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ORIGINAL RESEARCH

A novel cytochrome P450 1D1 gene in Nile tilapia fish (*Oreochromis niloticus*): partial cDNA cloning and expression following benzo-a-pyrene exposure

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Abstract To understand the detoxification and bioactivation mechanisms for organic contaminants, it is essential to identify the cytochrome P450 (CYP) complement. Therefore, this study aimed to clone a partial cDNA sequence of the novel CYP1D1 gene from the fish *Oreochromis niloticus* and examine whether intraperitoneal injection of benzo-a-pyrene (BaP), a potent AHR agonist, is capable of inducing CYP1D1 mRNA expression in different tilapia fish tissues. The cloned nucleotide sequence consisted of 713 bp representing a portion of the tilapia CYP1D1 cDNA ORF, encoding 237 amino acids. Amino acid sequence comparison of *O. niloticus* CYP1D1 with the sequences of CYP1D1 from other species showed that this gene shared the highest identity of 81% with *Fundulus heteroclitus* CYP1D1. Furthermore, analysis of the percent identities shared by the deduced amino acid sequence of *O. niloticus* CYP1D1 with the sequences of CYP1 from other species revealed that the highest identities were shared with fish CYP1As. Real-time PCR results revealed that the highest expression level of CYP1D1 mRNA was found in muscles, followed by gills, liver, and intestine, while there was no detectable expression recorded in bile acid. These results indicate that tilapia CYP1D1 plays an important role in the metabolism of xenobiotics, expanding our knowledge regarding the diversity of CYP1 genes in this important model fish species.

Keywords Tilapia · CYP1D1 · BaP · Cloning · Real-time PCR

Introduction

Aquatic ecosystems harbor large amounts of persistent pollutants, such as dioxins, planar polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). PAHs are a very large family of more than 100 compounds that contain at least 2 aromatic rings. The main source of PAH toxicity in wild-caught fish is oil spill exposure (Cousin and Cachot 2014).

Biomarkers in fish can be used as early warning signs of the deterioration of aquatic environments, which may allow environmental management to mitigate potential adverse impacts at higher levels of biological organization. To date, little is known about the aryl hydrocarbon receptor (AHR)-regulated genes responsible

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for the impacts of PAHs. However, genes in the cytochrome P450 1 (CYP1) family are considered to be among the most sensitive indicators involved in PAH bioactivation (Nebert and Russell 2002).

The induction of CYP1A by PAHs in fish has been used as a biomarker of exposure since the mid-1970s (Payne and Penrose 1975). Moreover, recently, CYP1B genes were also shown to be activated by PAHs in the same manner as the CYP1A family, and the associated protein products metabolize PAHs (Savas et al. 1994; Zhang et al. 1998; Hassanin et al. 2013).

CYP1C genes have been cloned from the genomes of several species, such as bird, frog, and fish genomes (Goldstone et al. 2007). In fish, PCB126, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and PAHs are capable of inducing CYP1Cs in both embryonic and adult stages (Jönsson et al. 2007; Zanette et al. 2009; Jönsson et al. 2010; Hassanin et al. 2012).

Human CYP1D1P (formerly known as CYP1A8P) has five nonsense mutations in the putative coding region of exons 2 and 7 and is, therefore, not expected to encode a functional protein (Uno et al. 2011). CYP1D1 was cloned from zebrafish and killifish (Zanette et al. 2009) and shared the highest amino acid sequence identity with CYP1A (55−56%). However, the discovery of functional CYP1D1 in fish introduced the possibility that the CYP1D1 in fish genomes, lacking nonsense mutations, could be expressed as a functional enzyme in other species. However, the low response of this gene towards different substrates and inducers raises doubts regarding the role of such genes in xenobiotic metabolism (Saad et al. 2016).

Benzo-a-pyrene (BaP) is a pervasive, cancer-causing, mutagenic PAH (Office of the Federal Registration (OFR) 1982; Tsuji and Walle 2007) that causes dramatic changes in fish biomarkers after short-term exposure (Peters et al. 1997; Gravato and Santos 2003).

Nile tilapia *(Oreochromis niloticus)* is considered an ideal model in toxicological research for many reasons, including ease of handling and maintenance in the laboratory. Furthermore, this species speedily responds to environmental alterations (Almeida et al. 2002; Figueiredo-Fernandes et al. 2006). The complete sequence of the tilapia CYP1D1 gene is available in the Ensembl database (http://asia.ensembl.org/Oreochromis_niloticus/Gene/Sequence?db=core;g=ENSONIG00000010740;r=GL831172.1:920994-929301;t=ENSONIT00000013514). In contrast to other CYP1 isoforms, the promoter region of the CYP1D1 gene contains few xenobiotic-responsive element (XRE)-like sequences (Kawai et al. 2012). Therefore, analysis of tilapia CYP1D1 can provide new insight into the relationship between this gene and AHRs.

Thus, this study aimed to clone a partial cDNA sequence of Nile tilapia CYP1D1 and characterize this sequence by sequence analysis, phylogenetic analysis, and expression pattern analysis in different tissues of fish treated with BaP.

Materials and methods

Fish handling and treatment

Forty male Nile tilapia (*O. niloticus*) with a mean weight of 500 g were purchased from a local Japanese fish farm and acclimated for 2 weeks, during which the fish were fed a commercial standard dry diet twice a day. The fish were then divided into two groups, namely the control and treated groups, with 20 fish in each group. Fish in the treated group were first anesthetized by immersing in crushed ice (Summerfelt and Smith 1990) followed by intracoelomic injection (in an area that is in front of but slightly off-center from the anus or vent) with a single dose of BaP (100 mg/kg body weight), which was suspended in corn oil before application and used fresh. Fish in the control group were injected with the same volume of corn oil as a vehicle after being anesthetized as mentioned above. Twenty-four hours later, the fish in both groups were killed; liver, intestine, gill, bile acid, and muscle tissue samples were collected, frozen in liquid nitrogen and stored at – 80 °C until analysis.

Cloning of a partial cDNA sequence of tilapia CYP1D1

Total RNA extraction and reverse transcriptase-assisted PCR

Total RNA was extracted from different tissues of tilapia using Isogen reagent (Nippon Gene Co., Ltd.) according to the manufacturer's protocol. The purity and concentration of the extracted RNA samples were determined spectrophotometrically as described by Sambrook and Russel (2001). The A260/A280 ratio ranged from 1.7 to 1.9, and mRNA reverse transcription was performed using Superscript II reverse transcriptase (Gibco BRL, USA) to generate 5′-RACE-ready and 3′-RACE-ready first-strand cDNA using the SMART^{TM} RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's protocol.

Oligonucleotide primers and PCR amplification of the tilapia CYP1D1 cDNA fragment

Two degenerate primers (Genenet.co.jp) were designed using highly conserved regions of both the *Fundulus heteroclitus* CYP1D1 sequence (accession number NM_001309918) and *Danio rerio* CYP1D1 sequence (accession number NM_001007310), which are retrievable from GenBank. The nucleotide sequences for the primers used were as follows: sense primer (5′–GGS AAY YTK YTC CAG VTK GR–3′; where S is G or C; Y is C or T; K is G or T; V is A, C or G; and R is A or G) and antisense primer (5′–AGR GCA TCW GTR ATR TCY CK–3′; where R is A or G; W is A or T; Y is C or T; and K is G or T). The PCR was performed as follows: 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 3 min. Then 1% agarose gels were used to examine the PCR products. The DNA band of the expected size was excised, purified using the GFX PCR DNA and Gel Band Purification Kit (GE Health Care, UK), and then cloned into the PT7Blue T-vector (Novagen, USA). The purified plasmids were directly sequenced by dye terminator cycle sequencing using the ABI PRISM Dye Terminator Cycle Sequencing Kit (PE Biosystems, USA) and an Applied Biosystems 3130xl DNA sequencer.

Phylogenetic analysis

To construct the CYP1D1 gene family phylogenetic tree for various fish species, the CYP1D1 mRNA sequences with the following GenBank accession numbers were obtained from the database and used: NM_001007310 (zebrafish CYP1D1), JX454650 (Indian medaka CYP1D1), FJ786961 (killifish CYP1D1), NM_001246671 (rhesus monkey CYP1D1), and NM_001126813 (tropical clawed frog CYP1D1).

Furthermore, to construct the phylogenetic tree of CYP1 gene families from different species, the CYP1 sequences with the following GenBank accession numbers were obtained from the database and used: AB015638 (Japanese eel CYP1A1), AB020414 (Japanese eel CYP1A9), AF210727 (zebrafish CYP1A1), X73631 (plaice CYP1A1), U14162 (scup CYP1A1), FJ389918 (tilapia CYP1A1), K03191 (human CYP1A1), BC125440 (mouse CYP1A1), NM_000761 (human CYP1A2), NM_009993 (mouse CYP1A2), HQ829968 (tilapia CYP1B1), XM_003978309 (fugu CYP1B1), AB048942 (common carp CYP1B1), HQ829969 (tilapia CYP1C1), AY444748 (Japanese eel CYP1C1), and L04751 (human CYP4A). Sequence alignment was performed by the Clustal W method using the Lasergene MegAlign program (Ver 5.52,2003, DNASTAR Inc).

Expression of CYP1D1 mRNA in different tissues of tilapia fish

Real-time quantitative PCR was performed using Fast Start Essential DNA Green Master (Roche, 06402712001) and a LightCycler Nano system (Roche Applied Science). A total of 30 tissue samples were used in this experiment. Three samples from each of liver, gills, intestine, bile acid, and muscle tissues were taken from fish in the control group, and a similar number of samples was obtained from the treated group. RNA reverse transcription was performed using the PrimeScript^{TM} 1st strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's protocol. Primers for *O. niloticus* CYP1D1 cDNA and internal control (*β-actin*) cDNA (accession number EU887951) are listed in Table 1. Each PCR consisted of 2 × Master Mix (10 μl), primers (10 μM each), cDNA template (2 μl) and ddH_2O to a final volume of 20 μl. Reactions were then performed as follows: initial denaturation at 95 °C for 10 min; 35 cycles of 95 °C for 10 s, 60 °C for

Table 1 Real-time PCR primers for the *Oreochromis niloticus* CYP1D1 and *β-actin* genes

Gene	Primer description	Sequence (5′-3′)	Location	Product size
CYP1D1	F	5′-AGGGATGGTGCAGGTGATTC-3′	357−376	113 bp
	f			
	R	5′-CCAAAACAGAGCGCACAGAC-3′	451−470	
β-Actin	F	5′-GGGTCAGAAAGACAGCTACGTT-3′	42−63	143 bp
	R	5′-CTCAGCTCGTTGTAGAAGGTGT-3′	164−185	

10 s, and 72 °C for 15 s; one cycle of 95 °C for 30 s; and one cycle of 60 °C for 20 s and 95 °C for 20 s. Each PCR run included an RT-negative control for each gene and a no-template control. All samples for each gene were run in triplicate on the same plate. Quantitative analysis of gene expression was performed using the geometric mean for the internal control genes according to a previously described equation (Hellemans et al. 2007).

Results

Partial nucleotide sequence of tilapia cytochrome P450 1D1 cDNA and its deduced amino acid residues

Tilapia CYP1D1 cDNA was identified using the two degenerate primers designed using the highly conserved regions of both the *F. heteroclitus* CYP1D1 and *D. rerio* CYP1D1 sequences as mentioned earlier. The obtained PCR fragment was then cloned and sequenced. The nucleotide sequence consisted of 713 bp,

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GGG AAC TTT CTC CAG GTT GGG GAG CAG ATT CAT CTC TCT TTA ACT GGG TTG AGG CTT CAG      60
 G   N   F   L   Q   V   G   E   Q   I   H   L   S   L   T   G   L   R   L   Q      20
TAT GGA GAC ATC TTC AAG CTC CGC CTT GGC TCT TTG ACT GTT GTC GTT CTG AGT GGG TAC     120
 Y   G   D   I   F   K   L   R   L   G   S   L   T   V   V   V   L   S   G   Y      40
AAC ACC ATC AGG CAG GCT CTG GTT CGA CAC GGG GAA GCT TTT GCG GGG CGA CCT AAC CTT     180
 N   T   I   R   Q   A   L   V   R   H   G   E   A   F   A   G   R   P   N   L      60
TTC ATC TTC TCT GCT ATA GCC AAT GGG ACC AGC ATG ACT TTC AAT GAG AAC TAC GGG CCT     240
 F   I   F   S   A   I   A   N   G   T   S   M   T   F   N   E   N   Y   G   P      80
GTG TGG CTG CTC CAT AAG AAG CTG TGT AAG AAT GCC CTC AGG TCT TTC TCC CAG GCT GAG     300
 V   W   L   L   H   K   K   L   C   K   N   A   L   R   S   F   S   Q   A   E     100
CCA AGG GGT TTT GGT GCC ACC TGC CTC TTA GAG GAG CAC ATA TGT GCA GAG GCT GCA GGG     360
 P   R   G   F   G   A   T   C   L   L   E   E   H   I   C   A   E   A   A   G     120
ATG GTG CAG GTG ATT CGA GAA AAA GCT GCT AAA GAG GAC ATG GAG GGT ATA GAC CCA GCA     420
 M   V   Q   V   I   R   E   K   A   A   K   E   D   M   E   G   I   D   P   A     140
ACG ACC TTG GTA ACC TCA GTG GCA AAT GTC GTC TGT GCG CTC TGT TTT GGG AAA CGG TAT     480
 T   T   L   V   T   S   V   A   N   V   V   C   A   L   C   F   G   K   R   Y     160
GAC TAC AGT GAT AAG GAG TTT CTC ACT ATT GTT GAT GTC AAC AAC GAG GTC CTG AAG CTC     540
 D   Y   S   D   K   E   F   L   T   I   V   D   V   N   N   E   V   L   K   L     180
TTT GCA GCG GGG AAC CTG GCT GAT TTC TTC CCC GTG TTT CGC TAC TTT CCG AGT CCA TCT     600
 F   A   A   G   N   L   A   D   F   F   P   V   F   R   Y   F   P   S   P   S     200
CTG AGA AAG ATA GTC CAG TAC GTT CGC AGG ATG AAC AGG TTC ATG GAG CGG AAC ATC GAG     660
 L   R   K   I   V   Q   Y   V   R   R   M   N   R   F   M   E   R   N   I   E     220
GAA CAC ATT GAC ACC TTT GAT AAG AAC CGT ATC CGA GAC ATT ACA GAT GCC CT             713
 E   H   I   D   T   F   D   K   N   R   I   R   D   I   T   D   A                 237
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Fig. 1 Partial nucleotide sequence (713 bp) of tilapia cytochrome P450 1D1 cDNA and its deduced amino acid (237) residues

representing a portion of the tilapia CYP1D1 cDNA ORF and encoding 237 amino acids (Fig. 1). The nucleotide sequence was registered in GenBank under accession number MF694263.

Amino acid sequence comparison

The percent identities shared by the deduced amino acid sequence of *O. niloticus* CYP1D1 with the sequences of CYP1D1 from other species are shown in Table 2. The highest identity was 81%, with *Fundulus heteroclitus* CYP1D1, followed by 68.8% with zebrafish CYP1D1, 47.7% with Western clawed frog CYP1D1, and 47.2% with both crab-eating macaque CYP1D1 and rhesus monkey CYP1D1. Moreover, the percent identities shared by the deduced amino acid sequence of *O. niloticus* CYP1D1 with the sequences of CYP1 from other species are shown in Table 3. The highest identities were found with fish CYP1As (43.2% with tilapia CYP1A1, 43.9% with Japanese eel CYP1A1, 42.8% with zebrafish CYP1A1, 41.5% with plaice CYP1A1, and 43.8% with scup CYP1A1), followed by fish CYP1B1s (36.8% with tilapia CYP1B1, 35.5% with fugu CYP1B1, and 34.9% with carp CYP1B1) and fish CYP1C1s (36.1% with tilapia CYP1C1 and 37.5% with Japanese eel CYP1C1).

Phylogenetic tree

Figure 2 shows that tilapia CYP1D1 is more closely related to *F. heteroclitus* CYP1D1 than to CYP1D1 genes from other species. Figure 3 shows that the amino acid sequence of tilapia CYP1D1 is more similar to those of CYP1As than to those of CYP1B1s or CYP1C1s.

CYP1D1 mRNA levels in different tissues of BaP-treated fish

Real-time RCR results (Fig. 4) revealed higher expression levels of CYP1D1 mRNA in different tissues (including muscles, gills, liver, intestine, and bile acid) of tilapia fish treated with BaP compared with the same fish tissues in the control group. The highest expression levels of CYP1D1 mRNA were observed in muscles (16.11), followed by gills (6.06), liver (5.94), and intestine (4.92), while very low expression levels were observed in bile acid (0.16), compared with the control sample.

Discussion

CYP1 family genes induced by AHRs play an important role in metabolism of the xenobiotic PAH. Therefore, understanding the evolution of fish CYP1s and their association with AHRs is important for the prediction and evaluation of the ability of fish to adapt to xenobiotic risk (Kawai et al. 2012).

In this study, we identified and cloned a partial nucleotide sequence of Nile tilapia CYP1D1 and investigated the effect of the environmental pollutant and AHR agonist BaP on the expression of this sequence.

Table 2 Percent identities of the deduced amino acid sequences of CYP1D1 gene subfamilies

	Crab-eating macaque CYP1D1	*Fundulus heteroclitus* CYP1D1	Rhesus monkey CYP1D1	Western clawed frog CYP1D1	Zebrafish CYP1D1
Tilapia CYP1D1	47.2	81.0	47.2	47.7	68.8
Crab-eating macaque CYP1D1		47.5	98.9	53.4	48.5
Fundulus heteroclitus CYP1D1			47.7	48.1	69.4
Rhesus monkey CYP1D1				53.4	48.7
Western clawed frog CYP1D1					48.3

Table 3 Percent identities of the deduced amino acid sequences of CYP1 gene families

	J.eel 1A9	Zebra 1A1	Plaice 1A1	Scup 1A1	Human 1A1	Mouse 1A1	Human 1A2	Mouse 1A2	Fugu 1B1	Carp 1B1	J.eel 1C1	Human 4A	Tilapia 1A1	Tilapia 1B1	Tilapia 1C1	Tilapia 1D1
J.eel 1A1	78.2	74.6	76.7	79.2	54.3	57.2	52.6	49.9	39.5	39.7	37.8	16.8	73.0	37.8	38.3	43.9
J.eel 1A9		72.9	73.7	75.0	54.5	57.3	53.0	50.7	38.7	39.1	38.3	17.6	70.0	38.1	37.9	42.7
Zebra 1A1			73.6	76.1	54.9	57.8	52.6	48.7	38.3	40.1	37.2	17.0	72.6	38.3	38.3	42.8
Plaice 1A1				84.8	55.1	56.6	52.0	49.5	39.2	39.7	38.6	18.2	80.0	37.0	38.2	41.5
Scup 1A1					55.9	57.8	53.6	51.1	38.0	39.0	37.6	16.9	79.5	36.3	37.8	43.8
Human 1A1						79.5	71.3	66.8	39.1	39.3	38.3	18.8	55.9	37.1	37.9	43.8
Mouse 1A1							65.8	69.3	39.5	38.9	38.5	18.3	55.5	38.0	38.9	45.2
Human 1A2								71.7	37.4	36.0	36.0	18.0	52.7	35.7	35.5	41.9
Mouse 1A2									38.4	37.4	38.6	17.3	50.1	38.8	38.2	41.7
Fugu 1B1										62.4	49.4	17.1	37.8	69.9	49.2	35.3
Carp 1B1											49.2	15.3	38.5	62.6	48.3	34.9
J.eel 1C1												16.2	37.3	48.7	80.2	37.5
Human 4A													18.1	16.0	16.2	17.3
Tilapia 1A1														37.8	37.6	43.2
Tilapia 1B1															50.2	36.8
Tilapia 1C1																36.1

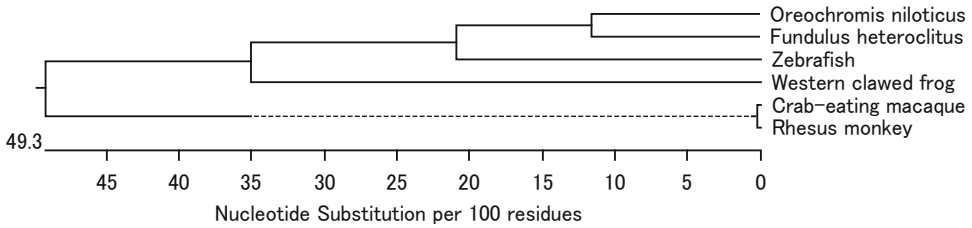

Fig. 2 Phylogenetic tree of CYP1D1 cDNAs using amino acid sequences

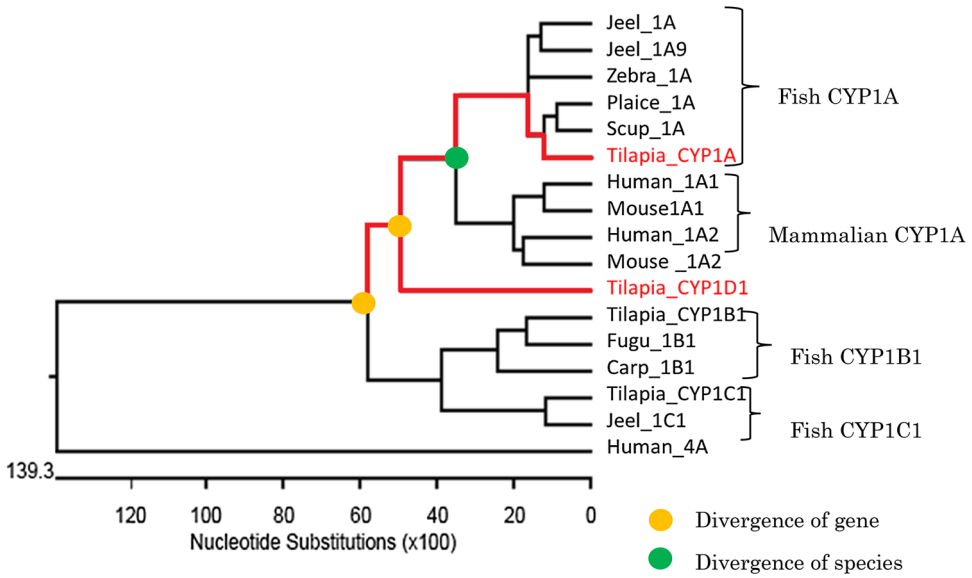

Fig. 3 Phylogenetic tree of CYP1 family cDNAs using amino acid sequences

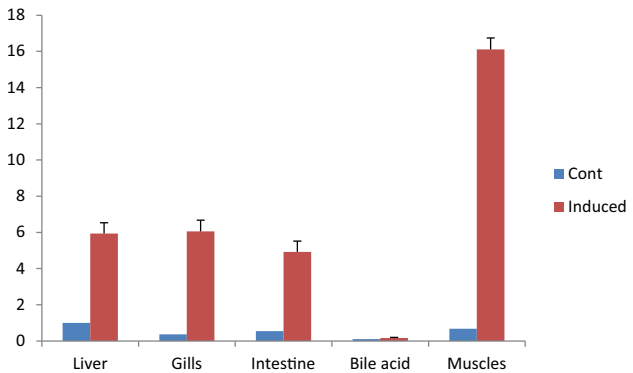

Fig. 4 CYP1D1 mRNA levels in different tissues of BaP-treated fish

Vertebrate CYP1 subfamilies include the CYP1A, CYP1B, CYP1C, and CYP1D subfamilies. Recently, the CYP1D subfamily was identified in non-mammalian vertebrates such as fish (Goldstone et al. 2009). In addition, five P450 type 1 family enzymes (CYP1A, CYP1B, CYP1C1, CYP1C2 and CYP1D) have been isolated from different fish species. CYP1D1 was first cloned in the freshwater fish species medaka (*Oryzias latipes*) by Goldstone et al. 2009. Since then, these enzymes have been isolated from the three-spined stickleback (*Gasterosteus aculeatus*), killifish, and zebrafish (Goldstone and Stegeman 2008; Zanette et al. 2009). Characterization of the CYP1D subfamily of genes can provide us with much information that will aid the understanding of CYP1 regulation and evolution (Kawai et al. 2012).

We successfully cloned a partial nucleotide sequence of the tilapia CYP1D1 cDNA ORF consisting of 713 bp, encoding 237 amino acids. Phylogenetic analyses grouped tilapia CYP1D1 together with predicted CYP1D1 sequences from other fish species. Furthermore, this CYP1D1 subfamily was placed in a common clade with CYP1As. Thus, CYP1As and tilapia CYP1D together constitute one clade, while CYP1Bs and CYP1Cs constitute another clade. This finding demonstrates a close evolutionary relationship between CYP1D1 and CYP1A and suggests a common ancestral origin for both of these genes (Goldstone et al. 2009).

The cytochrome P450 1 (CYP1) gene family is one of most highly responsive gene families to AHR agonist exposure (Jönsson et al. 2011). These genes are upregulated by AHR activation and induced by several components (Goldstone et al. 2009). Amino acid sequence domain analysis of fish CYP1B, CYP1C and CYP1D revealed the uniqueness of the catalytic functions or substrates of these proteins (Uno et al. 2012); however, all previous studies stated that the CYP1D1 gene family is expressed in fish but is not induced by AHR agonists such as PCB126, TCDD, or 6-formylindolo[3,2-b]carbazole (FICZ) (Goldstone and Stegeman 2008; Goldstone et al. 2009; Jönsson et al. 2009; Zanette et al. 2009).

The real-time PCR results in this study showed that CYP1D1 is induced in different Nile tilapia fish tissues by the AHR agonist BaP. Similar findings were reported by Scornaienchi et al. (2010), who observed low activity of zebrafish CYP1D1 towards BaP, with a distinct spectrum of BaP metabolites. The BaP-6,12- and BaP-1,6-dione metabolites were the major products of CYP1D1 metabolism. However, CYP1D1 produced no detectable BaP-7,8-diol and very little 3-OH-BaP, which were major metabolites of all other zebrafish CYP1s. This difference in regioselectivity towards BaP suggests that the function of CYP1D1 may be different from that of other CYP1s. Many previous studies have reported that the number of XRE regions upstream of fish CYP1D1 is lower than that found for other CYP1 family genes and that AhR ligands did not induce CYP1D1 gene expression (Goldstone et al. 2009; Zanette et al. 2009; Jönsson et al. 2009), indicating that fish CYP1D1 is regulated by different mechanisms and could be induced via other signal cascades.

The highest expression of tilapia CYP1D1 mRNA was reported in the muscles, followed by the gills, liver, and intestine. Our previous studies on the expression levels of the CYP1 family in tilapia following intraperitoneal injection of BaP showed distinct induction of CYP1A1 expression in the liver and intestine (Hassanin et al. 2009), while the highest expression levels of CYP1B1 and CYP1C1 were recorded in the liver, followed by the intestine and muscles (Hassanin et al. 2012, 2013). The differences in the expression of tilapia CYP1 isoforms are likely indicative of their roles in vivo.

In conclusion, to our knowledge, this is one of the first reports on the structure and expression pattern of CYP1D1 in Nile tilapia fish. Our results showed that tilapia CYP1D1 was induced by BaP, while all previous studies reported that CYP1D1 seems to have very minimal, if any, activity towards all other AHR agonists, indicating that the function of CYP1D1 is different from that of other CYP1s. This result will be of great importance for the identification of the possible contribution of CYP1D1 in the bioactivation or inactivation of ecological contaminants and for the possible application of this gene as a biomarker in fish.

Compliance with ethical standards

Conflict of interest None of the authors have any conflict of interest regarding the research described in this article.

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