Gonadal transcriptome sequencing of the critically endangered *Acipenser dabryanus* to discover candidate sex-related genes

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**ABSTRACT**

**Background:** *Acipenser dabryanus*, an endemic Chinese species, has been listed as a first-class protected animal in China. Sturgeons are among the oldest and most primitive group of existing fish in the world and occupy a special place in the evolutionary history of fish. Thus, a study of the reproduction and sex differentiation of sturgeon will be of great value for fish as well as the whole vertebrate group.

**Methods:** In this study, we conducted comparative analysis of the testes and ovaries transcriptomes of *A. dabryanus* to screen for sex-differentiation and sexual development-related genes.

**Results:** The transcriptome sequencing of six cDNA libraries generated 265 million clean reads, encompassing 79 Gb of sequences. The N50 and mean length of the identified 91,375 unigenes were 1,718 and 989 bp, respectively. A total of 6,306, 9,961, 13,170, 15,484, and 23,588 unigenes were annotated in the clusters of orthologous groups, gene ontology categories, Kyoto Encyclopedia of Genes and Genomes Pathway, euKaryotic orthologous groups, and NCBI non-redundant protein databases, respectively. A total of 5,396 differentially expressed genes were found between the two sexes, with 1,938 predicted to be up-regulated in ovaries and 3,458 in testes. A total of 73 candidate genes known to be involved in sex differentiation and sexual development were searched in the transcriptome of *A. dabryanus* of which 52 showed significant similarity. We highlighted six genes that are differentially expressed between the two sexes and may play important roles in sex differentiation and gonad maintenance. In addition, 24,271 simple sequence repeats (SSRs) and 550,519 single-nucleotide polymorphisms (SNPs) were detected.

**Discussion:** This work represents the first transcriptome study comparing the ovary and testis in *A. dabryanus*. The putative differentially expressed genes between the gonads provide an important source of information for further study of the sex-differentiation related genes and the sex-differentiation mechanism in sturgeons. The SSRs or SNPs identified in this study will be helpful in the discovery of sex-related markers in *A. dabryanus*.
INTRODUCTION

Sex is regarded as the queen of problems in evolutionary biology (Lewispye & Montalban, 2015). In the evolution of sex determination mechanisms in vertebrates, fish display diverse and complicated sex determination systems, and almost any sex determination mechanisms revealed in vertebrates has been discovered in fish (Pan et al., 2015). Therefore, the study of sex determination and differentiation in fish will help to reveal the formation and evolution of sex determination mechanisms of the whole vertebrate group. Sturgeons are among the oldest and most primitive group of existing fish in the world and are in the transition from cartilaginous fish to bony fish (Icardo et al., 2002). Therefore, considering the special evolutionary position of sturgeons, study on their reproduction and sex differentiation will be of great theoretical value for fish as well as vertebrates.

Acipenser dabryanus, also called Dabry’s sturgeon, is a freshwater sturgeon restricted to the Yangtze River system (Zen, 1990). The sexual maturity of A. dabryanus in males is reached at 4–6 years of age and in females at 6–8 years of age (Zhuang et al., 1997). Due to the heavy fishing, damming, and habitat degradation, the natural population of A. dabryanus has decreased dramatically in recent years and the wild species is almost extinct in the Yangtze River (Zhang et al., 2011; Li et al., 2015; Wu et al., 2014; Du et al., 2014). Consequently, A. dabryanus was listed as a first-class protected animal by the China Government (Wang, Yue & Chen, 1998) and also listed as a Critical Endangered species in the International Union for Conservation of Nature and Natural Resources Red List (www.iucnredlist.org/details/231/0). Thus, captive breeding of A. dabryanus may be the last chance for their survival and sustainability. However, it is very difficult to identify the sex of A. dabryanus based on secondary sexual characteristics during artificial propagation. Currently, growers usually wait 3–4 years before fish are sexed via an invasive surgical examination of the gonads (Doroshov, Moberg & Van Eenennaam, 1997).

This limits the effective protection and artificial propagation of A. dabryanus. Therefore, a comprehensive study on the sex-differentiation and reproduction of A. dabryanus is crucial for their sustainable development.

The development and growth of sturgeons are very slow (Grandi & Chicca, 2008). Gonadal sex differentiation occurs late, and the histological differentiation time differs according to species. The earliest gonadal sex differentiation was observed at 3 months in Acipenser gueldenstaedtii (Akhundov & Fedorov, 1991), while most other sturgeons begin early gonad differentiation between 6 months and 9 months of age (Grandi, Giovannini & Chicca, 2007; Wrobel et al., 2002; Flynn & Benfey, 2007). Subsequently, it takes years for gonadal development to reach sexual maturity both in the wild and on fish farms. In theory, genes involved in sex differentiation might not function and express in the gonads of adult females or males but in immature individuals. In Chinese sturgeons, the closest species to Dabry’s sturgeon, the oocytes were still in the primary oocyte growth stage in 5-year-old females (Yue et al., 2015). Therefore, a comprehensive transcriptome study of the gonads in the immature period is essential to the discovery of the early sex-differentiation genes.
Transcriptome screening is one of the most powerful and efficient methods for discovering functional genes (Vidotto et al., 2013) as well as genetic markers. In the present study, we used the next-generation Illumina HiSeq platform to sequence the transcriptomes of *A. dabryanus*. This is the first transcriptome study to compare the ovary and testis in *A. dabryanus*. Our research can provide abundant information on the genes involved in reproduction and the general mechanism of gonad differentiation and development based on the gene expression profile. Moreover, the simple sequence repeats (SSRs) and single-nucleotide polymorphisms (SNPs) identified by transcriptome sequencing can contribute to the discovery of genetic or sex-linked markers in *A. dabryanus*.

**MATERIALS AND METHODS**

**Ethical procedures**

All fish handling and experimental procedures were approved by the Animal Care and Use Committee of the Fishery Institute of the Sichuan Academy of Agricultural Sciences (20170226001A), and all animal collection and use protocols were carried out in accordance with the guidelines and regulations for the care and use of laboratory animals at the Fishery Institute of the Sichuan Academy of Agricultural Sciences.

**Sample materials**

To discover potential sex-differentiation and sexual development-related genes, three developmental stages, including the early sex differentiation phase 9-month-old, 1-year-old, and 3-year-old immature individuals, were sampled separately at the Fishery Institute of the Sichuan Academy of Agricultural Sciences. One male and one female sturgeon were collected for transcriptome sequencing of each developmental stage. After anaesthetization by 0.05% MS-222 (Sigma, St. Louis, MO, USA), the testis and ovary tissues were collected and immediately flash frozen in liquid nitrogen, then transferred to an ultralow freezer at −80°C until RNA extraction. Gonadal tissues (9 months old, 1 year old, and 3 years old) were fixed in Bouin’s solution, dehydrated by ethanol, cleared, and embedded in paraffin. Gonads were cut into eight-μm paraffin sections and stained with haematoxylin and eosin to identify the sex of Dabry’s sturgeon.

**RNA-Seq library preparation and sequencing**

Total RNA was extracted from gonads using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and examined on a 1% agarose gel. RNA was quality-controlled by using a NanoPhotometer® spectrophotometer (Implen, Westlake Village, CA, USA) and quantified using a Qubit® RNA Assay Kit in a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). The NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (Neb, Beverly, MA, USA) was used for library construction according to manufacturer’s instructions. Briefly, poly-A mRNA was purified from total RNA samples with Magnetic Oligo (dT) Beads. Fragmentation buffer was added to the purified mRNA to break them into a suitable size (about 200 bp). The short fragments of mRNA were then used for the first strand strands with random hexamer primers. DNA polymerase I,
dNTPs, RNase H, and buffer were added to the first strand and the second strand of cDNA were then synthesized. After purification with AMPure XP beads, double-stranded cDNA were further added with the “A” base and connected with sequencing adapters. Qubit2.0 (Thermo, Waltham, MA, USA) and Agilent 2100 (Agilent, Santa Clara, CA, USA) were used to check the concentration and insert size of the final cDNA libraries to ensure the quality. A total of six libraries for gonads in different developmental stages across the immature phase were prepared and sequenced separately using the Illumina HiSeq 2000. The libraries were named T1 (the library of the 3-year-old male), T2 (the library of the 3-year-old female), T3 (the library of the 1-year-old male), T4 (the library of the 1-year-old female), T5 (the library of the 9-month-old male), and T6 (the library of the 9-month-old female).

**Transcriptome assembly**

The raw reads were trimmed and quality controlled by the software WipeAadapter.pl (Biomarker Technologies Co., Ltd, Beijing, China) to remove reads with adaptors. Reads with more than 50% of bases having a Q-value ≤20 were filtered out by using the software Fas-tq_filter (Biomarker Technologies Co., Ltd, Beijing, China). To avoid missing short genes or those displaying low expression-levels, all clean reads were assembled together to perform the de novo assembly with program Trinity (Grabherr et al., 2011). Assembly completeness was estimated using the Bench-marking universal single-copy orthologs (BUSCO) analysis (Simão et al., 2015). The lineage dataset used in BUSCO is actinopterygii_odb9. The sequences assembled in Trinity were grouped together to conduct the annotation analysis. The reads of each individual were aligned to the assembled de novo transcriptome to obtain gene expression levels.

**Gene annotations**

All assembled sequences were used for BLAST (Altschul et al., 1997) searches and annotation against the NCBI non-redundant (Nr) protein (Deng et al., 2006), Swiss-Prot (Apweiler et al., 2004), gene ontology (GO) (Ashburner et al., 2000), clusters of orthologous groups (COG) (Tatusov, Galperin & Natale, 2000), euKaryotic orthologous groups (KOG) (Koonin et al., 2004), eggNOG (Huerta-Cepas et al., 2015), and protein family (Pfam) (Finn et al., 2013) databases using an e-value of 1e−5, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (Kanehisa et al., 2004) results were obtained by comparing with KEGG database using KOBAS2.0 (Xie et al., 2011). After predicting the amino acid sequence of unigenes, the software HMMER (Eddy, 1998) was used to compare them with Pfam database to get the annotation information of unigenes. The annotation tool TransDecoder was used to predict coding regions in the transcripts.

**Differential expression**

Clean reads from female samples including all three developmental stages (T2, T4, and T6) were grouped together as immature females to perform the following differential expression analysis and so were the male samples (T1, T3, and T5). Bowtie
was used to realign the original reads to the Trinity-assembled de novo transcriptome, and RSEM was used to obtain an abundance estimation for each sample (Li & Dewey, 2011). The expression profiles of the transcripts in different cDNA libraries were detected with the gene expression method of fragments per kilobase of transcript per million mapped reads (Trapnell et al., 2010). Differential expression analysis was performed using edgeR (Robinson, McCarthy & Smyth, 2010) in the OmicShare tools, a free online platform for data analysis (www.omicshare.com/tools). The default parameters of edgeR were used, and differential expression genes (DEGs) were selected according to log2 fold change ≥1 and P-value >0.05. Based on the gene expression level of the different samples, differentially expressed transcripts were annotated against the Nr protein, Swiss-Prot, GO, COG, KOG, eggNOG, Pfam, and KEGG databases to identify underrepresented and overrepresented terms in the male and female gonad tissues.

**Microsatellite and SNP discovery**

The microsatellite regions from the sequence data were identified using the program MISA, available online (http://pgrc.ipk-gatersleben.de/misa/). Six types of SSRs were examined: mono-, di-, tri-, tetra-, penta- and hexa-nucleotide repeats, and the compound SSR. The minimum repeat for each type of SSRs was set as 10, 6, 5, 5, 5, and 5 for mono-, di-, tri-, tetra-, penta-, and hexa-nucleotide, respectively.

STAR software (Dobin et al., 2013) was used to align the reads and unigenes of each specimen, and GATK (McKenna et al., 2010) was performed during SNP calling and used to identify SNPs. The identification standards satisfied the following conditions: no more than three continuous single-base mismatches within 35 bp and an SNP quality greater than 2.0 after deep normalization of the sequence.

**Validation of transcriptomic data**

To validate the expression profiles of the differentially expressed genes, eight putative genes were chosen and determined by real-time RT-PCR using the SYBR Green Real-time PCR Master Mix (Toyobo, Tokyo, Japan) and the IQ Multicolor Detection System (Bio-Rad, Hercules, CA, USA). Primers for real-time PCR are displayed in Table S1. To detected the most stably expressed housekeeping gene, β-actin, Gapdh, and EF-1 were selected as candidate genes. Cycle threshold (Ct) values of all three genes were calculated using ovaries and testes of different developmental stages as template. The software geNorm (Vandesompele et al., 2002) was used to check the most suitable housekeeping gene for Dabry’s sturgeon. The real-time PCR was performed using a volume of 20 μl containing one μl cDNA template, 10 μl 2 × SYBR Green real-time PCR Master Mix, 0.5 μl of each target gene primer (10 μM), and eight μl water. The PCR cycling conditions were as follows: 5 min at 95 °C; then 40 cycles of 20 s at 95 °C, 20 s at 55 °C, and 30 s at 72 °C; finally, 70–95 °C with a ramp up of 0.5 °C per 10 s was performed to generate a melting curve. Each sample was performed and analyzed in triplicates. The 2^ΔΔCt method was used to analyze the expression level of the differentially expressed genes (Livak & Schmittgen, 2001).
RESULTS

Histological analysis

We started the sample collection of gonads 5 months from hatching, and the gonads remained undifferentiated at the histological level at 8 months. In 9-month-old sturgeon, gonads started to differentiate and spermatophore can be observed in testis and oogonium began to form in ovary (Figs. 1A and 1D). The different type of epithelium between ovary and testis was the most striking characteristic used to distinguish the male from female (Chen et al., 2004). In the 1-year-old and 3-year-old sturgeon, it is more obvious to see the ruga type and cylindrical epithelium in ovary and smooth type epithelium in testis (Figs. 1B, 1E and 1F). The connective tissue surround the spermatogonia and form into a circular or elongated spermatophore (Figs. 1A and 1C). The connective tissue and microvessels are rich in ovary tissue, the follicular shape is irregular and loose and oogonium can be observed inside the follicle (Figs. 1D, 1E and 1F).

Sequencing and de novo assembly

Sequencing of the six cDNA libraries derived from the male and female gonads at 9 months, 1 year, and 3 years generated 265 million clean reads, encompassing 79 Gb of sequences. Transcriptome sequencing data have been deposited to the NCBI Sequence Read Archive database under accession numbers SRR6167299, SRR6172670, SRR6173479, SRR6175505, SRR6179331, and SRR6179394. The GC content frequency distribution of all reads ranged from 48.42% to 49.87%. The reads were assembled into 177,843 putative transcripts using Trinity with an N50 length of 1,908 bp and an average length of 1,210 bp. The N50 and mean length of the 91,375 unigenes were 1,718 and 989 bp, respectively (Table 1). Of the 91,375 unigenes, 63,271 unigenes (69.24%) were shorter than 1,000 bp,
while 28,104 unigenes were longer than 1,000 bp long and 11,945 unigenes had a length of over 2,000 bp. BUSCO analysis showed that 3,872 genes (1,211 complete and single-copy BUSCOs, 1,987 complete and duplicated BUSCOs, 674 fragmented BUSCOs) were identified in the BUSCO database while the other 712 genes were the missing BUSCOs (Table 1). The unigene sequences were assigned with an ORF predictor TransDecoder, with a total of 48,623 unigenes (53.2%) detected putative CDS and according amino acid sequence.

### Annotation analysis

Based on a comparison with protein sequences deposited in the Nr protein, Swiss-Prot, GO, COG, KOG, eggNOG, Pfam, and KEGG databases, we annotated a total of 24,978 unigenes within the Nr transcriptome of Dabry’s sturgeon. Significant matches were found for 6,306 unigenes in the COG database, 9,961 in the GO database, 13,170 in the KEGG database, 15,484 in the KOG database, 17,385 in the Pfam database, 13,515 in the Swiss-Prot database, 23,179 in the eggNOG database, and 23,588 in the Nr database (Table 2). Regarding homology with other species, 10,065 clusters (42.70%) were similar to *Lepisosteus oculatus*, followed by *Latimeria chalumnae*, *Oncorhynchus mykiss*, *Danio rerio*, *Esox lucius*, *Astyanax mexicanus*, *Chrysemys picta*, *Xenopus tropicalis*, *Callorhinchus milii*, *Stegastes partitus*, and other species (Fig. 2).

The three main categories of GO annotations were 22,525 (34.79%) for cellular components, 11,925 (18.41%) for molecular function, and 30,303 (46.80%) for biological processes. In the cellular component ontology, the most abundant terms were cell, cell part, organelle, and membrane, whereas in the molecular function ontology, the most abundant terms were binding and catalytic activity; in the biological process ontology, we found that the most abundant terms annotated with the unigenes were cellular process, single-organism process, metabolism process, and biological regulation (Fig. 3).

By mapping the unigenes to the COG database, we found that general function prediction only (2,092, 24.24%), replication, recombination and repair (1,033, 11.97%), translation (792, 9.18%), and signal transduction mechanisms (739, 8.56%) were the most frequently represented functional clusters in our transcriptome (Fig. 4). Only one

### Table 1 Summary of the *A. dabryanus* transcriptomes assembly.

| Statistic                              | Transcript  | Unigene |
|----------------------------------------|-------------|---------|
| Total number                           | 177,843     | 91,375  |
| Total length                           | 215,143,471 | 90,408,018 |
| Average length                         | 1,209.74    | 989.42  |
| N50 length                             | 1,908       | 1,718   |
| Complete single-copy BUSCOs            | 1,211       |         |
| Complete duplicated BUSCOs             | 1,987       |         |
| Fragmented BUSCOs                      | 674         |         |
| Missing BUSCOs                         | 712         |         |
| Total BUSCO groups searched            | 4,584       |         |
| Total BUSCO genes recovered            | 3,872 (84%) |         |

Chen et al. (2018), *PeerJ*, DOI 10.7717/peerj.5389
unigene was annotated to the category nuclear structure, and no unigene was found to belong to extracellular structures.

Furthermore, unigenes were annotated in the KOG database and clustered into 25 KOG categories (Fig. S1), with “general function prediction only” containing the greatest number of unigenes (3,357, 19.28%), followed by “signal transduction mechanism” (3,007, 17.27%) and posttranslational modification, protein turnover, and chaperones (1,328, 7.63%).

We also mapped the unigenes to the KEGG pathway database and found that all the unigenes mapped to 290 pathways (Table S2). Briefly, of these sequences with a KEGG annotation, most genes (355, 2.47%) were from the MAPK signaling pathway, followed by focal adhesion (343, 2.39%), actin cytoskeleton regulation (304, 2.12%), endocytosis (292, 2.03%), the calcium signaling pathway (257, 1.79%), and ribosomes (256, 1.78%).

**Table 2 Functional annotation of unigenes of the A. dabryanus transcriptome.**

| Database | Annotated number | 300–1,000 (bp) | ≥1,000 (bp) |
|----------|-----------------|----------------|-------------|
| COG      | 6,306           | 964            | 4,705       |
| GO       | 9,961           | 2,009          | 7,616       |
| KOG      | 15,484          | 3,020          | 11,708      |
| Pfam     | 17,385          | 3,019          | 13,499      |
| Swissprot| 13,515          | 2,527          | 10,545      |
| eggNOG   | 23,179          | 5,019          | 16,401      |
| Nr       | 23,588          | 5,453          | 16,844      |
| All annotated | 24,978   | 5,759          | 17,020      |

**Figure 2** BLAST top-hit species distribution of homologous sequences in the A. dabryanus transcriptome. The number percentage of unigenes which are homologous with other species were presented.
**Figure 3** Functional gene ontology classification of the *A. dabryanus* gonadal transcriptome. Gene ontology analysis was performed for three main categories: cellular component, molecular function, and biological process.

**Figure 4** Clusters of orthologous group classifications of the *A. dabryanus* gonadal transcriptome. A total of 6,306 unigenes were grouped into 24 COG classifications and no unigene was found to belong to extracellular structures.
Differentially expressed genes in different libraries and annotation analysis

To identify differentially expressed transcripts in males and females, the ovaries or testes of three developmental phases were grouped together as immature females and males to be compared. A total of 5,396 differentially expressed genes were found between the two sexes, with 1,938 predicted to be up-regulated in ovaries and 3,458 in testes (Fig. 5).

All unigenes identified as differentially expressed were annotated. GO annotations revealed that the DEGs were mostly allocated to the following terms: molecular function, biological process, cellular component, single-organism process, and cellular process. All DEGs were annotated into 21 COG categories, with “general function prediction only” containing the greatest number of DEGs, followed by replication, recombination and repair, posttranslational modification, protein turnover, chaperones and amino acid transport, and metabolism. By mapping the DEGs to the KOG database, we found that over half of the DEGs were classified into the general function prediction only, signal transduction mechanism, cytoskeleton and posttranslational modification, protein turnover, and chaperone categories. In the KEGG pathway analysis, most DEGs between the two sexes were from pathways for cell adhesion molecules, neuroactive ligand-receptor interactions, MAPK signaling pathway, focal adhesion, and Wnt signaling pathway.

Search for genes potentially involved in sex differentiation in Dabry’s sturgeon

We evaluated 73 candidate genes known to be involved in sex differentiation, sex determination, and sexual development to search for unigenes annotated in the transcriptome of Dabry’s sturgeon (Table S3). Significant matches were found for 52 of the 73 genes investigated (Table S3). Some genes shown to be sex-determining genes in other species, such as Dmrt1, Fem1, and Gsdf, were present in the gonad transcriptome
of Dabry’s sturgeon. The anti-Müllerian hormone genes were missing in Dabry’s sturgeon. The SRY (sex-determining region Y)-box genes Sox1, Sox3, Sox4, Sox6, Sox9, Sox11, Sox14, and Sox17; steroidogenic enzymes Cyp11a1, Cyp17a1, Cyp19a1a, and Fst; and sex hormone receptors Ar1, Ar2, Esr1, Esr2a, and Esr2b were found in Dabry’s sturgeon. Other essential genes involved in sex differentiation and sexual development (Foxl2, Bmp15, Gata4, Lhx1, Lhx9, Wnt, Nanos1) were also detected.

Identification of SNP and SSR markers
A large number of SSR and SNP markers were obtained. A total of 24,271 SSRs were identified from the transcriptomes of A. dabryanus. Disregarding mononucleotides, dinucleotides were the most abundant category, accounting for 61.53% of all SSRs, followed by trinucleotides (34.80%) and tetranucleotides (3.44%), as shown in Fig. 6. In contrast, penta (0.15%) and hexanucleotides (0.08%) were less abundant.

In total, 550,519 SNPs (453,779 from T1, 416,775 from T2, 485,960 from T3, 430,889 from T4, 489,734 from T5, and 480,501 from T6) were obtained. Regarding the type of SNPs, more heterozygous SNPs than homozygous SNPs were found in each library (Table 3).
The distribution of the identified SNPs is shown in Fig. 7, and the majority of the unigenes only had one SNP.

**Validation of gene expression by real-time PCR analysis**

The most commonly used reference genes β-actin, Gapdh, and EF-1 were firstly amplified in different tissues of Dabry’s sturgeon to check the most stably expressed one.
The average expression stability ($M$ value) was calculated according to the Ct value of the housekeeping genes. Genes with the lowest $M$ values have the most stable expression. In the present study, the $M$ value for $\beta$-actin, Gapdh, and EF-1 was 0.793, 1.36, and 0.861, respectively (Fig. S2). Therefore, $\beta$-actin was selected as the reference gene in Dabry’s sturgeon for the following qPCR analysis. To evaluate the transcriptome data, we chose eight differentially expressed genes (Fig. 8) potentially involved in sex differentiation, determination, or development for validation using real-time PCR. A comparison of the transcriptome data with the qPCR results revealed similar expression profiles, though the exact fold-change of these genes in different samples varied (Table 4). Dmrt1 was also checked by real-time PCR with no expression level found in the 3-year old female gonad, so we failed to do the further statistics analysis for the real-time PCR.

**DISCUSSION**

**Transcriptome efficiency**

Sturgeons are bony fish with remaining structural traits typical of the more primitive Chondrostei and are considered to be in the transitional position from Chondrostei to bony fish (Icardo et al., 2002). Thus, the study of the reproduction and sex-differentiation of sturgeons will enrich the research on evolutionary mechanism of the sex-determination and differentiation in fish. However, limited genetic information for Dabry’s sturgeon has hindered studies at the molecular level. This study was the first attempt at testicular and ovarian transcriptome sequencing in *A. dabryanus* using the Illumina HiSeq sequencing platform. The current study conducted deep sequencing of *A. dabryanus* encompassing over 10 Gb data for each library. In addition, we exclusively investigated gonads of immature phase to obtain information on the gene expression profile between the two genders. We used RNA-seq technology to identify a large number of unigenes annotated in various protein databases that can assist in the subsequent study of the gene expression profiling of *A. dabryanus*. After assembling, we obtained 91,375 unigenes with a mean length of 989 bp. The average length is much longer than that of the unigenes identified in previous studies (Chen et al., 2016; Hale, Jackson & Dewoody, 2010; Chen et al., 2018, PeerJ, DOI 10.7717/peerj.5389).

| Gene   | FPKM (T/O) | qPCR(T/O) |
|--------|------------|-----------|
| Wnt4   | 3.18       | 2.58      |
| Lhx1   | 0.29       | 0.16      |
| Zp     | 0.09       | 0.25      |
| Spata4 | 7.30       | 18.81     |
| Spata7 | 56.74      | 80.63     |
| Sox9   | 3.84       | 2.64      |
| Spata17| 28.19      | 38.50     |
| Spata13| 3.19       | 15.28     |
| Dmrt1  | 12.14      | –         |

Notes:

T/O, the relative expression level of genes in testis compared to that in ovary; T, testis; O, ovary. “–” means there was no expression of the gene found in 3-year-old ovary.
Yue et al., 2015). Moreover, completeness analysis showed that our A. dabryanus transcriptome assembly contained 84% of the BUSCO core eukaryotic gene set, indicating the acceptable quality and high integrity of our assembled data. The large numbers of unigenes obtained in this study will be useful for future analyses of genes related to sex differentiation, reproduction, and marker detection in Dabry’s sturgeon.

**Genes related to reproduction**

The *Dmrt1* gene is implicated in male determination and testis development in diverse metazoan phyla (Zhu et al., 2000; Hodgkin, 2002). In birds, which have ZW sex determination systems, previous studies have confirmed that *Dmrt1* was located on the Z chromosome but was absent from the W chromosome (Chue & Smith, 2011). However, the expression pattern of *Dmrt1* in fish is more complicated. In some species such as *Oryzias latipes* (Kobayashi et al., 2004), *Oreochromis niloticus* (Kobayashi et al., 2008), *Clarias gariepinus* (Raghuveer & Senthilkumaran, 2009), *Gobiocypris rarus* (Zhang, Zha & Wang, 2008), and *Paralichthys olivaceus* (Jo et al., 2007), *Dmrt1* was found to be specifically expressed in males, while in *D. rerio* (Guo et al., 2005), *O. mykiss* (Marchand et al., 2000), *Gadus morhua* (Johnsen et al., 2010), *Odontesthes bonariensis* (Fernandino et al., 2008), and *Silurus meridionalis* (Liu, Zhang & Wang, 2010), *Dmrt1* was found to be significantly up-regulated in males. Except for Adriatic sturgeon, which lacks the *Dmrt1* gene in both gonads (Vidotto et al., 2013), gonadal dimorphic *Dmrt1* expression has also been found in most sturgeons. The male-restricted and male-biased expression of *Dmrt1* was reported in Russian sturgeon (Chen et al., 2016) and lake sturgeon (Hale, Jackson & Dewoody, 2010), respectively, while no significant difference in its expression in the testes and ovaries was found in Amur sturgeon (Jin et al., 2015) and Chinese sturgeon (Yue et al., 2015). In the present study, *Dmrt1* expression was higher in males than in females (Fig. 8). The result indicated a strongly biased expression of *Dmrt1* in male Dabry’s sturgeons which is in accordance with the expression profile of *Dmrt1* in most other fish species.

The *Sox9* gene encodes a transcription factor that is critical for testis determination and development in vertebrates (Sinclair et al., 1990; Gubbay et al., 1990). Mutations in *Sox9* cause campomelic dysplasia, a human skeletal dysmorphology syndrome often associated with male to female sex reversal (Wagner et al., 1994; Foster et al., 1994). *Sox9* was also found to be expressed in fish and showed high expression levels in the testes compared with those in the ovaries in several species, such as *O. mykiss* (Takamatsu et al., 1997) and *Monopterus albus* (Zhou et al., 2003). Interestingly, the expression of *Sox9* in medaka was predominantly observed in the adult ovary, whereas in the testis, its expression was extremely low (Nakamoto et al., 2005; Yokoi et al., 2002), suggesting that the function of *Sox9* in the gonad is quite different in medaka and that the role of *Sox9* gene in sex-determination in fish is more complicated than in mammals.

In sturgeons, the expression of the *Sox9* gene did not differ significantly in the ovary and testis in most of the studied species, such as Chinese sturgeon (Yue et al., 2015), lake sturgeon (Hale, Jackson & Dewoody, 2010) and Amur sturgeon (Jin et al., 2015). However, a recent study of *Sox9* in Russian sturgeon found that this gene was specifically expressed...
in males and revealed that the Sox9 gene was responsible for the development of male gonads (Chen et al., 2016). In the present study, we found a significantly higher expression of Sox9 in males than in females in the transcriptome of Dabry’s sturgeon (Fig. 8). The result suggested that, similar to Dmrt1, the Sox9 gene might play a specific role in testis differentiation and maintenance in Dabry’s sturgeon.

The spermatogenesis-associated gene (Spata) was reported to be a candidate spermatocyte apoptosis-related gene involved in the regulation of apoptosis during spermatogenesis (Groh et al., 2013). Spata4 was previous confirmed to be specifically expressed in testis ranging from mammals to birds (Liu et al., 2004; Xie et al., 2007). While in D. rerio and O. mykiss, the expression of Spata4 is also restricted to gonads but with a slight expression level which can be detected in ovary (Liu et al., 2005a, 2005b).

In the present study, Spata4 showed a significantly higher expression level in males than females (Fig. 8), indicating that the function of this protein in sturgeon is in agreement with that in other fish. Besides Spata4, it is noticeable that all other genes belong to the Spata family demonstrated a male-bias expression profile, especially Spata7 and Spata17, whose expression level in males was nearly 60-fold and 30-fold higher than that in females (Fig. 8; Table 4), suggesting a potential role of this gene during spermatogenesis in Dabry’s sturgeon.

Besides male-dominant expressed genes, we also found a significant up-regulated gene in the ovary named zona pellucida protein (Zp). Zp is a specialized extracellular matrix surrounding the developing oocyte and plays important roles in oocyte development and protection, fertilization, and spermatozoa binding (Hinsch et al., 1994; Gupta et al., 2012). In mice, the presence of an intact Zp could significantly improve the post-vitrification survival of oocytes compared to oocytes without Zp (Choi et al., 2015). In Dabry’s sturgeon, the expression of Zp in the ovary was over 10-fold higher than in the testis. The present result was consistent with the expression profile of Zp in Chinese sturgeon which found Zp was the primarily up-regulated gene in the ovary (Fig. 8). This finding implies that the Zp gene in Dabry’s sturgeon as well as other sturgeons may have similar functions to those found in other vertebrate and mammalian species, potentially indicating an essential role of Zp in oocyte maintenance.

Fem1 is a sex-determining gene in Caenorhabditis elegans that functions in the signaling pathway that controls sex determination, and it is required in both germline and somatic tissues (Gaudet, VanderElst & Spence, 1996; Spence, Coulson & Hodgkin, 1990). Fem1 has also been found in humans, mice, and zebrafish, and the sequence shows high conservation across vertebrates, implying its conserved role in developmental functions (Ventura-Holman et al., 2003). In the transcriptome of Dabry’s sturgeon, Fem1 was identified but no differential expression was found between the sexes, consistent with previous studies in Amur sturgeon (Jin et al., 2015) and Chinese sturgeon (Yue et al., 2015) and suggesting that in sturgeons, Fem1 may not play a key role in sex differentiation.

Other genes involved in the formation and maintenance of germ cells were also found in the transcriptome of Dabry’s sturgeon. In mice, the LIM homeobox gene is essential for mouse gonad formation, and a lack of Lhx expression will lead to a failure to form a
discrete gonad (Birk et al., 2000). In Dabry’s sturgeon, we found LIM homeobox family genes Lhx1 and Lhx9. The Lhx1 gene was expressed significantly higher in females than in males (Fig. 8), while Lhx9 was not differentially expressed between the two gonad types. Lhx1 was found to be uniquely present in the testis transcriptome in Chinese sturgeon (Yue et al., 2015), contrary to the findings in the present study. Therefore, the accurate function of the LIM homeobox gene in sturgeons requires further investigation.

The Wnt genes are sources of differentiation-inducing signals during normal developmental events and Wnt4 has been clearly established to play a role in ovarian differentiation in mammals (Nusse & Varmus, 1992). In mice, the absence of Wnt4 in female embryos results in the masculinization of XX gonads (Vainio et al., 1999). Compared with the specific role discovered in mammals, little is known regarding the role in gonad differentiation of Wnt4 gene in fish. In a previous study, Wnt4 was not detected in Chinese sturgeon while in Dabry’s sturgeon, Wnt4 was identified in the ovary and testis with a significant sexual dimorphism in favor of male (Fig. 8). The male-dominant expression profile of Wnt4 was also observed in some other fish such as O. mykiss (Nicol et al., 2012) and Cynoglossus semilaevis (Hu et al., 2014). The different expression profile of Wnt4 between mammals and fish indicates that Wnt4 might play diverse roles in development of the reproductive system.

Identification of markers

Simple sequence repeats are tandem repeat DNA sequences that are highly polymorphic and are increasingly used as marker systems in molecular genetics studies, including research on parentage analysis (Poetsh et al., 2012), quantitative trait locus mapping (Keong et al., 2014), marker-assisted selection (Song et al., 2012) and population genetics (Remington et al., 2001). By transcriptome sequencing, we identified a large number of SSRs and analyzed the types and frequencies. In the transcriptome of Dabry’s sturgeon, dinucleotides were the most common microsatellites (accounting for up to 96.3%) if mononucleotide repeats were not taken into consideration, suggesting the existence of diverse dinucleotide repeat motif loci in Dabry’s sturgeon which are similar to those in other sturgeons such as Chinese sturgeon (Yue et al., 2015) and Russian sturgeon (Chen et al., 2016). Since Dabry’s sturgeon is a critically endangered species, SSRs identified from the transcriptome are useful markers for further assessment of genetic diversity and studies of population structure. Moreover, RAPD and AFLP have been applied in sturgeons to screen for sex-linked markers, but no sex-specific loci have been found (Wuertz et al., 2006; Keyvanshokooh, Pourkazemi & Kalbassi, 2007). Thus, the co-dominant microsatellite marker may be a more effective approach to search for sex-related markers in polyploid species.

CONCLUSION

In the present study, we conducted a comparative analysis of gonadal transcriptomes in Dabry’s sturgeon. A total of 5,396 differentially expressed genes were found between both sexes, with 1,938 up-regulated in ovaries and 3,458 in testes. A total of 52 candidate genes known to be involved in sex differentiation, determination, and sexual development were
found in the transcriptome of Dabry’s sturgeon. All genes belonging to the Spata family demonstrated a male-dominant expression profile, suggesting the crucial role of these genes during spermatogenesis in Dabry’s sturgeon. The male-biased expression of Dmrt1 and Sox9 in Dabry’s sturgeon also implied their potential role during testis development or differentiation. Similar to Chinese sturgeons, the expression of Zp in the ovary was significantly higher than in testis in Dabry’s sturgeon, indicating its potential conserved role in oocyte maintenance in vertebrate species. In addition, 24,271 SSRs and 550,519 SNPs were detected, which will assist further research on the discovery of sex-related markers.

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Competing Interests
The authors declare that they have no competing interests.

Author Contributions
• Yeyu Chen conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
Ya Liu conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
Quan Gong conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
Jiansheng Lai performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
Mingjiang Song performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
Jun Du conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft.
Xiaochuan Deng prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

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Data Availability
The following information was supplied regarding data availability:
Transcriptome sequencing data have been deposited to the NCBI Sequence Read Archive (SRA) database under accession numbers SRR6167299, SRR6172670, SRR6173479, SRR6175505, SRR6179331, and SRR6179394.

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