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CYP1A mRNA expression in redeye mullets (*Liza haematocheila*) from Bohai Bay, China

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ABSTRACT

Induction of cytochrome P4501A (CYP1A) has been used as a biomarker in fish for monitoring aromatic and organic contaminants. In this study, a partial of CYP1A gene in redeye mullet (*Liza haematocheila*) was isolated and sequenced, and then a real-time quantitative reverse-transcription polymerase chain reaction assay was developed for quantification of CYP1A mRNA normalized to β-actin. The developed method was applied to detect CYP1A mRNA expression in redeye mullets collected from Nandaihe (reference site) and Dashentang (impacted site) in Bohai Bay, China. CYP1A mRNA expression values were significantly elevated in redeye mullets from Dashentang compared to a reference site – Nandaihe, which was correlated with the contents of different environmentally relevant pollutants in tissues, particularly with PCBs and PBDEs.

1. Introduction

Biomonitoring for measurement on the direct biomolecular and physiological responses of individual organisms can provide valuable information on the environmental pollutions and improve the process of risk assessment. The bimolecular responses, such as vitellogenin (Vtg), cytochrome P4501A (CYP1A) and metallothionein (MT), indicate not only the presence of pollutants, but also the effects of the pollutants at the molecular level which may result in the adverse physiological disturbances in future (van der Oost et al., 2003).

CYP1A is a superfamily of hemoproteins in various species which catalyze oxidative metabolism of a wide variety of xenobiotics in both endogenous and exogenous origin (Nelson et al., 1996). As a ubiquitous detoxification system in organisms, CYP1A is also sensitive to environmentally relevant concentrations of a wide range of organic contaminants including dioxins, polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs) (Mehmood et al., 1996; Mansuy, 1998). These compounds can induce CYP1A expression through ligands binding to the arylhydrocarbon receptor (AhR) and the induction response can be monitored at the transcriptional level by measuring the change of CYP1A mRNA level (Cousinou et al., 2000; Rees and Li, 2004). Compared to the protein levels of CYP1A, the induction of CYP1A can occur within hours of exposure and the level of CYP1A mRNA decreases when induction response is eliminated. Moreover, the real-time quantitative polymerase chain reaction developed recently is at least 10-fold more sensitive in detecting CYP1A induction over 7-ethoxyresorufin O-deethlyase (EROD) activity and radioimmunoassay, and at least 100-fold more sensitive than Northern or slot blotting in measuring CYP1A RNA (Miller et al., 1999; Cousinou et al., 2000). Therefore, the changes of CYP1A gene expression level in fish are often used as a biomarker for biomonitoring organic contamination, especially PCBs and PAHs exposure, as evaluating the early biological effects (Miller et al., 1999; Roy et al., 2009).

The value of the CYP1A biomarker has been demonstrated in the recent field studies with fish exposed to environmental pollutants such as PAHs and PCBs (Stagg et al., 2000; Fisher et al., 2006; Quiros et al., 2007). Although the induction of CYP1A mRNA in fish does not provide detail information about the exact compounds and exposure concentrations, it may indicate particular chemicals classes such as planar aromatic compounds and the possible impacts on fish health. Furthermore, the change of CYP1A mRNA level can serve as a composite measure of exposure over time rather than a single point in time, and only a very small amount of tissue is required for CYP1A mRNA analysis. In order to design highly specific primers for development of a quantitative PCR assay, it must know the nucleotide sequence of the CYP1A gene of organism.

Bohai Bay in the north of China is one of the economically highly developed area recently. More than 1 billion tons of wastewater from industrial, agriculture and municipal sources was discharged into the bay every year (Meng et al., 2004). Notably, the organic compounds such as PCBs and PAHs were also discharged

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into this area (Hu et al., 2005; Wan et al., 2005, 2007). In this study, redeye mullet (*Liza haematocheila*) was chosen as a sentinel bioindicator because it possesses several excellent characteristics, such as the extreme environmental tolerance, very common in coastal and short-distance migration as a native species. A partial cDNA which encodes the CYP1A gene of the fish was cloned, and the real-time qPCR assay was developed for biomonitoring the coastal pollutants in Bohai Sea using CYP1A as a biomarker. In addition, PAHs, PCBs and PBDEs were analyzed for better understanding the potential causal agents to induce CYP1A.

2. Materials and methods

2.1. Sampling

Dashentang in the north of Bohai Bay is a highly contaminated industrial section of the bay. To compare the contaminated site, a relatively less impacted site in Nandaihe was chosen as a reference site (Fig. 1). Redeye mullets were captured by purse net from Dashentang and Nandaihe in September 2005. The fish was knocked-out by a sharp blow to the head, and body weights and lengths were measured in field (Table 1). The condition factor (CF: a measure of the body form) was calculated for individual fish as follows:

$$CF = W/L^3 \times 100$$

where W is bodyweight (g) and L is total length (cm). The sex of individual fish was determined based on the gonad morphology, and the fish was discarded if their sex could not be determined. The liver and gonads were weighed to determine the hepatosomatic index (HSI: liver weight as a percentage of body weight) and gonadosomatic index (GSI: gonad weight as a percentage of body weight). A partial of liver was frozen in liquid nitrogen in cryogenic vials immediately, subsequently stored in −80 °C after transporting to our laboratory until used for total RNA isolation and CYP1A mRNA analysis.

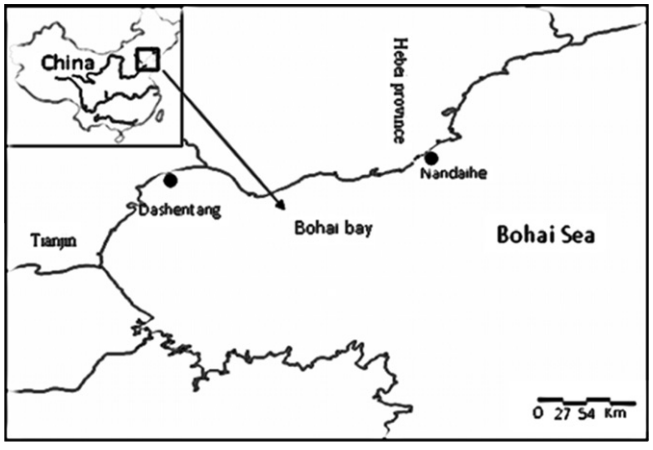

Fig. 1. Sampling sites for collecting the redeye mullet from Dashentang (39°10′41.66″N, 117°57′52.79″E) (impacted site) and Nandaihe (39°43′31.03″N, 119°24′48.17″E) (reference site) in Bohai Bay, north of China.

2.2. Total RNA isolation and first strand cDNA synthesis

About 20–30 mg of liver tissues ($n = 6$ for male and female, respectively) were pulverized in liquid nitrogen and extracted for total RNA isolation using Trizol® Reagent (Invitrogen Ltd., USA) according to the manufacturer's protocol. RNA samples were incubated at 37 °C with RNase-free DNase I (TakaRa Ltd., Dalian, China), and then re-suspended in 50–100 μl of diethylpyrocarbonate-treated water (DEPC–H_2O). RNA concentrations and purity were determined spectrophotometrically at 260 and 280 nm. One microliter of total RNA (about 1 μg) was mixed with 0.25 μl of dNTPs (2.5 mM each) and 0.25 μl of Oligo$(dT)_{15}$ (0.5 μg/μl). The mixture was heated at 70 °C for 10 min and quick chilled on ice. Then, 2 μl of 5× first strand buffer, 0.5 μl of dithiothreitol (0.1 M), and 0.25 μl of SuperScript III (Invitrogen Ltd., USA) were added. The reaction mixture was incubated at 25 °C for 5 min, 42 °C for 5 min, 50 °C for 45 min and inactivated at 70 °C for 15 min. A negative control without reverse transcriptase was performed in parallel. The synthesized cDNA was diluted 10 times with aqua sterilisa and stored at −20 °C until used for PCR.

2.3. Partial amplification and sequencing of CYP1A gene

To isolate CYP1A sequences, a partial of CYP1A was amplified using forward primer 5′-TC(c,g,t)GTGGC(c,t)AA(c,t)GT(a,g,c,-t)ATCTG-3′ and a reverse primer 5′-CA(g,c)CG-(c,t)TTGTG(c,t) TTCAT(g,t)GT-3′. The primers were designated according to a conserved region by alignment of available CYP1A sequences of other species in Genebank. The amplification conditions were 1 min at 95 °C initial denaturation, followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and performed in 50-μl reaction volumes. Products were separated on a 1.2% agarose gel and the expected fragment was purified, cloned, sequenced and blasted. Afterwards, the sequence obtained was aligned to the coding region of other species CYP1A using CLUSTALW algorithm (accession numbers for the genes used in the analysis are listed in Table 2).

Table 2
CYP1A genes and accession numbers of organisms and human used in the phylogenetic analysis.

Name (common name)	GenBank accession number
Liza haematocheila (Redeye mullet)	FJ827632
Liza saliens (Mullet)	AF072899
Anguilla japonica (Japanese eel)	AB020414
Dicentrarchus labrax (Sea bass)	U78316
Limanda limanda (Sand dab)	AJ001724
Stenotomus chrysops (Scup)	U14162
Chaetodon capistratus (Butterflyfish)	U19855
Opsanus tau (Toadfish)	U14161
Microgadus tomcod (Tomcod)	L41886
Oncorhyncus mykiss (Rainbow trout)	AF015660
Salmo salar (Atlantic salmon)	AF361643
Salvelinus fontinalis (Brook trout)	AF539414
Salvelinus namaycush (Lake trout)	AF539415
Danio rerio (Zebrafish)	AB078927
Mus musculus (Mouse)	Y00071
Homo sapiens (Human)	K03191

Table 1
Basic parameters of redeye mullets from Nandaihe and Dashentang. Values are mean with standard deviation.

Site	Sex	Weight (g)	Length (cm)	HSI (%)	GSI (%)	CF	*N*
Nandaihe	Female	25.51 ± 2.24	177.02 ± 45.84	1.85 ± 0.20	0.88 ± 0.11	1.06 ± 0.10	28
	Male	23.48 ± 2.63	176.08 ± 72.97	1.62 ± 0.33	0.44 ± 0.15	1.10 ± 0.09	25
Dashentang	Female	26.30 ± 2.14	198.63 ± 40.47	1.60 ± 0.53	0.68 ± 0.20	1.04 ± 0.08	33
	Male	24.10 ± 2.48	167.80 ± 57.26	1.94 ± 0.27	0.49 ± 0.10	1.11 ± 0.09	34

Phylogenetic tree and genetic distances were determined using the Neighbor-joining method.

2.4. Quantifying CYP1A mRNA in redeye mullets

The real-time qPCR assay for quantifying CYP1A mRNA was developed using β-actin (no. EF638008) as the endogenous control gene. The specific primers of CYP1A and β-actin were designed from the obtained sequences as following: CYP1A forward primer 5′-ACATCACAGACTCCCTCA-3′, reverse primer 5′-CTCCTGTATCTCT GGGTAA-3′ and β-actin forward primer 5′-GTGATGAAGCCCAG AGCAAGA-3′, reverse primer 5′-TGGTCACAATACCGTGCTCAAT-3′. Amplification of CYP1A and β-actin was done separately using the same volumes of the reverse transcriptase reaction products. Reactions contained 10 μl of SYBR® Green PCR master mix (Applied Biosystems), 250 nM each of forward and reverse primers, and 1 μl of cDNA template in final reaction volume of 20 μl. Reactions were performed using an ABI Prism® 7000 Sequence Detection System (Applied Biosystems) with the following conditions: initial at 95 °C for 2 min and then 40 cycles of 15 s at 95 °C, 60 s at 60 °C.

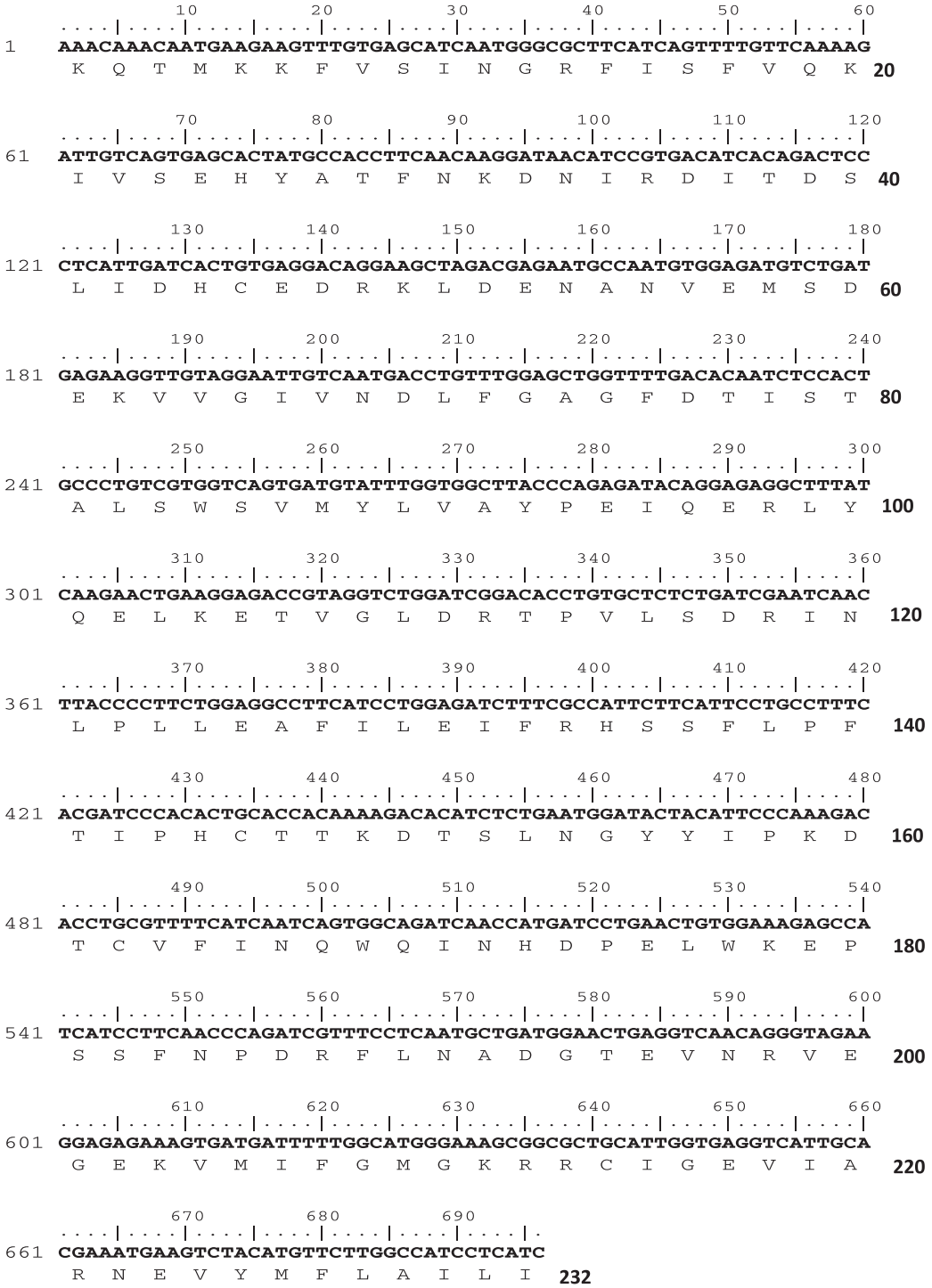

Fig. 2. Partial nucleotide and protein sequence of the redeye mullet CYP1A gene. Left numbers are nucleotide, and right numbers are amino acid residues.

A melting curve analysis after reaction was performed to check the specificity of the products. All samples were analyzed in duplicate and the ratio of CYP1A to β-actin was used to assess the expressions of target gene.

2.5. PAHs, PCBs and PBDEs analysis

PAHs, PCBs and PBDEs in muscles of redeye mullets were analyzed following methods that have been described and validated in previous reports (Wan et al., 2007; Zhao et al., 2010).

For PAHs, about 2 g of dried weight samples were spiked with 4 ng of a 2-flurobiphenyl (2-FBP) as a surrogate (Supelco, USA), and then mixed with anhydrous sodium sulfate for 24 h. The samples were extracted using *n*-hexane:acetone 50:50 (v/v) with ASE300 (DIONEX), and the extracts were purified with activated silica columns and then gel permeation chromatography. Afterwards, 1 μl of extract was used for analyzing PAHs by gas chromatography (Agilent 7890) coupled with mass spectrometry (Agilent 5975C) using an electron impact ionization source (EI) in the selected ion monitoring (SIM) mode. The ongoing precision and

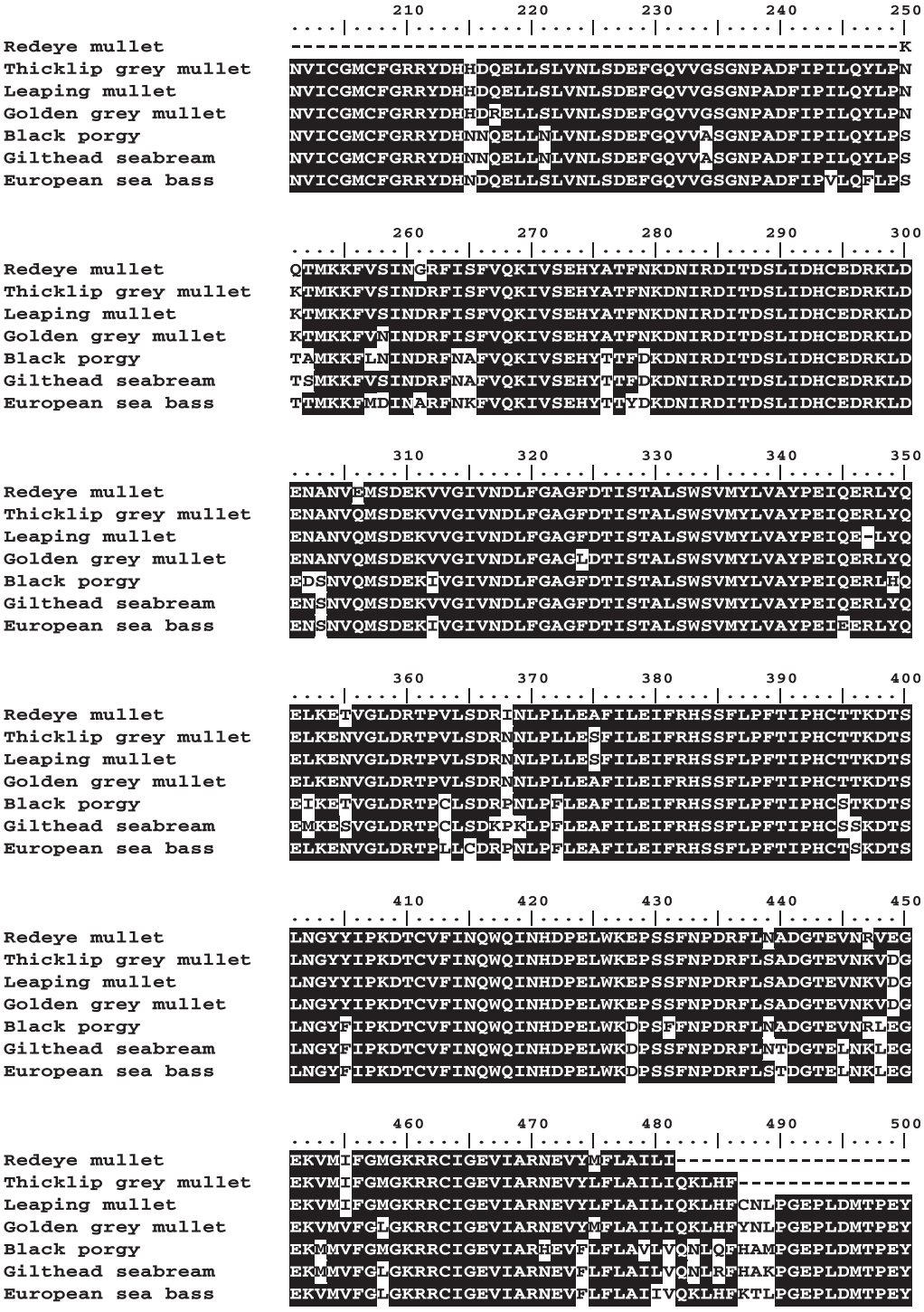

Fig. 3. Multiple sequence alignment of the partial amino acid sequence of redeye mullet CYP1A with those of thicklip grey mullet [ABD95933], leaping mullet [QW9683], golden grey mullet [O42231], black porgy [ABI54450], gilthead seabream [O42457], European sea bass [CAB63650].

recovery results were in the range of 60–105%, RSD of <15%, and quantification was performed by an external standard method.

PCBs and PBDEs were analyzed following the methods outlined in the US EPA 1668A (US EPA, 2003) and US EPA 1614 (US EPA, 2007). Briefly, approximately 2 g of dried weight samples were spiked with 1 ng of a $^{13}C_{12}$–PCBs mixture (US EPA 68A–LCS mixture) and a $^{13}C_{12}$–PBDEs mixture (EO-5277), and mixed with anhydrous sodium sulfate for 24 h. The samples were then extracted using *n*-hexane:acetone 50:50 (v/v), and the extracts were purified and fractionated with multi-layer silica gel columns. The PCB fractions were further purified with basic alumina columns, and the PBDE fractions were purified with Florisil columns. $^{13}C_{12}$-labeled injection internal standards EPA 68A-IS and EO-5275 were added to each PCBs fraction and PBDEs fraction, respectively. An aliquot of the extract was injected immediately into GC–MS using an Agilent 6890 gas chromatograph coupled to an Autospec Ultima mass spectrometer (Waters Micromass, Manchester, UK). Quality control was satisfied the requirement of the US EPA method 1668A and method 1614 with RSD of <30% ongoing precision and 50–130% of recovery. Quantification was performed using an isotope dilution method.

2.6. Data analysis

Statistical analyses were performed using SPSS 12.0 software (Chicago, IL, USA). Independent *t* test analysis was used to determine the difference between two treatment groups. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. CYP1A sequence and phylogenetic analysis

A 696-bp sequence corresponding to 232 amino acid sequence was amplified by PCR using degenerate primers from redeye mullet liver cDNA (Fig. 2), and the obtained sequence was confirmed by BLAST (http://blast.ncbi.nlm.nih.gov/) (GenBank accession number FJ827632). The deduced amino acid sequence shared with 99% sequence identity with the corresponding thicklip grey mullet (*Chelon labrosus*) (ABD95933), leaping mullet (*Liza saliens*) (AAD22398), golden grey mullet (*Liza aurata*) (AAB70307), black porgy (*Mylio macrocephalus*) (ABI54450), gilthead seabream (*Sparus auratus*) (O42457), and European seabass (*Dicentrarchus labrax*) (CAB63650) (Fig. 3). Multiple sequences aligned using the CLUSTALW algorithm followed by construction of a phylogenetic tree using the Neighbor-joining method, and the phylogenetic tree suggested that all of the family CYP1A genes in different organisms were highly related in progress of phylogenesis (Fig. 4).

3.2. Real-time quantifying CYP1A mRNA in redeye mullets

In this study, a 10-fold dilution series was carried out for the cDNA standard from 10^9 to 10^4 molecules and amplified for 40 cycles during PCR. The standard curves for both CYP1A and β-actin were linear over six orders of magnitude with the linear correlation ($r^2 > 0.99$) between the threshold cycle (Ct) and the number of copies of the target. The specificity of real-time RT-PCR with SYBR Green detection was ascertained by comparing the heat dissociation curves of the amplification products from different samples to those of the standards, and the ratios of CYP1A to β-actin cDNA were used to assess the CYP1A gene expression in samples.

The CYP1A mRNA was quantified in redeye mullets collecting from highly contaminated area in Dashentang and from the reference site in Nandaihe (Fig. 5). The CYP1A mRNA levels between males and females both in Nandaihe (0.022 copies/copy β-actin in six males and 0.025 copies/copy β-actin in six females) and Dashentang (0.11 copies/copy β-actin in six males and 0.13 copies/copy β-actin in six females) were not significantly different ($p > 0.05$). However, the CYP1A mRNA levels in both males and females from Dashentang were significantly higher than those from Nandaihe ($p < 0.05$).

3.3. PAHs, PCBs and PBDEs concentrations

The concentrations of PAHs and PCBs in redeye mullets from Nandaihe and Dashentang were detected (Fig. 6). The concentration of PAHs in redeye mullets from Dashentang (554.29 ng/g dw) was similar to that from Nandaihe (498.61 ng/g dw), and the concentration of phenanthrene was the dominant congener (275.56 ng/g dw in Dashentang and 255.32 ng/g dw in Nandaihe). The concentration of total PCBs in redeye mullets from Dashentang (68.58 ng/g dw) were more than five times higher than that from Nandaihe (13.61 ng/g dw), and the concentration of total PBDEs in redeye mullets from impacted site in Dashentang (1407.26 pg/g dw) were also about five times higher than that from reference site in Nandaihe (317.45 pg/g dw).

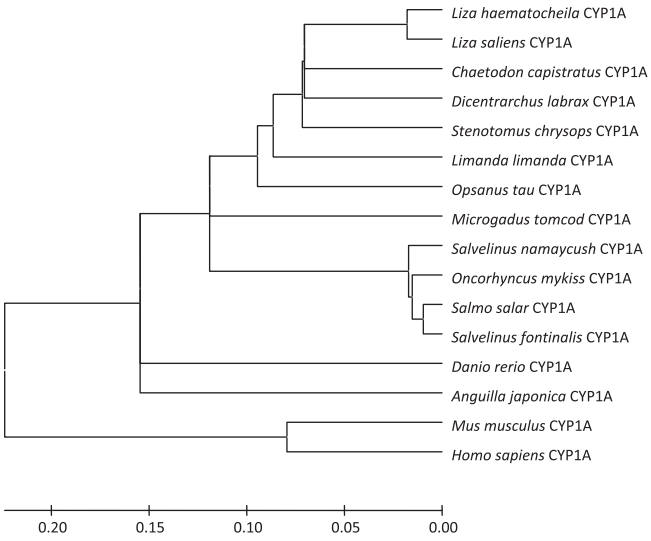

Fig. 4. Phylogenetic analysis of CYP1A gene. The phylogenetic tree and genetic distances were determined using the Neighbor-joining method.

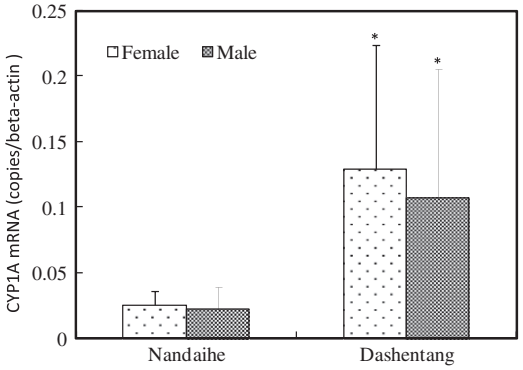

Fig. 5. Mean ratio of CYP1A/beta-actin mRNA copies in male and female redeye mullets collected from the reference site in Nandaihe and the impacted site in Dashentang. An asterisk (*) indicates significant difference between the two sites. Error bar represents the standard deviation.

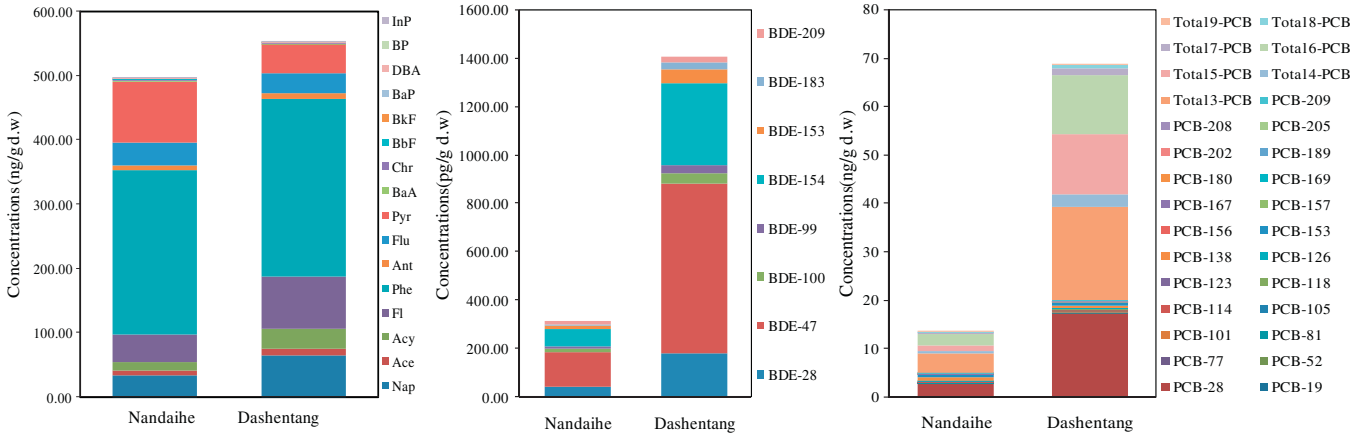

Fig. 6. Profile of PAHs, PCBs and PBDEs in redeye mullets from Nandaihe and Dashentang in Bohai Bay.

4. Discussion

The induction of CYP1A in fish exposure to contaminants that bind to aryl hydrocarbon receptor (AhR) is widely employed as a biomarker in pollution monitoring studies of the aquatic environment (Van den Heuvel et al., 1994; Whyte et al., 2000). CYP1A induction may be affected by size and age (Addison and Willis, 1983), maturity stage (Sleiderink et al., 1995), and as well as on light, pressure temperature, salinity (Kloepper-Sams and Stegeman, 1992; Rees and Li, 2004) and other factors. In this study, the redeye mullets for analyzing CYP1A mRNA expression were sampled at the same time and the similar size of the fish was selected in order to minimize these affecting factors (Table 1).

The results of this study showed that CYP1A mRNA expression levels in liver significantly increased in redeye mullets from Dashentang compared to those from the reference site in Nandaihe, suggesting that the level of AhR ligands in Dashentang would be higher than that in Nandaihe. Among these organic contaminants detected in this study, PAHs level in redeye mullets from Dashentang (554.29 ng/g dw) was similar to that in Nandaihe (498.61 ng/g dw). The total PAHs in redeye mullets from Nandaihe and Dashentang was similar to Red Sea fish from Red Sea coast of Yemen (422.1 ng/g dw) (DouAbul et al., 1997), and was higher than sand flathead from Port Phillip Bay (Victoria) (Nicholson et al., 1994) (55.7 ng/g dw) and English sole from Puget Sound (below 200 ng/g dw) (Landlot et al., 1987). This may be related with that the redeye mullets feed on the benthic organisms which had the highest PAHs concentrations in our previous investigation in Bohai Bay (Wan et al., 2007). Moreover, approximately 50% of the total PAHs in the sampling sites was phenanthrene which is a low-toxicity, non-CYP1A-inducing compound (Fig. 6a). The results were consistent with the predominant PAHs in sediments (Hu et al., 2010). Toxic equivalent (TEQ) of PAHs using the toxic equivalent factor (TEF) calculated according to Nisbet and LaGoy (1992) was 1.95 ng/g dw in Dashentang and 1.47 ng/g dw in Nandaihe. The similar concentrations in two sites indicated that PAHs was not responsible for the difference of CYP1A induction between Nandaihe and Dashentang.

PBDEs was also detected in the present study, and the concentration of PBDEs in redeye mullets from Dashentang was 1407.26 pg/g dw, which was much higher than that from Nandaihe (317.45 pg/g dw). Compared with other field investigations, PBDEs in redeye mullets from Dashentang was higher than the eels collected from a coastal lagoon in the north-western Mediterranean Sea (138.14–455.49 pg/g wet weight (ww)) (Mariottini et al., 2008) and from the inland Sea of Seto in Japan (<2–120 pg/g dw) (Akutsu et al., 2001), but was significantly lower than pike in Sweden (17,000 ng/g dw) (Andersson and Blomkvist, 1981) and American eels in American (1.2–407.9 ng/g dw) (Ashley et al., 2007). The most dominant congener was BDE-47 in both sites (Fig. 6b) which is considered the most abundant in aquatic biota and has the highest bioconcentration factor (BCF) (Wan et al., 2008). Relative induction potencies (REPs) of PBDEs congeners to TCDD for inducing CYP1A (Chen et al., 2001) were estimated to be 0.0013 in Dashentang, which was three times higher than that in Nandaihe (0.00032 pg TEQ/g dw). The results indicate that PBDEs was responsible for the difference of CYP1A induction between Nandaihe and Dashentang.

As one of the best-known inducer for CYP1A gene (Marohn et al., 2008), the previous investigations had demonstrated that CYP1A expression in wild fish or caged fish can discriminate the pollutions degree of PCBs among rivers in USA (Rees and Li, 2004; Brammell et al., 2010). The concentration of PCBs in redeye mullets from Dashentang (68.58 ng/g dw) was about five times higher than that from Nandaihe (13.61 ng/g dw) (Fig. 6c), which was higher than the marine fish in previous investigations in China (mean, 3600 pg/g ww, and range, 830–8040 pg/g ww (Yang et al., 2006); mean, 1133 pg/g ww, and range, 38.9–3514 pg/g ww (Shen et al., 2009)), but all were significantly lower than catfish (0.56–2.94 μg/g dw) from Lagos Lagoon, Nigeria (Adeyemi et al., 2009). The PCB profiles showed that the coplanar-PCBs including 12 congeners accounted for 12.90% of the total PCBs in Dashentang, but only 1.91% in Nandaihe. It should be noted that TEQ for PCBs calculated according to Van den Berg et al. (1998) was 0.56 ng/g dw in Dashentang, which was significantly higher than that in Nandaihe (0.00015 ng/g dw). Marohn et al. (2008) have reported the threshold of PCB which could induce CYP1A1 mRNA expression to be 0.05 ng TEQ/g dw based on the European eels (*Anguilla anguilla*) exposure to PCBs by an intraperitoneal injection. The TEQ for PCBs in Dashentang (0.56 ng/g dw) was more than 10 times higher than the threshold, whereas the TEQ in Nandaihe was much lower (0.00015 ng/g dw) than the threshold. Thus, the results of this study indicate that PCBs is the main Ah receptor agents to induce the higher CYP1A expression in redeye mullets from Dashentang. This phenomenon was also found in another study where PCBs and dioxin-like compounds but not PAHs induced CYP1A expression to a greater extent in actual environment on the basis of the fact that PAH concentrations did not correlate with EROD activity (Shaw et al., 2004).

The induction of CYP1A is not restricted to the classical AhR ligands such as PAHs and PCBs; other chemical classes including heterocyclic compounds such as the more polar amines also can induce CYP1A expression (Brack et al., 2000; Altenburger et al., 2003; Brack and Schirmer, 2003; Navas et al., 2003). So while it is impossible to

identify all of compounds for inducing CYP1A expression in wildfish, the overall data in the present study showed that PCBs and PBDEs were a part of responsibility for the difference of CYP1A induction between-sites according to the discriminant analysis.

5. Conclusion

CYP1A mRNA expression values were significantly elevated in redeye mullets from Dashentang of Baihe Bay compared to the fish from the reference site in Nandaihe. PCBs and PBDEs may be main responsible to the CYP1A induction in redeye mullets in Dashentang. And so the real-time RT-PCR assay developed in the present study for analysis of CYP1A expression in redeye mullet can be used to distinguish the degree of pollutions among sites and the redeye mullet is a promising sentinel species for EDC field investigating in coastal environment.

Acknowledgments

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