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Original research article

Molecular characterization and expression of the *feminization-1c* (*fem-1c*) in the freshwater mussel (*Hyriopsis cumingii*)

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ABSTRACT

The *feminization-1c (fem-1c)* gene has been shown to be associated with sex differentiation and determination in many metazoan species. It belongs to the *fem-1* family which is a member of the ANK superfamily. In this study, the full-length cDNA of the *fem-1c* (*Hcfem-1c*) gene was isolated from the freshwater mussel (*Hypriopsis cumingii*). The isolated *Hcfem-1c* cDNA was 2196 bp in length and encoded a putative protein of 621 amino acids that contains seven ANK domains. Phylogenetic analysis of the deduced HcFEM-1C protein showed that it clustered with the other invertebrates homologues, indicating that the sequence of HcFEM-1C was conserved during evolution. Quantitative real-time PCR (qPCR) expression revealed that the *Hcfem-1c* gene was expressed in the adductor muscle, foot, liver, gill, kidney, mantle, and gonads of male and female adult mussels (two years old). In the gonads *Hcfem-1c* was much less abundant in males than that in females. During early development of the gonads, *Hcfem-1c* transcripts were significantly increased in the primordial germ cell differentiation stage (5 months old). We hypothesized that *Hcfem-1c* probably regulates female gonad differentiation. *In situ* hybridization showed that a strong and specific signal concentrated in the female oocyte cell membrane and male follicular wall, indicating that *Hcfem-1c* gene may not only be involved in female gonad differentiation, but also participates in egg development. This study laid the foundations for a better understanding of gender differentiation mechanism in *H. cumingii*.

1. Introduction

Hypriopsis cumingii, a unique freshwater pearl mussel of China, occupies a large proportion of the pearl breeding industry. The pearls produced by *H. cumingii* are characterized for their smoothness, bright color and fine texture. *H. cumingii* pearl production accounts for 80% of the total output value of freshwater pearls (Wang, Bai, Liu, & Li, 2014). The pearl yield of the *H. cumingii* is affected by gender. The pearls produced by male mussels are superior to those produced by female mussels in terms of total weight, particle weight and diameter; thus, male mussels show a significant advantage in breeding pearls (Zhao et al., 2013). To date, there have been few studies on sex determination and sex differentiation in *H. cumingii*. Research on gender differentiation will help to improve the quality of breeding pearls and their economic value.

The *fem* gene is an important factor in gender regulation that was first identified in the nematode *Caenorhabditis elegans*, and its role is also very thorough (Zhang et al., 2013). The main characterized genes involved in nematode sex determination hierarchy are: *her-1* (hermaphroditization-1) > *tra-2* (*transformer-2*) > *fem-1* (feminization-1), *fem-2* (feminization-2), *fem-3* (feminization-3), *Cul-2* (Cullin-2) > *tra-1* (transformer-1) (Zanetti & Puoti, 2013). The specific regulatory mechanism is as follows: *fem-1/2/3* genes act upon the downstream TRA-1 protein directly. TRA-1 protein is the terminal factor of the regulatory pathway that promotes the development of individuals into females (Kuwabara, 2007; Zarkower & Hodgkin, 1992). The X/A (number of X chromosomes per set of autosomes) ratios determined two types of chromosomes in the nematode: XX and XO (Jäger, Schwartz, Horvitz, & Conradt, 2004; Trent et al., 1991). A low X/A ratio activates the transcription of the *her-1* gene, which was an inhibitory ligand of the TRA-2 protein. Freed from the influence of TRA-2 protein, the complexes formed by FEM-1/2/3 and CUL-2 proteins target the downstream transcription factor, TRA-1, for degradation and therefore, the

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Abbreviations

fem-1c	feminization-1c
EST	expressed sequence tag
qRT-PCR	quantitative real-time PCR
ORF	open reading frame
PBS	phosphate-buffered saline
RACE	rapid amplification of cDNA ends

individuals would develop into males. In contrast, a high X/A ratio represses the transcription of the *her-1* gene, leaving the TRA-2 protein in the active form, which could bind to FEM-1/2/3 and CUL-2 to regulate the expression of TRA-1 protein. In this case, the organism would develop into a hermaphrodite (Arur, Ohmachi, Berkseth, Nayak, Hansen, & Zarkower, 2011; Doniach & Hodgkin, 1984; Hodgkin & Brenner, 1977). Mutation of *fem-1* in the nematode led to a sex change from male to hermaphrodite (Krakow, Sebald, King, & Cohn, 2001; Schvarzstein & Spence, 2006; Starostina et al., 2007). Thus, the *fem-1* gene has a significant effect on the sex determination pathway of *C. elegans*. In addition, studies in silkworms, bees, and other invertebrates have confirmed that *fem* genes play a major role in gender regulation (Nagaraju, Gopinath, Sharma, & Shukla, 2014; Sakai, Aoki, & Suzuki, 2014).

Multiple *fem-1* gene isoforms have been described. They are characterized by the existence of ankyrin repeat domains which comprises typically 30 amino acids and is one of the most common protein−protein interaction motifs in nature (Bork, 1993; Du & Hu, 2002). In vertebrates, three highly conserved *fem-1* genes *fem-1a*, *fem-1b*, and *fem-1c* have been described (Itoh, Kampf, & Arnold, 2009; Venturaholman, Seldin, Li, & Maher, 1998). In humans, *fem-1c* is highly expressed in the testis and is associated with the formation of sperm (Venturaholman, Lu, Si, Izevbigie, & Maher, 2003). In *Locusta migratoria manilensis*, expression of the *fem-1c* gene gradually increases with the development of the testis and thus may be related to spermatogenesis (Shi et al., 2013). However, in aquatic animals there have been few studies on *fem-1c* (Ma, Liu, Lin, Li, & Qiu, 2016; Shi, Li, Gui, & Zhou, 2015). In this study, we have isolated the *fem-1c* gene of *H. cumingii*, to understand its role in development and to provide a theoretical basis to study sex differentiation of *H. cumingii*.

2. Materials and methods

2.1. Animal rearing and tissue sampling

The larvae and two years old *H. cumingii* used in this study were collected from a breeding farm in Jinhua City, Zhejiang Province, China, and transported to the laboratory. All the mussels were kept at 26 ± 2 °C in cage for two days and regularly fed with chlorella in the cage. Various tissues, including the adductor muscle, foot, liver, gill, kidney, mantle, gonad were sampled from adult mussels. Embryos were collected from the gills of mussels. Fertilized mussels were placed in a cool dry place for 4–5 h, and placed back into water. Fertilized eggs were discharged from the gills, and approximately 200 were collected into a centrifuge tube using a pipette. The larvae of *H. cumingii* cannot be defined accurately; therefore, the approximate stages were deduced from the time of embryonic development. The embryonic period was divided into the following periods: 0 day (fertilized eggs), 3, 6, 9 and 10 days. For RNA isolation, all samples were collected and immediately frozen in liquid nitrogen.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from the frozen preserved tissues using TRNzol (Tiangen, Shanghai, China), according to the manufacturer's instructions. A PrimeScript™ first-strand cDNA synthesis kit (TaKaRa, Dalian, China) was used to obtain cDNA, according to the manufacturer's protocol.

2.3. Cloning of Hcfem-1c

The full-length *Hcfem-1c* cDNA sequence was cloned by rapid amplification of cDNA ends (RACE). Primers for RACE were designed based on an expressed sequence tag (EST) sequence identified in GenBank under the accession number KY465663 (Table 1). The open reading frame (ORF) was cloned by RT-PCR using the primers Fem-1c-F and Fem1c-R. A SMARTer RACE 5′/3′ Kit (Clontech) was used to obtain the full-length cDNA sequence of *Hcfem-1c*, according to the manufacturer's instructions. Primers were designed according to the ORF of *fem-1c*; primers Fem-1c-3′ and Fem-1c-5′ were used to amplify the 5′ and 3′ untranslated regions (UTRs). For all PCRs, the program was: initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR product was purified and ligated into vector PGEM-T vector (Promega, Madison, USA) at 16 °C for 12 h, and then transformed into *Escherichia coli* DH5α competent cells. The plasmid inserts of positive clones were sequenced by the sequencing service unit of Sangon Biotech (Shanghai, China).

2.4. Sequence and phylogenetic analysis

The sequence of the cloned cDNA was confirmed using the BLAST algorithm at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The ORF of *Hcfem-1c* gene was determined using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The nucleotide and amino acid sequence identity and the prediction of conserved domains, were confirmed using the BLAST program against the non-redundant protein sequences database (GenBank, NCBI). The physical parameters of the HcFEM-1C protein were analyzed using

Table 1
Primers used in this study.

Name	Sequence (5′–3′)	Purpose	Temp. (°C)
Fem-1c-F	TGTATGCAAGGCTGATATTGAA	Partial fragment amplification of fem1c	55.2
Fem-1c-R	GGTTGGCTATTTCGATATCG	Partial fragment amplification of fem1c	53.9
Fem-1c-3′	TAGTCAAGTTTCTGGTGGCA	3′-RACE offem1c	59.2
Fem-1c-5′	GACTTTTGATGGAGAAAC	5′-RACE offem1c	68.5
Fem-1c-RT-F	ATGCATGTCTTATGCTTGATG	qPCR of fem1c	57.0
Fem-1c-RT-R	TGAACAAGCAAGATGAAG	qPCR of fem1c	57.0
EF1α-F	GGAACTTCCCAGGCAGACTGTGC	qPCR	58.0
EF1α-R	TCAAAACGGGCCGCAGAGAAT	qPCR	58.0

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