

Expression pattern of *dmrt4* from olive flounder (*Paralichthys olivaceus*) in adult gonads and during embryogenesis

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Abstract The *dmrt* (*doublesex* and *mab-3* related transcription factor) gene family comprises several transcription factors that share a conserved DM domain. *Dmrt1* is considered to be involved in sexual development, but the precise function of other family members is unclear. In this study, we isolated genomic DNA and cDNA sequences of *dmrt4*, a member of the *dmrt* gene family, from olive flounder, *Paralichthys olivaceus*, through genome walking and real-time reverse transcriptase (RT)-PCR. Sequence analysis indicated that its genomic DNA contains two exons and one intron. A transcriptional factor binding sites prediction program identified a sexual development-related protein, Sox9 (Sry-like HMG box containing 9) in its 5' promoter. Protein alignment and phylogenetic analysis suggested that flounder *Dmrt4* is closely related to tilapia *Dmo* (DM domain gene in ovary). The expression of *dmrt4* in adult

flounder was sexually dimorphic, as shown by real-time RT-PCR analysis, with strong expression in the testis but very weak expression in the ovary. Its expression was also strong in the brain and gill, but there was only weak or no expression at all in some of the other tissues tested of both sexes. During embryogenesis, its expression was detected in most developmental stages, although the level of expression was distinctive of the various stages. Whole mount in situ hybridization revealed that the *dmrt4* was expressed in the otic placodes, forebrain, telencephalon and olfactory placodes of embryos at different developmental stages. These results will improve our understanding of the possible role of flounder *dmrt4* in the development of the gonads, nervous system and sense organs.

Keywords *Paralichthys olivaceus* · *dmrt4* · Real-time reverse transcriptase-PCR · Whole mount in situ hybridization · Sexually dimorphic expression

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Introduction

Many basic cellular processes are shared across vast phylogenetic distances, whereas sexual development mechanisms are highly variable among phyla although the existence of two sexes is nearly universal in the animal kingdom. The only molecular similarity in sexual development found to date between phyla is the

dmrt (*Drosophila doublesex* and *Caenorhabditis elegans mab-3* related transcription factor) gene family, which contains a DM domain, a zinc-finger-like DNA-binding motif. Several members of this gene family have been cloned from both invertebrates and vertebrates, such as *Drosophila melanogaster*, *Caenorhabditis elegans*, fish, reptiles, birds and mammals, and found to be expressed in developing gonads as well as the adult ovary and/or the testis (Brunner et al. 2001; Cao et al. 2007; Guan et al. 2000; Kettlewell et al. 2000; Kim et al. 2003; Kondo et al. 2002; Ottolenghi et al. 2000a, b; Raymond et al. 1999; Smith et al. 1999; Veith et al. 2006; Winkler et al. 2004; Yamaguchi et al. 2006). There is accumulating evidence from different phyla that more than one *dmrt* gene is involved in sexual development. For example, the expression pattern of *dmrt1* is consistent with a role in testis differentiation (De Grandi et al. 2000; Guo et al. 2005; Lei et al. 2007; Marchand et al. 2000; Matsuda et al. 2003; Moniot et al. 2000; Nanda et al. 2002; Pask et al. 2003; Raymond et al. 1999; Shan et al. 2000; Shibata et al. 2002; Veith et al. 2003), while *dmrt3*, *dmrt5* and *dmrt7* exhibit sexually dimorphic expression in early embryonic gonads (Kim et al. 2003; Smith et al. 2002). At the present time, members of *dmrt* gene family, such as *dmrt1*, *dmrt2*, *dmrt3* and *dmrt5*, have been cloned in a number of fishes, including zebrafish, medaka, tilapia, fugu, platyfish, groupers and rainbow trout (Cao et al. 2007; Guan et al. 2000; Guo et al. 2005; Kondo et al. 2002; Marchand et al. 2000; Veith et al. 2006; Winkler et al. 2004; Xia et al. 2007; Yamaguchi et al. 2006).

One member of the *dmrt* gene family in particular, *dmrt4* (or tilapia *dmo*, DM domain gene in ovary), has different expression patterns in different species, and this variation in expression may be associated with gonad development or other development processes. *Xenopus dmrt4* (*Xdmrt4*) is initially expressed in the anterior neural ridge and then becomes progressively restricted to part of the telencephalon and the olfactory placode. Interference with *Xdmrt4* function results in the block of the Noggin-mediated induction of *neurogenin*, *Xebf2* and *NCAM* (neural cell adhesion molecule), and *Xdmrt4*-deficient embryos show impaired neurogenesis in the olfactory epithelium later in embryogenesis. *Xdmrt4* is an important regulator of neurogenesis in the olfactory

system upstream of Neurogenin and Xebf2 and is required for Noggin-mediated neutralization (Huang et al. 2005). Tilapia *dmo* (corresponding to *dmrt4* in other species) is highly expressed in the ovary but not expressed at all in the testis, suggesting that it might have important roles in gonadal development (Cao et al. 2007; Guan et al. 2000). Medaka *dmrt4* is expressed in the olfactory placodes, forebrain, telencephalon and nasal pits of embryos as well as in the gonads and some other tissues of adult fish from both sexes (Kondo et al. 2002; Winkler et al. 2004). *Dmrt4s* from fugu and mouse are expressed in the gonads of both sexes at similar levels and also in some other tissues (Kim et al. 2003; Yamaguchi et al. 2006). It is now generally accepted that members of the *dmrt* gene family, including *dmrt4* (or *dmo*), are structurally conserved and may play a (number of different) role(s) in sexual development, although their precise function is unclear. However, the expression patterns of *dmrt4s* have only been studied in freshwater fishes, including tilapia, medaka and fugu. Consequently, more information from maricultural fishes is required.

Olive flounder, *Paralichthys olivaceus*, is one of the most important maricultural fish in China. Since the female flounder grows faster than its male counterpart, producing all-female flounder may be an efficient approach to increasing the production of this cultured fish (Yamamoto 1999). The sexual development of fishes is influenced by various factors associated with their complex habitat and relative low position in the phylogenetic ranking of vertebrates. As there is little information on sex-linked markers and sex-related gene in flounder, it is necessary to study the molecular mechanisms of sexual development. A number of related studies have focused on cytochrome P450 aromatase (Cyp19) (Kitano et al. 1999, 2000), Mullerian inhibiting substance (Mis, or anti-Mullerian hormone, Amh) (Yoshinaga et al. 2004) and FoxL2 (fork head box L2) (Yamaguchi et al. 2007). However, there has been no report on any of the members of the *dmrt* gene family, including the *dmrt4* gene in flounder to date. In this paper, we isolated and characterized flounder *dmrt4* and analyzed its expression pattern. Our data may contribute to future studies on the roles of the flounder *dmrt* gene family in sexual development.

Materials and methods

Fish and embryo culture

Olive flounder brood stock was cultured at the Institute of Oceanology, Chinese Academy of Sciences, Qingdao city, China under controlled conditions [photoperiod: 14/10 h (light/dark); temperature $15 \pm 1^\circ\text{C}$; aerated seawater] and fed with commercial particle food twice daily. The fertilized eggs were obtained by mixing sperm and eggs collected from matured males and females by gentle stripping. The embryos and fries were cultured at $15 \pm 1^\circ\text{C}$ in 1-m^3 tanks under the same conditions as for the brood stock. Live adult flounders were bought from the Nanshan fish market in Qingdao.

Cloning of flounder *dmrt4* gene

The flounder *dmrt4* genomic sequence was obtained by overlapping DNA fragments cloned from flounder genome walking libraries by a PCR-based Genome-Walker method (Clontech, Palo Alto, CA). Briefly, flounder genomic DNA was completely digested with restriction enzymes *DraI*, *EcoRV*, *HpaI*, *PvuII*, *ScaI*, *SmaI* and *StuI* to yield blunt-ended DNA fragments. The digested DNA was then ligated with a DNA adaptor (Clontech). The resulting DNA fragments were used as templates for PCR amplification with two adaptor-specific primers together with two DM domain consensus primers or *dmrt4*-specific primers.

Specifically, the first part of the flounder *dmrt4* was cloned by one round of Nest-PCR with DM domain consensus primers (Dmrtpr5' or Dmrtpr3') and the adapter primers (AP1 or AP2) (Table 1). The remaining part of the *dmrt4* genomic sequences were cloned by another round of Nest-PCR using *dmrt4*-specific primers (Admrtpr5'/3' or Sdmrtpr5'/3') together with the adapter primers (Table 1). The PCR conditions for genome walking were: (1) first round of Nest-PCR: seven cycles of 94°C for 25 s, 72°C for 3 min; 32 cycles of 94°C for 25 s, 67°C for 3 min; 67°C for 7 min; (2) second round of Nest-PCR: five cycles of 94°C for 25 s, 72°C for 3 min; 20 cycles of 94°C for 25 s, 67°C for 3 min; 67°C for 7 min. All of the fragments were cloned into pUCm-T vector (Sangon, Shanghai) and sequenced, respectively. Analysis of full-length *dmrt4* genomic sequence was carried out using GENESCAN (www.genes.mit.edu) and DNASTAR (Madison, WI) software.

Total RNA isolation

Total RNA was extracted from the testis, ovary, kidney, brain, spleen, heart, gill, eye, stomach and intestinal tissues of adult flounder (total length 30 ± 2.0 cm) and flounder embryos of different developmental stages using Trizol (Invitrogen, Carlsbad, CA). The quantity and purity of the RNAs were checked by electrophoresis on a 1% agarose gel with ethidium bromide staining.

Table 1 Sequences of genome walking, RT-PCR and real-time RT-PCR primers

Primer name	Primer sequence (5'–3')
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
Dmrtpr5'	CAG(A/C)G(A/G)GTCATGGC(G/C)GC(G/T)CAGGT(G/T/C)GC(C/T)
Dmrtpr3'	(A/C)G(A/G)(A/C)G(A/G)CAGCAGGC(C/T)CA(A/G)GA(A/G)GAGGA(A/G)
Admrtpr'5'	TCCAAAAACATCAAAACTGGGAGCAGCTGG
Admrtpr'3'	TGGAGTGTGCTGGTTGTCGAGGCA
Sdmrtpr'5'	GTCTATGGTGAGAACGTGCAAAGGTG
Sdmrtpr'3'	ACATTGTCAAATCCATCGAGCTGGTG
Dm4ReF	TCCATCGAGCTGGTGTGAG
Dm4ReR	TGAGCGAGTTGGGTGATTAGA
Dm4cd5'	CTCAGGCAATGGAGAGCAGAATCAG
Dm4cd3'	TCATTTTCACTGTTCAGTCGTGCGTAC
ActReF	TGATGGTAGGAATGGGACAGAA
ActReR	GATGGGATACTTCAGGGTCAGG

RT, Reverse transcriptase;
F, forward primer; R,
reverse primer

All sequences were
presented in the 5'–3'
direction

cDNA cloning of flounder *dmrt4* gene

First-strand cDNA synthesis was carried out according to M-MLV RT Usage information (Promega, Madison, WI) using the DNase-treated (Promega) total RNA as template. The volume for each reaction was 25 μ l and contained 4 μ l isolated RNAs, 1 μ l AP-oligodT, 8 μ l RNase-free water, 1 μ l M-MLV RT, 1 μ l ribonuclease inhibitor (Promega), 5 μ l M-MLV buffer (10 mM Tris-HCl, 25 mM KCl, pH 8.3, 0.6 mM MgCl₂, 2 mM DTT) and 5 μ l dNTP. The reaction mixture was incubated at 70°C for 5 min and then at 42°C for 1 h.

Flounder *dmrt4* cDNA was cloned by RT-PCR with primers Dm4cd5' and Dm4cd3' to determine the intron–exon boundary. The fragments were cloned into the pBluescript II SK *Sma*I site (Stratagene, La Jolla, CA) and sequenced.

Transcriptional factor binding sites prediction

Transcriptional factor binding sites were predicted using MATCH PROGRAM (www.gene-regulation.com/pub/programs.html#match). The database is TRANSFAC 6.0 (Kel-Margoulis et al. 2002).

Protein alignment and phylogenetic analysis of flounder Dmrt4 protein

The sequence of the flounder Dmrt4 protein was obtained by translating *dmrt4* cDNA using a translate tool (www.expasy.ch). Protein alignment was done by CLUSTALW (www.ebi.ac.uk), and phylogenetic analysis was performed using CLUSTAL X and MEGA3 software with all reported Dmrt4 (or Dmo) sequences, including fugu Dmrt4 (GenBank accession no. NP_001033037.1), southern platyfish Dmrt4 (GenBank accession no. AAL83919.1), medaka Dmrt4 (GenBank accession no. BAB63259.1), Nile tilapia Dmo (GenBank accession no. AAF79932.2), blue tilapia Dmo (GenBank accession no. AAR34460.1), African clawed frog Dmrt4 (GenBank accession no. NP_001084923.1) and house mouse Dmrt4 (GenBank accession no. AAN77234.1). In the phylogenetic analysis, full-length protein sequences were used, no amino acid was deleted, gaps were not ignored and the distances were corrected using the Poisson method

for correcting distance. The phylogenetic tree was constructed using the neighbor-joining method, and the tree topology was evaluated by 1000 replications bootstraps.

Quantity analysis of flounder *dmrt4* mRNA expression by real-time RT-PCR

The expressions of *dmrt4* in different tissues of adult flounder and flounder embryos of different developmental stages were quantified by real-time RT-PCR. Total RNA isolation and first-strand cDNA synthesis were performed according to the methods mentioned above. Real-time RT-PCR was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems, Foster City, CA). The amplification was performed in a total volume of 20 μ l that contained 10 μ l of 2 \times SYBR Green Mix (Takara, Japan), 2 μ l diluted cDNA and 0.4 μ l of each primer (Dm4ReF and Dm4ReR) (Table 1). The real-time PCR program consisted of one cycle of 95°C for 6 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. The dissociation analysis of amplified products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. Following completion of the PCR program, data were analyzed with ABI 7300 SDS software (Applied Biosystems). To maintain consistency, the software automatically set the baseline. The comparative CT method was used to analyze the expression level of flounder *dmrt4*. The CTs for the amplified target *dmrt4* and the CTs for the internal control β -actin (primers ActReF and ActReF) were determined for each sample. Differences in the CT for the target and the internal control, denoted the Δ CT, were calculated to normalize the differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT-PCR. The group with the highest Δ CT value was used as the reference sample, called the calibrator. The Δ CT for each sample was subtracted from the Δ CT of the calibrator, and the difference was called $\Delta\Delta$ CT. The expression level of *dmrt4* could be calculated by $2^{-\Delta\Delta$ CT}, and the value stands for an n -fold difference relative to the calibrator. All data are given in terms of relative mRNA expression as the mean \pm standard error (SE). The results were subjected to t -test analysis,

and the *P* values of less than 0.05 or 0.01 were considered statistically significant.

Whole mount in situ hybridization

The plasmid clone containing the whole cDNA sequence of flounder *dmrt4* was used as a template to generate the antisense digoxigenin-labeled RNA probes using T3 RNA polymerase. RNA probes were made by in vitro transcription in the presence of digoxigenin-11-UTP* (Roche Applied Science, Germany). Hatching embryos were anesthetized with 0.2% MS222 (3-aminobenzoic acid ethyl ester) before fixation. Embryos were fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) and then stored in 100% methanol at -20°C. Embryos were dechorionated with fine forceps. Hatching stage embryos were treated with 10 µg/ml proteinase K for 10 min in PBS at room temperature. The embryos were then refixed with 4% paraformaldehyde for 30 min at room temperature. In situ hybridization was carried out as described by Du and Dienhart (2001). Embryos were hybridized with the *dmrt4* antisense RNA probes at a final concentration of 0.5–1 ng/µl. Hybridization was performed at 70°C overnight, and the signals were detected using a 2000-fold dilution of anti-DIG antibody (Roche Molecular Biochemicals, Indianapolis, IN) coupled to alkaline phosphatase; the color reaction was performed with fresh color reaction buffer containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP). Embryos in glycerol were photographed under the microscope (DM LB2, Leica) with a Nikon 4500 digital camera. Finally, the results of whole mount in situ hybridization were confirmed on a frozen cross section of these embryos along the anterior–posterior axis. Post-fixed embryos of the in situ hybridization procedure were washed with three changes of 1× PBS to remove the glycerol, transferred into 30% sucrose solution and incubated overnight at 4°C; they were then embedded in freezing agent (Microm Laborgerate GmbH, Walldorf, Germany) and sectioned into 5-µm-thick slices using a microtomy cryostat (Microm HM 505 E; Microm Laborgerate GmbH, Walldorf, Germany) at -20°C. The sections frozen in glycerol were also photographed under the microscope (DM LB2, Leica) with a Nikon 4500 digital camera.

Results

Isolation and characterization of the *dmrt4* gene from flounder

The flounder *dmrt4* gene was isolated by nest-PCR from flounder genome walking libraries as described in the Materials and Methods. A 889-bp fragment was amplified during the first-round nest-PCR using the DM domain consensus primers with the adaptor-specific primers. Sequence analysis revealed that this fragment is the homolog of *dmrt4*. The complete genomic sequence of flounder *dmrt4*, which was approximately 2.9 kb (Fig. 1a), was obtained following the second-round nest-PCR. Flounder *dmrt4* cDNA was obtained by RT-PCR, and its open reading frame (ORF) was found to span 1251 bp. Two exons and one intron of *dmrt4* were identified by comparing genomic sequences with the isolated cDNA sequences. Sequence analysis revealed conserved the consensus sequence GT ... AG at the exon–intron boundary (GenBank accession no., EF552054) (Fig. 1b). This structure is shared by all vertebrate *dmrt4*.

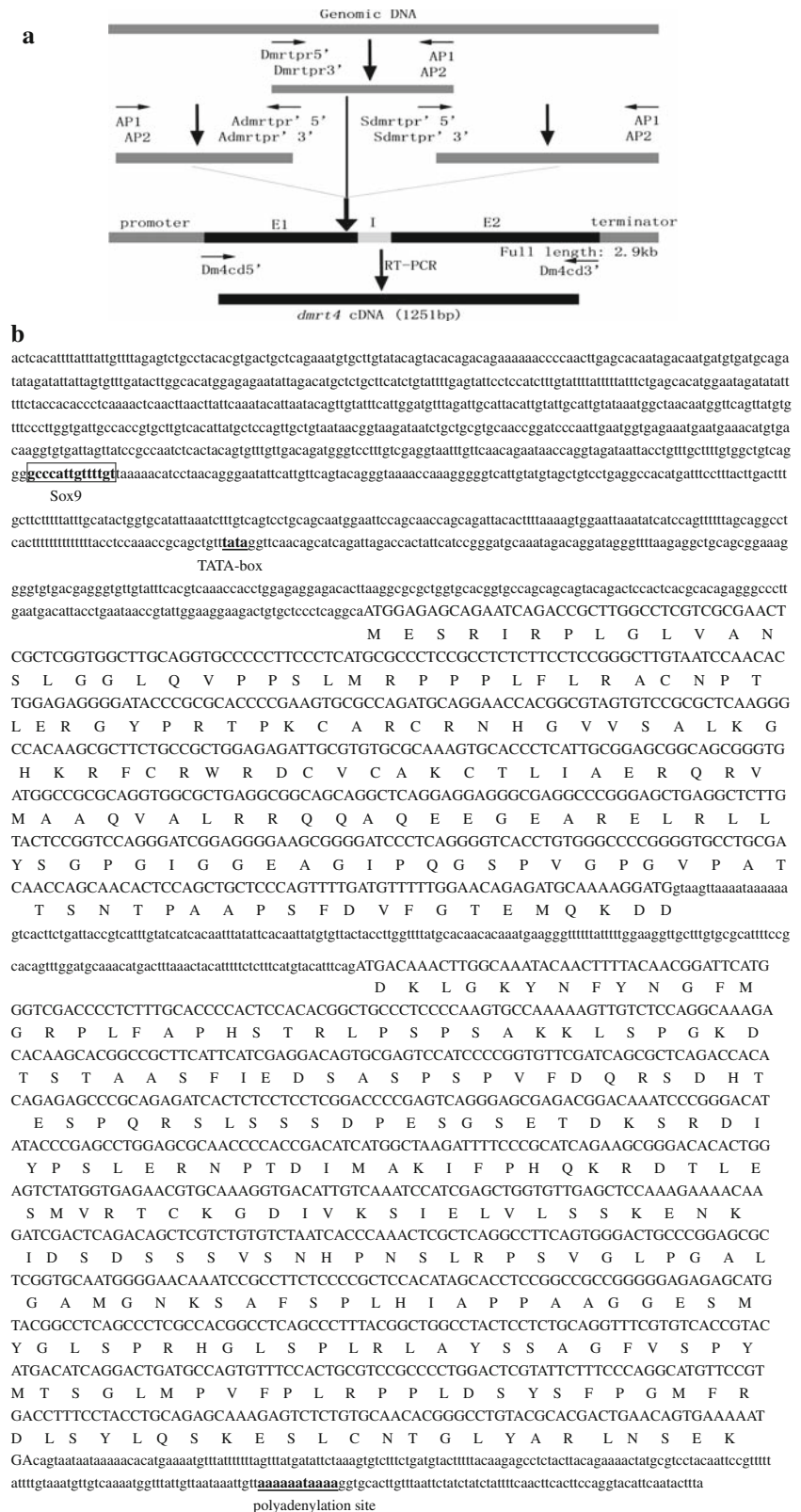
Prediction of the transcriptional factor binding sites identified a Sox9 (Sry-like HMG box containing 9) binding site (gccATTGTttgt; core match is 1.0, matrix match is 0.995) in the 5' promoter region of flounder *dmrt4*. A TATA-box (TATA) and polyadenylation site (AATAAA) were also found in the 5' promoter region and 3' un-translated region of flounder *dmrt4*, respectively (Fig. 1a).

Alignment and phylogenetic analysis of flounder Dmrt4 protein

Flounder *dmrt4* encodes a protein of 416 amino acids, including a highly conserved DM domain located at amino acids 44–102 close to the N-terminal and a DMA domain near the C-terminal region. The DM domain proteins with DMA domain are known as DmrtA proteins.

Protein alignments with all known fish Dmrt4 (or Dmo) revealed that Dmrt4 is highly conserved in fish (Fig. 2a). Flounder Dmrt4 shares 68, 69, 69, 73, 75% identity with fugu Dmrt4, southern platyfish Dmrt4, medaka Dmrt4, Nile tilapia Dmo and blue tilapia Dmo, respectively, but it shares relative low identity with African clawed frog Dmrt4 and house mouse

Fig. 1 Strategy for the cloning and analysis of the sequence of flounder *dmrt4*. **a** Strategy for flounder *dmrt4* genomic cloning and cDNA cloning [horizontal arrows indicate the locations of primers used in genome walking and reverse transcriptase (RT)-PCR]. **b** Genomic structure and deduced amino acid sequence of flounder *dmrt4*



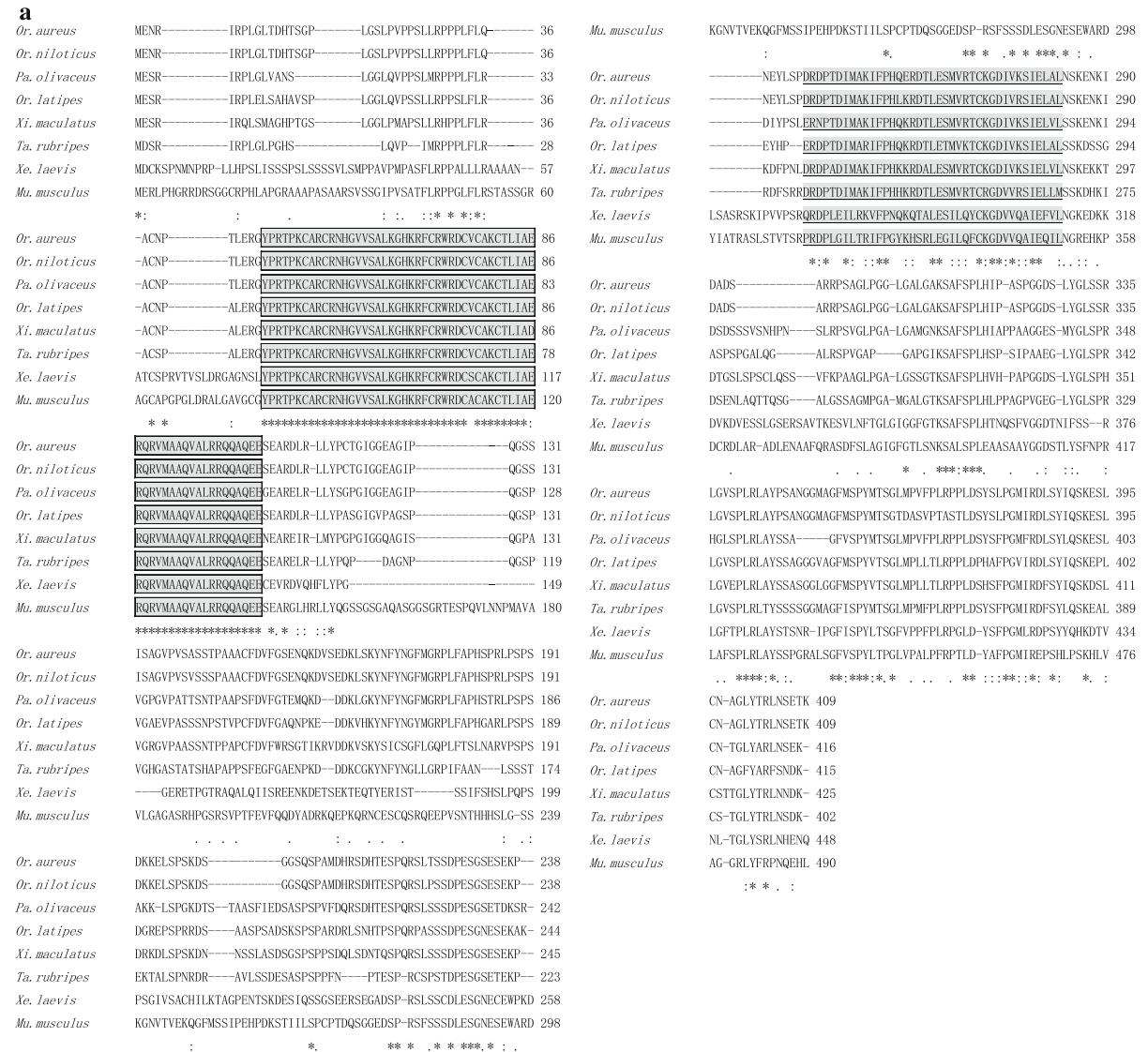


Fig. 2 Protein alignment and phylogenetic analysis of flounder Dmrt4 with Dmrt1-5 of other fish and vertebrates. **a** Comparison of the deduced amino acid sequence of flounder Dmrt4 with those of other vertebrates. The deduced protein sequences were used in the analysis using the CLUSTAL W sequence alignment program. The highly conserved zinc-finger-like DNA-binding motif, the DM domain, is *framed and shaded*, and the DMA domain is *underlined and shaded*. The asterisk indicates identical amino acids. **b** Phylogenetic analysis of flounder Dmrt4 relative to the Dmrt1-5 of other fish species and vertebrates. The arrow denotes flounder Dmrt4. *dm1* (*dmy*), Dmrt1 (*Dmy*) sequence, *dm2*, Dmrt2 sequence, *dm3*, Dmrt3 sequence, *dm4* (*dmo*), Dmrt4 (*Dmo*)

Dmrt4 (Table 2, Fig. 2a). The DM domain of flounder Dmrt4 exhibits more than 90% identity with those of other species. In addition, the DMA domain also

sequence, *dm5*, Dmrt5 sequence. The tree topology was evaluated by 1000 replications bootstraps, and numbers on each branch of the tree represent bootstrap support value. The GenBank accession numbers for these vertebrate Dmrt4 (or *Dmo*) proteins are: *Or. aureus*, *Oreochromis aureus* (blue tilapia *Dmo*, AAR34460.1); *Xi. maculatus*, *Xiphophorus maculatus* (southern platyfish Dmrt4, AAL83919.1); *Ta. rubripes*, *Takifugu rubripes* (*fugu* Dmrt4, NP_001033037.1); *Or. latipes*, *Oryzias latipes* (medaka Dmrt4, BAB63259.1); *Or. niloticus*, *Oreochromis niloticus* (Nile tilapia *Dmo*, AAF79932.2); *Xe. laevis*, *Xenopus laevis* (African clawed frog Dmrt4, NP_001084923.1); *Mu. musculus*, *Mus musculus* (house mouse Dmrt4, AAN77234.1)

exists in the Dmrt4 of other fishes and vertebrates (Fig. 2a). Phylogenetic analysis of flounder Dmrt4 protein with Dmrt1-5 proteins in other fish species

Fig. 2 continued

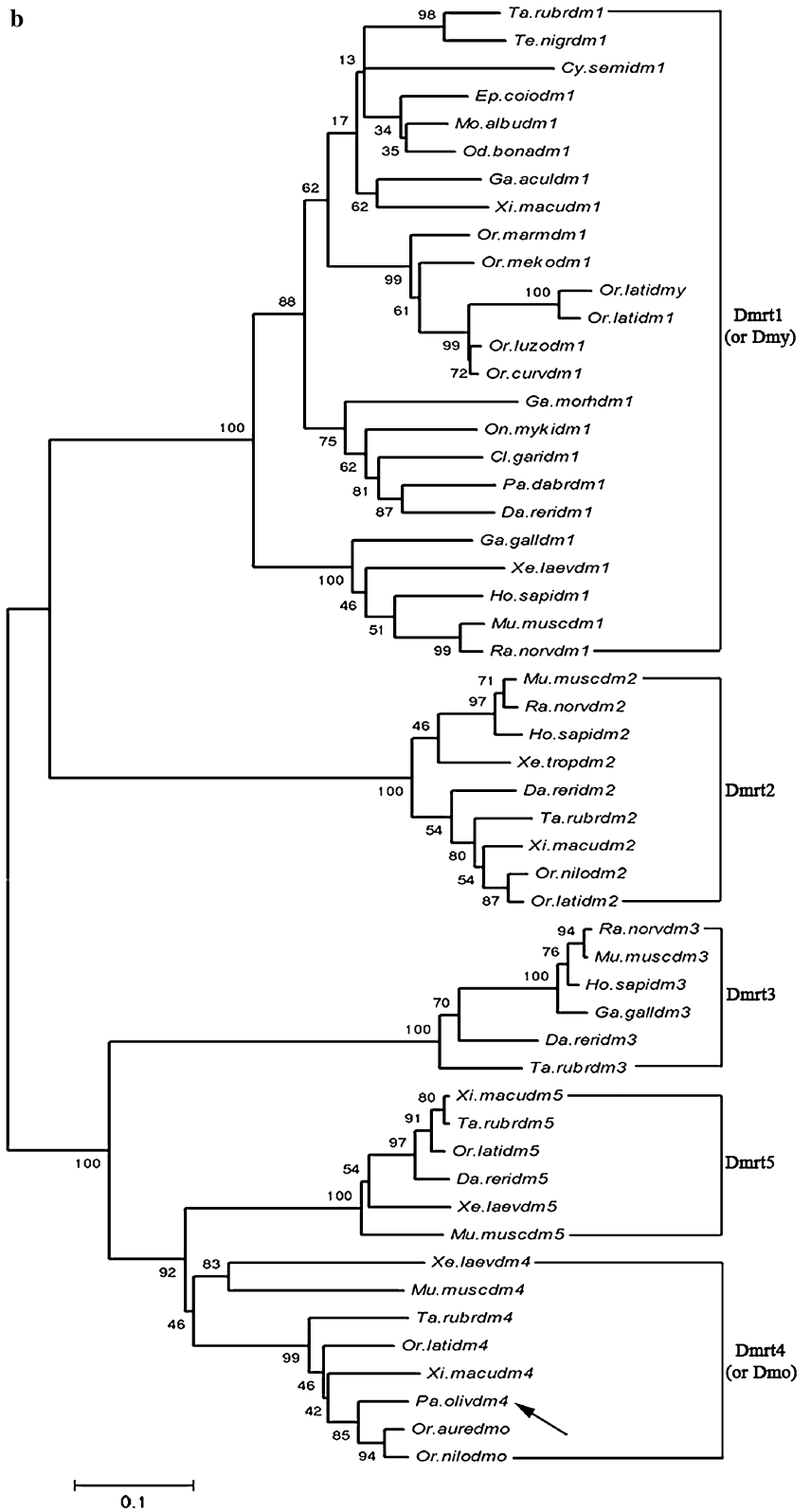


Table 2 Amino acid sequence identities of flounder Dmrt4 with various species

Source species ^a	<i>Pa.ol</i>	<i>Or.au</i>	<i>Or.ni</i>	<i>Ta.ru</i>	<i>Or.la</i>	<i>Xi.ma</i>	<i>Xe.la</i>	<i>Mu.mu</i>
<i>Pa.ol</i>	100							
<i>Or.au</i>	75	100						
<i>Or.ni</i>	73	96	100					
<i>Ta.rus</i>	68	65	64	100				
<i>Or.la</i>	69	70	68	64	100			
<i>Xi.ma</i>	69	68	66	61	66	100		
<i>Xe.la</i>	46	46	45	46	41	46	100	
<i>Mu.mu</i>	43	43	43	40	44	43	46	100

^a *Pa.ol*, *Paralichthys olivaceus* (flounder Dmrt4); *Or.au*, *Oreochromis aureus* (blue tilapia Dmo, GenBank accession no. AAR34460.1); *Xi.ma*, *Xiphophorus maculatus* (southern platyfish Dmrt4, GenBank accession no. AAL83919.1); *Ta.ru*, *Takifugu rubripes* (fugu Dmrt4, GenBank accession no. NP_001033037.1); *Or.la*, *Oryzias latipes* (medaka Dmrt4, GenBank accession no. BAB63259.1); *Or.ni*, *Oreochromis niloticus* (Nile tilapia Dmo, GenBank accession no. AAF79932.2); *Xe.la*, *Xenopus laevis* (African clawed frog Dmrt4, GenBank accession no. NP_001084923.1); *Mu.mu*, *Mus musculus* (house mouse Dmrt4, GenBank accession no. AAN77234.1)

and vertebrates confirmed that flounder Dmrt4 belongs to the Dmrt4 protein cluster and is closely related to Nile tilapia Dmo and blue tilapia Dmo (Fig. 2b). These data suggest that the flounder Dmrt4 may be the homolog of tilapia Dmo.

The distribution of the *dmrt4* gene in different tissues of adult flounder

Our analyses revealed that the relative mRNA abundance of *dmrt4* in different tissues of the adult flounder, as determined by real-time RT-PCR, is significantly higher in the testis, gill ($P < 0.05$) and brain ($P < 0.01$) than in the ovary and other tissues (Fig. 3a). In the adult flounder, *dmrt4* was strongly expressed in the testis and very weak expressed in the ovary. In addition, it was also strongly expressed in the brain and gill, but there was only weak or no expression at all in other tissues, including the kidney, spleen, heart, eye, stomach and intestine of both sexes.

The temporal and spatial expression analysis of *dmrt4* gene in flounder embryos

The relative mRNA abundance of *dmrt4* in flounder embryos at different developmental stages, as determined by real-time RT-PCR, was significantly higher at the 1–2 somites stage ($P < 0.05$), 3–4 somites stage ($P < 0.01$) and 5–6 somites stage ($P < 0.05$) (Fig. 3b). The level of *dmrt4* expression is relatively low at the gastrula stage, but it increases rapidly from

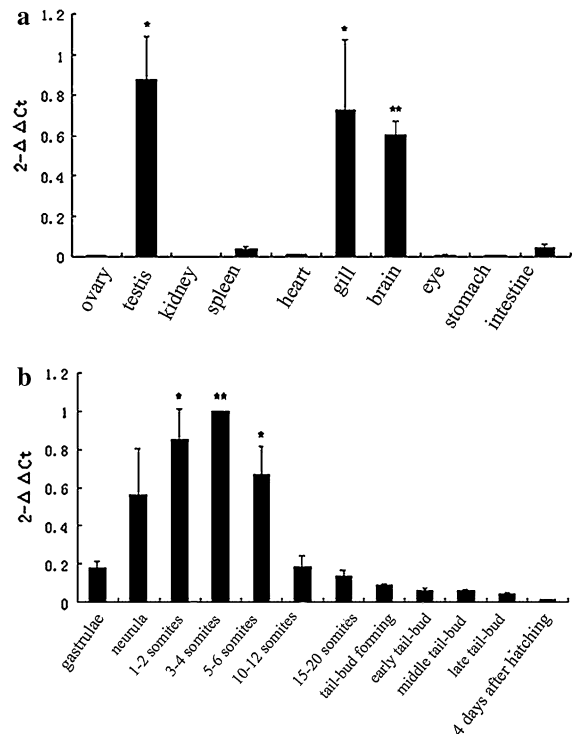
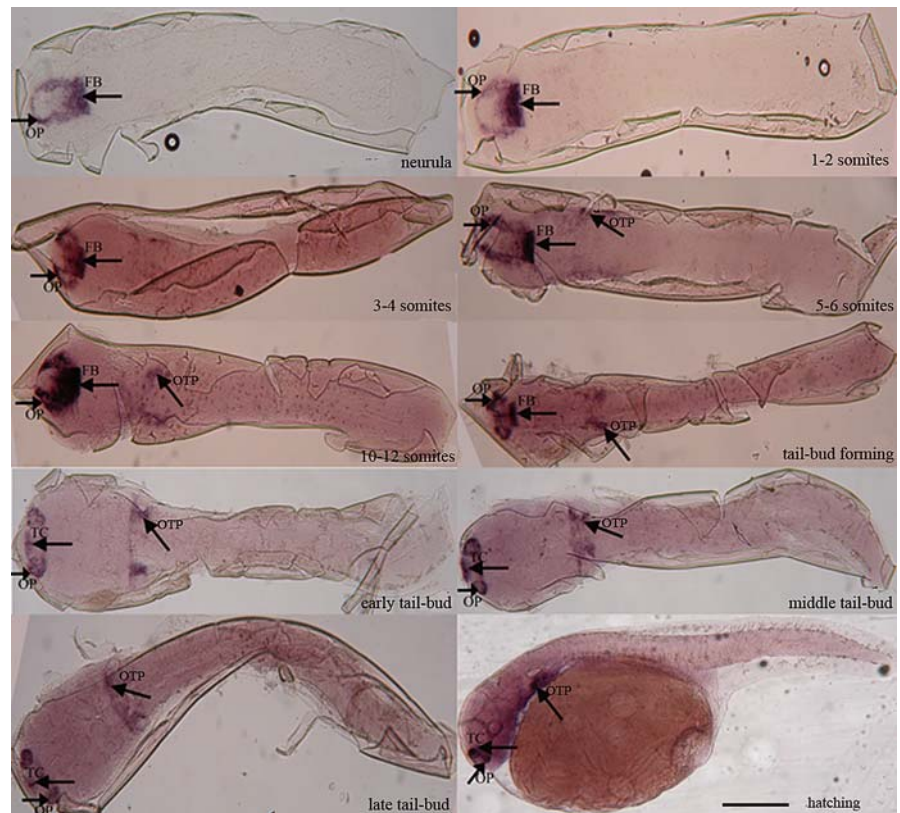


Fig. 3 Real-time RT-PCR analysis of flounder *dmrt4* expression. **a** Relative mRNA abundance of *dmrt4* in various tissues of adult flounder, **b** relative mRNA abundance of *dmrt4* in flounder embryos of various developmental stages. Filled box Relative expression quantity, vertical bars mean \pm standard error (SE) ($n = 3$). Significant differences across control are indicated: * $P < 0.05$, ** $P < 0.01$

this stage onwards, reaching peak expression at the 3–4 somites stage; thereafter, its expression decreases gradually until it reaches the lowest value.

Fig. 4 Spatial expression of *dmrt4* in flounder embryos of different developmental stages. Neurula to late tail-bud: anterior (left) and dorsal view; hatching: anterior (left) and lateral view. *Dmrt4* transcripts were detected in the otic placodes (OTP), telencephalon (TC), forebrain (FB) and olfactory placodes (OP). Scale bar: 0.6 mm



Embryos of ten different embryological stages were dechorionated and used in whole mount in situ hybridization to determine the spatial expression of *dmrt4* in flounder embryos of these different stages. The results shown in Fig. 4, which were confirmed by frozen sections (Fig. 5), show that flounder *dmrt4* was expressed in olfactory placodes from the neurula stage to the hatching stage (Fig. 5b). It was strongly expressed in the forebrain from the neurula stage to tail-bud forming (Fig. 5a), subsequently becoming restricted to—and relatively weakly expressed in—the telencephalon from the early tail-bud stage to hatching stage. From the 5–6 somites stage to the hatching stage, low-level transcription was also observed in the otic placodes (Fig. 5c). The designation of all of these embryological stages are in accordance to the designations of Tian et al. (2004).

Discussion

In our study, the full sequence of flounder *dmrt4* was cloned and characterized. It contains two exons and

one intron with the conserved consensus sequence GT...AG at the exon–intron boundaries. This structure is shared by all vertebrate *dmrt4*. According to the prediction of MATCH PROGRAM, the identified binding site of the sex-related protein Sox9 (Wagner et al. 1994) may act as a transcriptional factor binding site and play a role in the transcriptional regulation of flounder *dmrt4* expression, which itself may be related to gonad development. This gene encodes a protein which contains a DMA domain near the C-terminal region and a highly conserved DM domain adjacent to the N-terminal. The DM domain may act as the zinc-finger-like DNA-binding motif of transcriptional factor Dmrt4, and the DMA domain may function as the motif for the binding of other transcriptional co-factors and be indispensable to the role of Dmrt4 in the transcriptional regulation of other genes. Phylogenetic analysis confirmed that flounder Dmrt4 belongs to the Dmrt4 protein cluster and is most closely related to Nile tilapia Dmo and blue tilapia Dmo (shows 73 and 75% similarity, respectively) and that it may be a homolog (Cao et al. 2007; Guan et al. 2000).

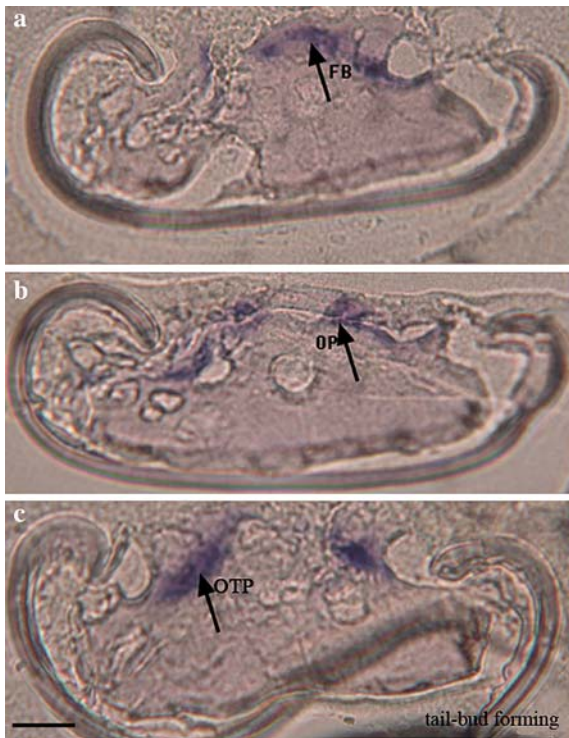


Fig. 5 The frozen cross section of a flounder tail-bud forming embryo after whole mount in situ hybridization along the anterior–posterior axis. Signals were detected in the forebrain (FB; a), olfactory placodes (OP, b) and otic placodes (OTP; c). Scale bar: 0.05 mm

Our analysis of *dmrt4* revealed that its expression is sexually dimorphic in the adult flounder. There is a significant difference in *dmrt4* expression in the male and female gonads, with expression much stronger in the testis and much weaker in the ovary. The expression pattern of tilapia *dmo* (corresponding to *dmrt4*) in the gonads is the opposite. In blue tilapia and Nile tilapia, *dmo* is highly expressed in the ovary, and there is no expression in the testis (Cao et al. 2007; Guan et al. 2000). However, *dmrt4* expression in the mouse and other fishes has been found to have somewhat different patterns compared with those of flounder and tilapia. Mouse *dmrt4* expression was detected by RT-PCR to be at similar levels in both XX and XY gonads from embryonic day 11.5 (E11.5) onwards (Kim et al. 2003). In medaka, *dmrt4* is located in a different chromosome, unlike the previously cloned *dmrt* genes, and its expression pattern is also different. Madaka *dmrt4* is expressed relatively strongly in the adult testis and the differentiating

gonads of larvae, while it is expressed very weakly in the adult ovary and is only visible in primary oocytes by in situ analysis (Kondo et al. 2002; Winkler et al. 2004). Fugu *dmrt4* is expressed in the gonads of both sexes (Yamaguchi et al. 2006). In addition, in the adult flounder, *dmrt4* is strongly expressed in the brain and gill, but it is only weakly or not expressed at all in the kidney, spleen, heart, eye, stomach and intestine of both sexes. The phenomena of *dmrt4* expression in other tissues have also been reported in mouse and other fishes even though their expression patterns are different. Mouse *dmrt4* expression is more widespread than those of other *dmrt* genes and has been detected from embryonic day 11.5 (E11.5) onwards by RT-PCR in the gonads as well as in many other tissues, including the kidney, heart, brain, stomach, intestine, bladder and lung (Kim et al. 2003). In blue tilapia, a slight expression of *dmo* in the brain of both sexes was also detected. Statistical analysis showed that in the brain, the mean *dmrt4* mRNA levels in the female were significantly higher than those in male, and real-time RT-PCR was unable to detect expression in the heart, liver, kidney, and muscle (Cao et al. 2007). In Nile tilapia, *dmo* was not detected in the kidney and brain (Guan et al. 2000). Medaka *dmrt4* is not considerably well expressed in the brain, but higher levels have been detected in the eyes, gills and kidney of both sexes (Kondo et al. 2002). In fugu, *dmrt4* is expressed in the spleens of both sexes, suggesting the involvement of this organ in the immune system (Yamaguchi et al. 2006). All these expression analyses imply that *dmrt4* expression can be detected not only in the male gonad in particular, but also in the brain and gill of adult flounder.

During flounder embryogenesis, *dmrt4* is expressed at most developmental stages, from the gastrula to larva after hatching, although the expression level at these different stages is distinctive. Based on our results of the real-time RT-PCR analysis, expression levels at the three earliest stages of embryogenesis are significantly higher than those at the later stages. From the results of whole mount in situ hybridization, confirmed by frozen sections, we determined that flounder *dmrt4* is expressed in the ectodermally derived olfactory placodes, neuroectodermal forebrain and telencephalon and otic placodes during embryogenesis. The expression level of *dmrt4* is always high in the olfactory placodes for all these

stages from neurula stage to the hatching stage, but it is dynamic in the telencephalon. While transcripts are distributed throughout the forebrain at early stages, remaining strongly expressed from the neurula stage to tail-bud forming stage, the expression domain becomes more and more restricted to a small area around the first ventricle in the dorsal telencephalon and is relatively weak at the early tail-bud stage. Low-level transcription was also observed in the otic placodes from the 5–6 somites stage to the hatching stage, but no hybridization signal was detected in primordial germ cells (PGCs). The analytic results of flounder *dmrt4* expression during embryogenesis obtained by real-time RT-PCR and whole mount in situ hybridization are in agreement. The temporal and spatial expressions of the *dmrt4* gene in flounder embryos are similar to those reported in medaka but different from those in mice. In medaka, there is a strong expression of *dmrt4* beginning at 1-day post-fertilization (1 dpf, gastrula) that decreases during the later stages of embryogenesis, i.e. during the early larval stages (from 3 dpf onwards); towards the end of embryogenesis, strong expression re-appears. The in situ hybridization result suggest that the medaka *dmrt4* expression signal was not detectable in PGCs, while it was detected in the olfactory placodes and telencephalon during the early stages of embryo and in the nasal pits during the late stages of embryo; low-level transcription was also found in the otic placodes (Winkler et al. 2004). In situ hybridization results confirmed that mouse *dmrt4* mRNA is detectable in the gonad of both sexes from E11.5. Expression, while widespread, is not ubiquitous, even within the urogenital tract and gonad. At E11.5, mouse *dmrt4* is expressed in the PGCs but not the mesonephros; at E12.5, it is expressed in the seminiferous tubules but not in the interstitial cells of the testis (Kim et al. 2003). As data on whole mount in situ hybridizations are still rather limited, more studies need to be done in the future.

Our results, taken together with those of other researchers, indicate that as a member of *dmrt* gene family, flounder *dmrt4* may be related to the development of the gonads, nervous system and sense organs, although its precise function remains as yet unclear. These results will facilitate future studies on the roles of the flounder *dmrt* gene family in sexual development.

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