Supplementary Materials

Supplementary Materials and Methods

Sequence homology comparation and phylogenetic tree construction

The full-length coding sequence of *Mauremys reevesii Cyp19a1* was accessed from a previous study (accession number KU821113). Alignment of deduced amino acid sequences was carried out using ClustalX (v2.1) and GeneDoc (v3.2). Multiple amino acid alignments for tree construction were performed using ClustalW (v2.1) and the phylogenetic tree was constructed using the neighbor-joining method in Mega v6.0. The accession numbers of amino acid sequences used for phylogenetic analysis included: *Homo sapiens* (AMNP_000094.2); *Mus musculus* (NP_031836.1); *Gallus gallus* (NP_001001761.2); *Alligator mississippiensis* (XM_019477372.1); *Pelodiscus sinensis* (XP_006135137.1); *Anolis carolinensis* (XP_016852003.1); *Python bivittatus* (XP_007422249.1); *Xenopus tropicalis* (NP_001090630.1); *Andrias davidianus* (ALL29317.1); *Danio rerio* (NP_571229.3).

Tissue-specific expression of *Cyp19a1*

Adult freshwater turtles (*M. reevesii*) were obtained from the Hanshou Turtle Farm (Hunan, China). To examine tissue-specific expression of *Cyp19a1*, we collected the heart, liver, brain, intestines, skin, kidneys, lungs, testes, and ovaries from male and female adult turtles for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis. Adult testes and ovaries were separately sectioned for hematoxylin and eosin (H&E) staining. Research was performed under approval from the Animal Ethics Committee at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001).

Egg collection and incubation

Freshly laid *M. reevesii* turtle eggs were obtained from the same turtle farm. For the various incubation experiments, fertilized eggs were randomized into plastic boxes with moist vermiculite and placed in incubators at different temperatures with a water potential of −220 kPa. In this species, incubation of eggs at 26 °C (MPT) generates all males, whereas incubation at 32 °C (FPT) generates all females (Hou, 1985; Tang et al., 2017). Embryos incubated for 7, 9, 11, 14, 17, 21, and 38 days at 32 °C approximately reached stages 16, 17, 18, 19, 20, 21, and 25, respectively. Embryos incubated at 26 °C for 12, 15, 18, 21, 25, 29, and 50 days corresponded to stages 16, 17, 18, 19, 20, 21, and 25, respectively. To determine sexually dimorphic expression patterns, eggs were constantly maintained in an incubator at 26 °C or 32 °C. For the temperature-shift experiments, eggs were moved at developmental stage 16 from an incubator at 26 °C to another at 32 °C and vice versa. For the temperature-gradient incubation experiments, groups of eggs were placed into incubators at 25, 26, 27, 28, 29, 30, 31, and 32 °C. Embryos were staged based on criteria established by Greenbaum (2002) in *Trachemys scripta*. We sampled gonads at stages 16, 17, 18, 19,

20, 21, and/or 25 from embryos incubated at MPT, FPT, MPT→FPT, and FPT→MPT and gonads at stages 18 and 21 from embryos incubated at a gradient temperature of 25 °C–32 °C for qRT-PCR analysis.

Exogenous estrogen and aromatase inhibitor treatments

To examine the role of *Cyp19a1*, a steroid estrogen (17β-estradiol, E8875, Sigma, USA) was administered to eggs incubating at MPT (26 °C), and a non-steroidal aromatase inhibitor (letrozole, PHR1540, Sigma, USA) was administered to eggs incubating at FPT (32 °C). 17β-estradiol or letrozole were dissolved in 95% ethanol at a concentration of 10 μg/μl, and 10 μl of the drug was applied topically to the eggshell in the region adjacent to the embryo at stage 16. Controls were treated with 10 μl of 95% ethanol. Gonad-mesonephros complexes were dissected from estrogen- and aromatase inhibitor-treated and control embryos at stage 25 for histological and immunohistochemical analysis. Gonads treated with estrogen were separated from the adjacent mesonephros at stages 17, 18, 19, 21, and 25, and preserved for qRT-PCR analysis.

RNA extraction and qRT-PCR

Gonads from embryos in each group were microdissected from the mesonephros, and 15 pairs of gonads were harvested for RNA extraction using TRIzon Reagent (CW0580, Cwbiotech, China). Each RNA sample was independently collected with 2–3 biological duplicates. First-strand cDNA was synthesized using 0.5 μg of RNA and the EasyQuick RT MasterMix (CW2019, Cwbiotech, China) based upon the manufacturer's protocols. The qRT-PCR analysis was performed using the Roche LightCycler 480 system with a SsoFast EvaGreen Supermix (#1725201, Bio-Rad, USA). Each sample was run in triplicate. After normalization with β-actin, relative RNA levels in samples were calculated using the comparative threshold cycle (Ct) method. Primer sequences for PCR are listed in Table S1.

H&E staining

Embryo gonad-mesonephros complexes and adult gonads were immersed in 4% paraformaldehyde (PFA) overnight at 4 °C and transferred to 70% ethanol. Tissues were wrapped in weighing paper and placed in processing cassettes, dehydrated through a serial alcohol gradient, embedded in paraffin wax blocks, and sectioned. Before staining, tissue sections (5 μm) were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in phosphate-buffered saline (PBS). After staining with H&E, the sections were dehydrated through increasing concentrations of ethanol and xylene and sealed with Permount™ Mounting Medium (G8590, Solarbio, China).

Immunofluorescence

After the gonad-mesonephros complexes from turtle embryos (at indicated stages) were immersed in 4% PFA overnight at 4 °C, they were moved through a MeOH

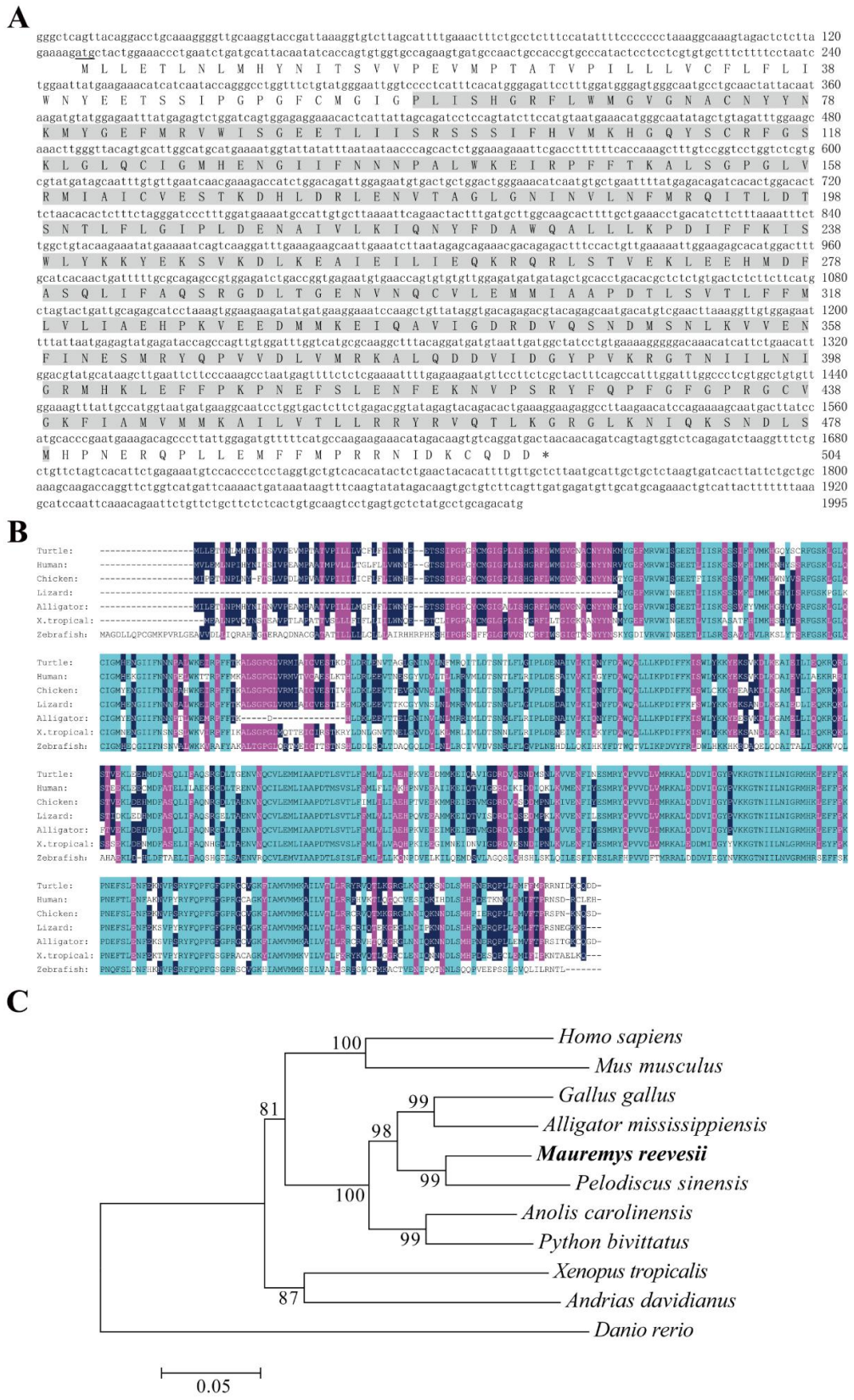
gradient, embedded in paraffin wax and sectioned. Paraffin sections (~5 μm) were first deparaffinized, and antigens were unmasked by microwaving sections in 10 mM/L citrate buffer, pH 6.0 (15 min). Sections were covered with primary antibodies and incubated overnight at 4 °C or 1 h at room temperature. Primary antibodies included rabbit anti-Sox9 (AB5535, 1:1 000, Chemicon, USA), rabbit anti-Vasa (ab13840, 1:200, Abcam, China), and mouse anti-β-catenin (C7207, 1:250, Sigma, USA). Secondary antibodies included Alexa Fluor 594 donkey anti-rabbit IgG (A21207, Invitrogen, USA), Alexa Fluor 594 donkey anti-mouse IgG (A21203, Invitrogen, USA), Alexa Fluor 488 donkey anti-rabbit IgG (A21206, Invitrogen, USA), and Alexa Fluor 488 donkey anti-mouse IgG (A21202, Invitrogen, USA) diluted at 1:250, which were used to detect primary antibodies. Nuclei were stained with DAPI. Gonadal sections were imaged using a confocal microscope (A1 Plus, Nikon, Japan).

Supplementary Results

Characterization of *Cyp19a1* gene in *M. reevesii*.

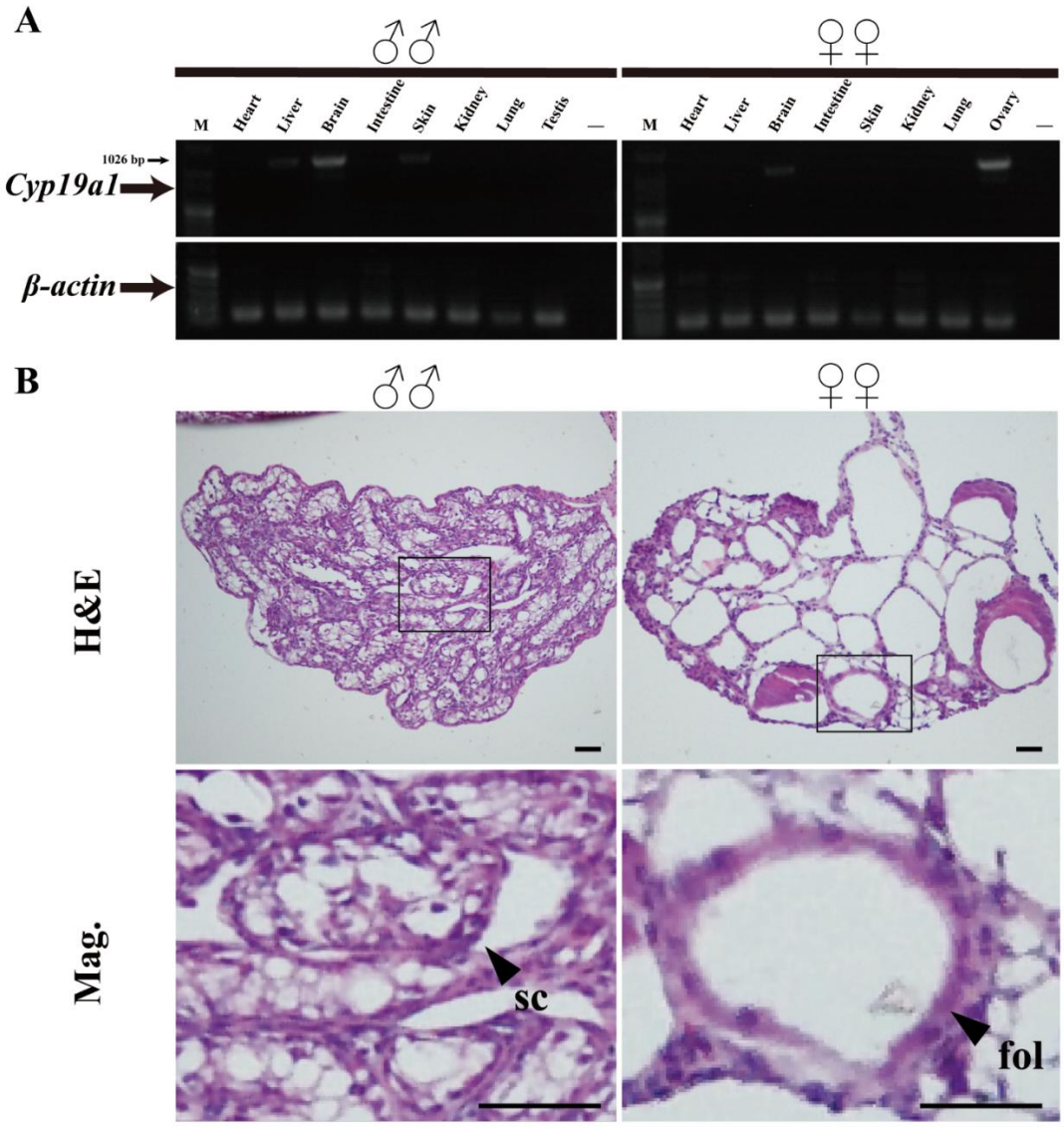
The complete cDNA sequence of *Cyp19a1* in *M. reevesii* was 1 995 base pairs (bp), with a 126 bp 5’ untranslated region (UTR), 355 bp 3’ UTR, and 1 515 bp open reading frame (ORF), which encoded a protein of 504 amino acids (Supplementary Figure S1A). The deduced amino acid sequence of *M. reevesii Cyp19a1* shared 84.9%, 84.4%, 75.1%, and 53.5% identity with chicken, lizard, human, and zebrafish, respectively (Supplementary Figure S1B), indicating that *M. reevesii Cyp19a1* was evolutionary more closely related to the chicken, lizard, and human than to fish (Supplementary Figure S1C).

Cyp19a1 mRNA was abundantly expressed in the ovary, but not in the testis of adult turtles (Supplementary Figure S2A). The adult testis had a dense medulla with seminiferous cords, while the ovary showed a developing follicular system (Supplementary Figure S2B).

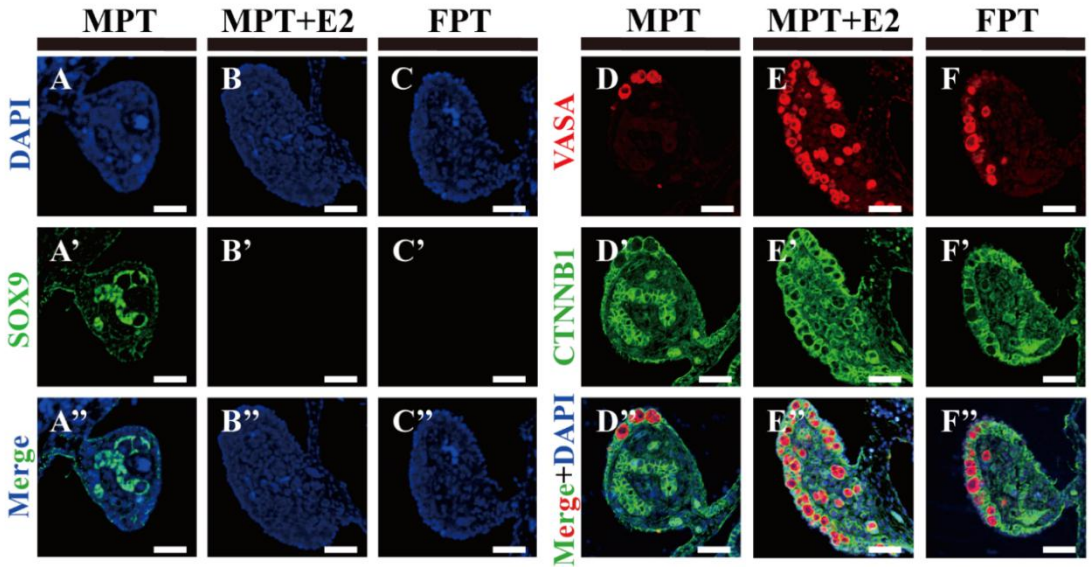


Supplementary Figure S1. Sequence and phylogenetic analyses of *M. reevesii Cyp19a1*. (A) Complete cDNA sequence of *M. reevesii Cyp19a1* and deduced amino

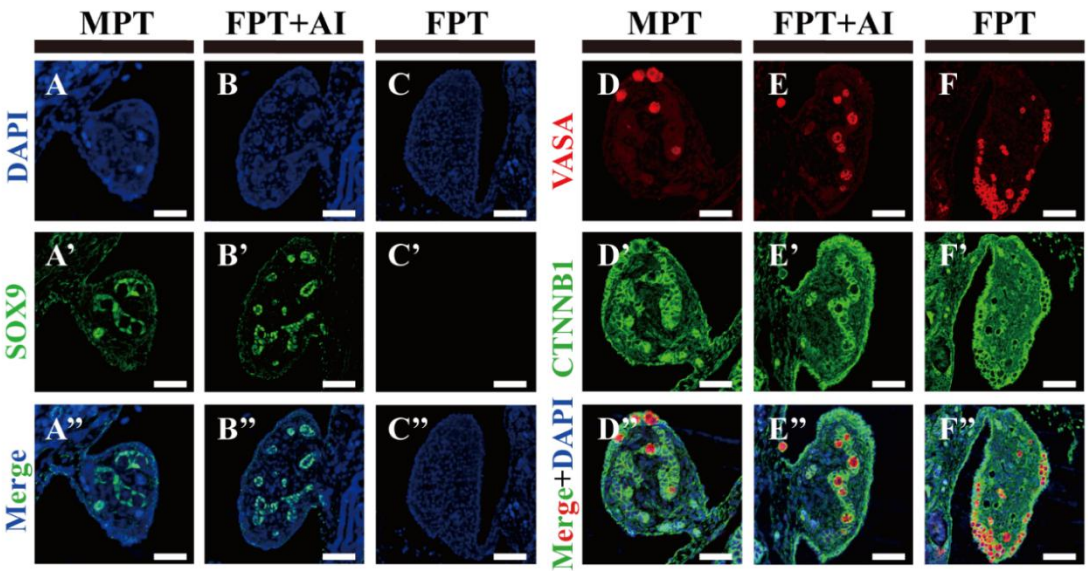
acid sequence. Start codon ATG is underlined, and stop codon is indicated by an asterisk. Highly conserved cytochrome P450 domain is in shadow. (B) Alignment of amino acid sequence of *M. reevesii Cyp19a1* with that of other typical species. (C) *Cyp19a1* phylogenetic tree analysis of *M. reevesii* and other species based on neighbor-joining (N-J) method. Numbers at major branch nodes are bootstrap percentage values based on 1 000 replicates. Each branch length scale in terms of genetic distance is indicated at bottom of tree.



Supplementary Figure S2. *Cyp19a1* expression and histology of testis and ovary in adult *M. reevesii*. (A) Expression of *Cyp19a1* mRNA in different tissues was analyzed by qRT-PCR, which showed specific high expression in ovary instead of testis. (B) H&E staining of adult testis and ovary sections. sc, seminiferous cord; fol, follicle; scale bars are 50 μm.



Supplementary S3. Expression of sex-specific marker genes in MPT gonads following estrogen treatment at stage 21. (A-C'') *Sox9* protein expression in gonadal sections from MPT, MPT with estrogen treatment, and FPT embryos. (D-F'') Distribution of germ cells in MPT gonads following estrogen treatment, determined by *Vasa* immunostaining. Scale bars are 50 μm.



Supplementary Figure S4. Masculinization of FPT *M. reevesii* embryos following aromatase inhibitor treatment at stage 21. (A-C'') Immunofluorescence of *Sox9* in transverse sections of MPT, FPT with aromatase inhibition, and FPT gonads. (D-F'') Immunofluorescence of *vasa* and *β-catenin* in MPT, FPT with aromatase inhibition, and FPT gonads. Scale bars: 50 μm.