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Nine co-localized cytochrome P450 genes of the *CYP2N*, *CYP2AD*, and *CYP2P* gene families in the mangrove killifish *Kryptolebias marmoratus* genome: Identification and expression in response to B[α]P, BPA, OP, and NP

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ABSTRACT

The *CYP2* genes are the largest and most diverse cytochrome P450 (CYP) subfamily in vertebrates. We have identified nine co-localized *CYP2* genes (~55 kb) in a new cluster in the genome of the highly resilient ecotoxicological fish model *Kryptolebias marmoratus*. Molecular characterization, temporal and tissue-specific expression pattern, and response to xenobiotics of these genes were examined. The *CYP2* gene clusters were characterized and designated *CYP2N22-23*, *CYP2AD12*, and *CYP2P16-20*. Gene synteny analysis confirmed that the cluster in *K. marmoratus* is similar to that found in other teleost fishes, including zebrafish. A gene duplication event with diverged catalytic function was observed in *CYP2AD12*. Moreover, a high level of divergence in expression was observed among the co-localized genes. Phylogeny of the cluster suggested an orthologous relationship with similar genes in zebrafish and Japanese medaka. Gene expression analysis showed that *CYP2P19* and *CYP2N20* were consecutively expressed throughout embryonic development, whereas *CYP2P18* was expressed in all adult tissues, suggesting that members of each CYP2 gene family have different physiological roles even though they are located in the same cluster. Among endocrine-disrupting chemicals (EDCs), benzo[α]pyrene (B[α]P) induced expression of *CYP2N23*, bisphenol A (BPA) induced *CYP2P18* and *CYP2P19*, and 4-octylphenol (OP) induced *CYP2AD12*, but there was no significant response to 4-nonylphenol (NP), implying differential catalytic roles of the enzyme. In this paper, we identify and characterize a *CYP2* gene cluster in the mangrove killifish *K. marmoratus* with differing catalytic roles toward EDCs. Our findings provide insights on the roles of nine co-localized CYP2 genes and their catalytic functions for better understanding of chemical-biological interactions in fish.

1. Introduction

Cytochrome P450 (CYP) represents a large gene superfamily that catalyzes oxidoreductive and peroxidative reactions with a vast array of endogenous and exogenous compounds in vertebrates (Nelson et al., 1997; Nelson et al., 2013). *CYPs* are considered the most divergent eukaryotic gene families, exhibiting functional heterogeneity within and between species (Nelson et al., 2013). Among 18 *CYP* gene families in vertebrates, the *CYP2* family is the largest and most complex; to date, over 40 *CYP2* subfamilies and hundreds of *CYP2* genes have been reported (Kubota et al., 2013; Genome Reference Consortium, 2016). Of the CYP2 family, the CYP2N, CYP2AD, and CYP2P subfamilies are diverse enzymes with overlapping expression and that catalyze the NADPH-dependent oxidation of fatty acids in the extra-hepatic tissues of many species including teleost fish (Oleksiak et al., 2000; Saad et al., 2016). In the zebrafish, *CYP2N*, *CYP2AD*, and *CYP2P* genes are co-located on chromosome 20 and show similar synteny to human *CYP2J2,* which is responsible for the oxidation of arachidonic acid to x-terminal alcohols, *cis*-epoxyeicosatrienoic acids (EETs), and mid-chain *cis-trans* conjugated dienols (HETEs) (Qu et al., 2001; Ma et al., 2002; Wang

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et al., 2007; Saad et al., 2016). These catalytic products (e.g., EETs) control critical biological functions including the regulatory role of peptide hormones in the pancreas, pituitary, and hypothalamus (Falck et al., 1983). However, the biological functions of the entire co-localized *CYP2* subfamily gene cluster with respect to xenobiotics has not yet elucidated in animals, including fish. Understanding the regulatory role of *CYP2* genes is critical because of their importance in the transformation of pharmacological, toxicological, and endogenous substrates (Kubota et al., 2013).

To date, several *CYP2* family members have been identified from zebrafish, rat, mice, rabbit, cow, and human and were shown to have different expression patterns in brain, heart, lung, liver, intestine, kidney, ovary, and testis in addition to diverse functions (reviewed in Wang et al., 2007). In the killifish *Fundulus heteroclitus*, the CYP2N subfamily was identified, characterized, and confirmed to function in CYP-derived arachidonate metabolism, similar to the role in mammals (Oleksiak et al., 2000). However, the types of *CYP2* subfamilies are different among teleost fish even though they are co-located on the same chromosome. For example, *CYP2V, CYP2AA*, and *CYP2AD* subfamilies were not identified in Japanese medaka and stickleback but were reported in zebrafish (Nelson, 2009; Kirischian et al., 2011). However, there are no reports on the CYP2 subfamilies of *CYP2Ns, CYP2ADs,* and *CYP2Ps* and no experimental evidence for xenobiotic-induced expression with a focus on ecotoxicological perspectives. As an ecotoxicology model, the physiological and molecular response to different xenobiotics belonging to the group of endocrine disrupting chemicals (EDCs), such as benzopyrene (B[α]P), bisphenol A (BPA), 4-octylphenol (OP), and 4-nonylphenol (NP), have been studied in the self-fertilizing mangrove killifish *Kryptolebias marmoratus* (Rhee et al., 2009; Kim et al., 2016). Thus, characterization of the co-localized *CYP2* cluster gene from *K. marmoratus* and analysis of gene expression in response to EDCs will provide insight into the biological importance of these co-localized *CYP2* members in fish.

K. marmoratus (previously *Rivulus marmoratus*) is an oviparous teleost that is used for diverse laboratory studies because of its small size (3–5 cm), short life cycle (12–16 weeks), and ease of maintenance in aquaria (Lee et al., 2008; Kim et al., 2016). As an androdioecious species, more than 60% of the hermaphrodites transform into secondary males within 3–4 years depending on environmental factors (e.g., temperature), which have a profound impact on determining the sex ratio in this species (Harrington, 1967). Combined with the literary evidence and available whole-genome data (Rhee and Lee, 2014; Kelley et al., 2016; Rhee et al., 2017), it is apparent that *K. marmoratus* represents a potential platform for assessing the harmful impact of diverse chemicals in the aquatic environment. Thus, elucidating the molecular interactions of co-localized *CYP2* cluster genes with EDCs will provide knowledge on their possible involvement in xenobiotic-induced signaling mechanisms that could lead to new directions in *CYP2* gene research.

This study focuses on the identification of nine co-localized *CYP2* cluster genes (*Km-CYP2*) in scaffold 9 of the *K. marmoratus* genome together with synteny analysis and evaluation of the phylogenetic relationship with Japanese medaka *Oryzias latipes*, pufferfish *Takifugu rubripes*, stickleback *Gasterosteus aculeatus*, and zebrafish *Danio Rerio* (outgroup). We also investigated the spatiotemporal expression pattern of the nine *Km-CYP2* genes and their interactions with EDCs (B[α]P, BPA, OP, and NP). Overall, this work provides new insights into the molecular interaction of EDCs with the *Km-CYP2N, Km-CYP2AD*, and *Km-CYP2P* members of the CYP2 family with the future perspective of potential applications in toxicogenomics in response to EDCs.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used for this study were of molecular biology grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), unless specifically stated otherwise. Animal experiments were performed according to guidelines of the Institutional Animal Care and Use Committee of the Animal and Plant Quarantine Agency (South Korea).

2.2. Fish rearing condition

The mangrove killifish *K. marmoratus* was reared at the aquarium facility at the Department of Biological Science, Sungkyunkwan University (Suwon, South Korea). Fish were maintained in 20-L glass tanks containing artificial seawater (ASW) (TetramMarine, Cincinnati, OH, USA) with confined environmental conditions of 25 ± 1 °C, 14 h/10 h light/darkness, 15 ppt salinity, and pH 8.0, controlled through an automated flow-through system. Each tank in the system housed 40 fish larvae that were maintained on a diet of brine shrimp *Artemia franciscan* provided twice a day.

2.3. Genome-wide identification of the Km-CYP2 gene cluster

The sequences of the *Km-CYP2* gene cluster in scaffold no. 9 were identified from the whole-genome database of *K. marmoratus* (Rhee et al., 2017; total scaffold no. 3072; total length, 680 Mb; N50 value, 2.2 Mb) using similar gene sequences from zebrafish as a query (*D. rerio*). The coding sequences were subjected to BLAST analysis to confirm sequence similarities. To obtain the sequences of all *Km-CYP2* cluster genes, a local BLAST search (BLASTp) was conducted with the fully assembled transcripts against the non-redundant (NR) database of the NCBI. BLASTp hits with an *E*-value $\leq 10^{-5}$ were considered significant. To obtain InterPro IDs from the fully assembled transcripts for additional GO annotations, a local InterProScan on Linux x64 (Ver. 5.8−49.0, https://code.google.com/p/interproscan/) was conducted with PROSITE, PRINTS, Gene3D, PfamA, and SuperFamily analyses. The results from a local BLAST search were mapped and annotated with GO terms using the automated annotation suite Blast2gocli. InterPro IDs were mapped to GO terms and merged with blast-derived GO annotations using Blast2go-cli. Further confirmation was achieved by polymerase chain reaction (PCR) amplification and sequencing. Gene-level synteny of the *Km-CYP2* gene cluster was compared with that of Japanese medaka (*O. latipes*), pufferfish (*T. rubripes*), stickleback (*G. aculeatus*), and zebrafish (*D. rerio*) collected from the published chromosome assembly information with further identification. The *Km-CYP* genes were kindly annotated by Prof. David R. Nelson (University of Tennessee Health Science Center) and Dr. Gared V. Goldstone (Woods Hole Oceanographic Institution).

2.4. Phylogenetic analysis

Phylogenetic analysis of the translated nine Km-CYP2 products was performed. The entire amino acid sequences of CYP2 cluster genes of *D. rerio* (DrCYP2) and *O. latipes* (OlCYP2) were retrieved from GenBank (Suppl. Table S1). Multiple alignments of deduced amino acid sequences of nine Km-CYP2s with those of DrCYP2 and OlCYP2 were performed using Clustal X 1.83 software (Thompson et al., 1997). Amino acid sequences from *K. mamoratus*, *O. latipes*, and *D. rerio* were aligned using MEGA software (ver. 6.0; Center for Evolutionary Medicine and Informatics, Tempe, AZ, USA). To establish a best-fit substitution model for phylogenetic analysis, a model showing the lowest score in the Bayesian Information Criterion (BIC; Schwarz, 1978) and the Akaike Information Criterion (AICc; Hurvich and Tsai, 1989; Posada and Buckley, 2004) was determined by maximum likelihood (ML) analysis. According to the results of the model test, the LG + G + I model was chosen to generate a phylogenetic tree using MEGA software. For phylogenetic analysis, full-length protein sequences were aligned, and a phylogenetic tree was obtained as described above with an additional bootstrapping test (1000 replicates)