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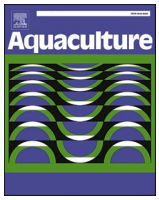

# Unique nucleolar dominance patterns in different ploidy hybrid lineages derived from *Cyprinus carpio* (♀) × *Megalobrama amblycephala* (♂)

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A B S T R A C T

Nucleolar dominance is one of the epigenetic phenomena that refers to the dominance effect exhibited by the suppression of ribosomal RNA gene loci from one parent in heterozygous organisms. In this paper, the genetic and expression changes of 45S rRNA genes in different generations of the crucian carp-like homodiploid fish lineage (2nNCRC, $F_1$, $F_2$, $F_3$, $F_5$) and the autotetraploid carp lineage (4nNC, $F_1$-$F_3$) formed by distant hybridization of common carp (*Cyprinus carpio*, COC) (♀) × blunt snout bream (*Megalobrama amblycephala*, BSB) (♂) were mainly studied. The results showed that the nucleolar dominance patterns were different in different generations of the 2nNCRC lineage and the 4nNC lineage. In the different generations of the 2nNCRC lineage, 2nNCRC-$F_1$ ($F_3$, $F_5$) exhibited the phenomenon of nucleolar dominance, while 2nNCRC-$F_2$ did not and expressed the mutant 45S rRNA. The establishment of the nucleolar dominance pattern presented a more complicated situation, which may be related to the inconsistent coping mechanism among different generations after suffering the effect of "genome shock" at the early stage of the formation of the 2nNCRC lineage. Among the different generations of the 4nNC lineage, 4nNC-$F_2$ existed the phenomenon of nucleolar dominance and expressed mutant 45S rRNA. However, 4nNC-$F_1$ ($F_3$) did not exist in the phenomenon of nucleolar dominance. The establishment of the nucleolar dominance pattern showed a process of instability-stability-instability, revealing the epigenetic instability of this lineage. This result may be related to the comprehensive effect caused by the continuous "genome shock" at the early stage of the formation of the 4nNC lineage, which can provide clues for further revealing the possible mechanism of occurrence and survival of polyploidization vertebrates. Moreover, this study found that the nucleolar dominance was biased towards BSB in the 2nNCRC lineage, while it was biased towards COC in the 4nNC lineage. The inconsistent results appeared in the different ploidy lineages formed by the same distant hybridization combination, which further verified that the dominance of transcription advantages species did not necessarily determine the biased characteristics of nucleolar dominance. Two lineages derived a mutant ITS1 sequence, which the mechanism may be related to some mechanisms triggered by the "genome shock" effect. This study will further fill a gap in studying nucleolar dominance in the early stage of the formation of homodiploid and polyploid vertebrates and provide important experimental data for studying fish epigenetics, which is of great significance in fish genetic breeding and biological evolution.

## 1. Introduction

Distant hybridization refers to a crossing between two different species or higher taxa, which can facilitate the transfer of genomes between different species and lead to phenotypic and genotypic changes in offspring. This change may be related to the compatibility and coordination of the biparental nuclei (Liu, 2022; Liu, 2010). Nuclear genes located on the chromosomes within the eukaryotic nucleus express the genetic information of organisms by directing the synthesis of proteins, thereby controlling the performance of biont traits. Thus, the study of nuclear genes is exceedingly valuable in distant hybridization. As the vital one of the nuclear genes, 45S rDNA is an effective gene for

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studying the phylogeny between species and a molecular marker for identifying the kinship between species (Lee et al., 2022). The 45S rDNA comprises tandem repeat units of the 18S–5.8S–26S rRNA genes and non-transcribed spacers and is a vital gene responsible for forming nucleolar organizer regions (Zhang et al., 2016). Tandem repeats and genetic instability are typical features of 45S rDNA. Tandem repeats can freely transfer from one locus to another on the chromosomes, which is a transposable factor. The genetic instability can lead to differences and even variations of 45S rDNA genotypes among closely related species or even within species (Huang et al., 2012). In thale cress (*Arabidopsis thaliana*), 45S rDNA, under the action of RNA polymerase I, was transcribed into 45S rRNA. The expression of 45S rRNA happened selectively silent during transcription (Layat et al., 2012).

Nucleolar dominance refers to the phenomenon that 45 rRNA genes of only one parent happen to the silence within hybrids (Layat et al., 2012). The discovery of the nucleolar dominance phenomenon was first in Hawksbeard (*Crepis*) (Navashin, 1934). Later, many data indicated that the phenomenon of nucleolar dominance was universal. The natural wild-type species had the phenomenon of nucleolar dominance, such as mesquite (*Prosopis juliflora*) (Tapia-Pastrana, 2020), purple false brome (*Brachypodium hybridum)* (Borowska-Zuchowska et al., 2019), thale cress (Chen et al., 1998), and fruit fly (*Drosophila melanogaster*) (Warsinger-Pepe et al., 2020). In addition, the artificial distant hybrid species also generated the phenomenon of nucleolar dominance, such as the distant hybridization of plants (Santos et al., 2020), invertebrates (Oliveira et al., 2006), amphibians (Reeder and Roan, 1984), fish (Xiao et al., 2016), and mammals (Gallardo et al., 2006). The distant hybrid species include both diploid and polyploid species. Among diploid hybrid species, the report of phenomenon of nuclear dominance was very rare in diploid hybrid plants, while it was widespread in diploid hybrid animals. For example, in invertebrates, the water flea (*Tigriopus californicus*) of interspecific hybridization existed the phenomenon of nucleolar dominance (Flowers and Burton, 2006). Among amphibians, the phenomenon of nucleolar dominance was found in the clawed frogs (*Xenopus laevis* and *Xenopus mulleri*) of interspecific hybrid (Honjo and Reeder, 1973; Macleod and Bird, 1982). In fish, the phenomenon of nucleolar dominance was discovered in the allodiploid fish lineage formed by distant hybridization of blunt snout bream (*Megalobrama amblycephala*, BSB) and topmouth culter (*Culter alburnus*, TC), and this lineage existed five nucleolar dominance patterns (Xiao et al., 2016). The diploid hybrids described above with nucleolar dominance are all allodiploid. At present, no report found about nucleolar dominance in autodiploid (homoploid) hybrid species. Among polyploid hybrid species, the abundant studies have identified the phenomenon of nucleolar dominance in allopolyploid hybrid plants. For example, allopolyploid hybrid species formed by thale cress (Lewis et al., 2004), wheat (Houchins et al., 1997), rape (*Brassica napus*) (Chen and Pikaard, 1997a), and so on (Pikaard, 2001) had the phenomenon of nucleolar dominance. However, few of these studies have involved the phenomenon of nuclear dominance in autopolyploid plants. In polyploid hybrid animals, the phenomenon of nucleolar dominance was observed in mammalian allotetraploid red rats (Gallardo et al., 2006). Subsequently, in fish, the phenomenon of nucleolar dominance was found in the allotetraploid fish $F_1$ and autotetraploid fish $F_2$ formed by distant hybridization of red crucian carp (*Carassius auratus red var.*, RCC) × blunt snout bream (*Megalobrama amblycephala*, BSB). The establishment of the phenomenon of nucleolar dominance began in $F_1$, and $F_2$ inherited the phenomenon from $F_1$. The nucleolar dominance pattern was a stable maternal inherited effect in the tetraploid hybrid fish lineage (Cao et al., 2018).

The knowledge of nucleolar dominance in autodiploid (homoploid) and autotetraploid species formed by distant hybridization is still limited. In the autodiploid (homoploid) and autopolyploid progeny formed by distant hybridization, the number of the genomes and chromosome sets of their parents is unequal, which leads to the increase of nuclear-nuclear incompatibility of parent, and which is not conducive to the stable heredity of biparental genetic material (Liu, 2022; Liu, 2010). The result also adds many uncertainties to the exploration of nucleolar dominance. This study by the blood and liver of the crucian carp-like homodiploid fish and the autotetraploid carp lineages formed by distant hybridization of common carp (*Cyprinus carpio*, COC) (♀) × blunt snout bream (*Megalobrama amblycephala*, BSB) (♂) as experimental materials to amplify the DNA sequence and cDNA sequence of the internal transcriptional spacer 1 (ITS1) of 45S rRNA, respectively. This study focuses on exploring the genetic variability and expression regularity of 45S rRNA and the nucleolar dominance in the early formative stages of distant hybrid lineages to reveal the influence of epigenetic phenomena on biological evolution and biodiversity. We will unfold the study of the phenomenon of nucleolar dominance in autodiploid (homoploid) and autopolyploid fish formed by distant hybridization to furthermore excavate the rare reasons for the formation of homoploid hybrid species and polyploid vertebrates and provide clues for revealing the possible survival mechanisms of homoploid species and polyploid vertebrates.

## 2. Materials and methods

### 2.1. Sample resources

The natural species contain common carp (abbreviated as COC, 2n = 100) and blunt snout bream (abbreviated as BSB, 2n = 48). The hybrid species contain the crucian carp-like homodiploid fish (abbreviated as 2nNCRC, 2n = 100) lineage ($F_1$, $F_2$, $F_3$, $F_5$), the autotetraploid carp (abbreviated as 4nNC, 4n = 200) lineage ($F_1$, $F_2$, $F_3$), and the allotetraploid fish (abbreviated as 4nCB, 4n = 148) formed by distant hybridization of COC (♀) × BSB (♂). The above hybrid fish came from the Engineering Research Center of Polyploid Fish Breeding and Reproduction of the State Education Ministry and the State Key Laboratory of Developmental Biology of Freshwater Fish at Hunan Normal University in Changsha, Hunan Province, China. According to Fig. 1, COC as the maternal parent and BSB as the paternal parent through distant hybridization to form four different ploidy hybrid $F_1$ progenies, including 2nNCRC, 4nCB, and so on (Wang et al., 2017). 4nCB as the maternal parent and 2nNCRC as the paternal parent through distant hybridization to form 4nNC-$F_1$ (Wang et al., 2019, 2020c). 2nNCRC-$F_1$, through successive self-inbreeding, formed the 2nNCRC lineage. 4nNC-$F_1$, through successive self-inbreeding, created the 4nNC lineage (Liu, 2022). In this experiment, three COC, three BSB, two 2nNCRC-$F_1$, three 2nNCRC-$F_2$, four 2nNCRC-$F_3$, three 2nNCRC-$F_5$, five 4nCB, five 4nNC-$F_1$, two 4nNC-$F_2$, and four 4nNC-$F_3$ were selected as experimental materials.

### 2.2. DNA extraction, PCR, cloning, and sequencing of ITS1 fragments of 45S rDNA

Total genomic DNA was extracted from the blood of COC, BSB, 2nNCRC ($F_1$, $F_2$, $F_3$, $F_5$), 4nCB, and 4nNC ($F_1$, $F_2$, $F_3$) by the Mini Best Universal Genomic DNA Extraction Kit (Takara), respectively. Two pairs of ITS1 primers, as follows: COC-ITS1: (F:5′-CTATCGGGAGGAAGTAAAGTCGT-3′, R:5′-TGATCCACCGCTAAGAGTTGTA-3′); BSB-ITS1: (F:5′-AAGGTTTCGTCCCCGAGA-3′, R:5′-GGAACGCACACGTACCTA-3′), were designed and synthesized according to the 45S sequences of COC (GenBank: JN628435.1) and BSB (GenBank: MG830472.1) to amplify different ITS1 sequences from different genomic DNA templates. PCR was performed in a total volume of 25 uL with 12.5 uL of LA mix (Takara), 9.5 uL of sterile ultrapure water, 0.5 uL of F and R primers each, and 2 uL of genomic DNA template. The PCR thermal program consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 45 s, with a final extension step at 72 °C for 10 min and storage temperature at 16 °C. A previous study indicated that the agarose gel density used to amplify the ITS1 sequence and perform electrophoresis was 2% to distinguish fragments with an interval of about 20 bp (Xiao et al., 2016). Finally,

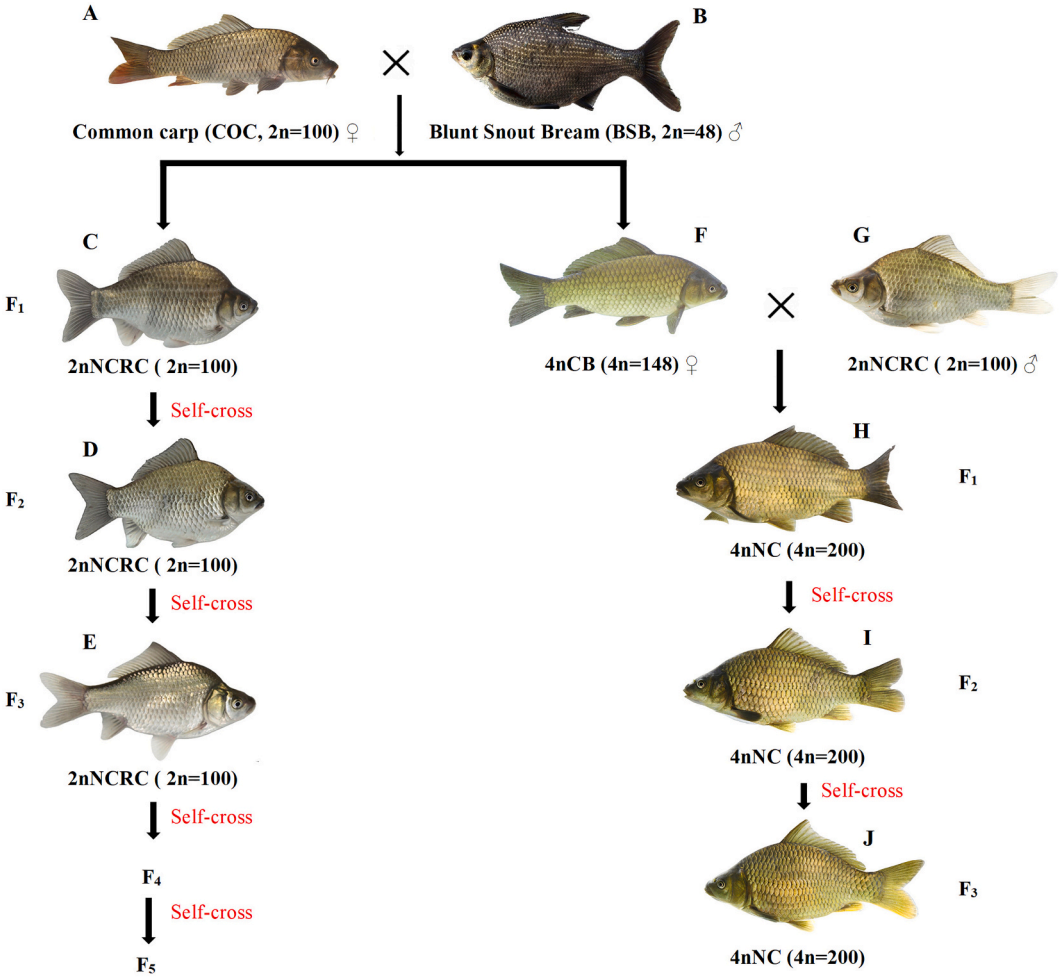


**Fig. 1.** The formative process of 2nNCRC lineage, 4nCB, and 4nNC lineage derived from *Cyprinus carpio* (♀) × *Megalobrama amblycephala* (♂). A: Common carp (COC, 2n = 100, ♀). B: Blunt snout bream (BSB, 2n = 48, ♂). C: The crucian carp-like homodiploid fish $F_1$ (2nNCRC-$F_1$, 2n = 100). D: The crucian carp-like homodiploid fish $F_2$ (2nNCRC-$F_2$, 2n = 100). E: The crucian carp-like homodiploid fish $F_3$ (2nNCRC-$F_3$, 2n = 100). The crucian carp-like homodiploid fish $F_4$ and $F_5$ were formed by self-crossing. F: The allotetraploid fish (4nCB, 4n = 148, ♀). G: The crucian carp-like homodiploid fish (2nNCRC, 2n = 100, ♂). H: The autotetraploid carp $F_1$ (4nNC-$F_1$, 4n = 200). I: The autotetraploid carp $F_2$ (4nNC-$F_2$, 4n = 200). J: The autotetraploid carp $F_3$ (4nNC-F3, 4n = 200). (note: figs. (A, B, C, D, E) were reported in Scientific Reports, 2017, 7: 4189; figs. (F, G, H, I, J) were reported in Aquaculture, 2020, 515).

continued exploration revealed that the optimal concentration of agarose gel for performing electrophoresis was 2.5%. The agarose gel was subsequently photographed under ultraviolet light to preserve the agarose gel image. PCR products were recycled and purified by the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech), which was directly cloned into the pMD18-T vector (Takara) to produce the plasmids. The plasmids were transformed into *E. coli* DH5a cells, which were preliminarily tested to see if they could be successfully cloned using PCR. Finally, to enhance the probability of detecting duplicated paralogs and to circumvent errors for PCR, 20 clones of each different ITS1 fragment from each sample were sequenced with vector-specific primers using the primer walking method on an ABI 3730XL automatic sequencer (ABI PRISM 3730, Applied Biosystems, CA, USA).

### 2.3. RNA extraction, PCR, cloning, and sequencing of ITS1 fragments of 45S rRNA

Total RNA was extracted from the liver of COC, BSB, 2nNCRC ($F_1$, $F_2$, $F_3$, $F_5$), 4nCB, and 4nNC ($F_1$, $F_2$, $F_3$) using Trizol Reagent (Sangon Biotech), respectively. The usage of the agarose gel electrophoresis to detect RNA integrity. The concentration and OD values of 1 uL RNA were measured using an enzyme-labeled instrument. The template RNA was reverse transcribed into cDNA by the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA quality was detected by the amplification of the β-actin gene. Next, the ITS1 sequence was amplified by COC-ITS1 primer in different cDNA templates. The following PCR and cloning procedures are consistent with Step 2.2.

### 2.4. Comparison and analysis of sequencing results

The sequencing results were opened from COC, BSB, 2nNCRC-$F_1$ ($F_2$-$F_3$, $F_5$), 4nCB, and 4nNC-$F_1$ ($F_2$-$F_3$) using the Editseq in DNASTAR 7.0 software (DNAStar Inc.). Sequencing results were saved by removing the Vector sequences from the known ITS1 primer sequences. We downloaded ITS1 sequences related to crucian carp from the NCBI website (https://www.ncbi.nlm.nih.gov/). Sequencing results for the same species imported into the BioEdit application were saved as a file. The file compared with COC, BSB, and crucian carp sequences using ClustalW programs (http://www.ebi.ac.uk/) was saved as another file. The obtained sequences were screened for ITS1 fragments using BLAST (http://www.ncbi.nlm.nih.gov) searches. Finally, the filtered sequences were exported using File's Graphicview in the Bioedit application and saved (Wang et al., 2021).

### 2.5. Detection and analysis by SNP

SNP refers to DNA sequence polymorphisms at the genome level caused by variations in a single nucleotide. SNP loci can quickly identify heterozygotes and homozygotes with significantly different peak patterns, mutations, or polymorphisms. The genetic and expression characteristics between hybrid fish and their parents can be recognized more clearly and intuitively by mapping SNP loci (Kwok and Duan, 2002). SNP loci were sought in COC, BSB, and crucian carp using the BioEdit application. The screened sequences of 2nNCRC-$F_1$ ($F_2$-$F_3$, $F_5$), 4nCB, and 4nNC-$F_1$ ($F_2$-$F_3$) were analyzed homology and variation by SNP loci of COC, BSB, and crucian carp.

## 3. Results

### 3.1. Inheritance and expression results of 45S rRNA ITS1 in the crucian carp-like homodiploid fish lineage

At the genetic level of ITS1 sequences, according to Figs. 2A, 3, Table 1, and the sequencing results, all individuals of 2nNCRC-$F_1$ inherited the specific ITS1 sequence of parent and derived the variant ITS1 sequence (this sequence type was highly homologous to crucian carp (GenBank: MH362747.1) (abbreviated as 2nCC)). According to Fig. 2B, Table 1, and the sequencing results, all individuals of 2nNCRC-$F_2$ inherited the specific ITS1 sequence of the original parent, and most of the individuals also derived the variant ITS1 sequence. Based on Fig. 2C, Table 1, and the sequencing results, most individuals of 2nNCRC-$F_3$ inherited the specific ITS1 sequence of the original parent.

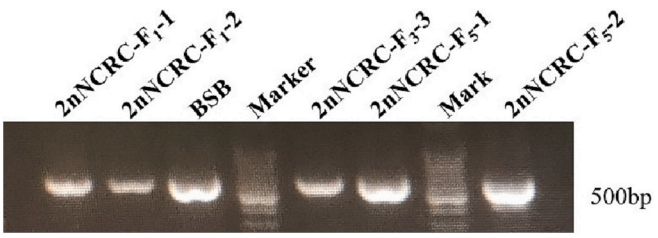


**Fig. 3.** DNA agarose gel electrophoresis of ITS1 sequences of different generations of 2nNCRC lineage and paternal parent BSB. By designing the specific ITS1 sequence primers of BSB, randomly selected five samples from different generations of 2nNCRC lineage as $F_1$–1, $F_1$–2, $F_3$–3, $F_5$–1, $F_5$–2, and further verified the specific ITS1 sequence results of the inherited paternal parent BSB of the 2nNCRC lineage.

However, a small number of individuals in 2nNCRC-$F_3$ inherited only the specific ITS1 sequence of BSB and derived a variant ITS1 sequence. According to Figs. 2D, 3, Table 1, and the sequencing results, all individuals of 2nNCRC-$F_5$ inherited the specific ITS1 sequence of the original parent, and a few individuals also derived a variant ITS1 sequence.

At the level of expression of 45S rRNA, according to Fig. 2E, Table 1, and the sequencing results, some individuals of 2nNCRC-$F_1$ expressed 45S rRNA of their parents, while others expressed only 45S rRNA of BSB. According to Fig. 2F, Table 1, and the sequencing results, all individuals of 2nNCRC-$F_2$ expressed 45S rRNA of the original parent, and most individuals also expressed the mutant 45S rRNA. According to Fig. 2G,

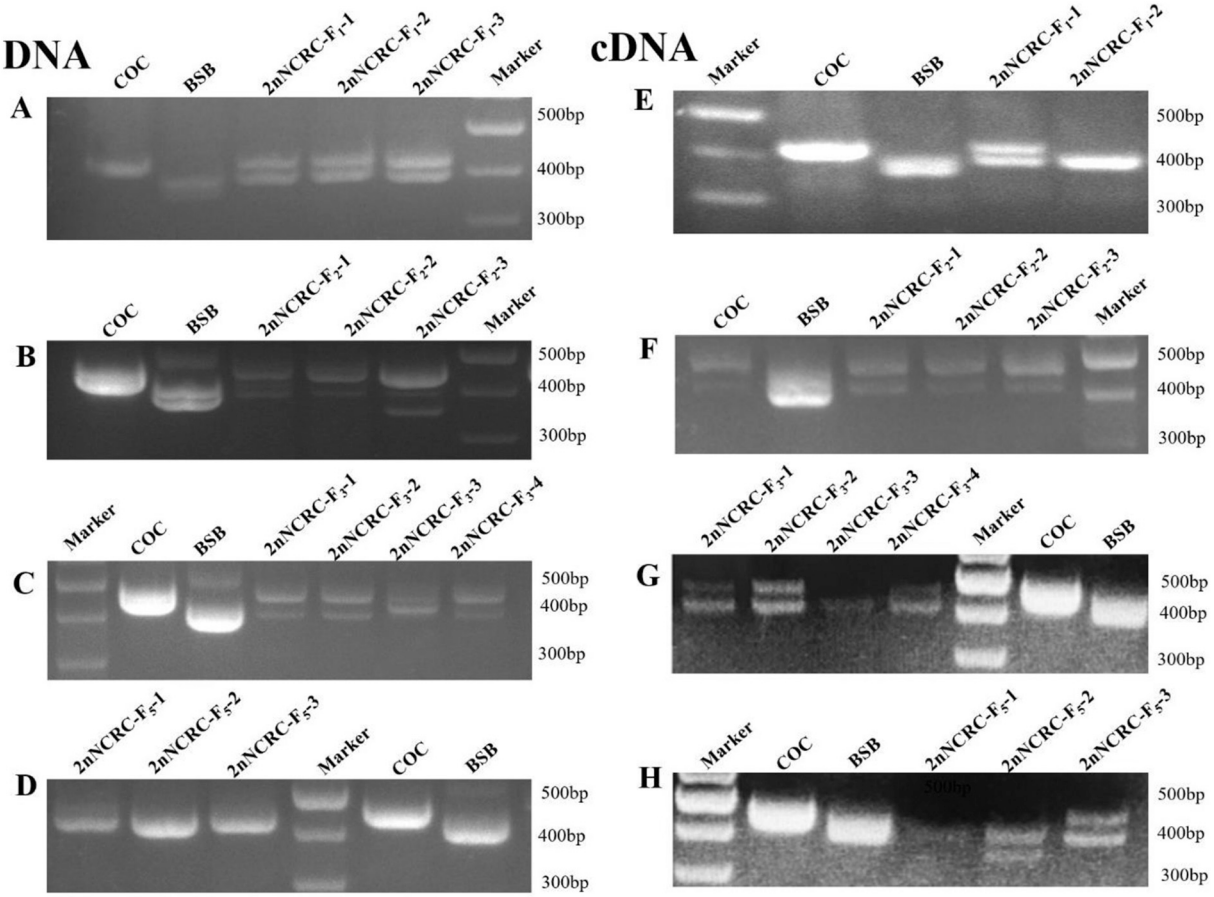


**Fig. 2.** DNA and cDNA agarose gel electrophoresis of ITS1 sequences of different generations of 2nNCRC lineage and their parents. A: Genetic type of ITS1 sequence of 2nNCRC-$F_1$. B: Genetic type of ITS1 sequence of 2nNCRC-$F_2$. C: Genetic type of ITS1 sequence of 2nNCRC-$F_3$. D: Genetic type of ITS1 sequence of 2nNCRC-$F_5$. E: 45S rRNA expression type of 2nNCRC-$F_1$. F: 45S rRNA expression type of 2nNCRC-$F_2$. G: 45S rRNA expression type of 2nNCRC-$F_3$. H: 45S rRNA expression type of 2nNCRC-$F_5$.

**Table 1**
Inheritance and expression types in the 2nNCRC lineage ("+" indicates the existence of corresponding types).

| Species name | ITS1 genetic type | | | 45S rRNA expression type | | |
|---|---|---|---|---|---|---|
| | COC | BSB | 2nCC | COC | BSB | 2nCC |
| COC | + | | | + | | |
| BSB | | + | | | + | |
| 2nNCRC-$F_1$-1 | + | + | + | + | + | |
| 2nNCRC-$F_1$-2 | + | + | + | | + | |
| 2nNCRC-$F_2$-1 | + | + | + | + | + | |
| 2nNCRC-$F_2$-2 | + | + | + | + | + | + |
| 2nNCRC-$F_2$-3 | + | + | | + | + | + |
| 2nNCRC-$F_3$-1 | + | + | | + | + | |
| 2nNCRC-$F_3$-2 | + | + | | + | + | |
| 2nNCRC-$F_3$-3 | | + | + | | + | |
| 2nNCRC-$F_3$-4 | + | + | + | + | + | |
| 2nNCRC-$F_5$-1 | + | + | | | + | |
| 2nNCRC-$F_5$-2 | + | + | | | + | |
| 2nNCRC-$F_5$-3 | + | + | + | + | + | |

Table 1, and the sequencing results, most individuals of 2nNCRC-$F_3$ expressed 45S rRNA of the original parent, while few only expressed 45S rRNA of BSB. According to Fig. 2H, Table 1, and the sequencing results, a few individuals of 2nNCRC-$F_5$ expressed 45S rRNA of the original parent, while most of the individuals expressed only the 45S rRNA of BSB.

Based on the sequencing results, we found SNP loci located at the 22, 31, 109 (or 83, 103) base loci of ITS1 sequences of COC, BSB, and 2nCC, respectively. According to Fig. 4, the genetic and expression types of 45S rRNA showed inconsistent results in different generations of the 2nNCRC lineage. There were two patterns in 2nNCRC-$F_1$: Firstly, 2nNCRC-$F_1$ inherited the ITS1 sequence of parent and the newly derived variant, that expressed the parent's 45S rRNA. Secondly, 2nNCRC-$F_1$ inherited the ITS1 sequence of parent and the newly derived variant, which expressed only the 45S rRNA of BSB. Therefore, nucleolar dominance existed in 2nNCRC-$F_1$. There were three patterns in 2nNCRC-$F_2$: Firstly, 2nNCRC-$F_2$ inherited the ITS1 sequence of the original parent, which expressed the 45S rRNA of the original parent and the mutant type. Secondly, 2nNCRC-$F_2$ inherited the ITS1 sequence of the original parent and the newly derived variant, which expressed the 45S rRNA of the original parent. Thirdly, 2nNCRC-$F_2$ inherited the ITS1 sequence of the original parent and the newly derived variant, which expressed the 45S rRNA of the original parent and the mutant type. Thus, there is no nucleolar dominance in 2nNCRC-$F_2$. Three patterns were presented in 2nNCRC-$F_3$: Firstly, 2nNCRC-$F_3$ inherited the ITS1 sequence of the original parent, which also expressed the 45S rRNA of the original parent. Secondly, 2nNCRC-$F_3$ inherited the ITS1 sequence of the original parent and the newly derived variant, which also expressed the 45S rRNA of the original parent. Thirdly, 2nNCRC-$F_3$ inherited the ITS1 sequence of BSB as well as the newly derived variant, which expressed only the 45S rRNA of BSB. Thus, nucleolar dominance existed in 2nNCRC-$F_3$. There were two patterns in 2nNCRC-$F_5$: Firstly, 2nNCRC-$F_5$ inherited the ITS1 sequence of the original parent and the newly derived variant, which also expressed the 45S rRNA of the original parent. Secondly, 2nNCRC-$F_5$ inherited the ITS1 sequence from the original parent, which expressed only the 45S rRNA of BSB. Consequently, there is nucleolar dominance in 2nNCRC-$F_5$. The results reveal that the nucleolar dominance patterns of the 2nNCRC lineage undergo a process of stability to instability to stability and gradually stabilize in 2nNCRC-$F_5$.

### *3.2. Inheritance and expression results of 45S rRNA ITS1 in the autotetraploid carp lineage*

At the genetic level of ITS1 sequence, according to Fig. 5A, Table 2, and the sequencing results, all individuals of 4nCB inherited the specific ITS1 sequence of parents and newly derived variant ITS1 sequence (this sequence type was highly homologous to crucian carp (GenBank: MH362747.1) (abbreviated as 2nCC)). According to Figs. 5B, 6, Table 2, and the sequencing results, all individuals of 4nNC-$F_1$ inherited the specific ITS1 sequence of the original parent, and some individuals also derived the new variant ITS1 sequence (this sequence type was highly homologous to crucian carp (GenBank: MH362747.1) (abbreviated as 2nCC)). According to Fig. 5C, Table 2, and the sequencing results, all individuals of 4nNC-$F_2$ inherited the specific ITS1 sequence of their original parent, and some individuals also derived the new variant ITS1 sequence. According to Figs. 5D, 6, Table 2, and the sequencing results, all individuals of 4nNC-$F_3$ inherited specific ITS1 sequence fragments from their original parent, and all individuals also inherited newly derived variant ITS1 sequences.

At the level of expression of 45S rRNA, all individuals of 4nCB, 4nNC-$F_1$, and 4nNC-$F_3$ expressed 45S rRNA from either the parent or the original parent, according to Figs. 5E, F, H, Table 2, and the sequencing results. However, according to Fig. 5G, Table 2, and the sequencing results, all individuals of 4nNC-$F_2$ expressed only the 45S rRNA of COC, and some individuals expressed the mutant 45S rRNA.

Based on the sequencing results, we found that SNP loci located at the 22, 31, 109 (or 83, 103) base loci of ITS1 sequences of COC, BSB, and 2nCC, respectively. According to Fig. 7, the genetic and expression types of 45S rRNA showed inconsistent results for different generations of the 4nNC lineage. There were two patterns in 4nNC-$F_1$: Firstly, 4nNC-$F_1$ inherited the ITS1 sequence of the original parent, which expressed 45S rRNA of the original parent. Secondly, 4nNC-$F_1$ inherited the ITS1 sequence of the original parent and the newly derived variant, which expressed the 45S rRNA of the original parent. As a result, there is no nucleolar dominance in 4nNC-$F_1$. Two patterns were presented in 4nNC-$F_2$: Firstly, 4nNC-$F_2$ inherited the ITS1 sequence of the original parent, which expressed only the 45S rRNA of COC. Secondly, 4nNC-$F_2$ inherited the ITS1 sequence of both the original parent and the newly derived variant, which expressed the 45S rRNA of COC and the mutant. Thus, nucleolar dominance existed in 4nNC-$F_2$. There was only one pattern in 4nNC-$F_3$: 4nNC-$F_3$ inherited the ITS1 sequence of the original parent and the newly derived variant, which expressed the 45S rRNA of the original parent. Consequently, there is no nucleolar dominance phenomenon in 4nNC-$F_3$. The results reveal that the nucleolar dominance patterns of the 4nNC lineage undergo a process from unstable to stable to unstable, which indirectly reflected the severe "genome shock" phenomenon in the early formative stage of the 4nNC lineage.

## 4. Discussion and conclusions

The ITS1 sequence between the 18S and 5.8S rRNAs of eukaryotes is a variable region of 45S rRNA, which can identify the genetic relationship between species (Lee et al., 2022), easily identify natural species and hybrid species (Baldwin et al., 1995), and identify the case of occurrence of significant variation (Havlová et al., 2016). Some individuals reportedly amplified the biparental ITS1 sequence in the hybrid $F_1$ of lineage of BSB × TC, indicating the hybridity of the hybrid lineages. Among the hybrid lineage, a few of individuals of $F_2$ amplified the ITS1 sequence of a single parent, suggesting that hybridization could lead to gene occurred mutation, recombination, and differentiation (Xiao et al., 2016). In addition, the rapid evolution and frequent recombination of the ITS1 sequence could lead to significant mutations in the genome of thale cress (Havlová et al., 2016). This study analyzed the genetic variation and expression levels of 45S rRNA in different ploidy lineages derived from distant hybridization of COC × BSB. In the 2nNCRC and 4nNC lineages, some individuals inherited and expressed 45S rRNA of parent (or original parent), reflecting the hybridity of these two lineages. In the 2nNCRC and 4nNC lineages, some individuals have newly derived and expressed mutant 45S rRNA, illustrating the variability in both lineages.

45S rRNA is a classic gene for studying gene structural composition and gene expression level between species, which also is a

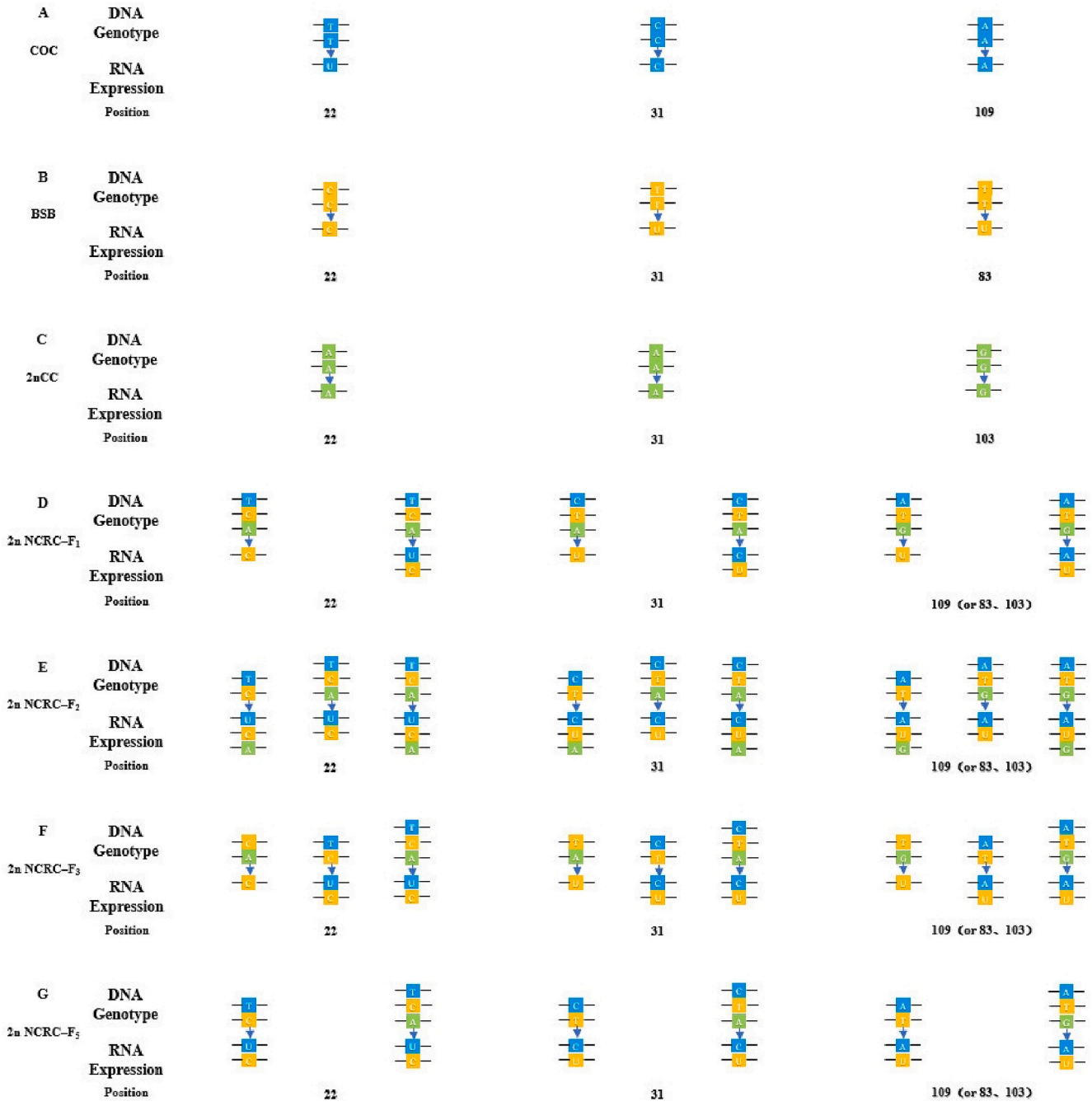


**Fig. 4.** Inheritance and expression pattern of 45S rRNA from 2nNCRC lineage and their parents. A: Genotype and expression of 45S rRNA gene in COC. B: Genotype and expression of 45S rRNA gene in BSB. C: Genotype and expression of 45S rRNA gene in 2nCC. D: Genotype and expression of 45S rRNA gene in 2nNCRC-$F_1$. E: Genotype and expression of 45S rRNA gene in 2nNCRC-$F_2$. F: Genotype and expression of 45S rRNA gene in 2nNCRC-$F_3$. G: Genotype and expression of 45S rRNA gene in 2nNCRC-$F_5$. Note: For each pattern of each fish, one sample is used to present.

representative gene for studying nucleolar dominance (Pikaard, 1999). The molecular basis of nucleolar dominance is that RNA polymerase I from the female or male parent exhibits reversible silencing during ribosomal RNA (rRNA) gene transcription (Pikaard, 2014; Tucker et al., 2010). The earliest nucleolar dominance was reported in the interspecific hybrid progeny nucleolus of hawksbeard (Navashin, 1934). This study showed that 2nNCRC-$F_1$ ($F_3$, $F_5$) expressed only the 45S rRNA of BSB, while 4nNC-$F_2$ expressed only the 45S rRNA of COC. The results are consistent with the hawksbeard, so there is the phenomenon of nucleolar dominance in the two lineages. Current knowledge of nucleolar dominance in distant hybrid species is widespread. The data indicated that in plants, the formation and differentiation of nucleolar dominance began with the $F_1$ generation of allotetraploid thale cress, and the steady establishment of nucleolar dominance took place in the $F_2$ generation (Chen et al., 1998; Pikaard, 2001). In invertebrates, the rapid formation of nucleolar dominance began with $F_1$ in the interspecific hybrids of water fleas, and nucleolar dominance happened with differentiation in the $F_2$ (Flowers and Burton, 2006). In mammals, nucleolar dominance also existed in the allotetraploid red rat (Gallardo et al., 2006). Current knowledge of nucleolar dominance in distant hybrid fish is not sufficiently comprehensive. Limited data indicated no nucleolar dominance in the $F_1$ generation of allodiploid fish (abbreviated as 2nBT) lineage derived from distant hybridization of BSB × TC, but the formation and differentiation of nucleolar dominance occurred in $F_2$ (Xiao et al., 2016).

**Table 2**
Inheritance and expression types in the 4nCB and 4nNC lineage ("+" indicates the existence of corresponding types).

| Species name | ITS1 genetic type | | | 45S rRNA expression type | | |
|---|---|---|---|---|---|---|
| | COC | BSB | 2nCC | COC | BSB | 2nCC |
| COC | + | | | + | | |
| BSB | | + | | | + | |
| 4nCB-1 | + | + | + | + | + | |
| 4nCB-2 | + | + | + | + | + | |
| 4nCB-3 | + | + | + | + | + | |
| 4nCB-4 | + | + | + | + | + | |
| 4nCB-5 | + | + | + | + | + | |
| 4nNC-$F_1$-1 | + | + | + | + | + | |
| 4nNC-$F_1$-2 | + | + | + | + | + | |
| 4nNC-$F_1$-3 | + | + | + | + | + | |
| 4nNC-$F_1$-4 | + | + | | + | + | |
| 4nNC-$F_1$-5 | + | + | | + | + | |
| 4nNC-$F_2$-1 | + | + | | + | | |
| 4nNC-$F_2$-2 | + | + | + | + | | + |
| 4nNC-$F_3$-1 | + | + | + | + | + | |
| 4nNC-$F_3$-2 | + | + | + | + | + | |
| 4nNC-$F_3$-3 | + | + | + | + | + | |
| 4nNC-$F_3$-4 | + | + | + | + | + | |

In addition, the allotetraploid fish (abbreviated as 4nRB) derived from distant hybridization of red crucian carp (*Carassius auratus* red var., RCC) × blunt snout bream (*Megalobrama amblycephala*, BSB) rapidly established nucleolar dominance in $F_1$. The allotetraploid fish (abbreviated as 4nRR) derived from distant hybridization of RCC × BSB stably inherited nucleolar dominance of 4nRB-$F_1$ in $F_2$ (Cao et al., 2018; Wang et al., 2022). This study showed that the rapid formation of the nucleolar dominance arose in 2nNCRC-$F_1$, suddenly disappeared in 2nNCRC-$F_2$, re-established in 2nNCRC-$F_3$, and finally gradually stabilized in 2nNCRC-$F_5$. The establishment of nucleolar dominance did not happen in 4nNC-$F_1$, it began to form in 4nNC-$F_2$, and suddenly disappeared in 4nNC-$F_3$. Two results confirm that the nucleolar dominance of the 2nNCRC and 4nNC lineages are different from the above-known distant hybridization fishes, which may be related to the complex formation background of these two hybrid lineages (Gu et al., 2022; Luo et al., 2019; Wang et al., 2021; Wang et al., 2020a; Wang et al., 2020b, 2020c; Wang et al., 2019; Wang et al., 2017).

BSB (2n = 48) as the maternal parent and TC (2n = 48) as the paternal parent through distant hybridization to form the 2nBT (2n = 48) lineage. The biparental chromosomal numbers were equal in the 2nBT lineage, which was conducive to the karyotype pairing and nuclear fusion of their parent. Finally, the result led to stably inheriting biparental genetic material in 2nBT (Xiao et al., 2014). However, in the 2nNCRC lineage, the biparental chromosomal numbers were not equal, and COC had more chromosomal numbers than BSB, which was not conducive to karyotypic pairing and nuclear fusion of their parent.

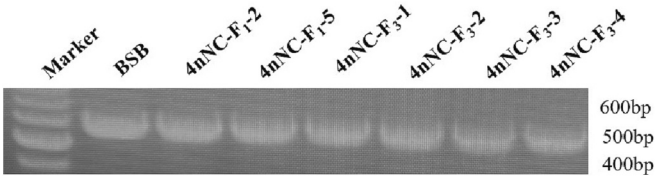


**Fig. 6.** DNA agarose gel electrophoresis of ITS1 sequences of different generations of 4nNC lineage and paternal parent BSB. By designing the specific ITS1 sequence primers of BSB, randomly selected six samples from different generations of 4nNC lineage as $F_1$–2, $F_1$–5, $F_3$–1, $F_3$–2, $F_3$–3, $F_3$–4, and further verified the specific ITS1 sequence results of the inherited paternal parent of 4nNC lineage.

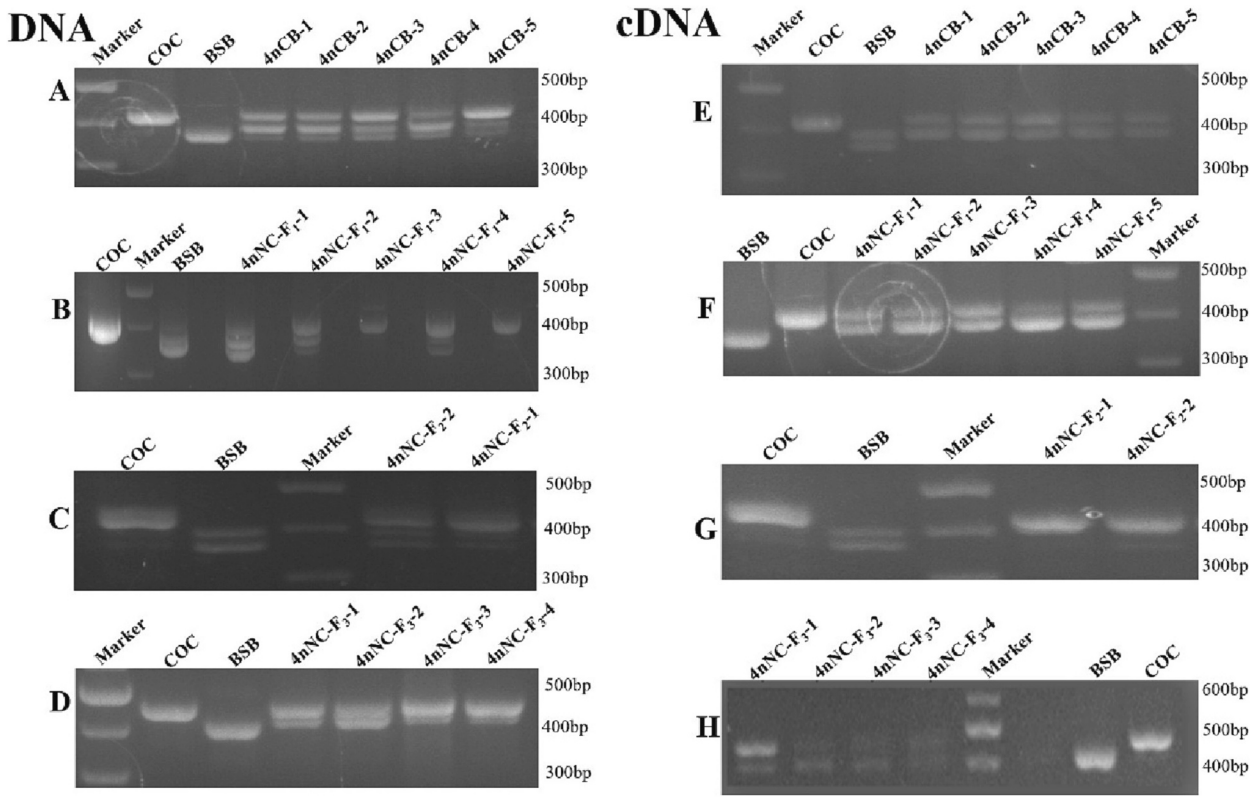


**Fig. 5.** DNA and cDNA agarose gel electrophoresis of ITS1 sequences of different generations of 4nCB and 4nNC lineage and their parents. A: Genetic type of ITS1 sequence of 4nCB. B: Genetic type of ITS1 sequence of 4nNC-$F_1$. C: Genetic type of ITS1 sequence of 4nNC-$F_2$. D: Genetic type of ITS1 sequence of 4nNC-$F_3$. E: expression type of 45S rRNA in 4nCB. F: expression type of 45S rRNA in 4nNC-$F_1$. G: expression type of 45S rRNA in 4nNC-$F_2$. H: expression type of 45S rRNA in 4nNC-$F_3$.

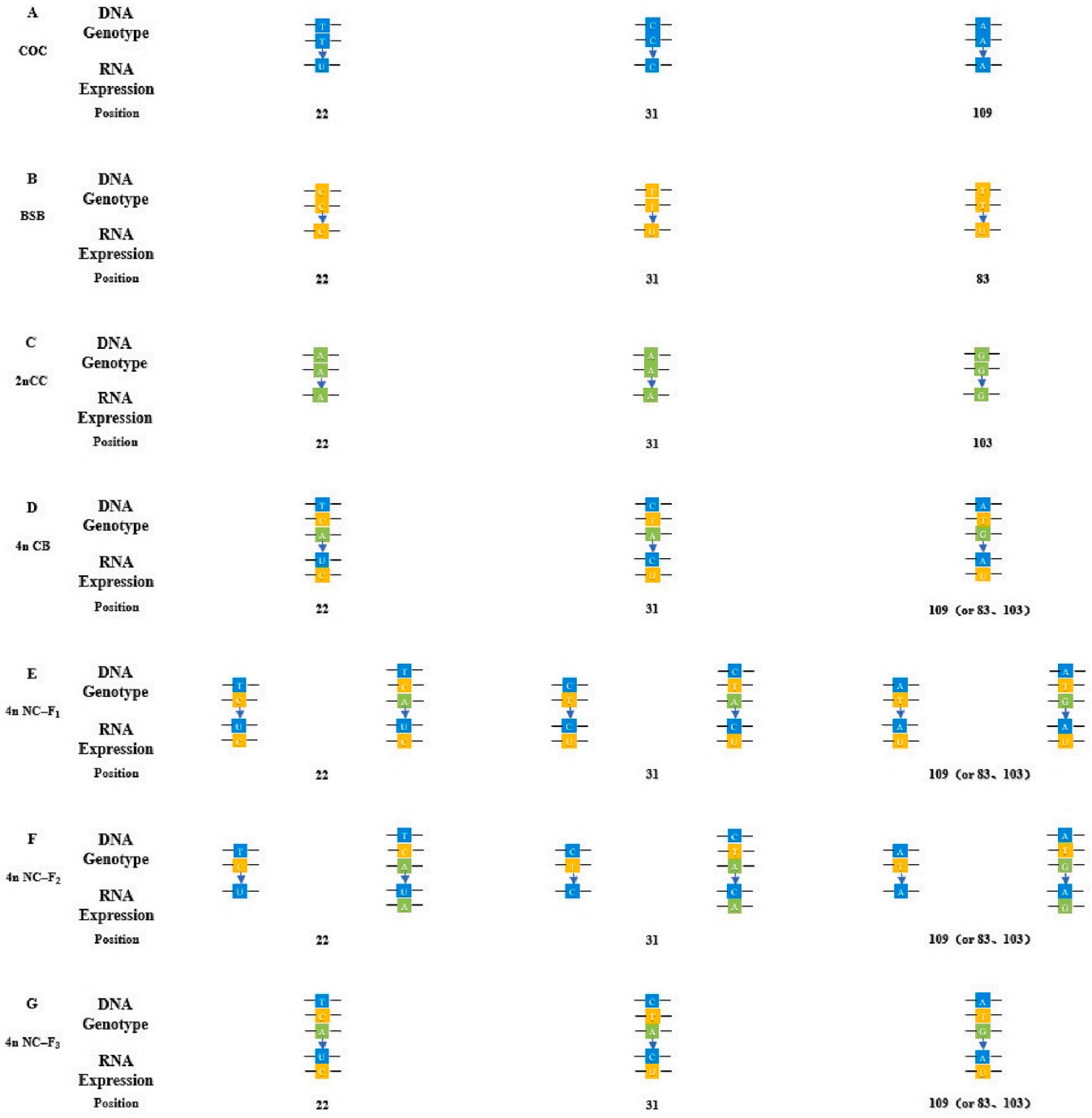


**Fig. 7.** Inheritance and expression patterns of 45S rRNA from 4nCB and 4nNC lineage and their parents. A: Genotype and expression of 45S rRNA gene in COC. B: Genotype and expression of 45S rRNA gene in BSB. C: Genotype and expression of 45S rRNA gene in 2nCC. D: Genotype and expression of 45S rRNA gene in 4nCB. E: Genotype and expression of 45S rRNA gene in 4nNC-$F_1$. F: Genotype and expression of 45S rRNA gene in 4nNC-$F_2$. G: Genotype and expression of 45S rRNA gene in 4nNC-$F_3$. Note: For each pattern of each fish, one sample is used to present.

Ultimately, this result led to unstable inheritance of the biparental genetic material in 2nNCRC lineage. In this complex genetic background, the nucleolar dominance patterns of the two hybrid diploid lineages are inconsistent. In the 2nBT lineage, the nuclear dominance pattern underwent a process from instability to stability, and the establishment of nuclear dominance only needed two generations. However, there was no previous report on the study of nucleolar dominance of autodiploid (homoploid) hybrids, which fills the gap in this study of nucleolar dominance of different hybrid species. In the 2nNCRC lineage, the nuclear dominance pattern showed a trend of stable-unstable-stable change, and the stable establishment of nuclear dominance required at least five generations. At the same time, the establishment of the nucleolar dominance pattern presented a more complicated situation, which may be related to the inconsistent coping mechanism among different generations after suffering the "genome shock" effect at the early stage of the formation of the 2nNCRC lineage. In these two diploid lineages, the possible reason for the different nucleolar dominance results is that the number of chromosomes of parent is unequal in the autodiploid species, which leads to the female genetic material being dominant, and the male genetic material can only be fragmented into the genetic material of the offspring. The number of chromosomes of parent is equal in the allodiploid species, which leads to the genetic

material of parent can well coexist, also resulting in a "genome shock" effect that is less significant than that of homodiploid hybrids. In the tetraploid fish lineages, 4nRB through self-inbreeding to form 4nRR (Qin et al., 2014), while 4nCB and 2nNCRC through hybridization to create 4nNC (Wang et al., 2020c). In different genetic backgrounds, the genome of the former is relatively genetically stable. At the same time, the genome of the latter may continue to be affected by the comprehensive effect caused by "genome shock" (McClintock, 1984; Natali et al., 1998). Both results eventually led to differences in the nucleolar dominance pattern between the 4nRR and 4nNC lineages. In the 4nRR lineage, there was a stable establishment of a nucleolar dominance pattern in $F_1$, and this pattern was a sustained stable hereditary trend. However, in the 4nNC lineage, the nucleolar dominance pattern showed a trend of unstable-stable-unstable change, and the stable establishment of nucleolar dominance needed at least three generations. The possible reason why the nucleolar dominance results of these two autotetraploid fish were different is that the latter was formed through two rounds of distant hybridization, while the former was formed through a round of distant hybridization of RCC × BSB. The nucleolar dominance of the latter is more significant than that of the former after suffering the "genome shock" effect (Shimizu, 2022). Therefore, it is relatively difficult for the 4nNC lineage to form a genetically stable lineage. The nucleolar dominance pattern is different in the different ploidy lineages formed by the same distant hybridization combination, which may be related to the inconsistent coping mechanism among homoploidy and polyploidy after suffering the "genome shock" effect at the early stage of the formation of the 2nNCRC and 4nNC lineages, and the mechanisms may include gene silencing and methylation (Comai et al., 2003; McClintock, 1984; Natali et al., 1998).

In the study of nucleolar dominance patterns of distant hybrid fish, there are not the same patterns among different distant hybrid fish, which may be related to the bias and randomness of nucleolar dominance (Chen et al., 1998). According to the report, the nucleolar dominance was biased towards the paternal parent in the $F_2$ generation of allotetraploid thale cress (Chen et al., 1998). The nucleolar dominance of the clawed frog (Honjo and Reeder, 1973; Macleod and Bird, 1982) and purple false brome (Idziak and Hasterok, 2008) were biased towards the species of transcription advantages. The nucleolar dominance of 4nRB-$F_1$ and 4nRR-$F_2$ was biased towards maternal parent RCC (Cao et al., 2018), showing obvious maternal genetic effects. However, there was no bias in the nucleolar dominance of the 2nBT lineage (Xiao et al., 2016). In this study, the nucleolar dominance was biased towards BSB in the 2nNCRC lineage, while biased towards COC in the 4nNC lineage. However, the biased results are inconsistent in the different ploidy lineages formed by the distant hybridization of COC and BSB, indicating that the transcriptionally dominant species are not necessarily predominant in the biased characteristic. According to previous research in the lab, there was a similar biased result in the study of the transcriptome of diploid fish and tetraploid fish formed by distant hybridization of red crucian carp (RCC) (2n = 100, ♀) and common carp (COC) (2n = 100, ♂). The expression of the diploid fish tended to be the original paternal parent, while the expression of the tetraploid fish tended to be the original maternal parent (Liu et al., 2016). In the randomness characteristic of nucleolar dominance, rRNA silencing was variable in the $F_1$ generation of the allotetraploid thale cress, demonstrating that nucleolar dominance was random (Chen et al., 1998). Furthermore, the randomness of nucleolar dominance existed in the interspecific hybrids $F_2$ of water fleas (Flowers and Burton, 2006) and 2nBT-$F_2$ (Xiao et al., 2016). This study showed that there was no randomness in the nucleolar dominance of 2nNCRC and 4nNC lineages. The bias and randomness of nucleolar dominance may be related to the mechanism of nucleolar dominance (Chen et al., 1998), which may influence the nucleolar dominance pattern of hybrid species. The mechanisms of nucleolar dominance included DNA methylation (Chen and Pikaard, 1997b), histone modification (Lawrence et al., 2004), and so on. The mechanism of nucleolar dominance plays an important role in the selective silencing of rRNA genes and may alter the nucleolar dominance pattern.

This study has shown that the variant ITS1 sequence, which was highly homologous to ITS1 of 2nCC, was presented in the 2nNCRC and 4nNC lineages. The previous research reported only proposed mutations of the nucleotide base site in the ITS sequence (Schubert and Wobus, 1985) rather than producing a new ITS1 sequence. For example, there are nucleotide base site mutations of the ITS sequences in scallion (*Allium*) (Schubert and Wobus, 1985), 4nRB, and 4nRR (Cao et al., 2018). The earliest theory mechanism of nucleotide base site mutation of the ITS sequences was that nucleotide base happened the "jump" phenomenon in the nucleolus organizing regions of the scallion (Schubert and Wobus, 1985). Previous researchers also proposed related speculative ideas in other different species, such as gene recombination (Havlová et al., 2016), adaptation to high-pressure environments (Lauro et al., 2007), and retrotransposon-driven amplification of rDNA (Havlová et al., 2016; Symonová et al., 2013). In the study of the nucleotide base site mutations of ITS sequences of 4nRR lineage, its researcher speculated about some possible mechanisms, such as the Exosome mediated quality control of misfolded pre-rRNAs following polyadenylation (Chekanova et al., 2007; Kadaba et al., 2006) and the non-functional rRNA decay led to reduced stability of mature rRNAs contained in fully assembled ribosomes and ribosomal subunits (LaRiviere et al., 2006). There is a correlation mechanism for nucleotide base-site mutations in the ITS1 sequence, so what is the correlation mechanism for the formation of a new variant ITS1 sequence? The formative mechanism of the ITS1 variant DNA sequence may be related to the mechanisms of gene recombination (Duarte et al., 2010; Taylor and Raes, 2004), exon shuffling (Moran et al., 1999; Van Rijk et al., 1999), retrotransposition (Betrán et al., 2002), mobile elements (Mao et al., 2015), lateral gene transfer (Ochman et al., 2000), and so on caused by the "genomic shock" effect. The results also showed that two lineages expressed mutant 45S rRNA, which may be related to pre-rRNA splicing (Melen, 1999; Yip et al., 2013).

This study found that the proportion of individuals with mutant ITS1 sequences was a gradually decreasing trend in different generations of the 2nNCRC lineage while was a gradually increasing trend in different generations of the 4nNC lineage (Table 3). In addition, this study also found that some individuals of 2nNCRC-$F_2$ expressed mutant 45S rRNA due to not existing nucleolar dominance. In contrast, other generations of the 2nNCRC lineage with nucleolar dominance did not express the mutant 45S rRNA. However, the opposite result to the 2nNCRC lineage existed in the 4nNC lineage. Some 4nNC-$F_2$ individuals expressed the mutant 45S rRNA due to the presence of nucleolar dominance, while other generations of the 4nNC lineage without nucleolar dominance did not express the mutant 45S rRNA. These phenomena may be related to the formation background of both lineages. The 2nNCRC lineage reportedly was a homoploid species formed by distant hybridization of COC × BSB (Wang et al., 2017). In the early formative stage of homoploid species, homoploid species can only form relatively unstable species due to the lack of genomic protection mechanisms. They can tend to

**Table 3**
Probability of occurrence of mutant ITS1 sequences in different generations of 2nNCRC lineage and 4nNC lineage.

| Generations | Total number of samples | The number of individuals with mutant sequences in the sample | The number of individuals with mutant sequences in the sample/Total number of samples |
|---|---|---|---|
| 2nNCRC-$F_1$ | 2 | 2 | 100.00% |
| 2nNCRC-$F_2$ | 3 | 2 | 66.67% |
| 2nNCRC-$F_3$ | 4 | 2 | 50.00% |
| 2nNCRC-$F_5$ | 3 | 1 | 33.33% |
| 4nNC-$F_1$ | 5 | 3 | 60.00% |
| 4nNC-$F_2$ | 2 | 1 | 50.00% |
| 4nNC-$F_3$ | 4 | 4 | 100.00% |

a relatively stable situation through continuous seif-inbreeding (Abbott et al., 2010; Mavárez and Linares, 2008; Yang et al., 2019). The 4nNC lineage was an autopolyploid species formed by distant hybridization of COC × BSB (Wang et al., 2020c). In the early formative stage of autopolyploid species, due to efficiently protecting the genomic integrity, autopolyploid species can quickly form relatively stable species (Abbott et al., 2010; Mavárez and Linares, 2008; Yang et al., 2019). Therefore, differences in the genetic composition and expression of the two species during the early formative stages of homoploid and polyploid species may cause these phenomena. These phenomena may also be related to the result of each other balance between maintaining the internal stability of the hybrid species genomes and continuous violent oscillations of genomes from different parents in the early stages of hybrid formation (Wang et al., 2021).

There are similarities and differences in the nucleolar dominance patterns between different ploidy hybrid lineages of COC × BSB and other hybrid species. The nucleolar dominance pattern of 2nNCRC and 4nNC lineages will further extend the knowledge of nucleolar dominance in homoploid and polyploid fish formed by distant hybridization. The patterns will also provide significant epigenetic data for the rare reasons for the formation of homoploid hybrids and the reasons for the survival of polyploid hybrids. In addition, the diversity of nucleolar dominance patterns reflects epigenetic diversity as well as genetic and species diversity. Consequently, nucleolar dominance is a buffering agent for distant hybridization and polyploidy formation. Nucleolar dominance also plays an important role in distant hybridization (Mao et al., 2015), polyploidy formation, and the evolution of species (Chandrasekhara et al., 2016).

## Author statement

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## Declaration of Competing Interest

The authors have no conflicts of interest.

## Data availability

Data will be made available on request.

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