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cDNA SEQUENCING AND EXPRESSION ANALYSIS OF *Dicentrarchus labrax* HEME OXYGENASE-1

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Abstract – The liver cDNA encoding heme oxygenase-1 (HO-1) was sequenced from European sea bass (*Dicentrarchus labrax*) (accession number no. EF139130). The HO-1 cDNA was 1250 bp in nucleotide length and the open reading frame encoded 277 amino acid residues. The deduced amino acid sequence of the European sea bass had 75% and 50% identity with the amino acid sequences of tetraodontiformes (*Tetraodon nigroviridis* and *Takifugu rubripes*) and human HO-1 proteins, respectively. A short hydrophobic transmembrane domain at the C-terminal region was found, and four histidine residues were highly conserved, including human his^{25} that is essential for HO catalytic activity. RT-PCR of mRNA from eight different European sea bass tissues revealed that, in a homeostatis state, the heme oxygenase-1 was abundant in the spleen and liver but not in the brain.

Key words: Heme oxygenase-1 cDNA, European sea bass, *Dicentrarchus labrax*, cloning, tissue expression.

INTRODUCTION

Heme oxygenase (HO), a microsomal enzyme, catalyzes the rate-limiting enzymatic step of heme degradation. HO cleaves heme to form three biologically active products: biliverdin, an effective anti-oxidant and antiviral complex (5), which is further converted to bilirubin by bilirubin reductase; carbon monoxide (CO) which is a newly identified signaling molecule (11); free iron (Fe^{2+}) which regulates certain gene expression, such as ferritin, transferrin and NO synthase (19). To date, three isoforms of HO have been identified: HO-1 (19), HO-2 (17) and HO-3 (21). These isozymes are the products of different genes, and HO-1 and HO-2 share a low amino acid similarity (43%) (19). The inducible isoform of HO, HO-1, is a 32 kDa heat shock protein (HSP-32) which not only plays an important role in heme degradation, but also acts as a protective system maintaining cellular homeostasis. HO-1 gene expression is regulated by various stress stimuli such as UV-light (12), trace metals (3,4) oxidative stresses (10), pesticides (23,29), lipopolysaccharide (31), heat shock (2), hypoxia (8) and hyperoxia (14). Recent studies have focused on the expression of HO-1 in several disease states (27) including transplant rejection, xenograft survival, acute renal injury (1), myocardial ischemia/reperfusion, atherosclerosis**.** Since HO-1 is ubiquitously distributed in mammalian tissues and plays a role in modulating tissue responses to injury in pathophysiological states, we have focused on this isoform in marine fish to understand its gene expression and tissue distribution after pollutant exposures. The model fish marine used for our study is the marine teleost fish, *Dicentrarchus labrax*. The European sea bass is a non-model organism but is an important species in aquaculture. Moreover, coastal areas, which are the most frequently used for aquaculture activities, are likely to be contaminated by a complex mixture of pollutants

including urban, industrial and agricultural wastewaters.

MATERIALS AND METHODS

Dicentrarchus labrax L.

Juvenile European sea bass were reared at "Dentech" a marine fish farm near Toulon, France. European sea bass (10-12 months), with average masses of 80-100 g, were maintained under laboratory conditions (34 ‰ salinity, ambient temperature of 22°C and natural photoperiod) for 1 month.

RNA isolation

Samples of liver, spleen, gill, intestine, kidney, heart, muscle and brain were collected from juveniles sedated in an ice bath and euthanized by cervical dislocation. Tissues were immediately submerged in RNA*later*® (Ambion, Applied Biosystems, France) and stored at -80°C until further analysis. Tissues (50-100 mg) were crushed in 1 ml of EXTRACT-ALL (Eurobio, France). When the homogenization is performed add 200 µl chloroform then vortex for 15 s. and leave at room temperature for 3 min. Then the sample is centrifuged at 12000g for 15 min. at 4°C. Following the centrifugation, the aqueous phase is transfered to a new tube and 500 µl of isopropanol is added and lead for 10 min. at room temperature. After a centrifugation (12000g, 10 min., 4°C), supernatant is removed and the RNA pellet is washed in 70% ethanol. Following centrifugation (7500g, 5 min., 4°C), supernatant is removed and RNA pellet is air dried for 10 min. The RNA pellet is redissolved in 50 µl of distilled water. The concentration and quality of the RNA samples were assessed by spectrophotometry and by 1.5% (w/v) agarose gel electrophoresis. Any DNA contamination was removed by digestion with 2U/µl DNase I, RNase-free (Ambion) at 37°C for 30 min.

Primers

The oligonucleotides used as reverse transcriptase - polymerase chain reaction (RT-PCR) primers are listed in Table 1. The non-specific primers were designed on a region of HO-1 cDNAs from *Gillichthys mirabilis* (GenBank accession no. AF266203). Specific primers were then suited to our new sea bass sequence.

Table 1. Primers used in RT-PCR, 3' and 5' RACE analysis for sequencing and expression analysis of *Dicentrarchus labrax* HO-1 in liver.

Primer	Sequence	cDNA position from liver sea bass
RT-PCR		
Tail	5' CCA CGC GTC GAC TAG 3'	
PolydTTVtail	5' CCA CGC GTC GAC TAG TAC TTT TTT TTT TTT TTT TTT TTV T 3'	*
HO1dirtail	5' CCA CGC GTC GAC TAG TAC yTs ATG mdG ArC TTy CAr ArG GG 3'	*
HO1dir	5' yTs ATG mdG ArC TTy CAr ArG GG 3'	216 → 237
HO1rev	5' CCd GAs AGG TCw CCs Ahy TAy CG 3'	525 ← 547
5' and 3' RACE		
HO1RT-2	5'(P)-GCT TAT ACT GTT GCA GGG TGA CTT 3'	*
HO1A1-2	5' CAT GAC TCT CCT TTG TCA CC 3'	176 ← 195
HO1A2-2	5' GAT CCC TGT CAG TAA TCT GC 3'	137 ← 156
HO1S1-2	5' CAG GGC AGA AAA CAC AGA GC 3'	197 → 216
HO1S2-2	5' GAT GCT GAG CTT CCA GAG GG 3'	218 → 237
tail2	5' CCA CGC GTC GAC TAG TAC AGC CAA TGA 3'	*
PolydTTVtail2	5' CCA CGC GTC GAC TAG TAC AGC CAA TGA TTT TTT TTT TTT TTT TTT TTV 3'	*
S1DlHO-v2	5' ACC CAT TTA CTT CCC GG 3'	344 → 360
HO1Dl_S658	5' CAG CTG TAT CGC AGC CGG ATG AAC AGC 3'	654 → 680
HO1Dl_S736	5' GCC TTT GAG TTT AAT ATT CAG GTC TTT GAT G 3'	732 → 762

The European sea bass HO-1 cDNA was sequenced in three fragments by RT and PCR amplification of the coding region and by rapid amplification of cDNA ends (RACE) analysis. The partial HO1 sequence of *Dicentrarchus labrax* was performed with three RT-PCR reactions using non-specific primers (polydTTVtail, HO1dirtail, HO1dir, HO1rev) designed from a conserved region of *Gillichthys mirabilis* HO-1 mRNA (GenBank accession no. AF266203). The cDNA was synthesized by incubating 1µg of liver total RNA in 20 µl reaction volume: 5X first-strand buffer, 200 U/µl Superscript II Reverse Transcriptase (Invitrogen), 5 mM of dNTPs, 100 mM of DTT, 40 U/µl RNase Inhibitor (Eurobio) and 50 µM of polydTTVtail at 42°C for 40 min then at 70°C for 15 min. The first PCR was performed using one µl of the first-strand mixture in a 50 µl total volume combining 10X PCR buffer, 50 mM of $MgCl_2$, 5 mM of dNTPs, 5 U/µl of Taq DNA Polymerase (Invitrogen) and the set of non specific primers (10 µM) polydTTVtail and HO1dirtail. Ten µl of DNA obtained was diluted to 500 µl with water and 1 µl was further amplified by a second PCR using the set of non-specific primers (10 µM) HO1dir and HO1rev. The amplification procedure of these two PCR consisted of 2 min at 94°C followed by 30 cycles at 94°C for 15 s (DNA denaturation), 71°C (first PCR) or 50°C (second PCR) (annealing) for 45 s, 72°C for 45 s (extension) and the extension phase of the last cycle was prolonged by 5 min. The amplified fragment (270 pb) was sequenced by Genome Express, France.

For the 5' RACE, 1 µg of total RNA of sea bass liver was reverse transcribed to cDNA with 5 U/µl AMV Reverse Transcriptase XL (Takara, 5' full RACE Core Set) and 200 µM of 5' end-phosphorylated RT primer (HO1RT-2) for the subsequent ligation, following the manufacturer's instructions. Ten µl of tailed cDNA were diluted with 100 µl of water. One µl of diluted tailed cDNA was then amplified by a first PCR using 10 µM of the internal specific primers (HO1A1-2, HO1S1-2). Ten µl of first PCR DNA products were diluted to 1000 µl with water and 1 µl was re-amplified with the same enzyme but with 10 µM of the external specific primers (HO1A2-2, HO1S2-2). These two PCR reactions were performed in 50 µl of total volume combining 5 U/µl Hot Master Taq DNA Polymerase (Eppendorf), 10X Hotmaster Taq Buffer, 5 mM of dNTPs, and following the amplification procedure : 2 min at 94°C, 30 cycles of 45 s at 94°C (DNA denaturation), 45 s at 55.8°C (first PCR) or 64.9°C (second PCR) (annealing), 2 min at 72°C (extension) and a final extension performed at 72°C for 5 min. The amplified fragment (488 bp) was sequenced by Genome Express, France.

For the 3' RACE, two steps (4 PCR reactions) were necessary to obtain the 3' end DNA sequence of sea bass HO-1. 2.53 µg of total liver RNA were reverse transcribed to cDNA in a 20 µl final volume combining 10X RNA PCR buffer, 25 mM of $MgCl_2$, 10 mM of dNTPs, 5 U/µl AMV Reverse Transcriptase XL (Takara, 3'-full RACE Core Set), 40 U/µl RNase inhibitor and 10 µM of an oligo $(dT)_{22}$ containing an anchor sequence (polydTTVtail2). The RT reaction was performed at 30°C for 10 min followed by a cycle at 50°C for 30 min. The enzyme denaturation phase was performed at 95°C for 5 min. A reaction without enzyme was included as negative control. For the first PCR, 20 µl of RT reaction were amplified in a final volume of 100 µl. The reaction mix included 10X Hot Master taq Buffer, 5 mM of dNTPs, 5 U/µl Hot Master Taq DNA Polymerase (Eppendorf) and a pair of 10 µM primers (polydTTVtail2, HO1S1-2). For the second PCR, the forward primer (S1DlHO-v2) was designed from the nucleotide sequence (488 bp) obtained with the 5' RACE amplification and the reverse primer (tail2) was complementary to the anchor sequence. The amplification conditions of these two PCR reactions were similar : 2 min at 94°C , 30 cycles of 45 s at 94°C (DNA denaturation), 45 s at 55°C (annealing), 2 min at 72°C (extension). The extension phase of the last cycle was prolonged by 5 min. As the result of this 3' RACE (step I), a single fragment (908 bp) was obtained and sequenced.

Because the amplification product was scarce, the DNA 3'end sequence was not obtained and a second 3' RACE amplification was performed with a new pair of specific primers: (polydTTVtail2 & HO1Dl_S658) to PCR3 reaction and (tail2 & HO1Dl_S736) to PCR4 reaction. The annealing temperature of amplification procedure was then modified: 63.1°C (PCR3) or 55°C (PCR4) for 45 s. As the result of this 3' RACE (step II), a 575 bp fragment was obtained and sequenced.

Tissue-specific expression of HO-1 gene

Total RNA (1 µg) was isolated from liver, spleen, gills, intestine, kidney, heart, muscle and brain of juvenile European sea bass as described above. The PCR amplification conditions were similar: 2 min at 94°C , 30 cycles of 45 s at 94°C (DNA denaturation), 45 s at 63°C (annealing), 2 min at 72°C (extension). The extension phase of the last cycle was prolonged by 5 min. The PCR was performed using specific primers (10 µM) for HO-1 (sense: HO1Dir201 5'-CCC TGA ATT TCT AGT TGC CCA TGC-3'; antisense: HO1Rev201 5'-TCC GTC AGC TCC ACG CTG TTC ATC-3'), resulting in a 201 bp PCR product corresponding to the amplification between positions +494 and +694 of the HO-1 mRNA sequence.

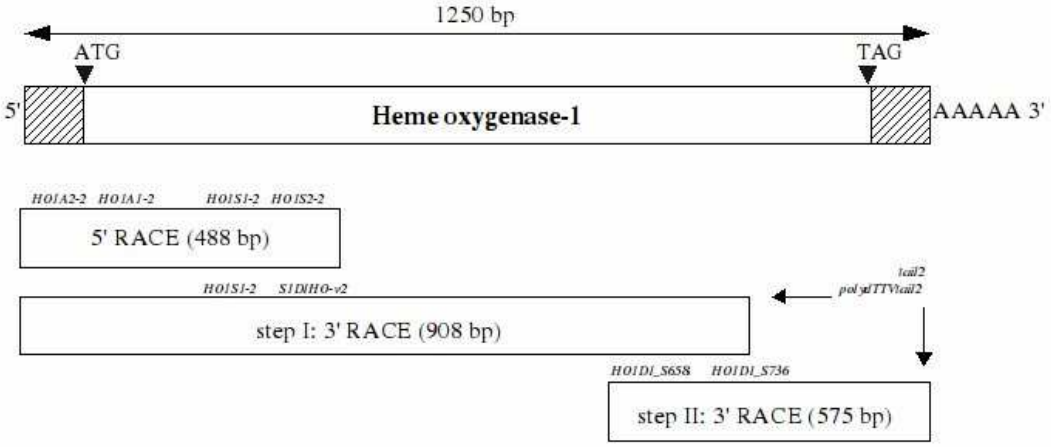
RESULTS AND DISCUSSION

Sequencing of European sea bass HO-1 cDNA

A partial sequence of the cDNA of *Dicentrarchus labrax* HO-1 was initially obtained by RT-PCR based on a region of *Gillichthys mirabilis* HO-1 mRNA (GenBank accession no. AF266203). The complete cDNA sequence was then obtained by 5' and 3' RACE. A 1250 bp cDNA was sequenced (Fig. 1) with a 5'-untranslated region (UTR) of 101 bp and a 3'-UTR of 318 bp, including the polyadenylation signal (AATAAA), typical of eucaryotic mRNAs, at nucleotide positions 1211-1216.

The Open Reading Frame (ORF) was 834 bp, corresponding to a deduced protein of 277 amino acid residues. Several conserved functional domains present in cloned HO-1 were also identified in the sea bass HO-1. The deduced HO-1 sequence had three potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr) at N^{227}, N^{233} and N^{249}. HO-1, an integral membrane protein of the smooth endoplasmic reticulum (15), was anchored to the sER membrane via hydrophobic residues at the carboxyl terminus. This portion of protein is not necessary for HO catalytic activity (Maines, 1997).

(A)



(B)

```
   1 ggagcacaaactgtgcaggacatccagaggaaaaacagtgaatctttgtgaatatagacc
  61 aaaccaccacatctacagaacttcatcctaactgggaaacaatggagacagaaaagagga
                                              M  E  T  E  K  R  T      7
 121 ctcagacaacagcagagcagattactgacagggatctgtcagaacaaatcaaaaaggtga
      Q  T  T  A  E  Q  I  T  D  R  D  L  S  E  Q  I  K  K  V  T     27
 181 caaaggagagtcatgtcagggcagaaaacacagagctgatgctgagcttccagaggggac
      K  E  S  H  V  R  A  E  N  T  E  L  M  L  S  F  Q  R  G  Q     47
 241 aagtcaccctgcaacagtataagctcctcctgtcctcgctgtatgagatctaccaggttt
      V  T  L  Q  Q  Y  K  L  L  L  S  S  L  Y  E  I  Y  Q  V  L     67
 301 tggaggaagagctggacaggaactccaaccaccctggcgttgcacccatttacttcccgg
      E  E  E  L  D  R  N  S  N  H  P  G  V  A  P  I  Y  F  P  A     87
 361 ctgaactggccaggctggaggccattgagaaagacctggaatacttctatggccaggact
      E  L  A  R  L  E  A  I  E  K  D  L  E  Y  F  Y  G  Q  D  W    107
 421 ggagagagaggattgttgtccctgcagctactaaaagatactgccacagactcagacaaa
      R  E  R  I  V  V  P  A  A  T  K  R  Y  C  H  R  L  R  Q  I    127
 481 ttggcgcagaaaaccctgaatttctagttgcccatgcttacacccggtacctgggtgacc
      G  A  E  N  P  E  F  L  V  A  H  A  Y  T  R  Y  L  G  D  L    147
 541 tgtctggaggtcaagtcctgggtcggatcgcccaaaagtccatggggctgaagagcagtg
      S  G  G  Q  V  L  G  R  I  A  Q  K  S  M  G  L  K  S  S  D    167
 601 atggtctgtcattctttgccttccctggtgtgtccagccccaacctgttcaaacagctgt
      G  L  S  F  F  A  F  P  G  V  S  S  P  N  L  F  K  Q  L  Y    187
 661 atcgcagccggatgaacagcgtggagctgacggaggaggagaggaacggcgtgctggagg
      R  S  R  M  N  S  V  E  L  T  E  E  E  R  N  G  V  L  E  E    207
 721 aggctgtcagagcctttgagtttaatattcaggtctttgatgatttacagaagatgctga
      A  V  R  A  F  E  F  N  I  Q  V  F  D  D  L  Q  K  M  L  N    227
 781 atgtcactgaaatacagaattgttcaacactctccaaaccagtgaagatgccccagatcc
      V  T  E  I  Q  N  C  S  T  L  S  K  P  V  K  M  P  Q  I  P    247
 841 ccggaaacatgaccaaaactacccctctgcttcggatggtcctaggacttttgtggctc
      G  N  M  T  K  T  T  P  L  L  R  M  V  L  G  L  F  V  A  L    267
 901 tggccacagtcagtatgggaatctatgctttttagagaaaacacacagcgagaaatactc
      A  T  V  S  M  G  I  Y  A  F                                   277
 961 tgtatttgctgttaaggtgttaaggtctttgcttttgtactgtaagattattaaaaggct
1021 tttaacgatttgtaaattacaaaataatgtgtaaaatatttgtaatttaactgagaacca
1081 catttataatatatattcaggaaagctgagggtattgatttttgaatatcacgatgttct
1141 aagactgtcatccagtgtgcgttaaaatgtgcaatatgtgcgctgttaatattgtaaata
1201 ttacctttttaataaaatgttttgttgaaataaaaaaaaaaaaaaaaaa
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Figure 1. (A) Schematic diagram showing strategy for isolating full length mRNA HO-1 in *Dicentrarchus labrax*. The initial fragment, highlighted in grey, was obtained from a region of *Gillichthys mirabilis* HO-1 mRNA (GenBank accession no. AF266203). (B) Nucleotide and deduced amino acid sequences of *Dicentrarchus labrax* Heme oxygenase-1. Numbers on the left side mark the nucleotide residue positions. Numbers on the right side mark the positions of amino acid residues (mature protein). The start (ATG) and stop (TAG) codons and polyadenylation signal (AATAAA) are in bold. The three boxes indicate the putative HO-2 binding region, the HO signature and the potential transmembrane domain, respectively. The three putative N-linked glycosylation (Asn-Xaa-Ser/Thr) sites are underlined. Histidine residues are in bold and double-underlined. The present sequence has been sent to the GenBank and given the accession number EF139130.

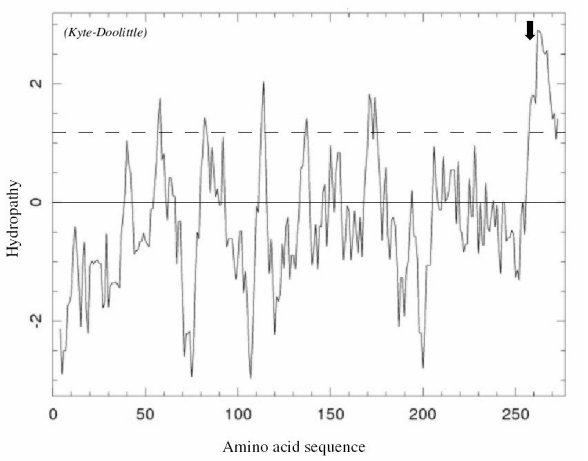

Figure 2. Hydrophobicity profile of the HO-1 amino acid sequence in European sea bass. The hydrophobicity profile was calculated according to Kyte and Doolittle (1982) with a window size of 19. Hydrophobic regions achieve a positive value and a window size of 19-21 yields, a plot in which transmembrane domains stand out sharply, with values of a least 1.6 at their center (http://darwin.cis.fiu.edu:8000/HydropathyPlots/).

A hydropathy profile (Fig. 2) revealed one hydrophobic region at the C-terminus (254-276 amino acid positions). The presence of this short hydrophobic transmembrane domain was also reported in *Rattus norvegicus* (Shibahara et al., 1985) and human (Xia et al., 2004). A BLAST research indicated that the deduced amino acid sequence of the cDNA shared a 75% identity (86% similarity) with *Takifugu rubripes* (GenBank accession no. O73688), 75% identity (85% similarity) with *Tetraodon nigroviridis* (GenBank accession no. CAF95107) and 64% identity (77% similarity) with *Danio rerio* (GenBank accession no. CAQ14965) suggesting that this sequence encodes *D. labrax* Heme Oxygenase-1. This result was confirmed by the presence of a Leu-Val-Ala-His-Ala-Tyr-Thr-Thr-Arg-Tyr-Leu-Gly sequence in *D. labrax* which forms a heme binding pocket. This domain sequence is found in diverse species ranging from bacteria to man. Outside the "HO signature" conserved among all species, a box of 23 amino acids could represent the specific binding region of HO-1 compared to HO-**2.** This HO-1 sequence may function to regulate HO activity, in certain tissues where multiple HO isoforms co-exist such as in the lungs and brain (Wen et al., 2003). The European sea bass "binding region" shows 55% of identity (86% of similarity) with the human binding region (Fig. 3)

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Human              58 YVALEEEIERNKESPVFAPVYF 79
European sea bass  64 YQVLEEELDRNSNHPGVAPIYF 85
                      * .****::**.: * .**:**
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Figure 3. Putative binding region of sea bass HO-1 to HO-2

Sequence comparison of HO-1 protein

The amino acid sequence deduced from the ORF of the European sea bass HO-1 cDNA was compared with the known amino acid sequences of HO-1 from 11 other species (Fig. 4).

The number of amino acid residues (mature protein) is within a narrow range (272-296) for all species except platypus (701). Alignment of amino acid sequences also indicated that the European sea bass HO-1 shares a 75% identity (85-86% similarity) with tetraodontiform (*Tetraodon nigroviridis, Takifugu rubripes).* The identity was lower (64%) with the HO-1 protein reported in cypriniform (*Danio rerio).* The Amino acid identities of the European sea bass sequence with those of human, African clawed frog (*Xenopus laevis*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), pig (*Sus scrofa*), chicken (*Gallus gallus*) and platypus (*Ornithorhynchus anatinus*) were 50, 49, 48, 48, 48, 49, 47 and 46% respectively (Table 2).

A conserved sequence of 23 amino acid residues (Fig. 5) was found in the European sea bass (amino acid positions at 132-153). This sequence was first defined in the rat HO-1 as the heme binding pocket (Maines and Gibbs, 2005) and is now known as the "heme oxygenase signature". The highest identity (100%) was found when the European sea bass region was compared with regions from perciform (long-jawed mudsucker), cypriniform (zebrafish), mammals (human, rat, mouse) and chicken.

```
                          132                 153
European sea bass         PEFLVAHAYTRYLGDLSGGQVL 22
Fugu                      PEYLIAHAYTRYLGDLSGGQVL 22
Pufferfish                PEYLIAHAYTRYLGDLSGGQVL 22
Long jawed Mudsucker      PELLVAHAYTRYLGDLSGGQVL 22
Zebrafish                 PELLVAHAYTRYLGDLSGGQVL 22
Human                     PELLVAHAYTRYLGDLSGGQVL 22
Mouse                     PELLVAHAYTRYLGDLSGGQVL 22
Rat                       PELLVAHAYTRYLGDLSGGQVL 22
Pig                       PELLVAHAYTRYMGDLSGGQVL 22
African clawed frog       PELLVSHAYTRYLGDLSGGQVL 22
Chicken                   PELLVAHAYTRYLGDLSGGQVL 22
Platypus                  PELLMAHAYTRYLGDLSGGQIL 22
                          ** *::******:*******:*

                          HO signature
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Figure 5. Alignment of the Heme binding pocket of heme oxygenase-1 in twelve species: European sea bass (*Dicentrarchus labrax),* fugu (*Takifugu rubripes),* pufferfish (*Tetraodon nigroviridis),* long-jawed mudsucker (*Gillichthys mirabilis),* zebrafish (*Danio rerio),* human (*Homo sapiens),* pig (*Sus scrofa),* rat (*Rattus norvegicus),* mouse (*Mus musculus),* chicken (*Gallus gallus),* platypus (*Ornithorhynchus anatinus),* African clawed frog (*Xenopus laevis).*

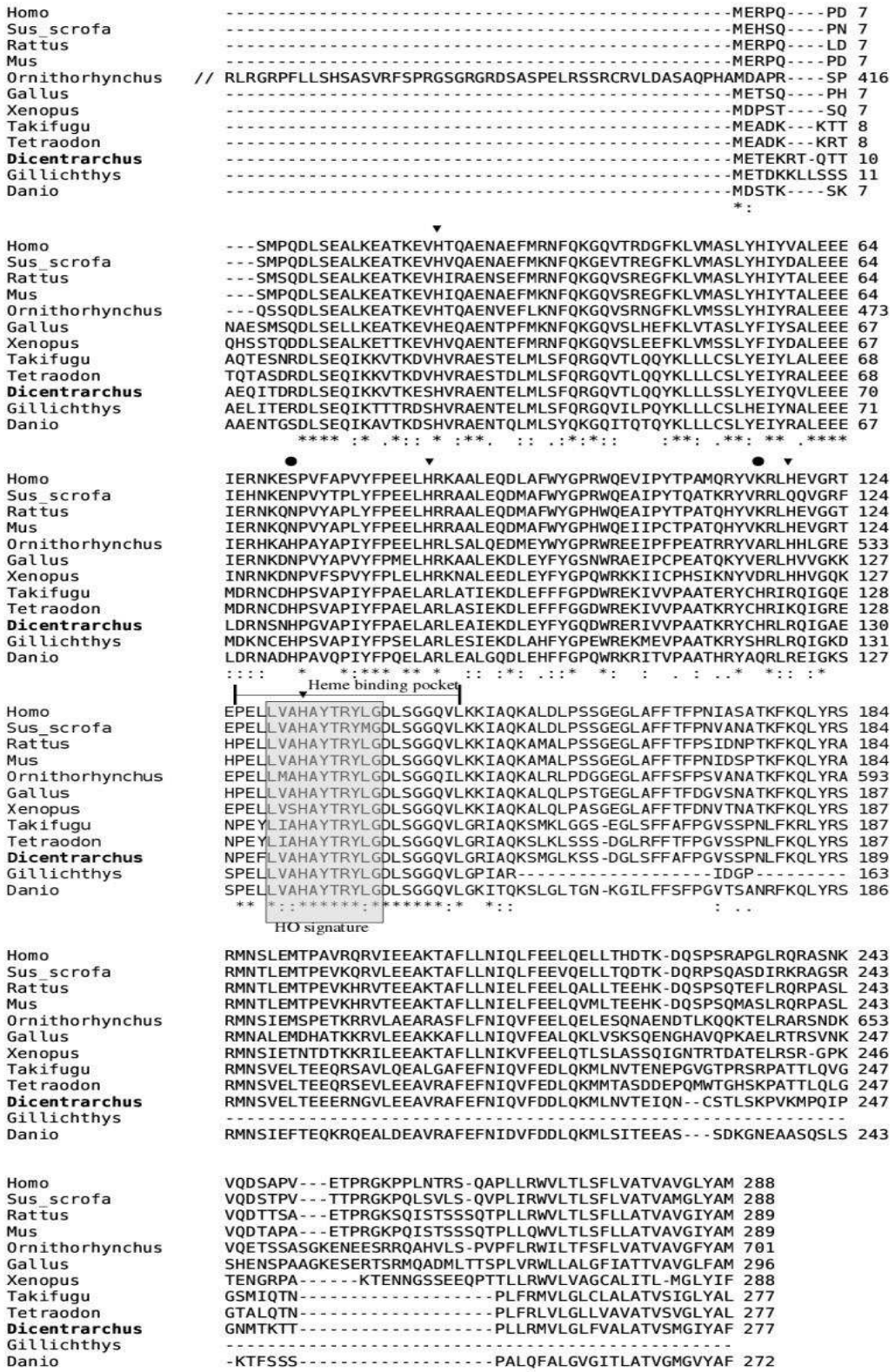

Figure 4. Amino acid sequence alignment (ClustalW2. http://www.ebi.ac.uk/) from *Dicentrarchus labrax* heme oxygenase-1(GenBank accession no. ABL74501) and *Takifugu rubripes* (GenBank accession no. O73688), *Tetraodon nigroviridis* (GenBank accession no. CAF95107), *Gillichthys mirabilis* (GenBank accession no. AAG13323), *Danio rerio* (GenBank accession no. CAQ14965), *Homo sapiens* (GenBank accession no. P09601), *Sus scrofa* (GenBank accession no. P32394), *Rattus norvegicus* (GenBank accession no. P06762), *Mus musculus* (GenBank accession no. NP_034572), *Gallus gallus* (GenBank accession no. NP_990675), *Ornithorhynchus anatinus* (GenBank accession no. XP_001520273), *Xenopus laevis* (GenBank accession no. AAH60435). Asterisk indicates a perfect match among all aligned sequences; (**:**) indicates strongly similar and (**.**) indicates weakly conserved. Histidine residues (His 25, 84, 119 and 132) conserved in mammals are indicated by the symbol (□) and the histidine residues in European sea bass with a different position are indicated by (●).

Table 2. Percent level of identity and similarity of HO-1 proteins from the GenBank database. Levels of identity and similarity of the HO-1 proteins were calculated using the NCBI Blastp (http://blast.ncbi.nlm.nih.gov/). The following GenBank database entries were used: *Dicentrarchus labrax* heme oxygenase-1 (GenBank accession no. ABL74501), *Takifugu rubripes* (GenBank accession no. O73688), *Tetraodon nigroviridis* (GenBank accession no. CAF95107), *Gillichthys mirabilis* (GenBank accession no. AAG13323), *Danio rerio* (GenBank accession no. CAQ14965), *Homo sapiens* (GenBank accession no. P09601), *Sus scrofa* (GenBank accession no. P32394), *Rattus norvegicus* (GenBank accession no. P06762), *Mus musculus* (GenBank accession no. NP_034572), *Gallus gallus* (GenBank accession no. NP_990675), *Ornithorhynchus anatinus* (GenBank accession no. XP_001520273), *Xenopus laevis* (GenBank accession no. AAH60435).

HO1	European sea bass	Fugu	Pufferfish	Long-jawed Mudsucker	Zebrafish	Human	Mouse	Rat	Pig	Platypus	African clawed Frog	Chicken
European sea bass		86	85	86	77	67	66	67	67	65	67	66
Fugu	75		93	87	76	65	69	66	67	67	67	66
Pufferfish	75	84		86	76	65	68	67	68	65	68	68
Long-jawed Mudsucker	74	74	73		84	73	74	74	75	76	78	75
Zebrafish	64	68	59	70		66	64	64	66	64	63	64
Human	50	47	50	54	46		91	89	92	80	74	75
Mouse	48	48	47	53	45	82		95	88	74	72	75
Rat	48	47	47	54	43	80	93		86	76	70	75
Pig	48	47	50	54	45	82	78	77		82	74	76
Platypus	46	45	45	52	46	63	62	63	65		72	77
African clawed Frog	49	49	48	55	48	61	58	57	60	57		75
Chicken	47	47	48	56	44	63	62	62	61	63	64	

IDENTITY

SIMILARITY

Histidine residues at positions 25, 84, 119 and 132 in human sequence are conserved in rat mouse, chicken (32), platypus, African clawed frog and pig. Two of these his residues (his^{84} and his^{119}) differ in their position in European sea bass: his^{87} and his^{122} respectively. These four histidine residues are important for heme binding. The his^{25} residue, conserved in all species, is essential for HO-1 catalytic activity (9,13).

Phylogenetic tree of HO-1

Phylogenetic analysis was performed to characterize the evolutionary relationship between European sea bass and various organisms. The deduced amino acid sequence of sea bass HO-1 was aligned with eleven known HO-1 proteins cloned to date using the ClustalW2 program and phylogenetic tree was performed using the Neighbor-Joining method with Phylowin (Fig. 6). The topology of the tree presented in this paper updates the evolutionary relationships of the eleven known heme oxygenase-1 proteins. The consensus tree shows two main branches, one of them clusters all teleosts and the other branch includes tetrapods. *Drosophila melanogaster* was used as an outgroup to root the tree. HO-1 is evolutionary conserved in the primary amino acid sequence. For human, rat and mouse, the extent of HO-1 identity is greater than 80%, as for perciformes and tetraodontiformes.

Tissue-specific expression of HO-1 mRNA

To investigate the tissue-specific distribution of mRNA expression of HO-1 in European sea bass, we performed RT-PCR using total RNA from eight tissues of juvenile sea bass: liver, spleen, gill, intestine, kidney, heart, abdominal skeletal muscle and brain (Fig. 7). The partial cDNA of RPL17 was used as an internal control (data not shown). In the homeostasis state, HO-1 mRNA was expressed in all tissues. Predominant expression was observed in spleen (18,33), which is a key organ to engulf and digest senescent erythrocytes and maintain the HO-1 in an induced state. Its basal transcript level is moderately abundant in liver (16), but increases rapidly after an organism exposure to various agents (24). The transcript level in the brain is the lowest among the seven tissues, what is in agreement with the data of HO-1 distribution (28). In the brain, HO-2 is constitutively expressed and is the predominant isoform

(21,25). Moreover HO-2 is relatively stable throughout all ages, whereas HO-1 was observed to be progressively down-regulated in an age-related manner (6,7).

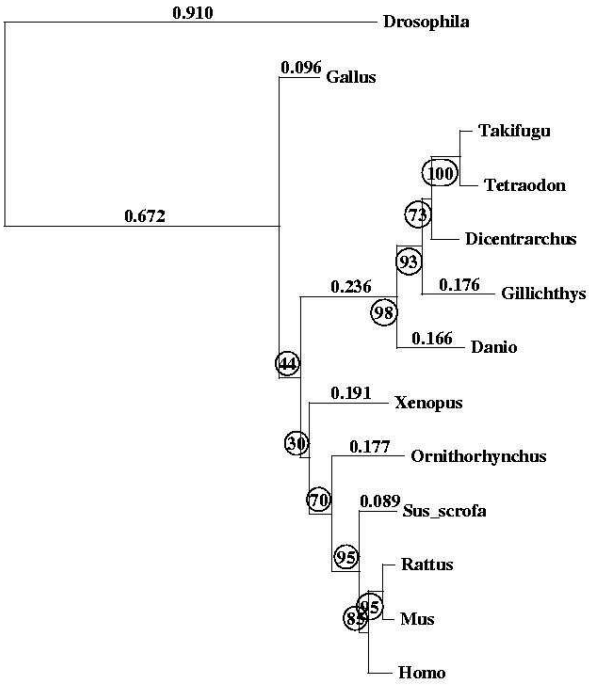

Figure 6. Phylogenetic tree for heme oxygenase-1. The amino acid sequences were aligned using the ClustalW2 program and the phylogenetic tree was performed using the Neighbor-Joining method with Phylowin. To check for the statistical validity of the different nodes in the generated tree, the bootstrapping method was used (Felsenstein, 1985) with a total of 1000 boostraps. HO-1 gene from *Drosophila melanogaster* (GenBank accession no. NP_524321) served as an outgroup to root the tree. Comparisons were made with the amino acid sequences of European sea bass (*Dicentrarchus labrax)*, fugu (*Takifugu rubripes)*, pufferfish (*Tetraodon nigroviridis)*, long-jawed mudsucker (*Gillichthys mirabilis)*, zebrafish (*Danio rerio)*, human (*Homo sapiens)*, pig (*Sus scrofa)*, rat (*Rattus norvegicus)*, mouse (*Mus musculus)*, chicken (*Gallus gallus)*, platypus (*Ornithorhynchus anatinus)*, African clawed frog (*Xenopus laevis).*

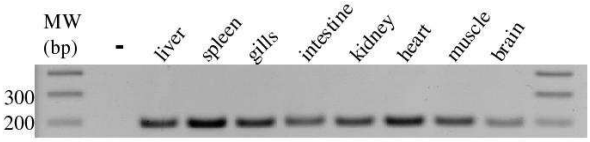

Figure 7. Tissue-specific expression of HO-1 mRNAs. Total RNA from various tissues was analyzed by RT-PCR using sequence specific primers for HO-1 as described in Materials and Methods. A 100 bp molecular weight marker (MW) was used to assess the size of the PCR products. The PCR product size was 201 bp. A negative control (-) was also included in the PCR reaction to discard false positives.

In conclusion, we have cloned and characterized the cDNA encoding heme oxygenase-1 in liver European sea bass (GenBank accession no. EF139130). In this paper we have also demonstrated the expression of this isoform in several tissues in a homeostasis state. However, its expression and regulation patterns, and roles of HO-1 in detoxification mechanisms require further study in *Dicentrarchus labrax*. Current research is ongoing to quantify HO-1 gene expression in various tissues of European sea bass exposed to heavy metal concentrations. This study is aimed at determining whether the gene expression of HO-1 can be use as a possible early biomarker of exposure in ecotoxicological studies.

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