Journal of Oceanology and Limnology
Vol. 37 No. 4, P. 1333-1341, 2019
https://doi.org/10.1007/s00343-019-8182-1

Functional analysis of the promoter of the *dmrt1* gene in Chinese tongue sole, *Cynoglossus semilaevis**

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Received Jul. 1, 2018; accepted in principle Aug. 14, 2018; accepted for publication Sep. 6, 2018

Abstract The *dmrt1* (*doublesex* and *mab-3* related transcription factor 1) gene is considered to play an essential role in testis differentiation and development among metazoan species. As an economically important marine fish in China, Chinese tongue sole (*Cynoglossus semilaevis*) possess a ZW/ZZ sex-determining system, and the females usually grow two to four times bigger than males. Previous studies have confirmed that Z-linked *dmrt1* gene is the male determining gene in *C. semilaevis* and TALENs-mediated knocking-out individuals showed increased growth phenotype. In order to investigate the function of *dmrt1* regulatory regions and its potential application, we have cloned the promoter of *C. semilaevis dmrt1*. The acquired sequence consisted of a 5′-upstream sequence and a partial exon1; functional analysis showed that there were two positive regulatory regions in the promoter, as well as one negative region and one neutral region. These positive regions were then combined to obtain several newly constructed promoters. Both in vitro and in vivo analysis confirmed one of them (*dmrt1-Δ3+Δ4*) showed elevated promoting activity compared to the full-length promoter and could drive exogenous gene expression in tongue sole gonads. Our results will provide useful information for understanding the regulatory mechanism of *dmrt1* gene during *C. semilaevis* sex determination, and the endogenous promoter will facilitate the transgenic research for analyzing sex- and growth-related genes.

Keyword: *Cynoglossus semilaevis*; *dmrt1*; promoter; transcription regulation; luciferase assay; gonad

1 INTRODUCTION

dmrt1 (*doublesex* and *mab-3* related transcription factor 1), first isolated from the human testis cDNA library, is homologous to the *Drosophila* sex regulatory gene *doublesex* and the nematode worm sex regulator *mab-3*, both of which contain a zinc finger-like DNA-binding motif (DM domain) (Raymond et al., 1998). With expression mainly in the developing gonads of male embryos, *dmrt1* is evolutionarily conserved among metazoan species including mammals, birds, reptiles and fishes, and plays an essential role in testis differentiation and development (Raymond et al., 1999, 2000; Jørgensen et al., 2008; Smith et al., 2009; Yoshimoto and Ito, 2011; Zhao et al., 2015).

In most mammals that employ an XX/XY sex-determining system, the *Sry* gene on the Y chromosomes triggers the male sex determination, while *dmrt1* is dispensable in the sex determination process but maintains Sertoli cell in postnatal testis instead (Gubbay et al., 1990; Sinclair et al., 1990; Zhao et al., 2015). Nevertheless, in several non-

* Supported by the Special Scientific Research Funds for Central Non-profit Institutes, Yellow Sea Fisheries Research Institute (No. 20603022016004), the Central Public-interest Scientific Institution Basal Research Fund, YSFRI, CAFS (No. 20603022017003), the National Natural Science Foundation of China (No. 31530078), the Taishan Scholar Climbing Project Fund of Shandong of China, National Natural Science Foundation Projects (No. 31802275), and the Post-doctoral Applied Research Project Fund of Qingdao City (No. ZQ51201617023)
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mammalian vertebrates, such as chicken and frog with a ZZ/ZW system, *dmrt1* is mapped to the Z chromosome and considered to be a male sex-determining gene (Koopman, 2009; Yoshimoto and Ito, 2011; Lambeth et al., 2014). Intriguingly, in a teleost fish medaka (*Oryzias latipes*) that uses an XX/XY system, the duplicated copy of *dmrt1* on the Y chromosome named *DMY*/*Dmrt1Y* is served as a male determinant (Matsuda et al., 2002; Nanda et al., 2002). *DMY*/*Dmrt1Y* is exclusively expressed in the somatic cells surrounding the germ cells in XY embryos during early gonadal formation and persists in a Sertoli cell in mature testis (Kobayashi et al., 2004). Over-expression of this particular gene can induce female-to-male sex-reversal (Matsuda et al., 2007), and knocking-down/out can result in fertile male-to-female sex-reversal (Paul-Prasanth et al., 2006; Luo et al., 2015; Chakraborty et al., 2016). In addition, a sexually dimorphic expression pattern of *dmrt1* has been detected mainly during testicular differentiation in male individuals in many other fish species such as rainbow trout, zebrafish, orange-spotted grouper and tilapia (Guan et al., 2000; Marchand et al., 2000; Xia et al., 2007; Jørgensen et al., 2008). However, functional evidence of this gene in fish is still rare so far.

Chinese tongue sole (*Cynoglossus semilaevis*) is an economically important marine flatfish that is widely distributed in Chinese coastal waters, and the body length and weight of adult females are two to four times that of males (Chen et al., 2009; Ji et al., 2011). Thus, studies of sex determination in *C. semilaevis* will have both theoretical and economic significance. Our previous studies in *C. semilaevis* have suggested that it has ZZ/ZW sex chromosome system and *dmrt1* is located on the Z chromosome with a corrupted pseudogenized copy on the W chromosome (Zhou et al., 2005; Chen et al., 2009, 2014). The *C. semilaevis dmrt1* is highly expressed in the germ cells and pre-somatic cells of the undifferentiated gonad at the "sex-determining stage" (approximately 60-dph) and persists at high levels during testis development in the male individual. In the sex-reversed ZW males (genotype ZW) induced by high temperature, this gene exhibited similar expression level to normal male (ZZ) (Chen et al., 2014; Shao et al., 2014). Furthermore, knocking-out of *dmrt1* in ZZ male using the TALENs technique causes an abnormal testis and disrupted spermatogenesis, and the *dmrt1*-deficient males show accelerate growth characteristics compared to wild-type males (Cui et al., 2017). All of these observations support the view that *dmrt1* is a male determining gene in *C. semilaevis*, which might function in a dosage-dependent manner. Therefore, investigation of the promoter of this gene could provide further information for understanding its sex-related regulation mechanism. Besides, identifying of this promoter could supply a suitable regulatory element to drive interesting gene expression through a transgenic approach in the *C. semilaevis*.

In this paper, we cloned the promoter sequence of *C. semilaevis dmrt1*, which included 5′-upstream sequence and partial exon1. To investigate the potential role of regulatory elements in gene expression, we have constructed truncated promoters and analyzed their promoting activity. The results showed that there were two positive regulatory regions in the promoter. Furthermore, we combined these positive regions to construct a new promoter, in vitro analysis showed its high driving activity (3.25 times versus that of the full length promoter), and in vivo analysis confirmed its improved promoting capability for exogenous gene expression in tongue sole gonads.

2 MATERIAL AND METHOD

2.1 Material

2.1.1 Ethics statement

The collection and handling of the *C. semilaevis* used in this study were approved by the Animal Care and Use Committee at the Chinese Academy of Fishery Sciences, and all the experimental procedures were performed in accordance with the guidelines for the Care and Use of Laboratory Animals at the Chinese Academy of Fishery Sciences.

2.1.2 Experimental fish maintenance

The *C. semilaevis* were kept in the Haiyang High-Tech Experimental Base (Haiyang, China). The fish were fed twice a day with commercial pellets and were reared in filtered seawater at 22–23°C.

2.2 Method

2.2.1 Acquisition and analysis of the *C. semilaevis dmrt1* promoter sequence

Cynoglossus semilaevis genomic DNA was extracted from caudal fins using a TIANamp Marine Animals DNA Kit (TIANGEN, China). The *dmrt1*

Table 1 Sequences of primers

Primer	Sequence (5′→3′)
dmrt-F	ggggtaccCACCAAGTTTACGGTTCTC
dmrt-F2	ggggtaccAAAGGATACAAAGCTTCA
dmrt-F3	ggggtaccGTGAATACCAGGCAGT
dmrt-F5	ggggtaccCAATTGCATACTTTATAACC
dmrt-R	ccctcgagCATGGTCCTTGTCTGTGGG
dmrt-Δ3-R	ggactagtGAAAGTGAAGATGCCTAA
dmrt-Δ4-R	ggggtaccGCATACTCAGCATCAACA
dmrt-Δ3+Δ4-R	ggggtaccGAAAGTGAAGATGCCTAA
Luc-qF	CAACTGCATAAGGCTATGAAGAGA
Luc-qR	ATTTGTATTCAGCCCATATCGTTT
β-actin-qF	GAGTAGCCACGCTCTGTC
β-actin-qR	GCTGTGCTGTCCCTGTA

Uppercase letters indicated the primer sequence and lowercase letters indicated the restriction enzyme sites with protective nucleotides.

promoter was isolated using specific primers (dmrt-F/R, Table 1) designed according to the archived *dmrt1* mRNA sequence (GenBank: EU070761.1) and the assembled *C. semilaevis* Z chromosome sequence (GenBank: NC_024328.1). The PCR cycling conditions were: 5 min initial denaturation at 94°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min; and a final 10 min extension at 72°C. The 2 935 kb PCR product was sub-cloned into the *pEASY*-T1 cloning vector (Transgene, China), transformed into *Trans*1-T1 cells (Transgene, China) and sequenced.

The putative transcriptional binding sites in the promoter sequences of *dmrt1* were predicted using the TFBIND online software (http://tfbind.hgc.jp/) (Tsunoda and Takagi, 1999). To analyze the similarity of *dmrt1* promoter among species, the corresponding sequence of zebrafish, medaka, frog, rat, and human were downloaded from the NCBI database and the multiple sequence alignment was conducted by the MAFFT online tool (https://www.ebi.ac.uk/Tools/msa/mafft/).

2.2.2 Construction of the luciferase transfection vectors

To evaluate the promoting activity of regulatory elements of *dmrt1*, six promoter-luciferase plasmids were constructed by serial-deletion, according to the distribution of predicted positive/negative transcription factors (Fig.1). The primers used were listed in Table 1, with a *Kpn* I and *Xho* I recognition site added to the 5′ end of each forward and reverse primer, respectively. Purified PCR products were digested and cloned into the *Kpn* I/*Xho* I site of the *pGL3-Basic* vector (Promega, CA, USA) which contains a firefly luciferase gene. In addition, *pGL-dmrt1-1* was digested to construct *pGL-dmrt1-4* and *pGL-dmrt1-6* with *Kpn* I/*Spe* I and *Kpn* I/*Pst* I, respectively, followed by end-blunting using T4 polymerase (TaKaRa, Dalian, China) and self-ligation with T4 ligase (Promega, WI, USA) (sketched in Fig.2).

For *pGL-dmrt1-Δ3* construction, dmrt-F and dmrt-Δ3-R (Table 1) were used to amplify the -2 932/-1 928 *dmrt1* promoter fragment and *Kpn* I and *Spe* I were used to delete the -2 932 to -1 326 sequence of *pGL-dmrt1-1*. Thus, the -2 932/-1 928 PCR product was ligated to the deleted *pGL-dmrt1-1* to form *pGL-dmrt1-Δ3*. For *pGL-dmrt1-Δ4* and *pGL-dmrt1-Δ3+Δ4* constructions, *dmrt-F/dmrt-Δ4-R* and *dmrt-F/dmrt-Δ3+Δ4-R* primers (Table 1) were designed to amplify the -2 932/-1 326 and -2 932/-1 928 *dmrt1* promoter fragments, respectively, similarly to the *pGL-dmrt1-Δ3* formation. The two PCR products were digested by *Kpn* I and then ligated separately to the *pGL-dmrt1-5* vector (-867/+3) (sketched in Fig.3). All plasmids were prepared using an EndoFree Plasmid Mini Kit (CWBIO, Beijing, China).

2.2.3 Cell culture

Human embryonal kidney (HEK) 293T cells were routinely cultured in DME/F-12 medium (HyClone, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Australia) and recombinant human FGF (Invitrogen, MD, USA) and maintained in 5% CO_2 at 37°C.

2.2.4 Transfection and luciferase assay

Transfection experiments were performed in 24-well culture plates. For the dual-luciferase assay, the cells were co-transfected with 800 ng of promoter-luciferase constructs and 40 ng of *pRL-TK* plasmid (Renilla luciferase gene driven by HSV thymidine kinase promoter) using Lipofectamine 2000 Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The *pGL-3 Basic* vector was used as negative control. At 48 hours post-transfection, cells were lysed and the activities of Firefly and Renilla luciferase were measured using dual-luciferase reporter gene assay kit (Beyotime, Shanghai, China) with a Varioskan Flash spectral scanning multimode reader (Thermo, Vantaa, Finland). Each experiment was performed in triplicate.

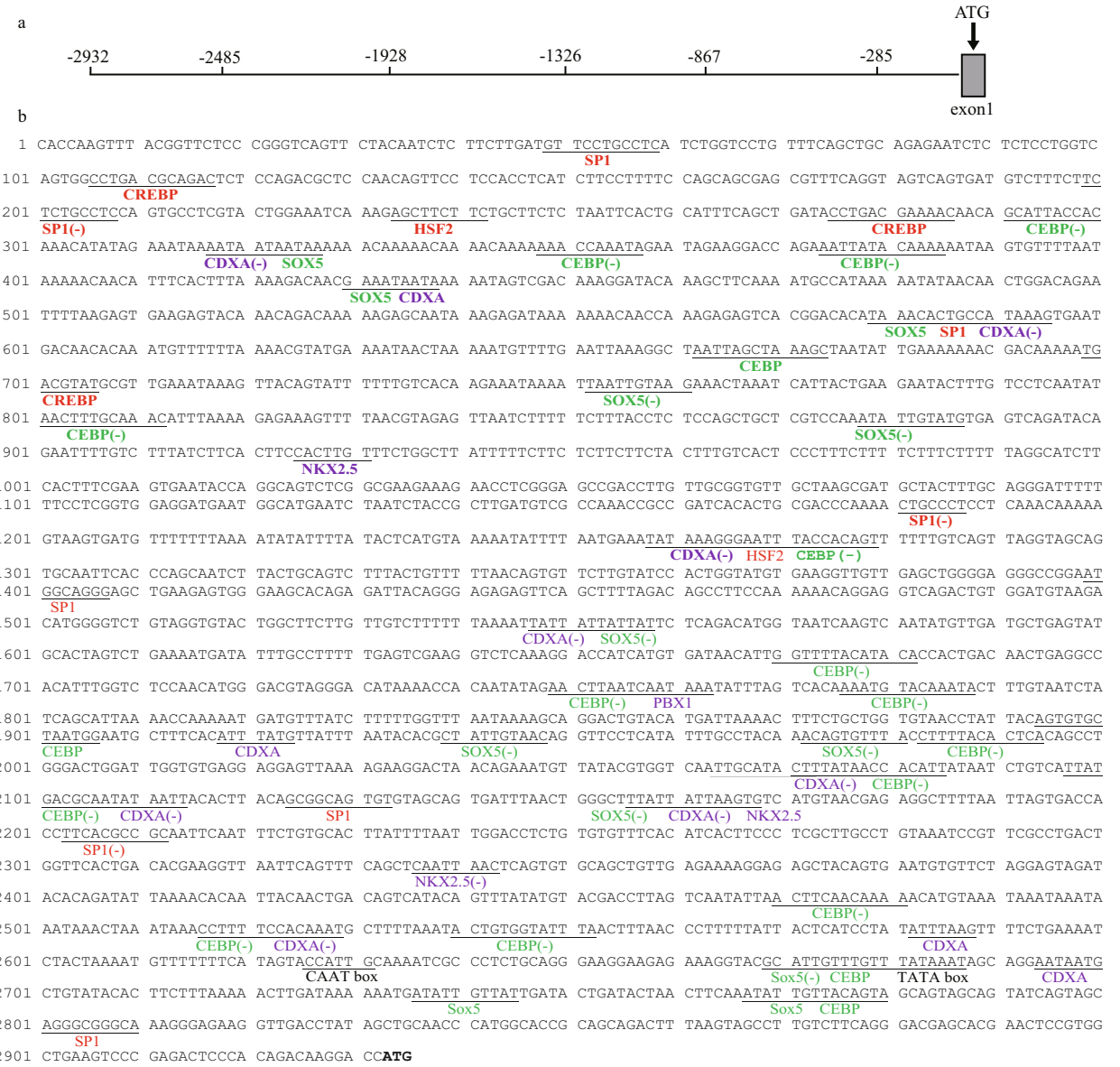

Fig.1 Nucleotide sequence of *C. semilaevis dmrt1* promoter region and the potential transcription factor binding sites

a. schematic map of the *C. semilaevis dmrt1* promoter. The box represented the exon1. The numbers above the line indicated the positions (the first base of ATG was set as +1); b. nucleotide sequence of the *C. semilaevis dmrt1* promoter and its potential functional components. Underlined boldface letters showed the CAAT box and TATA box. Putative positive factors were underlined in red, negative factors in green, neutral factors in purple, the ATG was in bold. (-) represented the location on the complementary chain.

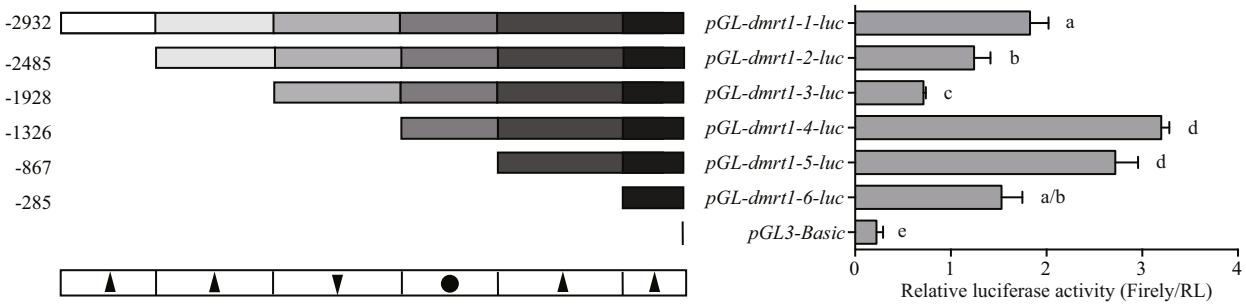

Fig.2 Luciferase assays of the *C. semilaevis dmrt1* truncated vectors

The left panel showed the schematic map of the *dmrt1* truncated promoter-luciferase plasmids and the effects of each region. The triangle, inverted triangle or circle represented the up-regulated, down-regulated or neutral effect of the corresponding region. The right panel exhibited the luciferase activities of corresponding truncated promoters. Statistical differences were indicated as different letters.

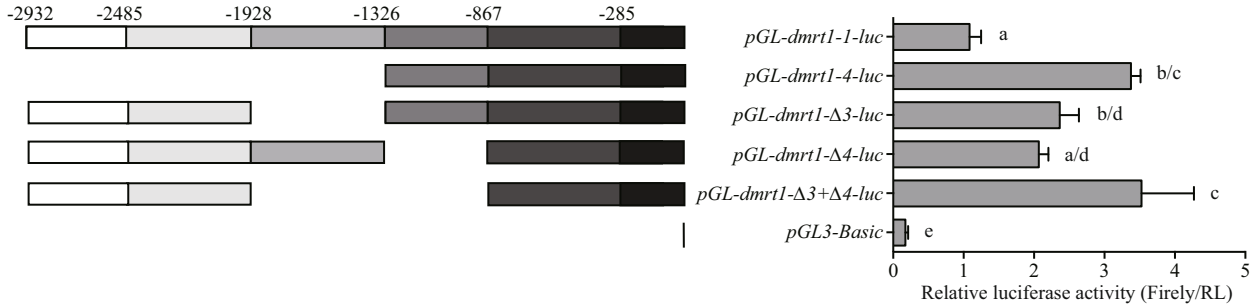

Fig.3 Luciferase assays of the new-constructed *C. semilaevis dmrt1* promoters

The left panel showed the schematic map of the region-deleted promoter-luciferase plasmids. The numbers above indicated the positions. The right panel exhibited the luciferase activities of corresponding promoters. Statistical differences were indicated as different letters.

2.2.5 In vivo analysis of promoters

To test whether candidate *dmrt1* promoters could drive the expression of reporter gene luciferase in *C. semilaevis* in vivo, plasmids *pGL-dmrt1-4*, *pGL-dmrt1-Δ3*, and *pGL-dmrt1-Δ3+Δ4* were injected into *C. semilaevis* separately. Test *C. semilaevis* were divided randomly into 7 groups (n=3 per group) according to plasmids injected: PBS as blank control; *pGL-3 Basic* vector as a negative control; *pGL-3 Control* vector containing an SV40 promoter as positive control; *pGL-dmrt1-1*; *pGL-dmrt1-4*; *pGL-dmrt1-Δ3* and *pGL-dmrt1-Δ3+Δ4*. Each group was kept in a separate 80 L tank for 2 days for acclimation. Then the injection was performed precisely into the gonad with a dose of 20 μg plasmid in a total volume of 40 μL with PBS (according to the preliminary experiments). At 48 h post injection, gonads of each *C. semilaevis* were sampled and stored in liquid nitrogen until RNA extraction.

2.2.6 qRT-PCR analyses of luciferase level

Total RNA of each *C. semilaevis* was extracted from gonads using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. After examined by agarose gel electrophoresis and quantified using NanoDrop 2000 Spectrophotometer (Thermo, USA), 2 μg RNA with DNA contamination removed was used to synthesis cDNA using FastQuant RT Kit (with gDNase) (TIANGEN, China). The amount of luciferase mRNA was determined by qRT-PCR using the 7500 Real-Time PCR System (ABI, USA). The 20 μL reactions contained 2× SYBR Premix Ex Taq, 2 pmol each primer (Luc-qF/R), 2 μL of 10× diluted cDNA templates and 0.4 μL of ROX Reference Dye. Each cDNA sample was amplified in triplicate with the cycling conditions: 95°C for 30 s, and 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by melt curve analysis. *β-actin* (β-actin-qF/R) was used as the internal control. Relative abundance of transcripts was calculated as $R=2^{-\Delta\Delta Ct}$.

2.2.7 Statistical analysis

The results of the luciferase assay experiment were expressed as mean±SD. The values were compared by one-way ANOVA followed by Bonferroni's multiple comparison test using GraphPad Prism 6.0 (GraphPad, USA). Statistical significant differences were defined as $P<0.05$.

3 RESULT

3.1 Acquisition and analysis of the *C. semilaevis dmrt1* promoter sequence

The *dmrt1* promoter sequence of 2 935 bp (-2 932/+3) was cloned from the *C. semilaevis* genomic DNA with specific primers dmrt-F/R (Table 1) and verified by sequencing. This sequence contained a 2 887-bp of a promoter sequence and a 48-bp of partial exon1. Intriguingly, although the coding region of *dmrt1* gene had high identities among species, the promoter sequence showed low similarity to the same regions of zebrafish, medaka, frog, rat and human (data not shown). Prediction of putative transcriptional binding sites revealed that the promoter sequence harbored potential binding sites for multiple transcription factors including the TATA box and the inverted CAAT box, as well as positive transcription factors Sp1, Egr1, and HSF2; negative transcription factors CEBP, Sox5; and neutral factors Cdxa and Nkx2.5 (Fig.1).

3.2 Luciferase assays of *C. semilaevis dmrt1* regulatory regions

To explore the promoter activity of the isolated *C. semilaevis dmrt1* sequence, we generated a set of

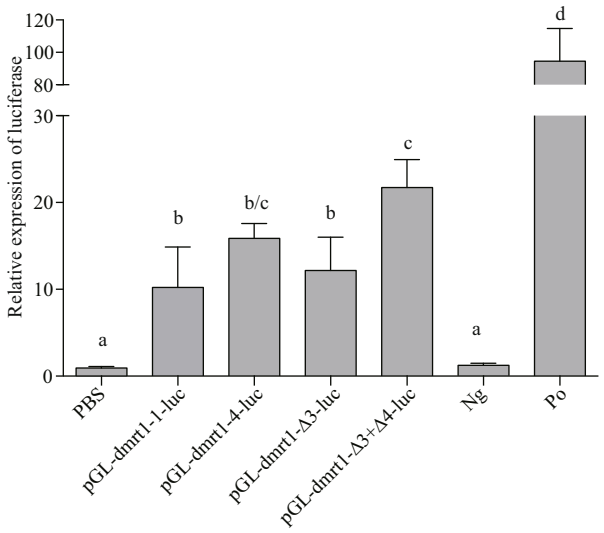

Fig.4 Expression of the luciferase gene in vivo after gonadal injection

Data are shown as mean±SD of three independent samples. Statistical differences are indicated as different letters. *pGL-3 Basic* vector was used as negative control and *pGL-3 Control* vector was used as the positive control. Note: the error bar of the positive control was clipped due to the *y*-axis limit.

truncated promoter-*luc* vectors according to the predicted transcription factor binding sites. Compared to the full-length promoter, four truncated fragments exhibited changes of promoter activities, either increased or decreased (P<0.05), while *pGL-dmrt1-6-luc* showed similar activity (Figs.1a, 2). The highest promoter activity was observed in *pGL-dmrt1-4-luc* (1.75 times of that of *pGL-dmrt1-1-luc*), while the lowest was in *pGL-dmrt1-3-luc* (39.14% of that of *pGL-dmrt1-1-luc*). Based on the results, we have made a pairwise comparison and classified the six regions into three categories by their regulatory behavior. Four of them showed positive effect including -2 932 to -2 485, -2 485 to -1 928, -867 to -285 and -285 to +3. One negative region was observed from -1 928 to -1 326, while region from -1 326 to 867 was rather neutral (Fig.2).

3.3 Construction of new *C. semilaevis dmrt1* promoter and in vitro activity analysis

To further analyze the regulatory role of the six regions mentioned above, precise deletion studies were performed to construct *pGL-dmrt1-Δ3-luc*, *pGL-dmrt1-Δ4-luc* and *pGL-dmrt1-Δ3+Δ4-luc* corresponding to a deletion of -1 928 to -1 326, -1 326 to -867, -1 928 to -867, respectively. Then their activities were analyzed in 293T cells (Fig.3). Deletion of the negative region (-1 928 to -1 326) resulted in the up-regulated activity of *pGL-dmrt1-Δ3-luc* (P<0.05), which confirmed the negative regulatory effect of this region. The specific deletion of the neutral region (-1 326 to -867) caused a slight but not significant increase, also consistent with the previous conclusion. The simultaneous deletion of the negative and neutral region (-1 928 to -867) significantly increased the activity, and the activity of the resulted of *pGL-dmrt1-Δ3+Δ4-luc* was ~3.25 times as that of *pGL-dmrt1-1-luc* (P<0.05).

3.4 In vivo activity of newly constructed *C. semilaevis* promoter

The in vivo activities of *pGL-dmrt1-1-luc*, *pGL-dmrt1-4-luc*, *pGL-dmrt1-Δ3-luc*, and *pGL-dmrt1-Δ3+Δ4-luc* were tested by injecting them precisely into the *C. semilaevis* gonads. The qRT-PCR was performed to detect the expression of the luciferase gene. The result confirmed the successful in vivo driving activities of each newly constructed promoter, and the activity of *dmrt1-Δ3+Δ4* was the highest among the tested constructs, which was 2.13 times of that of *dmrt1-1* (P<0.05) and 1.37 times of that of *dmrt1-4* (P>0.05). Although *dmrt1-4* and *dmrt1-Δ3* performed higher activities than *dmrt1-1*, there was no statistical significant difference, which was different from the in vitro test (Fig.4).

4 DISCUSSION

dmrt1 is a conserved transcription factor required for testis formation and somatic-cell masculinization in various vertebrate species (Yoshimoto and Ito, 2011; Luo et al., 2015; Zhao et al., 2015). Expression profiles of *dmrt1* showed a strict specificity in germ cells and somatic cells of embryonic gonads and postnatal testis (Smith et al., 1999; Raymond et al., 2000; Shetty et al., 2002; Xia et al., 2007). In Chinese tongue sole, since an efficient protocol for microinjection of flatfish embryos have been developed recently (Cui et al., 2017), the research of sex determining the mechanism through a transgenic approach becomes possible. Now an essential step in developing transgenic *C. semilaevis* is to identify a suitable regulatory element to drive interesting gene expression. Here we focus on the promoter of *dmrt1* mainly because (1) an endogenous promoter may be a better choice since its activity may be stronger in the native environment compared to an exogenous promoter (Friedenreich and Schartl, 1990; Sunilkumar et al., 2002), and (2) the expression profile of *dmrt1*

may provide a precise spatial and temporal control for target genes referred to sex-determining and growth.

The promoter region (-2 935 to +3) of *C. semilaevis dmrt1* has been isolated by PCR. This region shows low similarity with other species, which is consistent with a recent research regarding the molecular evolution of *dmrt1* promoters in a wide range of vertebrates including agnatha, bony fish, amphibian, reptile, bird, and mammal. There is no ncEx1 (non-coding first exon) in *dmrt1* gene of *C. semilaevis*, as in the chicken and mice. It has been reported that species without an ncEx1 have evolved an intronic derived promoter to regulate both the somatic- and germ-cell expression of *dmrt1* (Mawaribuchi et al., 2017). In addition, the *C. semilaevis dmrt1* promoter has a TATA box located ~200 bp upstream of the transcriptional start site (TSS). A CAAT box, which is essential for the promoter activity, was also found at the location of 257 bp upstream of the TSS (Quitschke et al., 1989).

In the *C. semilaevis dmrt1* promoter, we have predicted binding sites for multiple transcription factors. Among them, Sp1 and Egr1 have been reported as positive elements that are important for the *dmrt1* promoter function. Sp1 zinc finger transcription factors expressed in a variety of cells and tissues play a critical role in the maintenance of differentiated cells (Marin et al., 1997; Cook et al., 1999). The Egr proteins are thought to be involved in cellular growth and differentiation in a variety of tissues and a previous research in mice showed that Egr1 had a sexually dimorphic function in male and female fertility (Topilko et al., 1998; Tourtellotte et al., 1999). HSF2 is most abundantly expressed in certain cell types of mice testis and the expression level is elevated during spermatogenesis. The increased HSF2 will induce the formation of heterotrimers with HSF1 and then provide a switch that integrates the transcriptional activation of their common target genes during the maturation of male germ cells (Åkerfelt et al., 2010). On the other hand, CEBP and Sox5 are thought to be negative regulators of the *dmrt1* promoter. CEBP-α is a protein expressed in Sertoli cells and known to act as a transcriptional repressor (Slomiany et al., 2000; Lei and Heckert, 2002). Sox5 in zebrafish could bind the *dmrt1* promoter and inhibit *dmrt1* expression (Gao et al., 2005). These binding sites are useful for predicting the regulatory region in the *C. semilaevis dmrt1* promoter.

To characterize the regulatory elements of the *C. semilaevis dmrt1* promoter, we constructed a series of truncated promoter-*luc* vectors. The luciferase assay indicated that there were two positive regulatory regions, a distal regulatory region located at -2 932/-1 928 and a proximal region at -867/+3 (Fig.2). The similar situation has been found in the rat *dmrt1* promoter. It possesses a distal regulatory region -3.2 kb to -2.8 kb that is involved in Sertoli cell-specific expression of *dmrt1* and proximal sequences between -1.3 kb to 400 bp that is for germ cell-specific expression of *dmrt1* (Lei et al., 2009). However, we have demonstrated that there is no homology in the *dmrt1* promoter sequence between the tongue sole and rat, thus the transcription elements of *dmrt1* promoter should be investigated in future work.

Besides the two positive regulatory regions, there is a negative regulatory region (-1 928 to -1 326) and a region has no significant effect (-1 326 to -867). We have made separate and simultaneous deletion. The deletion of the negative regulation region (*dmrt1-4* and *dmrt1-Δ3*) showed significantly higher promoter activity than the full-length promoter (*dmrt1-1*) in the *in* vivo test. For in vitro test, higher activities were also observed for *dmrt1-4* and *dmrt1-Δ3* compared to *dmrt1-1*, but the differences were not statistically significant. The reason may be the complex situation of in vivo experiment. The absorption and utilization of exogenous plasmids may be different among individuals and thus could affect the significance of the differences. Intriguingly, both in vivo and in vitro experiments illustrated that simultaneous deletion of the two regions (*dmrt1-Δ3+Δ4*) performed a significantly higher promoter activity (Figs.3–4). The results suggest there may be an interaction between the two regions and together they have a higher negative effect. Through bioinformatics analysis, Sox5 and CEBP are most enriched elements located in the two regions. As the Sox5 can interact with CEBPs and cooperatively regulate the target genes involved in differentiation and development (Kfoury and Kapatos, 2009), we postulate that Sox5 and CEBP may be important elements for the negative regulatory effect. However, the hypothesis requires further investigation.

5 CONCLUSION

In summary, this study cloned the promoter sequence of *C. semilaevis dmrt1* and functional analysis showed that there were two positive regulatory regions, one negative regulatory region, and one neutral regulatory region. Furthermore, we

combined the positive regions and thus constructed several new promoters. After in vitro and in vivo analysis, one constructed promoter was confirmed with high driving activity and could drive reporter gene expression in the gonad. As the first report of the *C. semilaevis dmrt1* promoter, our results will not only contribute to understanding the regulatory mechanism of the *dmrt1* gene during *C. semilaevis* sex determination but also provide an endogenous promoter to facilitate the transgenic research for analyzing sex- and growth-related genes.

6 DATA AVAILABILITY STATEMENT

Based on previous studies, the *dmrt1* promoter sequence data that support the findings of this study have been deposited in GenBank with the accession code NC_024328.1 (https://www.ncbi.nlm.nih.gov/nuccore/NC_024328.1).

References

Åkerfelt M, Morimoto R I, Sistonen L. 2010. Heat shock factors: integrators of cell stress, development and lifespan. *Nat. Rev. Mol. Cell. Biol*., **11**(8): 545-555.

Chakraborty T, Zhou L Y, Chaudhari A, Iguchi T, Nagahama Y. 2016. *Dmy* initiates masculinity by altering *Gsdf**Sox9a2*/*Rspo1* expression in medaka (*Oryzias latipes*). *Sci. Rep*., **6**: 19 480.

Chen S L, Tian Y S, Yang J F, Shao C W, Ji X S, Zhai J M, Liao X L, Zhuang Z M, Su P Z, Xu J Y, Sha Z X, Wu P F, Wang N. 2009. Artificial gynogenesis and sex determination in half-smooth tongue sole (*Cynoglossus semilaevis*). *Mar. Biotechnol*., **11(**2): 243-251.

Chen S L, Zhang G J, Shao C W, Huang Q F, Liu G, Zhang P, Song W T, An N, Chalopin D, Volff J N, Hong Y H, Li Q Y, Sha Z X, Zhou H L, Xie M S, Yu Q L, Liu Y, Xiang H, Wang N, Wu K, Yang C G, Zhou Q, Liao X L, Yang L F, Hu Q M, Zhang J L, Meng L, Jin L J, Tian Y S, Lian J M, Yang J F, Miao G D, Liu S S, Liang Z, Yan F, Li Y Z, Sun B, Zhang H, Zhang J, Zhu Y, Du M, Zhao Y W, Schartl M, Tang Q S, Wang J. 2014. Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nat. Genet*., **46**(3): 253-260.

Cook T, Gebelein B, Urrutia R. 1999. Sp1 and its likes: biochemical and functional predictions for a growing family of zinc finger transcription factors. *Ann. N. Y. Acad. Sci*., **880(**1): 94-102.

Cui Z K, Liu Y, Wang W W, Wang Q, Zhang N, Lin F, Wang N, Shao C W, Dong Z D, Li Y Z, Yang Y M, Hu M Z, Li H L, Gao F T, Wei Z F, Meng L, Liu Y, Wei M, Zhu Y, Guo H, Cheng C H K, Schartl M, Chen S L. 2017. Genome editing reveals *dmrt1* as an essential male sex-determining gene in Chinese tongue sole (*Cynoglossus semilaevis*). *Sci. Rep*., **7**: 42 213.

Friedenreich H, Schartl M. 1990. Transient expression directed by homologus and heterologous prometer and enhancer sequences in fish cells. *Nucleic Acids Res*., **18**(11): 3 299-3 305.

Gao S, Zhang T, Zhou X, Zhao Y, Li Q, Guo Y Q, Cheng H H. 2005. Molecular cloning, expression of *Sox5* and its down-regulation of *Dmrt1* transcription in zebrafish. *J. Exp. Zool. B Mol. Dev. Evol*., **304B**(5): 476-483.

Guan G J, Kobayashi T, Nagahama Y. 2000. Sexually dimorphic expression of two types of DM (*Doublesex*/*Mab-3*)-domain genes in a teleost fish, the Tilapia (*Oreochromis niloticus*). *Biochem. Biophys. Res. Commun*., **272**(3): 662-666.

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Münsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. 1990. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature*, **346**(6281): 245-250.

Ji X S, Liu H W, Chen S L, Jiang Y L, Tian Y S. 2011. Growth differences and dimorphic expression of growth hormone (GH) in female and male *Cynoglossus semilaevis* after male sexual maturation. *Mar. Genomics*, **4**(1): 9-16.

Jørgensen A, Morthorst J E, Andersen O, Rasmussen L J, Bjerregaard P. 2008. Expression profiles for six zebrafish genes during gonadal sex differentiation. *Reprod. Biol. Endocrinol*., **6(**1): 25.

Kfoury N, Kapatos G. 2009. Identification of neuronal target genes for CCAAT/enhancer binding proteins. *Mol. Cell. Neurosci*., **40**(3): 313-327.

Kobayashi T, Matsuda M, Kajiura-Kobayashi H, Suzuki A, Saito N, Nakamoto M, Shibata N, Nagahama Y. 2004. Two DM domain genes, *DMY* and *DMRT1*, involved in testicular differentiation and development in the medaka, *Oryzias latipes*. *Dev. Dyn*., **231**(3): 518-526.

Koopman P. 2009. Sex determination: the power of *DMRT1*. *Trends Genet*., **25**(11): 479-481.

Lambeth L S, Raymond C S, Roeszler K N, Kuroiwa A, Nakata T, Zarkower D, Smith C A. 2014. Over-expression of DMRT1 induces the male pathway in embryonic chicken gonads. *Dev. Biol*., **389**(2): 160-172.

Lei N, Heckert L L. 2002. Sp1 and Egr1 regulate transcription of the *Dmrt1* gene in Sertoli cells. *Biol. Reprod*., **66**(3): 675-684.

Lei N, Karpova T, Hornbaker K I, Rice D A, Heckert L L. 2009. Distinct transcriptional mechanisms direct expression of the rat *Dmrt1* promoter in sertoli cells and germ cells of transgenic mice. *Biol. Reprod*., **81**(1): 118-125.

Luo D J, Liu Y, Chen J, Xia X Q, Cao M X, Cheng B, Wang X J, Gong W M, Qiu C, Zhang Y S, Ki Cheng, C H, Zhu Z Y, Hu W. 2015. Direct production of XY^{DMY-} sex reversal female medaka (*Oryzias latipes*) by embryo microinjection of TALENs. *Sci. Rep*., **5**: 14 057.

Marchand O, Govoroun M, D'Cotta H, McMeel O, Lareyre J J, Bernot A, Laudet V, Guiguen Y. 2000. DMRT1 expression during gonadal differentiation and

spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. *Biochim*. *Biophys*. *Acta*, **1493**(1-2): 180-187.

Marin M, Karis A, Visser P, Grosveld F, Philipsen S. 1997. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. *Cell*, **89**(4): 619-628.

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey C E, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M. 2002. *DMY* is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature*, **417**(6888): 559-563.

Matsuda M, Shinomiya A, Kinoshita M, Suzuki A, Kobayashi T, Paul-Prasanth B, Lau E L, Hamaguchi S, Sakaizumi M, Nagahama Y. 2007. *DMY* gene induces male development in genetically female (XX) medaka fish. *Proc*. *Natl*. *Acad*. *Sci*. *USA*, **104**(10): 3 865-3 870.

Mawaribuchi S, Musashijima M, Wada M, Izutsu Y, Kurakata E, Park M K, Takamatsu N, Ito M. 2017. Molecular evolution of two distinct *dmrt1* promoters for germ and somatic cells in vertebrate gonads. *Mol*. *Biol*. *Evol*., **34**(3): 724-733.

Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z H, Haaf T, Shimizu N, Shima A, Schmid M, Schartl M. 2002. A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc*. *Natl*. *Acad*. *Sci*. *USA*, **99**(18): 11 778-11 783.

Paul-Prasanth B, Matsuda M, Lau E L, Suzuki A, Sakai F, Kobayashi T, Nagahama Y. 2006. Knock-down of *DMY* initiates female pathway in the genetic male medaka, *Oryzias latipes*. *Biochem*. *Biophys*. *Res*. *Commun*., **351**(4): 815-819.

Quitschke W W, Lin Z Y, DePonti-Zilli L, Paterson B M. 1989. The beta actin promoter. High levels of transcription depend upon a CCAAT binding factor. *J*. *Biol*. *Chem*., **264**(16): 9 539-9 546.

Raymond C S, Murphy M W, O'Sullivan M G, Bardwell V J, Zarkower D. 2000. *Dmrt1*, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev*., **14**(20): 2 587-2 595.

Raymond C S, Parker E D, Kettlewell J R, Brown L G, Page D C, Kusz K, Jaruzelska J, Reinberg Y, Flejter W L, Bardwell V J, Hirsch B, Zarkower D. 1999. A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. *Hum*. *Mol*. *Genet*., **8**(6): 989-996.

Raymond C S, Shamu C E, Shen M M, Seifert K J, Hirsch B, Hodgkin J, Zarkower D. 1998. Evidence for evolutionary conservation of sex-determining genes. *Nature*, **391**(6668): 691-695.

Shao C W, Li Q Y, Chen S L, Zhang P, Lian J M, Hu Q M, Sun B, Jin L J, Liu S S, Wang Z J, Zhao H M, Jin Z H, Liang Z, Li Y Z, Zheng Q M, Zhang Y, Wang J, Zhang G J. 2014. Epigenetic modification and inheritance in sexual reversal of fish. *Genome Res*., **24**(4): 604-615.

Shetty S, Kirby P, Zarkower D, Graves J A M. 2002. DMRT1 in a ratite bird: evidence for a role in sex determination and discovery of a putative regulatory element. *Cytogenet*. *Genome Res*., **99**(1-4): 245-251.

Sinclair A H, Berta P, Palmer M S, Hawkins J R, Griffiths B L, Smith M J, Foster J W, Frischauf A M, Lovell-Badge R, Goodfellow P N. 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature*, **346**(6281): 240-244.

Slomiany B A, D'Arigo K L, Kelly M M, Kurtz D T. 2000. C/EBPα inhibits cell growth via direct repression of E2F-DP-mediated transcription. *Mol*. *Cell*. *Biol*., **20**(16): 5 986-5 997.

Smith C A, McClive P J, Western P S, Reed K J, Sinclair A H. 1999. Conservation of a sex-determining gene. *Nature*, **402**(6762): 601-602.

Smith C A, Roeszler K N, Ohnesorg T, Cummins D M, Farlie P G, Doran T J, Sinclair A H. 2009. The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. *Nature*, **461**(7261): 267-271.

Sunilkumar G, Connell J P, Smith C W, Reddy A S, Rathore K S. 2002. Cotton α-globulin promoter: isolation and functional characterization in transgenic cotton, *Arabidopsis*, and tobacco. *Transgenic Res*., **11**(4): 347-359.

Topilko P, Schneider-Maunoury S, Levi G, Trembleau A, Gourdji D, Driancourt M A, Rao C V, Charnay P. 1998. Multiple pituitary and ovarian defects in *Krox-24* (*NGFI-A*, *Egr-1*)-targeted mice. *Mol*. *Endocrinol*., **12**(1): 107-122.

Tourtellotte W G, Nagarajan R, Auyeung A, Mueller C, Milbrandt J. 1999. Infertility associated with incomplete spermatogenic arrest and oligozoospermia in *Egr4*-deficient mice. *Development*, **126**(22): 5 061-5 071.

Tsunoda T, Takagi T. 1999. Estimating transcription factor bindability on DNA. *Bioinformatics*, **15**(7-8): 622-630.

Xia W, Zhou L, Yao B, Li C J, Gui J F. 2007. Differential and spermatogenic cell-specific expression of *DMRT1* during sex reversal in protogynous hermaphroditic groupers. *Mol*. *Cell*. *Endocrinol*., **263**(1-2): 156-172.

Yoshimoto S, Ito M. 2011. A ZZ/ZW-type sex determination in *Xenopus laevis*. *FEBS J*., **278**(7): 1 020-1 026.

Zhao L, Svingen T, Ng E T, Koopman P. 2015. Female-to-male sex reversal in mice caused by transgenic overexpression of *Dmrt1*. *Development*, **142**(6): 1 083-1 088.

Zhou L Q, Yang A G, Liu X Z, Du W, Zhuang Z M. 2005. The karyotype of the tonguefish *Cynoglossus semilaevis*. *J*. *Fish*. *Sci*. *China*, **29**(3): 417-419. (in Chinese with English abstract)