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Duplication and divergence: Two new *Dmrt1* gene paralogs identified in autotetraploid *Carassius auratus*

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ABSTRACT

Autotetraploid *Carassius auratus* (4n = 200, RRRR, 4nRR), derived from the whole-genome duplication (WGD) of *Carassius auratus* red var. (2n = 100, RR, RCC), displays normal gonadal development and produces diploid gametes. The Doublesex and Mab-3-related transcription factor 1 (*Dmrt1*) gene plays a critical role in testis development in teleosts. To enhance understanding of how autotetraploidization affects gonadal development, the genetic differences, expression traits, epigenetic modifications, and expression localization of *Dmrt1* genes in RCC and 4nRR were examined. Two original *Dmrt1* genes (4nRR_*Dmrt1–1* and 4nRR_*Dmrt1–2*) from RCC were identified in 4nRR, and two paralogous genes (4nRR_*Dmrt1–3* and 4nRR_*Dmrt1–4*) were observed. Analyses demonstrated that males exhibited significantly elevated *Dmrt1* gene expression levels compared to females, both RCC and 4nRR, and this was negatively correlated with the *Dmrt1* promoter methylation. In the 4nRR testis, the original copy (4nRR_*Dmrt1–1*) showed the highest expression level at all developmental stages, ranging from 1.5- to 2.8-fold higher than the other copies. In the 4nRR ovary, the highest expression level was observed for the paralogous gene 4nRR_*Dmrt1–4*, which was 1.4- to 31-fold higher than the other copies. Furthermore, continuous expression and localization of the *Dmrt1* gene during spermatogenesis were observed in RCC and 4nRR, with no obvious signal detected in the ovary. In 4nRR, the two newly derived *Dmrt1* paralogs exhibited a significant expression pattern in the ovary. This observation suggests that WGD may have triggered a restructuring of gene expression regulatory mechanisms, thereby leading to the divergence of gonad-specific expression patterns. These data provide a theoretical basis for understanding gonadal development in polyploid teleosts and offer novel perspectives for fish breeding programs and studies on genetic evolution.

1. Introduction

Polyploidy is caused by the duplication of a single genome (autopolyploid) or the integration of two or more diverse genomes (allopolyploid), which affects genes, genomes, cells, tissues, organisms, and ecosystems (Kihara and Ono, 1926; Wendel, 2000). Polyploidy is common in the plant kingdom but relatively rare in the animal kingdom. Nevertheless, scientists have documented polyploidy in various invertebrates, including insects, and certain vertebrates, including fish, amphibians, and reptiles (Chen and Ni, 2006). The potential synthetic effects of genomic structural changes and altered expression may severely constrain the survival of vertebrate offspring (Liu et al., 2016). Changes in ploidy level have been identified as key events in the evolution of fish groups (Šlechtová et al., 2006), including *Acipenseridae* (Ludwig et al., 2001), *Cyprinidae* (Alves et al., 2001; David et al., 2003), and *Salmonidae* (Crespi and Fulton, 2004; Phillips and Ráb, 2001).

The processes of sex determination and differentiation in fish are intricate and dynamic mechanisms found in vertebrates. These processes are influenced by a combination of genetic, epigenetic, and environmental factors (Devlin and Nagahama, 2002). DNA methylation is an

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Table 1
Information on the primers used for CDS and DNA validation, methylation primers, and assessment of relative expression levels via qRT-PCR.

Primers	Forward sequence(5′-3′)	Reverse sequence (5′-3′)
For CDS cloning		
Dmrt1–1	ATGAGCGAGGAAGAGCAGAATAAC	TCATTTGGTTTGCCCCTCGATG
Dmrt1–2	ATGAGTGAGGAGGAGCAGAG	TTATTTGGTTACCCCATCAACG
For real-time qPCR		
RCC/4nRR_*Dmrt1–1*	GAGGAAGAGCTGGGCATTTG	CATCGAACGGCTCCCTAGAT
RCC_*Dmrt1–2*	GAGGAAGAGCTGGGTATTTG	CATGGAGCGATTCCCTAGGT
4nRR_*Dmrt1–2*	ATCCCAGGGCCACCATCA	GGGAAACGTTATGGCTGGAC
4nRR_*Dmrt1–3*	GAGGAAGAGCTGGGCATTTG	CATGGAACGGCTCCCTAGGG
4nRR_*Dmrt1–4*	ATCTCAGGGCCGCCATCG	CTGGGTGGCACACATGCAG
β-actin	GCCCTGCCCCATGCCATCCT	AGTGCCCATCTCCTGCTCGA
For methyl-specific PCR		
RCC_*Dmrt1–1*	TTGAAGTATGATTTTATGAGATAGT	ATCAATCTACATTTCTAACACTAAC
RCC_*Dmrt1–2*/ 4nRR_*Dmrt1–4*	TAGTTATTTAGTTTTAGTGGGAAGA	TCTAACACTAACAATCTCTCCAACT
4nRR_*Dmrt1–1*	GTATGATTTTATGAGATAGTTTGGT	ATCTACATTTCTAACACTAACAATC
4nRR_*Dmrt1–2*	TTAGTTTTAGTGGGAGAAATTGAAT	TCTAACACTAACAATCTCTCCATCT
4nRR_*Dmrt1–3*	TAGTTATTTAGTTTTAGTGGGAAGA	ATCAACCTACATTTCTAACACTAAC

epigenetic modification that involves the covalent addition of a methyl group to the carbon-5 position of cytosine in genomic CpG dinucleotides, catalyzed by DNA methyltransferases. Although CpH (H = A/C/T) methylation may also exist, in plants and animals, cytosine methylation occurs predominantly in the context of CpG dinucleotides (Gehring and Henikoff, 2007), and CpG methylation is more commonly observed at CpG sites within genomic promoter regions (Gonzalez-Recio, 2012). DNA methylation can influence chromosomal conformation and alter chromatin structure, thereby affecting the binding ability of DNA-binding proteins, histone modifications, and small RNAs (Zhang and Pradhan, 2014). DNA methylation plays a critical role in controlling genome activity; highly methylated regions tend to exhibit low transcriptional activity, which may impact the phenotype of animals (Gonzalez-Recio, 2012). DNA methylation in coding regions is associated with genes that are moderately to highly expressed and enriched (Zhang et al., 2006; Valverde et al., 2024; Bewick and Schmitz, 2017), whereas methylation in promoter regions is typically associated with gene repression or silencing (Kankel et al., 2003; Ding and Chen, 2018). DNA methylation is important in genome regulation (Finnegan et al., 2000); methylation can directly inhibit transcription by blocking the binding of transcription factors (Staiger et al., 1989), or indirectly through proteins that bind to methylated DNA (Sepehri et al., 2019). The Doublesex and Mab-3-related transcription factor 1 (*Dmrt1*) gene is a member of the prototypical *Dmrt* family, with a common DNA-binding motif known as the DM domain (Zhu et al., 2000). The *Dmrt1* gene has been identified as an important and conserved regulator of testis development in different species, including mammals (Raymond et al., 1999), birds (Smith et al., 2009), reptiles (Kettlewell et al., 2000), amphibians (Shibata et al., 2002), and fish (Herpin and Schartl, 2011). The *Dmrt1* gene expression is significantly elevated during testis differentiation in teleost species, including rainbow trout (*Oncorhynchus mykiss*) and lambari fish (*Astyanax altiparanae*) (Marchand et al., 2000; Adolfi et al., 2015). Similarly, higher levels of *Dmrt1* gene expression in the gonads of air-breathing catfish (*Clarias gariepinus*) during spermatogenesis suggest that it may play a role in this reproductive process (Raghuveer and Senthilkumaran, 2009). Furthermore, the *Dmrt1* gene is associated with testis development and may be important for ovary differentiation in zebrafish (Guo et al., 2005). Recent studies have revealed that in Scatophagidae fish, the *Dmrt1* gene has been recruited as the master sex-determining gene via divergent mechanisms: allelic variation versus gene duplication and translocation (Huang et al., 2025). Therefore, the *Dmrt1* gene is essential for gonadal development in teleosts.

The autotetraploid *Carassius auratus* (4n = 200, RRRR, 4nRR) lineage, which was caused by the hybridization of *Carassius auratus* red var. (2n = 100, RR, RCC) (♀) with *Megalobrama amblycephala* (BSB) (2n = 48, BB, ♂), has been demonstrated to develop normal gonads and produce diploid gametes. This lineage (F_2–F_{20}) was created through continuous self-crossing (Qin et al., 2018a; Qin et al., 2014). Additionally, 4nRR possesses four sets of chromosomes inherited from RCC, with significant phenotypic differences between RCC and 4nRR (Qin et al., 2018b; Qin et al., 2014). After polyploid formation, considerable and rapid changes in genome structure and gene expression occur (Leitch and Bennett, 1997). Therefore, the newly synthesized 4nRR serves as an excellent model species to study gonad development in polyploid teleosts.

In this study, we compared the genetic variation, expression characterization, epigenetic modification, and expression localization of the *Dmrt1* gene in RCC and 4nRR. This study was designed to comprehensively explore the properties of *Dmrt1* gene expression in 4nRR, reveal the genetic variation patterns during autotetraploidization.

2. Materials and methods

2.1. Sample collection

All RCC and 4nRR specimens were randomly obtained from Hunan Normal University, China. These fish were generated during the reproductive season in April 2022 at the University Engineering Research Center of Polyploid Fish Breeding and Reproduction, which operates under the State Ministry of Education. All individuals were reared in outdoor ponds (0.067 ha) under controlled conditions, including a suitable pH range (7.0–8.5), water temperature (22–24 °C), dissolved oxygen levels (5.0–8.0 mg/L), and provision of sufficient feed.

According to a detailed histological study on gonadal development in *Carassius auratus* red var. (Wu et al., 2009), this species reaches sexual maturity at approximately one year of age, and the gonads progress through well-defined stages: stage II at 4–5 months, stage III at 6–7 months, and stage IV at 7–10 months post-hatching. Notably, the ovarian development has two rapid growth phases, occurring at 4–8 months and 10–12 months of age. Accordingly, our three time points represent critical developmental windows: 5 months (early differentiation, stage II), 8 months (rapid growth phase, stages III–IV), and 12 months (functional maturity, stages V–VI). Furthermore, Gonadal development in 4nRR proceeded in a manner consistent with that of its diploid progenitor. Therefore, these time points allow for a systematic study of the entire process of gonadal development in RCC and 4nRR.

2.2. Statistical analysis

Data from qPCR were analyzed using an unpaired two-tailed Student's *t*-test. For quantitative analysis of Dmrt1 fluorescence intensity, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was performed. All statistical analyses were carried out using GraphPad Prism software. Statistical significance is represented by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns $P > 0.05$).

2.3. RNA extraction and complementary DNA (cDNA) synthesis

In this study, RNA was systematically extracted from the testis and ovary of RCC and 4nRR using an RNA extraction kit (Omega, R6934–01) following the detailed protocols. To ensure the quality of the extracted RNA, the concentration and purity were measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek) and 2% agarose gel electrophoresis. After confirming that the RNA quality, concentration, and purity were within acceptable ranges, the extracted gonadal RNA was reverse transcribed using a reverse transcription kit (Thermo Fisher Scientific, K1682) according to the manufacturer's instructions.

2.4. Coding sequence (CDS) region cloning

Primers for amplifying the *Dmrt1* gene from RCC (PRJNA487739) and 4nRR (PRJCA047597) were designed using primer software (version 5.0), with cDNA as the template (Table 1). All primers were synthesized by Tsingke (Beijing, China). Polymerase chain reaction (PCR) amplification was conducted in a 50 μL reaction system using LA Taq polymerase (TaKaRa, China). The resulting PCR products were cloned into the pMD18-T vector (TaKaRa, China) and transformed into *Escherichia coli* DH5α competent cells (Sangon, China). To minimize PCR-derived errors, at least 60 positive clones were selected using PCR screening and sent to Tsingke for sequencing. The nucleotide and amino acid sequences of the *Dmrt1* gene were aligned using BioEdit software.

2.5. Phylogenetic analysis

Phylogenetic analysis of the *Dmrt1* gene copies was performed using MEGA 11 software. Multiple sequence alignment was conducted with the ClustalW algorithm to ensure alignment accuracy. Subsequently, the Neighbor-Joining (NJ) method was employed to construct the phylogenetic tree, and the robustness of the tree was evaluated by 1000 bootstrap replicates to ensure the reliability of the results.

2.6. Gonad structures

Based on the characteristics of *Dmrt1* gene expression at various developmental stages, 10 females (5, 8, and 12 months old) and 10 males (5, 8, and 12 months old) from RCC and 4nRR were randomly sampled for histological analysis of gonadal structures and detailed scrutiny of gonadal structures using the method described by Qin et al. (Qin et al., 2018a).

2.7. Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was performed on the Prism 7500 Sequence Detection System (ABI) to investigate *Dmrt1* gene expression in RCC and 4nRR. RNA was extracted from gonadal tissues for qRT-PCR, using the *β-actin* gene as a reference gene. Three independent biological replicates ($n = 3$) were performed for each condition, with each sample run in triplicate technical replicates. To distinguish the four copies of the *Dmrt1* gene in 4nRR, a primer was designed using sequence-length and base differences between 4nRR_*Dmrt1–1* and 4nRR_*Dmrt1–3*, as well as 4nRR_*Dmrt1–2* and 4nRR_*Dmrt1–4* as the entry points (Table 1). As shown in Fig. 2, the black box indicates the highly conserved DM domain of the *Dmrt1* gene.

Table 2
The amplification efficiency data of the quantitative primers.

Gene	Efficiency (%)	R^2	Slope
RCC_*Dmrt1–1*	102.6	0.9921	−3.261
RCC_*Dmrt1–2*	94.2	0.9958	−3.468
4nRR_*Dmrt1–1*	93.0	0.9944	−3.501
4nRR_*Dmrt1–2*	93.5	0.9987	−3.487
4nRR_*Dmrt1–3*	94.7	0.9940	−3.455
4nRR_*Dmrt1–4*	96.1	0.9956	−3.419
β-actin	98.0	0.9980	−3.37

The red box marks a 6-bp insertion that is specific to 4nRR_*Dmrt1–3*, while the blue box indicates a 9-bp insertion present in both 4nRR_*Dmrt1–2* and 4nRR_*Dmrt1–4*. Data were analyzed using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen (Livak and Schmittgen, 2001). The detailed procedures followed those outlined by Duan et al. (Duan et al., 2014).

The amplification efficiency of all primers was validated via standard curve analysis using 5-fold serial dilutions of pooled cDNA. All primer pairs exhibited amplification efficiencies ranging from 93.0% to 102.6%, with correlation coefficients (R^2) > 0.99, which fully complies with the MIQE guidelines (acceptable range: 90–110%) (Bustin et al., 2009). Detailed primer amplification efficiency data are presented in Table 2. Specifically, the reference gene *β-actin* showed an amplification efficiency of 98.0%, and the RCC_*Dmrt1–1* primer pair achieved an efficiency of 102.6%, which remains within the acceptable range.

2.8. Methyl-specific PCR

The promoter regions of the *Dmrt1* gene were identified using Ensembl (http://asia.ensembl.org/index.html) with RCC and 4nRR genomes as reference. Methylation primers were designed by predicting the CpG islands in the promoter region using MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Table 1). Total genomic DNA was extracted from the gonads of 12-month-old RCC and 4nRR specimens using the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (version 5.0, TaKaRa, Japan), and its integrity and concentration were evaluated using the same protocol as for RNA analysis. Subsequently, the purified DNA was treated with bisulfite using the EZ DNA Methylation-Gold™ Kit (Zymo Research, United States). Bisulfite-treated DNA was used as a template for PCR amplification, and PCR products were cloned and sequenced to assess the methylation status of the *Dmrt1* genes. The resulting sequence methylation was analyzed using the BiQ Analyzer software.

2.9. Immunofluorescence localization

Gonads from 6- and 12-month-old individuals were fixed overnight in Bouin's solution, gradually dehydrated in a graded ethanol series, embedded in paraffin, and sectioned at 7 μm using a Leica RM2016 microtome. The primary antibody used for immunofluorescence was a rabbit polyclonal antibody against Dmrt1 (Abmart, TT0006). The secondary antibody used was goat anti-rabbit Alexa Fluor 488 (Sangon Biotech, D110061). Gonads were incubated with anti-Dmrt1 rabbit antibodies (dilution 1:150) at 4 °C overnight, then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:150) in the dark for 2 h. Signal detection was performed using a Leica microscope.

To quantify the relative expression levels of Dmrt1 in the gonads of RCC and 4nRR at different months of age, intensity measurements were performed according to the ImageJ User Guide. Images from the same gonadal samples were set with the same threshold. At least three images from three independent gonadal samples were measured for each experimental condition. Within each image, the fluorescence intensity of Dmrt1 was measured for each cell type and normalized to the background fluorescence intensity within the same image. The resulting data were analyzed using GraphPad Prism software, and the specific

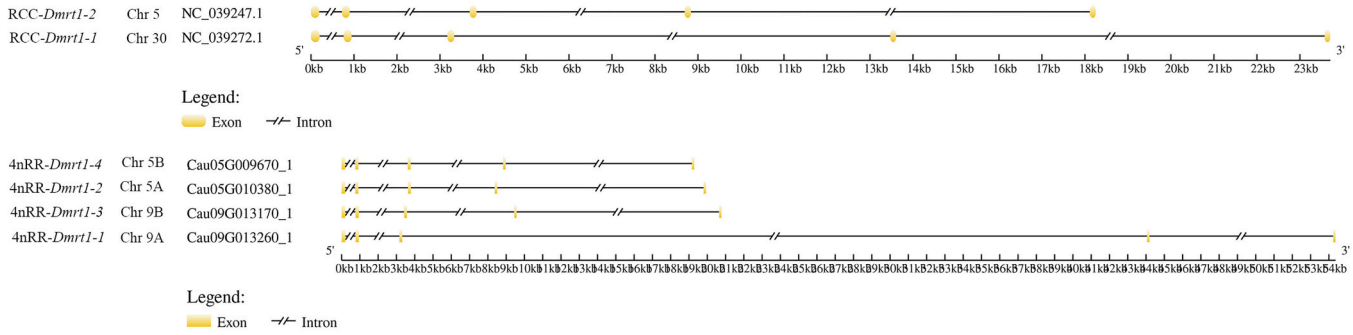


Fig. 1. Gene structures of different copies of the *Dmrt1* gene in RCC and 4nRR.

Table 3
Different copies of the *Dmrt1* gene in RCC and 4nRR.

Name	Name	Location	Intron number	Length (aa)	DM domain position	Length (bp)
RCC_*Dmrt1–1*	NC_039272.1	11,355,250–11,378,957	4	265	52–192	23,708
RCC_*Dmrt1–2*	NC_039247.1	17,324,664–17,342,915	4	268	52–192	18,252
4nRR_*Dmrt1–1*	Cau09G013260.1	22,251,319–22,305,685	4	265	52–192	54,367
4nRR_*Dmrt1–2*	Cau05G010380.1	19,086,262–19,106,196	4	268	52–192	19,935
4nRR_*Dmrt1–3*	Cau09G013170.1	22,099,717–22,120,499	4	267	52–192	20,783
4nRR_*Dmrt1–4*	Cau05G009670.1	17,952,006–17,971,301	4	268	52–192	19,296

Table 4
Exon and intron length of different copies of the *Dmrt1* gene in RCC and 4nRR.

Name	Exon1	Intron1	Exon2	Intron2	Exon3	Intron3	Exon4	Intron4	Exon5
RCC_*Dmrt1–1*	203	560	185	2228	160	10,142	139	9962	137
RCC_*Dmrt1–2*	194	530	181	2791	160	4840	148	9279	137
4nRR_*Dmrt1–1*	203	560	185	2217	163	40,730	139	10,041	137
4nRR_*Dmrt1–2*	194	549	181	2710	163	4580	148	11,281	137
4nRR_*Dmrt1–3*	197	561	191	2473	163	5858	139	11,072	137
4nRR_*Dmrt1–4*	194	548	181	2696	163	5052	148	10,185	137

statistical methods are described in the Statistical analysis section.

3. Results

3.1. Genetic variation of the Dmrt1 gene in 4nRR

Two copies of the *Dmrt1* gene (*Dmrt1–1* and *Dmrt1–2*) were obtained in RCC and 4nRR by DNA-level validation, and two paralogs (*Dmrt1–3* and *Dmrt1–4*) were found in 4nRR, both of which possessed the complete gene structure. Fig. 1 and Table 3 present that the *Dmrt1* gene comprises five exons and four introns, with a conserved DM domain. The primary differences in sequence length among copies were located within the intronic regions. The statistical analysis of exon and intron lengths is presented in Table 4. Although genomic sequences were acquired in this study, we amplified the CDS region to verify the accuracy of the four gene copies in 4nRR, given potential sequencing inaccuracies. Sequence comparison of the coding region revealed that the CDS length of the *Dmrt1* gene was relatively stable, ranging from 798 to 807 bp (Fig. 2). In 4nRR, *Dmrt1–1*, *Dmrt1–2*, *Dmrt1–3*, and *Dmrt1–4* were sequentially positioned on chromosomes 09 A, 05 A, 09 B, and 05 B, respectively. The sequence similarities between RCC and 4nRR were 99.7% for *Dmrt1–1* and 99.2% for *Dmrt1–2* (Table 5). The results revealed that autotetraploidization did not alter the functional domains or the *Dmrt1* gene structure, although it changed the sequence. As shown in Fig. 3, the phylogenetic tree is divided into four major lineages, and the several different copies from RCC and 4nRR are clustered within two of these lineages: one centered on *Dmrt1–1* and the other on *Dmrt1–2*. The additional *Dmrt1–3* copy in 4nRR was clustered within the *Dmrt1–1* lineage, while *Dmrt1–4* was grouped in the *Dmrt1–2* lineage. Notably, all *Dmrt1* copies in 4nRR preferentially clustered with their corresponding orthologs in RCC, rather than forming an independent tetraploid-specific clade. These results demonstrate that the *Dmrt1* genes in 4nRR originated from the genome duplication of RCC, and the copies maintained high evolutionary conservation after polyploidization.

3.2. Analysis of gonad development

RCC and 4nRR attained sexual maturity at one year of age, and their gonadal development exhibited similar morphological progression. Fig. 4 illustrates the gonadal microstructures of female and male individuals at different ages in RCC and 4nRR. In females of both fish types, the ovaries develop to stage II by 5 months of age, containing numerous phase II oocytes. During subsequent ovarian development, phase III oocytes (observed at 8 months) and phase IV oocytes (observed at 12 months) are present. In males, the testes reach stage II at 5 months, with abundant spermatogonia visible. After 8 months, large clusters of primary and secondary spermatocytes and spermatids are observed. At 12 months of age, the testes were predominantly filled with spermatozoa.

3.3. Differential expression of the Dmrt1 gene in the gonads

The qRT-PCR can detect differences in *Dmrt1* gene expression between males and females within the same developmental period in RCC and 4nRR, with expression levels significantly higher in the testis than in the ovary. Fig. 5 demonstrates the *Dmrt1* gene expression level in the gonads of RCC and 4nRR at different developmental stages. In both female and male RCC, *Dmrt1–1* expression was significantly higher than

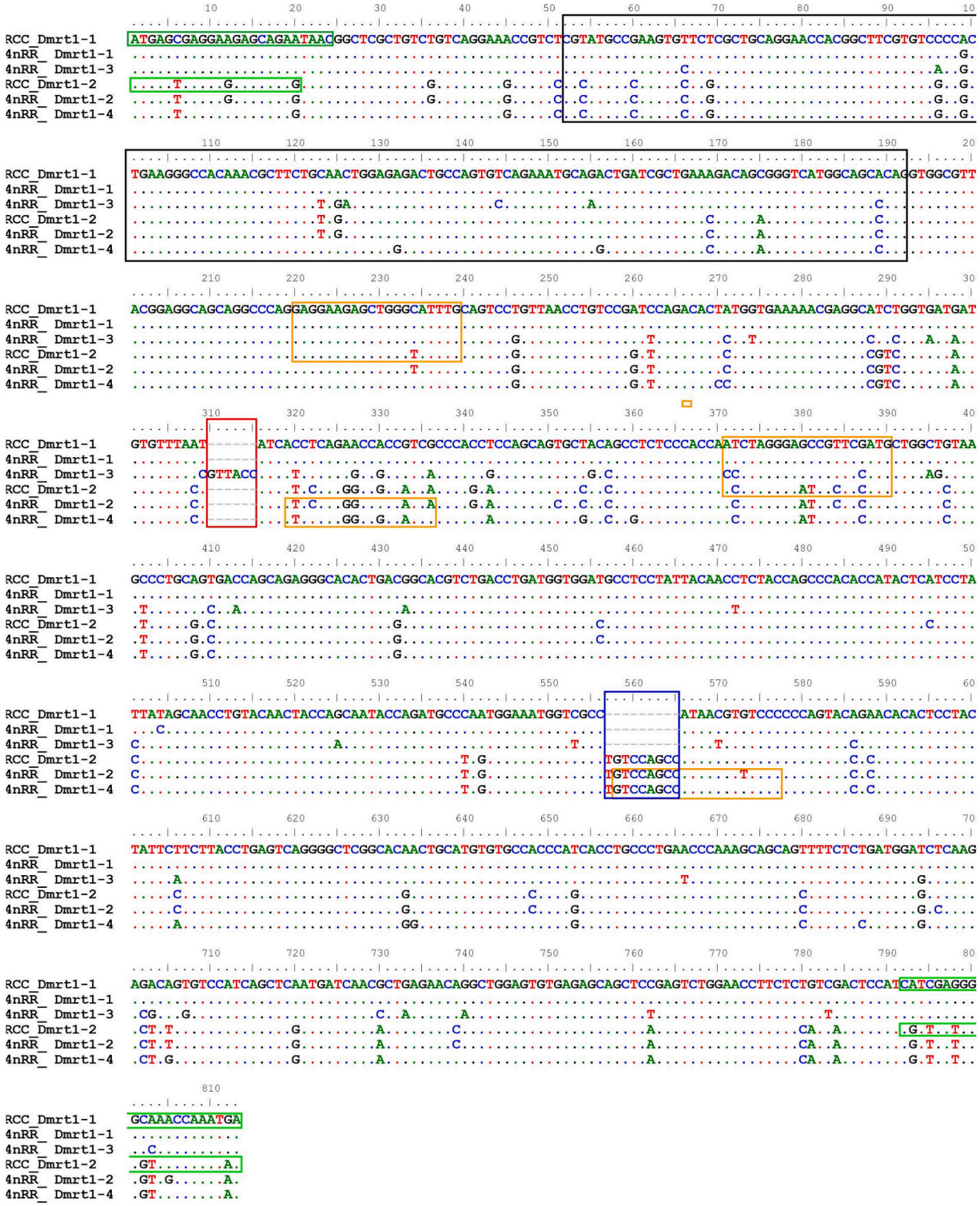


Fig. 2. Comparison of CDS of the *Dmrt1* gene in 4nRR and RCC.
The black box highlights the position of the DM domain, the red box presents the base insertion in 4nRR_*Dmrt1–3,* and the blue box represents sequence length differences among RCC_*Dmrt1–2*, 4nRR_*Dmrt1–2*, 4nRR_*Dmrt1–4*, and RCC_*Dmrt1–1*. The green boxes represent PCR primers, and the yellow boxes represent qPCR primers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Dmrt1–2 ($P < 0.001$), with females showing 5.3-, 8.5-, and 3.3-fold higher levels at 5, 8, and 12 months, respectively, and males showing 1.5-, 2.1-, and 3.4-fold higher levels. The original copy of the *Dmrt1* gene (*Dmrt1–1*) exhibits preferential expression in the 4nRR testis at various developmental stages, consistent with RCC, while the duplicated copies (*Dmrt1–3* and *Dmrt1–4*) are preferentially expressed in the 4nRR ovary. Moreover, in 4nRR females, the highest *Dmrt1* gene expression level is observed at 8 months of age. However, higher *Dmrt1* gene expression levels are consistently detected in 4nRR males, with a gradual increase from 5 to 8 months of age, and a decrease by 12 months of age.

Table 5
Similarity of CDS and amino acid sequence of *Dmrt1* gene in RCC and 4nRR.

Gene name		CDS size(bp)	Identity (%)		Number of amino acids	Identity (%)	
Dmrt1	RCC_*Dmrt1*–1	798	RCC_*Dmrt1*–1: RCC_*Dmrt1*–2	89.2%	265	RCC_*Dmrt1*–1: RCC_*Dmrt1*–2	87.6%
			RCC_*Dmrt1*–1: 4nRR_*Dmrt1*–1	99.7%		RCC_*Dmrt1*–1: 4nRR_*Dmrt1*–1	100%
			RCC_*Dmrt1*–1: 4nRR_*Dmrt1*–2	88.9%		RCC_*Dmrt1*–1: 4nRR_*Dmrt1*–2	87.3%
			RCC_*Dmrt1*–1: 4nRR_*Dmrt1*–3	92.7%		RCC_*Dmrt1*–1: 4nRR_*Dmrt1*–3	89.5%
			RCC_*Dmrt1*–1: 4nRR_*Dmrt1*–4	90%		RCC_*Dmrt1*–1: 4nRR_*Dmrt1*–4	88.4%
	RCC_*Dmrt1*–2	807	RCC_*Dmrt1*–2: 4nRR_*Dmrt1*–1	89.2%	268	RCC_*Dmrt1*–2: 4nRR_*Dmrt1*–1	87.6%
			RCC_*Dmrt1*–2: 4nRR_*Dmrt1*–2	99.2%		RCC_*Dmrt1*–2: 4nRR_*Dmrt1*–2	99.6%
			RCC_*Dmrt1*–2: 4nRR_*Dmrt1*–3	88.9%		RCC_*Dmrt1*–2: 4nRR_*Dmrt1*–3	84.4%
			RCC_*Dmrt1*–2: 4nRR_*Dmrt1*–4	97.2%		RCC_*Dmrt1*–2: 4nRR_*Dmrt1*–4	98.5%
	4nRR_ *Dmrt1*–1	798	4nRR_*Dmrt1*–1: 4nRR_*Dmrt1*–2	88.9%	265	4nRR_*Dmrt1*–1: 4nRR_*Dmrt1*–2	87.3%
			4nRR_*Dmrt1*–1: 4nRR_*Dmrt1*–3	92.7%		4nRR_*Dmrt1*–1: 4nRR_*Dmrt1*–3	89.5%
			4nRR_*Dmrt1*–1: 4nRR_*Dmrt1*–4	90.0%		4nRR_*Dmrt1*–1: 4nRR_*Dmrt1*–4	88.4%
	4nRR_ *Dmrt1*–2	807	4nRR_*Dmrt1*–2: 4nRR_*Dmrt1*–3	88.4%	268	4nRR_*Dmrt1*–2: 4nRR_*Dmrt1*–3	84%
			4nRR_*Dmrt1*–2: 4nRR_*Dmrt1*–4	96.7%		4nRR_*Dmrt1*–2: 4nRR_*Dmrt1*–4	98.1%
	4nRR_ *Dmrt1*–3	804	4nRR_*Dmrt1*–3: 4nRR_*Dmrt1*–4	89.1%	267	4nRR_*Dmrt1*–3: 4nRR_*Dmrt1*–4	84.8%
	4nRR_ *Dmrt1*–4	807			268		

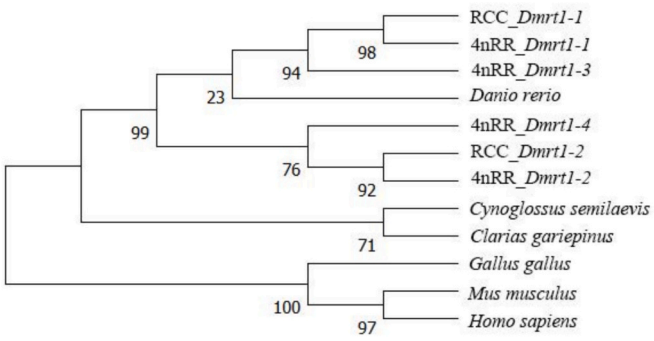


Fig. 3. The evolutionary relationship of *Dmrt1* genes in RCC and 4nRR.

3.4. Alteration of the DNA methylation status in Dmrt1

In 4nRR, in addition to the original *Dmrt1–1* and *Dmrt1–2*, the duplicated copies *Dmrt1–3* and *Dmrt1–4* were detected. Consequently, we assessed the DNA methylation status of *Dmrt1–1*, *Dmrt1–2*, *Dmrt1–3*, and *Dmrt1–4* in 4nRR, as well as that of *Dmrt1–1* and *Dmrt1–2* in RCC, at the time when their expression levels peaked in the gonads. We compared the methylation levels of the *Dmrt1* gene promoters in the gonads of RCC and 4nRR at 12 months of age (Fig. 6 and Table 6). The methylation patterns of each *Dmrt1* gene copy in 4nRR were consistent with those observed in RCC, with higher methylation levels in ovaries and lower levels in testes. By integrating methylation levels with *Dmrt1* gene expression, a double-Y-axis graph illustrated a significant negative correlation between these two factors (Fig. 7). We speculate that the sexually dimorphic expression of *Dmrt1* in the gonads of 4nRR and its original strain, RCC, may be regulated by promoter DNA methylation.

3.5. Immunofluorescence localization analysis

Immunofluorescence analysis revealed consistent Dmrt1 localization during the same developmental period in RCC and 4nRR. Fig. 8 presents the localization of Dmrt1 in gonads at different developmental periods in RCC and 4nRR. The localization results of the Dmrt1 in the gonadal cells of 4nRR during the non-breeding period (6-month-old) were consistent with those of RCC. In the testis, Dmrt1 was primarily expressed in primary and secondary spermatocytes and spermatids, with bright fluorescence signals detected in spermatids. No obvious signal was observed in the ovary. The localization results of the Dmrt1 in the gonadal cells of 4nRR during breeding (12-month-old) were also consistent with those of RCC. In the testis, it was primarily expressed in spermatozoa. No obvious signal was detected in the ovary.

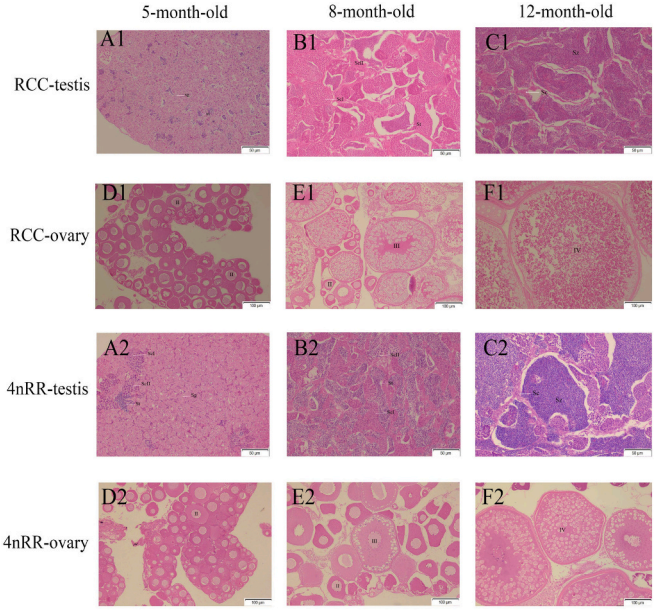


Fig. 4. Gonad microstructure of different age stages of females (5, 8, and 12-month-old) and males (5, 8, and 12-month-old) in both RCC and 4nRR. (A1)5 months old testis of RCC; (A2)5 months old testis of 4nRR; (B1)8 months old testis of RCC; (B2)8 months old testis of 4nRR; (C1)12 months old testis of RCC; (C2)12 months old testis of 4nRR; (D1)5 months old ovary of RCC; (D2)5 months old ovary of 4nRR; (E1)8 months old ovary of RCC; (E2)8 months old ovary of 4nRR; (F1)12 months old ovary of RCC; (F2)12 months old ovary of 4nRR; Sg, Spermatogonium; Sc, Sertolicell; St, Spermatid; Sz, Spermatozoa; ScI, Primary spermatocytes; ScII, Second spermatocytes; II, phaseIIoocyte; III, phase III oocytes; IV, phase IV oocytes.

To investigate the expression pattern of Dmrt1 in different ploidy cyprinid fish at different developmental stages, we performed quantitative analysis of Dmrt1 fluorescence intensity in testes and ovaries of 6-month-old and 12-month-old RCC and 4nRR (Fig. 9).

A highly conserved male-specific expression pattern of Dmrt1 was observed in both RCC and 4nRR across all tested developmental stages. In 6-month-old fish, the relative fluorescence intensity of Dmrt1 in testes was extremely significantly higher than that in ovaries in both RCC ($P < 0.0001$) and 4nRR ($P < 0.0001$). Specifically, the Dmrt1 expression level in 6-RCC-testes was approximately 3.6-fold higher than that in 6-RCC-ovaries, while 6-4nRR-testes showed approximately 3.0-fold higher expression than 6-4nRR-ovaries. In 12-month-old adult fish, the male-specific expression of Dmrt1 was further maintained. The Dmrt1

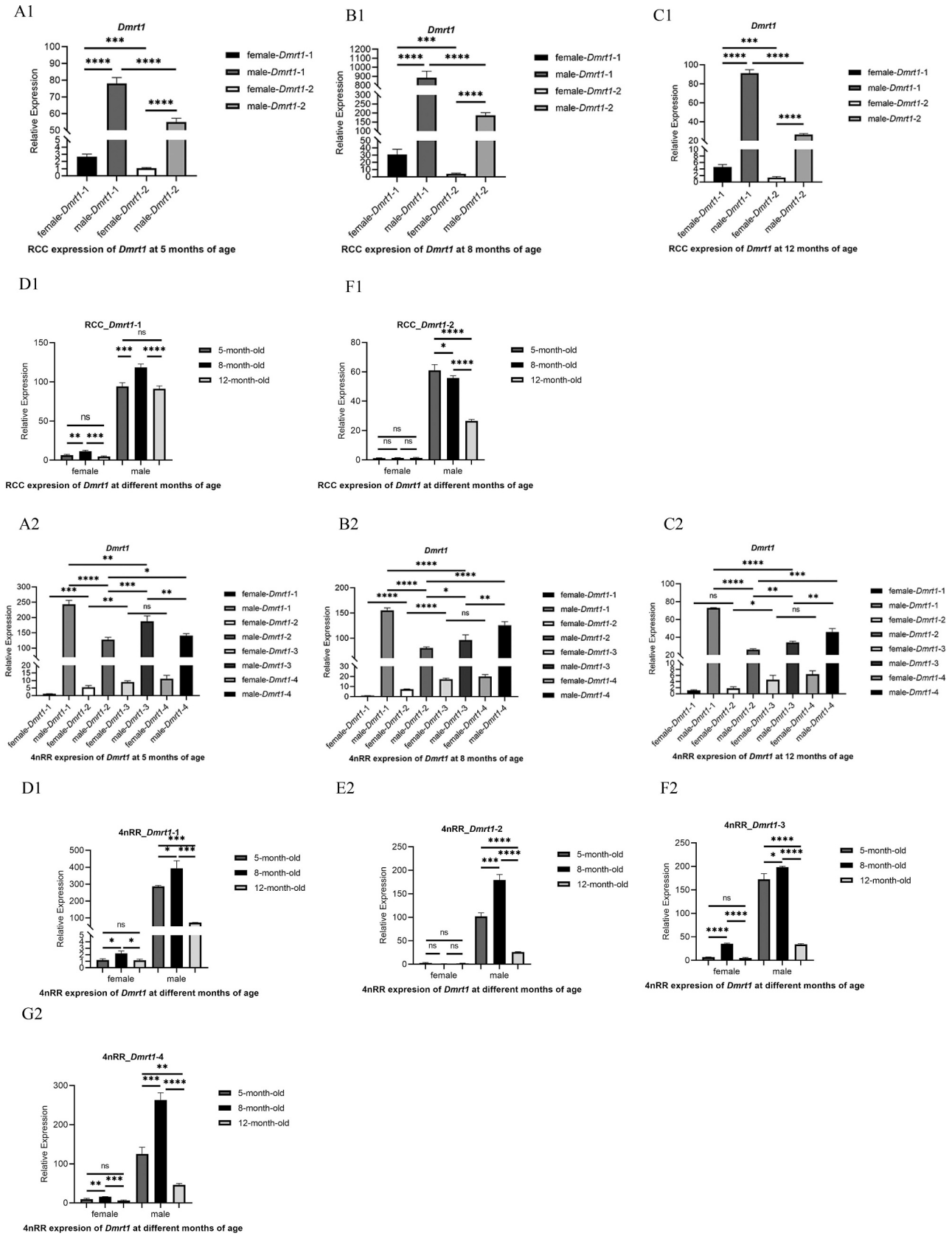


Fig. 5. Relative expression levels of *Dmrt1* in RCC and 4nRR at different age stages (5, 8, and 12-month-old) of females and males, and the differential expression characteristics between females and males in RCC and 4nRR.

A1-C1, Expression levels of Dmrt1 in RCC gonads at 5, 8, and 12 months of age; D1-E1, Expression trends of *Dmrt1* in RCC gonads for two replicates (RCC_*Dmrt1–1*, RCC_*Dmrt1–2*); A2-C2, Expression levels of *Dmrt1* in 4nRR gonads at 5, 8, and 12 months of age.D2-G2, Expression trends of *Dmrt1* in 4nRR gonads for four replicates (4nRR_*Dmrt1–1*, 4nRR_*Dmrt1–2*, 4nRR_*Dmrt1–3*, 4nRR_*Dmrt1–4*);(* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns $P > 0.05$).



Fig. 6. The CpG methylation status of different *Dmrt1* genes in testes and ovaries (12-month-old) of RCC and 4nRR.
A1-D1, RCC: (A1) RCC_*Dmrt1–1*- ovaries; (B1) RCC_*Dmrt1–1*- testes; (C1) RCC_*Dmrt1–2*- ovaries; (D1) RCC_*Dmrt1–2*- testes; A2-H2, 4nRR: (A2) 4nRR_*Dmrt1–1*- ovaries; (B2) 4nRR _*Dmrt1–1*- testes; (C2) 4nRR_*Dmrt1–2*- ovaries; (D2) 4nRR_*Dmrt1–2*- testes; (E2) 4nRR_*Dmrt1*–3- ovaries; (F2) 4nRR_*Dmrt1–3*- testes; (G2) 4nRR_*Dmrt1–4*- ovaries; (H2) 4nRR_*Dmrt1–4*- testes; Each box displays one CpG position, with the yellow region representing methylation and the blue region representing no methylation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 6
Methylation degree in the *Dmrt*1 promoter of RCC and 4nRR.

Name(type)	Methylated degree
RCC_*Dmrt1–1*- ovaries	0.079
RCC_*Dmrt1–1*- testes	0.005
RCC_*Dmrt1–2*- ovaries	0.055
RCC_*Dmrt1–2*- testes	0.005
4nRR_*Dmrt1–1*- ovaries	0.050
4nRR_*Dmrt1–1*- testes	0.005
4nRR_*Dmrt1–2*- ovaries	0.041
4nRR_*Dmrt1–2*- testes	0.000
4nRR_*Dmrt1–3*- ovaries	0.111
4nRR_*Dmrt1–3*- testes	0.005
4nRR_*Dmrt1–4*- ovaries	0.124
4nRR_*Dmrt1–4*- testes	0.005

fluorescence intensity in testes was extremely significantly higher than that in ovaries for both RCC ($P < 0.0001$) and 4nRR ($P < 0.0001$), with 12-RCC-testes showing ~11.9-fold higher expression than 12-RCC-ovaries, and 12-4nRR-testes showing ~5.0-fold higher expression than 12-4nRR-ovaries. Autotetraploidization did not change Dmrt1 expression and localization, ensuring normal function.

4. Discussion

Whole-genome duplication (WGD) is a pivotal driver of species evolution and phenotypic innovation, particularly in teleosts, where polyploidization events are frequent and associated with adaptive radiation (Song et al., 2012; Van de Peer et al., 2017). 4nRR is a rare fertile autopolyploid that derived from WGD of the diploid RCC during distant hybridization (Qin et al., 2014). Its ability to undergo "diploid-like chromosome pairing" and produce viable diploid gametes makes it an ideal model to dissect the molecular mechanisms underlying polyploid fertility (Qin et al., 2019). The *Dmrt1* gene, a conserved regulator of sex determination and gonad development in vertebrates (Herpin and Schartl, 2011), plays a critical role in spermatogenesis and testis differentiation (Bishop et al., 1992; Chen et al., 2016).

Gene duplication arising from WGD is a major source of genetic novelty, with duplicated genes typically following three evolutionary fates: pseudogenization, neofunctionalization, or subfunctionalization (Sandve et al., 2018; Ohno, 1970). After autotetraploidization, the copy number of the *Dmrt1* gene increased in the 4nRR lineage. In addition to the two original *Dmrt1* genes (*Dmrt1–1* and *Dmrt1–2*) inherited from RCC, two new paralogs (*Dmrt1–3* and *Dmrt1–4*) were derived. After polyploidization, insertions occurred in the CDS of the *Dmrt1* gene in the 4nRR compared to its diploid progenitor; however, the DM domain remained unaffected. The two retained parental copies (*Dmrt1–1* and *Dmrt1–2*) shared >99% nucleotide sequence similarity with their respective progenitors, while the two newly derived copies (*Dmrt1–3* and *Dmrt1–4*) shared >92% similarity with their respective progenitors. Both retained and newly generated copies of the *Dmrt1* genes were functional. Following tetraploidization, the rapid genomic reorganization that occurs in the newly established 4nRR genome provides a foundation for the adaptation of gene structure, expression, and function.

It is speculated that the expansion of *Dmrt1* gene copies and retention of intact functional domains in 4nRR reflect an adaptive response to WGD. Unlike non-essential genes that may undergo silencing or divergence, the role of the *Dmrt1* gene in gonad development requires dosage balance and functional stability. The maintenance of multiple functional copies may also provide a buffer against genetic drift, ensuring consistent expression, even in the genomic rearrangements common in newly formed polyploids (Chen, 2007; Liu et al., 2016).

Autotetraploidization alters genomic structure, thereby leading to dynamic and random variations in gene expression (Chen and Ni, 2006; Liu et al., 1998). In addition to the differences in expression levels within the gonads, the *Dmrt1* gene expression level varies among different copies. Regarding research on the changes in genomic structure and gene expression caused by polyploidization, Ohno (Ohno, 1970) proposed that after gene replication, one copy of a gene retains its original function, while the other copy can evolve into a new gene. Polyploidization alters genomic structure and gene expression, thereby increasing genomic flexibility. A small portion of repetitive copies is eventually retained as a source of functional innovation (Wolfe, 2001), which may provide variations absent in diploids and enhance the plasticity of the genome or species to adapt to the constantly changing environment (Chen, 2007; Parisod et al., 2010; Otto, 2007). *Dmrt1* gene expression in RCC and 4nRR exhibited strong sexual dimorphism, with significantly higher expression in testes than in ovaries ($P < 0.0001$) across all age stages (5, 8, and 12 months). This temporal trend indicates that Dmrt1 expression is developmentally regulated in the testes of RCC and 4nRR, correlating with the progression of spermatogenesis. Therefore, we speculate that the differences in expression levels of different *Dmrt1* copies in the gonads at various ages, as well as the altered expression of the *Dmrt1* gene in 4nRR compared with its progenitor RCC, allow the *Dmrt1* gene to maintain its normal function after autotetraploidization, thereby ensuring normal gonadal development and stable inheritance in 4nRR.

Epigenetic control is crucial for sex determination and differentiation, particularly via the CpG methylation of genes with sex dimorphic expression, which influence endocrine regulation directly or indirectly

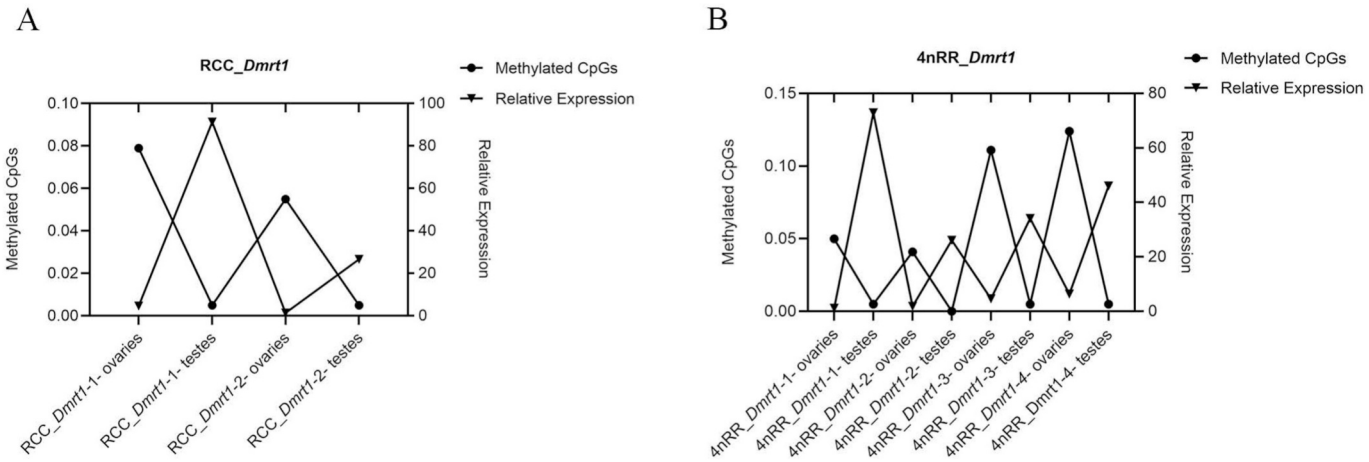


Fig. 7. Double Y-axis map of *Dmrt1* gene expression and promoter methylation levels in RCC and 4nRR.
A, RCC; B, 4nRR.

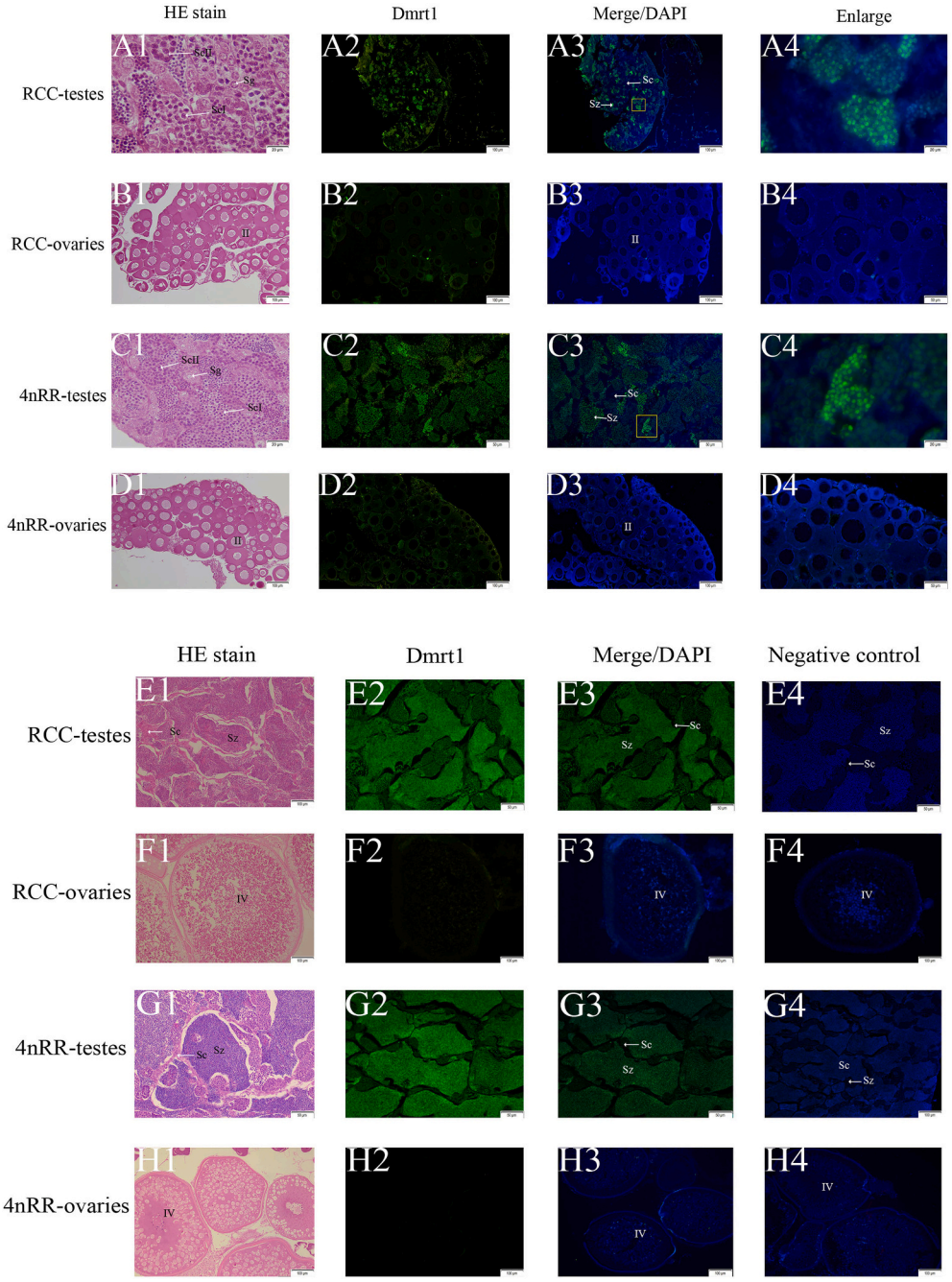


Fig. 8. Localization of *Dmrt1* in the gonads of RCC and 4nRR at different ages (6- and 12-month-old).
At 6 months of age: A1-A4, RCC- testes; B1-B4, RCC-ovaries; C1-C4, 4nRR-testes; D1-D4, 4nRR-ovaries; At 12 months of age: E1-E4, RCC- testes; F1-F4, RCC-ovaries; G1-G4, 4nRR-testes; H1-H4, 4nRR-ovaries; Sg, Spermatogonium; Sc, Sertolicell; St, Spermatid; Sz, Spermatozoa; ScI, Primary spermatocytes; ScII, Second spermatocytes; II, phaseIIoocyte; III, phase III oocytes; IV, phase IV oocytes.

(Kuroki and Tachibana, 2018). The methylation patterns of the *Dmrt1* gene promoter copies in 4nRR were consistent with those in RCC. Methylation level was relatively high in the ovaries and low in the testes. Methylation level was combined with the *Dmrt1* gene expression level, and a double-Y-axis plot was used to present a significant negative correlation between them. This inverse relationship aligns with findings in other teleosts, including Japanese flounder (*Paralichthys olivaceus*), where methylation of the *Dmrt1* gene promoters suppresses ovarian expression (Wen et al., 2014), and European sea bass (*Dicentrarchus labrax*), where methylation of sex-related gene promoters modulates temperature-dependent sex ratios (Navarro-Martín et al., 2011). After autotetraploidization, the DNA methylation patterns of each copy of the *Dmrt1* gene promoter in the 4nRR gonads were consistent with those of its original mother, RCC, indicating that epigenetic regulation of the gene promoter plays a significant role in polyploid gene expression and provides an important guarantee for the normal expression of the *Dmrt1* gene in the 4nRR gonads.

Immunofluorescence analysis confirmed that the *Dmrt1* gene localization was identical in RCC and 4nRR, with specific signals in testicular germ cells (primary or secondary spermatocytes, spermatids, and spermatozoa) and no detectable expression in ovaries. This cell-specific pattern is consistent with the established role of *Dmrt1* in spermatocyte differentiation and sperm maturation (Lei et al., 2007; Webster et al., 2017). In mammals, *Dmrt1* is expressed in both Sertoli cells and

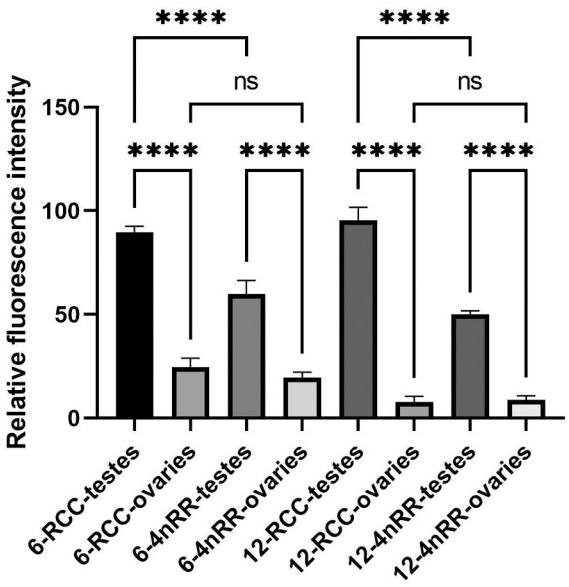


Fig. 9. Quantitative analysis of *Dmrt1* fluorescence intensity in testes and ovaries of 6-month-old and 12-month-old RCC and 4nRR.
Data are presented as the mean ± standard error of the mean (SEM). ****$P <$ 0.0001, ns $P > 0.05$.

germ cells. However, fish exhibit species-specific expression patterns. In some fish species, *Dmrt1* is restricted to Sertoli cells, for example, in Nile tilapia (Kobayashi et al., 2008), *Takifugu rubripes* (Yamaguchi et al., 2006), and medaka (Kobayashi et al., 2004); in others, it is expressed in both Sertoli cells and germ cells, for example, *Carassius auratus gibelio* (Li et al., 2014), *Halobatrachus didactylus* (Úbeda-Manzanaro et al., 2014); and in still others, it is restricted to certain germ cell types. In teleosts, Dmrt1 is not always localized to Sertoli cells; for example, in catfish (Raghuveer and Senthilkumaran, 2009), Atlantic cod (Johnsen et al., 2010), and orange-spotted grouper (*Epinephelus coioides*) (Xia et al., 2007), the *Dmrt1* gene is localized to germ cells. This is consistent with our results.

The retention of the *Dmrt1* gene localization pattern in 4nRR further confirms that polyploidization does not alter the cell-specific functions of this gene. The low expression level of the *Dmrt1* gene in ovaries also supports its role as a male-specific regulator, as ectopic expression in female germ cells could lead to sex reversal or oocyte dysfunction (Smith et al., 2009). Combined with expression and methylation data, these localization results demonstrate that the expression pattern of the *Dmrt1* gene is conserved in 4nRR, an essential factor for maintaining species' fertility and stable lineage.

In this study, the genetic variation, expression patterns, epigenetic modification, and spatial localization of the *Dmrt1* gene were systematically compared between RCC and 4nRR. The findings of this study revealed how autopolyploidization modulates a key sex-related gene to maintain gonadal function and provided insights into the evolution and breeding of polyploid fish.

CRediT authorship contribution statement

Qian Xiao: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Qingwen Xiao:** Investigation, Formal analysis, Data curation. **Xu Huang:** Investigation, Formal analysis. **Yanlin Fu:** Visualization, Investigation, Formal analysis. **Yuhan Yang:** Visualization, Investigation, Formal analysis. **Chongqing Wang:** Visualization, Investigation, Formal analysis. **Xiaowei Xu:** Visualization, Investigation, Formal analysis. **Xidan Xu:** Visualization, Investigation. **Kun Zhang:** Visualization, Investigation. **Yue Zhou:** Investigation. **Jinhai Bai:** Investigation. **Zhengkun Liu:** Writing – review & editing, Investigation. **Enkui Hu:** Writing – review & editing, Project administration. **Siyang Li:** Investigation. **Yuchen Jiang:** Investigation. **Ling Xiong:** Formal analysis. **Wanjing Peng:** Investigation, Formal analysis. **Zeyang Li:** Investigation. **Qinbo Qin:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization.

Ethics statement

In this study, all sample protocols were approved by the Institute of Experimental Animals, Hunan Province, China. Before tissue collection, all fish were deeply anesthetized with 100 mg/L MS-222 (Sigma-Aldrich, St. Louis, MO, United States).

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Data availability

Data will be made available on request.

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