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Effects of Incubation Temperature on the Expression of Sex-Related Genes in the Chinese Pond Turtle, *Mauremys reevesii*

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Keywords

Cyp19a1 · *Dmrt1* · *Foxl2* · RNA transcription · *Rspo1* · *Sf1* · *Sox9* · Temperature-dependent sex determination

Abstract

Despite widespread temperature-dependent sex determination (TSD) in reptiles, it is still unclear how the molecular network responds to temperature variation and drives the sexual fate. Profiling of sex-related genes is the first step in understanding the sex determination system in reptiles. In this study, we cloned the full-length coding sequences of *Cyp19a1*, *Foxl2*, *Rspo1*, *Sf1*, and *Sox9* in an Asian freshwater turtle (*Mauremys reevesii*) with TSD and identified the expression patterns of these genes and *Dmrt1* at different incubation temperatures to understand their roles in urogenital development. Our results showed that *Cyp19a1, Foxl2,* and *Rspo1* were expressed in the adrenal-kidney-gonadal complex at a high level in females, while *Sf1* and *Dmrt1* were highly expressed in males. In addition, *Foxl2* and *Rspo1* showed sex-dimorphic expression in the presumed early thermosensitive period (TSP), *Dmrt1* was upregulated at the beginning of the presumed TSP, and *Sox9* did not show sex-dimorphic expression until the end of the presumed TSP. These results suggest that *Foxl2* and *Rspo1* are probably upstream genes involved in female sex determination and that *Dmrt1* may be a key factor in male sex determination. Therefore, our study provides a solid foundation for further investigations on the molecular mechanism underlying sex determination in *M. reevesii*.

Vertebrate mechanisms of sex determination range between 2 main extremes: genotypic sex determination (GSD) and environment-dependent sex determination (ESD) [Bull, 1980; Wilhelm et al., 2007]. Mammals and birds exhibit GSD, but they have quite different key factors that control their sexual fate. The sex of a therian mammalian embryo is determined by the existence of the *Sry* gene located on the Y chromosome, with heteromorphic sex chromosomes (XY) determining an individual to be male [Kashimada and Koopman, 2010]. In birds, no homolog of *Sry* has been found. Dosage-dependent *DMRT1* on the Z chromosome is considered to determine the male fate, and heteromorphic sex chromosomes (ZW) determine a female [Smith and Sinclair, 2001]. In medaka fish, *dmy* (the DM-domain gene on Y chromosome) is required for male sex determination [Matsuda et al., 2002]. In contrast, GSD and ESD coexist in different

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lineages of reptiles, with GSD prevailing in squamates and ESD in all crocodiles, most turtles, and some lizards [Ferguson and Joanen, 1982; Janzen and Paukstis, 1991; Viets et al., 1993]. ESD mainly occurs as temperature-dependent sex determination (TSD) in reptiles, in which the sex of the offspring is determined by the incubation temperature in the thermosensitive period (TSP) [Viets et al., 1993].

The sex-determining system has been investigated in a number of reptiles, from turtles to crocodilians [Valenzuela, 2004]. These studies identified 3 models of TSD: (1) TSDIa – MF type, with males at low incubation temperatures and females at high temperatures [Willingham et al., 2000; Du et al., 2007, 2010]; (2) TSDIb – FM type, the opposite of TSDIa [Mitchell et al., 2006]; and (3) TSDII – FMF type, with females at both low and high temperatures and males at intermediate temperatures [Ferguson and Joanen, 1982]. Despite widespread occurrence of TSD in reptiles, it is still unclear how the molecular network responds to temperature and drives the sexual fate. Our understanding of the molecular network in reptiles is mostly based on previous studies that investigated sex determination in mammals, although recent transcriptomic approaches have examined the alligator (*Alligator mississippiensis*) [Yatsu et al., 2016], slider turtle (*Trachemys scripta*) [Czerwinski et al., 2016], and painted turtle (*Chrysemys picta*) [Radhakrishnan et al., 2017] genomes directly. Earlier studies suggested that in therian mammals the high expression of S*ry* upregulates the expression of *Sox9* and downstream genes such as *Dmrt1* [Bullejos and Koopman, 2001]. Then, *Sox9* interacts with *Sf1* to upregulate the expression of *Amh* (also known as *Mis*), which controls the regression of the female ductal primordium [Sekido and Lovell-Badge, 2008]. Previous studies have evaluated sex-related genes in different lineages of turtles [Bull et al., 1988; Valenzuela et al., 2013], lizards [Rhen and Crews, 2001; Endo et al., 2008], and in crocodiles [Western et al., 1999a; Urushitani et al., 2011] that undergo TSD by using a candidate gene approach. Although *Sry* has no homologs outside therian mammals, more than 15 homologous genes involved in the sex determination pathway of mammals were identified in reptiles [Capel and Tanaka, 2013]. Amongst these sex-related genes, *Sox9, Dmrt1,* and *Amh* are expressed at a high level in males, and *Foxl2, Cyp19a1,* and *Rspo1* are highly expressed in females [Crews et al., 2001; Shoemaker et al., 2007a; Smith et al., 2008; Valenzuela et al., 2013].

Research on the molecular networks in sex determination in reptiles (especially TSD) is still in the early stages. Importantly, from the existing data it is evident that genes involved in sex determination are expressed differently not only among distant vertebrate lineages but also among closely related species [Valenzuela et al., 2013]. For example, *Sox9* expression precedes *Amh* expression in mammals, but the opposite trend can be observed in birds and alligators [Western et al., 1999a]. Evidence shows that, even in related species, the key genes working as the sex determination trigger can be very different. For example, 2 closely related species of medaka have different key sex-determining genes, namely *dmy* in *Oryzias latipes* and *gsdf* in *O. luzonensis* [Myosho et al., 2012]. Accordingly, profiling of homologous sex-related genes in different species is important for understanding the sex determination system and its evolution in vertebrates. Yet, a vast majority of the studies of gene expression in TSD reptiles concentrated on few species, mostly turtles of the family Emydidae (e.g., *T. scripta, C. picta*), plus a few other turtles, crocodilians (e.g., *A. mississippiensis*) and lizards (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000479360). Most of these turtles are found in North America. For example, transcriptome analysis revealed that genes involved in steroid enzyme synthesis and brain development were sexually dimorphic expressed prior to TSP in *T. scripta* [Czerwinski et al., 2016], similar to what is observed for *Sf1*, *Wt1*, and *Dax1* involved in gonadal development in *C. picta* [Valenzuela et al., 2006; Valenzuela, 2008a, b], and it also revealed that *Cirbp* (cold-inducible RNA-binding protein) is involved in sex determination in *C. serpentina* [Schroeder et al., 2016]. But there are few studies on the molecular networks of TSD reptiles in other regions such as Asia [Hoshi and Nakao, 2008; Inamdar et al., 2015]. Thus, molecular studies across TSD reptiles representing other turtle families, reptilian lineages, and geographic regions are overdue and necessary if we are to fully understand the evolution of TSD.

As a step to help fill this gap, here we studied the sex-related genes of the Chinese three-keeled pond turtle, *Mauremys reevesii* (TSDIa – MF type). To our knowledge, this is the first such study in a chelonian of the family Geomydidae. Specifically, we cloned full open reading frames (ORFs) of sex-related genes such as *Cyp19a1* (*aromatase*), *Foxl2*, *Rspo1*, *Sf1*, and *Sox9* and investigated the expression patterns of these genes as well as of the previously characterized *Dmrt1* [Hoshi and Nakao, 2008] during the TSP at different incubation temperatures to assess the potential roles of these genes in the sex determination of *M. reevesii*. These genes were chosen because they play critical roles in sex determination in other vertebrates, including TSD

reptiles. For instance, *Rspo1* and *Sox9* sit on opposite sides of the balance in early vertebrate sex determination that is then tipped towards the male or female pathway [DiNapoli and Capel, 2008]. *Sf1* is key for the formation of the bipotential gonad and displays one of the earliest thermosensitive expression patterns in TSD turtles [Valenzuela et al., 2006]. *Cyp19a1* is indispensable for ovarian formation, and its activity is thermosensitive in TSD reptiles [Desvages and Pieau, 1992; Smith et al., 1995], and *Foxl2* and *Dmrt1* are candidate transducers of the environmental temperature into TSD and are critical for female and male development, respectively [Matsumoto and Crews, 2012]. Profiling of these genes and their potential functions in the sex-determining pathways provides a solid basis for further investigations on the molecular network underlying TSD in reptiles.

Table 1. Degenerate primer sequences used to clone the genes related to sex determination in *Mauremys reevesii*

	Sequence 5′–3′
Aromatase-dg-sense	GGGCTCAGTTACAGGACCT
Aromatase-dg-antisense	TGTCCAGATGGTCTTTCG
Foxl2-dg-sense	GAAGCCGGACCCSKCKCAG
Foxl2-dg-antisense	GAAGCCRGACTGCAGGTAC
Rspo1-dg-sense	TAGCCTGGGCAATCTTTACGAG
Rspo1-dg-antisense	CCATTGTAAAGTCCTTGGCATT
Sf1-dg-sense	GGACCGTGCAGAATAACAAG
Sf1-dg-antisense	TGTGTTAAGCCAAATCGATGC
Sox9-dg-sense	GAAGCAAGACCTGAAGCGAGA
Sox9-dg-antisense	ACGAGTTGGCCGGTGAGA

Table 2. RACE primer sequences used to clone the genes related to sex determination in *Mauremys reevesii*

	Sequence 5′–3′
Aromatase-3race-1-senses	CCTTTGGATGGGAGTGGG
Aromatase-3race-2-senses	GTCCTGGTCTCGTGCGTATG
Foxl2-5race-1-senses	CCTTCTCGAACATGTCCTCGCAG
Foxl2-5race-2-senses	GGGCACCTTGATGAAGCACTCG
Foxl2-3race-1-senses	GGCAGAACAGCATCCGCCACAACC
Foxl2-3race-2-senses	ACGGCTACCTCTCCCCGCCCAAG
Sf1-5race-1-senses	GACCTCAGGGATGTCGGGTGGGG
Sf1-5race-2-senses	TGGATGCCGTGGATGGTGGAGGAC
Sox9-5race-1-senses	GCTCAGGGTGGTCATCGTG
Sox9-5race-2-senses	GCCGCTGTAGGTGACTTGG

Materials and Methods

Egg Collection and Incubation

In May 2014, we obtained 120 freshly laid eggs from mixed 15–30 clutches of the three-keeled pond turtle (*M. reevesii*) from a private turtle farm in Hangzhou, Zhejiang province. The eggs were maintained in vermiculite and transported to the laboratory. All eggs were candled, and viable eggs with a white fertilization spot on top of the egg shell were randomly placed in trays (220 × 100 × 80 mm) containing 1:1 vermiculite and water. The trays were then transferred to 2 incubators (KB240; Binder, Germany) set at 26°C (male-producing temperature [MPT]) and 30°C (female-producing temperature [FPT]) [Du et al., 2007]. Additional eggs (5–10) laid the same day were opened to assess their developmental stage, and all embryos were at a stage earlier than stage 12 [Greenbaum, 2002] at the onset of the incubation experiments. The temperatures of the incubators were monitored daily with thermal data loggers (iButton Thermochron, DS 1921; MAXIM Integrated Products Ltd.). The egg trays were rotated twice a week within the incubator to avoid small temperature-gradient effects.

Tissue Collection

Embryonic development was monitored by periodic dissection of randomly selected eggs every other day after 1 week of incubation. The developmental stages of the embryos were determined according to the staging criteria previously established for *T. scripta* [Greenbaum, 2002]. A total number of 108 embryos were harvested across stages 15, 16, 17, 19, 21, and 23 (9 embryos per stage per temperature), and the adrenal-kidney-gonad (AKG) complex of each embryo was rapidly dissected and immediately frozen in liquid nitrogen. The AKG complex has been utilized as a tissue source to investigate the expression of genes involved in sexual development [Murdock and Wibbels, 2006]. While using the AKG complex can mask differential expression from the gonad alone when expression is present in the larger adrenal-kidney portion [Valenzuela et al., 2013], valuable insights can be gained from differential expression in AKG complex data that help develop a working hypothesis for future research. Here, we used the AKG complex as an initial step to explore the expression of sex-related genes in *M. reevesii*. While the presence of TSD in *M. reevesii* is well documented [Du et al., 2007], no study has determined the TSP for this turtle. Thus, we assumed that the target stages sampled here may encompass the early, middle, and late TSP in this species as it occurs in other cryptodiran turtles [Bull and Vogt, 1981], but we note that because the TSP can vary among closely related species [Bull and Vogt, 1981; Bieser and Wibbels, 2014; Gomez-Saldarriaga et al., 2016], further testing is needed to corroborate the validity of this assumption.

Total RNA Extraction and cDNA Synthesis

Three AKG complexes were pooled together as a sample and homogenized in a mortar with liquid nitrogen. Three of these pooled samples were extracted for each developmental stage at different incubation temperatures. Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's

Table 3. Real-time PCR primer sequences for the genes related to sex determination in *Mauremys reevesii*

	Sequence 5′–3′	Product length, bp[a]
β-actin-RT-sense	CTGCTCACTGAAGCCCCCC	201
β-actin-RT-antisense	GGCGTAGCCCTCGTAGATTGG	
Aromatase-RT-sense	TCCTTCTCGCTACTTTCAGCCATTTG	197
Aromatase-RT-antisense	ATAAGGGCTGTCTTTCATTCGGGTG	
Foxl2-RT-sense	CCGGCATCTACCAGTACATCATCAGC	116
Foxl2-RT-antisense	GGGCACCTTGATGAAGCACTCGTT	
Rspo1-RT-sense	TCCACCTGCCCAGAAGGCTACAC	140
Rspo1-RT-antisense	TCGTTGCCTTTCTTGAAGCCACAC	
Sf1-RT-sense	TGTCCTCCACCATCCACGGC	167
Sf1-RT-antisense	GGGAAAGGAGGGGTAATGGTAGC	
Dmrt1-RT-sense	TGGAAAGCAACAGCCCAACAC	174
Dmrt1-RT-antisense	ATGGCTGGTAAAAACTGCCGTAG	
Sox9-RT-sense	GACCATCACCCGCTCGCAG	137
Sox9-RT-antisense	GCGTGTACATGGGCCTTTGG	

[a] According to Udar et al. [2003].

protocol. The quality and quantity of each total RNA sample were assessed using a spectrophotometer (Thermo) at 260 and 280 nm. Then, the total RNA was treated with gDNA eraser to eliminate genomic DNA contamination, and single-stranded cDNA was reverse-transcribed using the RT reagent kit with gDNA eraser (TaKaRa Bio Inc.) according to the manufacturer's protocol.

Cloning of Turtle Gene Homologs

Degenerate primers for *Cyp19a1*, *Foxl2*, *Rspo1*, *Sf1*, and *Sox9* were designed on conserved regions based on the sequences of *T. scripta*, *C. picta*, *A. mississippiensis*, *Lepidochelys olivacea*, and human (Table 1). Primers were used to amplify cDNA fragments of 646 bp of *Cyp19a1* (annealing: 52°C, extension: 0.5 min), 415 bp of *Foxl2* (annealing: 58°C, extension: 0.5 min), 1,600 bp of *Sf1* (annealing: 56°C, extension: 1.5 min), 974 bp of *Sox9* (annealing: 59°C, extension: 1 min), and the full length of *Rspo1* (1,632 bp; annealing: 59°C, extension: 1.5 min). The PCR cycling conditions were as follows: 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 52–59°C for 30 s, 72°C for 0.5–1.5 min, and then 72°C for 7 min. Then, 5′- and 3′-rapid amplification of cDNA ends (RACE) were performed to obtain the full length of *Cyp19a1*, *Foxl2*, *Sf1*, and *Sox9* by using the SMART RACE Kit (Clontech, TaKaRa Bio) according to the manufacturer's instructions. The primers used for RACE were designed on the basis of the fragments of each gene (Table 2). The amplified fragments were separated using gel electrophoresis and then purified with the QuickGel Extraction Kit (CWBIO). The purified fragments were cloned into the pEASY-T1 vector (Transgen) and sequenced using M13F and M13R primers. For *Dmrt1*, we used the full length sequence (GenBank: AB365876.1) previously cloned by others [Hoshi and Nakao, 2008].

Quantitative Real-Time PCR

AKG complexes from 3 individual turtles were pooled into one sample, and 3 such samples were used to quantify gene expression via QRT-PCR per stage for each temperature treatment. Relative gene expression levels were assessed using SYBR Green (Bio-Rad) according to the manufacturer's protocol and the Roche LightCycler 480 real-time PCR cycler (Roche). All samples were amplified in triplicate, and the expression data from the target genes were normalized to the constitutive expression of β-actin. The relative gene expression levels were calculated using the equation $2^{-\triangle\triangle CT}$. The primers (Table 3) used to assay gene expression were designed using Primer Premier 5.0 (PremierBiosoft). The quantitative PCR cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 63°C for 20 s, and then a final melting curve from 65–95°C to assess that single products were amplified by the qPCR reactions. Specificity was verified further using agarose gel electrophoresis.

Statistical Analysis

We conducted factorial analysis of variance to detect the influence of temperature and developmental stage on the expression of the sex-related genes. The data are presented as means ± one standard error, and statistical significance was assessed at $\alpha = 0.05$.

Results

Isolation of cDNA

After performing RT-PCR and RACE, we obtained the full lengths of the 5 genes (available in NCBI GenBank). Overall, nucleotide and amino acid sequences from *M. reevesii* closely resemble those reported for other turtles and were increasingly disparate to those of alligator, chicken, mouse, and human as described below.

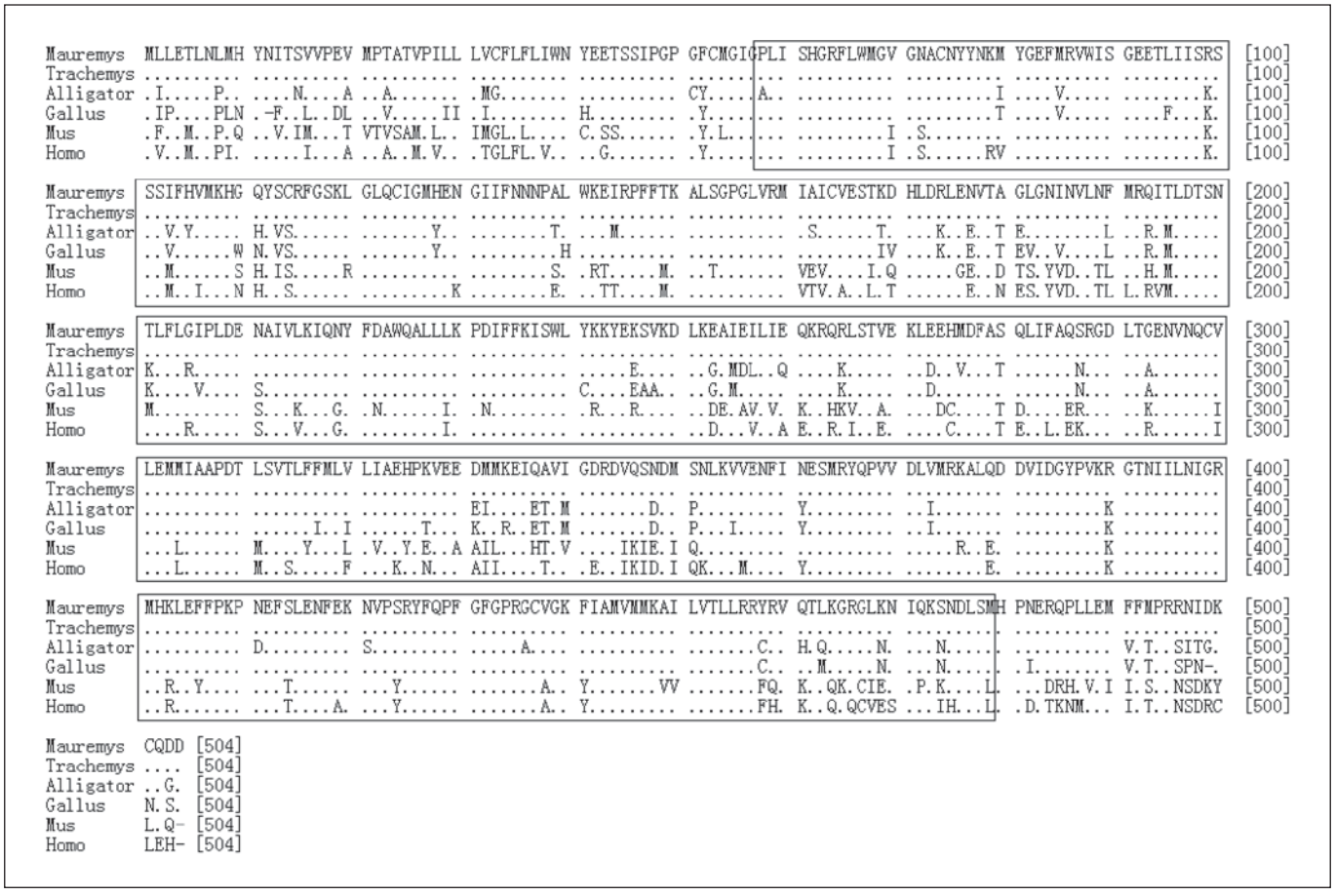

Fig. 1. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human CYP19A1. The box demarcates the conserved domain of the cytochrome P450 superfamily. Accession numbers for the sequences used are turtle (*Trachemys scripta*), AAG09376; alligator (*Alligator mississippiensis*), NP001274195; chicken (*Gallus gallus*), NP001001761; mouse (*Mus musculus*), NP031836; human (*Homo sapiens*), NP000064.

Cyp19a1

A total of 1,995 bp of nucleotide sequence (GenBank: KU821113) and 504 of deduced amino acid sequence were obtained (online suppl. Fig. 1). The deduced amino acid sequence was 97, 86, 82, 73, and 76% identical to *T. scripta*, alligator, chicken, mouse, and human aromatase, respectively (Fig. 1). The conserved domain of the cytochrome P450 superfamily was identical between *M. reevesii* and *T. scripta*.

Foxl2

A total of 2,224 bp of nucleotide sequence (GenBank: KU821114) and 301 of deduced amino acid sequence were obtained (online suppl. Fig. 2). The deduced amino acid sequence was 99, 84, 86, 70, and 66% identical to *C. picta*, alligator, chicken, mouse, and human FOXL2, respectively (Fig. 2). The forkhead domain was highly conserved across vertebrates, with only a single amino acid change at position 86 in mammals.

Rspo1

A total of 1,633 bp of nucleotide sequence (GenBank: KU821115) and 260 of deduced amino acid sequence were obtained (online suppl. Fig. 3). The deduced amino acid sequence was 98, 85, 87, 65, and 68% identical to *C. picta*, alligator, chicken, mouse, and human RSPO1, respectively (Fig. 3). The conserved domain of Furin-like repeats was identical between *M. reevesii* and *C. picta*.

Sf1

A total of 1,812 bp of nucleotide sequence (GenBank: KU821116) and 466 of deduced amino acid sequence

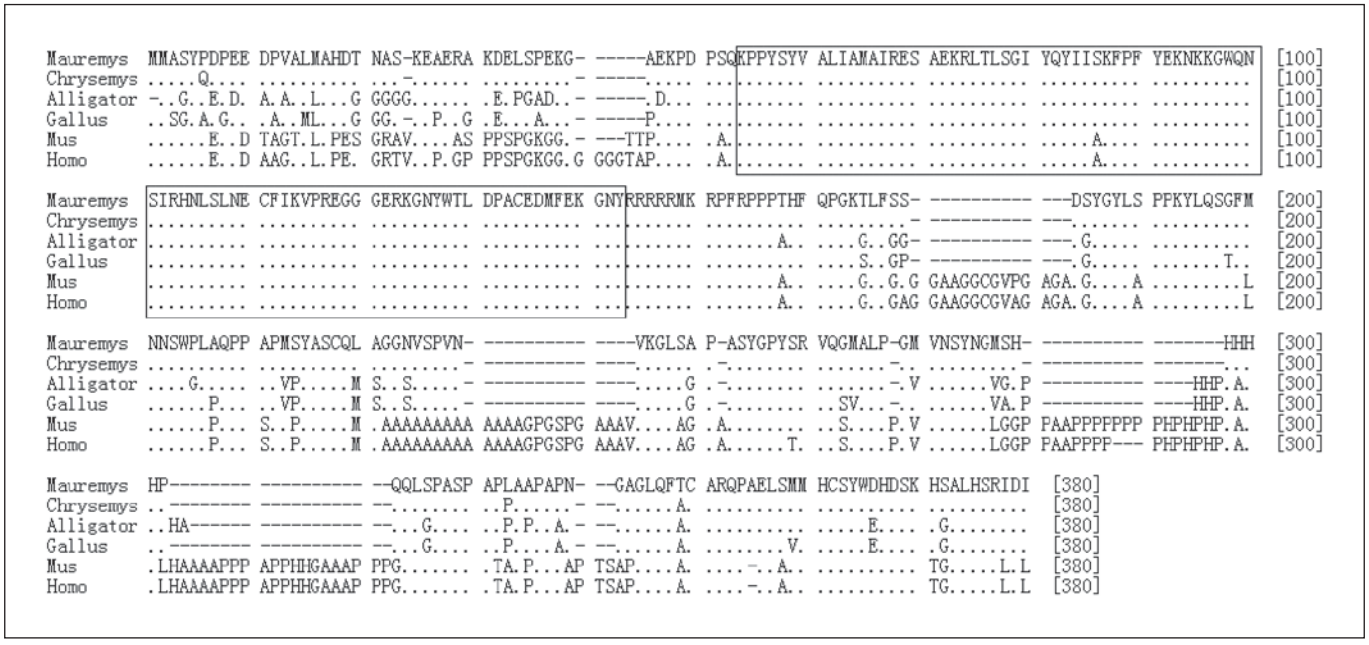

Fig. 2. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human FOXL2. The box demarcates the forkhead domain. Accession numbers for the sequences used are turtle (*Chrysemys picta*), XP005282573; alligator (*Alligator mississippiensis*), XP006258874; chicken (*Gallus gallus*), NP001012630; mouse (*Mus musculus*), NP036150; human (*Homo sapiens*), NP075555.

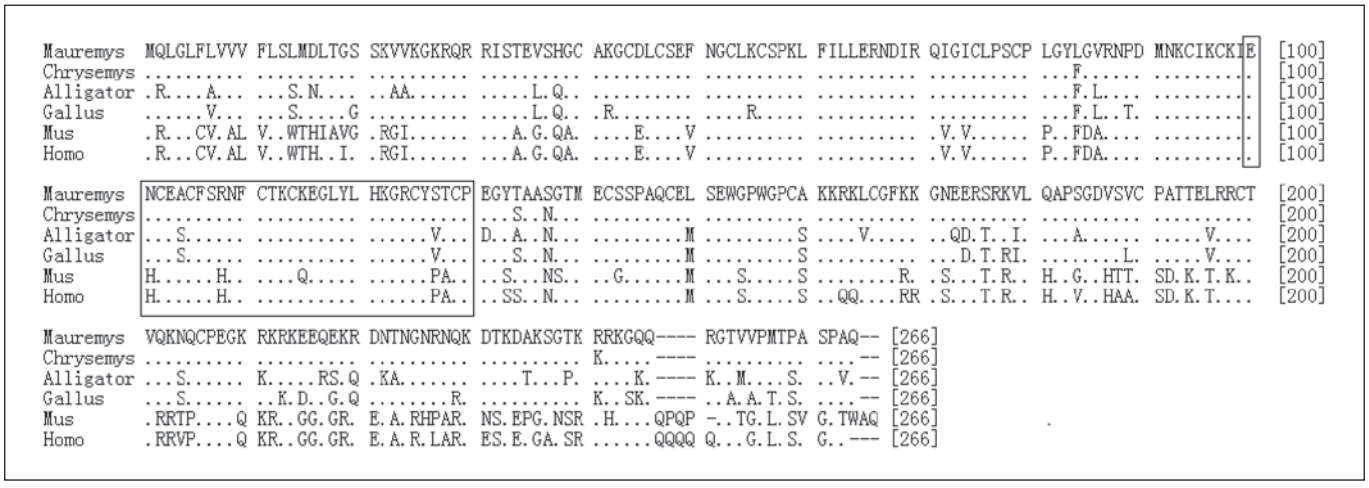

Fig. 3. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human RSPO1. The box demarcates the conserved domain of Furin-like repeats. Accession numbers for the sequences used are turtle (*Chrysemys picta*), XP005298408; alligator (*Alligator mississippiensis*), XP006264933; chicken (*Gallus gallus*), NP001305373; mouse (*Mus musculus*), NP619624; human (*Homo sapiens*), NP001033722.

were obtained (online suppl. Fig. 4). The deduced amino acid sequence was 98, 93, 88, 70, and 71% identical to *C. picta*, alligator, chicken, mouse, and human SF1, respectively (Fig. 4). The DNA-binding domain was identical between *M. reevesii* and *C. picta*; only 2 amino acid changes were identified in alligator and mammals, while the ligand-binding domain was less conserved.

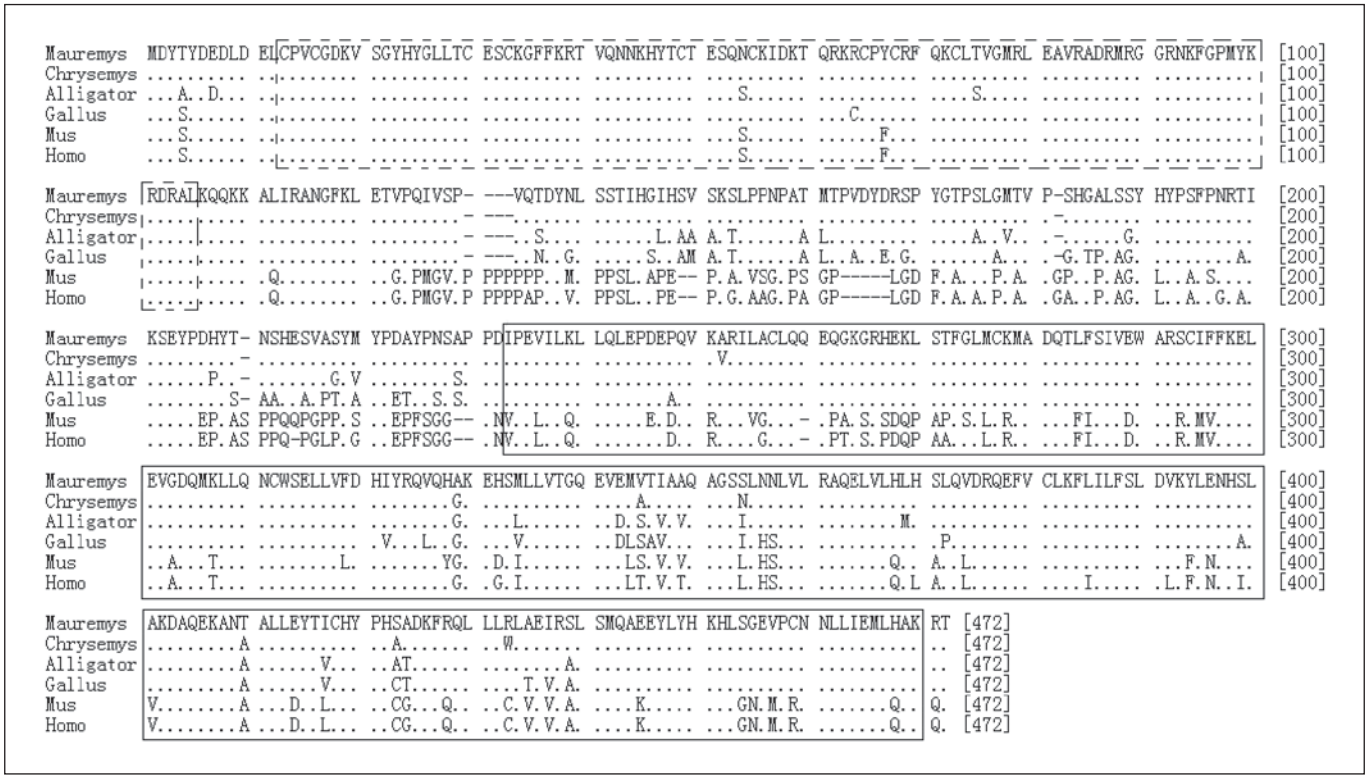

Fig. 4. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human SF1. The dashed box demarcates the N-terminal DNA-binding domain and the solid box demarcates the C-terminal ligand-binding domain. Accession numbers for the sequences used are turtle (*Chrysemys picta*), XP005279455; alligator (*Alligator mississippiensis*), XP006271373; chicken (*Gallus gallus*), NP990408; mouse (*Mus musculus*), NP620639; human (*Homo sapiens*), NP004950.

Sox9

A total of 2,053 bp of nucleotide sequence (GenBank: KU821117) and 301 of deduced amino acid sequence were obtained (online suppl. Fig. 5). The deduced amino acid sequence was 98, 95, 90, 85, and 85% identical to *T. scripta*, alligator, chicken, mouse, and human SOX9, respectively (Fig. 5). As expected, the SOX-TCF HMG-box was highly conserved between vertebrates.

Gene Expression Patterns at Different Incubation Temperatures

The expression of several putative genes involved in TSD was analyzed using RT-PCR (Fig. 6) across stages 15–23 (the morphological status of the developing gonad across stages 16–23 is shown in online suppl. Fig. 6), which we presume encompass the TSP in *M. reevesii*. The expression level of *Cyp19a1* was low throughout the presumptive TSP at MPT. However, it was low in the early presumptive TSP and increased after stage 17, reaching its peak at stage 21 at FPT. As a result, the expression level of *Cyp19a1* was significantly higher at FPT than at MPT ($F_{1, 24}$ = 44.40, $p < 0.001$; Fig. 6C). Similar to the expression level of *Cyp19a1*, the level of *Foxl2* was significantly higher at FPT than at MPT ($F_{1, 24}$ = 37.98, $p < 0.001$; Fig. 6A). At FPT, the expression level of *Foxl2* increased at stage 16 and remained high until the end of the presumptive TSP. *Rspo1* was another gene that showed high-level expression in females ($F_{1, 24}$ = 50.47, $p < 0.001$; Fig. 6B), with its expression reaching a peak at stage 19 of FPT but remaining low at MPT. In contrast, the expression level of *Sf1* was higher at MPT than at FPT ($F_{1, 24}$ = 5.16, p = 0.032; Fig. 6D). Similarly, the expression level of *Dmrt1* was higher at MPT than at FPT from stage 16 onwards, with a peak at stage 19 ($F_{1, 24}$ = 68.74, $p < 0.001$; Fig. 6E). Inconsistent with the expression levels of the other genes, the expression level of *Sox9* did not differ between MPT and FPT throughout the presumptive TSP ($F_{1, 24}$ = 1.04, p = 0.31; Fig. 6F).

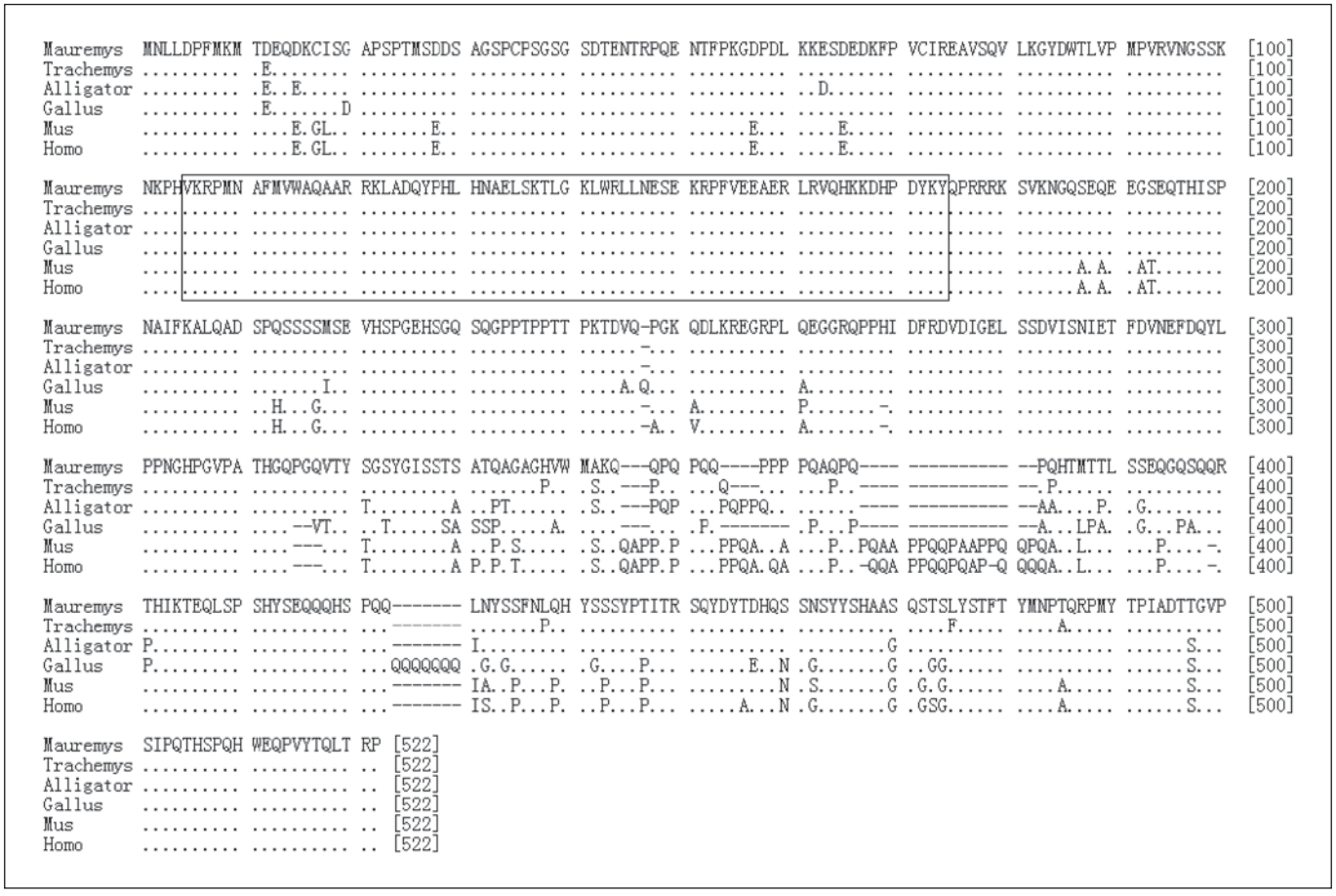

Fig. 5. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human SOX9. The box demarcates the conserved domain of the SOX-TCF HMG-box. Accession numbers for the sequences used are turtle (*Trachemys scripta*), ACG70782; alligator (*Alligator mississippiensis*), NP001274197; chicken (*Gallus gallus*), NP989612; mouse (*Mus musculus*), NP035578; human (*Homo sapiens*), NP000337.

Discussion

We obtained full-length ORFs of 5 genes that are candidates involved in the sex determination cascade of *M. reevesii* given their role in this developmental process in other vertebrates. In addition, the amino acid sequences were characterized. This information will help us to understand the functions of the gene products and provide a basis for further studies on how these genes have evolved. A recent study showed that a consistent amino acid shift at positions 54 and 57 in DMRT1 distinguished GSD and TSD species and accompanied the evolution of GSD in reptiles from an ancestral TSD condition, as turnover events occurred by 1 or 2 mutations at least 3 times among non-avian reptiles [Janes et al., 2014]. Thus, our study contributes in identifying amino acid sequences of other sex-related genes in additional reptiles, which should help to understand whether the inter-species difference in protein sequences accompanies the shift between TSD and GSD and to illuminate the evolution of sex determination mechanisms.

Our study also uncovered the AKG expression patterns of these genes during the presumptive TSP in *M. reevesii* and points to their potential roles in urogenital development and perhaps sex determination and differentiation. On one hand, *Foxl2*, *Rspo1,* and *Cyp19a1* are genes with female-biased expression involved in ovarian formation in vertebrates, and consistently, these genes show sex-dimorphic expression during the presumptive TSP, with significantly higher expression levels at FPT than at MPT in *M. reevesii* (Fig. 6A–C). In contrast, *Sf1* and *Dmrt1* are involved in testicular development in ver-

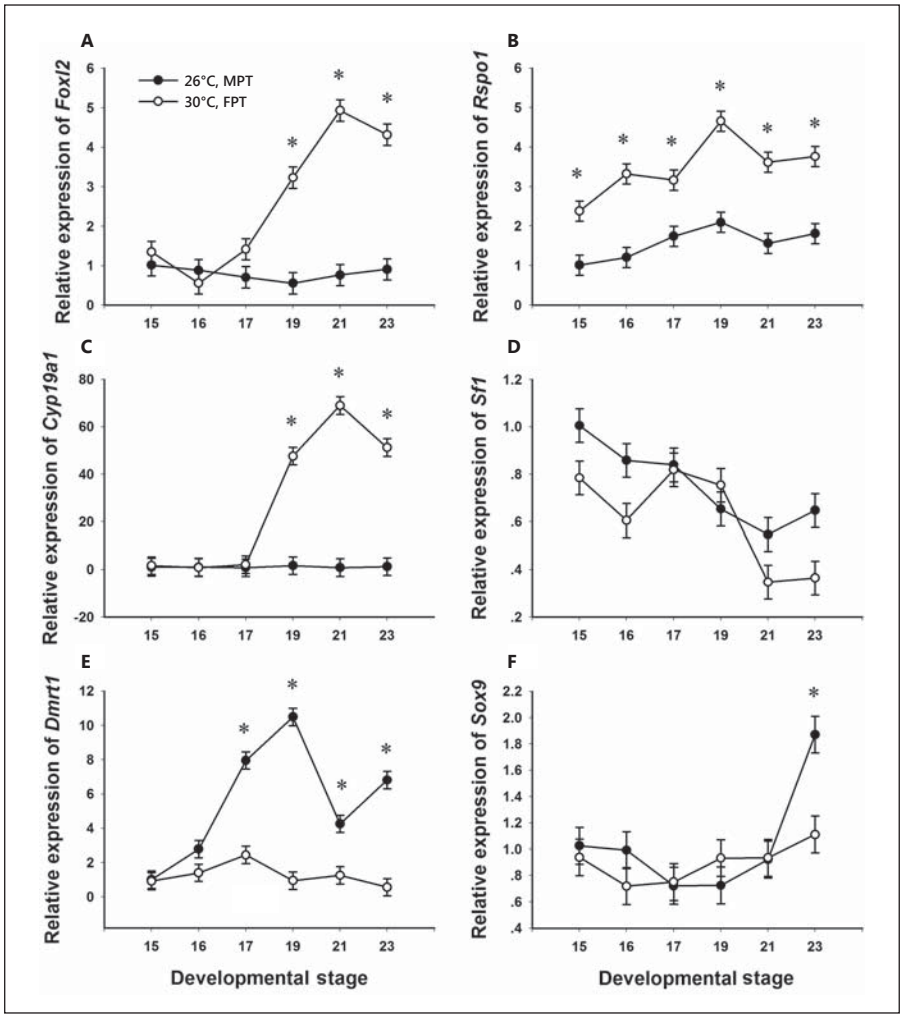

Fig. 6. Effects of temperature on the expressions of *Cyp19a1*, *Foxl2*, *Rspo1*, *Sf1*, *Dmrt1,* and *Sox9* in the adrenal-kidney-gonad (AKG) complex (n = 3 mixed samples) of *Mauremys reevesii* using quantitative PCR. Relative transcript abundance was normalized to the constitutively expressed gene β-actin. Expression levels at the female-producing temperature (FPT) are shown in open circles, levels at the male-producing temperature (MPT) are shown in black circles. The y-axis indicates fold changes of gene expression with respect to expression during stage 15 at MPT. The x axis indicates the developmental stage. Asterisks indicate significant difference between temperatures. Factorial ANOVA showed that temperature significantly affected the expression levels of the genes ($p < 0.0001$). The data are presented as mean fold changes ± standard error.

tebrates, and here they showed male-biased expression (Fig. 6D, E). As in other turtles, *Sox9* expression was male-biased only at the end of the presumptive TSP [Torres Maldonado et al., 2002; Shoemaker et al., 2007b], which is different from the expression pattern found in the developing gonads of mammals, perhaps because we examined AKG complexes and not gonads alone. Below, we discuss the roles of these genes in sex determination and differentiation in greater detail.

Genes with Female-Biased Expression

The 3 female-biased genes, *Foxl2*, *Rspo1,* and *Cyp19a1,* play an important role in female sexual development.

Foxl2 is a member of the forkhead-box transcription factor family, which is a key factor in female sex determination in both GSD and TSD. *Foxl2* deficiency causes premature ovarian failure and the blepharophimosis-ptosis-epicanthus inversus syndrome [Crisponi et al., 2001; Loffler et al., 2003]. In mammals, *Foxl2* is required to suppress ovary-to-testis transdifferentiation. Loss of *Foxl2* expression in the follicle leads to upregulation of *Sox9*, and follicle and theca cells reprogram to form Sertoli and Leydig cells [Uhlenhaut et al., 2009]. The *Foxl2* female-biased expression observed in our study mimics the pattern in other TSD reptiles [Matsumoto and Crews, 2012; Janes et al., 2013].

Rspo1 is located upstream in the Wnt pathway and is involved in early gonadal development [Bernard et al., 2012; Chassot et al., 2012]. *Rspo1* deficiency can lead to female-to-male sex reversal in mammals [Parma et al., 2006; Chassot et al., 2008]. Consistent with this role, *Rspo1* was found to be upregulated in our study during female development as in other TSD reptiles (Fig. 6B) [Smith et al., 2008] and fish [Zhang et al., 2011; Zhou et al., 2016].

Cyp19a1 is involved in various developmental and biochemical processes (e.g., secondary sexual characteristics, hormone secretion, and glucose homeostasis) in GSD vertebrates [Rigaudiere et al., 1989; Baghaei et al., 2003; Bader et al., 2012; Chen et al., 2015]. Aromatase, a gene product of *Cyp19a1*, is a key enzyme for estrogen biosynthesis [Kitano et al., 1999] and is a key factor in sex determination and ovarian differentiation [Bogart, 1987; Lambeth et al., 2013], because estrogen, the product of the aromatization of testosterone by aromatase, can override the effect of male-determining genes (e.g., *Sry*) and induce ovarian development [Kobayashi et al., 2003; Pask et al., 2010]. In some fish and reptiles, *Cyp19a1* shows female-biased expression, as found in our study (fig. 6C) [Gabriel et al., 2001; Ramsey et al., 2007], and environmental temperatures can affect *Cyp19a1* expression and in turn reverse the sex of GSD teleosts and TSD reptiles [Baroiller et al., 2009; Matsumoto and Crews, 2012]. In addition, the methylation level of *Cyp19a1* is highly correlated with its expression and temperature [Matsumoto et al., 2013, 2016]. Therefore, *Cyp19a1* appears to play a critical role in sex differentiation of TSD species.

Genes with Male-Biased Expression

Sf1 and *Dmrt1* show significantly male-biased expression in *M. reevesii* (Fig. 6D, E) as well as in some other species.

Sf1 encodes a nuclear orphan receptor involved in the formation of steroidogenic organs and in turn in sex determination [Parker and Schimmer, 1997; Morohashi, 1999]. The expression pattern of *Sf1* is not conserved among different lineages of vertebrates [Valenzuela et al., 2013]. It is highly expressed during testicular development in mammals [Hatano et al., 1994; Ikeda et al., 1994] and during ovarian development in birds and fish [Smith et al., 1999; Ijiri et al., 2008]. Similarly, the expression pattern of *Sf1* differs among reptiles with TSD. For example, *Sf1* is expressed in a female-biased way in *A. mississippiensis* [Western et al., 2000] but male-biased in turtles (*M. reevesii, T. scripta,* and *C. picta*) [Fleming et al., 1999; Crews et al., 2001; Valenzuela et al., 2013; this study]. Although in our result *Sf1* is more highly expressed in the entire AKG complex at MPT than at FPT, the dimorphic expression across stages differed from the pattern observed in other TSD turtles. Because *Sf1* is highly expressed in the adrenal, this could mask subtle differential expression in the gonad that might exist [Fleming et al., 1999], as observed in other TSD turtles [Fleming and Crews, 2001; Valenzuela et al., 2006, 2013].

Dmrt1 encodes a putative transcription factor containing a zinc-finger-like DNA-binding motif (DM domain) [Shen and Hodgkin, 1988], and its homologs have been found in all vertebrates [Raymond et al., 1999; Nanda et al., 2002; Koopman, 2009]. *Dmrt1,* or its homolog *dmy,* plays an important role in sex determination of birds [Koopman, 2009] and fish [Matsuda et al., 2002]. Similarly, our study indicated that *Dmrt1* may also be responsible for male sex determination in *M. reevesii* because of its male-biased expression during the early presumptive TSP (Fig. 6E), which is consistent with the findings of previous studies on other TSD reptiles [Murdock and Wibbels, 2006; Shoemaker et al., 2007b].

Sox9 is an important sex determination gene in mammals, because it is a direct target of *SRY* and can upregulate the down-stream target *Amh*, which leads to mullerian duct degeneration [Hiramatsu et al., 2010]. However, in *Xenopus tropicalis*, *Sox9* is regarded as a gene involved in sex differentiation rather than sex determination, because the expression of *Sox9* is first detectable when the gonads are well differentiated [El Jamil et al., 2008]. Our study detected monomorphic *Sox9* expression from stage 15 to 22 and dimorphic male-biased expression at the end of the presumptive TSP, suggesting that *Sox9* may be involved in late testis differentiation as previously shown in other turtles [Torres Maldonado et al., 2002; Shoemaker et al., 2007b; Diaz-Hernandez et al., 2012]. Alternatively, our use of AKG complexes could have masked earlier gonadal differential expression of *Sox9* as it has been reported in other TSD turtle studies [Shoemaker et al., 2007b; Valenzuela et al., 2013]. Further, in *L. olivacea*, *Sox9* is expressed in medullary cords of the bipotential gonads in both male and female embryos and is turned off in presumptive ovaries and remains expressed in presumptive testis [Diaz-Hernandez et al., 2012], a pattern that differs from other TSD turtles [Valenzuela et al., 2013]. However, discrepancies between our study and findings in other vertebrates may represent true interspecific differences in *Sox9* expression, which is known to be evolutionarily labile [Valenzuela et al., 2013]. For instance, a pattern similar to ours was found in *A. mississippiensis* [Western et al., 1999b], but a male-biased expression pattern was detected in some turtles (*L. olivacea* and *T. scripta*) [Torres Maldonado et al., 2002; Shoemaker et al., 2007b]. In addition, SRY along with SF1 binds to the enhancer of *Sox9* in mammals [Sekido and Lovell-Badge, 2008], while in TSD turtles which lack *SF1*, the regulation of *Sox9* is certainly different than in mammals.

In conclusion, vertebrate ovarian developmental genes *Cyp19a1*, *Foxl2,* and *Rspo1* exhibit consistent expression

patterns in the developing AKG complexes of *M. reevesii*, whereas the expression of *Sf1*, *Dmrt1*, and *Sox9* is consistent with their involvement in the male developmental pathway. However, whether discrepancies in the observed profiles for *Sf1* and *Sox9* are attributable to their masking expression in adrenal-kidney tissue remains uncertain. Taken together, these genes are important candidates involved in sex determination and differentiation in *M. reevesii,* and further research using gonadal tissue separated from the adrenal-kidney is warranted. Our study provides a solid basis for further investigations on the molecular mechanism of sex determination in this species as well as other vertebrates in general.

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Statement of Ethics

The research was performed under approval of the Animal Ethics Committee at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001).

Disclosure Statement

The authors have no conflicts of interest to declare.

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