Two cytochrome P450 aromatase genes in the hermaphroditic ricefield eel Monopterus albus: mRNA expression during ovarian development and sex change

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Abstract

Previously, the ricefield eel (Monopterus albus) was speculated to have only one cytochrome P450 aromatase gene. In this study, however, the cDNAs encoding two distinct cytochrome p450 aromatases, cp19a1a and cp19a1b, were isolated. The genomic organizations of both cp19 genes were conserved when compared with other teleosts. Northern blot detected an abundant expression of cp19a1a in the ovary, and cp19a1b in the hypothalamus. RT-PCR coupled with Southern blot showed that cp19a1a was expressed predominantly in the gonads of both sexes, with higher levels in the ovary than testis, while cp19a1b was expressed in all the tissues examined in the male, but only in the brain and pituitary in the female. The levels of cp19a1a mRNA in the ovary were increased significantly during vitellogenesis, but decreased significantly at mature stage. The levels of cp19a1b mRNA in the brain and pituitary did not vary significantly during vitellogenesis. As ovarian development shifted from vitellogenesis to maturation, the levels of cp19a1b mRNA was decreased significantly in the brain, but increased significantly in the pituitary. During natural sex change from female to male, the levels of cp19a1a mRNA in the gonad were significantly decreased. The levels of cp19a1b mRNA in the hypothalamus were significantly increased at the early intersexual phase, whereas the expression levels in the pituitary were significantly decreased at the intersexual phases. Taken together, these results showed a novel sexual dimorphism of cp19a1b mRNA tissue distribution, and both CYP19 genes were associated with the ovarian development and natural sex change of the ricefield eel.


Introduction

Hermaphroditism is widespread among teleosts (Devlin & Nagahama 2002). In the sequential hermaphroditic fish, sex change, the transformation of an individual from one sex to the other in adulthood, occurs naturally in its life cycle. It has been shown that estradiol (E2) administration induced male to female sex change of protandrous black porgy (Lee et al. 2000, 2001), while inhibition of aromatase, the enzyme complex for catalyzing the conversion of androgens to estrogens, induced sex change from female to male in the protogynous goby (Kroon & Liley 2000) and grouper (Bhandari et al. 2004), but blocked natural sex change in the protandrous porgy (Lee et al. 2001). In Gobiodon erythrospilus, a hermaphroditic fish that naturally exhibits bidirectional sex change, manipulation of E2 levels via the aromatase pathway induced adult sex change in each direction (Kroon et al. 2005). The above studies imply that aromatase plays important roles in the gonadal differentiation and sex change in hermaphroditic fish.

Cytochrome P450 aromatase (CYP19) is an essential part of the aromatase enzyme complex. In mammals, with the exception of pig (Choi et al. 1997, Graddy et al. 2000), CYP19 is encoded by a single copy of the CYP19 gene, and the tissue-specific expression was achieved by the use of tissue-specific promoters and alternative splicing of 5’untranslated exons (Simpson 2003). However, two distinct CYP19 genes, namely cp19a1a and cp19a1b, which were expressed mainly in the ovary and brain respectively, were identified in teleosts, such as Carassius auratus (Vagdez & Pfieffer 2004), Halichoeres tenuispinis (Choi et al. 2005), Fundulus heteroclitus (Greytak et al. 2005), and Epinephelus coioides (Zhang et al. 2004). In fishes such as rainbow trout and tilapia, the expression of cp19a1a was shown to be crucial for ovarian differentiation (Guiguen et al. 1999). In the protandrous gilthead seabream, differential expression of cp19a1a gene in the gonad was shown to be associated with sex reversal (Wong et al. 2006). In the protogynous grouper, gonadal cp19a1a expression was decreased during 17α-methyltestosterone (MT)-induced precocious sex change (Zhang et al. 2004, 2007). In juvenile sea
bass, on the other hand, females exhibited higher mRNA expression levels of cyp19a1b in the brain than males by the time of gonadal sex differentiation (Blázquez & Pfärrer 2004). These studies suggest that both cyp19 genes may be involved in the gonadal differentiation in teleosts, but more studies are needed, especially regarding cyp19a1b.

Ricefield eel Monopterus albus, a member of the order Synbranchiformes, is a protogynous hermaphrodite fish that changes sex from female functional, through an intersexual stage, to the functional male phase during its life cycle. This is an intriguing phenomenon and has been under extensive investigation; however, the mechanism of natural sex change in the ricefield eel remains unclear (Ye et al. 2007). The purpose of this study was to elucidate the involvement of cyp19 gene in the process of sex change in the ricefield eel. A previous study speculated that there was only one form of cyp19 gene in the ricefield eel (Yu et al. 2008). Our present study, however, identified two cyp19 genes, and results indicated that in addition to cyp19a1a in the gonad, cyp19a1b in the hypothalamus and pituitary was also associated with the ovarian development and sex change of the ricefield eel.

Materials and Methods

Animals and tissues

The ricefield eels used in this study were obtained from a local dealer in Guangzhou, Guangdong Province, P R. China. All procedures and investigations were reviewed and approved by the Center for Laboratory Animals of Sun Yat-Sen University, and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals. Fish were killed by decapitation, and tissues were dissected, frozen immediately in liquid nitrogen and stored at −70 °C until RNA extraction. The phenotypic sex and gonadal developmental stages of the ricefield eel were verified by histological sectioning of gonads and microscopic analysis. Ovarian development was classified as four stages in this paper, namely oocytes at previtellogenic stage (stage PV), oocytes at early vitellogenic stage (stage EV), oocytes at mid- to late-vitellogenic stage (stage MLV), and mature oocytes (stage OM). The transition of sex from female to male was classified into five sexual phases according to previous work (Chan & Phillips 1967, Chan et al. 1972), namely female stage (F; gonadal lamella filled with oocytes at vitellogenic stage), early intersexual stage (IE, appearance of small testicular lobules and some atretic oocytes in the gonadal lamella, which was mainly occupied by ovarian follicles at various stages of maturation), middle intersexual stage (IM, the gonad lamella was occupied by a significant amount of testicular tissues, with some remnant atretic ovarian follicles), late intersexual stage (IL, the gonad lamella was mainly occupied by testicular lobules, with a few atretic primary oocytes), and male stage (M, the gonad lamella was filled with testicular tissues, and devoid of ovarian tissues).

Isolation of total RNA

Total RNA was isolated from frozen tissues using TRIzol reagent (Invitrogen) and quantified based on the absorbance at 260 nm. The integrity of RNA was checked with agarose gel electrophoresis.

Oligonucleotides

The sequences of oligonucleotides used as PCR primers are listed in Table 1. Oligonucleotides CYP19F1, CYP19R1, and CYP19R2 were degenerate primers and designed based on the nucleotide sequences in highly conserved regions of previously identified vertebrate CYP19. Other oligonucleotides CYP19A1F1, CYP19A1F2, CYP19A1F3, CYP19A1F4, CYP19A1F5, CYP19A1R1, CYP19A1R2, CYP19A1R3, CYP19A1R4, CYP19A1R5, CYP19A1R6, CYP19A2F1, CYP19A2F2, CYP19A2F3, CYP19A2F4, CYP19A2F5, CYP19A2F6, CYP19A2R1, CYP19A2R2, CYP19A2R3, CYP19A2R4, CYP19A2R5, CYP19A2R6, β-actinF1, β-actinF2, β-actinR1, and β-actinR2 were gene

Table 1 Sequences of oligonucleotide primers used

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Cloning of cyp19a1a and cyp19a1b cDNAs

The initial fragments of cyp19a1a and cyp19a1b cDNAs were amplified with PCR from brain and ovary cDNAs respectively. The primers were CYP19F1 and CYP19R1 for cyp19a1a cDNA, and CYP19F1 and CYP19R2 for cyp19a1b cDNA. The brain and ovary cDNAs were transcribed from ricefield eel brain and ovary total RNA respectively, by ThermoScript RT System according to the manufacturer’s instructions. PCR and ovary total RNA respectively, by ThermoScript RT System (Invitrogen) according to the manufacturer’s instructions. PCR was performed in 25 μl final volume containing 2.5 μl 10X Ex Taq buffer, 2.5 mM MgCl2, 0.2 mM dNTP, 0.4 μM of each primer, and 1-25 U TaKaRa Ex Taq DNA Polymerase (TaKaRa, DaLian, China) and 0.5 μl of brain or ovary cDNA. After an initial 3-min denaturing step at 94 °C, 35 cycles of amplification were performed for 0.5 min at 94 °C, 0.5 min at 55 °C, and 1-5 min at 72 °C, and then followed by a final extension for 7 min at 72 °C. The target PCR products were gel purified using Gel Extraction System (Omega, USA) and cloned into pGEM-T Easy Vector (Promega). The plasmid DNA was sequenced with forward and reverse universal primers using the BigDye Terminator kit and an ABI Prism 3730 DNA sequencer (Perkin–Elmer, Wellesley, MA, USA).

After determining the nucleotide sequences of the initial fragments of the cyp19a1a and cyp19a1b cDNAs, we used the GeneRacer kit (Invitrogen) to isolate their 5'- and 3'-ends according to the manufacturer’s instructions. The oligonucleotides were CYP19A1F1 and CYP19A1F2 for 5'RACE, and CYP19A1R1 and CYP19A1R2 for 5'RACE of cyp19a1a; CYP19A2F1 and CYP19A2F2 for 3'RACE, and CYP19A2R1 and CYP19A2R2 for 5'RACE of cyp19a1b. The PCR products were cloned into pGEM-T Easy Vector (Promega) and sequenced as described previously. The full-length cDNA sequences were obtained by combining the 3'- and 5'-end sequences.

Cloning of cyp19a1a and cyp19a1b genes

Genomic DNA was extracted from the liver of the female ricefield eel by phenol/chloroform method. About 100 ng of genomic DNA was subjected to PCR with three sets of primer pair CYP19A1F3 and CYP19A1R3, CYP19A1F4 and CYP19A1R4, and CYP19A1F5 and CYP19A1R5 for cyp19a1a gene, and another three sets of primer pair CYP19A2F3 and CYP19A2R3, CYP19A2F4 and CYP19A2R4, and CYP19A2F5 and CYP19A2R5 for cyp19a1b gene. These primers were designed according to the cDNA sequences of CYP19. The PCR products were cloned and their sequences were determined and combined to obtain the genomic DNA sequences containing the full coding regions of cyp19a1a (EU841366) and cyp19a1b (EU840259) respectively.

Sequence analysis

The deduced amino acid sequences of the ricefield eel cyp19a1a and cyp19a1b were compared with previously published sequences of some representative vertebrates. The percentage identity of amino acid sequences was calculated using the Megalign of the DNASTar software package (DNASTAR, Inc., Madison, WI, USA). The alignment of amino acids sequences were performed using the ClutalX1.81 software (Plate–Forme de Bio-Informatique, Illkirch Cedex, France). The phylogenetic tree was generated by the maximum parsimony method of PHYLIP software package (http://evolution.genetics.washington.edu/phylip.html) and visualized by TREEVIEW1.5 (Eisen M; http://rana.lbl.gov/eisen/?page-id=42). The bootstrap values were estimated by 1000 replications and given at each branch point.

The intron–exon boundaries as well as the intron sequences of the ricefield eel cyp19a1a and cyp19a1b were determined by comparing the genomic sequences and the cDNA sequences.

Northern blot analysis

Total RNA (30 μg) isolated from the hypothalamus, ovary, and liver of the female ricefield eel at stage MLV was size separated on a 1.5% formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N+, Amersham Biosciences). The hybridization probes were a 1065 bp cDNA fragment for cyp19a1a (nt: 312–1376), and a 728 bp cDNA fragment for cyp19a1b (nt: 18–745). All probes were randomly labeled with [32P]dCTP using the Random Primer DNA Labeling kit (TaKaRa). Hybridization was performed using the Northern Max kit (Ambion, Austin, TX, USA). The membrane was hybridized overnight at 50 °C with the 32P-labeled cyp19a1b probe, washed twice with low stringency wash solution (equal to 2X SSC, 0.1% SDS) at 50 °C for 10 min, twice with high stringency wash solution (equal to 0.1X SSC, 0.1% SDS) at 50 °C for 15 min, and then exposed to a phosphor storage screen and visualized with a Typhoon 8600 Variable Mode Imager (Molecular Dynamics, Sunnyvale, CA, USA). The blot was then stripped with boiling solution (0-1% SDS), and the removal of the radioactive cyp19a1b probe was verified by exposing the stripped blot to the phosphor storage screen. After stripping, the same membrane was hybridized overnight at 50 °C with the 32P-labeled cyp19a1a probe, washed and exposed as described for cyp19a1b.

RT-PCR coupled with Southern blot analysis of tissue patterns of CYP19 mRNA expression

Total RNA (2 μg) isolated from different tissues of female (stage EV) or male ricefield eel was first treated with DNase I
(Invitrogen, Amplification Grade, CA, USA) to remove any genomic DNA contamination. The RNA was then reverse transcribed using oligo(dT) primer and Thermoscript RT-PCR System (Invitrogen) according to the manufacturer's instructions. The integrity of all RNA samples was verified by the successful amplification of β-actin.

The first-strand reaction (0.5 μl) was amplified for each target gene using the Biometra TGRADIENT thermal cycler. PCR was performed in 15 μl final volume containing 1.5 μl 10X reaction buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.2 μM of each primer, and 1-0 U Platinum Taq DNA Polymerase (Invitrogen). Water was used as a negative control in the RT-PCR. To detect mRNA expression in various tissues, the reaction mixture was heated at 94 °C for 2 min, followed by 34 cycles of amplification for CYP19, and 28 cycles for β-actin. The primers were CYP19A1F6 and CYP19A1R6 for cyp19a1a, CYP19A2F6 and CYP19A2R6 for cyp19a1b, and β-actinF1 and β-actinR1 for β-actin, which generated PCR products of 335, 157, and 484 bp respectively. The PCR products were separated on a 1.5% agarose gel and transferred onto a nylon membrane by the capillary method. The membrane was hybridized overnight at 48 °C with the same probes as used for the northern blot, followed by washing twice with low stringency wash solution at 48 °C for 10 min, with high stringency wash solution twice at 48 °C for 15 min, and then exposed to a phosphor storage screen and visualized with a Typhoon 8600 Variable Mode Imager (Molecular Dynamics). These experiments were repeated twice using samples from different individual fish at the same sexual stage, and similar results were obtained.

In vitro incubation of pituitary glands with E₂

The pituitary glands of the female ricefield eel (OM stage) were dissected and washed in M199 media (Sigma) on ice. Approximately five pituitary glands were placed in a 24-well tissue culture dish with 1 ml of M199 media (Sigma) containing 0-1 U/ml penicillin (Gibco) and 0-1 μg/ml streptomycin (Gibco), and then incubated at 25 °C in a humidified incubator under 5% CO₂. E₂ stock solutions were prepared in 100% ethanol at concentrations 10⁻¹-fold higher than the final. After pre-incubation for about 6h, the pituitary glands were treated with E₂ (1, 10, and 100 nM) or ethanol (0-1%, control group) for 24 h. Three replicates were made for each treatment. After completion of incubation, the pituitary glands were collected and total RNA were extracted with TRIzol reagent (Invitrogen) for real-time quantitative RT-PCR analysis of lhb mRNA expression.

Real-time quantitative PCR analysis of CYP19 and lhb mRNA expression

Real-time PCR analysis was established to quantify the mRNA expression levels of cyp19a1b in the brain and pituitary gland, and cyp19a1a in the ovary of the ricefield eel during ovarian development and sex change. The primers for real-time PCR analysis were CYP19A1F6 and CYP19A1R6 for cyp19a1a, CYP19A2F6 and CYP19A2R6 for cyp19a1b, LHF1 and LHR1 for lhb and β-actinF2 and β-actinR2 for β-actin.

The real-time PCR was performed on the Rotor-Gene 3000 detection system (Corbett Research, Sydney, Australia) in a volume of 20 μl containing 0-2 μM of each primer, 10 μl of 2X SYBR Green Master Mix (TOYOBO, Osaka, Japan), 1 μl of cDNA template which was transcribed from 1 μg total RNA using Thermoscript RT-PCR System (Invitrogen). The PCR cycling conditions were: 95 °C for 1 min; 40 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 45 s; 85 °C for 20 s for signal collection in each cycle. To assess the specificity of the PCR amplification, a melt-curve analysis was performed at the end of the reaction by increasing temperature from 55 to 99 °C and held for 5 s at every increment of 1 °C, and a single peak was observed. Data were produced by the Rotor-gene version 6-0-22 software. Products were also visualized on an ethidium bromide-stained agarose gel, and a single band confirmed the specificity of the PCR amplification. Finally, PCR products were cloned and sequenced to further confirm their authenticities as gene-specific amplicons.

The quantification of the mRNA expression level was performed using a standard curve with tenfold serial of dilution of plasmid containing corresponding DNA fragments from 10¹ to 10⁸ copies. The correlation coefficients and PCR efficiencies were not less than 0.997 and 80% respectively. To minimize variation due to the differences in RNA loading, each sample was normalized to the expression level of the house keeping gene β-actin, and the mRNA expression levels of target genes were presented as the copy number ratios to β-actin.

Statistical analysis

All data are presented as means ± S.E.M. The statistical significant differences were analyzed by one-way ANOVA followed by the Duncan’s multiple range test using the SPSS software package (SPSS, Inc., Chicago, IL, USA). Significance was set at P<0.05.

Results

Nucleotide and deduced amino acid sequences of cyp19a1a and cyp19a1b

The full-length cyp19a1a cDNA sequence (EU252487) obtained from the ricefield eel ovary consisted of 1828 bp including the poly (A) tail. This sequence included an open reading frame (ORF) of 1557 bp, a 54 bp 5’ untranslated region (UTR) and a 199 bp 3’ UTR. The ORF encoded a putative protein of 518 amino acids with a calculated molecular mass of about 58.3 kDa. The putative polyadenylation signal TATAAA was located 15 bases upstream the poly (A) tail.

The full-length cyp19a1b cDNA sequence (EU252488) obtained from the ricefield eel brain consisted of 2268 bp
including the poly (A) tail. This sequence included an ORF of 1506 bp, a 215 bp 5'UTR and a 532 bp 3'UTR. The ORF encoded a putative protein of 501 amino acids with a calculated molecular mass of 56.6 kDa. The putative polyadenylation signal ATTAAA was located 12 bases upstream the poly (A) tail.

**Amino acid sequence comparison**

The deduced amino acid sequences of the ricefield eel cyp19a1a and cyp19a1b were aligned with other vertebrate CYP19 homologues (Fig. 1). Both cyp19a1a and cyp19a1b of the ricefield eel had the conserved putative function domains,
namely the membrane spanning region, I-helix region, Ozol's peptide region, aromatase-specific region, and heme-binding region. The two ricefield eel CYP19 forms shared only 62.5% of overall amino acid sequence identity (Tables 2 and 3), but each isoform was 87.6 and 80.4% identical with the corresponding A1- or A2-isofrom of grouper, a perciform fish, and around 64% with those of zebrafish, a cypriniform fish. The two ricefield eel CYP19 forms shared low but similar degrees of overall sequence identities with frog, Pleurodeles, chicken, mouse, and human CYP19 (49.9–55.4%), but high

Ricefield eel/A1  QTVVECVSSTQTHLDL--------DLGHDVLSLLRCTVIDISNRLFLGVNPYNEKELLKLH  231
Grouper/A1   QTVVECVSATAQTHLDL--------DLGHDVLSLLRCTVIDISNRLFLDVPEYSEKELLKL  231
Zebrafish/A1 RTVA1CVSSTAKHLDNQLQMTPASGHDALSLLRAIVDISNRLFLVPLNAKDLVLK  219
Ricefield eel/A2  KSVECVSSTAEHDLQEMTDPYSVKDALSLLRAIVDISNRLFLVPLNAKDLV  218
Grouper/A2   KSVECVSSTAHKLQEMTDPYSVKDALSLLRAIVDISNRLFLVPLNAKDLV  218
Zebrafish/A2  KSVECVSSTANRQDLVQLFQTDASGHDVLNLRCVVDVSNRLFRPLKELEKL  218
Frog       QTENEC1RTSNHYDLNSVTEGLNQVLDLKLMLQLMTSNNLFRLPDEIEVLQIK  216
Chicken     RM1AC1CSTVHLDKEELVTEVNGVNLMLMRMLHTSNLFLVPLDEAIVLIQIK  217
Mouse       RMVECVSSTIKQHLGRELQTVGDSTYVDSLTLMRMLHTSNLFLGVPDEAIVQIK  218
Human       RMVTVCAESLTHDLRLEEVTSNGYVDVLTLLRVMDSLNTFLFLPDEAIYVIQIK  218

Figure 1 (continued)


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Figure 1 Alignment (by ClustalX1.8) of ricefield eel cyp19a1a and cyp19a1b amino acid sequences with other vertebrate CYP19 forms. The identical, highly conserved, and less conserved amino acid residues were indicated by (*), (:), and (.) respectively. Regions of putative functional domains were in gray box and indicated by roman numerals and overlines with arrowheads (I, the membrane-spanning region; II, the I-helix region; III, Ozol’s peptide region; IV, the aromatic region; V, the heme-binding region). The protein sequences were downloaded from Entrez (NCBI). For details, please refer to Fig. 2.
identities were evident in the putative function domains of all vertebrate CYP19.

**Phylogenetic analysis**

A phylogenetic tree of vertebrate CYP19 proteins (Fig. 2) was constructed by the maximum parsimony method of PHYLIP software package. This phylogenetic tree clearly segregated vertebrate CYP19 proteins into three branches, namely a teleost cyp19a1a branch, a teleost cyp19a1b branch, and a tetrapod CYP19 branch. The ricefield eel cyp19a1a and cyp19a1b belonged to the teleost cyp19a1a and cyp19a1b branches respectively, and were most closely related to the corresponding CYP19 isoforms of perciform fish.

**Genomic organization of cyp19a1a and cyp19a1b genes**

The ricefield eel cyp19a1a gene consisted of nine exons and eight introns, and cyp19a1b gene consisted of ten exons and nine introns (Fig. 3). Most of the intron–exon boundaries follow the consensus sequence of GT/AG for splicing donor and acceptor sites, except three in cyp19a1b gene. The genomic structures of the ricefield eel cyp19a1a and cyp19a1b gene were almost the same as those of tilapia, and also conserved when compared with those of zebrafish (Fig. 3). The initiation codon was located in the exon1 for cyp19a1a, and in the exon2 for cyp19a1b, which is also conserved among these fish.

**Tissue-specific and sexual dimorphic expression of cyp19a1a and cyp19a1b genes**

With northern blot analysis (Fig. 4), the expression of cyp19a1a was detected in the ovary but not in the hypothalamus and liver, while the expression of cyp19a1b was detected in the hypothalamus but not in the ovary and liver of the female ricefield eel. The tissue patterns of cyp19a1a and cyp19a1b mRNA expression were further analyzed in both the female and male ricefield eel with RT-PCR coupled with Southern blot (Fig. 5). The cyp19a1a was expressed predominantly in the gonads of both sexes, and gel image indicated that the expression level in the ovary was higher than that in the testis. The expression of cyp19a1b gene was detected at very low levels in the olfactory bulb and telencephalon of the female, and in the pituitary and optic tectum-thalamus of the male, but no signals were detected in other tissues examined in both sexes.

The cyp19a1b was expressed in all the tissues examined in males, but only in the brain and pituitary in females.

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**Table 2** Percent of overall and partial identities of the predicted amino acid sequence of cyp19a1b compared with CYP19 isolated from other vertebrates. The identity was calculated using MegAlign of DNAstar software package.

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**Table 3** Percent of overall and partial identities of the predicted amino acid sequence of cyp19a1a compared with CYP19 isolated from other vertebrates. The identity was calculated with MegAlign of DNAStar software package.

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<th></th>
<th>Overall identity</th>
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<th>Ozol’s peptide region</th>
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<td>79.2</td>
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male, expression of cyp19a1b was detected at higher levels in the olfactory bulb, telencephalon, hypothalamus, optic tectum-thalamus, medulla oblongata, and pituitary, but at lower levels in the cerebellum, testis, spleen, pancreas, heart, liver, and kidney, and at very low levels in the muscle, intestines, and blood. In the female, expression of cyp19a1b was detected at higher levels in the telencephalon, hypothalamus, optic tectum-thalamus, and pituitary, but barely detectable or undetectable in other tissues.

mRNA expression of cyp19a1a and cyp19a1b during ovarian development

Real-time PCR analysis was employed to quantify the mRNA expression levels of cyp19a1a in the ovary, and cyp19a1b in the hypothalamus (including optic tectum-thalamus) and pituitary during ovarian development (Fig. 6). In the ovary, the relative mRNA levels of cyp19a1a were low at PV and EV stages, but increased significantly at the vitellogenic stage, and then decreased significantly at mature stage. In the hypothalamus, the relative mRNA levels of cyp19a1b were maintained at similar levels from previtellogenic to vitellogenic stages, but decreased significantly when oocytes became mature. In the pituitary, the relative mRNA levels of cyp19a1b were also maintained at similar levels from previtellogenic to vitellogenic stages, but increased significantly when oocytes get mature.

To examine the possible hypophyseal effects of locally produced estrogens on lhb mRNA expression the in vitro incubated pituitary glands of mature female ricefield eel were treated with E2 of different concentrations. Results showed that E2 greatly stimulated hypophyseal lhb mRNA expression in a dose-dependant manner (Fig. 7). The expression level was increased about fourfold at a lower concentration (1 nM), and about six- to sevenfold at higher concentrations (10 and 100 nM).

mRNA expression of cyp19a1a and cyp19a1b during natural sex change

The relative mRNA expression levels of cyp19a1a in the gonad, and cyp19a1b in the hypothalamus and pituitary
during natural sex change were also determined with real-time PCR analysis (Fig. 8). In the gonad, the expression levels of cyp19a1a were decreased continuously and significantly from female phase, through intersexual phases, to male phase. In the hypothalamus, the mRNA expression levels of cyp19a1b peaked at the IE phase, but there were no significant differences among other phases. In the pituitary, there were relative RNAs expression levels of cyp19a1b were significantly decreased at the intersexual phases, but increased significantly at the male phase when compared with the intersexual phase. However, the expression level of cyp19a1b at the male phase was still significantly lower than that at the female phase.

Discussion

Our present study identified cDNAs encoding two different cytochrome P450 aromatases, namely cyp19a1a and cyp19a1b, in the ricefield eel, which concurs with the fact that most teleosts have two CYP19 genes, namely cyp19a1a and cyp19a1b (Piferrer & Blázquez 2005), and contradicts the previous report of only one form of CYP19 gene in this fish (Yu et al. 2008). The cyp19a1a identified in the present study shared 97.8% homology at the nucleotide level and 97.7% homology at the amino acid level with the previously reported one (Yu et al. 2008), having a total of 46 different nucleotides and 12 different amino acids, and these sequence differences could be probably due to the different geographical populations of the fish used, South China (Guangdong) in the present study and East China (Jiangsu) in the previous study. Phylogenetic analysis showed that the ricefield eel cyp19a1a and cyp19a1b were clustered with teleost cyp19a1a and cyp19a1b respectively. Multiple amino acid sequence alignments showed that both CYP19 proteins contained the putative functional domains as other vertebrate CYP19, including the membrane-spanning region, I-helix region, Ozol’s peptide region, the aromatic region, and the heme-binding region. As in the orange-spotted grouper (Zhang et al. 2004), the homology between the two forms of ricefield eel CYP19 was lower than the homology of each isoform with the corresponding forms of other teleosts. The two forms of ricefield eel CYP19 were more closely related to the corresponding forms of perciform fishes, conforming to the lineage relationship.

The presence of two CYP19 genes in the ricefield eel was further confirmed by the isolation of corresponding genomic DNA sequences. In contrast to seven introns in cyp19a1a gene reported previously (Yu et al. 2008), our present results showed that the genomic structures of CYP19 genes in the ricefield eel are well conserved when compared with other teleost fishes including tilapia and zebrafish, namely eight introns and nine exons in cyp19a1a, and nine introns and ten exons in cyp19a1b. The genomic structures of ricefield eel...
Expression of two CYP19 genes in ricefield eel · Y Zhang and others

**cyp19** genes in the present study were further confirmed by the cloning of **cyp19** genes from the fish caught in the wild of Taishan, Guangdong Province, and cultured in Hunan Province of P R China (data not shown).

It has been shown that two **cyp19** genes in teleosts were differentially expressed, with **cyp19a1a** mainly expressed in the ovary and **cyp19a1b** in the brain (Zhang et al. 2004, Piferrer & Blázquez 2005). Similarly, northern blot detected the expression of **cyp19a1b** in the hypothalamus rather than the ovary and liver, and the expression of **cyp19a1a** in the ovary rather than the brain, suggesting that two **cyp19** genes of the ricefield eel were also differentially expressed in these tissues. Interestingly, three transcripts were detected for **cyp19a1b** in the hypothalamus of the ricefield eel by northern blot, which were about 7.0, 4.0, and 1.7 kb in sizes respectively. In tilapia, two transcripts of 6.2 and 4.4 kb were also detected for **cyp19a1b** in the brain by northern blot (Chang et al. 2005). However, the biological significances and mechanisms of multiple **cyp19a1b** transcripts in these fish remain to be elucidated.

The tissue patterns of **CYP19** mRNA expression in the ricefield eel were further analyzed by RT-PCR and results showed differential expression of both **CYP19** genes in more extensive tissues of both sexes. The mRNA expression of **cyp19a1a** was primarily restricted to the gonads, with a higher level in the ovary than in the testis as indicated by gel images, but barely detectable or undetectable in the extragonadal tissues examined, which suggests that **cyp19a1a** may be primarily involved in the gonadal differentiation and development in the ricefield eel. This tissue expression pattern was in striking contrast to what has been reported in the previous study, where the expression of **cyp19a1a** was not only detected in the gonad, but also in the brain, liver, and pituitary, and even the expression level was higher in the brain than the gonad (Yu et al. 2008). The exact reasons for these discrepancies are not clear at present, which could be due to different genetic backgrounds of this fish from different geographical locations.

In addition to the hypothalamus, **cyp19a1b** was shown to be abundantly expressed in the pituitary gland, telencephalon, and optic tectum-thalamus of both sexes of the ricefield eel by RT-PCR analysis. This regional expression pattern of **cyp19a1b** in the brain appeared to be common in the teleost fish (Kazeto & Trant 2005), and was implicated in the control of reproductive activities (González & Piferrer 2003). However, the extraneural expression of **cyp19a1b** in the ricefield eel exhibited an obvious sexual dimorphism. In the female, the expression of **cyp19a1b** was not detected in tissues other than the brain and pituitary gland, whereas in the male, the expression of **cyp19a1b** was detected in all the tissues examined. By contrast, Atlantic halibut **cyp19a1b** was ubiquitously expressed in the adult male and female without sexual dimorphism (van Nes et al. 2005). Whether the extraneural expression of **cyp19a1b** in the male ricefield eel is of any physiological relevancy, and how it is regulated remains to be identified.

The mRNA expression of both **cyp19a1a** and **cyp19a1b** was detected in the testis of the ricefield eel. Overlapped expression of both **CYP19** genes were also observed in the testis of other teleosts, such as zebrafish (Sawyer et al. 2006) and wrasse (Choi et al. 2005). In mammals, it has been shown that estrogen is essential for spermatogenesis and male fertility (O’Donnell et al. 2001, Carreau et al. 2003). It is possible that the expression of **CYP19** genes and thus production of

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**Figure 5** Tissue distribution of **cyp19a1a** and **cyp19a1b** in the female (A) and male (B) ricefield eel as analyzed by RT-PCR coupled with Southern blot. Ob, olfactory bulb; Te, telencephalon; Hy, hypothalamus; Ot, optic tectum-thalamus; Ce, cerebellum; Mo, medulla oblongata; Pi, pituitary; Ov, ovary; Ts, testis; Mu, muscle; Sp, spleen; Pa, pancreas; He, heart; Li, liver; Ki, kidney; In, intestines; Bl, blood; RT−, RT minus; NC, negative control.

[caption image and table here]

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estrogen are also important for the reproduction of male teleosts. Consistent with this hypothesis, the expression of estrogen receptors were found in the testis of teleosts, such as rainbow trout (Nagler et al. 2007) and zebrafish (Menuet et al. 2002). The overlapped expression of both CYP19 genes in the testis of the ricefield eel may offer a more refined regulation of testicular estrogen production, and thus male reproductive function.

Real-time PCR analysis showed that the mRNA expression level of cyp19a1a in the ovary; b) cyp19a1b in the hypothalamus (including optic tectum-thalamus); and (C) cyp19a1b in the pituitary. PV, previtellogenic stage; EV, early vitellogenic stage; MLV, mid- to late-vitellogenic stage; OM, oocyte at mature stage. The mRNA expression levels of CYP19 were determined by real-time RT-PCR. Each bar represents the mean of normalized expression levels of replicates (n=5). Means marked with different letters are significantly different (P<0.05).

Figure 6 Relative mRNA levels of CYP19 in the ricefield eel during ovarian development. (A) cyp19a1a in the ovary; (B) cyp19a1b in the hypothalamus; and (C) cyp19a1b in the pituitary. PV, previtellogenic stage; EV, early vitellogenic stage; MLV, mid- to late-vitellogenic stage; OM, oocyte at mature stage. The mRNA expression levels of CYP19 were determined by real-time RT-PCR. Each bar represents the mean of normalized expression levels of replicates (n=5). Means marked with different letters are significantly different (P<0.05).

estrogen in stimulating the hepatic synthesis of vitellogenin in teleosts (Nagahama 1994). It is generally believed that the maturation of post-vitellogenic oocyte in teleosts is induced by maturation-inducing hormone (MIH), presumably 17α, 20β-dihydroxy-4-pregnane-3-one in some teleosts, and the steroidogenic shift from E2 to 17α, 20β-dihydroxy-4-pregnane-3-one is a prerequisite for the oocyte to undergo maturation (Senthilkumaran et al. 2004). The down-regulation of ovarian cyp19a1a expression in the ricefield eel at the mature stage was in agreement with this steroidogenic shift; however, the mechanism remains to be identified.

It is interesting to note that the expression levels of cyp19a1b in the pituitary was significantly increased at the mature stage when compared with MLV stage in the ricefield eel, suggesting that locally synthesized E2 in the pituitary may play a role in the oocyte final maturation. In catfish, a preovulatory induction of cyp19a1b in the pituitary was also detected, and suggested to be involved in the regulation of lhb subunit expression (Kazeto & Trant 2005). A half-site estrogen response element (ERE) is present in the promoter of lhb subunit (Yaron et al. 2001), and LH was shown to play a central role in the maturation of post-vitellogenic oocytes in lower vertebrates (Nagahama 1997). Based on these lines of evidence, it could be hypothesized that increased expression of cyp19a1b in the pituitary of the ricefield eel at mature stage might raise the local level of E2, which in turn increases the expression of lhb subunit, and LH promotes the final maturation of oocytes. In support of this hypothesis, it was observed that E2 up-regulated hypophyseal lhb subunit mRNA expression dose dependently in vitro in the female ricefield eel.

In the brain, however, the expression levels of cyp19a1b were significantly decreased at the mature stage when compared with MLV stage in the ricefield eel. This is in
Inhibition of non-mammalian vertebrates, such as fish, amphibians, reptiles, and birds (Piferrer & Blázquez 2005). In contrast to what has been reported in catfish, where the transcript abundance of cyp19a1b in the brain was significantly increased at the preovulatory stage (Kazeto & Trant 2005). In the protogynous wrasse, the expression of cyp19a1b in the brain was also gradually increased along reproductive seasons (Choi et al. 2005). It seems that the regulation of cyp19a1b gene in the brain of the ricefield eel is unique when compared with other teleosts. Consistent with this, we have found a mutated ERE (RGATCAnnnTGACCA instead of RGGTCAnnnTGACCY) in the 5’ flanking sequence of the ricefield eel cyp19a1b (unpublished data), while in other teleosts, this ERE site is perfectly conserved (Forlano et al. 2006).

Aromatase has been implicated on sex differentiation in non-mammalian vertebrates, such as fish, amphibians, reptiles, and birds (Piferrer & Blázquez 2005). Inhibition of aromatase activity has been shown to induce sex change from female to male in the protogynous honeycomb grouper (Bhandari et al. 2004, 2005) and the bidirectional sex-changing fish (Kroon et al. 2005). However, it is not clear whether only gonadal aromatase or both gonadal and brain aromatases were involved in these processes. To further clarify the involvement of CYP19 genes in the sex change of fish, the mRNA expression of cyp19a1a in the gonad and cyp19a1b in the brain and pituitary of the ricefield eel during natural sex change was examined using real-time PCR analysis. The gonadal mRNA level of cyp19a1a was continuously decreased from female phase, through intersexual phases, to male phase. These results are in agreement with the changes of gonadal cyp19a1a expression during MT-induced sex change from female to male in the orange-spotted grouper (Zhang et al. 2007), suggesting that the down-regulation of gonadal cyp19a1a gene is involved in the natural sex change of the ricefield eel.

It was demonstrated for the first time that the expression of cyp19a1b in the pituitary varied significantly during natural sex change of the ricefield eel, with highest levels at female phase, lowest levels at intersexual phases. The pituitary is an important component of reproductive endocrine axis in vertebrates and produces two gonadotropins, LH and follicle-stimulating hormone, which play central roles in gonadal development. It has been shown that mammalian LH induces a precocious sex change of the ricefield eel at the postspawning stage (Yeung et al. 1993), suggesting that the pituitary gonadotropins may be involved in the natural sex change of the ricefield eel. As stated above, locally synthesized E2 in the pituitary may regulate the production of LH, and thus may be involved indirectly in the sex change of the ricefield eel. However, the exact roles and mechanisms of locally synthesized E2 in the pituitary of the ricefield eel during sex change need further study.

During the natural sex change of the ricefield eel, the expression levels of cyp19a1b in the hypothalamus peaked at the IE phase. Similarly, a transient increase in the expression level of cyp19a1b gene in the hypothalamus at intersexual phase was also observed during MT-induced sex change of the orange-spotted grouper, a protogynous sex-changing fish (Zhang et al. 2004). In mammals, it has been shown that the conversion of androgens to estrogens in the brain is essential for brain sexual differentiation toward male pattern and activation of male sexual behavior (Lephart 1996, Balthazart & Ball 1998). In male Poecilia reticulate, a teleost fish, inhibition of cytochrome p450 brain aromatase reduced two male-specific sexual behaviors (Hallgren et al. 2006). In this context, it can be hypothesized that the increased expression of cyp19a1b in the hypothalamus at the IE phase may play a role in masculinizing the brain of the ricefield eel during sex change from female to male, which awaits further study.

In summary, cyp19a1a and cyp19a1b were differentially expressed in the gonad and brain of the ricefield eel, and the tissue distribution of cyp19a1b exhibited a novel sexual dimorphism. Both cyp19a1a and cyp19a1b may be involved...
in the ovarian development and natural sex change of the ricefield eel via the hypothalamus–pituitary–gonadal axis, but their exact roles remain to be identified.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

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