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Identification and expression of a factor of the DM family in the oyster *Crassostrea gigas*

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ABSTRACT

The Pacific oyster *Crassostrea gigas* is a successive not systematic protandric hermaphrodite. Searching for an ortholog to *Dmrt1*, a conserved sex determinism factor, we have identified the first complete cDNA of a DM factor in Lophotrochozoa which we have called *Cg-DMl* (*Crassostrea gigas DMRT-like*). It is 359aa long, with the DM domain common to all the family factors, and one DMA domain specific to members such as *Dmrt4* and *Dmrt5*. Its gene presents one intron of 598 bp. Real time PCR and *in situ* hybridization have shown that *Cg-DMl* was expressed in both sexes, with a significantly higher expression in male than in female gonads at the end of the adult gametogenetic cycle and that a significant peak of expression was observed in spat between 1 and 2 months of age. These results suggest that *Cg-DMl* may be involved in the development of the gonad and may constitute preliminary clues for future work in order to better understand DM protein evolution.

1. Introduction

Sex determining systems are highly variable among phyla and involve genetic and/or environmental factors (Torres-Maldonado et al., 2002). Nevertheless, all these systems involve a molecular cascade of genes coding transcriptional or mRNA splicing factors which, ultimately, orientate the gonad differentiation into a testis or an ovary. One of the most conserved genes of this molecular pathway is *Dmrt1*, the vertebrate ortholog of Doublesex (*dsx*) and *Mab-3* which regulates the male differentiation in *D. melanogaster* (Erdman and Burtis, 1993) and *C. elegans* (Shen and Hodgkin, 1988) respectively. *Dmrt1* encodes a putative transcription factor with a conserved zinc finger-like DNA-binding motif, termed DM domain. The family of the DM factors includes at least eight vertebrate and eleven invertebrate members with different tissue-specific expression patterns according to species and/or DM gene member (Hong et al., 2007). In all vertebrates examined to date *Dmrt1* expression is restricted to the differentiating and to the adult male gonads as observed in mice (Raymond et al., 2000), chicken (Smith et al., 1999), amphibians (Shibata et al., 2002), rainbow trout (Nanda et al., 2002) and *Lepidochelys olivacea* turtle (Torres-Maldonado et al., 2002). Other genes of the DM domain gene family are also expressed in gonads besides in other tissues, such as *Dmrt3*, *Dmrt5*, *Dmrt7* and *Dmrt8.2* in the mouse embryo (Kim et al., 2003; Veith et al., 2006), *Dmrt3* in the testis of the medaka (Winkler et al., 2004), *Dmrt5* in the brain and in the germinal cells of the zebrafish (Guo et al., 2004) and *Dmrt4* in the ovary of the tilapia (Guan et al., 2000).

The Pacific oyster *Crassostrea gigas* is a marine bivalve mollusc. Its development consists of three main periods: a rapid embryonic one (during 7 h post-fertilization (hpf)), a larval period with 5 different stages (16 hpf–20 days post-fertilization (dpf)), and a spat period (from 30 dpf) after the fixation and metamorphosis of larvae (around 22 dpf) (Galtsoff, 1964), whose gonadic development is observed around 3 month old (personal observations). In the adult, the gonad is a diffuse organ made of numerous tubules separated by connective tissue, the whole constituting the gonadic area (Franco et al., 2008) and evolving according to an annual reproductive cycle subdivided into four main stages (Heude-Berthelin et al., 2001; Chavez-Villalba et al., 2003): stage 0 where gonads are restricted to dispersing small clusters of germ cells during the inactive stage; stage I corresponding to mitosis of spermatogonia or oogonia which are indiscernible at this stage as is the sex of the animal; stage II where all the stages of spermatogenesis and growing oocytes are visible in male and female gonadal tubules, respectively; and stage III corresponding to the mature reproductive stage. The oyster is a successive and irregular protandric hermaphrodite without differentiable sexual chromosomes and some studies of controlled mating have shown that the sex ratio would be paternally influenced (Guo et al., 1998). Although the oyster *vasa*-like gene, a determinant of the germline, has been identified (Fabioux et al., 2004a), the factors implied in the adult sex

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change and in sex determination during the first gonadic development remain unknown in *C. gigas*.

The objective of this work was therefore to identify a DM factor in order to have a potential marker of the gonadic differentiation in *C. gigas*. In this paper we report isolating and molecular characterization of a DM domain gene, *Crassostrea gigas DMRT-like* (*Cg-DMl*). We also examined the gene expression and discussed the potential role of this factor in *C. gigas*.

2. Materials and methods

2.1. Experimental animals

Animals were purchased from a local oyster nursery (SATMAR, Gatteville, France). The developmental stages were obtained by mating adults from two families, one with a sexual imbalance rate in favour of the males (14 and 32% female ratio in 2004 and 2005 respectively) and the other in favour of the females (57% female ratio in 2006). The adult oysters we used in 2005 belong to two families presenting sexual imbalance rates, with female ratios in 2004 and 2005 of 80 and 63% for one and 14 and 32% for the other, respectively.

2.2. Reverse transcription, cloning and sequencing

Reverse transcription was performed using oligo(dT)$_{15}$ (Promega, Charbonnières, France), 1 µg mRNA, and 200 units of M-MLV RT. cDNAs were used as a template for PCR amplification with two degenerated primers, DMRT1-s (5′-TGCCAAAGTGTGCCAGATGT(A/C)G(A/C/G/T)AA(C/T)CA(C/T)GG-3′) and DMRT1-a (5′-GGATTCCCAGCTCCTCCTC(C/T)TG(A/C/G/T)GC(C/T)TG-3′), designed in the conserved DM domain of *Dmrt1* and corresponding to the amino acid sequences PKCARCRNHG and QAQEEELGI respectively. PCR was performed with 10 ng of male gonadic area cDNA and cycled 35×(45 s/94 °C, 45 s/60 °C, 60 s/72 °C). The resulting 200 bp fragment isolated was used to generate full-length cDNA by 5′- and 3′-RACE-PCR with the GeneRacer™ Kit (Invitrogen, Eragny sur Oise, France) using adaptor and gene specific primers T2S (5′-CGGGTGGTGTCCGGCCTTGAAAGGT-3′), T2SN (5′-CGGCCTTGAAAGGTCA-CAAACGGTACTG-3′), T2A (5′-GCGCACACACAGTCCCTCCATCTACAG-3′), T2AN1 (5′-CCCTCCATCTACAGTACCGTTTGTGA-3′) and T2AN2 (5′-TTCAAGGCCGGACACCACCCGTGAT-3′). The search of different splicing forms of mRNA was performed using 5′- and 3′-RACE-PCR with COM5 (5′-CATGCGAGCATCCGATAGATACCCACGC-3′), DMS (5′-CCATG-GAGTGGTGTCCGC-3′) and 5DMASB (5′GTTGCCTTTCTGCTATCAGGG-3′) primers. For amplification of full-length cDNA and genomic sequences of *Cg-DMl*, sense primers SPL52 (5′-CCAACGAAATCGGCAAACACACA-3′) and HDMSB (5′-GGGAAGTATGGGTGGAAGAGG-3′) and anti-sense primer 3UTRas (5′-TGACATATAAGGTCAGTGAAATAACC-3′) were designed in the 5′ and 3′ cDNA ends. *Cg-DMl* cDNA and genomic DNA were cloned into PCRII-Topo vector using a TA cloning kit (Promega), and sequenced using ABI cycle sequencing chemistry (GENOME express, Meylan, France).

2.3. Screening of genomic library

A genomic library of *C. gigas* was created in λ-DASH$_{II}$ (Stratagene, Amsterdam, The Netherlands) following the manufacturer's instructions and 1.8×10^6 independent clones with an insert size between 15 and 40 kbp were recovered (Herpin et al., 2002). After amplification, a total of 50000 recombinant λ-DASH phages were plated on XL1 MRA *E. coli* strain (Stratagene), adsorbed to 5 nitrocellulose membranes, eluted and screened at different stringencies with a digoxigenin-11-dUTP labelled DM domain probe (base 152–584 of *Cg-DMl* cDNA) synthesized using a DIG DNA labelling kit (Roche Diagnostics, Meylan, France). Hybridizations were done during 16 h in 1% blocking reagent (Roche Diagnostics), 50% formamide, 750 mM NaCl, 5 mM EDTA, 0.1% Tween 20, 75 mM sodium citrate pH 7, 100 µg/ml Torula yeast RNA at 35, 45 or 50 °C. Then, washes were performed under different combinations of temperatures (35 to 65 °C) and salinities (0.5× to 10×SCC). Positive clones were purified as described in Sambrook and Russell (2001) and a Southern blotting was performed on DNA digested by EcoRI using a DM probe. DNA of these clones was also blotted and subjected to differential hybridization using either a DM domain probe found in all the factors of the family, an HDM probe (HDM: "Hors domaine DM", out of the DM domain; base 584–693 of *Cg-DMl* cDNA) specific to *Cg-DMl* or an intron probe (base 441–1029 of the intronic sequence of *Cg-DMl*).

2.4. Phylogenetic analysis

A range of invertebrate and vertebrate protein sequences encoding *DMRT* homologs were aligned using the CLUSTALW software (Thompson et al., 1994). Phylogenetic analyses were performed by the neighbour-joining method (Saitou and Nei, 1987) using *MEGA* version 4 (Tamura et al., 2007).

2.5. Real time quantitative PCR

Quantitative RT-PCR analysis was performed using the iCycler apparatus (BioRad, Marnes-La-Coquettes, France). Total RNA was isolated from adult tissues and from developmental stages using Tri-Reagent (Sigma, Saint Quentin Fallavier, France) following the manufacturer's instructions. After DNase I treatment, 1 µg of total RNA was reverse transcribed. The iQ™ SYBR Green supermix PCR kit (BioRad) was used for real time monitoring of 5 ng template cDNA amplification for 40 cycles (15 s/95 °C, 15 s/60 °C). Accurate amplification of the target amplicon was assessed by constructing a melting curve. Amplification of cDNA was performed using primers specific to the DM domain (DMS 5′-CCATGGAGTGGTGTCCGC-3′, DMASB 5′-GTTGCCTTTCTGCTATCAGGG-3′) or HDM domain (HDMSB 5′-GGGAAGTATGGGTGGAAGAGG-3′, HDMASB 5′-AAGGTTTGGCGCCATTTGA-3′). A parallel amplification of the oyster elongation factor 1α (*EF1α*, GenBank accession no. BQ426516) reference transcript was performed using qfElongI (5′-ACCACCCTGGTGAGATCAAG-3′) and qrElongI (5′-ACGACGATCGCATTTCTCTT-3′) primers. The relative mRNA levels were normalized to 100 *EF1α* transcripts using the following formula $N = 100\times2^{(Ct_{EF1\alpha} - Ct_{Cg\text{-}DMl})}$ (Lelong et al., 2007). Statistical analyses were performed using two tailed Mann–Whitney *U*-test.

2.6. In situ hybridization

One-cm slices of oysters were fixed in Davidson's fixative. Some sections were stained with a Prenant–Gabe trichrome according to a classical protocol (Gabe, 1968) to illustrate the structure of the organs. For *in situ* hybridization experiments, 5 µm-slices were treated with 5% proteinase K in TE buffer for 30 min at 37 °C. After a pre-hybridization for 2.5 h at room temperature (RT) and 30 min at 52 °C, hybridization was performed overnight at 52 °C with DIG-labelled sense (control) or anti-sense riboprobe (400 ng/mL) in hybridization buffer. Probes were synthesized using a DIG RNA labelling kit (Roche Diagnostics) and with the *Cg-DMl* cDNA as a template. After extensive washing (2×SSC at RT and 0.5×SSC at 65 °C) and blocking in 1.5% blocking reagent for 1 h, detection was performed by 2 h incubation with an alkaline phosphatase-conjugated anti-DIG antibody diluted 1:500 in blocking solution, followed by 4 h incubation with 2% NBT/BCIP solution in darkness at RT. All reagents were made following the manufacturer's instructions (Roche Diagnostics). Sections were examined and pictures were taken with a Nikon Eclipse 80i microscope coupled to a Nikon DXM1200-C camera (Nikon, Champigny-Sur-Marne, France).

3. Results

3.1. Cg-DMl cloning and sequence analysis

A complete *DMRT-like* sequence of 1535 bp (Fig. 1A) (GenBank accession no. EU046234) shows a 5′ untranslated region (UTR) of 108 bp, an open reading frame (ORF) of 1080 bp with the ATG codon at

position 109 bp and a TAA stop codon at position 1186 bp, and a 3′ untranslated region of 347 bp including a single poly(A) signal (AATAAA) at position 1490 bp, 16 bp upstream of the poly(A) tail. The full-length, continuity and sequence of this cDNA were confirmed by new RT-PCR and sequencings. The deduced amino acid sequence is 359aa long, rich in serine (22%) and proline (28%), and contains the DM domain consensus sequence (from aa 26 to 81) with conserved cysteines and histidines characteristic of the DMRT protein family (Fig. 1A and C). Amino acid alignment indicates that besides the common DM domain, there are a conserved DMA domain found in *Dmrt4* and *Dmrt5* but not in *Dmrt1* and a short conserved domain of 7aa (RSAFSPI) near the C-terminus (Fig. 1A and C; Fig. 3). New 3′RACE-PCR experiments from mRNA of 1.5 month old juvenile oysters allowed us to identify two shorter cDNA isoforms ending at 720 bp and 511 bp with poly(A) tails starting at 488 bp and 696 bp respectively but without any conventional poly(A) signal identified. The sequences of the two shorter cDNAs were identical between themselves and to the full-length cDNA (Fig. 1A). PCR amplification of genomic DNA revealed the presence of only one intron 588 bp long as for *Dmrt4* and *Dmrt5* (Fig. 1A, B and C), with a GT/AG donor/acceptor site located between the nucleotides 484 and 485 of the cDNA (Fig. 1B).

Screening of the genomic DNA library using the conserved DM domain probe allowed us to retrieve 1 to 2 positive clones per 50,000 phages, which is representative of the size of the oyster's genome, at the different conditions of stringency tested. Analysis of the 8 clones by Southern blotting after EcoRI digestion and hybridization with the DM domain probe revealed 3 types of profiles illustrated by the clones 2A1, 2B1 and 3P1 which show only one band at a size of about 2000, 3000 or 9500 bp (Fig. 2A). Dot analysis of these clones using either the conserved DM domain probe (Fig. 2B, Line 1), the specific HDM probe (Fig. 2B, Line 3) or the intron probe (Fig. 2B, Line 2) did not allow us to observe any differential hybridization (Fig. 2B) and, consequently, to identify other oyster's *Dmrt* orthologs. Controls with a DNA sample from the DIG DNA labelling kit (Roche Diagnostics) and with *Cg-DMl* DNA inserted in a PCRII-Topo vector gave positive hybridizations while the plasmid alone gave no labelling (Fig. 2B).

3.2. Phylogenetic analysis

Phylogenetic analysis was performed to investigate the relationship between *Cg-DMl* and members of the *Dmrt* family. A complete protein sequence alignment of *Cg-DMl* and members of the *Dmrt* family from both protostomes and deuterostomes indicated that *Cg-DMl* was more closely related to the *Dmrt5* of the medaka with 38% identity. The corresponding phylogenetic tree confirmed that *Cg-DMl* was clustered with *Dmrt4* and *Dmrt5* (Results not shown). When this analysis was limited to the DM domain, the highest identity rates of *Cg-DMl* were observed with the Drosophila *Dmrt99B* and tilapia *DMO* (both 98%), the medaka *Dmrt4* and *Dmrt5* (98% and 96% respectively), and human and mouse *Dmrt5* (both 96%) (Fig. 3A). As also shown in Fig. 3A, sequence comparison of this domain among different species revealed a consensus sequence of 5aa ($K_{41}GHKR_{45}$) of the putative NLS (Nuclear Localization Signal) located in a conserved zinc module consisting of intertwined CCHC and HCCC Zn^{2+}-binding sites. With respect to the DMA domain and the short conserved motif of 7aa (RSAFSPI) of the protein (Fig. 3B), the first one shared highest levels of amino acid identity with the human *Dmrt4* and *Dmrt5* sequences (52% and 57% respectively), the coral *AmDMl* (55%), and the Drosophila *Dmrt93B* and *Dmrt99B* (44% and 36% respectively) while the second one showed highest identity rates with the human *Dmrt4* and *Dmrt5* (71% and 85% respectively), Drosophila *Dmrt93B* and *Dmrt99B* (57% and 71% respectively) and coral *AmDMl* (57%). The phylogenetic tree generated using the DM domain of oyster, human, mouse and invertebrates *Dmrt* (Fig. 4) provided evidence that *Cg-DMl* is grouped with *Dmrt5* with high bootstrap support (62). This cluster was closer to *Dmrt4* (bootstrap of 73) than to the other members of *DMRT* family, grouped together with a bootstrap support between 88 and 100.

3.3. Cg-DMl expression during the development and in the adult gonads

Real time quantitative RT-PCR experiments showed, during the development (Fig. 5) as in the adult gonads (Fig. 6), the same profile of expression with both DM and HDM primers, suggesting that mRNA expression only reflects *Cg-DMl* transcript levels. During the development of the oyster, *Cg-DMl* mRNA levels were significantly increased in spat between 1 and 2 months of age (Fig. 5). In adult tissues (Results not shown), *Cg-DMl* mRNA abundance was higher in gills (mean ± SEM: 0.298 ± 0.11), labial palps (0.21 ± 0.10) and mantle (0.13 ± 0.07) than in gonads (mix at various gametogenetic stages; 0.02 ± 0.01), adductor muscle (0.03 ± 0.03) and digestive glands (0.04 ± 0.03). A more extended analysis of *Cg-DMl* mRNA expression in the gonads of adults at different stages of the gametogenetic cycle revealed significantly increased levels in males at the end of the spermatogenetic cycle (mean ± SEM: 0.51 ± 0.47; stage III M) comparatively to females (mean ± SEM: 0.015 ± 0.008; stage III F) or to more precocious stages (Fig. 6). The variability of results observed in males at stage III, where some of them present a ten fold increase comparatively to others, was not related to histological differences of the gonads but only to individual variability. Likewise, no differences were observed between the individuals of the two families. Although the stage II male and female is not the stage where *Cg-DMl* mRNA is the most expressed, it illustrates best the cell localization of the mRNA expression by *in situ* hybridization, as seen on histological sections stained with a Prenant–Gabe trichrome, where tubules show all the types of germinal cells (Fig. 7A and D). In the male gonads (Fig. 7A–C) a cytoplasmic mRNA staining was detected in the spermatogonia and/or in the somatic cells surrounding them in the gonadal tubules (Fig. 7B). In the female gonads (Fig. 7D–H), a cytoplasmic mRNA staining, particularly intense in the juxtanuclear area, was observed in germ cells from oogonia (Fig. 7E and F) to the vitellogenic oocytes stages (Fig. 7G and H). Negative controls with the sense riboprobe gave sometimes a faint non significant signal (Fig. 7C, F and H). In the mantle (Fig. 8A–C), *Cg-DMl* mRNAs were detected in the cytoplasm of epithelial cells and large goblet cells with the anti-sense probe (Fig. 8B) but not with the sense probe (Fig. 8C). In the digestive gland (Fig. 8D–F) the mRNA expression was only found in the cytoplasm of clusters of young cryptic epithelial cells with the anti-sense riboprobe (Fig. 8E and F).

4. Discussion

We report here the molecular characterization of *Cg-DMl*, which is the first complete sequence of a DM factor identified in the Protostomia Lophotrochozoa phylum. The deduced serine- and proline-rich amino acid sequence presents a DM domain characteristic of the DMRT protein family and a conserved DMA domain near the C-terminus. The DMA domain is conserved with at least 50% identity in the branch of *Dmrt3*, *Dmrt4* and *Dmrt5* (Miller et al., 2003; Guo et al., 2004). The DM domain contained the putative NLS located in the zinc module consisting of intertwined CCHC and HCCC Zn^{2+}-binding sites. NLS of many transcription factors were reported within or in the vicinity of their DNA-binding domain, including zinc finger factors, homeodomain factors, HMG box factors, and helix–loop–helix factors (Ying et al., 2007). These results suggest that the DM domain of *Cg-DMl* may have, as in other species, bipotential functions, both DNA-binding and nuclear import (Zhu et al., 2000; Ying et al., 2007). Another conserved domain of 7aa present in the C-terminus was identified in the oyster *Dmrt* sequence and it corresponds to the first 7aa of the DMB domain found in zebrafish *Dmrt5* (Guo et al., 2004) and human *Dmrt3*, *4* and *5* (Kato et al., 2008). The genomic sequence of *Cg-DMl* only shows one intron, like *Dmrt4* and

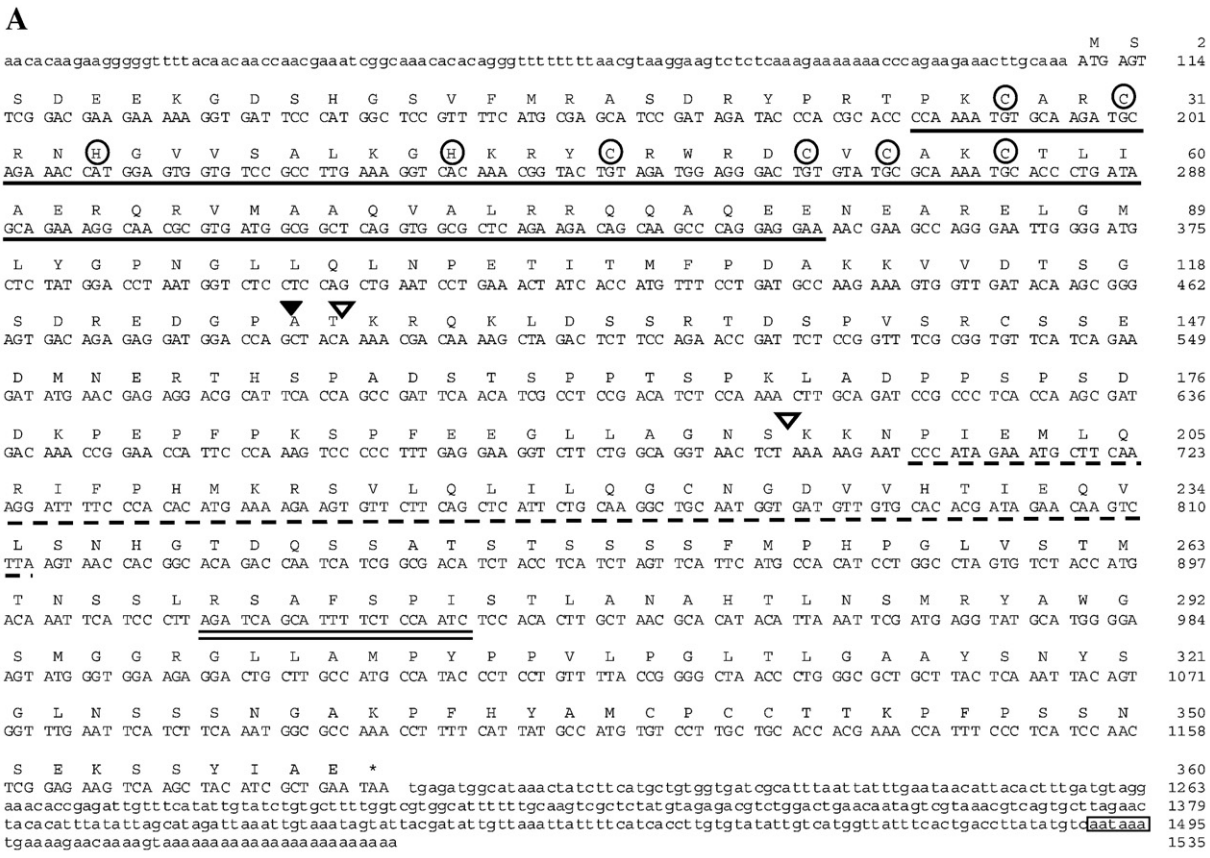

Fig. 1. (A) Nucleotide sequence of the *Cg-DMl* cDNA and its deduced amino acid sequence (EU046234). DM domain is underlined, the dashed and the double underlines indicate the DMA domain and the short 7aa conserved motif respectively. The polyadenylation signal is boxed. The black triangle indicates the intron/exon junction. The open triangles indicate the end of the two shorter cDNA isoforms. The cysteine and histidine conserved residues are circled. (B) Nucleotide sequence of the *Cg-DMl* intron. The donor/acceptor (GT/AG) sites are dark-shaded. (C) Schematic presentation of *Cg-DMl*, *Dmrt1*, *Dmrt4* and *Dmrt5* genes. Conserved domains [DM, DMA, DMB and rich in proline/serine (P/S)] are indicated by black boxes. The short conserved amino acid motifs (7 aa) are indicated by stripped boxes. The solid triangles indicate stop codons and the open triangle indicates the poly(A) signal. Lengths of the coding sequences and intron in base pairs (bp) are shown above, and the numbers below indicate amino acid positions.

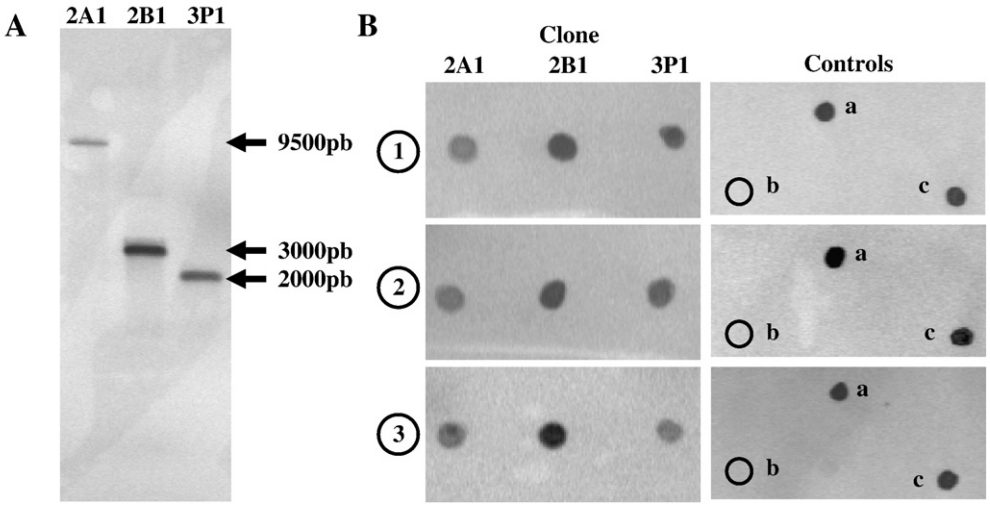

Fig. 2. (A) Southern blot analysis of three of the eight clones obtained from the screening of the genomic library of *C. gigas*. DNAs were digested with EcoR1 and hybridized with a digoxigenin-11-dUTP labelled DM domain probe. Lane 1: clone 2A1; lane 2: clone 2B1; and lane 3: clone 3P1. For each clone, only one band was observed, around 2000, 3000 or 9500 bp. (B) Differential hybridization of the DNA of the clones 2A1, 2B1 and 3P1 with either a DM domain probe found in all the factors of the family (Line 1), the *Cg-DMl* intron probe (Line 2) or a HDM probe specific to *Cg-DMl* (Line 3). a: positive control from the DIG DNA labelling kit; b: negative control with the plasmid alone; and c: positive control with the plasmid with *Cg-DMl* DNA. The absence of differential hybridization illustrates the apparent absence of other *Dmrt* orthologs than *Cg-DMl* in the oyster.

Dmrt5 genes (Guo et al., 2004; Yamaguchi et al., 2006) and in contrast with *Dmrt1* which presents 4 and 5 introns in mammals (Cheng et al., 2006) and fishes (Yamaguchi et al., 2006) respectively. All these results and phylogenetic analyses indicated that *Cg-DMl* was most closely related to *Dmrt4* and *Dmrt5* homologs. In the oyster, we have been unable to show the presence of other genes encoding DM factors or alternative splicing transcripts. However, 3′RACE-PCR experiments from mRNA of 1.5 month old spat allowed us to identify two additional differential polyadenylation at 488 bp and 696 bp without any AAUAAA signal. Even if we cannot exclude oligo(dT) mismatching during the reverse transcription step, AAUAAA-independent polyadenylation has been reported for *Dmrt1* isoforms in male germ cells of the zebrafish (Guo et al., 2005). In the vertebrates and invertebrates studied so far, many DM factors or splicing transcripts have been characterized within each species, at least three in *Ciona intestinalis* as in the mosquito *Anopheles gambiae* and coral, four in *Drosophila*, six in fishes, eight in human and eleven in *Caenorhabditis elegans* ((Volff et al., 2003; Hong et al., 2007)). In vertebrates, these factors, like *Dmrt4* and *5* are expressed

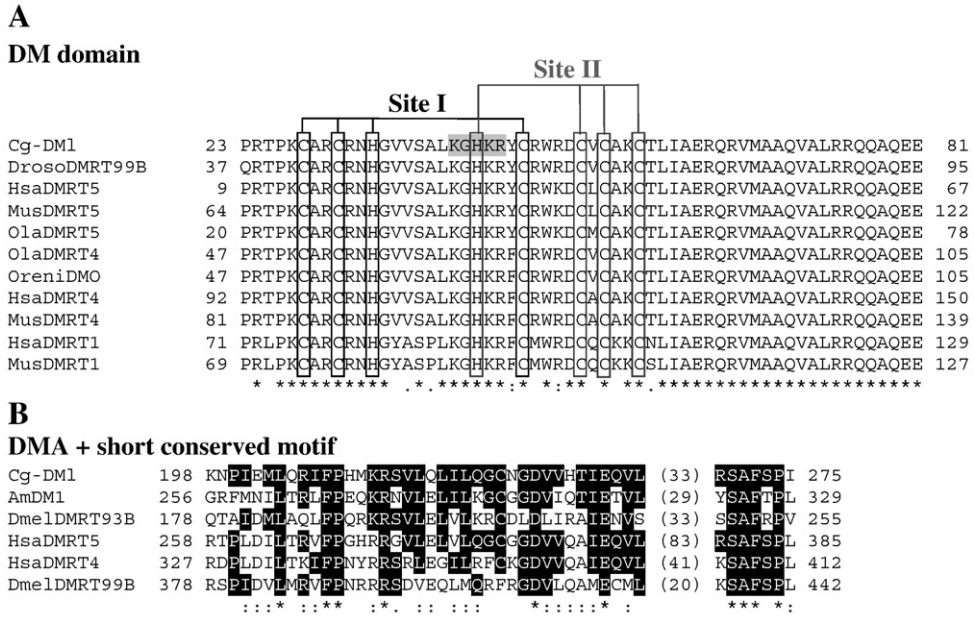

Fig. 3. (A) Alignment of amino acid sequences of the DM domain of oyster *Cg-DMl*, Drosophila *Dmrt99B*, mouse (Mus), human (Hsa) and medaka (Ola) *Dmrt4* and *Dmrt5*, tilapia (Oreni) *DMO* and mouse and human *Dmrt1*. Identical amino acids are indicated by asterisks. Amino acids with conserved similarities are indicated by dots or colons. The short conserved NLS (Nuclear Localization Signal) (KGHKR) is grey-shaded and the zinc module consisting of intertwined CCHC and HCCC Zn^{2+}-binding sites are indicated with grey (Site II) and black (Site I) boxes. (B) Alignment of amino acid sequences of the DMA domain and the short conserved motif of oyster *Cg-DMl*, coral *AmDML*, Drosophila *DmelDmrt93B* and *99B* and human (Hsa) *Dmrt4* and *Dmrt5*. Identical amino acids to oyster *Cg-DMl* are dark-shaded. Conserved amino acids between all sequences are indicated by stars. Amino acids with conserved similarities are indicated by dots or colons. Alignments were generated using CLUSTAL W. The numbers of the amino acid residues at the beginning and the end of the domains are indicated. (GenBank accession no. *Cg-DMl* (EU046234), *DrosoDMRT99B* (NP_524549), *HsaDMRT5* (NM_032110), *MusDMRT5* (AY145837), *OlaDMRT5* (AB083691), *OlaDMRT4* (AB055958), *OreniDMO* (AF203490), *HsaDMRT4* (BC130435), *MusDMRT4* (AF542047), *HsaDMRT1* (NM_021951), *MusDMRT1* (NM_015826), *AmDM1* (AF530064), *DmelDMRT93B* (NP_524428).

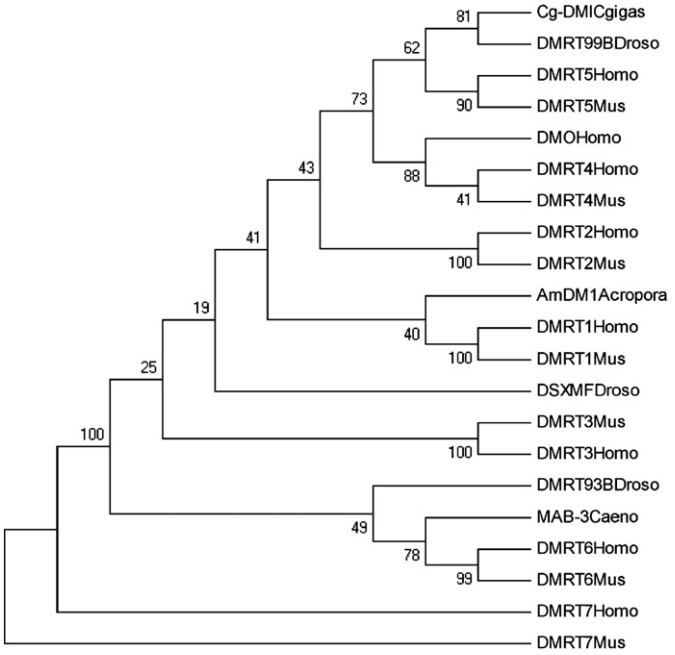

Fig. 4. Phylogenetic tree generated using the DM domain of the *DMRT* proteins showing that *Cg-Dml* is clustered together with *DMRT5*. Phylogenetic analysis was performed by neighbour-joining method (Saitou and Nei, 1987) using *MEGA4* (Tamura et al., 2007). Numbers in the branches represent the bootstrap values (%) from 100 replicates (Felsenstein, 1985). (GenBank accession no. EU046234, NP_524549, NM_032110, AY145837, AK096011, BC130435, AF542047, AF130729, AF539811, AF530064, NM_021951, NM_015826, NP_524272, AF541936, NM_021240, NP_524428, AF022388, AJ291671, AF542048, AJ291669, AF542046).

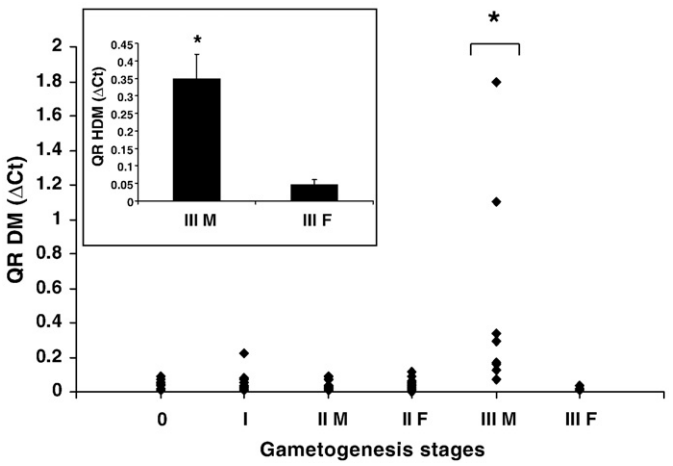

Fig. 6. Real time RT-PCR transcripts quantification relative to *EF1α* in the gonads of adults of *C. gigas* during the gametogenetic cycle with DM (diamonds) and HDM (insert) primers. Stage 0: undifferentiated gonad ($n=9$); Stage I: gonia mitosis and sex indiscernible ($n=29$); Stage II: active spermatogenesis (IIM, $n=21$) and growing oocytes (IIF, $n=58$); Stage III: male (IIIM, $n=8$) and female (IIIF, $n=5$) mature stage. QR: Relative Quantity, arbitrary units. These graphs show mRNA levels significantly increased (*:$P<0.05$) in males at the end of the spermatogenetic cycle compared to females or to more precocious stages.

in other tissues on top of the gonads, suggesting that they may control a broader range of biological processes (Hong et al., 2007). The presence, in *Cg-Dml*, of a DMA domain specific to *Dmrt4* and *5* in vertebrates, also found for the coral *AmDM1*, reinforces the hypothesis that the ancestral DM proteins possessed a DMA domain (Miller et al., 2003; Volff et al., 2003). Therefore, *Cg-Dml*, as an ancestral DM factor, might be involved in a large range of biological processes whereas evolution has led to diverse DM factors, some as *Dmrt4* and *5* which may also have large functions associated to the ancestral structure and others with a more specific role, such as *Dmrt1* in sex differentiation.

In order to know if *Cg-Dml* mRNA expression is gonad specific, i.e. in agreement with a role in the gonadic differentiation, we performed real time PCR in adult tissues. The expression was ubiquitous, in the gills, labial palps, mantle, adductor muscle, digestive gland and gonads. *Dmrt4* and *Dmrt5*, to whom *Cg-Dml* is more related, have also shown different tissue expressions, including gonads, depending on the species considered (Guan et al., 2000; Kim et al., 2003; Guo et al., 2004). By *in situ* hybridization, *Cg-Dml* mRNA expression was restricted to particular cells, the epithelial cells in the mantle and young cryptic epithelial cells which may renew the stock of old epithelial cells in the digestive gland (Galtsoff, 1964). DM factor expression has also been observed in potentially high proliferating tissues such as *Dmrt4* in the embryonic gonad, brain and olfactory system respectively in mice (Kim et al., 2003) and medaka fish (Winkler et al., 2004), and *Dmrt5* in the developing nervous system in the zebrafish (Guo et al., 2004).

Although *Cg-Dml* is expressed in several adult tissues, its involvement in the gonadic development of the oyster is not excluded. By *in situ* hybridization, in the testis, it is observed in the cytoplasm of spermatogonia and/or somatic cells surrounding them although it is impossible at this microscopic level to differentiate the germ cell and the somatic cell limits. However, these results are in agreement with the expression of *Dmrt5* and *Dmrt1* in testicular germ cells of the

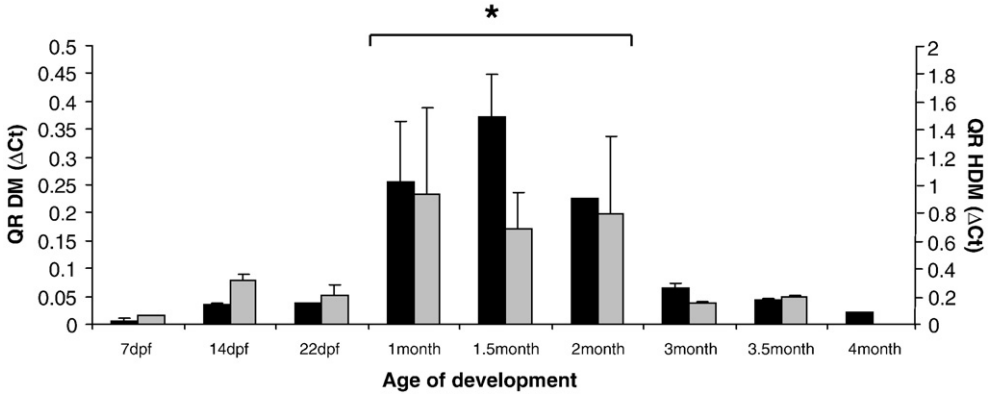

Fig. 5. Real time RT-PCR transcripts quantification relative to *EF1α* in developmental stages of *C. gigas* with DM (black bars) and HDM (grey bars) primers. Total RNAs were isolated from veliger larvae of 7 and 14 days post-fertilization (7 and 14 dpf), post-metamorphosis larvae (22 dpf) and 1 to 4 month old spat (1, 1.5, 2, 3, 3.5 and 4 months). QR: Relative Quantity, arbitrary units. Values are mean ± SEM of triplicates. These graphs show mRNA levels significantly increased (*:$P<0.05$) in spat between 1 and 2 months of age compared to the other developmental stages.

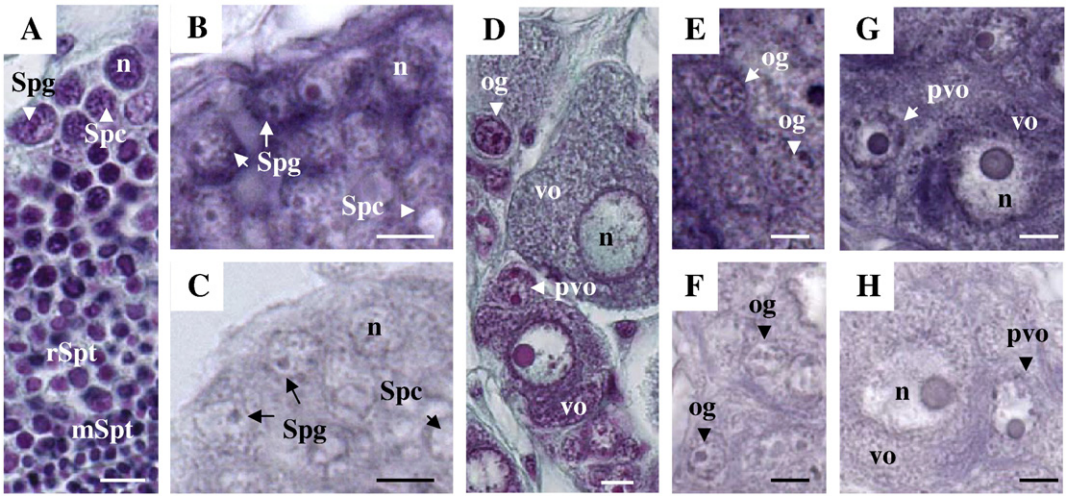

Fig. 7. Histological sections of gonads of stage II male (A) and female (D) stained with a Prenant–Gabe trichrome—*Cg-DMl* mRNA expression pattern by *in situ* hybridization with anti-sense riboprobe (B, E and G) and sense riboprobe (C, F and H). Male (A–C) and female (D–H) gonads with mRNA staining in the cytoplasm of the spermatogonia and/or of the somatic cells surrounding them (B) and in the oogonia (E), pre-vitellogenic and vitellogenic oocytes respectively (G). mSpt: mature spermatids, n: nucleus, og: oogonia, pvo: pre-vitellogenic oocyte, rSpt: round spermatids, Spc: spermatocytes, Spg: spermatogonia, and vo: vitellogenic oocyte. Bars: 5 µm.

zebrafish and the grouper (Guo et al., 2004, 2005; Xia et al., 2007) or of *Dmrt1* which is expressed either in spermatogonia and Sertoli cells (Raymond et al., 2000) or in Sertoli cells only (Winkler et al., 2004; Yamaguchi et al., 2006) depending on the species. Associated with this variable cell location of expression, *Cg-DMl* may have divergent roles in the gonad, such as an involvement in the proliferation of spermatogonia and differentiation of Sertoli cells as found for *Dmrt1* in vertebrates (Fahrioglu et al., 2007; Herpin et al., 2007; Kim et al., 2007; Lei et al., 2007). By real time PCR, *Cg-DMl* mRNA was expressed (i) in the adult gonads of both sexes as reported for *Dmrt1*, *2*, *3*, *4* and *5* in some vertebrates (Guan et al., 2000; Guo et al., 2004, 2005; Matsushita et al., 2007), (ii) with a significant increase in males, but not in females, at the end of the gametogenetic cycle, i.e. just before the resting period of the gonad. In hermaphrodites such as the black porgi (He et al., 2003) and the grouper (Xia et al., 2007), changes in expression profile of *Dmrt1* appeared correlated to the ovary/testis transition. In *C. gigas*, where only some individuals of a male population change to female, such a correlation between the *Cg-DMl* expression profile variation and sex transition remains to be precised. Considering that *Cg-DMl* is expressed in spermatogonia, the highest transcript levels should be observed for stage II as in stage III, although real time PCR results only mention an increase in stage III. This apparent disagreement might be explained, in stage III, by an increase of the number of spermatogonia as other germ cells, not only due to their proliferation as in stage II but also to an increase of the gonadic volume from 5–40% of the visceral mass in the less mature animals to 60% at stage III (Fabioux et al., 2004a). Whatever the testicular cells expressing *Cg-DMl*, we have observed that some males at stage III

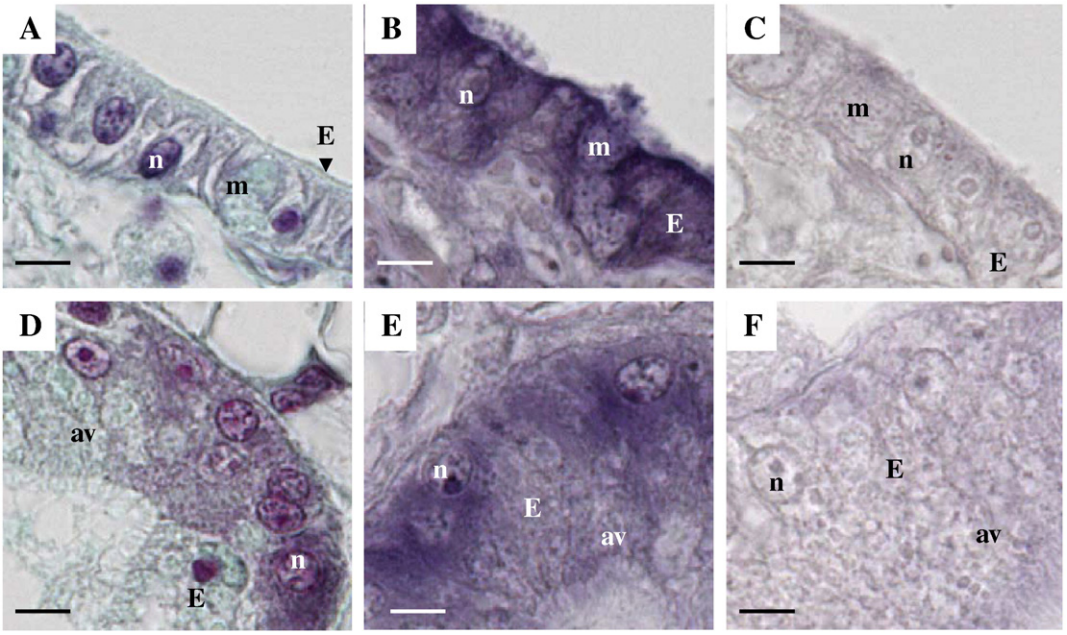

Fig. 8. Histological sections of the mantle (A) and the digestive gland (D) stained with a Prenant–Gabe trichrome—*Cg-DMl* mRNA expression pattern by *in situ* hybridization with anti-sense riboprobe (B and E) and sense riboprobe (C and F). Mantle (B) with *Cg-DMl* mRNA expression in the cytoplasm of epithelial cells and large goblet cells. Digestive gland (E) with mRNA staining in the cytoplasm of clusters of young cryptic epithelial cells. av: apical vesicles; E: epithelium, m: mucous, and n: nucleus. Bars: 5 µm.

present higher levels of *Cg-Dml* mRNA than others without any histological differences. This could be in agreement with Guo et al. (1998) who reported that two types of males could exist in the oyster.

During the development, *Cg-Dml* mRNA expression measured by real time PCR increased in spat between 1 and 2 months of age. This period of the development takes place between the larvae metamorphosis occurring around 22 days post-fertilization and the 3 month old spat stage which shows gonadic tissue (personal observations). To our knowledge, only one study by Fabioux et al. (2004b) deals with the development of the gonad during the organogenesis of the oyster by following the *in situ* expression of the oyster *vasa*-like gene, a determinant of the germline. These authors hypothesized that cells giving rise to putative primordial germ cells proliferate and migrate to the anterior and the posterior sides of the body during the late larval stages to differentiate again, in spat, into two groups of germinal stem cells which, at sexual maturity, would proliferate intensively to form the gonadal tubules. Therefore, the kinetic of the *Cg-Dml* mRNA expression appears in accordance with the development of the gonad in *C. gigas*.

Cg-Dml, as an ancestral DM factor, might be involved in a large range of biological processes and its role in cell proliferation and/or differentiation during the development of the gonad remains to be precised.

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