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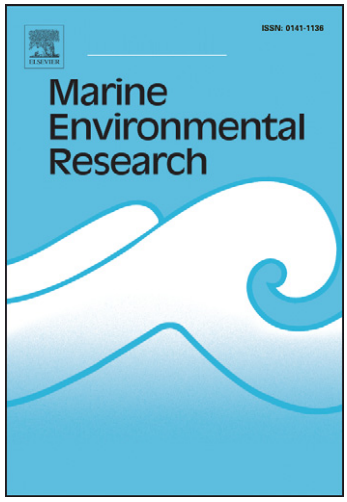

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Cloning a new cytochrome P450 isoform (CYP356A1) from oyster *Crassostrea gigas*

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Abstract

We have cloned the full-length cDNA of the first member of a new cytochrome P450 (CYP) family from the Pacific oyster *Crassostrea gigas*. This new *CYP* gene was obtained based on an initial 331 bp fragment previously identified among the list of the

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differentially expressed genes in oysters exposed to untreated domestic sewage. The full-length *CYP* has an open reading frame of 1500 bp and based on its deduced amino acid sequence was classified as a member of a new subfamily, CYP356A1. A phylogenetic analysis showed that CYP356A1 is closely related to members of the CYP17 and CYP1 subfamilies. Semi-quantitative RT-PCR was performed to analyze the *CYP356A1* expression in different tissues of the oyster (digestive gland, gill, mantle and adductor muscle). Results showed slightly higher *CYP356A1* expression in digestive gland and mantle, than the other tissues, indicating a possible role of the CYP356A1 in xenobiotic biotransformation and/or steroid metabolism.

Keywords: Cytochrome P450; Pacific oyster; Crassostrea gigas; biotransformation enzymes, CYP356A1, CYP17.

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The cytochromes P450 (CYP) superfamily is one of the largest and functionally most diverse protein families. CYP enzymes are associated with xenobiotic biotransformation and other processes, including homeostasis, hormone biosynthesis and degradation, and oxidative stress (Stegeman and Hahn, 1994). CYP enzymes are the most important oxidative (phase I) biotransformation enzymes in terms of catalytic versatility and breadth of xenobiotic biotransformations carried out (Guengerich, 1987). In this study we cloned the full-length cDNA of a member of a new CYP subfamily from the gill of Pacific oyster *Crassostrea gigas* which has been previously identified in the list of up-regulated genes in oysters exposed to domestic sewage (Medeiros et al., this volume).

An initial fragment of 331 bp was previously identified among the list of the differentially expressed genes in oysters exposed to untreated domestic sewage (Medeiros et al., this volume). Amplification of 5' and 3' cDNA ends were performed by SMART RACE (Clontech), using specific primers (forward 5'-CCAGAAGAATTTGACCCACTTCG-3' and reverse 5'-TTTGTAATCGGACGGAAGCTCTAC-3'). Reactions were set to 25 cycles of: 30 s at 94ºC, 30 s at 51ºC, and 2 min at 72ºC. PCR products were analyzed in 1.2% agarose gel and the 550 bp and 400 bp expected products were purified, cloned and sequenced on ABI3730 (Applied Biosystems). The results were analyzed using BioEdit software. Amplification of the internal region was carried out using the primers, forward 5'-GAAAGGCTCTCAGGCATTATCT-3' and reverse 5'-CCTCTTGACATTTTGCTTGG-3'. Amplification conditions were initial denaturation for 2 min at 94ºC, followed by 30 cycles: 30 s at 94ºC, 45 s at 47ºC, and 60 s at 72ºC. PCR product was directly sequenced on MEGABACE 1000 (GE Healthcare). Phylogenetic studies were carried out using Bayesian techniques as implemented in the software MrBayes, which estimates posterior

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probabilities using Metropolis-Hastings coupled Monte Carlo Markov chains (MC^3). Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out in order to analyze the *CYP* expression in different tissues (digestive gland, gill, mantle and adductor muscle) using the forward and reverse primers (forward: 5'-CCAGAAGAATTTGACCCACTTCG-3', reverse: 5'-TTTGTAATCGGACGGAAGCTCTAC-3'). In order to avoid individual variability, each tissue was pooled from 5 oysters and the total RNA was used for this analysis. The densitometry of products was quantified using Scion Image software.

The full-length sequence of the new *CYP* gene has 1500 bp (Fig. 1, Genbank access no. **ABR45717**). The deduced amino acid sequence shows conserved motifs typical of CYP enzymes, such as the heme group binding region, helix-C, helix-I and helix-K motifs (Fig. 1). This sequence was classified by the CYP Nomenclature Committee as a member of a new subfamily, CYP356A1. The phylogenetic analysis demonstrates a close relationship between the CYP356A1 and CYP1 and CYP17 subfamilies (Fig. 2a). CYP356A1 may be classified as an invertebrate CYP Clan 2 (*CYP17*-like) gene, sharing 32-36% amino acid identity (masking out regions of alignment uncertainty) with vertebrate CYP17s. In contrast, CYP356A1 shares a lower percentage identity (30-33%) with CYP1 and CYP2 genes. The CYP17 family is associated with steroid metabolism, while CYP1A is classically used as biomarker of exposure to polycyclic aromatic hydrocarbons (Hahn, 2002). No *CYP17* genes have been found in non-chordate invertebrates, although *CYP17* is present in amphioxus (Mizuta and Kubokawa, 2007), and *CYP17*-like genes were identified in the genome of the purple sea urchin, *Strongylocentrotus purpuratus* (Goldstone et al., 2006). Similarly, *CYP1* and *CYP1*-like genes have been detected in tunicates (CYP1E and

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CYP1F subfamilies; Goldstone et al submitted) and sea urchins (Goldstone et al., 2006; 2007), but no *CYP1* sequences have been found in non-deuterostome invertebrates.

Semi-quantitative RT-PCR results showed that higher expression of *CYP356A1* was observed in digestive gland and mantle when compared to gill and adductor muscle (Fig. 2b). The digestive gland and mantle are important tissues for both biotransformation and steroid metabolism.

Considering that the *CYP356A1* has been identified among the list of the up-regulated genes in oysters exposed to untreated domestic sewage (Medeiros et al., this volume), and that this gene was induced by 1.9-fold in oysters exposed to sewage under laboratory conditions (Medeiros et al., submitted) we suggest to test this parameter as a biomarker of exposure in field studies.

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Figure captions:

Fig. 1. Nucleotide and deduced amino acid sequences of CYP356A1 from *Crassostrea rhizophorae*. Some motifs of CYP signatures are indicated, including the C, K, and I helices, and the heme-binding region.

Fig. 2. (A) Phylogenetic relationships using Bayesian techniques. Phylogenetic relationships were estimated with uninformative prior probabilities using the WAG model of amino acid substitution and prior uniform gamma distributions approximated with four categories (WAG+I+G). Four incrementally heated, randomly seeded Markov chains were run for $3x10^6$ generations, and topologies were sampled every 100th generation. Burnin values were conservatively set at $1x10^6$ generations. (B) Semi-quantitative RT-PCR and densitometry of CYP356A1 in different tissues of oyster *Crassostrea gigas*. Pool of 5 individuals were used for the analysis in each tissue Legend: G, gill; DG, digestive gland; MT, mantle; M, muscle.

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Figure 1

```
   1  TAAGCAGTGGTATCAACGCAGAGTACGCGGGGGCATTCGATGAGAGAAAAACAGTAGAGA
                              M  L  K  L  S  M  N  T  Q  T  V  L  A  G
  61  ACACCAAAGAGAGCAATCATGTTGAAGTTGTCCATGAACACCCAGACCGTTTTAGCGGGA
       I  C  V  G  L  L  V  Y  Y  V  I  K  R  M  R  Y  R  L  P  P
 121  ATATGCGTTGGTCTTTTGGTATATTACGTCATCAAACGGATGCGGTATCGTCTGCCACCC
       G  P  W  C  I  P  L  V  G  H  Y  K  I  Y  S  S  P  E  M  H
 181  GGGCCATGGTGTATCCCTCTTGTTGGTCATTATAAAATTTATTCATCTCCCGAGATGCAC
       K  K  I  A  A  L  S  K  D  Y  G  P  V  V  R  I  S  F  G  P
 241  AAGAAAATCGCAGCGCTGTCCAAGGACTACGGCCCTGTCGTCCGAATTTCGTTTGGCCCC
       Q  T  W  V  V  L  N  D  I  N  T  V  V  E  A  M  V  K  R  K
 301  CAAACCTGGGTTGTGCTTAATGACATCAACACCGTGGTGGAAGCCATGGTCAAAAGGAAG
       A  D  F  A  G  R  P  H  F  T  S  G  D  V  F  T  E  G  G  K
 361  GCTGATTTTGCCGGGAGGCCGCACTTTACATCGGGTGATGTGTTCACAGAAGGAGGAAAG
       D  I  A  F  S  N  Y  S  A  S  W  K  F  H  R  K  I  A  G  K
 421  GATATAGCCTTCAGCAATTATTCAGCTTCCTGGAAATTCCATAGGAAAATAGCCGGAAAG
       A  L  R  H  Y  L  Q  G  D  L  L  E  N  M  I  Q  E  N  M  N
 481  GCTCTCAGGCATTATCTACAAGGAGATTTACTGGAAAACATGATTCAAGAGAACATGAAT
       K  F  L  N  K  M  A  E  E  K  E  P  F  M  F  K  E  Y  V  D
 541  AAATTTTTGAACAAGATGGCCGAGGAAAAAGAGCCGTTTATGTTTAAAGAATACGTCGAT
       L  M  V  F  H  Q  L  Y  T  I  C  F  G  E  K  R  P  T  D  D
 601  CTGATGGTTTTTCATCAACTATACACAATATGCTTTGGAGAAAAGCGTCCCACAGATGAC
       P  E  V  N  K  L  L  K  I  D  N  D  L  I  D  K  L  G  T  G
 661  CCGGAAGTGAATAAACTGCTTAAGATAGACAATGATTTGATTGACAAGCTAGGTACCGGG
       L  F  E  D  I  I  P  Y  F  K  D  I  Y  P  T  K  K  W  Q  M
 721  CTTTTTGAGGATATAATCCCCTATTTTAAAGACATCTATCCAACGAAAAAATGGCAGATG
       F  L  S  M  V  D  E  M  L  T  V  L  R  R  K  F  R  E  H  V
 781  TTTCTCTCCATGGTGGACGAAATGCTCACAGTTCTTAGAAGAAAATTTAGAGAGCATGTT
       E  T  F  Q  P  G  V  N  R  D  F  I  D  S  M  L  I  A  K  Q
 841  GAAACCTTCCAGCCAGGAGTCAACAGGGACTTCATTGACAGCATGTTAATCGCTAAACAG
       E  A  K  D  E  G  D  E  A  A  L  E  V  M  D  D  T  H  L  V
 901  GAAGCGAAGGATGAGGGCGATGAGGCGGCCCTGGAGGTCATGGATGATACGCACCTCGTT
       Q  T  I  S  D  I  F  F  A  G  V  D  T  T  R  F  T  M  D  W
 961  CAGACCATATCTGATATCTTCTTTGCGGGGGTAGACACTACTCGTTTCACAATGGACTGG
       F  V  Y  F  M  T  R  F  P  E  F  Q  A  K  C  Q  E  E  I  D
1021  TTCGTTTATTTCATGACACGATTTCCGGAATTCCAAGCAAAATGTCAAGAGGAAATTGAC
       R  V  V  G  S  E  Q  P  S  M  K  D  R  S  K  L  D  Y  T  E
1081  AGAGTTGTTGGATCAGAACAACCTTCAATGAAGGACAGAAGCAAATTGGATTACACCGAG
       A  C  L  F  E  S  M  R  L  S  N  V  V  G  I  G  L  P  H  M
1141  GCCTGTCTGTTTGAATCGATGCGGCTTTCGAATGTTGTAGGCATAGGGCTCCCACACATG
       T  I  C  D  S  Q  V  G  G  Y  D  V  P  K  G  T  T  V  V  I
1201  ACAATTTGTGATTCACAAGTTGGTGGATACGATGTCCCAAAAGGTACCACTGTAGTCATC
       N  H  W  A  L  H  H  D  P  K  Y  W  K  D  P  E  E  F  D  P
1261  AACCACTGGGCGCTTCACCATGACCCTAAATATTGGAAGGACCCAGAAGAATTTGACCCA
       L  R  Y  L  D  E  N  G  K  M  K  P  A  K  P  D  S  W  L  P
1321  CTTCGCTATCTCGATGAAAACGGTAAAATGAAACCCGCGAAACCAGATAGCTGGCTTCCC
       F  S  A  G  R  R  V  C  L  G  E  S  L  A  K  P  E  I  L  L
1381  TTCTCAGCCGGACGTAGAGTTTGCTTGGGAGAAAGTTTGGCCAAACCAGAAATCCTACTG
       M  C  A  N  L  L  Q  R  F  E  I  S  L  P  E  G  V  K  P  N
1441  ATGTGTGCCAATCTTCTACAGCGATTTGAAATAAGTCTCCCAGAGGGCGTGAAGCCGAAT
       L  E  H  R  L  P  G  F  G  V  E  L  P  S  D  Y  K  I  V  V
1501  TTAGAGCACCGACTTCCGGGCTTTGGCGTAGAGCTTCCGTCCGATTACAAAATCGTGGTG
       K  E  R  N  R  D  -
1561  AAAGAGAGAAATAGAGATTAAAGAATAATGGCGCTTGCGATCTTTTTTGTATTACAGACA
1621  CCACTAACACAACTGACTATTTAATATTAATACAATGT
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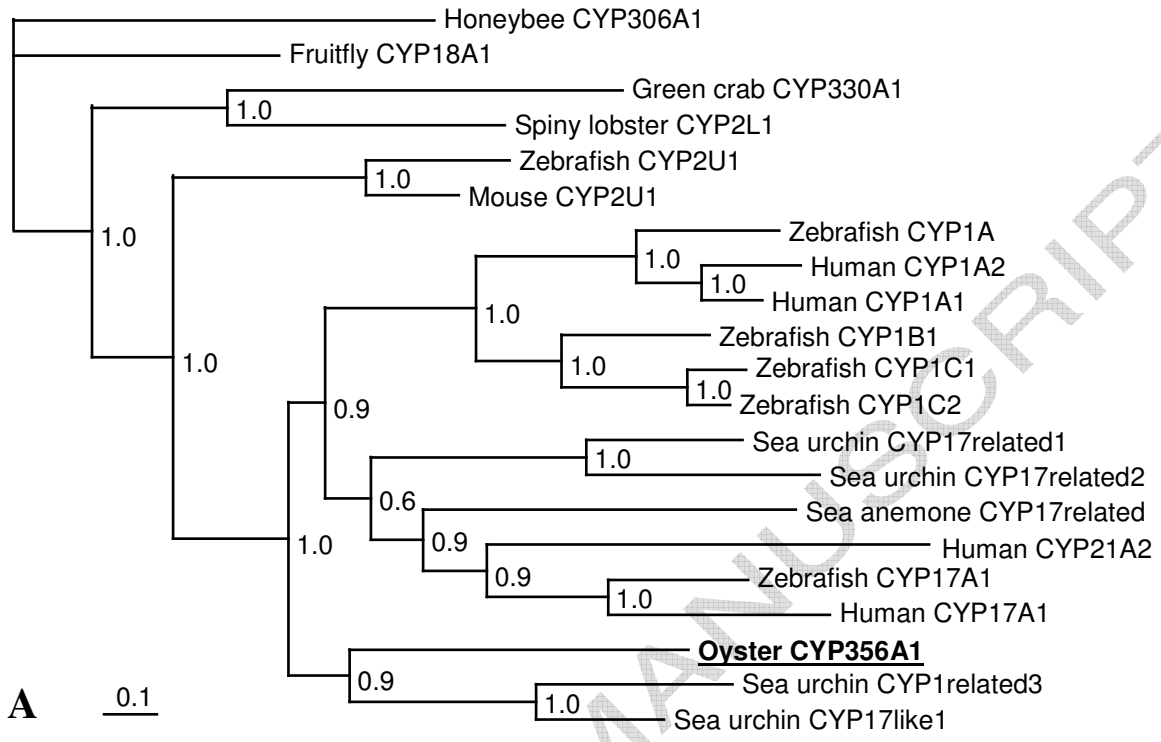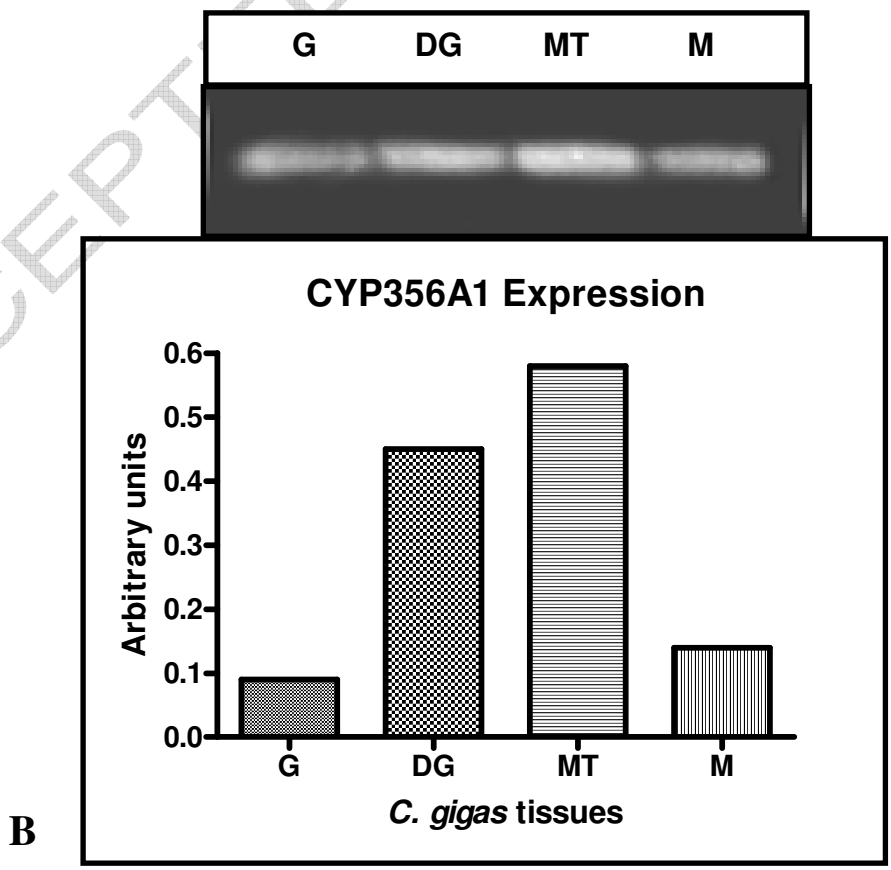
Figure 2