# Molecular cloning of doublesex and mab-3-related transcription factor 1, forkhead transcription factor gene 2, and two types of cytochrome P450 aromatase in Southern catfish and their possible roles in sex differentiation

**Zhihao Liu[1,*], Fengrui Wu[1,*], Baowei Jiao[1,3,*], Xiuyue Zhang[1], Chongjiang Hu[1], Baofeng Huang[1], Linyan Zhou[1,2], Xigui Huang[1], Zhijian Wang[1], Yaoguang Zhang[1], Yoshitaka Nagahama[2], Christopher H K Cheng[3]** and **Deshou Wang[1]**

[1]Key Laboratory of Eco-environments in Three Gorges Reservoir Region (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, Chongqing 400715, China

[2]Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan

[3]Department of Biochemistry and the Environmental Science Programme, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, China

(Requests for offprints should be addressed to C H K Cheng; Email: chkcheng@cuhk.edu.hk; D Wang; Email: wdeshou@swu.edu.cn)

*(Z Liu, F Wu and B Jiao contributed equally to this work)

## Abstract

To address the roles of doublesex and mab-3-related transcription factor 1 (*Dmrt1*), forkhead transcription factor gene 2 (*Foxl2*), and aromatase in sex differentiation of Southern catfish, the cDNA sequences of these genes were isolated from the gonads. *Dmrt1a* and *Dmrt1b* were found to be expressed in the gonads, being higher in the testis. A low expression level of *Dmrt1b* was also detected in the intestine and kidney of the male. *Foxl2* was found to be expressed extensively in the brain (B), pituitary (P), gill and gonads (G), with the highest level in the ovary, indicating the possible involvement of *Foxl2* in the B–P–G axis. Cytochrome P450 (*Cyp)19b* was found to be expressed in the brain, spleen, and gonads, while *Cyp19a* was only expressed in the gonads and spleen. All-female Southern catfish fry were treated with fadrozole (F), tamoxifen (TAM), and 17β-estradiol ($E_2$) respectively, from 5 to 25 days after hatching (dah). The expression levels of these genes were measured at 65 dah. In the F-, TAM-, and F+TAM-treated groups, *Dmrt1a* and *Dmrt1b* were up-regulated in the gonad, whereas *Foxl2* and *Cyp19a* were down-regulated, while the expression of *Cyp19b* in the gonad remained unchanged. Furthermore, down-regulation of *Foxl2* and *Cyp19b* was also detected in the brain. In the $E_2$-treated group, *Dmrt1a* and *Dmrt1b* were down-regulated to an undetectable level in the gonad, whereas *Foxl2* and *Cyp19b* were up-regulated in the brain. Consistent with the observed changes in the expressions of these genes, 56, 70, and 80% sex-reversed male individuals were obtained in the F-, TAM-, and F+TAM-treated groups respectively. These results indicate the significant roles of *Dmrt1*, *Foxl2*, and *Cyp19* in the sex differentiation of Southern catfish.

*Journal of Endocrinology* (2007) **194,** 223–241

## Introduction

The doublesex and mab-3 (DM)-related transcription factor 1 (*Dmrt1*) belongs to the DM domain gene family. Among the different phyla of the animal kingdom including vertebrates, it is the only gene found to be conserved in structure and function in male sex determination and differentiation. Deletion of a chromosome segment containing *DMRT1* resulted in sex reversal in humans (Raymond *et al.* 1999*a*). Furthermore, *DMRT1* was found to be expressed in the genital ridge before sex differentiation, specifically in the testis but not in the ovary (De Grandi *et al.* 2000, Shan *et al.* 2000*b*). Mouse *Dmrt1* was also found to be expressed in the genital ridge during the sensitive period of sex determination and its expression was found to persist during the whole process of gonad differentiation. However, it was also found to be expressed in both XY and XX embryos, being higher in XY than in XX. Mutation of *Dmrt1* led to failure in producing differentiated testes resulting in germ cell death indicating that *Dmrt1* is a prerequisite for testis differentiation in postnatal mouse (Raymond *et al.* 2000). Also, a number of reports have demonstrated the essential roles of *Dmrt1* in sex determination and differentiation in birds and reptiles (Nanda *et al.* 1999, 2000, Shan *et al.* 2000*a*, Torres *et al.* 2002, Smith *et al.* 2003). In fish, *Dmrt1* was found to be expressed exclusively during the early stages of male gonad differentiation, but not in the female gonad in Nile tilapia (Guan *et al.* 2000) and rainbow trout (Marchand *et al.* 2000). Further research revealed that treatment with exogenous estrogen down-regulated *Dmrt1* expression in rainbow trout (Marchand *et al.*


DOI: 10.1677/JOE-07-0135
0022–0795/07/0194–223 

2000). However, there is little information on the influence of aromatase inhibitor on *Dmrt1* expression in fish.

Winged helix/forkhead transcription factor gene 2 (*Foxl2*), a member of the forkhead (*FH*) gene family, is a newly isolated transcription factor expressed specifically in the eyelid and adult ovary (Cocquet *et al.* 2003, Loffler *et al.* 2003, Baron *et al.* 2004, Govoroun *et al.* 2004). Mutation of *Foxl2* gives rise to various diseases, such as blepharophimosis-ptosis-epicanthus inversus syndrome and premature ovarian failure in humans (Cocquet *et al.* 2003) as well as polled intersex syndrome (PIS) in goats (Pailhoux *et al.* 2001, 2002, Nikic & Vaiman 2004). During early human development, *Foxl2* plays a crucial role in female reproduction, especially in differentiation and proliferation of granulosa cells, ovarian development, and maintenance of ovarian function by regulating the transcription of certain target genes (Cocquet *et al.* 2003, Loffler *et al.* 2003, Pisarska *et al.* 2004, Yao 2005). Because of its expression in the early genital ridge and its inhibitory action on the male differentiation pathway (Ottolenghi *et al.* 2005), *Foxl2* is recognized as the earliest sex dimorphic marker of ovarian determination and differentiation in mammals. In mice, it is first detected in the developing ovaries at 13·5 days post conception (dpc) and its expression is detected throughout fetal development to the first day of postpartum (Loffler *et al.* 2003). In medaka (*Oryzias latipes*), *Foxl2* is not involved in ovarian determination, but in differentiation of the granulosa cells (Nakamoto *et al.* 2006). In tilapia (*Oreochromis niloticus*), expression of *Foxl2* begins early during differentiation of the gonads and persists until adulthood (Wang *et al.* 2004).

The roles of estrogen in fish reproduction have been particularly well studied because of the diverse reproductive strategies found in this group of vertebrates. The complex effects of estrogen on fish sex differentiation are mediated largely through changes in aromatase activity and expression. As a key enzyme responsible for estrogen synthesis, much research has been focused on this enzyme. Until now, two aromatase genes (*Cyp19a* and *Cyp19b*) have been identified in fish (Chiang *et al.* 2001, Kwon *et al.* 2001). Several reports have demonstrated the involvement of aromatase in fish gonad differentiation (Chang *et al.* 1997, Kitano *et al.* 1999) and oocyte maturation (Bobe *et al.* 2006).

In tilapia, *Cyp19a* was found to be highly expressed in females during early sex differentiation, but dramatically decreased in males between 15 and 27 days after hatching (dah; Kwon *et al.* 2001). A similar situation was found in rainbow trout where aromatase expression was found to be elevated by 100-fold in ovaries before sex differentiation (Guiguen *et al.* 1999). Previous findings strongly indicate that *Cyp19a* plays a decisive role in sex differentiation in these species. However, other reports also demonstrated the important role of *Cyp19b* in zebrafish gonad sex differentiation (Kishida & Callard 2001, Trant *et al.* 2001). It was found that estradiol, ethinylestradiol, and 17-methyltesterone could enhance the production of *Cyp19b* mRNA in zebrafish embryos. Recent reports on sea bass (Blazquez & Piferrer 2004) also revealed sex-related differences in the expression profiles of *Cyp19b* in the brain during sex differentiation. These results suggest that *Cyp19b* is also involved in fish sex differentiation. Since *Cyp19a* expression is mainly restricted to the gonads, this gene is probably directly involved in the sex differentiation of the gonads (Chang *et al.* 1997, Guiguen *et al.* 1999, Kitano *et al.* 1999, Kwon *et al.* 2001). On the other hand, since *Cyp19b* is mainly expressed in the brain, this gene is conceivably involved in sex differentiation in an indirect manner (Kwon *et al.* 2001, Chang *et al.* 2005, Kazeto & Trant 2005, Sawyer *et al.* 2006), probably through the brain-pituitary-gonad axis.

Both *Dmrt1* and *Foxl2* are important somatic markers related to the sexual phenotypes and are highly correlated with aromatase activity. Several reports have shown that Foxl2 and aromatase are co-localized in the somatic cells of the developing XX gonads at both the mRNA and protein levels (Nakamoto *et al.* 2006, Pannetier *et al.* 2006). Further investigations in mammals have indicated that Foxl2 activates *Cyp19* gene transcription by direct binding to the promoter (Pannetier *et al.* 2006). Our studies on tilapia have also demonstrated that the regulation of Foxl2 on *Cyp19a* gene transcription is in a female-specific manner (Wang *et al.* 2007). Although located upstream of *Cyp19* and considered the earliest known gene exhibiting sexual dimorphic expression patterns in ovarian somatic cells (Baron *et al.* 2005, Nakamoto *et al.* 2006), information on how *Foxl2* regulates sex differentiation, especially its regulation by other genes essential in sex differentiation, is scarce. Actually some reports in chicken (Hudson *et al.* 2005) have indicated the regulatory action of aromatase on *Foxl2*. Inspired by this study, we have therefore studied the effects of estrogen and aromatase on the expression of *Foxl2* and *Dmrt1* in Southern catfish.

Southern catfish is a good model for studying gonadal sex differentiation in fish. Under laboratory conditions, the fry develop into all-female adult fish without any treatment. Our previous work has revealed the histology of the gonad during the early stages of sex differentiation in this all-female population (Zhang *et al.* 2005). However, the mechanism of this process is still unknown. Here, we report the isolation of *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* cDNAs and their gene expression patterns in Southern catfish. We have also studied the influence of the aromatase inhibitor fadrozole (F), the estrogen receptor antagonist tamoxifen (TAM), and the natural estrogen 17β-estradiol ($E_2$) on the expression levels of these genes in this species. The involvement of these genes in sex differentiation in Southern catfish is discussed.

## Materials and Methods

### *Animals*

Adult Southern catfish, *Silurus meridionalis* Chen, obtained from the Jia Ling River in Chongqing, a tributary of the Yangtze River, were reared in aerated tanks until use. The fry used in the experiments were obtained by artificial

propagation using parental fish raised in our laboratory. To reduce variations, fry hatched from the same parental fish were used in the experiments. All animal experiments conformed to the Guideline for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

### *Chemicals and reagents*

The steroid hormone ($E_2$) was purchased from Sigma. The non-steroid aromatase inhibitor (F) and the estrogen receptor antagonist (TAM) were from Novartis Company (AG, Switzerland) and Egis (Budapest, Hungary) respectively. All the primers used in the present study were synthesized by Invitrogen Life Technologies.

### *Cloning of Dmrt1a and Dmrt1b, Foxl2, Cyp19a, and Cyp19b cDNAs*

Total RNAs from the Southern catfish tissues were prepared using Tripure Reagent (Boehringer, Mannheim, Germany). The first strand cDNA was synthesized with 5 μg total RNA from gonadal tissues using oligo-$dT_{18}$ primer and M-Mulv reverse transcriptase (Promega) according to the manufacturer's instructions. Using the cDNA as template, degenerate or gene-specific primers were added to the PCR mixtures. The PCR conditions consisted of 94 °C (2 min), followed by 36 cycles of 94 °C (30 s), 57–65 °C (45 s), and 72 °C (1–1·5 min). The reaction was ended by a further 10 min at 72 °C. PCR was performed on a PTC-100 thermal cycler (Bio-Rad). All the primers used in the present study are listed in Table 1. These primers were designed based on the conserved regions of the known *Dmrt1*, *Foxl2*, and *Cyp19* sequences. The PCR products were resolved on a 1·2% agarose gel and the target DNA fragments were purified using QIAquick Gel Extraction Kit (Qiagen). The fragments were then cloned into pGEM-T vector (Promega) and bi-directional sequencing was performed by the dideoxy chain termination method using an ABI PRISM 377 DNA genetic analyzer (Sangon, Shanghai, China).

Then, 5′- and 3′-RACE were performed using the SMART RACE Kit (Clontech) according to the manufacturer's instructions and using the primers (Table 1) designed according to the cloned cDNA fragment sequences. After sequencing of the RACE products, five pairs of primers designed in the untranslated regions (UTR; Table 1) were used to amplify the entire coding regions from the gonad first-strand cDNA. The products were then sequenced again to confirm their DNA sequences.

### *Sequence analyses*

Alignment of nucleotide sequences and their deduced amino acid (aa) sequences was performed using the multiple alignment software DNAStar and ClustalX. ClustalX was also employed to calculate and display the phylogenetic trees using the N-J method. The values represent bootstrap scores of 1000 trials, indicating the credibility of each branch. All the nucleotide and aa sequences used in the phylogenetic analysis, except those from the Southern catfish, were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). The GenBank accession numbers of these sequences are as follows: 1) Dmrt1: human (AF130728), mouse (NM_015826), dog (XP_851495), chicken (AAF19666), turtle (AB179697), tetraodon (AY152820), fugu (NM_001037949), pejerrey fish (AY319416), platyfish (AF529187), medaka (AF319994), rainbow trout (AF209095), African catfish (AF439561), Nile tilapia (AF203489), black seabream (AY323953), sturgeon (AY057061), zebrafish (NP_991191); 2) Foxl2: human (AF301906), goat (AY112725), mouse (AF522275), pig (AY340971), rabbit (AY340972), cattle (AY340970), Tammar wallaby (AY340969), chicken (AY487165), fugu (CAAB01001061), tetraodon (CAG06418), Nile tilapia (AY554172), zebrafish (XM_693823), rainbow trout (AY507927), medaka (AB252055), ciona (BAE06446); 3) Cyp19a: sea bass (CAC43178), gilthead seabream (AAL27699), black seabream (AAP23236), red seabream (BAB82524), Atlantic halibut (CAC36394), bastard halibut (BAA74777), Mozambique tilapia (AAD31031), Nile tilapia (AAO62625), channel catfish (AAB32613), goldfish (AAC14013), zebrafish (NP_571229); 4) Cyp19b: Nile tilapia (AAO62626), channel catfish (AAL14612), goldfish (AAB39408), zebrafish (NP_571717), fathead minnow (CAC38767); and 5) Cyp19: Atlantic stingray (AAF04617), African clawed frog (BAA90529), chicken (AAA48739), American alligator (AAK31803), red-eared slider turtle (AAG09376), mouse (NP_031836), rat (NP_058781), human (NP_112503), monkey (AAK58465), pig (AAB51387), cattle (NP_776730), goat (AAN23836), sheep (CAB40543).

### *Analysis of Dmrt1a, Dmrt1b, Foxl2, Cyp19a, and Cyp19b expression patterns by RT-PCR*

Total RNA (5 μg) was isolated from various tissues of adult Southern catfish, and treated with DNase I (Promega) to remove any possible genomic contamination. All treated total RNA was used to synthesize the cDNA. Reverse transcription was carried out using M-Mulv reverse transcription polymerase (Promega) and total RNAs from adult Southern catfish tissues according to the manufacturer's instructions. The synthesized cDNA was diluted by twofold until use. The quality of the synthesized cDNA was assured by standard RT-PCR together with the negative (in the absence of template), positive (in the presence of the cloned cDNA), and genomic DNA contamination (the template derived from an equal amount of RNA without reverse transcription) controls. Then RT-PCR was employed to analyze the expression patterns of *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b*. The PCR conditions for the target genes consisted of 94 °C (2 min), followed by 28–32 cycles of 94 °C (30 s), 57–65 °C (45 s), and 72 °C (1–1·5 min), ending with 10 min of extension at 72 °C. The following pairs of primers (Table 1)

**Table 1** Sequence of primers used in the present study

| | Sequence | Purpose |
|---|---|---|
| **Primer** | | |
| *Dmrt1*-F1 | 5′-CTGAAGGGCCACAAGCGCTT-3′ | cDNA fragment PCR |
| *Dmrt1*-R2 | 5′-TAGTAGGAGTGCATACGGTACTG-3′ | |
| *Foxl2*-F2 | 5′-TG(CT)GAGGA(CT)ATGTT(CT)GAGAAGGG-3′ | cDNA fragment PCR |
| *Foxl2*-R1 | 5′-CCCA(GA)TA(TCA)GA(GA)CA(GA)TGCATCAT-3′ | |
| b-F1 | 5′-TGGATCAA(G)C(T)GGAGAA(G)GAA(G)AC-3′ | cDNA fragment PCR |
| b-R1 | 5′-TTCAGA(G)ATG(T)TAGGTTG(TC)GTT(GC)CC-3′ | |
| a-F1 | 5′-TTGAAGCACTCGCAGTACACGT-3′ | cDNA fragment PCR |
| a-R1 | 5′-TGTATCTCAGTGAGGATGCGT-3′ | |
| *Dmrt1*-RACE-F1 | 5′-GGAGACAGCAGGCTCAGGAGGAGGA-3′ | RACE |
| *Dmrt1*-RACE-F2 | 5′-GATCGCCTTCCTCCAGCAGCACCGGAT-3′ | RACE |
| *Dmrt1*-RACE-F3 | 5′-CCTGGTGGTGGATGCTTCCTACTACAA-3′ | RACE |
| *Dmrt1*-RACE-F4 | 5′-GCTGTCCAGCCACAACATGTCCCAG-3′ | RACE |
| *Dmrt1*-RACE-R1 | 5′-TTGTAGTAGGAAGCATCCACCACCAGG-3′ | RACE |
| *Dmrt1*-RACE-R2 | 5′-CCGGTGCTGCTGGAGGAAGGCGATCTC-3′ | RACE |
| *Dmrt1*-RACE-R3 | 5′-TCCTCCTCCTGAGCCTGCTGTCTCCTTA-3′ | RACE |
| *Foxl2*-RACE-F1 | 5′-ACCGCCAACGTCACACTTCCAAGC-3′ | RACE and tissue distribution |
| *Foxl2*-RACE-F2 | 5′-CCTCCTGCGCCCATGTCCTACACGTC-3′ | RACE |
| *Foxl2*-RACE-F3 | 5′-ACCGGGCTTCAGTTCGCGTGCTCGC-3′ | RACE |
| *Foxl2*-RACE-R1 | 5′-GCGAGCACGCGAACTGAAGCCCGGT-3′ | RACE and tissue distribution |
| *Foxl2*-RACE-R2 | 5′-GTGTAGGACATGGGCGCAGGAGGTT-3′ | RACE |
| *Foxl2*-RACE-R3 | 5′-GCGAGCACGCGAACTGAAGCCCGGT-3′ | RACE |
| b-F2 | 5′-GAGATGGGGAGGCTCGTGGAGCA-3′ | RACE and tissue distribution |
| b-F3 | 5′-AGGCAGTGTGTGTTGGAGATGGTGA-3′ | RACE |
| b-R3 | 5′-CACTACACAGCGTAGGAAGTTCAGAGCA-3′ | RACE |
| b-R4 | 5′-CCTTGTTCATCCATACCGATGCAC-3′ | RACE |
| b-R5 | 5′-CTACAGTGAAGCCTTCAATGAC-3′ | RACE and tissue distribution |
| a-F1 | 5′-TTGAAGCACTCGCAGTACACGT-3′ | RACE and tissue distribution |
| a-F2 | 5′-CTGCAGAGGACATTGGAAATCTGCACC-3′ | RACE |
| a-F3 | 5′-AACTTGACAACCTGAACTTCACTGAG-3′ | RACE |
| a-R2 | 5′-TGAAGCTCCTTAGCAGCGTTCCTG-3′ | RACE and tissue distribution |
| a-R3 | 5′-TTAGAGCTTTAGTGAAGTACACGCGCACT-3′ | RACE |
| a-R4 | 5′-CCCAGTTTACTGCCGAACCTCGACGT-3′ | RACE |
| *Dmrt1*-UTR-F | 5′-AGCAGCAGCAGTAGCACGA-3′ | Full-length cDNA amplification and tissue distribution |
| *Dmrt1*-UTR-R | 5′-GTCAGTCACAGTAACACAGTGCAG -3′ | |
| *Dmrt1*-ASSR | 5′-CTCTGCACAGGAGGTCTGTAGTAC -3′ | |
| *Foxl2*-UTR-F | 5′-CTTCAGAGGCACTGCGTTTGTG-3′ | Full-length cDNA amplification |
| *Foxl2*-UTR-R | 5′-GGTGAGATTCCCGTGTTTGGTT-3′ | |
| b-UTR-F | 5′-AGCCTGCGAACTTCAGCCAGACTCACA-3′ | Full-length cDNA amplification |
| b-UTR-R | 5′-GCTTTCCATTCTTTTATTAGTTTCT-3′ | |
| a-UTR-F | 5′-ACACAGAACTCGGTTCTCCGTTCC-3′ | Full-length cDNA amplification |
| a-UTR-R | 5′-CGCCTTTATCTTCATTCCCTCACA-3′ | |
| β-Actin-F | 5′-CCATCTCCTGCTCGAAGTC-3′ | Internal control |
| β-Actin-R | 5′-CACTGCCCATCTACGAG-3′ | |

were used to amplify the respective cDNA fragments: *Dmrt1a* (*Dmrt1*-UTR-F and *Dmrt1*-UTR-R giving a 1143 bp cDNA fragment), *Dmrt1b* (*Dmrt1*-UTR-F and *Dmrt1*-ASSR giving a 1221 bp cDNA fragment), *Foxl2* (*Foxl2*-F2 and *Foxl2*-R1 giving a 379 bp cDNA fragment), *Cyp19a* (a-F1 and a-R1 giving a 689 bp cDNA fragment), and *Cyp19b* (b-F1 and b-R1 giving a 404 bp cDNA fragment). A 900 bp Southern catfish β-actin fragment was amplified (28 cycles) using a pair of β-actin primers (Table 1) as the internal control. All the PCR products were resolved on 1·0% agarose gels and then stained with ethidium bromide to visualize the bands.

As members of the DM domain gene family in one species are rather conserved, especially in the DM-domain, the two *Dmrt1* isoforms of Southern catfish are quite similar except at the UTRs. In order to ensure the specificity of the amplification of the two *Dmrt1* isoforms, the forward and reverse primers were designed at the 5′- and 3′-UTRs of the two isoforms respectively. This resulted in relatively large size amplicons. It is known that different size amplicons are amplified at different rates over a PCR run. Therefore, an initial validation study was performed in each case to ensure that the cycle number chosen for each target was within the linear portion of the PCR amplification. In fact the PCR

conditions that we have adopted are very close to similar studies reported in the literature. Generally speaking, the expression levels of transcription factors, such as *Dmrt1* and *Foxl2* are relatively low in the gonad. In the work done on lizard (Sreenivasulu *et al.* 2002) and mouse (Loffler *et al.* 2003), 36 and 35 cycles were used to measure the *Dmrt1* expression level respectively, while in the mouse (Loffler *et al.* 2003) and medaka (Nakamoto *et al.* 2006), 33 cycles and 30–35 cycles were used to measure the *Foxl2* expression levels respectively. As for aromatases, 30–35 cycles were used to measure the expression levels of these genes (Choi *et al.* 2005).

### *Drug treatment*

Southern catfish all-female fry (300 fry/aquarium) were reared in aerated 30×30×50 cm aquaria. The water temperature was kept at 28±1 °C. The drugs (F, TAM and $E_2$) were dissolved in 95% ethanol and added to the fish feed at the following concentrations: F, 100 μg/g; TAM, 25 μg/g; F+TAM, 100 μg/g+25 μg/g; $E_2$, 25 μg/g. The vehicle ethanol was added to the control feed. Drug treatment was applied to the fry from 5 to 25 dah, the critical period of sex differentiation in Southern catfish.

### *Analysis of drug treatment on Dmrt1a, Dmrt1b, Foxl2, Cyp19a, and Cyp19b gene expression levels by semi-quantitative RT-PCR*

Brains and gonads were dissected from individual fish of each experimental group at 65 dah. First strand cDNA was prepared from the tissues. The quality of the synthesized cDNA was checked as described in the tissue distribution studies. Semi-quantitative RT-PCR (Zhang & Gui 2004) was performed to measure the mRNA levels using the primers described in the previous section. A series of PCRs with different cycle numbers (from 22 to 36, with an interval of 2) were performed to determine the linear phase of the amplification. Based on these pilot experiments, 28 cycles for β-actin and 30, 32, 28, 30, 32 cycles for the target genes (*Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* respectively) were chosen and applied to the subsequent semi-quantitative RT-PCR analyses. These initial validation studies were essential to ensure the semi-quantitative nature of the mRNA quantitation so that the amount of the amplified product was proportional to the amount of the target template in the samples. Band intensities resulting from the PCR amplification were analyzed using the image analysis software Quantity One (Bio-Rad). *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* mRNA levels were expressed relative to that of β-actin in each sample. In our study, β-actin expression was found to be unaffected by the drug treatment, as reported previously for $E_2$ (Matsumura *et al.* 2004) and TAM (Parte *et al.* 2002).

Data analyses were performed using one-way ANOVA and the least significant difference on the GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA).

### *Histological studies*

Gonads of 120 dah fish from the drug treatment groups and the control group were isolated for histological observation as described previously (Zhang *et al.* 2005). Briefly, the gonads were dissected out and fixed in Bouin's solution for 24 h at room temperature, and subsequently dehydrated, embedded in paraffin, and then serially sectioned at 6 μm thickness. The sections were counterstained with hematoxylin–eosin.

## Results

### *Sequences of Southern catfish Dmrt1a, Dmrt1b, Foxl2, Cyp19a, and Cyp19b cDNAs*

The isolated *Dmrt1a* cDNA is 1347 bp long, with an open reading frame (ORF) of 891 bp encoding a putative protein of 296 aa (GenBank accession no. EF015487). Southern catfish Dmrt1a contains the characteristic 62 aa DNA-binding domain (the DM-domain). It exhibits the highest homology to those isolated from African catfish (78·3%), sturgeon (59·7%) and pejerrey fish (58·6%). It also shares around 48% homology to those isolated from mammals.

The isolated *Dmrt1b* cDNA is 1513 bp long, with an ORF of 816 bp encoding a putative protein of 271 aa (GenBank accession no. EF015488). *Dmrt1b* is a 3′-alternatively spliced form of the *Dmrt1* gene. It contains the same 62 aa DM-domain as in Dmrt1a, but with a different C-terminus.

The isolated Southern catfish *Foxl2* cDNA is 1455 bp long, with an ORF of 900 bp encoding a putative protein of 299 aa (GenBank accession no. EF015396) containing the characteristic 105 aa DNA-binding domain (the FH-domain). It shares the highest homology with those isolated from the Nile tilapia (79·9%) and fugu (79·8%), and the lowest homology to that from human (58·3%).

The isolated *Cyp19a* cDNA is 2168 bp long, with an ORF encoding a putative protein of 540 aa (GenBank accession no. AAP83133). The isolated *Cyp19b* cDNA is 2337 bp long, with an ORF encoding a putative protein of 507 aa (GenBank accession no. AAP83132). They share the highest homology with the channel catfish Cyp19a (83%) and Cyp19b (86%) respectively.

### *Sequence analyses*

Alignment of the Southern catfish Dmrt1 with those from other vertebrates shows that it is highly conserved, especially in the DM-domain (>86·2% homology), the male-specific motif and the proline/serine (P/S)-rich region near the C-terminus. In addition, the N-termini upstream of the DM-domain and the male-specific motif in Southern catfish

Dmrt1 isoforms are shorter by about 40 aa than the mammalian counterparts (Fig. 1). Based on an alignment of 17 Dmrt1 sequences, a phylogenetic tree was constructed using the fugu Dmrt2 as the outgroup. The high homology of this protein between Southern catfish and African catfish, as well as among other teleosts, is reflected in the tree (Fig. 2).

As shown in Fig. 3, Southern catfish Foxl2 is highly conserved in the FH domain. Outside the FH domain, the C-terminal region is more conserved than the N-terminal region. However, like other non-mammalian counterparts, the Southern catfish Foxl2 contains neither the 14-poly-alanine tract nor the glycine and proline repeats, which are present in all mammalian Foxl2 sequences (Fig. 3). Based on the alignment of 14 complete and 2 partial Foxl2 sequences, a phylogenetic tree was constructed using mouse Foxl1 as the outgroup. The high conservation of this protein in vertebrates, especially among mammals and fish respectively, is reflected in the tree (Fig. 4).

Alignment of the cloned Cyp19 sequences revealed that the Southern catfish aromatases exhibit high homology with the counterparts from other vertebrates. Regions responsible for membrane spanning, α-helix, Ozol's peptides (the steroid substrate-binding domain), aromatic residue-rich, and heme-binding could be identified (Fig. 5). These regions share high homologies with those from other species. It can be seen from the tree (Fig. 6) that the Southern catfish Cyp19a and Cyp19b are clustered into two different clades.

### *Expression of Dmrt1a, Dmrt1b, Foxl2, Cyp19a, and Cyp19b in Southern catfish tissues*

*Dmrt1a* was found to be exclusively expressed in the gonads, being higher in the testis than in the ovary. *Dmrt1b* was also expressed predominantly in the gonads, again being higher in the testis than in the ovary. However, a low expression of *Dmrt1b* was also detected in the intestine and kidney (Fig. 7). On the other hand, *Foxl2* was expressed in the brain, pituitary, gill, and gonads, with the highest level in the ovary.

*Cyp19a* was found to be expressed in the gonads and spleen, being highest in the ovary and lowest in the spleen. No expression was detected in other tissues. *Cyp19b* was expressed in the brain, gonads, and spleen in both male and female fish, being the highest in the brain and lowest in the spleen (Fig. 7).

### *Effect of drug treatment on gene expression*

The semi-quantitative RT-PCR results demonstrated that *Dmrt1a* and *Dmrt1b* mRNA levels were significantly increased in the gonad (Fig. 8), whereas *Foxl2* mRNA levels were decreased by treatment with F, TAM, or F+TAM both in the brain and in the ovary (Fig. 9). On the other hand, *Dmrt1a* and *Dmrt1b* mRNA levels in the $E_2$ treatment group did not exhibit any change (Fig. 8) in comparison with the control group, while *Foxl2* mRNA level was increased (Fig. 9).

The expression of *Cyp19a* mRNA in the gonad, as well as that of *Cyp19b* in the brain, was significantly down-regulated in groups treated with F, TAM, or F+TAM (Figs 10 and 11). In contrast, the expression of *Cyp19b* in the gonad of these groups did not show any significant change as compared with the control fish (Fig. 11). On the other hand, the expression of both *Cyp19a* and *Cyp19b* mRNA in the gonad was significantly up-regulated when treated with $E_2$ (Figs 10 and 11).

### *Gonad histology of the drug-treated fish*

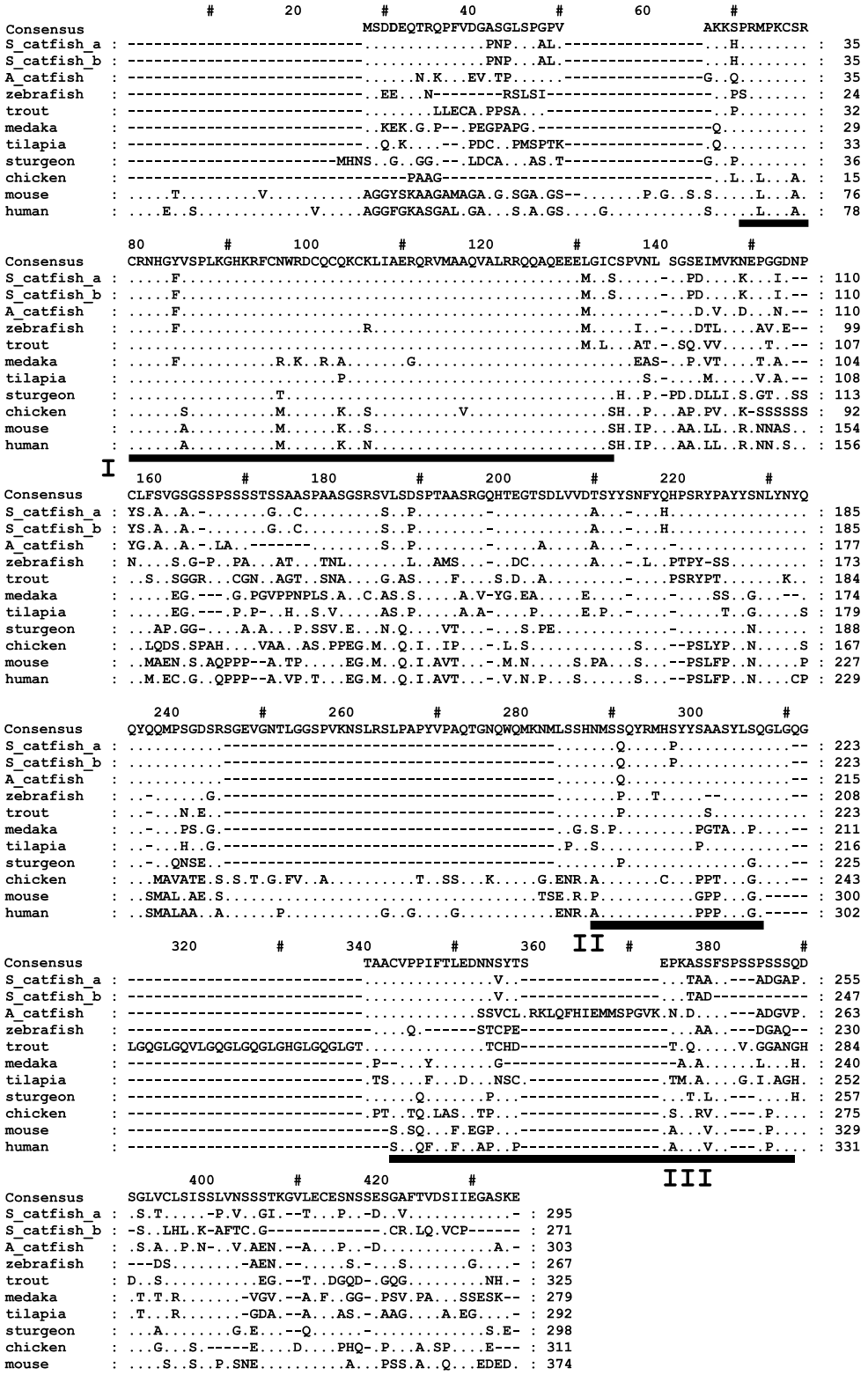
At 120 dah, the gonads of the drug-treated fish were studied histologically. Partial sex reversal was observed in the F, TAM, and F+TAM treatment groups. The ovarian walls of these fish were thickened and the ovarian cavity shrunk (Fig. 12D–F). In the F treatment group (Fig. 12D), many degenerating follicles were found and the oocyte number was decreased because of this degeneration, while the numbers of somatic cells were increased as compared with the control (Fig. 12A and B). In the TAM treatment group (Fig. 12E), some cava, which might be the space left behind by the degenerated follicles, were found. Moreover, elongated ellipse-shaped oocytes, which are different from the round- or ellipse-shaped oocytes in the control group, were also found. In the F+TAM treatment group (Fig. 12F), spermatocyst-like structure, spermatogonia, and primary spermatocytes were observed. Complete sex reversal was also observed in the F (56 out of 100 examined fish), the TAM (70 out of 100 examined fish), and the F+TAM (80 out of 100) treatment groups. These sex-reversed gonads showed typical testicular structures (Fig. 12C). On the other hand, the gonad histology of the $E_2$ treatment fish was the same as the control group.

## Discussion

### *Sequence analysis and tissue distribution of Dmrt1s, Foxl2, and Cyp19s in Southern catfish*

In the present study, Southern catfish *Dmrt1a* and *Dmrt1b* cDNAs were cloned and the encoded polypeptides were shown to contain the characteristic DM-domain shared by all DM proteins. The DM-domain in Southern catfish Dmrt1a and Dmrt1b exhibited very high homology (>86·2%) with

**Figure 1** Alignment of the deduced aa sequences of the Southern catfish Dmrt1 with those from other vertebrates. The sources of the sequences are described in the Materials and Methods section. Regions of high homology are underlined and indicated by Roman numerals: I, the DM-domain; II, the male-specific motif; III, the P/S rich region. The computer programs CLUSTALX and GeneDoc (http://www.psc.edu/biomed/genedoc) were used to construct this figure.



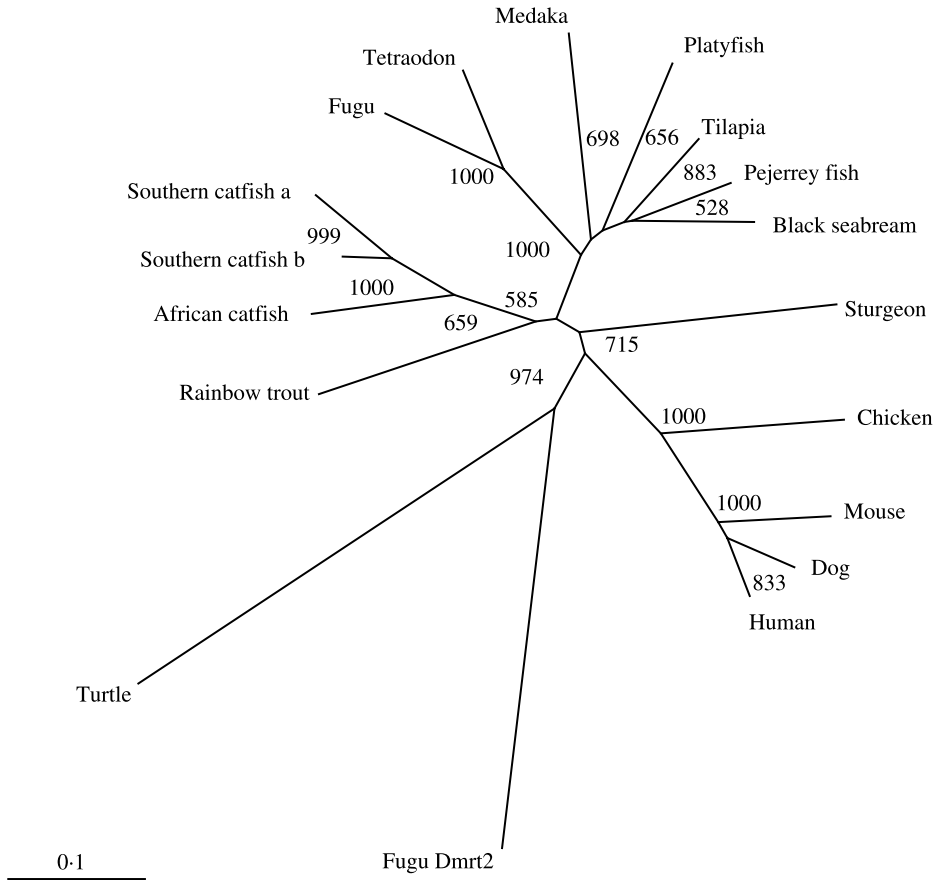



**Figure 2** Phylogenetic tree of Dmrt1 in vertebrates. The tree was rooted using fugu Dmrt2 (CAC42780) as the outgroup. Branch lengths are proportional to the number of aa changes on the branch. The sources of the sequences are described in the Materials and Methods section.

the counterparts from other vertebrates, suggesting that the DM-domain of Dmrt1 is highly conserved during evolution. In the tissue distribution studies, the Southern catfish *Dmrt1a* is expressed exclusively in the gonads, being higher in the testis than in the ovary. This is consistent with the results reported in other vertebrates (Raymond *et al.* 1999*a*,*b*, Guan *et al.* 2000, Kettlewell *et al.* 2000, Marchand *et al.* 2000, Nanda *et al.* 2000, Shibata *et al.* 2002, Torres *et al.* 2002, Smith *et al.* 2003). Due to its early expression in the testis and its high expression level during subsequent gonad development and spermatogenesis, *Dmrt1* is therefore believed to be essential for testis differentiation and maintenance of male characters in vertebrates (Raymond *et al.* 1999*a*, 2000, Guan *et al.* 2000, Kettlewell *et al.* 2000, Marchand *et al.* 2000, Shibata *et al.* 2002, Smith *et al.* 2003). Results of the present study, therefore, provide supporting evidence of the involvement of *Dmrt1* in male sex differentiation in fish.

We have successfully obtained the 3′-alternatively spliced isoform of *Dmrt1a*, and named it as *Dmrt1b*. The tissue distribution results demonstrated that *Dmrt1b* is also found in the gonads, again being higher in the testis than in the ovary. However, unlike *Dmrt1a*, which is exclusively expressed in the gonads, *Dmrt1b* is extensively expressed in other non-gonadal tissues including the intestine and kidney of the male fish, indicating that this isoform may have other functions. Some previous reports also demonstrated the expression of *Dmrt1* outside the gonad such as in the chicken Mullerian tract (Shan *et al.* 2000*b*, Smith *et al.* 2003). The Mullerian tract still belongs to the reproductive system, but the intestine and kidney are not part of the reproductive system. To our knowledge, this is the first report indicating that *Dmrt1* may operate in other non-reproductive systems as well.

In mammals, *Foxl2* is highly expressed in the ovary and is commonly regarded as female-specific (Cocquet *et al.* 2002, Loffler *et al.* 2003, Pannetier *et al.* 2003). In birds, expression of *Foxl2* is predominantly observed in the ovary (Govoroun *et al.* 2004). In fish, studies from tilapia and medaka demonstrated that the expression of *Foxl2* starts in the

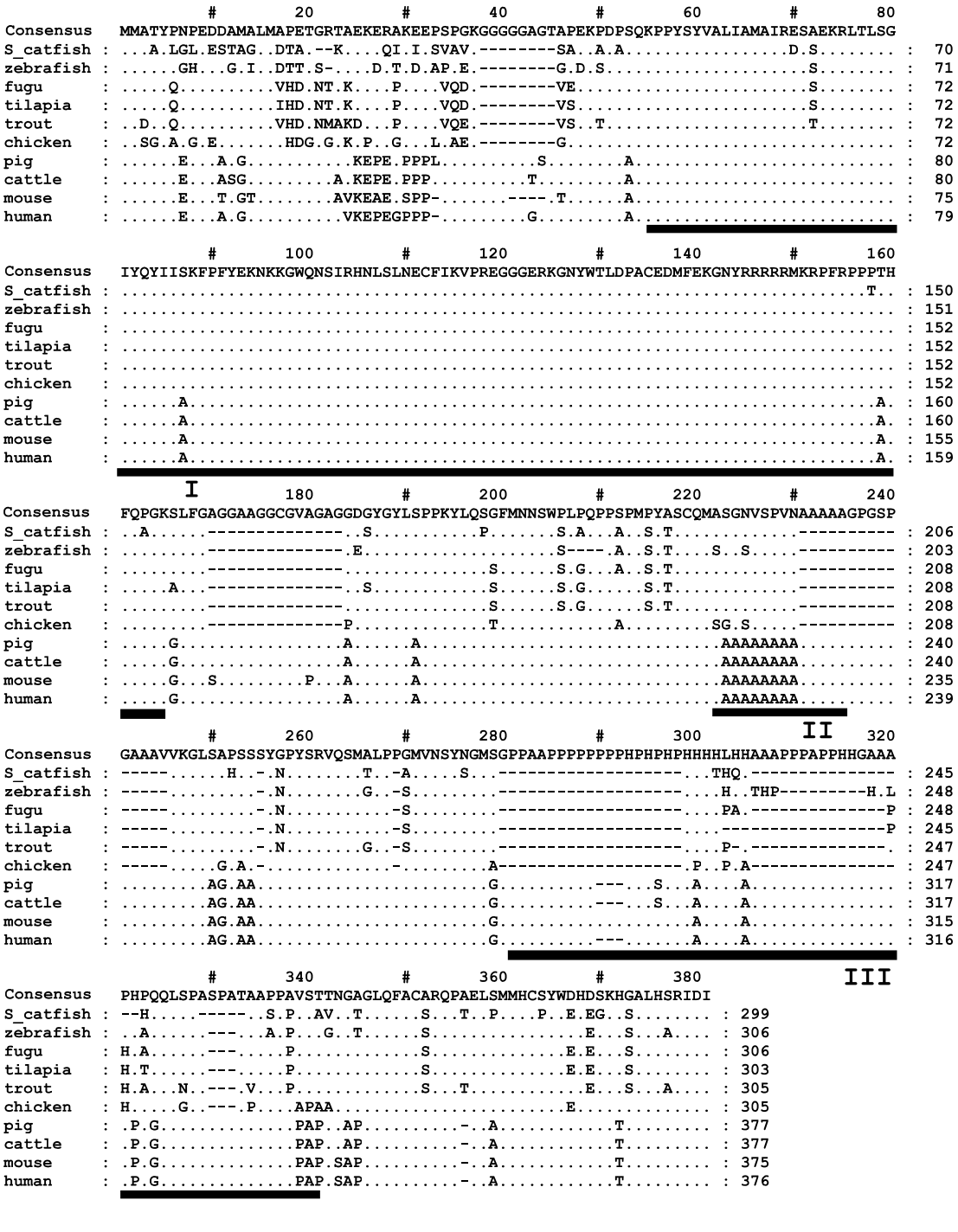



**Figure 3** Alignment of aa sequences of the Southern catfish Foxl2 with those from other vertebrates. The sources of the sequences are described in the Materials and Methods section. Regions of high homology are underlined and indicated by Roman numerals: I, the FH domain; II, the polyalanine tract; III, the glycine and proline repeats. The computer programs CLUSTALX and GeneDoc (http://www.psc.edu/biomed/genedoc) were used to construct this figure.

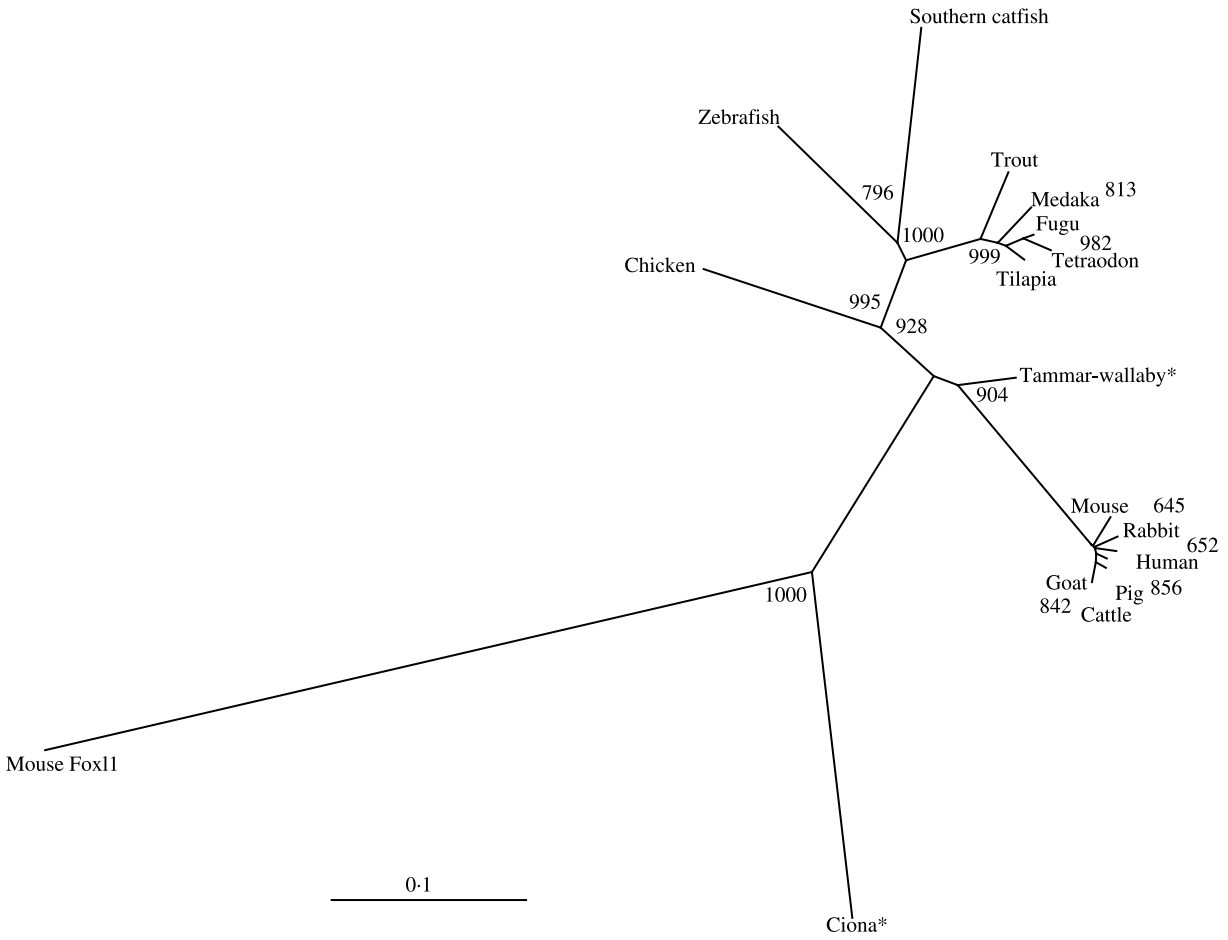
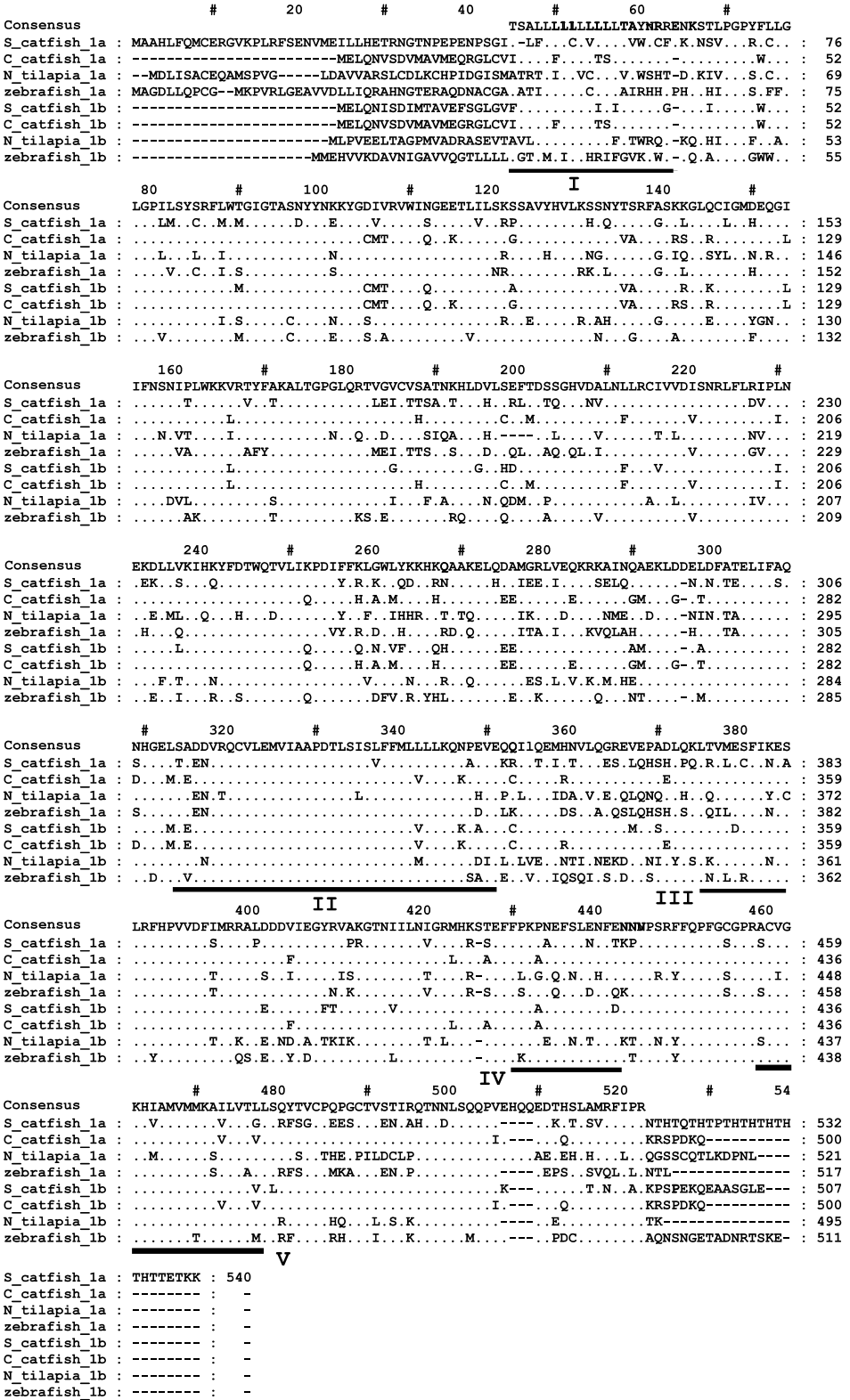



**Figure 4** Phylogenetic tree of Foxl2. The tree was rooted using mouse Foxl1 (NM_008024) as the outgroup. Branch lengths are proportional to the number of aa changes on the branch. Partial sequences (*) may yield artificial short branches. The sources of the sequences are described in the Materials and Methods section.

somatic cells surrounding the germ cells in XX gonads immediately after initiation of ovarian differentiation and is maintained in the granulosa cells throughout ovarian development. In adult ovary, *Foxl2* is expressed in the previtellogenic and vitellogenic follicles, but its expression ceases in the postvitellogenic follicles (Wang *et al.* 2004, Nakamoto *et al.* 2006). In the present study, the tissue distribution results revealed that the Southern catfish *Foxl2* expression is restricted to the brain (B), pituitary (P), gill, and gonads (G). This is consistent with our previous findings in tilapia (Wang *et al.* 2004). This expression pattern indicates that the B–P–G axis and the hormone synthesizing enzymes might be the main targets of *Foxl2*. Moreover, our results also demonstrated a lower expression level of *Foxl2* in the testis. Similar results have been reported in tilapia and rainbow trout (Baron *et al.* 2004, Wang *et al.* 2004). These results indicate that *Foxl2* might also play some roles in the male fish as well.

In the present study, the cDNAs of two types of aromatase have been isolated in Southern catfish. Homology analysis showed that they are highly conserved even though they are

**Figure 5** Alignment of the deduced aa of the Southern catfish Cyp19s with those other teleosts. The sources of the sequences are described in the Materials and Methods section. Regions of high homology are indicated by Roman numerals: I, the membrane spanning domain; II, the helical region; III, Ozol's peptide; IV, the aromatic region; V, the heme-binding region. Asterisks and dots indicate identical and similar aa residues respectively. The computer programs CLUSTALX and GeneDoc (http://www.psc.edu/biomed/genedoc) were used to construct this figure.



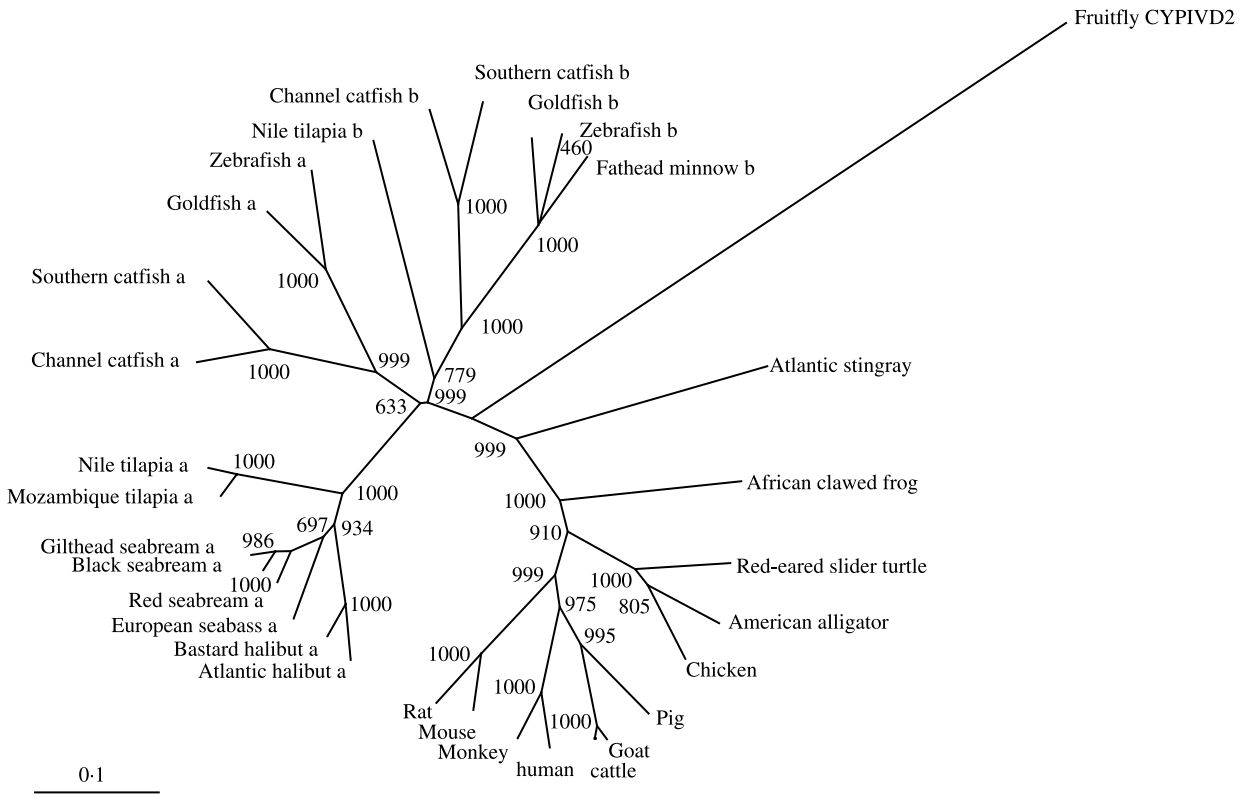



**Figure 6** Phylogenetic tree of Cyp19 in vertebrates. The tree was rooted using fruit fly CYPIVD2 as the outgroup. Branch lengths are proportional to the number of aa changes on the branch. The sources of the sequences are described in the Materials and Methods section.

encoded by two different genes. Tissue distribution analysis revealed that both *Cyp19a* and *Cyp19b* were expressed in the gonads and spleen, whereas *Cyp19b* was also expressed in the brain. Gonad and brain are the major tissues of estrogen production in fish as well as in other vertebrates. The high expression of both types of aromatase in the ovary and *Cyp19b* in the brain has been reported in several teleosts (Trant *et al.* 1997, Tchoudakova & Callard 1998, Kitano *et al.* 1999, Kwon *et al.* 2001). In our study, *Cyp19a* was not detected in the brain, similar to the situation found in zebrafish (Kishida & Callard 2001). In line with our findings, *Cyp19a* was also reported to be expressed in the spleen of tilapia (Chang *et al.* 2005). Spleen is not a steroidogenic tissue. Nevertheless, aromatase and *P450scc* (cholesterol side-chain cleavage enzyme) were found to be expressed in the spleen of human and mouse (Price *et al.* 1992, Morohashi *et al.* 1999). Screening of the major steroidogenic enzymes is needed to ascertain whether the teleost spleen is capable of carrying out the steroidogenesis process or not. However, our data indicate that at least the circulating steroid precursors could be converted to estrogen in this organ. Another interesting point worthy of further investigation is the reason for the need to synthesize steroids/estrogen in the spleen.

*Regulatory action of estrogen on Dmrt1s, Foxl2, and Cyp19s in Southern catfish*

In the present study, the all-female Southern catfish fry were treated with an aromatase inhibitor (F) and an estrogen receptor antagonist (TAM) during the sensitive period of sex differentiation (5–25 dah; Zhang *et al.* 2005). Expression of the Southern catfish *Dmrt1a* and *Dmrt1b* in the gonad was found to be the same in the $E_2$ treatment group as in the control group, whereas substantial up-regulation was detected in the F, TAM, and F+TAM groups (Fig. 8). While *Foxl2* expression in the gonads and brain was significantly up-regulated in the $E_2$ treatment group, it was down-regulated in the F, TAM and F+TAM groups (Fig. 9). Simultaneously, *Cyp19a* expression in the gonads and *Cyp19b* expression in the brain were also decreased in the F, TAM, and F+ TAM groups (Figs 10 and 11).

Several reports have revealed the regulatory action of estrogen on the expression of *Dmrt1*, *Foxl2*, and aromatase (Marchand *et al.* 2000, Kishida & Callard 2001, Kishida *et al.* 2001, Tsai *et al.* 2001, Baron *et al.* 2004, 2005, Menuet *et al.* 2005). In the work done on rainbow trout, *Dmrt1* (Marchand *et al.* 2000) and *Foxl2* (Baron *et al.* 2004) expression was down-regulated and up-regulated respectively when the fish

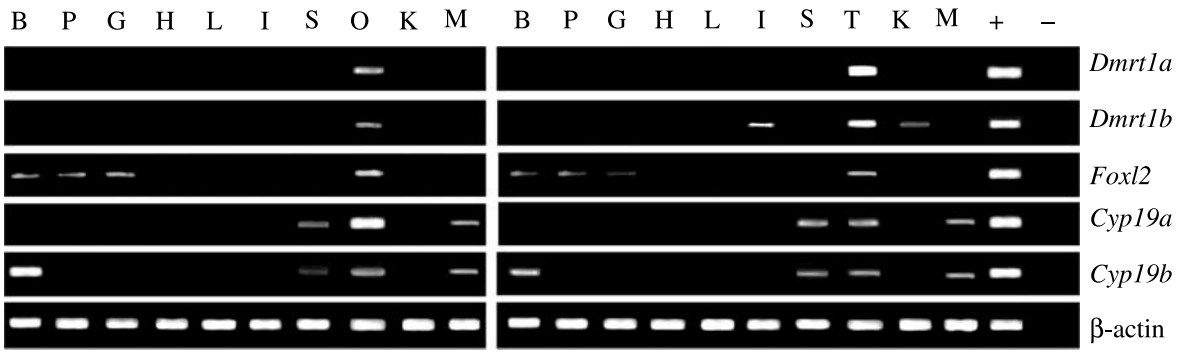



**Figure 7** RT-PCR analysis of *Dmrt1a, Dmrt1b, Foxl2, Cyp19a,* and *Cyp19b* gene expression in various tissues of the adult Southern catfish. B, brain; P, pituitary; G, gill; H, heart; S, spleen; L, liver; I, intestine; O, ovary; K, kidney; M, muscle; T, testis; +, positive control; −, negative control. β-Actin was used as the internal control.

was treated with estrogen, whereas in zebrafish and tilapia, estrogen treatment could up-regulate aromatase expression (Kishida & Callard 2001, Kishida *et al.* 2001, Tsai *et al.* 2001, Menuet *et al.* 2005). Other reports have shown that reduction in estradiol amount (by F treatment) increased *Dmrt1* expression (Smith *et al.* 2003) as well as decreased *Foxl2* (Hudson *et al.* 2005) and aromatase expression (Kishida & Callard 2001, Kishida *et al.* 2001, Tsai *et al.* 2001, Menuet *et al.* 2005) and enzyme activity (Melo & Ramsdell 2001, Lee *et al.* 2004). In the present study, both reduction in estrogen amount (by F treatment) and inhibition of estrogen signaling (by TAM treatment) resulted in up-regulation of *Dmrt1* expression but down-regulation of *Foxl2* and aromatase expression. On the other hand, $E_2$ treatment caused down-regulation of *Dmrt1* expression and up-regulation of *Foxl2* expression. These results provided additional evidence for the important role of estrogen on the expression of *Dmrt1*, *Foxl2*, and aromatase. However, there is no putative ER-binding site or estrogen response element (ERE) observed on the promoters of known *Dmrt1* and *Foxl2* genes. Therefore, the down-regulation of *Dmrt1* and up-regulation of *Foxl2* by estrogen treatment might be an indirect effect. This indirect regulatory action of estrogen on *Foxl2* expression might be mediated through a positive feedback mechanism as suggested in chicken (Hudson *et al.* 2005). Our results support this hypothesis, indicating the conservation of this regulatory mechanism among vertebrates. In our results, these regulatory phenomena were also observed in the brain, indicating that sex differentiation was controlled at different levels in the B–P–G axis.

Previous promoter analysis showed that the binding sites for several sex determining factors in mammals such as Ad4BP/SF-1, WT1, and SRY were present in the 5′-flanking region of *Cyp19a* but not *Cyp19b*. On the other hand, ERE was found only in the 5′-flanking region of *Cyp19b* but not *Cyp19a* in fish, indicating that these two aromatase genes might be regulated differently (Callard *et al.* 2001, Kazeto *et al.* 2001, Tchoudakova *et al.* 2001, Tong & Chung 2003, Chang *et al.* 2005, Kazeto & Trant 2005). In the present study, $E_2$ treatment up-regulated *Cyp19b* in the brain and *Cyp19a* in the gonad. However, $E_2$ treatment only caused a mere 1·4-fold up-regulation of *Cyp19b* in the brain, lower than the results reported by others (Menuet *et al.* 2005, Sawyer *et al.* 2006) where dramatic up-regulation of *Cyp19b* by $E_2$ treatment was reported both *in vitro* and *in vivo*. Elevated expression of *Cyp19b* was also observed in channel catfish and zebrafish embryos by estrogen treatment (Kishida & Callard 2001, Trant *et al.* 2001, Blazquez & Piferrer 2004). These discrepancies might be explained by the different time points employed for gene expression evaluation after drug treatment.

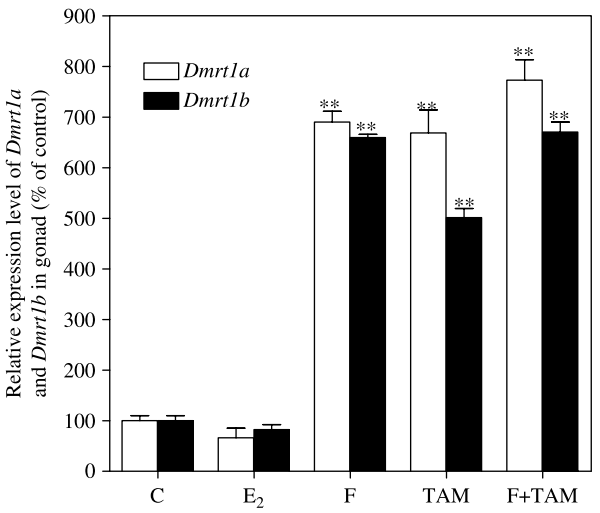



**Figure 8** Regulation of *Dmrt1a* (white column) and *Dmrt1b* (black column) gene expression in Southern catfish gonad by various drug treatments. Results obtained by semi-quantitative RT-PCR are expressed as mean values ± S.E.M. from five individual fishes. (**$P<$ 0·01 as compared with the respective control by one-way ANOVA). C, control; $E_2$, 17β-estradiol; F, fadrozole; TAM, tamoxifen; F+TAM, fadrozole+tamoxifen. The doses of the drugs used are described in the Materials and Methods section.

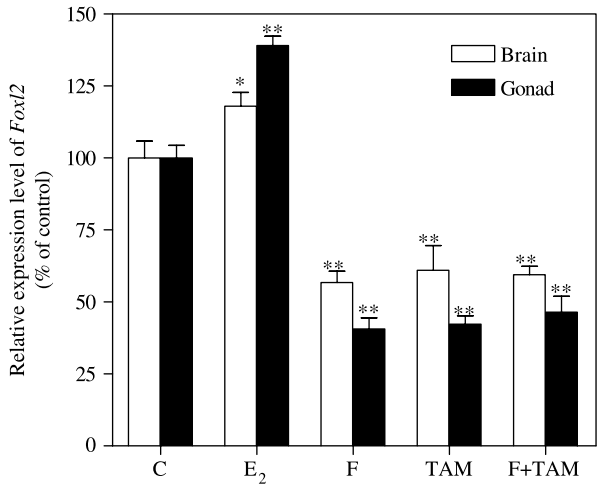



**Figure 9** Regulation of *Foxl2* gene expression in Southern catfish brain (white column) and gonad (black column) by various drug treatments. Results obtained by semi-quantitative RT-PCR are expressed as mean values ± S.E.M. from five individual fishes. (*$P$< 0·05; **$P$<0·01 as compared with the respective control by one-way ANOVA). C, control; $E_2$, 17β-estradiol; F, fadrozole; TAM, tamoxifen; F+TAM, fadrozole+tamoxifen. The doses of the drugs used are described in the Materials and Methods section.

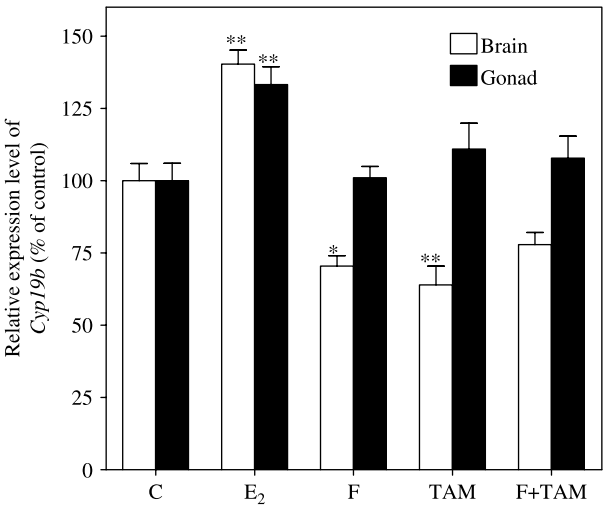



**Figure 11** Regulation of *Cyp19b* gene expression in Southern catfish brain (white column) and gonad (black column) by various drug treatments. Results obtained by semi-quantitative RT-PCR are expressed as mean values ± S.E.M. from five individual fishes. (*$P$< 0·05; **$P$<0·01 as compared with the respective control by one-way ANOVA). C, control; $E_2$, 17β-estradiol; F, fadrozole; TAM, tamoxifen; F+TAM, fadrozole+tamoxifen. The doses of the drugs used are described in the Materials and Methods section.

The dramatic up-regulation of *Cyp19b* during $E_2$ treatment is an acute event that occurs quickly. The presence of ERE in the *Cyp19b* promoter of channel catfish (Kazeto & Trant 2005) and zebrafish (Kazeto *et al.* 2001, Tchoudakova *et al.* 2001) supports this notion. In our case, the gene expression levels were checked at 65 dah, 40 days after the termination of $E_2$ treatment (which lasted from 5 to 25 dah). At 65 dah, the peak of the $E_2$-stimulated *Cyp19b* expression is probably already passed but the subsequent biological effects of this $E_2$-stimulated gene expression are still prominent.

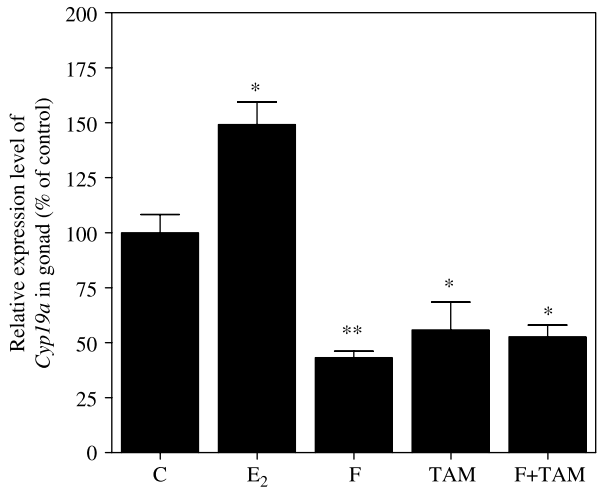



**Figure 10** Regulation of *Cyp19a* gene expression in Southern catfish gonad by various drug treatments. Results obtained by semi-quantitative RT-PCR are expressed as mean values ± S.E.M. from five individual fishes. (*$P$<0·05; **$P$<0·01 as compared with the respective control by one-way ANOVA). C, control; $E_2$, 17β-estradiol; F, fadrozole; TAM, tamoxifen; F+TAM, fadrozole + tamoxifen. The doses of the drugs used are described in the Materials and Methods section.

In contrast to the up-regulation of *Cyp19b* expression in the brain after $E_2$ treatment, *Cyp19b* expression in the gonad remains unchanged. This raises the following possibility in explaining this differential expression pattern in Southern catfish: either tissue-specific promoters are employed for *Cyp19b* transcription in the gonad and brain as in the case of human or perhaps only in the brain is there an appropriate cellular context for transcriptional activation by estrogen. Further studies on this interesting point are highly warranted.

### *The possible roles of Dmrt1s, Foxl2, and Cyp19s in the sex differentiation of Southern catfish*

Different from mammals, sex determination and differentiation of non-mammalian vertebrates depend less on the sex determination genes. On the contrary, hormones and environmental factors play very important roles. Among these factors, estrogen plays key roles in ovarian differentiation in non-mammalian vertebrates, including fish (Chang *et al.* 1997, Kwon *et al.* 2000, Lee *et al.* 2000, Kobayashi *et al.* 2003), amphibians (Yu *et al.* 1993), and birds (Elbrecht & Smith 1992, Smith & Sinclair 2004). As the key enzyme catalyzing the conversion of endogenous androgen to

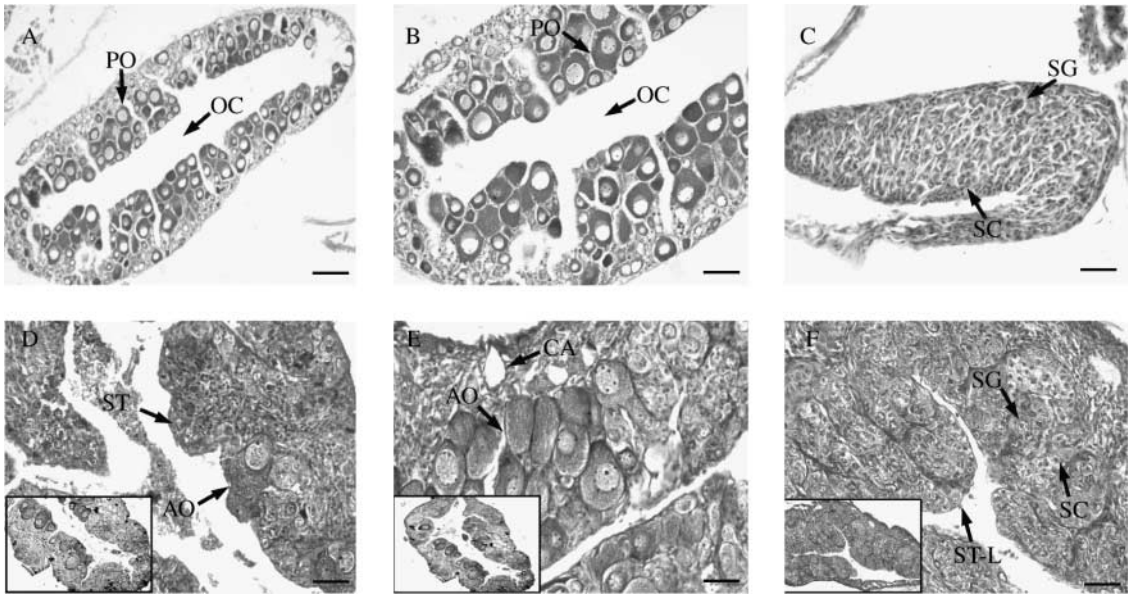



**Figure 12** Histological observation of control and drug-treated Southern catfish gonads. (A) Ovary of control fish at 120 dah; scale bar = 100 μm. (B) Part of A with high magnification; scale bar = 50 μm. (C) Typical testis observed in the fadrozole (F)-, tamoxifen (TAM)-, and F + TAM-treated fish; scale bar = 50 μm. (D–F) ovotestis observed in the F-, TAM-, and F + TAM-treated fish respectively; scale bar = 50 μm. The inserts in D, E, F show the waned ovary cavities for the three drug-treated groups respectively. PO, primary oocyte; OC, ovary cavity; ST, Sertoli cells; AO, atretic oocyte; CA, cavum; SC, spermatocyte; SG, spermatogonia; ST-L, spermatocyst-like structure.

estrogen, aromatase is highly related to gonad malformation, sterility, intersex, or sex reversal. Inhibition of aromatase gene expression and enzyme activity resulted in reduced estrogen production and subsequently male-to-female sex reversal, which has been reported in Coho salmon (*Oncorhynchus kisutch*; Afonso *et al.* 1999), bastard halibut (*Paralichthys olivaceus*; Kitano *et al.* 2000), and Nile tilapia (Kobayashi *et al.* 2003). Consequently, the maintenance of aromatase expression and activity is the prerequisite for ovarian differentiation and development.

Until now, several genes have been reported to be involved in aromatase regulation and sex differentiation of vertebrates, including *Dmrt1* and *Foxl2* through interaction with Ad4BP/SF-1.

The role of *Dmrt1* in sex determination and differentiation of vertebrates has been studied in several species (Nanda *et al.* 1999, 2000, Raymond *et al.* 1999*a*,*b*, Kettlewell *et al.* 2000, Marchand *et al.* 2000, Shan *et al.* 2000*a*, Smith *et al.* 2003). In mice, humans, chicken, and turtles, *Dmrt1* expression was found to be sexually dimorphic and restricted to the testis (Raymond *et al.* 1999*a*,*b*, Kettlewell *et al.* 2000, Shan *et al.* 2000*a*, Smith *et al.* 2003). Moreover, $Dmrt1^{-/-}$ knockout XY mice develop testicular differentiation failure (Raymond *et al.* 2000), while humans with chromosome 9p deletion develop sex reversal (Raymond *et al.* 1999*a*). In chicken treated with aromatase inhibitor, *Dmrt1* expression increased significantly. Aromatase inhibitor-treated ZW embryos endured sex reversal and developed as physiological males (Smith *et al.* 2003). These results suggest the important role of *Dmrt1* in male sex determination and differentiation of vertebrates.

Study of has Nile tilapia further revealed that *Dmrt1* can suppress Ad4BP/SF-1- and Foxl2-mediated *Cyp19a1* transcription. *Dmrt1* can also suppress Ad4BP/SF-1-mediated *StAR* and *p450c17*, but activate *Cyp11a1* and *Cyp11b* promoter activities. These results suggest that *Dmrt1* plays a decisive role in the testicular differentiation of Nile tilapia by down-regulating aromatase gene expression and possibly by shifting the entire steroidogenic pathway towards androgen production (Wang *et al.* 2006).

In the present study, Southern catfish *Dmrt1a* and *Dmrt1b* were found to be expressed mainly in the gonads. In addition, the expression of *Dmrt1a* and *Dmrt1b* was decreased by $E_2$ treatment but increased by F, TAM, or F + TAM treatment. Consistent with the increased expression of *Dmrt1a* and *Dmrt1b*, 50, 70, and 80% of sex-reversed male fish were obtained in the F, TAM, or F + TAM treatment groups respectively, while the expression of both *Dmrt1a* and *Dmrt1b* remained low in the female control (Fig. 8). This demonstrates that the expression of *Dmrt1a* and *Dmrt1b* is highly related to the male phenotype and reveals the pivotal role *Dmrt1* plays in Southern catfish testicular differentiation, probably mediated through regulating aromatase expression.

To date, no upstream regulators or downstream targets of *Dmrt1* have been identified, making it difficult to place *Dmrt1* in any gene regulatory pathway. Even though our data and those from others suggest a potential regulatory linkage between *Dmrt1* and *Cyp19*, further investigations are necessary to establish their casual relationship.

*Foxl2* has been shown to be involved in ovarian differentiation in previous reports (Baron *et al.* 2004, Ottolenghi *et al.* 2005, Nakamoto *et al.* 2006). In mouse, chicken, and turtle, representatives of three phylogenetically distant vertebrate groups that possess different mechanisms of sex determination, the expression of *Foxl2* was detected in the early ovaries of all these three species around the time of sex determination and *Foxl2* expression was sexually dimorphic in all cases (Loffler *et al.* 2003). These data suggested that *Foxl2* is a highly conserved early regulator of vertebrate ovarian differentiation. In fish, *Foxl2* expression was found to be sexually dimorphic in Nile tilapia (Wang *et al.* 2004, 2007), rainbow trout (Baron *et al.* 2004), and medaka (Nakamoto *et al.* 2006), supporting the hypothesis that *Foxl2* may play important role in ovarian differentiation in teleosts as well.

Further evidence for the involvement of *Foxl2* in the transcriptional regulation of *Cyp19* was reported recently in vertebrates. In goats suffering from PIS, Foxl2 was found to activate the *Cyp19* specific promoter 2, and *Cyp19* expression was reduced when *Foxl2* ceased to express in the primary stages of ovarian differentiation (Pannetier *et al.* 2006). In the early stages of gonad differentiation, Foxl2 and Ad4BP/SF-1 were found to be co-localized with Cyp19a in the ovary of tilapia. Promoter assays further revealed that the FH-domain of Foxl2 binds to the consensus ACAAATA sequence on the *Cyp19a* promoter, thereby activating the expression of *Cyp19a*. Furthermore, Foxl2 can interact with Ad4BP/SF-1 to enhance Ad4BP/SF-1-activated *Cyp19a* gene transcription in a female-specific manner (Wang *et al.* 2007).

In our study, the expression of *Foxl2* was found to be sexually dimorphic with dominant expression in the ovary, consistent with previous reports (Loffler *et al.* 2003, Baron *et al.* 2004, Wang *et al.* 2004, Nakamoto *et al.* 2006). Furthermore, *Cyp19a* and *Cyp19b* were also found to be expressed mainly in the ovary and brain respectively, being higher in female than in male. When treated with F and/or TAM, *Foxl2*/*Cyp19a* expression in the gonad and *Foxl2*/*Cyp19b* expression in the brain decreased significantly, similar to the results reported in chicken (Hudson *et al.* 2005). Gene expression study and histological analyses of the F-, TAM-, and F+TAM-treated fish revealed that the down-regulation of *Foxl2*, *Cyp19a*, and *Cyp19b* expression was highly correlated with the female-to-male sex reversal of Southern catfish in these groups. These results, together with the tissue distribution of *Foxl2*, *Cyp19a*, and *Cyp19b* mRNA in Southern catfish, strongly suggest that these genes are involved in Southern catfish ovarian differentiation and development. Furthermore, the down-regulation of *Foxl2* and *Cyp19b* in the brain of the F and/or TAM treatment groups also indicated that *Foxl2* and *Cyp19b* might regulate sex differentiation at the brain and pituitary levels. It is well known that brain sexualization in mammals is, at least in part, determined early in development through the testis-differentiating effects of *Sry*, whereas fish could retain the possibility of constantly adapting their brain gender to their gonadal gender. For these reasons, *Cyp19b* appears to be a critical gene whose disruption could grossly affect the functioning of the whole reproductive axis (Menuet *et al.* 2005).

Consistent with the changes in gene expression patterns in the drug-treated fish, obvious morphological changes, from partial to complete sex reversal, were also observed in the gonads of the F, TAM, and F+TAM treatment groups as compared with control fish. These features include incomplete/diminished ovarian cavity formation, elongation of the oocytes, degeneration and reduction of the follicles, proliferation of somatic cells, and appearance of testicular structures (Zhang & Xie 1996), such as spermatogonia, primary spermatocytes, etc. These results suggest that F and/or TAM treatment shrink the ovarian cavities in size probably by compression of the thickened ovary wall, as reported in golden rabbitfish (*Siganus guttatus*; Komatsu *et al.* 2006). However, F, TAM, and F+TAM treatment resulted in only 56, 70, and 80% fish being sex-reversed from female to male. On the other hand, 100% sex-reversed phenotypic males were reported in fathead minnow (*Pimephales promelas*; Zerulla *et al.* 2002) and zebrafish (Fenske & Segner 2004). Despite that 100% males were observed in the F-treated zebrafish, only 64% of the fish showed male-phenotypical expression of *Cyp19a*, indicating that the physiological regulation of estradiol synthesis was not irreversibly masculinized by the F treatment (Fenske & Segner 2004). In our study, though the F-, TAM-, and F+TAM-treated fish showed very low expression of *Cyp19a*, only 56, 70, and 80% males resulted respectively. The reason why all Southern catfish offspring obtained by artificial propagation under laboratory conditions are female remains elusive, making it unresolved at the moment why we were unable to generate 100% sex-reversed males in the F, TAM, and F+TAM treatment groups. Furthermore, since sex-linked genomic markers are not yet available for Southern catfish, it is not possible at this stage to ascertain whether those fish, which are still undergoing transition from female to male in the drug treatment groups, are genetic females or not.

In summary, the cDNAs of four genes, which showed clear sexual dimorphic expression pattern from fry to adult, were cloned from the Southern catfish. *Foxl2*, *Cyp19a* (gonad only), and *Cyp19b* are expressed higher in the female brain and gonad, thus favoring ovarian differentiation. On the other hand, *Dmrt1a* and *Dmrt1b* are expressed higher in the male gonad, thus favoring male sex differentiation. Treatment with F, TAM, and F+TAM of the all-female fry reversed the expression patterns of these genes and caused sex reversal of the treated fish. Taken together, our results indicate that *Dmrt1*, *Foxl2*,

*Cyp19a*, and *Cyp19b* are important genes implicated in the sex differentiation of Southern catfish.

## Acknowledgements

We are grateful to Professor Y M Tang for the help in cloning the *Cyp19a* and *Cyp19b* cDNAs. We thank the Novartis Company in Switzerland for providing the pure powder drug of fadrozole for our studies. This research was funded by grants from the Leading Program of State Education Ministry (No. 2004-104161), the Chongqing Natural Science Foundation (No. CSTC2004BB8450), and the Free Exploration Fund of the Key Laboratory of Eco-environments in the Three Gorges Reservoir Region (Ministry of Education); (No. 124470-20500312). We also thank The Chinese University of Hong Kong for the provision of Direct Grants. The sequences reported in this paper have been deposited in the GenBank database at NCBI and are assigned the following accession nos: EF015487, EF015488, EF015396, AAP83133, and AAP83132. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

## References

Afonso LO, Iwama GK, Smith J & Donaldson EM 1999 Effects of the aromatase inhibitor Fadrozole on plasma sex steroid secretion and ovulation rate in female coho salmon, *Oncorhynchus kisutch*, close to final maturation. *General and Comparative Endocrinology* **113** 221–229.

Baron D, Cocquet J, Xia X, Fellous M, Guiguen Y & Veitia RA 2004 An evolutionary and functional analysis of FoxL2 in rainbow trout gonad differentiation. *Journal of Molecular Endocrinology* **33** 705–715.

Baron D, Houlgatte R, Fostier A & Guiguen Y 2005 Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. *Biology of Reproduction* **73** 959–966.

Blazquez M & Piferrer F 2004 Cloning, sequence analysis, tissue distribution, and sex-specific expression of the neural form of P450 aromatase in juvenile sea bass (*Dicentrarchus labrax*). *Molecular and Cellular Endocrinology* **219** 83–94.

Bobe J, Montfort J, Nguyen T & Fostier A 2006 Identification of new participants in the rainbow trout (*Oncorhynchus mykiss*) oocyte maturation and ovulation processes using cDNA microarrays. *Reproductive Biology and Endocrinology* **4** 39–54.

Callard GV, Tchoudakova AV, Kishida M & Wood E 2001 Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of *cyp19* genes in teleost fish. *Journal of Steroid Biochemistry and Molecular Biology* **79** 305–314.

Chang XT, Kobayashi T, Kajiura H, Nakamura M & Nagahama Y 1997 Isolation and characterization of the cDNA encoding the tilapia (*Oreochromis niloticus*) cytochrome P450 aromatase (P450arom): changes in P450arom mRNA, protein and enzyme activity in ovarian follicles during oogenesis. *Journal of Molecular Endocrinology* **18** 57–66.

Chang XT, Kobayashi T, Senthilkumaran B, Kobayashi-Kajura H, Sudhakumari CC & Nagahama Y 2005 Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*). *General and Comparative Endocrinology* **141** 101–115.

Chiang EF, Yan YL, Tong SK, Hsiao PH, Guiguen Y, Postlethwait J & Chung BC 2001 Characterization of duplicated zebrafish *cyp19* genes. *Journal of Experimental Zoology* **290** 709–714.

Choi JY, Park JG, Jeong HB, Lee YD, Takemura A & Kim SJ 2005 Molecular cloning of cytochrome P450 aromatases in the protogynous wrasse, *Halichoeres tenuispinis*. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **141** 49–59.

Cocquet J, Pailhoux E, Jaubert F, Servel N, Xia X, Pannetier M, De Baere E, Messiaen L, Cotinot C, Fellous M *et al*. 2002 Evolution and expression of Foxl2. *Journal of Medical Genetics* **39** 916–921.

Cocquet J, De Baere E, Gareil M, Pannetier M, Xia X, Fellous M & Veitia RA 2003 Structure, evolution and expression of the FOXL2 transcription unit. *Cytogenetic and Genome Research* **101** 206–211.

Elbrecht A & Smith RG 1992 Aromatase enzyme activity and sex determination in chickens. *Science* **255** 467–470.

Fenske M & Segner H 2004 Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). *Aquatic Toxicology* **67** 105–126.

Govoroun MS, Pannetier M, Pailhoux E, Cocquet J, Brillard JP, Couty I, Batellier F & Cotinot C 2004 Isolation of chicken homolog of the *FOXL2* gene and comparison of its expression patterns with those of aromatase during ovarian development. *Developmental Dynamics* **231** 859–870.

De Grandi A, Calvari V, Bertini V, Bulfone A, Peverali G, Camerino G, Borsani G & Guioli S 2000 The expression pattern of a mouse doublesex-related gene is consistent with a role in gonadal differentiation. *Mechanisms of Development* **90** 323–326.

Guan G, Kobayashi T & Nagahama Y 2000 Sexually dimorphic expression of two types of DM (*Doublesex/Mab-3*)-domain genes in a teleost fish, the Tilapia (*Oreochromis niloticus*). *Biochemical and Biophysical Research Communications* **272** 662–666.

Guiguen Y, Baroiller JF, Ricordel MJ, Iseki K, Mcmeel OM, Martin SA & Fostier A 1999 Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). *Molecular Reproduction and Development* **54** 154–162.

Hudson QJ, Smith CA & Sinclair AH 2005 Aromatase inhibition reduces expression of *FOXL2* in the embryonic chicken ovary. *Developmental Dynamics* **233** 1052–1055.

Kazeto Y & Trant JM 2005 Molecular biology of channel catfish brain cytochrome P450 aromatase (CYP19A2): cloning, preovulatory induction of gene expression, hormonal gene regulation and analysis of promoter region. *Journal of Molecular Endocrinology* **35** 571–583.

Kazeto Y, Ijiri S, Place AR, Zohar Y & Trant JM 2001 The 5′-flanking regions of CYP19A1 and CYP19A2 in zebrafish. *Biochemical and Biophysical Research Communications* **288** 503–508.

Kettlewell JR, Raymond CS & Zarkower D 2000 Temperature-dependent expression of turtle Dmrt1 prior to sexual differentiation. *Genesis* **26** 174–178.

Kishida M & Callard GV 2001 Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development. *Endocrinology* **142** 740–750.

Kishida M, McLellan M, Miranda JA & Callard GV 2001 Estrogen and xenoestrogens upregulate the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **129** 261–268.

Kitano T, Takamune K, Kobayashi T, Nagahama Y & Abe SI 1999 Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). *Journal of Molecular Endocrinology* **23** 167–176.

Kitano T, Takamune K, Nagahama Y & Abe SI 2000 Aromatase inhibitor and 17α-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). *Molecular Reproduction and Development* **56** 1–5.

Kobayashi T, Kajiura-Kobayashi H & Nagahama Y 2003 Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, tilapia. *Cytogenetic and Genome Research* **101** 289–294.

Komatsu T, Nakamura S & Nakamura M 2006 Masculinization of female golden rabbitfish *Siganus guttatus* using an aromatase inhibitor treatment during sex differentiation. *Comparative Biochemistry and Physiology. Toxicology and Pharmacology* **143** 402–409.

Kwon JY, Haghpanah V, Kogson-Hurtado CM, McAndrew BJ & Penman DJ 2000 Masculinization of genetic female nile tilapia (*Oreochromis niloticus*) by dietary administration of an aromatase inhibitor during sexual differentiation. *Journal of Experimental Zoology* **287** 46–53.

Kwon JY, McAndrew BJ & Penman DJ 2001 Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia *Oreochromis niloticus*. *Molecular Reproduction and Development* **59** 359–370.

Lee YH, Lee FY, Yueh WS, Tacon P, Du JL, Chang CN, Jeng SR, Tanaka H & Chang CF 2000 Profiles of gonadal development, sex steroids, aromatase activity, and gonadotropin II in the controlled sex change of protandrous black porgy, *Acanthopagrus schlegeli* Bleeker. *General and Comparative Endocrinology* **119** 111–120.

Lee YH, Wu GC, Du JL & Chang CF 2004 Estradiol-17β induced a reversible sex change in the fingerlings of protandrous black porgy, *Acanthopagrus schlegeli* Bleeker: the possible roles of luteinizing hormone in sex change. *Biology of Reproduction* **71** 1270–1278.

Loffler KA, Zarkower D & Koopman P 2003 Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: *FOXL2* is a conserved, early-acting gene in vertebrate ovarian development. *Endocrinology* **144** 3237–3243.

Marchand O, Govoroun M, D'Cotta H, McMeel O, Lareyre J, Bernot A, Laudet V & Guiguen Y 2000 DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. *Biochimica et Biophysica Acta* **1493** 180–187.

Matsumura R, Takeuchi S & Takahashi S 2004 Effect of estrogen on melanocortin-3 receptor mRNA expression in mouse pituitary glands *in vivo* and *in vitro*. *Neuroendocrinology* **80** 143–151.

Melo AC & Ramsdell JS 2001 Sexual dimorphism of brain aromatase activity in medaka: induction of a female phenotype by estradiol. *Environmental Health Perspectives* **109** 257–264.

Menuet A, Pellegrini E, Brion F, Gueguen MM, Anglade I, Pakdel F & Kah O 2005 Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. *Journal of Comparative Neurology* **485** 304–320.

Morohashi K, Tsuboi-Asai H, Matsushita S, Suda M, Nakashima M, Sasano H, Hataba Y, Li CL, Fukata J, Irie J *et al.* 1999 Structural and functional abnormalities in the spleen of an *mFtz-F1* gene-disrupted mouse. *Blood* **93** 1586–1594.

Nakamoto M, Matsuda M, Wang DS, Nagahama Y & Shibata N 2006 Molecular cloning and analysis of gonadal expression of *Foxl2* in the medaka, *Oryzias latipes*. *Biochemical and Biophysical Research Communications* **344** 353–361.

Nanda I, Shan Z, Schartl M, Burt DW, Koehler M, Nothwang H, Grutzner F, Paton IR, Windsor D, Dunn I *et al.* 1999 300 Million years of conserved synteny between chicken Z and human chromosome 9. *Nature Genetics* **21** 258–259.

Nanda I, Zend-Ajusch E, Shan Z, Grutzner F, Schartl M, Burt DW, Koehler M, Fowler VM, Goodwin G, Schneider WJ *et al.* 2000 Conserved synteny between the chicken Z sex chromosome and human chromosome 9 includes the male regulatory gene *DMRT1*: a comparative (re)view on avian sex determination. *Cytogenetics and Cell Genetics* **89** 67–78.

Nikic S & Vaiman D 2004 Conserved patterns of gene expression in mice and goats in the vicinity of the polled intersex syndrome (PIS) locus. *Chromosome Research* **12** 465–474.

Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, Pilia G & Schlessinger D 2005 Foxl2 is required for commitment to ovary differentiation. *Human Molecular Genetics* **14** 2053–2062.

Pailhoux E, Vigier B, Chaffaux S, Servel N, Taourit S, Furet JP, Fellous M, Grosclaude F, Cribiu EP, Cotinot C *et al.* 2001 A 11.7-kb deletion triggers intersexuality and polledness in goats. *Nature Genetics* **29** 453–458.

Pailhoux E, Vigier B, Vaiman D, Servel N, Chaffaux S, Cribiu EP & Cotinot C 2002 Ontogenesis of female-to-male sex-reversal in XX polled goats. *Developmental Dynamics* **224** 39–50.

Pannetier M, Servel N, Cocquet J, Besnard N, Cotinot C & Pailhoux E 2003 Expression studies of the PIS-regulated genes suggest different mechanisms of sex determination within mammals. *Cytogenetic and Genome Research* **101** 199–205.

Pannetier M, Fabre S, Batista F, Kocer A, Renault L, Jolivet G, Mandon-Pepin B, Cotinot C, Veitia R & Pailhoux E 2006 FOXL2 activates P450 aromatase gene transcription: towards a better characterization of the early steps of mammalian ovarian development. *Journal of Molecular Endocrinology* **36** 399–413.

Parte P, Balasinor N, Gill-Sharma MK, Maitra A & Juneja HS 2002 Temporal effect of tamoxifen on cytochrome P450 side chain cleavage gene expression and steroid concentration in adult male rats. *Journal of Steroid Biochemistry and Molecular Biology* **82** 349–358.

Pisarska MD, Bae J, Klein C & Hsueh AJ 2004 Forkhead L2 is expressed in the ovary and represses the promoter activity of the steroidogenic acute regulatory gene. *Endocrinology* **145** 3424–3433.

Price T, Aitken J & Simpson ER 1992 Relative expression of aromatase cytochrome P450 in human fetal tissues as determined by competitive polymerase chain reaction amplification. *Journal of Clinical Endocrinology and Metabolism* **74** 879–883.

Raymond CS, Kettlewell JR, Hirsch B, Bardwell VJ & Zarkower D 1999*a* Expression of Dmrt1 in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. *Developmental Biology* **215** 208–220.

Raymond CS, Parker ED, Kettlewell JR, Brown LG, Page DC, Kusz K, Jaruzelska J, Reinberg Y, Flejter WL, Bardwell VJ *et al.* 1999*b* A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. *Human Molecular Genetics* **8** 989–996.

Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ & Zarkower D 2000 *Dmrt1*, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes and Development* **14** 2587–2595.

Sawyer SJ, Gerstner KA & Callard GV 2006 Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: gene specific tissue distribution, sex differences, developmental programming, and estrogen regulation. *General and Comparative Endocrinology* **147** 108–117.

Shan Z, Nanda I, Wang Y, Schmid M, Vortkamp A & Haaf T 2000*a* Sex-specific expression of an evolutionarily conserved male regulatory gene, *DMRT1*, in birds. *Cytogenetics and Cell Genetics* **89** 252–257.

Shan Z, Zabel B, Trautmann U, Hillig U, Ottolenghi C, Wan Y & Haaf T 2000*b* Fish mapping of the sex-reversal region on human chromosome 9p in two XY females and in primates. *European Journal of Human Genetics* **8** 167–173.

Shibata K, Takase M & Nakamura M 2002 The *Dmrt1* expression in sex-reserved gonads of amphibians. *General and Comparative Endocrinology* **127** 232–241.

Smith CA & Sinclair AH 2004 Sex determination: insights from the chicken. *Bioessays* **26** 120–132.

Smith CA, Katz M & Sinclair AH 2003 DMRT1 is upregulated in the gonads during female-to-male sex reversal in ZW chicken embryos. *Biology of Reproduction* **68** 560–570.

Sreenivasulu K, Ganesh S & Raman R 2002 Evolutionary conserved, *DMRT1*, encodes alternatively spliced transcripts and shows dimorphic expression during gonadal differentiation in the lizard, *Calotes versicolor*. *Mechanism of Development* **119** S55–S64.

Tchoudakova A & Callard GV 1998 Identification of multiple *CYP19* genes encoding different cytochrome P450 aromatase isozymes in brain and ovary. *Endocrinology* **139** 2179–2189.

Tchoudakova A, Kishida M, Wood E & Callard GV 2001 Promoter characteristics of two *cyp19* genes differentially expressed in the brain and ovary of teleost fish. *Journal of Steroid Biochemistry and Molecular Biology* **78** 427–439.

Tong SK & Chung BC 2003 Analysis of zebrafish cyp19 promoters. *Journal of Steroid Biochemistry and Molecular Biology* **86** 381–386.

Torres MLC, Landa PA, Moreno MN, Marmolejo VA, Meza MA & Merchant LH 2002 Expression profiles of *Dax1*, *Dmrt1*, and *Sox9* during temperature sex determination in gonads of the sea turtle *Lepidochelys olivacea*. *General and Comparative Endocrinology* **129** 20–26.

Trant JM, Lehrter J, Gregory T, Nunez S & Wunder J 1997 Expression of cytochrome P450 aromatase in the channel catfish, *Ictalurus punctatus*. *Journal of Steroid Biochemistry and Molecular Biology* **61** 393–397.

Trant JM, Gavasso S, Ackers J, Chung BC & Place AR 2001 Developmental expression of cytochrome P450 aromatase genes (*CYP19a* and *CYP19b*) in zebrafish fry (*Danio rerio*). *Journal of Experimental Zoology* **290** 475–483.

Tsai CL, Wang LH & Fang LS 2001 Estradiol and para-chlorophenylalanine downregulate the expression of brain aromatase and estrogen receptor-$\alpha$ mRNA during the critical period of feminization in tilapia (*Oreochromis mossambicus*). *Neuroendocrinology* **74** 325–334.

Wang DS, Kobayashi T, Zhou L & Nagahama Y 2004 Molecular cloning and gene expression of *Foxl2* in the Nile tilapia, *Oreochromis niloticus*. *Biochemical and Biophysical Research Communications* **320** 83–89.

Wang DS, Kobayashi T, Zhou LY, Ijiri S, Morohashi K & Nagahama Y 2006 Transcriptional regulation of aromatase (*Cyp19a1*) by Foxl2 and Dmrt1 in the gonads during sex differentiation in the Nile tilapia, *Oreochromis niloticus*. In *The 4th International Symposium on the Biology of Vertebrate Sex Determination*, April 10–16, pp 35–36. Kona, Hawaii, USA.

Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, Okubo K, Morohashi KI & Nagahama Y 2007 Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4BP/SF-1. *Molecular Endocrinology* **21** 712–725.

Yao HH 2005 The pathway to femaleness: current knowledge on embryonic development of the ovary. *Molecular and Cellular Endocrinology* **230** 87–93.

Yu NW, Hsu CY, Ku HH, Chang LT & Liu HW 1993 Gonadal differentiation and secretions of estradiol and testosterone of the ovaries of *Rana catesbeiana* tadpoles treated with 4-hydroxyandrostenedione. *Journal of Experimental Zoology* **265** 252–257.

Zerulla M, Lange R, Steger-Hartmann T, Panter G, Hutchinson T & Dietrich DR 2002 Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). *Toxicology Letters* **131** 51–63.

Zhang YB & Gui JF 2004 Molecular characterization and IFN signal pathway analysis of *Carassius auratus* CaSTAT1 identified from the cultured cells in response to virus infection. *Developmental and Comparative Immunology* **28** 211–227.

Zhang YG & Xie XJ 1996 Reproductive biology of *Silurus meridionalis* Chen: the development and annual change in the gonads. *Acta Hydrobiologica Sinica* **20** 8–11.

Zhang XY, Jiao BW, Wu TL, Jin CB & Wang DS 2005 Histological observation on gonadal sex differentiation in the Southern catfish, *Silurus meridionalis*. *Chinese Journal of Zoology* **40** 41–48.

Received in final form 4 April 2007
Accepted 10 April 2007
Made available online as an Accepted Preprint
16 April 2007