Identification of multiple *dmrt1*s in catfish: localization, dimorphic expression pattern, changes during testicular cycle and after methyltestosterone treatment

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Abstract

The double sex and mab-3 related (DM) transcription factor 1 (*dmrt1*) plays an important role in testicular differentiation. Here, we report cloning of multiple *dmrt1*s, a full-length and two alternative spliced forms from adult catfish (*Clarias gariepinus*) testis, which encode predicted proteins of 287 (*dmrt1a*), 253 (*dmrt1b*) and 233 (*dmrt1c*) amino acid residues respectively. Interestingly, *dmrt1c* lacks the majority of the DM domain. Multiple *dmrt1*s (*dmrt1a* and *dmrt1c*) were obtained from *Clarias batrachus* as well. Tissue distribution (transcript and protein) of catfish *dmrt1* revealed exclusive expression in testis. Semi-quantitative RT-PCR revealed the presence of multiple *dmrt1*s with high levels of *dmrt1a* in adult testis but not in ovary. Real-time RT-PCR analysis during testicular cycle showed higher levels of *dmrt1* transcripts in preparatory and pre-spawning when compared with spawning and post-spawning phases. Immunocytochemical and immunofluorescence localization revealed the presence of catfish Dmrt1 protein in spermatogonia and spermatocytes, which indicates plausible role in spermatogenesis. Histological analysis indicated initiation of gonadal sex differentiation in catfish around 40–50 days after hatching. The potential role for *dmrt1* in testicular differentiation is evident from its stage-dependent elevated expression in developing testis. Furthermore, dimorphic expressions of *dmrt1*s were evident at different stages of gonadal development or recrudescence in catfish. Treatment of methyl testosterone (MT) during early stages of gonadal sex differentiation resulted in adult males. Interestingly, we also obtained MT-treated fishes having ova-testis gonads. Analysis of *dmrt1*, *sox9a*, *foxl2* and *cyp19a1* expression patterns in MT-treated gonads revealed tissue-specific pattern. These results together suggest that multiple *dmrt1*s are testis-specific markers in catfish.

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Introduction

Several transcription factors like *sry*, *dmy*, *sox9*, *ad4BP/SF-1*, *dmrt1*, *dax1* and *wt1* have been identified to play an important role in sex determination and differentiation of vertebrates including teleosts (Swain & Lovell-Badge 1999, Hughes 2001, Matsuda *et al.* 2002, Wang *et al.* 2002, Kobayashi *et al.* 2004). Molecular similarity in sexual development across different phyla found so far is among *Drosophila doublesex*, *Caenorhabditis mab-3* and vertebrate *dmrt1* (Raymond *et al.* 1998). *Dsx* and *mab-3* related transcription factor 1 (*dmrt1*) belongs to gene family of putative transcription factors that share a highly conserved novel zinc finger DNA binding domain (DM domain) across different phyla (Raymond *et al.* 1998). *Dmrt1* has been cloned from several vertebrate species including mammals, birds, reptiles, amphibians and fishes (Nanda *et al.* 1999, De Grandi *et al.* 2000, Guan *et al.* 2000, Kettlewell *et al.* 2000, Nagahama 2005, Osawa *et al.* 2005) and has been implicated in testicular differentiation. Furthermore, *dmrt1* has been localized in 9p23.4 and monosomy at this region in XY individuals and manifests

feminization and gonadal dysgenesis (Ottolenghi & McElreavey 2000). In mice, *Dmrt1* expresses in both male and female embryonic genital ridges, but as the differentiation proceeds, it is gradually lost from the ovary and expressed only in the Sertoli and germ cells of testis. High expression of *Dmrt1* was observed in testis at 13.5 days post coitum and its expression is maintained throughout the adult after birth (Raymond *et al.* 1999, De Grandi *et al.* 2000). Localization of *Dmrt1* in the Z-chromosome of birds designated it as a male master testis-determining gene (Nanda *et al.* 1999). *Dmrt1* knockout mice showed its dispensability in females yet it is required in males for postnatal testicular development by effecting the differentiation of both Sertoli and germ cells (Raymond *et al.* 2000). These expression patterns indicate that *dmrt1* is likely to have a conserved role in the early stages of testis development. Studies from lower vertebrates are mostly done in teleosts to specify the conserved role of *dmrt1*. *DMY*, a Y-linked male sex-determination gene, similar to *sry* discovered in medaka has been shown to be a duplicate of autosomal gene *dmrt1* (Matsuda *et al.* 2002, Zhang 2004). Nevertheless, most of these reports are

from daily or fortnight breeders like zebrafish, medaka and tilapia (Guan *et al.* 2000, Kobayashi *et al.* 2004, Guo *et al.* 2005). Research reports on this line using annual breeders with a focus on seasonal reproductive cycle are limited (Marchand *et al.* 2000, Huang *et al.* 2005). In this regard, fish that undergo seasonal pattern of gonadal attenuation and recrudescence rather than continuously mature individuals may provide interesting highlights to understand the role of *dmrt1* not only during development but also during recrudescence. The fate of *dmrt1* transcripts after testicular differentiation is not clear at present. Such an attempt may provide more insights to understand its role in adult, if any. Although tracking of *dmrt1* may not provide a direct role to this issue but may contribute to understanding whether timing of *dmrt1* expression coincides with the beginning of testicular recrudescence vis-à-vis spermatogenesis. Catfish, *Clarias gariepinus* is an annual breeder, which takes 1 year to attain maturity. It is domesticated in south India and interestingly it exhibits seasonal pattern of reproductive cycle. Previously, we reported cloning of partial cDNA fragments of *dmrt1* from catfish testis that indicated the presence of more than one form of *dmrt1* (Raghuveer *et al.* 2005). In the present study, we aimed to clone full-length *dmrt1* cDNA from catfish testis and also explored the possibility of multiple forms. In addition, we also aimed to study the spatio-temporal expression pattern of *dmrt1*(s) during early stages of gonadal development in juveniles as well as at different phases of testicular recrudescence in adult catfish. We also intend to confirm the presence of *dmrt1*(s) from a closely related catfish species *Clarias batrachus* to augment our findings. Furthermore, our study was extended to localize *dmrt1* in juvenile and adult testis. Administration of methyltestosterone (MT) or ethynylestradiol (EE2) is a useful strategy to skew the sex of the population in question to study testicular and ovarian differentiation (Nagahama 2005). We used MT treatment strategy to explicitly define the role of *dmrt1* and endorse its importance as a candidate marker for testicular development.

Materials and methods

Animals and sampling

Catfish (*C. gariepinus*) at different age groups were reared in fresh water tanks under ambient photo-thermal conditions. Mature spermiating male and gravid female fishes were used for IVF to obtain catfish fries at different age groups. The newly hatched catfish fries were fed with live tube worms until 3 months. Juvenile fishes of 3–8 months old were fed with live tube worms and commercially available fish feed. Adult catfish (1-year old) were reared in the outdoor tanks of the laboratory and fed with minced goat liver in

addition to fish feed. Commonly referred to as the African or air-breathing catfish, this species is abundantly available in the ponds and lakes of Hyderabad. The seasonal reproductive cycle of catfish is divided into four phases (Swapna *et al.* 2006): preparatory (February–March), prespawning (April–June), spawning (July–October) and post-spawning/regressed phases (November–January). Adult gonads during the reproductive cycle of catfish were collected and a part of it was fixed in Bouin's fixative while remaining tissue was stored at -80°C for total RNA extraction. Juvenile catfish fries at different age groups (50, 100 and 150 days after hatching (dah)) were dissected and gonads were removed using fine forceps under stereozoom microscope (Leica, Wetzlar, Germany). Number of gonads of similar age group and same sex were pooled and stored at -80°C for total RNA extraction. Treatments of MT were given to catfish fingerlings during the critical period of gonadal differentiation as explained earlier (Raghuveer *et al.* 2005). In brief, about 250 catfish fries were treated with MT in pulse (3 h) at a dose of 500 $\mu\text{g/l}$ intermittently (six durations) till 21 dah. We used high doses of MT as low doses were found to be ineffective in our pilot studies. The MT-treated fishes were reared until they reach maturity and after (1 to 2 years). Then their gonads were dissected out for histology and total RNA extraction. Adult *C. batrachus* were obtained from local fish markets of Hyderabad as well as from north India. These were maintained and fed with minced goat liver *ad libitum* during acclimation in the laboratory for a fortnight before killing.

Cloning of full-length and alternatively spliced forms of *dmrt1*

Primers for rapid amplification of cDNA ends (RACE) were designed based on the sequence information of partial cDNA fragment of catfish *dmrt1* that was cloned earlier. SMART 5' and 3' cDNA templates were made from testis total RNA according to manufacturer's protocol using the SMART RACE cDNA amplification kit (Clontech, Tokyo, Japan). 5' RACE was performed using the DM domain primer, DMr1: 5'-GCTATCTC-CACTGGGCATCTGCTGGT-3' and universal primer A mix (UPM): 5'-CTAATACGACTCACTATAGGGCAAG-CAGTGGTATCAACGCAGAGT-3'. Touchdown PCR cycling conditions were as follows: each at 94°C (30 s) and 72°C (2 min) for 5 cycles, 94°C (30 s) and 70°C (2 min) for 5 cycles, 94°C (30 s), 68°C (30 s) and 72°C (2 min) for 27 cycles, in a 20 μl reaction mix containing $1\times$ PCR buffer, 100 μM dNTP mix, 1U PCR advantage *Taq* DNA polymerase (Clontech), 0.1 μM each primer, and 1 μl of the cDNA. After the primary PCR, nested PCR was performed using 5' nested primer DMr2:

5'-AATCATTTCTGCTCATCCTTCACC-3' and nested universal primer (NUP): 5'-AAGCAGTGGTATCAACG-CAGAGT-3'. The nested PCR conditions were as follows: 94 °C (30 s), 65 °C (30 s) and 72 °C (2 min) for 35 cycles. 3' RACE was performed using the DM domain primer DMf1: 5'-ATGCCGAAGTGCTCCC GGTGCAGG-3', and UPM using the same PCR conditions mentioned above. After 3' RACE, nested PCR was performed using 3' nested primer DMf2: 5'-GTCCCCGCCAGTTACA-GAAGCGCTTG-3' and NUP using the same PCR conditions described above. All PCR amplifications were done using thermal cycler (Applied Biosystems, Foster City, CA, USA). All the amplified cDNA fragments were gel-purified (Qiagen, Hamburg, Germany), cloned in TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) and nucleotide sequenced. We aimed to amplify the open reading frame (ORF) of *dmrt1* using primers *dmrt1orfF* and *dmrt1orfR* (Table 1) designed at ORF flanking region of *dmrt1* cDNA obtained by RACE. The PCR conditions used for ORF amplification were as follows: 94 °C (30 s), 60 °C (30 s) and 72 °C (1 min) for 35 cycles. The PCR amplified products were gel-purified, cloned in TOPO cloning vector (Invitrogen) and nucleotide sequenced.

Tissue distribution pattern of *dmrt1* by reverse transcription (RT)-PCR

RT-PCR was carried out to study expression pattern of *dmrt1* in different tissues of adult catfish. For this, total RNA was extracted from different tissues (brain, spleen, gill, heart, intestine, kidney, liver, testis and ovary) of adult catfish using the Sigma TRI-reagent (Sigma)

method. Reverse transcription was carried out using superscript-III reverse transcriptase (Invitrogen) with oligo d(T)₁₈ primers and 5 µg of total RNA at 50 °C. PCR amplification was done using thermal cycler (Applied Biosystems) under the following conditions: 94 °C (1 min), 58 °C (30 s), 72 °C (1 min), for 35 cycles using specific *dmrt1aF* and *dmrt1aR* primers (Table 1). *β-actin* was used as an internal endogenous control to test the quality of cDNA templates.

Expression pattern of multiple *dmrt1s* in developing and adult gonads by semi-quantitative RT-PCR

Semi-quantitative RT-PCR as described by Kwon *et al.* (2001) was carried out to study the expression patterns of multiple *dmrt1s* in adult and developing gonads. Total RNA was prepared from adult and developing gonads collected from juvenile catfish at 50, 100 and 150 dah using the Sigma TRI-reagent method. Reverse transcription was carried out using superscript-III reverse transcriptase (Invitrogen) with oligo d(T)₁₈ primers and 5 µg of total RNA at 50 °C. PCR amplification was done using thermal cycler (Applied Biosystems) under the following conditions: 94 °C (1 min), 60 °C (30 s), 72 °C (1 min), for 30 cycles using specific primers *dmrt1aF*, *dmrt1aR*, *dmrt1bF*, *dmrt1bR*, *dmrt1cF* and *dmrt1cR* designed for amplification of respective *dmrt1* forms (Table 1). Catfish *β-actin* was PCR amplified at 94 °C (1 min), 60 °C (30 s), 72 °C (1 min) for 28 cycles using specific *β-actinF* and *β-actinR* primers (Table 1) as an internal control.

Real-time quantitative RT-PCR (qRT-PCR) for *dmrt1s*

Pilot experiments for multiple *dmrt1s* expression during testicular cycle was done by semi-quantitative RT-PCR using ORF-specific primers. Later, expression of multiple *dmrt1s* was analyzed by relative qRT-PCR using SYBRGreen detection method. Total RNA was extracted from the testis samples of four different phases (preparatory, pre-spawning, spawning and post-spawning phases) using the Sigma TRI-reagent method, and RT was carried out using random hexamers in the presence of superscript-III reverse transcriptase (Invitrogen) according to manufacturer's protocol. Successful RT was confirmed for all samples by performing PCR amplification of internal control *β-actin*. *Dmrt1* and *β-actin* specific primers were designed such that one primer spanned the junction of two exons, giving a single cDNA PCR product and precluding amplification of genomic DNA. Similarly, catfish specific *β-actinF* and *β-actinR* primers (Table 1) were designed for use as normalization controls. One set of specific primers *dmrt1abRTF* and *dmrt1abRTR* (Table 1) were used to amplify the DM domain region, which will give both *dmrt1a* and *b* transcripts of same

Table 1 List of primers used for PCR amplification

Primers	Nucleotide sequence (5'–3')
<i>Dmrt1orfF</i>	GAAGCACGAGGCCGCGCAGAG
<i>Dmrt1orfR</i>	GTTGGTATACTCTCTGAGACTT
<i>β-actinF</i>	ACCGGAGTCCATCACAATACCACT
<i>β-actinR</i>	GAGCTGCGTGTGGCCCTGAG
<i>Dmrt1abRTF</i>	ATGGCCGCTCAGGTGGCTCTGCGG
<i>Dmrt1abRTR</i>	GCGGCTCCCAGAGGCAGCAGGAGA
<i>Dmrt1cRTF</i>	CCAGGGCCAGGTGGCTCTGCG
<i>Dmrt1cRTR</i>	GCGGCTCCCAGAGGCAGCAGGAGA
<i>Dmrt1aF</i>	ATGCCGAAGTGCTCCCGGTGC
<i>Dmrt1aR</i>	AGCGGCTCCCAGAGGCAGC
<i>Dmrt1bF</i>	AAGGATGAGCACCAGGGGACA
<i>Dmrt1bR</i>	TTACTTAGCAGCTCCCTCTAT
<i>Dmrt1cF</i>	CCAGGGCCAGGTGGCTCTGCG
<i>Dmrt1cR</i>	TTACTTAGCAGCTCCCTCTAT
<i>Sox9aF</i>	GCAGAGCTCAGCAAAACCCGG
<i>Sox9aR</i>	GCTGGAAGCGGGAGAGTCGG
<i>Foxl2F</i>	TGCGAGGACATGTTTGAGAAGGG
<i>Foxl2R</i>	TCCCAGTATGAGCAGTGCATCAT
<i>Cyp19a1F</i>	TTGGATCGGGAATTGGGACAGC
<i>Cyp19a1R</i>	AGCTTTAGCGAAGTAGCTGCG

size except *dmrt1c* form. Another set of specific primers *dmrt1cRTF* and *dmrt1cRTR* (Table 1) were used to amplify exclusively *dmrt1c* (DM-less form) only. Specific cDNA amplification was then carried out in triplicate using power SYBRGreen PCR Mastermix (Applied Biosystems) in a 7500 Fast Real-time PCR machine (Applied Biosystems) at 60 °C annealing temperature for 40 cycles according to the manufacturer's protocol. Melting-curve analysis was performed for each sample to check single amplification. During PCR, fluorescence accumulation resulting from DNA amplification was recorded using the sequence detector software (Applied Biosystems). Cycle threshold (CT) values were obtained from the exponential phase of PCR amplification, and *dmrt1* expression was normalized against β -actin expression, generating a Δ CT value (Δ CT = *dmrt1* CT – β -actin CT). Relative expression was then expressed according to the Default $2^{-\Delta$ CT.

Production of polyclonal antibody against Dmrt1 protein

Polyclonal anti-catfish Dmrt1 antibody was raised in rabbit using catfish partial Dmrt1 recombinant protein of the conserved DM-domain as antigen. The partial Dmrt1 protein was expressed in *Escherichia coli* DE3 LacI host using 0.5 M IPTG by cloning the partial *dmrt1* cDNA fragment of 340 bp into bacterial pET BLUE2 vector system (Novagen, Madison, WI, USA). The expressed fusion protein with the histidine tag (His-tag) was then purified using Ni-NTA Agarose column (Qiagen) according to the manufacturer's protocol. This purified recombinant protein was used to raise polyclonal antibody in rabbit after confirming by immunoblot using the monoclonal His-tag antibody. For raising polyclonal antibody against the purified protein, rabbit was first injected with Freund's complete adjuvant (Bangalore Genei Pvt Ltd, Bangalore, India) at the vola. Later after 1 week, 500 µg of the purified protein emulsified in Freund's complete adjuvant was injected into the swollen lymphoid node. Then two booster injections were given with the same antigen amount but using Freund's incomplete adjuvant (Bangalore Genei Pvt Ltd) for each week. After the last booster dose the rabbit was bled 2 weeks later and the serum sample was stored at –80 °C until use. Rabbit pre-immune serum was obtained by collecting 2 ml blood before antigen injection. All the rabbits in the present study were used following the guidelines of Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals and also after obtaining prior permission.

Western blot analysis was carried out to verify the specificity of the polyclonal Dmrt1 antibody raised in the rabbit. For this, different tissues (testis, ovary, gut, spleen, liver, muscle, heart, brain) of catfish were

homogenized in 250 µl homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 0.1% TritonX-100 and 0.1 mM phenyl-methylsulphonyl fluoride using a Sigma hand homogenizer. Of each sample 50 µg with a pre-stained marker was electrophoresed through a 15% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Pall-Life Sciences, NY, USA). Membrane was blocked in 5% skimmed milkpowder in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature. After blocking, membrane was washed three times for 15 min each in TBST solution and incubated with purified anti-catfish Dmrt1 antibody at 4 °C overnight in 0.5% skimmed milk powder/TBST solution. Blot was washed and incubated with secondary antibody alkaline phosphatase conjugated goat anti-rabbit IgG (Bangalore Genei Pvt Ltd) for 1 h. After washing, blot was developed using BCIP-NBT (Bangalore Genei Pvt Ltd).

Immunocytochemical and immunofluorescence localization of *dmrt1*

Testis sections from adult male catfish were deparaffinised in xylene, rehydrated in successively lower graded concentrations of ethanol and then treated with 0.1% H₂O₂ for 10 mins to prevent endogenous peroxidase reaction. The sections were washed in 0.1 M PBS with 0.1% Tween 20 (PBST) and then blocked with 10% normal goat serum in 0.1 M PBS for 10 min at room temperature. Sections were then incubated overnight at 4 °C in 1:1000 dilution of anti-catfish *dmrt1* antiserum or antiserum pre-absorbed with excess of peptide used for immunization. Later sections were washed thrice with PBST and incubated with biotin conjugated secondary antibody at room temperature for 1 h. The sections were incubated with 1:500 dilution of streptavidin labelled HRP conjugate for 15 min at room temperature. The sections were washed with PBS and colour was developed using commercially supplied 3' 3'-diaminobenzidine as chromogen and H₂O₂ as substrate for HRP. The sections were washed, dehydrated in graded ethanol, cleared in xylene and mounted using distrene-plasticizer-xylene mountant. Photomicrographs were taken using Olympus light microscope (Olympus, Japan). For immunofluorescence studies, testis sections were processed in the same way as mentioned above up to primary antibody incubation step except for 0.1% H₂O₂ treatment. The sections were then in PBST and incubated with FITC fluorescent-labelled secondary antibody at room temperature for 1 h. After that the sections were washed and mounted using 90% glycerol. Photomicrographs were taken immediately using Olympus fluorescence microscope (Olympus). Hematoxylin and propidium iodide (PI) was used as a counter stain for better clarity.

All reagents and secondary antibodies to perform immunocytochemistry were obtained from Bangalore Genei Pvt Ltd.

Expression patterns of *dmrt1*, *sox9a*, *foxl2* and *cyp19a1* in gonads of adult MT-treated fishes

Total RNA was extracted from gonadal tissues of adult MT-treated catfish using the Sigma TRI-reagent method. Reverse transcription was carried out using superscript-III reverse transcriptase (Invitrogen) with oligo d(T)₁₈ primers and 5 µg of total RNA at 50 °C. PCR amplification was done using thermal cycler (Applied Biosystems) under the following conditions: 94 °C (1 min), 55 °C (30 s), 72 °C (1 min), for 35 cycles using specific primers (Table 1) designed for *dmrt1a*, *sox9a*, *foxl2* (Sridevi & Senthilkumaran, unpublished observations) and *cyp19a1* (Rasheeda *et al.* 2005) genes of *C. gariepinus*. β -actin was used as an internal control to test the quality of cDNA templates.

Histology

The trunk region of catfish fries at different stages (30, 50, 75, 100, 150 dah) and gonads of adult MT-treated

fishes were fixed in Bouin's solution, dehydrated and embedded in paraplast (Sigma). For light microscopy, 4 mm thick sections were cut and stained with hematoxylin-eosin. All the photomicrographs for histological analysis were taken using Olympus light microscope.

Results

cDNA cloning of multiple alternative transcripts of catfish *dmrt1*

To isolate cDNA of *dmrt1* from catfish testis we performed 5' and 3' RACE using the partial *dmrt11* cDNA sequence data that was previously obtained from *C. gariepinus* (Raghuvver *et al.* 2005). After aligning the 5' and 3' end regions of *dmrt1* sequence that overlap in the DM-domain region, we obtained 1.1 kb full-length cDNA sequence of *dmrt1* (hereafter referred as *dmrt1a*) which encodes a putative protein of 287 amino acids (Fig. 1A). The amino acid sequence comparison of catfish *dmrt1a* with other vertebrate *dmrt1* proteins revealed considerable homology with other teleosts like zebrafish (61%), rainbow trout (59%), the eel (57%) and the Nile tilapia (54%). The phylogenetic analysis of

Figure 1 (A) Nucleotide sequence of catfish full-length *dmrt1a* cDNA and its deduced amino acid sequence. Nucleotides are numbered to the left. The DM domain is shaded, putative SY domain is underlined, * indicates stop codon. (B) Phylogenetic tree showing the evolutionary status of catfish *dmrt1*. Bootstrap analysis with 100 replicates was used to assess the strength of nodes in the tree. Phylogenetic analysis was done using CLC bio: CLC Main workbench software (accession numbers: Human NM_015826; Mice NM_021951.2; Rat NM_053706; Pig NM_214111; Zebrafish AY157562; Medaka AF319994; Rainbow trout NM_001124269; Eel AF421347; Fugu NM_001037949; Tilapia AF209095; Pufferfish AY135175; Catfish (*dmrt1a*) FJ596554). (C) Representative gel image showing multiple alternative spliced forms of catfish *dmrt1* amplified from adult testis by RT-PCR using ORF flanking primers.

vertebrate *Dmrt1* proteins (Fig. 1B) showed the existence of two main clades. The first clade represents mammalian *Dmrt1* sequences while the second one is for teleost *Dmrt1* sequences. Interestingly, we obtained multiple *dmrt1* transcripts (Fig. 1C) when we attempted to amplify the ORF region of *dmrt1* using specific primers designed at ORF flanking region of full-length *dmrt1a* cDNA. Sequence information of these multiple bands revealed that these products represented *dmrt1* transcripts generated by multiple alternative splicing. Two alternative spliced forms *dmrt1b* and *dmrt1c* were obtained in catfish along with *dmrt1a*, which encodes different size predicted proteins with 253, 233 and 287 amino acids respectively (Fig. 2). The nucleotide sequence data of multiple *dmrt1*s have been submitted to the GenBank and the accession numbers are *dmrt1a* (FJ596554), *dmrt1b* (FJ596555) and *dmrt1c* (FJ596556). The entire alternative splicing events of the *dmrt1* in catfish occurred within the ORF towards the 5' end region. Interestingly, the *dmrt1c* isoform is lacking most of the DM-domain region at 5' end. Cloning of *dmrt1a* and *dmrt1c* ORFs from a closely related species, *C. batrachus* confirmed our findings in *C. gariepinus*. The GenBank accession numbers of *C. batrachus dmrt1*s are FJ596557 and FJ596558.

Tissue distribution and expression pattern of *dmrt1* in adult and developing gonads

Tissue distribution pattern of *dmrt1* revealed exclusive expression in testis (Fig. 3A). Semi-quantitative RT-PCR analysis showed that *dmrt1* spliced forms a, b and c were detectable only in adult testis (Fig. 3B). Further the expression of *dmrt1a* was higher than those of *dmrt1b*

and *dmrt1c*. Stage-dependent increase in *dmrt1a* expression was observed in testis at different age groups of catfish (50, 100 and 150 dah). Expression of *dmrt1b* and *dmrt1c* were also evident in developing male gonads. By contrast, *dmrt1a* could not be detected in developing female gonads (Fig. 3C).

Dmrt1 expression in different phases of testicular cycle

Real-time qRT-PCR analysis showed that *dmrt1a* and *dmrt1b* expression was high during pre-spawning when compared with preparatory, spawning and post-spawning phases. A similar kind of expression pattern was true for *dmrt1c* which lacks the majority of the DM-domain (Fig. 4). This indicates that *dmrt1* transcripts are abundantly expressed during active spermatogenesis i.e. in preparatory and pre-spawning phases where spermatogonia and spermatocytes are more in number (Swapna *et al.* 2006). In addition, *dmrt1a* transcript is abundantly expressed in testis when compared with *dmrt1b* and *dmrt1c* transcripts. Studies from semi-quantitative RT-PCR pilot experiments revealed similar patterns of expression for multiple *dmrt1*s (densitometric quantification data not shown). Representative gel image was shown in the inlet of Fig. 4.

Immunocytochemical and immunofluorescence detection of *dmrt1* in testis

The polyclonal catfish *dmrt1* antibody raised in rabbit revealed an expected band of ~ 31 kDa by western blotting in testis but not in other tissues. This band

Figure 2 Amino acid sequence alignment of multiple *dmrt1*s from *Clarias gariepinus* and *C. batrachus* using EBI clustalW software. GenBank accession numbers (*C. gariepinus*: *dmrt1a* (FJ596554), *dmrt1b* (FJ596555), *dmrt1c* (FJ596556) and *C. batrachus*: *dmrt1a* (FJ596557), *dmrt1c* (FJ596558)). DM-domain is underlined. Box indicates loss of DM-domain due to alternative splicing in *dmrt1c*. Arrows showing splicing sites of the *dmrt1* intron–exon junctions.

Figure 3 (A) RT-PCR analysis in different tissues of adult catfish. (B) Semi-quantitative RT-PCR analysis ($n=3$) of multiple *dmrt1s* in adult testis and ovary. (C) Semi-quantitative RT-PCR ($n=3$) amplification of multiple *dmrt1s* in testis and ovary at different age groups of juvenile catfish (50, 100 and 150 days after hatching). β -actin was used as an internal endogenous control.

corresponds to the *dmrt1a* form which encodes a 31 kDa putative protein (Fig. 5). However, we could not detect the other two variants of *dmrt1* by western blot which may be due to their low expression levels as mentioned in the real-time PCR results. Absence of immunoreactivity using pre-absorbed antiserum confirms that the primary antibody of *dmrt1* is specific for catfish (Fig. 6A). Our immunocytochemical data revealed that *dmrt1* expression was observed in the surrounding regions of the testicular lumens filled with spermatozoa (Fig. 6B). At higher magnification, *dmrt1* immunoreactivity was detected in primary spermatogonia (SG1), secondary spermatogonia (SG2) and spermatocytes (SC), while spermatids/sperm (SP) did not show any immunoreactivity in both mature testis (Fig. 6C and D) and juvenile testis at 200 dah (Fig. 6E). We also carried out immunofluorescence studies, where *dmrt1* specific immunofluorescence signal (green) was detected in spermatogenic cells surrounding the lumens of adult testis except SP (Fig. 6F and G). But no *dmrt1* signal was detected in the pre-vitellogenic oocytes (PO) of ovary (Fig. 6H). Red fluorescence is due to PI indicating SP.

Histological observation of gonadal development in catfish and gonads of MT-treated fishes

Conventional histological methods were used to study the onset of gonadal sex differentiation in catfish.

Figure 4 Real-time quantitative RT-PCR analysis of multiple *dmrt1s* during different phases of testicular cycle in catfish. The relative expression of multiple *dmrt1s* to β -actin in gonads was analyzed by the Applied Biosystems software. Data from real-time PCR were expressed as mean $\pm$ S.E.M. ($n=3$). Common letters/numbers indicate means that are not significantly different. Means with different letters/numbers differ significantly ($P<0.05$). Significance between groups was tested by ANOVA followed by Student's-Newman-Keuls' test using Sigma stat 3.5 software. Inset of Fig. 4: Representative gel image showing semi-quantitative RT-PCR expression of multiple *dmrt1s* during different phases of testicular cycle (i. preparatory, ii. pre-spawning, iii. spawning and iv. post-spawning phases; $n=3$).

Figure 5 Western blot analysis (top panel) of *Dmrt1* protein expression in various tissues, such as heart (H), muscle (Mu), liver (L), kidney (K), spleen (S), brain (B), ovary (O) and testis (T) which revealed an ~ 31 kDa *Dmrt1* protein band in testis, which is consistent with the size calculated from the sequence (31 224 kDa) using Lasergene software.

A primitive gonad with primordial germ cells surrounded by supporting cells was observed in the abdomen region near coelomic cavity at 30 dah (Fig. 7A). Histological observations of gonadal

development in catfish revealed that ovarian differentiation precedes testicular differentiation. Female gonad at 50 dah (Fig. 7B) showed signs of development of ovarian cavity (OC) and few growing oocytes (Goc) already. The ovarian differentiation is completed at 75 dah showing PO (Fig. 7C) while testicular differentiation in catfish started around 50–75 dah wherein developing germ cells were observed (Fig. 7D and E). Differentiated gonads can be isolated from 75-day-old fingerlings and the proliferation of spermatogonia (SG) and oogonia (OG) can be observed at this age. The testicular development was clearly evident in 100 dah gonadal sections showing developing SG1, and SG2 (Fig. 7F). In male gonad at 200 dah, the lumens are filled with few SP indicating the progress of spermatogenesis (Fig. 7G). Female gonad at 150 dah showed PO (Fig. 7H). These observations indicated that morphological gonadal sex differentiation occurs around 40–50 dah as determined by the formation of an OC and the proliferation of OG. These events began earlier than the proliferation of SG.

Figure 6 Immunocytochemical localization of *dmrt1* in adult and juvenile testis using anti-catfish *dmrt1* serum. (A) Adult testis section incubated with pre-absorbed antiserum, which was used as a negative control. (B) Adult testis section showing *dmrt1* immunoreactivity in boundaries of lumens filled with spermatids/sperm (SP); arrow heads show DAB staining. (C) and (D) Adult testis section at higher magnifications. (E) Juvenile catfish testis section at 200 dah. Arrows indicate the position of the cell types: primary spermatogonia (SG1), secondary spermatogonia (SG2), spermatocytes (SC), spermatid/sperm (SP), counter stained with hematoxylin. Immunofluorescence localization of *dmrt1* in adult testis using anti-catfish *dmrt1* serum. (F) and (G) Adult testis section: arrows indicate the position of the spermatogenic cells. (H) Ovary section: previtellogenic oocyte (PO), counter stained with propidium iodide. All scale bars represent 50 µm. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-09-0011>.

Figure 7 The development and differentiation of catfish gonads (hematoxylin-eosin staining). (A) 30 dah primitive gonad showing primordial germ cells (PGCs). Inset showing another area where two PGCs are surrounded by supporting cells. (B) and (C) 50 dah and 75 dah female gonads. (D) and (E) 50 dah and 75 dah male gonads. (F) and (G) 100 dah and 200 dah male gonads. (H) 150 dah female gonad. Arrows indicate the position of the cells: primordial germ cells (PGCs), primary spermatogonia (SG1), secondary spermatogonia (SG2), spermatocytes (SC), spermatid/sperm (SP), growing oocyte (Goc), pre-vitellogenic oocyte (PO) and ovarian cavity (OC); broken circles indicate group of SG2 in Fig. 7G. All scale bars represent 50 µm. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-09-0011>.

Histological examination of gonads of MT-treated fishes (80%) revealed development of mature males with testes. However, 20% of the fish population appeared as intersex/bisexual, which is evident by the presence of ova-testis and ovary (Fig. 8A). In the same group, few fish had testis and ovary. On further evaluation, the cross-section of ova-testis showed the presence of spermatogenic cells, sperm and oocytes (Fig. 8B). Histological section of the mature ovary showed full grown vitellogenic follicles as well as pre-vitellogenic follicles (Fig. 8C). Representative histological examination of testis section from 80% of the group of mature males revealed the presence of various types of spermatogenic cells (Fig. 8D).

Expression of *dmrt1*, *sox9a*, *foxl2* and *cyp19a1* in gonads of MT-treated fishes

Ova-testis gonad of MT-treated fish showed expression of all the genes tested, i.e. *dmrt1*, *sox9a*, *foxl2*, *cyp19a1* (Fig. 9) as it contained both ovarian follicles and spermatogenic cells. On the other hand, MT-treated fishes with mature testes showed the expression of *dmrt1* and *sox9a* only (Fig. 9). *Foxl2* and *cyp19a1* expression are restricted to mature ovary of

MT-treated fish, which possess pre-vitellogenic and mature vitellogenic follicles (Fig. 9). The quality of first strand cDNA templates obtained from MT-treated gonads was tested using β -actin (data not shown).

Figure 8 (A) Representative photograph of MT-treated fish showing morphology of ova-testis and ovary. Histology of MT-treated fish gonads (hematoxylin-eosin staining): (B) Ova-testis, (C) Mature ovary, (D) Mature testis. Arrows indicate the position of the cells: spermatogonia (SG), spermatocytes (SC), spermatid/sperm (SP) growing oocyte (Goc) and Vitellogenic follicle (VTF). All scale bars represent 50 µm. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-09-0011>.

Figure 9 Representative gel image showing expressions of *dmrt1a*, *sox9a*, *foxl2* and *cyp19a1* in ova-testis, mature ovary and mature testis of MT-treated fishes.

Discussion

This report depicts cloning of multiple forms of *dmrt1* in *C. gariepinus*. We observed the expression pattern of *dmrt1* in developing testis and ovary by semi-quantitative RT-PCR and also during different phases of testicular cycle by real-time qRT-PCR. Among multiple *dmrt1s*, *dmrt1a*, the dominant form, was obtained using RACE. It showed a high degree of homology with other vertebrate species at the amino acid level in the DM domain region which is conserved across different phyla (Raymond *et al.* 1998). In addition to *dmrt1a*, we report the isolation of two different alternative spliced forms, *dmrt1b* and *dmrt1c* from adult catfish testis using RT-PCR amplification with ORF flanking primers. All splicing events occurred within the ORF region at the 5' end. Interestingly, *dmrt1c* lacks a major part of the DM-domain region. Multiple alternative splicing in *dmrt1* gene was familiar in diverse species (Burtis & Baker 1989, Sreenivasulu *et al.* 2002, Guo *et al.* 2005, Cheng *et al.* 2006, Lu *et al.* 2007, Zhao *et al.* 2007). To our knowledge, 5' end spliced variants were shown for the first time in a lower vertebrate species in this study. Similar kinds of splicing events were also reported in the gonads of mouse, where four different forms of *Dmrt1* exist and one of the forms is lacking the entire DM-domain region (Lu *et al.* 2007). This shows that the multiple alternative splicing, which give rise to different isoforms, is a common feature of the *Dmrt1* gene in vertebrates. The functional significance of alternative splicing events is unclear at present. Alternative spliced isoforms may provide various targets for different upstream and downstream interacting factors in sexual differentiation. Few targets that are regulated by DM-factors have been identified, for example, yolk protein genes in *Drosophila* and *Caenorhabditis elegans*, which is regulated by DSX and MAB-3 respectively (Yi & Zarkower 1999, Yi *et al.* 2000). The alternative spliced forms of *dmrt1b* or *dmrt1c* may regulate the activity of *dmrt1a* which is dominant. Although the isoform *dmrt1c* is lacking most of the DM domain region, the presence

of other regions (SY domain) may prevent the DNA binding of dominant isoform especially *dmrt1a*, thus acting as a negative regulator. Further identification of new targets associated with *dmrt1* will provide more information on how these spliced forms operate and regulate each other.

Tissue distribution pattern indicated exclusive expression of *dmrt1a* in testis. This was also true when analyzed for Dmrt1 protein by western blot. Semi-quantitative RT-PCR analysis of multiple *dmrt1s* further confirmed sexual dimorphism. Histological studies from our laboratory revealed that the gonadal sex differentiation in catfish starts around 40–50 dah. Based on this, we analyzed multiple *dmrt1s* expression pattern in developing gonads between 50 and 150 dah. Multiple forms of *dmrt1* exist in developing testis but not in developing ovary of catfish. Male-specific expression of *Dmrt1* was noticed in some species like human (Moniot *et al.* 2000), chicken (Nanda *et al.* 1999), frog (Osawa *et al.* 2005), garden lizard (Sreenivasulu *et al.* 2002) and some fish species (Fernandino *et al.* 2006, Ijiri *et al.* 2008, Xia *et al.* 2007, Kobayashi *et al.* 2008). In our study, we observed male-specific expression of multiple *dmrt1s* in developing and adult catfish. Taken together, these results indicate an important role for *dmrt1s* in an early testicular development and recrudescence (see below). By contrast, reports from zebrafish (Guo *et al.* 2005) and rice field eel (Huang *et al.* 2005) showed the expression of *dmrt1* in both male and female gonads. Nevertheless, the role of *dmrt1* in ovarian differentiation has not been defined properly in vertebrates.

The fate of *dmrt1* after testicular differentiation i.e. in adult testis has not been studied in detail so far in any of the lower vertebrates. In the present study, we quantified multiple *dmrt1s* at different phases of testicular cycle of catfish. Our real-time PCR data showed that *dmrt1a* and *b* was expressed abundantly during preparatory and pre-spawning phases where spermatogonia and spermatocytes are dominant (Swapna *et al.* 2006), when compared with other phases of catfish testicular cycle. This indicates that in catfish, expression of *dmrt1* is higher during the period of spermatogenesis and its expression decreases gradually thereafter during spawning/spermiation and post-spawning phases. In rainbow trout (Marchand *et al.* 2000) and pejerrey (Fernandino *et al.* 2006), *dmrt1* expression was found to be high throughout spermatogenesis but decreased at spermiation. Seasonal changes in *dmrt1* expression may contribute to the maintenance of testicular cycle. Furthermore, immunocytochemical and immunofluorescence data revealed that *dmrt1* was localized in primary spermatogonia, secondary spermatogonia and spermatocytes, while spermatid/spermatozoa did not show any immunoreactivity. Similar expression profile of *dmrt1* was

observed in zebrafish (Guo *et al.* 2005), red-spotted grouper (Xia *et al.* 2007). Localization pattern and up-regulation of *dmrt1* expression during testicular recrudescence indicates plausible role in spermatogenesis but not in spermiation/spawning.

This study also aimed to find out whether treatment of MT induces testis-specific expression pattern of *dmrt1* as observed in genetic female sex population of the Nile tilapia (Kobayashi *et al.* 2008). Exogenous hormone treatments during the critical period of sex differentiation resulted in complete sex change in the Nile tilapia (Nagahama 2005). Treatment of MT (1000 ng/l) in zebrafish and long term exposure to EE2 (5 ng/l) in eel during gonadal development resulted in intersex fish (Orn *et al.* 2003, Nash *et al.* 2004). A previous report (Raghuveer *et al.* 2005) from our laboratory showed that exogenous MT treatments given to catfish fries skewed the catfish population completely towards male development during the course of gonadal differentiation. However, in the present study, we obtained both masculinized and intersex/bisexual fish. The apparent discrepancy may be due to large sample size and also follow-up until adult. The dosage of MT used in the present study might also contribute to this effect. Expression of both male (*dmrt1* and *sox9a*) and female (*foxl2* and *cyp19a1*) sex-specific genes was observed in ova-testis gonad of MT-treated fish. On the contrary, dimorphic expression pattern of male and female sex-specific genes were observed in completely developed testis and ovary. Such dimorphic expression pattern was also reported during gonadal sex differentiation and/or hormone-induced sex reversal (Alam *et al.* 2008, Kobayashi *et al.* 2008, Ijiri *et al.* 2008). By contrast, in rainbow trout oestrogen treatment up-regulates female specific genes but does not suppress all male specific genes during male-to-female gonadal trans-differentiation (Vizziano-Cantonnet *et al.* 2008). These results together warrant a role for *dmrt1* in testicular differentiation/spermatogenesis.

In conclusion, we identified multiple forms of *dmrt1* from adult catfish testis. Stage-dependent elevation of *dmrt1*s expression in juveniles authenticates a pivotal role for *dmrt1* in testicular differentiation. MT-treatment studies further endorse *dmrt1* as a testis-specific gene. *Dmrt1* could be localized in SG and SC but not in SP. High expression of *dmrt1* during testicular recrudescence indicates its importance in the entraining of testicular cycle.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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