Organization and Expression Analysis of 5S and 45S Ribosomal DNA Clusters in Autotetraploid Fish Derived From Carassius Auratus Red Var. (♀) × Megalobrama Amblycephala (♂)

Chun Zhao
Hunan Normal University
Yuxin Zhang
Hunan Normal University
Huan Qin
Hunan Normal University
Chongqing Wang
Hunan Normal University
Xu Huang
Hunan Normal University
Li Yang
Hunan Normal University
Tingting Yu
Hunan Normal University
Xidan Xu
Hunan Normal University
Xiang Luo
Hunan Normal University
Qinbo Qin (qqb@hunnu.edu.cn)
Hunan Normal University
Shaojun Liu
Hunan Normal University

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Organization and expression analysis of 5S and 45S ribosomal DNA clusters in autotetraploid fish derived from *Carassius auratus red Var.* (♀) × *Megalobrama amblycephala* (♂)

Chun Zhao[1†], Yuxin Zhang[1†], Huan Qin[1], Chongqing Wang[1], Xu Huang[1], Li Yang[1], Tingting Yu[1], Xidan Xu[1], Xiang Luo[1], Qinbo Qin[1]*, Shaojun Liu[1]*

[1]State Key Laboratory of Developmental Biology of Freshwater Fish, Engineering Research Center of Polyploid Fish Reproduction and Breeding of the State Education Ministry, College of Life Sciences, Hunan Normal University, Changsha, 410081, Hunan People's Republic of China.

*Correspondence:

Qinbo, Qin, State Key Laboratory of Developmental Biology of Freshwater Fish, Engineering Research Center of Polyploid Fish Reproduction and Breeding of the State Education Ministry, College of Life Sciences, Hunan Normal University, Changsha, 410081, Hunan People's Republic of China. Email: qqb@hunnu.edu.cn. Telephone number, 186-7074-7358.

Shaojun, Liu, State Key Laboratory of Developmental Biology of Freshwater Fish, Engineering Research Center of Polyploid Fish Reproduction and Breeding of the State Education Ministry, College of Life Sciences, Hunan Normal University, Changsha, 410081, Hunan People's Republic of China. Email: lsj@hunnu.edu.cn. Telephone number, 136-3741-8159.

Authors E-mail:

Chun Zhao, Email: 3238431088@qq.com.

Yuxin Zhang, Email: 296984632@qq.com.

Huan Qin, Email: 1178373275@qq.com.

Chongqing Wang, Email: 1436236021@qq.com.

Xu Huang, Email: 2696177754@qq.com.

Li Yang, Email: 1430404360@qq.com.

Tingting Yu, Email: 768060178@qq.com.

Xidan Xu, Email: 1912533911@qq.com.

Xiang Luo, Email: 2977286314@qq.com.

Qinbo Qin, Email: qqb@hunnu.edu.cn.

Shaojun Liu, Email: lsj@hunnu.edu.cn.

Abstract

Background: Autotetraploid fish (4n = 200, RRRR) (abbreviated as 4nRR) are derived from whole genome duplication of red crucian carp (2n = 100, RR) (abbreviated as RCC). rDNA is often used to study molecular evolution of repeated sequences because it has high copy rate and special conserved coding regions in genomes. In this study, we determined the sequences (5S, ITS1-5.8S-ITS2 region), structure, methylation level (NTS and IGS), and expression level (5S and 18S) of 5S and 45S rRNA genes in 4nRR and RCC in order to elucidate the effects of autotetraploidization on ribosomal DNA (rDNA) in fish.

Results: Results showed that there was high sequence similarity of 5S, 5.8S and ITS1 region between 4nRR and RCC. This study also identified two different types of ITS2 region in 4nRR and predicted the secondary structure. It turns out that both secondary structures are functional. Compared with the diploid ancestor of RCC, there was no significant difference in NTS (5S) methylation level, but the expression level of 5S rRNA was lower in 4nRR, indicating that methylation had little effect on the expression level in 4nRR. IGS (45S) was hypermethylated in 4nRR compared to RCC, but the expression of 18S gene were no significantly different from that in RCC, indicating that methylation regulation affected gene expression in 4nRR.

Conclusion: These results demonstrate the effects of related structure and expression of autotetraploidization on rDNA. In addition, this study provides reference for studying the effect of autopolyploid on the evolution of species.

Key words: autotetraploid, ribosomal DNA, methylation, polyploidy

Background

Polyploidy studies have reported about different aspects of life such as genome duplication, gene expression, and subsequent evolution (Adams *et al.,* 2005; Hegarty *et al.,* 2013). Polyploids can be classified into autopolyploids and allopolyploids. The former presents two or more homologous chromosomes in a homopolyploid which may contribute to the formation of polyvalent bodies during meiosis, whereas the latter predominantly forms bivalent pairings (Comai, 2005; Qin *et al.,* 2019a). It is worth noting that most polyploidy associated studies mainly focus on plants and less on animals. In our previous studies, we developed allotetraploid hybrids (4n = 148, RRBB) (abbreviated as 4nRB) from the first generation of *Carassius auratus red var*. (2n = 100, RCC) (♀) × *Megalobrama amblycephala* (2n = 48, BSB) (♂) hybrids (Qin *et al.,* 2014a). In subsequent studies, abnormal chromosomal behavior during meiosis in allotetraploid hybrids (4nRB) led to the formation of autotetraploid sperm and autodiploid eggs, which eventually formed autotetraploid fish (4nRR) (Qin *et al.,* 2014b; Qin *et al.,* 2015). Current research has mainly focused on allopolyploids, with only few autopolyploid studies. As the first vertebrate to evolve, the genomes of fish have been comprehensively studied, and thus they can be used to better understand the evolution of vertebrate cell genome (Symonová and Howell, 2018).

Ribosomal DNA (rDNA) is commonly used to study the molecular evolution of multigene families. In eukaryotes, rDNA genes are mainly divided into two categories, 5S rDNA and 45S rDNA repeats. rDNA encodes rRNA that represents a highly conserved gene product in all cells (Pinhal *et al.,* 2011; Long and Dawid, 1980; Cao *et al.,* 2018). In animals, 45S rDNA contains 18S, 5.8S, 28S, and spacers (IGS, ITS1 and ITS2), while the 5S rDNA gene is a unit consisting of a gene transcription region (120bp) and a non-transcribed spacer (NTS). Previous studies have reported that the three types of 5S rDNA classes (type I: 203bp; type II: 340bp; and type III: 477bp) are differentiated using NTS types (NTS I, NTS II, and NTS III) (Long and Dawid, 1980; Korn and Brown, 1978; Qin *et al.,* 2019a). IGS is a transcriptional regulatory

sequence of rDNA which modulates cellular processes (Ruffini *et al.,* 2013; Fernández *et al.,* 2000). Previous analyses of rDNA repeats have mostly been carried out in invertebrates and plants. Therefore, information on 5S and 45S rDNA in vertebrates is scarce. One study reported that rRNA molecules must fold into secondary structures in order to function properly in ribosomes (Noller, 1984). ITS2 provides useful biological information at a higher taxonomic level, even in all eukaryotes, because it has a conserved secondary structure (Coleman, 2007). Many gene promoter regions are rich in CpG, commonly known as CpG islands. Studies have shown that cytosine methylation in CpG dinucleotide guanosine 5' plays an important role in gene expression regulation (Bird, 1992; James *et al.,* 1996). In this study, we analyzed the sequence, structure, methylation, and expression changes in 5S and 45S rDNA clusters between autotetraploid fish (4nRR) and its parental species (red crucian carp (RCC)). From an evolutionary perspective, comparing the arrangement of synthetic autopolyploids with parents of the 5S and 45S rDNA makes sense because of their similar genomic compositions. Our results will provide a new perspective for the organization and evolution of multigene families.

Results

Expression sequence analysis of 5S rRNA coding region and ITS1-5.8S-ITS2 sequence

A total of 40 copies of the gene sequences were analyzed from 4nRR and RCC. Amplification of the 5S rRNA coding region in 4nRR and RCC produced a 120 bp band. BLASTn alignment of the sequences detected a few base substitution changes when the autotetraploids (4nRR) were compared with the corresponding parents (RCC) (Fig. 1). Moreover, the coding region sequences of 4nRR had high sequence identity (average similarity of 97.5%) with corresponding sequences from RCC. Therefore, our preliminary analysis showed that the 5S rRNA coding region of 4nRR had high similarity with the corresponding parental species sequence (GenBank Accession Nos. MZ041022 and MZ041023).

It is well known that two specific sequences (called internal transcription spacers) separate the mature rRNA sequences: ITS1 (between 18S rRNA and 5.8S rRNA) and ITS2 (between 5.8S and 28S rRNA). We cloned and sequenced PCR products in order to compare the internal transcription region (ITS1-5.8S-ITS2) of 4nRR and RCC. We divided the ITS region into ITS1, 5.8S, and ITS2 regions for better comparison. BLASTn sequence alignments showed that the ITS1 and 5.8S rRNA of 4nRR had 100% similarity (Fig. 2) to RCC (ITS1: GenBank Accession Nos. MZ041015 and MZ041016; 5.8S: GenBank Accession Nos. MZ041020 and MZ041021). Nevertheless, we found two different types of ITS2 in 4nRR: type I (inherited from the parental species (RCC)) and type II (a newly formed type which was only expressed in tetraploid species) (Fig. 3) (GenBank Accession Nos. MZ041017-MZ041019). Figure 3 showed intraspecific variation of these sequences. Results indicated that type II ITS2 had obvious insertion, deletion and base substitution. These findings suggest that the ITS1 sequence is much more conserved than ITS2.

Prediction of ITS2 secondary structure

ITS2 usually has four helices, but not all eukaryotes have the same number of helices. Studies have shown that only helix II and helix III are recognizable and are essentially common in all organisms (Coleman, 2007). In this study, we predicted the secondary structure of ITS2 according to the two different sequences of type I and type II (Fig. 4). It turned out that both secondary structures were functional. The results showed that helix II (pyrimidine-pyrimidine) and helix III had high conservation in type I and type II of ITS2, especially the 5' side of helix III (CCGGTGG).

Expression analysis of 5S and 18S rRNA

We compared the expression of 5S and 18S rRNA genes in 4nRR using quantitative real-time PCR with RCC acting as the control group (Fig. 4). Results showed that the amount of 5S transcriptional products in

the liver tissues of 4nRR was significantly lower than that of RCC group (Fig. 5(A); $P < 0.05$). However, there was no significant difference in the expression of 18S rRNA gene between RCC and 4nRR (Fig. 5(B); $P > 0.05$). These results suggested that the effects of polyploidy on the expression levels of 5S and 18S rRNA genes were not consistent.

Methylation-Specific PCR of NTS (5S) and IGS (45S)

The results showed that only the extent of methylation difference in NTS II was observed because the 5S arrays of NTS I and NTS III had the same level of methylation in RCC and 4nRR (Fig. 6) (GenBank Accession Nos. MZ041027- MZ041032). However, there was no significant methylation difference of NTS II in RCC and 4nRR (85% and 92.5%, respectively) ($P > 0.05$). Figure 7 showed analysis of the IGS methylation status of 45S rRNA in liver tissues (GenBank Accession Nos. MZ041024-MZ041026). Our results indicated that there were two different types of IGS (4nRR I and 4nRR II) in 4nRR. Furthermore, 4nRR I had a similar methylation level with RCC ($P > 0.05$), while 4nRR II had a higher methylation level than RCC ($P < 0.05$). In general, the IGS methylation status of 4nRR was hypermethylated and the degree of IGS methylation was negatively correlated with the relative expression of genes

Discussion

Ribosome 5S and 45S rDNA genes play a critical role in ribosome folding and functionality (Pinhal *et al.,* 2011). Studies have shown that the ITS region is a useful genetic marker for the analysis of intraspecific variation (Kahn, 1996; Lee and Wen, 2001; Collins *et al.,* 1993). Our results indicated that the coding region of 5S rRNA gene, 5.8S rRNA gene and ITS1 region sequences were almost conserved in 4nRR. A previous study reported that the 5S rRNA gene (transcribed by RNA polymerase III) contained an internal control region (ICR) that acted as the promoter for the gene (Hallenberg *et al.,* 1994). Generally, variation in 5S

rDNA occurs in the NTS region, but the coding region remains unchanged (Martins *et al.,* 2001; Martins *et al.,* 2002). It has been reported that the ITS region (45S rDNA) participates in proper processing of ribosomal RNA sequences and forming mature functional rRNA subunits (Johansen *et al.,* 2006). Thus, based on these results, autopolyploidization has no significant effect on the organization of 5S and 5.8S rRNA genes. With regard to the ITS2 region, a comparison between RCC and 4nRR indicated that there were two types of ITS2 region in 4nRR. These mutations can be attributed to the weak selection pressure on any single copy of the gene, thereby allowing a degree of variation in the gene region (Kellogg and Appels, 1995; Allaby and Brown, 2001). In addition, hybridization is accompanied by genome changes in order to overcome threats to its survival (Mcclintock, 1984).

The ITS2 secondary structure presented in this study is consistent with other ITS2 structure predictions. ITS2 usually has four helices, with helix II and helix III being recognizable in almost all organisms. Helix II is very short, does not have any branches, and has a pyrimidine–pyrimidine mismatch. On the other hand, helix III is usually longer than helix II and often has branches. Previous studies reported that the largest absolute sequence conserved region in the entire ITS2 is located on the 5' side (YCGGTGGR) of helix III close to the tip (Coleman, 2007; Joseph, 1999; Coleman and Vacquier, 2002). Moreover, these conservative characteristics are preserved in type I and type II helices. The ITS2 conserved structural motifs is necessary for all aspects of ribosomal processing (Keller *et al.,* 2009). The helix of region I is highly similar in both types. Traditionally, helix IV is the most variable region in ITS2, thus, it is normal for the two types of 4nRR to be different, both secondary structures are functional (Young and Coleman, 2004; Coleman, 2007). These differences may also reflect differences in the formation of mature functional ribosomes because there are many steps involved in the production of a mature rRNA gene (Johansen *et al.,* 2006).

Newly formed polyploids undergo extensive genomic changes after genome merger and replication (Madlung *et al.,* 2013). Polyploidy significantly affects genome formation and other genetic aspects such as

gene expression. We found that there were no significant differences in expression of the 18S rRNA gene between 4nRR and RCC. However, the 5S rRNA gene showed significant differences. Moreover, all the genes were doubled in autotetraploid fish compared to RCC. Theoretically, if each gene was normally expressed, the total gene volume would be much higher than that of the diploid parent. This supports the findings of a previous study which reported that the origin of polyploid lineages is not consistent at the ploidy level of gene expression, with regard to increase or decrease (Church and Spaulding, 2009). Previous studies have shown that the genomic DNA locus of autotetraploids differ from those of diploids (Qin *et al.,* 2019b). However, these results do not explain whether the gene expression differences were caused by genomic DNA site changes or epigenetic silencing. For example, changes in DNA methylation, a common epigenetic phenomenon, can also regulate gene expression.

To verify whether the differences in RCC and 4nRR were dependent on the methylation status, we analyzed the NTS methylation pattern of the different 5S rRNA arrays of RCC and 4nRR using the genomic sequencing technique. Previous studies have associated cytosine methylation with the non-expression of a gene (Flavell *et al.,* 1986; Razin and Riggs, 1980). The 5S rRNA clusters of NTS I and NTS III in RCC and 4nRR were all methylated and they showed no difference in methylation status. Furthermore, although the methylation levels of NTS II varied, there was no significant difference. In summary, the methylation level in all 5S sequences is similar. These results indicate that methylation may not affect the binding of transcription factors to 5S rDNA, nor did it regulate transcription of the 5S rRNA gene. Thus, it may have no significant effect on expression of 5S rRNA gene. IGS, as a variable part of 45S rDNA, usually contains enough variation to allow examination of genetic relationships between closely related species (Fernández *et al.,* 2000; Penteado *et al.,* 1996; Nickrent and Patrick, 1998). There are two types of IGS in 4nRR; type I are hypomethylated, while type II are typehypomethylated. This ensures that the methylation level is consistent in the tetraploid. Among them, there was no significant difference between type I and RCC, while type II

showed significant difference and a higher methylation degree than RCC. The results showed that IGS methylation was negatively correlated with relative gene expression, and methylation inhibited the expression level to some extent. The emergence of two types of IGS can be attributed to the fact that the establishment of nucleolar dominance requires several generations of selection and screening during the homologous polyploidization process. It is possible that the inhibitory mechanism that controls nuclear dominance in hybrids also control the number of active 45S rRNA gene in pure breeds and may reflect the dose compensation mechanism (Wallace and Langridge, 1971; Pikaard, 1999; Cao *et al.,* 2018). However, regulation of the active 5S rRNA gene may be different. Our quantitative real-time PCR results indicated that the expression of 5S rRNA gene was low in all 4nRR individuals, while the expression level of 18S rRNA gene showed no significant difference between RCC and 4nRR. In our previous studies, we observed loss of chromosomal sites in the generation of the tetraploid system (Qin *et al.,* 2019b). As regulatory regions, NTS and IGS regulate gene expression in the late stage according to methylation. This phenomenon can explain why the number of chromosomes in autotetraploid fish increased but there was no positive increase in the expression level. In addition, 45S and 5S rRNA could not make much difference in number because they form the large and small subunits of the ribosome. Otherwise, the subunits would not be paired quantitatively.

rDNA is an important component of nuclear structure and an integral part of the mechanisms that maintain genomic integrity (Guetg and Santoro, 2012; Tsekrekou *et al.,* 2017; Grummt, 2013). This study has revealed the basic unity of rDNA sequences in the hybrid species and that 5S rRNA and ITS sequences are still conserved during the autopolyploidization process. One study reported that the high transcriptional and recombination rates of rDNA contribute to the diversity of the genome and formation of reproductive barriers (Symonová and Howell, 2018). Moreover, the repetitive nature of rDNA and other duplicated genes leads to a high degree of evolutionary dynamics (Terencio *et al.,* 2015; Charlesworth *et al.,* 1994). Therefore,

this tetraploid lineage can be an attractive model for elucidating genomic changes associated with quadrupling. Our results will expand the understanding of homologous polyploidy effects on ribosomal DNA and have important significance for the evolutionary study of polyploid crucian carps. In addition, the information on the sequence and structure of the autotetraploid fish (5S and 45S rRNA) provides a reference for further studies on the evolution of rDNA in fish and other vertebrates.

Conclusion

By comparing and analyzing the sequences, structures, expression levels and methylation levels of ribosomal RNA genes (5S rRNA, 45S rRNA) in autotetraploid fish (4nRR), we found that 5S rRNA, 5.8S rRNA and ITS1 were highly conserved, but autopolyploidization promoted the structural differentiation of ITS2. The expression levels and methylation results showed that the methylation of the 5S rRNA regulation region did not regulate the expression of the gene, but the 45S rRNA regulation region affected the expression of 18S rRNA gene in autotetraploid fish to some extent. Polyploidization is one of the main driving forces of biological evolution. The data from this study provide some references for studying the evolution of ribosomal DNA in autopolyploid species.

Materials and methods

Materials

Experimental fish were provided by the Engineering Center of Polyploid Fish Breeding of the National Education Ministry, Hunan Normal University.

Expression sequence and expression analysis of 5S rDNA

Our analysis involved sequencing of 30 clones for each accession. Genomic DNA was isolated from blood of all samples using genomic DNA extraction kit (Takara). PCR was then performed with a specific primer complementary to the 5S rRNA conserved coding region. The primers were synthesized according to the method described by Qin *et al.,* (2010). Primer sequences were; GCTATGCCCGATCTCGTCTGA (5′-3′) and CAGGTTGGTATGGCCGTAAGC (5′-3′). The PCR program included 30 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 35 s, and elongation at 72 °C for 35 s. Final extension was performed at 72 °C for 15 min. Moreover, RNA was extracted from liver tissues using Trizol reagent in accordance with the manufacturer's instructions (Invitrogen, San Diego, CA). Next, the RNA was reverse transcribed to cDNA using the PrimeScript™ RT reagent kit (Perfect Real Time, Takara) with a gDNA eraser. The 5S rRNA gene-specific primer (5′-CAGGTTGGTATGGCCGTAAG-3′) was then used to amplify the first-strand cDNA.

Amplification products were analyzed using 1-1.2% agarose gel electrophoresis stained with ethidium bromide. The PCR products were then cloned, followed by selection of clones with inserts of the predicted length (203 bp) for sequencing. Next, Bioedit and ClustalW software was used to analyze the sequence homology and variation of the amplified fragments of 4nRR and RCC. To determine gene expression differences, quantitative real-time PCR (Prism 7500 sequence detection system, ABI) was used to analyze the expression level of the target genes. Relative gene expression was normalized to the expression of β-actin gene, an endogenous control.

Expression sequence (ITS1-5.8S-ITS2) and expression (18S) analysis of 45S rDNA

For amplification of ITS1-5.8S-ITS2, the following primer was used: 5′-AGTCGTAACAAGGTTTCCGTAGGTG-3′ and 3′-TTATGGCCGTGCTCTGGCTAT-5′ (Cao *et al.,* 2018). PCR was carried out using the conditions described above but with exception of the annealing

temperature (57°C). Moreover, the 18S rRNA gene-specific primer (5′-CATCTAAGGGCATCACAGAC-3′) was used to amplify the first-strand cDNA. Sequences and expression analysis were conducted according to a previously described protocol (Cao *et al.,* 2020).

Secondary structure of ITS2 sequences

We conducted comparative sequence analysis to elucidate the secondary structure of ITS2 sequences. More information about species relatability and intraspeciality variation was obtained by examining the functional folding patterns or secondary structures of the rRNA regions of interest (Wesson *et al.,* 1993; Coleman, 2007). We determined the structure with the lowest free energy and compared the secondary structure of ITS2 cloned by 4nRR.

Methylation-Specific PCR

Using the common carp genome as a reference, we identified the spacer regions (NTS and IGS) of 5S and 45S rRNA genes in NCBI database. Sequences of the corresponding target NTS and IGS were retrieved from RCC genome ((DDBJ/EMBL/GenBank accession no. PRJNA289059) and 4nRR genome (unpublished), respectively. Genomic DNA was extracted from liver tissues using Sangon Animal Genomic DNA extraction kit (n=3 fish per treatment). Next, the extracted DNA was treated according to the methylcoded bisulfite conversion kit protocol (Thermo Fisher). Gene-specific primers for NTS (NTS I, NTS II, and NTS III) and IGS (Table 1) were designed using Primer 5.0 software. PCR products were ligated, transformed, and sequenced. Finally, sequences obtained from methylation results were retrieved using BiQ analyzer.

Abbreviations

RCC: *red crucian carp*

BSB: *Megalobrama amblycephala*

4nRB: allotetraploid hybrids

4nRR: autotetraploid fish

NTS: non-transcribed spacer

IGS: internal transcribed spacer

rDNA: ribosomal DNA

rRNA: ribosomal RNA

Declarations

Ethics approval and consent to participate

The study was approved by Ethics Committee of Hunan Normal University, all methods were carried out in accordance with relevant guidelines and regulations. This study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study were included in this published article and its supplementary information files. The sequence for these libraries have been uploaded to the NCBI Sequence Read Archive site (http://www.ncbi.nlm.nih.gov/sra/; accession nos.): ITS1 (GenBank Accession Nos. MZ041015 and MZ041016); ITS2 (GenBank Accession Nos. MZ041017-MZ041019); 5.8S (GenBank

Accession Nos. MZ041020 and MZ041021); 5S (GenBank Accession Nos. MZ041022 and MZ041023); IGS (GenBank Accession Nos. MZ041024-MZ041026); NTS (GenBank Accession Nos. MZ041027-MZ041032).

Competing interests

The authors declare that there are no competing financial interests.

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Authors' contributions

SL and QQ have designed of the work. CZ has contributed to this study for the design, in executing experiments and in writing manuscript. YZ, HQ, CW and XH have made substantial contributions to the acquisition and analysis of data. LY, TY, XX and XL have substantively revised the work.

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Figure captions

Fig. 1. Expression sequences of 5S rRNA coding regions from RCC and 4nRR.

Fig. 2. Expression sequences of ITS1 and 5.8S from RCC and 4nRR.

Fig. 3. Expression sequences of ITS2 from RCC and 4nRR.

Fig. 4. ITS2 RNA transcript secondary structures predictions in 4nRR. A is the secondary structure in RCC and type I. B is the secondary structure of type II expressed in 4nRR.

Fig. 5. Relative expression of the 5S and 18S genes in the livers of RCC and 4nRR during the breeding season. (A) is the relative expression levels of 5S in the liver. (B) is the relative expression levels of 18S in the liver.

Fig. 6. Sequencing results of methylation extent of NTS II of 5S rDNA, wherein yellow represents methylation and blue represents no methylation

Fig. 7. Sequencing results of methylation extent of IGS of 45S rDNA, wherein yellow represents methylation and blue represents no methylation.

Tables

Table 1 Primers used in methyl-specific PCR

Primer name	Sequence
For clonig sequence	
NTS-F	5′-GCTATGCCCGATCTCGTCTGA-3′
NTS-R	5′-CAGGTTGGTATGGCCGTAAGC-3′
IGS-F	5′-GGGTGGCGGCGTCTGATAGA-3′
IGS-R	5′-CCCAAACTTCAGGATTTGTGC-3′
For methyl-specific PCR	
NTS I-F	5′-CGGAAGTTAAGTAGGTTTGGGT-3′
NTS I-R	5′-GTAAACGAAAACTACTACAAAA-3′
NTS II-F	5′- GAATATTAGGTGTTGTAAGTT-3′
NTS II-R	5′-AACCGTAAACGAAATCTACTA-3′
NTS III-F	5′-TTGGGAATATTAGGTGTTGTAA-3′
NTS III-R	5′-TAAACGAAAACAACTACAAAAA-3′
IGS RCC-F	5′-GYGTTTGATAGAGGGTTAYGGGGTTT-3′
IGS RCC-R	5′-TAAAAACCCRTCAACCCCTCTCAAAC-3′
IGS 4nRR I-F	5′-GTTGTATTTYGGTTTTTTTGGGGGTT-3′
IGS 4nRR I-R	5′-TAATAAAAACCCGTCAACCCCTCTCA-3′
IGS 4nRR II-F	5′-TTTTGGTTTTYGGTGGTGTGGGGATT-3′
IGS 4nRR II-R	5′-TCTCAACRACRCCRAAACCCAAAAAC-3′

Additional files

Fig. 1. Expression sequences of 5S rRNA coding regions from RCC and 4nRR.

Fig. 2. Expression sequences of ITS1 and 5.8S from RCC and 4nRR.

Fig. 3. Expression sequences of ITS2 from RCC and 4nRR.

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Fig. 7. Sequencing results of methylation extent of IGS of 45S rDNA, wherein yellow represents methylation and blue represents no methylation.

Figures

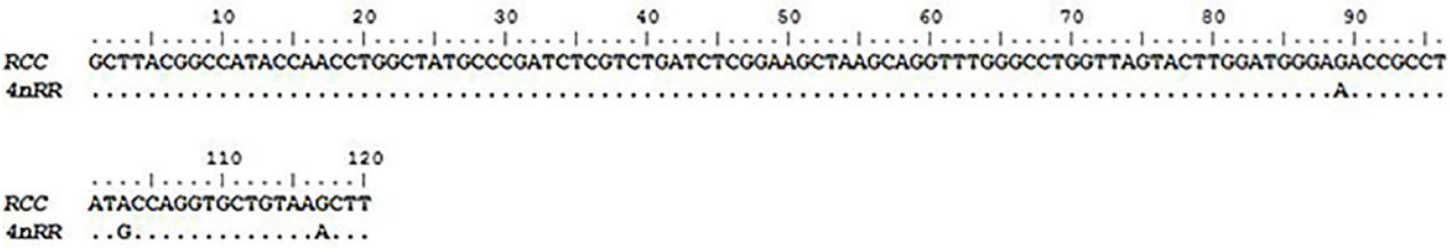

Figure 1

Expression sequences of 5S rRNA coding regions from RCC and 4nRR.

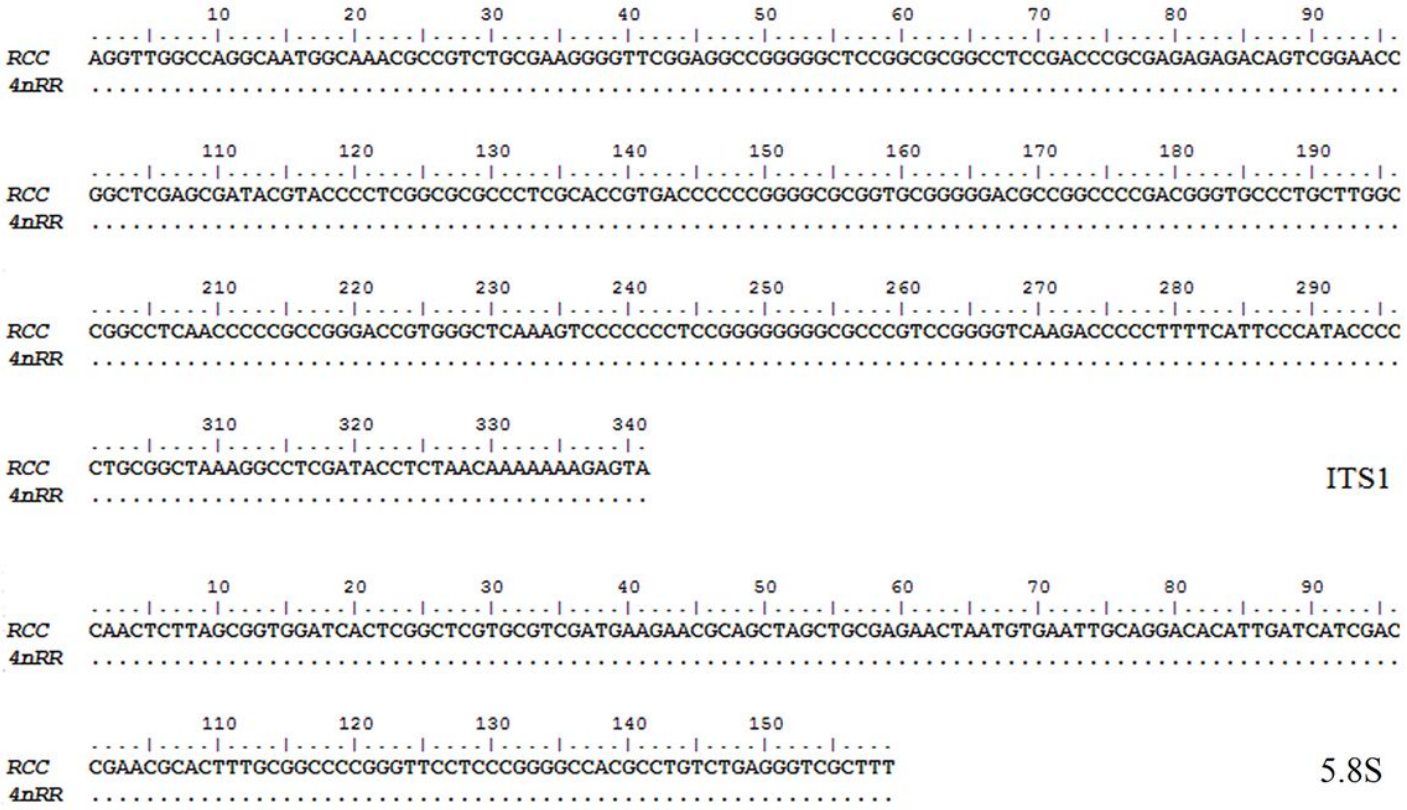

Figure 2

Expression sequences of ITS1 and 5.8S from RCC and

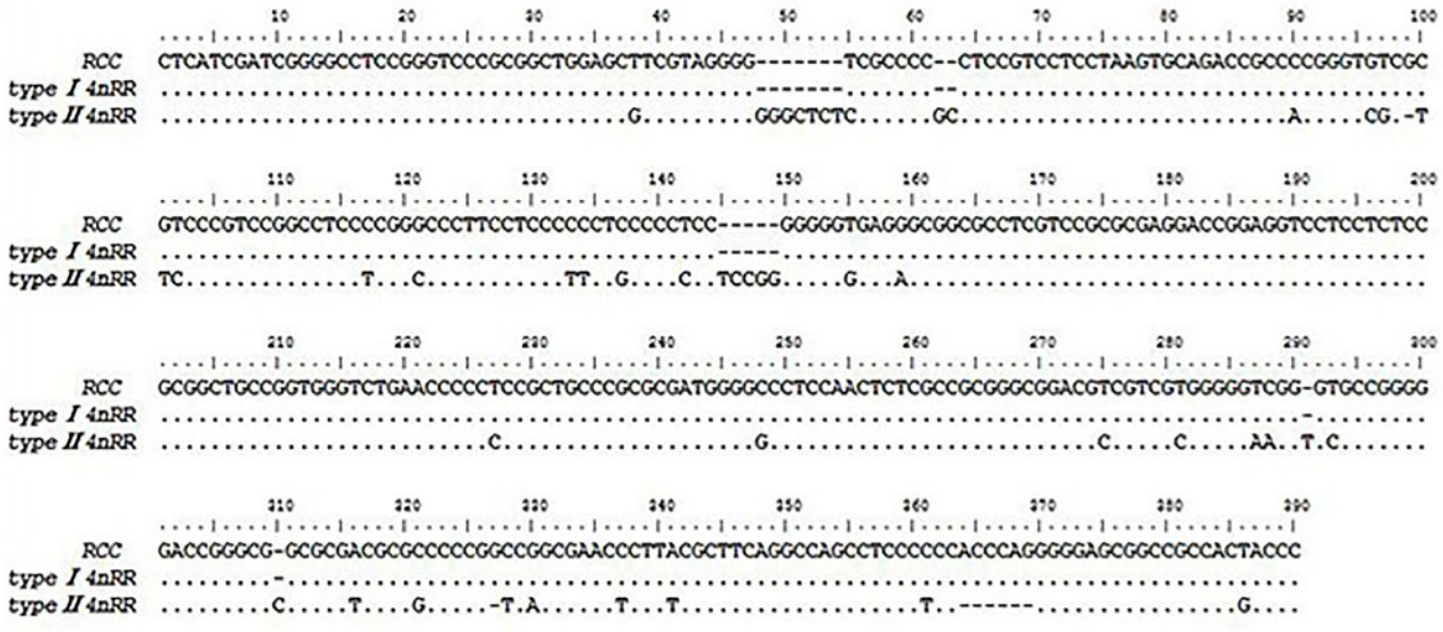

Figure 3

Expression sequences of ITS2 from RCC and 4nRR.

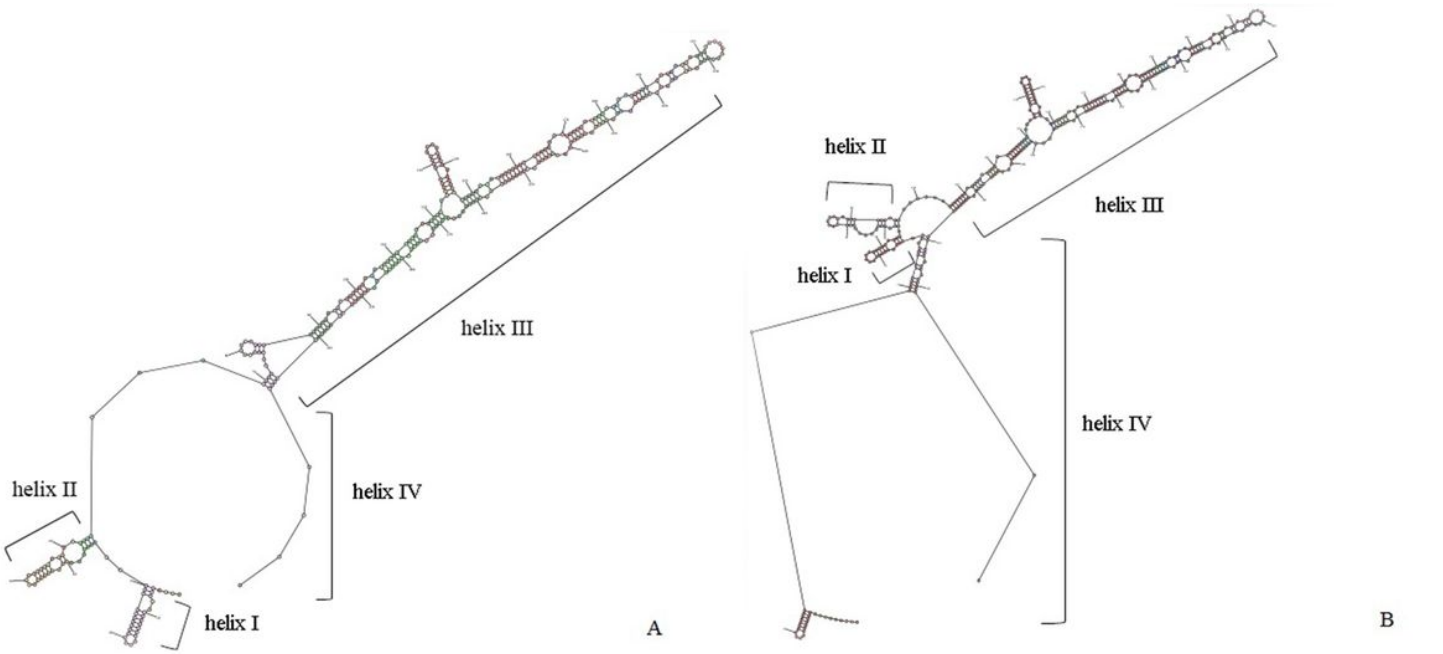

Figure 4

ITS2 RNA transcript secondary structures predictions in 4nRR. A is the secondary structure in RCC and type I B is the secondary stru cture of type II expressed in 4nRR

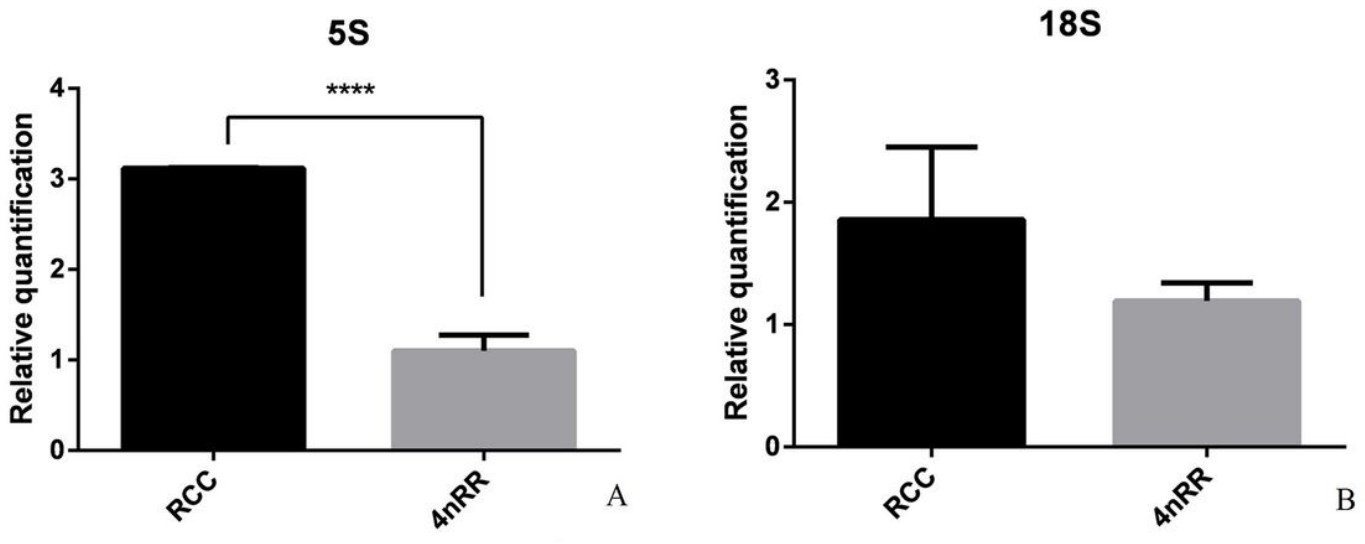

Figure 5

Relative expression of the 5S and 18S genes in the livers of RCC and 4nRR during the breeding season. (A) is the relative expression levels of 5S in the liver. (B) is the relative expression levels of 18S in the liver.

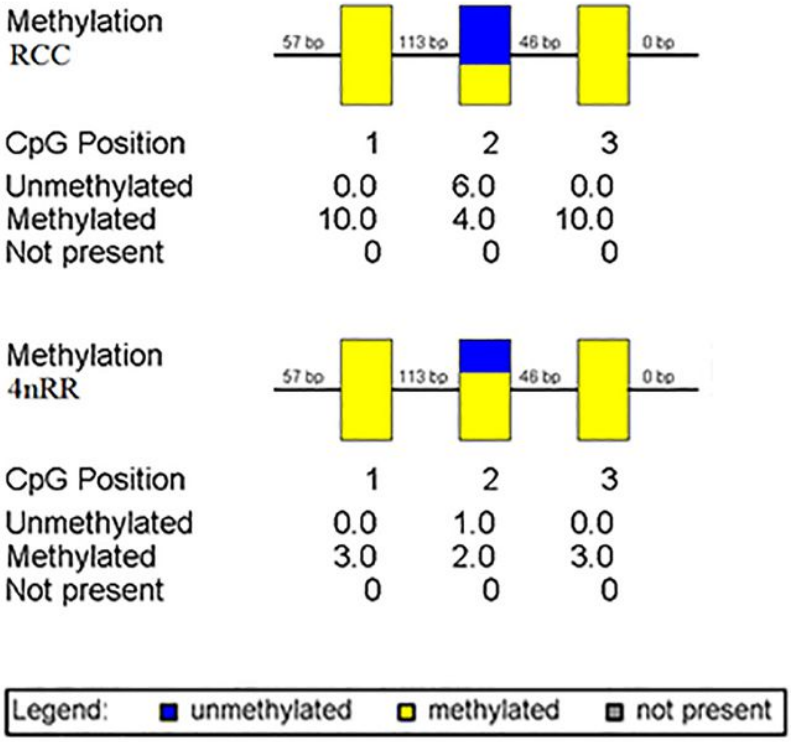

Figure 6

Sequencing results of methylation extent of NTS II of 5S rDNA, wherein yellow represents methylation and blue represents no methylation

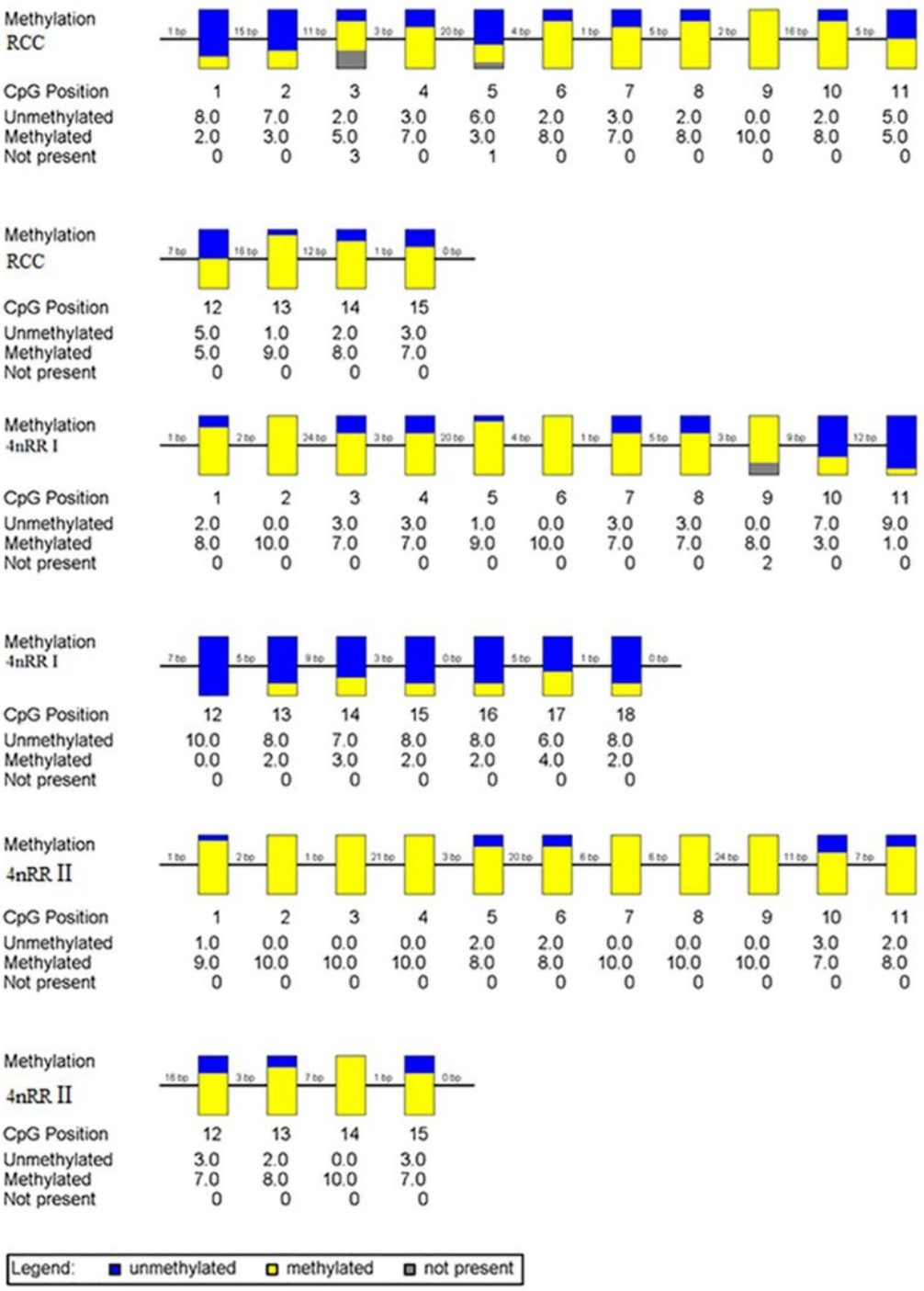

Figure 7

Sequencing results of methylation extent of IGS of 45S rDNA, wherein yellow represents methylatio n and blue represents no methylation.