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Molecular identification of steroidogenesis-related genes in scallops and their potential roles in gametogenesis

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Abstract

Sex steroids are crucial for controlling gametogenesis and germ cell maturation in vertebrates. It has been proposed that Yesso scallop (*Mizuhopecten yessoensis*) has the same sex steroids as those animals, but the scallop biosynthetic pathway is unclear. In this study, we characterized several steroidogenesis-related genes in *M. yessoensis* and proposed a putative biosynthetic pathway for sex steroids that is similar to that of vertebrates. Specifically, we identified several steroidogenesis-related gene sequences that encode steroid metabolizing enzymes: StAR-related lipid transfer (START) protein, 17α-hydroxylase, 17,20-lyase (*cyp17a*), 17β-hydroxysteroid dehydrogenase (*hsd17b*), and 3β-hydroxysteroid dehydrogenase (*hsd3b*). We sampled adult scallops throughout their reproductive phase to compare their degree of maturation with their intensity of mRNA expression. Semi-quantitative RT-PCR analysis revealed a ubiquitous expression of transcripts for steroid metabolizing enzymes (i.e., *star*, *cyp17a*, *hsd17b*, and *hsd3b*) in peripheral and gonadal tissues. Real-time PCR analysis revealed a high level of expression of *star3* and *cyp17a* genes in gonadal tissues at the early stage of cell differentiation in scallops. Interestingly, mRNA expression of *hsd3b* and *hsd17b* genes showed a synchronous pattern related to degree of gonad maturity. These results indicate that both *hsd3b* and *hsd17b* genes are likely involved in steroidogenesis in scallops. We therefore believe that these steroid-metabolizing enzymes allow scallops to endogenously produce sex steroids to regulate reproductive events.

Keywords : Transcriptome, Bivalve, Sex steroids, Gonadal maturation, Reproduction

1. Introduction

In vertebrates, sex steroids (e.g., androgens, estrogens, and progesterone) are synthesized from cholesterol and play crucial roles in several physiological functions, such as gonadal maturation [1], germ cell proliferation [1], and sexual behavior [2]. However, in invertebrates, little is known about the physiological origin of sex steroids, especially their biosynthetic pathways [3]. Previous papers have shown that mollusks contain key enzymes for metabolizing sex steroids: 17α-hydroxylase, C-20–22-lyase in *Ariolimax californicus* [4] and *Mytilus edulis* [5], 17β-hydroxysteroid dehydrogenase in *Crassostrea gigas* [6,7], *M. edulis* [5], and *Mizuhopecten yessoensis* [6], 3β-hydroxysteroid dehydrogenase in *C. gigas* [7] and *M. yessoensis* [6], 5α-reductase in *A. californicus* [4], and *M. edulis* [5], and aromatase in *Helix aspersa* [8] and *M. yessoensis* [6]. These studies imply the presence of a biosynthetic pathway for sex steroids in mollusks, suggesting an endogenous source of molluskan sex steroids. We are also aware that these steroids might be derived from exogenous sources, such as from microalgae, plankton, and marine vertebrates [9,10]. However, there is strong evidence towards the presence of a biosynthetic pathway for sex steroids in mollusks indicating that they synthesize some sex steroid themselves because many studies have revealed sex steroid biosynthesis in mollusks and the roles of those steroids in reproduction [3,11].

In recent years, sex steroids have been found in the gonadal tissues and digestive glands of several mollusk species [3,11,12]. In addition, a number of reports have described some of the physiological effects of vertebrate-type steroids on reproduction in mollusks. For examples, one *in vivo* experiment showed that ovary and testis levels of 17β-estradiol, testosterone, and progesterone influence egg and sperm release in the sea scallop *Placopecten magellanicus* [13]. Likewise, levels of 17β-estradiol and testosterone in ovaries and testes of Zhikong scallop (*Chlamys farreri*) influence calmodulin-2 and vitellogenin mRNA expression [14]. Furthermore, seasonal variations of 17β-estradiol levels involved in reproductive events have been observed in several mollusk species, such as *C. farreri* [14], *M. yessoensis*, and *C. gigas* [6]. Nevertheless, relative to vertebrates, fundamental evidence of the biosynthetic pathways for the above-described sex steroids has not been clearly elucidated in mollusks (including bivalves).

The advance of genome sequencing studies of mollusks [[15], [16], [17]] have uncovered steroidogenesis-related

molecules, such as steroidogenic enzyme genes [[18], [19], [20], [21]] and steroid receptors [22]. Consequently, an increasing number of steroidogenesis-related genes have been studied in mollusks, including *hsd17b8* in *C. farreri* [19], *hsd17b10* in *Mytilus galloprovincialis* [21], *hsd17b11* in *Haliotis diversicolor supertexta* [20], and *hsd17b12* in *Nucella lapillus* [18], *M. galloprovincialis*, and *H. diversicolor* [21,23]. However, previous studies were not sufficient to provide the entire pathway for sex steroid biosynthesis. To understand steroidogenesis in mollusks, we aim to identify a series of steroidogenesis-related genes expressed in gonadal tissues of scallop. To do this, we identify several steroidogenesis-related genes from Yesso scallop (*M. yessoensis*) transcriptome libraries and characterize deduced protein sequences to propose the biosynthetic pathway for sex steroids. In addition, we analyzed the mRNA expression of steroidogenesis-related genes in the testes and ovaries of scallops throughout their reproductive phase to better understand how sex steroid biosynthesis is related to reproduction in mollusks.

2. Materials and methods

2.1. Sample collection

Adult Yesso scallops were purchased from 2016 to 2017 from a local scallop farm in Ogatsu Bay, Miyagi, Japan) during their reproductive phase. To analyze the degree of maturation, a gonad index was used to quantify the extent of gonadal development and histology was used to observe gametogenesis. Soft body tissue and gonadal tissue were excised and then weighed to calculate the gonad index (GI) [i.e., GI = 100 * (gonadal weight/soft bodyweight], as described elsewhere in Nagasawa et al. [24]. Sex was distinguished by examining gonadal morphology and by histological observations [25,26]. Gonadal tissues (testis and ovary), peripheral tissues (Fig. S7)(cerebral and pedal ganglia, visceral ganglion, gill, mantle, adductor muscle, digestive gland, kidney and hemocytes) were collected and stored at −30 °C in RNAlater stabilization solution (Thermo Scientific, Waltham, MA) for later analysis.

2.2. Transcriptome survey and bioinformatic analysis

Local blasting was performed with Yesso scallop transcriptome data (NCBI accession number SRX047537) [15] which was generated by 454 GS FLX platform and our unpublished resource which was generated by ion torrent platform. Amino acid sequences of steroidogenesis-related genes such as *star*, *cyp17a*, *hsd17b*, and *hsd3b* etc. from *Crassostrea gigas*, *C. angulata*, *C. farreri*, and *Mimachlamys nobilis* were selected for query. Deduced amino acid sequences were obtained using EMBOSSTranseq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/ (11June2018)). The protein motifs or functional domain structures were analyzed using NCBI CDD (https://www.ncbi.nlm.nih.gov/cdd (11June2018)), Prosite [a Swiss-Prot protein database (http://www.expasy.org/prosite/ (11June2018))], or Pfam (http://pfam.xfam.org/ (11June2018)). Multiple sequence alignments were performed with ClustalX [27]. A phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates using the MEGA7 algorithm [28].

2.3. Histology

Testes and ovaries were dissected, fixed in Davidson's solution for 24 h at 4 °C, and then preserved in 70% ethanol until use. Fixed tissues were processed using the standard paraffin method. Each section was cut at 5 μm thicknesses and stained with Mayor's hematoxylin and eosin Y (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). Photographs were taken under a BX-53 Olympus light microscope (Tokyo, Japan) with a DP73 Olympus digital camera (Olympus Co., Tokyo Japan) using cellSens Standard 1.7 software.

2.4. RNA extraction and cDNA synthesis

Total RNA was isolated from dissected scallop tissues using an RNeasy mini kit (Qiagen, Tokyo, Japan) following the company's procedures. All samples were treated with DNase I (Qiagen, Tokyo, Japan) on a spin column. Quantity of RNA was analyzed with a NanoDrop ND-100 instrument (Thermo Scientific), whereas integrity was determined by electrophoresing the RNA on a 1% (w/v) agarose gel. First-strand cDNAs were synthesized from DNase-treated RNA (1 μg) using high-capacity cDNA reverse transcription kits (Applied Biosystems, Tokyo, Japan).

2.5. Rapid amplification of cDNA ends (RACE)-PCR

Full-length cDNAs of steroidogenesis-related genes were obtained by 5′RACE-PCR and 3′RACE-PCR using a SMARTer RACE5′/3′ kit (Clontech, CA, USA) with gene specific primers (Table 1) designed from contig sequences. The reaction

was set in a 10-μL solution (total volume) containing 1.9 μL of premixes (20 mM Mg^{2+} and 2.5 mM each of dNTPs and TakaRa Ex Taq HS), 0.8 μL of 10μM each of forward and reverse primers, 5.7 μL of ultrapure water, and 0.8 μL of cDNA. Touchdown PCR was performed using 5 cycles at 94 °C for 30 s and 72 °C for 3 min, followed by 5 cycles at 94 °C for 30 s, 70 °C for 30 s, and at 72 °C for 3 min. The sample was then subjected to 30 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 3 min before completing the final extension step at 72 °C for 5 min. The reaction was maintained at 4 °C before collecting the sample. Amplicons were examined using gel electrophoresis and purified using a QIA quick gel extraction kit (Qiagen, USA). DNA fragments were cloned into pGEM-T easy vector (Promega, USA) and then sequenced by Macrogen, Inc. (Seoul, South Korea).

2.6. Semi-quantitative RT-PCR (RT-PCR)

Tissue distributions of mRNA were determined for adult *M. yessoensis* during their growth stage (December) using RT-PCR with gene specific primer sets (Table 1). This growth stage is a transition between early developing phase and mature phase which are abundant of reproductive-related cells [26,29]. PCR was performed with TaKaRa Ex Taq HS polymerase (TaKaRa-Bio, Shiga, Japan) by pre-heating the sample at 95 °C for 5 min, followed by 35 cycles (for steroidogenesis-related genes) or 30 cycles (for reference genes) of 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C. An elongation step was performed at 72 °C for 5 min. Quantified products were examined with gel electrophoresis on a 2% (w/v) agarose gel. A photograph was taken under UV light with a AB-6932GXES digital camera (ATTO, Japan).

2.7. Quantitative real-time PCR (qPCR)

Testes and ovaries were analyzed with qPCR using a 7300 Real-Time PCR system (Applied Biosystems, Warrington, UK), as described elsewhere in Nagasawa et al. [30], to quantify mRNA levels of steroidogenesis-related genes in scallop gonadal tissues during their reproductive phase. Reactions were set in a total volume of 20 μL containing 10 μL of power SYBR PCR master mix, 2 μL of cDNA template, 0.8 μL of 10 μM each of forward and reverse gene specific primers (Table 1), and 6.4 μL of ultrapure water. The program was initially set at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Dissociation curve analysis was set at 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. Each amplicon was confirmed by examining melting curves and using gel electrophoresis. The cycle threshold (C_T) values were set at 0.2 to define the level of arbitrary fluorescence intensity on 7300 system SDS software. Amplification efficiency was examined against a standard curve constructed from a diluted series of pooled cDNA samples (1:1, 1:2, 1:4, 1:8, and 1:16). Three stable reference genes [DEAD-box RNA helicase (*heli*), ubiquitin (*ubq*), 60 s ribosomal protein L 16 (*rpl16*)] studied by Feng et al. [31]) were selected and validated using geNorm software (gemorm.cmgg.be). Two best reference genes (*rpl16* and *heli*) and geNorm stability values (M) for *rpl16* (0.617), *heli* (0.632), and *ubq* (0.716) were chosen and used for normalizing the data. The relative mRNA expression of steroidogenesis-related genes was calculated using the $2^{-\Delta\Delta Ct}$ formula [32] and normalized.

2.8. Statistical analysis

To assess the changes in mRNA expression levels of steroidogenesis-related genes among seasons and sexes, statistical analyses were conducted with a two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Significance level was set at $p < 0.05$ for all experiments.

3. Results

3.1. Gametogenesis during the reproductive phase of scallop

All scallops showed the lowest GI value at the beginning of reproductive season (September), which then gradually increased until it peaked at the mature stage (March) (Fig. 1A). There was no significant difference in GI index between sexes. The lowest GI value (6.92 ± 0.78) in males occurred in the early-differentiation stage (September), whereas the highest GI value (23.86 ± 1.35) occurred when scallops were mature (March). Likewise, in females the lowest GI (8.29 ± 0.52) occurred in the early-differentiation stage (September), whereas the highest GI value (24.10 ± 1.33) occurred when females were mature (March). Both males and females showed significant differences among maturation stages in histological characteristics, suggesting that gametogenesis in scallop gonadal tissues in both sexes is related to changes in GI (Figs. 1B– G). At the early-differentiation stage (September) in both sexes, there were only a few germ cells scattered along the thin acinar wall and lumen spaces were almost empty (Fig. 1B and E). At the growth stage, germ cells were clearly identifiable and attached along larger acinar walls (Fig. 1C and F). Proliferation of

germ cells was observed toward the edge of the lumen space. At this stage, we found spermatogonia and spermatocytes in males (Fig. 1C) and oogonia and oocytes (primary vitellogenic oocytes) in females (Fig. 1F). At the mature stage, spermatogonia and oogonia were somewhat present along cell walls and several germ cells fully occupied the lumen spaces (Fig. 1D and G). In March, we observed spermatocytes, spermatids, and spermatozoa in mature testes (Fig. 1D) and primary vitellogenic oocytes and ripe oocytes in mature ovaries (Fig. 1G).

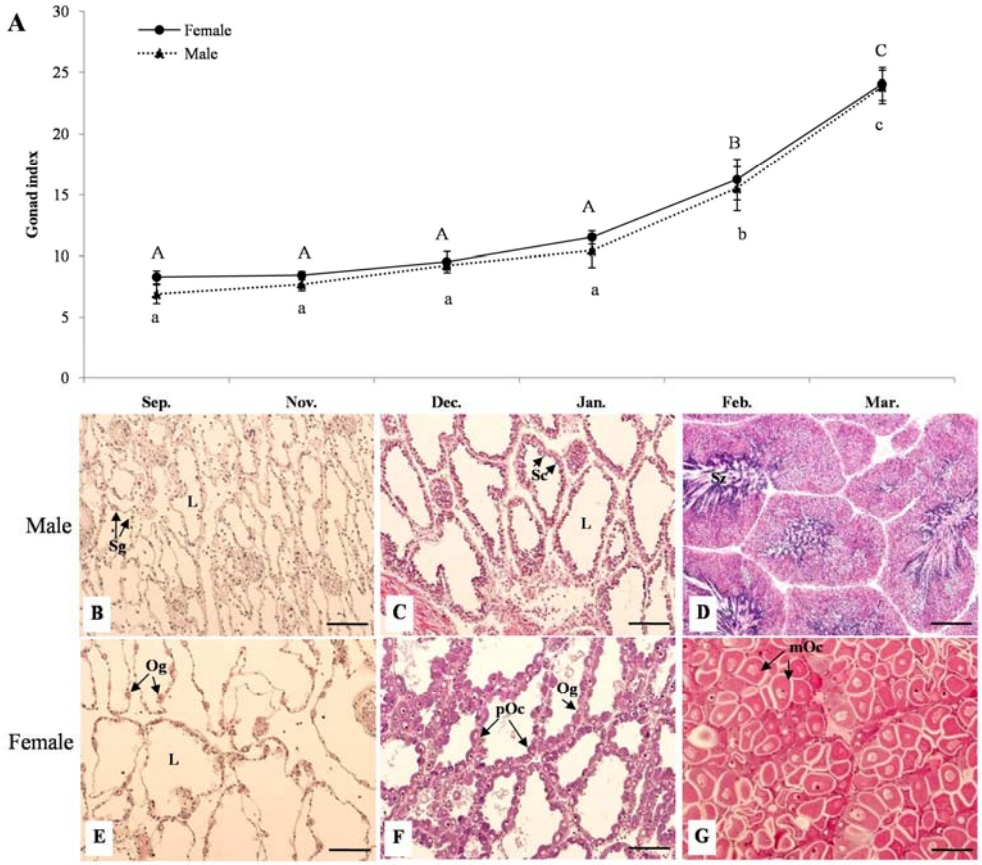

Fig. 1 Gonadal development and gametogenesis of Yesso scallop (*M. yessoensis*) during their reproductive phase. Gonad index increased during experimental period (A). Values represent the mean ± SEM, and significant differences among stages denote with different letters: upper case for females and lower case for males ($p < 0.05$). Illustration of histology: B–D represent spermatogenesis; E–G represent oogenesis. Sg: spermatogonia, Sc: spermatocytes, Sz: spermatozoa, Og: oogonia, pOc: previtellogenic oocyte, mOc: mature oocyte, L: lumen. Black scale bar is 50 μm.

3.2. cDNA cloning of the steroidogenesis-related genes

The cDNA of the scallop *star3* gene was 1585 bp long with an open reading frame (ORF) of 1446 bp (481 amino acids, GenBank accession no. MH040336). A multiple alignment analysis demonstrated that amino acid residues we observed in the scallop *star3* gene were conserved and shared with other vertebrate *star3* sequences, especially in the region of the cholesterol-binding START domain [33] (Fig. 2A). The deduced amino acid sequences of the *star3* cDNA gene showed 52%–65% homology among various vertebrate and invertebrate homologs.

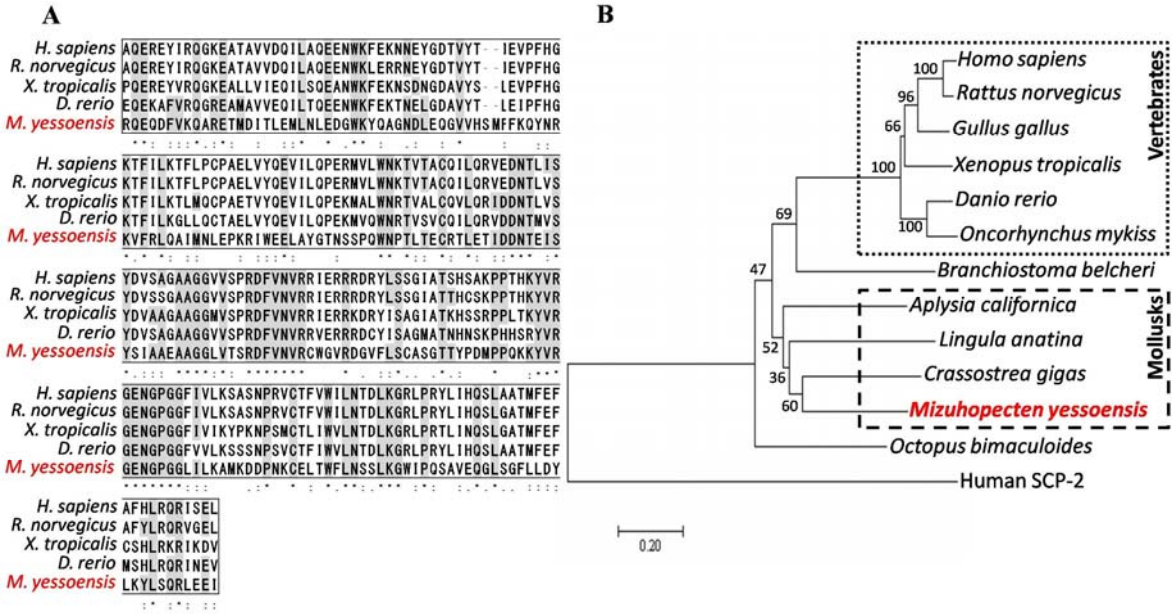

Fig. 2 Structural motif domains and phylogenetic tree of the scallop *star3* gene. Shaded regions denote STARD3 motif sequences of amino acids identical to those of vertebrates (A). Multiple sequence alignment is represented by various symbols: * = identical, = strong homology, and = less homology. Phylogenetic tree constructed with a neighbor-joining method based on the homologous sequences of vertebrates and invertebrates (B). Accession no: *Homo sapiens* (NP_006795.3), *Rattus norvegicus* (NP_001014251.1), *Gallus gallus* (XP_015155025.1), *Xenopus tropicalis* (NP_001006799.1), *Danio rerio* (NP_571737.1), *Oncorhynchus mykiss* (XP_021480476.1), *Branchiostoma* belcheri (XP_019636776.1), *Aplysia californica* (XP_005099198.1), *Lingula anatina* (XP_013403292.1), *Crassostrea gigas* (XP_011434195.2), *Mizuhopecten yessoensis* (MH040336), *Octopus bimaculoides* (XP_014785710.1), and human_SCP-2 (P22307.2). Bootstrap consisted of 1000 replicates.

The full-length cDNA of the *cyp17a1* gene was 2121 bp long, composed of an ORF of 1551 bp encoding 516 amino acids (GenBank accession no. MH040337). The scallop *cyp17a1* gene was conserved in Ozols' tridecapeptide region [34], Ono sequence area [35], and the heme ion binding site, indicating that the cDNA belonged to the *cyp17* gene [34,35] (Fig. 3A). The predicted scallop protein from the *cyp17a1* gene showed that the *cyp17a1* gene was 47%–63% homologous between vertebrates and invertebrates.

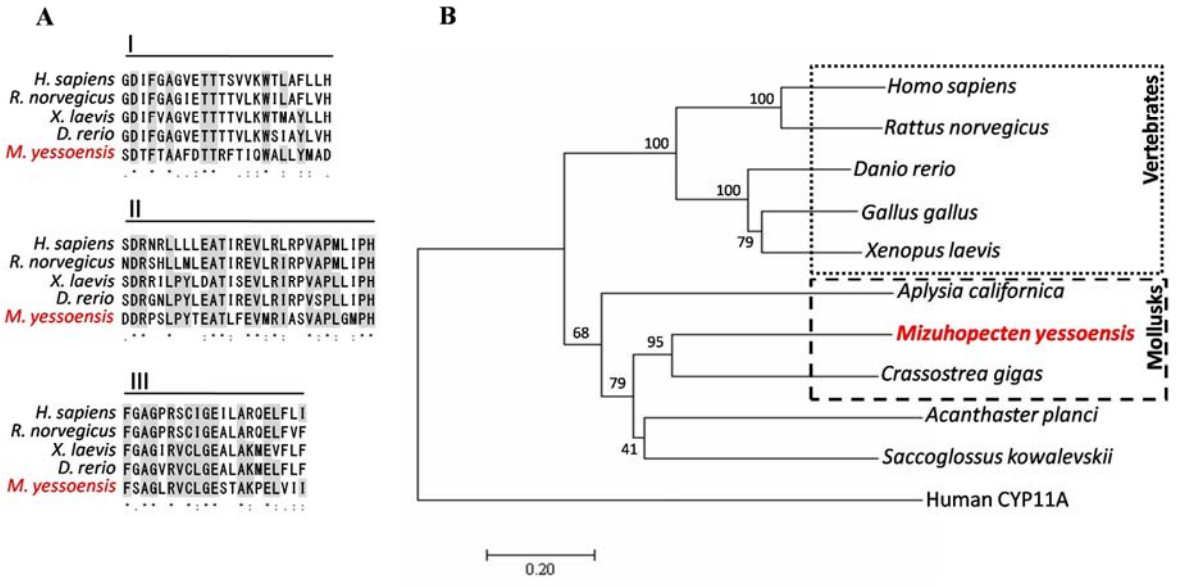

Fig. 3 Structural motif domains and phylogenetic tree of the scallop *cyp17a1* gene. Shaded regions indicate the motif sequences of amino acids identical to ono-sequence area (I), ozols' tridecapeptide area (II) and heme ion binding site (III) of vertebrates (A). Multiple sequence alignment represented by various symbols: * = identical, = strong homology, and = less homology. Phylogenetic tree constructed with a neighbor-joining method based on the homologous sequences of vertebrates and invertebrates (B). Accession no: *Homo sapiens* (AAA52151.1), *Rattus norvegicus* (NP_036885.1), *Xenopus laevis* (AAG42003.1), *Danio rerio* (AAP41821.1), *Gallus gallus* (AAA48997.1), *Aplysia californica*, (XP_012941324.1), *Mizuhopecten yessoensis* (MH040337), *Crassostrea gigas* (NP_001292231.1), *Acanthaster planci* (XP_022111252.1), *Saccoglossus kowalevskii* (XP_002730849.1), and human CYP11 A (AAA52162.1). Bootstrap consisted of 1000 replicates.

The cDNA of *hsd3b1* gene was 1667 bp long, composed of an ORF of 1203 bp encoding 400 amino acid residues (GenBank accession no. MH040341). Alignment of deduced amino acid sequences were conserved in the NAD-binding site (GxxGxxG) and active site (YxxxK) [36,37] (Fig. 4A). The amino acid sequence of the *hsd3b1* gene was 42%–68% homologous between vertebrates and invertebrates.

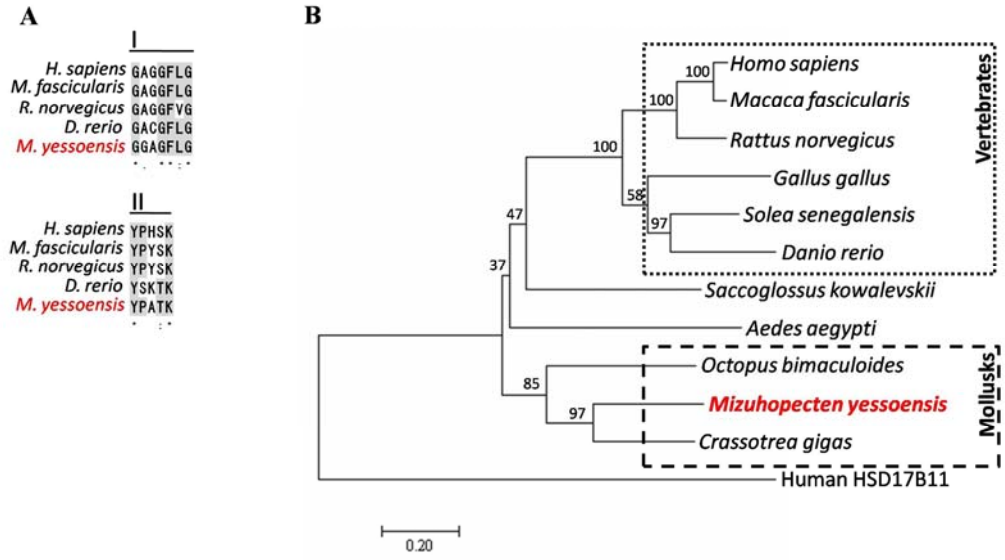

Fig. 4 Structural motif domains and phylogenetic tree of the scallop *hsd3b1* gene. Shaded regions indicate motif sequence of amino acids identical to NAD-binding site (I) and active site (II) of vertebrates.(A). Multiple sequence alignment represented by various symbols: * = identical, = strong homology, and = less homology. Phylogenetic tree constructed using a neighbor-joining method based on the homologous sequences of vertebrates and invertebrates (B). Accession no: *Homo sapiens* (NP_000853.1), *Macaca fascicularis* (NP_001270516.1), *Rattus norvegicus* (NP_001007720.3), *Gallus gallus* (XP_015149845.1), *Solea senegalensis* (ACN89887.1), *Danio rerio* (NP_997962.1 3), *Saccoglossus kowalevskii* (XP_002738669.1), *Aedes aegypti* (XP_001650664.1), *Octopus bimaculoides* (XP_014778473.1), *Mizuhopecten yessoensis* (MH040341), *Crassostrea gigas* (XP_011433629.1), and human HSD17B11 (NP_057329.3). Bootstrap consisted of 1000 replicates.

The cDNA encoding the scallop *hsd17b8* gene showed that it is 1096 bp long, composed of an ORF of 759 bp encoding 252 amino acids (GenBank accession no. MH040338). The *hsd17b8* gene homolog was conserved and showed in specific regions, such as in the cofactor binding domain, the structure stabilization site, and the catalytic site [16,18,38,39] (Fig. 5A). Our comparison of deduced amino acid sequences derived by comparing sequences with other vertebrates and invertebrates showed 67%–98% similarity.

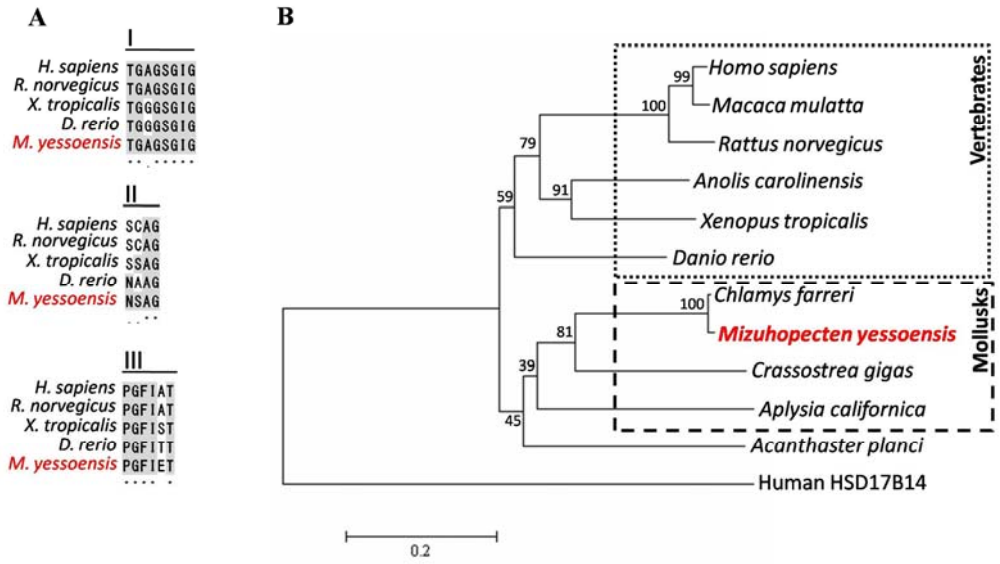

Fig. 5 Structural motif domains and phylogenetic tree analysis of the scallop *hsd17b8* gene. Shaded regions indicate motif sequences of amino acids identical to cofactor binding domain (I), structure stabilization domain (II) and catalytic site (III) of vertebrates. (A). Multiple sequence alignment represented by various symbols: * = identical, = strong homology, and = less homology. Phylogenetic tree constructed using neighbor-joining method based on the homologous sequences of vertebrates and invertebrates (B). Accession no: *Homo sapiens* (NP_055049.1), *Macaca mulatta* (NP_001108430.1), *Rattus norvegicus* (NP_997694.1), *Anolis carolinensis* (XP_008122613.2), *Xenopus tropicalis* (NP_001016671.1), *Danio rerio* (NP_001005292.2), *Chlamys farreri* (AHE80140.1), *Mizuhopecten yessoensis* (MH040338), *Crassostrea gigas* (XP_011430925.1), *Aplysia californica* (XP_005103566.1), *Acanthaster planci* (XP_022094727.1), and human HSD17B14 (NP_057330.2). Bootstrap based on 1000 replicates.

The homolog of the scallop *hsd17b11* gene is 1090 bp long. This cDNA showed a single 912 bp ORF encoding 303 amino acids (GenBank accession no. MH040339). Alignment of the putative protein with other species showed high similarity, which were based on important structural motifs of this enzyme in the cofactor binding domain, structure stabilization domain, and catalytic site [16,18,38,39] (Fig. 6A). When the entire deduced amino acid sequence was compared with other vertebrates and invertebrates, it showed 57%–71% similarity.

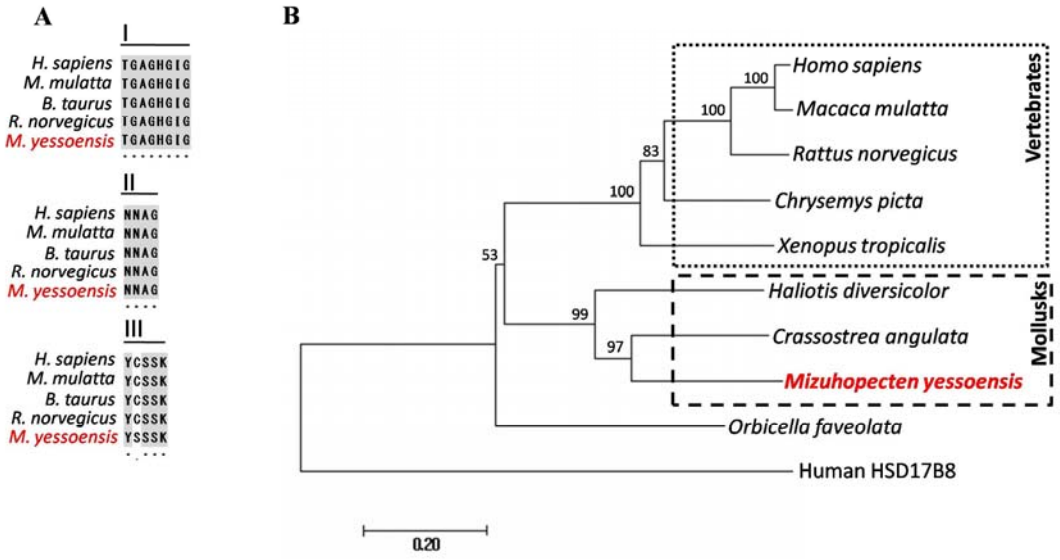

Fig. 6 Structural motif domains and phylogenetic tree analysis of the scallop *hsd17b11*gene. Shaded regions indicate motif sequences of amino acids identical to cofactor binding domain (I), structure stabilization domain (II) and catalytic site (III) of vertebrates (A). Multiple sequence alignment represented by * = identical: = strong homology, and = less homology. Phylogenetic tree constructed using neighbor-joining method based on the homologous sequences of vertebrates and invertebrates (B). Accession no: *Homo sapiens* (NP_057329.3), *Macaca mulatta* (EHH26026.1), *Rattus norvegicus* (NP_001004209.1), *Chrysemys picta* (XP_005292079.1), *Xenopus laevis* (NP_001011304.1), *Haliotis diversicolor* (ADV02385.1), *Crassostrea angulata* (AFK26305.1), *Mizuhopecten yessoensis* (MH040339), *Orbicella faveolata* (XP_020617493.1), and human HSD17B8 (NP_055049.1). Bootstrap consisted of 1000 replicates.

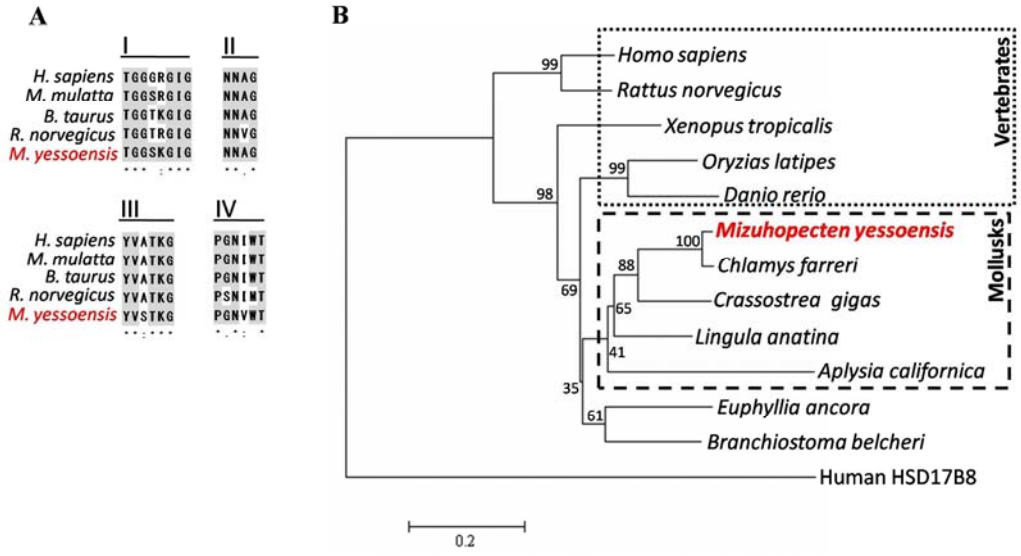

Fig. 7 Structural motif domains and phylogenetic tree analysis of the scallop *hsd17b14* gene. Shaded regions indicate motif sequences of amino acids identical to cofactor binding domain (I), structure stabilization domain (II), catalytic site (III) and a domain which determine the reaction's direction (IV) of vertebrates (A). Multiple sequence alignment represented by various symbols: * = identical, = strong homology, and = less homology. (B) Phylogenetic tree constructed using a neighbor-joining method based on the homologous sequence among various species. Accession no: *Homo sapiens* (NP_057330.2), *Rattus norvegicus* (NP_001178040.1), *Xenopus tropicalis* (XP_002935043.1), *Oryzias latipes* (XP_004071213.1), *Danio rerio* (NP_001003521.1), *Mizuhopecten yessoensis* (MH040340), *Chlamys farreri* (AGV08297.1), *Crassostrea gigas* (XP_011423932.1), *Lingula anatine* (XP_013414512.1), *Aplysia californica* (XP_012939218.1), *Euphyllia ancora* (AJP75149.1), *Branchiostoma* belcheri (XP_019638069.1), and human HSD17B8 (NP_055049.1). Bootstrap consisted of 1000 replicates.

3.3. Phylogenetic analysis

Our phylogenetic analysis of the six steroidogenesis-related genes showed that the deducted amino acid sequences of *M. yessoensis* clustered mainly with invertebrates and separated from vertebrate sequences. The scallop *star3* gene was located closer to the bivalve clade than to other mollusk species (Fig. 2B). The scallop *cyp17a1* was clustered within the *cyp17* clade with *cyp17* proteins of bivalves, echinoderms, acorn worms, and gastropods (Fig. 3B). The scallop *hsd3b1* gene was located in the clade of mollusk *hsd3b*1 homologs and was clearly distinguished from vertebrate, insect, and other invertebrate *hsd3b1* homologs (Fig. 4B). The scallop *hsd17b8* gene occurred within the invertebrate clade, which included *hsd17b8* homologs of bivalves, gastropods, and echinoderms. These sequences were positioned with a sister group comprising vertebrate *hsd17b8* gene sequences (Fig. 5B). The scallop *hsd17b11* gene was closest to the other mollusks and was clearly distinguishable from the vertebrate *hsd17b11* gene sequence (Fig. 6B). The scallop *hsd17b14* gene was positioned with mollusk *hsd17b14* homologs and formed a sister clade with cnidarians and the amphioxus *Branchiostoma belcheri* (Fig. 7B).

3.4. Tissue distribution of steroidogenesis-related genes

Steroidogenesis-related genes were ubiquitously expressed in peripheral tissues, especially in gonadal tissues of both sexes during the growth stage (Fig. 8). The *star3* gene was broadly expressed in all examined tissues of both sexes. For the *cyp17a1* gene in both sexes, transcripts were abundant in cerebral and pedal ganglia, visceral ganglion, digestive gland, and the kidney tissues, whereas *cyp17a1* mRNA was only weakly expressed in many other tissues. However, *cyp17a1* mRNA was not detectable in the adductor muscle of female scallops. Although *hsd3b1* mRNA showed

a sexually dimorphic distribution among tissues, *hsd3b1* mRNA was high in cerebral and pedal ganglia, visceral ganglion, testis, and ovary tissues. In contrast, mRNAs of *hsd17b8*, *hsd17b11*, and *hsd17b14* genes showed a ubiquitous distribution, including in testes and ovaries. Signals for the 60 s ribosomal protein L16 (from the *rpl16* gene, used as an internal reference gene) showed a highly-consistent expression in every tissue in scallops. In fact, all six steroidogenesis-related genes were highly expressed in the cerebral and pedal ganglia, visceral ganglion, digestive gland, and gonadal tissues of adult scallops.

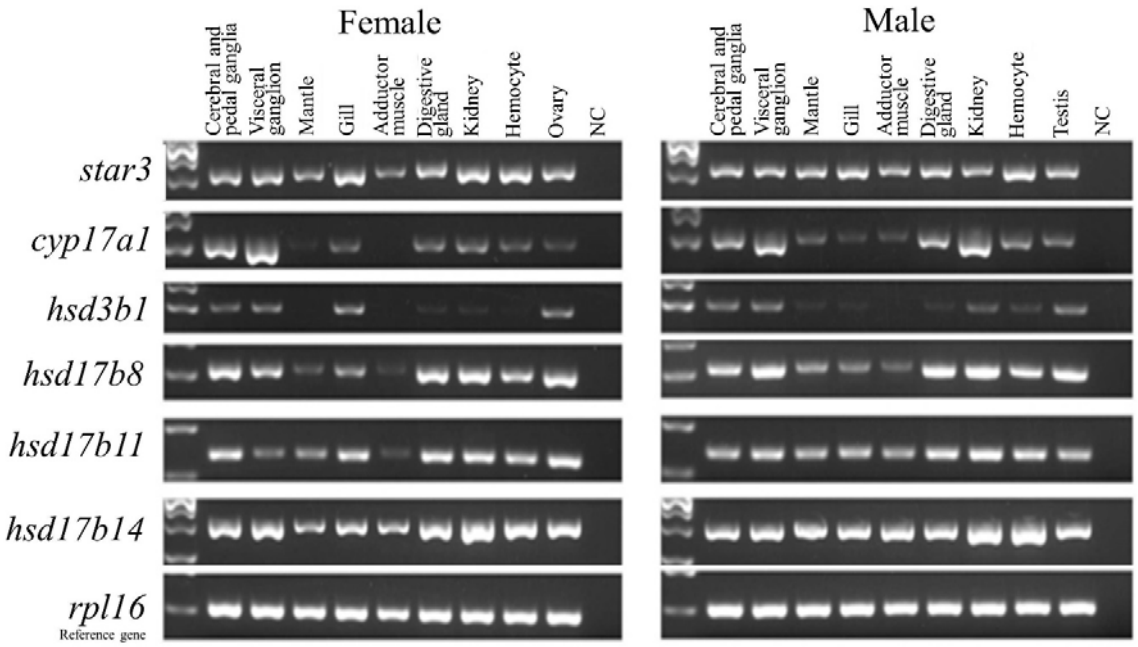

Fig. 8 Tissue distributions of steroidogenesis-related genes of *M. yessoensis*. Abbreviations: *star3* (StAR-related lipid transfer protein 3), *cyp17a1* (17α-hydroxylase, 17,20-lyase), *hsd17b8* (17β-hydroxysteroid dehydrogenase 8), *hsd17b11* (17β-hydroxysteroid dehydrogenase 11), *hsd17b1*4 (17β-hydroxysteroid dehydrogenase 14), *hsd3b1* (3β-hydroxysteroid dehydrogenase 1), *rpl16* [60s ribosomal protein L 16 (reference gene)], and NC (no RT-template).

3.5. Differential expression of steroidogenesis-related genes associated with sexual maturation during the reproductive phase

Changes in mRNA levels for six steroidogenesis-related genes (i.e., *star3*, *cyp17a1*, *hsd3b1*, *hsd17b8*, *hsd17b11*, and *hsd17b14*) were identified in gonadal tissues throughout the reproductive phase (Fig. 9). The *star3* gene was highly expressed at the early stage of scallop cell differentiation (September) in both testicular and ovarian tissues. Expression of *star3* mRNA in scallops slightly increased in ovaries at sexual maturity (March), whereas only low levels of mRNA were detected in mature testes. The *cyp17a1* gene was high expression at the early stage of cell differentiation (September and November) and lower expression at later stages in testes and ovaries. Before the sexual maturity, the expression of the *hsd3b1* gene was low, but the gene became most-highly expressed in scallop testes at sexual maturity (March); however, expression of mRNA remained low in ovaries. Notably, *hsd17b8* and *hsd17b11* gene expression patterns were similar to those in ovaries in that expression gradually increased as gonads matured during the reproductive phase. In contrast, *hsd17b8* and *hsd17b11* genes were not well expressed in scallop testes throughout their reproductive phase. The mRNA expression pattern of the *hsd17b14* gene was not associated with the maturity in reproductive phase and showed higher concentrations of mRNA in ovaries than in testes.

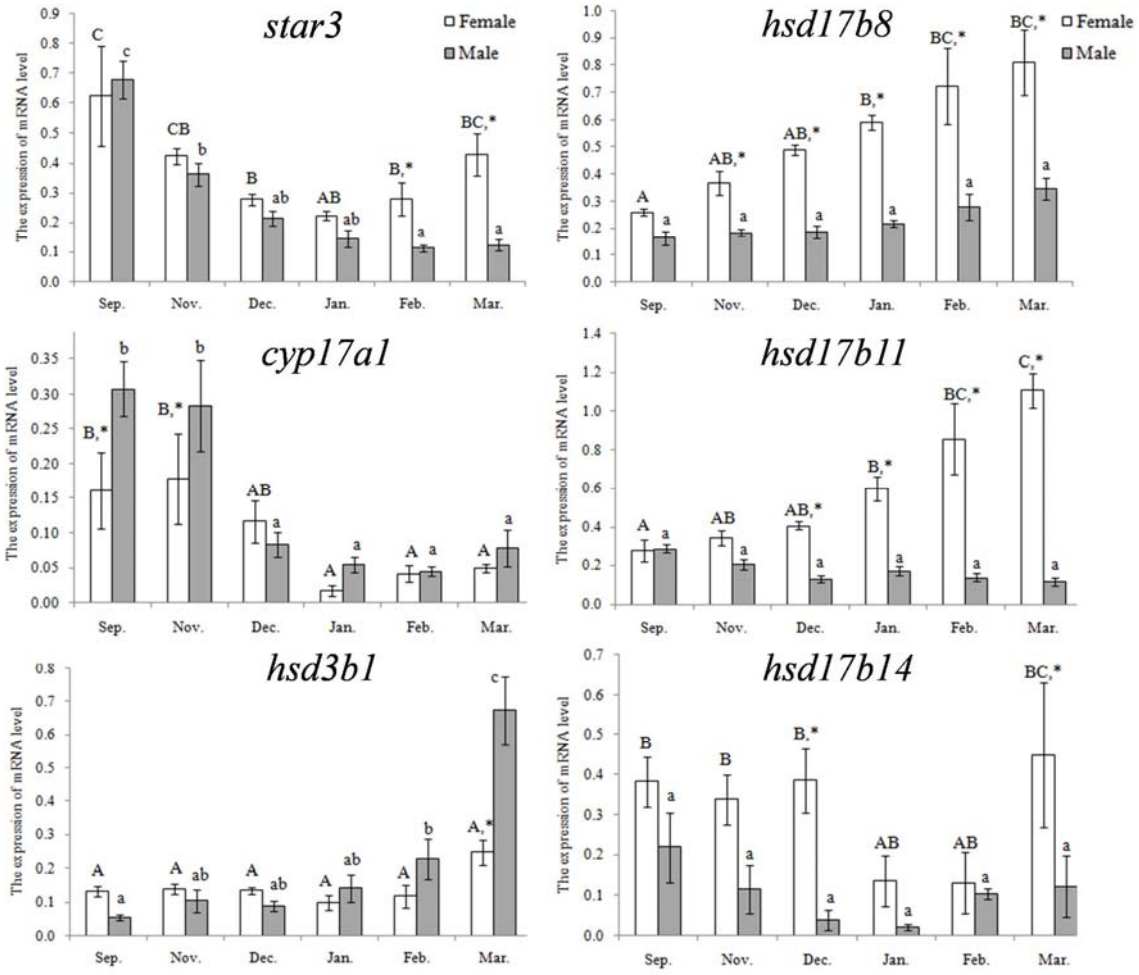

Fig.9 Seasonal profiles of steroidogenesis-related mRNA expression of M. yessoensis in gonadal tissues during their reproductive phase. The relative expression levels represented by the mean ± SEM. Significant differences in expression among months indicated by different letters and between sexes by asterisks ($p < 0.05$). Females denoted by unfilled bar and male by filled, dark-gray bars. Five samples were obtained from each sex at each sample date.

4. Discussion

Steroidogenesis-related enzymes are crucial for the biosynthesis of sex steroids. These enzymes are generally associated with lipid transfer proteins, cytochrome P450, and various oxidative enzymes [33,40]. Many studies have attempted to understand the biosynthetic processes driving steroidogenesis in mollusks and putative biosynthetic pathways have been proposed from an examination of steroid metabolism and biochemical changes in gonadal tissues [3,11]. However, the extent of involvement of endogenous sex steroids in reproductive events is still controversial [9,10]. To gain more insight into the influence of endogenous sex steroids in steroidogenesis in mollusks, we cloned several steroidogenesis-related genes from the scallop that are known to be the genes primarily responsible for sex steroid biosynthesis in vertebrates. Our putative biosynthesis pathway suggests that the scallop shares an almost identical biosynthetic pathway with vertebrates (Fig. 10).

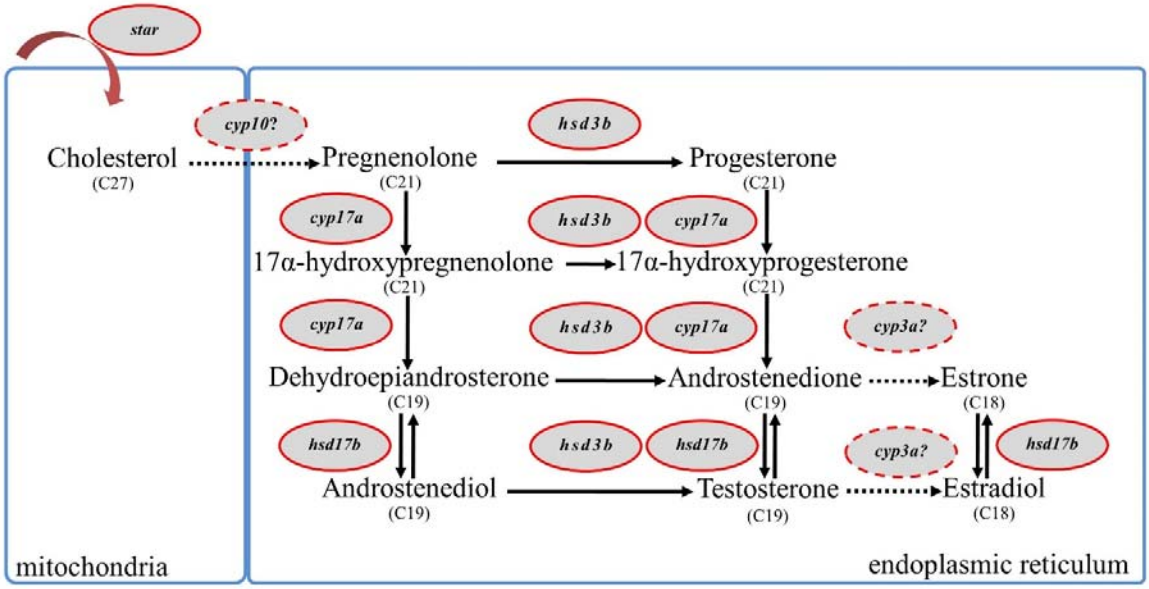

Fig. 10 The putative pathway for sex steroid biosynthesis in *M. yessoensis*. The pathway was deduced from known sex steroid metabolizing enzymes in vertebrates. Solid arrows indicate identified steroidogenesis-related genes, whereas dotted arrows indicate undiscovered genes in invertebrates. Question mark symbol indicates not available or replaced by other enzymatic genes. *star* = StAR-related lipid transfer protein; *cyp10* = cytochrome P450 10, which may replace *cyp11a*; *cyp17a* = 17α-hydroxylase; 17,20-lyase; *hsd17b* = 17β-hydroxylase dehydrogenase; *hsd3b* = 3β-hydroxysteroid dehydrogenase; *cyp3a* = cytochrome P450 3, which may replace *cyp19a.*

In our study, we identified the steroidogenic acute regulatory protein (StAR) in scallop. This gene contains StAR-related lipid transfer (START) domain 3 proteins involved in lipid transport [33,41]. StAR delivers cholesterol to the inner mitochondrial membrane to generate pregnenolone as a precursor of sex steroids [33,41] The existence of the scallop *star* may imply that if there is a biosynthetic pathway, cholesterol can be utilized as a resource precursor for steroid synthesis.

The cytochrome P450 enzymes play an important role in the oxidative metabolism of both exogenous and endogenous substrates [42]. In sex steroid biosynthesis, there are three types of cytochrome P450 genes encoding steroid metabolizing enzymes: *cyp11a*, *cyp17a*, and *cyp19a*. In this study, *cyp11a* and *cyp19a* genes were not in transcriptome libraries. Both *cyp* genes have been reported in studies of evolutionary relationships among vertebrates [43]. However, both the immunoreactivity and mRNA signaling of the *cyp11a* gene have been detected in the cytoplasm of basophilic cells in the digestive gland of *M. edulis* and *M. galloprovincialis* [44,45], suggesting that cholesterol-catabolizing enzymes may exist in bivalves. Recently, members of *cyp* gene families were reported in several bivalves, including *Mytilus* californianus [46], *M. galloprovincialis* [46], *C. gigas* [46], *C. virginica* [46], and *C. farreri* [47]. Huihui et al. [47] reported that *cyp10* genes belonged to mitochondrial *cyp* and the cluster of human *cyp11* and *cyp27.* The *cyp10* genes in mollusks have not been established so far, but the *cyp10* gene has been identified in the snail *Lymnaea stagnalis* [48]. In addition, molluscan the *cyp10* gene shows an evolutionary relationship with the *cyp11* gene family in vertebrates [46,47]. Hence, the *cyp10* gene would be a candidate for further work examining steroid metabolizing enzyme activities in scallops.

The aromatization reaction in vertebrates generates estrogens from androgens involving catalysis by aromatase enzyme [40], although the precise pathway for this reaction in invertebrates is still not clear. Cytochrome P450 aromatase activity has been previously identified in the scallop using ^{3}H-water assay, which identified immunoreactive cells [49]. Although the enzymatic activity generating estrogens showed synchrony with 17β-estradiol concentrations, it is premature to conclude whether or not conversion to 17β-estradiol from androgens was mediated by this pathway. This is because the *cyp19a* gene is conserved only vertebrate phyla [43] and the sequence of

this gene has not been clearly identified in invertebrates. We postulate that if P450 aromatase is not a member of the aromatizing gene family in invertebrate phyla [43], then invertebrates could possess other ancestral types of *cyp* genes for use in aromatization.To date, the *cyp1* and *cyp3* genes are thought to be associated with aromatization in mussels [50], humans [51] and fishes [52], suggesting their potential for converting androgens to estrogens [40]. In fact, several biocides have been manufactured that interrupt aromatase activity [53]. For example, tributyltin (TBT), a biocide in anti-fouling paint, is a well-known endocrine-disrupting compound that interrupts aromatase activity in mollusks [53]. TBT has also been shown to represses *cyp3a* mRNA expression in the mussel *M. edulis* [50]. The pathway might convert androgen to 17β-estradiol homolog in bivalves, which has been detected as a vertebrate 17β-estradiol [53] and might be primary ligand for bivalve receptors [54,55]. Conceivably, the invertebrate *cyp3a* gene may function as an aromatizing-like enzyme similar to the vertebrate P450 aromatase enzyme.

In our study, only a single cytochrome P450c17 (encoded by the *cyp17a* gene) was encountered in scallop gonadal tissues. The *cyp17a* gene possesses both 17α-hydroxylase and 17, 20 lyase within a single gene that are essential for the production of sex steroids [40,56]. In mollusks, the enzymatic activities of the *cyp17a* gene have been reported in several species (e.g., *Sepia officinalis* [57], *A. californicus* [4], and *M. edulis* [5]). These studies support our identified *cyp17a* sequence and our inference that the scallop *cyp17a* gene is very similar to other vertebrate homologs, in particular, the amino acid residues in the specific, conserved regions of P450c17 [34,35] This suggests that the scallop *cyp17a* homolog could provide a key enzyme involved in sex steroid biosynthesis.

Hydroxysteroid dehydrogenases (HSDs) are important enzymes for sex steroid production in vertebrates [40]. These enzymes belong to the short-chain alcohol dehydrogenase/reductase (SRD) super family and have two-types of metabolizing enzymes (i.e., 17β- and 3β- hydroxysteroid dehydrogenase) [40]. The 17β-hydroxysteroid dehydrogenase (from the *hsd17b* gene) metabolizes dehydroepiandrosterone to androstenediol, androstenedione to testosterone, and estrone to 17β-estradiol, or vice versa in vertebrates [38]. To date, 14 isoforms from animal phyla have been isolated from the *hsd17b* gene [58] and four isoforms have been reported in mollusks [[18], [19], [20], [21],23]. Our results show that at least three isoforms occur in *M. yessoensis*. The functional characterization of the *hsd17b8* gene has been studied in Zhikong scallop (*C. farreri*) [19], whereas the *hsd17b11* gene has been studied in abalone (*H. diversicolor*) [20]. Specifically, the *hsd17b8* gene in *C. farreri* intensely converts 17β-estradiol to estrone and weakly converts testosterone to androstenedione [19]. In *H. diversicolor*, the *hsd17b11* gene showed an ability to change 5α-androstane-3α, 17β-diol to androsterone and testosterone to androstenedione [20]. The function of the *hsd17b14* gene is to encode an oxidative enzyme catalyzing NAD^+-dependent reactions involved in the formation and inactivation of estrogens and androgens, reactions which has been well studied in vertebrates [59]. However, the functional activity of the *hsd17b14* gene has never been confirmed for invertebrates. In our study, we found that the *hsd17b8*, *hsd17b11*, and *hsd17b14* homologs in scallops contain typical, conserved domains belonging to the SDR family [40]. Therefore, we could deduce the amino acid residues that share relatively high homology with vertebrate sequences, which suggests that the scallop *hsd17b* gene could encode enzymes needed for essential activities in steroid biosynthesis, enzymes that are similar to those used by Zhikong scallop, abalone, and other vertebrates [19,20,59].

The 3β-hydroxysteroid dehydrogenase (encoded by the *hsd3b* gene) is a metabolizing enzyme that generates progesterone, 17α-hydroxyprogesterone, andostenedione, and testosterone in vertebrate biosynthetic pathways [40]. These 3β-HSD activities have been detected in Pacific oyster (*C. gigas*) via biochemical procedures and histochemical techniques [7] and in Yesso scallop (*M. yessoensis*) via immunohistochemistry approaches [6]. The 3β-HSD activities have been identified in several tissues of *C. gigas* and a strong signal has been shown in the elongated, epitheloid tissues in kidneys and in interstitial cells of gonadal tissues [7]. Furthermore, immunoreactivity against 3β-HSD has been detected in cells aligned along the outside of the germinal acini of the scallop ovary [6]. To date, there have been no studies focused on sequencing the *hsd3b* gene or determining its function in bivalves. In our study, we identified 3β-hydroxysteroid dehydrogenase (encoded by the *hsd3b1* gene) in scallop. The enzyme 3β-HSD is likely involved in sex steroid biosynthesis in bivalves because testosterone has been detected in bivalves [14,22,60]. This suggests that 3β-HSD may exert its influence similarly in both vertebrates and bivalves.

Sex steroids are synthesized in response to environmental cues in seasonal breeders [61]. Hormones 17β-estradiol and testosterone reach their highest levels in bivalves when gonads are mature [14,22,49], suggesting that they are associated with regulating bivalve reproduction. Recently, estrogens have been detected in both male and female scallops, suggesting that they regulate reproduction [14,49]. However, seasonal changes for steroidogenesis-related mRNA have not been reported in all scallop species. In our study, we identified the expression of mRNA of steroidogenesis-related genes in several tissues during growth and focused on their seasonal variation in gonadal tissues. Our results showed that all transcripts could be detected not only in ovaries and testes, but also in peripheral tissues,

which indicates that steroidogenesis-related genes might have other functions in addition to producing steroids. Other reports have shown a similar, ubiquitous distribution of some steroidogenesis-related genes (e.g., *hsd17b*, *star,* and *cyp17a*) in mammals, fishes, and bivalves [21,[61], [62], [63]], suggesting they might influence other physiological functions, such as lipid metabolism and/or detoxification.

In steroid biosynthesis, a precursor is defined as a fundamental limiting resource that regulates the synthesis of steroids. The first reaction in steroidogenesis, mediated by StAR which is composed of the lipid-binding domain (STARD), provides a key molecule that regulates the timing and rate of pregnelonone synthesis [40]. In our study, the *star3* mRNA was abundantly expressed in gonadal tissues at the early stages of testis and ovary differentiation in scallops. Concentrations of *star3* mRNA declined during the growth stage in scallops and then increased again in mature ovaries, but not in testes. This indicates that the *star3* gene may provide a crucial enzyme for steroid biosynthesis in ovaries at the early stages of cell differentiation and maturation, but only during the early stage of cell differentiation in testes. In the sequential series of events that regulate the biosynthesis of sex steroids, both the *star* gene and cytochrome P450c17 (encoded by the *cyp17a* gene) are critical for the production of sex steroids [36]. Furthermore, the *cyp17a1* gene supposedly tightly controls the expression of *star* [41]. In rainbow trout and Japanese eel, the transcript of the *cyp17a* gene is abundant in cells of mature ovaries, but has not been detected in immature gonads [56,64], whereas *cyp17a* mRNA changes little in zebrafish ovaries as they mature [65]. Unfortunately, information about *cyp17a* mRNA expression in mollusks is still not yet available. Despite this, a few studies that have shown enzymatic activities of cytochrome P450c17 in gonadal tissues of *Sepia officinalis* [57], *A. californicus* [4], and *M. edulis* [5]. In our study, *cyp17a1* mRNA was high at early stages of cell differentiation, but not at other later stages in both testes and ovaries. These findings suggested that the transcription and translation of *cyp17a1* are greater at the beginning of the reproductive season and suppressed toward sexual maturation.

17β-hydroxysteroid dehydrogenase (encoded by the *hsd17b* gene) is critical for metabolizing sex steroids in the last steps of androgen and estrogen syntheses [40]. In our study, a similar pattern of *hsd17b8* and *hsd17b11* mRNA expression was found in gonadal tissues throughout the reproductive phase, but a similar pattern was not found in the expression of the *hsd17b14* gene. Previously reported changes in expressions of *hsd17b8* appear to have been related to degree of sexual maturity in various mollusk species, such as *C. farreri* [19]. In *H. diversicolor*, *hsd17b11* mRNA shows a higher degree of expression at the spawning stage in testes and at non-reproductive stages in ovaries [20]. In addition, higher levels of 17β-estradiol have been detected in ovaries of scallops that undergo synchronous synthesis of vitellogenin during maturation [14,49]. Our results showed that the pattern of change in *hsd17b8* and *hsd17b11* mRNA were similar to that exhibited by maturing ovaries, suggesting that these enzymes may be involved in estrogen biosynthesis in scallop (as a rate-limiting enzyme).

Androgens are important in producing male characteristics and in sperm mobility in vertebrates [66]. In bivalves, detectable levels of testosterone in testes correspond to changes throughout the reproductive phase, with the highest concentrations occurring at sexual maturity when many germ cells are in testes [14,60]. In our study, we found that *M. yessoensis* showed an abundance of germ cells at sexual maturity when *hsd3b1* mRNA levels showed their highest expression in testes. Our results indicate that the *hsd3b1* gene in scallops may provide a metabolizing enzyme to synthesize testosterone from steroids.

5. Conclusions

The identification and characterization of steroidogenesis-related genes in Yesso scallop *M. yessoensis* provide new insights into the biosynthetic pathway for sex steroids in the scallop and related invertebrates. The expression of mRNA's orthologous to vertebrate steroidogenesis-related transcripts was associated with season and reproductive status. These findings support that hypothesis of scallops can synthesize sex steroids (endogenously) to regulate maturation of gonads. Further studies of functional characterization of the steroidogenesis-related genes and their associated signaling pathways within the gonads of Yesso scallop should provide additional insight into the function of steroidogenic enzymes and the role of endogenous steroid hormones in their physiology. The results described here are expected to serve as a model for related invertebrate species.

Conflict of interest

None declared

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Table and Figures

Table 1 Gene specific primers used for semi-quantitative RT-PCR and real-time qPCR analyses

Gene name	PCR type	Sequence)5′-3′(	Size)bp(	r^2	Efficiency)%(	Intron insertion	GenBank	Reference
	RACE	10x Universal primer A mix)UPM(	-	-	-	-	-	-
star3	RACE, RT	Fw: GCTGAAGACTAATCTGTGAAGTG	1585	-	-	-	MH040336	This study
		Rv: GACGACGATGATACAGGTAACG						
	qPCR, RT	Fw: CCTGGGCCGAGACCTGGTTC	221	0.999	83.68	Yes	-	This study
		Rv: GCGTCTGAAACCCACCACGATG						
cyp17a1	RACE	Fw: GTAGTGAGTAAGTGAAGGATGGA	2121	-	-	-	MH040337	This study
		Rv: CAATGACTGTAAGGGTGCCTGA						
	qPCR, RT	Fw: TCAAAGATTCTGTGTAGTGAGTAAGTG	269	0.995	91.17	Yes	-	This study
		Rv: TCTCAGACAGACGCGCACAAG						
hsd3b1	RACE	Fw: ACACCCTGCCTCGTATTACAG	1667	-	-	-	MH040341	This study
		Rv: AGCTAACTGAACACACCTGCTC						
	qPCR, RT	Fw: CGTAACTACGGTCTGTCGCA	208	0.997	86.81	No	-	This study
		Rv: GGCTGTACAGGGGTTGGTAG						
*hsd17b*8	RACE	Fw: AGAGTTCACAATGGCTTCCGC	1096	-	-	-	MH040338	This study
		Rv: TAATGTTGTGTGCTGCCTCAG						
	qPCR, RT	Fw: TCCGTGTAAACGCCATCTTAC	198	0.994	82.08	Yes	-	This study
		Rv: CACCTGCCACCTCCAGAGTG						
*hsd17b*11	RACE	Fw: GGAGTTGAGGAAGGACTGTG	1090	-	-	-	MH040339	This study
		Rv: TACGGCTATTACCAAAACTATCAC						
	qPCR, RT	Fw: CTATGGGTTTGCGGAGGCTA	153	0.997	90.83	Yes	-	This study
		Rv: GCAACCTCTTTCGGTGTCAAC						
*hsd17b*14	RACE	Fw: TAGTTCAATGGCGGAATCTGAGGTAC	1488	-	-	-	MH040340	This study
		Rv: TCGACAACATATTAACGGCGTTCAG						
	qPCR, RT	Fw: GCAAAGTGACAATTGTCACTG	237	0.994	91.37	Yes	-	This study
		Rv: TCCTTCCGTACTTCTCCACTG						
rpl16	qPCR, RT	Fw: CTGCCAGACAGACTGAATGATGCC	117	-	95.49	Yes	N.A.	Feng et al., 2013]25[
		Rv: ACGCTCGTCACTGACTTGATAAACCT						
heli	qPCR	Fw: CCAGGAGCAGAGGGAGTTCG	186	-	77.02	Yes	N.A.	Feng et al., 2013]25[
		Rv: GTCTTACCAGCCCGTCCAGTTC						
ubq	qPCR	Fw: TCGCTGTAGTCTCCAGGATTGC	184	-	80.05	Yes	N.A.	Feng et al., 2013]25[
		Rv: TCGCCACATACCCTCCCAC						