Identification of multiple dmrt1s 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 dmrt1s, 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 dmrt1s (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 dmrt1s 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 dmrt1s 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, foxI2 and cyp19a1 expression patterns in MT-treated gonads revealed tissue-specific pattern. These results together suggest that multiple dmrt1s 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 methytestosterone (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 photothermal 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 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 ug/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′-GCTATCTGC-ACCTGGGCATCTGCTGGT-3′ and universal primer A mix (UPM): 5′-CTAATACGACTCACTATAGGGCAAG-CAGTGATATCAACGAGATG-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 1X 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'-AATCATTTCTGGCTATCTCTTACC-3' and nested universal primer (NUP): 5'-AAGCAGTGGATACGCGACAGGAGGT-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 DM1: 5'-ATGCGAAGTGCTCTCCC GGTCGAGG-3', and UPM using the same PCR conditions mentioned above. After 3' RACE, nested PCR was performed using 3' nested primer DM2: 5'-GTCCCGCCAGGTACGAAGGCT-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 real-time 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

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequence (5'–3')</th>
</tr>
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<tbody>
<tr>
<td>dmrt1orfF</td>
<td>GAGGCAGAGGCCGCAGCAGAG</td>
</tr>
<tr>
<td>dmrt1orfR</td>
<td>GTTTGATACCTCCTGTAGACTT</td>
</tr>
<tr>
<td>β-actinF</td>
<td>ACCGGAGTCATCAACAAATCAGT</td>
</tr>
<tr>
<td>β-actinR</td>
<td>GAGCTCGGTGTGTCCTGAG</td>
</tr>
<tr>
<td>Dmrt1abRTF</td>
<td>ATGCGCCTGACCTGCTCGGG</td>
</tr>
<tr>
<td>Dmrt1abRTR</td>
<td>GCCGCTCCAGGAGCGCAGGAGAGA</td>
</tr>
<tr>
<td>Dmrt1cRTF</td>
<td>CCGGGCCAGGTGCGGCTCCG</td>
</tr>
<tr>
<td>Dmrt1cRTR</td>
<td>GGCGCTCCAGGAGCGCAGGAGAGA</td>
</tr>
<tr>
<td>Dmrt1aF</td>
<td>ATCGCGAAGTCCTCGCGGTCG</td>
</tr>
<tr>
<td>Dmrt1aR</td>
<td>AGGGCGTCCAGGAGCGACG</td>
</tr>
<tr>
<td>Dmrt1bF</td>
<td>AAGGATGAGCAGCACCGGACAAA</td>
</tr>
<tr>
<td>Dmrt1bR</td>
<td>TTACTTACAGCTCCCTTAT</td>
</tr>
<tr>
<td>Dmrt1cF</td>
<td>CCGGGCCAGGTGCGGCTCGG</td>
</tr>
<tr>
<td>Dmrt1cR</td>
<td>TTACTTACAGCTCCCTTAT</td>
</tr>
<tr>
<td>Sox9aF</td>
<td>GGCAGAGTCGACGCAAACCCCGG</td>
</tr>
<tr>
<td>Sox9aR</td>
<td>GGTGAGAGCCGAGGCCGTCG</td>
</tr>
<tr>
<td>Fox2F</td>
<td>TGGCAGAAGCTTTTGAGAAGGG</td>
</tr>
<tr>
<td>Fox2R</td>
<td>TTCCAGATGAGCAGTGCTCAT</td>
</tr>
<tr>
<td>Cyp19a1F</td>
<td>TTGGATGCGGAAATTTGGAGACG</td>
</tr>
<tr>
<td>Cyp19a1R</td>
<td>AGCTTACGCGAAGTACTCGG</td>
</tr>
</tbody>
</table>
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 ΔCT value (ΔCT = dmrt1 CT – β-actin CT). Relative expression was then expressed according to the Default 2−ΔCT.

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, Bangaluru, 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 phenylmethylsulphonyl 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 microscopy 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 *dmrt1* sequence data that was previously obtained from *C. gariepinus* (Raghuveer 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...
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 dmrt1 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 dmrt1s 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 dmrt1s 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 spermatagonia 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 dmrt1s (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...
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.
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 spermato genesis (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 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.

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 magnification. (E) Juvenile catfish testis section at 200 dah. Arrows indicate the position of the cell types: primary spermatogonia (SG1), secondary spermatogonia (SG2), spermatocytes (SC), spermatids/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 spermatogonic 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.
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 7 The development and differentiation of catfish gonads (hematoxylin-eosin staining). (A) 30 dah primitive gonad showing primordial germ cells (PGCs). Inlet 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), interstitial cells (IC), oogonia (OG), 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.

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.
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 (Sreenivasalu 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, immuno-cytochemical and immunofluorescence data revealed that dmrt1 was localized in primary spermatogonia, secondary spermatogonia and spermatocytes, while spermatid/spermatoozoa 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 spermatiation/spawning.

This study also aimed to find out whether treatment of MT induces testis-specific expression pattern of dmrt1 as observed in generic 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 masculanized 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 dmrt1s 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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