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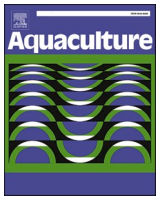

Characteristics of testis development in autotetraploid fish

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ABSTRACT

Fertile autotetraploid fish are an excellent germplasm resource for producing improved triploids. However, males produce more diploid gametes than females during production, which means that males are more valuable in application. To better understand the testis development of autotetraploid fish (4nRR) derived from the distant hybridization of *Carassius auratus* red var. (RCC, ♀) × *Megalobrama amblycephala* (BSB, ♂), all-male autotetraploid fish (AM4nRR) were generated. In this study, the tissue structure and transcriptome changes in different periods of gonadal development of AM4nRR were analyzed. Histological sectioning revealed a small number of gonium at 45 days post-hatching (dph), the number of germ cells greatly increased during 65–95 dph, and primary spermatocytes and secondary spermatocytes were observed at 105 dph. Meanwhile, white semen could be extruded from most individuals at 120 dph, and the DNA content of sperm from AM4nRR was approximately twice that from RCC as determined by flow cytometry. These results indicated that AM4nRR reached sexual maturity at 120 dph and produced diploid sperm. In addition, a total of 149.55 Gb of clean data and 56,188 unigenes were obtained from the testicular tissue of AM4nRR at different developmental periods (25 dph, 35 dph, 40 dph, 45 dph, 65 dph, 95 dph and 120 dph). Differential expression analysis showed that the number of differentially expressed genes (DEGs) among the three periods of 40 dph, 45 dph, and 65 dph was the lowest (72–275). However, the expression levels of partial DEGs at 45 dph changed abruptly compared with those at 40 dph and 65 dph according to hierarchical clustering analysis, which was consistent with the expression characteristics of gonadal development-related genes at the early period of sex differentiation. These results suggested that 40–65 dph might be a critical period for the initiation of testis development in autotetraploid fish. These findings not only provide a new gamete resource for the large-scale preparation of improved triploids but also provide a basis for further study of sex differentiation in autotetraploid fish.

1. Introduction

Polyploidization plays an important role in the evolution and formation of species and is considered a drastic method of genetic mutation. Due to the strong plasticity of fish chromosomes, polyploidization is more common in fish than in other vertebrates (Liu, 2010). For a long time, the preparation of tetraploid fish has been a research hotspot in the field of aquaculture. Tetraploid fish have been prepared using chemical and physical methods such as cytochalasin B, hydrostatic pressure, and heat/cold shock, but genetically stable tetraploid fish lineages have not been obtained (Bidwell et al., 1985; Chourrout and Nakayama, 1987; Chourrout et al., 1986; Gui et al., 1991; Refstie, 1981; Thorgaard et al., 1981; Zou et al., 2004). However, distant hybridization can effectively create genetically stable tetraploid lineages, and large-scale improved triploid fish can be prepared by interploidy crossing between tetraploid fish and diploid fish (Liu, 2010; Liu et al., 2022c; Wang et al., 2018). Triploid fish have obvious advantages in terms of growth rate, meat quality, and stress resistance, which are of great value in production applications and basic theoretical research (Liu et al., 2021). For example, by crossing an allotetraploid hybrid with Japanese crucian carp (*Carassius cuvieri*, ♀) and Xingguo red carp (*Cyprinus carpio* var. *singuonensis*, ♀), sterile triploid Xiangyun crucian carp (3n = 150) and

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triploid Xiangyun carp (3n = 150) are generated, respectively (Liu et al., 2022a; Yang et al., 1993). These two triploid fish have substantial economic value, and they are farmed and popular among consumers in 28 provinces and municipalities in China due to their growth superiority, high meat quality, strong stress resistance, infertility, and other advantageous traits (Liu et al., 2022b). As high-quality germplasm resources, fertile tetraploid fish, especially male tetraploid fish, can produce substantial numbers of diploid gametes, which ensures the large-scale preparation of improved triploids.

Gonadal development is the key to fish reproduction and breeding, as well as the continuation of populations and the development of new species. Gonadal development in fish has always been an interest of researchers. It is generally believed that the gonadal development of fish is affected by genetic factors, the external environment (chemical and physical factors, social environment, or sex ratio), and regulation of the nervous and endocrine systems. Moreover, gametogenesis in fish is a precise, highly complex and coordinated cell development process that is vulnerable to interference from other factors (Billard, 1986). Gametogenesis is a biological process in which diploid or haploid precursor cells divide and differentiate into mature haploid gametes (Ye et al., 2014). There are different forms of gametogenesis, including spermatogenesis (male) and oogenesis (female). Spermatogenesis is a highly complex and specialized process of cellular differentiation leading to the formation of functional spermatozoa for successful reproduction, involving mitotic cell division, meiosis, and the process of spermiogenesis (de Kretser et al., 1998; Renkawitz-Pohl et al., 2005). Although the size and shape of the mature sperm vary considerably among different species, the process of spermatogenesis is well conserved in all sexually proliferating organisms. There are >32,500 fish species in nature, making fish the largest and most diverse group of vertebrates (Wang et al., 2018). However, only a few species used for basic research and/or in aquaculture biotechnology have been studied in relation to spermatogenesis (Schulz et al., 2010).

Polyploidy can be classified into autopolyploidy and allopolyploidy. Autopolyploidy arises by multiplication of chromosome sets within a species, while allopolyploidy arises by multiplication of chromosome sets resulting from intergeneric or interspecific hybridization (Piferrer et al., 2009). In our previous study, we established an autotetraploid fish lineage (4nRR, RRRR, 4n = 200, F_2-F_{17}) by distant hybridization of *Carassius auratus* red var. (RCC, RR, 2n = 100, ♀) × *Megalobrama amblycephala* (BSB, BB, 2n = 48, ♂), which had four sets of chromosomes from RCC (Liu et al., 2006; Qin et al., 2016; Qin et al., 2014b). Although this 4nRR lineage was derived from the distant hybridization of RCC and BSB, 4nRR (F_2) resulted from the fertilization of the diploid eggs and diploid sperm from the F_1 hybrids (4nRB, RRBB, 4n = 148) (Qin et al., 2014b). The above diploid eggs and diploid sperm were autodiploid gametes containing only RCC chromosomes, which were produced by special chromosome behavior in germ cells of 4nRB (Qin et al., 2014a). 4nRR, as an important germplasm resource, reached sexual maturity in one year and was able to produce diploid gametes, which is of great significance for the production of excellent triploid and diploid fish (Qin et al., 2018). Male-sterile and female-fertile autotriploid crucian carp (3n = 150) were prepared by a cross between male 4nRR and female RCC (Hu et al., 2019; Qin et al., 2019). In addition, YY fish (2n = 100, ♂) were prepared by androgenesis and fertilized diploid sperm of 4nRR with haploid eggs of RCC irradiated by ultraviolet rays without chromosome doubling so that they inherited only the nuclear genome of 4nRR (Zhou et al., 2015).

Based on the establishment of an autotetraploid fish lineage (4nRR, 4n = 200, F_2-F_{17}) and YY fish, all-male autotetraploid fish (AM4nRR, 4n = 200) were artificially obtained via the hybridization of 4nRR (♀, F_{14}) × YY fish (♂, 2n = 100). In this study, the tissue structure and transcriptome changes in different periods of testis development of AM4nRR were analyzed. This study is important for fish genetic breeding and reproductive biology.

2. Material and methods

2.1. Ethics statement

The Administration of Affairs Concerning Animal Experimentation Guidelines states that approval from the Science and Technology Bureau of China and the Department of Wildlife Administration is not necessary when the fish in question are not rare or near extinction (first-class or second-class state protection level). Therefore, approval was not required for the experiments conducted in this study. All fish were raised in natural ponds and were euthanized using 2-phenoxyethanol (Sigma) before being dissected.

2.2. Animals and crosses

All fish in this study were cultivated in ponds at the State Key Laboratory of Development of Freshwater Fish of Hunan Normal University, Hunan, China. The culture conditions included a breeding density of 1500–1800 per 667 m^2, a feeding density with expanded feed (protein content 32%, fat content 3.0%) equal to 5% of the parent's body weight when the water temperature was 15–28 °C, and a dissolved oxygen concentration above 3 mg/L.

During the reproductive season (April–June), 20 female 4nRR (F_{14}) and 10 YY fish with weights >1 kg were selected as the maternal fish and paternal fish, respectively. A mixture of oxytocin hormones was used to supply artificial oxytocin, with an injection dose per kilogram of fish of 200 IU human chorionic gonadotropin (HCG), 10 μg luteinizing releasing hormone (LRH-A2) and 1 mg domperidone. When the female parental fish begin to spawn, they were removed for artificial insemination. Approximately 200,000 dark green eggs from 4nRR were fertilized with sperm of YY fish.

Embryos were produced by artificial fertilization and hatched in culture dishes at a water temperature of 24–26 °C. Since the eggs of 4nRR were demersal without stickiness, desticking was omitted. The fertilization rate (no. of embryos at the gastrula stage/no. of eggs) and the hatching rate (no. of hatched fry/no. of eggs) were calculated for a total of 6000 embryos, which were divided into three groups with 2000 embryos for each group. The embryo development process was completed in approximately 3 days, and the fry emerged from the egg membrane. Then, all the fry were transferred to microbiome-rich ponds for further culture with a breeding density of 50,000 per 667 m^2. When the fry had grown to 1–2 cm, we started feeding them puffed powder (protein content 32%, fat content 3.0%) at 5% of the parents' body weight when the water temperature was 15–28 °C. The dissolved oxygen concentration was maintained above 3 mg/L. When the fry had grown to 5–6 cm, they were transferred to new ponds for further culture under the same culture conditions as the adult fish.

2.3. Analysis of ploidy and appearance

To determine ploidy, flow cytometry was used to measure the DNA content of all fish in this study. Since the dye used in the flow detection was DAPI, the FL1 channel was chosen when we detected the mean DNA content. Cells were prepared from three types of tissues, including (1) the caudal fin of fish aged from 30 days post-hatching (dph) to 60 dph, (2) blood cells collected from 60 dph to adult fish, and (3) semen. All samples were treated according to the method described in a previous paper (Peng et al., 2020). In all experiments, RCC was used as a control fish.

At 10 months of age, 10 4nRR and 10 AM4nRR fish were selected randomly for morphological statistics. For the measurable traits, the whole length, body length, body width, tail length, tail width, head length and head width of 4nRR and AM4nRR were measured, and their values were converted into proportions, which reduced the effects caused by different body sizes. The countable traits analyzed included the numbers of lateral scales, upper lateral scales, lower lateral scales,

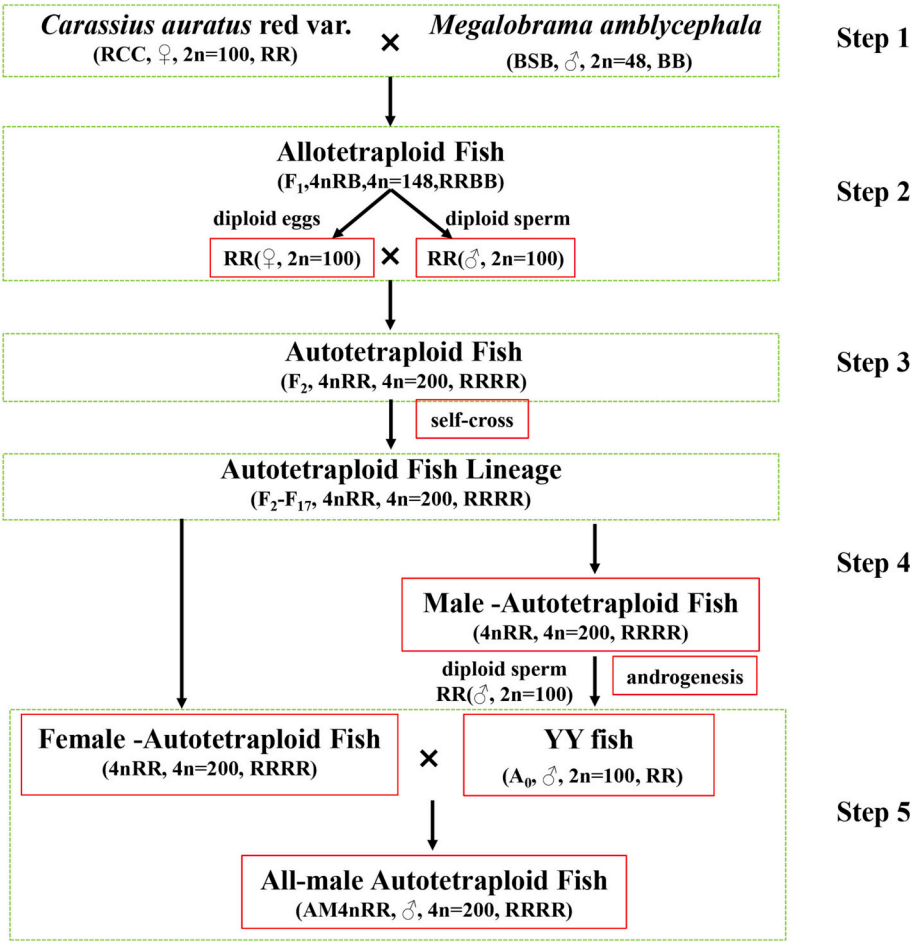

Fig. 1. The crossing procedure used to produce AM4nRR.

dorsal fins, abdominal fins, and anal fins.

2.4. Testicular structure and sperm phenotypes

Ten fish were sampled every 5 days from 20 to 120 dph. Undifferentiated gland and testis samples were excised from fish and fixed in Bouin's solution. After the larvae were dissected and Bouin's solution was added to the abdominal cavity, the early testis changed from transparent to yellowish, showing two thin lines. The fixed samples were dehydrated and paraffin-embedded. Then, 5–7 μm sections were prepared using a Leica RM2015 Microtome (Leica, Germany) and stained with hematoxylin-eosin (H–E). Sections were observed and photographed with a light microscope (OLYMPUS, Japan).

To observe the phenotype of sperm, semen was collected by gently squeezing the abdomen of AM4nRR at 120 dph. The sperm of RCC was used as the control. The semen samples were fixed in 2.5% glutaraldehyde for over 2 h. Ten microliters of semen was evenly coated onto clean slides and dried at room temperature. The slides were stained with Giemsa solution (Solarbio, China) for 10 min and observed under a light microscope to determine whether the sperm of AM4nRR had a normal head and tail structure. Furthermore, the remaining fixed semen was dripped onto a clean coverslip and dehydrated successively with alcohol concentrations of 50%, 70%, 80%, 90%, 95%, and 100%, each for 15 min. Then, the coverslips were dried with a freeze dryer (VD-250R, Japan), ion-sputtered with gold (Au^{+}), and observed and photographed under a scanning electron microscope (Jsm-6360, Japan) to determine the head diameter of the sperm.

2.5. RNA extraction, library construction, and sequencing

The testicular tissue of AM4nRR at different developmental periods (25 dph, 35 dph, 40 dph, 45 dph, 65 dph, 95 dph, and 120 dph) was excised under sterile conditions, frozen quickly in liquid nitrogen, and stored at −80 °C for further use. The tissue was analyzed histologically at the same periods. We prepared three sets of undifferentiated gland or testis samples as biological replicates for each group. Total RNA was isolated using RNAiso reagent (TaKaRa, Japan) following the manufacturer's protocol. The purity, concentration, and integrity of RNA samples were tested using advanced molecular biology equipment to ensure the use of qualified samples for transcriptome sequencing.

All cDNA libraries were constructed and sequenced by Biomarker Technologies Corporation (Beijing, China) with the Illumina HiSeq 2000 platform using paired-end technology according to the manufacturer's instructions. Clean data were obtained by removing reads containing adapters, reads containing poly-Ns and low-quality reads from the raw data. At the same time, the Q20, Q30, GC content, and sequence duplication levels of the clean data were calculated. All downstream analyses were based on clean data with high quality.

2.6. Data assembly and annotation

The clean data were assembled de novo using Trinity software (V2.5.1) to obtain the unigene libraries of AM4nRR at different periods of testis development (Grabherr et al., 2011). The unigene libraries were compared with 7 databases, namely, NR, SwissProt, GO, COG, KOG, eggNOG4.5, and KEGG, by BLAST software to obtain the annotation information of unigenes with an e-value of 1e-5. At the same time,

Table 1
The fertilization rate and the hatching rate of the hybridization between 4nRR and YY fish.

	Group 1 (2000 eggs)	Group 2 (2000 eggs)	Group 3 (2000 eggs)	Average rate
Fertilization rate	86.95%	89.50%	84.16%	86.87 ± 2.18
Hatching rate	76.50%	74.90%	77.35%	76.25 ± 1.02

Table 2
Mean DNA content of RCC and AM4nRR.

Fish type	Mean DNA content	Coefficient of variation (CV%)	Ratio	
			Observed	Expected
RCC	99.87 ± 6.25	6.26		
AM4nRR	211.43 ± 8.67	4.14	AM4nRR/2 RCC = 1.06[a]	1

[a] The observed ratio was not significantly different ($P > 0.05$) from the expected ratio.

HMMER software was used to compare unigene libraries with the Pfam database with an e-value of 1e-10.

2.7. Analysis of gene expression and differential expression

The unigene library was used as the reference sequence, and the reads obtained by sequencing from each period were aligned to the unigene library by Bowtie software. The expression level of unigenes was evaluated by RSEM. The expression abundance of the corresponding unigene was represented by the FPKM value. DESeq2 software was used to compare the expression levels of genes in undifferentiated glands and testis tissues at 25 dph, 35 dph, 40 dph, 45 dph, 65 dph, 95 dph, and 120 dph (e.g., 25 dph vs. 120 dph, a total of 21 groups). The screening conditions for differentially expressed genes (DEGs) were set as a false discovery rate (FDR) < 0.01 and a fold change (FC) ≥ 2. To further explore these DEGs, enrichment analysis was performed on the DEGs annotated to the GO, COG, KOG, eggNOG, and KEGG databases, and the numbers of upregulated and downregulated DEGs in 21 groups were statistically analyzed. The expression levels of DEGs in different periods of testis development were compared to analyze the changes in expression trends.

2.8. Quantitative real-time PCR verification

To examine the reliability of the RNA-seq results, some DEGs involved in testis development and spermatogenesis were selected for validation using quantitative real-time PCR (RT–qPCR) on a Prism 7500 Sequence Detection System (Applied Biosystems, USA). All the experiments in this stage were performed with three samples and three replicates to improve the accuracy of the results. cDNA was prepared from undifferentiated gland and testis samples (25 dph, 35 dph, 40 dph, 45 dph, 65 dph, 95 dph, and 120 dph). All primer sequences for *β-actin* (the internal control gene) and the DEGs for RT–qPCR are listed in Supplementary Table S1. RT–qPCR amplification was performed in a 10 μL reaction mixture containing 5 μL of SYBR Green Mix (Applied Biosystems, USA), 1 μL of cDNA, 0.4 μL of each primer, and 3.2 μL of ddH_2O. The RT–qPCR conditions were 50 °C for 5 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative expression of each DEG was calculated by using the $2^{-\Delta\Delta Ct}$ method. Analysis of variance and pairwise comparisons of the data were performed by SPSS 17.0 software.

Table 3
The measurable traits of 4nRR and AM4nRR.

Fish type	Whole length/ body length	Body length/ body width	Body length/ head length	Head length/ head width	Tail length/ tail width	Body width/ head width
4nRR	1.21 ± 0.02	2.24 ± 0.07	3.73 ± 0.01	1.09 ± 0.01	0.84 ± 0.03	1.88 ± 0.04
AM4nRR	1.22 ± 0.01	2.25 ± 0.05	3.71 ± 0.03	1.09 ± 0.03	0.85 ± 0.03	1.89 ± 0.01

Table 4
The countable traits of 4nRR and AM4nRR.

Fish type	No. of lateral scales	No. of upper lateral scale	No. of lower lateral scale	No. of dorsal fins	No. of abdominal fins	No. of anal fins
4nRR	29.52 ± 1.00 (29–32)	5.34 ± 0.52 (5–6)	6.84 ± 0.71 (5–7)	III + 18.26 ± 0.48 (III + 18–19)	8.65 ± 0.55 (8–9)	III + 5.42 ± 0.42 (III + 5–6)
AM4nRR	29.88 ± 1.21 (29–32)	5.45 ± 0.48 (5–6)	6.79 ± 0.68 (5–7)	III + 18.53 ± 0.37 (III + 18–19)	8.67 ± 0.45 (8–9)	III + 5.49 ± 0.47 (III + 5–6)

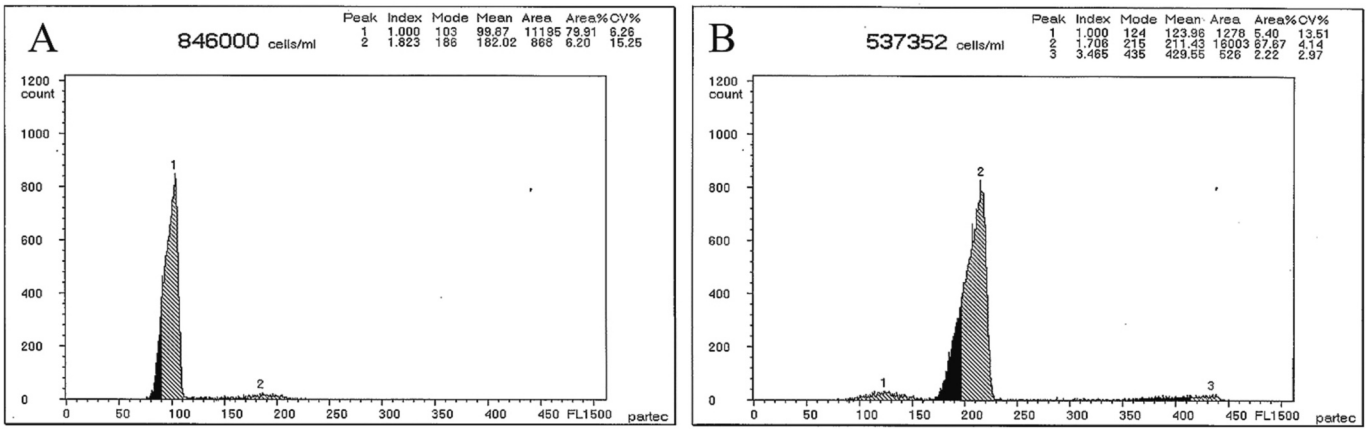

Fig. 2. Ploidy analysis of AM4nRR. (A) The mean DNA content of RCC (peak 1: 99.87). (B) The mean DNA content of AM4nRR (peak 2: 211.43).

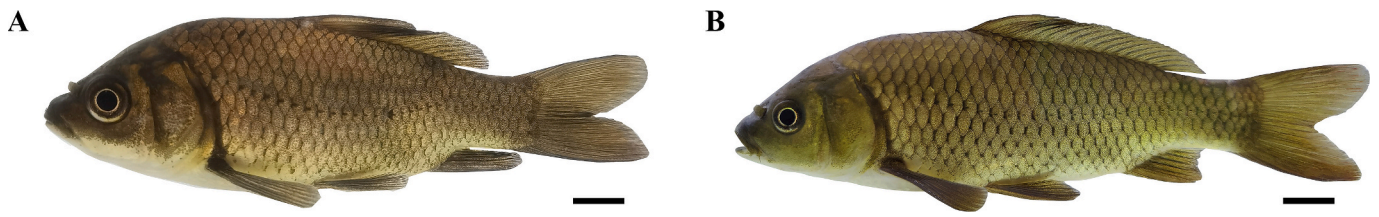

Fig. 3. Morphology of AM4nRR and 4nRR. (A) The morphological appearance of 4nRR. (B) The morphological appearance of AM4nRR. Bar = 2 cm.

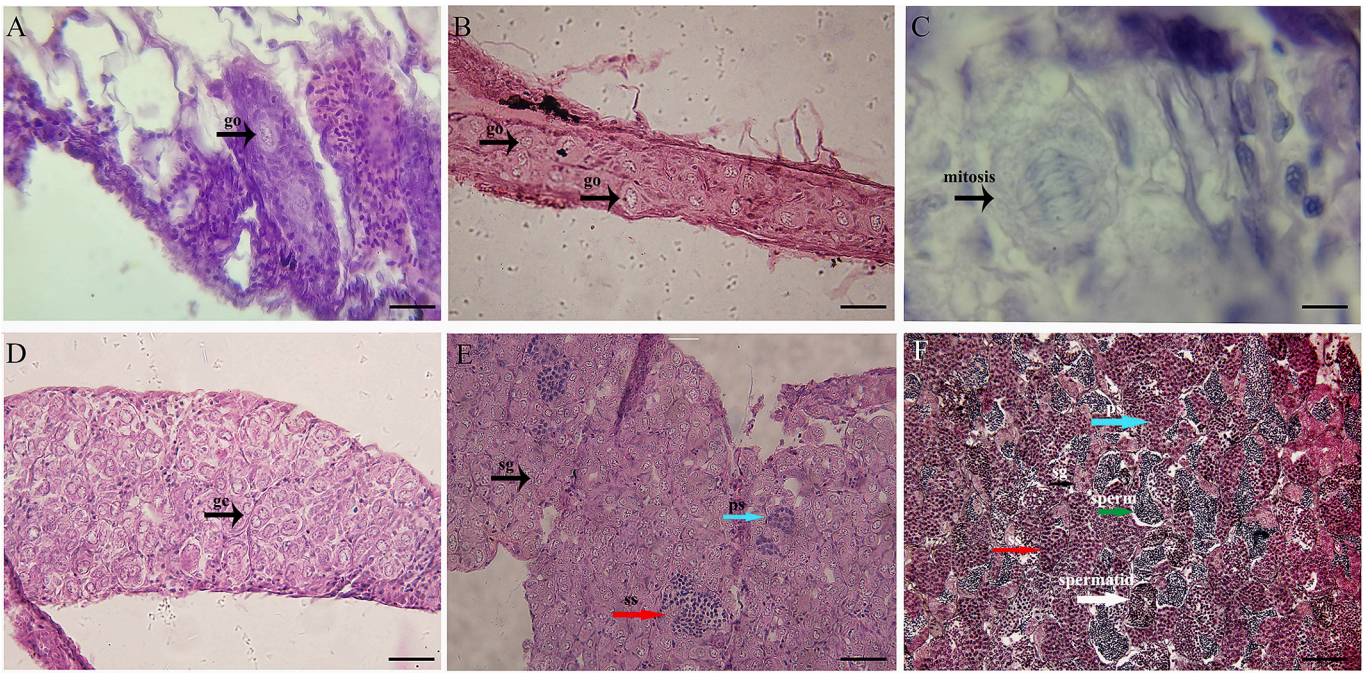

Fig. 4. Testis development of AM4nRR. (A) Histological section at 45 dph, where the black arrow shows the gonium (go). Bar = 10 μm. (B) Histological section at 65 dph, where the black arrows show the gonia (go). Bar = 20 μm. (C) Histological section at 70 dph, where the black arrow indicates mitosis of the gonium (go). Bar = 4 μm. (D) Histological section at 95 dph, where the black arrow shows many germ cells (gc) in clusters. Bar = 20 μm. (E) Histological section at 105 dph. The black arrow shows the spermatogonium (sg). The blue arrow shows primary spermatocytes (ps), and the red arrow shows secondary spermatocytes (ss). Bar = 20 μm. (F) Histological section at 120 dph. The green arrow shows sperm. The white arrow shows the spermatid. The black arrow shows the spermatogonium (sg). The blue arrow shows the primary spermatocyte (ps), and the red arrow shows the secondary spermatocyte (ss). Bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Formation of AM4nRR and measurement of DNA content and appearance

The crossing procedure is outlined in Fig. 1. AM4nRR was obtained via the hybridization of 4nRR (♀, 4n = 200) and YY fish (♂, 2n = 100). The average fertilization rate and hatching rate were 86.87% and 76.25% from 3 groups, respectively (Table 1). As shown in Table 2 and Fig. 2, the ploidy of AM4nRR samples was identified by measuring the DNA content of the caudal fin cells or blood cells using RCC as the control. The DNA content of AM4nRR was equal to double that in RCC ($P > 0.05$), suggesting that AM4nRR was a tetraploid fish.

The values of the measurable and countable traits of 4nRR and AM4nRR are shown in Table 3 and Table 4. The ratios of the measurable traits and countable traits were not significantly different ($P > 0.05$) between AM4nRR and 4nRR. 4nRR and AM4nRR had exactly the same appearance (Fig. 3).

3.2. Testicular development and sperm phenotypes of AM4nRR

To understand the testis development of AM4nRR, we collected ten fish every 5 days from 20 to 120 dph and performed histological sectioning. Fig. 4 shows some representative slices of gonads. A small number of gonium could be observed at 45 dph (Fig. 4A). During 65–95 dph, mitosis of the gonium was obvious, and the number of germ cells greatly increased (Fig. 4B-4D). At 95 dph, large numbers of germ cells appeared and were distributed in clusters (Fig. 4D). At 105 dph, primary spermatocytes and secondary spermatocytes were observed (Fig. 4E). White semen could be extruded from most individuals at 120 dph, and there were a few spermatogonia, many primary spermatocytes, secondary spermatocytes, spermatids and sperm in the paraffin sections (Fig. 4F). Flow cytometry analysis revealed that the mean DNA content of sperm from AM4nRR (100.51) was approximately twice that from RCC (49.25) (Fig. 5A and B). Furthermore, the phenotypes of the sperm from AM4nRR and RCC were compared via light microscopy and scanning electron microscopy. As shown in Fig. 5C and D, the sperm produced by AM4nRR and RCC and stained with Giemsa were well developed and had a normal head and tail. The head of AM4nRR sperm was larger than that of RCC sperm (Fig. 5E and F). At the same magnification, the mean diameter of RCC haploid sperm was 2.25 μm, whereas the mean diameter of AM4nRR sperm was 3.28 μm. Our results indicated that AM4nRR could produce normal diploid sperm (2n = 100). Our analysis of reproductive traits revealed that AM4nRR individuals were

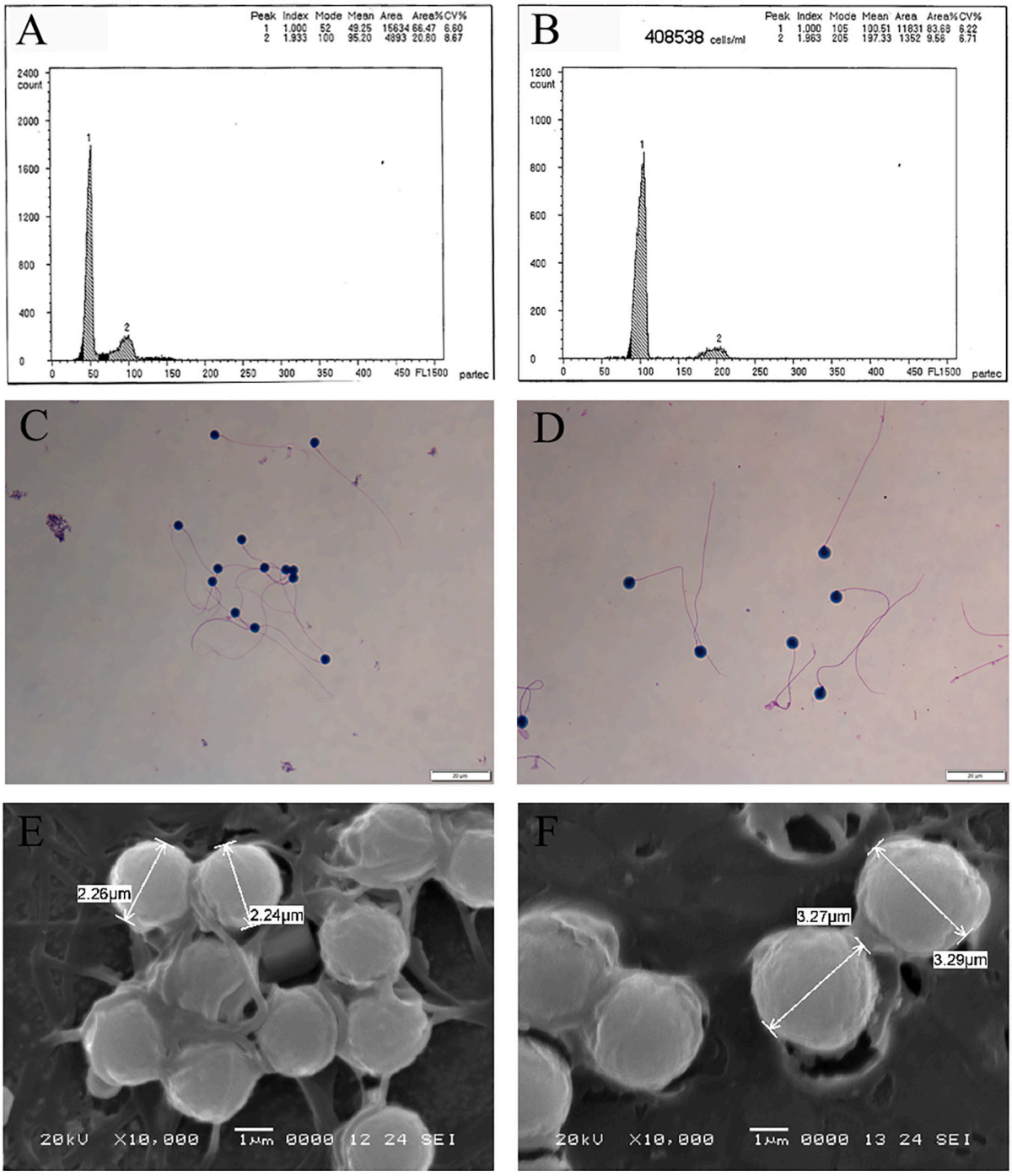

Fig. 5. DNA content and phenotype analysis of AM4nRR sperm. (A) The DNA content of RCC sperm (peak 1: 49.25). (B) The DNA content of AM4nRR sperm (peak 1: 100.51). (C) and (E) The phenotypes of the sperm from RCC. (D) and (F) The phenotypes of the sperm from AM4nRR.

fertile and reached sexual maturity at 120 dph.

3.3. Transcriptome sequencing and data analysis

The undifferentiated glands and testis samples (25 dph, 35 dph, 40 dph, 45 dph, 65 dph, 95 dph, and 120 dph) of AM4nRR were sequenced and quality controlled. The amount of clean data per sample was above 6.09 Gb, and a total of 149.55 Gb of clean data was obtained. The Q30 ratio of sequencing data was above 94.50%. A total of 56,188 unigenes were obtained after assembly. Among them, there were 23,796 unigenes with a length of >1 kb (Fig. S1). Functional annotation of the unigenes was performed, including comparison with the COG, GO, KEGG, KOG, Pfam, SwissProt, eggNOG4.5, and NR databases, and 31,863 unigene annotation results were obtained (Table 5).

3.4. Differential expression analysis

As shown in Table 6, with FDR < 0.01 and FC ≥ 2 as the screening conditions for DEGs, 21 groups of DEGs were obtained. According to the differential expression analysis, the number of DEGs between the three periods of 40 dph, 45 dph, and 65 dph was the lowest (72–275), and it was speculated that 40–65 dph may be the key period for sustainable

Table 5
Statistics of annotated unigenes.

Annotation Database	Annotated_Number	300 ≤length	length ≥1000
COG	7340	1326	6014
GO	18,986	5921	13,065
KEGG	17,155	5646	11,509
KOG	19,270	5223	14,047
Pfam	21,652	4884	16,768
Swissprot	17,380	4162	13,218
eggNOG	28,124	9237	18,887
Nr	31,442	11,443	19,999
All_Annotated	31,863	11,798	20,065

Table 6
Statistics of the number of DEGs.

Groups	Number of DEG	Up-regulated	Down-regulated
25 dph vs 35 dph	7579	3448	4131
25 dph vs 40 dph	8257	3770	4487
25 dph vs 45 dph	6540	2974	3566
25 dph vs 65 dph	5220	2493	2727
25 dph vs 95 dph	7812	3352	4460
25 dph vs 120 dph	15,226	7610	7616
35 dph vs 40 dph	2316	785	1531
35 dph vs 45 dph	1330	250	1080
35 dph vs 65 dph	1912	812	1100
35 dph vs 95 dph	7526	3559	3967
35 dph vs 120 dph	16,521	8473	8048
40 dph vs 45 dph	269	71	198
40 dph vs 65 dph	275	122	153
40 dph vs 95 dph	8257	4287	3970
40 dph vs 120 dph	16,601	8450	8151
45 dph vs 65 dph	72	69	3
45 dph vs 95 dph	4979	2593	2386
45 dph vs 120 dph	14,870	7557	7313
65 dph vs 95 dph	4389	2098	2291
65 dph vs 120 dph	13,886	6838	7048
95 dph vs 120 dph	11,369	5021	6348

development of the testis. In addition, GO enrichment analyses were performed on each group of DEGs, and functional genes related to testis development in the reproduction and reproductive process category were screened. The 293 DEGs in this category and their respective expression levels were obtained, and their expression trends in each stage of testis development were analyzed. It was found that the expression levels of some DEGs changed abruptly at 45 dph compared with 40 dph and 65 dph (Fig. 6). Furthermore, combined with histological sectioning, these results indicated that 40–65 dph might be a critical period for the initiation of testis development. These data suggested a dynamic shift in gene expression during testis development and provided a basis for further studying sex differentiation and spermatogenesis in tetraploid fish.

3.5. RT–qPCR verification

To verify the quality of the RNA-seq data and the reliability of the DEGs involved in testis development and spermatogenesis, 22 DEGs (*Amh*, *Dmrt1*, *Sox9a*, *Tex11*, *Piwil1*, *TSGA10*, *PiwiL2*, *Brdt*, *Brca2*, *Tdrd1*, *Edx1*, *Cyp17a*, *Smad2*, *Zar1*, *OVOL1*, *DDX4*, *Sas6*, *Ptgs1*, *Tdrd5*, *Spo11*, *Strbp*, and *Zp3*) were chosen for validation by RT–qPCR. The expression trends of these genes detected by RT–qPCR were basically consistent with those obtained from the RNA-seq data, implying that the RNA-Seq results were reliable (Fig. 7).

4. Discussion

At present, several genetic breeding technologies are used to prevent reductions in growth rates, resistance abilities, and fertility of fish, including hybridization, gynogenesis, androgenesis, selective breeding, transgenesis, and gene editing (Wang et al., 2018). Hybridization is a widely used technology in fish genetic breeding (Liu et al., 2020). Artificial androgenesis in fish is based upon fertilization of genetically inactivated eggs with normal sperm followed by re-diploidization and, in case of using gametes of two different species, it can be considered a special type of hybridization. The one-step and multistep breeding techniques of distant hybridization can obtain offspring with excellent traits (Wang et al., 2018). However, androgenesis, as a special hybridization, may be a potential technology to obtain excellent populations. In this study, AM4nRR (4n = 200) were artificially obtained via the interploidy cross of 4nRR (♀) and YY fish (♂) (Fig. 1 and Fig. 2). This process can be considered a special multistep breeding. AM4nRR reached sexual maturity at 120 dph, earlier than common male allotetraploids at 150 dph and male 4nRR at 1 year of age (Liu, 2014; Qin et al., 2018). We speculated that offspring resulting from androgenesis techniques might also have some improved fertility traits, such as a shorter time to sexual maturity. In tetraploid fish, the emergence of univalent, trivalent, and quadrivalent gametes during meiosis might prevent the formation of normal diploid gametes, while bivalent pairing could prevent the occurrence of abnormal meiosis and effectively improve the ability of tetraploid species to produce gametes, which is considered the best way to maintain the genetic stability of tetraploid species (Deniz, 2002; Sybenga, 1996). Therefore, meiosis in the newly synthesized AM4nRR fish was still in strict compliance with the bivalent pairing system, which could produce substantial numbers of diploid sperm for large-scale production of improved triploids.

There are three types of gonadal development and differentiation in fish: gonochorism, hermaphroditism, and intersexuality (Devlin and Nagahama, 2002). Many teleosts are gonochorists, defined as individuals that develop only as males or females and remain the same sex throughout their life cycle. Yamamoto summarized that there were two major types of gonochoristic species, including differentiated and undifferentiated gonochoristic species (Yamamoto, 1969). The former refers to early gonadal development from an indifferent gonad directly into the ovary or testis, such as in *Cyprinus carpio* L, *Esox masquinongy*, *Oncorhynchus kistuch*, and *Abramis brama* (Komen et al., 1992; Lin et al., 1997; Piferrer and Donaldson, 1989; Talikina, 1995), whereas the latter means that all gonads initially develop as ovaries, but in approximately half of the population, ovarian tissue subsequently degenerates and the gonads develop into testes, such as in *Gramma loreto* and *Danio rerio* (Asoh and Shapiro, 1997; Maack and Segner, 2003). Histological sectioning of AM4nRR showed the stages of sperm development during 45–120 dph, and mature sperm were observed at 120 dph (Fig. 3). Furthermore, during the period from 20 dph to 120 dph of AM4nRR, “ovarian-like” structures were not observed through continuous histological sectioning, and all surviving individuals of AM4nRR at 120 dph produced white semen. These results indicated that AM4nRR reached sexual maturity at 120 dph and might be a differentiated gonochoristic fish. These findings provide a cellular biological basis for studying testis development in bisexual fertile tetraploid fish and will improve our understanding of the process of testis development in polyploid fish.

The expression of many sex-determining genes or gonadal development-related genes fluctuates significantly in the early period of sex differentiation, showing a high level, and then decreases directly or is maintained in the juvenile stage (Heule et al., 2014). The time point of gene expression fluctuation also marks the time point of initial gonadal development. Sex-determining genes are mainly the genes that are transiently expressed in the undifferentiated glands and directly determine the development of the bipotential gonad into either a testis or an ovary (Mei and Gui, 2015). In this study, we found that the number of DEGs among the three periods of 40 dph, 45 dph, and 65 dph was the lowest (72–275), and the expression levels of some DEGs changed abruptly at 45 dph compared with 40 dph and 65 dph (Fig. 5). However, the 45 dph gonads were undifferentiated under histological sectioning. Similar results were found previously in Nile tilapia and Yellow River

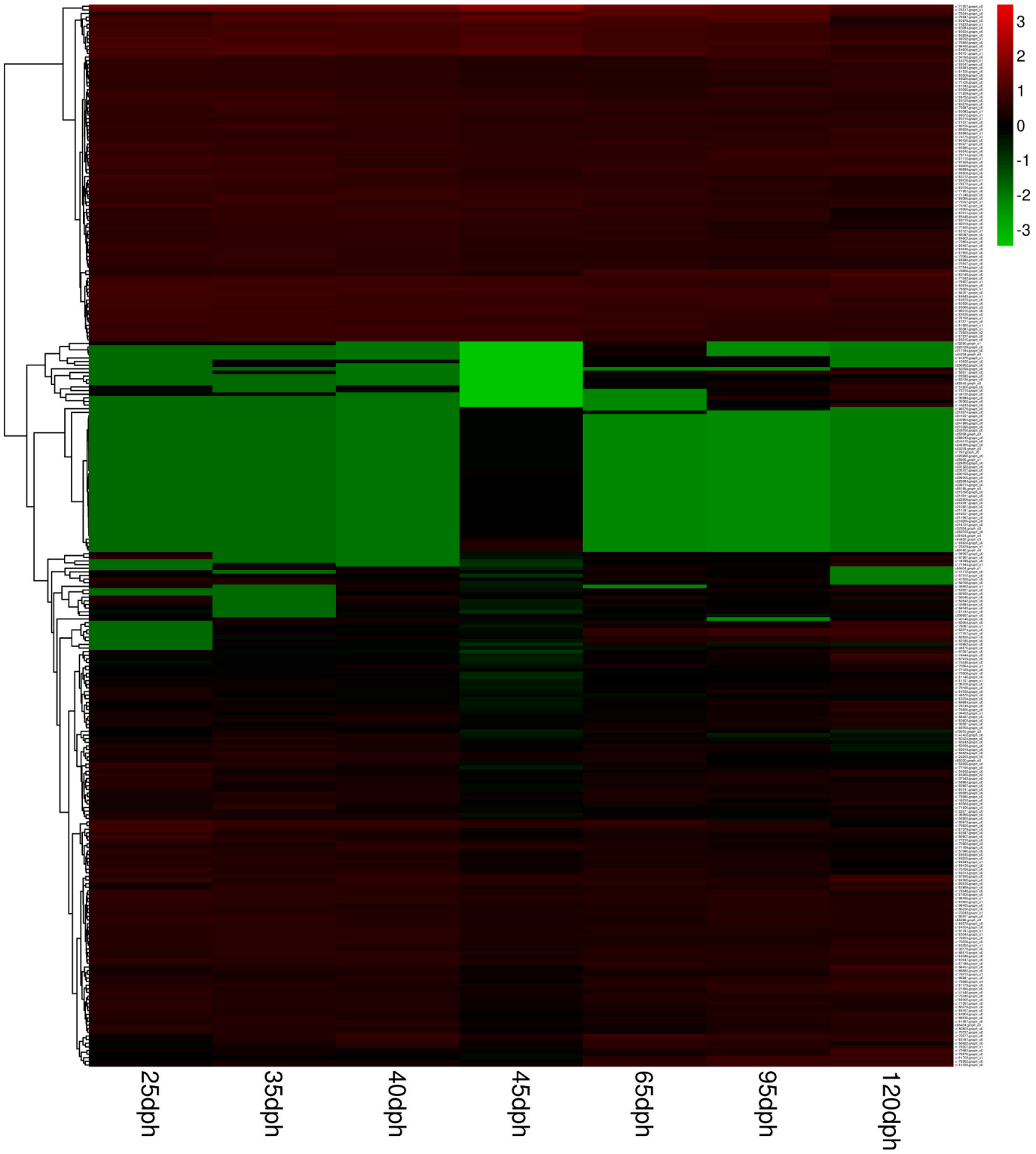

Fig. 6. Hierarchical clustering analysis of the relative expression data of 293 genes related to testis development in AM4nRR.

carp (Ijiri et al., 2008; Jia et al., 2016). It was speculated that these fluctuating genes might be related to sex determination and that 40–65 dph might be a critical period for the initiation of testis development or the undifferentiated glands developed into the testis during this period. Of course, further experiments will be required to verify this specific regulatory mechanism. In addition, meiosis and spermatogenesis are essential and complex processes in testis development that involve the regulation of multiple genes. Alterations in the expression and function of these genes might lead to spermatogenic deficiency and male infertility (Westerveld, 2008; Zhou et al., 2009). In this study, twenty-two DEGs were chosen from 293 DEGs related to testis development in the reproduction and reproductive process category to investigate the trends in expression. Based on the RNA-seq data and the results of RT–qPCR, most DEGs began to show a surge in expression from 95 dph to 120 dph, and many germ cells appeared under histological sectioning, including spermatogonia, primary spermatocytes, secondary spermatocytes,

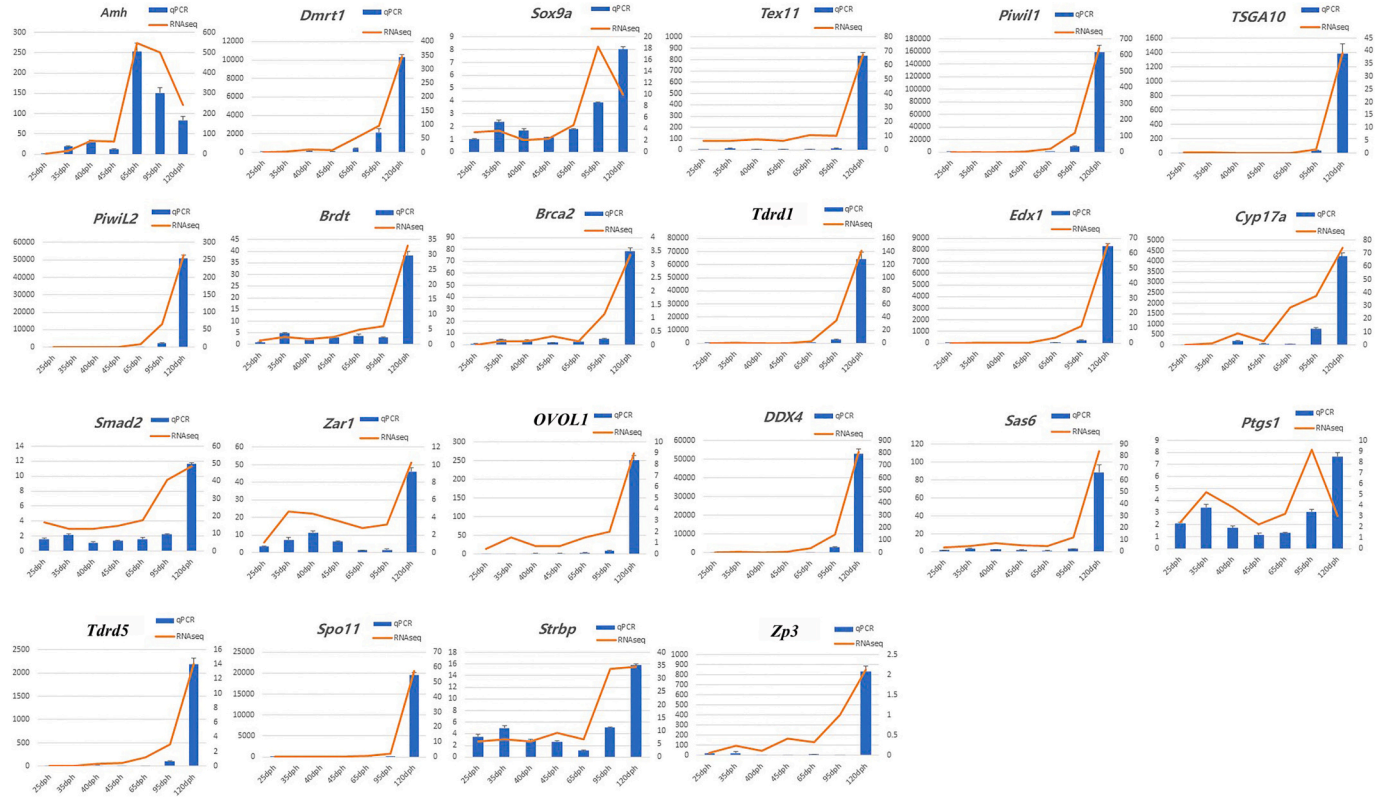

Fig. 7. Verification of the DEGs related to testis development using RT–qPCR. The Y-axis on the left represents the relative expression level of related genes in different periods of testis development in AM4nRR. The Y-axis on the right represents the expression level (FPKM) of these genes determined by RNA-seq. The X-axis represents different periods of testis development in AM4nRR.

spermatids, and sperm, during this period. These results indicated that meiosis was vigorous during this period, and many spermatogonia appeared and developed into spermatocytes. The normal expression of these genes might be the key factor in producing diploid sperm in AM4nRR.

In summary, the ploidy and sperm phenotypes of AM4nRR and the tissue structure and transcriptome changes in different periods of testis development of AM4nRR were analyzed. AM4nRR is an important germplasm resource that can be used to mass produce excellent triploid fish. This study provides a basis for better understanding testis development and further studying sex differentiation in autotetraploid fish.

Authors' contributions

Xu Huang, Qinbo Qin and Shaojun Liu designed the experiments, performed the analyses, and performed the technical discussions; Xu Huang and Qingwen Xiao performed the experiments and drafted the manuscript; Qinbo Qin, Ming Ma and Chang Wu modified the manuscript; Chang Wu and Chongqing Wang participated in transcriptome analysis and discussions; Xiaowei Xu participated in the sequence alignment; Yali Long collected the experimental materials; Xidan Xu and Yue Zhou participated in the histological sectioning. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

The clean data from the Illumina HiSeq 2000 were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database and are available for download (PRJNA933784, http://www.ncbi.nlm.nih.gov/bioproject/933784).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.740269.

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