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Research paper

Characterization of *vasa* in the gonads of different ploidy fish

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ABSTRACT

Vasa is an essential gene for germ cell development belonging to the DEAD-box family. In this study, we comparatively analyzed the expression characteristics of *vasa* in diploids, triploids, and tetraploids. The sequences showed high similarity among these fish and other vertebrates, with characteristic domains. Tissue expression analysis revealed that *vasa* was expressed exclusively in the gonad of different ploidy fishes. During embryogenesis, *vasa* expression was lower in diploid than in triploid and tetraploid fish, caused by doubling of the genome of tetraploids and abnormal gonads in the triploid fish. In adults, *vasa* mRNA levels were significantly lower in the testes of sterile triploid fish compared with fertile diploids and tetraploids. In the ovaries, triploid fish showed consistently high expression from the non-breeding season to the breeding season. Immunohistochemistry and western blotting results also supported the abnormal expression of *vasa* in triploid gonads. This study demonstrates, for the first time, that fish of different ploidy exhibit different expression patterns of *vasa* that contribute to the differentiation of gonadal development.

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1. Introduction

Vasa belongs to the DEAD-box (Asp-Glu-Ala-Asp) protein family, which plays a crucial role in germ cell formation (Raz, 2000). The DEAD-box proteins have a wide variety of functions in RNA processing in germ cells, such as RNA editing, RNA splicing, translation initiation, and RNA degradation (Luking et al., 1998; Rocak and Linder, 2004). *Vasa* is localized specifically in germline cells, indicating a particular function in gonads (Toyooka et al., 2000; Noce et al., 2001; Rocak and Linder, 2004). In addition, failure of spermatogenesis during meiosis has been observed in *vasa* gene knockout mice (Tanaka et al., 2000). Other studies have reported that *vasa* mutant female fruit flies (*Drosophila melanogaster*) can produce eggs, however, fertilization rates decreased significantly compared with the controls (Hay et al., 1990; Williamson and Lehmann, 1996).

The *vasa* protein was initially isolated from the pole cells of fruit flies. Subsequent studies have reported that *vasa* sequences were obtained in teleosts, including the catfish (*Clarias gariepinus*) (Raghuveer and Senthilkumaran, 2010), European sea bass (*Dicentrarchus labrax*) (Blazquez et al., 2011), rare minnow (*Gobiocypris rarus*) (Cao et al., 2012), Senegalese sole (*Solea senegalensis*) (Pacchiarini et al., 2013), Pacific bluefin tuna (*Thunnus orientalis*) (Nagasawa et al., 2009), ricefield eel (*Monopterus albus*) (Ye et al., 2007), grass carp (*Ctenopharyngodon idella*) (Li et al., 2010), and gibel carp (*Carassius auratus gibelio*) (Xu et al., 2005). These studies also indicated that, as in other animals, *vasa* proteins are expressed exclusively in fish gonads. A series of studies in zebrafish (*Danio rerio*) suggested that *vasa* expressed throughout embryogenesis in germ cells (Olsen et al., 1997). Differential expression of a *vasa* homolog in the gonads of tilapia (*Oreochromis niloticus*) during gametogenesis suggests a possible role for *vasa* in regulating the meiotic progression of male and female germ cells (Kobayashi et al., 2000). These studies prove that *vasa* is an exclusive gonadal marker and participates in germ cell development. Although research on the *vasa* gene structure and functions has been carried out in many organisms, the mechanism of expression still needs further study.

By intercrossing female red crucian carp (*Cassius auratus*) with male common carp (*Cyprinus carpio*), we created F_1 diploid hybrids and then F_2 hybrids by self-mating the F_1 generation. Intriguingly, F_2 hybrids could produce diploid gametes resulting in tetraploid descendants in the F_3 generation. To date, generations F_3–F_{24} have been produced (Liu et al., 2001). In addition, by intercrossing these tetraploids with diploids, we obtained triploid fish, which are potentially of high commercial value as they are sterile and grow rapidly (Song et al., 2012). In previous study, we showed that dysfunction in the hypothalamic–pituitary–gonadal axis contributes to the infertility of these triploids (Long et al., 2009). However, until now, details of *vasa* expression patterns and its biological function in aquatic animals have remained limited, especially in fish strains with different ploidies.

Here, we cloned full-length *vasa* cDNAs from the testes of fish with different ploidies. The tissue expression pattern was validated by

Abbreviations: RT-PCR, reverse-transcription polymerase chain reaction; RACE, rapid amplification of vasa cDNA ends.

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reverse-transcription polymerase chain reaction (RT-PCR). We also assayed the *vasa* mRNA expression differentiation in different ploidy fish during embryogenesis and in adults by real-time RT-PCR. Finally, we extended our research to *vasa* protein differentiation in fish with different ploidies using western blotting and immunohistochemistry. These results will improve our knowledge of *vasa* expression patterns in different ploidy fish and its function in sterile triploids.

2. Materials and methods

2.1. Animals and samples

Red crucian carp, common carp, triploids and tetraploids were obtained from the Engineering Research Center of Polyploid Fish Breeding and Reproduction of the State Education Ministry in Hunan Normal University. Before tissues excision, all fish were anesthetized using 2-phenoxyethanol. The tissues were obtained and stored at −80 °C until further analysis.

2.2. RNA extraction and cDNA cloning

Total RNA was prepared using TRIzol reagent (Invitrogen life technologies, Changsha, China) following the manufacturer's instructions. The integrity was checked on 1.2% agarose gels and the quantity of total RNA was assessed by spectrophotometry. The first-strand cDNA was synthesized with primer oligo $(dT)_{18}$ using the MMLV Reverse Transcriptase System. To isolate core partial *vasa* cDNA sequences, polymerase chain reaction (PCR) amplification was carried out using the first-strand cDNA of the testes of triploids and tetraploids as templates. The PCR conditions were as follows: 94 °C for 5 min, then 94 °C for 45 s, 58 °C for 30 s, and 72 °C for 2 min with 30 cycles using *vasa*-F and *vasa*-R degenerate primers (Table 1). The products were cloned and sequenced to obtain the central *vasa* cDNA of triploid and tetraploid fish.

2.3. 5′- and 3′-rapid amplification of vasa cDNA ends (RACE)

To isolate the full-length *vasa* cDNA, 5′ and 3′ RACE protocols were performed using SMART RACE cDNA Amplification kits (Clontech) following the manufacturer's instructions. The *vasa* cDNA 3′ ends were amplified using the sense primers *vasa*-3-1 and *vasa*-3-2 (Table 1). The *vasa* cDNA 5′ ends were amplified with the specific anti-sense primers *vasa*-5-1 and *vasa*-5-2. The primers were designed based on the central *vasa* cDNA sequences. All PCR runs were performed at 94 °C for 5 min, followed by 35 cycles of amplification at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 2 min, and an additional elongation at 72 °C for 10 min after the last cycle. The PCR amplified products were cloned and sequenced.

Table 1
Primers used for *vasa* cloning and expression analysis.

Primer name	Sequence (5′-3′)	Usage
Vasa-F	AGGAAACGGGAAGGCAAACAAT	Degenerate RT-PCR
Vasa-R	ATGTTCCCACAGCGTCCG	Degenerate RT-PCR
*Vasa*3race adaptor	CTGATCTAGAGGTACCGGATCC	3′RACE
*Vasa*3race adaptor + olig(T)	CTGATCTAGAGGTACCGGATCCTTTT TTTTTTTTTT	3′RACE
Vasa-3-1	CGCACGATGGTCTTTGTTGA	3′RACE
Vasa-3-2	GAATACGTCCATCGCATCGG	3′RACE
*Vasa*5race adaptor	GACTCGAGTCGACATCG	5′RACE
*Vasa*5race adaptor + (dT)17	ACTCGAGTCGACATCGATTTTTTTTT TTTTTTTT	5′RACE
Vasa-5-1	ATTGTTTGCCTTCCCGTTTC	5′RACE
Vasa-5-2	TCCTCTGCCCCTGAATCCA	5′RACE
Vasa-RT-F	CATGCCTTCAAAAGAGGATCG	RT-PCR and real-time PCR
Vasa-RT-R	ACCAACCACACCAACAGCAA	RT-PCR and real-time PCR
β-actin-F	GCCCTGCCCCATGCCATCCT	RT-PCR and real-time PCR
β-actin-R	AGTGCCCATCTCCTGCTCGA	RT-PCR and real-time PCR

2.4. Phylogenetic analysis

A homology search of the deduced amino acid sequence of the obtained cDNA was carried out using the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Amino acid sequences were aligned using the ClustalW2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) at default settings. A phylogenetic tree was constructed with the neighbor-joining (NJ) method using MEGA version 4 (Tamura et al., 2007). The bootstrapping method was used to check for the statistical validity of the nodes generated in the tree with 1000 bootstrap replicates performed for analysis.

2.5. Vasa tissue distribution

The distribution of *vasa* in various tissues of different ploidy fishes was analyzed by reverse transcription PCR (RT-PCR). Specific primers (Table 1) were designed based on the cDNA sequences and the gene of β-actin was used as an internal control. The synthesis of cDNAs of different tissues was as described above. The PCR conditions were 94 °C for 5 min followed by 30 cycles at 94 C for 30 s, 58 °C for 30 s, and 72 °C for 30 s and a single elongation step at 72 °C for 10 min.

2.6. Real-time RT-PCR

Real-time RT-PCR was performed to assay the mRNA expression of *vasa* in different ploidy fish. All the tests were repeated three times to improve the accuracy in a Prism 7500 Sequence Detection System (Applied Biosystems). We used β-actin as an endogenous control. The PCR conditions were 50 °C for 5 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Determination of vasa mRNA expression during embryogenesis

Red crucian carp ($n = 7$ for each stage), triploid ($n = 7$ for each stage), and tetraploid ($n = 7$ for each stage) embryos at different developmental stages were collected using a stereomicroscope. After total RNAs had been isolated, the cDNAs were synthesized and the *vasa* mRNA expression levels were determined by real-time RT-PCR.

2.8. Determination of vasa mRNA expression in adult fish

To analyze the *vasa* mRNA expression levels in adult individuals gonads of common carp, red crucian carp, triploids, and tetraploids, the testes and ovaries were collected during the breeding season (2 years old, April) and non-breeding season (2 years old, November) ($n = 8$ for each kind of fish). After total RNA isolation and cDNA synthesis, the *vasa* transcripts were assayed by real-time RT-PCR.

2.9. Western blotting

Adult gonad samples ($n = 5$ for each group) were homogenized on ice in RIPA buffer (Bio-Rad) according to the manufacturer's instructions. The samples were subjected to 15% SDS–PAGE in a Bio-Rad Mini-Protein electrophoresis system. The separated proteins were transferred to a nitrocellulose membrane. Then the membrane was blocked with 5% non-fat dry milk for 1 h at room temperature, and incubated with the primary anti-*vasa* antibody. The antibody was polyclonal antibody against gibel carp recombinant *vasa* protein from rabbit injection which was described before (Xu et al., 2005). The specific of anti-*vasa* antibody was confirmed by western blotting (Supplementary

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