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Expression profiling of the sex-related gene *Dmrt1* in adults of the Lusitanian toadfish *Halobatrachus didactylus* (Bloch and Schneider, 1801)

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ABSTRACT

Doublesex and mab-3 related transcription factor 1 (*Dmrt1*) gene is a widely conserved gene involved in sex determination and differentiation across phyla. To gain insights on *Dmrt1* implication for fish gonad cell differentiation and gametogenesis development, its mRNA was isolated from testis and ovary from the Lusitanian toadfish (*Halobatrachus didactylus*). The cDNA from *Dmrt1* was synthesized and cloned, whereas its quantitative and qualitative gene expression, as well as its protein immunolocalization, were analyzed. A main product of 1.38 kb, which encodes a protein of 295 aa, was reported, but other minority *Dmrt1* products were also identified by RACE-PCR. This gene is predominantly expressed in testis (about 20 times more than in other organs or tissues), specially in spermatogonia, spermatocytes and spermatids, as well as in somatic Sertoli cells, indicating that *Dmrt1* plays an important role in spermatogenesis. Although *Dmrt1* transcripts also seem to be involved in oogenesis development, and it cannot be excluded that toadfish *Dmrt1* could be functionally involved in other processes not related to sex.

1. Introduction

Lusitanian toadfish (*Halobatrachus didactylus*), is an eastern Atlantic marine teleost belonging to the family Batrachoididae. It is a sedentary, benthic species found in shallow waters along the coasts from the Bay of Biscay (Spain) to Ghana, as well as the western Mediterranean (Roux, 1986). This toadfish is a gonochoristic species with unknown sex-determination system, where heteromorphic sex chromosomes have not been found (Merlo et al., 2007; Palazón et al., 2003). Toadfish has a moderate commercial importance in southwestern Iberian countries; however, it has received special attention in multidisciplinary studies, as hematology, toxicology, feeding, ecology and even for experimental cardiological purposes (Aureliano et al., 2002; Borges et al., 2003; Campana et al., 2003; Coucelo et al., 2000; Desantis et al., 2007; Modesto and Canário, 2003a; Palazón-Fernández et al., 2011; Pendon et al., 1992; Sarasquete, 1983; Sarasquete et al., 1982; Soares et al., 2003, 2008; Vasconcelos and Ladich, 2008; Vasconcelos et al., 2007, 2011a,b). Different physiological aspects have been studied: reproductive biology (Palazón-Fernández et al., 2001); histological, histochemical and biochemical serum, hepatic and gonad compounds and reserve macromolecules (lipids, glucid cations, proteins, enzymes, etc.), during several reproductive cycles of toadfish (González de Canales et al., 1992; Muñoz-Cueto et al., 1996; Rosety et al., 1992); as well as morphometric changes and sex steroid levels during the annual reproductive cycle (Modesto and Canário, 2003b). More recently, interesting information has been reported about cytogenetic and molecular topics in Lusitanian toadfish and other Batrachoid fish species (Merlo et al., 2007, 2012; Úbeda-Manzanaro et al., 2010a,b). However, and according to our present knowledge, there is no available information about expression of sex-related genes in Batrachoididae fish species, which is considered one of the most highly evolved groups of marine teleosts (Modesto and Canário, 2003b). Different genetic, neurohormonal, physiological behavioral and environmental factors (e.g. temperature), among others, can strongly influence the course of sex determination and sex differentiation, as well as the gonad development in fish species (Devlin and Nagahama, 2002).

Doublesex and Mab-3 (DM) related transcription factor 1 (*Dmrt1*) belongs to a gene family characterized for possessing a highly conservative zinc-finger DNA-binding motif (DM domain), and *Dmrt1* is considered the first preserved gene in the sex-determination/sex-differentiation cascade between phyla (Erdman and Burtis, 1993; Marchand et al., 2000; Raymond et al., 1998). *Dmrt1* is known to play an important role in early testicular differentiation in metazoan, although it can work as an upstream or downstream regulator in the

Abbreviations: APA, alternative polyadenylation; bp, base pairs; cDNA, DNA complementary to RNA; DEPC, diethyl dicarbonate; IHC, immunohistochemistry; ISH, in situ hybridization; mRNA, messenger RNA; ORF, open reading frame; PBST, phosphate buffered saline with Tween 20; ppm, parts per million; qPCR, quantitative polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

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sex cascade depending on the species (Hodgkin, 2002; Zhu et al., 2000). Besides, multiple potential target genes for *Dmrt1* have been recently identified in mouse testis (Murphy et al., 2010), although its putative downstream targets are largely unknown for most species. *Dmrt1* genes (or *Dmrt1* paralogs) have risen to the top of the sex-determination hierarchy, in at least three distantly related clades: the *Dmrt1* paralog named *Dmrt1by* or *DMY* in some fish species of the genus *Oryzias* (Matsuda et al., 2002, 2003; Nanda et al., 2002); the paralog *DM-W* gene in some amphibian species of the genus *Xenopus* (Yoshimoto et al., 2008); and the Z-chromosome-linked gene *Dmrt1* in avian species (Chue and Smith, 2011; Smith et al., 2009). However, *Dmrt1*-related sex-determining systems reported in *Oryzias latipes* and *Oryzias curvinotus* (Matsuda et al., 2002, 2003; Nanda et al., 2002), and in *Xenopus laevis* (Yoshimoto et al., 2008), are an exception and appear to be a recent evolution event, considering that closely related species do not have in common these systems (Bewick et al., 2011; Kondo et al., 2003, 2004).

Interestingly, *Dmrt1* may play a key role as a downstream regulator in testis differentiation, development and spermatogenesis, as it was reflected by the male-biased expression of *Dmrt1* in all vertebrate groups, including fish species (Amberg et al., 2009; Kettlewell et al., 2000; Liu et al., 2010; Marchand et al., 2000; Nanda et al., 2002; Smith et al., 1999; Veith et al., 2003). The knowledge about regulatory factors involved in the reproductive sexual dimorphism, and the possible participation in development of different germ type cells of both male and female gametogenesis, is of great interest. In this paper, a first molecular and cellular approach to the *Dmrt1* gene is reported. For this purpose, we have isolated and cloned its mRNA and cDNA for quantitative and qualitative gene expression. We also have analyzed in parallel the protein immunolocalization, using a specific toadfish Dmrt1 antibody, on histological sections of testis, ovary, and several other somatic organs and tissues from adult toadfish specimens.

2. Materials and methods

2.1. Biological samples

Specimens from toadfish (n = 10) were collected from natural populations in the Bay of Cadiz (SW Spain). Fish were deeply anesthetized with 1500 ppm phenoxyethanol (Sigma, Spain) and slaughtered by decapitation according to REGA-ES110280000311 animal welfare procedures (ICMAN.CSIC). Immediately after dissection, the different organs and tissues (ovary, testis, heart, brain, muscle, liver, gill, intestine, swim bladder, spleen and kidney) were frozen in liquid nitrogen and stored at −80 °C until used.

For in situ hybridization (ISH) gonad tissues were fixed with 4% paraformaldehyde in DEPC treated phosphate-buffered saline-Tween 20 (PBST) solution overnight at 4 °C and stored in methanol at −20 °C after washing three times for 1 h with PBST. These samples were also used for immunohistochemistry (IHC). After paraffin embedding procedure, serial sections were stained with hematoxylin–eosin and hematoxylin–VOF (Sarasquete and Gutiérrez, 2005) for histomorphological tissue and cell characterizations in both gonads.

2.2. Nucleic acid extraction, cloning and phylogenetic analysis of Dmrt1

Tissue samples (100 mg) were homogenized in TriReagent (Sigma) using an ULTRA-TURRAX homogenizer (Ika), and total RNA was isolated according to the manufacturer's protocol. Contaminating genomic DNA was removed using DNase I (Fermentas).

The integrity of the total RNA was verified by 1% agarose/formaldehyde gel electrophoresis, and the quality and concentration of the RNA were assessed by UV spectrophotometry (A260nm/A280nm ratios > 1.7).

The full-length complementary DNA sequence from *Dmrt1* was obtained from gonadal RNA by 5′ and 3′ rapid amplification of cDNA ends (RACE) using the SMARTer RACE cDNA Amplification Kit (Clontech). Firstly, RACE-Ready cDNA was obtained according to the manufacturer's instructions. This cDNA was used to perform a PCR with degenerate primers, NDM1F and NDM1R (Table 1), designed from sequence alignment of teleost orthologues using ClustalW (http://www.genome.jp/tools/clustalw/) and localized at the conserved DM domain. PCR amplification was carried out in a Doppio thermocycler (VWR) using a total volume of 50 μL containing 2 μL of 5′-RACE-Ready cDNA, 5 μL 10× buffer, 3 mM $MgCl_2$ 300 pM of dNTP mix, 200 pM of each primer, and 2 U of Taq polymerase (Roche). Thermocycler conditions were 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 62 °C, and 1 min at 72 °C, and a final 10 min extension at 72 °C. The PCR product was purified and inserted into pGEM-T Easy Vector System (Promega). The positive clones were sequenced at the Sequencing Service from Biomedal (Spain).

Gene specific primers DMF1 and DMF2 were designed for nested 3′-RACE (see Table 1) using the obtained *Dmrt1* partial sequence. The amplification procedures were performed according to the manufacturer's instructions with modifications in the thermal cycling. Once the 3′-RACE was obtained, cloned and sequenced as above, gene specific primers DMR444 and DMRTRvN were designed for performing nested 5′-RACE using the new obtained sequence at the 3′ end. Different products were obtained, cloned and sequenced as before. The different obtained sequences were assembled in a single one using BioEdit 7.0.9.0 (Hall, 1999).

The putative amino acid sequence of the main Dmrt1 protein product was deduced using a translate tool (http://web.expasy.org/translate/). Multi-alignment of selected Dmrt1 proteins was performed with ClustalW algorithm and the molecular phylogenetic analysis was conducted using the neighbor joining method (Saitou and Nei, 1987) with MEGA 5.1 software (Tamura et al., 2011). The analysis was performed on vertebrates and included proteins belonging to other Dmrt sub-family present in fish, including the DMY paralogs from some species of the genus *Oryzias* and the fish-specific Dmrt2b paralogs. Bootstrap resampling (Felsenstein, 1985) was applied to assess support for individual nodes using 10,000 replicates, and the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965), uniform rates among sites and complete deletion option treatment of gaps and missing data. The branches with very low bootstrap confidence values were collapsed.

Table 1
Primers used for the sequencing and quantification of *Dmrt1* mRNA levels in *Halobatrachus didactylus*.

Primer name	Sequence (5′ → 3′)	Technique	R^2	E[a]
NDM1F	CCKCTGAAGGGMCACAARCGCTTC	Obtaining DM domain		
NDM1R	GCVACCTGVGCMGCCATGACYCKC	Obtaining DM domain		
DMF1	GGGACACAAACGCTTCTGCAACTGGAGG	3′-RACE		
DMF2	CTGCAACTGGAGGGACTGTCAGTGTCCA	3′ nested RACE		
DMR444	AGACATGCTGTGGGTAGACAGGCGACC	5′-RACE		
DMRTRvN	GCACTTTGGACACTGACAGTCCCTC	5′ nested RACE		
OLIGODMF	GAGTCACAGGGAGTCGGTCA	qPCR & ISH	0.9976	0.9993
DMREVQ	CGGGTTTCCATAGTAGGTGGAGT	qPCR & ISH		
EFF	CCGGTATCTCCAAGAACGGAC	qPCR	0.9956	1.0269
EFR	GCTCACCTCCTTGTTGATCTCA	qPCR		

K = G/T; M = A/C; R = A/G; Y = C/T; V = A/C/G.
[a] 100% Efficiency is E = 1.