Society of America

a voracious opportunistic predator consuming small fish and a large variety of invertebrates (Tortoneese 1986). Larvae are equally opportunistic predators, feeding in a lower size range.

Reproduction, Maturity, and Spawning

Adult sea bass reproduce sexually by external fertilization. The sea bass is gonochoristic; sex confirmation is possible only during the spawning season. The proportion of females resulting from individual crossings may vary from 1 to 70%. Sex differentiation starts usually 200 days post-hatching (dph), with females differentiating first. Temperature clearly influences sex ratio; high temperatures favor the development of males (Piferrer et al. 2005).

Sexual maturity occurs during the second year after birth for the males (23–30 cm), and during the third year (31–40 cm) for the females. Unlike gilthead sea bream, female gonads complete their maturation at the same time, and all eggs are released together in a short time. During the spawning season, each mature female may produce between a quarter and half a million eggs per kilogram of her body weight.

Reproduction takes place at sea and has not been observed in lagoons and estuaries. Juveniles and adults from the lagoons migrate offshore where they mate with individuals from the open sea. There is only one spawning season per year, which occurs in win-

ter in the Mediterranean Sea (December to March), and into June in the Atlantic Ocean. Spawning takes place in groups in mid-water, and the eggs can be found throughout the water column. Egg and larval development accelerates at higher temperatures. There is a difference in growth between sexes. Established “size-age” curves distinguishing male and female individuals show that at the same age, the females are larger than males (Fritsch 2005 and references therein).

Genome Size, Karyotype, and Sex Determination

The average diploid nuclear DNA content (2C) of *D. labrax* is 1.55–1.58 pg (Peruzzi et al. 2005; A. Libertini unpublished) corresponding to about 763 Mb (Dolezel et al. 2003). A+T base pairs represent 60.25% of the total genome (A. Libertini unpublished). The karyotype (Fig. 2) consists of 24 subtelocentric-acrocentric chromosome pairs (NF = 48) gradually decreasing in size. Size polymorphic NORs were detected on the terminal or near-terminal site of the short arms of a small pair (Cataudella et al. 1973; Aref'yev 1989; Vitturi et al. 1990; Sola et al. 1993; Cano et al. 1996). The smallest acrocentric pair was found to be heteromorphic for C-heterochromatin in males (Cano et al. 1996), suggesting an early differentiation stage of sex chromosomes in *D. labrax*. There are no recognizable heterochromosomes.

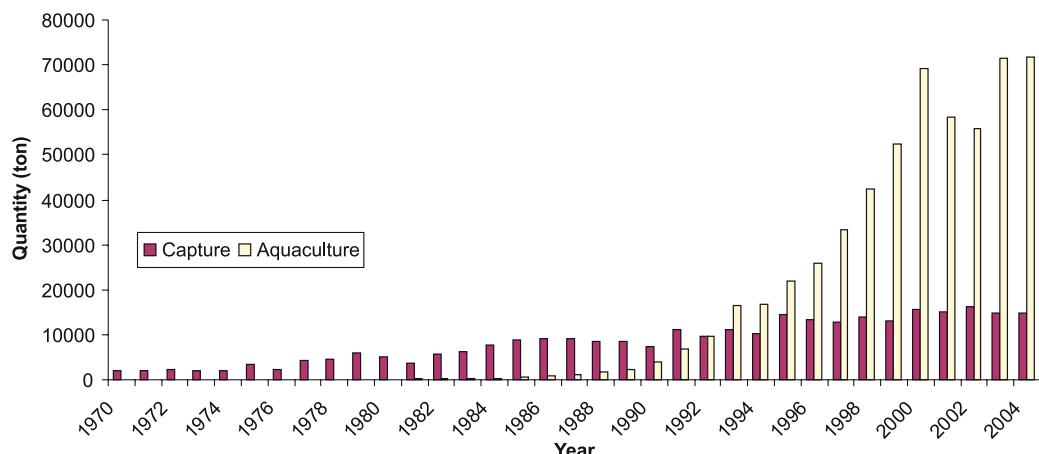


Fig. 3 Capture and aquaculture statistics of European sea bass from 1970 to 2004 (source: FAO 2006)

5.1.3 Economic Importance

European sea bass has a high commercial value, both from captures from wild stocks and from aquaculture production in the last 25 years (Fig. 3). In 2004, worldwide sea bass fishery production amounted to 14,933 metric tons with France (4,792 metric tons) and Italy (3,318 metric tons) accounting for 32% and 22% of the captures, respectively. Aquaculture production reached 71,649 metric tons in 2004, with Greece producing 55,691 metric tons, followed by Turkey (26,297 metric tons), Italy (6,831 metric tons), Spain (4,513 metric tons), and France (3,438 metric tons) (FAO 2006).

5.1.4 Biodiversity and Strain Evaluation

Genetic Markers Available

Initially, a few tens of allozymes were the choice of marker for population genetic studies and the occasional broodstock characterization (Benharrat et al. 1983; Allegrucci et al. 1997; Castilho 1998; Castilho and McAndrew 1998a; Sola et al. 1998; Ergüden and Turan 2005). RAPDs (Caccone et al. 1997) and mitochondrial DNA (mtDNA) markers (Patarnello et al. 1993; Caccone et al. 1997; Cesaroni et al. 1997) have also been developed. There are more than 200 polymorphic AFLP markers available (D. Chistiakov and F. Volckaert unpublished). More than 250 microsatel-

lite DNA markers have been isolated from special-purpose genomic libraries (García de León et al. 1995; Castilho and McAndrew 1998a; Castilho and McAndrew 1998b; Ciftci et al. 2002; Tsigenopoulos et al. 2003; Chistiakov et al. 2004; Chistiakov et al. 2005). This number is likely to increase through bioinformatic screening of EST libraries (see below) for sequences containing tandem repeats (SSR-ESTs). Single nucleotide polymorphisms (SNPs) are being data mined from EST sequences (E. Souche and F. Volckaert unpublished).

Population Structuring and Phylogeography

The spatiotemporal differentiation of sea bass populations is one of the best studied among European marine fishes. There are numerous genetic studies based on allozymes (Benharrat et al. 1983; Allegrucci et al. 1997; Castilho 1998; Castilho and McAndrew 1998a; Sola et al. 1998; Ergüden and Turan 2005), mitochondrial DNA (Patarnello et al. 1993; Caccone et al. 1997; Cesaroni et al. 1997), RAPDs (Caccone et al. 1997), microsatellites (Castilho and McAndrew 1998b; Naciri et al. 1999; Bahri-Sfar et al. 2000, 2005; Castilho and Ciftci 2005; Fritsch 2005; Katsares et al. 2005), and a few based on combinations of the above markers (Lemaire et al. 2000, 2005). These studies have led to the identification of three genetically distinct zones: the northeastern Atlantic Ocean, the western Mediterranean, and the eastern Mediterranean (Benharrat et al. 1983; Allegrucci et al. 1997; Bahri-Sfar et al. 2000; Lemaire et al. 2005). On the basis of microsatellite and mtDNA loci, transition zones have been localized at

the Almeria-Oran oceanic front (Naciri et al. 1999) between the Atlantic (the Alboran Sea included) and the western Mediterranean, and close to the Strait of Sicily (Bahri-Sfar et al. 2000) between the eastern and western Mediterranean. This was a surprise for this euryhaline and eurythermic demersal species, since adult migratory behavior has been reported up to several hundred kilometers. Nevertheless, the pattern seems related to climate cycling and sea level fluctuations during the Pleistocene that undoubtedly had a strong influence on the distribution of the species in the Atlantic Ocean and Mediterranean Sea (Patarnello et al. 2007).

There was no significant population structure either in the Atlantic Ocean (allozymes: Castilho 1998; Castilho and McAndrew 1998a; microsatellites: Naciri et al. 1999; Fritsch 2005) or in the western Mediterranean Sea (microsatellites: García de León et al. 1997; Naciri et al. 1999; Bahri-Sfar et al. 2000; mtDNA: Patarnello et al. 1993; Lemaire et al. 2005). On the contrary, the genetic structure of eastern Mediterranean sea bass populations is consistent with the subdivision of the region into several basins, e.g., the Adriatic, Ionian, and Aegean seas, the Libyco-Tunisian Gulf (Bahri-Sfar et al. 2000), and the Levantine basin (Castilho and Ciftci 2005). Interestingly, a high degree of morphological differentiation was detected between populations from the Aegean and the Black seas, but no allozymic differentiation was detected (Ergüden and Turan 2005). Cases in which individual samples do not match populations from the same geographical origin are not surprising, since fingerlings originating from the Atlantic and western basin have been and are being used to seed hatcheries all over the Mediterranean (Bahri-Sfar et al. 2005).

Chromosome Set Manipulation

D. labrax is one of the best-studied marine fishes with regard to chromosome set manipulation, but commercial production of manipulated sea basses has not been achieved yet. Triploid and meiotic diploid gynogens have been produced by retention of the second polar body with heat-, cold-, or hydrostatic pressure-shock since the early 1990s (Carrillo et al. 1993; Colombo et al. 1995; Gorshkova et al. 1996). Gonadal maturation in triploid sea bass was shown to be significantly impaired: females developed rudimentary ovaries, while males proved to be gametically

sterile, although sterility did not improve growth rate (Peruzzi et al. 2004 and references therein). The induction of gynogenesis produced mixed-sex stocks, indicating that the mechanism of sex determination in *D. labrax* does not correspond to a simple mono-factorial system with female homogamety (Peruzzi et al. 2004). Both diploid meiogynogenetic males and females were fully fertile and showed growth performances comparable with biparental diploids (Felip et al. 2002; Peruzzi et al. 2004; Francescon et al. 2005). In sea bass, tetraploid and double haploid offspring were also produced by preventing the first zygotic mitosis (Peruzzi and Chatain 2003; Francescon et al. 2004). Mortality in tetraploid sea basses was complete within the first month of life, while doubled haploids may survive and attain adulthood (Bertotto et al. 2004, 2005; A. Libertini unpublished). In the literature, there is no reference to androgenesis in *D. labrax*.

Pedigree Analysis and Strain Evaluation

Pedigree analysis based on parentage assignment from microsatellite marker profiling has been generally implemented in commercial breeding programs (García de León et al. 1998; Haffray et al. 2004; BASSMAP 2006; Dupont-Nivet et al. 2007; Saillant et al. 2006). Economic acceptance of this technology is high as the space to raise families separately is limiting.

As mentioned above, *D. labrax* exhibits a clear genetic structure in three clades. Strain testing between populations of different clade origin has been limited in scope. Gorshkov et al. (2004) screened "French," lagoonal (Lake Edku) and marine (Port Said) Egyptian sea basses and their crosses at the National Center for Mariculture (Eilat, Israel) in order to assess their performance under the same captive culture conditions. Strains and crosses varied significantly for traits of economic interest such as growth, survival, body composition, sex ratio, sexual maturation patterns of males, and frequencies of body shape abnormalities. There was no indication of heterosis; the potential of southeast Mediterranean sea bass for aquaculture is high. Mylonas et al. (2005) exposed northwest Mediterranean (French) sea bass at the Hellenic Center for Marine Research (Crete, Greece) and southeast Mediterranean (Lake Edku, Egypt) sea bass at the National Center for Mariculture to temperatures of 13, 17, and 21 °C. Higher growth was observed at higher temperatures, but fish of the French strain exposed at

13 and 17 °C during larval rearing showed compensatory growth once exposure to the lower temperature was finished. Their final size at 300 dph was similar or greater than the group exposed to 21 °C. Performance differences may be attributed to known genetic differences, but this remains to be tested.

Selection and Fitness

Some allozyme loci in *D. labrax* may exhibit patterns of allele frequencies shaped by adaptation to different environments (Allegrucci et al. 1997). When data from microsatellite and allozyme markers in Mediterranean lagoon and marine populations were compared (Lemaire et al. 2000), there was evidence that half of the allozymes used in the analysis underwent some sort of selection, and only a few allozyme loci seemed to be implicated in the differentiation between marine and lagoon samples. A transcriptome analysis of genes involved in salinity adaptation showed that they are tissue and environment dependent (Boutet et al. 2006). What the genetic correlates are of adaptation to varying salinity at the molecular level remains to be investigated under an evolutionary and ecologically functional genomic approach.

Reduced genetic diversity has been documented in hatchery populations, mainly as heterozygote deficiencies (Sola et al. 1998), reduced allelic richness, and differences in allele and genotype frequencies (Sola et al. 1998; Bahri-Sfar et al. 2005; Katsares et al. 2005). This may be due to the outcome of mass selection practices, the addition of related F₁ or F₂ individuals to the original broodstock, or founder effects. Mass spawnings are generally skewed with the participation of a couple of females and a few males (see below). Some of these aquaculture stocks were found to be largely outbred and open to wild broodstock (Bahri-Sfar et al. 2005).

5.1.5

Strain Testing, Breeding Objectives, and Classical Breeding

Strain and breed choice is one of the first steps in animal breeding. However, the most important decision in any animal improvement program is the choice of a breeding objective. A badly chosen objective means that any genetic progress (no matter how fast) will be

in the wrong direction. The difficulty comes from the nature of the problem, since it is a non-genetic one (Harris and Newman 1994). The breeding objective is a list of traits in which change is desired. Any trait included must be heritable. If not, there is no point of attempting to improve it since it will not be reflected in F₁. They also must have an economic value. It is wasteful to squander selection pressure on traits that, if improved will not affect income. On the other hand, selection criteria are those characteristics that are observed or measured in the animals and used in selection to improve the limits in the breeding objective. They must be heritable, easily (and cheaply) measured, and they must correlate with the traits in the breeding objective. In other words, the set of economic traits (breeding objectives) is not necessarily (and usually is not) the same set of traits that are recorded (selection criteria) in a genetic evaluation scheme (Harris and Newman 1994). A very comprehensive example is the feed conversion ratio (FCR). FCR can be a breeding objective for any genetic improvement program because of its economic importance, but it is not usually a selection criterion in a fish breeding program, since individual measurements of FCR are impossible under commercial rearing conditions.

There are a few cases where economic values have been calculated in fish (Gjerde 2007; Steine 2007), but not for the European sea bass. Usually, selection criteria are misused as breeding objectives without the appropriate economic balance that a breeding objective requires.

Genetic parameters are crucial for the definition of a breeding objective or selection criterion. The high cost of their estimation requires a careful design and sound planning. Vandepitte et al. (2001) simulated a quantitative trait for various mating designs in two strains of sea bass. Various mixed models were used to estimate the heritability, the difference between cross means, and the statistical power. The authors concluded that a factorial cross by sets of 40 sires/strain with 8 dams and a sample size of 2,000 progenies is appropriate for comparing sea bass strains and jointly estimating heritability.

Few heritability values and correlates for any phenotypic trait have been published. Saillant et al. (2006) followed growth from year one to two; heritability tended to increase with the age of the fish (from 0.21

at 341 dpf to 0.56 at 818 dpf). High genetic correlations among the weights recorded at various ages pointed to the stability of the genetic values throughout the growth phase.

To date, Dupont-Nivet et al. (2007) have analyzed the largest number of families (253 families; 33 sires \times 23 dams) bred at a single site (Palavas-les-Flots, France) and raised at four sites (Portugal, France, Italy, and Israel) for heritabilities and genotype by environment interactions. All fish showed a heritability of weight of 0.62; excluding highly deformed fish increased the heritability to 0.65. When analyzing the heritability of weight by site, values varied from 0.52 (France and Israel) to 0.64 (Portugal). Genetic correlations of weight between sites varied from 0.75 (Portugal–Israel) to 0.98 (Italy–Israel).

Heritabilities for several traits were estimated in another recent project (Chatziplis et al. 2007). For the target traits and population used, see below in the Section “QTLs Detected.” Body weight (at 757 dpf) showed moderate (0.21) heritability for a multiple-trait animal model. Such an estimate was lower than other heritability estimates, which generally range between 0.5–0.6 for 2–3-year-old European sea bass (Dupont-Nivet et al. 2007; Saillant et al. 2006), 0.4–0.5 for 2-year-old gilthead sea bream (C. Batargias unpublished), or 0.2–0.3 for Atlantic salmon at 2–3 years of age (Rye and Refstie 1995; Quinton et al. 2005). A possible explanation is the small sample size and the family structure. Sex was treated as a polygenic trait based on the absence of sex chromosomes and on the plasticity of its phenotype. Its heritability was estimated at 0.34 ± 0.20 and 0.43 ± 0.22 for a single- and multiple-trait animal model, respectively. These estimates, although higher, concur with other estimates that show a heritability of 0.23 on the observed scale and 0.53 on the underlying scale (Vandeputte et al. 2007).

Traits with high estimates of heritability, such as body weight and sex, can be easily selected in order to achieve strong genetic gains through classical breeding designs. Carefully designed breeding programs with pedigree information and BLUP estimates of breeding values can lead to a 10% genetic gain per generation without compromising the effective population size and level of inbreeding (C. Batargias unpublished).

5.2 Construction of Genetic Maps

Classical mapping efforts, either phenotypic, cytologic, or isoenzymatic, have never been attempted. A plausible explanation for this absence is the complexity of breeding marine fishes and hence the rather recent closure of the life cycle (Barnabé 1972). European sea bass has been a late addition to the growing list of fish taxa with genetic maps. The first report of a map is very recent (Chistiakov et al. 2004), but since that time, progress has been steady and considerable. A first-generation linkage map was published a year later (Chistiakov et al. 2005).

The linkage map of European sea bass has been developed using a single family of 50 full-sib progeny derived from two wild-caught fish from the northern Adriatic Sea. This structure is simple and powerful for mapping segregating markers and hence minimizes the amount of genotyping required, although markers that are not heterozygous in the parents cannot be mapped. The focus in the first-generation map has been on microsatellite markers. Because these markers are generally highly polymorphic, they provide a solid framework for mapping and are likely to be reliably informative in a range of sea bass populations. Such a map thus provides a good foundation for other research, such as QTL mapping, pedigree reconstruction, and diversity studies. Genotype profiles are verified and stored in the *ResBass* database (<http://www.resspecies.org>).

In the first-generation linkage map, 162 markers (from 174 that were polymorphic in the family) were assembled into 25 linkage groups (compared to the 24 chromosomes in the haploid karyotype of sea bass) (Fig. 4; Chistiakov et al. 2005). The sex-averaged map covered 815 centiMorgans (cM) with an average intermarker spacing of 5.0 cM. The map based on female meioses was 906 cM and that from male meioses was 567 cM, a 1.6-fold difference. There is considerable variation in the size of the linkage groups, which varied from 0.0 cM with 2 markers to 147 cM with 17 markers on the sex-averaged map. The mapping population will remain a long-term resource for sea bass genomics, and as markers are added to the map, it is expected to coalesce to 24 linkage groups equivalent to the 24 chromosomes. Dissemination of the linkage map of Euro-

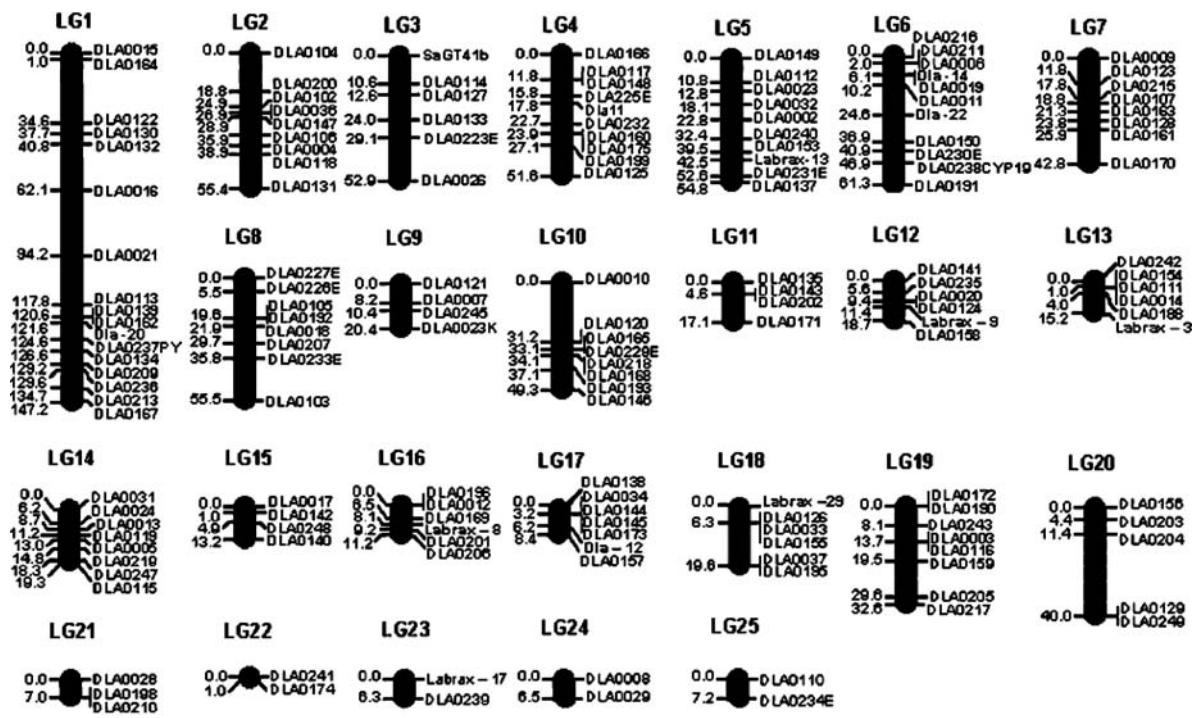


Fig. 4 Sex-averaged linkage map in Kosambi centi-Morgans of European sea bass consisting of 25 linkage groups (Chistiakov et al. 2005). With kind permission of the Genetics Society of America

pean sea bass is managed through the *Arkdb* database (<http://www.thearkdb.org>).

A draft updated map based on 369 microsatellite and AFLP markers has been assembled (D.A. Chistiakov, C.S. Tsigenopoulos, J. Lagnel, G. Yuanmei, B. Hellemans, C.S. Haley, F.A.M. Volckaert, G. Kotoulas submitted). The density of type I markers (associated with genes) has been increased ($n = 30$), and cross-linking the genetic map with the genome of other fishes will enhance functional and comparative genomic studies.

5.3 Gene Mapping

Gene mapping has just started, but considerable progress is expected in the coming years. So far, nucleotide sequences of known genes and mRNAs of *Dicentrarchus labrax* available from GenBank have been screened for microsatellites. Eight microsatellite-containing DNA sequences were found, encoding cytochrome P450 aro-

matase (CYP19; accession number AJ318516) and recombination-activating protein 1 (RAG1; AF137203), somatolactin (AJ277390), β -actin (AJ537421), interleukin 1 β (AJ269472), peptide Y (AJ005380), cytochrome P450 aromatase (AY138522), and HMG-CoA reductase (AY424801). Microsatellites located within the *RAG1* gene (Venkatesh et al. 1999) and at the 3'-untranslated region of mRNAs encoding aromatase and peptide Y were found to be polymorphic. The polymorphic markers were designated DLA0222RAG1, DLA0237PY, and DLA0238CYP19.

Two of those, DLA0237PY and DLA0238CYP19, being informative in the mapping population (Chistiakov et al. 2004), have been incorporated into the first-generation microsatellite-based map of sea bass (Chistiakov et al. 2005). These are the first two mapped genes of the European sea bass; they are situated in two separate linkage groups, LG1 (peptide Y) and LG6 (cytochrome aromatase P450) (Table 2).

Peptide Y is a member of a neuropeptide Y-like family, whose members exhibit a variety of hormonal activities (Cerda-Reverter et al. 2000). A functional role of peptide Y is not fully known, but an ortholog

Table 2 Genes mapped in *D. labrax* with flanking microsatellite markers

Marker	Gene	Protein	Linkage group	Position at the linkage group, cM*
DLA-20 [flanking marker 1]	PY	Peptide Y	1	121.6
			1	124.6
			1	126.6
DLA0230E [flanking marker 1]	CYP19	Cytochrome aromatase P450	6	40.9
DLA0238CYP19			6	46.9
DLA0191 [flanking marker 2]			6	61.3

* Genetic position of every marker is given for the sex-averaged linkage map (Chistiakov et al. 2005).

of peptide Y in tetrapods – pancreatic polypeptide PP – is shown to regulate food intake and blood pressure in rats (Balasubramaniam et al. 1992). In teleosts, expression of peptide Y was observed in the preoptic area of the brain, suggesting that this peptide may be involved in the control of pituitary secretion (Cerda-Reverter et al. 2000). This makes peptide Y a likely functional candidate for commercially significant QTLs, such as those responsible for control of growth and maturation in sea bass.

The *CYP19* gene encodes cytochrome P450 aromatase, an enzyme that converts C19 androgens to C18 estrogens (Dalla Valle et al. 2002). Since steroid hormones (androgens and estrogens) are critical in the regulation of gonadal development and energy homeostasis, the *CYP19* gene represents a suitable functional candidate in studies of QTLs associated with the reproductive traits (i.e., sex differentiation and maturation) in sea bass (Lambard et al. 2005; Simpson et al. 2005).

5.4 Studies on Quantitative Trait Loci (QTL)

Once a medium-density linkage map is available (D.A. Chistiakov, C.S. Tsigenopoulos, J. Lagnel, G. Yuanmei, B. Hellemans, C.S. Haley, F.A.M. Volckaert, G. Kottoulas submitted), the next step is the utilization of the map information for the detection of associations between the mapped markers and putative quantita-

tive trait loci (QTL) affecting commercial traits of interest. The available microsatellite markers were used for QTL screening on selected linkage groups of the sea bass genome. Sib pair analysis, combined with selective genotyping (Chatzilis et al. 2001), can be an efficient method for the detection of QTL associated with commercial traits of interest. The availability of large families, and the outbred structure of fish breeding programs, can increase substantially the power of such methods. This was the reason for its initial selection for the analysis for QTL trait association studies. However, the cost to create a specific family structure as a QTL mapping reference population can be prohibitive. Therefore, the use of pooled samples of fish from commercial breeding populations is the most economical option to map QTL. This was the case in a pilot QTL study (Chatzilis et al. 2007).

5.4.1 Target Traits

A production broodstock of 103 brooders consisting of 58 females and 45 males and maintained under late photoperiod shift was chosen for the production of F₁. After a day of natural mass spawning, a routine rearing procedure was followed without grading in order to preserve the spectrum of variance in the population. Two thousand, five hundred and four fish were anesthetized, PIT-tagged, fin-clipped for genotyping, weighed (Table 3a), photographed (Fig. 5), and sexed by stripping. Two-year-old sea bass were classified in two categories: the running males, indi-

Table 3a Descriptive statistics for body weight in the whole F₁ population (sampling in year two). Listed are mean body weight by sex and overall, standard deviation (SD), coefficient of variation (CV%), and minimum and maximum values

	n	Mean BW	SD	CV (%)	Min	Max
Males	1550	432.7	72.6	16.8	139	717
“Females” ^a	762	524.4	122.9	23.4	228	994
Sex not recorded	192	469.6	101.0	21.5	286	811.9
Overall	2504	463.5	101.7	21.9	139	994

^a see text for explanation.

Table 3b Descriptive statistics for body weight in the whole F₁ population (sampling in year 4). Column headings as in Table 3a

	n	Mean BW	SD	CV (%)	Min	Max
Males	1496	439.5	76.3	17.4	190	994
Females	458	564.4	121.3	21.5	286	913
Overall	1954	468.8	103.5	22.1	190	994

Table 4 Morphometric traits analyzed in a pilot QTL analysis of European sea bass

Number	Code	Description
1	SL	Standard length (Distance from tip of snout to caudal fin)
2	SNOP	Head length (Distance from tip of snout to operculum)
3	SNDO	Distance from tip of snout to insertion of dorsal fin
4	SNAN	Distance from tip of snout to insertion of anal fin
5	OPDO	Distance from operculum to insertion of dorsal fin
6	OPAN	Distance from operculum to insertion of anal fin
7	OPCA	Body length (Distance from operculum to caudal fin)
8	DOPV	Body depth (Distance between insertions of dorsal and pelvic fins)
9	DOAN	Distance between insertions of dorsal and anal fins
10	DOCA	Distance between dorsal and caudal fins
11	ANPV	Distance between insertions of anal and left pelvic fins
12	ANCA	Distance between anal and caudal fins

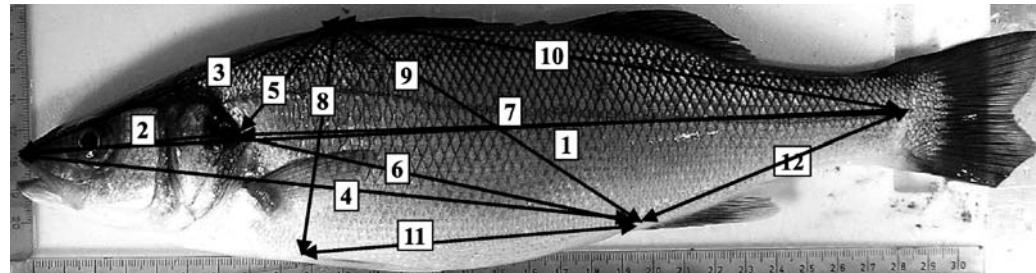


Fig. 5 Morphometric measurements taken on each individual. For a description of the measurements, see Table 4

Table 5 Mean phenotypic values of twelve morphometric traits of European sea bass. Measurements (cm) are taken from a digital picture. For a description of the measurements see Table 4 and Fig. 5

	SL	SNOP	SNDO	SNAN	OPDO	OPAN	OPCA	DOPV	DOAN	DOCA	ANPV	ANCA
Males	28.68	6.87	10.14	20.70	4.50	13.95	21.82	7.53	12.88	19.44	11.91	8.98
Females	30.74	7.46	10.93	22.33	4.83	15.00	23.29	8.12	13.90	20.77	12.76	9.55
Average	29.33	7.06	10.39	21.22	4.60	14.28	22.28	7.72	13.20	19.86	12.18	9.16

cated by the presence of sperm, and the “females,” indicated by the absence of sperm, hence including females and immature males. Females mature in their third year of age, complicating the distinction between females and immature males at their second year of age. In order to clarify the sex of each individual, a sexing procedure took place in year four (Table 3b).

In the first sampling (Table 3a), 67% of the progeny were recorded as males and 33% as “females” (see above), whereas in the more reliable second sampling (Table 3b), the female percentage dropped to 23%. Given the extreme bias towards males in cultured sea bass, the latter estimate of females is closer to reality and points to the uncertainty in sexing a sea bass population when it is done at an inappropriate time. The difference in mean body weight of the two sex groups corresponds to 28.5%, which is within the upper range reported for female harvest weight (Carrillo et al. 1995; Blazquez et al. 1999; Gorshkov et al. 1999; Saillant et al. 2001a, b), suggesting the sexual dimorphism is expressed before sexual maturity.

Morphometric traits were measured from digital pictures. Landmarks, operationally defined as relocatable coordinate positions on an object in a two-dimensional or three-dimensional Euclidean space, were placed at several positions of the body in order to cover all the biologically informative points of the body. Measures are listed in Table 4 and plotted on an individual fish in Fig. 5.

Twelve morphometric traits cover head and body length and depth. The average standard length (SL) was 29.33 cm, the average head length (SNOP) 7.06 cm, the average body length (OPCA) 22.28 cm, and the average body depth (DOPV) 7.72 cm (Table 5). Sexual dimorphism is highly significant ($P < 0.001$) in all traits.

5.4.2 Genome Scan

Each broodfish and progeny was genotyped at 13 loci, 7 loci of linkage group 1 (LG1) and 6 spanning other linkage groups (LG2, 14, 5, 18, and 17, respectively). Four hundred and twenty-four progeny assigned to just two parents were used for subsequent analysis. The main characteristics of the pedigree structure are that one female, which dominates the spawning day, was paired with 26 males which contributed unequally to the spawning. This was unexpected, since the expected spawning behavior was to have at least 2–3 females coupled with multiple males.

Unfortunately, such a family structure is not appropriate, in terms of power of detection of QTL, for the application of sib pair analysis. Nevertheless, there are alternative analyses for the detection of QTL within outbred populations that have been tested in farm animal populations. One of them could be the half-sib analysis described by Knott et al. (1996) or alternatively a variance component analysis method such as described by Heath et al. (1997).

Male farm animals are usually mated to a large number of females, producing half-sib sire families with a common parent, the male animal. In fish, the female parent is usually the common parent since her eggs are usually fertilized by a large number of males. However, the rationale behind the method remains the same, since genotypic and phenotypic information is available on the half-sib progeny and also genotypic information on the common parent (female parent in the case of fish). Any associations detected are based on the comparison between half-sib progenies that are carrying alternative marker alleles from the common parent.

Half-sib analysis does not utilize any information from the uncommon parent. However, variance component analysis calculates identity-by-descent (IBD),

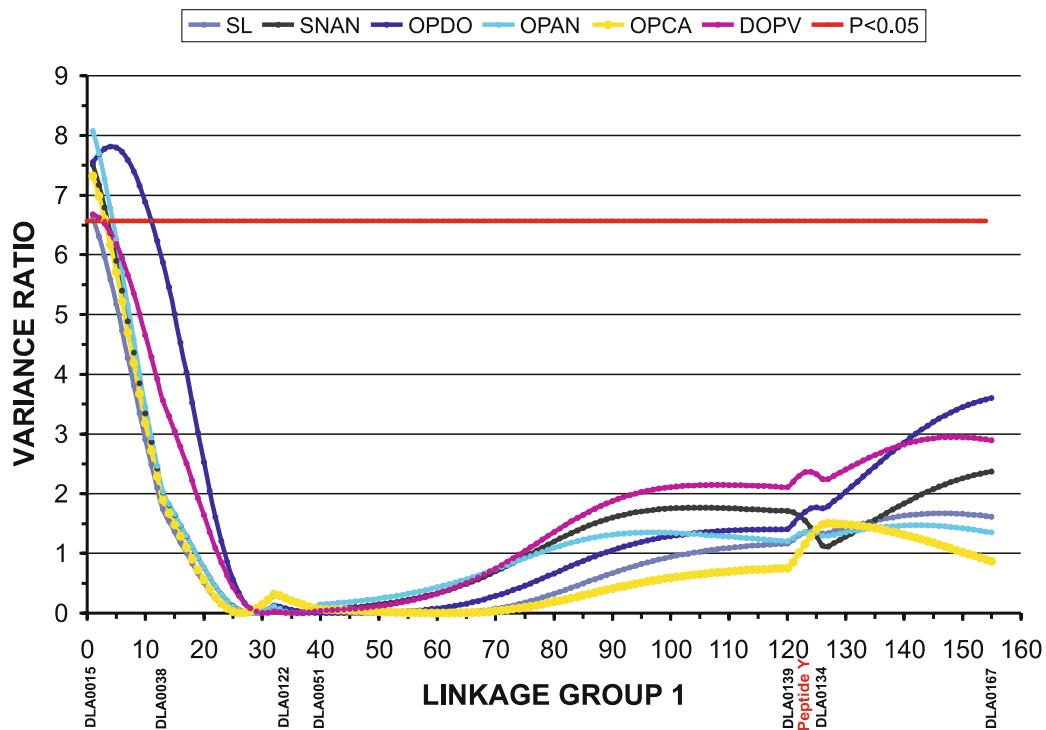


Fig. 6 Variance ratios from the half-sib analysis (Knott et al. 1996) of a single half-sib family that exceed the chromosome-wide threshold ($P < 0.05$; Churchill and Doerge 1994) for 6 morphometric traits of European sea bass. For abbreviations, see Table 4

based on the marker genotypes of offspring and parents on a genetic region (i.e., interval between two markers). The phenotypes of the offspring are analyzed using maximum likelihood techniques for sources of genetic variation from inside the genetic region under evaluation and outside the genetic region (polygenic effects). Any significant sources of variation within the genetic region under testing indicate the existence of a QTL in that region.

5.4.3 QTLs Detected

The reasons for performing a preliminary analysis on LG1 were its large size and its content of mapped genes (Cerdà-Reverter et al. 2000; Chistiakov et al. 2005). Two analyses – half-sib analysis (Knott et al. 1996) and variance component analysis (Heath et al. 1997; Seaton et al. 2002) – were performed for the detection of association between growth (bodyweight at slaughter) and seven microsatellite markers from the LG1 in a sample of 422 fish (Chatziplis et al. 2007).

The first results pointed to significant associations between the marker genotypes and the trait values quantifying body length and body depth. The associations did not map close to the candidate gene (peptide Y) but closer to the beginning of LG1, near marker DLA0015 (Fig. 6). Moreover, there was suggestive linkage between body weight and the same marker genotypes (Fig. 7). A more refined search of the linkage group one (i.e., more markers and more families), might identify more QTL affecting the growth of sea bass.

The preliminary status of the above-mentioned analysis is being expanded to all linkage groups and to complementary traits related to handling stress.

5.5 Marker-Assisted Breeding

Scientific breeding approaches applied to livestock have a long track record of success, particularly when applied to terrestrial species. However, while tradi-

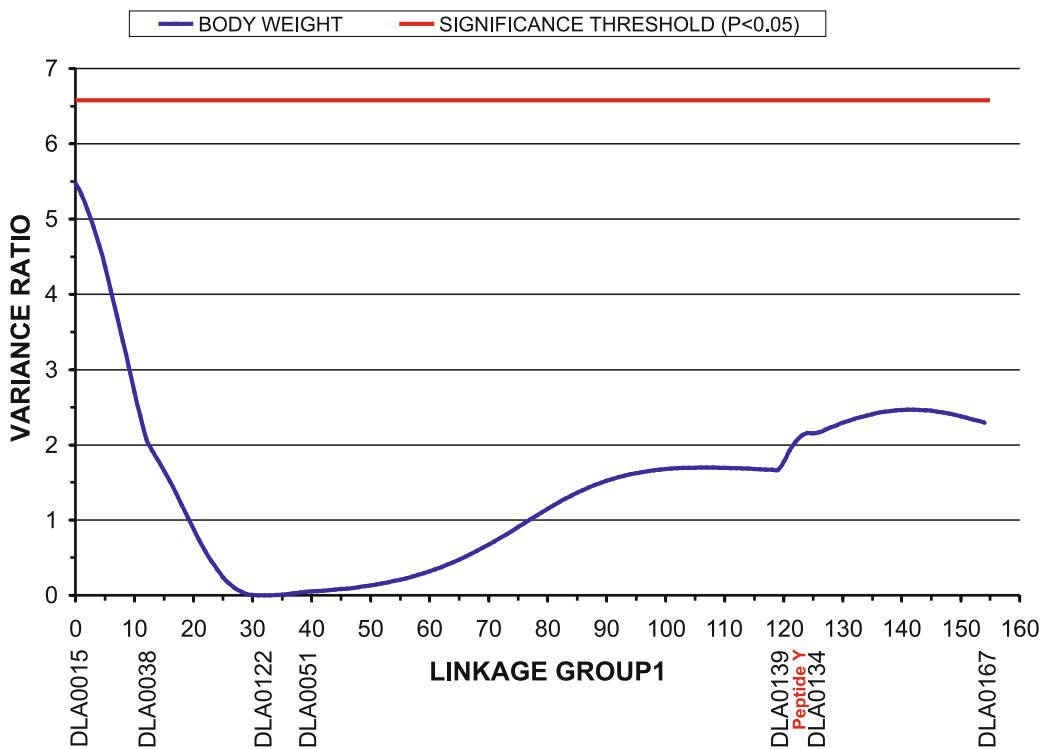


Fig. 7 Variance ratio from the analysis (Knott et al. 1996) of body weight suggesting a QTL near the microsatellite marker DLA0015 at the beginning of LG1 of European sea bass

tional selection approaches that predict genetic merit based on trait records and pedigree information are effective, they also have their limitations. Traditional methods cannot of course predict genetic merit with complete accuracy and are limited in effectiveness when traits are difficult or expensive to measure, expressed late in life, or only in particular environmental circumstances. Disease resistance, for example, is often difficult to improve by traditional methods. Marker-assisted selection is being increasingly used to overcome these problems in terrestrial livestock breeding. In this approach, genetic markers are identified that are associated with desired genetic variation (through genetic linkage or sometimes as causal genetic variants). Such markers then allow animals to be selected based upon genetic marker information. This can be done as soon as a sample can be collected for genotyping, without the need to wait until an individual expresses a particular trait or meets a particular environment (e.g., a disease). A number of studies have shown the extra progress such approaches can provide, particularly for traits that are normally difficult to improve by traditional methods (e.g., Hayes

and Goddard 2003). A prerequisite for the application of marker-assisted selection is the development of the appropriate genetic tools, initially genetic markers that cover the entire genome and genetic maps that relate the markers to the genome and each other. As additional QTLs of economic interest become available for European sea bass, they will be integrated in the classical commercial breeding schemes.

5.6 Advanced Research

Several other tools are under development. Owing to the paucity of sequence data available for Mediterranean aquaculture species, in particular the European sea bass, the EU Network of Excellence Marine Genomics Europe developed a progressive strategy to obtain genomics resources. As a first step, the production of 14 normalized cDNA libraries from a range of tissues have yielded 29,500 expressed sequence tags (EST) from which 17,700 contigs were obtained. With

the new data, the bioinformatic discovery of markers (SNPs, microsatellites) has been facilitated. Meanwhile, several cDNA libraries and hundreds of EST sequences have been generated by others (BASSMAP 2006; Chini et al. 2006). As a second step, cDNA microarrays are under development to be used in experiments with several objectives, including the identification of candidate genes for stress tolerance and disease resistance. Hundreds of SNPs and sequences containing tandem repeats (SSR-ESTs) have already been identified from the EST traces.

A large-insert BAC library has been produced corresponding to $13\times$ coverage of the genome of a European sea bass originating from the Adriatic Sea, with an average insert size of 164 kb (Whitaker et al. 2006). The ends of the BAC fragments, as well as a limited number of full BAC fragments, have been sequenced. They are being assembled in a physical map and compared to the genomes of other lower vertebrates (H. Kuhl and R. Reinhardt, in prep.). In analogy to the newly developed radiation hybrid (RH) physical map of gilthead sea bream (Senger et al. 2006), an RH mapping panel of European sea bass is being generated with the final aim of preparing an RH map (F. Senger and F. Galibert in prep.). The linkage and physical maps will be cross-linked and compared in detail with other animals.

All these resources bring European sea bass to the group of the top ten genome-rich fish species and shorten the gap between model organisms and aquaculture species. They represent a valuable resource for selective breeding, evolutionary and population genomics, and the interpretation of the transcriptome and proteome for the physiological evaluation of European sea bass.

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6 Japanese Flounder

C. Castaño Sánchez¹, K. Kobayashi¹, M.R.M. Coimbra^{1,2}, K. Fuji¹, T. Sakamoto¹, and N. Okamoto¹

¹ Faculty of Marine Science, Tokyo University of Marine Science and Technology, Konan 4, Minato, Tokyo 108-8477, Japan
e-mail: nokamoto@kaiyodai.ac.jp

² Departamento de Pesca e Aquicultura, Universidade Federal Rural de Pernambuco, Dois Irmãos, 52171-900 Recife-PE, Brazil

6.1 Introduction

6.1.1

Economic Importance and Current Status

The Japanese flounder (*Paralichthys olivaceus*) is widely distributed throughout the Japanese coast and is one of the most important commercial marine species in this country. Market landings of this species from the wild have been fluctuating around 8,000 t/year and have not increased over the last decade in spite of stock management efforts. Therefore, stock enhancement programs are being carried out nationwide to improve exploitable resources (Sekino et al. 2003; Watanabe et al. 2006).

The technologies of mass production were established during the 1980s. Formulated feeds have been developed for Japanese flounder, which have resulted in high feed conversion rates and growth to market size within 1–2 years (Seikai 2002). Sex manipulation is a common practice in the culture of *P. olivaceus*. As females grow faster than males, methods for all-female production were also developed (Yamamoto 1999). During the 1990s, Japanese flounder aquaculture production reached a plateau. Since then, annual Japanese flounder aquaculture production has been fluctuating at about the same level as the catch of wild Japanese flounder. At the same time, Japanese flounder production in other Asian countries has increased. Aquaculture production in Korea has already exceeded that of Japan, and production in China is increasing rapidly (Seikai 2002).

6.1.2 Breeding Objectives and the Utility of Molecular Mapping

Target traits for breeding improvement in aquaculture include disease resistance, rapid growth rate, temperature tolerance, salinity tolerance, better meat quality, and high feed conversion ratio. Among these traits, disease resistance is one of the most valuable, as disease is a serious problem in all aquaculture species. During seed production and breeding processes, Japanese flounder is vulnerable to several viruses, bacteria, and protozoan pathogens. High mortalities also occur during the grow-out phase due to viral, bacteria, and parasitic diseases (Muroga and Egusa 1996). Japanese flounder also present color abnormalities (Venizelos and Benetti 1999), which decrease its market price. These economically important traits are generally controlled by several associated genes, whose loci are referred to as quantitative trait loci (QTLs). Molecular markers, such as microsatellites, have been used in heritability and genetic variation studies. QTL analysis and selective breeding programs using molecular markers appear to have promise for improving quality and quantity of farm-raised Japanese flounder. In order to breed for a desired trait, it is essential to know the pedigrees of the trait. The highest priority should be given to critical traits, such as disease resistance. Methods to confirm the phenotypes must be carefully selected.

Mapping families produced by controlled matings are essential for QTL analysis. Resource families must be as different as possible from each other

in phenotype and genotype to achieve the highest variability of offspring. Backcross or F₂ families are commonly used as a mapping family. Recombination frequencies between the phenotypes of the individuals and alleles from the molecular markers are measured. A QTL is identified as a specific locus showing the lowest recombination frequency between the phenotype and a molecular marker. Genetic linkage maps showing the locations of genes or DNA fragments, such as microsatellites, are used as signposts to map QTL for particular traits. The accuracy with which the QTL position is known depends on the number of molecular markers. The higher the resolution of a linkage map, the more accurately it will be mapped.

Molecular markers showing the highest genotype/phenotype associations can be used for marker-assisted selection (MAS) programs, whose goal is to improve production. MAS programs have three distinct stages (I, II, and III) depending on the degree of genotype/phenotype associations. Stage I is used if a QTL is located by relatively distant flanking markers. The genotype/phenotype associations may be maintained only in certain specific families. This stage is the least favorable for MAS and should be avoided if possible. Stage II is used if a QTL has been fine-mapped to a region of closely linked markers. Specific haplotype blocks that maintain the genotype/phenotype associations in populations are identified. MAS is more promising at this stage. Stage III is the best for a MAS program and is achieved if causal genes have been identified. At this stage, MAS is replaced by gene-assisted selection (GAS).

6.2 Molecular Markers

6.2.1 Available Markers

Currently, microsatellites with dinucleotide repeats are the most commonly used among the available genetic markers for Japanese flounder. Several publications describe the isolation and characterization of these markers (Takagi et al. 1999; Coimbra et al. 2001; Sekino and Hara 2000, 2001a, b; Castaño Sánchez et al. 2006a). Recently, we submitted

a large number of dinucleotide repeats to GenBank (<http://www.ncbi.nlm.nih.gov>). In addition, Castaño Sánchez et al. (2007) reported the first set of tri- and tetranucleotide repeats for this species. A genomic bacterial artificial chromosome (BAC) library was constructed by Katagiri et al. (2000). In addition, 8,842 EST sequences are now available at the GenBank database (<http://www.ncbi.nlm.nih.gov>). However, there is still no record of EST markers being used in genetic mapping. Several genes have been isolated for the Japanese flounder but have not been mapped (e.g., Ooi et al. 2006; Takano et al. 2006; Hirono et al. 2007; Hwang et al. 2007). Additionally, Saitoh et al. (2000) sequenced the whole mitochondrial genome of *P. olivaceus*. To our knowledge, no attempts are being made to sequence the whole genome of Japanese flounder.

6.2.2

Application of Molecular Markers in Aquaculture and Population Studies

The genetic variability of cultured organisms might be less than in the wild (Allendorf and Phelps 1980). Therefore, genetic monitoring of hatchery-rearing populations is required to minimize the genetic loss of cultured organisms. Since cultured organisms are being released to enhance natural resources, maintaining genetic variability is important for Japanese flounder, not only to prevent inbreeding and bottleneck effects, but also to protect the genetic structure of natural stocks. Accordingly, microsatellite, intersimple sequence repeat (ISSR), and mitochondrial DNA-based markers have already been used for population and hatchery studies. For example, Sekino and Hara (2001b) used microsatellite markers to examine population structure. Subsequently, Sekino et al. (2002) found a loss of genetic variation for microsatellites and mtDNA-based markers in hatchery strains of Japanese flounder. They observed bottleneck effects in the analyzed strains. Therefore, Sekino et al. (2004) developed a microsatellite-based kinship estimator to help breeders retain genetic variation in closed and non-pedigreed captive populations. Microsatellite and mtDNA-based markers have also been used for tracing the populations, determining the parentage of organisms to be released to the sea, genetically tagging released organisms, and analyzing the

genetic variability of Japanese flounder (Hara and Sekino 2003; Sekino et al. 2003, 2005).

Liu et al. (2005, 2006) produced two strains of Japanese flounder for use in selective breeding programs. One strain was resistant to *Vibrio anguillarum*, and the other was susceptible. Both strains showed significant genetic losses compared with the wild populations. Using microsatellite markers, Shikano (2005) found that albinism in *P. olivaceus* might be heritable and that color variation among albinos was partly caused by additive genetic effects. Furthermore, the number of alleles per locus was significantly lower in albinos than in wild individuals, and most albinos tended to be closely related to each other.

6.3 Genetic Linkage Maps

6.3.1 First-Generation Genetic Linkage Map

Coimbra et al. (2003) constructed the first genetic linkage map for a flatfish (Japanese flounder) using a hybrid population bred from a cross between two established strains (one strain was susceptible and the other resistant to lymphocystis disease). The male was a phenotypic male produced by gynogenesis. Forty-four individuals of the F₁ progeny were used to construct the map, which was based on 352 amplified fragment length polymorphism (AFLP) and 111 microsatellite markers. Because recombination sites tended to be different in males and females, sex-specific maps were produced. The haploid chromosome number of this species is 24 (Sakamoto and Nishikawa 1980). However, 25 linkage groups were identified in the male map, spanning 741.1 cM. A total of 88 microsatellites and 140 AFLPs were linked to the male map, with an average distance between markers of 8 cM. Linkage group sizes ranged from 0 to 66 cM. In the female map, 27 linkage groups were identified, extending from 0 to 63.6 cM. The average distance between the 101 microsatellites and the 193 AFLPs included in the female map was 6.6 cM.

Haldane (1922) suggested that when meiotic recombination rates vary between sexes, recombination is usually suppressed in the heterogametic sex. In several fish species, recombination appears to be re-

duced in sires. In zebrafish, Singer et al. (2002) found that recombination rates in male meiosis are dramatically suppressed. The same tendency was observed in other species. The F:M ratio was found to be 3.25:1 in rainbow trout (Sakamoto et al. 2000), 3.92:1 and 8.26:1 in Atlantic salmon (Gilbey et al. 2004 and Moen et al. 2004, respectively), 6.4:1 in brown trout (Gharbi et al. 2006), 1.69:1 in Arctic charr (Woram et al. 2004), and 1.48:1 in the European sea bass (Chistiakov et al. 2005). Using segregation data of the first-generation linkage map of the Japanese flounder, Coimbra et al. (2003) found a higher recombination rate in males, with a F:M ratio of 1:7.4. However, in that study the male was gynogenetically sex-reversed, so that it was unclear whether the ratio was influenced by the genetic origin of the male. In addition, only a few markers from 16 linkage groups were used to perform the analyses. Thus, a small number of markers were used to calculate the recombination rates. Further studies are needed to elucidate the differences in recombination rates between sexes in Japanese flounder.

6.3.2 Second-Generation Genetic Linkage Map

The primary use of genetic linkage maps of Japanese flounder is in selective breeding programs. Accordingly, the map requires markers that could be analyzed in different families or populations as well as the family used for making a map. The first-generation map was built using a large number of AFLP markers. Muller and Wolfenbarger (1999) indicated that AFLPs are most useful in clonal species or analysis of controlled crosses. There was a need for maps based mainly on codominant markers, such as microsatellites, so that they could be used to study different families or populations. Consequently, second-generation maps were constructed for males and females by Kobayashi (2002) in his master's thesis.

To construct the second-generation maps, wild adult Japanese flounder were caught in the Setonai Sea (western Japan) and were crossed in captivity. Sex-specific genetic linkage maps (A2 maps) were built by genotyping 46 of the F₁ hybrid population using Map Manager QT (Manly et al. 2001) to determine linkage at a minimum LOD score of 3.0 and then establish the orders of the markers in the map. Figure 1 represents the A2 male and female maps. A total

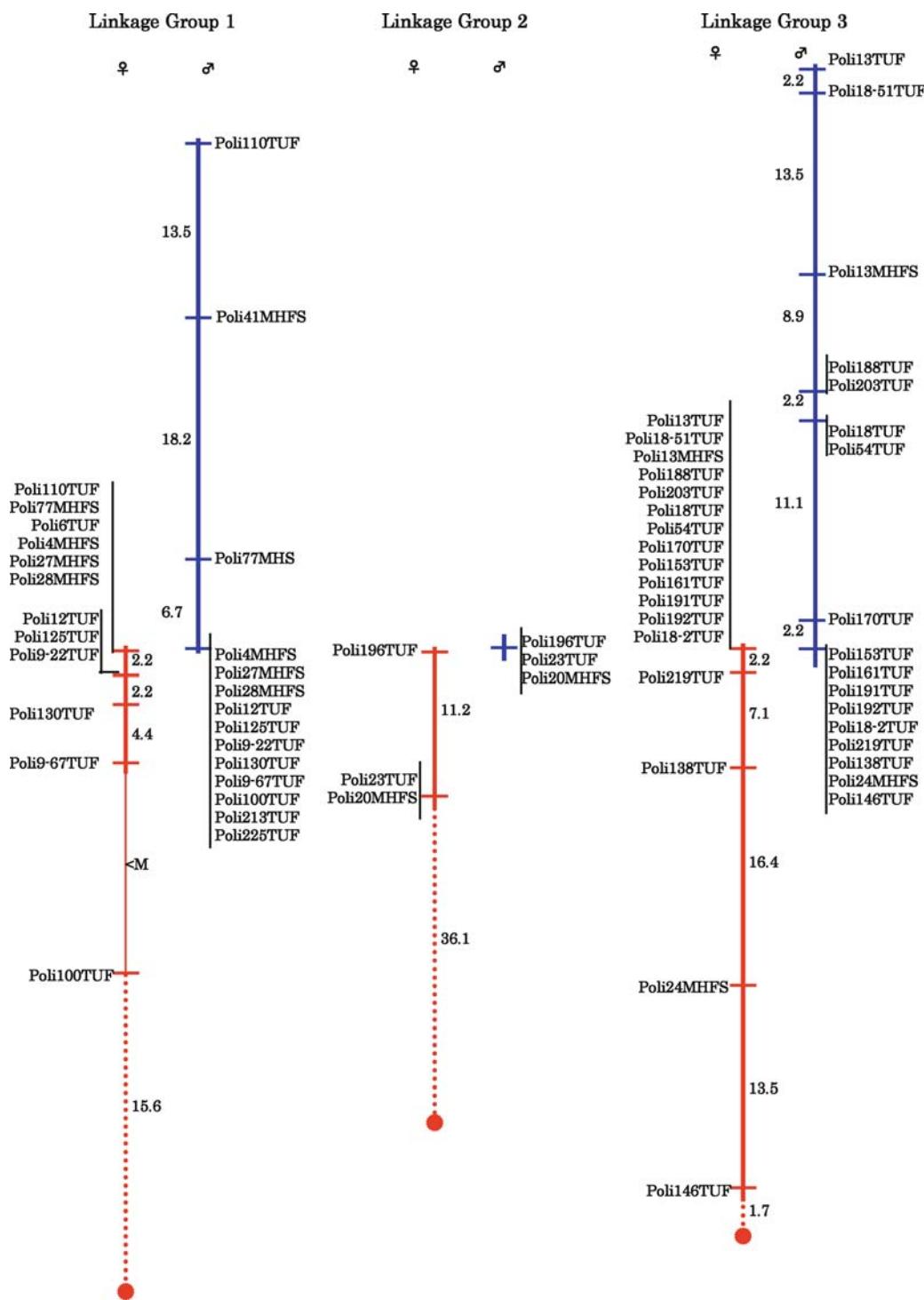
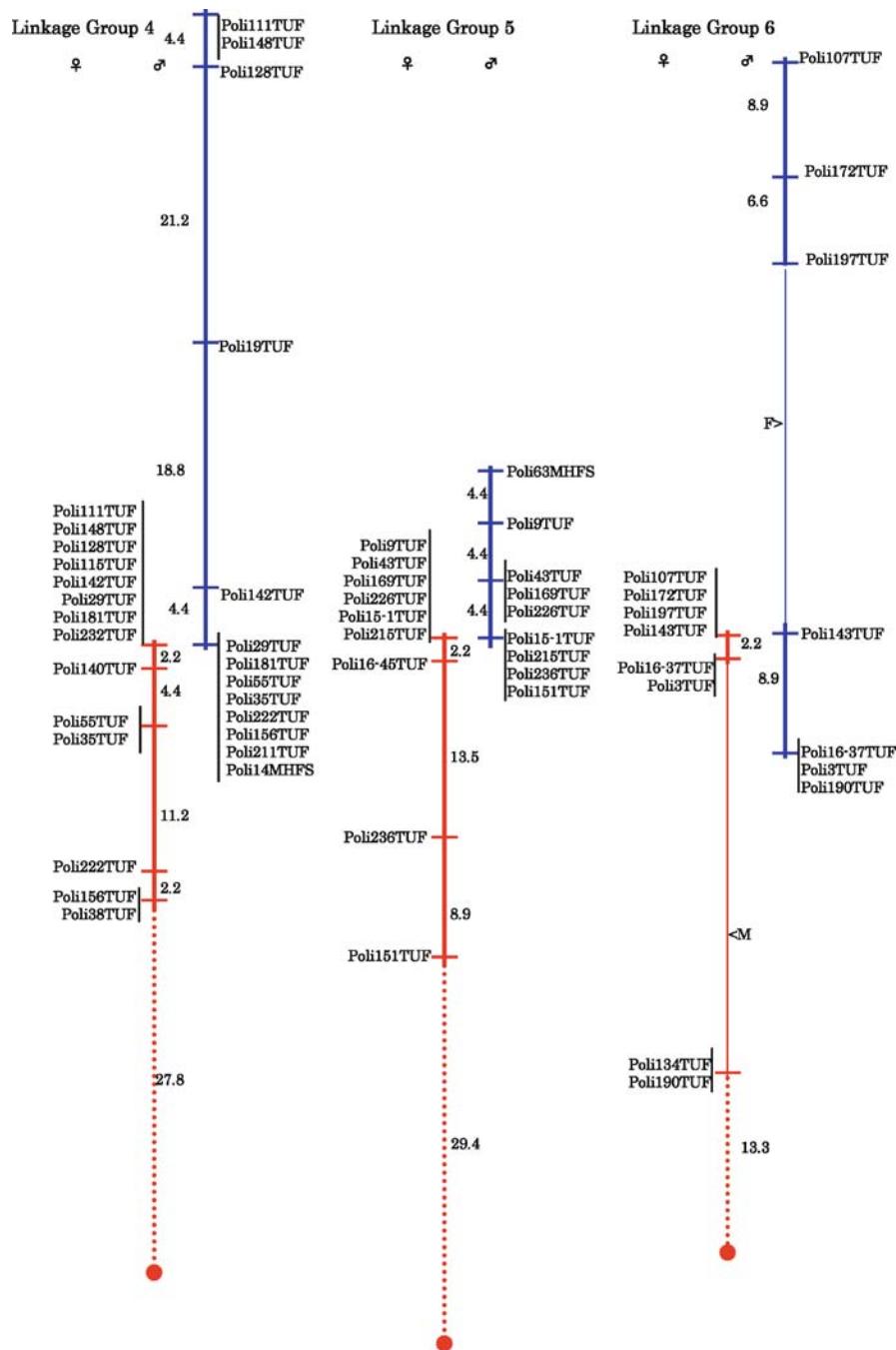
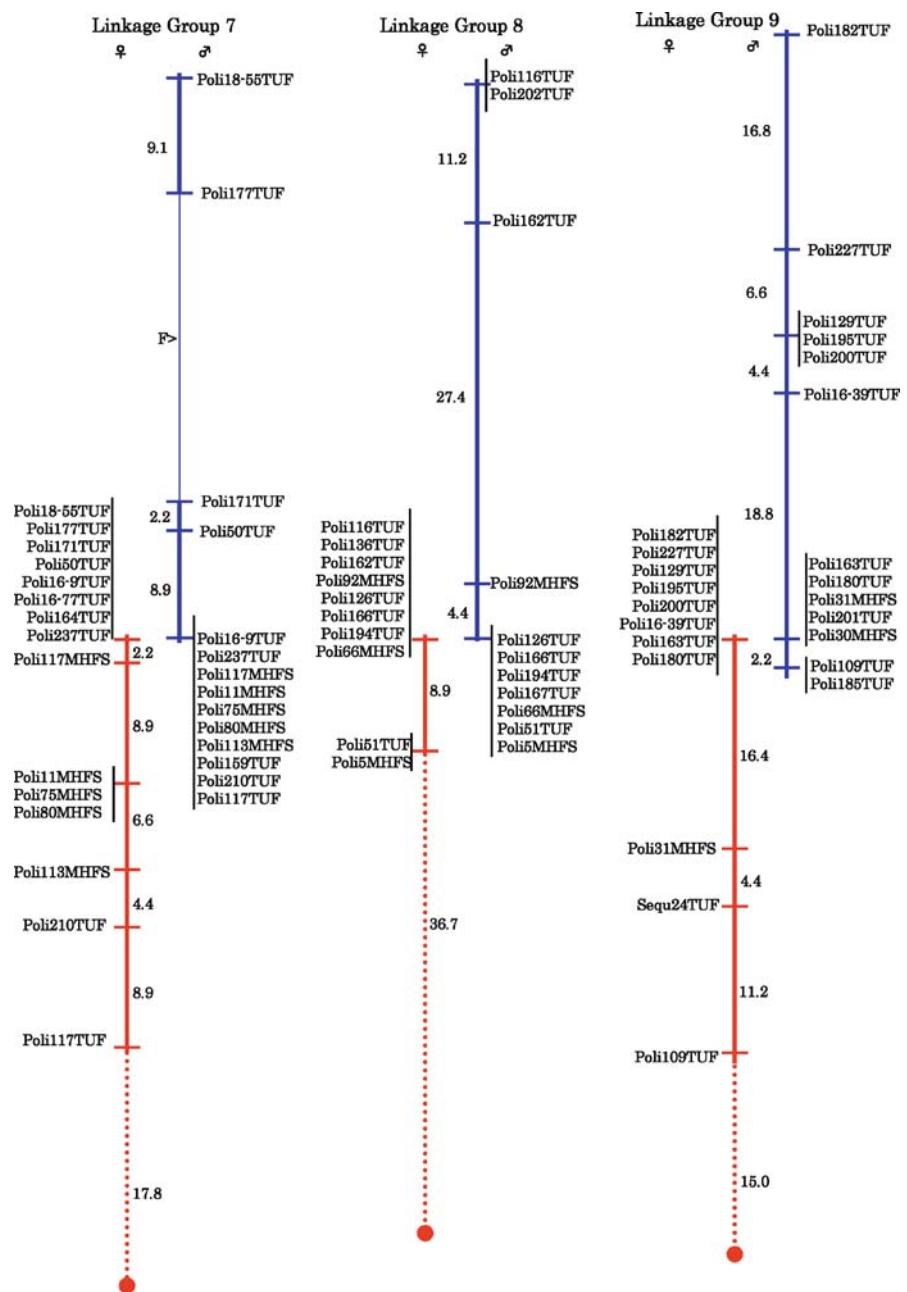
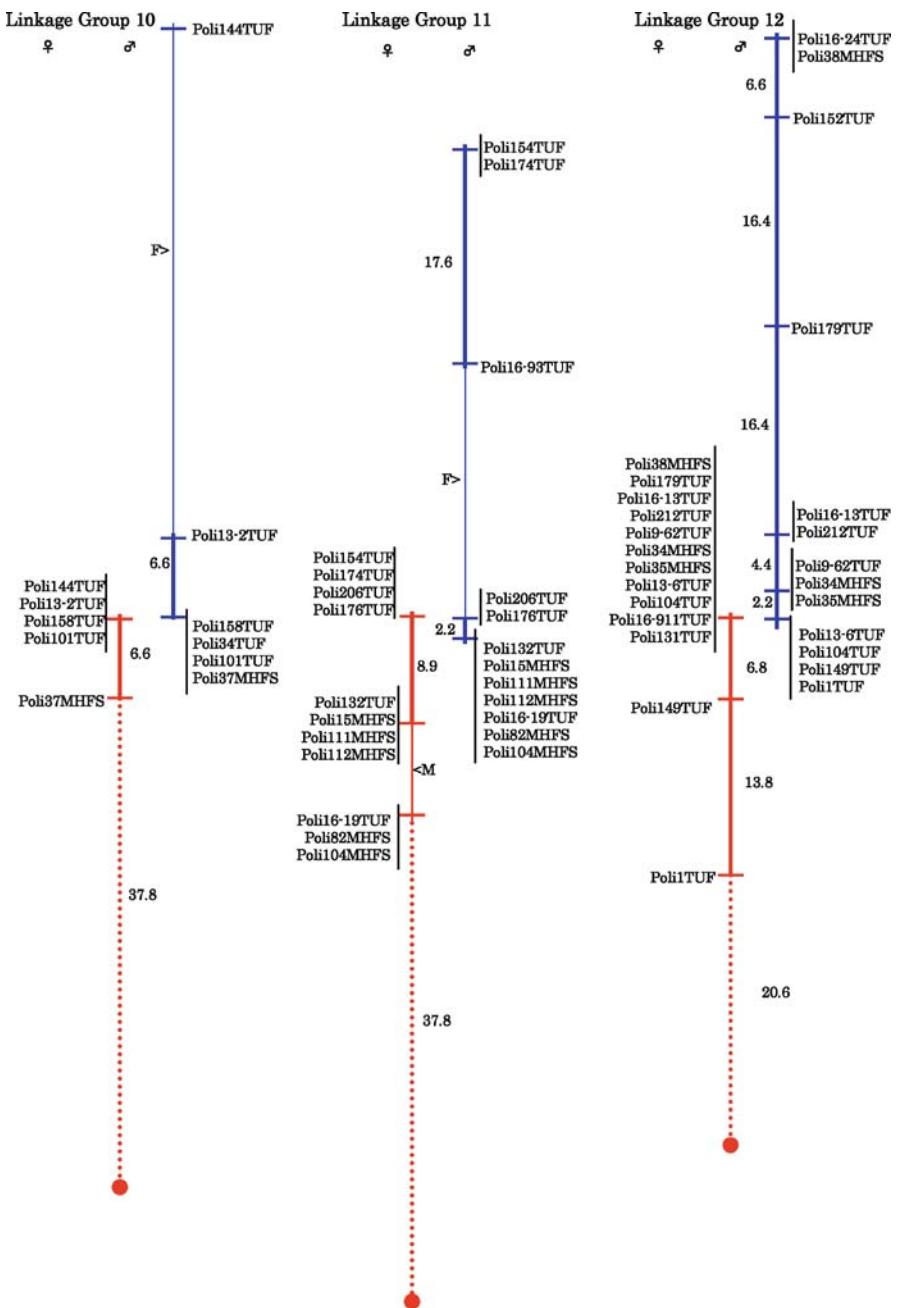
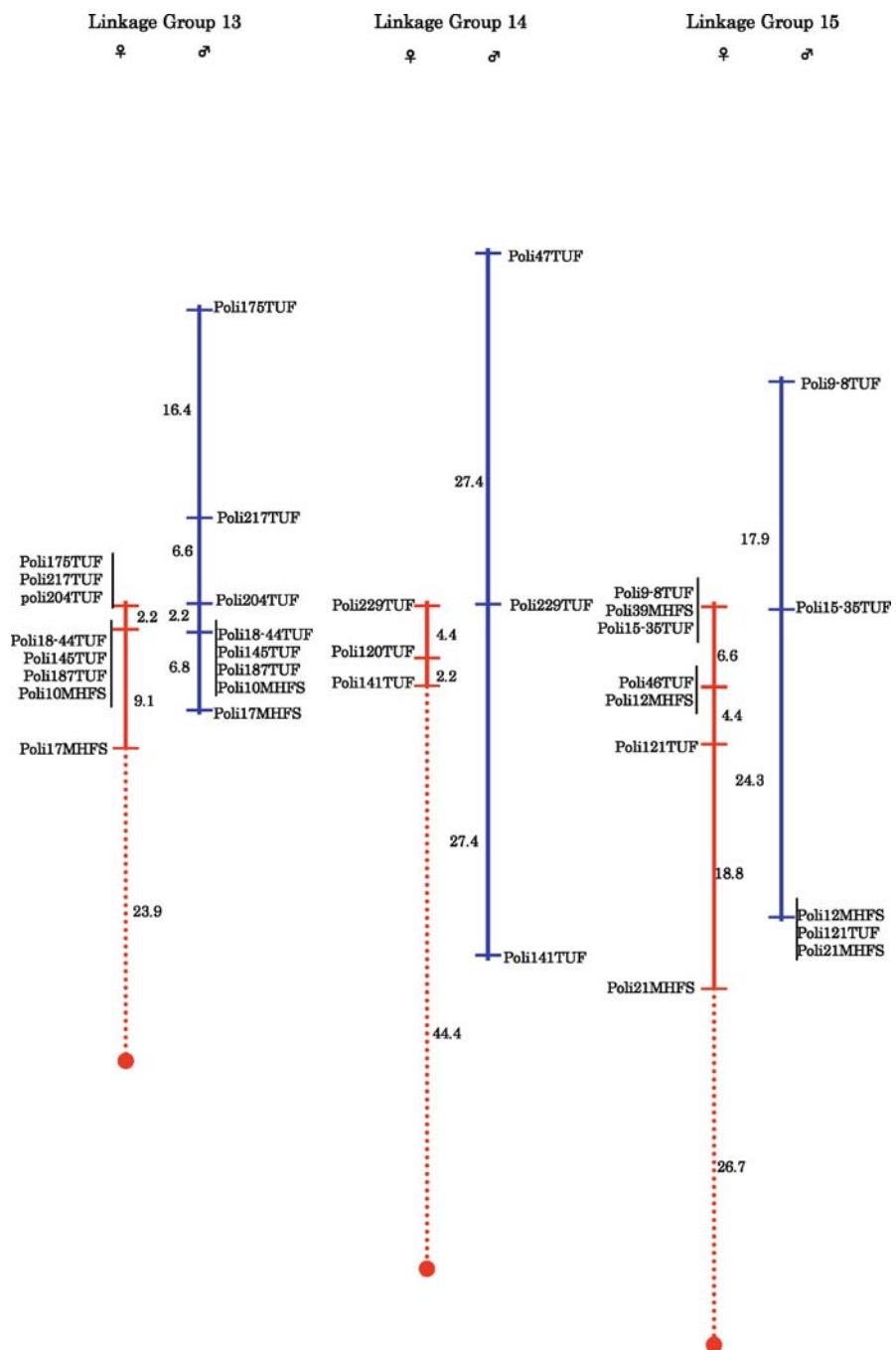


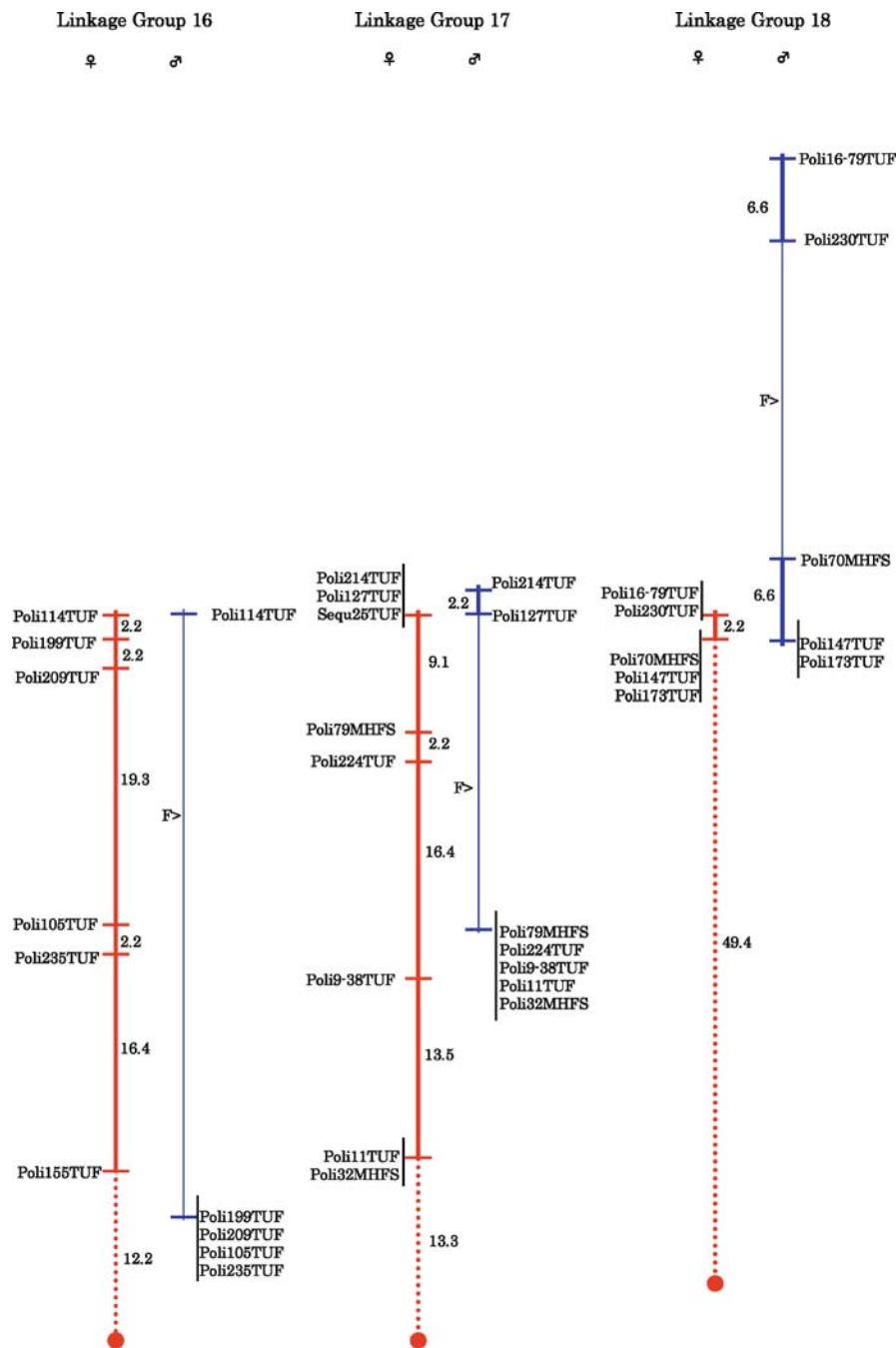
Fig. 1 Genetic linkage maps of Japanese flounder made by conjugation of the A2 maps and the centromere map. The symbols <M and >F on the maps indicate the parts of the maps that were connected based on linkages identified in the linkage map of the opposite sex. Positions of the centromeres were estimated from the equation $D = (100)(y/2)$, where y is the number of heterozygous recombinants per total number of samples. If the frequency of heterozygous recombinants is 100%, marker–centromere distance is estimated as 50 cM

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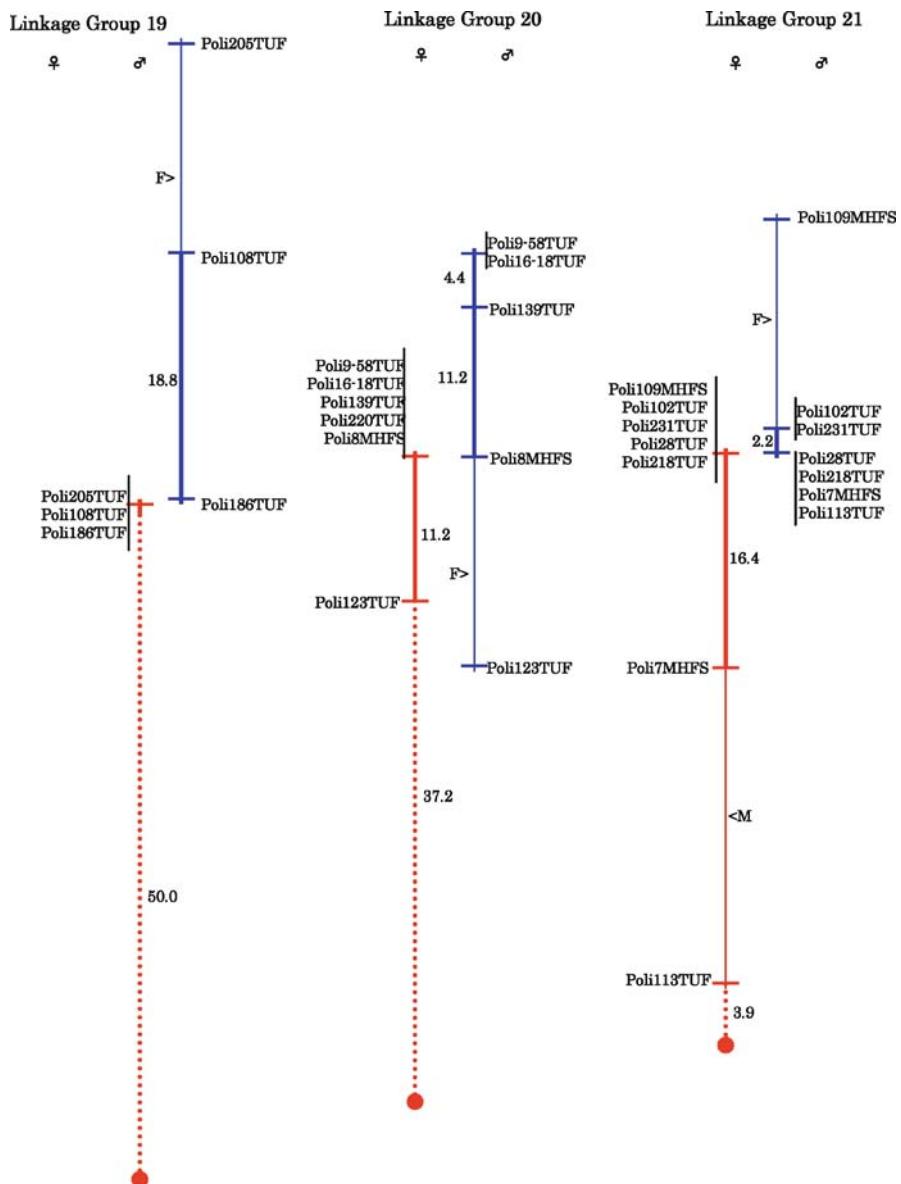
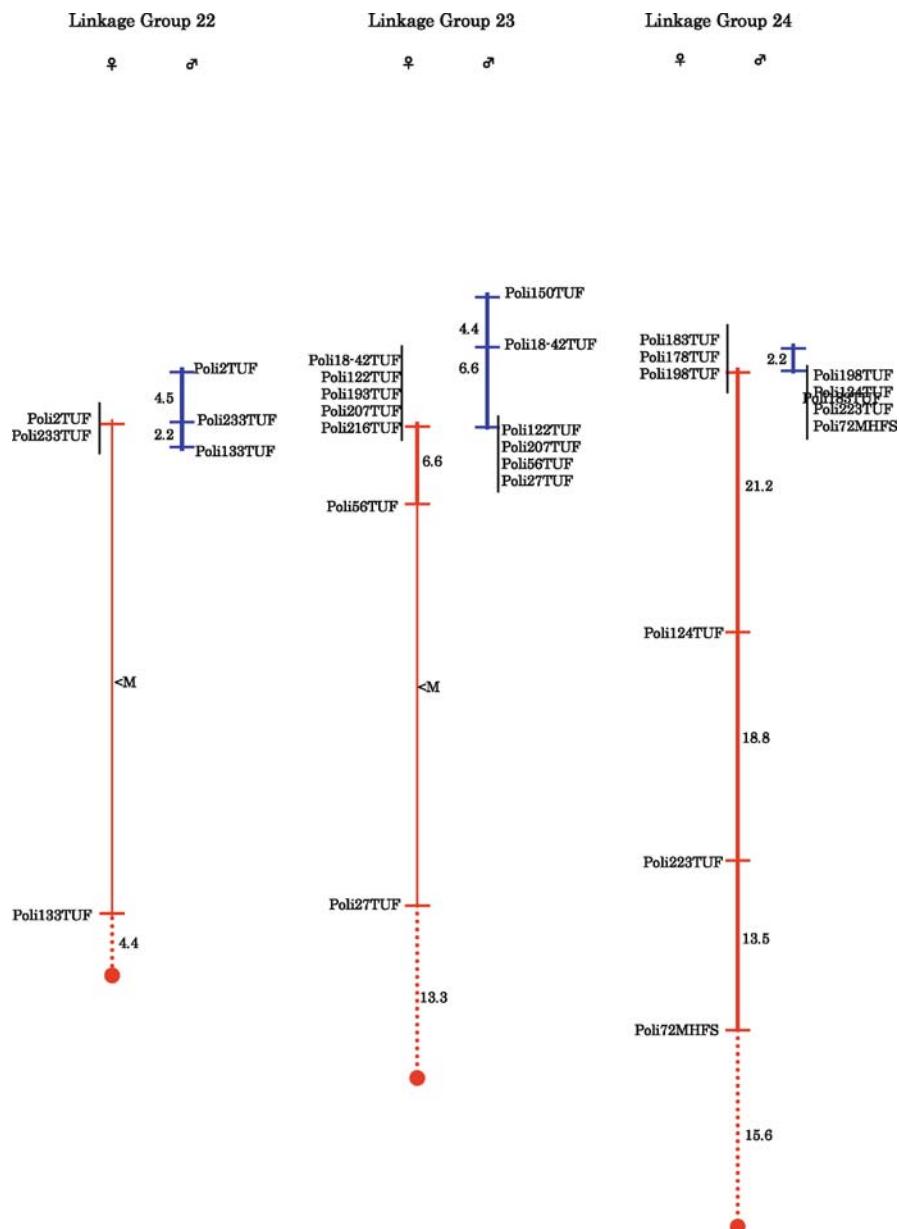


Fig. 1 (continued)

**Fig. 1** (continued)

of 230 microsatellite markers are included in the A2 map. The number of linkage groups in both the male and female maps is 24, which is the same as the number of chromosomes of the species (Sakamoto and Nishikawa 1980). Linkage group sizes ranged from 0 to 53.3 cM. The 204 markers included in this map are separated by an average of 4.1 cM. The male map spans a total of 837.5 cM. As in the previous version of the map, the A2 female map is shorter than the male map, covering 645.5 cM. The female map has 208 markers, with an average inter-marker distance of 3.1 cM. Linkage group lengths varied from 2.2 to 50.0 cM. The Japanese flounder map is still the only published map of a flatfish species. Although the A2 map has better resolution, it still has some gaps between adjacent markers. Therefore, there is still a need to construct a more detailed map for the Japanese flounder, which would cover most of the genome of this species.

6.3.3

Marker-Centromere Map

Kobayashi (2002) constructed a microsatellite-centromere map for Japanese flounder using gynogenetic diploids, which were produced by suppressing the second meiotic division, as suggested by Yamamoto (1999). Eggs were obtained from the dam of the A2 family used for constructing the second-generation map. Microsatellite–centromere distances were obtained from an analysis of genotyping data of 90 gynogenetic diploids. The distances were calculated using the method of Allendorf and Leary (1984). The estimated distance from a microsatellite to a centromere was calculated according to the equation $D = (100)(y/2)$, where y is the number of heterozygous recombinants per total number of samples.

To determine centromere–telomere orientation, two to nine markers were analyzed in each linkage group. The chromosomes of Japanese flounder are acrocentric (Sakamoto and Nishikawa 1980). Accordingly, the centromeres were graphically placed at the end of each linkage group of the female map (A2 mapping family, Fig. 1). After orienting the linkage groups with their corresponding centromeres at the end, we observed that recombination events tended to occur more frequently in centromeric regions in

the female and, conversely, in telomeric regions in the male progeny. Recombination events in fish species usually occur one time per chromosome arm, indicating the existence of interference after the formation of a single chiasma (Thorgaard et al. 1983). Complete interference has been detected for several fish species (e.g., Thorgaard et al. 1983; Guyomard et al. 1984; Sakamoto et al. 2000). High levels of interference were also observed for Japanese flounder. The limited number of recombination events in fish makes genetic linkage maps accurate.

6.4

Application of Genetic Linkage Maps and Future Prospects

6.4.1

Quantitative Trait Loci Studies

P. olivaceus genetic linkage maps have already been used to perform QTL analysis. One of the priorities for selective breeding programs for Japanese flounder is to breed organisms that are resistant to the main pathogens affecting the culture ponds. Japanese flounder is susceptible to several pathogens, including lymphocystis disease virus (LDV) and a pathogenic bacterium that produces edwardsiellosis (Muroga and Egusa 1996). Individuals affected with LDV typically present hypertrophied cells on skin, fins, and/or mouth. The hypertrophied lymphocystis cells accumulate in tumor-like nodules; therefore, affected fish lose commercial value. In addition, lymphocystis nodules located on the mouth interfere with feeding and could lead to starvation. LD is consequently costly to farmers. There are presently no medicines or commercially available vaccines for LD. Therefore, Fuji et al. (2006a) attempted to identify the LD-resistance locus by linkage analysis. To accomplish this, phenotypically selected LD-resistant (KP-B) and LD-susceptible lines (KP-A) were used to generate a hybrid KP-BA and, subsequently, a backcross family (KP-BAA), which were exposed to LDV-contaminated water. Fifty of the microsatellite markers included in the first generation map (Coimbra et al. 2003) were used in the analysis, and one locus showing highly significant association with LD resistance was detected. The authors found a lymphocystis disease-resistant

locus located close to the markers Poli.9-8TUF, Poli.9-35TUF, and Poli.121TUF on linkage group (LG) 15. Poli.9-8TUF explained 50% of the total phenotypic variation in the 136 individuals screened. We are using the results obtained to work with farmers in preliminary selective breeding programs.

Edwardsiellosis is a common bacterial disease that affects Japanese flounder culture (Savan et al. 2004). We are currently trying to identify QTLs related to edwardsiellosis resistance so they can be used in a selective breeding program. Studies are also being conducted to identify QTLs associated with color abnormalities, such as pseudo-albinism. Two QTLs associated with pseudo-albinism were identified on LG 5 and LG 24 (Fuji et al. 2006b).

6.4.2 Future Prospects

Although Japanese flounder genetic linkage maps have already been used to identify QTLs and to guide selective breeding, more detailed maps are needed to achieve more precise results. We are presently building high-density, sex-specific genetic linkage maps for Japanese flounder. The maps are being constructed mainly using microsatellite markers consisting of di-, tri- and tetranucleotide repeats; additionally, EST markers and one gene were also used (Castaño Sánchez et al. 2006b).

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7 Shrimp

Arun K. Dhar¹, Betsy Read², and Robert A. Bullis¹

¹ Advanced BioNutrition Corporation, 7155 Columbia Gateway Drive, Columbia, MD 21046, USA

e-mail: adhar@abn-corp.com

² College of Arts and Sciences, California State University, San Marcos, CA 92096, USA

7.1 Introduction

The international shrimp farming industry is just over 30 years old, but estimated global production of farm-raised shrimp has already reached 2.5 million metric tons in 2005 (FAO 2006). Farmed-raised shrimp are among the most important agricultural commodities, in terms of export earnings, for many tropical and subtropical countries with large coastal regions. The importance of this growing industry is reflected by these production numbers and by the millions of people employed by this industry worldwide. Despite high levels of production, shrimp farmers have experienced significant economic losses in recent years, due primarily to the presence of diseases that now plague the industry. In particular, viral diseases have had, and will continue to have, a profound impact on the growth of the industry. In Asia, mortalities due to white spot syndrome virus (WSSV), yellowhead virus (YHV), and Taura syndrome virus (TSV) since their global emergence in 1994 have resulted in economic losses of over US \$1 billion per year (Lightner et al. 1998; Lightner 2003). In South America, TSV has resulted in losses greater than US \$400 million per year (Brock et al. 1997; Dhar et al. 2004). These viruses, and other known viruses of penaeid shrimp (Table 1), have had a devastating impact on shrimp farming regions worldwide.

Diseases due to viruses such as WSSV, TSV, and infectious hypodermal and hematopoietic necrosis virus (IHHNV), were all transferred with live shrimp stocks well before their etiology and pathogenesis were understood and before the development of spe-

cific viral diagnostic tests. In the aftermath of the resulting viral pandemics, the industry moved rapidly to develop biosecurity programs to protect the industry. These efforts, as well as integrated research programs dedicated to improving the health of seed stocks, have helped restore production levels to those achieved before these diseases emerged as significant impediments to profitability (Moss 2005). This chapter provides an overview of the development of the specific pathogen free (SPF) domestication program in shrimp and the current status of genomic research in shrimp.

7.1.1 **Biosecurity, Domestication, and Development of Specific Pathogen Free Shrimp**

Biosecurity, as it is applied to shrimp aquaculture, is defined as the exclusion of specific pathogens from cultured shrimp stocks in broodstock facilities, hatcheries, and farms, or from entire countries or regions, for the purpose of disease prevention (Lightner 2003). In the wake of the global viral epizootics described above, and particularly in response to the spread of WSSV and TSV in Asia and the Americas, the shrimp farming industry adopted new practices for biosecurity (Lee and O'Bryan 2003). These principles include: control of shrimp stocks from a list of excludable diseases and pathogens of concern, availability of sensitive and specific diagnostics methods, biosecure environmental controls to prevent the introduction of new pathogens, and disinfection and eradication methods to contain and eradicate existing disease outbreaks (Lightner 2003). Almost all of these tools and principles have emerged in response

Table 1 Major viruses of penaeid shrimp

Virus	Genome	Virion Size	
<i>DNA Viruses</i>			
Infectious Hypodermal & Hematopoietic Necrosis Virus (IHHNV)	ssDNA	20 nm	Systemic parvovirus
Hepatopancreatic Parvovirus (HPV)	ssDNA	22–24 nm	Enteric parvovirus
Spawner-isolated Mortality Virus (SMV)	ssDNA	20 nm	Parvovirus
Lymphoidal Parvo-like Virus (LPV)	ssDNA	25–30 nm	Parvo-like virus
Baculovirus penaei (BP)	dsDNA	55–75 × ~300 nm	Tetrahedral baculovirus
Monodon-type Baculovirus (MBV)	dsDNA	~75 × 300 nm	Spherical baculovirus
Baculoviral Midgut Gland Necrosis Virus (BMN)	dsDNA	~75 × 300 nm	Nonoccluded baculovirus
Hemocyte Rod-shaped Virus (HRV)	dsDNA	90 × 640 nm	Baculo-like virus
White Spot Syndrome Virus (WSSV)	dsDNA	130 × 350 nm	Whispovirus
Iridovirus (IRIDO)	dsDNA	136 nm	Iridovirus
<i>RNA Viruses</i>			
Taura Syndrome Virus (TSV)	ssRNA	30 nm	Cricket paralysis-like virus
Yellowhead/Lymphoid Organ/Gill associated virus (YHV/LOV/GAV)	ssRNA	44 × 173 nm	Okavirus
Lymphoid Organ Vacuolization Virus (LOVV)	ssRNA	55 nm	Toga-like virus
Infectious myonecrosis virus (IMNV)	dsRNA	40 nm	Totivirus
Reo-like Viruses (REO III & IV)	dsRNA	50–70 nm	Reo-like virus

to viral diseases of economic importance. Many of the principles adopted by the industry are identical to those used successfully in other animal industries (Bullis and Pruder 1999).

Of the principles stated above, the most important is stock control, defined as the use of captive or domesticated stocks that have been cultured under controlled conditions and have been subjected to a rigorous and active disease surveillance program. Because previously, large segments of the industry grew shrimp collected from the wild or that were the offspring of wild-caught broodstock (Argue and Alcivar-Warren 1999), the industry unfortunately is responsible for transferring most shrimp viruses from one geographical region to another. Once a list of excludable pathogens had been developed, and suitable diagnostic tools were made available for detection (Table 2), it was possible to eliminate those pathogens from domesticated or wild-caught stocks (Lightner and Redman 1998). It also became possible to list shrimp as free of specifically listed pathogens (SLPs; i.e., those pathogens of concern that can be detected) and certify them as specific pathogen free (SPF; i.e., of any particular pathogens).

Development of SPF stocks has followed the International Council for the Exploration of the Seas (ICES) guidelines that serve to eliminate both horizontally and vertically transmitted pathogens (Sinderman 1990). Briefly, that process consists of the following components: (1) identify stocks of interest; (2) evaluate health status and disease history; (3) acquire and test samples for specifically listed pathogens; (4) import and quarantine (F_0) population and monitor F_0 stock; (5) produce an F_1 generation from F_0 stock; (6) destroy F_0 stock; (7) culture F_1 stock through critical stages, monitor health, and test for SLPs; and (8) if SLPs, pests, or other significant pathologies are not detected, the F_1 stock may be defined as SPF and released from quarantine. In industry vernacular, these released stocks are referred to as “high health” (HH) when they leave the biosecure environment of quarantine (Moss et al. 2001a; Lightner 2003).

Following the development of domesticated SPF lines of shrimp, it became possible to develop improved stocks through the processes of natural and artificial selection. Nucleus breeding centers, broodstock providers, and even some shrimp farmers routinely breed the survivors of natural or

Table 2 Diagnostic and epidemiological characteristics of six Office International de Epizooties (OIE) notifiable and listed viral diseases of major commercially important species of penaeid shrimp (*P. vannamei*, *P. monodon*, *P. japonicus*, *P. merguiensis*, and *P. indicus*)

Pathogen	Diagnosis	Virulence Susceptible	Resistant	Transmission Horizontal	Vertical	Reservoirs	Stability In Water	Enzootic
White Spot Syndrome Virus	BA BF LM GP PCR	All Penaeids	None	Ingestion Contact	Unlikely	Many Non-Penaeids	Hours–Days	Yes
Yellowhead Virus	BA LM GP PCR	<i>P. monodon</i> <i>P. vannamei</i>	<i>P. merguiensis</i>	Ingestion Contact	Unlikely	A Few Penaeids	Days	Yes
Taura Syndrome Virus	BA BFLM Ab GP PCR	<i>P. stylirostris</i> <i>P. vannamei</i>	<i>P. stylirostris</i>	Ingestion Contact	No	No Non-Penaeids	Weeks–Months	Yes
Infectious hypodermal and hematopoietic necrosis virus	BA BFLM GP PCR	<i>P. stylirostris</i>	<i>P. vannamei</i> <i>P. monodon</i> <i>P. indicus</i>	Ingestion Contact	Possible	No Non-Penaeids	Weeks–Months	Yes
Monodon baculovirus	BF LM GP PCR	<i>P. monodon</i> <i>P. indicus</i>	<i>P. merguiensis</i>	Ingestion Contact	No	No Non-Penaeids	Days	Yes
Baculovirus penaei	BP LM GP PCR	Larval	<i>P. vannamei</i>	Adults	Ingestion Contact	No	No Non-Penaeids	Days
			<i>P. stylirostris</i>					

Adapted from Lotz and Lightner, 1999.

? = Unknown BA = bioassay PCR = Polymerase Chain Reaction

BF = bright field microscopy of tissue impression smears, wet mounts, stained whole mounts.

LM = light microscopy examination of stained histological sections.

Ab = antibody based test, e.g. ELISA.

GP = gene probe based test, e.g. dot blot hybridization or in situ hybridization.

Table 3 Half-sib heritability estimates (h^2 half-sib) for commercially important traits of *Penaeus vannamei* (adapted from Moss et al., 2001)

Trait	h^2 half-sib (SE)
Sex Ratio	-0.002 ± 0.012
Taura Virus Resistance	0.09 ± 0.03
Percent Tail	0.15 ± 0.12
NH3 Tolerance	0.16 ± 0.10
Growth on Fishmeal Containing Diets	0.40 ± 0.06
Growth on Vegetable Diets	0.40 ± 0.30

induced disease outbreaks with the expectations that offspring will possess characteristics capable of conferring viral disease resistance (Moss et al. 2001). Such offspring are referred to as specific pathogen resistant (SPR) stocks (Lightner 1995). Success in the development of SPR stocks has resulted in the availability of *Penaeus stylirostris* resistant to IHHNV (Clifford 1998) and *Penaeus vannamei* resistant to TSV (Argue and Alcivar-Warren 1999; Moss et al. 2001a). Much additional work has been accomplished for *P. vannamei* at the Oceanic Institute in Hawaii, where Moss and co-workers screen more than 80 genetically selected families per year for improved characteristics (www.oceanicinstitute.org). Heritability estimates (h^2) for half-sib matings are now available for *P. vannamei* and provide a useful tool for breeders wishing to improve the characteristics of their own stocks of shrimp in response to the major issues of concern to the industry, such as viral disease resistance, growth at high densities, or the continued use of fishmeal and fish oil in shrimp diets (Table 3). Estimates of h^2 are low for fitness traits, such as viral disease resistance, and phenotypes with h^2 estimates less than 0.15 are often difficult to improve by selection (Tave 1993). Nonetheless, *P. vannamei* lines resistant to several strains of TSV have become a valuable tool for researchers studying viral pathogenesis in this species.

Integrated research programs dedicated to improving the health of seed stocks (www.usmsfp.org, www.aims.gov.au) have also developed methods to control gonadal maturation (Wyban and Sweeny 1991), techniques for artificial insemination (Argue

and Alcivar-Warren 1999), and fluorescent elastomer alphanumeric tags to mark individual animals for accurate identification in large containment systems (Arce et al. 1999). Individual shrimp may also be identified with DNA fingerprinting (Moore et al. 1999).

Other molecular techniques applied to the selective breeding of shrimp include: (1) assessment of genetic diversity to avoid inbreeding, (2) the measurement of effective population size, (3) tracking maternal and paternal lineages using mitochondrial and nuclear DNA markers, and (4) establishing markers linked to growth and viral disease resistance (Moore et al. 1999; Xu et al. 2003). The following sections review the status of molecular genetic technologies for the development of genetic markers and linkage maps as well as the molecular approaches used to identify differentially expressed genes in viral pathogenesis in shrimp.

7.2

Genetic Markers and Linkage Maps in Shrimp

7.2.1

Genetic Markers

Random Amplified Polymorphic DNA (RAPD)

RAPD fingerprinting has already been found to be very useful in shrimp, a species in which genomic information is limited. The RAPD method was used to identify population-specific markers in specific pathogen free populations of *P. vannamei* (Garcia et al. 1994), as well as to determine the genetic heterogeneity and population structure of wild *P. monodon* in the Gulf of Thailand and Andaman Sea (Tassanakajon et al. 1998a; Klinbunga et al. 2001) and wild *P. stylirostris* in the Gulf of California, Mexico (Aubert and Lightner 2000). RAPD markers have also been identified in families of *P. stylirostris* shrimp that are resistant to IHHNV (Hizer et al. 2002) and baculovirus penaei (BP) in *P. vannamei* (Alcivar-Warren et al. 1997). These markers could be used for the construction of high-density linkage maps in shrimp and to facilitate map-based cloning and marker-assisted selection of quantitative trait loci.

Microsatellites

In shrimp, microsatellites were first identified in *P. vannamei* while sequencing a population-specific RAPD marker (Garcia et al. 1994). Since then, efforts have been made to develop and use microsatellite markers for addressing different questions in shrimp aquaculture and population studies in *P. vannamei*, *P. monodon*, *P. japonicus*, *P. schmitti*, and *P. setiferus* (Garcia et al. 1995; Bagshaw and Bucholt 1997; Tassanakajon et al. 1998b; Wolfus et al. 1999; Xu et al. 1999; Supungul et al. 2000; Ball and Chapman 2003; Maggioni et al. 2003; Valles-Jimenez et al. 2004). Microsatellite markers were used to assess genetic diversity in SPF populations in a *P. vannamei* breeding program (Wolfus et al. 1999; Cruz et al. 2003). They were also used to assess the population structure of *P. monodon* in different geographic regions in the Philippines (Xu et al. 2001); to evaluate the genetic variation and population structures in *P. vannamei* from Mexico to Panama (Valles-Jimenez et al. 2004), in *P. schmitti* along the coastal zone in Brazil (Maggioni et al. 2003), and in *P. setiferus* along the Atlantic coast of the US and in the Gulf of Mexico (Ball and Chapman 2003). Candidate microsatellite markers have also been associated with resistance to Taura syndrome disease in *P. vannamei* (Xu et al. 2003). The feasibility of using microsatellite markers in developing linkage maps for shrimp has been debated due to the low abundance and the difficulties in getting a large number of usable microsatellites in *P. japonicus*. Hence, it has been suggested that microsatellites may not be appropriate markers for genome mapping in shrimp (Tassanakajon et al. 1998b; Moore et al. 1999). However, in recent years, a large number of useful microsatellites were generated in *P. vannamei* and *P. monodon*, either by constructing DNA libraries and sequencing a large number of clones (Meehan et al. 2003), or by mining databases of expressed sequence tags (ESTs) (Perez et al. 2005; Maneeruttanarungroj et al. 2006). These microsatellites are currently being used for linkage mapping and other genetic studies in shrimp.

Amplified Fragment Length Polymorphism (AFLP)

So far, the application of AFLP markers in genetic studies in shrimp has been very limited. AFLP markers were first used for linkage mapping in *P. japonicus*, *P. monodon*, and *P. vannamei* (Moore et al. 1999; Wilson et al. 2002; Li et al. 2003; Zhang et al. 2006). In

P. japonicus, many polymorphic AFLP markers were found to be consistent across families, making it possible to combine the marker analysis from different families in constructing a single linkage map for this species (Moore et al. 1999).

7.2.2

Linkage Maps of Shrimp

Until recently, shrimp farming was primarily dependent on the use of wild-caught broodstock. An increase in the demand for seafood, with shrimp as the primary choice, and the declining trend in capture fisheries have led to efforts to develop captive breeding in shrimp. Two centers where captive breeding programs have been successfully initiated include the US Marine Shrimp Farming Program (USMSFP) in Hawaii and the Australian Institute of Marine Science (AIMS) in Townsville, Australia. The success in captive breeding paved the way for initiating genetic improvement programs in shrimp. Breeding objectives have primarily focused on enhancing growth and survivability as opposed to combating viral pathogens. To verify the pedigree and to maintain genetic diversity, RAPD and microsatellite markers were used. Mapping families were developed for *P. vannamei* by the USMSFP and for *P. monodon* and *P. japonicus* by AIMS. The estimated physical size of the shrimp genome is 2.3×10^9 bp, which corresponds to approximately 70% of the human genome ($\sim 3 \times 10^9$ bp and 3,000 cM) (Chow et al. 1990). Shrimp appear to contain a large number of small chromosomes, and therefore the identification of individual chromosomes by morphological means is difficult. The haploid chromosome numbers (*n*) reported for penaeid shrimp varies from 43 to 46, depending on the species. For example, in *P. vannamei*, *n* = 46 (Campos Ramos 1996) and *n* = 44 (Chow et al. 1990) have been reported, whereas in *P. monodon*, *n* = 44 (Wilson et al. 2002), and in *P. japonicus*, *n* = 43 (Li et al. 2003) have been observed.

So far, low-density linkage maps have been constructed for *P. monodon* (Wilson et al. 2002; Maneeruttanarungroj et al. 2006), *P. japonicus* (Li et al. 2003), and *P. vannamei* (Zhang et al. 2006). These maps were constructed using AFLP and microsatellite markers. The *P. monodon* map was initially constructed using three mapping families and 116 AFLP

markers. The map contained 8 major and 11 minor linkage groups (<http://www.aims.gov.au/shrimpmmap>) with a total genome length of 1,412 cM. The average interval between adjacent markers was 22.4 cM. The anticipated haploid linkage group for *P. monodon* is 44 (Wilson et al. 2002). Therefore, this map was an initial draft with large gaps between markers. Recently, Maneeruttanarungroj and colleagues (2006) added 144 new markers to the *P. monodon* map, including 36 microsatellite markers obtained by mining EST data available in the public databases. The number of linkage groups in the current *P. monodon* male map is 47, and in the female map, it is 36. The addition of new markers increased the resolution of *P. monodon* maps, with the average interval between the markers being 7.0 cM and 8.0 cM for male and female maps, respectively (Maneeruttanarungroj et al. 2006). The *Penaeus monodon* genome size in recombination units corresponds to 2,000 cM (Wilson et al. 2002). Therefore, additional markers are needed to condense the existing maps and to match the expected haploid chromosome number of 44.

In *P. japonicus*, one paternal and one maternal map were constructed using 227 and 125 AFLP markers, respectively (Li et al. 2003). The paternal map contained 43 linkage groups with genome coverage of 1,781 cM, and the maternal map contained 31 linkage groups with genome coverage of 1,026 cM. Both maps had an average density of markers of approximately 1 per 10 cM. The haploid chromosome number for *P. japonicus* is 43, with an estimated genome size of 2,300 cM (Moore et al. 1999). Therefore, more markers are needed to increase the resolution of both the maps.

Recently, a genetic linkage map has been published for *P. vannamei* (Zhang et al. 2006). The map was constructed using both microsatellite and AFLP markers. There are 45 linkage groups for both male and female maps with an average of 7.1 and 5.9 markers per group, respectively. The estimated length of the female map is 5,444 cM, and for the male map, it is 4,626 cM (Zhang et al. 2006).

The confirmation of sex-linked markers on the maternal, but not paternal, map has led to the suggestion that the female is heterogametic (Li et al. 2003; Zhang et al. 2006). This highlights the potential for using the map-based information to manipulate sex ratios in shrimp. In shrimp, females are bigger than males and, therefore, developing all-female families will have a significant impact on yield at the farm level where the population contains both males and

females. As the resolution of shrimp genetic maps increases by the addition of new markers, it will be possible to identify genes that govern sex determination in shrimp and to exploit this information to develop all-female progenies at the farm level. Such a development will have a huge impact on the global production of farmed shrimp.

Linkage maps serve as a foundation of genetic knowledge for any species. Worldwide, there are at least six commercially important species of penaeid shrimp (*P. vannamei*, *P. stylirostris*, *P. setiferus*, *P. monodon*, *P. japonicus*, and *P. chinensis*) and one freshwater prawn (*Macrobrachium rosenbergii*). As linkage maps are developed for at least some of these species, it will be worthwhile to determine how much synteny exists among these species and among other crustaceans. Existence of macro- and microcolinearity among these species will expedite the development of high-density linkage maps, marker-assisted breeding in these species, and the determination of the evolutionary relationship among different species of shrimp and other crustaceans.

The resolution of a linkage map is dependent on the number of polymorphic markers available and also on the extent of genetic recombination. Since the levels of genetic recombination vary from one region of a chromosome to another, high-density genetic mapping may not be possible for some regions of the chromosome. As a result, there may be gaps in the maps and markers linked to genes located within regions with low levels of recombination. This problem is overcome by constructing a physical map of the chromosome that contains overlapping or contiguous DNA fragments along the entire chromosome. Bacterial artificial chromosome (BAC) and P1-derived artificial chromosomes (PAC) could be used for constructing such a physical map. Once BAC or PAC libraries are made, expressed sequence tags (ESTs) that are isolated from different tissues and different species of shrimp (see Section 7.3.1) could be anchored (e.g., by fluorescent in situ hybridization techniques) to the chromosomes.

7.3 Efforts in Reverse Genetics

Forward genetics approaches have been traditionally used for gene discovery in aquatic and other agri-

cultural species. This involves cloning the gene for a given trait when the phenotype and the position of the locus in the genetic map are known (from phenotype to gene). However, in an aquacultural species like shrimp (i.e., *Penaeus* sp.), where the domestication process has begun only recently and the genetic map is in its infancy, reverse genetics can be a powerful approach for gene discovery. In reverse genetics, a set of candidate genes with a known sequence, whose expression is modulated in response to a given treatment or altered by point mutation (e.g., deletion or insertion) in the coding or regulatory region, is identified first. The transcriptional activity of the gene is used as a first clue in terms of linking the gene to a specific biological process. The analysis of the diversity of an mRNA population produced by a cell under certain biotic or abiotic stress or developmental conditions (also known as transcriptome analysis) is performed using a variety of methods. In the following section, particular methods that have been applied to shrimp are described.

7.3.1 Expressed Sequence Tag (EST) Analysis

The EST approach has been used across a wide variety of plants and animals, including many aquaculture species, to assess tissue-specific transcriptome profiles, discover novel genes, and identify EST-associated microsatellite markers. A GenBank database search revealed that for the five major commercially important shrimp species combined (*Penaeus vannamei*, *P. setiferus*, *P. stylirostris*, *P. japonicus*, and *P. monodon*), there are fewer than 30,000 ESTs currently available (Fig. 1). In contrast, many more ESTs are available for other commercially important aquaculture species (e.g., catfish, rainbow trout, salmon, tilapia, and oyster; Fig. 1). The number of ESTs for different species of shrimp is continuously increasing as more sequences are added to the database from various cDNA sequencing projects.

The first report on ESTs in shrimp was published in 1999 and examined the gene expression patterns across various tissues (i.e., cephalothorax, eyestalk, and pleopod) in black tiger shrimp, *P. monodon* (Lehnert et al. 1999). The putative identities of many of these ESTs revealed the occurrence of tissue-specific expression including many novel ($n = 61$) genes. Since then,

a number of papers describing ESTs from different species of shrimp have been published. Several putative immune genes were isolated from the hemocyte and hepatopancreas cDNA libraries from cultured specific pathogen free Pacific white shrimp (*P. vannamei*) and wild Atlantic white shrimp (*P. setiferus*) (Gross et al. 2001). Among the ESTs, antimicrobial peptides were most prevalent in the hemocyte libraries, and lectins represented the most abundant transcripts in the hepatopancreas cDNA libraries for both species of shrimp (Gross et al. 2001). Immune genes have also been isolated by EST analysis from a hemocyte cDNA library of *P. monodon*, including genes that are involved in the clotting system and the prophenoloxidase-activating system, as well as antioxidant enzymes, antimicrobial peptides, and serine protease inhibitors (Supungul et al. 2002).

Rojtinnakorn and colleagues (2002) compared the mRNA expression profiles of healthy and WSSV-infected kuruma prawns (*P. japonicus*) by EST analysis of hemocyte cDNA libraries. The defense genes accounted for 2.7% ESTs in the healthy shrimp library and 15.7% in the WSSV-infected library. Among the immune genes, genes that encode protease inhibitors, apoptotic peptides, and tumor-related proteins were found only in the infected library and not in the healthy library, suggesting that these proteins are involved in defense mechanisms against WSSV infection in kuruma prawns (*P. japonicus*) (Rojtinnakorn et al. 2002). Comparison of the transcriptome profiles of healthy and WSSV-infected *P. monodon* revealed that WSSV infection not only modulates the expression of immune genes but also the expression of genes involved in many basic cellular metabolic processes (Leu et al. 2006). Suppression subtractive hybridization (SSH) was used to isolate the differentially expressed genes in the hepatopancreas and hemocytes of shrimp (*P. japonicus*) that survived after white spot syndrome virus challenge (He et al. 2005; Pan et al. 2005). Genes that showed similarity to interferon-like protein and (2'-5') oligo (A) synthetase-like proteins, which are a part of the interferon pathways in vertebrates, were isolated from the hemocyte SSH library.

Large-scale EST sequencing projects for the purpose of gene discovery and functional genomic studies have been undertaken in *P. monodon* (<http://pmonodon.biotech.or.th>) (Tassanakajon et al. 2006). More than 10,000 ESTs were generated from

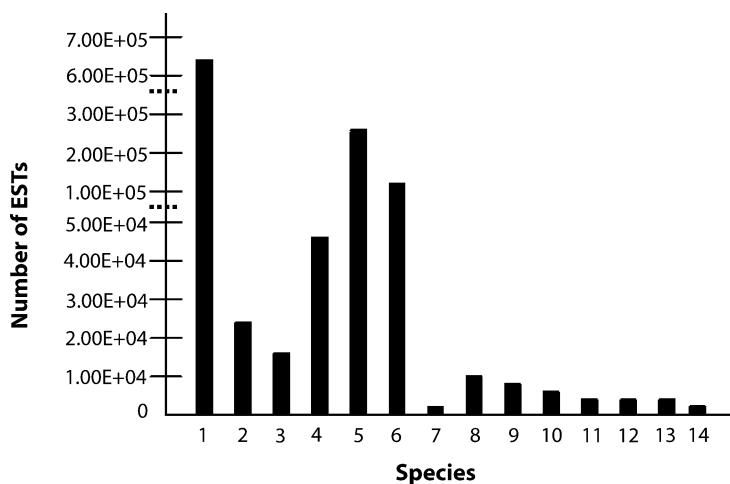


Fig. 1 A bar diagram showing the number of ESTs available at the NCBI database for different aquaculture species. For comparison, two non-aquaculture species, zebrafish and Japanese puffer fish, were included in the list. 1 zebrafish, *Danio rerio*, 2 Japanese puffer fish, *Takifugu rubripes*, 3 blue catfish, *Ictalurus furcatus*, 4 channel catfish, *Ictalurus punctatus*, 5 rainbow trout, *Oncorhynchus mykiss*, 6 salmon, *Salmo salar*, 7 Nile tilapia, *Oreochromis niloticus*, 8 oyster, *Crassostrea gigas*, 9 oyster, *Crassostrea virginica*, 10 Pacific shrimp, *Penaeus vannamei*, 11 white shrimp, *Penaeus setiferus*, 12 kuruma prawn, *Marsupenaeus japonicus*, 13 black tiger shrimp, *Penaeus monodon*, and 14 blue shrimp, *Penaeus stylirostris*

various shrimp tissues and more than 50% of the ESTs had no similarities to the database entries, indicating the huge potential of ESTs for gene discovery in shrimp. These ESTs are valuable resources for genome mapping as well as for developing therapeutics for viral and bacterial diseases in shrimp.

7.3.2 mRNA Differential Display

The shrimp genome, like that of any other invertebrate or vertebrate species, contains thousands of genes, the expressions of which are temporally and spatially governed by a regulatory network. Modulation in this intricate regulatory circuit leads to a diseased state displaying a variety of developmental and pathological conditions. Therefore, isolating and characterizing genes that are differentially expressed under various conditions (e.g., healthy vs. diseased and resistant vs. susceptible) is critical to understanding these biological processes. An RNA fingerprinting method, mRNA differential display, has been widely used for isolating differentially expressed genes characteristic of different pathological and/or developmental conditions (Liang and Pardee 1997; Liang 2002; Yang and Liang 2004). The method involves isolating

RNA, reverse transcribing mRNA using an anchored primer, and then amplifying the cDNA using an arbitrary primer and the same anchored primer used for cDNA synthesis. The amplified products are subjected to denaturing polyacrylamide gel electrophoresis for high-resolution separation, and the differentially expressed cDNAs are excised from the gel. Theoretically, 80 arbitrary primers in combination with three one-base (G/T/C) anchored oligo-dT primers (240 primer combinations) could detect 96% of the expressed genes in a cell $[1 - (0.96)^n]$, where n is the number of arbitrary primers]. Currently, there are several vendors selling kits for differential display. The protocols are generally very similar. While the cDNA amplicons (tags) generated by the mRNA differential display method are short and can be readily resolved, when subjected to denaturing polyacrylamide gel electrophoresis, they are at the same time long enough to uniquely identify an mRNA transcript. Since oligo-dT-anchored primers amplify predominantly the 3' end of the mRNA and the 3' non-coding regions are very diverse, the nucleotide sequences of the differentially expressed cDNAs often do not show similarity with the GenBank database entries. On the other hand, the heterogeneity of the 3' end of the mRNA allows one to differentiate members of a gene family.

In shrimp, the mRNA differential display method has been used to identify differentially expressed genes in WSSV-infected shrimp (Astrofsky et al. 2002; Luo et al. 2003). An RNA fingerprint of healthy and WSSV-infected shrimp is shown in Fig. 2. While comparing the expression profiles of hepatopancreas in healthy and WSSV-infected shrimp, Astrofsky et al. (2000) isolated several differentially expressed transcripts, and fewer than 20% of the genes showed similarity with database entries. As the number of entries in the EST database is increased and the genomes of many other species are sequenced, the possibility of finding similarity in the differentially isolated genes in species like shrimp will increase. Luo et al.

(2003) identified a novel gene containing a C-type lectin domain, which showed antiviral activity, while comparing the mRNA fingerprints of WSSV-sensitive and WSSV-resistant *P. monodon*. This indicates the potential of using the differential display technique in surveying the transcriptomes of shrimp under different treatment conditions.

7.3.3 Microarray Analysis

Microarray analysis is revolutionizing our understanding of the dynamics of gene expression. The power of the DNA microarray method lies not so much in its quantitative nature but rather in its ability to monitor the expression of thousands of genes simultaneously (Schena et al. 1998). The technology is exceedingly valuable and has tremendous potential as a means of addressing many of the important issues in genetic studies in shrimp. For example, shrimp gene expression profiles can be used to identify genes that are important to nutritional requirements, growth rates, fecundity, and/or disease resistance. The identification of target genes is a necessary first step in efforts aimed at developing strategies for effective breeding programs, nutritional supplementation, vaccination, or disease therapy.

In various species of shrimp, gene expression studies, using differential display and suppressive subtractive hybridization, have been applied to study different aspects of viral pathogenesis (Astrofsky et al. 2002; Luo et al. 2003; He et al. 2005; Zhao et al. 2007). Although differential display and SSH are less expensive than microarray analysis, they are labor intensive and require extensive screening procedures. The potential of microarray analysis in shrimp is only beginning to be realized. Dhar and colleagues (2003) printed a small array of 100 elements containing 47 unique ESTs obtained from a WSSV-infected hepatopancreas cDNA library and 37 elements from differential display screening of WSSV-infected shrimp to demonstrate the feasibility of employing microarray analysis to identify genes involved in viral pathogenesis. A total of 24 differentially expressed genes were identified, 16 of which were up-regulated and 8 of which were down-regulated. An expression profile of three of the differentially expressed genes, an immune gene (C-type lectin-1), a structural gene (40S ribosomal pro-

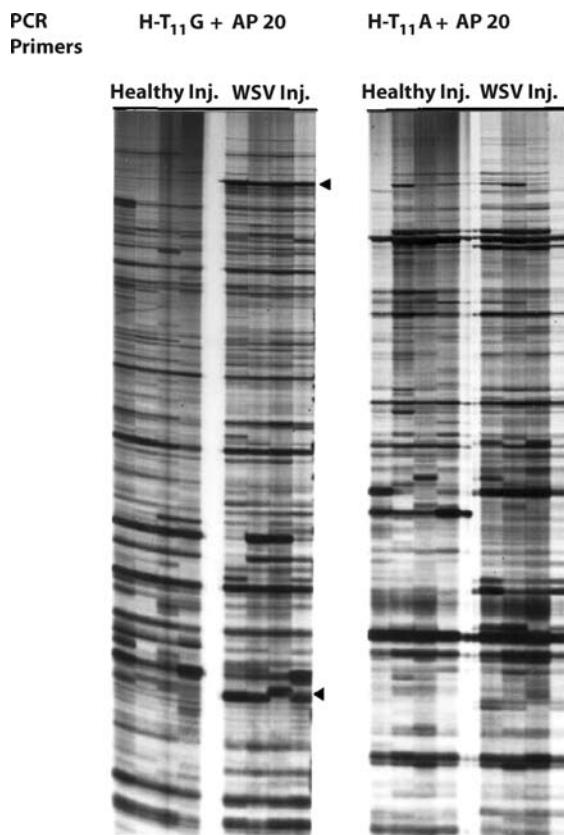


Fig. 2 An RNA fingerprint of healthy and white spot syndrome virus (WSSV)-infected shrimp using the mRNA differential display method. One base-anchored oligodT primer used for cDNA was synthesized (H-T11G and H-T11A), and the primers for RT-PCR (H-T11G or H-T11A with AP20) were taken from RNAimage kit of GenHunter Corp., Nashville, Tennessee. The arrows indicate the candidate differentially expressed genes

tein), and a gene involved in lipid metabolism (fatty acid binding protein), was generated in healthy and WSSV-infected shrimp by real-time PCR. This was the first such study to demonstrate the effectiveness of shrimp-based microarrays and how they might be exploited to address problems limiting shrimp aquaculture production.

Khadijah and co-workers (2003) successfully designed a WSSV-specific DNA microarray to measure WSSV gene expression in specific pathogen free and WSSV-infected shrimp. The DNA chips produced some interesting but troublesome results, suggesting that the SPF shrimp (developed and grown under biosecure conditions for six generations by the BIOTEC group in Thailand) were carriers of WSSV and were actively expressing viral genes. Some WSSV genes were highly expressed in the SPF shrimp; however, these viral genes were not among those highly expressed in infected shrimp, possibly indicating differences in viral pathogenesis in the two host systems. Further structural analysis of the WSSV proteins revealed signatures of known regulatory proteins, suggesting that these viral proteins may modulate host and/or viral transcription to affect viral latency and/or pathogenesis. These results of Khadijah and co-workers are promising in that they may lead to a better understanding of the establishment of viral latency in asymptomatic carriers and the molecular mechanisms of WSSV-induced mortality, which may ultimately enable researchers to develop therapeutic modalities to prevent viral outbreak.

To evaluate WSSV gene expression during infection in shrimp, Marks and colleagues (2005) printed an array containing nearly all of the putatively identified WSSV open reading frames (ORFs) based on the published sequences of WSSV-TH, WSSV-CN, and TH-96-II genomes. RNA was then isolated from the gill tissue of WSSV-TH-infected *P. monodon* at 0, 8, 20, 32, and 48 h post infection, and the expression of viral transcripts was monitored. Microarray results demonstrated that most of the WSSV-predicted ORFs (79%) are transcriptionally active, with transcription coordinately regulated in a classical cascade of "putatively early" and "putatively late" gene products. The "putatively early" transcripts reached maximal expression at 20 h post infection and included genes involved in nucleotide metabolism, DNA replication, and protein modification; while those of the "putative late" class reached maximal expression 48 h post in-

fection and were defined primarily by genes encoding major and minor virion structural proteins. Investigators also sought to characterize the transcriptomes of WSSV isolates WSSV-TH and TH-96-II. These isolates exhibit difference in virulence and are distinguished by the presence of two large genomic fragments in TH-96-II of ~5.3 kb and ~13.2 kb, encoding 10 additional ORFs that are absent in WSSV-TH. Microarray experiments conducted 2 days post infection in both *P. monodon* and in the crayfish *Astacus leptodactylus* showed that most genes encoded by the 13.2 kb fragment in TH-96-II are transcriptionally active. Furthermore, expression of most ORFs shared by the two viral isolates occurs at similar levels in the two crustacean species. Taken together, these results suggest the gene products of the added TH-96-II ORFs are not essential for infection, but are likely to have a functional role in both species. Transcription profiling has provided insight into the basic biology of WSSV. Future work aimed at testing the effect of drugs on the viral replication and gene expression will be important and again may elucidate targets for the development of effective treatments against WSSV.

The utility of cDNA microarrays in identifying candidate immune genes involved in WSSV pathogenesis has been demonstrated in recent studies (Wang et al. 2006; Robalino et al. 2007). Comparison of transcriptome profiles on healthy and virus-infected shrimp showed that WSSV infection broadly activates the expressions of many immune-related genes such as heat-shock proteins and antimicrobial peptides while compromising the expression of many other genes, such as those involved in antioxidation and signal transduction (Wang et al. 2006; Robalino et al. 2007). It is often difficult to determine the biological significance of the gene expression modulation from transcript-abundant data, particularly when there is only a limited set of probes available for microarray analysis. However, as large numbers of ESTs are generated from different tissues/organs involved in defense and metabolic processes, and more comprehensive microarrays are utilized, a network of genes involved in key pathways governing WSSV pathogenesis will emerge. This will help in identifying critical targets for developing therapeutics against WSSV and potentially other viral and bacterial diseases in shrimp.

The use of microarrays in shrimp aquaculture has been minimal, owing largely to the lack of com-

mercially available microarrays containing shrimp sequences. Biologically meaningful expression profiling has been performed across species using heterologous cDNA microarrays (Tsoi et al. 2003; Renn et al. 2004). Tsoi and co-workers (2003) demonstrated the feasibility of using human microarrays to facilitate the discovery of differentially expressed genes in Atlantic salmon liver during *Aeromonas salmonicida* infection. In another very elegant study, researchers showed quantitative functional analysis of heterologous hybridization across widely divergent fish species to a single microarray platform based on cichlid fish cDNAs (Renn et al. 2004). These types of strategies overcome some of the restrictions imposed by less-studied systems that are of importance but for which there is only limited sequence information.

Microarrays have accelerated our understanding of the complex molecular processes governing host pathogen interactions in mammalian systems and have led to improvements in the diagnosis, treatment, and prevention of infectious diseases (Bryant et al. 2004). Similar kinds of studies will continue to contribute to our knowledge of genetic evolution, the determinants of pathogenicity, and the complexities of the development and activation of innate immunity in shrimp. Global gene expression profiling may also be applied to facilitate our understanding of molecular adaptations that allow some shrimp to tolerate extremes in environmental conditions and/or certain stocks to be more resistant to bacterial infection or viral-induced mortality. The biological insights that can be gained from transcription profiling have broad applications not only for identifying molecular markers for shrimp breeding programs, but also for the improvement of future shrimp farming in terms of monitoring and maintaining the health and welfare of shrimp and the quality of shrimp products.

and in South America. With the increase in growth and potential, shrimp farming is also facing a growing number of challenges. These include the emergence of viral diseases, lack of an assured supply of genetically improved stocks, and concern over the use of unsustainable global resources (e.g., fishmeal and fish oil) in prepared diets, among many others. Developing genetic maps for shrimp and identifying markers for qualitative and quantitative traits will be very useful for developing shrimp breeding programs to address the above issues. So far, only very low-density linkage maps have been developed for three commercially important species of shrimp (*P. monodon*, *P. japonicus*, and *P. vannamei*). To develop high-density linkage maps to increase the genome coverage and eventually to use the resulting maps to identify genes governing qualitative and quantitative traits is going to take some time. Meanwhile, the reverse genetics approach could accelerate the process of identifying candidate genes that might be associated with qualitative and quantitative traits. Comparing the gene expression profiles of healthy and virus-infected animals, virus-resistant and virus-susceptible animals, high-growth and low-growth families, and families with differential growth characteristics will allow the identification of candidate genes and the molecular pathways that are involved in their processes. These candidate genes could be used as potential markers for developing genetically superior lines of shrimp and for developing therapeutics against viral and bacterial diseases, enhancing the profitability and sustainability of the shrimp farming industry.

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7.4 Future Perspectives

Over the past few decades, shrimp aquaculture has undergone a transformation from subsistence farming to a major industry providing jobs directly and indirectly to millions of people around the globe. Shrimp exports are a major source of foreign earnings for many countries with large ocean boundaries in Asia

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8 Oysters

Ximing Guo, Yongping Wang, Lingling Wang, and Jeong-Ho Lee

Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA, e-mail: xguo@hsrl.rutgers.edu

8.1 Introduction

Oysters are marine bivalves belonging to the families Ostreidae and Gryphaeidae (Bivalvia, Mollusca). They are benthic, sessile filter-feeders widely distributed in world estuaries. As filter-feeders and reef-builders, oysters are keystone species in many estuary ecosystems. Oysters can reach sexual maturity in 0.5–2 years and live for up to 20 years. Some species are protandric and dioecious, while others are simultaneous hermaphrodites. Fertilization is external and produces free-swimming larvae that can disperse over large distances. Hermaphroditic species usually brood larvae in the gill chamber.

Oysters are important aquaculture species. As a group, oysters account for a significant portion of world aquaculture production. In 2004, world aquaculture production of oysters amounted to 4.6 million metric tons, second only to the production of cyprinids (FAO 2006). The major oyster farming countries are China, France, Korea, the US, Japan, and Australia.

The exact number of extant oyster species is unknown, as many of the reported species may be synonymous (Carriker and Gaffney 1996). This is because oyster classification based on shell characteristics is unreliable. The true number of living oyster species may be between 40 and 60. Aquaculture production mainly comes from six species: the Pacific oyster (*Crassostrea gigas*), the eastern oyster (*C. virginica*), the Hong Kong oyster (*C. hongkongensis*), the Portuguese oyster (*C. angulata*), the European flat oyster (*Ostrea edulis*), and the Sydney rock oyster (*Saccostrea glomerata*). The first four species probably account for more than 90% of the total production.

The Pacific oyster, while native to northeastern Asia, has been introduced to and cultured in many countries in North and South America, Europe, and Africa. It is probably the most important cultured oyster by value. The eastern oyster is cultured on the Atlantic coast of North America and the Gulf of Mexico. The Hong Kong oyster is cultured in southern China. The Portuguese oyster is cultured in China and Europe.

Oyster farming has a long history that dates back 2,000 years in some regions. Despite its long history and large production, oyster farming remains technologically unsophisticated. Most of the oyster production today is still based on wild seed. Hatchery technologies for commercial production became available in the early 1960s (Loosanoff and Davis 1963) and are becoming increasingly important for some species and regions. The farming of the Pacific oyster outside its native range relies exclusively on hatchery-produced seed. The eastern oysters farmed along the Atlantic coast of the US are mostly hatchery produced.

Hatchery production of oyster seed opens the possibility of genetic improvement through traditional selective breeding as well as new genomic approaches. Selective breeding has been successful in improving the performance of oysters. In the eastern oyster, selective breeding has focused on resistance to two major diseases: MSX (multinucleated sphere X, caused by *Haplosporidium nelsoni*) and Dermo (caused by *Perkinsus marinus*) (Ford and Tripp 1996). Strong resistance to MSX and moderate resistance to Dermo are obtained after 4–5 generations of mass selection (Ford and Haskin 1987; Calvo et al. 2003; Guo et al. 2003). In the Pacific oyster, family-based selection and line crossing have led to improvements in yield (Langdon and Evens 2003). Breeding in the Sydney rock oyster has resulted in a significant reduction in time to market and dual resistance to winter mortality and the QX

(Queensland unknown) disease caused by *Marteilia sydneyi* (Nell and Perkins 2006).

Oyster breeding so far has been practiced without much knowledge of the genome (Guo 2004). Information about the genomic architecture of major economic traits and advanced genomic technologies may greatly enhance the efficiency of selection. Considerable effort has been invested in studying the genome of oysters. This chapter provides a review and discussion of recent advances.

8.2 The Oyster Genome

8.2.1 Chromosome Number

The oyster genome is small and represented by a low haploid number and a conserved karyotype. All *Crassostrea* oysters studied so far have a haploid number of 10 chromosomes (Nakamura 1985). In comparison, most clams and scallops have a haploid number of 19, and deviations from the modal number often involve chromosome losses. The fact that clams and scallops have nearly twice as many chromosomes as oysters, and that some can tolerate considerable chromosome loss, suggest that they may be ancient tetraploids derived from a whole-genome duplication event (Wang and Guo 2004). If the genome duplication hypothesis is true, oysters should represent the diploid lineage and have simple genomes that are not complicated by recent duplications. The hypothesis is in agreement with molecular data, which places oysters at a basal position in phylogenetic trees of bi-

valves (Adamkewicz et al. 1997; Giribet and Wheeler 2002).

8.2.2 Genetic Length

The oyster genome has a short genetic length. Genetic length is determined by the haploid chromosome number and the number of meiotic crossovers per chromosome. Theoretically, one crossover at a chromosome equals to 50 centiMorgan (cM) in genetic distance, as 50% of the progeny would be recombinants. Cytogenetic studies on meiosis find mostly only one chiasma per synapsed chromosome pair in oysters. The average crossover frequency in *C. virginica* is estimated to be 1.03 per chromosome in males and 1.16 per chromosome in females, which translates into total genetic lengths of 515 and 580 cM, respectively (Z. Wang and Guo unpublished; Table 1). Similarly, low crossover frequency has been observed in the Pacific oyster. The estimated genetic length for *C. gigas* is 525 cM for males and 615 for females (Table 1).

8.2.3 Genome Size

The oyster's genome size as measured in DNA content is among the smallest for all bivalve molluscs. According to published reports, the haploid DNA content (C-value) is 0.69 pg for *C. virginica* (Hinegardner 1974) and 0.91 pg for *C. gigas* (González-Tizón et al. 2000). An unpublished study using flow cytometry and two species (mice and zebrafish) as reference standards estimated the C-value for *C. virginica* as 0.753 pg and

Table 1 Properties of the eastern (*C. virginica*) and Pacific (*C. gigas*) oyster genomes

Parameters	<i>C. virginica</i>	<i>C. gigas</i>
Haploid chromosome number	10	10
Crossover per meiosis (male/female)	10.3/11.6	10.5/12.3
Expected genetic map length (cM)	515/580	525/615
Haploid DNA content (pg)	0.721	0.891
Haploid genome size (Mbp)	667	824
Location of major rRNA loci	2 p	10 q

that for *C. gigas* as 0.873 pg (K. Buono and Guo unpublished). The averages of the reported and our estimates, 0.721 pg for *C. virginica* and 0.891 pg for *C. gigas*, are represented in Table 1 as C-values for the two species. These C-values correspond to genome sizes of 667 Mbp for *C. virginica* and 824 Mbp for *C. gigas*. C-values have been estimated for 90+ bivalve molluscs, and they range from 0.65 to 5.4 pg (Gregory 2005).

8.3 Construction of Genetic Maps

8.3.1 Brief History

Foltz (1986) conducted the first linkage study in the eastern oyster by genotyping 11 allozyme loci in 10 full-sib families. Segregation analysis detected two linkage groups: one with four loci and the other with three. A similar study in the Pacific oyster with 14 allozyme loci also detected two linkage groups (McGoldrick and Hedgecock 1997).

Gene-centromere mapping of allozyme loci has been conducted in the Pacific oyster using gynogenetic diploids (Guo and Gaffney 1993). Gynogenetic diploids were produced by blocking the release of the second polar body (PB2) in eggs fertilized with ultraviolet-inactivated sperm (Guo et al. 1993). Gyno-

genetic diploids produced in this way offer an opportunity to map genes in relation to their centromeres. A crossover between a locus and its centromere followed by the retention of PB2 creates a heterozygote at the locus (half-tetrad). The proportion of heterozygotes provides a measure of recombination frequency and hence genetic distance from the centromere. The gene-centromere recombination frequency for the seven allozyme loci studied was high, averaging 0.74, suggesting that the distribution of crossovers is not random, but skewed toward the centromere. Random distribution would produce an average gene-centromere recombination frequency of 0.50.

The high gene-centromere recombination rate is not unusual for bivalves. In the dwarf-surfclam *Mulinia lateralis*, a similar study found that the gene-centromere recombination rate is 100% for most allozyme loci, suggesting there is always one and only one crossover occurring close to the centromere (Guo and Allen 1996). The high gene-centromere crossover frequency (or high proportion of heterozygotes) cannot be explained by selective mortality of homozygotes, as triploids gave the same estimates. These findings indicate that there is mostly one crossover per chromosome, which preferentially occurs close to the centromere. Such a biased distribution of crossovers may have important implications in genome mapping, because a genetic distance may not accurately predict physical distance.

Table 2 Type and number of genetic markers in the eastern oyster, *Crassostrea virginica*

Type	Number of Loci	Source
Allozyme	32	Buroker 1983
Restriction fragment length polymorphism (RFLP)	4	Karl and Avise 1992
Amplified fragment length polymorphism (AFLP)	396	Yu and Guo 2003; 2006
Single-strand conformation polymorphism (SSCP)	2	Yu and Guo 2003
Microsatellite (MS) – genomic	7	Brown et al. 2000
	1	Yu and Guo 2003
	18	Reece et al. 2004
MS – expressed sequence tags	8	Carlson and Reece 2007
	53	Wang and Guo 2007
	66	Wang et al. unpublished
Single nucleotide polymorphism (SNP)	46	Lee and Guo 2006; in prep.
	58	Zhang and Guo unpublished

8.3.2

First-Generation Maps

The lack of suitable genetic markers has been the main obstacle to mapping oyster genomes. For a long time, allozymes were the only type of genetic markers available for oysters (Buroker 1983; Table 2). Allozymes are not well suited for genome mapping as they are few in number and low in polymorphism. Several types of DNA markers can be used for genome mapping. They include restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), microsatellites (MS) and single nucleotide polymorphisms (SNPs) (Liu and Cordes 2004). AFLPs are popular markers for genome mapping in agricultural species, where the number of codominant markers is often limited. AFLPs combine the specificity of restriction sites and the ease of PCR-based assays (Vos et al. 1995). They were used to construct the first-generation maps for many aquaculture species (Liu 2007). The main advantage of AFLPs is the ease of obtaining a large number of markers quickly without prior knowledge of DNA sequences.

The first oyster genetic map was constructed in the eastern oyster using primarily AFLPs along with a few MS and type I markers (Yu and Guo 2003). The map was constructed with a “pseudo-testcross” family involving two parents from a stock that had been selected for disease resistance for about ten generations. Seventeen AFLP primer pairs were used, producing 282 segregating markers. Moderately dense male and female linkage maps were constructed using a backcross model. The male framework map consists of 114 markers spanning 647 cM. The female map has 84 markers covering 904 cM. Both maps have 12 linkage groups, two more than the haploid chromosome number. The observed genome coverage is about 84%. The female map is considerably longer than the male map, which is not unexpected. Six distorted markers formed a cluster on LG8 of the male map, suggesting that they are linked to a locus that affects larval survival in the eastern oyster (Yu and Guo 2003).

An AFLP-based genetic map has also been constructed for the Pacific oyster (Li and Guo 2004). The Pacific oyster map was constructed with a backcross family involving the Miyagi and Hiroshima strains: (Miyagi × Hiroshima) × Miyagi. The two strains have clear differences in shell and growth charac-

teristics (Imai and Sakai 1961). Three hundred and eighty-three AFLP markers were obtained and used for map construction. The female framework map consists of 119 markers in 11 linkage groups, spanning 1,030.7 cM, with an average interval of 9.5 cM per marker. The male map contains 96 markers in 10 linkage groups in accordance with the haploid number, covering 758.4 cM, with 8.8 cM per marker. The genome coverage is 82% for the female map and 81% for the male map. The female map has four clusters of distorted loci that are homozygote deficient, pointing to loci that are important for survival.

While AFLP markers are efficient markers, they are dominant and cannot be easily transferred among populations. Maps constructed with AFLPs alone may not be very useful unless codominant markers are added. The addition of codominant markers to the first-generation AFLP-based maps is essential.

8.3.3

Mapping with Codominant Markers

Codominant markers, such as MS and SNPs, are superior markers for genome mapping. MS are highly polymorphic and can easily be mapped in a given population. SNPs can be developed from any gene and offer the chance of identifying and mapping functional polymorphisms. Mapping with codominant markers has been a challenge in oysters, because there have been few codominant markers available. The development of codominant markers has been expensive and slow.

A large set of MS markers have been developed for the Pacific oyster (Li et al. 2003) and used for the construction of the first MS-based oyster map (Hubert and Hedgecock 2004). The MS map was constructed with 11-day-old larvae from three families. The use of larvae was intended to avoid segregation distortions, which are pervasive in oysters (Launey and Hedgecock 2001; Li and Guo 2004). The male linkage map consists of 88 loci in 10 linkage groups with a total genetic length of 616.1 cM. The female map has 86 loci and covers 770.5 cM. The average marker interval of the MS map is between 8 and 10 cM. As the map was constructed from the segregation in three families, differences in marker order have been observed

and taken as evidence for polymorphism in chromosomal rearrangement. Such an interpretation should be viewed with caution, because linkage mapping is a dynamic process. Any given order is not absolute but assigned with a probability. Even with the same data set, adding or removing one of the markers often causes changes in the estimated marker order.

In the eastern oyster, progress has been made on the development of codominant markers. More than 250 codominant markers are now available for this species (Table 2). They include 153 MS and 104 SNP markers. Most of the MS markers (127 of 153) are developed from expressed sequence tags (Carlsson and Reece 2007; Wang and Guo 2007). ESTs proved to be valuable resources for the development of MS markers. More than 200 MS-containing sequences are identified from a collection of 9,101 ESTs, of which 127 have been successfully converted to MS markers in the eastern oyster. Most of the EST-derived MS markers are located in the 5'UTR region and are highly polymorphic. Some are less variable and may be indels instead of true MS. Because they are part of or immediately adjacent to expressed genes, EST-derived MS are excellent markers for comparative genome mapping and for the mapping of functional genes.

SNPs are also excellent markers for genome mapping for two reasons. First, SNPs are highly abundant in the genome and can be developed for almost any gene. Second, SNPs are amenable to high-throughput genotyping using highly efficient array-based plat-

forms. In oysters, SNPs are especially abundant. The SNP frequency in the Pacific oyster is estimated to be one per 40 bp (Curole and Hedgecock 2005). In the eastern oyster, we sequenced seven gene fragments with a combined length of 1,691 bp in 19 individuals from diverse geographic origins. On average, we found 1.65 SNPs per 100 bp per individual, or one SNP per 61 bp (C Saout and Guo unpublished). By mining the EST database and resequencing, 104 SNPs have been developed for the eastern oyster (Lee and Guo 2006; L Zhang and Guo unpublished). Several methods for SNP genotyping have been tested, including single base extension, restriction enzyme digestion, tetra-primer amplification and T_m -shift. Among all these methods, the T_m -shift assay proves to be most reliable and cost effective. The T_m -shift assay is based on allele-specific amplification and melting curve analysis (Wang et al. 2005a). The allele-specific primers carry GC-rich tails of different length that produce T_m -shifts during the melting curve analysis (Fig. 1). It does not require labeled primers. It can be performed on real-time PCR systems with SYBR green. The cost can be reduced to less than \$0.30 per genotype if homemade assays are used.

Some of the newly developed MS and SNP markers have been added to the AFLP-based maps of the eastern oyster. The addition of 64 codominant markers to the AFLP-based maps has allowed the identification of homologies and the construction of an integrated genetic map. The integrated map of the eastern oyster

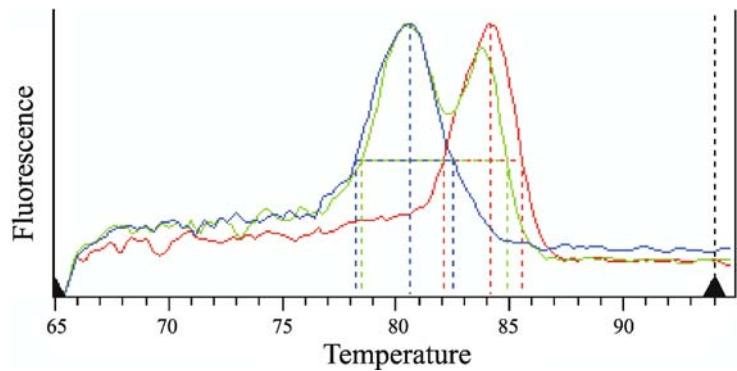
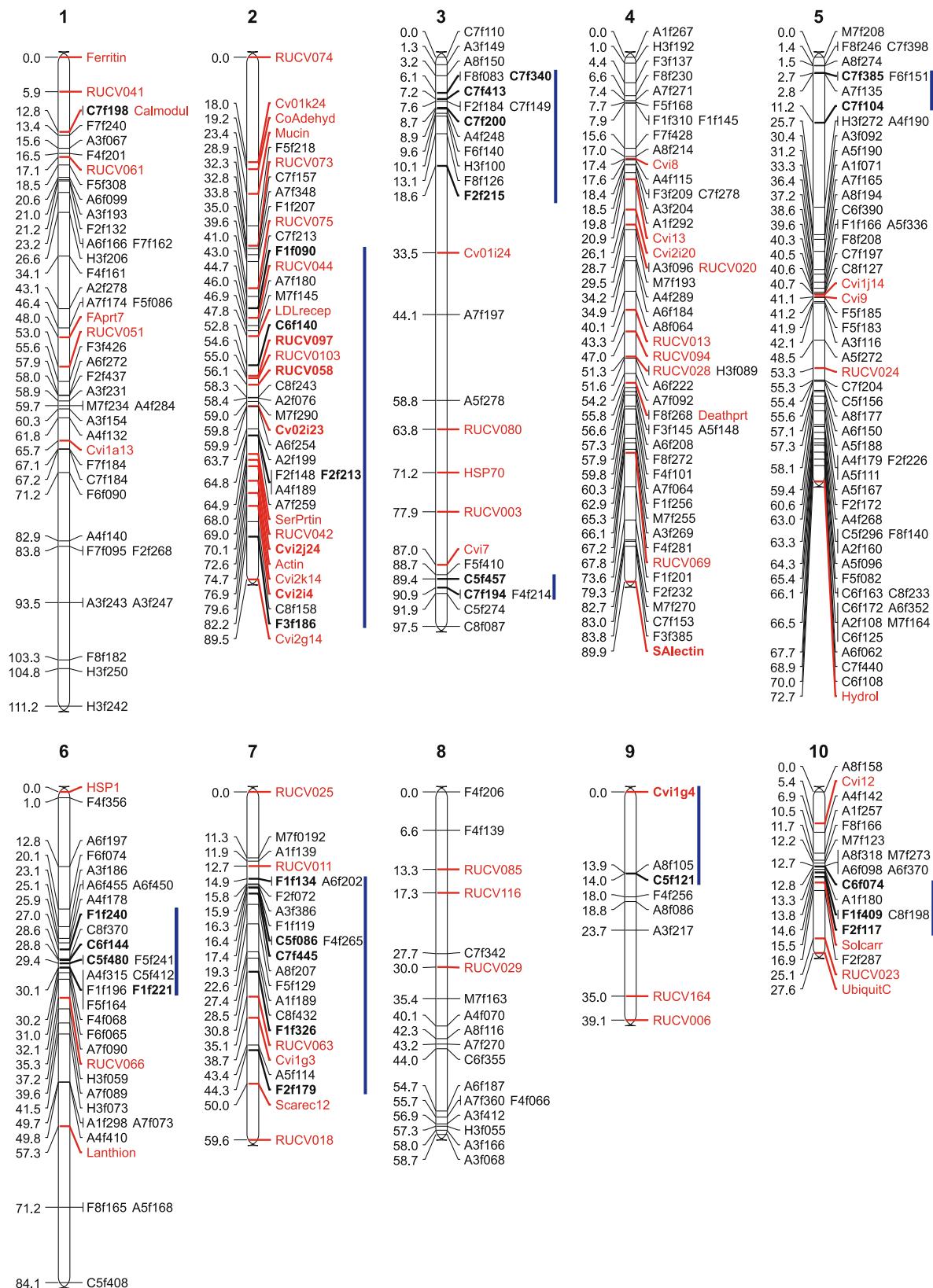


Fig. 1 SNP genotyping with melting curve analysis where two alleles are amplified with tails of different length, producing different melting temperatures. The blue and red curves are two homozygotes, and the green curve is a heterozygote

Fig. 2 An integrated genetic map of the eastern oyster with 249 AFLP, 47 MS, and 17 SNP markers. Codominant markers are in red, and markers showing significant frequency shifts after disease-caused mortalities are bold. Blue bars designate genomic regions containing disease-resistance genes



contains 313 markers, including 249 AFLPs, 47 MS, and 17 SNPs (Fig. 2). The map has ten linkage groups in accordance with the haploid number and a total genetic length of 729.9 cM. The average marker interval is 2.3 cM. It contains the mapping position of 47 genes and expressed sequences, represented by 17 SNPs and 30 EST-derived MS. The 64 codominant markers provide a framework for the map that should allow integration with future maps. More codominant markers are being added to the integrated genetic map.

The integrated eastern oyster map is based on the AFLP-based map of the DNE1 family (Yu and Guo 2006). DNE1 is an outcross family with a wild and a selected oyster as parents. Two other maps are being constructed with codominant markers and better designed backcross and F₂ families. The use of multiple families is important for the mapping of SNPs, which may not segregate in a given family. With the use of a large number of codominant markers, all these maps are being integrated for the construction of a high-density consensus map for the eastern oyster. The consensus map should have 250+ codominant and 250+ AFLP markers and a resolution of close to 1 cM.

While codominant markers are preferable, AFLPs can still play an important role in genome mapping. AFLPs are highly efficient markers and can be used to fill gaps and saturate maps at low cost, which is important when codominant markers cannot provide sufficient coverage. With limited resources, the best strategy is to use a combination of codominant and AFLP markers. The codominant markers can provide links among maps, while AFLPs help to saturate the map.

8.3.4 Comparative and Cytogenetic Mapping

Comparative mapping between the Pacific and eastern oysters has not been possible, as only a small fraction of the MS markers are transferable between the two species (Li et al. 2003). The continued development and mapping of type I markers should enable comparative mapping between the two oysters in the future. Comparative mapping may provide insights about how genome changes occurred, since the two species diverged.

At the present time, the only recognized difference between the two genomes is the size of the chro-

mosome carrying major ribosomal RNA genes. It is the second largest chromosome in the eastern oyster and the smallest chromosome in the Pacific oyster (Wang et al. 2004; Table 1). This difference holds true for all five Pacific species and three Atlantic species studied so far. Clearly, the divergence in the major rRNA-bearing chromosome occurred after the Atlantic–Pacific break, but before speciation within each ocean.

A preliminary cytogenetic map has been constructed for the eastern oyster using fluorescence in situ hybridization (FISH) of selected genes and DNA fragments (Wang et al. 2005b; Guo et al. 2007). The mapped loci include the major (18S-5.8S-28S) rRNA, the minor (5S) rRNA, an unknown sequence, and nine P1 clones (Fig. 3). They cover eight of the ten chromosomes. Additional BAC clones are being added to the cytogenetic map. Sequence tags and SNPs are being developed from the mapped loci for linkage mapping. The cytogenetic map can be used to map genes to chromosomes, anchor linkage and physical maps, and conduct comparative studies on chromosomal rearrangement.

8.4 Gene Mapping

The development of type I markers has facilitated the mapping of functional genes in the eastern oyster. Forty-seven type I markers have been placed on the integrated map (Fig. 2), providing genome locations of the genes they represent. The mapped markers include 17 SNPs and 30 EST-derived MS. Many of these genes are involved in host defense.

Host-defense genes have been targeted for marker development in the eastern oyster as part of our strategy of identifying disease-resistance genes. Putative host-defense genes are obtained by two ways. First, we have constructed suppression subtractive hybridization (SSH) libraries to identify oyster genes that are differentially expressed upon challenge with *P. marinus*. Sequencing and analysis of SSH libraries have identified 107 eastern oyster and 69 Pacific oyster genes that are up-regulated upon challenge with *P. marinus* (Tanguy et al. 2004). Most of these genes have been resequenced for SNP discovery. Secondly, we have searched the public database for genes that may

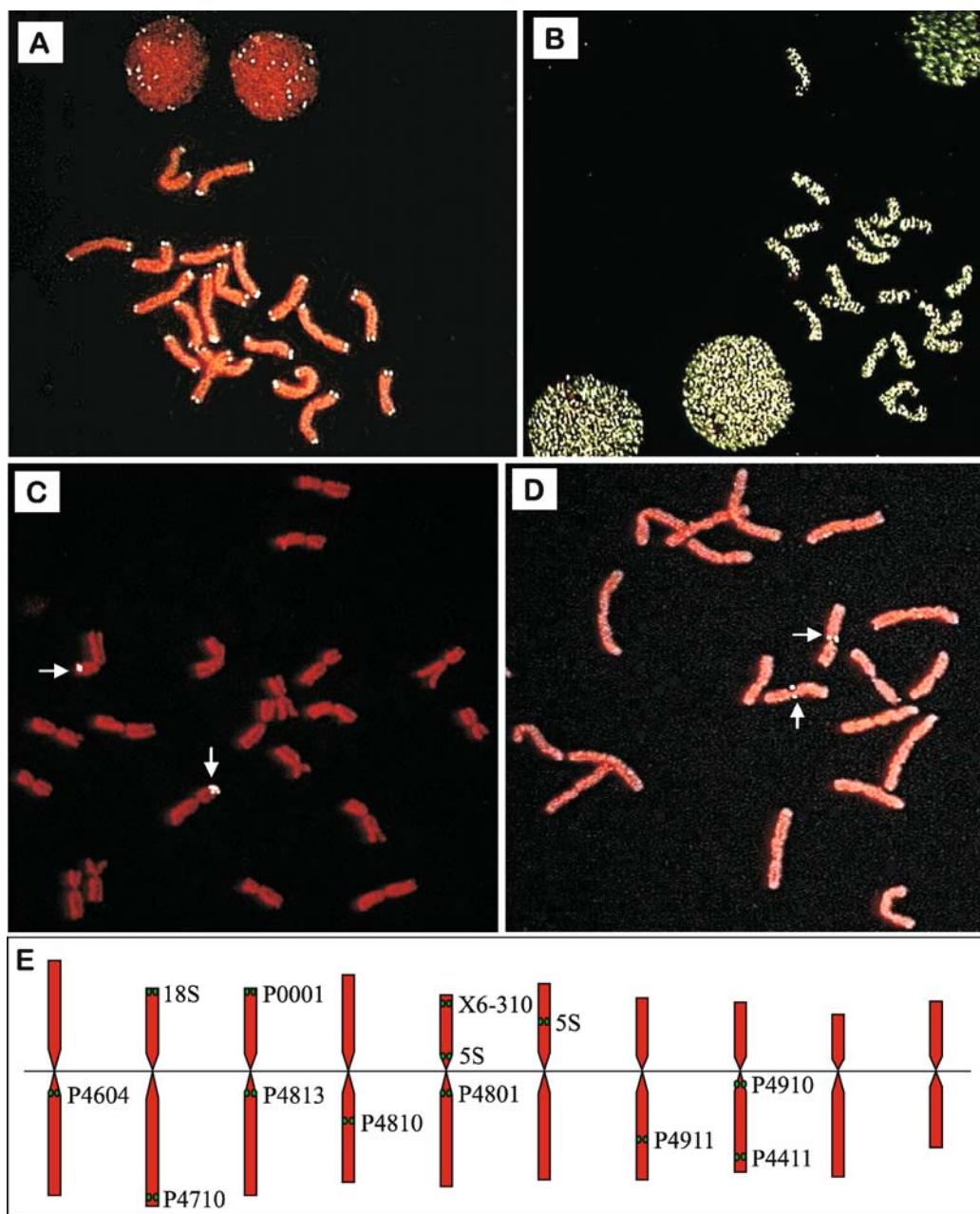


Fig. 3 Chromosomal mapping of DNA fragments in the eastern oyster with fluorescence *in situ* hybridization: A the vertebrate telomeric sequence, B an interspersed repetitive element, C the major rRNA genes, D a P1 clone, and E a preliminary cytogenetic map

be involved in host defense in other organisms. Some of these genes have multiple copies in the database that contains SNPs. Others are selected for resequencing and SNP development.

The mapped genes include ferritin, calmodulin, fatty acid binding protein, 3-hydroxyacyl-CoA de-

hydrogenase, mucin 5, LDL receptor, serine protease inhibitor 1, actin, three heat shock proteins, death-associated protein, sialic acid-binding lectin, hydrolase, lanthionine synthetase, scavenger receptor, solute carrier 3, ubiquitin C, tetraspanin protein (RUCV019), DUF614 protein (RUCV028), and other

genes of unknown functions (Fig. 2). Some of these genes are clearly important for host defense. Lectins and protease inhibitors are well-known host-defense genes (Kang et al. 2006). Ferritin may be important for defense against *P. marinus*. As an iron-binding protein, ferritin may sequester iron that is needed by the parasite for proliferation (Gauthier and Vasta 1994). Scavenger receptor, solute carrier 3, fatty-acid receptor, and ubiquitin may play a role in the transportation, endocytosis, destruction, and cleanup of foreign organic molecules that are often created following infections (Janeway et al. 2004). Some of the genes, such as heat shock proteins, may be involved in the general response to stress and diseases. The mapping of these proteins is important for the identification of disease-resistance genes in the eastern oyster. They may be interrogated as candidate genes if they are closely linked to a disease-resistance QTL. Variations at these genes may directly affect host defense or indirectly through linkage to regulatory elements nearby (Sutter et al. 2007).

8.5 QTL Mapping and Marker-Assisted Breeding

The identification and mapping of economically important QTLs is one of the main objectives of oyster genomics. QTL mapping is the first step toward marker-assisted selection. It may be argued that some of the traits, such as growth, can be effectively selected for without the assistance of markers. Nevertheless, an understanding of the genome structure of all production traits is important. For some traits, such as disease resistance, marker-assisted selection may be particularly effective.

8.5.1 Growth and Shell Characteristics

In the Pacific oyster, studies have been conducted to map growth heterosis QTL, shell color, and shell shape. Mapping in an F₂ family with 186 AFLPs and 51 MS has identified four QTLs affecting growth (Hedgecock et al. 2004; Curole and Hedgecock 2007). A single QTL has been identified for shell color, and two have

been identified as affecting shell shape (Curole and Hedgecock 2007).

8.5.2 Disease Resistance

In the eastern oyster, mapping efforts have focused on disease-resistance genes/QTL. Disease resistance is the most important trait for the eastern oyster farming industry. The eastern oyster is affected by three major diseases: MSX, Dermo, and JOD (caused by *Roseovarius crassostreae*) (Ford and Tripp 1996; Maloy et al. 2007). Any one of these diseases can cause up to 80–90% mortality in naive oysters. The development of a disease-resistant strain has been a top priority of the eastern oyster industry.

Selective breeding has produced strains that show strong resistance to MSX, but improvement in Dermo resistance has been slow. Currently, selection is based on field exposure, which becomes ineffective in years when the exposure is low or absent. The inability to maintain constant selection pressure presents a major challenge for breeding disease resistance in oysters. The problem can be solved by marker-assisted selection. If disease-resistance genes or markers are identified, they can be targeted in years when the diseases are absent. Even when diseases are present, disease-resistance markers can be used to increase selection pressure and efficiency.

There are several challenges in mapping disease-resistance genes in oysters. First, disease resistance is difficult to measure and quantify. Infection intensity may not be a good measure of resistance. Resistant oysters can be more tolerant to infections than susceptible ones. The only reliable measure of resistance is probably survival. Second, it is difficult to collect tissues from susceptible oysters, as death is discovered only when oysters gap and tissues are already rotten. Finally, there are no highly inbred and well-characterized lines needed for the construction of mapping populations.

To overcome these challenges, a family-based association mapping strategy has been used to map disease-resistance genes in the eastern oyster (Guo et al. 2004). In this approach, families are produced and deployed for field exposures to diseases (MSX cannot be artificially transmitted in the lab). Samples are collected and archived regularly. Two samples,

one immediately before and the other after disease-inflicted mortalities, are selected for genotyping. A large number of markers are screened in the before and after mortality samples. Markers that show significant frequency shifts after disease-inflicted mortalities are considered as affected markers. When two or more affected markers cluster closely together on the genetic map and all show frequency shifts in the same direction, they are considered as linked to a disease-resistance gene or QTL. The lack of well-defined inbred lines is compensated for by mapping in multiple families, so that most QTLs are detected.

Using the family-based association strategy, we identified ten disease-resistance QTLs in one family (DNE1): seven on the female map and three on the male map (Yu and Guo 2006). The addition of 64 codominant markers has integrated the two maps and consolidated the QTL into eight: two on LG3 and one each on LG2, LG5, LG6, LG7, LG9, and LG10 (Fig. 2). On LG6, for example, four affected markers are clustered within a 3 cM region, and all four markers show frequency shifts in the same direction in accordance with linkage phase. On LG2, nine affected markers, including four AFLPs and ten codominant markers, are clustered together. Some of the codominant markers are mapped in another family (HB4), showing the same pattern of clustering and frequency shifts after disease-caused mortalities. There is no question that there is at least one major disease-resistance gene on LG2. Based on the degree of frequency shift, Cvi2i23 is the most strongly affected marker and likely the closest to the resistance gene (Fig. 2).

The clustering of the affected markers is not an artifact caused by segregation distortion. The mapping analysis was conducted with JoinMap 4.0 using independence LOD scores, which are not affected by segregation distortion. The clustering was observed in the before-mortality sample when there was no segregation distortion, suggesting that these loci are truly linked to each other. The markers in the cluster are affected by disease-caused mortalities because of their close linkage to a major disease-resistance gene.

8.5.3 Marker-Assisted Breeding

QTL mapping is at its early stages in oysters. There has been no marker-assisted selection yet. The mapped

QTLs need to be validated and mapped at high resolutions before marker-assisted selection can occur. Association studies are being conducted for the validation and fine-mapping of the disease-resistance genes in the eastern oyster. Markers have been used for parentage assignment and monitoring inbreeding which are important in oyster breeding (Curle and Hedgecock 2007).

8.6 Advanced Genomic Resources

8.6.1 Physical Mapping

BAC libraries have been constructed for the eastern and Pacific oysters (Cunningham et al. 2006). The Pacific oyster library consists of 73,728 clones with an average size of 152 kb. The eastern oyster library contains 55,296 clones with an average insert size of 150 kb. Both libraries have a genome coverage of 11.8 \times . These BAC libraries are valuable resources for oyster genomics. They are essential for physical and chromosome mapping, genome sequencing, positional cloning of genes and their regulatory elements, and some other genomic studies. A physical map is being constructed for the Pacific oyster using BAC fingerprinting (P. Gaffney, pers. comm.).

8.6.2 ESTs and Microarrays

ESTs are valuable resources for gene discovery and the development of genetic markers. The number of oyster ESTs has been increasing steadily, although it still lags behind other major aquaculture species. Currently, GenBank has 5,315 ESTs for the Pacific oyster and 14,650 for *C. virginica* (as of September 10, 2007). These ESTs and the oyster transcriptome have been partially characterized in several studies (Jenny et al. 2002; Gueguen et al. 2003; Peatman et al. 2004; Quilang et al. 2007; Tanguy et al. 2004). The *C. virginica* ESTs have been used for the development of MS and SNP markers (Lee and Guo 2006; Wang and Guo 2007). The number of available ESTs remains inadequate for either species. Further efforts are needed to significantly increase the oyster EST database.

A cDNA microarray has been constructed for the eastern and Pacific oysters (Jenny et al. 2007). The microarray has 27,496 features, representing 4,460 ESTs from the eastern oyster, 2,320 from the Pacific oyster, and 16 non-oyster DNAs as controls. Although the coverage is low, this is the first oyster microarray, and it will undoubtedly open new areas of research in oyster genomics. An oligo-based microarray is under construction for the eastern oyster that may provide higher gene coverage (Z. Liu, pers. comm.).

8.6.3 Whole-Genome Sequencing

A case for sequencing the genome of the Pacific oyster has been made by a consortium of oyster researchers (Hedgecock et al. 2005). The Pacific oyster has been proposed as a candidate for genome sequencing because of its importance as an aquaculture species worldwide. A similar case can also be made for the sequencing of the eastern oyster genome. The genome of the eastern oyster is 20% smaller than that of the Pacific oyster. The eastern oyster is economically and culturally important for the United States. Diseases have devastated eastern oyster populations along the mid-Atlantic coast, causing serious economic and ecological damages. A genome sequence for the eastern oyster would greatly advance research and development in many areas. So far, the genome of only one mollusc, the gastropod owl limpet *Lottia gigantea*, has been sequenced (Chapman et al. 2007). The sequencing of the oyster genome, a bivalve, would provide a better representation of Mollusca.

The exceptionally high levels of polymorphism in oysters may pose a challenge to sequence assembly. The problem can be overcome by sequencing relatively inbred individuals and with the aid of physical and genetic maps. With the advent of the new sequencing technologies, sequencing the oyster genomes may no longer be prohibitive. Eventually, the genomes of both oyster species should be sequenced for comparative analyses.

8.7 Conclusions and Future Prospects

Considerable progress has been made in oyster genomics. Now, we have hundreds of codominant markers, basic genetic maps, an expanding EST database,

BAC libraries, and a cDNA microarray. QTLs have been identified and mapped for some economically important traits, including growth and disease resistance. A physical map for the Pacific oyster is under construction. Still, oyster genomics faces some challenges and limitations in resources. First, the lack of well-characterized inbred lines continues to limit QTL mapping efforts. Work is needed to establish inbred lines with unique traits such as fast or slow growth, resistance or susceptibility to specific diseases. These lines are essential for the construction of reference populations needed for QTL mapping. Second, the number of ESTs currently available is grossly inadequate. Several hundred thousand oyster ESTs are needed for a reasonable coverage of the transcriptome, for gene discovery, marker development, and the development of high-density microarrays. Finally, we need to adopt SNPs as the next generation markers. The development of an SNP array that offers deep genome coverage would be most valuable for future QTL mapping and genome-wide association studies.

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