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Molecular identification of *dmrt1* and its promoter CpG methylation in correlation with gene expression during gonad development in *Culter alburnus*

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Abstract Dmrt1, a member of the Dmrt family, is an important transcription regulator of gender determination. To study the biological function of *dmrt1* in sexual differentiation and its potential implication in breeding technology, we obtained the full-length cDNA and proximal promoter sequence of *dmrt1* in *Culter alburnus,* and analyzed the impact of promoter CpG methylation on the gene expression pattern of *dmrt1* during gonad development. *Dmrt*1 was 922 bp in length and consisted a 150 bp 5′-UTR, a 28 bp 3′-UTR, and a 744 bp open reading frame (ORF). Based on the coding sequence of the *dmrt1* gene, the deduced amino acid sequence was detected, and the protein structure of this gene was predicted in *C. alburnus*. The results indicate that the structure and function of *dmrt1* were highly conservative compared to other vertebrates. The expression level of *dmrt1* mRNA in different tissues was explored by qRT-PCR, which was only highly expressed in the testes and almost undetectable in other tissues. The CpG methylation pattern of the *dmrt1* promoter was studied using DNA sequencing of sodium bisulfite in adult testes and ovaries, and it was found that *dmrt1* promoter CpGs were not methylated in the testes, whereas hypermethylated in the ovaries. These findings demonstrate that DNA methylation can regulate sexual dimorphic expression of *dmrt1*, and therefore epigenetic modifications may play a critical role in the gonad differentiation of *C. alburnus.*

Keywords *Culter alburnus* · *Dmrt1* · CpG methylation · Gene expression · Sex determination

Yongyi Jia and Jianbo Zheng contributed equally to this work.

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Introduction

The sex determination and differentiation mechanisms of fish are currently the most complex and dynamic process of all vertebrates, regulated by genetic, epigenetic, or environmental factors (Devlin and Nagahama, 2002). Dmrt1, belonged to the Dmrt (Doublesex and Mab-3-related transcription factor) family, is a significant transcriptional regulator that contains a common DNA-binding motif known as the DM domain (Raymond et al. 1998; Smith et al. 2009; Herpin and Schartl, 2011). To date, Dmrt1 has been found to play a vital role in sex determination, differentiation, and maintenance of organ functions in a variety of species, including fish, mammals, reptiles, birds, and amphibians (Kettlewell et al. 2000; Grandi et al. 2000; Kondo et al. 2002; Aoyama et al. 2003).

In addition, accumulated studies have demonstrated that epigenetic regulation has an indispensable role in sex determination, especially the methylation modification of CpG dinucleotide in sex-related gene promoter (Navarro-Martín et al. 2011; Zhang et al. 2014; Tachibana, 2015). For example, Wang et al. found that the increase of DNA methylation level of the *cyp19a1a* promoter was correlated with mRNA expression level, and the epigenetic control of *cyp19a1a* expression may affect estrogen synthesis and sex reversal in *Nile tilapia* (Wang et al. 2017). Besides, many studies have reported that CpG methylation of sex-related gene promoters has an important influence on sex ratio through their spatial and temporal expression (Nishino et al. 2004; Wen et al. 2014; Mamta et al. 2016). Although these puzzles remain to be elucidated in the future, these data strongly suggest that DNA methylation-mediated control of gene expression involves in sex determination.

Culter alburnus is one of the most important freshwater aquaculture species in China, and females in *C. alburnus* grow faster than males, which shows substantial sexual dimorphism. However, key regulatory genes and mechanisms involved in sex determination and differentiation in *C. alburnus* remain unclear. These outstanding issues limited the application of sex-controlled breeding technology in *C. alburnus* breeding.

In the current study, we first reported the sequence features of the *dmrt1* gene in *C. alburnus*. Then, the expression pattern of *dmrt1* mRNA in different tissues was analyzed via quantitative real-time PCR. Moreover, we examined the state of CpG methylation in the *dmrt1* promoter of *C. alburnus* in the ovaries and testes by examining the correlation between CpG methylation and sexual dimorphic expression of *dmrt1*. In conclusion, our results suggest that differential DNA methylation may be crucial for the formation of sex phenotypes, and this study provides an important basis for further study of the genetic mechanisms of sex determination in *C. alburnus* and other species.

Materials and methods

Animal sample preparation and ethics statement

Three male and three female *C. alburnus* adults were collected from the Balidian breeding base of Zhejiang Institute of Freshwater Fisheries (Huzhou, Zhejiang Province). All samples (including the brain, liver, kidney, testis, spleen, ovary, eye, heart, muscle) were immediately frozen and stored at − 80 °C for RNA and DNA extraction. This study was approved by the Ethics Committee of Laboratory Animal Center of Zhejiang University (Zju201306-1-11-060).

RNA isolation and cDNA synthesis

Total RNA from different tissues was isolated using the Trizol Kit (Sangon, China) according to the user manual. We synthesized the cDNA using the HiFiScript cDNA Kit (Cwbio, China) and stored at − 20 °C until use.

Molecular cloning cDNA and promoter of *dmrt1*

Primer Premier 5.0 was used to design a conserved region-specific primer pair (*dmrt1*-F, *dmrt*1-R) for the *dmrt1* gene according to published sequences from other species. Intermediate fragments were obtained by PCR with the following procedure: 94 °C for 5 min; 32 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and 72 °C for 5 min. Next, the PCR product was purified using a Gel Extraction Kit (Axygen, USA) and cloned into pMD18-T vector (Takara, Japan) for sequencing. Based on the above intermediate sequence, rapid amplification of cDNA ends (RACE) PCR was used to obtain full-length cDNA of *dmrt1* following the manufacturer's instructions with gene-specific primers (RACE-F1, RACE-F2, RACE-R1, and RACE-R2 for 3'/5' RACE, respectively).

Genomic DNA was extracted from the gonadal tissue of *C. alburnus*, and a genomic walking library was constructed according to the GenomeWalker Universal Kit's protocol (Clontech, Japan). Specific reverse primers (SRP1, SRP2) were designed from the previously cloned cDNA sequence, and the promoter fragment purification had the same method as we used above. All primers used in this study were shown in Table 1.

Sequence analysis

The open reading frame (ORF) for *dmrt1* was analyzed by Jellyfish software (3.3.1) and translated into amino acid sequences. The full-length amino acid sequence alignments between some fish and other vertebrates were performed using DNAMAN software. The three-dimensional structure of Dmrt1 protein was predicted

with SWISS-MODEL (https://swissmodel.expasy.org/). The phylogenetic tree was constructed with the neighbor-joining method of MEGA 5.0 software.

Gene expression profile in different tissues by qRT-PCR

Total RNA from different tissues was extracted using a Trizol Kit (Sangon, China) and then treated with *Dnase* I to remove contaminated genomic DNA. Real-time quantitative PCR of *dmrt1* was performed as previously described (He et al. 2014), and *β-actin* was employed as an internal standard. The primer sequences for PCR were as follows: 5'-GCC GCC TGT CCA GCC ATA ATG-3′ (*dmrt1*-RT-F); 5′-CAC ATG CAG CCG TGT CGA TCC-3′ (*dmrt1*-RT-R); *β-actin*-F, 5′-TCC CTT GCT CCT TCC ACC A-3′; *β-actin*-R, 5′-GGA AGG GCC AGA CTC ATC GTA-3′. Each test and its endogenous *β-actin* control were performed in triplicate. The melting curves were analyzed after amplification to identify specific products in all PCR reactions. Relative expression levels were calculated by comparative threshold (Ct) method and plotted as histograms with GraphPad Prism5 program software.

Bisulfite sequencing of the *dmrt1* CpG island

The analysis region of the *dmrt1* CpG island was predicted by the online software MethPrimer (http://www.urogene.org/methprimer/) in *C. alburnus*. The genomic DNA was subjected to bisulfite salt treatment according to the instructions of the CpGenomeTM DNA Modification Kit (Chemicon, USA). The primers for amplifying the bisulfite converted sequence of the *dmrt1* promoter CpG island were 5′-ATT TTG TTT TTA GTG GGA AGA AAT T-3' (BSP-F) and 5'-TCC TCA CTC ATA CCT ACA AAT AAT AAT AA-3' (BSP-R). PCR products were then purified and cloned into pMD18-T Simple Vector (Takara, USA) for sequencing.

Table 1 Primers used in this study.

Primers name	Sequences (5'-3')
*dmrt*1-F	CCAGGACGCCCAAGTGCTCCCGG
*dmrt*1-R	TTCTTCCTCCTGGGCCTGCTGCCT
RACE-R1	TCCCTCCAGTTGCAGAAGCGTTTGTGG
RACE-R2	GGGCTGCCATGACCCGCTGTCTTTCAG
RACE-F1	GGAGGGACTGCCAGTGTCAGAAATG
RACE-F2	CAGCGGGTCATGGCAGCCCAGGTGG
SRP1	CGAGCACTTGGGCATACGGGACGGTTT
SRP2	GCCGTTACTCTGCTCTTCCTCACTCAT
*dmrt*1-RT-F	GCCGCCTGTCCAGCCATAATG
*dmrt*1-RT-R	CACATGCAGCCGTGTCGATCC
β-actin-F	TCCCTTGCTCCTTCCACCA
β-actin-R	GGAAGGGCCAGACTCATCGTA
BSP-F	ATTTTGTTTTTAGTGGGAAGAAATT
BSP-R	TCCTCACTCATACCTACAAATAATAATAA

Results

Cloning and molecular characteristics of *dmrt1* in *C. alburnus*

The complete cDNA sequence of *dmrt1* gene was obtained in *C. alburnus* through the following procedure. First, a pair of degenerate primers was used to identify the intermediate fragments of the expected size of *dmrt1*, which were based on the reported sequence of other species. Subsequently, we amplified the full-length cDNA sequence via 5'−/3'-RACE, and finally obtained proximal promoter sequence of *dmrt1* on the basis of the first exon and 5'-UTR sequence through the chromosome walking. Sequence analysis revealed that the total length of *dmrt1* was 922 bp, including a 150 bp 5'-UTR, a 28 bp 3'-UTR, and a 744 bp open reading frame (ORF) that encoded a 247-amino acid protein with a predicted molecular mass of 27.1 kDa (Fig. 1). Additionally, a 397 bp promoter region of the *dmrt1* gene was cloned, containing 14 CpG sites, Sry binding sites, Sox13 binding sites, and AhR:Arnt binding sites (Fig. 1). The cDNA sequence and partial promoter region have been submitted to GenBank with accession number MG860531.

Structure prediction and phylogenetic analysis of Dmrt1 protein

The full-length amino acid sequence alignment revealed that the DM domain was highly conserved among different vertebrates (Fig. 2). In addition, we employed SWISS-MODEL to construct the three-dimensional structure of the Dmrt1 protein (Fig. 3a). The phylogenetic relationships among different animal Dmrt1 protein sequences were analyzed, including *Danio rerio*

Sry

taaggtctggtcaactaactacattttgttgttctgtaaacaacataaattatttgcttattaccacttacatttttacaactttgtgataggcttacctaa

acaaactggctgaagcccatataaatctaaatgtaaccatgctttaaaacgccatcctcgcatgttttgtgtcttatgatctatgaattcatgagaca

Ahr:arnt

catggggagtcactttgcttccagtgggaagaaactgaacatcagctcccacgcgcgctggaacgcacaggttgctcgcgccaaatatttgcc

Sox13

tattgtatcaaacctcagacccccctccttataaagttttataatccgcggtcgtgtcatattcgaagcagaagaggccgaccgtcatcaccacc

tgtaggcATGAGTGAGGAAGAGCAGAGTAACGGCTCGCTGTCCGTCAGGAAACCGTCCCG

M S E E E Q S N G S L S V R K P S R

TATGCCCAAGTGCTCGCGCTGCAGAAACCACGGATTCGTGTCTCCGCTGAAGGGCCAC

M P K C S R C R N H G F V S P L K G H

AAACGCTTCTGCAACTGGAGGGACTGCCAGTGTCAGAAATGCAGACTAATCGCTGAA

K R F C N W R D C Q C Q K C R L I A E

AGACAGCGGGTCATGGCAGCCCAGGTGGCCTTACGGAGGCAGCAGGCCCAGGAGGA

R Q R V M A A Q V A L R R Q Q A Q E E

AGAAATGGGCATTTGCAGTCCGGTTAACCTGTCCGGTTCAGACACTCTGGTGAAGAAC

E M G I C S P V N L S G S D T L V K N

GAGGTCGTGGGTGACCATGTGTTTACCATCAGCTCAGGACCGCCATCACCCACCACCA

E V V G D H V F T I S S G P P S P T T

GCAGCGCTACCGCCTCTCCCACTAACATAGAGAGCCGTTCCATGCTGGCTCTAAGCTCT

S S A T A S P T N I E S R S M L A L S S

GGAATGACCAGCAGAGGGCACACTGAGGGCCCATCTGAACTGATGGTGGATGCCTCCT

G M T S R G H T E G P S E L M V D A S

ATTACAACCTCTACCAGCCCACACCGTACACGTCCTACTATAGCAACCTGTACAACTAC

Y Y N L Y Q P T P Y T S Y Y S N L Y N Y

CAGCAATACCAGATGCCCAGTGGAAATGGCCGCCTGTCCAGCCATAATGTGCCCCCTC

Q Q Y Q M P S G N G R L S S H N V P P

CGTACCGCACACACTCCTACTATTCATCTTACCTGAGTCAGGGGATCGACACGGCTGCA

P Y R T H S Y Y S S Y L S Q G I D T A A

TGTGTCCCACCCAGCACCTGCTCTGAGCCCAAAGCAGCAGGTGGCATTTCCTCCAGCG

C V P P S T C S E P K A A G G I S S S

TGATCCTCTGCCAGCCTCTCCCTCCCCCACTCCATCATCTGGCAATGGACTAGactggacgtt

V I L C Q P L P P P L H H L A M D -

actgaggttacctgagat

Fig. 1 Nucleotide sequence and putative amino acid sequence of *dmrt1* in *C. alburnus*. CpG sites are highlighted in red. Three transcription factor binding sites are shown in the yellow background. The deduced amino acids are shown in purple. Initiation codon (ATG) and stop codon (TAG) are shown in green

(AF439562), *Oryzias curvinotus* (AB091696), *Mus musculus* (AF202778), *Cynoglossus semilaevis* (ABS31368), *Homo sapiens* (AF130728), *Xenopus laevis* (NM001096500), *Bos taurus* (ACN86339), *Cyprinus carpio* (KF713504), *Oncorhynchus mykiss* (AF209095), *Pelodiscus sinensis* (KF924758), *Crocodylus palustris* (ACD74914), *Epinephelus coioides* (ABK15558), *Acanthopagrus schlegelii* (AY323953), *Monopterus albus* (AAP80398), *Silurus meridionalis* (ABM54575), *Paralichthys olivaceus* (ACD62474), *Paramisgurnus dabryanus* (ABK88911), *Xiphophorus maculatus* (AAN65377), *Tetraodon nigroviridis* (AAN74844), *Pan troglodytes* (XM003312019), *Podarcis siculus* (ACU40920), and *C. alburnus*. The results indicate that the phylogenetic tree consisted mainly of two branches, one composed of fish, and the other composed of mammals, reptiles, birds, and amphibians (Fig. 3b). Further, the *C. alburnus* Dmrt1 protein sequence was closest in evolution to the sequences of *D. rerio* and *C. carpio*. In general, the phylogenetic relationship of the *C. alburnus dmrt1* gene was consistent with the evolutionary position of its species.

Gene expression patterns in different adult tissues of *C. alburnus*

Specific primers were designed via the cDNA sequence of *dmrt1*, and gene expression profiles in

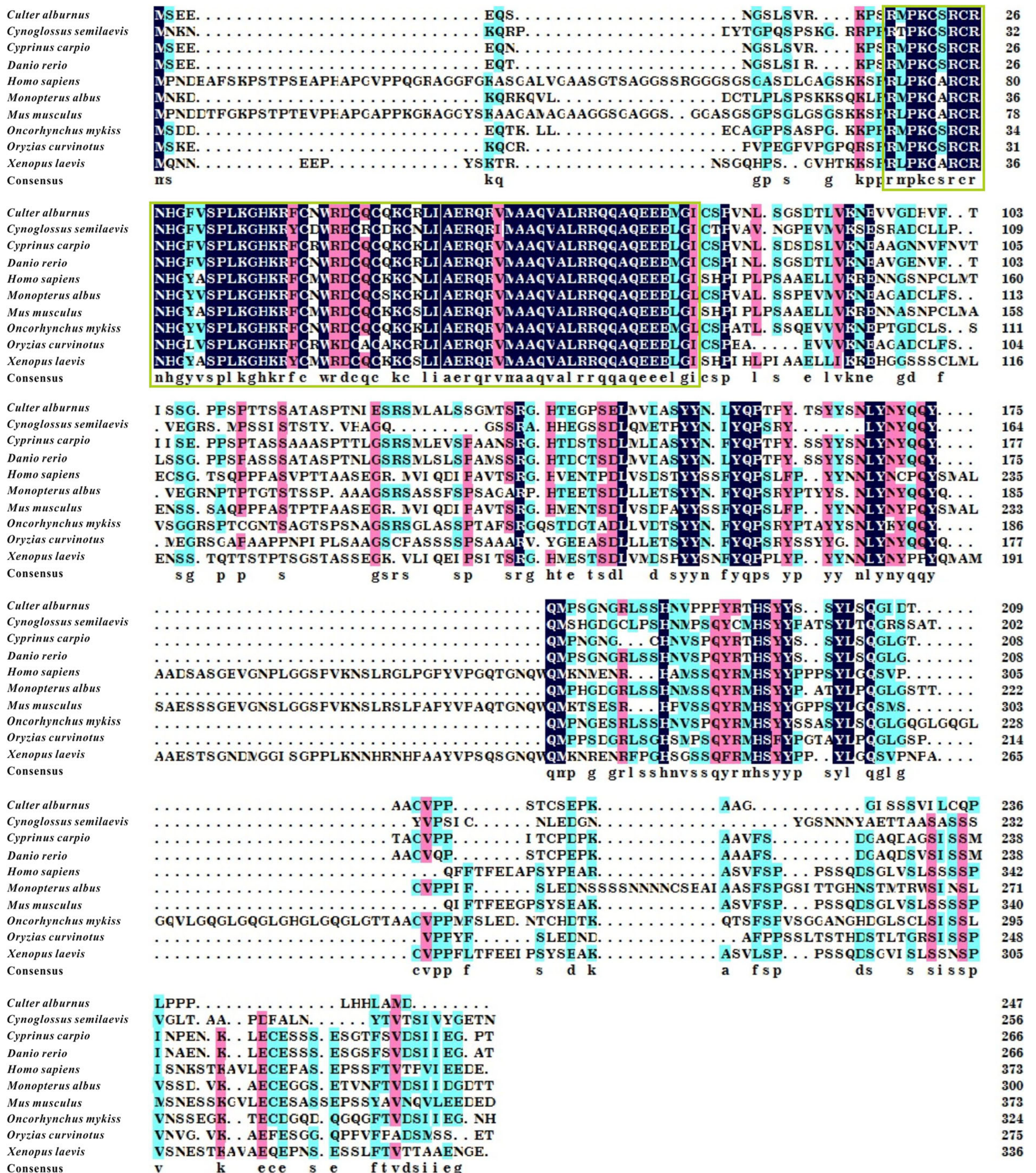

Fig. 2 The amino acid sequence alignment of different Dmrt1 proteins. The green rectangle under alignment represents a conserved DM domain. The black regions represent the same amino acid, the red one represented more than 50% similarity, and the blue one represents more than 33% similarity

different tissues were subjected to quantitative real-time PCR. The data showed that its expression was extremely high in adult testes, while its expression was particularly challenging to detect in the eye, brain, heart, liver, spleen, kidney, muscle, and ovaries (Fig. 4). These results strongly

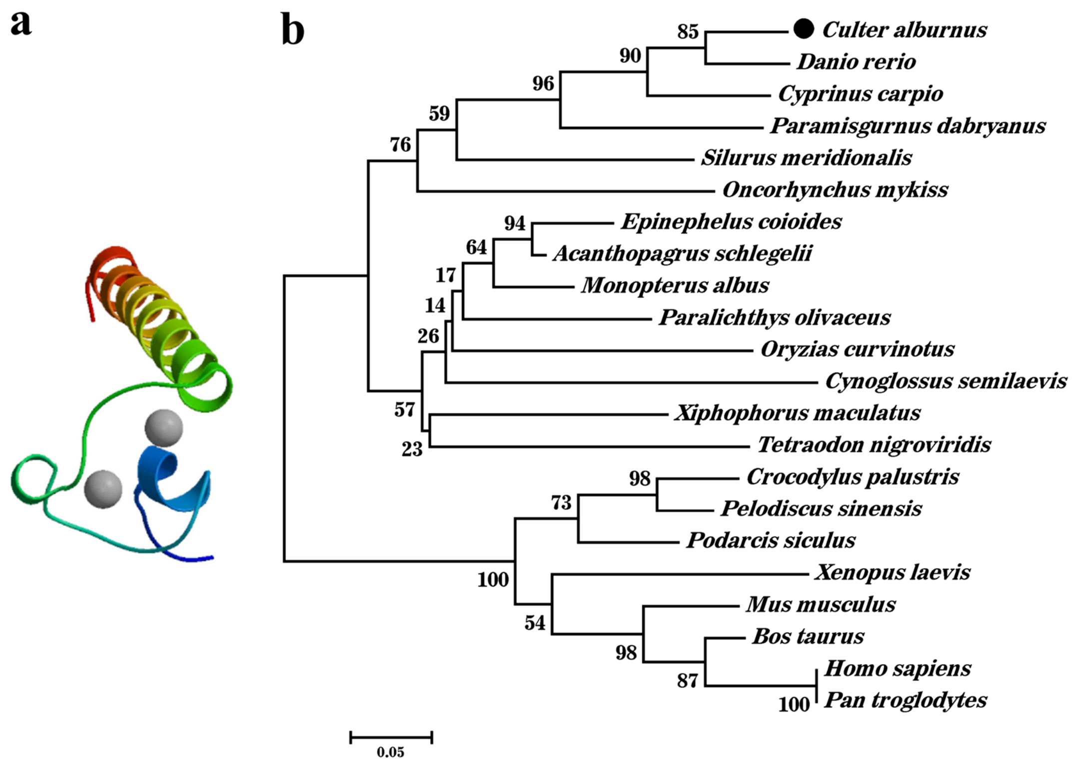

Fig. 3 Structure features and phylogenetic tree analysis of Dmrt1 protein in *C. alburnus*. **a** The three-dimensional structure of Dmrt1 using SWISS-MODEL software. **b** The phylogenetic tree of Dmrt1 was established by the neighbor-joining method using Mega 5.1 software. Five hundred bootstrap replicates were carried out. Each (%) bootstrap value is displayed on the branch point

suggest that *dmrt1* may play a crucial role in testicular development.

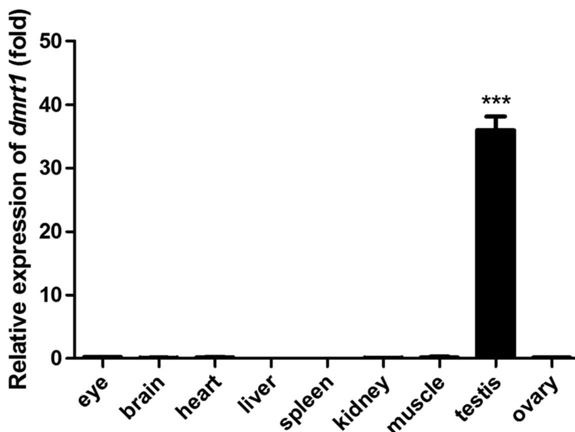

Fig. 4 Gene expression analysis of *dmrt1* in different tissues of *C. alburnus*. The *dmrt1* gene is highly expressed in testes. However, its expression is difficult to detect in the eye, brain, heart, liver, spleen, kidney, muscle, and ovary. *β-actin* is the internal control. Values were means of three replicates ± SD, and statistical analysis was performed by unpaired *t* test. ***$p < 0.001$

Differential CpG methylation pattern of *dmrt1* promoter in gonadal tissues

To better explore and understand the mechanisms of sex determination and differentiation in *C. alburnus*, we examined whether *dmrt1* expression was involved in DNA methylation in testes and ovaries. Thus, CpG islands were identified using online software MethPrimer (Fig. 5a) and analyzed by bisulfite sequencing in the *dmrt1* promoter region of the *C. alburnus* transcription factor. Our results showed that among 12 CpG sites located near the translation start site, none of the CpG sites were methylated in testes (Fig. 5b; Fig. S1), while the methylation level in adult ovaries was exceptionally high (Fig. 5c; Fig. S1). These results show that *C. alburnus* may be involved in DNA methylation-mediated control of *dmrt1* gene expression during gonadal development, and observed the inverse relationship between methylation level and gene expression.

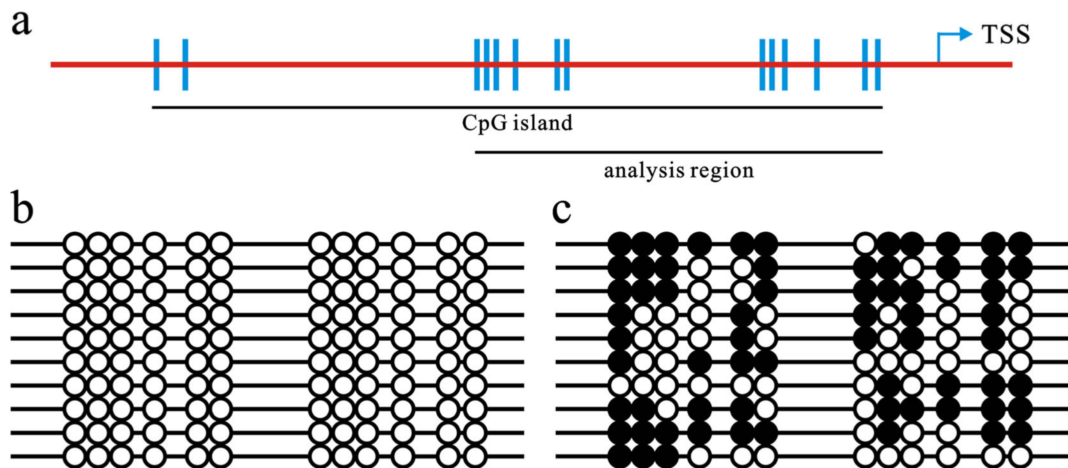

Fig. 5 Differential CpG methylation of *dmrt1* promoter in testes and ovaries. **a** The position of *dmrt1* promoter CpG island, with each vertical line representing the position of the CpG dinucleotide. **b**, **c** Bisulfate sequencing results of *dmrt1* promoter CpG island in the genomes of *C. alburnus* testes and ovaries. Open and filled circles indicate unmethylated or methylated positions. Ten clones were analyzed for each sample

Discussion

Dmrt1 is another important male-related gene responsible for testicular development discovered after *sry* and *sox9* (Koopman, 2001; She and Yang, 2016; Rahmoun et al. 2017). In the present study, we obtained the full-length cDNA sequence of *C. alburnus dmrt1* by RT-PCR and RACE methods, and identified CpG islands in its promoter region by chromosome walking. The structure prediction, phylogenetic tree, quantitative expressions, and CpG methylation patterns of *dmrt1* were successively analyzed.

The Sry and Sox13 transcription factor binding sites were distributed in the *dmrt1* promoter region (Fig. 1) and were encoded by sex-related genes, indicating that *C. alburnus dmrt1* was actively participated in gonadal differentiation and development. Besides, the protein encoded by this gene also contains a highly conserved DM domain shared by the *dmrt* gene family in *C. alburnus* (Fig. 2), suggesting that it had similar functional properties as reported in other species (Raymond et al. 1999; Marchand et al. 2000; Nanda et al. 2002; Raymond et al. 2000). Neighbor-joining phylogenetic tree analysis shows that the *C. alburnus* Dmrt1 protein gathers in a cluster with other teleostean proteins (Fig. 3b). These results reveal that the molecular evolutionary relationship of *dmrt1* is basically consistent with the evolutionary status of traditional species, indicating the *dmrt* gene family is the most conservative gendered gene family.

The expression profile of *C. alburnus dmrt1* shows high testicular levels and other tissue low levels similar to previous studies (Marchand et al. 2000; Kobayashi et al. 2004; Yamaguchi et al. 2006; Anne et al. 2008; Kobayashi et al. 2008), demonstrating its vital role in testicular development. In addition, we discovered that the modification level of CpG methylation in the *dmrt1* promoter region in the ovaries was impressively high, and this modification appeared to cease in the testes, which observed the inverse relationship between CpG methylation level and gene expression. These results manifest methylation-mediated sexual dimorphic expression of *dmrt1* involved in gonadal development in *C. alburnus*.

In conclusion, our study demonstrates that differential DNA methylation is essential for driving gonads to form ovaries or testes through regulating sexual dimorphic expression of *dmrt1* in *C. alburnus*. This research also provides an important basis for the future investigation of the genetic mechanism of sex determination in *C. alburnus* and other species. Understanding the molecular genetic mechanism of sex determination is critical to the development of feasible aquaculture practical applications in *C. alburnus* breeding.

Author contributions Yongyi Jia, Jianbo Zheng, Zhimin Gu, and Liqiao Chen conceived and designed the experiments. Jianbo Zheng, Meili Chi, Shili Liu, and Yongyi Jia performed the experiments. Jianbo Zheng, Wenping Jiang, Shun Cheng, and Yongyi Jia analyzed the data. Yongyi Jia, Jianbo Zheng, Zhimin Gu, and Liqiao Chen wrote the paper.

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Compliance with ethical standards This study was approved by the Ethics Committee of Laboratory Animal Center of Zhejiang University (Zju201306-1-11-060).

Conflict of interest The authors declare that they have no conflict of interest.

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