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Dmrt1 (doublesex and mab-3-related transcription factor 1) expression during gonadal development and spermatogenesis in the Japanese eel

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ABSTRACT

Dmrt1, doublesex- and mab-3-related transcription factor-1, has been suggested to play critical roles in male gonadogenesis, testicular differentiation and development, including spermatogenesis, among different vertebrates. *Vasa* is a putative molecular marker of germ cells in vertebrates. In this study, we cloned the full-length *dmrt1* cDNA from Japanese eel, and the protein comprised 290 amino acids and presented an extremely conserved Doublesex and Mab-3 (DM) domain. Vasa proteins were expressed in gonadal germ cells in a stage-specific manner, and were expressed at high levels in PGC and spermatogonia, low levels in spermatocytes, and were absent in spermatids and spermatozoa of Japanese eels. Dmrt1 proteins were abundantly expressed in spermatogonia B cells, spermatocytes, spermatids, but not in spermatozoa, spermatogonia A and Sertoli cells. To our knowledge, this study is the first to show a restricted expression pattern for the Dmrt1 protein in spermatogonia B cells, but not spermatogonia A cells, of teleosts. Therefore, Dmrt1 might play vital roles at the specific stages during spermatogenesis from spermatogonia B cells to spermatids in the Japanese eel. Moreover, the Dmrt1 protein exhibited a restricted localization in differentiating oogonia in the early differentiating gonad (ovary-like structure) of male Japanese eels and in E2-induced feminized Japanese eels. We proposed that dmrt1 may be not only required for spermatogenesis but might also play a role in oogenesis in the Japanese eel.

1. Introduction

Dmrt1, doublesex- and mab-3-related transcription factor-1, contains a common zinc finger-like DNA-binding motif (Doublesex and Mab-3 domain, DM domain) (Erdman and Burtis, 1993) and has been reported to exhibit sexual dimorphic expression during gonad development. Dmrt1 is normally expressed at higher levels in the testes than in the ovaries. Dmrt1 has been described as an important and conserved regulator of testicular development in different species, including mammals (mouse, *Mus musculus*; Raymond et al., 2000), birds (chicken, *Gallus gallus*; Nanda et al., 2000), reptiles (red-eared slider turtle, *Trachemys scripta elegans*; Kettlewell et al., 2000), amphibians (frog, *Rana rugosa*; Shibata et al., 2002), and teleosts (tilapia (*Oreochromis niloticus*); Guan et al., 2000). For example, studies using a *Dmrt1* knockout mouse revealed that the gene is indispensable for testis differentiation after sex determination, but is not necessary for ovary development (Raymond et al., 2000). In teleosts, *dmrt1* expression is significantly increased during testicular differentiation in rainbow trout (*Oncorhynchus mykiss*) and lambari fish (*Astyanax altiparanae*) (Marchand et al. 2000; Adolfi et al., 2015). The *dmrt1* gene is expressed at high levels throughout spermatogenesis in rainbow trout, and then is substantially reduced during spermiation. Notably, *dmrt1* expression was not detected during the differentiation of rainbow trout ovaries (Marchand et al. 2000). The expression of *dmrt1* was exclusively restricted to the testis and varied according to spermatogenetic stages in pejerrey (*Odontesthes bonariensis*). Therefore, *dmrt1* was suggested to be involved with the initiation and maintenance of the spermatogenetic cycle in adult pejerrey (Fernandino et al. 2006). Dmrt1 may play a role in spermatogenesis in air-breathing catfish (*Clarias gariepinus*). Higher *dmrt1* expression was detected in the gonads of air-breathing catfish during the period of spermatogenesis, and then slowly decreased during spawning/spermiation and postspawning phases. Furthermore, the air-breathing

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catfish Dmrt1 protein was localized in spermatogonia and spermatocytes using immunocytochemical and immunofluorescence staining (Raghuveer and Senthilkumaran, 2009). However, *dmrt1* expression was detected in ovaries. Guo et al. (2005) observed *dmrt1* expression in developing germ cells in both the testis and ovary using *in situ* hybridization and suggested that the *dmrt1* gene is not only correlated with testis development but may also be important for ovarian differentiation in zebrafish (*Danio rerio*). According to a recent study by Webster et al. (2017), *dmrt1* is not only expressed in both germ cell lines but also documented in Sertoli cells of the testes in zebrafish.

Germ cells in gonads are important for the study of gonadal sex differentiation and development in fish. For example, Kurokawa et al. (2007) evidenced germ cells are indispensable for sexual dimorphism in the medaka gonad. Vasa, an ATP-dependent RNA helicase, belongs to the DEAD (Asp-Glu-Ala-Asp)-box helicase family and is involved in gametogenesis, germ cell specification, and stem cell biology (for a review, see Lasko, 2013). *Vasa* is a putative molecular marker of germ cells in many organisms, including teleosts (for example: zebrafish: Yoon et al. 1997; tilapia: Kobayashi et al., 2000; turbot (*Scophthalmus maximus*): Robledo et al., 2015; silver catfish (*Rhamdia quelen*): Ricci et al., 2018). In gibel carp (*Carassius auratus gibelio*), Xu et al. (2005) documented the highest level of the Vasa protein in spermatogonia, reduced levels in spermatocytes, low levels in spermatids, and the absence of the protein in sperm. In addition, the highest expression of gibel carp Vasa is observed in oogonia and persists throughout oogenesis.

Eels are catadromous basal teleosts with a mysterious migratory life cycle, including reproduction in the ocean and a long growth phase in continental waters. Leptocephali larvae are carried by the ocean current toward the coast and metamorphose into glass eels, which enter estuaries and freshwaters and then develop into yellow eels. After several years of the growth phase, yellow eels undergo a prepubertal secondary metamorphosis called silvering, and transform into silver eels (Rousseau et al., 2013). Eels are gonochoristic species; Colombo and Grandi (1996) indicated that gonadal differentiation occurs during the yellow eel phase. Although studies in several *Anguilla* species showed they have heteromorphic sex chromosomes (Passakas, 1981; Wiberg, 1983; Park and Kang, 1979; Park and Grimm, 1981), the gender of eels was not believed to be associated with the heteromorphic sex chromosomes (Wiberg, 1983). Conversely, environmental factors, such as density, temperature, pH and social interaction, were suggested to override genetic sex determination in eels (review: Tesch, 2003; Davey and Jellyman, 2005). Gonadal development in eels is generally related to body size rather than age, according to previous studies (Colombo and Grandi, 1996; Oliveira and McCleave, 2000; Jellyman, 2001; Jeng et al., 2018). Furthermore, as shown in our previous study, male Japanese eels seem to differentiate through an intersexual stage. Conversely, no degenerated testicular tissues were observed during ovarian differentiation in E2-induced feminized Japanese eels (Jeng et al., 2018). Geffroy et al. (2016) observed significantly higher *dmrt1* expression in the gonads of intersex and male European eels than in the gonads of female European eels. In our previous study, the expression of *dmrt1* transcripts was significantly increased in Japanese eels during testicular development. Moreover, E2 inhibited the *dmrt1*-dependent formation of testicular structures and induced the formation of ovarian structures in Japanese eels (Jeng et al., 2018). Thus, *dmrt1* may be involved in testicular development in eels. However, the cellular localization of Vasa and Dmrt1 proteins within the gonads of Japanese eels remains unclear. In the present study, we aimed to clone the full-length *dmrt1* cDNA from Japanese eel testis and elucidate the cellular distribution and functions of the Vasa and Dmrt1 proteins in the gonads of Japanese eel.

2. Materials and methods

2.1. Animals

In experiments 1 and 2, elvers (*A. japonica*) with a body length of 9.54 ± 0.96 cm and a body weight of 0.88 ± 0.34 g were purchased from a commercial eel dealer in Pingtung in southern Taiwan and transferred to the university culture station. The experimental fish were reared in indoor 2.5 ton tanks with one ton of aerated freshwater (300 elvers/tank) under natural light and temperature conditions.

In experiment 3, nine 3-4-yr-old male eels (body weight of 461.4 ± 38.0 g, body length of 66.9 ± 3.5 cm) were obtained from an aquaculture farm in Taiwan and transferred to the university culture station. The eels were treated with human chorionic gonadotrophin (HCG) to induce spermatogenesis.

All procedures and investigations were approved by the College of Life Science of the National Taiwan Ocean University Institutional Animal Care and Use Committee (Affidavit of Approval of Animal Use Protocol: No. 104008) and performed in accordance with standard guiding principles.

2.2. Experimental design

We performed the three experiments listed below to further elucidate the cellular localization of the Vasa and Dmrt1 proteins in gonads during sex differentiation and spermatogenesis in Japanese eels.

2.2.1. Experiment 1: Cellular localization of the Vasa and Dmrt1 proteins in gonads during the testicular differentiation and development of Japanese eels (control group)

The experimental elvers were reared in two 2.5 ton tanks and fed a commercial eel feed powder (Tung Li Feed Industrial Co., Ltd., Ping-Tung County, Taiwan) for 240 days (Apr. 8, 2015 to Dec. 15, 2015). Gonadal tissues were collected for hematoxylin/eosin staining and immunohistochemistry (IHC). In teleosts, spermatogenesis consists of primordial germ cells (PGCs), spermatogonia (SG), spermatocytes, spermatids and spermatozoa; oogenesis consists of PGCs, oogonia (OG), primary oocytes (PO), previtellogenic oocytes, vitellogenic oocytes, and mature oocytes. Based on our previous histological analysis (Jeng et al., 2018), the gonads of the eels (control group) collected during testicular differentiation were divided into undifferentiated (named S1, the gonads only had PGCs), differentiating (named S2, the gonads resembled ovaries and contained PGCs, OG and PO) and S3 (intersexual gonads, the gonads exhibited a male-like testicular structure with OG, PO, with/without degenerating oocytes (DO) and SG) and differentiated (testis) (named S4, the gonads with SG and other male germ cells) stages.

2.2.2. Experiment 2: Cellular localization of the Vasa and Dmrt1 proteins in the gonads of E2-induced feminized Japanese eels

The expression of *dmrt1* is maintained at low levels during ovarian development in E2-induced feminized Japanese eels, according to our previous study (Jeng et al., 2018). We treated elvers with E2 to feminize Japanese eels and further clarify the localization of the Vasa and Dmrt1 proteins in the ovary. According to our previous study (Jeng et al., 2018), an E2-containing diet (10 mg of E2/kg of feed; Sigma-Aldrich Corp., St. Louis, MO) was prepared. E2 was orally administered to 300 elvers for 180 days. Gonadal tissues were collected for IHC. According to our previous study (Jeng et al., 2018), the E2-treated eels were also divided into three groups: undifferentiated (named FS1, the gonads only contained PGCs), differentiating (named FS2, containing PGCs, OG and PO) and differentiated eels (named FS3, containing OG and PO).

2.2.3. Experiment 3: The expression of dmrt1 in HCG-treated male eels

We administered HCG (Gona-5000 injection, China Chemical & Pharmaceutical Co. Ltd., Taipei, Taiwan) to induce gonadal development in male Japanese eels and to assess the expression of the *dmrt1*

transcripts and the cellular distribution of the Dmrt1 protein in the testis during spermatogenesis. Nine 3–4-yr-old male eels were divided into two groups: a control (n = 5) and HCG-treated group (n = 4). HCG was administered as described in a previous study (Jeng et al., 2012). Gonadal tissues were collected for gene expression and IHC analyses.

2.3. Sampling procedures

Eels were anesthetized with 800 ppm of 2-phenoxyethanol before sacrifice. The body weight and body length were measured. The body segment containing the gonadal tissue (when the fish was small) or gonadal tissue (a piece of gonad in the bigger fish) was fixed overnight with 4% paraformaldehyde in PBS (phosphate-buffered saline) for histological observations and IHC. For gene expression studies, gonadal tissues were collected and stored at −80 °C for the quantitative real-time PCR analysis (qPCR).

2.4. Cloning of the full-length dmrt1 cDNA from Japanese eel

The full-length *dmrt1* cDNA was cloned using RT-PCR and rapid amplification of cDNA ends (RACE). Specific primers were designed for RACE based on the partial transcriptome sequence. The primers for Japanese eel *dmrt1* were: 5′RACE-R494: 5′GATTACGCCAAGCTTGGCA TCTGGTACTGCTGGTAGTTGT'3, and 3′RACE-F441: 5′GATTACGCCAA GCTTCCGCTACCCTGCCTACTACAGCAACC'3. All procedures were performed according to users' manuals (SMARTer™ RACE cDNA Amplification Kit, Clontech, Tokyo, Japan). PCR products were separated on a 1.2% agarose gel, cloned into the pGEM®-T Easy vector, and sequenced. Dmrt1 protein sequences from different species were aligned using the Multiple Sequence Alignment tool (CLUSTALW, http://www.genome.jp/tools-bin/clustalw). Phylogenetic trees were constructed with Molecular Evolutionary Genetics Analysis (MEGA) software 7.0 using the neighbor-joining (NJ) method, yielding an unrooted consensus tree with 1000 bootstrap replicates.

2.5. Production of antibodies against Japanese eel Vasa and Dmrt1

Two synthetic 15-mer (NH2-C-FQRENGRQPAVQSQP) and 14-mer peptides (NH_2-C-NPRGKVFASTDTRK) derived from the C-terminal fragment of eel Vasa (GenBank: ASV71763.1) and a synthetic 14-mer (NH_2-C-SMSSGKKPPRMPKC) derived from the N-terminal fragment of eel Dmrt1 (Fig. 1) were coupled to keyhole limpet hemocyanin. These antigens were then used to produce polyclonal antibodies in guinea pigs (Vasa) and rabbits (Dmrt1). The antibodies were prepared by Yao-Hong Biotechnology Inc. (Taiwan) and ICON Biotechnology Co., Ltd. (Taiwan), respectively.

2.6. Protein extraction and Western blotting

Protein extraction and Western blotting were conducted using methods reported in a previous study (Jeng et al., 2012). Japanese eel testes were homogenized with a sonicator in a modified RIPA (radioimmunoprecipitation assay) buffer (75 mM Tris base, pH 7.4, 200 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.5% Na-deoxycholate and 1% NP-40 supplemented with a cocktail of protease inhibitors (Roche, Mannheim., Germany) and phenylmethylsulfonyl fluoride (PMSF)). Lysates were incubated on ice for 1 h and then centrifuged at 10,000*g* for 20 min. The concentrations of the extracted proteins contained in the supernatants were measured using the Bio-Rad protein assay kit (Bio-Rad Co., Hercules, CA). Extracted proteins (50 mg) were resolved on 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with TBST (150 mM NaCl, 20 mM Tris pH 7.6 and 0.1% Tween-20) containing 5% skim milk powder for 1 h. The membrane was then incubated with the guinea pig (or rabbit) polyclonal antibody against Japanese eel Vasa (1:1000) (or Dmrt1, 1:1000) for 2 h at room temperatures. Membranes were incubated with an alkaline phosphatase-conjugated goat anti guinea pig (or rabbit) IgG (1:5,000) (AnaSpec Inc., Fremont, CA) for 1 h at room temperature. Finally, the proteins were visualized using the BCIP/NBT liquid substrate system (Sigma).

2.7. Double immunohistochemical staining for Japanese eel Vasa and Dmrt1

IHC staining was performed using the method described in a previous study (Jeng et al., 2012), with slight modifications. The gonads of Japanese eel were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Transverse paraffin sections (5 μm) were rehydrated through a graded series of ethanol solutions, rinsed with PBS and then incubated with 3% H_2O_2 in PBS to block endogenous peroxidase activity. Sections were treated with 10 mM sodium citrate (Sigma) pH 6.0 for 30 min at 80 °C to expose the antigens of the target protein. Nonspecific binding was blocked by incubating the sections with PBS containing 0.2% Triton and 5% skim milk powder. For the double detection of Vasa and Dmrt1, sections were incubated with a mixture of guinea pig polyclonal antibodies against eel Vasa and rabbit polyclonal antibodies against eel Dmrt1. Sections were then exposed to a mixture of Alexa Fluor 488-conjugated goat anti-guinea pig (1:150; Invitrogen Molecular Probes) or Alexa Fluor 594-conjugated goat anti-rabbit (1:150; Invitrogen Molecular Probes) antibodies. Finally, the slides were mounted with Vectashield mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) that permits the visualization of cell nuclei. Sections were observed under an epifluorescence microscope (Olympus Provis) equipped with a DP71 digital camera. Images were processed with the Olympus Analysis Cell software.

2.8. Histology

The eel body segment or gonadal tissue was dehydrated and embedded in Paraffin. The sections with 5-μm were stained with hematoxylin and eosin. The gonadal status was classified according to light microscopic examination. The study in Japanese eels by Jeng et al. (2018) was used as a reference for the characterization of gonadal histological structure.

2.9. RNA extraction and reverse transcription

Total RNA was extracted from the eel gonadal tissues by homogenization in Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The quantity of RNA was estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). The 260/280 nm ratios of total RNA ranged from 1.8 to 2.0. Reverse transcription (RT) was performed using SuperScript® III Reverse Transcriptase (Invitrogen) with oligo $(dT)_{12–18}$ primers (Promega, Madison, WI) according to the manufacturer's protocol. The first-strand cDNAs were used for cloning and qPCR.

2.10. Quantification of gene transcripts using real-time quantitative PCR

The qPCR analyses of the expression of the *dmrt1* and *ef1a* (elongation factor 1 alpha) transcripts were conducted as described in a previous study (Jeng et al., 2018). The quantification of gene expression in standards (plasmids with cDNA sequence) and samples was conducted simultaneously using qPCR (Applied Biosystems 7300 Real-Time. PCR System; Applied Biosystems, Foster City, CA) with SYBR green I as a dsDNA minor-groove binding dye; *ef1a* was chosen as an internal control gene. No amplification was observed in non-template reactions and melting curves indicated the amplification of a single amplicon for each gene. The slopes of the respective standard and sample curves of the log (cDNA concentrations) vs. Ct (the calculated fractional cycle number at which the PCR-fluorescence product is detectable above a threshold) were −3.3 to −3.5, indicating an

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     AAGCAGTGGTATCAACGCAGAGTACATGGGGGCGGAACTAGATTGGCATTCTTTTGTTTCATTTACTATTTTTGTCCAGTAGTTATTTTT
     TAACTCTAGAAAGTTAAGGGCCTTTTTTTATAAGCTGCAACATCTAAGGTGGGTTTAACGCTTTTCATCGGAA
   1 ATGAGCGACGACGAACAGGCCAAGCAGTCCTTGGAATGTGCTGGATCCATGTCTTCTGGCAAAAAACCGCCGAGAATGCCTAAGTGTTCC
     M  S  D  D  E  Q  A  K  Q  S  L  E  C  A  G  S  M  S  S  G  K  K  P  P  R  M  P  K  C  S

  91 CGTTGCAGAAACCATGGTTATGTGTCACCTTTGAAGGGACATAAACGATTTTGTAACTGGAGGGATTGTCTATGTCAGAAATGCAAATTG
     R  C  R  N  H  G  Y  V  S  P  L  K  G  H  K  R  F  C  N  W  R  D  C  L  C  Q  K  C  K  L

 181 ATCGCAGAACGGCAGAGGGTGATGGCGGCCCAGGTGGCACTCCGCAGACAGCAGGCCCAAGAGGAGGAGATGGGCATCTGCAGTCCAGTC
     I  A  E  R  Q  R  V  M  A  A  Q  V  A  L  R  R  Q  Q  A  Q  E  E  E  M  G  I  C  S  P  V

 271 ACCTTGTCCAGCACGGAGGTCATGGTGAAGAATGAAGCCACAGGTGACAGGGCTTGCTCCTTCTCCGCAGGGGAAAAGTCTCCCCCTCTC
     T  L  S  S  T  E  V  M  V  K  N  E  A  T  G  D  R  A  C  S  F  S  A  G  E  K  S  P  P  L

 361 CAAAACAACGAAGCCACATCACCCTCTGCTACAGGCAACCGACCCGCCATGCCGTCCAGTCCCACATCGGCCAGCAGGGGGCACCCTGAG
     Q  N  N  E  A  T  S  P  S  A  T  G  N  R  P  A  M  P  S  S  P  T  S  A  S  R  G  H  P  E

 451 GGTTCATCTGATCTGGTGGTGGACGCATCCTACTACAACTTCTACCAGCCCTCCCGCTACCCTGCCTACTACAGCAACCTGTACAACTAC
     G  S  S  D  L  V  V  D  A  S  Y  Y  N  F  Y  Q  P  S  R  Y  P  A  Y  Y  S  N  L  Y  N  Y

 541 CAGCAGTACCAGATGCCCAGCAACGAGGGTCGCCTGTCTGGCCACAGCGTGTCCCCGCAGTACCGGATGCACTCGTACTACTCCGCGGCC
     Q  Q  Y  Q  M  P  S  N  E  G  R  L  S  G  H  S  V  S  P  Q  Y  R  M  H  S  Y  Y  S  A  A

 631 TCCTACCTGAGCCAGGGCCTGGCAGCCCCTGGCTGCGTGCCCCCCATCTTCACCCTGGAGGACAACGCCTCCTTCCCTGAGCCCAAGGCT
     S  Y  L  S  Q  G  L  A  A  P  G  C  V  P  P  I  F  T  L  E  D  N  A  S  F  P  E  P  K  A

 721 GCGATGTTCGCTCCTGGCAGTGGGCATGACGCTGGCCTGCCCTGCCTGTCCATCAACCCCTTGGTCAACTCTGAAACCAAGCAGGAGTGC
     A  M  F  A  P  G  S  G  H  D  A  G  L  P  C  L  S  I  N  P  L  V  N  S  E  T  K  Q  E  C

 811 GAGACCAGTTCTGAGTCTGAGGTCTTCGCCGTGAACGTTGTCATCGATGGGCCCAGTGAGTGA 873
     E  T  S  S  E  S  E  V  F  A  V  N  V  V  I  D  G  P  S  E  *
     GGCCTTCCAGGTGACAGCCCCTCCCCACCACAGCAGCTAAATCAAGCTGAAAATCACTGATGATCACCAGCAGCACTGTCAATGCGAAAG  963
     GGTCTGTGGCGTTTCCATAGCCCCCTGTATTCTGTATTTGTTTTCAAGTTTCATACTTTCATTCATTATTTTTGTGCCAGGTGAAATAAA 1053
     TAGAATTGCATCATTATGGAGTATAAGGCATTCAGCAACATTGACTCTCAATGCGTTGCATAACAATGGCATTTTATAGTTTTGCAGAAA 1143
     TCGCCTTGCTCTAAGACAATTAATGAAATTTGAGTTTAGAATTTTTTAAGTTATTAATAATCATAGTTCTGAACAAAGCAAAATGTCAGT 1233
     TTTGTCAGTGGCGCTACAAAATTCATTTTGTATTCAAATTGCTCAGCACTGTGAATATTCATTTTGGCACATTCTCCATGCATTTTACAA 1323
     ATAAGCACGGAATGCTCAGACTGTGCTACAGTATGTTTTTGTCATATAGTTGTATCACCTTTAAATGTATTATGAATTATTGTAATGCTC 1413
     TTAATAATAGTGTACTTATGTTCATGTTCATGTCAGTACAGTATATCTGTAGTCTAAAATTAACATTTCATAATGGATAACTACACCCAC 1503
     TG 1505
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Fig. 1. Nucleotide and deduced amino acid sequences of *Anguilla japonica* Dmrt1. Start and stop codons are shown in bold, the DM domain is underlined. The boxed sequence indicates a typical polyadenylation signal.

amplification efficiency of 90–100%. Gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method, and the highest value in each comparison was set to 1 to calculate the relative expression.

2.11. Data analysis

The transcript levels are presented as means ± SD. The values analyzed using Student's *t*-test to evaluate significant differences ($P < 0.05$) between treatment groups. All statistical analyses were performed using SPSS10.0 for Windows.

3. Results

3.1. Cloning and sequence analysis of Japanese eel dmrt1

The 873-bp open reading frame (ORF) that encoded 290 amino acids, a 162-bp 5′-untranslated region (UTR), and a 632-bp 3′-UTR of the *dmrt1* transcript were cloned in this study. The characteristic conserved DM domain comprises amino acids 24–87, and the 3′-UTR contains a potential polyA signal (Fig. 1). Moreover, the deduced amino acid sequence of Japanese eel Dmrt1 was compared to amino acid sequences from other teleost Dmrt1 proteins, and the DM domain corresponded to the homologous domains of teleost sequences (Fig. 2).

The phylogenetic analysis of the Dmrt1 sequences (Fig. 3) revealed the existence of two main clades: one represented by the mammalian, bird, reptilian and amphibian Dmrt1 sequences, and the second by Dmrt1 sequences from fish. The Japanese eel (superorder Elopomorpha) Dmrt1 clustered in the fish clade together with Dmrt1 from rainbow trout (superorder Protacanthopterygii) and Atlantic cod (superorder Paracanthopterygii).

3.2. Specificity of the Japanese eel Vasa and Dmrt1 antibodies

Western blotting of testis extracts using the Japanese eel Vasa or Dmrt1 antibody yielded a single band of the expected size (63 KDa and 32 KDa, respectively), as shown in Fig. 4A and B. This band disappeared following preabsorption with the peptides that were used to generate the antibodies (data not shown).

3.3. Cellular localization of the Vasa and Dmrt1 proteins in eel gonads

3.3.1. Cellular localization of the Vasa and Dmrt1 proteins during testis differentiation in eels

In this study, transverse sections of gonads at various stages (S1 to

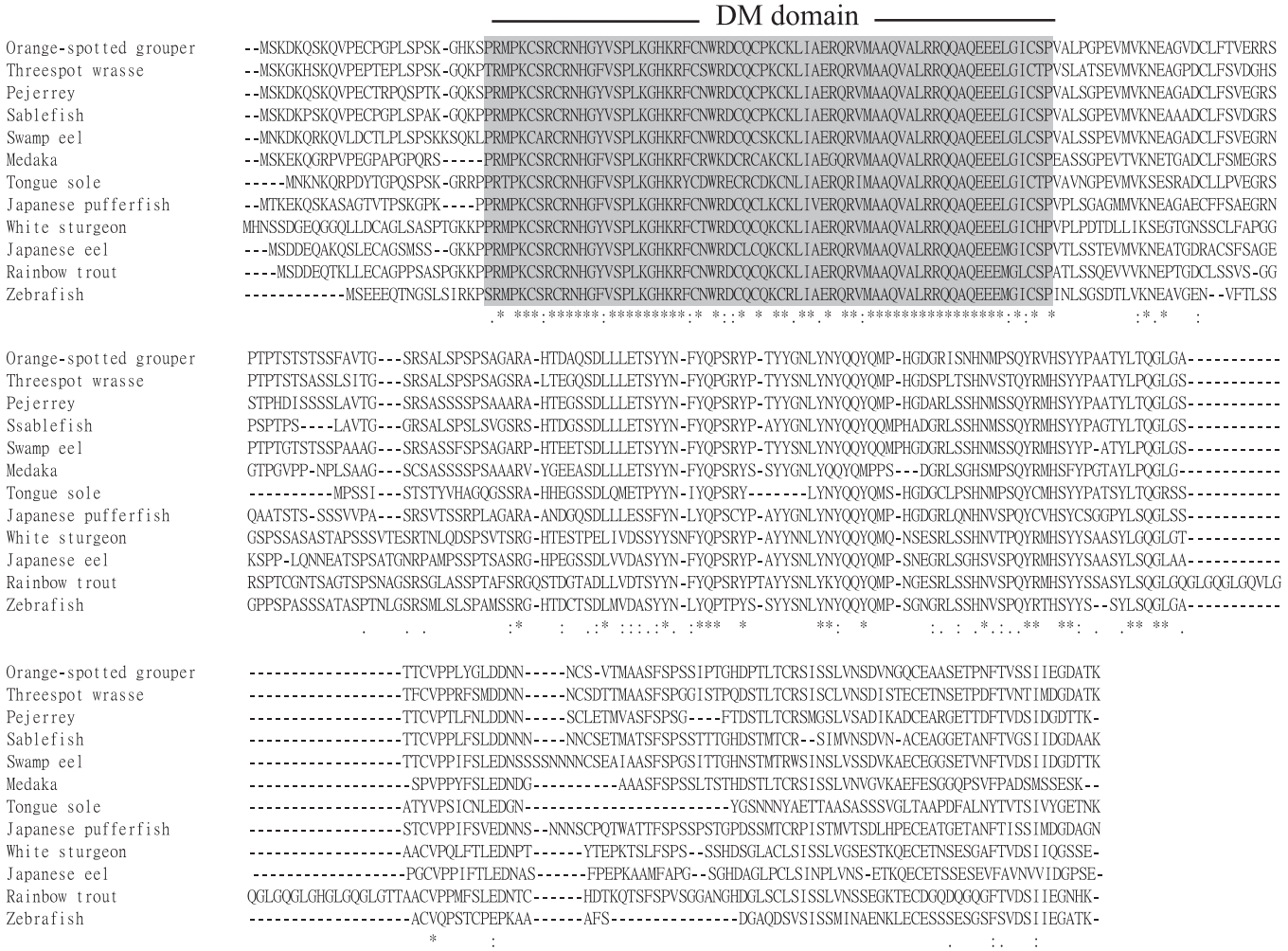

Fig. 2. Deduced amino acid sequence of *Anguilla japonica* Dmrt1 aligned with sequences from other fish. The DM domain is indicated. Asterisks corresponds to 100%, two spots to 80% and one spots to 60% amino acid sequence identity, respectively. The GenBank accession numbers are: orange-spotted grouper (*Epinephelus coioides*), ABK15558.1; threespot wrasse (*Halichoeres trimaculatus*), BAQ20799.1; pejerry (*Odontesthes bonariensis*), AAP84606.3; sablefish (*Anoplopoma fimbria*) AGH69790.1; swamp eel (*Monopterus albus*), AAP80398.1; medaka (*Oryzias latipes*) AAL02165.1; tongue sole (*Cynoglossus semilaevis*) ABS31368.1; Japanese pufferfish (*Takifugu rubripes*), BAE16952.1; white sturgeon (*Acipenser transmontanus*), AAL18252.1; rainbow trout (*Oncorhynchus mykiss*), AAG17544.1, three-spined stickleback (*Gasterosteus aculeatus*), AAW62304.1; and zebrafish (*Danio rerio*), AAQ04555.1.

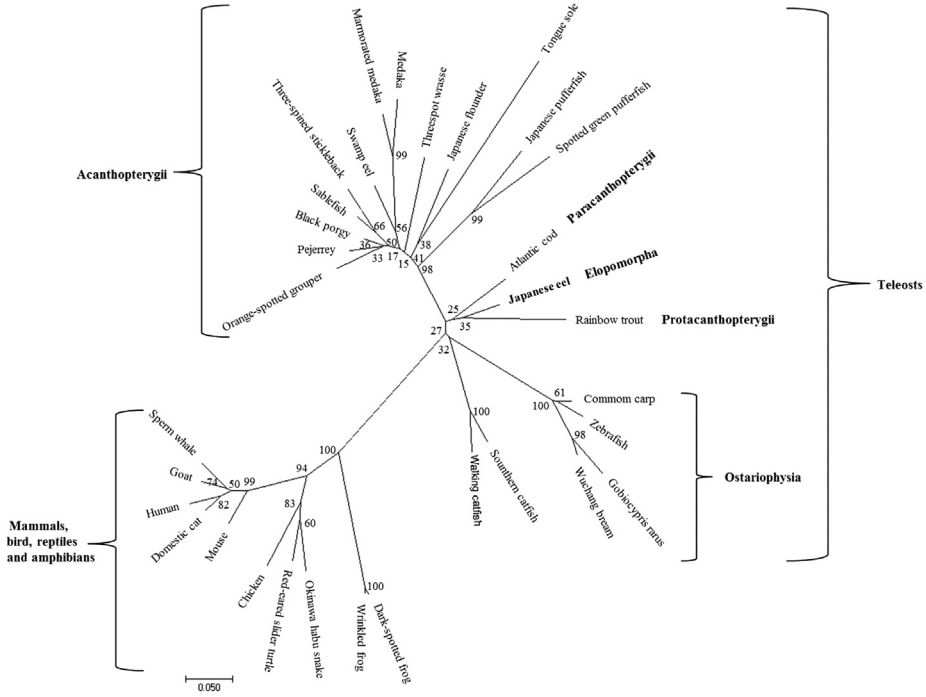

Fig. 3. Phylogenetic tree of Dmrt1 in vertebrates. The values represent bootstrap scores of 1000 trials, indicating the credibility of each branch. GenBank accession numbers not listed in the legend for Fig. 2: domestic cat (*Felis catus*), XP_023099083.1; human (*Homo sapiens*), AAD40474.1; goat (*Capra hircus*), AOV81585.1; sperm whale (*Physeter catodon*), XP_023982652.1; mouse (*Mus musculus*), AAF12826.1; chicken (*Gallus gallus*), AAF19034.1; Okinawa habu snake (*Protobothrops flavoviridis*), BAF36482.1; red-eared slider turtle (*Trachemys scripta*), ARW59277.1; dark-spotted frog (*Pelophylax nigromaculatus*), ABS45107.1; wrinkled frog (*Rugosa rugose*), BAF31129.1; Barramundi perch (*Lates calcarifer*), AKI32577.1; walking catfish (*Clarias batrachus*), ACR77514.1; southern catfish (*Silurus meridionalis*), ABM54574.1; common carp (*Cyprinus carpio*), XP_018938149.1; *Gobiocypris rarus*, AFA45126.1; Wuchang bream (*Megalobrama amblycephala*), AHF72546.1; Atlantic cod (*Gadus morhua*), ADH94552.1; Japanese pufferfish (*Takifugu rubripes*), BAE16952.1; spotted green pufferfish (*Tetraodon nigroviridis*), AAN74844.1; marmorated medaka (*Oryzias marmoratus*), AAS91466.1; sablefish (*Anoplopoma fimbria*), AGH69790.1; three-spined stickleback (*Gasterosteus aculeatus*) AAW62304.1; and black porgy (*Acanthopagrus schlegelii*), AAP84972.1.

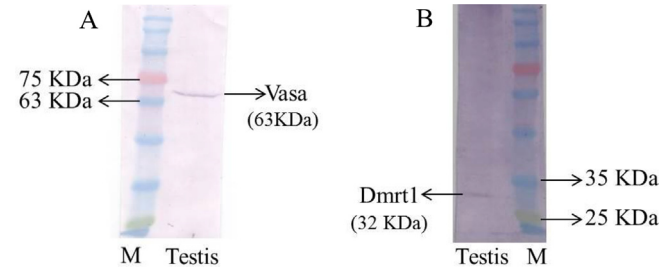

Fig. 4. Western blot analysis of Vasa and Dmrt1 expression in testis extracts. (A) An incubation with the Vasa antibody (1:1000 dilution) yielded a single band at the expected size of 63 KDa. (B) An incubation with the Dmrt1 antibody (1:1000 dilution) yielded a single band at the expected size of 32 KDa. M: protein markers; Testis: testis extracts.

S4) of sexual differentiating male eels stained with hematoxylin and eosin were shown in Fig. 5A–D. Vasa-positive cells, but no Dmrt1 signal, were observed in the gonads at the S1 stage (Fig. 5E). Vasa protein signals were detected in germ cells (oogonia and/or spermatogonia) and primary oocytes, while positive signals for the Dmrt1 protein were specifically observed in some but not all of the oogonia in the gonads at the S2 stage (Fig. 5F). In this study, we found some oogonia with vasa-positive and Dmrt1-negaive signals (early oogonia, defined as oogonia-I, OG-I) and some oogonia with Vasa-positive and Dmrt1-positive signals (late or differentiating oogonia, defined as oogonia-II, OG-II). Vasa proteins were presented in spermatogonia A and B, primary oocytes and degenerating oocytes. Dmrt1 proteins were specifically expressed in spermatogonia B cells, but no Dmrt1 signal was observed in the spermatogonia A cells in the gonads at the S3 and S4 stages (Fig. 5G and H). Testis sections that only contained spermatogonia A cells were analyzed to verify the location of Dmrt1 proteins. We found that strong Vasa protein signals were found and no positive-Dmrt1 signal was observed in these spermatogonia A cells (Fig. 5I–L).

3.3.2. The expression of the Vasa and Dmrt1 proteins during eel spermatogenesis

We analyzed the expression of the *dmrt1* transcripts in the testis using real-time PCR to further elucidate the expression profile of the *dmrt1* transcripts and cellular localization of the Dmrt1 protein in the testis of the Japanese eel. HCG induces testis development in eels (Miura et al., 1991). In the present study, the histological analysis showed the testis section of control eel only contained spermatogonia A and B, the testis section of HCG-treated eel contained spermatogonia A and B, spermatocytes, spermatids and spermatozoa (Fig. 6A). The gonadosomatic index (GSI%) was 0.08 ± 0.03% in the control group and 3.58 ± 1.82% in the HCG group (Fig. 6B). The levels of the *dmrt1* transcripts in the testis were significantly increased in the HCG-treated eels compared to the control eels (Fig. 6C). No significant difference was observed in the internal control gene *ef1a* transcript levels between the gonadal tissues from the control and HCG groups (Fig. 6D).

The result of double IHC staining showed that the Vasa proteins were present in spermatogonia (both A and B cells) and spermatocytes, but no clear signal was observed in spermatids and spermatozoa; furthermore, the Dmrt1 protein was expressed in spermatogonia B cells, spermatocytes, spermatids, but not in spermatozoa and spermatogonia A cells (Fig. 7A–D).

3.3.3. The expression of the Vasa and Dmrt1 proteins during ovarian development in E2-induced feminized Japanese eels

Although the expression of the *dmrt1* transcripts was maintained at low levels during ovarian development in E2-induced feminized Japanese eels (Jeng et al., 2018), the Dmrt1 protein was detected in the gonads of E2-induced feminized Japanese eels using IHC in the present study. No Dmrt1 protein signal was observed in the primordial germ cells of the gonad at the FS1 stage (Fig. 7E) or in primary oocytes at the FS2 and FS3 stages (Fig. 7F and G). Oogonia with Dmrt1-negative (OGI) and Dmrt-positive (OG-II) signals were present in the ovaries at the FS2 and FS3 stages (Fig. 7F and G). The Vasa protein was expressed in the oogonia and primary oocytes in the ovaries at the FS2 and FS3 stages (Fig. 7F and G).

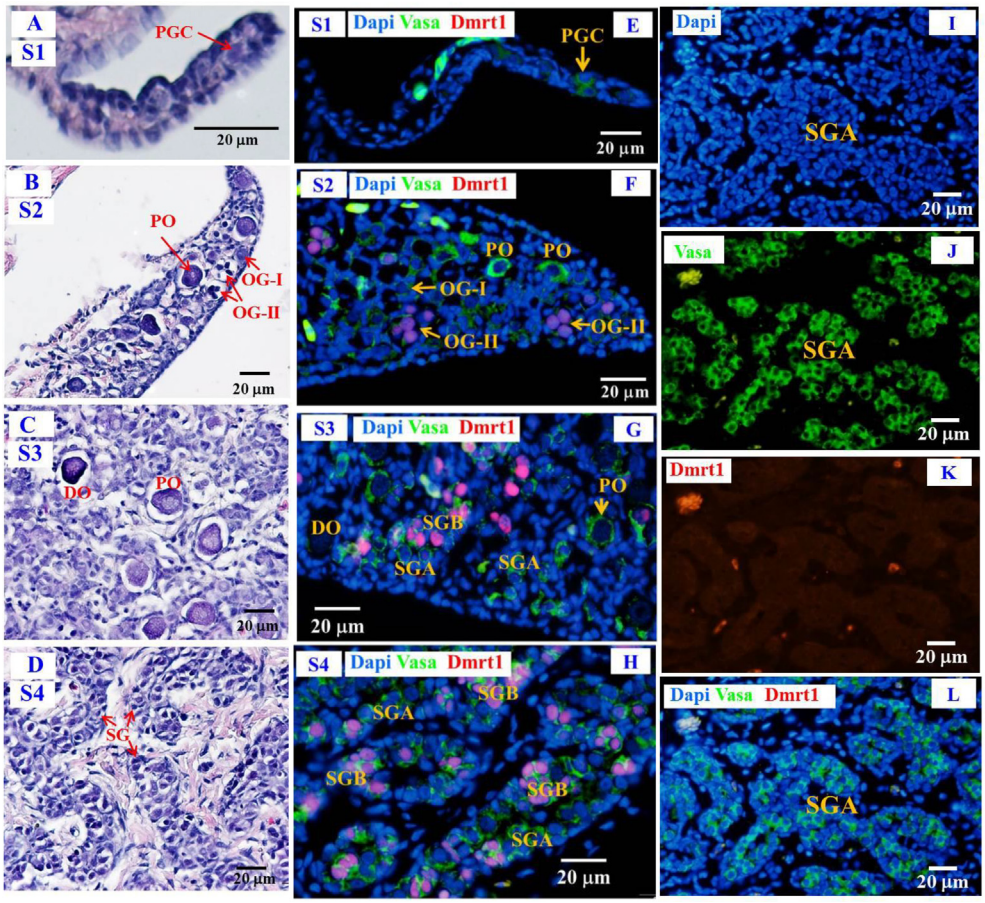

Fig. 5. Transverse sections staining with hematoxylin and eosin (A - D) and immunofluorescence staining for the Vasa (green) and Dmrt1 (red) proteins in Japanese eel gonads at various stages of testicular differentiation (E - L). (A, E) S1 stage, undifferentiated gonad. (B, F) S2 stage, early differentiating gonad. (C, G) S3 stage, late differentiating gonad. (D, H) S4 stage, differentiated testis. (I-L) Testis with only spermatogonia A cells. PGC, primordial germ cell; OG-I, early oogonia; OG-II, late oogonia; PO, primary oocyte; DO, degenerating oocyte; SGA, spermatogonia A cell; SGB, spermatogonia B cell.

4. Discussion

4.1. Characterization of the dmrt1 cDNA and phylogeny

The Dmrt1 protein from Japanese eel comprised 290 amino acids, contained an extremely conserved DM domain, similar to the Dmrt1 protein from other fish species, as shown in Fig. 1. The DM domain contains six cysteine residues, which are probably related to the formation of the unusual intertwined zinc-finger motif (Zhu et al., 2000; Johnsen et al., 2010). Moreover, the residues lysine[92] and arginine[93]

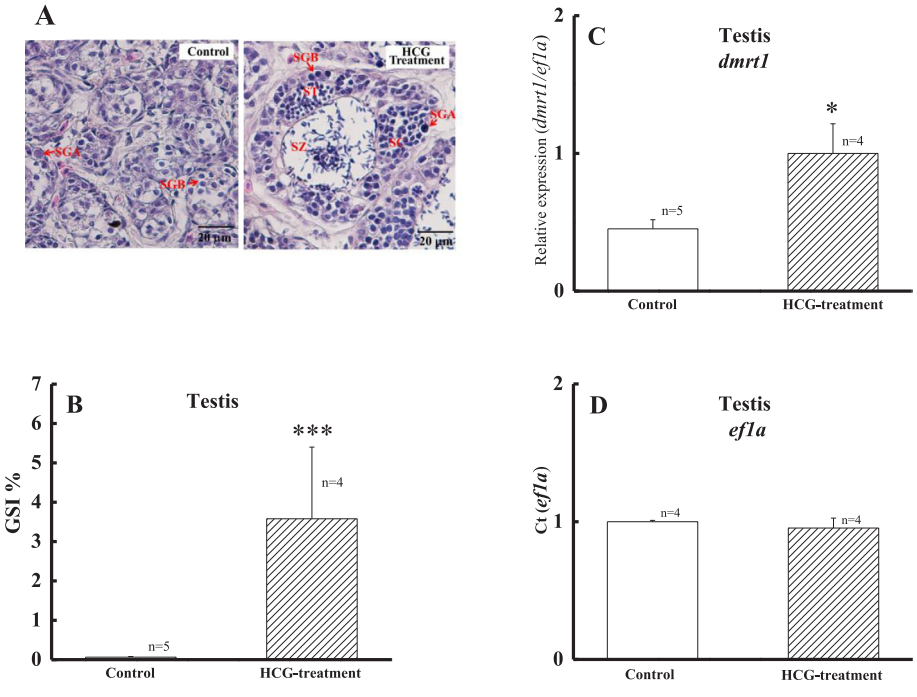

Fig. 6. The transverse sections gonads stained with hematoxylin and eosin (A), gonadosomatic index (GSI %) (B), the profile of *dmrt1* expression (C), and the internal control gene *efla* expression (D) in the control and HCG-treated male Japanese eels. Data are presented as means ± standard deviations (SD). The number in the figure represents the number of fish in each group. Asterisks indicate significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$) between the control male eels and HCG-treated male Japanese eels. SGA, spermatogonia A cell; SGB, spermatogonia B cell; SC, spermatocyte; ST, spermatid; SZ, spermatozoa.

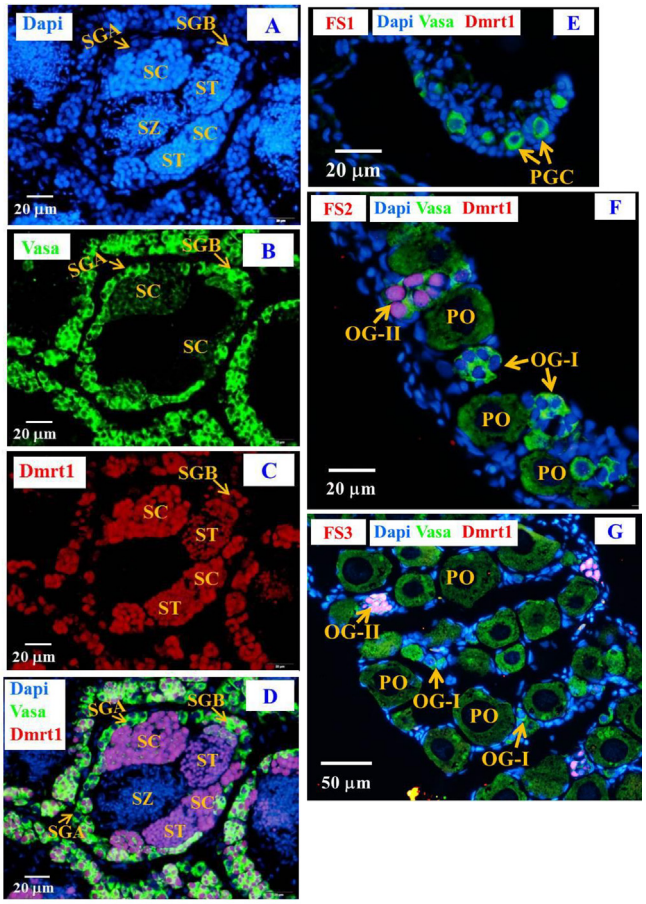

Fig. 7. Immunofluorescence staining for the Vasa (green) and Dmrt1 (red) proteins in the testis of Japanese eel during spermatogenesis (A–D) and in the gonads of estradiol (E2)-induced feminized Japanese eels in various stages of sexual differentiation (E–G). (E) FS1 stage, undifferentiated gonad. (F) FS2 stage, differentiating ovary. (G) FS3 stage, differentiated ovary. SGA, spermatogonia A cell; SGB, spermatogonia B cell; SC, spermatocyte; ST, spermatid; SZ, spermatozoa; PGC, primordial germ cell; OG-I, early oogonia; OG-II, late oogonia; PO, primary oocyte.

within the DM domain are critical for DMRT1 nuclear localization in humans (Ying et al., 2007). These residues are highly conserved in teleost Dmrt1 proteins, including Japanese eel (lysine[47] and arginine[48]). The C-terminal regions of DMRT1 in different species were highly variable; this region functions as a transactivation domain, as indicated by Yoshimoto et al. (2006) and Murphy et al. (2007).

The present phylogenetic analysis of the Dmrt1 sequences from diverse vertebrates showed that Dmrt1 of teleosts and other species separated into two main clusters. The Dmrt1 proteins from Japanese eel, rainbow trout and Atlantic cod were clustered in the same clade, but with low bootstrap values. This result was consistent with the study by Johnsen et al. (2010), who indicated that the branching of teleost Dmrt1 proteins normally followed the conventional phylogeny, but several nodes presented low bootstrap values. However, Fernandino et al. (2006) showed that Dmrt1 in the acanthopterygian species did not show a clear correspondence with traditional systematic phylogeny based on morphology.

4.2. Stage-specific expression of the Vasa protein in germ cells in the gonads of Japanese eel

Vasa was suggested to be a specific germ cell marker and suitable for germ cell determination in many species. In the present study, the Vasa protein exhibited the stage-specific expression in the germ cells in Japanese eel gonads. The Vasa protein was expressed at high levels in PGC and spermatogonia, low levels in spermatocytes, and was absent in spermatids and spermatozoa; moreover, the protein was also expressed in oogonia and oocytes in the differentiating testes and ovary of Japanese eels. Thus, Vasa is a suitable germ cell marker for eels. Stage-specific expression of the Vasa protein in the gonads at different developmental stages has also been reported in various species. In fetal and adult male and female human gonads, the VASA protein is expressed in the gonadal germ cells, and the highest expression is observed in spermatocytes and mature oocytes (Castrillon et al., 2000). As shown in the study by Lee et al. (2018), VASA is a putative marker of undifferentiated spermatogonia and differentiated spermatocytes in the prepubertal and postpubertal porcine testis, respectively. Rzepkowska and Ostaszewska (2013) observed Vasa aggregates in the spermatogonia of Siberian sturgeon (*Acipenser baerii*), and a weak Vasa-positive signal also was detected in secondary spermatocytes and early spermatids. Moreover, the Vasa protein was detected in all oogonia clustered in ovarian nests of Russian sturgeon (*Acipenser gueldenstaedtii*; Rzepkowska and Ostaszewska, 2013. Li et al. (2009) indicated that Vasa was not essential for PGC proliferation, motility, identity and survival, but was necessary for cell autonomous PGC migration in medaka. Differences in *vasa* RNA expression in the testis of tilapia have been reported (Kobayashi et al., 2000). Strong *vasa* signals were detected in spermatogonia and lower levels were detected in early primary spermatocytes of tilapia (Kobayashi et al., 2000). No *vasa* signals were observed in secondary spermatocytes, spermatids or spermatozoa of tilapia (Kobayashi et al., 2000). Therefore, tilapia *vasa* was suggested to have a differential function in the regulation of the meiotic progression of germ cells (Kobayashi et al., 2000). We speculated that eel Vasa may be involved in PGC proliferation and may play a similar role as the tilapia protein in the regulation of the meiotic progression of germ cells.

4.3. Expression of the Dmrt1 protein during gonad development and spermatogenesis in Japanese eel

Dmrt1 has been suggested to play critical roles in male gonadogenesis, testicular differentiation and development, including spermatogenesis, among different vertebrates (for a minireview, see Herpin and Schartl, 2011). Furthermore, *dmrt1* is expressed in both the somatic and germ cells in the testes of some animals, for instance in mouse (Lei et al. 2007), chicken (Omotehara et al. 2014), lizard (*Calotes versicolor*, Sreenivasulu et al. 2002), and frog (*Xenopus laevis*, Mawaribuchi et al., 2017). Mouse DMRT1 was suggested to play essential roles in two different processes in the developing gonads: somatic cell masculinization and germ cell development (Zarkower, 2013).

The cellular localization of Dmrt1 appears to differ among teleost fishes. The *Dmrt1* mRNA and protein were also detected in both the germ cells (spermatogonia) and somatic Sertoli cells of platyfish (*Xiphophorus maculatus*; Veith et al., 2006), gibel carp (Li et al., 2014), and zebrafish (Webster et al., 2017); furthermore, the Dmrt1 protein from gibel carp was suggested to be involved in testis differentiation and spermatogenesis (Li et al., 2014). In the present study, Dmrt1 proteins were localized in spermatogonia B cells, spermatocytes, and spermatids, but not in spermatogonia A cells and spermatozoa. Moreover, Dmrt1 expression was not observed in the somatic Sertoli cells in Japanese eels. To our knowledge, this study is the first to show that the Dmrt1 protein was specifically expressed in spermatogonia B cells and was not localized in spermatogonia A cells in teleosts. Therefore, Dmrt1 might play vital roles in specific stages of spermatogenesis from spermatogonia B cells to spermatids in Japanese eels. The result was similar to a study of the red-spotted grouper (*Epinephelus akaara*; Xia et al., 2007). The Dmrt1 protein was present in spermatogonia, primary spermatocytes and secondary spermatocytes, but not in the Sertoli cells of the red-spotted grouper (Xia et al., 2007). Accordingly, grouper Dmrt1 was suggested to play a specific role at the specific stages from

spermatogonia to spermatocytes (Xia et al., 2007). However, the precise functions of Dmrt1 during spermatogenesis require further studies. Conversely, Dmrt1 was only expressed in the Sertoli cell of pufferfish (*Takifugu rubripes*, Yamaguchi et al., 2006), Nile tilapia, (Kobayashi et al., 2008), black porgy (Acanthopagrus schlegelii, Wu et al., 2012) and three-spot wrasse (*Halichoeres trimaculatus*, Kobayashi et al., 2014). Our previous study using black porgy (a protandrous hermaphroditic fish) suggested that *dmrt1* expressed in Sertoli cells functions as a key regulator of initial testis differentiation and is also important in testis growth/development and in the control of sexual fate (Wu et al., 2012).

4.4. Expression of the Dmrt1 protein in the ovary of E2-induced feminized Japanese eels

Although a strong male-biased expression of *dmrt1* seems to be a general trend, *dmrt1* was expressed to some extent in the ovaries of different species. In Atlantic cod, the *dmrt1* mRNA signal was detected in spermatogonia, the maturing oocytes and in the presumptive oogonia in the immature ovary (Johnsen et al., 2010). Therefore, *dmrt1* from Atlantic cod was proposed to be associated with the early stages of spermatogenesis and oogenesis (Johnsen et al., 2010). In addition to the developing germ cells in the testis, the *dmrt1* mRNA was also detected in developing oocytes, including early perinucleolus stage oocytes, late yolk vesicle stage oocytes, and oil drop stage oocytes, using *in situ* hybridization in zebrafish (Guo et al. 2005). Webster et al. (2017) further indicated that Dmrt1 is an essential regulator of the normal transcription of the *amh* (anti-Müllerian hormone, proposed to be a key testis-specific gene) and *foxl2* (forkhead box L2, suggested to be an ovary-associated gene) genes in zebrafish. Therefore, *dmrt1* was proposed to be important in testis development and ovarian differentiation in zebrafish.

Based on our IHC results, the Dmrt1 protein was only localized in some oogonia (may be late oogonia or differentiating oogonia) in the ovaries of E2-induced feminized Japanese eels, and no positive signal was observed in the primary oocytes. *Dmrt1* was suggested to directly regulate the expression of the meiotic inducer *Stra8* and plays an important role in promoting oogenesis in the fetal mouse ovary; *Dmrt1* is also required for the formation of normal numbers of ovarian follicles in the juvenile mouse ovary (Krentz et al., 2011). However, according to Matson et al. (2011), DMRT1 suppresses the expression of *Foxl2* and reprograms granulosa cells into Sertoli-like cells in adult mice. We suggest that *dmrt1* is not only critical in spermatogenesis but also play a role in oogenesis in Japanese eel. The possible role for *dmrt1* in regulating female meiosis and testicular differentiation during gonadal development requires further investigation.

4.5. Summary

In summary, the Vasa protein was expressed in gonadal germ cells in a stage-specific manner, suggesting that Vasa is a suitable germ cell marker in Japanese eels. The full-length Japanese eel *dmrt1* cDNA was cloned in this study, and the deduced amino acid sequence of the DM domain corresponds to the homologous domains in teleost Dmrt1 sequences. Dmrt1 proteins were abundantly expressed in spermatogonia B cells, spermatocytes, and spermatids, but not in spermatozoa, spermatogonia A cells and Sertoli cells. Dmrt1 proteins also exhibited a restricted localization in differentiating oogonia in early differentiating gonads (ovary-like structures) of male eels and in E2-induced feminized Japanese eels. We propose that Dmrt1 might play vital roles at specific stages of spermatogenesis ranging from spermatogonia B cells to spermatids, and might also play a role in oogenesis in the Japanese eel.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.03.012.

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