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Distant hybridization leads to different ploidy fishes

LIU ShaoJun

Key Laboratory of Protein Chemistry and Fish Developmental Biology of Education Ministry of China, College of Life Sciences, Hunan Normal University, Changsha 410081, China

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Distant hybridization makes it possible to transfer the genome of one species to another, which results in changes in phenotypes and genotypes of the progenies. This study shows that distant hybridization or the combination of this method with gynogenesis or androgenesis lead to different ploidy fishes with genetic variation, including fertile tetraploid hybrids, sterile triploid hybrids, fertile diploid hybrids, fertile diploid gynogenetic fish, and their derived progenies. The formations of the different ploidy fishes depend on the genetic relationship between the parents. In this study, several types of distant hybridization, including red crucian carp (*Carassius auratus* red var.) (2*n*=100, abbreviated as RCC) (♀)×common carp (*Cyprinus carpio* L.) (2*n*=100, abbreviated as CC) (♂), and RCC (2*n*=100) (♀)×blunt snout bream (*Megalobrama amblycephala*) (2*n*=48, abbreviated as BSB) (♂) are described. In the distant hybridization of RCC (♀)×CC (♂), bisexual fertile F_3–F_{18} allotetraploid hybrids (4*n*=200, abbreviated as 4*n*AT) were formed. The diploid hybrid eggs and diploid sperm generated by the females and males of 4*n*AT developed into diploid gynogenetic hybrids and diploid androgenetic hybrids, respectively, by gynogenesis and androgenesis, without treatment for doubling the chromosome. Improved tetraploid hybrids and improved diploid fishes with genetic variation were derived from the gynogenetic hybrid line. The improved diploid fishes included the high-body RCC and high-body goldfish. The formation of the tetraploid hybrids was related to the occurrence of unreduced gametes generated from the diploid hybrids, which involved in premeiotic endoreduplication, endomitosis, or fusion of germ cells. The sterile triploid hybrids (3*n*=150) were produced on a large scale by crossing the males of tetraploid hybrids with females of diploid fish (2*n*=100). In another distant hybridization of RCC (♀)×BSB (♂), different ploidy fishes were obtained, including diploid bisexual fertile natural gynogenetic fish (2*n*=100), sterile triploid hybrids (3*n*=124), and bisexual fertile tetraploid hybrids (4*n*=148). Furthermore, two kinds of pentaploid hybrids (5*n*=172 and 5*n*=198) were formed. The biological characteristics and the mechanisms of formation of the different ploidy fish were compared and discussed at the cellular and molecular level. The results indicated distant hybridization or the combination of this method with gynogenesis or androgenesis affects the formation of different ploidy fish with genetic variation.

distant hybridization, genetic variation, gynogenesis, androgenesis, diploid, triploid, tetraploid, evolutionary biology, genetic breeding

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It has been suggested that hybridization has facilitated speciation and adaptive radiation in animals and plants [1]. Currently, more than 28000 fish species have been identified, which is more than the number of species of all other vertebrate groups combined [2]. Ohno [3] put forward the theory that the two rounds of tetraploidization occurred during the evolution of vertebrates. However, this conjecture lacked the direct support of tetraploidization events. Distant hybridization of fishes is defined as the distant crossing between two different kinds of fishes belonging to different species, subfamilies, families, or orders. Distant hybridization is also called remote hybridization. Distant

email: lsj@hunnu.edu.cn

hybridization makes it possible to transfer the genome of one species to another. Thus, it is a useful strategy for altering the genotypes and phenotypes of the offspring. Distant hybridization results in genome-level alterations, including diploid, triploid, tetraploid hybrids, and diploid gynogenetic fish with subgenome-level alterations, such as the formation of the microchromosomes, depending on their parents' genetic relationship, including their similarity with regard to their chromosomal numbers. Phenotypically, the distant hybrids exhibit heterosis in appearance, growth rate, survival rate, and disease resistance resulting from the combination of the beneficial traits from both parents. Distant hybridization between fishes has been previously reported [4,5]. But they did not focus on the relationship between the distant hybridization and the formation of the different ploidy fishes.

Polyploids are organisms with one or more additional chromosome sets. Polyploidy is widespread in plants. For example, approximately 70% of angiosperms have polyploidy in their history [6]. We investigated more than 300 fish species' chromosomal numbers and found that all the chromosomal numbers were even, and there were approximate ploidy relationships between two different species, suggesting that the tetraploidization occurred during the evolution of fishes. However, it is difficult to determine what the real parents were in such presumed tetraploid fishes because the assumed tetraploidization events occurred millions of years ago. Thus the establishment of an artificial tetraploid lineage is of fundamental significance to evolutionary biology. By means of inter-ploidy crossing, i.e. crossing between tetraploid fish and diploid fish, sterile triploid fish were generated on a large scale. The sterility of the triploids prevents them from mating with other fish, which is useful for avoiding genetic mixing and safeguarding fish genetic resources. The development of gonads in the triploid fish was abnormal because of sterility, leading to the smaller size of the gonads. It is possible that the reduced energy of the gonad development translates into increased energy of flesh development. The sterile triploid fish had the potential to grow faster than the diploid control fish.

Generally, artificial gynogenesis is achieved from haploid eggs, activated by sterilized heterogenous sperm and treated with the process for diploidization. The eggs develop into the diploid progenies possessing genetic material mainly from the maternal fish. Artificially induced gynogenesis is a significant feature of fish genetic breeding, monosexual breeding, and studies about fish sex determination. It was generally thought that the genetic effect of one generation of gynogenesis was equal to that of several generations of sister-brother inbreeding. The effect of heterogenous sperm leads to the presence of hybrid traits and heterosis in the gynogenetic offspring. There have been many reports concerning fish artificial gynogenesis using haploid eggs [4,7], which have to be subjected to harmful treatment for diploidization. Artificial gynogenesis has been induced in a number of fish species, e.g. rainbow trout [8], tilapia [9], fighting fish [10], white bass [11], red crucian carp [12], and Japanese crucian carp [13]. The main advantage of gynogenesis using diploid eggs is that it avoids the treatment for doubling the chromosomes, thus not only simplifying the technological process, but also increasing the survival rate of the gynogenetic fish. The establishment of a 4*n*AT line in the present study provided an excellent platform for the gynogenesis of diploid eggs and the androgenesis of diploid spermatozoa.

Natural gynogenesis has been reported in fishes [14,15]. There is a type of natural gynogenetic fish (*Poecilia formosa*), which generally produces all-female offspring. However, a rare male of this naturally gynogenetic species was found. Unfortunately, it was not feasible to confirm what the male natural gynogenetic fish's father was. A previous study [16] concluded that this species was of hybrid origin. However, direct evidence was lacking to support this conclusion. Our results showed that the distant hybridization of RCC (♀)×BSB (♂) led to the formation of both male and female natural gynogenetic fish whose paternal fish was clear. The bisexual fertile gynogenetic fish had advantages over the mono-sexual fertile gynogenetic fish because the former was easier to bread in order to obtain progeny.

Our investigation of distant hybridization or the combination of this method with gynogenesis or androgenesis, is significant in both evolutionary biology and genetic breeding.

1 The different ploidy fishes from the distant hybridization of RCC (♀)×CC (♂)

In the catalog, RCC (*Carassius auratus* red var.) and CC (*Cyprinus carpio* L.) are members of different genera, and both are diploid bisexual fertile fish with 100 chromosomes. Crossing between these two species is categorized as distant hybridization. Phenotypically, the main difference between these two fishes is that CC have two pairs of barbels while RCC have no barbels. In addition, the body color of CC is grey, while that of RCC is red. The F_1 and F_2 hybrids of RCC (♀)×CC (♂) were diploid hybrids with 100 chromosomes. Interestingly, the males and females of diploid F_2 hybrids were able to generate unreduced diploid spermatozoa and diploid eggs, respectively, which were fertilized to form the allotetraploid hybrids in the F_3. In the F_3, there were fertile tetraploid males and females, which generated diploid sperm and eggs. The diploid sperm and eggs of the F_3 were fertilized to produce the tetraploids in the F_4. The tetraploid F_{18} was subsequently formed and the F_3–F_{18} tetraploid hybrids (abbreviated as the 4*n*AT) were formed in succession. The diploid F_1 and F_2 reached maturity at the age of 2 years, while 4*n*AT were mature at the age of 1 year. Thus it took us more than 20 years to establish this tetraploid hybrid line [17,18]. Under natural conditions, the

4*n*AT were able to reproduce tetraploid progeny. Over 20 years, we found that each generation of the 4*n*AT was fertile and could survive at least 4 years under normal conditions. The life span of the 4*n*AT was similar to that of the diploid control (RCC).

The formation of the F_3–F_{18} tetraploid hybrids indicated that their tetraploidy was stably inherited from one generation to another. The formation of this allotetraploid hybrid line is presented in Figure 1.

The establishment of the bisexual fertile tetraploid hybrid line has the following advantages: (i) it is used to produce sterile triploid hybrids by inter-ploidy level crossing on a scale large. (ii) It will become a new species and provides an excellent model system for investigating the genetic relationship between the tetraploids and their parents, at the cellular and molecular levels. (iii) Its diploid eggs and diploid spermatozoa are excellent materials for studying developmental biology because they do not require chromosome doubling treatment during gynogenesis or androgenesis.

The sterile triploid hybrids (3*n*=150, abbreviated as the 3*n*JC) were produced on a large scale by crossing the males of the 4*n*AT with the females of diploid Japanese crucian carp (*Carassius auratus cuvieri*). The 3*n*JC had many advantages, e.g. sterility, stronger disease resistance, faster growth rate, and good-quality flesh. They were widely cultured in China and readily accepted by fish farmers and customers. The sterility of the 3*n*JC prevented them from mating with other fishes and safeguarded natural fish genetic resources. Their sterility made the energy required for gonadal development transfer into flesh development. Thus, the sterile triploid fish had the potential to grow faster than the diploid control (RCC). Over 20 years, we found that the 3*n*JC could survive at least 6 years under normal conditions, and that all the 3*n*JC at the age of 1 to 6 years were sterile.

Their sterility also made the triploid hybrids good vectors for the development of transgenic triploid fish, which reduced the environmental risk of the transgenic fish.

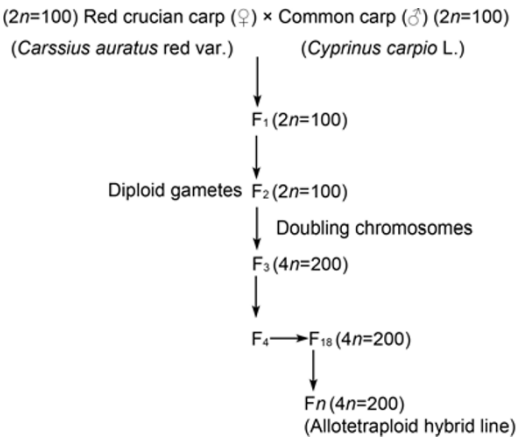

Figure 1 The establishment of the allotetraploid hybrid line of RCC (♀)× CC (♂).

In terms of evolution, various changes in genotypes and phenotypes took place in the 4*n*AT. The 4*n*AT meet the essential conditions for becoming a new species, e.g. the change of the inherited material from 100 to 200 chromosomes, the stable inheritance of tetraploidy, and reproductive isolation. When the 4*n*AT mated with diploid fish, their progeny were all sterile triploids, demonstrating reproductive isolation. Therefore, the 4*n*AT have the potential to become a new species with 200 chromosomes. This is the first reported case of the artificial creation of bisexual fertile allotetraploid hybrids in fish [17–20].

Genotypically, the chromosome number in the somatic cells changed from the 100 of the parents to 200 of the 4*n*AT. The 4*n*AT comprised two sets of chromosomes from RCC and two sets from CC, which was confirmed by examination of the chromosome number, karyotype analysis, FISH (fluorescence *in situ* hybridization), and DNA content. In the 4*n*AT, 100 bivalents formed as the chromosomes paired during the first meiosis of the germ cells, which resulted in the formation of the diploid gametes (Figure 2). This meant that each pair of homologous chromosomes came from either RCC or CC. The 4*n*AT are allotetraploid hybrids instead of autotetraploids; thus, only bivalent pairing occurred, not multivalent pairing, during meiosis I in the 4*n*AT. In the RCC control, 50 bivalents formed as the chromosomes paired during the first meiosis of the germ cells, which resulted in haploid gametes [21].

At the molecular level, the 4*n*AT also presented varied traits. For example, using the HMG-*sox* DNA marker [22,23], 4*n*AT presented 4 DNA bands (215, 628, 918 and 1957 bp) while RCC indicated 3 DNA bands (215, 617 and 1958 bp) and CC showed 2 DNA bands (215 and 918 bp), in which the 215-bp fragment belonged to *Sox*11, the 617 and 628 bp fragments belonged to *Sox*9a, the 918 bp fragment belonged to *Sox*9b, and the 1957-bp and 1958-bp fragment belonged to *Sox*4.

DNA fingerprinting analysis indicated that the 4*n*AT

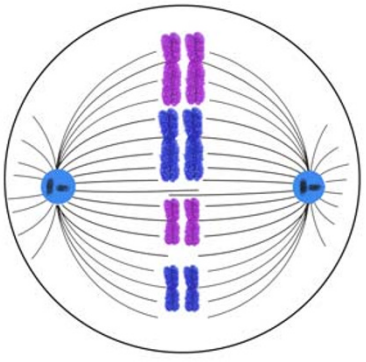

Figure 2 A model indicating the bivalent pairing of homologous chromosomes, instead of multivalent pairing, during the first meiosis of the germ cells of the 4*n*AT. The mauve chromosomes represent those from RCC, while the blue chromosomes represent those from CC. The long and short chromosomes represent the nonhomologous chromosomes.

contained sequence deletions that were mostly from the male parent's genome. On the other hand, several new DNA bands that were absent in their parents were found in the 4*n*AT, which could be related to the phenotypic variation of the 4*n*AT. Some nucleotide mutations were found in the *cyclin A1* and *B1* genes of 4*n*AT, which resulted in amino acid substitutions and would have an evolutionary effect [24].

The whole mitochondrial (mt) DNA genome of the 4*n*AT and their original parents, RCC and CC, were sequenced. The mtDNA nucleotide identity between the 4*n*AT and their maternal fish (RCC) was noticeably higher than that between the 4*n*AT and their paternal fish (CC), suggesting that the mt genome of the 4*n*AT was strictly maternally inherited and that the mt DNA of RCC was stably inherited in this tetraploid hybrid line [25].

However, the 3*n*JC generated by crossing Japanese crucian carps (♀)×the 4*n*AT (♂) possessed a recombinant mtDNA fragment (12759 bp) derived from the paternal fish. It was concluded that mtDNA recombination had occurred via fusion of the maternal and paternal mtDNAs [26].

Phenotypically, both the somatic cell size and the germ cell size of the 4*n*AT changed. For example, the size of the erythrocytes of 4*n*AT, 3*n*JC, and diploid RCC increased with increasing ploidy level [27]. The 4*n*AT contained 33% special erythrocytes with a dumbbell nucleus while the CC and RCC had no such special erythrocyte. With respect to the germ cell size, the diameter (2.4 μm) of the head of the diploid sperm of the 4*n*AT was markedly larger than that (1.9 μm) of the haploid sperm of CC. Likewise, the diameter (0.17 cm) of the diploid egg of the 4*n*AT was evidently larger than that (0.13 cm) of the haploid eggs of RCC. Other phenotypic changes also took place in the 4*n*AT. For example, 4*n*AT had two pairs of short barbels while the CC had two pairs of longer barbels and RCC had no barbel. The lateral scale number (30–34) of 4*n*AT was intermediate between that of CC (34–39) and that of RCC (28–30).

Although changes in the genotypes and phenotypes occurred in the 4*n*AT, the 4*n*AT appeared to be physiologically normal. For example, the 4*n*AT possessed normal pituitary and gonad structures, which established the normal hypothalamus-pituitary-gonad (HPG) axis. The histological and ultrastructure of the pituitary in 4*n*AT, 3*n*JC, and diploid RCC were compared. The results showed that there were 6 endocrine cell types in the pituitaries of these 3 kinds of fishes. In the same type of pituitary cells, the cell size gradually increased with increasing ploidy level. During the breeding season, the 4*n*AT had a higher proportion of gonadotropin cells (GTH) than the 3*n*JC, and diploid RCC had a higher proportion of GTH than the 3*n*JC. This contributed to the earlier sexual maturity of the 4*n*AT and the sterility of the 3*n*JC. Among the 3 kinds of fishes, the proportion of somatotropin (STH) cells in the 3*n*JC was the highest, whereas that in the 4*n*AT was the lowest, which was related to the faster growth rate of the 3*n*JC and slower growth rate of the 4*n*AT. In addition, in the GTH cells of meso-adenohypophysis after the breeding season, there were many endocrine particles in the 3*n*JC. In contrast, these endocrine particles were released from the cells in 4*n*AT and diploid RCC, suggesting that the sterility of the 3*n*JC might be related to unreleased hormone particles in the GTH cells [28].

Gonadotropin-releasing hormone (GnRH), gonadotropin hormone (GTH), and gonadotropin hormone receptor (GTHR) are the primary signal molecules of the HPG axis. We compared the expression of *Gnrh2*, *Gthβ*, and *Gthr* in 4*n*AT, 3*n*JC, and diploid RCC. The *Gnrh2* gene was expressed in the midbrains, pituitaries, and gonads, the *Gthβ* gene was expressed in the pituitaries, and the *Gthr* gene was mainly expressed in the gonads. These results indicated that all 3 genes participated in the regulation of gonadal development. Using a real-time polymerase chain reaction and *in situ* hybridization, the expression level of *Gthr* in the gonads of the 3*n*JC was shown to be lower than that of the 4*n*AT and diploid RCC. This would weaken the combination of GTHR in the 3*n*JC's gonads with GTH released from the pituitary and lead to the sterility of the 3*n*JC because the gonads could not produce enough sex steroids. In addition, the low expression of *Gthr* in the 3*n*JC probably affected the down-regulation of *Gthβ*, which subsequently affected the down-regulation of *Gnrh2*. Thus the expression levels of *Gnrh2* and *Gthβ* genes in the 3*n*JC were the highest after the breeding season. The results indicated that the differential expression of *Gnrh2*, *Gthβ*, and *Gthr* in 3*n*JC and 4*n*AT could explain their sterility and fertility to a certain extent [29].

However, the original reason for the sterility of the 3*n*JC (3*n*=150) was the disordered chromosome pairing during the first meiosis of the germ cells, where there existed 50 paired bivalents and 50 unsynapsed univalents. By contrast, chromosome pairing during the first meiosis of the germ cells was normal in 4*n*AT and RCC, where there were respectively 100 bivalents and 50 bivalents [21]. The abnormal gonadal structures and abnormal expressions of some genes, which lead to the sterility of the 3*n*JC, were the consequences of the disorder of the chromosome pairing.

Dmc1 (disrupted meiotic cDNA) is a functionally specific gene that encodes a protein required for homologous chromosome synapsis during meiosis. The full-length cDNA of *Dmc1* in diploid RCC was 1375 bp long, while it was 1383 bp long in 3*n*JC and 1379 bp long in 4*n*AT. *Dmc1* was expressed only in the gonads of these 3 kinds of fishes. The expression of this gene during the breeding season markedly differed among the three different ploidy fishes. The high expression of *Dmc1* in female 3*n*JCs might be associated with their abnormal meiosis and sterility [30].

Three kinds of abnormal gonadal structures within the reproductive season were observed in 3*n*JC. The first was the testis-like gonads containing many spermatids, but no mature spermatozoon. The second was the ovary-like go-

nads containing many oogonia, a few primary ova, but no mature ova. The third was fat tissue-like gonads containing only fat tissue and no germ cells. The growth rate of fish with the third type was faster than that of fish with the second type, and fish with the second type grew faster than fish with the first type. The above results indicated that the energy normally used for gonadal development could be transferred into flesh development [31,32].

Our previous study [17] indicated that female F_2 generated 3 sizes of eggs: large eggs with the diameter of 0.2 cm, middle-sized eggs with a diameter of 0.17 cm, and small eggs with a diameter of 0.13 cm, respectively accounting for 50%, 35.9%, and 14.1%. The middle-sized eggs were regarded as diploids because their size was equal to that of the diploid eggs generated by the 4*n*AT. The small-sized eggs were regarded as haploid eggs because they were the same size as the eggs of RCC. The large-sized eggs were determined to be triploid eggs because the tetraploid offspring (4*n*=200) were obtained when the female F_2 hybrids were mated with the males of RCC [33]. Similarly, certain male F_2 hybrids produced more than 3 types of sperm, including haploid, diploid, and tetraploid sperm, respectively accounting for 40%, 48.75% and 2.5% [19]. It was the fertilization of diploid eggs and diploid sperm produced by F_2 hybrids that led to the formation of the tetraploids found in F_3. Larger spermatogonia with 2 nuclei and larger-size oogonia with 2 nuclei were respectively found in the testes and ovaries of F_2 hybrids. Furthermore, 200 chromosome spreads identified as tetraploid were found in the spermatogonia of the F_2 testis [34]. In the testes of F_2 hybrids, spermatids with 2 nuclei and mature spermatozoa with 2 nuclei were observed. The above results indicated that F_2 hybrids did generate the diploid gametes that resulted in the formation of the tetraploid hybrids. The total number of mature eggs in F_2 hybrids was fewer than that of their maternal fish (RCC), and that of the 4*n*AT. The total amount of the spermatozoa in F_2 hybrids was also less than that of their paternal fish (CC), and that of the 4*n*AT. The semen of F_2 hybrids was water-like, suggesting fewer sperm in the semen, while the semen of the CC and the 4*n*AT was thickly white. It required 2 years for F_2 hybrids to reach maturity, while it took 1 year for the RCC and the 4*n*AT to mature. The above phenomena indicated that the reproductive ability of F_2 hybrids was lower than that of their parents and that of the 4*n*AT. To overcome this reproductive limitation, whilst being driven by the evolutionary dynamic, the diploid F_2 hybrids evolved into the tetraploid hybrids by generating the unreduced gametes, which had an advantage in reproduction over the diploid F_2 hybrids. The formation of the unreduced gametes in diploid hybrids was the key evolutionary element in the formation of the tetraploid hybrids.

The mechanism of the formation of the unreduced gametes was related to premeiotic endoreduplication, endomitosis, or fusion of germ cells. Endoreduplication occurs in a cell performing multiple S-phases without entering mitosis and without undergoing cytokinesis, which results from arresting cells in the G_2-phase before they enter mitosis. Endoreduplication results in a giant cell with a single giant nucleus. Endomitosis is similar to endoreduplication. However, endomitosis results from arresting cells within the M-phase before they complete mitosis. Endomitosis results in a single giant cell with a single multilobulated nucleus, each lobe presumably containing a diploid genome. With time, individual lobes separate from one another to produce a multinucleated cell [35]. Endomitosis and endoreduplication have been reported in the somatic cells of various organisms [35]. However, this is rarely reported for the germ cells. The fusion of germ cells means 2 diploid germ cells such as 2 diploid oogonia fuse to form a tetraploid germ cell, or 2 haploid germ cells such as 2 haploid spermatids fuse to form a diploid germ cell.

2 Distant hybridization of RCC (♀)×CC (♂) combined with gynogenesis or androgenesis

In 1999, the first generation of diploid gynogenetic hybrids (2*n*=100, abbreviated as G_1) of RCC (♀)×CC (♂) were obtained by artificial gynogenesis from the eggs of the 4*n*AT (F_8–F_{10}). They were activated by UV-treated sterilized sperm of scatter scale carp without treatment for doubling the chromosomes, and developed into living gynogenetic hybrids. Interestingly, like the diploid F_2 hybrids of RCC (♀)×CC (♂), G_1 was also able to produce the unreduced diploid eggs that developed into the second generation of the diploid gynogenetic hybrids (2*n*=100, abbreviated as G_2), after activation by sterile sperm, but without treatment for doubling the chromosomes. In the same way, G_3, G_4, and G_5 were formed, establishing a diploid gynogenetic clonal hybrid line (G_1–G_5, 2*n*=100) [36,37] (Figure 3). Each generation of this line reached maturity at the age of 2 years. Thus, it took us 10 years to establish this line. All the fish of this line are female, suggesting that the sex-determining type of the female 4*n*AT is XXXX, and that of this diploid gynogenetic line is XX. The main reproductive trait of this line is that they could generate unreduced eggs. For example, G_2 produced three types of eggs: 2.47% small-size eggs (diameter=0.13 cm), 95.60% middle-size eggs (diameter=0.17 cm), and 1.89% unusually large-size eggs (diameter=0.2 cm). The middle-size eggs were regarded as diploid eggs because the 4*n*AT also produced the same-sized diploid eggs. By contrast, RCC only produced one kind of egg with a diameter of 0.13 cm. With the increasing generation of this line, the percentage of unreduced diploid eggs increased. For example, G_2 produced 95.6% diploid eggs, G_3 generated 97.5% diploid eggs, and G_4 produced 98.7% diploid eggs.

Although G_1–G_5 resulted from gynogenesis, they showed hybrid traits and heterosis, suggesting that the diploid eggs generated from both the 4*n*AT and this diploid gynogenetic

hybrid line were hybrid eggs. Like the 4*n*AT, G_1–G_5 had an intermediate appearance between RCC and CC. For example, G_1–G_5 had 2 pairs of short, not readily visible barbels whereas CC had 2 pairs of visible barbels and RCC had no barbels. The number of lateral scales of G_1–G_5 was 31–32 while that of CC was 36–37 and that of RCC was 29. The formation of G_1–G_5 indicated a diploid gynogenetic hybrid clonal line (G_1–G_5–G_n) had been established that possessed the ability to generate unreduced diploid eggs, providing evidence that gynogenetic fish in nature probably originated from distant hybridization combined with gynogenesis. G_1–G_5 were genetically and phenotypically similar to the F_2 hybrids of RCC (♀)×CC (♂). Thus, the establishment of the diploid gynogenesis clonal line provided an excellent platform for studies about how diploid hybrids produced diploid eggs.

The formation of the unreduced diploid eggs from the diploid gynogenetic hybrid line was also related to premeiotic endoreduplication, endomitosis, or fusion of germ cells, which might involve in selected genes of the cell cycle, such as *cdc2* and *cyclinB*. We are currently investigating the molecular mechanism of the formation of the unreduced gametes, including constructing a forward suppression subtractive hybridization complementary DNA (cDNA) library in this diploid gynogenetic hybrid line to identify the related genes [38].

G_1–G_5 had stronger disease resistance and a faster growth rate than the 4*n*AT. For example, they grew 20.6% faster than the 4*n*AT. Using diploid eggs of this gynogenetic line, improved tetraploid hybrids (abbreviated as G_1×4*n*AT) were

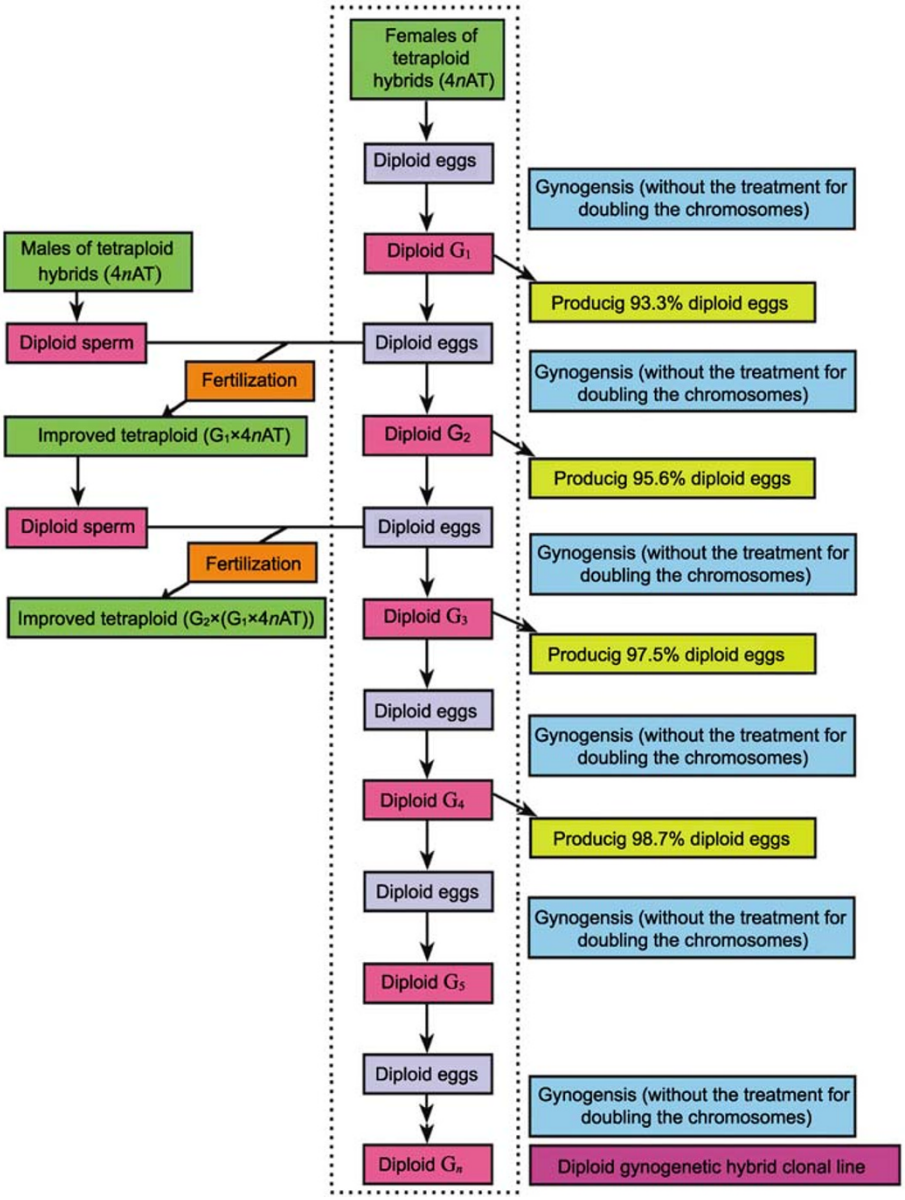

Figure 3 The establishment of the diploid gynogenetic hybrid line of RCC (♀)×CC (♂).

produced by crossing this all-female gynogenetic line with the males of the 4*n*AT. G_1×4*n*AT had stronger disease resistance than the 4*n*AT and grew 12.7% faster than the 4*n*AT [39]. G_1×4*n*AT also showed stronger reproductive ability, including the spawning rate and amount of semen than the 4*n*AT. More than 200000 G_1×4*n*AT were annually produced, and were crossed with the diploid fishes to annually generate the 300–400 million sterile triploid hybrids.

Interestingly, 98% of G_1×4*n*AT were tetraploids and they generated tetraploid progeny, while the other 2% of G_1× 4*n*AT displayed high body traits and generated 3 kinds of bisexual fertile diploid fish: high-body RCC, high-body fork-like-tailed goldfish, and gray CC. The high-body RCC produced three kinds of progeny: high-body RCC, high-body grey crucian carp and high-body color crucian carp [39]. The presence of high-body RCC indicated phenotypic variation compared with the original parent fish, RCC. The presence of high-body goldfish suggested that the goldfish in nature probably originated from the distant hybridization of RCC (♀)×CC (♂) combined with the gynogenesis.

The females of the diploid high-body RCC were mated with the males of the improved tetraploid (G_1×4*n*AT) to produce, on a large scale, improved triploid hybrids [40] (Figure 4).

The above results indicated that the distant hybridization of RCC (♀)×CC (♂) or the combination of this method with gynogenesis leads to formation of tetraploid hybrids, diploid gynogenetic hybrids, and derived diploid high-body crucian carp, high-body goldfish, high-body color crucian carp and high-body grey crucian carp. This suggests that distant hybridization and gynogenesis were a critical factor in the formation of the new types of bisexual fertile tetraploid hybrids and new types of bisexual fertile diploids with genetic variation, which are significant in evolutionary biology and genetic breeding.

The diploid hybrid spermatozoa, generated by the 4*n*AT, developed into diploid androgenetic hybrids (2*n*=100, abbreviated as A_0) after they entered the UV-radiated eggs of goldfish (*Carassius auratus*). Both the males and females were found in A_0 with the sex ratio (female: male) of 1:1, suggesting the sex-determining types of the males of the 4*n*AT belonged to XXXY. The males and females of A_0 reached sexual maturity at the age of 2 years. Improved tetraploids (4*n*=200) were obtained by mating the males with the females of A_0, suggesting that diploid sperm and diploid eggs were respectively generated by the males and females of A_0 [41,42].

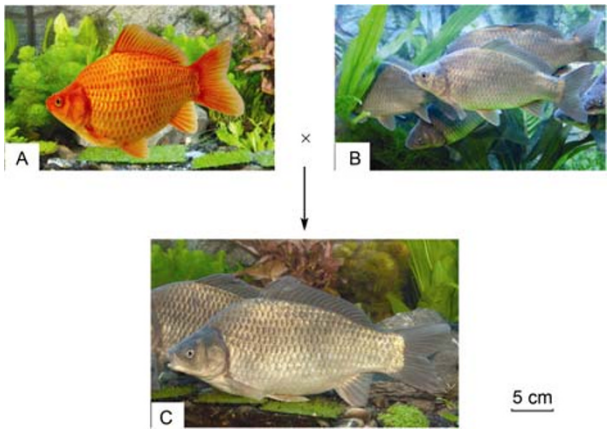

Figure 4 The females of the high-body RCC (A) were mated with the males of the improved tetraploid hybrids (B) to generate the improved triploid hybrids (C).

3 The different ploidy fishes from the distant hybridization of RCC (♀)×BSB (♂)

To investigate the distant hybridization between the parents with different chromosomal number, we designed another distant hybridization of RCC (♀) (2*n*=100)×BSB (*Megalobrama amblycephala*) (♂) (2*n*=48). Compared with the distant hybridization of RCC (♀)×CC (♂), this distant hybridization had the same maternal fish, RCC, while the paternal fish was different. In this hybridization, the paternal fish, BSB, has 48 chromosomes instead of the 100 chromosomes in CC. In the catalog, RCC and BSB belong to different subfamilies (Cyprininae subfamily and Cultrinae subfamily). In the first generation of RCC (♀)×BSB (♂), we obtained sterile triploid hybrids (3*n*=124, abbreviated as the 3*n*RB) and bisexual fertile tetraploid hybrids (4*n*=148, abbreviated as 4*n*RB), as well as the bisexual fertile natural gynogenetic RCC (2*n*=100, abbreviated as 2*n*GRCC). The 3*n*RB possessed 2 sets of chromosomes from RCC and 1 set of chromosomes from BSB. The 4*n*RB had 2 sets of chromosomes from RCC and 2 sets of chromosomes from BSB. 2*n*GRCC and their offspring presented 2 sets of chromosomes from RCC with 1–3 microchromosomes from BSB. The females of the 4*n*RB were able to generate unreduced tetraploid eggs. These tetraploid eggs were respectively fertilized with haploid sperm of BSB and RCC, to form 2 kinds of pentaploid hybrids (5*n*=172 and 5*n*=198) [43,44] (Figure 5). The formation of the unreduced tetraploid eggs was also due to premeiotic endoreduplication, endomitosis, or fusion of germ cells of oogonia, as in F_2 hybrids of RCC (♀)×CC (♂) and diploid gynogenetic hybrid line (G_1–G_5).

In this hybridization, only the 2*n*GRCC, 3*n*RB, and 4*n*RB hybrids, but no diploid hybrids (abbreviated as 2*n*RB), were found to survive. However, diploid hybrid embryos with 74 chromosomes were detected by examining the chromosome number of the embryos. The diploid hybrids probably could not survive because of the large difference in chromosome number between RCC (2*n*=100) and BSB (2*n*=48), which inhibited the diploid hybrid embryos from developing into living fish. Natural gynogenesis, tetraploidization, and triploidization were helpful in overcoming the obstacle to form living progeny.

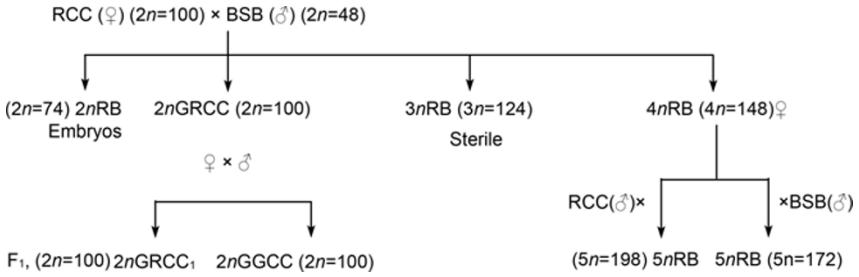

Figure 5 The formation of the different ploidy fishes in the distant hybridization of RCC (♀)×BSB (♂).

We hypothesized that natural diploid gynogenetic RCC came from the arrest of the ejection of the second polar body of the RCC's egg, which resulted in the chromosomes' spontaneous doubling. The formation of the male 2*n*GRCC resulted from genetic leakage of the paternal fish in the form of the microchromosomes, including the paternal male-determining gene. The 3*n*RB hybrids resulted from the retention of the second polar body of the fertilized eggs and the 4*n*RB hybrids resulted from the inhibition of the first cleavage of the fertilized eggs.

Regarding the phenotypic variation in the polyploid hybrids of this hybridization, the feeding habits of the 3*n*RB and the 4*n*RB, like that of BSB, were herbivorous, suggesting that the polyploid offspring had genetic material from BSB. Their herbivorous feeding habit provided them with wider feeding resources, which was beneficial in aquaculture. The presence of unusual erythrocytes with dumbbell nuclei, which seemed to be two nuclei, was used as a cytological marker to distinguish the polyploid hybrids from their diploid parents. With increasing ploidy level, the percentage of unusual erythrocytes increased [45]. The most striking variation trait in the 4*n*RB hybrids was the presence of the barbels, which were absent in their parents. CC have 2 pairs of barbels, thus, the presence of barbels in the 4*n*RB provided evidence that the barbels in CC probably originated from hybridization.

We examined the genotypic variation in the polyploid hybrids of this hybridization by analysis of HMG-*sox* DNA markers [22,23]. The 4*n*RB hybrids not only shared several DNA fragments found in both RCC and BSB, but also possessed a newly formed 918-bp DNA fragment, indicating that the 4*n*RB hybrids not only had altered chromosomal ploidy levels, but had also altered DNA sequences. The 4*n*RB hybrids shared the common and different fragments, providing genetic evidence that the 4*n*RB hybrids came from RCC and BSB, but were different from RCC and BSB. The specific 712-bp DNA fragment of BSB was absent in the 3*n*RB and 4*n*RB hybrids, providing further genetic evidence that the 3*n*RB and 4*n*RB hybrids were different from BSB [43].

Transposons are a vital component of species evolution. A novel transposon termed *Tte1* was detected in the 4*n*RB [46], suggesting that the 4*n*RB was also a good polyploid model species for investigating evolution.

Besides the 3*n*RB and 4*n*RB hybrids, the females and unexpected males of 2*n*GRCC with the 1:1 sex ratio were also found in the distant hybridization of RCC (♀)×BSB (♂). Both the females and males of 2*n*GRCC were fertile, and they mated with each other to generate the 2*n*GRCC$_1$ and another variant gray crucian carp (2*n*GGCC). The 2*n*GRCC and their offspring were shown to be diploids (2*n*=100) with one to three microchromosomes, by examining the chromosomal metaphases. The evidences for the male's genetic effect in 2*n*GRCC were provided by means of chromosomal metaphases, fluorescence *in situ* hybridization, *Sox*-HMG DNA markers, and microsatellite DNA markers. The genotypic variances of 2*n*GRCC resulted in altered phenotypes that were quite different from their maternal parent. The formation of the bisexual 2*n*GRCC and their progeny indicated that distant hybridization could generate bisexual diploid gynogenetic fish with genetic variation, which is of significance in both fish genetic breeding and evolutionary biology [44].

4 Comparisons of the different methods of formation of the different ploidy fishes

By a long-term investigation, we found that distant hybridization was the ideal way to form the different ploidy fishes which actually existed in nature. Therefore, studies concerning distant hybridization are of substantial value in evolutionary biology. The main effect of distant hybridization is to create genetic variation in the hybrid progeny which is not available through inbreeding. For example, the incompatibilities of the genetic material between RCC and CC led to the formation of the unreduced gametes by endoreduplication, endomitosis, or the fusion of germ cells in the F_2 hybrids of RCC (♀)×CC (♂). It was also driven by the evolutionary reproductive dynamic, because the F_1 and F_2 hybrids of RCC (♀)×CC (♂) had abnormal gonadal development and needed 2 years to reach maturity, while the 4*n*AT had better gonadal development and only required 1 year to reach maturity. In the cross of RCC (♀)×BSB (♂), the incompatibilities of the genetic material between RCC and BSB led to the formation of triploid hybrids or natural

gynogenetic fish by inhibiting the second polar body and to the formation of tetraploid hybrids by inhibiting the first cleavage.

Based on the above results, it was concluded that it was more probable to form the diploid or triploid hybrids than the tetraploid hybrids in the first generation of the distant hybridization, when the parents had the same chromosome number. For example, in the first generation of the distant hybridization of RCC (♀) (2*n*=100)×CC (♂) (2*n*=100), diploid hybrids were found. In the first generation of the distant hybridization of BSB (♀) (2*n*=48)×*Xenocypris davidi* Bleeker (♂) (2*n*=48), both diploid (2*n*=48) and triploid hybrids (3*n*=72) were observed (data not published). In the first generation of the distant hybridization of BSB (♀) (2*n*=48)×predatory carp (*Erythroculter ilishaeformis*) (♂) (2*n*=48), both diploid (2*n*=48) and triploid hybrids (3*n*=72) were observed (data not published). In the first generation of the distant hybridization of grass carp (*Ctenopharyngodon idellus*) (♀) (2*n*=48)×BSB (♂) (2*n*=48), diploid (2*n*=48) and triploid hybrids (3*n*=72) were observed (data not published). In the first generation of the distant hybridization where the parents had different chromosomal numbers, it was more probable to form tetraploid hybrids, triploid hybrids, or natural diploid gynogenetic fish than diploid hybrids (sometimes, only the diploid embryos were observed), e.g. in the first generation of the distant hybridization of RCC (♀) (2*n*=100)×BSB (♂) (2*n*=48), and in the first generation of the distant hybridization of RCC (♀) (2*n*=100)×*Xenocypris davidi* Bleeker (♂) (2*n*=48) (data not published). However, these conclusions are not definitive, as other exceptional events exist. For example, we found living diploid hybrids (2*n*=74) in the first generation of the distant hybridization of RCC (♀) (2*n*=100)×predatory carp (*Erythroculter ilishaeformis*) (♂) (2*n*=48) (data not published). Therefore, the formation of the different ploidy fishes mainly depends on the genetic relationship between the parents.

Certain distant hybridizations generate surviving progeny while other hybridizations do not. The mechanism is unclear. However, we can formulate a few guidelines. For example, we think that the formation of the offspring of the distant hybridization is related to the chromosome numbers of the parents. In general, when the chromosome number of the maternal fish is equal to or larger than that of the paternal fish, the distant hybridization forms the living progeny, e.g. the distant hybridization of RCC (♀) (2*n*=100)×BSB (♂) (2*n*=48). When the chromosomal number of the maternal fish is less than that of the paternal fish, the distant hybridization hardly produces living progeny, e.g. the distant hybridization of BSB (♀) (2*n*=48)×RCC (♂) (2*n*=100), and the distant hybridization of *Xenocypris* davidi Bleeker (♀) (2*n*=48)×RCC (♂) (2*n*=100) (data not published).

Like the F_2 hybrids of RCC (♀)×CC (♂) and the diploid gynogenetic hybrid line (G_1–G_5), the F_1 hybrids (4*n*RB) of RCC (♀)×BSB (♂) had the ability to generate both reduced and unreduced gametes. The formation of unreduced gametes which is related to endoreduplication or endomitosis or the fusion of germ cells, is the basic dynamic of the formation of different polyploid fishes and is of fundamental significance in evolutionary biology and genetic breeding.

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