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cDNA cloning, characterization and expression of cytochrome P450 family 1 (CYP1A) from Javanese medaka, *Oryzias javanicus* by environmental conditions

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Cytochrome P450 family 1 (CYP1A) subfamily genes are the most well studied CYP genes in vertebrates; however, information on *CYP1A* genes in Javanese medaka is relatively scarce. In the present study, full-length cDNA of CYP1A was cloned from the fish liver exposed to 500 ppb β-naphthoflavone for 24 h, which is 2439 bp contained in an open reading frame of 1593 bp encoding a protein of 530 amino acids. Real time polymerase chain reaction (RT-PCR) was used to measure the quantitatively tissue expression of the gene by environmental stress conditions. The results indicate that the highest levels of the *CYP1A* gene transcript was in intestine and the lowest in liver of the fish that fed on fuel oil-contaminated feed. Javanese medaka CYP1A transcripts were detected in the gill, muscle and intestine when transferred from seawater to freshwater with the highest level of expression in gill and muscle. CYP1A gene expression in the tissues tends to be down-regulated in Javanese medaka starved for one week.

Key words: Cytochrome P450, CYP1A, Javanese medaka, heavy fuel oil, salinity, starvation, cloning, expression.

INTRODUCTION

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, including 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 studied

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the most. Mammalian species contain only the CYP1A and CYP1B subfamilies, while the CYP1C subfamily has been detected only in non-mammalian vertebrates (Goldstone et al., 2007). Mammals have two *CYP1A* genes (CYP1A1 and CYP1A2), while most fish have a single *CYP1A* gene (Goldstone et al., 2007).

Javanese medaka (*Oryzias javanicus)* is one of more than 14 species belonging to the genus *Oryzias* distributed in the estuarine regions of East and Southeast Asia (Iwamatsu, 1998; Kinoshita et al., 2009). Medaka has been used as an experimental test fish because it has several advantages, including its ability to adapt to different environmental conditions. Therefore, the fish has been widely used in assessing environmental risk, toxicity testing for new pollutants in various environments, biological response testing and sensitive molecular biomarkers testing (Koyama et al., 2007; Woo et al., 2009)

Baker (1970) and Williams et al. (1994) reports that crude oil and its refined petroleum products contain several toxic organic and inorganic components, which constitutes a significant health risk for both human and other organisms. The ingestion of petroleum hydrocarbon has been reported to induce oxidative stress (Val and Almeida-Val, 1999) by 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). Petroleum also cause 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).

Salt stress is an aquacultural and environmental problem considered worldwide. Euryhaline fish, such as the Javanese medaka, can live in both fresh and seawater. However, there are 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 be mediated immediately by 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 a better understanding of their physiological and toxicological functions.

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, derived in a high percentage from protein synthesis, and mobilize their endogenous reserves to obtain the energy required to maintain the vital processes (Miriam and Ana, 2011). Starvation may influence 7-ethoxyresorufin-*O*-deethylase (EROD) activities in fish (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, which are 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 (1993), 3 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).

Earlier studies have demonstrated that β-naphthoflavone (BNF) is able to induce (Zhang et al., 1990) the activities of the hepatic CYP1A, UDP-GT and GST in rainbow trout (*Onchorhynchus mykiss*). CYP1A and phenol UDP-GT were induced by BNF in sea bass (*Dicentrarchus labrax*) (Novi et al., 1998). In dab (*Limanda limanda*), only CYP1A was induced; whereas, phase II activities were unchanged (Lemaire et al., 1996). In gilthead seabream *(Sparus aurata*), a proterandrous hermaphrodite species, a hepatic CYP1A isoform has been demonstrated to be inducible by benzo[*a*]pyrene and BNF (Şen and Arinç, 1997). *Takifugu obscurus* CYP1A showed a positive response to BNF exposure (Kim et al., 2008). BNF is an aryl hydrocarbon receptor agonist. AhR is a ligand activated transcription factor that forms a hetero-dimer with the AhR nuclear translocator (ARNT). The AhR-ARNT hetero-dimer binds to XREs. This binding initiates the transcription of several genes of biotransformation enzymes including CYP1A (Whitlock, 1990). From report, Fish differ from mammals in having not one, but at least two *AhR* genes (Hahn, 2002). However, the induction mechanism of CYP1A in fish is believed to be similar to mammals (Pollenz et al., 2002). BNF-induced expression of CYP1A has been reported in many fish species (Sarasquete and Segner, 2000; Chung-Davidson et al., 2004; Jonsson et al., 2007). The mechanism of CYP1A inducted by BNF and other inducers involves the activation of the AhR, which initiates gene transcription (Whitlock, 1990). Here, we report on cDNA cloning and sequence analysis of one of the dominant isoforms of cytochrome P450 gene *CYP1A* from Javanese medaka exposed to 500 ppb BNF over a period of 24 h. We also studied expression patterns of CYP1A mRNA in different tissues caused by environmental factors.

Table 1. Oligonucleotide primers used for CYP1A cloning and real time PCR.

Primer name	Description	Location	Primer sequences (5'-3')
Dgp_CYP1A 1F	Degenerate PCR	957-976	GACTCCCTBATTGAYCACTG
Dgp_CYP1A 2R	Degenerate PCR	1229-1248	TGCCACTGRTTGATGAAGAC
Java_CYP1A_GSP 3F	RACE PCR	203-227	ACATCGGCCTGGACCGAAATCCTAC
Java_CYP1A_GSP 2R	RACE PCR	306-331	CCTTTGTTGAGCAGTGTGGGATTGTG
Java_CYP1A 1F	Real-time PCR	1163-1182	CATTCACAATCCCACACTGC
Java_CYP1A 2R	Real-time PCR	1269-1288	ATGGATCCTGCCACAGTTTC
Java_actin 1F	β-actin RT PCR	346-365	AGGGAGAAGATGACCCAGAT
Java_actin 2R	β-actin RT PCR	447-466	CAGAGTCCATGACGATACCA

MATERIALS AND METHODS

Treatment of fish

Javanese medaka (*Oryzias javanicus*) was cultured in the aquarium facility at the Marine Biotechnology Laboratory, Faculty of Fisheries, in Kagoshima University. Fish were acclimatized to laboratory conditions for one week before the experiment, during which they were fed twice daily on a fine fish commercial diet. After the acclimatization period, eight 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 the tissue using ISOGEN reagent (Nippon Gene, Japan), according to the manufacturers' 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 (2001) (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 CYP1A

Degenerate inosine-containing primers were designed from highly conserved regions, based on the alignment of CYP1A sequences from other fish species. All the primers are listed in Table 1**.** PCR was performed using an Astec PC320 PCR system (Astec Bio, Japan) and TaKaRa Ex Taq polymerase (Takara Bio, Japan) by 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 extension for 2 min at 72°C. The 5' and 3' ends of the CYP were obtained by rapid amplification of cDNA ends (RACE) using the SMART™ RACE cDNA amplification kit (Clontech Takara Bio, Japan) per 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 *E. 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 Laser gene sequence analysis software (DNAStar ver 5.2). 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 clustal W program with MegAlign in DNAStar. DNA sequences and their GeneBank accession numbers that were retrieved from the database were used in the analysis presented in Table 2. Phylogenetic and molecular evolutionary analyses were constructed by web-based software using the neighbor-joining method (Dereeper et al., 2010). MatGAT (James et al., 2003) was used to calculate similarity and identity of deduced amino acid sequences of Javanese medaka CYP1s with other CYP1 family members.

CYP1A expression in various organs of Javanese medaka

Medaka fish and experimental design

Javanese medaka fish were acclimatized to laboratory conditions for one week before the experiment during which they were fed twice daily on the medaka commercial diet (Kyorin, Japan), and all fish were fed well at the initiation of the treatments. Natural seawater (33-34 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 an oil-contaminated feed at levels of 0% (control) and 1%. Thirty (30) 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 two days prior to freshwater transfer, and the change in salinity was

Table 2. Gene Bank accession numbers of the CYP1A cDNAs used

Specie	Gene name	Accession number
Tilapia (*Oreochromis niloticus*)	CYP1A	FJ389918
Three-spined stickleback (*Gasterosteus aculeatus*)	CYP1A	HQ202281
Plaice (*Pleuronectes platessa*)	CYP1A1	X73631
Japanese eel (*Anguila japonica*)	CYP1A	AB015638
Rainbow trout (*Oncorhynchus mykiss*)	CYP1A1	U62796
	CYP1A2	U62797
Oyster toadfish (*Opsanus tau*)	CYP1A	U14161
Atlantic tomcod (*Microgadus tomcod*)	CYP1A1	L41917
European flouder (*Platichthys flesus*)	CYP1A	AJ132353
Zebrafish (*Danio rerio*)	CYP1A	AB078927
Leaping mullet (*Liza saliens*)	CYP1A	AF072899
Atlantic salmon (*Salmo salar*)	CYP1A	AF361643
Japanese medaka (*Oryzias latipes*)	CYP1A	AY297923
Indian medaka (*Oryzias melastigma*)	CYP1A	JQ905051

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 (15) 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 was sampled after 24 h.

Starvation experiment

The fish were acclimated to laboratory conditions for one week prior to the study and all fish were fed 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™ first strand cDNA Synthesis Kit (Takara Bio, Japan), following the supplier's protocol. Gene-specific primers for CYP1A 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 CYP1A within a given organ, and to calculate changes in fold-induction in response to experimental conditions, using β-actin as a reference gene.

RESULTS

Characterization of CYP1A cDNA from Javanese medaka

The full-length CYP1A cDNA was cloned from Javanese medaka liver exposed to 500 ppb β-naphthoflavone for 24 h. The results showed that the full-length cDNA of CYP1A from Javanese medaka contains 2439 bp with an open reading frame of 1593 bp. The deduced protein sequence has 530 amino acid residues with an estimated molecular weight of 60.43 kDa. A 141 bp 5' untranslated region precedes the start codon, and a long 705 bp 3' untranslated region follows the stop codon. Three putative polyadenylated signal sites (AATAAA) were found in the long 3' untranslated region (Figure 1).

Similarity and identity analysis of the CYP1A cDNA

Table 3 shows the percentage of similarity and identity of deduced amino acid sequences of Javanese medaka CYP1A with other published CYP1A subfamily sequences. Results indicate that CYP1A shared the highest amino acid identity with Indian medaka (*Oryzias melastigma*) CYP1A (93.4%), followed by 90% with Japanese medaka (*Oryzias latipes*) CYP1A, and 79.1% with Leaping mullet (*Liza saliens*) CYP1A1. A lower overall identity was recorded to Atlantic tomcod (*Microgadus tomcod*) CYP1A1 (64.1%). In terms of similarity, Javanese medaka CYP1A shared the highest amino acid similarity with Indian

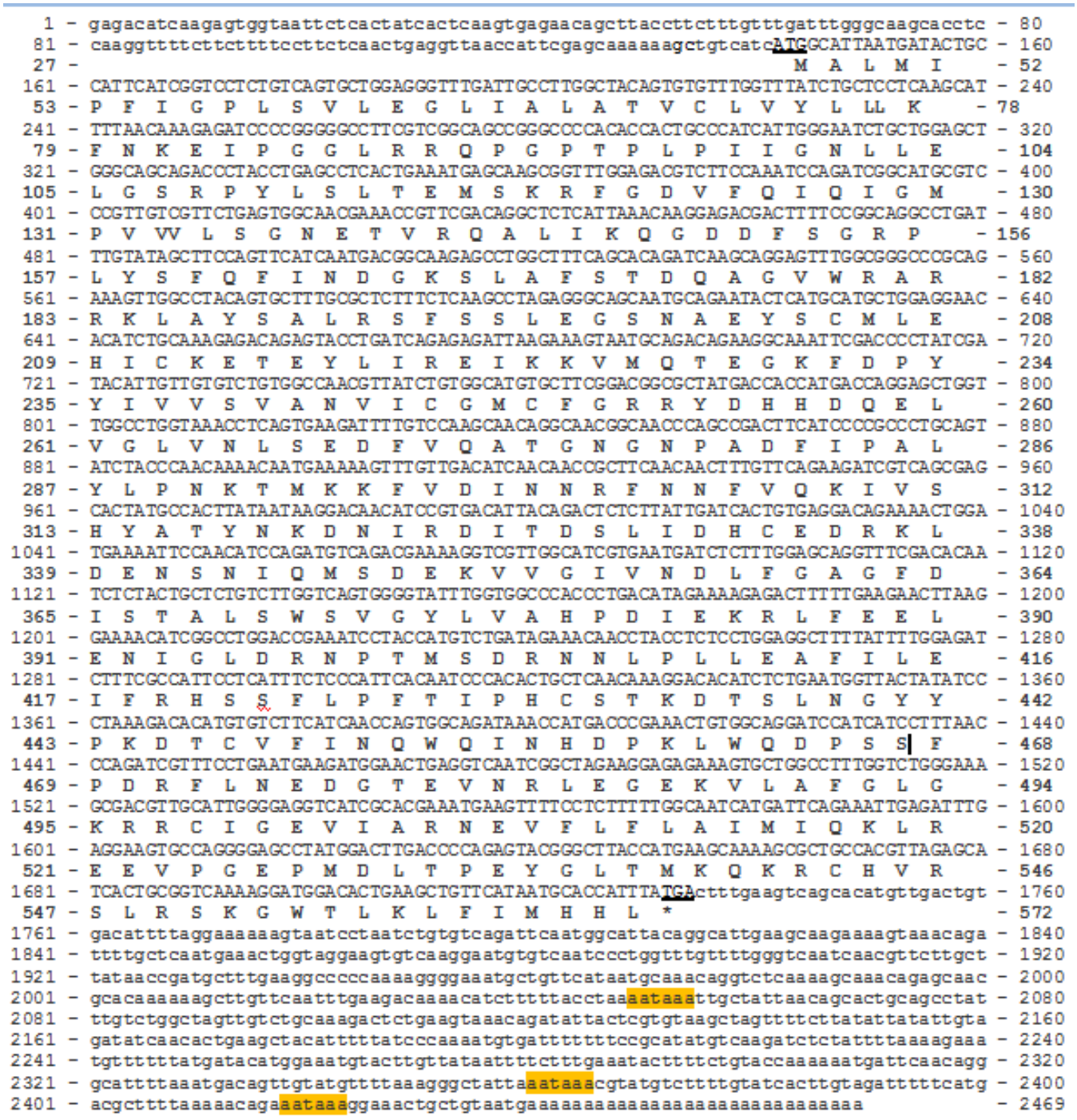

Figure 1. Full-length cDNA of CYP1A and the deduced amino acid residues. The coding sequences are shown in uppercase letter whereas lowercase for untranslated regions. The predicted amino acid sequences are in bold letters. The translation start codon and termination codon are underlined and the putative polyadenylation signal (aataaa) is colored in orange.

medaka (*Oryzias melastigma*) (96.6%), followed by Japanese medaka (*Oryzias latipes*) CYP1A (95.8%). The lowest similarity was noted in tomcod CYP1A1 (80.8%).

Phylogenetic analysis of the CYP1A enzyme

The phylogenetic tree based on the amino acid

Table 3. Percent similarity and identity (upper triangle) of deduced amino acid sequence of Javanese medaka CYP1A with other CYP1A subfamily members

Parameter	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Java_CYP1A		76.8	75.5	75.7	70.9	74.7	73.6	74.3	64.1	76	68.3	79.1	74.2	90	93.4
Tila_CYP1A	88.7		76.8	80.2	72.7	75.3	74.7	77.4	64.8	80.6	72.4	83.3	74.7	77.9	77.9
Stickle_CYP1A	90	91		86.2	76.4	80.8	80.5	78.1	70.6	86	72.6	82.1	80.7	75.2	76
Plaice_CYPIA1	88.5	92.3	93.5		76.6	80.1	79.3	81.6	70	97.9	73.5	85.4	80.1	77.4	77
Jeel_CYP1A	88.3	88.7	91.6	90.8		80.7	79.5	74.5	68.3	76.6	74.6	77	81.2	73.1	72
Rainbow_CYP1A2	89.1	89.3	91.8	91	92.3		98.1	77.6	70.1	80.7	77.6	81	97.1	75.1	74.7
Rainbow_CYP1A1	88.3	88.7	91.4	90.2	91.8	99		77	69.2	79.9	76.8	80.3	96.6	73.9	74.3
Toadfish_CYP1A	88.7	91.9	91.7	93.1	90.2	90.6	89.8		68.2	82	74.3	80.2	77.6	77	76.6
Tomcod_CYP1A1	80.8	82	81.8	83.3	82.3	82.8	82	82.9		70.6	63.1	69.1	70.7	65.2	65.5
Flouder_CYP1A	89.4	92.5	93.5	98.5	90.6	91	90.2	92.9	83.5		73.1	85	80.5	78.3	77.4
Zebra_CYP1A	84.7	87.3	88.5	89.1	89.6	90.6	90	88.7	80.5	88.9		74.7	76.8	69.9	69.1
Mullet_CYP1A1	90.8	93.5	92.3	93.7	90.6	91.6	91	93.3	85.4	93.9	89.1		80.7	78.9	80
Salmon_CYP1A	88.5	89.1	91.6	91	92.1	98.9	97.7	90.2	82.8	90.4	90.6	91.4		74.9	74.9
*O. latipes*_CYP1A	95.8	90.4	91.2	89.8	89.4	89.5	88.7	90.2	82	91	86.2	92.3	89.3		93.1
*O. melastigma*_CYP1A	96.6	91	91.2	89.8	89.6	90.2	89.5	90.2	82.3	90.8	86	92.3	89.5	97.9	

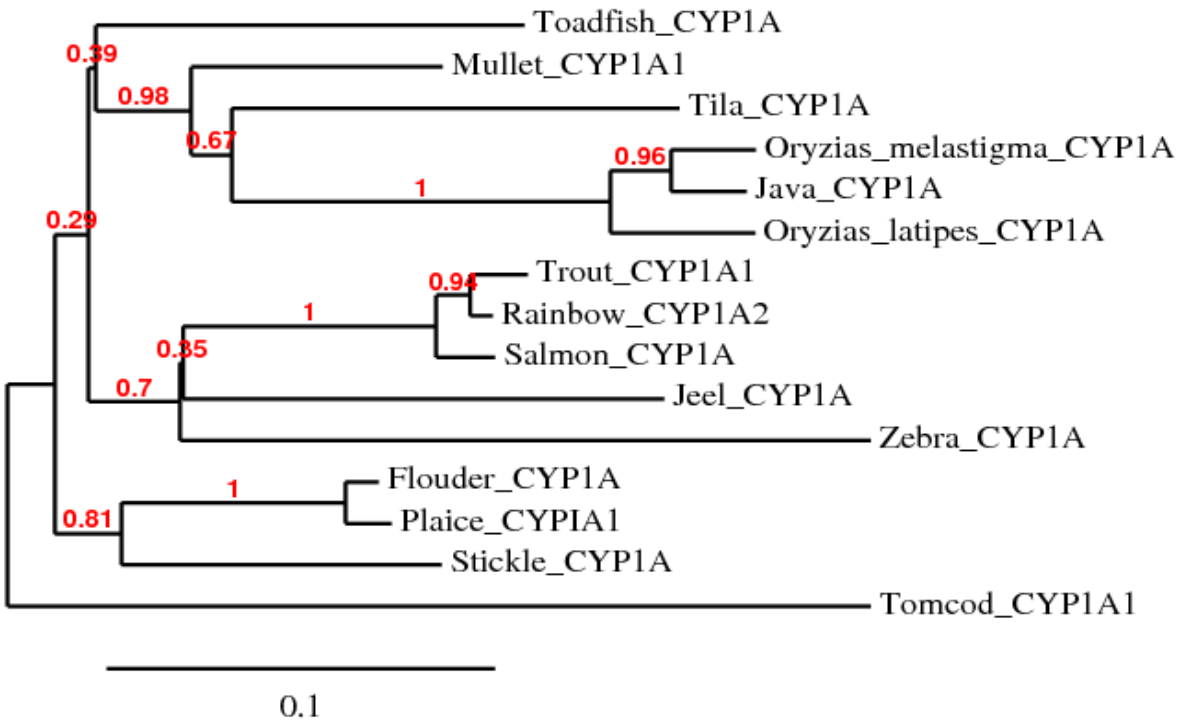

Figure 2. Phylogenetic tree of Javanese medaka CYP1A constructed by the neighbor-joining using percent identity of deduced amino acid sequences. The building of the tree also involves a bootstrapping process repeated 100 times to generate a majority consensus tree. *Microgadus tomcod* was used as an outgroup.

sequences, was used to assess the relationship of CYP1A in Javanese medaka with those of other fish species (Figure 2). The phylogenetic tree, with 14 representatives of full-length CYP1A subfamily protein sequences, indicates that the CYP1A clustered with Japanese medaka (*Oryzias latipes*) CYP1A and Indian

medaka (*Oryzias melastigma*) CYP1A1, indicated the evolutionary relatedness.

Functional domain identification

The deduced amino acid sequences of CYP1A shares a number of characteristics with other cytochrome P450s. Javanese medaka CYP1A N-terminal consists of a proline-glycine rich region as PGPTPLPI. In addition, sequence alignment of Javanese medaka CYP1A enzymes with those of the CYP1A subfamily sequences indicated that Javanese medaka CYP1A contain the five structural conservations around heme-binding core for all cytochrome P450s and six separate substrate recognition sites (SRSs) (Figure 3). The signature motif (FxxGxRxCxG) of the heme-binding core appeared as FGLGKRRCIR. The heme-interacting region of Helix C (WxxxR) was presented as WRARR. The highly conserved residues in Helix I ((A/G) GxxT) showed as GFDTY. The structural conservation played a role in the stabilization of the core structure of cytochrome P450s by hydrogen bond; Helix K (ExxR) was found in the Javanese medaka CYP1A as EIFR. The invariant sequence (PxxFxPE/DRF) proximal to the heme-binding motif is demonstrated as PSSFNPDRF. Figure 3 identifies the six putative substrate recognition sites, (SRS1, SRS2, SRS3, SRS4, SRS5 and SRS6) in the medaka CYP1A gene. The substrate recognition sites (SRSs) of the orthologous Javanese medaka and other species CYP1A subfamily were inferred by the sequence alignment used by (Gotoh, 1992).

CYP1A mRNA expression in Javanese medaka

Effect of oil-contaminated feed on CYP1A expression

Real time PCR results showed that the highest expression rates of the CYP1A 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>muscle>gill>liver). Although the liver is a major site of expression, not all genes were predominantly expressed in this tissue (Table 4).

Effect of salinity shock on CYP1A expression

Javanese medaka CYP1A transcripts were detected in most of the tissues examined, including gill, muscle and intestine, but not liver (Table 5). The highest and lowest levels of expression of CYP1A from Javanese medaka were found in gill and liver, respectively (gill > muscle > intestine>liver).

Effect of starvation on CYP1A expression

Real-time PCR results showed that the CYP1A gene expressed in the tissues analyzed tended to be down regulated in Javanese medaka starved for one week (Table 6). Starvation had no effect on CYP1A induction in all examined tissues, except gill.

DISCUSSION

Identification of a new CYP1A subfamily expands the diversity of CYP1 genes and presents an opportunity to increase our understanding of the physiological and toxicological significance members of this gene family. In this study, the full-length cDNA, namely CYP1A, were cloned from Javanese medaka. The coding sequences of the identified cDNA were around 1500 bp, which translated into a 500 amino acid protein with a molecular weight around 58 to 60 kDa. Nomenclature of cytochrome P450 is generally based on the identity and evolutionary relationship shared with other cytochrome P450 sequences at the time of discovery, such that genes are grouped in the same family and subfamily when they have > 40% and > 55% amino acid sequence identity, respectively (Nelson 2006). In the present study, Javanese medaka CYP1A was placed in CYP1A family, due to its high identity with most of the CYP1A family members (93.4% with *Oryzias melastigma* CYP1A and 90% with *Oryzias latipes* CYP1A). In addition, molecular phylogenetic analysis shows that Javanese medaka CYP1A and CYP1A subfamily members from other species were grouped in one clade (Figure 2).

The characteristics of the N-terminal region of medaka CYP1A protein exactly match that of the microsomal P450 proteins. At the N-terminal region of medaka CYP1A, 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 sequence of the Javanese medaka CYP1A gene. 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 the heme-binding motif (PxxFxPE/DRF). (Feyereisen 2005) CYP protein has a strongly conserved region surrounding the heme core structure and possesses poorly conserved N- and C-termini regions (Hasemann et al. 1995). Therefore, these structural

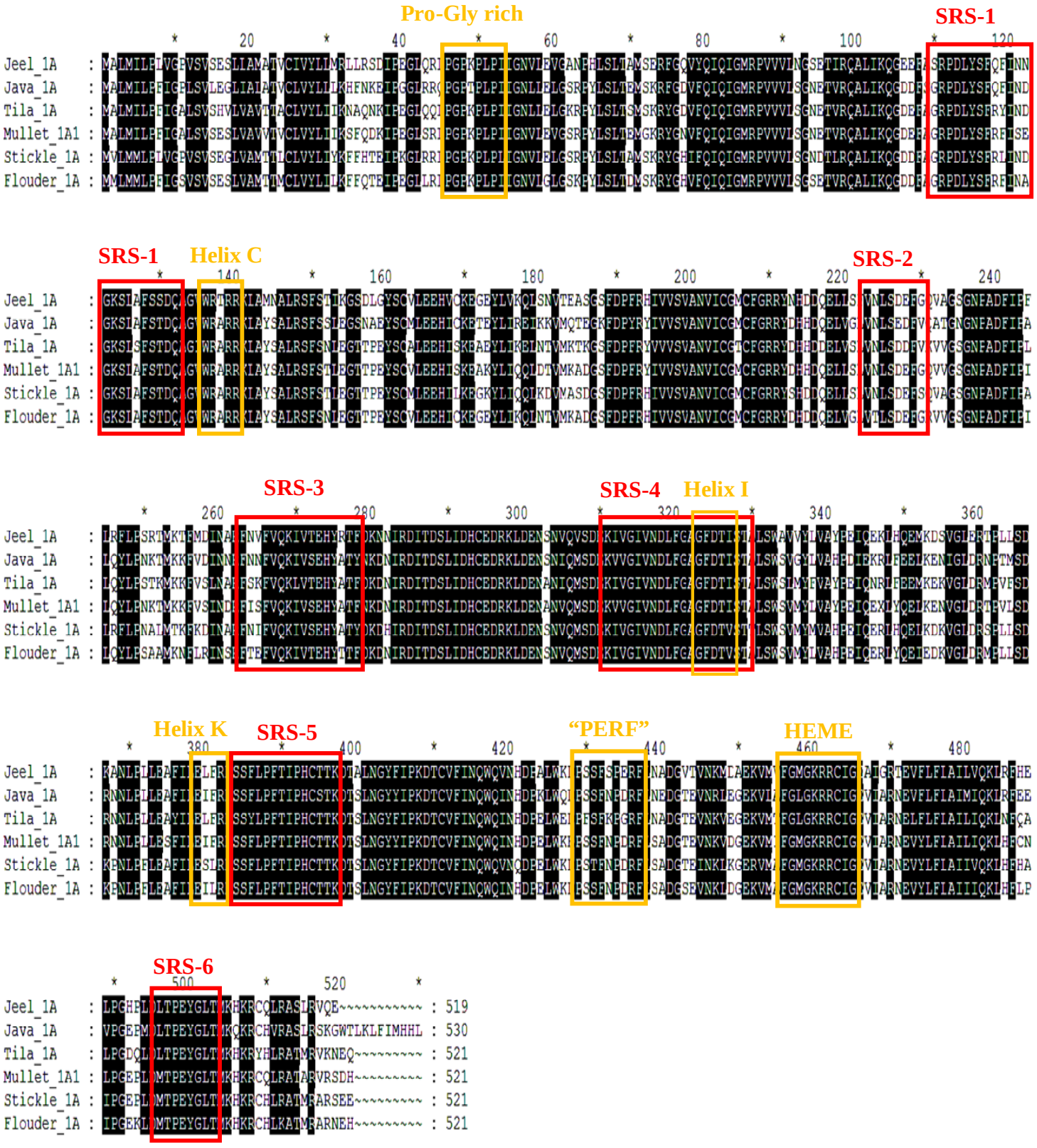

Figure 3. Amino acid sequence alignments of Javanese medaka CYP1A with orthologues. Orange boxes indicate the conserved motif region and red boxes indicates substrate recognition sites (SRS).

Table 4. CYP1A mRNA expressions in Javanese medaka by oil-contaminated feed

Sample Name	Oil-contaminated feed		β-actin		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	Mean Ct	Std Dev Ct	Mean Ct	Std Dev Ct			
L.ctr	19.62	0.12	20.34	0.15	-0.72	1.74	0.30
L.ind	19.50	0.11	18.48	0.11	1.02		
G.ctr	23.94	0.22	18.46	0.25	5.48	-2.21	4.64
G.ind	25.65	0.19	22.38	0.25	3.27		
M.ctr	20.21	0.35	15.09	0.10	5.13	-2.41	5.32
M.ind	16.84	0.16	14.12	0.05	2.72		
I.ctr	20.94	0.13	14.40	0.11	6.54	-10.28	1243.96
I.ind	24.19	0.06	27.92	0.07	-3.74		

Amount of CYP1A 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). L. ctr = liver control; L. ind = liver induced; G. ctr = gill control; G. ind = gill induced; M. ctr = muscle control; M. ind = muscle induced; I. ctr = intestine control; I. ind = intestine induced.

Table 5. CYP1A mRNA expressions in Javanese medaka by salinity shock.

Sample name	Salinity shock		β-actin		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	Mean Ct	Std Dev Ct	Mean Ct	Std Dev Ct			
L.ctr	16.75	0.14	14.61	0.13	2.14	0.68	0.62
L.ind	17.94	0.06	15.12	0.10	2.82		
G.ctr	21.40	0.06	16.05	0.07	5.35	-4.64	24.98
G.ind	20.76	0.08	20.05	0.05	0.70		
M.ctr	22.71	0.09	15.25	0.07	7.47	-2.67	6.35
M.ind	25.63	0.18	20.83	0.12	4.80		
I.ctr	18.87	0.14	10.68	0.12	8.19	-0.80	1.74
I.ind	17.57	0.11	10.17	0.05	7.40		

Amount of CYP1A 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). L. ctr = liver control; L. ind = liver induced; G. ctr = gill control; G. ind = gill induced; M. ctr = muscle control; M. ind = muscle induced; I. ctr = intestine control; I. ind = intestine induced.

consensus sequences have been used as a guideline for cytochrome P450 identification.

In this study, Figure 3 shows the location of the SRS regions within the Javanese medaka CYP1A protein; results shows that SRS1, SRS4, SRS5, SRS6 were highly similar between these CYP1A subfamilies. These SRSs have shown to be conserved among CYP1A orthologous. In contrast, 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).

The real-time PCR results of this study demonstrate the largest levels of CYP1A mRNA transcript in response to 1% fuel oil-contaminated feed observed in intestine, followed by muscle and gill, respectively. The induction of the CYP1A 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 defensive mechanism against the pollutants entering from the external environment (Hassanin et al., 2009). Chris (2001) studied the specific expression of the CYP1A gene in intestine, gills and liver of tilapia exposed to coastal sediments; there was a large increase in CYP1A transcripts in intestine and liver, respectively (Chris et al., 2001). In addition, Hassanin (2009) reported that benzo[a]pyrene (100 mg/kg injection) induced CYP1A1 mRNA expression in intestine and liver of tilapia, with no detectable expression in the gill and kidney (Hassanin et al., 2009). Similar results were reported by Neilson (2000) and Wong (1996), who found a high level of expression of CYP1A in

Table 6. CYP1A mRNA expressions in Javanese medaka by starvation.

Sample name	Starvation		β-actin		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	Mean Ct	Std Dev Ct	Mean Ct	Std Dev Ct			
L.ctr	26.73	0.32	28.62	0.20	-1.89	5.00	0.03
L.ind	17.30	0.19	14.19	0.09	3.12		
G.ctr	19.39	0.11	12.09	0.07	7.30	-0.54	1.45
G.ind	20.46	0.18	13.71	0.31	6.76		
M.ctr	25.35	0.18	20.69	0.09	4.66	2.79	0.14
M.ind	25.56	0.42	18.10	0.11	7.46		
I.ctr	16.51	0.20	9.88	0.10	6.64	1.38	0.38
I.ind	19.83	0.10	11.81	0.40	8.02		

Amount of CYP1A 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). L. ctr = liver control; L. ind = liver induced; G. ctr = gill control; G. ind = gill induced; M. ctr = muscle control; M. ind = muscle induced; I. ctr = intestine control; I. ind = intestine induced.

intestine, with no detectable expression in gills, of tilapia exposed to polycyclic aromatic hydrocarbons (PAHs) (Neilson, 2000). The induction of CYP enzymes in fish liver was first seen as an indicator of aquatic contamination in the 1970s (Zanette et al., 2009). Since then, many studies have shown that CYP genes in vertebrate liver strongly induced by certain organic contaminants that represent a risk to humans and wildlife (Zanette et al., 2009). In this study, no constitutive expression was found in liver. While the lack of induction in the liver was unexpected, it may be because the fish was fed the contaminated food in a short time and thus rapid biotransformation of heavy oil in the liver lowered the concentrations in this tissue.

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 conditions for 24 hours. 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 (*Oreochromis mossambicus*) acclimated to seawater from freshwater (Fiol and Kültz, 2005). Fiol (2007) indicated that euryhaline fish can sense and quantify changes in external salinity and activate appropriate compensatory responses (Fiol and Kültz, 2007). After seawater transfer, some parameters are expressed differently with salinity, such as transcription factors, and blood parameters (osmotic pressure, glucose, and cortisol), which the levels of which increased when salinity increased (McGuire et al., 2010). The outcome of the study showed that the expression levels of the biomarker family genes also increased when salinity decreased. Following transfer to freshwater, CYP1A 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 CYP1A gene involvement in another unexpected physiological function of the Javanese medaka, that is, acclimation to changes in salinity.

Vertebrates differ in their ability to tolerate starvation. Some small birds and mammals may only tolerate one day of starvation, (Mosin 1984; Blem 1990) whereas, some snakes and frogs are reported to survive nearly two years of starvation (de Vosjoli et al., 1995) . 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 showed that CYP1A genes expressed in the tissues analyzed were down-regulated in Javanese medaka starved for 1 week. Several researchers 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 CYP1A gene on Javanese medaka was not influenced by a 1-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 CYP1A content in *Salvelinus 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). In this study, the transcription rate of the CYP1A gene 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 prepare themselves for food digestion (Sánchez-Paz et al., 2003).

Conclusion

In summary, this paper identified and cloned the CYP1A gene in Javanese medaka, an important model species used extensively in environmental toxicology studies. The highest transcript levels in response to 1% heavy fuel oil-contaminated feed were found in the intestine and the lowest in liver. The highest transcript levels were found in gill and muscle when the fish was transferred from seawater to freshwater. The CYP1A gene expressed in the tissues studied was down-regulated in Javanese medaka starved for 1 week. The CYP1A gene from Javanese medaka increased the set of potential biomarkers of environmental stress conditions in fish.

Conflict of Interests

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

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