Two unisexual artificial polyploid clones constructed by genome addition of common carp (*Cyprinus carp*) and crucian carp (*Carassius auratus*)

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Abstract A polyploid hybrid fish with natural gynogenesis can prevent segregation and maintain their hybrid vigor in their progenies. Supposing the reproduction mode of induced polyploid fish being natural gynogenesis, allopolyploid hybrid between common carp and crucian carp into allopolyploid was performed. The purpose of this paper is to describe a lineage from sexual diploid carp transforming into allotriploid and allotetraploid unisexual clones by genome addition. The diploid hybrid between common carp and crucian carp reproduces an unreduced nucleus consisting of two parental genomes. This unreduced female pronucleus will fuse with male pronucleus and form allotriploid zygote after penetration of related species sperms. Allotriploid embryos grow normally, and part of female allotriploid can produce unreduced mature ova with three genomes. Mature ova of most allotriploid females are provided with natural gynogenetic trait and their nuclei do not fuse with any entrance sperm. All female offspring are produced by gynogenesis of allotriploid egg under activation of penetrating sperms. These offspring maintain morphological traits of their allotriploid maternal and form an allotetraploid unisexual clone by gynogenetic reproduction mode. However, female nuclei of rare allotriploid female can fuse with penetrating male pronuclei and result in the appearance of allotetraploid individuals by means of genome addition. All allotetraploid females can reproduce unreduced mature eggs containing four genomes. Therefore, mature eggs of allotetraploid maintain gynogenetic trait and allotetraploid unisexual clone is produced under activation of related species sperms.

Keywords: carp, unreduced, genome addition, allopolyploid, gynogenesis, clone.

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The application of hybrid vigor and crossbreeding is conventional and proved effective. Nevertheless, the phenotype of the progeny of hybrids, which carry hybrid vigor and produce their offspring through bisexual reproduction, will segregate inevitably and their hybrid vigor will decrease in subsequent generations. The more serious consequence might result in destroying completely those endemic populations when hybrids are released into open water bodies, because hybrids will cross with their related species at random. Therefore, a vigor hybrid biotype with gynogenesis is advantageous. In nature, there are a few of fishes reproducing unisexually their offspring by gynogenesis. These include silver crucian carp (*Carassius auratus gibelio*)[1,2] found in Russia and Northeast of China, *Carassius auratus* var. Sugu found in South China[3], *Carassius*

auratus langsdorfii of Japan[4], *Poecilia formosa* and *Poeciliopsis 2monacha-lucida* in southern America[5—9]. Cellular and isozymic evidence shows some unisexual allopolyploid fish, amphibian and lizard originated from hybridization[5,8—12]. Even some gynogenetic polyploid fishes have been reported in nature, and some successful attempts have been made to trace the origin of unisexual fish. But unfortunately, the question of whether a new unisexual biotype may be induced from bisexual population by an artificial method has rarely been explored. Allotriploid reproduced by backcrossing of diploid hybrid between common carp and crucian carp and fertility of allotriploid hybrid between common carp and crucian carp was reported, however, gynogenetic trait of backcrossing allotriploid was never found[13—15]. Early in the 1970s, a possible approach for preventing the offspring with vigor from genetic segregation by means of inducing hybrid into triploid was advanced on the supposition that the reproduction mode of this triploid hybrid might be natural gynogenesis[16]. Herein, we report a lineage from sexual diploid carp transforming into two new unisexual clones by genome addition with emphasis on oogenesis and spermatogenesis of diploid hybrid between common carp and crucian carp and their allopolyploid.

1 Materials and methods

1.1 Experimental fishes

A red variety of common carp (*Cyprinus carpio* L.), mirror carp and red crucian carp (*Carassius auratus* L.) were collected from the Guanqiao Experimental Station of Institute of Hydrobiology, the Chinese Academy of Sciences. Induced fish including their diploid hybrid, allotriploid and allotetraploid were rared in ponds of the same station.

1.2 Induced spawning and incubation

Parental fish were injected intraperitoneally with pituitary suspensions of common carp or human chorionic gonadotropin (HCG) to induce spawning. Fertilized eggs were scattered on nylon gauze net and incubated in numbered plastic tanks. Incubation temperature of water was 20—25℃. Swimming fries of different experimental group were separately cultured in numbered ponds.

1.3 Cytological and histological observation of gonad

Testes and ovary specimens were rinsed with distilled water, chopped into 5 mm. For microscopic observation specimens were fixed in Bouin's fluid, embedded in paraffin, sectioned into 10 μm thickness, stained with Hematoxylin and eosin. For ultramicroscopic observation specimens were prefixed in 2.5% glutaraldhyde in 0.1 mol/L phosphate buffer (pH 7.2) at 4℃ for 24 h. After several washes in the same buffer, the samples were post-fixed for 2 h at 4℃ in 1% osmium tetroxide in the same buffer, dehydrated in a graded series of acetone and embedded in Epon-812. Thin sections (0.4—0.5 μm) were stained with uranium acetate and lead citrate, then examined in an H-7000FA transmission electron microscope.

1.4 Genetic inactivation of sperms

Milt of related species maintained in diluted Hank's solution was irradiated with ultraviolet light. Milt samples in a 9 cm diameter Petri dish on a minivibrator were irradiated with an ultraviolet lamp (15 W, 2350 Å) for 15 min. Depth of milt samples in the Petri dish was less than 2 mm and vertical distance from surface of milt samples to ultraviolet lamp was 17 cm.

1.5 Ploidy determination

The chromosome counts of gastrular cell were conducted according to the direct routine air-drying technique with Giemsa staining[17]. Methods of peripheral blood cell and renal cell culture were used to study chromosome counts of fingerling over 100 g[17,18].

2 Results

2.1 Reproductive traits of diploid hybrid

Red common carp embryos are transparent, without any melanic pigment. This is different from embryos of hybrid between red common carp (red variety) and red crucian carp. And the embryos of hybrid between red common carp and red crucian carp are viable and develop and grow normally. Chromosome count revealed the hybrid was diploid ($2n$ = 100). Sex ratio of hybrid between red common carp and red crucian carp is about 1 ∶ 1. There is no difference between hybrid male and normal male crucian carp or common carp at 2 years old in external morphology of testes at spawn season. However, pink color instead of white color in normal mature testes of common carp was found. Light microscope observation on sections of diploid hybrid testes revealed that primary spermatocytes, secondary spermatocytes and abnormal spermatids at different metamorphosis stages occurred in spermatangium. However, functional spermatozoon was never observed. Electron microscope examination showed that primary spermatocyte would be abortion caused by the abnormal meiosis, such as the defeat of the synaptonemal complex without pairing of chromosomes, known as unequal nuclear division. However, few spermatids passed through meiosis, but they would follow abnormal spermiogenesis, and 1—3 vacuoles occurred in nucleus of spermatid (fig. 1).

Diploid hybrid females between common carp and crucian carp are fertile. Observations under the light microscope and the electron microscope revealed that some oocytes in diploid hybrid could pass through the entire developmental courses of oogenesis, including yolk accumulation, the formation of follicular layer, zona radiata, chorion and micropyle. Embryos of these mature ova develop normally after fertilization by sperm of related species. At the same time, some aborted oocytes at stage II could be found in mature ovaries and some oocytes stopped at first meiosis and two unequal nuclei occurred in one oocyte (fig. 2).

Diploid hybrid mature eggs activated by inactive sperm develop normally. Additionally, embryos without any haploid symptom can pass hatchery stage, even without artificial diploidization treatment. Morphological traits of these offspring are identical with their maternal. Chromosome

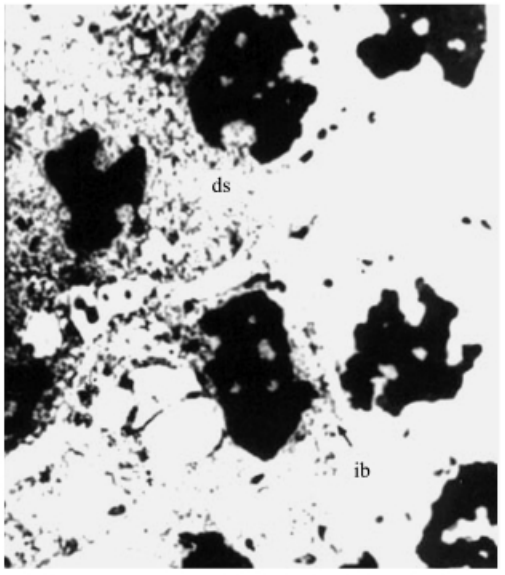

Fig. 1. Transmission electromicrograph image of sectioned testis of male diploid hybrid between *Cyprinus carpio* and *Carassius auratus* showing large amount of abnormal spermatocytes. ds, Abnormal spermatocyte at anaphase; ib, intercellular bridge. × 8000.

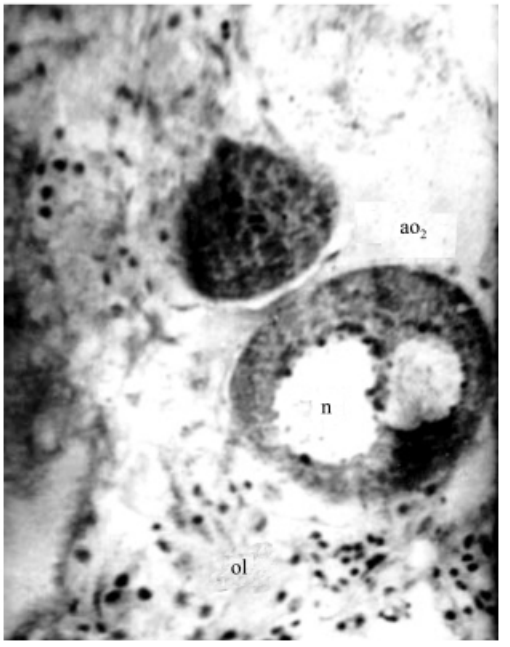

Fig. 2. Light microscope image of sectioned ovary of female diploid hybrid between *Cyprinus carpio* and *Carassius auratus* showing abnormal oocytes at II stage. ao_2, Aborted oocyte at II stage; n, nucleus; ol, ovarian lamellae. × 600.

count revealed that these offspring consisted of 100 chromosomes. This indicated that the female pronucleus of diploid hybrid mature egg maintained two genomes ($2n$ = 100). When back-crossing diploid hybrid egg with normal non-irradiated sperm of scattered scaled mirror carp, morphological features of offspring appeared to be hybrid between red common carp and scattered scaled mirror carp, while the chromosome count of fingerlings showed that all offspring were triploid ($3n$ = 150). These results suggested that female pronucleus of diploid hybrid could fuse with male pronucleus and form allotriploid individual which consists of 2 genomes from common carp and 1 genome from crucian carp.

2.2 Development of primary oocyte and spermcyte and reproduction mode of allotriploid

The ratio between male and female individuals of allotriploid original generation is 1∶1. During reproduction season, the testes of three years old male allotriploid are fully developed. Pink organ extends throughout most of the length of the abdominal cavity. Electron microscope observation revealed that spermatangium was filled with primary spermatocytes, secondary spermatocytes and spermatids. However, normal spermatozoon was never detected. At the same time, a large amount of abnormal spermatocytes were found, such as degrading sperm with pycnosis nucleus and long triangle head with numerous vacuoles (figs. 3 and 4). Light micro

scope observation of mature ovary section revealed that ovary was filled with a large amount of mature oocytes at stage IV. The oocytes at stages I, II and III were observed. Transmission electron microscope observation found that oocyte at stage I still remained at pachytene stage of first meiosis.

Mature eggs of allotriploid can develop normally and pass hatchery stage after being activated by inactive sperms of related species. Chromosome count of these gynogenetic embryonic cells revealed that embryonic cells consisted not only of triploid ($3n = 150$), but also of haploid ($1n = 50$), diploid ($2n = 100$), and aneuploid ($1.5n = 75$). The ploid percentages of offspring from different female individual were different, the highest percentage of allotriploid embryos was 76.5%, and the lowest was 0. However, in cultured cells of gynogenetic fingerling only allotriploid can be detected. When non-irradiated sperms of related species, such as red crucian carp (*Carassius auratus* var. red, $2n = 100$), mirror carp (*Cyprinus carpio* var. mirror, $2n = 100$), wild common carp (*Cyprinus carpio* L., $2n = 100$), Chinese blunt snout bream (*Megalobrama amblycephala*, $2n = 48$), gudgeon topmouth (*Pseudorasbora parva*, $2n = 50$) and *Hemiculter leucisculus* ($2n = 48$), were used as an activator, all viable offspring from allotriploid mature eggs were allotriploid ($3n = 150$) and their morphological traits were identical with their maternal. No diploid or aneuploid fingerling was found. This suggested that female pronucleus of almost all of mature allotriploid eggs might not fuse with penetrated male pronucleus, but these eggs containing three genomes gynogenetically developed after activation by related sperms.

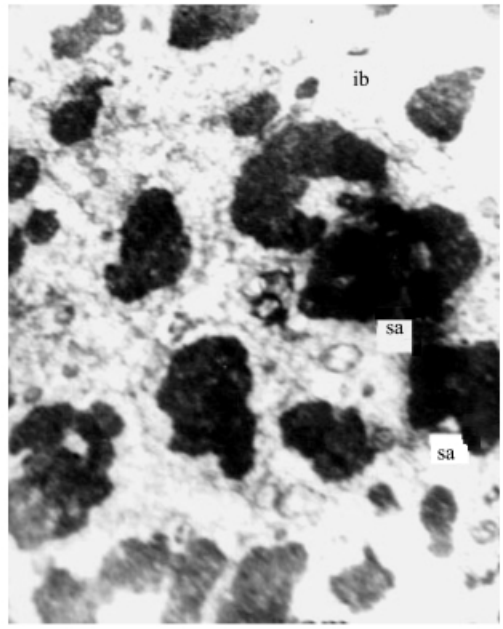

Fig. 3. Transmission electromicroscope image of sectioned testis of male allotriploid carp showing the structure of degenerating spermatocytes. sa, Degenerating spermatocyte; ib, intercellular bridge. × 3500.

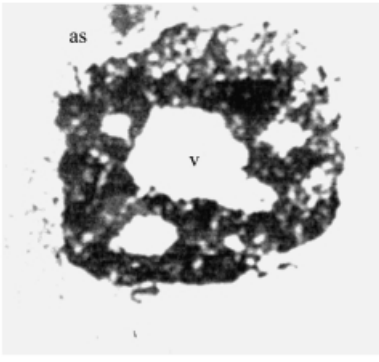

Fig. 4. Transmission electromicroscope image of sectioned testis of male allotriploid carp showing the nucleus at intermediate stage of pycnosis of the degenerating spermatocyte. as, Aborted spermatocyte; v, vacuole. × 10000.

2.3 Allotetraploid produced by allotriploid female pronucleus fused with male pronucleus

In 1997, a brood of allotetraploid was detected in offspring from one individual mature al-

lotriploid female whose eggs were inseminated with non-irradiated sperm of red crucian carp. These allotetraploid fries and fingerlings were nursed separately. External morphological traits of these allotetraploid fingerlings were similar to gibel carp (*Carassius auratus gibelio*), except for yellowish body color instead of gray. Ten fingerlings were randomly taken for serving in kidney cell culture. Chromosome count from 50 metaphases revealed that chromosome numbers were from 185 to 210 with mode of 200. This shows that all individuals tested were allotetraploid ($4n$ = 200).

2.4 Reproduction mode of allotetraploid female

Allotetraploid females become sexually mature at one round year age. The ratio of female and male was 1∶1 in the original generation produced by triploid female pronucleus fused with male pronucleus of bisexual red crucian carp, but all female offspring were found in the second and next generations, since they were all gynogenetic offspring. Inactive sperms of related species were used as activator for stimulating allotetraploid eggs, embryos developed and hatchery normally[19]. Chromosome count of fingerlings revealed that offspring of allotetraploid egg activated by inactive sperms of related species were all allotetraploid. Also, when normal sperms of common carp, crucian carp, Chinese blunt snout bream (*Megalobrama amblycephala*, $2n$ = 48), gudgeon topmouth (*Pseudorasbora parva*, $2n$ = 50), *Erythroculter dabryi* ($2n$ = 48) and *Hemiculter leucisculuus* ($2n$ = 48) were used for inseminating allotetraploid eggs, all offspring produced were also allotetraploid ($4n$ = 200). The external morphological traits of allotetraploid offspring were identical to their maternal, no matter what kind of sperms was used. Furthermore, 28 primers of RAPD were used for amplifying DNA of allotetraploid maternal and their offspring, altogether 147 bands were produced, no any difference was detected[20]. To date, 4 generations of all female allotetraploid carp were produced by means of allotetraploid carp egg stimulated by inactive or normal sperms of related species. These results suggest that allotetraploid carp egg developed gynogenetically when they were activated by any kind of sperm.

3 Discussion

3.1 Lineage from bisexual diploidy into unisexual allopolyploidy

Under activation of inactive sperm and without artificial diploidization treatment egg of diploid hybrid between common carp and crucian carp could develop into diploid offspring. However, when this kind of egg was backcrossed with normal sperms of common carp or crucian carp, allotriploid occurred in all offspring. These results showed that female pronucleus of diploid hybrid without capacity of natural gynogenesis but could fuse with penetrating male pronucleus then a new allotriploid was produced by genome addition. Female pronuclei of most allotriploid individuals could not fuse with penetrating male pronuclei and it yielded gynogenetically allotriploid female progeny after cross with male of bisexual species[21—23]. After 7 generations of gynogenetic reproduction from female allotriploid carp an allotriploid gynogenetic clone was established.

Nevertheless, female pronucleus of rare individuals of allotriploid carp can fuse with penetrating male pronucleus and allotetraploid offspring was obtained[19]. With capacity of natural gynogenesis allotetraploid yielded another allotetraploid clone (fig. 5).

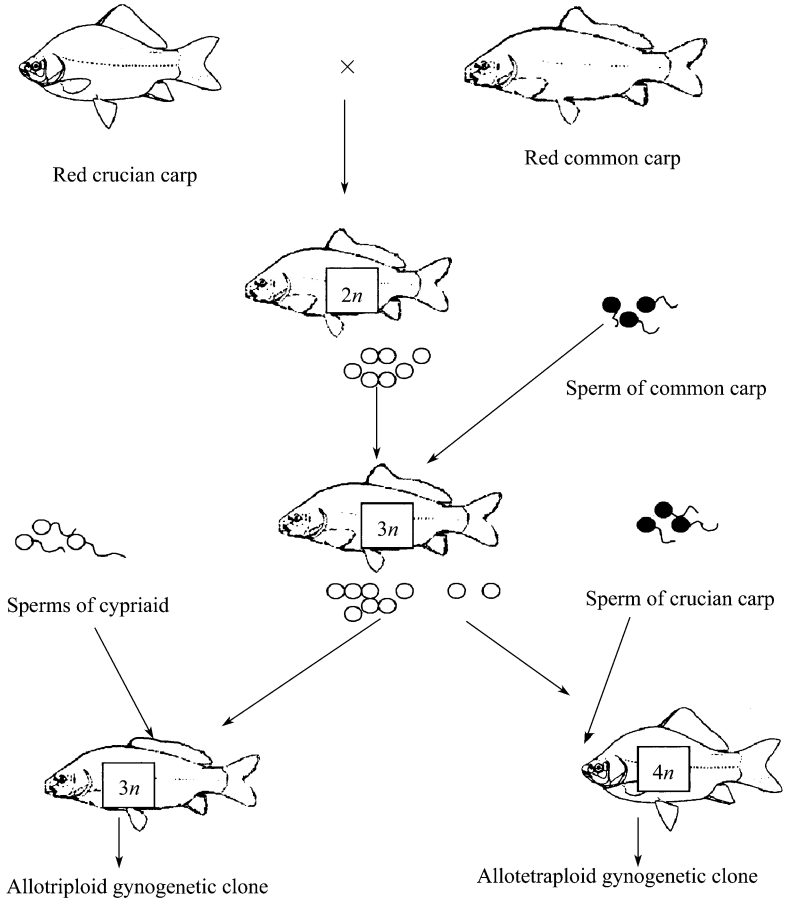

Fig. 5. A diagram illustrating establishment of two unisexual clones.

3.2 Regarding unreduced oocyte

Minority of oocytes of diploid hybrid between red common carp and red crucian carp become decay, but a majority of them can develop normally into mature egg. After the eggs were activated by inactive sperm they not only could develop normally, but also could pass hatchery stage. Without any artificial diploidization treatment, hatchery ratio of these artificial gynogenetic embryos could be compared with that of those eggs crossed with normal crucian carp sperm. Chromosome counting revealed that all artificial gynogenetic fingerlings were diploid. These results suggested that there were two sets of chromosome remaining in mature egg of diploid hybrid

between common carp and crucian carp. Aspects of gonad maturation in triploid fish have not been examined in detail. Some scientists have assumed that premeiotic endomitosis involving chromosome duplication without subsequent cell division could take place in oocytes in gynogenetic diploid or triploid fishes[6,10,14]. However, according to Cherfas the conjugation of homologous chromosomes, crossover and reductional division did not occur in the course of maturation. Even the egg undergoes two maturational divisions, but the first meiosis is abortive and the second maturation division is completed soon after the penetration of the sperm, when the only polar body is separated and triploid group of female chromosomes remains in the egg[24].

The facts of external morphological traits and isozyme patterns of artificial gynogenetic offspring produced by diploid hybrid between common carp and crucian carp being identical with their mother demonstrated that the female produced unreduced egg and female nucleus was deficient in first meiosis. The identity of external morphological traits and isozyme patterns of gynogenetic fingerling of allotriploid carp with its mother also intimated female nucleus of allotriploid carp without first meiosis. The minority of oocyte of allotriploid carp could pass entire oogenesis process and become a normal mature egg, but majority of oocyte was abortive. It was assumed that abortive oocytes might be blocked at first meiosis stage. By which mechanism mature egg passes through entire oogenesis process still remains to be further studied.

3.3 Regarding transforming of reproduction mode

Numerous gynogenetic triploid fishes have been found in the nature, including *Poecilia formosa*, *Poeciliopsis monach-lucida*, *Phoxinus eos-neogaeus*, *Menidia clarkhubbsi*, *Rutilus alburnoides*, *Carassius auratus gibelio* and *Carassius auratus langsdorfii*[1,2,4,7–9,25–27]. The analysis of chromosomal complements and electrophoretic data have indicated that the triploid form originated from crosses of diploid hybrid females with males of related species[5,10]. Based on studies with unisexual fishes of the genus *Poeciliopsis* Schultz suggested that normal meiotic processes often were disrupted in interspecific hybrids and these hybrids produced unreduced ova that, upon backcrossing with one of the sexual ancestors, lead to new polyploid (allotriploid) biotypes[28]. Quattro et al. referred to this pathway for origin of allotriploid biotypes from allodiploid ancestors as the “genome addition”[29]. Ova produced from these hybrid original allopolyploid female are usually provided natural gynogenesis. Moreover, with more than 2 genomes these kinds of ova can normally divide and develop into viable offspring after activation by male pronucleus. According to our results, oogonia of diploid hybrid would develop into unreduced oocyte due to the abortive first meiosis. Female pronucleus of this kind of mature egg would fuse male pronucleus penetrating into egg and allotriploid progeny was obtained. Part of oocyte with three genomes from female individuals of allotriploid could develop into mature, and its female pronucleus could not fuse with penetrating male pronucleus but it yielded gynogenetic allotriploid progeny. Biological traits of these allotriploid offspring were similar to their allotriploid mother. It was found that female pronucleus of rare individuals of allotriploid carp could fuse with penetrating male

pronucleus. In this case, the allotetraploid offspring were obtained from three female genomes and one paternal genome. Thus, a lineage of transforming bisexual diploid carp into two new gynogenetic clones of allotriploid and allotetraploid by means of cross and genome addition was established. The data from this lineage revealed that hybrid constitution of chromosomal complement might prevent the reduction of the female chromosomes in the course of oocyte maturation. Furthermore, genome addition (allotriploid and allotetraploid) of female pronucleus and male pronucleus might induce female nucleus change from gametogamy to pseudogamy, and bisexual was changed into unisexual in the reproduction mode. Also, these results provide additional evidence about hypothesis of hybrid originating of polyploid fishes.

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