A DOCTORAL DISSERTATION

DMRT in Protogynous Wrasse, *Halichoeres tenuispinis*

: Molecular Identification and Characterization of Putative Regulatory Region

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GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

August, 2008

자성선숙성 자웅동체어류인 놀래기 (*Halichoeres tenuispinis*)의 *DMRT*

: 유전자 분석 및 조절영역의 특성에 관한 분자생물학적 연구

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정 형 복

이 논문을 이학 박사학위 논문으로 제출함

2008년 8월

정형복의 이학 박사학위 논문을 인준함

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2008년 8월

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A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

August, 2008

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August 2008

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LIST OF ABBREVIATIONS

17α, 20β-DP	17α, 20β-dihydroxy-4-pregnen-3-one
Dax1	dosagesensitive sex reversal, adrenal hypoplasia congenita, critical region on the X-chromosome, gene 1
DMO	*Doublesex-Mab3* domain containing gene in Ovary
DMRT	*Doublesex-Mab3* Related Transcription factor
DMY	DM-domain gene on the Y chromosome
dsx	Doubsex
DW-ACP	DNA-Walking Annealing Control Primer
GSI	Gonado-Somatic Index
GSP	Gene Specific Primer
Mab3	Male abnormal 3
Mis	Mullerian-inhibiting substance / Anti-Mullerian Hormone
ORF	Open-Reading Frame
P450scc	Cytochrome P450, family 11, subfamily A, polypeptide 1
PCR	Polymerase Chanin Reaction
PIP	Percentage Identity Plot
RACE	Rapid Amplification of cDNA Ends
SF1	Steroidogenic Factor 1
Sox9	SRY-like HMG-box 9
SRY	Sex-determining Region Y
TDF	Testis Determining Factor
TSP	Target Specific Primer
TSPN	Nested Target Specific Primer
UTR	Un-Translated Region
VBP	Vitellogenin Binding Protein
Wt1	Wilms′ tumor gene 1

ABSTRACT

The physiological, behavioral, and functional sex of fish may be determined or induced, and is under the control of various genetic, social, and environmental factors. Fishes exhibit diverse reproductive strategies, including gonochorism, sequential hermaphroditism (protogynous and protoandrous), or synchronous hermaphroditism, with some self-fertilizing species. Despite this diversity, the genes than control sex determination and differentiation are conserved in many animal species. During development, steroid-producing cells convert pregnenolone to testosterone, which functions in sex-hormone synthesis and eventually results in functionally mature sexes. Androgens and estrogens are also conserved as hormonal regulators. Other examples of conserved genes include those encoding 3β-hydroxysteroid dehydrogenase, 11 β-hydroxylase, Dax, Hox, Wnt, SF, and aromatases. Sex determination in fish is regulated by DMY, a homolog of the mammalian sex-determining gene SRY. DMY is also located on the Y chromosome and is a duplicate copy of the *DMRT* gene, which contains a conserved DM domain region and male-specific motif found in *Drosophila melanogaster doublesex* (*dsx*) and *Caenorhabditis elegans mab3*. These male-specific DM domain-containing genes encode novel zinc-finger transcription factors and play key roles in sex development and/or determination.

This study was performed to gain insight into sexual differentiation in the protogynous wrasse *Halichoeres tenuispinis*. First, a full-length cDNA from the wrasse testis (*DMRT*) and partial cDNA from the ovary (*DMO*) were isolated by cDNA library screening or rapid amplification of cDNA ends (RACE). Wrasse *DMRT* was 3,119 bp long and contained the DM domain and the male-specific motif, but not the DMA or DMB domain. It was highly

homologous to *DMRT* cDNAs isolated in other species. Two cDNAs were identified for *DMRT*: a short sequence and a second, longer sequence with a relatively long 5′-untranslated region (UTR) and additional nucleotide insertions. The *DMO* cDNA was 545 bp long; the first exon showed 100% nucleotide sequence homology to wrasse *DMRT* cDNA, but the 5′-UTR was split into two pieces showing near identity to the *DMRT* genomic sequence. The deduced amino acid sequence of wrasse *DMRT* included a zinc finger DNA-binding motif and confirmed that the DM domain is highly conserved within phyla. The predicted tertiary structure of the wrasse DM domain showed strong similarity to DM domains in *D. melanogaster dsx* and human *DMRT*. Northern blot analysis identified a 3.2-kb transcript roughly equivalent in size to the *DMRT* nucleotide sequence detected in the testis, but not in the ovary, confirming that this sequence is male-specific in protogynous wrasse. Southern blot analysis suggested that the wrasse genome contains two copies of the *DMRT* gene. To analyze *DMRT* structure, contigs were generated from wrasse genomic DNA and re-arranged according to the *DMRT* cDNA sequence. The open reading frame (ORF) consisted of five exons and four introns. The first through fifth exons encoded 73-, 58-, 53-, 63-, and 53-amino acid sequences, respectively. Donor-acceptor splice sites (GT-AG) were identified at all exon-intron junctions. To better understand the transcriptional regulation of *DMRT*, DNA walking was used to clone a 1,721-bp sequence from the 5′-flanking region of wrasse testis genomic DNA, and 21 putative regulatory sites were identified. Seventeen regions harboring GATA1, AP4, GATA2, GATAx, Sox5, AP1, C/EBP, Dof2, AP1, STATx, C/EBP, Dof3, GATA1, GATA3, AP1, Dof1, SRY, C/EBP alpha, C/EBP beta, TATA, and CAP binding sites were amplified from the 5′-flanking region. To evaluate transcriptional regulation, 5′-deletion and 5′-flanking region mutants for these 17 regions were constructed, ligated into luciferase-expressing pGL3-Basic or pGL3-Enhancer

vectors, and then transiently transfected into Cos-1 and TM4 cells. In both cell lines, pGL3-Enhancer chimeric mutants showed significant regulatory activity, but not pGL3-Basic chimeric mutants. Distal GATA binding sites (-1,721 to -1,362) and the proximal SRY binding region (-330 to -123) were important for transcriptional regulation of the wrasse *DMRT* gene. Regulatory activities of the distal and proximal regions were 81- and 17-fold higher, respectively, than that of the non-chimeric luciferase vector, which was used as an internal control. Although several DM domain-containing genes have been isolated in fishes, the function and signaling mechanisms of these genes remain unclear. Further studies are required to identify regulators of the *DMRT* gene and to determine the role of *DMRT* during sex reversal in protogynous wrasse.

Key words: Wrasse, *DMRT, DMO,* Protogynous hermaphrodite, DMY, ORF, cDNA library, RACE, Northern blot, Southern blot, Gene structure, PCR contig, Promoter, Luciferase, SRY, GATA, Regulation of gene expression, Cos-1, TM4, Transfection

INTRODUCTION

1. Background of Research

The plasticity of fish physiology offers an enormous opportunity for the molecular study of sex determination, differentiation, and development. The physiological, behavioral, and functional sex of fish may be determined or induced, and is under the control of various genetic, social (Sunobe and Nakazono, 1993), and environmental factors (Nakamura *et al.*, 1998). Fishes exhibit diverse reproductive strategies, including gonochorism, sequential hermaphroditism (protogynous and protoandrous; Sadovy and Shapiro, 1987), or synchronous hermaphroditism (Atz, 1964), with some self-fertilizing species (Soto *et al.*, 1992; Cole and Noakes, 1997).

In hermaphroditic fishes, both male and female sex can be observed in an individual during the reproductive season. Hermaphroditism and the karyotype of fish were previously described by Delvin and Nagahama (2002). In protandrous hermaphrodites, individuals begin life as males and then, given the proper cue, undergo sex reversal (Hattori, 1991; Godwin *et al.*, 1996). In protogynous hermaphrodites, female sex occurs first (Reinboth and Brusle-Sicard, 1997; Shapiro *et al.*, 1993). In nature, sex reversal is triggered by social and/or environmental cues (Warner *et al.*, 1991; Munday *et al.*, 1998; Oliveira *et al.*, 2002) and involves re-organization of gonadal cell types, duct systems, hormonal profiles, and sex-specific behaviors; this process may be relatively rapid, sometimes occurring within only a few weeks (Godwin, 1994). *Halichoeres tenuispinis* is a protogynous wrasse that commonly occurs in the northwestern Pacific, and was first described by Gunther in 1862. This species spawns in the summer (June-July) and undergoes a color change

during sex reversal (Lee *et al.*, 1993).

Endogenous hormones play critical roles in sex determination, differentiation, and development, which involve complex interactions between gonadotropins and steroids produced in the pituitary and the gonads, respectively (Bieniarz and Epler, 1992; Nagahama, 1994). Testis development occurs in two stages: testis formation and the maintenance and differentiation of the Wolffian duct. These events are regulated by many factors, including steroids and their associated genes (Barsoum and Yao, 2006). In general, the steroid estradiol produces a female phenotype (Yamamoto, 1969), whereas testosterone and 11-ketotestosterone produce a male phenotype (Jiang *et al.*, 1996; Nagahama, 1999). Moreover, exogenous steroids can suppress or influence the genetically programmed path of sex determination or development; for example, exogenous estradiol inhibited the production of 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) *in vitro* (Vizziano *et al.*, 1996). Several steroid-related genes, such as P450scc (Takahashi *et al.*, 1992), 11β-hydroxylase (Jiang *et al.*, 1996), the androgen and estrogen receptors (Kim *et al.*, 2002), and aromatase (Chang *et al.*, 1997; Choi *et al.*, 2005) have been cloned in fish. These genes play important roles in sexual maturation; for example, the 11β-hydroxylase gene is more highly expressed in the developing testis than in the ovary (Liu *et al.*, 2000).

Despite such diversity and plasticity, many animals appear to share some similarities regarding the molecular mechanism of sex determination, development, and differentiation. Although the upstream regulators of these pathways are extremely diverse, the downstream regulators appear to be at least partially conserved (Raymond *et al.*, 1998). During animal development, steroid-producing cells convert pregnenolone to testosterone, which functions in sex hormone synthesis and leads to the development of functionally mature sexes. In mammals, the *SRY* gene on the Y chromosome was identified as a sex-determining gene (Sinclair *et al.*, 1990). SRY interacts with the regulatory

region of the aromatase gene promoter (Haqq *et al*., 1993) and binds directly to the *Sox9* gene promoter (Kent *et al*., 1996). SRY also interacts with *Dax1*, *SF1*, *Wt1*, *Lim1*, and *Wnt4* in the control of sex determination and gonadal development (McElreavey *et al*., 1993; Koopman, 1999; Goodfellow and Camerino, 1999; Kim *et al*., 2006; Wilhelm *et al*., 2007). The role of *Dax1*, an antagonist of SRY, was examined in species that lack sex chromosomes but express the SRY-related gene, *doublesex* and *mab3*-related transcription factor 1 (*DMRT*1; Raymond *et al*., 1998; Western *et al*., 2000; Sugita *et al*., 2001). Recent studies have reported the presence of small germline-specific RNAs (29-30 nucleotides) that are highly abundant in mouse, rat, and human testis and bind to the murine Piwi protein (Aravin *et al*., 2006; Girard *et al*., 2006). This expression profile in the testis implies another possible mechanism for *SRY*-independent sex determination and development. In additional, positional cloning identified a DM domain-containing *SRY* homolog, designated *DMY*, located on the Y chromosome in the region essential for male germline cell development (Matsuda *et al*., 2002; Nanda *et al*., 2002). *DMY* is a duplicate copy of the autosomal *DMRT*1 gene, which contains conserved regions of the *doublesex* (*dsx*) and *mab3* genes and shows sex-specific expression (Raymond *et al*., 1998; Smith *et al*., 1999; Matsuda *et al*., 2003; Kobayashi *et al*., 2004). *DMY* spans approximately 280 kb of the Y-specific region and is considered to be a testis determining factor (TDF) in fish. However, although many studies have confirmed the role of *DMY* as a TDF in fish, it does not seem to be universal (Veith *et al*., 2003; Volff *et al*., 2003). Several genes containing the DM DNA-binding domain have been isolated and characterized in humans (Raymond *et al*., 1998), medaka (Matsuda *et al*., 2003; Winkler *et al*., 2004), chicken (Nanda *et al*., 2000), frog (Shibata *et al*., 2002), turtle (Kettlewell *et al*., 2000; Torres Maldonaldo *et al*., 2002), mouse (Smith *et al*., 1999, 2002), tilapia (Guan *et al*., 2000), platyfish (Kondo *et al*., 2002; Veith *et al*., 2003), orange-spotted grouper (Xia *et al*.,

2007), and rainbow trout (Marchand *et al.*, 2000).

DMRT is thought to function as a transcription factor for downstream genes in the sex-determining pathway (Burtis *et al.*, 1991; Shen and Hodgkin, 1988). *DMRT* expression is readily up-regulated in the gonad during embryogenesis and female-to-male sex reversal via treatment with aromatase inhibitors or steroids. In addition, an SRY binding site was identified in the 5′-flanking region of the tilapia *DMRT1* gene, but not in the tilapia *DMO*, implying that *DMRT1* may play the role of the *Sox9* gene in tilapia sex determination and/or development (Guan *et al.*, 2000). For these reasons, *DMRT1* is considered to be a good starting point for the investigation of sex determination or differentiation pathways (Richard-Mercer *et al.*, 1995; Marchand *et al.*, 2000; Smith *et al.*, 2003). *DMRT1* is dominantly expressed in the testis, but, as an indicator of its functional diversity, ovarian expression has also been reported (Guo *et al.*, 2005). *DMRT* is also involved in phenotypic disorders, such as growth retardation in humans (Ounap *et al.*, 2004; Hong *et al.*, 2007). Several types of DM domain-containing genes were isolated from the medaka (*Oryzias latipes*) autosome, some of which showed non-sex-specific expression profiles. For example, *DMRT2* is expressed in early somites, *DMRT3* in dorsal interneurons, and *DMRT4* in the developing olfactory system (Winkler *et al.*, 2004; Huang *et al.*, 2005). Although *SRY* is a primary TDF, a gain-of-function mutation in the *DMRT* gene leads to testis induction without SRY, suggesting that the *DMRT* gene plays a role in an as yet unknown male sex determination pathway. In the genital ridge of chicken embryos, *DMRT1* showed sex-specific expression in the ZW chromosome system, and its expression level differed among tissues (Raymond *et al.*, 1999). In turtles, which show temperature-dependent sex determination, *DMRT1* was up-regulated in the testis and down-regulated in the ovary during the sex-determining period, suggesting that *DMRT1* functions in SRY-independent, temperature-dependent sex determination

(Shoemaker *et al.*, 2007). In salamanders, aromatase was up-regulated and *DMRT* was down-regulated during genetic male to phenotypic female sex reversal, implying that *DMRT*s have a male-biased function (Sakata *et al.*, 2006). *DMRT*s are not only differentially expressed in different stages of the gonad, but they are also restricted to specific stages and cell types during spermatogenesis, indicating that the *DMRT* genes are sex-, tissue-, and stage-specific regulators (Xia *et al.*, 2007).

DM domain-containing genes are zinc-finger transcription factors that play key roles in sex development and/or determination. *DMRT1* isolated from the testis has a DM domain in the 5′-region of the open reading frame (ORF), in addition to a conserved male-specific motif in the 3′-region. This motif is a homolog of the *dsx* male splicing variant (dsx^m) found in *D. melanogaster*, which is expressed in a male-specific manner (Raymond *et al.*, 1998). In contrast, the ovary-specific DM domain-containing gene, *DMO*, lacks this motif (Guan *et al.*, 2000; Marchand *et al.*, 2000). Regardless of the function of the DM domain in sex determination and differentiation, *DMRT*s lacking the DM domain were reported to function in various tissues with somewhat different results (Ottolenghi *et al.*, 2002; Veith *et al.*, 2006; Kato *et al.*, 2008). In addition to the DM domain, conserved DMA domains were found in *DMRT* genes isolated from several species, including *Daphnia magna*, which switches between sexual and asexual reproduction. Thus, the function of the DM domain remains unclear (Ottolenghi *et al.*, 2002; Kato *et al.*, 2008). The DM domain binds to DNA as a dimer, allowing the recognition of pseudopalindromic sequences (Erdman *et al.*, 1996; Yi and Zarkower 1999; Zhu *et al.*, 2000). In the *dsx* gene, the DM domain sequence showed a novel zinc module containing CCHC and HCCC zinc-binding sites (see Figs. 8 and 10). Nuclear magnetic resonance (NMR) analysis revealed that these sites share the consensus sequence C-x(2)-C-x(2)-H-x(8)-H-x(3,4)-C-x(4)-C-x-C-x(2,3)-C (Zhu *et al.*, 2000). In the *dsx* DM domain, cysteine residues are essential for

DNA binding and chelate zinc in a distinct manner compared to the classical zinc finger (Erdman and Burtis, 1993; Erdman *et al.*, 1996). DNA sequence recognition is dependent upon the basic C-terminal tail, which contacts the minor groove of the target sequence (Zhu *et al.*, 2000). Although the C-terminal tail of *DMRT* is disordered, it forms a nascent α-helix at low temperatures and is required for DNA recognition and biological function (Zhu *et al.*, 2000).

Several isoforms of the *DMRT1* gene have been identified. In zebrafish, three *DMRT1* cDNAs generated by alternative splicing, designated *DMRT1a*, *DMRT1b*, and *DMRT1c*, encode proteins of 267, 246, and 132 amino acids, respectively (Guo *et al.*, 2005). In addition, the number of exons varies among *DMRT* genes. In zebrafish, the *DMRT1a*, *DMRT1b*, and *DMRT1c* genes showed seven, five, and three exons, respectively (Guo *et al.*, 2005), whereas zebrafish *DMRT5* consisted of only two exons (Guo *et al.*, 2004). Medaka *DMRT2* and platyfish *DMRT2* consisted of three exons, whereas medaka *DMRT4* and platyfish *DMRT4* consisted of two exons (Kondo *et al.*, 2002). In Takifugu rubripes, the *DMRT1* gene showed genome-wide conservation in comparison to medaka, and a percentage identity plot (PIP) revealed significant homology between the fugu *DMRT1* genomic region and the human genome (Brunner *et al.*, 2001).

The presence of an SRY binding site in the promoter region of *DMRT1* (Guan *et al.*, 2000) suggested that *DMRT1* may play a role similar to that of *Sox9*, which is also up-regulated by SRY binding (Clarkson and Harley, 2002; Koopman, 1999). Repeat sequences of GATA and GACA are associated with sex chromosomes in *Poecilia reticulata* (Nanda *et al.*, 1992). Based on the degree of sequence similarity in the 5′-flanking region among humans, pigs, and mice, the *DMRT1* gene is thought to be regulated in a conserved fashion (Boyer *et al.*, 2002). Although these homologous regions were physically separated, aligned regions showed over 60% sequence homology. However,

additional studies have indicated that although the *DMRT* gene is conserved among species, its regulation may differ significantly. In the rat, *DMRT1* was expressed in Sertoli cells under the regulation of GATAx, GATA4, SP1, and Egr1 (Lei and Heckert, 2002, 2004); in contrast, this gene is expressed in spermatogonia, primary spermatocytes, and secondary spermatocytes in orange-spotted grouper (Xia *et al*., 2007). These data suggest that *DMRT* expression may be regulated via different mechanisms among species. GATA, which is a primary regulator of the *DMRT* gene, also shows a synergistic effect with vitellogenin-binding protein (VBP) in the regulation of the vitellogenin (vtg) gene (Teo *et al*., 1999).

2. Objectives

The plasticity of sex differentiation in hermaphroditic fish offers a unique opportunity to examine the molecular mechanisms of natural sex reversal. Despite abundant evidence that exogenous factors can determine or induce functional sex in hermaphroditic fish, many questions remain regarding the mechanism of this complicated process. Moreover, although many studies have examined the various roles of *DMRT* genes in sex determination and/or differentiation, very little information is available regarding transcriptional regulation of this gene and the roles of *DMRT* subtypes and isoforms in hermaphroditic fish.

To understand the molecular mechanisms of sex determination and sex differentiation in hermaphroditic fish, we examined the structure and regulation of sex determination-related genes in the protogynous wrasse *Halichoeres tenuispinis*. Full-length *DMRT* and partial *DMO* cDNAs were isolated by screening a cDNA library or by rapid amplification of cDNA ends (RACE) and then characterized. The full-length wrasse *DMRT1* cDNA (3,119 bp) contained both the conserved DM domain and the male-specific motif.

The partial *DMO* cDNA (545 bp) contained most of the DM domain region. Regarding the structure of wrasse *DMRT*, contiguous sequences (contigs) were generated from genomic DNA, and five exons were identified. Southern blot analysis was performed to verify the gene copy number for wrasse *DMRT*, and Northern blot analysis was performed to detect transcripts. To examine the regulation of the wrasse *DMRT* gene, a 1,721-bp sequence from the 5′-flanking region was isolated and then recombined with the luciferase reporter gene to construct wrasse *DMRT*-luciferase chimeric mutants. Luciferase assays revealed that the proximal and distal regions, which contain SRY and GATA binding sites, respectively, were important for the transcriptional regulation of wrasse *DMRT*.

MATERIALS AND METHODS

1. Experimental animals

Wrasse (*H. tenuispinis*) were collected at the same location on the coastline of Jeju Island, Korea, throughout the reproductive season (May to June). After anesthetizing on ice, body length, body weight, and gonad weight were determined for each individual and were used to calculate the gonado-somatic index (GSI). The gonads, brain, liver, kidney, spleen, muscle, gill, and heart were then dissected out and stored in liquid nitrogen until RNA and DNA extraction.

2. Total RNA, mRNA and genomic DNA isolation

Total RNA was extracted from each tissue using RNAzol B (TEL-TEST, Friendswood, TX, USA) or Tri-reagent (MRC, Cincinnati, OH, USA), according to the manufacturer's protocol. Approximately 100 mg of frozen tissue were homogenized in 1 *m*L of cold RNAzol B or Tri-reagent in 1.5-*m*L tubes using a pestle, vortexed, mixed with 200 μL of chloroform, and then vortexed again. Homogenates were incubated on ice for 5 min and then centrifuged at 12,000 × g for 15 min at 4℃. The upper aqueous phase was transferred into a clean tube and incubated with 0.5 *m*L of isopropanol for 10 min at room temperature. The RNA precipitate was centrifuged at 12,000 × g for 5 min at 4℃, after which the supernatant was removed and washed in 1 *m*L of 75% diethyl pyrocarbonate (DEPC)-treated EtOH and centrifuged at 7,500 × g for 5 min at 4℃. After removing the DEPC-treated EtOH, the RNA pellet was air-dried at room temperature and dissolved in 50-100 μL of

DEPC-treated water. To prevent DNA contamination, total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA), according to the manufacturer's protocol. To quantify RNA and assess its quality, absorbance was measured at 260 and 280 nm. RNA solutions with A260/280 ratios of 1.6-1.8 were used for further experiments. Messenger RNA from the testis and ovary was isolated using the PolyATract mRNA Isolation System (Promega), according to the manufacturer's protocol. Isolated mRNA was annealed with biotinylated oligo (dT) primer by heating at 65℃ for 10 min. After adding 3 μL of Biotinylated-Oligo(dT) Probe and 13 μL of 20 × SSC, the samples were incubated at room temperature for 10 min. Annealed oligo (dT)-mRNA hybrids were labeled with streptavidin-paramagnetic particles and captured using a magnetic stand. The captured RNA particles were washed four times in 300 μL of 0.1 × SSC and then eluted in 250 μL of DEPC-treated water. Genomic DNA was isolated from 10 mg of testis and liver using a Wizard Genomic DNA Purification kit (Promega). Tissues were homogenized in 600 μL of nuclei lysis solution and incubated at 65℃ for 25 min. Nuclear lysates were treated with RNase and protein precipitation solution, and, after centrifuging at 13,000 × g for 4 min, the DNA-containing supernatant was transferred to a new tube. DNA was precipitated in 600 μL of isopropanol, centrifuged at 13,000 × g for 1 min, and washed with 70% EtOH. DNA was dissolved in 100 μL of DNA rehydration solution and used in further experiments.

3. cDNA synthesis

Polymerase chain reaction (PCR) used to synthesize cDNA from 0.5 μg of total RNA using an AdvanTage RT-for-PCR kit (Clontech, Palo Alto, CA, USA). Total RNA was diluted in 6.25 μL of DEPC-treated water and annealed with 0.5 μL of oligo (dT) primer (0.5 μg/μL) by incubating at 70℃

for 2 min. Then, 2 μL of 5× reaction buffer, 0.5 μL of dNTP mix (10 mM each), 0.25 μL of RNase inhibitor (10 U), and 0.5 μL of MMLV-reverse transcriptase were added to the annealed RNA and incubated at 42℃ for 60 min. Synthesis was terminated by heating at 94℃ for 5 min, and the purified DNA was then diluted in 40 μL of nuclease-free water.

4. Oligonucleotide primers and probes

Primer sets for the amplification of DM domain-containing cDNA fragments were designed based on the conserved *DMRT* cDNA DM domain regions from *Oncorhynchus mykiss* (accession number AF209095), *Oreochromis niloticus* (AF203489, AF203490), *Danio rerio* (AF305094), and *H. tenuispinis* (AY177711) stored in GenBank (Table 1). Next, cDNA library screening was performed using radio-labeled PCR product amplified using degenerate primers (Table 1) as an oligonucleotide probe. Specific intron- and exon-spanning primers were used to amplify the target sequence during Northern and Southern blotting, respectively (Table 2). The probes were prepared using a HexaLabel^{TM} DNA Labeling kit (Fermentas, Hanover, MD, USA), and 100 ng of purified target-specific PCR products were labeled with 50 μCi of [α-^{32}P]-dCTP using exo- Klenow fragment. The sizes of probes used in Northern and Southern blotting for *DMRT* and β-actin were 416, 411, and 510 bp, respectively. The primers used in RACE were designed based on the DM domain region of wrasse *DMRT* cDNA (Table 2). The primer sets used to generate contigs for the ORF of the *DMRT* gene from wrasse genomic DNA were designed by comparing the genomic DNA sequences of *O. latipes DMRT1a* (AY157712) and *F. rubripes DMRT1* (AJ295039) to the wrasse *DMRT* cDNA sequence (AY177711) (Table 3). Seventeen promoter clones were generated using specific primer sets that were designed to amplify putative regulatory regions (Table 4). Nested target-specific primers (TSP) were designed to isolate the promoter region from genomic DNA (Table 5).

Table 1. Primer sets used in this study. Cloning of *DMRT* and *DMO* cDNA fragments, synthesizing probes to screening cDNA library, and sequencing of wrasse *DMRT* were done by using these primers

Primer	Sequence	Description
DM1 F	5′-CCCMGGATGCCCAAGTGCTCY-3′	Sense primer for *DMRT* cDNA fragment and probe synthesis
DM1 R	5′-YTCYTCTTGRGCCTGCTGCCK-3′	Antisense primer for *DMRT* cDNA fragment
DM2 F	5′-CGSTGYAGGAAYCACGGMTAC-3′	Sense primer for *DMRT* cDNA fragment
DM2 R	5′-CAGAGCMACCTGGGCCGCCAT-3′	Antisense primer for *DMRT* cDNA fragment and probe synthesis
DM3 F	5′-CATCACAGGGAGTCGCTC-3′	First internal sense primer for sequencing *DMRT*
DM3 R	5′-GCTTGGGGAACAGCATAG-3′	First internal antisense primer for sequencing *DMRT*
DM4 F	5′-GCGATGCTGCTAAATAAG-3′	Second internal sense primer for sequencing *DMRT*
DM4 R	5′-TCCTTTTTGCTCATTCGG-3′	Second internal antisense primer for sequencing *DMRT*
DMO1 F	5′-GGTTTTGTGTCTCCCCTGAA-3′	Sense primer for *DMO* cDNA fragment
DMO1 R	5′-GCCATGACTCTCTGCCTCTCAGCT-3′	Antiense primer for *DMO* cDNA fragment
DMO2 F	5′-CTTTTGCCGMTGGAGRGACTGC-3′	Sense primer for *DMO* cDNA fragment
DMO2 R	5′-GCTTGCCAGAGTCACAGGA-3′	Antiense primer for *DMO* cDNA fragment

* R (A, G), Y (C, T), M (A, C), K (G, T)

Table 2. Primers used for RACE PCR, 5′-flanking region cloning, and probes synthesis for Northern and Southern blotting

Primer	Sequence	Description
RDM1	5′-GCCATGACTCTCTGCCTCTCAGCT-3′	Gene-Specific Primer for 5′-RACE
RDM2	5′-CTGCCTTCTCAGAGCAACCT-3′	Nested primer for 5′-RACE
RDM3	5′-TAAGTGCTCCCGCTGTCG-3′	Gene-Specific Primer for 3′-RACE
RDM4	5′-GGTTTTGTGTCTCCCCTGAA-3′	Nested primer for 3′-RACE
5′-RACE	5′-GTCTACCAGGCATTCGCTTCAT-3′	5′-RACE primer
3′-RACE	5′-CTGTGAATGCTGCGACTACGAT-3′	3′-RACE primer
TDM1	5′-GCCATGACTCTCTGCCTCTCAGCT-3′	First Target-Specific Primer for cloning 5′-flanking region
TDM2	5′-CAGGGGAGACACAAAACCGTGG-3′	Second Target-Specific Primer for cloning 5′-flanking region
TDM3	5′-CCGACAGCGGGAGCACTTAGGC-3′	Third Target-Specific Primer for cloning 5′-flanking region
DW-ACP1	5′-[ACP^{TM}]-AGGTC-3′	1st ACP^{TM} primer for 1st DNA Walking PCR
DW-ACP2	5′-[ACP^{TM}]-TGGTC-3′	2nd ACP^{TM} primer for 1st DNA Walking PCR
DW-ACP3	5′-[ACP^{TM}]-GGGTC-3′	3rd ACP^{TM} primer for 1st DNA Walking PCR
DW-ACP4	5′-[ACP^{TM}]-CGGTC-3′	4th ACP^{TM} primer for 1st DNA Walking PCR
DW-ACPN	5′-[ACPN^{TM}]-GGTC-3′	Nested ACP^{TM} primer for 2nd DNA Walking PCR
Uni-primer	5′-TCACAGAAGTATGCCAAGCGA-3′	Universal primer for 3rd DNA Walking PCR
PDM1 F	5′-AGAGACTGCCAGTGCCCTAA-3′	Sense primer for Northern probe synthesis
PDM1 R	5′-GAGTGCATGCGGTACTGAGA-3′	Antisense primer for Northern probe synthesis
PDM2 F	5′-ACCTCTGTGCCCATCAAAAG-3′	Sense primer for Southern probe synthesis
PDM2 R	5′-GGTCCACATCTAAACTGCTGTG-3′	Antisense primer for Southern probe synthesis
β-actin F	5′-ACTACCTCATGAGAGTCCTG-3′	Sense primer for β-actin probe synthesis
β-actin R	5′-TTGCTGATCGACATCTGCTG-3′	Antisense primer for β-actin probe synthesis

5. cDNA library construction and screening

A cDNA library was constructed from testis RNA using a SMART™ cDNA Library Construction kit (Clontech). First-strand cDNA was synthesized from 1 μg of total RNA using SMART IV Oligonucleotide, CDS III/3′ PCR primer, and PowerScript™ Reverse Transcriptase, and then amplified via long distance (LD) PCR (pre-heated for 20 s at 95℃, followed by 20 cycles at 95℃ for 5 s and 68℃ for 6 min) using an Advantage 2 PCR kit (Clontech). The amplified cDNA mixture was treated with 40 μg of Proteinase K and digested with 200 U of SfiI restriction enzyme. cDNA purification was performed using CHROMA SPIN-400 Columns, and five peak fractions were collected after verifying by electrophoresis on a 1.1% agarose gel. Purified cDNA was ligated to a λTriplEx2 vector and packed into a λ phage using Gigapack III Gold Packaging Extract (Stratagene, La Jolla, CA, USA). Phages packed with the unamplified cDNA library were transduced into *Escherichia coli* XL1-Blue, and 17.1×10^6 pfu/*m*L were acquired. Next, 6.8×10^4 phages were amplified to a final titer of 1.5×10^{10} pfu/*m*L, and 3.5 μL of the diluted cDNA library (1.5×10^4 pfu/μL) were used for screening. Phages containing the cDNA library were transduced into *E. coli* XL1-Blue and cultured for 12 h on plates containing 4 *m*L of LB/$MgSO_4$, and then plaques were blotted onto positive-charged nylon membrane (Osmonics, Westborough, MA, USA). This cDNA replica was hybridized in 50% deionized formamide, 5× SSC, 0.02% SDS, and 1% Denhardt's solution at 37℃ for 12 h with 10 ng of ^{32}P-labeled probe synthesized using the HexaLabel™ DNA Labeling kit (Fermentas). The membrane was washed under low- (2× SSC, 0.1% SDS, at room temperature) and high-stringency conditions (0.1% SSC, 0.1% SDS, at 65℃), and then exposed to X-ray film at -70℃ for 2 days. λTriplEx2 extracted from positive plaques obtained via screening was converted to a pTriplEx2 plasmid in *E. coli* BM25.8 by in vivo

Table 3. Primer sets used for construction of PCR contigs

Primer	Sequence	Description
DMST1 F	5′-CCGACAGCGGGAGCACTTAGGC-3′	1st sense primer used for construction of PCR contig
DMST1 R	5′-TCACAGAAGTATGCCAAGCGA-3′	1st antisense primer used for construction of PCR contig
DMST2 F	5′-GCCGCTCGAGCTCCAGATTGGCATCG-3′	2nd sense primer used for construction of PCR contig
DMST2 R	5′-GGCAATGCCGTTGAACTTTACTG-3′	2nd antisense primer used for construction of PCR contig
DMST3 F	5′-CAAACTCATTAAAGCTGCATGG-3′	3rd sense primer used for construction of PCR contig
DMST3 R	5′-CATTTCACACTCTGAGCTGAGC-3′	3rd antisense primer used for construction of PCR contig
DMST4 F	5′-TCACAGAAGTATGCCAAGCGA-3′	4th sense primer used for construction of PCR contig
DMST4 R	5′-GAGTGCATGCGGTACTGAGA-3′	4th antisense primer used for construction of PCR contig
DMST5 F	5′-CAGATGCCTCATGGAGACAAC-3′	5th sense primer used for construction of PCR contig
DMST5 R	5′-AGGCTGCCATGGTCTCAG-3′	5th antisense primer used for construction of PCR contig
DMST6 F	5′-TCTGTGTGCCACCTCGCTTCA-3′	6th sense primer used for construction of PCR contig
DMST6 R	5′-TCACAGAAGTATGCCAAGCGA-3′	6th antisense primer used for construction of PCR contig
DMST7 F	5′-CAGTTTGGCCTTAGTCTTATTCC-3′	7th sense primer used for construction of PCR contig
DMST7 R	5′-TCACAGAAGTATGCCAAGCGA-3′	7th antisense primer used for construction of PCR contig

* Universal primer from DNA Walking PCR was respectively named as DMST1 R, DMST4 F, DMST6 R, and DMST7 R for convenience of explanation.

Table 4. Primers used for *DMRT*-luciferase chimeric mutants construction

Primer	Position	Sequence	Linker	Description
ProLu1 F	-122	5′-GCCGCTCGAG CTCCAGATTGGCATCG -3′	Xho I	Sense primer for region 1
ProLu1 R	+50	5′-GCCCAAGCTT AGAGGTTCGGTGAGCTCCG -3′	Hind III	Antisense primer for region 1
ProLu2 F	-330	5′-GCCGCTCGAG ACACACGAGAAATACACAAAC -3′	Xho I	Sense primer for region 2
ProLu2 R	-123	5′-GCCCAAGCTT GCTGTAAGGTCACGGTCTC -3′	Hind III	Antisense primer for region 2
ProLu3 F	-489	5′-GCCGCTCGAG GTTTGCTTTCAGTCCCAG -3′	Xho I	Sense primer for region 3
ProLu3 R	-331	5′-GCCCAAGCTT GTGTCGGGGGGCTACTTAAC -3′	Hind III	Antisense primer for region 3
ProLu4 F	-676	5′-GCCGCTCGAG TATACAATTACAATGACCAGCGTC -3′	Xho I	Sense primer for region 4
ProLu4 R	-490	5′-GCCCAAGCTT CCAACTTGTTGCAACTATGAGTC -3′	Hind III	Antisense primer for region 4
ProLu5 F	-868	5′-GCCGCTCGAG ATCTCTCTTCAAAAACCCACTG -3′	Xho I	Sense primer for region 5
ProLu5 R	-677	5′-GCCCAAGCTT CGATAACTTACCTGGTTCTGACGTG -3′	Hind III	Antisense primer for region 5
ProLu6 F	-1,090	5′-GCCGCTCGAG TATCATAATACCTCACCATATG -3′	Xho I	Sense primer for region 6
ProLu6 R	-869	5′-GCCCAAGCTT CAGACTGTACCATTATGAGCTG -3′	Hind III	Antisense primer for region 6
ProLu7 F	-1,361	5′-GCCGCTCGAG ACGGCTGTTTGAATGTCTTC -3′	Xho I	Sense primer for region 7
ProLu7 R	-1,091	5′-GCCCAAGCTT GTATTCCCTTTGGGTTTGTTGC -3′	Hind III	Antisense primer for region 7
ProLu8 F	-1,547	5′-GCCGCTCGAG TATAGCCAATCACTGGGTGTC -3′	Xho I	Sense primer for region 8
ProLu8 R	-1,342	5′-GCCCAAGCTT GAAGACATTCAAACAGCCCTG -3′	Hind III	Antisense primer for region 8
ProLu9 F	-1,721	5′-GCCGCTCGAG GTCTGAGTCGGTATTGC -3′	Xho I	Sense primer for region 9
ProLu9 R	-1,548	5′-GCCCAAGCTT GAAATCAGCAACCCAGATAGC -3′	Hind III	Antisense primer for region 9

Table 5. Primers used for cloning unknown sequences in genomic DNA for the construction of PCR contigs

Primer	Sequence	Description
TSPa 1	5′-GCCATGACTCTCTGCCTCTCAGCT-3′	Target-specific primer used for DNA Walking 1st PCR-a
TSPa 2	5′-CAGGGGAGACACAAAACCGTGG-3′	Nested Target-specific primer used for DNA Walking 2nd PCR-a
TSPa 3	5′-CCGACAGCGGGAGCACTTAGGC-3′	Nested Target-specific primer used for DNA Walking 3rd PCR-a
TSPb 1	5′-ATGATGTTGAGCCTTTTCTGCA-3′	Target-specific primer used for DNA Walking 1st PCR-b
TSPb 2	5′-TTCCTCCTCTCCAGGTTGCTCTG-3′	Nested Target-specific primer used for DNA Walking 2nd PCR-b
TSPb 3	5′-TCAGGAGGAGGAACTTGGGATTTG-3′	Nested Target-specific primer used for DNA Walking 3rd PCR-b
TSPc 1	5′-GAAAGCTCCTCCTTCACTCATGA-3′	Target-specific primer used for DNA Walking 1st PCR-c
TSPc 2	5′-GGGTGAACTGCAGGACAAAATGC-3′	Nested Target-specific primer used for DNA Walking 2nd PCR-c
TSPc 3	5′-CAGGACGGAAACAGAAGGAGCTG-3′	Nested Target-specific primer used for DNA Walking 3rd PCR-c
TSPd 1	5′-TCCGACTCTGAGCGTCTCCT-3′	Target-specific primer used for DNA Walking 1st PCR-d
TSPd 2	5′-TTTGGGGACAGGGTGCGACT-3′	Nested Target-specific primer used for DNA Walking 2nd PCR-d
TSPd 3	5′-TCCGCTCTGGTGGCATGTGA-3′	Nested Target-specific primer used for DNA Walking 3rd PCR-d
DW-ACP1	5′-[ACP^{TM}]-AGGTC-3′	1st ACP^{TM} primer for 1st DNA Walking PCRs
DW-ACP2	5′-[ACP^{TM}]-TGGTC-3′	2nd ACP^{TM} primer for 1st DNA Walking PCRs
DW-ACP3	5′-[ACP^{TM}]-GGGTC-3′	3rd ACP^{TM} primer for 1st DNA Walking PCRs
DW-ACP4	5′-[ACP^{TM}]-CGGTC-3′	4th ACP^{TM} primer for 1st DNA Walking PCRs
DW-ACPN	5′-[ACPN^{TM}]-GGTC-3′	Nested ACP^{TM} primer for 2nd DNA Walking PCRs
Uni-primer	5′-TCACAGAAGTATGCCAAGCGA-3′	Universal primer for 3rd DNA Walking PCRs

excision and circularization, which resulted from Cre recombinase-mediated site-specific recombination at the *loxP* sites. Plasmids from BM25.8 were isolated and sequenced.

6. RACE

RACE was performed using a CapFishing™ Full-length cDNA Premix kit (Seegene, Seoul, Korea). First-strand full-length cDNA was synthesized using 3 μg of total RNA from adult ovary. Next, 2 μL of 10 μM dT-adaptor were incubated at 42℃ for 60 min with 20 U of RNasin RNase inhibitor (Promega) and 200 U of SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), followed by the addition of 3 μL of preheated 10 μM CapFishing™ adaptor and 60 U of SuperScript™ III Reverse Transcriptase (Invitrogen). The samples were incubated at 42℃ for 30 min, followed by inactivation at 70℃ for 15 min and 94℃ for 5 min. The samples were then diluted in 180 μL of nuclease-free water. 5′- and 3′-RACE-PCR was performed using 5 μL of the first-strand, full-length cDNA as a template, 25 μL of SeeAmp™ Taq Plus Master Mix, 10 pmol of 5′- or 3′-RACE primer, and target-specific primers, for a final reaction volume of 50 μL.

Amplification was performed using the following parameters: pre-heating at 94℃ for 3 min, followed by 30 cycles of 94℃ for 40 s, 58℃ for 40 s, and 72℃ for 1 min, and post-extension at 72℃ for 5 min. cDNA fragments amplified via RACE were separated on an agarose gel. The expected products were isolated using a Gel Extraction kit (SolGent, Daejeon, Korea), ligated into pGEM-T easy vector (Promega), and then cloned in *E. coli* JM109 (Promega). Plasmids containing each cDNA fragment were purified using an Exprep GeneAll Plasmid Quick kit (GeneAll Biotechnology, Seoul, Korea) and sequenced.

7. Sequencing and sequence analysis

The products acquired via cDNA library screening, RACE, and subcloning were sequenced using the 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) or entrusted to Genotech (Daejeon, Korea), a private organization. Cloned plasmid DNA was purified using an Exprep GeneALL Plasmid Quick kit (GeneAll Biotechnology) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and a MyCycler™ thermal cycler (BIO-RAD, Hercules, CA, USA) under the following conditions: 30 cycles of 96℃ for 15 s, 50℃ for 8 s, and 60℃ for 4 min. DNA sequences were analyzed using BLASTN (National Center for Biotechnology Information, National Institutes of Health, USA). Deduced amino acid sequence and multiple sequence alignment results were acquired using the MultAlin server (Corpet, 1988). CLUSTAL W (Thompson *et al.*, 1994) was used to compare nucleotide sequence homology, using the default settings. Motif analysis and prediction of functional and regulatory sites in wrasse DM domain-containing genes were performed using the PROSITE database (http://www.ebi.ac.uk/ppsearch/) hosted by the European Bioinformatics Institute (Tables 6 and 7). Folding in the amino acid sequence was predicted using the PHYRE Protein Fold Recognition server (version 2.0; Bennett-Lovsey *et al.*, 2008), and tertiary structure was predicted using SWISS-MODEL (Arnold *et al.*, 2006) and the ExNRL-3D/ExPDB database (see Fig. 10). A phylogenetic tree was constructed using PHYLIP (version 3.67) with 1,000 bootstrap replicates. Scientific names and accession numbers for the DM-containing genes used in multiple sequence alignment and phylogenetic tree analysis are as follows : *dsx* (*Drosophila melanogaster*, AAA17840), *mab3* (*Caenorhabditis elegans*, NM_001027293), H.sDMT (*Homo sapiens*, NM_021951), H.sDMO (*H. sapiens*, NM_022160), M.mDMT (*Mus musculus*, NP_783578), G.gDMT (*Gallus gallus*, AF123456), A.mDMT (*Alligator*

Table 6. Putative functional sites in wrasse *DMRT* cDNA

Position	Residues	Pattern	Description	Reference
31-61	CSRCRNHGFV-SPLKGHKRFC-SWRDCQCPKC	C-x(2)-C-x(2)-H-x(8)-H-x(3,4)-C-x(4)-C-x-C-x(2,3)-C	DM DNA-binding domain signature	Smith *et al.* 1999
197-200	NVSS	N-{P}-[ST]-{P}	N-glycosylation site	Miletich *et al.* 1990
51-53	SWR	[ST]-x-[RK]	Protein kinase C phosphorylation site	Kishimoto *et al.* 1985
51-54 229-232 256-259 292-295	SWRD SVEE TPQD TILD	[ST]-x(2)-[DE]	Casein kinase II phosphorylation site	Pinna L.A. 1990
22-27 132-137 217-222 219-224	GQKPSR GSRSAL GLGSTF GSTFCV	G-{EDRKHPFYW}-x(2)-[STAGCN]-{P}	N-myristoylation site	Grand R.J.A. 1989

Table 7. Putative regulation sites on the wrasse *DMRT* promoter region

Name	Position	Sequences	Score	Description
GATA-1	-1,640 / -1,631 -612 / -599	CACCATCATC CTCATATCTATTA	98 86	GATA-binding factor 1
AP-4	-1,598 / -1,589	CCCCGCTGTG	89	Activator protein 4
GATA-2	-1,569 / -1,560	AGCTATCTGG	95	GATA-binding factor 2
GATAx	-1,385 / -1,375	GTTATTTATCT	88	GATA binding site
sox-5	-1,338 / -1,329	TAATTGTTAA	94	SOX5
AP-1	-1,244 / -1,235, -1,172 / -1,162, -514 / -506	CTGACTGACC, TATGACACAGT, CAGACTCAT	87 85 85	Activator protein 1
C/EBP	-1,162 / -1,150, -986 / -974	TCAGTGGTAATGT, ACCTCATCAAATA	87 87	CCAAT/enhancer binding protein

Table 7. Putative regulation sites on the wrasse *DMRT* promoter region (Continued)

Name	Position	Sequences	Score	Description
Dof-2	-1,119 / -1,109	TATTAAAGCAA	98	Dof2-Single zinc finger transcription factor
STATx	-994 / -986	TTAAGGGAA	90	Signal transducer and activator of transcription
Dof-3	-623 / -613	TTGCTTTTCAC	92	Dof 3-Single zinc finger transcription factor
GATA-3	-610 / -602	CACTATCTA	92	GATA-binding factor 3
Dof-1	-451 / -441	TGCTTTATTT	91	Dof1/MNB1a- single zinc finger transcription factor
SRY	-315 / -309	AAACAAA	100	SEX Determining region Y gene product
C/EBP alpha	-305 / -292	ATATTTTGCAATGA	92	CCAAT/enhancer binding protein alpha
C/EBP beta	-305 / -292	ATATTTTGCAATGA	94	CCAAT/enhancer binding protein beta
TATA	-276 / -267	ACTTTAAAAC	88	Retroviral TATA box
cap	-245 / -238	AAAAATGA	87	Cap signal for transcription initiation

mississippiensis, AF192560), E.cDMT (*Epinephelus coioides*, EF017802), O.aDMT (*Oreochromis aureus*, DQ185027), T.rDMT (*Takifugu rubripes*, NM_001037949), X.mDMT (*Xiphophorus maculatus*, AF529187), M.aDMT (*Monopterus albus*, AF421347), C.gDMT (*Clarias gariepinus*, AF439561), D.rDMT (*Danio rerio*, AF439562), O.nDMT (*O. niloticus*, AF203489), O.nDMO (*O. niloticus*, AF203490), O.mDMT (*Onocorhynchus mykiss*, AF209095), P.jDMT (*Pseudolabrus japonicus*, DQ062159), H.tDMT (*Halichoeres tenuispinis*, AY177711), and H.tDMT1 (*H. tenuispinis*, this study).

8. Northern blotting

Northern blotting was performed using 0.5 μg of wrasse testis or ovary poly(A) mRNA. Poly(A) mRNAs were separated against RNA markers on a 1.1% formaldehyde-agarose gel at 55 V for 4 h (Promega) in 1× MOPs buffer. Separated transcripts were then transferred onto a positively charged Nytran Supercharge nylon membrane (Schleicher & Schuell BioScience, Keene, NH, USA) in neutral transfer buffer under a 50-mbar vacuum created using a Pharmacia LKB VacuGene Pump (Amersham Pharmacia Biotech, Uppsala, Sweden) for 2 h. The transcripts were immobilized using a GS Gene LinkerTM UV Chamber (BIO-RAD) at 120 mJ/cm^2. After pre-hybridization without probe for 1 h at 68℃, the fixed transcripts were hybridized with a specific, ^{32}P radio-labeled (15 μCi), intron-spanning, oligonucleotide probe for wrasse *DMRT* cDNA using ExpressHyb^{TM} Hybridization Solution (Clontech) at 68℃ for 4 hours. Unbound and non-specific probe was removed by washing for 30 min under low-stringency conditions (2× SSC, 0.05% SDS, room temperature), and for 50 min under high-stringency conditions (0.1% SSC, 0.1% SDS, at 50℃). Following hybridization, the membrane was exposed to X-ray film (Biomax-MS, Kodak, NY, USA) at -70℃ for 2 days. After verifying the transcript signal on the film, probe was removed from the

membrane by incubating at 68℃ for 1 h in 50% deionized formamide, 0.1× SSC, 0.1% SDS; the membrane was then re-used for β-actin detection. β-actin hybridization and detection were performed as described above.

9. Southern blotting

Wrasse testis genomic DNA (100 μg) was single-digested with *Hind*III, *Sac*I, *Pst*I, *Sfi*I, *BamH*I, and *Sma*I separately, and double-digested with *EcoR*I and *Hind*III. The DNA fragments were purified via ethanol precipitation and quantified using Gene Quant II (Amersham Pharmacia Biotech), and 25 μg of the purified DNA were used for Southern blotting. In the positive control, 100 ng of *EcoR*I-digested pGEM-T easy vector (Promega) containing probe sequence was used. DNA was fractionated on a 0.7% agarose gel in 0.5× TBE buffer at 30 V for 19 h, or until the loading dye (bromophenol blue) migrated 13 cm from the loading slot. Fractionated DNA was denatured by gentle agitation in an alkaline transfer buffer (0.4 N NaOH and 1 M NaCl), and then transferred to a positively charged Nytran Supercharge nylon membrane (Schleicher & Schuell BioScience) under a 50-mbar vacuum created using a Pharmacia LKB VacuGene Pump (Amersham Pharmacia Biotech) for 4 h. Transferred DNA was incubated in neutralization buffer II (0.5 M Tris-HCl [pH 7.2] and 1M NaCl) and then immobilized using a GS Gene Linker^{TM} UV Chamber (BIO-RAD) at 150 mJ/cm^2. Fixed DNA was hybridized with a specific, ^{32}P radio-labeled (5 μCi), exon-spanning, oligonucleotide probe for wrasse *DMRT* genomic DNA using ExpressHyb^{TM} Hybridization Solution (Clontech) at 60℃ for 15 h. Unbound and non-specific probe was removed by washing for 45 min under low-stringency conditions (2× SSC, 0.05% SDS, at room temperature), and for 60 min under high-stringency conditions (0.1× SSC, 0.1% SDS, at 50℃). Following hybridization, the membrane was exposed to X-ray film (AGFA CP-BU NEW 100 NIF, Agfa-Gevaert, Mortsel, Belgium) at -70℃ for 5 days.

10. PCR contig construction from genomic DNA

Contigs for the ORF of the *DMRT* gene were generated via PCR using wrasse genomic DNA, target-specific primers, and DNA Walking SpeedUp™ Premix kit (Seegene, Seoul, Korea) or via direct subcloning using genomic DNA from wrasse testis. Target-specific nested primer sets were designed based on the wrasse *DMRT* cDNA sequence (Table 5), followed by genome walking. The first PCR (pre-heating at 94℃ for 5 min, followed by 30 cycles of 94℃ for 30 s, 55℃ for 30 s, 72℃ for 100 s, and post-extension at 72℃ for 7 min) was performed using 100 ng of genomic DNA as a template, 10 pmol of target-specific primers, 25 μL of 2× SeeAmp™ACP™ Master Mix II, and four types of 10 pmol DNA Walking Annealing Control Primer (DW-ACP) for separate reactions. Initial PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega). A second, nested PCR (pre-heating at 94℃ for 3 min, followed by 35 cycles of 94℃ for 30 s, 58℃ for 30 s, 72℃ for 100 s, and post-extension at 72℃ for 7 min) was performed using 5 μL of the purified initial PCR products as template, 10 pmol of DNA Walking Annealing Control Nested Primer (DW-ACPN), 10 pmol of target-specific first nested primer, and 10 μL of 2× SeeAmp™ACP™ Master Mix II, for each of the four reactions. A third nested PCR (pre-heating at 94℃ for 3 min, followed by 30 cycles of 94℃ for 30 s, 63℃ for 30 s, 72℃ for 100 s, and post-extension at 72℃ for 7 min) was performed using 1 μL of second PCR products as a template, 10 pmol of Universal Primer (Uni-primer), 10 pmol of target-specific second nested primer, and 10 μL of 2× SeeAmp™ACP™ Master Mix II, for each of the four reactions. PCR products were verified on a 1.5% agarose gel, DNA bands were extracted using a Gel Extraction kit (SolGent), ligated into pGEM-T easy vector (Promega), and then cloned in *E. coli* JM109 (Promega). Plasmids containing each DNA contig were purified using an

Exprep GeneAll Plasmid Quick kit (GeneAll Biotechnology) and sequenced. Direct subcloning was performed using primer sets spanning unidentified regions between contigs (Table 3) and TaKaRa LA Taq^{TM} (TaKaRa Bio Inc., Otsu, Shiga, Japan). Briefly, 50 ng of genomic DNA were used as a template with 10 pmol of sense primer, 10 pmol of anti-sense primer, 20 mM of dNTP mix, 5 μL of 10× LA PCR Buffer II (Mg^{2+} free), 5 μL of 25 mM $MgCl_2$, and 2.5 U of TaKaRa LA Taq, to make a final reaction volume of 50 μL. Twenty-eight contigs were cloned using the method described above, and new primers sets were selected to minimize the number of contigs and possible errors. Finally, seven contigs were cloned, sequenced, and assembled, and the structure of the wrasse *DMRT* ORF was determined.

11. Cloning the putative promoter region of wrasse *DMRT* and construction of *DMRT*-Luciferase chimeric mutant clones

The putative promoter region of the wrasse *DMRT* gene was isolated using target-specific nested primers and DNA Walking SpeedUp^{TM} Premix kit (Seegene, Seoul, Korea). Four anti-sense nested primers (Table 2) were designed to amplify from +212 in the first exon, and DNA walking PCR reactions were performed as described above. A 1,826-bp product was cloned, and the upstream region from -1,721 to the start codon was sequenced and analyzed using the PROSITE, BLOCKS, ProDom, PRINTS, and Pfam databases (Bioinformatics Center Institute for Chemical Research). Twenty-one putative regulatory regions in the wrasse *DMRT* promoter were identified (Tables 7 and 8). To examine the regulatory activity of these regions, the sequence from position -1,771 to -50 was compartmentalized into 17 regions. Primer sets were designed to amplify each region (Table 4) with linker sequences compatible to the *Xho*I and *Hind*III restriction sites of the pGL3 Luciferase Reporter Vector (Promega; see Fig. 16). These 17 regions

Table 8. Nucleotide sequence of 5′-flanking region from wrasse *DMRT* gene, and a sites of putative regulatory regions

	Sequence	Name
-1,721	TGAGTCGGTATTGCGGTTCAC	
-1,700	TTGCTCGCATGACCCTATAAACAGTGCATTGGAGAATTGTAACAGTTTAA	
-1,650	AGTGCCAGATCACCATCATCATAATTTCAATTAGGTCTCATTTTATTTCA	GATA1
-1,600	GCCCCCGCTGTCAAAAGTAATCCATTTATCCAGCTATCTGGGTTGCTGAT	AP4, GATA2
-1,550	TTCTATAGCCAATCACTGGATGTCAGGGAGAGCACTCATGCTAATAGAAA	
-1,500	GGTTAAATGTTTTAATAACAGACTCCATAACTGCATCATAATTTCTCATT	
-1,450	ATTACTTTTGGGGTGATTAAAGTCAGTGTAGCCATGCTGATTTCAGCAGC	
-1,400	CAATGAAGTGTAACAGTTATTTATCTTACACAGCTTTTCAGGGCTGTTTA	GATAx
-1,350	AATGTCTTCTTTTAATTGTTAACTTTTCATTGCCTCTCTATTTGGCTGCA	Sox5
-1,300	GGGATTTTAATTGGTAGCTTCGTTCCACTCCCTGCCGGTAAATTTACTTG	
-1,250	GCTTGACTGACTGACCCTTGTGGAGGTTAATTCAGTTTCAGCTCAGCCAA	AP1
-1,200	GCCTGTAAATTAATATGAGAGTGTAAGCTCACTACAAATCAGTGGTAATG	C/EBP
-1,150	TAAGAGCAACAGTAAATATATTTCAGTGCAATTATTAAAGCAACAAACCCA	Dof2
-1,100	AAGGGAATACTATCATAATACCTCACCATATGACACAGTTCAGACTATAG	AP1
-1,050	AGGTACAGAAATATTTAGAGGAACTTGTTACCACCAAGGATAACTCCCCA	
-1,000	CTGAAATTAAGGGA*A**CCTCATCAAATA*TCTTCAGATCTAAGATCAAATAC	STATx, C/EBP
-950	CTCCTTGCACCCATCCCTTTATCATCAATCAAAAAATAACATGATCTGAG	
-900	AGCCCCCCGCCAGCTCATAATGGTACAGTCTGATCTCTCTTCAAAAACCC	
-850	ACTGGATATCCTCTCTTCCTGTTTGTCTCTTTCAAAGCAGTGGGAGCCTA	
-800	TTCACAAACATTTGAATTAAATCAAAATCGAATGCATAAATGTATCAATT	
-750	CCTATAAGTAGACTATTTATAAATAATGACGGTTTTTCAACTGCACTTTC	
-700	ACGTCAGAACCAGGTAAGTTATCGTATACAATTACAATGACCAGCGTCTG	
-650	TTTTCAAGAATTTATGTTTCCAGTTCTTTGCTTTTCAC*C**TCACTATCTAT*	Dof3, GATA1, GATA3
-600	*TA*AATATTTTTTTAAATAGTGCAACTTTATATAGATATTGTTGTCTTCTC	
-550	TGTGTTGGGGACACATTTTATGGGTGCGAGTTATTACAGACTCATAGTTG	AP1
-500	CAACAAGTTGGGTTTGCTTTCAGTCCCAGGATCTTAAGACTTACTGTAAA	
-450	TGCTTTATTTATTTTTTAATAACTCCACAAAGAAATAACTCTGGAAATAA	Dof1
-400	CACTGATTAAGAGGGATGAAGTGAAGCCTCTCTATTTCTTTTAAAAACTG	
-350	GTTAAGTAGCCCCCCCACACACACGAGAAATACACAAACAAAATA*ATATT*	SRY, C/EBPα and β
-300	*TTGCAATGA*AAAATGTAACATCTAACTTTAAAACGTGTTCGCTCATGTTG	TATA
-250	TGTGAAAAAATGATGGAGTGTGGGGCTCGTCCTGTCCCTTCACCTCTCTC	CAP
-200	CCCCGCTTCCAGCTTGTATCCTCCCACATCACAACATCACCACATCACCA	
-150	GGGCGGAGAGAGACCGTGACCTTACAGCCTCCAGATTGGCATCGGTACAG	
-100	ACTTTAACAAGCCGAACCTACCCTGCTGGGACAAATTTCAACACAGTAGC	
-50	TAAAGACGAACAGTTGGGCAGGTTTTGCAGTAGACGTTTTTATTTTAGAC	
+ 1	*ATG*AGTAAAGACAAGCAGAGCAAGCAGGTGCCGGAGCTCACCGAACCTCT	

were amplified from wrasse genomic DNA, double-digested with *Xho*I and *Hind*III, ligated into linearized pGL3-Basic or pGL3-Enhancer luciferase reporter vector, and transfected into the *E. coli* JM109 strain. All clones were double-digested with *Sma*I (cleaving at the middle of the vector) and *Hind*III (cleaving at the insert-vector junction) and run on a 1% agarose gel to detect possible multiple insertions, and then sequenced. All plasmid DNAs were prepared in nuclease- and endotoxin-free water (Amresco, Solon, OH, USA).

12. Cell culture, transient transfection, and luciferase assay

The African green monkey kidney cell line (Cos-1) and an epithelial mouse testis cell line (TM4) were purchased from the Korean Cell Line Bank. Both cell lines were cultured in Dulbecco's Modified Eagle's Medium (D-MEM; GIBCO BRL, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/*m*L), and streptomycin (100 μg/*m*L) under 5% CO_2 at 37℃. For transient transfection, Cos-1 and TM4 cells were seeded into 96-well culture plates (NUNC, Roskilde, Denmark) at densities of 2×10^4 and 1.5×10^4 cells per well, respectively. Non-recombinant pGL3-Basic luciferase reporter vector was used as a negative control, and non-recombinant pGL3-Control luciferase reporter vector was used as a positive control. *DMRT*-luciferase pGL3-Basic or Enhancer vectors were used to evaluate the regulatory activity of each region. Synthetic *Renilla* luciferase reporter vector (phRL-TK; Promega) was included as a control to normalize transfection efficiency. Triplicate transfections were carried out for each chimeric recombinant using FuGENE 6 Transfection Reagent or FuGENE HD Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA).

Thirty-three hours (Cos-1) or immediately (TM4) after seeding, 0.3 μg of

negative control vector (non-recombinant pGL3-Basic or pGL3-Enhancer vectors), positive control vector (non-recombinant pGL3-Control vector), or *DMRT*-luciferase chimeric pGL3-Basic or Enhancer luciferase reporter vectors were co-transfected with 50 ng of phRL-TK vector per well using FuGENE 6 or HD Transfection Reagent (reagent [μL]: plasmid [μg] ratio = 1:6 for Cos-1 cells, 3:1 for TM4 cells). Cells were incubated for an additional 27 (Cos-1 cell) or 110 (TM4 cells) h after transfection, and then lysed and assayed for both firefly and *Renilla* luciferase activity using the Dual-Glo™ Luciferase Assay System (Promega). Luminescence was measured using FLUOstar Optima (BMG Labtech, Offenburg, Germany). Detailed treatments are described in Table 9. The data represent the ratio of firefly to *Renilla* luciferase activity in wrasse *DMRT*-luciferase chimeras relative to the ratio of firefly to *Renilla* luciferase activity in the negative control vectors (non-recombinant pGL3-Basic or pGL3-Enhancer) and that of the promoter- and enhancer-harboring vector (non-recombinant pGL3-Control).

Table 9. Overall scheme of transient transfection and luciferase assay

Transfection point		33 hours (Cos-1) or immediately (TM4) after cell seeding									
Name		pGL3-Vectors			17 wrasse *DMRT*-luciferase chimeric mutants (Basic or Enhancer)						
		Control 5256 bp	Enhancer 5064 bp	Basic 4818 bp	IN 1	IN 2	IN 3	IN 4	IN 5	IN 6	IN 7
Insert Size (bp)		0	0	0	174	206	271	222	192	187	159
DNA (μg) : Fugene (μL) Ratio		1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3
Repetition of treatment		4	4	4	4	4	4	4	4	4	4
Numbers of cell (/well. ×1,000)		20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15
Mutants (Luciferase)	Treated amount (ng)	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300
	Concentration (ng/μL)	220	240	100	85	210	170	185	125	245	210
	Treated volume (μL)	1.36	1.25	3	3.52	1.42	1.76	1.62	2.4	1.22	1.42
phRL-TK (Renilla)	Treated amount (ng)	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50
	Concentration (ng/μL)	160	160	160	160	160	160	160	160	160	160
	Treated volume (μL)	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31
FBS-free DMEM (μL/repetition)		40	40	40	40	40	40	40	40	40	40
Fugene reagent (μL/repetition)		1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05
Incubation after transfection (hrs)		27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110
Luc. substrate (1:1 diluted)		50	50	50	50	50	50	50	50	50	50
Stop & Glo substrate (1:99 diluted)		50	50	50	50	50	50	50	50	50	50

* number / number in table = Cos-1 / TM4 experiment.

Table 9. Overall scheme of transient transfection and luciferase assay (continued)

Transfection point		33 hours (Cos-1) or immediately (TM4) after cell seeding									
Name		17 wrasse *DMRT*-luciferase chimeric mutants (Basic or Enhancer)									
		IN 8	IN 9	DE 1	DE 2	DE 3	DE 4	DE 5	DE 6	DE 7	DE 8
Insert Size (bp)		208	172	1771	1599	1441	1252	1065	873	651	380
DNA (μg) : Fugene (μL) Ratio		1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3	1 : 3
Repetition of treatment		4	4	4	4	4	4	4	4	4	4
Numbers of cell (/well. ×1,000)		20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15	20 / 15
Mutants (Luciferase)	Treated amount (ng)	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300	300 / 300
	Concentration (ng/μL)	240	245	135	235	220	110	190	290	295	300
	Treated volume (μL)	1.25	1.22	2.22	1.27	1.36	1.72	1.57	1.03	1.01	1
phRL-TK (Renilla)	Treated amount (ng)	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50	200 / 50
	Concentration (ng/μL)	160	160	160	160	160	160	160	160	160	160
	Treated volume (μL)	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31	1.25 / 0.31
FBS-free DMEM (μL/repetition)		40	40	40	40	40	40	40	40	40	40
Fugene reagent (μL/repetition)		1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05	1.5 / 1.05
Incubation after transfection (hrs)		27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110	27 / 110
Luc. substrate (1:1 diluted)		50	50	50	50	50	50	50	50	50	50
Stop & Glo substrate (1:99 diluted)		50	50	50	50	50	50	50	50	50	50

* number / number in table = Cos-1 / TM4 experiment.

RESULTS

1. Isolation of DM domain-containing genes

1) *DMRT*, a DM domain-containing gene isolated from the wrasse testis

After screening 5.25 × 10^4 pfu of our wrasse testis cDNA library, we obtained five weakly positive plaques during the first screening, and over 200 strongly positive plaques during the second screening (Fig. 1). After sequencing, we obtained a 3,119-bp full-length cDNA containing a 184-bp 5′-untranslated region (UTR), a DM domain, and the male-specific motif (Fig. 2). Nucleotide sequencing of the DM domain and male-specific motif region revealed 100% identity to a previously characterized wrasse *DMRT* cDNA (AY177711). Sequence comparison revealed differences of two bases in the ORF, 56 bases in the 3′-UTR, and 184 bases in the 5′-UTR (Fig. 3). In contrast to the extremely short 5′-UTR in our previously isolated wrasse *DMRT* cDNA sequence and many other *DMRT*s from various species (human, NM181872.1 and NM006557.3; fugu, AB201461 and AB201464; black porgy, DQ399799), the cDNA isolated here had a relatively long 5′-UTR. In addition, this new sequence contains stretches of six and eleven nucleotides that are absent from the previously isolated wrasse *DMRT* cDNA (Fig. 3).

2) *DMO*, a DM domain-containing gene isolated from the wrasse ovary

Full-length first-strand cDNA was generated from the wrasse ovary to isolate potential DM domain-containing genes. A partial sequence was obtained using specific primers for the first exon of wrasse *DMRT* and degenerate primers. Based on this partial sequence, target-specific primers were selected and RACE-PCR was performed as described in the Materials

Figure 1. Positive plaques from the second screening of a wrasse testis-derived cDNA library. Lambda phages from initial positive plaques were transduced into the bacterial lawn. A plaque replica was hybridized with radioactive probe, exposed to X-ray film, and then analyzed.

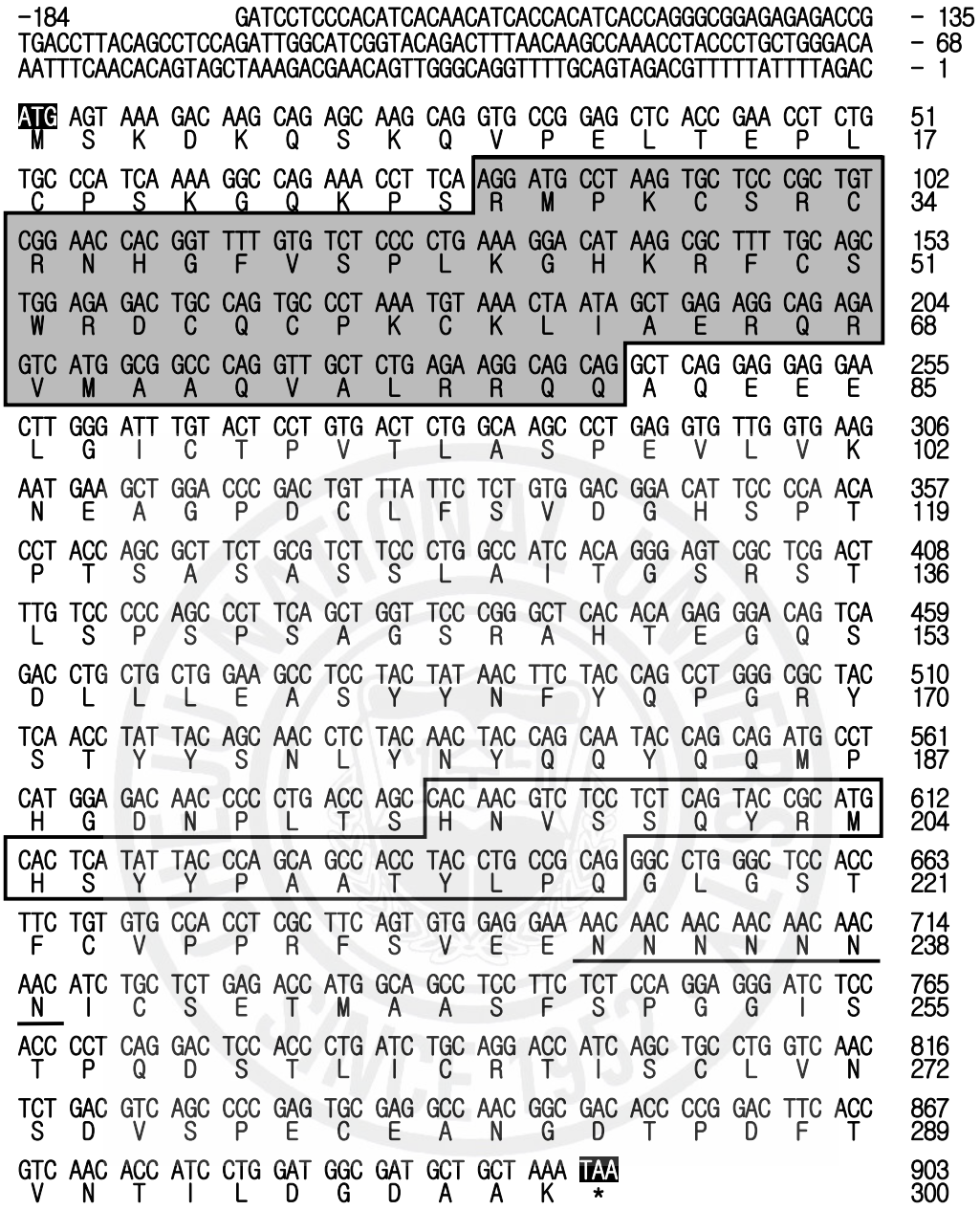

Figure 2. Nucleotide and deduced amino acid sequences for wrasse *DMRT1* cDNA. *DMRT1* (3,119 bp) encoded a 300-amino acid protein containing the DM domain and male-specific motif. Black boxes, start and stop codons; grey box, DM domain; white box, male-specific motif; underlined sequence, asparagine-rich region.

GAGAAGCTAAGATTAGCATCACATGACGAAGCATCATGAGATCAGACAAATTTGATGTTT 963
TGCAAAAAGTTTGAAAGTTTAGGTTGCTTCAGCTCCTTCTGTTTCCGTCCTGAAAGCACA 1023
GCATGTGTTCTGATTCTTTTACAAAGCACAGTTAGCATTTTGTCCTGCAGTTCACCCTTA 1083
ACCTTAAAGCTCTTACATCAATCATTACAGTAGTTCACGAGTGAAGGAGGAGCTTTCAGA 1143
CTAAAAGACGTGCAAGTTCACAAGTAACACCTTGCAAATCTTTCAGTGGGTCTGATCTCT 1203
TTTATTTTTTGACTAACTCTTTAAAGAAACACTTTAAAGATGGTTAAATTTACCCTGAAT 1263
CCAGGACAGATCAGGTTTTTCAGACTGAAAAACCTGATCTGTCCTGATCATGTTCCTGTG 1323
TGAAGCTCAACACTTGCTTTAAGCCTCTGAAAGCAACAGTTGGTACAAAGTTCAGCTTTT 1383
TTACAGCACAAAATGCAGAATAATGGTTTTAAAAATGTACTTGTCTTGTTCTTTTTCACA 1443
CCATTTCAGTCATGTCTGTTCACAATGAACCAGCAGTTCATTACTTCATAGTCTGACAAT 1503
CAGAATATAGCAGATATATTTTTTAGAGCTTAAATCTAAAATCAGTTTGAATACAACCAA 1563
AGCACTTATTTAGAAAACCAAAGTGGTGAAAATTGGCACAAAGAAGAAATCTTTTCTTCC 1623
CCTTCTTTTATGATACGTCTCAATTATTGACAAATGTTGCATGTAGATGCATGTAGCGTG 1683
TTTTAAAATCTATTTTCAAAGCAGTGAACCAGAAGTTTTCATAGCTCTAACCAGTCAACG 1743
ATGGTTTGGTGAGTGTTCGAAAAATGTCAGGACATCCTGGAAACTCAACATGATTCTTTT 1803
TTTGTCTAAATCTGACACAAGATACTTCAGAAGCACAAAAACATTTTTGAAAACAATTCA 1863
TTGATGTTTTGTGTTAGTAGTTAATCTTGTAAAGGATTTAATGTGGTTTCTAAATGACCT 1923
AAATCATACATTAAATATTTCTCTGAGGTTATTTTTGGAGTAAACTTGAGATAGTTAAGG 1983
TTTGTAGCTGGATATTTTTTTTTCTGTTTATCTCCGTTTAAAAAATTTTCAACCGAATGA 2043
GCAAAAAGGGAGGAAACAACTGATAAACGCCTTTCCAAACTATGTCCCGGACATTTTATG 2103
GCCCCCCCCCCCAAAAAGAACGATCAGATAACTTTTTAAAAAAGTTTTATAAAGTCGTTT 2163
TTAAAACAGCTTTTCTTAGGTACGAACGAATGTGTCACTGAGTTAAAGTTTGGAGAACTT 2223
CTCTGACTCTGAGGGTCTCCTCCTTTGGGGACAGAGTGCGACTCGAAGGAGAAAAAAGGA 2283
ATCGTGAATCATGTTTCCCTCCGCTGGGGGGGCATGTGAGGAGCACCTCCCGCTTATTTA 2343
TGTCACCGATCCAGGGGCATCGTTTATATCGGAGGATTTGCATCTCTAGGGTAGAAATTA 2403
CCTCTTTCAGACCAGGAGGTTTGTTTTTCAGTTACAGACATTTGTAAAGAGAGCAGACCT 2463
GAGAATTTTCAGTGTCAGCCTCTCAAATGTTAGTGTCTAACGTAAAGGTCTGACTTTAGA 2523
TCTTCAGGGCCAATTCTAGTCCTAAAAAAATTCTATGCTGTTCCCCAAGCCTGAAATAAT 2583
CTCAGATTAATCAGAGACATTCCCAGTAAGAAGCCTTTGTAATCTGTTTATCTTTTTGAC 2643
GCTGTTATTTTTTGTCAACCTAAAATACTTTTTAACGTCTTTCCCTCATGAAATAATGAA 2703
AAAAACCCCCAGTGTTGCCCACTTTTACTCTGCTTTGTTTAAACGACTTCAAGAGATTTT 2763
CAAACTTTTCTGCCTGTGGGAAGCCGACCTCTCCACCACTGTTCTGCAGACTGTTTATGT 2823
GTTTCGTGTTTTATGGAGATCAATTTTCACTCTGCAAGCTTTCCAAGTGTTTGTTTGTGT 2883
TCGTGTTTGCATAAAACAGCACCAAAAAAAAAAAAAAAAAAAAAAAAAAA 2935

Figure 2. Nucleotide and deduced amino acid sequences for wrasse *DMRT1* cDNA (Continued).

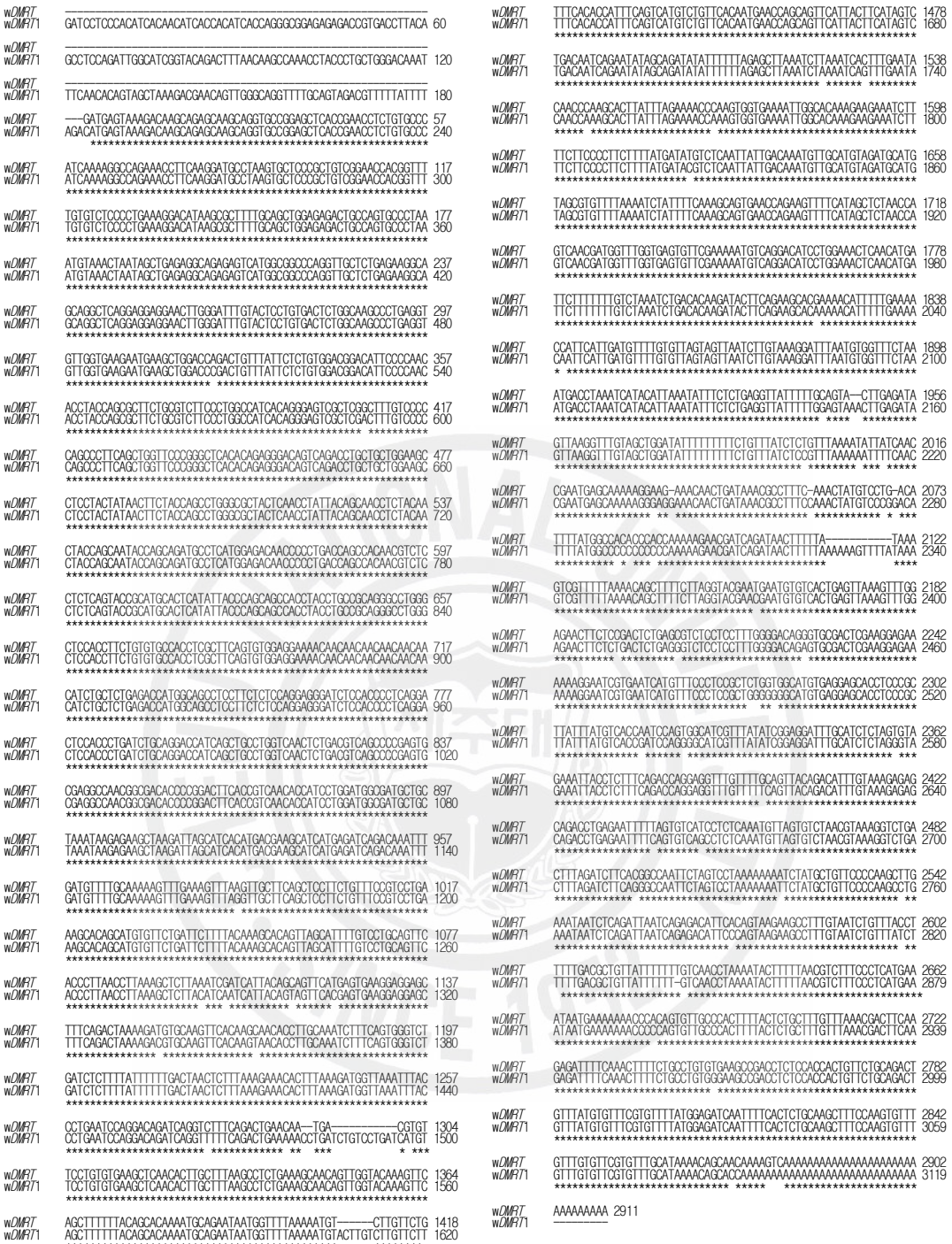

Figure 3. Nucleotide sequence comparison for wrasse *DMRT* (AY177711) and the newly isolated wrasse *DMRT1*. Wrasse *DMRT1* possessed an additional 184 bp in the 5′-UTR region. The remainder of the gene showed near identity to the previously submitted wrasse *DMRT* sequence.

and Methods. A partial wrasse *DMO* cDNA (545 bp) was isolated and cloned into pGEM-T easy vectors, followed by nucleotide sequencing. The product contained most of the DM domain region and 333 bp of the 5′-UTR (Fig. 4). When this sequence was compared to the genomic DNA sequence of *DMRT*, 212 bp of the first exon region showed 100% homology, whereas the 5′-UTR was divided into two regions. The sequence from positions -1 to -192 of *DMO* was identical to the same region of genomic DNA, and, with the exception of six bases, the *DMO* sequence from positions -193 to -333 was identical to that of -692 to -831 in the genomic DNA (Fig. 5). However, despite this high degree of homology, no donor-acceptor splice sites (GT-AG) were found at the putative exon-intron junctions.

2. Characterization of DM domain-containing cDNAs

1) Homology and phylogenetic analysis

An amino acid sequence alignment for wrasse DMRT and various DM domain-containing proteins (from humans to *C. elegans*) revealed that the DM domain region is highly conserved within phyla (Fig. 6). Multiple alignments were not performed for wrasse *DMO* cDNA, and the two wrasse DMRTs isolated here differed by only one amino acid. Wrasse DMRT showed 60 and 43% homology with rainbow trout and tilapia DMRT, respectively. Moreover, homology within the DM domain region of *DMRT*s was approximately 80%. As shown in Figure 7, the phylogenetic tree constructed for *DMRT*s showed three main branches for *DMO*s, human and chicken *DMRT*s, and fish *DMRT*s (Fig. 7).

2) Prediction of the two- and three-dimensional structures of wrasse DMRT

The results of our motif analysis and predicted functional sites within wrasse DM domain-containing genes are summarized in Table 6. A cysteine-

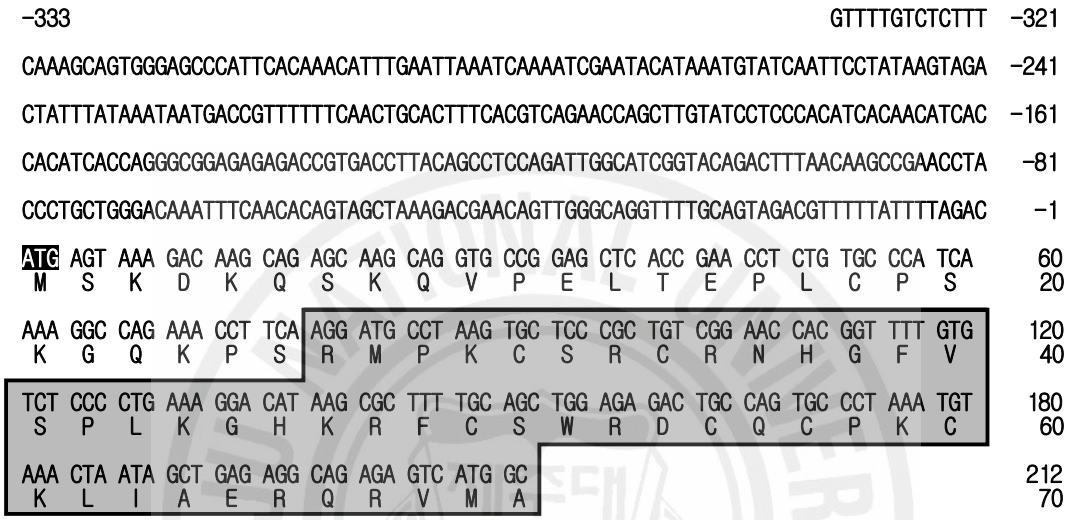

Figure 4. Nucleotide and deduced amino acid sequences for the wrasse *DMO* gene. The partial sequence included 212 bp of the putative ORF and 333 bp of the 5′-UTR. The 5′-UTR was longer than that of wrasse *DMRT*, but the partial ORF was identical to wrasse *DMRT*. Black box, start codon; grey box, DM domain.

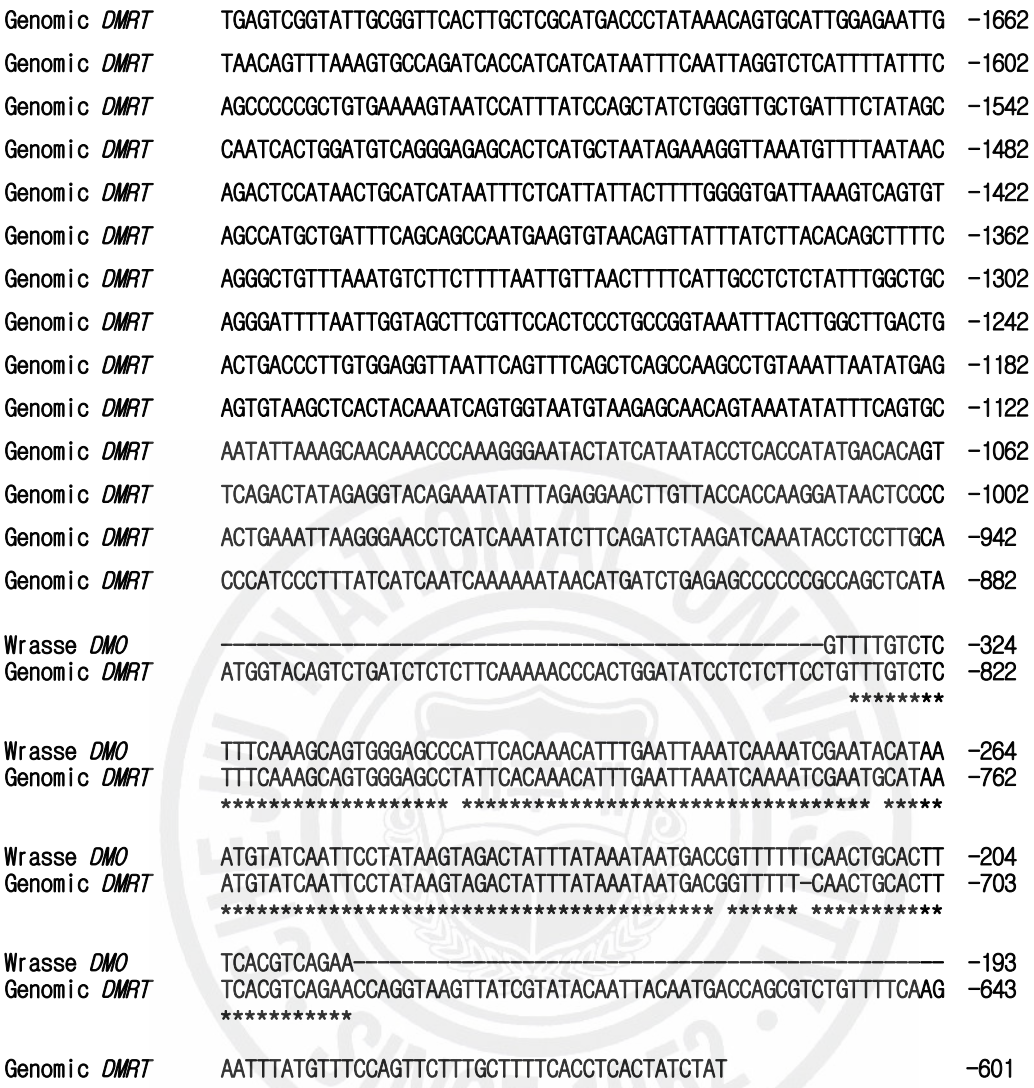

Figure 5. Nucleotide sequence comparison for wrasse *DMO* cDNA and *DMRT* genomic DNA. The first exon (212 bp) of *DMO* showed 100% homology to *DMRT*, but the 5′-UTR was divided into two regions (-1 to -192 and -193 to -333) that were identical to regions -1 to -192 and -692 to -831 in *DMRT*, respectively, with the exception of six bases. No donor-acceptor splice sites (GT-AG) were identified in the exon-intron junction. Black box, start codon.

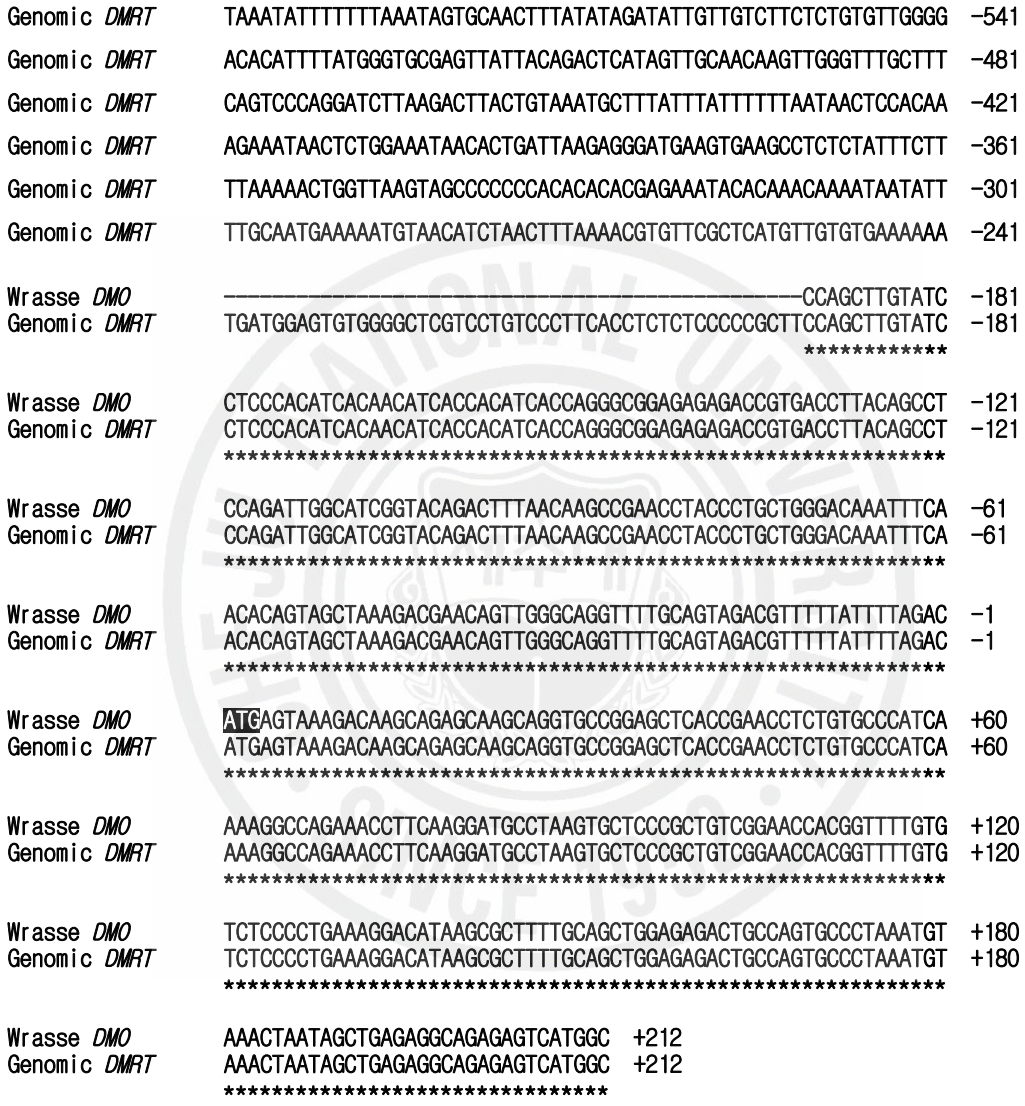

Figure 5. Nucleotide sequence comparison for wrasse *DMO* cDNA and *DMRT* genomic DNA (Continued).

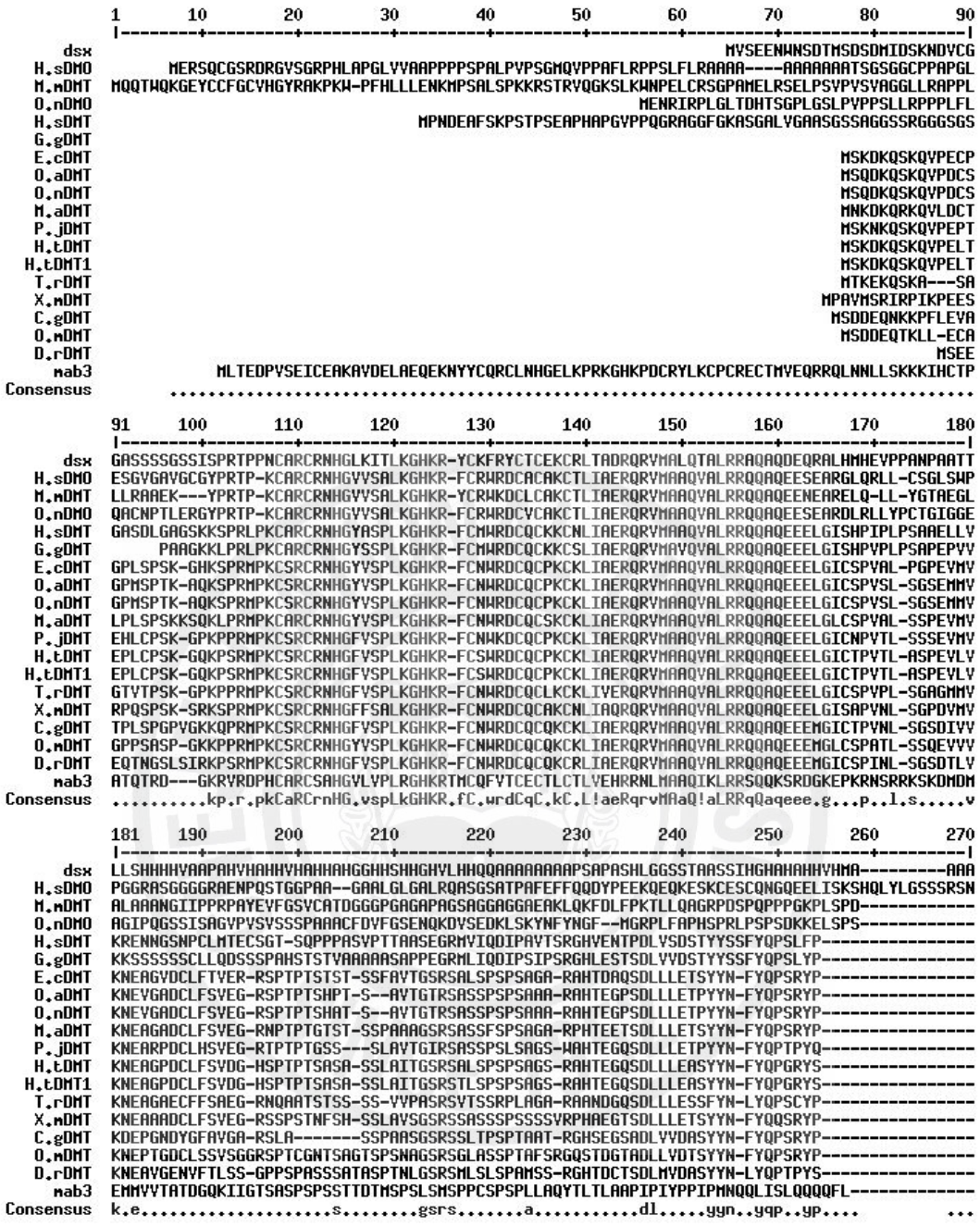

Figure 6. Multiple sequence alignment for various DMRTs. Conserved amino acids are noted in consensus sequence (capital letters, conserved in all species; small letters, highly conserved), and full scientific names of all species are listed in the Materials and Methods. The amino acid sequence of the DM domain and male-specific motif region were highly conserved within phyla.

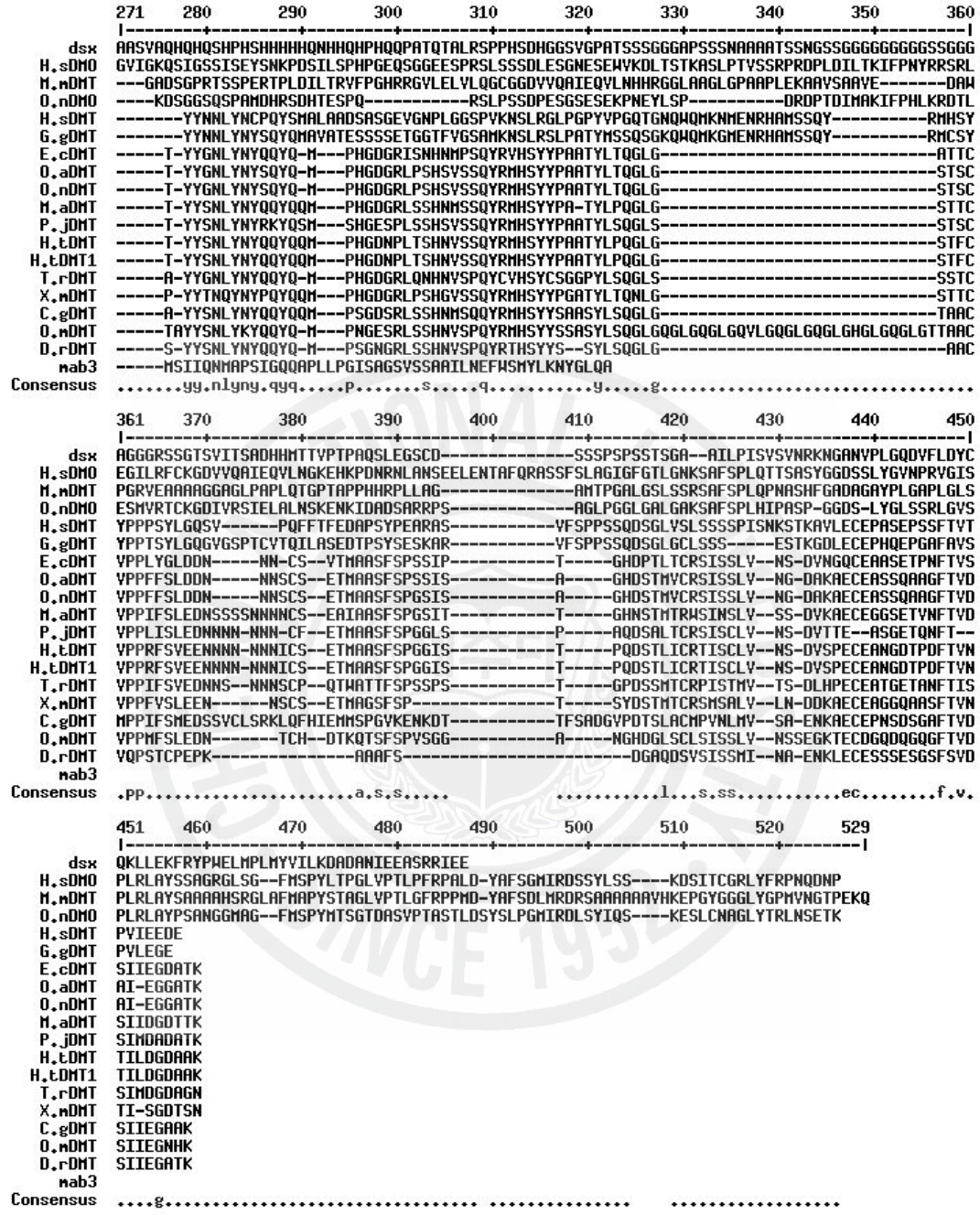

Figure 6. Multiple sequence alignment for various DMRTs (Continued).

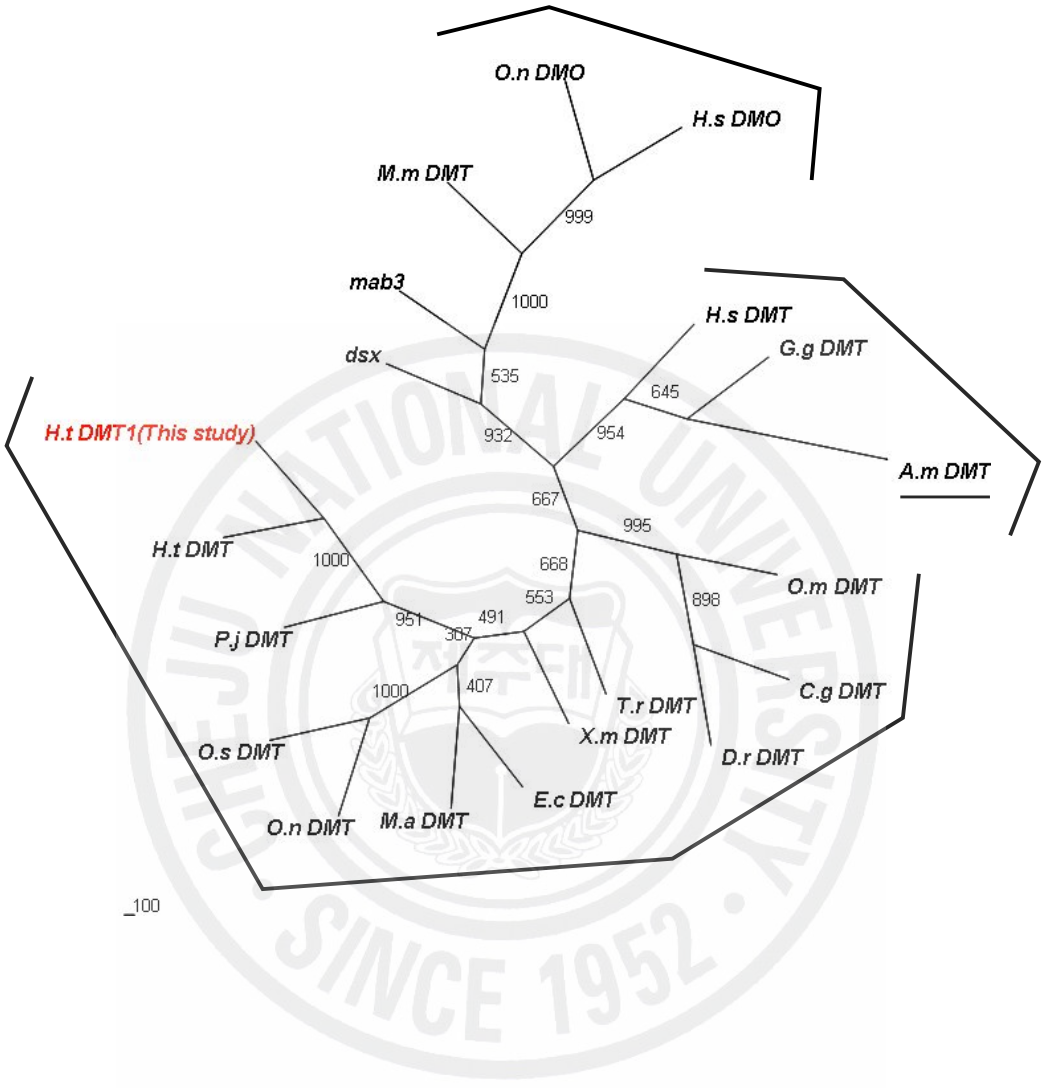

Figure 7. Phylogenetic analysis for the *DMRT* gene. The distance and bootstrap values (1,000 replicates) are represented by bar and internal edge labels, respectively. *Alligator mississippiensis DMRT1* was used as an outgroup. Full scientific names of all species are listed in the Materials and Methods.

rich 30-amino acid domain with a zinc finger DNA-binding motif was identified as the DM domain. Two intertwined CCHC and HCCC zinc-binding sites with six cysteine residues were identified (Figs. 8 and 9). As shown in Figure 10, the three-dimensional (3D) structure of the wrasse DM domain, which was predicted using SWISS-MODEL (Arnold *et al*. 2006), showed high tertiary similarity to the DM domains of *D. melanogaster* DSX and human DMRT, based on the sequences within the RCSB Protein Data Bank (http://www.rcsb.org/). Other putative functional sites, such as N-glycosylation, protein kinase C phosphorylation, casein kinase II phosphorylation, and N-myristoylation sites, were identified (Table 6).

3. Identification of transcripts and gene copy numbers in the wrasse *DMRT* gene

Northern blot analysis was performed to verify the presence of wrasse *DMRT* transcripts in the testis and ovary. A 3.2-kb transcript, which corresponded to the known size of *DMRT* cDNA, was strongly detected in the testis using a ^{32}P-labeled intron-spanning oligonucleotide probe; however, expression was not detected in the ovary (Fig. 11). These results confirm that *DMRT* is a male-specific gene in protogynous wrasse. In addition, a 1.2-kb transcript was weakly detected in the testis, but not in the ovary, suggesting that this small *DMRT* transcript may also function in the wrasse testis. Overall, these results indicate that the wrasse *DMRT* gene is involved in testis determination or differentiation.

Southern blot analysis was used to determine the gene copy number for wrasse *DMRT*s. Genomic DNA was treated with several restriction enzymes and then fractionated. Positive signals were detected at 2.1 kb in the *Eco*RI/*Hind*III double-digested fraction, and at 2.2, 4.2, 2.8, and 5 kb in the *Hind*III-, *Sac*I-, *Pst*I-, and *BamH*I single-digested fractions, respectively (Fig. 12, left panel). However, no bands were detected in the double-digested *Sfi*I

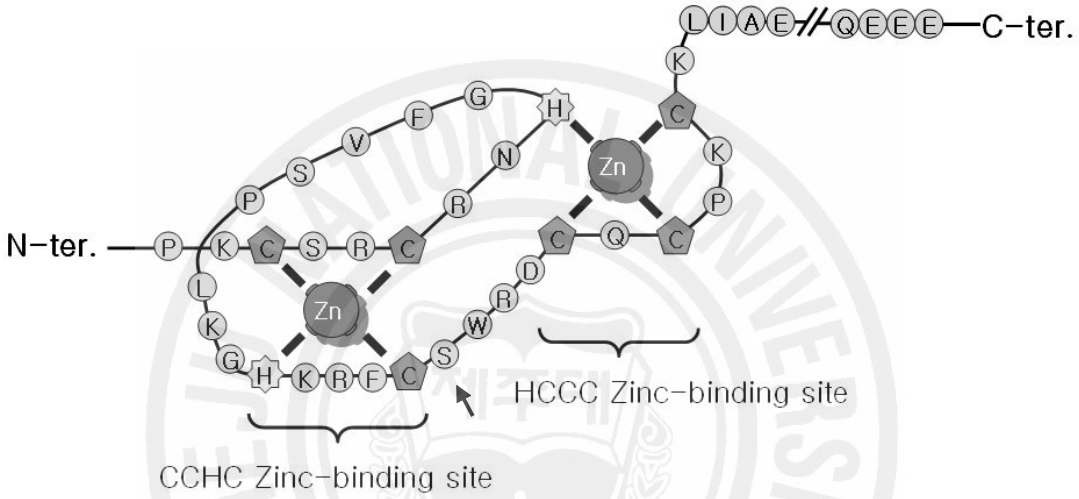

Figure 8. Schematic representation of the zinc module in the wrasse DM domain. CCHC and HCCC zinc-binding sites are intertwined. The 51st residue in wrasse DMRT was serine, whereas other teleost species exhibit a histidine residue (arrow). The amino acid sequence of the wrasse DM domain was analyzed using the PROSITE database (release 20.15). C, conserved cysteine residue involved in zinc binding; H, histidine; Zn, zinc atom.

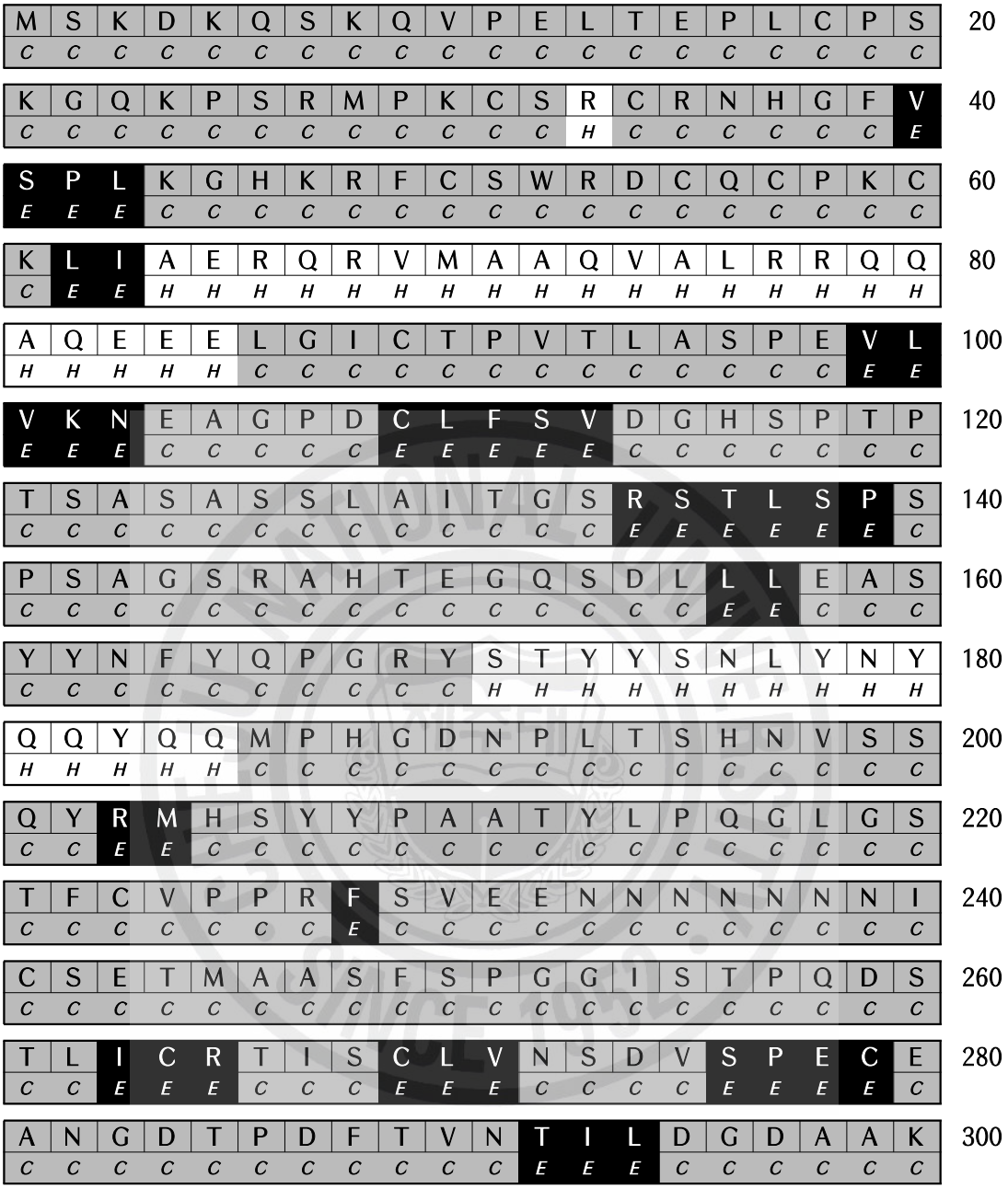

Figure 9. Secondary structure of wrasse DMRT. White boxes, alpha-helix forming amino acids; black boxes, beta-strand forming amino acids; grey boxes, coiling amino acids.

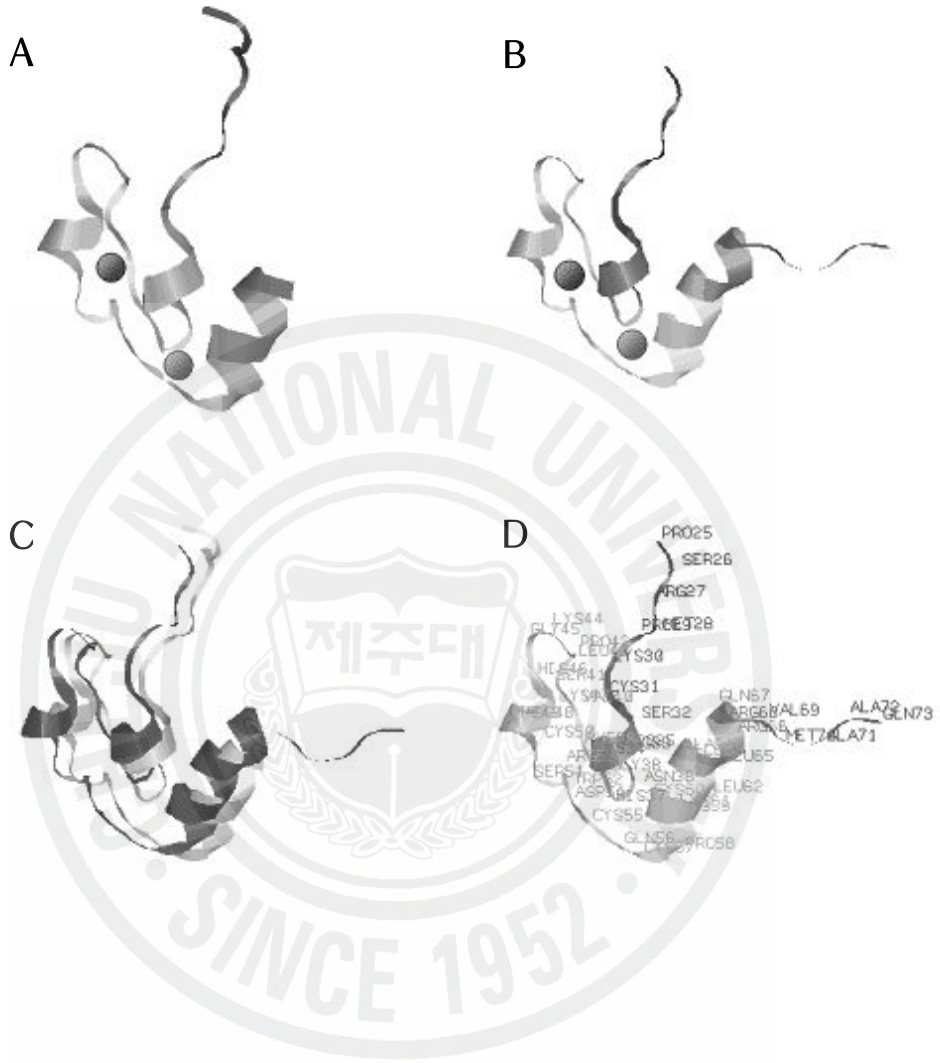

Figure 10. Predicted tertiary structure of the DM domain in wrasse DMRT and *D. melanogaster* doublesex (DSX). Three-dimensional images showing the DM domains of DSX (A) and wrasse DMRT (B) bound to a zinc atom (sphere). The tertiary structure of DM domains from wrasse DMRT (black) and DSX (grey) overlapped (C). Amino acids are labeled at sequential positions (D).

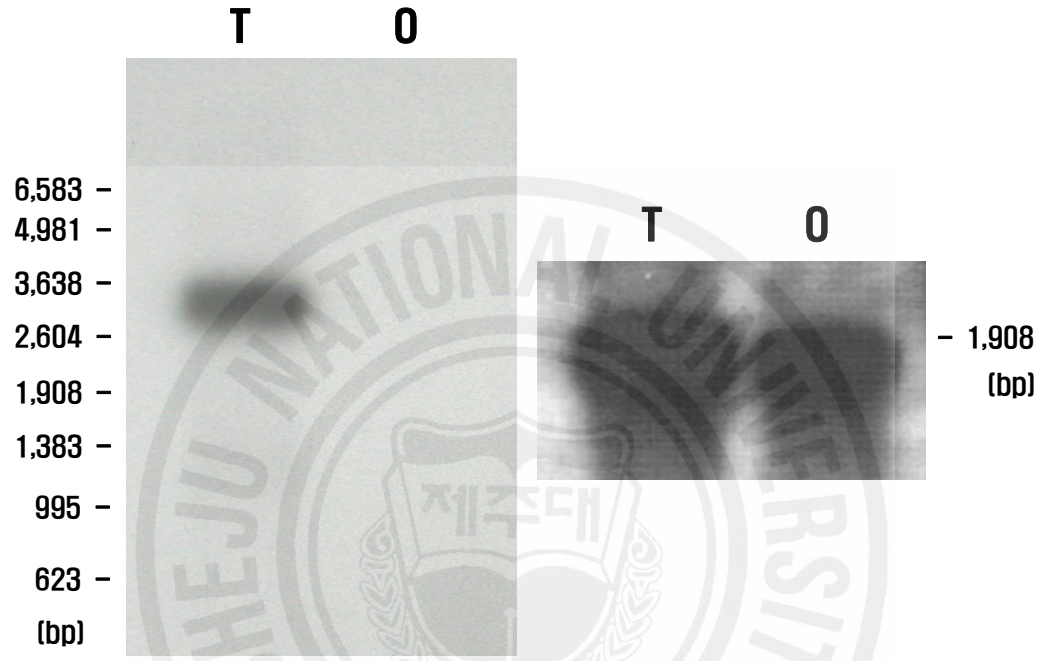

Figure 11. Northern blot analysis of *DMRT* transcription in wrasse testis and ovary. ^{32}P-labeled probe was hybridized with wrasse *DMRT* or β-actin transcripts immobilized on a nylon membrane and then exposed to X-ray film. A 3.2-kb *DMRT* transcript was detected in the testis (T), but not in the ovary (O; left panel), confirming that *DMRT* is a male-specific gene in protogynous wrasse. β-actin transcription was used as an internal control (right panel). RNA markers were used as a size marker.

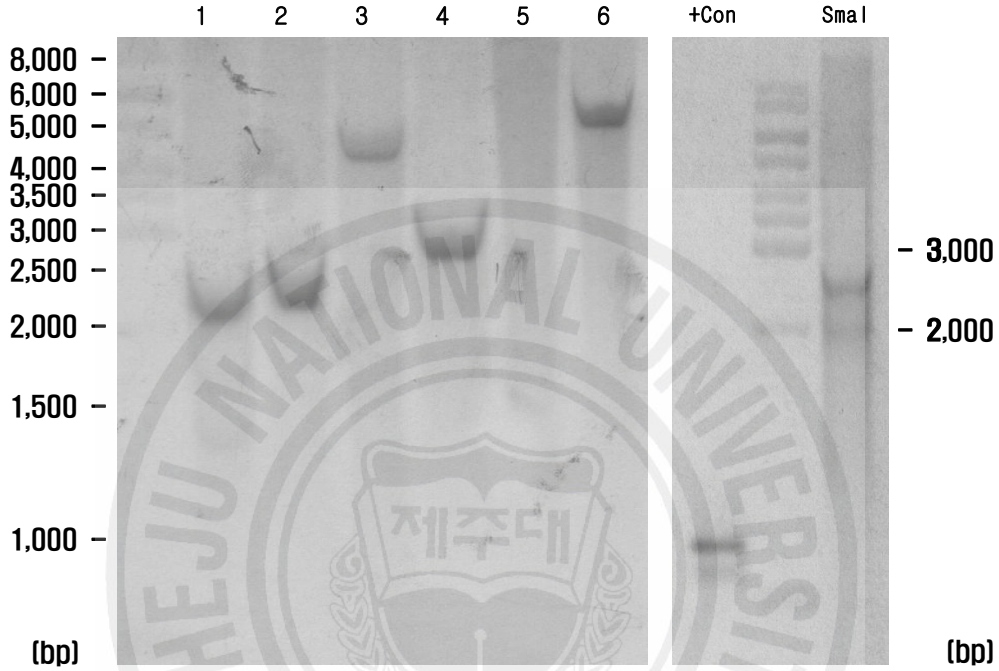

Figure 12. Southern blot analysis for wrasse *DMRT*. Wrasse genomic DNA was digested with several restriction enzymes, fractionated on an agarose gel, transferred to a nylon membrane, and then hybridized with gene-specific, ^{32}P-labeled probe. In each lane, fractionated DNA was digested with *EcoR*I and *Hind*III (1), *Hind*III (2), *Sac*I (3), *Pst*I (4), *Sfi*I (5), or *BamH*I (6) (left panel), and insert-digested plasmid DNA was applied as a positive control (+Con). The *Sma*I-digested fraction showed two 2- and 2.5-kb bands with differing signal intensities.

fraction, presumably because *Sfi*I restriction sites are rare in genomic DNA. In the *EcoR*I/*Hind*III fraction, weak signals were also detected at 1 and 1.5 kb, which may have been the result of non-specific hybridization. In the *Sma*I-digested fraction, the presence of 2.5- and 2-kb bands indicated the possible existence of other wrasse *DMRT*s (Fig. 12, right panel).

4. Gene structure

The genomic structure of the wrasse *DMRT* ORF was surveyed via DNA walking and target-specific primers (Table 8). PCR contigs were constructed from testicular genomic DNA using primers based on the wrasse *DMRT* cDNA sequence, which produced 28 overlapping products (data not shown). Based on these 28 short contigs, primer sets amplifying seven long contigs were selected, and the products were sequenced (Table 3). After sequence assembly, we identified five exons and four introns within the ORF of wrasse *DMRT* (Figs. 13 and 14). In the seven-contig primer set, the first primer set amplified from the 5′-flanking region to the middle of the first exon, producing a 1,826-bp product spanning -1721 to +105 bp. The second primer set amplified from the proximal 5′-flanking region into the second intron, producing a 1,541-bp product spanning the entire first intron and second exon. The third, fourth, fifth, sixth, and seventh primer sets amplified from the second to third intron (1,652-bp product spanning the entire third exon), from the third intron to the mid-fourth exon (973-bp product), from end to end of the fourth exon (190-bp product), from the mid-fourth exon into the fourth intron (898-bp product), and from the fourth intron into the 3′-UTR (1,695-bp product spanning the entire fifth exon), respectively (Fig. 13). Splicing junctions were pinpointed by identifying donor-acceptor splice sites (GT-AG) at the 5′- and 3′-ends of each intron (Mount, 1982). Interestingly, the 5′-flanking region in *DMRT* genomic DNA contained the two 5′-UTR segments identified in wrasse *DMO* cDNA (Fig. 14).

Figure 13. Gene structure and sequence for wrasse *DMRT*. Five exons consisting of 219, 175, 158, 190, and 158 bp were identified. The nucleotide sequences of the 5′-flanking region examined in the promoter assay and the unidentified 3′-UTR (shaded) were conjugated. Italics, start and stop codons; black boxes, exons; dots, unknown sequence.

Figure 13. Gene structure and sequence for wrasse *DMRT* (Continued).

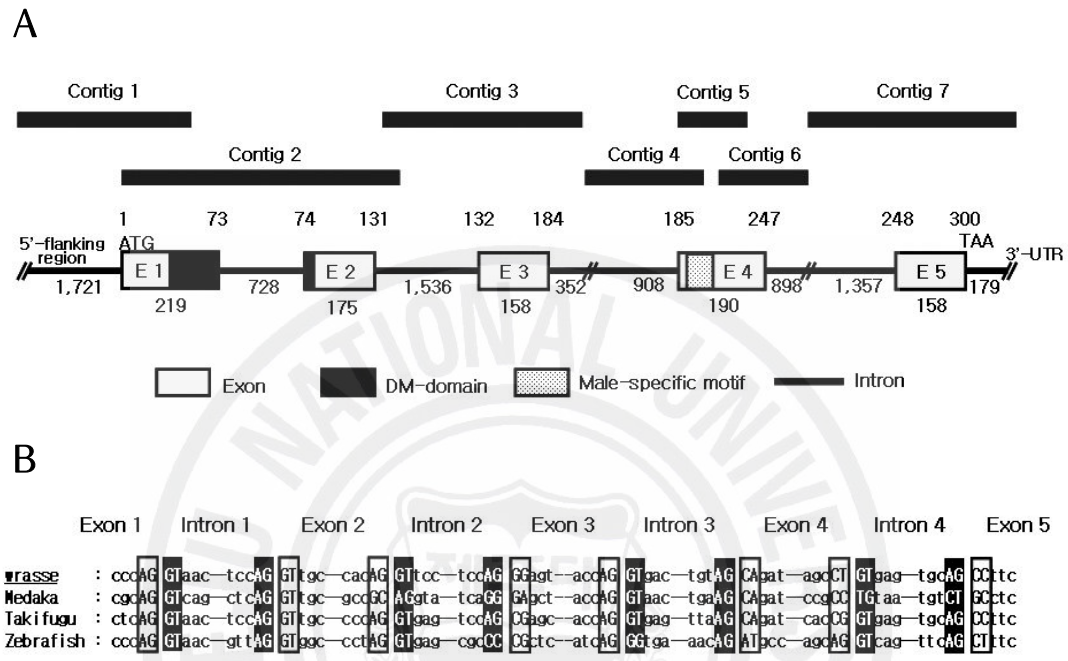

Figure 14. Schematic representation of wrasse *DMRT* gene structure and comparison of exon-intron junction. (A) Six contigs were generated from genomic DNA. Numbers under each solid bar (exon) and line (intron) represent the nucleotide sequence length (bp). Numbers on the bars indicate amino acid position. (B) Nucleotide sequence comparison at the *DMRT* gene exon-intron junction in four fishes. Acceptor-donor splice sites are indicated in empty and black boxes.

5. Characterization of wrasse *DMRT* transcriptional regulation

1) Putative regulatory sites in the promoter

As shown in Figure 13, a 1,826-bp product containing 1,721 bp of the region upstream from the start codon was cloned and sequenced. Putative regulatory sites within this sequence were identified using the Motif Library Database (Bioinformatics Center Institute for Chemical Research), and 21 putative sites scoring over 85 were selected. The selected putative regulatory sites (GATA1, AP4, GATA2, GATAx, Sox5, AP1, C/EBP, Dof2, AP1, STATx, C/EBP, Dof3, GATA1, GATA3, AP1, Dof1, SRY, C/EBP alpha, C/EBP beta, TATA, and CAP) are summarized in Table 7. In the promoter assay, the 5′-flanking region was amplified as 17 distinct regions (Fig. 15): first region (-122 to +50), harboring no putative regulatory sites; second region (-331 to -123), harboring TATA box, CAP, SRY, C/EBP alpha, and C/EBP beta binding sites; third region (-489 to -332), harboring a Dof1 binding site; fourth region (-676 to -490), harboring Dof3, GATA1, GATA3, and AP1 binding sites; fifth region (-868 to -677), harboring no putative regulatory sites; sixth region (-1,090 to -869), harboring STATx and C/EBP binding sites; seventh region (-1,361 to -1,091), harboring Sox5, AP1, C/EBP, and Dof2 binding sites; eighth region (-1,547 to -1342), harboring GATAx binding sites; ninth region (-1,721 to -1,548), harboring GATA1, AP4, and GATA2 binding sites. The tenth to seventeenth regions were 5′-deletion mutants for regions 1 - 9. Each PCR product was ligated into the *Xho*I/*Hind*III site of pGL3-Basic and pGL3-Enhancer vectors (Fig. 16). Successful recombinants were selected by PCR, digested with *Hind*III/*Sma*I, and sequenced to exclude multiple insertions (Fig. 16). Schematic representations are presented in Figures 17 and 18.

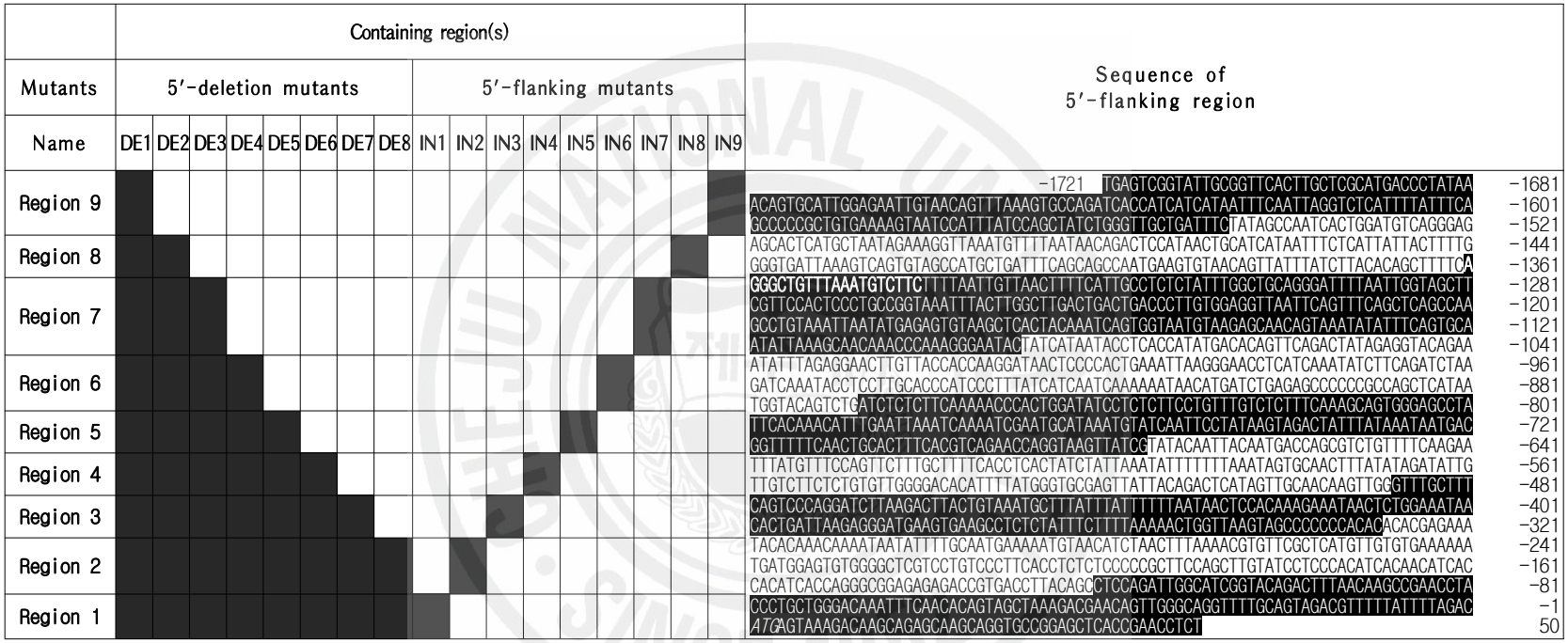

Figure 15. Selection of 5′-flanking regions for construction of wrasse *DMRT*-luciferase chimeras. 5′-deletion mutants and 5′-flanking mutants were prepared to examine the role of putative regulatory sites, using the primers indicated in Table 4. Shaded or non-shaded sequences represent each region amplified by PCR (right panel). The regions recombinated into each construct are marked in black boxes (left panel).

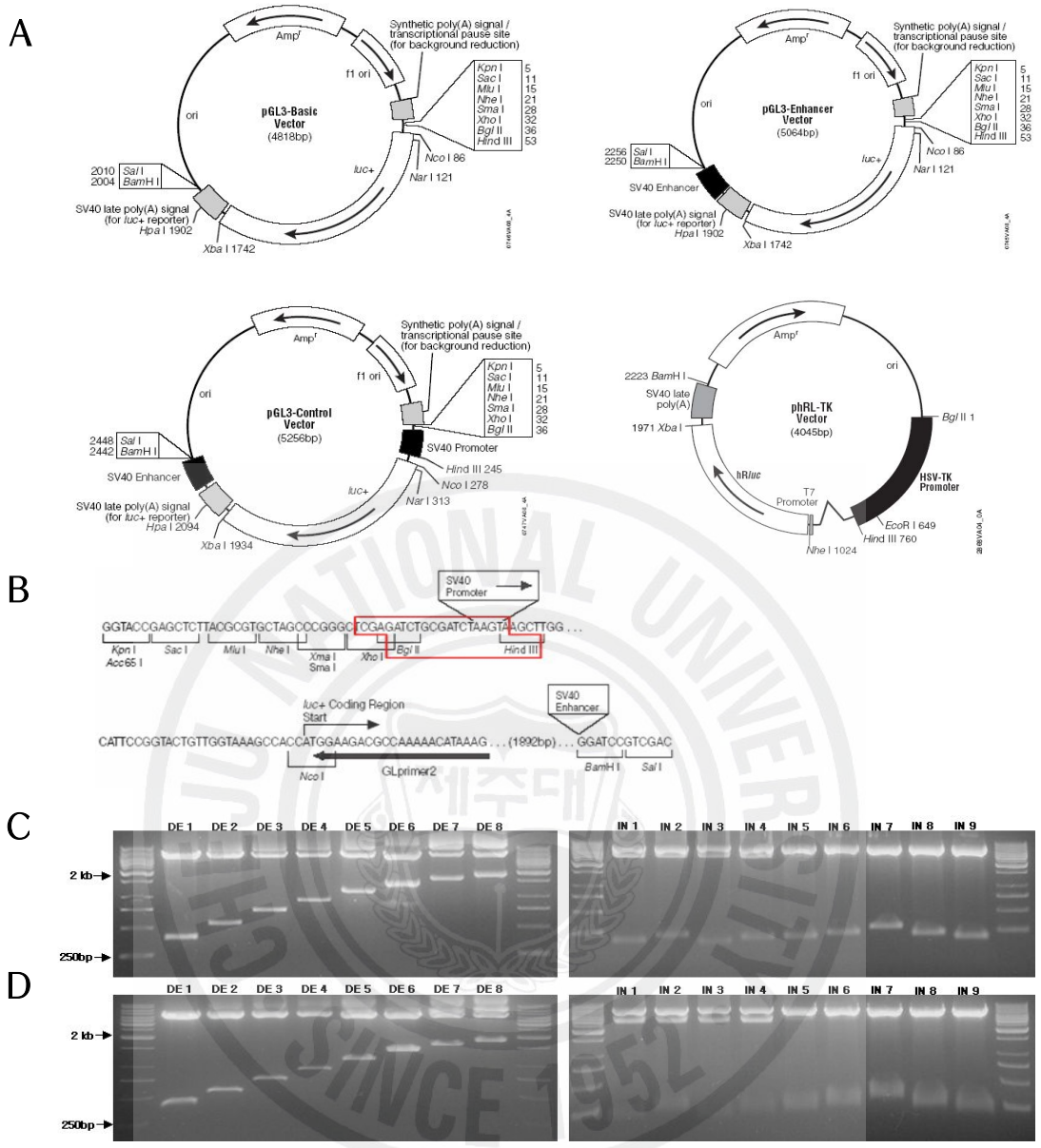

Figure 16. pGL3 and phRL-TK vector maps and multiple cloning sites (MCS) in pGL3 vectors. (A) pGL3-Basic and pGL3-Enhancer vectors were used to express *DMRT*-luciferase chimeras and as internal controls. pGL3-Control vector was used as a positive control, and phRL-TK vector was used to normalize firefly luminescence. (B) The *Xho*I and *Hind*III sites were selected, and chimeric vectors were digested using *Xho*I/*Hind*III and *Hind*III/*Sma*I. Vector maps and MCS draw was acquired from the manufacturer's web site (http://www.promega.com). (C,D) Verification of PCR products.

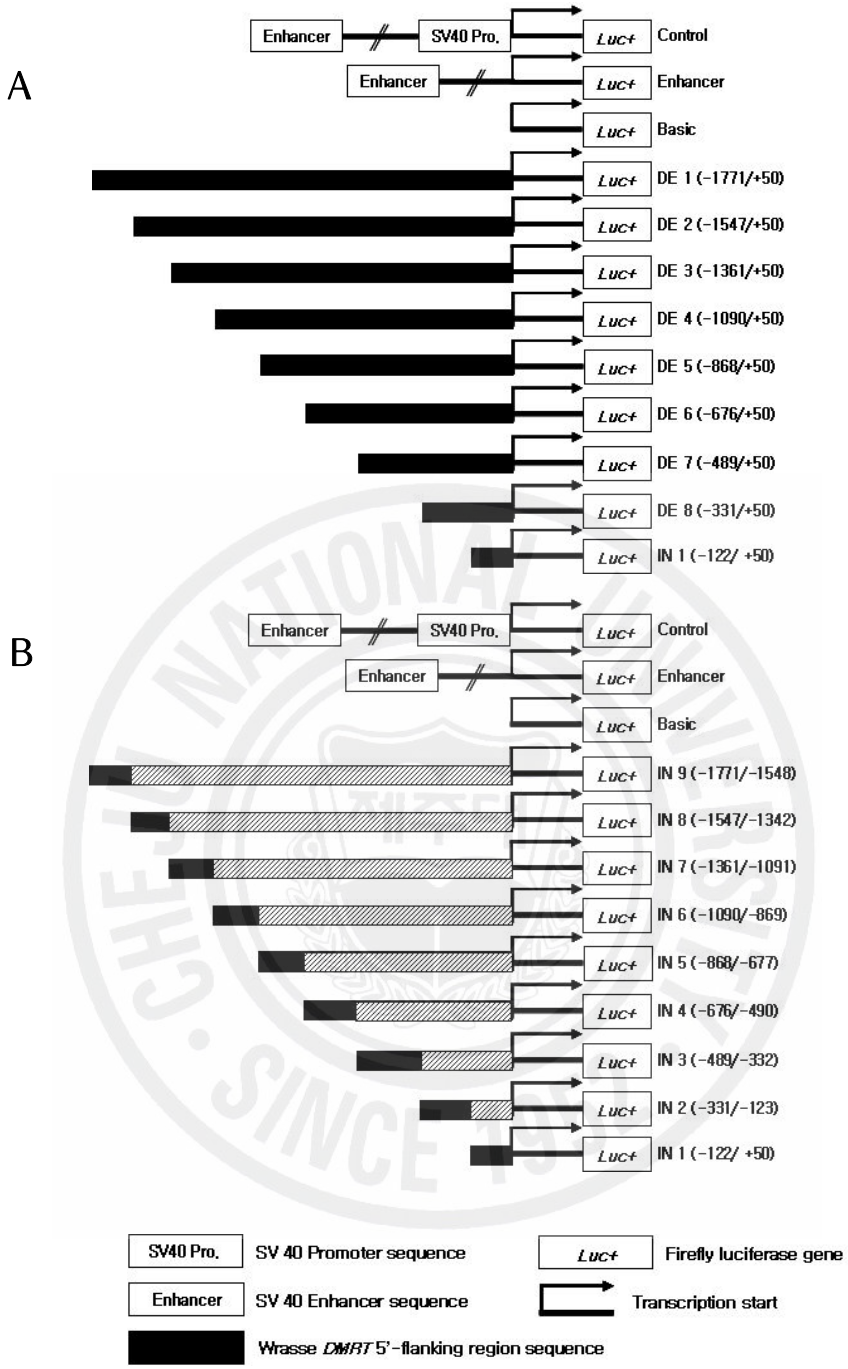

Figure 17. Schematic representation of wrasse *DMRT*-luciferase chimeras inserted into pGL3-Basic vector. To examine transcriptional activity, 5′-deletion mutants (A) and 5′-flanking mutants (B) were inserted upstream from firefly luciferase gene, without a promoter or enhancer sequence.

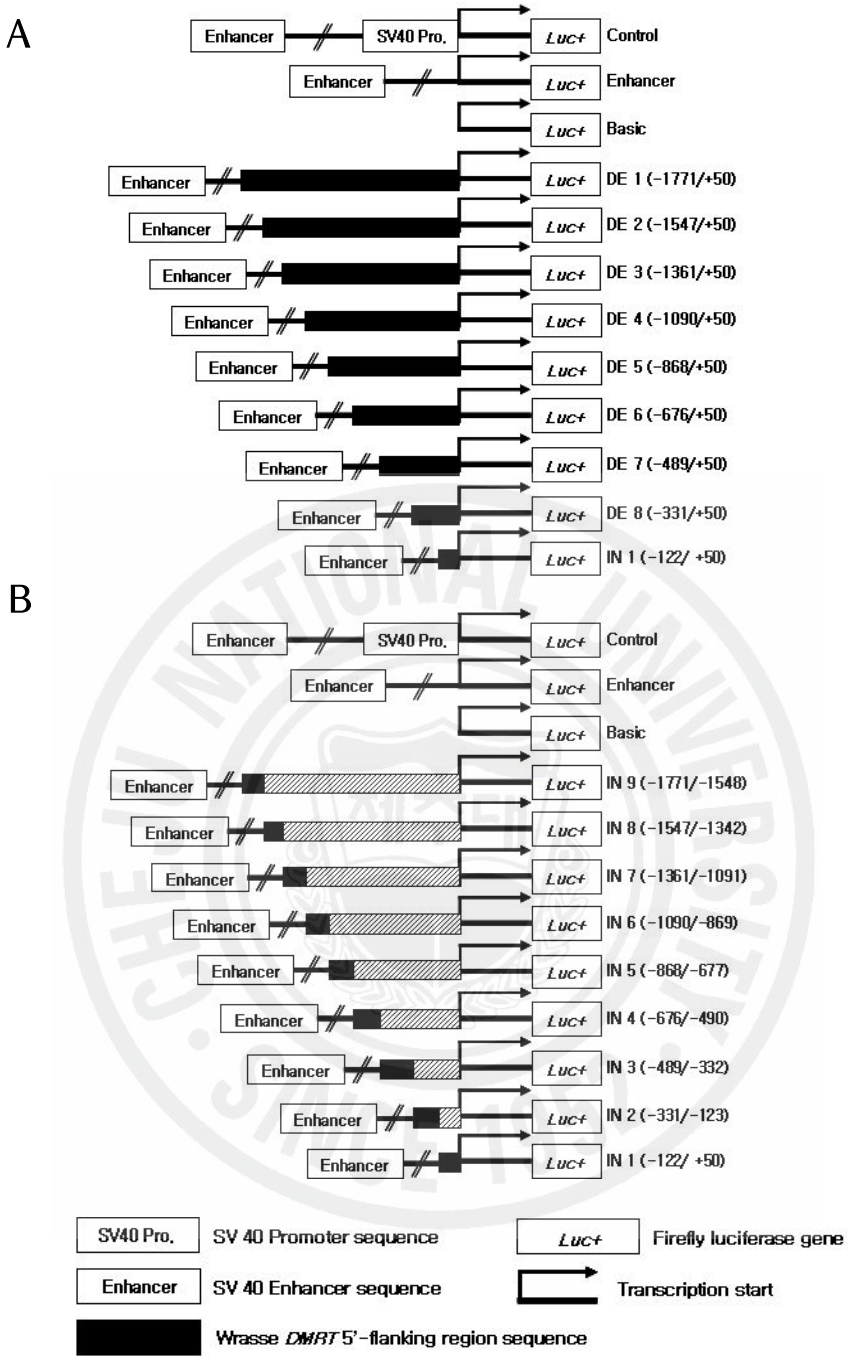

Figure 18. Schematic representation of wrasse *DMRT*-luciferase chimeras inserted into pGL3-Enhancer vector. To examine transcriptional activity, 5′-deletion mutants (A) and 5′-flanking mutants (B) were inserted downstream from an enhancer sequence and upstream from the firefly luciferase gene.

2) Transcriptional activity within the 5′-flanking region

To evaluate transcriptional regulation, the 17 recombinant clones described above were positioned upstream of the firefly luciferase reporter gene. The transcriptional activity of each recombinant clone was evaluated in the Cos-1 and TM4 cell lines. *DMRT*-luciferase recombinants cloned into pGL3-Basic vector did not show significant expression compared to non-chimeric pGL3-Basic vector in either cell line (Figs. 19A, 19B, 20A, and 20B). However, *DMRT*-luciferase chimeras in pGL3-Enhancer vectors showed significant transcriptional activity (Figs. 19C, 19D, 20C, and 20D). In the Cos-1 cell, three *DMRT*-luciferase chimeric recombinants (regions 2, 8, and 9) showed transcriptional activation of the luciferase gene. Proximal region 2 contained a putative SRY binding site, which increased transcriptional activity approximately 17-fold compared to non-chimeric vector. Distal regions 8 and 9 contained GATA1, AP4, GATA2, and GATAx binding sites, which increased transcriptional activity approximately 28- and 81-fold, respectively, compared to non-chimeric vector. In TM4 cells, proximal region 2 and distal regions 8 and 9 were also important for transcriptional regulation of the wrasse *DMRT* gene. Proximal region 2 increased transcriptional activity approximately 12-fold, and distal regions 8 and 9 increased transcriptional activity approximately 27- and 29-fold, respectively, compared to non-chimeric vector.

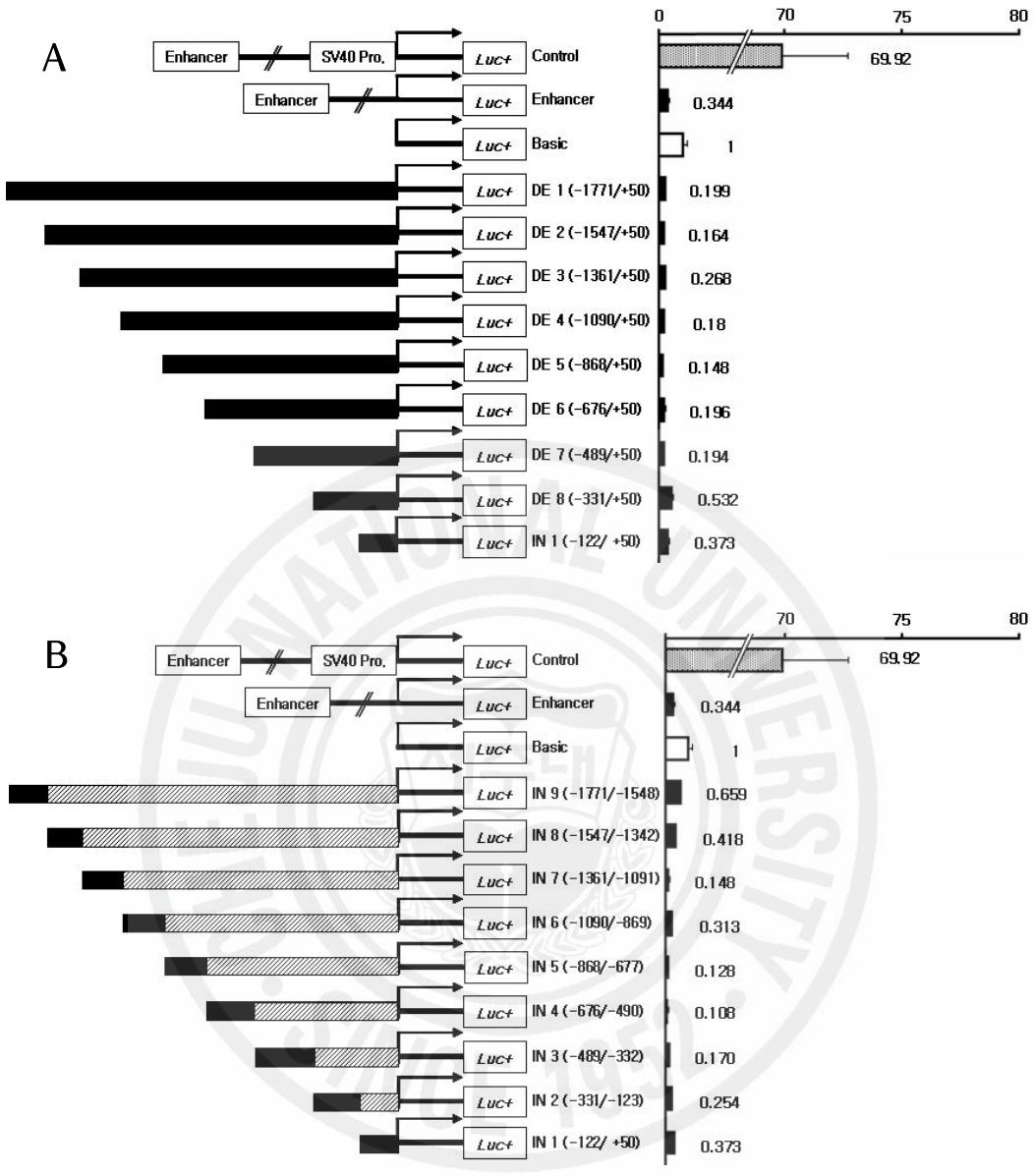

Figure 19. Regulatory activity of *DMRT*-luciferase chimeras in Cos-1 cells. 5′-deletion mutants (A, C) and individual mutants (B, D) were inserted into pGL3-Basic (A, B) or pGL3-Enhancer (C, D) vector and transfected into Cos-1 cells. Non-chimeric pGL3-Basic and Enhancer vectors were used as internal and negative controls, respectively. pGL3-Control plasmid DNA was used as a positive control. Each value represents the mean ± standard error for three replicates.

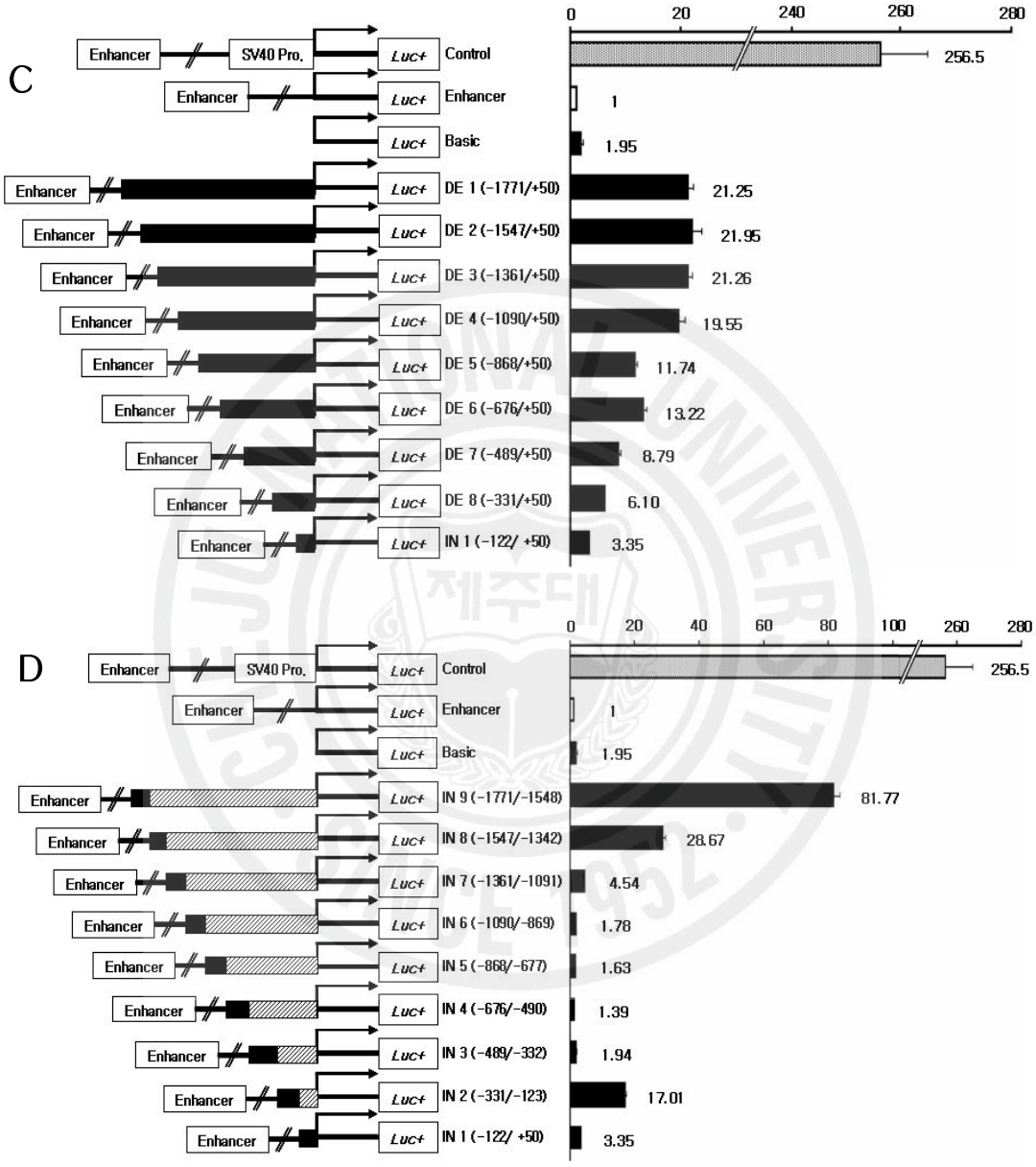

Figure 19. Regulatory activity of *DMRT*-luciferase chimeras in Cos-1 cells (continued).

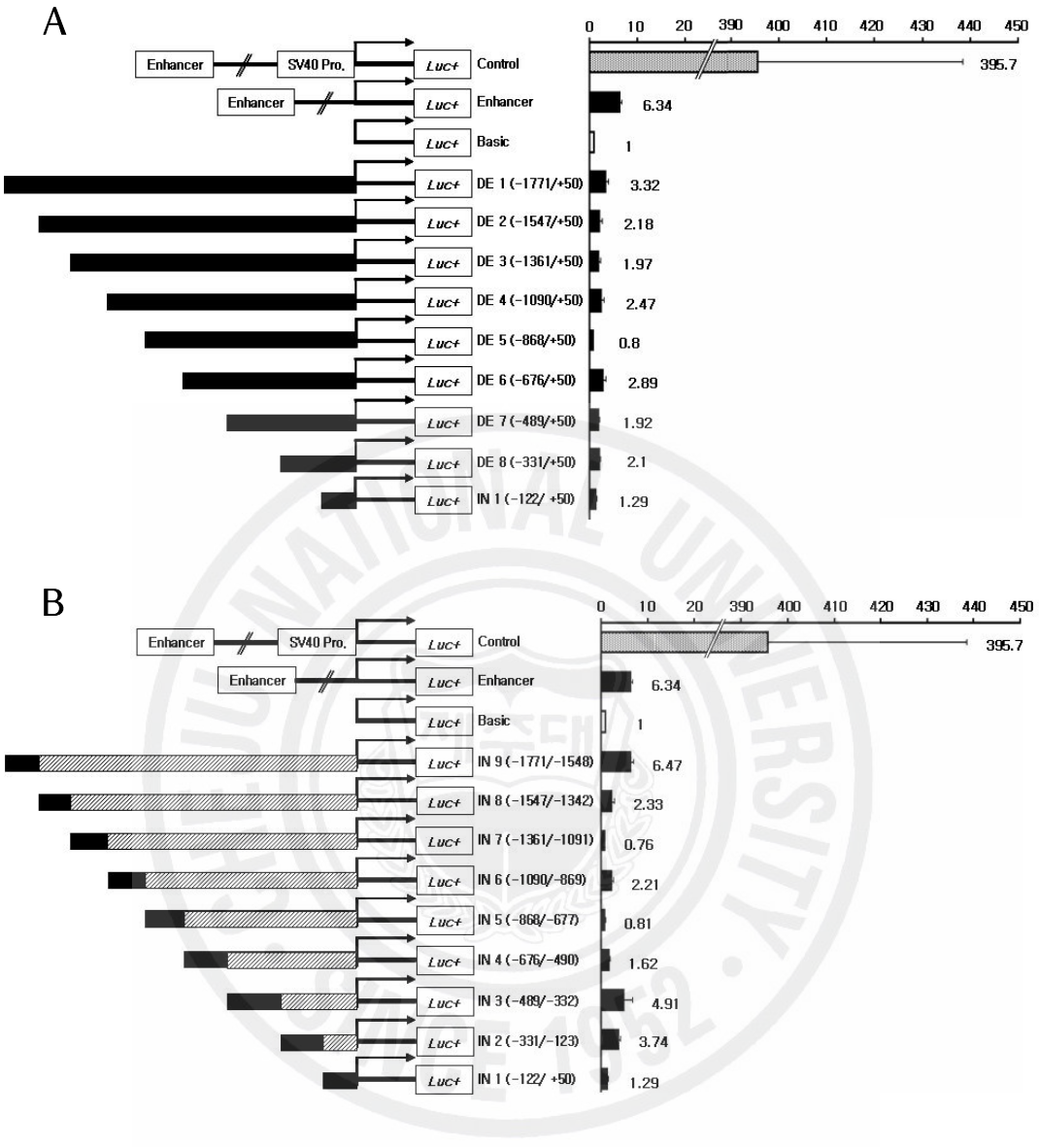

Figure 20. Regulatory activity of *DMRT*-luciferase chimeras in TM4 cells. 5′-deletion mutants (A, C) and 5′-flanking mutants (B, D) were inserted into pGL3-Basic (A, B) or pGL3-Enhancer (C, D) vector and transfected into TM4 cells. Non-chimeric pGL3-Basic and Enhancer vectors were used as internal and negative controls, respectively. pGL3-Control plasmid DNA was used as a positive control. Each value represents the mean ± standard error for three replicates.

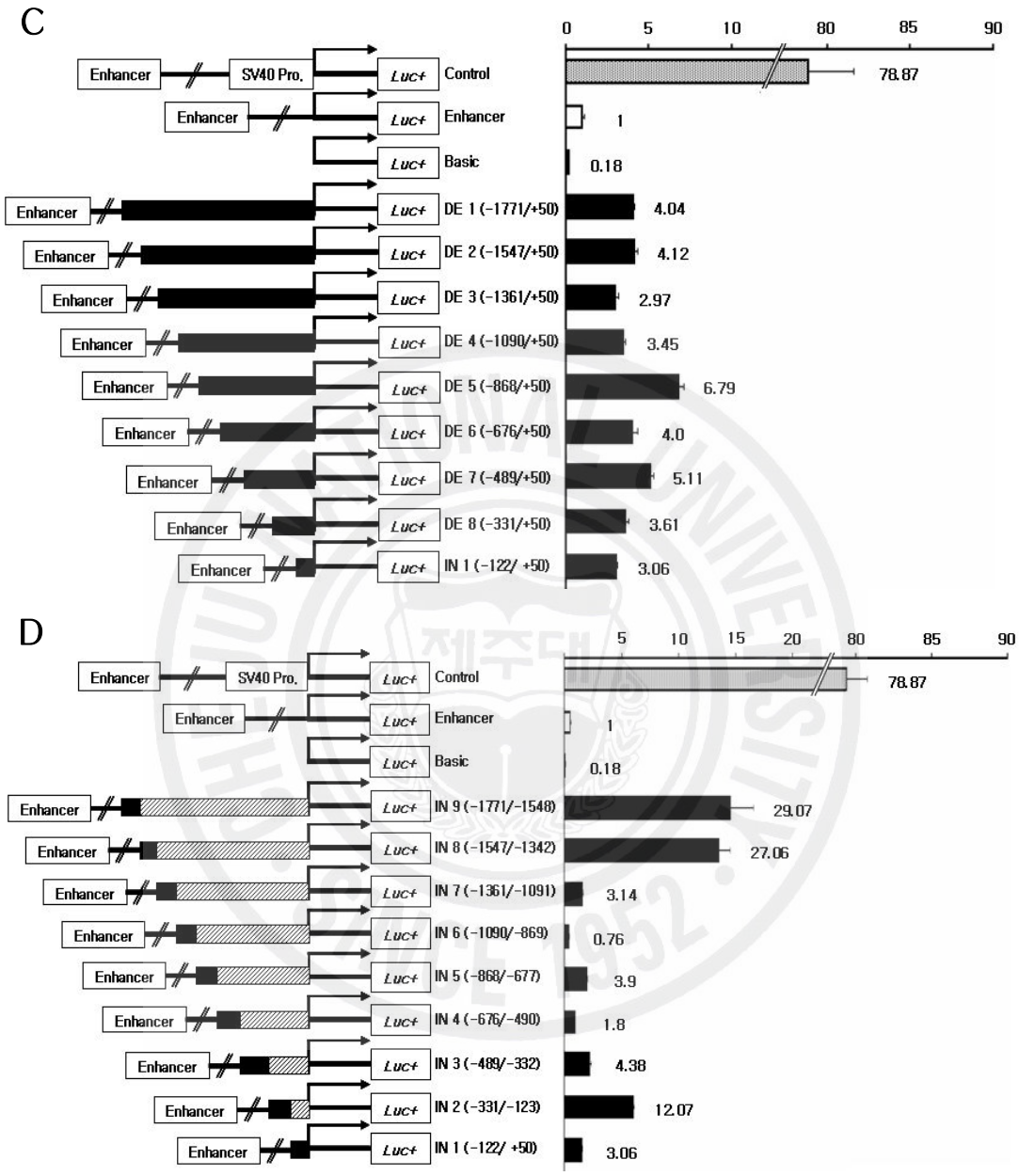

Figure 20. Regulatory activity of *DMRT*-luciferase chimeras in TM4 cells (continued).

DISCUSSION

1. *H.tenuispinis* expresses DM domain-containing genes

In this study, we isolated a full-length cDNA for wrasse *DMRT* from a testis-derived cDNA library. The cDNA was 3,119 bp long, including 184 bp of 5′-UTR and 2,032 bp of 3′-UTR. This cDNA contained a DM domain and the male-specific motif, and it was confirmed that the DM domain is conserved within phyla (Raymond *et al.*, 1998). The low-efficiency CATAAA poly(A) signal sequence, rather than the high-efficiency AATAAA sequence, was identified 14 bp upstream of the poly(A) tail. In addition, previous studies have identified many other *DMRT* isoforms as a result of alternative splicing (Winkler *et al.*, 2004; Guo *et al.*, 2005; Huang *et al.*, 2005). However, these cDNAs do not differ significantly in size or sequence, except in the 5′-UTR region. Interestingly, we isolated two highly similar *DMRT* cDNAs from the wrasse testis. Because some species show multiple copies of the *DMRT* gene, it is possible that the two *DMRT*s identified here originate from different regions of the genome (Brunner *et al.*, 2001). In addition to the DM domain and male-specific motif, wrasse *DMRT* cDNA also exhibited an asparagine-rich region and several short conserved motifs; however, the DMA and DMB domains identified in *D. magna* and *D. rerio* were absent (Kato *et al.*, 2008; Guo *et al.*, 2004).

A 545-bp partial cDNA for wrasse *DMO* was isolated from the ovary via 5′-RACE. This partial sequence contained most of the DM domain region and 333 bp of the 5′-UTR. Upon comparison with the genomic *DMRT* sequence, 212 bp of the first exon of *DMO* showed 100% homology with *DMRT*, but the 5′-UTR was divided into two aligned regions. Identical homology among

the first exons and divided 5′-UTRs may be the result of low- or non-conservancy at the exon-intron junction (Mount, 1982; Sinclair *et al.*, 1990), rather than two independent genes. Therefore, wrasse *DMO* and *DMRT* may be alternatively spliced forms of the same gene. In silkworms, alternative splicing of the same primary transcript is used to produce male- and female-specific mRNAs (Ohbayashi *et al.*, 2001).

However, the absence of donor-acceptor splice sites at the putative exon-intron junction in wrasse *DMO* suggests that *DMO* is located at a different site in the genome (Sinclair *et al.*, 1990). In addition, a report revealed that the 5′-flanking regions in mouse, human, and pig share high sequence homology, with the insertion and deletion of non-homologous sequences (Boyer *et al.*, 2002). Thus, if wrasse *DMRT* and *DMO* reside at different loci in the genome, insertion and deletion of sequences may have occurred.

Phylogenetic analysis of the wrasse *DMRT* gene showed high homology in the DM domain region and male-specific motif, confirming earlier evidence that this gene is evolutionarily conserved (Erdman and Burtis, 1993; Raymond *et al.*, 1998, 1999). Amino acid sequence alignment revealed that the DM domain region was highly conserved within phyla. The two wrasse DMRTs were differ by only one amino acid, and the partial cDNA for wrasse DMO was excluded from the multiple sequence alignment analysis. The deduced amino acid sequence for wrasse DMRT showed 60 and 43% homology with rainbow trout and tilapia DMRTs, respectively. Moreover, the DM domain retained approximately 80% homology compared to DMRTs from other species. A phylogenetic tree constructed for DMRTs showed three main branches for DMOs, human and chicken DMRTs, and fish DMRTs.

Expression analyses confirmed that *DMRT* is a male-specific gene in protogynous wrasse (Guan *et al.*, 2000; Marchand *et al.*, 2000), suggesting that wrasse *DMRT* plays a sex-related function in testis determination or

differentiation. Northern blot analysis detected two transcripts in the testis, one strongly positive and one weak, which may be the result of homology between the probe sequence and other *DMRT* isoforms. However, the dominant, specific transcript was only detected in wrasse testis, although several smaller (0.7–0.9 kb) isoforms have been reported in other fish (Guo *et al.*, 2005). These results demonstrate that wrasse *DMRT* is almost exclusively restricted to the testis. Previous reports have shown that *DMRT* isoforms are conserved in the 5′-region, but are variable in the 3′-region (Kondo *et al.*, 2002; Guo *et al.*, 2005). Thus, a probe targeting the male-specific motif would be more efficient in detecting the 3.2-kb transcript. Our Northern blot analysis was performed using mRNA from wrasse collected in May, during pre-reproductive gonad development. We previously observed that wrasse *DMRT* is strongly expressed during May and then down-regulated during June (data not shown), indicating that *DMRT* expression is time-dependent. In addition, alternatively spliced isoforms were differentially expressed in different tissues and different developmental periods (Winkler *et al.*, 2004; Guo *et al.*, 2005; Huang *et al.*, 2005). However, we were not able to confirm the existence of other wrasse *DMRT* isoforms via Northern blot analysis. Although a second 1.2-kb transcript was detected, we cannot exclude the possibility that it was a degradation product of the major transcript. If the 1.2-kb band was a true transcript, it would suggest that wrasse express another *DMRT* isoform, which may be differentially expressed in a time-dependent manner or under the control of some unknown activator. Designing specific probes for conserved regions, such as the DM domain region or the 5′-end of the ORF, would increase the probability of finding other *DMRT* transcripts (Guo *et al.*, 2005).

2. Genomic structure of the wrasse *DMRT* gene

Southern blotting was used to determine the gene copy number of wrasse *DMRT*. Positive signals were detected in various restriction enzyme-digested DNA fractions. However, no band smaller than 10 kb was detected in the *Sfi*I-digested fraction, presumably because *Sfi*I restriction sites are relatively rare in genomic DNA (average fragment size is 30 kb in the human genome; Sambrook and Russell, 2001). The *Sma*I-digested fraction revealed 2.5- and 2-kb bands, suggesting the existence of additional wrasse *DMRT* subtypes. However, these bands were neither straight nor sharp, which may reflect the large amount of DNA initially loaded (to compensate for the relatively low radioactivity [5 μCi] of the probe) or migration inhibition by residual proteins. Considering that DMY is a duplicate copy of the *DMRT* gene, these low-molecular-weight bands may indicate the existence of an additional *DMRT* gene located at a different locus of the wrasse genome (Matsuda *et al*., 2002). Moreover, sequence variations of *DMRT*s have been found frequently at the 3′ region, and are <0.7 kb with one intron, and sometimes no intron, containing *DMRT* mRNAs (Kondo *et al*., 2002; Guo *et al*., 2005). The probe used in this study was relatively long (400 bp) and spanned the exon-intron junction. Considering these factors, the weak signal detected in this study may represent another copy of the *DMRT* gene. Further studies are required to confirm the number and location of *DMRT*s within the wrasse genome using fluorescent *in situ* hybridization (FISH) and Southern analysis using PCR contigs (Matsuda *et al*., 2002; Nanda *et al*., 2002).

DNA walking with target-specific primers was used to examine the *DMRT* ORF in wrasse genomic DNA (Table 8). The wrasse *DMRT* gene consisted of five exons and four introns within the ORF. As predicted, donor-acceptor splice sites were identified at the exon-intron boundaries (Mount, 1982); these sites were also conserved in mammals and other fishes (Guo *et al*., 2005).

Interestingly, the 5′-flanking region of genomic wrasse *DMRT* DNA contained both of the 5′-UTR segments identified in wrasse *DMO* cDNA. Numerous examples of alternative splicing have been documented in both lower and higher vertebrates (Brunner *et al*., 2001; Kondo *et al*., 2002; Guo *et al*., 2004, 2005; El-Mogharbel *et al*., 2007). Wrasse and platypus *DMRT1* has five exons (El-Mogharbel *et al*., 2007), whereas *DMRT*s of medaka and platyfish has distinct isoforms consisting of two and three exons (Kondo *et al*., 2002). Zebrafish genomic *DMRT1* has seven exons, which are then assembled into three distinct isoforms containing three, five, and five exons (Guo *et al*., 2005). Notably, no intron was found within the 1.7-kb *DMRT* gene in orange-spotted grouper (Xia *et al*., 2007). These examples of alternative splicing strongly suggest the existence of two or more *DMRT* isoforms in wrasse.

Motif analysis using the database showed that the cysteine-rich DM domain consisted of intertwining CCHC and HCCC zinc-binding sites containing six cysteine residues. The 3D structure of the wrasse DM domain predicted using SWISS-MODEL (Arnold *et al*. 2006) showed high tertiary similarity to the DM domain in *D. melanogaster dsx* (Zhu *et al*., 2000).

3. Transient transfection and promoter assay

To examine transcriptional regulation of the *DMRT* gene, DNA walking and specific nested primers designed against the first exon were used to clone the 5′-flanking region from wrasse testis genomic DNA. A 1,721-bp region upstream of the start codon was cloned and sequenced, and a number of putative regulator binding sites (GATA1, AP4, GATA2, GATAx, Sox5, AP1, C/EBP, Dof2, AP1, STATx, C/EBP, Dof3, GATA1, GATA3, AP1, Dof1, SRY, C/EBP alpha, C/EBP beta, TATA, and CAP) were identified via database searches.

To examine promoter activity, *DMRT*-luciferase chimeric mutants containing these putative regulatory binding sites were transfected into Cos-1 and TM4 cells. It was previously reported that the *DMRT*s 5′-flanking regions in mammals (human, mouse, and pig) showed over 60% homology (Boyer *et al.*, 2002). However, the 5′-flanking region (1,721 bp) isolated from wrasse *DMRT* showed low sequence homology compared to human, mouse, pig, and fugu (data not shown), and no significant sequence homology was found even within fish *DMRT*s. Wrasse and fugu *DMRT* shared less than 10% sequence homology in the 5′-flanking region, suggesting significant differences in transcriptional regulation between these species. Phylogenetic analysis suggested that the wrasse *DMRT* gene has diverged greatly from fugu *DMRT*, with deletion and/or insertion of nucleotides.

Wrasse *DMRT*-luciferase chimeras lacking an enhancer sequence showed extremely low expression in both Cos-1 and TM4 cells. In mouse and rat, *DMRT* was expressed in Sertoli cells (Lei and Heckert, 2002); however, in orange-spotted grouper, *DMRT* was expressed only in germline cells, including spermatogonia, primary spermatocytes, and secondary spermatocytes (Xia *et al.*, 2007). These results suggest that *DMRT* expression is controlled via different mechanisms among vertebrates. Moreover, stable cell lines (MSC-1 and TM4) showed much lower *DMRT* expression than primary cultured Sertoli cells (Lei and Heckert, 2004), which may explain why wrasse *DMRT* expression was so low in vectors lacking the enhancer sequence. In contrast, *DMRT*-luciferase chimeras linked to an enhancer sequence showed dramatic increases in *DMRT* expression in both Cos-1 and TM4 cells (up to 30% of the positive control). These results suggest that even though the *DMRT* gene itself is conserved from lower to higher vertebrates, its regulation is not. Because *Sox9*, which is regulated by SRY (Kent *et al.*, 1996), was repressed in grouper Sertoli cells (Xia *et al.*, 2007), it is possible that *DMRT1*, which also contains an SRY binding site, has functionally

replaced *Sox9* in the wrasse testis.

In this study, the GATA1, AP4, GATA2, GATAx, SRY, C/EBP alpha, and C/EBP beta binding sites regulated the transcription of *DMRT* in Cos-1 and TM4 cells. Among these, the AP4, C/EBP alpha, and C/EBP beta sites were relatively less important as transcriptional regulators, because the functionally similar AP1 and C/EBP binding sites of wrasse *DMRT* showed almost no transcriptional activity. The GATA1 and GATA3 sites located at positions -612 to -599 showed low transcriptional activity, which may reflect competition at these overlapping sites, and/or their relatively low prediction scores (distal GATA1, 98; proximal GATA3, 86). Notably, the Sox5 binding site showed low transcriptional activity, which supports our hypothesis that *DMRT1* could functionally replaced *Sox9* in wrasse. Interestingly, although STAT was reported to be a testis-specific regulator in mammals (Guan *et al*., 2000), the STATx binding site showed low transcriptional activity in wrasse, providing further evidence that the wrasse *DMRT* gene is regulated via a unique mechanism. All 5′-deletion mutants showed relatively low transcriptional activities compared to the 5′-flanking mutants, which may reflect the fact that the enhancer sequence was not specific to the wrasse *DMRT1* promoter region.

Based on our results, we hypothesize that the *DMRT* genes function in two molecular pathways (Fig. 21). First, because *DMRT* is able to interact with SRY, it may function as a positive competitor or direct activator of *Sox9* and/or downstream gene(s), such as an *Amh*. In general, many sex determination- and differentiation-related genes are activated by the synergistic effect of transcription factors and/or enhancers. Thus, it is important to consider the possibility of cooperation among several regulatory sites (Teo *et al*., 1999; Zhu *et al*., 2000; Miyamoto *et al*., 2008).

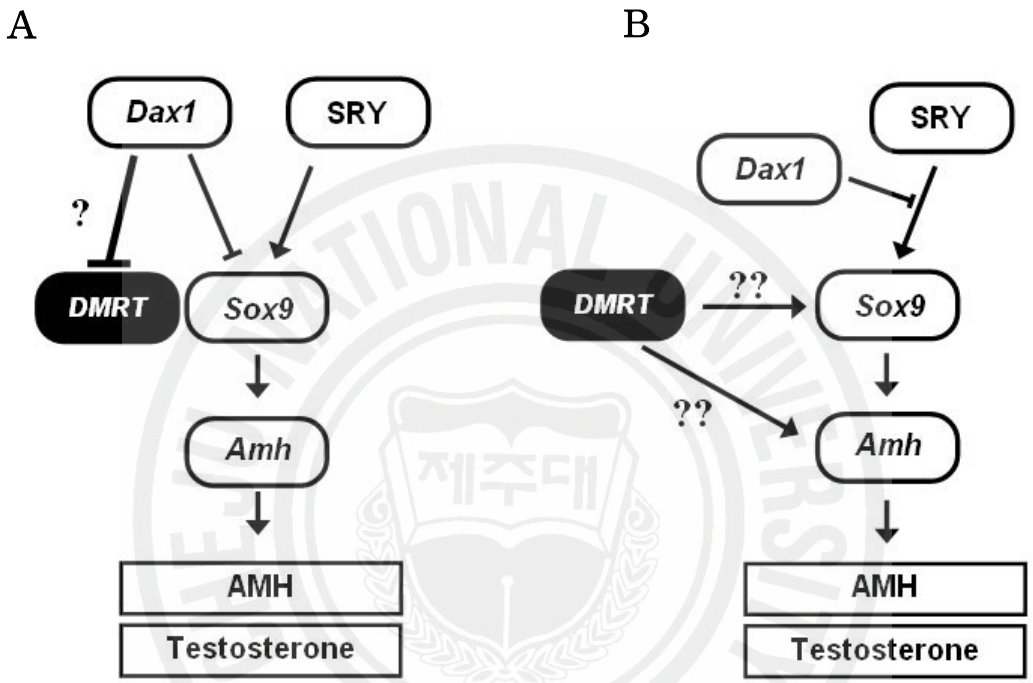

Figure 21. Putative functional mechanism of wrasse DMRT. DMRT may function as a positive competitor for *Sox9* (A) or as a direct activator of *Sox9* and/or downstream gene(s), such as an *Amh* (B).

4. Conclusions

In this study, two cDNAs for DM-domain containing genes were isolated from the testis and ovary of the protogynous wrasse *H. tenuispinis*. The full-length *DMRT* (3,119 bp) contained the conserved DM domain and male-specific motif. In contrast, the partial *DMO* cDNA isolated from the ovary was 545 bp long and contained most of the DM domain. A 3.2-kb transcript was detected in testis, but not in the ovary, confirming that *DMRT* plays a male-specific role in protogynous wrasse. Our results suggest that wrasse possess two copies of the *DMRT* genes, one of which consists of five exons. GATA binding sites in the distal region (-1,721 to -1,362) and the proximal SRY-binding region (-330 to -123) regulated wrasse *DMRT* gene transcription in Cos-1 and TM4 cells. This study provides basic insight into the roles of *DMRT* genes in protogynous fish. Our results indicate that *DMRT* plays a testis-specific function in *H. tenuispinis*, although the existence of an ovarian subtype (*DMO*) suggests that these DM domain-containing genes participate in a complex regulatory mechanism in protogynous fish.

Because wrasse reverse their functional sex after sexual development, one would expect to observe dramatic changes in *DMRT* and/or *DMO* expression in the gonads of adult females undergoing sex reversal. Because *DMRT* is up-regulated during testis determination and development, it is important to identify the exogenous factors and gene(s) that regulate *DMRT* expression. Furthermore, because sex reversal in wrasse involves social cues, it is also important to determine the relationship between visual information and gonadotropin expression. Additional research is required to understand the relationship between endogenous hormones and *DMRT* expression. Endogenous hormones do not seemed to act directly upon *DMRT* expression, but rather upon upstream genes such as GATA, which we identified as a

regulator of *DMRT* in this study.

Although several types of DM domain-containing genes have been isolated from fishes, the function and signaling mechanisms of these genes remain unclear. Further studies using DNase I footprinting and the luciferase reporter assay are required to identify regulators of the *DMRT* gene. Furthermore, *DMRT*-knockout using RNA interference (RNAi) may help to determine the role of *DMRT* in protogynous wrasse during sex reversal. Finally, yeast two-hybrid screening and the electrophoretic mobility shift assay (EMSA) may prove useful in identifying molecules that interact with *DMRT*.

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국 문 초 록

어류에서 생리적, 행동학적, 그리고 기능적 성 역할은 유전적, 사회적, 그리고 다양한 환경적 요소의 영향을 받는다. 어류는 종에 따라, 자웅이체, 동시자웅동체, 그리고 순차적인 성 전환을 일으키는 자성선숙성 자웅동체와 웅성선숙성 자웅동체 등 다양한 성 체제를 가지고 있다. 성 체제 결정에 많은 가변요인들이 영향을 미칠 수 있지만, 성 결정 및 분화 경로에 있어 보편적으로 나타나는 중요한 인자들이 존재한다. 동물의 문 (phyla) 수준에서 보존적인 스테로이드방출세포가 발생초기 단계에 작용하여 pregnenolone을 testosterone으로 전환시키고, 기능적 성 발현을 이끄는 성 호르몬 합성에 작용한다. Androgen과 estrogen을 포함하여, 3β-hydroxysteroid dehydrogenase, 11β-hydroxyase, Dax1, Sox9, Wt1, SF1, 그리고 aromatases와 같은 유전자들도 문 (phyla) 수준에서 보존적으로 발견되고 있다. 특히, 포유류에서 처음으로 보고된 SRY 유전자는 성 결정인자로서 작용하는 것으로 밝혀졌다. 어류에서도 포유류의 SRY와 유사한 기능을 갖는 유전자가 발견되었고, Y 염색체상에 존재함으로 인해 DMY라 명명되었다. DMY는 *Drosophila melanogaster*의 *doublesex* 유전자와 *Caenorhabditis elegans*의 *mab3* 유전자에 공통적인 서열인 DM-domain으로 갖고 있으며, 수컷 특이적인 발현양상을 보인다고 알려진 *DMRT*의 Y 염색체 복사본이라고 보고된 바 있다. DM-domain을 함유하는 유전자들은 zinc-finger 전사인자로서, 성 발달이나 성 결정에 중요한 역할을 담당한다. 본 연구는 자성선숙성 자웅동체어류인 놀래기 (*Halichoeres tenuispinis*)에서 성 결정 및 분화의 분자적 기작을 이해하기 위한 기초자료를 제공하기 위하여 수행하였다. 본 연구에서 cDNA library screening 및 RACE 법을 통하여 놀래기 정소에서 DM-domain을 함유하는 유전자의 전체 cDNA 서열을, 난소에서는 일부의 서열을 밝혀냈다. 본 연구에서 분리해 낸 놀래기 *DMRT* 유전자는 3,119 bp의 크기로서, DM-domain과 male-specific motif를 함유하고 있지만 DMA 및 DMB domain은 없었다. 놀래기의 난소에서 분리해 낸 DM-domain 함유 유전자인 *DMO*의 부분서열에는 놀래기 *DMRT* 유전자의

첫 엑손부위와 5'-UTR 영역을 포함하고 있었다. *DMO* cDNA의 545 bp의 서열 중 212 bp는 놀래기 *DMRT* 유전자와 100%의 염기서열 상동성을 나타내었다. 놀래기 genomic DNA의 서열과 비교했을 때 놀래기 *DMO*의 5'-UTR 부위 서열은 두 부분으로 나뉘어졌으나, 상응하는 부위의 염기서열에는 차이가 없었다. 여러 동물에서 보고된 DMRT 단백질들의 아미노산 서열을 비교해 본 결과, DM-domain 영역이 문(phyla) 수준에서 매우 보존적임을 확인할 수 있었다. 아미노산의 3차 구조를 예측한 결과, 놀래기 DM-domain은 *Drosophila melanogaster*의 *doublesex*와 사람의 *DMRT*에 존재하는 DM-domain 영역과 입체적으로 매우 유사한 구조를 갖고 있음을 확인할 수 있었다. *DMRT* 전사체를 확인하기 위하여 수행한 Northen blot 분석 결과, *DMRT* cDNA의 크기와 일치하는 약 3.2 kb 크기의 전사체가 정소에서는 검출되었다. 그러나, 난소에서는 전사체가 검출되지 않아, 자성선숙성 자웅동체어류인 놀래기에서 *DMRT* 유전자는 정소 특이적인 기능을 담당하는 것으로 추론할 수 있었다. Southern blot 분석 결과, 놀래기의 *DMRT*는 게놈상에 두 개의 좌위에 존재할 것으로 판단되었다. 놀래기 정소의 genomic DNA에서부터 여러 PCR-contig들을 확보하여 *DMRT*의 cDNA 서열과 상응하게 배열하여 *DMRT* 유전자 구조를 분석하였다. 그 결과, *DMRT* ORF 영역이 5개의 엑손과 4개의 인트론으로 구성되어 있음을 확인하였다. 놀래기 *DMRT* 유전자의 1번 엑손은 73개의 아미노산을 암호화하고 있었으며, 2번 엑손은 58개, 3번과 4번, 그리고 5번 엑손은 각각 53개, 63개, 53개의 아미노산을 암호화하고 있었다. 또한, 모든 엑손-인트론 연결부위에는 보존된 splicing donor 및 acceptor 서열이 존재하였다. *DMRT* 유전자의 전사조절에 관여하는 인자들을 확인하기 위하여 Genomic DNA로부터 DNA-Walking 방법을 사용하여 1,721 bp 길이의 5'-flanking 부위를 분리하여 스물 한 곳의 발현조절부위를 예측하였다. 5'-flanking 부위를 주형으로 사용하여 CAP, TATA box, SRY, C/EBP alpha, C/EBP beta, Dof1, AP1, Dof3, GATA1, GATA3, STATx, C/EBP, AP1, Dof2, C/EBP, AP1, sox5, GATAx, AP4, GATA2, 그리고 GATA 1을 부위별로 포함하는 PCR을 수행하였다. 각 PCR 산물을 루시페라아제 발현벡터인 pGL3-Basic, pGL3-Enhancer vector에 cloning하여 *DMRT*-루시페라아제 혼성체를 구성하였다. 혼성체는 5'-UTR 부위가 조금씩 줄어드는 5'-deletion 방

식과 특정 조절부위만을 개별적으로 혼성화시키는 두 방법으로 구성하여 Cos-1 세포주와 TM4 세포주에 각기 주입함으로 그 전사 활성정도를 측정하였다. 두 종류의 세포 모두에서 pGL3-Basic vector 혼성체들은 전사활성을 나타내지 않았다. 그러나, pGL30-Enhaner vector 혼성체들을 전사활성을 나타내었으며, 이를 통해 말단부의 GATA 결합부위와 (-1,721 bp～-1,362 bp) 기저부의 SRY 결합부위가 (-330 bp～-123 bp) 놀래기 *DMRT*의 발현에 중요한 역할을 담당할 것으로 추측되었다. 말단부 및 기저부를 함유한 혼성 luciferase vector는 비혼성 luciferase vector에 비해, 전사활성이 최대 81배와 17배가 높은 것으로 확인되었다. 다양한 어류에서 DM-domain을 함유하는 유전자들이 보고되고 있지만, 그 기능과 신호전달의 기전은 아직도 분명히 밝혀지지 않았다. 따라서, 앞으로 다른 형태의 성결정 및 분화 관련 유전자들을 포함하여, 정소특이적인 발현을 나타내는 *DMRT* 유전자의 전사조절 인자를 밝혀내고, 그 작용경로를 밝히는 연구가 필요하다.

주요어: DM-domain 함유 유전자, *DMRT*, *DMO*, 자성선숙성 자웅동체, DMY, ORF, RACE, 노던블럿, 써던블럿, 유전자구조, PCR contig, 프로모터, 루시페라아제, SRY, GATA, 유전자발현조절, Cos-1, TM4

감사의 글

교수님들과 선배님들이 어려워 쩔쩔매던 그 때는 추억으로 접히고, 어느 덧 ‘후배들’ 앞에서 ‘잔소리’를 늘어놓는 선배로, 그리고 이제는 울타리 안에서의 선배 자리를 물려주어야 할 시간이 제게도 돌아왔습니다. 항상 부족했고 스스로도 만족스럽지 못했지만, 여러분들의 크신 도움으로 학위과정을 마무리할 수 있게 되었습니다. 저의 모습을 오랫동안 지켜봐 주시고 지도해주셨던 여러 교수님들과 띠 동갑이 훌쩍 넘는 새내기 후배들, 실험실에서 먹고 자며 어려운 일들을 함께 겪으며 고민했던 선・후배님들, 그리고 지금의 제 자신을 있게 해 준 생명과학과 가족들 모두에게서 받은 큰 사랑에 감사의 마음을 가질 수 있었던 소중한 시간이었습니다. 이러한 관심과 격려로 인해 이루어 진 그간의 결과를 부끄러운 마음으로 내놓습니다.

본 논문이 나오기까지 격려와 지도, 때로는 질타로써 저를 이끌어주시고 아껴주신 김세재 교수님께 진심으로 감사의 마음을 전합니다. ‘실험실 가족’의 수장이자 아버지로서 보여주셨던, 제자들이 부끄러워질 열정과 헌신의 모습을 저 또한 잊지 않고 기억할 것입니다. 가슴이 아프기도 했지만, 제게 부족한 부분들을 정확히 짚어주시고 자극을 주시던 그 말씀을 깊이 새기어, 더욱 발전하는 모습을 보이리라 다짐합니다. 감기로 인해 힘든 수업을 받을 때, 쉬었다 하자시며 조용히 음료를 건네주셨던 유머와 근엄의 오문유 교수님. 자칫 어려울 수 있는 내용을 정말 쉽게 설명하여 주셨던 오덕철 교수님. 학부시절, 복학 후의 첫 과제물에서 오기와 자존심을 일깨워주셨던 ‘호랑말코’ 이용필 교수님. 미소와 함께 풍부한 현장의 경험과 지식을 제공하여 주셨던 김문홍 교수님. 본 논문의 심사위원장을 기꺼이 맡아 여러 부분의 조언과 새로운 시작에 필요한 격려를 아끼지 않아 주셨고, 깊이 있는 내용으로 공부의 재미를 알게 해 주셨던 김원택 교수님. 이모같은 편안함으로 삶의 여러 이야기들을 들려주셨던 이화자 교수님. 함께 공부하자시며 제자들의 의견을 존중해 주셨던 고석찬 교수님. 심사위원으로서 본 논문을 다듬고 조언해 주셨고, 우리 생명과학과에 새로운 바람을 일으키시는 이선령 교수님. 교수님들의 가르치심과 조언, 그리고 격려의 말씀들을 발판삼아 떳떳한 제자가 될 것을 약속드리며, 깊은 감사의 마음을 전합니다. 또한, 석・박사과정의 연구주제에 대한 기본 아이디어와 함께, 아무것도 모르던 학부시절에 직접 해부까지 하시며 숨은 지식도 아낌없이 내어 주셨고, 본 논문의 심사위원까지 맡아주신 이영돈 교수님께 진심으로 감사의 마음을 전합니다. 십 년이 넘는 시간을 실험실에서 함께 부대끼며 정말 많은 실전지식들을 넘겨주셨고, 본 논문의 심사위

원까지 맡아 꼼꼼하게 챙겨주신 친형 같은 박지권 박사님께도 감사의 마음을 전합니다.

각 처에서 관련 분야를 자랑스럽게 이끌어 가시는 정완석 박사님, 강신해 박사님, 문상욱 박사님, 김기옥 박사님, 김성철 박사님, 박영철 선생님, 이동헌 박사님, 고미희 박사님, 오유성 박사님, 강봉조 박사님, 문명옥 박사님, 정용환 박사님, 송관필 박사님, 오주형 박사님, 오순자 박사님, 박수영 박사님, 그리고 한상현 박사님께도 감사의 마음을 전합니다. 해양과환경연구소를 통해 알게 된 김병호 박사님, 나오수 선배님, 임봉수 박사님, 송영보 박사님, 박용주 박사님, 이치훈 박사, 허상우 선생, 허성표 선생, 류용운 선생, 강형철 선생, Sao 선생, 김수용, 김병훈, 학생께서도 제게 많은 도움과 따뜻한 마음을 주셨습니다. 동문으로서의 자부심과 사랑을 느끼게 해 주신 김관배 선배님, 진기탁 선배님, 김청식 선배님, 오진보 선배님, 김익현 선배님, 강맹수 선배님, 김미량 선배님, 이창훈 선배님, 이정배 선배님, 오충헌 선배님, 한경용 선배님, 양인석 선배님, 김이사벨 선배님, 최진석 선생, 양경철 선생, 김성윤 선생께도 감사드립니다.

어디에 내 놓아도 자랑스러운 실험실 가족들인 최수연 박사님, 황일선 선생님, 진영준 선생, 고희철 선생, 오대주 선생, 황준호 선생, 강성일 선생, 현경만 선생, 이주엽 선생, 김무한 선생, 신혜선 선생, 홍윤석, 김효민, 양윤실, 홍혜진, 강민지, 윤선아, 최재영, 박소현, 이지훈 후배들께 미안함과 더불어 실험실의 '가족'으로서의 긍지를 당부합니다. 표현은 잘 못하지만, 항상 걱정해주고 진심어린 마음으로 지켜봐 준 윤지현 박사에게도 저의 깊은 마음을 전합니다.

보고싶은 친구들인 명근, 민준, 인철, 일남, 기인, 정근, 경호, 서형, 성일, 준범, 창민, 종승, 대현, 영엽, 재식, 수영, 그리고 영철이, 잘 해주지 못해 미안한 동기들인 강정찬 선생, 고민홍 선생, 김민우 선생, 김병삼 선생, 김병석 선생, 김상범 박사, 성철우 선생, 윤병준 선생, 윤훈석 박사, 현문일 선생, 힘든 연구의 길을 택해 정열을 불태우는 정상배 선배님, 전형식 선생님, 김병수 선생님, 현화자 선생, 이종철 선생, 송국만 선생, 윤원종 선생, 강민철 선생, 장민호 선생, 양경식 선생, 이창훈 선생, 김지영 선생, 고운철 선생, 이가은 선생, 한은규 선생, 임은영 선생, 이세영 선생, 그리고 짧은 지면에 다 적지 못하는 여러 선·후배님들께서도 많은 도움을 주셨습니다.

먼 곳에서도 바로 옆에 있는 듯, 필요한 격려와 도움을 주시는 누님. 무뚝뚝하나 따뜻한 마음으로 아낌없이 베푸시는 멋진 매형. '형제'로서 더 이상의 말이 필요 없는 형님. 중·고교 동창이라 아쉬웠지만, 그 생각이 이제는 부끄러운 형수님. 그리고 제가 하는 모든 일들을 항상 믿고 존중해 주시는 아버지와 어머니께 감사의 마음으로 이 논문을 바치며 사랑한다는 말을 전합니다.