Two cytochrome P450 aromatase genes in the hermaphrodite 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, *cyp19a1a* and *cyp19a1b*, were isolated. The genomic organizations of both *cyp19* genes were conserved when compared with other teleosts. Northern blot detected an abundant expression of *cyp19a1a* in the ovary, and *cyp19a1b* in the hypothalamus. RT-PCR coupled with Southern blot showed that *cyp19a1a* was expressed predominantly in the gonads of both sexes, with higher levels in the ovary than testis, while *cyp19a1b* was expressed in all the tissues examined in the male, but only in the brain and pituitary in the female. The levels of *cyp19a1a* mRNA in the ovary were increased significantly during vitellogenesis, but decreased significantly at mature stage. The

levels of *cyp19a1b* mRNA in the brain and pituitary did not vary significantly during vitellogenesis. As ovarian development shifted from vitellogenesis to maturation, the levels of *cyp19a1b* mRNA was decreased significantly in the brain, but increased significantly in the pituitary. During natural sex change from female to male, the levels of *cyp19a1a* mRNA in the gonad were significantly decreased. The levels of *cyp19a1b* 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 *cyp19a1b* mRNA tissue distribution, and both *CYP19* genes were associated with the ovarian development and natural sex change of the ricefield eel.

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Introduction

Hermaphroditism is widespread among teleosts (Devlin & Nagahama 2002). In the sequential hermaphrodite 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 erythrosipilus*, 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 hermaphrodite 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 *cyp19a1a* and *cyp19a1b*, which were expressed mainly in the ovary and brain respectively, were identified in teleosts, such as *Carassius auratus* (Tchoudakova & Callard 1998), *Oncorhynchus mykiss* (Tanaka *et al.* 1992, Valle *et al.* 2002), *Danio rerio* (Kishida & Callard 2001), *Oreochromis niloticus* (Kwon *et al.* 2001), *Dicentrarchus labrax* (Blázquez & Piferrer 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 *cyp19a1a* was shown to be crucial for ovarian differentiation (Guiguen *et al.* 1999). In the protandrous gilthead seabream, differential expression of *cyp19a1a* gene in the gonad was shown to be associated with sex reversal (Wong *et al.* 2006). In the protogynous grouper, gonadal *cyp19a1a* expression was decreased during 17 α -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 & Piferrer 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 functional female, 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 Guiding Principles 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 gonadal 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, CYP19A1F6, 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

	Sequence (5'-3')
Primer name	
CYP19F1	WCSGCRGTGCAACTAGTACAA
CYP19R1	CATCTCCARCACRCACTG
CYP19R2	GGCTGGAAMWAAACGWCKVGG
CYP19A2R1	CTGTCTGCCAGGTCTCAAAGTA
CYP19A2R2	CGTGTCCAGAGGGAGCAGTC
CYP19A2F1	GGAGGATGATCTTGACTTTG
CYP19A2F2	CTAATCTTTGCCCAGAACCACGG
CYP19A1R1	GTAGTTGCTGGCTGTGCCTAT
CYP19A1R2	GCAGACGAGCAGCATCAAGG
CYP19A1F1	CGCCGTTACTTCCAGCCATTC
CYP19A1F2	ACTGCCTCCACAGACCAACA
GR5P	CGACTGGAGCACGAGGACACTGA
GR5NP	GGACACTGACATGGACTGAAGGAGTA
GR3P	GCTGTCAACGATACGCTACGTAACG
GR3NP	CGTACGTAACGGCATGACAGTG
CYP19A1F3	CGCTTGGACCGTTTCATTAGGC
CYP19A1F4	TCACCTAGACGACCTGGACAAT
CYP19A1F5	CGGACACTTTATCCATCAGCCTC
CYP19A1R4	CACAACAGTGTCAATTTCTCT
CYP19A1R5	GCTCAGAGTGTGTTGACAGCTACC
CYP19A2F3	TCAGTGGAAGTGCTGATGCTCT
CYP19A2F4	GAGTGTATTGGGATGGAAGGAA
CYP19A2F5	AGAGTTGAAGATAGTGGAGGAG
CYP19A2R3	CTGTCTGCCAGGTCTCAAAGTA
CYP19A2R4	TAGCCTTCAAAGACTGAAACTG
CYP19A2R5	CACATATTTTCAGAGCTGACTGG
CYP19A1F6	CATACTCAGCAGGTTCATCAGCG
CYP19A1R6	TCTCATTGACAGGTACACCA
CYP19A2F6	CAGAGGACAGTGGCGATCTGTG
CYP19A2R6	CATTGAGTGGCACCCTGAGGAA
LHF1	TCCTGGTGTGGACCCGACT
LHR1	CGTATGCTGTCTATTGTGG
β-actinF1	CGTACCACCGGAATTGTCA
β-actinR1	GTACCACGACAGCACAG
β-actinF2	GCGTGACATCAAGGAGAAGC
β-actinR2	CTCTGGGCAACGGAACCTCT

specific, and these primers were denoted at the end by either 'R' or 'F' to indicate reverse or forward primers respectively. Oligonucleotides GR3P and GR3NP were adapter primers for the 3'RACE, and oligonucleotide GR5P and GR5NP were adapter primers for the 5'RACE. Oligonucleotides β -actinF1, β -actinF2, β -actinR1, and β -actinR2 were designed according to ricefield eel β -actin (AY647143), and LHF1 and LHR1 according to ricefield eel luteinizing hormone (LH) β -subunit (lhb, EU840258).

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 (Invitrogen) according to the manufacturer's instructions. PCR was performed in 25 μ l final volume containing 2.5 μ l $10\times$ *Ex Taq* buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μ M of each primer, and 1.25 U *TaKaRa Ex Taq* DNA Polymerase (TaKaRa, DaLian, China) and 0.5 μ l 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 3'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 ClustalX1.81 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 μ g) 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 [α - 32 P]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 32 P-labeled *cyp19a1b* probe, washed twice with low stringency wash solution (equal to $2\times$ SSC, 0.1% SDS) at 50 °C for 10 min, twice with high stringency wash solution (equal to $0.1\times$ 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 32 P-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 μ g) 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 μ l) was amplified for each target gene using the Biometra TGRADIENT thermal cycler. PCR was performed in 15 μ l final volume containing 1.5 μ l 10 $\times$ reaction buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.2 μ M 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 μ g/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 10³-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 μ l containing 0.2 μ M of each primer, 10 μ l of 2 $\times$ SYBR Green Master Mix (TOYOBO, Osaka, Japan), 1 μ l of cDNA template which was transcribed from 1 μ g 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 $\pm$ 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,

Figure 1 (*continued*)

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-isoform 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

Ricefieldeel/A1	QTVEVCVSSTQTHLDDL----DNLGHVDVLSLLRCTVIDISNRLFLGVPVNEKELLLKIH	231
Grouper/A1	QTVEVSVSATQTHLDDL----DGLGHVDVLSLLRCTVVNISNRLFLDVPVSEKELLLKIL	231
Zebrafish/A1	RTMEICTTSTNSHLDDLSQLTDAQQLDILNLLRCIVVDVSNRLFLGVPPLNEHDLLQKIH	238
Ricefieldeel/A2	RTVAICVSSTAKHLDNLQEMTAPSGHVDALSLLRAIVVDISNRLFLRVPLNAKDLLVKIK	219
Grouper/A2	KSVGICVSSTAHLDSLQEMTDPSKHVDALSLLRAIVVDVSNKFLRVPLNAKDLLLKIN	218
Zebrafish/A2	KSVEVCVSATNRQLDVLQEFDTASGHVDVLNLLRCIVVDVSNRLFLRIPLNEKELLIKIH	218
Frog	QTTENCIRSTNHYLDNLSNVTNELGNVDVLKLMRLIMLDTSNLFLRIPLDESEIVLKIQ	216
Chicken	RMIAICVESTIVHLDKLEEVTEVGNVNLMMRRIMLDTSNKFLGVPPLDESAIVLKIQ	217
Mouse	RMVEVCVESIKQHLDRLGEVDTSGYVDVLTLMRHIMLDTSNMFLGIPLDESAIVKIKI	218
Human	RMVTVCAESLKTHLDRLEEVTNESGYVDVLTLLRRVMLDTSNLFLRIPLDESAIVKIKI	218
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Ricefieldeel/A1	KYFETWQGVLPKPDIFYKFGWIYKRHKAAARELQNAIKSLVEQKRRDMEQADKLDN-INF	290
Grouper/A1	KYFDTWQTVLIKPDVYKFDWIHQRHKTAAQELQDAIESLVEQKRRDMEQADKLDN-INF	290
Zebrafish/A1	KYFDTWQTVLIKPDVYFRDLWLHKKHKRDAQELQDAITALIEQKKVQLVHAEKLDH-LDF	297
Ricefieldeel/A2	NYFETWQTVLIKPDIFFKIGWLCNRHKRASKELQDAMESLLEIKRQMINETDKLDDDLDF	279
Grouper/A2	NYFDTWQTVLIMPDIFFKIGWLYNKHKRAAQELQDTMEALLECKRKMNHCEKLDEAFDF	278
Zebrafish/A2	RYFSTWQTVLIQPDIFFKLDFVYRKVHLAAKELQDEMCKLVEQKRQAINTEKLDE-MDF	277
Frog	KYFDAWQALLKPDIFFKISWLYKKEKSANDLKEATELLIEQKRQKLSSEKLDDEDMDF	276
Chicken	NYFDAWQALLKPDIFFKISWLCKKYEAAKDLKGAMEILIEQKRQKLSSTVEKLDEHMD	277
Mouse	GYFNAWQALLIKPNIFFKISWLYRKYERSVKDLKDEIAVLVEKRRHKVSTAEKLEDCMD	278
Human	GYFDAWQALLIKPDIFFKISWLYKKEKSVDKDKDAIEVLIAEKRCRISTEEKLEECMD	278
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Ricefieldeel/A1	TAELIFAQNHGELSAENVTCVLEMVIAAPDLSISLFFMLLLKQNPDELQLQEI	350
Grouper/A1	TAELIFAQNHGELSAENVRCVLEVIAAPDLSISLFFMLLLKQNPDELQLQGI	350
Zebrafish/A1	TAELIFAQSHGELSAENVRCVLEMVIAAPDLSISLFFMLLLKQNPDELKILQEMDS	357
Ricefieldeel/A2	ATELIFAQNHGELSDNVRCVLEMVIAAPDLSVSLFFMLLLKQNPDELKIVEEMT	339
Grouper/A2	ATELIFAQNVGELSDNVRCVLEMVIAAPDLSISLFFMLLLKQNPDELRIVEEMNT	338
Zebrafish/A2	ATELIFAQNHDELSDVDVRCVLEMVIAAPDLSISLFFMLLLKQNSAVEEQIVQEIQS	337
Frog	SSELIFAQNHGDLTAENVNQCILEMLIAAPDTMSVSLFFMLVLIQHPKIEEGIMNEMDK	336
Chicken	ASQLIFAQNRGDLTAENVNQCILEMLIAAPDTMSVTLFIMLILIAADPTVEEKMREIET	337
Mouse	ATDLIFAERRGDLTKENVNQCILEMLIAAPDTMSVTLFIMLLVAEYPEVEAAILKEIHT	338
Human	ATELILAERKRGDLTRENVNQCILEMLIAAPDTMSVSLFFMLFLIAKHPNVEEAIKEIQT	338
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Figure 1 (continued)

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.

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.

	Overall identity	Membrane spanning domain	I-helix region	Ozol's peptide region	Aromatic region	Heme-binding region
Ricefield eel/A1	62.5	46.2	82.9	65.2	66.7	91.7
Grouper/A1	62.9	30.0	86.0	69.6	66.7	91.7
Grouper/A2	80.4	69.2	82.9	78.3	91.7	91.7
Zebrafish/A1	62.1	38.5	85.7	60.9	66.7	87.5
Zebrafish/A2	63.5	53.8	82.9	73.9	75.0	87.5
Frog	53.4	30.8	68.6	69.6	75.0	79.2
Pleurodeles	53.3	35.7	71.4	69.6	83.3	75.0
Chicken	52.1	30.8	65.7	65.2	83.3	87.5
Mouse	49.9	46.2	57.1	73.9	75.0	75.0
Human	51.1	38.5	62.9	69.6	75.0	83.3

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 *cyp19a1a* 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. In the

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.

	Overall identity	Membrane spanning domain	I-helix region	Ozol's peptide region	Aromatic region	Heme-binding region
Ricefield eel/A2	62.5	46.2	82.9	65.2	66.7	91.7
Grouper/A1	87.6	68.8	94.3	95.7	83.3	100
Grouper/A2	61.1	50.0	88.6	78.3	66.7	83.3
Zebrafish/A1	64.0	46.7	97.1	78.3	66.7	95.8
Zebrafish/A2	58.7	26.7	88.6	78.3	83.3	79.2
Frog	55.4	26.7	71.4	56.5	83.3	83.3
Pleurodeles	52.5	50.0	65.7	56.5	83.3	70.8
Chicken	51.9	40.0	68.6	47.8	83.3	83.3
Mouse	51.7	40.0	62.9	60.9	83.3	75.0
Human	52.3	40.0	65.7	60.9	83.3	79.2

Figure 2 Phylogenetic analysis of vertebrate CYP19. The phylogenetic tree was generated by the maximum parsimony method of PHYLIP software package and visually depicted by TREEVIEW1.5. The CYP19 from *Drosophila melanogaster* (CAA80549) served as an outgroup to root the tree. The bootstrap values (%) were given at each branch point. The protein sequences were downloaded from Entrez (NCBI).

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, and at lower levels in the olfactory bulb, cerebellum, and medulla oblongata, 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 pre-vitellogenic 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 E_2 of different concentrations. Results showed that E_2 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

Figure 3 The schematic showing the relative positions of introns and exons of teleost *CYP19* genes. The exons are boxed and indicated by roman numerals at the bottom, and the introns are depicted by lines. The translated exons are shaded. The genomic structure was determined by comparing the sequences of the corresponding cDNA and genomic DNA, and the sequences of tilapia and zebrafish were downloaded from *Entrez* (NCBI): Nile tilapia *cyp19a1a* gene (AF472620) and cDNA(U72071), Nile tilapia *cyp19a1b* gene (AF472621) and cDNA(AF295761); Zebrafish *cyp19a1a* gene (NM131154) and cDNA(NC007129), Zebrafish *cyp19a1b* gene (NM131642) and cDNA(NC007136).

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, the relative mRNA 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

Figure 4 Northern blot analysis of *cyp19a1a* and *cyp19a1b* mRNA. Total RNA (30 µg) from the hypothalamus (Hy), ovary (Ov), and liver (Li) of female ricefield eel was separated on a 1% agarose gel (A), and hybridized with a *cyp19a1a* cDNA probe (B), or with a *cyp19a1b* cDNA probe (C) randomly labeled with [α -³²P]dCTP. The positions of 18S and 28S RNA were indicated by arrows and used as markers.

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.

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

Figure 6 Relative mRNA levels of *CYP19* in the ricefield eel during ovarian development. (A) *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 ($P<0.05$).

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* was significantly increased in the ovary of the ricefield eel during vitellogenesis, but then decreased significantly at mature stage. The similar patterns of *cyp19a1a* expression during oogenesis were also observed in red seabream (Gen *et al.* 2001) and gobiid fish (Kobayashi *et al.* 2004). The increase of ovarian *cyp19a1a* expression during vitellogenesis is in agreement with the role of gonadal

Figure 7 The effects of E_2 on *lhb* mRNA expression in the *in vitro* incubated pituitary of ricefield eel. The pituitary glands were taken from mature female ricefield eel and incubated for 24 h in the presence or absence (control, containing 0.1% ethanol) of E_2 (concentration at 1, 10, and 100 nM). The mRNA expression levels of *lhb* were determined by real-time RT-PCR. Each bar represents the mean of normalized expression levels of replicates ($n=3$). 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 E_2 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 E_2 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 E_2 , 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 E_2 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

Figure 8 Relative mRNA levels of *CYP19* in the ricefield eel during the natural sex change. (A) *cyp19a1a* in the gonad; (B) *cyp19a1b* in the hypothalamus; and (C) *cyp19a1b* in the pituitary. F, the female stage; IE, the early intersexual stage; IM, the middle intersexual stage; IL, the late intersexual stage; M, the male 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 ($n=4-5$). Means marked with different letters are significantly different ($P<0.05$).

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 (RGATCAnnn-TGACCA 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 E_2 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 E_2 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 reticulata*, 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.

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