The New Vertebrate CYP1C Family: Cloning of New Subfamily Members and Phylogenetic Analysis

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ABSTRACT

Two novel CYP1 genes from teleost fish constituting a new subfamily have been cloned. These paralogous sequences are designated CYP1C1 and CYP1C2. Both genes were initially obtained from untreated scup *Stenotomus chrysops* tissues by RT-PCR and RACE. Scup CYP1C1 and CYP1C2 code for 524 and 525 amino acids, respectively, and share 80-81% identity at the nucleotide and amino acid levels. Orthologues of CYP1C1 and CYP1C2 were identified in genome databases for other fish species, and both CYP1B1 and CYP1C1 were cloned from zebrafish (*Danio rerio*). Phylogenetic analysis shows that CYP1Cs and CYP1Bs constitute a sister clade to the CYP1As. Analysis of sequence domains likely to have functional significance suggests the two CYP1Cs in scup may have catalytic functions and/or substrate specificity that differ from each other and from those of mammalian CYP1Bs or CYP1As. RT-PCR results indicate that CYP1C1 and CYP1C2 are variously expressed in several scup organs.

Keywords: Cytochrome P450, CYP1A, CYP1B1, CYP1C, PAH, planar aromatic hydrocarbons

INTRODUCTION

Members of the cytochrome P450 1 (CYP1) family of monooxygenases are well known for catalyzing the oxidation and often bioactivation of a wide variety of common environmental carcinogens and promutagens. Mammalian CYP1A1 and CYP1A2 are active in the biotransformation of planar aromatic hydrocarbons and both aryl and heterocyclic amines, respectively [1-3], while CYP1B1 substrate specificity encompasses that of the two CYP1As [4-6]. CYP1B1 is the only known member of the CYP1B subfamily in humans and presumably other mammals, and exhibits the highest catalytic activities of all CYP1 enzymes for several of these substrates [4], which may potentially determine susceptibility to carcinogenesis by some planar aromatic hydrocarbons (PAHs) [7, 8]. For example, studies with CYP1B1-null mice established CYP1B1 as the sole CYP1 enzyme responsible for the carcinogenicity of the PAH pro-carcinogen 7,12-dimethylbenz[a]anthracene in mice [9]. CYP1A1 is able to metabolize this substrate but that does not mediate its carcinogenicity [9]. CYP1B1 is also involved in the metabolism of estradiol [10, 11], apparently playing a role in estrogen-induced carcinogenesis [12].

CYP1As and CYP1Bs are regulated by the ligand-activated aryl hydrocarbon receptor transcription factor (AHR) [13, 14]. CYP1A induction is widely used as a biomarker of exposure to AHR agonists but the role played by CYP1B1 in xenobiotic metabolism and carcinogenesis argues that CYP1B or any other CYP1 family genes also must be considered when assessing exposure to contaminants in environmental systems.

In this study, we investigated the presence of additional CYP1 genes in early diverging vertebrates, initially in the teleost fish scup, *Stenotomus chrysops*. At the outset

of these studies, the CYP1B subfamily contained only human, rat, and mouse CYP1B1. 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 [15]. In a preliminary report we described the occurrence of CYP1B-like sequences in the fish species scup and plaice [16]. Subsequently, the full-length plaice sequence was obtained and classified as a CYP1B1 [17]. Here we report on the full-length sequences of the novel scup CYP1B-like sequences, obtained from liver tissue of untreated fish by RT-PCR and RACE reactions. These sequences appear to represent a sister clade to the CYP1Bs, defining a novel CYP gene subfamily, CYP1C, consisting of two paralogous genes, CYP1C1 and CYP1C2. Orthologues of these genes have subsequently been identified in other fish species, and we have also cloned full length CYP1C1, as well as CYP1B1, from zebrafish. Inspection of amino acid sequences and domains known to play functional or structural roles among CYP1 genes suggest that the CYP1Cs will differ functionally from each other and from other CYP1s. The occurrence of multiple CYP1 subfamilies in fish expands the possible distribution of functions and regulation that in mammals are restricted to the CYP1As and CYP1Bs.

MATERIALS AND METHODS

Animals and Animal Treatment

Scup were caught with traps in waters near Woods Hole Harbor, MA, USA and held in flowing filtered seawater at 16-20°C until use. Scup used for determining constitutive expression of CYP1 genes were depurated for more than one and a half

years. All fish were killed by severing the spinal cord and their organs were removed and frozen in liquid nitrogen immediately after death. The studies were conducted in accordance with principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

RNA Isolation

Total RNA was isolated using RNA STAT-60™ (Tel-Test, Friendswood, TX) and poly(A)+ RNA was prepared by one pass over a mini-oligo(dT)-cellulose spin column (5 prime- 3 prime Inc, Boulder, CO). DNA contamination was removed using DNA-free™ (Ambion, Austin, TX). RNA concentrations and purity were determined spectrophotometrically by measuring absorbance at 260 nm and 280 nm.

RT-PCR Conditions and Primer Design

Scup: Degenerate inosine-containing primers were designed in highly conserved regions based on an alignment of rat, human, and mouse CYP1B1 genes. All primers are listed in Supplemental Information. RT-PCR was performed using the GeneAmp RNA-PCR kit (Perkin-Elmer). Reverse transcription of liver mRNA (1μg) was primed with random hexamers (final concentration 2.5 μM). Standard reaction conditions were modified to include 5 mM $MgCl_2$ solution, 20 units (U) RNase inhibitor, and 50 U MuLV reverse transcriptase. PCR reactions (100 μl) were as per the kit instructions with the following modifications: 2 mM $MgCl_2$, 0.5 Unit of AmpliTaq Gold™, and 1 μM each primer. Samples were heated to 95°C for 10 min, followed by 35 cycles of 15 s at 95°C and 30 s at 40°C or 50°C, and a final extension step of 10 min at 72°C. An optional 60 s extension step at 72°C was added to each cycle when expected band size was bigger than

700 bp. Nested PCR reactions were prepared with 1 µl of the template DNA either straight or diluted 1/10.

Zebrafish: 5' RACE ready cDNA was generated per kit instructions using the SMART™ RACE kit (Clontech) with mRNA isolated from untreated zebra fish liver using the MicroPoly(A) Purist™ kit (Ambion). CYP1B1 was PCR amplified using vent polymerase (New England Biolabs, Beverly, MA) using the the 3' and 5' UTR primers listed in the supplemental information. CYP1C1 mRNA was PCR amplified using the primers listed in the supplemental information.

RACE Conditions and Primer Design

Scup: Race primers were designed after obtaining CYP1B-like sequences from scup liver with nested RT-PCR. All RACE primers are listed in Supplemental Information. A library of adaptor-ligated ds cDNA from scup liver was constructed using the Marathon™ cDNA Amplification kit (Clontech Laboratories Inc, Palo Alto, CA). The adaptor-ligated cDNA was diluted 1/250 in tricine-EDTA and one microliter of that dilution was used for both 5' and 3'RACE reactions. RACE were performed according to the manufacturer's protocol. The final step of nested-RACE was shortened to 20 cycles instead of 25.

PCR, RACE and SQRT-PCR products of expected size were separated on ethidium bromide-stained agarose gels, purified over a silica matrix with a Geneclean® III Kit (Bio 101, Vista, CA), cloned into pGem®-T Easy vector (Promega, Madison, WI) and sequenced in both directions by using either the SequiTherm or SequiTherm Excel long-read cycle sequencing kits (Epicentre Technologies, Madison, WI) and an

automated DNA sequencer (LI-COR Inc., Lincoln, NE) or with an ABI 3730XL capillary sequencer (Applied Biosystems, Foster City, CA).

Zebrafish: CYP1C1 cDNA was cloned into JM109 cells using the pGEM T (Promega) vector. CYP1B1 cDNA was cloned using the pGEM T vector after A-tailing with standard procedures using *Taq* polymerase. Transformed colonies were grown in a BioBlock (Fisher Scientific) and plasmid DNA was purified using the standard protocol for the GeneMachines™ RevPrep Orbit. The resulting DNA was sequenced by standard protocols on an ABI 3730XL capillary sequencer using Big Dye Terminator (ABI) and M13 forward and reverse primers or the internal primers described in supplemental information. For CYP1B1, the forward internal primer was used in addition to the M13 primers to get clean sequence for the center of the transcript. The CYP1C1 sequence submitted is the consensus sequence of 7 full length clones.

Cloning of Scup Actin

Degenerate actin primers (see Supplemental Information for sequences) were generously provided by Dr. Mitchell Sogin (Marine Biological Laboratory, Woods Hole, MA). RT-PCR was performed using the Gene-Amp RNA-PCR kit (Perkin-Elmer). RT-PCR reactions were carried out as described above for the CYP1C genes, but using 200 ng of liver mRNA template, 1 µl of each PCR primer (100 ng/µl stock solution) and an annealing temperature of 52°C.

Semiquantitative RT-PCR

Specific primers were designed to amplify similar size fragments from scup CYP1C1, CYP1C2, CYP1A and actin genes (214-239 bp long). Both CYP1C amplicons span 5'utr and ORF regions; the CYP1A and actin amplicons are located within their

respective ORF. All primer sequences are given in Supplemental Information. The identity of the products amplified by these primers was confirmed by sequencing. The semiquantitative RT-PCR was performed using the Gene-Amp RNA-PCR kit (Perkin-Elmer). Reverse transcription was primed with random hexamers (final concentration 2.5 μM). One RT master mix was prepared for each sample in order to assure consistency by minimizing intra-sample variability. RT minus reactions were also conducted to control for DNA contamination and efficacy of the DNase-free treatment. The PCR reactions were carried out according to the kit instructions. Ten microliters of each sample in an experiment were separated on the same 2% agarose gel, stained 25 min in ethidium bromide (1mg/ml in TAE buffer), and destained 45 min in distilled and deionized water before quantification by spot densitometry (with auto-background subtraction) using ChemImager™ 4000 software as described in the manual's protocol.

Sequence Analyses

Sequence assembly was performed using MacVector™ 6.5 and AssemblyLIGN™ 1.0 (Oxford Molecular Ltd., Oxford, England). Amino acid sequences were aligned and analyzed using ClustalW [18] and GCG/Seqlab (Accelrhys, San Diego, CA). Cloned sequences have been submitted to GenBank (scup CYP1C1 **AF131885**, scup CYP1C2 **AF235138**, zebrafish CYP1C1 **XXXXXXXX**, zebrafish CYP1B1 **AY534681**)

CYP1C sequences were predicted from the *Takifugu rubripes* (v3.0; [19]), *Danio rerio* (Zv4; [20]), and *Tetraodon nigroviridis* (v6.0; [21]) genomes using BLAST to identify the region and Genescan to improve the gene predictions [22]. CYP1C sequences from carp (*Cyprinus carpio*) were provided by T. Itakura (personal communication).

Phylogenetic trees were constructed using Bayesian methods after masking ambiguous positions in the alignment (452 characters used out of 571). Trees were rooted using known CYP2 genes (see Table 6 in Supplemental Information for accession numbers). Bayesian phylogenies were constructed with MrBayes 3.0 [23]. Briefly, differential heated Metropolis-coupled Monte Carlo Markov chains (MC^3) using the JTT model of amino acid substitution [24] and prior uniform gamma distributions approximated with four categories were run for 3 000 000 generations and sampled every 100^{th} generation. Analysis of the MC^3 parameter output using BOA 1.0 [25] indicated that this degree of sampling was sufficient to avoid sampling autocorrelation. Four parallel, differentially heated Markov chains were used to sample a larger tree space, and in order to confirm the Bayesian results, three independent, randomly seeded analyses of the data set were performed with identical results. Calculations of the MC^3 burn-in values using BOA confirmed the burn-in estimates (conservatively set at 200 generations) derived from the rough parameter plots produced by MrBayes. The analysis of functional divergence was performed using the likelihood ratio method of Knudsen and Miyamoto [26], which identifies amino acid positions within assigned subfamilies that exhibit substitution rates that are faster or slower than the substitution rates observed in the subfamily as a whole.

RESULTS

Isolation of CYP1B-like Sequences from Scup

Degenerate inosine-containing primers were designed in regions where human, rat, and mouse CYP1B1 share a high percentage of nucleotide identity and where there is a low percentage of nucleotide identity with their respective CYP1A1 sequences. mRNA from scup tissues including liver and head kidney was screened with all possible

combinations of the primers (Supplemental Table 1). The same primers also amplified fragments from scup testis, spleen, heart, gill, kidney, gut, eye, and muscle (data not shown). The brightest bands were obtained with liver mRNA and consequently, this organ was chosen as the source of RNA for the rest of the cloning experiments. Upon sequencing, a BLAST search against all Genbank sequences identified these bands as fragments of a CYP1B-like gene.

Identification of Two Novel CYP1 Genes

A library of adaptor-ligated ds cDNA was generated from scup liver, using tissue from the same animal from which the internal CYP1B-like fragments had been isolated. The complete 3' end of the ORF as well as more than 400 bp of 3'UTR sequence were obtained by 5' and 3' RACE reactions using nested combinations of the primers (listed in Supplemental Information). The 5'RACE reactions generated bands corresponding to two similar yet distinct sequences. Nested RACE reactions generated a full length ORF that was initially classified as a CYP1B gene, based on preliminary phylogenetic analyses (data not shown). A total of 9 and 13 partial clones were sequenced for the two new scup CYP1B-like genes, respectively. The presence of two distinct and novel CYP1 genes in scup was confirmed by sequencing the two full length ORFs, using specific primers designed in the 5' and 3' ends of the two genes.

Phylogenetic analysis of the two full-length sequences confirmed the two novel scup genes to be part of the CYP1 family, distinct from both mammalian and fish CYP1As and sufficiently different from CYP1B genes to warrant consideration of establishing a new subfamily. As genomic information became available in other fish, homologues of the scup sequences were identified in two *Tetraodontiformes* species

(*Takifugu rubripes* [19] and *Tetraodon nigroviridis* [21]). With this information, the two scup genes were recognized as sufficiently distinct as to constitute a new subfamily, CYP1C, and were named CYP1C1 and CYP1C2. The phylogenetic relationship of the CYP1Cs to other CYP1s is shown in Figure 1.

Cloning of CYP1s from Danio rerio

The full length CYP1B1 from *Danio rerio* reported here was originally cloned as part of an effort to examine the expression of CYP1 genes in zebrafish. Subsequent to the discovery of the new CYP1Cs in scup, two zebrafish CYP1Cs were identified in the preliminary zebrafish genome assembly (Zv4; [20]). One of these corresponding genes in zebrafish was cloned and sequenced, and is orthologous to the scup CYP1C1 (Figure 1). CYP1B1, CYP1C1 and CYP1C2 are in the current zebrafish genome assembly (Zv4), but have not been assembled into a supercontig or linkage group.

Sequence Comparisons for Structural and Functional Inferences

Amino acid identities between CYP1C1 and CYP1C2 and other members of the CYP1 family are shown in Table 1. Full length scup CYP1C1 and CYP1C2 share 80-81% nt and a.a. identity with each other and 71-75% nt and 75-86% a.a. identity with other fish CYP1Cs, falling to 48-49% nt and 36-39% a.a identity with fish and mammalian CYP1As. The zebrafish CYP1C genes are similarly divergent from the CYP1B1 and CYP1A subfamilies (see Table 1). The 44-50% a.a. identity between the CYP1Cs and any CYP1Bs is at the lower end of the percentage amino acid identity for genes to be classified to the same subfamily [15], and that this difference occurs within a species is consistent with the classification of the CYP1Cs in a distinct subfamily.

Scup CYP1C1 and CYP1C2 code sequences of 525 and 524 a.a. respectively, shorter than the mammalian CYP1Bs by 8-19 a.a. Zebrafish CYP1C1 and CYP1C2, respectively, code for 523 and 514 amino acids. Sequences are shown aligned in Figure 2 along with regions of interest for structural and functional inferences. The highly conserved P450 fingerprint consensus sequence F(G/S)xGx(H/R)xCxGxx(I/L/F)A [27], is identical between the two scup CYP1Cs and shares 64% identity with mammalian CYP1Bs. Figure 2 identifies the six putative substrate recognition sites in CYP1C1 and CYP1C2, as inferred by sequence alignment with the CYP2 genes used by Gotoh [28].

Expression of CYP1 Genes in Scup Liver and Head Kidney

Relative expression of the CYP1A and CYP1C genes and β-actin was measured in liver and head kidney of three untreated male scup (Figure 3). The liver was chosen for its prominent role in xenobiotic and endogenous substrate (e.g. steroid) metabolism by CYPs. The head-kidney was chosen for its potential capacity for xenobiotic metabolism [29] and for its adrenal-like physiological role in fish [30]; CYP1B1 is expressed in mammalian adrenal gland [31]. CYP1 expression levels were normalized to β-actin data, which remained relatively constant across all samples (data not shown). In general, CYP1 expression levels were higher in liver than in head kidney; this difference was most pronounced for CYP1A. CYP1C2 expression was lower than CYP1A or CYP1C1 expression in liver. The same trend was evident in head kidney.

DISCUSSION

Identification of a new CYP1 subfamily, the CYP1Cs, expands the diversity of CYP1 genes and presents an opportunity to increase our understanding of the

physiological and toxicological significance of members of this CYP gene family. The involvement of CYP1A and CYP1B enzymes in the metabolism and activation of numerous environmental carcinogens is well known [3, 32]. Endogenous substrates are also increasingly being identified for these enzymes, including compounds such as heme oxidation products, estradiol, retinoids and arachidonic acid [33-39]. Knowledge of the distribution of catalytic functions among the CYP1 enzymes in early diverging vertebrates could help to elucidate further the breadth of CYP1 activities and perhaps point to the physiological role and regulation of CYP1 genes in vertebrates in general.

Classification of P450 genes follows a general rule including: (1) that P450 sequences should have >40% a.a. identity to be classified within the same family, and (2) that (mammalian) P450s with >55% a.a. identity are classified in the same subfamily [15]. The increasing number of new P450 sequences from lower vertebrates, invertebrates, and prokaryotes has made classification more intricate, and the cut-off for genes belonging to the same subfamily was lowered to >46% a.a identity to accommodate evolutionary distance [15]. The classification of genes as orthologous requires a conclusive amount of data that can include sequence analyses and genomic characteristics (number of exons, intron/exon boundaries) as well as information on gene regulation, enzymatic function, and immunological cross-reactivities. The difficulty in determining orthologous relationships is compounded in gene families where divergence has led to numerous subfamilies and divergence of genes in a subfamily occurs independently within a taxon, as well as where there is gene conversion. Generally, there is difficulty encountered when available information on each member is limited [40, 41].

The classification of these new genes within the CYP1 family has been spared some difficulty, as the single CYP1B gene in plaice was clearly much more closely related to the mammalian CYP1B1s than were the two CYP1B-related (now CYP1C) sequences in scup. The two scup genes share 44-48% and 36-39% a.a. identity with mammalian CYP1Bs and CYP1As, respectively, and clearly belong to the CYP1 family based on sequence and phylogenetic analysis (Figure 1). Thus, despite the distinctions, the scup sequences initially were classified as CYP1B2 and CYP1B3. When the *Takifugu* genome became available, that fish genome contained a close homologue (orthologue) of mammalian and plaice CYP1B1, as well as apparent orthologues of the two novel CYP1 sequences in scup, consistent with classifying these later sequences to a different subfamily, CYP1C, and establishing the scup sequences reported here as the type sequences for the CYP1Cs. The cloning of both a CYP1B1 and a CYP1C from zebrafish supports the distinction between these subfamilies.

Sequences homologous to the CYP1Cs have been derived from genome projects including the zebrafish (*Danio rerio*), and two fugu genomes (*Takifugu rubripes* and *Tetraodon nigroviridis*). These genomic sequences will require cloning to confirm their expression and sequences. However, our cloning results and evidence from EST studies indicates that CYP1Cs are expressed in zebrafish, killifish (*Fundulus heteroclitus*), salmon (*Salmo salar*), and stickleback (*Gasterosteus aculeatus*) [42]. Moreover, our results indicate that CYP1Cs are expressed in a number of organs, and others have detected CYP1C expression in gill (El Kady and Itakura, personal communication); CYP1B1 also is highly expressed in gill tissue of some fish [17]. 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. We have not been able to identify a CYP1C in any mammalian genome thus far (*Homo sapiens, Mus musculus, Rattus norvegicus, Pan troglodytes, Bos taurus* and *Canis familiaris*).

Sequence characteristics

Based on our alignment of the two CYP1Cs and the identification of regions of interest for structural and functional inferences, both enzymes are likely to have catalytic activities that are different from other CYP1 enzymes, and are likely to be slightly different from each other. A likelihood ratio test for functional divergence [26] identified a number of sites throughout both proteins that exhibit substitution rates that are faster than the average of the protein as whole, indicating that these sites are either unnecessary for the function of the enzyme or play a role in the evolving function of these proteins. Significant rate differences between the two paralogous genes CYP1C1 and CYP1C2 means that the function of these sites are probably different between the two genes.

Substrate recognition sites (SRS), described in the CYP2 proteins by Gotoh [28], were identified as six areas likely to or known to contain substrate-binding residues inferred by alignment with and comparisons to bacterial CYPs. The six SRS may differ in their relative importance among CYPs but are likely to correspond to regions containing substrate-contacting residues in most CYP genes. Based on the analysis of functional divergence (see Figure 2), it is likely that SRS1, SRS3, and SRS4 contribute the most to any differences in substrate recognition, as these three SRS regions include both the largest number of absolute amino acid differences and the sites with the most

significant substitution rate differences between CYP1C1 and CYP1C2. SRS4 is composed of the threonine-rich center of the I helix, which interacts with the heme-complexed oxygen [43, 44]. The I-helix contains a conserved threonine residue (at position 330 in Figure 2) preceded by two a.a. that play a role in substrate specificity [45]. The two members of the subfamily contain residues with very different chemical properties in the first of these positions (position 328 in Figure 2): glutamine (polar) versus leucine (aliphatic). This observation suggests that these two enzymes may have slightly different substrate specificity. Of note, the CYP1C1s share this glutamine residue with the CYP1B1s, although the preceding residue (position 327) is a serine in the CYP1B1s rather than a glycine, as in all of the CYP1Cs.

Substitution rate differences within SRS2 and SRS6 between the CYP1Cs and mammalian and fish CYP1B1 sequences suggest that these two regions may play a role in differentiating the substrate specificities of both the CYP1Bs and the CYP1Cs. SRS1 and SRS4 exhibit striking patterns of slowed substitution rates relative to the gene set as a whole (see Supplemental Information), suggesting that these two regions may play a role in determining a broader type of substrate or cofactor recognition that is a general feature of both the CYP1B and CYP1C subfamilies.

The regulation of CYP1C genes is likely to share some features with the other CYP1 subfamilies. Both CYP1C1 and CYP1C2 were expressed in liver and in head kidney, a teleost counterpart to the adrenal, where CYP1B1 is highly expressed in mammals. Expression of the two scup CYP1Cs in other organs warrants further study: distinct expression patterns of the two CYP1Cs in multiple organs would suggest distinct physiological roles. Furthermore, at least one canonical xenobiotic response element

(XRE; also know as dioxin response element, DRE) can be found in the region upstream of each zebrafish CYP1C gene (-452 and -567 bp relative to translation start for CYP1C1 and CYP1C2, respectively).

The presence of two CYP1Cs in fish reflects a gene duplication event, although the phylogenetic origin of this event is yet to be determined. A duplication event is not likely specific to the *Perciformes*, as the occurrence of CYP1Cs in fugu indicates a broader distribution within the fishes. The presence of *both* CYP1Cs in carp [46] and in zebrafish extends the gene duplication event to at least the early Cretaceous (100-125 Ma; [47, 48]). Only one CYP1C gene has been found thus far in the Japanese eel, *Anguilla japonica* [46], suggesting that the CYP1C subfamily in fish originated at least as early as the late Jurassic (ca 150 Ma; [47, 48]). The *Anguilla* CYP1C gene clusters within the CYP1C1 subclade, rather than basal to the overall CYP1C clade, indicating that either *Anguilla* has lost an expressed CYP1C2, or that the gene remains to be cloned. Identification and sequencing of CYP1C genes in other fish as well as in species that evolved prior to and subsequent to the fish/tetrapod branching will be necessary to date the origin of the CYP1C subfamily, as well as the CYP1C duplication. Interestingly, there are CYP1B/1C related genes in the genomes of the urochordates *Ciona intestinalis* and *Ciona savignyi.*, suggesting that the CYP1B/1C line extends back much further than the origin of the *Vertebrata* (Goldstone *et al.* unpublished data).

Substrates of the teleost CYP1Cs have yet to be determined. Analysis of catalytic activities of expressed CYP1Cs should shed light on whether the teleost CYP1Cs are functionally more like the CYP1Bs than the CYP1As, or possess different activites.

The study of P450 genes structure provides information necessary for drawing structural-functional inferences as well as insights on the evolution of this complex superfamily of enzymes. In these respects, the structural and functional analysis of the CYP1C genes in fish, the largest and earliest diverging vertebrate group, should provide information essential in aquatic toxicology, and could illuminate the diversity of roles of the CYP1 gene family. Likewise, studies are needed to address the potential influence of sex and sexual maturity on the expression of the scup CYP1Cs, and to determine the possible regulation by AHR agonists, as compared to other members of the mammalian and fish CYP1 family. We believe such studies of multiple CYP1Cs in fish may provide an evolutionary perspective and insight into the endogenous and toxicological significance of the CYP1 family.

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Figure 1. Phylogenetic tree showing the relationship between the CYP1A, CYP1B and CYP1C subfamilies. Bayesian MC^3 and maximum parsimony analyses retrieved identical topologies. Trees were monophyletically rooted with known CYP2 genes. Values shown at branch points are Bayesian posterior probabilities resulting from 3 000 000 generations of MC^3. See Supplemental Information for a table of Accession Numbers.

Figure 2. Amino acid alignment of scup, zebrafish, and fugu CYP1C1 and CYP1C2. Grey shading indicates amino acid differences. Sites with blue or red shading indicate positions that exhibit slower or faster substitution rates than the average for all positions, respectively, determined using the likelihood ratio test of Knudsen and Miyamoto (see text). Positions with both blue and red highlighting indicate a significant rate difference exists between the two subfamilies ($p<0.05$). The positions of the substrate recognition sites (SRS) from Gotoh (1992) are indicated with dashed lines.

Figure 3. Expression levels of CYP1A, CYP1C1, and CYP1C2 in liver and head kidney normalized to β-actin as determined by RTPCR (error bars=1σ, n=3). CYP expression tended to be greater in liver than in head kidney (Tukey's HSD, $p<0.01$), and CYP1C2 was expressed at a significantly lower level than CYP1A or CYP1C1 (Tukey's HSD, $p<0.01$).

Table 1. Percent amino acid identities. CYP1Cs fall within the CYP1B/1C subclade cutoff value of 40% but are distinct from the CYP1A1/1A2 clade.

Table 1

Table 1. Percent amino acid identities.

	STENOT_1C1	STENOT_1C2	DANIO_1C1	CYPRIN_1C1	FUGU_1C1	ANGUIL_1C1	FUGU_1C2	DANIO_1C2	CYPRIN_1C2	FUGU_1B1	PLEURO_1B	DANIO_1B	RATTUS_1B1	MUS_1B1	HOMO_1B1	HOMO_1A1	MUS_1A1	HOMO_1A2	MUS_1A2	PLEURO_1A	DANIO_1A	STENOT_1A
STENOT_1C1	100%	80%	78%	80%	86%	82%	74%	75%	74%	50%	48%	49%	46%	47%	49%	37%	37%	36%	37%	38%	38%	37%
STENOT_1C2		100%	74%	75%	76%	78%	83%	77%	76%	48%	47%	50%	45%	46%	47%	38%	39%	37%	39%	38%	37%	37%
DANIO_1C1			100%	86%	77%	79%	71%	76%	74%	49%	48%	48%	47%	46%	48%	38%	38%	37%	37%	37%	37%	36%
CYPRIN_1C1				100%	77%	82%	72%	77%	77%	49%	48%	50%	47%	47%	49%	39%	38%	37%	37%	38%	37%	37%
FUGU_1C1					100%	80%	73%	71%	71%	50%	47%	49%	47%	48%	50%	38%	39%	37%	38%	39%	39%	39%
ANGUIL_1C1						100%	74%	75%	76%	50%	49%	50%	48%	48%	48%	38%	38%	36%	38%	38%	38%	38%
FUGU_1C2							100%	73%	72%	48%	47%	49%	45%	45%	46%	37%	37%	35%	36%	38%	36%	36%
DANIO_1C2								100%	88%	49%	47%	50%	46%	46%	47%	38%	39%	37%	38%	37%	37%	36%
CYPRIN_1C2									100%	47%	45%	49%	45%	45%	46%	38%	37%	37%	37%	37%	35%	35%
FUGU_1B1										100%	72%	63%	50%	50%	52%	38%	38%	38%	37%	38%	37%	36%
PLEURO_1B											100%	62%	47%	47%	49%	38%	38%	38%	38%	38%	38%	38%
DANIO_1B												100%	51%	52%	54%	38%	38%	36%	37%	39%	39%	38%
RATTUS_1B1													100%	93%	80%	38%	38%	38%	37%	37%	38%	38%
MUS_1B1														100%	81%	39%	39%	39%	37%	38%	38%	38%
HOMO_1B1															100%	39%	39%	37%	37%	37%	38%	38%
HOMO_1A1																100%	80%	72%	67%	57%	56%	57%
MUS_1A1																	100%	67%	71%	57%	58%	58%
HOMO_1A2																		100%	72%	53%	53%	54%
MUS_1A2																			100%	50%	50%	52%
PLEURO_1A																				100%	74%	85%
DANIO_1A																					100%	76%
STENOT_1A																						100%

Figure 1

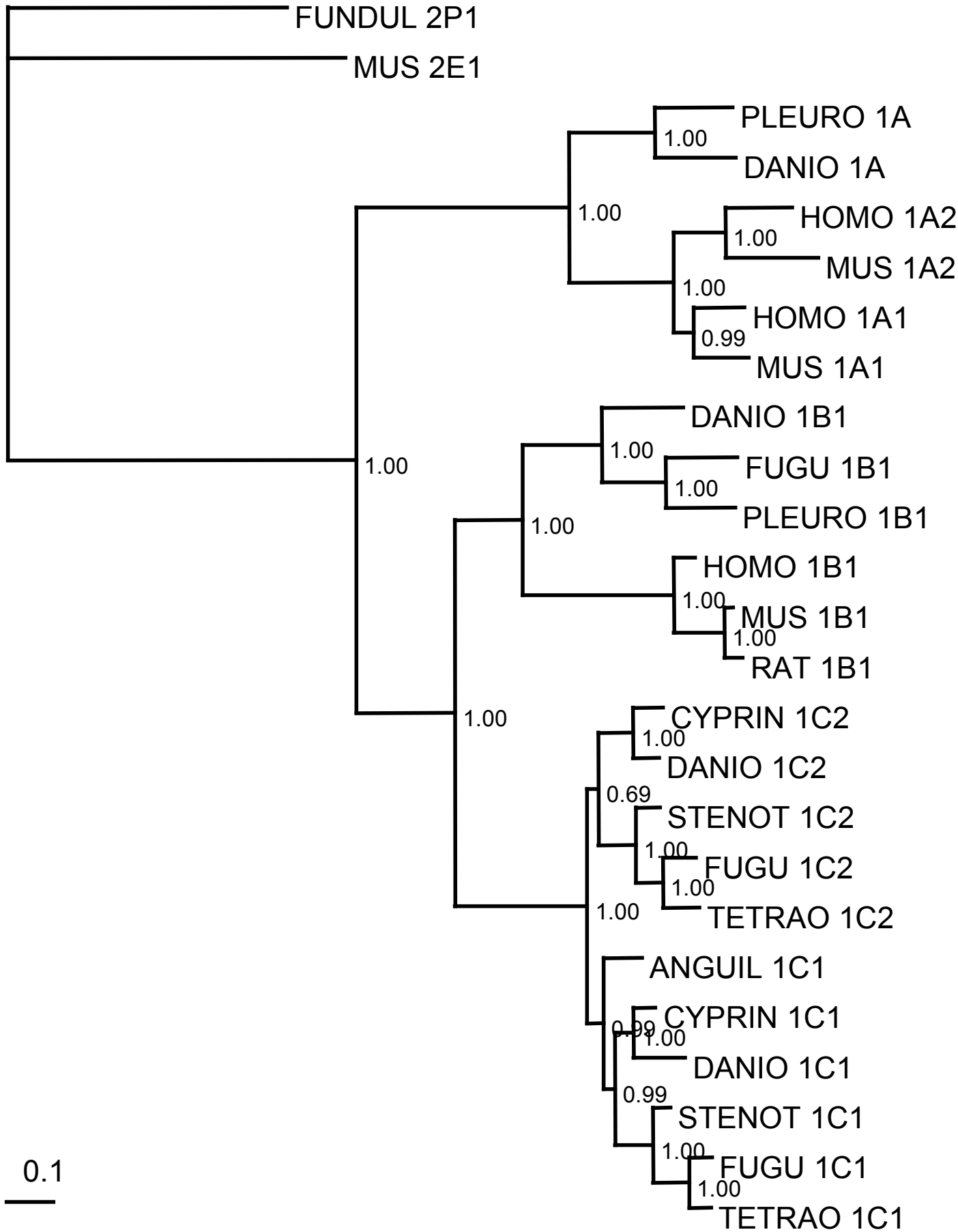
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Figure 2

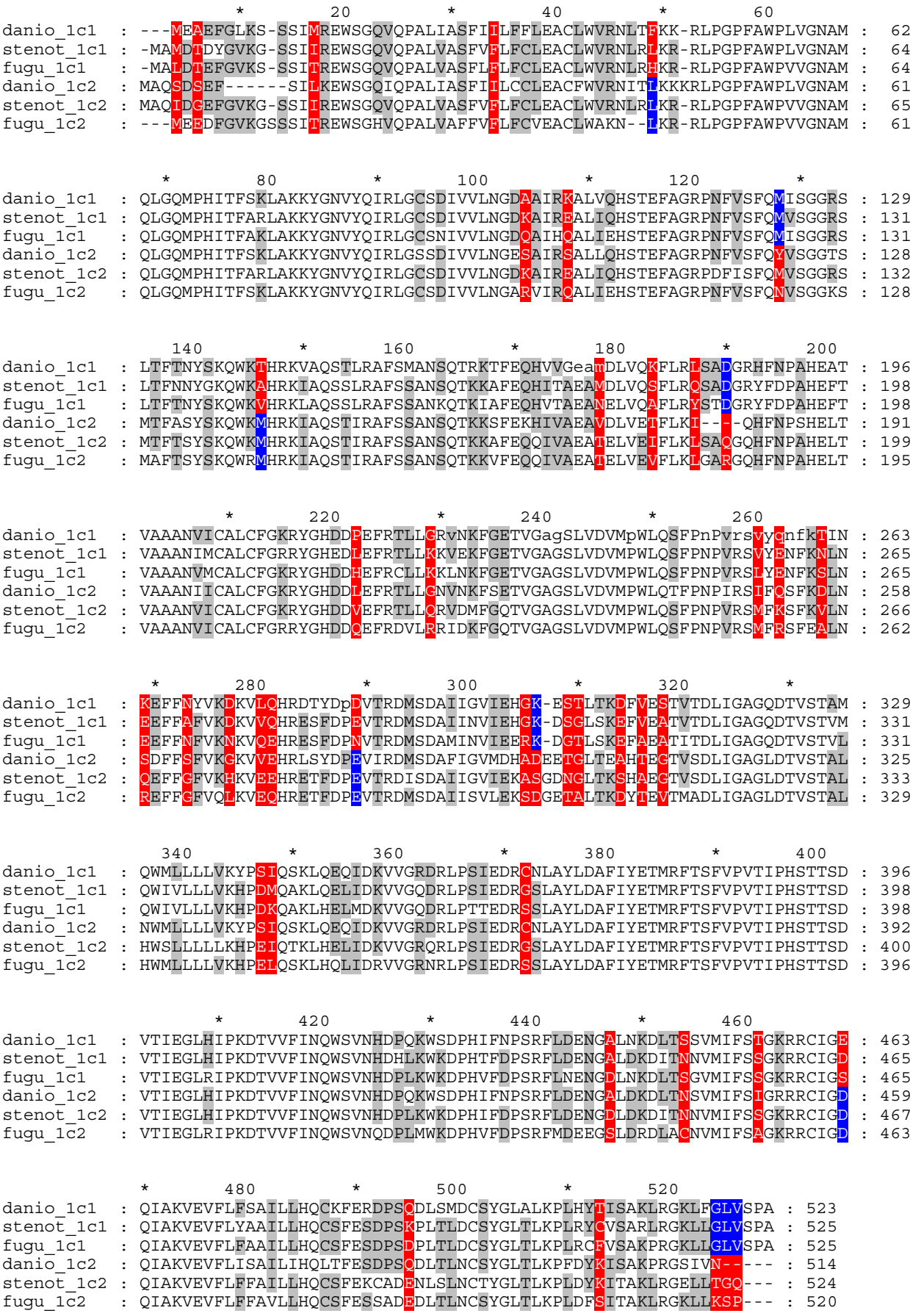
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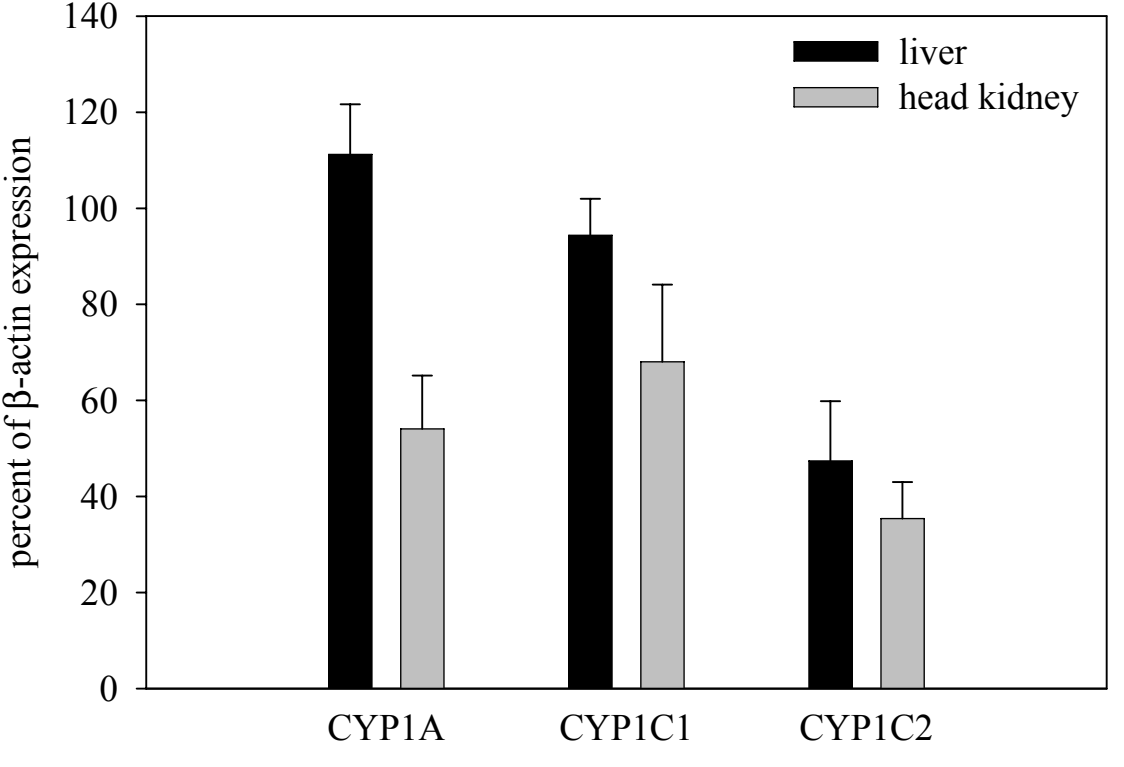
Figure 3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liver
head kidney
CYP1A
CYP1C1
CYP1C2

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