The chromosomal constitution of fish hybrid lineage revealed by 5S rDNA FISH

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Abstract

Background: The establishment of the bisexual fertile fish hybrid lineage including the allodiploid and allotetraploid hybrids, from interspecific hybridization of red crucian carp (Carassius auratus red var. 2n = 100, 2n = AA) (♀) × common carp (Cyprinus carpio L. 2n = 100, 2n = BB) (♂), provided a good platform to investigate genetic relationship between the parents and their hybrid progenies.

Results: The chromosomal inheritance of diploid and allotetraploid hybrid progenies in successive generations, was studied by applying 5S rDNA fluorescence in situ hybridization. Signals of 5S rDNA distinguished the chromosomal constitution of common carp (B-genome) from red crucian carp (A-genome), in which two strong signals were observed on the first submetacentric chromosome, while no major signal was found in common carp. After fish hybridization, one strong signal of 5S rDNA was detected in the same locus on the chromosome of diploid hybrids. As expected, two strong signals were observed in 4nF3 tetraploid hybrids offspring and it is worth mentioning that two strong signals were detected in a separating bivalent of a primary spermatocyte in 4nF3. Furthermore, the mitosis of heterozygous chromosomes was shown normal and stable with blastular tissue histological studies.

Conclusions: We revealed that 5S rDNA signal can be applied to discern A-genome from B-genome, and that 5S rDNA bearing chromosomes can be stably passed down in successive generations. Our work provided a significant method in fish breeding and this is important for studies in fish evolutionary biology.

Keywords: Interspecific hybridization, Heterozygous chromosomes, FISH, 5S rDNA

Background

In general, interspecific hybridization and polyploidy in plants were potent evolutionary mechanisms [1, 2]. Recently, new genetic evidence suggested that hybrid speciation was more common in plants and animals than we thought, and it has played a very constructive part in animal evolution [3, 4]. As fertile hybrids, the integrity and inheritance of heterozygous genome was a focused issue, which concerned to mitotic and meiotic stability of hybrid offspring and formation of fertile progenies. Now the inheritance rule of heterozygous genome had been widely studied in hybrid plants, while as there was a limit to the material, related research only focused on such a few hybrid vertebrates as fish and frog [5, 6].

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fish were produced. Until now, the F$_{24}$-F$_{24}$ AT has been formed through successive breeding. Our previous study has indicated that the AT has inherited large amount of genetic material from their original parents with some variations [11–15]. However, the chromosomes constitution and inheritance pattern of hybrid lineage were still unknown. Identification of all genome chromosomes was crucial to understanding the genome constitution and inheritance rule of the hybrid genome. It has been widely studied in hybrid plant, using by fluorescence in situ hybridization (abbreviated as FISH), and genomic in situ hybridization (abbreviated as GISH) [16–18]. However, due to the lack of chromosome-specific molecular probes, the current reported studies were few in hybrid fish related to chromosomal localization.

The 5S rDNA in higher eukaryotes was organized in tandem repeat units that consisted of highly conserved 120 bp transcribing sequence and variable non-transcribed spacers (abbreviated as NTS) [19]. Variations in NTS, related to insertions-deletions, minirepeats and pseudo genes, were often species specific and have successfully been served as markers in evolutionary studies [20–22]. In addition, due to the numerous copies of repeated sequences, their chromosomal localization was easily detected by FISH. Analysis of 5S rDNA sequences and chromosomal localization of them were an effective method of genetic diversity monitoring, which were widely used to explore the phylogeny relationship among closer species and polyploidy origin [23–25]. Masaru and Hideo [26] also found that the 5S rDNA could be candidates for phylogenetic molecular markers for the crucian carp.

In the current study, based on our establishment of 2nF$_1$, 2nF$_2$, 4nF$_3$ and 4nF$_{22}$ hybrid lineage of RCC×CC, a comparative analysis of 5S rDNA fragments among all samples were carried by PCR and related sequences were screened out as probes to determine the chromosomal localization of the 5S rDNA for 2nF$_1$, 2nF$_2$, and 4nF$_3$ fish by FISH, which elucidated the inheritance rules of heterozygous chromosomes in course of polyploid hybrid fish origin and propagation. Furthermore, cytological observation of early embryos in blastula stage was used to determine the mitotic stability in hybrid offspring.

**Methods**

**Ethics statement**

All experiments were approved by Animal Care Committee of Hunan Normal University and followed guidelines statement of the Administration of Affairs Concerning Animal Experimentation of China. All samples are raised in natural ponds and all dissections are performed under sodium pentobarbital anesthesia, and all efforts are made to minimize suffering.

**Fishes and genomic DNA samples**

Specimens of RCC (2n = 100), CC (2n = 100), 2nF$_1$ (2n = 100), 2nF$_2$ (2n = 100), 4nF$_3$ (4n = 200) and 4nF$_{22}$ (4n = 200) were obtained from the Engineering Center of Polyploid Fish Breeding of National Education Ministry located at Hunan Normal University. Total genomic DNA was isolated from the peripheral blood cells according to the standard phenol-chloroform extraction procedures described by Sambrook et al. [27] with minor modifications.

**PCR amplification, cloning and sequencing**

One pair of primers (5'-TATGCCCGATCTCGTCT GATC-3' and 5'-CAGGGTGTATGGCCGTAAGC-3') [26] was synthesized to amplify the 5S rDNA repeats directly from genomic DNA by PCR. The genomic DNA of RCC, CC, 2nF$_1$, 2nF$_2$, 4nF$_3$ and 4nF$_{22}$ were used as a template for subsequent PCR (2 to 10 individuals for each of the 6 samples). The PCR cycling conditions were: 5 min at 94 °C, 30 cycles of denaturation at 94 °C for 30s, annealing at 56 °C for 30s, and extension 72 °C for 1 min, ending with 10 min of extension at 72 °C. The PCR products were analyzed in 1.2 % agarose gels stained by ethidium bromide, purified by Gel Extraction Kit (Sangon), cloned into the pMD18-T vector (Takara), and transferred into E. coli DH5α. Then the positive clones were sequenced by Sangon. The sequences were analyzed by ClustalW2.

**Chromosome preparation**

The kidney cells in RCC, CC, 2nF$_1$, 2nF$_2$ and 4nF$_3$ were used for the chromosome observation at the metaphase of mitosis and the testis cells in 4nF$_3$ were used to observe the process of meiosis. The concanavalin A was injected into the abdominal cavity of the samples for one to three times at the dosage of 2-8 μg/g. The interval time was 12–24 h. Two to six hours prior to harvest, colchicines (2-4 μg/g) were used to arrest the chromosome at the metaphase. All the kidney tissue in the above samples was ground in 0.8 % NaCl. The hypotonic treatment was accomplished with 0.075 mol/L KCl for 40–60 min, followed by fixation in 3:1 methanol-acetic acid (three changes). The cells were spread on clean slides.

**Probe preparation of the 5S rDNA sequence and fluorescence in situ hybridization**

FISH is used to assess chromosomal location of 5S rDNA of RCC, CC, 2nF$_1$, 2nF$_2$, and 4nF$_3$ fish. The hybridization was performed according to the method described by Masaru and Hideo with minor modifications [26]. In brief, the purified PCR
product of related 5S rDNA fragments of RCC labeled with Dig-11-dUTP by PCR DIG Probe Synthesis Kit (Roche, Germany) were used as probes. After pretreatment with 2 × SSC for 30 min, 70 %, and 100 % ethanol for 5 min each, the slides with chromosome metaphase spreads of all samples were denatured in 70 % deionized formamide /2 × SSC for 2 min at 75 °C, dehydrate in a 70 % (−20 °C, to avoid DNA renaturation) and 100 % ethanol series for 5 min each, and then air-dry. The probe was prepared by adding labeled DNA with 20 × SSC, deionized formamide and dehydrating in boiling water for 5 min. Hybridizations were allowed to proceed under a sealed cover slip in a moist chamber at 37 °C overnight. The next day the slides were washed; twice for 15 min in 2 × SSC with 50 % formamide and then in 2 × SSC and 1 × SSC for 5 min each. After a series of post-hybridization washing were performed, the spectrum signals were achieved with 10ul of 1ug ml−1 FITC-conjugated anti-digoxigenin antibody from sheep (Roche, Germany). Then the slides were washed three times for 5 min each in TNT (formulas of eluate: 0.1 M Tris–HCl, 0.15 M NaCl,0.05 % Tween 20). After slides were placed in 10ul anti-fade solution containing 0.5 μg/ml of 4, 6-diamidino-2-phenylindole (abbreviated as DAPI), they were viewed using a Leica inverted DMIRE2 microscope image system (Leica, Germany). Images were captured with CW4000 FISH software (Leica, Germany). Good-quality metaphase spreads were photographed and used for analysis of karyotypes.

Cytological observations of early embryos
After 6 h after fertilization, the F3 embryos in blastula stage were fixed in Smith solution for 4 to12 h, and then are washed by alcohol for 2 to 3 times. The paraffin-embedded sections were cut at 6 μm and stained with Harris hematoxylin and eosin. The structure of zygote was observed under a light microscope and photographed with Pixera Pro 600 ES.

Results
Analysis of 5S rDNA fragments
The electrophoretic band pattern of DNA fragments amplified with the primers of 5 s rDNA was distinctive between RCC and CC. RCC exhibited two major DNA bands (approximately 200 bp and 340 bp) and some repeated ladder-like bands, CC exhibited other two major DNA bands (approximately 200 bp and 400 bp) and some repeated ladder-like bands as well. As their hybrid offspring, 2nF1 and 4nF22 exhibited three major DNA bands (approximately 200 bp, 340 bp and 400 bp) and some repeated ladder-like bands. Consensus sequences of the 5 s rDNA repeat units were obtained. All of the typical sequences were deposited at GenBank under the accession numbers (KM359661- KM359679) (Table 1).

The NTS sequence of 5S rDNA also showed an extensive variation between RCC and CC. In RCC, different-sized 5S rDNA units contained one 120 bp coding region and a variable NTS. In CC, there was only one kind of fragment of 5 s rDNA (202 bp, 340 bp, 477 bp and 613 bp) were characterized by different lengths of NTS. In CC, there was only one kind of fragment of 5 s rDNA (202 bp) with 120 bp coding region and one 82 bp NTS unit. The 400 bp fragment from amplified DNA bands was just two repeats of 202 bp unit. In 2nF1 and 4nF22, like their original parents, two kinds of fragments of 5 s rDNA (approximately 200 bp and 340 bp) both contained similar coding region and NTS (Table 1).
120 bp coding region and one monomeric 82 bp repeat unit were identified. The similarity of 82 bp-unit among different clones of RCC was 95.1 % and the similarity of 82 bp-unit among different clones of CC was 91.5 %, while the 82 bp-unit of RCC was different from 82 bp-unit of CC with lower similarity of 52.4 %-68.3 % (Table 2). Sequence alignments showed high similarity of 340 bp units between RCC, 2nF1, and 4nF22, even in gibel carp, Carassius auratus (abbreviated as Cag) [25] (Fig. 2 and Table 3), so the 340 bp-fragments were screened out as a probe to assess chromosomal location of 5S rDNA of RCC, CC, 2nF1, 2nF2, and 4nF3 fish.

### Table 1

| Species | Length(bp) | Coding sequence | NTS | Accession no. |
|---------|------------|-----------------|-----|--------------|
| RCC     | 201        | 119             | 82 = 0*138 + 82ₐRCC-a | KM359661 |
|         | 202        | 120             | 82 = 0*138 + 82ₐRCC-b | KM359662 |
|         | 340        | 121             | 219 = 1*137 + 82 | KM359663 |
|         | 339        | 121             | 218 = 1*136 + 82 | KM359664 |
|         | 477        | 121             | 356 = 2*137 + 82 | KM359665 |
|         | 613        | 120             | 493 = 3*137 + 82 | KM359666 |
| CC      | 202        | 120             | 82 = 0*138 + 82ₐCC-a | KM359667 |
|         | 204        | 121             | 83 = 0*138 + 82ₐCC-b | KM359668 |
|         | 203        | 121             | 82 = 0*138 + 82 | KM359671 |
|         | 201        | 120             | 82 = 0*138 + 82 | KM359672 |
|         | 341        | 120             | 219 = 1*137 + 82 | KM359673 |
|         | 339        | 120             | 219 = 1*137 + 82 | KM359674 |
| 2nF1    | 201        | 119             | 82 = 0*138 + 82 | KM359675 |
|         | 202        | 120             | 82 = 0*138 + 82 | KM359676 |
|         | 338        | 119             | 219 = 1*137 + 82 | KM359677 |
|         | 477        | 120             | 357 = 2*138 + 82 | KM359678 |
|         | 639        | 122             | 504 = 3*137 + 93 | KM359679 |

RCC-ₐ, RCC-ₐ, CC-ₐ, CC-ₐ: indicate the different approximately 82 bp-units. The alignment data of them was showed in Table 2.

### Table 2

| Sequence name | RCC-ₐ | RCC-ₐ | CC-ₐ | CC-ₐ |
|---------------|-------|-------|------|------|
| RCC-ₐ         | -     | 95.1 %| 68.3 %| 53.7 %|
| RCC-ₐ         | -     | -     | 68.3 %| 52.4 %|
| CC-ₐ          | -     | -     | -    | 91.5 %|
| CC-ₐ          | -     | -     | -    | -    |

55 rDNA localization

The diploid RCC, CC, 2nF1, and 2nF2 samples showed 100 chromosomes which were classified into 22 metacentric (abbreviated as M), 34 submetacentric (abbreviated as SM), 22 subtelocentric (abbreviated as ST) and 22 telocentric (abbreviated as T) chromosomes. The tetraploid 4nF3 samples revealed 200 chromosomes, which were classified into 44 metacentric (M), 68 submetacentric (SM), 44 subtelocentric (ST) and 44 telocentric (T) chromosomes [7, 28]. No difference was observed between the females and the males.

Two signals of 340 bp-fragment were clearly detected in the first SM chromosomes pair of RCC, with a few weak signals being detected in other chromosomes (Fig. 3a, b), while the CC fish has no major signal. As to their hybrid lineage offspring, one strong signal was found in the first SM chromosome of 2nF1 and 2nF2 fish (Fig. 3c, d) and two strong signals were detected in the same chromosomes of AT (Fig. 3e, f), also with a few weak signals being detected in other chromosomes.

Chromosomal localization of 340 bp-fragment in metaphase of meiosis spreads in primary spermatocytes of 4nF3 indicated that two strong signals being located on a separating bivalent (Fig. 3g, h).

### Cytological observation of early embryos in blastula stage

Cytological observation of mitosis in F3 early embryo cells showed that F3 performed normal and stable mitosis, with normal distribution of chromosomes.
Discussion

Different 5 s rDNA classes were reported in several mammals and fish species, and generally two different type of 5 s rDNA units have been characterized by distinct NTS types and base substitution [29, 30]. In our work, the construction of different-sized 5S rDNA units were further elucidated as containing one 120 bp coding region and one 82 bp repeat unit, which were spaced at regular interval with zero, one, two, or three 138 bp-IPR repeat units (Table 1), this result revealed the assembly rule of NTS in RCC, which enriched the current data of molecular organization analysis of 5 s rDNA.

Modern cytogenetic analyses, such as FISH and GISH have been widely applied for studies in many processes of chromosome evolution, including structural rearrangements, as well as extensive studies on phylogenetic and genomic relationships [31]. In this work, chromosome inheritance of hybrid lineage progenies of RCC × CC were investigated by detecting the signal of 340 bp fragments of 5 s rDNA on chromosomes in successive generations, i.e. RCC, CC, 2nF1, 2nF2, and 4nF3 fish. The 340 bp fragments was highly conserved between RCC and their hybrid lineage progenies, but not in CC genome, which made the 340 bp fragments in RCC a suitable indicator to monitor chromosomal inheritance in RCC, CC and their hybrid progeny, as it was recently showed in other hybrid fish [32, 33]. Besides that, our results revealed that the chromosomal localization of the 340 bp fragments of RCC could serve as a suitable genetic marker to distinguish the chromosomal constitution of CC from RCC, as two strong detected signals on the first pair of larger-sized SM chromosomes were observed, but not in CC chromosomes. The other weak FISH signals were also 5S rDNA locus with less copy of repeat units.

Since there have been very few studies on chromosomal inherence behavior in fish hybridization, we looked into the 5S rDNA bearing chromosome in successive hybrid generation. The strong signal with same locus as observed in RCC were also found in 2nF1 and 2nF2 fish and the number of signal doubled in 4nF3 fish. Furthermore, two strong signals were also detected in a separating bivalent of a primary spermatocyte in 4nF3, which promised that two 5S rDNA sites would equally distributed to each gamete and made every gamete acquire one 5S rDNA bearing chromosome. Those results may suggest that 2nF1 and 2nF2 fish contained one 5S rDNA bearing chromosome from A-genome and one 5S rDNA bearing chromosome from B-genome, and 4nF3 fish possessed two 5S rDNA bearing chromosomes from A-genome and two 5S rDNA bearing chromosomes from B-genome. Additional studies was made in order to investigate the mitosis, results demonstrated that in hybridization the mitosis process maintained normal and stable, which demonstrated the compatibility of genome between RCC and CC, and promised the genetic stability of hybrid progeny. Based on the fish analysis and cytological observation, we speculate that chromosomes of RCC and CC may equally pass down to hybrid generation (Fig. 5).

The chromosomal rearrangements having occurred less frequently was a promising result supporting the development of stable allotetraploid hybrid lines,
Fig. 3 Mitotic metaphase spreads and karyotypes of RCC, 2 nF₂ and 4 nF₃ fish with FISH signals of 5S rDNA. (a) Two strong and a few weak signals were detected in RCC fish; the boxes indicated two major signals of 5S rDNA (green). (b) The karyotype of RCC fish revealed two strong signals located on the first pair of larger-sized SM chromosomes. (c) One strong signal and a few weak signals in 2nF₂ fish (green); the box indicated the one major signal of 5S rDNA (green). (d) The karyotype of 2nF₂ fish show one strong signal on the first group of larger-sized SM chromosomes. (e) The FISH analysis of 4nF₃ fish exhibited the double signal feature of two strong and a few weak signals (green), the boxes indicated the two major signals (green). (f) The karyotype of 4nF₃ fish showed two strong signals on the first group of larger-sized SM chromosomes. All metaphase chromosomes were counterstained with DAPI and appear blue. (g) The chromosome pairing in Meiosis I of spermatocytes in 4nF₃ showed 100 bivalents under light microscope. (h) Two signals of 5S rDNA located on a separating bivalent. M: metacentric; SM: submetacentric; ST subtelocentric; T: telocentric. (a-f) Bar = 3 μm; (g,h) Bar = 5 μm.
which were also proved in allohexaploid *Brassica* line [34] and the lineage of *X.laevis* [35]. However, the allotetraploid *Festuca pratensis × Lolium perenne* hybrid of three generations shared various rDNA loci profiles with chromosomal rearrangements [31], indicating a tendency of *F. pratensis* genome-like chromosomes to be less stable in hybrid of three generations. In our work, the strong FISH signals of 5S rDNA in RCC passed down stably in successive hybrid generation, which implied that chromosomal rearrangements have occurred less frequently in area of highly repeated sequence of 5S rDNA. Thus it can be seen that genomic contribution was different in different type of hybridized combinations, which will affect the genetic stability of hybrid progenies. Further studies were necessary to prove or reject this hypothesis as well as for deeper understanding of the mechanisms responsible for the fate of heterozygous chromosomes in hybrid lineage progenies.

**Conclusions**

Few studies focused on chromosomal inheritance in successive generations of hybrid fish. We executed the 5S rDNA FISH analysis in successive generations of diploid and allotetraploid hybrid progenies and revealed 5S rDNA signal can be applied to discern A-genome from B-genome, and that 5S rDNA bearing chromosomes respectively coming from A-genome and B-genome can be stably passed down in successive hybrid generations.

**Abbreviations**

AT: Allotetraploid hybrid fish; RCC: Red crucian carp; CC: Common carp; 2nF₁: The first generation of diploid hybrid fish of RCC × CC; 2nF₂: The second generation of diploid hybrid fish of RCC × CC; 4nF₃: The first generation of tetraploid hybrid fish of RCC × CC; Cag: Gibel carp, *Carassius auratus*; FISH: Fluorescence in situ hybridization; GISH: Genomic in situ hybridization; NTS: Non-transcribed spacers; IPR: Interposed region; DAPI: 4, 6-diamidino-2-phenylindole; M: Metacentric; SM: Submetacentric; ST: Subtelocentric; T: Telocentric.

**Competing interests**

The authors declared no conflict of interest.

**Authors’ contributions**

CZ, LY and SL designed the study, carried out the analyses, performed the technical discussions, prepared and draft the manuscript. YC, JX and MT participated in data simulation and discussions. YW and YX were involved in the statistical analysis. All authors read and approved the final manuscript.

**Acknowledgments**

This work is supported by the National Natural Science Foundation of China (30901100/31430088), the Cooperative Innovation Center of Engineering and New Products for Developmental Biology of Hunan Province (20134486),

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**Fig. 4** Cytological observations of early embryos in blastula stage. (a) Early embryo cells in metaphase of mitosis; (b) Early embryo cells in anaphase of mitosis; (c) Early embryo cells in telophase of mitosis; the arrowheads showed the normal distribution of chromosomes and structure of spindle body. Bar = 10 μm.

**Fig. 5** Schematic presentation of chromosome transmission in hybrid fish from RCC, CC to 2nF₁, and to 2nF₂, as well as to 4nF₃ and 4nF₂₄.
major international cooperation projects of the National Natural Science Foundation of China (31210103918), training Program of the Major Research Plan of the National Natural Science Foundation of China (91331105), the National High Technology Research and Development Program of China (2011AA100403), the National Key Basic Research Program of China (2012CB229305), the fund of education department of Hunan province (15A116), The fund of sci-techn innovation and entrepreneur for Hunan young talents, the Project Supported by Science and Technology Program of Hunan Province (No.2015JC3065; No.2014FJ3084) and the construct program of the key discipline in Hunan province and China.

Received: 10 June 2015 Accepted: 16 November 2015 Published online: 03 December 2015

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