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Gene cloning and expression analysis of AhR and CYP4 from *Pinctada martensii* after exposed to pyrene

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Abstract Pyrene, a typical polycyclic aromatic hydrocarbon, is a common pollutant in the marine environment. Polycyclic aromatic hydrocarbons initiate cellular detoxification in an exposed organism via the activation of the aryl hydrocarbon receptor (AhR). Subsequent metabolism of these xenobiotics is mainly by the cytochrome P450 enzymes of the phase I detoxification system. Full-length complementary DNA sequences from the pearl oyster *Pinctada martensii* (pm) encoding AhR and cytochrome P4 were cloned. The *P. martensii* AhR complementary DNA sequence constitutes an open reading frame that encodes for 848 amino acids. Sequence analysis indicated PmAhR showed high similarity with its homologues of other bivalve species. The cytochrome P(CYP)4 complementary DNA sequence of *P. martensii* constitutes an open reading frame that encodes for 489 amino acids. Quantitative real-time analysis detected both PmAhR and PmCYP4 messenger RNA expressions in the mantle, gill, hepatapancreas and adductor muscle of *P. martensii* exposed to pyrene. The highest transcript-band intensities of PmAhR and PmCYP4 were observed in the gill. Temporal expression of PmAhR and PmCYP4 messenger RNAs induction was observed in gills and increased between 3 and 5 days post exposure; then returned to control level. These results suggest that messenger RNAs of PmAhR and PmCYP4 in pearl oysters might be useful parameters for monitoring marine environment pyrene pollution.

Keywords Pyrene · cDNA cloning · AhR · CYP4 · *Pinctada martensii* · mRNA expression

Introduction

The aryl hydrocarbon receptor (AhR) is a tyical member of transcription factors containing a basic helix-loop-helix (bHLH) and a Per-Arnt-Sim (PAS) domain that regulates the expression of genes in a ligand-dependent manner (Hankinson 1995; Schmidt and Bradfield 1996). AhR can bind and be activated by a variety of chemicals (Denison and Heath-Pagliuso 1998), including a diverse array of anthropogenic compounds present in the environment, such as polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs) and low affinity-binding natural compounds (Denison et al. 2002). The study by Liu and Zhang (2004) showed that the AhR mRNA expressions in gills of the clam *Ruditapes philippinarum* correlated with Benzo(a)pyrene (BaP) concentrations, which suggested it's use as a molecular biomarker of BaP exposure. Normally, the non-ligand AhR exists in the cytoplasmic matrix as a complex with two molecules of HSP90, the

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X-associated protein 2 (XAP2; (Meyer et al. 1998)) and p23 (a co-chaperone protein of 23 kDa) (Kazlauskas et al. 1999). Upon binding to a ligand like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or PAHs, the AhR dissociates from the complex, dimerizes to Arnt (AhR nuclear translocator), and interacts with the xenobiotic responsive element (XRE) so as to control the expression of a specific target genes like Cytochrome P (CYP)1A (Hahn 1998; Schmidt and Bradfield 1996).

Cytochrome P450 system contains 22 subfamilies which encode isozymes that show both constitutive and inducible expression (Simpson 1997) and have been detected in virtually all organisms examined from bacteria to mammals (Scott 1999). Cytochrome P450 enzymes play an essential role in an organism's cellular detoxification system, particularly in metabolizing xenobiotics, such as drugs and chemical compounds including environmental pollutants. Carcinogens (e.g. PAHs) are known to exert their mutagenic and carcinogenic effects only after cytochrome P450-mediated oxidation to polar intermediates (Simpson 1997). CYP4, one of the oldest members of the cytochrome P450 family, is regulated by xenobiotics (Reddy and Rao 1986). Pan et al. (2011) suggested that the induction of CYP4 of *R. philippinarum* could be used as a potential biomarker of PAH pollution in the marine environment.

PAHs are ubiquitous pollutants. Ecological risk indicates that the threat of pyrene is higher than other selected PAHs (Dong et al. 2009; Feng et al. 2009; Wu et al. 2013). The pearl oyster *P. martensii* is one of the most important commercial species from the South China Sea and is the main species for artificial pearl production. The pearl oyster accumulates marine chemical compounds like PAHs due to its filtering habit and low metabolic rate. To date, the research in bivalves has focused on the effect of PAHs on AhRs and gene expression, but little attention has been paid to study of PAHs effect on pearl oysters. The objective of the present study was to investigate PAH-induced stress in the pearl oyster by evaluating the role of the cytochrome P450 system in the detoxification of pyrene. The complete PmAhR and PmCPY4 cDNAs were cloned and characterized in *P. martensii* and the transcriptional changes of both genes in various tissues in response to pyrene exposure were investigated. Results obtained were assessed in the selection of useful biomarkers for monitoring the pollution of PAHs in the marine environment.

Materials and methods

Exposure experiments

Healthy pearl oysters *P. martensii* (aged 1.5 years and height 5.78 ± 0.26 cm), exhibiting normal foot movement and siphoning, were collected from Li'an harbor in Hainan and acclimated to laboratory condition in tanks with aerated sand-filtered seawater (salinity 30 ‰, pH 8.0) at 25 ± 1 °C for 3 days before test. During the acclimation period, the seawater was renewed completely every day and the physical condition was maintained. The animals were not fed.

Pyrene (Sigma, USA) was dissolved in acetone with the initial concentrations and stored in amber glass bottles covered by silver paper. These solutions were then added to the each tank with 20 L seawater to reach finial exposure concentrations of 0, 4, 8, 16, 32 μg L^{-1} which were based on the primary test with the final acetone concentration of 0.001 %. The pearl oysters were randomly divided into five treatment groups with three replicates for each treatment and each tank had 18 individuals. All the experimental conditions were the same as in acclimation. The exposure experiment lasted for 7 days. Three oysters from each replicate were randomly sampled at 0, 3, 5, 7 days. Gills of each oyster from same tank were excised and mixed together, then frozen in liquid nitrogen immediately and stored at −80 °C until used for RNA isolation. The gills of untreated organisms were collected from another three oysters to clone AhR and CYP4 genes. The mantles, gills, hepatopancreas and adductor muscles of untreated oysters were sampled to detect the tissue distribution of gene expressions.

RNA isolation and reverse transcription

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and the contaminated genomic DNA was eliminated using RNase-free DNase (Fermentas, USA) following the protocols. RNA quantity, purity and integrity were clarified by RNA electrophoresis on 1.0 % agarose gel in 1 × TAE buffer (Tris mol L^{-1}, Acetic acid mol L^{-1}, EDTA 0.1 mol L^{-1} pH 8.0) with ethidium bromide (EB) and the UV absorbance ratio at 260 and 280 nm (Nanodrop2000, USA). cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Thermo, USA) at 42 °C for 60 min with oligo dT-adaptor primer (Table 1) following the manuals.

Cloning the target sequences

Degenerate primers (Table 1) were designed in cDNA conserved regions of AhR or CYP4 coding sequences (National Center for Biotechnology Information, NCBI, USA) based on the sequences [AhR: soft-shell clam *Mya. arenaria* (AAF70378.1), mussel *Dreissena. polymorpha* (AAZ8370 0.1), mouse *Mus. musculus* (AAA02896.1), clam *R. philippinarum* (ACM16807.3), scallop *A. farreri* (ACL80140.3), fruit fly *Drosophila melanogaster* (AAD09205.1); CYP4: mouse *Mus. musculus* (AAH08996.1), clam *Meretrix.*

Table 1 Oligo nucleotide primers used in this study

Primer name	Primer sequence (5′-3′)	Application
PmAhR_F	AAYCCNAGYAARCGNCAYC	Degenerate primer
PmAhR_R	AARTCNGGYTTRCTRTTYTTRTA	
PmCYP4_F	ACNCCAGCNTTYCAYTTTGAC	
PmCYP4_R	TTYCTTGRNCCTGCWGAAAADGG	
PmAhR_GSP1	TGGACGGCAAATCAAGCAAAGAGAGTGG	5′RACE
PmAhR_NGSP1	CCTCCCCGTCACAGGTAACAACGAATAG	
PmCYP4_GSP1	TGGGGACTGAAGAGCTTTGGCGCAT	
PmCYP4_NGSP2	AGTTTGGACACCGCCTTCATACGA	
SMARTer II A	AAGCAGTGGTATCAACGCAGAGTACXXXXX	
5′CDS primer	(T)25 V N	
PmAhR_GSP2	GCTGCTCACCAAGAACTGATAAAGACGG	3′RACE
PmAhR_NGSP2	TAGTTCTGGGCTCATCGCTTACCGCTGG	
PmCYP4_GSP2	AGGCGGTGTCCAAACTCATAGGTC	
PmCYP4_NGSP2	ATCCTCAGCAGCTTGACAAACGGT	
3′CDS primer A	AAGCAGTGGTATCAACGCAGAGTAC(T)30VN	
NUP	AAGCAGTGGTATCAACGCAGAGT	
UPA	CTAATACGACTCACTATAGGGC	
PmAhR_QF	CTGTGACGGGGAGGTATTCT	RT-PCR & real-time primer
PmAhR_QR	TGGTTTTGTCTTGAGGTAGGG	
PmCYP4_QF	GATGGCTGACGCTGTAGTGT	
PmCYP4_QR	CGGTATCTGGATTTCCTTGA	
GAPDH_QF	TTTTGGCATTGAGGAAGGTTTG	
GAPDH_QR	CAGTGGAGGATGGTATGATGTTAG	
M13-47	CGCCAGGGTTTTCCCAGTCACG	Vector primer
RV-M	GAGCGGATAACAATTTCACAC	

meretrix (AGC92781.1), clam *R. philippinarum* (ACM16 804.2), scallop *A. farreri* (ACL80141.1), clamworm *Nereis. aibuhitensis* (ADI52567.1), oyster *Crassostrea. gigas* (EKC42662.1), zebra fish *Danio. rerio* (NP_954686.1)]. These sequences were aligned and an internal fragment of each gene was amplified using degenerate primers.

PCR reaction was operated in a total volume of 25 μL using MyCycler Thermal Cycler (Bio-Rad, USA), containing the necessary reaction buffer. For AhR amplication, it was conducted at 95 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min as the final reaction. For CYP4 amplification, a touch-down PCR cycles were conducted: a 3 min denaturation at 95 °C followed by 35 cycles of 94 °C for 30 s, annealing at 60, 58, 56, 54 °C for 1 min at first 4 cycles and 52 °C for 1 min for the rest 31 cycles, 72 °C for 1 min, and a final cycle of 72 °C for 10 min. PCR products were analyzed by electrophoresis on 1.0 % agarose gel stained with goldview in 1 × TAE buffer. The PCR products were purified (Axygen, USA) and inserted into pMD 18-T vector (Takara, Dalian, China). Then the vectors were transformed into *Escherichia coli* DH5α and incubated overnight at 37 °C. Positive clones were identified by blue/white screening and PCR screening with M13-47and RV-M primers, and then were sent to be sequenced (Sangon Biotech).

RACE-PCR

Full lengths of target genes were obtained by using the SMART RACE cDNA amplification kit (Clontech, CA, USA). Gene specific primers (GSPs) were designed from the former cDNA fragment respectively. For 5′RACE, amplifications were performed on cDNA using 1 μM of SMARTer II A primer and 5′CDS primer, then the PCR was performed with UPA primer coupled with1 μM of gene specific primers PmAhR_GSP1 and1 μM PmCYP4_GSP1, followed by the nested PCR with NUP and PmAhR_NGSP1, PmCY P4_NGSP1, the PCR condtions: 1 min for denaturation at 95 °C, then 25 cycles of 95 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min, 72 °C for 10 min; for nested-PCR amplication, 1 min for 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 65 °C, 3 min at 72 °C, then a final cycle of 10 min at 72 °C, respectively. In 3′RACE, the PCR reactions were performed with PmAhR_GSP2, PmCYP4_GSP2 and 3′CDS primer, followed by PmAhR_NGSP2, PmCYP4_NGSP2

and NUP. The preparation methods of cDNA and amplification conditions of both RACE-PCRs were conducted as suggested by the manuals.

Sequence analysis and database comparison were performed using BLAST search of the GenBank (NCBI). Multiple sequence alignments were performed with ClustalW. The unrooted trees were built by MEGA5.1 using Neighbour-joining method, consisting of 1,000 trials with bootstrap.

PmAhR and PmCYP4 tissue expression

The mRNA expression of PmAhR and PmCYP4 genes in the specific tissues (mantle, gill, hepatopancreas, adductor muscle) of pearl oysters were detected by RT-PCR. The fragment of PmAhR and PmCYP4 were amplified by using specific primers (Table 1). Before taking semi-quantitative RT-PCR assays, the template cDNA was detected by control gene adding different volumes to make the product more equal in image. The amplification conditions were as follows: 95 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and 1 cycle of 72 °C for 7 min. Products were analyzed by electrophoresis on 2.0 % agarose gel stained with EB in 1 × TAE.

Quantitative real-time PCR of PmAhR and PmCYP4 mRNAs in gills

The expression level of PmAhR and PmCYP4 mRNAs were acquired by real-time PCR (qPCR), which was performed with SYBR Premix Ex TaqTM II kit (Takara, Dalian, China) using ABI 7500 (Applied Biosystems). The method for checking the quality of total RNA of tissues is same as described above. The specific primers PmAhR-QF and PmAhR-QR and PmCYP4-QF and PmCYP4-QR for qPCR were designed by Primer Premier 5 software (Canada premier, inc.) to amplify a 167 bp fragment of PmAhR gene and a 132 bp of PmCYP4, respectively. The house keeping primers PmGAPDH-QF and PmGAPDH-QR were used to amplify a 134 bp gene fragment as the internal control.

The PCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s, 72 °C for 30 s. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that specific product was amplified and detected. The method used in this experiment was Delta–delta Ct method (Livak and Schmittgen 2001).

Data analysis

The deduced amino acids of genes were aligned by DNAMAN and these sequences were used to construct polygenetic trees by Mega5.1 (neighbor-joining method). Data of gene expression were analyzed by Microsoft excel and the significant difference were performed by one-way ANOVA with Duncan-test (SPSS Inc.), taking $p < 0.05$ as significant, $p < 0.01$ as highly significant.

All primers used in the experiment were listed in Table 1.

Results

PmAhR and PmCYP4 cDNA and the deduced amino acid sequences analysis

A 968-bp partial PmAhR cDNA was amplified using PCR with degenerate primers. Based on this fragment, the complete sequence of PmAhR cDNA with 4,253 bp was obtained by RACE PCR (Supplementary Fig. S1), containing a 5′UTR (Untranslated Region) of 372 bp, a 3′UTR of 1,334 bp with a poly A tail, and an open reading frame (ORF) of 848 amino acids with predicted 95.63 kDa protein and the theoretical pI of 8.61 (Genbank accession number: KJ010544). Multiple alignment analysis of the amino acid sequences revealed high identity to other reported invertebrates AhRs (Supplementary Fig. S2). It showed 74.7 % identity with scallop *A. farreri* AhR (ACL80140.3), 62.7 % with soft-shell clam *M. arenaria* AhR(AF261769_1), 61.5 % with mussel *D. polymorpha* AhR (AAZ83700.1) and 43.5 % with clam *R. philippinarum* AhR (ACM16807.3).

The sequence showed that PmAhR contained a bHLH motif and two PAS domains. Three putative nuclear localization sequences (NLS) were indentified in the PmAhR homologue sequence ($K^{14}RRRR^{18}$; $P^{35}SKRHRE_{41}$; K^{411}-$KQKDQVRDAFVQNNGRKK^{429}$) (Ikuta et al. 1998). In addition, a putative nuclear export sequence was also present ($S^{62}KLDKLSILRL^{72}$). However, PmAhR did not contain a Q-rich region, which has been shown to be important for transactivation in the mammalian AhRs (Fukunaga et al. 1995; Jain et al. 1994), which is same as previous findings that all the bivalves AhRs so far examined also lack this Q-rich region, except for soft-shell clam *M. arenaria* AhR (Butler et al. 2001).

Phylogenetic analyses showed that PmAhR homologue grouped with marine invertebrate AhRs, of clam *R. philippinarum*, scallop *C. farreri* and *C. gigas* etc.; while AhRs of teleost *F. heteroclitus*, zebra fish *D. rerio*, mouse *M. musculus* and human *H. sapiens* etc. were grouped in a separate clade (Fig. 1).

A cDNA fragment of 921 bp was sequenced from *P. martensii*. From the fragment, the 1,855 bp full-length cDNA sequence of PmCYP4 was obtained (Genbank accession number: KJ010543), which contained 1,497 bp of an open reading frame with 489 amino acids, encoding a predicted 58.0 kDa protein and theoretical pI of 8.02, similar size with other bivalves CYP4 s. The signature motif of

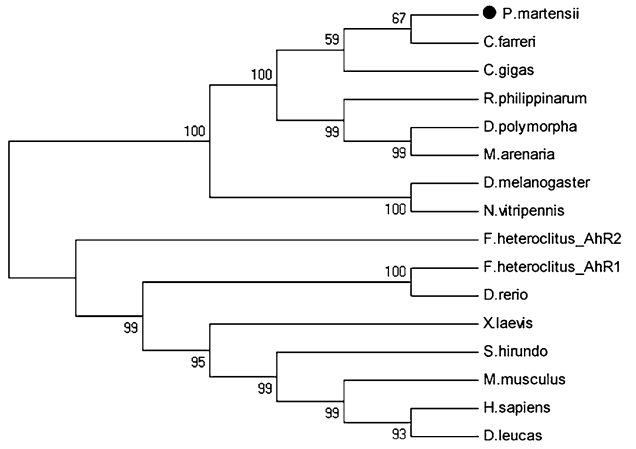

Fig. 1 Neighbor-joining phylogenetic tree of AhR protein sequences from *P. martensii* and other selected species. The tree was constructed by MEGA5.1. The Bootstrap value is 1,000, the number on each branch shows the percentage of bootstrap value

CYP, FxxGxRxCxG (F^{438}SAGPRNCIG^{447}), was identified in the vicinity of the C-terminus, which was corresponded to the heme-binding domain and served as the fifth ligand to the heme iron (Werck-Reichhart and Feyereisen 2000). Another conserved motif, EVDTFMFEGHDTT, was present at amino acid residues 301–313 (Supplementary Fig. S3), including the unique glutamic acid residue E^{308} of CYP4 family (Rewitz et al. 2004).

Multiple alignments demonstrated that the deduced amino acid sequence of the PmCYP4 homologue shared significant similarities with other reported CYP4s (Supplementary Fig. S4). The analyses showed that the amino acid sequence of PmCYP4 showing the highest similarity (50.2 %) with the sequences from clam scallop *A. farreri* CYP4 (ACL80141.1), 42.6 % with gastropod *Cyphoma gibbosum* CYP4 (ACD75825.1), 41.6 % with clam *M. meretrix* CYP4 (AGC92781.1), 40 % with oyster *C. gigas* CYP4 (EKC42662.1), 36.9 % with zebra fish *D. rerio* CYP4T (NP_954686.1), 35.3 % identity with mouse *M. musculus* CYP4b1 (AAH08996.1), 34.7 % with human *H. sapiens* CYP4 (AAL57720.1), and 22.9 % with fruit fly *D. melanogaster* CYP4aa1 (NP_611067.2) .

The phylogenetic relationships between the CYP450 subfamily genes including representative mammals, fish and invertebrates were showed in Fig. 2. PmCYP4 was clustered in a branch with other CYP4s, while other families of CYP1s, CYP2s and CYP3s genes were clustered on separate evolutionary clades.

Tissue distributions of PmAhR and PmCYP4 mRNAs in pearl oyster

PmAhR and PmCYP4 transcripts were examined in mantles, gills, hepatopancreases, adductor muscles of pearl oyster by RT-PCR (Fig. 3). PmAhR and PmCYP4 showed the greatest transcript in gills compared to other tissues, the lowest mRNA level was observed in adductor muscles.

PmAhR and PmCYP4 mRNA expression in gill after pyrene exposure

The mRNA levels were evaluated by qPCR in gills exposed to pyrene with the nominal concentrations of 0, 4, 8, 16, 32 μg L^{-1} (Fig. 4). PmAhR mRNA expression level in 32 μg L^{-1} concentration group was induced significantly ($p < 0.01$) after 3 days exposure; its expression level in all exposed groups were induced dramatically at 5 days exposure ($p < 0.01$), then fell back to control level at 7 days. PmCYP4 mRNA expression showed a similar trend with the expression of PmAhR at 3 days, which was significantly induced in the highest concentration group; the highest expression level of PmCYP4 was observed in 8 μg L^{-1} concentration group at 5 days ($p < 0.01$), then decreased to normal level at 7 days exposure.

Discussion

AhR as an important receptor in biotransfomation of varieties of compounds has been widely studied in mice, humans and other mammals (Hahn 1998). With the growing use of non-mammalian species like bivalves as experimental models in ecotoxicology research, it is increasing important to understand the mechanism of AhR pathway in bivalves. However, to date, there were only few AhR cDNA sequences identified from bivalves (Butler et al. 2001). In this study, a full-length PmAhR cDNA sequence of *P. martensii* has been cloned, and the sequence analysis indicated PmAhR showed high similarities with its homologues of other bivalve species (Fig. 2). Like other known AhRs, PmAhR also possesses a bHLH motif and two PAS domains (Dolwick et al. 1993; Fukunaga and Hankinson 1996; Fukunaga et al. 1995; Swanson and Yang 1996; Whitelaw et al. 1993), which suggests that the cloned PmAhR is an AhR homolog referring to the bHLH-PAS family classification criterion (Atchley and Fitch 1997). In addition, the sequence P^{35}SKRHR^{40} involved in xenobiotic response element (XRE) half site binding was presented in PmAhR bHLH motif (Swanson and Yang 1996). Importantly, two putative NLS are conserved with the human AhR (K^{14}RRRR^{18}; P^{35}SKRHRE^{41}) (Ikuta et al. 1998) and a third theoretical bipartite NLS (K^{411}KQKDQVRDA FVQNNGRKK^{429}) was similar to an NLS from *M. arenaria* (Butler et al. 2001), which is not apparent in mammalian AhRs. In addition, a putative nuclear export sequence is also presented (Butler et al. 2001), as in mammalian AhRs (S^{63}LDKLSVLRL^{72}, with a human V to

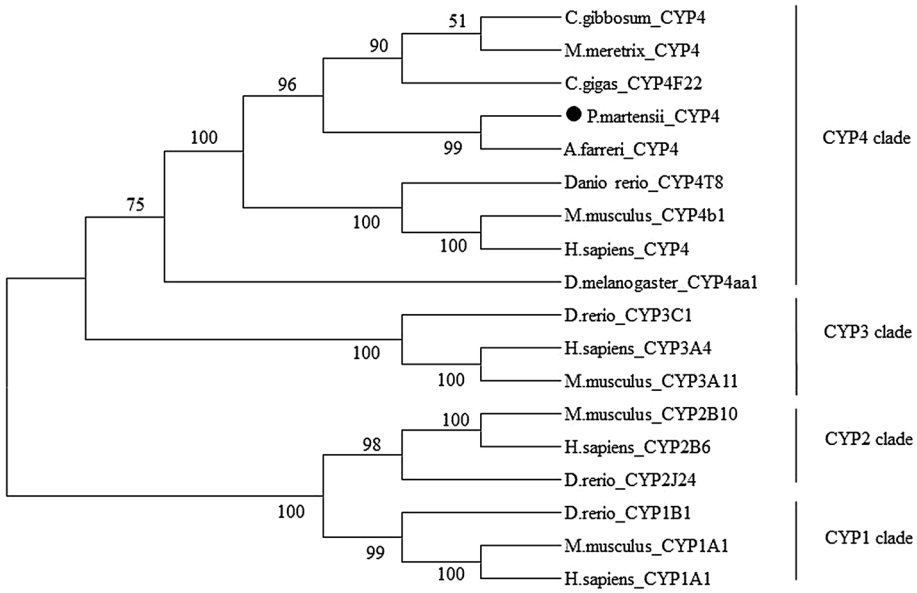

Fig. 2 Phylogenetic tree of CYP450 from different species. Bootstrap values from 1,000 replicates were displayed as percentages

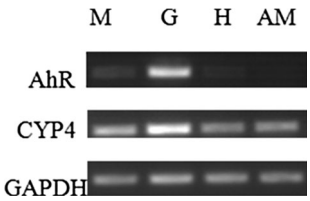

Fig. 3 The oyster AhR and CYP4 transcriptswere examined in four different tissues from *P. martensii*. Representative ethidiumbromide stained agarose gel showing RT-PCR products of the expected sizes (167, 132 bp; respectively) from total RNA isolated from themantle (M), gill (G), hepatopancreas (H) and adductor muscle (Am) of *P. martensii*. GAPDH levels of *P. martensii* were shown for comparison and the expected size was 134 bp

pearl oyster I substitution) (Ikuta et al. 1998). Based on these characteristics, it is predicted that the PmAhR homolog may play a similar role in organism as published in vertebrates.

Multiple analysis showed that the putative ligand-binding domain of the PmAhR (a.a. 219–382) is most closely related to that of the *C. farreri* and *R. philippinarum* (a.a. 220–383), the *M. arenaria* and *D. polymorpha* (a.a. 223–386). However, the C-terminal region of the PmAhR displays little similarity with other AhR sequences and mammalian AhRs have a prominent Q-rich region, which has previously been demonstrated to be important for the transactivation function (Jain et al. 1994), while PmAhR does not include a Q-rich region, which is consistent to other research on bivalves (Liu et al. 2010) except *M. arenaria* (Butler et al. 2001). It is reported that most of the examined fish AhRs exhibited transactivation activity, even though they lack Q-rich regions (Liu et al. 2010), and they were considered to have an unidentified functional transactivation domain. It is predicted that the function domain of the AhR has been altered with evolution. Therefore, we are not able to predict PmAhR lacks the transactivation activity. Phylogenetic analysis showed that PmAhR was grouped with all bivalves AhRs and clustered on a separate clade, providing further evidence that PmAhR belongs to AhR family.

P450 is involved in both synthetic and catabolic reactions. They metabolize endogenous substances including vitamins, steroids, fatty acids and prostaglandins, and also play an important role in metabolizing xenobiotics to detoxified forms, or in some cases to harmful reactive intermediates (Simpson 1997). CYP4 is one of the oldest P450 families having evolved about 1.25 billion years ago, just after the formation of the steroid biosynthetic genes. This family contains 22 subfamilies, which encode isozymes that show both constitutive and inducible expression (Simpson 1997). CYP4 expression is also regulated by xenobiotics (Reddy and Rao 1986). CYP4 enzymes might also be involved in the metabolism of exogenous compounds in invertebrates, including bivalves (Danielson 2002; Jørgensen et al. 2005; Rewitz et al. 2006; Scott et al. 1994; Snyder 1998; Tian et al. 2014). CYP4 genes appear to be highly conserved during evolution and exist in both vertebrates and invertebrates (Nelson 1998). In this study, we isolated a full-length CYP4 cDNA from mollusc *P. martensii* and PmCYP4 sequence shares varieties of common characteristics among different sequences from the P450 superfamily. The signature motif, FxxGxxxCxG, is present at the PmCYP4 amino acids 438–447 (Supplementary Fig. S3), which is essential for heme binding (Yang et al. 2008). Another motif, E^{300}-VDTFLFEGHDTT^{313}, reported as invariant among CYP4 family members, is also conserved in PmCYP4, which is consistent to what reported in other studies (He et al. 2002;

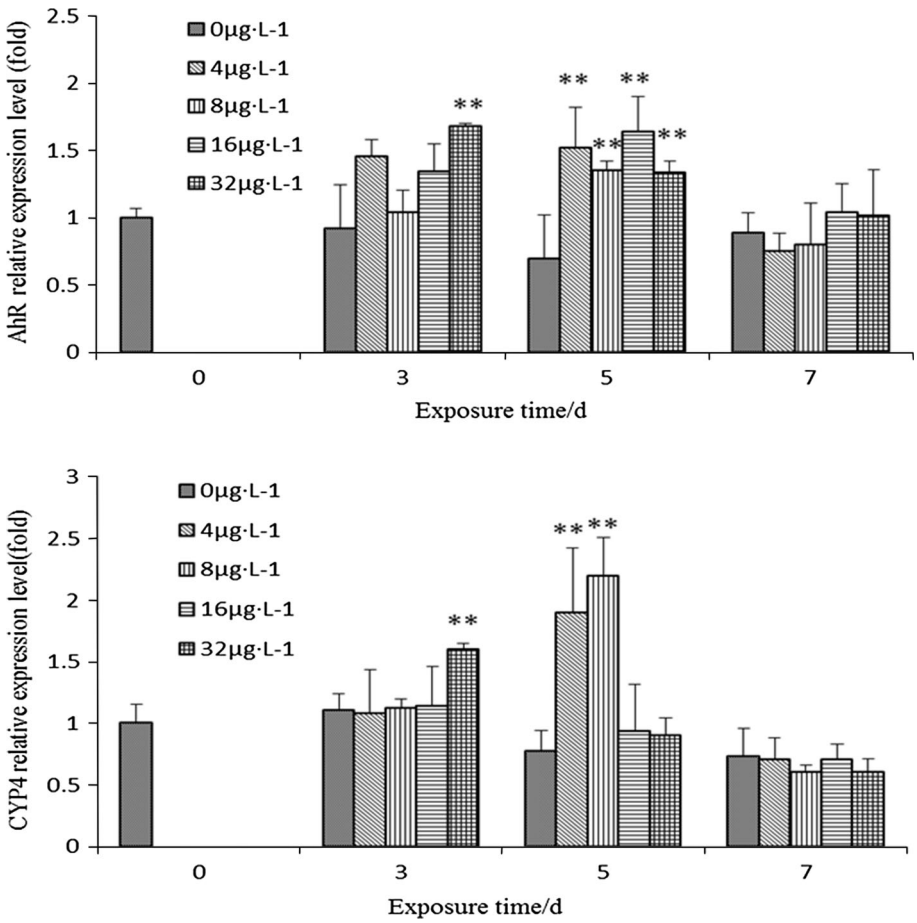

Fig. 4 Temporal expressions of PmAhR and PmCYP4 transcripts in gills after exposed to pyrene were measured by SYBR Green quantitative real-time RT-PCR and results are expressed as normalized fold expressions according to respective GAPDH mRNA levels for the same sample. *Vertical bars* represented the mean ± SD (n = 3). Significant differences from control are indicated with asterisk at $p < 0.01$

Liu and Zhang 2004). Phylogenetic analysis showed that PmCYP4 was clustered with other bivalves CYP4s in another subclade departed from other P450 clans, indicating these members of mollusk CYP4 may function similarly.

The results of RT-PCR showed PmAhR and PmCYP4 transcripts were detected in different tissues (mantle, gill, hepatopancreas, adductor muscle) examined, the highest band intensities of PmAhR and PmCYP4 were observed in the gill. PmAhR tissue transcription result here is consistent with the result of *M. arenaria* (Butler et al. 2001), while PmCYP4 transcription is different from results in other studies (Miao et al. 2011; Pan et al. 2011; Zhou et al. 2010).

AhR as a ligand-activated transcription factor mediates many of the biological and toxic effects of PAHs, TCDD and other structurally diverse ligands via causing gene expression. TCDD is a widely investigated AhR ligand, which is one of the most toxic chemicals. Studies suggest that AhR plays an important and perhaps primary role in the effect of TCDD toxicity on organisms because of its transcriptional function (Butler et al. 2004; Kim et al. 2002; Ohi et al. 2003). Kim et al. (2005) found that AhR expression level of Baikal seal (*Pusa sibirica*) was up-regulated when exposed to PAHs. Kann et al. (2005) reported that AhR mRNA expression levels of mouse embryo fibroblasts were down-regulated by benzo(a)pyrene. Vrzal et al. (2009) observed dexamethasone showed a significant decreasing effects on the AhR mRNA expression of human hepatocytes. A study by Butler et al.(2001) showed that AhR of *M. areaneria* lacks TCDD and beta-naphthoflavone binding sites; Liu et al.(2010) revealed that expression levels of AhR in *R. philippinarum* were induced by BaP and the absolute expression levels of AhR showed temporal and dose-dependent response. In this study, PmAhR mRNA expression levels exposure to pyrene were analyzed, showing a notable temporal-dependent relationship, which is similar to what reported by Bo et al. (2010).

In vertebrates and fish, cytochrome P4501A (CYP1A) induction via AhR is a potent indicator of a persistent exposure of pollutants, such as PAHs, PCBs, dioxins etc., and therefore is widely accepted as an environmental biomarker (Goksøyr and Förlin 1992; Miller et al. 1999; Taysse et al. 1998). However, to date, there are little known about CYP1A of bivalves, the study by Chaty suggested that P450 isozymes other than CYP1A could be induced after exposure to chemicals like PCBs in mussels (Chaty et al. 2004). It is reported that BaP induced CYP4A gene

expression in rat hepatoma cells (Kim et al. 2005). Miao et al. (2011) analyzed the expression of CYP4 in *C. farreri* after exposed to BaP, the results showed that CYP4 of *C. farreri* was significantly decreased in gills and digestive gland during the exposure. While, CYP4 gene of *C. farreri* can be up-regulated by BaP in a dose-dependent manner after 10 days exposure (Tian et al. 2014). Rewitz et al. (2004) reported one of the CYP4 genes expression of *N. virens* was induced by benz(a)anthracene (2.6-fold). The study by Pan et al. (2011) showed that there was an increased expression of CYP4 mRNA after exposure to B(a)P for 10 days in *R. philippinaruman*, whereas the analysis of CYP4 expression in *R. philippinaruman* by Boscolo Papo et al. (2014), did not evidence differences among BaP treatments. The current study showed that CYP4 mRNA expression in *P. martensii* of the highest concentration group was induced significantly in earlier sampling days, while the lower concentration groups were induced after 5 days treatment, which suggests that CYP4 gene expression in the pearl oyster is inducible by PAHs, suggesting CYP4 enzymes in *P. martensii* may play a role in the metabolism of environmental pollutant pyrene. Interestingly, the changes of PmCYP4 and PmAhR gene expressions were similar with the induction of the pollutant in this study; therefore, we speculate that PmCYP4, as same as vertebrates CYP1A, might be able to metabolize PAHs via AhR in pearl oyster. However, the responsible regulation mechanisms of AhR pathway in xenobiotic response elements are still poorly understood, further studies are needed to reveal the underlying mechanisms.

In summary, a full-length AhR and CYP4 genes were cloned in pearl oyster *P. martensii* for the first time. The qPCR results presented here demonstrate that pyrene exposure resulted in an increasing expression level of PmAhR and PmCYP4 genes, which suggests that mRNA expressions of both genes could be used as potential molecular biomarkers of pyrene exposure.

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Conflict of interest The authors declare that there are no conflicts of interest for their work.

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