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Pf-Dmrt4, a potential factor in sexual development in the pearl oyster *Pinctada fucata**

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Abstract The mechanisms of sex determination and sex differentiation in the pearl oyster *Pinctada fucata* are currently poorly understood. We therefore investigated the roles of orthologs of the *Dmrt* gene family, key players in male gonad differentiation in mammals, in *P. fucata* sex differentiation and sexual development. *Pf-Dmrt4* exhibits features typical of the *Dmrt* family, and displays significant homologies to the DMRT4 cluster. *Pf-Dmrt4* mRNA expression in the gonads during a gametogenic cycle, measured by quantitative polymerase chain reaction, was maximal in mature individuals. *Pf-Dmrt4* expression, demonstrated by in situ hybridization, was localized in the spermatozoa, spermatids, oocytes and vitellogenic oocytes. Knockdown of *Pf-Dmrt4* with double-stranded RNA resulted in decreased mRNA expression levels. And Pf-Dmrt4-dsRNA-injected groups showed spawning-stage male gonads, with ruptured follicles and released spermatozoa. Our results enhance the understanding of sex determination and differentiation in *P. fucata* and suggest that *Pf-Dmrt4* could be involved in male gonadal development, and maintenance of male gonadal function.

Keyword: *Pinctada fucata*; *Pf-Dmrt4*; gene expression; sexual development

1 INTRODUCTION

Sexual reproduction is a remarkable phenomenon in biology and the primary source of genetic variation required for evolutionary survival. It can be found in almost all types of multicellular animals. The mechanisms of sex determination and differentiation vary among phyla, possibly as a result of long-term evolution of the genes involved in the sex determination pathways (Marín and Baker, 1998). Bivalves belong to the second largest phylum and display highly diverse sexual phenotypes. The mollusc pearl oyster *Pinctada fucata* exhibits complex modes of sexual reproduction that consists of protandric dioecy, occasional sex change and hermaphroditism. Environmental factors such as population density, water temperature, and sex ratios play important roles in sex determination and differentiation in bivalves (Yu et al., 2007, 2011), though the function of genetic factors remains unclear.

The *Drosophila doublesex* (Burtis and Baker, 1989) and *Caenorhabditis elegans mab-3* (Shen and Hodgkin, 1988) genes are widely conserved and have been implicated in sex determination (Raymond et al., 1999). The *Dmrt* (double-sex and mab-3-related transcription factor) gene family encodes proteins with conserved DM domains (a common zinc finger-like DNA-binding motif) and is represented by multiple members in both vertebrates and invertebrates (Volff et al., 2003). To date, eight members of the family have been reported in humans (Ottolenghi et al., 2002) and seven have been found in mice (Kim et al., 2003). DM-domain family members have been shown to regulate sexual development in vertebrates and invertebrates, and the function of the *Dmrt* gene

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family in sex regulation has been highly conserved during evolution (Hodgkin, 2002).

Apart from *Dmrt1*, DM-domain family members are expressed during development in tissues other than the gonads, suggesting that these proteins may control a broader range of developmental processes (Hong et al., 2007). Some *Dmrt4* and *Dmrt5* orthologs play key roles in sexual development in different animals such as zebrafish (Guo et al., 2004; Winkler et al., 2004), blue tilapia (Cao et al., 2010), *Chlamys farreri* (Feng et al., 2010) and *Crassostrea*. *gigas* (Naimi et al., 2009).

As a highly conserved gene family, *Dmrt* genes have been cloned and studied in various vertebrates (Ottolenghi et al., 2002; Aoyama et al., 2003) and several invertebrates, including fruit flies (Crémazy et al., 2001), nematodes (Volff et al., 2003), sea squirts (Leveugle et al., 2004) and freshwater prawns (Peng et al., 2005). In molluscs, orthologs of the DM-domain transcription factors have been isolated from the oyster *C*. *gigas* (*CgDMl*) (Naimi et al., 2009), the scallop *Ch*. *farreri* (*Cfdmrt4-like*) (Feng et al., 2010), the abalone *Haliotis asinina* (*HADMRT1-like*) (Klinbunga et al., 2009), the pearl oyster *Pinctada martensii* (*pmDmrt2* and *pmDmrt5*) (Yu et al., 2009, 2011), and the scallop *Ch*. *nobilis* (*dmrt5*) (Shi et al., 2014). Gene expression analysis of some of these orthologs suggests the possible involvement of these genes in gonad differentiation and development (Naimi et al., 2009; Feng et al., 2010). Sex determination and differentiation in molluscs appear to be complex because of the diverse range of species and underlying molecular mechanisms involved.

Recently, the genomics of some economically marine bivalves are studied to provide better understanding of the molecular mechanisms underlying their different reproductive strategies such as the black-lip pearl oyster *P*. *margaritifera* (Teaniniuraitemoana et al.,2014), the Pacific oyster *C*. *gigas* (Zhang et al., 2014) and the pearl oyster *P*. *fucata* (Takeuchi et al., 2012; Du et al., 2017). The available transcriptome is a powerful and novel resource to study sexual pathway in marine mollusks.

It is an essential step to identify and characterize sex-specific genes to explore the molecular pathways of sexual development in the pearl oyster *P*. *fucata*. This study therefore investigated the genetic mechanisms responsible for sex differentiation and gonad development of *Pf-Dmrt4* in *P*. *fucata*, which is an economically important species for pearl production in China. The full-length cDNA sequence of Pf-Dmrt4 during gonad differentiation was characterized, and the spatio-temporal expression patters of *Pf-Dmrt4* were studied during gonad differentiation and in adult tissue using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The cellular localization and the sex-steroid –induced expression of the gene were also investigated.

2 MATERIAL AND METHOD

2.1 Experimental animals and tissue sampling

Pearl oysters *P*. *fucata* were obtained from Daya Bay in Shenzhen (Guangdong Province, China), cultivated in floating net cages in the sea under natural conditions. All specimens were collected between May, 2012 and August, 2013. Testes and ovaries were excised, frozen immediately in liquid nitrogen and maintained at -80°C until RNA extraction for *Pf-Dmrt4* cloning. Ovary, testis, heart, digestive gland, adductor muscle, mantle, and gill tissues were also dissected and stored in the same manner for tissue-distribution analysis. Gonad tissues from different stages were dissected, fixed in Bouin's solution at 4°C overnight, and submitted to histological observation to determine the gonadal stages. Gonad samples were fixed with 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS) overnight at 4°C and then stored in methanol at -20°C for in-situ hybridization. Gametes were obtained and passed through a 100-μm screen to remove tissue debris. The eggs were mixed with sperms at 25–26°C in filtered seawater which contained 0.005%–0.006% (v/v) ammonia. After fertilization, embryos and different stages of larvae were then prepared for RNA extraction.

2.2 Cloning and sequencing of *Pf-Dmrt4*

Total RNA was extracted from testes using an E.Z.N.A. Mollusc RNA Kit according to the manufacturer's protocol (Omega, Japan). The quantity and quality of RNA were assayed by UV spectrophotometry (OD_{260}/OD_{280}). A ReverTra Ace-First-strand cDNA Synthesis Kit (Toyobo, Japan) was used to synthesize first-strand cDNA from 1 μg total RNA. All primers for *Pf-Dmrt4* used in the present study are listed in Table 1. PCR primers were designed using Primer Premier 5.00 (Premier Biosoft International, Palo Alto, CA, USA). All primer pairs were designed to eliminate false-positive bands of potential genomic DNA contamination. A fragment

Table 1 Primers used for *Pf-Dmrt4* cloning and functional analysis

No.	Primer name	Usage	Sense/Antisense	Primer sequence (5′→3′)
1	df1	RT-PCR (partial)	Sense	5′-GCAGTTTGGCTTTGTCTATTTGAT-3′
2	dr1	RT-PCR (partial)	Antisense	5′-TGGGCAACAGGGATACATAGCATAA-3′
3	dmrt5′-1	5′RACE	Antisense	5′-TCTCGTCATCTGAACTCATCTTGC-3′
4	dmrt3′-1	3′RACE	Sense	5′-TCATCGCCCGACAGATTGGA-3′
5	dmrt3′-2	Nested 3′RACE	Sense	5′-GCCTCACTTTCACTTGGAGATTGTA-3′
6	UPM mixture	5′ and 3′RACE	Sense	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ 5′-CTAATACGACTCACTATAGGGC-3′
7	NUP	5′ and 3′RACE	Sense	5′-AAGCAGTGGTATCAACGCAGAGT-3′
8	df2	RT-PCR (ORF)	Sense	5′-CTCCAAACATACGGACATCAGAA-3′
9	dr2	RT-PCR (ORF)	Antisense	5′-ATCTCCAAGTGAAAGTGAGGCAA-3′
10	df3	Real-time PCR	Sense	5′-GACAAAAGAAATGCCTCGC-3′
11	dr3	Real-time PCR	Antisense	5′-TGTCAGAGCCACTTTCCG-3′
12	18S-f	Real-time PCR	Sense	5′-CGTTTCAACAAGACGCCAGTAG-3′
13	18S-r	Real-time PCR	Antisense	5′-ACGAAAAAAAGGTTTGAGAGACG-3′
14	df4	In-situ hybridization	Sense	5′-CGAATTCAAAATCTTCGGAAAGTGGCTC-3′
15	dr4	In-situ hybridization	Antisense	5′-GAAGCTTTTGGGCAACAGGGATACATAG-3′
16	df5	RNAi	Sense	5′-GCGTAATACGACTCACTATAGGGAGACTGAGGAGACAGCAAGCACA-3′
17	dr5	RNAi	Antisense	5′-GCGTAATACGACTCACTATAGGGAGATGGAGATGACGTGCTGATGG-3′
18	GFPf	RNAi	Sense	5′-GCGTAATACGACTCACTATAGGGAGAATGGTGAGCAAGGGCGAGGAG-3′
19	GFPr	RNAi	Antisense	5′-GCGTAATACGACTCACTATAGGGAGATTACTTGTACAGCTCGTCCATG-3′

Restriction sites of Hind III and EcoRI are underlined. The T7 promoter sequence is double-underlined.

obtained from the transcriptome of *P. fucata* was amplified using a pair of specific primers df 1 and dr1. BD SMART RACE cDNA Amplification Kit (Clontech, USA) was used to synthesize SMART cDNA. The resulting 1 384-bp fragment was used to generate full-length cDNA by 5′- and 3′ rapid amplification of cDNA ends. The cycling conditions for all PCR were: 3 min at 94°C for denaturation, 35–40 cycles at 94°C for 30 s, 55–60°C for 30 s and 72°C for 1–2 min, followed by a further elongation at 72°C for 10 min. The PCR products were purified with The E.Z.N.A. Gel Extraction Kit (Omega BioTek, USA), subcloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequenced (Applied Biosystems, USA).

2.3 Tissue distributions of *Pf-Dmrt4* in *P. fucata*

Tissue expression patterns of *Pf-Dmrt4* mRNA were analyzed by real-time qPCR. Expression analysis was performed in ovary, testis, gill, mantle, heart, digestive gland, and adductor muscle tissues collected from three pearl oysters and stored at -80°C. Reactions were performed in triplicate. Real-time qPCR were carried out using a Roche LightCycler 480 real-time PCR system, with SYBR green master mix (Toyobo, Japan) following the manufacturer's instructions. All the qPCR primers are presented in Table 1. Real-time PCR was performed as follows: 94°C for 1 min, and 40 cycles of 94°C for 15 s, 15 s at 55°C and 72°C for 1 min.

2.4 Expression patterns of *Pf-Dmrt4* in embryonic and larval *P. fucata*

The developmental expression patterns of *Pf-Dmrt4* were analyzed in five developmental stages, which were collected and stored at -80°C: blastula-stage embryo, trochophore, D-shaped larva, umbo larva and metamorphosis stage. Total RNA extraction and real-time qPCR were the same as described above and three repetitions were analyzed.

2.5 Expression patterns of *Pf-Dmrt4* in *P. fucata* gonads during gametogenesis

Gonadal sections were fixed with Bouin's fixative , 6 μm slices were prepared, and processed with conventional histological analysis. The sections were stained with hematoxylin-eosin and examined microscopically to classify the natural gonadal development. The gametogenic expression patterns of *Pf-Dmrt4* were analysed by real-time PCR in

cDNAs from four stages of ovary and testis (growing stage, mature stage, spawning stage and resting stage), synthesized as described above. Five replicates of each sample were analyzed.

2.6 Expression of *Pf-Dmrt4* mRNA following methyltestosterone injection

The effect of methyltestosterone (MT) on the mRNA expression levels of *Pf-Dmrt4* were investigated by injecting male oysters with MT (purchased from Guangzhou Lifescience Biotechnology Co. Ltd., Guangzhou, China) at the gonadal mature stage. MT was dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) and added into modified Herbst's artificial seawater (ASW) to a concentration of 1 μg/μL. A 100-μL aliquot of suspension was injected into the adductor muscle of male oysters. The animals in Negative control group were injected with 100 μL of ASW containing 1% DMSO. Eight oysters were randomly collected before injection (blank control). A further eight oysters were collected every 6 h after injection for 3 times, respectively, and the gonads were dissected and fixed for RNA extraction and real-time PCR.

2.7 In situ hybridization

The expression of *Pf-Dmrt4* in gonads were localized using the gene-specific primers designed for amplification (Table 1). Digoxigenin -labeled RNA probes were prepared from a 482-bp fragment of the *Pf-Dmrt4* cDNA that had been amplified and subcloned into pGEM-T Easy vectors (Promega) as a template. The probes were labeled with a DIG RNA labelling kit (Roche Diagnostics, Switzerland). Transcriptions were performed using SP6 (sense) or T7 (antisense) RNA polymerase. Paraformaldehyde-fixed gonad samples were embedded in paraffin, sectioned (10 μm), de-paraffinized, and then rehydrated. In situ hybridization was performed as described by Miyashita et al. (2008), with some modifications. Gonad sections were hybridized with DIG-labeled RNA probes, and hybridized signals were visualized with NBT/BCIP system and alkaline phosphatase-conjugated anti-DIG antibody using a DIG Nucleic Acid Detection Kit (Roche Diagnostics) following the manufacturer's protocol.

2.8 RNA interference

RNA interference was performed as described by Suzuki et al. (2009), with some modifications. The primers used to generate *Pf-Dmrt4* and green fluorescent protein (GFP) double-stranded (ds) RNAs are shown in Table 1. The T7 promoter sequence is underlined. RiboMAXTM Large Scale RNA Production System (T7) kit (Promega, USA) was uesd to synthesize and purify the dsRNA. RNase-free DNase I (TaKaRa, Japan) was used to digest template DNA. Synthesized dsRNA was diluted to 10, 20 and 40 μg/100 μL with PBS, and 100 μL of each solution was injected into the adductor muscles of 2-year-old oysters with mature testes. PBS 100 μL or 20 μg *GFP* dsRNA in 100 μL PBS were used as blank and negative controls. Each treatment selected 7 oysters. Total RNA was extracted from the gonad of each individual 7 days after injection and used for first-strand cDNA synthesis. The expression levels were quantified by real-time qPCR, using *18S* as a reference gene. The *Pf-Dmrt4* qPCR primers were designed to amplify the region that did not overlap the region targeted by dsRNA. Gonads were dissected from the injected-oyster groups 7 days after injection, fixed in Bouin's solution at 4 °C overnight, and submitted to histological observation at the light-microscope level.

2.9 Statistical analysis

Homology analysis of the deduced amino acid (aa) sequence was conducted using the public NCBI-BLAST database. Motif analysis was performed using InterPro Scan on the ExPASy Proteomics Server. ClustalX (1.81) was used for multiple sequence alignments of amio acids. A phylogenetic tree was constructed by the neighbor-joining method using MEGA.6. Three-dimensional model of Pf-DMRT4 were predicted by SWISS Model on http://swissmodel.expasy.org/. All reference sequences were downloaded from GenBank and public genome resources. Quantitative data were shown as mean±S.E.M. Significant differences were estimated by one-way ANOVA followed by Duncan's multiple range tests.

3 RESULT

3.1 Isolation and characterization of *Pf-Dmrt4*

A full-length cDNA encoding *Pf-Dmrt4* was isolated from *P. fucata* (GenBank accession number: KM272582). The cDNA sequence of *Pf-Dmrt4* was 1 711 bp, containing an open reading frame of 1 104 bp, 5′ untranlated region (UTR) of 199 bp and 3′UTR of 408 bp with a 30-bp polyA tail. The deduced aa sequence was 367 aa and contained a DM domain consensus sequence (from 23–81 aa) with conserved

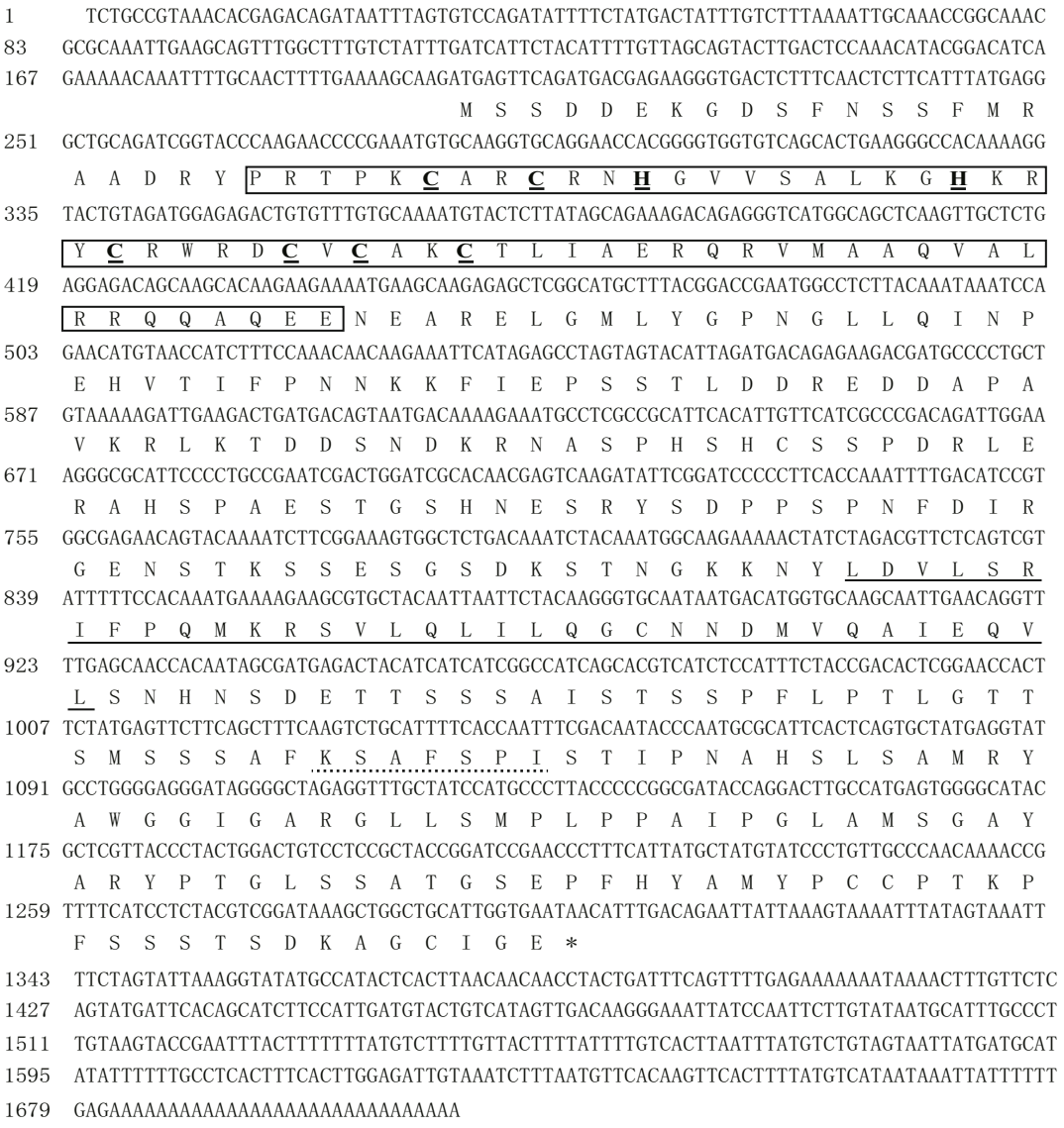

Fig.1 Nucleotide and deduced amino acid sequences of *Pf-Dmrt4*

DM domain is boxed, the underlined and the dashed lines indicate the DMA domain and the short motif, respectively. The cysteine and histidine conserved residues are bolded and underlined.

cysteines and histidines characteristic of the DMRT protein family, and a DMA domain (from 207–242 aa) (Fig.1).

The amino acid sequence of the conserved DM domain of *P. fucata* Pf-DMRT4 was aligned with known sequences in various members of this family in different species (Fig.2a). The highest identity rate was 100% with *P. martensii* DMRT5, followed by *Ch. farreri* DMRT4-like, *C. gigas* DMI and *Ch. nobilis* DMRT5 (98%). Sequence comparison revealed a conserved zinc module consisting of intertwined CCHC and HCCC Zn^{2+}-binding sites, and a consensus sequence of five aa (KGHKR) of the putative nuclear localization signal (NLS). There was a conserved DMA domain and a short conserved domain of seven aa (KSAFSPI) near the C-terminus in DMRT4 and DMRT5, but not in DMRT-like types (Fig.2b). The DMA domain of *P. fucata* DMRT4 displayed highest homologies of 74% with Cg-DMI, 68% with *Ch. farreri* DMRT4-like, 66% with *Ch. nobilis* DMRT5 and 65% with *Oryzias latipes* DMRT5. The seven-aa conserved domain shared the highest levels of aa identity with *Ch. farreri* DMRT4-like and *Ch. nobilis* DMRT5 (100%), Cg-DMI and *H. sapiens* DMRT4 (85.71%) and *H. sapiens* DMRT5 (71.43%).

The phylogenetic tree generated using the complete protein genes of DMRT from various species showed that *P. fucata* DMRT4 was most closely related to *P. martensii* DMRT5, then clustered with *Ch. farreri* DMRT4-like, *C. gigas* DMI and *Ch. nobilis* DMRT5

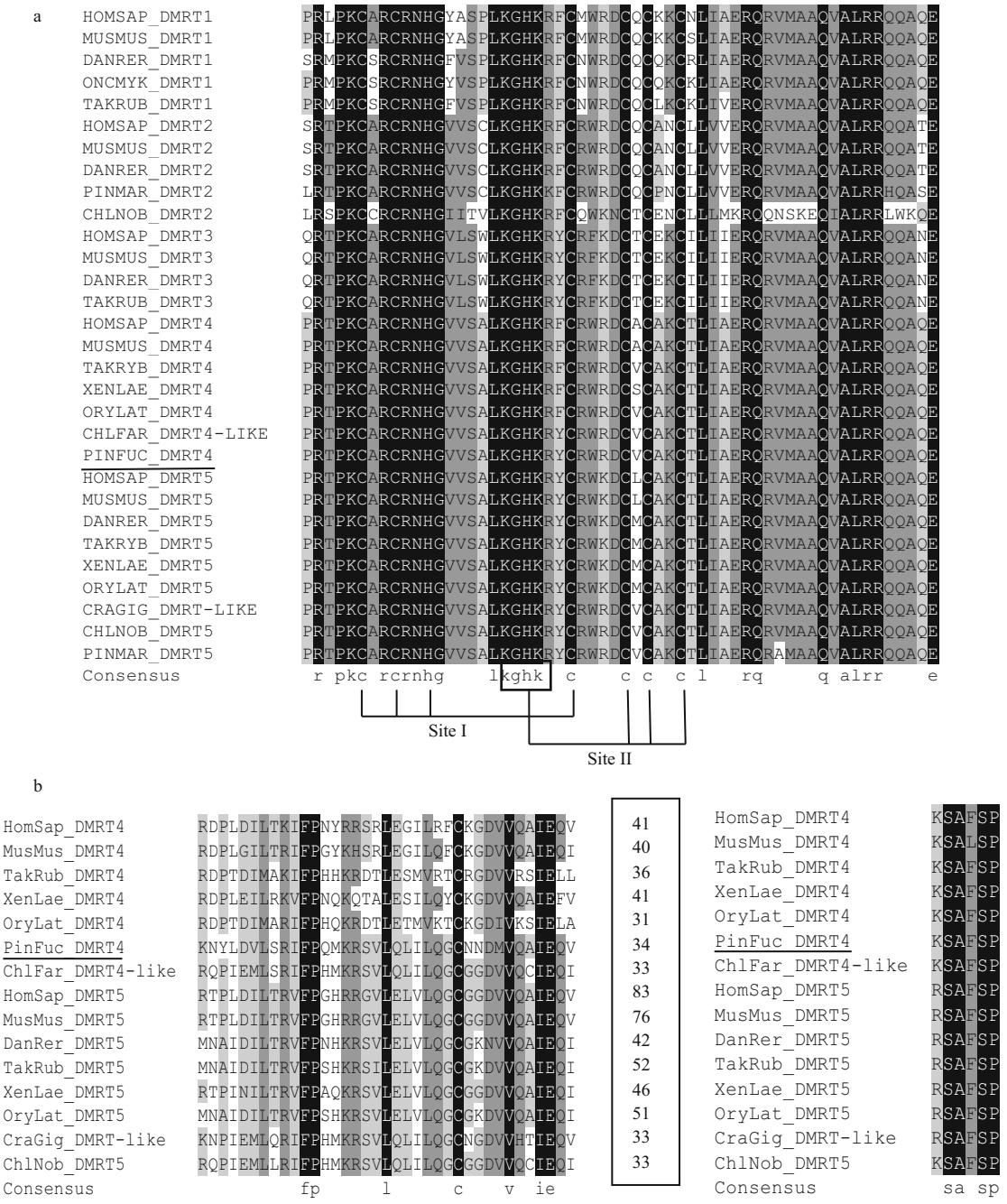

Fig.2 Alignment of amino acid sequences of the DM domain of *P. fucata* DMRT4 with other DMRTs of different species (a) and alignment of amino acid sequences of the DMA domain and the short conserved motif from different species (b)

In a: identical residues are shown in black, dark and light gray represent strongly or weakly conserved similar amino acids. The short conserved signal NLS (Nuclear Localization Signal) (KGHKR) is underlined and the zinc module consisting of intertwined CCHC and HCCC Zn^{2+}-binding sites (Site I and II) are indicated with black boxes. In b: identical amino acids to *Pf-Dmrt4* is underlined. The numbers of the amino acid residues between DMA domain and the short conserved motif are indicated in the black box. (GenBank accession numbers were listed in Supplementary Material 1).

(Fig.3a). *P. fucata* DMRT4 was grouped with the mollusc DMRT4 and DMRT5 clusters, but more closely with DMRT4, implying that *P. fucata* DMRT4 may be a member of the DMRT4 cluster. Two transcripts encoding orthologs of the DM domain transcription factor were identified in *P. margaritifera*, PinMarg DMRT1 and PinMarg DMRT2 were clustered with OryLat DMRT1 and PinMar DMRT2, then grouped with DMRT1 isoform X1 and X2 which were found in the genomic database in *C. gigas*.

A model of the structure of the Pf-DMRT4 was predicted by SWISS Model and the yellow indicated zinc-finger-like with DNA binding modules of DM domain (Fig.3b).

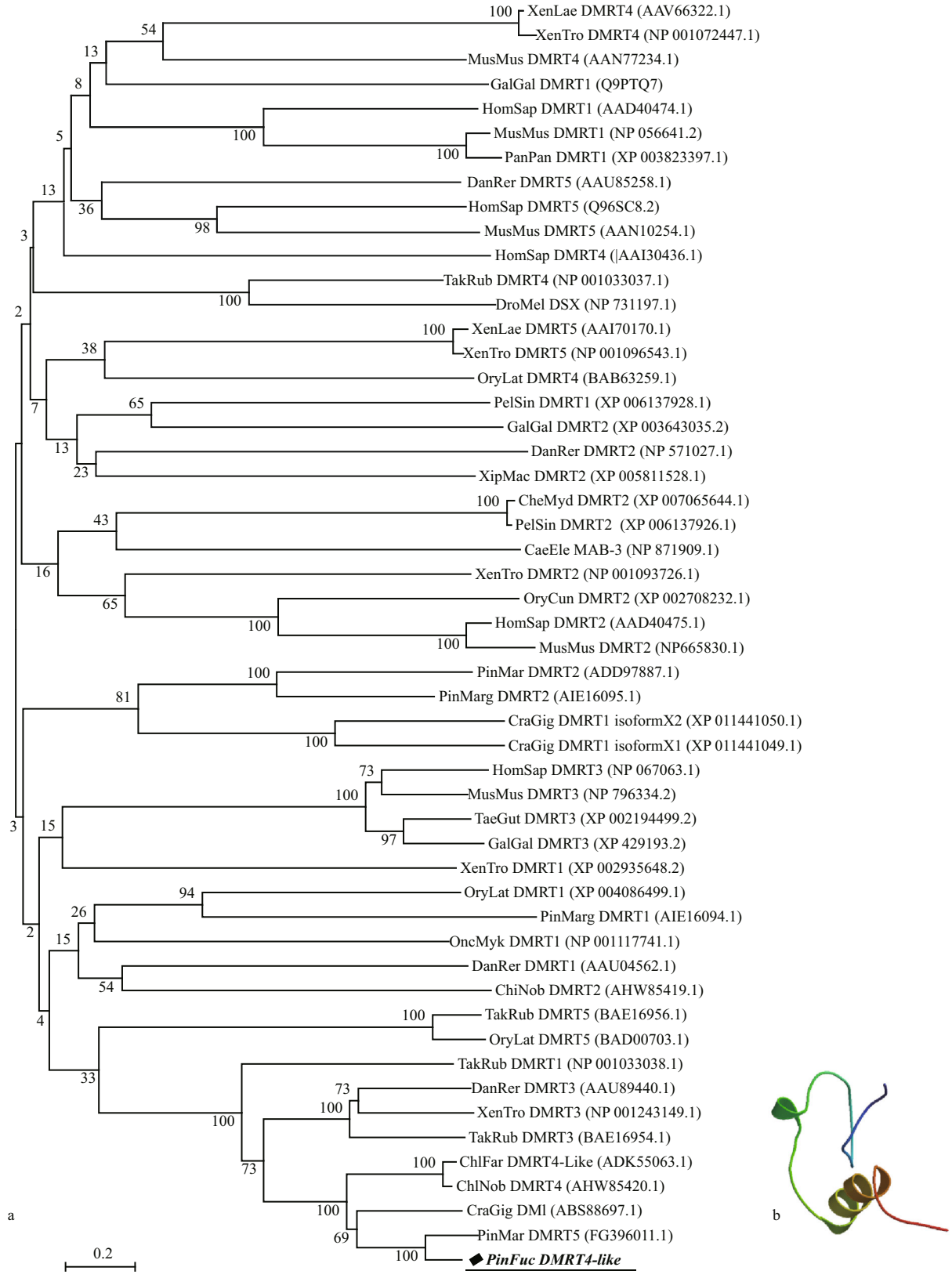

Fig.3 The phylogenetic tree of the DMRT proteins was constructed by MEGA.6 using the neighbor-joining method (a) and structure model of Pf-DMRT4 protein (b)

In a: the number shown at each branch displays the bootstrap value. Each sequence is denoted by the species followed by its subtype. Abbreviations of the species were listed in Supplementary Material 2 and the corresponding GenBank accession numbers of the reference sequences are shown in the phylogenetic tree. In b: yellow indicated zine-fmger-like with DNA binding modules of DM domain.

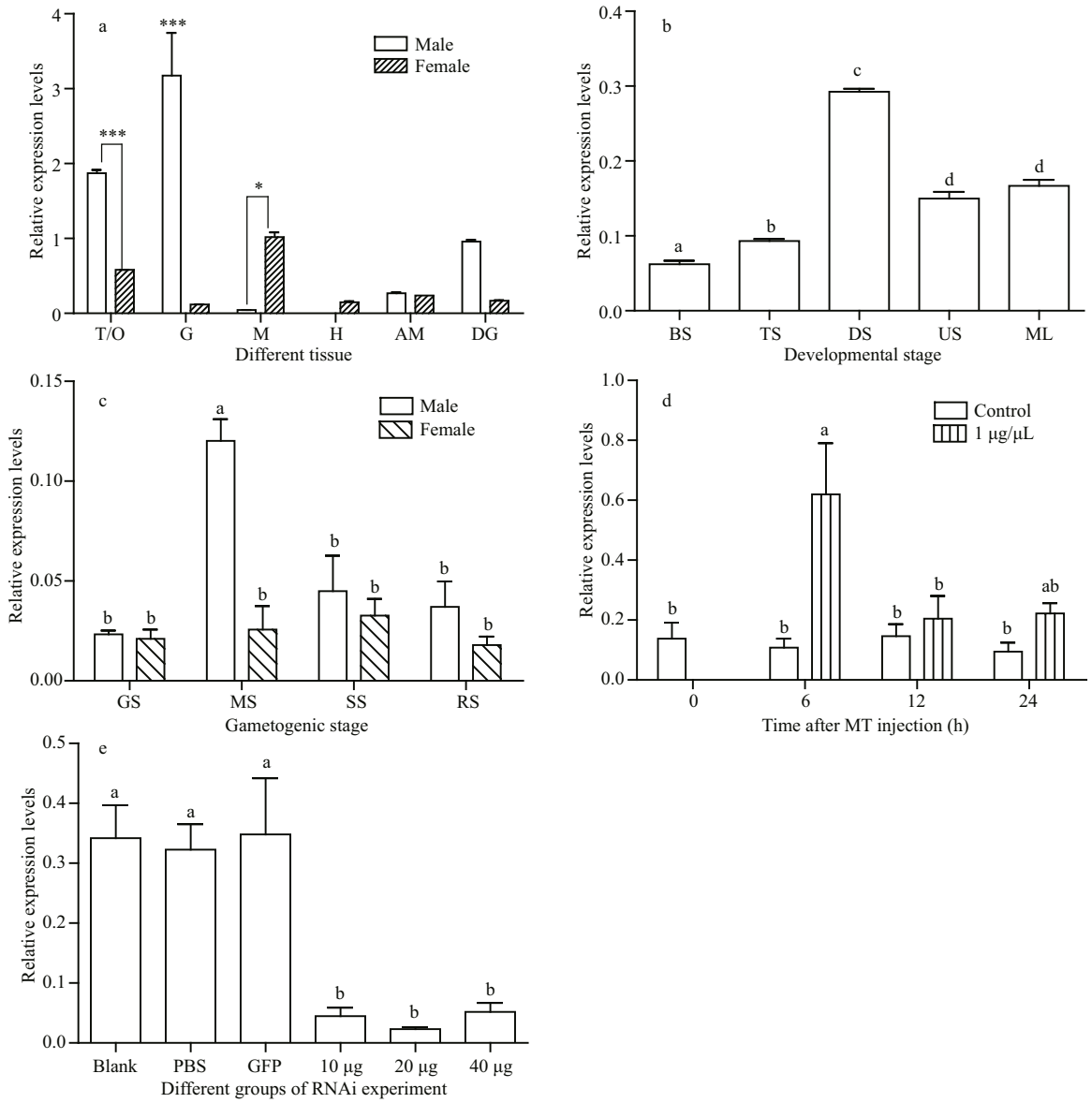

Fig.4 Expressions of *Pf-Dmrt4* mRNA in tissues of *P. fucata* (a); expression pattern of *Pf-Dmrt4* mRNA in embryonic and larval stages of *P. fucata* (b); *Pf-Dmrt4* mRNA temporal expression in adult gonadic areas of *P. fucata* along a gametogenetic cycle (c); levels of mRNA expression for *Pf-Dmrt4* mRNA in the gonad of *P. fucata* after MT injection (d); expression levels of *Pf-Dmrt4* knocked down by RNAi (e)

In a: the mRNA levels were quantified by real-time RT-PCR in: T: testis (male) or O: ovary (female); G: gill; M: mantle; H: heart; AM: adductor muscle; DG: digestive gland. The results are expressed as fold-change. Each bar represents the mean±S.E.M. of three samples. *: $P<0.05$; ***: $P<0.001$. *18S* (AY877529) was used as the reference gene. In b: BS: blastula stage; TS: trochophore stage; DS: D-shaped larva stage; US: umbo larva stage; ML: metamorphosis of larva. Each value represents the mean±SEM ($n=3$). ***: $P<0.001$. In c: GS: growing stage; MS: mature stage; SS: spawning stage; RS: resting stage. Each bar represents the mean±S.E.M. of five samples. Value without common notation (a, b) differ significantly ($P<0.05$). In d: each value represents the mean±SEM ($n=10$). Means not sharing the same superscript are significantly different ($P<0.05$). In e: the expression levels of *Pf-Dmrt4* mRNA in the testis were measured by real-time quantitative PCR 7 days after injection. Seven oysters were used in each group. Means not sharing the same superscript are significantly different ($P<0.05$).

3.2 *Pf-Dmrt4* expression pattern in tissues

Pf-Dmrt4 mRNA expression levels were detected in all tested tissues by real time qRT-PCR. The expression profiles using df3, dr3 and Sfi, Sr1 primers suggested that mRNA expression reflected *Pf-Dmrt4* transcript levels. *Pf-Dmrt4*mRNA levels were significantly higher in gills and testis, moderate in digestive gland, and low in mantle, adductor muscle and heart tissues. In female tissues, *Pf-Dmrt4* mRNA levels were higher in the mantle and ovary compared with other tissues. *Pf-Dmrt4* expression levels were higher in testis than in ovaries (Fig.4a).

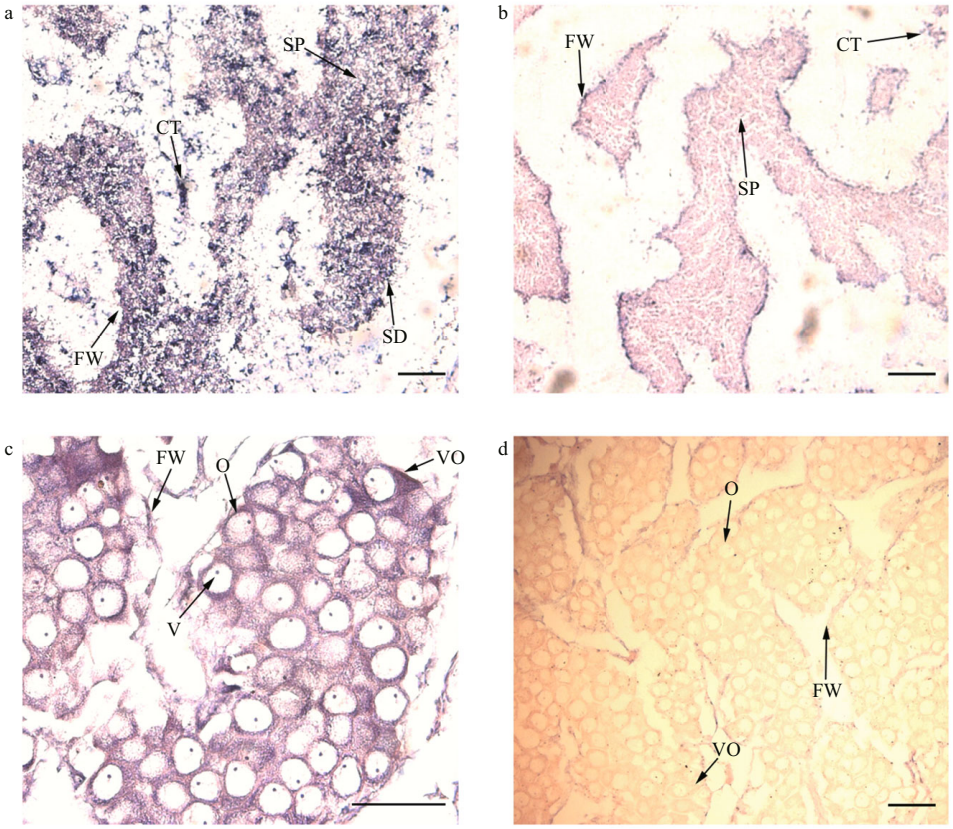

Fig.5 *Pf-Dmrt4* mRNA cellular expression patterns by in-situ hybridization in the gonadic area during the mature stage

In males, *Pf-Dmrt4* mRNA expression is found in spermatozoa and to a lesser extent in spermatid (a). In females, cytoplasmic staining is observed in oocytes and vitellogenic oocytes (c). Whatever the sex, a somatic staining cannot be excluded. No staining is observed with the sense probe (b, d). SP: spermatozoa; CT: connected tissue; N: nucleus; O: oocyte; VO: vitellogenic oocytes; FW: follicular wall. Bars: 200 μm.

3.3 *Pf-Dmrt4* expression patterns in different developmental stages of *P. fucata*

Pf-Dmrt4 mRNAs were expressed in all embryonic and larval developmental stages examined (Fig.4b). *Pf-Dmrt4* expression was highest in D-shaped larvae.

3.4 Temporal expression patterns of *Pf-Dmrt4* during the adult gametogenic cycle

The gametogenic cycle can be divided into four stages: growing stage (active spermatogenesis and growing oocytes), mature stage (mature germ cells and almost no follicular internal), spawning stage (ruptured follicular, spawning mature sperms and oocytes) and resting stage (irregular shape of follicular, contains few spermatogonia and oogonia, degenerate oocytes and sperms can be seen occasionally). *Pf-Dmrt4* mRNA expression levels in gonad were detected by real-time qPCR at all adult gametogenic stages (Fig.4c). *Pf-Dmrt4* mRNA levels were significantly higher in the mature stage compared with the other three stages in the male gametogenic cycle. However, there were no significant differences in *Pf-Dmrt4* expression among the different stages of the female gametogenic cycle.

3.5 Effects of MT injection on *Pf-Dmrt4* expression

Pf-Dmrt4 mRNA expression in the gonads during a gametogenic cycle, measured by quantitative polymerase chain reaction, was maximal in mature male testis. Therefore, mature male testis were used in the MT injection experiment.

Pf-Dmrt4 mRNA levels in the testis were significantly increased 6-h post-injection with 1 μg/μL of MT (Fig.4d), being almost 3- fold higher than in control groups, respectively.

3.6 Localization of *Pf-Dmrt4* transcripts in the gonads of *P. fucata*

We determined the cellular localizations of *Pf-Dmrt4* transcripts in mature *P. fucata* by *in situ* hybridization. *Pf-Dmrt4* mRNA in the male gonads was detected in the cytoplasm of spermatozoa and to a lesser extent in spermatids (Fig.5a). In the female

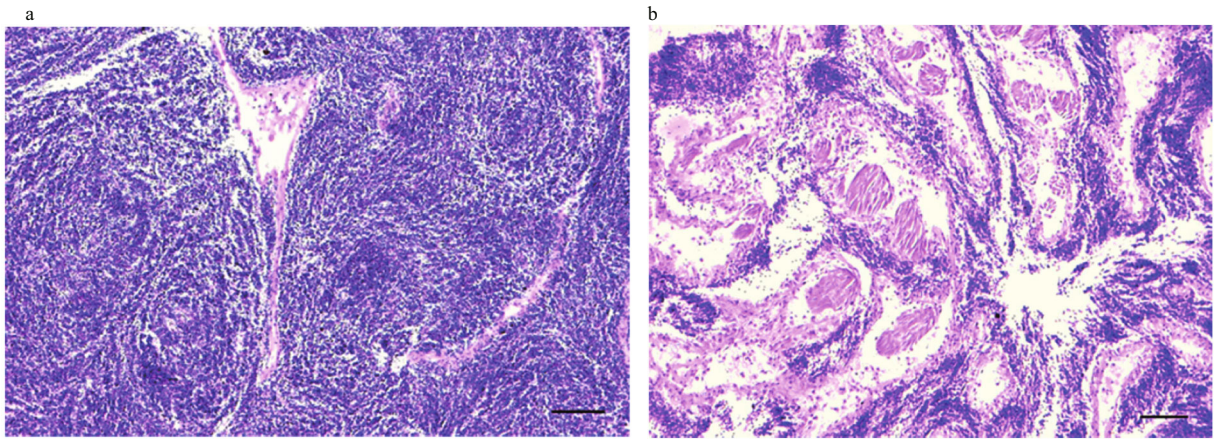

Fig.6 Light microscope images of the testis of the pearl oysters injected with PBS (a), 20 μg of *Pf-Dmrt4* (b) dsRNA in the magnification of 100, respectively

Bars: 200 μm.

gonads (Fig.5c), cytoplasmic mRNA staining was observed in oocytes and vitellogenic oocytes. Negative controls with the sense riboprobe sometimes produced a faint, insignificant signal (Fig.5b, d).

3.7 RNA interference knockdown of *Pf-Dmrt4*

Pf-Dmrt4 expression levels were decreased by *Pf-Dmrt4* dsRNA (10, 20, and 40 μg) respectively (Fig.4e). Expression levels in the groups injected with 20 μg *Pf-Dmrt4* dsRNA were suppressed to approximately 70% and 50% of those in the PBS- and GFP-dsRNA-injected groups, respectively.

Male gonads of pearl oysters in each injection group were observed under an optical microscope. The gonad structure was normal (mature stage) in the PBS-injected groups (Fig.6a), while *Pf-Dmrt4*-dsRNA-injected groups showed spawning-stage male gonads (Fig.6b), with ruptured follicles and released spermatozoa.

4 DISCUSSION

4.1 Structure and molecular characteristics of *Pf-Dmrt4*

The *Dmrt*-family gene *Pf-Dmrt4* from the gonads of *P. fucata* was cloned. The deduced aa sequence of Pf-Dmrt4 is serine- and proline-rich, includes a DM domain characteristic of the DMRT protein family, a conserved DMA domain near the C-terminus, and a short seven-aa motif (KSAFSPI). The DM domain contains the putative NLS (KGHKR) located in the zinc module, consisting of intertwined CCHC and HCCC Zn^{2+}-binding sites. The NLS is characterized as a transcription regulator of the DMRT family and plays a part in DNA-binding and nuclear import (Zhang et al., 2001; Ying et al., 2007). The DMA domain is conserved in the branch of DMRT3, DMRT4 and DMRT5 (Guo et al., 2004). Wen et al. (2009) assumed that the DMA domain may act as a motif to bind other transcriptional co-factors and can probably be essential for the role of DMRT4 to regulate other genes in transcriptional level.

Yu et al. (2009) previously cloned PinMarDmrt5 from *P. martensii*, which is largely identical to *Pf-Dmrt4*. However, the deduced PinMarDmrt5 product of 206 aa lacks the DMA domain and the short conserved motif. Sequence alignment of the two genes revealed that PinMarDmrt5 lacked four nucleotides causing a frame shift. The subsequent nucleotide sequence resulted in a stop codon, thus explaining the short product with no DMA domain or conserved motif. *P. fucata* DMRT4 displayed higher homologies with human DMRT4 and DMRT5 than with human DMRT1–3.

The availability of the *P. fucata* genome (Takeuchi et al., 2012; Du et al., 2017) provide an opportunity for a comprehensive study of sex-determining pathways in this species. In the genome of the *P. fucata*, a dmrtA2-like gene was investigated. While pacific oyster genome encodes three DM domain containing genes: *Cg19568*, *Cg01830*, and *Cg15952* (named *CgDMl*) (Zhang et al., 2014). They found that high expression in testis supports a possible role for *Cg19568* (named *CgDsx*) in determining or promoting male-specific development. It contains a DM domain showing closest homology (45%, E-value=9e-13) to *Dsx* isoform A found in *D. melanogaster*. *Pmarg-dmrt2* sequence was seen to share the highest amino acid identity with *P. martensii dmrt2* and zebrafish *Dmrt2* (100% and 95%, respectively), and *pmarg-*

dmrt with *P. martensii dmrt2* and mouse *Dmrt4* (59% and 58%, respectively). The other two DM domain genes both show the highest homology to DmrtA2 from many species. Phylogenetic analysis showed that *Pf-Dmrt4* was grouped with DMRT4 clusters and more closely with human DMRT4. *Pf-Dmrt4 is* a DM domain gene and a close relative of CgDMl. Volff et al. (2003) proposed a simpler nomenclature (*Dmrt1–8*) based on human genes, with no suggestions of structural or phylogenetic relationships between different genes. We therefore named it as *Pf-Dmrt4*.

4.2 Potential function of *Pf-Dmrt4*

It was reported that ancestral DM proteins possessed a DMA domain (Miller et al., 2003; Volff et al., 2003), and the presence of a DMA domain in *Pf*-DMRT4 reinforces the hypothesis. *Pf*-DMRT4 as an ancestral DM factor, could be engaged in a number of biological processes, including functions associated with the ancestral structure, whereas evolution has led to diverse DM factors.

Pf-Dmrt4 was expressed in all the tested adult tissues, which is similar to *DMl* in *C. gigas* (Naimi et al., 2009), *Cf-dmrt4-like* in *Ch. farreri* (Feng et al., 2010), and related genes in other species with different expression patterns (Kim et al., 2003; Guo et al., 2004). Differences in DMRT4 expression among species might reflect variations in gene function. In *Takifugu rubripes*, *Dmrt4* is expressed in the spleen, suggesting an involvement in the immune system (Yamaguchi et al., 2006). Additionally, *Pf-Dmrt4* mRNA expression levels were highest in male gills, as for *dmrt5* in *Ch. nobilis* (Shi et al., 2014). While, *dmrt5* was expressed highest in gills but no sexual difference has been detected in *Ch. farreri* (Feng et al., 2010) and in *C. gigas* (Naimi et al., 2009). These findings suggested that *Pf-Dmrt4* is involved in the respiratory system.

Pf-Dmrt4 mRNA was also expressed widely in embryonic and larval stages. Its detection at an early stage resembled the situations in *Ch. farreri* (Feng et al., 2010), *Ch. nobilis* (Shi et al., 2014), and some other vertebrates such as medaka (Winkler et al., 2004). *Cf-dmrt4-like* expression was ubiquitous during embryonic development and peaked at the 8–16-cell stage (Feng et al., 2010), while *Ch. nobilis dmrt5* expression peaked at the blastula stage (Shi et al., 2014). *Pf-Dmrt4* mRNA expression peaked at the D-shaped larva stage, and this stage is a crucial period to build the larva structure, indicating that *Pf-Dmrt4* may play a role in early embryonic development, especially in ontogenesis of larva.

Pf-Dmrt4 may also be involved in gonadal development in the pearl oyster. It was expressed in both sexes, but at higher levels in the testis than in the ovary, as reported for *Dmrt4* in *Ch. farreri* (Feng et al., 2010), *DMl* in *C. gigas* (Naimi et al., 2009), medaka (Winkler et al., 2004), and the Japanese pufferfish (Yamaguchi et al., 2006). *Pf-Dmrt4* expression levels increased significantly during the mature stage in the testis, but there were no significant differences in mRNA expression in the female gonad, consistent with an increase in *Cg-DMl* in males at the mature stage (Naimi et al., 2009). The number of spermatogonia increase in the mature stage (stage III), as a result not only of their proliferation but also of an increase in gonad volume from 5%–40% of the visceral mass in less-mature animals to 60% at this stage (Fabioux et al., 2004). This indicates that *Pf-Dmrt4* may be involved in regulating male gonadal development in *P. fucata*.

In situ hybridization of the testis revealed that *Pf-Dmrt4* was expressed in the cytoplasm of spermatozoa and spermatids, though it was impossible to differentiate between germ cell and somatic cell limits at this microscopic level. In *C. gigas*, *Cg-DMl* was observed in the cytoplasm of spermatogonia and/or the surrounding somatic cells (Naimi et al., 2009), in agreement with the expression of *Dmrt5* in testicular germ cells in zebrafish (Guo et al., 2004). Different expression patterns exist in different species (Raymond et al., 2000; Winkler et al., 2004). Variations in *Pf-Dmrt4* localization suggest that it may play divergent roles in the gonad, including involvement in the proliferation of spermatozoa. Knockdown of *Pf-Dmrt4* by RNA interference caused morphological changes in the male gonad, indicating that *Pf-Dmrt4* might be involved in maintaining the structure of the male gonad in *P. fucata*.

Sex steroids play an important role in bivalve reproduction and can promote gamete development. Estrogen injection can affect gametogenesis and gametogenesis-related metabolic pathways in invertebrates (Wang and Croll, 2003; Croll and Wang, 2007). MT significantly upregulated *Pf-Dmrt4* expression levels in mature-stage male gonads. Though the effects of sex steroid hormones on *Dmrt4* or *Dmrt5* are largely unknown, Shi et al. (2014) showed that *Dmrt5* mRNA in *Ch. nobilis* male gonads was significantly increased by injection with three different concentrations of MT. Similar findings were observed for *Oreochromis aureus Dmrt4*, implying

that *Dmrt4* might be a direct downstream gene under the control of steroids (Cao et al., 2010), and that *Pf-Dmrt4* might play a similar role.

5 CONCLUSION

Pf-Dmrt4 is involved in the sex-differentiation pathway in the pearl oyster *P. fucata*. *Pf-Dmrt4* is involved in a wide range of biological processes, including cell proliferation and/or regulating male gonadal development and maintaining the structure of the mature testis in *P. fucata*. More studies are needed to clarify the precise physiological functions of *Pf-Dmrt4*.

6 DATA AVALABILITY STATEMENT

Sequence data that support the findings of this study have been deposited in National Center for Biotechnology Information (GenBank) with the primary accession code KM272582.

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Supplementary Material 1 GenBank accession numbers of the reference sequences used in the Fig.2

Species name	Protein name	Accession number or reference
Chlamys farreri	ChlFar-DMRT4-like	ADK55063.1
Chlamys nobilis	ChlNob-DMRT5-like	AHW85420.1
Chlamys nobilis	ChlNob-DMRT2	AHW85419.1
Crassostrea gigas	CraGig-DMl	ABS88697.1
Danio rerio	DarRer-DMRT1	AAU04562.1
Danio rerio	DarRer-DMRT2	NP_571027.1
Danio rerio	DarRer-DMRT3	AAU89440.1
Danio rerio	DarRer-DMRT5	AAU85258.1
Homo sapiens	HomSap-DMRT1	AAD40474.1
Homo sapiens	HomSap-DMRT2	AAD40475.1
Homo sapiens	HomSap-DMRT3	NP_067063.1
Homo sapiens	HomSap-DMRT4	AAI30436.1
Homo sapiens	HomSap-DMRT5	Q96SC8.2

To be continued

Supplementary Material 1 Continued

Species name	Protein name	Accession number or reference
Mus musculus	MusMus-DMRT1	NP_056641.2
Mus musculus	MusMus-DMRT2	NP_665830.1
Mus musculus	MusMus-DMRT3	NP_796334.2
Mus musculus	MusMus-DMRT4	AAN77234.1
Mus musculus	MusMus-DMRT5	AAN10254.1
Oncorhynchus mykiss	OncMyk-DMRT1	NP_001117741.1
Oryzias latipes	OryLat-DMRT4	BAB63259.1
Oryzias latipes	OryLat-DMRT5	BAD00703.1
Pinctada martensii	PinMar-DMRT2	ADD97887.1
Pinctada martensii	PinMar-DMRT5	FG396011.1
Takifugu rubripes	TakRub-DMRT1	NP_001033038.1
Takifugu rubripes	TakRub-DMRT3	BAE16954.1
Takifugu rubripes	TakRub-DMRT4	NP_001033037.1
Takifugu rubripes	TakRub-DMRT5	BAE16956.1
Xenopus laevis	XenLae-DMRT4	AAV66322.1
Xenopus laevis	XenLae-DMRT5	AAI70170.1

Supplementary Material 2 Abbreviations of the species used in Fig.3

Abbreviation	Species	Abbreviation	Species	Abbreviation	Species
AllSin	*Alligator sinensis*	CriGri	*Cricetulus griseus*	OryLat	*Oryzias latipes*
AzuFar	*Azumapecten farreri*	DanRer	*Danio rerio*	PanPan	*Pan paniscus*
CaeEle	*Caenorhabditis elegans*	DroMel	*Drosophila melanogaster*	PanTro	*Pan troglodytes*
CalMil	*Callorhinchus milii*;	EquCab	*Equus caballus*	PelSin	*Pelodiscus sinensis*
ChaMyd	*Chelonia mydas*	GalGal	*Gallus gallus*	PhyCat	*Physeter catodon*
CheMyd	*Chelonia mydas*	HomSap	*Homo sapiens*	PinMar	*Pinctada martensii*
ChlFar	*Chlamys farreri*	IctTri	*Ictidomys tridecemlineatus*	PinMarg	*Pinctada margaritifera*
ChlNob	*Chlamys nobilis*	MusMus	*Mus musculus*	TaeGut	*Taeniopygia guttata*
ChrBel	*Chrysemys picta bellii*	OdoDiv	*Odobenus rosmarus divergens*	TakRub	*Takifugu rubripes*
ClaGar	*Clarias gariepinus*	OncMyk	*Oncorhynchus mykiss*	XenLae	*Xenopus laevis*
CraGig	*Crassostrea gigas*	OryCun	*Oryctolagus cuniculus*	XenTro	*Xenopus tropicalis*