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Short Communication

Isolation and sequencing of doublesex/male abnormal 3 (DM) related transcription factor (*Dmrt*) genes from the Asian toad *Bufo gargarizans* (Cantor, 1842)

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Abstract

The doublesex/male abnormal 3 (dsx/mab-3 or DM) domain gene family involved in sexual development encodes putative transcription factors including a DNA-binding homology motif, the DM domain. We used highly degenerate primers to clone and sequence seven distinct DM related transcription factor (*Dmrt*) genes from the Asian toad (*Bufo gargarizans* Cantor, 1842). A database search for the cloned sequences revealed the following percentage identity with the homologous *Dmrt* genes of the human: *BgDmrt1* = 97%, *BgDmrt2* = 97%, three isoforms of *BgDmrt*3 (*BgDmrt3a* = 93%, *BgDmrt3b* = 95%, *BgDmrt3c* = 100%) and two isoforms of *BgDmrt5* (*BgDmrt5* = 97%, *BgDmrt5* = 91%). Based on DM domain amino acid sequence similarities we constructed a phylogenetic tree which grouped vertebrate and invertebrate *Dmrt* genes into seven distinct subfamilies. The DM domains of both human and the newly-discovered *Bufo gargarizans* genes contained two conserved zinc-chelating sites (CCHC and HCCC), except *BgDmrt3b*, which contained the CCRC and HCCC sites.

Key words: Bufo gargarizans, *Dmrt* genes, DM domain, SSCP.

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The DM domain gene family has some members with a conserved DNA-binding DM domain encoding putative transcription factors related to the sexual regulators Doublesex (DSX) from *Drosophila melanogaster* and Male abnormal 3 (MAB-3) from *Caenorhabditis elegans* (Erdman and Burtis, 1993; Raymond *et al.*, 1998). The DM domain has a highly intertwined structure that chelates two zinc atoms, and makes specific DNA contacts predominantly in the minor groove (Zhu *et al.*, 2000). Doublesex and MAB-3 related transcription factor (*Dmrt*) genes have recently been cloned from a wide range of vertebrates, including fish, amphibians, reptiles, birds and mammals (Mastuda *et al.*, 2002; Smith *et al.*, 2002; Kettlewell *et al.*, 2000; Shibata *et al.*, 2002). These genes have been found to play essential roles in a variety of sex developmental processes, and at least some of these functions have been conserved. For example, *Dmrt1* gene knock-out mice have severe defects in testis differentiation and *DMY* mutant medaka fish (*Oryzias latipes*) show sex reversal during which XY fish carrying a mutant DM-domain gene on the Y chromosome (the *DMY* gene) develop not as males but as females (Raymond *et al.*, 2000; Mastuda *et al.*, 2002). The frog (*Rana rugosa*) *Dmrt1* gene is expressed in the differentiating testis but is not detectable during ovarian differentiation (Shibata *et al.*, 2002). The zebrafish (*Danio rerio*) *Terra/Dmrt2* gene is an early left-sided expressed gene that links left-right patterning with bilateral synchronization of the segmentation clock (Saude *et al.*, 2005). The *Dmrt3* gene is primarily expressed in the forebrain, neural tube and nasal placode of both mice and chickens (Smith *et al.*, 2002), while the expression of *Dmrt5* in adult zebrafish is restricted to brain cells and developing germ cells (Guo *et al.*, 2004).

The widely distributed Asian toad (*Bufo gargarizans* Cantor, 1842), also called the Chusan Island toad, plays an important ecological role in the environments where it lives. Although Shang (1983) showed that *Bufo gargarizans* has a ZZ/ZW type chromosomal sex determination system, the molecular mechanisms of sex determination and differentiation remain unclear for this species. As a prelude to understanding the involvement of *Dmrt* genes in the sexual development of this toad, we cloned the DM domain gene family of *B. gargarizans* using degenerate primers from genomic DNA. In the present paper we report the cloning and nucleotide sequencing of seven different *Dmrt* genes from *B. gargarizans*. The phylogenetic relationships between the DM domain genes of verte-

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brates and invertebrates were also examined based on DM domain amino acid sequences.

We obtained three female and two male *B. gargarizans* from Wuhu city (Anhui province, China) and humanly sacrificed them. Total genomic DNA was isolated from muscle tissues by routine protocols (Sambrook *et al.*, 1989). To amplify the conserved DM domains we used a pair of degenerate primers D1 (5'-TGCG(AGC)(AC)G(AG)TGC(AC)G(AG)AA(CT)CACGG-3') and D2 (5'-C(GT)(GC)AG(GC)GC(GC)ACCTG(GC)GC(AGCT)GCCAT-3') (Ren *et al.*, 2001), the expected amplification fragment length being 140 bp with these primers. The polymerase chain reaction (PCR) was carried out in a 25 μL reaction mixture containing 50 ng of genomic DNA, 1.5 mM Mg^{2+}, 200 μM of each dNTP, 0.2 μM of each primer and 1 unit of Taq DNA polymerase (Promega, Peking, China). The cycling conditions were as follows: 32 cycles of 30 s at 94 °C, 40 s at 62 °C and 40 s at 72 °C , followed by 7 min elongation at 72 °C. The PCR products were cloned using the pMD 18-T Vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* strain DH5α (Sangon, Shanghai, China) and 115 white clones were transferred to a plate of clones from an initial culture plate of lysogeny broth (LB) media containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactoside (IPTG) and 75 positive clones with insert PCR fragment were confirmed using colony PCR (Shen *et al.*, 2000). The distinctive positive clones were screened using single-strand conformation polymorphism (SSCP) (Nie *et al.*, 1999) and sequenced using the universal sequencing primer on an ABI377 auto-sequencer (Applied Biosystems). All DNA sequences were analyzed using the basic local alignment search tool (BLAST) and CLUSTAL X1.8 programs and the phylogenetic tree was constructed using the molecular evolutionary genetic analysis (MEGA) program V2.1 (Kumra *et al.*, 2001).

Using the *B. gargarizans* genomic DNA as template we obtained a 140 bp PCR fragment and we obtained seven distinct positive clones from the DNA of the male and female toads, although there was no difference between the clones in respect to the sex of the toads from which the clones originated (Figure 1).

Database searches and phylogenetic analysis of the clones uncovered seven unreported *Dmrt* sequences, representing distinct genes, which we named by adding the prefix *Bg* (*Bufo gargarizans*) and submitted to GenBank as *Bufo gargarizans* doublesex and mab -3 related transcription factor (*BgDmrt*) genes. The genes and their GenBank accession numbers and sequence identity to homologous human *DMRT* genes (in parentheses) are: *BgDmrt1* (DQ217562, 97%), *BgDmrt2* (DQ217563, 97%), *BgDmrt3a* (DQ217564, 93%), *BgDmrt3b* (DQ217565, 95%), *BgDmrt3c* (DQ217566, 100%), *BgDmrt5a* (DQ217569, 97%) and *BgDmrt5b* (DQ217571, 91%). Note that *BgDmrt3* has three isoforms and *Dmrt5* has two isoforms. The putative amino acid sequences of the *BgDmrt* genes and the alignment between corresponding human *DMRT* sequences are shown in Figure 2. The DM domains of both human and the newly-discovered *B. gargarizans* genes contained two conserved zinc-chelating sites (CCHC and HCCC), except *BgDmrt3b*, which contained the CCRC and HCCC sites. It is probable that the DM domain genes act as a transcription regulator in sex determination and other developmental processes, mediated by the DNA-binding zinc motif (Zhu *et al.*, 2000).

The putative amino acid sequences of the seven clones were compared with 34 published *Dmrt* gene sequences accessible at the NCBI BLAST server (Table 1), the DM-domain neighbor-joining phylogenetic tree being shown in

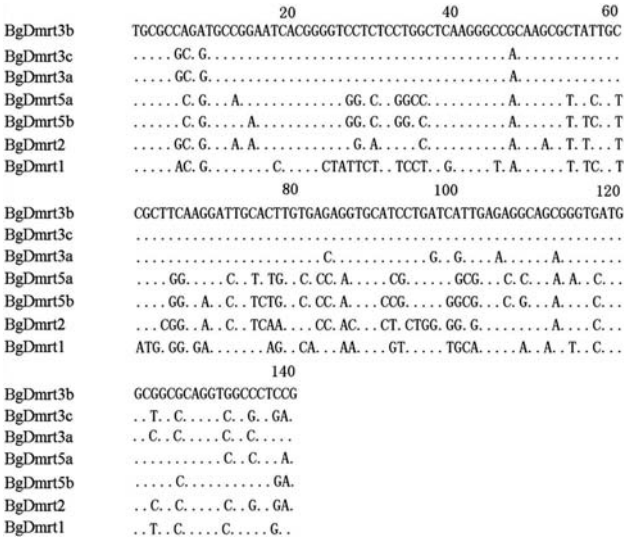

Figure 1 - The nucleotide sequences of seven doublesex/male abnormal 3 (dsx/mab-3 or DM) related transcription factor (*DMRT*) gene DM domains from *Bufo gargarizans* (*Bg*). Dots indicate identities with *BgDmrt3b*.

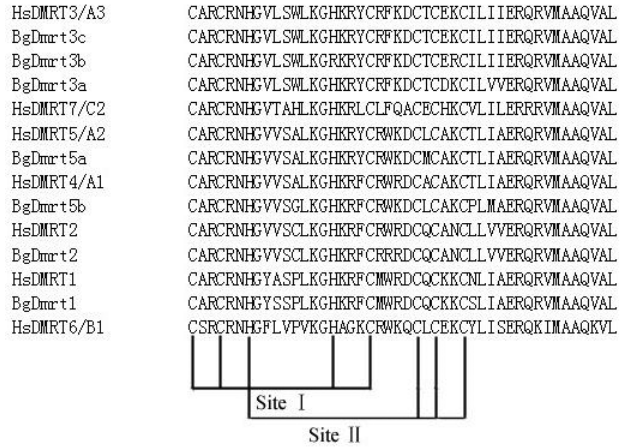

Figure 2 - Multiple alignment of the amino acid sequences of the doublesex/male abnormal 3 (dsx/mab-3 or DM) domains from toad (*Bufo gargarizans* or Bg) and human (*Homo sapiens* or Hs) DM related transcription factors (DMRTs). Two DM domain Zn-chelating sites, SiteI (CCHC) and siteII (HCCC), are also shown.

Table 1 - Doublesex/male abnormal 3 (dsx/mab-3 or DM) related transcription factor (DMRT) domain sequences included in this analysis.

DM domain sequence (synonym) and species*	GenBank number	DM domain sequence (synonym) and species*	GenBank number
Human		**Animal** (continued)	
DMRT1	NM-021951	Dmrt4	
DMRT2	AF130729	*M. musculus*	AF542047
DMRT3 (DMRTA3)	NM-021240	*E. brenchleyi*	AAY26899
DMRT4 (DMRTA1)	AJ290954	(Dmo) *O. niloticus*	AAF79932
DMRT5(DMRTA2)	AJ301580		
DMRT6 (DMRTB1)	AJ291671	Dmrt5	
DMRT7 (DMRTC2)	AJ291669	*M. musculus*	AY145837
		E. brenchleyi	AAY26900
Animal		*O. latipes*	BAD00703
Dmrt1		Dmrt6	
Mus musculus	NM-015826	*M. musculus*	AF542048
Gallus gallus	AF211349		
Oncorhynchus mykiss	AAG17544		
Eremias brenchleyi	AAY26895	Dmrt7	
Takifugu rubripes	CAC42778	*M. musculus*	AF542046
(DMY)*Oryzias latipes*	AB071534		
		F10C1.5	
Dmrt2		*Caenorhabditis elegans*	AAA93409
M. musculus	AF539811		
Oreochromis niloticus	AA074518	Dmrt99B	
Chrysemys scripta elegans	AAG15567	*Drosophila melanogaster*	NP_524549
T. rubripes	CAC42780		
(Terra) *Danio rerio*	AF080622	Dmrt11E	
		D. melanogaster	NM_078591
Dmrt3			
M. musculus	AAN77230	Mab-3	
G. gallus	XP427822	*C. elegans*	†
T. rubripes	AJ295039		
O. latipes	AAL02164		

**M. musculus* = mouse; *G. gallus* = chicken; *O. mykiss* = trout; *E. brenchleyi* = a wall lizard; *T. rubripes* = Japanese pufferfish; *O. latipes* = medaka fish; *O. niloticus* = tilapia; *C. s. elegans* = red-eared turtle; *D. rerio* = zebrafish; *C. elegans* = nematode; *D. melanogaster* = fruit fly.
†Raymond *et al*., 1998.

Figure 3. Phylogenetic analysis showed that all these *Dmrt* genes were clustered into seven different subfamilies (*Dmrt1-7*), and confirmed the identity of the *BgDmrt* genes identified by us. However, the *BgDmrt5b* was not included in the *Dmrt5* group, implying more accumulation of amino acid substitutions in *BgDmrt5b* than in other members of the *Dmrt5* cluster. We also compared four different invertebrate DM domain proteins with other vertebrate DM domain proteins using Mab-3 from the nematode *Caenorhabditis elegans* as the outgroup and found that Dmrt2 was related to Dmrt11E from the fly *Drosophila melanogaster*, Dmrt4 was related to *Drosophila melanogaster* Dmrt99B and Dmrt5 was related to *Caenorhabditis elegans* F10C1.5, suggesting that both the *Dmrt2* and *Dmrt4/Dmrt5* groups are ancient because they both contain invertebrate sequences. Ottolenghi *et al.* (2002) used structural analysis to show that the human genes *DMRTA1*, *DMRTA2* and *DMRTA3*, also called *DMRT4, DMRT5* and *DMRT3* respectively (the alternative nomenclature for human *DMRT* genes follows Volff *et al.* (2003), likewise for *DMRTB1* and *DMRTC2*, which was called *DMRT6* and *Dmrt7* respectively), all have a DM domain and a DMRTA domain, phylogenetic analysis showing a relationship between *DMRTA1* and *DMRTA2* while *DMRTA3* was more distant. Our neighbor-joining analysis supported a relationship between vertebrate *Dmrt4* and

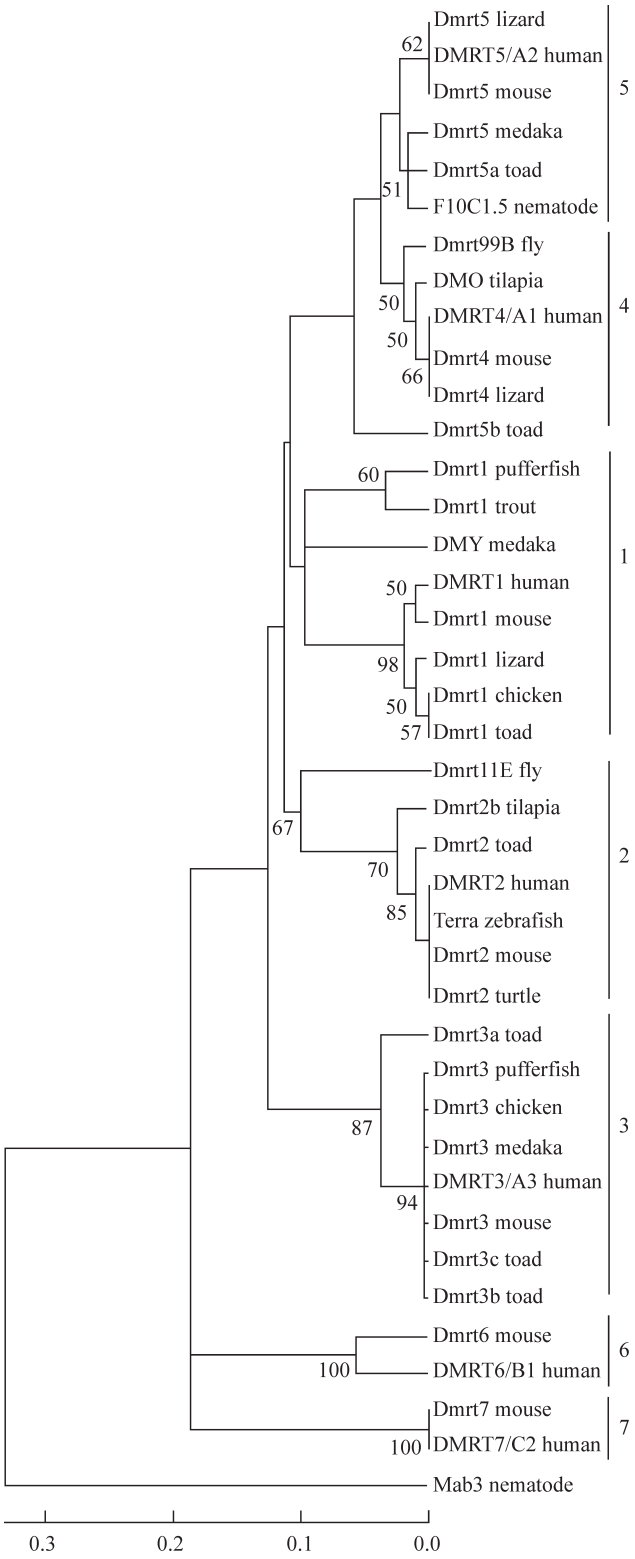

Figure 3 - Neighbor-joining phylogenetic tree (1000 replicates) of some vertebrate and invertebrate doublesex/male abnormal 3 (dsx/mab-3 or DM) related transcription factor (*DMRT*) gene family members based on amino acid sequence data. Nematode Mab-3 sequences were the outgroup. Branches with less than 50% support have been collapsed. The toad DM domain sequences in the tree were the *Bufo gargarizans* sequences obtained during the research described in this paper.

Dmrt5 but, in contrast, no closer phylogenetic relationship could be detected between *Dmrt3* and *Dmrt4/Dmrt5*, although this does not preclude a close relationship. Volff *et al.* (2003) have pointed out that different *Dmrt* genes can be under very different evolutionary constraints. In our analysis the short branches in the *Dmrt3* group suggests very strong sequence conservation, while the fact that the branches in the *Dmrt1* group appear to be longer indicates much more relaxed evolutionary constraints for these sequences.

This is the first report describing *Dmrt* genes from *B. gargarizans*. Several of the clones obtained in this study indicate that *Dmrt* genes that are duplicated in *B. gargarizans* are present in mammals as single copies. For example, the human genome contains single copy of *DMRT3* (*DMRTA3*), whereas this gene is present in at least three copies in *B. gargarizans*. Intriguingly, the *BgDmrt3a* gene is divergent from other *Dmrt3* genes in the phylogenetic tree and the zinc-chelating site I of *BgDmrt3b* is cystine, cystine, arginine, cystine (CCRC) not cystine, cystine, histidine, cystine (CCHC). According to Prince and Pickett (2002), gene duplication is a mechanism by which new gene functions may be acquired. The very recent duplication of the *Dmrt1* has apparently led to the formation of the master male-determining *DMY* gene in the medaka fish, and similar scenarios might have generated new paralogs of other *Dmrt* genes in different taxa (Mastuda *et al.*, 2002; Volff *et al.*, 2003). The *Dmrt* gene duplication in *B. gargarizans* may have been the result of such a process, unlike the situation in teleost fish, which may have been the result of the ancient duplication of the whole genome. However, how these *Dmrt* genes function in the sexual development *B. gargarizans* still needs further experimental exploration.

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Internet Resources

BLAST at http://www.ncbi.nlm.nih.gov/BLAST/.

CLUSTAL X1.8 at http://www.igh.cnrs.fr/bin/clustalxguess.cgi.

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