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Transcriptomic analysis of gonads in Malabar red snapper (*Lutjanus malabaricus*) reveals genes associated with gonad development

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ABSTRACT

Malabar red snapper (*Lutjanus malabaricus*) is an important and high-value aquaculture species in Singapore that is attracting substantial interest and new industry investment. The consistent production of good quality eggs is an important step for establishing a selective breeding program for the species in Singapore. To achieve improved spawning outcomes, reproductive studies are needed to address the challenges in Malabar red snapper seed production, such as limited spawning success in closed culture systems. However, despite its rising profile as an aquaculture candidate and the need to improve broodstock husbandry, currently there are scarce genomic resources for the species, particularly related to genes involved in sex determination and differentiation. Herein, we report the first gonadal transcriptomes of adult L. *malabaricus* generated using RNA-Seq of testes ($n = 6$) and ovaries ($n = 6$). A total of 14,421 significant differentially expressed genes (DEGs) were found by comparing the gene expression profiles of ovaries and testes, including 6499 upregulated female-biased and 7922 downregulated male-biased DEGs. These DEGs included genes known to be involved in reproductive processes such as male-biased *dmrt1, spat4, odf3b and sox9,* and female-biased *cyp19a1, zp4, sox3, bmp15 and esr1*. The expression level of 16 selected DEGs were validated by RT-qPCR. This study lays the basis for further research in genes involved in sex determination and differentiation, sex control and breeding in Malabar red snapper.

1. Introduction

Malabar red snapper (*Lutjanus malabaricus*) is a commercially important food fish species, which has been widely accepted by consumers in many countries due to its high nutritional value and favourable eating quality. *L. malabaricus* is also commonly referred to as Malabar blood snapper, saddletail snapper, scarlet seaperch, or saddletail seaperch, and is found over a broad distribution throughout the Indo-Pacific region (Salini et al., 2006). Adults of this species inhabit both coastal and offshore reefs (McPherson et al., 1988), whereas juveniles tend to inhabit inshore silty and coarse sand/rubble estuarine areas (Fry et al., 2009). Malabar red snapper is primarily piscivorous, although its diet also includes small amounts of benthic crustaceans, cephalopods and other benthic invertebrates (Kailola, 1993). Due to the decrease in capture fisheries caused by over exploitation of wild fish stock (Pauly et al., 1998), farming of Malabar red snapper has been increasing to fulfil the market demand attracted by its high market value. Identifying different red snapper species within the Lutjanidae family is challenging due to various factors, including the distinct appearances exhibited by juveniles and adults, the abundance of species, and the similarities in morphologies observed among different species. Thus, the majority of production data of red snapper are recorded under the *Lutjanus* genus. Farmed red snapper is mainly produced in China, Hong Kong SAR and Southeast Asian countries such as Malaysia and Philippines (FAO, 2020). Malabar red snapper is also identified as a promising tropical species for coastal and marine aquaculture in Singapore (Shen et al., 2021).

One of the main challenges in the developing Malabar red snapper farming industry is that seedstock is derived from broodstock fish that naturally spawn in open net cages with no control over pairings; thus industry has not yet been able to access high quality and genetically improved snapper seedstock through breeding programmes, which has

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achieved great success in a number of key aquaculture species such as salmon, tilapia, carp, shrimp and oyster (Zenger et al., 2019). However, some prerequisites are needed to instigate selective breeding programmes, including understanding genetic mechanisms of sex determination and differentiation in the species under selection: an understanding of genetic control of sex determination and differentiation can lead to the development of targeted manipulation of sex control, reproduction, and sex ratios of broodstock fish to achieve improved breeding outcomes (Budd et al., 2015).

In contrast to mammals and birds, which have highly conserved master genes that dictate the development of their proto-gonads into either testes or ovaries, the process of sex differentiation and determination in fish is plastic and influenced by a range of genetic, epigenetic, and environmental factors (Devlin and Nagahama, 2002; Budd et al., 2015). Sex determination also varies among different species from strict gonochorism to simultaneous or sequential hermaphroditism. The sex determination system of Malabar red snapper is deemed to be gonochoristic, since gonochorism has been observed in all lutjanids studied so far (Heupel et al., 2010; Newman et al., 2016) and no individuals exhibiting hermaphroditism or evidence of transitional gonad states between sexes were found in large samples examined from wild populations (McPherson et al., 1992; Newman, 2002; Fry et al., 2009). Based on data collected from wild caught fish, the sexual maturity of Malabar red snapper is typically reached at a length of 32–50% of their maximum size, which was reported to be 645 mm standard length (SL) in northern Australia and 740 mm SL in eastern Indonesia (Fry et al., 2009). Furthermore, males tend to reach sexual maturity at a slightly smaller size than females, with an average length of around 240 mm SL for males compared to 250–300 mm SL for females. Malabar red snapper also exhibited differential growth between sexes, whereby males were observed to attain a larger size than females of the same age from the age of 9 years on average, possibly due to the onset of sexual maturity (Newman, 2002). Male and female snapper do not exhibit obvious phenotypic dimorphism in colouration, morphology or size individually, meaning that presently in order to determine the sex of an individual it is necessary to examine the gonads directly through dissection or cannulation.

Within the literature, there are no studies reporting on the molecular mechanisms of sex determination and sex differentiation in Malabar red snapper, or any snappers. Traditionally, the study of genes involved in sex determination and differentiation utilize hybridization-based microarrays, sequencing-based EST libraries and chemical tag-based serial analysis of gene expression (SAGE). With the advent of Next Generation Sequencing (NGS), RNA sequencing (RNA-Seq) has become the efficient and dominant approach for comparative transcriptome studies between gonadal types (Tao et al., 2018; Sun et al., 2013; Poon et al., 2023). In the present study, RNA-Seq was performed on testes and ovaries of adult Malabar red snapper, and we report the first gonad transcriptome for each sex of the species. Significant differentially expressed genes (DEGs) and genetic pathways associated with reproduction were identified by comparative transcriptomic analysis. Furthermore, RT-qPCR assays for 16 sex-biased genes were developed, and two of the most important sex-determining genes, *dmrt1* and *cyp19a1* were fully characterized and compared with other teleosts.

2. Materials and methods

2.1. Ethics statement

The study was carried out at James Cook University in Singapore, following the approval from the Institute Animal Care and Use Committee (IACUC) under the reference number 2021-A010.

2.2. Experimental design and sample collection

In total, 21 Malabar red snapper were sampled from two different batches of adult Malabar red snapper at a coastal farm in Singapore in November 2021 and July 2022. The seasonal seawater temperature fluctuation in Singapore is very low (< 3 °C) and has unnoticeable impact on fish gonadal development. Twelve individual samples of testes ($n = 6$; body weight 1.80–2.53 kg; body length 48–52 cm) and ovaries (n = 6; body weight 1.65–2.15 kg; body length 46–50 cm) were selected and used for the present study. The gender of the fish was determined by observing milt or eggs in the gonads after the fish had been euthanised using 200 ppm AQUI-S® and dissected. To identify the stage of gonad development, a portion of each gonad sample was fixed in 10% neutral buffered formalin containing 4% formaldehyde and stored at room temperature before standard histology procedures. Another portion of each sample was preserved immediately in RNAlater® tissue storage solution (Sigma-Aldrich) and stored at −80 °C before RNA isolation.

2.3. Gonadal histology and RNA isolation

The gonad tissue samples were prepared by dehydrating them in ascending series of ethanol, cleared using xylene, and then embedded in paraffin wax. Gonad sections (5 μm thick) were cut and mounted onto glass slides. The slides were then dewaxed in xylene and rehydrated in descending series of ethanol and stained with hematoxylin and Eosin (H&E). Finally, the slides were dehydrated in ascending series of ethanol, cleared with xylene and placed under a coverslip. The slides were examined under an Olympus BX53 System Microscope (Olympus). Histological criterion for classifying the stage of gonads were derived from past publications (Kjesbu et al., 2003; Guiguen et al., 1994; Fry et al., 2009; Shinkafi et al., 2011) and are described in Fig. 1.

Total RNA extraction was performed using Trizol (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. In total, 12 RNA samples were obtained from six testes and six ovaries. All RNA samples were treated with a TURBO DNA-free™ Kit (Thermo Fisher Scientific) to remove contaminating DNA. RNA quantity and quality were assessed by a Nanodrop (Thermo Fisher Scientific) and Agilent 2200 Bioanalyzer (Agilent Technologies), respectively, prior to RNA sequencing. The RNA integrity number (RIN) of samples used for library construction included 10 samples with the RIN ranging from 7 to 9.9, and two samples with a RIN of 5.2 which passed further quality controls and hence were also included in the analyses.

2.4. Library preparation and sequencing

Libraries for RNA sequencing were prepared using 1 μg of total RNA extracted from both testis and ovary samples. Fragmentation of mRNA was achieved through divalent cations and high temperature. Random primers were employed for priming. Subsequent to the synthesis of first strand cDNA and second-strand cDNA, the purified double-stranded cDNA underwent treatment to repair both ends and introduce a dA-tailing in one reaction, followed by a T-A ligation to attach adaptors to both ends. Size selection of Adaptor-ligated DNA was carried out using VAHTS™ DNA Clean Beads (Vazyme). PCR amplification was then conducted on each sample using P5 and P7 primers with subsequent validation of the PCR products. Finally, libraries with different indices were multiplexed and loaded on an Illumina Novaseq instrument and a 2 × 150 paired-end (PE) configuration was used for sequencing as per manufacturer's instructions.

2.5. Processing of sequence reads and mapping to the reference genome

Cutadapt (v1.9.1) was used to process the raw reads. The following parameters were applied: phred cut-off of 20, error rate of 0.1, adapter overlap of 1 bp, minimum length of 75 bp, and a proportion of N of 0.1. Adapter sequences were removed, and any 5′ or 3′ end bases containing unknown nucleotides with quality values below 20 were trimmed. Additionally, any reads that were <75 bp long after trimming were

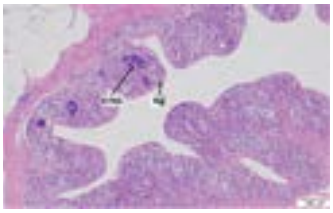
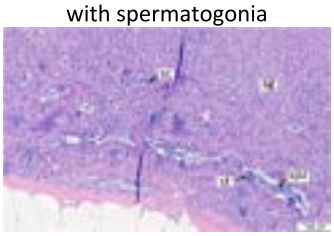
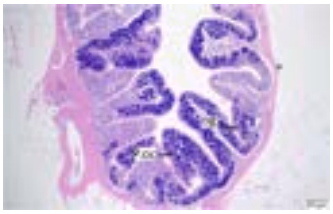
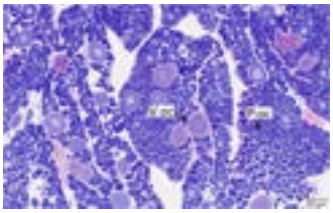
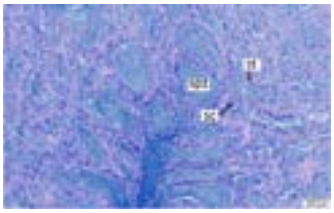
Maturity Stage	Male	Female
Immature (I)	Mostly spermatogonia with few spermatocytes, spermatids, and spermatozoa	Mostly oogonia with few primary growth oocytes
		
Maturing (II)	More spermatocytes, spermatids, and spermatozoa with spermatogonia	Mostly oogonia and previtellogenic oocytes
		
Mature (III)	Mostly spermatocytes and spermatids with more spermatozoa	Vitellogenic oocytes < 50% within histological cross-section
		
Spawning (IV)	Mostly spermatozoa	Vitellogenic oocytes > 50% within histological cross-section
		
Spent (V)	Empty sperm ducts with few spermatozoa (pictures not available)	Oocytes atresia (pictures not available)

Fig. 1. Histological criteria for classifying the gonadal stages of Malabar red snapper.
sg = spermatogonia, sc = spermatocytes, st = spermatids, spz = spermatozoa, cno = chromatin nucleolus oocytes, P.oc = previtellogenic oocytes, V.oc = vitellogenic oocytes (animals from the present study had gonads in Stage II – Maturing and III – Mature). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

discarded (Martin, 2011). Filtered data were subsequently aligned to a reference Malabar red snapper genome (unpublished data) using HISAT2 (v2.0.1) with default parameters (Kim et al., 2015). Filtered data were also assembled using StringTie (v1.3.3b) to predict alternative splicing (Pertea et al., 2015), and DEXSEq. (V1.18.4) was used for differential exon usage (DEU) analysis to detect the differential expression of genes on exon level with significant adjusted *p*-value <0.05 (Li et al., 2015). Results from both analyses were verified by mapping raw reads to the respective gene's coding sequence (CDS) in Geneious Prime 2022.2.1 (https://www.geneious.com) and visual inspection of the mapped reads for several representative sex-related genes such as *dmrt1*, *cyp19a1*, *amh*, *gsdf* and *sox3*.

2.6. Gene differential expression, gene ontology and pathway enrichment analysis

The level of gene expression was measured by counting the number of reads mapped to each gene with HTSEq. (v0.6.1) (Anders et al., 2014). Fragments per kilo bases per million reads (FPKM) was calculated to normalise the total number of exon fragments for each gene to total read counts and gene length, so that the expression of genes with different sequencing depths and length were comparable. Gene differential analysis was conducted using the Bioconductor package DESeq2 (V1.6.3) (Love et al., 2014). The outcomes of the DESeq2 analysis were subsequently analysed to identify genes exhibiting significant differential expression based on the criteria of fold change >2 and q-value (i.e., adjusted *p*-value to estimate the false discovery rate for multiple testing

using Benjamini-Hochberg) below 0.05. Differential Gene Ontology (GO) enrichment analysis was performed using GOSEq. (v1.34.1) to find GO terms with a significant q-value <0.05 (Young et al., 2010). KEGG pathway enrichment analysis (http://www.genome.jp/kegg/) was conducted using KEGG pathway units and a hypergeometric test to identify the pathways significantly enriched among of the differentially express genes compared to the transcriptome background (Kanehisa and Goto, 2000). Candidate sex-associated DEGs in the gonads of Malabar red snapper were selected based on the enrichment analyses and available literatures. Protein-protein interaction network of these DEGs was constructed using *Danio rerio* as the reference organism with minimum required interaction score of 0.400 (Szklarczyk et al., 2019).

2.7. RT-qPCR validation of selected DEGs

Sixteen DEGs known to be involved in reproductive processes were selected for RT-qPCR validation using the RNA samples described above, which include eight male-biased DEGs (i.e., *ddx4*, *piwil1*, *sox9*, *piwil2*, *brdt*, *spata7*, *dmrt1* and *odf3b*) and eight female-biased DEGs (i.e., *zp3*, *cyp19a1*, *sox3*, *bmp15*, *hsd17b12a*, *figla*, *cyp1b1* and *cyp26a*). Reverse transcription was carried out using the SensiFAST cDNA Synthesis Kit (Bioline) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was reverse-transcribed into cDNA using random primers and the reverse transcriptase enzyme. Specific primers were designed for the target genes using Geneious Prime 2022.2.1 based on the gene sequences obtained from the transcriptome. RT-qPCR was performed using the KAPA SYBR® FAST qPCR Master Mix (2×) Kit (Roche) on the StepOnePlus™ Real-Time PCR System thermal cycler (Applied Biosystems). Each reaction consisted of 10 μl of KAPA SYBR FAST qPCR Master Mix (2×), 0.4 μl of each primer (10 μM), 2 μl of cDNA template, 0.4 μl of 50× ROX High and 6.8 μl of nuclease-free water. The cycling conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s and 64 °C (for *dmrt1* and *cyp19a1*) or 60 °C (for all the other genes) for 30 s. A dissociation curve was generated at the end of each run to confirm the specificity of the amplification. The threshold cycle (Ct) values were determined using the StepOne™ Software v.2.3 (Applied Biosystems). Four candidate reference genes (i.e., *actb*, *ef1a*, *rpl8* and *gadph*) were chosen and their expression stability was evaluated using RefFinder (http://blooge.cn/RefFinder/). *actb* was determined to be the most stable reference gene and used as the internal control to calculate the relative gene expression levels using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The expression levels of the target genes were compared between male and female groups using the Student's *t*-test. A *p*-value of <0.05 was considered statistically significant. In addition, two of the most important sex related genes *dmrt1* and *cyp19a1* were fully characterized using both raw RNA-Seq reads and Sanger sequencing. RT-PCR was conducted using primers spanning all exons of *dmrt1* and *cyp19a1* for both testis and ovary samples to detect possible alternative splicing of these two genes. For this, PCR amplification was performed using Taq PCR Core Kit (Qiagen) in a thermal cycler with the following parameters: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 10 min. The primer sequences for RT-qPCR and RT-PCR are provided in Supplementary Table S1. Both RT-qPCR and RT-PCR products were sent for Sanger sequencing to confirm the amplicons matched expected gene sequences.

2.8. Characterization of dmrt1 and cyp19a1 genes in Malabar red snapper

Further to RNA-Seq and RT-qPCR, the two most extensively studied sex-biased genes in teleosts, namely *dmrt1 (male-biased)* and *cyp19a1* (female-biased) were also fully characterized. The introns and exons of *dmrt1* and *cyp19a1* in Malabar red snapper were annotated by mapping their mRNA sequences to the reference genome (unpublished). To prepare other sequences needed to generate the phylogenetic trees based on 15 fish species, as well as human and chicken, the mRNA sequences of *dmrt1* and *cyp19a1* in 14 organisms were downloaded from National Center for Biotechnology Information (NCBI) and trimmed to include only coding sequences (CDS), including Asian seabass (*Lates calcarifer*), Atlantic salmon (*Salmo salar*), channel catfish (*Ictalurus punctatus*), chicken (*Gallus gallus*), common carp (*Cyprinus carpio*), European sea bass (*Dicentrarchus labrax*), giant grouper (*Epinephelus lanceolatus*), gray mullet (*Mugil cephalus*), human (*Homo sapiens*), medaka (*Oryzias latipes*), milkfish (*Chanos chanos*), Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*). Since the CDS of *dmrt1* and *cyp19a1* in crimson red snapper (*Lutjanus erythropterus*) and cobia (*Rachycentron canadum*) are not available in NCBI, the entire gene sequence (exons and introns) and/or cDNA sequences were downloaded and the exons of both genes were annotated by aligning the sequence to that of Malabar red snapper (accession number: crimson red snapper *dmrt1*, OQ735404 and *cyp19a1*, OQ735405; cobia *dmrt1*, OQ735403 and *cyp19a1*, OQ735406). The consensus phylogenetic trees and amino acid sequence alignment of *dmrt1* and *cyp19a1* were generated for 15 fish species, human and chicken using Geneious Prime 2023.0.1. The Tamura-Nei genetic distance model and neighbor-joining method were used to construct the phylogenetic trees with no outgroup selected. To assess the statistical support for each branch, the bootstrap resampling method with 1000 replicated and a minimum support threshold of 50% was utilized. All CDS of *dmrt1* and *cyp19a1* were translated and aligned using the MUSCLE algorithm implemented in Geneious Prime with default parameters.

3. Results

3.1. Histology and staging of Malabar red snapper gonads

Images of a representative male and female Malabar red snapper sampled in this study, together with their gonads and gonadal histological cross-sections are shown in Fig. 2. In the histological sections of testis, mainly spermatocytes and spermatids were observed with a relatively small number of spermatozoa present (Fig. 2g and h); in the sections of ovary, there were mainly previtellogenic oocytes and vitellogenic oocytes were <50% (Fig. 2e and f). Therefore, both male and female gonads were assessed to be in Stage III (mature) gonad development.

3.2. Sequencing data filtering and alignment to the reference genome

RNA-Seq of the 12 libraries representing 6 testis and 6 ovary samples generated 422,948,200 (63.44 Gb) and 420,796,142 (63.12 Gb) raw sequence reads for male and female Malabar red snapper, respectively (Supplementary Table S2). The raw sequencing data was submitted to the NCBI database's Sequence Read Archive (SRA) and can be accessed using the BioProject accession number PRJNA952938. After data filtering, a total of 420,536,608 (62.26 Gb) clean sequence reads were obtained for testes and 416,068,714 (61.11 Gb) clean reads were obtained for ovaries. The percentage of bases with Qphred quality scores higher than 20 (i.e., Q20) and 30 (i.e., Q30) ranged from 97.09% to 98.11% and 92.70% to 94.93% respectively, which is an indication of high quality. Filtered data were subsequently aligned to the reference genome (unpublished data) and the total mapped reads per sample varied from 89.60% to 92.04% (Supplementary Table S3). Visual inspection of mapped reads against CDS of genes with predicted alternative splicing events or differential exon usage as well as other sex-related DEGs showed no evident alternative splicing events within or between sexes in these genes.

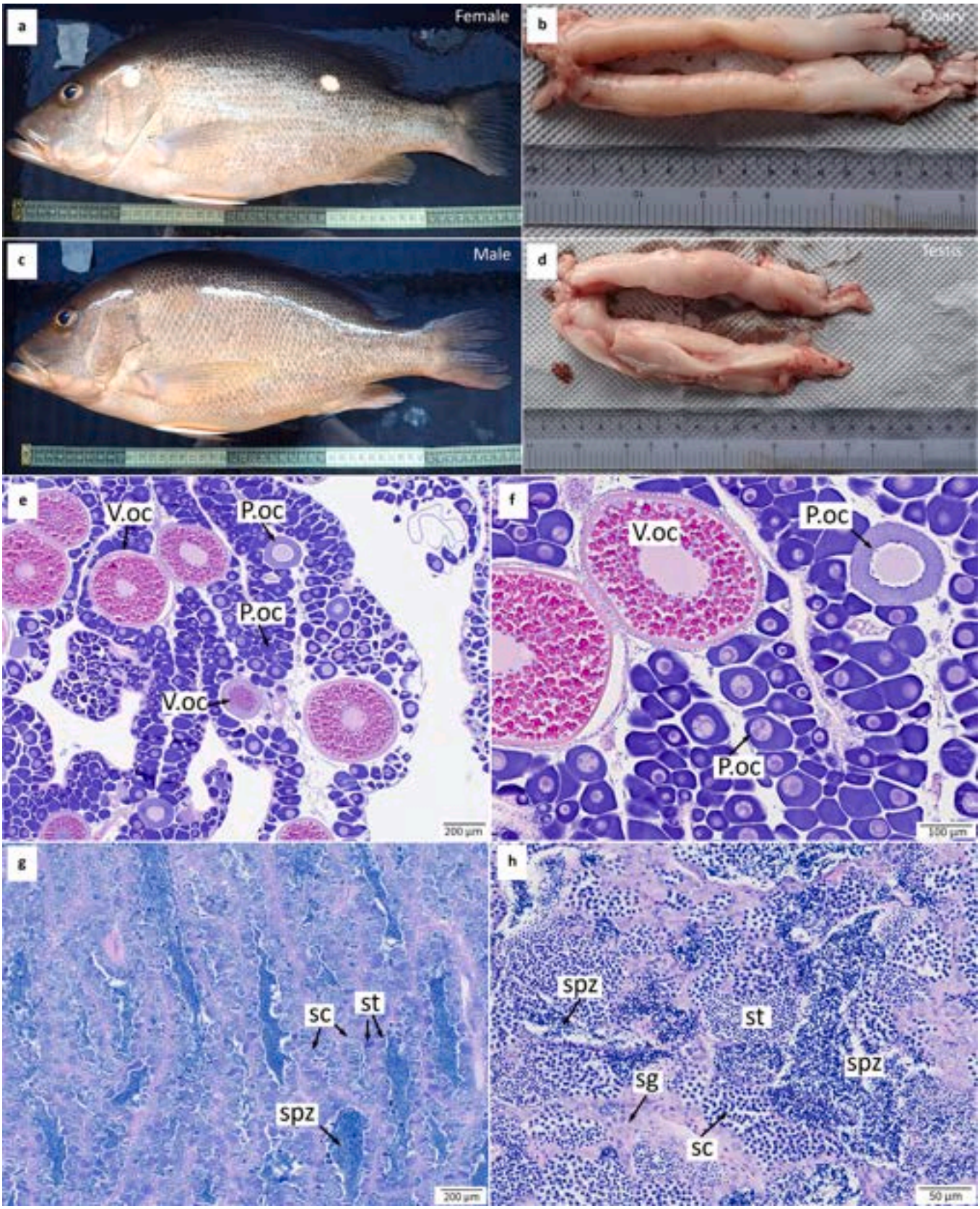


Fig. 2. Morphology and histology of representative Malabar red snapper ovary and testis.
(a) Female and (c) male red snapper and (b and d) their gonads; (e and f) histology of the ovary. P.oc = previtellogenic oocytes, V.oc = vitellogenic oocytes; (g and h) histology of the testis. sg = spermatogonia, sc = spermatocytes, st = spermatids, spz = spermatozoa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Determination of differentially expressed genes (DEGs) and correlation of samples

In total, 14,421 DEGs were identified by comparing the gene expression level of testes and ovaries according to the criteria of fold-change >2 and q-value <0.05. By comparing ovaries to testes, 6499 DEGs were upregulated and 7922 DEGs were downregulated (Fig. 3a). The heatmap generated using all the DEGs identified in ovaries and testes revealed that the transcriptomic profiles of testes was distinct compared to that of ovaries (Fig. 3b). A Pearson correlation was performed between each pair of samples. The correlation coefficients (*r*) of samples within the same sex were >0.85 (Fig. 3c), which indicates similar expression profiles among all biological replicates, including the two ovary samples with relatively low RIN value. In addition, Principal Component Analysis (PCA) showed that the testis and ovary samples clustered together with other samples within their respective gonad groupings (Fig. 3d). The *p* values for PC1 (= 49.6%) and PC2 (= 14.1%) were 1.91E-09 and 0.84 respectively, indicating that the gene expressions profiles in testes (observed in PCA quadrants I and II) and ovaries (observed in PCA quadrants III and IV) were significantly different.

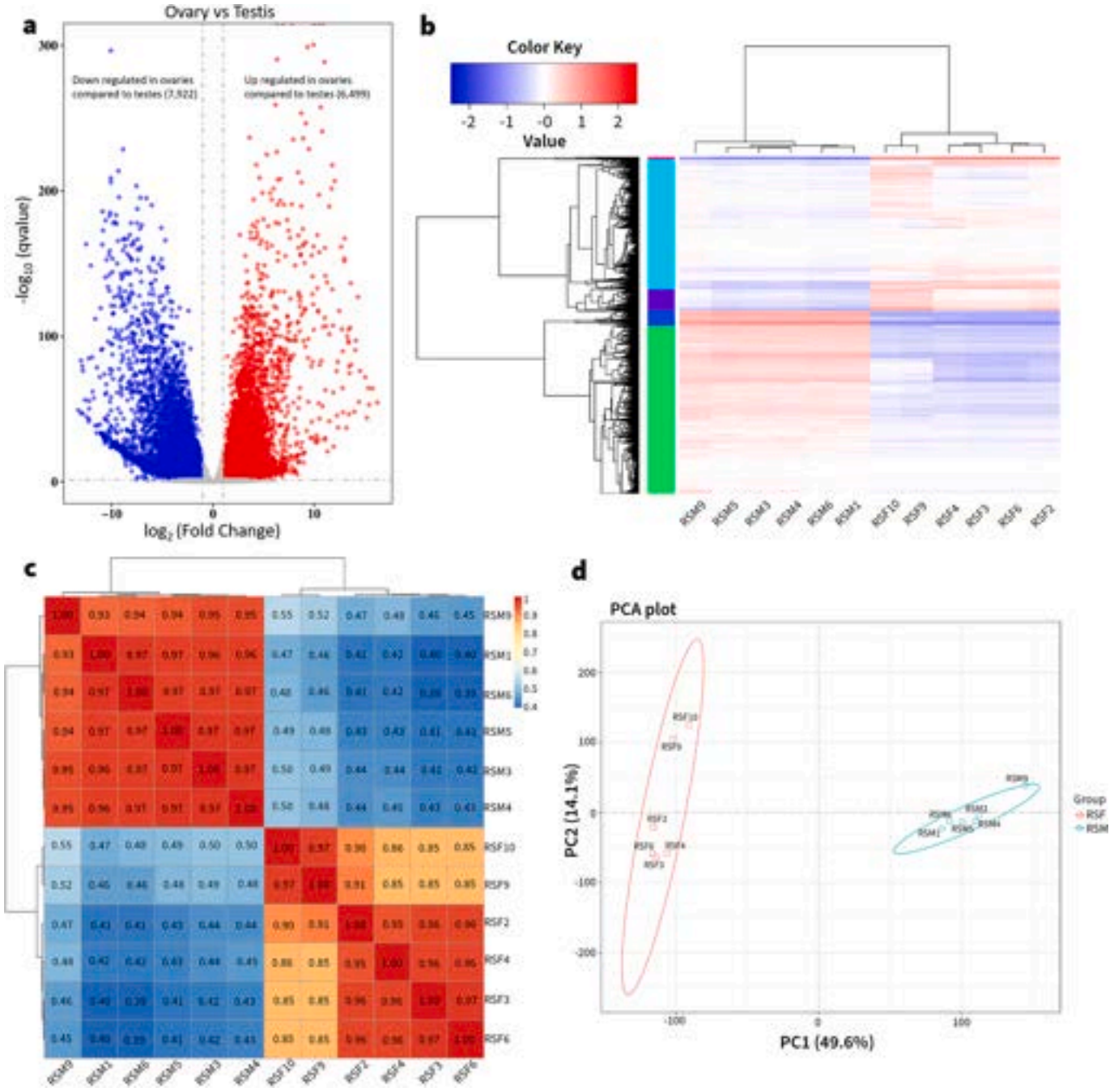


Fig. 3. Determination of DEGs and correlation of samples.
(a) Volcano plot of DEGs in ovaries versus testes; (b) Heatmap for cluster analysis of DEGs. Males (RSM1, 3, 4, 5, 6, 9) and females (RSF2, 3, 4, 6, 9, 10) are represented by columns and genes by rows. The colour blue indicates down-regulated gene expression while red indicates up-regulated expression; (c) Pearson correlation of gene expression between samples; (d) PCA plot of all 12 samples using two main principal components. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Gene Ontology (GO) and KEGG pathway analysis

GO and KEGG pathway enrichment analyses were performed for studying the functions of DEGs identified between testes and ovaries. A total of 41 GO terms were significantly enriched ($p < 0.05$) and categorized into molecular function, cellular component and biological process. The top three most prominent GO terms were integral component of membrane (GO:0016021), nucleus (GO:0005634) and DNA binding (GO:0003677) by the number of DEGs in each term; and structural constituent of ribosome (GO:0003735), translation (GO:0006412) and ribosome (GO:0005840) by p value (Fig. 4a and Supplementary Table S4). There were also several DEGs with sex- and reproduction-related GO terms, such as male sex determination (GO:0030238), ovarian follicle development (GO:0001541), germ cell development (GO:0007281) and spermatogenesis (GO:0007283). KEGG pathway enrichment analysis showed that 363 pathways were enriched, out of which nine were significant ($q < 0.05$) (Fig. 4b and Supplementary Table S5). The most prominent pathway was ribosome (ko03010), which is under Genetic Information Processing and Translation in BRITE functional hierarchies. The other most significantly enriched pathways included melanogenesis (ko04916), penicillin and cephalosporin biosynthesis (ko00311), monobactam biosynthesis (ko00261) and lysine biosynthesis (ko00300).

3.5. Analysis of representative DEGs involved in reproductive process of Malabar red snapper

Based on the enrichment analyses and available literatures, 66 DEGs involved in sex determination, sex differentiation, gonadal development and reproduction of teleosts were selected for further analysis (Table 1 & Fig. 5a), including 35 male-bias and 29 female-bias DEGs. These DEGs included genes known to be involved in reproductive processes such as *sry*-box containing proteins (*sox2, sox3, sox30, sox4, sox7, sox8, sox9, sox10 and sox11*), tektins (*tekt1, tekt2, tekt3 and tekt4*), spermatogenesis-associated proteins (*spata1*, *spata2*, *spata4*, *spata6* and *spata7*), synaptonemal complex proteins (*sycp2* and *sycp3*), piwi-like proteins (*piwil1* and *piwil2*), Cytochrome P450 family members (*cyp11b*, *cyp19a1*, *cyp1a1*, *cyp1b1*, *cyp2d15*, *cyp2j2* and *cyp3a40*), wnt signalling components (*wnt1*, *wnt3a*, *wnt4a*, *wnt5a*, *wnt5b*, *wnt8a* and *wnt8b*), zona pellucida sperm-binding proteins (*zp1*, *zp2*, *zp3* and *zp4*), outer dense fiber proteins (*odf2* and *odf3b*), ATP-dependent RNA helicases (*ddx4*, *ddx5* and *ddx17*) etc. Protein-protein interaction network of these DEGs is shown in Fig. 6.

3.6. RT-qPCR validation of selected DEGs

The expression profiles of eight male-biased genes (*ddx4*, *piwil1*, *sox9*, *piwil2*, *brdt*, *spata7*, *dmrt1* and *odf3b*) and eight female-biased genes (*zp3*, *cyp19a1*, *sox3*, *bmp15*, *hsd17b12a*, *figla*, *cyp1b1* and *cyp26a*) were further evaluated by RT-qPCR to validate the relative expression levels obtained by transcriptomic dataset, and to provide a species-specific molecular resource for L. *malabaricus*. The evaluation of stabilities of the four reference genes (i.e., *actb*, *ef1a*, *rpl8* and *gapdh*) can be found in Supplementary Fig. S6. The RNA-Seq and RT-qPCR results showed a strong positive correlation (Pearson correlation coefficient $r = 0.97$, $p =$ 1.4E-10) in expression profiles of all eight male-biased DEGs and seven female-biased DEGs (with the exception of *cyp1b1*, Fig. 5b and c), which suggested the validity of the comparative transcriptomic analysis for these sex related DEGs.

3.7. Characterization of dmrt1 and cyp19a1 genes

The RT-PCR amplification of *dmrt1* and *cyp19a1* produced a single amplicon with expected size and sequence for *dmrt1* in testis and *cyp19a1* in ovary respectively, indicating that no alternative splicing was detected in both genes. Mapping the mRNA of *dmrt1* and *cyp19a1* of Malabar red snapper to the reference genome revealed that there were five exons in *dmrt1* and nine exons in *cyp19a1* (Supplementary Fig. S7a and b), and the length of the CDS is 909 bp for *dmrt1* and 1560 bp for *cyp19a1* (GenBank accession numbers: *dmrt1*, OQ735401 and *cyp19a1*, OQ735402). According to the phylogenetic trees for Malabar red snapper and 14 other fish species, both *drmt1* and *cyp19a1* had the closest genetic distance to crimson red snapper, followed by European seabass and giant grouper (Supplementary Fig. S7c and d). The amino acid sequence alignment of *dmrt1* and *cyp19a1* in Malabar red snapper and 14 other fish species are shown in Supplementary Fig. S7e and f. The

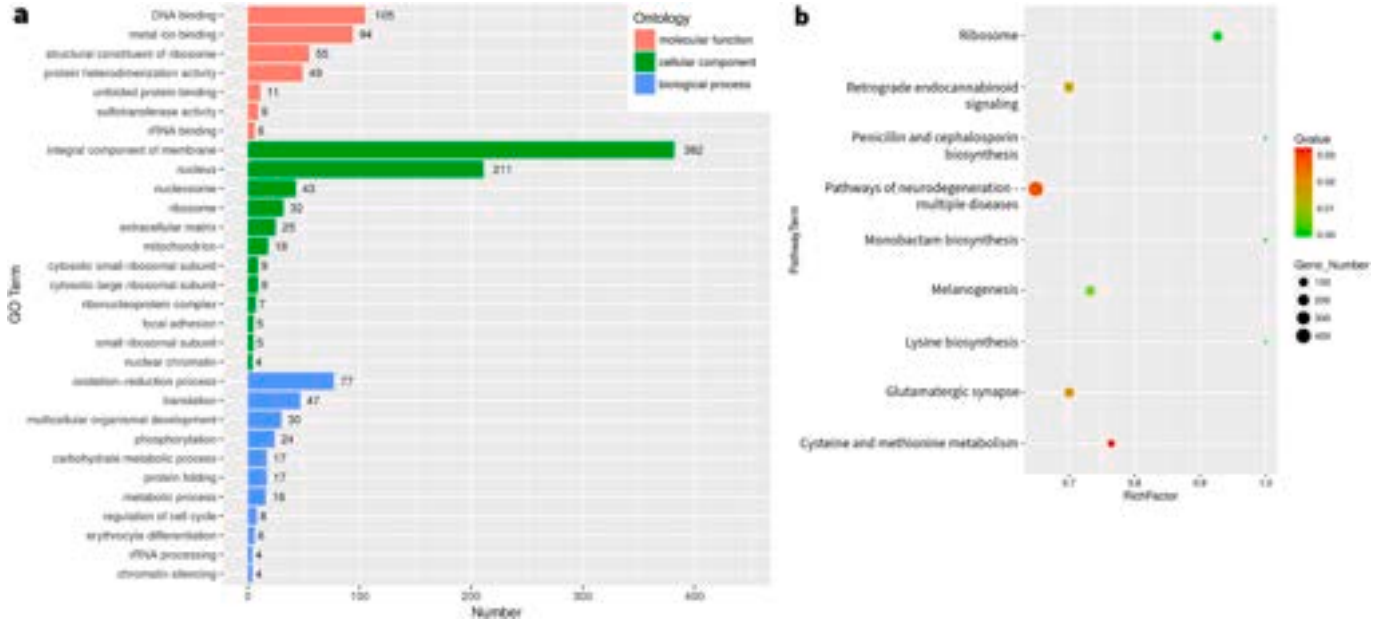


Fig. 4. GO and KEGG enrichment analysis between testes and ovaries.
(a) top 30 significantly enriched GO terms, X axis represents the number of DEGs within each GO term and Y axis specifies enriched GO terms; (b) significantly enriched KEGG pathways, X axis represents Rich Factor and Y axis specifies KEGG pathways. The size of the dot is proportional to the number of DEGs present in the pathway. The colours represent different q-values.

Table 1
Candidate sex-associated DEGs identified in the gonads of Malabar red snapper.

Gene Name	Gene Description	log2FoldChange	padj	Sex-bias	Gene ID
sox30	sry-box containing protein 30	−12.04	2.1E-149	Male	Scaffold_11.g13211
tekt1	tektin-1	−10.62	3.0E-141	Male	Scaffold_18.g21373
odf3b	outer dense fiber protein 3	−10.23	6.2E-143	Male	Scaffold_4.g04315
tekt2	tektin-2	−10.02	7.6E-122	Male	Scaffold_15.g18118
spata4	spermatogenesis-associated protein 4	−9.90	3.4E-88	Male	Scaffold_5.g05612
sycp2	synaptonemal complex protein 2	−8.44	1.8E-157	Male	Scaffold_4.g04569
tekt4	tektin-4	−8.35	6.3E-18	Male	Scaffold_3.g02904
klhl10	Kelch-Like Protein 10	−7.71	1.1E-62	Male	Scaffold_15.g18161
dmrt1	doublesex- and mab-3-related transcription factor 1	−6.86	4.3E-90	Male	Scaffold_10.g11930
spata7	spermatogenesis-associated protein 7	−5.12	7.0E-39	Male	Scaffold_23.g26116
cyp11b	Cytochrome P450 11b	−4.47	5.0E-06	Male	Scaffold_6.g07199
wnt3a	Wnt Family Member 3a	−4.17	3.0E-04	Male	Scaffold_13.g15633
sox10	sry-box containing protein 10	−3.92	2.0E-04	Male	Scaffold_3.g03341
rsph10b	radial spoke head 10 homolog B	−3.91	4.0E-32	Male	Scaffold_19.g22631
odf2	outer dense fiber protein 2	−3.82	5.1E-71	Male	Scaffold_18.g20983
cyp2d15	Cytochrome P450 2d15-like	−3.79	9.2E-08	Male	Scaffold_12.g13913
tekt3	tektin-3	−3.67	1.1E-06	Male	Scaffold_19.g22157
sycp3	synaptonemal complex protein 3	−3.65	9.2E-26	Male	Scaffold_12.g14987
brdt	bromodomain testis-specific protein	−3.54	2.5E-32	Male	Scaffold_7.g08053
sox9	sry-box containing protein 9	−3.54	1.1E-16	Male	Scaffold_17.g20305
wnt1	Wnt Family Member 1	−3.31	5.16E-03	Male	Scaffold_4.g04797
wnt8a	Wnt Family Member 8a	−3.24	6.6E-03	Male	Scaffold_5.g06340
bmp7	bone morphogenetic protein 7-like	−2.88	2.3E-03	Male	Scaffold_2.g02233
wnt5b	Wnt Family Member 5b	−2.86	2.7E-18	Male	Scaffold_21.g24097
sox2	sry-box containing protein 2	−2.67	5.8E-03	Male	Scaffold_7.g08884
zp1	zona pellucida sperm-binding protein 1	−2.61	3.9E-02	Male	Scaffold_12.g14255
wnt8b	Wnt Family Member 8b	−2.34	3.8E-02	Male	Scaffold_16.g19009
amh	anti-müllerian hormone	−2.11	1.7E-02	Male	Scaffold_7.g08366
piwil2	Piwi-like protein 2	−2.01	5.4E-17	Male	Scaffold_10.g12478
cyp2j2	Cytochrome P450 2j6-like	−1.98	7.0E-03	Male	Scaffold_7.g08277
piwil1	Piwi-like protein 1	−1.82	2.3E-09	Male	Scaffold_10.g11638
spata6	spermatogenesis-associated protein 6	−1.75	6.8E-17	Male	Scaffold_7.g08742
ddx17	probable ATP-dependent RNA helicase ddx17	−1.67	3.5E-25	Male	Scaffold_17.g20012
cyp1a1	Cytochrome P450 1a1	−1.55	3.2E-02	Male	Scaffold_1.g00962
ddx5	probable ATP-dependent RNA helicase ddx5	−1.42	6.4E-26	Male	Scaffold_3.g02765
ddx4	probable ATP-dependent RNA helicase ddx4	−1.26	1.8E-03	Male	Scaffold_24.g26804
dmrta1	doublesex- and mab-3-related transcription factor a1	1.21	3.1E-05	Female	Scaffold_22.g24893
wt1-a	Wilms tumor protein homolog A	1.22	9.1E-03	Female	Scaffold_1.g00667
esr1	estrogen receptor 1	1.48	2.9E-02	Female	Scaffold_14.g16670
sox7	sry-box containing protein 7	1.51	1.0E-02	Female	Scaffold_14.g16882
spata2	spermatogenesis-associated protein 2	1.56	3.4E-04	Female	Scaffold_16.g18630
nr5a2	nuclear receptor subfamily 5 group A member 2	1.69	3.5E-03	Female	Scaffold_10.g12675
sox11	sry-box containing protein 11	1.74	1.1E-14	Female	Scaffold_14.g16358
sox8	sry-box containing protein 8	2.29	3.2E-03	Female	Scaffold_17.g20298
nr0b1	nuclear receptor subfamily 0 group B member 1	2.86	6.0E-17	Female	Scaffold_9.g10799
esr2	estrogen receptor 2b	3.16	3.2E-24	Female	Scaffold_23.g25717
wnt4a	Wnt Family Member 4a	3.29	1.0E-10	Female	Scaffold_2.g02363
sox4	sry-box containing protein 4	3.32	6.0E-22	Female	Scaffold_6.g06904
wt1	Wilms tumor protein homolog	3.54	5.9E-15	Female	Scaffold_12.g13968
gdf9	growth/differentiation factor 9	3.60	2.4E-34	Female	Scaffold_5.g05832
emx2	homeobox protein emx2	3.99	2.4E-11	Female	Scaffold_16.g19185
cyp26a1	Cytochrome P450 26A1	4.66	3.8E-41	Female	Scaffold_19.g21931
cyp1b1	Cytochrome P450 1b1-like	4.77	3.1E-08	Female	Scaffold_23.g26399
figla	factor in the germline alpha	4.83	1.3E-97	Female	Scaffold_10.g12522
spata1	spermatogenesis-associated protein 1	5.61	4.4E-98	Female	Scaffold_7.g08651
hsd17b12a	very-long-chain 3-oxoacyl-CoA reductase-A	6.04	3.8E-36	Female	Scaffold_12.g14850
wnt5a	Wnt Family Member 5a	6.41	9.0E-53	Female	Scaffold_4.g04715
bmp15	bone morphogenetic protein 15	6.76	3.4E-49	Female	Scaffold_22.g25198
foxl2	forkhead box protein l2a	7.20	1.7E-35	Female	Scaffold_8.g09257
cyp3a40	Cytochrome P450 3a40	9.03	4.7E-21	Female	Scaffold_4.g04709
sox3	sry-box containing protein 3	9.18	4.4E-247	Female	Scaffold_5.g06133
zar1	zygote arrest protein 1	9.75	2.6E-156	Female	Scaffold_7.g08246
cyp19a1	Cytochrome P450 19a1	9.81	1.0E-30	Female	Scaffold_1.g00327
zp2	zona pellucida sperm-binding protein 2	12.82	2.4E-90	Female	Scaffold_14.g16737
zp3	zona pellucida sperm-binding protein 3	12.98791032	3.38E-154	Female	Scaffold_17.g20177
zp4	zona pellucida sperm-binding protein 4	13.56	8.4E-107	Female	Scaffold_13.g15527

dmrt1 amino acid sequence in Malabar red snapper exhibits the highest similarity to that of crimson red snapper (100%), followed by cobia (80%) and European seabass (77.7%). Similarly, the *cyp19a1* amino acid sequence in Malabar red snapper displays the highest similarity to that of crimson red snapper (99.6%), followed by European seabass (91.1%) and giant grouper (90.4%).

4. Discussion

Sex determination and differentiation is a broad research topic which studies the developmental processes by which an undifferentiated zygote develops down a male or female pathway. Understanding of sex determination and differentiation mechanisms in fish could benefit

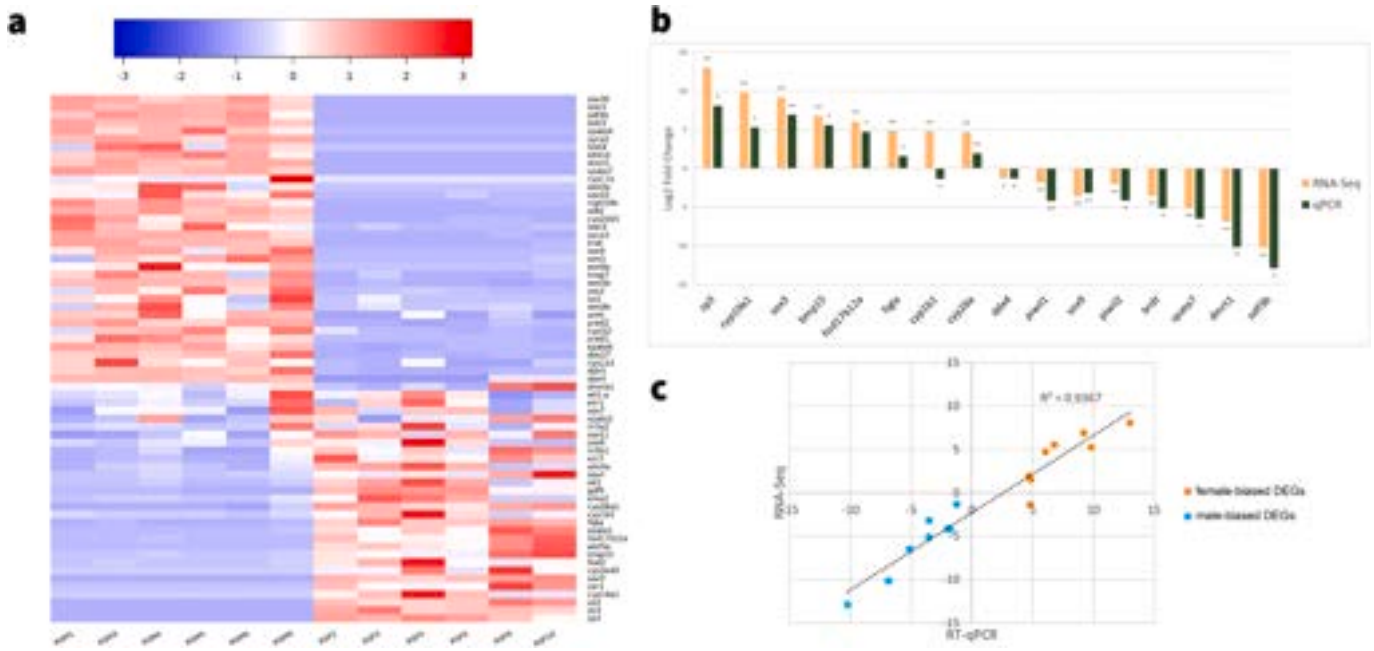


Fig. 5. Representative DEGs involved in reproductive processes of Malabar red snapper.
(a) heatmap of 66 representative DEGs in males (RSM1, 3, 4, 5, 6, 9) and females (RSF2, 3, 4, 6, 9, 10); (b) RT-qPCR validation of selected DEGs, * indicates $P < 0.01$ and ** indicates $P < 0.001$; (c) correlation between qPCR and RNA-Seq results. The numbers in both x and y axis represent log2FoldChange values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Protein-protein interaction network of candidate sex-associated DEGs in Malabar red snapper.
Orange nodes represent female-biased DEGs and blue nodes represent male-biased DEGs. Edges represent associations between nodes, and line thickness indicates the confidence of associations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aquaculture production via better broodstock management, prevention of unwanted reproduction to avoid waste of energy in fish, and sex-specific production if the farmed species exhibits sexual growth dimorphism (Wang, 2019). The present study reports a comparative transcriptomic analysis of ovary and testes in adult Malabar red snapper. The primary objective of this study was to identify DEGs between gonadal type of the Malabar red snapper and to provide fundamental information for further study on the molecular mechanisms underlying sex-specific gonadal function and reproductive biology. The transcriptomic analysis reported herein revealed a total of 14,421 significant DEGs, of which 6499 genes were upregulated in ovaries and 7922 genes upregulated in testes. DEGs known to play a crucial role in the gonadal development, sex differentiation/determination and reproduction in vertebrates were identified for Malabar red snapper. Further, RT-qPCR of 16 key DEGs were developed and has become a valuable resource for future L. *malabaricus* research, which can be used in further developmental studies to allow for a greater understanding of their reproductive biology such as the exact timing of their sex differentiation and gonadal maturation.

The GO and KEGG analysis of DEGs revealed that several biological processes, molecular functions and genetic pathways were enriched in female and male gonads, with ribosome being the most enriched GO term and KEGG pathway. Significant enrichment in ribosome related terms and pathways is probably because the formation, differentiation, and function of germ cells across species is controlled by stage-specific regulation of mRNA translation at both individual transcript and global levels, and it is necessary for oocytes to generate and accumulate a suitable quantity of ribosomes to sustain the development of the early embryo until zygotic transcription begins (Marianne et al., 2021). In the Protein-protein interaction network (Fig. 6), several DEGs showed significant association with other sex-related genes, such as male-biased *dmrt1*, *amh* and *cyp11b*, and female-biased *cyp19a1*, *foxl2*, *gdf9* and *bmp15*. These genes could be ideal candidates for further studies to reveal the underlying mechanisms of the sex determination and differentiation. For instance, the DEGs' protein locations and functions in gonads could be investigated using technologies such as protein electrophoresis, western blotting and protein mass spectrometry.

In previous studies of sex differentiation/determination systems in vertebrates, SRY/*sry* has been identified as the sex-determining gene for males in most mammals, but no corresponding homolog has been detected in other species (Herpin and Schartl, 2015). However, many downstream genes and pathways are conserved in vertebrate species. For example, *dmrt1Yb* has been identified as the master sex determining gene in Japanese Medaka (Nagahama et al., 2002). Male-restricted or

male-biased *dmrt1* expression was also reported in a considerable number of fish species, including North African catfish, rare minnow, Nile tilapia, medaka, olive flounder, lake sturgeon, zebrafish, Atlantic cod, pejerrey, Rainbow trout, shovelnose sturgeon and southern catfish with functions related to male gonadogenesis and further differentiation (Herpin and Schartl, 2011). In the present study, male-biased expression pattern of *dmrt1* (log2FC = −6.9) was observed in Malabar red snapper, suggesting that *dmrt1* is also likely involved in male gonadogenesis, differentiation and maintenance as reported in the other teleost species (Herpin and Schartl, 2011).

Cyp19a1 encodes aromatase which convert androgens into estrogens. *Cyp19a1* is known to be involved in the differentiation and development of the ovary in other finfish species such as Asian seabass (Guiguen et al., 2010; Domingos et al., 2018) and salmon (Piferrer et al., 1994), and plays a crucial role in sex determination (Omura, 1999; Zheng et al., 2019). For example, *cyp19a1a* and *bmp15* were reported to be necessary for the maintenance of the female gonad in zebrafish based on the findings that mutation in either gene could cause fish to develop as male (Dranow et al., 2016). In the present study, females showed higher expression levels of both *cyp19a1* and *bmp15* in comparison to males, with log2FC values of 9.8 and 6.8, respectively, indicating both genes played significant roles in ovarian development and sex maintenance of Malabar red snapper. Another seven CYP gene members were also identified in this study, including male-biased *cyp11b*, *cyp1a1*, *cyp2d15*, *cyp2j2*, and female-biased *cyp1b1*, *cyp3a40* and *cyp26a1*. The CYP gene superfamily encompasses numerous genes that encode P450 enzymes, which play crucial roles in metabolizing exogenous substances such as drugs and chemical carcinogens, environmental contaminants, as well as endogenous substrates such as vitamins, steroids, fatty acids, and prostanoids, and in the detoxification of the aforementioned exogenous chemicals (Uno et al., 2012). For instance, *cyp11b* encodes steroid 11-beta-hydroxylase, which is involved in the synthesis of 11-ketotestosterone, a spermatogenesis-inducing hormone (Jiang et al., 1996). Male-biased expression pattern of *cyp11b* was also reported in many other fish species such as zebrafish (Wang and Orban, 2007), rainbow trout (Baron et al., 2005), Japanese flounder (Meng et al., 2020), European sea bass (Rocha et al., 2009), and midshipman fish *Porichthys notatus* (Arterbery et al., 2010), and in early testicular development of the protandrous hermaphodite Asian seabass (Banh et al., 2017). This is consistent with the higher expression level of *cyp11b* in the testis of Malabar red snapper compared to the ovary, which revealed its importance in testis development and spermatogenesis in Malabar red snapper. The bone morphogenetic protein (BMP) family bolong to the transforming growth factor-beta (TGF-β) superfamily, which is known to have an impact on reproduction of various organisms (Monsivais et al., 2017). According to the research by Ogino et al. (2014), *bmp7* contributes to the development of papillary processes, which are male secondary sex characteristics in medaka. Similarly, our study showed that *bmp7* was overexpressed in the testis (log2FC = −2.88) of Malabar red snapper. However, Baron et al. (2005) found that *bmp7* was involved in ovary development in rainbow trout. Therefore, it is possible that *bmp7* plays a regulatory role in the development of both male and female gonads in teleost species. Another member of the TGF-β superfamily, *gdf9*, was found upregulated in the Malabar red snapper ovaries (log2FC = 3.6), which agrees with its female-biased expression in other species such as Chinese tongue sole (Shi et al., 2022) and zebrafish (Chen et al., 2022).

In teleosts, *dmrt1* and *cyp19a1* are two of the extensively investigated sex-biased genes which are prominently involved in male and female sex determination and differentiation respectively (Rajendiran et al., 2021). Therefore, the introns and exons of *dmrt1* and *cyp19a1* were annotated for L. *malabaricus* and *L. erythropterus* in this study. It is worth noting that the Intron 7 of *cyp19a1* in both species starts with the dinucleotide "GC" instead of the canonical "GT". The GC-AG introns are rare and non-canonical splice site combinations, only represent 0.7% of introns in human (Burset et al., 2000), 0.6% in *Caenorhabditis elegans* (Farrer et al., 2002), 1.2% in plants (Pucker and Brockington, 2018), and 1.0–1.2% in fungi (Rep et al., 2006). The GC-AG introns were reported to be associated with long non-coding RNAs and protein-coding genes, and it may play a role in gene expression regulation (Abou Alezz et al., 2020). Based on the alignment of the deduced amino acid sequences of *dmrt1* and *cyp19a1*, high similarity among 17 selected vertebrate species were observed (Supplementary Fig. S7e & f), which agrees with the view that both *dmrt1* and *cyp19a1* genes are highly conserved across vertebrates and even invertebrate species (Herpin and Schartl, 2015; Callard et al., 2011). In the phylogenetic trees of *dmrt1* and *cyp19a1* (Supplementary Fig. S7c and d), it was as expected that Malabar red snapper fell into the same clade as its sister-species, the crimson red snapper which also belongs to the *Lutjanidae*; however, it was striking that the similarities of the deduced amino acid sequences between the two species were 100% for *dmrt1* and 99.6% for *cyp19a1*, respectively. Both Malabar and crimson red snapper are commercially important species in the Indo-Pacific region. The morphology of the two species is remarkably alike in appearance, especially in their early life stages; thus, it is challenging to differentiate them, and they are generally referred to as "redfish" or "red snapper" (McPherson et al., 1992; Fry et al., 2009), which may be explained by the particularly high similarities in their genomes. In addition, the *dmrt1* and *cyp19a1* of Malabar red snapper also had close genetic relationship to other gonochoristic species such as European seabass and cobia, whose gonad development systems are better studied (Ribas et al., 2019; Geffroy et al., 2021; Soloperto et al., 2022; Shen et al., 2023; Ma et al., 2022). Such knowledge from closely related species could provide valuable reference and insights for further investigation of sex determination and differentiation in Malabar red snapper.

Several DEGs from the *Sox* gene family were identified in this study. *Sox* genes are a group of transcription factors which play crucial roles during reproduction and gonad development in many fish species. For example, *Sox3* was speculated to be the evolutionary precursor of *Sry* (Herpin and Schartl, 2015) and identified as the master sex-determining genes in Indian ricefish *Oryzias dancena* (Takehana et al., 2014). The known functions of *sox* genes were mainly related to sex determination, differentiation and gonadal development in fish (Hu et al., 2021). In the present study, *sox* genes were found to be expressed in both sexes, with *sox2*, *sox9*, *sox10* and *sox30* being upregulated in testis and *sox3*, *sox4*, *sox7*, *sox8* and *sox11* being upregulated in ovary. These findings suggest that *sox* genes also play a significant role in the development and differentiation of gonads in both male and female Malabar red snapper.

The canonical Wnt signalling pathway plays a key role in the regulation of various developmental processes, including in fish sex determination and differentiation (Wu and Chang, 2009; Nicol and Guiguen, 2011). Several DEGs from the Wnt family were revealed in this study, including male-biased *wnt1*, *wnt3a*, *wnt5b*, *wnt8a* and *wnt8b*, as well as female-biased *wnt4a* and *wnt5a*. *Wnt4* plays a pivotal role in ovarian differentiation in mammals as *Wnt-4*-mutant females were masculinized (McMahon et al., 1999). Ovary-predominant *wnt4* expression during gonadal differentiation was also found in black porgy (Wu and Chang, 2009), but not in rainbow trout (Nicol et al., 2012). Furthermore, the differentiation of Sertoli cells was impaired in the testes of *wnt4* mutant mice, indicating *Wnt4* has a significant function in the development of the mammalian testis as well (Jeays-Ward et al., 2004). In this study, *wnt4a* was up regulated in the ovaries of Malabar red snapper (log2FC = 3.29), making it an ideal candidate gene to study the Wnt signalling pathway in ovary differentiation in Malabar red snapper.

There were also a few groups of DEGs which play specific functions in reproduction. For example, *tekt1*, *tekt2*, *tekt3* and *tekt4* encode Tektins which played a part in the development of sperm flagella and/or sperm mobility (Iguchi et al., 2002). Zona pellucida sperm-binding proteins *zp2*, *zp3* and *zp4* were highly expressed in ovaries which suggested that they could play an important role in egg envelope hardening at fertilization, protecting embryos, and facilitating embryo hatching as reported in other teleosts (Sano et al., 2022) In contrast, *spata4*, *spata6* and *spata7* which were highly expressed in testes could be crucial for spermatogenesis in males as reported in other vertebrates, including fish

(Agarwal et al., 2020). Some other sex-specific genes include estrogen receptors (*esr1* and *esr2*), Piwi-like proteins (*piwil1* and *piwil2*) which are required for spermatogenesis (Litwack, 2018), and *zar1* which is a gene expressed in the ovaries and has been conserved throughout evolution in vertebrates (Wu et al., 2003).

5. Conclusions

This study details the first transcriptome and comparative gene expression profiles in female and male gonads in adult Malabar red snapper. The information of DEGs and candidate sex-associated genes is valuable for further research on gene functions and networks related to gonadal development, sex determination and differentiation in Malabar red snapper and could be used for the broader Lutjanidae family as a whole. Our findings also lay the foundation for further studies to understand fish spawning and sex control such as establishing a balanced sex ratio for broodstock management, prevention of precocious maturation, or production of monosex populations when a certain sex is more valuable, as it is the case of faster growing females. One future research area could be comparison of differential gene expression in gonads of different stages, or gonads under various hormonal / environmental treatments, which could help to reveal more details in the mechanisms of sex differentiation in Malabar red snapper.

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Credit authorship contribution statement

Bing Liang: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Dean R. Jerry:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Xueyan Shen:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Joyce Koh:** Investigation, Writing – review & editing. **Celestine Terence:** Investigation, Writing – review & editing. **Maria G. Nayfa:** Writing – review & editing. **Vu Nguyen:** Writing – review & editing. **Grace Loo:** Writing – review & editing, Funding acquisition. **Shubha Vij:** Writing – review & editing, Funding acquisition. **Jose A. Domingos:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

Data availability

The raw reads used in this article have been deposited into the Sequence Read Archive (SRA) of the NCBI database under BioProject accession number: PRJNA952938.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741258.

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