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Autotriploid origin of *Carassius auratus* as revealed by chromosomal locus analysis

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In the Dongting water system, the *Carassius auratus* (Crucian carp) complex is characterized by the coexistence of diploid forms (2*n*=100, 2nCC) and polyploid forms. Chromosomal and karyotypic analyses have suggested that the polyploid *C. auratus* has a triploid (3*n*=150, 3nCC) and a tetraploid origin (4*n*=200), respectively. However, there is a lack of direct genetic evidence to support this conclusion. In this paper, analysis of the 5S rDNA chromosomal locus revealed that the 3nCC is of triploid origin. Analysis of the species-specific chromosomal centromere locus revealed that 3nCC individuals possess three sets of *C. auratus*-derived chromosomes. Our results provide direct cytogenetic evidence suggesting that individuals with 150 chromosomes are of autotriploid origin within the *C. auratus* complex. It marks an important contribution to the study of polyploidization and the evolution of vertebrates.

autotriploids, chromosomal loci, polyploidization, evolution

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INTRODUCTION

Crucian carp (*Carassius auratus*) is distributed throughout Eurasia. Although three types of ploidy have been documented in *C. auratus gibelio* (Cherfas, 1966; Zhou and Gui, 2002; Zhu et al., 2006), *C. auratus* was thought to be presented only in the diploid form (2*n*=100; 2nCC, indicates the C. auratus with 100 chromosomes) (Yu, 1989; Zan and Song, 1980). Since the 1980s, triploid (3*n*=150; 3nCC, indicates the *C. auratus* with 150 chromosomes) and tetraploid (4*n*=200; 4nCC) forms have been documented (Xiao et al., 2011). Thus, *C. auratus* is also characterized by the coexistence of diploids and polyploids. In *C. auratus* complex populations (Jakovlić and Gui, 2011; Jiang et al., 2013; Luo et al., 2014), the diploids and the polyploids are quite similar morphologically, but differ markedly in their modes of reproduction. The diploid form reproduces sexually, whereas the polyploid form has dual reproduction modes, with both unisexual gynogenesis and sexual reproduction (Koboyasi, 1971; Mezhzherin and Kokodiy, 2010; Yang et al., 1992). The chromosomal and karyotypic diversity documented in the *C. auratus* complex suggests that the polyploid forms (3nCC and 4nCC) have triploid and tetraploid origin (Xiao et al., 2011; Yu et al., 1992; Zan, 1982). There is, however, a lack of direct genetic evidence to support this conclusion. Similarly, it is unclear whether the supernumerary set of chromosomes originated from similar or closely related individuals ("autopolyploid"), or from a different species ("allopolyploid").

The 5S rDNA and centromere sequences have numerous copies and show obvious species-specificity. Their location on the chromosomes is easily detected by fluorescence *in-situ* hybridization (FISH) with labeled 5S rDNA or species-specific centromere probes (He et al., 2012; Martins

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and Galetti, 2001; Murakami and Fujitani, 1998; Qin et al., 2014a, 2014b, 2014c). In a previous study using 5S rDNA and species-specific centromere probes, we demonstrated that tetraploid hybrids (4*n*=148, AABB) of *C. auratus* var. red (2*n*=100, AA) (♀)×*Megalobrama amblycephala* (2*n*=48, BB) (♂) can generate autotriploid gametes with three sets of crucian carp-derived chromosomes (3*n*=150, AAA) and autodiploid gametes with two sets of crucian carp-derived chromosomes (2*n*=100, AA) (Qin et al., 2014b, 2014c). By using 5S rDNA and species-specific centromere probes, we provide direct cytogenetic evidence showing that *C. auratus* with 150 chromosomes is autotriploid in origin. This study possesses great significance for the study of polyploidization and the evolution of vertebrates.

RESULTS

Examination of chromosome number

The distribution of chromosome numbers in 2nCC and 3nCC individuals is presented in Table 1. Among the 2nCC individuals, 92% of the chromosomal metaphases had 100 chromosomes (Figure 1A), indicating that they are diploid with 100 chromosomes. Among the 3nCC individuals, 83.5% of the chromosomal metaphases possessed 150 chromosomes (Figure 1B), indicating that they are triploids with 150 chromosomes. Several uncounted micro-chromosomes were found in 3nCC individuals (Figure 1B, arrows). Over the past three years (2012–2014), we have examined 300 *C. auratus* samples from the Dongting water system. In these samples, 2nCC and 3nCC individuals accounted for 22% and 78%, respectively, of the catch, whereas no tetraploid *C. auratus* (4*n*=200) were found.

Table 1 Examination of chromosome numbers

Fish type	No. of metaphase	Distribution of chromosome numbers			
		<100	100	<150	150
2nCC	200	16	184		
3nCC	200			33	167

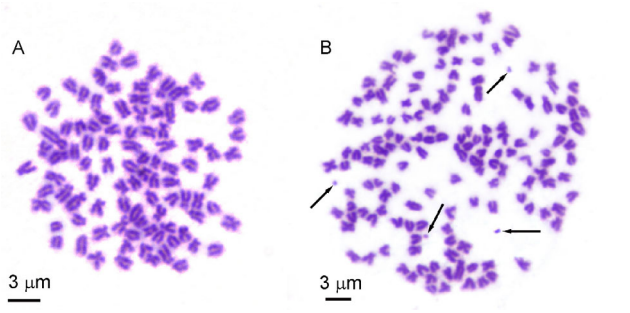

Figure 1 Chromosome spreads at metaphase in 2nCC and 3nCC. A, The metaphase chromosome spreads of 2nCC individuals possess 100 chromosomes. B, The metaphase chromosome spreads of 3nCC individuals possess 150 chromosomes, in some samples uncounted micro-chromosomes were found (indicated by arrows).

Fluorescence *in-situ* hybridization

The 5S rDNA probe (340 bp) hybridized with the metaphase chromosomes of 2nCC and 3nCC, in both *Cyprinus carpio* and *Megalobrama amblycephala*. The results of the FISH are shown in Table 2. Hybridization of the 5S rDNA probe yielded two large 5S rDNA loci and two small loci in 86% of the chromosomal metaphases from 2nCC individuals (Figure 2B). The chromosomal locus map revealed that two large 5S rDNA loci were located on a homologous submetacentric chromosome, while two small 5S rDNA loci

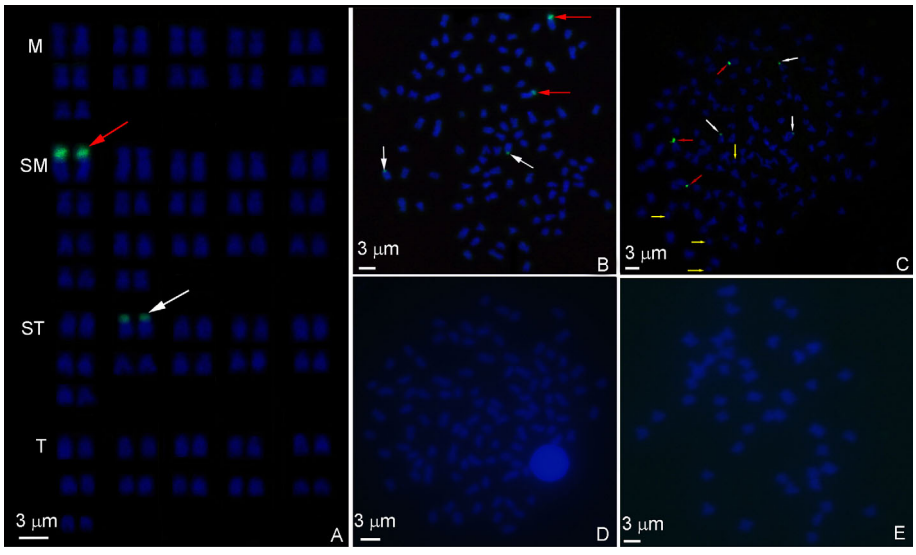

Figure 2 Examination of hybridizing signals by the 5S rDNA probe. A, The karyotype of 2nCC indicated that one pair of large 5S rDNA loci was located on a homologous submetacentric chromosome (red arrows), and that one pair of small 5S rDNA loci was located on a homologous subtelocentric chromosome (white arrows). B, There were two large 5S rDNA loci (red arrows) and two small 5S rDNA loci (white arrows) in 2nCC. C, There were three large 5S rDNA loci (red arrows) and three small 5S rDNA loci (white arrows) in 3nCC. Yellow arrows indicate micro-chromosomes. D, No 5S rDNA locus was found in *Cyprinus carpio*. E, No 5S rDNA locus was found in *Megalobrama amblycephala*.

were located on a homologous subtelocentric chromosome (Figure 2A). Hence, 2nCC individuals possess one large and one small 5S rDNA locus per chromosome set. Hybridization of the 5S rDNA probe yielded three large 5S rDNA loci and three small loci in 79% of the chromosomal metaphases from 3nCC individuals (Figure 2C), suggesting that 3nCC individuals have three sets of chromosomes. However, no 5S rDNA locus was found in *C. carpio* or *M. amblycephala* (Figure 2D and E).

The species-specific centromere probe hybridized with 100 chromosomes in 2nCC individuals (Figure 3A), but none in *C. carpio* (Figure 3C), *M. amblycephala* (Figure 3D), *Ctenopharyngodon idella*, or *Erythroculter ilishaeformis* (Table 2), suggesting that this probe is a species-specific marker of *C. auratus* chromosomes. Among the 3nCC samples, the centromere probe hybridized to 150 chromosomes in 84.5% of the metaphase spreads (Figure 3B), suggesting that 3nCC individuals possessed 150 *C. auratus*-derived chromosomes. Our results provide direct evidence that 3nCC is of autotriploid origin.

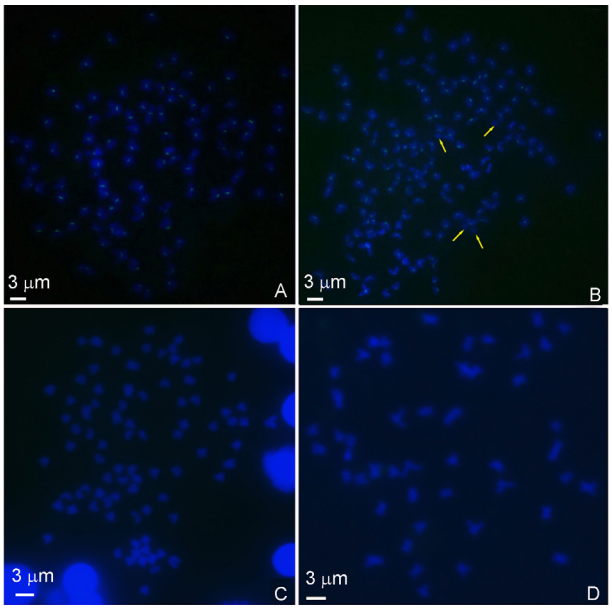

Figure 3 Examination of hybridizing signals by the species-specific centromere probe. A, The centromere probe hybridized to 100 chromosomes in 2nCC. B, The centromere probe hybridized to 150 chromosomes in 3nCC, yellow arrows indicate micro-chromosomes. C, No chromosome was hybridized by the centromere probe in *Cyprinus carpio*. D, No chromosome was hybridized by the centromere probe in *Megalobrama amblycephala*.

Table 2 Examination of chromosomal locus number

Fish type	No. of chromosomes	Centromere probe	5S rDNA gene probe	
		No. of loci	No. of large loci	No. of small loci
2nCC	100	100	2	2
3nCC	150	150	3	3
Cyprinus carpio	100	0	0	0
Megalobrama amblycephala	48	0	0	0
Ctenopharyngodon idella	48	0	No test	
Erythroculter ilishaeformis	48	0	No test	

DISCUSSION

Teleosts are widely believed to have undergone an additional round of whole-genome duplication, referred to as the 3R hypothesis, in contrast with mammals. This third round of whole-genome duplication is specific to ray-finned fish, possibly occurring about 360 million years ago, thus preceding the teleost divergence (Venkatesh, 2003). The chromosome number of common carp and crucian carp ($2n$=100) is twice that of most other *Cyprinidae*. Thus, it has been assumed that *C. carpio* and *C. auratus* have undergone a fourth round of genome duplication (Luo et al., 2007; Wang et al., 2012). In recent years, many studies have revealed that polyploid *C. auratus* have undergone several successive rounds of genome polyploidy and that they have experienced an additional, more recent genome duplication event (Gui and Zhou, 2010; Li et al., 2014; Luo et al., 2014). In the Dongting water system, the *C. auratus* complex is characterized by the coexistence of diploids (2nCC) and polyploids (3nCC and 4nCC), which are quite similar morphologically but differ markedly in their modes of reproduction (Koboyasi, 1971; Mezhzherin and Kokodiy, 2010; Yang et al., 1992). Although triploid *C. auratus* did not arise through whole genome duplication of the diploid *C. auratus* ($2n$=100) genome, genome duplication may still occur contemporarily and may take place spontaneously in nature. Tetraploid ($4n$=200) individuals have been documented in *C. auratus* populations in the Dongting water system (Xiao et al., 2011), but were not detected during our sampling. This is likely because tetraploid individuals are rare in the mixed diploid-polyploid *C. auratus* populations, and were therefore not detected during our limited sampling.

The chromosomal and karyotypic diversity reported in the *Ca. auratus* complex suggests that the polyploid *C. auratus* (3nCC and 4nCC) have a triploid and tetraploid origin (Yu et al., 1992; Zan, 1982). However, to our knowledge, to date, there was no direct genetic evidence for this. Although analyses of both mitochondrial and nuclear DNA sequences have revealed that recurrent autoploidization occurs in all sampled populations of the *C. auratus* complex (Luo et al., 2014), our most important finding was the direction of cytogenetic evidence for the autotriploid origin of *C. auratus* with 150 chromosomes. What was the possible origin of the autotriploid forms in the *C. auratus* complex? Several researchers have speculated that the formation of polyploid fish in nature is attributable to hybridization. For example, *C. auratus gibelio* are believed to have originated from the ancient hybridization of a diploid female crucian carp and a

male common carp (Fan et al., 2001). Usually, because chromosomes originate from two different species, distant hybridization generally induces formation of allopolyploids but not autopolyploids (Liu, 2010). Indeed, distant hybridization can also induce autopolyploids through a special meiosis mechanism (Qin et al., 2014c, 2015; Xu et al., 2015). In the previous study, we obtained fertile allotetraploid fish (4*n*=148, AABB) from interspecific hybridization of red crucian carp (2*n*=100, AA) (♀)×blunt snout bream (2*n*=48, BB) (♂) (Liu et al., 2007; Qin et al., 2010). Interestingly, complete separation of the parental genomes during meiosis occurred in the allotetraploid fish, which gave rise to autotriploid gametes (AAA), autodiploid gametes (AA), and haploid gametes (A) (Qin et al., 2014b). Consequently, the diploid sperm and eggs of the allotetraploid were fertilized to produce autotetraploid progeny (4*n*=200, AAAA). Thereafter, F_2–F_8 generations of the autotetraploid were formed (Qin et al., 2014c). Thus, we speculate that autotriploid crucian carp in the Dongting water system were derived from hybridization of diploid female crucian carp and a male of a different species.

MATERIALS AND METHODS

Source of samples

C. auratus individuals were collected from the Yuanjiang River and Xiangjiang River in the Dongting water system, Hunan Province from 2012 to 2014. Fish were reared and bred at the Reproduction of State Education Ministry at Hunan Normal University. Fish were treated in accordance with the Care and Use of Agricultural Animals in Agricultural Research and Teaching guidelines, and their use was approved by the Science and Technology Bureau of China. Approval from the Department of Wildlife Administration was not required for the experiments conducted in this paper. Fish were heavily anesthetized using 100 mg L^{-1} MS-222 (Sigma-Aldrich, USA) before dissection.

Preparation of chromosome spreads

To determine ploidy, chromosomal preparations were obtained from peripheral blood cell cultures of 15-month-old fish. Briefly, 0.2 mL blood was collected from each fish, using a syringe soaked in 0.1% sodium heparin. Cells were cultured in nutrient solution at 25.5°C and 5% CO_2 for 68–72 h, then colchicine was added 3.5 h before harvest. Cells were harvested by centrifugation, subjected to hypotonic treatment with 0.075 mol L^{-1} KCl at 26°C for 25–30 min, and then fixed in methanol-acetic acid (3∶1, v/v) with three changes. Cells were dropped onto cold slides, air-dried, and stained for 30 min in 4% Giemsa solution. Chromosome preparations were examined under an oil lens at a magnification of 3330×. Good-quality metaphase spreads were photographed. For each fish sample, 200 metaphase spreads (20 metaphase spreads in each sample) were examined.

Fluorescence *in-situ* hybridization (FISH)

The probes for FISH of the 5S rDNA and species-specific centromeres were constructed for 2nCC and amplified by PCR using the primers 5′-GCTATGCCCGATCTCGTCTGA-3′ and 5′-CAGGTTGGTATGGCCGTAAGC-3′, and the primers 5′-TTCGAAAAGAGAGAATAATCTA-3′ and 5′-AACTCGTCTAAACCCGAACTA-3′, respectively. The FISH probes were produced by Dig-11-dUTP labeling (using a nick translation kit, Roche, Germany) of purified PCR products. FISH was performed according to the method described by He et al., 2012. For each type of fish, 200 metaphase spreads (20 metaphase spreads in each sample) of chromosomes were analyzed.

Authors' contributions *Qinbo Qin and Shaojun Liu designed the experiments; Juan Wang, Min Hu and Shennan Huang performed the experiments; Qinbo Qin performed the statistical analysis and wrote the manuscript. All authors read and approved the final manuscript.*

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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