New Cytochrome P450 1B1, 1C2 and 1D1 Genes in the Killifish *Fundulus heteroclitus*: Basal Expression and Response of Five Killifish *CYP1s* to the AHR Agonist PCB126

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Abstract

Knowledge of the complement of cytochrome P450 (CYP) genes is essential to understanding detoxification and bioactivation mechanisms for organic contaminants.We cloned three new *CYP1* genes, *CYP1B1*, *CYP1C2* and *CYP1D1*, from the killifish *Fundulus heteroclitus*, an important model in environmental toxicology. Expression of the new *CYP1s* along with previously known *CYP1A* and *CYP1C1* was measured by qPCR in eight different organs. Organ distribution was similar for the two *CYP1Cs*, but otherwise patterns and extent of expression differed among the genes. The AHR agonist 3,3_,4,4_,5-pentachlorobiphenyl (PCB126) (31 pmol/g fish) induced expression of *CYP1A* and *CYP1B1* in all organs examined, while *CYP1C1* was induced in all organs except testis. The largest changes in response to PCB126 were induction of *CYP1A* in testis (□700-fold) and induction of *CYP1C1* in liver (□500-fold). *CYP1B1* in liver and gut, *CYP1A* in brain and *CYP1C1* in gill also were induced strongly by PCB126 (>100-fold). *CYP1C1* expression levels were higher than *CYP1C2* in almost all tissues and *CYP1C2* was much less responsive to PCB126. In contrast to the other genes, *CYP1D1* was not induced by PCB126 in any of the organs. The organ-specific response of *CYP1s* to PCB126 implies differential involvement in effects of halogenated aromatic hydrocarbons in different organs. The suite of inducible CYP1s could enhance the use of *F. heteroclitus* in assessing aquatic contamination by AHR agonists. Determining basal and induced levels of protein and the substrate specificity for all five CYP1s will be necessary to better understand their roles in chemical effects and physiology.

1. **Introduction**

Cytochrome P450 (CYP) enzymes catalyze oxidative metabolism of thousands of drugs, environmental pollutants, and endogenous compounds. Environmental pollutants such as halogenated hydrocarbons, polycyclic aromatic hydrocarbons (PAH), herbicides, and pesticides (Nebert and Russell, 2002) include many substrates of mammalian CYP1s (CYP1A1, CYP1A2, CYP1B1), and of fish CYP1As (e.g., Schober et al., 2006). While metabolism often results in detoxification, the action of CYP1 enzymes also can generate toxic metabolites that contribute to increased risks of cancer, birth defects, and other toxic effects (Nebert and Karp, 2008). Expression of mammalian CYP1A1 and fish CYP1As can be induced strongly by PAH, planar polychlorinated biphenyl (PCB), dibenzo-*p*-dioxin (PCDD), and dibenzofuran (PCDF) congeners, and some natural products, via activation of aryl hydrocarbon receptor (AHR) (Hahn, 2002). These features have led to widespread use of CYP1A gene expression as a marker of environmental exposure to AHR agonists in humans and wildlife (e.g., Stegeman, 1986; Fujita et al., 2001; Lambert et al., 2006).

Most fish have only one *CYP1A* gene and one *CYP1B1* gene (Goldstone et al., 2007). Fish also have two CYP1Cs, the paralogous *CYP1C1* and *CYP1C2* (Godard et al., 2005). Like *CYP1A* and *CYP1B1*, the *CYP1Cs* are induced to varying degrees by AHR agonists in zebrafish (Jönsson et al., 2007a; Jönsson et al., 2007b). More recently we have identified a fifth *CYP1* gene in teleosts, *CYP1D1* (Goldstone et al., 2007; Goldstone and Stegeman, 2008). Zebrafish *CYP1D1* and *CYP1A* share a relatively high percent identity and have similar gene structures. However, neither PCB126 nor TCDD induced transcription of zebrafish *CYP1D1*. CYP1D1 protein is expressed in zebrafish liver, and heterologously expressed CYP1D1 is catalytically active with ethoxyresorufin, albeit at significantly lower rates than CYP1A (Goldstone et al.,

2009). It is possible that chemicals and receptors other than AHR are involved in regulating these various *CYP1s*, and that they act on distinct sets of substrates, which could vary among species.

To date, all five *CYP1s* have been examined in detail only in zebrafish. We sought to determine if the identity of the full suite of *CYP1* genes occurs and is similarly regulated in the Atlantic killifish *Fundulus heteroclitus.* This species is one of the most abundant estuarine fishes along the Atlantic coast of North America, and frequently is used in toxicological studies, including developmental toxicology and environmental carcinogenesis (Burnett et al., 2007). Biological and ecological features (small size, rapid development, small home range, tolerance of varied conditions) and adaptation to high levels of contaminants have spurred interest in this species as an alternative non-mammalian vertebrate model species (Burnett et al., 2007; Matson et al., 2008).

Regulation of CYP1A-like activities has been studied in *F. heteroclitus* for 30 years (e.g., Stegeman, 1978) and *CYP1A* was cloned a decade ago (Morrison et al., 1998). More recently, a killifish *CYP1C1* was reported (Wang et al., 2006). Here, we report on the identification and cloning of the full-length sequences of three new *F. heteroclitus CYP1* genes*, CYP1B1*, *CYP1C2,* and *CYP1D1*. Basal expression and regulation by the AHR agonist PCB126 were compared in different organs for the three new genes, and for *CYP1A* and *CYP1C1*. It appears that orthologous *CYP1* genes are similarly expressed in *Fundulus* and in zebrafish. Identifying substrates of the CYP1s in this model will help to achieve a general understanding of their involvement in chemical effects and their roles in endogenous functions, and may contribute to understanding the resistance to AHR agonist toxicity seen in some populations.

2. Materials and Methods

2.1 Fish collection and maintenance

Killifish were obtained from Scorton Creek (Massachusetts, USA) in May 2008, using minnow traps. Scorton Creek has been used as a reference site for studies of *F. heteroclitus* for years (Bello et al., 2001). Fish were acclimated at 20 °C in flowing seawater for one month and were fed twice a day with Omega One Freshwater Flakes™ during the acclimation period. The procedures used in these experiments were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution.

2.2 Cloning of new CYP1s

Liver, brain and eye were dissected from one randomly selected untreated fish. Total RNA was isolated using Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories Inc., Hercules, CA), which includes elimination of genomic DNA by DNAse treatment. The RNA quantity and quality was determined spectrophotometrically (Nanodrop ND 1000; NanoDrop Technologies, Wilmington, DE). cDNA was synthesized from 2 μg of total RNA, using the Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA), anchored oligo(dT) primer (MWG Biotech, Inc., High Point, NC) and RNasin RNase inhibitor (Promega Corp., Madison, WI).

Degenerate primers were designed using highly conserved regions of *CYP1B1* and *CYP1D1* sequences from other fish species (Supplemental Table 1), avoiding conserved regions present in other CYP1 subfamilies. Gene-specific primers for *CYP1C2* were designed against a sequence fragment in GenBank. PCR reactions were carried out for *CYP1B1* and *CYP1C2* using brain cDNA and for *CYP1D1* using liver cDNA. PCR products were resolved on a 1% agarose gel and then isolated, ligated into the pGEM-T Easy Vector (Promega), and transformed into

114 *E.coli* (TOP 10 Kit, Invitrogen). Plasmids were purified from cultures of positive clones
115 (QiaPrep™, Qiagen) and were sequenced (MWG Biotech).
116 The 5' and 3' ends of *CYP1B1*, *CYP1C2* and *CYP1D1* were obtained by rapid
117 amplification of cDNA ends (RACE) with the BD Smart™ RACE cDNA Amplification Kit
118 (Clontech) using the RACE kit and gene-specific primers described in Supplemental Table 1.
119 Gel-purified 3' and 5' RACE products were cloned and sequenced as described above.
120 Nucleotide sequences were translated and aligned with other CYP1 family members as
121 before (Jönsson et al., 2007b; Goldstone et al., 2009). Predicted medaka (*Oryzias latipes*) and
122 stickleback (*Gasterosteus aculeata*) *CYP1* gene sequences were derived from the Ensembl
123 genomes (Release 49). Phylogenetic trees were constructed by analyzing predicted amino acid
124 sequences using maximum likelihood (RAxML 7.0.3; (Stamatakis, 2006b)), and Bayesian
125 methods (MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003)). Regions of alignment uncertainty
126 were excluded from phylogenetic analysis (Kreil and Ouzounis, 2003) by automatic masking
127 using a custom-written script. The WAG-CAT model of amino acid substitution with a gamma
128 distribution of substitution rates (PROTMIXWAG) was used in all likelihood analyses, based on
129 likelihood tests using RAxML (Stamatakis, 2006a). MrBayes estimates posterior probabilities
130 using Metropolis-Hastings coupled Monte Carlo Markov chains (MC3). MC3 estimates were
131 performed with the WAG model of amino acid substitution and prior uniform gamma
132 distributions approximated with four categories (WAG+I+Γ). Four incrementally heated,
133 randomly seeded Markov chains were run for $3x10^6$ generations, and topologies were sampled
134 every 100th generation. Burn-in value was set to 10^6 generations. Putative functional domains
135 (i.e., representing substrate recognition sites) were evaluated for similarity to domain sequences
136 in orthologous *CYP1* genes in other species. The amino acid sequence similarities between

various CYP1s were plotted using GCG (v. 10.3; Accelrys, San Diego, CA). Similarity scores were calculated using the BLOSUM62 amino acid similarity matrix (Henikoff and Henikoff, 1992).

2.3 Experimental Treatment

Twenty male fish (3-5 g whole body weight) were acclimated for 24 hr in recirculating, filtered and aerated water in two 10-gallon aquaria (10 fish per aquaria) at 18 °C. After acclimation the fish were weighed and 10 fish were injected intraperitoneally with 3,3',4,4',5-pentachlorobiphenyl (PCB126) dissolved in DMSO at the dose of 31 pmol/g fish (10 μg/kg fish). Ten fish were also injected with an equivalent volume of DMSO alone. Previous studies in our laboratory using scup (*Stenotomus chrysops*), showed that injection of this PCB126 dose caused high-level induction in gene expression and catalytic function (EROD) of CYP1A with absence of mortality (unpublished). The same PCB126 dose was also injected in pink snapper (*Pagrus auratus*) in another study, which gave similar results in CYP1A induction (Tugiyono, 2002). At 48 hrs after injection, control and PCB126 treated fish were killed by cervical transection and liver (n = 10), gill (n = 8), gut, heart, kidney, eye, brain, and testis (n = 4) of individual fish were dissected and immediately placed in RNAlater (Ambion). The samples were held for 24h at 4 °C, and then stored at -20 °C. Previous studies in our lab showed higher induction of *CYP1A* gene expression in fish exposed to AHR agonists using 48hs exposure. This was also observed by Kim et al. (2008) who tested a variety of exposure times, and analyzed *CYP1A* in different pufferfish organs.

2.4 Quantification of CYP1 Transcripts

Total RNA was extracted and cDNA was synthesized as described above. Gene-specific primers for the new *F. heteroclitus CYP1s* and for *F. heteroclitus CYP1A*, *CYP1C1*, and *β-actin*

designed with Primer3 (Rozen and Skaletsky, 2000) were obtained from MWG Biotech (Primer sequences are shown in Table 1). Real-time PCR was performed using iQ SYBR Green Supermix (according to the manufacturer's instructions) and an iQ Real-Time PCR Detection System (Bio-Rad). For each sample, gene expression was analyzed in triplicate with the following protocol: 95°C for 3 min and 40 cycles of 95°C for 15 s and 62°C for 1 min. Melt curve analysis was performed on the PCR products at the end of each PCR run to ensure that a single product was amplified. The $E^{\Delta ct}$ method 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 PCB126 treatment. The efficiency of the PCR reactions for each gene was calculated using the standard curves generated from dilutions ($10^{2} – 10^{9}$ molecules) of plasmid (pGEM-T Easy Vector) containing fragments of a given target gene (Schmittgen and Livak, 2008). Jönsson et al. (2007b) compared data normalized to different house-keeping genes *(β-actin* or *ARNT*) using the $E^{\Delta ct}$ method and showed that results differ between these normalizations. Organ-specific differences in Ct values for *β-actin* and other reference genes in fish have been shown before (McCurley and Callard, 2008), and produce conflicting qPCR results when using the Δ^{ct} $^{housekeeping - ct\ target}$ normalizing method. Therefore, to compare relative expression levels between different organs, molecule numbers for *CYP1s* and *β-actin* were calculated based on standard curves and relative *CYP1* transcript abundance was determined for a specific amount of total RNA from each organ using *β-actin* only as an efficiency control to normalize individual samples within a specific organ, but not among different organs.

2.5 Statistics

Equality of variance was tested, accepting $P>0.05$ (Bartlett's test) (Prism 4, GraphPad Software Inc., San Diego, CA). Data were logarithmically transformed to improve equality of

variances. The differences among transcript levels for *CYP1s* in different organs were evaluated using one-way analysis of variance (ANOVA, $P<0.05$), followed by Tukey's test for multiple comparisons (Prism 4, GraphPad Software Inc., San Diego, CA). Differences between the means of control and PCB126 treated groups were analyzed using Student's t-Test and data are presented as fold difference between treated and control groups.

3. **Results**

3.1 Cloning of CYP1B1, CYP1C2 and CYP1D1 transcripts in F. heteroclitus.

Using degenerate primers based on CYP1B1 and CYP1D1 in other teleosts, we were able to amplify partial sequences (about 700 bp) from *F. heteroclitus*. Aligning the amino acid sequences predicted from these cloned PCR products with other fish CYP1 amino acid sequences resulted in preliminary classification of the new *Fundulus* sequences as *CYP1B1* and *CYP1D1*.

The alignment of fish CYP1s included a predicted translation of a 588 bp sequence obtained from GenBank (Accession AF235140) that was annotated as *F. heteroclitus CYP1B1*. However, alignment of the predicted amino acid sequence with other CYP1 sequences indicated that this 588 bp sequence was likely a *CYP1C2*, and not a *CYP1B1*. This putative *CYP1C2* sequence and the cloned fragments of the other two genes were used as starting points for full-length sequencing using PCR reactions primed with RACE and gene specific primers. Full-length sequences were obtained for all three genes (GeneBank accession FJ786959, FJ786960 and FJ786961, respectively).

3.2 Sequence analysis

206 The open reading frames for the three new *Fundulus* genes give translated proteins of 537,
207 524 and 535 amino acids, 10-15 amino acids longer than the corresponding zebrafish CYP1B1,
208 CYP1C2, and CYP1D1, respectively (Supplemental Figure 1). The full-length killifish
209 sequences display 61%, 69% and 67% pair-wise identity to CYP1B1, CYP1C2 and CYP1D1 of
210 zebrafish, respectively, increasing to 69%, 74%, and 75% identity when ambiguously-aligned
211 regions of a large set of CYP1s are masked (Table 2). Notably, our annotation of these
212 subfamilies and genes is in agreement with the CYP classification based on the amino acid
213 percent identities suggested by (Nelson et al., 1996), accepting identities higher than 55 % for the
214 same subfamily.
215 Phylogenetic analysis of the deduced amino acid sequences for the five *F. heteroclitus*
216 CYP1s was done together with members of the CYP1 family from zebrafish and other selected
217 species. These include medaka and stickleback, where five CYP1 sequences were found in their
218 genomes. Figure 1 shows that the killifish sequences clustered with the other teleost CYP1s,
219 with the CYP1As and CYP1D1s appearing in one clade, and the CYP1B1s and the CYP1Cs
220 occurring in another. In all cases, the *F. heteroclitus* CYP1s clustered most closely with the
221 predicted CYP1 orthologues of medaka. Interestingly, as in previous analyses (Goldstone et al.,
222 2007; Goldstone et al., 2009), in this analysis zebrafish CYP1C1 and CYP1C2 clustered together
223 rather than with their respective orthologues. This general result may be due to gene conversion,
224 as seen in avian CYP1As (Goldstone and Stegeman, 2006), although the taxonomic sampling of
225 the CYP1Cs is not yet sufficient to conclude this with any certainty.
226 In general, the corresponding CYP1s in *Fundulus* and zebrafish exhibit high degrees of
227 amino acid sequence similarity (Supplemental Figure 2). However, this similarity varied along
228 the length of the proteins, with the lowest similarities (BLOSUM62-based score) observed in the

membrane anchor, D helix, and the disordered H-I loop regions. The substrate recognition sites (SRS) of the orthologous zebrafish and *Fundulus* CYP1s are 74-84% identical, with SRS 2 and 3 displaying the largest number of differences (Figure 2). Comparisons of *F. heteroclitus* CYP1A and CYP1D1 sequences along the length of the proteins showed a marked likeness between CYP1A and CYP1D1 in SRSs 4 and 5 and a marked difference in SRSs 2 and 3 (Figure 3). These results are similar to the variation in sequence similarities observed between zebrafish CYP1A and CYP1D1 (Goldstone et al., 2009).

3.3 CYP1 transcript expression in organs of F. heteroclitus

Figure 4 shows the differences in expression levels among the *CYP1* genes in a given organ. Relative levels of expression are calculated and expressed as $\Delta^{\text{ct } \beta\text{-actin} - \text{ct } target}$. In liver, heart, kidney, gut, eye and gill, *CYP1A* was the highest expressed transcript. In liver, *CYP1A* was ~300 times higher than *CYP1D1*, ~1,300 times higher than *CYP1B1* and *CYP1C1*, and ~30,000 times higher than *CYP1C2*. In brain, *CYP1B1* and *CYP1D1* were the most highly expressed transcripts. In testis, *CYP1C1* was the most highly expressed transcript, ~1,000 and 3,000 times higher than *CYP1A* and *CYP1B1*, respectively. *CYP1C2* was expressed at the lowest levels among the five *CYP1* genes in most of the organs examined (liver, heart, kidney, eye, brain and kidney).

Figure 5 shows relative transcript levels of the five *CYP1s* measured by qPCR, comparing the levels of a given gene in the eight organs we sampled from control fish: liver, kidney, gut, heart, gill, eye, brain and testis. The analysis shows that the highest levels of *CYP1A* transcript were observed in liver. Levels of *CYP1B1* transcript were similarly high in brain, heart, gill, eye and kidney. Earlier studies observed differences in the basal levels of two reference genes, *β*-

actin and *ARNT2,* among the different organs (Jönsson et al., 2007b). Thus, in order to avoid erroneous comparisons of specific *CYP1* transcripts between different organs based on a given reference gene, we calculated transcript abundances based on standard curves. Individual sample correction for *β-actin* levels were organ-specific to allow for *CYP1* transcript comparison among organs. The highest levels of both *CYP1C1* and *CYP1C2* were in kidney, and of *CYP1D1* were in brain and kidney. The lowest levels of *CYP1A* were observed in testis, brain and eye; *CYP1B1* in testis, gut and liver; *CYP1C1* and *CYP1C2* in liver and gill; *CYP1D1* in liver and gut. *CYP1C1* and *CYP1C2* showed generally similar patterns of basal expression levels among organs.

3.4 PCB126 effects on CYP1 expression

No mortality was observed in *F. heteroclitus* injected with PCB126 or the carrier DMSO. PCB126 induced the expression of *CYP1A* and *CYP1B1* in all eight organs we examined (liver, heart, kidney, gut, eye, brain, gill and testis) (Figure 6). The most substantial changes in *CYP1* expression in response to PCB126 were in liver, where *CYP1C1* was induced ~500-fold and *CYP1B1*~200-fold, and in testis, where *CYP1A* was induced ~700-fold. *CYP1B1* in liver and gut, *CYP1A* in brain and *CYP1C1* in gill also were induced strongly (~100-fold in each case).

CYP1C1 also was induced in all organs except testis. Although *CYP1C1* and *CYP1C2* had similar transcript profiles in the different organs of control fish, *CYP1C2* was much less responsive to PCB126 compared with *CYP1C1*. Strikingly, in contrast to the other four genes, *CYP1D1* was not significantly induced by this dose of PCB126 in any of the organs examined.

4. Discussion

4.1 Identification of new CYP1 genes in Fundulus heteroclitus

With the cloning and sequencing of three new *CYP1* genes the *CYP1* family in *F. heteroclitus* is expanded to four subfamilies and five genes, *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2* and *CYP1D1*. Previously we established that the same set of CYP1 subfamilies and genes occurs in zebrafish, and that the five zebrafish CYP1s are expressed at the level of transcription (Jönsson et al., 2007a; Jönsson et al., 2007b; Goldstone et al., 2009). The five genes in *Fundulus* appear to be orthologues of those in zebrafish, and likewise all five are expressed in many organs.

Molecular phylogenetic analysis shows that *Fundulus CYP1A* and *CYP1D1* are grouped together in one clade, while *CYP1B1* and the *CYP1Cs* are in another. This result is fully consistent with our prior observation that the *CYP1Cs* and *CYP1Bs* are sister subfamilies occurring in one monophyletic clade (Goldstone et al., 2007; Jönsson et al., 2007b) and that the *CYP1As* and *CYP1Ds* are in another (Goldstone et al., 2009). The common molecular phylogeny for the *CYP1* genes in several species thus supports the hypothesis that the *CYP1As* and *CYP1Ds* diverged from a common *CYP1A/CYP1D* ancestor, and the *CYP1Bs* and *CYP1Cs* from a common *CYP1B/CYP1C* ancestor (Goldstone et al., 2007; Goldstone et al., 2009).

Killifish *CYP1C1* and *CYP1C2* are very closely related phylogenetically. A similarly close relationship occurs between *CYP1C1* and *CYP1C2* genes in fugu (Godard et al., 2005), and in zebrafish, where the two genes are located immediately adjacent to one another on chromosome 17 (Jönsson et al., 2007b). These observations suggest that the two *CYP1C* paralogs resulted from an independent duplication subsequent to a divergence of the *CYP1B* and *CYP1C* lines. Further, the observations are consistent with the idea that this gene duplication occurred subsequent to the branching of fishes from the vertebrate line, and that it occurred more recently than the whole genome duplication thought to have taken place in the fish lineage.

The phylogenetic analyses also show that the killifish CYP1 sequences are more closely related to the CYP1 sequences in medaka and stickleback than to those in zebrafish. These similarities could be expected, since zebrafish is in the superorder *Ostariophysi* while medaka, stickleback and Atlantic killifish are in the superorder *Acanthopterygii*. These two superorders were separated about 290 million years ago, while orders *Beliniformes* (medaka) and *Cyprinodontiformes* (Atlantic killifish) were separated more recently, around 153 million years ago (Steinke et al., 2006).

4.2 CYP1 expression in F. heteroclitus

When compared to other *CYP1s*, higher levels of *CYP1A* transcript were observed in most of the organs analyzed, except brain and testis. Relatively higher levels of *CYP1A* were also observed in the organs of abdominal cavity (e.g. liver, kidney and gut) in zebrafish, and could be associated with the role of these organs in nutrient uptake and processing of body waste products, e.g., detoxification of endogenous metabolites and food-derived AHR agonists (Jonsson et al., 2007b). Although *CYP1A* was the most abundant transcript in eye and heart, *CYP1B1* and *CYP1C1* were also expressed in substantial levels in these organs. Yin et al. (2008) showed that the basal level of *CYP1B1* in zebrafish embryos is regulated by an AHR2-independent pathway, and that CYP1B1 is not directly involved in pericardial edema generated by dioxin toxicity. It is well known that *CYP1B1* is important for normal eye development in mammals (Choudhary et al., 2006) and that mutations in this gene are correlated with glaucoma in human populations (Ohtake et al., 2003). The physiological importance of *CYP1B1* and *CYP1C1* basal levels in the adult fish eye and heart remains to be elucidated.

Similar to the present study, previous studies have shown greater levels of *CYP1B1* and *CYP1D1* expression in brain of adult zebrafish compared to the other *CYP1*s (Jönsson et al.,

2007b; Goldstone et al., 2009). In developing zebrafish *CYP1B1* expression was seen in the brain before hatching (Yin et al., 2008). In rat brain as well the basal levels of expression of *CYP1B1* were higher than *CYP1A*s, in four distinct regions of the brain (Desaulniers et al., 2005). While both *CYP1B1* and *CYP1D1* are expressed in brain the significance of this expression to brain function is unknown. The relatively higher levels of *CYP1B1* expression in brain suggest that these *CYP1s* could play particular roles in this organ in vertebrates including fish and mammals. The same may be true for CYP1D1 in species where this gene is expressed (see below).

High levels of all five *CYP1s* were observed in kidney. *CYP1C1*, *CYP1C2* and *CYP1D1*, were particularly high when compared to their levels in other organs. There could be an important function for those CYP1s in detoxification; high levels of gene expression for many phase-I, II and III biotransformation enzymes, as well as receptors involved in the regulation of those genes, are observed in the kidney and participate in the elimination of many xenobiotics, drugs and endogenous compounds (Xu et al., 2005).

Comparing the levels of expression of each CYP1 in different organs of killifish showed similar patterns for the two *CYP1C*s, with higher levels in kidney, brain and testis, and lower levels in liver and gill. The relative levels of expression of *CYP1C1* in kidney, liver, testis and eye observed by Wang et al., (Wang et al., 2006), in a different population of *F. heteroclitus*, are similar to the *CYP1C* results presented here. However, while there are similarities in the organ-specific patterns of expression of the *CYP1C*s, *CYP1C2* was expressed at much lower levels than *CYP1C1*. Thus, the two *CYP1C*s could be regulated by similar organ-specific pathways, but may yet function differently in a given organ.

343 The determination that these five *CYP1s* are expressed in *Fundulus* as they are in
344 zebrafish is important, perhaps especially so in the case of *CYP1D1*. This is because in humans
345 *CYP1D1* is a pseudogene (*CYP1D1P*) (Goldstone et al., 2009) and it appears to be a pseudogene
346 in some other species as well (unpublished data), and thus, finding a *CYP1D1* gene does not
347 necessarily mean that it will be expressed. The *CYP1D1* locus may have been lost from the
348 genome of still other species, including some fish; we have been unable to detect a *CYP1D* in
349 pufferfish genomes. In some species CYP1D1 functions may be accomplished by other CYPs,
350 possibly other CYP1s. It will be interesting to determine whether CYP1D1 has unique substrate
351 specificities.
352 Generally, it appears that there are catalytic similarities among fish CYP1As. The
353 substrate specificities of the other CYP1s in *Fundulus* are not yet known, and are only poorly
354 known in zebrafish. We have expressed the five zebrafish CYP1s in yeast, and determined that
355 the zebrafish CYP1Cs and CYP1D1 act on some of the same alkoxyresorufin substrates that are
356 oxidized by CYP1A (Goldstone et al., 2009 and unpublished). In the eel *Anguilla japonica,*
357 CYP1C1 appears to metabolize the CYP1A substrates 7-ethoxyresorufin and 7-ethoxycoumarin,
358 although with lower enzymatic activity than CYP1A. Interestingly, there were differences
359 between the eel CYP1A and CYP1C1 in the products formed from one substrate; eel CYP1C1
360 produced two products from flavanone whereas CYP1A produced just one (Uno et al., 2008).
361 Studies with heterologously-expressed enzymes can indicate substrate specificities of the
362 different CYP1s. However, to understand the role of a particular CYP in a given organ will
363 require determining the levels of protein expressed, and contributions to substrate turnover by
364 use of specific inhibitors, or inhibitory antibodies. We have obtained antibodies to zebrafish
365 CYP1D1 and have determined that CYP1D1 protein is expressed in zebrafish liver (Goldstone et

al., 2009). Unfortunately, the antibodies to zebrafish CYP1D1 do not recognize a protein in *Fundulus* liver (unpublished data). Determining the catalytic function of the various CYP1s with multiple substrates will be essential to establishing the physiological roles of these enzymes. At present this would be merely a matter of speculation. However, the expression patterns suggest to us that these enzymes will be shown to have distinct physiological roles in fish.

4.3 Response of CYP1s to PCB126 in F. heteroclitus

The five CYP1s in killifish differed in their responses to treatment of the animals with the potent AHR agonist PCB126. The observation that CYP1A is induced in all organs was expected. Previous studies have shown that CYP1A in fish is induced to high levels of expression in detoxification organs (e.g., liver, gastrointestinal tract, gill and kidney), and that CYP1A is induced in some cell types, often in endothelium, in all organs (e.g., Smolowitz et al., 1992). Immunohistochemical studies have also shown that CYP1A protein levels are strongly induced in all organs of *Fundulus* exposed to AHR agonists (VanVeld et al., 1997). The present study also shows that *CYP1B1* was induced in all organs examined. The most significant changes in *CYP1* expression in response to PCB126 were induction of *CYP1A* (~700-fold) in testis and induction of *CYP1C1* (~500-fold) in liver. *CYP1B1* in liver and gut, *CYP1A* in brain and *CYP1C1* in gill also were strongly induced (>100-fold). Low basal levels of the respective *CYP1* gene expression, together with a strong induction by AHR agonists, could explain the higher fold-increases over control values observed in some organs.

In contrast to *CYP1A*, *CYP1B1* and *CYP1C1*, expression of *CYP1C2* transcript was weakly induced, and only in three of the organs analyzed (liver, heart and gill). The lesser responsiveness of *CYP1C2* to a potent AHR agonist is reminiscent of the results obtained with

zebrafish, in which the responsiveness of *CYP1C2* to PCB126 was largely absent from adults (Jönsson et al., 2007a; Jönsson et al., 2007b). The similarity of results observed in killifish and zebrafish, even considering that there were different exposure routes for PCB126 in the experiments with adult fish, suggest a silencing of the induction response of CYP1C2 in adults of these fish species. Distinct from the other CYP1s, *CYP1D1* was not induced in any of the eight organs we examined here. In like fashion, there was no induction of *CYP1D1* in zebrafish adults treated with PCB126 or TCDD (Goldstone et al., 2009).

It might be argued that response of CYP1s in *Fundulus* embryos could differ from that in adults. In zebrafish, the only notable difference between adult and embryonic responses to PCB126 was that *CYP1C2* was induced in zebrafish embryos. However, in zebrafish the responsiveness of *CYP1C2* to PCB126 decreased with developmental age. The developmental pattern of expression and the response of the five *CYP1* genes to AHR agonists during embryonic development in *Fundulus* is under investigation.

The mechanism involved in the lesser induction response of *CYP1C2* in adult fish is not known. However, the lack of induction of *CYP1D1* in zebrafish was suggested to be related to a lack of functional AHR response elements (AHREs), which are binding sites for the AHR /ARNT in the promoter regions (Goldstone et al., 2009). In zebrafish, there are only two putative AHREs (of unproven function) in the promoter of zebrafish *CYP1D1*, in contrast to the 22 putative AHREs and three proven functional AHREs for *CYP1A* (ZeRuth and Pollenz, 2007; Goldstone et al., 2009). A variety of other response elements were identified in the promoter region of zebrafish *CYP1D1* (Goldstone et al., 2009) and thus the possibility of induction via other receptors and agonists still remains to be elucidated, both for the *CYP1Cs* and *CYP1D1* in *Fundulus* and in zebrafish.

412 The induction of CYP enzymes in fish liver was first suggested as an indicator of aquatic
413 contamination in the 1970s (e.g., Payne, 1976). Since then, many studies have shown that
414 CYP1As in vertebrate liver (often measured by activity assay and protein detection by Western
415 blot) are strongly induced by some organic contaminants that represent risk for human and
416 wildlife (e.g., PAHs, coplanar PCBs, polychlorinated dibenzofurans, and dibenzodioxins)
417 (Bucheli and Fent, 1995). The results of the present study, along with the results of Wang et al.,
418 (Wang et al., 2006), suggest that in addition to *CYP1A*, other *CYP1s*, notably *CYP1B1* and
419 *CYP1C1*, have potential to be additional sensitive biomarkers of exposure to AHR agonist
420 contaminants in *F. heteroclitus* and likely other fishes. Lesser responsiveness by *CYP1C2* in
421 adults and the lack of *CYP1D1* induction by potent AHR agonists in two different species
422 suggests that these two genes would not be suitable markers for exposure to such chemicals.
423 Nevertheless, CYP1C2 and CYP1D1 may still be involved in the toxicity of AHR agonists or
424 other chemicals, if such chemicals are substrates for these enzymes, or if these enzymes have
425 important endogenous substrates and are inhibited by xenobiotics. Thus, all five CYP1 isoforms
426 could be involved chemical effects, and perhaps in the resistance to AHR agonist toxicity
427 observed in some *Fundulus*. Studies of the expression of the five CYP1 genes are under way in
428 *F. heteroclitus* populations that have developed resistance to toxic effects of and a lack induction
429 of *CYP1A* by halogenated AHR agonists (Elskus et al., 1999; Bello et al., 2001) .
430 In summary, we identified and cloned three new *CYP1* genes, *CYP1B1*, *CYP1C2* and
431 *CYP1D1,* in the Atlantic killifish *Fundulus heteroclitus*, a vertebrate model used extensively in
432 environmental toxicology studies. Substantial differences in the levels of expression for the five
433 *CYP1s* were observed in the examined organs. We also showed that these *CYP1s* differ in the
434 response to the potent AHR agonist PCB126. The organ-specific differences in basal levels, and

435 in fold-induction by PCB126, suggest that regulatory mechanisms for the five *CYP1* genes could
436 differ. Finally, these new *CYP1* family members increase the set of potential biomarkers of
437 aquatic contamination in fish, and may aid in elucidating possible mechanisms of toxicity of
438 AHR agonists, and the physiological roles of this important gene family.
439

440 **5. Acknowledgements**

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448 **Figure captions:**

449 **Figure 1.** Phylogenetic tree of selected CYP1 amino acid sequences. In all cases the *Fundulus*
450 sequences cluster most closely with the medaka sequences, as expected based on taxonomic
451 relationships. Numbers at the nodal points are support values derived from Bayesian
452 phylogenetic and maximum likelihood bootstrap analyses ($3x10^6$ generation and 100 replicates,
453 respectively; Bayes/ML support). Sequences presented in this phylogenetic tree include
454 predicted sequences for medaka (MEDAKA) and stickleback (GASAC), as well as new and
455 previously published sequences for killifish (FUNHE), zebrafish (DANIO), human (HOMO) and
456 mouse (MUSMU). See supplemental data for accession numbers and references.

457 **Figure 2.** Alignment of substrate recognition sites (SRS 1-6) of *Fundulus heteroclitus* (FUNHE)
458 and zebrafish *Danio rerio* (DANRE) CYP1. Residues that are identical to *Fundulus* CYP1A
459 sequence are indicated by a dot.

460 **Figure 3.** Similarity between CYP1A and CYP1D1 protein sequence in *Fundulus heteroclitus*
461 using a BLOSUM62-based score. A 10-residue running average similarity is displayed. The
462 substrate recognition sites (SRS) are indicated with a yellow bar. Helix and sheet designations
463 are marked below the figure by red and blue, respectively.

464 **Figure 4.** Comparison among different *CYP1* transcript levels of *Fundulus heteroclitus* from
465 control group in a given organ: liver, heart, kidney, gut, eye, brain, gill or testis. Equal letters
466 indicate absence of difference among groups (ANOVA – Tukey HSD, $p<0.05$; n=4-10). Relative
467 levels for *CYP1* transcripts were determined by qPCR using the $E^{\Delta Ct}$ method and *beta-actin* as
468 housekeeping gene (see Methods). Data are presented in a logarithmic scale.

Figure 5. Organ-specific expression of *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2* or *CYP1D1* in control *Fundulus heteroclitus*. Equal letters indicate absence of difference among organs for a given *CYP1* (ANOVA – Tukey HSD, $p<0.05$; n=4-10). Levels for *CYP1* and *beta-actin* gene expression were determined by qPCR using standard curves. *Beta-actin* was employed to normalize individual gene expression within but not between each organ group. Gene expression is presented as relative levels.

Figure 6. Fold induction of *CYP1s* 48-hours after injection with PCB 126 (31 nmol/g fish) relative to fish injected with DMSO (control) in *Fundulus heteroclitus*. Analyses were done in liver, heart, kidney, gut, eye, brain, gill or testis. The numbers of molecules for CYP1 transcripts, as well as for the housekeeping gene beta-actin, were determined by qPCR using standard curves. (*$p<0.05$, **$p<0.005$ and ***$p<0.001$, comparison between control and PCB injected groups using Student's t-Test; n=4-10).

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667

668 **Table 1.** Primers employed in the *CYP1A*, *CYP1B1, CYP1C1, CYP1C2, CYP1D1* and *β-actin*

669 qPCR reactions in *Fundulus heteroclitus*.

primer name	Primer sequence 5' - 3'	Location
1A forward	CTTTCACAATCCCACACTGCTC	1301 - 1322
1A reverse	GGTCTTTCCAGAGCTCTGGG	1404 - 1423
1B1 forward	ATATTTGGAGCCAGCCAGGACACG	629 - 652
1B1 reverse	CGCACCTGCATCTCAGGGTACTTG	691 - 714
1C1 forward	TCTGGACGCCTTCATCTACGA	1296 - 1316
1C1 reverse	GTGACGTCCGATGTGGTTGA	1360 - 1379
1C2 forward	GCAGGCTGCCATCTGTTGAGGACA	1257 - 1280
1C2 reverse	CGAAGCTGGTGAAACGCATTGTCT	1317 - 1340
1D1 forward	CTTTCACCATCCCTCACTGCACCA	1322 - 1345
1D1 reverse	GTCTCCGGATCACCCCAAAGATCC	1428 - 1451
β-actin forward	TGGAGAAGAGCTACGAGCTCC	*
β-actin reverse	CCGCAGGACTCCATTCCGAG	*

* no full sequence available

670

671

Table 2. Identities between *Danio rerio* (DANRE) and *Fundulus heteroclitus* (FUNHE) *CYP1* sequences. Values for amino acid identities are presented in the right-top and nucleotide identities in the left-bottom of the table. Regions of ambiguously aligned sequences were masked.

	FUNHE_1A	DANRE_1A	FUNHE_1D1	DANRE_1D1	FUNHE_1B1	DANRE_1B1	FUNHE_1C1	DANRE_1C1	FUNHE_1C2	DANRE_1C2
FUNHE_1A		0.73	0.47	0.48	0.41	0.43	0.41	0.40	0.41	0.40
DANRE_1A	0.70		0.48	0.48	0.42	0.43	0.42	0.42	0.41	0.41
FUNHE_1D1	0.56	0.56		0.75	0.40	0.40	0.39	0.40	0.41	0.41
DANRE_1D1	0.56	0.55	0.70		0.38	0.39	0.38	0.40	0.41	0.42
FUNHE_1B1	0.50	0.51	0.52	0.48		0.69	0.53	0.56	0.53	0.55
DANRE_1B1	0.51	0.51	0.50	0.48	0.68		0.54	0.56	0.55	0.56
FUNHE_1C1	0.49	0.50	0.50	0.49	0.61	0.59		0.81	0.70	0.75
DANRE_1C1	0.49	0.50	0.50	0.51	0.60	0.60	0.72		0.73	0.83
FUNHE_1C2	0.50	0.49	0.50	0.50	0.59	0.58	0.71	0.69		0.74
DANRE_1C2	0.49	0.50	0.51	0.51	0.60	0.60	0.69	0.82	0.70	

Supplemental data for Zanette et al. "Three new P450 1 genes in the killifish *Fundulus heteroclitus*: basal expression and response of the full complement of killifish *CYP1s* to the AHR agonist PCB126"

Supplemental Table 1. Degenerate primers employed in the amplification of cDNA fragments of the new *CYP1B1* and *CYP1D1* genes and specific primers used for Smart-RACE cloning of full-length sequences of *CYP1C2*, *CYP1B1* and *CYP1D1* in *Fundulus heteroclitus*.

primer name	Primer sequence 5' - 3'	Location
1B1 degenerate forward	GCBGGSAGCATCGTGGACGTG	374 - 394
1B1 degenerate reverse	TTGGACAGCTCCTCDCCRATGCA	1058 - 1080
1D1 degenerate forward	GRATGAAYGGATTCATGGAGCGCA	947 - 970
1D1 degenerate reverse	GGTGCYBAGATCCAGCTBCTGACC	1627 - 1650
1B1 3' forward 1	ATATTTGGAGCCAGCCAGGACACG	629 - 652
1B1 3' forward 2	CTGCCGTACGTCATGGCCTTCATC	782 - 805
1B1 5' reverse 1	CCACCGAGAAGATGAGCACGTTG	1024 - 1046
1B1 5' reverse 2	TCAAATGTCTCCGGGTTGGACCAC	949 - 972
1C2 3' forward 1	GCAACGGTGGCAGATTTGATCGGCGCAGGC	1121 - 1150
1C2 3' forward 2	TGACAGGGTGGTGGGGAGAGGCAGGC	1237 - 1262
1C2 5' reverse 1	TGAGGGTCCTTCCACCTCAGGGGGTCGTG	1442 - 1470
1C2 5' reverse 2	GGGGGATGGTGACAGGGACGAAGCCGGT	1331 - 1358
1D1 3' forward 1	GCGCAGGATCCAGGAACACATCAAC	966 - 990
1D1 3' forward 2	GGGTTGCAGTGGAGCCTGTTGTACCT	1147 - 1172
1D1 5' reverse 1	CATCTCCAAACGTGCGACTCCATCC	1551 - 1575
1D1 5' reverse 2	GTCTCTTGTGGTGCAGTGAGGGATGG	1328 - 1353

692

693 Supplemental Figure captions for Zanette et al. “Three new P450 1 genes in the killifish
694 *Fundulus heteroclitus*: basal expression and response of the full complement of killifish *CYP1s*
695 to the AHR agonist PCB126”

696 **Supplemental Figure 1.** Alignment of *Fundulus heteroclitus* (FUNHE) and zebrafish *Danio*
697 *rerio* (DANRE) CYP1s. Substrate recognition sites (SRS 1-6) and the heme-binding domain are
698 designated by rectangles. A dot indicates residues that are identical to the *Fundulus* CYP1A
699 sequence.

700

701 **Supplemental Figure 2.** Pairwise similarities between *Fundulus* and zebrafish CYP1 ortholog
702 pairs of protein sequences using a BLOSUM62-based score. A 10-residue running average
703 similarity between *Fundulus* and zebrafish orthologs is presented, with the different zebrafish-
704 *Fundulus* CYP1 pairwise comparisons offset by cumulative 5 point intervals for presentation
705 purposes.

Fig 1

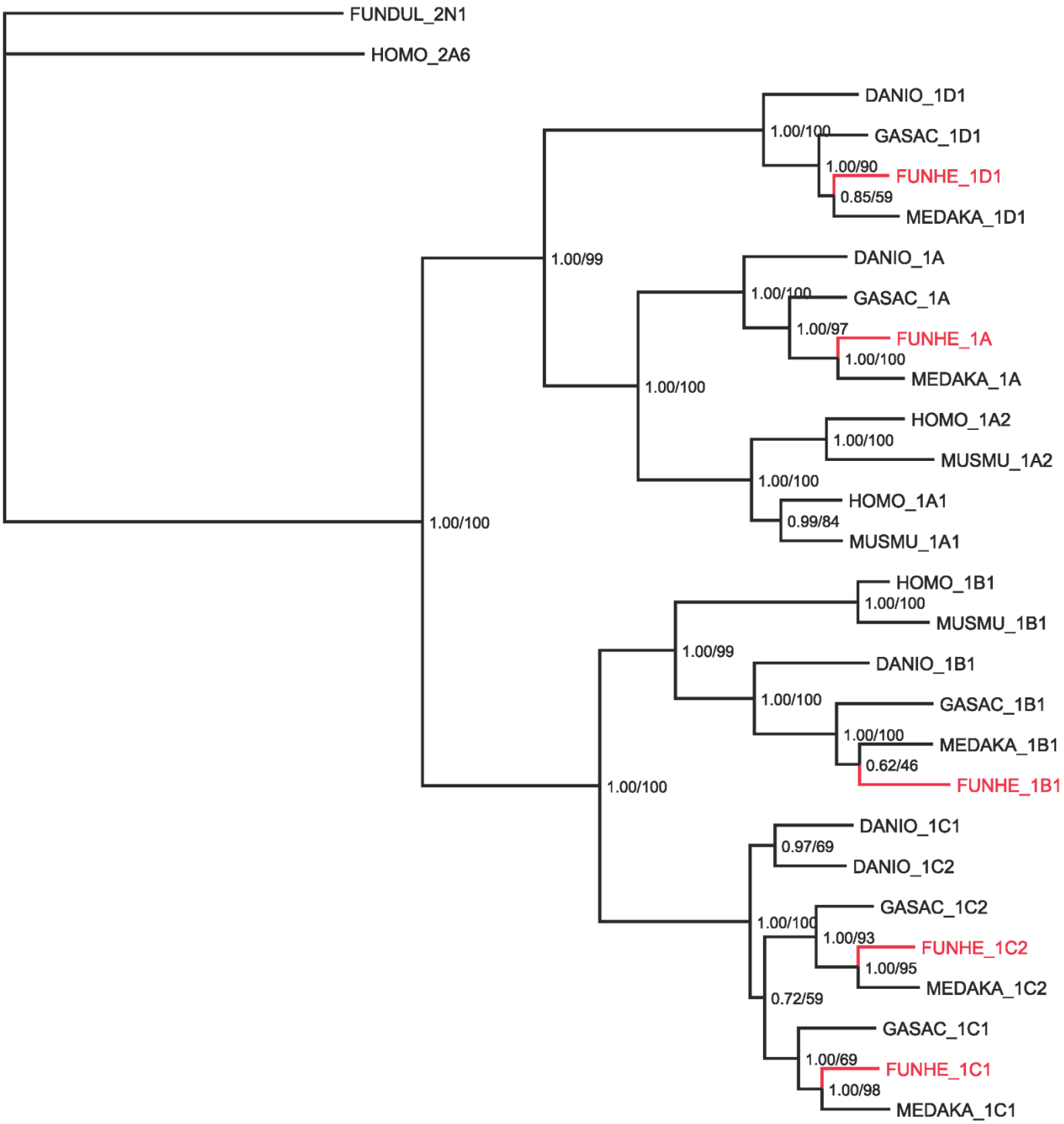

FUNDUL_2N1
HOMO_2A6
DANIO_1D1
1.00/100
GASAC_1D1
1.00/90
FUNHE_1D1
0.85/59
MEDAKA_1D1
1.00/99
DANIO_1A
1.00/100
GASAC_1A
1.00/97
FUNHE_1A
1.00/100
MEDAKA_1A
1.00/100
HOMO_1A2
1.00/100
MUSMU_1A2
1.00/100
HOMO_1A1
0.99/84
MUSMU_1A1
1.00/100
HOMO_1B1
1.00/100
MUSMU_1B1
1.00/99
DANIO_1B1
1.00/100
GASAC_1B1
1.00/100
MEDAKA_1B1
0.62/46
FUNHE_1B1
1.00/100
DANIO_1C1
0.97/69
DANIO_1C2
GASAC_1C2
1.00/100
1.00/93
FUNHE_1C2
1.00/95
MEDAKA_1C2
0.72/59
GASAC_1C1
1.00/69
FUNHE_1C1
1.00/98
MEDAKA_1C1

Fig 2

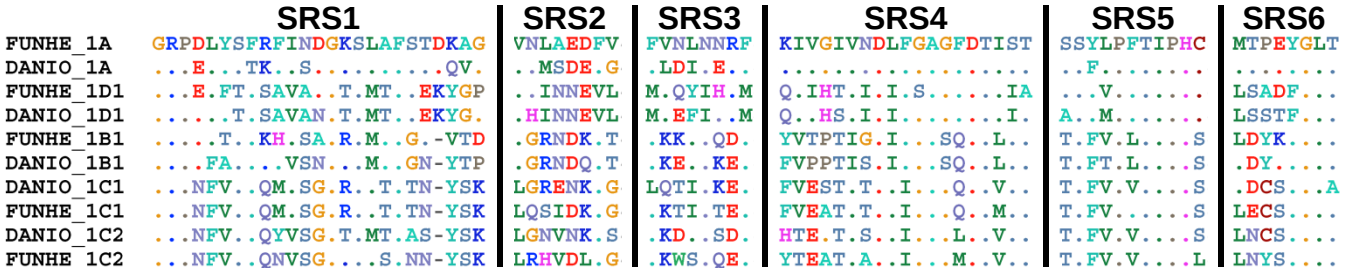

SRS1
SRS2
SRS3
SRS4
SRS5
SRS6
FUNHE_1A GRPDLYSFRFINDGKSLAFSTDKAG VNLAEDFV FVNLNNRF KIVGIVNDLFGAGFDTIST SSYLPFTIPHC MTPEYGLT
DANIO_1A ...E...TK..S.........QV. ..MSDE.G .LDI.E.. ....F..... .....
FUNHE_1D1 ...E.FT.SAVA..T.MT..EKYGP ..INNEVL M.QYIH.M Q.IHT.I.I.S.....IA ...V..... LSADF...
DANIO_1D1 .....T.SAVAN.T.MT..EKYG. .HINNEVL M.EFI..M Q..HS.I.I.....I. A..M..... LSSTF...
FUNHE_1B1 .....T..KH.SA.R.M..G.-VTD .GRNDK.T .KK..QD. YVTPTIG.I...SQ..L.. T.FV.L....S LDYK....
DANIO_1B1 ....FA....VSN...M..GN-YTP .GRNDQ.T .KE..KE. FVPPTIS.I...SQ..L.. T.FT.L....S .DY.....
DANIO_1C1 ...NFV..QM.SG.R..T.TN-YSK LGRENK.G LQTI.KE. FVEST.T..I...Q..V.. T.FV.V....S .DCS...A
FUNHE_1C1 ...NFV..QM.SG.R..T.TN-YSK LQSIDK.G .KTI.TE. FVEAT.T..I...Q..M.. T.FV.....S LECS....
DANIO_1C2 ...NFV..QYVSG.T.MT.AS-YSK LGNVNK.S .KD..SD. HTE.T.S..I...L..V.. T.FV.V....S LNCS....
FUNHE_1C2 ...NFV..QNVSG....S.NN-YSK LRHVDL.G .KWS.QE. YTEAT.A..I...M..V.. T.FV.V....L LNYS....

Fig 3

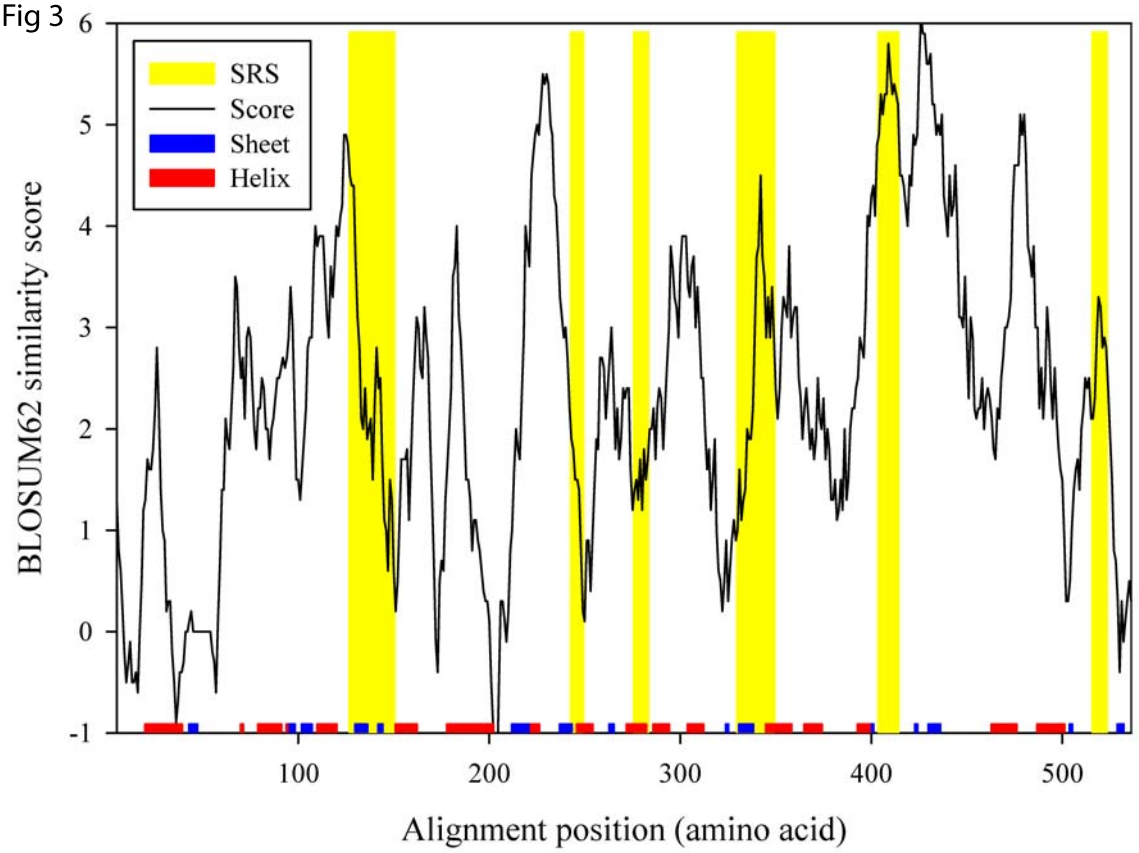

SRS
Score
Sheet
Helix
BLOSUM62 similarity score
6
5
4
3
2
1
0
-1
100
200
300
400
500
Alignment position (amino acid)

Fig 4

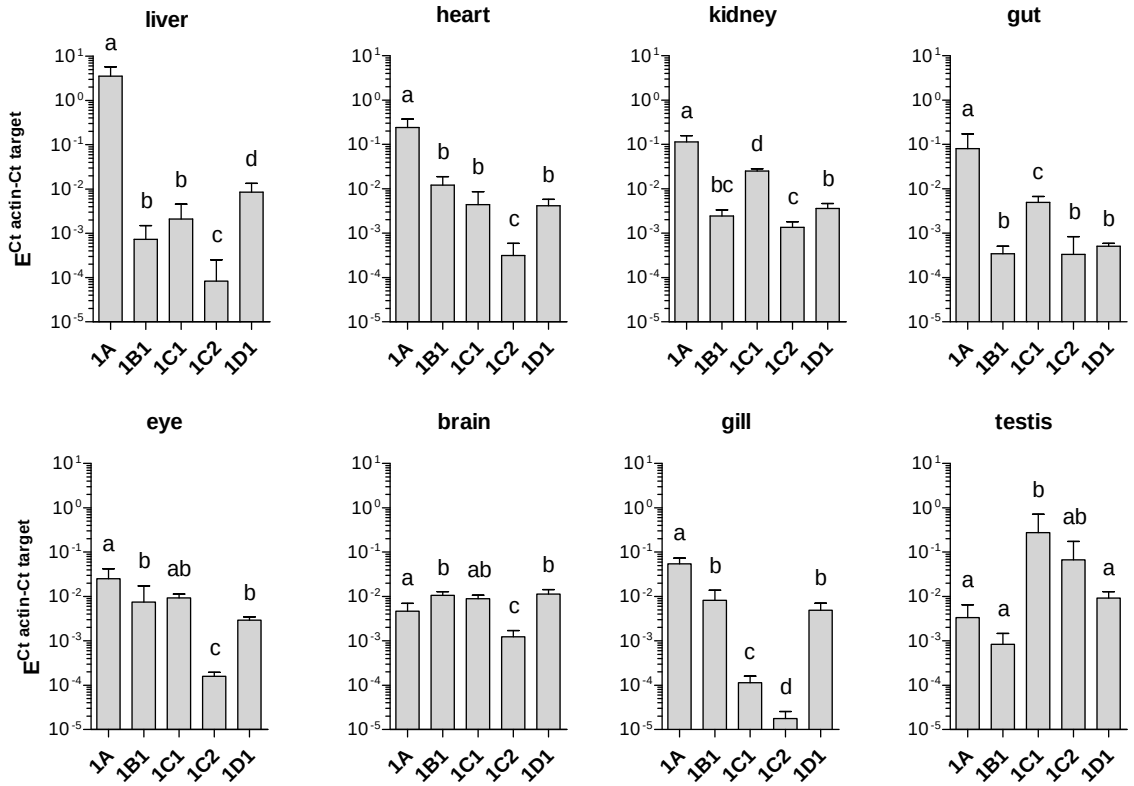

liver
heart
kidney
gut
eye
brain
gill
testis
E^Ct actin-Ct target
1A
1B1
1C1
1C2
1D1

Fig 5

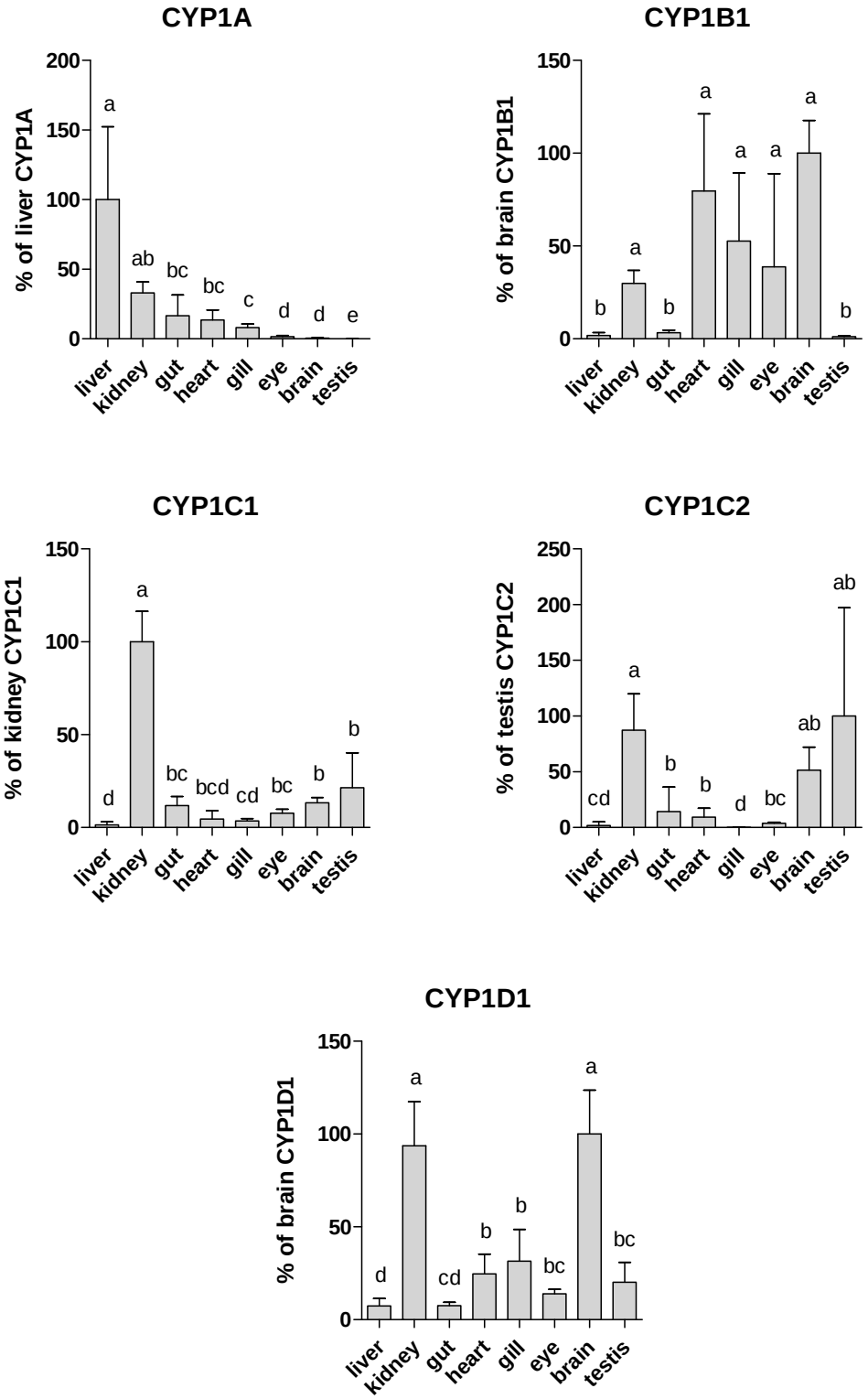
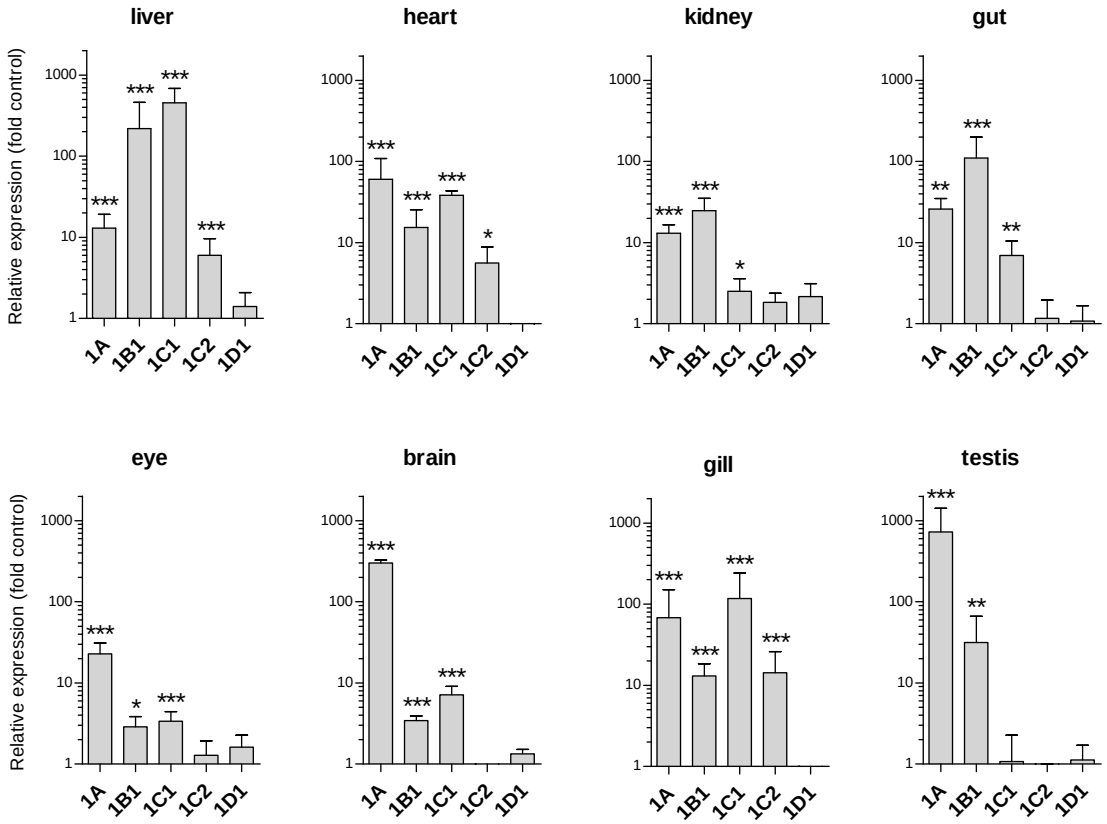

CYP1A
% of liver CYP1A
200
150
100
50
0
a
ab
bc
bc
c
d
d
e
liver
kidney
gut
heart
gill
eye
brain
testis
CYP1B1
% of brain CYP1B1
150
100
50
0
b
a
b
a
a
a
a
b
CYP1C1
% of kidney CYP1C1
150
100
50
0
d
a
bc
bcd
cd
bc
b
b
CYP1C2
% of testis CYP1C2
250
200
150
100
50
0
cd
a
b
b
d
bc
ab
ab
CYP1D1
% of brain CYP1D1
150
100
50
0
d
a
cd
b
b
bc
a
bc

Fig 6


liver
heart
kidney
gut
eye
brain
gill
testis
Relative expression (fold control)
1000
100
10
1
***
**
*
1A
1B1
1C1
1C2
1D1

Supplementary figure 1