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Gonadal transcriptome analysis of the common carp, *Cyprinus carpio*: Identification of differentially expressed genes and SSRs☆

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ABSTRACT

Common carp (*Cyprinus carpio*) is a world-wide freshwater fish of eutrophic waters. *C. carpio*, have various reproductive traits, including early sexual maturity, that may make them excellent, large, realistic, aquaculture model species. In the present work, *de novo* assembly of gonadal (testicular and ovarian) transcriptomes from juvenile common carp was performed to identify genes involved in gonadal development. A total of 81,757 and 43,257 transcripts with average lengths of 769 and 856 bp, were obtained from the immature testicular and ovarian transcriptomes, respectively. About 84,367 unigenes were constructed after removing redundancy involving representation of transcripts in both gonadal transcriptomes. Gene ontology (39,171 unigenes), clusters of orthologous group's analysis (6651 unigenes) and Kyoto encyclopedia of genes, and genomes automatic annotation server analysis (4783 unigenes) were performed to identify potential genes along with their functions. Furthermore, 18,342 (testis) and 8693 (ovary) simple sequence repeats were identified. About 298 differentially expressed genes were identified, of which 171 and 127 genes were up-regulated in testis and ovary, respectively. Quantitative real-time reverse transcription PCR was performed to validate differential expression of selected genes in testis and ovary. Nearly 809 genes related to reproduction were identified, sex-wise expression pattern of genes related to steroid synthesis, endocrine regulation, germ cell maintenance and others

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Abbreviations: ad4bp/sf-1, adrenal 4 binding protein/steroidogenic factor-1; *amh*, anti-Mullerian hormone; *amhr2*, anti-Mullerian hormone receptor type 2; *amhy*, Y chromosome-linked anti-Mullerian hormone; *ar*, androgen receptor; *atm*, Ataxia telanglectasia mutated; COG, clusters of orthologous groups; Ct, cycle threshold; *ctnnbip1*, catenin beta interacting protein 1; *cux1*, cut like homeobox 1; *cux2a*, cut like homeobox 2a; *cyp11b1*, cytochrome P450, family 11, subfamily b, polypeptide 1; *cyp17*, steroidogenic cytochrome P450 17-hydroxylase/lyase; *cyp19a1*, cytochrome P450, family 19, subfamily a, polypeptide 1; *cxcl12*, C-X-C motif chemokine ligand 12; *cxcr4*, C-X-C motif chemokine receptor 4; *dax1*, dosage-sensitive sex-reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; DEG, differentially expressed genes; *dmrt1*, doublesex and mab-3 related transcription factor 1; *DMY*, Y-specific DM-domain; dph, days post hatch; *egr2b*, early growth response 2b; *er*, estrogen receptor; *fam101b*, family with sequence similarity 101, member B; *fam192a*, family with sequence similarity 192, member A; *fam210b*, family with sequence similarity 210, member B; *fbox43*, F-box only protein 43; *fem1b*, fem-1 homolog B; *fgfr1a2*, fibroblast growth factor receptor 1-A-like; *foxk2*, forkhead box k2; *foxl2*, forkhead box L2; FSH, follicle stimulating hormone; *fshr*, follicle stimulating hormone receptor; *fstl3*, follistatin-like 3 (secreted glycoprotein); *fzd8*, frizzled class receptor 8; *gata4*, GATA binding protein 4; *gata6*, GATA binding protein 6; *gdf9*, growth differentiation factor 9; GnRH, gonadotropin-releasing hormone; GO, gene ontology; *gsdf*, gonadal somatic cell-derived factor; *hsd3b*, hydroxy-delta-5-steroid dehydrogenase, 3 beta; *hsd3b7*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7 *hsd11b1* hydroxysteroid 11-beta dehydrogenase 1; *hsd11b2*, hydroxysteroid 11- beta dehydrogenase 2; *hsd17b2*, hydroxysteroid 17- beta dehydrogenase 2; *hsd20b2*, hydroxysteroid 20- beta dehydrogenase 2; *igf1ra*, insulin-like growth factor 1a receptor; *ipo4*, importin 4; KASS, KEGG, automatic annotation server; KEGG, Kyoto encyclopedia of genes and genomes; *kiss2*, kisspeptin2; *lhcgr*, luteinizing hormone/choriogonadotropin receptor; *mapk*, mitogen-activated protein kinase; *mis*, Mullerian inhibiting substance; *msl1b*, male-specific lethal 1 homolog b; NGS, next generation sequencing; *pcna*, proliferating cell nuclear antigen; *plzf*, promyelocytic leukemia zinc finger; *prlhr2a*, prolactin releasing hormone receptor 2a; PPA, protein phosphatase 2A; *pou5f1*, POU class 5 homeobox 1; qRT-PCR, quantitative real time reverse transcription PCR; RA, retinoic acid; RAR-RXR, retinoic acid receptor/retinoid x receptor heterodimers; *rspo1*, R-spondin 1; sdY, *sexually dimorphic on the Y chromosome*; *smad3b*, SMAD family member 3b; *sox9a*, SRY-box 9a; *SRY*, sex-determining region Y; SSR, simple sequence repeat; *StAR*, steriodogenic acute regulatory protein; *sycp1*, synaptonemal complex protein 1; *sycp3*, synaptonemal complex protein 3; *tac3a*, tachykinin 3a; *tcf3a*, transcription factor 3a; *TGF-β*, transforming growth factor-beta; *wnt*, wingless-type MMTV integration site family; *wt1*, Wilms tumor 1; *zar1*, zygote arrest 1; *zp2*, zona pellucida sperm-binding protein 2

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factors related to gonadal differentiation was observed, and expression analysis of *nanos*, *ad4bp/sf-1,* and *gdf9* was performed. The present study identified certain important genes/factors involved in the gonadal development of *C. carpio* which may provide insights into the understanding of sex-differentiation and gonadal development processes.

1. Introduction

The common carp, *Cyprinus carpio* belonging to family Cyprinidae is native to western Asia and was translocated or introduced throughout the world. Certain native wild populations were considered vulnerable to extinction in 2008 by the International Union for Conservation of Nature. The common carp has become one of the most important food fishes with many strains and varieties in different parts of the world (Xu et al., 2012). There are abundant strains and local populations of common carp found in India, including many hybrid populations. The Indian common carp spawn in two peak breeding periods, from January to March and from July to August. Furthermore, at different stages, the gonads show discrete changes during the reproductive cycle. Due to its economic and ecological importance, different genetic and genomic studies were performed in the common carp (Ji et al., 2012; Kongchum et al., 2010). In spite of the reports being available on different aspects of reproduction and breeding (Routray et al., 2007), the molecular mechanisms underlying gonadal maturation and breeding of carp in tropical or subtropical climates has not been clearly understood. In comparison with the bisexual reproduction in higher vertebrates, teleosts have different approaches for sexual reproduction, like unisexuality (Schartl et al., 1995), hermaphroditism (Warner, 1984) and gonochorism (Dipper and Pullin, 1979). In addition, there are two mechanisms of sex-determination, genetic and environmentally regulated (Janzen, 1995). Teleost fishes are an excellent model for studying the evolution of sex chromosomes as they have a broad range of sexual developmental systems, with an absence of morphologically distinct genders in most species. A master set of sex-determination and gonadal phenotype-related genes have been identified in multiple fish species, including, *amhy* in *Odontesthes hatcheri*, *amhr2* in *Takifugu rubripes*, *dmrt1* in *Cynoglossus semilaevis* and *Clarias gariepinus, DMY* and *dmrt1* in *Oryzias latipes*, *gsdf* in *O. luzonensis*, and *sdY* in *Oncorhynchus mykiss* (Chen et al., 2014; Hattori et al., 2012; Kamiya et al., 2012; Matsuda et al., 2002; Myosho et al., 2012; Nanda et al., 2002; Raghuveer and Senthilkumaran, 2009; Yano et al., 2012). Several conserved genes playing critical roles in sexual development of mammals were also analyzed in fish, including *ad4bp/sf-1*, *dax1*, *foxl2*, *gata4*, *mis*, *sox3*, *sox9a*, *sox9b*, *StAR, wt1, cyp11b1*, *cyp17*, *cyp19a1*, *hsd3b*, and *hsd11b* (Raghuveer et al., 2011; Sandra et al., 2010). These genes act together in a complex network to direct gonadal development. In addition, hormones and various biological pathways, such as estrogen signaling pathway, steroid hormone biosynthesis (Baroiller et al., 1999), *TGF-β* signaling pathway (Liu et al., 2017), and *wnt* signaling pathway (Amberg et al., 2013; Prathibha and Senthilkumaran, 2017) also play a vital role in the gonadal differentiation. Although much is known about the process of sex-differentiation in fish, the precise mechanisms of sex-differentiation as well as those involved in primary sex-determination remain undefined in several teleosts (Devlin and Nagahama, 2002). Fish gonadal sex-differentiation shows varied strategies and physiological regulations compared to mammals (Baron and Guiguen, 2003). The differentiation of bi-potential gonad to either testis or ovary depends on the regulation of steroidogenic pathway. In lower vertebrates, sex-steroids (androgens and estrogens) play a critical role in gonadal differentiation (Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002). However, the mechanism of action of these steroids on gonad sex-differentiation is not well understood. Unlike sex-determining systems, the genes involved in gonadal differentiation appear to be relatively conserved. Nevertheless, studies on the functional connections of these genes to relevant pathways are minimal in fish. Novel sex-related genes are required to elucidate the complex mechanisms of gonadal development in fishes. Over the last decade, NGS technologies have provided operative tools for high throughput sequencing, which has enhanced the efficacy and speed of gene discovery. Compared to whole genome sequencing, NGS via RNA-Seq affords a cost effective approach to retrieve transcriptome sequences and molecular markers that can reveal DEG associated with phenotypic traits and gender. DEG between testis and ovary gives a prospective information to recognize and understand the molecular basis of gonadal development and growth. Groups of gonadal development-related genes have been revealed by scrutiny of sex-biased transcriptomes in several species including, gilthead sea bream (Calduch-Giner et al., 2013), the Nile tilapia (Tao et al., 2013), the Japanese flounder (Zhang et al., 2016), the Russian sturgeon (Hagihara et al., 2014), Southern bluefin tuna (Bar et al., 2016), and yellow catfish (Lu et al., 2014). The transcriptome of common carp (Ji et al., 2012) has been sequenced and assembled employing NGS platforms, and differences between the ovary and testis transcriptomes of adult common carp from the China's yellow river have been explored using suppression subtractive hybridization (Chen et al., 2015). However, ideally such differences will be fully explored only by using a comprehensive set of transcriptomes from every tissue through each life stage across every major habitat (Ji et al., 2012). Furthermore, complete annotation and functional validation of the common carp genome or transcriptome have not been done with reference to gonadal stage and function. Hence, in this study, gonadal transcriptomes of juvenile common carp were sequenced to obtain a critical overview of genes potentially involved in gonadal growth and development. In addition, DEG and biological pathways were also identified by comparing testis and ovary transcriptomes. These data provide information on marker genes found expressed in gonads sex-wise at specific reproductive stages, genes related to steroid synthesis, endocrine regulation, germ cell maintenance and others factors, which are crucial for acquiring valuable insights into mechanisms of gonadal differentiation in fish. The present study also identified a major group of reproduction-related genes, potential players in gonadal development and characterized SSRs to be employed for genetic improvement purposes. The large number of reproduction-related genes identified in this study also provide direction for future genetic and functional genomic research in common carp.

2. Materials and methods

2.1. Animals and sampling

Common carp used for this study were reared at the aquaculture facility of Karnataka Veterinary Animal and Fisheries Sciences University, Bangalore, India. Nearly 2,000 offsprings were raised in a 2000 m^2 pond and fed four times daily under standard feeding regime (3% feed/bodyweight ratio daily). The oxygen level was retained at 3 mg/L or above. Animals were procured and during acclimatization, fishes were maintained under natural photo-thermal conditions by feeding commercial carp food *ad libitum* and kept in 1 m^3 tanks with filtered tap water, until experimentation. Fifteen juvenile carps, 110 to 130 dph were randomly selected and gonadal tissues were dissected out. A portion of the gonadal tissue was fixed in Bouin's fixative (15:5:1, saturated picric acid, formaldehyde and glacial acetic acid) for histological observation, and the remaining tissue was snap frozen in liquid nitrogen and stored at −80 °C for RNA-Seq. To perform qRT-PCR various tissues from adult female (brain, kidney, liver, muscle, and ovary) and adult male (brain, kidney, liver, muscle, and testis) fishes were collected, snap frozen in liquid nitrogen and stored at −80 °C. Whenever required gonadal tissues (n = 3) were pooled to obtain one biological sample and necessary experimental sets were chosen according to the analysis. Fish sampling was performed following the general procedures and with the endorsement of the Institutional Animal Ethics Committee, University of Hyderabad (CPCSEA, Inst. Reg# No#151/1999 dt.22.07.1999).

2.2. Histology

The male and female gonads of juvenile common carp, 110 to 130 dph were fixed in Bouin's solution, dehydrated and embedded in paraplast (Sigma). Sections of 5 μm were cut using a microtome (Leica, Wetzler, Germany), stained with hematoxylin-eosin and photomicrographs were taken using Olympus CX41 bright field light microscope (Olympus, Tokyo, Japan).

2.3. Total RNA isolation and cDNA library construction

The gonads of the juvenile common carp were staged by histology. Total RNA was prepared from immature gonad tissue (testis and ovary) samples of carp individually using TRI reagent (Merck, USA; Cat No. T9424). The concentration and integrity of RNA were examined with Ribogreen method using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA with RIN values > 7.0 were selected. Equal amounts of the high quality RNA from gonads were then pooled together for mRNA isolation, cDNA synthesis, and sequencing. Library preparation was performed following NEB Next Ultra RNA Library Prep Kit (New England Biolabs, USA; Cat No. E7530S) following the manufacturer's instructions. In brief, one μg of total RNA was used to isolate mRNA using NEB Next Poly (A) mRNA Magnetic Isolation Module which was time-dependently fragmented using RNA binding buffer provided in the kit. Then first strand cDNA was prepared using ProtoScript II Reverse Transcriptase, following which the second strand cDNA was synthesized, purified using AMPure XP Beads, end-repaired using NEBNext End Prep Enzyme Mix, and NEBNext Adaptor ligated using Blunt/TA Ligase. These adapter-ligated fragments were subjected to 12 cycles of enrichment and barcoding and the products were purified using Ampure XP beads.

2.4. Sequencing and assembly

Sequencing was done using Illumina Next Seq 500 Sequencer with 75 PE chemistry. The paired-end reads obtained after massive parallel sequencing were subjected to several filters to obtain the potential reads that are specific to identify the transcripts. First, a quality check using FASTQC was performed. The low quality reads (mean Phred_score < 20) were removed and the reads with adapter sequences were trimmed. If the length of the trimmed read was below 50 bp, then such reads were discarded. All processed reads were then assembled into transcripts without any reference (*de novo*) using Trinity software (trinityrnaseq20140413p1).

2.5. Functional annotation and ontology

The homology search was done for assembled transcripts' sequences against *T. rubripes*, *Danio rerio*, and (partially annotated) *C. carpio* protein sequences downloaded from UniProt database (http://www.uniprot.org/uniprot/). NCBI-blast2.2.29 was used for homology search between the sequences. BLASTX program was used to perform similarity searches. Gene annotation was assigned to the RNA-Seq transcripts based on the top BLAST hit. GO annotation analysis was then performed. The annotation results were categorized into biological process, cellular components, and molecular function. KEGG pathways were assigned to those assembled contigs using the online KAAS (http://www.genome.jp/tools/kaas). COG annotation for transcripts of testis and ovary were based on homology (https://www.ncbi.nlm.nih.gov/COG).

2.6. Identification of DEG

The reads for both gonadal tissues were separately aligned to the unigene sequences of *C. carpio* and read count profiles were generated. DESeq "R" package was used for differential gene expression. The package DESeq provide methods for testing for differential expression by use of the negative binomial distribution and a shrinkage estimator for the distribution's variance. The threshold for the P value was < = 0.05. DEG between male or female gonads were identified.

2.7. SSRs detection

SSRs were identified by mapping all clean reads to the assembled transcript reference using MIcroSAtellite identification tool (MISA, version 1.0, http://pgrc.ipkatersleben.de/misa/). The minimum repeat number used for every unit was ten for mononucleotide, six for dinucleotide, five for tri-, tetra-, penta- and hexa-nucleotide microsatellites.

2.8. Experimental validation by qRT-PCR

Few candidate DEG were validated by qRT-PCR to verify the expression profile obtained from the transcriptome data. Tissue distribution pattern of different transcripts was analyzed using SYBR Green detection method. For this, total RNA was extracted from different tissues (brain, kidney, liver, muscle, ovary, and testis) of adult common carp using TRI (Sigma) method. qRT-PCR was performed by following

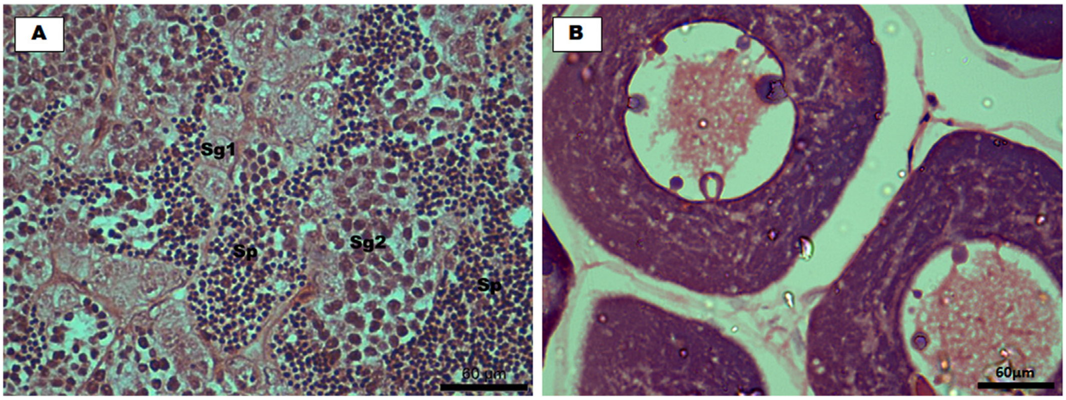

Fig. 1. Histology of gonads A-Immature testis showing Sg1: Primary spermatogonia, Sg2: Secondary spermatogonia, Sp: Sperms/spermatids; B-Immature ovary at perinucleolar-oil droplet stage (Scale: 60 μm).

the method described in Rajakumar and Senthilkumaran (2014). All reactions were performed in triplicate for 3 different samples using specific primers (Table S1). qRT-PCR amplification was carried using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, USA; Cat No. 4367659) in an ABI Step One Plus real time PCR system (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The expression was normalized against 18SrRNA expression, used as an endogenous control, generating a ΔCt value (ΔCt = Target Ct $-$18SrRNA Ct). Relative expression was then calculated according to equation $2^{\wedge-\Delta Ct}$.

3. Results

3.1. Histological observation

The maturation level of gonads were histologically determined. Upon observation the differentiating (110 to 130 dph) testis (Fig. 1A) showed presence of primary spermatogonia, secondary spermatogonia and spermatids/sperms indicating the progression from meiotic to spermiogenic phase of spermatogenesis. The juvenile ovary (110 to 130 dph) showed several nucleoli appearing at the periphery of nucleus indicating the perinucleolar stage (Fig. 1B) of primary oocyte growth.

3.2. Sequencing and reads assembly

A total of 17.06 and 14.87 million paired-end reads with 94.1% and 93.6% high quality bases (Phredscore $>$ 20) were generated from high throughput sequencing of the immature testis and ovary cDNA libraries, respectively. The transcriptome sequences generated from this study have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE112157 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE112157). Nearly, 81,757 and 43,257 transcripts with average lengths of 769 and 856 bp, and N50 of 1364 and 1453 bp, were obtained after *de novo* assembly for testis and ovary, respectively (Table 1). From these, 84,367 unigenes were constructed after removing redundancy which was a representation of all testicular and ovarian transcriptomes. The total size of the transcriptome was 62.9 and 37 Mbp for testis and ovary, respectively. All transcripts longer than 201 bp were selected and nearly 18,472 and 12,574 transcripts longer than 1000 bp of testis and ovary, respectively were obtained. About, 62,231 unigenes (50%) showed significant hits compared to known proteins. Of these, only 39,171 unigenes could be fully annotated with the available GO data.

3.3. Unigene annotation

The unigenes with counterparts in public protein databases were annotated with GO, which offers a dynamically organized vocabulary and classified relationships to symbolise information regarding the categories of Biological process, Cellular component and Molecular function. Analysis of GO term distribution showed that 'regulation of transcription, DNA templated [GO: 0006355]', 'integral component of membrane [GO: 0016021]', and 'ATP binding [GO: 0005524]' were the most common annotation terms within the three GO categories, respectively. For Biological process, 'regulation of transcription' (1745 unigenes) and 'transcription' (1668 unigenes) were the predominant groups. Under Cellular component, 'integral component of membrane' (12,405 unigenes) represented the most abundant classification followed by 'nucleus' (6951 unigenes). Under Molecular function, 'ATP binding' (6821 unigenes) and 'zinc ion binding' (4686 unigenes) represented nearly half of the category (Fig. 2). To conduct further functional prediction and classification, all unigenes were compared with proteins from the COG database. In total, 6651 unigenes were classified into 26 categories (Fig. 3). The top three classifications are general function prediction only (1089 unigenes), signal transduction mechanisms (813 unigenes); and translation, ribosomal structure, and biogenesis (760 unigenes). Following the most abundant three groups were posttranslational modification, protein turnover, chaperones (674 unigenes) and amino acid transport and metabolism (Table S2).

KAAS was used to perform pathway analysis for functional categorization. Enzyme Commission (EC) numbers were assigned (Table S3) with 3012 enzyme codes for 4782 unigenes. Pathways were categorized into 6 main groups (Table 2), metabolism (1440 unigenes), organismal systems (175 unigenes), environmental information processing (917 unigenes), genetic information processing (1511 unigenes), cellular processes (697 unigenes) and human diseases (42 unigenes). The majority of the mapped unigenes represented (Fig. 4) signal transduction (13.1%, 627 unigenes), followed by folding, sorting and degradation (11.7%, 563 unigenes) and translation (11.2%, 538 unigenes). KAAS annotation identified 96 genes related to reproduction, distributed in 7 pathways (Table 3). These genes covered the major processes of reproduction, including *wnt* signaling pathway, GnRH signaling pathway, steroid biosynthesis, oocyte meiosis, steroid hormone biosynthesis, neuroactive ligand-receptor and insulin signaling pathway (Table S3).

3.4. Identification of SSRs

A total of 18,342 and 8693 SSRs (Table S4) were finally obtained from testis and ovary, respectively. Within SSRs in the testis, the most abundant type of repeat motif was mono (10,938), di-nucleotide repeats (3748), followed by tri (2127), quadra- (1 9 4), penta- (6), and *hexa*-nucleotide (4) repeat units (Fig. 5). Within SSRs in the ovary, the most abundant types of repeat motif were mono (4805) and di-nucleotide repeats (1919), followed by tri- (1298), quadra- (87), penta- (13), and hexa- nucleotide (1) repeat units ((Fig. S1A)). The most abundant repeat motif in SSRs (Fig. S1B) was A/T (18,115), followed by AC/GT (3792), AG/CT (1603), AT/AT (1281) and ATC/ATG (7 8 7).

3.5. DEG

Differential expression between testis and ovary were analysed to identify the genes involved in sex-differentiation. Based on the applied criteria (twofold or greater change and $p < = 0.05$), there were 171 unigenes showing higher expression in testis than in ovary, and 127 unigenes showed higher expression in ovary than in testis. There was no significant difference in the expression of most unigenes between testis and ovary. In order to visualize comprehensive similarities and differences in gene expression between testis and ovary, a scatterplot was generated using R package and clear differences in gene expression between testis and ovary could be observed (Fig. S2). The DEG report has been uploaded in NCBI's GEO (GEO Series accession number GSE112157).

Table 1
Summary of *C. carpio* gonadal transcriptome.

Transcriptome Assembly	Testis	Ovary
Transcripts generated	43,257	81,757
Maximum transcript length	14,732 bp	14,333 bp
Minimum transcript length	201 bp	201 bp
Average transcript length	856 bp	769 bp
Total transcripts length	37 Mbp	62 Mbp
Transcripts > 200 bp	43,257	81,757
Transcripts > 500 bp	21,462	34,010
Transcripts > 1000 bp	12,574	18,472
Transcripts > 10 Kbp	2	3
N50 value	1453 bp	1364 bp
Number of reads used	1,38,84,178	1,01,99,309
Total number of reads	1,62,67,344	1,39,16,372
Percentage of reads used	85.35	73.29

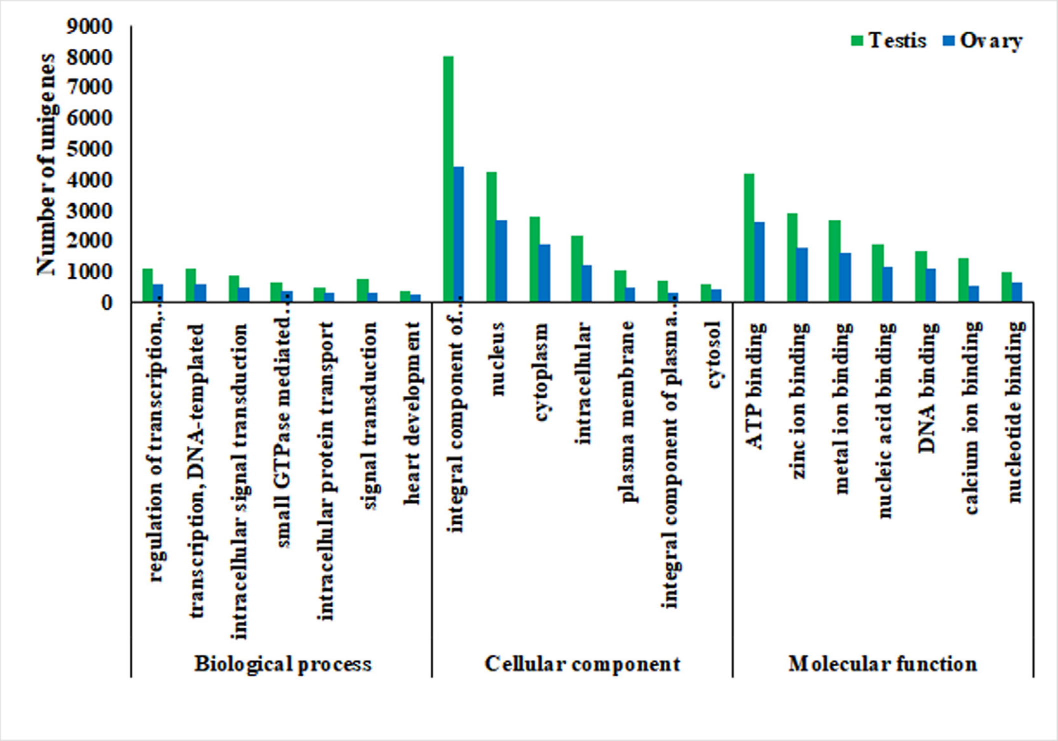

Fig. 2. GO analysis of the *C. carpio* testicular and ovarian transcriptomes of immature carp. The distribution of GO terms portrayed in the three categories: Biological process, cellular component and molecular function.

3.6. DEG validation in gonads

Twelve DEG such as *amh*, *egr2b*, *dax1*, *dmrt1*, *fbox43*, *foxk2*, *fzd8*, *gata6*, *mapk*, *sox9a*, *wt1*, and *zp2* related to sex-differentiation, were chosen to verify the changes in their expression levels in gonads. Expression of *amh*, *dax1*, *dmrt1*, *gata6*, *sox9a*, and *wt1* was higher in testis, whereas *egr2b*, *fbox43*, *foxk2*, *fzd8*, *mapk*, and *zp2* were found to be elevated in ovary, (Fig. 6). These results reiterate the differential gene expression pattern observed in gonadal transcriptome analysis.

3.7. Identification of reproduction- related genes

Nearly, 809 genes related to reproduction were identified by literature supported searching (Table S5). The gonadal transcriptome data was used to profile the expression of several reproduction-related genes. Sex-steroid synthesis related genes such as *StAR*, *hsd3b7*, *hsd17b*, *hsd20b2*, *cyp19a*, and *er* showed higher expression levels in ovary than in testis. The exceptions are *hsd11b*, and *ar* which showed higher expression in testis (Fig. 7A). Genes involved in various gonadotropin pathway receptors such as *lhcgr*, *fgfr1a2*, *tac3a*, *notch2* and *fshr*, *prlhr2a*, *kiss2* were moderately over-expressed in the testis and ovary,

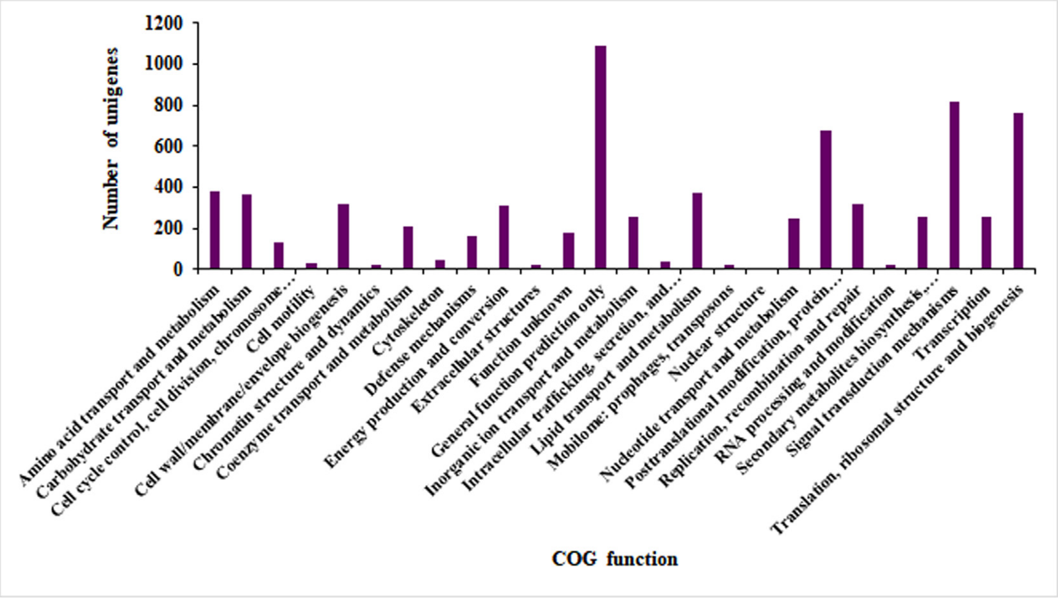

Fig. 3. COG classification of *C. carpio* testicular and ovarian transcriptomes of immature carp.

Table 2
KAAS mapping for gonadal transcriptome of *C. carpio*.

KAAS categories represented	Number of KO	Unique sequences
Metabolism		
● Amino acid metabolism	104	140
● Biosynthesis of other secondary metabolites	1	1
● Carbohydrate metabolism	126	183
● Energy metabolism	105	170
● Glycan biosynthesis and metabolism	128	174
● Lipid metabolism	145	217
● Metabolism	119	180
● Metabolism of cofactors and vitamins	76	109
● Metabolism of other amino acids	16	47
● Metabolism of terpenoids and polyketides	18	25
● Nucleotide metabolism	123	188
● Xenobiotics biodegradation and metabolism	5	6
Cellular Processes		
● Cell growth and death	93	142
● Cell motility	37	60
● Cellular community	44	84
● Transport and catabolism	226	411
Organismal Systems		
● Circulatory system	22	30
● Development	5	16
● Endocrine system	40	67
● Immune system	42	56
● Sensory system	5	6
Human Diseases		
● Endocrine and metabolic diseases	4	7
● Infectious diseases	19	35
Genetic Information Processing		
● Folding, sorting and degradation	327	563
● Replication and repair	120	149
● Transcription	143	261
● Translation	339	538
Environmental Information Processing		
● Membrane transport	19	22
● Signal transduction	371	627
● Signaling molecules and interaction	190	268
Total	**3012**	**4782**

Table 3
Reproduction-related pathways identified in common carp gonadal transcriptome.

Description	Number of genes in the pathway
Wnt signaling pathway	47
Steroid biosynthesis	14
Oocyte meiosis	12
Insulin signaling pathway	10
Steroid hormone biosynthesis	8
GnRH signaling pathway	3
Neuroactive ligand-receptor interaction	2

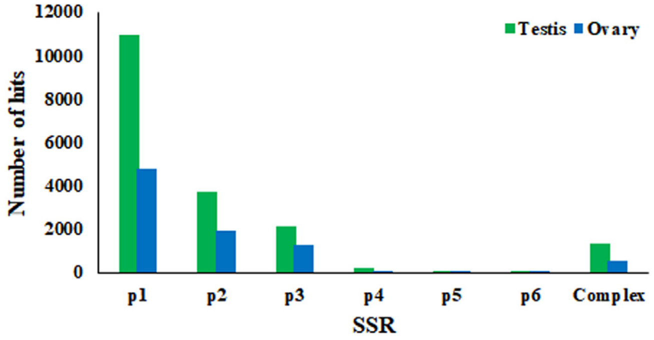

Fig. 5. Distribution of SSRs identified from common carp testis and ovary transcriptomes of immature carp.

respectively (Fig. 7B). Some of the genes involved in germ cell maintenance were highly expressed in ovary, namely *vasa*, *pou5f1*, *ipo4*, *pcna*, and *notch1* and other germ cell related genes like *sycp3*, *piwi*, *plzf*, *cxcr4*, and *cxcl12* showed higher expression in testis (Fig. 7C). Another set of genes that were found to be over-expressed in testicular tissue included various factors related to gonadal differentiation such as *igf1ra*, *wt1*, *rspo1*, *ctnnbip1*, *tcf3a*, *msl1b*, and *atm* while *fem1b* and *smad3b* showed higher expression in ovary (Fig. 7D).

Candidate genes with well-known functions, like *nanos*, germline gene, autosomal *ad4bp/sf-1* and ovary specific *gdf9*, were selected for tissue distribution analysis. Expression of *nanos* was high in brain and kidney followed by testis and ovary (Fig. 8) while *gdf9* was found to be high in ovary followed by brain, testis, kidney, muscle and liver (Fig. 8).

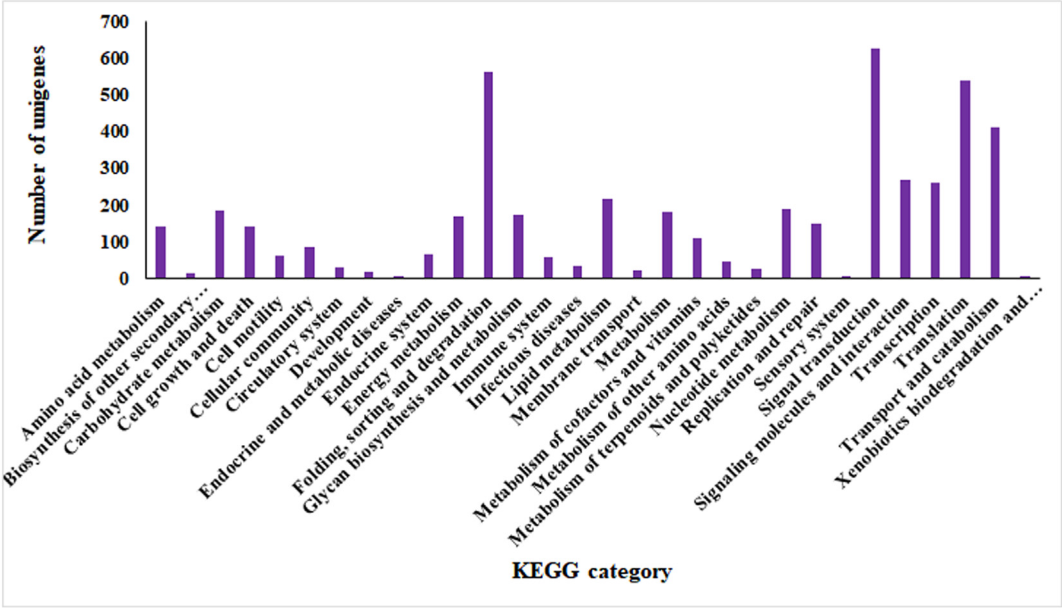

Fig. 4. KEGG categorization of *C. carpio* testicular and ovarian transcriptomes of immature carp.

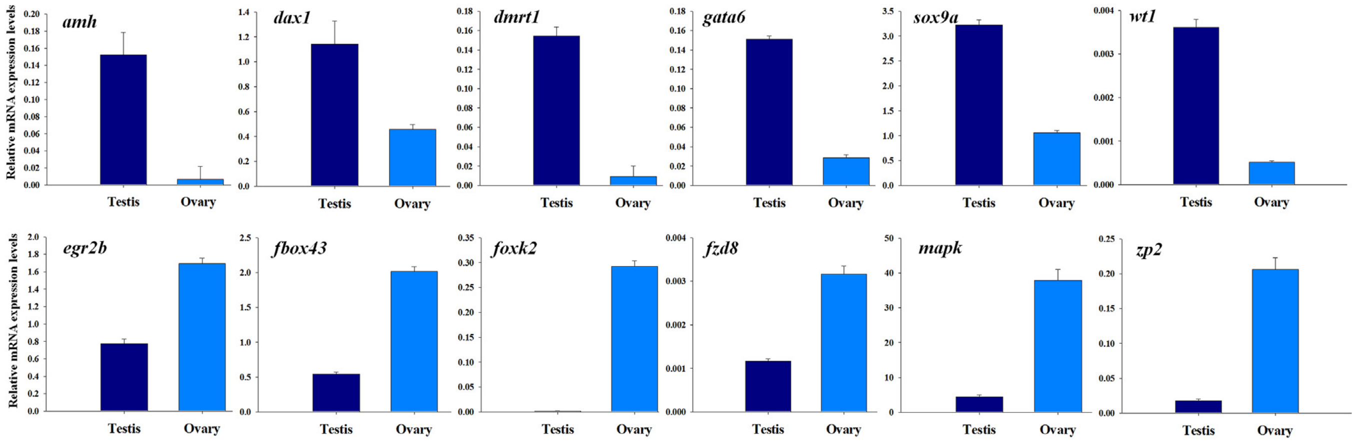

Fig. 6. Validation of immature *C. carpio* gonadal tissue transcriptome results by qRT-PCR using 12 selected DEG in testis and ovary. qRT-PCR fold changes are normalized by changes in 18SrRNA values ($P < 0.05$; ANOVA followed by Student–Newman–Keuls' test). All data from qRT-PCR studies were expressed as mean ± SEM.

Expression of *ad4bp/sf-1* was found to be high in testis, followed by kidney, brain, and ovary based on the qRT-PCR analysis (Fig. 8).

4. Discussion

With advancement of NGS technology, the information on DEG between testis and ovary is growing, and these genetic databases offer libraries facilitating the identification of corresponding genes in other fish species. Though the available gonadal transcriptomic data continues to increase, there is a need for novel sex-related genes to understand the convoluted process of gonadal development in fishes. To gain insight into the molecular fundamentals of gonad development and growth, in this study, the gonadal transcriptome of *C. carpio* was obtained after *de novo* assembly of RNA-Seq data from developing testis and ovary of immature fish. Several new players showing dimorphic gene expression were identified. Candidate genes for sex-differentiation and development, showing differential gene expression, were further tested to validate our findings. Further, the major group of reproduction-related genes identified were similar with previous reports indicating that these genes are functional in fishes. The data obtained in this study adds to the transcriptomic profile available for the family Cyprinidae.

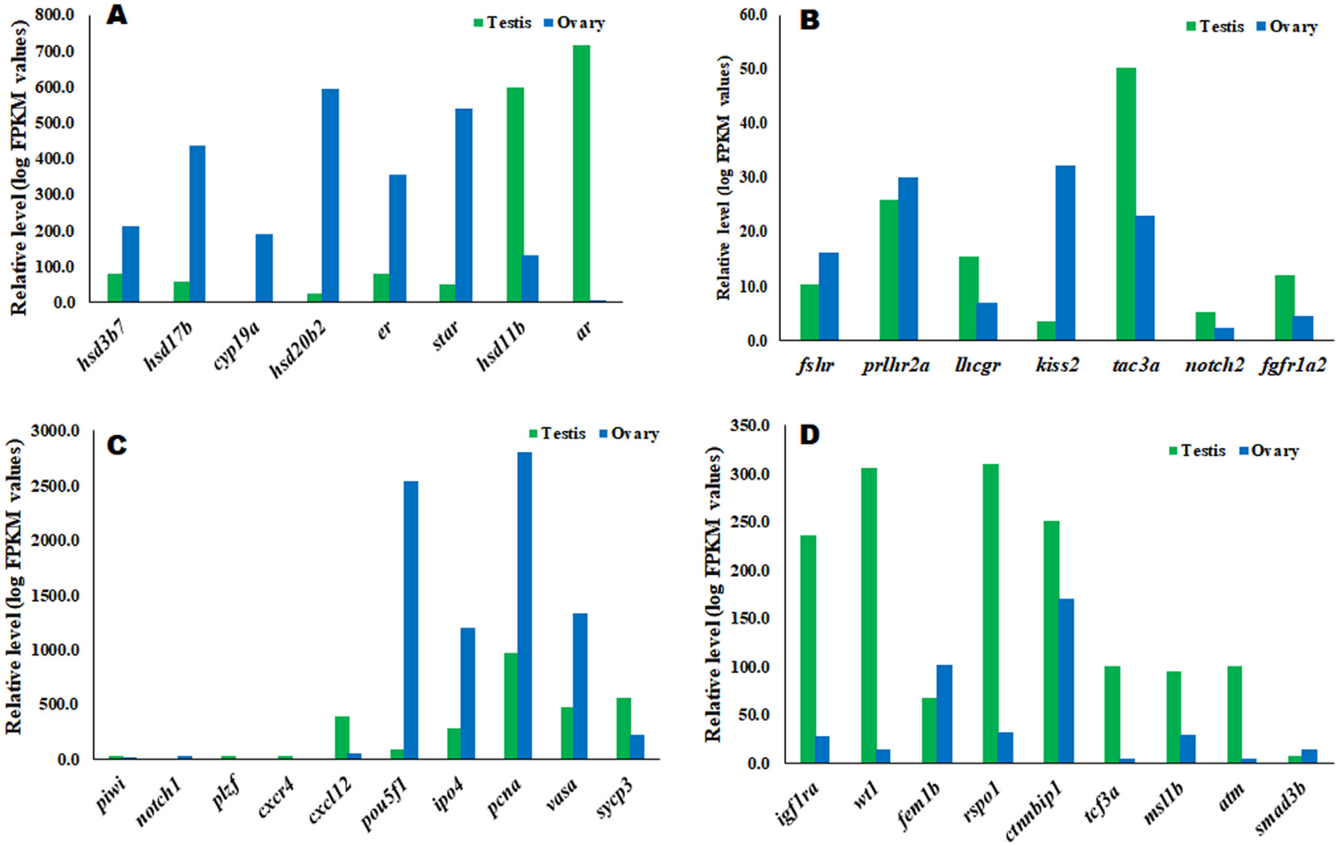

Fig. 7. Relative levels of transcripts of genes potentially involved in gonadal differentiation in juvenile common carp (A-steroid synthesis, B-endocrine regulation, C-germ cell maintenance, D-other factors).

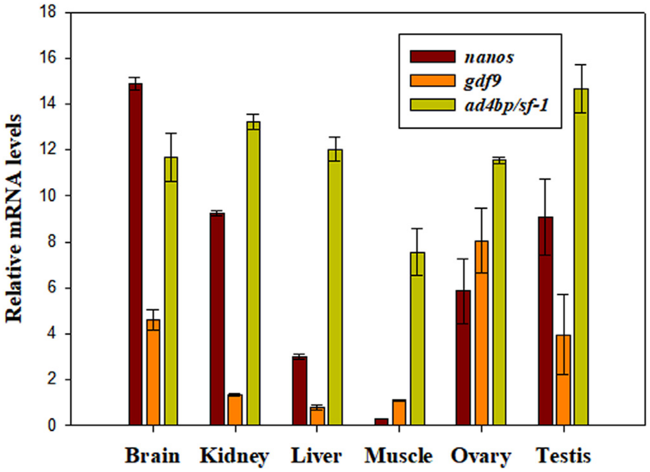

Fig. 8. Tissue distribution analysis of *nanos, gdf9* and *ad4bp/sf-1* in common carp. All other details are same as Fig. 6.

4.1. GO, COG, KEGG

One of the essential features of transcriptome analysis is to associate individual sequences and related expression information with biological functions. The juvenile stage is a transcriptionally active stage as the bipotential gonad differentiates into a specific and functionally active gonad, either testis or ovary. GO, COG and mapped unigenes were majorly involved in translational machinery, indicating that juveniles are at a crucial phase of gonadal development. The KAAS pathway analysis is based on sequence similarities and accommodates bi-directional best hit information, enabling it to attain a high degree of accuracy in comparison to a manually curated KEGG GENES database. Hence, KASS can facilitate research on the relationship between different genes obtained from the transcriptome (Moriya et al., 2007). Transcripts encoding enzymes or other proteins involved in reproductive pathways, for example, *hsd3b*, GnRH, serine/threonine-PP2A, corticosteroid *hsd11b*, sterol 14-demethylase, lanosterol synthase and many more were detected. Pathway analysis mapped some of the genes involved in the *wnt* signaling pathway, oocyte meiosis and steroid hormone biosynthesis, all of which play a pivotal role in sex-differentiation and gonadal development (Baroiller et al., 1999; Prathibha and Senthilkumaran, 2017). Recognizing these pathways permits the analysis of reproduction mechanisms in *C. carpio*, although most of the genes present in the putative KEGG pathways that were identified were not found in the present study.

4.2. SSRs

SSRs or microsatellites are polymorphic regions existing in genomic DNA consisting of 2 to 6 bp repeated core sequences (Queller et al., 1993) and important to perform research on evolution, molecular ecology, quantitative trait loci analysis, genetic diversity, gene mapping, population genetic analysis comparative genomics, and marker-assisted selective breeding. The SSRs identified in the current study, provide a valuable resource for molecular research in common carp.

4.3. DEG

Studies have suggested that the sex-biased genes, primarily or absolutely expressed in one sex, drive the phenotypic differences in males and females (Assis et al., 2012; Ellegren and Parsch 2007) and cause the phenotypic sexual dimorphism in zebrafish (Small et al., 2009). In juveniles, a group of genes are differentially expressed to fulfil the requirement of gonadal development. A total of 298 genes showed significant differences between the testicular and ovarian transcriptomes, including 171 and 127 genes showing higher expression in testis and ovary, respectively. The higher number of testis versus ovary up-regulated genes found in the common carp was in accordance with the result obtained in *Oreochromis niloticus* (Tao et al., 2013). The histological observations depict (Fig. 1A) active stages of spermatogenesis in testis with evidence of the presence of spermatocytes and spermatids in the spermatogenic cysts, and the increase in mass of the testis during this time is largely due to materials synthesized locally coinciding with our transcriptome data showing more number of transcripts and DEG in testis. On the other hand, the ovary (Fig. 1B) was observed to be in late primary oocyte growth or early secondary growth prior to the onset of vitellogenesis. Possibly, at this stage the intense transcriptional activity in the oocyte subsides as it transits into the main phase of growth, which is due to acquisition of materials (such as neutral lipid precursors and yolk proteins) produced in other tissues due to which the number of transcripts and DEG may be lesser in ovary in comparison to testis. Overall, the difference of gene numbers between female and male might imply that male gonadal development may require a larger number of early expressing transcripts compared with females in teleosts, triggering an early testis upregulated gene expression pattern before any female pattern emerges. DEG were investigated exposing a number of genes that might be enriched in sex-related biological pathways. Among DEG, a few candidate genes like *amh*, *dmrt1*, *sox9a*, associated with testicular differentiation (Kamiya et al., 2012; Kobayashi et al., 2008; Nagahama 2005; Raghuveer and Senthilkumaran, 2009; Raghuveer and Senthilkumaran, 2010a), *dax1* and *gata6* involved in gonadogenesis (Liu et al., 2016; Wang et al., 2002), *wt1* regulating SRY (Hossain and Saunders, 2001), *zp2* and *mapk* playing roles in oocyte development (Chang et al., 1997; Ponza et al., 2011), *foxk2* involved in gonadal development (Yuan et al., 2014), *fbox43* having a role in oocyte meiosis (Tung et al., 2005), *fzd8* receptor of *wnt* signaling (von Schalburg et al., 2006) and *egr2b* expressed in murine granulosa cells (Jin et al., 2016) were selected and their differential expression was validated by qRT-PCR to indicate their critical role in gonadal development. The previous study by Chen et al. (2015) also identified differential expression of *wt1* and *zp3* in testis and ovary of adult common carp using suppression subtractive hybridization. Thus, *wt1* and *zp2*, *zp3* show dimorphic expression from juvenile stage and is consistent up to adulthood.

4.4. Identification of new players from DEG

New players like *cux1*, *dmrt2a*, *fam192a*, *fstl3*, *retinoic acid receptor RXR-beta-A*, *stathmin*, *sycp1* and *zar1* also showed dimorphic gene expression pattern. CDP/Cut (CCAAT displacement protein) is a transcription factor involved in the regulation of cell growth and differentiation-related genes (Nepveu, 2001). Vanden Heuvel et al. (1996) reported that *cux1* represents an example of a transcription factor that undergoes testis-specific alternative splicing during spermatogenesis. Previous studies have reported *cux* is required for reproductive functions and its genetic ablation resulted in reduced male fertility (Luong et al., 2002) and impaired lactation (Tufarelli et al., 1998) in mice. Dimorphic expression pattern of *cux2a* was observed, indicating its role in reproduction of fishes as well.

Gene expression of *dmrt1* is associated with the testicular development in many species (Raghuveer and Senthilkumaran, 2009). Differential expression pattern of *dmrt2a* was observed, coinciding with previous report by Peng et al. (2016) in which *dmrt2b* showed dimorphic expression pattern, suggesting these new players' *dmrt2a* and *dmrt2b* might potentially be associated with sex differentiation in common carp.

A recent transcriptomic study on developmental gonads in protandrous black porgy (Zhang et al., 2018) reported up-regulated expression of *fam101b* in ovary coinciding with *fam192a* and *fam210b* showing dimorphic expression in common carp, elucidating their importance in ovarian development.

Follistatin is a FSH-suppressing protein that is produced in the pituitary and upon binding to *activin*, neutralizes the FSH stimulatory actions of *activin* (Nakamura et al., 1990). Follistatin, that encodes a TGF-β superfamily binding protein, inhibits formation of the XY-specific coelomic vessel in XX gonads, a feature normally associated with testis differentiation (Yao et al., 2004). Follistatin displayed early sexually dimorphic expression profile in rainbow trout (Vizziano et al., 2007). Incidentally, *fstl3* showed dimorphic expression pattern, revealing its crucial role in common carp gonadal differentiation.

RA is an active derivative of vitamin A that diffuses through tissues and binds to heterodimers of the nuclear receptors RAR-RXR, which recognize RA-response elements in DNA to control the expression of RA-target genes (Duester 2008). RA signaling stimulates differentiation of spermatogonial germ cells and induces meiosis in male but not female gonads (Duester 2013). RA receptor RXR-beta-A showed differential gene expression in juvenile carp coinciding with report of Rodríguez-Marí et al. (2013) in zebrafish suggesting that the continuous availability of retinoic acid to germ cells in bipotential gonads prohibits the sexually dimorphic onset of meiosis and is consistent with the initial development of meiotic oocytes in juvenile teleosts. *Stathmin* is a neuron-enriched gene mediating tubulin depolymerization (Sobel et al., 1989). Studies demonstrated that maternal *stathmin* accumulates in oocytes and is redistributed in pre-implantation embryos (Koppel et al., 1999). Chu et al. (2006) observed intense expression of *stathmin* in ovary of tilapia. Dimorphic expression pattern of *stathmin* observed in the present study indicates its functional significance in gonads of common carp.

Sycp3 is a meiosis-specific component of the synaptonemal complex, required for the synapsis of homologous chromosomes, and is a marker of meiosis in mammals (Page et al., 2006). Its importance in gametogenesis and fertility has been studied in a few teleost species such as medaka and zebrafish (Iwai et al., 2006; Ozaki et al., 2011). Laldinsangi and Senthilkumaran (2018) demonstrated that *sycp3* have a potential role in the development and maintenance of testicular function in catfish. Dimorphic expression pattern of *sycp1* revealed its crucial role during gonadal development of common carp as well.

Ovary-specific maternal factor, *zar1* plays an essential role during oocyte-to-embryo transition (Wu et al., 2003). Expression of *zar1* was identified in gonads of carp, coinciding with a recent transcriptome study in tilapia (Tao et al., 2018) that reported correlation of *zar1* with the expression of genes previously known to be involved in sex differentiation. Further investigations including functional analysis of these genes in female and male fishes are needed to substantiate our findings.

4.5. Genes related to reproduction

A large number of genes (8 0 9) related to reproduction have been identified by gonadal transcriptome analysis. Corticosteroids (*hsd3b7*, *hsd17b*, *hsd11b*) and their receptors (*ar* and *er*) play crucial role in the regulation of reproduction by acting as transcription factors in somatic cells of gonads (Milla et al., 2009). Steroid synthesis gene *hsd3b7*, expressed in Leydig cells and responsible for biosynthesis of steroids, mainly progesterone and testosterone, was moderately over-expressed in ovary. In addition, *hsd17b*, that regulate the concentration of biologically active androgens and estrogens and involved in synthesis of estradiol, was also over-expressed in ovary. Other steroid related genes like, the sterol transfer coding gene *StAR*, genes coding for *er* and *cyp19a1* (Barney et al., 2008) which are responsible for estradiol-17β biosynthesis in females, followed similar expression pattern as reported in transcriptome analysis of the Russian sturgeon (Hagihara et al., 2014). On the other hand, *hsd11b*, an enzyme which converts gonad maturation inhibiting cortisol or corticosterone into inactive cortisone or 11-dihydrocorticosterone, respectively and gene coding for *ar* showed higher expression in testis, as that of the expression pattern in the Nile Tilapia gonadal trancriptome analysis (Tao et al., 2013). Key marker genes of germ cells namely, *vasa*, *pou5f1* (Raghuveer and Senthilkumaran, 2010b; Wang et al., 2011) playing role in stem cell maintenance and regulation, and *pcna*, playing crucial role in meiosis (Miura et al., 2002) were over-expressed in the ovary, indicating their germ cell regulation in the female ovary. Other germ cell maintenance genes such as *piwi*, *notch1*, *plzf*, *cxcr4*, *cxcl12*, *ipo4*, and *sycp3* were moderately over-expressed in testis like that of the transcriptome of Southern bluefin tuna (Bar et al., 2016). The expression pattern of other genes/factors related to gonadal differentiation like *hsd20b2, lhcgr*, *fgfr1a2*, *tac3a*, *notch2, fshr*, *prlhr2a*, *kiss2*, *igf1ra*, *wt1*, *rspo1*, *ctnnbip1*, *tcf3a*, *msl1b*, *fem1b*, *smad3b*, and *atm* between testis and ovary were comparable to the transcriptome analysis of previous reports (Bar et al., 2016; Hagihara et al., 2014; Shi et al., 2015; Wang et al., 2017).

4.6. Expression analysis of candidate genes

Few candidate genes including *nanos* (germline), *ad4bp/sf-1* (autosomal) and *gdf9* (ovary specific) were selected for further expression analysis. The germline gene*, nanos*, is a RNA binding protein that plays significant roles in early development and, more precisely, in primordial germ cell development (Tsuda et al., 2003). Tissue distribution analysis revealed high expression of *nanos* in brain and kidney followed by testis and ovary, indicating a plausible role for this gene in various physiological processes including gonadal development. Incidentally, *gdf9*, belonging to the TGF-β superfamily was highly expressed in ovary followed by brain, testis, kidney, muscle, and liver, suggesting that it plays a role in ovarian development and differentiation (McGrath et al., 1995). The orphan nuclear receptor, *ad4bp/sf-1* is an essential factor for mammalian sexual differentiation, endocrine organogenesis and steroidogenesis (Morohashi et al., 1992). In teleosts, *ad4bp/sf-1* is critical for transcriptional regulation of *cyp19a1a* (Hu et al., 2001; Wang et al., 2007; Yoshiura et al., 2003) and other genes encoding steroidogenic enzymes (Nakamoto et al., 2012; Val et al., 2003). The qRT-PCR analysis revealed expression of *ad4bp/sf-1* to be high in testis, followed by kidney, brain, and ovary evidencing the importance of *ad4bp/sf-1* in testicular differentiation. Taken together, present study provides an array of marker genes to understand the molecular basis of sex-differentiation and gonadal development in teleosts.

5. Conclusion

Overall, analysis of common carp gonadal transcriptome using NGS RNA-Seq technology identified diverse regulatory mechanisms/ pathways of sexual development and provided a valuable resource for evaluating differential gene expression patterns. The dimorphic expression of sex-related genes offers additional insights into teleostean gonadal differentiation.

Conflicts of interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.12.004.

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