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Full Length Research Paper

Molecular characterization of cytochrome P450 1B1 and effect of benzo(a) pyrene on its expression in Nile tilapia (*Oreochromis niloticus*)

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Cytochrome P4501 (CYP1) family enzymes are most active in hydroxylating a variety of environmental contaminants including Polyaromatic Hydrocarbons (PAH), planar polychlorinated biphenyls and arylamines. CYP1B which belongs to the cytochrome P450 superfamily of genes, is involved in the oxidation of endogenous and exogenous compounds, and could potentially be a useful biomarker in fish for exposure to arylhydrocarbon receptors (AhR) ligands. In this study, a new complementary DNA (cDNA) of the *CYP1B* subfamily encoding *1B1* was isolated from Nile tilapia (*Oreochromis niloticus*) liver after intracoelomic injection with benzo (a) pyrene (BaP). The full-length cDNA was 2107 base pair (bp) long and contained a 5' noncoding region of 29 bp, an open reading frame of 1527 bp coding for 508 amino acids and a stop codon, and a 3' noncoding region of 551 bp, respectively. The deduced amino acid sequence of Nile tilapia CYP1B1 shows similarities of 79.7, 70.3, 65.7, 65.4, 65.0, and 63.7% with Plaice CYP1B1, Japanese eel CYP1B1, zebra fish CYP1B1, common carp CYP1B1, common carp CYP1B2 and Channel catfish CYP1B1, respectively. The phylogenetic tree based on the amino acid sequences clearly shows tilapia CYP1B1 and Plaice CYP1B1 to be more closely related to each other than to the other CYP1B subfamilies. Furthermore, real-time PCR was used for measuring BaP induction of CYP1B1 mRNA in different organs of tilapia (*O. niloticus*), using β-actin gene as internal control, and the results revealed that there was a large increase in CYP1B1 mRNA in liver (22.8), intestine (2.0) and muscles (1.3).

Key words: *Oreochromis niloticus*, benzo (a) pyrene, CYP1B1 cDNA, sequence analysis, real-time PCR.

INTRODUCTION

CYP enzymes constitute a unique superfamily of heme-containing proteins that are bound to the membrane of the endoplasmic reticulum and play a crucial role as an oxidation-reduction compound of the monooxygenase system. This system is involved in the oxidative metabolism of a wide variety of xenobiotics such as drugs, carcinogens, and environmental disrupters, as well as endogenous substrates such as steroids and fatty acids (Nebert

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and Gonzalez, 1987; Nelson et al., 1996). Most chemical carcinogens in the environment are chemically inert in them and require metabolic activation by CYP enzymes to exhibit carcinogenicity in experimental ani-mals and humans (Conney, 1982; Guengerich and Shimada, 1991). Previously, *CYP1A* enzymes were thought to be the only enzymes responsible for the metabolic activation of most carcinogenic PAHs to reactive electrophiles in mice, rats and rabbits (Conney, 1982).

However, recently *CYP1B* genes have been isolated from mammals and, in common with the *CYP1A* family, they are transcriptionally activated by PAH, and their protein products metabolise PAH (Savas et al., 1994; Zhang et al., 1998). Indeed, metabolism and carcinogenesis studies have recently shown *CYP1B1* to be a critical and necessary enzyme in the activation of several xenobiotics, most notably the PAH 7, 12-dimethyl- benzanthracene (DMBA) (Shimada et al., 1996; Buters et al., 1999), they reported that *CYP1B1* is located exclusively at extra hepatic sites and mediates the carcinogenesis of DMBA.

Shimada et al. (1996, 2002) also reported that *CYP1B1* participates with *CYP1A1* and *CYP1A2* in the activation of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin, benzo (a) pyrene and related carcinogens causing initiation of cancers in human and mice.

Phylogenetic analysis of CYP1 family sequences indicated that mammalian CYP1A and CYP1B lines diverged before the evolutionary emergence of mammals, suggesting the possible existence of CYP1B in fishes (Nelson et al., 1996). In a preliminary report, Godard et al. (2000) described the occurrence of CYP1B-like sequences in the fish species scup and plaice. Subsequently, the full-length plaice sequence was obtained and classified as a CYP1B1 (Leaver and George, 2000).

For carp (*Cyprinus carpio*), two CYP1B sequences have been submitted to GeneBank namely CYP1B1 and CYP1B2 (El-Kady et al., 2004a, 2004b). BaP, a member of the PAH family, is rapidly metabolized often into unstable byproducts such as epoxides, which have mutagenic and cytotoxic effects (Miller and Ramos, 2001). Thus, it elicit toxic effects at least in part by activating the AhR (Denison and Nagy, 2003), and AhR activation has been shown to affect the transcription of CYP isozymes 1A and 1B1 (Nebert et al., 2000). Therefore, in this study, the fish *Oreochromis niloticus* was used as a model organism to investigate the central hypothesis that CYP1B is involved in the molecular mechanisms of BaP-mediated toxicity.

Successful completion of this aim will provide a greater molecular understanding of this important P450 gene and its role in the mechanisms of action of PAHs. This research will further define the utility of *O. niloticus* fish as a model organism for studying PAH-associated toxicities. To achieve such a purpose, cDNA of the *CYP1B1* gene was isolated from the liver of *O. niloticus* fish after intracoelomic injection with BaP and sequenced. Phylogenetic analysis was performed to assess the relation-ship of this newly identified CYP1B1 gene with other CYP1B family members, and the expression pattern of CYP1B1 mRNA was determined in liver, intestine and muscle of tilapia using real -time PCR.

MATERIALS AND METHODS

Treatment of fish

Nile tilapia with a mean weight of 500 g were obtained from a local fish farm and were treated with a single intracoelomic injection of BaP (100 mg/ kg body weight) suspended in corn oil. Simultaneously, with the treated fish, control fish of similar mean weight was intracoelomicly injected with an equivalent volume of the vehicle (corn oil). The treated and control fish were killed 24 h after the injection and samples of liver, intestine and muscles were collected, immediately frozen in liquid nitrogen and stored at -80°C.

RNA isolation

Total RNA was isolated from 2 g of each of the samples of frozen liver, intestine and muscles according to the Standard Acid Guanidinium Thiocyante Phenol Choloroform (AGPC) extraction method (Chomczynski and Sacchi, 1978). Total RNA concentration and purity were determined spectrophotometrically as described by Sambrook and Russel (2001), and A_{260}/A_{280} ratio were between 1.7 and 1.9. Poly $(A)^+$ RNA was purified using an Oligotex-dt30 <super> mRNA purification kit (Takara, Japan).

Reverse transcriptase-assisted polymerase chain reaction

Reverse transcription of mRNA was performed with Superscript II reverse transcriptase (Gibco BRL,USA) to generate 5'-RACE-Ready and 3'- RACE-Ready first strand cDNA using a SMART™ RACE cDNA amplification kit (Clontech, USA) according to the manufacture's protocol.

Oligonucleotide primers and PCR amplification of tilapia *CYP1B1* cDNA fragment

Degenerate primers (Genenet.co.jp) were designed based on the conserved regions of four fishes CYP1B sequences retrievable from GenBank (Table 1). The sense primer (5'- GGR AGC ATM GTG GAY GT -3'; where R is for A or G; M for A or C and Y is for T or C) and the antisense primer (5'- GTG SGG RAT GGT KAS RGG -3'; where S is for G or C; R is for A or G, and K for G or T). PCR reactions contained cDNA (2.5 µl), 200 µM each of dNTPs, 100 µM each of degenerate primers, 0.25 µl Taq Polymerase Mix, and 10× reaction buffer in a final volume of 50 µl. The cycling conditions were as follows: Initial denaturation step at 94°C for 150 s followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min and ended by a final extension step at 72°C for 5 min. PCR products were analyzed on 1% agarose gels. The DNA band of expected size was excised with a scalpel, purified using GFX PCR DNA and a gel band purification kit (GE Health Care, UK), the PCR products obtained were cloned into PT7BlueT- vector (Novagen, USA). Purified plasmids were directly sequenced by dye terminator cycle sequencing using an ABI PRISM dye terminator cycle3130 xl DNA sequencer.

Table 1. *CYP1B* genes used in designing of degenerate primers.

Gene	Specie	Accession number
CYP1B1	common carp	AB048942
CYP1B2	common carp	AY437775
CYP1B1	Japanese eel	AY518340
CYP1B1	channel catfish	DQ088663

Table 2. Oligonucleotide primers used in PCR amplification of Tilapia CYP1B1 cDNA fragments.

Primer	Nucleotide Sequence	Nucleotide location
F1	5'- GGACGTTATGCCCTGGCTGCAGTA -3'	662-685
F2	5'- ACTTCCCCAACCCCATCAAAACCA -3'	685-708
R1	5'- CGGTGTAGCCCATGATGGATGTG-3'	1139-1161
R2	5'- TCCTGCTGGAGACGCACCTGTATC-3'	974-997

Table 3. Real-Time PCR primers of *Oreochromis niloticus* CYP1B1 and β-actin genes.

Gene	Primer description	Sequence (5'-3')	Location	Product size
CYP1B1	F	5'- TTACGTCATGGCCTTCATCTAC -3'	1058- 1079	122 bp
	R	5'- ATGACTGTGTTCTTTGGTACGG -3'	1159-1180	
β-actin	F	5'-GGGTCAGAAAGACAGCTACGTT-3'	42-63	143 bp
	R	5'-CTCAGCTCGTTGTAGAAGGTGT-3'	164-185	

3' and 5' RACE PCRs for full length cDNA

Four gene-specific primers (GSP) were designed based on the sequence obtained from PCR with degenerate primers. The primer pairs GSP-F1 and GSP-R1 (Genenet.co.jp) in combination with the universal primer mix included in a RACE PCR kit were used for the initial 5'- and 3'-RACE, respectively (Table 2). Initial 5'- and 3'-RACE PCR reactions were diluted by 50-fold using tricine EDTA buffer and a 5 µl aliquot of diluted PCR reactions was used in each of 50 µl nested-PCR reactions using primers GSP-F2 and GSP-R2 in combination with the nested universal primer mix included in a RACE PCR kit for 5'- and 3'-RACE, respectively. The initial RACE PCR reactions contained the same components as in the degenerate primer PCR except that the final concentration of each primer was 20 µM. PCR conditions were 5 cycles of denaturation for 30 s at 94°C and annealing for 3 min at 72°C; 5 cycles of denaturation at 94°C for 30 s, annealing at 70°C for 30 s, and extension for 3 min at 72°C and finally 35 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s and extension at 72°C for 3 min. Nested-PCR reactions contained the same components as in the initial PCR except that diluted initial PCR products (5 µl) were used instead of the Race cDNA as the template and the nested universal primer (10 µM) was used instead of the universal primer mix.

The cycle conditions for the nested-PCR were as follows: 35 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 3 min. PCR products were purified, cloned and sequenced as previously described. Sequence has been deposited in the GenBank/NCBI data bank with an accession number HQ829968.

Phylogenetic analysis

DNA sequences with the following GenBank accession numbers were retrieved from the database and used in the phylogenetic analysis: AB048942 (common carp CYP1B1), AY437775 (common carp CYP1B2), AY518340 (Japanese eel CYP1B1), DQ088663 (channel catfish CYP1B1), AJ249074 (Plaice CYP1B1) and AY727864 (zebra fish CYP1B1). In order to determine homology among CYP1B family cDNAs or deduced amino acid sequences from various species, sequence alignment was performed by the CLUSTAL W method using Laser gene Megalign program (Ver 5.52,2003, DNASTAR Inc).

CYP1B1 expression in different organs of *Orochromis niloticus* using real -time PCR

Reverse transcription

Reverse transcription of the RNA samples isolated from liver, intestine and muscles was performed using Primescript™ RT reagent kit (Takara, Japan) according to the manufacturer's instructions. Reactions were incubated for 15 min at 37°C then 5 s at 85°C to inactivate the reverse transcriptase. RT products were stored at 4°C for further PCRs.

Primer design for real- time PCR reaction

Primers for *O. niloticus CYP1B1*cDNA and β-actin cDNA (accession no. EU887951), as an internal standard (Table 3) were designed using Laser gene primer select program (Ver5.52, 2003, DNASTAR Inc), with melting temperatures (T_m) ranging from 58 to 60°C, and amplicon lengths of 50 to 150 bp. Optimal programmed primer annealing temperatures were designed closely so that the optimal annealing temperatures were close enough to run all reactions under the same thermal parameters.

Table 4. Percent identities of deduced amino acid sequences of fish *CYP1B* gene subfamilies.

	Plaice CYP1B1	J.eel CYP1B1	Zebrafish CYP1B1	Common carp CYP1B1	Common carp CYP1B2	Ch.catfish CYP1B1
O. niloticus CYP1B1	79.7	70.3	65.7	65.4	65.0	63.7
Plaice CYP1B1		67.2	63.2	63.1	63.0	62.2
J.eel CYP1B1			64.0	64.5	64.6	62.1
Zebrafish CYP1B1				84.6	85.0	67.1
Common carp CYP1B1					92.3	67.6
Common carp CYP1B2						68.0

Real-time PCR conditions and analysis

Each PCR reaction consisted of 10 µl of SYBR® Premix Ex Taq™ II (2X), 10 µM of each primer, 2 µl of cDNA template and double distilled water to a final volume of 20 µl. Reactions were then analyzed on an ABI 7300 Real-Time PCR System under the following conditions: 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 3 min. All standard dilutions, no template controls and induced samples were run in triplicates. The fluorescence signals were measured at the end of each extension step. The threshold cycle (C_t) was determined for each sample using the exponential growth phase and the baseline signal from the fluorescence versus cycle number plots. To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run. The amount of CYP1B1 mRNA, normalized to β-actin mRNA, was given by the formula 2-ΔΔCT; where CT is the threshold cycle indicating the fractional cycle number at which the amount of amplified CYP1B1 reached threshold. The ΔCT value is determined by subtracting the average β-actin CT value from the average CYP1B1 CT value. Then, the calculation of ΔΔCT involves subtraction of the ΔCT value of the calibrator (in our case the calibrator was average ΔCT value of control fish response in the BaP studies) from ΔCT value of each sample. Accordingly, CYP1B1 mRNA levels were reported as fold change in abundance relative to the average calibrator response.

Statistical analysis

The statistical differences between the groups were determined, and the data expressed as mean ± standard deviation. Excel (Microsoft, NY) were used to analyze the data, and Student's t test was used for the comparisons. A P-value <0.05 was considered significant. At least three determinations were carried out for each data point.

RESULTS AND DISCUSSION

Nucleotide sequence analysis of CYP1B cDNA

An important recent finding in fish is that they have CYP1B and now CYP1C genes, CYP1B was first cloned in plaice (*Pleuronectes platessa*) (Leaver and George, 2000), then cloned in carp (El-Kady et al., 2004a, 2004b), channel catfish (*Ictalurus punctatus*) (Kristine et al., 2006) and zebrafish (Hou-Chu Yin, 2008). In both plaice and channel catfish, only a single isoform of CYP1B has been identified, whereas both CYP1B1 and CYP1B2 genes have been cloned in carp (*Cyprinus carpio*). In this study, a new cDNA of the CYP1B subfamily encoding CYP1B1 was isolated from Nile tilapia. The nucleotide sequence (Figure 1) contained a 5' noncoding region of 29 bp, an open reading frame of 1527 bp coding for 508 amino acids and a stop codon, and a 3' noncoding region of 551 bp. The predicted molecular weight was 57.67 KDa. The sequence had one polyadenylation signal (AAGAAA) and a poly A tail of 30 nucleotides. This sequence was aligned with the previously mentioned sequences by CLUSTAL W (Thompson et al., 1994) using Lasergene Megalign program, version 5.52, 2003 (DNASTAR Inc).

Comparison of amino acid sequences

Table 4 shows the percent identities of deduced amino acid sequences of *O*. *niloticus* CYP1B1 with the other fish CYP1B genes. The highest identity was 79.7% with plaice CYP1B1, followed by 70.3% with Japanese eel CYP1B1, 65.7% with zebra fish CYP1B1, 65.4% with common carp CYP1B1, then ended by 65% with common carp CYP1B2 and 63.7% with channel catfish CYP1B1. These results suggested that the obtained amplification product corresponds to tilapia CYP1B1 as it has more than 55% amino acid identity with other fishes CYP1Bs.

Phylogenetic analysis

The phylogenetic tree based on the amino acid sequences were used to assess the relationship of CYP1B1 of *O*. *niloticus* with those of other fish species.

Figure 2 clearly shows tilapia CYP1B1 and plaice CYP1B1 are more closely related to each other than to the other CYP1B subfamilies. As sequences from other species accrue, it is clear that many teleost fish possess a complement of three CYP1 subfamilies, CYP1A, CYP1B and CYP1C, and that the CYP1Bs and CYP1Cs together constitute a sister clade to the CYP1As (Godard et al., 2005).

Figure 1. Nucleotide sequence (2107 bp) of cytochrome CYP1B1 cDNA and its deduced amino acids (509) residues. Consensus sequence for polyadenylation signal (AAGAAA) is in bold. The stop codon, TAA, is marked with an asterisk.

Characteristic structural features of *Oreochromis niloticus* CYP1B1 protein

Substrate recognition sites (SRSs)

Sequence alignment of tilapia (*O. niloticus*) CYP1B1 protein with those of common carp CYP1B1 (accession no. AB048942), common carp CYP1B2 (accession no. AY437775), Plaice CYP1B1 (accession no. AJ249074), Japanese eel CYP1B1 (accession no. AY518340), channel catfish CYP1B1 (accession no. DQ088663), zebra fish CYP1B1 (accession no. AY727864) indicated

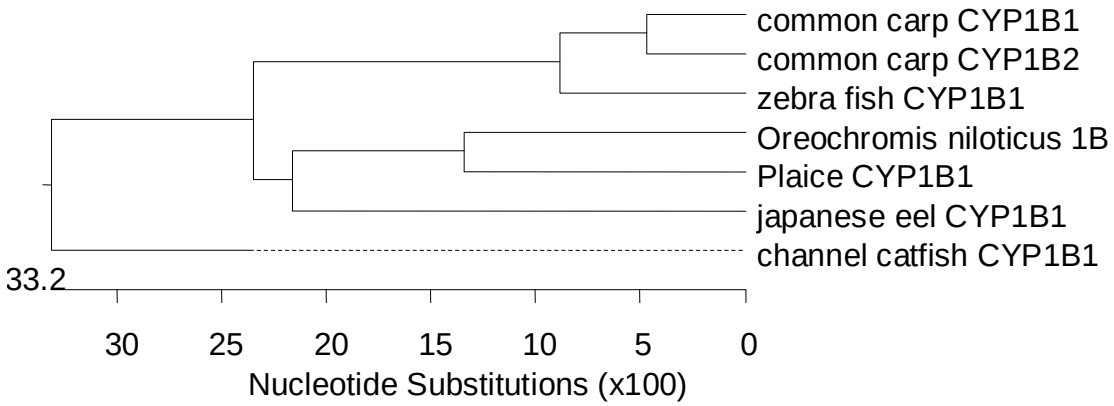

Figure 2. Phylogenetic tree of fish CYP1B1 cDNAs using the amino acid sequences of fishes.

that *O. niloticus* protein contain six separate substrate recognition sites (SRSs) (Figure 3). Location and amino acids sequences of the substrate recognition sites (SRSs) according to Gotoh (1992) are indicated as follows (Table 5).

Highest structural conserved regions: Deduced amino acid sequence of *O. niloticus* CYP1B1 possesses all major functional domains characteristics of previously discovered CYP1B1 molecules including (Figure 3):

1) A putative heme-binding cysteine at position 484 in the typical signature sequence of 477- FSLGKRRCIG-486, this sequence is highly conserved among teleost CYP1B1s, but an F477Y change (at position 477) exists in channel catfish CYP1B1. Also, L479M (at position 479) changed to M in channel catfish CYP1B1 and common carp CYP1B2 but changed to V (L479V) in common carp CYP1B1.
2) Threonine residue present in the I-helix at positions 348 in a signature sequence 344-ASQDT-348, this conserved threonine is proposed to play a crucial role in the binding of oxygen.
3) The proline rich region which is postulated to be crucial for the correct conformation of microsomal CYPs is found in the region downstream of the amino-terminal signal anchor sequences in a signature sequence 70-PPGPLAWP-77.
4) Arginine residue which is integral to enzymatic function in a signature sequence of 448-WSQPDIFDPERF-459.
5) The absolutely conserved Glu-X-X-Arg motif in helix K, this region is probably needed to stabilize the core structure.

CYP1B1 mRNA level in different tissues of BaP treated fish

PAHs are belonging to persistent organic pollutants (POPs), which have become the focus of concern because of their ever-growing level in the environment and wildlife. PAHs mainly result from incomplete combustion of organic materials and present in the air, soil and water (Gelboin, 1980; Chu et al., 2003; Chen et al., 2004). BaP, the most potent carcinogen in the PAH family, is the unique recognized carcinogen (a group 1 carcinogen) by the International Agency for Research on Cancer (IARC, 1983; Straif et al., 2005). BaP can be metabolically activated by CYP enzymes and epoxide hydrolase to form DNA adducts, thus exerting its mutagenic and carcinogenic effects. In the meantime, the metabolic enzymes are also involved in the degradation and final elimination of BaP. Therefore, the equilibrium and modulation of the metabolic enzyme level are of great significance to determine the damaging effects of BaP under different micro environments (Nahrgang et al., 2009; Vondrácek et al., 2009; Shi et al., 2010). Therefore, in this study, expression patterns of CYP1B1 mRNA were determined in liver, intestine and muscle of tilapia after intracoelomic injection of BaP using real -time PCR. Results revealed that there was a large increase in CYP1B1 mRNA in liver (22.8), intestine (2.0) and muscles (1.3) (Tables 6, 7 and Figure 4). The liver was chosen for its prominent role in xenobiotic and endogenous substrate (for example, steroid) metabolism by CYPs. Also, the induction of CYP1B1 in liver and intestine provided a defensive mechanism against the pollutants entering from the external environment. Concerning with CYP1B1 expression levels in other fish species, El-kady et al. (2004a) stated that carp exposed to 3- methylcholanthrene had CYP1B1 messenger RNA (mRNA) expression in liver, intestine and gill.

Kristine et al. (2006) study the induction of CYP1B mRNA expression in BaP-exposed catfish (20 mg/kg intra peritonealy after 4 days) and found that BaP exposure significantly induced CYP1B message in blood (10.7 fold), gonad (17.4 fold) and liver (13 fold) of laboratory catfish. Also, Wolinska et al. (2011) evaluated lethal and sub-lethal effects of BaP on mRNA expression of CYP1B1 in zebrafish (Danio rerio) larvae exposed for 48 h to a BaP concentration of 0.50 µmol•l, transcript quantification performed on the pools of zebra fish larvae revealed signi-

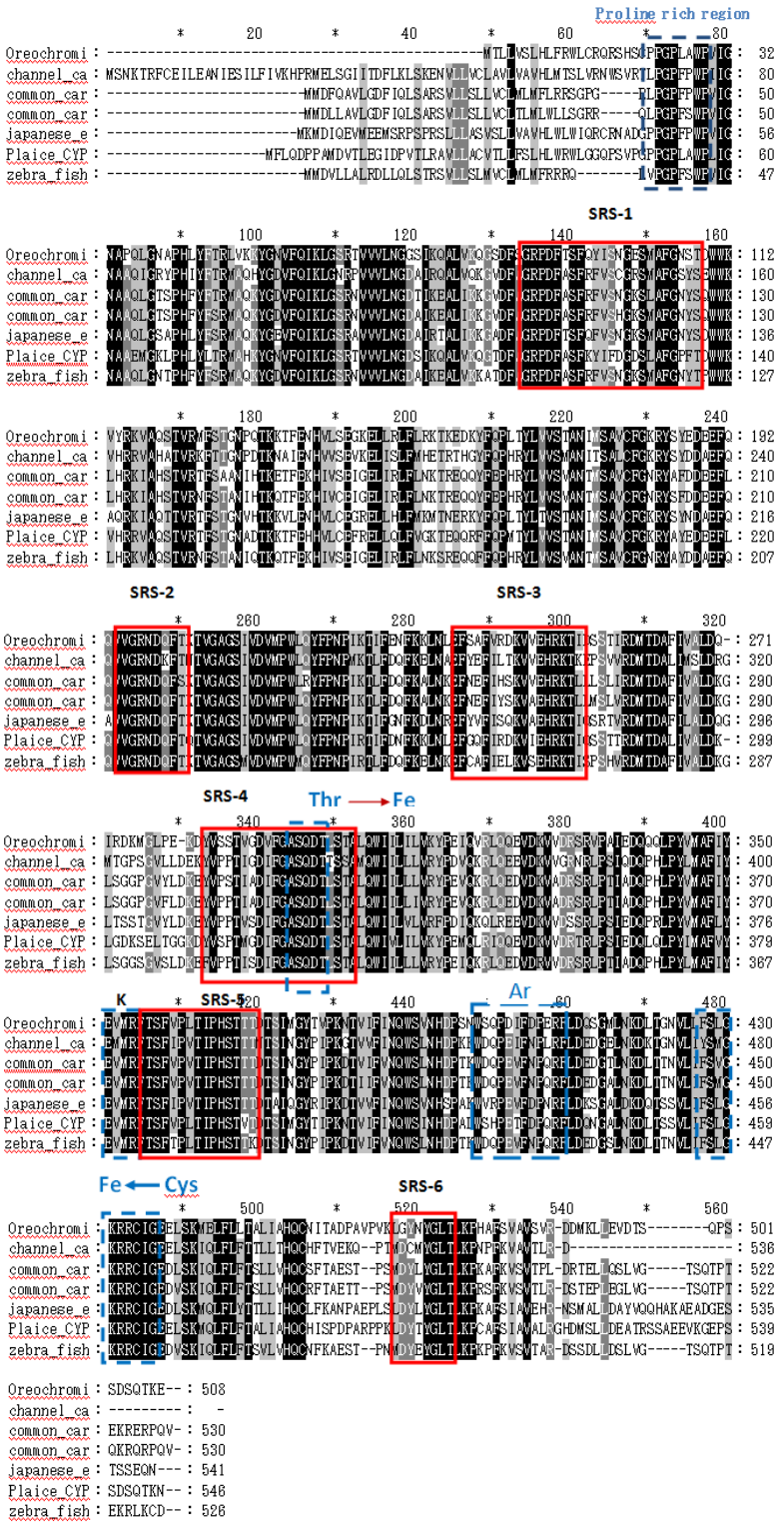

Figure 3. Sequence alignment of Tilapia CYP1B1 protein with those of other fishes CYP1B1 and CYP1B2. The sequences were aligned using Clustal W. The amino acid sequences in the red boxes indicate the positions corresponding to the Substrate Recognition Sequences (SRSs) in CYP1B. The amino acid sequences in the blue dashed boxes indicate proline rich region, K region, conserved threonine residue, Arginine residue and a putative heme-binding region respectively.

Table 5. Location and amino acid sequences of the six SRSs in *oreochromis niloticus* CYP1B1protein.

	Location		Amino acid sequence
	from	To	
SRS -1	134	156	GRPDFTSFQYISNGESMAFGNST
SRS-2	243	251	VGRNDQFT
SRS-3	286	301	FSAFVRDKVVEHRKTI
SRS-4	333	351	YVSSTVGDVFGASQDTLST
SRS-5	406	419	TSFVPLTIPHSTTT
SRS-6	517	524	LGYNYGLT

Table 6. Real time PCR results for Tilapia CYP1B1 and β-actin gene.

Sample name	Tilapia CYP1B1				β- actin gene			
	Mean C_t	Std Dev C_t	t-value	Pr	Mean C_t	Std Dev C_t	t-value	Pr
L. cont	30.2[a]	0.88	8.77	<0.005	18.80a	0.17	1.34	>.0.025
L. ind	25.92[b]	0.4			19.03a	0.23		
I. cont	28.78[a]	0.81	3.1	>0.025	17.54[a]	0.1	5.52	≤ 0.005
I .ind	27.33[a]	0.07			17.06[b]	0.11		
M. cont	21.33[a]	0.12	10.21		17.91[a]	0.15	4.23	
M. ind	23.48[b]	0.34		<0.005	19.64[b]	0.69		<0.025

Table 7. Amount of CYP1B1 mRNA, normalized to β-actin mRNA.

Sample name	Tilapia CYP1B1 average C_t	β-actin gene average C_t	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta Ct}$
L. cont	30.20	18.80	11.40	-4.51	22.8
L.ind	25.92	19.03	6.89		
I. cont	28.78	17.54	11.24	-0.97	2.0
I.ind	27.33	17.06	10.27		
M. cont	21.33	17.91	3.42	0.42	1.3
M. ind	23.48	19.64	3.84		

Where: L.cont: Liver control, L.ind: Liver induced, I.cont: Intestine control, I.ind: Intestine induced, M.cont: Muscle control M. ind: Muscle induced.

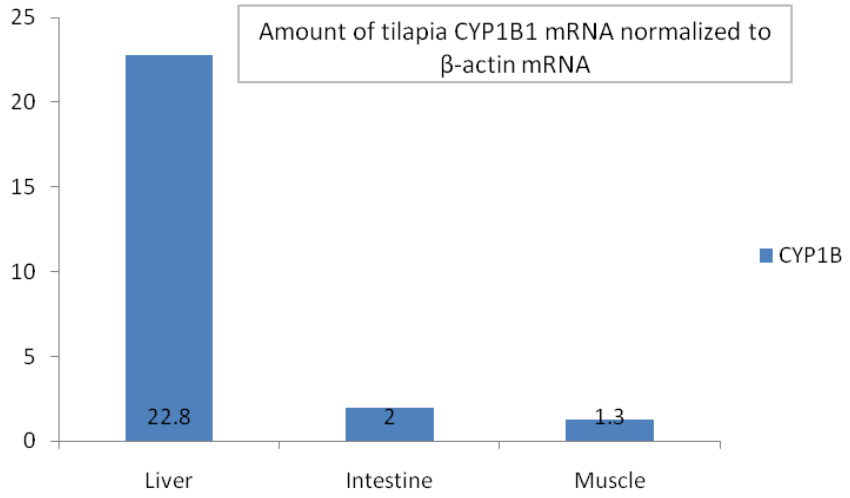

Figure 4. Tilapia CYP1B1 mRNA normalized to β-actin mRNA.

ficant mRNA accumulation [ER = 26.11 ($p<0.001$)]. The expression of CYP1B1 gene is regulated by AhR, which forms an active transcription factor heterodimer with the AhR nuclear translocator (ARNT) after ligand-binding such as BaP, and consequently induces the expression of the CYP1B1. Then, BaP is metabolized to form biologically active 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo (a) pyrene (BPDE) which can form several kinds of adducts with DNA (Nahrgang et al., 2009; Vondrácek et al., 2009). These adduct may subsequently lead to replication errors or mutations if not repaired in time.

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