Short Communication

Characterization and sexual dimorphic expression of Cytochrome P450 genes in the hypothalamic–pituitary–gonad axis of yellow catfish

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\textbf{Abstract}

Yellow catfish (Pelteobagrus fulvidraco) is an important freshwater fish species in China. In particular, an all-male population has been commercially produced for the males grow faster than females. However, the molecular mechanisms underlying sexual dimorphism of body size and sex differentiation are still unclear in yellow catfish. This study attempts to characterize and analyze the expression of Cytochrome P450 (CYP) family members that have been shown to play an important role in sex differentiation and metabolism in teleosts. A total of 25 CYP genes were identified from our transcriptomes by de novo sequencing and Solexa sequencing, including 17 genes with complete open reading frame (ORF). Phylogenetic analyses were conducted to compare these genes with their counterparts from other teleosts. In the tissues of hypothalamic–pituitary–gonad (HPG) axis, most of the genes were expressed at uniform level in both sexes. However, multiple CYP genes displayed sexual dimorphic expression, such as \textit{cyp2ad2}, \textit{cyp4b}, \textit{cyp8a}, \textit{cyp11b2}, \textit{cyp17a} and \textit{cyp27a} expressed at higher level in testis than in ovary, whereas \textit{cyp2g}, \textit{cyp7a}, \textit{cyp8b}, \textit{cyp19a1a} and \textit{cyp26a} expressed at higher level in ovary than in testis. The expression response of six CYP genes in ovary was also assessed after 17α-methyltestosterone (MT) treatment. Testis-biased expressed \textit{cyp11b2} and \textit{cyp17a} were significantly up-regulated, while \textit{cyp11a} and \textit{cyp19a1a} were reduced in ovary after MT treatment. Our work is helpful for understanding molecular evolution of CYP genes in vertebrates and the mechanism of sexual dimorphism in teleosts.

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\section*{1. Introduction}

The Cytochrome P450 (CYP) super-family genes encode multiple enzymes with diverse functions in vertebrates. They usually synthesize or metabolize endogenous molecules and interact with exogenous chemicals from the diets or environments (Baldwin et al., 2009). Recently, CYP family members are also reported as essential factors for sex differentiation. \textit{Cyp19a1a}, a gene that is highly expressed in ovaries and aromatizes androgens into estrogens, plays a conserved role for ovary development in teleosts (Heule et al., 2014; Huang et al., 2009). The sexual dimorphic expression of \textit{Cyp19a1a}, \textit{Cyp1a1}, \textit{Cyp2e1}, and \textit{Cyp7b1} has been partially explained by different patterns of epigenetic modifications (Penaloza et al., 2014; Zhang et al., 2013), although some microRNAs are potentially correlated with the female-biased expression of \textit{Cyp26b1} via post-transcriptional regulation (Xie et al., 2013). Transcriptional factors \textit{Sf1} and \textit{Sox9} are co-expressed with \textit{Cyp26b1} in Sertoli cells and up-regulate its expression to ensure the male fate of germ cells, whereas \textit{Cyp26b1} and \textit{Sf1} are co-expressed in Leydig cells. The FOXL2 antagonizes \textit{Cyp26b1} expression in ovaries (Kashimada et al., 2011). Male-biased expression of \textit{Cyp2CT1} in adult rats is irreversibly imprinted shortly after birth that was likely resulted by growth hormone other than the usual testosterone (Das et al., 2014). \textit{Cyp17}, an enzyme for androgens and estrogens synthesis in teleosts (Attard et al., 2008), plays a key role for ovarian development in \textit{Cynoglossus semilaevis} (Chen et al., 2010). Sex-specific expression of \textit{cyp19a1a} in \textit{XX} gonad at 5 dah and \textit{cyp11b2} in \textit{XY} gonad from 35 dah was observed during early gonad differentiation in Nile tilapia (Jiiri et al., 2008). However, the regulation and function of CYPs in sexual dimorphism are still unclear.

Sexual dimorphism is widely defined as the morphological, physiological and behavioral differences between individuals of...
different sexes in the same species (Desjardins and Fernald, 2009; Leinonen et al., 2011). Sex dimorphic growth pattern and male priority phenomenon have been observed in yellow catfish, and an all-male population has been produced in this fish by crossing YY super-male and XX female (Dan et al., 2013; Gui and Zhu, 2012; Wang et al., 2009). However, the exact molecular mechanism for sexual dimorphism remains unclear. Yellow catfish will provide a promising research model to study candidate genes and possible pathways responsible for sexual dimorphism and sex differentiation (Chen et al., 2014; Jing et al., 2014; Mei and Gui, 2015).

Here, we reported the identification and characterization of yellow catfish CYP genes and investigated their phylogenetic relationships to other vertebrates. We also examined the sexual dimorphic expression pattern of CYPs in hypothalamus-pituitary-gonad axis of yellow catfish. Our study will provide a clue to reveal molecular mechanism of sexual dimorphism and sex differentiation in teleosts.

2. Materials and methods

2.1. Experimental animals and hormone treatment

One-year-old sexually mature yellow catfish individuals with similar size were collected from our breeding center at Jiangzhou, Hubei province, China. All fish were acclimated in the laboratory facility for one week, the water was aerated and temperature was maintained at 26 °C. The genetic sex of experimental fish was determined as described previously (Dan et al., 2013). Three kinds of tissues including pituitary, hypothalamus and gonads of each sex were collected from 3 individuals. The experimental operations were conducted as the requirement of the institution animal care and use committee of Huazhong Agricultural University.

For the hormone treatment experiment, healthy female individuals were injected with 17α-methyltestosterone (MT, sigma–Aldrich) and male with 17α-ethinylestradiol (EE2, sigma–Aldrich) behind the pectoral fin. The MT and EE2 were dissolved in 5% ethanol/95% saline prior to injection. Control groups were injected only with 5% ethanol/95% saline. In previous studies, injection of 1–100 µg MT per gram body weight into fish body has been shown to affect gonadal development and induce female-to-male sex inversion (Kelly et al., 1996; Sarter et al., 2006; Tan-Fermin et al., 1994). When different doses of MT (0, 1, 10 and 100 µg/g body weight) were injected into female yellow catfish, the levels of 17β-estradiol (E2) and 11-ketotestosterone (11-KT) in the blood were measured using the fish E2 ELISA Kit and 11-KT ELISA Kit (Enzyme-linked Biotechnology Company, Shanghai) as described previously (Parikh et al., 2006). We found that 10 µg MT/g body weight is an optimal dose that gives a relative more stable effect on endogenous sex hormones, as indicated by a durative reduction of endogenous E2 and increase of endogenous 11-KT. Moreover, 10 µg EE2/g body weight is a usual concentration for fish injection (Carrera et al., 2007; Jing et al., 2014), that had significant estrogenic effect on male darkbarbel catfish (Pelteobagrus vachelli), a close relative species of Pelteobagrus fulvidraco (Li et al., 2009). For the following experiment, both MT and EE2 were injected at a dose of 10 µg per gram body weight. Gonadal tissues were sampled at 0, 12, 24, 36, 48, 72, 96 and 120 h post treatment (4–5 fish individuals per group). The collected tissues were immediately stored in liquid nitrogen container for RNA extraction.

2.2. Sequence analysis

Our 454 pyrosequencing library was constructed from multiple tissues of yellow catfish (NCBI accession number: SRP032172), while Solexa sequencing data (unpublished data) was generated from libraries constructed from gonads. The assembled sequences were performed local BLAST searches against the NCBI non-redundant (nr), STRING and GENE databases (cutoff value E ≤ 1e–5). Gene names were assigned to each sequence based on the highest alignment score among BLAST matches. A set of CYP family genes were retrieved and translated using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted ORFs were then verified by BLASTP against nr protein sequence database. Finally, the output protein sequences were put into The Simple Modular Architecture Research Tool website (SMART) (http://smart.embl-heidelberg.de/) to predict the conserved domains based on sequence homology.

2.3. Phylogenetic analysis

Seventeen Cyp genes with full length were identified in P. fulvidraco, and ninety-five other Cyp genes were obtained from GenBank including cyp1a1, cyp1b1, cyp4b, cyp4v, cyp7a, cyp7b, cyp8a, cyp8b, cyp11a, cyp11b, cyp17a, cyp19a1a, cyp19b1b, cyp20a, cyp26a, cyp27 and cyp51. The coding sequences of total 112 CYP genes were initially aligned in MEGA6 by MUSCLE method with default parameters (Tamura et al., 2013). Poorly aligned positions were removed by Gblocks (Castresana, 2000). After Gblocks treatment, the best-fit nucleotide substitution model among 88 models was chosen in jModelTest (Darriba et al., 2012). As a result, the parameters (i.e. partition, -lnL proportion of invariable sites and Gamma distribution shape) were optimized using with the Akaike Information Criterion (AIC) (Posada and Buckley, 2004), and the model GTR + I + G was determined as the most likely model of nucleotide substitution. Phylogenetic relationships were reconstructed in Maximum Likelihood (ML) and Bayesian inference (BI). In ML pipeline, PhyML 3.0 (Guindon et al., 2010) were performed with parameters obtained from AIC (The proportion of invariable was fixed to 0.007 and the shape of the gamma distribution parameter was fixed to 1.173). The number of distribution categories was set to 4 and the median approximation was performed for each rate class. The random BioNJ tree and the SPR moves were regarded as starting tree and topological searching operation. Each internal branch of the phylogeny was estimated using non-parametric bootstrap with 1000 replicates. The Bayesian analysis was performed in MrBayes with following settings by AIC: Isst nst = 6 rates = gamma, prset statefreqpr = fixed (0.2275, 0.2747, 0.2840, 0.2139), revmatpr = fixed (rAC = 1.9346, rAG = 3.4645, rAT = 1.6537, rCG = 1.1964, rCT = 4.0525, rGT = 1.0000), Shapepr = fixed (1.1730), pinvarpr = fixed (0.0070). The Markov chains Monte Carlo search was performed with 4 heated chains for 50,000,000 generations, with Samplefreq = 1000. The initial 1250 trees were burn-in and calculated posterior probabilities by the rest of stable trees.

2.4. RNA extraction, reverse transcription and quantitative RT-PCR (qRT-PCR)

The total RNA was extracted using miRNeasy Mini Kit (QIAGEN, USA) and treated with RNase-free DNase (Qiagen) according to the manufacturer’s instruction. 1 µg total RNA was reverse transcribed using GoldScript cDNA Synthesis Kit (Invitrogen, USA). Primers were designed using Primer 5.0 and all were listed in Table 1. The primers were confirmed without primer dimers in qPCR test and the sequences were also confirmed by sequencing. qRT-PCRs were performed on a Bio-Rad PCR system by CFX96 Optics Module (Bio-Rad, Singapore) with SYBR Green I Dye as described previously (Wang et al., 2013; Zhong et al., 2014). All qPCR reactions were performed in 20 µL reactions, containing 10 µL 2 × SYBR green master mix (Bio-rad, USA), 0.5 µL (10 µM) of each primers, 1 µL the RT synthetic...
cDNA template and 8 μL double distilled water. Samples were run with the following program parameters: 95 °C for 1 min, followed by 39 cycles of 95 °C for 15 s, 56 °C for 20 s, 72 °C for 20 s, 77 °C for 5 s, 79 °C for 5 s and 81 °C for 5 s, ended with the melt curve 65–95 °C. Negative control (no-template reaction) was always included.

Specificity of amplification for each reaction was analyzed by dissociation curves using CFX manager software (Bio-Rad). Six housekeeping genes (β-actin, rp17, GAPDH, e1fa, b2m and 18S rRNA) were selected to check their transcription stability after sex hormone treatment. Using geNorm software for measurement (Vandesompele et al., 2002), β-actin (M value = 1.039 < 1.5) was found as the most stable followed by elfa (M value = 1.087). Therefore, β-actin was selected as the internal reference gene for normalization by 2^−DDCt method (Mei et al., 2008). These data represent the results of three independent experiments performed in triplicate.

2.5. Data analysis

The heat map profile of each gene expression in hypothalamic–pituitary–gonad (HPG) axis was drawn by using R program. The relative expression was depended on the quantitative Real-time PCR analysis. The average relative expression of a CYP gene with middle expression level was set as 1, and all other gene expressions were normalized to its level. The heat map was drawn with the log base for ten of each value. The gene expression levels in the heat map were shown from low (green), middle (black) to high (red) color. The heat map was shown as mean ± SD. Significant differences between control and treated groups were assessed by the one-way ANOVA analysis of variance followed by turkey t-test. A probability (P) of <0.05 was considered statistically significant.

Table 1
Identification of CYP genes in the yellow catfish transcriptome and primers used for quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank accession no.</th>
<th>Sequence integrity</th>
<th>Primer sequences (5′−3′)</th>
<th>Product size (bp)</th>
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<td>F: GTCCACAGGAAAGTCTTCCA R: ACCTCAGGTTCCACCATTT</td>
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</table>
3. Results

3.1. Identification and phylogenetic analysis of CYP genes in yellow catfish

From 454 pyrosequencing and Solexa sequencing data, a total of 25 CYP genes, such as cyp1a1, cyp1b1, cyp2g, cyp2j, cyp2r1, cyp2AD, cyp4a, cyp4b, cyp4f, cyp4v, cyp5a, cyp7a, cyp7b, cyp8a, cyp8b, cyp11a, cyp11b2, cyp17a, cyp19a1a, cyp19a1b, cyp20a, cyp24a, cyp26a, cyp27a and cyp51, were identified in yellow catfish with high levels of similarities in P450 domain (Table 1). Among all, 17 CYP genes including cyp1a1, cyp1b1, cyp4b, cyp4v, cyp7a, cyp7b, cyp8a, cyp8b, cyp11a, cyp11b2, cyp17a, cyp19a1a, cyp19a1b, cyp20a, cyp26a, cyp27a and cyp51 had full CDS (coding sequence), whereas other 8 genes, such as cyp2g, cyp2j, cyp2r1, cyp2AD, cyp4a, cyp4f, cyp5a and cyp24a, were with partial CDS in our transcriptomes. And, all of them were confirmed by BlastX and ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Phylogenetic analysis was conducted to investigate the evolutionary relationship of CYP family genes in yellow catfish. Phylogenetic tree were constructed using amino acid sequences of P450 domain from 17 CYP genes with full CDS, while 8 genes with partial CYP CDS were skipped (Fig. 1). Extremely similar phylogenetic tree was created by Maximum Likelihood (ML) and Baysian (BI) method. A total of 112 CYP proteins from yellow catfish and other teleosts were obtained from NCBI and ensemble database and were categorized into 11 clades when cyp19 proteins were treated as outgroup. All CYP genes with high bootstrap/posterior probability were clustered into appropriate clades and duplicated genes were clustered into the same clade. For instance, six cyp1a1 and seven cyp1b1 genes formed the clade of cyp1. Cyp11 and cyp27 genes were clustered together adjacent to the branch of cyp1 and cyp17 genes, and this cluster relation was consistent with the evolutionary status of P450 genes in channel catfish and mammals. Moreover, cyp7 and cyp8 were clustered together in many vertebrates (Eggertsen et al., 1996; Zhang et al., 2014).

Similarly, the branches of cyp7 and cyp8 genes were next to the branches of cyp26 and cyp51 genes, and this big cluster was adjacent to the branch of cyp4 and cyp20 genes. The phylogenetic relation between cyp4, cyp26 and cyp51 was very similar to the phylogeny in the eukaryotes including non-deuterostome metazoans (Albalat and Canestro, 2009; Eggertsen et al., 1996; Nelson,
In each branch, yellow catfish was adjacent to the species of Cypriniformes that was consistent to the traditional classification method by morphology and mitochondrial evolution events (Ladewig de Panepucci et al., 1984; Peng et al., 2006).

3.2. Sexual dimorphic expression of CYP genes in hypothalamic–pituitary–gonad (HPG) axis

To assess sexual dimorphic expression profiles of CYP genes, we performed qRT-PCR analysis in hypothalamic–pituitary–gonad (HPG) axis. The average relative expression of cyp5a in female hypothalamus was set as 1, and all other gene expressions were normalized to its relative expression. Furthermore, graphical heat map was constructed using $10^{\log_{10}}$ (normalized value) to show sexually dimorphic expression of all CYP genes (Fig. 2). As shown in the gonads, the expression of cyp2AD, cyp4b, cyp8a, cyp11b2, cyp17a and cyp27a were obviously higher in testes than in ovaries, whereas cyp2g, cyp7a, cyp8b, cyp19a1a and cyp26a showed more expression in ovaries than in testes. In hypothalamus, the expression of cyp4a, cyp5a, cyp7a, cyp8b and cyp17a were higher in males than in females, while cyp2g, cyp4v and cyp11a showed higher expression in females than in males. In the pituitary, we figured out higher expression levels of cyp4a, cyp7a, cyp8a, cyp26a, cyp27a in male than in female, whereas cyp1a1, cyp1b1, cyp4f, cyp4v, cyp8b, cyp11a, cyp17a showed advantageous expression in female pituitary.

3.3. Expression changes of several selected CYP genes in ovaries after MT treatment

For yellow catfish, male individuals exhibit much faster growth rate than female individuals under the same culturing condition.
biased expressed genes

20%) and reduced endogenous E2 (about 20%). And, the testis-female fish, MT treatment elevated the level of 11-KT (about nious levels of E2 (76–86 ng/L) and 11-KT (60–74 ng/L) in control in ovaries of yellow catfish. In comparison with normal endoge-

remained under the normal level.

24 hpt (65.6% reduction), and recovered around 72 hpt. However, the ovary-biased expression of ovary-biased expressed genes cyp11b2 and cyp17a were significantly up-regulated in ovary after MT treatment. As shown in Fig. 3, the expression of cyp11b2 was significantly increased to more than fivefolds at 96 h post treatment (hpt), and cyp17a was up-regulated to about threefold at 24 hpt and normally at 48 hpt. In contrast, the ovary-biased expressed genes cyp8b, cyp11a and cyp19a1a were significantly down-regulated in ovary after MT treatment. Cyp8b had a sharp decrease (67.7%) at 12 hpt and then was recovered to normal level at 48 hpt. The expression of cyp11a and cyp19a1a were respectively decreased at 36 hpt (45.4% reduction) and 24 hpt (65.6% reduction), and recovered around 72 hpt. However, the expression of cyp19a1a remained under the normal level. Unexpectedly, the ovary-biased cyp26a was induced by MT treatment at 72 hpt and recovered to normal level at 120 hpt.

3.4. Expression of several selected CYP genes in testes after EE2 treatment

To determine whether EE2 mediates the expression of CYP genes, 1-year old male individuals were injected with EE2 and their testes were collected for RT-PCR analysis (Fig. 4). After EE2 treatment, the testis-biased gene cyp11b2 was significantly down-regulated at 36 hpt and up-regulated to about twofolds at 96 and 120 hpt. The expression of ovary-biased genes cyp26a, cyp19a1a, cyp8b and cyp11a were increased after EE2 treatment. Among them, cyp26a was gradually increased and reached to about 5-fold at 36 and 48 hpt, finally fell back to normal level after 72 hpt. The expression level of cyp19a1a was significantly increased to more than 8-folds at 36 hpt and remained under the normal level after 48 hpt. Cyp8b and cyp11a respectively had an increasing trend from 72 hpt (223%) and 96 hpt (207%), finally reached up to peak at 120 hpt.

4. Discussions and conclusion

CYP genes are involved in many biological processes including the metabolism of a number of exogenous and endogenous compounds (Nebert et al., 2013). CYP family has been characterized in several teleost fishes with whole genome sequence, such as zebrafish (Goldstone et al., 2010), Japanese pufferfish (Nelson, 2003) and channel catfish (Zhang et al., 2014). In channel catfish, the CYP genes are also involved in responses against disease (Zhang et al., 2014). In spite of their importance, only cyp1a and cyp2ad were characterized from yellow catfish and correlated to fish detoxification and xenobiotic metabolism (Kim et al., 2008; Ku et al., 2014). In this study, we characterized and systematically analyzed CYP genes in yellow catfish, and assessed sexually dimorphic expression patterns of these genes in hypothalamic–pituitary–gonad (HPG) axis.

From the expression pattern of CYP genes in hypothalamic–pituitary–gonad (HPG) axis (Fig. 2), we found that multiple CYP genes exhibited a notable sex difference. Interestingly, expression of CYP genes in fish has been shown to be modulated by sex hormone (Navas and Segner, 2001). Therefore, a set of CYP genes that have sex-biased expression in gonads were examined to check their response to sex hormone treatment (Fig. 3 and 4). MT treatment elevated the level of endogenous 11-KT and reduced endogenous E2, while EE2 treatment had an opposite effect. Significantly, we found that ovary-biased genes cyp11a, cyp8b and cyp19a1a significantly decreased upon MT treatment and increased upon EE2 treatment, whereas testis-biased genes cyp11b2 consistently

increased after MT treatment and significantly decreased after EE2 treatment. The elevation of endogenous 11-KT in testis by MT was positively correlated with the expressions of testis-biased genes, whereas the elevation of endogenous E2 by EE2 had an opposite effect on gene expression. These trends indicate that there is an appropriate cellular context in ovary and testis for transcriptional inhibition and activation by MT and EE2.

Cyp19a1a is a critical factor for converting androgen to estrogen and is the terminal steroidogenic enzyme in the biosynthesis pathway of estrogen (Guiguen et al., 2010). Moreover, MT functions as an inhibitor of aromatase in some fish species including Japanese flounder (Kitano et al., 2000) and orange-spotted grouper (Zhang et al., 2007), for it could affect local estrogen formation by regulating aromatase expression or by inhibiting aromatase action (Bhandari et al., 2006). Cyp19a1a has the ability to convert cholesterol to pregnenolone (Uno et al., 2012), and its expression is regulated by trans-regulators such as SF-1, DAX-1, TreP-132, LBP and GATA (Shih et al., 2011). Previous reports indicate that androgen treatment reduces the expression of cyp11a in the testis of rainbow trout (Baron et al., 2005), whereas EE2 up-regulates the expression of cyp11a in the oocytes of Atlantic salmon (Vang et al., 2007). Our results also indicate that MT treatment down-regulates the expression of cyp11a in the ovary of yellow catfish (Fig. 3).

Cyp11b2 is an enzyme responsible for the synthesis of 11-ketotestosterone (Jiri et al., 2008). Another testis-biased gene, cyp17a, is inhibited by EE2 in the testis of fathead minnow and rainbow trout (Baron et al., 2005; Filiby et al., 2007). Additionally, CYP17 is also required for androgen production in human (Lee-Robichaud et al., 1999). Our results suggest that MT might have a vital effect on the hypothalamic–pituitary–gonad (HPG) axis, by which results in a reduced secretion of gonadotropin and finally leads to a fading of steroidogenic enzymes in the rainbow trout, Oncorhynchus mykiss (Filby et al., 2007). Mol. Reprod. Dev. 71, 471–479.


