



Full-length gonad transcriptome analysis of Amur sturgeon Dmrt family genes: identification, characterization, and expression patterns during gonadal differentiation

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Abstract The regulatory mechanisms that govern sex differentiation in sturgeon are still poorly understood. The doublesex and Mab-3-related transcription factor (Dmrt) gene family is known for its extensive roles in sex determination and differentiation across vertebrates. This study aimed to identify new members of sturgeon Dmrt family genes and core actors in the gonadal differentiation of Amur sturgeon. A full-length gonad transcriptome database was exploited to identify Dmrt gene orthologs. Analyses of phylogenetic relationships and selection pressure were performed, and tissue expression profiles and spatiotemporal expression patterns in gonads were then analyzed using real-time PCR. In total, five Dmrt family genes were identified from the full-length gonad transcriptome, including Dmrt2,

DmrtA1, DmrtA2, DmrtB1a, and DmrtB1b. Phylogenetic analysis showed that these genes were clustered into clades corresponding to the doublesex/Mav-3 (DM) genes of vertebrates. Furthermore, the analysis of evolutionary selective pressure indicated that DmrtB1a and DmrtB1b were subject to positive selection, suggesting the existence of adaptive evolution in sturgeon. The extensive tissue expression profiling of each Dmrt family gene revealed typical characteristics. Remarkably, according to a spatiotemporal expression pattern analysis, in later stages, DmrtB1b expression increased in testes and was significantly higher in testes than in ovaries at 24 months after hatching (M) and 36 M. This study provides a genetic resource of full-length Dmrt family genes and increases the understanding of Dmrt functions in sex differentiation in sturgeon.

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Abbreviations

Dmrt	Doublesex and Mab-3-related transcription factor
DM	Doublesex/Mab-3
ORF	Open reading frame
CDS	Coding sequences
UG	Undifferentiated gonad
M	Months after hatching
Ka	Nonsynonymous substitutions

Ks	Synonymous substitutions
HE	Hematoxylin and eosin staining

Introduction

Sturgeons (Acipenseriformes) are an ancient fish group that originated during the Devonian period, with a history of over 200 million years; therefore, they constitute idea model species for studying development and evolution in vertebrates (Bemis et al. 1997; Saito et al. 2014). Sturgeon oocytes are highly prized as a value source of caviar food, referred to as “black gold”; however, wild sturgeon resources have declined in abundance due to habitat destruction and heavy exploitation. To date, in an updated January 2021 press release, the International Union for Conservation of Nature and Natural Resource (IUCN) Red List identified sturgeons as one of the most endangered animal groups, as some 85% of sturgeon species are on the verge of extinction (<http://www.iucnredlist.org/search>). Fortunately, efforts to develop a sturgeon aquaculture industry for artificial production have been made worldwide, which will not only ensure a stable supply of sturgeon products but will aid in the conservation of sturgeon stocks.

Although sturgeons are dioecious, it is difficult to distinguish females and males using morphological characteristics in larval, juvenile, or even adult stages. The aquaculture water temperature does not affect the sex ratio (i.e., the sex differentiation of sturgeon shows no relationship with the breeding environment). More excellent female stocks or female gonadal development regulation technologies are urgently desired in the sturgeon aquaculture industry. Although the genomes of two sturgeon species have been decoded, including the American paddlefish (*Polyodon spathula*) (Cheng et al. 2021) and the Sterlet (*Acipenser ruthenus*) (Du and Stöck 2020), no sex chromosomes have been found. The regulatory mechanisms of sex differentiation in sturgeon are still poorly understood (Wang et al. 2019).

The existence of doublesex and Mab-3-related transcription factor (Dmrt) genes was first formally reported in the fruit fly, *Drosophila* (Hildreth 1965), in which a primary transcript common to the two sexes but produced male and female mRNAs (Burtis and Baker 1989). Dmrt family genes are characterized by a doublesex/Mab-3 (DM) domain, which consists

of six conserved intertwined CCHC and HCCC Zn (2+) binding sites and α -helix structures (Erdman and Burtis 1993; Zhu et al. 2000). In mouse, eight Dmrt genes (Dmrt1 to Dmrt8) have been identified (Veith et al. 2006a). Dmrt genes have been intensively studied because they represent major transcription factors of the sex determination and differentiation pathways. To date, Dmrt genes have been suggested to be sex determination genes in medaka fish, *Oryzias latipes* (Matsuda et al. 2007); African clawed frog, *Xenopus laevis* (Yoshimoto et al. 2008); chicken, *Gallus gallus* (Smith et al. 2009); and daphnia, *Daphnia magna* (Kato et al. 2011). Meanwhile, Dmrt genes have been suggested to play a core role in gonad development (i.e., downstream of the sex differentiation cascade) because changes in their expression levels induce sexual abnormalities (Webster et al. 2017). For example, the loss of Dmrt1 causes mouse spermatogonia to precociously exit the normal spermatogonial program and enter meiosis (Matson et al. 2010).

The number of Dmrt gene orthologs varies between species. For example, five, six, five, seven, five, and eight Dmrt family genes have been identified in zebrafish (*Danio rerio*), Japanese pufferfish (*Takifugu rubripes*), Atlantic cod (*Gadus morhua*), largemouth bass (*Micropterus salmoides*), zebra finch (*Taeniopygia guttata*), and mouse (*Mus musculus*), respectively (Dong et al. 2020; Johnsen and Andersen 2012; Veith et al. 2006a; Yamaguchi et al. 2006; Zhou et al. 2008). Dmrt1–5 have been widely reported in all vertebrates, while Dmrt7 (DmrtC2) and Dmrt8 (DmrtC1) are only present in mammals. An earlier study implied that Dmrt6 (DmrtB1) may be missing in teleost fish (Veith et al. 2006b). Since a DmrtB1 ortholog was first reported to exist in a coelacanth (*Latimeria chalumnae*), studies have shown that Nile tilapia (*Oreochromis niloticus*) and five other teleost fish species, (i.e., largemouth bass (*Micropterus salmoides*), Asian seabass (*Lates calcarifer*), channel catfish (*Ictalurus punctatus*), spotted gar (*Lepisosteus oculatus*), and African coelacanth (*Protopterus amphibius*)) also carry DmrtB1 genes in their genome (Dong et al. 2020; Zhang et al. 2014b). Evolutionarily, it has been hypothesized that DmrtB1 probably separated from the ancestor of the Dmrt1–2 and Dmrt3–5 clades during the first round of vertebrate genome duplication (Johnsen and Andersen 2012). Additionally, synteny analysis in a genome-wide comparison indicated that all six other Dmrt

family genes (Dmrt1, Dmrt2a, Dmrt2b, and Dmrt3–5) are relatively conserved, but apart from DmrtB1 gene (Dong et al. 2020). Ancestral vertebrate genomes likely underwent two rounds (1R and 2R) of whole-genome duplication (WGD). Teleost fishes then underwent a teleost-specific round of WGD (Hoegg et al. 2004). Furthermore, Acipenseriformes (sturgeons) experienced up to three additional lineage-specific WGDs (Lebeda et al. 2020; Ludwig et al. 2001), resulting in the polyploidy patterns observed in sturgeons. Polyploidy provides beneficial genetic flexibility and broad adaptive responses; i.e., additional gene copies theoretically allow evolution under reduced selective constraint and the acquisition of novel gene functions that contribute to adaptation (Panchy and Lehti-Shiu 2016). The members of the Dmrt gene family in sturgeon are unclear. A previous study indicated that sexually dimorphic expression of Dmrt1 was not observed in the gonads of juvenile sturgeon (age ≤ 4 years old) (Okada et al. 2017). Therefore, the core Dmrt genes involved in sex differentiation in sturgeon are still poorly understood because limited gene information is currently available.

In the present study, a scientific hypothesis is proposed that whether there are other members of the sturgeon Dmrt gene family, which maybe play the core actors in gonadal differentiation in Amur sturgeon, *Acipenser schrenckii*. To verify the hypothesis, we found five additional Dmrt family genes. The characteristics of these genes, including their sequence structure, phylogenetic relationship, and selection pressure, were analyzed. Furtherly, we examined the expression distribution of five Dmrt family genes in seven main tissues, together with the spatiotemporal expression patterns in the gonads of *A. schrenckii* during early sex differentiation. As a result, DmrtB1a and DmrtB1b are reported for the first time in sturgeon, and the spatiotemporal expression pattern of DmrtB1b may shed light on its core role in the gonads of Amur sturgeon during gonadal differentiation.

Materials and methods

Identification of Dmrt family genes in Amur sturgeon

A full-length gonad transcriptome dataset was reported by the previous study (Zhang et al. 2020)

and deposited in the NCBI database (accession number SRR9656303). Full-length Dmrt transcript candidates were further screened from NCBI non-redundant protein sequence (NR) database annotations using the Diamond BLASTX method according to E -value $< 1 \times 10^{-5}$. After BLAST alignment and sequence homology assessment, five Dmrt genes with full-length sequences were identified and named Dmrt2, DmrtA1, DmrtA2, DmrtB1a, and DmrtB1b. The sequences of all the five Dmrt family genes were uploaded to the GeneBank database of NCBI, and the accession numbers were ON051541 (Dmrt2), ON051542 (DmrtA1), ON051543 (DmrtA2), ON051544 (DmrtB1a), and ON051545 (DmrtB1b).

Sequence analysis and structural characterization

The open reading frames (ORFs) of five Dmrt genes were predicted using the NCBI ORFfinder program (www.ncbi.nlm.nih.gov/orffinder/) and the simple modular architecture research tool (SMART) was used to predict the conserved domains based on sequence homology (<http://smart.embl-heidelberg.de/>). Subsequently, the amino acid size, molecular weight (MW), and theoretical isoelectric point (PI) of each Dmrt protein were calculated by using ProtParam of the ExpASY database (<http://web.expasy.org/protparam/>).

The mature structure of each Dmrt protein was analyzed. The secondary structure was predicted using online PSIPRED software (<https://bioinf.cs.ucl.ac.uk/psipred/>). A tertiary protein structure model was constructed and further processed by using the SWISS-MODEL procedure (<https://swissmodel.expasy.org/interactive/>) and PyMOL annotation software. The subcellular localizations of five Dmrt proteins were revealed using the PredictProtein tool (<https://www.predictprotein.org/>).

Sequence alignment and phylogenetic analysis

Phylogenetic analysis was conducted with reference Dmrt proteins from 18 species, including *A. schrenckii*, *A. transmontanus*, *A. ruthenus*, *A. baerii*, *Parus major*, *Columba livia*, *Mus musculus*, *Homo sapiens*, *Bos taurus*, *Takifugu rubripes*, *Paralichthys olivaceus*, *Oryzias latipes*, *Danio rerio*, *Oreochromis niloticus*, *Xenopus laevis*, *Nipponia nippon*, *Anas platyrhynchos*, and *Gallus gallus*. Dmrt accession numbers of 18 typical

species from eight classes are listed in Table S1. The amino acid sequences of Dmrt genes were aligned using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). A phylogenetic tree was reconstructed based on the maximum likelihood method using the MEGA X package, and the number of bootstrap test repetitions was 1000.

Furthermore, a phylogenetic tree of DmrtB1 amino acid sequences was reconstructed using mouse Dmrt8 as an outgroup. In addition to the DmrtB1a and DmrtB1b amino acid sequences of *A. schrenckii* employed for alignment, DmrtB1 sequences from five actinopterygian (ray-finned) fishes (*Oreochromis niloticus*, *Micropterus salmoides*, *Lates calcarifer*, *Ictalurus punctatus*, and *Lepisosteus oculatus*) and three sarcopterygian fishes (*Protopterus amphibious*, *Latimeria chalumnae*, and *Latimeria menadoensis*) were also included. The accession numbers of the DmrtB1 nucleotide and protein sequences from the eight species and mouse, as an outgroup, are listed in Table S2. The neighbor-joining method was used to reconstruct the phylogenetic tree by using the MEGA 7.0 package with the following parameter settings: Jones-Taylor-Thornton (JTT) model and all site treatment. The values represent the bootstrap scores from 1000 trials, indicating the credibility of each branch.

Substitution rate estimation and comparison (Ka/Ks analysis)

The average nonsynonymous substitutions (Ka) and synonymous substitutions (Ks) of DmrtB1 and the corresponding Ka/Ks ratios were calculated to test selection pressure at the codon-based nucleotide sequence level using KaKs_Calculator version 2.0 (Wang et al. 2010). The accession numbers of DmrtB1 genes with complete open reading frame (ORF) sequences from eight teleost fishes are listed in Table S2. Thus, the Ka, Ks, and Ka/Ks values of all paired comparison groups based on the ORF sequences of DmrtB1 genes were determined. The Ka/Ks ratio of each pair was calculated using three different algorithms, including the YN, gYN, and MYN algorithms.

Experimental Amur sturgeons and histological features of gonads

A total of 22 healthy Amur sturgeons were sampled from the Engineering and Technology Center of

Sturgeon Breeding and Cultivation of the Chinese Academy of Fishery Science (Beijing, China). All Amur sturgeon individuals were from four developmental stages during early sex differentiation as follows: (1) Amur sturgeons were at 5 months after hatching (5 M) ($N=4$, sex-undifferentiated state). The average body weight (BW) and body length (BL) were 70.5 ± 8.4 g and 20.1 ± 0.6 cm, respectively; (2) 12 M Amur sturgeons ($N=6$, 3 females and 3 males; BW 130.8 ± 17.6 g; BL 26.9 ± 1.5 cm); (3) 24 M Amur sturgeons ($N=6$, 3 females and 3 males; BW 658.3 ± 86.1 g; BL 48.2 ± 1.9 cm); and (4) 36 M Amur sturgeons ($N=6$, 3 females and 3 males; BW 2475.0 ± 521.2 g; BL 67.8 ± 3.4 cm). Before sampling, all experimental Amur sturgeon individuals were anesthetized with eugenol in water for 1–3 min, following the AVMA guidelines (2013). The main tissues from each Amur sturgeon individual, including the brain, intestine, muscle, liver, heart, spleen, gill, and gonads (undifferentiated gonads (UGs), testis, and ovary), were sampled and then immediately preserved into liquid nitrogen until total RNA extraction.

The histological analyses of each gonads were previously performed using hematoxylin and eosin (HE) staining. The gonad tissues from 5 M were in a sex-undifferentiated state. At 12 M, ovarian differentiation was recognizable by a folded or invaginated epithelium contained few oogonia clusters, whereas testis differentiation features were evident with a smooth epithelium with single spermatogonial cells. At 24 M, ovaries were in the majority of growth oocytes (diameter approximately 50 μm) and testes began to present obvious seminal lobules. The gonads of 3-year-old (i.e., 36 M) *A. schrenckii* were visible at the early gametogenesis stage. The ovary was filled with the advantaged primary oocyte with bigger diameter (100–500 μm) and abundant nucleolus, whereas the testis was alveolate seminiferous lobule filled with increasing spermatogonia cells (Zhang et al. 2020).

Expression assay of five Dmrt family genes using real-time PCR method

Total RNA was extracted from each tissue using the RNAiso reagent (TaKaRa, Tokyo, Japan). RNA purity was detected using 1.5% gel electrophoresis. RNA concentration and purity were measured using a microplate reader (Thermo Fisher Scientific).

The RNA quality criteria for the RNA samples were clear bands of 28S and 18S rRNAs and OD 260/280 > 1.8. Qualified RNAs were used for further expression assays using real-time PCR.

A tissue expression profiling assay was performed on seven main tissues of six 3-year-old Amur sturgeons (i.e., 6 biological replicates), including the brain, intestine, muscle, liver, heart, spleen, and gill. Then, we further analyzed the spatiotemporal expression patterns in the gonads during their differentiation in four stages, ranging from UGs ($N=4$) to differentiated gonads, including 12 M (3 testes and 3 ovary), 24 M (3 testes and 3 ovary), and 36 M (3 testes and 3 ovary).

Briefly, 200 ng of qualified RNAs from each sample was reverse transcribed using a First-strand cDNA Synthesis Kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. The reactions were incubated at 42 °C for 60 min and 70 °C for 15 min and then held at 4 °C. Blank and template-free reactions served as negative controls. The primers of five Dmrt family genes were designed using Oligo 7 software. The primer sequences used in these assays are listed in Table S3. Real-time PCR was performed using the Applied Biosystems Quant-Studio™ 5 platform (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. A total of 0.5 µl of cDNA was used as the template in a 20 µl reaction mixture, along with 10 µl of the SYBR® Green Master Mix (Applied Biosystems, Carlsbad, USA), 0.5 µl of each primer (10 µM), and 8.5 µl of ultrapure water under the following conditions: 50 °C for 2 min for Heated-labile Uracil-DNA Glycosylase (UDG) activation and then 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s.

Each targeted gene was analyzed in triplicate wells in more than three Amur sturgeon individuals (biological replicates). The expression levels of targeted genes were calculated using the relative quantity ($2^{-\Delta\Delta CT}$) method after normalization against the β -actin gene. Furthermore, the values obtained in intestines (as reference tissues for tissue expression profiling analysis) and UGs (as reference tissues for spatiotemporal expression pattern analysis) were normalized to 1. The mean and standard error of the measurements ($M \pm SEM$) were calculated from the technological and biological replicates. Statistical significance was measured using the independent sample t -test in SPSS 17.0, with $P < 0.05$ indicating significance.

Results

Sequence characteristics and conservation analysis of the five Dmrt family genes of *A. schrenckii*.

The ORF of Dmrt2 has a total length of 1046 bp, encoding 348 amino acids. The ORFs of DmrtA1 and DmrtA2 are 1353 bp and 1338 bp and encode 450 and 445 amino acids, respectively. The ORFs of DmrtB1a and DmrtB1b are 1104 bp and 1122 bp and encode 367 and 373 amino acids, respectively. The detailed characteristics of the five Dmrt genes are summarized in Table 1.

According to domain composition, the five Dmrt family genes could be divided into two groups: the first group contained DmrtA1 and DmrtA2, with both DM and DMA domains, while the second group contained Dmrt2, DmrtB1a, and DmrtB1b, all of which had only the DM domain (Fig. 1). The schematic illustration of amino acid conservation among the five Dmrt family genes showed that their DM domain sequences were highly conserved and the DMA

Table 1 Sequence characteristics of the five Dmrt family genes and the encoded proteins in *A. schrenckii*

Gene	Full-length (bp)	ORF (bp)	5'UTR (bp)	3'UTR (bp)	Amino acids (aa)	Molecular weight (kDa)	Isoelectric point (PI)
Dmrt2	2291	1046	324	921	348	38.7	8.57
DmrtA1	1888	1353	381	769	450	48.9	8.70
DmrtA2	2853	1338	480	1035	445	47.8	8.35
DmrtB1a	2434	1104	10	1113	367	39.5	7.19
DmrtB1b	2348	1122	72	1155	373	40.9	9.80

domains of DmrtA1 and DmrtA2 were also highly conserved; however, the degree of variation among amino acid sequence of the DM and DMA domains and other regions was relatively high. A schematic illustration of the amino acid sequence conservation of the five genes in *A. schrenckii* is shown in Supplementary Fig. 1.

Advanced protein structure prediction for the five Dmrt family genes of *A. schrenckii*.

The secondary structures of all five Dmrt family proteins consisted of 4-peptide α -helices (4.63 to 10.06%), β -sheets (1.33 to 3.45%), and coils (83.11 to 93.73%). The secondary structure types and their corresponding proportions among the five Dmrt family proteins of *A. schrenckii* are shown in Fig. 2 and Table S4.

The tertiary structure analysis of the five Dmrt family proteins showed that DmrtA1 and DmrtA2 existed as monomers and that the other three Dmrt proteins formed homotrimers. Through further analysis, we found that serine (Ser)-rich regions were only distributed in the α -helices of Dmrt2, DmrtA1, and DmrtA2, while redundant alanine (Ala) residues were mainly distributed in β -sheets or junctions between β -sheets and α -helices (Fig. 3).

Sequence similarity comparison and phylogenetic analysis

The similarity of the amino acid sequences among the DM domains and coding sequences (CDSs) of the five Dmrt family genes was analyzed. We found that the similarity among DM domains was higher than that among CDSs. For example, the amino acid sequence similarity was highest between the DM domains of DmrtB1a and DmrtB1b, at 98.2%, followed by that between DmrtA1 and DmrtA2 (94.4%) and Dmrt2 and DmrtA2 (88.9%). In contrast, the degree of CDS similarity between Dmrt2 and DmrtB1a was the same as that between Dmrt2 and DmrtB1b, at 51.9%. The results of the comparisons of amino acid similarity among the five Dmrt family genes (DM domains vs. CDS) are listed in Table S5.

Subsequently, the phylogenetic tree of the five Dmrt family genes was reconstructed based on the amino acid sequence of the DM domains. The phylogenetic analysis revealed that the Amur sturgeon DmrtA2, DmrtA1, and Dmrt2 sequences aggregated into single branches and then clustered together with the corresponding sequences of other teleost fishes. Additionally, we found that DmrtA2 showed the earliest evolutionary divergence from the Dmrt family and that DmrtA1 presented the closest phylogenetic relationship with DmrtA2, followed by Dmrt2. Importantly, Amur sturgeon DmrtB1a and DmrtB1b, which were the only two novel Dmrt family genes

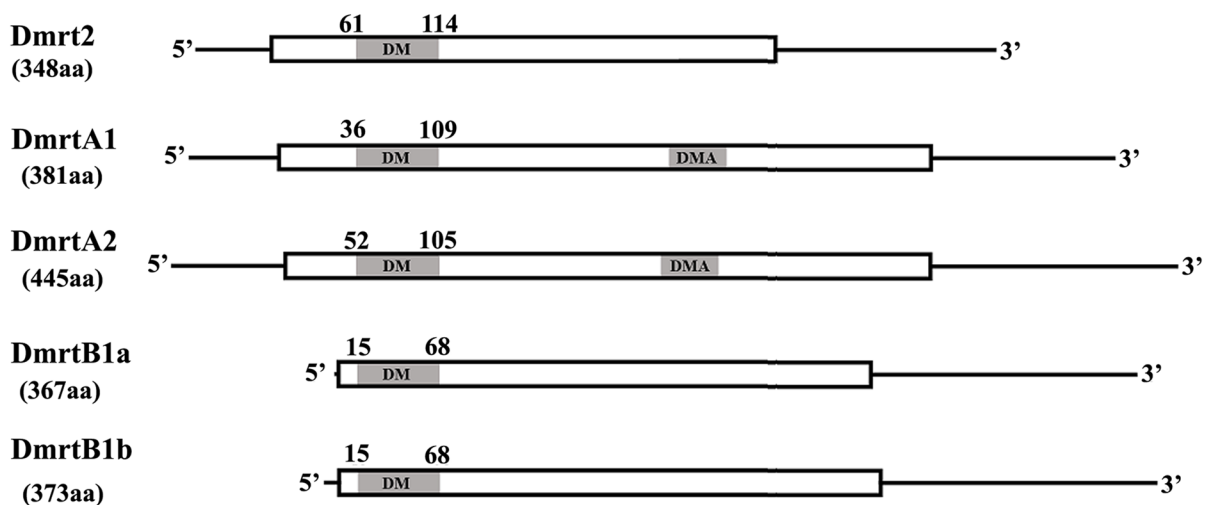


Fig. 1 Schematic illustration of the primary structures of the five Dmrt family genes in *A. schrenckii*

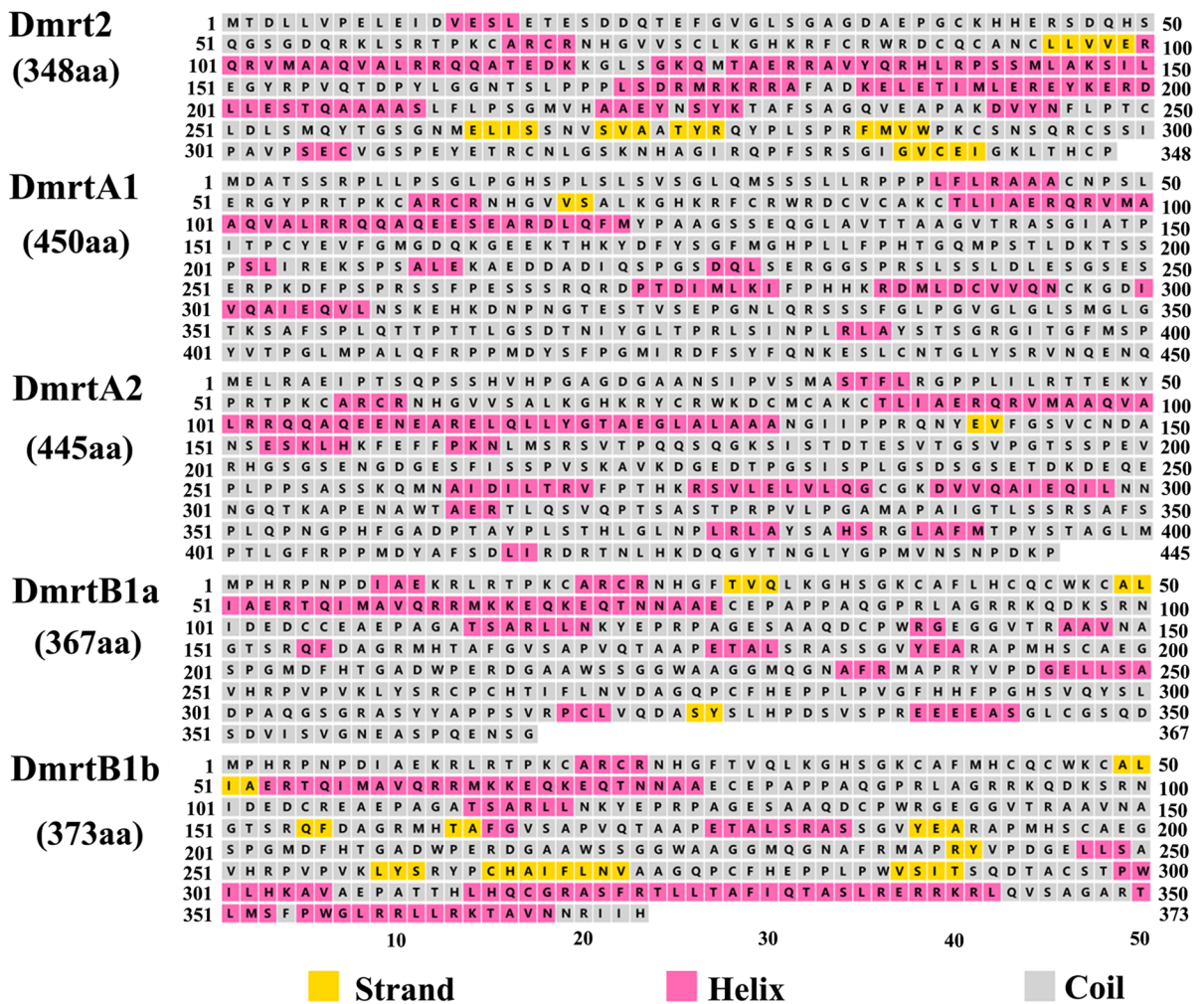


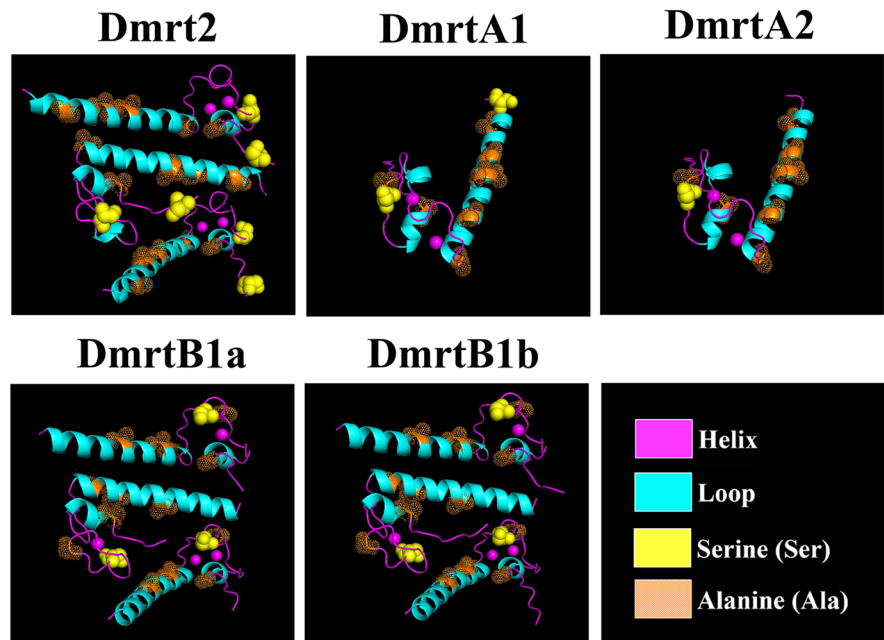
Fig. 2 Schematic illustration of the secondary structures of the five Dmrt family proteins in *A. schrenckii*

in sturgeon, aggregated into a single clade and then clustered together with the sequences of other vertebrates (Fig. 4).

Furthermore, using the mouse Dmrt8 amino acid sequence as the outgroup, we reconstructed protein-based phylogenetic tree of DmrtB1 genes reported from nine teleost fishes, including six actinopterygian fishes and three sarcopterygian fishes. As shown in the phylogenetic tree (Fig. 5a), it was clear that the DmrtB1 proteins from three Perciformes species (*O. niloticus*, *M. samoides*, and *L. calcarifer*) were clustered into one clade, while the DmrtB1 proteins from one Acipenseriformes species (*A. schrenckii*), two Coelacanthiformes species

(*L. chalumnae*, *L. menadoensis*), one Dipnoi species (*P. amphibius*), one Siluriformes species (*I. punctatus*), and one Lepidosteiformes species (*L. oculatus*) were clustered into another clade. Additionally, the DmrtB1 proteins from Acipenseriformes (*A. schrenckii*) and Siluriformes (*I. punctatus*) showed the closest phylogenetic relationship. Intriguingly however, the phylogenetic relationships of the DmrtB1 proteins between Acipenseriformes (*A. schrenckii*) and three sarcopterygian fishes were closer than those between Acipenseriformes (*A. schrenckii*) and three Perciformes species as they belonged to the actinopterygians.

Fig. 3 Schematic illustration of the tertiary structures of the five Dmrt family proteins in *A. schrenckii*



DmrtB1a and DmrtB1b were positive selection

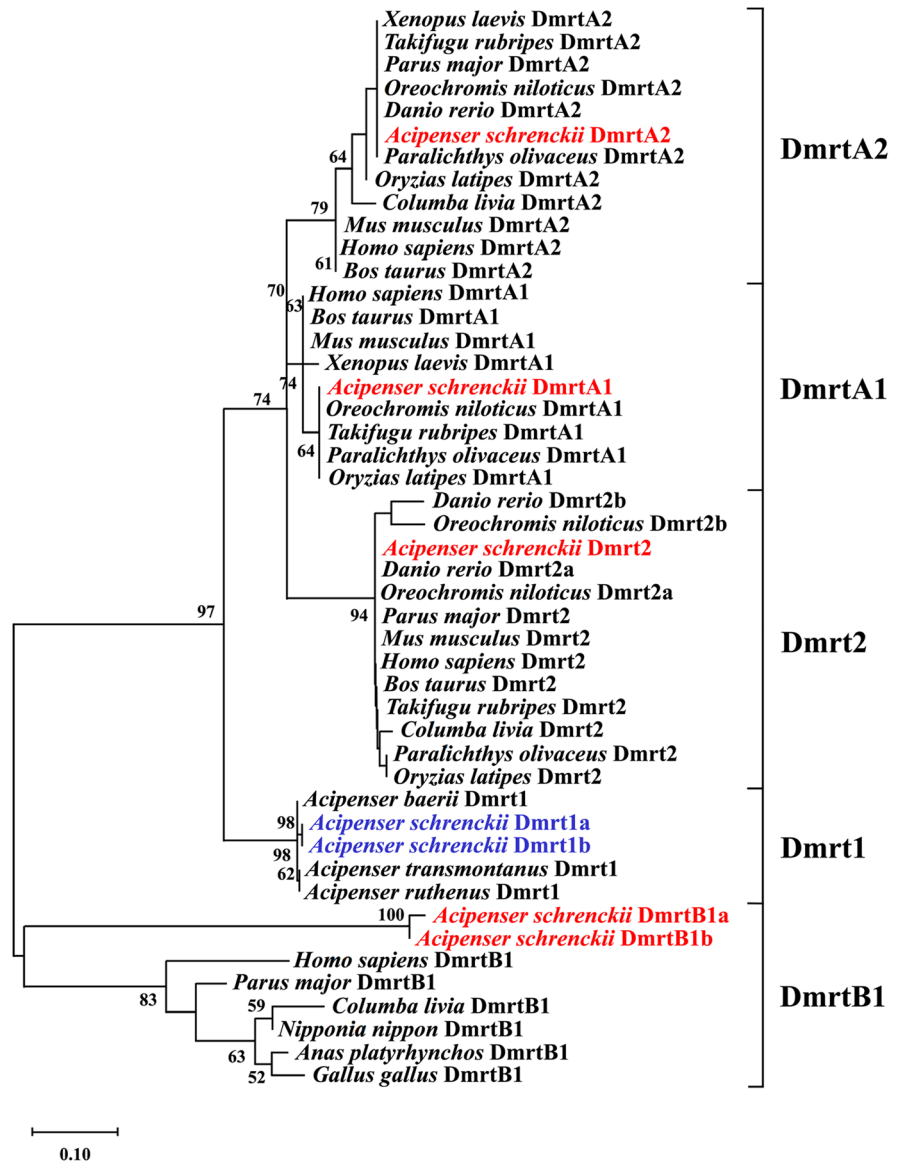
Ka and Ks assessments provide very important markers used in studies of evolutionary selection on genes. The Ka/Ks ratio can be used to assess whether genes are under selection pressure, where a $Ka/Ks > 1$ indicates probable positive selection, while Ka/Ks values close to 1 represent neutral evolution or relaxed selection, and $Ka/Ks < 1$ (especially less than 0.5) indicates purifying selection. First, the Ka/Ks ratios of *A. schrenckii* were assessed against eight related teleost species: *O. niloticus*, *M. salmoides*, *L. calcarifer*, *I. punctatus*, *L. oculatus*, *P. amphibious*, *L. chalumnae*, and *L. menadoensis*. The results showed average Ka/Ks values > 1 for DmrtB1a (1.49) and DmrtB1b (1.73), indicating positive selection (Fig. 5b). The relatively Ka/Ks ratios indicating potential adaptive evolution may reflect specific selection pressure on *A. schrenckii*. Alternatively, they could be a sign of increased variability of these particular proteins within a broader group of species. The DmrtB1 gene Ka/Ks ratios were also calculated for the rest of the paired sequence comparisons among the eight fish species (average Ka/Ks ratio – 1.728). The Ka/Ks cross test showed that positive DmrtB1 gene selection events were widespread in teleost fish.

Tissue expression profiles and spatiotemporal expression patterns in gonads

To reveal the expression characteristics of the five Dmrt family genes, expression analysis of seven main tissues of 3-year-old *A. schrenckii* individuals was first performed using real-time PCR. Tissue expression profiles of the five Dmrt family genes are shown in Fig. 6. Here, we observed two obvious expression characteristics. (1) Relative expression was lowest in the brain, but each Dmrt gene was expressed in all seven tissues, which may indicate that the five Dmrt genes have extensive biological functions. (2) Dmrt2, DmrtA1, DmrtA2, and DmrtB1a were the most highly expressed in gills, but the expression level of DmrtB1b was the highest in the liver.

Subsequently, spatiotemporal expression patterns in the gonads during sex differentiation and further development were analyzed using real-time PCR. The results of the spatiotemporal expression pattern analysis are present in Fig. 7. The expression level of Dmrt2 was highest in 5 M gonad (UGs) and there were no differences between the testis and ovary in any of the three examined differentiated stages. DmrtA1 and DmrtA2 showed similar spatiotemporal expression patterns, with relatively higher expression levels in 5 M and in the ovaries in the other three

Fig. 4 Maximum likelihood analysis showing phylogenetic relationships based on the amino acid sequences of the Dmrt gene DM domains among 19 species. The numbers on each branch represent the bootstrap values obtained with 1000 replicates



developmental stages. In the 36 M gonads, the relative expression of DmrtA1 and DmrtA2 was significantly higher in ovaries than in testes ($P < 0.05$). In contrast, the expression levels of DmrtB1a gradually increased in the testes from 12 to 36 M, and the significantly different expression levels were observed between testes than ovaries at 36 M ($P < 0.05$). Most interestingly, the expression pattern of DmrtB1b was impressive during the gonadal differentiation of *A. schrenckii*. The expression of DmrtB1b was lowest in UGs. Thereafter, the expression level of DmrtB1b was first increased at 12 M, but then decreased in the

ovaries at 12 M and 36 M, while it increased in testes and the testes showed significantly higher expression than the ovaries at 24 M and 36 M ($P < 0.05$).

Discussion

The Dmrt gene family produces a large number of transcripts, which show highly conserved biological functions during sex determination and differentiation across vertebrates. One aim of the present study was to identify novel Dmrt gene family members in

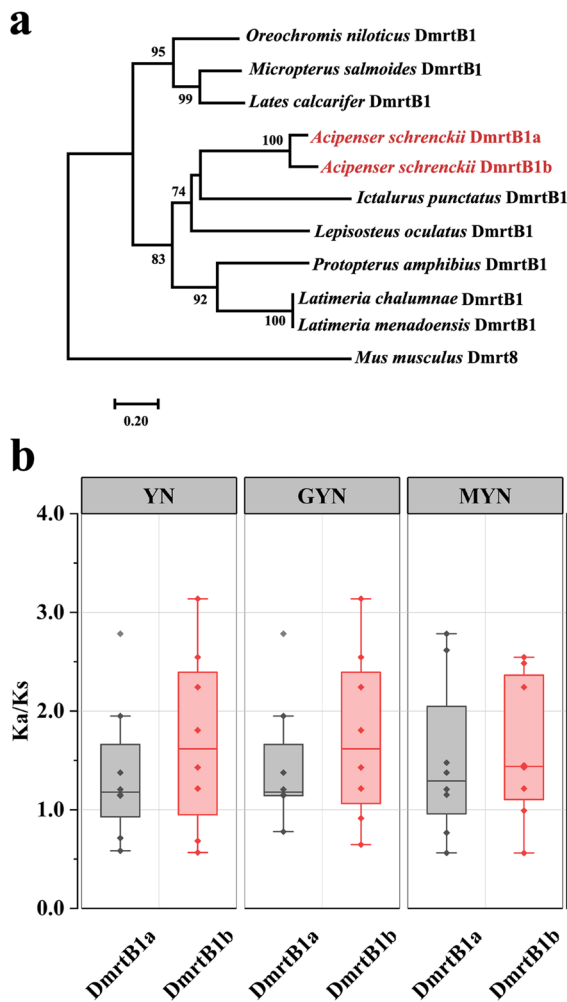


Fig. 5 Phylogenetic tree construction and evolutionary pressure analysis for DmrtB1. **a** The protein-based phylogenetic tree of DmrtB1 genes was reconstructed based on all nine teleost fishes with reported sequences, including six actinopterygian fishes and three sarcopterygian fishes, using the mouse Dmrt8 amino acid sequence as the outgroup. **b** Boxplot showing Ka/Ks ratios of DmrtB1 genes assessed at the codon-based nucleotide sequence level. The open reading frame (ORF) sequences of eight teleost fish species (the accession numbers in Table S2) were aligned against the reference sequence of *A. schrenckii*

sturgeon. Dmrt genes can be divided into several different subfamilies according to protein domain, such as DmrtA, DmrtB, and DmrtC. In the DmrtA subfamilies, three members, DmrtA1 (Dmrt4), DmrtA2 (Dmrt5), and DmrtA3 (Dmrt3), also show conserved DMA domains, in addition to the DM domain (Ottolenghi et al. 2002). In comparison

with short-read sequencing, the methodological advantages of PacBio Isoform sequencing (Iso-Seq) achieve better completeness of both the 5' and 3' end sequences of full-length cDNA molecules. The further annotation of full-length transcripts can play a critical role in gene discovery (Zhang et al. 2019). Therefore, five additional full-length Dmrt family genes were successfully identified in *A. schrenckii*, which were designated Dmrt2, DmrtA1, DmrtA2, DmrtB1a, and DmrtB1b. These findings provide a genetic basis for better understanding the structural and functional properties of these genes in sturgeon species.

The Dmrt gene family has undergone frequent, independent gene replication events during the course of evolution; thus, the kinds and members of Dmrt family genes may be different between phyla. Variation is even observed within vertebrates, among which mammals include some genes that are absent from teleost fish (Volf et al. 2003); for example, no fish orthologs of Dmrt7 and Dmrt8 have been identified (Zafar et al. 2019). In the present study, five Dmrt family genes with full-length cDNA sequences were identified as new family members from sturgeon, in addition to Dmrt1a and Dmrt1b reported previously (Okada et al. 2017). According to the phylogenetic relationship analysis (Fig. 4), the five Dmrt family genes belong to four different Dmrt subfamilies. We also found that the phylogenetic positions of Dmrt2, DmrtA1, and DmrtA2 were relatively ancient, which was consistent with a previous study (Volf et al. 2003).

Some Dmrt family genes in different species may show novel expression patterns and new biological functions. For example, Dmrt1 gave rise to the master male-determining gene found in medaka fish (Matsuda et al. 2007) but not in more divergent fish. In zebrafish, the duplicated Dmrt2b and Dmrt2a with structural variation and expression diversification suggests their diverse roles, as Dmrt2b functions in specification of branchial arches, and Dmrt2a functions in somitogenesis (Zhou et al. 2008). In the present study, extensive tissue expression profiling revealed interesting and common expression characteristics of the five Dmrt family genes. Among these genes, Dmrt2, DmrtA1, DmrtA2, and DmrtB1a were highly expressed in gills, but DmrtB1b showed the most abundant expression in the liver. Contrasting expression patterns of these genes in tissues other

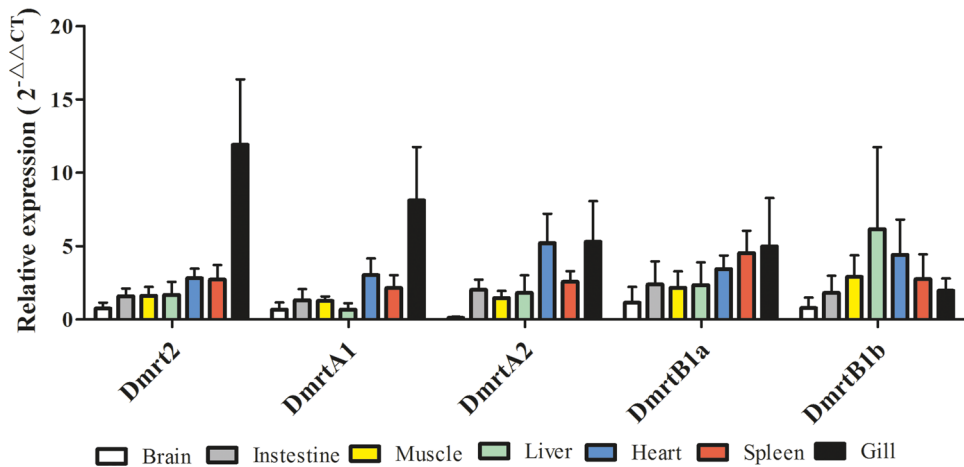


Fig. 6 Tissue expression profiling of the five Dmrt family genes in seven main tissues of 3-year-old *A. schrenckii* individuals

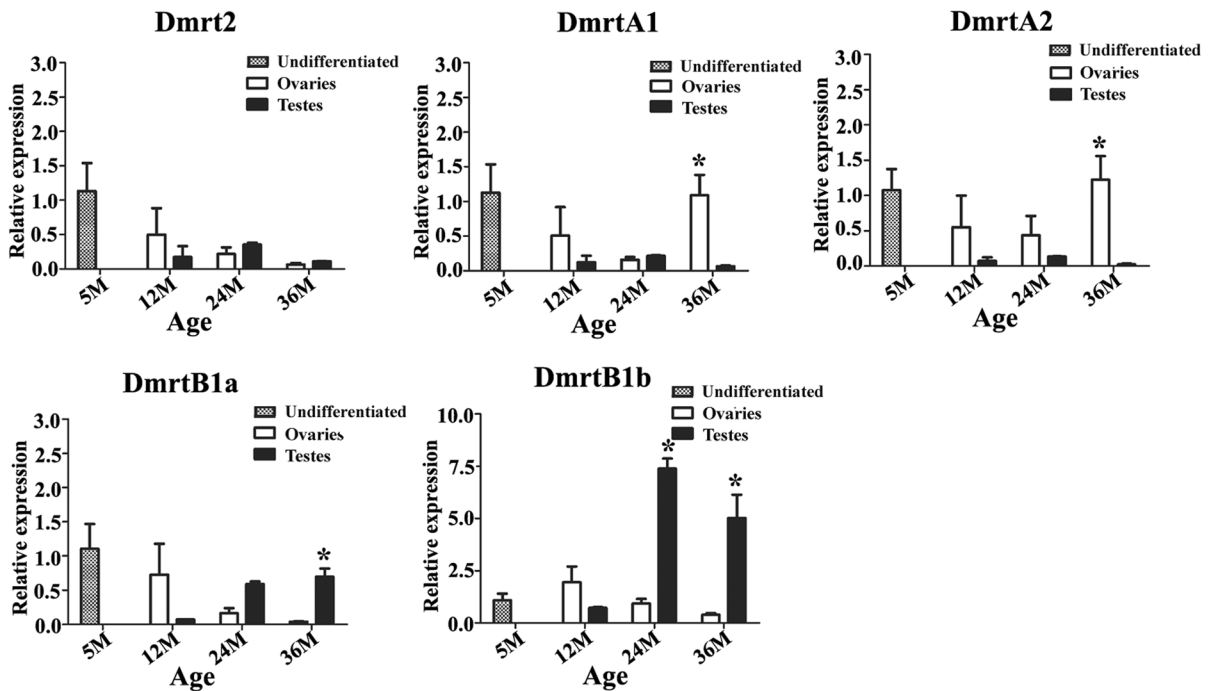


Fig. 7 Spatiotemporal expression patterns in the gonads during gonadal differentiation in *A. schrenckii*. Asterisks (*) above a column represent significant differences between the testis

and ovary at the same developmental stage. $P < 0.05$ as evaluated by the *t*-test in SPSS 17.0

than the gonads were initially reported; thus, their functions may be worthy of further investigation.

The roles of Dmrt family genes in sex determination and differentiation vary among vertebrates. For example, Dmrt1 has been identified as

a sex determination gene in *O. latipes* (Matsuda et al. 2007), *X. laevis* (Yoshimoto et al. 2008), and *G. gallus* (Smith et al. 2009), but no role of this gene has been identified in sturgeon (Okada et al. 2017). In the present study, five additional Dmrt family genes

with full-length cDNA sequences were identified for the first time, which represents the newest genetic information on Dmrt family genes in sturgeon. Further analysis revealed the spatiotemporal expression patterns of the five Dmrt family genes in gonads in stages ranging from UGs to the ovaries or testes of 3-year-old individuals. Our results showed that the expression of Dmrt2 was highest in UGs, whereas it appeared to play no role in early gonadal development in *A. schrenckii*. However, the role of Dmrt2 varies in other teleost fish. For example, the expression of Dmrt2 is higher in male gonads during early gonadal development in Chinese tongue sole (*Cynoglossus semilaevis*) (Zhu et al. 2019), and Dmrt2 has been shown to be involved in sex differentiation in swamp eel (Sheng et al. 2014). However, Dmrt2 is also reported to play a significant role in premature ovarian differentiation and maintenance in orange-spotted grouper (*Epinephelus coioides*) (Lyu et al. 2019). DmrtA1 was demonstrated to be absent in the zebrafish genome, but DmrtA2 was found to be preferentially expressed in the adult testis (Xu et al. 2013). In the present study, it was interesting to find that DmrtA1 and DmrtA2 were highly expressed in UGs and in the ovaries in the other three developmental stages, with significantly higher expression being observed in ovaries than in testes in 36 M *A. schrenckii*. Our results are consistent with the role of DmrtA2 in normal female germ cell development in humans (Poulain et al. 2014).

Dmrt1 shows testis-dominant expression in adults (i.e., 4-year-old *A. schrenckii*) but not in the gonads of juvenile individuals (Okada et al. 2017). However, DmrtB1b presented the lowest expression in UGs in the present study, after which it increased in testes and presented significantly higher expression in testes than in ovaries in 24 M and 36 M *A. schrenckii*. According to related studies, 24 M and 36 M correspond to the stages of spermatogonial cell proliferation and early gametogenesis, respectively, in *A. schrenckii* (Zhang et al. 2020). The above observation may suggest that DmrtB1b acts as a core regulatory factor in the sexual differentiation of *A. schrenckii*; DmrtB1a and DmrtB1b were reported in sturgeon for the first time in the present study. A functional analysis of DmrtB1^{-/-} mice showed that DmrtB1 played a key role in coordinating the transition in gametogenic programs from spermatogonial differentiation and mitosis to spermatocyte development and meiosis

(Zhang et al. 2014a). The mammalian results may suggest core roles of DmrtB1b in male gonad differentiation in sturgeon, which is worth further study.

Conclusions

A total of five new members of the Dmrt gene family were identified in sturgeon based on a full-length gonad transcriptome database; these genes were designated Dmrt2, DmrtA1, DmrtA2, DmrtB1a, and DmrtB1b. These five Dmrt proteins all contain a conserved DM domain and/or DMA domain and exhibit similar mature structures. Phylogenetic analysis indicated that these genes were clustered into clades corresponding to other DM domain genes of vertebrates. Some interesting characteristics of DmrtB1a and DmrtB1b were also found; for example, they showed closer phylogenetic relationships between Acipenseriformes (*A. schrenckii*) and three sarcopterygian fish groups and positive selection pressure in sturgeon. Extensive tissue expression profiling of each Dmrt family gene showed typical characteristics. Remarkably, the spatiotemporal expression pattern of DmrtB1b may shed light on its core role in the gonads of Amur sturgeon during sex differentiation and further testis development. Overall, this study not only provides a genetic resource of full-length Dmrt family genes but also increases available knowledge for the further investigation of Dmrt functions in sex differentiation in sturgeon.

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Author contribution X. J. Zhang and J. P. Chen conceived and designed the study. X. J. Zhang, G. Y. Li, J. B. Zhou, M. Lv, and L. M. Li performed the experiments. X. J. Zhang and G. Y. Li analyzed the sequence data. X. J. Zhang contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary files.

Code availability Not applicable.

Declarations

Ethics approval All experimental animal procedures followed the principles of Guide for Care and Use of Laboratory Animal and were approved by the Ethics Committee of Laboratory Animal of Institute of Zoology, Guangdong Academy of Science (No: GIZ20200828).

Consent to participate Consent was obtained from individual participants.

Consent for publication All authors have approved the final manuscript.

Conflict of interest The authors declare no competing interests.

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