Shaojun Liu *Editor*

Fish Distant Hybridization

Science Press
Beijing

Springer

Fish Distant Hybridization

Shaojun Liu
Editor

Fish Distant Hybridization

Science Press
Beijing

Springer

Editor
Shaojun Liu
State Key Laboratory of Developmental
Biology of Freshwater Fish, College of
Life Sciences
Hunan Normal University
Changsha, Hunan, China

ISBN 978-981-16-5066-6 ISBN 978-981-16-5067-3 (eBook)
https://doi.org/10.1007/978-981-16-5067-3

Jointly published with Science Press
The print edition is not for sale in China (Mainland). Customers from China (Mainland) please order the print book from: Science Press.

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

More than 32,000 fish species have been found in nature representing the largest group of vertebrates. Intriguingly, the numbers of chromosomes in many fishes generally increase by double which provides important clues for the investigation of the fish hybridization, polyploidization, and diversity of chromosomes. These fish species also provide abundant natural resources to study fish distant hybridization and breeding.

Distant hybridization is defined as crosses between two different species or higher-ranking taxa. By distant hybridization, two different species can be combined, accelerating the variations both in genotype and phenotype in the offspring of the distant hybridization. If these variations are inheritable, new fish lineages or even new species can be formed. Researches on distant hybridization pose important significances on heredity, breeding, and biological evolution. Are the abundant and varied fish resources in nature related to distant hybridization? What are the biological traits of progenies from distant hybridization? What are the rules regarding the genetics and reproduction biology of the distant hybridization? In plants, Mendel's laws provide an important genetic basis for intraspecific hybridization. However, the phenomena described in Mendel's laws rarely appear in distant hybridization. So, some unknown rules including the genetic rule and reproduction rule in distant hybridization deserve to be explored.

Based on a large number of fish distant hybridization experiments, we summarize the rules of fish inheritance and reproduction, and further establish one-step and multistep breeding technologies that are suitable for interspecific hybridization and intraspecific hybridization. This book contains 12 chapters. We review the progress of animal distant hybridization and polyploids. In addition, we also introduce the results regarding fish distant hybridization and polyploid fishes through the long-term and systematic investigation. Via designing and performing fish distant hybridization by considering the parents' traits such as chromosome numbers, phylogenetic relationship, fertility, appearances, feeding habits, growth rates, and stress resistance, we have generated a series of important fish lineages derived from distant hybridization. For example, we have produced allotetraploid lineage (F_3–F_{29}) derived from red crucian carp $\times$ common carp, autotetraploid lineage (F_2–F_{15}) derived from red crucian carp $\times$ blunt snout bream, allodiploid lineage (F_1–F_6) derived from blunt snout bream $\times$ topmouth culter, allodiploid lineage (F_1–F_3)

derived from topmouth culter × blunt snout bream, crucian carp-like homodiploid lineage (F_1–F_7) and autotetraploid lineage (F_2–F_5) derived from common carp × blunt snout bream, red crucian carp-like homodiploid lineage (F_1–F_3) and goldfish-like homodiploid lineage (F_1–F_2) derived from koi carp × blunt snout bream, autodiploid lineage (F_2–F_5) derived from Japanese white crucian carp × blunt snout bream, and allodiploid lineage (F_1–F_5) derived from Japanese white crucian carp × red crucian carp. In addition, we have generated the sterile triploid fish derived from the allotetraploid fish × diploid fish, and the triploid fish have fast growth performance, strong stress resistance, and other superiorities, which have been widely farmed and brought significant economic, social, and ecological benefits. Furthermore, we have generated up-mouth bream hybrid fish derived from backcross of the blunt snout bream × topmouth culter hybrid lineage with blunt snout bream, which have advantages in growth performance, strong stress resistance, and high-quality meat.

We have established the diploid gynogenetic hybrid clone lineage, diploid androgenetic hybrid clone lineage, and the improved tetraploid lineage derived from red crucian carp × common carp as well as the improved diploid crucian carp and improved triploid Xiangyun crucian carp II. Additionally, we have generated the gynogenetic red crucian carp, gynogenetic Japanese white crucian carp, gynogenetic goldfish, and gynogenetic blunt snout bream using sperm from different species.

On the basis of successfully producing a series of fishes with different ploidy levels, the book introduces the basic research results of these fishes at individual, organism, cell, and molecular levels, including the results of fish appearances, DNA contents, karyotypes, cytology of fertilization, embryonic development, growth development, meat quality, intermuscular bone, structures of pituitary, endocrine characteristics, nuclear genomes, mitochondrial genomes, mRNA expressions, and protein expressions. The formations of the allotetraploid lineage, autotetraploid lineage, allodiploid lineage, and natural gynogenetic diploid lineage derived from the distant hybridization provide important evidence for demonstrating these pipelines that can generate new lineages or even new species. In these fishes derived from the distant hybridization, the critical biological phenomena such as production of unreduced gametes, gene variations, and gene recombinations in the progenies have been found.

The reproductive traits in fish distant hybridization are summarized. For example, the unreduced gametes, including the diploid and triploid gametes derived from the hybrids of fish distant hybridization, are produced, which result in the formation of the tetraploid lineages. On the other hand, the reduced haploid gametes, derived from the hybrids of fish distant hybridization, are produced, which result in the formation of the diploid lineages.

The genetic rules in fish distant hybridization are elucidated. For example, when the number of maternal chromosomes is equal (or almost equal) to the number of paternal chromosomes, the F_1 hybrids can easily survive, and the allotetraploid lineages and allodiploid lineages can be established. When the number of the maternal chromosomes is significantly larger than that of the paternal chromosomes, autotetraploid lineages and autodiploid lineages can be established. When the

number of the maternal chromosomes is significantly fewer than that of the paternal chromosomes, it is difficult for the F_1 hybrids to survive.

Furthermore, one-step breeding technology and multistep breeding technology are established to produce a series of improved fish. For example, via one-step breeding technology, the hybrids with a high survival rate, strong resistance, and fast growth rate are obtained from Japanese white crucian carp × red crucian carp. Via multistep breeding technology, improved hybrids are produced by crossing the female of the Japanese white crucian carp (♀) × red crucian carp (♂) lineage with the male Japanese white crucian carp, which have the advantages such as higher body height and faster growth rate.

Combined with figures, this book presents a comprehensive context and can be used as a reference book for the research in fish genetic breeding, aquaculture, genetics, developmental biology, zoology, and animal evolution. If shortcomings exist in the book, we sincerely look forward to receiving comments and advices from readers and peers.

Hunan, China
October 8, 2020

Shaojun Liu

Book Review I

Fish is the largest group of vertebrates, and their genetic composition and reproductive behaviors are very diverse. It is known that distant hybridization and chromosomal polyploidy induction are feasible in plants and have been widely used for breed improvement. For example, the triploid seedless fruits have been widely welcomed by consumers. In general, distant hybridization and polyploidy-induction breeding are difficult to achieve in vertebrates. However, these restrictions have been broken in fish and they are successfully applied to aquaculture, with one of the outstanding contributions from Professor Shaojun Liu at Hunan Normal University, Changsha city, Hunan province, China.

Professor Shaojun Liu successfully carried forward his father Yun Liu's academic legacy by investigating in depth the distant hybridization and polyploid-induction breeding of farmed fish to a whole new stage. Professor Shaojun Liu and his team have used techniques such as distant hybridization and gynogenesis to alter the karyotypes of farmed fish with different ploidy. This monograph records their theoretical findings and practical experiences in detail. With the application of various physical and chemical factors to fertilized fish eggs, they successfully induced and then selected a number of lineages of tetraploid and diploid fish. These new lineages enriched resources of the farmed fish germplasm with potential for breeding. They have used these new lineages to produce improved triploid and diploid farmed fish. Theoretically, they systematically studied biological mechanisms, at chromosomal level, of the formation of new strains of distant hybrid fish as well as biological characteristics of fish with altered karyotypes, including genetic characteristics, reproductive behavior, and growth performance. They elucidate the genetic rules regarding the formation of the tetraploid and diploid fish lineages derived from distant hybridization. They also demonstrate the reproductive rules regarding the unreduced and reduced gametes, which resulted in the formation of the tetraploid and diploid fish. Furthermore, they establish one-step and multistep breeding strategies, which are used to produce lineages of farmed fish with improved farming traits. It is because of these outstanding achievements that Professor Shaojun Liu was elected to the Chinese Academy of Engineering in 2019. In addition, this book provides a comprehensive review of advances in animal studies of distant hybridization and polyploidy technology.

In summary, this is a book with distinctive features in animal genetics and breeding, and reproductive biology, which is hereby recommended to researchers and students engaged in animal breeding and reproductive biology.

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

Zuoyan Zhu

Peking University
Beijing, People's Republic of China
September 6, 2020

Book Review II

Fish is the largest group in vertebrates and the numbers of chromosomes in many fishes are in ploidy-level relationship. Is this relationship associated with distant hybridization? This question is worth studying. Via designing the crosses of fish distant hybridization, Professor Shaojun Liu and his research team have obtained fertile tetraploid and diploid fish lineages, providing suitable research models and systematic platform for investigation of distant hybridization, which have significance in fish genetic breeding and biological evolution.

Professor Shaojun Liu and his colleagues have spent more than 30 years on developing different ploidy fishes by distant hybridization and chromosomal ploidy manipulation. They have obtained a large amount of innovative results showing academic significance and application prospect in basic and applied research in the field of distant hybridization. In this field, they have carried out systematic and effective experiments to establish the fertile tetraploid and diploid fish lineages derived from distant hybridization, which are used as the new germplasm resources to produce improved triploids and diploids. They elucidate the genetic rules at the chromosome level for the formation of the tetraploid and diploid lineages derived from fish distant hybridization. They also demonstrate the reproductive rules on the occurrence of the unreduced and reduced gametes, which result in the formation of the tetraploid and diploid lineages derived from fish distant hybridization. On the other hand, they establish the one-step breeding technology and multistep breeding technology used to produce a series of improved fish.

I am extremely impressed by Professor Liu's persistence in studying fish distant hybridization and polyploid fishes. He enjoys a high level of popularity in this research area because of his profound scientific attainments in fish genetic breeding. The tireless pursuit of genetic breeding prompts him to finish this book.

This book summarizes research progress of animal distant hybridization and polyploid organisms. It also provides a detailed description of the fruitful results yield by Liu's scientific research team during long-term and systematic researches of fish distant hybridization. The book consists of exquisite pictures and detailed descriptions, which is an outstanding scientific work on fish distant hybridization. It provides theoretical and practical guidance for animal genetic breeding.

Here, I sincerely recommend *Fish Distant Hybridization* to scientists and students who engage in aquaculture and animal genetic breeding.

School of Life Sciences, Sun Yat-Sen University Haoran Lin
Guangzhou, People's Republic of China
September 10, 2020

Contents

The Research Advances in Animal Distant Hybridization and Polyploid Organisms

1

Shaojun Liu, Shi Wang, Qingfeng Liu, Chang Wu, Yi Zhou, Min Tao, Chun Zhang, Qinbo Qin, and Kaikun Luo

Abstract

Distant hybridization leads to genetic changes which contribute to rapid evolution and heterosis for higher growth rates, better adaptability, and stronger disease resistance in offspring. Some studies show that widespread distant hybridizations occur in animals in nature. Hybridization events bring new genetic diversity for animal populations which are favorable for genetic breeding. After hybridization, phenotypic variations are driven by interaction of subgenomes such as genetic recombination. In addition, the new genotypes provide possibility for speciation. Simultaneously, polyploidization derived from the distant hybridization is widespread in natural organisms including autopolyploid and allopolyploid. Distant hybridization can generate new lineages including the diploid lineages and tetraploid lineages. Through distant hybridization, a series of diploid and polyploid hybrid fish lineages have been generated, and they can be used as the new germplasm resources to produce the improved fish, which have great significance in biological evolution, genetics, and breeding. In this chapter, we give a brief introduction to animal distant hybridization and polyploid organisms.

Keywords

Distant hybridization · Polyploid · Breeding · Speciation · Fish · Genome duplication

S. Liu (✉) · S. Wang · Q. Liu · C. Wu · Y. Zhou · M. Tao · C. Zhang · Q. Qin · K. Luo
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_1

1.1 The Research Advances in Animal Distant Hybridization

Distant hybridization is defined as a crossing between two different species or higher-level taxa, which can promote the transfer of genomes between species and lead to phenotypic and genotypic changes in progeny. In terms of genotypes, distant hybridization can lead to changes in genetics, such as the production of new diploid, triploid, and tetraploid. At the level of genomic DNA, distant hybridization leads to changes in DNA recombination, deletion, and insertion in offspring. With regard to phenotypes, distant hybridization can produce heterosis with higher growth rate, better adaptability, as well as stronger disease resistance (Liu 2010).

A number of fertile and stable allopolyploid plants were obtained by interspecific hybridization, including allotetraploid *Raphanobrassica* derived from chromosome doubling and allohexaploid wheat (*Triticum aestivum*) generated by hybridization (Liu 1991). Researchers thought that gibel carp (*C. gibelio*) has undergone several rounds of genome polyploidy and experienced an additional, more recent genome duplication event. In addition, gibel carp are under an evolutionary trajectory of diploidization (Gui and Zhou 2010). Under natural conditions, diploid, triploid, and tetraploid crucian carp have been confirmed to exist (Xiao et al. 2011). More and more studies have shown that the evolution of polyploid fish is related to distant hybridization (Meyer et al. 2006; Saitoh et al. 2010; Nolte et al. 2005). Distant hybridization has been shown to favor speciation and evolutionary radiation. The allopolyploidization caused by distant hybridization is a potential driving force for the occurrence of new species (Grant et al. 2005; Mallet 2007). The progeny of distant hybridization contains genomes from different species, and the interaction of two genomes provides a basis for gene recombination in offspring. The formation of fertile distant hybrid lineages may create new variations and even new species.

In Sect. 1.1 of Chap. 1, we provide an overall introduction and review of distant hybridization in animals, especially in the formation of fertile distant hybrid lineages and their characteristics of genetic variation. The applications of distant hybrid lineages in aquaculture are also introduced. This overview can provide a useful overall reference for the in-depth study of animal hybridization (including fish hybridization) in the future.

1.1.1 The Animal Distant Hybridization Profile

1.1.1.1 Hybridization Between Phyla

Hybridization between different phyla has been found in several hybrids, including sea urchin (*A. crassispina*, ♀) from Echinodermata × mollusk (*Acmaea nanshaensis*, ♂) from Mollusca, sea urchin (♀) × blue mussel (*Mytilus edulis*, ♂) from Mollusca, and sea urchin (♀) × annelid (*Nereis succinea*, ♂) from Annelida. The larvae of all three kinds of hybrids were similar to those of sea urchins. Although sperm from the paternal parent entered into the egg, the fusion of distant male and female nuclei was difficult. Even if the phenomenon of fusion has occurred, the chromosomes from paternal parent were eventually degraded and not merged into

the nuclei of the offspring. Therefore, these hybrid larvae resembled the larvae of sea urchin, the maternal parent (Zhu 1961).

1.1.1.2 Hybridization Between Classes

Interclass hybridization has been observed in Echinodermata between sea urchin (♀) and *C. hesperus* (♂), and their larval offspring were similar to the maternal parent (Loeb 1903).

1.1.1.3 Hybridization Between Orders

Successful hybrid fry were generated by crossing between blunt snout bream (*Megalobrama amblycephala*, ♀) from Cypriniformes and Chinese perch (*Siniperca chuatsi*, ♂) from Perciformes. Hybrids between silver carp (*Hypophthalmichthys molitrix*, ♀) from Cypriniformes and red seabream (*Pagrosomus major*, ♂) from Perciformes were reported.

1.1.1.4 Hybridization Between Families

Hybridization between families has been reported between blue tilapia (*Oreochromis aureus*, ♀) and Chinese perch (*Siniperca chuatsi*, ♂). The survival rate of fry was as low as 0.3–0.5% recorded (Yu et al. 2003b; Yang et al. 2004).

Among amphibians, *Hyla arborea* (♀) in southern France could cross with *Pelobates cultripes* (♂). However, only a few of these hybrids could undergo metamorphosis. In addition, these hybrids had similar appearances to their maternal parent, *Hyla arborea*. Hybridization has also been reported between *Hyla arborea* (♀) and *B. calamita* (♂). In this case, the paternal parent chromosomes were eliminated and did not involve in embryonic development. However, relatively good development of hybrid progeny was subsequently found (Zhu 1934). In contrast, most embryos from the hybridization of *B. calamita* (♀) × *Hyla arborea* (♂) died during the blastocyst and gastrula stages. Only a handful of the tadpoles could pass through metamorphosis, and these individuals were all female (Zhu 1961).

These hybridizations between different phyla, classes, orders, or families have not been found to simultaneously generate fertile females and males in the F_1 generation. Thus, it is not possible to obtain self-crossing lineages using these hybrids. In this book, the term distant hybrid lineage refers to subsequent generations that derived from self-mating of F_1 hybrids.

1.1.1.5 Hybridization Between Subfamilies

In fish, several hybridizations have been reported between different carp subfamilies: bighead carp (*Hypophthalmichthys nobilis*, ♀) × blunt snout bream (♂) and blunt snout bream (♀) × bighead carp (♂); bighead carp (♀) × grass carp (*C. idella*, ♂) and grass carp (♀) × bighead carp (♂) (Guo et al. 1966); grass carp (♀) × blunt snout bream (♂) (He et al. 2013); grass carp (♀) × silver carp (♂) and silver carp (♀) × grass carp (♂); grass carp (♀) × common carp (*C. carpio*, ♂) (Ye et al. 1989); grass carp (♀) × black Amur bream (*Megalobrama terminalis*, ♂) (Liu 1987a, b); black carp (*Mylopharyngodon piceus*, ♀) × black Amur bream (♂) (Liu et al. 1981;

Chen 1984); common carp (*Cyprinus carpio* var. *xingguonensis*, ♀) × grass carp (♂) (Wu et al. 1981; Liu et al. 1987; Wu et al. 1988); silver carp (♀) × blunt snout bream (♂) and blunt snout bream (♀) × silver carp (♂) (Fisheries experimental station of Beijing 1973; Pan 1987; Zhu et al. 1993); silver carp (♀) × Bleeker's yellow tail (*Xenocypris davidi*, ♂); and common carp (♀) × silver carp (♂) and silver carp (♀) × common carp (♂) (Gui et al. 1993; Wang and Liu 1986).

Studies in red crucian carp (*Carassius auratus* red var., ♀) × blunt snout bream (♂) (Liu et al. 2007b), blunt snout bream (♀) × Bleeker's yellow tail (♂) (Hu et al. 2012), and common carp (♀) × blunt snout bream (♂) (Wang et al. 2017) demonstrated that distant hybrid lineages could be generated by hybridization between subfamilies. Additionally, autotetraploid fish lineage with genetic variation characteristics was obtained by the crossing of red crucian carp (♀) × blunt snout bream (♂) (Qin et al. 2014) and hybridization of common carp (♀) × blunt snout bream (♂) (Wang et al. 2020b).

1.1.1.6 Hybridization Between Genera

Examples of intergeneric hybrids in birds included western capercaillie (*Tetrao urogallus*, ♀) × black grouse (*Lyrurus tetrix*, ♂) (Zhu 1961), chicken (*Gallus gallus*, ♀) × common pheasant (*Phasianus colchicus*, ♂) (Zhu 1961), helmeted guineafowl (*Numida meleagris*, ♀) × Indian peafowl (♂) (Zhu 1961), helmeted guineafowl (*Numida meleagris*, ♀) × chicken (♂) (Zhu 1961), chicken (♀) × Indian peafowl (*Pavo cristatus*, ♂) (Zhu 1961), and chicken (♀) × coturnix (*Coturnix coturnix*, ♂) (Yu et al. 2003a).

Antelope-cattle hybridization is an example of crossing between different genera of mammals. Goats (*Capra hircus*) and sheep (*Ovis aries*), which belong to different genera, can form hybrids through natural insemination or artificial insemination. For example, a living hybrid was obtained by the crossing of goat (♀) × sheep (♂) in France in 1948 (McGovern 1973).

Successful crossing between camel (*Camelus ferus*) and the South American llama (*Lama glama*) was achieved by artificial insemination. The hybrid was created at a Dubai Camel Breeding Center in 1995. The length of the hybrid's ear was intermediate between those of the parents. The hybrid, similar to the camel, had a long tail and strong legs adapted for desert walking. In contrast, the hybrid, similar to llama, lacked a hump and had fluffy hair.

Examples of hybridization between fish genera include red crucian carp (♀) × common carp (♂) (Liu et al. 2001b; Liu 2010; Sun et al. 2003), gibel carp (♀) × common carp (♂) (Jiang et al. 1983), silver carp (♀) × bighead carp (♂) and bighead carp (♀) × silver carp (♂) (Zhang et al. 1979), white Amur bream (*Parabramis pekinensis*, ♀) × black Amur bream (♂) (Department of biology SU, Taiyuan agriculture fawcss 1973), blunt snout bream (♀) × white Amur bream (♂) (Lin 1984), black carp (♀) × grass carp (♂), goldlined seabream (*Rhabdosargus sarba*, ♀) × red seabream (♂) (Jiang et al. 1997; Qu et al. 2000), yellowfin seabream (*Sparus latus*, ♀) × goldlined seabream (♂) (Qu et al. 2000), goldlined seabream (♀) × blackhead seabream (*Sparus macrocephalus*, ♂) (Qu et al. 2000), small-scale yellowfin (*Plagiognathops microlepis*, ♀) × Bleeker's yellow tail (♂) (Zhang et al.

1990), and mud carp (*Cirrhinus molitorella*, ♀) × *Sinilabeo decorus tungting* (♂) (Zhang et al. 1984).

Hybrid F_2 and subsequent generations from the distant hybridization of red crucian carp (♀) × common carp (♂) (Liu et al. 2001b), common carp (♀) × red crucian carp (♂) (Wang et al. 2020b), blunt snout bream (♀) × topmouth culter (*Culter alburnus*, ♂) (Xiao et al. 2014), and topmouth culter (♀) × blunt snout bream (♂) (Ren et al. 2019) had been obtained. Further, an allotetraploid hybrid lineage from red crucian carp (♀) × common carp (♂) was generated.

A research team led by Professor Sifa Li at Shanghai Ocean University obtained fertile F_1 hybrids by using the distant hybridization between reciprocal crosses of GIFT tilapia *Oreochromis niloticus* (♀) × *Sarotherodon melanotheron* (♂). The F_2 offspring could be generated from the F_1 hybrids.

1.1.1.7 Interspecific Hybridization

Chlamys farreri (♀), a type of scallop, was crossed with *Chlamys nobilis* (♂) to obtain an F_1 hybrid (Penglai Red) (Liu et al. 2006). Cytological studies on the fertilization process of the hybridization between *Patinopecten yessoensis* (♀) and *Chlamys farreri* (♂) showed that the fertilization effect of reciprocal crosses was normal. Therefore, heterologous sperm not only activated the egg but also contributed to the genetic composition (Yang et al. 2002a).

In fish, examples of interspecific hybridization include Mozambique tilapia (*Oreochromis mossambicus*, ♀) × Nile tilapia (*Oreochromis niloticus*, ♂) and Nile tilapia (♀) × Mozambique tilapia (♂) (Liu et al. 1985; Wan et al. 1987), Southern catfish (*Silurus meridionalis*, ♀) × Amur catfish (*Silurus asotus*, ♂) (Wang et al. 2004a), and black Amur bream (♀) × blunt snout bream (♂) and blunt snout bream (♀) × black Amur bream (♂) (Yang et al. 2002b).

The research team of Guifeng Li from Sun Yat-sen University had studied distant hybridization in mandarin fish. They directly crossed the big-eye mandarin fish (*Siniperca kneri*, ♀) with Chinese perch (*Siniperca chuatsi*, ♂) to obtain F_1 hybrids. Self-mating of the F_1 hybrids had produced F_2 progeny (Lu et al. 2013).

In amphibians, a variety of interspecific hybridization have been observed. Both reciprocal crosses between *Rana nigromaculata* and *Rana plancyi fukienensis* could produce juvenile frogs (Ting 1956). Only a few tadpoles were obtained by the crossing of a *Rana guentheri* (♀) × *Rana rugulosa* (♂) (Zhu 1934). Normal offspring was obtained by the reciprocal cross of *Microhyla pulchra* (♀) × *Microhyla ornata* (♂) (Zhu 1934).

Examples of interspecific hybrids include mules, the F_1 offspring of *Equus caballus* (♀) × *Equus asinus* (♂), and hinnies, the corresponding offspring of *Equus asinus* (♀) × *Equus caballus* (♂). Few mule individuals were fertile (Zong et al. 1985). Reciprocal cross-generated hybrids of *Equus zebra* (♀) and *Equus caballus* (♂) showed similar appearances to *Equus zebra*.

An example of an interspecific bovine hybrid is that of *B. grunniens* (♀) × *B. taurus domestica* (♂). Male hybrids were infertile, while females were fertile. In the Russian Altai region, using these hybrids and different varieties of

domestic cattle, ideal dual-purpose (milk and meat) offspring had been generated (Ivanova 1960).

The most representative example of interspecific hybridization between sheep species is the cross between *Ovis ammon* and *Ovis aries*. In addition, the cross between *Ovis musimon* and *Ovis aries* had long been discovered in the former Soviet Union. In addition, *Ovis ammon arkal* was crossed with *Ovis merino* to create a new variety (Bunch et al. 1976).

Examples of interspecific hybridization of other mammals are the offspring of lion (*Panthera leo*, ♀) × tiger (*Panthera tigris*, ♂) called tigons and the offspring of tiger (♀) × lion (♂) called ligers. It seems that female hybrids of liger were more likely to survive than males (Gray 1954). The offspring of lion (♀) × leopard (*Panthera pardus*, ♂), referred to as a leopon, was first bred in 1910 in an Indian zoo. The leopon exhibited a similar body size to a lion, and the male leopon had 20 cm long mane. Grolar bear is the hybrid progeny of *Ursus maritimus* (polar bear) (♀) and *Ursus arctos* (grizzly bear) (♂). Although grizzly bear and polar bear are genetically similar, they usually avoid each other in the wild. On April 16, 2006, an American hunter killed a grolar bear in the Canadian Arctic. This was the first time that a hybrid bear had been found in the wild. Before this discovery, hybrid bears could only be found in zoos. However, no distant hybrid lineages were reported subsequently.

1.1.2 The Formation of Fish Distant Hybrid Lineages

Based on the above evidences, fertile females and fertile males have not been simultaneously found in hybrid F_1 in other animals except for fish. Only several fertile female individuals are obtained in hybrid F_1 from goat × sheep, horse × donkey, cattle × domestic yak, and lion × tiger hybridizations, while no fertile male can be found. Thus, fertile lineages are only formed in fish, not in other animals which may be due to the following reasons: (1) Long-term systematic studies on F_1 distant hybrid progeny have been neglected because of the influence of the traditional view that interspecies reproductive isolation barriers cause infertility or reduce fertility in distant hybrids. (2) Although some hybrids have poorly developed testes at the beginning generations of hybridization such as those in F_1 or F_2, and only watery semen can be produced, the sperm in the watery semen still have the possibility to fertilize and form progeny. The partial fertility of poorly developed gonad is easily ignored during breeding. (3) As an early-diverging vertebrate group, fish have a lower degree of genetic differentiation than other vertebrates, thus increasing the survival rates of distant hybrids. (4) The number of fish species is much greater than those of other vertebrates (mammals, birds, reptiles, and amphibians), which provides greater opportunities for distant hybridization to occur. (5) The majority of fish species exhibit external fertilization types, which is convenient for the investigation of hybridization mechanisms and the observation of embryonic development.

Distant hybridization has been well studied in fishes, which are the largest vertebrate group with more than 32,000 species. Previous studies (Meyer et al. 2006; Saitoh et al. 2010; Song et al. 2012; Yu et al. 1989) regarding the chromosome data for 318 fish species indicate that the majority of chromosomal number are even, especially 44, 48, 50, and 100 (Song et al. 2012). More specifically, based on the statistical results, 26 species have 44 chromosomes; 119 species have 48 chromosomes; 77 species have 50 chromosomes; and 19 species possess 100 chromosomes. Two fish species, *Monopterus albus* (Synbranchiformes) and *Liobagrus marginatus* (Siluriformes), show the smallest number of chromosomes ($2n = 24$), and *A. sinensis* has the greatest number of chromosomes ($2n = 264$). Chromosome numbers among fish generally increase by double, which suggests that most fish experience polyploidization and distant hybridization. Considering the large number of such species, some past polyploidization events may be related to distant hybridization. However, due to the long evolutionary history of fish, it is difficult to observe direct evidence of polyploid generation through distant hybridization. In our previous study, tetraploid fish lineages are successfully established via distant hybridization, which provide a research model and relevant evidence for studying the origin of new fish species through distant hybridization. The tetraploid lineages also provide a systematic experimental platform for investigating polyploidization in fish. Thus, these lineages have great significance in biological evolution and fish breeding studies.

The production of fish polyploids via distant hybridization is not only affected by the number of parental chromosomes but also by the compatibility and interaction between parental DNA and cytoplasm. Selecting parental fish with the same number of chromosomes for distant hybridization is conducive to the correct pairing of chromosomes and the proper distribution of genetic materials in cells during mitosis and meiosis. At the cellular and molecular levels, this compatibility is ultimately conducive to the common stable inheritance of genetic material. Conversely, in parents with different numbers of chromosomes, the nuclear-nuclear and nuclear-cytoplasmic incompatibility between the two species increases, resulting in abnormal chromosome pairing and unstable inheritance.

The selection of parents for distant hybridization experiments needs to consider factors such as the number of chromosomes, appearances, feeding habits, reproductive characteristics, phylogenetic relationships, growth rates, disease resistance, and other factors. When designing hybrid combinations, parents with different desirable traits are selected to produce hybrids with a combination of advanced traits. For example, in the crossing of red crucian carp with blunt snout bream, we were concerned about the respective chromosome numbers ($2n = 100$ and 48, respectively), different feeding habits (red crucian carp is omnivorous, while blunt snout bream is herbivorous), and short period of sexual maturity (1–2 years for blunt snout bream). In the experiment involving topmouth culter and blunt snout bream, the same parental chromosome numbers ($2n = 48$ for both parents), different feeding habits (topmouth culter is carnivorous and blunt snout bream is herbivorous), and short sexual maturity period (1–2 years for both) of the two species were considered. It is not easy to design a distant hybridization study since the selection of appropriate

parental combinations requires long-term effort to evaluate the properties of different progeny. If the breeding bottlenecks are eliminated in F_1 and F_2 hybrid generations, a fertile lineage can be produced, making cross-breeding possible.

Triploid fish cultivation has many advantages. Because triploids are sterile, they do not contaminate natural fish germplasm resources. In addition, they also show the advantages of fast growth rates, strong disease resistance, and good meat quality, which improves quality and yield, reduces or eliminates the use of medicines, and leads to lower production costs. The development of infertile triploids from crosses of tetraploid fish and diploid fish is one of the ideal methods to produce triploid fish.

The development of tetraploid fish has always been a research hotspot in aquaculture. The researchers used cytochalasin B to induce tetraploid rainbow trout (*Oncorhynchus mykiss*) (Refstie 1981). American researchers used heat shock induction to produce tetraploid rainbow trout (Thorgaard et al. 1981) and tetraploid Amur catfish (*Silurus asotus*) (Bidwell et al. 1985). French researchers had used two methods—hydrostatic pressure and heat shock—to induce tetraploid rainbow trout (Chourrout et al. 1986; Chourrout and Nakayama 1987). In China, researchers had used a variety of methods: A crucian carp transparent-colored tetraploid variety (Gui et al. 1991) was generated using hydrostatic pressure treatment combined with cold shock; heat shock was applied to generate tetraploids of Japanese white crucian carp (♀) × red crucian carp (♂) hybrids (Chen et al. 1997); tetraploid blunt snout bream was induced via the heat shock method (Zou et al. 2004); and hydrostatic pressure was used to produce tetraploid Japanese flounder (*Paralichthys olivaceus*) (Li et al. 2012). Unfortunately, none of these described methods has successfully generated fertile tetraploid offspring (both male and female were fertile), which can form lineage. Nevertheless, this research provides useful experimental data and results for developing artificial tetraploids.

The tetraploid fish artificially produced by chemical or physical methods is different from the naturally produced tetraploid fish. These chemical or physical methods may lead to incomplete chromosomal doubling, resulting in mixed haploid-diploid or aneuploid individuals. In addition, these methods may adversely affect important cytoplasmic factors in fish. Such chromosomal and cytoplasmic changes are likely to lead to reduced fertility and survival rates.

The previous results have proven that distant hybridization is an ideal method for producing fertile tetraploid lineages because it mirrors tetraploid fish formation in nature.

1.1.3 The Genetic Variability of Fish Lineages Derived from Distant Hybridization

Hybridization and genetic introgression between species are common phenomena during biological evolution, especially for the rapid radiation (Seehausen 2004; Comai 2005; Mallet 2007; Liu 2010; Liu et al. 2001a). Dozens of interspecies hybridization have been established, such as the red crucian carp (♀) × common carp (♂), common carp (♀) × red crucian carp (♂), red crucian carp (♀) × blunt

snout bream (♂), blunt snout bream (♀) × topmouth culter (♂), and so on. The phenotypic and genotypic characteristics of offspring derived from the distant hybridization are very different from the parental species. Here, we discuss the phenotypic and genotypic variations of offspring derived from the distant hybridization including the tetraploid lineage derived from the red crucian carp (♀) × common carp (♂), the tetraploid lineage derived from the red crucian carp (♀) × blunt snout bream (♂), the reciprocal crosses of blunt snout bream (♀) × topmouth culter (♂), the diploid lineage derived from the common carp (♀) × blunt snout bream (♂), and the diploid lineage derived from the koi carp (♀) × blunt snout bream (♂).

1.1.3.1 The Phenotypic and Genotypic Variation in the Tetraploid Lineage of Red Crucian Carp (♀) × Common Carp (♂)

Our laboratory has established an allotetraploid hybrid lineage (F_3–F_{29}, $4n = 200$) derived from the red crucian carp (♀) × common carp (♂) (Liu et al. 2001a; Liu et al. 2001b; Sun et al. 2003) and autotetraploid fish lineage (F_2–F_{15}, $4n = 200$) (Liu et al. 2007b). The approaches in generating the two different types of tetraploid represent two important routes to produce tetraploid fish. Unlike the autotetraploid produced by colchicine- and hydrostatic-based methods, in which the sperm and ovum come from the same species, the autotetraploid fish lineage derived from distant hybridization between red crucian carp and blunt snout bream involves the hereditary basis from both parents. However, the autotetraploids derived from the same species have no hybrid genetic materials. The allotetraploid hybrid lineage ($4n = 200$) derived from red crucian carp (♀) × common carp (♂) and the autotetraploid fish lineage ($4n = 200$) derived from red crucian carp (♀) × blunt snout bream (♂) had the same maternal parent (red crucian carp), whereas their original paternal parents were different. These two tetraploid lineages are of great significance for fish genetic breeding and biological evolution.

The newly formed polyploid genome is unstable and undergoes rapid reconstruction to achieve a more harmonious genomic coexistence in the nucleus (Comai 2005; Chen and Ni 2006). *Brassica* plants are an example, in which multiple genome rearrangements and segmental deletions occurred within five generations after polyploid formation (Song et al. 1995). Genomic structural changes mainly included deletion, insertion, duplication, translocation, and transposition events (Chen and Ni 2006). By using chromosome-specific fluorescent in situ hybridization (FISH) markers, at least nine reciprocal translocations were detected in tetraploid tobacco (*Nicotiana tabacum*), indicating that most tobacco chromosomes were related to both parental genomes (Kenton et al. 1993). Through genomic in situ hybridization (GISH) analysis, five reciprocal translocations were found in the genome of tetraploid oat (*A. sativa*). A similar result was found in hexaploid *A. sativa*, with 18 detected reciprocal translocations (Jellen et al. 1994). It was confirmed that the allotetraploid hybrid lineage possessed the genetic material from both of their parents and verified the hybridity of allotetraploid hybrid lineage by using the FISH and other biotechnology (Liu et al. 2010).

The studies on the *HoxC9a* (Song 2013), *Sox9a*, *Sox9b* (Liu et al. 2007a), and other related genes in allotetraploid hybrid lineage showed that the allotetraploid lineage inherited the genetic material from both original parents, including red crucian carp (maternal) and common carp (paternal), indicating that the genetic material from different parents could be inherited simultaneously in tetraploid fish lineages.

We cooperated with the lab of Yaping Zhang at Yunnan University to systematically study gene mutations in liver tissue transcriptomes and genetic recombination phenomena in the hybrids of red crucian carp (♀) × common carp (♂) (including diploid and tetraploid hybrids). The gene recombination and the existence of genetic variation were verified within the diploid F_1, F_2, and tetraploid groups (Liu et al. 2016). In Sect. 3.5, Chap. 3, we have provided a detailed introduction for these results.

It is found that genetic variation and genetic recombination not only happen at the transcriptional level but also at the genomic DNA level in F_1 hybrids. For example, evidences for gene mutation and recombination had been found in the F_1 of the hybrid lineage derived from blunt snout bream (♀) × topmouth culter (♂), revealing the *HoxD9a* recombination in the F_1 hybrids (Song 2013). In the diploids and triploids of the F_1 hybrids of the lineage of grass carp and blunt snout bream, 5S rDNA (demonstrating they are real hybrids) from maternal grass carp and paternal blunt snout bream was not only found, but two different 5S rDNA recombination events from the parents were also found (He et al. 2013). Genomic DNA recombination is detected in the F_1 generation of distant hybrid somatic cells (liver cells). These DNA recombinations in the somatic cells of F_1 generation derived from distant hybrid can be explained by the following reasons: For distant hybridization, the genetic material in somatic cell comes from different types of genome in parents, some genes in the different types of genome may activate DNA endonucleases in this particular environment of distant hybridization, and the subsequent DNA fragments can be exchanged and reconnected through DNA recombination repair mechanisms, generating recombinant DNA. Why the recombinant DNA can easily happen in somatic cells of the F_1 generation derived from distant hybridization? We conjecture that the long-term separation of the two parental genomes is harmful to their coexistence in somatic cells from the distant offspring. Thus, the recombination of two different genomes can make the hybrid genes, which can be inherited in the offspring, which is beneficial to the genetic variation.

The DNA recombination associated with distant hybridization is an important type of variation in somatic cells in the diploid F_1. These variations play a key role in the formation of the hybrid lineage. In addition, genetic variation promotes the adaptive evolution for the new hybrid lineage including the survival and reproduction with the genetic background of distant hybridization.

The genetic variation including DNA recombination in both diploid lineages and tetraploid lineages derived from the distant hybridization would be inherited by the next generation and lead to further variation with the continuation of the hybrid lineage. In the distant hybridization lineage, the formation of a tetraploid hybrid lineage not only doubles the genetic material but also changes the pairing model of

chromosomes during the meiosis, which is beneficial for the reproduction and survival of tetraploids. In addition, somatic cell DNA recombination can be continued in the tetraploid hybrid lineage. The trend of DNA recombination in the allotetraploid derived from distant hybridization is also beneficial for tetraploid fish derived from the diploid fish, and finally leading to the formation of the new diploidization fish with the doubled genetic material and variation.

More studies are needed to research what kinds of genes are most likely to switch and in what proportions. More in-depth research remains to be conducted on this topic.

Somatic cell DNA recombination is easily detected in fish distant offspring, because of the heterogenous maternal and paternal genomes. On the contrary, in more closely related fish hybrids or self-mating offspring, it is unclear whether DNA recombination of somatic cell occurs at genomic or transcriptional levels. Such DNA recombination is not easily identified, because of the high genetic similarity in self-crossing or closer hybridization. More studies are needed in this area.

The alterations of genotypic may lead to changes in performance. The cell volume of polyploids is generally increased, accompanied by genome doubling and increases in genetic material. Polyploid animals and plants have found many ways to fit for the increased cell size (Mable 2004; Gregory and Mable 2005). Polyploid plants have the same number of cells as diploids but exhibit larger body sizes.

The traits of allotetraploid offspring derived from red crucian carp (♀) × common carp (♂) differed in different ways from those of its original parents. For instance, red crucian carp lacked barbels and common carp possessed two pairs of barbels. The allotetraploid hybrids had one pair of barbels. The length/height and head length/snout length of the allotetraploid hybrid lineage were intermediate between those of its original parents. The length/head length of the allotetraploid hybrid lineage was, however, lower than that of its original parents. In allotetraploid offspring of red crucian carp (♀) × common carp (♂), the numbers of vertebra, numbers of gill rakers, numbers of lateral line scales, and numbers of pharyngeal teeth were between those of the original parents (Liu 2010; Liu et al. 2001b).

Diploid, triploid, and tetraploid fish also have differences at the cellular level. As the ploidy level increases, the nucleus and cell diameter of erythrocytes and other types of cells increase (Lu et al. 2009; Liu et al. 2004). In allotetraploid hybrids, some erythrocyte nuclei exhibited a particularly dumbbell-shaped appearance (Liu et al. 2003).

1.1.3.2 The Phenotypic and Genotypic Changes in the Tetraploid Hybrid Lineage Derived from Red Crucian Carp (♀) × Blunt Snout Bream (♂)

The allotetraploids ($4nF_1$, $4n = 148$) were obtained in F_1 hybrids of red crucian carp (♀) × blunt snout bream (♂), and the F_2 ($4nF_2$) generation was obtained from F_1 self-crossing and showed genetic variation. The allotetraploids ($4nF_1$, $4n = 148$) of red crucian carp (♀) × blunt snout bream (♂) contained two sets of red crucian carp chromosomes and two sets of blunt snout bream chromosomes. However, the $4nF_2$

and its self-crossing offspring (F_2–F_{15}, $4n = 200$) were autotetraploid and contained four sets of red crucian carp chromosomes.

In appearance, red crucian carp has red body color, while blunt snout bream has silvery white body color, and neither presents any barbels. In contrast, the autotetraploid hybrids exhibited a gray body color and a pair of barbels, showing obvious evidence of genetic changes.

With respect to genotypes, the autotetraploid hybrids showed significant genetic variation. We used a centromere-specific repetitive sequence of red crucian carp as a FISH probe to confirm red crucian carp and blunt snout bream chromosomes in the genomes of $4nF_1$ and $4nF_2$. The chromosomes of red crucian carp showed fluorescence signals, whereas those of blunt snout bream did not show any signal. The 100 chromosomes of $4nF_1$ that were from red crucian carp had fluorescence signals. The $4nF_2$ would be expected to exhibit 200 signals labeled chromosomes. However, only 100 chromosomes showed the signal, explaining the presence of significant genetic variation with respect to the other 100 chromosomes. And, the 5S rDNA gene cluster of $4nF_1$ and $4nF_2$ showed significant genetic variation. Red crucian carp had three types of 5S rDNA structural units (types I, II, and III), while only one (type IV) was found in blunt snout bream. These four types of 5S rDNA structural units had different base composition and sequence length. The $4nF_1$ inherited three 5S rDNA structural units from the maternal parent, while the paternal type was completely lost, showing the $4nF_1$ genome with obvious variation. For $4nF_2$ genome, a dimeric structure was formed by connecting types I and II. However, no similar dimeric structure was identified in the red crucian carp and $4nF_1$ genomes. This result demonstrated that $4nF_2$ chromosomes underwent reorganization causing 5S rDNA combinations.

We identified the genetic mutations of the *HoxD*4*a* in allotetraploid ($4nF_1$, $4n = 148$) and autotetraploid hybrids ($4nF_6$, $4n = 200$) in the distant hybrid lineage derived from red crucian carp (♀) × blunt snout bream (♂). This mutation could be genetically passed from $4nF_1$ to $4nF_6$. In addition, we also found that two kinds of different *HoxD*4a genes from maternal red crucian carp and paternal blunt snout bream were recombined, and this recombination change could be genetically passed from $4nF_1$ to $4nF_6$. The results showed that both allotetraploid ($4n = 148$) and autotetraploid hybrids ($4n = 200$) in the distant hybrid lineage derived from red crucian carp (♀) × blunt snout bream (♂) contained genetic material from their original parents.

We comparatively analyzed the phenotypes and genotypes of the allotetraploid hybrid lineage derived from red crucian carp (♀) × common carp (♂) ($4n = 200$) and the autotetraploid hybrid lineage derived from red crucian carp (♀) × blunt snout bream (♂) ($4n = 200$). These two types of tetraploid hybrids have different arrangements of the pharyngeal teeth: the allotetraploid hybrids had two rows, whereas the autotetraploid hybrids had only one. Regarding genotypes, transcriptome analysis revealed that the two lineages have similarities and differences.

These two types of tetraploid fish lineages exhibited the same chromosome number ($4n = 200$). They had the same maternal parent red crucian carp but different

paternal parents (common carp and blunt snout bream, respectively) which led to difference in hepatic mRNA expressions in the two kinds of tetraploid fish in same conditions. Thereinto, the autotetraploids had more expressed genes compared to allotetraploids.

In co-expressed genes, the two tetraploids have differences in the nucleotide sequences of various genes. One example showing obvious differences is the highly expressed homologous gene *bhlhe40* (1881 bp). The sequence differences of this gene can be used as one of molecular markers for identifying these two kinds of tetraploids. Functionally, this gene is widely expressed in various tissues and is thought to be involved in the control of cell differentiation.

Two ways can form different ploidy fishes: (1) distant hybridization using parents with the same chromosome numbers and (2) distant hybridization using parents with different chromosome numbers. Investigation of principle in formation of different ploidy fishes, especially in the formation of allotetraploid lineages containing two subgenomes from parents (such as allotetraploid lineage of red crucian carp (♀) × common carp (♂), $4n = 200$) and autotetraploid lineages only containing maternal genome with variations (such as autotetraploid lineage of red crucian carp (♀) × blunt snout bream (♂), $4n = 200$), plays an important role in fish genetic breeding and biological evolution. In fish genetic breeding studies, the formation of these fish demonstrates that tetraploid fish can be produced by these two ways, providing abundant tetraploid parents for production of triploid fish. In biological evolution studies, the formation of these tetraploid fish suggests that polyploid fish may evolve from these two ways in the wild. The successful exploration of the two ways provides suitable model systems for research on association between fish distant hybridization and polyploidization.

1.1.3.3 The Changes in the Phenotype and Genotype of the Blunt Snout Bream × Topmouth Culter Lineage

Morphologically, lateral line scales, dorsal fin rays, and anal fin rays of diploid F_1 and F_2 hybrids of blunt snout bream (♀) × topmouth culter (♂) were numerically intermediate between blunt snout bream and topmouth culter, whereas the numbers of pelvic fin rays in diploid F_1 and F_2 hybrids of blunt snout bream (♀) × topmouth culter (♂) were more than those in their parents (Xiao 2013).

Gene structure analysis showed that blunt snout bream (♀) × topmouth culter (♂) F_1 and topmouth culter (♀) × blunt snout bream (♂) F_1 inherited specific *its1* from both parents, while several individuals of blunt snout bream (♀) × topmouth culter (♂) F_2 and topmouth culter (♀) × blunt snout bream (♂) F_2 showed specific *its1* lost from their parents. In hybrid F_1 including diploid F_1 hybrids of blunt snout bream (♀) × topmouth culter (♂), triploid F_1 hybrids of blunt snout bream (♀) × topmouth culter (♂) and diploid F_1 hybrids of topmouth culter (♀) × blunt snout bream (♂) inherited and expressed specific 18S rRNA from both parents.

In diploid F_2 hybrids of blunt snout bream (♀) × topmouth culter (♂), some individuals inherited and expressed specific 18S rRNA from both parents, some individuals inherited specific 18S rRNA from both parents but only expressed

specific 18S rRNA from blunt snout bream, and some individuals only inherited and expressed specific 18S rRNA from topmouth culter.

In diploid F_2 hybrids of topmouth culter (♀) × blunt snout bream (♂), some individuals inherited and expressed specific 18S rRNA from both parents, some individuals inherited specific 18S rRNA from both parents but only expressed specific 18S rRNA from blunt snout bream, some individuals inherited specific 18S rRNA from both parents but only expressed specific 18S rRNA from topmouth culter, and some individuals only expressed and inherited specific 18S rRNA from blunt snout bream (Xiao 2013).

In blunt snout bream, topmouth culter, and their hybrids, the internal control regions (ICRs) in the coding areas including an A frame, internal element (IE), and C frame were all presented in the 5S rDNA sequence. The coding areas in the 5S rDNA sequences of blunt snout bream and topmouth culter were highly conserved. For diploid F_2 hybrids of blunt snout bream (♀) × topmouth culter (♂), diploid F_1 hybrids of topmouth culter (♀) × blunt snout bream (♂), and diploid F_2 hybrids of topmouth culter (♀) × blunt snout bream (♂), the type II of 5S rDNA presented a T → C transition at nucleotide position 73. In diploid F_1 hybrids of blunt snout bream (♀) × topmouth culter (♂), a G → A transition occurred in type I 5S rDNA at nucleotide position 93 (Xiao 2013).

Comparison and analysis of NTS (nontranscribed intergenic spacer) sequences in blunt snout bream, topmouth culter, diploid F_1 and F_2 hybrids of blunt snout bream (♀) × topmouth culter (♂), triploid F_1 hybrids derived from blunt snout bream (♀) × topmouth culter (♂), and diploid F_1 and F_2 hybrids of topmouth culter (♀) × blunt snout bream (♂) revealed some insertion sequences and base substitutions. Compared with the parental NTS-I sequences, a poly(A) sequence was inserted into the NTS-I sequence of the hybrids; in diploid F_1 and F_2 hybrids of topmouth culter (♀) × blunt snout bream (♂), a new short variation sequence, CATTTT, was inserted. The sequence variation between NTS II of topmouth culter and NTS II of the hybrids comprised abundant single nucleotide substitutions or deletions (Xiao 2013).

Various nucleotide substitutions and evidence of recombination in F_1 derived from blunt snout bream (♀) × topmouth culter (♂) were found by analyzing *Hox* gene sequences. Intron regions of the *HoxD9a* gene in blunt snout bream (♀) × topmouth culter (♂) F_1 showed exchanges and recombination. The first portion of its intron sequence was consistent with that of blunt snout bream, and the latter portion matched that of topmouth culter. This exchange-restructuring phenomenon in blunt snout bream (♀) × topmouth culter (♂) provided evidence of genetic diversity and genetic variation and provided key data about the ability of offspring to adapt to the environment and the process of biological evolution (Song 2013).

1.1.3.4 The Changes in the Phenotype and Genotype of the Lineages Derived from Common Carp (♀) × Blunt Snout Bream (♂) and Koi Carp (♀) × Blunt Snout Bream (♂)

In terms of phenotype, the crucian carp-like homodiploid fish lineage originated from the common carp (♀) × blunt snout bream (♂) had undergone significant variation compared with parents. Similarity, the red crucian carp-like homodiploid fish lineage and the goldfish-like homodiploid fish lineage originated from the koi carp (♀) × blunt snout bream (♂) had also undergone significant variation compared with parents. For example, compared with maternal parent having two pairs of barbels, the crucian carp-like homodiploid fish lineage, the red crucian carp-like homodiploid fish lineage, and the goldfish-like homodiploid fish lineage had no barbels. Regarding the measured and countable traits, the crucian carp-like homodiploid fish lineage, the red crucian carp-like homodiploid fish lineage, and the goldfish-like homodiploid fish lineage were significantly different from their parents (Wang et al. 2018; Wang et al. 2017).

In terms of genotype, fluorescence in situ hybridization (FISH) and 5S rDNA analyses showed that the genotype of the crucian carp-like homodiploid fish lineage differed from those of its parents but was similar to that of diploid crucian carp (Wang et al. 2017). The mitochondrial DNA organization and nucleotide composition of the crucian carp-like homodiploid fish lineage had more similarities to those of the existing diploid crucian carp than those of the parents (Wang et al. 2020a). The microsatellite DNA and 5S rDNA analyses showed that the red crucian carp-like homodiploid fish lineage and the goldfish-like homodiploid fish lineage were closely related to goldfish and red crucian carp, respectively. A twin tail of the goldfish-like homodiploid fish lineage was related to a base mutation in *chordinA* from G to T in the goldfish-like homodiploid fish lineage, indicating that the red crucian carp-like homodiploid fish lineage and the goldfish-like homodiploid fish lineage could be used to study gene variation and function (Wang et al. 2018). FISH also revealed that the genotype of the red crucian carp-like homodiploid fish lineage and the goldfish-like homodiploid fish lineage differed from those of its parents but was closely similar to that of red crucian carp and goldfish (unpublished data).

1.1.4 The Application of the Lineages Derived from the Fish Distant Hybridization

The distant hybrid lineage is very useful for studies of genetic breeding and biological evolution. Improved fish can be obtained by crossing the hybrid lineage and common diploid fish. Examples of the result obtained include the generation of new types of bream. This bream was produced from the backcrossing of female improved hybrid fish-blunt snout bream (♀) × topmouth culter (♂) and male blunt snout bream (refer to Chap. 8). Similarly, using the new types of bream as maternal parent and topmouth culter as paternal parent, second round of backcrossing was performed to obtain a new type of hybrid culter with similar appearance to topmouth culter but fewer intermuscular bones of the body. Similar second round of

backcrossing, using blunt snout bream as paternal or maternal parent, was performed to obtain other improved hybrid breams. Other types of high-quality hybrid fish could be produced by crossing female blunt snout bream or topmouth culter individuals with male individuals of blunt snout bream (♀) × topmouth culter (♂) or topmouth culter (♀) × blunt snout bream (♂) lineages. Allotetraploid fish could be mated with diploid fish to generate sterile triploid fish. This way could be used to produce fast-growing, highly resistant, sterile triploid crucian carp (triploid Xiangyun crucian carp) and triploid carp (triploid Xiangyun carp). We had also combined the methods of distant hybridization and gynogenesis to obtain improved tetraploid fish populations. A kind of improved triploid fish with improved characteristics (triploid Xiangyun crucian carp II) was obtained in large-scale production after mating these fish with related diploid fish (Chen et al. 2009).

Sterile allotriploid fish in F_1 and allotetraploid fish ($4nF_1$, $4n = 148$) were formed by distant hybridization between parents with different chromosome numbers. Subsequent self-mating of this allotetraploid fish led to the generation of fertile autotetraploid fish lineage (F_2–F_{15}, $4n = 200$). A large scale of triploid fish can be produced by mating autotetraploid fish and diploid fish. Natural gynogenetic red crucian carp with genetic variation characteristics could also be produced in the first generation of red crucian carp (♀) × blunt snout bream (♂). Subsequent self-mating led to the formation of a diploid red crucian carp lineage showing genetic mutation that could be used to produce improved diploid hybrids (refer to Chap. 6).

The common carp (♀) × blunt snout bream (♂) belonging to distant hybridization between parents with different chromosome numbers resulted in the production of a crucian carp-like homodiploid fish ($2nF_1$, $2n = 100$) and allotetraploid fish ($4nF_1$, $4n = 148$). Furthermore, a new autotetraploid fish ($4n = 200$) was produced by the hybridization of the allotetraploid fish ($4n = 148$) (♀) × the crucian carp-like homodiploid fish ($2n = 100$) (♂). Subsequently, a new autotetraploid fish lineage with genetic variation (F_2–F_5, $4n = 200$) was established by successive self-crosses (Wang et al. 2020b).

In addition, the koi carp (♀) × blunt snout bream (♂) belonging to distant hybridization between parents with different chromosome numbers resulted in the production of a red crucian carp-like homodiploid fish ($2nF_1$, $2n = 100$) and gynogenesis koi carp ($2nF_1$, $2n = 100$). The self-mating of red crucian carp-like homodiploid fish produced goldfish-like homodiploid fish with twin tails ($2nF_1$, $2n = 100$). Goldfish-like homodiploid fish had greater genomic DNA variations, which resulted in the changes of phenotype. The goldfish-like homodiploid fish was used as the new fish resources to mate with other goldfish to produce new types of goldfish. The formation of goldfish-like homodiploid fish was very significant in fish genetic breeding and evolutionary biology (Wang et al. 2018).

The Japanese white crucian carp (♀) × red crucian carp (♂) belonging to interspecific hybridization between parents with same chromosome numbers resulted in the formation of an allodiploid hybrid lineage (WR-F_1–F_5, $2n = 100$). A kind of improved diploid fish (abbreviated as WR-II, $2n = 100$) was produced by backcrossing between the WR-F_1 (♀) × Japanese white crucian carp (♂), and the WR-II exhibited a higher body and smaller head compared with its original parents.

The formation of the lineage (WR-F_1–F_5) and WR-II brought the new germplasm resources to produce improved fish with advantageous traits (Liu et al. 2019).

1.1.5 The Genetic Rules and Reproductive Rules in Fish Distant Hybridization

Through long-term and systematic studies on distant hybridization of fish, the research team of the authors made many distant crosses in which a lot of fertile lineages and improved fish were obtained. According to the obtained results, it is concluded that reproductive barriers between species in fish can be broken and the fertile lineages can be established. The results derived from the large number of distant crosses laid a solid foundation for exploring the genetic and reproductive rules in fish distant hybridization. The main rules regarding genetics and reproduction related to fish distant hybridization are obtained and are described in Chap. 12. Furthermore, the one-step and multistep breeding technologies are established and are applied, which are also described in Chap. 12.

1.2 The Research Advances in Polyploid Organisms

Polyploid is an organism with three or more complete sets of chromosomes. Studies showed that all kinds of angiosperm were paleopolyploids and had experienced one or more rounds of polyploidy (Otto 2007). Ohno put forward the hypothesis of genome duplication according to the indexes of genome size and isozyme complexity, inferring that two rounds (2R) of whole-genome duplication (WGD) had happened during early vertebrate evolution (Ohno 1970b). From cephalochordates to jawless vertebrates, the first duplication occurred; after the divergent evolution of hagfish and lampreys, the second duplication occurred (Holland et al. 1994). Studies of *Hox* gene clusters strongly supported this hypothesis (Meyer and Van de Peer 2005). Data about vertebrate genome sequencing, phylogenetic trees, and genomic map positions offered more evidence to support the hypothesis of genome duplication (Dehal and Boore 2005; Blomme et al. 2006). In addition, the ray-finned fish was reported to have experienced a third genome duplication after their separation from the tetrapods (Amores et al. 1998; Taylor et al. 2001; Volff 2005). The polyploids produced via genome duplication exhibited changes in gene regulation, genome structure, and expression and subsequently developed into new diploids or paleopolyploids (Soltis et al. 2004; Comai 2005; Kassahn et al. 2009). The systematic discussion and summary for the polyploidy genetic influence in the types and occurred ways in plant and animal polyploids have significant theoretical and practical value for genetic breeding. Here, we show a systematic description of the studies of polyploid organisms, particularly polyploid fish.

1.2.1 Polyploid Plants

1.2.1.1 The Frequency of Polyploidy in Various Groups of Plants

Polyploids in bryophytes contain approximately 53% of the total species. For example, *Plagiomnium medium* had been reported as an allotetraploid plant (Soltis et al. 1993). The frequency of polyploidy in pteridophytes had been reported to be 95% (Grant et al. 2005). Approximately 38% of plants of gymnosperm were polyploids. California redwood (*Sequoia sempervirens*, $2n = 6X = 66$) was the only natural hexaploid conifer in the world (Ahuja and Neale 2005; Hair 1968). Via one round of polyploidization and hybridization, the ancestors of these trees produced AAB-type plants, after which the genome doubled, producing the hexaploid redwood. Furthermore, there were triploid or tetraploid species of *Cryptomeria*, cypress, red pine, spruce, and larch.

There were many researches on angiosperm polyploids, and it had happened for more than a century. A mutant of *Oenothera lamarckiana*, *gigas*, was found to be a type of tetraploid plant in the twentieth century (Gates 1909). Despite many attempts, it was difficult to determine the actual frequency of polyploids in various plant lineages. The chromosome numbers of angiosperm ancestors were 7–9 and that any $n \geq 14$ chromosomes flowering plant had undergone polyploidization during angiosperm evolution (Grant et al. 2005). Grant speculated that 47% of flowering plants originated from polyploidy, and he thought that 58% of monocots and 43% of dicots were polyploids (Grant et al. 2005). The analyses of genome indicated that an early polyploidization event might have predated the radiation of flowering plants, indicating that 100% of angiosperms were paleopolyploids (Bowers et al. 2003; Otto 2007).

1.2.1.2 The Molecular Biological Research on Polyploidy Plants

Comparisons of diversification rates suggested that the doubling of genome might have led to a dramatic increase in the numbers of species in several angiosperm lineages, including Solanaceae, Poaceae, Brassicaceae, and Fabaceae (Soltis et al. 2009). Research of MADS-box gene duplication and studies of protein-protein interaction networks among MADS-box proteins (Veron et al. 2007) also supported the occurrence of genome doubling early in angiosperm history. In plant genome research, the way of examining the frequency distribution of the synonymous mutation rate (*Ks*) for pairs of duplicate genes had been used to expressed sequence tags (ESTs) from a set of angiosperm (Albert et al. 2005), and researchers found evidence of ancient genome-wide duplication in *Persea americana* (Lauraceae), *Nuphar advena* (Nymphaeaceae), *Saruma henryi* (Aristolochiaceae), and *Liriodendron tulipifera* (Magnoliaceae). Furthermore, in California poppy (*Eschscholzia californica*, Papaveraceae) and sweet flag (*A. americanus*, Acoraceae), independent genome duplications were detected (Cui et al. 2006). Under current research, the formation of diploid, triploid, tetraploid, and hexaploid plants was thought to have occurred within the long-term natural variability of sweet flag (*A. calamus*) development (Zhang and Wang 2006).

Whole-genome sequencing helps to conduct comprehensive and systematic research on the polyploidy phenomenon. Numerous duplicate genes were found in *A. thaliana* by complete sequencing, indicating two or three rounds of genome-wide duplication (Paterson et al. 2000). Additional evidence indicated that *Arabidopsis* also experienced an earlier whole-genome duplication (WGT) before the two rounds of whole-genome replication (Severin et al. 2011). The evolutionary process of soybean (*Glycine max*) was also found to have this pattern, which had three rounds of polyploidization, comprising two rounds of tetraploidy and one round of hexaploidy. Genomic sequencing data showed that there was a common ancestor of grape (*Vitis vinifera*), poplar (*Populus papaya*), and *Arabidopsis* which experienced a hexaploidy process, while this ancestor did not participate in the evolutionally process of *Oryza sativa* (Jaillon et al. 2007). Studies of the papaya (*Carica papaya*) genome provided further support to the occurrence of genome duplication (Ming et al. 2008).

1.2.2 Polyploid Animals

Polyploidy is also widespread in animals. About 200 examples of polyploids from insects and vertebrates have been reported, not including the number of polyploids in other invertebrate groups (Otto 2007; Gregory and Mable 2005).

1.2.2.1 Polyploids in Invertebrates

In invertebrates, the polyploids of *Turbellaria* and *Oligochaeta* are usually hermaphrodites. *Pontoporeia affinis*, a glacial relict crustacean, is a polyploid created by bisexual reproduction. *A. salina* of Branchiopoda and *Trichoniscus* sp. of terrestrial isopod are haploid created by monogenesis (Wu 1988). Different subspecies of diploids ($2n = 2$) and tetraploids ($2n = 4X = 4$) were found in *Parascaris equorum* of Nematomorpha (Liu 1991).

It is estimated that there are 2.5–three million species of insects around the world, but less than 100 are considered as polyploids (Ye 1998). For instance, tetraploid *Saga pedo* in Orthoptera Tettigoniidae, triploid *Psychoda parthenogenetica* in Psychodidae, and tetraploid *Diprion* sp. in Hymenoptera Tenthredinidae have been discovered. The most abundant is polyploids of Coleoptera Curculionidae. A total of 38 triploids, 17 tetraploids, 5 pentaploids, and 2 hexaploids have been reported in parthenogenetic subspecies or species. Commonly, the types of reproduction in polyploid insects have been reported as parthenogenesis. Most of them don't have the ability to fly and exhibit a small body. The environment of their living environment is stable and they have a longer life stage (Li 2002).

1.2.2.2 Polyploids in Vertebrates

Humans and other terrestrial vertebrates may have shared a common fish ancestor that lived 360–450 million years ago, and it contains a genome with 12 chromosomes (Naruse et al. 2004; Volff 2005). In the early state of vertebrate evolution, through various non-polyploidy chromosome rearrangements and two rounds of

whole-genome duplication, new species with haploid chromosome number of 20–30 were formed (Woods et al. 2005). The Actinopterygii fish species occupy over half of all vertebrate species. The group experienced another round of fish-specific genome duplication (Amores et al. 1998; Taylor et al. 2001; Volff 2005). Genome duplication provided a good environmental adaptation and has played a key role in the evolution of fish and terrestrial vertebrates (Ohno 1970a).

Some polyploid fish showed reproductive type as parthenogenesis and usually associated with hybridization events, because fish also are lower vertebrates (Gui and Zhou 2010). For example, gibel carp could be obtained by gynogenesis. In addition, two types of triploid gibel carp, with 156 or 162 chromosomes, were identified by the analysis of karyotype (Zhou and Gui 2002). Some research suggested that the gibel carp was during the evolutionary trajectory of diploidization. The coexistence of populations of different ploidies (diploid, triploid ($2n = 3X = 150$) and tetraploid ($2n = 4X = 200$)) was found among crucian carp in the water system from Dongting (Xiao et al. 2011). The studies had shown that in triploid fish, several microchromosomes were observed and triploid fish could produce hybrids by gynogenesis or sexual reproduction, but tetraploid fish could only produce all-female offspring by gynogenesis. A similar situation also existed in Dianchi high-back crucian carp ($2n = 3X = 162$) in Yunnan, China (Luo 1991). From the perspective of whole-genome duplication, these kinds of triploids should be considered as hexaploids. Furthermore, some monometrosis triploids of Cyprinodontiformes usually are of hybrid origin. For instance, the Amazon molly (*Poecilia formosa*) is a hybrid by the cross of *Poecilia latipinna* and *Poecilia mexicana*. When its gynogenetic diploid fish were backcrossed with *Poecilia mexicana*, gynogenesis triploid fish could be generated (Lampert and Schartl 2008; Luo 1991).

In some studied fish families, all members were derived from tetraploids (Zan 1985), as observed in Salmonidae, Coregonidae, Catostomidae, and Thymallidae. Additionally, the fish in Siluridae, Callichthyidae, and Loricariidae also had the origin of triploidy or tetraploidy.

In Cyprinidae, some certain fish groups originated from polyploidy (Zan 1985). Many species, which belonged to *Cyprinus*, *Carassius*, or *Barbus*, were tetraploid fish ($2n = 4X = 100$). The *Schizothorax grahami* and *Schizothorax taliensis* that belonged to Schizothoracinae were hexaploids. The *Misgurnus fossilis* that belonged to Cobitidae was tetraploid. The coexistence of natural diploids and polyploids has been found in *Cobitis taenia*, *Cobitis elongatoides* (Ráb et al. 2000; Boron and Kotusz 2000), and *Misgurnus anguillicaudatus* (Abbas et al. 2009).

In reptiles and amphibians, the reports regarding polyploids are increasing. Nearly all the polyploids are associated with existing diploid relatives, and numerous polyploids were similar to diploid species in morphology, but obvious polyploid families or genera were infrequent (Wu 1988). Among anurans, both *Ceratophrys dorsata* ($2n = 8X = 104$) found in Brazil and *Ceratophrys ornata* found in Argentina were octoploids. The *Xenopus ruwenzoriensis* ($2n = 6X = 108$) found in Uganda was hexaploid. Both *Xenopus vastitus* ($2n = 4X = 72$) found in Africa and *Hyla versicolor* ($2n = 4X = 48$) found in America were tetraploids. The

B. viridis ($2n = 4X = 44$) found in Xinjiang, China, was also a tetraploid. In anurans, only a few triploids have been reported, including *Rana esculenta* ($2n = 3X = 39$) found in North America. In *Ambystoma* of Caudata, *A. platineum*, *A. tremblayi*, and *Ambystoma texanumx laterale* were triploid gynogenetic individuals ($2n = 3X = 42$); however, both *Pseudobranchus striatus* ($2n = 4X = 64$) and *Siren lacertina* ($2n = 4X = 52$) were sexual tetraploids (Li 1980).

All polyploids found in reptiles were triploids and they produced parthenogenetic offspring (Li 1992). *Cnemidophorus neomexicanus*, *Cnemidophorus uniparens*, and *Cnemidophorus exsanguis* ($2n = 3X = 69$) from Teiidae were triploids which were found in Central America. In Gekkonidae, *Gehyra variegate ogasauarisimae* ($2n = 3X = 63$), *Hemidactylus garnotii* ($2n = 3X = 70$), and *Heteronotia binoei* ($2n = 3X = 63$) were triploids, which came from different countries such as Japan and Australia. The triploids belonging to Agamidae including *Leiolepis tripoida* ($2n = 3X = 54$) and *Leiolepis belliana* ($2n = 3X = 54$) were widely distributed in Malaysia.

Polyploidy has never been observed in birds and mammals (Comai 2005). Some researchers thought that the golden hamster (*Mesocricetus auratus*) is a polyploid, but it has been proven to be a pseudotetraploid (Li 1980). In chicken embryos, 0.9% are triploids or tetraploids. However, ploidy changes in birds and mammals are typically fatal, which lead to polyploids dying early during development (Otto 2007).

The existence of all the natural polyploid animals discussed above has been indirectly speculated, and there is no direct evidence to verify their formation process. The polyploid animals were produced by distant hybridization (such as artificial polyploid fish). It is helpful to study the formation process of polyploids.

1.2.3 The Occurrence of Polyploids

1.2.3.1 The Mechanisms of Polyploid Formation

Three main routes to formation of polyploid organisms were chromosome doubling, unreduced gametes, and polyspermy (Otto and Whitton 2000; Ramsey and Schemske 1998).

The failure of cell division in mitosis may lead to chromosome doubling in plants and animals directly. It may occur in the zygote, early embryo, or meristem in plants and lead to the production of polyploidy tissues and the generation of polyploids ultimately (Soltis et al. 2004; Ramsey and Schemske 1998). The classic case is *Primula kewensis* (Newton and Pellew 1929), a tetraploid belonging to *Primula*. It originated from fertile tetraploid shoots by the chromosome doubling of sterile F_1 hybrid of *Primula floribunda* and *Primula verticillata*. The naturally occurring zygote chromosome doubling generated the tetraploid of *Oenothera lamarckiana* (Gates 1909). On the other hand, in animals, during the distant cross of red crucian carp and blunt snout bream, the first cleavage of some zygotes in the F_1 was inhibited, causing the doubling of chromosome number and producing tetraploids (Liu et al. 2007b; Liu 2010).

In plants, the major mechanisms of polyploidization were the combination of reduced and unreduced gametes, or the fusion of two unreduced gametes (Otto and Whitton 2000). For instance, the triploids and pentaploids originated from the open-pollinated diploid progenies, *Crepis capillaris*, which were produced by the fusion of unreduced ($2n$ and $4n$) and reduced (n) gametes (Ramsey and Schemske 1998). During the hybridization between *Raphanus sativus* ($2n = 18$) and *B. oleracea* ($2n = 18$), the allotetraploid offspring (*Raphanobrassica*, $2n = 4X = 36$) could be produced in F_2, because unreduced gametes were generated by a handful of stem cells in the F_1 (Karpechenko 2010). The allotetraploid *Tragopogon* and autotetraploid *Dactylis glomerata* could also be produced in a similar way (Soltis et al. 2004).

In animals, the progenies from distant cross events may obtain the ability to produce unreduced gametes in terms of reproductive characteristic. The unreduced gametes could be produced from some hybrids in the F_2 of red crucian carp ($2n = 100$) × common carp ($2n = 100$). Then, the combination of unreduced eggs and spermatozoa produced viable allotetraploid fish ($4n = 200$, or $2n = 4X = 200$) in the F_3 (Liu et al. 2001a; Liu et al. 2001b; Sun et al. 2003; Liu 2010). The diploid gynogenetic clonal lineage generated by the gynogenesis of the allotetraploid eggs could produce unreduced diploid eggs stably (Zhang et al. 2005). The behavior of germ cells before meiosis, which might involve the premeiotic fusion of germ cells, endoreduplication, or endomitosis, was the potential mechanism to form unreduced gametes (Liu et al. 2001b; Ullah et al. 2009; Liu et al. 2010).

After the chromosome doubling of germ cells, normal meiosis is carried out and results in unreduced gametes. The F_1 hybrids ($4n = 148$) of red crucian carp (♀) × blunt snout bream (♂) whose parents belong to different subfamilies could produce unreduced diploid eggs and diploid sperm. These two types of unreduced gametes underwent fertilization and generated the autotetraploid fish ($4n = 200$) with genetic variation (Qin et al. 2014).

The abnormal meiosis was a potential reason to produce unreduced gametes. The unreduced gametes could be produced by immature cytokinesis, namely, the meiotic division without reentry of the polar body into the female pronucleus or the second meiotic division. Another possibility is that the second polar body was released abortively during the second meiosis or the sister chromatids could not separate normally. In the hybrids of red crucian carp and blunt snout bream, the triploids formed by the retention of second polar body (Liu et al. 2007b; Liu et al. 2010).

Polyspermy refers to an egg fertilized by two or more sperm at the same time (Ramsey and Schemske 1998). It has been described in some polyploidy orchids and is the most common mechanism leading to human triploids (Otto and Whitton 2000). However, it was not regarded as a common mechanism for the formation of polyploidy.

1.2.3.2 The Polyploidy Promoted by Hybridization

During the process of speciation, the phenomenon of hybridization and gene flow (introgression) were frequent, especially in the fast radiation group, which accelerates the process of the origination and evolution of species (Comai 2005; Seehausen 2004; Mallet 2005; Liu 2010; Liu et al. 2001a). Hybridization served as a

catalyst in terms of speciation and major evolutionary innovations. The somatic chromosome doubling of hybrid offspring may lead to allopolyploidy (Mallet 2005). Chromosome pairing of meiosis in hybrid diploids was often unsuccessful; however, it was usually overcome by producing unreduced gametes in hybrids (Otto 2007). The generation of polyploids from hybrid lineages was promoted after the fusion and formation of unreduced gametes. Current research shows that whole-genome replication is beneficial to biological evolution. We thought that distant hybridization is an important driving force for polyploidy that accompanied genome replication.

Chapter 1 and other chapters will provide a detailed description of the research addressing these two distant hybridization methods for producing different ploidy fishes. In summary, we believe that distant hybridization plays an important role in the evolution and genetic breeding of fish according to previous researches.

1.2.3.3 The Parthenogenesis and the "Triploid Bridge"

The parthenogenetic polyploids in animals enriched the types of polyploid animals (Li 1980, 1991, 1992). During a long period of evolution, those unreduced eggs derived from distant cross can develop into diploid populations by gynogenesis. The diploids may produce unreduced eggs, which are fertilized with haploid sperm to form triploids. Through the way of gynogenesis, triploids can maintain their fertility. In addition, by backcrossing to diploids using their unreduced triploid eggs, tetraploids would be generated (Zan 1985). Using triploids as ladders, the "triploid bridge" was formed by the indirect route of tetraploid formation (Soltis et al. 2004; Mallet 2007). Higher-ploidy populations could have evolved from tetraploids. Several higher-polyploidy plants were generated through the "triploid bridge" (Harlan and deWet 1975). For instance, the backcrossing of spontaneous triploids and diploids obtained 1% of tetraploid progenies (*Populus tremula*). Similarly, the triploid apple varieties, a spontaneous polyploids, could produce a small number of tetraploid progenies (Ramsey and Schemske 1998). Moreover, higher-polyploidy animals were also generated through the "triploid bridge." For example, the combination of fertile autotriploid eggs from female allotetraploid fish and haploid sperm from male crucian carp-like homodiploid fish produced a large number of autotetraploid fish (Wang et al. 2020b). All the above-mentioned triploid and tetraploid fish produced unreduced triploid eggs during reproduction. Allotriploid fish produced by mating allotetraploid with diploid are all infertile. Although these infertile allotriploids are at an evolutionary dead end, their infertility is significant for aquaculture applications.

1.2.3.4 The Biological Habits Promote Polyploids

Both reproduction systems and growth habits of species had an effect on the production of unreduced gametes (Ramsey and Schemske 1998). The proportion of polyploids is higher in perennial herbs with vegetative reproduction. It is possible that the existence of vegetative reproduction reduced the selective pressure for zoogamy, thus providing a loose environment for producing unreduced gametes and other nonfunctional gametes, and further accelerated the production of unreduced gametes.

1.2.3.5 The Environmental Factors Affect the Occurrence of Polyploids

The environment, particularly the temperature, had an important effect on polyploid formation (Otto and Whitton 2000). The chromosome doubling could be promoted by radiation acting and environmental factors such as cold or heat stimulation on diploid fertilized eggs. When these factors act on meiosis of diploids, they can inhibit the release of the polar body, causing the production of polyploids. In China, the plateau uplift of the Qinghai-Tibet Plateau led to rapid changes in temperature and humidity, and the southeastern margin of the plateau and its surrounding areas of the plateau became a major region for polyploid fish formation (Zan 1985). In plants, a dramatic increase was observed in diploid pollen production of lilium (*Uvularia grandiflora*) after the abnormal cold period (Belling 1925). In potato (*Solanum tuberosum*), at different temperatures, the frequency of diploid gametes was almost twice as different (McHale 1983).

1.2.4 The Genetic Effects of Polyploidy and Changes in Gene Expression

1.2.4.1 The Genomic Change after Polyploid Formation

The rapid genomic recombination and instable genome are the most characteristic features of new polyploidy, polyploidy is the attempt to achieve the harmonious coexistence of multiple genomes in a single nucleus (Comai 2005; Chen and Ni 2006). For example, in the hybrids of polyploid *Brassica*, extensive genomic rearrangement and fragment loss were observed within five generations (Song et al. 1995). The structural changes included genomic deletions, insertions, duplications, translocations, and transpositions (Chen and Ni 2006). At least nine intergenomic chromosomal rearrangements in allotetraploid tobacco (*Nicotiana tabacum*) were identified, and the majority of chromosomes in the tobacco genome possessed the parental genes (Kenton et al. 1993). Similarly, through GISH analysis, five intergenomic translocations in allotetraploid oat (*Avena*) were detected and approximately 18 in the allohexaploid (Jellen et al. 1994).

In this chapter, the phenotypic and genotypic changes are introduced in allotetraploid hybrid lineage derived from red crucian carp (♀) × common carp (♂), autotetraploid fish lineage derived from red crucian carp (♀) × blunt snout bream (♂), and diploids hybrid lineage derived from blunt snout bream (♀) × topmouth culter (♂). The results showed that changed genotypes and phenotypes of different ploidy fish formed by distant hybridization are beneficial to the evolution of these hybrids and their application in fish genetic breeding.

1.2.4.2 The Changes in Gene Expression in Polyploids

The polyploids went through changes in gene expression except the changes of genome structure (Kellogg 2003; Lee and Chen 2001), such as gene silencing, downregulation or upregulation of expression, neofunctionalization, nonfunctionalization, and subfunctionalization. Both genetic and epigenetic mechanisms in this context played important roles (Chen and Tian 2007). Genetic

changes resulted from changes in DNA sequence, leading to the loss of genes or permanent changes in genomic DNA. Epigenetic alterations including histone modification, DNA methylation, dosage compensation, and RNA interference affected gene expression and showed obvious phenotypic effects (Liu and Wendel 2003). Treatment with a DNA methyltransferase inhibitor altered the morphology of allotetraploid *Arabidopsis* and its gene expression through DNA demethylation, demonstrating the epigenetic regulation of transcriptional expression of allopolyploids (Madlung et al. 2002). In addition, the activation and inhibition of transcriptional elements can also lead to the alterations in gene expression and further promote the rapid recombination of the polyploidy genome (Fedoroff 2000). Recent studies have investigated the epigenetic changes in polyploid fish. The global DNA methylation state of allotetraploid fish of red crucian carp (♀) × common carp (♂) differed from that of their parents (Xiao et al. 2013). The miRNA profile of ovary tissue showed the difference between allotetraploid fish of red crucian carp (♀) × common carp (♂) and red crucian carp. The differentiated miRNA mainly targeted such as metabolism-related genes, but not fertility-related genes (Zhou et al. 2015). The allotriploid fish, as another kind of polyploid fish, exhibited sterile characteristics. The DNA methyltransferase gene, *dnmt*3, showed different expression patterns in the ovary of allotriploid fish and red crucian carp (Zhou et al. 2019b). The histone methylation state of H3 displayed significant change in the ovary of allotriploid fish when compared with red crucian carp (Zhou et al. 2019a).

1.2.4.3 Diploidization

In order to maintain stability of genome after merging and doubling, the allopolyploid usually experienced diploidization to eliminate widespread genomic incompatibilities (Doyle et al. 2008). Cytologically, during meiosis incomplete pairing of homologous chromosomes formed which resulted in the unfunctional gametes, but allopolyploids usually exhibited bivalent chromosome pairing, a diploid-like meiotic behavior, but not multivalent chromosome pairing (Otto 2007). In allopolyploid wheat, some particular factors like *Ph1* acted as a "local editor" to ensure specific centromere binding among homologous chromosomes (Ma and Gustafson 2005). The allotetraploids of *Arabidopsis* were capable of homologous pairing during the first three generations after they were formed. It may be that the parents provided genes that controlled the pairing behavior, or the characteristics of the parental chromosomes prevented homoeologous pairing. Moreover, the elimination of genomic sequences and rearrangement of chromosome benefited from diploidization of cytology (Ma and Gustafson 2005), as the demonstrations in many polyploid plants. In autopolyploids, consequent genetic changes, gene sequence changes, and chromosome rearrangement might increase pairing fidelity over time, resulting in disomic inheritance ultimately (Otto 2007). Genetically, gene expression in polyploids was decreased to avoid redundancy of duplicated genes (Ma and Gustafson 2005).

The changes in functional diversity and gene expression could be promoted by the presence of a large number of epigenetic modifications such as repetitive

sequences, transposable elements, and DNA methylation in polyploids, further accelerating the process of diploidization (Soltis et al. 2004). Diploidization of inheritance and function facilitates the slow transition from polyploids to new diploids. There are still many questions regarding diploidization, and further research is required. Studies on artificial polyploids have shown their diploidization process, but it is still unknown whether these polyploids will become diploids. In short, the fertility and stability of polyploids were ensured by the diploidization behavior, which is conducive to their survival and reproduction.

1.2.4.4 The Changes Promote the Evolution of Polyploids

A large number of redundant genes can protect polyploids from the harmful effects of mutations. Polyploids could mask harmful recessive alleles though the expression dominant alleles. Diversification of gene functions could be promoted by changing redundant copies of essential or important genes (Comai 2005). In addition, hybrid polyploids could merge the advantageous traits of parents and further present heterosis by integrating different genomes. Compared with similar diploid organisms, it shows better environmental adaptability. Polyploids can innovate and improve its function through rapid or slow changes in genome and gene expression to achieve success in speciation and evolution.

1.2.5 Polyploid Breeding

1.2.5.1 The Application of Polyploid Breeding in Plants

The research on biochemical characters and growth in natural polyploid plants revealed that polyploids, particularly allopolyploids, presented several obvious advantages, such as faster metabolism, larger vegetative organs, enhanced stress resistance, and more secondary metabolites (Ma et al. 2008). Artificial polyploid breeding is becoming increasingly common in plants. For vegetable crops (Yang et al. 2006), fruit trees (Wang et al. 2004b), horticultural plants (Sun and Zhang 2004), and medicinal plants (Zhang et al. 2009), by the application of distant hybridization, artificial selective breeding, the induction of breeding by physico-chemical factors, somatic hybridization, protoplast culture, and tissue culture, more polyploid lineages were established and widely applied.

One of the well-known applications in polyploid breeding was seedless triploid watermelon (Yuan et al. 2009). The acquisition of tetraploid parents was the key to produce triploid watermelon. The treatment of colchicine was the most common method. When treating diploid watermelon seeds and seedlings with colchicine, it hindered the formation of spindle fibers. By this way, tetraploid watermelons were obtained with doubled genome. Triploid seeds were produced by ploidy interbreeding between tetraploid and diploid watermelons. Triploid seedless watermelons presented obvious advantages, such as larger fruit, increased stress resistance, a higher sugar content, and a higher yield, which were valued for consumers and growers. Thus, China has become the largest producing country of seedless watermelon.

Some other polyploid lineages have been established by the breeding technology of hybridization and selective breeding in *Fragaria* (X = 7), *Prunus* (X = 8), *Malus* (X = 17), and *Pyrus* (X = 17) (Wang et al. 2004b). By the ways of hybridization, artificial induction, and tissue culture to induce chromosome duplication, polyploid cucumber (*Cucumis sativus*), cabbage (*B. rapa pekinensis*), lily (*Lilium brownii*), chili pepper (*Capsicum*), and lettuce (*Lactuca sativa*) had been produced, some of which such as cabbage had been widely planted and applied (Yang et al. 2006). Among horticultural plants, polyploid balsamine (*Impatiens balsamina*), Armeniaca mumeBeauty mei (*Prunus blireana* 'Meiren'), *Cymbidium goeringii*, *Dendrobium devonianum*, mandala (*Datura stramonium*), and common calla (*Zantedeschia aethiopica*) were acquired (Sun and Zhang 2004). The formed polyploid medicinal plants, such as *Twotooth achyranthes*, *Glycyrrhiza pallidiflora*, *Isatis tinctoria*, *Radix angelicae dahuricae*, *Lonicera japonica*, and *Salvia miltiorrhiza*, had the characteristics of increased stress resistance and higher output (Zhang et al. 2009).

1.2.5.2 The Application of Polyploid Breeding in Animals

The polyploid breeding of animals had played an important role in aquatic organisms, and the polyploid breeding had experienced tremendous progress in marine fish, freshwater fish, crustaceans, and shellfish. A lot of polyploids has been produced by biological (nuclear transfer, distant hybridization, cell fusion), chemical (the use of chemical inducers such as caffeine, colchicine, polyethylene glycol, and cytochalasin), or physical (hydrostatic pressure shock and temperature shock) methods (Shen and Yao 2004).

The generation of triploid fish is mainly through two ways. The first one is direct production including hybridization, physical, and chemical methods, and the other one is indirect production by the hybridization of tetraploid fish and diploid fish. It is difficult to guarantee the production of 100% triploids via the former method, but the latter method can achieve this goal. The artificial induction of polyploidy fish by chemical or physical methods was based on the inhibition of the first cleavage of fertilized eggs or the retention of the first or the second polar body of oocytes, thus obtaining tetraploids or triploids (Hu and Li 2009).

Polyploid breeding using temperature shock treatment or cytochalasin in shellfish and crustacean often resulted in high mortality during larval development or at the stage of early embryo (Song et al. 2010; Wang et al. 2004c). The treatment of hydrostatic pressure shock and temperature shock was usually used to induction of polyploid fish. Heat shock was performed in yellow catfish (*Pelteobagrus fulvidraco*) and 57% triploids were successfully obtained (Song et al. 2010). By the way of hydrostatic pressure shock, the transparent triploid colored crucian carp was obtained. In addition, through the combination of cold shock and hydrostatic pressure shock, tetraploid embryos were produced (Gui et al. 1990).

Although triploid fish can be obtained by the above methods, they cannot guarantee 100% triploid offspring. There were no reports on the production of fertile tetraploid fish, through inhibiting the first cleavage of fertilized eggs. Each generation of tetraploid broodstock must be processed manually, so the cost was increased.

However, the preparation of polyploids through artificial physical and chemical factors was different from the formation of polyploids in nature, which might result in the formation of aneuploidy individuals. The chemical inducers induced the formation of polyploid, which often influenced the genetic material or other structures in the cytoplasm, affecting the development and activity of the individuals, and were prone to produce deformities and chimeras.

Hybridization could accelerate the process of speciation in nature. In fish, artificial hybridization corresponds to the laws of natural evolution and accelerated the formation of polyploid populations. It has been proven that the most practical and effective biological method to obtain allopolyploid fish was distant hybridization. In the cross of grass carp and common carp, several allotetraploid fish were obtained (Wu et al. 1988). In addition, the compound tetraploid allogynogenetic gibel carp was identified in an artificial group (Gui et al. 1992). By crossing hybrids of Xingguo red carp × red crucian carp with scattered scale mirror carp, an artificial composite triploid was acquired, but only the females of the complex triploid were fertile (Wu et al. 1993). Although these studies did not form tetraploid populations contained fertile female and fertile male simultaneously, nor were put into production, they provided useful reference data for polyploid breeding by distant hybridization.

In the distant hybridization of red crucian carp and common carp, partial fertile diploid fish were found in the first generation, when the parents had the same chromosome number. The F_2 hybrids obtained by self-crossing of the F_1 were all diploid fish. The allotetraploid hybrids ($4n = 200$ or $2n = 4X = 200$, abbreviated as $4n$AT) were found among the F_3 hybrids obtained by self-crossing of F_2 (Liu 2010; Liu et al. 2001b). To date, the $4n$AT has been propagated to F_{29} and formed a stable tetraploid lineage. It is the first fertile allotetraploid lineage (containing fertile female and male simultaneously) with stable genetic traits in the world that provided abundant tetraploid resources for producing massive sterile triploid fish. Through the crossing of male $4n$AT with female diploid crucian carp or common carp, large-scale sterile triploid fish ($3n = 150$, or $2n = 3X = 150$) were produced. These triploid fish have been promoted and farmed in China widely and easily accepted by fish farmers and have produced significant economic, social, and ecological benefits. In addition, by crossing sex-reversal diploid Japanese white crucian carp with female $4n$AT, the all-female triploid fish was obtained. The all-female triploid fish was the first example of polyploid fish in the world by the combined way of sex reversal, gynogenesis, and ploidy breeding (Luo et al. 2011). The fertile and genetically stable allotetraploid lineage with the inheritance of heterosis may become a new species (Liu 2010; Liu et al. 2001b). The fertile allotetraploids provide an excellent model system to explore the polyploid origin in nature and the evolution regarding fish.

The distant hybridization of red crucian carp (♀) × blunt snout bream (♂), which were parents with different chromosome numbers, had also made remarkable progress (Liu et al. 2007b; Liu 2010; Qin et al. 2010). In the first generation, two types of offspring were obtained, including sterile triploid fish ($3n = 124$, or $2n = 3X = 124$) and fertile tetraploid fish ($4n = 148$, or $2n = 4X = 148$). The tetraploid fish could produce reduced and unreduced gametes. Thus, the autotetraploid lineage

($4n = 200$, or $2n = 4X = 200$) could be established by the self-mating of the tetraploid fish. The first generation of tetraploid hybrids has been propagated to F_{15} by self-mating. A new type of triploid fish ($3n = 150$, or $2n = 3X = 150$) was obtained by crossing the tetraploid fish and diploid crucian carp. Two kinds of pentaploid hybrids ($5n = 198$ and $5n = 172$) could be produced using the eggs of the first generation of tetraploid hybrids ($4n = 148$) and the sperm of the red crucian carp and blunt snout bream, respectively. In addition, we have performed systematic research on distant hybridization, such as crosses of red crucian carp (♀) × topmouth culter (♂), red crucian carp (♀) × Bleeker's yellow tail (♂), blunt snout bream (♀) × topmouth culter (♂), blunt snout bream (♀) × Bleeker's yellow tail (♂), and grass carp (♀) × blunt snout bream (♂) (Liu 2010). We hope that more hybrids may be generated to accelerate the development of aquaculture and provide information related to evolutionary theory.

1.2.5.3 The Significance of the Study of Polyploids

Based on the in-depth study regarding polyploid organisms, researchers believed that polyploidization had played an important role in the evolutionary history of organisms. Traditional research mostly focuses on artificially produced allopolyploid model plants and crop plants, such as *Arabidopsis*, *Brassica*, cotton (*Gossypium*), wheat (*Triticum*), tobacco (*Nicotiana*), and corn (*Zea mays*), and understood the characteristics of the genome and gene changes following polyploidization. Recently, with the rapid development of genome sequencing, the techniques of modern molecular biology and bioinformatics were usually used to study wild polyploids. However, researchers hold different views regarding the exact time of polyploidy, the frequency, rate, and ploidy times of different organisms in nature. Therefore, it is very necessary to carry out more research on additional species, especially polyploid model animals, to reveal the above problems.

Due to the lack of models, research advances in polyploid animals are relatively slow. In some model plants, the study of the effects of epigenetics on genotype and phenotype has reached a new height. However, there are relatively few studies on epigenetics in polyploid animals. The lineages, including the allotetraploid lineage of red crucian carp (♀) × common carp (♂), the autotetraploid lineage of red crucian carp (♀) × blunt snout bream (♂), and the autotetraploid lineage of common carp (♀) × blunt snout bream (♂), strictly established good models for the study of polyploid animals. The ongoing work involving bacterial artificial chromosome library construction, whole-genome sequencing, transcriptome sequencing, the identification of gene clusters, and methylation assays will provide strong data and theoretical support for the study of animal polyploidization.

Although a lot of studies concerning polyploidization have been reported, further in-depth study regarding the associated mechanisms of polyploid formation was required. There is no doubt that continuing studies of polyploid plants and animals will be of great significance in biological evolution, genetics, and breeding.

References

Abbas K, Li MY, Wang WM, Zhou XY (2009) First record of the natural occurrence of hexaploid loach *Misgurnus anguillicaudatus* in Hubei Province, China. J Fish Biol 75(2):435–441

Ahuja M, Neale D (2005) Evolution of genome size in conifers. Silvae Genetica 54:126–137

Albert VA, Soltis DE, Carlson JE, Farmerie WG, Wall PK, Ilut DC, Solow TM, Mueller LA, Landherr LL, Hu Y, Buzgo M, Kim S, Yoo M-J, Frohlich MW, Perl-Treves R, Schlarbaum SE, Bliss BJ, Zhang X, Tanksley SD, Oppenheimer DG, Soltis PS, Ma H, DePamphilis CW, Leebens-Mack JH (2005) Floral gene resources from basal angiosperms for comparative genomics research. BMC Plant Biol 5:5

Amores A, Force A, Yan Y-L, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang Y-L, Westerfield M, Ekker M, Postlethwait JH (1998) Zebrafish *hox* clusters and vertebrate genome evolution. Science 282(5394):1711–1714

Bidwell CA, Larry Chrisman C, Libey GS (1985) Polyploidy induced by heat shock in channel catfish. Aquaculture 51(1):25–32

Blomme T, Vandepoele K, De Bodt S, Simillion C, Maere S, Van de Peer Y (2006) The gain and loss of genes during 600 million years of vertebrate evolution. Genome Biol 7(5):R43–R43

Boron A, Kotusz J (2000) The preliminary data on diploid-polyploid complexes of the genus *Cobitis* in the Odra River basin, Poland (Pisces, Cobitidae). Folia Zool 49:79–84

Bowers JE, Chapman BA, Rong J, Paterson AH (2003) Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. Nature 422(6930):433–438

Bunch TD, Foote WC, Juan Spillett J (1976) Sheep-goat hybrid karyotypes. Theriogenology 6(4):379–385

Chen M, Yang X, Yu X, Chen H, Yi Y, Liu H (1997) Chromosome ploidy manipulation of allotetraploids and their fertility in Japanese phytophagous crucian carp (JPCC)(♀) × red crucian carp (RCC)(♂). Acta Hydrobiol Sinica 21(3):197–206

Chen S (1984) Investigation on the inter-subfamily hybridization (*Mylopharyngodon piceus* R. (♀) × *Megalobrama terminalis* R. (♂)) I. comparative cytogenetic study on *Mylopharyngodon piceus* R. (♀), *Megalobrama terminalis* R. (♂) and their F_1 generation. J Nat Sci Hunan Norm Univ 4:71–80

Chen S, Wang J, Liu S, Qin Q, Xiao J, Duan W, Luo K, Liu J, Liu Y (2009) Biological characteristics of an improved triploid crucian carp. Sci China C Life Sci 52:733–738

Chen ZJ, Ni Z (2006) Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. BioEssays 28(3):240–252

Chen ZJ, Tian L (2007) Roles of dynamic and reversible histone acetylation in plant development and polyploidy. Biochim Biophys Acta 1769(5–6):295–307

Chourrout D, Chevassus B, Krieg F, Happe A, Burger G, Renard P (1986) Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females — potential of tetraploid fish. Theor Appl Genet 72(2):193–206

Chourrout D, Nakayama I (1987) Chromosome studies of progenies of tetraploid female rainbow trout. Theor Appl Genet 74(6):687–692

Comai L (2005) The advantages and disadvantages of being polyploid. Nat Rev Genet 6(11):836–846

Cui L, Wall PK, Leebens-Mack JH, Lindsay BG, Soltis DE, Doyle JJ, Soltis PS, Carlson JE, Arumuganathan K, Barakat A, Albert VA, Ma H, dePamphilis CW (2006) Widespread genome duplications throughout the history of flowering plants. Genome Res 16(6):738–749

Dehal P, Boore JL (2005) Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biol 3(10):e314

Department of biology SU, Taiyuan agriculture fawcss (1973) Preliminary study on artificial hybridization of white Amur bream × black Amur bream. Freshw Fisher 5:6–9

Doyle JJ, Flagel LE, Paterson AH, Rapp RA, Soltis DE, Soltis PS, Wendel JF (2008) Evolutionary genetics of genome merger and doubling in plants. Annu Rev Genet 42(1):443–461

Fedoroff N (2000) Transposons and genome evolution in plants. Proc Natl Acad Sci 97(13):7002–7007
Fisheries experimental station of Beijing (1973) A preliminary summary of fish introduction and hybridization test. Freshw Fisher 3:15–18
Gates RR (1909) In: Engelmann W (ed) The stature and chromosomes of Oenothera gigas De Vries. Leipzig, Berlin
Grant PR, Grant BR, Petren K (2005) Hybridization in the recent past. Am Nat 166(1):56–67
Gray A (ed) (1954) Mammalians hybrids commonwealth. Cambridge University Press, New York
Gregory TR, Mable BK (2005) CHAPTER 8 - polyploidy in animals. In: Gregory TR (ed) The evolution of the genome. Academic Press, Burlington, pp 427–517
Gui J, Liang S, Sun J, Huang W, Jiang Y (1990) Studies on genome manipulation in fish I. induction of triploid transparent colored crucian carp (*Carassius auratus* transparent colored variety) by hydrostatic pressure. Acta Hydrobiol Sinica 14(4):336–344
Gui J, Liang S, Zhu L, Jiang Y (1992) Preliminary proof of the mode of gynogenesis of artificial tetraploid gibel carp. Science Bulletin 9:836–838
Gui J, Liang S, Zhu L, Sun J, Jiang Y (1993) Cytogenetic analysis of developmental difference in hybrid embryos between reciprocal crosses in distant hybridization of fishes. Zool Res 14(2):171–177
Gui J, Sun J, Liang S, Huang W, Jiang Y (1991) Studies on genome manipulation in fish II. Tetraploidy induced by hydrostatic pressure treatment and combination of hydrostatic pressure and cold treatments in transparent colored crucian carp. Acta Hydrobiol Sinica 15(4):333–342
Gui J, Zhou L (2010) Genetic basis and breeding application of clonal diversity and dual reproduction modes in polyploid *Carassius auratus gibelio*. Sci China Life Sci 53(4):409–415
Guo H, Tu F, Wang B (1966) Preliminary observation of *Ctenopharyngodon idellus* and *Aristichthy nobilis* artificial hybridization and their offspring. Chin J Zool 4:188–189
Hair JB (1968) The chromosomes of the Cupressaceae. N Z J Bot 6(3):277–284
Harlan JR, deWet JMJ (1975) On Ö. Winge and a prayer: the origins of polyploidy. Bot Rev 41(4):361–390
He W, Xie L, Li T, Liu S, Xiao J, Hu J, Wang J, Qin Q, Liu Y (2013) The formation of diploid and triploid hybrids of female grass carp × male blunt snout bream and their 5S rDNA analysis. BMC Genet 14(1):110
Holland PWH, Garcia-Fernàndez J, Williams NA, Sidow A (1994) Gene duplications and the origins of vertebrate development. Development 1994(Supplement):125–133
Hu J, Liu S, Xiao J, Zhou Y, You C, He W, Zhao R, Song C, Liu Y (2012) Characteristics of diploid and triploid hybrids derived from female *Megalobrama amblycephala* Yih × male *Xenocypris davidi* Bleeker. Aquaculture 364-365:157–164
Hu L, Li J (2009) Reviews on the progresses of researches on fish polyploid breeding. Fish Sci Technol 5:7–10
Ivanova EE (1960) The hybridization between species of *Bos grunniens* and large footed animals. The Soviet Academy of Sciences Press, Moscow
Jaillon O, Aury J-M, Noel B, Policriti A, Clepet C, Casagrande A, Choisne N, Aubourg S, Vitulo N, Jubin C (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449(7161):463–467
Jellen EN, Gill BS, Cox TS (1994) Genomic *in situ* hybridization differentiates between a/D- and C-genome chromatin and detects intergenomic translocations in polyploid oat species (genus *Avena*). Genome 37(4):613–618
Jiang S, Li J, Qu Y, Zhong C, Yu D (1997) Studies on hybridization female silver bream *Rhabdosargus sarba* and × male red bream *Pagrosomus major*. Mar Sci 5:33–38
Jiang Y, Liang S, Chen B, Yu H, Shan S, Yang D, Lin S, Shen G (1983) Biological effect of heterologous sperm on gynogenetic offspring in Carassius auratus gibelio. Acta Hydrobiol Sinica 8(1):1–13
Karpechenko G (2010) The production of Polyploid gametes in hybrids. Hereditas 9:349–368

Kassahn KS, Dang VT, Wilkins SJ, Perkins AC, Ragan MA (2009) Evolution of gene function and regulatory control after whole-genome duplication: comparative analyses in vertebrates. Genome Res 19(8):1404–1418

Kellogg EA (2003) What happens to genes in duplicated genomes. Proc Natl Acad Sci 100(8):4369–4371

Kenton A, Parokonny AS, Gleba YY, Bennett MD (1993) Characterization of the *Nicotiana tabacum* L. genome by molecular cytogenetics. Mol Gen Genet 240(2):159–169

Lampert KP, Schartl M (2008) The origin and evolution of a unisexual hybrid: *Poecilia formosa*. Philos Trans R Soc B 363(1505):2901–2909

Lee H-S, Chen ZJ (2001) Protein-coding genes are epigenetically regulated in *Arabidopsis* polyploids. Proc Natl Acad Sci 98(12):6753–6758

Li S (1980) Polyploid in vertebrates. Chin J Zool 2:52–54

Li S (1991) Chromosomes of amphibian and its revolution. Chin J Zool 26(2):47–52

Li S (1992) Parthenogenetic reptiles. Chin J Zool 27(1):41–44

Li S (2002) Polyploid insects. Knowledge Insects 39(2):147–151

Li W, Chen S, Ji X, Xie M, Xu Y, Deng H (2012) Induction and identification of tetraploid fry in *Cynoglossus semilaevis*. J Fishery Sci China 19(2):196–201

Lin Y (1984) A comparative of the karyotypes in Chinese bream, herbivorous bream and their hybrid. Zool Res 5(3):65–66

Liu B, Wendel JF (2003) Epigenetic phenomena and the evolution of plant allopolyploids. Mol Phylogenet Evol 29(3):365–379

Liu G, Bao Z, Hu J, Wang S, Yao B, Zhan A (2006) ISSR analysis of two species of scallop (*Chlamys farreri*, *C .nobilis*) and their intra-and inter-species mating descendants. J Ocean Univ China 36(1):71–75

Liu G, Wu W, Lin L, Xu D, Zheng Y (1987) A cytological study on the cross fertilization of red common carp with grass carp. J Fish China 11(1):17–21

Liu J, Liu S, Tao M, Li W, Liu Y (2007a) Isolation and expression analysis of testicular type Sox9b in allotetraploid fish. Mar Biotechnol 9(3):329–334

Liu Q, Liu J, Liang Q, Qi Y, Tao M, Zhang C, Qin Q, Zhao R, Chen B, Liu S (2019) A hybrid lineage derived from hybridization of *Carassius cuvieri* and *Carassius auratus* red var. and a new type of improved fish obtained by back-crossing. Aquaculture 505:173–182

Liu Q, Wang Y, Liu S, Guo X, Luo K, Zhang C, Liu Y (2004) Comparison of blood and blood cells in different ploidy cyprinid fishes. Prog Nat Sci 14(10):1111–1117

Liu R, Wang H, Chen J (1985) Investigation on sexual difference composition of serum protein of two tilapia and their hybrid. J Fish China 9(3):265–273

Liu S (1987a) Studies of insemination cytology in hybridization between grass carp and freshwater bream. J Fish China 11(13):225–232

Liu S (1987b) Studies on cytogenetics of *Ctenopharyngodon idellus*, *Megalobrama terminalis* and their triploid F1 hybrid. Acta Hydrobiol Sinica 11(1):52–58

Liu S, Cao Y, He X, Li J, Liu Y (2001a) The formation of allotetraploid hybrids of common carp with red crucian carp and evolutionary significance of tetraploidization in vertebrate. Eng Sci 3(12):33–41

Liu S, Liu Y, Zhou G, Zhang X, Luo C, Feng H, He X, Zhu G, Yang H (2001b) The formation of tetraploid stocks of red crucian carp × common carp hybrids as an effect of interspecific hybridization. Aquaculture 192(2):171–186

Liu S, Luo J, Chai J, Ren L, Zhou Y, Huang F, Liu X, Chen Y, Zhang C, Tao M, Lu B, Zhou W, Lin G, Mai C, Yuan S, Wang J, Li T, Qin Q, Feng H, Luo K, Xiao J, Zhong H, Zhao R, Duan W, Song Z, Wang Y, Wang J, Zhong L, Wang L, Ding Z, Du Z, Lu X, Gao Y, Murphy RW, Liu Y, Meyer A, Zhang Y-P (2016) Genomic incompatibilities in the diploid and tetraploid offspring of the goldfish × common carp cross. Proc Natl Acad Sci 113(5):1327–1332

Liu S, Qin Q, Wang Y, Zhang H, Zhao R, Zhang C, Wang J, Li W, Chen L, Xiao J, Luo K, Tao M, Duan W, Liu Y (2010) Evidence for the formation of the male gynogenetic fish. Mar Biotechnol 12(2):160–172

Liu S, Qin Q, Xiao J, Lu W, Shen J, Li W, Liu J, Duan W, Zhang C, Tao M, Zhao R, Yan J, Liu Y (2007b) The formation of the polyploid hybrids from different subfamily fish crossings and its evolutionary significance. Genetics 176(2):1023–1034

Liu S, Sun Y, Zhou G (2003) The mature gonads of allotetraploid crucian carp group and ultrastructural observation of red blood cell. Prog Nat Sci 13(2):194–197

Liu SJ (2010) Distant hybridization leads to different ploidy fishes. Sci China Life Sci 53(4):416–425

Liu Y, Chen S, Wang Y (1981) Cytological study on the fertilization of the egg of *Mylopharyngodon piceus* with the sperm of *Megalobrama terminalis*. Acta Hydrobiol Sinica 7(3):329–340

Liu Z (ed) (1991) Genetics (in Chinese). Higher Education Press, Beijing

Loeb J (1903) The fertilization of the egg of the sea-urchin by the sperm of the starfish. Univ. Cal. Pub. Physiology I, Oakland

Lu W, Liu S, Long Y, Tao M, Zhang C, Wang J, Xiao J, Chen S, Liu J, Liu Y (2009) Comparative study of erythrocytes of polyploid hybrids from various fish subfamily crossings. Cell Tissue Res 336(1):159–163

Lu X, Sun J, Wang H, Luo D, Hou X, Liu L, Li G (2013) Observations on embryonic development of reciprocal hybrids of *Siniperca kneri* Garman × *Siniperca chuatsi* Basilewsky and F_2 of *S. kneri* females × *S. chuatsi* males F1. J Fisheries Sci China 20(5):975–981

Luo J (1991) Polyploidy fishes and fish breeding using polyploidization. Zhujiang Fishery 17:69–74

Luo K, Xiao J, Liu S, Wang J, He W, Hu J, Qin Q, Zhang C, Tao M, Liu Y (2011) Massive production of all-female diploids and triploids in the crucian carp. Int J Biol Sci 7(4):487–495

Ma H, Zhang J, Li Z (2008) Research progresses on breeding technology of plant polyploids. Protect Forest Sci Technol 1(82):43–46

Ma XF, Gustafson J (2005) Genome evolution of allopolyploids: a process of cytological and genetic diploidization. Cytogenet Genome Res 109:236–249

Mable BK (2004) 'Why polyploidy is rarer in animals than in plants': myths and mechanisms. Biol J Linn Soc 82(4):453–466

Madlung A, Masuelli RW, Watson B, Reynolds SH, Davison J, Comai L (2002) Remodeling of DNA methylation and phenotypic and transcriptional changes in synthetic Arabidopsis allotetraploids. Plant Physiol 129(2):733–746

Mallet J (2005) Hybridization as an invasion of the genome. Trends Ecol Evol 20(5):229–237

Mallet J (2007) Hybrid speciation. Nature 446(7133):279–283

McGovern P (1973) The effect of maternal immunity on the survival of goat × sheep hybrid embryos. J Reprod Fertil 34(2):215–220

McHale NA (1983) Environmental induction of high frequency 2*n* pollen formation in diploid Solanum. Can J Genet Cytol 25(6):609–615

Meyer A, Salzburger W, Schartl M (2006) Hybrid origin of a swordtail species (Teleostei: *Xiphophorus clemenciae*) driven by sexual selection. Mol Ecol 15(3):721–730

Meyer A, Van de Peer Y (2005) From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). BioEssays 27(9):937–945

Ming R, Hou S, Feng Y, Yu Q, Dionne-Laporte A, Sw JH, Senin P, Wang W, Ly BV, Lewis KLT, Salzberg SL, Feng L, Jones MR, Skelton RL, Murray JE, Chen C, Qian W, Shen J, Du P, Eustice M, Tong E, Tang H, Lyons E, Paull RE, Michael TP, Wall K, Rice DW, Albert H, Wang M-L, Zhu YJ, Schatz M, Nagarajan N, Acob RA, Guan P, Blas A, Wai CM, Ackerman CM, Ren Y, Liu C, Wang J, Wang J, Na J-K, Shakirov EV, Haas B, Thimmapuram J, Nelson D, Wang X, Bowers JE, Gschwend AR, Delcher AL, Singh R, Suzuki JY, Tripathi S, Neupane K, Wei H, Irikura B, Paidi M, Jiang N, Zhang W, Presting G, Windsor A, Navajas-Pérez R, Torres MJ, Feltus FA, Porter B, Li Y, Burroughs AM, Luo M-C, Liu L, Christopher DA, Mount SM, Moore PH, Sugimura T, Jiang J, Schuler MA, Friedman V, Mitchell-Olds T, Shippen DE, dePamphilis CW, Palmer JD, Freeling M, Paterson AH, Gonsalves D, Wang L, Alam M (2008)

The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). Nature 452(7190):991–996

Naruse K, Tanaka M, Mita K, Shima A, Postlethwait J, Mitani H (2004) A medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. Genome Res 14(5):820–828

Newton nCF, Pellew nC (1929) Primula kewensis and its derivatives. J Genet 20(3):405–467

Nolte AW, Freyhof J, Stemshorn KC, Tautz D (2005) An invasive lineage of sculpins, *Cottus* sp (Pisces, Teleostei) in the Rhine with new habitat adaptations has originated from hybridization between old phylogeographic groups. Proc R Soc B Biol Sci 272(1579):2379–2387

Ohno S (1970a) The enormous diversity in genome sizes of fish as a reflection of Nature's extensive experiments with gene duplication. Trans Am Fish Soc 99(1):120–130

Ohno S (1970b) Evolution by gene duplication. Springer-Verlag, Heidelberg, Berlin

Otto SP (2007) The evolutionary consequences of polyploidy. Cell 131(3):452–462

Otto SP, Whitton J (2000) Polyploid incidence and evolution. Annu Rev Genet 34:401–437

Pan G (1987) Preliminary studies on the hybrid between *Hypophthalmichthys molitrix* and *Megalobrama amblycephala*. Freshw Fish 1:17–19

Paterson AH, Bowers JE, Burow MD, Draye X, Elsik CG, Jiang C-X, Katsar CS, Lan T-H, Lin Y-R, Ming R, Wright RJ (2000) Comparative genomics of plant chromosomes. Plant Cell 12(9):1523–1539

Qin Q, He W, Liu S, Wang J, Xiao J, Liu Y (2010) Analysis of 5S rDNA organization and variation in polyploid hybrids from crosses of different fish subfamilies. J Exp Zool B Mol Dev Evol 314(5):403–411

Qin Q, Wang Y, Wang J, Dai J, Xiao J, Hu F, Luo K, Tao M, Zhang C, Liu Y, Liu S (2014) The autotetraploid fish derived from hybridization of *Carassius auratus* red var. (female) × *Megalobrama amblycephala* (male). Biol Reproduct 91(4):93, 1–11

Qu Y, Li J, Zhou H (2000) Larval development and growth of intergeneric crossing of Sparidae fishes. J Fishery Sci China 7(2):110–112

Ráb P, Rábová M, Bohlen J, Lusk S (2000) Genetic differentiation of the two hybrid diploid-polyploid complexes of loaches, genus Cobitis (Cobitidae) involving C-taenia, C-elongatoides and C. spp. in the Czech Republic: karyotypes and cytogenetic diversity. Folia Zool 49:55–66

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst 29(1):467–501

Refstie T (1981) Tetraploid rainbow trout produced by cytochalasin B. Aquaculture 25(1):51–58

Ren L, Li W, Qin Q, Dai H, Han F, Xiao J, Gao X, Cui J, Wu C, Yan X, Wang G, Liu G, Liu J, Li J, Wan Z, Yang C, Zhang C, Tao M, Wang J, Luo K, Wang S, Hu F, Zhao R, Li X, Liu M, Zheng H, Zhou R, Shu Y, Wang Y, Liu Q, Tang C, Duan W, Liu S (2019) The subgenomes show asymmetric expression of alleles in hybrid lineages of *Megalobrama amblycephala* × *Culter alburnus*. Genome Res 29(11):1805–1815

Saitoh K, Chen W-J, Mayden RL (2010) Extensive hybridization and tetrapolyploidy in spined loach fish. Mol Phylogenet Evol 56(3):1001–1010

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19(4):198–207

Severin AJ, Cannon SB, Graham MM, Grant D, Shoemaker RC (2011) Changes in twelve homoeologous genomic regions in soybean following three rounds of polyploidy. Plant Cell 23(9):3129–3136

Shen A, Yao W (2004) Research progresses on triploid breeding of aquatic animal. J Hydroecol 3(133):1–3

Soltis D, Albert V, Leebens-Mack J, Bell C, Paterson A, Zheng C, Sankoff D, dePamphilis C, Wall P, Soltis P (2009) Polyploidy and angiosperm diversification. Am J Bot 96:336–348

Soltis D, Soltis P, Rieseberg D (1993) Molecular data and the dynamic nature of polyploidy. Crit Rev Plant Sci 12:243–273

Soltis DE, Soltis PS, Tate JA (2004) Advances in the study of polyploidy since plant speciation. New Phytol 161(1):173–191

Song C, Liu S, Xiao J, He W, Zhou Y, Qin Q, Zhang C, Liu Y (2012) Polyploid organisms. Sci China Life Sci 55(4):301–311

Song K, Lu P, Tang K, Osborn TC (1995) Rapid genome change in synthetic polyploids of Brassica and its implications for polyploid evolution. Proc Natl Acad Sci 92(17):7719–7723

Song L, Yang Y, Wang W, Liu X, Yuan L (2010) Induction of triploidy in yellow catfish *Pelteobagrus fulvidraco* by heat shock. Fish Sci 29(6):352–255

Song Z (2013) Microsatellite DNA analysis and the evolution of Hox gene clusters in diploid hybrids derived from blunt snout bream × Culter and its original parents. Hunan Normal University, Changsha

Sun M, Zhang S (2004) The application of polyploid breeding in garden crop. Jiangsu Agric Sci 1: 67–72

Sun Y, Liu S, Zhang C, Li J, Huang W, Zhang J, Luo K, Zhou G, Liu Y (2003) The chromosome number and gonadal structure of F_9~F_{11} allotetraploid crucian-carp. Acta Genet Sin 30(5):414–418

Taylor JS, Van de Peer Y, Braasch I, Meyer A (2001) Comparative genomics provides evidence for an ancient genome duplication event in fish. Philos Trans R Soc Lond Ser B Biol Sci 356(1414):1661–1679

Thorgaard GH, Jazwin ME, Stier AR (1981) Polyploidy induced by heat shock in rainbow trout. Trans Am Fish Soc 110(4):546–550

Ting H-P (1956) Hybridization experiments of Peking anurans. J Fujian Norm Univ 2:1–8

Ullah Z, Lee CY, Depamphilis ML (2009) Cip/kip cyclin-dependent protein kinase inhibitors and the road to polyploidy. Cell Div 4(1):10

Veron AS, Kaufmann K, Bornberg-Bauer E (2007) Evidence of interaction network evolution by whole-genome duplications: a case study in MADS-box proteins. Mol Biol Evol 24(3):670–678

Volff JN (2005) Genome evolution and biodiversity in teleost fish. Heredity 94(3):280–294

Wan S, Huang E, Qi C, Wei Y (1987) Comparative experiment of production performance between all-male hybrid tilapia (Mozambique Tilapia × Nile tilapia) and Nile tilapia. Freshw Fish 2:15–16

Wang C, Zou G, Luo X, Pan G, Yang G, Zhu C (2004a) Comparative study on the electrophorgram of isozymes and proteins of *Silurus meridionalis, S asotus* and their hybrid. J Huazhong Agric Univ 25(3):281–285

Wang H, Liu R (1986) The study of the hybridization of *Hypophthalmichthys molitrix*♀ × *Cyprinus carpio* L♂ species. J Nanjing Univ (Natural Sciences) 1:88–94

Wang S, Jiao N, Zhao L, Zhang M, Zhou P, Huang X, Hu F, Yang C, Shu Y, Li W, Zhang C, Tao M, Chen B, Ma M, Liu S (2020a) Evidence for the paternal mitochondrial DNA in the crucian carp-like fish lineage with hybrid origin. Sci China Life Sci 63(1):102–115

Wang S, Ye X, Wang Y, Chen Y, Lin B, Yi Z, Mao Z, Hu F, Zhao R, Wang J, Zhou R, Ren L, Yao Z, Tao M, Zhang C, Xiao J, Qin Q, Liu S (2017) A new type of homodiploid fish derived from the interspecific hybridization of female common carp × male blunt snout bream. Sci Rep 7(1):4189

Wang S, Zhou P, Huang X, Liu Q, Lin B, Fu Y, Gu Q, Hu F, Luo K, Zhang C, Tao M, Qin Q, Liu S (2020b) The establishment of an autotetraploid fish lineage produced by female allotetraploid hybrids × male homodiploid hybrids derived from *Cyprinus carpio* (♀) × *Megalobrama amblycephala* (♂). Aquaculture 515:734583

Wang T, Zhang J, Qi Y, Pang H (2004b) Advances on polyploid breeding of fruit crops in China. J Fruit Sci 21(6):592–597

Wang Y, Yang C, Luo K, Zhang M, Qin Q, Huo Y, Song J, Tao M, Zhang C, Liu S (2018) The formation of the goldfish-like fish derived from hybridization of female koi carp × male blunt snout bream. Front Genet 9:437

Wang Z, Li K, Yu R, Zheng X, Wang R (2004c) Progress of tetraploid breeding in Molluscs. J Ocean Univ China 34(2):195–200

Woods IG, Wilson C, Friedlander B, Chang P, Reyes DK, Nix R, Kelly PD, Chu F, Postlethwait JH, Talbot WS (2005) The zebrafish gene map defines ancestral vertebrate chromosomes. Genome Res 15(9):1307–1314

Wu C, Ye Y, Chen R, Liu X (1993) An artificial multiple triploid carp and its biological characteristics. Aquaculture 111(1):255–262

Wu M (1988) Heredity and evolution of animal polyploids. Chin J Zool 23(5):48–51

Wu W, Li C, Liu G, Xu D, Liu C, Xie J, Shan C (1988) Studies on tetraploid hybrid between red common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idellus*) and its backcross triploid. Acta Hydrobiol Sinica 12(4):356–363

Wu W, Lin L, Xu D (1981) A tetraploidy hybrid crossing red carp Cyprinus carpio L. with grass carp Ctenopharyngoden idella Cuv. Et Val. Acta Hydrobiol Sinica 7(3):433–436

Xiao J (2013) Establishment of hybrid strains between blunt snout bream and topmouth culter and their genetic characteristic research. Hunan Normal University, Changsha

Xiao J, Kang X, Xie L, Qin Q, He Z, Hu F, Zhang C, Zhao R, Wang J, Luo K, Liu Y, Liu S (2014) The fertility of the hybrid lineage derived from female *Megalobrama amblycephala* × male *Culter alburnus*. Anim Reprod Sci 151(1):61–70

Xiao J, Song C, Liu S, Tao M, Hu J, Wang J, Liu W, Zeng M, Liu Y (2013) DNA methylation analysis of allotetraploid hybrids of red crucian carp (*Carassius auratus* red var.) and common carp (*Cyprinus carpio* L.). PloS One 8(2):e56409

Xiao J, Zou T, Chen Y, Chen L, Liu S, Tao M, Zhang C, Zhao R, Zhou Y, Long Y, You C, Yan J, Liu Y (2011) Coexistence of diploid, triploid and tetraploid crucian carp (*Carassius auratus*) in natural waters. BMC Genet 12(1):20

Yang A, Qingyin W, Liu Z, Zhang Y (2002a) Cytological observation on cross fertilization of *Chlamys farreri* and *Patinopecten yesoensis* with fluorescent microscope. Marine Fisheries Res 23(3):1–4

Yang H, Li S, Zou S (2002b) A primary study on inheritance of morphological traits from *Megalobrama amblycephala*, *Megalobrama terminalis* to their reciprocal hybrids(F_1). J Shanghai Fish Univ 11(4):305–309

Yang H, Xia D, Liu L, Wu T (2004) Studies on hereditary relationship between Oreochromis aurea (♀), Siniperca chuatsi (♂) and their offspring. J Fish China 28(5):594–598

Yang Y, Zhuang Y, Chen L (2006) Vegetable polyploid and polyploidy breeding. Acta Agric Univ Jiangxiensis 28(4):534–538

Ye M (1998) Polyploidy phenomenon and formation in animals and plants. Bull Biol 33(2):21–23

Ye Y, Wu Q, Chen R (1989) Studies on cytology or crosses between grass carp and carp-asynchronization between nucleus and cytoplasm in distant hybridization of fishes. Acta Hydrobiol Sinica 13:234–239

Yu C, Zhao Z, Li D, Li Y (2003a) Early growth performance measurement for chicken (♂) quail (♀) and their hybrids. J Shihezi Univ (Natural Science) 7(1):11–14

Yu J, Xia D, Yang H, He Y, Wu T (2003b) Morphology of the progenies of *Oreochromis aurea* (♀) × *Sinipperca chuatai* (♂). J Fish China 27(5):431–435

Yu X, Zhou D, Li Y, Li K, Zhou M (eds) (1989) Chromosomes of Chinese fresh-water fishes. Science Press, Beijing

Yuan J, Dang X, Zhan Y (2009) Advances on polyploid breeding in watermelon. Chin J Tropical Agric 29(3):65–70

Zan R (1985) Polyploidy of fish and its function in evolution. J Yunnan Univ 7(2):235–243

Zhang C, Sun Y-D, Liu S, Liu Y (2005) Evidence of the unreduced diploid eggs generated from the diploid gynogenetic progeny of allotetraploid hybrids. Acta Genet Sin 32(2):136–144

Zhang J, Liu X, Wang Z, Jin G (1984) A comparative study on the karyotypes among the hybrid fish (*Sinilabeo decorus* Tung-ting ♂ × *Cirrhinus molitorella* ♀) and its parental fishes. Acta Hydrobiol Sinica 8(3):313–321

Zhang W, Wang Y (2006) Progress of polyploid breeding technology applied in medical plants scale. Guiding J Trad Chin Med 12(2):83–85

Zhang X, Liu J, Wang L (2009) Polyploidy breeding and its application research progress of medicinal plants. J Jilin Norm Univ 4:128–131

Zhang Y, Tan Y, Ou Yang H (eds) (1990) Pond fish culture in China. Science Press, Beijing

Zhang Z, Qiu Q, Hu M, Lin K (1979) Observations on the embryonic and larval development of the backcross hybrids of *Aristichthys nobilis* (♀) × (*Aristichthys nobilis* ♀× *Hypophthalmichthys molitrix*♂) (♂). Acta Zool Sin 25(2):108–117

Zhou L, Gui JF (2002) Karyotypic diversity in polyploid gibel carp, *Carassius auratus gibelio* Bloch. Genetica 115(2):223–232

Zhou R, Shang R, Gong D, Xu X, Liu S (2019a) Characterization of H3 methylation in regulating oocyte development in cyprinid fish. Sci China Life Sci 62(6):829–837

Zhou R, Shang R, Gong D, Xu X, Tang Q, Tao M, Zhao R, Liu S (2019b) Characterization of de novo DNA methyltransferase dnmt3 regulating sterility in female allotriploid fish. Aquaculture 504:345–353

Zhou R, Wu Y, Tao M, Zhang C, Liu S (2015) MicroRNA profiles reveal female allotetraploid hybrid fertility. BMC Genet 16(1):119

Zhu L, Gui J, Liang S, Jiang Y (1993) Isozyme expression of distant hybridization offspring and artificial triploid in silver carp (*Hypophthalmichthys molitrix*). Acta Hydrobiol Sinica 17(4):293–297

Zhu X (1934) Research on Guangzhou frogs interbreed. Nat Sci Sun Yat-sen Univ 6:219–262

Zhu X (1961) Discussion about the fertilization process of animal hybridization. Chin Sci Bull 7:1–7

Zong E, Fan G, Yin H, Wang B, Zhang T, Sun M, Jiao S (1985) A study on the chromosomes of interspecific F2 hybrids between horse and ass. Sci Agric Sin 1:83–86

Zou S, Li S, Cai W, Zhao J, Yang H (2004) Establishment of fertile tetraploid population of blunt snout bream (*Megalobrama amblycephala*). Aquaculture 238(1):155–164

The Formation of Allotetraploids of Red Crucian Carp × Common Carp

2

Shaojun Liu, Yi Zhou, Min Tao, Chun Zhang, Qinbo Qin, Rurong Zhao, Chenchen Tang, and Kaikun Luo

Abstract

To date, researchers have exerted great efforts to develop new fertile diploid and tetraploid animals and used them as new germplasm resources to generate the improved diploid and triploid varieties on a large scale. Fertile allotetraploid animals can form new lineages with changes in ploidy providing the possibility to become new species. On the other hand, allotetraploid hybrids can be used for producing large numbers of sterile triploid hybrids. However, production of new allotetraploid animal groups through distant hybridization is a long-term and systematic project that requires perseverance and unremitting effort. Through more than 30 years of continuous investigation, a fertile allotetraploid hybrid lineage ($4n = 200$) has been generated from red crucian carp (*Carassius auratus* red var., $2n = 100$, ♀) × common carp (*Cyprinus carpio* L., $2n = 100$, ♂) hybridization. The establishment of the lineage provides an allotetraploid model to investigate the occurrence and evolution of polyploid fish in nature and provides sufficient tetraploid resources to produce sterile triploid fish. Via crossing allotetraploid hybrid (♂) with Japanese crucian carp (*Carassius cuvieri*, ♀) and Xingguo red carp (*Cyprinus carpio* var. *singuonensis*, ♀), sterile triploid Xiangyun crucian carp ($3n = 150$) and triploid Xiangyun carp ($3n = 150$) are generated, respectively. In addition, a gynogenetic diploid hybrid lineage has been established through the gynogenesis of the allotetraploid fish. Furthermore, an improved tetraploid hybrid lineage and an improved red crucian carp lineage have been developed. By using these two improved lineages, the improved triploid Xiangyun crucian carp II has been produced. Currently, triploid Xiangyun crucian carp, triploid Xiangyun carp, and triploid Xiangyun crucian carp II are

S. Liu (✉) · Y. Zhou · M. Tao · C. Zhang · Q. Qin · R. Zhao · C. Tang · K. Luo
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_2

farmed in 28 provinces and municipalities in China. These fishes are welcomed by breeders and generate remarkable economic, social, and ecological benefits. This chapter mainly introduces the techniques to produce allotetraploid hybrids by distant hybridization of red crucian carp (♀) × common carp (♂) and the biological characteristics of the hybrid offspring.

Keywords

Allotetraploid · Hybrids · Red crucian carp · Common carp · Chromosomes · Unreduced diploid gametes

2.1 The Red Crucian Carp and Common Carp Used as the Parents of the Allotetraploid Hybrids

2.1.1 The Biological Characteristics of Red Crucian Carp

Red crucian carp (RCC) (*C. auratus* red var.), a variation of crucian carp, belongs to Cyprinidae, Cyprininae, and *Carassius*. This fish has a relatively shorter, smaller head compared with common carp (CC) (*Cyprinus carpio* L.). It has a round blunt snout without barbel. RCC is covered by large scales and a lateral line could be easily observed. The growth rate is slower than CC. The length of adult fish is 15–20 cm. RCC is an omnivorous fish in red color. It shows high viability, high fertility, and beautiful shape. The red color is a special genetic marker of the RCC, which is convenient for conducting relevant genetic analysis for hybridization. The RCC is a normal diploid fertile fish, which has 100 chromosomes with a karyotype of 22m + 34sm + 22st + 22t (Liu et al. 2001). It is not only used as an aquarium fish but is also the important germplasm resources for fish breeding. RCC lays adhesive eggs. The males reach the sexual maturity at 8–10 months old, while the females reach the sexual maturity at 1 year old.

2.1.2 The Biological Characteristics of Common Carp

Common carp (CC) belonging to Cyprinidae, Cyprininae, *Cyprinus*, is an important freshwater farmed fish that is widely distributed around China. This species presents different kinds of subspecies, including Xiangjiang River wild common carp (XRWCC), Hebao carp, Yellow River carp, Xingguo red carp, scattered scale mirror carp, and etc., because of differences in its geographical distribution. The XRWCC in the Xiangjiang River of Hunan province, China, used as the paternal parent of tetraploid hybrids, has two pairs of barbels. The XRWCC is an omnivorous fish, whose juveniles consume macrozooplankton as a staple food but the adult individuals feed on benthonic animals as well as aquatic plants and algae. The

XRWCC presents a lot of excellent characteristics including high adaptability, enduring difficult environmental conditions, moderate size, and an affordable price. Under common conditions, male fishes could produce mature sperm at 1 year old and female fishes could generate mature eggs at 1–2 years old. The XRWCC lays adhesive eggs. The chromosome number of this kind of carp is 100, with a karyotype of 22m + 34sm + 22st + 22t (Liu et al. 2001).

2.2 The Biological Characteristics of F_1, F_2, and F_3 Hybrids of Red Crucian Carp and Common Carp

The F_1 hybrids were produced by the mating of female RCC and male XRWCC. During the breeding season, watery semen could be produced from 4.7% of F_1 males, and mature eggs could be stripped out from 44.3% of F_1 females. The F_2 hybrids were produced via the self-mating of fertile F_1 hybrids. Among F_2 hybrids, there were only a few fertile individuals. The appearance of F_1 and F_2 hybrids was intermediate to that of RCC and CC. The growth rate of the F_2 hybrids was faster than that of their maternal parent and exhibited stronger disease resistance. The fertile male and female individuals of F_1 and F_2 hybrids could produce mature gametes at 2 years old. The F_3 hybrids were produced via F_2 self-mating. In F_3, several fertile female allotetraploid and male allotetraploid hybrids were found.

2.2.1 The Biological Characteristics of F_1 Hybrids

Studies on the biological characteristics of F_1 hybrids have been conducted by appearance measurement, histological analyses, chromosome assays, flow cytometry analyses, etc. The fertilization rate of F_1 hybrid was 99.3%. The hatching rate of F_1 hybrid was 92.5%. The survival rate of F_1 hybrids past hatching was 80.0%, and only a few deformed individuals were observed. The appearance of the adult F_1 hybrids was intermediate to those of RCC and CC. The color of F_1 individuals was gray; they exhibited two pairs of barbels, although they were shorter than those of CC; the number of vertebrae and the scale formula was also intermediate to those of RCC and CC (Table 2.1). The F_1 hybrids were diploid with 100 chromosomes ($2n = 100$), and the chromosome karyotype formula was 22m + 34sm + 22st + 22t (Liu et al. 2001).

2.2.2 The Biological Characteristics of F_2 Hybrids

Via self-mating of F_1 hybrids, F_2 hybrids could be generated. Although the male fertile F_1 hybrids produced watery semen, the mature sperm from the watery semen could fertilize the mature eggs of the fertile female F_1 hybrids to form F_2 hybrids, which had an important biological foundation for the establishment of distant hybridization lineages of RCC and CC.

Table 2.1 Biological characteristics of F_1 hybrids, F_2 hybrids, allotetraploid F_3 hybrids, and their parents

Fish characteristics	RCC (maternal parent)	F_1 hybrids	F_2 hybrids	Allotetraploid F_3 hybrids	CC (paternal parent)
Chromosome numbers	100	100	100	200	100
DNA content	2*n*	2*n*	2*n*	4*n*	2*n*
Shape and color	Round and short shape; red	Intermediate to RCC and CC; gray	Intermediate to RCC and CC; gray	Intermediate to RCC and CC; gray	Long shape; gray
Forms and numbers of barbels	None	Two pairs of barbels; Shorter than those in CC	Two pairs of barbels; Shorter than those in CC	Only two pairs of small bases of barbels existed	Two distinct pairs of barbels
Lateral line scale numbers	29	32	32	31–32	36–37
Vertebra numbers	28–29			33–34	36–37
Pharyngeal teeth	1 line	2 lines	2 lines	2 lines	3 lines

2.2.2.1 The Incubation, Growth Performance, Appearance, and Chromosome Ploidy of F_2 Hybrids

The fertilization rate of F_2 hybrid was 18.0%. The hatching rate of F_2 hybrid was 5.4%. The percentage of abnormal hatched fry reached 90.0%, and the survival rate was less than 40.0%. The appearance of the adult F_2 hybrids was intermediate to those of RCC and CC. Their color was gray, and they had two pairs of barbels, though they were shorter than those of CC. The measurable properties of the vertebrae and fish scales were also intermediate to those of RCC and CC. We concluded that most of F_2 hybrids were diploid ($2n = 100$) by evaluating the chromosomes of kidney cells, and the karyotype was 22m + 34sm + 22st + 22t. In addition, 5% of triploid hybrids were identified from the F_2.

2.2.2.2 Gonadal Development of F_2 Hybrids

The gonad of F_2 hybrids could be divided into three types of gonads: the testis, ovary, and adipose types. The proportion of females and males was close to 1:1, and there were only a few gonads with adipose type. In the breeding season, the gonadosomatic index of the normal ovary of 2-year-old female hybrids was 11.0–20.0%. Oocytes in phases I, II, III, and IV could be observed. Among these phases, phase IV oocytes exhibited micropyle and micropylar cells, and several degenerated oocytes could be observed, which were not synchronized from phases III to IV. The F_2 hybrids were multiple spawning type.

The testis development of some male F_2 hybrids was normal according to the developmental rules of germ cells. The testis showed a complete developmental process, following the sequence as spermatogonia → primary spermatocyte →

secondary spermatocyte → spermatid → sperm. Therefore, F_2 hybrids could release watery semen. Their fertility was higher than that of F_1 hybrids. In the adipose type of hybrid F_2, no germ cell was found (Zhang et al. 2008).

2.2.2.3 Gamete Characteristics of F_2 Hybrids

F_2 hybrids produced mature eggs in various sizes, whose adhesiveness was lower than that in RCC and CC. Diploid female F_2 hybrids exhibited three kinds of eggs, with diameters of 0.20 cm, 0.17 cm, and 0.13 cm, respectively. The proportions of the three egg types were 50.0%, 35.9%, and 14.1%. Several eggs had diameters of 0.17 cm which were similar to the size of eggs from allotetraploid hybrids (F_3–F_{29}) considered as diploid eggs. Those with the 0.13 cm diameter were similar to the haploid eggs of RCC and Japanese crucian carp which were considered to be haploid eggs. Several eggs had diameters of 0.20 cm which may be higher ploidy than diploid eggs and thus, were considered as triploid eggs.

The result of scanning electron microscopy showed that the male F_2 hybrids produced a variety of different sizes of sperm. The head diameters of the smallest sperm were only 1.32 μm, while the head diameters of the largest sperm were about 18.39 μm. The diameters of several sperm head were 2.48–2.85 μm. Most of the diameters of sperm head were 1.85–2.15 μm. The diameters of sperm head from CC were about 1.9 μm, while the diameters of sperm head from allotetraploid RCC × CC (F_3–F_{29}) were about 2.4 μm. Based on these results, the sperm with a head diameter of 1.85–2.15 μm were haploid sperm. The sperm with a head diameter of 1.40 μm might be aneuploid sperm, and the sperm with a head diameter greater than 3.00 μm were more likely to be polyploid (Liu et al. 2001). After the diploid eggs and sperm of F_2 hybrids encountered each other, a normal fertilization process was observed. Thus, F_3 allotetraploid hybrids could be produced via the self-mating of F_2 individuals.

2.2.3 The Biological Characteristics of F_3 Hybrids

The F_2 fish could produce diploid eggs and sperm, leading to the generation of allotetraploid F_3 hybrids.

2.2.3.1 The Hatching, Growth Performance, and Chromosome Ploidy of the F_3 Hybrids

The fertilization rate of the F_3 hybrid was 40.0%. The hatching rate of the F_3 hybrid was 6.0%. The malformation rate of the F_3 embryos after hatching reached 90.0% and survival rate was lower than 10.0%. The appearance of the surviving adult fish was intermediate to that of RCC and CC. They exhibited a gray body and two pairs of barbels, which were shorter than those of CC. Their vertebra number, scale formula, and other countable characters were also intermediate to those of RCC and CC. Some biological traits of F_3 hybrids were listed in Table 2.1.

The chromosome analysis using kidney cells indicated that three ploidies of F_3 hybrids were generated, including diploid ($2n = 100$), triploid ($3n = 150$), and

tetraploid ($4n = 200$) fish, accounting for 73.3%, 20.0%, and 6.7% of the F_3 hybrids, respectively. There were several fertile female and male individuals among the tetraploid F_3 hybrids.

2.2.3.2 The Gonadal Development of F_3 Hybrids

The gonadal development of the diploid F_3 hybrids was similar to that of the F_1 and F_2 hybrids. Triploid F_3 hybrids included female and male individuals, both of which were infertile. However, allotetraploid F_3 hybrid individuals exhibited normal gonadal development. In the breeding season, mature eggs could be squeezed out by pressing the abdomen of the female allotetraploid hybrids. The eggs of allotetraploid hybrids were significantly larger than those of normal diploid RCC. The histological assays showed that a large number of mature eggs with the yolk and a few smaller primary oocytes in phase I and II were presented in the ovaries of allotetraploid hybrids. When the belly of the male tetraploid hybrids was squeezed, white semen would flow out. Observations in histological sections showed that the male allotetraploid hybrid's testes were filled with testis lobules. Spermatogonia, spermatids, and mature sperm were observed in the testis lobules. Both spermatogonia and Sertoli cells were found in the testes. In the Sertoli cells, particulate matter could be found in the cytoplasm. These particles might be associated with hormone synthesis. The head diameter of the diploid sperm of the allotetraploid hybrids was 2.40 μm.

Under the transmission electron microscope, the diploid sperm of tetraploid hybrid and haploid sperm of CC exhibited similar structures. The sperm was composed of one head and one tail. The head was round or oval and was full of dense nuclear material. The front part of the tail contained several mitochondria. The central axial filament of the tail was typical of a "9 + 2" arrangement of microtubules. The fertilization of eggs and sperm from allotetraploid hybrids was normal in comparison with common fishes. After normal embryonic development, the progenies were survival.

Therefore, allotetraploid F_3 hybrids could reproduce normally, which lays an important foundation for establishing a stable population of allotetraploid hybrids.

2.2.4 The Mating Test of F_2 Hybrids with Other Fishes

Based on the observation that F_2 hybrids exhibited three different sizes of eggs, mating experiments involving F_2 hybrids (♀) × RCC (♂) and F_2 hybrids (♀) × allotetraploid hybrids (♂) were designed and performed. Through chromosome assays and flow cytometry analysis, fishes with three different ploidies, including tetraploid, triploid, and diploid fishes, were determined in the progenies of F_2 hybrids (♀) × RCC (♂). Tetraploid and triploid fishes were generated from the progenies of F_2 hybrids (♀) × allotetraploid hybrids (♂). The presence of fishes with different ploidies in the two mating tests suggested that F_2 hybrids could produce triploid, diploid, and haploid eggs (Liu et al. 2006).

2.2.5 The Significance of the F_2 Hybrids Producing Unreduced Gametes

Allotetraploid hybrids were generated among the F_3 hybrids because F_2 hybrids could produce unreduced gametes. It was the first report on the artificial formation of the allotetraploid lineage in which both the fertile males and fertile females existed in vertebrates. It also provided important evidence that distant hybridization can result in the formation of the allotetraploid lineage in vertebrates. The establishment of allotetraploid hybrid lineage provided the foundation of diploid gamete resources to generate the sterile triploid fishes. There was no doubt that the formation of tetraploid lineage had important value for basic research and further applications.

The mechanism whereby the diploid F_2 hybrids produced unreduced gametes was an essential and interesting issue that deserved to be elucidated. The studies on gonadal development, chromosome behavior of germ cells, gamete size and fertilization cytology behavior of F_2 hybrids, as well as biological characteristics such as the chromosome numbers of tetraploid F_3 hybrids and progenies derived from F_2 and other fishes confirmed that F_2 hybrids could produce diploid gametes and that the formation mechanism was relevant to the endoreduplication or fusion of germ cells at the early stage. In diploid F_2 hybrids, the production of diploid gametes could help overcome the incompatibility of the genetic materials of their parents. This led to the formation of fertile female and male allotetraploid F_3. The normal gonadal development of allotetraploid F_3 was observed and the age at sexual maturity was 1 year old which was 1 year earlier than that of F_2. Subsequently, the hybrid strain would reproduce allotetraploid hybrids instead of diploid hybrids, which was an inevitable result of biological selection. When parents exhibited the same number of chromosomes, the formation of fertile hybrid lineages was also observed following the crossing of blunt snout bream (♀) × topmouth culter (♂), topmouth culter (♀) × blunt snout bream (♂), or koi carp (♀) × color crucian carp (♂).

References

Liu S, Liu Y, Zhou G, Zhang X, Luo C, Feng H, He X, Zhu G, Yang H (2001) The formation of tetraploid stocks of red crucian carp × common carp hybrids as an effect of interspecific hybridization. Aquaculture 192(2):171–186

Liu S, Sun Y, Luo K, Liu Y (2006) Evidence of different ploidy eggs produced by diploid F_2 hybrids of *Carassius auratus* (♀) × *Cyprinus carpio* (♂). Acta Genet Sin 33(4):304–311

Zhang C, Liu S, Sun Y, Xiao J, Qin Q, Wang J, He W, You C, Liu Y (2008) Chromosomal studies of germ cells in diploid and polyploid fish produced by distant crossing. J Mol Cell Biol 41(1):53–60

3 The Basic Biological Features of Allotetraploid Lineage of Red Crucian Carp × Common Carp

Shaojun Liu, Yi Zhou, Rong Zhou, Chun Zhang, Liujiao Yuan, Min Tao, Qinbo Qin, Rurong Zhao, Chenchen Tang, and Kaikun Luo

Abstract

The allotetraploid lineage of red crucian carp (*Carassius auratus* red var., ♀) × common carp (*Cyprinus carpio* L., ♂) has been propagated to generation 27 (F_3–F_{29}) and formed a fertile allotetraploid lineage. The formation of the allotetraploid lineage provides new germplasm resource for aquaculture and suitable model for studying polyploidization. This established allotetraploid lineage provides a new approach for fish breeding. By distant hybridization, this novel allotetraploid fish with two subgenomes shows changes in phenotypes and genotypes. The comprehensive investigation of the features of this allotetraploid lineage provides evidences for the role of distant hybridization in polyploidization events. This chapter mainly introduces the appearance, chromosome ploidy, gonadal development and breeding, diploid gametes, growth performance, and other biological features of the allotetraploid hybrids.

Keywords

Allotetraploid · Hybrids · Polyploid · Meiosis · Embryonic development · Sex determination

S. Liu (✉) · Y. Zhou · R. Zhou · C. Zhang · L. Yuan · M. Tao · Q. Qin · R. Zhao · C. Tang · K. Luo
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_3

3.1 The Appearance and Development of Allotetraploid Hybrids of Red Crucian Carp × Common Carp

3.1.1 The Appearance of Allotetraploid Hybrids of Red Crucian Carp × Common Carp

The allotetraploid fish could be easily identified from red crucian carp, common carp, and diploid hybrids of red crucian carp × common carp (F_1–F_2) according to appearances. Allotetraploid fish had two short pairs of barbels which were shorter than that in common carp and diploid hybrids of red crucian carp × common carp (F_1–F_2). Similar to that of common carp, the body color of allotetraploid fish was gray. The body shape of allotetraploid fish was intermediate to that of red crucian carp and common carp with little difference from that of diploid hybrids of red crucian carp × common carp (F_1–F_2). The growth rate of allotetraploid fish was slower than that of diploid red crucian carp × common carp (F_1–F_2). The number of lateral line scales of allotetraploid fish was 30–34 which was intermediate to that of red crucian carp (number of lateral line scales = 28–30) and common carp (number of lateral line scales = 34–39). The appearance of allotetraploid fish showed the inheritance and variation from red crucian carp and common carp (Liu et al. 2001b).

3.1.2 The Embryogenesis of Allotetraploid Hybrids of Red Crucian Carp × Common Carp

After the eggs were fertilized by sperm from allotetraploid, they began to undergo normal development. At 21 °C, the fertilized eggs would be hatched at 80 h post-fertilization. The early embryonic development process was as follows.

3.1.2.1 Zygote Period

The mature fertilized egg of allotetraploid was round and adhesive, and its diameter was 1.70 ± 0.10 mm. After water swelling, the fertilized egg would be enlarged and perivitelline space clearly expanded. The fertilized egg was light in color and transparent, and normal cleavage would be formed. The protoplasm of the fertilized egg aggregated at the animal pole and gradually bulged, then the blastodisc was formed after 20–40 min.

3.1.2.2 Cleavage Period

At 80 min after fertilization, the blastodisc began to undergo its first cleavage (longitudinal division), and two blastomeres of the same size were formed. At 2 h 10 min after fertilization, the blastodisc was divided longitudinally in the vertical direction of the first division and four cells with similar sizes were formed; at 2 h 45 min after fertilization, the third fission was complete (two longitudinal divisions) and eight blastomeres neatly arranged in two rows were formed; at 3 h 20 min after fertilization, the fourth cleavage (two longitudinal divisions) was complete, forming the 16-cell stage embryo; at 3 h 50 min after fertilization, the fifth fission was

complete (transverse division), forming the 32-cell stage embryo; a multilayer blastodisc was formed and bulged after the sixth horizontal division (forming the 64-cell stage).

3.1.2.3 Blastula Period

At 7 h 10 min after fertilization, the volume of the cells decreased, and blastula was formed as the result of continuous blastodermal cell division; at 11 h 40 min after fertilization, the blastodermal cells expanded downward and became slightly flattened; at 13 h 30 min after fertilization, the blastodermal cells spread over the yolk, and the blastoderm started to downward forming the late blastula.

3.1.2.4 Gastrula Period

At 14 h 50 min after fertilization, the 1/3 epiboly stage appeared and early gastrula-stage embryo began to form; 2 h 30 min later, the gastrula extended downward to 1/2 epiboly stage to form the mid-gastrula embryo; at 23 h 20 min after fertilization, the cells surrounded the lower 3/4 of the yolk, forming the late gastrula-stage embryo.

3.1.2.5 Neurula Period

At 27 h 30 min after fertilization, the cells continued to extend downward and curve inward, leaving only the yolk plug, although the blastopore had not yet closed, and the germ ring shrank markedly, forming the neurula; at 29 h 10 min after fertilization, the blastopore closed.

3.1.2.6 Organogenetic Period

At 31 h 20 min after fertilization, a pair of elliptical optic primordia appeared on either side of the rostral side of the embryo. Approximately 1 h later, the optic vesicle could be clearly observed, and myomeres appeared; at 34 h 40 min after fertilization, 6–8 pairs of myomeres in the center of the embryo could be clearly observed and the brain had differentiated into three parts (the forebrain, midbrain, and hindbrain); 2 h later, the tail bud appeared; at 39 h after fertilization, the muscles in the middle-rear portion of the embryo began to wriggle slowly, and a portion of the embryonic heart began to beat; the contraction of the heart began to speed up, the blood circulation formed, and the embryo began to wriggle rapidly, at 40–58 h after.

3.1.2.7 Hatching Period

At 62 h 30 min after fertilization, the tail of the embryo proper wriggled strongly and broke through the egg membrane with the swinging motion of its tail. Under normal conditions, the tail was the first part of the embryo to break through the egg membrane. It took 12–16 h to complete the process from egg membrane rupture until the embryo proper became completely deciduate. The fry with yolk sacs were able to move freely after hatching for 1–2 days.

The above results showed that embryos of the allotetraploid hybrids presented a normal embryonic development process. This process might guarantee the survival of the offspring. These offspring laid an important biological foundation for the natural breeding of fertile allotetraploid hybrids (Liu et al. 2001a).

3.2 Ploidy of Allotetraploid Hybrids

We performed a statistical analysis of the chromosome numbers of allotetraploid ploidy. The results showed that F_3–F_{21} allotetraploid hybrids were tetraploid having 200 chromosomes. To date, this allotetraploid lineage has been propagated to F_{29}. The allotetraploids had two sets of red crucian carp chromosomes and two sets of common carp chromosomes, in accordance with the analysis of chromosome karyotype. The karyotype formula was 44 m + 68sm + 44st + 44 t, corresponding to the doubling of the formula of F_1 and F_2 hybrids (22 m + 34sm + 22st + 22 t). In addition, in the No. 1 submetacentric chromosomes of F_1 and F_2 hybrids, the ratio of the long arm to the short arm (RLS) of one chromosome was higher than that of the other. It was obvious that the higher-RLS chromosome came from common carp and the lower came from red crucian carp. Similarly, the RLS values of two chromosomes were higher than those of two other chromosomes among the No. 1 submetacentric chromosomes of F_3 and F_8 allotetraploid hybrids, and the higher-RLS chromosomes were from common carp, while the two lower-RLS chromosomes were from red crucian carp (Sun et al. 2003; Liu 2010).

The erythrocyte DNA contents of F_2, F_3, and F_4 hybrids were measured by flow cytometry technique, using the erythrocyte DNA contents of diploid Japanese crucian carp as control. Comparative results were presented in Table 3.1, which showed that F_2 hybrids were diploids, while F_3 and F_4 hybrids were tetraploids.

The nuclear volume of erythrocyte was determined (Table 3.2). The results showed that the ratio of erythrocyte nuclear volume in F_3 hybrid to the erythrocyte

Table 3.1 Measurement of the erythrocyte DNA contents of Japanese crucian carp and F_2, F_3, and F_4 hybrids (Liu et al. 1999)

Fish	Average value of DNA content	DNA content ratio
Japanese crucian carp	48.51	
F_2	45.03	0.93[a]
F_3	98.68	2.03[b]
F_4	108.48	2.23[c]

[a]The ratio was not significantly different ($P > 0.05$) from a 1:1 ratio
[b]No significant difference was found with 2:1 ratio ($P > 0.05$)
[c]No significant difference was found with 2:1 ratio ($P > 0.05$)

Table 3.2 Nuclear volume of erythrocyte from F_3 hybrids and Japanese crucian carp (Liu et al. 2001b)

Fish	Major axis (μm)	Minor axis (μm)	Volume (μm^3)	Volume ratio with Japanese crucian carp
Japanese crucian carp	6.37 ± 0.56	3.37 ± 0.56	36.92 ± 8.86	
F_3 allotetraploid hybrids	9.71 ± 1.38	3.81 ± 0.63	73.87 ± 17.64	2.00[a]

[a]No significant difference was found with 2:1 ratio ($P > 0.05$)

nuclear volume in diploid Japanese crucian carp was close to 2:1. The measured results were in accord with the observed chromosome numbers and DNA contents.

The results proved that the allotetraploid hybrids presented intermediate characteristics between red crucian carp and common carp. Allotetraploid hybrids exhibited normal development in terms of fertilization cytology. The chromosome number of the allotetraploid hybrids was $4n = 200$. Both female and male hybrids presented a steady inheritance, which ensured allotetraploid transmission from one generation to another. Allotetraploid hybrids formed a very large allotetraploid fish group. They were regarded as suitable model organisms for studying the evolution of vertebrates because of their clear family formation process. In addition, they had provided important tetraploid fish breeding resources for the mass production of sterile triploid fish.

3.3 The Meiosis of Germ Cells in Allotetraploid Hybrids and the Formation of Diploid Gametes

3.3.1 Meiosis of Fish Germ Cells

Species exhibiting sexual reproduction produces mature germ cells in which the number of chromosomes per cell is halved by meiosis. Chromosomes are copied only once in meiosis while the cells go through two rounds of division. In addition, exchange and recombination occur between homologous chromosomes during this time. Meiosis results in the number of chromosomes in mature germ cells being halved compared to that in primordial germ cells. Meiosis is a complicated process. Briefly, it involves one round of DNA duplication and two cell divisions. In the first cell division, the homologous chromosomes generally separate into two cells, and the number of chromosomes reduces by half. In the second cell division, the sister chromatids separate into two cells, and the number of chromosomes is not changed. One spermatocyte could be transformed into four spermatids, which then metamorphose into four sperm. One oocyte transforms into one mature egg and three polar bodies in the process of meiosis. This type of meiosis is observed in normal fishes. Spermatocytes finish all the meiosis process before being discharged out as sperm, while oocytes are blocked at the metaphase of the second meiotic division. After fertilization, mature eggs release the second polar body, then finished meiosis.

Meiosis plays an important role in maintaining constant of chromosome numbers, allocation and recombination of genetic material, and evolutionary development. It not only maintains the genetic stability of species but also gives rise to variability in gametes because of the exchange of homologous chromosomes and recombination of nonhomologous chromosomes. This laid a foundation for the formation of the genetic diversity in fish.

3.3.2 The Diploid Gametes Produced by Allotetraploid Hybrids

Allotetraploid hybrids have been propagated to generation 27 (F_3–F_{29}). The fertility of both sexes was proved by using a series of detection methods. Some relevant morphological measurements and studies of molecular genetics and population genetics have been executed and demonstrated that these allotetraploids inherited the genetic material of their parental species and maintained genetic stability in their offspring with the hybrid subgenomes.

The features of the appearance, scale formula, body color, and pharyngeal teeth of allotetraploid hybrids were intermediate between those of red crucian carp and common carp. Chromosome karyotype studies also showed that the allotetraploid hybrids contained not only the genome of red crucian carp but also that of common carp. The *Sox* genes of the allotetraploid hybrids and the original parents were amplified by using degenerate primers targeting conserved *Sox* HMG box regions. The results showed that the amplifications of the allotetraploids exhibited bands that were both shared with and different from those in the original parents. We also compared the sequence homology of the allotetraploids and the original parents on the basis of the gene sequence of *cyclin B*, and their similarities showed that the allotetraploids retained the genetic material of the original parents after multigenerational breeding. All of these results illustrated that allotetraploids possessed hybrid genomes which came from their original parents. As the basis of sexual reproduction, meiosis is an important contributor to genetics, evolution, and diversity. How do these allotetraploids, with nonhomologous chromosomes, maintain stable chromosome numbers in subsequent offspring via meiosis during sexual reproduction and assure that genetic features were stably inherited in every generation?

3.3.2.1 Meiosis of the Germ Cells of Allotetraploids

The meiosis of allotetraploids was crucial to maintain their genetic stability. When the germ cells of allotetraploids underwent meiosis, chromatin was transformed into sister chromatids via replication, giving rise to the question of how chromosome synapsis occurred during the process of cell division.

Allotetraploids had four sets of chromosomes. We represented the chromosomes from red crucian carp as An ($n = 1, 2,..., 50$) and those of common carp as Bn ($n = 1, 2,..., 50$), so the chromosome composition of allotetraploids could be represented as AnAnBnBn ($n = 1, 2,..., 50$). Theoretically, AnAn or BnBn can be regarded as homeologous chromosomes when An and Bn were considered partially homeologous chromosomes. During meiosis, it was likely that AnAn or BnBn bivalents (Fig. 3.1) or AnBn bivalents formed homeologous chromosome pair. Many other forms, such as An or Bn univalents, AnAnBn or AnBnBn trivalents, or AnAnBnBn tetravalents, could also be formed. However, univalents, trivalents, and tetravalents were detrimental to the production of normal diploid gametes and tetraploid offspring, because their existence could lead to disorders of meiosis. Relevant genome analyses, especially studies in plants, had shown that allopolyploids were more likely to form bivalents and not multivalents than autopolyploids. The probability of homologous multivalent formation in

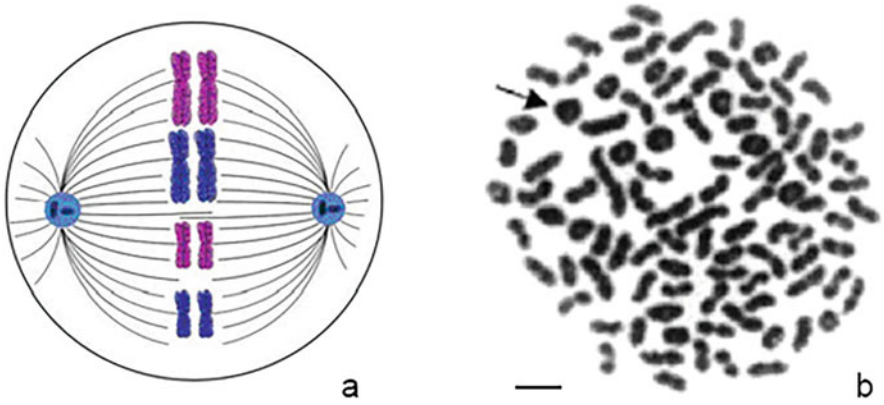

Fig. 3.1 The observations of chromosomes in meiosis in allotetraploids (Liu 2010). (**a**) A diagram of bivalent pairing of homologous chromosomes at the first meiosis in allotetraploids. The mauve chromosomes represented those from red crucian carp and the blue chromosomes represented those from common carp. (**b**) One hundred bivalents were formed at the first meiosis in allotetraploids, and the arrow indicated ringlike bivalents. Bar = 3 μm

autopolyploids was 3.5 times that in allopolyploids (Ramsey and Schemske 1998). In addition, some homologous chromosomes would show priority in pairing on the basis of bivalents. For example, the *Ph* gene maintained the pairing of some homologous chromosomes in wheat, corn, and cotton (Griffiths et al. 2006).

During meiosis in allotetraploids, 100 bivalents formed via chromosome pairing, and no or fewer univalents, trivalents, and tetravalents were formed (Fig. 3.1). The primary spermatocytes (primary oocytes) of allotetraploids, with 4 sets of chromosomes, predominantly showed the formation of 100 A_nA_n and B_nB_n bivalents rather than 25 $A_nA_nB_nB_n$ tetravalents or other multivalent forms during meiosis. The A_nA_n bivalents or B_nB_n bivalents were separated after the first meiosis, resulting in half the number of chromosomes, and then A_nB_n secondary spermatocytes (or secondary oocytes) ($2n = 100$) were formed. The sister chromatids of the secondary spermatocytes (or secondary oocytes) separated in the second meiosis. Finally, diploid spermatids (or eggs) ($2n = 100$) were formed, and A_nB_n bivalents in heterologous male and female gametes were produced. After fertilization, the A_nB_n male and female gametes would transform into new $A_nA_nB_nB_n$ allotetraploid offspring to ensure that allotetraploid hybrids were transmitted through successive generations in a stable way.

However, gene recombination might occur between A_n and B_n chromosomes because both crucian carp and common carp exhibited 100 chromosomes, even though they belonged to different genera. Working cooperatively with Professor Zhang YP's laboratory at Yunnan University, our laboratory identified gene recombination and variation in the liver transcriptome of allotetraploids. We also observed these phenomena in some nuclear genes of allotetraploids. Recombination might

occur in germ cells or somatic cells. Gene recombination between A_n and B_n chromosomes had no influence on homologous pairing between A_nA_n and B_nB_n chromosomes. Further studies are still needed to analyze gene recombination in allotetraploids.

3.3.2.2 Features of Diploid Gametes Generated by Allotetraploids

Diploidy of Gametes from Allotetraploids

The average diameters of red crucian carp and Japanese crucian carp eggs were 0.13 ± 0.01 cm and 0.14 ± 0.01 cm, respectively. It meant that these two diploid fishes possessed similar-size eggs. The diameter of the eggs of the allotetraploid hybrids was 0.17 ± 0.01 cm, and these eggs were obviously larger than the haploid eggs produced by diploid fish.

Sperm from allotetraploid hybrids and common carp were observed with scanning electron microscopy. In addition, the volume of the sperm head was calculated using the formula $(4/3)\pi a^3$ (where a represented the semidiameter). The average diameter of the haploid sperm head produced by diploid red crucian carp was 1.90 μm, and its volume was 3.59 μm^3, while the average diameter of the sperm head generated by allotetraploids was 2.40 μm (Fig. 3.2), and its volume was 7.23 μm^3. The volume of the latter was two times that of the former, and we could speculate that the DNA content of diploid sperm generated by allotetraploids was two times that of haploid sperm produced by diploid red crucian carp, which provided important evidence that allotetraploids could generate diploid sperm.

The ratio of the average DNA content of sperm generated by tetraploid fish to that of sperm generated by red crucian carp was 2.18, which further verified the diploidy of the sperm generated by allotetraploids (Liu et al. 2005).

In addition, we produced triploid Xiangyun crucian carp ($3n = 150$) and triploid Xiangyun carp ($3n = 150$), which came from hybrid of male allotetraploid hybrids respectively with female diploid Japanese crucian carp ($2n = 100$) and Xingguo red carp ($2n = 100$). These results proved that diploid gametes ($2n = 100$) were generated by allotetraploids (Liu et al. 2001b). Activation of the eggs of female allotetraploids by the sperm of scattered mirror carp was used to generate G_1 hybrids, in which the first generation consisted of all-female gynogenetic diploids ($2n = 100$), on a large scale without chromosome duplication in the genetic breeding of fish. The diploidy of the G_1 fish demonstrated that diploid eggs could be produced by female allotetraploids (as discussed in Chap. 5). Fertile diploid offspring produced via androgenesis (A_0) were successfully activated by fertilizing sperm from male allotetraploids and the eggs of haploid goldfish with genetic material treated with UV, without chromosome doubling of the male nucleus. The diploidy of A_0 showed that allotetraploids were able to produce diploid sperm (refer to Chap. 5) (Sun et al. 2007).

The results described above showed that allotetraploids could generate diploid gametes in a stable way. In addition, we proved that diploid eggs and sperm were in a

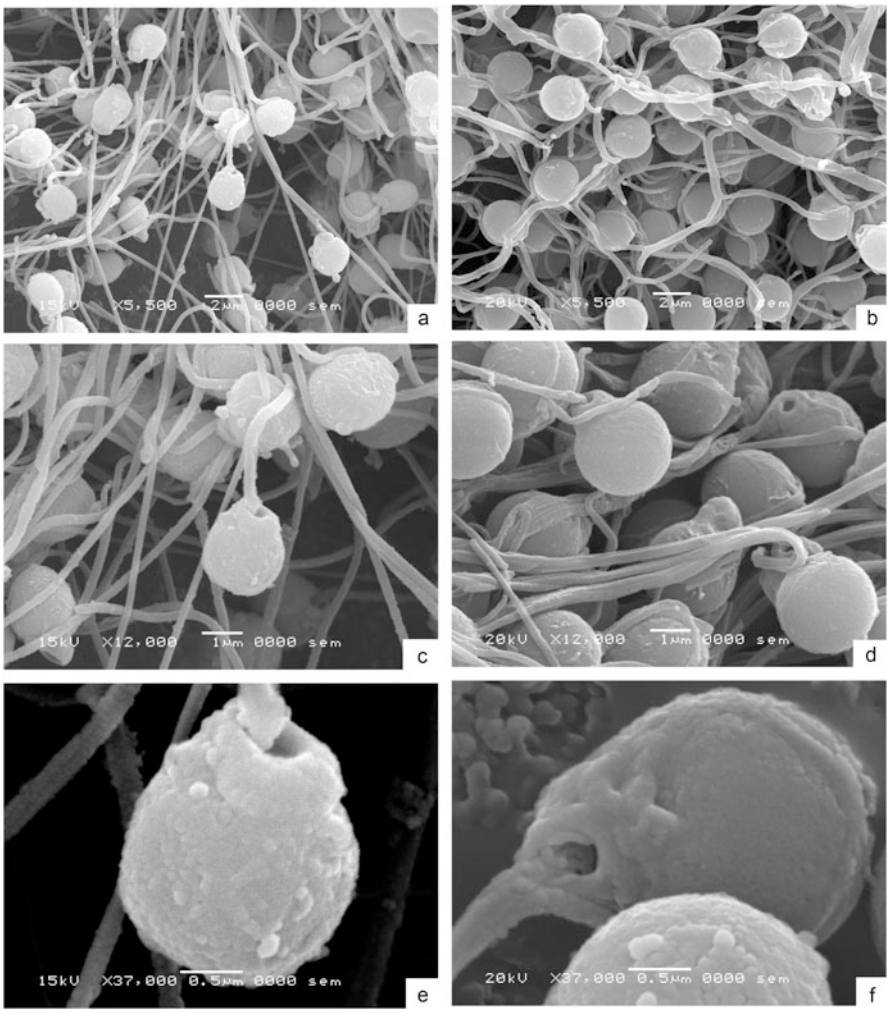

Fig. 3.2 The scanning electron microscopy observations of sperm from red crucian carp and allotetraploids at different magnifications. (**a**, **c**, **e**). Analysis of haploid sperm from red crucian carp by scanning electron microscopy. (**b**, **d**, **f**). Analysis of diploid sperm from allotetraploid by scanning electron microscopy

normal condition by observing the microstructure and ultrastructure of the gonad of allotetraploids and their mature eggs and sperm. They could produce normal tetraploid offspring after fertilization. The occurrence of full diploid gametes provided a major hereditary basis for self-multiplication from allotetraploids cultivated by artificial feeding.

Genome Heterology of Diploid Gametes of Allotetraploids

A gynogenetic diploid hybrid lineage was established after the gynogenesis of female allotetraploid eggs. This lineage has been propagated to G_{10}. The production of offspring ($2n = 100$) by gynogenesis remained an obvious trait of hybrids. For example, the length of the barbels in the offspring produced by gynogenesis was intermediate to those of the original parents with only the base of barbels, and the number of lateral line scales (31–32) was intermediate to those of common carp (36–37) and red crucian carp (29). The examination of the features of the gynogenetic diploid hybrids showed that they possess hybrid genomes of both common carp and red crucian carp. In addition, the diploid hybrids not only retained the hybrid features of their parents but they also presented a unique reproductive mode in that they could produce diploid eggs.

Fertile diploid offspring produced by androgenesis (A_0) were activated via the androgenesis of male allotetraploids, and these offspring also retained the hybrid traits of the parents, such as possessing two pairs of short barbels with characteristics intermediate to those of the original parents. In recent years, both A_0 females and males had been shown to produce diploid eggs and sperm, respectively (refer to Chap. 5).

Offspring produced via androgenesis and gynogenesis from the diploid gametes of allotetraploids presented the hybrid traits of the original parents in terms of their appearance, which demonstrated the heterology of diploid gametes. Additionally, our laboratory proved that diploid hybrids produced via both androgenesis and gynogenesis could produce unreduced gametes after years of experimental research and production practices, which demonstrated the hybrid essence of diploid gametes from allotetraploids. Previous researches had shown that the frequency of diploid heterozygote-generated unreduced gametes was very high (Ramsey and Schemske 1998; Otto 2007). As the common carp and red crucian carp belonged to different genera, and they presented differences related to distant genetic relationships, the incompatibility between nonhomologous chromosomes, which respectively came from common carp and red crucian carp, would result in unnormal pairing during meiosis. However, if the early germ cells of hybrids conducted endoreduplication directly, or normal meiosis began after germ cell fusion or without the first meiosis, the problem of homologous chromosome pairing in hybrids would be resolved, and unreduced gametes would be generated. Thus, diploid hybrids produced via both androgenesis and gynogenesis could generate unreduced gametes, which reflected the heterology of the nature of inheritance and the heterology of the diploid gamete genome of allotetraploids.

According to the analysis described above, we could speculate that the meiosis of allotetraploids occurred as follows: the homologous chromosome pairing occurred between the chromosomes coming from the same fish species. In detail, 100 chromosomes from red crucian carp were paired to form 50 bivalents, and 100 chromosomes from common carp were paired to form the other 50 bivalents during meiosis I in the first oocytes (spermatocytes) of allotetraploids, and all the 100 bivalents were separated to form secondary oocytes (spermatocytes) in which every cell possessed 50 chromosomes from common carp and 50 chromosomes from

red crucian carp. During meiosis II, the sister chromatids were separated to form eggs (or spermatids) in which every cell possessed 50 chromatids from common carp and 50 chromatids from red crucian carp. At last, hybrid diploid gametes were produced; they mated each other to form new allotetraploid offspring with two sets of genomes from common carp and two sets of genomes from red crucian carp. In this way, the allotetraploid hybrids were stably transmitted through successive generations.

3.4 The Sex Determination Mechanism of Allotetraploid Hybrids of Red Crucian Carp (♀) × Common Carp (♂)

A large number of allotetraploid hybrids of red crucian carp (♀) × common carp (♂) had been formed by self-mating. Observations of the gonadal development of allotetraploid red crucian carp (♀) × common carp (♂) and breeding practices had proven that both of the female and male allotetraploid were fertile. Normal offspring could be produced by self-mating from allotetraploid (Liu et al. 2001b; Sun et al. 2003).

Allotetraploid hybrids of red crucian carp (♀) × common carp (♂) played an important role in research on modes of sex inheritance because of their special status in the study of evolution and heredity. Researchers had investigated the genetic sex hereditary mechanism of allotetraploid hybrids of red crucian carp (♀) × common carp (♂) produced by gynogenesis and androgenesis, generating important data at the chromosomal and molecular levels.

3.4.1 The Chromosomal Sex Determination in Female Allotetraploid Hybrids of Red Crucian Carp (♀) × Common Carp (♂)

Allotetraploid hybrids of red crucian carp (♀) × common carp (♂) could produce diploid eggs. The diploid eggs were activated with crucian carp sperm treated with UV radiation, and a group of gynogenetic G_1 hybrids showing normal development was obtained. The individuals of the G_1 group ($2n = 100$) were diploid, and all were females. Thirty percent of G_1 individuals showing normal gonadal development could produce mature eggs. The sexual maturation time of the G_1 fish was approximately 2 years, which was longer than that of female red crucian carp. Interestingly, the G_1 fish produced diploid eggs; without treatment for chromosome doubling, we obtained gynogenetic hybrids (G_2) with these diploid eggs activated by red crucian carp sperm treated with UV. The G_2 individuals ($2n = 100$) were all females exhibiting normal gonadal development. These fish could also produce a large number of diploid eggs after sexual maturity. These diploid eggs produced further generations via gynogenesis to form an all-female diploid gynogenetic clonal hybrid lineage (discussed in Chap. 5). The diploid hybrid gynogenetic group proved that the sex chromosome karyotype of male allotetraploid red crucian carp (♀) × common carp (♂) was XXXX (Liu et al. 2004b; Yan et al. 2005). This result was in accord

with the theory that the sex determination type was XX in the all-female gynogenetic offspring of red crucian carp and common carp (Wang et al. 2008).

3.4.2 The Chromosomal Sex Determination in Male Allotetraploid Hybrids of Red Crucian Carp (♀) × Common Carp (♂)

We had also conducted research on the androgenesis of diploid sperm produced by male allotetraploid hybrids of red crucian carp (♀) × common carp (♂) in a diploid androgenetic clonal hybrid lineage produced by our group. Without treatment for chromosome doubling, androgenetic groups (A_1) were produced by crossing goldfish eggs treated with UV and diploid sperm from allotetraploid hybrids of red crucian carp (♀) × common carp (♂). The resulting individuals were all diploid. Similar to the G_1 group, the A_1 group reached sexual maturity in 2 years. The ratio of females to males was 1.14:1. The ratio in their offspring was 0.82:1. The P values of the statistics showed that there was no distinct difference of the ratio of males to females in the androgenetic groups and their offspring from a 1:1 ratio (discussed in Chap. 5). We speculated that the sex determination type of the parents was XXXY, with a male to female ratio of the androgenetic offspring close to 1:1. However, the possibility of XXYY male tetraploids could not be excluded based on the present evidence because we chose the experimental fish in which to conduct androgenesis randomly. Theoretically, the number of male individuals should be markedly higher than that of female individuals in the androgenetic offspring of XXYY male tetraploid fish.

The results showed that the sex chromosome type of the female allotetraploid red crucian carp (♀) × common carp (♂) was XXXX. Theoretically, there were two potential sex chromosome types in male allotetraploid red crucian carp (♀) × common carp (♂): XXXY and XXYY (Fig. 3.3). Among these systems, the XXXY type had been proven to exist, while type XXYY has not, and further research is required.

3.4.3 The Sex-Determining Gene and Sex Inheritance of Allotetraploid Hybrids of Red Crucian Carp (♀) × Common Carp (♂)

A great deal of experimental data had shown that the *Sry* gene was the master sex determination gene in mammals (da Silva et al. 1996). The *Sox* gene family, which was related to the *Sry* gene, had become an important research focus in the study of fish sex determination mechanisms (O'Brien and Degnan 2000; Ezaz et al. 2004). In addition, both genetic and environmental factors regulate fish sex determination and differentiation (Conover and Kynard 1981; Nagler et al. 2004). As mentioned previously, *Sox9* was relevant to sex determination, and the studies on *Sox9* in allotetraploid red crucian carp (♀) × common carp (♂) were discussed below.

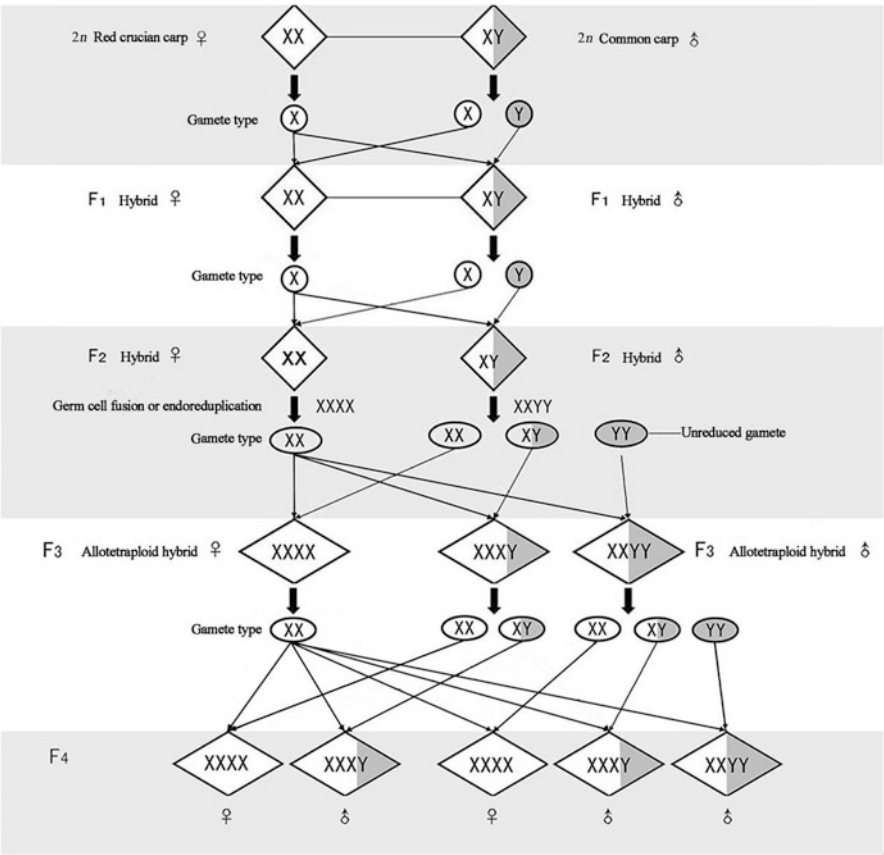

Fig. 3.3 The genetic map of chromosomal sex determination in allotetraploid hybrids of red crucian carp (♀) × common carp (♂)

We amplified the *Sox* gene from the genomic DNA of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) and their original parents, red crucian carp and common carp. Sequence alignment showed that the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) exhibited two fragments (600 bp and 900 bp), which were consistent with the fragments found in their parents (600 bp fragment in red crucian carp and 900 bp fragment in common carp). All of these fragments contained one intron, and the insertion site of the intron conformed to the GT-AG rule. We proved that the 600 bp fragment of the tetraploid fish was a *Sox9a* gene with a 413 bp intron in the conserved HMG region, and the resulting cDNA was 217 bp. Three of the fragments (the 900 bp fragment from allotetraploid fish, the 600 bp fragment from red crucian carp, and the 900 bp fragment from common carp) presented the highest homology to *Sox9* sequences of other species. The three gene fragments were designated *Atsox9b*, *Rcsox9a*, and *Ccsox9b* on the basis of the general rules of *Sox* gene nomenclature. To further verify the existence of

introns, we designed a pair of specific primers to amplify the cDNA sequences corresponding to the conserved HMG regions of these three genes by RT-PCR and compared the products to the corresponding genome sequences. Finally, we identified their positions and sizes.

The sizes of the HMG box introns of *Atsox9a* and *Atsox9b* of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) were 413 bp and 703 bp, respectively, while that of *Rcsox9a* from red crucian carp was 401 bp, and that of *Ccsox9b* from common carp was 714 bp. We detected 7 variable nucleotide sites, 390 conserved nucleotide sites, and additional sites that were inserted or deleted in the sequence comparison of the introns of *Atsox9a* and *Rcsox9a*. There was a 14 bp sequence (AAGGAAATGCTGAT) insertion at the 127–140 bp position and a 2 bp (TA) deletion at 311–312 bp position in the nucleotide sequence of the *Atsox9a* intron. *Atsox9a* presented a similar intron nucleotide sequence to *Rcsox9a*, with a similarity rate of 94.4%. We detected 3 variable nucleotide sites, 689 conserved nucleotide sites, and other sites that were inserted or deleted in the sequence comparison of the introns of *Atsox9b* and *Ccsox9b*. There was a deleted 11 bp sequence (ATGCACATCAT) at 217–227 bp in the nucleotide sequence of *Atsox9b*. *Atsox9b* showed high similarity to *Ccsox9b* in the intron sequence, with a similarity rate of 97.8%. In addition, the G + C content of the introns was lower than that of the exons (Liu et al. 2007c).

The phylogenetic tree of the *Sox9* gene HMG box intron sequences of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂), red crucian carp, common carp, *Danio rerio*, and *Oncorhynchus keta* was constructed via the NJ analysis method. The allotetraploid hybrids of red crucian carp (♀) × common carp (♂) were clustered with the original parents, red crucian carp and common carp, while they were separated from *Danio rerio* and *Oncorhynchus keta*. Sister groups were composed of *Sox9a* of red crucian carp with *Sox9a* of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) and *Sox9b* of common carp with *Sox9b* of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂). The results demonstrated that the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) present a closer genetic relationship to red crucian carp and common carp than to other fishes. This analysis also showed that the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) are the hybrid offspring of red crucian carp and common carp and that the hybrids possess the genetic traits of their parents (Liu et al. 2007c).

The intron splice sites of the four *Sox9* genes conformed by the GT-AG rule. Some of the *Sox9* genes exhibited higher similarity in their intronic HMG motif sequences, although the similarity was lower compared to the exon sequences. The sequence similarity of the introns of *Atsox9a* and *Rcsox9a* was 94.4%, while that of their exons was 99.5%. The phylogeny of the HMG box intron sequences of the *Sox9* genes of different fishes indicated that their evolutionary relationships were similar to the results of traditional taxonomy; that was the allotetraploid hybrid of red crucian carp (♀) × common carp (♂), red crucian carp, common carp, and zebrafish

clustered into the Cyprinidae group, while *Oncorhynchus keta* was separated from Salmonidae. These results showed that the intron sequences could provide useful evolutionary information, which meant that they can be regarded as genetic markers for studying the evolutionary relationships between related species.

Among the over 40 *Sox* genes that were discovered, *Sox5*, *Sox9*, *Sox17*, and *Sox20* had more or less introns. The conserved intronic motif of the *Sox9* gene that directly determines sex type might have important functions. Takase et al. (Takase et al. 2000) reported that introns in the *Sox9* gene of frogs exhibit two splicing patterns, and the two kinds of protein produced might induce two different developmental differentiation mechanisms. *Sox9a* of red crucian carp and *Sox9a* of tetraploid fish exhibit identical amino acid sequences in HMG motif to *Sox9b* of common carp and *Sox9b* of tetraploid fish, and the corresponding introns possess relatively high similarity. Therefore, we studied the splicing patterns of the *Sox9* gene intronic HMG box motif, which have important implications for the further study of the variation and function of the *Sox9* genes of vertebrates.

We obtained the sequences of the *Sox9a* and *Sox9b* genes from the testis of allotetraploid hybrids of red crucian carp (♀) × common carp (♂) via the rapid amplification of cDNA end (RACE) method. We found that the *Sox9b* of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) produces two differentially expressed transcripts via the alternative splicing of introns. Northern hybridization analysis indicated that the gene was expressed in the testis, brain, and heart. No signals were found in the ovary. The results showed that the *Sox9b* of allotetraploid fish participated in the testicular development of male tetraploid fish and exhibits functions in the development of the heart and brain.

After determining the expression of *Sox9b* in the testis but not in the ovary (Liu et al. 2007b), we cloned and analyzed a portion of the sequence of the *Sox4* gene of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) and its promoter fragment in the 5′-flanking regulatory region (Liu et al. 2007a). The *Sox4* gene was expressed in the human brain, heart, and testis at high levels, and its function was closely related to gonadal functions (Farr et al. 1993). The *Sox4b* gene of *Danio rerio* was transiently expressed in endocrine cells and associated with the occurrence of α endocrine cells (Mavropoulos et al. 2005). The cloned *Sox4* gene promoter region from tetraploid fish harbored a transcription factor-binding site for *Sry*, which indicated that the *Sry* gene might be involved in the expression and regulation of *Atsox4*. Thus, the expression of the *Atsox4* gene played a significant role in the gonadal development of male tetraploid fish.

3.5 The Evolutionary Significance of Allotetraploid Hybrids of Red Crucian Carp (♀) × Common Carp (♂)

3.5.1 Functions of Polyploidy in Evolution

Ohno et al. proposed the evolution theory of gene duplication in 1968 and suggested that the *Gnathostomata* experienced two rounds of tetraploidy during evolution (Ohno et al. 1968). They found that the DNA content of *Gnathostomata* members was four times than that of invertebrates, following the "one to four rule." They believed that tetraploidy first occurred in the evolution from early Cephalochordata to Agnatha based on that rule. The evidence for this hypothesis was that *B. lanceolatum* (a representative of *Cephalochordata* and a transitional species between non-vertebrates and agnathan vertebrates) has only one *Hox* gene, while *Lampetra japonica* (a representative of the agnathan vertebrates) has two or three independent *Hox* genes. Tetraploidy occurred for the second time in Vertebrata, in accordance with the gene duplication theory. From fishes to mammals, at least four independent *Hox* genes existed in *Gnathostomata*. The four relevant *Hox* genes were located on different chromosomes among mammals. Thus, if there was one gene in invertebrates, four similar genes could be found in vertebrates.

Four protein-coding or hormone genes with two similar but different forms were found in common carp: hemoglobin alpha-I chain and hemoglobin alpha-II chain, prolactin-I and prolactin-II, gonadotropin alpha-II chain and gonadotropin alpha-II chain, and somatropin-I and somatropin-II. In some known vertebrates, these four genes belonged to the same type (Ohno 1999), providing evidence that common carp underwent chromosome doubling (tetraploidy). People generally believed that common carp evolved from grass carp by chromosome doubling because it presented two times of the number of chromosomes found in grass carp, which conformed to the theory of Ohno. It had been speculated that common carp evolved from grass carp via a chromosome doubling event that occurred 15 million years ago. In addition, *Oncorhynchus mykiss* was considered an old tetraploid fish because it had four *LDH* genes.

However, these hypotheses were all indirect inferences. The formation of allotetraploid red crucian carp (♀) × common carp (♂) might provide direct evidence of the tetraploidy of vertebrates. We performed a systematic analysis of the genetic relationships of allotetraploid hybrids of red crucian carp (♀) × common carp (♂) and their parents. The results showed that DNA recombination, deletion, and insertion occurred in the allotetraploid red crucian carp (♀) × common carp (♂). The genetic variability and fertility of hybrid lineages are two important outcomes for genetic breeding and evolution studies. Thus, the allotetraploid generated from distant hybridization provided a valuable resource that deserves exploration.

In this book, the hybridization derived allotetraploid hybrids of red crucian carp (♀) × common carp (♂) ($4n = 200$) lineage and autotetraploid hybrids ($4n = 200$) lineage were introduced (discussed in Chap. 6). Due to its variations of genetic structures compared to their parents, these lineages had more potentiality and certain advantages. The autotetraploid derived from colchicine and high hydrostatic

pressure methods is different from the autotetraploid derived from red crucian carp × blunt snot bream hybridization. The autotetraploid derived from red crucian carp × blunt snot bream hybridization ($4n = 200$) had parents which were two different species and this promised genetic basis of hybridization, while the autotetraploid derived from colchicine and high hydrostatic pressure methods only had one species as its parents lacking genetic basis of hybridization.

3.5.2 The Formation of Allotetraploid Hybrids of Red Crucian Carp (♀) × Common Carp (♂) Provided Direct Evidence of Polyploid Evolution

Taxonomically, red crucian carp and common carp belong to different genera. Both of diploid red crucian carp and common carp have 100 chromosomes. The F_1 and F_2 hybrids of red crucian carp (♀) and common carp (♂) were diploid which had 100 chromosomes. Intriguingly, the F_2 hybrids could produce diploid gametes and generate allotetraploid hybrids of red crucian carp (♀) × common carp (♂) of the F_3 generation. The female and male F_3 hybrids were fertile. Subsequently, F_4 hybrids were obtained. To date, F_3–F_{29} allotetraploid red crucian carp (♀) × common carp (♂) lineage was consecutively produced. The F_1 and F_2 hybrids reached maturity at 2 years, while allotetraploid hybrids of red crucian carp (♀) × common carp (♂) reached maturity at 1 year. Under natural conditions, the allotetraploids of red crucian carp (♀) × common carp (♂) were able to produce progeny. Over more than 20 years, we found that each generation of the allotetraploid red crucian carp (♀) × common carp (♂) was fertile and the individuals could survive for at least 4 years. Their life span was similar to that of the diploid control (red crucian carp). The formation of the allotetraploid red crucian carp (♀) × common carp (♂) lineage was represented in Fig. 3.4.

The establishment of the fertile allotetraploid hybrids of red crucian carp (♀) × common carp (♂) lineage provided direct evidence related to polyploid evolution:

1. The lineage had potential to be a new species and also provided a suitable model for investigating the genetic relationship between allotetraploid hybrids and their parents.
2. The diploid gametes from the allotetraploids were excellent materials for gynogenesis or androgenesis because no chromosome doubling treatment was required in the manipulation.

The allotetraploid hybrids of red crucian carp (♀) × common carp (♂) exhibited the essential factors necessary for becoming a new species. The chromosome sets were changed from 100 to 200. When the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) were mated with diploid fish, their progeny were all sterile triploid fish, demonstrating reproductive isolation. The genetic stability (the tetraploidy could be inherited) and reproductive isolation from the parental species

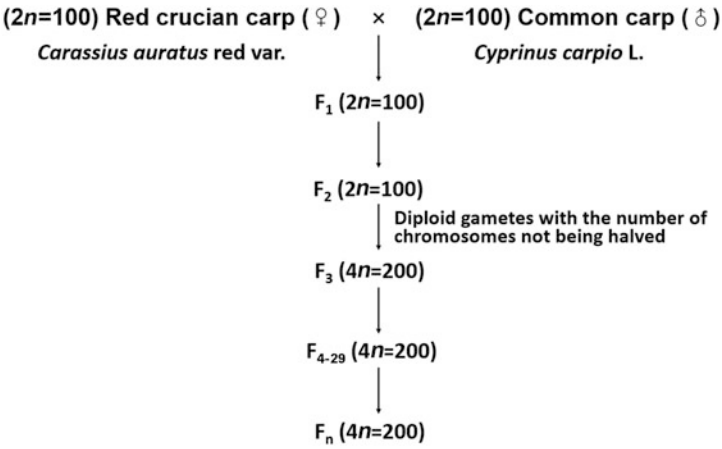

Fig. 3.4 The establishment of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) lineage of red crucian carp (♀) × common carp (♂)

promised this lineage to be a new species. Thus, allotetraploid hybrids of red crucian carp (♀) × common carp (♂) were the first reported case of the artificial creation of fertile allotetraploids (both male and female offspring were fertile) in fish (it was also the first case in vertebrates).

The somatic and germ cell size showed changes under tetraploidy. For example, the size of the erythrocytes in allotetraploid hybrids of red crucian carp (♀) × common carp (♂), allotriploid hybrids of red crucian carp (♀) × common carp (♂), and diploid red crucian carp increased with increasing ploidy levels. The allotetraploid hybrids of red crucian carp (♀) × common carp (♂) contained 33% of distinctive erythrocytes with a dumbbell-shaped nucleus, while the common carp and red crucian carp exhibited no such distinctive erythrocytes. The diploid sperm from allotetraploid hybrids of red crucian carp (♀) × common carp (♂) was larger than that of common carp. Similarly, the diameter of the diploid eggs from allotetraploid hybrids of red crucian carp (♀) × common carp (♂) was larger than that of red crucian carp.

At the molecular level, the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) also presented varied traits. For example, regarding the HMG-sox DNA marker, the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) presented four DNA bands (215, 628, 918, and 1957 bp), while red crucian carp exhibited three DNA bands (215, 617, and 1958 bp), and common carp contained two DNA bands (215 and 918 bp). The sequencing results suggested that 215 bp fragment was *Sox11*, the 617 and 628 bp fragments were *Sox9a*, the 918 bp fragment was *Sox9b*, and the 1957 bp and 1958 bp fragments were *Sox4*. The DNA fingerprinting result suggested that the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) had sequence deletions from male parent (Liu et al. 2004a). On the other hand, several new DNA bands were found in the allotetraploid red crucian carp (♀) × common carp (♂), which could be related to the genotypic

variation of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂). Some nucleotide mutations were found in the *CyclinA1* and *B1* genes of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) that resulted in amino acid substitutions. The mitochondrial (mt) DNA genomes of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) and their original parents, red crucian carp and common carp, were sequenced. The mtDNA nucleotide identity between the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) and the maternal fish species (red crucian carp) was markedly higher than that between the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) and the paternal fish species (common carp), suggesting that the mitochondrial genome of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) was strictly maternally inherited and that the mtDNA of red crucian carp was stably inherited in allotetraploid hybrids of red crucian carp (♀) × common carp (♂) lineage (Guo et al. 2007).

The cytosine methylation patterns of red crucian carp, common carp, and the allotetraploid are determined by using MSAP (methylation-sensitive amplification polymorphism) assays. We found a 38.31% (136) change in the methylation status at 355 randomly selected CCGG sites and these changes were related to metabolism or cell cycle regulation (Xiao et al. 2013). At the same time, the analysis of significant differences indicated that the methylation content of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂) was relatively higher than that of the original parents (Xiao et al. 2013). Gene silencing might occur upon gene methylation. The increase in the methylation level observed in the allotetraploid red crucian carp (♀) × common carp (♂) on the basis of genomic levels showed that the increased gene number resulting from chromosome doubling could be dealt with via gene methylation during the origination of the allotetraploid hybrids of red crucian carp (♀) × common carp (♂). The allotetraploid hybrids of red crucian carp (♀) × common carp (♂) harbored two sets of chromosomes from red crucian carp and two sets from common carp, which was confirmed by the analysis of chromosome numbers, karyotypes, and DNA contents. In the allotetraploid red crucian carp (♀) × common carp (♂), 100 bivalents formed as the chromosome paired during the first meiosis and formed diploid gametes. Only bivalent pairing occurred (not multivalent pairing) during the first meiosis in the allotetraploid red crucian carp (♀) × common carp (♂), and each pair of homologous chromosomes came from either red crucian carp or common carp.

Based on the two reference genomes of red crucian carp and common carp, we performed the systematic analyses of liver transcriptomes in the four generations (F_1, F_2, F_{18}, and F_{22}) of hybrid offspring and their inbred parents in allotetraploid lineage of red crucian carp (♀) × common carp (♂) (Liu et al. 2016). In the four generations of hybrid offspring, we found 9.67–11.06% of chimeric genes, which were obtained from the recombination of orthologous sequences between maternal red crucian carp and paternal common carp (Fig. 3.5, patterns 1–5) (Liu et al. 2016). Some of these chimeric genes, such as *CSNK*, *CDC*, *RAD*, and *UBE*, were associated with the regulation of cell cycle and DNA damage response and repair (via recombination). These chimeras and mutation may induce from DNA repairing including

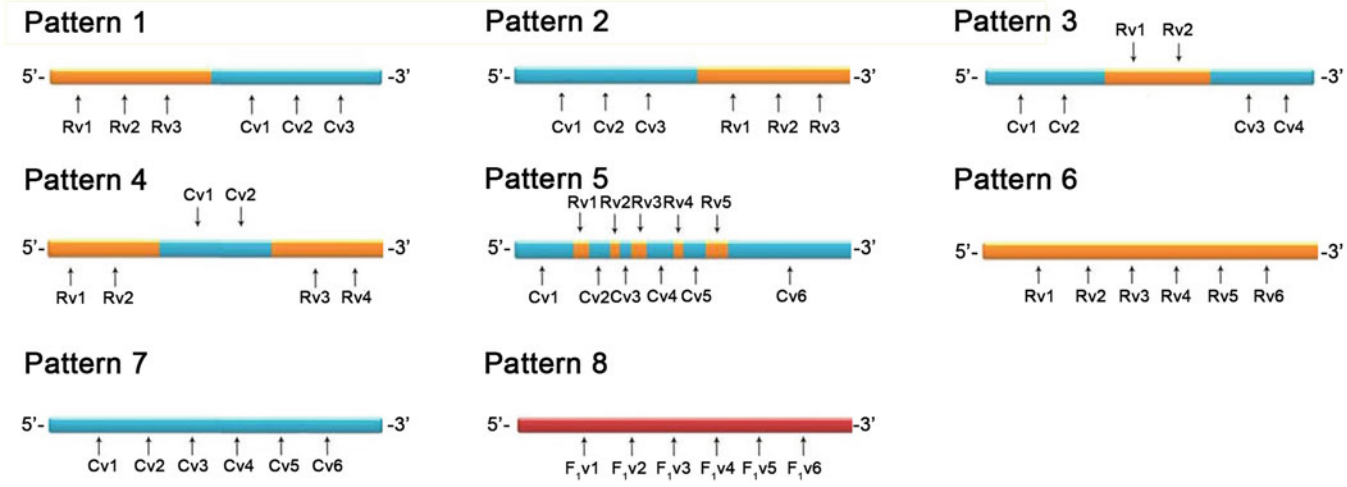

Fig. 3.5 The patterns of chimeric genes in hybrids of red crucian carp and common carp. Orange bars represent the red crucian carp-specific variants; blue bars represent common carp-specific variants; and red bars represent offspring-specific variants

recombination or nonhomologous end-joining or even transposon activity. Several genes were chimeric and differentially expressed genes which had function in mutagenesis and repair pathways. Meanwhile, we also found some paternal-origin or maternal-origin genes in allotetraploid lineage of red crucian carp (♀) × common carp (♂) (Liu et al. 2016). Among these, maternal-origin genes (Fig. 3.5, pattern 6) were less common than paternal-origin genes (Fig. 3.5, pattern 7). We further detected 1.02–1.16% of genes with mutations unique to offspring (Fig. 3.5, pattern 8). The diploid offspring (F_1 and F_2) exhibited paternal-biased expression based on the analyses of differential expression, while the tetraploids (F_{18} and F_{22}) exhibited maternal-biased expression.

We speculated that the genes showing recombination in hybrids could ultimately face two different destinies on the basis of these results: ① accumulated sequence mutations might lead to the occurrence of early termination, giving rise to pseudogenization. ② Some new functions arose and became fixed as result of natural selection. Hybridization between species and polyploidization were important drivers of evolution. The appearance of allelomorphic genes after hybridization played a major role in the maintenance of gene stability and the generation of new species. There were other evidences of nuclear DNA recombination in allotetraploid hybrid of red crucian carp (♀) × common carp (♂). ① The *Hoxc9a* gene of the allotetraploid hybrid of red crucian carp (♀) × common carp (♂) exhibited the subtypes of red crucian carp and common carp but also exhibited a recombinant subtype. Exchange and rearrangement occurred in the intron region, and the first half of the nucleotide sequence was similar to that of common carp, while the sequence of the other half was the same as that of red crucian carp (Zou 2012). ② Recombination had occurred in the nuclear *GnRH2* gene of the allotetraploid red crucian carp (♀) × common carp (♂) (Wang 2013). ③ The results of BAC library and long PCR analyses proved that gene recombination occurred in the four genes of the allotetraploid hybrid of red crucian carp (♀) × common carp (♂) (Wang et al. 2015).

3.6 The Epigenetic Changes in Allotetraploid Hybrids of Red Crucian Carp (♀) × Common Carp (♂)

Hybridization and polyploidization are the two driving forces to speciation in plants and low animals. The gene redundancy with genome duplication leads to genomic recombination and gene expression changes (Otto 2007; Chen 2007). The gene expression changes would be resulted from epigenetic change in polyploidization.

3.6.1 DNA Methylation

The cytosine methylation levels in the allotetraploid hybrid of red crucian carp (♀) × common carp (♂) with its original parents were assayed by methylation-sensitive amplification polymorphism (MSAP) method (Xiao et al. 2013). Using the isoschizomers including *Msp*I and *Hpa*II, the restriction site (CCGG) could be

identified with different sensitivities to methylation statutes (McClelland et al. 1994). After *Msp*I digestion, only fully methylated CCGG site (CmCGG) could be detected by PCR, while externally hemi-methylated CCGG site (mCCGG) could be detected by PCR after *Hpa*II digestion. An absence of bands after both treatments indicated full methylation of all cytosine, full methylation of the external cytosine, hemi-methylation of the internal cytosine, or nonexistence of the site (caused by a genetic mutation). The results of MSAP analysis showed that 355 groups of bands were detected and could be assigned to 61 categories.

The ratio of all the four kinds of methylation status between red crucian carp and common carp was not significantly different from 1:1, revealing that the red crucian carp and the common carp possessed the same methylation level at the 355 CCGG sites. The ratios were 1.35 and 1.46 for allotetraploid hybrid of red crucian carp (♀) × common carp (♂)/red crucian carp and allotetraploid hybrid of red crucian carp (♀) × common carp (♂)/common carp, respectively. The number of methylations at internal cytosine were significantly higher than 1. Thus, the methylation levels were increased in allotetraploid hybrid of red crucian carp (♀) × common carp (♂)/red crucian carp when compared to its parents. The sites with methylation changes were in genomic fragments and genes related to metabolism or cell cycle regulation (Xiao et al. 2013). Thus, in allotetraploid hybrids, DNA methylation may lead to gene expression changes and affect phenotypes.

3.6.2 MicroRNA

The fertile characteristic of allotetraploid fish is crucial for stable inheritance to retain genetic characteristics. microRNA as a group of transcriptional regulators is also involved in gonadal development. The miRNA profiles of diploid red crucian carp and allotetraploid hybrid of red crucian carp (♀) × common carp (♂) ovaries were compared (Zhou et al. 2015). Without surprise, the miRNAs related to ovary maturation were abundant in the tested tissues. Meanwhile, 34 upregulated miRNAs and 9 downregulated miRNAs were found in allotetraploid hybrids of red crucian carp (♀) × common carp (♂) when compared to red crucian carp. The predicted target genes of these differentially expressed miRNAs were genes associated with metabolic, cytoskeleton, and defense systems. The study provided epigenetic evidence for female allotetraploid hybrids of red crucian carp (♀) × common carp (♂) fertility and phenotypic changes resulting from increased ploidy.

References

Chen ZJ (2007) Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. Annu Rev Plant Biol 58(1):377–406

Conover DO, Kynard BE (1981) Environmental sex determination: interaction of temperature and genotype in a fish. Science 213(4507):577–579

Ezaz MT, Harvey SC, Boonphakdee C, Teale AJ, McAndrew BJ, Penman DJ (2004) Isolation and physical mapping of sex-linked AFLP markers in Nile tilapia (*Oreochromis niloticus* L.). Mar Biotechnol 6(5):435–445

Farr CJ, Easty DJ, Ragoussis J, Collignon J, Lovell-Badge R, Goodfellow PN (1993) Characterization and mapping of the human SOX4 gene. Mamm Genome 4(10):577–584

Griffiths S, Sharp R, Foote TN, Bertin I, Wanous M, Reader S, Colas I, Moore G (2006) Molecular characterization of Ph1 as a major chromosome pairing locus in polyploid wheat. Nature 439(7077):749–752

Guo X, Liu S, Liu Y (2007) Evidence for maternal inheritance of mitochondrial DNA in allotetraploid: full length research article. DNA Seq 18(4):247–256

Liu J, Li W, Liu S, Tao M, Long Y, Liu Y (2007a) Cloning and analysis of partial sequences of 5′regulation region from Sox4 in allotetraploid hybrids derived from red crucian carp and common carp. Prog Nat Sci 17(9):1174–1180

Liu J, Liu S, Tao M, Li W, Liu Y (2007b) Analysis of intron sequence variability of the conservative HMG-box of Sox9 genes in allotetraploids and their original parents. Prog Nat Sci 17(5):537–543

Liu J, Liu S, Tao M, Li W, Liu Y (2007c) Genetic variation analysis of HMG conserve regions in Sox9 genes from allotetraploid red crucian carp × common carp and its original parents. Prog Nat Sci 17(3):313–319

Liu J, Liu S, Zhang C, Sun Y, Yan J, Liu Y (2004a) Cloning and analysis of the splicing site of introns of Sox9a gene of allotetraploid. Prog Nat Sci 14(6):461–465

Liu Q, Wang Y, Liu S, Guo X, Luo K, Zhang C, Liu Y (2004b) Comparison of blood and blood cells in different ploidy cyprinid fishes. Prog Nat Sci 14(10):1111–1117

Liu S (2010) Distant hybridization leads to different ploidy fishes. Sci China Life Sci 53(4):416–425

Liu S, Feng H, Liu Y, Zhou G, Zhang X, Luo C, He X (1999) The measurement of DNA content of the tetraploid F_3-F_4 hybrids of red crucian carp (♀) × common carp (♂) and their triploid offspring and other related diploid fish. J Nat Sci Hunan Norm Univ 22(4):61–68

Liu S, Li S, Liu Y (2001a) The early embryonic development of allotetraploid hybrids of red crucian carp (♀) × common carp (♂). J Nat Sci Hunan Norm Univ 24(1):55–57

Liu S, Liu Y, Zhou G, Zhang X, Luo C, Feng H, He X, Zhu G, Yang H (2001b) The formation of tetraploid stocks of red crucian carp × common carp hybrids as an effect of interspecific hybridization. Aquaculture 192(2):171–186

Liu S, Luo J, Chai J, Ren L, Zhou Y, Huang F, Liu X, Chen Y, Zhang C, Tao M, Lu B, Zhou W, Lin G, Mai C, Yuan S, Wang J, Li T, Qin Q, Feng H, Luo K, Xiao J, Zhong H, Zhao R, Duan W, Song Z, Wang Y, Wang J, Zhong L, Wang L, Ding Z, Du Z, Lu X, Gao Y, Murphy RW, Liu Y, Meyer A, Zhang Y-P (2016) Genomic incompatibilities in the diploid and tetraploid offspring of the goldfish × common carp cross. Proc Natl Acad Sci 113(5):1327–1332

Liu S, Zhao R-R, Liu J-H, Sun Y-D, Zhang C, Luo K-K, Liu Y (2005) Comparison of DNA content of blood cells and sperm among the different ploidy level fish. Acta Zool Sin 51(2):360–364

Mavropoulos A, Devos N, Biemar F, Zecchin E, Argenton F, Edlund H, Motte P, Martial JA, Peers B (2005) sox4b is a key player of pancreatic α cell differentiation in zebrafish. Dev Biol 285(1):211–223

McClelland M, Nelson M, Raschke E (1994) Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. Nucleic Acids Res 22(17):3640–3659

Nagler JJ, Cavileer T, Steinhorst K, Devlin RH (2004) Determination of genetic sex in Chinook salmon (*Oncorhynchus tshawytscha*) using the male-linked growth hormone pseudogene by real-time PCR. Mar Biotechnol 6(2):186–191

O'Brien EK, Degnan BM (2000) Expression of POU, sox, and Pax genes in the brain ganglia of the tropical abalone *Haliotis asinina*. Mar Biotechnol 2(6):545–557

Ohno S (1999) Gene duplication and the uniqueness of vertebrate genomes circa 1970–1999. Semin Cell Dev Biol 10(5):517–522

Ohno S, Wolf U, Atkin NB (1968) Evolution from fish to mammals by gene duplication. Hereditas 59(1):169–187

Otto SP (2007) The evolutionary consequences of polyploidy. Cell 131(3):452–462

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst 29(1):467–501

da Silva SM, Hacker A, Harley V, Goodfellow P, Swain A, Lovell-Badge R (1996) Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat Genet 14(1):62–68

Sun Y, Zhang C, Liu S, Duan W, Liu Y (2007) Induced interspecific androgenesis using diploid sperm from allotetraploid hybrids of common carp × red crucian carp. Aquaculture 264(1):47–53

Sun Y-D, Liu S, Zhang C, Li J-Z, Huang W-R, Zhang J, Luo K-K, Zhou G-J, Liu Y (2003) The chromosome number and gonadal structure of F_9~F_{11} allotetraploid crucian-carp. Acta Genet Sin 30(5):414–418

Takase M, Noguchi S, Nakamura M (2000) Two Sox9 messenger RNA isoforms: isolation of cDNAs and their expression during gonadal development in the frog *Rana rugosa*. FEBS Lett 466(2–3):249–254

Wang D (2013) Expression and sex determination mechanism of reproductive genes of HPG axis in different ploidy fish. Hunan Normal University, Changsha

Wang J, Qin Q, Chen S, Liu S, Duan W, Liu J, Zhang C, Luo K, Xiao J, Liu Y (2008) Formation and biological characterization of three new types of improved crucian carp. Sci China Ser C Life Sci 51(6):544–551

Wang J, Ye LH, Liu QZ, Peng LY, Liu W, Yi XG, Wang YD, Xiao J, Xu K, Hu FZ, Ren L, Tao M, Zhang C, Liu Y, Hong YH, Liu SJ (2015) Rapid genomic DNA changes in allotetraploid fish hybrids. Heredity 114(6):601–609

Xiao J, Song C, Liu S, Tao M, Hu J, Wang J, Liu W, Zeng M, Liu Y (2013) DNA methylation analysis of allotetraploid hybrids of red crucian carp (*Carassius auratus* red var.) and common carp (*Cyprinus carpio* L). PloS One 8(2):e56409

Yan J, Liu S, Sun Y, Zhang C, Luo K, Liu Y (2005) RAPD and microsatellite analysis of diploid gynogens from allotetraploid hybrids of red crucian carp (*Carassius auratus*) × common carp (*Cyprinus carpio*). Aquaculture 243(1):49–60

Zhou R, Wu Y, Tao M, Zhang C, Liu S (2015) MicroRNA profiles reveal female allotetraploid hybrid fertility. BMC Genet 16(1–4):119

Zou L (2012) The evolution study on *Hox* genes in allotetraploid and its original parents. Hunan Normal University, Changsha

The Basic Biological Characteristics of Sterile Allotriploid Fish

4

Shaojun Liu, Min Tao, Yi Zhou, Rong Zhou, Jialin Cui, Lu Huang, Shi Wang, Qiubei Wang, Rurong Zhao, Chenchen Tang, and Kaikun Luo

Abstract

The formation of allotetraploids of red crucian carp (*Carassius auratus* red var., ♀) × common carp (*Cyprinus carpio* L., ♂) fills a gap for producing both fertile female and male allotetraploids in vertebrates, which is important for genetic breeding and evolutionary studies. In fish breeding, this lineage provides the valuable resources of diploid gametes for the production of allotriploids. In the early 1990s, using diploid gametes generated from the allotetraploids and haploid gametes generated from Japanese white crucian carp (*Carassius cuvieri*) or Xingguo red carp (*Cyprinus carpio* var. *singuonensis*), triploid Xiangyun crucian carp and triploid Xiangyun carp were produced, respectively. Triploid Xiangyun crucian carp and triploid Xiangyun carp are farmed and popular among consumers in 28 provinces and municipalities in China due to their growth superiority, high meat quality, strong resistance, infertility, and other advantageous traits. Farming of these triploids achieves significant economic, social, and ecological benefits. In addition, new types of allotriploid fish are produced by crossing the male allotetraploids with female goldfish or red crucian carp, which have advantageous traits in growth rate, shape, and meat quality. On the other hand, the diploid eggs and diploid sperm from allotetraploid fish are used to establish gynogenetic and androgenetic diploid hybrid lineages, respectively. Subsequently, the improved tetraploid hybrids are generated from the gynogenetic and androgenetic diploid hybrid lineages. By crossing the improved allotetraploid hybrids with improved red crucian carp, triploid Xiangyun crucian carp II

S. Liu (✉) · M. Tao · Y. Zhou · R. Zhou · J. Cui · L. Huang · S. Wang · Q. Wang · R. Zhao · C. Tang · K. Luo
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_4

is produced. This chapter mainly introduces the morphological traits, reproductive physiology, genetic characteristics, and applications of the allotriploid fish.

Keywords

Allotriploid fish · Sterile · Fast growth · Morphological traits · Genetic characteristics

4.1 The Formation, Appearance, and Karyotype of Triploid Xiangyun Crucian Carp and Triploid Xiangyun Carp

4.1.1 The Formation and Morphological Traits of Triploid Xiangyun Crucian Carp and Triploid Xiangyun Carp

By crossing between female Japanese white crucian carp (*Carassius cuvieri*, $2n = 100$) and male allotetraploids of red crucian carp (*Carassius auratus* red var., ♀) × common carp (*Cyprinus carpio* L., ♂) hybridization, triploid Xiangyun crucian carp ($3n = 150$) was formed. By crossing between female Xingguo red carp (*Cyprinus carpio* var. *singuonensis*, $2n = 100$) and male allotetraploids of red crucian carp (♀) × common carp (♂) hybridization, triploid Xiangyun carp ($3n = 150$) was generated.

Triploid Xiangyun crucian carp had a flat-sided body with a humped back and the abdomen was slightly pointed. The upper part of the body side was steel gray and the lower side was silver gray with a white belly. The snout was blunt. The terminal mouth was blunt and oblique with the thick lower lip. Either side of the lower jaw had only one pair of very short barbels. The appearance of triploid Xiangyun crucian carp was shown in Fig. 4.1.

Triploid Xiangyun carp was spindle-shaped with a high back and round belly. Generally, the back was gray-black, while the belly was silver-gray. Triploid Xiangyun carp had a terminal mouth. Two pairs of barbels were found at the corners of the mouth.

Fig. 4.1 The appearance of triploid Xiangyun crucian carp. Bar = 5 cm

4.1.2 The Karyotype and DNA Content of Triploid Xiangyun Crucian Carp and Triploid Xiangyun Carp

The chromosome numbers of kidney cells in triploid Xiangyun crucian carp and triploid Xiangyun carp were mainly from 145 to 150 (Zhang et al. 2005; He 1999). The modes ($3n = 150$) of triploid Xiangyun crucian carp and triploid Xiangyun carp accounted for 78.0% and 78.6% of all the chromosome numbers in cells. These shreds of evidence confirmed that triploid Xiangyun crucian carp and triploid Xiangyun carp were both triploids with chromosome numbers as $3n = 150$. The karyotypes were both 33 m + 51sm + 33st + 33 t. Atypia chromosomes and specific chromosomes with satellites and secondary constrictions were not found due to the different gonadal development appearances.

The DNA contents were analyzed using the blood of triploid Xiangyun crucian carp and triploid Xiangyun carp by flow cytometry, respectively. The DNA content in the blood sample was tested by flow cytometry. The analysis showed that the DNA content of triploid Xiangyun crucian carp was 1.53 times that of diploid common carp. The DNA content of triploid Xiangyun carp was 1.48 times that of diploid common carp. All these ratios had no significant difference to 1.5:1 ($P > 0.05$). The result was identical to the chromosome number ($3n = 150$) of triploid Xiangyun crucian carp and triploid Xiangyun carp which proved that triploid Xiangyun crucian carp and triploid Xiangyun carp were triploids (Liu et al. 1999).

4.2 The Mechanism of Sterility and Rapid Growth Appearance in Allotriploid Fish

4.2.1 The Sterility of Triploid Xiangyun Crucian Carp and Triploid Xiangyun Carp

In terms of genetics, triploid Xiangyun crucian carp had three sets of chromosomes. Therefore, the chromosome pairing was abnormal during meiosis and could not form normal gametes. In terms of reproductive physiology, triploid Xiangyun crucian carp showed gonadal infertility. The gonads of triploid Xiangyun crucian carp could be classified into three types including testis, ovary, and fat-like type, and all of them could not generate normal gametes. More than 10 years of cultivation experiments had also proved that triploid Xiangyun crucian carp and triploid Xiangyun carp were sterile.

Triploid Xiangyun crucian carp had genetic characteristics with three sets of chromosomes, resulting in biological characteristic changes of the hypothalamus, pituitary, and gonadal axis (the reproductive axis) and ultimately leading to infertility. The related infertility characteristics and infertility mechanisms were shown as follows.

4.2.1.1 The Gonadal Development of Triploid Xiangyun Crucian Carp

During the breeding season, the gonadal structures of triploid Xiangyun crucian carp at the age of 1 and 2 were similar which could be divided into testis, ovary, and fat-like types (Liu et al. 2002). The results of histological sections were shown in Fig. 4.2a–c.

Testis type: The testes of triploid Xiangyun crucian carp were white and brown observed with naked eyes. Bilaterally asymmetrical testes were common. Several individuals only had un-well developmental testis or even no testis could be found on one side, while well developmental testis could be observed on the other side. The shapes of the testes had uneven thickness. Under the light microscope, the testes were full of seminiferous lobules. Spermatids were found to be distributed in seminiferous lobules. Vacuoles could be found in several spermatids and showed degenerative forms with abnormal mature sperm. The observation results showed that intracellular substances were disintegrating and disappearing in degenerative spermatids by the transmission electron microscopy. At the same time, mature sperm could be found in testes of Japanese white crucian carp.

Ovary type: Bilaterally asymmetrical ovaries were common with uneven thickness. Under the light microscope, the cells in ovaries were cystically arranged with small volumes. Under the electron microscopy, these small cells were in a lower differentiated degree and no yolk was found. Since there were no obvious oocyte characteristics, it was difficult to identify which were spermatogonia or oogonia. These small cells were inlaid with a few primary oocytes at phase II and a few large degraded oocytes at phase III. In the degenerative oocytes, the yolk was dissolved. Irregular curve and inward shrinkage could be observed at the outside of oocytes. In the ovaries of Japanese white crucian carp, most oocytes were large with the abundant yolk. No cystic-arranged small cell structure could be found in ovaries from Japanese white crucian carp.

Fat-like type: Only two white adipose tissues on two sides of the dorsal mesentery in the gonads were observed under naked eyes. This type had neither testicular structures nor ovarian structures. Cells were full of fat particles. The cytoplasm and nucleus were pulled aside. When preparing histological sections, fat particles in the

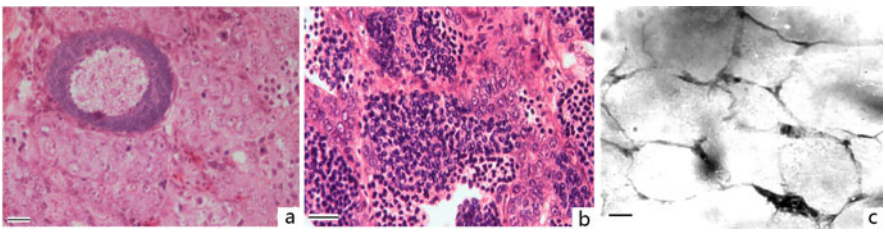

Fig. 4.2 The gonadal development of triploid Xiangyun crucian carp. (**a**) Ovary type gonad in which there were a lot of cystic-arranged small cells with lower differentiation degree. Small number of primary oocytes could be seen in the small cells. Bar = 20 μm. (**b**) Testis type gonad. Spermatids were in seminiferous lobules. Some spermatids were vacuolated and degenerated. Bar = 50 μm. (**c**) Fat-like type gonad. Structures of adipocyte could be observed. Bar = 25 μm

adipocyte were easily dissolved by organic solvent (such as xylene) and became blurred. Under the electron microscope, the fat particles in adipocyte could be observed. In these adipose tissues, no evidence of germ cells could be found.

The gonads of triploid Xiangyun carp also included the three types mentioned above (Liu et al. 2000).

The gonad weight and gonadosomatic index of triploid Xiangyun crucian carp and Japanese white crucian carp in the same period were measured. The gonadosomatic index of the ovary in triploid Xiangyun crucian carp was lower than that in diploid Japanese white crucian carp. Similarly, gonadosomatic index of testis in triploid Xiangyun crucian carp was lower than that in diploid Japanese white crucian carp. The "gonadosomatic index" (fat-like gonad weight/body weight) of fat-like gonads in triploid Xiangyun crucian carp was lower than the average value of gonadosomatic index in ovary and testis of diploid Japanese white crucian carp. In the same reproductive stage, the gonadosomatic index of ovary and testis in diploid Japanese white crucian carp was 2.85 and 1.94 times of that in triploid Xiangyun crucian carp. The average gonadosomatic index in ovary and testis of diploid Japanese white crucian carp was 5.60 times of "gonadosomatic index" of fat-like gonads in triploid Xiangyun crucian carp (Liu et al. 2002). Combined with the results of the gonadal histological section, it could be concluded that triploids had no normal gonadal development and could not produce mature sperm and eggs. In addition, more than 10 years of cultivation had also proved the infertility of triploid Xiangyun crucian carp.

In addition, the ovary development of triploid Xiangyun crucian carp undertook more suppression than testis, which was consistent with the observation from histological sections. Ovaries of triploid Xiangyun crucian carp were mainly occupied with small cells with a lower degree of differentiation, while only a few bigger oocytes with yolk were observed. Therefore, the ovarian gonadosomatic index was low. In the testis of triploid Xiangyun crucian carp, the testes were mainly occupied with spermatids. Though there were not a large number of mature sperm, the testicular structures were similar to the testis of diploid fish. Thus, the difference of testicular gonadosomatic index between triploid and diploid fish was smaller compared to that of ovarian gonadosomatic index. No germ cell existed in the fat-like gonad of triploid Xiangyun crucian carp. The "gonadosomatic index" had significant differences from the average gonadosomatic index of ovary and testis in diploid Japanese white crucian carp (Liu et al. 2002).

4.2.1.2 The Chromosome Behavior of Triploid Xiangyun Crucian Carp in Meiosis

Triploid Xiangyun crucian carp and triploid Xiangyun carp were allotriploids with genome $3n = 150$. Since the gonads were sterile, they could not produce mature sperm and eggs. Thus, these triploid fishes also could not reproduce itself. The results from chromosome pairing observation of triploid Xiangyun crucian carp spermatocytes in the first meiotic metaphase indicated that most chromosomes formed paired bivalent and unpaired univalent (Zhang et al. 2005). The bivalents

were thick and deeply stained. Several ring-shaped bivalents were found. Univalents were slender and lightly stained without pairing. During the meiosis of triploid Xiangyun crucian carp, the numbers of the bivalent and univalent formation were consistent with the result of somatic cell detection that was $3n = 150$ (Zhang et al. 2005).

A similar result was reported. For example, in artificial autotriploid transparent-colored crucian carp, the underdeveloped ovaries had a large number of bivalents and univalent but a few trivalents and ploy valents. It had been regarded that the disorder of chromosome pairing affected the development and growth of germ cells, leading to the block of gonadal development (Gui et al. 1990). In autotriploid, the three chromosome sets had high homology and it was easy to form trivalents. However, the chromosome sets in the allotriploids shared low homology and it had less possibility to form trivalents.

During meiosis, the coexistence of bivalents and univalents in triploid Xiangyun crucian carp would easily lead to aneuploid spermatid which contributed to abortion and abnormal development of mature sperm. In terms of the chromosome, the three sets of chromosomes were disordered in the pairing and separation stages during meiosis, which can cause aneuploid gametes. The suppressed development of germ cells led to nondevelopment or abortion which gave a better explanation of why both testis and ovary types in triploid Xiangyun crucian carp were sterile (Zhang et al. 2008). In the testis of triploid Xiangyun crucian carp, some primary spermatocytes could develop to spermatids and then aborted. In a few cases, some triploid fish could produce a small amount of sperm, but they could not form survived offspring due to the chromosomal aneuploidy of the sperm. Oocytes with yolk were in the ovary from triploid Xiangyun crucian carp but the overall development was abnormal and degenerated. Ultimately, normal mature eggs could not be generated.

4.2.1.3 The Pituitary Structure of Triploid Xiangyun Crucian Carp

The microstructure, ultrastructure, and histochemical characteristics of pituitaries in diploid red crucian carp ($2n = 100$), triploid Xiangyun crucian carp ($3n = 150$), and allotetraploids of red crucian carp (♀) × common carp (♂) ($4n = 200$) were analyzed and compared in the breeding season and after breading season. The results showed that pituitaries of the three fishes all had six different types of secretory cells. The sizes of pituitaries were significantly different among various ploidy fishes. The volumes of pituitary secretory cells and nuclei in allotetraploids of red crucian carp (♀) × common carp (♂) were significantly larger than those in diploids and triploids. The volumes of pituitary secretory cells and nuclei in triploid fish were slightly larger than those in diploid fish (Long et al. 2006). In addition, the studies on blood and blood cells of tetraploid, triploid and diploid fishes showed that erythrocytes and their nucleus, neutrophils and monocytes also presented a proportional relationship of 4:3:2. With the increase of ploidy, the ratios of erythrocyte minor axis to major axis were decreased (Liu et al. 2004a). Allotetraploids of red crucian carp (♀) × common carp (♂) contained four sets of chromosomes ($4n = 200$). Meanwhile, the number of chromosomes or DNA content was two times that of their original diploids parents (common carp and red crucian carp, $2n = 100$). Similarly, the

volumes of triploid pituitary cells and nuclei were larger than those of diploid fish. As for germ cells, the volume of diploid sperm head generated from allotetraploids of red crucian carp (♀) × common carp (♂) was two times that of haploid sperm volume of common carp. The diameters of ovulated diploid mature eggs [(0.17 ± 0.01) cm] were also significantly larger than the diameters of haploid eggs generated from diploid red crucian carp [(0.13 ± 0.01) cm] (Liu et al. 2001).

During the breeding season, somatotropic hormone cells (STH cells) in diploids pituitary accounted for 25% of meso-adenohypophysis. STH cells accounted for 38% of meso-adenohypophysis in triploids, while STH cells accounted for 20% of meso-adenohypophysis in tetraploids. Compared with the diploids, STH cell number of triploids was 1.5 times that of the diploids (Long et al. 2006). Previous experiments proved that triploids grew faster with a high survival rate. Its growth rate was 21.78% and 70.83% faster than that of Japanese white crucian carp and Pengze crucian carp, respectively. However, allotetraploids of red crucian carp (♀) × common carp (♂) grew slowly compared with diploids and triploids. Therefore, the proportion of STH in pituitary may relate to the growth rate. The higher proportion of STH cells may lead to a fast growth rate. Comparative analysis of gonadotropic hormone cells (GTH cells) in different ploidy fishes showed that GTH cells of tetraploid fishes had the largest proportion of cells. This may be because the sexual maturity age of allotetraploids of red crucian carp (♀) × common carp (♂) was earlier than diploid crucian carp and diploid common carp.

During the breeding season, GTH cells in meso-adenohypophysis in tetraploids were similar to diploids. The GTH cells were full of a large amount of closely arranged secretory granules and globules. Since GTH had a close association with gonadal development, allotetraploid fish had normal development of testis and ovary and produced mature sperm and eggs which was similar to diploids. Both allotetraploid females and males were fertile. However, in GTH cells of triploid meso-adenohypophysis, the number of secretory granules and globules was far less compared to diploids and tetraploids which were distributed in the endoplasmic reticulum. It was speculated that the degeneration of secretory granules and globules in GTH cells or the increase of some inhibited factors that suppressed secretory granules led to the great difference between triploid fishes and other fishes including diploids and tetraploids. Besides, the structures of GTH cell indifferent ploidy fishes were significantly different after the breeding season. In diploid and tetraploid GTH cells, a large amount of vacuole-shaped structures could be observed. This was because the appearance, structure, and number of GTH cells would be changed with their reproduction activities, which meant vacuole structures were found due to a release of a large number of granules after egg ovulation. However, only a few vacuoles could be found in triploids and most of the secretory granules and globules were not released out. Therefore, it could be inferred that some factors suppressed the release of secretory globules and granules in triploid GTH cells.

The Comparison of the Pituitary Histochemistry Structure

Histological observation was conducted to the pituitaries of red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂). There was no significant difference in appearance. Their pituitaries were white in color, and round or chicken heart-shaped, which were located at the ventral of interbrain, connecting with the hypothalamus through pituitary stem. They were composed of two parts: neurohypophysis and adenohypophysis. Nerve fibers, capillaries, and glial cells constituted neurohypophysis. The extremely fine branches of nerve fibers were distributed in every part of the pituitary gland (adenohypophysis), gliocytes, and capillary network linked with each other closely. The pituitary gland consisted of pro-adenohypophysis, meso-adenohypophysis, and meta-adenohypophysis.

By histochemical staining, six types of endocrine cell in the pituitaries of the three kinds of fishes were with litter difference (Long et al. 2006).

The Volume Comparisons of Pituitary Cells and Nuclei

The same type of cells in pituitaries of three ploidy level fishes had obvious difference in cell size. The diameters of GTH and STH increased with their ploidy levels increasing (Long et al. 2006). Apart from STH and GTH, there were other four secretory cells and their nuclei diameters increased with the ploidy level being higher.

Generally, the size of the nucleus and the number of chromosomes increased proportionately. And in order to maintain a constant karyoplasmic ratio, cells would enlarge proportionately with the nuclei enlarging. Therefore, polyploid cells and nuclei were usually bigger than those of diploids. But organs and bodies of polyploids were not necessarily larger than diploids. It was not to say tetraploids were twice the size of the diploids of the same age. It was because the number of cells reduced while cells were enlarging, which maintained the sizes of the bodies or organs more or less. Allotetraploids of red crucian carp (♀) × common carp (♂) contained four sets of chromosomes ($4n = 200$), and their chromosome numbers or DNA contents were twice that of their original diploid parents (red crucian carp and common carp, $2n = 100$). Thus, it might be due to the tetraploids having completed four sets of chromosomes with high genetic materials that the volumes of the pituitary secretion cells and nuclei were significantly larger than those of diploids. This might also explain why triploid pituitary cells and nuclei were larger than those of diploids.

The Analysis on Proportion of Acidophile and Basophil Cells

Acidophile and basophil cells took different proportions in meso-adenohypophysis of different ploidy fishes. During the breeding season, basophils (e.g., GTH cells) were obviously more than eosinophils (e.g., STH cells) in meso-adenohypophysis of three kinds of fishes. This was because STH cells and GTH cells took different proportions with the change of reproductive season. In breeding season, GTH cells increased and enlarged, coupled with their increased secretory granules and decreased STH cell. Diploid STH cells accounted for 25.0% of the

meso-adenohypophysis and triploid STH cells for 38.0%, but the tetraploid STH cells accounted for 20.0%. Compared with the diploids, the amount of triploid STH cells was 1.5 times. Feeding experiments have shown that triploids grew fast and had a high rate of survival. However, allotetraploids grew more slowly than diploids and triploids. Therefore, the authors supposed that the growth rate might be affected by the proportion of STH cells in pituitary. In addition, through a comparative analysis on GTH cells of different ploidy fishes, we found GTH cells accounted for the largest proportion in allotetraploid fish. The age of sexual maturity for allotetraploids of red crucian carp (♀) × common carp (♂) was earlier than the diploid crucian carp and diploid common carp which might be related to the larger proportion of GTH cells and strong secretive ability in allotetraploid fish.

4.2.1.4 The Ultrastructure Comparison of Pituitary Glands

GTH cells and STH cells in different ploidy fishes had not much difference in appearance. The ultrastructural features of STH cells and GTH cells in meso-adenohypophysis of three fishes were shown in Table 4.1.

As observed from the GTH cell ultrastructure of allotetraploids of red crucian carp (♀) × common carp (♂) meso-adenohypophysis, we found it developed well in breeding season like diploid red crucian carp. A large number of secretory granules and globules were full of the cells and arranged tightly. Because GTH cells had a close relationship with gonadal development, allotetraploids of red crucian carp (♀) × common carp (♂) could produce mature spermatozoa and eggs normally just like diploid red crucian carp. Previous studies showed that the morphology, structure, and number of GTH cells changed coupled with the change of reproductive activities. For example, vacuoles appeared in degenerate GTH cells after spawning because a large number of granules were released from cells (Liu 1993). We also found that, after breeding season, a large amount of vacuoles occurred in GTH cells of diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂), which indicated that they can conduct normal internal secretion activities and promote the gonadal development.

During breeding season, secretory granules and globules disseminated in the GTH cell endoplasmic reticulum of triploid Xiangyun crucian carp were far less than in diploids and allotetraploids of red crucian carp (♀) × common carp (♂) of the same period. Presumably, this might be associated with the stunted gonadal development of triploid Xiangyun crucian carp. Degeneration of secretory granules and globules in GTH cells or the inhibition of the increase of secretory granules may result in the difference among triploids, diploids, and allotetraploids of red crucian carp (♀) × common carp (♂). In addition, after the breeding season, only a small number of vacuoles could be observed in the triploid Xiangyun crucian carp GTH cells. Since something inhibited the release of secretory granules and globules, most of them would not discharge. Considering the correlation of the development of GTH cells and gonad, we could conclude that discharge inhibition of secretory granules and globules in or after the breeding season was related with the stunted gonad of sterile triploids (Long et al. 2006).

Table 4.1 The ultrastructural features of STH and GTH cells in meso-adenohypophysis of three different ploidy fishes

	STH			GTH		
	Diploid red crucian carp	Triploid Xiangyun crucian carp	Allotetraploid fish of red crucian carp (♀) × common carp (♂)	Diploid red crucian carp	Triploid Xiangyun crucian carp	Allotetraploid fish of red crucian carp (♀) × common carp (♂)
Cell shape	Close to round	Close to round or irregular	Round	Oval or microscler	Oval	Oval
Number of granules	Many			Many (large and small ones)		
Endoplasmic reticulum	Surround the nuclei like a ring			Distribute in the cytoplasm like bubble or pool		

4.2.1.5 The Comparative Analysis of HPG Axis Related Genes

Research on *GtHβ*

We analyzed the localization of *Lhβ* and *Fshβ* in pituitaries of different ploidy fish during the breeding season by using in situ hybridization (Long et al. 2009a). The locations of the two genes in triploid Xiangyun crucian carp were similar to those in tetraploids. *Fshβ* was found in the center of pituitary, namely, meso-adenohypophysis, but not in the pro-adenohypophysis and meta-adenohypophysis. *Lhβ* gene mainly existed in the meso-adenohypophysis.

The Comparative Analysis of Gonadotropin Genes

Differential gene expression analysis of *GtHβ* genes was carried out in red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂). The results of real-time PCR showed that both *Fshβ* and *Lhβ* genes were only expressed in the pituitaries but not in other tissues (Long et al. 2009a). By in situ hybridization, we found that *Fshβ* and *Lhβ* genes were all expressed in the meso-adenohypophysis (the center of pituitary) among different ploidy fishes. This was consistent with the fact that GTH cells produced gonadotropin and GTH cells of different ploidy fishes were mainly located at the meso-adenohypophysis. The above results indicated that these two genes were all able to promote the maturation of gametes and gonads.

Real-time PCR analysis was conducted in pituitaries of different ploidy fishes. We found that *Fshβ* and *Lhβ* genes showed great differential expression before and after the breeding season. In nonbreeding season, the expressions of *Fshβ* genes in allotetraploids of red crucian carp (♀) × common carp (♂) were slightly lower than in the diploids. It was contrary to this during breeding season. But the *Fshβ* gene was highly expressed in the pituitary of the triploid Xiangyun crucian carp before and after the breeding season. The *Lhβ* gene had similar expression patterns in these three fishes (Fig. 4.3a–d).

In diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂), with the gonadal development its *Fshβ* and *Lhβ* mRNA levels increased and decreased when gonads were degenerated. This was quite unlike salmonids which were of continuity. Such a phenomenon also happened in goldfish and common carp. It was supposed that this maybe due to the complete synchronism of ovary maturation of salmon fishes. They only produced one egg in the spawning season, while common carps were not completely synchronized, the ovaries of which contained a number of oocytes at different stages.

It was noteworthy that regardless of before or after the breeding season, the expressions of *Fshβ* and *Lhβ* genes in triploid Xiangyun crucian carp were higher than in diploid and tetraploid fishes. Combined with the previous ultrastructure of pituitaries, we could consume that after the breeding season the secretory granules and globules in GTH cells of triploid Xiangyun crucian carp could not be discharged normally like diploids and tetraploids. And gonadotropins of *Fshβ* and *Lhβ* mRNA at the molecular level after breeding season could not decrease normally which resulted in a higher expression than that in diploids and tetraploids.

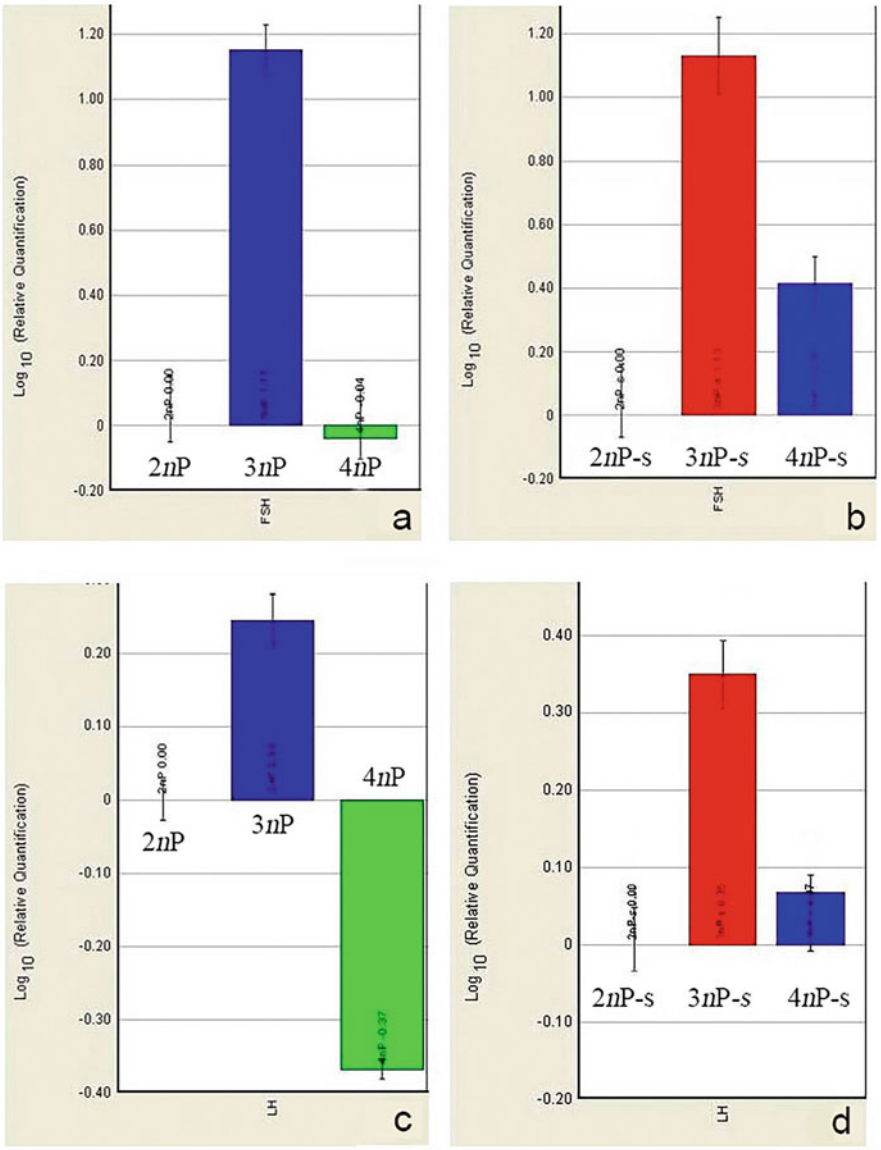

Fig. 4.3 The results of real-time PCR showed the expression of *Fshβ* and *Lhβ* in the pituitaries of different ploidy fishes (Long et al. 2009a) (partially quoted). (**a**) In nonbreeding season, expressions of *Fshβ* genes in the pituitaries of different ploidy fishes. (**b**) In breeding season, expressions of *Fshβ* genes in the pituitaries of different ploidy fishes. (**c**) In nonbreeding season, expressions of *Lhβ* genes in the pituitaries of different ploidy fishes. (**d**) In breeding season, expressions of *Lhβ* genes in the pituitaries of different ploidy fishes. *2n*P, 3*n*P, and 4*n*P represented pituitaries of diploid red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂) in nonbreeding season, respectively. *2n*P-s, 3*n*P-s, and 4*n*P-s represented pituitaries of diploid red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂) in breeding season, respectively. Note: On basis of diploid pituitaries, the original content of *Fshβ* and *Lhβ* mRNA in diploid pituitaries was 1.

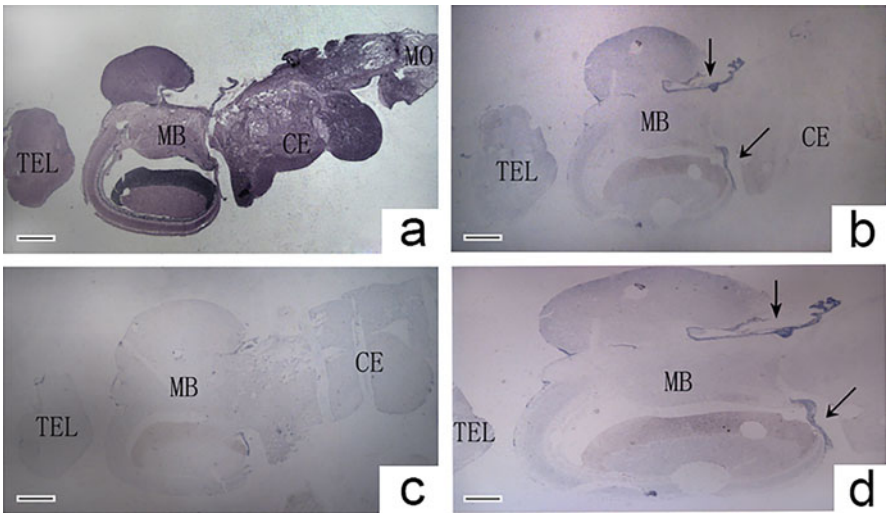

Fig. 4.4 The localization observation of *Gnrh2* genes in the midbrain of allotetraploids of red crucian carp (♀) × common carp (♂) (in situ hybridization). (**a**) The hematoxylin-eosin (HE) staining observation of sagittal section of allotetraploids of red crucian carp (♀) × common carp (♂) brain tissue. TEL: telencephalon. MB: midbrain. CE: epencephalon. MO: medulla oblongata. (**b**) *Gnhr2* gene expression in allotetraploids of red crucian carp (♀) × common carp (♂) by antisense RNA probe. Arrows indicated positive signals of *Gnhr2* mRNA localized in the posterior of midbrain or between midbrain and epencephalon. (**c**) Allotetraploids of red crucian carp (♀) × common carp (♂) brain tissue in situ hybridization by sense RNA probe. No positive signal was found in it. (**d**) Detection of *Gnhr2* gene expression in the brain of allotetraploids of red crucian carp (♀) × common carp (♂) by antisense probe. Arrows indicated positive signals of *Gnhr2* mRNA localized in the posterior of midbrain. Bar is 1000 μm in (**a**, **b**, and **c**) and 500 μm in (**d**)

The pituitary structure of triploid Xiangyun crucian carp was different from diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂), which was related to the fast growth and sterility characteristics of the triploid Xiangyun crucian carp. In addition, a comparative study to gene structure and expression characteristics of *GtHβ* also explained the fertility of tetraploid fish and sterility of triploid fish (Long et al. 2009a; Long et al. 2009b).

In situ hybridization was used to make a research on the localization of *Gnrh2* genes in the brains of different ploidy fishes. Localization of *Gnrh2* gene in allotetraploids of red crucian carp (♀) × common carp (♂) was shown in Fig. 4.4a–d. The results showed that *Gnhr2* genes displayed similar localization in diploid red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂), mainly in the posterior of midbrain or between the midbrain and epencephalon.

In addition, we also analyzed the localization of *Fshr* and *Lhr* genes in ovaries and testes of different ploidy fishes during the breeding season by using in situ hybridization (Long et al. 2009a).

The results of in situ hybridization revealed that *Fshr* mRNA was localized in the follicle cells, granule cells, and outer space of radiation membrane of ovaries in

diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂) while occurring in the sustentacular and interstitial cells of testes. The localization of *Lhr* gene in gonads of diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂) was similar to that of *Fshr* genes. However, the localizations of *Fshr* and *Lhr* genes in triploid Xiangyun crucian carp were quite different from diploids and tetraploids. There were only a few stage III oocytes in triploid ovaries. And the positive signals of *Fshr* and *Lhr* genes only could be observed in the follicle cells, granule cells, and outer space of radiation membrane of stage III oocytes. These signals were weaker than in diploids and tetraploids. But in triploid testes, no positive signal was observed in the sustentacular cells and interstitial cells.

4.2.1.6 Molecular Features of Sterile Allotriploid Fish

Dmc1 (disrupted meiotic cDNA) was a specific gene in the meiotic prophase I. The *Dmc1*-encoded protein is the necessary part for homologous chromosome pairing in meiosis. In the breeding season, *Dmc1* genes were cloned from ovary and testis of diploid red crucian carp, triploid Xiangyun crucian carp, and allotetraploid red crucian carp (♀) × common carp (♂). The results showed that *Dmc1* gene in the testes of different ploidy fishes was all expressed. *Dmc1* gene of triploid Xiangyun crucian carp was highly expressed in the ovary, but only low expression of *Dmc1* gene was found in diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂).

The results of real-time PCR showed that in the breeding season, *Dmc1* gene had higher expression in the testis compared to that in the ovary at the same period. Among the different ploidy fishes, the highest expression of *Dmc1* gene was found in the testis and ovary in triploid fish. Diploid fish had the medium expression of *Dmc1* gene in the testis and ovary. Allotetraploid fish had the lowest expression of *Dmc1* gene in the testis and ovary. During the nonbreeding season, higher expression of *Dmc1* gene was found in the testis compared to the ovary in the same kind of fish. Among the three different ploidy fishes, the highest expression was found in allotriploid ovary, followed by tetraploid ovary. Diploid ovary had the lowest expression of *Dmc1* gene. In the testis, expressions of the *Dmc1* gene in the three fishes were similar. (Tao et al. 2008).

The results of real-time PCR showed that *Dmc1* gene was expressed the most in the testis and ovary in triploid Xiangyun crucian carp among different ploidy fishes, followed by diploid red crucian carp, while the allotetraploids of red crucian carp (♀) × common carp (♂) had the lowest expression. Especially the expression of *Dmc1* gene was higher in allotriploids than that in diploid and allotetraploid fish, significantly. According to the normal development, gonads of diploid and allotetraploid fish in the breeding season were generally well-developed. Most of the cells were no longer in primary oocytes and primary spermatocytes. But gonadal cells of triploid Xiangyun crucian carp could not reach the mature phase most especially in the ovary, which are usually undeveloped. Thus, it could be proposed that *Dmc1* expression in different ploidy fishes had no relationship with ploidy levels but related to the development degree of gonads. Because triploid Xiangyun crucian

carp was sterile, cells in meiotic prophase were the most expressed in the breeding season. According to previous studies, the sexual maturity of allotetraploids of red crucian carp (♀) × common carp (♂) was earlier than that of diploid red crucian carp and its sperm and eggs were formed earlier. Thus, allotetraploids of red crucian carp (♀) × common carp (♂) had the lowest number of cells in meiotic prophase I. Combined with the results of the real-time PCR, these pieces of evidence suggested that *Dmc1* genes in different ploidy fishes were specifically expressed in meiotic prophase I which was similar to those in other species. Meanwhile, *Dmc1* gene was overexpressed in the triploid Xiangyun crucian carp which was significantly higher than that in diploid and allotetraploid fishes. This may relate to the disorder of homologous chromosome pairing and sterility in allotriploid fish (Tao et al. 2008).

In addition, fish gonadal development and gamete maturation were mainly controlled by the hypothalamic-pituitary-gonadal axis (HPG axis) which is the crucial axis in regulation by nervous and endocrine systems. Brain-derived gonadotropin releases hormone (GnRH) to stimulate the pituitary to secrete gonadotropins (GTH) which combines with specific gonadotropin receptors (GtHR) in gonads to promote the secretion of sex steroids and induces gonadal maturation. Comparison of *Gnrh*, *Gth*, and *Gthr* gene expression would be helpful for discussing the sterile mechanism of triploid Xiangyun crucian carp.

By real-time PCR and Western blot, the *Gnrh2* gene expressions in brains from diploid red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂) were analyzed from mRNA and protein levels. From Fig. 4.5a, b and Fig. 4.6a, b, during the nonbreeding season, the mRNA and protein expressions of *Gnrh*2 were the highest in the brain from triploid Xiangyun crucian carp, followed by diploid red crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂) had the lowest expression. During the breeding season, mRNA and protein expression of *Gnrh2* had the highest expression in brains from allotetraploids of red crucian carp (♀) × common carp (♂), followed by diploid red crucian carp, and the lowest expression was found in triploid Xiangyun crucian carp. Thus, compared to diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂), the expression of *Gnrh*2 in triploid Xiangyun crucian carp was lowest, which may lead to the unnormal release of secretory granules, and finally contribute to dysfunctional gonadal development and even sterility. However, during the nonbreeding season, high expression of *Gnrh*2 in triploid Xiangyun crucian carp was found which may be due to the fact that the gene could not be downregulated normally by some unknown factors (Long et al. 2009b; Wang 2013).

In bony fishes, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) have similar structures with FSH and LH in mammals, respectively. Both FSH and LH are composed of α subunit and β subunit. The two gonadotropins (GTHs) share the same α subunits, while they have different β subunits. By real-time PCR, the expressive differences of *Fshβ* and *Lhβ* of the breeding and nonbreeding

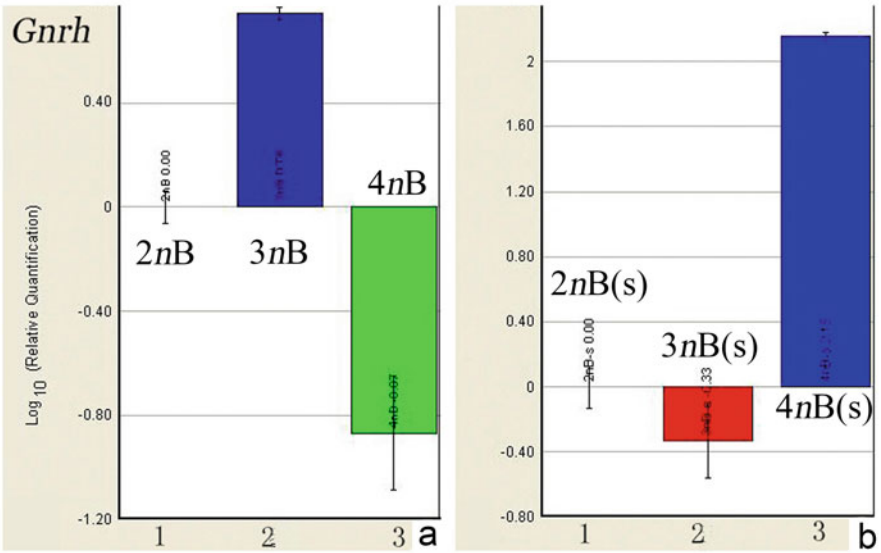

Fig. 4.5 The results of real-time PCR showed the gene expression of *Gnrh2* in brains of different ploidy fishes (partially quoted from reference) (Long et al. 2009a). (**a**) During the nonbreeding season, the expression profiles of *Gnrh2* in the brain of different ploidy fishes. (**b**) During the breeding season, the expression profiles of *Gnrh2* in the brain of different ploidy fishes. In (**a**) and (**b**), 1 indicated the midbrain of diploid red crucian carp, 2 indicated the midbrain of triploid Xiangyun crucian carp, and 3 indicated the midbrain of allotetraploids of red crucian carp (♀) × common carp (♂) (the midbrain of diploid fish was the calibrator sample that had a relative quantification value of 1)

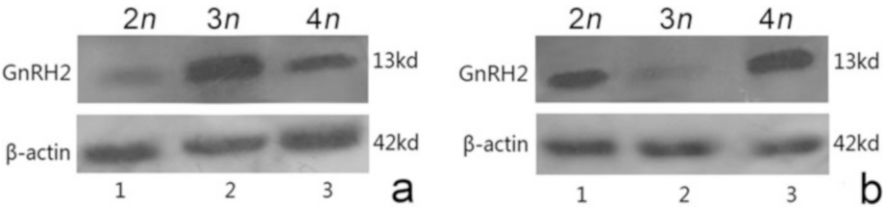

Fig. 4.6 The Western blot result of GnRH2 protein in brains of different ploidy fishes. (**a**) During the nonbreeding season, expression of GnRH2 protein in brains of different ploidy fishes. (**b**) During the breeding season, expression of GnRH2 protein in brains of different ploidy fishes. In (**a**) and (**b**), 1 indicated diploid midbrain, 2 indicated the midbrain of triploid Xiangyun crucian carp, and 3 indicated the midbrain of allotetraploids of red crucian carp (♀) × common carp (♂)

season in the pituitaries of different ploidy fishes were analyzed. *Fshβ* of allotetraploids of red crucian carp (♀) × common carp (♂) had lower expression than that of diploids during the nonbreeding season but a higher expression than that of diploids during the breeding season. However, *Fshβ* expression of the pituitary in triploid Xiangyun crucian carp was the highest during the nonbreeding and breeding

season. The *Lhβ* expressions in the three different ploidy fishes were similar with *Fshβ* expression (Long et al. 2009a; Long et al. 2009b).

It was noteworthy that regardless of nonbreeding or breeding season, the expressions of *Fshβ* and *Lhβ* in pituitary from triploid Xiangyun crucian carp were higher than those of diploid and tetraploid fishes. From the observation of pituitary ultrastructure, it could be found that after breeding season, the secretory granules and globules in GTH cells from triploid Xiangyun crucian carp could not be normally expelled like diploids and tetraploids (Long et al. 2009a). *Fshβ* and *Lhβ* mRNA content of triploid Xiangyun crucian carp after breeding season could not reduce normally which was consistent with its morphological traits.

Through in situ hybridization, the location of *Fshr* and *Lhr* in the ovaries and testes of different ploidy fishes in breeding season was analyzed. *Fshr* was located in follicle cells, granulosa cells, and outside layer of zona radiata in ovaries. While *Fshr* was distributed in Sertoli cells and Leydig cells in the testes from diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂) oocytes. The *Lhr* distributions in diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂) were similar to the distribution of *Fshr*. However, the location of *Fshr* and *Lhr* in the gonad of triploid Xiangyun crucian carp was different from that in diploid and tetraploid fishes. There were only a few oocytes in stage III in the ovary from triploid Xiangyun crucian carp. Positive hybridization signals of *Fshr* and *Lhr* could only be found in follicular cells, granulosa cells, and outside layer of zona radiata in oocytes in stage III. And these signals were weaker in triploid fish than those in diploids and tetraploids. In testicular lobules from triploid Xiangyun crucian carp, positive signal could not be observed in Sertoli cells and Leydig cells as that in the testis in diploid and tetraploid fish.

Vasa is a member of the DEAD-box family which encodes an ATP-dependent RNA helicase and plays an important role in processes of germ cell proliferation and differentiation (Linder et al. 1989). The research on *vasa* gene in red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂) would provide data to explore the molecular mechanism of sterile triploid fish. By real-time PCR, the expression of *vasa* in different tissues from triploid Xiangyun crucian carp was carried out. The result showed that *vasa* was specifically expressed only in the testis and ovary (Yu et al. 2015). While no expression signal was detected in the pituitary, heart, brain, spleen, liver, kidney, gills and muscle, which indicated *vasa* played an important role in the process of gonadal development of triploid fish (Liu 2012a).

In addition, real-time PCR was used to make a comparative study on *vasa* expression levels in gonads from diploid common carp, diploid red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂) (Liu 2012a). The result indicated that differences were found by comparing the sterile triploid fish and fertile fishes including common carp, red crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂). *Vasa* gene were expressed in testes from common carp, red crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂), and the expression level in the nonbreeding season was higher than that in the breeding season. Interestingly, comparing the

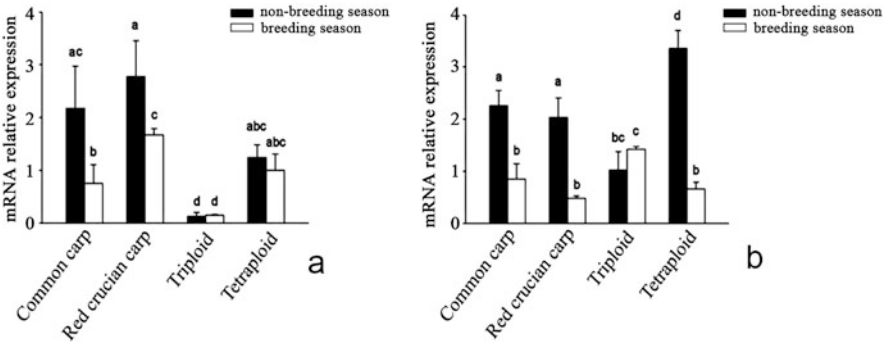

Fig. 4.7 The results of real-time PCR of *vasa* expression in the gonad of different ploidy fishes during breeding and nonbreeding season. (**a**) *Vasa* gene expression in testes in different ploidy fishes. (**b**) *Vasa* gene expression in the ovary in different ploidy fishes. Note: Different letters showed significant differences among groups ($P < 0.05$)

breeding and nonbreeding seasons, there was no significant difference in testicular expression of *vasa* gene in triploid Xiangyun crucian carp, while triploid Xiangyun crucian carp had significantly lower expression compared to common carp, red crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂) in testes (Fig. 4.7a). *Vasa* was expressed in ovaries from common carp, red crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂), and the expression levels in the nonbreeding season were all higher than that in the breeding season. However, in the ovary from triploid Xiangyun crucian carp, the *vasa* gene had higher expression during the breeding season than that during the nonbreeding season (Fig. 4.7b).

Previous study (Liu et al. 2000) showed that during the breeding season, the testis of triploid fish was full of immature spermatids but no mature sperm was found. Meanwhile, it also showed that the transcript of *vasa* gene in sperm from oligozoospermia patients was 1/5 of those of *vasa* gene in normal sperm (Xu et al. 2005) Therefore, it could be speculated that the sterility of male triploid fish had a certain relevance with the low expression of *vasa* gene. In addition, the previous study showed that the ovary of triploid Xiangyun crucian carp could not develop normally (Liu et al. 2000). During the breeding season, there were a large number of cystically arranged oogonia with small volumes and a few primary oocytes in the ovary of triploid Xiangyun crucian carp. The oocytes were mainly stagnated in stage I and stage II, while only a few oocytes in stage III and stage IV could be found. In diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂), the oocytes were mainly in stage I, stage II, and stage IV during the breeding season. During the nonbreeding season, oocytes of triploid crucian carp were mainly in stage I, while in red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂), oocytes were mainly in stage I and stage II. Another study in catfish, *Clarias gariepinus*, showed that the *vasa* gene was expressed mainly in stages I and II with the highest expression in stage II (Raghuveer and Senthilkumaran 2010). The

abnormal increase in *vasa* expression in ovary of triploid fish was associated with the main components of stage I and stage II oocytes. Compared to fertile red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂), the *vasa* gene expression in triploid Xiangyun carp was significantly abnormal with the reproductive cycle. Thus, the abnormal expression of *vasa* gene was related to the sterility of female triploid fish.

By using real-time PCR and Western blot, the expression of Piwi in different ploidy fishes like diploid red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂) was investigated (Zhou et al. 2012; Zhou et al. 2014). PIWI-interacting RNAs (piRNAs) as a new class of non-coding RNA were only found to be expressed in gonad specifically. By combining piRNA with Piwi proteins, they regulated gene expression in transcriptional and protein translation levels. Currently, the study on piRNA was only limited to a small number of animals (Burgess 2013). Similar to zebrafish, *Piwi* families contained two genes including *Piwil-1* and *Piwil-2* in common carp and crucian carp. The expression patterns of *Piwil-1* and *Piwil-2* in common carp and crucian carp were similar to those of *Piwil-1* and *Piwil-2* in zebrafish (Houwing et al. 2007; Zhou et al. 2012). The tissue expression of *Piwi* study showed *Piwi* was specifically expressed only in the testis and ovary from different ploidy fishes (Zhou et al. 2014). In addition, the *Piwi* expression in the testis of triploid Xiangyun crucian carp was lower than that in diploid red crucian carp both in the breeding season and nonbreeding season, while allotetraploids of red crucian carp (♀) × common carp (♂) had lower expression of *Piwi* only during the nonbreeding season. In ovaries, higher expression of *Piwi* in triploid Xiangyun crucian carp was observed compared to diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂) either during breeding season or nonbreeding season (Zhou et al. 2014).

Except for the abnormal expression of *Piwi*, piRNA expression in female triploid Xiangyun crucian carp also significantly increased. By cloning small RNA sequences in ovaries, 61, 72, and 66 piRNAs were obtained from diploid red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂), respectively.

Seven piRNA sequences were identified in the three fishes simultaneously. Interestingly, although only 12 identified piRNA could map to the transcribed mRNA sequence, the located gene functions were varied. For example, *ikbkg* plays an important role in immune function. *Zp2.5* is associated with the egg development. *Tll10* is a gene that encodes a tubulin tyrosine ligase. *Ttnb* encodes the protein which is a component of muscle fiber. *Zbtb48* encodes the protein which is a transcription factor and belongs to zinc finger protein. These genes have wide functions in some biological activities. Among them, *zp2.5* is directly involved in the final maturation of egg development. After studying on the ovaries of three fishes during the breeding season by real-time PCR, five of the seven piRNAs in triploid Xiangyun crucian carp were significantly higher than that in diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂), while the other two piRNAs had no significant expression difference in the three fishes. These results demonstrated that the expression amount of piRNAs had a correlation with the *Piwi*

expression. In addition, in vivo injection and in vitro culture proved that E2 suppressed the expression of piRNA (Zhou et al. 2014) .

A quantitative analysis of *Lhr* mRNA in ovaries from different ploidy fishes showed that *Lhr* expression in triploid Xiangyun crucian carp was significantly lower than in diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂) (Zhou et al. 2014), which was consistent with the result of in situ hybridization of *Lhr* previously reported (Long et al. 2009b).

These clues suggested that the abnormal increase of *Piwi*-piRNA signal pathway in sterile female triploid Xiangyun crucian carp was driven by the dysfunction of HPG axis, especially the abnormal expression of *Lhr* in the ovary. The HPG axis suppressed *Piwi*-piRNA signal pathway. During the breeding season, the relevant factors of the HPG axis were activated which inhibited the expression of piRNA. The inhibition of piRNA relieved the suppression of gonadal developed genes by piRNAs and ultimately led to the success of ovulation. In triploid Xiangyun crucian carp, the expression of HPG axis was dysfunctional and E2 could not normally be released, and therefore, HPG axis could not inhibit Piwi-piRNA signaling pathway normally. Even in the breeding season, piRNA and *Piwi* were both highly expressed in triploid fish which led to inhibition of transcriptional or genomic activities. Finally, oocytes could not be mature and successfully ovulated (Zhou et al. 2012; Zhou et al. 2014).

4.2.1.7 The Epigenetic Features of Sterile Allotriploid Fish

Histone post-modifications, including phosphorylation, acetylation, methylation, ubiquitination, SUMOylation, and crotonylation, are important epigenetic marks. Usually, they involve different biological processes via two main models, one is regulating gene expression by altering interactions between histones and DNA to influence the structure of chromatin (i.e., its compactness or looseness), and the other is recruiting different proteins (Strahl and David 2000; Tan et al. 2011; Xu et al. 2009). Till now, few studies have been reported on histone modifications involved in regulating meiotic impairment during oocyte early development. Comparative analysis was performed between the sterile female allotriploid fish and fertile female diploid red crucian carp (Zhou et al. 2019).

Twenty different post-modifications on various residues of histone H3 were detected by ELISA. Most histone H3 post-modifications levels were comparable. The levels of H3K4me3, H3K9me3, H3K79me, and H3K79me3 were higher in the ovaries of allotriploid fish than in those of red crucian carp (Zhou et al. 2019). In yeast, H3K4me3 occurs near double-strand break that initiate homologue recombination during the prophase stage of meiosis (Valérie et al. 2009). As a heterochromatin marker, H3K9me3 is enriched at unsynapsed trivalents during impaired SC formation (Naumova et al. 2013). In mice, during normal meiosis prophase I, histone H3K79me is uniformly present, while when meiosis is perturbed or arrested, its level increases (Ontoso et al. 2014). H3K79me3 increased from pachynema onward (Ontoso et al. 2014). Therefore, different histone modification detected above was consistent with the characteristics of pachytene arrest in allotriploid fish. However, to understand how these post-modifications regulate different gene expression in

oocytes during developmental arrest in fish, more studies, such as chromatin immunoprecipitation (ChIP), are needed.

The previous study showed that a variety of reproduction-related genes (*Dmc1*, *Gnrh2*, *Gth*, *Gthr*, *vasa*, etc.) had abnormal expression in triploid Xiangyun crucian carp, Piwi and piRNA in triploid Xiangyun crucian carp were also abnormally expressed, and the histone modification state of ovary in triploid Xiangyun crucian carp changed. This was closely related to the sterile phenotype of triploid Xiangyun crucian carp. In terms of sterility, triploid Xiangyun crucian carp and other sterile triploid fishes were similar in organisms, cells, and tissues as well as molecular characteristics. As for individuals, the sterility of triploid Xiangyun crucian carp showed that it could not form and ovulate normal eggs and sperm resulting in no progenies. As for cell and tissue levels, the sterility of triploid Xiangyun crucian carp was represented as dysfunctional pairing of homologous chromosomes and could not form gametes with reproductive function. As for gonads, the ovary, testis, and fat-like gonad were all abortive. As for pituitary, during the breeding season, the sterility showed that hormonal granules in pituitary could not be released and represented as characteristics of endocrine disorders. As for the molecular level, several genes such as *Dmc*1, *Gnrh*2, *Gth*, *Gthr*, *vasa*, *Piwi*, etc., had abnormal expression in triploid Xiangyun crucian carp. As for the epigenetic state, some histone modifications, such as H3K4me3, H3K9me3, H3K79me, and H3K79me3, had changed in the ovary of triploid Xiangyun crucian carp. Therefore, the sterility mechanism of sterile triploid fish was associated with its abnormality in genetic (chromosome), epigenetic (histone modification), endocrine (HPG axis), and reproduction (gonadal development).

4.2.2 The Rapid Growth Mechanism of Triploid Xiangyun Crucian Carp and Triploid Xiangyun Carp

Since the dysfunction of the sterile gonad in triploid Xiangyun crucian carp, the energy used in gonadal development might be transferred to the energy for growth which led to fast growth. Several specificities of triploid Xiangyun crucian carp may relate to their sterility and fast growth.

Growth and development of fish were controlled by the growth hormone/insulin-like growth factor axis. Growth hormone (GH) secreted by pituitary cells was combined with tissue-specific growth hormone receptor (GHR) to activate a series of biological cascades which induced transcription of downstream target genes. Insulin-like growth factor 1 (IGF-1) in the liver could stimulate tissue growth, especially muscle.

GH promotes growth, gonadal development, energy metabolism, osmoregulation, social behavior, and immunity as well as other physiological activities that were mediated by IGF-1 in the liver. Through a comparative study of *Gh*, *Ghr*, and *Igf-1* expression, the endocrine mechanism of fast growth in triploid Xiangyun crucian carp was investigated.

The tissue expression of *Gh*, *Ghr*, and *Igf-1* in triploid Xiangyun crucian carp was analyzed by real-time PCR. *Gh* was only specifically expressed in the pituitary, not in other tissues. *Ghr* and *Igf-1* had the same expression patterns expressed in all tested tissues with the highest expression in the liver. The liver was the major secretion tissue for IGF-1. IGF-1 was produced by biological cascade from the combination of GH and GHR; thus, the GHR content in the liver was higher in the liver compared with other tissues. In other tissues, IGF-1 primarily acted as an autocrine and paracrine hormone (Zhong et al. 2012).

The results of real-time PCR revealed the different expression levels of *Gh* in the pituitary, *Ghr*, and *Igf-1* in the liver as well as *Igf-1* in muscle in different ploidy fishes. Before and after breeding season, the highest expression of *Gh* in the pituitary was found in triploid Xiangyun crucian carp, while the lowest expression was observed in allotetraploids of red crucian carp (♀) × common carp (♂). As for the *Ghr* and *Igf-1* in the liver, triploid Xiangyun crucian carp had the highest expression, following medium expression in diploid red crucian carp, and the lowest in allotetraploids of red crucian carp (♀) × common carp (♂). Similar results were found in *Igf-1* from muscle. The highest expression of *Igf-1* in muscle was observed in triploid Xiangyun crucian carp, while the lowest expression was shown in allotetraploids of red crucian carp (♀) × common carp (♂) (Zhong et al. 2012).

The result of in situ hybridization demonstrated that *Igf-1* was widely expressed in the liver; however, stronger signals were found in triploid fish compared to the other two fishes (diploids and allotetraploids) (Fig. 4.8a–f). The clues above proved that *Gh*, *Ghr*, and *Igf-1* had a higher expression in triploid Xiangyun crucian carp, which may be related to the pituitary structure differences of triploid Xiangyun crucian carp (Zhong et al. 2012).

The results of real-time PCR and tissue in situ hybridization showed that the expressions of *Gh* in pituitary, *Ghr* in liver, and *Igf-1* in the liver and muscle were the highest in triploid Xiangyun crucian carp, followed by diploid red crucian carp, and the lowest expressions of these genes were found in allotetraploids of red crucian carp (♀) × common carp (♂). Especially the expressions of *Gh* in the pituitary and *Igf-1* in the liver in triploid Xiangyun crucian carp were significantly higher than that in other two fishes (diploids and allotetraploids). The somatotrophs (STH) cells (STH cells are the key cells which secrete GH) had different proportion in the pituitary in experimental fish which were determined by analyses of pituitary structures in diploid red crucian carp, triploid Xiangyun crucian carp, and allotetraploids of red crucian carp (♀) × common carp (♂). During the breeding season, STH cells accounted for 25.0%, 38.0%, and 20.0% in diploids, triploids, and tetraploids, respectively. Compared with the diploid fish, the number of STH cells in triploid fish was 1.5 times that of diploid fish. Previous shreds of evidence showed that the growth rate of triploid Xiangyun crucian carp was 21.8% and 70.8% faster than that of Japanese white crucian carp and Pengze crucian carp, respectively. While allotetraploids of red crucian carp (♀) × common carp (♂) grew slower than diploid red crucian carp and triploid Xiangyun crucian carp which demonstrated that the proportion of STH cells in the pituitary may relate to the growth rate. The growth hormone was considered to be secreted by STH cells in the pituitary. In addition, the

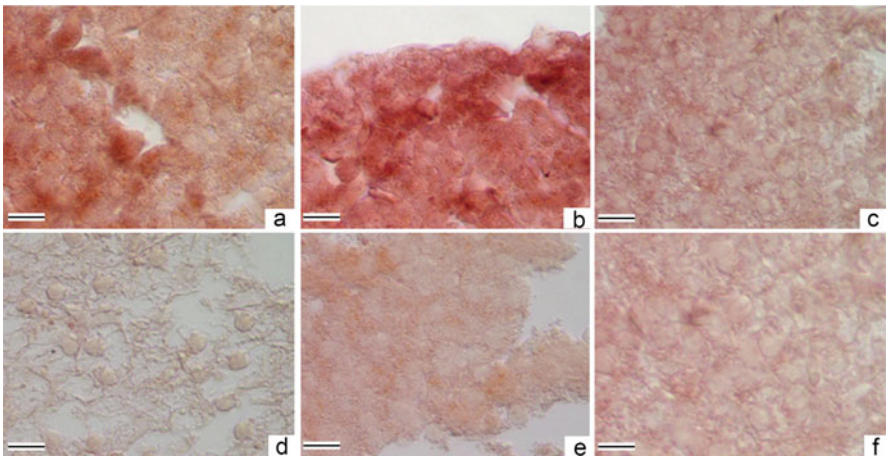

Fig. 4.8 The distribution of *Igf-1* expression in livers from different ploidy fishes. (**a**) Detection of *Igf-1* expression using probes of antisense RNA in the liver from diploid red crucian carp liver. (**b**) Detection of *Igf-1* expression using probes of antisense RNA in the liver from triploid Xiangyun crucian carp. (**c**) Detection of *Igf-1* expression using probes of antisense RNA in the liver from allotetraploids of red crucian carp (♀) × common carp (♂). (**d**) Control of *Igf-1* expression using probes of sense RNA in the liver from diploid red crucian carp liver. (**e**) Control of *Igf-1* expression using probes of sense RNA in the liver from triploid Xiangyun crucian carp. (**f**) Control of *Igf-1* expression using probes of sense RNA in the liver from allotetraploids of red crucian carp (♀) × common carp (♂). Bar = 20 μm

expression of GH in triploid Xiangyun crucian carp was much higher than that of diploid red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂), which may due to the different proportion of pituitary in different ploidy fishes. Presumably, more STH cells may secrete excessive GH, resulting in rise of GH concentration in the pituitary. To bind the excessive GH, the liver tissue secreted more GHR, thereby promoting excessive secretion of downstream factor IGF-1 in cascade reaction. With IGF-1 being released into the blood, the IGF-1 concentration was increased in muscle which promoted the growth of muscle and led to a faster growth rate of triploid Xiangyun crucian carp.

In addition, from the perspective of nutriology, the rapid growth characteristic of triploid Xiangyun crucian carp could also be explained. Glutamate dehydrogenase (GDH) was one of the important joint points connecting amino acid metabolism and glucose metabolism and played an important role in amino acid metabolism. The research showed that during breeding and nonbreeding seasons, *Gdh* gene mRNA expression in the liver of triploid Xiangyun crucian carp was higher than in red crucian carp, indicating triploid Xiangyun crucian carp had a higher metabolic efficiency for amino acids compared to red crucian carp with amino acid metabolism advantage (Liu et al. 2012; Liu 2012b).

4.3 The Application of Triploid Xiangyun Crucian Carp and Triploid Xiangyun Carp

Allotriploid hybrids not only have advantages including faster growth and stronger disease resistance but also exhibit sterility which is important for controlling the excessive multiplication of farmed fish and protecting natural germplasm resources. After more than 20 years of research and application, the allotriploid fish such as triploid Xiangyun crucian carp and triploid Xiangyun carp have advantages such as fast growth, good meat quality, high edible rate, strong disease resistance, and infertility. After popularization in China, the triploid Xiangyun crucian carp and triploid Xiangyun carp are popular among most farmers and consumers which produce significant economic and social benefits.

4.3.1 The Characteristics of Triploid Xiangyun Crucian Carp and Triploid Xiangyun Carp

4.3.1.1 Fast Growth Rate

Cultivation experiments showed that the growth rate of triploid Xiangyun crucian carp was 3–5 times faster than that of the local crucian carp. Compared to Japanese white crucian carp and Pengze crucian carp, the growth rate of triploid Xiangyun crucian carp was 21.8% and 70.8% faster than that of those, respectively. The farming experiment performed by Beijing Aquatic Product Technology Promotion Department showed that the growth rate of triploid Xiangyun crucian carp was 1.5 times faster than that of normal crucian carp. The cultivation test of Daxian County in Sichuan Province demonstrated that the growth rate of triploid Xiangyun crucian carp was 3 times faster than that of normal crucian carp. The triploid Xiangyun crucian carp fry (the fry had been cultivated in summer which was born in spring) grew to 300–400 g in regions south of the Yangtze River and 200–250 g in regions north of the Yangtze River (e.g., Beijing City and Liaoning Province) at the end of the same year, while juveniles (the fish had been cultivated in winter which was born in spring) attained 400–450 g when they become marketable fish. The maximum individual of triploid Xiangyun crucian carp was 2700 g. The polyculture generally yielded 150–200 kg per mu (666.7 m^2). If triploid Xiangyun crucian carp served as main farmed fish, the yield was 600–1200 kg per mu.

The growth rate of triploid Xiangyun carp was 30.0% to 40.0% faster than that of common carp. Comparative cultivation in the Qionghu Lake Fishery of Yuanjiang City in Hunan Province showed that the growth rate of triploid Xiangyun carp cultured in the cage was 45.8% faster than that of common carp. Cultivation in different ponds showed that the growth rate of triploid Xiangyun carp was 29.4% faster than that of common carp. The cultivation test of Daxian County in Sichuan Province showed that the growth rate of triploid Xiangyun carp was 40.0% faster than that of common carp. The triploid Xiangyun carp fry (the fry had been cultivated in summer which was born in spring) generally grew to 500–600 g at the end of the same year, while juveniles (the fish had been cultivated in winter

which was born in spring) attained 600–750 g when they became marketable fish. The polyculture yielded 150–350 kg per mu. According to market requirements in Hunan Province, farmers would control the size of triploid Xiangyun carp within 600–700 g to improve yield and benefits by increasing breeding density.

4.3.1.2 Sterility

Triploid Xiangyun crucian carp and triploid Xiangyun carp are allotriploid fish with chromosome sets as $3n = 150$. Their gonads are sterile, which could not produce mature sperm and eggs and also could not reproduce itself. This characteristic makes triploid Xiangyun crucian carp and triploid Xiangyun carp unable to mate with other common carp or crucian carp. Therefore, they would not cause differentiation and degeneration of quality. Meanwhile, they could not interfere with the germplasm resources of common carp, crucian carp, and other fish in natural waters.

4.3.1.3 Strong Feeding Ability

The triploid Xiangyun crucian carp has an omnivorous and plankton feeding habit. The feed coefficient is 1.3–1.4 when fed with pellet feed containing 30.0% to 37.0% proteins which contributes to the low farming cost. This is associated with the sterility of triploid Xiangyun crucian carp. Due to its sterility, the nutrition intake transfers to the energy for growth in maximum and decreases the energy for gonadal development. Although triploid Xiangyun carp is omnivorous, the ability to filter plankton is not obvious.

4.3.1.4 Strong Disease Resistance and High Survival Rate

The survival rates of triploid Xiangyun crucian carp and triploid Xiangyun carp fry (the fry had been cultivated in summer which was born in spring) are over 90.0%. The survival rates of juveniles (the fish had been cultivated in winter which was born in spring) are over 95.0%. When silver carp, bighead carp, grass carp, and crucian carp cultured in the same pond died due to hypoxia, few triploid Xiangyun crucian carp would die simultaneously.

4.3.1.5 The Beautiful Shape and High-Quality Meat

The edible parts of triploid Xiangyun crucian carp and triploid Xiangyun carp are 10.0% to 15.0% more than that of the general crucian carp and common carp and have few intercostal spinules. The nutritive values of fish mainly depend on protein and fat contents in muscle. In addition, the evaluation of protein quality depends on the amino acid composition and essential amino acid (EAA) content for humans. By comparison with other fish, triploid Xiangyun crucian carp and triploid Xiangyun carp are newly economical fish which contain low water content, low ash content and high nutritional value.

4.3.2 The Promotion and Application of Triploid Xiangyun Crucian Carp and Triploid Xiangyun Carp

Years of aquaculture prove that sterile triploid Xiangyun crucian carp and triploid Xiangyun carp have advantages such as fast growth, nice meat, high edible rate, strong disease resistance, low temperature resistance, and hypoxia resistance. They are also easy to catch and fish. Therefore, they can be cultivated in ponds, lakes, reservoirs, rice fields, net cages, and intensive and industrial freshwaters. They have been promoted nationwide and produced significant social and economic benefits.

4.4 The Triploid Fish Derived from Hybridization of Allotetraploid Fish [Red Crucian Carp (♀) × Common Carp (♂)] with Other Diploid Fish

Goldfish (*C. auratus*), red crucian carp, and Japanese white crucian carp all belong to the genus *Carassius*, Cyprinidae family, with 100 somatic chromosomes. Their biological characteristics have similarities as well as differences. For example, they are similar in genetic and physiological characteristics, but their appearances are significantly different. The red goldfish and red crucian carp are all in red, but red goldfish have double bifurcated tails and higher dorsal. The body colour of Japanese white crucian carp is silver. The paternal parent allotetraploids of red crucian carp (♀) × common carp (♂) respectively match the three maternal parents, and then we obtain three kinds of triploid crucian carps which are similar in genetic and physiological characteristics. For example, they are all sterile allotriploid fish, but different in respect to appearance.

Two kinds of triploid crucian carps from the crossing of male allotetraploids of red crucian carp (♀) × common carp (♂) with female diploid goldfish and red crucian carp, respectively, will be introduced later. The authors make a systematical research on the appearance, growth rate, chromosome number, karyotype, gonadosomatic index (GSI), and gonadal structure of these two triploid crucian carps which further enrich the research about polyploidy fishes.

4.4.1 The Allotetraploid Fish [Red Crucian Carp (♀) × Common Carp (♂)](♂) × Goldfish (♀)

4.4.1.1 The Early Embryonic Development of Allotetraploid Fish [Red Crucian Carp (♀) × Common Carp (♂)](♂) × Goldfish (♀)

Triploid crucian carp was obtained after crossing of allotetraploid fish [red crucian carp (♀) × common carp (♂)](♂) with goldfish (♀). The early embryonic development of triploid crucian carp was observed. The mature eggs of goldfish were yellow, round, and sticky with egg diameters ranging from 1.3 to 1.4 mm. The cleavage began 60 min after fertilization, in which the water temperature was (20 ± 1)°C. And the cleavage time lasted 60–180 min. It gradually entered into

the early, middle, and late blastula period after 3 h 30 min, 10 h later into the gastrula period, and 15 h 30 min later into the neurula period. The organs began to form after 19 h 30 min and 63–75 h after fertilization the fry hatched out. In addition, the section observation of embryos of triploid crucian carp showed that cell division in blastula period was vigorous, and cells in the gastrula period exhibited normal division, cytomorphosis, and migration. Combined with observation of the appearance development of triploid crucian carp embryos, it indicated that the triploid fish had a normal early embryonic development which provided an important biological basis for studying developmental biology of triploid fish and their application in production (Yi et al. 2006).

Zygote period: Mature eggs of goldfish were yellow, round, and sticky with egg diameters in 1.3–1.4 mm. They were smaller than the allotetraploid eggs [allotetraploid egg diameter (1.7 ± 0.1) mm]. The ooplasm was distributed evenly. Fertilized eggs expanded after absorbing water and became shining bright. The perivitelline space was amplified. The egg cytoplasm began to focus on the animal pole and gradually uplifted to form a blastoderm, 40 min after fertilization.

Cleavage period: The animal pole of cells began to crack and the blastoderm split into two similar blastomere lengthways and then it entered into the two-cell period approximately 60 min after fertilization. From 70 to 85 min, the blastoderm continued its second longitudinal crack along the vertical direction of the first division and this was the four-cell period. The blastoderm continued twice longitudinal cracks and entered into the eight-cell period in which eight blastomeres were arranged neatly into two rows after 110 min. The embryos were in the 16-cell period when blastomeres were arranged in a square shape in blastoderm about 110–144 min later. Embryos in multicellular period were found, and most of them were in the 64-cell period after 180 min later. The embryos entered into the multicellular period, and few were in the early blastula period after 3 h 45 min.

Blastula period: Cells continued to split with an increasing number, 4 h 30 min after fertilization. The size of cells also became correspondingly smaller. The embryos gradually entered into the morula period. When it came to 6 h, a small number of embryos died. In the 7 h 30 min, more than half of the embryos reached the late blastula period.

Gastrula period: Germ layer began to conduct epiboly approximately 10 h after fertilization. The embryos slowly entered into the gastrula period and the germ ring occurred. In the mid-gastrula period, embryonic shield continued to extend after 12 h 30 min.

Neurula period: Embryos entered into the early neurula period when yolk plug was not fully coated, 15 h 30 min after fertilization. In the 17 h 20 min, the yolk was completely coated, and yolk plug disappeared.

Organogenetic period: When it came to 19 h 30 min, embryos entered into the organogenetic period. And 20 h later, optic vesicle appeared. Sarcomere appeared after 21 h 30 min later. The brain was already differentiated into forebrain, midbrain, and afterbrain and the tail bud appeared after 22 h 30 min. In the 28 h, blastokinesis appeared and it moved into the muscle effector period. In the beginning the blastokinesis was with low frequency, but gradually it began to accelerate. Embryos

curled and extended with the reduction of yolk 31 h later. After 34 h, the heart began to beat with an average frequency of 39 beats/min and it gradually increased to 70 beats/min later. Blood started to flow after 47 h 30 min. And 49 h 30 min later, all embryos began their blood circulation.

Hatching period: The embryo tail was so considerably twisted out of the egg membrane during 63–75 h after fertilization. However, the newly hatched juvenile fish had a lot of yolk which could only swim for a short time in water. Finally, 1–2 days after fertilization, juveniles developed into free-swimming larvae.

Histological observation of early embryonic development: Section results showed that 6 h after fertilization, the cells in blastula period underwent vigorous mitosis and cell proliferation was normal. Cells deformed and readied to migrate under the section observation in the early gastrula period about 11 h after fertilization. After 15 h later, some cells in the late gastrula period of embryos were still proliferated under the section observation and some cells already deformed and migrated. And the migration direction was very clear (Yi et al. 2006).

4.4.1.2 The Appearance and Ploidy of Allotetraploid Fish [Red Crucian Carp (♀) × Common Carp (♂)](♂) × Goldfish (♀)

In appearance, the triploid crucian carp was steel gray with a single tail. Liu et al. studied the morphological traits of this triploid crucian carp, allotetraploids of red crucian carp (♀) × common carp (♂), goldfish, diploid Japanese white crucian carp, and triploid Xiangyun crucian carp (Liu et al. 2004b). From the ratio of body depth and body length, the length and number of barbels, and the number of dorsal fins and lateral lineage scales, the appearance of triploid crucian carp was just between its parent allotetraploids of red crucian carp (♀) × common carp (♂) and goldfish which showed the characteristics of hybridization. And the ratio of body depth and body length, barbel length and number of triploid Xiangyun crucian carp also presented the same trait, while the ratio of body depth and body length of new triploid crucian carp was significantly higher than that of the triploid Xiangyun crucian carp, reflecting the body of triploid crucian carp was wider than the triploid Xiangyun crucian carp, and it enriched the triploid fish types. After detecting renal cell chromosomes, the authors found the new triploid crucian carp had 150 chromosomes with karyotype 33 m + 51sm + 33st + 33 t (Liu et al. 2004b).

4.4.1.3 The Growth and Fertility of Allotetraploid Fish [Red Crucian Carp (♀) × Common Carp (♂)] (♂) × Goldfish (♀)

The average quality of allotetraploid fish [red crucian carp (♀) × common carp (♂)] (♂) × goldfish (♀) at 1 year of age was 350 g, and the largest one reached up to 550 g. From December of that year to the second year in July, all triploid crucian carps would be checked monthly, but no sperm or eggs could be squeezed out. The sex ratio of male and female triploid crucian carp was 1:1 which was detected in breeding season. The average quality of females was about 387.3 g, while the average quality of males was 328.6 g. This showed that females grew faster than males at 1 year old.

The mean value of triploid crucian carp ovary index was 4.1, and the mean value of testis index was 3.5. It could be viewed with eyes about the surface of triploid crucian carp where only a few egg granules could be observed. But mature egg granules were spread on the surface of red crucian carp ovary. Triploid crucian carp testis was white. No semen flew out when the testis was cut with scissors. Histological section showed that most triploid crucian carp ovaries were occupied by small volume of underdeveloped oogonia which usually grouped together to form cystic structures. Among these small cells, there were some primary oocytes in which no mature eggs were observed. The triploid crucian carp testis was mainly made up of many small fine tubes, some of which contained no spermatids, but spermatids can be seen in some other tubes in irregular shapes. They were not clear and began to degrade and disintegrate. Mature sperm could not be observed.

The ovary, testis, and fat gonad index of triploid crucian carp were lower than the ovary and testis gonad index and the two mean values of Japanese white crucian carp, respectively, which indicated that this triploid crucian carp was similar to triploid Xiangyun crucian carp in the gonadal development and they were both suppressed (Liu et al. 2004b).

4.4.1.4 The Meat Quality of Allotetraploid Fish [Red Crucian Carp (♀) × Common Carp (♂)] (♂) × Goldfish (♀)

The analysis of nutrient and amino acids in the new triploid crucian carp muscle showed that the content of amino acids of dry sample was 102.4 mg/g and the content of essential amino acids (*Thr*, *Val*, *Met*, *Ile*, *Leu*, *Phe*, *Lys*, *Trp*) for humans was 45.2 mg/g, accounting for 44.2% of the total amino acid content. The content of two kinds of semi-essential amino acids (*His*, *Arg*) was 11.68 mg/g, accounting for 11.4% of the total amount. And the content of four kinds of flavor amino acids (*Asp*, *Glu*, *Gly*, *Ala*) was 31.6 mg/g, accounting for 30.8% of the total amount. By comparing the nutrient content in the muscle of new triploid crucian carp and its paternal parent allotetraploids of red crucian carp (♀) × common carp (♂), maternal parent goldfish, and other types of fish, the content of four kinds of flavor amino acids was found to be much higher than those in its parents. The content of essential amino acids for humans was also much higher than in triploid Xiangyun crucian carp (triploid Xiangyun carp) and other similar fish (Xiang et al. 2006).

4.4.2 The Allotetraploid Fish [Red Crucian Carp (♀) × Common Carp (♂)](♂) × Red Crucian Carp (♀)

A new triploid crucian carp was made by crossing between male allotetraploids of red crucian carp (♀) × common carp (♂) and female diploid red crucian carp. This triploid crucian carp was flat in its body side in the shape of fusiform. Its body color was steel gray with no barbels. Most of their measurable and accountable traits were between their parents and some of the measurable traits were beyond its parents which reflected the hybridization characteristics.

The analysis of karyotype showed the new triploid crucian carp chromosome number was 150 which was consistent with the chromosome number of triploid Xiangyun crucian carp.

The gonadal development of the new triploid crucian carp lagged behind common diploid fish and their germ cells showed degradation. Two types could be observed from the new triploid crucian carp gonads: (1) The gonad in the belt was located on both sides of the swim bladder. It was pale yellow, and petal-shaped leaflet occurred after fixation and it took on ovarian features. But most of the germ cells in the ovary were in the oogonia period, and in some cells there were vesicular structures and they began to degenerate. (2) Only two adipose tissues were in the gonad. Neither testis nor ovary was found (Shen et al. 2006). In this new triploid fish, no testis gonad was found which might result from the randomness of sampling.

Regarding genetic composition, this triploid crucian carp was obtained from fertilization of diploid sperm of allotetraploid fish [red crucian carp (♀) × common carp (♂)] and haploid eggs of red crucian carp. So they both had genomes from common carp and red crucian carp and red crucian carp genomes might account for the majority. Genetic heterozygosity led to some distinct hybridization characteristics in their appearances. For example, the ratios of body depth/body length, caudal peduncle depth/caudal peduncle length, and head height/body depth were between their parents. Because genetic genes of red crucian carp accounted for the most, many appearance features were close to the maternal parent, such as measurable traits like ratios of body length/overall length, head length/body length, and head height/head length. Therefore, their body size was closer to that of red crucian carp. In the new triploid crucian carp, no individuals with barbels were detected, which was a noteworthy and interesting phenomenon. The crossing of red crucian carp and allotetraploids of red crucian carp (♀) × common carp (♂) was similar to the process of backcross. The progenies only had genetic genes from common carp and red crucian carp and the red crucian carp took the advantages. Therefore, it had a large possibility to own the red crucian carp characteristics (without barbels). It could be concluded that species with a closer genetic relationship with red crucian carp had a large possibility to have no barbels. Compared with several hybrids, we could find that hybrids had no barbels when its cytoplasm and nucleus both contained red crucian carp genetic materials. The formation of barbels whether they had a relationship with the genetic materials in red crucian carp or not required further research. The formation of the triploid fish provided good material for studying the barbel development (Shen et al. 2006).

References

Burgess DJ (2013) Defining piRNA expression. Nat Rev Genet 14(5):301

Gui J, Liang S, Sun J, Huang W, Jiang Y (1990) Studies on genome manipulation in fish, I. induction of triploids transparent colored crucian carp (*Carassius auratus transparent* colored variety) by hydrostatic pressure. Acta Hydrobiol Sinica 14:336–344

He X (1999) Researches on tetraploid gene bank and chromosomes of triploid Xiangyunnesis crucian carp and common carp. Hunan Normal University, Changsha

Houwing S, Kamminga L, Berezikov E, Cronembold D, Girard A, Elst H, Filippov D, Blaser H, Raz E, Moens C (2007) A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. Cell 129(1):69–82

Linder P, Lasko PF, Ashburner M, Leroy P, Nielsen PJ, Nishi K, Schnier J, Slonimski PP (1989) Birth of the D-E-A-D box. Nature 337(6203):121–122

Liu Y (1993) Propagation physiology of main cultivated fish in China. Agricultural Publishing House, Beijing, pp 22–23

Liu G (2012a) *Vasa* gene expression analysis in different polyploidy hybrids progeny of red crucian carp (♀) × common carp (♂). Hunan Normal University, Changsha

Liu Q, Wang Y, Liu S, Guo X, Luo K, Zhang C, Liu Y (2004a) A comparative study on blood and hemocyte of different polyploid crucian carp (♀) × common carp (♂). Prog Nat Sci 14(10): 1111–1117

Liu S, Cao Y, He X, Li J, Liu Y (2001) The formation of tetraplpoid hybrids of common carp with red crucian carp and the evolutionary significance of teraploidzation in vertebrate. Eng Sci 3:33–41

Liu S, Feng H, Liu Y, Zhou G, Zhang X (1999) The measurement of DNA content of the tetraploids F_3 - F_4 hybrids of red crucian carp (♀) × common carp (♂) and their triploid offspring and other related diploid fish. J Nat Sci Hunan Norm Univ 22(4):61–68

Liu S, Hu F, Zhou G, Zhang X, He X, Feng H, Liu Y (2000) Gonadal structure of triploid crucian carp produced by crossing allotetraploid hybrids of *Carassium auratus* red var.(♀) × *Cyprinus carpio* (♂) with Japanese crucian carp (*Carassius auratus cavieri*). Acta Hydrobiol Sinica 24: 301–306

Liu S, Sun Y, Li S, Feng H, Li J, Zhou G, Zhang X, Liu Y (2002) Analysis of gonadosomatic indexes of the triploid crucian carp. J Fish China 2:111–114

Liu S, Sun Y, Zhang C, Luo K, Liu Y (2004b) Triploid crucain carp-allotetraploid hybrids (♂) × goldfish (♀). Acta Genet Sin 31(1):31–38

Liu Z (2012b) Studies on cloning and expression of genes related to protein metabolism of different ploidy hybrids derived from red crucian carp (♀)× common carp (♂). Hunan Normal University, Changsha

Liu Z, Zhou Y, Liu S, Zhong H, Zhang C, Kang X, Liu Y (2012) Characterization and dietary regulation of glutamate dehydrogenase in different ploidy fishes. Amino Acids 43(6):2339–2348

Long Y, Liu S, Huang W, Zhang J, Sun Y, Zhang C, Song C, Liu J, Liu Y (2006) Comparative studies on histological and ultra-structure of the pituitary of different ploidy level fishes. Sci China Ser C Life Sci 49(5):446–453

Long Y, Tao M, Liu S, Zhong H, Chen L, Tao S, Liu Y (2009a) Differential expression of *Gnrh2*, *Gthβ*, and *Gthr* genes in sterile triploids and fertile tetraploids. Cell Tissue Res 338(1):151–159

Long Y, Zhong H, Liu S, Min T, Chen L, Xiao J, Liu Y (2009b) Molecular characterization and genetic analysis of *Gnrh2* and *Gthβ* in different ploidy level fishes. Prog Nat Sci: Mater Int 11: 119–129

Naumova A, Fayer S, Leung J, Boateng K, Camerini-Otero R, Taketo T (2013) Dynamics of response to asynapsis and meiotic silencing in spermatocytes from robertsonian translocation carriers. PLoS One 8:e75970

Ontoso D, Kauppi L, Keeney S, San-Segundo P (2014) Dynamics of DOT1L localization and H3K79 methylation during meiotic prophase I in mouse spermatocytes. Chromosoma 123(1–2):147–164

Raghuveer K, Senthilkumaran B (2010) Cloning and differential expression pattern of vasa in the developing and recrudescing gonads of catfish, *Clarias gariepinus*. Comp Biochem Physiol A Mol Integr Physiol 157(1):79–85

Shen J, Liu S, Sun Y, Zhang C, Luo K, Tao M, Zeng C, Liu Y (2006) A new type of triploid crucian crap - red crucian carp (♀) × allotetraploid fish (♂). Prog Nat Sci 16(12):1348–1352

Strahl B, David A (2000) The language of covalent histone modifications. Nature 6765(403):41–45

Tan M, Luo H, Sangkyu L, Fulai J, Yang J, Emilie M, Thierry B, Cheng Z, Rousseaux S, Rajagopal N, Lu Z, Ye Z, Zhu Q, Wysocka J, Ye Y, Khochbin S, Ren B, Zhao Y (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 6(146):1016–1028

Tao M, Liu S, Long Y, Zeng C, Liu J, Liu L, Zhang C, Duan W, Liu Y (2008) The cloning of *Dmc1* cDNAs and a comparative study of its expression in different ploidy cyprinid fishes. Sci China Ser C Life Sci 51(1):38–46

Valérie B, Nicolas R, Waka L, Sandrine B, Vincent G, Alain N (2009) Histone H3 lysine 4 trimethylation marks meiotic recombination initiation sites. EMBO J 28(2):99–111

Wang D (2013) Expression and sex determination mechanism of reproductive genes of HPG Axis in different ploidy fish. Hunan Normal University, Changsha

Xiang B, Liu S, Zhang C, Sun Y, Duan W, Shen J, Luo K, Tao M, Zeng C, Liu Y (2006) The analysis of nutritional component and amino acid composition of muscle in a new type of triploid crucian carp (*carassius auratus*). J Nat Sci Hunan Norm Univ 29:85–88

Xu D, Bai J, Duan Q, Costa M, Dai W (2009) Covalent modifications of histones during mitosis and meiosis. Cell Cycle 8(22):3688–3694

Xu H, Gui J, Hong Y (2005) Differential expression of *vasa* RNA and protein during spermatogenesis and oogenesis in the gibel carp (*Carassius auratus gibelio*), a bisexually and gynogenetically reproducing vertebrate. Dev Dyn 233(3):872–882

Yi N, Zhang C, Wang Y, Liu S, Liu Y (2006) The early embryonic development of triploid crucain carp coming from allotetraploid hybrids (♂) × goldfish(♀). J Nat Sci Hunan Norm Univ 29:87–91

Yu F, Zhong H, Gang L, Liu S, Zhang Z, Zhou Y, Tao M, Liu Y (2015) Characterization of vasa in the gonads of different ploidy fish. Gene 574:337–344

Zhang C, Liu S, Sun Y, Xiao J, Liu Y (2008) Chromosomal studies of germ cells in diploid and polyploid fish produced by distant crossing. J Mol Cell Biol 41(1):53–60

Zhang C, He X, Liu S, Sun Y (2005) Chromosome pairing in meiosis I in allotetraploid hybrids and allotriploid crucian carp. Acta Zool Sin 51(1):89–94

Zhong H, Zhou Y, Liu S, Tao M, Long Y, Liu Z, Zhang C, Duan W, Hu J, Song C (2012) Elevated expressions of GH/IGF axis genes in triploid crucian carp. Gen Comp Endocrinol 178(2):291–300

Zhou R, Shang R, Gong D, Xu X, Liu S (2019) Characterization of H3 methylation in regulating oocyte development in cyprinid fish. Sci China Life Sci 62(6):829–837

Zhou Y, Wang F, Liu S, Zhong H, Liu Z, Tao M, Zhang C, Liu Y (2012) Human chorionic gonadotropin suppresses expression of Piwis in common carp (*Cyprinus carpio*) ovaries. Gen Comp Endocrinol 176(2):126–131

Zhou Y, Zhong H, Liu S, Yu F, Hu J, Zhang C, Tao M, Liu Y (2014) Elevated expression of Piwi and piRNAs in ovaries of triploid crucian carp. Mol Cell Endocrinol 383(1–2):1–9

5 The Gynogenesis and Androgenesis of the Diploid Gametes Derived from the Allotetraploid Fish

Shaojun Liu, Chun Zhang, Yi Zhou, Shi Wang, Zhuangwen Mao, Dingbin Gong, Linmei Han, and Xin Gao

Abstract

Artificial gynogenesis is a good approach to improve the fish germplasm. Generally, in this approach, eggs are stimulated by genetically inactivated sperm (generally, sperm from different species are used), and the chromosomes in eggs are doubled which results in offspring containing maternal genetic material. In this process, the haploid eggs undergo a treatment for doubling the chromosomes, which possibly damage the "fertilized" eggs and will reduce the survival rate of the gynogenetic progeny. By using diploid eggs from tetraploid fish, the doubling chromosome procedure is unnecessary which simplifies the operation process and improves the survival rate of gynogenetic progeny. Besides gynogenesis, diploid gametes could be used to improve androgenesis approach. Artificial androgenesis is the fertilization of genetically inactivated eggs with normal sperm. During common induced process of androgenesis, the chromosomes from sperm nuclei need to be doubled. On the contrary, using diploid sperm from tetraploid fish, the chromosome doubled process is unnecessary to obtain diploid androgenetic progeny. In our previous studies, the allotetraploid lineage of red crucian carp (*Carassius auratus* red var., RCC, ♀) × common carp (*Cyprinus carpio* L., CC, ♂) has been established and lasted for 27 generations (F_3–F_{29}). Both the female and male individuals produced stable diploid eggs and sperm. This chapter introduces gynogenesis and androgenesis using diploid gametes derived from allotetraploids of red crucian carp × common carp (4*n*AT).

S. Liu (✉) · C. Zhang · Y. Zhou · S. Wang · Z. Mao · D. Gong · L. Han · X. Gao
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_5

Keywords

Gynogenesis · Androgenesis · Diploid eggs · Diploid sperm · Improved allotetraploid fish · Improved triploid fish

5.1 The Gynogenesis of the Diploid Eggs from 4*n*AT

5.1.1 The Establishment of the Diploid Gynogenetic Hybrid Lineage

The ultraviolet-irradiated sperm were used to activate the diploid eggs (derived from 4*n*AT) to produce diploid gynogenetic progeny without the treatment for doubling the chromosomes of eggs. Interestingly, diploid gynogenetic progeny could generate unreduced diploid eggs steadily which had been used to produce the gynogenetic lineage.

5.1.1.1 The Formation of the Gynogenetic Lineage

The sperm of male scattered scale carp (*Cyprinus carpio specularis*, SSC) and blunt snout bream (*Megalobrama amblycephala,* BSB) were respectively diluted with Hank's solution (1:4) and then poured into pre-cooling culture dishes. Next, the culture dishes were placed under two ultraviolet lamps (15 W), in a rocking machine (30–40 r/min) with the distance between two points being 10–12 cm. Diluted sperm were exposed to the ultraviolet light for 20–50 min to destroy the genetic material of sperm and the suspension was stirred by a rocking machine. The ultraviolet-irradiated sperm were stored in a tube and all processes were conducted in the dark at 4 °C. Afterward, ultraviolet-irradiated sperm were mixed with the diploid eggs of 4*n*AT in a dry culture dish and then water was added for further development of eggs. Finally, the diploid eggs were incubated in still water at 20–21 °C.

In this way, a large number of the first-generation all-female diploid gynogenetic hybrids of RCC × CC ($2n = 100$, G_1) had been produced (Liu et al. 2004). Most of the eggs (93.3%) produced by G_1 were diploid eggs. These eggs were used to produce the second-generation all-female diploid gynogenetic hybrids of RCC × CC ($2n = 100$, G_2) activated by genetically inactivated sperm of SSC or BSB (Zhang et al. 2005), in which most of the eggs (95.6%) produced by G_2 were also diploid eggs. Following the method above, the gynogenetic lineage has been established (Fig. 5.1) (Liu et al. 2007). The appearance and chromosome spread of G_1 were shown in Fig. 5.2a, b.

5.1.1.2 The Development of the Embryo in the Third-Generation Diploid Gynogenetic Hybrids of RCC × CC ($2n = 100$, G_3)

The embryo development of G_3 was observed. The matured eggs squeezed from G_2 were round and sticky and the fertilized eggs were in deep yellow with low transparency. During 15 minutes after fertilization, the fertilized eggs absorbed water and swelled, and the perivitelline space increased obviously. Multicellular

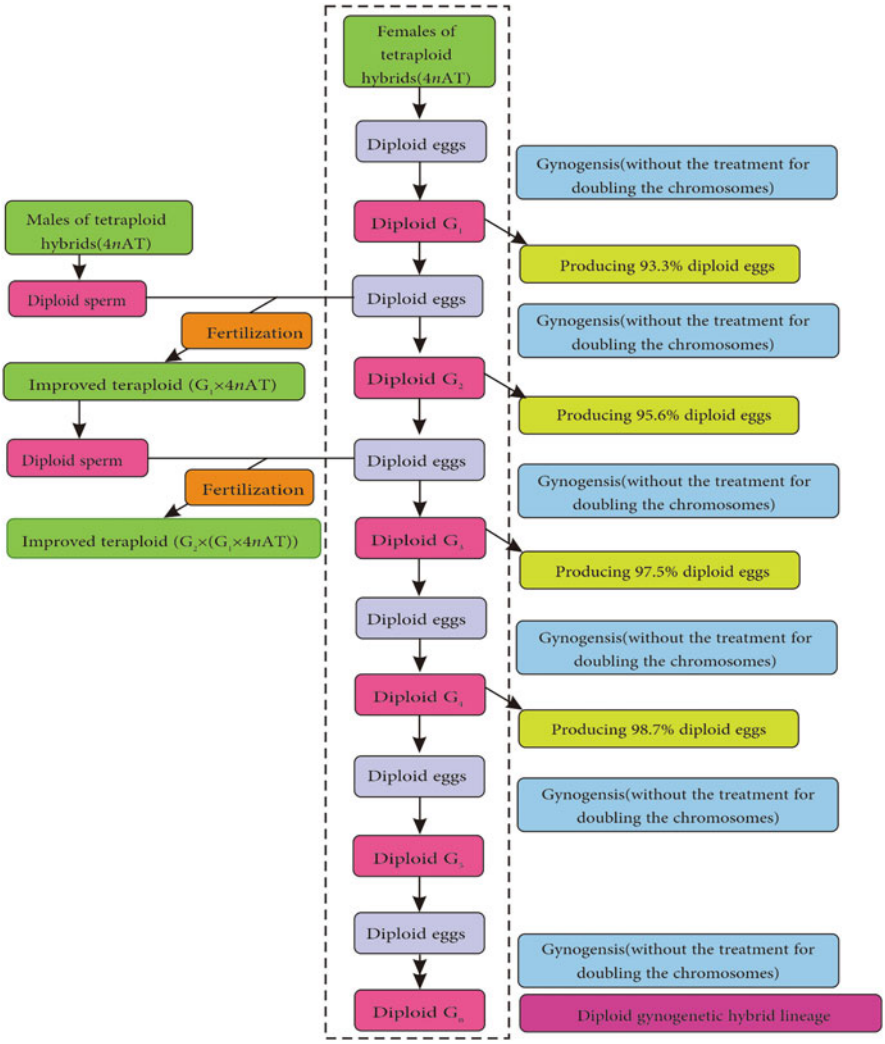

Fig. 5.1 The establishment of the gynogenetic lineage (Liu et al. 2007)

proembryo (morula) were formed in 6.3 hours after fertilization. Approximately 12.7 hours after fertilization, blastodermal cells were laid on the yolk and began to encircle downward to yolk. At this time, placenta was arcuate and yolk syncytial layer became round, which meant that it reached the later phase of blastocyst (low blastocyst) and began to gastrulation. Approximately 29 hours after fertilization, blastoderm cells continued to encircle downward and rolled inward, and the embryo of neural plate occurred with its front depressed to form neural groove. Germ layer encircled downward totally leaving tiny yolk plug, and blastopore had not occluded yet when the germ ring shank obviously and the neurula was formed. Approximately

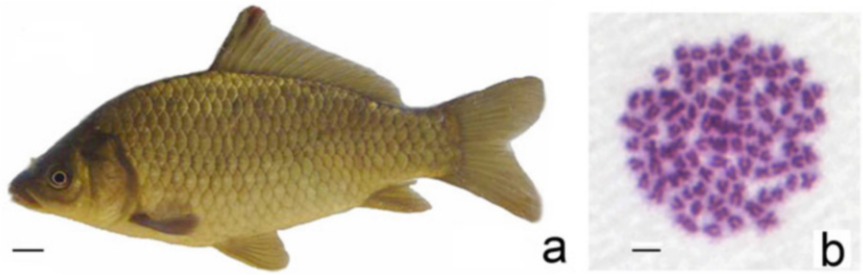

Fig. 5.2 The appearance and chromosome spread in G_1 (Liu et al. 2007). (**a**) The appearance of G_1. Bar = 3 cm. (**b**) The metaphase chromosome spread of mitosis cells in G_1 ($2n = 100$). Bar = 3 μm

32 hours after fertilization, the front of thick neural plate swelled and brain vesicle formed, while the tail was narrow and thin. At this time the head and tail were distinguished, indicated the start of organogenesis. Approximately 79–86 hours after fertilization, the activity of the embryo was obviously decreased. Later, the tail began to swing heavily. Approximately 89 hours after fertilization, the embryo broke through egg membrane. Approximately 12–16 hours later, the embryo hatched out (the hatching rate was 26.1%). The average length of newly hatched fry was 5.50 mm with an elliptical yolk sac in length of 1.50 mm below the abdomen. Approximately 1–2 days later, they could swim freely. These results proved that diploid eggs generated by G_2 had a normal embryonic development without the treatment of chromosome duplication and could be used to produce a large amount of healthy gynogenetic progeny. The G_3 had a higher fertilization rate and hatching rate, which indicated that the survival rate of gynogenetic progeny was improved by using diploid eggs without the treatment for doubling the chromosomes (Wang et al. 2005).

5.1.1.3 The Growth Characteristics of the Diploid Gynogenetic Hybrids of RCC × CC

In appearance, the gynogenetic lineage was similar to the second generation of hybrids in RCC × CC. It was gray with a single tail and had two pairs of inconspicuous barbells on the head, with good body shapes. The average weight of 8-month-old G_2 was 275.0 g, which was 20.6% faster than that of 4*n*AT in weight gain under similar raising conditions (Zhang et al. 2005). Besides, stress resistance and anti-disease ability of the gynogenetic lineage were stronger than those of 4*n*AT. The mortality rate of the gynogenetic lineage was lower than that of 4*n*AT. Especially in breeding season, a number of 10.0–15.0% individuals of 4*n*AT would die after spawning, while the mortality rate of the gynogenetic lineage was no more than 5.0%.

5.1.1.4 The Fertility of the Diploid Gynogenetic Hybrids of RCC × CC

During the breeding season, 1-year-old individuals of the gynogenetic lineage were all female but no egg could be squeezed out, in which a few primary oocytes and many oogonia-like cells are arranged in nests; 2-year-old individuals of the gynogenetic lineage possessed normal mature ovaries, which contained many mature eggs full of yolk. There was a micropyle on the mature ovum which indicated that the gynogenetic lineages were fertile and reached their sexual maturity in 2 years. The gonad-somatic index (ovary weight/body weight) of fertile gynogenetic lineage was 6.22 ± 0.51, which was higher than that of 4*n*AT (5.70 ± 1.03) (Liu et al. 2004). Generally speaking, 4*n*AT reached sexual maturity in 1 year and their matured eggs (or sperm) could be squeezed out in next breeding season. However, 1-year-old gynogenetic lineage could not produce mature eggs no matter the ovary developed normal or not. In 2-year-old gynogenetic lineage, 30.0% of individuals could produce eggs which could further develop into viable individuals after fertilization. In 3-year-old gynogenetic lineage, the fertile individuals were accounting for over 80.0%. The results indicated that the fertility of gynogenetic lineage developed with age. In addition, the gynogenetic lineage could produce mature gametes from March to June, while 4*n*AT could produce mature gametes mainly in April and early May.

5.1.1.5 The Other Biological Researches on the Diploid Gynogenetic Hybrids of RCC × CC

The Random Amplified Polymorphic DNA (RAPD) Analysis

Genomic DNA polymorphism was determined by the method of RAPD. A total of 34 random primers were used for DNA amplification in 4 samples of G_1, the tenth generation of 4*n*AT and SSC, respectively. About 3–10 distinct and bright bands, ranging from 0.5 to 2.1 kb, were amplified by random primers in three types of fish. A total of 1054, 1044, and 964 bands were amplified in G_1, the tenth generation of 4*n*AT and SSC, respectively. Each primer amplified 7.87, 7.68, and 7.20 bands in G_1 on average, the tenth generation of 4*n*AT and SSC, respectively. The average genetic similarity coefficients of G_1 to the tenth generation of 4*n*AT, G_1 to SSC, and the tenth generation of 4*n*AT to SSC were 0.97, 0.60 and 0.59, respectively. It indicated that the genetic material of G_1 mainly inherited from maternal parent (the tenth generation of 4*n*AT). However, two specific DNA bands were amplified in G_1, which were the same to paternal parent (SSC) but different to maternal parent – the tenth generation of 4*n*AT, by two pairs of primers (S28 and S128). These results indicated that G_1 had inherited the genetic material from heterologous sperm (Yan et al. 2005).

The Mechanism of the Unreduced Gametes Generated by Diploid Gynogenetic Hybrids of RCC × CC

Normally, the gametes of bisexual diploid fish had a half number of chromosomes of somatic cells. After the combination of male and female gametes, the chromosome number of oosperm was equal to that of parents to maintain the genetic stability between parents and their progeny. The production of unreduced gametes had also

been reported in other animals, for example, the *Oryzias latipes* (Sakaizumi et al. 1993), hybrids of common carp and crucian carp (Cherfas et al. 1994), hybrids of Atlantic salmon × brown trout (Galbreath and Thorgaard 1995), loach (Zhang et al. 1998), silver crucian carp (Ding and Jiang 1991), snake (Beçak et al. 2003), and so on. There were some different explanations about the generation mechanism of unreduced gametes. For example, Cherfas (Cherfas et al. 1994) and Beçak (Beçak et al. 2003) put forward that the multiploid gametes produced for nucleus DNA of some primordial germ cells conducted pre-meiotic endoreduplication in proliferating phase, while there was no division for cytoplasm; and some other scholars suggested that the diploid gametes might be resulted by retaining the second polar body. According to the mechanism research of the unreduced gametes derived from G_2, the author thought that chromosome doubling of germ cells might be resulted by fusion, pre-meiotic endoreduplication, endomitosis, and other approaches before the first meiosis. Germ cells with chromosome doubling could produce unreduced gametes (including diploid unreduced gametes) after normal meiosis. The establishment of fertile gynogenetic lineage provided an excellent platform for studying the mechanism of diploid eggs generated by diploid fish. Experiments showed that the production of diploid eggs in diploid gynogenetic fish of RCC × CC was related to pre-meiotic endoreduplication and endomitosis in germ cells. Evidences were shown as follows.

The Fertilization Cytology Observation of G_3

First, the eggs were activated by ultraviolet-irradiated sperm. Two minutes later, the chromosomes in eggs remained at the metaphase of meiosis II, and the spindles occurred (Fig. 5.3a). Three to 8 min after activation, the spindles had moved to the edge of the cytoplasm (Fig. 5.3b, c, d). Nine to 10 min after activation, the meiosis developed to the anaphase and telophase (Fig. 5.3e, f). Forty minutes after activation, the female pronucleus was formed (Fig. 5.3g). Seventy minutes after activation, the eggs reached to mitosis anaphase of the first cleavage and dragged by the spindles, and the two sets of chromosomes were moving to the two poles, respectively (Fig. 5.3h). The second polar body was observed to be released by "fertilization egg" during this period, which denied the deduction that chromosome doubling occurred by retaining the second polar body.

.

The Observation on Chromosomes of Gonad Germ Cell and Kidney Cell

In the early phase of oogonium chromosome spreads in G_2, mitosis occurred but no bivalent chromosome was formed. However, in mitotic metaphase, three types of chromosome spreads had been found including 100 chromosomes ($2n = 100$), 200 chromosomes ($4n = 200$), and 380 chromosomes which was near 400, suggesting that this chromosome spread was probably octoploid ($8n = 400$) (Zhang et al. 2005). It indicated that the oogonia of G_2 were mixed of diploid, tetraploid, and octoploid cells, in which the tetraploid oogonia could develop into diploid eggs via normal meiosis. One hundred bivalent chromosomes were detected in spermatocyte of $G_1 \times 4n$AT (the improved allotetraploid fish which came from G_1

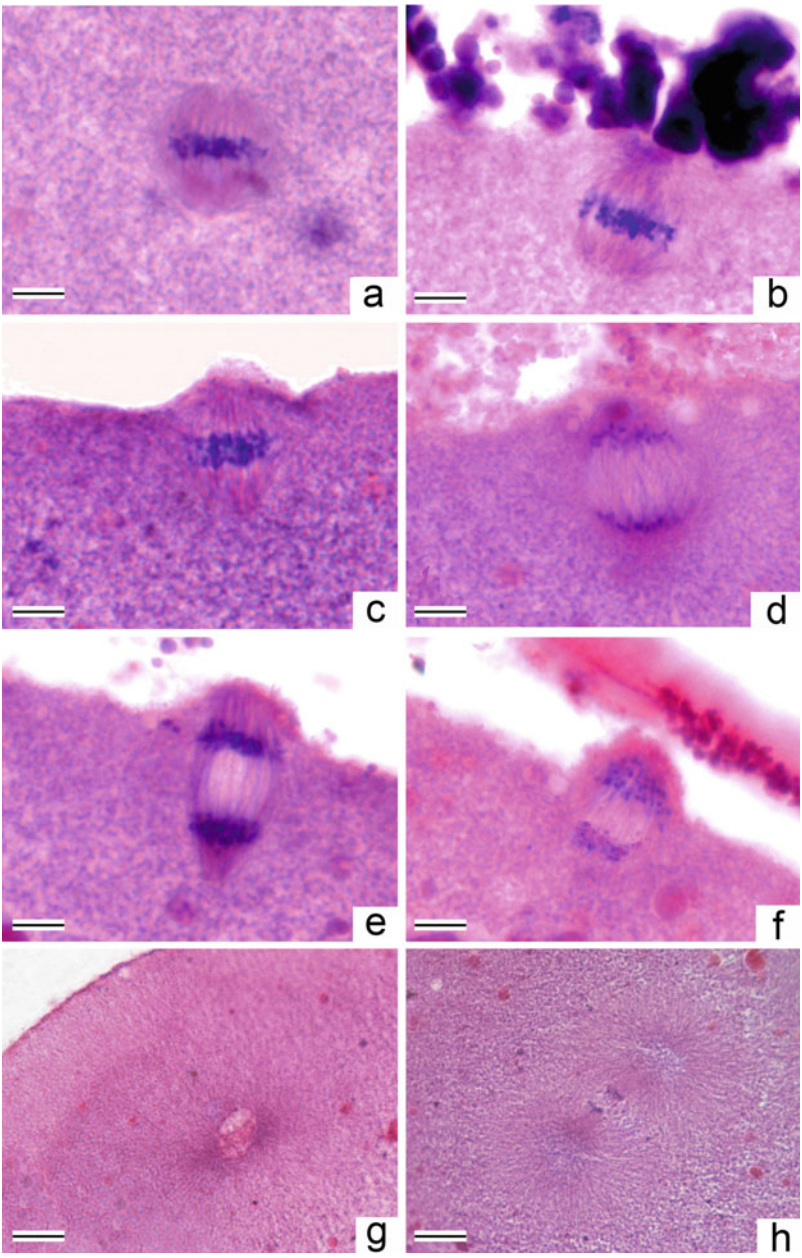

Fig. 5.3 The gynogenetic cytological observation of G_3 (Liu et al. 2007). (**a**) 2 **min** after activation, the chromosomes of eggs were at the metaphase of meiosis II and the spindles occurred. (**b**–**d**) 3–8 **min** after activation, the spindles at the metaphase of meiosis II had moved to the edge of the cytoplasm. (**e**) 9 **min** after activation, the eggs developed to the telophase, and the second polar body was formed. (**f**) 10 **min** after activation, the second polar body was formed and extruded from the cytoplasm. (**g**) 40 **min** after activation, the female pronucleus was formed. (**h**) 70 **min** after activation, the eggs reached to mitosis anaphase of the first cleavage. Bar = 10 μm

crossing with 4*n*AT), and no univalent, trivalent, and tetravalent chromosomes had been detected, which indicated that $G_1 \times 4n$AT could form stable diploid gametes (Zhang et al. 2005).

The Measurement of DNA Content for Oocyte in the Unmatured Diploid Gynogenetic Hybrids (G_3) of RCC × CC

The DNA content of blood cells in RCC was used as a control to detect the DNA content of early ovary germ cells of the unmatured G_2 measured by flow cytometry. The results showed that only one peak was shown in early ovary of diploid RCC. However, two peaks were shown in the unmatured G_2, and the ratio of DNA content of the first peak to that of the second peak was 1:2.1, which was similar to 1:2 ratio ($P > 0.05$). It demonstrated the chromosome number of some germ cells in early ovary of G_2 had already doubled. The results provided the evidence for chromosome doubling in the early germ cells of G_2.

5.1.1.6 The Application of the Gynogenetic Lineage

After long-term research, the gynogenetic lineage has been established, which had an important application value. The diploid gynogenetic lineage was fertile and could produce diploid eggs steadily, which provided an excellent material for studying the formation mechanism of the diploid gamete. Besides, it had advantages in growth rate, anti-disease ability, and fertility, which provided high-quality diploid egg resources for the development of new type of improved tetraploid hybrids. In this way, a new type of improved allotetraploid fish ($G_1 \times 4n$AT) was generated by crossing diploid gynogenetic fish of RCC × CC (♀) with allotetraploid fish of RCC × CC (♂). This new type of improved allotetraploid fish was fertile and had 200 chromosomes (Zhang et al. 2005), which provided that it was tetraploid ($4n = 200$). The improved triploid fish could be produced by crossing diploid eggs from diploid gynogenetic fish of RCC × CC with haploid sperm. In addition, the gynogenetic diploid fish were all female, which provided the excellent animal material to study sex determination.

5.1.2 The Formation of the Improved Allotetraploid Fish Using the Diploid Gynogenetic Hybrid Clone Lineage

The improved allotetraploid fish of RCC × CC with the benefits in fecundity and growth rate could be obtained by crossing diploid eggs generated from the gynogenetic lineage with diploid sperm produced by 4*n*AT.

5.1.2.1 The Formation of the Improved Allotetraploid Fish of RCC × CC ($G_1 \times 4n$AT)

Three types of different sizes of eggs with a diameter of 0.13 cm, 0.17 cm, and 0.20 cm could be generated in G_1. The eggs with the diameter of 0.17 cm

(accounting for 93.33%), which were the same to those produced by 4*n*AT, were considered as diploid eggs; the eggs with the diameter of 0.13 cm, which were the same to those produced by diploid RCC, were considered as haploid eggs. Furthermore, it needed further identification that the eggs with the diameter of 0.20 cm were polyploid eggs or not. A large number of surviving progeny ($G_1 \times 4n$AT), with embryonic hatching rate of 13.9%, were produced by crossing the diploid eggs generated by G_1 and diploid sperm generated by 4*n*AT. The improved allotetraploid fish appeared in two different types. Among them, thirty percent of individuals presented an obvious advantage in growth but no egg or sperm could be squeezed out, which were triploid hybrids, generated by the combination of haploid eggs with diploid sperm. The other 70.0% individuals of the improved tetraploid hybrids of RCC × CC could produce eggs or sperm, which were detected to be tetraploid fish according to the results of chromosomes number and DNA contents ($4n = 200$). The karyotype formula of chromosome in $G_1 \times 4n$AT was 44 m + 68sm + 44st + 44 t. They were generated by crossing diploid eggs in G_1 with diploid sperm in 4*n*AT (Zhang et al. 2005).

5.1.2.2 The Biological Characteristics and Application of the Improved Allotetraploid Fish of RCC × CC ($G_1 \times 4n$AT)

In appearance, $G_1 \times 4n$AT was intermediate between RCC and CC, which was similar to 4*n*AT with gray body and two pairs of short barbels. It had been proved that $G_1 \times 4n$AT had improved characteristics in fecundity, growth rate, stress resistance, and body shape compared to 4*n*AT. The sperm production of male $G_1 \times 4n$AT was obviously higher than that of male 4*n*AT. A male $G_1 \times 4n$AT in the weight of 10–150 g could stream out 3–5 ml of semen, while a male 4*n*AT could only stream out 1–3 ml of semen. Spawning period of female $G_1 \times 4n$AT was 2 months longer than that of female 4*n*AT, and the egg laying amount of female $G_1 \times 4n$AT was 30.0% higher than that of female 4*n*AT, which enhanced the number of tetraploid fish and provided enough parents to produce triploid fish in a large scale; the growth rate of $G_1 \times 4n$AT was 12.7% faster than that of 4*n*AT under the same circumstances; and the stress resistance of $G_1 \times 4n$AT was stronger than that of 4*n*AT, as the survival rate after spawning in $G_1 \times 4n$AT was 10.0–15.0% higher than that in 4*n*AT. A large amount of $G_1 \times 4n$AT produced in this way, with many benefits in biological characteristics, had been used to produce improved triploid fish in a large scale. In the past, 4*n*AT were used as parents to produce triploid fish; a total of 60 million individuals could be produced per year. Nowadays, $G_1 \times 4n$AT has been used as parents and has produced triploid fish reaching a scale of 400 to 500 million per year.

5.2 Androgenesis of the Diploid Sperm from 4*n*AT

5.2.1 The Overview of Artificial Induced Androgenesis in Fish

Artificial induced androgenesis in fish was usually defined as that haploid sperm fertilized with genetically inactivated eggs and underwent a treatment such as cold shock for doubling the chromosomes in the first cleavage period of "fertilized" eggs. The treatment of cold shock would damage the "fertilized" eggs, which obviously reduced the success rate of androgenesis. If the diploid sperm was produced by the 4*n*AT and the genetically inactivated eggs were "fertilized", the normal developing diploid androgenetic progeny could be directly obtained without the treatment of chromosome doubling. In this way, bisexual fertile androgenetic progeny had a higher survival rate, which laid a biological foundation for establishing a subsequent hybrid lineage.

Androgenetic individuals were rarely reported in nature, but were occasionally reported in fish hybridization. For example, Stanley JG (Stanley 1976) detected that androgenetic diploid grass carp progeny are produced with a lower occurrence rate of 0.02% in the hybrids of CC (♀) × grass carp (♂). Five percent of androgenetic individuals had been found in the hybrids of crucian carp (♀) × allogynogenetic sliver crucian carp (♂), in which the hatching rate was 5.0–8.0% (Yu et al. 2000). The androgenetic progeny were able to survive and reach to sexual maturity. The reports involved in artificial androgenesis were mainly focused on starry flounder, chum salmon, rainbow trout, brook trout, CC, crucian carp, Nile tilapia, loach, zebrafish, and yellow catfish. To date, there has been no report regarding the artificial androgenetic fish produced in a large number by haploid sperm. The main reason of lower survival rate of androgenetic individuals was that the UV irradiation and cold shock severely disrupted their development.

5.2.1.1 The Method of Inactivating the Genetic Material of the Eggs

The genetic material of eggs could be inactivated by ionizing radiation, UV irradiation, or egg over-maturing, which were similar to the method of inactivating genetic material used in gynogenesis. However, the treatment effects for eggs were worse than that for sperm, because the genetic material of eggs was protected by egg membranes and the volume of an egg was larger than that of sperm.

Ionizing radiation could induce chromosome breakage by damaging the mitochondrial DNA, messenger RNA, and other structures in the cytoplasm. In spite of this, ionizing radiation was still an effective source of radiation. By treating the matured eggs of CC and crucian carp with 20–25 KR γ ray and fertilizing them with the sperm of red carp, BSB, or grass carp, respectively, the androgenetic fish of red carp, BSB, and grass carp were induced successfully (Ye et al. 1990). The UV irradiation led to break hydrogen bonds resulted in DNA deforms, which affected DNA replication and transcription. But UV penetration was weak, which made it difficult in practical application as some eggs were not transparent and their direction could not be identified easily. On the other hand, the lower penetration of UV caused less damage to eggs. Adopting dose of UV to inactivate eggs had been

researched under dry condition. High UV radiation dose would cause a lot of damage to the egg cytoplasm, with the dose of 72 mJ/cm^2, only 11.2–20.0% of haploid individuals hatched in CC (Bongers et al. 1994). In addition, if the matured eggs of loach radiated with 210 mJ/cm^2 in the ovarian fluid of artificial synthetic, more than 94.0% of haploid individuals could be produced (Zhao et al. 1999).

Over-matured or aging eggs also could induce haploid androgenesis. After ovulation for 4–5 weeks, the remaining eggs of rainbow trout were used to fertilize with normal sperm, and all the haploid embryos (100.0%) were finally produced. A considerable number of embryos possessed reduced chromosomes and fatal haploid syndrome occurred (Yamazaki 1981). Chromosomal aberration induced by over-matured or aging eggs was similar to that of treating by radiation, which demonstrated that over-matured or aging eggs still pose a damage to egg chromosomes. However, experimental data about artificial fertilization cytology of the four Chinese farmed carps showed that chromosomes in the metaphase of meiosis II in over-matured eggs were broken down with the female nucleus genetic material inactivated, and egg cytoplasm denaturalized, which was bad for androgenesis. Normal mitosis would never occur in fertilization between full over-matured eggs and normal sperm, which make it difficult to conduct androgenesis by inactivating the genetic material of over-matured eggs (Liu 1993).

In addition, some researchers tried to rip out chromatin of nucleus mechanically with micromanipulation. Diploid androgenetic loach had been produced combining this method with nuclear transfer techniques (Liu et al. 1987).

5.2.1.2 The Male Nucleus Chromosome Doubling

Androgenetic haploid would die in hatching period or late hatching stage because of haploid syndrome, which made the doubling of haploid chromosomes be required for producing viable diploid androgenetic fish. As described in the method of doubling the female nuclear chromosomes, the male nucleus chromosomes could be doubled by refraining the first cleavage, not by refraining from the release of the second polar body. The diploid androgenetic fish had been achieved in rainbow trout (Scheerer et al. 1991) and loach (Parsons and Thorgaard 1985) with hydrostatic pressure. The androgenetic CC, sliver crucian carp, Nile tilapia, large-scale loach, zebrafish, and other fish had been produced with the method of heat shock. Furthermore, the method of cold shock was reported in large-scale loach.

Interestingly, the diploid sperm generated by allotetraploid fish were used to induce androgenesis, which could enhance the survival rate of androgenetic fish because the sperm possessed two sets of genomes, and diploid androgenetic fish could be produced without the treatment of chromosome doubling.

5.2.1.3 The Survival Rate and Fertility of the Androgenetic Fish

Androgenetic fish enjoyed a very low survival rate because of the damage of refraining the first cleavage and the genomes being in homozygotic state. Furthermore, in the process of inducing androgenesis, the negative effects of irradiated eggs and the effect of doubling the chromosomes of male nucleus on the embryo could

also reduce the survival rate of androgenetic fish, and androgenetic fish derived from cytoplasmic-nuclear hybridization, that is, the heterogeneous female egg cytoplasm, regulated the gene expression of the sperm genetic material. Therefore, if there was an error in the coordination of the egg cytoplasm and sperm nucleus gene expression, it would also affect the embryo's ability to live. The survival rate of androgenetic fish decreased obviously in the first year, especially in larval stage, which was reported on the studies of androgenesis (CC, rainbow trout, zebrafish, brook trout, Nile tilapia, and loach).

Many researchers had made many meaningful explorations to inactivate the genetic material of egg nucleus in a more effectively way, reducing damages on ooplasm as far as possible, and improving the survival rate of androgenetic fish. Adopting UV dose to inactivate the eggs of CC in dry condition, Bongers found that if the dose of 24–72 mJ/cm^2 was used to radiate eggs, which would cause a lot of damage to the egg cytoplasm, only 11.2–20.0% of haploids hatched (Bongers et al. 1994). If the dose of 40–104 mJ/cm^2 was used to radiate eggs and rotation was conducted while irradiating, 40.4–56.6% of haploid androgenetic hybrids hatched. If the dose of 250 mJ /cm^2 was used to radiate eggs and soaked with ovarian fluid and stirred while radiating them, 53.9% of haploid androgenetic fish hatched. In addition, putting the eggs of loach into a box made of cellophane, Arai radiated the eggs from up-and-down side with two UV lamps, respectively, 90.0% of haploid syndrome fry hatched with the radiation dose of 112–126 mJ/cm^2 (Arai et al. 1995). The success rate of producing surviving diploid fish by male nuclear genome doubling was extremely low, because the eggs that had become very fragile after irradiation were treated with physical shock and it was easy to cause serious damage to the eggs, especially in the egg cytoplasm. It was necessary to improve and optimize the male nucleus chromosome doubling technique.

There were only few detailed introductions on the gonadal development and fertility of androgenetic fish. The side effects caused by the operation of the androgenetic fish were more serious than that of the gynogenetic fish. Androgenetic fish itself was a karyoplasm hybrid, so their fertility was worse than that of gynogenetic fish. But some researchers had reported that some individuals of androgenetic rainbow trout, zebrafish, and CC developed their sexual maturity. The study about genetic analysis on the androgenetic rainbow trout was performed, and the results indicated that super-male individuals not only were able to survive but also could generate normal sperm, which mated with common rainbow trout to produce all-male progeny (Scheerer et al. 1991).

5.2.2 Androgenesis of the Diploid Sperm

Researches on androgenesis were mainly focused on diploid fish, while there are only a few studies on the androgenesis of diploid sperm induced by tetraploid fish, as it was hard to produce fertile tetraploid fish and the resources of diploid sperm were relatively small. There was a very low survival rate of androgenetic fish because of

the negative effect of chromosome doubling. If the androgenesis were induced with diploid sperm generated by tetraploid, the negative effect of treatment for chromosome doubling could have been avoided, which enhanced sharply the survival rate of androgenetic fish and provided an excellent approach to solve the bottleneck questions in researching androgenetic fish. Bisexual fertile diploid androgenetic fish (XX, XY, YY) were produced successfully by fertilizing the genetically inactivated haploid eggs of goldfish (C. *auratus* var. GF) with the diploid sperm generated by 4*n*AT, which didn't require chromosome doubling (Sun et al. 2007).

5.2.2.1 The Inducing Method of the Androgenetic Fish (AN_0)

After obtaining the haploid eggs of GF and diploid sperm of 4*n*AT through conventional method, Hank's liquid was used to dilute the semen of allotetraploid fish (1:4), which then were preserved in a refrigerator with a temperature of 4 °C. The eggs were directly squeezed into the petri dish containing the ovarian fluid in batches and were shaken to make it evenly spread. The egg granules were squeezed in batches into a petri dish with ovarian fluid and shaken to evenly spread them with ovarian fluid covering the eggs. Putting the petri dish on the ice bag to maintain its low temperature and laying them on the shaking table rotated with an angle of 5–10, the eggs were irradiated by UV rays for inactivating the genetic materials of the egg nucleus. During irradiation, the eggs were stirred by a rocking machine. The height between UV light and the petri dish was about 9.0–10.0 cm. The formula of ovarian fluid was 4.11 g/L calf serum, 118.0 mmol NaCl, 12.7 mmol KCl, 3.8 mmol Na_2HPO_4, 0.7 mmol $MgCl_2{\cdot}6H_2O$, 2.7 mmol $CaCl_2$, 5.5 mmol tyrosine, and 5.5 mmol glycine, using $NaHCO_3$ to adjust pH value to 8.14. The dose of UV radiation ranked from 240 mJ/cm^2, 300 mJ/cm^2, 360 mJ/cm^2, 420 mJ/cm^2, 480 mJ/cm^2, 540 mJ/cm^2 to 600 mJ/cm^2, and the responding radiation time was 120–300 s, respectively.

The UV-treated eggs were "fertilized" with the prepared sperm, taking some eggs without UV treatment fertilized with the sperm of allotetraploid fish as control. The experimental group and control group were not treated for chromosome doubling, and each experiment was repeated three times.

5.2.2.2 The Identification of the Androgenetic Fish

The Comparison of Survival Rate and Appearance

Hatching rates were 87.6%, 47.0%, 87.2%, and 86.9% in female A, B, C, and D individual of GF × 4nAT, respectively. The low hatching rates of eggs from female B individuals suggested the egg quality is poor, which might be due to over-matured eggs. In experimental groups, under different irradiation duration, the hatching rates and survival rates from hatching to start of feed had varied. Results were presented in Table 5.1.

There was no significant difference in hatching rates and survival rates of eggs from female A, C, and D group ($P < 0.05$), while the hatching and fertility rates of eggs from female B were obviously lower than that of another 3 groups of GF ($P < 0.05$), which demonstrated that egg quality posed an influence on the hatching

Table 5.1 The hatching rates (%) in relation to the females and the UV dose administered (Sun et al. 2007)

	UV dose (mJ/cm^2)				
Female parent	240	300	360	420	480
A*	3.7; 3.9; 4.1	3.8; 4.0; 4.5	3.1; 3.4; 3.7	2.4; 2.8; 2.9	0.6; 0.9; 1.0
B**	1.7; 1.9; 2.1	1.2; 1.4; 1.5	1.1; 1.2; 1.4	0.6; 0.7; 0.9	0.0; 0.0; 0.4
C*	3.4; 3.6; 3.9	3.6; 3.7; 4.0	2.9; 3.1; 3.3	2.1; 2.3; 2.4	0.4; 0.5; 0.6
D*	3.4; 3.5; 3.8	3.4; 3.6; 3.9	2.7; 2.9; 3.0	1.9; 2.1; 2.2	0.5; 0.7; 0.7

* Indicating under the same UV dose, hatching rates of different females do not differ significantly ($P > 0.05$); ** indicating the hatching rates of female B were significantly lower than the rates of other females under the same UV dose ($P < 0.05$); hatching rate = (number of hatching fry/number of eggs) × 100%

Table 5.2 The survival rates (%) from hatching to start of feeding in relation to the female and the UV dose administered (Sun et al. 2007)

	UV dose (mJ/cm^2)				
Female parent	240	300	360	420	480
A	39.3 ± 1.1	41.3 ± 2.6	36.9 ± 1.0	23.0 ± 3.5	16.5 ± 4.7
B	32.1 ± 0.9	27.4 ± 1.3	12.8 ± 1.2	19.0 ± 2.0	0
C	33.5 ± 2.4	38.6 ± 0.7	37.4 ± 0.9	20.1 ± 1.4	24.6 ± 5.1
D	40.7 ± 3.0	39.1 ± 0.9	32.7 ± 2.3	19.5 ± 0.8	23.4 ± 3.3
Pond number	1#	2#	3#	4#	5#

The survival rates from hatching to start of feeding of different females have significant difference ($P < 0.05$); survival rates from hatching to start of feeding expressed as (number of normal fry at start feeding/total number of fries at hatching ± SD) × 100%

rates of androgenetic fish. Until at higher UV dose of 420 mJ/cm^2 or 480 mJ/cm^2, the hatching rates of eggs treated at UV dose of 240, 300, and 360 mJ/cm^2 didn't differ significantly ($P > 0.05$). It showed that longstanding UV radiation caused damages on eggs and destroyed mtDNA and maternal mRNA, and then affected the hatching rate of the embryo. There was no significant difference in different experimental group ($P > 0.05$) with the same radiation dose treatment. There was no significant difference in the survival rates from hatching to first feeding of groups treated with UV dose of 240, 300, and 360 mJ/cm^2, but lower survival rate was significant in the groups receiving UV dose of 420 and 480 mJ /cm^2 ($P < 0.05$) (Table 5.2). With irradiation dose up to 600 mJ/cm^2, no more than 0.1% of individuals get survived. The highest hatching rate (mean 4.5%) and survival rate from hatching to start of feeding (41.3 ± 2.6%) were recorded for female A at a UV dose of 300 mJ/cm^2, although it was not significantly different from the hatching rate and survival rate obtained by treatment with UV dose of 240 mJ/cm^2, 360 mJ /cm^2, or 420 mJ/cm^2 ($P > 0.05$).

After 1 month, the survival rate of the experimental groups averaged 75.7%, 75.0%, 69.5%, 62.4%, and 67.0% (corresponding to pond 1#, 2#, 3#, 4#, 5#), while

Table 5.3 The number of different size of fish at the age of 5 months and their average weight in each pond (Sun et al. 2007)

Pond number	Individuals with large size		Individuals with small size		Proportion of small size individuals (%)
	Number	A Average weight (g)	Number	Average weight (g)	
1#	4	105 ± 14	7	55 ± 8	63.6
2#	4	92 ± 11	14	60 ± 12	77.8
3#	3	89 ± 9	9	48 ± 10	75.0
4#	2	85 ± 11	5	50 ± 9	71.4
5#	3	111 ± 13	2	76 ± 5	40.0

Table 5.4 The comparison in external morphological characteristics of AN_0, 4*n*AT, GF, G, and GF × 4*n*AT (partly referenced) (Sun et al. 2007)

Shape index	GF	4*n*AT	AN_0	G	GF × 4*n*AT
Body length/ body height	0.73 ± 0.05	0.39 ± 0.10	0.41 ± 0.06	0.42 ± 0.06	0.50 ± 0.13
Number of dorsal fins	I + 17	III + 18/19	III + 18	III + 18	II + 18
Barbel	None	Two pairs	Two pairs	Two pairs	One pair
Number of lateral line scales	25–26 6/7	30–34 6/7	31–33 6/7	31–34 6/7	30–31 6/7

the survival rate in the control group was 79.3%. Five months later, the proportion of fish with different size was recorded in each pond (Table 5.3).

In the control group, there was a formal resemblance between GF × 4*n*AT individuals. However, two morphological types of individuals were found in the experimental groups. "Large" individuals with higher mean weight were morphologically similar to GF × 4*n*AT fish, whereas "small" individuals with lower mean weight were morphologically similar to diploid gynogenetic fish (G). Concrete details of comparison are discussed in Table 5.4.

Comparing the weight of androgenetic individuals in smaller size with that of 4*n*AT in the same period, we found the growth rate of androgenetic fish was 8.0–13.0% faster than that of common 4*n*AT.

2. The Observation on Chromosomes and DNA Content Measurement.

The chromosome number of small individuals mainly ranged from 95 to 100, which showed that they were diploid ($2n = 100$). The "large" individuals were triploid with 150 chromosomes ($3n = 150$), suggesting unsuccessful inactivation of maternal genome. In the control group, all GF × 4*n*AT individuals were triploid ($3n = 150$), and their chromosome number ranged from 145 to 150 (90.6%) (Table 5.5). The tetraploid fish ($4n = 200$) was produced in self-mated progeny of diploid androgenetic fish and hybrids (AN_0 × 4*n*AT) of diploid androgenetic fish (♀) with allotetraploid fish (♂) (Fig. 5.4).

Table 5.5 The chromosome number distribution of AN_0, GF × 4*n*AT, and its self-mated progeny (AN_1) (Sun et al. 2007)

Fish type	Number of detected fish	Number of chromosomes						
		< 95	95–100	< 145	145–150	< 195	195–200	> 200
AN_0	5	22	228	0				
GF × 4*n*AT	10			47	453			
AN_1	5					22	223	5

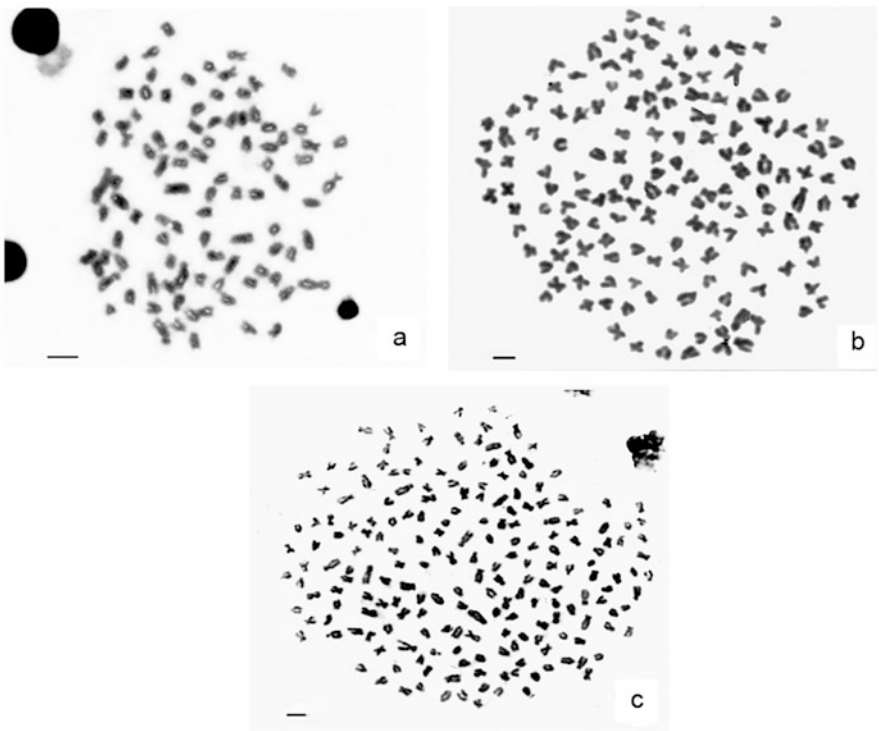

Fig. 5.4 The metaphase chromosome spreads of AN_0, GF × 4*n*AT, and AN_1 (Sun et al. 2007). (**a**) The metaphase chromosome spread of AN_0 ($2n = 100$). (**b**) The metaphase chromosome spread of GF × 4*n*AT ($3n = 150$). (**c**). The metaphase chromosome spread of AN_1 ($4n = 200$). Bar = 3 μm

The DNA content of red blood cells from all the detected fish was measured with flow cytometer, which correspond with the evaluation of metaphase chromosome spread results. The DNA of diploid RCC ($2n = 100$) was used as the standard; the DNA content of red blood cells from androgenetic fish in small size was equal to that of diploid RCC, was half that of 4*n*AT, and two thirds that of hybridization fish in controlled group, while the DNA content of "large" individual in experimental groups was the same as that of hybridization fish in the controlled group (Table 5.6).

Table 5.6 The comparison of DNA content of red blood cells from AN_0, GF × 4*n*AT, GF, and RCC (Sun et al. 2007)

Fish type	Average DNA content	Ratio	
		Observed	Expected
RCC	73.04		
AN_0	84.90	AN/RCC = 1.16	1
GF × 4*n*AT	103.19	(GF × 4*n*AT)/RCC = 1.41	1.5
GF	77.99	GF/RCC = 1.07	1

The observed ratio was not significantly different ($P > 0.05$) from the expected ratio

During the breeding season, 1-year-old fish in both the androgenetic experimental and the control group were unable to extrude eggs or semen. Five androgenetic fish were selected with small size in the experimental group randomly. By observing its gonadal structure with section, the results showed that the gonad of androgenetic fish was still in immature stage. There were a few of oogonia and a large number of oogonium-like cells distributed in the ovary and no matured oocyte was found; spermatogonium and spermatocytes are arranged regularly in the seminiferous tubules of testis tissue, but matured sperm could not be found. Sexually mature individuals were observed in 2-year-old androgenetic fish, meanwhile, female was able to release eggs and male could release watery semen. Sectioning showed that the ovary was in normal development and was filled with oocyte in stages III–IV. Its ovarian structure was similar to that of diploid gynogenetic fish, while its testis development, to some extent, was refrained. All gonads of hybridization fish in the controlled group were in abnormal development. No individual was able to release sperm or eggs, among 2-year-old fish, which was similar to the report on the gonadal development of triploid fish.

In the breeding season, male and female individuals of diploid androgenetic fish of sexual maturity produced a large amount of AN_1,while thousands of hybrids (AN_0 × 4*n*AT) were obtained by crossing female diploid androgenetic fish with male 4*n*AT. The new tetraploid fish represented obvious growth traits and strong stress resistance, compared to common 4*n*AT, which were similar to diploid gynogenetic fish.

5.2.3 Formation of the Improved 4*n*AT

5.2.3.1 The Formation of AN_1

Similar to G_1, G_2, and G_3, the AN_0 group still retained the traits of hybrids of RCC × CC with two pairs of short barbels, the length of which were intermediate to RCC (no barbels) and CC (two pairs of obvious barbels). Germ cells of diploid AN_0 would become abnormal, because they possessed the characteristics of RCC and CC, such as there existed tetraploid AN_1-4*n* and triploid AN_1-3*n* in A_1 self-mated hybrids of diploid androgenetic fish AN_0, which demonstrated that diploid AN_0 could produce diploid gametes. Researches showed that the reason why the gynogenetic

lineage could generate diploid gametes was probably that pre-meiotic endoreduplication and germ cell fusion happened in early germ cells. The principle of diploid eggs and sperm generated by female and male AN_0, respectively, was similar to that of diploid eggs produced by the gynogenetic lineage. In addition, the female and male individuals could generate diploid eggs and sperm, which mated with each other to produce 4*n*AT progeny. The above results showed that diploid hybrids could produce diploid gametes. On the other hand, whether it is the gynogenetic lineage or male and female diploid hybrids of AN_0, all of them reached sexual maturity in 2 years, while their original maternal parent (RCC) could reach its sexual maturity in 1 year, which showed that there might exist chromosomal doubling mechanism-endoreduplication, endomitosis, or cell fusion, which caused the formation mechanism of reduced gametes and delayed the age of sexual maturity of hybrids.

5.2.3.2 The Appearance of AN_1

There were morphological differences among tetraploid, triploid, and diploid hybrids of AN_1. AN_1-4*n* had two pairs of shorter barbels, while AN_1-3*n* and AN_1-2*n* had no barbels. AN_1-4*n* had fusiform appearance and their body outline was cycloid, the color of the dorsum of AN_1-4*n* was gray, but the color of the belly was yellow or white. The color of the dorsum of AN_1-3*n* and AN_1-2*n* was silvery white and gray, respectively. These different morphological traits could easily distinguish them from one another. In addition, comparison on the morphometric characteristics and countable characteristics of AN_1-4*n*, AN_1-3*n*, AN_1-2*n*, 4*n*AT, and RCC were represented in Tables 5.7 and 5.8.

5.2.3.3 The Gonadal Development of AN_1

In 10-month-old AN_1, tetraploids (AN_1-4*n*) account for 85%, triploids (AN_1-3*n*) account for 10.0%, and diploids (AN_1-2*n*) account for 5.0%, suggesting that most of the gametes were diploid generated by AN_0. AN_1-4*n* were bisexual fertile, but contemporaneous AN_1-3*n* and AN_1-2*n* are all sterile (Duan et al. 2007). The reason

Table 5.7 The ratios of measurable characteristics of AN_1-4*n*, AN_1-3*n*, AN_1-2*n*, 4*n*AT and RCC (Duan et al. 2007)

Fish type	Body length/ total length	Body height/ body length	Head length/ total length	Head height/ head length	Caudal peduncle depth/caudal peduncle length	Head height/ body height
AN_1-4*n*	0.81	0.42	0.25	0.90	1.05	0.55
AN_1-3*n*	0.82	0.43	0.26	0.81	0.99	0.52
AN_1-2*n*	0.82	0.41	0.26	0.88	1.07	0.56
4*n*AT	0.80	0.37	0.28	0.80	0.95	0.83
RCC	0.82	0.46	0.27	0.93	1.20	0.54

Table 5.8 The countable characteristics of AN_1-4*n*, AN_1-3*n*, AN_1-2*n*, 4*n*AT, and RCC (Duan et al. 2007)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins	Barbel
AN_1-4*n*	30–32	5–6	6–7	III + 18–19	8–9	III + 6–7	Two pairs
AN_1-3*n*	31–32	5–6	6–7	III + 17–18	8–9	III + 6–7	None
AN_1-2*n*	31–32	5–7	6–7	III + 18–19	7–9	III + 6	None
4*n*AT	30–34	5–7	5–8	III + 17–18	7–9	III + 6	Two pairs
RCC	28–30	5–6	5–6	III + 18–19	8–9	III + 5–6	None

for the sterility of 10-month-old AN_1-2*n* was that the gonadal development of AN_1-2*n* was similar to that of F_1, F_2, and the diploid gynogenetic hybrids (G_1, G_2) of RCC × CC. They are all sterile at the age of 1, while fertile at the age of 2, showing the feature of delayed gonadal development. Delayed gonadal development was seemingly caused by the pre-meiotic endoreduplication or cell fusion. Triploid hybrids of distant crossing are the reason why AN_1-3*n* were sterile. In the ovaries of AN_1-4*n*, most mature oocytes were in stage IV. Similarly, the testes of AN_1-4*n* are full of abundant mature spermatozoa. In females and males of AN_1-4*n*, the mature eggs and white semen could be squeezed out, respectively. Except for the figure of AN_1-4*n* germ cells being larger compared to diploid fish, the shape of AN_1-4*n* germ cells was similar to that of diploid fish. For example, the stage IV oocytes of AN_1-4*n* had two-ply follicular cells and had a micropyle on the oolemma. Diploid sperm of AN_1-4*n* comprise head and tail, and the tail has the structure of "9 + 2" microtubule construction to ensure normal movement of the sperm. It is mitochondria that provided motility of the diploid sperm. The head diameter of diploid sperm from AN_1-4*n* (2.40 μm) was obviously larger than that of RCC (1.90 μm) under scanning electron microscope, and it was diploid sperm. We can conclude that bisexual fertile tetraploids could be acquired by androgenesis, and the AN_1-4*n* tetraploids could produce diploid gametes so that the tetraploidy of tetraploid population could be steadily maintained in the future. The formation of bisexual fertile tetraploid fish producing diploid gametes stably by androgenesis was never reported before.

5.2.3.4 The Production of the Improved 4*n*AT

Tetraploid fish (AN_1-4*n*) were generated by the self-mating of AN_0. Compared to common tetraploid fish, the appearance of AN_1-4*n* had been improved after conducting a study on their measurable and countable traits, such as desirable traits in the aspects of body depth/body length, head height/body depth, and caudal peduncle depth/caudal peduncle length, and it fully showed that using androgenesis could well genetically improve allotetraploid hybrids. The improved tetraploid fish

both enriched the resource of tetraploid hybrids and provided better parents to produce triploid fish (Duan et al. 2007). In addition, the proportion of male fish was increased obviously in (AN_1-4n) hybrids generated by self-mating AN_0. This was because that there existed the super-male in the parents AN_0, and generally, multi-super-male self-mating with multi-female individuals, super-male (YY) and non-super-male (XY) might exist in AN_0 super-male in the same time. Theoretically, full male hybrids will be generated, if super-male AN_0 were crossed separately with female individuals. There need further efforts in studying super-male individuals.

5.2.4 Application of the Androgenetic Fish

As biotechnological methods, both the gynogenesis and androgenesis were of great significance in the research on the sex determination, protecting endangered species, monosexual breeding, and building the genetic pure line. Inbreeding from generation to generation was needed to build a pure line among the traditional breeding methods. It was a long-term, costly, and rigorous job. In particular, it was much more difficult to purify and rejuvenate those fish with a longer age of sexual maturity, such as silver carp, trout, grass carp, and sturgeon, using the traditional lineage breeding method. The genetic material of androgenetic fish mainly came from their male parents, and every gene was in the homozygous state; therefore, 2–3 generations were needed to build a pure lineage with better results.

Androgenetic fish also had a huge potential in protecting endangered species. Combined with sperm cryopreservation technology, androgenesis could become one of the effective measures to preserve germplasm resources and to rehabilitate endangered animal species, because it could be done only by fertilizing sperm with heterogeneous inactivated eggs in consanguinity.

The genetic material of androgenetic fish totally came from sperm, and their sex determination was controlled by sex chromosomes of sperm. As for heterogenous fish with male gametes, the proportion of female (XX) and super-male fish (YY) of androgenetic fish with the two sperm of X and Y was 1:1.

It was mentioned above that there existed super-male (YY) in allotetraploid androgenetic AN_0, and they could generate diploid sperm (YY) with the reproductive model by forming unreduced gametes, and then the sperm are fertilized with diploid eggs (XX) generated by female tetraploid fish to form full male tetraploid hybrids (XXYY). Therefore, the super-male fish AN_0 will be identified and all-male tetraploid fish produced. In this aspect, the super-male fish AN_0 (YY) had been produced and identified in androgenetic autotetraploid ($4n = 200$) AN_0 of hybrid lineage of RCC and blunt snout bream, then all-male tetraploid fish (XXYY) are generated. The details were discussed in Chap. 6, Section 4.

5.3 The Formation of the Improved Diploid Crucian Carp Derived from the Improved 4*n*AT

The improved allotetraploid fish ($G_1 \times 4n$AT) possessed excellent straits in growth and reproduction, which came from the improved diploid eggs backcrossed with the diploid sperm of allotetraploid RCC (♀) × CC (♂) and from the improved diploid eggs derived from the gynogenesis of allotetraploid fish. Those improved groups enriched the genetic resources of allotetraploid fish. Interestingly, 98.0% of hybrids generated by backcrossing of $G_1 \times 4n$AT were tetraploid ($4n = 200$). Their body shape and body color were similar to that of 4*n*AT, with the body height/body length ratio value of 0.36, while that in 2.0% of hybrids was 0.48, which was obviously higher than the former. It presented the obvious feature of high body. The improved allotetraploid fish (2%) with high body self-mated to generate three kinds of bisexual fertile diploid fish including artificial double-tailed goldfish (discussed in this chapter, Section 4), gray common carp, and the first generation of high-body red crucian carp. The first generation of high-body red crucian carp were further differentiated after self-mating and formed three new types of high-body crucian carp including high-body color crucian carp (ICCC), high-body gray crucian carp (IGCC), and the second generation of high-body red crucian carp (IRCC). The formation of high-body crucian carp was presented in Fig. 5.5. These three types of high-body crucian carp tend to form three distinctly improved diploid high-body lineages after self-mated to the next generation. The formation of these lineages had important significance in studying the biological evolution and fish genetic breeding (Wang et al. 2008).

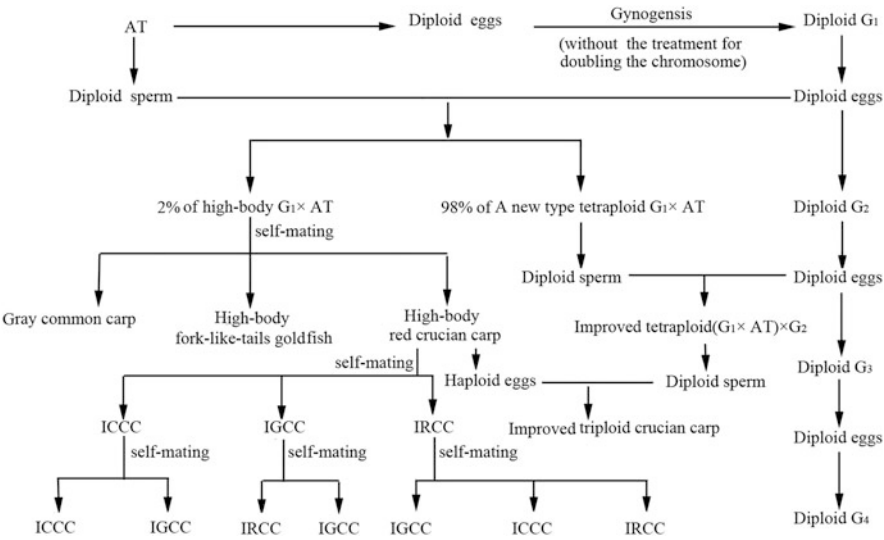

Fig. 5.5 The formation of improved diploid crucian carp (Wang et al. 2008)

5.3.1 The Biological Characteristics of IRCC, IGCC, and ICCC

5.3.1.1 The Appearance of IRCC, IGCC, and ICCC

The appearance of IRCC, IGCC, and ICCC was presented in Fig. 5.6a, b, c, d. Comparison of morphological traits of IRCC, IGCC, ICCC, and common RCC was presented in Table 5.9. Body height/body length of common RCC was 0.41, while that of high-body crucian carp was 0.53, which indicated obvious high-body feature; body length/total length of common RCC was 0.78, while that of high-body crucian carp was 0.82, which presented the feature of long body and short tail; head length/body length of common RCC was 0.29, while that of high-body crucian carp was 0.26; head height/body height of common RCC was 0.60, while that of high-body crucian carp was 0.49, showing the characteristic of small head. Using SPSS for statistical analysis of the comparable morphological traits, we found that significant differences ($P < 0.01$) existed in body length/total length and body height/body length values of IRCC, IGCC, and ICCC, compared to those of common RCC, which reflected the shape characteristics of small head, high body, and short tail. However, there were no significant differences in the body length/total length or body height/body length between IRCC, IGCC, and ICCC ($P > 0.01$). The countable traits of IRCC, IGCC, and ICCC were presented in Table 5.10. The table showed that these three types of high-body crucian carp were similar to common RCC in countable traits.

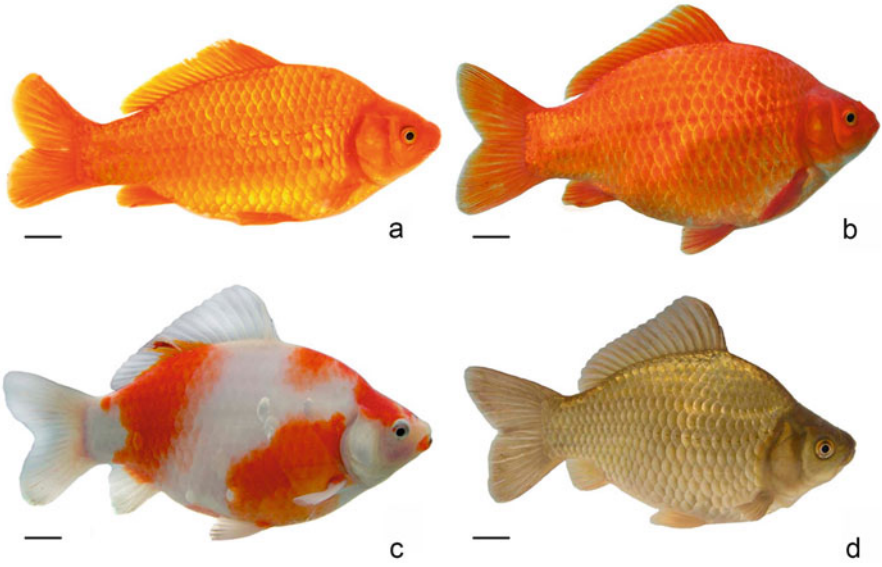

Fig. 5.6 The appearance of common RCC, IRCC, IGCC, and ICCC (Wang et al. 2008). (**a**) The appearance of RCC. (**b**) The appearance of IRCC. (**c**) The appearance of IGCC. (**d**) The appearance of ICCC. Bar = 2 cm

Table 5.9 The ratios of measurable characteristics of common RCC and high-body red crucian carp (Wang et al. 2008)

Fish type	Body height/body length	Body length/total length	Head length/body length	Head height/head length	Caudal peduncle depth/caudal peduncle length	Head height/Body height
RCC	0.41 ± 0.02	0.78 ± 0.01	0.29 ± 0.03	0.85 ± 0.03	1.24 ± 0.02	0.60 ± 0.03
IRCC	0.54 ± 0.02	0.82 ± 0.01	0.26 ± 0.01	0.86 ± 0.04	1.58 ± 0.03	0.49 ± 0.02
ICCC	0.51 ± 0.02	0.81 ± 0.02	0.25 ± 0.01	0.89 ± 0.02	1.87 ± 0.04	0.47 ± 0.02
IGCC	0.54 ± 0.01	0.83 ± 0.01	0.26 ± 0.02	0.88 ± 0.01	1.20 ± 0.01	0.53 ± 0.03

Table 5.10 The countable characteristics of RCC and high-body red crucian carp (Wang et al. 2008)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins
RCC	28.9 ± 0.88 (28–30)	5.4 ± 0.51 (5–6)	6.5 ± 0.25 (6–7)	III + 18.9 ± 0.69 (III + 18–20)	8.6 ± 0.24 (8–9)	III + 6.6 ± 0.24 (III + 6–7)
IRCC	28.2 ± 0.79 (27–29)	5.3 ± 0.48 (5–6)	6.6 ± 0.24 (6–7)	III + 16.4 ± 0.24 (III + 16–17)	8.5 ± 0.25 (8–9)	III + 6.7 ± 0.21 (III + 6-7)
ICCC	27.7 ± 0.48 (27–28)	5.5 ± 0.53 (5–6)	6.7 ± 0.21 (6–7)	III + 17.5 ± 0.25 (III + 17–18)	8.6 ± 0.24 (8–9)	III + 6.8 ± 0.16 (III + 6–7)
IGCC	28.9 ± 0.74 (27–29)	5.8 ± 0.78 (5–7)	6.3 ± 1.21 (5–8)	III + 17.6 ± 0.24 (III + 17–18)	8.1 ± 0.69 (7–9)	III + 6.5 ± 0.25 (III + 6–7)

III The number of spines

5.3.1.2 The Diploidy of the Improved Red Crucian Carp, Gray Crucian Carp, and Color Crucian Carp

The ploidy of IRCC, IGCC, and ICCC was measured. The results indicated the mode of chromosome number of these three types of high-body crucian carp most varied from 95 to 100, which accounted for 93.0%, 91.0%, and 95.0% respectively. It showed that these three types of high-body fish were diploid fish ($2n = 100$) (Wang et al. 2008).

5.3.1.3 The Gonadal Development of IRCC, IGCC, and ICCC

As the gonadal development of these three types of high-body crucian carp was basically synchronous, take the gonadal structure of IRCC as an example. White semen could be squeezed out from 7-month-old male IRCC and nearly mature ova could be squeezed out from 9-month-old female IRCC. After sample dissection, two normal ovaries were found to be oblong and soft in female samples, which almost occupy the abdominal cavity. Section observation indicated that the two ovaries of 7-month-old IRCC were in stage IV and those oocytes were filled with yolk, in which the micropyle could be observed. The milk-white testes of male sample developed well and were bilateral symmetry (Wang et al. 2008). The spermatogonia, spermatids, and numerous mature spermatozoa could be found in seminiferous tubules by observing the ultrastructure, in which the spermatozoa were consisted of a head and a tail and some connecting piece. In the head, the round nucleolus was compact chromatin. The connecting piece consists of centriole complex and "sleeve-shape" elements which were closely attached to the nucleus and had abundant mitochondria and vesicles. The tail stretched out from the "sleeve-shape" element and the axoneme of the tail with "9 + 2" microtubules was link to the basal body. The head size of IRCC's spermatozoa was no different with that of RCC, so it was being determined as haploid sperm (Wang et al. 2008).

5.3.1.4 The Biological Features of the Self-Mated Hybrids of IRCC, IGCC, and ICCC

Self-mating experiments were conducted among 1-year-old IRCC, IGCC, and ICCC, respectively. The fertilization rate and hatching rate were calculated. Then, these three types of high-body crucian carp were cultivated separately. Two months later, the body shape, body color, ploidy, gonadal development, and other aspects of their hybrids were observed. Self-mating experiments suggested that all samples had high fertilization rate and hatching rate. The fertilization rates of IRCC, IGCC, and ICCC were 89.8%, 91.3%, 93.7%, respectively; the hatching rate of IRCC was 85.4%, and 100,000 self-mated hybrids were produced. The hatching rate of ICCC was 87.4%, and 700,000 self-mated hybrids were generated. The hatching rate of IGCC was 88.2%, and 300,000 self-mated hybrids were produced (Table 5.11). In addition, high-body crucian carp could spawn many times from early April to the late August through ovarian renovation, while the common diploid RCC was just in April.

The self-mated hybrids of these three high-body crucian carp were all diploid with the feature of high body. The body color of progeny began to differentiate at

Table 5.11 The fertilization rate and hatching rate of these three types of high-body crucian carp (Wang et al. 2008)

Fish type	Number of parents	Fertilization rate	Hatching rate	Number of adult fish
IRCC	40 (♀)/20 (♂)	89.8%	85.4%	100,000
ICCC	100 (♀)/50 (♂)	91.3%	87.4%	700,000
IGCC	60 (♀)/35 (♂)	93.7%	88.2%	300,000

Table 5.12 The differentiated ratio of self-mated hybrids of these three types of high-body fish (Wang et al. 2008)

	Parent		
Progeny	IRCC	ICCC	IGCC
IRCC	40.0%	40.0%	20.0%
ICCC	–	75.0%	25.0%
IGCC	50.0%	–	50.0%

1 month old and gradually stabilized at 4 months old. Self-mated hybrids of IRCC were still differentiated into IRCC, IGCC, and ICCC, with the proportion of 2:1:2; self-mated hybrids of ICCC were differentiated into ICCC and IGCC, with the proportion of 3:1; self-mated hybrids of IGCC were differentiated into IGCC and IRCC, with the proportion of 1:1 (Table 5.12). We found that these self-mated hybrids all had the normal bisexual gonadal development, which was similar to their parents.

5.3.2 The Significance of the Formation of New Type of High-Body Crucian Carp

5.3.2.1 The Significance in Biological Evolution

The formation of 2.0% of high-body tetraploid hybrids with special high-body appearance derived from distant hybrid and gynogenesis demonstrated that allogynogenesis played a special role in the improved tetraploid fish.

Interestingly, the character differentiation was not observed in 27 generations of self-mated hybrids of 4*n*AT (F_3–F_{29}). However, three types of appearance were formed with obvious differentiation in body color and body shape by self-mating with the 2.0% of high-body improved 4*n*AT, which was IRCC, high-body artificial double-tailed goldfish, and steel gray common carp with two pairs of barbels. They all were bisexual fertile. We speculated that there might exist natural gynogenesis and androgenesis in the self-mating process of 2.0% of high-body improved 4*n*AT, which might be relevant to the gynogenesis of their ancestors. Furthermore, the production of self-mated progeny of the 2.0% of high-body improved 4*n*AT might be relevant to their ancestors which came from distant hybridization of RCC and CC.

Color differentiation happened in the self-mated hybrids of IRCC, further proving that character segregation would happen in the course of self-mating of gynogenetic distant hybridization progeny, which played an important role in biological evolution.

5.3.2.2 The Significance in Genetic Breeding

High-body crucian carp was bisexual fertile, which had propagated to G_7 by self-mating, and three types of high-body crucian carp lineages have been formed with stable characteristic of high body in a large scale. The number of chromosomes of this group was 100 ($2n = 100$). This provided a genetic breeding foundation to establish new diploid high-body crucian carp lineages. All of the results showed that high-body crucian carp had some improved traits, such as small head, high body, and short caudal peduncle. The value of body height/length reached 0.53, compared to that of common RCC (body height/length was 0.41), which showed the obvious improved feature of high body. The high-body triploid crucian carp could be produced by crossing the male 4*n*AT with the female high-body diploid crucian carp, which improved the yield of meat as well as the aquaculture benefit. Furthermore, reproductive advantages occurred in high-body crucian carp and its progeny in the process of artificial propagation, such as they possessed higher fertilization rate and hatching rate, higher gamete yields, and longer idiophase. In a word, the formation of these diploid high-body crucian carp groups provided excellent genetic resources for genetic breeding.

The formation of these three high-body crucian carps could provide foundation to build three new diploid crucian carp lineages with fast growth, excellent aquaculture traits, and strong fertility. It posed an important significance in theory and application to establish a bisexual fertile diploid crucian carp lineage with genetic feature of high-body, which was beneficial to produce improved triploid fish in a large scale. Character differentiation of the 2.0% of self-mated hybrids of high-body improved 4*n*AT and self-mated hybrids of diploid IRCC laid a foundation to study fish genetic characteristics. The formation of diploid high-body crucian carp proved that distant hybridization and gynogenesis posed a significant effect on fish genetic breeding and improvement.

5.4 The Formation of the Experimental Twin-Tail Goldfish

Combined with gynogenesis, improved high-body diploid crucian carps were artificially bred through distant hybridization of RCC and CC. In addition, the high-body experimental twin-tail goldfish were differentiated from the self-mated hybrids of the 2.0% of high-body improved 4*n*AT. The GF, a variant of crucian carp (*C. auratus*), belongs to the order Cypriniformes, family Cyprinidae, and genus *Carassius*. Previous studies had speculated that GF originated from wild population of crucian carp. At the beginning, wild crucian carp mutated into red/yellow individuals then this variant was divided into several lineages, including grass goldfish (long tail), dragon-eye goldfish (bulging eye), oval goldfish (no dorsal fin), wen goldfish (forked tail), and so on. The earliest record about goldfish was in the Jin Dynasty (265–420 A.D.). The earliest goldfish, red squama fish, type of the red/yellow, which were same as wild crucian carp living in natural water area, were found in Mount Lu. The diversity of phenotypic lineages of GF differs from crucian carp in their body color and shape and the appearance of the back, head, tail, anal fin, eyes,

opercula, scales, and nares film. One of the more crucial features of the GF was the bifurcated tail. In contrast, crucian carp had an undivided tail. The formation of GF could be divided into four evolution stages: stage I, the wild red/yellow crucian carp developed into RCC in semi-domestication; stage II, the beginning of domestication when the GF was started to be raised in pond; stage III, the period of basin raising; and stage IV, artificial selection stage. It had gone through more than 1000 years from wild crucian carp to GF with various lineages. Currently, there were more than 240 GF lineages cultured in the world and more than 140 GF lineages in China. Although there is interest in the evolutionary history of GF, less evidence supported the hypothesis that they originated from wild crucian carp. In this research, a new GF bred from distant hybridization provides a new idea about their evolutionary origin.

5.4.1 The Preparation of the Experimental Twin-Tail Goldfish

Similar to the formation of IRCC, IGCC, and ICCC, 2.0% of high-body improved allotetraploid hybrid progeny were differentiated into high-body artificial GF with bifurcated tail, which self-mated to form an artificial GF lineage (EG). Individuals in this group had many kinds of body color and shape, such as red, white, gray, and flower color. The formation of EG was presented in Fig. 5.7.

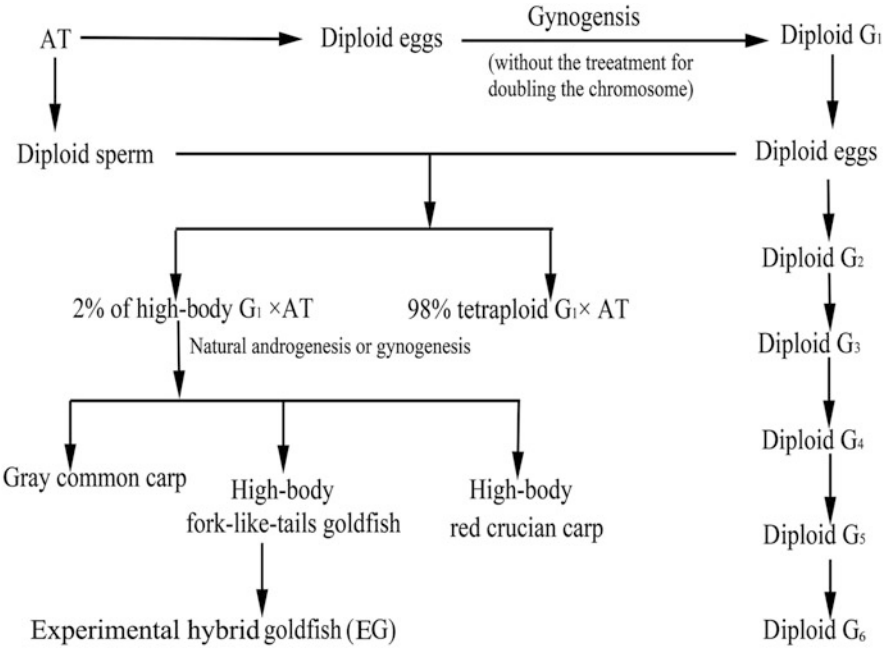

Fig. 5.7 The breeding path of EG (Wang et al. 2014)

The phenotypes, chromosome numbers, DNA contents, and gonadal structures of EG individuals were determined, and some biological features of genetic breeding were researched on the basis of the cellular level. In addition, the genetic constitution and the relationship between EG, RCC, and CC from the perspective of molecule were evaluated by using 5S rDNA, SSR, and mtDNA detection (Wang et al. 2014). Our results provided direct evidence to prove GF derived from crucian carp, and supplied an important biological foundation for studying its evolutionary process. Meanwhile, the formation of the EG posed an important significance in the genetic breeding of aquarium fish.

5.4.2 The Biological Characteristics of the Experimental Twin-Tail Goldfish

5.4.2.1 The Morphological Traits of the Experimental Twin-Tail Goldfish

Three types of EG were presented in Fig. 5.8a, b, c. Their morphological traits were similar to those of GF, and the relevant comparable traits were presented in Table 5.13. The countable traits of EG, RCC, and GF were nearly in the same level, and the results were shown in Table 5.14.

5.4.2.2 The DNA Content and Ploidy Detection

Taking RCC as control group, the DNA content of EG was measured, and results showed the ratio of DNA content of red blood cells from EG to that of RCC was 1.0. Furthermore, the majority of chromosome detection of EG individuals also showed that the chromosome number ranged between 95 to 100 (accounting for 93.0%), meaning that EG were diploid ($2n = 100$) (Wang et al. 2014).

5.4.2.3 The Observation on the Gonadal Structure

Six samples of 5-month-old EG were randomly selected to observe gonadal tissue section, which revealed that the ovaries of 5-month-old EG females were in stage III. In males, a large amount of matured sperm was occupied in the testis. White semen and mature eggs could be squeezed out from 7-month-old males and 9-month-old females, respectively (Wang et al. 2014).

Fig. 5.8 The appearance of EG (Wang et al. 2014). (**a**) Red individual. (**b**) Mixed color individual. (**c**) Black individual. Bar = 1 cm

Table 5.13 The ratios of measurable characteristics of EG, RCC, CC, and GF

Fish type	Body length/ total length	Body height/ body length	Head length/ body length	Caudal peduncle length/body length	Head height/ head length	Caudal peduncle depth/caudal peduncle length	Head height/ body height
GF	0.41 ± 0.02	0.77 ± 0.01	0.29 ± 0.03	0.11 ± 0.01	0.85 ± 0.03	1.24 ± 0.02	0.60 ± 0.03
RCC	0.54 ± 0.02	0.82 ± 0.01	0.26 ± 0.01	0.20 ± 0.03	0.86 ± 0.04	1.58 ± 0.03	0.48 ± 0.02
CC	0.34 ± 0.01	0.82 ± 0.07	0.25 ± 0.02	0.16 ± 0.02	0.81 ± 0.07	0.87 ± 0.10	0.59 ± 0.01
EG	0.51 ± 0.02	0.81 ± 0.02	0.25 ± 0.01	0.12 ± 0.01	0.89 ± 0.02	1.87 ± 0.04	0.47 ± 0.02

Table 5.14 The countable characteristics of EG, RCC, CC, and GF

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins
GF	28.9 ± 0.88 (28–30)	5.4 ± 0.51 (5–6)	6.5 ± 0.25 (6–7)	III + 18.9 ± 0.69 (III + 18–20)	8.6 ± 0.24 (8–9)	III + 6.6 ± 0.24 (III + 6–7)
RCC	28.2 ± 0.79 (27–29)	5.3 ± 0.48 (5–6)	6.6 ± 0.24 (6–7)	III + 16.4 ± 0.24 (III + 16–17)	8.5 ± 0.25 (8–9)	III + 6.7 ± 0.21 (III + 6–7)
CC	36.0 ± 1.22 (35–38)	5.3 ± 0.43 (5–6)	5.3 ± 0.43 (5–6)	III + 17.7 ± 0.83 (III+ 17–19)	8.6 ± 0.43 (8–9)	III + 6.3 ± 0.43 (III +6–7)
EG	27.7 ± 0.48 (27–28)	5.5 ± 0.53 (5–6)	6.7 ± 0.21 (6–7)	III + 17.5 ± 0.25 (III + 17–18)	8.6 ± 0.24 (8–9)	III + 6.8 ± 0.16 (III + 6–7)

5.4.2.4 The Mitochondrial DNA Sequencing

Complete mitochondrial genome sequence of EG was obtained by sequencing directly. The overall length was 16,579 bp. Genome sequences of mitochondrial DNA in GF, RCC, and CC obtained from GenBank were compared. The similarity ratios between EG and its original parents RCC and CC were 99.5% and 89.4%, respectively, by the analysis of jellyfish, which conformed to the rule of mitochondrial maternal inheritance. The similarity ratio between EG and GF reached 99.4% (Table 5.15), which represents a fairly high degree of homology. The phylogenetic trees of EG, RCC, (AY714387), CC (X61010), GF (AB111951), grass carp (EU391390), blunt sount bream (EU434747), Japanese white crucian carp (AB045144), ginbuna carp (AB006953), and zebrafish (NC002333) were built in accordance with the overall sequence of mitochondrial DNA. The results of this neighbor-joining tree were similar to that of the evolutionary tree built with the NTS region of 5S rDNA, which demonstrated the affinity between EG, GF, and RCC. Therefore, it could be speculated that there is possibility that a large variety of GF enjoyed the same origin as EG.

5.4.2.5 The Microsatellite Analysis

The genomic DNA of EG and GF was expanded with three pairs of microsatellite primer (common carp microsatellite primer MFW1, bighead carp microsatellite primers HLJY3940 and HLJY2526). The three different primers enjoyed a high consistency in EG and GF with the same amplified band. In addition, the isozygoty of EG only happened in some few microsatellite loci; most of the individuals showed heterozygosity in most loci, which was relevant to the fact that GF was generated by artificial breeding with exchanging and recombination of chromosome fragments. From microsatellite amplification pattern of EG, the results could be observed: (1) The amplify band of EG was similar to its original male parent or female parent in some primers, while EG not only posed the band of EG original parents but also expanded new specific band. (2) Even four isoforms within one gene locus were found in EG individuals. (3) There were duplicate genes in some loci of EG individuals. The GF was highly heterozygous in a gene, similar to EG, which had a complicated genome origin. Therefore, we could speculate that GF, probably the same as EG, came from constantly hybridized lineages.

Table 5.15 The comparison of mitochondrial DNA homology of GF, RCC, CC, and EG

Fish type	RCC	CC	GF
EG	99.5%	89.4%	99.4%
GF	99.6%	89.3%	–

5.4.3 The Significance of the Formation of the Experimental Twin-Tail Goldfish

The forked tail, which differed from that of crucian carp, was a unique trait of EG. In our study, in terms of appearance, the EG possessed a variety of body colors and a bifurcated tail, with differentiation never existing in other morphological traits, such as dorsal fins and operculum. Compared to common crucian carp, there was another obvious difference in the appearance of GF, which was shortened caudal peduncle. In this study, compared to the average ratio of caudal peduncle length to body length of RCC which was 0.20, the ratio of GF (0.12) was more similar to that of GF (0.11).

There were individuals with a variety of caudal fins, body shapes, and body colors in EG, and other morphological features such as dorsal fins, anal fins, and eye were not differentiated. Therefore, we could speculate that during the evolutionary process of GF, the body color and tail fins might had been differentiated earlier than the other characteristics, such as the head type, fins shape, eye type, the opercula, and the number of scales. Some Japanese scholars surmised that the loss of the dorsal fins marks the beginning of the artificial selection process, followed by a variety of other characteristics, such as the shape of eyes (Komiyama et al. 2009).

In addition, previous studies on the evolutionary relationship among GF, RCC, and CC were only in phenotype, while in physiological and biochemical characteristics, direct and powerful evidences were lacking. The formation of EG provided important biological evidence to study the origin and evolutionary process of GF, which also added new material to study the evolutionary biology.

5.5 The Formation of the Improved Triploid Crucian Carp

Improved triploid fish was obtained by interploidy crossing between improved allotetraploid and diploid fish, in which the biological traits including morphological features, fertility, and growth rate had been improved respectively by gynogenesis, androgenesis, backcrossing, selective breeding, and so on. A typical example was the improved triploid crucian carp (ITCC), also named Xiangyun crucian carp II, which was derived from crossing between the improved 4*n*AT and IRCC, both of which were improved by fish distant hybridization and gynogenesis (Chen et al. 2009). The formation of improved triploid crucian carp was presented in Fig. 5.9.

5.5.1 The Biological Characteristics of the Improved Triploid Crucian Carp

5.5.1.1 The Ploidy Detection of Improved Triploid Crucian Carp

The results of ploidy testing and analysis of improved triploid crucian carp and its parents showed that the chromosome number of $G_1 \times 4n$AT was mainly distributed between 190 and 200 (accounting for 92.0%), indicating that $G_1 \times 4n$AT was

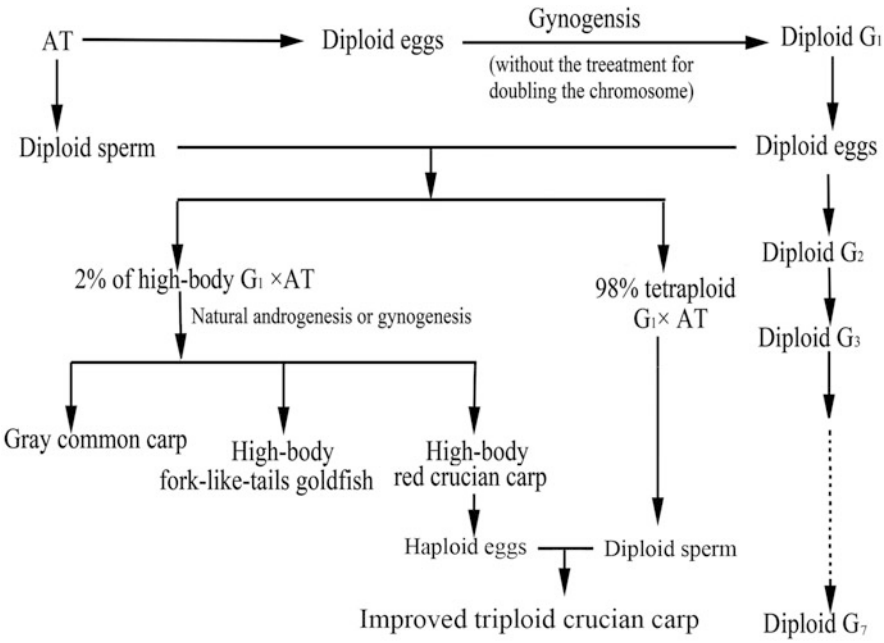

Fig. 5.9 The cultivating way of improved triploid crucian carp

tetraploid ($4n = 200$) (Fig. 5.10a). The chromosome number of IRCC was mainly distributed from 95 to 100 (accounting for 94.0%), which indicated that IRCC was diploid ($2n = 100$) (Fig. 5.10c). The chromosome number of ITCC was mainly distributed from 145 to 150 (94.0%), indicating that it was triploid ($3n = 150$) (Fig. 5.10e).

Based on the analysis of karyotype using Levan's standards, each karyotype formula of $G_1 \times 4n$AT, ITCC, and IRCC was similar, consisting of 11 metacentric (m) chromosomes, 17 submetacentric (sm) chromosomes, 11 acrocentric subtelocentric (st) chromosomes, and 11 telocentric (t) chromosomes. Therefore, the chromosome karyotypes of $G_1 \times 4n$AT, ITCC, and IRCC were 44 m + 68sm + 44st + 44 t (Fig. 5.10b), 22 m + 34sm + 22st + 22 t (Fig. 5.10d), and 33 m + 51sm + 33st + 33 t (Fig. 5.10f), respectively.

The DNA content of red blood cells from ITCC was detected, taking RCC as control group. The result showed that the average DNA content of red blood cells from RCC was 46.73, while that of ITCC was 64.44. The average DNA content of red blood cells from ITCC was 1.38 times than that of RCC, which was close to the expected ratio (1:1.5). It was similar to the chromosome result of $3n = 150$, which proved ITCC to be triploid.

5.5.1.2 The Observation on the Gonadal Structure of ITCC

Two gonadal structure types were observed in 20 samples of 1-year-old ITCC (Chen et al. 2009). The first type was ovary-like gonad, containing many oogonium-like

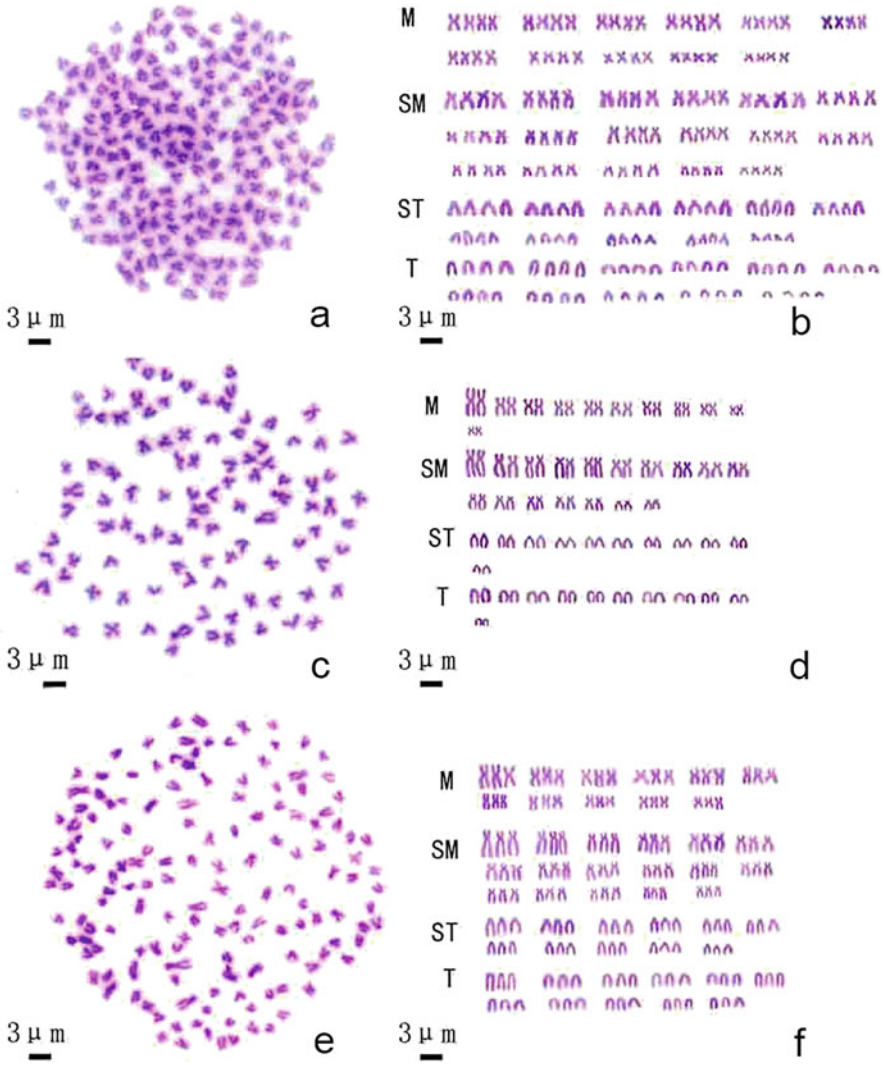

Fig. 5.10 The metaphase chromosome spreads and karyotypes of $G_1 \times 4n$AT, IRCC, and ITCC (Chen et al. 2009). (**a**) The metaphase chromosome spread of $G_1 \times 4n$AT ($4n = 200$). (**b**) The karyotype of $G_1 \times 4n$AT (44 m + 68sm + 44st + 44 t). (**c**) The metaphase chromosome spread of IRCC ($2n = 100$). (**d**) The karyotype of IRCC (22 m + 34sm + 22st + 22 t). (**e**) The metaphase chromosome spread of ITCC ($3n = 150$). (**f**) The karyotype of ITCC (33 m + 51sm + 33st + 33 t). Bar = 3 μm

cells, in which several oocytes in stage II were found. By ultrastructure observation, the chromatin of the oogonium-like cells was pyknotic and distributed aside, with the cell membrane invaginating, which indicated that the oogonium-like cells were disintegrating. Another type was testis-like gonad with abnormal spermatogenesis procedure, in which many spermatogonia with heteromorphous and cavitation

nuclei, or with a few sperm that lacked tails or nuclei, were observed by ultrastructural observation. In addition, ultrastructural observation showed that GTH cells in the ITCC pituitary remained to have many endocrine particles during breeding season, and only very few particles were discharged to form a hollow structure, while the STH cells still generated hormone particles abundantly which were released outside of the cells (Chen et al. 2009). These results showed that the gonadal development of ITCC was refrained, which portended that ITCC was sterile.

5.5.1.3 The Observation on Morphological Features of ITCC

The ITCC possessed the appearance of mouth terminal and arc-body shape with round scale, in which the back was gray, the abdomen was off-white, and the tail fins were gray, with short barbels (Fig. 5.11a, b, c). The ITCC had similar morphological features with its parents, but also enjoyed some obvious differences. The average ratio of body length to total length of ITCC was 0.82, which presented the feature of long body but short tail; the average ratio of body height to body length was 0.45, which showed the obvious feature of high body; the average ratio of head height to body height was 0.53, which indicated the feature of the small head. These comparable traits demonstrated that ITCC was mostly affected by its female parent such as smaller head, shorter caudal peduncle, and higher body. The number of lateral line scales of ITCC was 30–31 and that of scales below and above lateral line was 6–7 and 6, respectively. The number of dorsal fins was III + 17–19, the number of pelvic fins was 9, the number of pectoral fins was 16–17, and the number of anal fins was

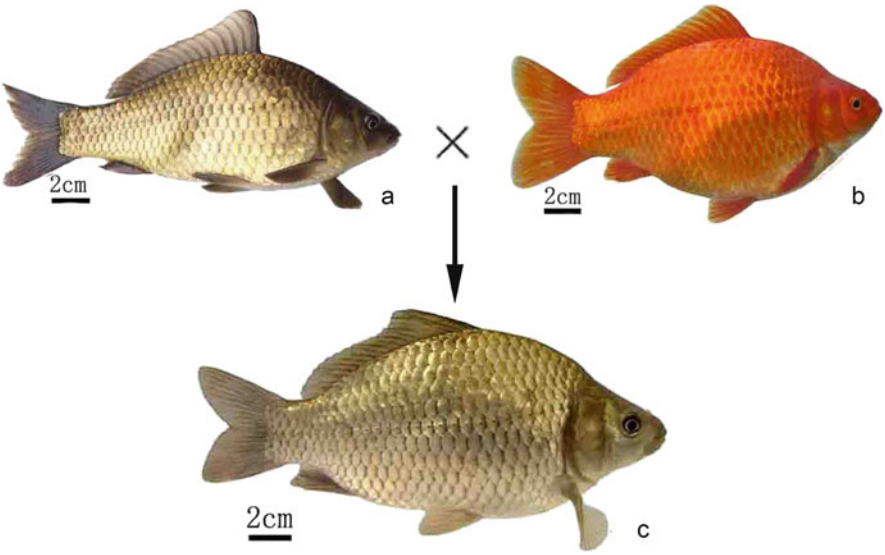

Fig. 5.11 The formation of ITCC (Chen et al. 2009). (**a**) The improved allotetraploid fish ($G_1 \times 4n$AT). (**b**) The improved red crucian carp (IRCC). (**c**) The improved triploid crucian carp (ITCC). Bar = 2 cm

III + 6. It was suggested that all of the countable traits of ITCC generally also fall in between those of its male and female parents, which showed the trait of hybridization. Countable and measurable traits were presented in Tables 5.16 and 5.17.

5.5.1.4 The Growth Rate of ITCC

In 2006, ITCC and Xiangyun crucian carp produced at the end of March of that year were cultured separately, the aquaculture water surface was 0.33 acres, and the stocking density was 3000 per acre. Twenty ITCC and 20 Xiangyun crucian carp were randomly selected to measure the weight and the average value of the weight at the beginning of each month from July to December was calculated. Results showed that the growth rate of ITCC was similar to that of Xiangyun crucian carp (Table 5.18).

The ITCC and common RCC produced at the end of March of that year were cultured in a same pond, the aquaculture water surface was 0.33 acres, and the stocking density was 3000 ITCC and 3000 common RCC per acre. Twenty ITCC and 20 RCC were randomly selected to measure the weight and the average value of the weight at the beginning of each month from July to December was calculated. Results showed that the growth rate of ITCC was 1.43 times than that of common RCC in the same pond (Table 5.19).

The same batch of ITCC and local crucian carp were selected for culture in a same pond, the aquaculture water surface was 0.33 acres, and the stocking density was 3000 ITCC and 3000 local crucian carp per acre. Twenty ITCC and 20 local crucian carp were randomly selected to measure their weight and the average value of the weight at the beginning of each month from July to December was calculated. Results showed that the growth rate of ITCC was 4.21 times than that of local crucian carp in the same pond (Table 5.20).

Breeding experiments from 2006 to 2013 showed that 1-year-old ITCC was able to reach to 500 g (with the heaviest reaching 900 g) and 2-year-old ITCC was able to reach to 1250 g (with the heaviest reaching 3000 g).

5.5.1.5 The Observation on Intermuscular Bones of ITCC

Intermuscular bones (IB) were unique to teleost fish, which were closely relevant to the fish evolution. Depending on the site of attachment from dorsal to ventral, IB were divided into three categories: epineural bone (EN), which attaches to the neural arches; epipleural bone (EP), which attaches to the hemal arches or ribs; and epicentral bone, which attaches to the central vertebrae (Meng et al. 1987). The existence of IB had an important influence on the economic, edible, and nutritive value of fish. Taking wild *C. auratus* (WCC, $2n = 100$) and *C. auratus* var. PengZe (PZCC, $3n = 150$) as a control group, the number, morphology, and distribution of IB in ITCC, IRCC, and $G_1 \times 4n$AT with conventional measurement and dissection method were documented (Li et al. 2013). The number of IB in WCC, PZCC, and $G_1 \times 4n$AT fell within the range from 78 to 83 ($\bar{x} = 81$), 80 to 86 ($\bar{x} = 84$), and 77 to 84 ($\bar{x} = 82$), respectively, while it showed a lower count in ITCC and IRCC, ranging from 77 to 82 ($\bar{x} = 79$) and 58 to 77 ($\bar{x} = 71$), respectively. Given different ploidy fish with different size and number of IB, further statistics of the

Table 5.16 The countable characteristics of IRCC, ITCC, and $G_1 \times 4n$AT (Chen et al. 2009)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins	Barbel
IRCC	28.2 ± 0.79 (27–29)	5.3 ± 0.48 (5–6)	6.6 ± 0.24 (6–7)	III+(16.4 ± 0.24) (III + 16–17)	8.5 ± 0.25 (8–9)	III +(6.7 ± 0.21) (III + 6–7)	None
ITCC	30.9 ± 0.74 (29–31)	5.8 ± 0.78 (5–7)	6.5 ± 0.25 (6–7)	III+(17.9 ± 0.69) (III + 17–19)	8.1 ± 0.69 (7–9)	III +(6.5 ± 0.25) (III + 6–7)	None
$G_1 \times 4n$AT	31.2 ± 0.79 (30–33)	5.4 ± 0.51 (5–6)	6.3 ± 1.01 (5–7)	III+(16.4 ± 0.24) (III + 16–17)	8.6 ± 0.24 (8–9)	III +(6.6 ± 0.24) (III + 6–7)	One pair

Table 5.17 The countable characteristics of IRCC, ITCC, and $G_1 \times 4n$AT (Chen et al. 2009)

Fish type	Body length/total length	Body height/body length	Head length/body length	Head height/head length	Caudal peduncle depth/caudal peduncle length	Head height/ body depth
IRCC	0.82 ± 0.01	0.53 ± 0.02	0.26 ± 0.01	0.87 ± 0.04	1.40 ± 0.03	0.45 ± 0.02
ITCC	0.82 ± 0.01	0.45 ± 0.02	0.27 ± 0.01	0.88 ± 0.02	1.25 ± 0.02	0.53 ± 0.03
$G_1 \times 4n$AT	0.83 ± 0.01	0.36 ± 0.01	0.27 ± 0.01	0.93 ± 0.03	0.80 ± 0.02	0.71 ± 0.03

Table 5.18 The comparison of the growth of ITCC and Xiangyun crucian carp (mean weight, g) (Chen et al. 2009)

Fish type	July	August	September	October	November	December
ITCC	48	142	227	322	387	460
Xiangyun crucian carp	47	146	230	315	390	456

Table 5.19 The comparison of the growth of ITCC and RCC (mean weight, g) (Chen et al. 2009)

Fish type	July	August	September	October	November	December
ITCC	42	130	189	274	332	380
RCC	40	100	150	230	248	265

Table 5.20 The comparison of the growth of ITCC and local crucian carp (mean weight, g) (Chen et al. 2009)

Fish type	July	August	September	October	November	December
ITCC	40	120	206	275	343	400
Local crucian carp	10	45	65	80	92	95

average count of IB in each sarcomere, ranked in order from lowest to highest, was 0.608 (IRCC), 0.633 (ITCC), 0.653 ($G_1 \times 4n$AT), 0.673 (PZCC), and 0.721 (WCC). The number of IB did not differ significantly between ITCC and $G_1 \times 4n$AT, and $G_1 \times 4n$AT and PZCC. But the number of IB in ITCC was significantly different from that in IRCC, WCC, and PZCC, and the same results were obtained between IRCC and other four fish species. The five groups of fish possessed seven forms of IB, including non-forked (I-shape), one-end-unequal-bi-fork, one-end-equal-bi-fork (Y-shape), one-end-multi-fork, two-end-bi-fork, two-end-multi-fork, and tree-branch forms (Fig. 5.12a, b, c). Generally, the more forward the IB, the more complex the form. The number of IB was not exactly equal between the left and right sides of the body, but on the whole, the number of IB on both sides was similar.

The number of IB of ITCC was less than that of WCC and PZCC under the premise of maintaining the basic physiological function of its nutrition, morphology, and activity; therefore, the edible value of ITCC was higher and enjoyed the popularity among consumers. The study of IB in different types of ploidy fish is of great significance to the developmental biology and genetic improvement of fish skeleton, which provides basis for further production of none- or less-IB fish.

5.5.2 The Significance of the Formation of the Improved Triploid Crucian Carp

The distant crossing could break through barriers between species, integrate their beneficial characteristics, expand the genetic variation, and produce new variants or species, which might enhance offspring viability, growth potential, stress resistance,

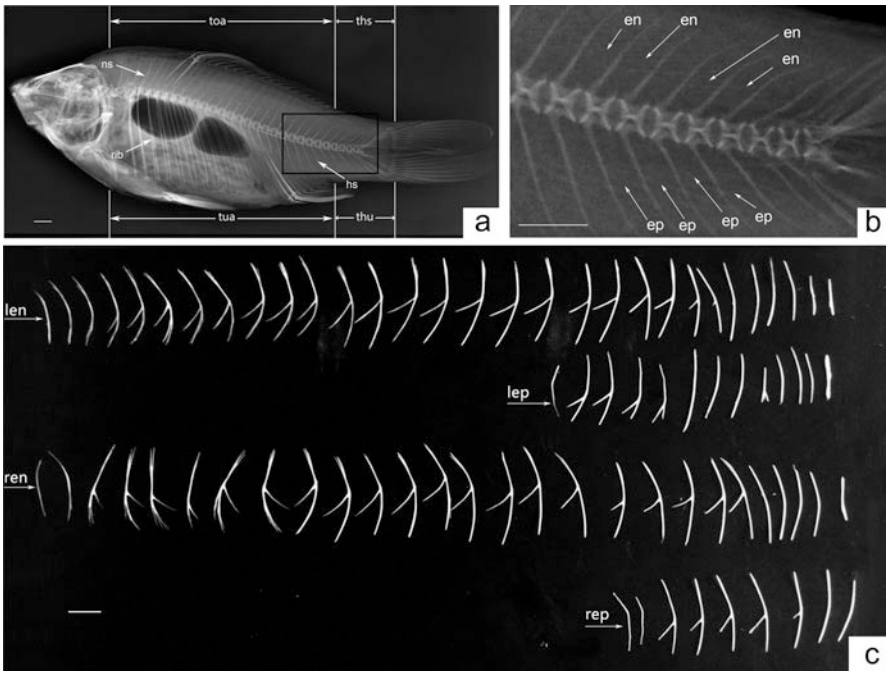

Fig. 5.12 The ITCC X-ray image and IB morphology (Li et al. 2013). a. The skeleton X-ray diagram of ITCC whole-mount. Tua: trunk under axis; toa: trunk on axis; ths: tail hindquarters shaft; thu: tail hindquarters shaft under muscle; ns: neural spine; hs: hemal spine. b. The enlarged view of the panel highlighted in A. ep: epipleural bon; en: epineural bone. c. The IB were arranged from anterior region to tail; ren: right epineural bones; rep: right epipleural bones; len: left epineural bones; lep: left epipleural bones. Bar = 1 cm

yield, and quality. Gynogenesis was an important purification breeding technology in fish, which can make the offspring effectively inherit the excellent traits of its female parent and to stabilize heredity. In addition, compared to its female parent, allogynogenetic effect would change some features of the gynogenetic offspring, such as morphology, growth rate, and even genetic characteristic. The improved 4*n*AT and improved diploid RCC were obtained by a combination of distant hybridization and gynogenesis, which passed some qualified characteristics to their hybrid offspring: ITCC.

Compared to the triploid Xiangyun crucian carp, ITCC not only possessed faster growth vigor, which was similar to that of triploid Xiangyun crucian carp, but also enjoyed some improved features in appearance. The average ratio of body height/body length of Xiangyun crucian carp was 0.41, while that of ITCC was 0.45. The ITCC showed obvious features of the high and thick back with small abdomen, which enhanced greatly the rate of flesh content. In addition, the appearance of ITCC was more similar to that of WCC, including the two sides of the body back being gray and having a light-yellow abdomen which made the whole fish body bright. The

formation of these improved traits was related to the preparation process of its parents and the biological features posed by its parents.

The ITCC was affected greatly by its female parent, for example, these measurable traits including body length/total length, head length/body length, and head height/head length, were deviated to its female parent; for its male parent, this was caused by its male parent tetraploid hybrids having the gene of both RCC and CC, and its female parent provided one set of chromosomes, which made the gene of RCC took a priority in ITCC and posed a greater influence. The other morphological features of ITCC were intermediate in its parents, such as body depth/body length and caudal peduncle depth/caudal peduncle length, but compared to CC, the original male parent of improved tetraploid hybrid fish, the traits were more deviated to RCC, and body shape was more close to RCC, which more likely to satisfy the demand of the market.

Many scholars had researched on the growth rate of triploid fish. Some reports indicated that the growth rate of triploid fry were not faster than that of diploid fish with the culture experiment of artificial induced triploid channel catfish (Wolters et al. 1982). In autotriploid rainbow trout, the growth rate of triploid individuals was slower than that of diploid fish before 2 years old, while that became faster after sexual maturity (Chourrout et al. 1986). Some experiment results showed that the growth rate of allotriploid fish was faster than that of diploid fish (Refstie et al. 1982). Raising fish in a pond with the same environmental and raising condition, it could be observed that the growth rate of triploid *Silurus asotus* was faster than that of diploid *Silurus asotus* from juvenile fish to adult (Yin et al. 1996). Some work had reported that the triploid Xiangyun crucian carp had obvious growth advantages (Liu et al. 2001). The growth rate of sterile triploid fish was fast which may be relevant to the inhibition of gonadal development. Sterile triploid fish cannot propagate because the homologous chromosomes of its germ cells were not able to pair in meiosis. The reason why it had a fast growth rate was that the gonadal development was refrained, and the energy for gonadal development was transformed into growth energy.

Systematic research on the formation of ITCC and its biological features provided the important biological theory to the genetic breeding of polyploid fish. Currently, there were about ten kinds of crucian carp of scale cultivation in China, which mainly came from selected breeding and hybrid breeding. The deficiencies of the former were its purity would become lower and lower with the increasing number of the generations, and the appearance and meat quality of simple hybrids of crucian carp and CC were different from those of WCC. Utilizing the distant hybridization in combination with gynogenesis, improved tetraploid and diploid fish were produced by selected breeding in multi-generations. The ITCC was generated by interploidy hybridization, which could overcome the general problem which existed in selected breeding and common hybrid breeding, and formed a new and improved variety with excellent traits. At the same time, as the parents of ITCC, improved 4*n*AT and IRCC had the features of higher productivity and stronger stress resistance capability. For example, the semen volume of improved 4*n*AT was 1.6–3.0 times as many as that of common tetraploid hybrids; IRCC had the traits of the large amount of eggs and long production period (from early April to end of August); improved 4*n*AT and IRCC

had the traits of lower death rate and stronger stress resistance capability. All these traits laid a foundation for the industrialized production of ITCC.

References

Arai K, Ikeno M, Suzuki R (1995) Production of androgenetic diploid loach Misgurnus anguillicaudatus using spermatozoa of natural tetraploids. Aquaculture 137(1–4):131–138

Beçak ML, Beçak W, Pereira A (2003) Somatic pairing, endomitosis and chromosome aberrations in snakes (Viperidae and Colubridae). An Acad Bras Cienc 75(3):285–300

Bongers A, In't Veld E, Abo-Hashema K, Bremmer I, Eding E, Komen J, Richter C (1994) Androgenesis in common carp (*Cyprinus carpio* L.) using UV irradiation in a synthetic ovarian fluid and heat shocks. Aquaculture 122(2–3):119–132

Chen S, Wang J, Liu S, Qin Q, Xiao J, Duan W, Luo K, Liu J, Liu Y (2009) Biological characteristics of an improved triploid crucian carp. Sci China Ser C Life Sci 52(8):733–738

Cherfas N, Gomelsky B, Emelyanova O, Recoubratsky A (1994) Induced diploid gynogenesis and polyploidy in crucian carp, *Carassius auratus gibelio* (Bloch), × common carp, *Cyprinus carpio* L., hybrids. Aquac Res 25(9):943–954

Chourrout D, Chevassus B, Krieg F, Happe A, Burger G, Renard P (1986) Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females—potential of tetraploid fish. Theor Appl Genet 72(2):193–206

Ding J, Jiang Y (1991) Comparative cytological studies on the oocyte maturation in gynogenetic crucian carp and amphimictic red carp. Acta Hydrobiol Sinica 15(2):97–102

Duan W, Qin Q, Chen S, Liu S, Wang J, Zhang C, Sun Y, Liu Y (2007) The formation of improved tetraploid population of red crucian carp × common carp hybrids by androgenesis. Sci China Ser C Life Sci 50(6):753–761

Galbreath PF, Thorgaard GH (1995) Sexual maturation and fertility of diploid and triploid Atlantic salmon x brown trout hybrids. Aquaculture 137(1–4):299–311

Komiyama T, Kobayashi H, Tateno Y, Inoko H, Gojobori T, Ikeo K (2009) An evolutionary origin and selection process of goldfish. Gene 430(1–2):5–11

Li L, Zhong Z, Zeng M, Liu S, Zhou Y, Xiao J, Wang J, Liu Y (2013) Comparative analysis of intermuscular bones in fish of different ploidies. Sci China Life Sci 56(4):341–350

Liu H, Yi Y, Chen H (1987) The birth of the androgenetic homozygous diploid loach. Acta Hydrobiol Sinica 11(3):241–246

Liu S, Duan W, Tao M, Zhang C, Sun Y, Shen J, Wang J, Luo K, Liu Y (2007) Establishment of the diploid gynogenetic hybrid clonal line of red crucian carp × common carp. Sci China Ser C Life Sci 50(2):186–193

Liu S, Liu Y, Zhou G, Zhang X, Luo C, Feng H, He X, Zhu G, Yang H (2001) The formation of tetraploid stocks of red crucian carp × common carp hybrids as an effect of interspecific hybridization. Aquaculture 192(2):171–186

Liu S, Sun Y, Zhang C, Luo K, Liu Y (2004) Production of gynogenetic progeny from allotetraploid hybrids red crucian carp × common carp. Aquaculture 236(1–4):193–200

Liu Y (ed) (1993) The reproductive physiology of cultured fishes in China. Agric Press, Beijing

Meng Q, Su J, Li W (eds) (1987) Comparative anatomy of fish. Science Press, Beijing

Parsons JE, Thorgaard GH (1985) Production of androgenetic diploid rainbow trout. J Hered 76(3):177–181

Refstie T, Stoss J, Donaldson EM (1982) Production of all female coho salmon (*Oncorhynchus kisutch*) by diploid gynogenesis using irradiated sperm and cold shock. Aquaculture 29(1–2):67–82

Sakaizumi M, Shimizu Y, Matsuzaki T, Hamaguchi S (1993) Unreduced diploid eggs produced by interspecific hybrids between *Oryzias latipes* and *O. curvinotus*. J Exp Zool 266(4):312–318

Scheerer PD, Thorgaard GH, Allendorf FW (1991) Genetic analysis of androgenetic rainbow trout. J Exp Zool 260(3):382–390

Stanley JG (1976) Production of hybrid, androgenetic, and gynogenetic grass carp and carp. Trans Am Fish Soc 105(1):10–16

Sun Y, Zhang C, Liu S, Duan W, Liu Y (2007) Induced interspecific androgenesis using diploid sperm from allotetraploid hybrids of common carp × red crucian carp. Aquaculture 264(1):47–53

Wang J, Liu S, Xiao J, Tao M, Zhang C, Luo K, Liu Y (2014) Evidence for the evolutionary origin of goldfish derived from the distant crossing of red crucian carp × common carp. BMC Genet 15(1):33

Wang J, Qin Q, Chen S, Liu S, Duan W, Liu J, Zhang C, Luo K, Xiao J, Liu Y (2008) Formation and biological characterization of three new types of improved crucian carp. Sci China Ser C Life Sci 51(6):544–551

Wang Y, Liu S, Yi N, Sun Y, Zhang C, Wang J (2005) Observation on early embryonic development of gynogenetic progeny from allotetraploid hybrids. J Nat Sci Hunan Norm Univ 28(3):58–61

Wolters WR, Libey GS, Chrisman CL (1982) Effect of triploidy on growth and gonad development of channel catfish. Trans Am Fish Soc 111(1):102–105

Yamazaki F (1981) Chromosome variations in salmonids. Chromosomal aberration by overriping and irradiation. Kaiyo Kagaku 13(1):71–80

Yan J, Liu S, Sun Y, Zhang C, Luo K, Liu Y (2005) RAPD and microsatellite analysis of diploid gynogens from allotetraploid hybrids of red crucian carp (*Carassius auratus*) × common carp (*Cyprinus carpio*). Aquaculture 243(1–4):49–60

Ye Y, Wu Q, Chen R (1990) Study on γ-ray (^{60}Co) induced androgenesis in fishes. Acta Hydrobiol Sinica 14(1):91–92

Yin H, Pan W, Sun Z (1996) Morphological characters and growth of triploid catfish (*Parasilurus asotus L.*). Chin J Fisher 9(2):23–26

Yu H, Wu J, Li P, Guan H, Xu H, Zhang S (2000) The androgenetic allogynogenetic silver crucian carp and its preliminary application. J Fishery China 24(1):17–22

Zhang C, Sun Y-D, Liu S, Liu Y (2005) Evidence of the unreduced diploid eggs generated from the diploid gynogenetic progeny of allotetraploid hybrids. Acta Genet Sin 32(2):136–144

Zhang Q, Arai K, Yamashita M (1998) Cytogenetic mechanisms for triploid and haploid egg formation in the triploid loach Misgurnus anguillicaudatus. J Exp Zool 281(6):608–619

Zhao Z, Wu Q, Gao G, Huang F (1999) Study on the development of androgenetic haploid of *Paramisgurnus dabryanus*. Zool Res 20(3):230–234

6 The Formation and Biological Characteristics of the Different Ploidy Fishes Derived from the Hybridization of Red Crucian Carp × Blunt Snout Bream

Shaojun Liu, Ming Wen, Xu Huang, Yi Zhou, Qinbo Qin, Kaikun Luo, Fangzhou Hu, Chongqing Wang, Huifang Tan, Yude Wang, and Qingfeng Liu

Abstract

Distant hybridization is a very effective way in genetic breeding, which can effectively alter genetic composition and phenotypic characteristics of the progenies. The formation of allotetraploid hybrid lineage after distant hybridization between red crucian carp and common carp with the same number of chromosomes, and how a large number of triploid hybrids came into being after mating tetraploids with diploids were previously elaborated (References: Chaps. 1–5). The maternal parent of the distant hybridization of red crucian carp (*Carassius auratus* red var.) × blunt snout bream (*Megalobrama amblycephala*) is red crucian carp, and it is mainly omnivorous and has been introduced detachedly in the first section of the second chapter in this book. The paternal parent, blunt snout bream, belongs to Cypriniformes, Cyprinidae, *Culter* subfamily, and *Megalobrama*, which is herbivorous and one of the major cultured freshwater fish. This chapter mainly describes how to use the two parents from different subfamilies with different number of chromosomes to generate different ploidy fishes, such as allotetraploid ($4n = 148$), autotetraploid ($4n = 200$), autotriploid and allotriploid ($3n = 150$), allopentaploid ($5n = 172$; $5n = 198$), allotriploid ($3n = 124$), gynogenetic red crucian carp ($2n = 100$), and other fishes, through distant hybridization as well as the biological characteristics of these different ploidy fishes.

S. Liu (✉) · M. Wen · X. Huang · Y. Zhou · Q. Qin · K. Luo · F. Hu · C. Wang · H. Tan · Y. Wang · Q. Liu
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_6

Keywords

Red crucian carp · Blunt snout bream · Distant hybridization · Polyploidization · Biological characteristics

6.1 The Formation and Major Biological Characteristics of Different Ploidy Hybrids in F_1 Derived from the Hybridization of Red Crucian Carp × Blunt Snout Bream

During the breeding season, both red crucian carp and blunt snout bream in sexual maturity were selected for distant hybridization experiments. Two groups were designed in the hybridization experiments. One group took the females of red crucian carp as the maternal parents, and the males of blunt snout bream as the paternal parents. The maternal and paternal parents were reversed in the second group. The fertilized eggs were developed in the water with appropriate temperature, and 2000 embryos were selected randomly to detect fertilization rate and hatching rate in each cross. In the cross combination of red crucian carp (♀) and blunt snout bream (♂), high fertilization rate (60.0%) and hatching rate (50.0%) were detected. Besides, the different ploidy fishes (2n, 3n, 4n) were detected in their offspring, and their proportions were 33.0%, 15.4%, and 51.6%, respectively. However, in the cross combination of blunt snout bream (♀) and red crucian carp (♂), there was no survival offspring.

6.1.1 The Characteristics of Genetic Construction of Different Ploidy Fishes

6.1.1.1 The Chromosome Number and Karyotype

The chromosome number and karyotype of different ploidy hybrids and their parents were observed and analyzed. Of all examined blunt snout bream, 93.0% observed at mitotic metaphase had 48 chromosomes, and its karyotype was 18 m + 22 sm + 8 st (Table 6.1). A pair of the largest submetacentric chromosomes at chromosomal mitotic metaphase of blunt snout bream was observed, which could be used to identify the chromosome source of the experimental fish. Of all examined red crucian carp, 96.0% observed at mitotic metaphase had 100 chromosomes (Table 6.1), and its karyotype was 22 m + 34 sm + 22 st + 22 t. Of all examined gynogenetic red crucian carp, 94.5% observed at mitotic metaphase had 100 chromosomes (Table 6.1), and its karyotype was 22 m + 34 sm + 22 st + 22 t (Fig. 6.1a). Of all examined triploid hybrids, 77% observed at mitotic metaphase had 124 chromosomes (Table 6.1), and its karyotype was 31 m + 45sm + 26st + 22 t (Fig. 6.1b). In these metaphases, only one large submetacentric chromosome from blunt snout bream was observed. Of all examined tetraploid hybrids, 73% observed at mitotic metaphase had 148 chromosomes (Table 6.1). Its karyotype was

Table 6.1 Examination of chromosome number in blunt snout bream (BSB), red crucian carp (RCC), gynogenetic red crucian carp (GRCC), triploid hybrids (3*n*RB), and tetraploid hybrids (4*n*RB) (Liu et al. 2007)

Fish type	Mitoses number	Distribution of chromosome number									
		<48	48	<100	100	<124	124	<148	148	<172	172
BSB	200	14	186								
RCC	200			8	192						
GRCC	200			11	189						
3*n*RB	200					46	154				
4*n*RB	200							54	146		

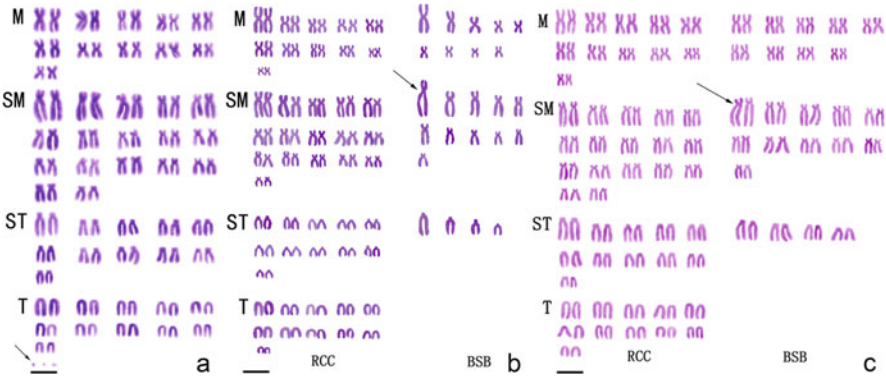

Fig. 6.1 The karyotype of gynogenetic red crucian carp, triploid hybrids, and tetraploid hybrids of red crucian carp (RCC) × blunt snout bream (BSB) (partially quoted from Qin et al. (2014c)). (**a**) The karyotype formula of gynogenetic red crucian carp was 22 m + 34 sm + 22st + 22 t, which consisted of two sets of chromosomes from red crucian carp. (**b**) The karyotype formula of triploid fish was 31 m + 45 sm + 26 st + 22 t, which consisted of two sets of chromosomes from red crucian carps and one set from blunt snout bream. The arrow indicated the largest submetacentric chromosomes from blunt snout bream. (**c**) The karyotype formula of tetraploid fish was 40 m + 56sm + 30 st + 22 t, which consisted of two sets of chromosomes from red crucian carp and two sets from blunt snout bream. A pair of the largest submetacentric chromosomes from blunt snout bream was highlighted with black arrows. Bar = 3 μm

Table 6.2 Mean DNA content of blunt snout bream (BSB), red crucian carp (RCC), gynogenetic red crucian carp (GRCC), triploid hybrids (3*n*RB), and tetraploid hybrids (4*n*RB) (Liu et al. 2007)

Fish type	Mean DNA content	Ratio	
		Observed	Expected
BSB	42.92		
RCC	52.19		
GRCC	52.87	GRCC/RCC = 1.01[a]	1
3*n*RB	72.63	3*n*RB/(RCC + 0.5BSB) = 0.99[a]	1
4*n*RB	97.05	4*n*RB/(RCC + BSB) = 1.02[a]	1

[a]The observed ratio was not significantly different ($P > 0.05$) from the expected ratio

40 m + 56 sm + 30 st + 22 t (Fig. 6.1c), and the two largest submetacentric chromosomes from blunt snout bream were observed (Liu et al. 2007).

6.1.1.2 The Detection of DNA Content

The DNA content of red crucian carp and blunt snout bream was used as the controls, and the DNA content among different ploidy fishes of red crucian carp × blunt snout bream was checked by flow cytometry (Table 6.2). The mean DNA content of gynogenetic red crucian carp was similar to the red crucian carp, indicating that it contained two sets of chromosomes from red crucian carp (Table 6.2). The mean DNA content of triploid fish was equal to the sum of that of red crucian carp and half of blunt snout bream, indicating that it contained two sets

of chromosomes from red crucian carp and one set of chromosomes from blunt snout bream. The mean DNA content of tetraploid hybrids was equal to the sum of that of red crucian carp and blunt snout bream, showing that it had two sets of chromosomes from red crucian carp and two sets of chromosomes from blunt snout bream (Liu et al. 2007).

6.1.1.3 Fluorescence in Situ Hybridization

To analyze the genetic composition of different ploidy fishes of red crucian carp × blunt snout bream, we employed the species-specific centromere probes (263 bp, JQ086761) of fluorescence in situ hybridization (FISH) to identify the chromosomal composition in these hybrids. This centromere probe is hybridized to all 100 chromosomes in red crucian carp ($2n = 100$) (Fig. 6.2a; Table 6.3), but none in blunt snout bream ($2n = 48$) (Fig. 6.2b; Table 6.3). Thus, chromosomes originated from red crucian carp and blunt snout bream could be distinguished by FISH with the centromere probe. For the gynogenetic red crucian carp ($2n = 100$), the probes are hybridized to 100 chromosomes (Fig. 6.2c; Table 6.3), implying that two sets of red crucian carp-derived chromosomes existed. For triploid hybrids ($3n = 124$), the probes are hybridized to 100 chromosomes and the rest of 24 chromosomes had no hybridization signal, indicating that two sets of red crucian carp-derived

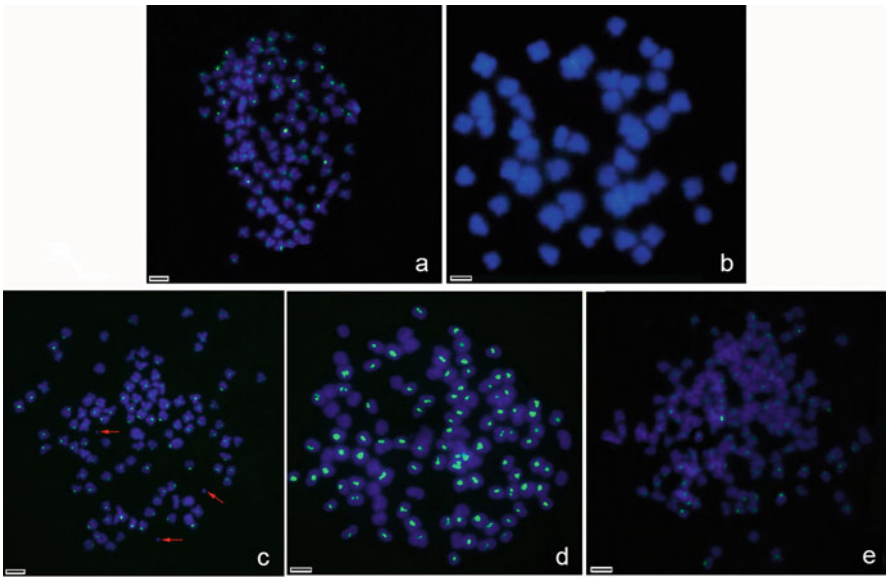

Fig. 6.2 Examination of hybridization signals using FISH in red crucian carp, blunt snout bream, and their hybrid offspring (Qin et al. 2014b; Dai 2014). (**a**) One hundred chromosomes in red crucian carp ($2n = 100$) had hybridization signals. (**b**) Non-hybridization signals in blunt snout bream. (**c**) One hundred chromosomes in gynogenetic red crucian carp ($2n = 100$) had hybridization signals, and red arrows indicated micro-chromosomes. (**d**) One hundred chromosomes in triploid hybrids ($3n = 124$) had hybridization signals. (**e**) One hundred chromosomes in tetraploid hybrids ($4n = 148$) had hybridization signals. Bar = 3 μm

Table 6.3 Examination of hybridization signals using FISH in red crucian carp (RCC), blunt snout bream (BSB), and their ploidy hybrid offspring (gynogenetic red crucian carp = GRCC; triploid fish = 3*n*RB; tetraploid fish = 4*n*RB) (Qin et al. 2014b)

Fish type	Number of fish	Chromosome number of mitosis metaphase	Signal number of mitosis metaphase
RCC	10	200	100
BSB	10	200	0
GRCC	10	200	100
3*n*RB	10	200	100
4*n*RB	10	200	100

chromosomes and one set of blunt snout bream-derived chromosomes were present (Fig. 6.2d; Table 6.3). For tetraploid hybrids ($4n = 148$), the probe was hybridized to 100 chromosomes and the rest of 48 chromosomes had no hybridization signal, indicating that two sets of red crucian carp-derived chromosomes and two sets of blunt snout bream-derived chromosomes were present (Fig. 6.2e; Table 6.3).

Taken together, we could infer that diploid hybrids contained two sets of chromosomes completely derived from red crucian carp, which implied they were natural gynogenetic red crucian carp. The triploid fish contained two sets of red crucian carp-derived chromosomes and one set of blunt snout bream-derived chromosome, which were allotriploid. The tetraploid hybrids contained two sets of red crucian carp-derived chromosomes and two sets of blunt snout bream-derived chromosomes, which were allotetraploid. No viable allodiploid hybrids were found in the cross combination of red crucian carp (♀) and blunt snout bream (♂), but some embryos with 74 chromosomes could be detected before hatching.

Our previous study showed that there were no univalents, trivalents, and tetravalents, but 100 divalents formed after homoeologous pairing, which resulted in generating diploid gamete ($2n = 100$) with 100 chromosomes during meiosis of allotetraploid fish ($4n = 200$) (Zhang et al. 2005; Liu et al. 2010). The diploid gynogenetic hybrid lineages were established by gynogenesis, and this process does not need to double the chromosomes of diploid eggs produced by allotetraploid hybrids with treatment (Liu et al. 2004, 2007). This result demonstrated that during distant hybridization the allotetraploid hybrids (AABB) formed by chromosome doubling and the homologous chromosomes (AA or BB) formed by the chromosome doubling of allotetraploid hybrids have overcome the homologous pairing disorder of allotetraploid hybrids. Some scholars also believed that the offspring of allotetraploid hybrids were formed through chromosome replication, and it restored bivalent pairing system through pairing of two pairs of different sources of homologous chromosomes (Comai 2005).

Therefore, homologous chromosomes were in pairs in allopolyploid hybrids, while nonhomologous chromosomes were hindered to be paired (Sybenga 1996). So it presented diploid pairing mode strictly during meiosis, so that each site was subject to disomic inheritance (Wu et al. 2001; Soltis and Soltis 2000). More importantly, the bivalent pairing system could prevent the occurrence of abnormal

meiosis and effectively improved the ability to produce gametes of tetraploid species (Sybenga 1996).

To overcome the inter-chromosomal/intergenic incompatibilities, three approaches were applied. Firstly, the 148 ({50 + 24} × 2) chromosomes of the tetraploid hybrids were formed by spontaneously doubling the 74 (50 + 24) chromosomes of the diploid hybrids, in which each chromosome from red crucian carp/blunt snout bream had its own homologous chromosome. With this strategy, the inter-chromosomal/intergenic incompatibilities were greatly reduced. Secondly, the triploid fish possessing 124 (50 × 2 + 24) chromosomes were generated, in which each chromosome from red crucian carp had its own homologous chromosome, and the inter-chromosomal/intergenic incompatibilities were released to a certain extent. Thirdly, through retaining the second polar body, red crucian carp with 100 (50 × 2) chromosomes were generated during the hybridization (Liu et al. 2007).

6.1.2 The Appearance of Different Ploidy Fish in F_1

In morphological traits, the different ploidy hybrids showed obvious differences from their parents. In terms of the parent appearance, red crucian carp had a red body color with no barbels, and blunt snout bream had a silver-gray body color with no barbels. Diploid gynogenetic red crucian carp in the F_1 hybrids had a red body color with no barbels. Allotriploid hybrids had a steel-gray body color with no barbels. Allotetraploid hybrids had a steel-gray body color with a pair of barbels (Liu et al. 2007, 2010; Qin 2010). For the feeding habit, like blunt snout bream, all hybrids were herbivorous. Measurable traits and countable traits of different ploidy fishes and their parents were employed to further identify the morphological differences.

6.1.2.1 The Morphological Traits of Diploid Gynogenetic Red Crucian Carp

For the measurable traits, the ratio of body length/overall length and head length/body length in gynogenetic red crucian carp was between those of red crucian carp and blunt snout bream, which were significantly different from those of red crucian carp and blunt snout bream. The ratio of body height/body length and tail height/tail length in gynogenetic red crucian carp was significantly larger than those of red crucian carp and blunt snout bream, whereas the ratio of head height/body height in gynogenetic red crucian carp was significantly smaller than that of red crucian carp and blunt snout bream. Obviously, the ratio of head height/head length of gynogenetic red crucian carp was higher than that of red crucian carp and blunt snout bream (Table 6.4) (Liu et al. 2010).

For the countable traits, apart from the number of dorsal fins of gynogenetic red crucian carp was intermediate to that of red crucian carp and blunt snout bream, the number of lateral scales, number of scales above lateral line, number of scales below lateral line, number of pelvic fins, and number of anal fins in gynogenetic red crucian carp were similar to those of red crucian carp and markedly different from those of blunt snout bream ($P < 0.01$) (Table 6.5) (Liu et al. 2010).

Table 6.4 Comparison of the measurable traits among blunt snout bream (BSB), red crucian carp (RCC), and gynogenetic red crucian carp (GRCC) (Liu et al. 2010)

Appearance	Fish type		
	RCC	BSB	GRCC
Body length/overall length	0.78 ± 0.03	0.84 ± 0.04	0.80 ± 0.03
Body height/body length	0.41 ± 0.03	0.43 ± 0.04	0.52 ± 0.04
Head length/body length	0.29 ± 0.01	0.21 ± 0.02	0.26 ± 0.02
Head height/head length	0.85 ± 0.02	0.88 ± 0.02	0.92 ± 0.03
Tail height/tail length	1.24 ± 0.01	0.93 ± 0.01	1.41 ± 0.01
Head height/body height	0.60 ± 0.01	0.49 ± 0.01	0.46 ± 0.01

Table 6.5 Comparison of countable traits among blunt snout bream (BSB), red crucian carp (RCC), and gynogenetic red crucian carp (GRCC) (Liu et al. 2010)

Appearance	Fish type		
	RCC	BSB	GRCC
Number of lateral scales	29.22 ± 0.65 (28–30)	50.94 ± 0.94 (49–52)	29.56 ± 0.51 (29–30)
Number of scales above lateral line	5.61 ± 0.5 (5–6)	9.67 ± 0.49 (9–10)	6.72 ± 0.46 (6–7)
Number of scales below lateral line	6.28 ± 0.46 (6–7)	10.05 ± 0.64 (9–11)	7.44 ± 0.51 (7–8)
Number of dorsal fins	III + 18.89 ± 0.58 (18–20)	III + 8.67 ± 0.49 (8–9)	III + 16.33 ± 0.49 (16–17)
Number of pelvic fins	8.67 ± 0.49 (8–9)	9.06 ± 0.64 (8–10)	8.67 ± 0.49 (8–9)
Number of anal fins	III + 6.17 ± 0.38 (6–7)	III + 25.89 ± 0.68 (25–27)	III + 6.77 ± 0.43 (6–7)

6.1.2.2 The Morphological Traits of Allotriploid and Allotetraploid Hybrids

Comparing the measurable traits of allotriploid fish to those of blunt snout bream, all ratios were significantly different ($P < 0.01$). However, comparing allotriploid hybrids to red crucian carp, most of these ratios were significantly different except for the ratio of body length/head length. Besides, comparing allotetraploid hybrids to blunt snout bream, all ratios also showed markedly different ($P < 0.01$). And between allotetraploid hybrids and red crucian carp, apart from the ratio of body length/body height and head length/head height, which were not markedly different ($P > 0.01$), ratios of other measurable traits were significantly different. In addition, the result of comparison between allotriploid and allotetraploid fish showed that except for the significant difference of head length/head height, there were no significant differences of other measurable traits (Table 6.6) (Liu et al. 2007).

Comparing the countable traits of allotriploid hybrids to those of blunt snout bream, all of them were markedly different ($P < 0.01$). However, comparing allotriploid hybrids to red crucian carp, except for the number of lateral scales, dorsal fins, and pelvic fins, the other traits were markedly different. Moreover, comparing allotetraploid fish to blunt snout bream, all countable traits were

Table 6.6 Comparison of measurable traits among blunt snout bream (BSB), red crucian carp (RCC), triploid (3*n*RB), and tetraploid hybrids (4*n*RB) (Liu et al. 2007)

Fish type	Overall length/body length	Body length/body height	Body length/head length	Head length/head height	Tail length/tail height	Head height/tail height
3*n*RB	1.31 ± 0.03	1.67 ± 0.03	3.70 ± 0.03	1.11 ± 0.04	0.71 ± 0.04	2.31 ± 0.04
4*n*RB	1.18 ± 0.02	2.18 ± 0.02	3.83 ± 0.03	1.08 ± 0.04	0.75 ± 0.04	1.92 ± 0.02
RCC	1.22 ± 0.02	2.18 ± 0.02	3.72 ± 0.03	1.07 ± 0.03	0.82 ± 0.03	1.84 ± 0.03
BSB	1.19 ± 0.03	2.37 ± 0.03	4.75 ± 0.04	1.14 ± 0.03	1.08 ± 0.04	2.09 ± 0.04

markedly different ($P < 0.01$). And comparing allotetraploid fish to red crucian carp, except for the number of dorsal fins and pelvic fins, the other traits were markedly different. In addition, the result of comparison between allotriploid and allotetraploid hybrids showed that except for the notable difference of the number of lateral scales, scales below lateral line, and barbels, there were no notable differences of other countable traits (Table 6.7) (Liu et al. 2007).

Morphological traits of allotriploid and allotetraploid hybrids were obviously different from their parents. Tables 6.6 and 6.7 indicated that most of the morphological traits of allotriploid and allotetraploid hybrids were significantly different from those of both red crucian carp and blunt snout bream, implying that these variation traits occurred in the polyploid hybrid offspring. Interestingly, barbels are present in allotetraploid hybrids but not in allotriploid hybrids and their parent. In addition, allotriploid hybrids and allotetraploid hybrids differed significantly in morphological traits. For example, allotetraploid hybrids had barbels and 31 to 32 lateral scales, but allotriploid hybrids had only 28 to 30 lateral scales without barbels.

Obvious differences of the measurable and countable traits presented in allotriploid and allotetraploid fish compared with their parents indicated the distant hybridization greatly affects the traits of offspring. Obviously, the barbels were present in allotetraploid hybrids, which were absent in their parents, red crucian carp, and blunt snout bream. The genomic changes of the chromosomes, DNA fragments, or sequences had the potential to lead phenotype changes.

6.1.3 The Reproductive Traits of Different Ploidy Fish in F_1

Diploid gynogenetic red crucian carp, allotriploid, and allotetraploid hybrids differed greatly in reproductive characteristics due to the difference of genetic composition. Gynogenetic red crucian carp was fertile and sexually matured at the age of 1. The allotriploid hybrids were completely sterile, while the allotetraploid hybrids were fertile and sexually matured at the age of 2.

6.1.3.1 The Reproductive Traits of Gynogenetic Red Crucian Carp

The female to male ratio in gynogenetic red crucian carp was approximately 1:1 ($P > 0.05$). By testing gonad of the 6-month-old gynogenetic red crucian carp, similar gonadal development of gynogenetic red crucian carp and the ordinary red crucian carp was observed. At the age of 1, gynogenetic red crucian carp could reach sexual maturity and produced mature gametes. The following description was mainly about testis structure and sperm shape: the mature testis of a 6-month-old male with many lobules containing a lot of spermatozoa and some spermatogonia (Fig. 6.3a, b). The sperm consisted of head and tail. The head had compact nuclear material and was wrapped by plasma membrane. There were some mitochondria at the neck between the head and tail of the sperm. The central axis of the sperm tail had a typical "9 + 2" microtubule structure (Fig. 6.3c, d). Mating female and male of gynogenetic red crucian carp, the fertilization and hatching rates were 91% and 75%,

Table 6.7 Comparison of countable traits among blunt snout bream (BSB), red crucian carp (RCC), triploid hybrids (3*n*RB), and tetraploid hybrids (4*n*RB) (Liu et al. 2007)

Fish type	Number of lateral scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins
3*n*RB	29.45 ± 0.51 (28–30)	6.60 ± 0.50 (6–7)	7.70 ± 0.47 (7–8)	III + 18.10 ± 0.79 (III + 17–19)	8.35 ± 0.49 (8–9)	III + 6.30 ± 0.47 (III + 6–7)
4*n*RB	31.65 ± 0.49 (31–32)	6.55 ± 0.51 (6–7)	6.45 ± 0.51 (6–7)	III + 18.70 ± 0.98 (III + 17–20)	8.60 ± 0.50 (8–9)	III + 6.40 ± 0.68 (III + 5–7)
RCC	29.20 ± 0.70 (28–30)	5.60 ± 0.50 (5–6)	5.70 ± 0.47 (5–6)	III + 18.65 ± 0.49 (III + 18–19)	8.55 ± 0.51 (8–9)	III + 5.65 ± 0.49 (III + 5–6)
BSB	50.90 ± 0.91 (49–52)	9.65 ± 0.49 (9–10)	10.05 ± 0.69 (9–11)	III + 8.65 ± 0.49 (III + 8–9)	9.10 ± 0.55 (8–10)	III + 25.85 ± 0.59 (III + 25–27)

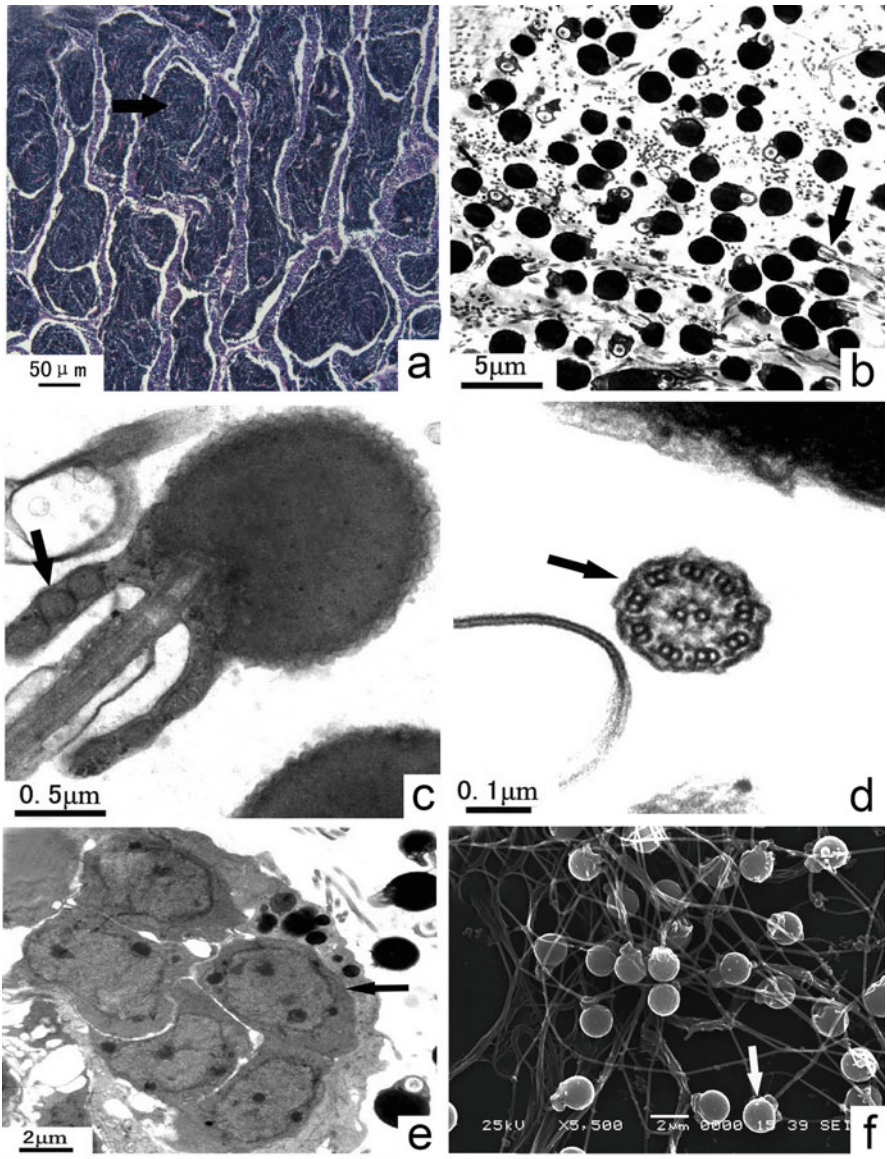

Fig. 6.3 The sperm appearance and structures of the mature testis of gynogenetic red crucian carp (quoted from Liu et al. (2010)). (**a**) Many lobules containing a lot of sperm (arrow) in the normal mature testis of gynogenetic red crucian carp were observed by histology. (**b**) Numerous mature sperm in lobules consisting of the head and tail were observed (arrow) in the mature testis of gynogenetic red crucian carp through ultrathin section. (**c**) The head of the mature sperm of gynogenetic red crucian carp had compact nuclear material and was wrapped by plasma membrane. At the neck between the head and tail of the sperm, there were some mitochondria (arrow). (**d**) A typical "9 + 2" microtubule structure (arrow) was observed in the tail of the sperm of gynogenetic red crucian carp. (**e**) The spermatogonia (arrow) of gynogenetic red crucian carp possessed the nucleus and the cytoplasm. A lot of ribosomes, endoplasmic reticulum, and mitochondria were in the cytoplasm. (**f**) Sperm (arrow) of gynogenetic red crucian carp were observed under the electron scanning microscope

respectively. The ratio of males to females of that mating offspring was close to 1:1 ($P > 0.05$). There were a large number of ribosomes, endoplasmic reticulum, mitochondria, and electron-dense materials in the cytoplasm of spermatogonia (Fig. 6.3e). Electron-dense materials often combined with the mitochondria to form complex. Supported cells adhere to spermatogonia, and some striking particulates in various sizes were within its cytoplasm. Most volume of the sperm cell was occupied by nucleus, but organelles in the cytoplasm were few (Fig. 6.3e). The spermatozoa of gynogenetic red crucian carp showed the normal appearance with the head and the tail as that of red crucian carp using scanning electron microscope. The diameter of the head of the spermatozoa of gynogenetic red crucian carp was 1.9 μm, which was equal to that of red crucian carp (Fig. 6.3f) (Liu et al. 2010).

Previously, we conducted artificial gynogenesis of red crucian carp with the following steps: activate the eggs of red crucian carp to develop, sterile sperm of blunt snout bream was treated with UV, then the ejection of the second polar body was inhibited so as to double the egg's chromosomes using cold shock (0–4 °C) for 30 min. The artificial gynogenetic progenies were all females, and no micro-chromosomes were observed at the metaphase. The dominating differences between the natural gynogenesis generated from hybridization and the artificial gynogenesis are the former does not undergo genetic material inactivation of sperm using UV treatment and chromosome doubling of eggs induced by cold shock treatment. Our research showed the UV-untreated sperm of blunt snout bream could enter into the egg of red crucian carp. Therefore, the genome of the sperm of blunt snout bream was able to recombine with the genome of red crucian carp, resulting in genetic variances. However, due to incompatibilities of inter-chromosomes and/or inter-genes from parents, a majority of the male genome of blunt snout bream were inactivated and eventually degraded, and a little DNA fragment may form the micro-chromosomes to incorporate into the genome of the egg. The egg with a micro-chromosome containing the male-determining gene from the paternal fish would develop into the male gynogenetic fish after spontaneous chromosome doubling. The fertility of the male gynogenetic red crucian carp was supported by observing the testis structure, the sperm's appearance, and the viability of self-crossing offspring.

Intriguingly, one to three micro-chromosomes were found in male diploid gynogenetic individuals and some females, implying that the micro-chromosome(s), derived from the paternal fish, in the female did not possess the male-determining gene. Moreover, one to three micro-chromosomes were found in progenies of gynogenetic red crucian carp, indicating that the micro-chromosomes were inherited from gynogenetic red crucian carp. In gynogenetic red crucian carp groups, the sex ratio of female to male was approximately 1:1. It implied that around 50% of eggs of red crucian carp eventually remained that micro-chromosome containing the male-determining gene. Similarly, in gynogenetic red crucian carp, a micro-chromosome with the male-determining gene was inherited into the next generation to form 50% of males.

6.1.3.2 The Reproductive Traits of Allotriploid Hybrids

Allotriploid fish had three sets of chromosomes, which might cause abnormal gonadal development due to chromosome pairing disorder during meiosis. In the breeding season, we observed the gonadal structure of allotriploid hybrids at the age of 2 and found ovary-type and testis-type gonadal structures: (1) the ovary-type gonad was pale yellow and presented as petal-shaped leaflet after fixation. A large number of underdeveloped and small ovogonium-like cells were observed in the ovary, some of which were vacuolated inside and began to be degraded (Fig. 6.4a). (2) Testis-type gonad was white and no semen was observed when dissected from adult fish with scissors. These testes were mainly composed of seminiferous tubules, some of which had no spermatid, but some had a few. The shape of spermatid was irregular with obscure outer surface, and these spermatids were in degradation and disintegration. We did not observe mature sperm (Fig. 6.4b). The sterile allotriploid hybrids in this chapter were similar to sterile Xiangyun crucian carp, Xiangyun crucian carp II in Chaps. 4 and 5, and other types of sterile allotriploid fishes (triploid blunt snout bream × Bleeker's yellow tail, in Chap. 11). They all could not produce mature gametes (Qin et al. 2014a).

6.1.3.3 The Size of Gametes Produced by Allotetraploid Hybrids

For allotetraploid hybrids, female and male were both fertile. The 2-year-old female and male allotetraploid hybrids, respectively, could produce mature eggs and watery semen. A small number of surviving offspring were produced by fertilizing watery semen with eggs. The spermatozoa of male allotetraploid fish were in different sizes. The average diameter of the large-size spermatozoa, accounting for 38%, was 4.5 μm. The average diameter of the medium-size spermatozoa, accounting for 56%, was 3.8 μm. There were 6% of small-size spermatozoa with mean diameter of 2.4 μm (Fig. 6.5a). In addition, the eggs of female allotetraploid fish have three

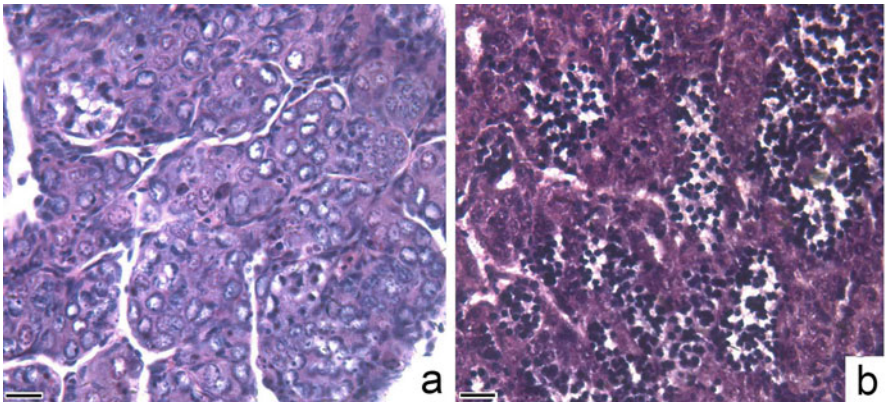

Fig. 6.4 The gonadal structure of allotriploid hybrids (quoted from Qin et al. (2014a)). (**a**) The ovary of allotriploid hybrids. (**b**) The testis of allotriploid hybrids. Bar = 20 μm

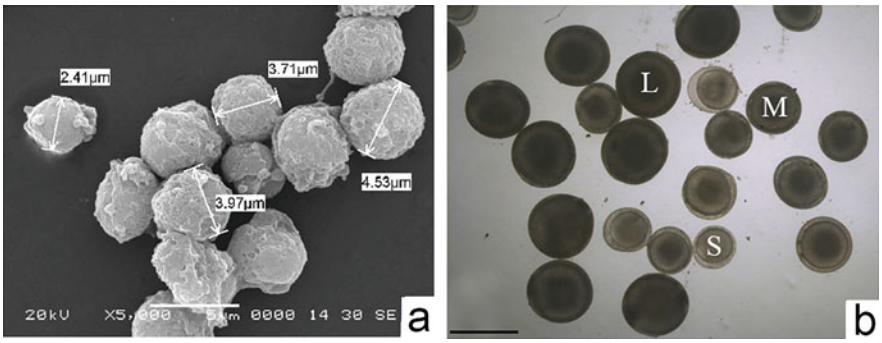

Fig. 6.5 Sperm and eggs of allotetraploid hybrids (Qin et al. 2014b). (**a**) Spermatozoon with different sizes of allotetraploid fish. (**b**) Three sizes of eggs of allotetraploid fish: the large eggs (L), the medium eggs (M), and the small eggs (S). Bar = 2.0 cm

different sizes. There were 89% of larger eggs with mean diameter of 0.20 cm. And there were 7% of medium-size eggs with mean diameter of 0.17 cm. There were 4% of small-size eggs with mean diameter of 0.13 cm (Fig. 6.5b). These results showed that the gametes of allotetraploid hybrids were obviously polymorphic in size (Qin et al. 2014b).

Generally, diploid animals through meiosis could produce the half-reduced gametes. Most of diploid fish, for example, generate haploid gametes. Studies had shown that the average head diameter of sperm produced by red crucian carp, common carps, and other diploid fish was approximately 1.9 μm, and the average diameter of haploid eggs was approximately 1.3 cm. The average head diameter of diploid sperm produced by allotetraploid hybrids was about 2.4 μm, and diploid egg was about 1.9 cm (refer to Chaps. 2 and 3). This result showed that the size of gametes will be changed due to the changes of ploidy level. Different sizes of eggs and sperm of allotetraploid hybrids had different genetic composition. Fertilization of diploid eggs and sperm will produce new tetraploid fishes with 200 chromosomes (Sect. 6.3 of this chapter). Besides, allotetraploid hybrids might produce gametes with higher ploidy levels than diploid gametes (Sect. 6.2 of this chapter).

6.1.4 The Cellular and Molecular Biological Characteristics of Different Ploidy Fish in F_1

To further study the genetic relationship and differences between gynogenetic red crucian carp, allotriploid fish, allotetraploid hybrids, and their parents, we have made a comparative analysis of erythrocyte nuclei, microsatellite sequences, *sox* genes, and *5S rDNA* genes among these fishes.

6.1.4.1 The Erythrocyte Nuclear Volume, Size, and Other Traits

We have detected the erythrocyte nuclear of red crucian carp, blunt snout bream, allotriploid hybrids, and allotetraploid fish. Twenty erythrocytes of each fish were randomly selected to test the minor and major axis of nuclei as well as the mean volume of erythrocyte (Table 6.8). The mean volume of erythrocyte nuclear was regularly increased with ploidy increasing. The average erythrocyte nuclear volume ratio of the allotriploid hybrids to the sum of that of red crucian carp and half of blunt snout bream was nearly 1:1 ($P > 0.05$), implying that allotriploid fish were triploid. The average erythrocyte nuclear volume ratio of allotetraploid hybrids to the sum of red crucian carps and the blunt snout breams was approximately 1:1 ($P > 0.05$), indicating that allotetraploid hybrids were tetraploid fish (Liu et al. 2007).

Figure 6.6a–d has shown erythrocyte nucleus traits of red crucian carp, blunt snout bream, allotriploid fish, and allotetraploid fish. With the same magnification, the nuclei of erythrocytes in allotriploid fish and allotetraploid fish were obviously larger than that of their parents. The erythrocyte nuclear volume of polyploid hybrids regularly increases with chromosome ploidy increasing. In addition, the nuclear appearance between the polyploidy hybrids and their parents is also different. For example, merely mononuclear erythrocyte could be observed in red crucian carp and blunt snout bream. But in allotriploid hybrids and allotetraploid hybrids, abnormal erythrocytes with two nuclei were found, which accounted for 3.4% and 9.4%, respectively. It indicated that the percentage of unusual erythrocytes with two nuclei increased as the ploidy level increased (Liu et al. 2007).

6.1.4.2 The Microsatellite Analysis

Using five pairs of microsatellite primers (MFW1, MFW7, MFW15, MFW17, and MFW21), PCR amplification was conducted with ten females of red crucian carp, ten males of blunt snout bream, and five females and five males of gynogenetic red crucian carp. We found MFW7 could effectively distinguish gynogenetic red crucian carp and its parents (red crucian carp and blunt snout bream) (Fig. 6.7). In the gynogenetic red crucian carp genome, there existed DNA fragments (arrows 4 and 5), which also presented in maternal parent, red crucian carp. It suggested that gynogenetic red crucian carp inherited those DNA fragments from its parent, red crucian carp. On the other hand, DNA fragments in similar size (arrows 1 and 7) are present in both gynogenetic red crucian carp and blunt snout bream, suggesting that gynogenetic red crucian carp inherited these DNA fragments from blunt snout bream. Intriguingly, in gynogenetic red crucian carp, new DNA fragments (arrows 2, 3, and 6) were found, but absent in red crucian carp and blunt snout bream, suggesting the DNA structure variation occurred in gynogenetic red crucian carp. Three males and one female of gynogenetic red crucian carp had unique microsatellite DNA fragment from blunt snout bream, which indicated that these DNA fragments may have no relationship with sex determination (Liu et al. 2010). It was significant to research on genetic material differences between female and male of gynogenetic red crucian carp, which related to sex determination.

Table 6.8 The average erythrocyte nuclear volume ratio of blunt snout bream (BSB), red crucian carp (RCC), allotriploid fish (3*n*RB), and allotetraploid fish (4*n*RB) (Liu et al. 2007)

Fish type	Major aix (μm)	Minor aix (μm)	Volume (μm^3)	Volume ratio	
				Expected	Observed
BSB	5.07 ± 0.47	2.44 ± 0.80	15.76 ± 1.92		
RCC	4.99 ± 0.27	2.95 ± 0.20	22.71 ± 2.42		
3*n*RB	6.96 ± 0.70	3.22 ± 0.33	37.64 ± 6.32	3*n*RB/(RCC + 0.5BSB) = 1.23[a]	1
4*n*RB	7.46 ± 0.56	3.27 ± 0.34	42.01 ± 8.31	4*n*RB/(RCC + BSB) = 1.09[a]	1

[a]Representing no significant difference between actual value and expected value ($P > 0.05$)

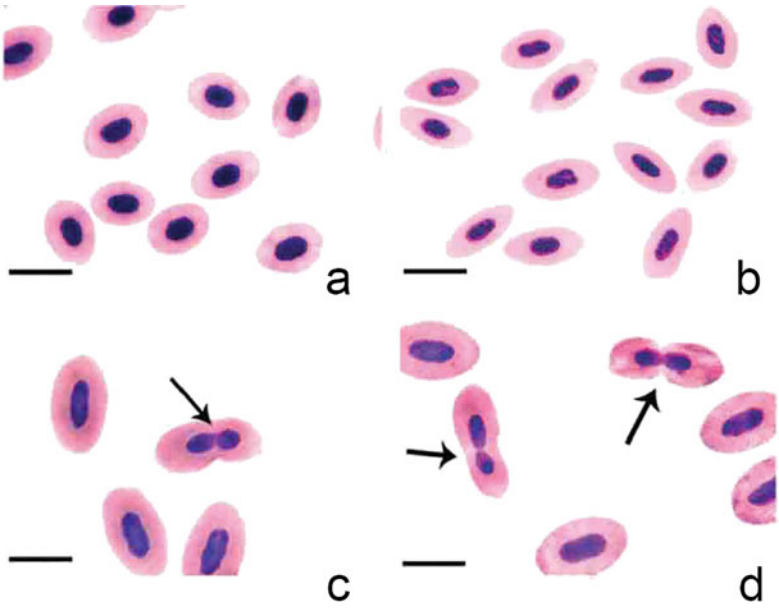

Fig. 6.6 Erythrocytes of red crucian carp, blunt snout bream, allotriploid hybrids, and allotetraploid hybrids (Liu et al. 2007). (**a**) Normal mononuclear erythrocyte in red crucian carp. (**b**) Normal mononuclear erythrocyte in blunt snout bream. (**c**) Normal mononuclear erythrocytes and unusual binuclear erythrocytes (arrows) in allotriploid hybrids. (**d**) Normal mononuclear erythrocytes and unusual binuclear erythrocytes (arrows) in allotetraploid hybrids. Bar = 0.01 mm

6.1.4.3 The Relevant *Sox* Genetic Characteristics

We conducted PCR amplification of HMG-*sox* DNA and examined the sequence of the amplified DNA fragments. In red crucian carp, three DNA fragments with 215 bp, 617 bp, and 1958 bp were amplified. In blunt snout bream, two DNA fragments with sizes 215 bp and 712 bp were amplified. In allotriploid hybrids, three DNA fragments with sizes 215 bp, 616 bp, and 1955 bp were amplified. In allotetraploid hybrids, four DNA fragments with sizes 213 bp, 616 bp, 918 bp, and 1959 bp were amplified. All these amplified sequences were deposited into GenBank, and the accession numbers were shown in Table 6.9. Sequence comparison showed the 215 bp DNA fragment of red crucian carp, blunt snout bream, and allotriploid fish was a part of *sox*11 gene, while the 213 bp DNA fragment of allotetraploid fish was a part of *sox*1 gene. And the 616 bp or 617 bp DNA fragment of red crucian carp, allotetraploid hybrids, and allotriploid fish was a part of *sox*9a gene, while the 712 bp DNA fragment of blunt snout bream belonged to *sox*9a gene. A 918 bp DNA fragment newly derived from allotetraploid hybrids was a part of *sox*9b gene. And the 1955 bp, 1958 bp, or 1959 bp DNA fragments of allotriploid hybrids, allotetraploid hybrids, and red crucian carp belonged to *sox*4 gene (Liu et al. 2007).

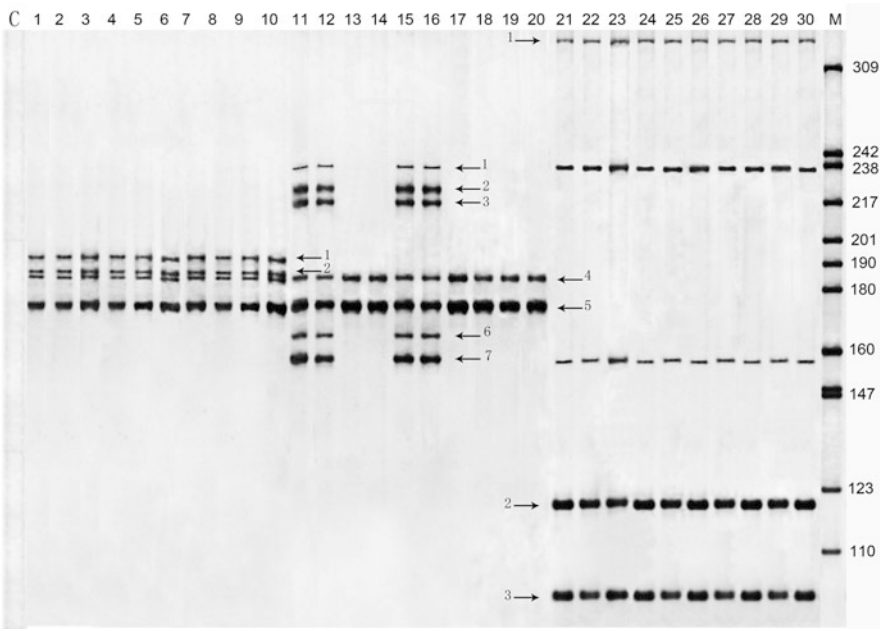

Fig. 6.7 Profile of microsatellite electrophoresis of PCR amplification using primer MFW7 with ten females of red crucian carp, ten males of blunt snout bream, and five female and five male gynogenetic red crucian carp (Liu et al. 2010). Lanes 1 to 10 stand for red crucian carp and arrows 1 and 2 refer to special DNA bands of red crucian carp; lanes 11 to 20 stand for gynogenetic red crucian carp, among which lanes 11 to 15 are males and lanes 16 to 20 are females. Arrows 1 and 7 refer to the common band of gynogenetic red crucian carp and blunt snout bream. Arrows 2, 3, and 6 are unique DNA bands of gynogenetic red crucian carp. Arrows 4 and 5 are the common band of gynogenetic red crucian carp and red crucian carp. Lanes 21 to 30 stand for the blunt snout bream. Arrows 1, 2, and 3 are special DNA bands of blunt snout bream. C means negative control; M stands for *pBR322* DNA/*Msp*I marker

Table 6.9 The GenBank sequence accession number of *sox* gene in red crucian carp (RCC), blunt snout bream (BSB), allotriploid (3*n*RB), and allotetraploid (4*n*RB) hybrids (Liu et al. 2007)

	GenBank sequence accession number			
DNA fragment	RCC	BSB	3*n*RB	4*n*RB
213 bp or 215 bp	EF219273	EF219276	EF370039	EF370036
616 bp, 617 bp, or 712 bp	EF219274	EF219277	EF370038	EF370035
918 bp	–	–	–	EF370034
1955 bp, 1958 bp, or 1959 bp	EF219275	–	EF370037	EF370033

Table 6.10 showed the similarity of *sox* gene sequence amplified from red crucian carp, blunt snout bream, and allotriploid hybrids. For the 215 bp or 213 bp fragment, similarity between red crucian carp and blunt snout bream was 84.0%, blunt snout bream and allotriploid hybrids was 86.0%, red crucian carp and allotriploid hybrids was 96.0%, blunt snout bream and allotetraploid hybrids was 69.0%, and red crucian

Table 6.10 The HMG-box similarity of *sox* gene in red crucian carp (RCC), blunt snout bream (BSB), allotriploid hybrids (3*n*RB), and allotetraploid hybrids (4*n*RB) (Liu et al. 2007)

DNA fragment	RCC vs. BSB (%)	RCC vs. 3*n*RB (%)	BSB vs. 3*n*RB (%)	RCC vs. 4*n*RB (%)	BSB vs. 4*n*RB (%)
213 bp or 215 bp	84	96	86	65	69
616 bp, 617 bp, or 712 bp	72	99	73	99	72
918 bp	–	–	–	–	–
1955 bp, 1958 bp, or1959 bp	–	99	–	100	–

carp and allotetraploid hybrids was 65.0%. It indicated that these DNA fragments in allotriploid hybrids and allotetraploid hybrids had great similarity (Liu et al. 2007).

In terms of 1958 bp DNA fragment of red crucian carp, 1955 bp DNA fragment of allotriploid hybrids, and 1959 bp DNA fragment of allotetraploid hybrids, red crucian carp had 99.0% similarity with allotriploid hybrids and 100% similarity with allotetraploid hybrids. It demonstrated that this kind of DNA fragments in allotetraploid hybrids and allotriploid hybrids was highly similar with red crucian carp. In addition, a specific 918 bp sequence of allotetraploid hybrids was submitted to GenBank, and the accession number was EF370033 (Table 6.9) (Liu et al. 2007).

From the numbers of DNA band, red crucian carp had three different DNA bands and blunt snout bream had only two, which indicated their different DNA characteristics. Among hybrids of red crucian carp (♀) and blunt snout bream (♂), the allotetraploid fish not only inherited all DNA bands from the red crucian carp and blunt snout bream but also owned its unique 918 bp DNA band, which indicated that both ploidy level and DNA sequence had changed in allotetraploid hybrids. This study offered genetic evidence to support that allotetraploid fish was derived from hybridization of red crucian carp and blunt snout bream. In addition, a specific 712 bp DNA band in blunt snout bream was absent in allotriploid hybrids and allotetraploid hybrids, which further illustrated that allotriploid hybrids and allotetraploid hybrids were different from blunt snout bream at molecular level (Liu et al. 2007). Due to the genome of allotriploid and allotetraploid hybrids possessing red crucian carp-derived chromosomes, the DNA fragment amplified from these hybrids had higher similarity with red crucian carp than blunt snout bream.

6.1.4.4 The *5S rDNA* Organization and Genetic Variation Features

In higher eukaryotes, the *5S rDNA* multigene family consists of tandemly repeated units; each unit is a highly conserved coding sequence (5S) with 120 bp. It encodes the *5S rRNA* and a highly variable non-transcribed intergenic spacer (NTS) (Pasolini et al. 2006). A number of researchers believed that even among unrelated species, 5S genes were highly conserved. On the contrary, NTS sequences exhibited species-specific with higher sequence-change rate (Pendas et al. 1995). Therefore, it is widely used in phylogeny studies (Nelson and Honda 1985; Leah et al. 1990; Sajdak et al. 1998). The evidence of the genetic variation and evolutionary

characteristics of the *5S rDNA* multigene family of the polyploid fish would greatly facilitate studying the evolutionary patterns of this multigene family in vertebrates.

The *5S rDNA* Organization

Using the specific primers, *5S rDNA* fragments of blunt snout bream, red crucian carp, allotriploid fish, and allotetraploid fish were amplified and sequenced (Qin et al. 2010). Sequence alignment analysis exhibited all fragments of red crucian carp, blunt snout bream, allotriploid hybrids, and allotetraploid fish were verified to be *5S rDNA* sequences. It includes the 3′ end of the coding region (21 bp), the whole NTS region, and the 5′ end of the coding region (99 bp) (Fig. 6.8a). For red crucian carp, three monomeric *5S rDNA* classes (class I, 203 bp; class II, 340 bp; and class III, 477 bp) were identified (Qin et al. 2010). For blunt snout bream, merely one monomeric *5S rDNA* (class IV, 188 bp) was identified (Fig. 6.8b). Four monomeric *5S rDNA* classes, with three from red crucian carp (class I, class II, and class III) and one from blunt snout bream (class IV), were identified in the allotriploid fish. And three monomeric 5S rDNA classes, with two from red crucian carp (class II and class III) and one novel *5S rDNA* class (class I-L), were identified in the allotetraploid fish. The class I-L and class I (a part of NTS: 83 bp) of maternal parent red crucian carp (class I) exhibited low similarity, but had the same structure. Besides, the 376 bp sequence from blunt snout bream and the allotriploid fish consisted of two classes of IV sequences (Fig. 6.8c). The 406 bp sequence from the allotetraploid fish was comprised of two class I-L sequences (Fig. 6.8d) (Qin et al. 2010). All the *5S rDNA* sequences had been deposited into the NCBI database and the sequence accession number is shown in Table 6.11.

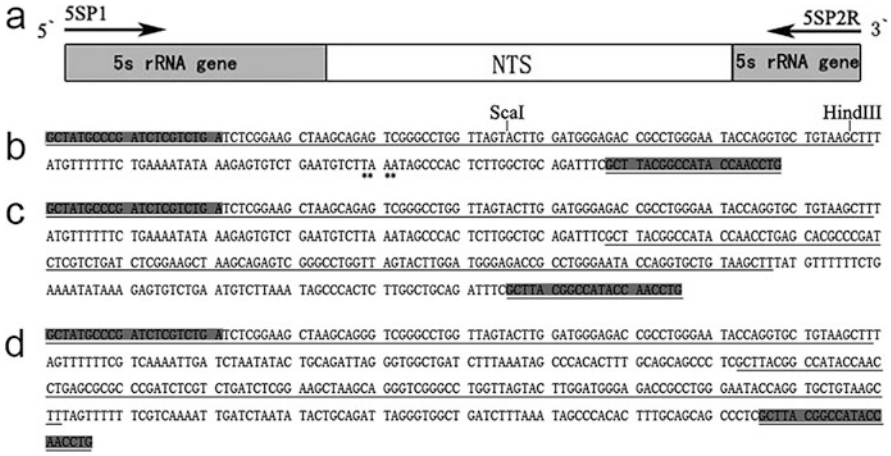

Fig. 6.8 Monomeric and dimeric *5S rDNA* (quoted from Qin et al. (2010)). (**a**) Schematic of higher eukaryotic *5S rRNA* genes. (**b**) Monomeric *5S rDNA* of blunt snout bream. (**c**) Dimeric *5S rDNA* of blunt snout bream. (**d**) Dimeric *5S rDNA* of allotetraploid fish. Primers are highlighted with gray shade and the coding sequences were underlined. The *Hind* III and *Sca* I enzyme-cutting sites were also shown

Table 6.11 Sequence accession numbers of the *5S rDNA* in allotriploid fish (3*n*RB), allotetraploid fish (4*n*RB), red crucian carp (RCC), and blunt snout bream (BSB) (Qin et al. 2010)

	Accession numbers			
DNA fragment	RCC	BSB	3*n*RB	4*n*RB
188 bp	–	GQ485554	GU329956	–
203 bp	GQ485555	–	GU329954	GU329957
339 bp or 340 bp	GQ485556	–	GU329953	GU329958
477 bp or 495 bp	GQ485557	–	GU329955	GU329959

Table 6.12 NTS sequences identified among common carp (CC), allotriploid fish (3*n*RB), allotetraploid fish (4*n*RB), red crucian carp (RCC), and blunt snout bream (BSB) (Qin et al. 2010)

NTS types (bp)	RCC and 3*n*RB (%)	BSB and 3*n*RB (%)	RCC and 4*n*RB (%)	CC and 4*n*RB (%)
68	–	100	–	–
83	97.5	–	73.8	98.7
220, 219	98.6	–	98.1	–
357, 375	93.0	–	92.8	–

The NTS Sequence

Sequence alignment of the NTS sequences of allotriploid fish and allotetraploid fish, and their parents showed several base substitutions and/or insertions–deletions were identified in the hybrid offspring. Comparison of NTS-I sequences of the allotriploid fish and red crucian carp exhibited high similarity with 97.5%, and the similarity of NTS-I-L sequence between allotetraploid fish and red crucian carp was 73.8% (Table 6.12) (Qin et al. 2010). On the contrary, alignment of the NTS-I-L sequence from allotetraploid fish and the NTS sequence from common carp exhibited an average similarity of 98.7%. Alignment of NTS-II sequences among red crucian carp, allotriploid fish, and allotetraploid fish showed that the sequence identity between allotriploid hybrids and red crucian carp was 98.6%, and the sequence identity between allotetraploid and red crucian carp was 98.1%. This result indicated that the sequence homology of allotriploid fish and allotetraploid fish to red crucian carp was high (Table 6.12) (Qin et al. 2010). NTS-III sequences of allotriploid fish and allotetraploid fish originated from the NTS-III of red crucian carp due to an insertion of an 18 bp poly A (Qin et al. 2010). And the similarity between allotriploid fish and red crucian carp was 93.0% and the similarity between allotetraploid hybrids and red crucian carp was 92.8% (Table 6.12). The NTS-IV sequence of the allotriploid fish and blunt snout bream showed a 100% similarity, which revealed that the sequence homology between allotriploid fish and blunt snout bream was high (Table 6.12) (Qin et al. 2010).

The Deletion of Parental-Specific 5S rDNA Fragments

The restriction endonucleases *Hind* III and *Sca* I were applied to digest genomic DNA of red crucian carp, blunt snout bream, allotriploid fish, and allotetraploid fish following with Southern hybridization using a probe of the *5S rDNA* sequence

(Class IV) from blunt snout bream. This probe specifically hybridized with the genomic DNA from the blunt snout bream and the allotriploid fish but did not hybridize with red crucian carp and allotetraploid fish. This hybridization result demonstrated that paternal-specific *5S rDNA* was absent in allotetraploid fish, but present in allotriploid fish (Table 6.12) (Qin et al. 2010).

6.2 The Formation of Backcross and Gynogenesis Offspring of Allotetraploid Red Crucian Carp (♀) × Blunt Snout Bream (♂)

It has been mentioned in the previous section that female and male allotetraploid fish were fertile, and the gamete size presented significant polymorphism. To determine the ploidy level and genetic composition characteristics of these different sized gametes, we used allotetraploid hybrids to perform backcross and gynogenesis. The chromosome numbers, karyotype, and DNA content of offspring were detected and analyzed to deduce the genetic composition of different ploidy of gametes.

6.2.1 The Formation and Genetic Composition of Backcross Offspring

We selected female allotetraploid hybrids and, respectively, backcrossed with male blunt snout bream and red crucian carp, which both produced numerous hybrid offspring. We also made an analysis to the genetic composition of these hybrids about their chromosome numbers and DNA content.

6.2.1.1 The Chromosome Numbers and Karyotype

The backcross of female allotetraploid hybrids and male blunt snout bream produced pentaploid hybrid with 172 chromosomes. At the chromosome mitosis metaphase, the three largest submetacentric chromosomes were detected, which are similar to those found in blunt snout bream. It indicated pentaploid fish had three sets of chromosomes from blunt snout bream and two sets from red crucian carp (Fig. 6.9a). The karyotype formula of pentaploid hybrids was 49 m + 67 sm + 34 st + 22 t (Fig. 6.9b), indicating that they possessed 49 metacentric chromosomes (22 from red crucian carp and 27 from blunt snout bream), 67 submetacentric chromosomes (34 from red crucian carp and 33 from blunt snout bream), 34 subtelocentric chromosomes (22 from red crucian carp and 12 from blunt snout bream), and 22 telocentric chromosomes from red crucian carp (Liu et al. 2007).

Two different ploidy fishes were detected in the backcross progenies of allotetraploid hybrids (♀) × red crucian carp (♂). One was the pentaploid hybrid with 198 chromosomes. It possessed the largest two submetacentric chromosomes from blunt snout bream, indicating pentaploid hybrids had three sets of red crucian carp-derived chromosomes and two sets of blunt snout bream-derived chromosomes (Fig. 6.10a). The other was the triploid hybrid ($3n = 150$). And the largest

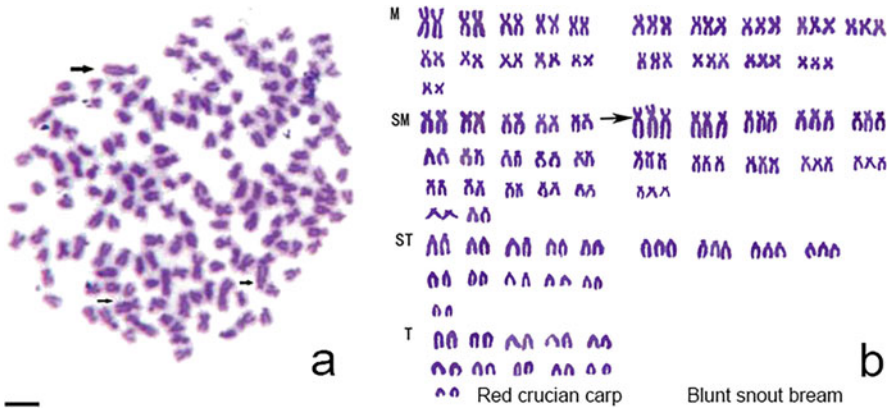

Fig. 6.9 Chromosome mitosis metaphase and karyotype of pentaploid hybrids obtained from backcrossing female allotetraploid hybrids with male blunt snout bream (quoted from Liu et al.(2007)). (**a**) The 172 chromosomes of pentaploid hybrid, in which the largest submetacentric chromosomes were marked by arrows. (**b**) The pentaploid hybrid chromosomes consisted of two sets of red crucian carp-derived chromosomes and three sets of blunt snout bream-derived chromosomes, and its karyotype was 49 m + 67sm + 34 st + 22 t. And the largest submetacentric chromosomes were marked by arrows. Bar = 3 μm

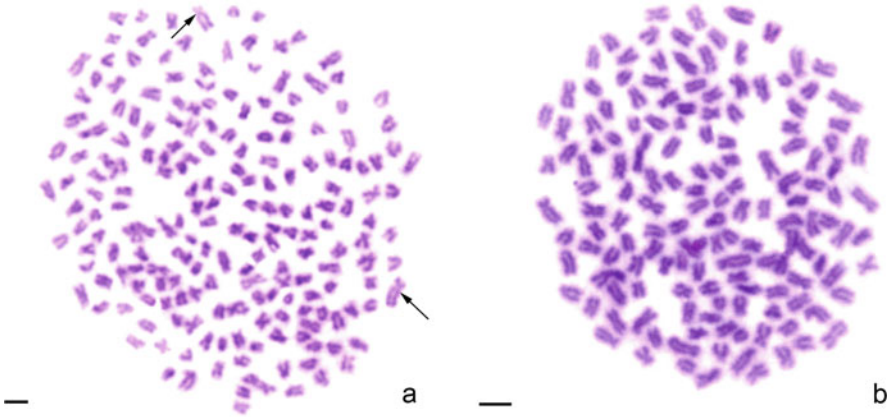

Fig. 6.10 Chromosome spreads of backcross progenies of allotetraploid hybrids (♀) × red crucian carp (♂) (quoted from Qin et al. (2014b)). (**a**) The largest two submetacentric chromosomes (arrows) from blunt snout bream were identified in pentaploid hybrids with 198 chromosomes. (**b**) The largest submetacentric chromosome from blunt snout bream wasn't identified in triploid hybrids with 150 chromosomes. Bar = 3 μm

submetacentric chromosomes from blunt snout bream weren't found, indicating triploid hybrids had only three sets of red crucian carp-derived chromosomes (Fig. 6.10b) (Qin et al. 2014b).

6.2.1.2 The Detection of DNA Content

The DNA contents of red crucian carp and blunt snout bream were used as the controls. Table 6.13 showed the distribution of DNA content of all the samples. The mean DNA content of triploid fish was equal to one and half times of that of red crucian carp, indicating that it contained three sets of red crucian carp-derived chromosomes. The mean DNA content of pentaploid fish was equal to that of the sum of one and half times red crucian carp and blunt snout bream, indicating that pentaploid fish contained three sets of red crucian carp-derived chromosomes and two sets of blunt snout bream-derived chromosomes.

6.2.1.3 Fluorescence in Situ Hybridization

Chromosome fluorescence in situ hybridization was conducted using *5S rDNA* (340 bp, GQ485556) of red crucian carp as a probe. Hybridization signals using the 340 bp probe were detected in the red crucian carp chromosome mitosis metaphase (Fig. 6.11a, b and Table 6.14), but no hybridization signal in blunt snout bream (Fig. 6.11c and Table 6.14). Through chromosome map of *5S rDNA* analysis, the 340 bp probe had two pairs of loci in the red crucian carp genome. One pair of homologous submetacentric chromosomes was stronger, whereas the other was weaker (Fig. 6.11c). This indicated a strong and a weak signal represented one set of red crucian carp-derived chromosomes. The chromosome mitosis metaphase of pentaploid hybrids had three strong signals and three weak signals (Fig. 6.11d and Table 6.14), indicating its genome had three sets of red crucian carp-derived chromosomes. The chromosome mitosis metaphase of triploid hybrids had three strong signals and three weak signals (Fig. 6.11e and Table 6.14), indicating its genome also possessed three sets of red crucian carp-derived chromosomes.

6.2.2 The Formation and Genetic Composition of Gynogenetic Offspring

To further understand the genetic composition of gamete of allotetraploid hybrids, the gynogenetic offspring of allotetraploid hybrids were established, and chromosome analysis and FISH were used to examine their genetic composition.

Table 6.13 The mean DNA content of red crucian carp (RCC), blunt snout bream (BSB), triploid fish (3*n*RBR), and pentaploid fish (5*n*RBR)

Fish type	Mean DNA content	Ratio	
		Observed	Expected
BSB	78.65		
RCC	106.97		
3*n*RBR	150.26	3*n*RBR/1.5RCC = 0.93[a]	1
5*n*RBR	227.6	5*n*RBR/(1.5RCC + BSB) = 0.95[a]	1

[a]Representing that there was no significant difference between the observed and expected ratio ($P > 0.05$)

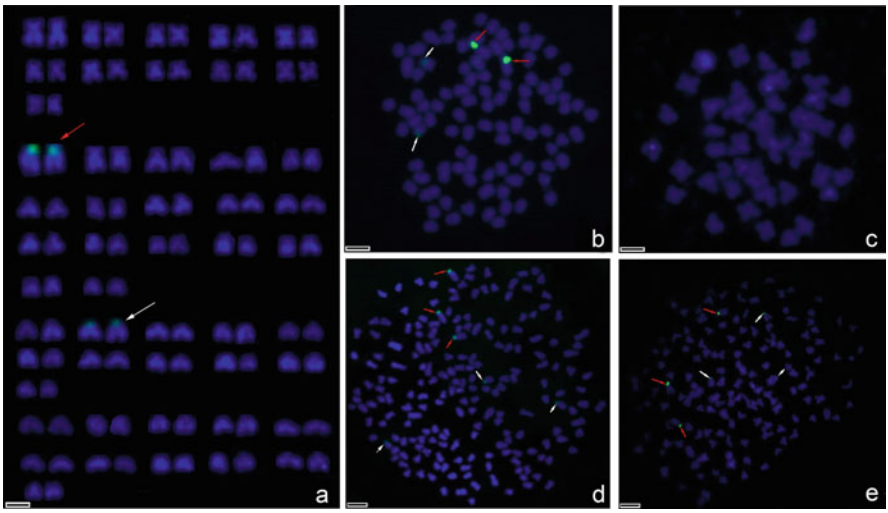

Fig. 6.11 Inspection of hybridizing signals by FISH in red crucian carp, blunt snout bream, triploid fish, and pentaploid fish (partially quoted from Qin et al. (2015) and Dai (2014)). (**a**) The FISH hybridization in red crucian carp chromosome mitosis metaphase (karyotype) using *5S rDNA* sequence as a probe. A pair of strong signals was observed in one pair of homologous submetacentric chromosomes (red arrows). A pair of weak signals was observed in one pair of homologous subtelocentric chromosomes (white arrows). (**b**) The red crucian carp chromosome meiosis had two strong and two weak signals. (**c**) No fluorescence signals were found in blunt snout bream. (**d**) The chromosome mitosis metaphase of pentaploid hybrids had three strong and three weak fluorescence signals. (**e**) The chromosome mitosis metaphase of triploid hybrids had three strong and three weak fluorescence signals. The red arrows represented strong signals, while the white ones represented weak signals. Bar = 3 μm

Table 6.14 Inspection of hybridizing signals by FISH in blunt snout bream (BSB), red crucian carp (RCC), triploid fish (3*n*RBR), and pentaploid fish (5*n*RBR)

Fish type	Fish number	Number of metaphase	Hybridization signals	
			Strong signals	Weak signals
RCC	10	200	2	2
BSB	10	200	0	0
3*n*RBR	10	200	3	3
5*n*RBR	10	200	3	3

6.2.2.1 The Preparation of Producing Gynogenetic Offspring of Allotetraploid Hybrids

Gynogenetic offspring of allotetraploid fish were produced by artificial gynogenesis. In gynogenesis process, eggs were activated using UV-treated sterilized sperm from blunt snout bream, without chromosomes doubling treatment. The statistics of hatching rate and survival rate of the progenies were shown in Table 6.15. Hybridization of the blunt snout bream sperm without UV irradiation and eggs of

Table 6.15 The relationship of hatching rate and survival rate with different UV irradiation time

Egg source	Sperm source	UV irradiation time (min)	Hatching rate (%)	Survival rate (%)
Tetraploid hybrids in F_1	Blunt snout bream	28	28.56 ± 1.23	20.56 ± 1.24
		40	11.65 ± 2.67	9.65 ± 2.65
		45	5.29 ± 1.25	4.12 ± 1.48

Hatching rate = (the hatched fry number/fertilized eggs number) × 100%; survival rate = (normal fish number at the start of feeding/total hatched fry number) × 100%

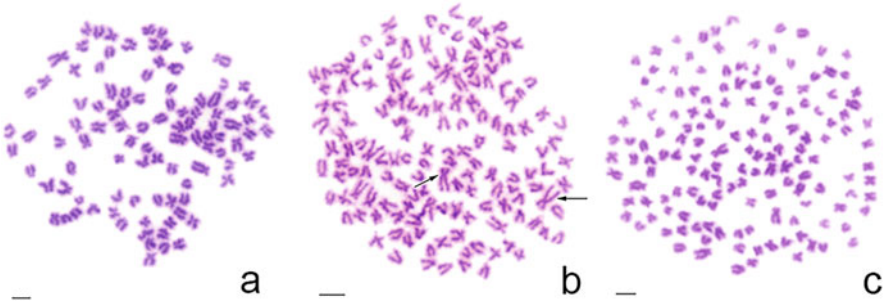

Fig. 6.12 The chromosome metaphase spreads of gynogenetic offspring (G_1–1, G_1–2, and G_1–3) (quoted from Qin et al. (2014b)). (**a**) The chromosome metaphase spreads of G_1–1 with 100 chromosomes showed that the largest submetacentric chromosome from blunt snout bream was not identified. (**b**) The chromosome metaphase spreads of G_1–2 with 148 chromosomes with the largest 2 submetacentric chromosomes (arrows) from blunt snout bream identified. (**c**) The chromosome metaphase spreads with 150 chromosomes showed that the largest submetacentric chromosomes from blunt snout bream were not identified. Bar = 3 μm

allotetraploid hybrids was used as a control group, and the hatching rate and survival rate were 65.5% and 53%, respectively.

6.2.2.2 The Morphological Traits

There were two types of gynogenetic progenies, which accounted for 8% and 92%, respectively. One was with red body color (G_1–1), and the other was steel gray. The one in red with no barbels was similar to red crucian carp in phenotype. But the one in steel gray had two different phenotypes of fish. Some with a pair of short barbels (G_1–2) were similar to allotetraploid hybrids in phenotype, while others had no barbels (G_1–3). In the control cross (allotetraploid hybrids (♀) × blunt snout bream (♂)), the body color of the hybrid was silvery white with no barbels and the growth rate was significantly slower than gynogenetic progenies. Gynogenetic offspring and hybrid offspring (allotetraploid hybrids (♀) × blunt snout bream (♂)) were easy to distinguish according to body color and barbel features.

6.2.2.3 The Chromosome Number

The chromosome number of gynogenetic offspring (G_1–1) with a red body color was 100 (Fig. 6.12a). The largest submetacentric chromosome from blunt snout bream

was not found in the metaphase spreads. It implied that this gynogenetic fish was diploid containing two sets of red crucian carp-derived chromosomes. In the gynogenetic offspring (2n = 148) with barbels (G_1–2), the largest two submetacentric chromosomes from blunt snout bream were found, which implied that it was allotetraploid (Fig. 6.12b) with two sets of red crucian carp-derived chromosomes and two sets of blunt snout bream-derived chromosomes. The gynogenetic offspring (G_1–3) whose body color was steel gray with no barbels had 150 chromosomes, and the largest submetacentric chromosome from blunt snout bream was not found in the metaphase spreads, indicating that it was triploid hybrids (Fig. 6.12c) with three sets of red crucian carp-derived chromosomes (Qin et al. 2014b).

6.2.2.4 Fluorescence in Situ Hybridization

In gynogenetic offspring of allotetraploid hybrids, the *5S rDNA* (340 bp, GQ485556) of red crucian carp was utilized as a probe to conduct chromosome fluorescence in situ hybridization. The signals of 340 bp probe in red crucian carp and blunt snout bream chromosome metaphase spreads were shown in Fig. 6.11. In the chromosome metaphase spreads of diploid gynogenetic offspring (G_1–1), two strong and two weak signals were observed. It indicated that its genome had two sets of red crucian carp-derived chromosomes. In the chromosome metaphase spreads of tetraploid gynogenetic offspring (G_1–2), two strong and two weak signals were observed, which indicated that its genome had two sets of red crucian carp-derived chromosomes. There were three strong and three weak signals in the metaphase spreads of gynogenetic triploid offspring (G_1–3), indicating that its genome possessed three sets of red crucian carp-derived chromosomes.

The specific repeats (263 bp, JQ086761) of red crucian carp were used as a probe to further conduct chromosome FISH in gynogenetic offspring from allotetraploid hybrids. In the chromosome mitosis metaphase of gynogenetic diploid offspring (G_1–1), 100 chromosomes were marked on the fluorescence signal, indicating that its genome possessed two sets of red crucian carp-derived chromosomes (Fig. 6.13a). In

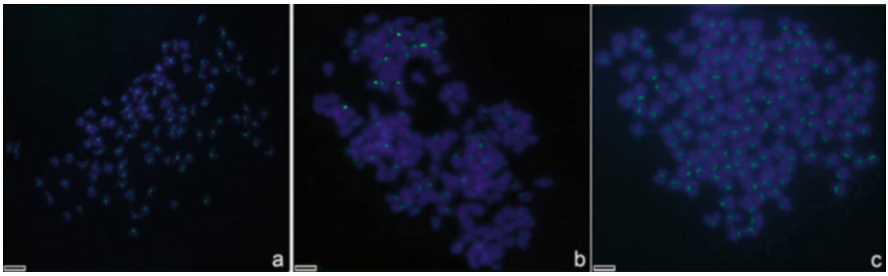

Fig. 6.13 Inspection of hybridizing signals using FISH with centromere probe in gynogenetic offspring of allotetraploid hybrids (G_1–1, G_1–2, and G_1–3) (quoted from Qin et al. (2014b)). (**a**) One hundred hybridizing signals were identified in gynogenetic diploid offspring (G_1–1). (**b**) One hundred hybridizing signals were identified in gynogenetic tetraploid offspring (G_1–2). (**c**) One hundred and fifty hybridizing signals were identified in gynogenetic triploid offspring (G_1–3). Bar = 3 μm

the chromosome mitosis metaphase of gynogenetic tetraploid offspring (G_1–2), 100 chromosomes were labeled hybridization signals, while 48 chromosomes were not marked with the hybridization signal (Fig. 6.13b), indicating that its genome possessed two sets of red crucian carp-derived chromosomes and two sets of blunt snout bream-derived chromosomes. In the chromosome mitosis metaphase of gynogenetic triploid offspring (G_1–3), 150 chromosomes were labeled with fluorescence signal, indicating that all of its chromosomes from the red crucian carp (Fig. 6.13c) (Qin et al. 2014b).

6.2.2.5 Fertility

During the breeding season, we detected the fertility of gynogenetic progenies at the age of 1. The gynogenetic diploid fishes (G_1–1) and triploid fishes (G_1–3) could produce mature eggs but not semen, which indicated no male individuals in gynogenetic diploid and tetraploid fish. Gynogenetic tetraploids (G_1–2) could not produce eggs and sperm at the age of 1. The fertile triploids (G_1–3) might be autotriploid fish.

During meiosis, abnormal chromosome behavior in allopolyploids due to the mixture of genomes from two different species was reported, which resulted in reproducing aneuploid gametes (Riera-Lizarazu et al. 2000; Li et al. 1998; Kasha and Kao 1970). For example, among progenies generated from crossing between *cruciferous brassica* and *violaceus*, the paternal and maternal genomes were completely or partially segregated during meiosis. When parental genome segregation occurred during the meiosis of hybrids, the chromosomes of *brassica* and *violaceus* might be separated into two groups and formed two types of gametes which included chromosome of *brassica* or *violaceus* (Li and Heneen 1999). Using the UV-treated sterilized sperm of blunt snout bream, allotetraploid fish eggs were activated to generate gynogenetic offspring without being subjected to chromosome doubling treatment. And then we produced the backcross progenies of allotetraploid hybrids (♀) × red crucian carp (♂). The genetic composition of the gynogenetic offspring and backcross progenies was evaluated by analyzing chromosome numbers and loci to study chromosome behavior of allotetraploid fish in the process of meiosis. This provided direct proof that allotetraploid hybrids could generate different gametes, including diploid gametes ($2n = 100$; AA) with 2 sets of red crucian carp-derived chromosomes, triploid gametes ($3n = 150$; AAA) with 2 sets of red crucian carp-derived chromosomes, and tetraploid gametes ($4n = 148$; AABB) with 2 sets of red crucian carp-derived chromosomes and 2 sets of blunt snout bream-derived chromosomes. With the knowledge of the genetic composition of these gametes, we could infer that allotetraploid hybrids ($4n = 148$) presented abnormal chromosomal behavior by ignoring the rule of homologous pairing during the meiosis. In allotetraploid hybrids, a number of germ cells could produce unreduced tetraploid gametes ($4n = 148$, AABB) through chromosome doubling by premeiotic endoreduplication, endomitosis, or fusion. After chromosome doubling, parents' chromosomes might be separated during meiosis in germ cells, resulting in the formation of gametes with two sets of red crucian carp-derived chromosomes ($2n = 100$, AA) and/or three sets of red crucian carp-derived genome ($3n = 150$, AAA). The parent red crucian carp chromosome might be doubled based on

synchronous genome doubling mechanism (the number of chromosomes increases in a manner of $2^n \times 100$, n is the number of replication) and non-synchronized parental genome doubling mechanism (the number of chromosome increases in a manner of $n \times 100$, n is the number of single parent genome replication) (Chap. 2). In that way, germ cells with different ploidies ($2n = 100$, 200, 300, 400, or higher ploidy levels) could be formed. After doubling, germ cells with 200 and 300 chromosomes could generate gametes with 100 and 150 chromosomes, respectively, through normal process of meiosis. Theoretically, gametes containing one or more sets of blunt snout bream-derived chromosomes could be generated due to complete parental genome separation during meiosis. However, it was not observed in our study. In terms of genetic stability, parental genome separation in hybrids could effectively reduce the incompatibility of genetic material and improve the survival rate and fertility of hybrids, which provided a good condition for improving fertility of progenies of allotetraploid hybrids.

6.3 The Establishment and Biological Characteristic of Autotetraploid Lineage Derived from the Hybridization of Red Crucian Carp × Blunt Snout Bream

The genetic material of allotetraploid hybrids in F_1 is derived from two species, and abnormal chromosome behavior led to the formation of gametes with special genomes. By observing the size of gametes of allotetraploid hybrids in F_1 and analyzing the genetic composition of the backcross progenies and gynogenetic offspring, we have offered a preliminary deduction about the genetic composition of parts of the allotetraploid hybrid gametes. And we found that autotetraploid fish with higher fertility and variant phenotype were formed through self-mating offspring of allotetraploid hybrids. Throughout years of genetic breeding, we have established an autotetraploid fish lineage (F_2–F_{15}) with genetic stability. This chapter will expound the formation mechanism, genetic composition, reproduction characteristics, and genetic diversity systematically.

6.3.1 The Genetic Construction and Variation Analysis

The chromosome of allotetraploid hybrids and their parents was described in the first section of Chap. 6. The autotetraploid fish (F_2–F_{15}) have 200 chromosomes (Fig. 6.14a) in which the largest submetacentric chromosome from blunt snout bream was not found, indicating chromosomes of autotetraploid fish consisted of four sets of red crucian carp-derived chromosomes (Fig. 6.14b). And its karyotype was 44 m + 68 sm + 44 st + 44 t. In addition, using the sum of red crucian carp and blunt snout bream DNA content as control, we found the ratio of the mean DNA content of autotetraploid and red crucian carp was close to 2:1, which also indicated it consisted of four sets of red crucian carp-derived chromosomes (Qin et al. 2014c).

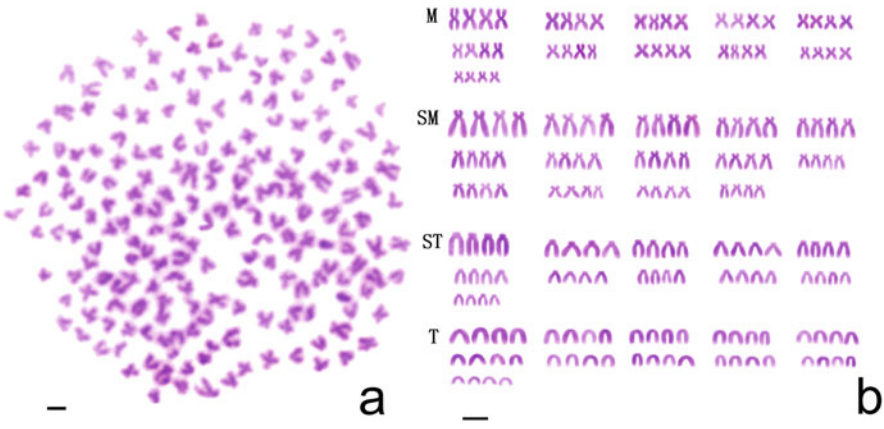

Fig. 6.14 Chromosome spreads at metaphase and karyotype of autotetraploid fish (partially quoted from Qin et al. (2014c)). (**a**) Autotetraploid fish possessed 200 chromosomes and the largest submetacentric chromosomes from blunt snout bream were not found. (**b**) The karyotype of autotetraploid fish was 44 m + 68sm + 44 st + 44 t. Bar = 3 μm

Two different structural units (340 bp and 477 bp) of *5S rDNA* of red crucian carp were used as molecular probes to conduct chromosome fluorescence in situ hybridization (FISH). One pair of strong signals and one pair of weak signals using the 340 bp probe in red crucian carp were identified (Fig. 6.15a), but no hybridization signal in blunt snout bream (Fig. 6.15b). And two strong signals and two weak signals were identified in the chromosome mitosis metaphase of allotetraploid hybrids (F_1), which showed the allotetraploid hybrids contained two sets of red crucian carp-derived chromosomes (Fig. 6.15c). For autotetraploid fish (F_2), two strong signals and six weak signals were identified in the chromosome mitosis metaphase. Though two strong signals became weak, the signals were still twice that of red crucian carp. This result also showed autotetraploid fish contained four sets of red crucian carp-derived chromosomes (Fig. 6.15d).

At mitosis metaphase of red crucian carp, eight hybridization signals were observed using the probe with 477 bp (Fig. 6.16a), and no signal was found in blunt snout bream (Fig. 6.16b). Eight signals were found in the chromosome mitosis metaphase of allotetraploid hybrids in F_1 (Fig. 6.16c), indicating that it contained two sets of red crucian carp-derived chromosomes. Sixteen signals were found in the chromosome mitosis metaphase of autotetraploid hybrids (F_2) (Fig. 6.16d), which was twice the number of red crucian carp. It indicated that autotetraploid fish (F_2) contained four sets of red crucian carp-derived chromosomes.

In addition, the red crucian carp-specific repetitive sequence (263 bp, JQ086761) was applied to conduct fluorescence in situ hybridization on the chromosome mitosis metaphase of red crucian carp, blunt snout bream, allotetraploid fish, and autotetraploid fish. According to the description of the first section, the probe could mark all

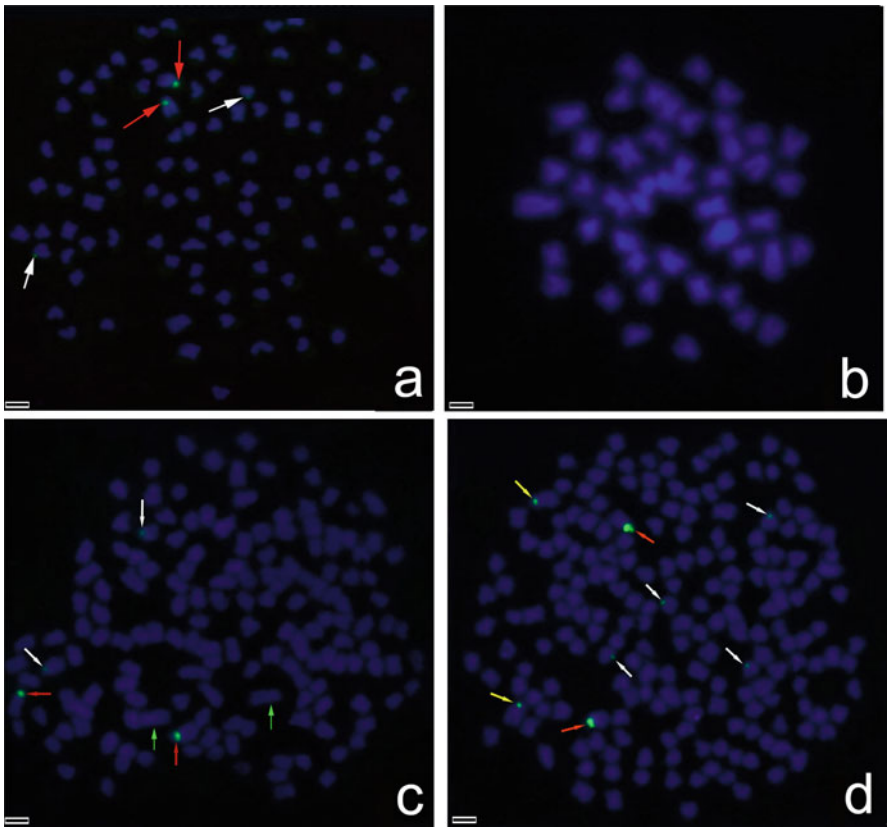

Fig. 6.15 Inspection of hybridizing signals using FISH (340 bp) in red crucian carp, blunt snout bream, allotetraploid fish (F_1), and autotetraploid fish (F_2) (partially quoted from Dai (2014)). (**a**) In red crucian carp, two strong signals (red arrows) and two weak signals (white arrows) were observed in the chromosome mitosis metaphase. (**b**) In blunt snout bream, no signal was found in the chromosome mitosis metaphase. (**c**) In allotetraploid hybrids, two strong signals (red arrows) and two weak signals (white arrows) were observed in the chromosome mitosis metaphase. (**d**) In autotetraploid fish, two strong signals (red arrows) and six weak signals (white arrows and yellow arrows) were observed in the chromosome mitosis metaphase. Bar = 3 μm

the chromosomes of red crucian carp, but didn't work in blunt snout bream, indicating this probe was effective to distinguish origination of chromosomes between blunt snout bream and red crucian carp. In the chromosome mitosis metaphase of allotetraploid hybrids, 100 chromosomes derived from red crucian carp were marked with florescent signals and 48 chromosomes derived from blunt snout bream were not marked with signals. Theoretically all chromosomes of the autotetraploid progenies can be marked with fluorescence signals, but only 100 chromosomes in the chromosome mitosis metaphase were marked and the another 100 chromosomes had no fluorescence signals, which implied that repetitive sequence could be changed or deleted in the 100 chromosomes.

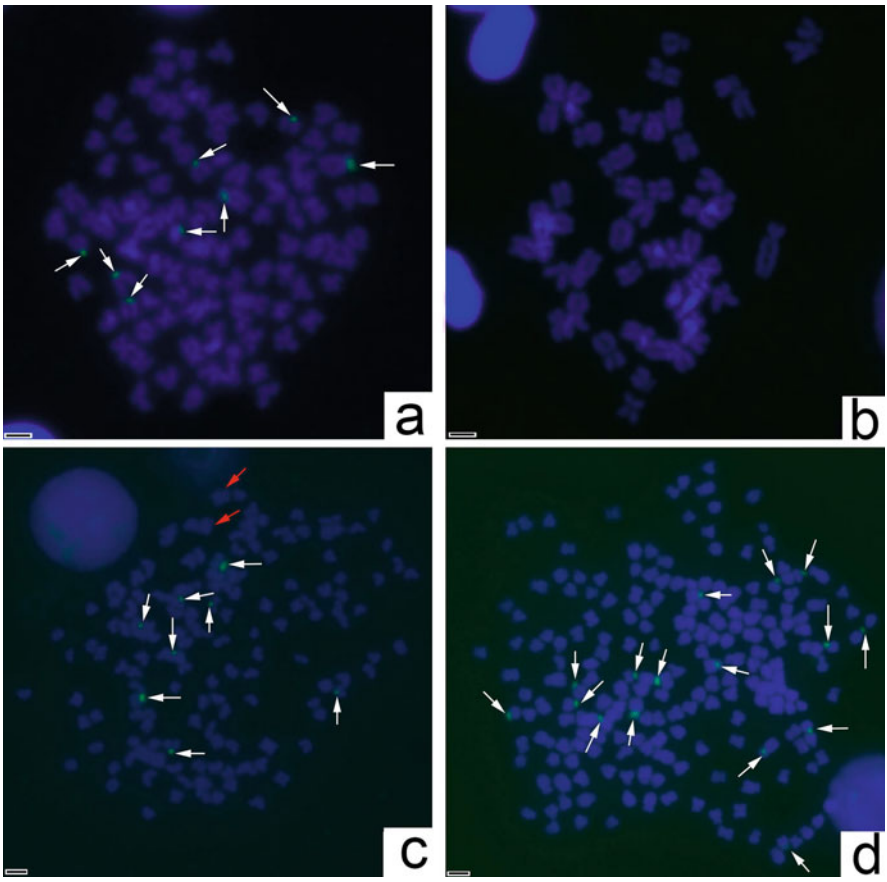

Fig. 6.16 Inspection of hybridizing signals by FISH (477 bp) in red crucian carp, blunt snout bream, allotetraploid fish (F_1), and autotetraploid fish (F_2) (partially quoted from Qin et al. (2014c)). (**a**) Eight signals were observed in the chromosome mitosis metaphase of red crucian carp (white arrows). (**b**) No signals were observed in the chromosome mitosis metaphase of blunt snout bream. (**c**) Eight signals were found in the chromosome mitosis metaphase of allotetraploid hybrids (white arrows). And the red arrows head to the largest submetacentric chromosome from blunt snout bream. (**d**) Sixteen signals were found in the chromosome meiosis metaphase of autotetraploid fish (white arrows). Bar = 3 μm

6.3.2 The Morphological Characteristics

Significantly different of ratios of measurable traits were observed between allotetraploid hybrids and blunt snout bream ($P < 0.01$). Except for body length/body height and head length/head height ($P > 0.01$), allotetraploid hybrids differ greatly from red crucian carp in other measurable traits. All the measurable traits of autotetraploid were significantly different to blunt snout bream, while there was no distinct difference with red crucian carp except body length/body height and head

width/tail width ($p < 0.01$). Significant differences between allotetraploid fish and autotetraploid fish were not observed except overall length/body length, body length/body height, body length/head length, and tail length/tail width ($p < 0.01$) (Table 6.16) (Qin et al. 2014c).

Significantly different ($P < 0.01$) ratios of countable traits between allotetraploid hybrids and blunt snout bream were observed. Apart from the numbers of dorsal fins and pelvic fins, other countable traits of allotetraploid hybrids differ greatly from red crucian carp ($P < 0.01$). All the countable traits of autotetraploid hybrids were significantly different to blunt snout bream ($P < 0.01$), while there was no distinct difference with red crucian carp except the numbers of scales below lateral line. There was no significant difference between allotetraploid and autotetraploid fish ($4nF_2$–F_{15}) except the number of lateral scales, scales above lateral line, dorsal fins, and anal fins ($P < 0.01$) (Table 6.17) (Liu et al. 2007).

Morphological traits of allotetraploid hybrids and autotetraploid hybrids were obviously different from red crucian carp and blunt snout bream (Fig. 6.17a–d). Interestingly, the difference was the presence of barbels in allotetraploid hybrids but not in red crucian carp and blunt snout bream, and the barbels transmitted to autotetraploid fish in a stable way. Data in Tables 6.16 and 6.17 supported that some morphological traits of allotetraploid fish and autotetraploid fish were different from red crucian carp and blunt snout bream. And parts of traits also were different between allotetraploid hybrids and autotetraploid fish (Qin et al. 2014c).

6.3.3 The Fertility and Sperm Size

The first section of this chapter had introduced that the female and male of allotetraploid hybrids sexually matured at the age of 2 and produced mature eggs and watery semen (sperm concentration was lower). Only a small number of offspring were produced by mating of female and male allotetraploid hybrids.

The ovaries of the 10-month-old allotetraploid hybrids contained a small number of stage II oocytes (Qin et al. 2014b), and most of the germ cells were in the form of oogonia (Fig. 6.18a). This suggested that the development of ovary lagged. The testes of the 10-month-old allotetraploid hybrids contained numerous spermatogonia and a few sperm, and their development also delayed (Fig. 6.18b). The 10-month-old autotetraploid fish had normal gonadal structure in which the lobules contained a large amount of mature sperm (Fig. 6.18c) and the ovaries contained many stage IV oocytes (Fig. 6.18d), indicating females and males of autotetraploid fish only needed 1 year to reach sexual maturity. In the breeding season, female and male of autotetraploid fish could produce numerous mature eggs and white semen, which fertilized each other and obtained numerous offspring ($4nF_3$) (Qin et al. 2014c).

The head diameter of haploid sperm of red crucian carp was 1.91 ± 0.11 μm (Fig. 6.19a, c). The head diameter of sperm of autotetraploid fish and its progenies was 2.41 ± 0.12 μm (Fig. 6.19b, d). The head diameter of sperm of red crucian carp was smaller than that of autotetraploid fish, which indicated that the sperm of autotetraploid fish were diploid (Qin et al. 2014c).

Table 6.16 Comparison of the measurable traits among red crucian carp (RCC), blunt snout bream (BSB), allotetraploid (4*n*RB), and autotetraploid fish ($4nF_2$) (Qin et al. 2014c)

Fish type	Overall length/body length	Body length/body height	Body length/head length	Head length/head height	Tail length/tail height	Head width/tail height
RCC	1.22 ± 0.02	2.18 ± 0.02	3.72 ± 0.03	1.07 ± 0.03	0.82 ± 0.03	1.84 ± 0.03
BSB	1.19 ± 0.03	2.37 ± 0.03	4.75 ± 0.04	1.14 ± 0.03	1.08 ± 0.04	2.09 ± 0.04
4*n*RB	1.18 ± 0.02	2.18 ± 0.02	3.83 ± 0.03	1.08 ± 0.04	0.75 ± 0.04	1.92 ± 0.02
$4nF_2$	1.23 ± 0.02	2.23 ± 0.08	3.73 ± 0.02	1.08 ± 0.02	0.84 ± 0.02	1.88 ± 0.06

Table 6.17 Comparison of countable traits among red crucian carp (RCC), blunt snout bream (BSB), allotetraploid (4*n*RB), and autotetraploid (4*n*F_2–F_8) fish (Liu et al. 2007)

Fish type	Number of lateral scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins
RCC	29.20 ± 0.70 (28–30)	5.60 ± 0.50 (5–6)	5.70 ± 0.47 (5–6)	III + 18.65 ± 0.49 (III + 18–19)	8.55 ± 0.51 (8–9)	III + 5.65 ± 0.49 (III + 5–6)
BSB	50.90 ± 0.91 (49–52)	9.65 ± 0.49 (9–10)	10.05 ± 0.69 (9–11)	III + 8.65 ± 0.49 (III + 8–9)	9.10 ± 0.55 (8–10)	III + 25.85 ± 0.59 (III + 25–27)
4*n*RB	31.65 ± 0.49 (31–32)	6.55 ± 0.51 (6–7)	6.45 ± 0.51 (6–7)	III + 18.70 ± 0.98 (III + 17–20)	8.60 ± 0.50 (8–9)	III + 6.40 ± 0.68 (III + 5–7)
4*n*F_2–F_8	29.54 ± 1.03 (29–32)	5.36 ± 0.50 (5–6)	6.81 ± 0.75 (5–7)	III + 18.27 ± 0.46 (III + 18–19)	8.63 ± 0.50 (8–9)	III + 5.45 ± 0.52 (III + 5–6)

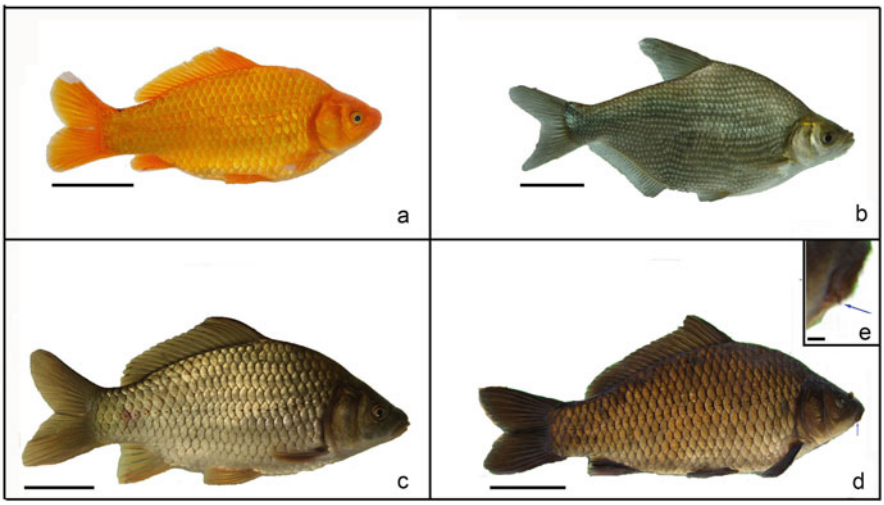

Fig. 6.17 The appearance of red crucian carp, blunt snout bream, allotetraploid hybrids, and autotetraploid fish (Qin et al. 2014c). (**a**) Red crucian carp. (**b**) Blunt snout bream. (**c**) Allotetraploid hybrids. (**d**) The appearance of autotetraploid fish and barbels indicated by an arrow. (**e**) The amplification of D ($4nF_2$–F_{15}) and barbels indicated by an arrow. Bar = 8 cm

6.3.4 The Establishment and Application of Autotetraploid Fish

Up to now, autotetraploid fish has propagated to the 15th generations (F_{15}). The genetic composition, reproductive characteristics, and morphological traits of each generation of autotetraploid fishes showed that we have established an autotetraploid lineage with stable genetic traits ($4nF_2$–$4nF_{15}$). This tetraploid fish could reproduce under natural conditions. In addition, the average fertilization rate of artificial reproduction of autotetraploid fish was approximately 75% and the hatching rate was approximately 60%. Autotetraploid fish could stably produce over 3000,000 progenies annually, which laid a solid foundation for the commercial application of autotetraploid lineage.

Using autotetraploid fish lineage generated by distant hybridization of female red crucian carp × male blunt snout bream, we produced autotriploid fish (Fig. 6.20a) and allotriploid fish (Fig. 6.20b) by mating that autotetraploid fish with gynogenetic red crucian carp and common carp respectively. The sterility of allotriploid hybrids facilitates avoidance of contamination of natural wild fish resources through mating with other wild fish. In addition, autotriploid fish could be applied as a model to study productive patterns of distant hybrid polyploids, and fertile female autotriploid fish also offered an important resource for fish breeding.

Allotetraploid hybrids in F_1 could produce gametes with polymorphic genetic composition including gametes with one, two, or three sets of red crucian carp-derived chromosome. As a result, the autotetraploid fish containing four sets of red crucian carp-derived chromosomes were generated by self-mating of allotetraploid

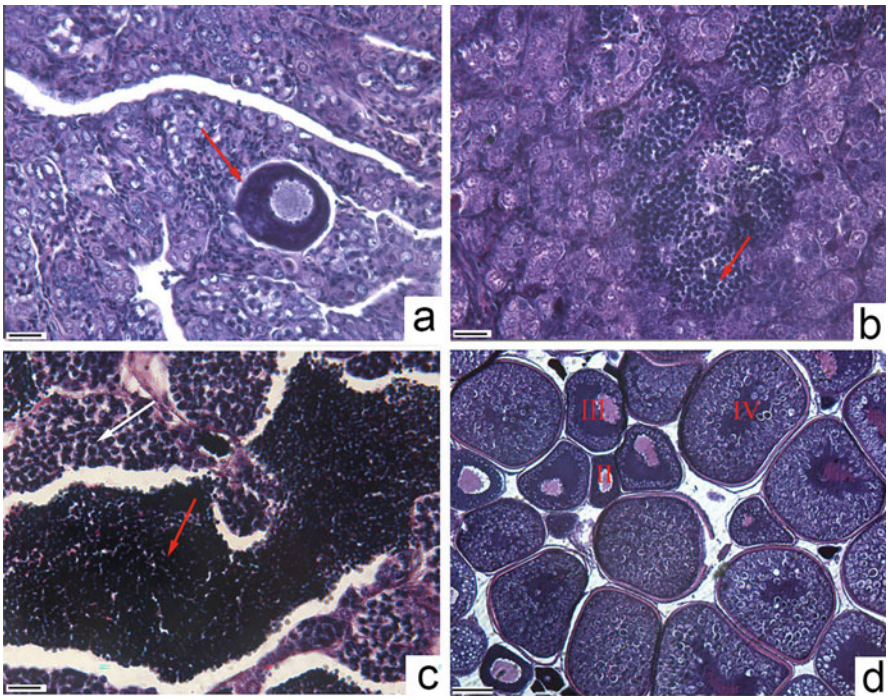

Fig. 6.18 The gonadal structure of allotetraploid hybrids and autotetraploid fish (quoted from Qin et al. (2014c)). (**a**) The ovary of allotetraploid hybrids with a few phase II oocytes (arrows). (**b**) The testis of allotetraploid hybrid with a few sperm (arrows). (**c**) The mature testis of autotetraploid fish with spermatids (white arrows) and mature spermatozoa (red arrows). (**d**) The mature ovary of autotetraploid fish in which there are full of phase IV oocytes and also contain oocytes at phase II and phase III. Bar = 20 μm

hybrids. From the genetic stability, separation of parent genome in progenies could effectively reduce incompatibility of genetic material and improved the survival rate and fertility of hybrid fish. And the allotetraploid hybrids sexually matured at the age of 2 and the fertility of male individuals was far inferior to that of the red crucian carp and blunt snout bream. But the autotetraploids sexually matured at the age of 1 and both female and male individuals had similar reproductive ability with red crucian carp. Due to exclusion of chromosomes of blunt snout bream, the genetic material incompatibility of autotetraploid fish was significantly reduced.

In autotetraploid or allotetraploid fish, the emergence of univalent, trivalent, and quadrivalent during meiosis might prevent forming normal diploid gametes, while bivalent pairing was considered to be the best way to maintain genetic stability of tetraploid species (Bilal, 2002). The head diameter of diploid sperm of autotetraploid fish was 2.40 μm (Fig. 6.19d), which was significantly greater than that of the red crucian carp (Fig. 6.19c). We obtained triploid fish with 150 chromosomes through hybridization between autotetraploid fish and improved diploid crucian carp. All results confirmed that the coexistence of four sets of red crucian carp-derived

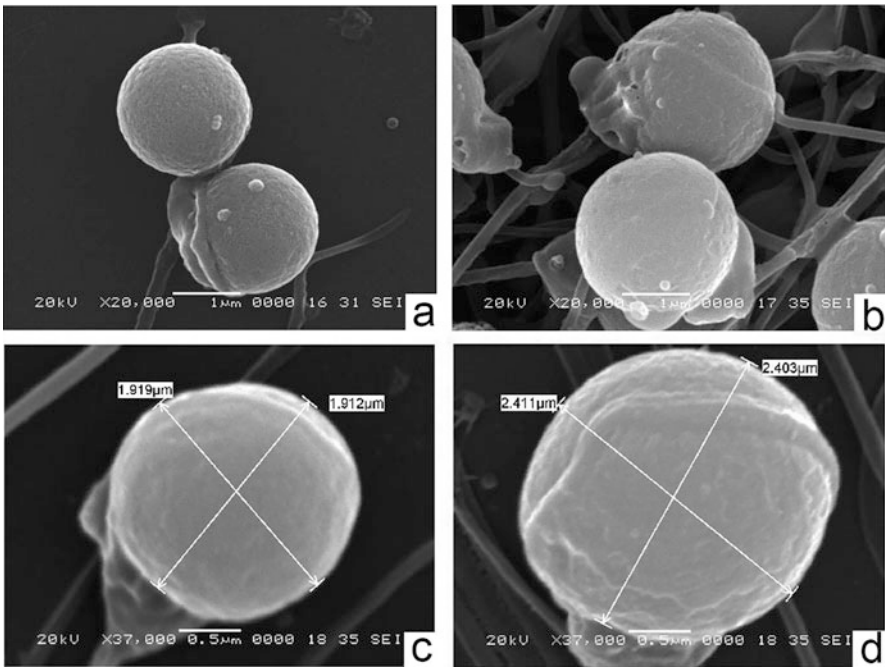

Fig. 6.19 The spermatozoa of red crucian carp and autotetraploid fish (quoted from Qin et al. (2014c)). (**a**) The spermatozoa of red crucian carp (×20,000). (**b**) The spermatozoa of autotetraploid fish (×20,000). (**c**) The spermatozoa of red crucian carp (×37,000). (**d**) The spermatozoa of autotetraploid fish (×37,000)

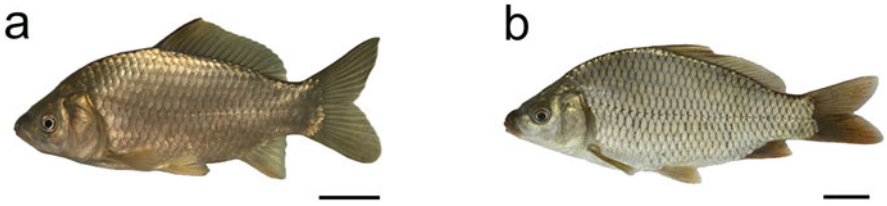

Fig. 6.20 The appearance of autotriploid hybrids and allotriploid hybrids (partially quoted from Hu et al. (2019)). (**a**) Autotriploid hybrids. (**b**) Allotriploid hybrids. Bar = 5 cm

chromosomes in autotetraploid fish did not incur disorder of chromosome pairing, and the meiosis was still in strict compliance with bivalent pairing system. What caused the bivalent pairing of autotetraploid fish during meiosis? In polyploid plants, the increasing bivalents and improving fertility were closely related to the deletion of special, low-copy, and non-coding DNA sequences (Ozkan and Feldman 2009). The deletion of DNA sequences could turn certain portion of the common sequence of homologous chromosomes into specific chromosomal sequences, which facilitated to increase the difference among homologous chromosomes and reduce the chance

of pairing during meiosis. It guaranteed strict pairing between two homologous chromosomes to lay a foundation for reviving from polyploidy to diploidy after polyploidization, which was conducive to the rapid establishment of polyploid species (Feldman et al. 1997). In our study, we used the specific repetitive sequence of red crucian carp (JQ086761) as a probe to conduct fluorescence in situ hybridization. All the chromosomes of red crucian carp were marked with fluorescence signals, and the blunt snout bream chromosomes were not marked with fluorescence signals. Therefore, this repetitive sequence may function as a special marker to track red crucian carp chromosomes. In the chromosome metaphase of allotetraploid hybrids, 100 red crucian carp-derived chromosomes were marked with fluorescence signals and 48 from blunt snout bream-derived chromosomes were not marked with fluorescence signals. Theoretically, autotetraploid fish with 4 sets of red crucian carp-derived chromosomes had 200 chromosomes marked in chromosome metaphase. But the results indicated that only 100 chromosomes were marked, and another 100 chromosomes were not marked, which illustrated the repetitive sequence of the 100 chromosomes has been mutated through DNA deletion or recombination DNA. We speculated that DNA variation in some autotetraploid fishes could quickly and effectively accelerate the differentiation of these homologous chromosomes and facilitated the formation of bivalent pairing during meiosis. The appearance of autotetraploid fish is significantly different from the original maternal parent red crucian carp. Generally, the genotype change often leads to phenotypic change. So, it is safe to infer that genotype changes during the process of genetic material doubling of autotetraploid fish would result in genotype changes, which contributed to the bivalent pairing during meiosis.

We made a research on gene structure of *HoxD4a* in allotetraploid fish and autotetraploid fish and found that *HoxD4a* gene derived from red crucian carp both in allotetraploid hybrids and autotetraploid fish had mutated. This variation generated in the first generation could be inherited to the second generation. And two different *HoxD4a* genes derived from maternal parent red crucian carp were found, and paternal parent blunt snout bream had undergone recombination in the two types of tetraploid fish. Such variation generated from the first generation also could be inherited to the second generation. These results had provided molecular evidence for the presence of genetic material from original parents in allotetraploid hybrids ($4n = 148$) and autotetraploid fish ($4n = 200$). In addition, by analysis of the gene structure of *HoxA2b* and *HoxB5b*, the recombination of red crucian carp DNA fragments and blunt snout bream DNA fragments in allotetraploid and autotetraploid fishes was also observed.

Compared to allotetraploid fish ($4n = 200$) generated from red crucian carp (♀) × common carp (♂), autotetraploid fish ($4n = 200$) generated from red crucian carp (♀) × blunt snout bream exhibited distinct differences in phenotype and genotype. One of the main differences in the phenotype of two tetraploid fishes was that allotetraploid fish had two rows of pharyngeal teeth, but autotetraploid fish had only one row of pharyngeal teeth. In terms of genotype, there were some differences in nucleotide sequences of these two types of tetraploid fishes. For example, the homologous gene *Bhlhe40* (length, 1881 bp) of these two tetraploid

fishes showed differences in the gene structure, which could be used as molecular markers to identify these two tetraploid fishes.

6.4 The Study of Androgenesis of Autotetraploid Fish and Preparation of All-Male Tetraploid Fish

As mentioned above, we have successfully established an autotetraploid lineage (F_2–F_{15}). To explore the sex determination system of this autotetraploid fish and produce all-male tetraploid fish, we made a research on androgenesis of autotetraploid fish (F_5). Considering that autotetraploid fish produce diploid gametes, diploid sperm was not treated with chromosome doubling to improve the survival rate of their self-mating offspring. Androgenetic fish (A_0) were obtained by mixing semen produced by autotetraploid fish with eggs of red crucian carp treated by UV (Reference: Sect. 5.2.2). Interestingly, androgenetic fish produce unreduced gametes which are diploid. Then an all-male tetraploid fish (XXYY) population was obtained by fertilizing mature eggs produced by female A_0 with white semen produced by a male A_0 (Fig. 6.21).

In addition, we also obtained all-male tetraploid fish (XXYY) population by fertilization of white semen produced by the male individual and eggs produced by gynogenetic diploid hybrids originated from red crucian carp (♀) × common carp (♂) clone system (G_6) (Reference: 5.2.4).

These results demonstrated that there exist sex-determining type XXYY and type XXXY in the males of autotetraploid lineage (F_2–F_{15}). The existence of two sex-determining types of XXYY and XXXY in male autotetraploid fish shows a special mechanism of sex determination, which needs further systematic research in the future.

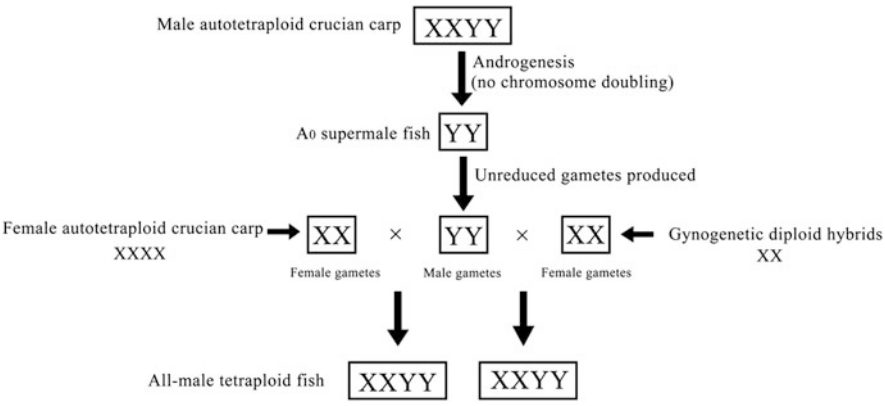

Fig. 6.21 The study on androgenesis of autotetraploid fish and preparation of all-male tetraploid fish

In production, we utilized male tetraploid fish to mate with female diploid fish to produce a large scale of sterile triploids. The successful development of all-male tetraploids is undoubtedly of great value in the production.

References

Comai L (2005) The advantages and disadvantages of being polyploid. Nat Rev Genet 6(11):836–846

Dai J (2014) Studies on molecular biological characteristics of the polyploidy hybrids of red crucian carp (*Carassius auratus* red var.)(♀) × blunt snout bream (*Megalobrama amblycephala*) (♂). Master's thesis, Hunan Normal University, Changsha

Feldman M, Liu B, Segal G, Abbo S, Levy AA, Vega JM (1997) Rapid elimination of low-copy dna sequences in polyploid wheat: a possible mechanism for differentiation of homoeologous chromosomes. Genetics 147(3):1381–1387

Hu F, Fan J, Qin Q, Huo Y, Wang Y, Wu C, Liu Q, Li W, Chen X, Liu C, Tao M, Wang S, Zhao R, Luo K, Liu S (2019) The sterility of allotriploid fish and fertility of female autotriploid fish. Front Genet 10:377

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225(225):874–876

Leah R, Frederiksen S, Engberg J, Sorensen PD (1990) Nucleotide sequence of a mouse 5S rRNA variant gene. Nucleic Acids Res 18(24):7441

Li Z, Heneen WK (1999) Production and cytogenetics of intergeneric hybrids between the three cultivated *Brassica* diploids and *Orychophragmus violaceus*. Theor Appl Genet 99(3):694–704

Li Z, Wu J, Liu Y, Liu H, Heneen W (1998) Production and cytogenetics of the intergeneric hybrids *Brassica juncea* × *Orychophragmus violaceus* and *B. carinata* × *O. violaceus*. Theor Appl Genet 96(2):251–265

Liu S, Sun Y, Zhang C, Luo K, Liu Y (2004) Production of gynogenetic progeny from allotetraploid hybrids red crucian carp×common carp. Aquaculture 236(1–4):193–200

Liu S, Qin Q, Xiao J, Lu W, Shen J, Li W, Liu J, Duan W, Zhang C, Tao M, Zhao R, Yan J, Liu Y (2007) The formation of the polyploid hybrids from different subfamily fish crossings and its evolutionary significance. Genetics 176(2):1023–1034

Liu S, Qin Q, Wang Y, Zhang H, Zhao R, Zhang C, Wang J, Li W, Chen L, Xiao J, Luo K, Tao M, Duan W, Liu Y (2010) Evidence for the formation of the male gynogenetic fish. Mar Biotechnol 12(2):160–172

Nelson DW, Honda BM (1985) Genes coding for 5S ribosomal RNA of the nematode *Caenorhabditis elegans*. Gene 38(1–3):245–251

Ozkan H, Feldman M (2009) Rapid cytological diploidization in newly formed allopolyploids of the wheat (Aegilops-Triticum) group. Genome 52(11):926–934

Pasolini P, Costagliola D, Rocco L, Tinti F (2006) Molecular organization of 5S rDNAs in Rajidae (Chondrichthyes): structural features and evolution of piscine 5S rRNA genes and nontranscribed intergenic spacers. J Mol Evol 62(5):564–574

Pendas AM, Moran P, Martinez JL, Garcia-Vazquez E (1995) Applications of 5S rDNA in Atlantic salmon, brown trout, and in Atlantic salmon × brown trout hybrid identification. Mol Ecol 4(2):275–276

Qin Q (2010) Studies on formation of the polyploidy hybrids of red crucian carp (*Carassius auratus* red var.)(♀) × blunt snout bream (*Megalobrama amblycephala*)(♂) and their biological characteristics. Doctoral thesis, Hunan Normal University, Changsha

Qin Q, He W, Liu S, Wang J, Xiao J, Yun L (2010) Analysis of 5S rDNA organization and variation in polyploid hybrids from crosses of different fish subfamilies. J Exp Zool B Mol Dev Evol 314 (5):403–411

Qin Q, Dai J, Liu S (2014a) Genetic composition and breeding traits observation of allotetraploid hybrids derived from red crucian carp × blunt snout bream. J Fish China 38(3):356–361

Qin Q, Wang Y, Wang J, Dai J, Liu Y, Liu S (2014b) Abnormal chromosome behavior during meiosis in the allotetraploid of *Carassius auratus* red var. (♀) × *Megalobrama amblycephala* (♂). BMC Genet 15:95

Qin Q, Wang Y, Wang J, Dai J, Xiao J, Hu F, Luo K, Tao M, Zhang C, Liu Y, Liu S (2014c) The autotetraploid fish derived from hybridization of *Carassius auratus* red var. (female) × *Megalobrama amblycephala* (male). Biol Reprod 91(4):1–11

Qin Q, Wang J, Wang Y, Liu Y, Liu S (2015) Organization and variation analysis of 5S rDNA in gynogenetic offspring of *Carassius auratus* red var. (♀) × *Megalobrama amblycephala* (♂). BMC Genetics 16:26

Riera-Lizarazu O, Vales MI, Ananiev EV, Rines HW, Phillips RL (2000) Production and characterization of maize chromosome 9 radiation hybrids derived from an oat-maize addition line. Genetics 156(1):327–339

Sajdak SL, Reed KM, Phillips RB (1998) Intraindividual and interspecies variation in the 5S rDNA of coregonid fish. J Mol Evol 46(6):680–688

Soltis PS, Soltis DE (2000) The role of genetic and genomic attributes in the success of polyploids. Proc Natl Acad Sci U S A 97(13):7051–7057

Sybenga J (1996) Chromosome pairing affinity and quadrivalent formation in polyploids: do segmental allopolyploids exist? Genome 39(6):1176–1184

Wu R, Gallo-Meagher M, Littell RC, Zeng ZB (2001) A general polyploid model for analyzing gene segregation in outcrossing tetraploid species. Genetics 159(2):869–882

Zhang C, Sun Y-D, Liu S, Liu Y (2005) Evidence of the unreduced diploid eggs generated from the diploid gynogenetic progeny of allotetraploid hybrids. Acta Genet Sin 32(2):136–144

7 The Formation and Biological Characteristics of the Different Ploidy Fish Derived from the Hybridization of Japanese White Crucian Carp × Blunt Snout Bream

Shaojun Liu, Fangzhou Hu, Yi Zhou, Chang Wu, Haitao Zhong, and Kaikun Luo

Abstract

Distant hybridization occurs widely in fish and is a useful strategy to produce different ploidy fish. In this chapter, we introduce the distant hybridization of female Japanese white crucian carp (*Carassius cuvieri*, $2n = 100$, JCC) and male blunt snout bream (*Megalobrama amblycephala*, $2n = 48$, BSB). In the offspring of this cross, we obtain androgenetic blunt snout bream ($2n = 48$, ADBSB), sterile allotriploid fish ($3n = 124$, 3*n*JB), and fertile allotetraploid fish ($4n = 148$, 4*n*JB). Moreover, the male 4nJB and female 4nJB are mated, and the viable autotetraploid fish ($4n = 200$, 4*n*AUJB), autotriploid fish ($3n = 150$, 3*n*AUJB), and diploid fish ($2n = 100$, 2*n*JB) are produced. Furthermore, we describe the appearance, chromosome ploidy, gonadal development, gametes, and other biological features of the different ploidy fish derived from this cross. The formation of these fish is of great significance in aquaculture, and it also provides a good model for studying the gene structure and expression variations of the progenies derived from the distant hybridization.

Keywords

Allotriploid · Allotetraploid · Androgenesis · Autotetraploid · Sterile · Morphological traits · Genetic characteristics

S. Liu (✉) · F. Hu · Y. Zhou · C. Wu · H. Zhong · K. Luo
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_7

7.1 The Formation and Major Biological Characteristics of the Different Ploidy Fish in F_1 Derived from the Hybridization of Japanese White Crucian Carp × Blunt Snout Bream

During the breeding season, both sexually mature Japanese white crucian carp (*Carassius cuvieri*, $2n = 100$, JCC) and blunt snout bream (*Megalobrama amblycephala*, $2n = 48$, BSB) were selected for distant hybridization experiments. The hybridization experiment was divided into two groups. In the first group, the females of JCC were used as the female parent, and the males of BSB were used as the male parent; in the second group, the females of BSB were used as the female parent and males of JCC were used as the male parent. The fertilized eggs were developed in the appropriate water temperature, and 5000 embryos were selected randomly to detect fertilization and hatching rate in each cross. The hatched fry was transferred to the pond for further culture. The cross combination of JCC (♀) and BSB (♂), high fertilization rate (>80.0%), and hatching rate (>54.0%) were observed. Besides, androgenetic blunt snout bream (ADBSB), allotriploid fish (3*n*JB), and allotetraploid fish (4*n*JB) were detected in these offspring, and the rates were 0.03–0.06%, 34.47–38.39%, and 61.55–65.48%, respectively. However, in the cross of BSB (♀) and JCC (♂), there was no survival offspring.

7.1.1 The Characteristics of Genetic Construction of Different Ploidy Fish

7.1.1.1 Chromosomal Number and Karyotype

The chromosomal number and karyotype of different ploidy fish and their parents were detected and analyzed. Of all examined samples in JCC, 92.5% of chromosomal metaphases possessed 100 chromosomes, and the karyotype formula was 22 m + 34 sm + 22 st + 22 t (Table 7.1). Of all examined samples in BSB, 95.5% of chromosomal metaphases had 48 chromosomes, and the karyotype formula was 18 m + 22 sm + 8 st (Table 7.1). A pair of the largest submetacentric chromosomes in chromosomal mitotic metaphase of BSB was observed, which could be used to identify the chromosome source of the test fish. Of all examined samples in ADBSB,

Table 7.1 Examination of chromosomal number distribution among BSB, JCC, ADBSB, 3*n*JB, and 4*n*JB (Hu et al. 2018)

Fish type	Number of metaphase	Distribution of chromosome number							
		<48	48	<100	100	<124	124	<148	148
BSB	200	9	191						
JCC	200			15	185				
ADBSB	200	7	193						
3*n*JB	200					38	162		
4*n*JB	200							21	179

96.5% of chromosomal metaphases had 48 chromosomes, and the karyotype formula was 18 m + 22 sm + 8 st (Table 7.1). Of all examined samples in 3*n*JB, 81.0% of chromosomal metaphases possessed 124 chromosomes, and the karyotype formula is 31 m + 45 sm + 26 st + 22 t (Table 7.1). In these metaphases, one of the largest submetacentric chromosomes from BSB was included. Of all examined samples in 4*n*JB, 89.5% of chromosomal metaphases possessed 148 chromosomes, and the karyotype formula is 40 m + 56 sm + 30 st + 22 t (Table 7.1). In these metaphases, a pair of the largest submetacentric chromosome from BSB was included.

7.1.1.2 Detection of DNA Content

The average DNA content of JCC and BSB were used as the control, and the distribution of average DNA content among different ploidy fish was shown in Table 7.2. The average DNA content of ADBSB was similar to that of the BSB, indicating that it had two sets chromosomes completely derived from BSB (Table 7.2); the average DNA content of 3*n*JB was equal to the sum of that of JCC and half of BSB (Table 7.2), indicating that it had two sets of chromosomes derived from JCC and one set of chromosomes derived from BSB; the average DNA content of 4*n*JB was equal to the sum of that of JCC and BSB (Table 7.2), indicating that it had two sets of chromosomes derived from JCC and two sets chromosomes derived from BSB.

In summary, the DNA content and chromosomal number show that the ADBSB, 3*n*JB, and 4*n*JB were diploid fish, allotriploid fish, and allotetraploid fish, respectively.

In the offspring of JCC (♀) × BSB (♂), we observed an extremely low percentage (0.03–0.06%) of ADBSB. The corresponding mechanism of the formation ADBSB was unclear. In general, the survival of androgenetic offspring was extremely low; this might be attributable to the homozygosity of these individuals. Thus, androgenesis looks like an accidental event that can't be easily detected unless large numbers of offspring were produced and examined. For 4*n*JB, we observed about 34.47–38.39% in the offspring of this cross combination. The specific mechanism of the formation 4*n*JB may be due to somatic chromosome doubling after fertilization. For 3*n*JB, we observed about 61.55–65.48% in the offspring of JCC (♀) × BSB (♂). The 3*n*JB formation probably due to the second polar body

Table 7.2 The DNA content distribution of JCC, BSB, ADBSB, 3*n*JB, and 4*n*JB (Hu et al. 2018)

Fish type	Mean DNA content	Ratio	
		Observed	Expected
BSB	68.8		
JCC	94.72		
ADBSB	74.55	ADBSB/BSB = 1.08[a]	1
3*n*JB	124.3	3*n*JB/(JCC + 0.5BSB) = 0.96[a]	1
4*n*JB	157.43	4*n*JB/(JCC + BSB) = 0.96[a]	1

[a]The observed ratio was not significantly different ($P > 0.05$) from the expected ratio

extrusion was inhibited during the second division of meiosis. The similar situation also was observed in triploid *Crepis capillaris* (Ramsey and Schemske 1998).

7.1.2 The Morphological Characteristics of Different Ploidy Fish in F_1

The ADBSB, 3*n*JB, and 4*n*JB differed significantly in morphological traits. For example, ADBSB looked like BSB; 4*n*JB had a pair of short barbels and six to eight pelvic fins; 3*n*JB had no barbels and only eight to nine pelvic fins.

The measurable traits and countable traits of different ploidy hybrids and their parents were examined (Tables 7.3 and 7.4). For ADBSB, all measurable traits were not significantly different ($P > 0.05$) with BSB, but significantly different ($P < 0.05$) from those of JCC. For the measurable traits between 3*n*JB and BSB, except for the ratio of tail length/tail height, which was not significantly different ($P > 0.05$), other ratios were significantly different ($P < 0.05$). Between 3*n*JB and JCC, except for the ratio of body length/head length, which was not significantly different ($P > 0.05$), other ratios were significantly different ($P < 0.05$). Between 4*n*JB and BSB, apart from the ratio of body length/body weight, which was not significantly different ($P > 0.05$), other ratios were significantly different ($P < 0.05$). Between 4*n*JB and JCC, all ratios were significantly different ($P < 0.05$). On the other hand, between 3*n*JB and 4*n*JB, except for the ratio of body length/head length, which was not significantly different ($P > 0.05$), other ratios were significantly different ($P < 0.05$) (Table 7.3).

For ADBSB, all countable traits were not significantly different ($P > 0.05$) with BSB, but significantly different ($P < 0.05$) from those of JCC. For 3*n*JB, all countable traits were significantly different ($P < 0.05$) with BSB; except for the number of lateral scales and upper lateral scales, which were not significantly different ($P > 0.05$), other countable traits were significantly different ($P < 0.05$) with JCC. For 4*n*JB, all countable traits were significantly different ($P < 0.05$) with BSB; except for the number of upper lateral scales, which was not significantly different ($P > 0.05$), other countable traits were significantly different ($P < 0.05$) with JCC. On the other hand, between 3*n*JB and 4*n*JB, except for the number of dorsal fins and anal fins, which were significantly different ($P < 0.05$), other countable traits were not significantly different ($P > 0.05$) (Table 7.4).

7.1.3 The Reproductive Traits of Different Ploidy Fish

The ADBSB, 3*n*JB, and 4*n*JB differed greatly in their reproductive characteristics due to their different genetic composition. Our analyses of reproductive traits revealed that the ADBSB and 4*n*JB were fertile and reached sexual maturity at the age of 2, but the 3*n*JB were sterile.

Table 7.3 Comparison of the measurable traits among JCC, BSB, ADBSB, 3*n*JB, and 4*n*JB (Hu et al. 2018)

Fish type	Overall length/body length	Body length/body width	Body length/head length	Head length/head width	Tail length/tail width	Body width/head width
BSB	1.19 ± 0.03	2.37 ± 0.03	4.75 ± 0.04	1.14 ± 0.03	1.08 ± 0.04	2.09 ± 0.04
JCC	1.24 ± 0.02	2.22 ± 0.15	3.70 ± 0.21	1.17 ± 0.06	0.81 ± 0.01	1.78 ± 0.09
ADBSB	1.18 ± 0.06	2.36 ± 0.06	4.73 ± 0.14	1.15 ± 0.22	1.08 ± 0.25	2.10 ± 0.21
3*n*JB	1.16 ± 0.03	2.28 ± 0.13	3.80 ± 0.25	1.03 ± 0.07	1.05 ± 0.08	1.72 ± 0.11
4*n*JB	1.21 ± 0.03	2.35 ± 0.12	3.81 ± 0.14	1.12 ± 0.06	1.02 ± 0.08	1.83 ± 0.10

Table 7.4 Comparison of the countable traits among JCC, BSB, ADBSB, 3*n*JB, and 4*n*JB (Hu et al. 2018)

Fish type	Number of lateral scales	Number of upper lateral scales	Number of lower lateral scales	Number of dorsal fins	Number of pelvic fins	Number of anal fins
BSB	50.90 ± 0.91 (52–49)	9.65 ± 0.49 (9–10)	10.05 ± 0.69 (9–11)	III + 8.65 ± 0.49 (III 8–9)	9.00 ± 0.55 (8–10)	III + 25.85 ± 0.59 (III 25–27)
JCC	33.15 ± 0.35 (32–34)	7.5 ± 0.42 (6–8)	7.6 ± 0.31 (5–7)	III + 6.45 ± 0.31 (III 6–7)	9.05 ± 0.75 (8–10)	III + 19.35 ± 0.86 (III 18–20)
ADBSB	50.90 ± 0.24 (52–49)	9.47 ± 0.12 (9–10)	9.42 ± 0.69 (9–11)	III + 8.44 ± 0.38 (III 8–9)	9.00 ± 0.13 (8–10)	III + 26.22 ± 0.37 (III 25–27)
3*n*JB	33.45 ± 0.74 (32–34)	7.7 ± 0.51 (7–8)	7.45 ± 0.60 (7–9)	III + 7.20 ± 0.61 (III 6–8)	7.65 ± 0.67 (6–8)	III + 17.25 ± 1.07 (III 15–20)
4*n*JB	32.05 ± 0.22 (32–33)	7.1 ± 0.31 (7–8)	7.95 ± 0.22 (7–8)	III +7.15 ± 0.37 (III 7–8)	8.70 ± 0.47 (8–9)	III +17.35 ± 0.49 (III 17–18)

7.1.3.1 Reproductive Traits of ADBSB

Gonadal development was not examined for ADBSB because their number is relatively few. Only two individuals of ADBSB are survival at 2 years old. In the breeding season, the white semen can be stripped out from the male ADBSB at the age of 2. The scanning electron microscope showed that the well-developed haploid sperm produced by male ADBSB had normal heads and tails. The mature sperm from ADBSB and eggs from normal BSB could fuse to form viable offspring.

7.1.3.2 Reproductive Traits of 4*n*JB

The observation of gonadal development is an important way for the assessment of fertility, especially in hybrid progenies. Histological sectioning was a frequently used technology to survey gonadal development. The histological sectioning of the testes of 18-month-old 4*n*JB results showed that they were at stage IV, in which the seminiferous tubules were filled with secondary spermatocytes (Fig. 7.1a). The

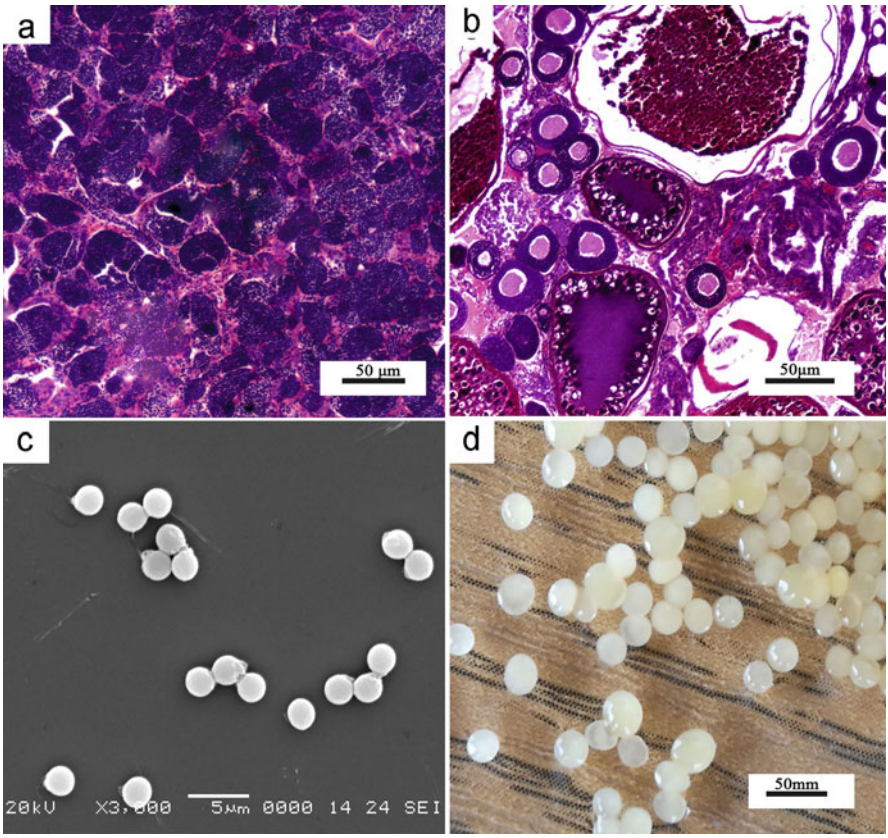

Fig. 7.1 Ovarian and testis microstructure and gamete appearance of 4*n*JB (Hu et al. 2018). (**a**) The testis microstructure of 4*n*JB. Bar = 20 μm. (**b**) The ovarian microstructure of 4*n*JB. Bar = 20 μm. (**c**) The sperm appearance of 4*n*JB. (**d**) The egg appearance of 4*n*JB

histological sectioning results of 18-month-old 4*n*JB ovaries showed that they were rich in oocytes in synchronized development and were characterized by the location of the yolk nucleus near the cell nucleus (Fig. 7.1b). In addition, water-like semen could be stripped out from male 4*n*JB and mature eggs could be stripped out from female 4*n*JB at the age of 2 during the breeding season. The water-like semen from male 4*n*JB included a small number of normal spermatozoa (Fig. 7.1c). In addition, the female 4*n*JB produced three sizes of eggs. The average diameter of the larger-size eggs was 0.20 cm, accounting for 89%. The average diameter of the medium-size eggs was 0.17 cm, accounting for 7%. The average diameter of the small-size eggs was 0.13 cm, accounting for 4% (Fig. 7.1d). The results showed that the gametes of 4*n*JB were obvious polymorphism in their sizes. The male 4*n*JB and female 4*n*JB were important genetic resources to produce viable autotetraploid fish ($4n = 200$), autotriploid fish ($3n = 150$), and diploid fish ($2n = 100$) (details shown in another section) by mating. These results suggested that 4*n*JB were a fertile allotetraploid hybrid fish, but they had lower fertile than that of their parents.

7.1.3.3 Reproductive Traits of 3*n*JB

Three types of gonadal structure were found in 3*n*JB after the observation of histological sectioning. The first type was the ovary-like gonad, which comprised a few small growing oocytes and some nests of undeveloped and small cells (Fig. 7.2a). The second type was the testis-like gonad, which comprised some lobules containing a large number of spermatids. However, only the degenerated spermatids were found, but no mature spermatozoa (Fig. 7.2b). For the third type, fat tissue replaced the location of gonad, and neither testes nor ovaries were observed. In addition, no gamete could be stripped out from the 1- or 2-year-old males or females of 3*n*JB in the breeding season. These results indicated that the 3*n*JB derived from distant hybridization were sterile.

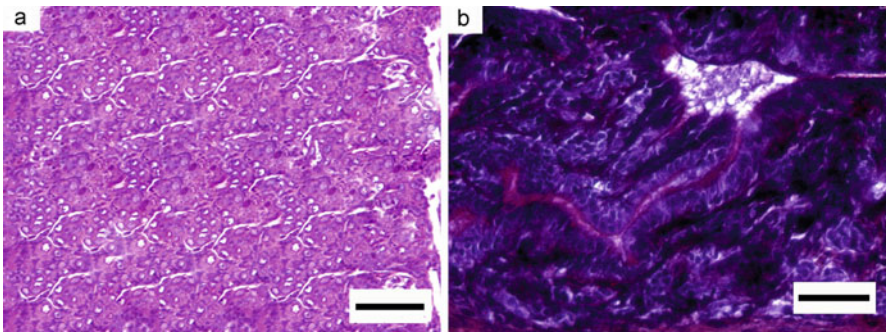

Fig. 7.2 Ovarian and testis microstructure of 3*n*JB (Hu et al. 2018). (**a**) The ovarian microstructure of 3*n*JB. (**b**) The testis microstructure of 3*n*JB. Bar = 20 μm

7.1.4 The Cellular and Molecular Biological Characteristics of Different Ploidy Fish in F_1

7.1.4.1 Molecular Organization of *5S rDNA*

The *5S* primer pair described in Qin et al. 2010 was used to amplify the *5S rDNA* genes directly from genomic DNA of JCC, BSB, ADBSB, 3*n*JB, and 4*n*JB. The results of PCR showed three DNA bands in JCC, two DNA bands in BSB, two DNA bands in ADBSB, three DNA bands in 3*n*JB, and four DNA bands in 4*n*JB, respectively.

The analyses of sequencing results showed that three different *5S rDNA* fragments in JCC were 203 bp, 340 bp, and 486 bp, respectively; two different *5S rDNA* fragments in BSB were188 bp and 376 bp respectively; two different *5S rDNA* fragments in ADBSB were 188 bp and 376 bp, respectively; five different *5S rDNA* fragments in 3*n*JB were 188 bp, 203 bp, 340 bp, 376 bp, and 496 bp, respectively, and four different *5S rDNA* fragments in 4*n*JB were 203 bp, 340 bp, 406 bp, and 494 bp, respectively (Table 7.5).

The sequencing results suggested that both JCC and BSB were highly conserved in their 5S RNA regions (Fig. 7.3) but exhibited significant variation in their NTS regions (Fig. 7.4). In JCC, the three monomeric *5S rDNA* classes (designated class I, 203 bp; class II, 340 bp; and class III, 486 bp, respectively) were distinct by different NTS sequences (designated NTS-I, 83 bp; NTS-II, 220 bp; and NTS-III, 366 bp, respectively) (Fig. 7.4a–c). In BSB, the only monomeric *5S rDNA* class (designated

Table 7.5 The results of *5S rDNA* sequences among JCC, BSB, BSB, 3*n*JB, and 4*n*JB (Hu et al. 2018)

Fish type	Number of sequenced clones	DNA fragments			
		~200 bp[a]	~340 bp[a]	~400 bp[a]	~500 bp[a]
JCC	30	Ten sequenced clones of 203	Ten sequenced clones of 340	–	Ten sequenced clones of 486
BSB	20	Ten sequenced clones of 188	Ten sequenced clones of 376	–	–
ADBSB	40	Twenty sequenced clones of 188	Twenty sequenced clones of 376	–	–
3*n*JB	60	Fifteen sequenced clones of 203 Five sequenced clones of 188	Eight sequenced clones of 340 Twelve sequenced clones of 376	–	Twenty sequenced clones of 496
4*n*JB	80	Twenty sequenced clones of 203	Twenty sequenced clones of 340	Twenty sequenced clones of 406	Twenty sequenced clones of 494

[a]The approximate size of PCR bands on the agarose gel

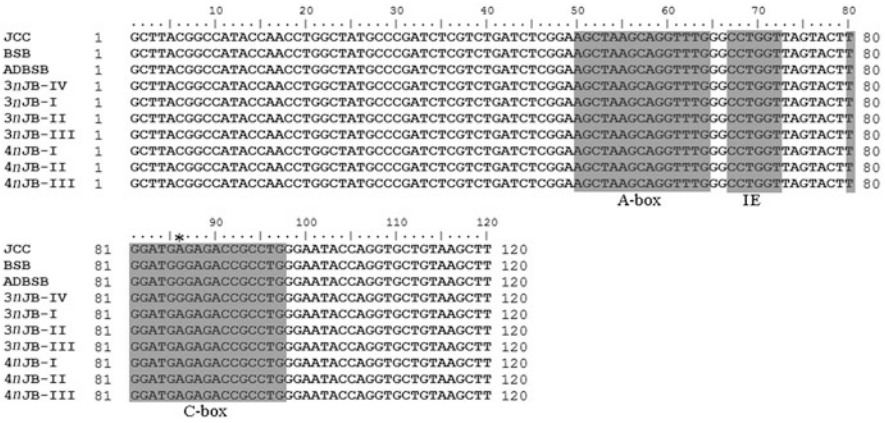

Fig. 7.3 The coding regions of *5S* rDNA in JCC, BSB, ADBSB, 3*n*JB, and 4*n*JB. Asterisks represent variable sites of the *5S* coding regions and shaded areas represent internal control regions (Hu et al. 2018)

class IV, 188 bp) was characterized by one NTS sequence (designated NTS-IV, 68 bp). The ADBSB also had only one monomeric *5S rDNA* completely from BSB; the 3*n*JB had four monomeric *5S rDNA* classes, with three of those from JCC (Fig. 7.4) and one from BSB (class IV). The 4*n*JB had three monomeric *5S rDNA* classes, completely from JCC (Fig. 7.4). The 406 bp DNA fragment from the 4*n*JB was a dimeric *5S rDNA* of class I sequences.

7.1.4.2 Fluorescence In Situ Hybridization

The probe used for fluorescence in situ hybridization (FISH) was the *5S* gene of JCC and amplified by PCR. The results of FISH (Fig. 7.5) showed that two strong and two weak fluorescence signals were found in JCC (Fig. 7.5a) and 3nJB (Fig. 7.5d), two strong and one weak fluorescence signals were found in 4nJB (Fig. 7.5e), and no fluorescence signal was found in BSB (Fig. 7.5b) and ADBSB (Fig. 7.5c). The FISH analysis also identified the heredity and variation at the chromosome level in the hybrids.

7.2 The Formation and Biological Characteristics of Autotetraploid, Autotriploid, and Diploid Fish in F_2 from the Hybridization of JCC × BSB

It has been mentioned in the previous section that both female and male of 4*n*JB ($4n = 148$) were fertile, and their gamete size presented significant polymorphism. In order to determine the genetic composition of these different sized gametes and fertility of 4*n*JB, we used female and male 4*n*JB to mating, and three different ploidy fish were obtained, including diploid fish (2*n*JB, $2n = 100$), autotriploid fish (3*n*AUJB, $3n = 150$), and autotetraploid fish (4*n*AUJB, $4n = 200$). The appearance,

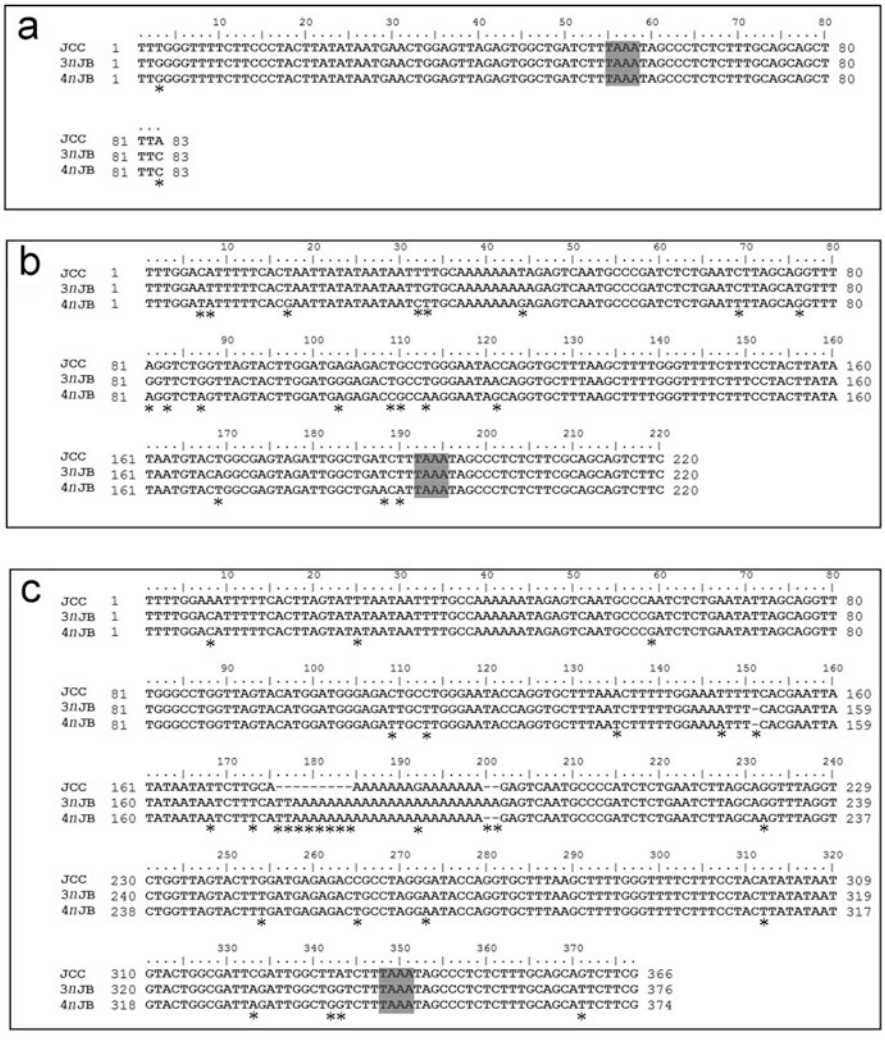

Fig. 7.4 The nucleotide sequence alignment of the NTS sequences in JCC, 3*n*JB, and 4*n*JB (Hu et al. 2018). (**a**) The NTS-I sequences in JCC, 3*n*JB, and 4*n*JB. (**b**) The NTS-II in JCC, 3*n*JB, and 4*n*JB. (**c**) The NTS-III in JCC, 3*n*JB, and 4*n*JB. The shaded area represents TATA elements. The asterisks represent variable sites of NTS

chromosome ploidy, gonadal development and breeding, gametes, growth phenotype, and other biological features of these different ploidy offspring were detected and analyzed.

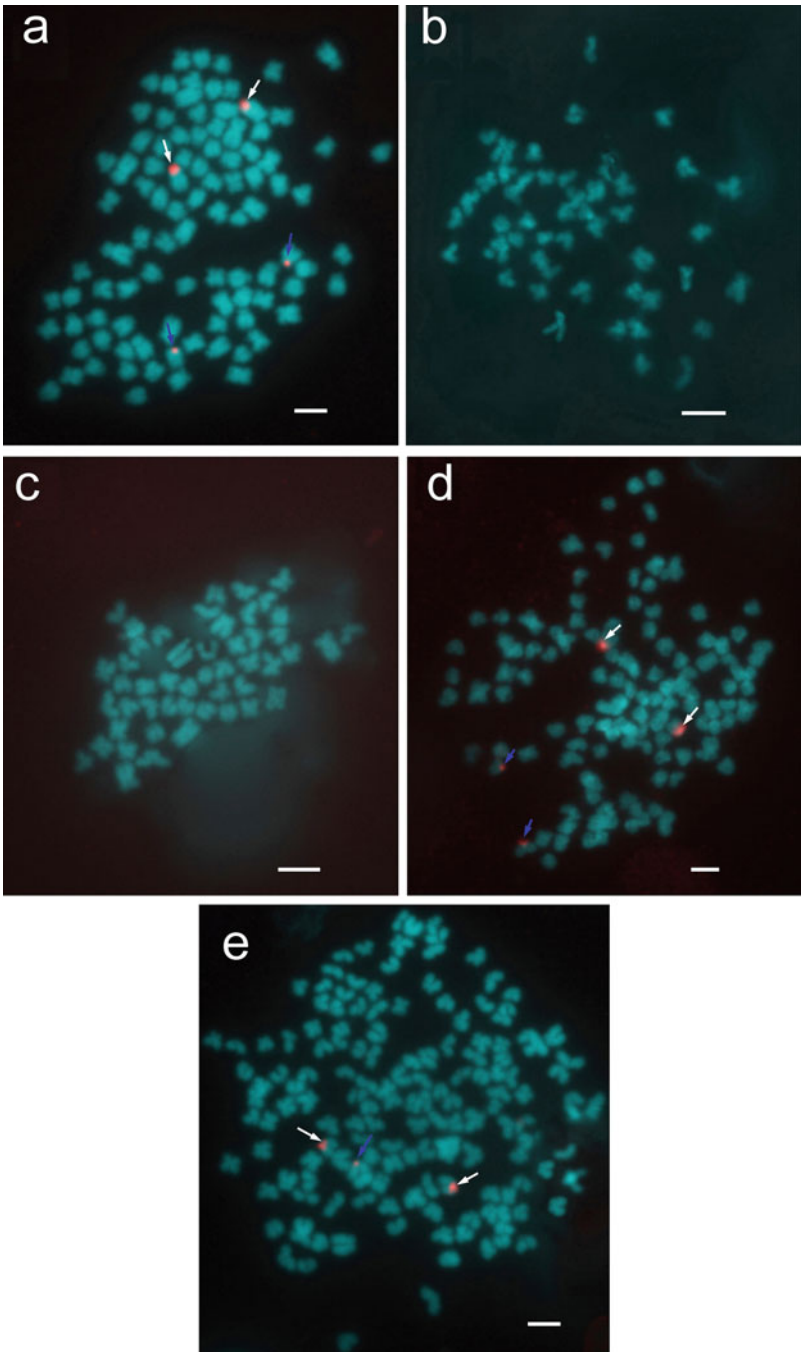

Fig. 7.5 Examination of fluorescence signals among JCC, BSB, ADBSB, 3*n*JB, and 4*n*JB (Hu et al. 2018). (**a**) Two strong fluorescence signals (white arrows) and two weak (blue arrows) hybridizing signals were found in JCC. (**b**) BSB has no *5S* gene locus. (**c**) ADBSB has no *5S* gene locus. (**d**) Two strong (white arrows) and two weak (blue arrows) hybridizing signals were found in 3*n*JB. (**e**) Two strong (white arrows) and one weak (blue arrows) hybridizing signals were found in 4*n*JB. Bar = 3 μm

7.2.1 The Characteristics of Genetic Construction of Different Ploidy Fish in F_2

7.2.1.1 Chromosomal Number and Karyotype

The chromosomal number and karyotype of different ploidy fish from mating of female 4*n*JB and male 4*n*JB were detected and analyzed. Of all examined samples in 2*n*JB, 94% of chromosomal metaphases had 100 chromosomes, and the karyotype formula was 22 m + 34 sm + 22 st + 22 t (Table 7.6). Of all examined samples in 3*n*AUJB, 82.5% of chromosomal metaphases possessed 150 chromosomes, and the karyotype formula was 33 m + 51 sm + 33 st +33 t (Table 7.6). Of all examined samples in 4*n*AUJB, 79.5% of chromosomal metaphases possessed 200 chromosomes, and the karyotype formula was 44 m + 68 sm + 44 st + 44 t (Table 7.6).

7.2.1.2 Detection of DNA Content

The sum DNA content of JCC was used as the control, and the distribution of DNA content among different ploidy fish was shown in Table 7.7. The mean DNA content of 2*n*JB was similar to JCC, indicating that it contained two sets of chromosomes completely from JCC (Table 7.7); the mean DNA content of 3*n*AUJB was equal to the sum of that of 1.5 times of JCC (Table 7.7), indicating that it contained three sets of chromosomes from JCC; the mean DNA content of 4*n*AUJB was equal to the sum of that of two times of JCC (Table 7.7), indicating that it had four sets of chromosomes from JCC.

Table 7.6 Examination of chromosome number in 2*n*JB, 3*n*AUJB, and 4*n*AUJB

Fish type	Number of metaphase	Distribution of chromosome number					
		<100	100	<150	150	<200	200
2*n*JB	200	12	188				
3*n*AUJB	200			35	165		
4*n*AUJB	200					41	159

Table 7.7 Mean DNA content of JCC, 2*n*JB, 3*n*AUJB, and 4*n*AUJB

Fish type	Mean DNA content	Ratio	
		Observed	Expected
JCC	94.72		
2nJB	98.5	2*n*JB/JCC = 1.04[a]	1
3*n*AUJB	146.34	3*n*AUJB/1.5JCC = 1.03[a]	1
4*n*AUJB	193.22	4*n*JB/2JCC = 1.02[a]	1

[a]The observed ratio was not significantly different ($P > 0.05$) from the expected ratio

7.2.2 The Morphological Characteristics of Different Ploidy Fish in F_2

The 2*n*JB, 3*n*AUJB, and 4*n*AUJB differ significantly in morphological traits. For example, 2*n*JB looked like JCC with white-gray body color; 3*n*AUJB had a pair of short barbels with a yellow-gray body color; 4nAUJB fish had a pair of short barbels with a steel-gray body color.

The measurable traits and countable traits of JCC, BSB, 4*n*JB, 2*n*JB, 3*n*AUJB, and 4*n*AUJB were examined (Tables 7.8 and 7.9). For measurable traits between 2*n*JB and JCC, except for the ratio of body length/body width and body width/head width, which were significantly different ($P < 0.05$), other ratios were not significantly different ($P > 0.05$). For measurable traits between 2*n*JB and BSB, all ratios were significantly different ($P < 0.05$). For the measurable traits between 3*n*AUJB and BSB, except for the ratio of head length/head width, which was not significantly different ($P > 0.05$), other ratios were significantly different ($P < 0.05$). Between 3*n*AUJB and JCC, except for the ratio of head length/head width, which was not significantly different ($P > 0.05$), other ratios were significantly different ($P < 0.05$). Between 4*n*AUJB and BSB, except for the ratio of head length/head width, which was not significantly different ($P > 0.05$), other ratios were significantly different ($P < 0.05$). Between 4*n*AUJB and JCC, except for the ratio of whole length/body length, which was not significantly different ($P > 0.05$), other ratios were significantly different ($P < 0.05$) (Table 7.8).

For countable traits between 2*n*JB and JCC, except for the number of lateral scales, the number of lower lateral scales, and the number of upper lateral scales, which were significantly different ($P < 0.05$), other data were not significantly different ($P > 0.05$). Between 2*n*JB and BSB, all data were significantly different ($P < 0.05$). For the countable traits between 3*n*AUJB and BSB, all data were significantly different ($P < 0.05$). Between 3*n*AUJB and JCC, except for the number of lower lateral scales, which was not significantly different ($P > 0.05$), other data were significantly different ($P < 0.05$). Between 4*n*AUJB and BSB, all data were significantly different ($P < 0.05$). Between 4*n*AUJB and JCC, except for the number of anal fins, which was not significantly different ($P > 0.05$), other data were significantly different ($P < 0.05$) (Table 7.9).

7.2.3 The Reproductive Traits of Different Ploidy Fish in F_2

The 2*n*JB, 3*n*AUJB, and 4*n*AUJB presented different reproductive characteristics. The 2*n*JB and 4*n*AUJB were fertile and reached sexual maturity at 1 year of age, but the 3*n*AUJB were sterile.

7.2.3.1 Reproductive Traits of 2*n*JB

The ovaries of the 8-month-old 2*n*JB were well developed and contained many stage IV oocytes (Fig. 7.6a). The testes of the 10-month-old 2*n*JB also well developed and contained a number of secondary spermatocytes in the seminiferous tubules

Table 7.8 Comparison of the measurable traits among 2*n*JB, 3*n*AUJB, 4*n*AUJB, 4*n*JB, BSB, and JCC

Fish type	Overall length/body length	Body length/body width	Body length/head length	Head length/head width	Tail length/tail width	Body width/head width
BSB	1.19 ± 0.03	2.37 ± 0.03	4.75 ± 0.04	1.14 ± 0.03	1.08 ± 0.04	2.09 ± 0.04
JCC	1.24 ± 0.02	2.22 ± 0.15	3.70 ± 0.21	1.17 ± 0.06	0.81 ± 0.01	1.78 ± 0.09
4*n*JB	1.21 ± 0.03	2.35 ± 0.12	3.81 ± 0.14	1.12 ± 0.06	1.02 ± 0.08	1.83 ± 0.10
2*n*JB	1.25 ± 0.08	2.18 ± 0.06	3.74 ± 0.11	1.15 ± 0.10	0.80 ± 0.04	1.86 ± 0.08
3*n*AUJB	1.28 ± 0.06	2.31 ± 0.05	3.84 ± 0.15	1.16 ± 0.10	0.95 ± 0.08	1.82 ± 0.05
4*n*AUJB	1.23 ± 0.13	2.24 ± 0.18	3.76 ± 0.08	1.18 ± 0.08	0.84 ± 0.06	1.86 ± 0.16

Table 7.9 Comparison of the countable traits among 2*n*JB, 3*n*AUJB, 4*n*AUJB, 4*n*JB, BSB, and JCC

Fish type	Number of lateral scales	Number of upper lateral scales	Number of lower lateral scales	Number of dorsal fins	Number of pelvic fins	Number of anal fins
BSB	50.90 ± 0.91 (52–49)	9.65 ± 0.49 (9–10)	10.05 ± 0.69 (9–11)	III + 25.85 ± 0.59 (III 25–27)	8.65 ± 0.49 (8–9)	III +25.70 ± 0.55 (III 25–27)
JCC	33.15 ± 0.35 (32–34)	7.53 ± 0.42 (6–8)	7.60 ± 0.31 (5–7)	III + 19.35 ± 0.86 (III 18–20)	6.45 ± 0.31 (6–7)	III +9.05 ± 0.75 (8–10)
4*n*JB	32.05 ± 0.22 (32–33)	7.14 ± 0.31 (7–8)	7.95 ± 0.22 (7–8)	III +17.35 ± 0.49 (III 17–18)	7.15 ± 0.37 (7–8)	III +8.70 ± 0.47 (III 8–9)
2*n*JB	30.65 ± 0.64 (29–31)	7.84 ± 0.42 (7–9)	7.66 ± 0.31 (7–8)	III + 19.45 ± 0.44 (III 18–20)	6.55 ± 0.38 (6–7)	III +9.35 ± 0.25 (8–10)
3*n*AUJB	30.52 ± 1.47 (30–32)	5.48 ± 0.84 (5–6)	7.26 ± 0.32 (7–8)	III + 16.96 ± 0.35 (III + 15–17)	7.34 ± 0.30 (7–9)	III + 6.55 ± 0.78 (III + 5–7)
4*n*AUJB	29.54 ± 1.03 (29–32)	5.36 ± 0.50 (5–6)	6.81 ± 0.75 (5–7)	III + 18.27 ± 0.46 (III + 18–19)	5.45 ± 0.52 (III + 5–6)	III + 8.63 ± 0.50 (8–9)

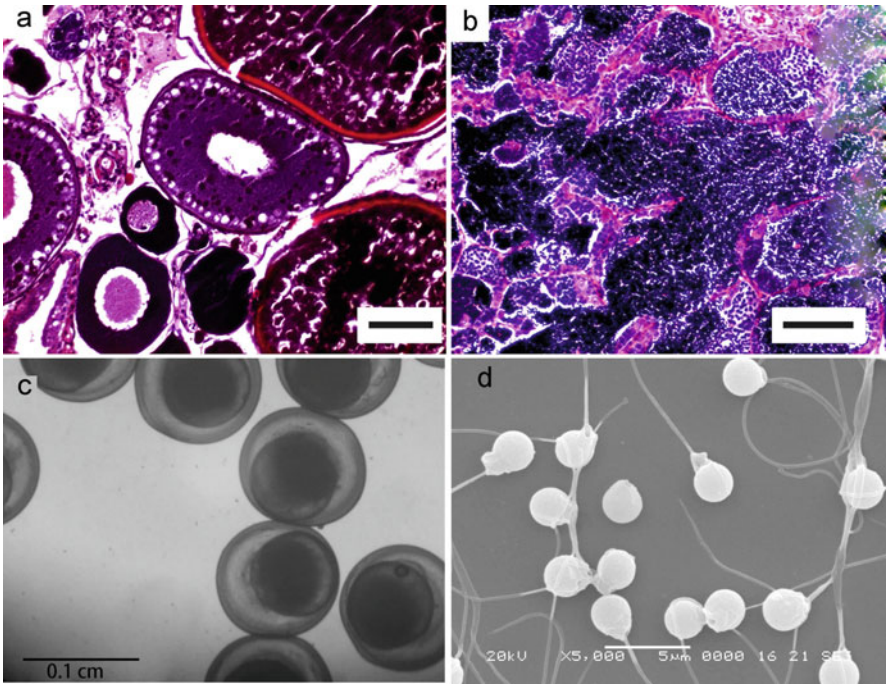

Fig. 7.6 The gonadal structure and gamete appearance of 2*n*JB. (**a**) The ovarian microstructure of 2*n*JB. (**b**) The testis microstructure of 2*n*JB. (**c**) The appearance of the eggs of 2*n*JB. (**d**) The appearance of the sperm of 2*n*JB

(Fig. 7.6b). In addition, in the breeding season, white semen and mature eggs could be produced by male and female of 2*n*JB, respectively. The female 2*n*JB produced normal eggs and the mean diameter of the eggs was 0.13 cm (Fig. 7.6c). In addition, the white semen from male 2*n*JB included many normal spermatozoa and the mean head diameter of the spermatozoa was 2.0 μm (Fig. 7.6d). The results showed that the 2*n*JB could produce haploid gametes. The male 2*n*JB and female 2*n*JB were mated and viable diploid offspring were produced, and to date, the population of 2*n*JB has been extended to the fifth generations (F_2–F_6).

7.2.3.2 Reproductive Traits of 3*n*AUJB

During the breeding season, both female and male 3*n*AUJB could not produce mature eggs and semen, respectively, at the age of 2. The ovarian development of 2-year-old 3*n*AUJB was pale yellow and presented as petal-shaped leaflet after fixation. A large number of underdeveloped and small ovogonium-like cells were observed in ovary, some of which were vacuolated inside and began to degrade (Fig. 7.7a). The testes of 2-year-old 3*n*AUJB were mainly composed of seminiferous tubules, some of which had no spermatid but some remained. These spermatids were shaped irregular and obscure outer surface and presented degradation and

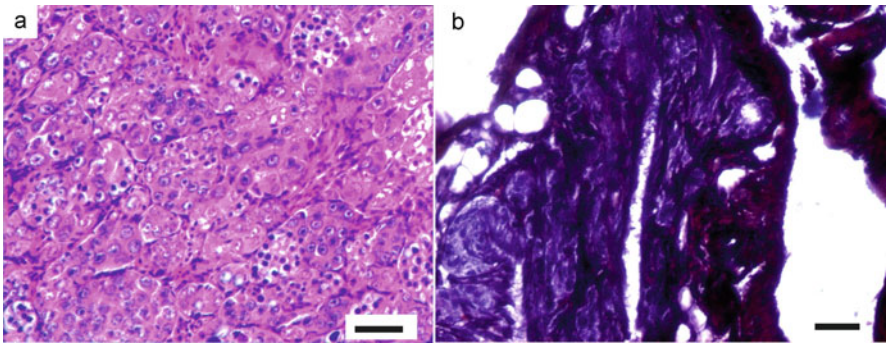

Fig. 7.7 The gonadal structure of 3*n*AUJB. (**a**) The ovarian microstructure of 3*n*AUJB. (**b**) The testis microstructure of 3*n*AUJB. Bar = 20 μm

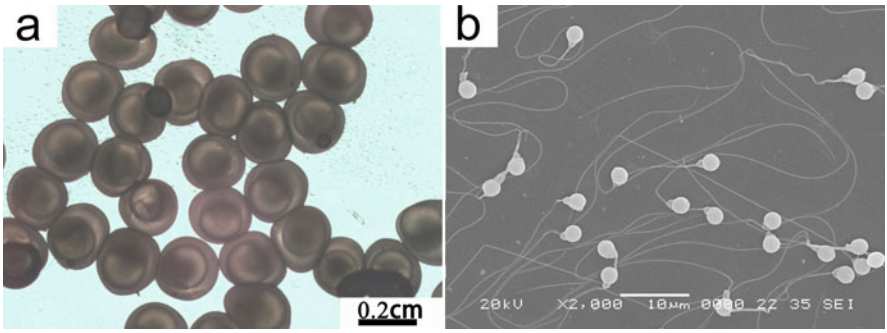

Fig. 7.8 The gamete appearance of 4*n*AUJB. (**a**) The appearance of the eggs of 4*n*AUJB. (**b**) The appearance of the sperm of 4*n*AUJB

disintegration phenomenon. No mature sperm were observed (Fig. 7.7b). The result indicated that the both female and male 3*n*AUJB were sterile.

7.2.3.3 Reproductive Traits of 4*n*AUJB

Gonadal development of 4*n*AUJB was not examined because number of 4*n*AUJB was relatively few. Only seven individuals of 4*n*AUJB fish were survival at 1 year old. In the breeding season, the white semen could be produced by male 4*n*AUJB individuals at the age of 1 and large numbers of eggs could be produced by female 4*n*AUJB (Fig. 7.8). The scanning electron microscope indicated that the diploid sperm produced by male 4*n*AUJB were well-developed. The mature sperm and eggs could fuse to form second generation.

References

Hu F, Wu C, Zhou Y, Cao L, Xiao J, Wang S, Wu Y, Ren L, Liu Q, Li W, Wen M, Tao M, Qin Q, Zhao R, Luo K, Liu S (2018) Production of androgenetic, triploid and tetraploid hybrids from the interspecific hybridization of female Japanese crucian carp and male blunt snout bream. Aquaculture 491:50–58

Qin Q, He W, Liu S, Wang J, Xiao J, Liu Y (2010) Analysis of 5S rDNA organization and variation in polyploid hybrids from crosses of different fish subfamilies. J Exp Zool B Mol Dev Evol 314B(5):403–411

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst 29:467–501

8 The Formation and Biological Characteristics of the Different Ploidy Fishes Derived from the Hybridization of Blunt Snout Bream × Topmouth Culter

Shaojun Liu, Li Ren, Chang Wu, Dingbin Gong, Yi Zhou, Min Tao, Chun Zhang, Qinbo Qin, and Kaikun Luo

Abstract

By distant hybridization, not only the establishments of the fertile tetraploid lineages, as mentioned in the previous chapters, are very important, but also the establishments of the fertile diploid lineages are very important in fish genetic breeding and biological evolution. In this chapter, we introduce the intergeneric hybridization of blunt snout bream (*Megalobrama amblycephala*, BSB) and topmouth culter (*Culter alburnus*, TC). The differences between this cross and the other crosses as mentioned in the previous chapters are listed as below: the cross of red crucian carp (*Carassius auratus* red var., RCC, $2n = 100$, ♀) and common carp (*Cyprinus carpio* L., $2n = 100$, ♂) is intergeneric hybridization, and both of the two parents are omnivorous and have the same chromosome number ($2n = 100$); the parents of the cross of RCC (♀) × BSB ($2n = 48$, ♂) and the cross of Japanese white crucian carp (*C. cuvieri*, $2n = 100$, ♀) × BSB ($2n = 48$, ♂) belong to different subfamilies and have different chromosome numbers ($2n = 100$; $2n = 48$). RCC and Japanese white crucian carp are omnivorous, while BSB is herbivorous. However, in the cross of BSB and TC, both BSB and TC have 48 chromosomes, and they are herbivorous and carnivorous, respectively. Here, we introduce the successful establishments of the reciprocal hybrid lineages of BSB and TC and their research results in morphology, cytology, biochemistry, and molecular biology.

Keywords

Distant hybridization · Reciprocal crosses · Herbivorous · Fertile · Allodiploid

S. Liu (✉) · L. Ren · C. Wu · D. Gong · Y. Zhou · M. Tao · C. Zhang · Q. Qin · K. Luo
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_8

8.1 The Formation of the Two Reciprocal Crosses of BSB × TC

Using hybrid breeding technology, we obtained two types of F_1 hybrids in the cross of BSB (♀) × TC (♂) and the cross of TC (♀) × BSB (♂). There were diploid and triploid progenies in F_1 hybrids of BSB (♀) × TC (♂) ($2nBT_1$ and $3nBT_1$, respectively). However, we only found diploid progenies in F_1 hybrids of TC (♀) × BSB (♂) ($2nTB_1$). Interestingly, the most of $2nBT_1$ and $2nTB_1$ individuals were highly fertile. White semen could be stripped out from 15-month-old male F_1 hybrids of these two crosses, which contained a large number of matured sperm; 15-month-old female F_1 hybrids of these two crosses presented the normal development in ovaries, which included a large amount of oocytes in period II. Some dark green eggs could be stripped out in the reproductive season of the next year. F_2 hybrids were obtained by self-crossing of F_1 hybrids. F_2 hybrids of BSB (♀) × TC (♂) ($2nBT_2$) and F_2 hybrids of TC (♀) × BSB (♂) ($2nTB_2$) were fertile. The F_3 hybrids were produced by self-crossing from F_2. Until now, the hybrid lineage of BSB (♀) × TC (♂) (F_1-F_6) and the hybrid lineage of TC (♀) × BSB (♂) (F_1-F_3) were established (Xiao 2013; Xiao et al. 2014), providing new germplasm resources for fish genetic breeding and the studies of the genetics in fish hybridization.

8.1.1 Introduction of the Hybrid Parents

The basic features of BSB have been introduced in Chap. 7. TC, as the other parent of this cross, is described as follows: in the categories, *Culter alburnus* belongs to *Culter* (Cypriniformes, Cyprinidae, Cultrinae). In shape, it is linear with a low and long body. It has a flat head with a bumped back. The lower jaw stands before the mouth. It possesses big and round eyes and its body is covered with small scales.

The male TC reaches its sexual maturity in 2 years, while the female reaches its sexual maturity in 2–3 years. Wild TC is a carnivorous fish. It feeds on plankton and aquatic insects at the fry stage. TC, with a weight of over 50 g, prefers small shrimps and delicate plants. TC is a kind of large fish with a fast growth, whose meat is pure white and quite delicious. Therefore, it enjoys the reputations in the Yangtze River and Taihu Lake in China because of its important breeding value.

The main differences between BSB and TC are as follows: in the categories, BSB belongs to *Megalobrama*, but TC belongs to *Culter*. Hybridization between these two fishes is intergeneric hybridization. In feeding habit, BSB is herbivorous, whereas TC is carnivorous. In addition, in shape, BSB is a spindle with a higher body width, and TC is a linear with a longer body.

8.1.2 Preparation for F_1 Hybrids

About 5–6 months before the breeding season, the healthy BSB and TC (weights over 1 kg, sexual maturity, well body shape, and bright body color) were bred in a designated pond. In particular, 1 month before the breeding season, the running water was used to stimulate gonadal development in each 2–3 days.

In late May to late June, selecting healthy parents with well gonad development, we injected them with mixed oxytocin, which included luteinizing hormone-releasing hormone analogue (LRH-A) and human chorionic gonadotropin (HCG). The dose of LRH-A was 10 μg/kg and that of HCG was 600 IU/kg. Firstly, we injected the female parents. About 4–5 h later, we injected the male parents with half of that dose. Immediately, we put the male and female parents into a pond with a proportion of 1:(1.25–1.5) to increase their contact opportunities, which could improve the spawning rate of these fishes. The running water was putted into the pond 3–4 h before spawning. Then, these fishes were kept in silent water for spawning smoothly.

The parents were divided into two groups: the first one was female BSB and male TC and the second one was female TC and male BSB. After putting mixed sperm and eggs into a clean and dry ceramic bowl, which was stirred with a dry feather for 2–3 min, we promptly placed the fertilized eggs on the mesh evenly with the feather. Finally, we put them into the hatchery. Cultured in net cage for 1–2 days after hatching, fry were moved into a pond when they was able to swim. About 3–4 days later, we splashed soybean milk into the pond 2–3 times a day until fry could ingest fodder.

By distant hybridization, both of the crosses of BSB (♀) × TC (♂) and TC (♀) × BSB (♂) exhibited the high fertilization rates (78.0% and 75.4%, respectively) and the high hatching rates (62.0% and 68.5%, respectively). These results are shown in Table 8.1 (Xiao 2013; Xiao et al. 2014).

8.1.3 Preparation for F_2 and F_3 Hybrids

The F_2 hybrids were produced by self-crossing of the F_1 hybrids, which were derived from the reciprocal crosses of BSB × TC when they were 2 years old. We found that both of the two diploid F_1 hybrids had the high spawning rates (95.3% and 91.5%, respectively) and male diploid F_1 hybrids could produce a large number of mature sperm. A huge number of the F_2 hybrids from the reciprocal crosses of BSB × TC could be obtained by self-crossing of the F_1 hybrids. The F_3 hybrids were produced by self-crossing of the F_2 hybrids derived from BSB (♀) × TC (♂) and TC (♀) ×

Table 8.1 The rates of fertilization and hatching in the hybrids of BSB and TC (Xiao 2013; Xiao et al. 2014)

	Fertilization rate (%)	Hatching rate (%)
BSB (♀) × TC (♂)	78.0	62.0
TC (♀) ×BSB (♂)	75.4	68.5

BSB (♂), respectively, when they reached sexual maturity (Xiao 2013; Xiao et al. 2014).

8.2 The Biological Characteristics of the Hybrids of BSB (♀) × TC (♂)

8.2.1 The Ploidy and the Karyotype Analysis of F_1 and F_2 Hybrids

Taking the parents as controls, we confirmed that there were two types of fish with different ploidy levels in the F_1 hybrids of BSB (♀) × TC (♂) based on DNA content and distribution of chromosome counts (Fig. 8.1). They were diploids with 48 chromosomes ($2n = 48$) (Fig. 8.1c) and triploids with 72 chromosomes ($3n = 72$) (Fig. 8.1d). Only diploids with 48 chromosomes ($2n = 48$) were detected in $2n\text{TB}_2$ (Fig. 8.1e, g). The DNA content of the different hybrids was shown in Table 8.2, and the chromosome numbers were presented in Table 8.3. The results of karyotype analysis showed that BSB had a similar karyotype with TC (18m+22sm +8st) (Fig. 8.2a, b). All the diploid hybrids had a similar karyotype with their original parents (18m+22sm+8st) (Fig. 8.2c, e–g). And the karyotype of $3n\text{BT}_1$ was 27m +33sm+12st (Fig. 8.2d) (Xiao 2013; Xiao et al. 2014).

8.2.2 The Appearance and the Feeding Habit of F_1 and F_2 Hybrids

The appearance of two reciprocal hybrids of BSB × TC was presented in Fig. 8.3. Some characteristics of the hybrids, such as lateral line scales, upper lateral line scales (Table 8.4), and body length/head height (Table 8.5), were intermediate between those of their original parents and were significantly different from their original parents ($p < 0.01$), which reflected obvious hybrid phenotype. In addition, we detected some variations in phenotypes (Table 8.5).

In dietary habit, BSB was herbivorous and its dental formula of pharyngeal teeth was 2.5.3/(4-3)/4.5.2 (Table 8.6). TC was carnivorous and its dental formula of pharyngeal teeth was 2.4.5/(4-5)/4.4.2 (Table 8.6). The $2n\text{BT}_1$, $2n\text{BT}_2$, $2n\text{TB}_1$, and $2n\text{TB}_2$, among the hybrids derived from BSB and TC, were herbivorous, and their dental formula was 2.5.3/(4-3)/4.5.2 (Table 8.6), which was similar to BSB (Liu 2013). The dietary habits of the hybrids were inherited from herbivorous BSB (Xiao 2013).

8.2.3 Fertility Detection of F_1 and F_2 Hybrids

Well-developed gonads were observed in the two reciprocal diploid F_1 and F_2 hybrids of the cross of BSB × TC. The ovaries of the 15-month-old ones were flat and flesh red. The results of paraffin section showed that the oocytes were rich in ovaries, which were at stage II (Fig. 8.4a, e, g, i). The testis of male diploids was gray

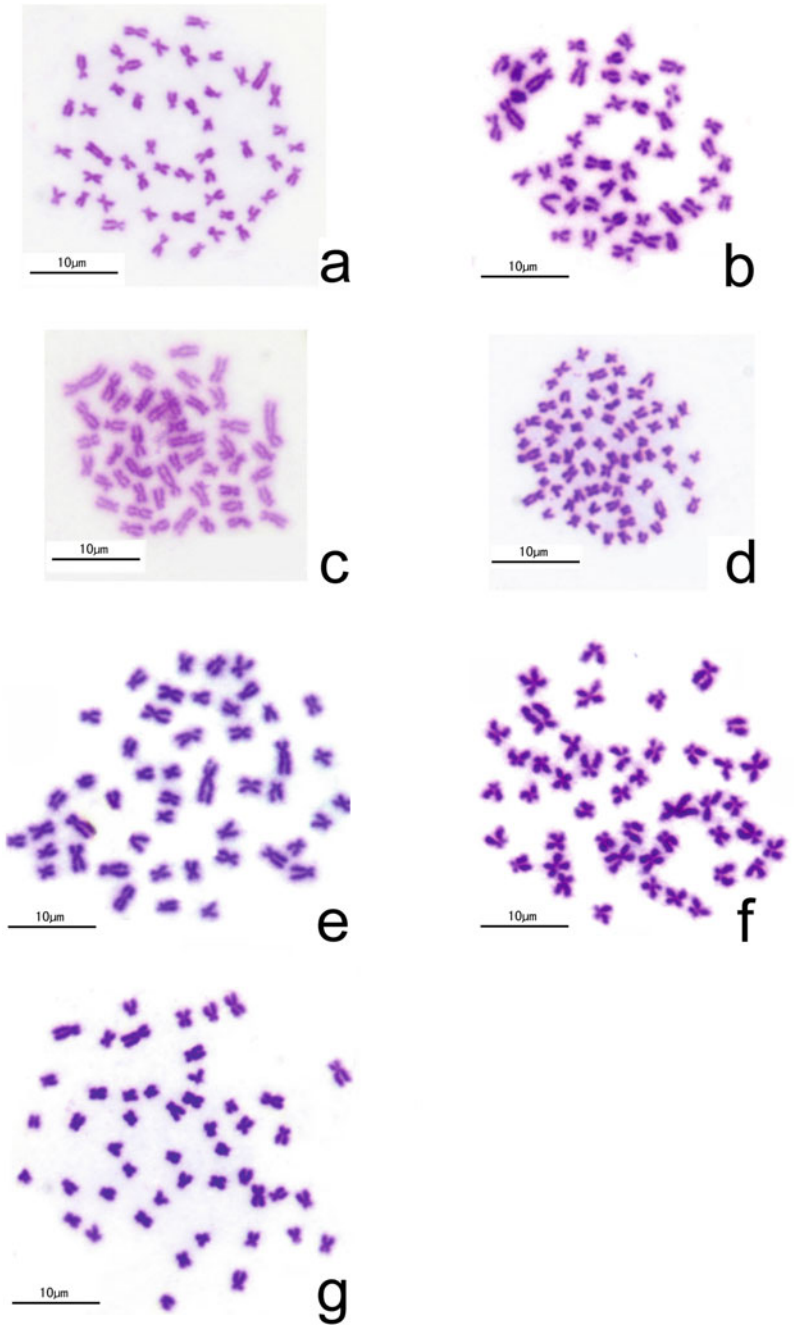

Fig. 8.1 The chromosome spreads at metaphase in BSB, TC, and their hybrids (Xiao 2013; Xiao et al. 2014). (**a**) The chromosome spread at metaphase in BSB ($2n = 48$). (**b**) The chromosome spread at metaphase in TC ($2n = 48$). (**c**) The chromosome spread at metaphase in $2n\mathrm{BT}_1$ ($2n = 48$).

Table 8.2 The erythrocyte DNA content of BSB, TC, and their hybrids (Xiao 2013; Xiao et al. 2014)

Fish type	Mean DNA content	Ratio	
		Observed	Expected
BSB	74.55		
TC	68.78		
$2n\text{BT}_1$	67.20	$2n\text{BT}_1/(0.5\ \text{BSB} + 0.5\ \text{TC}) = 0.94^a$	1
$3n\text{BT}_1$	102.97	$3n\text{BT}_1/(\text{BSB} + 0.5\ \text{TC}) = 0.95^a$ $3n\text{BT}_1/(0.5\ \text{BSB} + \text{TC}) = 0.97^a$	1
$2n\text{BT}_2$	68.73	$2n\text{BT}_2/(0.5\ \text{BSB} + 0.5\ \text{TC}) = 0.96^a$	1
$2n\text{TB}_1$	69.01	$2n\text{TB}_1/(0.5\ \text{BSB} + 0.5\ \text{TC}) = 0.96^a$	1
$2n\text{TB}_2$	70.28	$2n\text{TB}_2/(0.5\ \text{BSB} + 0.5\ \text{TC}) = 0.98^a$	1

[a]The observed ratio was not significantly different ($p > 0.05$) from the expected ratio

Table 8.3 The chromosome number in BSB, TC, and their hybrids (Xiao 2013; Xiao et al. 2014)

Fish type	Number of samples	Number of metaphase spreads	Chromosome number			Chromosome number		
			<45	<45	48	<68	68–71	72
BSB	10	100	1	4	95			
TC	10	100	1	5	96			
$2n\text{BT}_1$	10	100	1	5	94			
$2n\text{BT}_2$	10	100	1	4	95			
$2n\text{TB}_1$	10	100	2	5	93			
$2n\text{TB}_2$	10	100	1	5	94			
$3n\text{BT}_1$	10	100				2	7	91

white and white semen can be stripped out from small amount of individuals. Studies of paraffin section indicated that a large number of spermatids were observed in the seminiferous tubules (Fig. 8.4b, f, h, j). A large number of sperm observed by scanning electron microscope reflected the normal development of the testis (Fig. 8.5a–h). An asymmetric development in the ovary was detected in 15-month-old $3n\text{BT}_1$. The results of paraffin section showed that most of the germ cells were still in the period of oogonium, and only a part of oogonium developed into stage II (Fig. 8.4c), because they were not in synchronized development. There were a large number of tubular structures in 15-month-old testes. However, there were no spermatogonia or sperm in them (Fig. 8.4d) (Xiao 2013; Xiao et al. 2014).

Fig. 8.1 (continued) (**d**) The chromosome spread at metaphase in $3n\text{BT}_2$ ($3n = 72$). (**e**) The chromosome spread at metaphase in $2n\text{BT}_2$ ($2n = 48$). (**f**) The chromosome spread at metaphase in $2n\text{TB}_1$ ($2n = 48$). (**g**) The chromosome spread at metaphase in $2n\text{TB}_2$ ($2n = 48$)

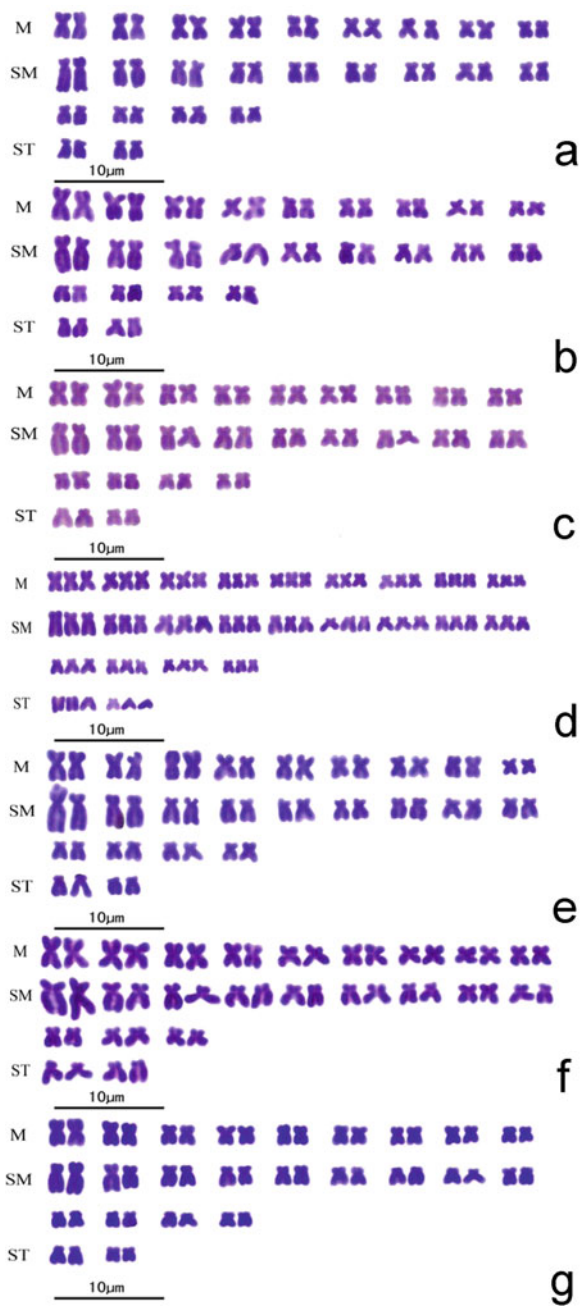

Fig. 8.2 The karyotype in metaphases of BSB, TC, and their hybrids (Xiao 2013; Xiao et al. 2014). (**a**) The karyotype of BSB (18m +22sm+8st). (**b**) The karyotype of TC (18m+22sm +8st). (**c**) The karyotype of $2n\text{BT}_1$ (18m+22sm+8st). (**d**) The karyotype of $3n\text{BT}_1$ (27m +33sm+12st). (**e**) The karyotype of $2n\text{BT}_2$ (18m +22sm+8st). (**f**) The karyotype of $2n\text{TB}_1$ (18m +22sm+8st). (**g**) The karyotype of $2n\text{TB}_2$ (18m +22sm+8st)

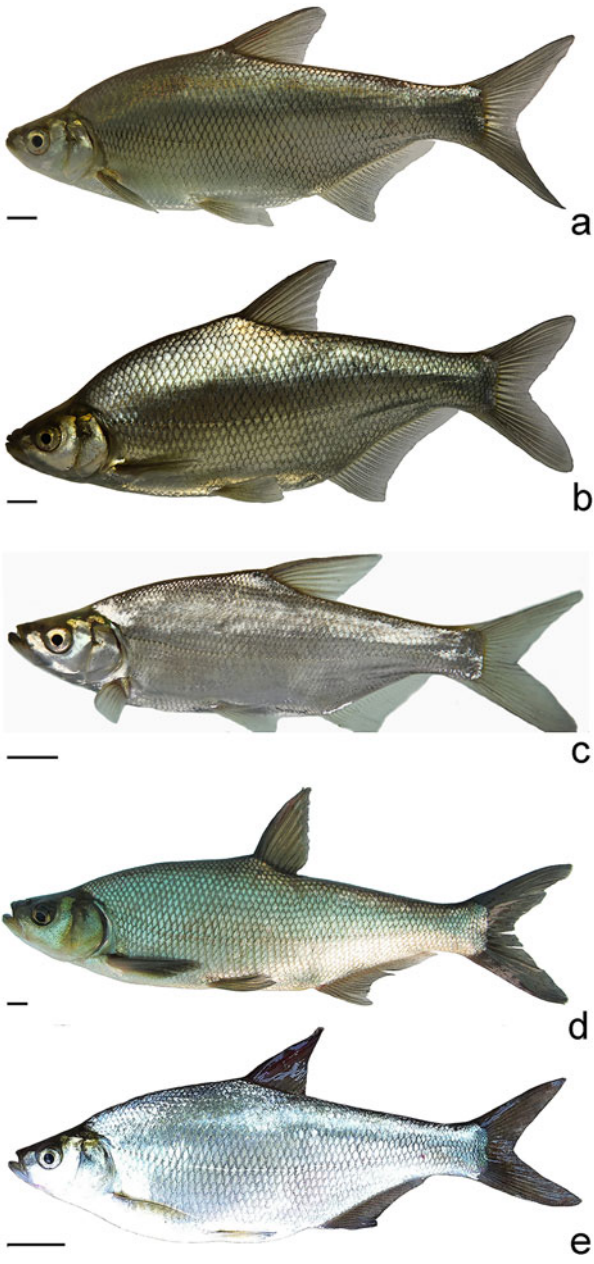

Fig. 8.3 The appearance of the two reciprocal crosses of BSB × TC (Xiao 2013). (**a**) The appearance of $2n\mathrm{BT}_1$. (**b**) The appearance of $3n\mathrm{BT}_1$. (**c**) The appearance of $2n\mathrm{BT}_2$. (**d**) The appearance of $2n\mathrm{TB}_1$. (**e**) The appearance of $2n\mathrm{TB}_2$. Bar = 1 cm

Table 8.4 The comparison on countable traits of BSB, TC, and their hybrids (Xiao 2013)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins
BSB	50.9 ± 0.99 (49–52)	9.4 ± 0.52 (9–10)	9.9 ± 0.86 (9–11)	III + 8.4 ± 0.51 (III + 8–9)	8.9 ± 0.86 (8–10)	III + 25.7 ± 0.67 (III + 25–27)
TC	86.6 ± 3.09 (80–92)	17.1 ± 1.20 (16–19)	6.5 ± 0.53 (6–7)	III + 7.0 ± 0.00 (III + 7)	9.0 ± 0.00 (9)	III + 21.9 ± 0.99 (III + 20–23)
$2nBT_1$	62.1 ± 2.23 (59–64)	12.4 ± 0.70 (11–13)	10.1 ± 0.57 (9–11)	III+8.0±0.00 (III+8)	9.1 ± 0.32 (9–10)	III + 23.3 ± 0.67 (III + 22–24)
$3nBT_1$	63.7 ± 1.95 (61–68)	13.1 ± 0.86 (12–14)	10.7 ± 0.67 (9–11)	III + 8.0 ± 0.00 (III + 8)	9.6 ± 0.52 (9–10)	III + 24.2 ± 0.92 (III + 23–26)
$2nBT_2$	60.8 ± 2.57 (57–64)	12.7 ± 0.67 (12–14)	10.1 ± 0.57 (9–11)	III + 8.1 ± 0.31 (III + 8–9)	9.2 ± 0.42 (9–10)	III + 22.5 ± 1.43 (III + 20–25)
$2nTB_1$	64.5 ± 2.59 (60–69)	13.1 ± 0.74 (12–14)	10.1 ± 0.57 (9–11)	III + 8.0 ± 0.00 (III + 8)	9.1 ± 0.32 (9–10)	III + 23.0 ± 0.94 (III + 21–24)
$2nTB_2$	60.7 ± 3.40 (55–64)	13.0 ± 1.05 (11–15)	9.7 ± 0.67 (9–10)	III + 8.0 ± 0.00 (III + 8)	9.2 ± 0.42 (9–10)	III + 22.3 ± 1.16 (III + 21–24)

Table 8.5 The comparison on measurable traits of BSB, TC, and their hybrids (Xiao 2013)

Fish type	Overall length/body length	Body length/body depth	Body length/head length	Head length/head height	Caudal peduncle length/caudal peduncle depth	Body depth/head height
BSB	1.18 ± 0.02	2.36 ± 0.04	4.61 ± 0.03	1.19 ± 0.06	1.04 ± 0.03	2.08 ± 0.05
TC	1.18 ± 0.03	3.97 ± 0.23	5.03 ± 0.31	2.18 ± 0.27	1.17 ± 0.03	2.27 ± 0.01
$2nBT_1$	1.19 ± 0.01	3.33 ± 0.11	4.96 ± 0.10	1.83 ± 0.54	1.37 ± 0.09	2.73 ± 0.04
$3nBT_1$	1.19 ± 0.02	3.27 ± 0.11	4.98 ± 0.12	1.78 ± 0.09	1.34 ± 0.08	2.72 ± 0.16
$2nBT_2$	1.17 ± 0.02	3.34 ± 0.16	5.01 ± 0.11	1.68 ± 0.09	1.50 ± 0.14	2.53 ± 0.14
$2nTB_1$	1.19 ± 0.02	3.47 ± 0.24	5.02 ± 0.19	1.63 ± 0.21	1.40 ± 0.07	2.56 ± 0.07
$2nTB_2$	1.19 ± 0.02	3.30 ± 0.18	4.93 ± 0.11	1.75 ± 0.07	1.51 ± 0.19	2.62 ± 0.14

Table 8.6 The dental formula of pharyngeal teeth of BSB, TC, and their hybrids (Liu 2013)

Fish type	Dental formula of pharyngeal teeth
TC	2.4.5/(4-5)/4.4.2
BSB	2.5.3/(4-3)/4.5.2
$2nBT_1$	2.5.3/(4-3)/4.5.2
$2nBT_2$	2.5.3/(4-3)/4.5.2
$2nTB_1$	2.5.3/(4-3)/4.5.2
$2nTB_2$	2.5.3/(4-3)/4.5.2

8.2.4 Fertility of F_1 Hybrids

The two diploid F_1 hybrids were obtained from reciprocal crosses of BSB and TC. These hybrids didn't exhibit a low fertility or sterility, which was always observed in most of the other F_1 hybrids led by distant hybridization. We found that both of the two reciprocal diploid F_1 hybrids of BSB and TC had the high induced spawning rates (95.3% and 91.5%, respectively), while a large number of sperm were observed in the diploid F_1 male hybrids with sexual maturity. Meanwhile, the high fertilization rates and hatching rates were observed in the self-crossed F_1 hybrids (Table 8.7) (Xiao 2013; Xiao et al. 2014). Two million $2nBT_2$ and 0.3 million $2nTB_2$ were obtained in the year 2011.

8.2.5 The Molecular Genetic Characteristics of the Hybrids

8.2.5.1 The *5S* rDNA Genetic Mutation of the Hybrid Lineages of BSB and TC

The coding region of *5S* rDNA sequences of BSB and TC was highly conserved. In these five hybrids derived from the hybridization of BSB and TC, nearly all of them inherited the genes from their parents. Only T→C transition of *5S* rDNA was found in the 73 nucleotide sites of $2nBT_2$, $2nTB_1$, and $2nTB_2$. And G→A transition of *5S* rDNA was detected in the 93 nucleotide sites of $2nBT_1$ (Kang 2013).

Some mutations occurred in nontranscribed spacer (NTS) of *5S* rDNA in the conserved coding region. Some base substitutions and insertions were detected in NTS regions by comparative analysis of the five hybrids and their original parents. A fragment of poly(A) sequence was inserted in the NTS-II sequence of all the hybrids, compared to the NTS-I of their original parents, and an additional fragment of CATTTT variation sequence was inserted in the diploid hybrids of TC (♀) and BSB (♂). Sequence variations of the NTS-II in all hybrids mainly referred to base substitutions and deletions, compared to the NTS-II in TC (Kang 2013).

8.2.5.2 The Variation on Genotypes and Phenotypes of *45S* rDNA

The two reciprocal diploid F_1 hybrids of BSB and TC inherited and expressed specific *45S* rDNA of their original parents (Figs. 8.6 and 8.7), while the genetic features and expression patterns of *45S* rDNA genes were different from the ones in the F_2 hybrids. Some individuals of the F_2 hybrid of BSB and TC inherited the *45S* rDNA gene from their original parents, but they only expressed the *45S* rRNA of one

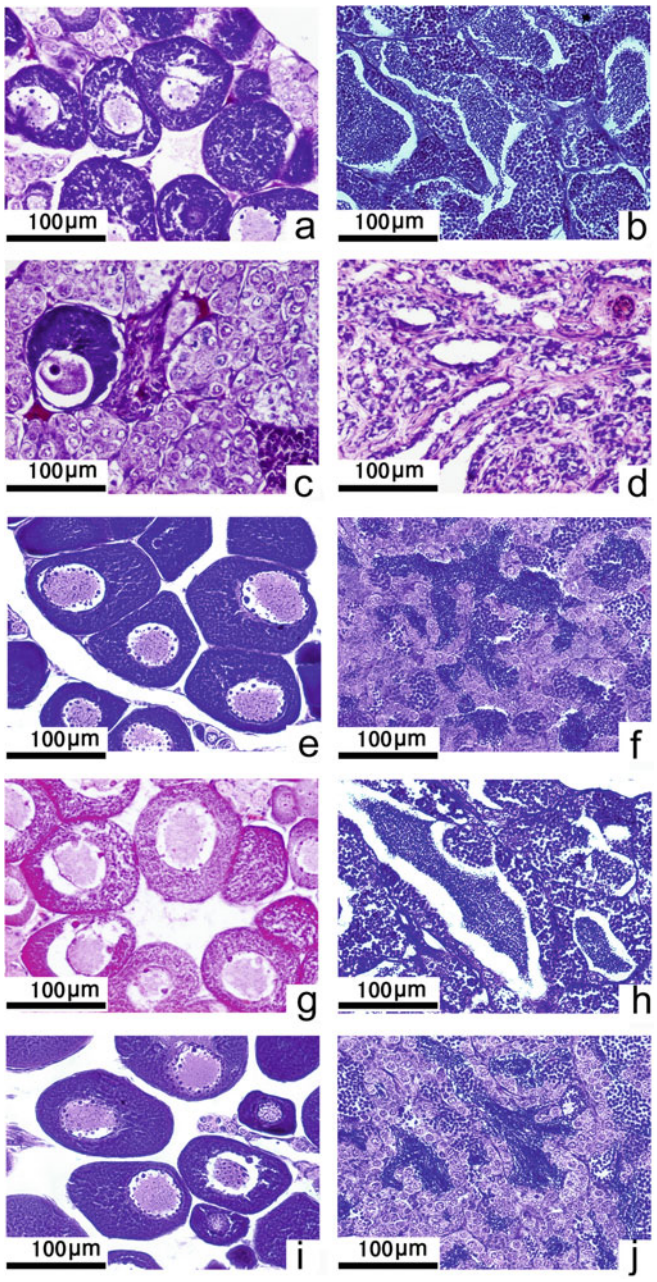

Fig. 8.4 The histological section of gonadal development in the two reciprocal hybrid lineages of BSB and TC. (**a**) The histological section of the ovary of 15-month-old $2nBT_1$. (**b**) The histological section of the testis of 15-month-old $2nBT_1$. (**c**) The histological section of the ovary of 15-month-old $3nBT_1$. (**d**) The histological section of the testis of 15-month-old $3nBT_1$. (**e**) The histological section of the ovary of 15-month-old $2nBT_2$. (**f**) The histological section of the testis of 15-month-

of the original parents (Figs. 8.6 and 8.7). In addition, some individuals of the F_2 hybrid of BSB and TC only inherited the *45S* rDNA gene from one of the original parents (Figs. 8.6 and 8.7).

8.2.5.3 The Analyses of Microsatellite DNA and *Hox* Gene Family in the Two Reciprocal Hybrid Lineages of BSB and TC

Some analyses of the genetic structure were performed in BSB, TC, and their F_1 hybrids based on the 15 pairs of microsatellite primers, which were designed by the microsatellite DNA of BSB. These results showed that 73 alleles were amplified in BSB, TC, and their F_1 hybrids. The F_1 hybrids exhibited higher heterozygosity and genetic diversity compared with their parents. Meanwhile, amplification sites of primer TTF08 in these three samples were different, so it can be considered as a molecular genetic marker to distinguish $2n\text{BT}_1$ and their original parents. We built neighbor-joining method with the clustering algorithm to calculate genetic similarity and genetic distance of these three samples, which demonstrated that $2n\text{BT}_1$ exhibited a closer genetic relationship with BSB (Nei 1978; Song 2013).

We conducted PCR amplification on DNA sequences of part of *hox* genes in BSB, TC, and $2n\text{BT}_1$ hybrids with specific degenerate primers of *hox* genes designed by the *hox* genes sequence of zebrafish, and we obtained 700–1600 bp fragments in these three samples. They exhibited the structure of "exon 1-intron-exon 2," while the separated bases of the two ends of intron were conformed to "GT-AG" rule.

Alignment of the *hox* gene sequences in BSB, TC, and $2n\text{BT}_1$ hybrids showed that base substitutions and deletions occurred in *hox* genes of $2n\text{BT}_1$ hybrids and there was a DNA recombination event in the *hoxd9a* gene of $2n\text{BT}_1$ hybrids. Recombination event was detected in intron region. The former part of nucleotide sequences in *hoxd9a* gene was the same to the ones in BSB, while the latter part of nucleotide sequences was the same as the ones in TC (Song 2013).

8.2.5.4 The Allelic Recombinant Genes Detected in the Hybrid Lineages

From F_1 to F_3 in the two reciprocal hybrid lineages of BSB and TC, 348–8940 recombinant reads (0.0033–0.0624% in all uniquely mapped reads) supported a distribution of 145 to 974 allelic recombinant genes in all transcriptomes. An average of only 0.0166% recombinant reads was detected; the average number of recombinant reads per gene was 5.93. A gradually increasing trend of the number of allelic recombinant events was found from F_1 to F_3 in hybrid lineage of TC (♀) and BSB (♂). However, this trend was not observed in hybrid lineage of BSB (♀) and TC (♂). To validate the allelic recombinant events from Illumina data, 14 genes were confirmed at the genomic DNA level using Sanger sequencing, while most of the

Fig. 8.4 (continued) old $2n\text{BT}_2$. (**g**) The histological section of the ovary of 15-month-old $2n\text{TB}_1$. (**h**) The histological section of the testis of 15-month-old $2n\text{TB}_1$. (**i**) The histological section of the ovary of 15-month-old $2n\text{TB}_2$. (**j**) The histological section of the testis of 15-month-old $2n\text{TB}_2$

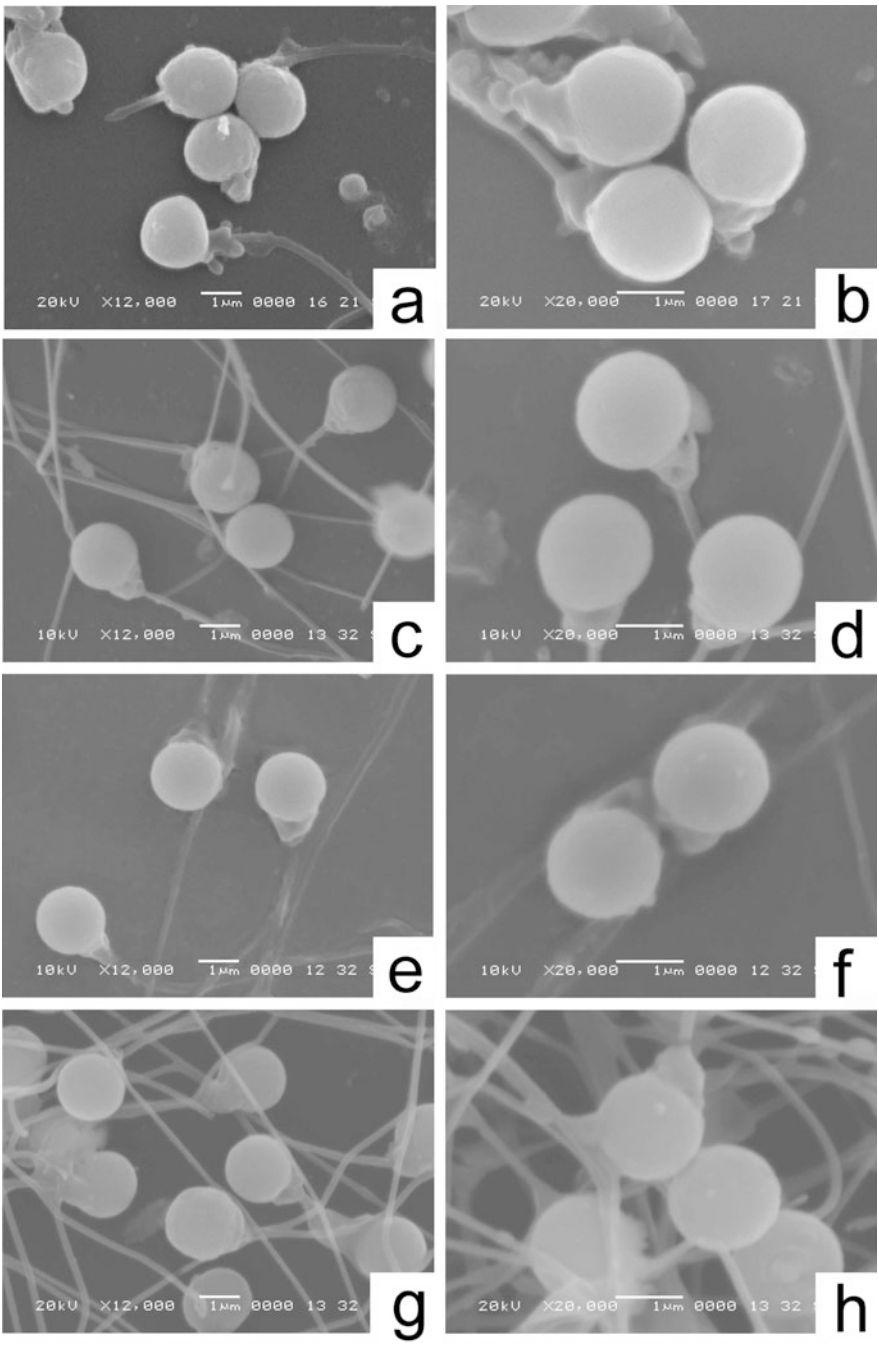

Fig. 8.5 The morphological structures of sperm in the two reciprocal hybrid lineages of BSB and TC (Xiao 2013; Xiao et al. 2014). (**a**, **b**) The structure of sperm of $2n\mathrm{BT}_1$ under the scanning electron microscope. (**c**, **d**) The structure of sperm of $2n\mathrm{BT}_2$ under the scanning electron

Table 8.7 The fertilization rates and hatching rates observed in self-crossing of $2n\text{BT}_1$ and $2n\text{TB}_1$ (Xiao 2013; Xiao et al. 2014)

Fish type	Fertilization rate (%)	Hatching rate (%)
$2n\text{BT}_1$	82.5	73.6
$2n\text{TB}_1$	80.8	71.3

allelic recombinant genes (48 of 49 in $2n\text{BT}_3$ and 50 of 52 in $2n\text{TB}_3$) detected by PacBio sequencing were consistent with the Illumina data (Ren et al. 2019).

8.2.6 Application of Up-Mouth Bream

As we mentioned above, the establishment of the fertile hybrid lineages had some important application values in the fishery. Some varieties with heterosis could be produced by backcrossing the fertile hybrid lineages with their original parents. They exhibited some advantages in faster growth, stronger stress resistance, meat quality, and appearance. Up-mouth bream (BTB) was obtained by the cross of $2n\text{BT}_1$ (♀) × BSB (♂), which grew faster (20%) than BSB, indicating an obvious heterosis of growth rate in BTB (Xiao 2013). High density cultivation was suited to BTB. In feeding habits, BTB was a herbivorous fish, whose food came from natural plants. Currently, BTB was widely bred in China. In addition, the other hybrids with heterosis could be obtained from the backcrossing of the two reciprocal hybrid lineages of BSB and TC.

8.3 The Formation and the Biological Characteristics of the Improved Hybrid Culter

8.3.1 The Formation and the Ploidy of the Improved Hybrid Culter

During the breeding season (May to June), the mature female BTB and mature male TC were collected. The improved hybrid culter (BTBT) was produced by the hybridization between female BTB and male TC. The rates of fertilization and hatching were calculated using 10,000 embryos, and the results showed 88.17% fertilization rate and 75.05% hatching rate. Lastly, BTBT were transferred to ponds for growth. The results of DNA contents and chromosome numbers showed that BTBT had 48 chromosomes and it was a diploid (Table 8.8, Fig. 8.8a–d) (Wu et al. 2020).

Fig. 8.5 (continued) microscope. (**e**, **f**) The structure of sperm of $2n\text{TB}_1$ under the scanning electron microscope. (**g**, **h**) The structure of sperm of $2n\text{TB}_2$ under the scanning electron microscope

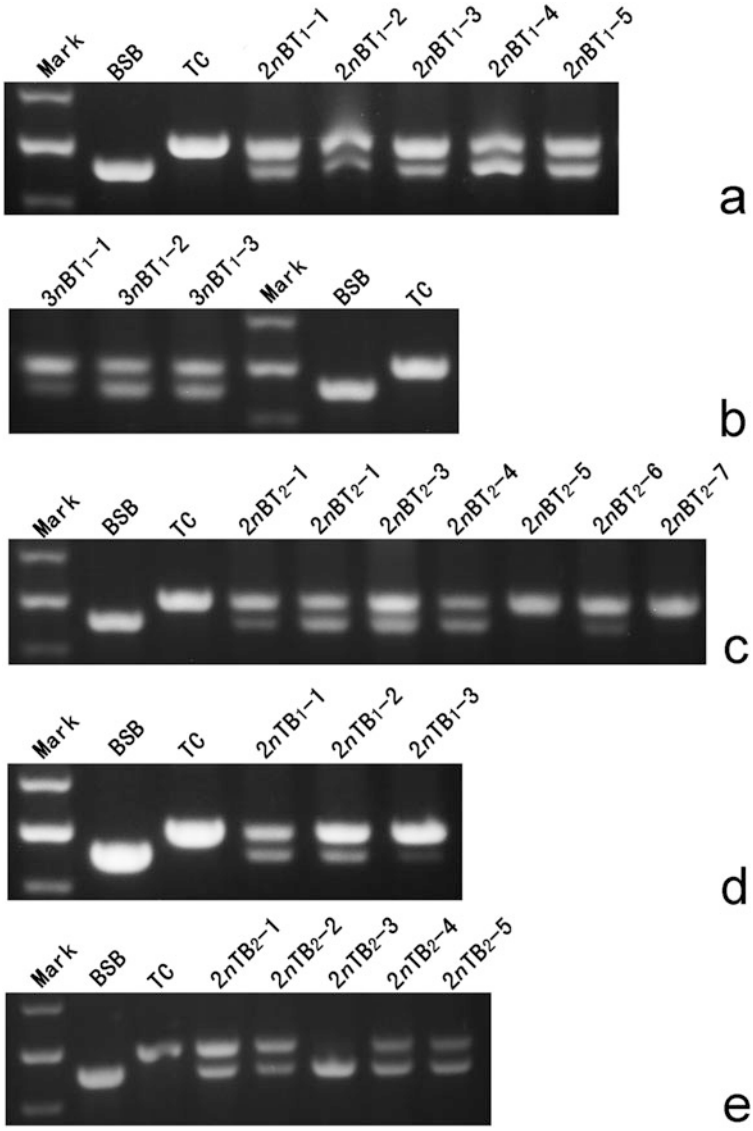

Fig. 8.6 The gel electrophoresis images of internal transcribed spacer (ITS)-I sequences of 45*S* rDNA from two hybrid lineages of BSB and TC and their original parents (Xiao 2013). (**a**) The ITS-I of $2nBT_1$. (**b**) The ITS-I of $3nBT_1$. (**c**) The ITS-I of $2nBT_2$, the band of BSB was deleted in samples 5 and 7. (**d**) The ITS-I of $2nTB_1$. (**e**) The ITS-I of $2nTB_2$, the band of TC was deleted in sample 3. Mark: 100 bp DNA marker

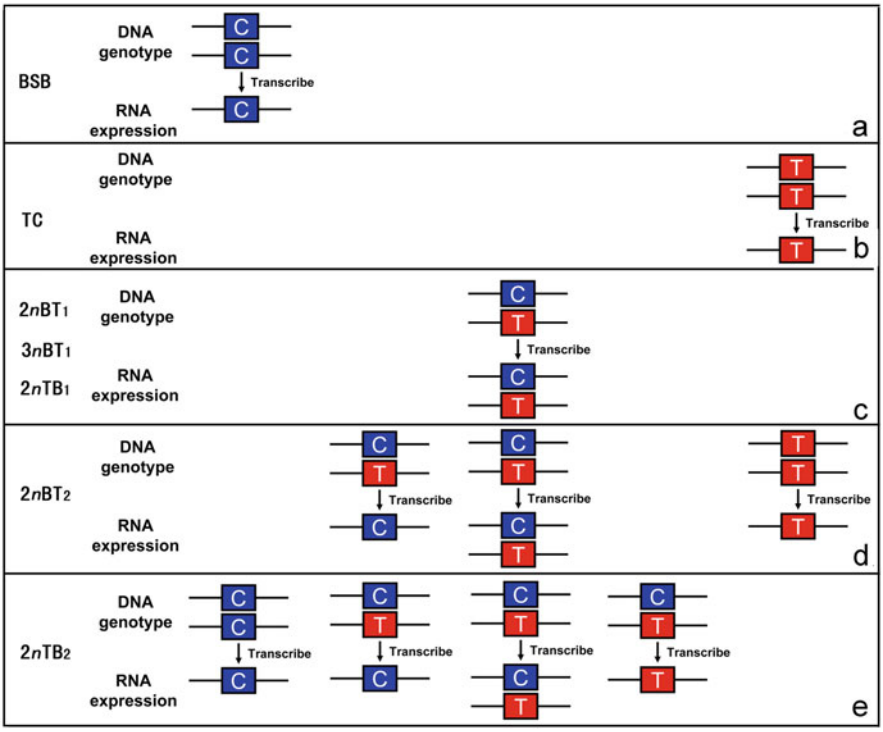

Fig. 8.7 The schematic diagrams of genotypes and phenotypes of TC, BSB, and their two reciprocal hybrid lineages (Xiao et al. 2016). (**a**) The *45S* rRNA in BSB. (**b**) The *45S* rRNA in TC. (**c**) The *45S* rRNA in $2n\text{BT}_1$, $3n\text{BT}_1$, and $2n\text{TB}_1$. (**d**) The *45S* rRNA in $2n\text{BT}_2$. (**e**) The *45S* rRNA in $2n\text{TB}_2$

8.3.2 The Appearance and Fertility of the Hybrid Culter

The appearance of BSB, TC, BTB, and BTBT was shown in Fig. 8.9a–d. The measurable traits including body height/head height, body length/body height, caudal peduncle length/caudal peduncle depth, and head length/head height of BTBT were between those of BSB and TC (Table 8.9), indicating the hybrid characteristics in BTBT. However, some measurable traits of BTBT such as body length/body height and head length/head height were similar to those in TC. In addition, the countable traits including the numbers of lateral line scales, scales above lateral line, and scales below lateral line in BTBT were between those in BSB and TC (Table 8.10). Although the hybrid features were detected in most of morphological traits, the appearance of BTBT was more similar to TC than BSB, especially in the body and head (Wu et al. 2020).

The histological sectioning results regarding BTBT gonads were shown in Fig. 8.10. In female 15-month-old BTBT, the yolk nucleus located near the cell nucleus and the ovaries were rich in oocytes, meaning they were at stage II

Table 8.8 The mean DNA contents of BSB, TC, BTB, and BTBT (Wu et al. 2020)

Fish type	Mean DNA content	Ratio	
		Observed	Expected
BSB	57.89		
TC	58.80		
BTB	62.66		
BTBT	57.94	BTBT/BSB = 1.00[a]	1
		BTBT/TC = 0.99[a]	
		BTBT/BTB = 1.08[a]	

[a]There is no significant difference ($p > 0.05$) between the observed ratio and expected ratio

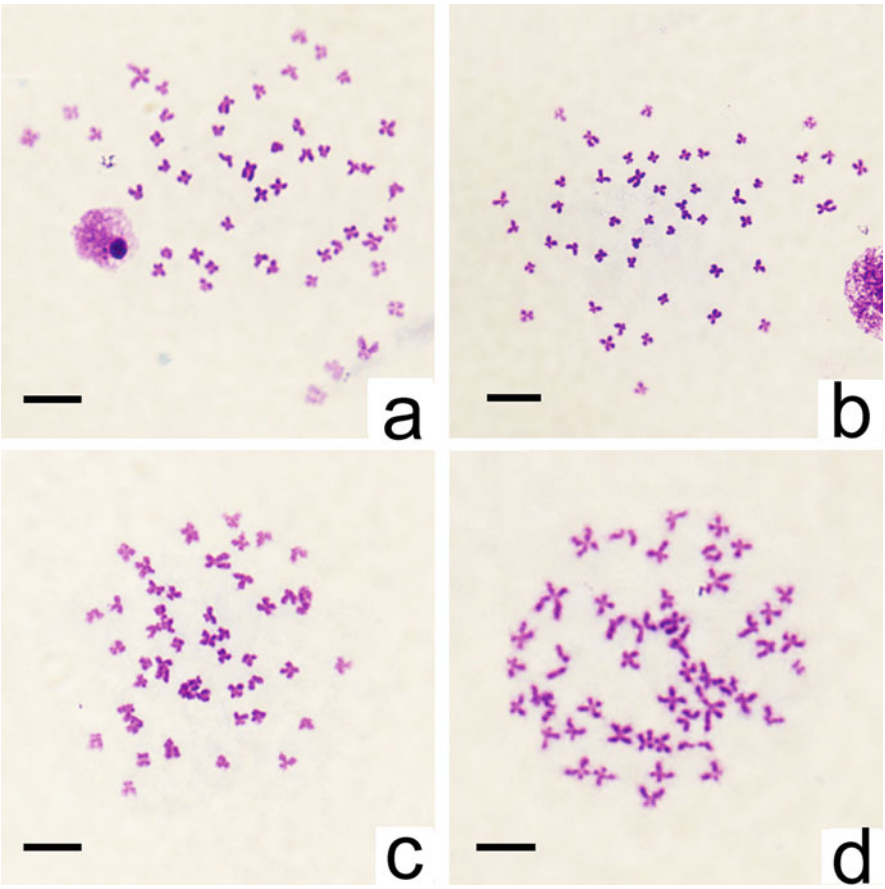

Fig. 8.8 The BSB, TC, BTB, and BTBT chromosome distribution at metaphase (Wu et al. 2020). (**a**) The BSB had 48 chromosomes observed. (**b**) The TC had 48 chromosomes. (**c**) The BTB had 48 chromosomes. (**d**) The BTBT had 48 chromosomes. Bar = 20 μm

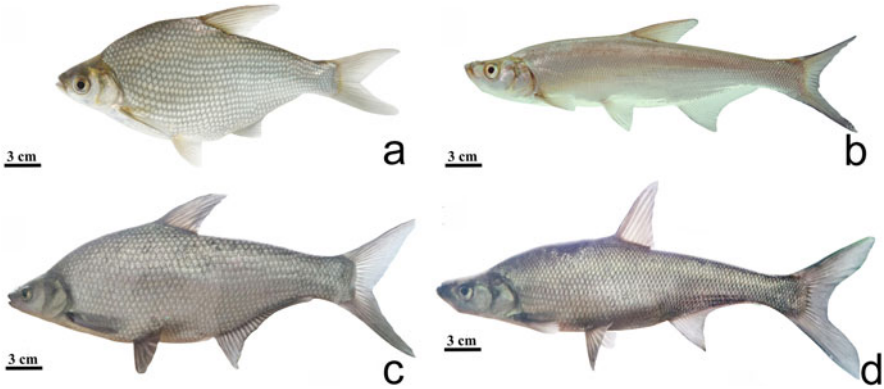

Fig. 8.9 The appearance of BSB, TC, BTB, and BTBT (Wu et al. 2020). (**a**) The appearance of BSB. (**b**) The appearance of TC. (**c**) The appearance of BTB. (**d**) The appearance of BTBT

(Fig. 8.10a). In male 15-month-old BTBT, white semen could be extruded easily. Meanwhile, the histological sectioning results showed that secondary spermatocytes were filled in seminiferous tubules, meaning the testes were at stage IV (Fig. 8.10b). In addition, a large number of offspring could be produced by self-crossing of BTBT (Wu et al. 2020).

8.3.3 The Application of the Hybrid Culter

To detect the number of intermuscular bones (abbreviated as IBs), the way of X-ray detection was performed. The IB numerical analysis was further performed, in which samples were divided into left or right epineural bones (abbreviated as Len or Ren) and left or right epipleural bones (abbreviated as Lep or Rep). The results of X-ray and IB numerical analyses were presented in Figs. 8.11a–h and 8.12a–d, respectively. The skeletal X-rays showed the specific locations of IBs. The mean IB numbers of BSB were 120.0 ± 3.58, the mean IB numbers of TC were 135.6 ± 2.42, the mean IB numbers of BTB were 123.4 ± 2.15, and the mean IB numbers of BTBT were 127.8 ± 2.04, respectively. Furthermore, the IB numbers of BTB were between BSB and TC, and the IB numbers of BTBT were between BTB and TC. Therefore, the BTBT had fewer IBs than that in TC (Wu et al. 2020).

8.3.4 The Molecular Genetic Characteristics of the Hybrid Culter

The genetic composition of BTBT was detected by the ITS-I of *45S* rDNA using the primers described in Xiao et al. (2016). In the genome of both BTB and BTBT, different ITS-I sequences derived from BSB and TC genome were found, respectively (named as BTB-T, BTB-B, BTBT-T, and BTBT-B, respectively) (Fig. 8.13),

Table 8.9 The measurable traits of BSB, TC, BTB, and BTBT (Wu et al. 2020)

Fish type	Overall length/body length	Body length/body depth	Body length/head length	Head length/head height	Body depth/head height	Caudal peduncle length/caudal peduncle depth
BSB	1.18 ± 0.03	2.36 ± 0.04	4.52± 0.21	1.21 ± 0.12	2.10 ± 0.21	1.07 ± 0.10
TC	1.18 ± 0.02	4.02 ± 0.23	5.11 ± 0.25	2.05 ± 0.25	2.37 ± 0.12	1.24 ± 0.12
BTB	1.19 ± 0.03	2.76 ± 0.17	4.62 ± 0.32	1.43 ± 0.18	2.25 ± 0.18	1.21 ± 0.08
BTBT	1.18 ± 0.01	3.80 ± 0.15	4.48 ± 0.19	1.80 ± 0.11	2.11 ± 0.06	1.13 ± 0.05

Table 8.10 The countable traits of BSB, TC, BTB, and BTBT (Wu et al. 2020)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins
BSB	50.9 ± 0.99 (49–52)	9.4 ± 0.52 (9–10)	9.9 ± 0.86 (9–11)	III + 8.4 ± 0.51 (III+8–9)	8.9 ± 0.86 (8–10)	III + 25.7 ± 0.67 (III + 25–27)
TC	86.6 ± 3.09 (80–92)	17.1 ± 1.20 (16–19)	6.5 ± 0.53 (6–7)	III + 7.0 ± 0.00 (III + 7)	9.0 ± 0.00 (9)	III + 21.9 ± 0.99 (III + 20–23)
BTB	56.9 ± 2.42 (53–61)	12.0 ± 0.47 (11–13)	10.1 ± 0.74 (9–11)	III + 8.0 ± 0.00 (III+8)	9.0 ± 0.00 (9)	III + 25.5 ± 1.08 (III + 24–27)
BTBT	69.0 ± 2.45 (66–71)	13.5 ± 0.49 (12–14)	9.5 ± 0.80 (9–11)	III + 8.2 ± 0.40 (III+8–9)	9.0 ± 0.00 (9)	III + 25.2 ± 0.75 (III + 24–26)

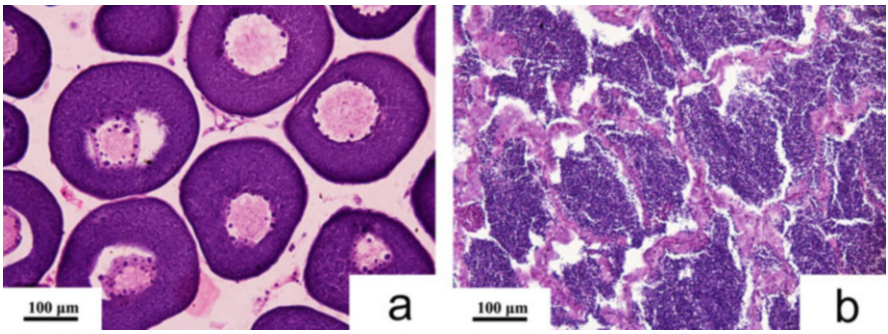

Fig. 8.10 The gonadal development of BTBT (Wu et al. 2020). (**a**) The gonadal development of 15-month-old BTBT ovary. (**b**) The gonadal development of 15-month-old BTBT testis

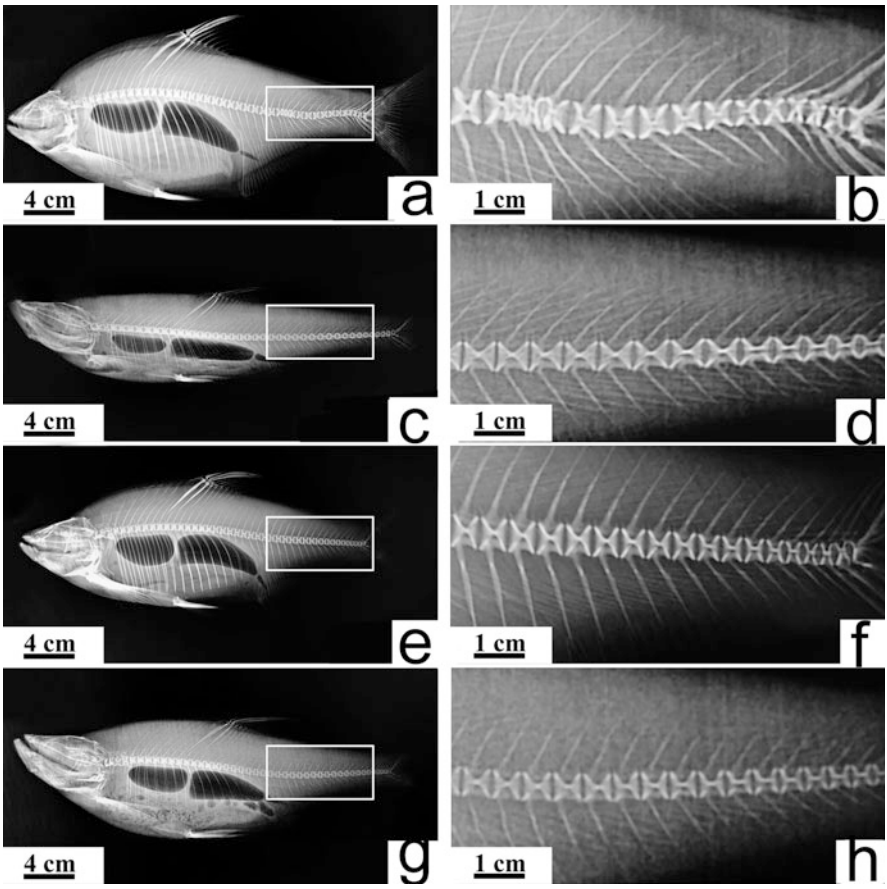

Fig. 8.11 The skeleton diagrams of BSB, TC, BTB, and BTBT by X-ray (Wu et al. 2020). (**a**, **b**) The skeleton diagram of BSB. (**c**, **d**) The skeleton diagram of TC. (**e**, **f**) The skeleton diagram of BTB. (**g**, **h**) The skeleton diagram of BTBT

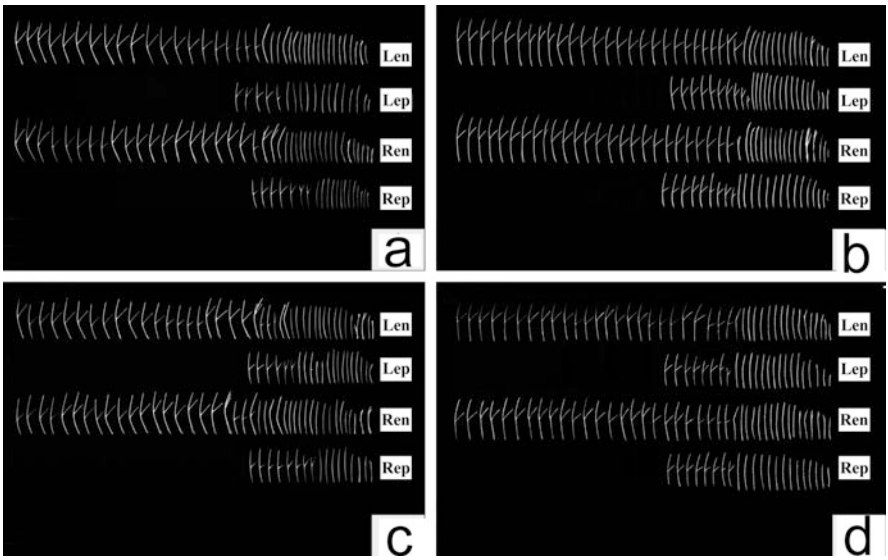

Fig. 8.12 The IB morphology of BSB, TC, BTB, and BTBT (Wu et al. 2020). (**a**) The IB morphology of BSB. (**b**) The IB morphology of TC. (**c**) The IB morphology of BTB. (**d**) The IB morphology of BTBT

illustrating that both BTB and BTBT had the hybrid genomes. In addition, one TC species-specific site of ITS-I was integrated into the sequence of BTBT-B at position 135; one BSB species-specific site of *18S* rDNA was integrated into that of BTB-T and BTBT-T at position 495, meaning recombination events could occur during the process of hybridization (Wu et al. 2020).

8.4 The Formation of the Improved Hybrid Bream

As we mentioned above, the establishments of the fertile hybrid lineages show some important application values in the fishery. The two types of hybrid bream were successfully obtained from the backcrossing of BTB with BSB (abbreviated as BBTB and BTBB, respectively). The number of lateral line scales in the two types of hybrid bream was different with the one in BSB. Although some hybrid features of morphological traits were found in BBTB and BTBB, the appearance of them was more similar to that of BSB than the one of TC. Moreover, PCR amplification was conducted on the DNA sequences of parts of *hox* gene in BSB, TC, BBTB, and BTBB using the specific degenerate primers, which were synthesized based on the *hox* gene of common carp. The DNA recombination events between BSB and TC were found in the *hoxd10a* gene of the two types of hybrid bream.

Fig. 8.13 The sequence alignment of partial *45S* rDNA in BSB, TC, BTB, and BTBT (Wu et al. 2020). The shaded area represents partial *18S* rDNA and *5.8S* rDNA of *45S* rDNA. The yellow area represents complete ITS-I of *45S* rDNA. The red box represents recombination site. The primers are underlined

References

Kang X (2013) Fertilization cytology of hybrids derived from *Megalobrama amblycephala* and *Erythroculter ilishaeformis* Bleeker and studies on its relevant molecular biology. Hunan Normal University, Changsha

Liu W (2013) The morphological characteristics study of Pharyngeal teeth and hypopharyngeal about several kinds of fish under the distant hybrid strains. Hunan Normal University, Changsha

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89(3):583–590

Ren L, Li W, Qin Q, Dai H, Han F, Xiao J, Gao X, Cui J, Wu C, Yan X, Wang G, Liu G, Liu J, Li J, Wan Z, Yang C, Zhang C, Tao M, Wang J, Luo K, Wang S, Hu F, Zhao R, Li X, Liu M, Zheng H, Zhou R, Shu Y, Wang Y, Liu Q, Tang C, Duan W, Liu S (2019) The subgenomes show asymmetric expression of alleles in hybrid lineages of *Megalobrama amblycephala* × *Culter alburnus*. Genome Res 29(11):1805–1815

Song Z (2013) Microsatellite DNA analysis and the evolution of Hox gene clusters in diploid hybrids derived from blunt snout bream × Culter and its original parents. Hunan Normal University, Changsha

Wu C, Huang X, Chen Q, Hu F, Zhou L, Gong K, Fu W, Gong D, Zhao R, Zhang C, Qin Q, Tao M, Liu S (2020) The formation of a new type of hybrid culter derived from a hybrid lineage of *Megalobrama amblycephala* (♀) × *Culter alburnus* (♂). Aquaculture 525:735328

Xiao J (2013) Establishment of hybrid strains between blunt snout bream and topmouth culter and their genetic characteristic research. Hunan Normal University, Changsha

Xiao J, Kang X, Xie L, Qin Q, He Z, Hu F, Zhang C, Zhao R, Wang J, Luo K, Liu Y, Liu S (2014) The fertility of the hybrid lineage derived from female *Megalobrama amblycephala* × male *Culter alburnus*. Anim Reprod Sci 151(1):61–70

Xiao J, Hu F, Luo K, Li W, Liu S (2016) Unique nucleolar dominance patterns in distant hybrid lineage derived from *Megalobrama Amblycephala* × *Culter Alburnus*. BMC Genet 17(1):150

9 The Formation and Biological Characteristics of Different Ploidy Fishes Derived from Common Carp × Blunt Snout Bream

Shaojun Liu, Yude Wang, Shi Wang, Yi Zhou, Chun Zhang, and Min Tao

Abstract

Distant hybridization refers to hybridization between two species or higher-level taxa. It can combine the different species genomes and contribute to significant changes in phenotypes and genotypes of hybrid offspring. This chapter mainly describes two distant crosses where the parents have different numbers of chromosomes leading to different ploidy fishes. In the offspring of first cross of common carp (*Cyprinus carpio* L., ♀) × blunt snout bream (*Megalobrama amblycephala*, ♂), we obtain the allotetraploid hybrids ($4n = 148$), autotetraploid fish lineage ($4n = 200$), autotriploid fish ($3n = 150$), crucian carp-like homodiploid lineage ($2n = 100$), improved diploid common carp lineage ($2n = 100$), improved diploid scattered mirror carp lineage ($2n = 100$), and other different ploidy fishes. In the offspring of the second cross of koi carp (*C. carpio haematopterus*, ♀) × blunt snout bream (♂), we obtain the red crucian carp-like homodiploid lineage ($2n = 100$), gynogenetic koi carp ($2n = 100$), and goldfish-like homodiploid lineage ($2n = 100$). These results are very useful in fish genetic breeding and evolutionary biology.

Keywords

Distant hybridization · Common carp · Koi carp · Blunt snout bream · Homodiploid lineage · Autotetraploid fish lineage

S. Liu (✉) · Y. Wang · S. Wang · Y. Zhou · C. Zhang · M. Tao
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_9

9.1 The Formation and Major Biological Characteristics of Different Ploidy Hybrids in F_1

During the breeding season, we pick common carp (abbreviated as COC) and blunt snout bream (abbreviated as BSB) which are mature in sexuality to perform two distant hybridization experiments about reciprocal cross. The first group takes COC and BSB as female and male parent, respectively, to conduct cross experiment. And the second group takes COC and BSB as male and female parent, respectively, to conduct cross experiment. In both two experiments, their fertilized eggs were placed in water for embryo development, and 2000 embryos were randomly selected from each experiment group to detect the fertilization rate and hatching rate. Eventually the hatched fry was raised in a large pool. The first cross experiment of COC (♀) and BSB (♂) showed that its fertilization rate had reached 85.8% and its hatching rate had reached 72.1%. Meanwhile we also obtained improved diploid common carp (abbreviated as 2*n*IDC), improved diploid scattered mirror carp (abbreviated as 2*n*IDMC), crucian carp-like homodiploid fish (abbreviated as 2*n*NCRC), and allotetraploid hybrids (abbreviated as 4*n*CB) which accounted for 76.4%, 17.5%, 1.7%, and 4.4%, respectively. However, in the COC (♂) and BSB (♀) cross experiment, no offspring survived.

9.1.1 Characteristics of Genetic Construction of Different Ploidy Hybrids

9.1.1.1 Chromosome Number and Karyotype

We have made a test on the chromosome number and karyotype of COC, BSB, and their different ploidy offspring (Wang et al. 2017). Over 95.5% of COC had 100 chromosomes in the metaphase (Table 9.1; Fig. 9.1a), and its karyotype formula was 22 m + 34 sm + 22 st + 22 t. About 93.5% of BSB had 48 chromosomes in the mitotic metaphase (Table 9.1), and its karyotype formula was 18 m + 22 sm + 8 st. Among them, there was a pair of the largest submetacentric chromosomes which marked to identify the chromosome source of the test fish (Fig. 9.1b). Ninety-two percent of 2*n*NCRC had 100 chromosomes in the metaphase (Table 9.1). The

Table 9.1 Chromosome test results of COC, BSB, 2*n*NCRC, 2*n*IDC, 2*n*IDMC, and 4*n*CB (Wang et al. 2017)

	Distribution of chromosome number						
Fish type	Number of metaphase	<48	48	<100	100	<148	148
COC	200			9	191		
BSB	200	13	187				
2*n*NCRC	200			16	184		
2*n*IDC	200			12	188		
2*n*IDMC	200			15	185		
4*n*CB	200					38	162

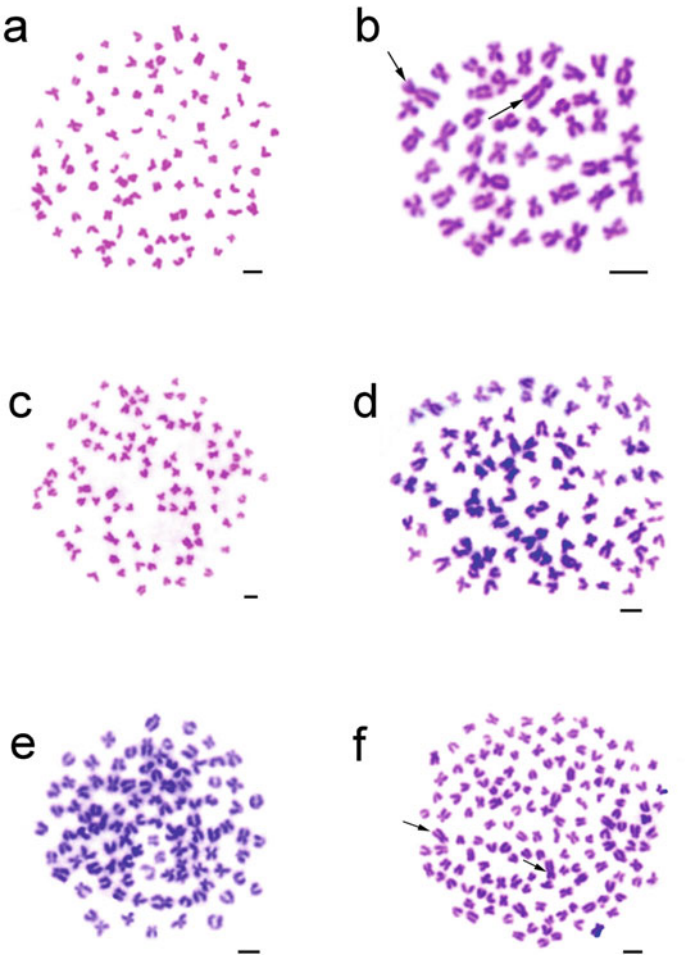

Fig. 9.1 Chromosome spreads at metaphase in different ploidy F_1 hybrids of COC (♀) × BSB (♂) (Wang et al. 2017). (**a**) Chromosome spreads at metaphase of COC had 100 chromosomes ($2n = 100$). (**b**) Chromosome spreads at metaphase of BSB had 48 chromosomes ($2n = 48$), and the arrows indicated the largest pair of submetacentric chromosomes. (**c**) Chromosome spreads at metaphase of 2*n*NCRC had 100 chromosomes ($2n = 100$). (**d**) Chromosome spreads at metaphase of 2*n*IDC had 100 chromosomes ($2n = 100$). (**e**) Chromosome spreads at metaphase of 2*n*IDMC had 100 chromosomes ($2n = 100$). (**f**) Chromosome spreads at metaphase of 4*n*CB had 148 chromosomes ($4n = 148$), and the arrows indicated the largest pair of submetacentric chromosomes. Bar = 3 μm

biggest submetacentric chromosome could not be found from BSB (Fig. 9.1c), and its karyotype formula was 22 m + 34 sm + 22 st + 22 t. Ninety-four of 2*n*IDC had 100 chromosomes in the metaphase (Table 9.1). The biggest submetacentric chromosome could not be found from BSB (Fig. 9.1d), and its karyotype formula was 22 m + 34 sm + 22 st + 22 t. A total of 92.5% of 2*n*IDMC had 100 chromosomes in

the metaphase (Table 9.1). The biggest submetacentric chromosome could not be found from BSB (Fig. 9.1e), and its karyotype formula was 22 m + 34 sm + 22 st + 22 t. Eighty-one percent of 4*n*CB had 148 chromosomes in the mitotic metaphase (Table 9.1), and there was a pair of chromosome from the largest submetacentric chromosome of BSB (Fig. 9.1f), and its karyotype formula was 40 m + 56 sm + 30 st + 22 t (Wang et al. 2017).

9.1.1.2 DNA Content Detection

We take the DNA content in COC and in BSB as the comparison to test the DNA content in different ploidy fishes of COC (♀) × BSB (♂) F_1. All the samples tested were presented in Table 9.2. The mean DNA content of 2*n*NCRC was similar to COC, indicating that it contained two sets of chromosomes from COC (Table 9.2); the mean DNA content of 2*n*IDC was similar to COC, indicating that it contained two sets of chromosomes from COC (Table 9.2); the mean DNA content of 2*n*IDMC was similar to COC, indicating that it contained two sets of chromosomes from COC (Table 9.2); the mean DNA content of 4*n*CB was in line with the sum of the mean DNA content of COC and BSB, indicating that it had two sets of chromosomes from COC and two sets of chromosomes from BSB (Table 9.2) (Wang et al. 2017).

9.1.1.3 Fluorescence In Situ Hybridization

To explore the genetic variation of different ploidy hybrids of COC (♀) × BSB (♂), 5S rDNA sequence probes (203 bp, GQ485556) of fluorescence in situ hybridization (FISH) were used to recognize the chromosomal variation in these hybrids. The previous studies had shown that this probe could label four fluorescence signals (two strong and two weak) on 100 chromosomes of red crucian carp (RCC) and wild crucian carp (CRC) (Qin et al. 2015). In this study, this probe did not have any hybridization signal in the chromosome spreads at metaphase of COC and BSB (Fig. 9.2a, b) (Wang et al. 2017). In the different ploidy hybrids of COC (♀) × BSB (♂), two strong signals were displayed in 2*n*NCRC (Fig. 9.2c), four signals (two strong and two weak) were presented in 4*n*CB (Fig. 9.2f), and there was no signal in 2*n*IDC or 2*n*IDMC (Fig. 9.2d, e) (Wang et al. 2017). The FISH results showed the heredity and variation of the chromosomes in the different ploidy hybrids at the

Table 9.2 The results of mean DNA content in BSB, COC, and their different ploidy F_1 hybrids (Wang et al. 2017)

The type of fish	Mean DNA content	Ratio	
		Observed	Expected
BSB	65.98		
COC	101.72		
2*n*NCRC	99.17	2*n*NCRC/COC = 0.97[a]	1
2*n*IDC	104.28	2*n*IDC/COC = 1.03[a]	1
2*n*IDMC	98.36	2*n*IDMC/COC = 0.97[a]	1
4*n*CB	162.64	4*n*CB/(COC + BSB) = 0.97[a]	1

[a]There is no significant difference between the observed and expected values

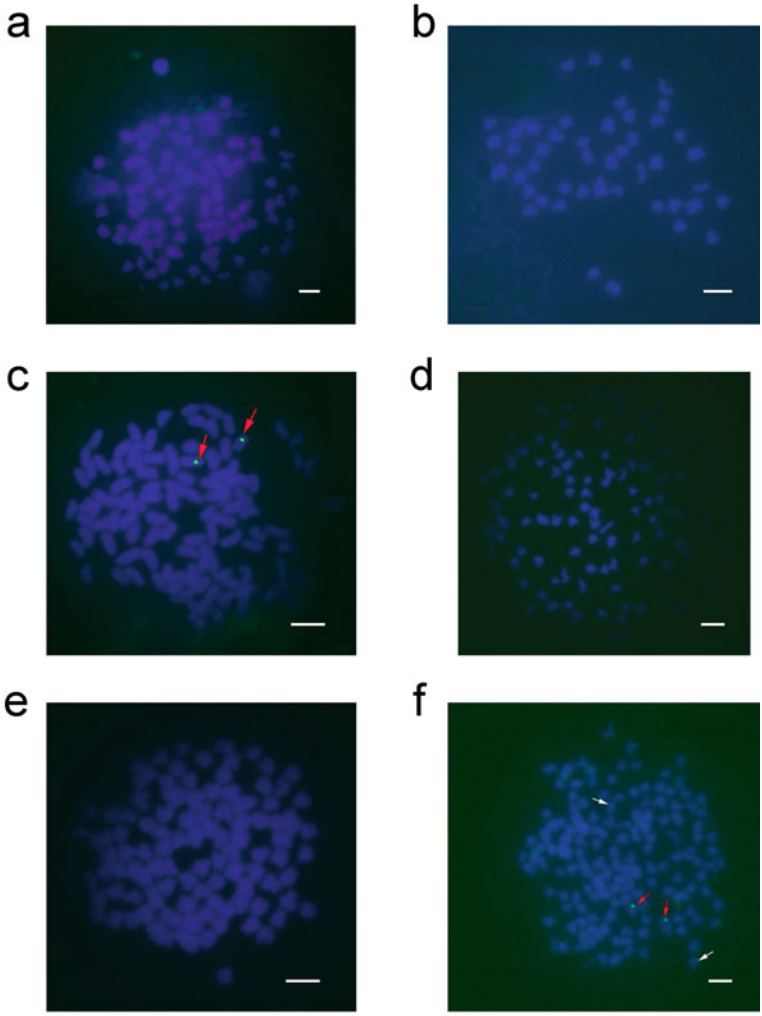

Fig. 9.2 The result of FISH signals in COC, BSB, and their different ploidy F_1 hybrids (Wang et al. 2017). (**a**) The COC did not have signals. (**b**) The BSB did not have signals. (**c**) The 2*n*NCRC had two strong fluorescence signals (red arrows). (**d**) The 2*n*IDC did not have signals. (**e**) The 2*n*IDMC did not have signals. (**f**) The 4*n*CB had four signals (two strong (red arrows) and two weak (white arrows)). Bar = 3 μm

molecular level and revealed that 2*n*NCRC and 4*n*CB have large variations at the chromosomal level. The FISH analysis also indicated that 2*n*NCRC and 4*n*CB were more chromosomally similar to RCC and CRC than its parents.

9.1.2 Appearance of Different Ploidy Fishes in F_1

For morphological traits, 2*n*NCRC showed obvious differences from their parents (Fig. 9.3a–f). The major morphological differences between COC and RCC, CRC, or Japanese white crucian carp (JCC) were that COC had two pairs of barbels and the number of lateral line scales was 35–38, but the other types of crucian carp had no barbels and the number of lateral line scales was 27–34. Furthermore, BSB had no barbels and the number of lateral line scales was 49–52 (Tables 9.3 and 9.4; Fig. 9.3). Obvious differences of traits ($P < 0.01$) were found in terms of the qualitative and quantitative traits between 2*n*NCRC and COC (Tables 9.3 and 9.4; Fig. 9.3), the most obvious characteristic being that 2*n*NCRC had no barbels, unlike CC. Moreover, 2*n*NCRC had fewer lateral line scales than COC ($P < 0.01$; Table 9.3). However, the HL/BL ratio of 2*n*NCRC was significantly greater than those of COC and BSB. The BD/BL and HH/BD ratios ($P < 0.01$) of 2*n*NCRC were between those of COC and BSB. Interestingly, the qualitative and quantitative traits of 2*n*NCRC, containing the loss of barbel and a few lateral line scales, were similar to those of CRC (Tables 9.3 and 9.4; Fig. 9.3). However, some morphological differences between 2*n*NCRC and CRC, including the number of lateral line scales, were detected: the number of lateral line scales was found in 2*n*NCRC between that of CRC and that of COC, although it was closer to that of CRC. A comparison research of the qualitative and quantitative traits between the hybrid progenies and their parents contributes to identify the similarities and differences between them. This morphological analysis showed that 2*n*NCRC was more phenotypically similar to CRC than its parents.

The number of vertebrates was reflected by uppercase Roman numerals, and the number of soft fins was indicated by Arabic numerals. The numbers before and after the symbol "+" presented the mean and standard deviation of the numbers of fins, and "–" indicated the range of the measured number of fins.

9.1.3 Reproductive Traits of Different Ploidy Fishes in F_1

The productive traits showed that the sexual maturity of COC and BSB was 2 years, while the sexual maturity of CRC and its varieties (RCC and JCC) was at 1 year old. The sexual maturity of 2*n*NCRC was at 1 year old, yet 2*n*IDC, 2*n*IDMC, and 4*n*CB reach sexual maturity at 2 years old. The ovaries of 6-month-old 2*n*NCRC were fully developed. Abundant proliferation for the development of phase II oocytes ((Fig. 9.4a) was displayed by many oogonia found. Moreover, a large number of eggs were stripped from 1-year-old 2*n*NCRC females. The white semen could be stripped out from male 2*n*NCRC individuals at 6 months and from male 2*n*IDC and 2*n*IDMC individuals at the age of 18 months. The spermatozoa of 6-month-old 2*n*NCRC, 18-month-old 2*n*IDC, and 18-month-old 2*n*IDMC with the scanning electron microscope showed that the heads and tails of the sperm produced by these male hybrids were well-developed (Fig. 9.4b–d). The size of the head of 2*n*NCRC sperm (Fig. 9.4b) was similar to that of 2*n*IDC and 2*n*IDMC sperm

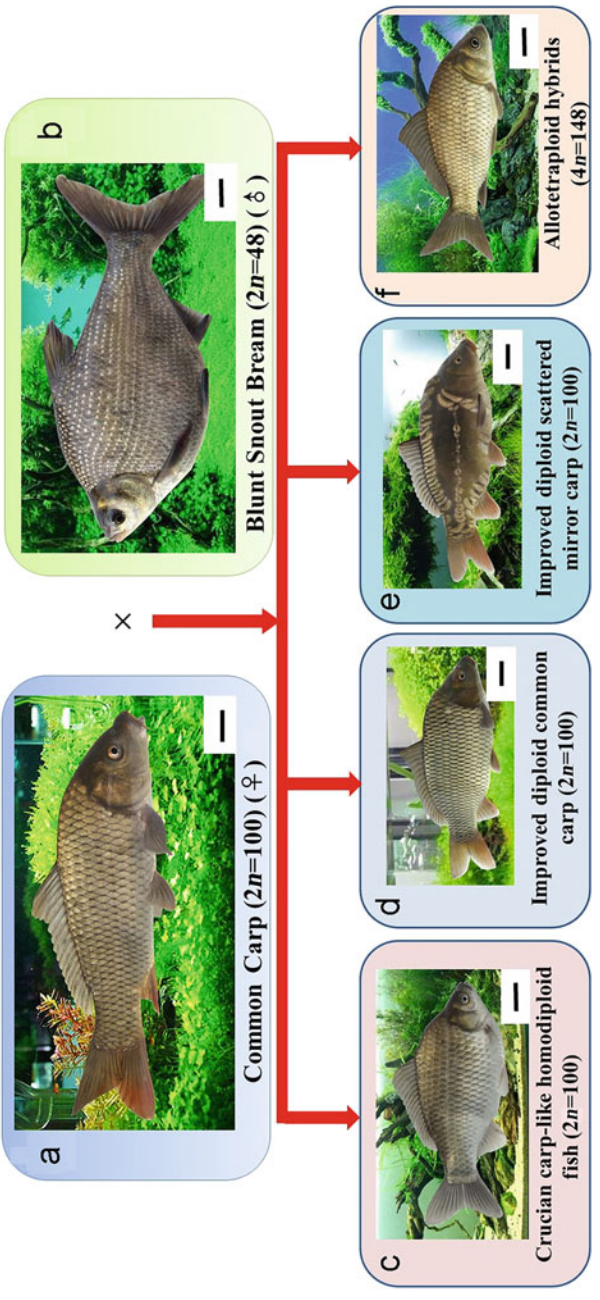

Fig. 9.3 Morphological traits of different ploidy hybrids in F_1 from COC (♀) × BSB (♂) (partially quoted from Wang et al. (2017)). (**a**) The appearance of common carp (COC). (**b**) The appearance of blunt snout bream (BSB). (**c**) The appearance of crucian carp-like homodiploid fish (2*n*NCRC). (**d**) The appearance of improved diploid common carp (2*n*IDC). (**e**) The appearance of improved diploid scattered mirror carp (2*n*IDMC). (**f**) The appearance of allotetraploid hybrids (4*n*CB). Bar = 5 cm

Table 9.3 Comparison of the countable traits among the in COC, BSB, and their different ploidy F_1 hybrids (Wang et al. 2017)

Fish type	No. of lateral line scales	No. of scales above lateral line	No. of scales below lateral line	No. of dorsal fin	No. of pelvic fin	No. of anal fin
COC	36.35 ± 1.43 (35–38)	5.37 ± 0.45 (5–6)	5.30 ± 0.43 (5–6)	III + 17.62 ± 0.89 (III + 17–19)	8.58 ± 0.51 (8–9)	III + 6.37 ± 0.39 (III + 6–7)
BSB	50.60 ± 1.20 (49–52)	9.48 ± 0.55 (9–10)	10.19 ± 0.97(9–11)	III + 8.50 ± 0.52 (III + 8–9)	9.18 ± 0.69 (8–10)	III + 25.90 ± 0.88 (III + 25–27)
2*n*NCRC	29.35 ± 0.38 (29–30)	6.43 ± 0.52 (6–7)	7.36 ± 0.56 (7–8)	III + 18.34 ± 1.28 (III + 17–20)	9.08 ± 1.69 (7–11)	III + 7.66 ± 1.25 (III + 6–9)
2*n*IDC	36.50 ± 0.50 (36–37)	5.75 ± 0.38 (5–6)	6.00 ± 1.00 (5–7)	III + 18.30 ± 1.32 (III + 17–20)	9.39 ± 1.27 (8–11)	III + 8.52 ± 1.11 (III + 7–10)
2*n*IDMC	\	\	\	III + 18.34 ± 1.32 (III + 17–20)	9.00 ± 1.00 (8–10)	III + 8.51 ± 1.12 (III + 7–10)
4*n*CB	30.94 ± 0.86 (30–32)	6.00 ± 0.00 (6)	6.45 ± 0.42 (6–7)	III + 17.94 ± 1.67 (III + 16–20)	8.36 ± 1.29 (7–10)	III + 7.99 ± 1.67 (III + 6–9)
RCC	29.15 ± 0.79 (28–30)	5.65 ± 0.36 (5–6)	6.35 ± 0.49 (6–7)	III + 19.05 ± 0.85 (III + 18–20)	8.39 ± 0.49 (8–9)	III + 6.38 ± 0.37 (III + 6–7)
JCC	33.14 ± 0.86 (32–34)	7.24 ± 1.06 (6–8)	6.03 ± 0.82 (5–7)	III + 19.14 ± 0.78 (III + 18–20)	9.16 ± 0.89 (8–10)	III + 6.49 ± 0.41 (III + 6–7)
CRC	28.48 ± 1.28 (27–30)	5.85 ± 0.83 (5–7)	6.00 ± 0.00 (6)	III + 17.02 ± 0.89 (III + 16–18)	8.45 ± 1.32 (7–10)	III + 7.54 ± 1.57 (III + 6–9)

Table 9.4 Comparison of the measurable traits among the in COC, BSB, and their different ploidy F_1 hybrids (Wang et al. 2017)

Fish type	BL/TL	BD/BL	HL/BL	HH/HL	CPD/CPL	HH/BD
COC	0.83 ± 0.07	0.34 ± 0.01	0.24 ± 0.02	0.81 ± 0.07	0.86 ± 0.11	0.60 ± 0.01
BSB	0.84 ± 0.04	0.41 ± 0.04	0.20 ± 0.04	0.88 ± 0.03	0.93 ± 0.04	0.49 ± 0.04
2*n*NCRC	0.84 ± 0.02	0.41 ± 0.02	0.26 ± 0.01	0.88 ± 0.02	0.88 ± 0.03	0.56 ± 0.01
2*n*IDC	0.83 ± 0.01	0.35 ± 0.01	0.27 ± 0.02	0.81 ± 0.03	0.84 ± 0.02	0.67 ± 0.03
2*n*IDMC	0.83 ± 0.05	0.34 ± 0.02	0.28 ± 0.02	0.81 ± 0.06	0.87 ± 0.10	0.66 ± 0.03
4*n*CB	0.83 ± 0.02	0.38 ± 0.02	0.27 ± 0.01	0.88 ± 0.02	0.89 ± 0.01	0.62 ± 0.01
RCC	0.81 ± 0.03	0.45 ± 0.01	0.27 ± 0.01	0.85 ± 0.04	1.27 ± 0.02	0.51 ± 0.02
JCC	0.81 ± 0.02	0.45 ± 0.03	0.27 ± 0.02	0.85 ± 0.06	1.23 ± 0.02	0.51 ± 0.03
CRC	0.81 ± 0.03	0.37 ± 0.03	0.27 ± 0.02	0.95 ± 0.10	1.08 ± 0.15	0.69 ± 0.07

Note: BL/TL (body length to total length), BD/BL (body depth to body length), HL/BL (head length to body length), HH/HL (head height to head length), CPD/CPL (caudal peduncle depth to caudal peduncle length), HH/BD (head height to body depth)

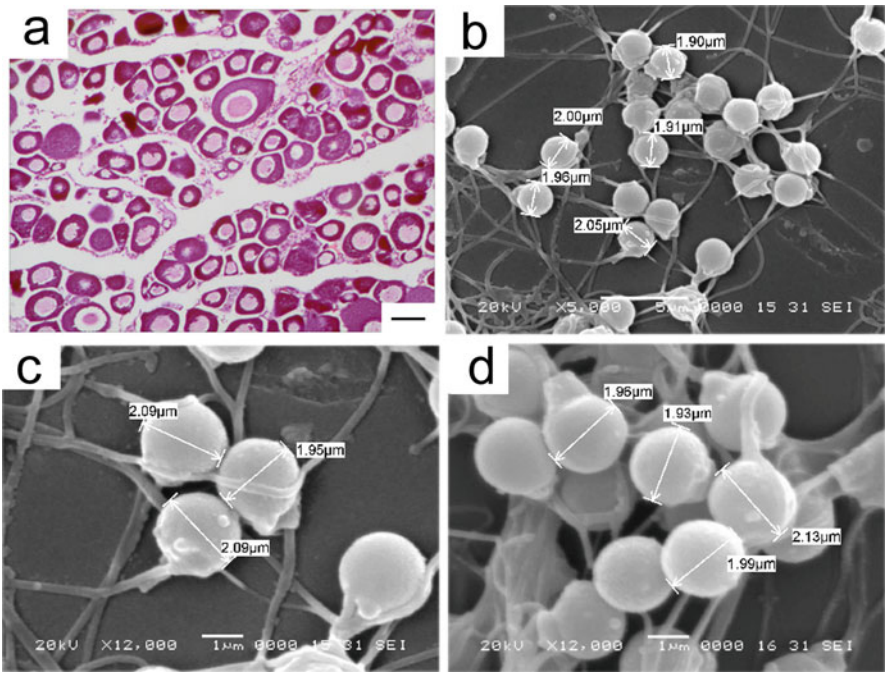

Fig. 9.4 Ovarian microstructure of 2*n*NCRC and structure of 2*n*NCRC, 2*n*IDC, and 2*n*IDMC sperm (Wang et al. 2017). (**a**) Ovarian microstructure of 2*n*NCRC. Bar = 20 μm. (**b**) Structure of 2*n*NCRC sperm under a scanning electron microscope. Bar = 5 μm. (**c**) Structure of 2*n*IDC sperm under a scanning electron microscope. Bar = 1 μm. (**d**) Structure of 2*n*IDMC sperm under a scanning electron microscope. Bar = 1 μm

(Fig. 9.4c, d). The mean diameter of 2*n*NCRC haploid sperm was 1.96 μm, while those of 2*n*IDC and 2*n*IDMC haploid sperm were 2.04 μm and 2.00 μm, respectively. The ploidy of the gametes could be identified by the size of the mature gametes. The individuals of 2*n*NCRC males and females were found to show normal gonadal development (Fig. 9.4) and produced mature sperm and eggs, which could fuse to form F_2. At present, 2*n*NCRC can be stably inherited from one generation to the next generation and forms a stable lineage (F_1–F_2–F_n). The result showed that 2*n*NCRC was similar to CRC in terms of reproductive traits (reaching sexual maturity at 1 year old).

9.1.4 Molecular Biological Characteristics of Different Ploidy Fishes in F_1

Comparative analyses of 5S rDNA, *Hox* genes, and mitochondrial (mt) DNA sequence structures were made by us to study the genetic relationship and differences between F_1 hybrids and their parents (COC and BSB).

9.1.4.1 5S rDNA Organization and Genetic Variation Features

5S rDNA has usually been used as a genomic DNA marker to trace evolutionary events (Pinhal et al. 2008; Ubeda-Manzanaro et al. 2010) and so that the 5S rDNA in COC, BSB, and 2*n*NCRC can be analyzed.

The class I 5S rDNA has two 5S rDNA bands (203 and 406 bp) in COC. The 406-bp band is likely to be an indicator of the 203-bp class I 5S rDNA duplication. BSB also presented two bands (188 bp and either 376 or 374 bp) belonging to class II (188 bp) and class II-V_1 (374 bp). The 376-bp band is likely to be a copy of the 188-bp class II 5S rDNA, and the 374-bp band is a variant band.

The 2*n*NCRC presented four bands: the first band was found to belong to class I-V_1 (196 or 205 bp), the second band belongs to class I-V_2 (339, 340, or 341 bp), the third band was classified as class II-V_1 (374, 398, 406, or 410 bp), and the fourth band was classified as class I-V_3 (478, 480, or 493 bp). BLAST results showed that the 2*n*NCRC 5S rDNA class I-V_1 is a variant of the 203-bp 5S rDNA class I of its female parent (COC) (Wang et al. 2017). It is interesting to note the 196-bp 5S rDNA class I-V_1 of 2*n*NCRC showed high nucleotide identity (92.10%) with RCC 203-bp 5S rDNA (GenBank Accession No. GQ485555.1). However, 7-bp deletion and 9-bp mutations were detected, showing substantially higher identity (80.50%) than that between 2*n*NCRC 196-bp 5S rDNA class I-V_1 and COC 203-bp 5S rDNA class I (Wang et al. 2017). Similarly, the identity of high nucleotide (99.50%) was observed between 2*n*NCRC 205-bp 5S rDNA class I-V_1 and JCC 205-bp 5S rDNA (GenBank Accession No. KR706447.1), and there was only one mutation in 2*n*NCRC (Wang et al. 2017). This similarity was significantly higher (78.30%) than that between 2*n*NCRC 205-bp 5S rDNA class I-V_1 and COC 203-bp 5S rDNA class I. The 340-bp 5S rDNA class I-V_2 shown in 2*n*NCRC band showed high nucleotide identity (98.50%) with CRC 340-bp 5S rDNA (GenBank No. DQ659273.1), which included 5-bp mutations, and this similarity was found to be higher (78.10%) than that between 2*n*NCRC 340-bp 5S rDNA class I-V_2 and COC 203-bp 5S rDNA class I (Wang et al. 2017). While the 92.40% was observed between 2*n*NCRC 493-bp 5S rDNA class I-V_3 and CRC 501-bp 5S rDNA (GenBank No. GU188690.1), which showed a 12-bp deletion, three (1-bp, 1-bp, and 2-bp) insertions, and 22 mutations, the lower 83.74% between 2*n*NCRC 493-bp 5S rDNA class I-V_3 and COC 203-bp 5S rDNA class I was found (Wang et al. 2017). The result showed that 2*n*NCRC is closely related to CRC at the genomic DNA level and was further proved by the complete 5S rDNA analyses. Additionally, a high 95.70% was observed between 2*n*NCRC 374-bp 5S rDNA class II-V_1 and BSB 374-bp 5S rDNA class II-V_1, where there were a 2-bp deletion, three (4-bp, 1-bp, and 1-bp) insertions, and four mutations. A lower identity was observed (82.27%) between 2*n*NCRC 374-bp 5S rDNA class II-V_1 and COC 203-bp 5S rDNA class I (Wang et al. 2017). These results showed the recombination of BSB genomic DNA into 2*n*NCRC genome, which leads to obvious changes in 2*n*NCRC genome. Moreover, elimination of part of the paternal 5S rDNA unit (BSB 188-bp 5S rDNA class II) in 2*n*NCRC during hybridization gave rise to genomic shock in the offspring of the distant hybridization.

The altered 5S rDNA structure of 2*n*NCRC is very close to that of CRC, providing further evidence that 2*n*NCRC is more closely related to CRC at the genomic DNA level.

9.1.4.2 Relevant *Hox* Gene Genetic Characteristics

The *Hox* clusters in COC, BSB, 2*n*IDC, and 2*n*IDMC are shown in Tables 9.5 and 9.6. The 2*n*IDC was found to undergo extremely significant mutations as the first generation of distant hybridization by the *Hox* gene cluster; for instance, in *HoxA4a*, there were five putative clusters and seven recombinant clusters in2nIDC (Tables 9.5 and 9.6); in *HoxD4a*, there were six putative clusters and one recombinant cluster in 2nIDC (Tables 9.5 and 9.6); in *HoxD10a*, there were five putative clusters and two recombinant clusters in 2nIDC (Tables 9.5 and 9.6). On the contrary, as the first generation of a distant hybridization, the significant mutations were not found in the *Hox* genes of 2*n*IDMC; almost all of the *Hox* gene clusters were originated from the female parent, COC, not including five recombinant clusters (Tables 9.5 and 9.6). Among these *Hox* gene clusters, all copies of *HoxB4a* in COC, BSB, 2*n*IDC, and 2*n*IDMC were pseudogenes containing a stop codon that prematurely lead to the expression of a full-length functional product (Tables 9.5 and 9.6). The copies of *HoxB1ai* in COC, 2*n*IDC, and 2*n*IDMC were pseudogenes due to stop codons (Tables 9.5 and 9.6). These results revealed that the *Hox* gene family in cyprinid fishes had undergone rapid evolution, with some genes gradually becoming pseudogenes, and some genes completely pseudogenized. In addition, we also found pseudogenes in the recombinant clusters, for example, *HoxC4aii* + *HoxC4ai* in 2*n*IDMC (Tables 9.5 and 9.6).

Our results provide a good model for genetic variation by showing large genotypic differences in 2*n*IDC originated from the distant hybridization COC and BSB. The number of *Hox gene* clusters in 2nIDC is about twice that in COC, except for the recombinant clusters (Tables 9.5 and 9.6). Among the 12 *Hox* genes, some of them orthologous to zebrafish genes were present as two copies in COC (except for *HoxA11b*, *HoxB1b*, *HoxB4a*, and *HoxC6b*), one copy in BSB, and 2–6 copies (not including recombinant clusters) in 2*n*IDC. The proliferation of such a rich diversity in gene copy number further reveals that distant hybridization as a catalyst accelerates the formation of species (Mallet 2007). One of the highlights of this study is the development of 2*n*IDMC originated from the distant hybridization of female COC and male BSB, which has a significant difference in phenotype compared to its parents; determining the mechanisms that triggered these new phenotypes to appear will help to understand the impact of hybridization on the speciation processes.

9.1.4.3 Analysis of Genetic Variations of mtDNA in Different Generations of 2*n*NCRC

To stable inheritance, the mtDNA sequence mutation occurred in different generations of 2*n*NCRC, which will be discussed in detail in this section. Figure 9.5 and Tables 9.7 and 9.8 directly showed the genetic variations in these eight mt structural regions (genes) (CR, *12S rRNA*, *16S rRNA*, *COI*, *Cytb*, NADH2 (*ND2*),

Table 9.5 The bands of PCR amplification (nonrecombinant bands) in COC, BSB, 2*n*IDC, and 2*n*IDMC (Luo et al. 2019)

Genes	Species	Locus	Size (bp)	Exon 1 (bp)	Intron (bp)	Exon 2 (bp)
HoxA4a	COC	*HoxA4ai*	1177	89–500	501–970	971–1177
		HoxA4aii	1182	89–500	501–975	976–1182
	BSB	*HoxA4a*-BSB	1188	89–500	501–981	982–1188
	2*n*IDC	*HoxA4ai*	1177	89–500	501–970	971–1177
		HoxA4aii	1182	89–500	501–975	976–1182
		HoxA4aiii	1184	89–500	501–977	978–1184
		HoxA4a-1	1181	89–500	501–974	975–1181
		HoxA4a-BSB	1188	89–500	501–981	982–1188
	2*n*IDMC	*HoxA4ai*	1177	89–500	501–970	971–1177
		HoxA4aii	1182	89–500	501–975	976–1182
HoxA9a	COC	*HoxA9ai*	817	1–381	382–620	621–817
		HoxA9aii	891	1–381	382–694	695–891
	BSB	*HoxA9b*	879	1–381	382–682	683–879
	2*n*IDC	*HoxA9ai*	817	1–381	382–620	621–817
		HoxA9aii	867	1–381	382–670	671–867
	2*n*IDMC	*HoxA9ai*	817	1–381	382–620	621–817
HoxA2b	COC	*HoxA2bi*	1490	1–314	315–905	906–1490
		HoxA2bii	1475	1–314	315–890	891–1475
	BSB	*HoxA2b*	1479	1–311	312–894	895–1479
	2*n*IDC	*HoxA2bi*	1490	1–314	315–905	906–1490
		HoxA2bii	1475	1–314	315–890	891–1475
		HoxA2biii	1486	1–314	315–901	902–1486
		HoxA2b-1	1448	1–314	315–863	864–1448
	2*n*IDMC	*HoxA2bi*	1490	1–314	315–905	906–1490
		HoxA2bii	1475	1–314	315–890	891–1475
HoxA11b	COC	*HoxA11bi*	1440	3–590	591–342	1343–1440
	BSB	*HoxA11b*	1703	3–602	603–1605	1606–1703
	2*n*IDC	*HoxA11bi*	1440	3–590	591–1342	1343–1440
		HoxA11bii	1401	3–590	591–1303	1304–1401
	2*n*IDMC	*HoxA11bi*	1439	3–590	591–1342	1343–1439
HoxB1a	COC	*HoxB1ai*[a]	1510	–	–	–
		HoxB1aii	1526	1–462	463–1250	1251–1526
	BSB	*HoxB1a*	1522	1–459	460–1246	1247–1522
	2*n*IDC	*HoxB1ai*[a]	1510	–	–	–
		HoxB1aiii	1484	1–450	451–1208	1209–1484
	2*n*IDMC	*HoxB1ai*[a]	1510	–	–	–
		HoxB1aii	1525	1–462	463–1249	1250–1525
HoxB4a[a]	COC	*HoxB4ai*[a]	1631	–	–	–
	BSB	*HoxB4a*[a]	1617	–	–	–
	2*n*IDC	*HoxB4ai*[a]	1630	–	–	–
		HoxB4aii[a]	1613	–	–	–
	2*n*IDMC	*HoxB4ai*[a]	1630	–	–	–

(continued)

Table 9.5 (continued)

Genes	Species	Locus	Size (bp)	Exon 1 (bp)	Intron (bp)	Exon 2 (bp)
HoxB1b	COC	*HoxB1bi*	731	1–477	478–565	566–731
	BSB	*HoxB1b*-BSB	751	1–477	478–585	586–751
	2*n*IDC	*HoxB1bi*	731	1–477	478–565	566–731
		HoxB1bii	733	1–477	478–567	568–733
		HoxB1b-BSB	751	1–477	478–585	586–751
	2*n*IDMC	*HoxB1bi*	731	1–477	478–565	566–731
HoxB5b	COC	*HoxB5bi*	1191	1–561	562–985	986–1191
		HoxB5bii	1190	1–564	565–984	985–1190
	BSB	*HoxB5b*-BSB	1227	1–564	565–1021	1022–1227
	2*n*IDC	*HoxB5bi*	1191	1–561	562–985	986–1191
		HoxB5bii	1190	1–564	565–984	985–1190
		HoxB5biii	1196	1–561	562–990	991–1196
		HoxB5b-BSB	1226	1–564	565–1020	1021–1226
	2*n*IDMC	*HoxB5bi*	1191	1–561	562–985	986–1191
		HoxB5bii	1190	1–564	565–984	985–1190
HoxC4a	COC	*HoxC4ai*	1169	1–410	411–928	929–1169
		HoxC4aii	1176	1–410	411–935	936–1176
	BSB	*HoxC4a*	1125	1–410	411–933	934–1125
	2*n*IDC	*HoxC4ai*	1169	1–410	411–928	929–1169
		HoxC4aii	1176	1–410	411–935	936–1176
		HoxC4aiii	1173	1–410	411–932	933–1173
		HoxC4a-1	1179	1–410	411–938	939–1179
	2*n*IDMC	*HoxC4ai*	1169	1–410	411–928	929–1169
		HoxC4aii	1176	1–410	411–935	936–1176
HoxC6b	COC	*HoxC6bi*	942	2–392	393–763	764–942
	BSB	*HoxC6b*-BSB	922	2–392	393–737	738–922
	2*n*IDC	*HoxC6bi*	949	2–392	393–763	764–949
		HoxC6bii	964	2–392	393–778	779–964
		HoxC6b-BSB	923	2–392	393–737	738–923
	2*n*IDMC	*HoxC6bi*	949	2–392	393–763	764–949
HoxD4a	COC	*HoxD4ai*	942	1–315	316–717	718–942
		HoxD4aii	944	1–315	316–719	720–944
	BSB	*HoxD4a*-BSB	911	1–306	307–686	687–911
	2*n*IDC	*HoxD4ai*	942	1–315	316–717	718–942
		HoxD4aii	944	1–315	316–719	720–944
		HoxD4aiii	952	1–315	316–727	728–952
		HoxD4a-1	937	1–315	316–712	713–937
		HoxD4a-2	960	1–315	316–735	736–960
		HoxD4a-BSB	911	1–306	307–686	687–911
	2*n*IDMC	*HoxD4ai*	942	1–315	316–717	718–942
		HoxD4aii	944	1–315	316–719	720–944

(continued)

Table 9.5 (continued)

Genes	Species	Locus	Size (bp)	Exon 1 (bp)	Intron (bp)	Exon 2 (bp)
HoxD10a	COC	*HoxD10ai*	1551	1–589	590–1321	1322–1551
		HoxD10aii	1546	1–592	593–1316	1317–1546
	BSB	*HoxD10a*-BSB	1574	1–592	593–1344	1345–1574
	2*n*IDC	*HoxD10ai*	1554	1–589	590–1324	1325–1554
		HoxD10aii	1546	1–592	593–1316	1317–1546
		HoxD10aiii	1495	1–592	593–1265	1266–1495
		HoxD10a-1	1480	1–592	593–1250	1251–1480
		HoxD10a-BSB	1574	1–592	593–1344	1345–1574
	2*n*IDMC	*HoxD10ai*	1554	1–589	590–1324	1325–1554
		HoxD10aii	1546	1–592	593–1316	1317–1546

[a]Denotes a pseudogene

NADH 4 (*ND4*), and NADH 5 (*ND5*)) in different generations of 2*n*NCRC. In these structural regions (genes) (only the consistent base sites of different generations of 2*n*NCRC are counted here), most of the base loci were conserved: the conserved loci in the CR accounted for 67.61%; the conserved loci in the two types of rRNAs (*12S rRNA* and *16S rRNA*) were 88.76 to 90.36%; and the conserved sites in the five protein-coding genes (*COI*, *Cytb*, *ND2*, *ND4*, and *ND5*) were 71.33–79.75%. In these structural regions (genes), 15.93% of the base loci in the CR, 5.83 to 6.71% of the base sites in the two types of rRNAs, and 6.90–12.50% of the five protein-coding genes were the same as those in maternal parent COC. It is commonly believed that animal mtDNA follows the mechanism of maternal inheritance. Recombination of mtDNA usually occurs in most plant, fungal, and protist species, but is traditionally considered rare or absent in animals (Gillham 1994; Rokas et al. 2003). In this study, the mtDNA sequence of 2*n*NCRC did not follow the maternal genetic mechanism, instead showing some embedding of paternal base sites due to the influence of distant hybridization. For example, 4.33% of the base sites in the CR, 0.52 to 1.90% of the base sites in the two types of rRNAs, and 3.68–5.87% of the five protein-coding genes were the same as those in paternal parent BSB. The paternal mtDNA fragments were stably inserted into the eight mt structural regions (genes) of different generations (F_1–F_3) of 2*n*NCRC to form chimeras. In addition, some base sites were mutated: the mutated sites in the CR accounted for 7.26%; the mutated sites in the two types of rRNAs were 1.57 to 2.56%; and the mutated sites in the five protein-coding genes were 6.37–7.26%. Most of these mutated sites were the same as those in CRC, showing that due to the influence of distant hybridization, 2*n*NCRC had a tendency to mutate toward CRC at the mtDNA level.

It is worth noting that the eight mt structural regions (genes) of 2*n*NCRC showed inconsistent base sites in different generations (Fig. 9.5; Tables 9.7 and 9.8). That is, some of the base sites of the first generation of 2*n*NCRC were not stably inherited by the second generation, resulting in inconsistent sites, but almost all the base sites

Table 9.6 PCR amplification bands (recombinant bands) in COC, BSB, 2*n*IDC, and 2*n*IDMC (Luo et al. 2019)

Genes	Species	Locus	Size (bp)	Exon 1 (bp)	Intron (bp)	Exon 2 (bp)
HoxA4a	2*n*IDC	*HoxA4ai* + *HoxA4aii*	1182	89–500	501–975	976–1182
		HoxA4aii + *HoxA4a-1* + *HoxA4ai*	1182	89–500	501–975	976–1182
		HoxA4ai + *HoxA4aiii*	1184	89–500	501–977	978–1184
		HoxA4ai + *HoxA4a*-BSB	1188	89–500	501–981	982–1188
		HoxA4ai + *HoxA4a*-BSB + *HoxA4ai*	1182	89–500	501–975	976–1182
		HoxA4a-BSB + *HoxA4ai*	1182/1188	89–500	501–975/981	976/982–1182/1188
		HoxA4a-BSB + *HoxA4aii*	1188	89–500	501–981	982–1188
	2*n*IDMC	*HoxA4aii* + *HoxA4ai*	1182	89–500	501–975	976–1182
HoxA2b	2*n*IDC	*HoxA2bi* + *HoxA2bii*	1490	1–314	315–905	906–1490
HoxB5b	2*n*IDC	*HoxB5bi* + *HoxB5bii* + *HoxB5bi*	1190	1–564	565–984	985–1190
		HoxB5bi + *HoxB5bii*	1190	1–564	565–984	985–1190
	2*n*IDMC	*HoxB5bii* + *HoxB5bi*	1194	1–564	565–988	989–1194
HoxC4a	2*n*IDC	*HoxC4aii* + *HoxC4ai*	1174	1–410	411–933	934–1174
		HoxC4aii + *HoxC4aiii*	1173	1–410	411–932	933–1173
	2*n*IDMC	*HoxC4aii* + *HoxC4ai*[a]	1169	1–410	411–928	929–1169
HoxD4a	2*n*IDC	*HoxD4aiii* + *HoxD4a-1*	937	1–315	316–712	713–937
	2*n*IDMC	*HoxD4ai* + *HoxD4aii*	944	1–315	316–719	720–944
HoxD10a	2*n*IDC	*HoxD10aii* + *HoxD10ai*	1554	1–589	590–1324	1325–1554
		HoxD10ai + *HoxD10a-1*	1494	1–588	589–1264	1265–1494
	2*n*IDMC	*HoxD10aii* + *HoxD10ai*	1545	1–592	593–1315	1316–1545

[a]Denotes a pseudogene

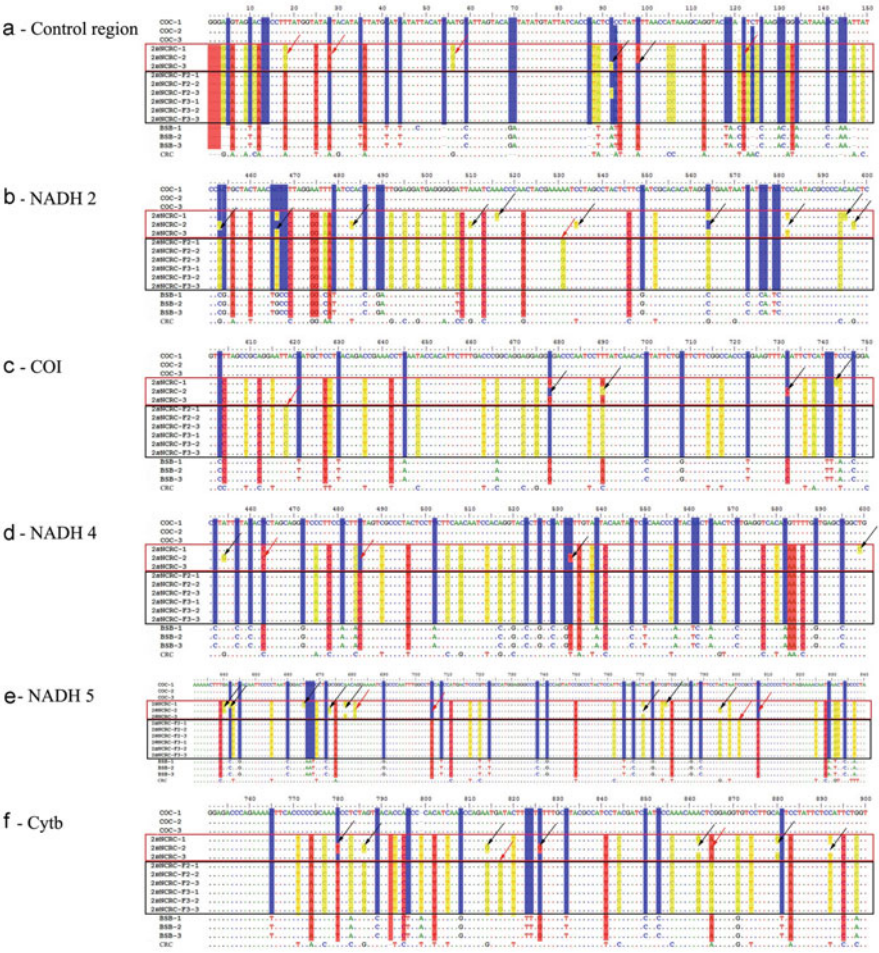

Fig. 9.5 The alignment of nucleotide sequence of the mtDNA structural regions (genes) of three COC, three BSB, three 2*n*NCRC-F_1, three 2*n*NCRC-F_2, three 2*n*NCRC-F_3, and one CRC (Wang et al. 2020a). (**a**) The alignment of partial nucleotide sequence CR. (**b**) The alignment of partial nucleotide sequence of *ND2*. (**c**) The alignment of partial nucleotide sequence of *COI*. (**d**) The alignment of partial nucleotide sequence of *ND4*. (**e**) The alignment of partial nucleotide sequence of *ND5*. (**f**) The alignment of partial nucleotide sequence of *Cytb*. Dots mean sequence consistency, and hyphens indicated insertions/deletions. Blue showed loci from COC that were different from BSB; red showed loci from BSB that were different from COC; yellow showed mutations that differed from both COC and BSB. The red box showed the nucleotide sequence alignment of 2*n*NCRC-F_1; the black box indicated the nucleotide sequence alignment of 2*n*NCRC-F_2–F_3. The red arrow showed that the base sites in the mtDNA structural regions (genes) were inconsistent between 2*n*NCRC-F_1 and 2*n*NCRC-F_2–F_3; the black arrow represented the polymorphic base sites in mtDNA structural regions (genes) of 2*n*NCRC-F_1

Table 9.7 Base site composition of the eight mtDNA structural regions (genes) of 2*n*NCRC in different generations (Wang et al. 2020a)

Name of gene	Size (bp)	Number (percentage) of conserved base sites	Number (percentage) of base sites of maternal consistency			Number (percentage) of base sites of paternal consistency			Number (percentage) of mutation sites		
			F_1–F_3	F_1	F_2–F_3	F_1–F_3	F_1	F_2–F_3	F_1–F_3	F_1	F_2–F_3
Control region	923	624 (67.61%)	147 (15.93%)	2 (0.22%)	4 (0.43%)	40 (4.33%)	1 (0.11%)	5 (0.54%)	67 (7.26%)	4 (0.43%)	9 (0.98%)
12S rRNA	954	862 (90.36%)	64 (6.71%)	0 (0.00%)	0 (0.00%)	5 (0.52%)	0 (0.00%)	0 (0.00%)	15 (1.57%)	1 (0.10%)	1 (0.10%)
16S rRNA	1681	1492 (88.76%)	98 (5.83%)	0 (0.00%)	1 (0.06%)	32 (1.90%)	1 (0.06%)	1 (0.06%)	43 (2.56%)	1 (0.06%)	4 (0.24%)
ND2	1047	759 (72.49%)	111 (10.60%)	0 (0.00%)	5 (0.48%)	57 (5.44%)	2 (0.19%)	0 (0.00%)	76 (7.26%)	2 (0.19%)	12 (1.15%)
COI	1551	1237 (79.75%)	107 (6.90%)	0 (0.00%)	8 (0.52%)	66 (4.26%)	0 (0.00%)	3 (0.19%)	101 (6.51%)	2 (0.13%)	6 (0.39%)
ND4	1381	1035 (74.95%)	125 (9.05%)	2 (0.14%)	9 (0.65%)	81 (5.87%)	2 (0.14%)	3 (0.22%)	88 (6.37%)	4 (0.29%)	8 (0.58%)
ND5	1824	1301 (71.33%)	228 (12.50%)	5 (0.27)	6 (0.33%)	94 (5.15%)	2 (0.11%)	5 (0.27%)	125 (6.85%)	4 (0.22%)	19 (1.04%)
Cytb	1141	875 (76.69%)	95 (8.33%)	0 (0.00%)	8 (0.70%)	42 (3.68%)	2 (0.18%)	3 (0.26%)	74 (6.49%)	3 (0.26%)	15 (1.31%)

Table 9.8 Polymorphic base sites of the eight mitochondrial structural regions (genes) of 2*n*NCRC-F_1 (Wang et al. 2020a)

Name of gene	Size (bp)	Number of unstable base sites of F_1			
		Maternal or paternal	Maternal or mutant	Paternal or mutant	Conserved or mutant
Control region	923	1 (0.11%)	4 (0.43%)	1 (0.11%)	14 (1.52%)
12S rRNA	954	0 (0.00%)	0 (0.00%)	0 (0.00%)	6 (0.63%)
16S rRNA	1681	1 (0.06%)	1 (0.06%)	1 (0.06%)	5 (0.30%)
ND2	1047	1 (0.10%)	5 (0.48%)	0 (0.00%)	17 (1.62%)
COI	1551	8 (0.52%)	4 (0.26%)	2 (0.13%)	7 (0.45%)
ND4	1381	5 (0.36%)	4 (0.29%)	2 (0.14%)	13 (0.94%)
ND5	1824	4 (0.22%)	4 (0.22%)	1 (0.05%)	26 (1.43%)
Cytb	1141	8 (0.70%)	3 (0.26%)	0 (0.00%)	13 (1.14%)

were stably inherited from the second generation by the third generation. For example, 4.88% of the base sites in the CR, 0.84% to 0.95% of the base sites in the two types of rRNAs, and 2.58–4.82% of the five protein-coding genes were inconsistent between 2*n*NCRC-F_1 and 2*n*NCRC-F_2–F_3. Figure 9.5 and Tables 9.7 and 9.8 also showed that the eight mt structural regions (genes) of 2*n*NCRC-F_1 formed by the hybridization of female COC and male BSB and different individuals in the same population (2*n*NCRC-F_1) showed various modes of genetic variation. Among the same base sites, some were inherited from the maternal parent COC, while others were inherited from the paternal parent BSB or were mutated. According to statistics, 2*n*NCRC-F_1 had 2.17% polymorphic base sites in the CR, 0.48 to 0.63% polymorphic base sites in the two types of rRNAs, and 1.35–2.20% polymorphisms in the five protein-coding genes. Our results showed rapid homoploid speciation in the first generation of distant hybridization and showed the instability of the newly established homoploid mt genome (Wang et al. 2020a).

9.2 Establishment and Biological Characteristic Study of Autotetraploid Fish Lineage

At present, we have established an autotetraploid fish lineage artificially ($4n = 200$, F_1–F_5, abbreviated as 4*n*NC) produced by the hybridization of female 4*n*CB and male 2*n*NCRC derived from the distant crosses of female COC and male BSB. The 4*n*CB (♀) × 2*n*NCRC (♂) hybridization had a high fertilization rate (84.0%), a high hatching rate (72.5%), and a moderately high survival rate (58.2%). This new type of autotetraploid fish was obtained through distant hybridization and mating between different ploidy sister taxa and had the advantages of high hatching and survival rates. This 4*n*NC lineage had four sets of chromosomes from COC. The sexual maturity of the females and males of 4*n*NC lineage (F_1–F_4) was at 1 year of age, which produced a large number of mature eggs and a large amount of white semen, respectively, thus ensuring the expansion and application of the population. We

expect to use this 4*n*NC lineage to prepare sterile triploid common carp with excellent traits on a large scale, which has great value for the commercial production of common carp.

9.2.1 Characteristics of Genetic Construction of Autotetraploid Fish Lineage

9.2.1.1 Chromosome Number and Karyotype

In Sect. 9.1.1, ploidy analyses of the number of chromosomes showed that 2*n*NCRC had 100 chromosomes, which was the same as that of COC, and that 4*n*CB had 148 chromosomes, which was the same as the total number of chromosomes in COC and BSB (Wang et al. 2017). The chromosomes were counted in 200 metaphase spreads for each sample of COC, BSB, and 4*n*NC lineage (F_1–F_4) (Table 9.9). In COC samples, 92.5% of the chromosomal metaphases had 100 chromosomes (Table 9.9; Fig. 9.6a), and no larger submetacentric chromosomes were found in COC, which was the same as the patterns described in our previous study (Liu et al. 2001). We tested BSB samples and observed that they had 48 chromosomes (90.0% of the chromosomal metaphases) (Table 9.9; Fig. 9.6b), and a large pair of submetacentric chromosomes was found in BSB, as described in previous studies (Liu et al. 2007). In the 4*n*NC lineage (F_1–F_4) samples that we tested, 86.0–89.0% of chromosomal metaphases had 200 chromosomes, lacking the large submetacentric chromosomes from BSB (Table 9.9; Fig. 9.6c–f), showing that they were tetraploid and had four sets of COC-derived chromosomes.

9.2.1.2 Detection of DNA Content

With the sum of the DNA contents of COC and BSB as the control group, the DNA content distribution of 2*n*NCRC, 4*n*CB, and 4*n*NC lineage (F_1–F_4) was shown in Table 9.10. The average DNA content of 2*n*NCRC was the same as that of COC ($P > 0.05$), showing that 2*n*NCRC had two sets of COC-derived chromosomes. The average DNA content of 4*n*CB was equal to the sum of the DNA content of COC and BSB ($P > 0.05$), showing that 4*n*CB had two sets of chromosomes from COC

Table 9.9 The chromosome numbers in BSB, COC, and 4*n*NC lineage (F_1–F_4) (Wang et al. 2020b)

	Distribution of chromosome number						
Fish type	Number of metaphase	<48[a]	48	<100[a]	100	<200[a]	200
BSB	200	20	180				
COC	200			15	185		
4*n*NC-F_1	200					28	172
4*n*NC-F_2	200					22	178
4*n*NC-F_3	200					27	173
4*n*NC-F_4	200					25	175

[a]The chromosome number was less than what they should be, owing to the loss of chromosomes in the procedure of chromosome preparation

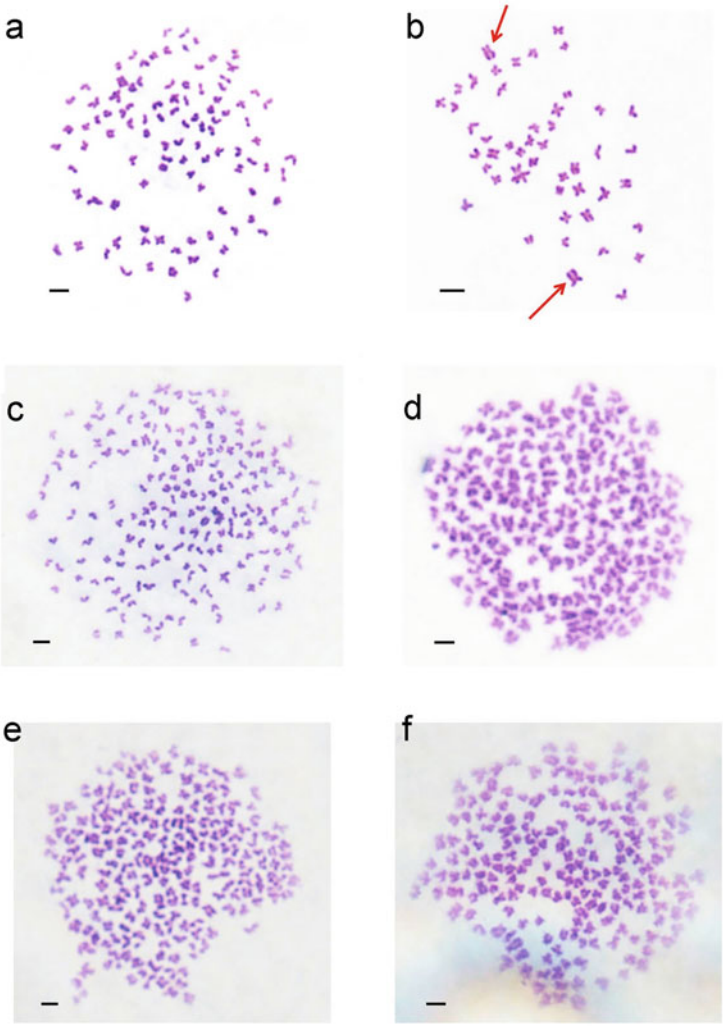

Fig. 9.6 The chromosome spreads at metaphase in COC, BSB, and 4*n*NC lineage (F_1–F_4) (Wang et al. 2020b). (**a**) The 100 chromosomes of COC had no large submetacentric chromosomes. (**b**) The 48 chromosomes of BSB had a pair of the largest submetacentric chromosomes (red solid arrows). (**c**) The 200 chromosomes of 4*n*NC-F_1 had no large submetacentric chromosomes. (**d**) The 200 chromosomes of 4*n*NC-F_2 had no large submetacentric chromosomes. (**e**) The 200 chromosomes of 4*n*NC-F_3 had no large submetacentric chromosomes. (**f**) The 200 chromosomes of 4*n*NC-F_4 had no large submetacentric chromosomes. Bar = 3 μm

and two sets of chromosomes from BSB (Wang et al. 2017). The average DNA content of 4*n*NC lineage (F_1–F_4) was twice that of COC ($P > 0.05$), indicating that 4*n*NC lineage (F_1–F_4) had four sets of COC-derived chromosomes (Table 9.10).

Table 9.10 The mean DNA content in BSB, COC, 2*n*NCRC, 4*n*CB, and 4*n*NC lineage (F_1–F_4) (Wang et al. 2020b)

Fish type	DNA content[a]	Ratio	
		Observed	Expected
BSB	67.29		
COC	105.40		
2*n*NCRC	107.34	2*n*NCRC/COC = 1.02[b]	1
4*n*CB	157.81	4*n*CB/(COC + BSB) = 0.91[b]	1
4*n*NC-F_1	209.03	4*n*NC-F_1/COC = 1.98[b]	2
4*n*NC-F_2	200.26	4*n*NC-F_2/COC = 1.90[b]	2
4*n*NC-F_3	196.55	4*n*NC-F_3/COC = 1.86[b]	2
4*n*NC-F_4	198.45	4*n*NC-F_4/COC = 1.88[b]	2

[a]The intensity of fluorescence (unit, channel)
[b]The observed ratio was not significantly different ($P > 0.05$) from the expected ratio

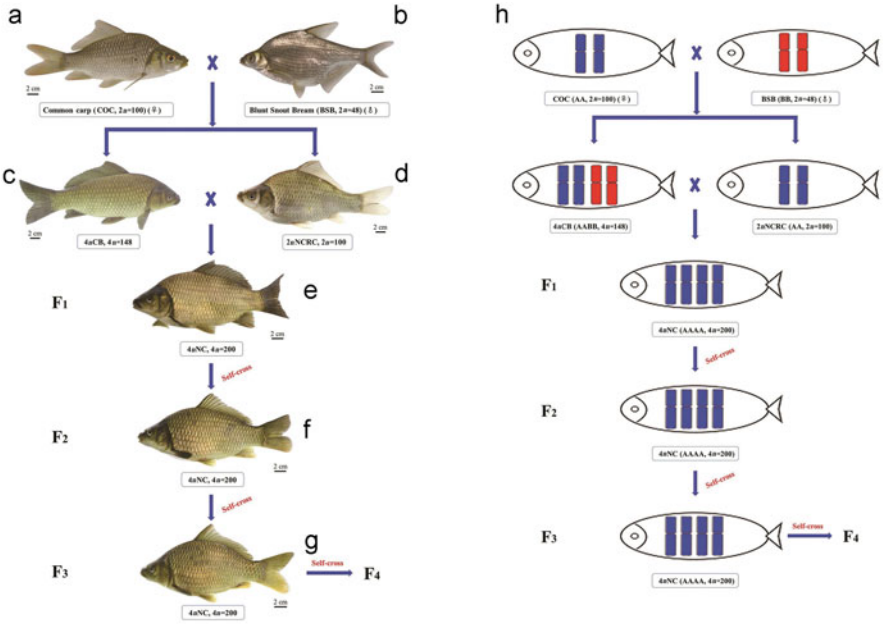

Fig. 9.7 Crossing procedure and appearance of COC, BSB, 4*n*CB, 2*n*NCRC, and 4*n*NC-F_1–F_4 (Wang et al. 2020b). (**a**) The appearance of COC. (**b**) The appearance of BSB. (**c**) The appearance of 4*n*CB. (**d**) The appearance of 2*n*NCRC. (**e**) The appearance of 4*n*NC-F_1. (**f**) The appearance of 4*n*NC-F_2. (**g**) The appearance of 4*n*NC-F_3. (**h**) Formation of experimental fish. The chromosomes of COC and BSB were marked in blue and red color, respectively. Bar = 2 cm

9.2.2 Appearance of Autotetraploid Fish Lineage

As shown in Fig. 9.7, there were obvious differences in morphological characteristics between 4*n*NC lineage (F_1–F_3) (Fig. 9.7e–g) or COC (Fig. 9.7a),

BSB (Fig. 9.7b), 4*n*CB (Fig. 9.7c), and 2*n*NCRC (Fig. 9.7d). Tables 9.11 and 9.12 exhibited the values for the countable and measurable traits in COC, BSB, 4*n*CB, 2*n*NCRC, and 4*n*NC lineage (F_1–F_3). For the countable traits (Table 9.11), between 4*n*NC lineage (F_1–F_3) and COC, except for the numbers of lateral line scales and scales below lateral line, all traits had significant differences ($P < 0.01$). Except for the number of pelvic fins, the other countable traits were significantly different ($P < 0.01$) between 4*n*NC lineage (F_1–F_3) and BSB. Between 4*n*NC lineage (F_1–F_3) and 2*n*NCRC, except for the number of lateral line scales, there were no significant differences in other countable traits ($P > 0.01$). In addition, there were no significant differences in the countable traits ($P > 0.01$) between 4*n*NC lineage (F_1–F_3) and 4*n*CB.

For the measurable traits (Table 9.12), between 4*n*NC lineage (F_1–F_3) and COC, except for BD/BL, the other traits had significant differences ($P < 0.01$). Except for BL/TL, BD/BL, HH/HL, and CPD/CPL, the other measurable traits were significantly different ($P < 0.01$) between 4*n*NC lineage (F_1–F_3) and BSB. In addition, there were no significant differences in the measurable traits ($P > 0.01$) between 4*n*NC lineage (F_1–F_3) and 2*n*NCRC. Except for HH/BD, there were no significant differences ($P > 0.01$) between 4*n*NC lineage (F_1–F_3) and 4*n*CB in other measurable traits.

9.2.3 Reproductive Traits of Autotetraploid Common Carp

Our previous analysis of reproductive traits showed that COC, BSB, and 4*n*CB were sexually mature at 2 years of age, while 2*n*NCRC was sexually mature at 1 year of age (Liu et al. 2001, 2007; Wang et al. 2017). The ovaries of 22-month-old 4*n*CB females and 10-month-old 2*n*NCRC females developed well and contained oocytes in stages II, III, and IV (Fig. 9.8a, c). The testes of 22-month-old 4*n*CB males contained a lot of spermatids, which developed into abnormal sperm (Fig. 9.8b). During the second breeding season, 4*n*CB females produced a large number of mature eggs, but 4*n*CB male gonads had poor gonadal development and only squeezed out a small amount of water-like semen. As expected, 4*n*CB males and females derived from self-crossing had no surviving offspring. The testes of 10-month-old 2*n*NCRC males contained more lobules with many mature spermatozoa (Fig. 9.8d). During the breeding season, a large amount of white semen could be stripped from the testes of 2*n*NCRC males. The combination of 4*n*CB (♀) × 2*n*NCRC (♂) successfully avoided the problem of poor quality of male 4*n*CB semen leading to fertilization failure. Furthermore, we successfully developed an autotetraploid fish with a high fertilization rate (84.0%), a high hatching rate (72.5%), and a medium survival rate (58.2%) through artificial crosses between female 4*n*CB and male 2*n*NCRC. The ovaries of 10-month-old 4*n*NC-F_3 females developed well and contained oocytes in stages II, III, and IV (Fig. 9.8e). The testes of 10-month-old 4*n*NC-F_3 males contained more lobules with many mature spermatozoa (Fig. 9.8f). During the breeding season, a large number of mature

Table 9.11 Comparison of the countable traits among BSB, COC, 2*n*NCRC, 4*n*CB, and 4*n*NC lineage (F_1–F_3) (Wang et al. 2017, 2020b)

Fish type	No. of lateral line scales	No. of scales above lateral line	No. of scales below lateral line	No. of dorsal fin	No. of pelvic fin	No. of anal fin
COC	36.35 ± 1.43 (35–38)	5.37 ± 0.45 (5–6)	5.30 ± 0.43 (5–6)	III + 17.62 ± 0.89 (III + 17–19)	8.58 ± 0.51 (8–9)	III + 6.37 ± 0.39 (III + 6–7)
BSB	50.60 ± 1.20 (49–52)	9.48 ± 0.55 (9–10)	10.19 ± 0.97 (9–11)	III + 8.50 ± 0.52 (III + 8–9)	9.18 ± 0.69 (8–10)	III + 25.90 ± 0.88 (III + 25–27)
2*n*NCRC	29.35 ± 0.38 (29–30)	6.43 ± 0.52 (6–7)	7.36 ± 0.56 (7–8)	III + 18.34 ± 1.28 (III + 17–20)	9.08 ± 1.69 (7–11)	III + 7.66 ± 1.25 (III + 6–9)
4*n*CB	30.94 ± 0.86 (30–32)	6.00 ± 0.00 (6)	6.45 ± 0.42 (6–7)	III + 17.94 ± 1.67 (III + 16–20)	8.36 ± 1.29 (7–10)	III + 7.99 ± 1.67 (III + 6–9)
4*n*NC lineage (F_1–F_3)	32.00 ± 1.00 (31–33)	6.35 ± 0.39 (6–7)	6.67 ± 0.46 (6–7)	III + 17.67 ± 1.62 (III + 16–19)	8.00 ± 1.00 (7–9)	III + 7.00 ± 0.00 (III + 7)

Table 9.12 Comparison of the measurable traits among BSB, COC, 2*n*NCRC, 4*n*CB, and 4*n*NC lineage (F_1–F_3) (Wang et al. 2017, 2020b)

Fish type	BL/TL	BD/BL	HL/BL	HH/HL	CPD/CPL	HH/BD
COC	0.83 ± 0.07	0.34 ± 0.01	0.24 ± 0.02	0.81 ± 0.07	0.86 ± 0.11	0.60 ± 0.01
BSB	0.84 ± 0.04	0.41 ± 0.04	0.20 ± 0.04	0.88 ± 0.03	0.93 ± 0.04	0.49 ± 0.04
2*n*NCRC	0.84 ± 0.02	0.41 ± 0.02	0.26 ± 0.01	0.88 ± 0.02	0.88 ± 0.03	0.56 ± 0.01
4*n*CB	0.83 ± 0.02	0.38 ± 0.02	0.27 ± 0.01	0.88 ± 0.02	0.89 ± 0.01	0.62 ± 0.01
4*n*NC lineage (F_1–F_3)	0.85 ± 0.02	0.39 ± 0.01	0.26 ± 0.02	0.85 ± 0.05	0.99 ± 0.10	0.57 ± 0.03

Note: BL/TL (body length to total length), BD/BL (body depth to body length), HL/BL (head length to body length), HH/HL (head height to head length), CPD/CPL (caudal peduncle depth to caudal peduncle length), HH/BD (head height to body depth

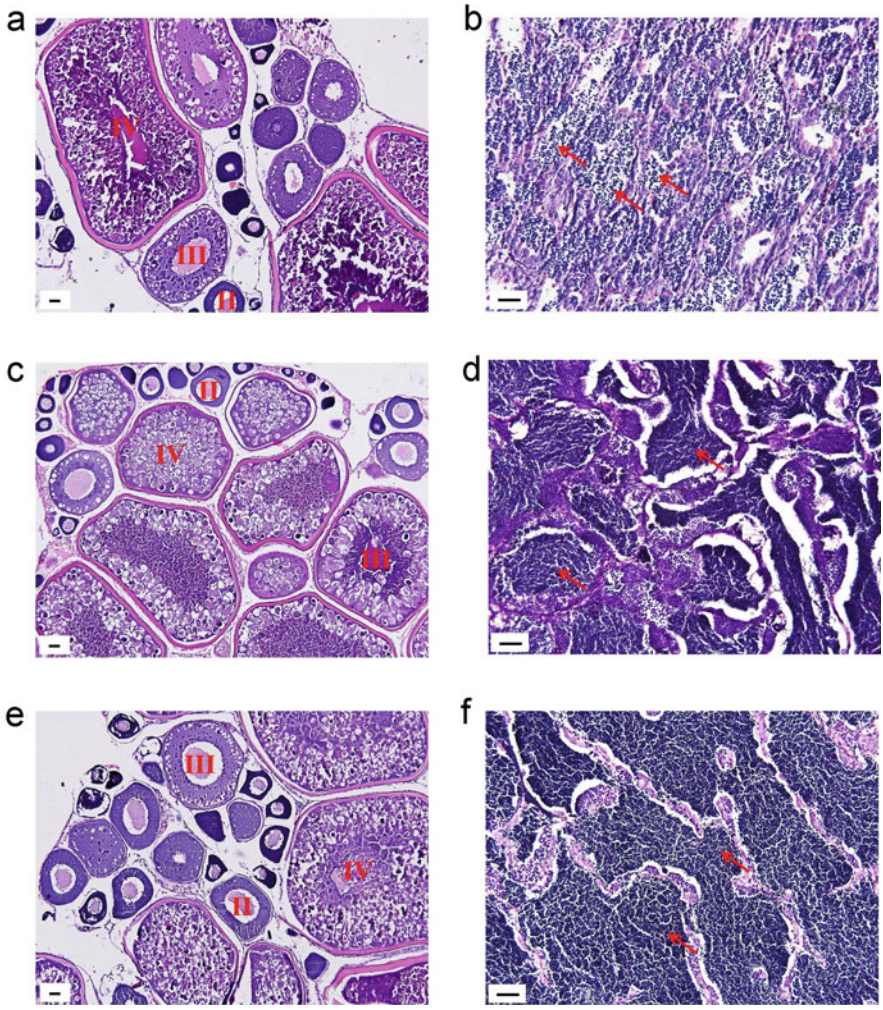

Fig. 9.8 The gonadal structure of 4*n*CB, 2*n*NCRC, and 4*n*NC-F_3 (Wang et al. 2020b). (**a**) The ovary of a 22-month-old 4*n*CB developed well and contained oocytes in stages II, III, and IV. (**b**) In the testis of a 22-month-old 4*n*CB, many spermatids developed into abnormal sperm (red arrow). (**c**) The ovary of a 10-month-old 2*n*NCRC developed well and contained oocytes in stages II, III, and IV. (**d**) The testis of a 10-month-old 2*n*NCRC contained many lobules with many mature spermatozoa (red arrow). (**e**) The ovary of a 10-month-old 4*n*NC-F_3 developed well and contained oocytes in stages II, III, and IV. (**f**) The testis of a 10-month-old 4*n*NC-F_3 contained many lobules with many mature spermatozoa (red arrow). Bar = 20 μm

eggs and a large amount of white semen were produced by female 4*n*NC and male 4*n*NC, respectively.

Scanning electron microscopy was performed on the spermatozoa of 10-month-old 4*n*NC-F_3 and 22-month-old COC. As shown in Fig. 9.9, the heads and tails of the sperm produced by COC and 4*n*NC-F_3 were well developed. In addition, the size of

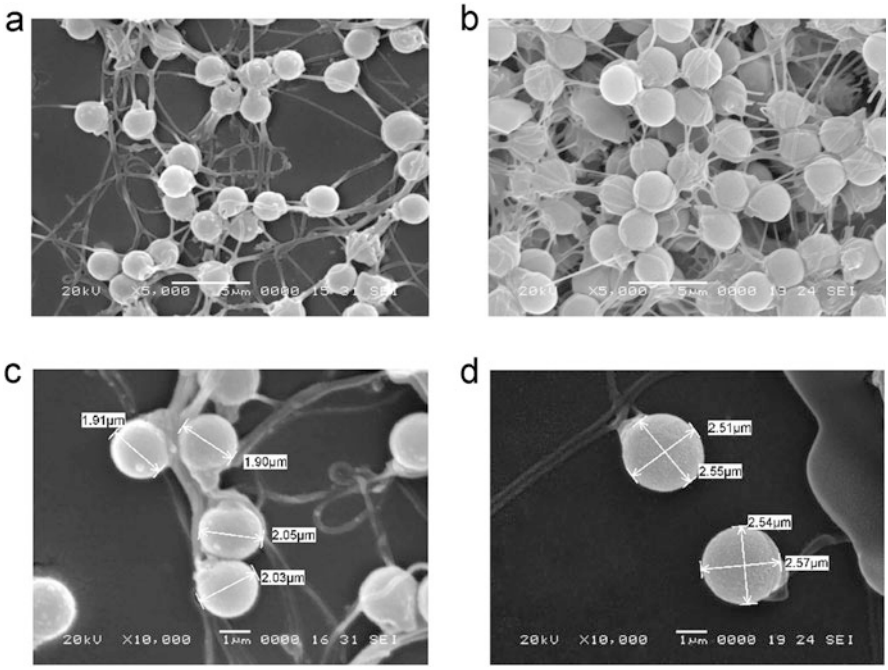

Fig. 9.9 The spermatozoa of COC and 4*n*NC-F_3 (Wang et al. 2020b). (**a**) The spermatozoa of COC. Bar = 5 μm. (**b**) The spermatozoa of 4*n*NC-F_3. Bar = 5 μm. (**c**) The spermatozoa of COC. Bar = 1 μm. (**d**) The spermatozoa of 4*n*NC-F_3. Bar = 1 μm

the head of 4*n*NC-F_3 sperm (Fig. 9.9b, d) was larger than the head of COC sperm (Fig. 9.9a, c). The average diameter of COC haploid sperm was 1.97 μm, while the average diameter of 4*n*NC-F_3 sperm was 2.54 μm. The spermatozoa volume of 4*n*NC-F_3 (8.58 μm^3) was two times larger than that of COC (4.00 μm^3), and there was no significant difference ($P > 0.05$) from the ratio of 2:1, suggesting that 4*n*NC-F_3 produced diploid spermatozoa.

At the level of reproductive biology, female 4*n*CB ($4n = 148$) had abnormal chromosome behavior during meiosis, resulting in the production of gametes with complete maternal chromosomes. The establishment of 4*n*NC ($4n = 200$, AAAA) was obtained by the fertilization of autotriploid eggs ($3n = 150$, AAA) by female 4*n*CB ($4n = 148$, AABB) and haploid sperm ($n = 50$, A) by male 2*n*NCRC ($2n = 100$, AA) (Fig. 9.7h). The formation of autotriploid eggs is related to the mechanism of genomic doubling by premeiotic endoreduplication, endomitosis, or oogonia fusion of female allotetraploid fish (Qin et al. 2014, 2015, 2016). The results of chromosome and DNA content levels showed that 200 chromosomes of 4*n*NC-F_1–F_4 individuals originated from COC. The newly established 4*n*NC lineage could produce normal diploid eggs and diploid sperm, thus maintaining tetraploidy from one generation to the next (F_1–F_5).

9.2.4 Cellular Biological Characteristics of Autotetraploid Common Carp

Figure 9.10 presented the nuclear characteristics of erythrocytes in COC and 4*n*NC-F_1. The erythrocyte nucleus of 4*n*NC-F_1 was larger than those in COC. This result was similar to the previous results in our laboratory (Liu et al. 2001, 2007). In addition to the difference in nuclear size, there were also differences in nuclear appearance between 4*n*NC-F_1 and COC. For example, normal erythrocytes with one nucleus and no unusual erythrocytes with two nuclei were found in COC (Fig. 9.10a). However, in 4*n*NC-F_1, unusual erythrocytes with two nuclei were observed (Fig. 9.10b). Table 9.13 showed the measured results of the mean erythrocyte nuclear volume in COC and 4*n*NC-F_1. The average nuclear volume of erythrocyte of 4*n*NC-F_1 was two times larger than that of COC, and there was no significant difference ($P > 0.05$) from the ratio of 2:1, suggesting that 4*n*NC-F_1 was tetraploid.

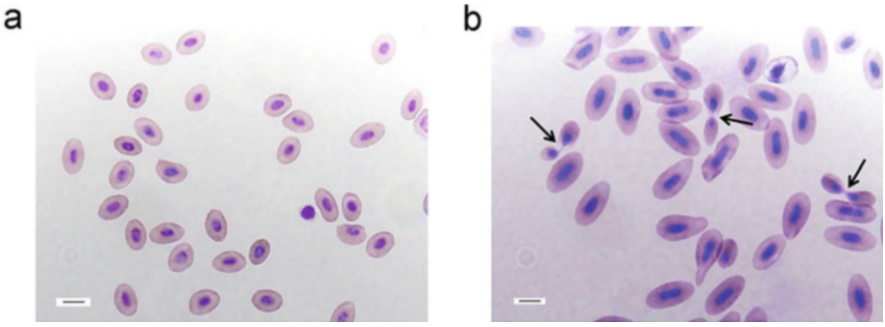

Fig. 9.10 Erythrocytes of COC and 4*n*NC-F_1 (Wang et al. 2020b). (**a**) Normal erythrocytes with one nucleus in COC. (**b**) Normal erythrocytes with one nucleus and unusual erythrocytes with two nuclei (arrows) in 4*n*NC-F_1. Bar =10 μm

Table 9.13 The average erythrocyte nuclear volume measurements for COC and 4*n*NC-F_1 (Wang et al. 2020b)

Fish type	Major axis (μm)	Minor axis (μm)	Volume (μm^3)	Volume ratio	
				Observed	Expected
COC	6.37 ± 0.58	4.28 ± 0.54	60.59 ± 13.82		
4*n*NC-F_1	9.70 ± 1.09	4.78 ± 0.43	114.43 ± 12.77	2.00[a]	2

[a]The observed ratio was not significantly different ($P > 0.05$) from the expected ratio

9.3 The Formation and Biological Characteristics of Koi Carp (♀) × Blunt Snout Bream (♂) Hybrids

9.3.1 Introduction of Hybrid Parents

Koi carp (KOC, $2n = 100$), the maternal parent, is a variety of ornamental carp of *C. carpio*, and most individuals of these species are also characterized by red or colorful bodies. Its body side is flat and spindle shaped, and its body surface is covered with scales. It can ingest mollusks, aquatic plants, algae, rice, and wheat food. Under natural conditions, the age of sexual maturity is two winter years. Blunt snout bream (BSB, $2n = 48$), the paternal parent, belongs to Cyprinidae (family), Cultrinae (subfamily), and *Megalobrama* (genus). The basic information of the paternal parent had been introduced in detail in Chap. 6 of the first section of the book. BSB is a suitable species to cross with KOC. Compared with KOC, BSB possess different chromosome number, different body color (gray) and the same age of sexual maturity (2 years) (Fig. 9.11a, b).

9.3.2 Preparation of F_1 Hybrids

During the breeding season (April–July), KOC and BSB were selected as the maternal parents and paternal parents, respectively. The cross experiment was performed in these two groups: in the first group, KOC and BSB were used as the maternal parent and paternal parent, respectively; and in the second group, the

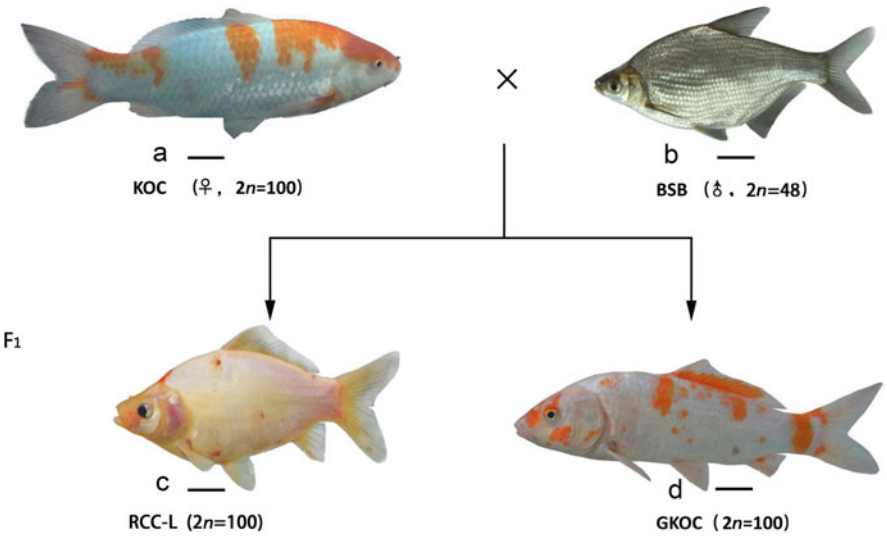

Fig. 9.11 The formation procedure and the appearance of KOC, BSB, RCC-L, and GKOC (Wang et al. 2018). (**a**) KOC. (**b**) BSB. (**c**) RCC-L. (**d**) GKOC. Bar = 3 cm

maternal parent and paternal parent were reversed. The mature eggs were fertilized with semen, and the embryos were developed in culture dishes at a water temperature of 18–23 °C. In the first group, the KOC (♀) × BSB (♂) resulted in two types of offspring: red crucian carp-like homodiploid fish (RCC-L) and gynogenetic koi carp (GKOC), 99% of RCC-L and 1% of GKOC existed (Fig. 9.11a–d). In the second group, the cross of BSB (♀) × KOC (♂) did not produce any living progeny. At present, the RCC-L lineage has been bred to the F_4 generation.

9.3.2.1 Major Biological Characteristics of RCC-L-F_1

The biological characteristics of RCC-L-F_1 were studied by means of shape measurement, paraffin sectioning, scanning electron microscopy, chromosome sectioning, and flow cytometry. The RCC-L-F_1 progeny showed a 95.3% fertilization rate, 90.2% hatching rate, and 85.3% survival rate, and there were only a very small number of deformed individuals. The body color of red pattern on white background of RCC-L-F_1 was similar to that of maternal parent KOC. The number of offspring's vertebrae, scale type, and other countable characters were significantly different from that of maternal parent KOC and paternal parent BSB and were similar to those of red crucian carp. According to the chromosome testing of kidney tissues cells, RCC-L-F_1 was diploid ($2n = 100$) (Fig. 9.12a), and its karyotype was 22 m + 34 sm + 22 st + 22 t (Fig. 9.12b) (Wang et al. 2018).

9.3.2.2 Observation of Gonadal Structure of RCC-L-F_1

The gonads of RCC-L-F_1 developed normally. At 11 months of age, the female diploid RCC-L-F_1's ovary was flat and pale yellow, and its paraffin section showed that there were a large number of phase II oocytes in the small growth phase in the

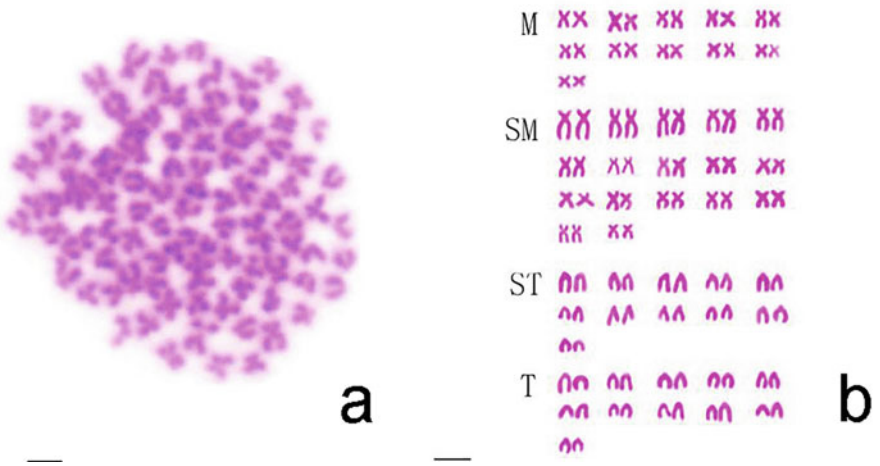

Fig. 9.12 Chromosome spreads and the karyotype of RCC-L-F_1 (Wang et al. 2018). (**a**) Chromosome spreads of RCC-L-F_1. (**b**) The karyotype of RCC-L-F_1. Bar = 3 cm

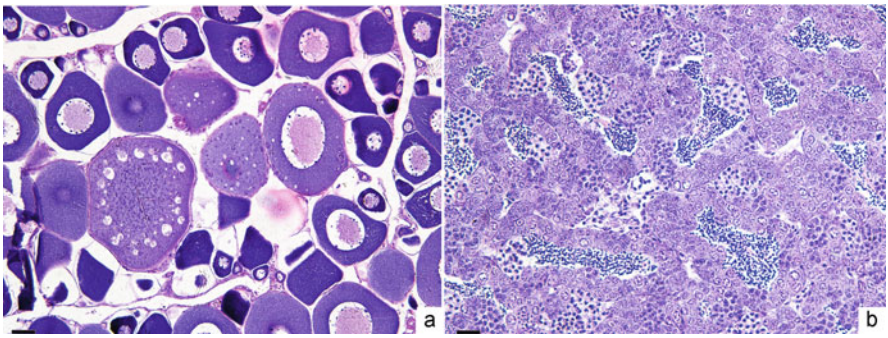

Fig. 9.13 The gonadal structure of diploid RCC-L-F_1. (**a**) The ovary of RCC-L-F_1. (**b**) The testis of RCC-L-F_1. Bar = 20 μm

Table 9.14 The fertilization and hatching rates of the self- mating of KOC, BSB, and RCC-L-F_1 (Wang et al. 2018)

	Fertilization rate (%)	Hatching rate (%)
The self- mating of RCC-L-F_1	95.3	90.2
The self-mating of KOC	95.6	85.3
The self-mating of BSB	92.9	88.2

ovary (Fig. 9.13a); the male diploid RCC-L-F_1 could squeeze out white semen, and its testis was banded. Paraffin sections showed that there were seminiferous tubules in the testis, and there were a large number of sperm cells (Fig. 9.13b) (Wang et al. 2018).

9.3.2.3 The Fertility of RCC-L-F_1

Unlike most distant hybrid F_1 generations with low sterility or low fertility, RCC-L-F_1 has a better fertility, and the time of sexual maturity was obviously 1 year earlier than that of the maternal parent KOC and the paternal parent BSB. When using artificial induction to reproduce, it was found that female diploid RCC-L-F_1 had a higher induction rate, male diploid F_1 also had a higher sperm production (Table 9.14), and the offspring had a higher fertilization rate and a hatching rate. In 2016, 300,000 RCC-L-F_1 fry was obtained.

9.3.3 Characteristics of Genetic Construction of F_1 Hybrids

Using full-length transcriptome and resequencing technology, the liver tissue of KOC was analyzed for orthologous genes and genomic data comparison. The results showed that in RCC-L-F_1, 48.20% of the genes were chimera and 3.70% of homologous genes were mutant. In the full-length transcriptome of RCC-L-F_1, 63.70% of the genetic material came from KOC, and 12.30% of the genetic material came from the BSB. The data of liver cDNA resequencing of RCC-L-F_1 were

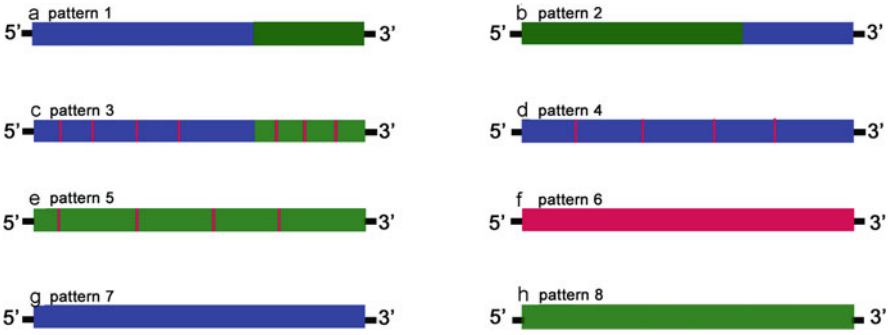

Fig. 9.14 The gene chimeric model (Wang et al. 2020c). Schematic diagrams of gene patterns for the offspring arising from the hybridization of KOC (K) and BSB (B). The heredity materials derived from KOC; The heredity materials derived from BSB; The heredity materials occurred variation

compared to the genomes of COC and BSB, respectively. The results showed that the genetic material of RCC-L-F_1 mainly came from the maternal parents KOC, accompanied by the insertion of the genetic material of the paternal parent BSB (Fig. 9.14) (Wang et al. 2020c).

9.3.4 Appearance and Feeding Habits of F_1 Hybrids

In terms of appearance, KOC was in red or colorful bodies with barbels. BSB was in silver gray with no barbels. The individuals were generally distinguished from KOC by their body colors and shapes. F_1 hybrids were more like the female parent; the body color was white inlaid with red. Regarding feeding habits, RCC-L similar to KOC was omnivorous.

Table 9.15 showed the trait values for KOC, BSB, and RCC-L. In terms of the measured traits, RCC-L and their progeny had HH/BH values between and dramatically different from those of KOC and BSB. In addition, RCC-L and their progeny had HL/BL values significantly greater than those of KOC and BSB and BL/WL values signally lower ($P < 0.05$) than those of KOC and BSB. RCC-L exhibited BH/BL values similar to that of BSB but different from that of KOC. The CPH/CPL values of RCC-L were between that of KOC and that of BSB and markedly different from both, because CPH/CPL values in GF-L were lower than that in KOC or BSB. The RCC-L and RCC had similar CPH/CPL values.

Table 9.15 The phenotypes including the measurable traits (the average ratios of body length to overall length (BL/OL), body depth to body length (BD/BL), head length to body length (HL/BL), head height to head length (HH/HL), caudal peduncle height to caudal peduncle length (CPD/CPL), and head height to body depth (HH/BH), and the countable traits (number of lateral scales, number of dorsal fins, number of pelvic fin, number of anal fins and their progeny and their parents) (Wang et al. 2018)

		Types of fish				
Phenotypes	KOC	BSB	RCC-L	GF-L	RCC	GF
BL/OL	0.86 ± 0.01	0.84 ± 0.04	0.82 ± 0.05	0.65 ± 0.01	0.82 ± 0.02	0.60 ± 0.02
BD/BL	0.38 ± 0.01	0.43 ± 0.04	0.43 ± 0.03	0.69 ± 0.04	0.41 ± 0.02	0.72 ± 0.06
HL/BL	0.25 ± 0.02	0.21 ± 0.02	0.26 ± 0.02	0.36 ± 0.01	0.31 ± 0.03	0.45 ± 0.04
HH/HL	0.96 ± 0.03	0.88 ± 0.02	0.61 ± 0.06	1.13 ± 0.03	0.88 ± 0.06	0.90 ± 0.06
CPD/CPL	0.80 ± 0.09	0.93 ± 0.01	0.83 ± 0.20	0.15 ± 0.03	0.93 ± 0.11	0.18 ± 0.05
HH/BH	0.63 ± 0.05	0.49 ± 0.01	0.61 ± 0.06	0.60 ± 0.08	0.65 ± 0.02	0.70 ± 0.06
No. of lateral line scales	35.5 ± 0.71	50.94 ± 0.94	26.65 ± 1.14	29.67 ± 1.52	28.60 ± 1.14	28.90 ± 0.08
No. of scales above lateral line	7.50 ± 0.71	9.67 ± 0.49	6.90 ± 0.31	7.33 ± 0.58	5.40 ± 0.55	5.40 ± 0.51
No. of scales below lateral line	5.50 ± 0.69	10.05 ± 0.64	5.40 ± 0.50	6.33 ± 0.58	5.36 ± 0.89	6.50 ± 0.25
No. of dorsal fin	III + 20.5 ± 0.68	III + 8.67 ± 0.49	III + 17.9 ± 1.02	III + 14.67 ± 0.90	III + 18.60 ± 0.55	III + 14.67 ± 1.02
No. of pelvic fin	11.5 ± 0.65	9.06 ± 0.64	8.60 ± 0.68	9.33 ± 0.65	7.80 ± 0.84	8.60 ± 0.24
No. of anal fin	III + 9.5 ± 0.62	III + 25.89 ± 0.68	III + 7.15 ± 0.59	III + 7.67 ± 0.58	III + 6.42 ± 0.55	III + 6.6 ± 0.24

9.4 The Formation and Biological Characteristic Study of Goldfish-Like Homodiploid Fish in F_2 from Koi Carp (*C. carpio haematopterus*, ♀) × Blunt Snout Bream (*Megalobrama amblycephala*, ♂) Hybridization

9.4.1 Morphological Characteristics

The goldfish-like homodiploid fish with twin tails (GF-L; $2n = 100$) was produced by self-mating of red crucian carp-like homodiploid fish (RCC-L; $2n = 100$) which derived from the distant crossing of koi carp (*C. carpio haematopterus*, KOC; $2n = 100$; ♀) with blunt snout bream (*Megalobrama amblycephala*, BSB; $2n = 48$; ♂). The phenotypes and genotypes of GF-L and RCC-L were very similar to those of GF and RCC, respectively. The most special was that GF-L own split twin tails (Fig. 9.15a–c) (Wang et al. 2020c).

9.4.2 Characteristics of Genetic Construction of Goldfish-Like Homodiploid Fish

Among GF-L, 90.0% of the chromosomal metaphase spreads had 100 chromosomes with a karyotype of 22 m + 34 sm + 22 st + 22 t, indicating that GF-L was diploid. Among GF, 90.0% of the metaphases had 100 chromosomes. Compared with the parent KOC, it had the same karyotype. Compared with ordinary goldfish, the DNA content and chromosome number were highly consistent (Wang et al. 2018).

Microsatellite patterns (SSR) were used to study the genetic relationship between GF-L and RCC-L, the original parents KOC and BSB. From the results of amplification, the GF-L had the same band as the original maternal parent koi carp, as well as the same band as the original paternal parent BSB, and there were also variable bands (Fig. 9.16) (Wang et al. 2018).

Through the analysis of orthologous gene, it was found that 46.50% of the genes in GF-L were chimeras and 8.30% of the homologous genes were mutated. In the full-length transcriptome of GF-L liver, 68.20% of the genetic materials are derived from KOC, and 11.90% of the genetic materials came from BSB. The resequencing data of the goldfish-like fish liver tissue were compared to the COC and BSB

Fig. 9.15 Morphological characteristics of GF-L in different generations (F_1–F_3) (Wang et al. 2020c). (**a**) GF-L-F_1. (**b**) GF-L-F_2. (**c**) GF-L-F_3. Bar = 4 cm

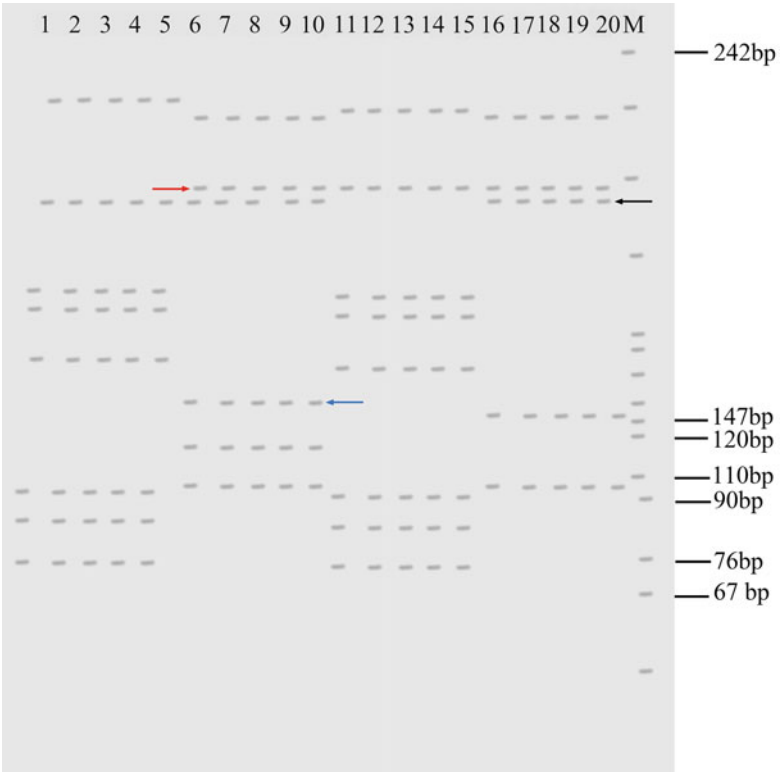

Fig. 9.16 Electrophotogram of microsatellite DNA patterns produced by the primer MFW2 in KOC, RCC-L, BSB, and GF-L (Wang et al. 2020c). Lanes 1–5 represented KOC. Lanes 6–10 represented RCC-L. Lanes 11–15 represented BSB. Lanes 16–20 represented GF-L. The black arrow indicated the DNA bands derived from KOC. RCC-L and GF-L commonly had this type of band, but BSB did not have this band. The red arrow indicated the DNA bands derived from BSB. RCC-L and GF-L commonly had this type of band, but KOC did not have this band. The blue arrow showed the band found only in RCC-L. M represented the pBR322 DNA/Mspl Marker

genomes, respectively. The results showed that the genetic material of GF-L mainly came from the maternal parent KOC, accompanied by the insertion of the genetic material of the paternal parent BSB (Fig. 9.14).

9.4.3 Comparison of Mitochondrial Genomic Protein-Coding Genes Among GF-L, RCC-L, GF, RCC, KOC, and BSB

The codon usage and amino acid distribution modes of the 13 protein-coding genes in GF-L, RCC, KOC, and BSB were mostly similar. However, the codon usage and amino acid distribution mode of GF-L were almost the same as those of GF, and those of RCC-L were similar to those of RCC, but quite different from those of KOC

and BSB. There were significant differences in the use of the UUU, UUC, CUA, AUU, and AUC codons between GF-L and KOC. GF-L was very different from BSB in the use of the UUU, UUC, UUA, AUU, AUC, and GCU codons. For instance, there were 108 UUU codons in GF-L, 76 in KOC, and 73 in BSB. Leu was the most frequent amino acid residue in GF-L, KOC, and BSB, while Cys was the least frequent.

9.4.4 Reproductive Traits of Goldfish-Like Homodiploid Fish

The 1-year-old GF-L was able to produce normal mature gametes. We stripped white semen from 10-month-old male GF-L and mature ova from 10-month-old female GF-L. In the testes of 1-year-old GF-L, we observed numerous mature spermatozoa, spermatids, and spermatogonia in the seminiferous tubules (Fig. 9.17a, c, e). Observation of the gonadal tissue sections revealed that the ovaries of 8-month-old female RCC-L were at stages III and IV, indicating that GF-L were fertile (Fig. 9.17b, d, f). Compared with the RCC-L, the GF-L had the same sexual maturity time (1 year); and compared with the original parent KOC and BSB, its sexual maturity was obviously 1 year earlier.

9.4.5 Establishment and Application of Goldfish-Like Homodiploid Fish

The male and female RCC-L that reached sexual maturity at 1 year were mated to produce the second generation. The self-mating of RCC-L produced 98% of RCC-L-F_2 and 2% of GF-L with split twin tails. The self-mating of GF-L produced the next generation of GF-L-F_2 with split twin tails. The lineage had now been bred to the F_4 generation. As a new type of fish, GF-L presented very beautiful phenotype, such as twin tails, blue eyes, and a white body accompanied by red spots. These phenotypes were very different from any other GF phenotype, showing that the GF-L lineage had great potential in the ornamental market. Compared with a GF, GF-L had more advantages. A female GF-L could produce tens of thousands of eggs and a male GF-L could produce 1–2 ml of semen, which were important factors in increasing the productivity of GF. A large number of new types of GF with high ornamental value could be obtained by crossing GF-L with ordinary GF. In summary, the formation of GF-L was very important to both evolutionary biology and fish genetic breeding (Wang et al. 2020c).

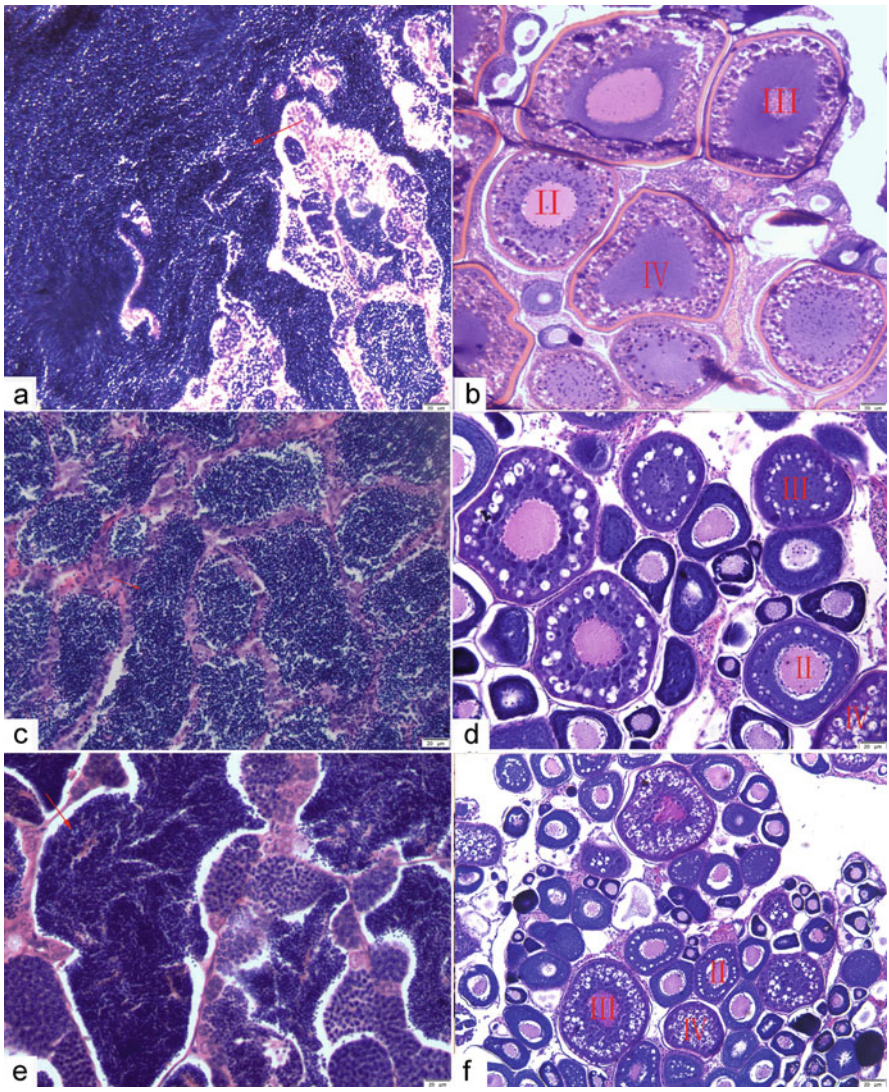

Fig. 9.17 Gonadal structure of BSB, KOC, RCC-L, and GF-L (Wang et al. 2020c). (**a**) Mature testis of BSB. (**b**) Mature ovary of KOC. (**c**) Mature testis of RCC-L. (**d**) Mature ovary of RCC-L. (**e**) Mature testis of GF-L. (**f**) Mature ovary of GF-L. Through scanning electron microscopy, the morphological characteristics of GF-L sperm were scanned, and it was found that the GF-L sperm had normal head and tail. The GF-L had a higher fertilization rate (92.35%) and hatching rate (85.36%)

References

Gillham NW (1994) Organelle genes and genomes. Oxford University Press

Liu S, Liu Y, Zhou G, Zhang X, Luo C, Feng H, He X, Zhu G, Yang H (2001) The formation of tetraploid stocks of red crucian carp× common carp hybrids as an effect of interspecific hybridization. Aquaculture 192(2):171–186

Liu S, Qin Q, Xiao J, Lu W, Shen J, Li W, Liu J, Duan W, Zhang C, Tao M, Zhao R, Yan J, Liu Y (2007) The formation of the polyploid hybrids from different subfamily fish crossings and its evolutionary significance. Genetics 176(2):1023–1034. https://doi.org/10.1534/genetics.107.071373

Luo K, Wang S, Fu Y, Zhou P, Huang X, Gu Q, Li W, Wang Y, Hu F, Liu S (2019) Rapid genomic DNA variation in newly hybridized carp lineages derived from Cyprinus carpio (female symbol) x Megalobrama amblycephala (male symbol). BMC Genet 20(1):87. https://doi.org/10.1186/s12863-019-0784-2

Mallet J (2007) Hybrid speciation. Nature 446(7133):279–283. https://doi.org/10.1038/nature05706

Pinhal D, Gadig OB, Wasko AP, Oliveira C, Ron E, Foresti F, Martins C (2008) Discrimination of shark species by simple PCR of 5S rDNA repeats. Genet Mol Biol 31(1):361–365

Qin Q, Wang Y, Wang J, Dai J, Liu Y, Liu S (2014) Abnormal chromosome behavior during meiosis in the allotetraploid of Carassius auratus red var.(♀)× Megalobrama amblycephala (♂). BMC Genet 15(1):95

Qin Q, Wang J, Dai J, Wang Y, Liu Y, Liu S (2015) Induced all-female autotriploidy in the allotetraploids of Carassius auratus red var. (female symbol) x Megalobrama amblycephala (male symbol). Mar Biotechnol 17(5):604–612. https://doi.org/10.1007/s10126-015-9647-7

Qin Q, Lai Z, Cao L, Xiao Q, Wang Y, Liu S (2016) Rapid genomic changes in allopolyploids of Carassius auratus red var. (female symbol) x Megalobrama amblycephala (male symbol). Sci Rep 6:34417. https://doi.org/10.1038/srep34417

Rokas A, Ladoukakis E, Zouros E (2003) Animal mitochondrial DNA recombination revisited. Trends Ecol Evol 18(8):411–417. https://doi.org/10.1016/s0169-5347(03)00125-3

Ubeda-Manzanaro M, Merlo MA, Palazon JL, Sarasquete C, Rebordinos L (2010) Sequence characterization and phylogenetic analysis of the 5S ribosomal DNA in species of the family Batrachoididae. Genome 53(9):723–730. https://doi.org/10.1139/g10-048

Wang S, Ye X, Wang Y, Chen Y, Lin B, Yi Z, Mao Z, Hu F, Zhao R, Wang J, Zhou R, Ren L, Yao Z, Tao M, Zhang C, Xiao J, Qin Q, Liu S (2017) A new type of homodiploid fish derived from the interspecific hybridization of female common carp x male blunt snout bream. Sci Rep 7(1):4189. https://doi.org/10.1038/s41598-017-04582-z

Wang Y, Yang C, Luo K, Zhang M, Qin Q, Huo Y, Song J, Tao M, Zhang C, Liu S (2018) The formation of the goldfish-like fish derived from hybridization of female koi carp x male blunt snout bream. Front Genet 9:437. https://doi.org/10.3389/fgene.2018.00437

Wang S, Jiao N, Zhao L, Zhang M, Zhou P, Huang X, Hu F, Yang C, Shu Y, Li W, Zhang C, Tao M, Chen B, Ma M, Liu S (2020a) Evidence for the paternal mitochondrial DNA in the crucian carp-like fish lineage with hybrid origin. Sci China Life Sci 63(1):102–115. https://doi.org/10.1007/s11427-019-9528-1

Wang S, Zhou P, Huang X, Liu Q, Lin B, Fu Y, Gu Q, Hu F, Luo K, Zhang C, Tao M, Qin Q, Liu S (2020b) The establishment of an autotetraploid fish lineage produced by female allotetraploid hybrids × male homodiploid hybrids derived from Cyprinus carpio (♀) × Megalobrama amblycephala (♂). Aquaculture 515:734583. https://doi.org/10.1016/j.aquaculture.2019.734583

Wang Y, Tan H, Zhang M, Zhao R, Wang S, Qin Q, Wang J, Zhang C, Tao M, Ma M, Chen B, Liu S (2020c) The hybrid genome of a new goldfish-like fish lineage provides insights into the origin of the goldfish. Front Genet 11:122. https://doi.org/10.3389/fgene.2020.00122

The Formation and Biological Characteristics of the Hybrids Derived from the Hybridization of Japanese White Crucian Carp × Red Crucian Carp

10

Shaojun Liu, Qingfeng Liu, Yi Zhou, Jing Wang, and Xuanyi Zhang

Abstract

As described in the previous chapters, many fish lineages or populations were obtained by hybridization, backcrossing, gynogenesis (androgenesis), self-crossing. These fertile lineages or populations are very useful in genetics, breeding, and biological evolution. In this chapter, we introduce a new hybrid lineage derived from the hybridization of Japanese white crucian carp (*Carassius cuvieri*, ♀, $2n = 100$, WCC) × red crucian carp (*Carassius auratus* red var., ♂, $2n = 100$, RCC), and the improved fish (WR-II) obtained by backcrossing of the hybrids and the WCC. Both WCC and RCC are important economic fish. WCC is characterized by fast growth, silver body color, poor meat quality, and weak stress resistance. RCC is characterized by perfect meat quality, strong stress resistance, and red body color. Hybridization can combine genomes of parent species, which may lead to changes in phenotype and genotype of the hybrids. And hybridization is accompanied by the emergence of advantageous traits. Therefore, we choose WCC and RCC as parents to produce a new kind of fish with strong stress resistance, fast growth, and good meat quality. After a lot of experimental research, we choose the hybrid combination in which the WCC is used as the maternal parent and RCC is used as the paternal parent to produce high-quality hybrid fish. Through successive breeding, we establish a new hybrid lineage and a new kind of improved fish (WR-II). In this chapter, we make a detailed introduction of the biological traits and genetic characteristics of distant hybridization of female WCC × male RCC and the new type of improved fish (WR-II).

S. Liu (✉) · Q. Liu · Y. Zhou · J. Wang · X. Zhang
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_10

Keywords

Distant hybridization · Hybrid lineage · Backcrossing · Fertility · Genetic characteristics

10.1 The Formation of Hybrid Lineage of WCC (♀) × RCC (♂)

Using artificial hybridization breeding technology, the F_1 hybrids (WR-F_1) of female WCC × male RCC were produced. We have only found diploid hybrids in this cross combination. Interestingly, all the WR-F_1 hybrids were highly fertile. White semen can be squeezed out from 10-month-old male WR-F_1, which contained abundant matured sperm; dark green eggs can be stripped out from female WR-F_1 (12 months old) in the breeding season. During the breeding season (April to May), WR-F_2 hybrids were obtained by self-crossing from WR-F_1. WR-F_2 hybrids were also bisexual fertile. Similarly, WR-F_3 hybrids were obtained by self-crossing of WR-F_2, WR-F_4 hybrids were obtained by self-crossing of WR-F_3, and WR-F_5 hybrids were obtained by self-crossing of WR-F_4. So, we obtained a hybrid lineage by the hybridization of female WCC × male RCC (Liu et al. 2019a).

10.1.1 Introduction of Hybrid Parents

The basic features of parents have been introduced in the previous chapters. Here, we mainly introduce the differences between WCC and RCC. In the catalog, the WCC belongs to *C. cuvieri*, *Carassius*, Cyprinidae, and the RCC belongs to *C. auratus*, *Carassius*, Cyprinidae. So, the hybridization between the two fish is interspecific hybridization. WCC is characterized by strong reproductive ability, small head, white body color, and quick growth. RCC is characterized by strong stress resistance, red body color, and perfect meat quality. However, WCC has poor meat quality and RCC shows slow growth. Besides, the red body color of RCC is restricted in sales (Wang et al. 2015). Thus, through this hybrid combination, we expect to obtain a new kind of hybrid fish that has the advantageous traits of both WCC and RCC.

10.1.2 Preparation of F_1 Hybrids

Before carrying out this hybrid combination, we first worked on the selective breeding of WCC and RCC. The excellent WCC and RCC were obtained after five generations of selective breeding. 5–6 months before the breeding season, WCC and RCC with weight over 1 kg, obvious sexual maturity characteristics, good shape, disease-free, and not injured were selected as parents for distant hybridization. In particular, 1 month before breeding period, we used running water to stimulate them every 2–3 days to promote sound gonadal development.

During the breeding season, when the water temperature was stable at 18–25 °C, the parent fish were injected with a mixed oxytocin combining LRH-A with HCG to hasten parturition. The dose of LRH-A was 10 μg/kg and that of HCG was 600 IU/kg, the maternal fish (WCC) was injected firstly, and 4–5 h later, the paternal fish (RCC) was injected with half of that dose. Immediately after the injection, the paternal and maternal parent fish were placed in the same spawning pond at a ratio of 1: (1.5–2.5) to increase their contact opportunities and increase spawning rate. Then we flushed water into the pond, especially 3–4 h before spawning. We stopped flushing water when it reached to the proper line, letting fish stay in water until spawn and produce sperm smoothly.

Females with large spawning and good spawning quality and males with large sperm production were selected for artificial insemination (artificial insemination Sect. 8.1.2). The fertilized eggs were placed in an incubation tank with a water temperature of 20–24 °C for hatching under running water. After the fry were fully hatched, they were first cultivated in the incubation tank for 1–2 days. The fry would be transferred to a pre-fertilized pond for rearing when they were with black dot in waist and able to swim. Three to four days later, we splashed fine soybean milk into the pond, 2–3 times a day, until fry can ingest fodder.

10.1.3 Preparation of F_2 to F_5 Hybrids

During the breeding season, we chose mature females and mature males for self-breeding. We produced WR-F_2 hybrids by self-crossing of WR-F_1. We cultured the fry of WR-F_2 under the same conditions as those of WR-F_1. In the breeding season, we obtained the WR-F_3 hybrids by the self-mating of WR-F_2. Similarly, the WR-F_4 hybrids and WR-F_5 hybrids were produced using the same ways. Thus, a hybrid lineage was formed by the hybridization of female WCC × male RCC (Fig. 10.1) (Liu et al. 2019a).

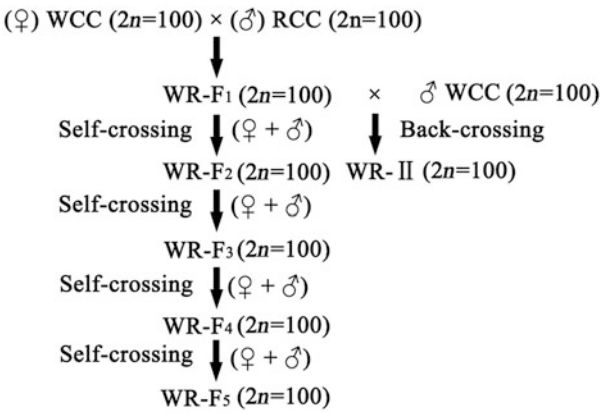

Fig. 10.1 The breeding procedure about the formation of the hybrid lineage and WR-II (Liu et al. 2019a)

10.2 The Biological Characteristics of Hybrids of WCC (♀) × RCC (♂)

10.2.1 The Morphological Traits of F_1 to F_4 Hybrids

The phenotypes of experimental fish were illustrated in Fig. 10.2a–f. The body color was gray in the hybrid offspring. The countable traits and the measurable traits of the hybrid lineage were shown in Tables 10.1 and 10.2. After analysis, there is no significant difference ($p > 0.05$) between the hybrid lineage and its parents for all countable traits and all measurable traits. However, the mean body weight of 2-year-old WR-F_1 (750 g) was significantly higher than the RCC (410 g) ($P < 0.05$), but similar to the WCC (762 g) (Liu et al. 2018).

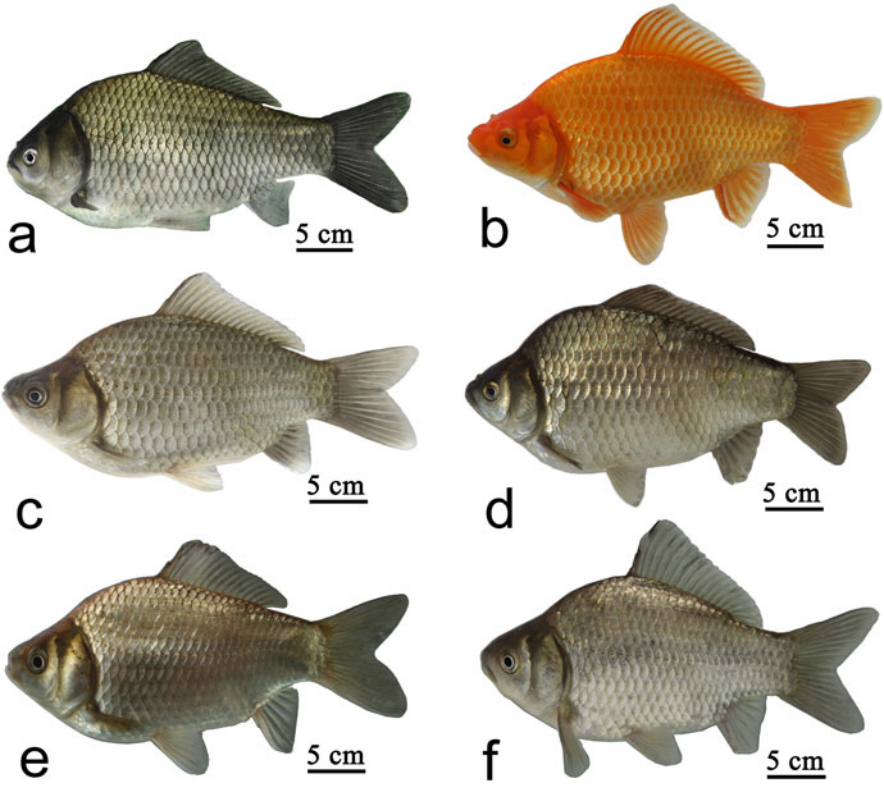

Fig. 10.2 The appearance of WCC, RCC, and their hybrids (Liu et al. 2019a). (**a**) The appearance of WCC. (**b**) The appearance of RCC. (**c**) The appearance of WR-F_1. (**d**) The appearance of WR-F_2. (**e**) The appearance of WR-F_3. (**f**) The appearance of WR-F_4. Bar = 5 cm

Table 10.1 Morphological feature of WCC, RCC, and WR-F_1–F_4 (Liu et al. 2019a)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins
WCC	32–34	6–8	5–7	III+18–20	8–10	III+6–7
RCC	28–30	5–6	6–7	III+18–20	8–9	III+6–7
WR-F_1	30–32	5–7	6–7	III+18–20	8–10	III+6–7
WR-F_2	30–31	6–7	6–7	III+17–18	8–9	III+6–7
WR-F_3	30–31	6–7	6–7	III+17–18	8–9	III+6–7
WR-F_4	29–30	7–8	6–7	III+17–19	8–9	III+6–7

Table 10.2 The ratios of measurable feature of WCC, RCC, and WR-F_1–F_4 (Liu et al. 2019a)

Fish type	Overall length/body length	Body length/body height	Body length/head length	Head length/head height	Caudal peduncle length/caudal peduncle depth	Head height/caudal peduncle depth
WCC	1.24 ± 0.02	2.22 ± 0.15	3.70 ± 0.21	1.17 ± 0.06	0.81 ± 0.01	1.78 ± 0.09
RCC	1.23 ± 0.02	2.20 ± 0.16	3.72 ± 0.27	1.18 ± 0.04	0.79 ± 0.02	1.80 ± 0.07
WR-F_1	1.25 ± 0.03	2.20 ± 0.15	3.70 ± 0.25	1.19 ± 0.06	0.82 ± 0.02	1.81 ± 0.12
WR-F_2	1.24 ± 0.04	2.21 ± 0.13	3.73 ± 0.16	1.18 ± 0.07	0.79 ± 0.05	1.80 ± 0.03
WR-F_3	1.24 ± 0.02	2.24 ± 0.12	3.73 ± 0.24	1.19 ± 0.02	0.81 ± 0.01	1.80 ± 0.06
WR-F_4	1.24 ± 0.01	2.23 ± 0.09	3.75 ± 0.26	1.19 ± 0.07	0.81 ± 0.02	1.80 ± 0.04

10.2.2 The Ploidy Analysis of F_1 to F_4 Hybrids

We used the DNA contents of WCC and RCC as the controls. The results of the DNA content from hybrids and their parents were shown in Table 10.3. All the DNA content of the tested hybrids was equal ($p > 0.05$) to the sum of half of the contents for each of WCC and RCC, implying that the hybrid lineage was diploid fish like their parents. We showed the chromosome numbers of the hybrid lineage in Table 10.4, and 100 metaphase spreads of each type of hybrid fish were tested in this study. According to our results, most of the tested metaphase spreads (more than 94%) had 100 chromosomes, showing that the hybrids were diploid fish with 100 chromosomes (Fig. 10.3a–d).

Table 10.3 Mean DNA content of WCC, RCC, and WR-F_1–F_4 (Liu et al. 2019a)

Fish type	Mean DNA content	Ratio	
		Observed	Expected
WCC	101.89		
RCC	103.17		
WR-F_1	104.09	WR-F_1/(0.5WCC+0.5RCC) = 1.02[a]	1
WR-F_2	102.15	WR-F_2/(0.5WCC+0.5RCC) = 1.00[a]	1
WR-F_3	98.06	WR-F_3/(0.5WCC+0.5RCC) = 0.96[a]	1
WR-F_4	104.71	WR-F_4/(0.5WCC+0.5RCC) = 1.02[a]	1

[a]The observed ratio was not significantly different ($P > 0.05$) from the expected ratio

Table 10.4 Chromosome number of mitotic metaphase in WR-F_1–F_4 (Liu et al. 2019a)

Fish type	Number of samples	Number of metaphase spreads	Chromosome number[a]		
			<97	97–99	100
WR-F_1	10	100	1	5	94
WR-F_2	10	100	1	3	96
WR-F_3	10	100	1	4	95
WR-F_4	10	100	1	4	95

[a]Values represent the number of individuals counted with the specified chromosome number

10.2.3 The Fertility Detection of F_1 to F_4 Hybrids

The ovaries and the testes of the hybrid lineage were observed at 10 months old. The results showed that the ovaries of offspring (WR-F_1–F_4) developed normally and were mainly composed of oocytes at stage II or III (Fig. 10.4a–d); the testes of offspring were full of mature sperm (Fig. 10.5a–d). Furthermore, the white sperm or mature ova could be squeezed out of 1-year-old hybrid fish. The sperm of the male hybrids had normal heads and tails (Fig. 10.6a–d), meaning that they possessed normal sperm morphology.

10.2.4 The Reproductive Capacity of F_1 to F_4 Hybrids

We observed the reproductive capacity of the hybrid lineage by detecting the fertilization rates and hatching rates from the hybrid lineage. The results showed that the hybrid lineage had high fertilization rates and high hatching rates, which showed a good reproductive capacity (Table 10.5).

10.2.5 The Application of Hybrids

As mentioned, the establishment of fertile hybrid lineage had an important application value in production. Many kinds of new species with heterosis could be produced by hybridization of fertile hybrid lineage with other fish. A new kind of

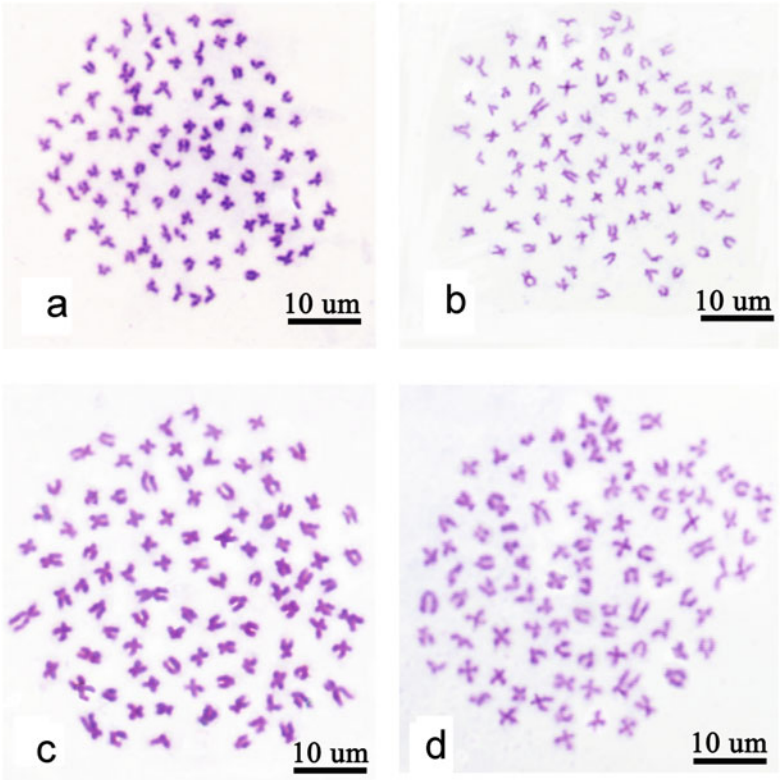

Fig. 10.3 The metaphase chromosome spreads of WR-F_1–F_4 (Liu et al. 2019a). (**a**) The metaphase chromosome spreads of WR-F_1 ($2n = 100$). (**b**) The metaphase chromosome spreads of WR-F_2 ($2n = 100$). (**c**) The metaphase chromosome spreads of WR-F_3 ($2n = 100$). (**d**) The metaphase chromosome spreads of WR-F_4 ($2n = 100$). Bar = 10 μm

improved fish (WR-II) (Figs. 10.1 and 10.7) was produced by backcrossing of WR-F_1 and the WCC. WR-II was diploid fish with 100 chromosomes. WR-II showed higher body and smaller head compared to those of its parents, and the average body weight of 1-year-old WR-II was 556 g (Liu et al. 2019a). We observed the gonadal development of WR-II at 10 months old. The results showed that the ovaries of WR-II were normal and the oocytes were mainly at stage II or III; the testes of WR-II possessed mature sperm. Furthermore, the white sperm or mature ova could be squeezed out of 1-year-old WR-II. WR-II was a good quality fish suitable for large-scale production. The formation of WR-II also brought new germplasm resources to produce triploid fish by the hybridization of diploid fish with tetraploid fish.

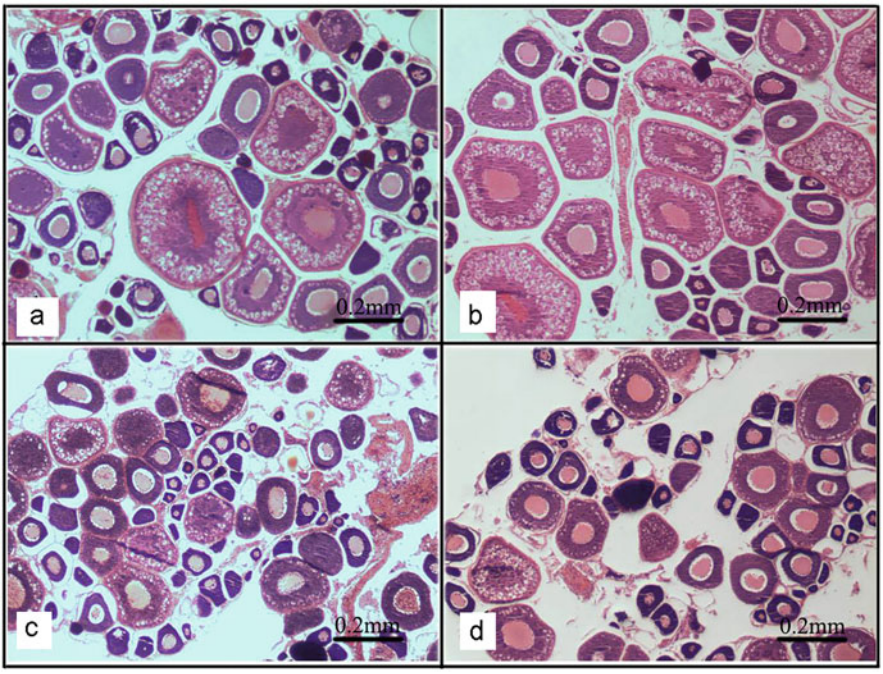

Fig. 10.4 The ovary microstructure of WR-F_1–F_4 (Liu et al. 2019a). (**a**) The histology of ovaries from WR-F_1. (**b**) The histology of ovaries from WR-F_2. (**c**) The histology of ovaries from WR-F_3. (**d**) The histology of ovaries from WR-F_4. Bar = 0.2 mm

10.3 The Molecular Genetic Characteristics of F_1 to F_4 Hybrids and WR-II

10.3.1 The Nucleotide Sequence of 5S rDNA in F_1 to F_4 Hybrids and WR-II

We used PCR (polymerase chain reaction) to amplify 5S rDNA of the hybrid lineage and WR-II. The same patterns with three bands were produced in all detected fish (Liu et al. 2019a). We revealed the sizes of the three fragments by Sanger sequencing, and the results showed that they were 203 bp, 340 bp, and 471 bp, respectively.

We confirmed that these fragments were all 5S rDNA repeat units by using BLASTn. Each repeat unit comprised a whole NTS region, a 3′ end of the coding region (100–120 bp in Fig. 10.8), and a large 5′ coding region of the adjacent unit (1–99 bp in Fig. 10.8). Different NTS types (NTS-83, NTS-220, and NTS-351) were categorized from three fragments of 5S rDNA (class I, class II, and class III for 203 bp, 340 bp, and 471 bp). In the coding regions, we identified all the internal control regions (including A box, internal element, and C box) (Fig. 10.8). We also examined the TATA box control element in all the NTS sequences, and the TATA

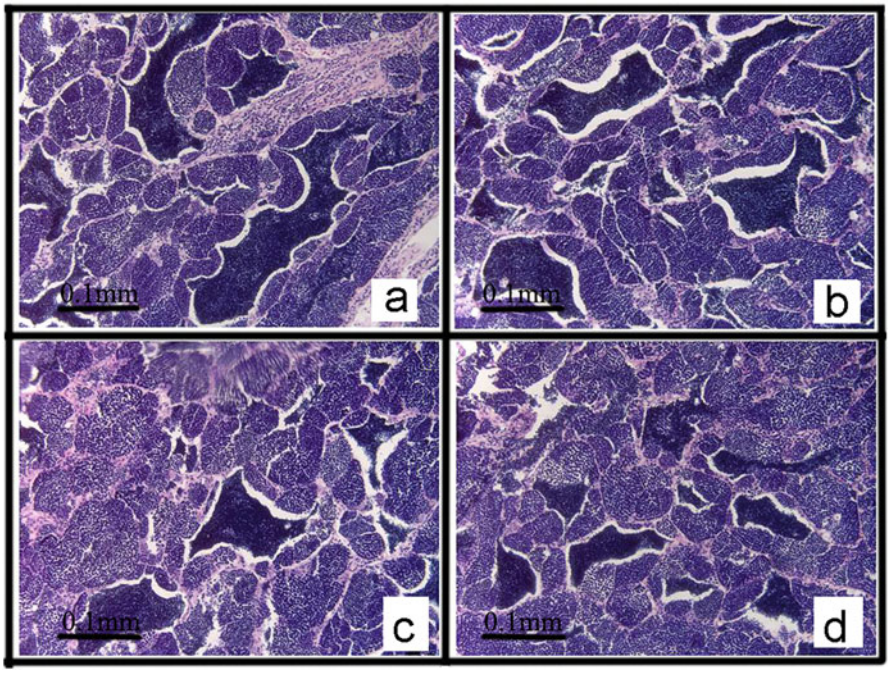

Fig. 10.5 The testis microstructure of WR-F_1–F_4 (Liu et al. 2019a). (**a**) The histology of the testis from WR-F_1. (**b**) The histology of the testis from WR-F_2. (**c**) The histology of the testis from WR-F_3. (**d**) The histology of the testis from WR-F_4. Bar = 0.1 mm

was modified to TAAA (Figs. 10.9 and 10.12). By analyzing the data in the coding region of the hybrid lineage, more mutant bases were found in class I rather than in class II and class III when compared with its parents. The coding region of WR-II was mainly inherited from WR-F_1 but with some mutant bases (Fig. 10.8). In terms of the NTS sequence data, the NTS-83 of the hybrid lineage has many mutant bases compared with its parents, and these mutant bases were stable in different generations (Fig. 10.9a); the NTS-220 of WR-F_1 mainly inherited the genetic features of RCC, but the NTS-220 of WR-F_2, WR-F_3, and WR-F_4 mostly inherited the genetic features from WCC and accompanied some mutant bases (Fig. 10.9b). The NTS-351 of WR-F_1 had some bases from WCC, some bases from RCC, and a mutant base (position 7 in Fig. 10.10), which showed chimeric features; the NTS-351 of WR-F_2, WR-F_3, and WR-F_4 had chimeric characteristics accompanied by some mutant bases. In the WR-II, the NTS-83 and NTS-220 were mainly inherited from the WR-F_1 bases and have mutant bases (NTS-83, position 4 in Fig. 10.9a; NTS-220, position 215 in Fig. 10.9b); however, the NTS-351 of WR-II was mainly inherited from its male-specific (WCC) bases and has three mutant bases (Fig. 10.10) (Liu et al. 2019a).

In general, the 5S rDNA of the hybrid lineage and WR-II inherited the characteristics from their parents, accompanied by the appearance of mutant bases

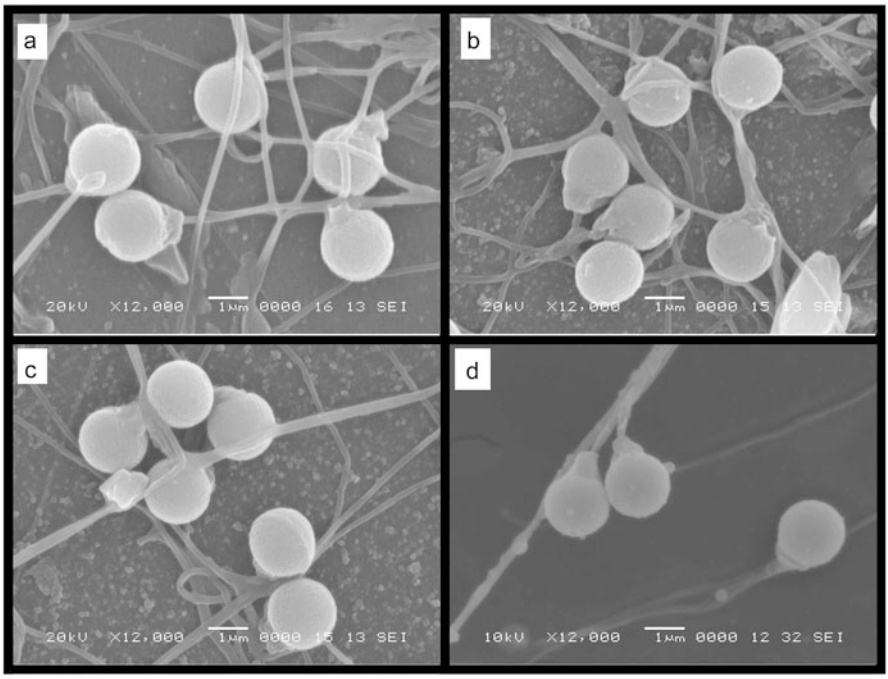

Fig. 10.6 The sperm structures of WR-F_1–F_4 under the scanning electron microscope (Liu et al. 2019a). (**a**) The structure of sperm from WR-F_1. (**b**) The structure of sperm from WR-F_2. (**c**) The structure of sperm from WR-F_3. (**d**) The structure of sperm from WR-F_4

Table 10.5 The fertilization rates and hatching rates of WR-F_1–F_4 (Liu et al. 2019a)

Fish type	Fertilization rate (%)	Hatching rate (%)
WR-F_1	90.2	81.5
WR-F_2	91.3	82.1
WR-F_3	90.8	82.7
WR-F_4	90.7	82.4

Fig. 10.7 The appearance of WR-II. Bar = 5 cm

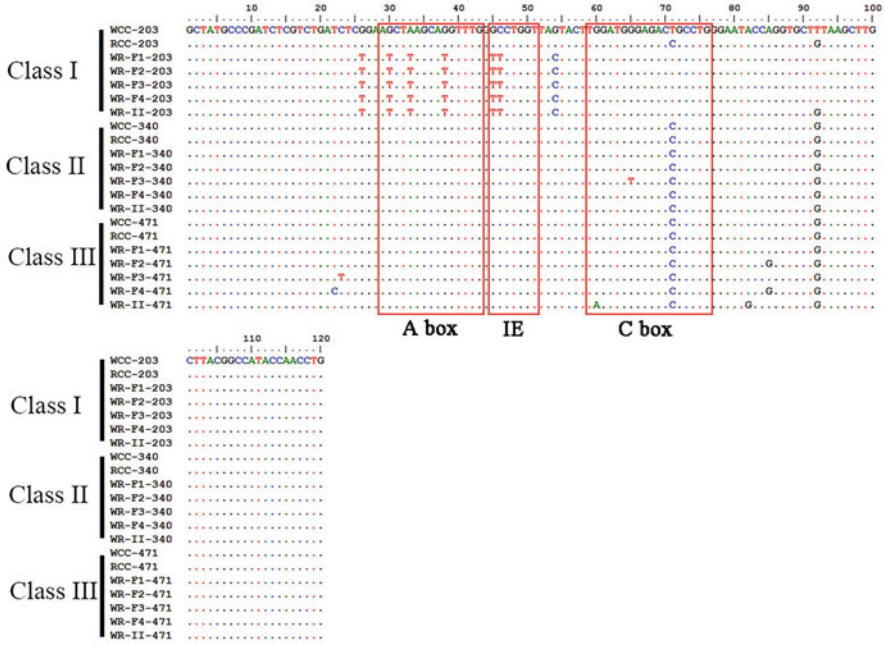

Fig. 10.8 Nucleic acid sequence alignment of 5S rDNA coding region of WR-F_1–F_4 and WR-II. The boxes showed the ICRs (Liu et al. 2019a)

and chimeric characteristics. The results of the studies revealed the heredity and variability of the hybrid lineage and WR-II from the molecular level.

10.3.2 The Liver Transcriptome Analysis of F_1 to F_2 Hybrids and Parents

6712 orthologous genes were identified among the transcriptomes from WR-F_1 and its parents; 4475 orthologous genes were identified among the transcriptomes from WR-F_2 and its parents. We identified chimeric patterns based on the distribution of variations and these orthologous genes. Eight gene patterns were classified in the hybrids (Fig. 10.11, Table 10.6), and these eight gene patterns were classified into three categories, including biparental origin genes, chimera, and mutant. Patterns 1–3 were included in the first category, patterns 4–6 were included in the second category, and patterns 7–8 were included in the third category.

In the first category, patterns 1–2 comprised genes that had a single chimeric fragment and without mutations; pattern 3 included genes that had chimeric fragments and with mutations (Fig. 10.11a–c). 19.04% of genes of WR-F_1 were

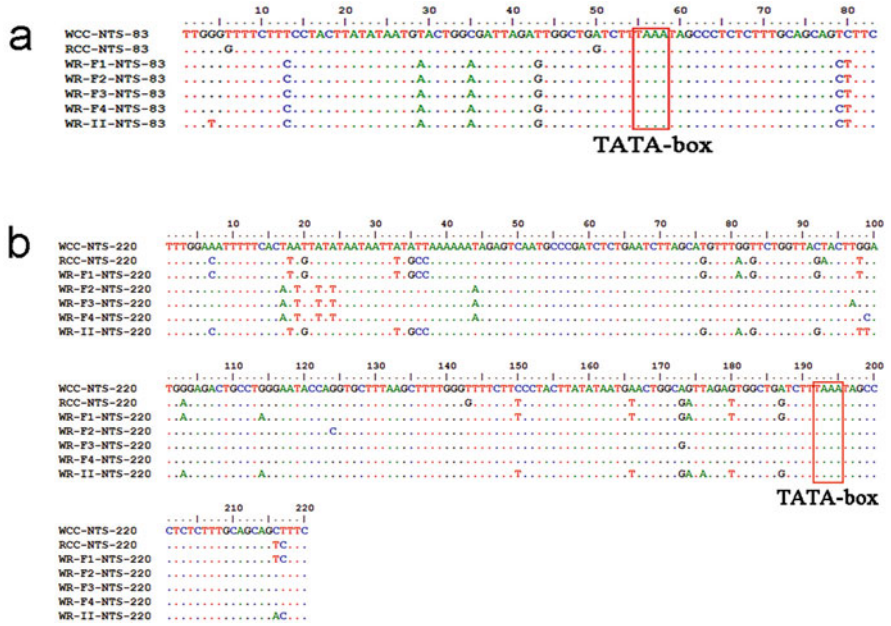

Fig. 10.9 Nucleic acid sequence alignment of the NTS (83 and 220) of hybrid lineage and WR-II (Liu et al. 2019a). (**a**) Nucleic acid sequence alignment of the NTS-83 sequences of hybrid lineage and WR-II. (**b**) Nucleic acid sequence alignment of the NTS-220 sequences of hybrid lineage and WR-II. The boxes included NTS upstream TATA-like sequences

included in the first category, and 4.18% of genes of WR-F_2 were included in the first category.

In the second category, patterns 4–6 included genes that are derived from both parents and with mutations (Fig. 10.11d–f). 6.90% of genes of WR-F_1 and 5.05% of genes of WR-F_2 were included in the second category.

In the third category, pattern 7 included genes from the WCC, while pattern 8 included genes from the RCC (Fig. 10.11g–h). 31.08% of genes of WR-F_1 were included in the third category, and 36.20% of genes of WR-F_2 were included in the third category (Liu et al. 2018).

We used PCR amplification and Sanger sequencing to confirm the chimeric genes at the genomic level. 22 of the 30 tested genes were validated by Sanger sequencing, indicating 73% success rate for the bioinformatics of patterns. And we confirmed 17 genes from 23 characterized chimeric genes (74%) by Sanger sequencing. The formation mechanisms of chimeric genes in the hybrids are poorly understood. Some research reports speculated that the genetic mechanisms might include chromosomal rearrangement, gene insertion or loss, and the distribution of transposable elements (Nei and Nozawa 2011; Rieseberg 2001).

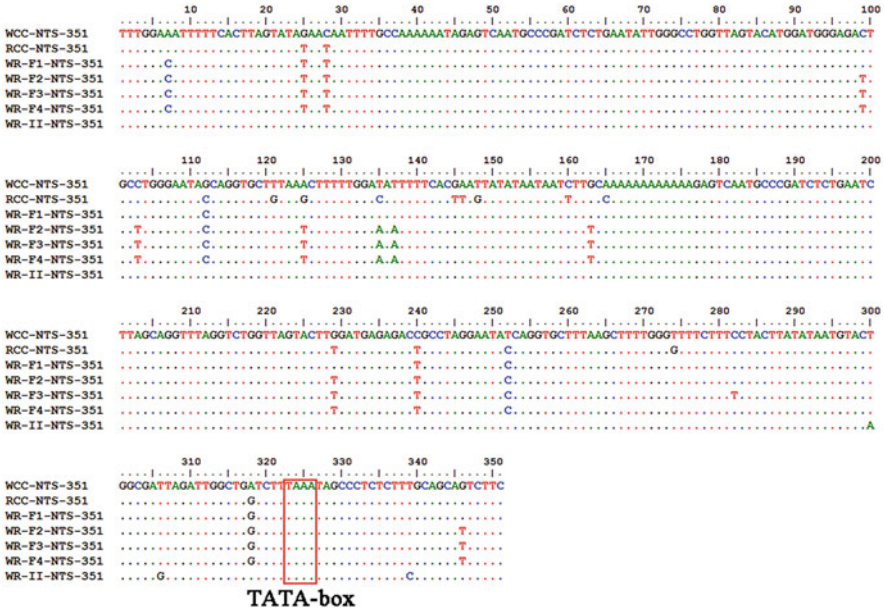

Fig. 10.10 Nucleic acid sequence alignment of the NTS-351 sequences of hybrid lineage and WR-II. The boxes included the NTS upstream TATA-like sequences (Liu et al. 2019a)

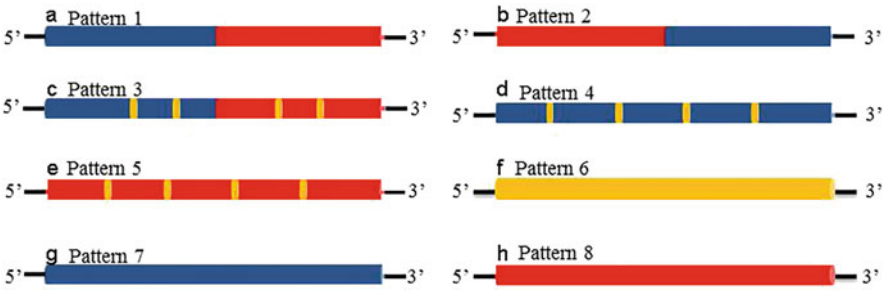

Fig. 10.11 Schematic diagrams of gene patterns of WR-F_1 and WR-F_2 (Liu et al. 2018). Blue bars indicated that offspring inherited the specific fragment of WCC. Red bars indicated that offspring inherited the specific fragment of RCC. Yellow bars indicated that offspring had specific mutations. (**a–c**) Chimeric genes had single or multiple chimeric fragments consisting of continuous, alternating variations from parent-specific variants, either with or without offspring-specific mutations. (**d–f**) Genes derived from progenitors but with mutations unique to offspring. (**g–h**) Genes were derived exclusively from one parent

We analyzed the functions of chimeric genes and mutant genes by means of bioinformatics. In this research, 1731 of chimeric genes and mutant genes from WR-F_1 were enriched by GO and pathway enrichment. Through GO enrichment, some chimeric and mutant genes were related to immune and developmental processes, and metabolism was identified, such as growth (GO:0040007), developmental

Table 10.6 Gene numbers of each pattern in WR-F_1 and WR-F_2 (Liu et al. 2018)

Categories	Chimeric genes	Genes with specific mutations	Genes of biparental origin
Patterns	1–3	4–6	7–8
Gene number (WR-F_1)	1278	463	2086
Percentage (WR-F_1) (%)	19.04	6.90	31.08
Gene number (WR-F_2)	187	226	1620
Percentage (WR-F_2) (%)	4.18	5.05	36.20

process (GO:0032502), metabolic process (GO:0008152), immune system process (GO:0002376), etc. According to these results, we hypothesized that the rapid growth and strong disease resistance of WR-F_1 might be related to some chimeric and mutant genes.

In conclusion, chimeric genes provided new ways for researchers to explore the genetic mechanism of heterosis.

10.4 The Functional Study of Tyrosinase Gene in F_1 Hybrids and WCC

As mentioned above, WR-F_1 had a gray body color (Fig. 10.12c) in adulthood, and it was different from WCC (silver, Fig. 10.12a) and RCC (red, Fig. 10.12b). We used microscope to observe the body color of WR-F_1, WCC, and RCC. The results of electron microscopic showed that the tail of WCC had much melanin (Fig. 10.12d), the tail of RCC had no melanin (Fig. 10.12e), and the tail of WR-F_1 had less melanin (Fig. 10.12f). Briefly, WR-F_1 had more melanin than that in RCC, but less than that in WCC; so the body color of WR-F_1 was a hybrid trait. Compared to RCC, the body color of WR-F_1 was more similar to WCC (Liu et al. 2019b).

Tyr (tyrosinase) is an important enzyme in the pathway of melanin synthesis and the mutation of *tyr* gene can cause oculocutaneous albinism (OCA) (Oetting and King 1999). Previous studies reported that *tyr* knockout mice showed both a total albinism and mosaic color in 9 days of birth (Zhang et al. 2016). Disruption of *tyr* gene in zebrafish embryos led to mosaic pigmentation patterns and some of these embryos were almost unpigmented (Jao et al. 2013). In order to evaluate the function of *tyr* in WR-F_1 and WCC, we had targeted disruption of *tyr* in WR-F_1 and WCC by crispr/cas9. At 4 days post-fertilization (dpf) and 120 dpf, we observed the body color of the mutant WCC, mutant WR, and their control fish. The results showed that the mutant WCC and mutant WR-F_1 had a significant decrease in melanin content compared with their respective WTs, so that some mutant WCC and WR-F_1 had pink body color (Figs. 10.13a–g and 10.14a–g). We detected the level of TYR from the skin of 120 dpf WT-WCC and mutation WCC. The results showed that there was no

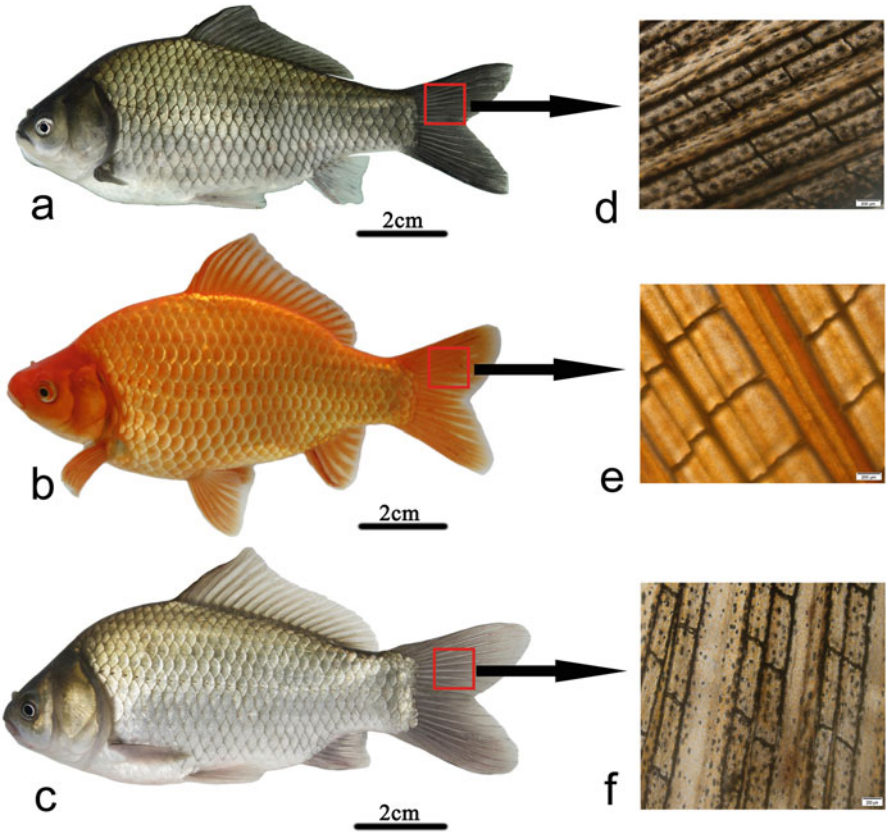

Fig. 10.12 The body color of WCC, RCC, and WR-F_1 and microscopic observation of their tail fin (Liu et al. 2019b). (**a**) WCC had a silver body color. The body color of WCC with silver color. (**b**) RCC had a red body color. (**c**) WR had a gray body color. (**d**) The tail fin of WCC had much melanin. (**e**) The tail fin of RCC had no melanin. (**f**) The tail fin of WR-F_1 had less melanin

TYR protein in mutation WCC compared with that in the WT-WCC, implying that the mutation affects the normal expression of TYR protein (Liu et al. 2019b). Moreover, the microscope observation results showed a significant melanin reduction in the mutant WCC compared with that in WT-WCC (Fig. 10.15a–d).

In general, *tyr* gene plays a vital role in the regulation of melanin in WCC and WR-F_1. Furthermore, these models will play an important role in studying the pigmentation of non-model fish.

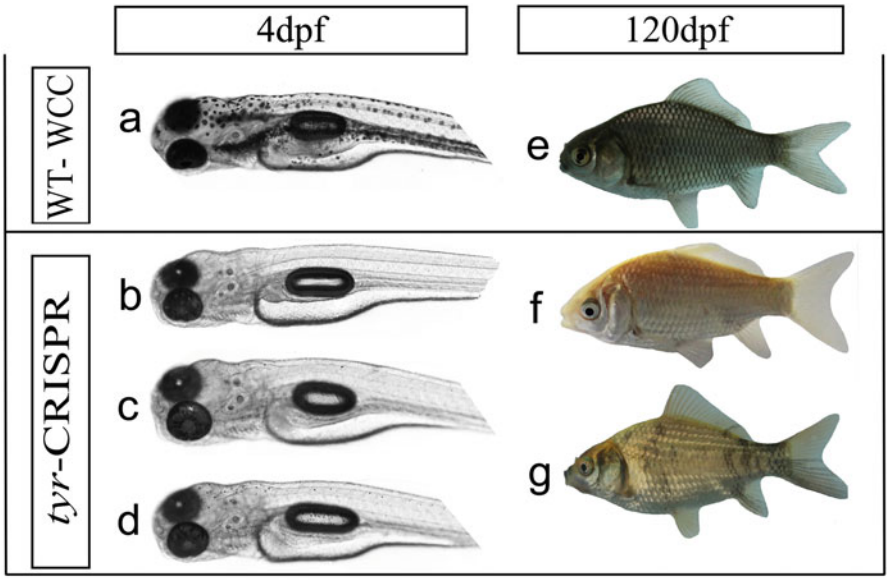

Fig. 10.13 WCC showed decreased melanin due to tyrosinase (*tyr*) disruption (Liu et al. 2019b). (**a**) WT-WCC exhibited obvious melanin. (**b–d**) Mutant WCC exhibited little melanin at 4 dpf. (**e**) WT-WCC had a black body color. (**f**) Mutant WCC had pink color. (**g**) Mutant WCC had mosaic melanin at 120 dpf

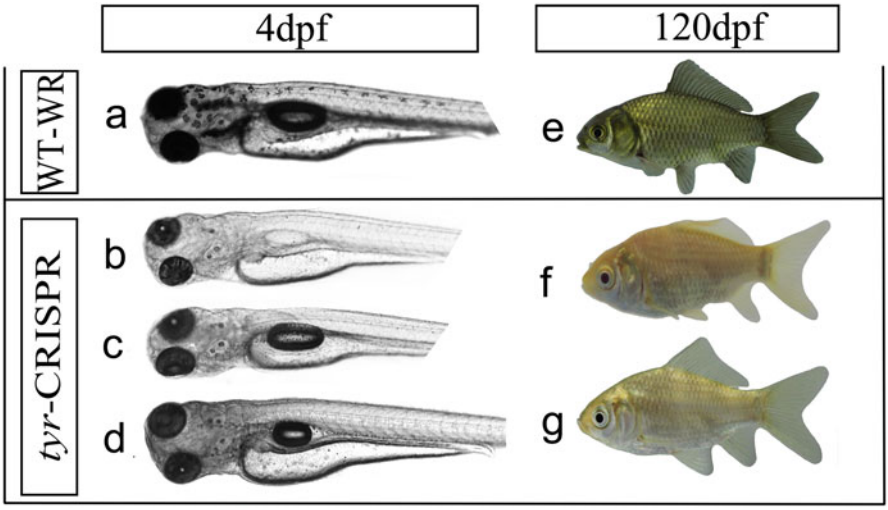

Fig. 10.14 WR-F_1 showed decreased melanin due to tyrosinase (*tyr*) disruption (Liu et al. 2019b). (**a**) WT-WR exhibited obvious melanin. (**b–d**) Mutant WR-F_1 exhibited little melanin at 4 dpf. (**e**) WT-WR-F_1 had a gray body color. (**f**) Mutant WR-F_1 had pink color. (**g**) Mutant WR-F_1 had mosaic melanin at 120 dpf

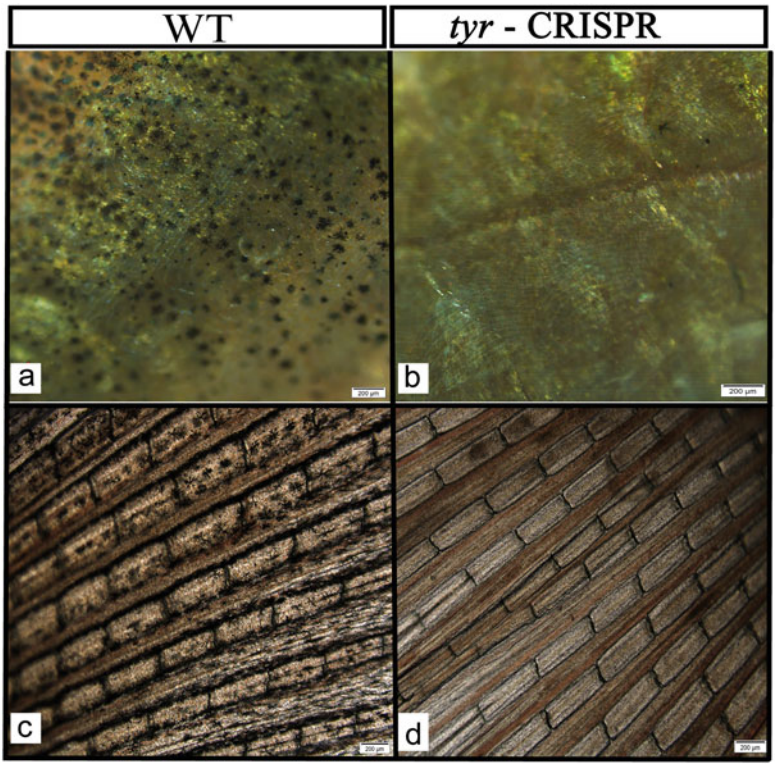

Fig. 10.15 Microscopy observation of the skin and tail fin from WT-WCC and mutant WCC (Liu et al. 2019b). (**a**) The skin of WT-WCC with abundant melanin. (**b**) The skin of mutant WCC with no melanin. (**c**) The tail fin of WT-WCC with abundant melanin. (**d**) The tail fin of mutant WCC with no melanin

References

Jao L-E, Wente SR, Chen W (2013) Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proc Natl Acad Sci 110(34):13904–13909

Liu Q, Qi Y, Liang Q, Xu X, Hu F, Wang J, Xiao J, Wang S, Li W, Tao M, Qin Q, Zhao R, Yao Z, Liu S (2018) The chimeric genes in the hybrid lineage of *Carassius auratus cuvieri* (♀) × *Carassius auratus* red var. (♂). Sci China Life Sci 61(9):1079–1089

Liu Q, Liu J, Liang Q, Qi Y, Tao M, Zhang C, Qin Q, Zhao R, Chen B, Liu S (2019a) A hybrid lineage derived from hybridization of *Carassius cuvieri* and *Carassius auratus* red var. and a new type of improved fish obtained by back-crossing. Aquaculture 505:173–182

Liu Q, Qi Y, Liang Q, Song J, Liu J, Li W, Shu Y, Tao M, Zhang C, Qin Q, Wang J, Liu S (2019b) Targeted disruption of tyrosinase causes melanin reduction in *Carassius auratus cuvieri* and its hybrid progeny. Sci China Life Sci 62(9):1194–1202

Nei M, Nozawa M (2011) Roles of mutation and selection in speciation: from Hugo de Vries to the modern genomic era. Genome Biol Evol 3:812–829

Oetting WS, King RA (1999) Molecular basis of albinism: mutations and polymorphisms of pigmentation genes associated with albinism. Hum Mutat 13(2):99–115

Rieseberg LH (2001) Chromosomal rearrangements and speciation. Trends Ecol Evol 16(7): 351–358

Wang J, Xiao J, Zeng M, Xu K, Tao M, Zhang C, Duan W, Liu W, Luo K, Liu Y, Liu S (2015) Genomic variation in the hybrids of white crucian carp and red crucian carp: evidence from ribosomal DNA. Sci China Life Sci 58(6):590–601

Zhang X, Liang P, Ding C, Zhang Z, Zhou J, Xie X, Huang R, Sun Y, Sun H, Zhang J, Xu Y, Songyang Z, Huang J (2016) Efficient production of gene-modified mice using *Staphylococcus aureus* Cas9. Sci Rep 6(1):32565

11 The Formation and Biological Characteristics of the Different Ploidy Fishes Derived from the Hybridization of other Cyprinid Fishes

Shaojun Liu, Qianhong Gu, Shi Wang, Yi Zhou, Qingfeng Liu, Yude Wang, Shengnan Li, and Dingbin Gong

Abstract

In the previous chapters, we introduced the formation and biological characteristics of some fertile tetraploid and diploid lineages and improved varieties derived from the distant hybridization. In this chapter, the formation and biological characteristics of other distant hybridization combinations are introduced. The parents of these combinations include Bleeker's yellow tail (*Xenocypris davidi*, YT), blunt snout bream (*Megalobrama amblycephala*, BSB), red crucian carp (*Carassius auratus* red var., RCC), improved color crucian carp (*Carassius auratus*, ICCC), topmouth culter (*Culter alburnus*, TC), grass carp (*Ctenopharyngodon idella*, GC), and koi carp (*Cyprinus carpio haematopterus*, KOC). These hybrid combinations include reciprocal cross between BSB × YT, RCC (♀) × YT (♂), RCC (♀) × TC (♂), GC (♀) × BSB (♂), KOC (♀) × ICCC (♂), and GC (♀) ×TC (♂). These hybrid crosses also produce different ploidy fishes which would enrich our knowledge regarding the distant hybridization and support more evidence for the formation of the genetic rules and reproductive rules in fish distant hybridization.

Keywords

Diploid · Triploid · Polyploid · Morphological traits · Genetic characteristics · Transcriptome

S. Liu (✉) · Q. Gu · S. Wang · Y. Zhou · Q. Liu · Y. Wang · S. Li · D. Gong
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_11

11.1 The Formation and Biological Characteristics of Blunt Snout Bream × Bleeker's Yellow Tail

11.1.1 The Characteristics of Genetic Construction of Different Ploidy Hybrids

Using blunt snout bream (*Megalobrama amblycephala*, BSB) and Bleeker's yellow tail (*Xenocypris davidi*, YT) as parents, diploid BSB (♀) × YT (♂) F_1 (2*n*BY), triploid BSB (♀) × YT (♂) F_1 (3*n*BY), diploid YT (♀) × BSB (♂) F_1 (2*n*YB), and triploid YT (♀) × BSB (♂) F_1 (3*n*YB) were obtained in the F_1 hybrids (Hu et al. 2012). The flow cytometer was employed to detect the mean DNA content of red blood cells from F_1 hybrids, and the DNA content of red blood cells from parents (BSB and YT) was used as the control. The data of DNA content between hybrids and their parents was shown in Table 11.1. There were two types of individuals with obviously different mean DNA content in the F_1 hybrids. The mean DNA content in the first type of the hybrids was equal to the average value of the DNA content from BSB and YT ($P > 0.05$), implying that these hybrids were 2*n*BY and 2*n*YB. The mean DNA content in the second type of the hybrid was equal to the sum of the DNA content of BSB and half of YT ($P > 0.01$) or was equal to the sum of the DNA content of YT and half of BSB ($P > 0.01$), suggesting that they were 3*n*BY and 3*n*YB (Hu 2013).

Metaphase chromosome preparation from cultured peripheral blood cells showed that both 2*n*BY (Fig. 11.1c) and 2*n*YB possessed 48 chromosomes ($2n = 48$), which were the same as those of BSB (Fig. 11.1a) and YT (Fig. 11.1b), while 3*n*BY (Fig. 11.1d) and 3*n*YB had 72 chromosomes ($3n = 72$).

Taking F_1 hybrids derived from BSB (♀) × YT (♂) as example, metaphase chromosome spread in 2*n*BY (Fig. 11.1c) had two large submetacentric chromosomes, and the karyotype of 2*n*BY was 18m+26sm+4st, which was similar to that of BSB and YT, while 3*n*BY possessed three large submetacentric chromosomes with the karyotype 27m+39sm+6st (Hu 2013).

Table 11.1 Mean DNA content of BSB, YT, 2*n*BY, 3*n*BY, 2*n*YB, and 3*n*YB (Hu et al. 2012)

Fish type	Mean DNA content	Ratio	
		Observed ratio	Expected ratio
BSB	70.39		
YT	63.48		
2*n*BY	65.88	2*n*BY/(0.5BSB+0.5YT) = 0.98[a]	1
3*n*BY	93.13	3*n*BY/(BSB+0.5YT) = 0.91[a]	1
2*n*YB	65.28	2*n*YB/(0.5BSB+0.5YT) = 0.98[a]	1
3*n*YB	96.92	3*n*YB/(YT+0.5BSB) = 0.98[a]	1

[a]Indicated that the observed ratio was not significantly different ($P > 0.05$) from the expected ratio

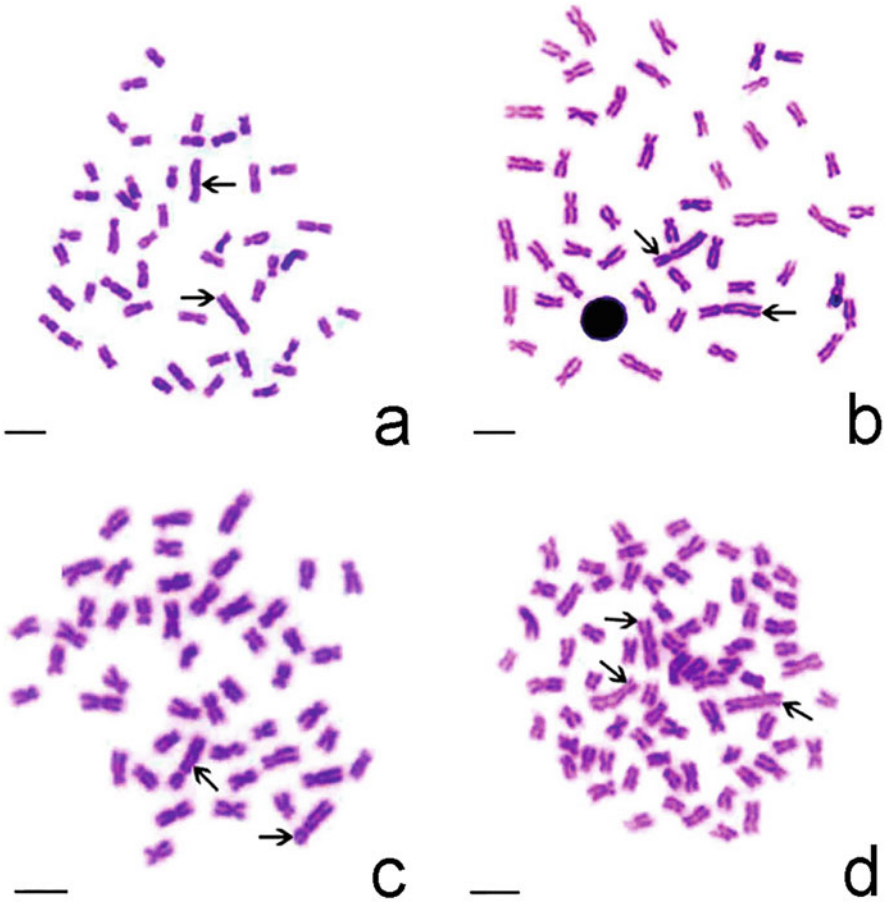

Fig. 11.1 Metaphase chromosome spread of BSB, YT, 2*n*BY, and 3*n*BY. (**a**) Metaphase chromosome spread in BSB ($2n = 48$). The arrow indicated a pair of large submetacentric chromosomes. (**b**) Metaphase chromosome spread in YT ($2n = 48$). The arrow indicated a pair of large submetacentric chromosomes. (**c**) Metaphase chromosome spread in 2*n*BY ($2n = 48$). The arrow indicated a pair of large submetacentric chromosomes. (**d**) Metaphase chromosome spread in 3*n*BY ($3n = 72$). The arrow indicated three large submetacentric chromosomes. Bar = 5.00 μm

11.1.2 The Appearance of Different Ploidy Fishes in F_1

Morphological traits of 2*n*BY, 3*n*BY, 2*n*YB, 3*n*YB, and their parents were presented in Figs. 11.2 and 11.3.

Seven countable traits (lateral line scales, scales above lateral line, scales below lateral line, dorsal fin, pectoral fin, pelvic fin, and anal fin) of BSB, YT, 2*n*BY, 3*n*BY, 2*n*YB, and 3*n*YB were counted. In addition, seven measurable traits (overall length, the length of body, the length of head, the length of caudal peduncle, head height, body depth, and caudal peduncle depth) were measured. Subsequently, the

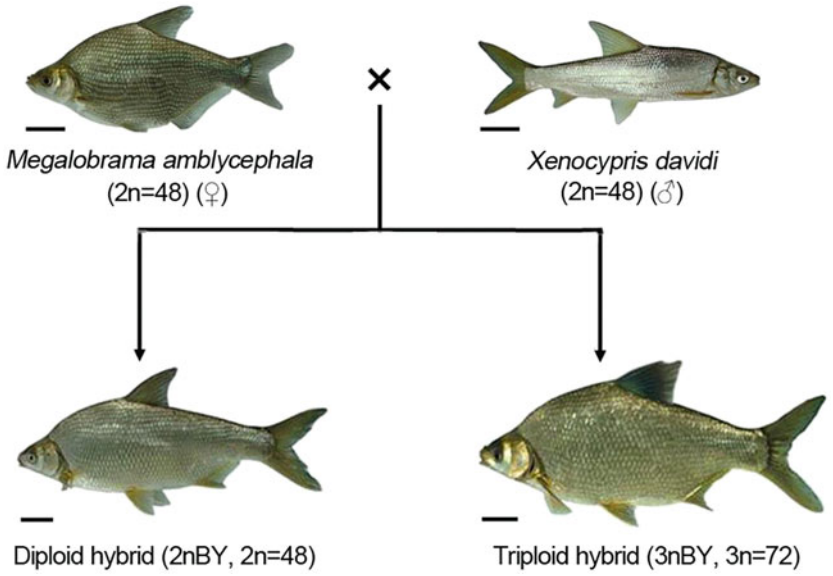

Fig. 11.2 The appearance of BSB, YT, 2*n*BY, and 3*n*BY. Bar = 1.00 cm

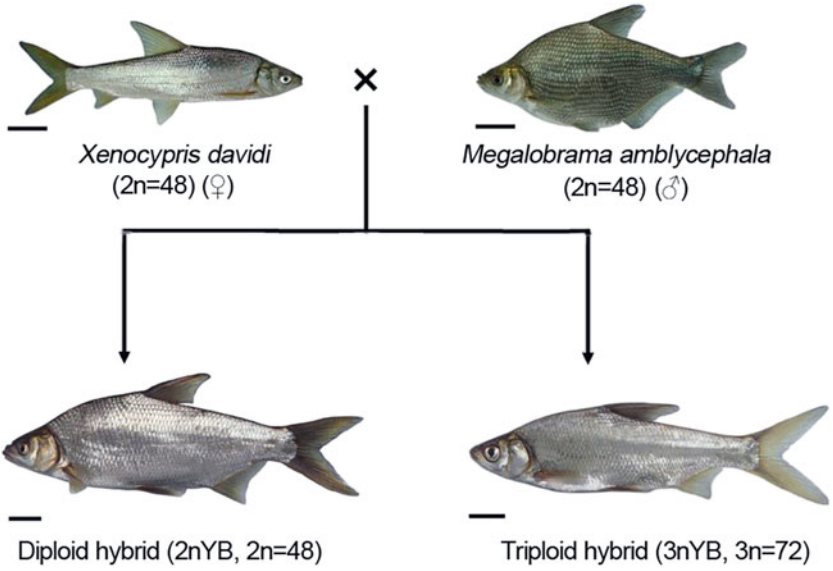

Fig. 11.3 The appearance of YT, BSB, 2*n*YB, and 3*n*YB. Bar = 1.00 cm

ratios of the overall length to body length (OL/BL), body length to head length (BL/HL), body length to body depth (BL/BD), head length to head height (HL/HH), caudal peduncle length to caudal peduncle depth (CPL/CPD), and body depth to

Table 11.2 Countable traits of BSB, YT, 2*n*BY, 2*n*YB, 3*n*BY, and 3*n*YB (Hu 2013)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins*	Number of pectoral fins	Number of pelvic fins	Number of anal fins*
BSB	52–57	11–14	8–11	III+7–8	15–18	9–10	III +23–31
2*n*BY	55–66	11–13	8–11	III+7–9	15–18	8–10	III +15–18
3*n*BY	55–63	12–14	9–10	III+7–8	15–18	9–10	III +18–20
2*n*YB	56–66	11–13	7–11	III+7–8	15–19	8–10	III +15–18
3*n*YB	61–64	11–12	8–9	III+7	17–18	9	III +14–17
YT	60–67	10–12	6–8	III+7–9	15–18	8–9	III +11–13

The upper Roman numerals in the column of * represent spines and Arabic numerals represent soft fins

head height (BD/HH) were calculated. Significant difference analysis and variance analysis were performed using SPSS software.

The seven countable traits of 2*n*BY, 3*n*BY, 2*n*YB, 3*n*YB, and their parents were presented in Table 11.2. The six countable traits (lateral line scales, scales above lateral line, scales below lateral line, dorsal fin, pectoral fin, and pelvic fin) in 2*n*BY and 3*n*BY were intermediate between BSB and YT. For instance, the number of lateral line scales in 2*n*BY, 3*n*BY, 2*n*YB, and 3*n*YB were 55–66, 55–63, 56–66 and 61–64 respectively, which were intermediate between 52–57 in BSB and 60–67 in YT with partial overlap with BSB and YT. Nevertheless, the number of anal fin in the four hybrids including III+15–18 in 2*n*BY, III+18–20 in 3*n*BY, III+15–18 in 2*n*YB, and III+14–17 in 3*n*YB were smaller than III+23–31 in BSB but larger than III+11–13 in YT. Consequently, the number of anal fin in the four hybrids had no overlap with that in BSB or YT, which could be used as one of the distinctive appearance characteristics to distinguish the hybrid offspring from their parents.

The comparison on the measurable traits among 2*n*BY, 3*n*BY, 2*n*YB, 3*n*YB, and their parents were presented in Table 11.3. The ratios of OL/BL in 2*n*BY and 3*n*BY were insignificantly different from that in BSB and YT ($P > 0.05$), but were significantly smaller than that in 2*n*YB and 3*n*YB ($P < 0.05$). The ratios of BL/HL in 2*n*BY, 3*n*BY, 2*n*YB, and 3*n*YB were 4.83, 4.76, 4.70, and 4.59, respectively, which were insignificantly different from those in BSB ($P > 0.05$), but were obviously smaller than those in YT ($P < 0.05$). There existed no significant differences in the BL/BD between 2*n*BY and 2*n*YB ($P > 0.05$), but they were significant different with 3*n*BY, 3*n*YB, and their parents in the BL/BD ($P < 0.05$). There were no significant differences in the HL/HH among these six kinds of fish ($P > 0.05$). The BD/HH in 2*n*BY was not significantly different with that in 2*n*YB ($P > 0.05$), but was significantly smaller than that in BSB (2.45) and 3*n*BY (2.33),

Table 11.3 Measurable characteristics of BSB, YT, 2*n*BY, 2*n*YB, 3*n*BY, and 3*n*YB (Hu 2013)

Fish type	Overall length/ body length	Body length/ head length	Body length/ body depth	Head length/ head height	Body depth/ head height	Caudal peduncle length/caudal peduncle depth
BSB	1.22 ± 0.02	4.65 ± 0.28	2.23 ± 0.16	1.17 ± 0.06	2.45 ± 0.16	0.99 ± 0.09
2*n*BY	1.23 ± 0.02	4.90 ± 0.31	2.86 ± 0.19	1.24 ± 0.07	2.13 ± 0.13	1.37 ± 0.10
3*n*BY	1.23 ± 0.03	4.76 ± 0.34	2.51 ± 0.16	1.23 ± 0.07	2.33 ± 0.13	1.13 ± 0.13
2*n*YB	1.27 ± 0.05	4.70 ± 0.36	2.80 ± 0.18	1.21 ± 0.14	2.02 ± 0.18	1.32 ± 0.14
3*n*YB	1.25 ± 0.02	4.59 ± 0.23	3.24 ± 0.15	1.24 ± 0.10	1.77 ± 0.21	1.53 ± 0.13
YT	1.22 ± 0.02	5.12 ± 0.13	3.70 ± 0.15	1.27 ± 0.04	1.75 ± 0.08	1.54 ± 0.13

and significantly larger than that in YT (1.75) and 3*n*YB (1.77). No significant difference was found comparing the CPL/CPD in 2*n*BY and 2*n*YB ($P > 0.05$), but they were both obviously larger than that in BSB and 3*n*BY ($P < 0.05$) and notably smaller than that in YT and 3*n*YB ($P < 0.05$). These results indicated that the body depths of F_1 hybrids increased compared to that of YT. Compared with other hybrids, except for the number of anal fin, the BL/BD, BD/HH, and other characteristics in 3*n*BY were more similar to those in BSB, suggesting that 3*n*BY had similar morphological characteristics with BSB.

11.1.3 The Reproductive Traits of Different Ploidy Fishes in F_1

The gonadal structures of 2*n*BY, 3*n*BY, 2*n*YB, 3*n*YB, and their parents (20–26 months old) were assayed via preparing paraffin sections. The results suggested that 2*n*BY had three types of gonads: ovary type (accounting for 36.4%), testis type (accounting for 52.7%), and intersexual gonad (accounting for 10.9%), while there were only two types of gonads in 3*n*BY—ovary type (35.7%) and testis type (64.3%). Similar to the 2*n*BY, three types of gonads were observed in the 2*n*YB including ovary type, testis type, and intersexual gonad and accounted for 44.8%, 37.9%, and 17.3%, respectively. In 3*n*YB, two types of gonads including ovaries (33.3%) and testes (66.7%) were observed. According to the proliferation and differentiation of germ cells in 2*n*BY, there were three types (Type I, Type II, and Type III) of ovaries, while four types (Type I, Type II, Type III, and Type IV) of testes and two different types in intersexual gonads (Type I and Type II). In 3*n*BY, two different types of ovaries (Type I and Type II) and three different types of testes (Type I, Type II, and Type III) were observed. The 2*n*YB and 3*n*YB had similar gonadal types with 2*n*BY and 3*n*BY. The only difference was that in 3*n*YB, no Type

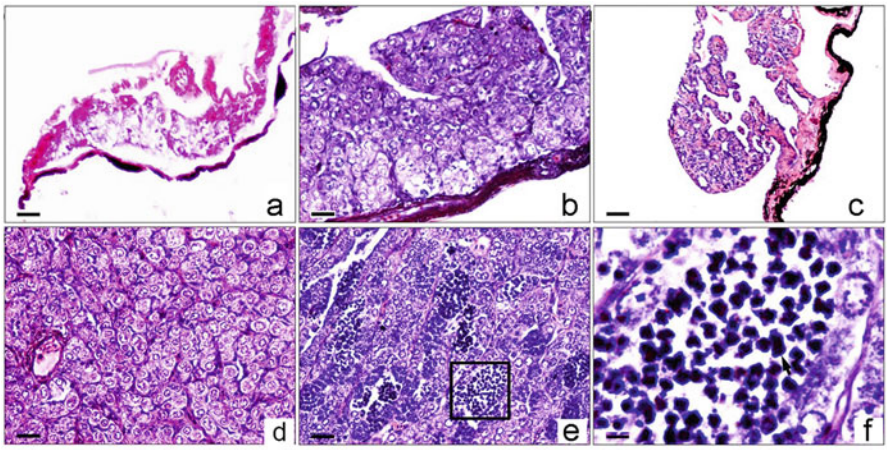

Fig. 11.4 Gonadal microstructure of 3*n*BY (Hu et al. 2012). (**a**) Type I 3*n*BY ovary, bar = 25 μm. (**b**) Type II 3*n*BY ovary, bar = 25 μm. (**c**) Type I 3*n*BY testis, bar = 50 μm. (**d**) Type II 3*n*BY testis, bar=25 μm. (**e**) Type III 3*n*BY testis, bar = 25 μm. (**f**) The part in the black frame was magnified from (**e**). The arrow showed degenerative spermatid with condensed nuclear substance. Bar = 5 μm

II ovary could be observed, while Type III and Type IV were found. The following content will introduce the different types of gonads of 3*n*BY and 2*n*BY sequentially.

The gonadal microstructure of 3*n*BY was presented in Fig. 11.4. There were two types of ovaries: in Type I, no proliferation and differentiation of oogonia could be observed in these types of ovaries (Fig. 11.4a), and in Type II, oogonia proliferated via mitosis, but could not differentiate into other types of oocytes (Fig. 11.4b). Three types of testes were viewed in 3*n*BY: in Type I, there existed testis-like structures in the gonads, but the germ cells were undifferentiated showing degeneration and abortion (Fig. 11.4c); in Type II, abundant spermatogonia were filled in the testes without developing and differentiating cells, and no typical seminiferous tubules could be found (Fig. 11.4d); and in Type III, there were very few light-stained spermatogonia but many other types of germ cells including deep-dyed homotype primary spermatocytes, secondary spermatocytes, and spermatids, which were arranged in piles in seminiferous tubules from 3*n*BY. However, the nuclear materials were condensed and solidified and no mature sperm could be formed in spermatids (Fig. 11.4e, f) (Hu 2013).

The microstructures of gonad in BSB, YT, and 2*n*BY were presented in Fig. 11.5. The normally developed ovaries of 20-month-old BSB mainly consisted of oocytes in phase III (Fig. 11.5a). However, three types of ovaries were observed in 2*n*BY. In Type I, the oogonia in 2*n*BY were not proliferated and aborted, which are similar to Type I ovary in 3*n*BY (Fig. 11.4a). In Type II, the germ cells in ovaries remained at the stage of oogonia, almost without oocytes (Fig. 11.5b). In Type III, ovaries were partially developed with abundant proliferated oogonia and several oogonia developed into oocytes in phase II and phase III, while most of germ cells remained in the early stage of oogonia (Fig. 11.5c, d).

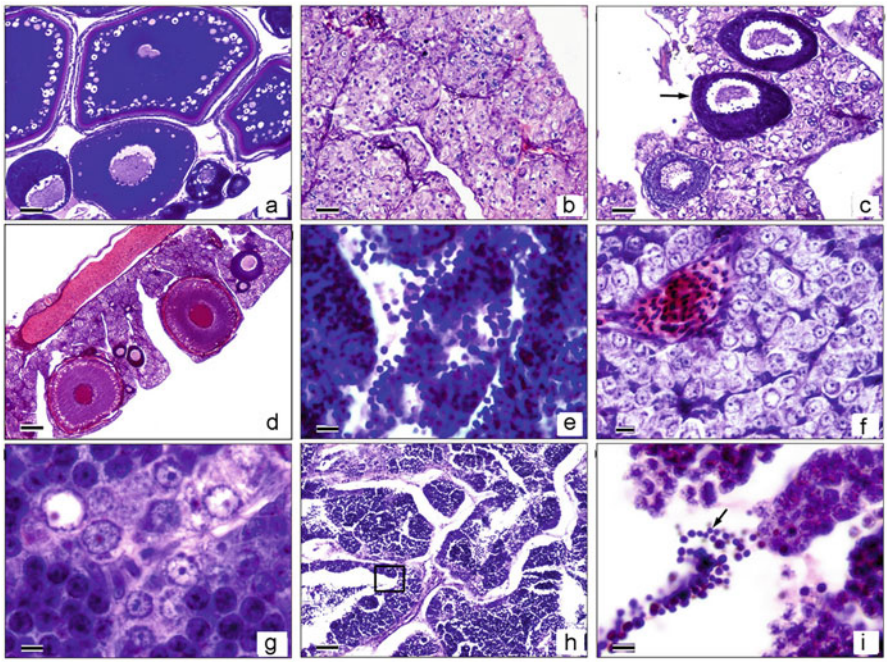

Fig. 11.5 Gonadal microstructures of BSB, YT, and 2*n*BY (partially quoted) (Hu 2013). (**a**) The ovary of 20-month-old BSB, bar = 50 μm. (**b**) Type II 2*n*BY ovary, bar = 25 μm. (**c**) Type III 2*n*BY ovary, bar = 25 μm. (**d**) Type III 2*n*BY ovary, bar = 100 μm. (**e**) The matured testis of 2-year-old YT, bar = 5 μm. (**f**) Type II 2*n*BY testis, bar = 25 μm. (**g**) Type III 2*n*BY testis, bar = 5 μm. (**h**) Type IV 2*n*BY testis, bar = 50 μm. (**i**) The part in the black frame was magnified in (**h**). The arrow showed spermatid. Bar = 5 μm

In testes from 2-year-old YT, they were filled with mature sperm (Fig. 11.5e). Compared with the mature testes in YT, 20–26-month-old 2*n*BY showed four types of testes. In Type I, the aborted testes were similar to the Type I in 3*n*BY (Fig. 11.4c). In Type II, the undifferentiated testes were filled with spermatocytes (Fig. 11.5f). In Type III, the germ cells of testes were proliferated and differentiated with various kinds of sperm cells (Fig. 11.5g), but no mature sperm could be observed. In Type IV, partially testes were developed, and the seminiferous tubules were filled with spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids. However, no semen could be squeezed out of these testes with only few sperm generated from spermatids (Fig. 11.5h, i).

The intersexual gonadal microstructure of 2*n*BY was presented in Fig. 11.6. They consisted of ovary-like and testes-like tissues and could be divided into two types. In Type I, the gonads appeared to be the testis; however, there were several oocytes in phase II (Fig. 11.6a, b) among the spermatogonia and spermatocytes. In Type II, the gonads seemed to be ovaries and were mainly composed of oogonia and the phase II oocytes, but the spermatogonia and spermatocytes also could be found in gonads

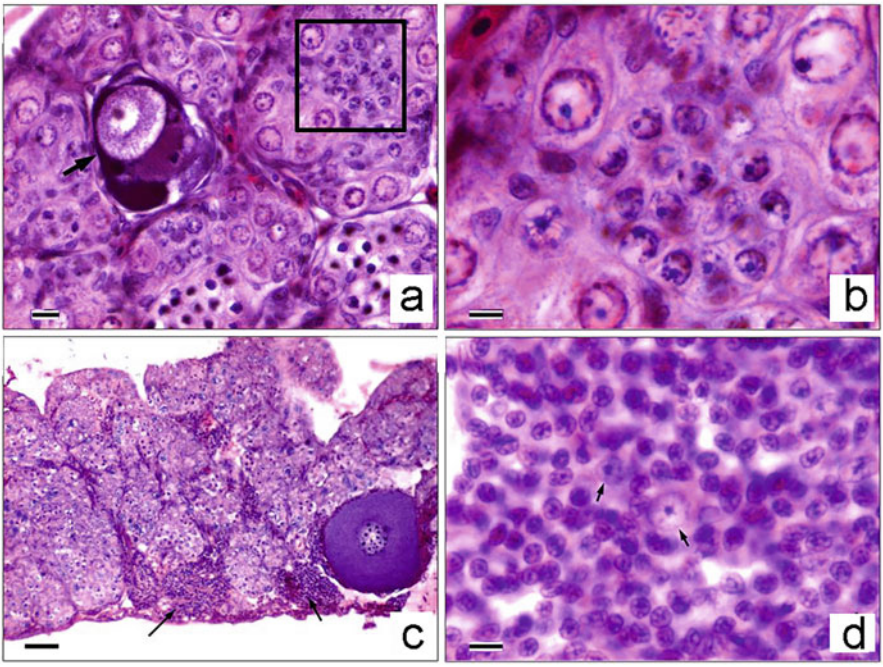

Fig. 11.6 Microstructure of intersexual gonad in 2*n*BY (Hu 2013). (**a**) Intersexual gonad appeared to be testes in 2*n*BY, bar = 100 μm. (**b**) The part in the black square was magnified in (**a**), bar = 5 μm. (**c**) Intersexual gonad appeared to be ovaries in 2*n*BY. The arrows indicated testis-like tissues, bar = 50 μm. (**d**) The magnified part in the area of the right arrow from (**c**). The arrows showed a sperm at oogonium and a spermatocyte, bar = 5 μm

(Fig. 11.6c, d) (Hu 2013). Consequently, the 2*n*BY was absolutely fertile and the F_2 population was produced successfully in our laboratory.

Production of F_2 hybrids is one of the bottlenecks in the process of establishing fish distant hybrid lineage. Generation of F_2 hybrids is of great significance for the establishment of lineage and even formation of new species. Researchers have been focusing on the fertility of male and female of F_1 hybrids derived from BSB (♀) × YT (♂). Fortunately, a very few of male and female fertile individuals were obtained from 2*n*BY with years of continuous practice, careful observations, and systematic studies. Through self-cross using the fertile individuals of 2*n*BY, the F_2 population was produced successfully, which laid a firm foundation for the establishment and continuity of hybrid lineage derived from BSB (♀) × YT (♂).

11.1.4 The Molecular Biological Characteristics of Different Ploidy Fish in F_1

11.1.4.1 The *Sox*-HMG Sequence Analysis

The conserved region of HMG box in *Sox* gene could be applied as a useful molecular genetic marker for distinguishing closely related species and hybrids and making comparison on the genetic composition among hybrids and their parents (Chen et al. 2009). The known primers *Sox* P1F and *Sox* P1R were used to amplify the *Sox*-HMG DNA fragments from BSB, YT, and their hybrids F_1. The results of the amplified products showed that the *Sox*-HMG DNA fragments from 2*n*BY, 3*n*BY, 2*n*YB, and 3*n*YB contained two fragments approximately 200 bp and 700 bp in length.

Sequencing results indicated that the fragments from the location of approximately 200 bp were actually 215 bp in the six assayed fishes (BSB, YT, 2*n*BY, 3*n*BY, 2*n*YB, and 3*n*YB). These amplified fragments contained two genes including *Sox1* and *Sox11*. The fragments with approximately 700 bp in length in BSB and YT were 714 bp (BSB714) and 710 bp (YT710), respectively. In 2*n*BY, the fragments with approximately 700 bp in length contained two sequences including 714 bp (2*n*BY714) and 709 bp (2*n*BY709). In 3*n*BY, the fragments with approximately 700 bp in length contained two sequences including 714 bp (3*n*BY714) and 710 bp (3*n*BY710). In 2*n*YB and 3*n*YB, the fragments with approximately 700 bp in length contained three sequences including 714 bp (2*n*YB714 and 3*n*YB714), 709 bp (2*n*YB709 and 3*n*YB709), and 708 bp (2*n*YB708 and 3*n*YB708). All of the fragments with approximately 700 bp in length were the *Sox9* gene.

Homology of the two different 215bp DNA fragments and the approximately 700 bp fragments from BSB, YT, 2*n*BY, 3*n*BY, 2*n*YB, and 3*n*YB were compared to investigate their relationships. The *Sox1* genes from BSB, 2*n*BY, and 3*n*BY shared 100% similarity which was higher than the similarity between YT and the hybrids (2*n*BY and 3*n*BY) (99.5% for both). The *Sox11* from BSB shared 98.6% similarity with 2*n*BY and 99.5% similarity with 3*n*BY, respectively, which were higher than the similarities of *Sox11* from YT compared to the hybrids (98.1% for 2*n*BY and 99.0% for 3*n*BY). The similarity of the 714 bp fragments between BSB and 2*n*BY was 99.5%. The similarity of the fragments between BSB714 and 2*n*BY709 was 92.2%. The similarity of the fragments between BSB714 and 3*n*BY710 was 91.9%. The similarity of the fragments between YT710 and 2*n*BY709 was 98.4%. The similarity of the fragments between YT (710) and 3*n*BY710 was 99.2%.

The neighbor-joining (NJ) phylogenetic tree was constructed using the fragments from the location of approximately 700 bp (*Sox9*). The NJ phylogenetic tree could be divided into two main clusters. One cluster included the 714 bp fragments of BSB and the four hybrids. The other one included 710 bp DNA fragments of YT, 710 bp DNA fragments of 3*n*BY, 709 bp and 708 bp DNA fragment from the four hybrids. These results indicated that the 714 bp DNA fragments of BSB (♀) × YT (♂) and YT (♀) × BSB (♂) hybrids were from BSB, while the 709 bp fragments of 2*n*BY, the 710 bp fragments of 3*n*BY, and the 708 bp fragments and 709 bp fragments of 2*n*YB and 3*n*YB were from YT (Hu 2013).

11.1.4.2 The *5S rDNA* Repeat Sequence Analysis

According to the report about *5S rDNA* in cyprinid fishes, a pair of specific primer for *5S rDNA* was designed as *5S* P1F 5′-GCTATGCCCGATCTCGTCTGA-3′ and *5S* P2R 5′-CAGGTTGGTATGGCCGTAAGC-3′. The coding sequence and nontranscribed spacer (NTS) of *5S rDNA* were amplified from the nuclear genome DNA in peripheral blood cell of BSB, YT, BSB (♀) × YT (♂), and YT (♀) × BSB (♂) hybrid F_1. It was similar to that of blunt snout bream (Qin et al. 2010). Two fragments located in 200 bp and 400 bp were obtained by amplifying the nuclear genomes of the hybrids and their parents.

The analysis of *5S rDNA* fragments in BSB (♀) × YT (♂) and YT (♀) × BSB (♂) hybrid F_1 showed that the 2*n*BY and 2*n*YB inherited the four fragments with different sizes including 188 bp, 206 bp, 376 bp, and 394 bp from male and maternal parents (Table 11.4). However, three new DNA fragments including 221 bp, 216 bp, and 405 bp were found in 3*n*BY besides the 188 bp and 376 bp DNA fragments. In 3*n*YB, the fragment with 394 bp disappeared, and only three fragments including 188 bp, 210 bp, and 377 bp were observed (Table 11.4). In addition, the alignment showed that 376 bp *5S rDNA* fragments of BSB and YT were formed by doubling of their own 188 bp DNA fragment, respectively. However, a new type of 376 bp DNA fragment was found in 2*n*YB and triploid 3*n*YB, both of which were combined with one 188 bp DNA fragment of BSB and one 188 bp DNA fragment of YT.

Sequencing results of the repetitive sequence in *5S rDNA* showed that two kinds of base insertion occurred in the NTS of repetitive unit in part of 188 bp DNA fragment from BSB and YT. An 18 bp "TTCAAAAAAAAAAAAAAA" fragment was inserted in BSB, while an 18 bp "GAAAGAAAGAAAGAAAAA" fragment was inserted in YT. There was only "GAAAGAAAGAAAGAAAAA" fragment inserted in the four hybrids either in the fragments about 200 bp or 400 bp in most cases. However, in 3*n*BY, the fragments about 200 bp in length include 211 bp and 216 bp DNA fragment. The 211 bp DNA fragment was formed by inserting "TTCAAAAAAAAAAAAAAA" in the 188 bp fragment, while the 216 bp DNA fragment was formed by inserting "GAAAGAAAGAAAGAAAAA" in the 188 bp fragment. In addition, the recombination of repetitive unit from BSB and YT was found in 2*n*BY and 3*n*BY (Hu 2013).

11.1.4.3 The Mitochondrial Genome Sequence Analysis

The complete mitochondrial DNA (mtDNA) sequences of the four hybrids were obtained by multi-fragment cloning and assembling. The length of mtDNA sequence

Table 11.4 The *5S rDNA* sequencing results of BSB, YT, 2*n*BY, 2*n*YB, 3*n*BY, and 3*n*YB (Hu 2013)

Fish type	Fragment about 200 bp	Fragment about 400 bp
BSB	188 bp	376 bp, 394 bp
2*n*BY	188 bp, 206 bp	376 bp
3*n*BY	188 bp, 206 bp	376 bp, 394 bp
2*n*YB	188 bp, 211 bp, 216 bp	376 bp, 405 bp
3*n*YB	188 bp, 206 bp	376 bp, 394 bp
YT	188 bp, 206 bp	377 bp

of 2*n*BY (GenBank accession number, GU949545) and 3*n*BY (GenBank accession number, GU949546) was 16,623 bp, which was identical to that of maternal BSB (GenBank accession number, EU434747). The homology of the mtDNA in hybrids with maternal BSB was 99.8% and that of hybrids with paternal YT (GenBank accession number, GQ289558) was only 92.3% due to the mutations of 20 nucleotides. The length of mtDNA sequences of 2*n*YB (GenBank accession number, HQ651067) and 3*n*YB was 16,630 bp, which was identical to that of maternal YT. The homology of the mtDNA in 2*n*YB and maternal YT was 99.7% with 47 different nucleotides, and that of the mtDNA in 3*n*YB and maternal YT was 99.8% with 37 different nucleotides.

The multiple sequence alignment of mtDNA using MEGA 4.1 showed that there were 1332 variable positions (accounting for 8%) and 15,307 conserved positions in the mtDNA sequence in the parents and hybrids. Taking mtDNA sequences of common carp (*Cyprinus carpio*) (GenBank accession number, X61010) and grass carp (*Ctenopharyngodon idella*, GC) as outgroup, the sequence homology was analyzed and compared by constructing a NJ phylogenetic tree using MEGA 4.1. The result showed that the mitochondrion of 2*n*BY and 3*n*BY originated from the same maternal BSB, while the mitochondrion of 2*n*YB and 3*n*YB came from the same maternal YT (Hu 2013).

The two chains of mtDNA from BSB (♀) × YT (♂) hybrids and YT (♀) × BSB (♂) hybrids, similarly to other Cyprinidae, contained one heavy chain and one light chain. The basic constitutions were as follows: 2 noncoding regions including 1 D-loop and 1 replication origin of L-strand; 2 *rRNA*s including 12S *rRNA* and 16S *rRNA*; 13 mitochondrial protein coding genes including 7 NADH dehydrogenase subunits (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6*), 3 cytochrome C oxidase subunits (*COI*, *COII*, and *COIII*), 2 ATP enzyme subunits (*ATPase*8 and *ATPase*6), and 1 *Cytb* gene; and 22 tRNAs.

Comparison on the mtDNA sequences in parents and hybrids indicated that 2*n*BY and 3*n*BY had identical mtDNA sequencing. The variable sites of these two fishes and their maternal BSB were distributed in nine regions, such as *NADH2* and *COI*. The 47 variable sites of 2*n*YB and its maternal YT were distributed in 17 regions such as 16S *rRNA*, *NADH1*, and *NADH5*. The 37 variable sites of 3*n*YB and its maternal YT were distributed in 16 regions, such as *D-loop*, *rRNA*, and *ND1*. Analyses of amino acid sequences in BSB (♀) × YT (♂) hybrids showed that the mutation of *COI* and *Cytb* led to one mutation of amino acid, respectively. Meanwhile, there existed one variation of amino acid in the *ND5* from 2*n*YB. One variation of amino acid from coding region of each gene including *ND2*, *COI*, *ND5*, and *ND6* was found in 3*n*YB. The base transition and transversion were evaluated and the ratio of transition to transversion was calculated (the overall transition/transversion bias, R), according to the base substitution model, using the mtDNA sequences from the hybrids.

Their base substitution model was mainly on transition, which was similar to most of the vertebrates. The R value between BSB and YT was 5.078; the R value between BSB and BSB (♀) × YT (♂) was 280.716; the R values between YT and 2*n*YB, YT and 3*n*YB, and 2*n*YB and 3*n*YB were 38.665, 14.301, and 10.531,

respectively. Thus, the R values by comparing the hybrids or comparing the hybrid and their parents were much higher than the R values by comparing the parents (Hu 2013).

11.1.4.4 The Transcriptome Sequencing Analysis on the Testis in 2*n*BY and 3*n*BY

To determine the differentially expressed genes in the testes from fertile 2*n*BY and sterile 3*n*BY, the transcriptomes from fertile 2*n*BY and sterile 3*n*BY testes were analyzed using the next-generation high-throughput sequencing technology. After filtering, 52,091,274 and 51,496,376 clean reads were obtained in the testes from 2*n*BY and 3*n*BY, respectively. A total of 102,298 and 81,212 unigenes were generated by the de novo assembly from 2*n*BY and 3*n*BY, respectively. Using these unigenes, 60,315 CDS (coding sequence) from diploid and 48,078 CDS from 3*n*BY were extracted, respectively.

The unigenes from the two transcriptomes were annotated by six different databases including NT, NR, Swiss-Prot, COG, GO, and KEGG. A total of annotated unigenes from 2*n*BY and 3*n*BY were 84,558 and 67,623, which accounted for 82.66% and 83.72% of the overall output unigenes (102,298 from 2*n*BY and 81,212 from 3*n*BY), respectively. For the annotated proteins, proteins annotated from NR database were the most which were 58,230 (accounting for 68.86% of the total annotated proteins) in 2*n*BY and 46,295 (68.46%) in 3*n*BY (Hu 2013).

The unigenes for 2*n*BY and 3*n*BY were assigned to 25 categories by COG annotation. The functional classification of COG in the two libraries was similar. Replication, recombination and restoration, transcription factor, transcript, ribosome structure and biosynthesis, cell cycle control, cell division, and chromosome segregation had the most unigenes, besides the general functions. The genes in these groups were associated with the process of gonadal sex differentiation and development, as well as gametogenesis. Meanwhile, 41,359 genes (40.43%) in 2*n*BY and 32,284 genes (39.75%) in 3*n*BY were annotated in 259 KEGG pathways which provided important evidences for analyzing the functions of differential expressed genes in comprehensive and systematic way.

The transcriptomic sequencing analysis on the testis in 2*n*BY and 3*n*BY obtained large information about gene expression. Meanwhile, the classified functions and metabolic pathways of these genes would help to better understand the regulatory network and molecular biological mechanisms behind the specificity of gonadal development of hybrids. These data, meanwhile, provided useful information for revealing the fertile mechanism of partial 2*n*BY and the sterile mechanism of 3*n*BY.

Rational combination of parents in fish distant hybridization is one of the major determinants to produce new hybrid lineages with heterosis. In the hybridized combination of BSB × YT, the standard body shape, beautiful body color, and healthy BSB and YT were selected by artificial breeding as parents. BSB had high body depth and high-quality meat but low meat yields, while YT had long body shape and high meat yields with lower survival rates after reproduction. Using BSB and YT as parents was to produce hybrids with comprehensive superiorities from

both parents and provided important experimental data to study the rule of fish distant hybridization and the formation of hybrid lineages in a systematic way.

Years of consecutive hybridization and breeding experiments have proven that the combination of BSB (♀) × YT (♂), under the same experimental condition, had higher fertilization rate and hatching rate ($P < 0.05$) than that of the control group of self-crossing YT hatched in the same batch. Furthermore, this hybridized combination was easy to the operation of artificial reproduction and was conducive to the large-scale production of fish fry. The hybrids of BSB (♀) × YT (♂) had several advantageous traits such as high body depth, high meat yields, and fast growth. This hybrid was designated as a new aquatic product which was validated by the annual conference of the national appraisal committee of aquatic protospecies and improved varieties (the National Aquatic Protospecies and Improved Species Examination Committee) in December 2011. The new aquatic products was named as "BSB × YT hybrid" and the variety registration number was GS-02-001-2011. The main effects of distant hybridization are prone to generate large amount of genetic mutations. There were diploid ($2n = 48$) and triploid ($3n = 72$) in the hybrids derived from the hybridization between BSB and YT. On the appearances, the four hybrids showed higher body depth than YT. The appearances of 3*n*BY were more similar to its maternal BSB, but were significantly different from other hybrids. These results provided an important guiding significance in the experiments of fish distant hybridization, in particular, the hybridization between the two species of subfamily Cultrinae and Xenocyprininae. In addition, the formation of F_1–F_2 hybrids of BSB (♀) × YT (♂) provided a major foundation to establish a new hybrid lineage with mutation in genotype and phenotype. It is of great significance for the further study on these hybrids.

11.2 The Formation and Biological Characteristics of Red Crucian Carp × Bleeker's Yellow Tail

11.2.1 The Chromosome Number of Different Ploidy Hybrids

RCC with 100 chromosomes belonged to the Cyprininae, and YT with 48 chromosomes belonged to the Xenocyprininae. The different ploidy progenies (Fig. 11.7) were obtained from the cross of female red crucian carp (*Carassius auratus* red var., RCC) × male YT. The fertilization and hatching rates of the hybrid combination were 59.2% and 48.7%, respectively. But no living offspring in the reciprocal cross (YT (♀) ×RCC (♂)) could be generated.

The ploidy levels of hybrids of RCC (♀) × YT (♂) by flow cytometry and chromosome preparation in peripheral blood cells were detected. Research results demonstrated that there were three different ploidy fishes in F_1 hybrids, including natural gynogenetic diploid RCC (2*n*NGR, $2n = 100$) (Fig. 11.8a), triploid hybrids (3*n*RY, $3n = 124$) (Fig. 11.8b), and tetraploid hybrids (4*n*RY, $4n = 148$) (Fig. 11.8c). Hybrids of this hybridization combination were similar to that of RCC (♀) × BSB (♂), introduced in Chap. 6. The tetraploid hybrids, triploid hybrids,

Fig. 11.7 Appearance of F_1 hybrids of RCC (♀) × YT (♂). (**a**) Natural gynogenetic diploid RCC from RCC (♀) × YT (♂). (**b**) Triploid hybrids from RCC (♀) × YT (♂). (**c**) Tetraploid hybrids from RCC (♀) × YT (♂). Bar = 1 cm

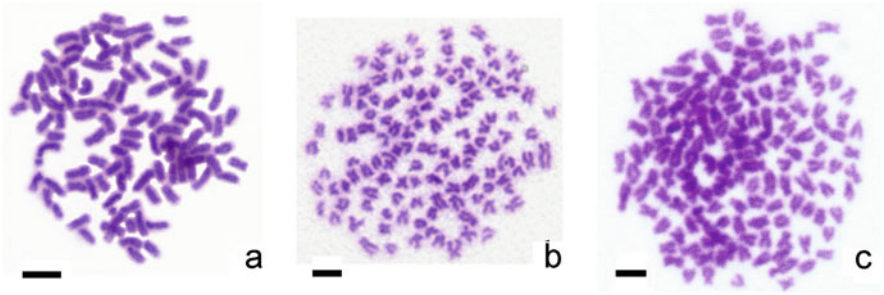

Fig. 11.8 Chromosome spread at metaphase in different ploidy F_1 of RCC (♀) × YT (♂). (**a**) Chromosome spread at metaphase of 2*n*NGR ($2n = 100$). (**b**) Chromosome spread at metaphase of 3*n*RY ($3n = 124$). (**c**) Chromosome spread at metaphase of 4*n*RY ($4n = 148$). Bar = 3 μm

and natural gynogenetic diploid fish were obtained, and there were no living diploid hybrids from these two subfamily distant hybridization combinations. In addition, no living hybrids were produced in these two reciprocal-cross combinations.

11.2.2 The Appearance Features of Different Ploidy Fishes in F_1

Seven countable traits of polyploid hybrids and their parents were detected, and relevant comparative data were presented in Table 11.5. Comparison results showed that five countable traits of lateral scales, scales above lateral line, dorsal fins, pectoral fins, and anal fins in hybrids were intermediate between those in their parents, which presented hybrid features, while abdominal fins and scales below lateral line were similar to paternal parent.

11.2.3 The Reproductive Traits of Different Ploidy Fishes in F_1

We had made a microscopic observation on the gonadal structure of polyploid hybrids with paraffin section technology. The ovarian microstructure observation

Table 11.5 Comparison on the countable traits between RCC, YT, and their polyploid hybrids (Hu 2013)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pectoral fins	Number of pelvic fins	Number of anal fins
RCC	28–30	6	7	III +18–20	8–9	13	III+6
4*n*RY	31–33	6–7	7–9	III +17–18	14–16	8–9	III+6
3*n*RY	30–34	7	8–9	III +16–19	16–17	7–9	III+6–7
YT	60–67	10–12	6–8	III+8–9	16–18	8–9	III +11–13

showed that the ovaries of 2-year-old 4*n*RY mainly entered a large growing period of phase III oocytes, and it could be seen that many phase II oocytes were entering the small growing period (Fig. 11.9a), and several oogonia could also be seen. The results indicated that the female gonad of 4*n*RY developed normally and matured for 2 years. The testis of 2-year-old 4*n*RY developed normally, and some lightly stained spermatogonia, as well as deeply stained piles of homotypic primary spermatocytes, secondary spermatocytes, and spermatids could be seen in the seminiferous tubules (Fig. 11.9b).

Two types of gonadal structure were found in 2-year-old 3*n*RY: one was fat-like type; the other was testis-like type (Fig. 11.9c). In the testis-like type, the seminiferous tubule could be seen some lightly stained spermatogonia, as well as deeply stained piles of primary spermatocytes and secondary spermatocytes, but no matured sperm was found. In the breeding season, the 2-year-old males and females of 3*n*RY could not squeeze out semen and eggs. These results suggested that 3*n*RY were sterile.

11.3 The Formation and Biological Characteristics of Red Crucian Carp × Topmouth Culter

11.3.1 The Chromosome Number of Different Ploidy Hybrids

Different ploidy hybrids were successfully produced by the distant hybridization of female RCC (Cyprininae, Cyprinidae, $2n = 100$) and male topmouth culter (*Culter alburnus*, Cultrinae, Cyprinidae, $2n = 48$, TC) (Fig. 11.10a–e). The fertilization rate and hatching rate of the hybrid combination were 83.6% and 74.6%, respectively, while no living hybrids in reciprocal cross were found (He et al. 2012).

The ploidy of hybrids was detected by flow cytometry and chromosome preparation in peripheral blood cells. The results showed that three kinds of hybrid offspring with different ploidy were produced by the crossing of female RCC and male TC

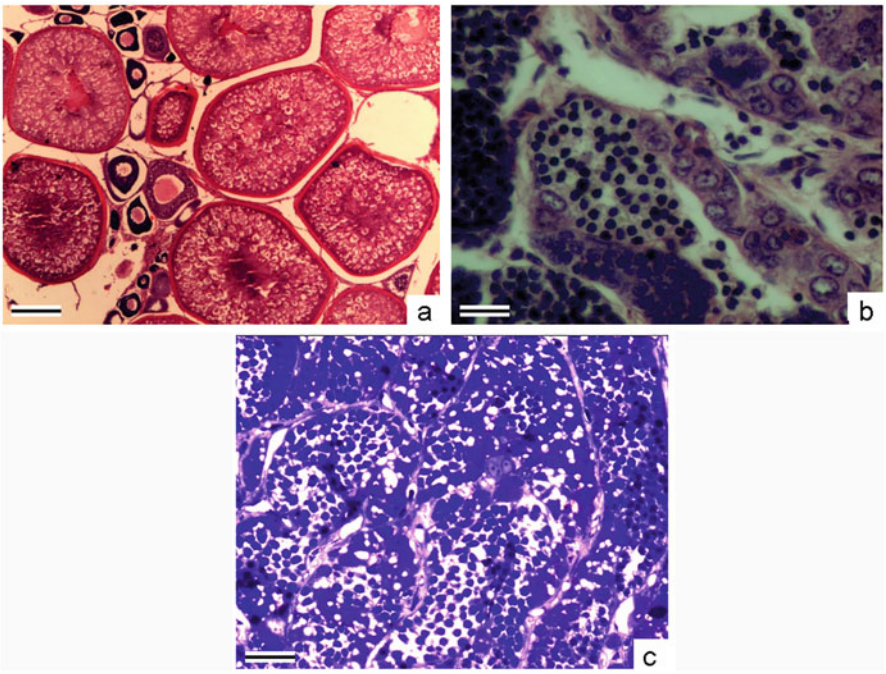

Fig. 11.9 The gonadal microstructure of 4*n*RY and 3*n*RY. (**a**) The ovarian microstructure of 2-year-old 4*n*RY, Bar = 80 μm. (**b**) The testis microstructure of 2-year-old 4*n*RY, Bar = 20 μm. (**c**) The testis-like gonadal microstructure of 3*n*RY. Bar = 20 μm

(Fig. 11.11a–e), including diploid hybrids of female RCC × male TC (2*n*RT) (2*n* = 74) (Fig. 11.11c), triploid hybrids of female RCC × male TC (3*n*RT) (3*n* = 124) (Fig. 11.11d), and tetraploid hybrids of female RCC × male TC (4*n*RT) (4*n* = 148) (Fig. 11.11e). The combination of this distant hybridization posed similarities and differences with those of combinations of RCC (♀) × BSB (♂) and RCC (♀) × YT (♂). For example, both tetraploid (4*n* = 148) and triploid (3*n* = 124) hybrids existed in these three combinations, but living diploid hybrids (2*n* = 74) were only generated from RCC (♀) × TC (♂).

11.3.2 The Appearance Features of Different Ploidy Fishes in F_1

We had detected seven countable traits of different ploidy hybrids and their parents (Table 11.6). The results showed that five countable traits (lateral scales, scales above lateral line, dorsal fins, pectoral fins, and anal fins) of 4*n*RT, 3*n*RT, and 2*n*RT were intermediate between RCC and TC, which presented hybrid features, while scales below lateral line and abdominal fins were more similar to RCC and TC, respectively.

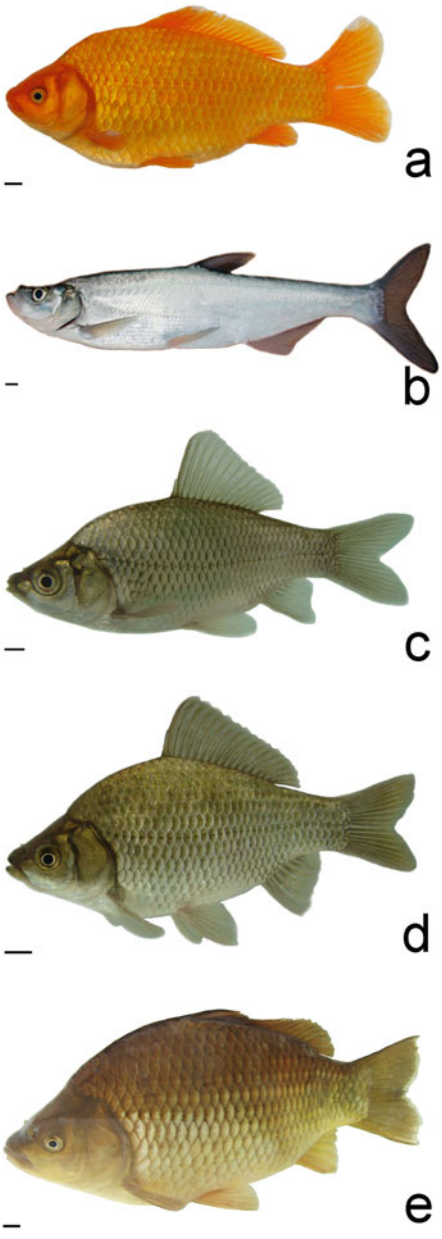

Fig. 11.10 Appearance of RCC, TC, and their different ploidy offspring (He et al. 2012). (**a**) RCC. (**b**) TC. (**c**) Diploid hybrids of female RCC × male TC (2*n*RT). (**d**) Triploid hybrids of female RCC × male TC (3*n*RT). (**e**) Tetraploid hybrids of female RCC × male TC (4*n*RT). Bar = 1 cm

11.3.3 The Reproductive Traits of Different Ploidy Fish in F_1

We have made a microscopic observation on the gonadal structure of the different ploidy hybrids by paraffin section technology. The results showed that the

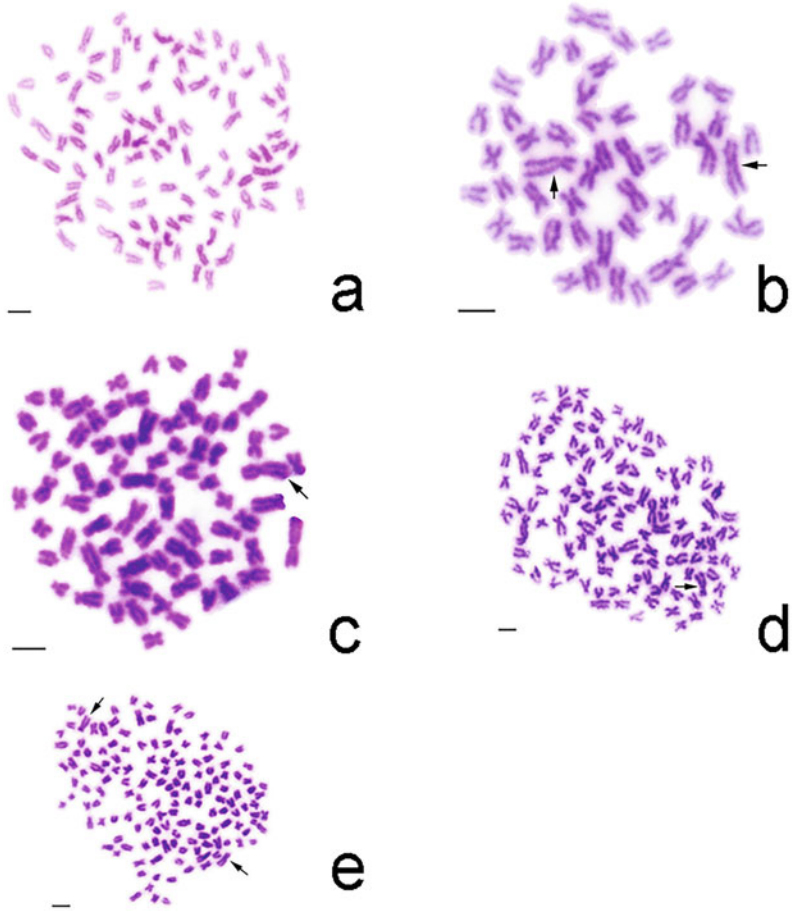

Fig. 11.11 Chromosome spread at metaphase of RCC, TC, and their hybrid offspring (He et al. 2012). (**a**) Chromosome spread at metaphase of RCC ($2n = 100$). (**b**) Chromosome spread at metaphase of TC ($2n = 48$). (**c**) Chromosome spread at metaphase of 2*n*RT ($2n = 74$). (**d**) Chromosome spread at metaphase of 3*n*RT ($3n = 124$). (**e**) Chromosome spread at metaphase of 4*n*RT ($4n = 148$). Bar = 3 μm

seminiferous tubule of 2-year-old 2*n*RT was filled with spermatogonia and primary spermatocytes, secondary spermatocytes, and spermatids (Fig. 11.12a, b), but no matured sperm was found. In addition, 2*n*RT with ovarian structure were not found (He 2012).

No matured sperm was found in the testis of 2-year-old 3*n*RT (Fig. 11.12c). In the breeding season, the 2-year-old males and females of 3*n*RT could not squeeze out semen and eggs. These results suggested that 3*n*RT were sterile (He 2012).

Table 11.6 Comparison on the countable traits of RCC, TC, and their hybrids (He et al. 2012)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pectoral fins	Number of pelvic fins	Number of anal fins
RCC	28–30	6	7	III +18–20	8–9	13	III+6
4*n*RT	30–33	6–7	7–8	III +17–19	16	9	III+6
3*n*RT	30–33	7–8	8–9	III +15–16	15–17	9	III+6–7
2*n*RT	33–37	7–8	8–10	III +14–18	16–17	7–9	III+7–8
TC	80–92	16–20	6–7	III+7	15–16	9	III +20–23

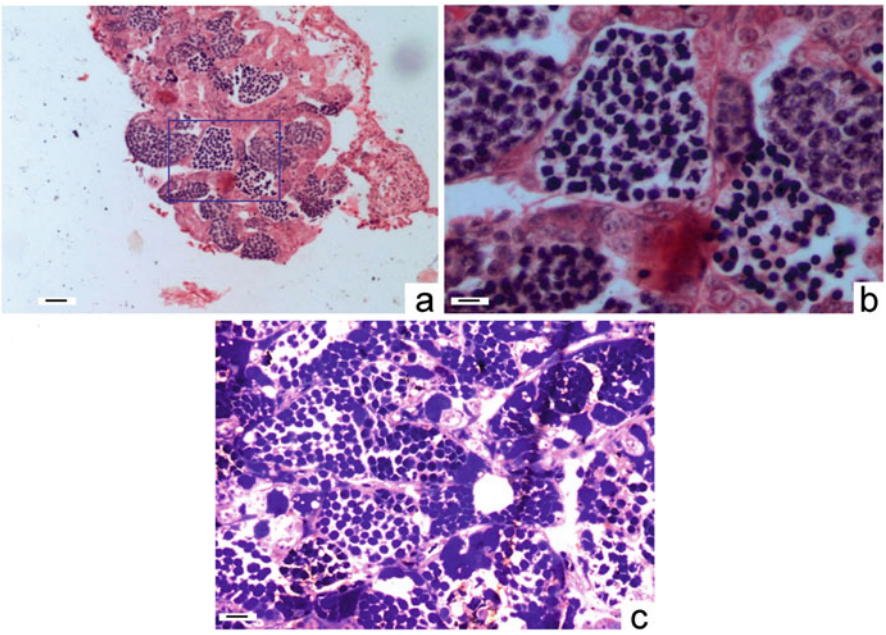

Fig. 11.12 The gonadal microstructure of 2*n*RT and 3*n*RT. (**a**) The testis microstructure of 2*n*RT. Bar = 100 μm. (**b**) The part in (**a**) is magnified in (**b**). Bar = 20 μm. (**c**) The testis microstructure of 3*n*RT. Bar = 20 μm

11.4 The Formation and Biological Characteristics of Grass Carp × Blunt Snout Bream

11.4.1 The Chromosome Number of Different Ploidy Hybrids

Different ploidy hybrids (Fig. 11.13a–d) were successfully obtained by crossing of female GC (*C. idella*, Leuciscinae, Cyprinidae, $2n = 48$) and male BSB (*M. amblycephala*, Cultrinae, Cyprinidae, $2n = 48$). The fertilization rate and hatching rate of the combination were 95.0% and 80.0%, respectively (He et al. 2013).

The ploidy of hybrids and their parents was detected by flow cytometry and chromosome preparation in peripheral blood cells (Fig. 11.14a–d). The results showed that two different ploidy level hybrids offspring were produced by the crossing of female GC and male BSB, including diploid hybrids of female GC ×

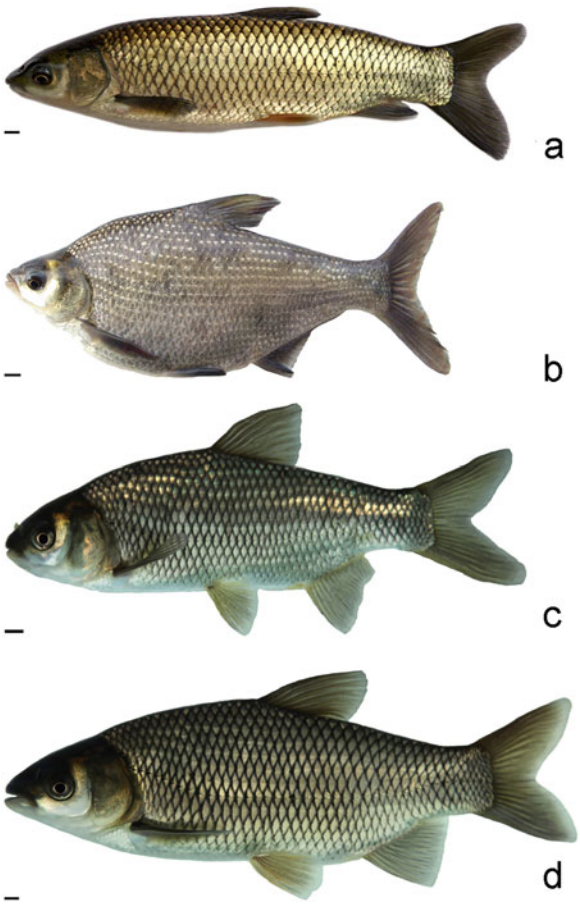

Fig. 11.13 Appearance of GC, BSB, and their different ploidy offspring (He et al. 2013). (**a**) GC. (**b**) BSB. (**c**) Diploid hybrids of female GC × male BSB (2*n*GB). (**d**) Triploid hybrids of female GC × male BSB (3*n*GB). Bar = 1 cm

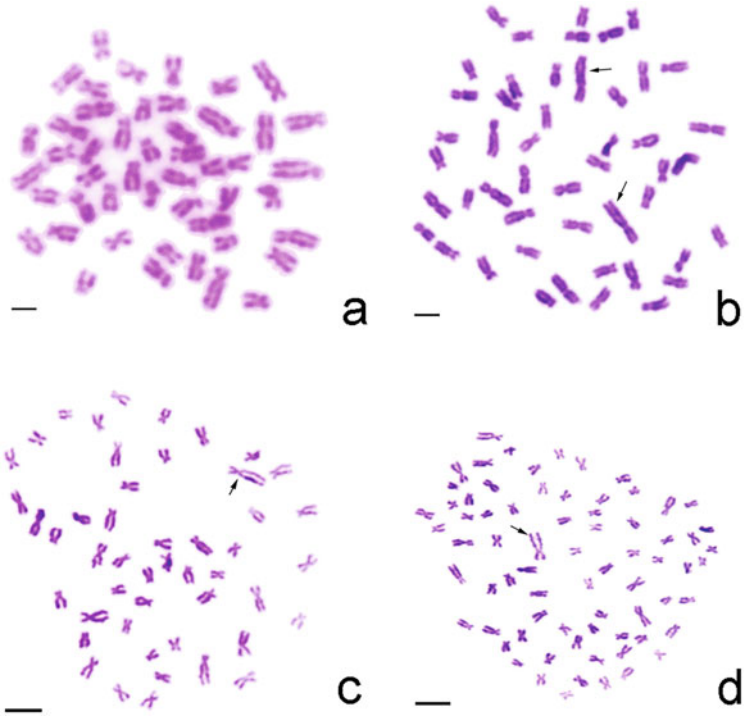

Fig. 11.14 Chromosome spread at metaphase of GC, BSB, and their hybrid offspring (He et al. 2013). (**a**) Chromosome spread at metaphase of GC ($2n = 48$). (**b**) Chromosome spread at metaphase of BSB ($2n = 48$). (**c**) Chromosome spread at metaphase of 2*n*GB ($2n = 48$). (**d**) Chromosome spread at metaphase of 3*n*GB ($3n = 72$). Bar = 10 μm

Table 11.7 Comparison on the size of erythrocyte of 2*n*GB and 3*n*GB (unit: μm, μm^2)

Fish type	Major axis (*A*)	Minor axis (*B*)	Area ($S = \pi \times A \times B/4$)
GC	9.89 ± 0.89	5.71 ± 0.43	44.09 ± 2.78
2*n*GB	9.12 ± 0.91	5.79 ± 0.35	41.37 ± 4.01
3*n*GB	11.01 ± 0.60	6.29 ± 0.48	54.67 ± 3.28

male BSB (2*n*GB) ($2n = 48$) (Fig. 11.14c) and triploid hybrids of female GC × male BSB (3*n*GB) ($3n = 72$) (Fig. 11.14d) (He et al. 2013).

11.4.2 The Appearance Features of Erythrocyte of Different Ploidy Fishes in F_1

Taking GC as control group, a comparison on the size of erythrocyte of 2*n*GB and 3*n*GB was performed with blood smear method. The results were presented in Table 11.7.

The results showed that the size of erythrocyte of 2*n*GB was similar to that of maternal GC; the size of erythrocyte of 3*n*GB was obviously larger than that of maternal GC and 2*n*GB. In addition, dumbbell-like nucleus erythrocytes were found in 3*n*GB, but these dumbbell-like nucleus erythrocytes could not be found in 2*n*GB, which could be regarded as a biological marker to distinguish 2*n*GB from 3*n*GB.

11.4.3 The Molecular Biological Characteristics of Different Ploidy Fishes in F_1

In the hybrid offspring, 2*n*GB and 3*n*GB not only inherited one type of *5S rDNA* class from their parents (GC and BSB), respectively, but also generated a novel chimera of *5S rDNA*. This result implied the occurrence of a reorganization event (Qin et al. 2010).

There was only one *5S rDNA* constitution unit in GC and BSB, which was named as type I (180 bp) and type II (188 bp), respectively. The highly conserved in the *5S rDNA* coding regions but exhibited large variation in the NTS regions was identified in GC and BSB. In the hybrid offspring, 2*n*GB not only inherited type I and type II from GC and BSB, respectively, but also generated a new chimera of type I and type II and a 10 bp poly A insertion in the type II sequence (He et al. 2013). The 3*n*GB showed similar results to that of 2*n*GB, but no insertion of the 10 bp poly A (He et al. 2013).

The internal control regions (ICRs) (including A box, internal element (IE), and C box) with highly conserved were all identified in the *5S rDNA* coding regions of GC, BSB, 2*n*GB, and 3*n*GB. Even so, a species-specific nucleotide site within A box (position 60) was yet observed between GC and BSB. In the NTS sequence, there was only one type in both GC and BSB, designated as NTS-I (60 bp) and NTS-II (68 bp), respectively. Except for a substitution at position-1 (C → T) in 2*n*GB and a substitution at position-36 (G → A) in 2*n*GB and 3*n*GB, the NTS-I sequence of GC, 2*n*GB, and 3*n*GB showed a high homology among them. Moreover, the NTS-II sequence of BSB, 2*n*GB, and 3*n*GB also showed a high homology among them. In a word, 2*n*GB and 3*n*GB not only inherited type I and type II of *5S rDNA* from their parents, respectively, but also generated a chimera of type I and type II, suggesting that somatic recombination has occurred. Besides, a 10 bp poly A insertion in the type II sequence of chimera in 2*n*GB and its absence in 3*n*GB were also detected. We speculated that, to improve fertility, it was necessary to insert a poly A into the type II sequence of chimera in 2*n*GB, despite the fact that there was no direct data available to explain the possible relationship between them.

Hybridization is considered to be an effective way to promote the adaptive radiation evolution and speciation of animals and plants. It has been proved that hybridization can lead to alterations in chromosomal structure, gene expression, and genome size. In the present study, one new chimera composed of type I and type II was observed in the genome of 2*n*GB and 3*n*GB, and nucleotide variation, including insertion-deletion and substitutions, was also detected in the NTS regions of these hybrid offspring (He et al. 2013). In a word, these results demonstrated that the

influence of hybridization, polyploidization, and variation of *5S rDNA* would result in rapid genomic changes.

In feeding habits, GC was herbivorous, same with that of BSB, whereas both the 2*n*GB and 3*n*GB were omnivorous; in disease resistance, BSB enjoyed a higher disease resistance ability, while GC was easy to get caught in illness in a certain growth stage; in hypoxia resistance, GC enjoyed a high hypoxia-resistant ability than BSB; in reproductive biology, the age of sexual maturity of GC was 4–5 years, while that of BSB was 2 years. It is our goal to produce hybrids with the advantages of GC and BSB and to establish a fertile hybrid lineage and even produce tetraploid fish. Currently, a batch of 4- or 5-year-old F_1 hybrids was produced and their biological traits should be further studied comprehensively and systematically.

11.5 The Formation and Biological Characteristics of Koi Carp × Improved Color Crucian Carp

11.5.1 The Formation and Chromosome Number of F_1

The hybrids were successfully produced by the hybridization between female koi carp (*Cyprinus carpio haematopterus*, Cyprininae, Cyprinidae, $2n = 100$, KOC) (Fig. 11.15a) and male improved color crucian carp (*C. auratus*, Cyprininae, Cyprinidae, $2n = 100$, ICCC) (Fig. 11.15b). In this combination, the maternal parent KOC was a variation of common carp (*C. carpio*, CC) with pink body color. In terms of the origin of paternal parent ICCC, referred to in Sect. 5.3, Chap. 5, it was a variation of crucian carp. This combination could be regarded as a similar reciprocal cross as the hybridization of female RCC × male CC introduced in Chap. 2. The similarity of these two combinations was inter-genera hybridization with the number of chromosomes of both parents being 100, while the difference of this hybridization combination was that the body color of F_1 hybrids was not gray but was golden yellow (Fig. 11.15c), even though the body color of their parents was related to red. The goals of conducting this hybridization are to explore whether allotetraploid fish can also be selected or not in the reciprocal cross, and to discuss the changes in body color in hybridization. The fertilization and hatching rates of F_1 hybrids were 62.2% and 58.7%, respectively (Xie 2013).

Fig. 11.15 Appearances of KOC (♀), ICCC (♂), and their F_1 hybrids. (**a**) KC. (**b**) ICCC. (**c**) Hybrids of KOC (♀) × ICCC (♂) (KI). Bar = 1 cm

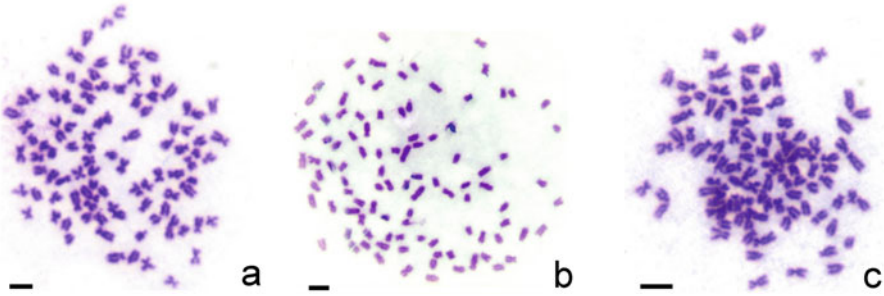

Fig. 11.16 Chromosome spread at metaphase of KOC (♀), ICCC (♂), and their F_1 hybrids. (**a**) Chromosome spread at metaphase of KOC ($2n = 100$). (**b**) Chromosome spread at metaphase of ICCC ($2n = 100$). (**c**) Chromosome spread at metaphase of hybrids of KOC (♀) × ICCC (♂) ($2n = 100$). Bar = 3 μm

Table 11.8 Comparison on the countable traits of KOC (♀), ICCC (♂), and their F_1 hybrids (Xie 2013)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pectoral fins	Number of pelvic fins	Number of anal fins
KOC	36–37	6–7	6–7	III +17–18	8–9	8–9	III+5–6
F_1	28–37	5–6	6–7	III +14–19	16	7–9	III+6–7
ICCC	27–28	5–6	5–7	III +17–18	15–17	8–9	III+6–7

The flow cytometry of KOC, ICCC, and their hybrids were shown in Fig. 11.16. The results of KOC (Fig. 11.16a) and ICCC (Fig. 11.16b) were similar to the hybrid KOC (♀) × ICCC (♂) (Fig. 11.16c) and its chromosome number was 100 ($2n = 100$) (Xie 2013).

11.5.2 The Appearance Features of Fish in F_1

We had detected seven countable traits of F_1 hybrids and their parents were presented in Table 11.8. The results indicated that four countable traits of F_1 hybrids (dorsal fins, pectoral fins, pelvic fins, and anal fins) were intermediate between their parents, which presented hybrid features. Scales below lateral line were similar to paternal parent, while scales above lateral line and lateral scales were similar to maternal parent, respectively (Xie 2013).

11.5.3 The Reproductive Traits of Fish in F_1

We had made a microscopic observation on the gonadal structure of hybrids with paraffin section technology. The results showed that several 2-year-old males and females had normal gonadal development. At this stage, the ovaries of 2-year-old hybrids entered stage III, and the ovaries were mainly occupied by a large growing period of phase III oocytes. Many phase II oocytes entering the small growing period could be observed (Fig. 11.17a). Besides, several oogonia could also be found in 2-year-old ovary. The results indicated that the female gonad of hybrids developed normally and achieved sexual maturity at 2 years old. The testis of 2-year-old hybrids developed normally, and some lightly stained spermatogonia as well as deeply stained and piles of homotypic primary spermatocytes, secondary spermatocytes, and spermatids could be seen in the seminiferous tubules (Fig. 11.17b). In the breeding season, 2-year-old female and male hybrid individuals who reached sexual maturity could be artificially induced spawning and sperm releasing, and living F_2 diploid and triploid hybrids were produced by self-mating, which suggested fertile male and female individuals existed in F_1 hybrids (Xie 2013).

In the breeding season, the 2-year-old or more than 2-year-old mature F_1 hybrids were selected for artificially fertilization and the F_2 hybrids could be successfully obtained (Fig. 11.18).

The number of chromosomes of F_1 hybrids was shown in Fig. 11.19a. Diploid and triploid fish were found in the F_2 hybrids by chromosome ploidy detection, and the results demonstrated that the number of chromosomes of the diploid and triploid fish was 100 (Fig. 11.19b) and 150 (Fig. 11.19c), respectively (Xie 2013).

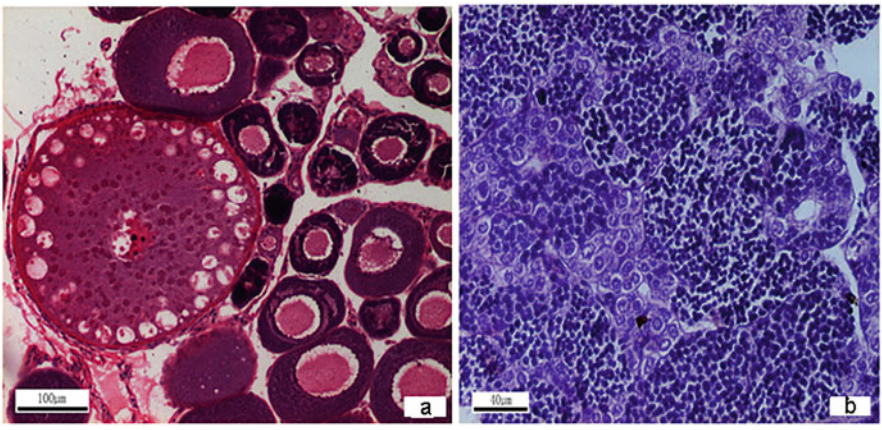

Fig. 11.17 The gonadal microstructure of F_1 hybrids. (**a**) Ovary of 2-year-old F_1 hybrids. (**b**) Testis of 2-year-old F_1 hybrids

Fig. 11.18 Appearances of F_1 hybrids and their self-mated hybrids. (**a**) F_1 hybrids. (**b**) Diploid fish in F_2 hybrids. (**c**) Triploid fish in F_2 hybrids. Bar = 1 cm

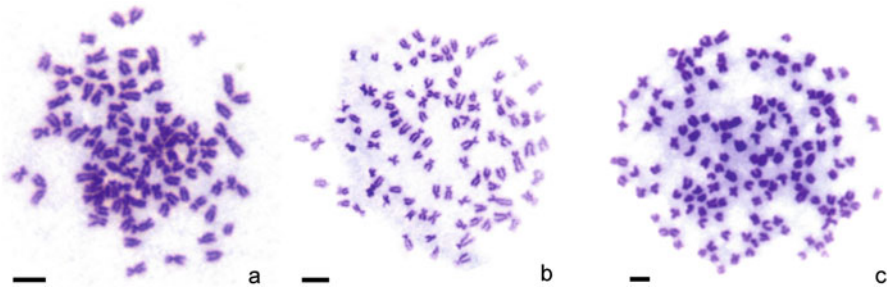

Fig. 11.19 Chromosome spread at metaphase of F_1 hybrids and their self-mated hybrids. (**a**) Chromosome spread at metaphase of F_1 hybrids ($2n = 100$). (**b**) Chromosome spread at metaphase of diploid F_2 hybrids ($2n = 100$). (**c**) Chromosome spread at metaphase of triploid F_2 hybrids ($3n = 150$). Bar = 3 μm

11.6 The Formation and Biological Characteristics of Grass Carp × Topmouth Culter

The basic biological characteristics of paternal parent in the distant hybridization of GC (♀) × TC (♂) have been introduced in Chap. 8, while the maternal parent was described as follows. As one of the four major Chinese carps, GC (*C. idella*, $2n = 48$) is one of larger farmer fishes with fast growth and reaches its sexual maturity at 4–5 years old. In feeding habits, adult GC was a typical herbivore, although it was carnivorous around 1.5 months after hatching (Li et al. 2015). For the herbivority, rapid growth rates, and high economic value, the GC becomes a global aquaculture species with worldwide distribution (Chilton and Muoneke 1992; Wang et al. 2015).

11.6.1 The Characteristics of Genetic Construction of Different Ploidy Hybrids

In the breeding season (from May to June), we selected female GC and male TC as the maternal fish and paternal fish, respectively. Surprisingly, this hybridization combination produced a high fertilization and hatching rate (Wu et al. 2019). Moreover, by the crossing of GC (♀, Fig. 11.20a) × TC (♂, Fig. 11.20b), two

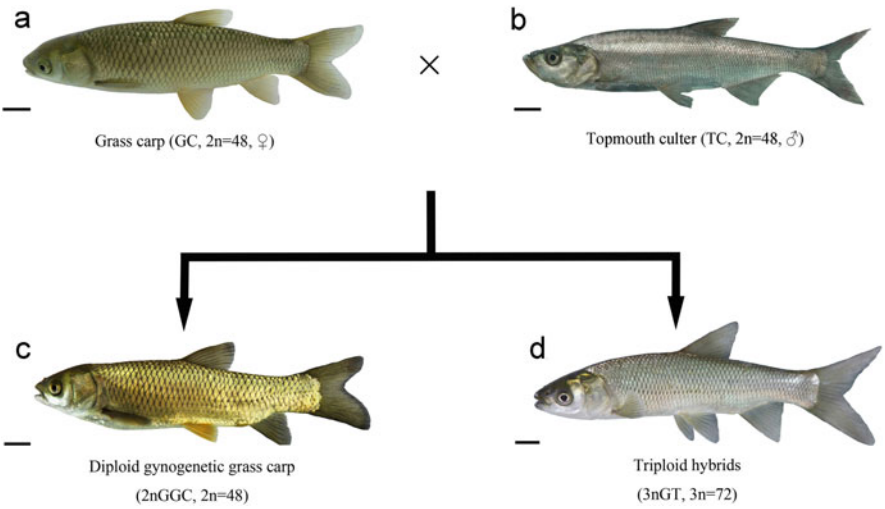

Fig. 11.20 The crossing procedure and appearances of GC, TC, 2*n*GGC, and 3*n*GT. (**a**) GC. (**b**) TC. (**c**) 2*n*GGC. (**d**) 3*n*GT. Bar = 2 cm

Table 11.9 Mean DNA content of GC, TC, 2*n*GGC, and 3*n*GT (Wu et al. 2019)

Fish type	Mean DNA content	Ratio	
		Observed	Expected
GC	60.59		
TC	71.31		
2*n*GGC	60.41	2*n*GGC/GC = 0.99[a]	1
3*n*GT	97.09	3*n*GT/(GC+0.5TC) = 1.00[a]	1

[a]There is no significant difference ($P > 0.05$)

kinds of hybrid offspring, namely, diploid gynogenetic GC (*2n*GGC, Fig. 11.20c) and triploid hybrids of GC (♀) × TC (♂) (3*n*GT, Fig. 11.20d), were obtained. 2*n*GGC and 3*n*GT comprised 0.07% and 99.93% of the offspring at 10 months old, respectively (Wu et al. 2019).

To measure the mean DNA content of erythrocytes of F_1 hybrids, we collected and measured the DNA content of offspring by flow cytometry (Partec). Using the DNA content of female and paternal parents as the controls, the distribution of the DNA content in GC, TC, and their offspring was shown in Table 11.9. Two kinds of ploidy offspring, including diploids and triploids, were detected by flow cytometry. The mean DNA content of diploids was equal to that of GC, implying that 2*n*GGC possessed two sets of chromosomes from GC. The mean DNA content of triploids was equal to the sum of GC and half of TC, indicating that 3*n*GT possessed one set of chromosomes from TC and two sets of chromosomes from GC (Wu et al. 2019).

To further determine ploidy, metaphase chromosome preparations were performed using kidney tissue of 2*n*GGC, 3*n*GT, and their parents GC and

Table 11.10 Examination of chromosome number of GC, TC, 2*n*GGC, and 3*n*GT (Wu et al. 2019)

Fish type	Number in metaphase	Distribution of chromosome number			
		<48	48	<72	72
GC	150	12	138		
TC	150	9	141		
2*n*GGC	300	14	286		
3*n*GT	300			21	279

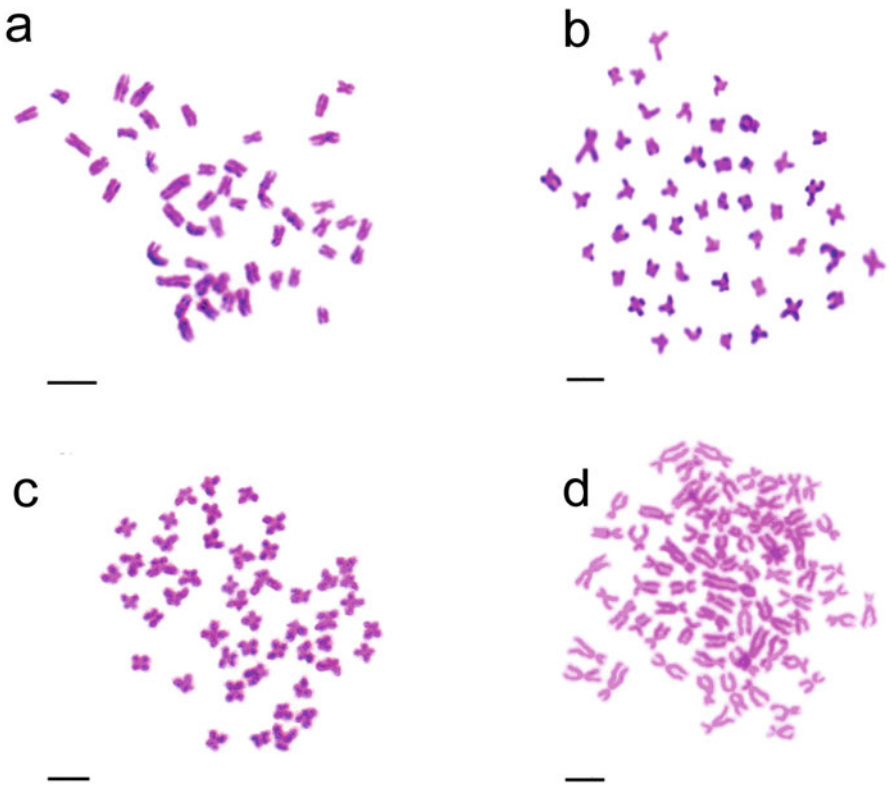

Fig. 11.21 Metaphase chromosome spreads of GC, TC, 2*n*GGC, and 3*n*GT. (**a**) Metaphase chromosome spreads of GC ($2n = 48$). (**b**) Metaphase chromosome spreads of TC ($2n = 48$). (**c**) Metaphase chromosome spreads of 2*n*GGC ($2n = 48$). (**d**) Metaphase chromosome spreads of 3*n*GT ($2n = 72$). Bar = 20 μm

TC. The chromosome number and distribution of all the samples were shown in Table 11.10 and Fig. 11.21. For diploid GC and TC, 92% and 94% of the chromosomal metaphase spreads had 48 chromosomes, respectively (Fig. 11.21a, b). In the hybrid offspring of GC (♀) × TC (♂), which was similar in appearance to GC, 95% of the chromosomal metaphase spreads possessed 48 chromosomes, implying that they were diploids with 48 chromosomes (Fig. 11.21c). In the hybrid offspring of

GC (♀) × TC (♂), whose appearance was intermediate between GC and TC, 93% of the chromosomal metaphase spreads possessed 72 chromosomes, suggesting that they were triploids with 72 chromosomes (Fig. 11.21d) (Wu et al. 2019).

11.6.2 The Appearance Features and Amino Acid Composition of Different Ploidy Fish in F_1

The countable and measurable traits in 2*n*GGC, 3*n*GT, and their parents were presented in Tables 11.11 and 11.12, respectively. There was no significant difference in countable traits between 2*n*GGC and GC, except for the anal fins. However, there were significant differences in all the countable traits between 2*n*GGC and TC

Table 11.11 The countable traits of GC, TC, 2*n*GGC, and 3*n*GT (Wu et al. 2019)

Fish type	Number of lateral line scales	Number of scales above lateral line	Number of scales below lateral line	Number of dorsal fins	Number of pelvic fins	Number of anal fins
GC	42.31 ± 0.89 (39–44)	6.43 ± 0.23 (6–7)	4.45 ± 0.34 (4–5)	III + 7.00 ± 0.00 (III + 7)	8.00 ± 0.00 (8)	III + 7.00 ± 0.00 (7)
TC	85.47 ± 2.58 (80–92)	17.62 ± 1.02 (16–20)	6.53 ± 0.47 (6–7)	III + 7.00 ± 0.00 (III + 7)	9.00 ± 0.00 (9)	III + 21.87 ± 1.01 (20–23)
2*n*GGC	43.71 ± 1.21 (42–45)	6.60 ± 0.23 (6–7)	4.64 ± 0.27 (4–5)	III + 7.00 ± 0.00 (III + 7)	8.00 ± 0.00 (8)	III + 8.00 ± 0.00 (8)
3*n*GT	53.89 ± 1.43 (51–56)	11.02 ± 0.96 (9–12)	6.37 ± 0.24 (6–7)	III + 7.00 ± 0.00 (III + 7)	8.00 ± 0.00 (8)	III + 10.64 ± 0.35 (10–11)

Table 11.12 The measurable traits of GC, TC, 2*n*GGC, and 3*n*GT (Wu et al. 2019)

Fish type	Overall length/ body length	Body length/ body width	Body depth/ head height	Head length/ head width	Head height/ head width	Caudal peduncle length/caudal peduncle depth
GC	1.19 ± 0.28	6.61 ± 0.20	1.45 ± 0.01	1.45 ± 0.07	1.01 ± 0.06	1.07 ± 0.05
TC	1.16 ± 0.03	10.21 ± 0.66	1.67 ± 0.14	2.64 ± 0.14	1.5 ± 0.21	1.18 ± 0.02
2*n*GGC	1.17 ± 0.10	6.66 ± 0.16	1.50 ± 0.04	1.48 ± 0.06	1.01 ± 0.07	0.98 ± 0.04
3*n*GT	1.21 ± 0.13	9.12 ± 0.57	1.21 ± 0.04	2.11 ± 0.15	1.30 ± 0.11	1.10 ± 0.09

($P < 0.01$), apart from the dorsal fins. For 3*n*GT, the number of lateral line scales, scales above lateral line, and anal fins were intermediate between those of GC and TC, but the number of scales below lateral line closely resembled that of TC, and the number of pelvic fins was similar to that of GC, indicating that 3*n*GT exhibited a variable hybrid-type shape for these countable traits.

For the measurable traits between 2*n*GGC and GC (Table 11.12), there existed no significant differences, implying that the appearance of 2*n*GGC closely resembled that of GC. The value of overall length/body length in 3*n*GT was significantly greater than that in both parents ($P < 0.05$); however, the ratio of body depth/head height was significantly lower compared with that in both parents ($P < 0.05$), implying the variable phenotype for 3*n*GT was found. Besides, the other measurable traits of 3*n*GT, including the value of body length/body width, head length/head width, head height/head width, and caudal peduncle length/caudal peduncle depth, were numerically intermediate between those of GC and TC (Wu et al. 2019).

The amounts of 17 amino acids, including 7 essential amino acids, 2 semi-essential amino acids, and 8 nonessential amino acids, present in 2*n*GGC, 3*n*GT, and their parents were illustrated in Table 11.13. The moisture contents of the four types of fish varied from 80.00% to 81.46% in fresh muscle. The total amino acid (TAAs) contents reached 53.74 g/100 g, 63.31 g/100 g, 58.76 g/100 g, and 61.90 g/100 g in GC, TC, 2*n*GGC, and 3*n*GT, respectively. Meanwhile, the total essential amino acids (EAAs), with values ranging from 19.83 to 23.57 g/100 g dry muscle, comprised 39.45–40.10% of TAAs. Besides, the total delicious amino acids (DAAs), which ranged from 21.20 to 25.08 g/100 g, are composed of approximately 40% of TAAs. The amount of TAAs was higher in TC than that in GC ($P < 0.05$), and the amounts of the total EAAs and DAAs between GC and TC were also significantly different. The glutamic acid ranging from 8.68 to 10.48 g/100 g was the most common amino acid found in all samples of fish muscle, while the cysteine was the least common amino acid detected in muscle, with values ranging from 0.47 g/100 g to 0.55 g/100 g (Wu et al. 2019).

The physical characteristics of the pharyngeal teeth in 2*n*GGC, 3*n*GT, and their parents GC and TC were shown in Fig. 11.22. The results indicated that GC possessed two rows of teeth on the pharyngeal bone, with four major pharyngeal teeth and two lateral pharyngeal teeth, and many striated grooves were found on the surface of the pharyngeal teeth (Fig. 11.22a, b). While in TC, three rows of needle-sharp teeth on the pharyngeal bone, including four major pharyngeal teeth, one middle pharyngeal tooth and three lateral pharyngeal teeth, were found (Fig. 11.22c, d). In the offspring, the appearance of pharyngeal teeth was not significantly different between 2*n*GGC (Fig. 11.22e, f) and GC, while 3*n*GT had four main pharyngeal teeth and three lateral pharyngeal teeth with striated grooves (Fig. 11.22g, h).

To verify the feeding habit of the hybrid offspring, grass was used to fed them to estimate whether they were herbivorous or not. The results showed that the TC was carnivorous, while both 2*n*GGC and 3*n*GT were herbivorous (Wu et al. 2019).

Table 11.13 The mean amino acid contents of 2*n*GGC and 3*n*GT muscle and their parents GC and TC (g/100 g) (Wu et al. 2019)

Type	Amino acid name	GC (g)	TC (g)	2*n*GGC (g)	3*n*GT (g)
Wet weight		3.58	3.19	2.40	3.68
Dry weight		0.69	0.59	0.48	0.71
Moisture content		80.83	81.46	80.00	80.55
Essential amino acids	Thr	2.28	2.66	2.84	2.69
	Met	1.88	2.18	1.76	2.11
	Ile	2.38	2.90	2.45	2.80
	Leu	4.29	5.20	4.69	5.08
	Lys	4.06	4.66	6.16	4.62
	Val	2.71	3.27	2.98	3.14
	Phe	2.22	2.69	2.51	2.54
Semi-essential amino acids	His	1.67	2.28	1.87	2.03
	Arg	3.16	3.34	2.30	3.47
Nonessential amino acids	Asp[a]	6.42	8.27	6.80	7.77
	Glu[a]	8.68	9.97	10.48	10.14
	Gly[a]	2.75	3.05	2.64	2.96
	Ala[a]	3.34	3.80	3.64	3.82
	Cys	0.55	0.54	0.47	0.53
	Tyr	1.98	2.42	1.98	2.22
	Pro	2.29	2.26	2.40	2.47
	Ser	3.06	3.67	2.79	3.52
∑TAAs		53.74	63.15	58.76	61.90
∑EAAs		19.83	23.57	23.38	22.97
∑DAAs		21.20	25.08	23.56	24.68
∑EAAs/∑TAAs		36.89	37.32	39.79	37.12
∑DAAs/TAAs		39.45	39.72	40.10	39.88

[a]Means delicious amino acid; ∑DAAs means the total delicious amino acids; ∑EAAs means the total essential amino acids; ∑TAAs means the total amino acids; tryptophan is destroyed in acid hydrolysis and is not detected

11.6.3 The Molecular Biological Characteristics of Different Ploidy Fish in F_1

A pair of primers was used to amplify the *5S rDNA* by PCR (Qin et al. 2010), and distinctive band patterns separated by gel electrophoresis were produced. There were three different sizes (approximately 180 bp, 360 bp, and 540 bp) in GC, two in TC (approximately 180 bp and 360 bp), three in 2*n*GGC (approximately 180 bp, 360 bp, and 540 bp), and three in 3*n*GT (approximately 180 bp, 360 bp, and 540 bp). The results indicated that these sequences, which were longer than 200 bp, were a simple unit consisting of a NTS region (approximately 60 bp) and a 120 bp CDS of the *5S* gene. Comparison on the first bands (approximately 180 bp) from the four types of fish revealed that the GC had a 180 bp fragment with a 68 bp NTS, TC had a 188 bp fragment with a 76 bp NTS, 2*n*GGC had a 180 bp fragment with a 68 bp NTS, and

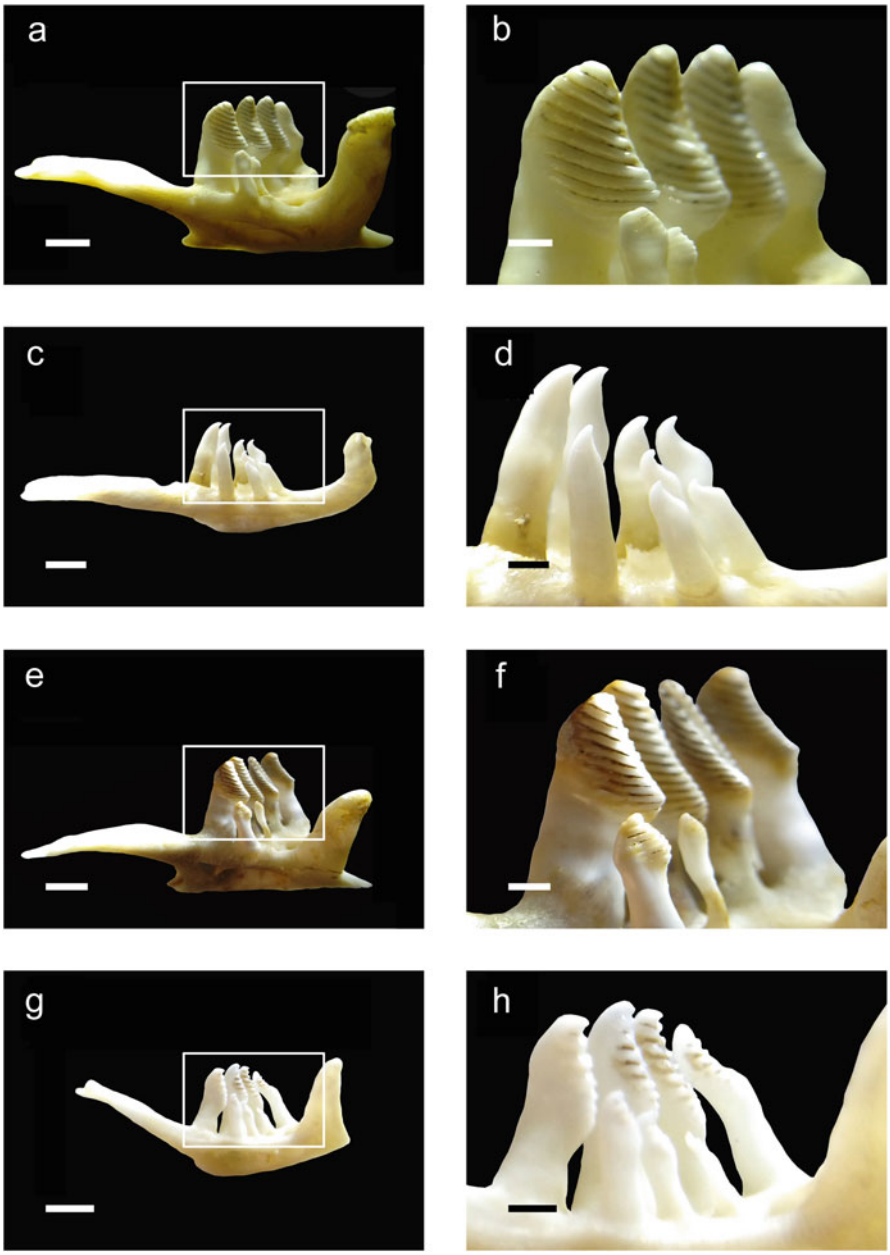

Fig. 11.22 The appearance of the pharyngeal teeth in GC, TC, 2*n*GGC, and 3*n*GT. (**a**) The pharyngeal teeth of GC possessed four major pharyngeal teeth, one middle pharyngeal tooth, and three lateral pharyngeal teeth. Bar = 2.1 cm. (**b**) The same as (**a**). Bar = 0.7 cm. (**c**) The pharyngeal teeth of TC possessed four main pharyngeal teeth and two lateral pharyngeal teeth. Bar = 2.1 cm. (**d**) The same as (**c**). Bar = 0.7 cm. (**e**) The pharyngeal teeth of 2*n*GGC possessed the same appearance as GC. Bar = 2.1 cm. (**f**) The same as (**e**). Bar = 0.7 cm. (**g**) The pharyngeal teeth of 3*n*GT possessed four major pharyngeal teeth and three lateral pharyngeal teeth. Bar = 2.1 cm. (**h**) The same as (**g**). Bar = 0.7 cm

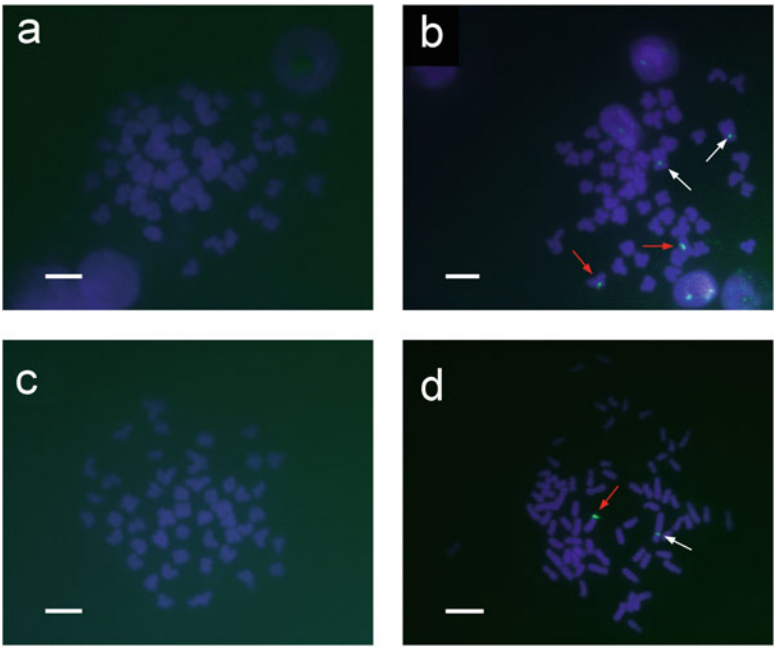

Fig. 11.23 Examination of hybridizing signals by fluorescence in situ hybridization (probe TC-188) in GC, TC, 2*n*GGC, and 3*n*GT. (**a**) GC had no hybridizing signal. (**b**) TC had two strong hybridizing signals (red arrow) and two weak hybridizing signals (white arrow). (**c**) 2*n*GGC had no hybridizing signal. (**d**) 3*n*GT had one strong hybridizing signal (red arrow) and one weak hybridizing signal (white arrow). Bar = 3 μm

3*n*GT had a 180 bp fragment with a 68 bp NTS and a 188 bp fragment with a 76 bp NTS (Wu et al. 2019). Besides, two significant SNPs were found in this sequencing result, which had great significance for distinguishing offspring from their parents.

Fluorescence in situ hybridization probes with Dig-11-dUTP (Roche, Germany) were constructed for the 188 bp *5S rDNA* unit of TC (TC-188), and the results were presented in Fig. 11.23. There were two strong signals and two weak signals in TC (Fig. 11.23b), one strong signal and one weak signal in 3*n*GT (Fig. 11.23d), and no signals in GC (Fig. 11.23a) or 2*n*GGC (Fig. 11.23c). This result suggested the genetic origin of 2*n*GGC and 3*n*GT at the chromosome level (Wu et al. 2019).

References

Chen L, Li W, Liu S, Tao M, Long Y, Duan W, Zhang C, Xiao J, Qin Q, Luo K, Liu J, Liu Y (2009) Novel genetic markers derived from the DNA fragments of *Sox* genes. Mol Cell Probes 23(3): 157–165

Chilton EW, Muoneke MI (1992) Biology and management of grass carp (*Ctenopharyngodon idella*, Cyprinidae) for vegetation control: a North American perspective. Rev Fish Biol Fish 2(4):283–320

He W (2012) Studies on formation of the different ploidy-level hybrids of Red Crucian Carp (*Carassius auratus* red var.) (♀) × Topmouth Culter (*Erythroculter ilishaeformis* Bleeker) (♂) and their biological characteristics. Hunan Normal University, Changsha

He W, Qin Q, Liu S, Li T, Wang J, Xiao J, Xie L, Zhang C, Liu Y (2012) Organization and variation analysis of *5S rDNA* in different ploidy-level hybrids of red crucian carp × topmouth culter. PLoS ONE 7(6):e38976

He W, Xie L, Li T, Liu S, Xiao J, Hu J, Wang J, Qin Q, Liu Y (2013) The formation of diploid and triploid hybrids of female grass carp × male blunt snout bream and their *5S rDNA* analysis. BMC Genet 14(1):110

Hu J (2013) Studies on genetic characters and gonad transcriptome of diploid and triploid hybrids derived from *Megalobrama amblycephala* Yih × *Xenocypris davidi* Bleeker. Hunan Normal University, Changsha

Hu J, Liu S, Xiao J, Zhou Y, You C, He W, Zhao R, Song C, Liu Y (2012) Characteristics of diploid and triploid hybrids derived from female *Megalobrama amblycephala* Yih × male *Xenocypris davidi* Bleeker. Aquaculture 364-365:157–164

Li L, Liang XF, He S, Sun J, Wen ZY, He YH, Cai WJ, Wang YP, Tao YX (2015) Transcriptome analysis of grass carp (*Ctenopharyngodon idella*) fed with animal and plant diets. Gene 574(2): 371–379

Qin Q, He W, Liu S, Wang J, Xiao J, Liu Y (2010) Analysis of *5S rDNA* organization and variation in polyploid hybrids from crosses of different fish subfamilies. J Exp Zool Part B 314(5): 403–411

Wang Y, Lu Y, Zhang Y, Ning Z, Li Y, Zhao Q, Lu H, Huang R, Xia X, Feng Q, Liang X, Liu K, Zhang L, Lu T, Huang T, Fan D, Weng Q, Zhu C, Lu Y, Li W, Wen Z, Zhou C, Tian Q, Kang X, Shi M, Zhang W, Jang S, Du F, He S, Liao L, Li Y, Gui B, He H, Ning Z, Yang C, He L, Luo L, Yang R, Luo Q, Liu X, Li S, Huang W, Xiao L, Lin H, Han B, Zhu Z (2015) The draft genome of the grass carp (*Ctenopharyngodon idellus*) provides insights into its evolution and vegetarian adaptation. Nat Genet 47(6):625–631

Wu C, Huang X, Hu F, Ouyang Y, Zhao L, Wang S, Li W, Fan J, Zhang C, Ren L, Qin Q, Luo K, Tao M, Liu S (2019) Production of diploid gynogenetic grass carp and triploid hybrids derived from the distant hybridization of female grass carp and male topmouth culter. Aquaculture 504: 462–470

Xie X (2013) The formation of the hybrids line between koi carp and colour crucian carp and its biological characteristics. Hunan Normal University, Changsha

The Summary of Fish Distant Hybridization 12

Shaojun Liu, Shi Wang, Qingfeng Liu, Yi Zhou, Chun Zhang, Min Tao, and Kaikun Luo

Abstract

If distant hybridization breaks reproductive barriers between species, it is possible to produce new variants or lineages that integrate characteristics from different species and exhibit the new characteristics their parents do not have. It is very significant to form the new lineages with genotypic and phenotypic changes derived from the distant hybridization, which are fertile in both males and females. However, fertile lineages derived from distant hybridization must overcome the key constraint of reproductive barriers. In this book (reference: Chaps. 1–11), we demonstrated that distant hybridization is an important pathway for new variants or lineages by overcoming reproductive barriers and summarized effective measures to overcome reproductive barriers to create fertile lineages through fish distant hybridization. In this chapter, we summarize the different combinations of fish distant hybridization and describe the main rules regarding inheritance and reproduction associated with fish distant hybridization. Furthermore, we introduce the one-step breeding technology and multistep breeding technology that are suitable for interspecific hybridization and intraspecific hybridization. In addition, we describe the utilization of fertile lineages derived from fish distant hybridization. On the other hand, we discuss the relationship between the rules derived from the fish distant hybridization and Mendel's laws which are generally applied to close hybridization. The purpose of this book is to provide a comprehensive reference for the establishment of fertile hybrid fish lineages by overcoming reproductive barriers and to clarify the important role of fish distant hybridization in the fields of genetic breeding, reproductive biology, and evolutionary biology.

S. Liu (✉) · S. Wang · Q. Liu · Y. Zhou · C. Zhang · M. Tao · K. Luo
State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, Hunan, China
e-mail: lsj@hunnu.edu.cn

S. Liu (ed.), *Fish Distant Hybridization*,
https://doi.org/10.1007/978-981-16-5067-3_12

Keywords

Distant hybridization · Lineage · Genetic variation · Reproductive barriers · Breeding technology

12.1 The Summary of Fish Distant Hybridization Groups

Through long-term and systematic studies on distant hybridization of fish, we have conducted 37 distant crosses and obtained surviving offspring in the 30 hybrid combinations with the freshwater fish parents with 100, 50, and 48 chromosomes (Table 12.1). About 132 populations and lineages were obtained by self-crossing, backcrossing, hybridization, and gynogenesis (or androgenesis) from these combinations.

12.1.1 The Establishment of Tetraploid Fish Lineages

We designed a group of distant hybridization experiments in which there were 100 maternal chromosomes ($2n = 100$) and 48 paternal chromosomes ($2n = 48$) and developed autotetraploid fish lineages through the distant hybridization combinations such as red crucian carp ($2n = 100$, ♀) × blunt snout bream ($2n = 48$, ♂) and common carp ($2n = 100$, ♀) × blunt snout bream ($2n = 48$, ♂) (Table 12.1) (Qin et al. 2014; Wang et al. 2020a). The autotetraploid fish lineage ($4n = 200$, 4*n*AU) derived from red crucian carp ($2n = 100$, ♀) × blunt snout bream ($2n = 48$, ♂) propagated to F_{15}. In addition, a new autotetraploid fish lineage ($4n = 200$) derived from common carp ($2n = 100$, ♀) × blunt snout bream ($2n = 48$, ♂) propagated to F_5 (Wang et al. 2020a). In F_1 of these hybrid lineages, there were fertile allotetraploids ($4n = 148$) (Wang et al. 2020a; Liu et al. 2007), which produced homologous diploid or homologous triploid gametes. These special gametes led to the formation of autotetraploid fish (Qin et al. 2014; Wang et al. 2020a).

In addition, we conducted intergeneric hybridization studies on female red crucian carp ($2n = 100$) × male common carp ($2n = 100$). Some fertile both females and males diploid individuals ($2n = 100$) had been found in F_1 of this cross, and they produced F_2 hybrids ($2n = 100$) by self-mating. The unreduced diploid eggs and sperm could be produced in the female and male individuals of F_2 hybrids, respectively. After fertilization, they formed fertile tetraploid individuals ($4n = 200$) in F_3, which subsequently formed an allotetraploid fish lineage (4*n*AT, F_3–F_{29}) ($4n = 200$) by continuously self-mating (Liu et al. 2001; Liu et al. 2016; Wang et al. 2015). On the other hand, we obtained the allodiploid hybrids (F_2) derived from the distant hybridization of female koi carp ($2n = 100$) and male colored crucian carp ($2n = 100$), which could produce unreduced sperm; and another new type of allotetraploid fish ($4n = 200$) was obtained by the hybridization between the female 4*n*AT and the male allodiploid hybrids (F_2) (Wang et al. 2020b). Furthermore, a new type of

Table 12.1 The distant hybridization experiments of freshwater fish (37 combinations, 30 combinations form surviving offspring)

Phylogenetic relationship		Combination of the number of parental chromosomes	Hybridized combination	Ploidies of F_1	Tetraploid and diploid fish lineages	Number
Different numbers of parental chromosomes (100, 50, and 48)	Subfamily	100 × 48	Common carp (♀) × blunt snout bream (♂) No survival offspring after blunt snout bream (♀) × common carp (♂)	$4n = 148$; $2n = 100$	Autotetraploid fish lineage (F_2–F_5, $4n = 200$) Autodiploid fish lineage (F_1–F_7, $2n = 100$)	1–2
			Red crucian carp (♀) × blunt snout bream (♂) No survival offspring after blunt snout bream (♀) × red crucian carp (♂)	$4n = 148$; $3n = 124$; $2n = 100$	Autotetraploid fish lineage (F_2–F_{15}, $4n = 200$)	3–4
			Japanese white crucian carp (♀) × blunt snout bream (♂) No survival offspring after blunt snout bream (♀) × Japanese white crucian carp (♂)	$4n = 148$; $3n = 124$	Autodiploid fish lineage (F_2–F_5, $2n = 100$)	5–6
			Red crucian carp (♀) × topmouth culter (♂) No survival offspring after topmouth culter (♀) × red crucian carp (♂)	$4n = 148$; $3n = 124$; $2n = 100$		7–8
			Red crucian carp (♀) × Bleeker's yellow tail (♂) No survival offspring after Bleeker's yellow tail (♀) × red crucian carp (♂)	$4n = 148$; $3n = 124$; $2n = 100$		9–10

(continued)

Table 12.1 (continued)

Phylogenetic relationship		Combination of the number of parental chromosomes	Hybridized combination	Ploidies of F_1	Tetraploid and diploid fish lineages	Number
			Goldfish (♀) × Bleeker's yellow tail (♂) No survival offspring after Bleeker's yellow tail (♀) × goldfish (♂)	$3n = 124$		11–12
			Koi carp (♀) × blunt snout bream (♂) No survival offspring after blunt snout bream (♀) × koi carp (♂)	$2n = 100$	Autodiploid fish lineage (F_1–F_4, $2n = 100$)	13–14
			Koi carp (♀) × bighead carp (♂)	$4n = 148$; $3n = 124$; $2n = 100$		15
		100 × 50	Red crucian carp (♀) × Chinese rare minnow (♂)	$4n = 150$; $3n = 125$		16
The same number of parental chromosomes (100 or 48)	Subfamily	48 × 48	Blunt snout bream (♀) × Bleeker's yellow tail (♂) and Bleeker's yellow tail (♀) × Blunt snout bream (♂)	$2n = 48$; $3n = 72$	Allodiploid fish lineage (blunt snout bream (♀) × Bleeker's yellow tail (♂), F_1–F_2, $2n = 48$)	17–18
			Grass carp (♀) × blunt snout bream (♂) and blunt snout bream (♀) × grass carp (♂)	$2n = 48$; $3n = 72$		19–20
			Bleeker's yellow tail (♀) × topmouth culter (♂) and topmouth culter (♀) × Bleeker's yellow tail (♂)	$2n = 48$; $3n = 72$		21–22

			Blunt snout bream (♀) × *Elopichthys bambusa* (♂) and *Elopichthys bambusa* (♀) × blunt snout bream (♂)	$2n = 48$; $3n = 72$		23–24
			Grass carp (♀) × topmouth culter (♂)	$2n = 48$; $3n = 72$		25
	Genus	48 × 48	Blunt snout bream (♀) × topmouth culter (♂) and topmouth culter (♀) × blunt snout bream (♂)	$2n = 48$; $3n = 72$	Two allodiploid fish lineages (blunt snout bream (♀) × topmouth culter (♂), F_1–F_6, $2n$=48; topmouth culter (♀) × blunt snout bream (♂), F_1–F_3, $2n = 48$)	26–27
			Blunt snout bream (♀) × *Culter mongolicus* (♂) and *Culter mongolicus* (♀) × blunt snout bream (♂)	$2n = 48$; $3n = 72$	Allodiploid fish lineage (blunt snout bream (♀) × *Culter mongolicus* (♂), F_1–F_2, $2n = 48$)	28–29
			Silver carp (♀) × bighead carp (♂) and bighead carp (♀) × silver carp (♂)	$2n = 48$		30–31
			Grass carp (♀) × *Elopichthys bambusa* (♂) and *Elopichthys bambusa* (♀) × grass carp (♂)	$2n = 48$; $3n = 72$		32–33
		100 × 100	Koi carp (♀) × red crucian carp (♂) and red crucian carp (♀) × koi carp (♂)	$2n = 100$	Allodiploid fish lineage (koi carp (♀) × red crucian carp (♂), F_1–F_2, $2n = 100$)	34–35
	Species (Interspecies)	100 × 100	Japanese white crucian carp (♀) × red crucian carp (♂) and red crucian carp (♀) × Japanese white crucian carp (♂)	$2n = 100$	Allodiploid fish lineage (Japanese white crucian carp (♀) × red crucian carp (♂), F_1–F_5, $2n = 100$)	36–37

Modified from Wang et al. (2019)

allotetraploid fish ($4n = 200$) was obtained through the cross between the female $4n$AU and the male $4n$AT (Hu et al. 2020).

12.1.2 The Establishment of Diploid Fish Lineages

We designed a group of distant hybridization experiments with the same number of chromosomes ($2n = 100$ or $2n = 48$) of both parents (Table 12.1), in which six fertile allodiploid fish lineages were developed. For example, two allodiploid fish lineages are derived from blunt snout bream (♀) × topmouth culter (♂) and topmouth culter (♀) × blunt snout bream (♂) (Ren et al. 2019; Xiao et al. 2014), and the other four types of allodiploid fish lineages are derived from blunt snout bream (♀) × Bleeker's yellow tail (♂) (Hu et al. 2012), blunt snout bream (♀) × *Culter mongolicus* (♂), koi carp (♀) × red crucian carp (♂) (Wang et al. 2020b), and Japanese white crucian carp (♀) × red crucian carp (♂) (Liu et al. 2019). These six allodiploid fish lineages integrated the genomes of both parents and showed hybrid characteristics of being an intermediate type between parents in genotypes and phenotypes.

In addition, we have established some autodiploid fish lineages by distant hybridization experiments in which the parents had the different number of chromosomes ($2n = 100$ and $2n = 48$). For example, an improved diploid white crucian carp lineage with gray-white body color was developed from Japanese white crucian carp (♀) × blunt snout bream (♂); a crucian carp-like homodiploid fish lineage with gray body color was developed from common carp (♀) × blunt snout bream (♂). The hybrid genome of the above two autodiploid fish lineages was mainly derived from the maternal parent genome, but some DNA fragments from the paternal parent (blunt snout bream) were also inserted. These two autodiploid fish lineages reflected the genetic characteristics derived from the paternal parent (blunt snout bream), such as a high back shape and good meat quality (Liu et al. 2017). In addition, we have successfully established the third autodiploid fish lineage of color crucian carp (or red crucian carp)-like fish lineage derived from koi carp (♀) × blunt snout bream (♂). The color crucian carp (or red crucian carp)-like fish lineage presented the traits of fast growth rate and a high back. Furthermore, a new type of goldfish with a twin tail and red and white body color was derived from the self-mating offspring of the color crucian carp-like homodiploid fish, which created a new type of goldfish germplasm resource with the characteristics of a high back. The hybrid genome of the color crucian carp (or red crucian carp)-like fish and the new type of goldfish was, respectively, derived from the maternal parent (koi carp) genome, but some DNA fragments from the paternal parent (blunt snout bream) were also inserted. The establishment of the lineages, including the color crucian carp (or red crucian carp)-like fish and the new type of goldfish, provided direct evidence for the evolutionary pathway of koi carp-color crucian carp-goldfish initiated by distant hybridization, which was of great significance in genetic breeding and evolutionary biology (Wang et al. 2018).

12.2 The Summary of the Main Genetic Rules and Reproductive Rules in Fish Distant Hybridization

12.2.1 The Genetic Rules in Fish Distant Hybridization

The number and karyotype of the parent's chromosomes have an important influence on the success of distant hybridization. Chromosomes are the carrier of genetic material, which can directly represent the cytogenetic characteristics of organisms, and are an important basis for studying the genetic rules between hybrid offspring and their parents. Through long-term and systematic research, we had carried out 37 distant hybridization experiments with 100, 50, and 48 chromosomes and obtained 30 hybrid combinations with surviving offspring (Table 12.1). Furthermore, we obtained about 132 populations and lineages by self-crossing, backcrossing, hybridization, and gynogenesis (androgenesis) from these combinations. These populations or lineages laid a solid foundation for exploring the genetic and reproduction rules of fish distant hybridization and for the establishment of the technologies of fish distant hybridization. According to results from our extensive researches on fish distant hybridization, we summarize the following genetic rules at the chromosome level.

When the number of maternal chromosomes is equal (or almost equal) to the number of paternal chromosomes, the allodiploid lineages and the allotetraploid lineages can be established by overcoming reproductive barriers (Fig. 12.1). For instance, the cross between red crucian carp ($2n = 100$, ♀) and common carp ($2n = 100$, ♂) resulted in an allotetraploid lineage (F_3–F_{29}), because the F_2 hybrids produced unreduced gametes (Liu et al. 2001). In addition, the cross between blunt snout bream ($2n = 48$, ♀) and topmouth culter ($2n = 48$, ♂) resulted in an allodiploid lineage (F_1–F_6) (Xiao et al. 2014).

When the number of maternal chromosomes is significantly greater than the number of paternal chromosomes, the autodiploid lineages and the autotetraploid lineages can be established by overcoming reproductive barriers (Fig. 12.2). For example, the cross between red crucian carp ($2n = 100$, ♀) and blunt snout bream ($2n = 48$, ♂) resulted in an autotetraploid lineage and an autodiploid lineage (Qin et al. 2010). The cross between common carp (♀, $2n = 100$) and blunt snout bream (♂, $2n = 48$) resulted in a crucian carp-like homodiploid lineage (F_1–F_7) (Wang et al. 2017).

When the number of maternal chromosomes is significantly less than that of the paternal parent, the hybrid offspring is unlikely to survive (Qin et al. 2014; Liu et al. 2016; Wang et al. 2019; Liu et al. 2020). However, when the numbers of chromosomes of the parents are in a ploidy relationship, the offspring can survive even if the number of maternal chromosomes is significantly less than that of the paternal parent (Guo et al. 2006; Chen et al. 2009). In this case, the coordination of the parents is good, for example, the hybridization of Japanese white crucian carp ($2n = 100$, ♀) and allotetraploid hybrids ($4n = 200$, ♂) to obtain the triploid fish (Guo et al. 2006), the crossing of red crucian carp ($2n = 100$, ♀) and autotetraploid hybrids ($4n = 200$, ♂) to obtain the autotriploid fish, and the hybridization of

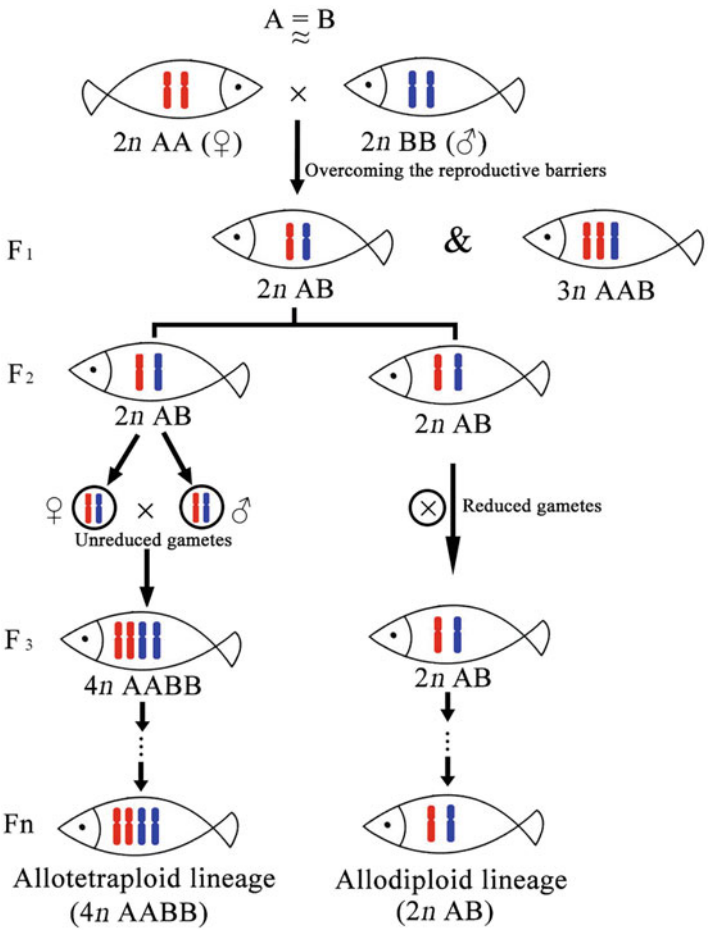

Fig. 12.1 The fertile lineages are produced when the number of chromosomes in maternal parent is equal to that in paternal parent (Liu et al. 2020)

common carp ($2n = 100$, ♀) and autotetraploid hybrids ($4n = 200$, ♂) to obtain the allotriploid fish (Hu et al. 2019).

The genetic rules summarized above showed the effect of maternal chromosome number and paternal chromosome number on distant hybridization. The chromosome number matching degree of hybrid parents will affect the nuclear-nuclear and nuclear-cytoplasmic compatibility of F_1 offspring (Table 12.2). The nuclear-nuclear and nuclear-cytoplasmic compatibility correlated with the survival rate of F_1 offspring. In F_1 offspring, the nuclear-nuclear and nuclear-cytoplasmic compatibilities include the compatibility between the maternal nuclear material (including the genome) and the paternal nuclear material (including the genome), between the maternal nuclear material (including the genome) and the cytoplasm, and between the paternal nuclear material (including the genome) and the cytoplasm.

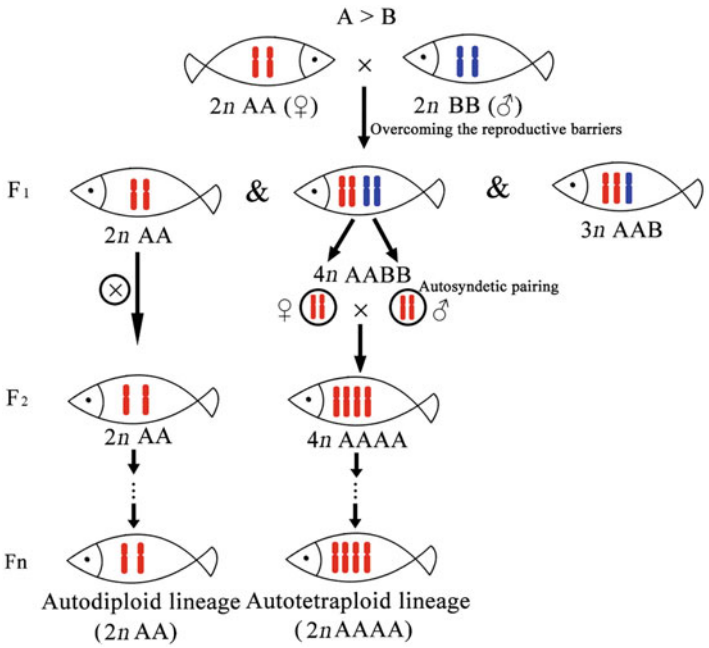

Fig. 12.2 The fertile lineages are produced when the number of chromosomes in maternal parent is larger than that in paternal parent (Liu et al. 2020)

Table 12.2 The relationship among maternal and paternal chromosomes and the compatibility degree of nuclear-nuclear and nuclear-cytoplasmic in distant hybrid F_1

Number of parental chromosomes	Compatibility			Overall compatibility	Survival rate
	Maternal nucleus-cytoplasm	Maternal nucleus-paternal nucleus	Paternal nucleus-cytoplasm		
♀ > ♂	√√	√	√	Normal	Normal
♀ = ♂	√√	√√	√√	Good	Good
♀ < ♂	√	×	×	Poor	Low

× poor compatibility, √ normal compatibility, √√ good compatibility

When the number of maternal chromosomes is equal (or almost equal) to that of paternal parent, the compatibility among maternal genome-paternal genome, paternal genome-cytoplasm, and maternal genome-cytoplasm is better, and the survival rate of F_1 offspring is good.

When the number of maternal chromosomes is significantly larger than the number of paternal chromosomes, the maternal genetic material is dominant in F_1 hybrids, and the hybrids have a certain developmental ability. In this situation, the compatibility among maternal genome-paternal genome, paternal genome-cytoplasm, and maternal genome-cytoplasm is not better than that in the first case

but better than the third case; in this case, the F_1 hybrids possess a considerable proportion of survival rate.

When the number of maternal chromosomes is significantly fewer than the number of paternal chromosomes, the maternal genetic material is not dominant in F_1 offspring, and the physiological development capability of the offspring is poor. In this situation, the compatibility among maternal genome-paternal genome, paternal genome-cytoplasm, and maternal genome-cytoplasm in F_1 offspring is bad, so the survival rate of F_1 offspring is very low. However, when the numbers of chromosomes of the maternal and paternal parents are ploidy, the compatibility among maternal genome-paternal genome, paternal genome-cytoplasm, and maternal genome-cytoplasm is better, and the survival rate of F_1 offspring is good.

Many transcriptome and genomic sequencing studies from existing distant hybrid fish lineages have been carried out. At the molecular level, we have found that the genetic characteristics of these hybrid fish lineages were the same, that is, these hybrid fish lineages not only inherited the genetic material from the maternal parent but also inherited the genetic material from the paternal parent with varying degrees.

For allotetraploid and allodiploid fish lineages derived from distant hybridization, the hybrid offspring have two subgenomes from its parents. For instance, many chimeric genes (>9%) were found in the orthologous genes of the transcriptomes of allotetraploid fish (Liu et al. 2016); similarly, many chimeric genes were found in bacterial artificial chromosome (BAC) libraries of allotetraploid fish (Ye et al. 2017). In addition, 19.04% of chimeric genes have been found among orthologous genes in the transcriptomes of allodiploid fish derived from Japanese white crucian carp (*Carassius cuvieri*, ♀) × red crucian carp (♂) (Liu et al. 2018). The allodiploid fish and allotetraploid fish derived from distant hybridization have two sets and four sets of genomes from their parents, respectively. At the DNA level, the genetic material from both parents is synthesized in the form of chimeric genes, which is conducive to the re-diploidization of hybrid fish lineages, and is the genetic basis for overcoming reproductive isolation. We also found the chimeric genes and 13 gene expression patterns in the allodiploid hybrid lineages derived from blunt snout bream × topmouth culter. Their gene expression patterns present symmetric expressions including the additive expression of parents and asymmetric expressions including the dominant expression of one parent (Ren et al. 2019).

The hybrid genome of autotetraploid and autodiploid fish lineages is mainly controlled by the maternal genome, and the DNA fragments of paternal are inserted into them. For example, we have distinguished the original paternal DNA fragments in autotetraploid fish and autodiploid red crucian carp derived from red crucian carp (♀) × blunt snout bream (♂) (Chen et al. 2018; Wang et al. 2017; Wang et al. 2018; Wang et al. 2020a). These lineages are also promoted re-diploidization by these inserted DNA fragments.

Distant hybridization combinations including parents with identical chromosome numbers performed by our laboratory are listed in Table 12.1. In the blunt snout bream (♀) × topmouth culter (♂) and topmouth culter (♀) × blunt snout bream (♂), in which the parents have 48 chromosomes and belong to different genera, blunt snout bream (♀) × topmouth culter (♂) has already been bred to the sixth

generation, and topmouth culter (♀) × blunt snout bream (♂) has already been bred to the third generation. A cross between blunt snout bream (♀, Cultrinae) × Bleeker's yellow tail (♂, Xenocyprininae), in which the parents have 48 chromosomes and they come from different subfamilies, has already been bred to the F_2 generation. Four-year-old F_1 offspring of grass carp (♀, Leuciscinae) × blunt snout bream (♂, Cultrinae) was produced from parents in different subfamilies with identical chromosome numbers ($2n = 48$). Whether these hybrids can establish a distant hybrid lineage remains to be studied. An intergeneric cross of red crucian carp (♀) × common carp (♂) (both $2n = 100$) has been generated for 29 generations. Some F_2 female and male individuals from red crucian carp (♀) × common carp (♂) offspring were able to produce diploid eggs and sperm; they generated male and female allotetraploid lineages in F_3, laying the foundation for the extension of the allotetraploid lineage. The similar cross of koi carp (♀) × red crucian carp (♂) has been developed to the F_2 generation.

As shown in Table 12.1, the successful hybridization events between fish species with different chromosome numbers performed by our laboratory include red crucian carp (♀) × blunt snout bream (♂), common carp (♀) × blunt snout bream (♂), red crucian carp (♀) × topmouth culter (♂), and so on. A F_1 allotetraploid ($4n = 148$) population was obtained from red crucian carp (♀) × blunt snout bream (♂); breeding of the F_2 and subsequent generations resulted in the establishment of an autotetraploid fish lineage (F_2–F_{15}, $4n = 200$) with changed characteristics. The distant hybridization with the parents with different chromosomes between red crucian carp (♀) and blunt snout bream (♂) yielded some female and male F_1 individuals that could produce diploid eggs ($2n = 100$) and diploid sperm ($2n = 100$); fertilization of these diploid gametes generated female and male autotetraploid fish ($4n = 200$) and provided a basic for the extension of the autotetraploid fish lineage. Another distant hybridization with the parents with different chromosomes between common carp (♀) and blunt snout bream (♂) also yielded a new type of autotetraploid fish lineage ($4n$=200), in which the fertilization of the autotriploid eggs obtained from female allotetraploid fish ($4n = 148$) and haploid sperm obtained from male crucian carp-like homodiploid fish ($2n = 100$) led to formation of the autotetraploid fish (Wang et al. 2020a).

The parents of distant hybridization with identical or different chromosome numbers can be used to establish hybrid lineages of different ploidy fish (Fig. 12.3). These results provide important evidence for the hypothesis that distant hybridization may play a crucial role in the formation of fish chromosomal diversity.

A systematic study of fish distant hybridization proved that distant hybridization could generate allotetraploid lineages, autotetraploid lineages, allodiploid lineages, homodiploid lineages, and natural gynogenetic diploid lineages. These results provide direct evidence supporting the hypothesis that these five different pipelines make it possible to produce new lineages even species by artificial hybridization, and show some important indirect evidence to prove that new species can be produced in these five ways under natural circumstances. On the other hand, we find that the fish distant hybridization can also result in the natural androgenetic diploid fish, which is also a very special pathway to form new type of fish (Hu et al. 2018).

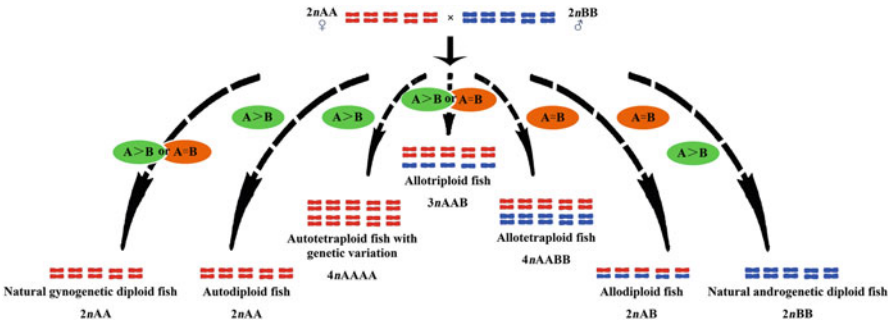

Fig. 12.3 Schematic diagram illustrating the different offspring derived from different distant hybridization crosses. Note: Blue and red show the chromosomes from different parents. By distant hybridization and subsequent selection, red crucian carp (AA, ♀) × common carp (BB, ♂) could generate the allotetraploid lineage (4*n*AABB), while red crucian carp (AA, ♀) × blunt snout bream (BB, ♂) could produce the autotetraploid lineage (4*n*AAAA), sterile allotriploid fish (3*n*AAB), or natural gynogenetic diploid fish (2*n*AA) lineage. Moreover, common carp (AA, ♀) × blunt snout bream (BB, ♂) could produce the autotetraploid lineage (4*n*AAAA) or the homodiploid lineage (2*n*AA). On the other hand, blunt snout bream (AA, ♀) × topmouth culter (BB, ♂) could produce the allodiploid lineage (2*n*AB)

12.2.2 The Reproductive Rules in Fish Distant Hybridization

According to research results from our extensive researches on fish distant hybridization, we summarize the reproduction rules at the gamete level.

Both the females and males of the diploid and tetraploid lineages produced by distant hybridization are fertile. Through a lot of research, we found that unreduced gametes produced by allodiploid fish ($2n = 100$) are a significant reason for the establishment of allotetraploid fish lineages ($4n = 200$); for example, unreduced gametes were produced from the F_2 hybrids derived from red crucian carp (♀) × common carp (♂), allotetraploid hybrids were produced in F_3 by self-crossing of F_2 hybrids, and an allotetraploid lineage (F_3–F_{29}) was established by self-crossing (Liu et al. 2001). The production of unreduced gametes might be related to the nuclear replication mechanism in germ cells (Liu et al. 2001; Liu et al. 2016). The key to the formation of autotetraploid is that allotetraploid could produce diploid gametes and triploid gametes; for instance, allotetraploid offspring ($4n = 148$) were produced in the F_1 hybrids of red crucian carp (♀) × blunt snout bream (♂), autodiploid gametes were obtained by the allotetraploid fish ($4n = 148$), and the autotetraploid ($4n = 200$) fish lineage was established (Qin et al. 2014). Other allotetraploid hybrids ($4n$=148) were produced in the F_1 hybrids of common carp (♀) × blunt snout bream (♂), the triploid eggs were produced by the female allotetraploid hybrids ($4n = 148$), and the autotetraploid fish lineage was formed by the hybridization of the female allotetraploid fish ($4n = 148$) with the male crucian carp-like homodiploid fish ($2n = 100$) (Wang et al. 2020a). On the other hand, haploid gametes obtained from diploid

hybrid fish are the important reason for the formation of the diploid fish lineages. The production of meiotic gametes may be related to the normal meiotic mechanism of germ cells (Liu et al. 2001; Liu et al. 2007).

12.2.3 The Distant Hybridization and Mendel's Laws

At meetings of the Brunn Natural History Society, the geneticist Gregor Mendel introduced his research findings on "Experiments in plant hybridization". Mendel summarized the law of free combination and the law of separation through the analyses on the seven pairs of traits in the hybridization experiments with peas. Unfortunately, the phenomenon described by Mendel's laws rarely occurs in distant hybridization. To better understand the similarities and differences between close hybridization and distant hybridization, we discuss the contact between Mendel's laws in close hybridization and the related rules in distant hybridization.

12.2.3.1 Differences

1. The experimental basis of Mendel's laws comes from the pea close hybridization, which produces hybrid offspring with genetic variation. In Mendel's experiments, the hybrid parents have a close genetic relationship and the chromosome numbers of the parents are the same ($2n = 14$). The chromosome number of F_1 hybrids is the same as that of parents ($2n = 14$). However, in the distant hybridization, the relationship between parents is far. The number of chromosomes of both parents is the same or different. The hybrid offspring have different ploidies; for example, in the hybrid combination with red crucian carp ($2n = 100$) as the maternal parent and blunt snout bream ($2n = 48$) as the paternal parent, the offspring of tetraploid hybrid fish ($4n = 148$), triploid hybrid fish ($3n = 124$), and gynogenetic diploid red crucian carp ($2n = 100$) were produced (Qin et al. 2014a); and tetraploid hybrid offspring ($4n = 148$), crucian carp-like homodiploid fish ($2n = 100$), improved diploid common carp ($2n = 100$), and improved diploid scattered mirror carp ($2n = 100$) are derived from the distant hybridization of common carp (♀, $2n = 100$) and blunt snout bream (♂, $2n = 48$) (Wang et al. 2017; Wang et al. 2020a).
2. The results of ploidy and separation for offspring of reciprocal cross in the Mendel's pea hybridization experiments are consistent, which indicated that the nuclear-cytoplasmic compatibility of both parents is better, and the genetic contribution rate of each parent is equal. But the results of the progenies of reciprocal cross produced through distant hybridization are different, which reflected that the parental nuclear-cytoplasmic compatibility is inconsistent, and maternal dominances are significant in distant hybridization.
3. In Mendel's experiments of pea hybridization, the hybrid progenies have good fertility and produce haploid gametes. But the hybrid offspring derived from distant hybridization can be fertile or infertile; in addition to haploid gametes,

diploid and triploid gametes can also be produced by the progenies derived from distant hybridization. For instance, the F_1 tetraploid hybrids ($4n = 148$) derived from fish distant hybridization can produce diploid and triploid gametes (Qin et al. 2014; Wang et al. 2020a).

4. In Mendel's experiments, the fertile hybrid lineage has the genome derived from the same species because their parents belong to the same species. In the fish distant hybridization, because their parents belong to different species, there existed the subgenomes in the allodiploid lineage or allotetraploid lineage; there also existed the hybrid genome mainly derived the maternal parent and inserted with the DNA fragments derived from the paternal parent in the autodiploid lineage and autotetraploid lineage.
5. In Mendel's experiments of pea hybridization, F_1 offspring can produce enough male and female gametes with the same type. The gametes can be fertilized according to random combination after self-crossing, and the F_2 offspring is diploid, whose ploidy is consistent with that of the parents, and the separation phenotype ratio is 3:1. However, due to the difference in fertility of the offspring, the sperm and egg numbers of F_1 hybrids produced by fish distant hybridization are not enough, and the ploidy of gametes may be inconsistent, so it is difficult to produce gametes with the same type, and it is difficult to obtain the result of the F_2 generation with a 3:1 phenotype ratio, and the ploidy of the F_2 hybrids formed may also be diversified.

12.2.3.2 Consistencies

In the early generations (at least approximately 50 generations) of progeny derived from distant hybridization, the hybrid genome mainly derived from maternal genome with inserted parental DNA fragments or two subgenomes derived from the parents will change violently and overcome the shock effect. With the increase of generations, the hybrid genome or the two subgenomes will tend to be balance and re-diploid, eventually evolving into almost the stable genome, but the two subgenomic traces will always be retained. Therefore, distant hybridization's special characteristics only exist in its early generations, and it is suitable for Mendel's laws when the genetic structure of hybrid offspring becomes stable. Similarly, in terms of the formation of gametes, the unreduced gametes produced at the early generations of distant crossing result in the formation of different ploidy fishes. After the tetraploid and diploid lineages stabilize, those lineages will be re-diploidized and they will stably produce the reduced gametes, which will be consistent with Mendel's laws both in genetics and reproduction.

12.3 The Summary of Main Technologies in Fish Distant Hybridization and Their Applications

12.3.1 The Establishment of One-Step Breeding Technology and Multistep Breeding Technology

We established one-step and multistep breeding technology based on long-term and systematic research on fish distant hybridization (Fig. 12.4). Batches of improved triploid fish and diploid fish were developed by these two technologies. Practice has proved that one-step and multistep breeding technology have a general guiding role in hybrid breeding of fish.

12.3.2 One-Step Breeding Technology

The one-step breeding technology is defined as: to generate F_1 hybrids with heterosis under the conditions that both parents of the hybridization have the same (or almost same) chromosome numbers. The F_1 offspring of this type of hybrid combination is consistent in shape and growth rate. Several F_1 hybrids with obvious heterosis were selected and bred through systematic research of phenotypic characteristics, chromosome numbers, karyotypes, and other biological characteristics of many F_1 hybrids and their parents. For example, F_1 hybrids derived from blunt snout bream (♀) × Bleeker's yellow tail (♂) presented obvious heterosis, such as small head, consistent body shape, high survival rate, and high meat rate. The growth rate of F_1 hybrids derived from blunt snout bream (♀) × Bleeker's yellow tail (♂) was 20–40% faster than that of its parents (Hu et al. 2012). Diploid and triploid hybrids were found in F_1 hybrids of grass carp (♀) × blunt snout bream (♂), and they showed many advantages such as strong stress resistance and fast growth rate.

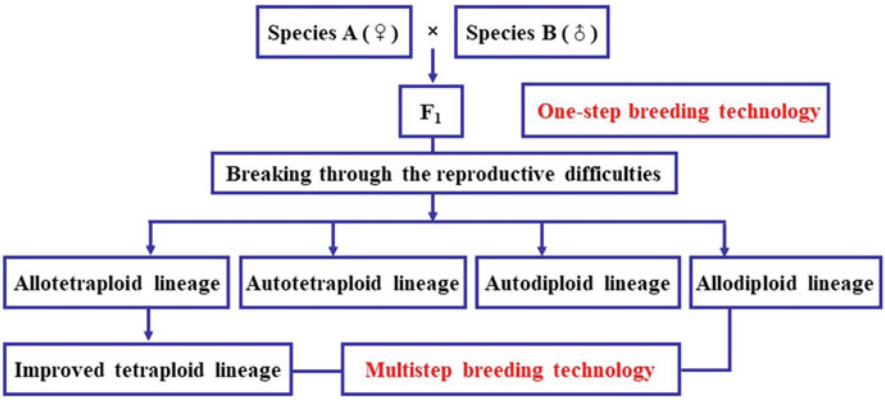

Fig. 12.4 Route map of one-step and multistep breeding technology (Wang et al. 2019)

Among them, the triploid hybrids derived from grass carp (♀) × blunt snout bream (♂) had a 30–40% faster growth rate than common grass carp (He et al. 2013).

Japanese white crucian carp and red crucian carp with the same chromosome number are different species or different subspecies, depending on different classification. Through a comparative study of reciprocal cross, the F_1 offspring derived from Japanese white crucian carp (♀) × red crucian carp (♂) showed obvious heterosis, such as strong resistance, high survival rate, fast growth rate (more than 30% faster than that of their parents), meanwhile their shape and color, were very similar to those of the natural wild crucian carp. Those hybrids could keep their scales and retain a good appearance during transportation. In addition, the contents of protein and flavorful amino acids in the meat of those hybrids were higher than those of the parents (Liu et al. 2017; Wang et al. 2015).

12.3.3 Multistep Breeding Technology

The multistep breeding technology is defined as: firstly, to establish fertile hybrid lineages derived from distant hybridization, including tetraploid and diploid lineages, and then, using the established lineages to develop improved triploid and diploid fish. Namely, by breaking through the reproductive barrier of F_1 hybrids, new types of fertile lineages such as diploid fish lineages and tetraploid fish lineages can be formed, which are used as the new fish germplasm resources to further produce the improved fish.

Application of tetraploid fish lineages: an allotriploid hybrid (with two sets of red crucian carp chromosomes and one set of common carp chromosome), which showed obvious heterosis, such as infertility, good meat quality, strong resistance, and rapid growth, was produced by mating female diploid common carp with male autotetraploid hybrid fish lineage derived from red crucian carp (♀) × blunt snout bream (♂). The meat flavor of the allotriploid hybrids was similar to that of crucian carp. The infertility of allotriploid hybrids has protected the intellectual property rights of seedling production and noninterference in natural fish resources.

Application of diploid fish lineages: an improved diploid up-mouth bream hybrid, which had obvious heterosis, such as beautiful appearance, less intermuscular spines, tender meat, and herbivore feeding, was produced by mating female allodiploid hybrid fish lineage derived from blunt snout bream (♀) × topmouth culter (♂) with male diploid blunt snout bream. The content of carbohydrate in the meat of the improved diploid up-mouth bream hybrid was lower than that of its parents, while the contents of protein, flavorful amino acids, and unsaturated fatty acids in the meat of this improved hybrid fish were higher than those of the parents (He et al. 2014). This kind of improved hybrid fish also had the advantages of higher low-oxygen tolerance, higher survival rate, faster growth rate (more than 20% faster than that of its parents), and stronger anti-disease resistance. An improved crucian carp hybrid with advantages such as higher back and faster growth was produced by crossing female allodiploid hybrid fish lineage derived from Japanese white crucian carp (♀) × red crucian carp (♂) with male diploid Japanese white crucian carp (Liu

et al. 2019). The diploid lineages and improved diploid hybrids mentioned above both showed a bisexual fertility, which can be used as the new fish germplasm resources for further breeding investigation.

12.3.4 The Applications of the Genetic and Reproductive Rules and One-Step and Multistep Breeding Technologies in Fish Distant Hybridization

Based on the genetic rules and reproductive rules of fish distant hybridization, the one-step and multistep breeding technology play a universal guiding role in fish genetic breeding. This genetic rule is aimed at the situation where there is the same or different number of parental chromosomes, which is universal because it covers all possible types of hybridization combination, including interspecific hybridization and intraspecific hybridization. In this study, the genetic compositions of all offspring of crosses can be predicted by the chromosome numbers of parents. The genetic rules involved in this book are very well organized and can effectively guide the implementation of pre-design by informing the hybrid offspring which are relatively easy to form, which are difficult to generate, and which are not feasible. It is easy to obtain many hybrid progenies if the chromosome numbers of both parents are equal (or almost equal), avoiding the death of offspring by design mistakes. In this case, hybrid F_1 has a high survival rate, a large number of individuals, and obvious heterosis advantages. It can be directly applied to production. This is the implementation of one-step breeding technology. Furthermore, if these offspring are both sexually fertile, different types of allodiploid and allotetraploid fish lineages can be established and then mated with different diploid fish to further produce excellent new varieties. This is the implementation of multistep breeding technology. When the number of maternal chromosomes is significantly larger than the number of paternal chromosomes, hybrid F_1 has a low survival rate and rare individuals, and heterosis is not obvious. The use of heterosis is not occurring in the first generation of hybridization, but the lineages established later will be more useful for further breeding. Generally speaking, it is necessary to break through the difficulties of reproductive isolation of hybrid progenies to create fertile hybrid fish lineages. Once the difficulty of reproductive isolation is broken, different types of autodiploid and autotetraploid fish lineages can be obtained, and these new fish lineages can be further used as important core parents to develop excellent fish, which is the implementation of multistep breeding technology.

Breeding means exploring individuals or populations with phenotypic and genotypic variations. Some of these individuals or populations with changed characteristics are directly used as improved varieties for production, and some are used as new germplasm resources to develop more improved varieties.

Through long-term research, the genetic rules and reproductive rules of fish distant hybridization have been explored, and one-step breeding technology and multistep breeding technology that are suitable for interspecific hybridization and intraspecific hybridization have been formed. We have produced a series of

improved fish by using these two breeding technologies, which proves that these two breeding technologies have good universality, scientific, and practical. The fact of successful hybrid breeding conducted in the past is consistent with these two breeding technologies, which fully shows that the two breeding technologies have good application effects and prospects.

References

Chen J, Luo M, Li S, Tao M, Ye X, Duan W, Zhang C, Qin Q, Xiao J, Liu S (2018) A comparative study of distant hybridization in plants and animals. Sci China Life Sci 61(3):285–309

Chen S, Wang J, Liu S, Qin Q, Xiao J, Duan W, Luo K, Liu J, Liu Y (2009) Biological characteristics of an improved triploid crucian carp. Sci China Ser C 52:733–738

Guo X, Liu S, Liu Y (2006) Evidence for recombination of mitochondrial DNA in triploid crucian carp. Genetics 172(3):1745–1749

He W, Xie L, Li T, Liu S, Xiao J, Hu J, Wang J, Qin Q, Liu Y (2013) The formation of diploid and triploid hybrids of female grass carp × male blunt snout bream and their 5S rDNA analysis. BMC Genet 14(1):110

He Z, Liu S, Xiao J, Hu F, Wen M, Ye L, Zhang C, Xu K, Tao M, Luo K, Liu Y (2014) Muscle nutrients of the backcross progeny of female diploid F_1 hybrid (blunt snout bream × topmouth culter) × male blunt snout bream and its parents. J Fish China 38(10):1797–1792

Hu F, Fan J, Qin Q, Huo Y, Wang Y, Wu C, Liu Q, Li W, Chen X, Liu C, Tao M, Wang S, Zhao R, Luo K, Liu S (2019) The sterility of allotriploid fish and fertility of female autotriploid fish. Front Genet 10(377):26

Hu F, Wu C, Zhou Y, Cao L, Xiao J, Wang S, Wu Y, Ren L, Liu Q, Li W, Wen M, Tao M, Qin Q, Zhao R, Luo K, Liu S (2018) Production of androgenetic, triploid and tetraploid hybrids from the interspecific hybridization of female Japanese crucian carp and male blunt snout bream. Aquaculture 491:50–58

Hu F, Zhong H, Fan J, Wu C, Wang S, Wang Y, Luo K, Zhao R, Liu J, Qin Q (2020) A new type of tetraploid fish derived via female autotetraploid × male allotetraploid hybridization. Aquaculture 524:735244

Hu J, Liu S, Xiao J, Zhou Y, You C, He W, Zhao R, Song C, Liu Y (2012) Characteristics of diploid and triploid hybrids derived from female *Megalobrama amblycephala* Yih × male *Xenocypris davidi* Bleeker. Aquaculture 364-365:157–164

Liu Q, Liu J, Liang Q, Qi Y, Tao M, Zhang C, Qin Q, Zhao R, Chen B, Liu S (2019) A hybrid lineage derived from hybridization of *Carassius cuvieri* and *Carassius auratus* red var. and a new type of improved fish obtained by back-crossing. Aquaculture 505:173–182

Liu Q, Liu J, Yuan L, Li L, Tao M, Zhang C, Qin Q, Chen B, Ma M, Tang C, Liu S (2020) The establishment of the fertile fish lineages derived from distant hybridization by overcoming the reproductive barriers. Reproduction 159(6):R237–R249

Liu Q, Qi Y, Liang Q, Xu X, Hu F, Wang J, Xiao J, Wang S, Li W, Tao M, Qin Q, Zhao R, Yao Z, Liu S (2018) The chimeric genes in the hybrid lineage of *Carassius auratus cuvieri* (♀) × *Carassius auratus* red var. (♂). Sci China Life Sci 61(9):1079–1089

Liu Q, Wang J, Xiao J, Chen X, Qi Y, Li W, Tao M, Zhang C, Qinbo Q, Luo K, Liu S (2017) Muscle nutrient of *Carassius auratus cuvieri* (♀) × *Carassius auratus* red var. (♂) and its parents. J Fish China 41(7):1133–1138

Liu S, Liu Y, Zhou G, Zhang X, Luo C, Feng H, He X, Zhu G, Yang H (2001) The formation of tetraploid stocks of red crucian carp × common carp hybrids as an effect of interspecific hybridization. Aquaculture 192(2):171–186

Liu S, Luo J, Chai J, Ren L, Zhou Y, Huang F, Liu X, Chen Y, Zhang C, Tao M, Lu B, Zhou W, Lin G, Mai C, Yuan S, Wang J, Li T, Qin Q, Feng H, Luo K, Xiao J, Zhong H, Zhao R, Duan W, Song Z, Wang Y, Wang J, Zhong L, Wang L, Ding Z, Du Z, Lu X, Gao Y, Murphy RW, Liu Y,

Meyer A, Zhang Y-P (2016) Genomic incompatibilities in the diploid and tetraploid offspring of the goldfish × common carp cross. Proc Natl Acad Sci 113(5):1327–1332

Liu S, Qin Q, Xiao J, Lu W, Shen J, Li W, Liu J, Duan W, Zhang C, Tao M, Zhao R, Yan J, Liu Y (2007) The formation of the polyploid hybrids from different subfamily fish crossings and its evolutionary significance. Genetics 176(2):1023–1034

Qin Q, He W, Liu S, Wang J, Xiao J, Liu Y (2010) Analysis of 5S rDNA organization and variation in polyploid hybrids from crosses of different fish subfamilies. J Exp Zool Part B 314(5): 403–411

Qin Q, Wang Y, Wang J, Dai J, Xiao J, Hu F, Luo K, Tao M, Zhang C, Liu Y (2014) The autotetraploid fish derived from hybridization of *Carassius auratus* red var. (female) × *Megalobrama amblycephala* (male). Biol Reprod 91(4):93, 1–11

Ren L, Li W, Qin Q, Dai H, Han F, Xiao J, Gao X, Cui J, Wu C, Yan X, Wang G, Liu G, Liu J, Li J, Wan Z, Yang C, Zhang C, Tao M, Wang J, Luo K, Wang S, Hu F, Zhao R, Li X, Liu M, Zheng H, Zhou R, Shu Y, Wang Y, Liu Q, Tang C, Duan W, Liu S (2019) The subgenomes show asymmetric expression of alleles in hybrid lineages of *Megalobrama amblycephala* × *Culter alburnus*. Genome Res 29(11):1805–1815

Wang J, Xiao J, Zeng M, Xu K, Tao M, Zhang C, Duan W, Liu W, Luo K, Liu Y (2015) Genomic variation in the hybrids of white crucian carp and red crucian carp: evidence from ribosomal DNA. Sci China Life Sci 58(6):590–601

Wang S, Tang C, Tao M, Qin Q, Zhang C, Luo K, Zhao R, Wang J, Ren L, Xiao J (2019) Establishment and application of distant hybridization technology in fish. Sci China Life Sci 62(1):22–45

Wang S, Ye X, Wang Y, Chen Y, Lin B, Yi Z, Mao Z, Hu F, Zhao R, Wang J, Zhou R, Ren L, Yao Z, Tao M, Zhang C, Xiao J, Qin Q, Liu S (2017) A new type of homodiploid fish derived from the interspecific hybridization of female common carp × male blunt snout bream. Sci Rep 7(1):4189

Wang S, Zhou P, Huang X, Liu Q, Lin B, Fu Y, Gu Q, Hu F, Luo K, Zhang C (2020a) The establishment of an autotetraploid fish lineage produced by female allotetraploid hybrids × male homodiploid hybrids derived from *Cyprinus carpio* (♀) × *Megalobrama amblycephala* (♂). Aquaculture 515:734583

Wang Y, Yang C, Luo K, Zhang M, Qin Q, Huo Y, Song J, Tao M, Zhang C, Liu S (2018) The formation of the goldfish-like fish derived from hybridization of female koi carp × male blunt snout bream. Front Genet 9:437

Wang Y, Zhang M, Tao S, Xie X, Tan H, Cao L, Wang J, Qin Q, Zhang C, Tao M (2020b) Unreduced diploid sperm from diploid hybrids and formation of a new type of tetraploid hybrid. Aquaculture 515:734584

Xiao J, Kang X, Xie L, Qin Q, He Z, Hu F, Zhang C, Zhao R, Wang J, Luo K, Liu Y, Liu S (2014) The fertility of the hybrid lineage derived from female *Megalobrama amblycephala* × male *Culter alburnus*. Anim Reprod Sci 151(1):61–70

Ye L, Jiao N, Tang X, Chen Y, Ye X, Ren L, Hu F, Wang S, Wen M, Zhang C (2017) Chimeras linked to tandem repeats and transposable elements in tetraploid hybrid fish. Mar Biotechnol 19(4):401–409