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Macrophage migration inhibitory factor (MIF) family in arthropods: cloning and expression analysis of two MIF and one D-dopachrome tautomerase (DDT) homologues in Mud crabs, *Scylla paramamosain*

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Abstract

The macrophage migration inhibitory factor (MIF) family, consisting of MIF and D-dopachrome tautomerase (DDT) in vertebrates, is evolutionarily ancient and has been found across Kingdoms including vertebrates, invertebrates, plants and bacteria. The mammalian MIF family are chemokines at the top of the inflammatory cascade in combating infections. They also possess enzymatic activities, e.g. DDT catalysis results in the production of 5,6-dihydroxyindole (DHI), a precursor of eumelanin. MIF-like genes are widely distributed, but DDT-like genes have only been described in vertebrates and a nematode. In this report, we cloned a DDT-like gene, for the first time in arthropods, and a second MIF in mud crab. The mud crab MIF family have a three exon/two intron structure as seen in vertebrates. The identification of a DDT-like gene in mud crab and other arthropods suggests that the separation of MIF and DDT preceded the divergence of protostomes and deuterostomes. The MIF family is differentially expressed in tissues of adults and during embryonic development and early life. The high level expression of the MIF family in immune tissues, such as intestine and hepatopancreas, suggests an important role in mud crab innate immunity. Mud crab DDT is highly expressed in early embryos, in megalops and crablets and this coincides with the requirement for melanisation in egg chorion tanning and cuticular hardening in arthropods, suggesting a potential novel role of DDT in melanogenesis via its tautomerase activity to produce DHI in mud crab. The clarification of the presence of both MIF and DDT in this report paves the way for further investigation of their functional roles in immunity and in melanogenesis in mud crab and other arthropods.

Key words: mud crab *Scylla paramamosain*; macrophage migration inhibitory factor (MIF); D-dopachrome tautomerase (DDT); cloning; gene organisation; expression analysis

1. Introduction

The macrophage migration inhibitory factor (MIF) family consists of MIF and D-dopachrome tautomerase (DDT) in vertebrates [1-2]. The MIF and DDT genes are located in close proximity on the same chromosome in the tetrapod genome and have a similar three exon/two intron structure. Their proteins share a similar homotrimer tertiary structure, binding to the same receptor complex, and are involved in various physiological and pathophysiological functions in vertebrates [1-3]. The MIF family is evolutionarily ancient and has been found across Kingdoms including vertebrates and invertebrates, plants and bacteria.

MIF was one of the first cytokines discovered 50 years ago as a T-cell derived soluble cytokine which inhibited the migration of macrophages. Over the years, the number of functions attributed to this protein increased significantly, positioning MIF at the top of the inflammatory cascade in combating infection and in immunoinflammatory and autoimmune diseases [4]. It is produced and stored intracellularly by a wide variety of cells, including monocytes/macrophages and T cells of the immune system, as well as in the pituitary gland [5]. MIF release is induced by stress, infections, PAMPs, cytokines and the immunosuppressive glucocorticoids, and is implicated in a wide range of biological activities. It functions as an upstream activator of innate immunity that regulates subsequent adaptive responses, counter-regulates the anti-inflammatory effect of glucocorticoids [4] and up-regulates *Toll-like receptor* expression by immune cells in response to the microbial products [6]. It can also control the c-Jun N-terminal activation domain-binding protein-1 (JAB-1) transcriptional effects [7], the suppression of the activation-induced p53-dependent apoptosis [7], the modulation of glycolysis and insulin resistance in insulin target cells [2], and embryonic development [8]. The first receptor identified for MIF was CD74, the membrane-expressed form of the invariant chain and an MHC class II chaperone. CD74 binds MIF with high affinity but by itself is not able to induce intracellular signaling and requires the recruitment of signaling-competent co-receptors, such as CD44, CXCR2, CXCR4 and

CXCR7 [9]. Apart from the chemokine-like functions, MIF acts as a chaperone protein [10] and an enzyme with D-dopachrome tautomerase and oxidoreductase activities [11].

DDT was originally described for its D-dopachrome tautomerase activity detectable in the cytoplasm of human melanoma, human liver and rat organs [12]. It is now clear that DDT and MIF are functional homologs in mammals that share not only an overlapping spectrum of activities but also distinct functions [1-2,13]. Both DDT and MIF possess the characteristic N-terminal proline-1 (after cleavage of the initiating methionine) which is the basis of their enzymatic tautomerase activities. Although both family members tautomerize the model substrate D-dopachrome, their products are different; MIF catalyzes a pure tautomerization to generate 5,6-dihydroxyindole-2-carboxylic acid whereas DDT catalysis results in an additional de-carboxylation to produce 5,6-dihydroxyindole (DHI) [1]. DHI is a precursor of eumelanin, thus it is involved in three physiologically important processes in invertebrates including defensive immune reactions, wound healing and cuticular hardening (sclerotization) [14]. Recently, DHI itself has been shown to have antiviral, antibacterial, antifungal and antiparasitic activities, which indirectly portray the role of DDT in defense in invertebrates [3,15]. Therefore, the non-physiologic catalytic activities of MIF or DDT with respect to D-dopachrome tautomerase activity may be proposed as a vestigial property of these proteins originating from their ancestral position in innate immunity and the melanotic encapsulation response in invertebrates [1,3].

At present, *MIF-like genes* have been cloned and characterized in many species from bacteria [16], plants [17], invertebrates and vertebrates. However, DDT-like genes have only been described in vertebrates and recently in the nematode *Caenorhabditis elegans* [18]. MIF-like genes have been described recently in Arthropods, such as white leg shrimp *Litopenaeus vannamei* [19], mud crab *Scylla paramamosain* [20], Chinese mitten crab *Eriocheir sinensis*[21], and the lone star tick *Amblyomma americanum* [22]. However, the presence of DDT-like genes in Arthropods is not clear. The mud crab is a commercially important crustacean and widely distributed along the coastline of the southern China and Pacific region [23]. Analysis of mud crab

transcriptome data identified three distinct contigs related to the MIF family, leading to the cloning of a DDT-like gene, for the first time in arthropods, and a second MIF (designated as spMIF2, with the previously reported mud crab MIF renamed as spMIF1) in mud crab [20]**.** The molecular characterization and expression analysis of the three mud crab MIF family members are reported.

2. Materials and methods

2.1. Experimental crabs

Mud crabs were purchased from a local aquarist in Xiamen city, China. The crabs were fed twice a day with commercial food and were acclimated for at least one week before any experiments.

2.2. RNA extraction and cDNA synthesis

Total RNAs were extracted from different tissues or developmental stages of mud crab using TRIzol® (Invitrogen, CA, USA) and treated with RNase-free DNase I (New England Biolabs Inc.) as described previously [23]. For analysis of gene expression, total RNA was reverse transcribed using a GoScript™ Reverse Transcription System (Promega, USA). The resultant cDNA samples were diluted 10 times with TE buffer and stored at −20 °C ready for real-time PCR analysis. For 3'-rapid amplification of cDNA ends (RACE) and 5'-RACE, SMART cDNA samples were synthesized from hepatopancreas total RNA using a SMARTer™ RACE cDNA Amplification kit (Clonetech, USA) following the manufacturer's instructions.

2.3. Cloning of full-length MIF family members in mud crab

Analysis of a mud crab transcriptome database [23] identified three divergent contigs homologous to MIF of mitten crab (E7CW70) and other species. Two of the cDNA contigs had complete Open Reading Frames (ORFs) with in frame stop codons before the main ORF. The other had only a partial ORF. To obtain the complete ORF, 3'- and 5'-RACE was conducted using primers detailed in Table 1. The cloning of PCR products and sequences analysis was performed as described previously [23]. The resultant cDNA sequences

were designated as spMIF1, spMIF2 and spDDT, according to homology and phylogenetic tree analysis, which will be described later.

2.4. Cloning of genomic DNA of MIF family members in mud crab

Genomic DNA was extracted from mud crab muscle using the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Japan) according to the manufacturer's protocol. The genomic sequences were amplified using LA Taq DNA polymerase (TaKaRa, Japan). Three spMIF1 primer pairs (gMIF1-F1/MIF1-GSPR2, gMIF1-F2/MIF1-FL-R and gMIF1-F3/gMIF1-R), two spMIF2 primer pairs (gMIF2-F1/gMIF2-R1 and gMIF2-F2/gMIF2-R2) and one DDT primer pair (Table 1) were used for amplification.

2.5. Sequence analysis

Gene organisation was predicted at NCBI using the Spidey program (http://www.ncbi.nlm.nih.gov/spidey/). The protein sequences were retrieved from the Expasy or NCBI protein databases. Global sequence comparisons were performed using the MatGAT program (V2.02) [24] using the scoring matrix BLOSUM60 with a gap open penalty of 10 and gap extension penalty of 1. Amino acid sequence multiple alignments were produced using CLUSTALX 2.1 [25] and shaded using BOXSHADE (http://www.ch.embnet.org/software/ BOX_form.html). The signal peptide was predicted using SignalP 4.0 [26] and non-classical secretion was predicted by SecretomeP 2.0 [27]. Phylogenetic trees were constructed from a CLUSTAL-generated multiple alignment of selected full-length MIF and DDT sequences by the neighbour-joining and maximum likelihood methods using MEGA6 [28] and were bootstrapped 10, 000 times.

2.6. Real-time qPCR

147 The transcript expression of mud crab MIF family members was quantified by real-time PCR using a
148 LightCycler 480 II real-time PCR system and a SYBR Green I master kit (Roche, Germany). The expression
149 of β-actin, a widely used housekeeping gene for mud crab gene expression analysis, was used to normalize the
150 expression levels across tissues and developmental stages. Primers were listed in Table 1 and the PCR
151 reaction was performed as previously described [23]. A serial 10-fold dilution of reference containing equal
152 molar amounts of PCR products of each gene was run along with the cDNA samples in the same 96-well PCR
153 plate. Each sample was tested in triplicate. Melting curve analysis was performed for specificity. The
154 transcript expression levels were calculated using the integrated software of the LightCycler 480 system.
155

156 **2.7. Tissue distribution of transcript expression of MIF family members in mud crab**

157 Eight adult mud crabs (150 ± 5 g, with carapace length of 56 ± 3 mm) were used in this experiment. The
158 crabs were anesthetized by putting in an ice bath for 5 to 10 min before sacrifice. Hemolymph was collected
159 from the base of the fourth leg with a 5-ml syringe containing 2.5 ml of a cold anticoagulant solution (NaCl
160 140 mmol/L; glucose 100 mmol/L; citric acid 26 mmol/L; trisodium citrate 30 mmol/L; pH 5.0), followed by
161 centrifugation at 500 g at 4 °C for 5 min. The resulting hemocyte pellets were re-suspended in 1 ml TRIzol for
162 total RNA extraction. Meanwhile, for each individual crab, ten other tissues/organs including gills, heart,
163 hepatopancreas, stomach, intestine, testis, seminal vesicle, ejaculatory duct, muscle and brain, were collected
164 separately and were homogenized immediately in TRIzol using a shaker (Retsch MS100, Japan) with glass
165 beads, then stored at -80 ºC until further experimentation.
166

167 **2.8. Gene expression ontogeny of MIF family members in mud crab**

168 Live embryos at different developmental stages were obtained from a local breeding farm near Xiamen,
169 China. Eggs, zoea and megalops were pooled to obtain 100 mg of materials and individual crablets were used
170 for RNA extraction. The samples were homogenized in TRIzol as above. Each development stage contained
171 four samples.

2.9. Statistical analysis

Differences of transcript expression of mud crab MIF family members between groups of tissues and developmental stages were analyzed using a one-way Analysis of Variance (ANOVA) and the least significant difference post hoc test using the IBM SPSS Statistics 23 package (SPSS, Chicago, IL). Significance was set at $p < 0.05$.

3. Results and Discussion

3.1. Molecular characterization of MIF family members in mud crab

Three cDNA sequences (**supplementary Fig. S1**) encoding for spMIF1, spMIF2 and spDDT, respectively, were cloned and their features are summarized in **Table 2**. The spMIF1 cDNA had 99% identity to the recently reported mud crab MIF (Acc. No. JX131610) [20] and encoded the same 120 amino acids (aa) with only one aa difference (H41 to R41). The spMIF2 cDNA also encoded for 120 aa but shared only 62.5% identity to spMIF1 at the aa level. The spDDT cDNA encoded for 115 aa and had identities of 28.3% and 27.5% to spMIF1 and spMIF2, respectively (**Table 2**). No signal peptide could be predicted by SignalP4.0 [26] but a NN-score of 0.575, 0.573 and 0.807 was predicted for spMIF1, spMIF2 and spDDT, respectively by SecretomeP [27], suggesting that the mud crab MIF family members may be secreted non-classically as seen with MIF family members in vertebrates [1-2].

The classification of MIF and DDT in mud crab is supported by homology analysis (**supplementary Table S1**), phylogenetic tree analysis (**Fig. 1**), conserved gene organisation (**Fig. 2**), and conserved structure as seen in the multiple alignment (**Fig. 3**). On the one hand, the spMIF1 and spMIF2, along with MIF molecules from other arthropods, i.e. mitten crab, shrimp, water flea *Daphnia pulex* and lone star tick, shared higher identities to vertebrate MIFs (26.5-44.8%) than to vertebrate DDT (21.2-35.5%, **Table S1**). On the

other hand, the spDDT, along with water flea and tick DDT molecules had higher identities to vertebrate DDT (25.2-42.4%) than to vertebrate MIFs (22.4-29.9%) (**Table S1**). It is worth noting that the family members have higher homology in vertebrates than in arthropods, e.g. mammalian MIFs share 63.2-71.3% identities to MIFs from other vertebrates, whereas mud crab MIFs have only 30.0-52.5% identities to water flea MIFs (**Table S1**).

To confirm the classification of MIF and DDT in arthropods, a phylogenetic tree analysis was constructed using MIF and DDT molecules from selected vertebrates and arthropods, with bacterial MIF-like molecules from *Prochlorococcus marinus* [16] as an outgroup. Both neighbour-joining and maximum-likelihood trees showed similar topology. In agreement with the homology analysis (**Table S1**), vertebrate MIF and DDT molecules clustered together first in a maximum-likelihood phylogenetic tree (**Fig. 1**). The arthropod MIFs grouped with vertebrate MIF molecules, and the arthropod DDTs grouped with vertebrate DDTs with high bootstrap support, suggesting that arthropods do have two types of MIF family members, namely MIF and DDT, as seen in vertebrates. The presence of both MIF and DDT in arthropods and nematodes, and in vertebrates re-enforces the idea that the separation of MIF and DDT function preceded the divergence of protostomes and deuterostomes [18].

Vertebrate MIF family members have a conserved three exon/two intron gene organization with the first intron phase 0 and the second intron phase II [29-30]. Analysis of the gene organization in arthropods in the database revealed that water flea MIF1 and MIF2 have only two exons but water flea DDT has the same structure as seen in vertebrate MIF family members (**Fig. 2**). To gain further insight into the evolution of the MIF family, the genomic organization of mud crab MIF family members was determined by cloning of their genomic DNA sequences using primers designed in the 5'- and 3'-UTRs. Whilst two genomic sequences of 4.7 kb and 5.4 kb were amplified for spMIF1, a single DNA fragment of 1.3 kb and 2.5 kb, was obtained for spMIF2 and spDDT, respectively. The genomic DNA sequences were deposited at GenBank under accession

numbers KJ728660 and KJ728661 (spMIF1), KJ728663 (spMIF2) and KJ728665 (spDDT). All mud crab MIF family genes shared the same three exon/two intron gene structure as seen in vertebrates (**Fig. 2**). The two spMIF1 genomic sequences differed only in the intron I, and may represent two alleles of the same gene. The lone star tick MIF gene and water flea DDT gene also had the three exon/two intron structure but the water flea MIF1 and MIF2 had their second intron missing (**Fig. 2**). A three coding exon/two intron structure is found in MIF-like genes in plants [17], nematodes [31] and arthropods in this report, in addition to vertebrates [30], indicating that a three exon/two intron gene structure might have existed in the early ancestral MIF/DDT gene, and that independent intron loss or gain had happened in different species/lineages, e.g. loss of intron II in water flea MIF1 and MIF2.

A multiple alignment of MIF family members from arthropods and selected vertebrates revealed not only general conservation of aa sequences, but also lineage specific characteristics (**Fig. 3**). All the MIF family members possess the characteristic N-terminal proline-1 (after cleavage of the initiating methionine) which is the basis of their enzymatic tautomerase activity. Both MIF and DDT from humans and mice have a β1α1β2β3β4α2β5β6 structure [13]. Five distinct aa in human MIF are implicated in the binding and catalysis of its substrate (P1, K32, I64, Y95 and N97, according to the human mature MIF, **Fig. 3**) [32] that were conserved in vertebrate MIF molecules (Fig. 3). The first four residues were also conserved in arthropod MIF molecules but only the first three of these five were conserved in DDT molecules from both vertebrates and arthropods. There are three conserved cysteine residues (C3-5, Fig. 3) across vertebrate MIFs, with C3 and C4 crucial for forming the catalytic centre of MIF oxidoreductase activity [11]. Only C3 is conserved across all the MIF family members. However, molecule and lineage specific cysteine residues exist, ie a cysteine residue (C1, **Fig. 3**) was conserved in α1 helix in vertebrate DDTs only and a cysteine residue (C2) in β2 sheet was present in all arthropod MIFs except the lone star tick MIF, but is missing in all the other MIF family members. The lineage-specific conservation of critical residues may reflect a lineage-specific evolution that may change substrate preference of the enzyme. It is noteworthy that DDT catalysis results in the production

247 of DHI, a precursor of eumelanin, and itself possessing antiviral, antibacterial, antifungal and antiparasitic
248 activities [3, 14-15]. The identification of DDT paves the way for further investigation into its functional roles
249 in the production of DHI, melanogenesis and in immunity in mud crab and other arthropods.
250
251
252 **3.2. Differential transcript expression of mud crab MIF family members *in vivo***
253 To shed light on the functional roles in mud crab, the expression of the three MIF family members was
254 comparatively studied in eleven tissues/organs from eight cultivated crabs (**Fig. 4**). The expression of all the
255 three MIF family members was detectable in all the tissues examined but with distinct patterns. The spMIF1
256 transcript was highest in hepatopancreas (HP) followed by testis, where the expression was 300-fold lower
257 than in HP, and only low levels were detectable in other tissues (**Fig. 4**). The SpMIF2 transcript was highest in
258 intestine and muscle, with high expression levels also detectable in HP, testis, ejaculatory duct and brain, but
259 the lowest levels in hemocytes. The SpDDT transcript was highest in intestine and hepatopancreas, followed
260 by heart, brain, stomach, testis, ejaculatory duct and hemocytes (**Fig. 4**). Comparatively, the spMIF1 transcript
261 was lower than spMIF2 and spDDT in all tissues except in HP, where the expression of spMIF1 was 8- and
262 11-fold higher than spMIF2 and spDDT, respectively. The spMIF2 transcripts were less varied and the highest
263 in each tissue except in intestine, heart and hemocytes where no significant differences were seen between
264 spMIF2 and spDDT, and in HP (**Fig. 4**).
265
266 The two MIF paralogues, spMIF1 and spMIF2 are differentially expressed, with spMIF1 preferentially
267 expressed in HP and spMIF2 preferentially expressed in other tissues (**Fig. 4**). Differential expression of
268 paralogues in different tissues is a common scenario that may indicate subfunctionalisation/
269 neofunctionalisation [33-34]. MIF transcript expression is high in liver, intestine, muscle and immune tissues
270 and showed species-specific patterns. In our comparative expression analysis of tissue distribution, three
271 tissues, HP, intestine and muscle stand out that highly express the MIF family members, (**Fig. 4**). The

intestine represents the largest compartment of the immune system that is continually exposed to antigens and immunomodulatory agents from the diet and the commensal microbiota, and is the port of entry for many clinically important pathogens [35]. The intestine highly expressed both spMIF2 and spDDT. Vertebrate liver is enriched with macrophages and natural killer cells and is a predominant innate immune organ that plays a vital role in the host defense against microorganisms [3, 36]. Crab HP is a functional counterpart of vertebrate liver, and has been implicated in host defense in invertebrates [19]. Mud crab HP preferentially expressed spMIF1, and also highly expressed spMIF2 and spDDT. High level expression of MIF in HP has also been reported in other crustaceans such as white leg shrimp, mitten crab and mud crab [19-21]. The high level expression of mud crab MIF family members in HP and intestine, and of other crustacean MIFs in HP suggests the MIF family members are significant innate immune molecules in mud crab and other crustaceans.

Another interesting finding is the preferential expression of spMIF2 in muscle. Although first described as a T cell-derived cytokine, MIF has been shown recently to be released by a variety of cell types [4]. Mouse MIF is expressed in different types of skeletal muscle and in C2C12 myotubes, a skeletal muscle cell line, and is implicated as a negative regulator of glucose transport in skeletal muscle [37]. The significance of muscle spMIF2 expression warrants further investigation in mud crab.

3.3. Transcript expression ontogeny of mud crab MIF family members

MIF family members are involved in embryonic development in vertebrates [38], but little is known of their function in arthropods. We examined the transcript expression of MIF family members in embryonic developmental stages (blastula, gastrula, eye placode and pigment), an early larval stage (zoea), the postlarval megalop and juvenile crablets in mud crab (**Fig. 5**). The developmental stages were as described previously [39]. The expression of spMIF1 was the lowest amongst the three MIF family members during the embryonic and post-larval stages. The SpMIF1 transcript was the lowest in the embryonic stages, but increased gradually and reached the highest level in the larval stage with a drop at the megalop stage but increasing again in

297 crablets (**Fig. 5**). Consistent with the high constitutive expression in adult tissues, the spMIF2 transcript was
298 high during the early developmental stages to juveniles, with higher levels detected at the pigmented stage and
299 in megalops (**Fig. 5**). SpDDT transcript expression was also high during early embryonic stages (blastula,
300 gastrula, eye placode) but was decreased at the pigmented and zoeal stages, with a surge in expression in
301 megalops and juvenile crablets (**Fig. 5**).
302
303 The low level of spMIF1 transcripts in juvenile crabs may reflect the fact that spMIF1 is only significantly
304 expressed in the HP as seen in adult crabs (**Fig. 4**) and the relatively small portion of HP in crablets. One
305 interesting finding of the ontogeny expression analysis is the surge of spMIF2 and spDDT in megalops (**Fig.**
306 **5**). The postlarval megalop stage represents both a change in appearance and a change in life style, and is a
307 transitional phase between the passive planktonic existence of the larvae and the more active benthic life of
308 the juvenile crab, and hence may require a change of defense mechanisms against increased pathogen
309 repertoires. Thus the heightened expression of spMIF2 and spDDT in megalops may have an important role in
310 host defense against changing pathogen repertoires. The kinetics of spDDT ontogenic expression is
311 particularly interesting. DDT catalysis results in the production of DHI [1], a precursor of eumelanin involved
312 in insect egg chorion tanning [40] and cuticular hardening [14, 41]. In the pigment stage of embryonic
313 development, chorion tanning must be stopped to get ready for hatching. This coincides with the decrease of
314 spDDT expression. The cuticle or exoskeleton, is an extracellular layer covering the outer surface and protects
315 the insect. Melanisation is one of the major biochemical events involved in insect cuticle hardening or
316 sclerotisation [41]. Although the physiological role of DDT and/or MIF in the production of DHI and
317 eumelanin remains to be determined, the high level expression of spDDT in early embryos, in megalops and
318 crablets, coinciding with the requirement for melanisation at these stages, may suggest a link of their
319 tautomerase activity and melanogenesis in mud crab.
320

321 **4. Conclusions**

Three MIF family members, two MIF paralogues and one DDT-like gene, have been characterised in mud crab. Comparative analysis suggests that the ancestral MIF gene may have had a three exon/two intron structure, and that the separation of MIF and DDT appears to have preceded the divergence of protostomes and deuterostomes. The MIF family members are differentially expressed in tissues of adult mud crabs and during embryonic development and early life. The high level expression of MIF family members in immune tissues, such as intestine and hepatopancreas, suggests an important role in innate immunity. Mud crab DDT is highly expressed in early embryos, in megalops and crablets, and this coincides with the requirement for melanisation, suggesting a potential novel role of DDT in melanogenesis via its tautomerase activity, to produce DHI in mud crab. The clarification of the presence of both MIF and DDT paves the way for further investigation of their functional roles in immunity and in melanogenesis in mud crab and other arthropods.

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Figure legend:

Fig. 1. Phylogenetic tree analysis of mud crab MIF family members with MIF family members from selected vertebrates and arthropods. The tree was constructed using full-length amino acid multiple alignments and the maximum-likelihood method within the MEAG6 program [28]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. The mud crab MIF family members cloned in this report are shaded. The accession numbers of other MIF family members follow the common species and molecule name.

Fig. 2. Comparison of gene organizations of the MIF family members in arthropods and vertebrates. The number in parenthesis is the length (bp) of the gene across the complete open reading frame. The black box with a number represents the coding region of an exon and its size (bp). The black bar represents an intron with the intron size (bp) over the bar and intron phase below. The gene organization of mud crab MIF family members was predicted using the Spidey program based on the cDNA and genomic DNA sequences reported

in this study (**Table 2**). The gene organizations of other MIF family members were extracted from ENSEMBL genes DAPPUDRAFT_308977 (water flea MIF1), DAPPUDRAFT_309058 (water flea MIF2), DAPPUDRAFT_93282 (water flea DDT), ENSG00000240972 (human MIF), ENSG00000099977 (human DDT), ENSMUSG00000033307 (mouse MIF), ENSMUSG00000001666 (mouse DDT), ENSGALG00000006326 (chicken MIF), ENSGALG00000006350 (chicken DDT), ENSDARG00000071336 (zebra fish MIF) ENSDARG00000044751 (zebrafish DDT), ENSXETG00000022357 (frog MIF) and ENSXETT00000048361 (frog DDT).

Fig. 3. Multiple amino acid sequence alignment of MIF family members from vertebrates and arthropods (A) and conserved residues important for enzyme activities (B). The amino acid sequences of the MIF family members from arthropods and selected vertebrates were aligned using ClustalW2 and BOXshaded. The secondary structure of human MIF family members is shown at the top of the alignment. Conserved residues important for human MIF tautomerase activity are indicated by black arrow heads and conserved cysteine residues by red arrow heads. The accession numbers of the sequences used are as in Fig. 1.

Fig. 4. Tissue expression profiles of mud crab MIF family members *in vivo*. The expression of spMIF1, spMIF2 and spDDT in eleven different tissues from eight crabs was determined by real-time PCR. Transcript levels were first calculated using a serial dilution of references in the same PCR run and expressed relative to that of β-actin. Data are shown as means + SEM (n=8). Expression ratios between different MIF family members are shown below, with shaded values indicating a significant difference ($p \leq 0.05$). SV=seminal vesicle, ED=ejaculatory duct and HP= Hepatopancreas.

Fig. 5. Expression profiles of mud crab MIF family members during embryonic and early developmental stages. Embryos at blastula, gastrula, eye placode and pigment stages, larvae (zoea), postlarval megalops and crablets were collected as described in the Materials and Methods. The expression of

MIF family members was first calculated using a serial dilution of references in the same PCR run and normalised to that of β-actin. Data are shown as means + SEM of four pools of embryos or larvae. Expression ratios between different MIF family members are shown below the graphs, with shaded values indicating a significant difference ($p \leq 0.05$). The expression levels between different developmental stages are statistically significant ($p < 0.05$) where letters over the bars are different. EP=Eye placode.

Table 1 Primers used for PCR cloning and real-time PCR analysis

Gene	Primer name	Sequence (5' to 3')	Application
spMIF1	MIF1-GSPF1	GCCTCAGCAAGATGGTCAGCGAGATG	3'-RACE
	MIF1-GSPF2	TCGCCTTCATGGACAAACATCTCGGGAT	3'-RACE
	MIF1-GSPR1	CAGGTCCTGGAACGCCATGAACATTCT	5'-RACE
	MIF1-GSPR2	CAGCCTGCCGATGGAACTCAGTCTG	5'-RACE
	gMIF1-F1	GAAGCCAGGAACGCTGCCACGAT	Genomic PCR
	gMIF1-F2	TCGCCTTCATGGACAAACATCTCGGGAT	Genomic PCR
	gMIF1-F3	GCGGTGGTCAGACTGAGTTCCAT	Genomic PCR
	gMIF1-R3	GGAGATCCCGAGATGTTTGTCCA	Genomic PCR
	MIF1-FL-R	CTGGTCAGTCTGTCTGTAGGAGTAGG	Genomic PCR
	MIF1 F	GAAGCCAGGAACGCTGCCACGAT	Real-time PCR
	MIF1 R	GTCCTGGAACACCATGAACATTCTATCA	Real-time PCR
spMIF2	gMIF2-R1	CGCCAGGAACTCAGTCATTGCTT	Genomic PCR
	gMIF2-F2	AGTGTCCGTAAGGCTTCAAT	Genomic PCR
	gMIF2-R2	CATCATCAGCCCATCATCTTC	Genomic PCR
	MIF2 F	GGAGGAGCGAGTGTCCGTAAGG	Real-time PCR
	MIF2 R	CCCATCATCAGCCCATCATCTTCC	Real-time PCR
spDDT	gDDT-F1	GAGAAGCCACCACCATGCCTATC	Genomic PCR
	gDDT-R1	ACAGCCTGATGTGAGTCGGTAAGT	Genomic PCR
	DDT F	AAGTCAGAGGCTTACTGCATGGTTC	Real-time PCR
	DDT R	CCAAGGGTTGTCTCCAGGTAGGG	Real-time PCR
β-actin	β-actin F	GCCCTTCCTCACGCTATCCT	Real-time PCR
	β-actin R	GCGGCAGTGGTCATCTCCT	Real-time PCR

Table 2 Summary of sequence analysis of MIF family members in mud crab

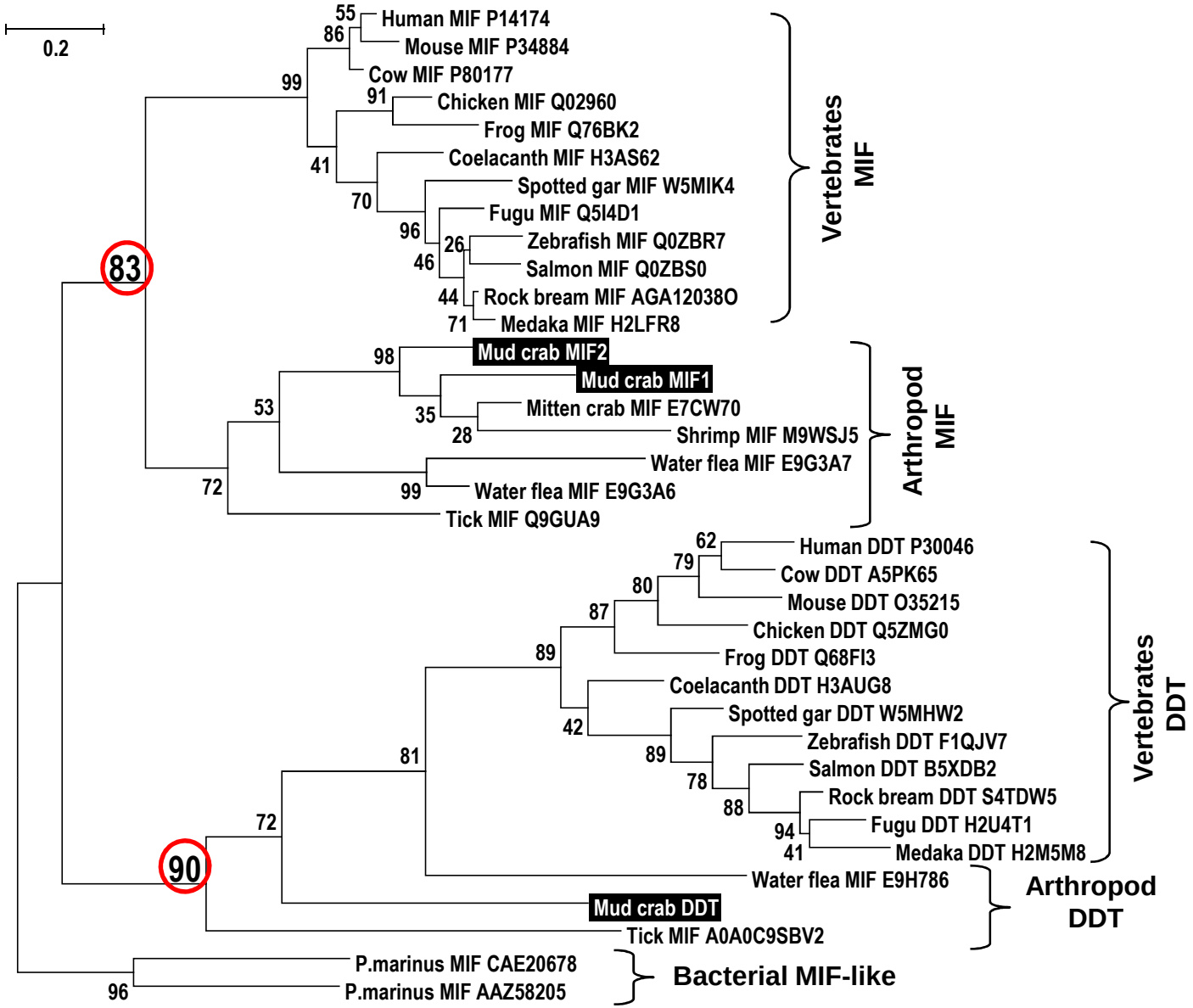
	Features	spMIF1	spMIF2	spDDT
cDNA	GenBank ID [1]	KJ728659	KJ728664	KJ728662
	Length (bp)	735	975	460
	ORF (bp)	363	363	348
Genomic DNA	GenBank ID [1]	KJ728660 KJ728661	KJ728663	KJ728665
	No. of Exons	3	3	3
Protein	Full length (aa)	120	120	115
	Signal peptide	No	No	No
	NN sore[2]	0.575	0.573	0.807
	No of cysteine	2	3	4
	MM/pI [3]	13.4/6.4	13.3/6.7	12.5/6.5
AA identity (%)	spMIF1	100.0	62.5	28.3
	spMIF2	62.5	100.0	27.5
	spDDT	28.3	27.5	100.0

Notes

1 Accession number in Genbank.

2 NN-score predicted by SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP/). Non-classically secreted proteins should obtain an NN-score exceeding the normal threshold of 0.5, but not at the same time be predicted to contain a signal peptide.

3 Theoretical molecular weight (kDa) and pI of the full length protein.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Cow MIF P80177
Chicken MIF Q02960
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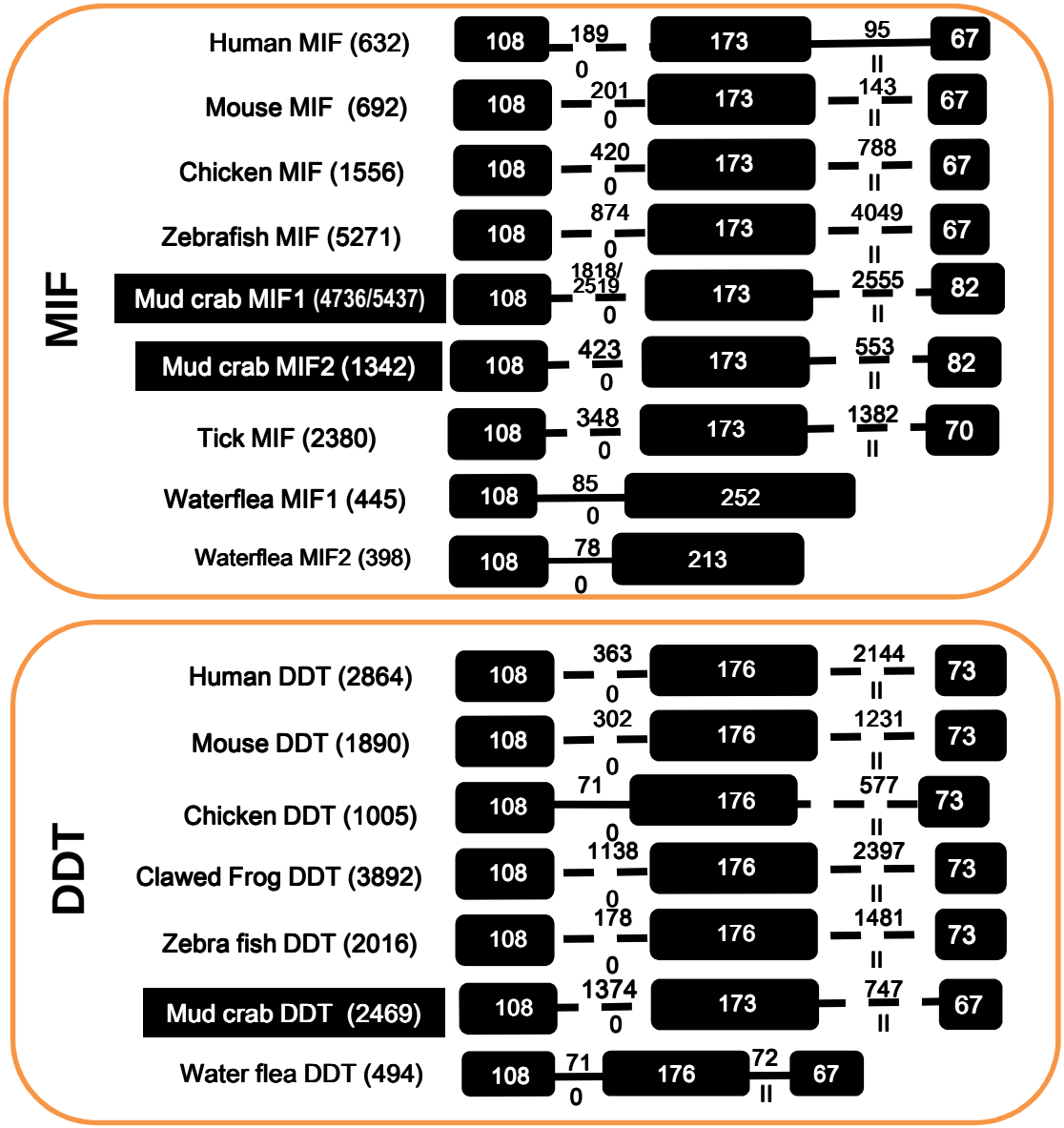
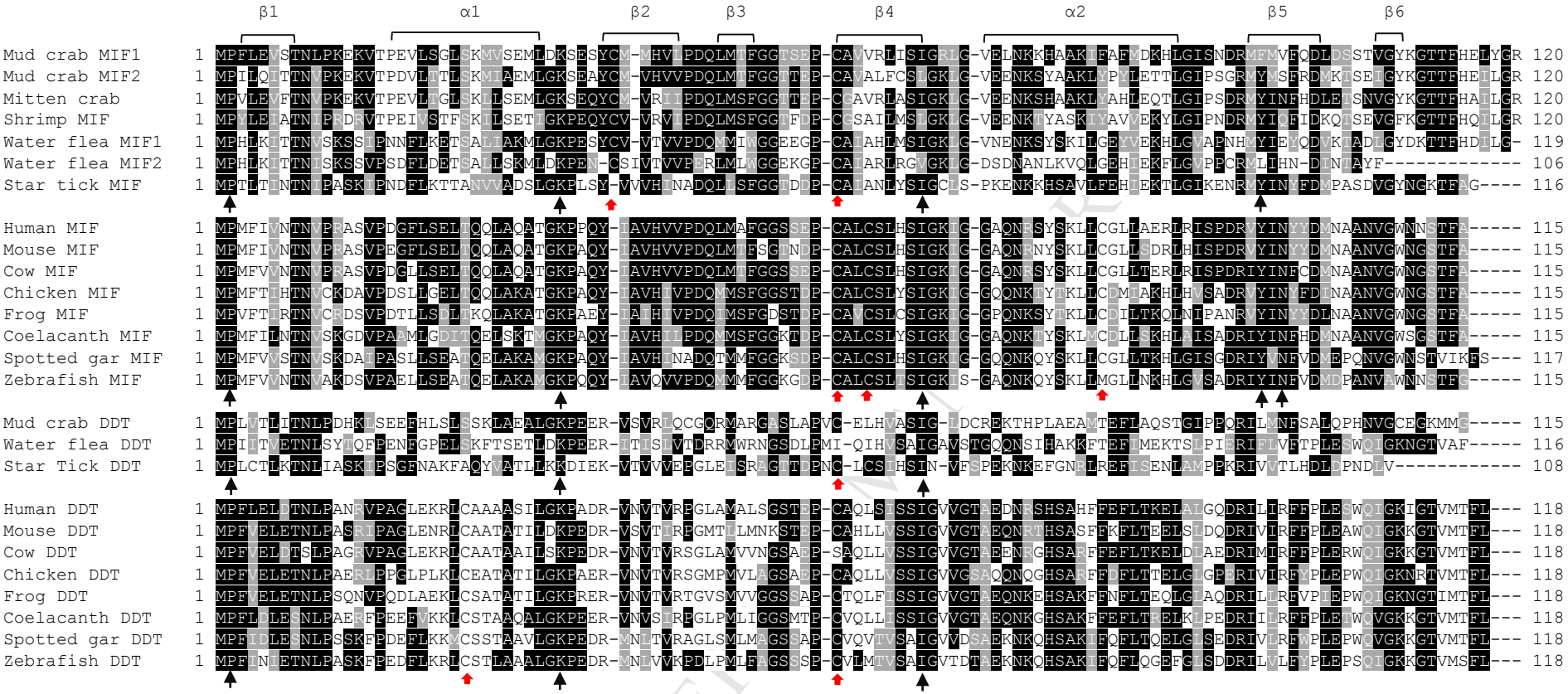
Spotted gar MIF W5MIK4
Fugu MIF Q5I4D1
Zebrafish MIF Q0ZBR7
Salmon MIF Q0ZBS0
Rock bream MIF AGA12038O
Medaka MIF H2LFR8
Vertebrates MIF
Mud crab MIF2
Mud crab MIF1
Mitten crab MIF E7CW70
Shrimp MIF M9WSJ5
Water flea MIF E9G3A7
Water flea MIF E9G3A6
Tick MIF Q9GUA9
Arthropod MIF
Human DDT P30046
Cow DDT A5PK65
Mouse DDT O35215
Chicken DDT Q5ZMG0
Frog DDT Q68FI3
Coelacanth DDT H3AUG8
Spotted gar DDT W5MHW2
Zebrafish DDT F1QJV7
Salmon DDT B5XDB2
Rock bream DDT S4TDW5
Fugu DDT H2U4T1
Medaka DDT H2M5M8
Vertebrates DDT
Water flea MIF E9H786
Mud crab DDT
Tick MIF A0A0C9SBV2
Arthropod DDT
P.marinus MIF CAE20678
P.marinus MIF AAZ58205
Bacterial MIF-like
83
90



A.



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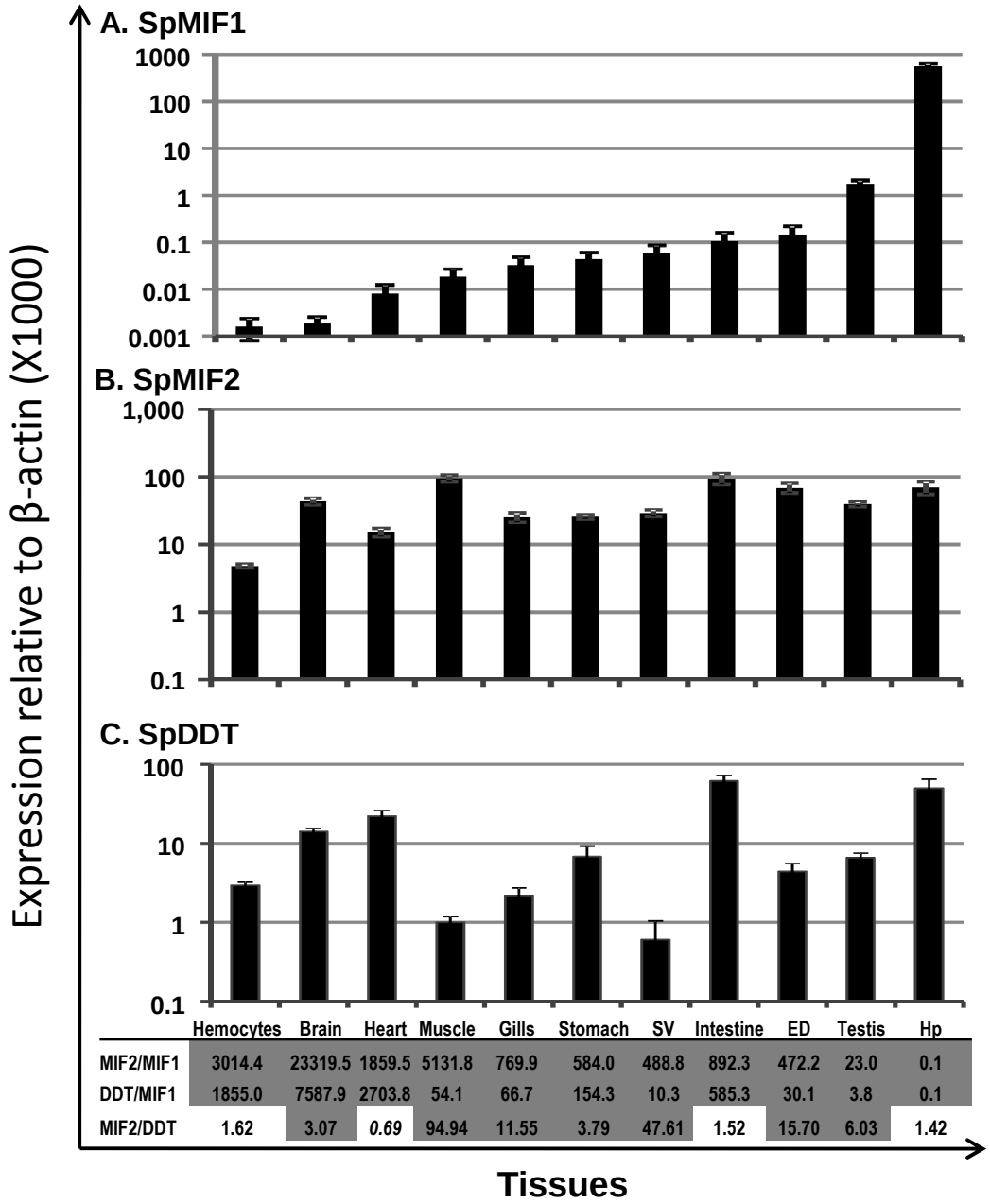
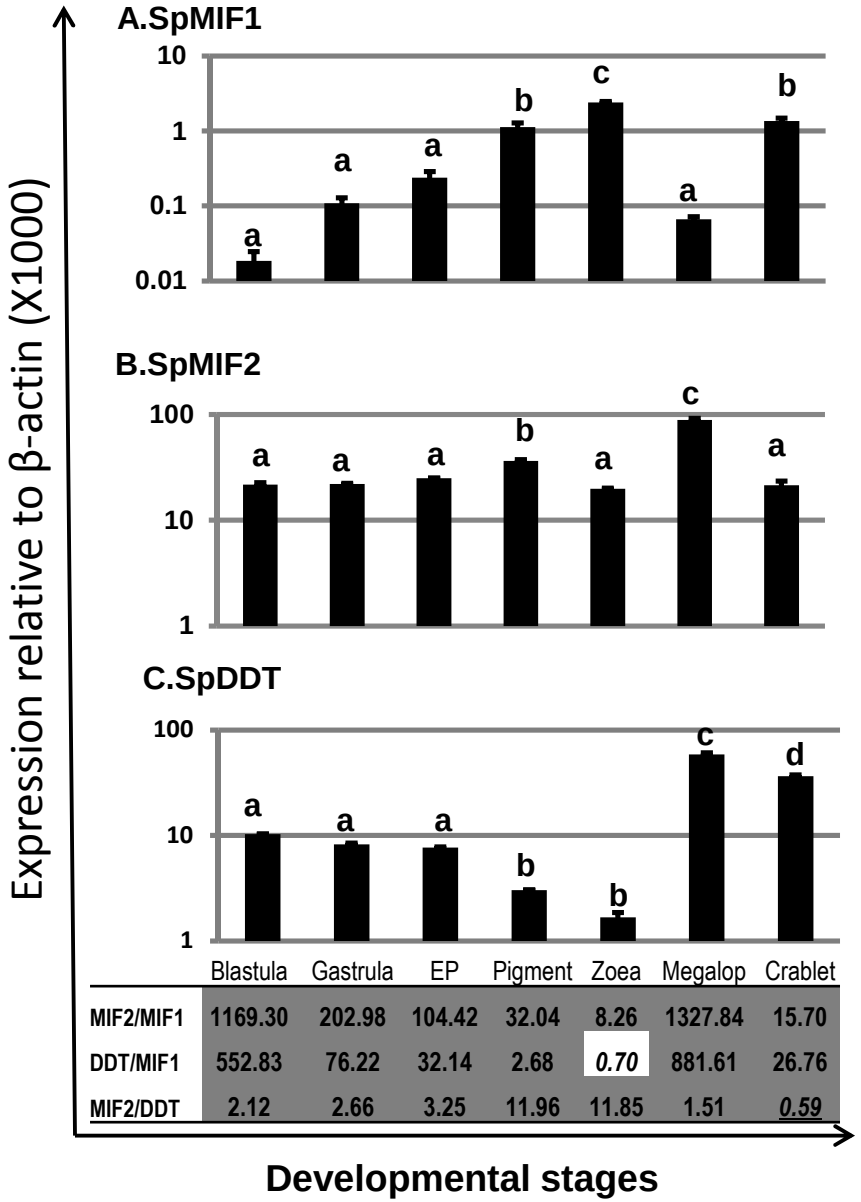


	Hemocytes	Brain	Heart	Muscle	Gills	Stomach	SV	Intestine	ED	Testis	Hp
MIF2/MIF1	3014.4	23319.5	1859.5	5131.8	769.9	584.0	488.8	892.3	472.2	23.0	0.1
DDT/MIF1	1855.0	7587.9	2703.8	54.1	66.7	154.3	10.3	585.3	30.1	3.8	0.1
MIF2/DDT	1.62	3.07	*0.69*	94.94	11.55	3.79	47.61	1.52	15.70	6.03	1.42



	Blastula	Gastrula	EP	Pigment	Zoea	Megalop	Crablet
MIF2/MIF1	1169.30	202.98	104.42	32.04	8.26	1327.84	15.70
DDT/MIF1	552.83	76.22	32.14	2.68	*0.70*	881.61	26.76
MIF2/DDT	2.12	2.66	3.25	11.96	11.85	1.51	*0.59*

Highlights

- The separation of MIF and DDT preceded the divergence of protostomes and deuterostomes.
- Two MIF paralogues and one DDT like gene are present in the mud crab *Scylla paramamosain*.
- Mud crab MIF family members have a three exon/two intron structure.
- The MIF family is differentially expressed in tissues of adults and during development.
- The MIF family is highly expressed in immune tissues, intestine and hepatopancreas.