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Original Article

Evolution and gene expression of matrix metalloproteinase gene family during gonadal development in *Scatophagus argus*

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Abstract: Background: During the reproductive cycle of *Scatophagus argus* (*S. argus*), their gonads undergo degeneration and re-maturation including the degradation of proteins in the extracellular matrix (ECM). Matrix metalloproteinases (MMPs), a family of zinc proteases, play a crucial role in ECM degradation. Objective: Our aim was to identify the MMP gene family of *S. argus* and determine their gene expression levels across various stages of gonadal development. Methods: The MMP gene family of *S. argus* in the genome was identified by using basic Local Alignment Search Tool (BLAST) and HMMER. Phylogenetic tree and synteny analysis were performed to investigate the evolutionary past of the MMP gene family. The gonads of 18 *S. argus* (9 males and 9 females) were dissected and a quantitative reverse transcription polymerase chain reaction (RT-qPCR) was conducted to investigate the expression levels of MMP genes across different stages of gonadal development. Results: Twenty-three MMP family genes were identified in the genome of *S. argus*. We divided the MMP gene family into 4 categories and found that teleosts exhibit a higher MMP gene copy number relative to other vertebrates. By sampling *S. argus* at different stages of gonadal development, we observed an upregulation in relative expression levels of 11 MMP genes in the testis or ovary. Ten MMP genes (*mmp2*, *9*, *14a*, *15a*, *15b*, *16a*, *17a*, *23b* and *24*) showed higher mRNA expression in the testis compared to the ovary and *mmp28* had higher expression during ovarian development. The tissue distribution results demonstrated that the gills exhibited the lowest relative expression level among all tissues examined. However, 6 genes (*mmp2*, *9*, *14a*, *15a*, *15b*, and *16a*) had relatively high expression in all tissues. Conclusion: The result suggested that teleost-special whole genome duplication was mainly responsible for the formation of the MMP gene family in teleosts. Expression patterns of MMP genes indicated that *mmp2*, *9*, *14a*, *15a*, *15b*, *16a*, *17a*, *23b* and *24* played a vital role in testicular development while *mmp28* was more important for ovarian development. Limitaion: Further studies are needed to determine their protein expressions in gonadal development and precise mechanism in gonadal differentiation. The study enhances our understanding of the MMP gene family in evolution of teleost and provides valuable insights for further research on MMPs in *S. argus*.

Keywords: MMP gene family, bioinformatics analysis, gonadal MMP gene expression

Introduction

Matrix metalloproteinases (MMPs) are a group of zinc-dependent enzymes that play a crucial role in the degradation of various proteins within the extracellular matrix (ECM). This process is essential for maintaining the stability of biological processes [1]. MMPs belong to the metzincins superfamily, characterized by a zinc-binding motif HEXXHXXGXX (H/D) followed by a conserved methionine Met-turn [2, 3].

MMPs are widely present in most eukaryotes, with few exceptions in basal metazoan clades [4]. The first identified vertebrate member of MMPs, MMP1, was initially discovered for its enzymatic activity in tadpole tail resorption back in 1962 and was described as a depolymerizing enzyme [5, 6]. Currently, there are more than 26 MMP genes found in vertebrates, of which 24 distinct MMP genes are identified in the human [7], with 23 MMP genes in mice [8], and 25 genes encoding MMPs in zebrafish

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(*Danio rerio*) [9, 10]. Invertebrates, on the other hand, possess a larger number of homologous genes [11]. Certain species have been discovered to harbor unique MMP genes or proteins such as *zmp2* in *Caenorhabditis elegans* [12], *mmp30* in zebrafish [10], HMMP in hydra (*Hydra vulgaris*) [13], and MMP18 in African clawed frog (*Xenopus laevis)* [14].

Each MMP typically consists of three common domains: a hemopexin-like C-terminal domain, a pro-peptide domain, and a catalytic domain [15]. The transcription and enzyme activity levels tightly regulate the proteolytic activity of MMPs [16, 17]. Most members of the MMP gene family have similar cis-acting binding sites in their gene promoter region, resulting in coordinated activation or inhibition of MMPs by various stimulating factors such as inflammatory cytokines, growth factors, glucocorticoids, or retinoic acid [18]. Additionally, endogenous inhibitors of MMPs, primarily including tissue inhibitors of metalloproteases (TIMPs) and α2-macroglobulin, can impede MMP activities by binding to the hemopexin domain at the C-terminal domain [19]. Based on their domain organization and substrate preference, they are classified into 6 categories: gelatinases, collagenases, matrilysins, membrane-type (MT)-MMPs, stromelysins, and others [15, 20].

Currently, there exists a well-established comprehension regarding the involvement of MMPs in diverse biological processes, including inflammation and immunity, cell migration, nervous system functioning, and notably reproduction [21-25]. Normal physiological processes involved in reproduction, such as the development of a functioning gonad and the release of an oocyte capable of fertilization during ovulation, rely on the dynamic remodeling of ECM. This remodeling process is regulated by a range of signaling molecules and multiple metalloproteases, such as MMPs. Numerous studies have reported a significant correlation between MMPs and reproductive processes in teleost fish over several decades. In medaka (*Oryzias latipes*), the expression of *mmp2* was higher 12 hours before ovulation compared to the ovulation period, while the relative expression of *mmp9* was higher during the ovulation period than 12 hours before ovulation [26]. The development of mature organs through migration and development of primordial germ cells (PGCs) from bipotential gonads is a remarkable process involving tissue remodeling [27, 28]. In the process of gonad formation in zebrafish, somatic cells positioned ahead of the germ cells release three different MMPs (MMP2, MMP9, and MMP14) to regulate the extracellular matrix surrounding the germ cells during their migration [29]. In the study by Chaves-Pozo, distinct expression patterns of *mmp9* and *mmp13* were observed in *Sparus aurata* during the male phase of the reproductive cycle when the testis undergoes abrupt morphological changes [30]. The mRNA expression of the *mmp9* remains constant during early spermatogenesis and progressively intensifies towards later stages, while the *mmp13* exhibits elevated expression during spermatogenesis followed by a gradual decline, reaching its lowest level during interphase.

Scatophagus argus (*S. argus*), also known as spotted scat, is a tropical euryhaline species belonging to the family Scatophagidae of order Perciformes. It exhibits a wide distribution range encompassing China, the Malay Archipelago, Indo Pacific, Philippines, and Australia [31, 32]. This omnivorous fish species displays evident sexual dimorphism in terms of adult body length and weight [33-36]. In recent years, due to its high protein content, palatability, and remarkable stress resistance, *S. argus* has shown significant commercial potential in the fish market and emerged as a popular candidate for marine aquaculture [37-39]. Our research group previously reported on the genome sequencing of *S. argus* using 2 sex-linked molecular markers (*dmrt1* and *dmrt3*), which revealed an XX-XY sex-determination system in this species [36]. Additionally, we have studied the gonadal transcriptome of female and male adult *S. argus* [40]. Moreover, we have reported on the isolation, expression pattern, and gene regulation of significant gonadal expressed genes in *S. argus*, including *amhrII*, *gsdf*, *nr0b1a*, *nr0b1b*, *igf3*, *foxl2* and *pgr* [40-45]. Having focused on reproduction regulation for the past few years, we have successfully obtained fertilized eggs by artificial induction of spawning, involving the injection of sex hormone mixtures during the reproductive period and subsequent artificial breeding. However, natural reproduction has not yielded any matured eggs yet, and further investigation is required to elucidate the reproductive regula-

tion of *S. argus*. Considering the role of MMPs in medaka on ovulation [46], we propose to explore whether MMPs exhibit a similar function in *S. argus*.

This study employed bioinformatics analysis to perform gene identification, phylogenetic tree construction, gene structure analysis, and synteny analysis of the MMP gene family in *S. argus*. Furthermore, a quantitative reverse transcription polymerase chain reaction (RT-qPCR) was conducted to investigate the tissue distribution and expression patterns of 11 MMPs during different stages of gonadal development. These findings provide valuable insights into understanding the involvement of the MMPs in *S. argus* on reproductive regulation during gonadal development.

Materials and methods

Animals and sampling

All *S. argus* used in this study were sourced from Zhanjiang Donghai Island Cultivation Base (Zhanjiang, Guangdong, China) and raised in water at 24°C-25°C. They were fed a commercial floating diet twice daily at 9:00 and 19:00. Before dissection, the fish were anesthetized using 100 mg/L of tricaine methane sulfonate (MS 222, Sigma, Saint Louis, MO). This experiment involving *S. argus* was conducted with the approval of the Animal Research and Ethics Committee of Guangdong Ocean University.

Gene identification

The MMP genes and MMP protein sequences of vertebrates, including *Anolis carolinensis* (*A. carolinensis*), *Callorhinchus milii* (*C. milii*), *Danio rerio* (*D. rerio*), *Gallus gallus*, *Gracilinanus agilis* (*G. agilis*), *Hippocampus comes* (*H. comes*), *Homo sapiens* (*H. sapiens*), *Larimichthys crocea* (*L. crocea*), *Macaca mulatta* (*M. mulatta*), *Mus musculus* (*M. musculus*), *Oryzias latipes* (*O. latipes*), *Salmo salar* (*S. salar*), *Takifugu rubripes* (*T. rubripes*), and *Xenopus tropicalis* (*X. tropicalis*) were collected from the Genebank (https://www.ncbi.nlm.nih.gov/genbank/) of NCBI and Ensembl database (http://asia.ensembl.org/index.html) in vertebrates. The sequences of MMPs in *H. sapiens* and *M. musculus* were reported previously [16, 47, 48]. Additionally, the MMP26 protein sequences from *G. agilis* and *M. mulatta*, as well as Mmp30 protein sequences of *T. rubripes* and *S. salar* were used as supplements.

To identify the MMP gene family members in *S. argus*, the HMMER3.0 (http://www.hmmer.org/) was performed using the Hidden Markov Model (HMM) profiles of Peptidase M10 (PF00413), Hemopexin (PF00045), and PG binding 1 (PF01471) domain downloaded from Pfam (http://pfam.xfam.org/). Moreover, the mRNA sequences of the MMP gene family in *L. crocea* and *D. rerio* were used as the reference compared to the genome of *S. argus* by the Basic Local Alignment Search Tool (BLAST)+ 2.9.0 program. We integrated the results obtained from HMMER and BLAST, followed by further analysis of potential sequences using the Pfam and Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/cdd/).

Phylogenetic tree

To investigate the evolutionary past of the MMP gene family in *S. argus*, the MMP protein sequences of vertebrates were aligned using the Clustalx method, and then Prottest 3.4.2 (https://github.com/ddarriba/prottest3) was performed to estimate the model, gamma shape (4 rate categories) and proportion of invariable sites of Phylogenetic tree. The WAG+I+G+F model with 100 bootstraps was employed to generate the phylogenetic tree using PhyML software (http://www.atgc-montpellier.fr/phyml/binaries.php), with BIONJ as the base tree for likelihood calculations and nearest neighbor interchange (NNI) as the topology search algorithm. Finally, iTOL (https://itol.embl.de/) was used to display and annotate phylogenetic trees.

Gene structure and synteny analysis

The generic feature format version 3 (GFF3) of *L. crocea* and *Oryzias latipes* were downloaded from the ensemble (http://asia.ensembl.org/index.html). GSDS 2.0 (http://gsds.gao-lab.org) was used to display the gene structure of MMPs in *S. argus*, *L. crocea*, and *O. latipes* to compare their changes in gene structure among three different fish species [49].

The locations and orientation of MMPs in vertebrates and their adjacent genes were determined using the Ensembl genome browser for synteny analysis. Synteny analysis of MMPs

Table 1. The *mmps* primer sequences used in qPCR

Gene	Primer name	Primer sequence (5'-3')
mmp2	*mmp2*-F	TTAGCTGTCGTCGGATTGTTCTTA
	mmp2-R	GACTGCTCGCACTTTGTCCTCA
mmp9	*mmp9*-F	CCTCTGACTTTTACTCGCCTCTTT
	mmp9-R	GCACCATTCGCATTCCCATAG
mmp11b	*mmp11b*-F	GTGTCCCAGACTATCCCACCTTA
	mmp11b-R	GACTGCTCGCACTTTGTCCTCA
mmp14a	*mmp14a*-F	CTCAAACATCAACGCCGCTTAC
	mmp14a-R	GCCCAGGTCTTTCAGGCTCTT
mmp15a	*mmp15a*-F	GAAGCTGACGTACTGCCAGGATA
	mmp15a-R	TCACGGTCTGTAGTGCGGGT
mmp15b	*mmp15b*-F	CCCATGCCCATCAGTCACTTC
	mmp15b-R	GACCATAATCCACCAGCTCCAG
mmp16a	*mmp16a*-F	ACCCCAAGGACATCACCCAGT
	mmp16a-R	GGTTCTCATTTCCTCATTGTAGCG
mmp17a	*mmp17a*-F	TGCGGTTGCAGTCCATGAGT
	mmp17a-R	GGGTTGCCTGGTCGAGTTGTA
mmp23b	*mmp23b*-F	GCCACTGCTGACATCACCATAG
	mmp23b-R	GCTCGCCGTTCAGACCATC
mmp24	*mmp24*-F	CTACAATGAGGAGAAGCGCACC
	mmp24-R	CCCATCCAGTCACGCAAAAT
mmp28	*mmp28*-F	ATGTCGCCGTACTACAGGAAACT
	mmp28-R	TGCTGTGAAGCTCTGTGAACTCC
β-actin	*β-actin*-F	GAGAGGTTCCGTTGCCCAGAG
	β-actin-R	CAGACAGCACAGTGTTGGCGT

and their adjacent genes in *S. argus* was performed using the BLAST+ 2.9.0 program based on the reference genome database of *S. argus* [50].

RT-qPCR

The gonads of eighteen *S. argus* (9 males and 9 females) were dissected. Each gonad was separated into two portions. One portion was immersed in Bouin's solution for histological analysis using the hematoxylin and eosin staining (H&E), while the other portion was snap-frozen in liquid nitrogen and stored at -80°C for RNA extraction. According to the histological observation of gonadal development at different stages (stage II, III, IV ovary [51] and stage III, IV, V testis [42]) by H&E, 8 gonads of males (n=9) and females (n=9) of *S. argus* were divided into 6 groups. An additional 6 fish (3 females and 3 males) were used for the tissue distribution analysis. Tissues including stomach, liver, kidney, intestine, heart, spleen, gonad, pituitary gland, hypothalamus, gill, head, and kidney were dissected and immediately frozen at -80°C to maintain RNA preserve RNA integrity for subsequent extraction.

Total RNA was extracted from each tissue using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, 1% agarose gel electrophoresis was performed to assess RNA sample degradation and contamination. The concentration and quality of the RNA were further evaluated by UV spectrophotometry at 260 nm (Nanodrop 2000c, Thermo Scientific, Waltham, MA, USA), ensuring an OD260/280 ratio between 1.8 and 2.0.

The expression patterns of 11 MMPs in the testes, ovaries, and their tissue distribution were performed based on the gonadal RNA-sequencing (RNA-seq) of adult *S. argus* by our groups [40]. Total RNA extraction, reverse transcription, and RT-qPCR were performed as previously described [52]. LightCycler® 480 Instrument (Roche) was utilized to conduct real-time PCR assay using the SYBR Green Master Mix (Toyobo, Japan). The RT-qPCR procedure included denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The fluorescent data collection was performed at 72°C. To determine the relative abundance of MMPs transcripts, the $2^{-\Delta\Delta Ct}$ method was employed with *β-actin* as a reference gene. The specific primer sequences utilized for qPCR are listed in **Table 1**.

Statistical analysis

The data were presented as the mean ± standard error (SE). Independent-sample t-tests were conducted to assess significant differences among the groups. A two-way analysis of variance (ANOVA) with Duncan's test was utilized to analyze the expression level of MMP mRNA in gender and gonads at various developmental stages. The significance level was set at α=0.05. All statistical analyses were performed using SPSS 16.0. The tissue distribution of MMP mRNA expression was clustered using Heatmap Illustrator 1.0 (HemI 1.0) for hierarchical clustering.

Results

Identification of MMP genes in S. argus and other species

Using the homology-based search in *S. argus* genome databases, a total of 23 potential genes encoding MMP proteins were successfully isolated, compared with 24, 25, and 21 MMP genes in *L. crocea*, zebrafish, and medaka, respectively. Among them, two subtypes of 8 MMP genes were identified, including *mmp11*, *mmp13*, *mmp14*, *mmp15*, *mmp16*, *mmp17*, *mmp20*, and *mmp23*. However, only one gene was found in tetrapods. In chicken, lizard, and Xenopus, 15, 16, and 19 MMP genes were also isolated, respectively. In tetrapods, *mmp1*, *mmp3*, *mmp7*, *mmp8*, and *mmp27* were identified, while they were absent in *S. argus* and other teleost genomes. In addition, *mmp10*, *mmp12*, and *mmp26* were only observed in mammals.

Phylogenetic analysis of MMP gene family in vertebrates

A total of 225 MMP proteins from different vertebrates were selected to build a phylogenetic tree and used to predict protein sequences of *S. argus*. Based on both sequence and structural analysis, the MMP family proteins in vertebrates can be classified into four types in the phylogenetic tree (**Figure 1**).

The first type emcompasses furin-susceptible MMPs, including MMP11, MMP19, MMP21, MMP23, and MMP28. Except for MMP19, all the members of this group possess a furin-susceptible site in their propeptide. The second type consists of membrane type (MT)-MMPs, which are characterized by a transmembrane structure that differentiate them from other MMPs. Type I MT-MMPs, including MMP14, MMP15, MMP16, and MMP24, are clustered into a group in the pink region. MT-MMP with glycosylphosphatidylinositol (GPI) anchored proteins, including MMP17 and MMP25, are grouped together in the blue region (**Figure 1**). The third type includes gelatinase, MMP2 and MMP9, characterized by three head-to-tail cysteine-rich repeats within their catalytic domain. The fourth type emcompasses MMP proteins with relatively simple domain structures, including MMP1, MMP3, MMP7, MMP8, MMP10, MMP12, MMP13, MMP20, MMP26, MMP27, and MMP30. MMP7 and MMP26 are distinguished by having a catalytic domain while lacking the additional domains. MMP1, MMP3, MMP10, MMP12, MMP13, MMP20, and MMP27 only containes the Hemopexin domain as an extra domain. Among these proteins, Mmp30 is an unique protein that belongs to teleost fish, showing a close relation to MMP20 in the phylogenetic tree.

Some proteins were not clustered with their protein counterparts. Specifically, Mmp7 and Mmp12 of *H. comes*, Mmp3, Mmp8, Mmp10, and Mmp12 of *C. milii*, as well as MMP3, MMP7, and MMP13 of *X. tropicalis* were grouped together in dark green region (**Figure 1**).

Synteny analysis of MMP genes in S. argus

A syntenic analysis of the local chromosome region of MMPs in vertebrates (including human, mouse, green anole, tropical clawed frog, eastern brown snake (*Pseudonaja textilis*), zebrafish), *L. crocea*, *S. argus*, and *O. latipes* was conducted in this study. The results revealed that the location of MMPs and their adjacent genes can be categorized into four situations according the conservation. The first was observed in *mmp2* as a representative of genes coding gelatinases. Its upstream genes (*slc6a2* and *lpcat2*) and downstream genes (*irx5* and *irx3*) exhibit a strong conserved synteny, except for *irx5* and *irx3* in green anole and *slc6a2* in tropical clawed frog (**Figure 2A**). The second was observed in *mmp28* as a representative of genes coding furin-activatable MMPs. It shows poorly conserved synteny with the variable surrounding genes in vertebrates. Only its upstream genes *pex12*, *ras10b*, and *cul3b* exhibit conserved synteny in tetrapods and teleosts, respectively. Similarly, its downstream gene, *taf15,* shows conserved synteny in vertebrates except for human and *por* in non-mammals (**Figure 2B**). The third was observed in genes coding the membrane-type *mmps* (*mt-mmps*). In teleosts, two subtypes of *mmp16* were identified, named *mmp16a* and *mmp16b,* while tetrapods including human, mouse, green anole. and frog have only a single copy. In tetrapods, *mmp16*, its upstream genes *cpne3,* and *cngb3*, as well as downstream genes *ripk2* and *osgin2* showed conserved synteny. Similarly, teleosts showed conserved synteny for *mmp16a* along with its upstream genes *cgnb3*

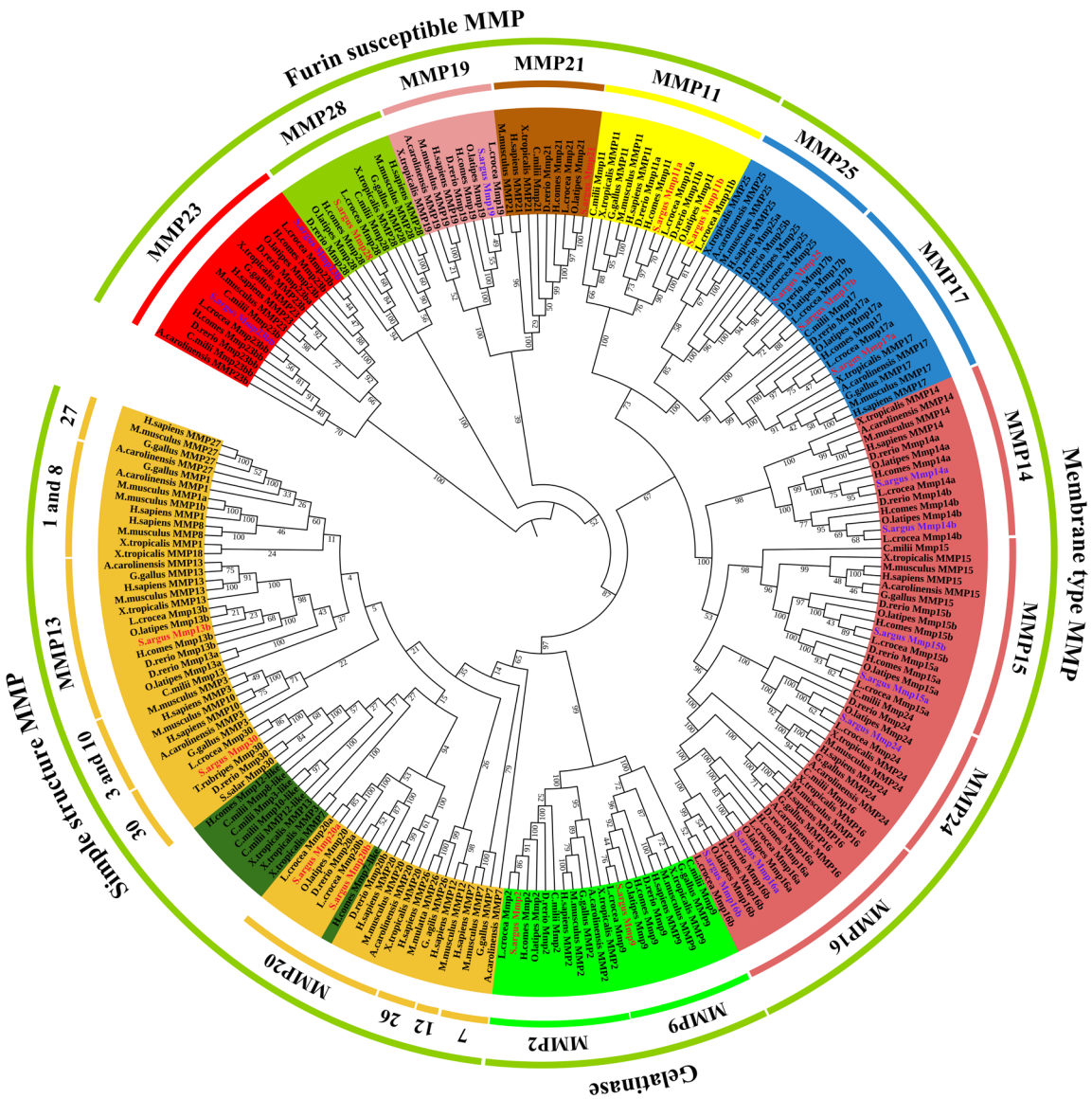

Figure 1. MMPs Phylogenetic tree of various vertebrates. The MMPs were divided into four specific evolutionary branches in the evolution process. (1) Secreted furin MMP, including MMP11, 19, 21, 23, 28; (2) Membrane-type MMP, including MMP14, 15, 16, 17, 24, 25; (3) Gelatinase, including MMP2 and MMP9; (4) Simple structure MMP, including MMP1, 3, 7, 8, 10, 12, 13, 20, 26, 27. *A. carolinensis: Anolis carolinensis*, *C. milii*: *Callorhinchus milii*, *D. rerio*: *Danio rerio*, *G. agilis*: *Gracilinanus agilis*, *G. gallus*: *Gallus gallus*, *H. apiens*: *Homo sapiens*, *H. comes: Hippocampus comes*, *L. crocea*: *Larimichthys crocea*, *M. mulatta*: *Macaca mulatta*, *M. musculus*: *Mus musculus*, *O. latipes*: *Oryzias latipes*, *S. salar*: *Salmo salar*, *S. argus*: *Scatophagus argus*, *T. rubripes*: *Takifugu rubripes*, *X. tropicalis*: *Xenopus tropicalis*.

and *cngb3-like*, as well as downstream genes *pomp* and *ramp3.* However, this conservation was not observed in zebrafish. As for the synteny of *mmp16*, its upstream and downstream genes are conserved in teleosts, except for downstream gene *cables1* in zebrafish (**Figure 2C**). The fourth was observed in genes coding the simple structure type *mmps*. The gene cluster included *mmp7*, *mmp12*, *mmp13*, *mmp20*, and *mmp27*. Their upstream genes *dync2h1* and *dcun1d5,* and downstream gene *tmem123* showed conserved synteny in tetrapods, except for tropical clawed frogs. In addition, *mmp13* and *mmp20*, their upstream genes *gucy2f* and *tsku*, and downstream gene *tbrg1* showed conserved synteny in teleosts, including *L. crocea*, *S. argus*, and *O. latipes*. However, this conservation was not observed in zebrafish and coelacanth (**Figure 2D**). Interestingly, the *mmp13* and *mmp20* were lost in teleosts.

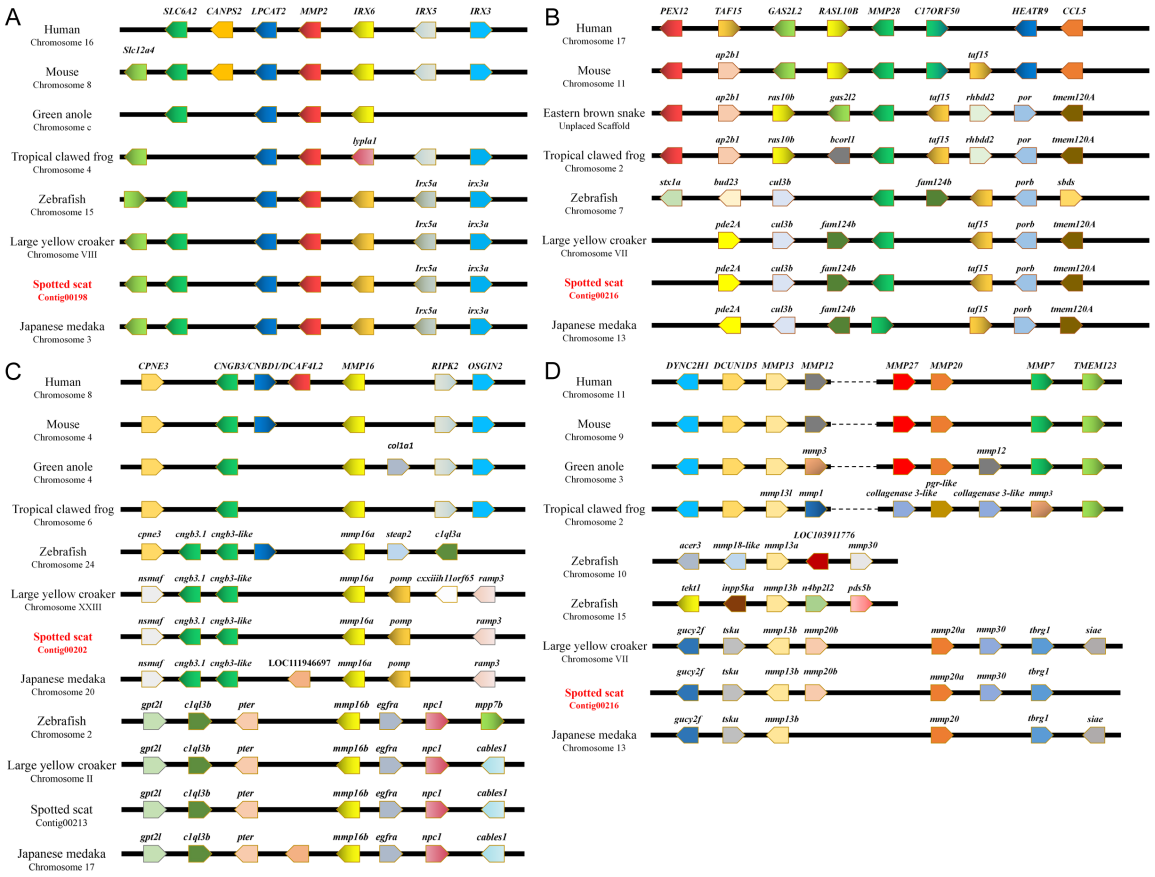

Figure 2. The syntenic analysis of *mmps* in vertebrates. Genomic regions flanking *mmps* were analyzed in representative vertebrate species including tetrapods (human, mouse, green anole, tropical clawed frog and eastern brown snake) and actinopterygians (zebrafish tilapia, large yellow croaker, spotted scat and Japanese medaka). Different colors represented different genes and arrows indicated their transcriptional direction. The distances between genes were not proportional to the actual distance on chromosomes and the bar lengths were not proportional to the distances between genes. Different genes were represented in different colors and their names were placed on the top. The dotted line indicated that the two regions were on the same chromosome and were close to each other.

Gene structure of MMP genes in S. argus

The gene structure of 23 MMP genes in *L. crocea*, *O. latipes*, and *S. argus* was analyzed using GSDS 2.0 (**Figure 3**). Among them, most *mmps* exhibited similar gene structures among the three species, except for *mmp21*, *mmp23bb*, and *mmp24*. *mmp21* showed the presence of 7 exons and 6 introns in *S. argus* and *O. latipes*, which differed from 6 exons and 5 introns in *L. crocea*. In *S. argus*, *mmp23bb* consisted of 8 exons and 7 introns and differed from the 7 exons and 6 introns in *L. crocea*. *mmp24* consisted 11 exons and 10 introns in *O. latipes* and differed from 10 exons and 9 introns in *L. crocea* and *S. argus*. The number of exons and introns of other MMP gene subtypes were the same except for *mmp23b* and *mmp23bb*.

Expression of gonadal MMP genes in S. argus by RNA-seq and RT-qPCR

Consistent with the gonadal RNA-Seq of *S. argus*, RT-qPCR analysis showed the expression of 11 MMP genes in the gonads of female and male *S. argus*, with the sex dimorphism expression (**Figure 4**). Among these MMP genes, the mRNA expression levels of *mmp2*, *mmp9*, *mmp11b*, *mmp14a*, *mmp15a*, *mmp15b*, *mmp16a*, *mmp17a*, *mmp23b*, and *mmp24* were significantly higher in the testes compared to the ovaries. Only *mmp28* mRNA expression was significantly expressed in the ovaries in *S. argus*.

Furthermore, the expression profiles of MMP genes in gonads at different gonadal develop-

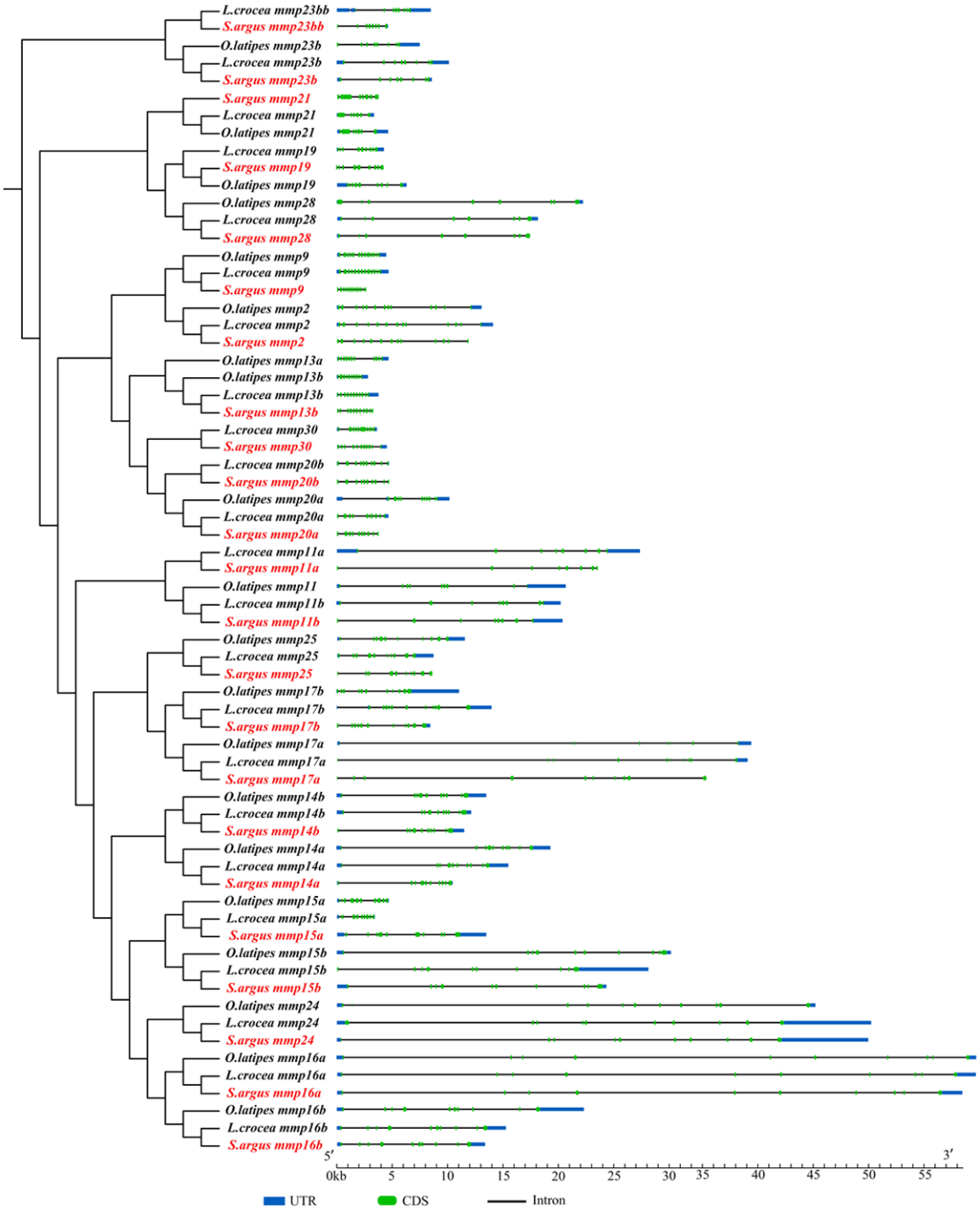

Figure 3. Gene structure of *S. argus mmps*. The untranslated region (UTR) and coding sequence (CDS) were represented by blue square and green ellipse respectively. The intron were represented by black line. *L. crocea*: *Larimichthys crocea*, *O. latipe*: *Oryzias latipe*, *S. argus*: *Scatophagus argus*.

ment stages (female: II, III, and IV; male: III, IV, and V) were also evaluated by RT-qPCR. The mRNA expression levels of *mmp2*, *mmp11b*, *mmp14a*, *mmp15a*, *mmp15b*, *mmp16a*, *mmp17a*, *mmp23b*, and *mmp24* were higher in the testes from stage III to V compared to females from stage II to IV. However, it is worth noting that *mmp28* exhibited high expression specifically in ovaries (**Figure 5A-K**). Furthermore, there was a significant increase in the mRNA

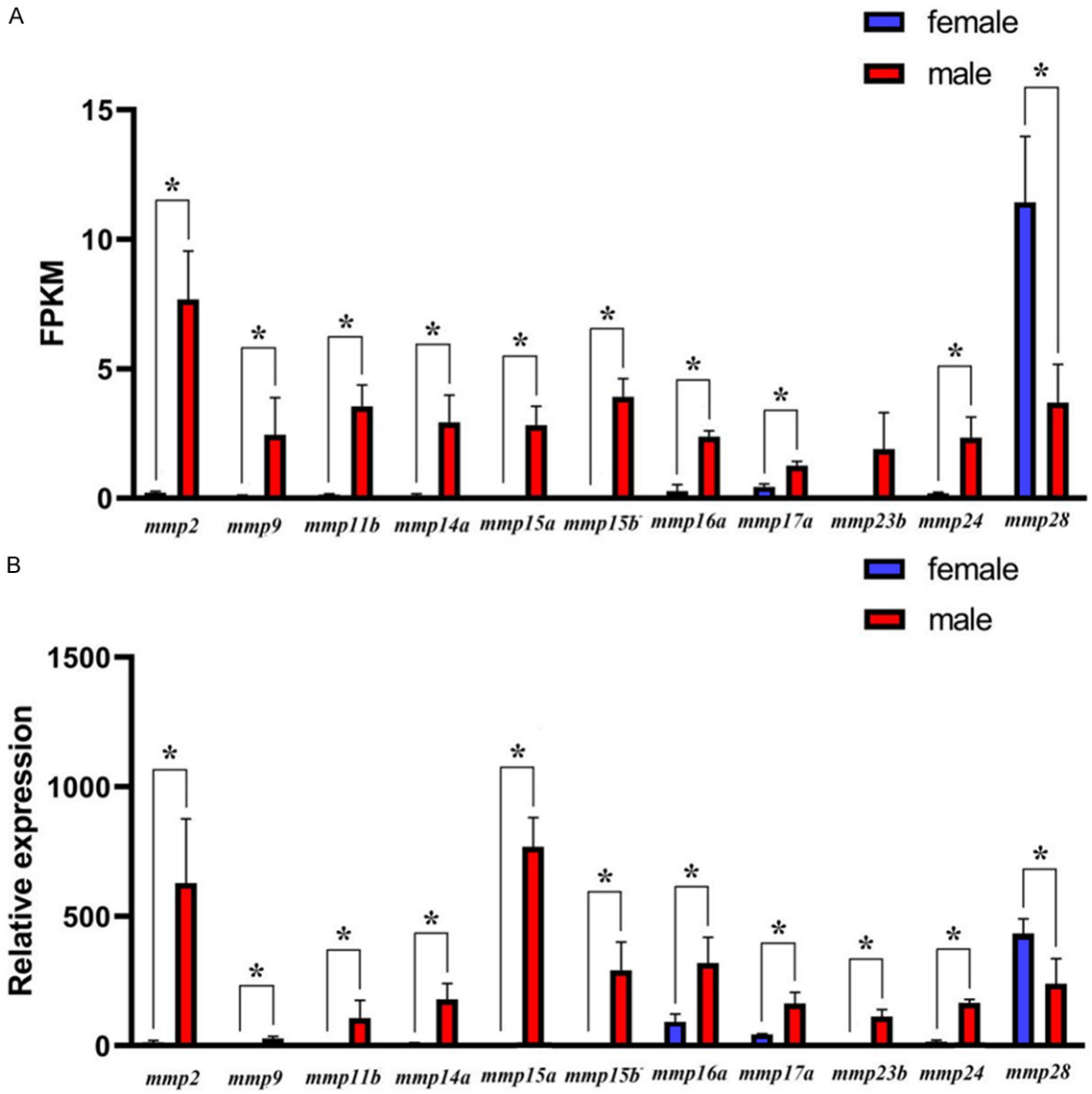

Figure 4. The relative expression of *mmp*s in gonad. A: RNA-Seq; B: qPCR. FPKM: Fragments Per Kilobase of exon model per Million mapped fragments. "*": Asterisk indicated statistical difference at $P < 0.05$ between female and male of each *mmp*s as determined by independent-sample T test.

expression levels of *mmp2*, *mmp15a*, *mmp16a*, and *mmp24* in males at stages IV and V compared to stage III. In contrast, no significant differences were observed among females from stages II to IV except for *mmp24*. Moreover, the mRNA expression level of gonadal *mmp11b* was significantly up-regulated in females at stages III and IV and in males at stage V when compared with females at stage II and males at stage III, respectively. The expression levels of *mmp17a* and *mmp23b* were significantly elevated at stage IV, while *mmp9* and *mmp14a* exhibited a significant increase at stage V in males compared to other stages.

Tissue distribution of MMP genes in S. argus by RT-qPCR

In this study, the tissue distribution of 11 MMP genes from 12 tissues was assessed using RT-qPCR. The relative expression levels of MMPs in gonads and kidneys exhibited sex dimorphism, as evidenced by the absence of clustering between male and female samples (**Figure 6**). Specifically, among 10 MMP genes (*mmp2*, *mmp9*, *mmp11b*, *mmp14a*, *mmp15a*, *mmp15b*, *mmp16a*, *mmp17a*, *mmp23b*, and *mmp24*), their relative expression levels were significantly higher in testis compared to the

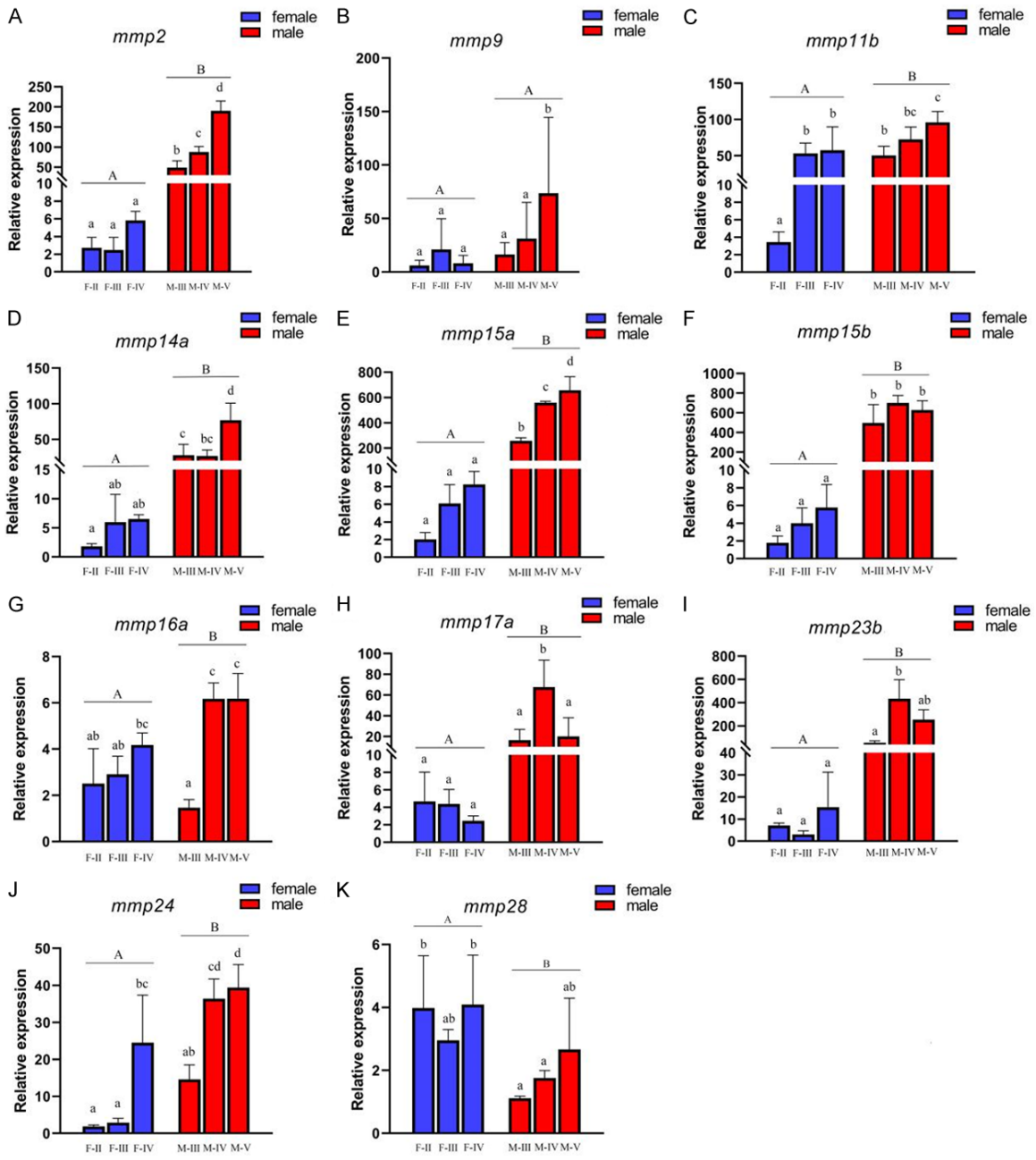

Figure 5. The relative expression of 11 selected *mmps* during different developmental phases gonad in *S. argus*. Data were presented as the mean ± standard error (SE). Different lowercase letters above the error bars indicated statistical differences at $P < 0.05$ between different development stages, different capital letters indicated significant differences at $P < 0.05$ between female and male spotted scat as determined by two-way ANOVA followed by Duncan's post-hoc test.

ovary. Conversely, the ovary showed a higher relative expression level of *mmp28* than the testis. The relative expression level of *mmp24* in the kidney was observed to be significantly low in both males and females of *S. argus*. The relative expression levels of MMP genes in the ovary, as well as in the gills of both males and females, were lower than those in other tissues.

Among the 11 MMP genes, the relative expression levels of 6 MMP genes (*mmp2*, *mmp9*, *mmp14a*, *mmp15a*, *mmp15b*, and *mmp16a*) were higher in most tissues and were grouped

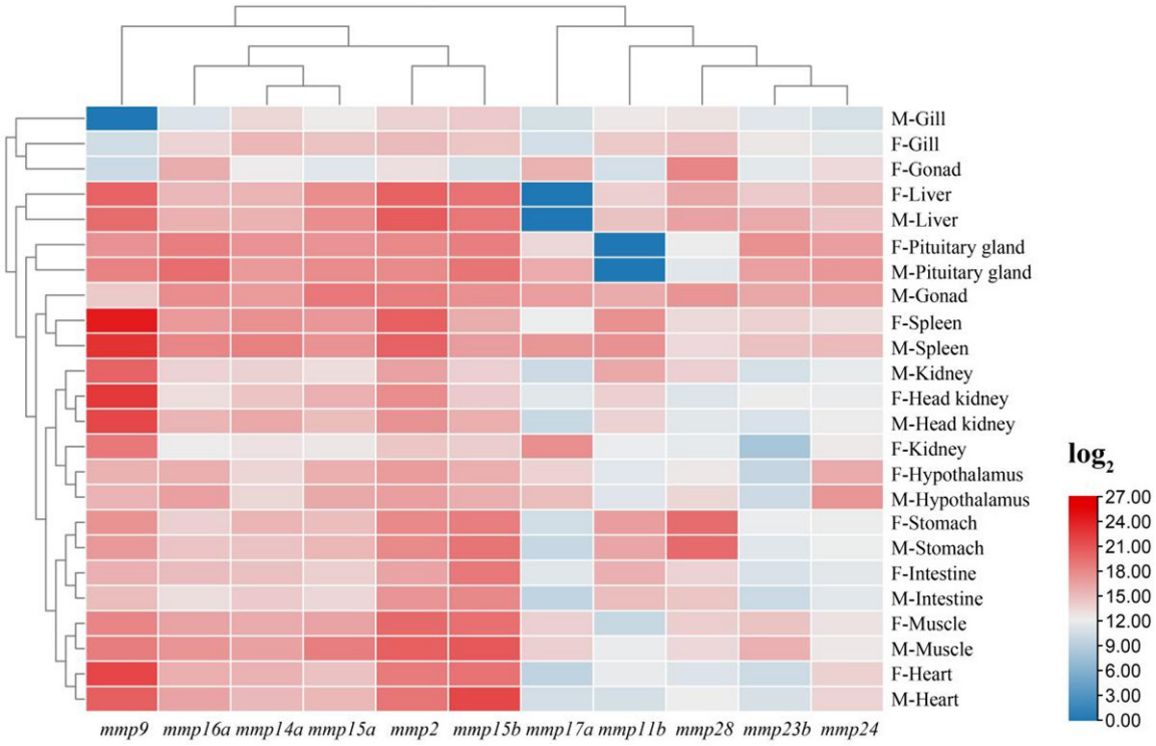

Figure 6. The tissue distribution of 11 selected *mmps* in adult female and male *S. argus*. The $\log_2$ values of the relative expression levels of *mmps* generated by qPCR were used to construct the heat map using TBtools. The color scale represents expression levels, warmer colors indicated higher expression. F: female, M: male.

into one group. The remaining five MMP genes (*mmp11b*, *mmp17a*, *mmp23b*, *mmp24*, and *mmp28*) displayed lower relative expression in most tissues and were also grouped together. *mmp9* was clustered into a single category and its relative expression in most tissues was higher than that of other MMP genes. Furthermore, due to their significantly lower relative expression in all 12 tissue types, *mmp23b* and *mmp24* were clustered into a separate group.

Discussion

Evolution of the MMP gene family

The phylogenetic tree analysis revealed that the insertion of a furin protein recognition site is a crucial mutation in the evolutionary process of the MMP gene family, leading to the division of MMP activation into two distinct modes. MMPs containing a furin protein recognition region are activated intracellularly, while those lacking this site require extracellular activation. Notably, MMP19 represents a unique branch within this group, sharing structural similarities with classical MMPs but showing close phylogenetic relatedness to MMP21. This suggests that the loss of the furin protein recognition site occurred during the evolution of MMP19. Among these types of MMPs, MMP23 exhibits significant divergence from other members due to its replacement of the hemopexin-like domain with a cysteine and proline-rich domain and an interleukin-1 type II receptor domain [53]. Furthermore, based on their positioning in the dark green region on the phylogenetic tree, it can be inferred that *X. tropicalis* and *C. milii* possess ortholog genes encoding proteins resembling collagenase, indicating their close relationship.

Gene replication is a fundamental process for expanding the size of gene families during evolution [54, 55]. It serves as the foundation for generating novel functional genes, thereby playing a crucial role in gene family formation [56, 57]. The whole genome duplication (WGD) has happened 2 times during the evolution of the common ancestor of tetrapod and teleost [57-60], with a teleost-special WGD (TS-WGD) happened during evolution of teleost, leading to an expansion of teleost's genome [61]. As an

indispensable gene family of vertebrates, the subtype genes of MMPs are also likely to have been influenced by WGD. It has been reported that *mmp13a* and *mmp13b* have been found in zebrafish [10].

In this study, the significant expansion of the MMP gene family can be attributed to two WGD events during early vertebrate evolution, leading to the emergence of a distinct gene cluster between *mmp7* and *mmp13*. The duplicated MMP genes remained after WGD and differentiated into new functions in the process of vertebrate evolution, which is similar to the formation process of globin family genes [62-64]. Although some genes were lost within this gene cluster in teleosts, several others have been retained and subsequently evolved into new MMP genes variants such as *mmp20a*, *mmp20b*, and *mmp30*.

The number of MT-MMP genes is the most significant difference between teleosts and other vertebrates. In teleosts, these MT-MMP genes are present in multiple copies, which may come from the specific WGD event of teleosts [61, 65]. However, these duplicated MT-MMP genes did not form a new type of MT-MMP genes during the evolution of teleosts. By comparing the MMP gene structure across three teleost species, we found little variation in the number of exons and introns in the MMP genes, indicating the stability of the MT-MMP genes in teleosts. Nevertheless, further investigation is required to elucidate the relationship between function and expression of these duplicated MT-MMP genes.

MMP gene expression in gonads

In this study, a total of 11 MMP genes exhibited differential expression in the developing gonads of females (phase II, III, and IV) and males (phase III, IV, and V) in *S. argus*. Specifically, *mmp2*, *9*, *14a*, *15a*, *15b*, *16a*, *17a*, 23b and *24* significantly expressed in testis, while *mmp28* showed significant expression in ovaries. Furthermore, the expression levels of these MMP genes displayed an increasing trend during the gonadal development phases. By integrating the expression patterns of these genes with their known functions in other vertebrates, this study aims to elucidate potential roles that MMP genes may play within the gonad of *S. argus*.

In the first case, the expression of MMP genes is associated with gonad structure formation in *S. argus*. Santana and Quagio-Grassiotto have detected the expression levels of MMP proteins in the testis of *Pimelodus maculatus* during the reproductive cycle [66]. The results suggested that the production and secretion of MMP proteins may be responsible for the proliferation of fibroblast and epithelial cells in the interstitial compartment of the testis. In a recent study on *Synbranchus marmoratus*, Talita found high expression levels of MMP2, 9, and 14 during gonadal remodeling, leading to significant alterations in the testicular interstitial compartment [67]. The action of these MMP proteins during the remodeling of teleost gonad structure is consistent with the increase in the expression of the MMP genes in our study. The substantial increase in the proliferation of fibroblast and epithelial cells in the testis of *S. argus* may explain this higher expression level of MMP genes in the testis than that in the ovary.

The involvement of MMPs in reproductive function has been confirmed for decades in higher vertebrates [24, 68, 69]. In mammals, MMPs play a crucial role in regulating cell migration during female ovulation and implantation of fertilized eggs through their interaction with tissue inhibitors of TIMPs [69]. After hCG treatment, granulosa cells in rats exhibited an eight-fold increase in the relative expression of *mmp14* at 8 hours, while the relative expression of *mmp25* peaked and subsequently decreased. Additionally, the relative expression of *mmp16* increased 16-fold at 12 hours after hCG treatment [70]. Therefore, it is plausible that the expression patterns of MMP genes are associated with reproduction in *S. argus*. Hagiwara et al. highlighted the link between the nuclear progestin receptor and LH-induced expression of MMP15 mRNA and the protein in *O. latipes* [71, 72]. The inhibition of MMP15 mRNA and protein expression specifically in the granulosa cells of developing follicles may impede follicle rupture during ovulation, highlighting the involvement of MMPs in teleost reproduction.

In our study, *mmp28* exhibited a unique expression pattern among the 11 MMP genes, with higher relative expression levels observed during ovarian development phases compared to those in the testis. Unlike its increasing expression in the testis, *mmp28* maintained consis-

tently high levels throughout all stages of ovarian development, suggesting additional functions beyond shaping gonadal structures. Previous studies have reported that mammalian *mmp28* induces epithelial-to-mesenchymal transition (EMT), a transformation process associated with folliculogenesis in the ovary [73-75]. The granulosa cells undergo an EMT as they activate and develop during follicle growth. The high expression of *mmp28* in the ovary may indicate its potential function in follicle development in the ovary of *S. argus*.

Estrogen plays an important role in the sex determination, differentiation, and gonad development of vertebrates and is also the significant regulatory factor in the MMP gene family. Previous studies have demonstrated that 17β-estradiol (E_2) can modulate the expression of *mmp2* and *mmp9* in endometrial epithelial cells [76]. Estrogen has the potential to enhance vascular endothelial growth factor expression and activate the Erk 1/2-Elk1 signaling pathway, thereby regulating MMP2 and MMP9 expression. Furthermore, Liarte et al. reported that E_2 could up-regulate the expression of MMP9 and MMP13 in the testis cells of *Sparus aurata* in vitro [77]. Using PROMO analysis, we identified several putative estrogen receptor binding sites within the upstream regions of all 11 MMP promoters in *S. argus*. These findings suggest the direct involvement of estrogen in regulating MMP genes expression in *S. argus*. Future experimental investigations will focus on elucidating the estrogen-mediated regulatory pathways governing MMPs in *S. argus*.

MMP gene expression in non-gonadal tissues

Besides the gonads, MMPs also have a wide range of functions in physiological processes such as ECM degradation in immune response, inflammation, and muscle tissue in teleosts. The ability of the MMP13 in rainbow trout (*O. mykiss*) to degrade gelatin and skin type I collagen, as well as cleave the non-helical regions of muscle type V collagen, has been demonstrated [78]. The systematic elevation of *mmp9* and *mmp13* levels in Atlantic salmon (*S. salar*) infected with the salmon louse (*L. salmonis*) suggests their induction by leukocyte influx or activation of resident immune cells like macrophages [79, 80].

In our study, analysis of tissue distribution revealed significantly higher relative expression levels for six MMP genes (*mmp2*, *9*, *14a*, *15a*, *15b*, and *16a*) compared to others across 11 tissues except for gill. This finding implies that these particular MMPs are primarily responsible for ECM degradation in *S. argus*. Their high expression in muscle and heart tissues may be attributed to their rich collagen content. Furthermore, high relative expression levels were also observed in immune organs including the spleen, head, and kidney indicating their crucial involvement in inflammation and immune responses exhibited by *S. argus*.

Conclusion

In this study, we identified 23 MMP genes from the genome of *S. argus*, including 7 MMP genes with two copies and a fish-special *mmp30*. Additionally, bioinformatics analysis was conducted to investigate the MMP gene family. We found that there are a large number of copies of the MMP gene in teleosts compared with other vertebrates. These MMP genes including *mt-mmps*, *mmp13* and a special MMP gene *mmp30* in teleosts may be the result of whole-genome duplication events unique to teleosts. During different stages of gonadal development, *mmp2*, *9*, *14a*, *15a*, *15b*, *16a*, *17a*, 23b and *24* showed higher mRNA expression in the testis compared to the ovary suggesting that they play a vital role in testicular development. *mmp28* had higher expression during ovarian development, indicating that it is more important for ovarian development. In the future, further studies are needed to determine their specific functions in gonadal development and precise mechanism in gonadal differentiation. The tissue distribution results demonstrated that 6 genes (*mmp2*, *9*, *14a*, *15a*, *15b*, and *16a*) had relatively high expression in all tissues. This finding implied that these particular MMPs are primarily responsible for ECM degradation in *S. argus* in non-gonadal tissues. Overall, the study enhances our understanding of the MMP gene family in evolution of teleost and provides valuable insights for further research on MMPs in *S. argus*.

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Disclosure of conflict of interest

None.

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