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Cloning and tissue expression of cytochrome P450 *1B1* and *1C1* genes from Javanese Medaka, *Oryzias Javanicus*, under environmental stress conditions

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Cytochrome P450 1 (CYP1) is widely used as an indicator of exposure to environmental contaminants. In the study, two full-length complementary DNAs encode for CYP1B1 and CYP1C1 were cloned from medaka liver exposed to 500 ppb β-naphthoflavone for 24 h. *CYP1B1*, having 1984 bp, contains an open reading frame of 1551 bp encoding a protein of 517 amino acids; while, *CYP1C1* having 2601 bp consists of an open reading frame of 1578 bp encoded for 525 amino acid residues. The highest levels of these CYP1 genes transcript were observed in intestine and the lowest in liver from the fish fed on fuel oil-contaminated feed. Javanese medaka CYP1B1 and CYP1C1 transcripts were detected in the gill, muscle and liver when transferred from seawater to freshwater with the highest level of expression in gill and muscle. Starvations for one week tended to down- regulate the Javanese medaka CYP1 expression.

Key words: Cytochrome P450, Javanese medaka, salinity, starvation, heavy fuel oil, cloning, expression.

INTRODUCTION

Cytochrome P450 (CYP) super family can be found in nearly all organisms, including animals, plants, fungi, lower eukaryotes and bacteria(Bernhardt, 2006; Nebert and Dalton, 2006; Nelson, 2010). Cytochrome P450 is widely used as an indicator of exposure to environmental contaminants (Hahn et al., 1998). CYP proteins play a critical role in the oxidative metabolism of endogenous compounds, and xenobiotic (exogenous) compounds, in-cluding pharmaceuticals and environmental toxins (Diotel et al., 2010). The cytochrome CYP1 family consists of four known subfamilies of vertebrate species (CYP1A, CYP1B, CYP1C and CYP1D) but only CYP1A and CYP1B enzymes appear to be present in all vertebrates and have been investigated the most. Mammals have twoCYP1A genes (*CYP1A1* and *CYP1A2*) while most fish have a single CYP1A gene (Goldstone et al., 2007). Almost all vertebrates have a single CYP1B gene, the *CYP1B1*. There are two CYP1C genes found in fishes

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(*CYP1C1* and *CYP1C2*), while all non-mammalian vertebrates have only a single CYP1C1 (Goldstone et al., 2007). Given their various degree of responsiveness to pollutants, expression patterns of the *CYP1A*, *CYP1B*, and *CYP1C* genes in fish could become useful biomarkers in environmental monitoring (Jonsson et al., 2010).

Javanese medaka (*Oryzias javanicus*) is used extensively as experimental test fish because it has several advantages; it has a unique osmotic adaptation mechanisms, and a wide range of salinity tolerance (Inoue and Takei, 2002). Another advantage is that medaka can naturally tolerate to low temperature, it can survive at 40°C in summer and 4°C in winter without any thermostatic regulator (Kinoshita et al., 2009). Therefore, the fish has been widely used in assessing environmental risk, toxicity test for new pollutants in various environments, biological response testing and sensitive molecular biomarkers testing (Koyama et al. 2007; Woo et al. 2009).

Salinity changes are considered as aquacultural and environmental problem worldwide. Euryhaline fish, such as the Javanese medaka, can live in both fresh and seawater. In freshwater, these fish are hyperosmotic relative to their external medium. Many changes in gene expression and protein activity observed at least 12 to 18 h after exposure to a hyperosmotic environment, suggesting that these effects may be mediated by immediate early gene transcriptional factors (Fiol and Kültz, 2005). Indeed, the involvement of such transcriptional factors displaying rapid changes to gene expression after hyperosomotic stress has recently been described in tilapia (*Oreochromis mossambicus*) transferred from freshwater to seawater(Fiol and Kültz, 2005). Functional genomic studies of fish stress responses, particularly the identification of a core set of stress-related transcripts of CYP1 genes, are crucial for better understanding in their physiological and toxicological functions.

Heavy fuel oil products have been reported to contain several toxic organic and inorganic components such as polycyclic aromatic hydrocarbons, atoms of sulfur, nitrogen, oxygen as well as metals such as; iron, vanadium, sodium, nickel, chromium and other metals present in small amounts (Baker, 1970; Williams et al., 1994; Irwin et al., 1998) which constitutes a significant health risk to both people and other organisms. The ingestion of petroleum hydrocarbon has been reported to induce oxidative stress (Val and Almeida-Val, 1999) generating free radicals, (Achuba and Osakwe, 2003) which leads to lipid peroxidation (Halliwell, 1994) that damages critical cellular macromolecules like DNA, lipids and proteins (Breimer, 1990; Romero et al., 1998; Souza et al., 1999). Heavy fuel oil also causes an increased prevalence of morphological abnormalities, and reductions in growth and recruitment causing Blue Sac Disease (BSD) in fish, with symptoms of edema, hemorrhaging, deformities and induced CYP1A enzyme(Marty et al., 1997). Fish affected in this way were unable to feed and eventually starved or were consumed by predators.

All animals face the possibility of limitations in food resources that could ultimately lead to starvation-induced mortality. Starvation is a situation undergone and tolerated by many species of fish in their natural environments in response to several factors. To survive these periods of unfavorable feeding conditions, fish reduce their energy expenditures, which in a high percentage are derived from protein synthesis, and mobilize their endogenous reserves to obtain the energy required to maintain the vital processes (Miriam and Ana, 2011). Starvation has been reported to have pro-oxidant effects, and both the inadequate neutralization of the reactive oxygen species (ROS) generated by oxidative metabolism and the reduced level of antioxidant defenses, both enzymatic and non-enzymatic, may be responsible for some of the detrimental effects of starvation. In addition, starvation may influence 7-ethoxyresorufin-O-deethylase (EROD) activities in fish (Andersson et al., 1985; Jorgensen et al., 1999). Starvation promotes the mobilization of lipids from adipose tissue and/or liver lipid droplets, and, thus the mobilization of the lipophilic toxicants is stored in these tissues (Sancho et al., 1998). The effects of starvation on EROD activities are contradictory; in rainbow trout starved for 6 or 12 weeks, a single intraperitoneal injection of benzo[a]pyrene decreased renal EROD activities after six weeks and hepatic EROD activities after 12 weeks of starvation (Andersson et al., 1985). However, in a study by Vigano et al. (1993), three weeks of starvation had no influence on hepatic EROD activities, (Vigano et al., 1993) whereas increased liver EROD activities were observed in starved Arctic charr (*Salvelinus alpinus*) previously exposed to the PCB mixture Aroclor 1260 (Jorgensen et al., 1999).

Real-time PCR (qRT-PCR) is a sensitive, reproducible and high-throughput method that can show subtle changes in relative quantities of a large number of genes, consuming small sample amounts (Bustin, 2002; Bustin and Nolan, 2004). Expression patterns of CYP1 genes in fish could become useful biomarkers in environmental monitoring (Jonsson et al., 2010). In the study, we report on cDNA cloning and sequence analysis of the dominant isoforms of cytochrome P450 genes, CYP1B1 and CYP1C1 from Javanese medaka exposed to 500 ppb BNF over a period of 24 h, and examined their level of inducibility by environmental stress factors in various fish organs using real-time PCR.

MATERIALS AND METHODS

Treatment of fish

Javanese medaka was held in the aquarium facility at the Marine Biotechnology Laboratory, Faculty of Fisheries, in Kagoshima University. The fish were cultured in laboratory conditions for one week before the experiment, during which they fed twice daily on a fine fish commercial diet. After the acclimatization period, eight

Table 1. Oligonucleotide primers used for CYP1B1, 1C1 cloning and real time PCR.

Primer name	Description	Location	Primer sequence (5'-3')
Dgp_CYP1B 1F	Degenerate PCR	697-715	GTGGAYGTGATGCCYTGGC
Dgp_CYP1B 2R	Degenerate PCR	1230-1250	TGRTTSAHRGACCACTGGTTG
Dgp_CYP1B 3F	Degenerate PCR	646-665	SAGGTGGTGGGYAGRAAYGA
Dgp_CYP1B 4R	Degenerate PCR	1107-1127	ACYTCRTARAYGAARGCCATG
Dgp_CYP1C 1F	Degenerate PCR	697-715	GTGGAYGTGATGCCYTGGC
Dgp_CYP1C 2R	Degenerate PCR	1230-1250	TGRTTSAHRGACCACTGGTTG
Java_CYP1B_GSP 1F	RACE PCR	3-28	GGATGTGATGCCTTGGCTCCAGTATT
Java_CYP1B_GSP 2R	RACE PCR	335-359	CCCACCACTCTGTCCACCTCCTTCT
Java_CYP1B_GSP 3F	RACE PCR	21-46	CCAGTATTTCCCCAACCCCATCAAGA
Java_CYP1B_GSP 6R	RACE PCR	309-333	CAGACGCCGCTGCATCTCAGGATAC
Java_CYP1C_GSP 1F	RACE PCR	131-155	ACCCCGAGGTGACCCGAGACATAAG
Java_CYP1C_GSP 2R	RACE PCR	458-483	AATGGTGACGTCAGAGGTGGTGGAGT
Java_CYP1C_GSP 3F	RACE PCR	172-196	GTGATTGAGCACGGAGAGGACAGCA
Java_CYP1C_GSP 4R	RACE PCR	404-429	GAAGCGCATGGTCTCGTAGATGAAGG
Java_CYP1B 1F	Real-time PCR	1188-1207	GAGCTACACCATCCCCAAGA
Java_CYP1B 2R	Real-time PCR	1301-1320	CTTGTTCAGCTTCCCCTCTG
Java_CYP1C 1F	Real-time PCR	1244-1263	ACCAGTTCTCCGTCAACCAC
Java_CYP1C 2R	Real-time PCR	1362-1381	GCCGTTTACCTGTGGAGAAA
Java_actin 1F	β-actin RT PCR	346-365	AGGGAGAAGATGACCCAGAT
Java_actin 2R	β-actin RT PCR	447-466	CAGAGTCCATGACGATACCA

adult fish were exposed to 500 ppb β-naphthoflavone for 24 h. Liver was dissected, immediately frozen in liquid nitrogen, and stored at -80°C until further use.

RNA preparation and cDNA synthesis

Total RNA was extracted from liver using ISOGEN reagent (Nippon Gene, Japan) according to the manufacturer's protocol. The concentration of RNA was determined by a spectrophotometer (Gene Spec V, Hitachi, Japan). Total RNA concentration and purity were determined spectrophotometrically as described by (Sambrook and Russell, 2001), and the A260/A280 ratio was between 1.7 and 1.9. Poly $(A)^+$ RNA was purified using an Oligotex™ -dT30 <Super> mRNA Purification Kit (Takara Bio, Japan). First strand cDNA was synthesized by the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Japan).

cDNA cloning and sequencing of CYP1 family cDNAs

Degenerate inosine-containing primers were designed from highly conserved regions, based on the alignment of CYP1B and CYP1C sequences from other fish species. All the primers are shown in Table 1. PCR was performed using an Astec PC320 PCR system (Astec Bio, Japan) and TaKaRa Ex Taq polymerase (Takara Bio, Japan) using the following PCR program: initial denaturation step at 94°C for 2 min 30 s and subsequent 35 cycles of amplification (94°C, 30 s; 50°C, 30 s; 72°C, 30 s) and a final extension step for 2 min at 72°C. The 5' and 3' ends of the CYP1 family cDNA were obtained by rapid amplification of cDNA ends (RACE) using the SMART™ RACE cDNA Amplification Kit (Clontech Takara Bio, Japan) following the supplier's protocol. Gene-specific primers (GSP) were designed based on the sequence obtained from PCR with degenerate primers (Table 1). For cloning, DNA bands of the expected size were excised from the gel, purified using the illustra GFX DNA and Gel Band Kit (GE Health Care, UK), and sub-cloned using pT7 Blue T vector (Novagen, USA) with the Ligation-Convenience Kit (Nippon Gene, Japan). Ligated DNA was transformed into JM109 *Escherichia coli* cells. Purified plasmids were directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA).

DNA sequencing data was retrieved and edited by Lasergene sequence analysis software (DNAStar ver5.2). Sequence homology searches were carried out using the Basic Local Alignment Search Tool (BLAST) program at http://www.ncbi.nlm.nih.gov/BLAST/, whereas sequence alignment was performed using the ClustalW program with MegAlign in DNAStar. DNA sequences with their GeneBank accession numbers that were retrieved from the database and used in the analysis are shown in Table 2. Phylogenetic and molecular evolutionary analyses were constructed by web-based software using the neighbor-joining method (Dereeper et al., 2008; Dereeper et al., 2010). MatGAT (James et al., 2003) was used to calculate similarity and identity of deduced amino acid sequence of Javanese medaka CYP1s with other CYP1 family members.

Expression of CYP1B1 and CYP1C1 in various organs of Javanese medaka

Animal and experimental design

Javanese medaka fish were acclimatized to laboratory conditions for one week before the experiment during which they were fed

Table 2. Gene Bank accession numbers of the CYP1 cDNAs used.

Species	cDNA	Accession number
Tilapia (*Oreochromis niloticus*)	CYP1B1	HQ829968
	CYP1C1	HQ829969
Scup (*Stenotomus chrysops*)	CYP1C1	AF131885
Plaice (*Pleuronectes platessa*)	CYP1B1	AJ249074
Rat (*Rattus norvegicus*)	CYP1B1	U09540
Human (*Homo sapiens*)	CYP1B1	U03688
Rainbow trout (*Oncorhynchus mykiss*)	CYP1C1	NM_001185031
Zebrafish (*Danio rerio*)	CYP1C1	NM_001020610
Chicken (*Gallus gallus*)	CYP1C1	JN656933
	CYP1B1	XM_001233594
Japanese eel (*Anguila japonica*)	CYP1B1	AY518340
	CYP1C1	AY444748
Carp (*Cyprinus carpio*)	CYP1B1	AB048942
	CYP1C1	AY437776
Killifish (*Fundulus heteroclitus*)	CYP1B1	FJ786959
	CYP1C1	DQ133570

twice daily on the medaka commercial diet (Kyorin, Japan); all fish were feeding well at the initiation of the treatments. Natural seawater (33 to 34 ppt) was used for all the experiments. The water temperature was kept at 23±0.5°C. Water pH value ranged from 7.4 to 7.6. The experimental systems were continuously aerated to ensure that the dissolved oxygen levels were adequately maintained.

Oil-contaminated feed experiment: Heavy fuel oil (bunker C) was used in the experiment. The oil contents of carbon and sulfur residues were <4 and <2%, respectively (Koyama and Kakuno, 2004). Javanese medaka was fed with an oil-contaminated feed at levels of 0% (control) and 1%. Thirty medaka fish were divided into two groups and kept in a 12 L tank. Feed was given *ad libitum* during the experiment cycle. Tissue samples were collected after 24 h.

Salinity shock experiment: Adult Javanese medaka cultured in seawater were starved for 2 days prior to freshwater transfer, and the change in salinity was ensured by direct transfer from seawater to either freshwater or seawater (control group). Tap water was dechlorinated, and aerated several days prior to its use in the salinity shock experiment. Fifteen medaka fish per group (control and treated) were kept in a 12 L tank. During the freshwater transfer experiments, fish either kept in seawater or transferred into freshwater were sampled after 24 h.

Starvation experiment: The fish were acclimated to laboratory conditions for one week prior to the study and all fish were feeding well at the initiation of the treatments. Two groups (15 medaka fish per group) of adult Javanese medaka were either starved or fed (control group) for 1 week. Fish were kept in a 12 L tank. Fish in the control group were fed twice daily with medaka commercial pellets (Kyorin, Japan).

Reverse transcription, primer design and real-time PCR

Total RNA was isolated from liver, gill, muscle, and intestine using QuickGene RNA Tissue Kit S II (RT-2) (Fujifilm, Japan), according to the manufacturer's instruction. Reverse transcription of mRNA was performed with PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio, Japan), following the supplier's protocol. Gene-specific primers for CYP1s and β-actin, as an internal control gene (accession no. JQ905607), were designed by the web-based software Primer3Plus (Andreas et al., 2007) with a product size between 50 to 150 bp, T_m ranging from 57 to 63°C and all the default parameters (Table 1). Real-time PCR was performed using FastStart Essential DNA Green Master Kit and a LightCycler® Nano system (Roche Applied Science). For each sample, gene expression was analyzed in triplicate with the following protocol: initial holding at 95°C for 10 min, 3-step amplification in 45 cycles (95°C for 10 s, 60°C for 10 s and 72°C for 15 s), holding stage at 95°C for 30 s and melting at 60°C for 20 s and 95°C for 20 s. Melt curve analysis was performed at the end of each PCR run to assure that the single product was amplified. The $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001) was used to compare the expression levels of the different CYP1s within a given organ, and to calculate changes in fold-induction in response to treatments, oil-contaminated feed, salinity and starvation, using β-actin as a reference gene.

RESULTS AND DISCUSSION

Cloning of CYP1B1 and CYP1C1 from Javanese medaka

Two full-length CYP1 cDNAs were cloned from Javanese medaka liver exposed to 500 ppb β-naphthoflavone for 24 h. CYP1B1 from Javanese medaka contains 1984 bp, a 5' noncoding region of 106 bp, an open reading frame of 1551 bp, and a 3' noncoding region of 300 bp including the polyA tail. The deduced protein sequence has 517 amino acid residues with an estimated molecular weight of 58.64 kDa. Three putative polyadenylated signal sites (AATAAA) can be found in the long 3' untranslated region

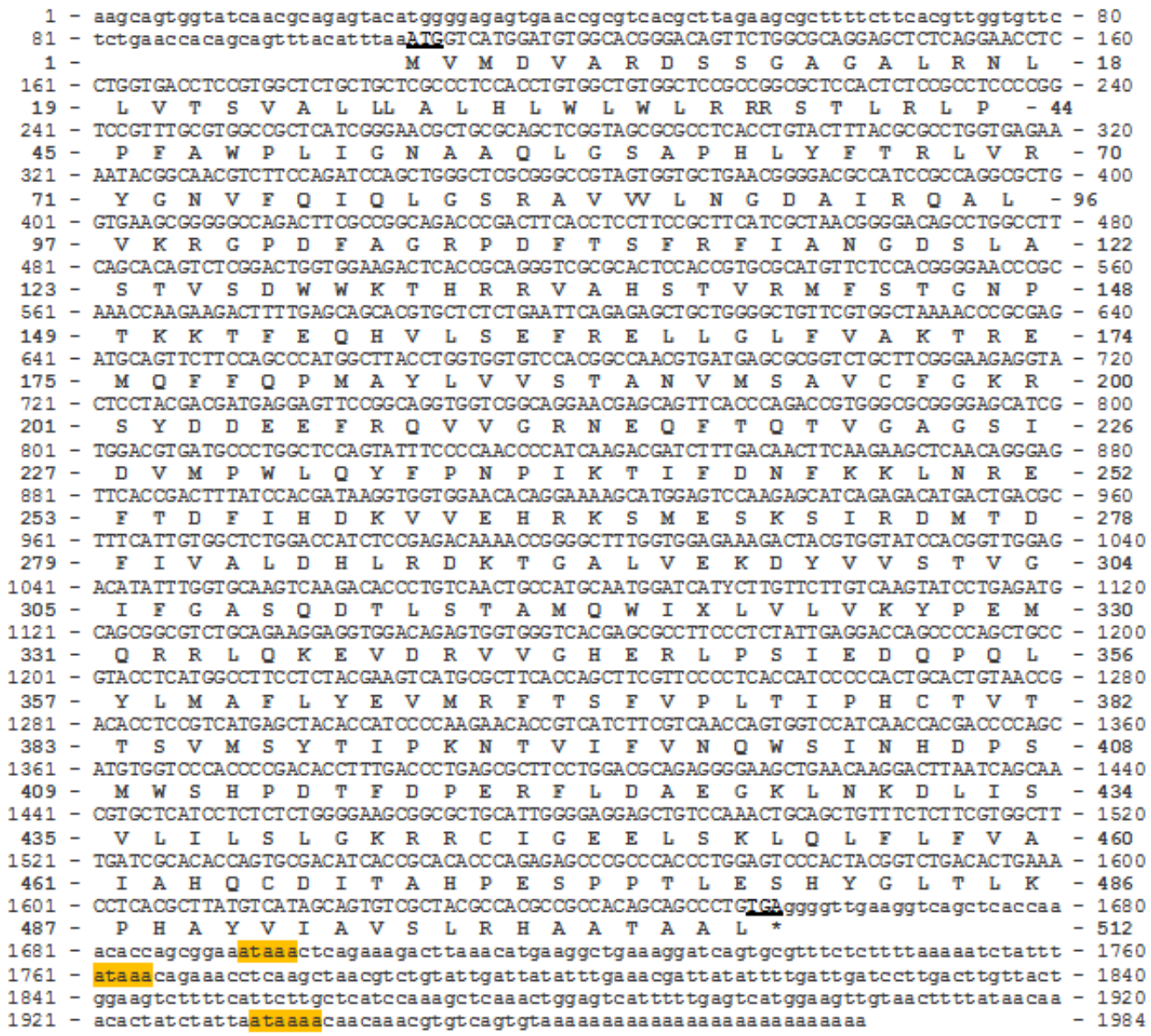

Figure 1A. Full-length cDNA of CYP1B1 and its deduced amino acid residues. The coding sequences are shown in uppercase letter whereas lowercase for untranslated regions. The predicted amino acid sequences are in bold letters. The translation start codon and termination codon are underlined and the putative polyadenylation signal (aataaa) is colored in orange.

(Figure 1A). The full-length cDNA of CYP1C1 from Java medaka contains 2601 bp with an open reading frame of 1578 bp. The deduced protein sequence has 525 amino acid residues with an estimated molecular weight of 59.21 kDa. A 216 bp 5' untranslated region precedes the start codon, and 807 bp 3' untranslated region follows the stop codon. Three putative polyadenylated signal sites (AATAAA) were found in the long 3' untranslated region (Figure 1B). Table 3 shows the percentage of similarity and identity of deduced amino acid sequences of Javanese medaka CYP1B1 and CYP1C1 with other published cytochrome P450 sequences. Results indicates that Javanese medaka CYP1B1 showed highest similarity and identity to CYP1B1 from tilapia (*Oreochromis niloticus*) 69.3 and 84.5%, respectively. The Javanese medaka CYP1C1 deduced amino acid sequence shows highest similarity and identity to the tilapia CYP1C1 82.3 and 95.4%, respectively. The phylogenetic tree, based on the amino acid sequences was used to assess the relationship of CYP1 of Javanese medaka with those in other fish species (Figure 2). The phylogenetic tree, with representatives of full-length CYP1 protein sequences, indicates

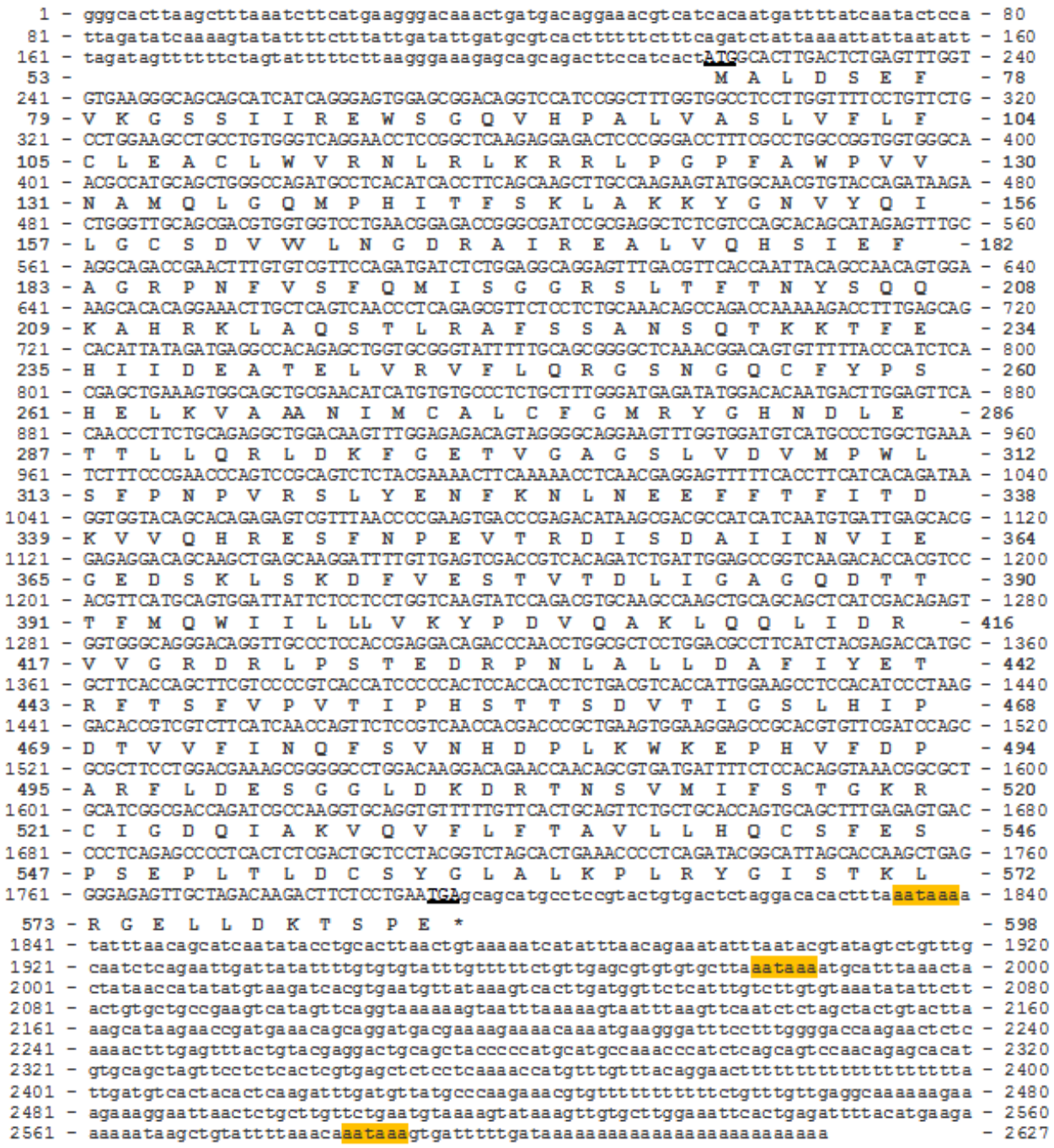

Figure 1B. Full-length cDNA of CYP1C1 and the deduced amino acid residues. The coding sequences are shown in uppercase letter whereas lowercase for untranslated regions. The predicted amino acid sequences are in bold letters. The translation start codon and termination codon are underlined and the putative polyadenylation signal (aataaa) is colored in orange.

that Javanese medaka CYP1B1 and tilapia (*O. niloticus)* CYP1B1 formed a cluster in the phylogenetic tree, indicating the evolutionary relatedness. The phylogenetic tree of Javanese medaka CYP1C1 and other CYP1 families and subfamilies revealed that CYP1C1 belonged to a cluster with CYP1C1 from scup (*Stenotomus chrysops*) and tilapia.

The deduced amino acid sequences of CYP1B1 and

Table 3. Percent similarity and identity (upper triangle) of deduced amino acid sequence of Javanese medaka CYP1s with other CYP1family members.

	1	2	3	4	5	6	7	8	9	10	11	12
Java_CYP1B1		61.9	63.3	69.3	68.3	69.1	51.5	49.7	49.1	50	48.6	48.7
Carp_CYP1B1	78.1		66.1	63.2	60.9	60.6	51.5	48.4	49.5	49.6	48.9	49.4
Jeel_CYP1B1	78.7	80.9		65.9	65	64.4	50.2	48.4	49.5	48.7	49	47.8
Tila_CYP1B1	84.5	80.2	79		72.2	68.2	48.8	48	49	48.7	48.6	46
Plaice_CYP1B1	79.3	77.1	80.8	83.3		68.9	47.8	47.3	48.2	47.1	47	45.6
Killi_CYP1B1	81.2	77.3	79.6	81.4	83.2		49.5	48.7	49.4	48.5	48.4	46.6
Java_CYP1C1	70.1	71.7	70.1	70.3	69.2	69.8		74.9	78.7	82.1	82.3	69.4
Carp_CYP1C1	71.4	71.1	70.7	71	69.2	71.3	89.5		82.5	80.6	78.2	67.9
Jeel_CYP1C1	70.3	71.1	70.7	71.9	69.8	71.1	90.7	92.8		82.5	80.1	70.2
Scup_CYP1C1	71.4	71.1	69.9	71.2	69.8	72.1	93	92.4	94.7		85.9	72.3
Tila_CYP1C1	70.3	71.7	70.9	70.3	68.9	70.2	93.2	91.3	92.6	95.4		68.6
Killi_CYP1C1	69.8	70.4	69	68.7	67.8	69.8	85.7	84.7	84.6	86.3	85	

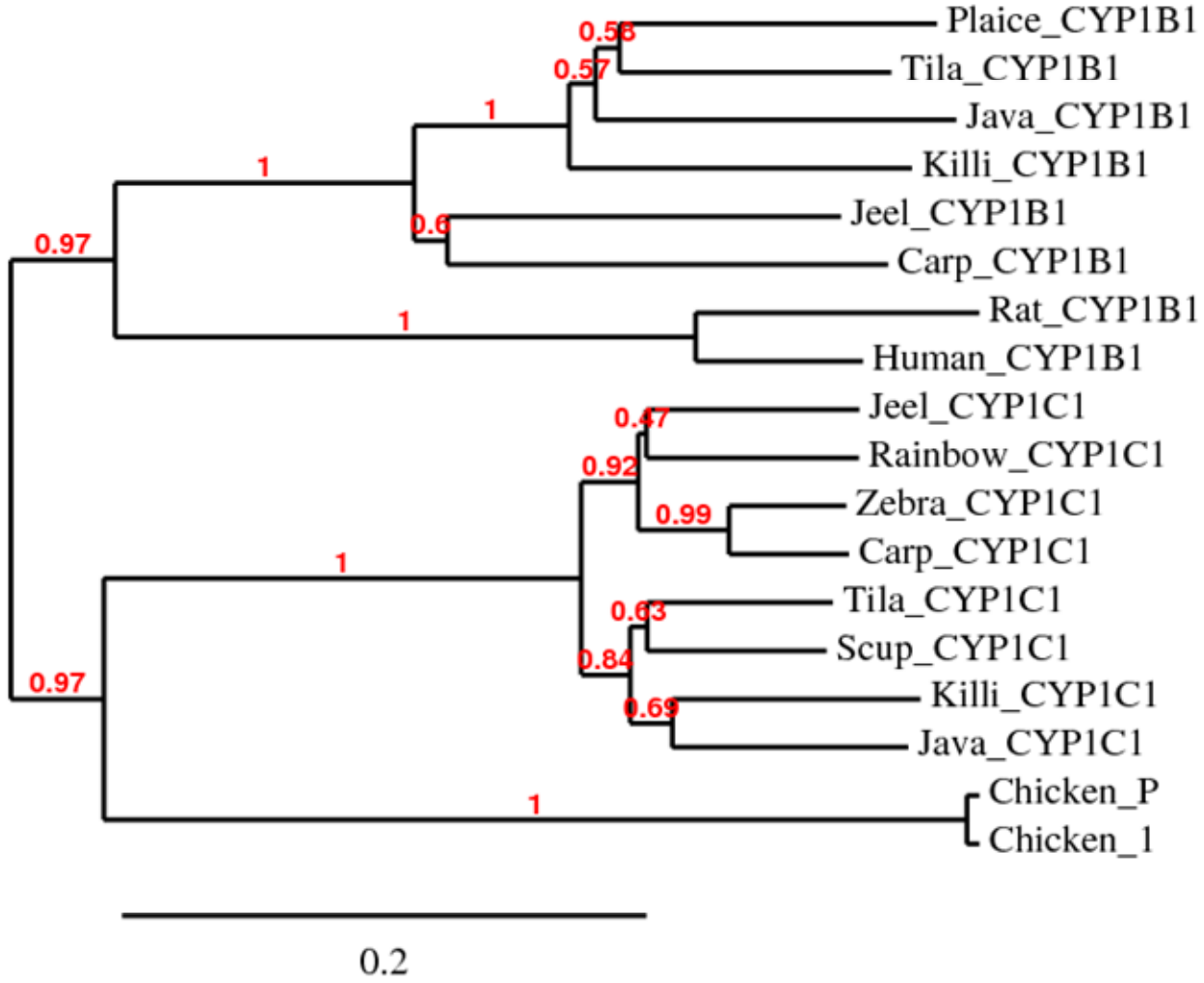

Figure 2. Phylogenetic tree of Javanese medaka CYP1s family genes constructed by the neighbor-joining using percent identity of deduced amino acid sequences of other species with accession numers. The building of the tree also involves a bootstrapping process repeated 100 times to generate a majority consensus tree.

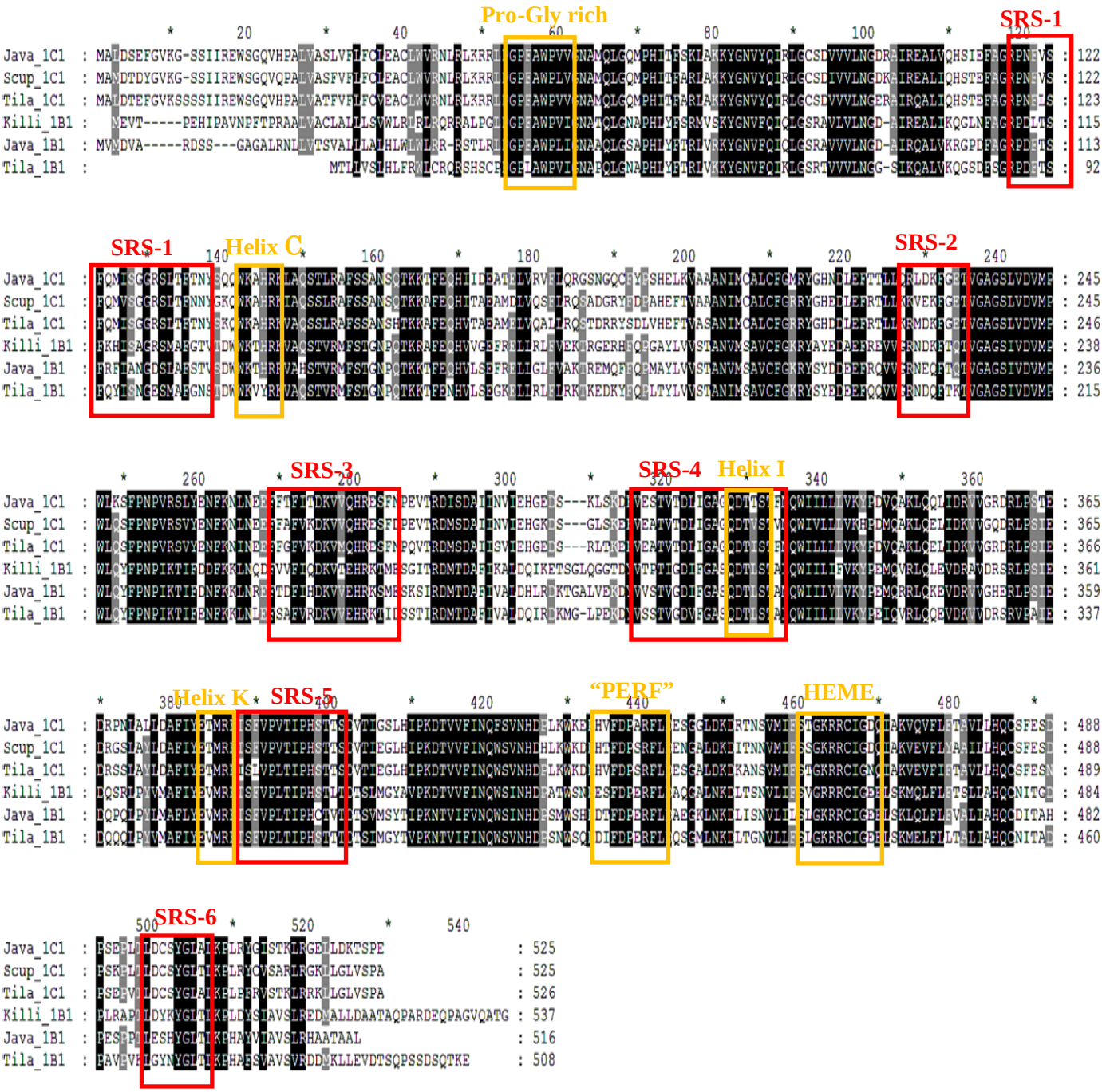

Figure 3. Amino acid sequence alignments of Javanese medaka CYP1s with orthologues. The alignment was constructed using the CYP1s amino acid sequences of tilapia (*Oreochromis niloticus*) and Japanese eel (*Anguilla japonica*) by ClustalW program. Orange boxes indicate the conserved motif region and red boxes indicates substrate recognition sites (SRS).

CYP1C1 shares a number of characteristic domains with other cytochrome P450s. The N-terminal of the Javanese medaka CYP1s consists of a proline-glycine rich region as PGPFAWPL in CYP1B1 and PGPFAWFV in CYP1C1. In addition, sequence alignment of Javanese medaka CYP1 enzymes with those of CYP1 family indicated that Javanese medaka CYP1B1 and 1C1 contain the five structural motifs around heme-binding core for all cytochrome P450 and six separate substrate recognition sites (SRSs) (Figure 3). The signature motif (FxxGxRxCxG) of the heme-binding core appeared as LSLGKRRCIR in CYP1B1 and FSTGKRRCIR in CYP1C1. The heme-inte-

Table 4. CYP1B1 mRNA expressions in Javanese medaka by environmental conditions.

Sample	Fold induction		
	Oil-contaminated feed	Salinity shock	Starvation
Liver	0.28 ± 0.04	1.44 ± 0.06	0.00 ± 0.00
Gill	7.24 ± 1.66	13.42 ± 0.70	1.00 ± 0.23
Muscle	2.84 ± 0.15	27.82 ± 1.51	0.15 ± 0.01
Intestine	11104,45 ± 76.67	0.60 ± 0.02	2.11 ± 0.33

Amount of CYP1B1 mRNA, normalized to β-actin mRNA. Relative fold induction was calculated by the equation $2^{-\Delta\Delta Ct}$ using data from carrier control. Data shows ± standard errors of the mean (n=3).

racting region of Helix C (WxxxR) was presented as WKTHR in CYP1B1 and WKAHR in CYP1C1. The highly conserved residues in Helix I ((A/G)GxxT) showed as SQDTL in CYP1B1 and GQDTT in CYP1C1. The structural motif played a role in the stabilization of the core structure of cytochrome P450s by hydrogen bond, Helix K (ExxR), was found in all of the Javanese medaka P450 proteins: EVMR in CYP1B1 and ETMR in CYP1C1. The invariant sequence (PxxFxPE/DRF) proximal to the heme-binding motif is demonstrated as PDTFDPERF in CYP1B1 and PHVFDPARF in CYP1C1. The characteristics of the N-terminal region of medaka CYP1s proteins exactly match that of the microsomal P450 proteins. At the N-terminal region of medaka CYP1s, a proline-glycine rich region was found, which allows CYPs to function at the ER-membrane. For efficient folding and proper assembly of the P450 proteins, the subsequent proline-glycine rich region acts as a rigid hinge for connecting the membrane anchor and the large catalytic domain, and hence designates the orientation of the catalytic domain in the cytoplasmic side of the ER membrane (Kusano et al., 2001; Kemper, 2004). Moreover, several conserved structural elements can be identified from the deduced protein sequences of the Javanese medaka CYP1 members. The structural conservation around the heme-binding core includes: the heme-binding motif (FxxGxRxCxG), helix C (WxxxR), helix I ((A/G)GxxT), helix K (ExxR), and the invariant sequence prior to heme-binding motif (PxxFxPE/DRF) (Feyereisen, 2005). CYP protein have a strongly conserved region surrounding the heme core structure and possesses poorly conserved N- and C-termini region (Hasemann et al., 1995). The more conserved regions include helices D, E, K, L, J and C-termini of helix I (Hasemann et al., 1995; Graham and Peterson, 1999; Rewitz et al., 2006d).

Rewitz et al. (2006d) stated that these characteristic signatures sequence are conserved for all P450s due to functional significance (Rewitz et al., 2006d). Therefore, these structural consensus sequences have been used as a guideline for cytochrome P450 identification. Previously, Gotoh et al. (1992) identified six regions on CYP2 that are putative substrate recognition site (SRS) for many further studies involving the determination of SRS regions (Godard et al., 2005; Wang et al., 2006). The flexible substrate recognition sites are located near the substrate access channel and catalytic site (Wade et al., 2004). Conformational changes of these regions indicated different kind of substrates in their catalytic centers and facilitate the enzymatic reaction (Gotoh, 1992; Johnson, 2003; Pylypenko and Schlichting, 2004), this feature explains the ability of an individual cytochrome P450s to catalyze a broad spectrum of substrate. In this study, Figure 3 shows the location of the

SRS regions within the Javanese medaka CYP1B1, and CYP1C1 proteins; results shows that SRS1, SRS4, SRS5, SRS6 were highly similar between these CYP1 genes. These SRSs have showed to be conserved among CYP1 orthologous. In contrast, SRS1, SRS2 and SRS3 exhibit low sequence similarity. These results are similar to the variation in sequence similarities observed between zebrafish CYP1A and CYP1D1 (Goldstone et al., 2009) and in CYP1 genes in killifish (Zanette et al., 2009). The six SRSs may differ in their relative importance among CYPs, but are likely to correspond to regions containing substrate-contacting residues in most CYP genes. These SRS regions have been proposed as crucial for defining substrate specificity for individual CYP isoforms (Gotoh, 1992).

CYP1 mRNAs expression in Javanese medaka

Effect of oil-contaminated feed on CYP1s expression

Real-time PCR results showed that the highest expression rates of the CYP1s gene from the Javanese medaka transcript in response to 1% heavy fuel oil-contaminated feed were observed in intestine, and the lowest in liver (intestine>gill>muscle>liver for CYP1B1; intestine>muscle>gill>liver for CYP1C1). Although the liver is a major site of expression, not all genes were predominantly expressed in this tissue (Tables 4 and 5). The induction of the CYP1s in intestine may reflect functions associated with the role of the organ in nutrient uptake and processing of body waste products, that is, detoxification of endogenous metabolites and providing a

Table 5. CYP1C1 mRNA expressions in Javanese medaka by environmental conditions.

Sample	Fold induction		
	Oil-contaminated feed	Salinity shock	Starvation
Liver	0.44 ± 0.37	1.08 ± 0.33	0.00 ± 0.00
Gill	1.51 ± 0.35	7.32 ± 1.07	0.49 ± 0.13
Muscle	1.60 ± 0.29	2.94 ± 0.50	0.02 ± 0.01
Intestine	7490,14 ± 131.01	0.88 ± 0.04	0.41 ± 0.09

Amount of CYP1C1 mRNA, normalized to β-actin mRNA. Relative fold induction was calculated by the equation $2^{-\Delta\Delta Ct}$ using data from carrier control. Data shows ± standard errors of the mean (n=3).

defensive mechanism against the pollutants entering from the external environment (Nebert et al., 2000; Hassanin et al., 2009). Abeer et al. (2012) determined the expression patterns of CYP1C1 gene in liver, intestine, and muscle of tilapia, and found that there was a large increase in CYP1C1 mRNA in liver, intestine and muscle of tilapia injected benzo[a]pyrene (100 mg/kg body weight) (Abeer et al., 2012). The induction of CYP enzymes in fish liver was first seen as an indicator of aquatic contamination in the 1970s (Payne, 1976; Zanette et al., 2009). Since then, many studies have shown that CYP genes in vertebrate liver are strongly induced by certain organic contaminants that represent a risk to humans and wildlife (Bucheli and Fent, 1995; Zanette et al., 2009). Although the liver is a major site of expression, not all genes were predominantly expressed in this tissue. In our studies, the CYP1s constitutive expression was down-regulated in liver. In carp, CYP1C1 has been reported by Northern blot in gills but not in kidney, liver, or intestine (Itakura et al., 2005). In the present study, we found that CYP1s mRNA was significantly induced by 1% heavy oil-contaminated feed in all tissue selected except liver. While, the lack of induction in the liver was unexpected, it may be because the fish were fed with contaminated food in a short time and thus rapid biotransformation of heavy oil in the liver lowered the concentrations in this tissue. Similar to the present study, previous studies have shown that the *Fundulus heteroclitus* exposed to BaP caused the lowest induction of CYP1C1 in female and male liver (Wang et al., 2006). Jonsson et al. (2007) found that CYP1B1, and CYP1C1 transcripts were induced in gills but not in liver of zebrafish exposed to β-naphthoflavone in the water, while similar exposure to PCB126 induced these genes in both organs (Jönsson et al., 2007). Comparing the levels of expression, each CYP1 in different organs of zebrafish, killifish, and mouse showed similar patterns for the Javanese medaka CYP1B1, with lower level in the liver and gill (Choudhary et al., 2005; Jönsson et al., 2007; Zanette et al., 2009). The relative levels of expression of CYP1B1 in brain, gill, kidney and liver observed by Gao et al. (2011) in three-spined stickleback were explored to aryl hydrocarbon receptor (AHR) which is similar to the CYP1B1 results presented here (Gao et al., 2011). The relatively low inducibility in liver tissue was supported by Hoffmann and Oris (2006) demonstrating that zebrafish exposed to 1.5 and 3.1 g/L waterborne BaP for 56 days, there was a dose-related increase in CYP1B mRNA expression in heads but not in liver (Hoffmann and Oris, 2006).

Effect of salinity shock on CYP1s expression

Javanese medaka CYP1B1 and CYP1C1 transcripts were detected in most of tissues examined including gill, muscle and liver (Tables 4 and 5). For CYP1B the highest and lowest levels of expression were found in muscle and liver, respectively (muscle>gill>liver>intestine). The highest level of CYP1C1 transcript expression was found in gill, while the lowest was found in the intestine (gill>muscle>liver>intestine). Many changes to gene expression and protein activity are observed at least 12 to 18 h after exposure to a hyperosmotic environment, suggesting that these effects may be mediated by immediate early gene transcriptional factors (Fiol and Kültz, 2005). In this study, we directly transferred the fish from seawater to freshwater and kept them in the stressful condition for 24 h. This result is also consistent with several previous studies. The involvement of such transcriptional factors displaying rapid changes in gene expression after hyperosomotic stress has been described in the gills of tilapia (*O. mossambicus*) acclimated to seawater from freshwater (Fiol and Kültz, 2005). The findings of the present study clearly show that CYP1 genes expression is up-regulated after freshwater transfer, suggesting that this biotransformation enzyme (Stegeman and Hahn, 1993; Buhler and Wang-Buhler, 1998; Rifkind, 2006) may be involved in the acclimation of Javanese medaka to freshwater. Fiol and Kültz (2007) indicated that euryhaline fish can sense and quantify changes in external salinity and activates appropriate compensatory responses (Fiol and Kültz, 2007). After seawater transfer, some parameters are expressed diffe-

rently with salinity, such as transcription factors, and blood parameters, the levels of which increased when salinity increased (Fiol and Kültz, 2005; McGuire et al., 2010). The outcome of the study presented that the expression levels of the biomarker family genes also increased when salinity decreased. Following transfer to freshwater, CYP1 mRNA levels rose, suggesting that this enzyme may play an important role in the salinity stress response developed at the level of the gill and muscle. The present study clearly suggests CYP1 genes involvement in another unexpected physiological function of the Javanese medaka, that is, acclimation to changes in salinity.

Effect of starvation on CYP1s expression

Real-time PCR results showed that starvation of the Javanese medaka for one week tended to be down-regulated in CYP1s expression (Tables 4 and 5). CYP1B1 expressed in intestine and gill, but not in muscle and liver. CYP1C1 gene expressed in all the tissues analyzed was down- regulated in Javanese medaka starved for one week. There are many reports on the effects of starvation on the content of nutritional components and on energy production pathways in fish (Shul'man, 1974; Love, 1980). Vertebrates differ in their ability to tolerate starvation. Some small birds and mammals may only tolerate one day of starvation (Baggott, 1975; Mosin, 1984; Blem, 1990), whereas some snakes and frogs are reported to survive nearly two years of starvation (Grably and Peiery, 1981; de Vosjoli et al., 1995). The mechanism by which starvation exerts its effects on these parameters is often discussed in terms of metabolic reorganization in response to changes in nutritional state. It is well- known that a substantial part of the dietary requirement of fish is derived from protein sources, whereas a larger proportion of carbohydrates and fat are necessary in homeothermic animals (Cowey and Sargent, 1972). Furthermore, several continuous days of starvation in mammals is a physiological abnormality, whereas fish are generally adapted for extensive periods of starvation. Therefore, the mechanism by which starvation exerts its effects on mammals is probably quite different from that of fish (Andersson et al., 1985; Quabius et al., 2002). The results of the present study shows that Javanese medaka starved for one week tends to down- regulate on CYP1 genes expression. Experimental induction of cytochrome P450 in fish by several xenobiotics is well- studied. Several researches have demonstrated that the induced activity may vary with sex, stage of sexual maturity, food availability and ambient temperature (Hansson et al., 1980; Forlin et al., 1984; Forlin and Haun, 1990; Quabius et al., 2002). However, the present study indicates that the level of induction of the CYP1 genes in Javanese medaka was down-regulated by a one-week period of starvation. Nutritional status (that is, long-term food deprivation) influenced both tissues concentrations and biomarker responses. Food deprivation did not appear to influence hepatic EROD activities or CYP1 gene content in *S. alpinus* held for 141 days either under a restricted feeding regime or without food (Jorgensen et al., 1999). Cytochrome P450-dependent activities towards selected substrates were decreased to varying extents, whereas the liver cytochrome P450 content was not affected by starvation (Andersson et al., 1985). The lower levels of CYP1 induction observed in this study may have results from a non-specific loss of microsomal proteins due to an increased demand for proteins needed for energy production (Quabius et al., 2002).

According to Barclay et al. (1983); Dall and Smith (1986), muscle protein is the main protein reserve during starvation periods (Barclay et al., 1983; Dall and Smith 1986). Most studies on nitrogen metabolism during starvation of decapods indicate that total protein values generally show a significant reduction, confirming that shrimp, as other crustaceans, are well adapted to use protein as a source of energy (Claybrook, 1983). The transcription rate of housekeeping genes or those expressed in a constitutive manner, such as h-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cytochrome c oxidase (COX), is constant by food deprivation (Yamada et al., 1997). In this study, the transcription rate of the CYP1 genes was down- regulated by starvation for a 1-week period. This might be a response to the acclimatization strategy in which organisms were fed at certain times each day, so that they prepared themselves for food digestion (Sánchez-Paz et al., 2003).

Conclusion

In summary, we cloned two CYP1 family genes, *CYP1B1* and *CYP1C1* in Javanese medaka, an important model fish widely used in environmental toxicology studies. Javanese medaka CYP1B1 and CYP1C1 transcripts were detected in all the gill, muscle and liver with the highest transcript levels found in gill and muscle, when the fish transferred from seawater to freshwater. The highest transcript levels were found in intestine and the lowest in liver in response to 1% heavy fuel oil-contaminated feed. Starvation of Javanese medaka for one week was down- regulated on CYP1 genes induction in most of tissue analyzed. Cloning of the CYP1 genes and given their various degrees of responsiveness to pollutants, expression patterns in fish could become useful biomarkers in environmental monitoring.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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