**De Novo** Transcriptome Analysis of Two Seahorse Species (*Hippocampus erectus* and *H. mohnikei*) and the Development of Molecular Markers for Population Genetics

Qiang Lin¹*, Wei Luo¹, Shiming Wan², Zexia Gao²

1 Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, Guangdong, 510275, China, 2 Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education/Key Lab. of Freshwater Animal Breeding, Ministry of Agriculture, College of Fisheries, Huazhong Agricultural University, Freshwater Aquaculture Collaborative Innovation Center of Hubei Province, Wuhan, 430070, China

* linqiangzsu@163.com

**Abstract**

Seahorse conservation has been performed utilizing various strategies for many decades, and the deeper understanding of genomic information is necessary to more efficiently protect the germplasm resources of seahorse species. However, little genetic information about seahorses currently exists in the public databases. In this study, high-throughput RNA sequencing for two seahorse species, *Hippocampus erectus* and *H. mohnikei*, was carried out, and de novo assembly generated 37,506 unigenes for *H. erectus* and 36,113 unigenes for *H. mohnikei*. Among them, 17,338 (46.23%) unigenes for *H. erectus* and 17,900 (49.57%) for *H. mohnikei* were successfully annotated based on the information available from the public databases. Through comparing the unigenes of two seahorse species, 7,802 candidate orthologous genes were identified and 5,268 genes among them could be annotated. In addition, gene ontology analysis of two species was similarly performed on biological processes, cellular components, and molecular functions. Twenty-four and twenty-one unigenes in *H. erectus* and *H. mohnikei* were annotated in the biosynthesis of unsaturated fatty acids pathways, and both seahorses lacked the Δ12 and Δ15 desaturases. Total of 8,992 and 9,116 SSR loci were obtained from *H. erectus* and *H. mohnikei* unigenes, respectively. Dozens of SSR were developed and then applied to assess the population genetic diversity, as well as cross-amplified in a related species, *H. trimaculatus*. The *H_C* and *H_E* values of the tested populations for *H. erectus*, *H. mohnikei*, and *H. trimaculatus* were medium. These resources would facilitate the conservation of the species through a better understanding of the genomics and comparative genome analysis within the *Hippocampus* genus.
Introduction

Seahorses belong to the family Syngnathidae, genus *hippocampus*, which is an iconic and ecologically important species globally, and so far they are relevant in many issues in marine conservation [1, 2]. Seahorses are frequently captured as trawl by catch, and their habitats are vulnerable to destruction, which leads to great pressure on wild stocks of seahorses [1, 3]. The heavy trade of seahorses for the Chinese traditional medicine, and ornamental and curio markets has caused a dramatic decline in many tropical source regions throughout the world [4, 5]. Therefore, in Endangered Species of Wild Fauna and Flora, seahorses have been listed in Appendix II of the Convention on International Trade [6].

Fortunately, seahorse aquaculture sprung up over the past ten years has been deemed as an effective strategy to partially decrease fishing pressure on wild seahorse population [2, 7–9]. However, the low germplasm resources of seahorses are still restricting the development of seahorse culture [10]. Developing predominant varieties and improving aquaculture techniques for seahorses are the important strategies to relieve the stress imposed by overfishing. So far, the relevant researches mainly focus on the biology [11], conservation [1], and aquaculture of seahorse species [9, 12, 13]. One of the key obstacles for selective breeding of seahorses is the lack of knowledge of their transcriptomic or genomic information, and availability of molecular markers.

In the recent years, the high-throughput sequencing technology has become a quite effective approach for a large amount of genes discovery on a genome-wide scale for non-model organisms [14]. RNA-seq and DNA-seq have opened the way to study functional and genetic information for many organisms [15]. As to a species of interest with no or little available genomic resources, transcriptome sequencing could offer a cost-effective method for high-throughput genes discovery. Compared to the full genome sequencing projects, transcriptome sequencing projects are more computationally tractable, but still can generate sufficient resources to meet the requirements of many non-model species’ researching projects [16].

Microsatellites (SSRs), which have many useful properties, such as good reproducibility, high polymorphism and codominant inheritance, have been widely used in population genetics, germplasm resource analysis [15–18] and molecular assisted selection (MAS) [19, 20]. Currently, only a few genetic markers are available for seahorses, including mitochondrial DNA (mtDNA) [21, 22] and microsatellites [23–25] developed by traditional methods. These data, however, have partially limited applicability for the breeding and germplasm improvement of seahorses.

The conservation and management of seahorse populations are of great international concern [26]. Among the seahorse species, the lined seahorse (*Hippocampus erectus*) and Japanese seahorse (*H. mohnikei*) have been suggested as the candidate species for aquaculture [2, 5, 8]. These two seahorse species are differed significantly from their morphology and distributions. *H. erectus* is larger in body size than that of *H. mohnikei* and it exists widely in the western Atlantic Ocean, and especially the area around the Gulf of Mexico, and *H. mohnikei* is distributed around the area of the North-western Pacific Ocean [1, 27]. Due to the insufficiency of available genomic or transcriptomic resources in the genus *Hippocampus*, Illumina Solexa sequencing technology was utilized to characterize the transcriptomes of *H. erectus* and *H. mohnikei*, and then the SSR markers were developed according to the transcriptome data to study the population genetics in seahorses.

Materials and Methods

Sample collection and preparation

*H. erectus* were sampled from a cultured population in Florida, United States (n = 40), and all the seahorses were cultured in indoor re-circulating holding tanks (90×80×60 cm) at Vero...
Beach marine laboratory, and the samples were approved only for the experimental usage by Florida Institute of Technology. *H. mohnikei* were collected from Yangmadao, China (n = 27), and *H. trimaculatus* were sampled along the China’s coast (n = 72). All the locations for seahorses sampling were absolutely approved that there were no any endangered or protected species by the local Marine and Fishery Bureaus (A201201I03) and Chinese Academy of Sciences (KZCX2-EW-QN206). Besides a few specimens (n = 17) were obtained with the help of the local fishers and buyers, most samples were captured by researchers using trawl boats. Cyt b and COI sequences from the tissue of dorsal fin (0.3×0.4 cm) of the seahorses were used to complete the species identifications before the further study [28], and all specimens were kept in 95% alcohol to extract genomic DNA for the evaluation of genetic diversity of seahorses (Table 1). The seahorses utilized in this study have been fully approved for the use of research work by the South China Sea Institute of Oceanology, Chinese Academy of Sciences (SCSIO-CAS). All samples used have received animal ethics approval for experimentation by the CAS (2011250).

The obtained seahorse samples for sequencing were sponsored by Zhanjiang Seahorse Center of the SCSIO-CAS, and the seahorses were kept in recirculating holding tanks (70×50×40 cm), and the seawater was from South China sea and treated with double sand filtration with the temperature 26±2°C, pH 8.1±0.2, and salinity of 31±0.5‰ for two weeks prior to sampling. All the seahorses were fed twice a day (0900 and 1400 h) with frozen *Mysis* sp. and live *Artemia* and uneaten food were siphoned out daily. One year old *H. erectus* and *H. mohnikei* were used for cDNA library construction through the euthanasia. Samples of the tissues, including brain, pituitary and ovary (development at stage V) were collected from three seahorses for each species, and all the seahorses were sacrificed after being anaesthetized for 30 min by MS-222 (Vivify Nature, China), which is approved for fish by FDA (Food and Drug Administration, USA). The sampling method was approved by the equivalent animal ethics committee of the CAS, and all the sampling procedures were specifically approved as part of obtaining the field permit. All samples were frozen immediately in liquid nitrogen, and stored at -80°C until use.

**RNA isolation**

Three seahorses in each species were used for RNA isolation, and fresh tissues including brain, pituitary and ovary were dissected and isolated RNA immediately. Total RNA was isolated using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). The quantity and quality of total RNA of each tissue sample was measured using a Nanodrop 2000 (Thermo Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies). For each species, the 0.50 μg

---

Table 1. Sampling location and sample size (n) of *H. erectus, H. mohnikei* and *H. trimaculatus. H. erectus* were cultured and approved only for the experimental usage by Florida Institute of Technology (FIT).

| Species       | Location         | Latitude  | Longitude | n  | Provider            |
|---------------|------------------|-----------|-----------|----|---------------------|
| *H. erectus*  | Vero Beach, FL, USA | 27.39°N   | 80.23°W   | 40 | Researcher in FIT   |
| *H. mohnikei* | Yangmadao        | 37.50°N   | 121.65°E  | 27 | Researcher          |
| *H. trimaculatus* | Fuzhou        | 26.08°N   | 119.14°E  | 23 | Researcher          |
|               | Quanzhou         | 24.53°N   | 118.36°E  | 3  | Buyer               |
|               | Shantou          | 23.02°N   | 116.28°E  | 16 | Researcher          |
|               | Yangjiang        | 21.65°N   | 112.20°E  | 1  | Researcher          |
|               | Haikou           | 20.03°N   | 110.13°E  | 4  | Fisher              |
|               | Qinghai          | 19.25°N   | 110.46°E  | 15 | Researcher          |
|               | Sanya            | 18.15°N   | 109.27°E  | 10 | Fisher              |

DOI:10.1371/journal.pone.0154096.t001
RNA from each tissue of brain, pituitary and ovary was combined and mixed into a single pool for the final usage. The RNA pools were DNase-treated using Turbo DNA-free (Ambion) and then purified with RNeasy Mini Kit (QIAGEN).

**cDNA library preparation and sequencing**

The construction of the libraries and the RNASeq were performed by Shanghai OE Biotech Company (Shanghai, China). The poly (A) mRNA purification, mRNA fragmentation and the cDNA library preparation for transcriptome sequencing were conducted using TruSeq RNA Sample Preparation kit v2 (Illumina Inc. San Diego, CA, USA; Catalog IDs 15025062). Then, the paired-end cDNA library with an insert size of 200 bp was prepared in accordance with Illumina’s protocols. The cDNA libraries were sequenced on the Illumina HiSeq2000 genomic sequencer platform at Shanghai OE Biotech Company, China. The sequencing of *H. erectus* and *H. mohnikei* were performed respectively.

**Transcriptome data processing and assembly**

For assembly, the clean reads were obtained through removing raw reads with adaptor and unknown nucleotides above 5% or those that were of low quality (containing more than 50% based with Q-value \( < 20 \)) were removed to obtain clean reads using a custom Perl script by Shanghai OE Biotech Company, China. Then the clean sequence data obtained for *H. erectus* and *H. mohnikei* were used to do *de novo* assembly, respectively. The Trinity software [14] (http://trinityrnaseq.sourceforge.net/, Version r2013_08_14) were used for *de novo* transcriptome assembly with default parameters using Kmer = 25, which had been used in many publications related to transcriptome data analysis [15, 16]. The contigs and unigenes with length of less than 200 bp were discarded due to a low annotation rate [16, 29]. The clean reads of the *H. erectus* and *H. mohnikei* were submitted to the NCBI Sequence Read Archive under the accession number of SRX535337, with SRR1275087 for *H. erectus* and SRR1280030 for *H. mohnikei*. The assembled unique sequences were deposited in the NCBI TSA DataSets under accession number of SUB531774.

**Functional annotation, CDS prediction and orthologous genes identification**

To annotate the transcriptomes of *H. erectus* and *H. mohnikei*, functional annotations were performed by sequence comparison with public databases included the NCBI non-redundant protein database (Nr, by December 2014) (http://www.ncbi.nlm.nih.gov), Swiss-Prot database (http://www.expasy.ch/sprot), Clusters of orthologous groups for eukaryotic complete genomes database (KOG) (ftp://ftp.ncbi.nih.gov/pub/COG/KOG/kyva) and Kyoto encyclopedia of genes and genomes (KEGG) pathway database (http://www.genome.jp/kegg/) using BLASTX alignment with an E-value of \( 1e^{-5} \), respectively. Based on the results of Nr database annotation, Blast2GO program [30] were used to perform GO annotation of unigenes and then WEGO [31] software was used to perform GO classification, describing biological processes, molecular functions and cellular components for every transcript.

The coding sequences (CDS) for unigenes were predicted by BLASTX and ESTScan. The transcript sequences were searched against the Nr, KOG, KEGG and Swiss-Prot protein databases using BLASTX (e-value \( < 10^{-5} \)). The best alignment results were used to determine the sequence direction of unigenes. When a transcript could not be aligned to any database, ESTScan Program [32] was used to predict coding regions and determine sequence direction.

The putative orthologous genes between the unigenes of *H. erectus* and *H. mohnikei* were searched by BLASTN program of BLAST 2.2.28+ software with E-value of \( 1e^{-5} \). Through using
the reciprocal best hit method with the BLASTN algorithm, the two unigenes from *H. erectus* and *H. mohnikei* with the best BLAST results (≥ 60%) were taken as the candidate orthologous genes.

SSRs identification and cross-amplification

All the obtained unigenes were used to search for microsatellite makers using MISA program [33] based on the criteria of a repeat threshold of six for di-, five for tri-, tetra-, penta- and hexa-nucleotide repeats. Considered the requirements for primer design, the sequences without containing 50-bp sequence on both sides of the microsatellite repeat were discarded for further analysis.

To validate the SSR makers, 10 individuals of two species were used at 87 and 94 random SSRs loci for *H. mohnikei* and *H. trimaculatus*, respectively. Total DNA was isolated from the dorsal fin of seahorse by using the Phenol/Chloroform procedure. PCR amplifications were conducted in a final volume of 10 µL containing 50 ng templates DNA, 1× PCR buffer, 2.0 mM MgCl₂, 2.5 mM dNTPs, 2.5 µM of each primer, and 1 U Taq polymerase. The PCR reaction cycling profile was 95°C for 5 min followed by 30 cycles of 35 s at 95°C, 40 s at primer-specific annealing temperature, 45 s at 72°C, and a final extension at 72°C for 8 min. The amplified PCR products were separated on 8% non-denaturing polyacrylamide gels at 150 V lasting 2 h and visualized via silver-staining.

To determine the cross utility of microsatellite markers developed in this study, cross-species amplification of the identified markers was assessed between *H. mohnikei* and *H. trimaculatus*. The identified markers for *H. mohnikei* and *H. trimaculatus* were also tested in another related seahorse’s species, *H. trimaculatus*.

Genetic diversity analysis in seahorses

The identified polymorphic SSR markers were employed to analyze the genetic diversity of *H. erectus*, *H. mohnikei*, and *H. trimaculatus* populations. The software Popgene 32 was utilized to calculate the number of alleles (*Nₐ*), observed heterozygosity (*Hₒ*), expected heterozygosity (*Hₑ*), and Hardy-Weinberg equilibrium (HWE). The results were adjusted for multiple simultaneous comparisons using a sequential Bonferroni correction. Polymorphism information content (PIC) values were calculated using the formula PIC = 1 - Σ*Pᵢ*², in which *Pᵢ* is the frequency of the *i*th allele [32].

Results

Illumina sequencing and de novo assembly

The total of 50,948,904 and 49,476,082 raw sequencing reads were generated for *H. erectus* and *H. mohnikei* using Illumina paired-end sequencing technology, respectively. After quality control of raw data, which removing and trimming the low-quality reads, adapters, poly-A tails, and reads containing more than 5% unknown nucleotides, approximately 4,090,677,279 bp and 3,960,288,422 bp of high-quality data were achieved, yielding total of 42,642,934 and 41,331,060 clean reads for *H. erectus* and *H. mohnikei*, respectively. The overview of the sequencing and assembly statistics are shown in Table 2.

The clean reads obtained for *H. erectus* and *H. mohnikei* were used to assemble the transcriptome for each species using the Trinity de novo assembler, respectively. The total of 337,628 contigs consisting of 673,692,247 bp for *H. erectus* and 283,894 contigs consisting of 463,004,720 bp for *H. mohnikei* were assembled firstly. In *H. erectus*, the size of the contigs ranged from 201 to 23,393 bp, with a mean length of 1,995 bp and N50 length of 3,572 bp (Fig 1).
As to *H. mohnikei*, the maximum size of the contigs was 24,257 bp, with a mean length of 1,631 bp and N50 length of 3,004 bp (Fig 1). Among these contigs, 188,279 (55.77%) for *H. erectus* and 139,806 (49.25%) for *H. mohnikei* were longer than 1,000 bp. These contigs were further assembled into unigenes using paired-end joining and gap-filling methods. The total of 37,506 unigenes with a mean length of 1,317 bp for *H. erectus* and 36,113 unigenes with a mean length of 1,335 bp for *H. mohnikei* were obtained in the study. There were 14,843 unigenes (39.58%) for *H. erectus* and 14,880 unigenes (41.20%) for *H. mohnikei* longer than 1,000 bp, as well as 7,353 unigenes (19.60%) for *H. erectus* and 7,320 (20.27%) for *H. mohnikei* longer than 2,000 bp. The size distribution of these unigenes was shown in Fig 1. In this study, the coding sequences (CDS) from *H. erectus* and *H. mohnikei* unigenes sequences were also detected and a total of 27,487 and 27,446 CDSs were obtained, respectively, among them, 7,211 (26.23%) and 7,367 (26.84%) CDSs were longer than 1,000 bp for *H. erectus* and *H. mohnikei*, respectively (Fig 1). These unigenes have been deposited in the NCBI Sequence Read Archive (SRA) database (accession number: SRR2922398, SRR2922418).

### Functional annotation

The total of 17,338 unigenes (46.23%) for *H. erectus* and 17,900 unigenes (49.57%) for *H. mohnikei* were annotated based on the information available from public databases including Nr, Swiss-Prot protein, KOG and KEGG using BLASTX with an E-value cut-off of $<10^{-5}$ (Table 3; S1 File). Among them, 5,500 unigenes for *H. erectus* and 5,709 unigenes for *H. mohnikei* showed significant matches to all four databases. Unigenes of *H. erectus* that were annotated in public databases are as follows: 17,268 (46.04%), 15,674 (41.79%), 13,169 (35.11%) and 6,403 (17.07%) unigenes in Nr, Swiss-Prot, KOG and KEGG, respectively. For *H. mohnikei*, there were 17,810 (49.32%), 16,428 (45.49%), 13,607 (37.68%) and 6,601 (18.28%) unigenes significantly matched to Nr, Swiss-Prot, KOG and KEGG among 36,113 assembled unigenes, respectively, which were similar to *H. erectus*. Furthermore, about 53.77% of unigenes (20,168) for *H. erectus* and 50.43% of unigenes (18,213) for *H. mohnikei* did not show any matches to known genes.

Our results showed that approximately 80% of unigenes over 1,000 bp in length in the both species had BLAST matches against the Nr database; however, only about 25% of unigenes

---

**Table 2. Summary of Illumina paired-end sequencing and assembly for *H. erectus* and *H. mohnikei*.**

| Database          | *H. erectus* | *H. mohnikei* |
|-------------------|--------------|---------------|
| Total raw reads   | 50,948,904   | 49,476,082    |
| Total clean reads | 42,642,934   | 41,331,060    |
| Number of contigs | 337,628      | 283,894       |
| Average length of contigs (bp) | 1,995 | 1,631 |
| Max length of contigs (bp) | 23,393 | 24,257 |
| Min length of contigs (bp) | 201 | 201 |
| Contig size N50 (bp) | 3,572 | 3,004 |
| Number of unigenes | 37,506 | 36,113 |
| Average length of unigenes (bp) | 1,317 | 1,335 |
| Max length of unigenes (bp) | 23,393 | 27,745 |
| Min length of unigenes (bp) | 201 | 201 |
| Transcript size N50 (bp) | 2,215 | 2,205 |
| Number of unigenes > = 1000 bp | 14,855 | 14,891 |

doi:10.1371/journal.pone.0154096.t002
Fig 1. Length distribution of all contigs, unigenes and CDSs for *H. erectus* and *H. mohriakei* (Unit: bp).

doi:10.1371/journal.pone.0154096.g001

Table 3. Summary of annotation percentage of *H. erectus* and *H. mohriakei* unigenes as compared to public database.

| Database   | *H. erectus* | *H. mohriakei* |
|------------|--------------|----------------|
|            | Number of unigenes | Annotation percentage (%) | Number of unigenes | Annotation percentage (%) |
| Nr         | 17,268       | 46.04           | 17,810           | 49.32            |
| SwissProt  | 15,674       | 41.79           | 16,428           | 45.49            |
| KEGG       | 6,403        | 17.07           | 6,601            | 18.28            |
| KOG        | 13,169       | 35.11           | 13,607           | 37.68            |
| All annotated unigenes | 17,338       | 46.23           | 17,900           | 49.57            |
| Total unigenes | 37,506       |                 | 36,113           |                 |

doi:10.1371/journal.pone.0154096.t003
with lengths shorter than 1,000 bp in both species generated BLAST matches (S1 File). The same tendency was also observed in BLAST results against the SwissProt database. The e-value distribution of the top hits in the Nr database revealed that 78.28% of the mapped unigenes for *H. erectus* and 78.57% of the mapped unigenes for *H. mohnikei* showed significant homology (e-value < 10^{-50}). The species distribution of the best matches for each sequence was as shown in Fig 2. For both species, zebra mbuna (*Maylandia zebra*) provided matches for 37.17% and 37.36% of sequences for *H. erectus* and *H. mohnikei*, respectively, followed by Nile tilapia (*Oreochromis niloticus*) (25.72% for *H. erectus* and 26.09% for *H. mohnikei*), pufferfish (*Takifugu rubripes*) (10.19% for *H. erectus* and 10.26% for *H. mohnikei*) and medaka (*Oryzias latipes*) (9.15% for *H. erectus* and 8.75% for *H. mohnikei*).

After the BLAST between the unigenes of the two seahorse species, a total of 7,802 pairs of putative orthologous genes were obtained (S2 File), using the reciprocal best hit method with the BLASTN algorithm. Among these unigenes, a total of 5,268 genes could be annotated based on the information available from public databases including Nr, Swiss-Prot, KOG and KEGG.

**Gene ontology and KOG classification**

Based on the Nr annotation, Gene Ontology (GO) classification was used to classify the functions of all unigenes. The total of 14,645 and 15,197 unigenes were assigned to one or more GO...
terms for *H. erectus* and *H. mohrikei*, respectively (S3 File). These unigenes were found to be involved in biological process, cellular components and molecular functions in *H. erectus* and *H. mohrikei*, respectively (Fig 3). In both species, unigenes related to cellular process (11,574 unigenes for *H. erectus* and 11,986 unigenes for *H. mohrikei*), cell (11,194 unigenes for *H. erectus* and 11,655 unigenes for *H. mohrikei*), and binding (10,262 unigenes for *H. erectus* and 10,645 unigenes for *H. mohrikei*) were all the most abundant genes in the biological processes, cellular components, and molecular functions, respectively.

All unigenes were subjected to a search against the KOG database for functional prediction and classification. In total, 13,169 unigenes for *H. erectus* and 13,607 unigenes for *H. mohrikei* were annotated and both grouped into 25 KOG classifications (Fig 4). However, some of these unigenes were assigned to multiple KOG classifications, and altogether 26,679 and 27,737 functional annotations were obtained for *H. erectus* and *H. mohrikei*, respectively. Among the 25 KOG categories, the cluster for signal transduction mechanisms [T] was the largest group in both tested species, with 7,264 in *H. erectus* and 7,694 in *H. mohrikei*, respectively.

**Metabolic pathways by KEGG analysis**

To identify the biological pathways in seahorses, unigenes were compared against the KEGG database and the corresponding pathways were established. Among the 37,506 unigenes of *H. erectus*, 6,403 (17.07%) had significant matches in the database and were assigned to 334 pathways. The pathway in cancer was involved in most unigenes (539), followed by PI3K-Akt
signaling pathway (463), focal adhesion (371), MAPK signaling pathway (339), and cAMP signaling pathway (332) (Fig 5; S4 File). Meanwhile, 6,601 (18.27%) unigenes of *H. mohnikei* were annotated to 335 pathways. The top three pathways with the greatest number of unigenes were the same to *H. erectus*, with pathways in cancer (528), PI3K-Akt signaling pathway (489) and focal adhesion (380) (Fig 5; S4 File).

To further understand the molecular interaction and reaction networks, the pathways of biosynthesis of unsaturated fatty acids in *H. erectus* and *H. mohnikei* were assigned based on KEGG database. Twenty-four and twenty-one unigenes in *H. erectus* and *H. mohnikei* were annotated to this pathway, respectively (S5 File). These unigenes encode 14 key enzymes involved in this pathways, including 3-oxoacyl-[acyl-carrier protein] reductase, enoyl-CoA hydratase/long-chain 3-hydroxyacyl-CoA dehydrogenase and 3-oxoacyl-[acyl-carrier protein] reductase. However, seahorses lacked the Δ12 and Δ15 desaturases (Fig 6).

**SSR marker discovery**

The potential SSRs were detected in all of the 37,506 assembled unigenes in *H. erectus* and 36,113 unigenes in *H. mohnikei* using MISA software. Of these unigenes, a total of 8,992 and 9,116 SSRs were identified for *H. erectus* and *H. mohnikei*, respectively, defined as di-to penta-nucleotide SSRs, with a minimum of six repetitions for dinucleotides and five repetitions for the remaining motifs. Of all the motif types, dinucleotides (5,508 for *H. erectus* and 5,284 for *H. mohnikei*) were the most frequent, followed by tri- (3,142 for *H. erectus* and 3,351 for *H. mohnikei*) and tetra-nucleotides (328 for *H. erectus* and 460 for *H. mohnikei*) (Table 4).
Transcriptome of Two Seahorse Species and Population Genetics Study

Fig 5. Top 15 KEGG pathways with the highest sequence numbers in H. erectus and H. mohnikei.
doi:10.1371/journal.pone.0154096.g005

Fig 6. Biosynthesis of unsaturated fatty acids pathways identified in H. erectus. Red boxes represent unigenes which are annotated to this pathway. Biosynthesis of unsaturated fatty acids pathways identified in H. mohnikei is the same as H. erectus.
doi:10.1371/journal.pone.0154096.g006
Among the microsatellites detected, the dominant classes of repeating sequences in the unigenes of both species were AC/GT (4,050 for *H. erectus* and 4,164 for *H. mohnikei*), followed by AG/CT (737 for *H. erectus* and 771 for *H. mohnikei*) and AAG/CTT (644 for *H. erectus* and 623 for *H. mohnikei*) repeats (Fig 7).

### SSRS identification and cross-amplification

In order to develop new molecular markers for seahorses, all the unigenes contained microsatellites were used to design primers. A total of 4,023 (44.7%) and 4,511 (49.5%) microsatellites could successfully design the primer pairs for PCR amplification using MISA software (S1 Table). In this study, 129 (including 57 di-, 45 tri-, and 27 tetra-nucleotide repeats) SSR loci of

| Seahorse species | Number | Di- | Tri- | Tetra- | Penta- | Hexa- | Total |
|------------------|--------|-----|------|--------|--------|-------|-------|
| *H. erectus*     | Number | 5,508 | 3,142 | 328 | 10 | 4 | 8,992 |
|                  | Length of motif | 15.86 | 16.95 | 20.85 | 25.00 | 34.50 | 9,116 |
|                  | Proportion | 61.25% | 34.94% | 3.65% | 0.11% | 0.04% |       |
| *H. mohnikei*    | Number | 5,284 | 3,351 | 460 | 13 | 8 |       |
|                  | Length of motif | 16.02 | 16.96 | 20.54 | 19.23 | 24.75 |       |
|                  | Proportion | 57.96% | 36.76% | 5.05% | 0.14% | 0.09% |       |

Among the microsatellites detected, the dominant classes of repeating sequences in the unigenes of both species were AC/GT (4,050 for *H. erectus* and 4,164 for *H. mohnikei*), followed by AG/CT (737 for *H. erectus* and 771 for *H. mohnikei*) and AAG/CTT (644 for *H. erectus* and 623 for *H. mohnikei*) repeats (Fig 7).

### Table 4. Characteristics of SSRs in *H. erectus* and *H. mohnikei*.

| Seahorse species | Number of motif repeats | Total |
|------------------|-------------------------|-------|
|                  | Di- | Tri- | Tetra- | Penta- | Hexa- |       |
| *H. erectus*     | 5,508 | 3,142 | 328 | 10 | 4 | 8,992 |
| *H. mohnikei*    | 5,284 | 3,351 | 460 | 13 | 8 |       |

Fig 7. Characteristics of top 15 dominant SSR motifs in *H. erectus* and *H. mohnikei*.

doi:10.1371/journal.pone.0154096.g007
randomly selected to check the successful amplification proportion of these SSRs. The results showed that 69 (53.49%) and 56 (50.91%) primer pairs of *H. erectus* and *H. mohnikei*, respectively, successfully amplified fragments; however, just 20 (15.50%) and 17 (15.45%) showed polymorphism in the tested populations for *H. erectus* and *H. mohnikei*, respectively, (S6 File; S7 File).

To test the cross utility of markers developed in this study, polymorphic markers from *H. erectus* and *H. mohnikei* were cross-amplified in each other and also tested in another related species, *H. trimaculatus*. Out of the 20 polymorphic SSRs loci from *H. erectus*, 14 (70.00%) and 16 (80.00%) were successfully amplified, and 4 (20.00%) and 8 (40.00%) showed polymorphism in *H. mohnikei* and *H. trimaculatus*, respectively (S1 Table). In addition, out of the 17 polymorphic SSRs loci of *H. mohnikei*, 10 (58.82%) and 10 (58.82%) were successfully amplified and 2 (11.76%) and 4 (23.53%) showed polymorphism in *H. erectus* and *H. trimaculatus*, respectively (S1 Table).

### Genetic diversity in seahorse populations

In the *H. erectus* cultured population, the number of alleles (\( N_A \)) per locus varied from 2 to 8, with a mean value of 4.65. The mean observed (\( H_o \)) and expected heterozygosity (\( H_e \)) were 0.53 and 0.62, respectively. The PIC values were ranged from 0.28 to 0.77, with a mean value of 0.56. In the Yangmadao population of *H. mohnikei*, the \( H_o \) and \( H_e \) values were ranged from 0.22 and 0.863, and from 0.17 and 0.84, respectively. The mean \( N_A \) was found to be 4.06, and the mean PIC value was 0.48. In the *H. trimaculatus* population along China’s coast, the \( H_o \) and \( H_e \) were ranged from 0.48 to 0.79 and from 0.52 to 0.71, respectively, and the mean \( N_A \) was 3.25, respectively. It was found that 10 loci out of 20 polymorphic SSRs loci for *H. erectus* deviated significantly from the Hardy-Weinberg equilibrium in its population (\( P < 0.05 \)), as well as 5 loci out of 17 polymorphic SSRs loci for *H. mohnikei* and 4 loci out of 8 polymorphic SSRs loci for *H. trimaculatus* (S1 Table).

### Discussion

The transcriptome exhibits the complete expressed RNA unigenes in the cell, and its characterization would be essential to understand the functional complexity of an organism [16]. The present study reported the transcriptome databases for seahorses *H. erectus* and *H. mohnikei*. Due to the unavailable of reference genome, the reads produced by Illumina HiSeq\textsuperscript{TM} 2000 (paired-end reads with 100 bp) were assembled using the *de novo* assembler Trinity [29]. The assembly results indicated that the length distribution pattern and mean length of unigenes (1,317.47 bp for *H. erectus* and 1,334.83 bp for *H. mohnikei*) were longer than those of recently studied aquatic species, such as Nile tilapia (*Oreochromis niloticus*) (618 bp) [34], Eastern Oyster (*Crassostrea virginica*) (1,023 bp) [35] and blunt snout bream (*Megalobrama amblycephala*) (692.9 bp) [36]. These results suggested that the transcriptome sequencing data for *H. erectus* and *H. mohnikei* in the present study were well assembled.

The values of annotation rate of unigenes for *H. erectus* (46.23%) for *H. mohnikei* (49.57%) based on the information available from public databases were similar to the other previously reported fish species, such as rohu carp (*Labeo rohita*) (47.45%) [37], mud loach (*Misgurnus anguillicaudatus*) (43.76%) [38]; while much lower than silver carp (*Hypophthalmichthys molitrix*) (63.2%) [39]. Many studies have suggested that the longer sequences were more likely to obtain BLAST matches in the protein databases [40, 41]. In our study, approximately 80% of unigenes over 1,000 bp in length in the both species had BLAST matches against the Nr database; however, only about 25% of unigenes with lengths shorter than 1,000 bp in the both
species generated BLAST matches, which maybe too short to obtain statistically meaningful matches. Meanwhile, for some unigenes, the absence of homologous sequences in the public databases may suggest their possible specific roles in seahorses. Moreover, it was found that the unigenes from both tested species had the best matches with *M. zebra* and *O. niloticus*, which both belonged to cichlid fish species. This result was consistent with the ray-finned fish phylogenetic tree based on nine nuclear genes [42]. We believe that the large-scale sequencing efforts on seahorse genome in the near future, as well as transcriptome will increase the coverage of our dataset even further.

Gene annotation and pathway analyses are helpful to predict potential genes and their possible functions at a transcriptome level. GO annotation and KOG classification analyses showed that the unigenes from both species presented a similar transcriptome, indicating that these two seahorses were closely genetically-related. Gene function categories associated with cellular processes, cells, and bindings are highly represented in both transcriptomes. These results are similarly found in *O. mykiss* [43] and *H. molitrix* [39]. Based on the KEGG pathway database, organismal systems represented the dominant pathway and contained 9 different subcategories, mainly including the nervous system, sensory system, immune system, excretory system, endocrine system, and system digestive system. These results indicated a large amount of genes involved in maintaining ordinary physiological function. It is unclear whether or not the pathway in human cancer is involved in a mass of unigenes in both seahorse species, which is similar to clawed frog (*Xenopus tropicalis*) [44] and frog (*Bombina maxima*) [45]. These results would provide the basic information for future studies, such as gene cloning, expression analysis and gene-associated markers identification.

Seahorse has high medicinal effect partly because of high content of highly unsaturated fatty acids (PUFA). Like other marine fish [46, 47], seahorse also lacks the Δ12 and Δ15 desaturases and so cannot form linoleic (18:2n-6) and α-linolenic (18:3n-3) acids from 18:1n-9. The biochemistry of PUFA synthesis, including pathways and reaction mechanisms, has been well described in fish [48]; however, until recently, little is known of the genes and gene products involved and of the factors affecting their expression in seahorse. In this study, many genes, which code the key enzymes in the pathway of biosynthesis of unsaturated fatty acids, were identified by blast against KEEG database, promoting the research on the metabolism and gene regulation of unsaturated fatty acids in seahorses and in other fish species.

Based on the transcriptome data, dinucleotide repeats were the most frequent SSR motif type in both *H. erectus* and *H. mohnikei*. This finding is consistent with results reported for channel catfish (*Ictalurus punctatus*) [49] and *M. amblycephala* [15]. Among the dinucleotide repeats, AC is the most frequent motif in our dataset, which was the same as *I. punctatus* [49], Japanese pufferfish (*Fugu rubripes*) [50], okaloosa darter (*Etheostoma okaloosae*) [51], and Tarim schizothoracin (*Schizothorax biddulphi*) [52]. Polymorphic SSR markers have become as one of the most popular genetic markers in some applications, such as in genetic diversity analysis, the genetic maps construction, quantitative trait loci mapping, marker assisted selection breeding and comparative genomics [49, 53]. Development of SSR markers is becoming more and more important with the tremendous development and rapid cost reduction of high through-put sequencing technology, which is because high through-put transcriptome sequencing not only produces an amount of sequence data for marker identification, but also because the obtained markers are gene-based [53]. The markers developed from transcriptome data are advantageous for their functional variation and useful for associated genetic studies [54]. So far, only a few SSR markers have been developed for seahorses [23–25, 55]. In the present study, a large amount of sequences containing SSR loci were obtained, and dozens of SSR markers were subsequently developed within a short time period. Generally, EST-derived SSR are more transferable between species than random genomic SSRs [56], and cross-species
amplification is both fast and inexpensive [55]. As the results showed in the present study, a middle rate of cross-amplification was found among the three seahorse species, suggesting the possibility of using primers inter-specifically among seahorse species.

The evaluation of genetic diversity might provide a guide for germplasm resource protection and breeding for seahorses. In the present study, the Ho values and He of the three seahorses were medium. This result was similar to the three populations of *H. trimaculatus* along the coasts of India by using 12 SSRs [25]. In addition, a large proportion of loci were found to deviate significantly from the Hardy-Weinberg equilibrium in this study. Singh also detected significant deviation in allele frequencies from Hardy-Weinberg equilibrium at a few loci [25]. The reasons for deviation from Hardy-Weinberg equilibrium in both researches were deficiency of heterozygotes at these loci. This deficiency of heterozygotes may due to the high inbreeding rate, relatively weak ability of swimming/mobility [1], and special mating pattern, i.e., “monogamy” [57]. Moreover, as seahorses are particularly vulnerable to human activities because of their lengthy durations of parental care, small brood sizes and faithful pair bonds [1], and the interference of human activities in near-coast waters, such as overfishing, habitat destruction and environment pollution, has a serious influence on the seahorse population genetic structure [22].

**Conclusion**

This study addressed the application of high through-put sequencing technology for seahorse *de novo* transcriptome sequencing and the most comprehensive study of seahorse transcriptome data. The large number of assembled unigenes and detected SSR markers obtained from the seahorse transcriptome indicated that Illumina paired-end sequencing could be used as a cost-effective and fast approach to discover novel genes and molecular markers for non-model organisms. Based on the sequences with SSR marker, dozens of SSR makers were developed, and they could be utilized in genetic diversity analysis and MAS. In addition, the transcriptome resources available for seahorses would facilitate the conservation management of the seahorse species through a better understanding of its biology, genomics, as well as comparative genome analysis within the *Hippocampus* genus.

**Supporting Information**

**S1 File. The annotation of unigenes of *H. erectus* and *H. mohnikei*.** The unigenes annotated based on the information available from public databases including NCBI non-redundant protein (Nr), Swiss-Prot protein, eukaryotic Orthologous Groups (KOG), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) for *H. erectus* (Table A in S1 File) and *H. mohnikei* (Table B in S1 File).

(XLS)

**S2 File. The candidate orthologous genes for *H. erectus* and *H. mohnikei* (Table A in S2 File).**

(XLS)

**S3 File. GO annotations for *H. erectus* and *H. mohnikei*.** The file contains 14,645 unigenes with a total of 11,484 term occurrences for *H. erectus* (Table A in S3 File), and 15,197 unigenes with a total of 11,543 term occurrences for *H. mohnikei* (Table B in S3 File) assigned to one or more GO terms.

(XLS)

**S4 File. Unigenes of *H. erectus* and *H. mohnikei* mapped to the KEGG pathway database.** The file contains 6,403 unigenes for *H. erectus* (Table A in S4 File) and 6,601 unigenes for *H.
mohnikei (Table B in S4 File) mapped to the KEGG pathway database.

S5 File. Unigenes annotated to biosynthesis of unsaturated fatty acids pathways in *H. erectus* (Table A in S5 File) and *H. mohnikei* (Table B in S5 File).

S6 File. The designed primer for the detected SSRs of *H. erectus* (Table A in S6 File) and *H. mohnikei* (Table B in S6 File).

S7 File. Part of the page photos of SSR markers identified in this study.

S1 Table. Characteristics of SSR isolated from *H. erectus* and *H. mohnikei*, and application in genetic diversity analysis. Characteristics of 20 polymorphic microsatellite loci isolated from *H. erectus* and cross-amplification in *H. mohnikei* and *H. trimaculatus*, and population genetic diversity of *H. erectus* (Table A in S1 Table); Characteristics of 17 polymorphic microsatellite loci isolated from *H. mohnikei* and cross-amplification in *H. erectus* and *H. trimaculatus*, and population genetic diversity of *H. mohnikei* (Table B in S1 Table); The population genetic diversity of *H. trimaculatus* (Table C in S1 Table).

Acknowledgments

We are very grateful to Dr. Zhang Yanhong for performing the RT-PCR to validate the genes gained from the transcriptomes. This study was funded by the Outstanding Youth Foundation in Guangdong Province (S2013050014802), the National Science Fund for Excellent Young Scholars (41322038), and the National Natural Science Foundation of China (41576145, 41306148). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

Conceived and designed the experiments: QL ZG. Performed the experiments: WL SW. Analyzed the data: QL WL. Contributed reagents/materials/analysis tools: QL SW ZG. Wrote the paper: QL WL ZG.

References

1. Lourie SA, Vincent ACJ, Hall HJ (1999) The taxonomy of Vietnam’s exploited seahorses (family Syngnathidae). Biol J Linn Soc 66: 231–256
2. Koldewey HJ, Martin-Smith KM (2010) A global review of seahorse aquaculture. Aquaculture 302: 131–152
3. Vincent ACJ (1996) The international trade in seahorses. Traffic International, Cambridge.
4. Martin-Smith KM, Vincent ACJ (2005) Seahorse declines in the Derwent estuary, Tasmania in the absence of fishing pressure. Biol Conserv 123: 533–545
5. Vincent ACJ, Foster SJ, Koldewey HJ (2011) Conservation and management of seahorses and other Syngnathidae. J Fish Biol 78: 1681–1724 doi: 10.1111/j.1095-8649.2011.03003.x PMID: 21651523
6. IUCN. (2011) IUCN red list of threatened species. [http://www.iucnredlist.org/]
7. Woods CMC (2000) Preliminary observations on breeding and rearing the seahorse, *Hippocampus abdominalis* (Teleostei: Syngnathidae) in captivity. New Zeal J Mar Fresh 34: 475–485
8. Lin Q, Lin JD, Huang LM (2009a) Effects of substrate color, light intensity and temperature on the survivorship and skin color change of the juvenile seahorse Hippocampus erectus Perry, 1810. Aquaculture 298: 157–161
9. Lin Q, Lin JD, Zhang D, Wang YB (2009b) Weaning of juvenile seahorses Hippocampus erectus Perry, 1810 from live to frozen food. Aquaculture 291: 224–229
10. Lin Q, Li G, Qin G, Gu N, Sun H, Feng P, et al. (2012) The dynamics of reproductive rates, offspring survivorship and growth in seahorses, Hippocampus erectus Perry, 1810. Biol. Open 1 (4): 391–396 doi: 10.1242/bio.2012398 PMID: 23214349
11. Vitturi R, Libertini A, Campolmi M, Calderazzo F, Mazzola A (1998) Conventional karyotype, nucleolar organizer regions and genome size in the five Mediterranean species of Syngnathidae (Pisces, Syngnathiformes). J Fish Biol 52: 677–687
12. Qin G, Zhang Y, Huang L, Lin Q (2014) Effects of water current on swimming performance, ventilation frequency, and feeding behavior of young seahorses (Hippocampus erectus). J. Exp. Mar. Biol. Ecol. 461: 337–343
13. Zhang YH, Qin G, Lin JD, Lin Q (2015) Growth, survivorship, air-bubble disease and attachment of the feeble juvenile seahorses Hippocampus kuda Bleeker, 1852. Journal of the World Aquaculture Society. 46 (3) 293–301.
14. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29: 64–652 doi: 10.1038/nbt.1883 PMID: 21572440
15. Gao Z, Luo W, Liu H, Zeng C, Liu XL, et al. (2012) Transcriptome analysis and SSR/SNP markers information of the blunt snout bream (Megalobrama amblycephala). PLoS ONE 7: e42637 doi: 10.1371/journal.pone.0042637 PMID: 22880060
16. Haas BJ, Papanicolaou A, Yassour M, Levin JZ, Thompson DA, et al. (2014) De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc 123: e783–e789
17. Huang W, Liang X, Qu CM, Cao JJ, Zhao C, et al. (2013) Development and characterization of novel polymorphic microsatellite loci in Siniperca scherzeri Steindachner and Siniperca chuatsi (Basilewsky). Mol Biol Rep 40: 751–756 doi: 10.1007/s11033-012-1958-4 PMID: 23224590
18. Wang W, Yi Q, Ma L (2014) Sequencing and characterization of the transcriptome of half-smooth tongue sole (Cynoglossus semilaevis). BMC Genomics 15: 470 doi: 10.1186/1471-2164-15-470 PMID: 24924151
19. Reid DP, Sztanto A, Glebe B, Danzmann RG, Ferguson MM (2005) QTL for body weight and condition factor in Atlantic salmon (Salmo salar): comparative analysis with rainbow trout (Oncorhynchus mykiss) and Arctic char (Salvelinus alpinus). Heredity 94: 166–172 PMID: 15483654
20. Luo W, Zeng C, Yi SK, Robinson N, Wang WM, et al. (2014) Heterosis and combining ability evaluation for growth traits of blunt snout bream (Megalobrama amblycephala) when crossbreeding three strains. Chinese Sci Bull 59: 857–864
21. Teske PR, Cherry MI and Matthee CA (2003) Population genetics of the endangered Knysna seahorse, Hippocampus capensis. Mol Ecol 12: 1703–1715 PMID: 12803625
22. Panithanarak T, Karuwancharoen R, Na-Nakorn U, Nguyen TTT (2010) Population genetics of the spotted seahorse (Hippocampus kuda) in Thai waters: implication for conservation. Zool Stud 49: 546–574
23. Jones AG, Kvarnemo C, Moore GI, Simmons LW, Avise JC (1998) Microsatellite evidence for monogamy and sex-biased recombination in the Western Australian seahorse Hippocampus angustus. Mol Ecol 7: 1497–1505 PMID: 9819904
24. Galbusera PHA, Gillemot S, Jouk P, Tesk PR, Hellemans B, et al. (2007) Isolation of microsatellite markers for the endangered Knysna seahorse Hippocampus capensis and their use in the detection of a genetic bottleneck. Mol Ecol Notes 7: 638–640
25. Singh KV, Gopalakrishnan A, Lakra WS, Sobti RC (2012) Microsatellite loci to determine population structure in the yellow seahorse (Hippocampus kuda) and the three-spotted seahorse (H. trimaculatus). Marine Biodivers 42: 481–488
26. Lourie SA (2004) Phylogeography of Southeast Asian seahorses in a conservation context. PhD thesis. McGill University, Montreal, Canada
27. Lourie SA, Foster SJ, Cooper EW, Vincent AC (2004) A guide to the identification of seahorses. Project Seahorse and TRAFFIC North America
28. Zhang Y, Pham NK, Zhang HX, Lin JD, Lin Q (2014) Genetic variations in two seahorse species (Hippocampus mohriki and Hippocampus trimaculatus): Evidence for Middle Pleistocene population expansion. PloS One 9: e105494 doi: 10.1371/journal.pone.0105494 PMID: 25144384
Transcriptome of Two Seahorse Species and Population Genetics Study

29. Shawn T O, Scott JE (2013) Assessing De Novo transcriptome assembly metrics for consistency and utility. BMC genomics 14: 465 doi: 10.1186/1471-2164-14-465 PMID: 23837739

30. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674–3676 PMID: 16081474

31. Ye J, Fang L, Zheng H, Zhang Y, Chen J (2006) WEGO: a web tool for plotting GO annotations. Nucleic Acids Res 34: 293–297

32. Iseli C, Jongeneel CV, Bucher P (1999) ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences. Proceedings of the International Conference for Intelligent Systems for Molecular Biology 1: 138–148

33. Thiel T, Michalek W, Varshey RK, Graner A. (2003) Exploiting EST databases for the development of cDNA derived microsatellite markers in barren (Hordeum vulgare L.). Theor Appl Genet 106: 411–422. PMID: 12589540

34. Zhang R, Zhang LL, Ye X, Tian YY, Sun CF, et al. (2013) Transcriptome profiling and digital gene expression analysis of Nile tilapia (Oreochromis niloticus) infected by Streptococcus agalactiae. Mol Biol Rep 40: 5657–5668 doi: 10.1007/s11033-013-2667-3 PMID: 24068429

35. Zhang L, Li L, Zhu Y, Zhang G, Guo X (2014) Transcriptome analysis reveals a rich gene set related to innate immunity in the Eastern oyster. Mar Biotechnol 16: 17–33 doi: 10.1007/s10126-013-9526-z PMID: 23907648

36. Tran NT, Gao ZX, Zhao HH, Yi SK, Chen BX, et al. (2015) Transcriptome analysis and microsatellite discovery in the blunt snout bream (Megalobrama amblycephala) after challenge with Aeromonas hydrophila. Fish Shellfish Immun 45: 72–82

37. Robinson N, Sahoo PK, Baranski M, Mahapatra KD, Jatindra N, et al. (2012) Expressed sequences and polymorphisms in rohu carp (Labeo rohita, Hamilton) revealed by mRNA-seq. Mar Biotechnol 14: 620–633 PMID: 22298294

38. Long Y, Li Q, Zhou B, Song GL, Li T, et al. (2013) De Novo assembly of mud loach (Misgurnus anguillicaudatus) skin transcriptome to identify putative genes involved in immunity and epidermal mucus secretion. PLoS One 8(2): e56998 doi: 10.1371/journal.pone.0056998 PMID: 23437293

39. Fu B, He S (2012) Transcriptome analysis of silver carp (Hypophthalmichthys molitrix) by paired-end RNA sequencing. DNA Res 19: 131–142 doi: 10.1093/dnares/dsr046 PMID: 22279088

40. Parchman TL, Geist KS, Grahnken JA, Benkmann CW, Buerkle CA (2010) Transcriptome sequencing in an ecologically important tree species: assembly, annotation, and marker discovery. BMC Genomics 11: 180 doi: 10.1186/1471-2164-11-180 PMID: 20233449

41. Li D, Deng Z, Qin B, Liu X, Men Z (2012) De novo assembly and characterization of bark transcriptome using Illumina sequencing and development of EST-SSR markers in rubber tree (Hevea brasiliensis Muell, Arg.). BMC Genomics 13: 192 doi: 10.1186/1471-2164-13-192 PMID: 22607098

42. Near TJ, Sandel M, Kuhn KL, Unmack PJ, Wainwright PC, et al. (2012) Nuclear gene-interred phylogenies resolve the relationships of the enigmatic Pygmy Sunfishes, Elassoma (Teleostei: Percomorpha). Mol Phylogenet Evol 63: 388–395 doi: 10.1016/j.ympev.2012.01.011 PMID: 22293156

43. Salem M, Rexroad CE, Wang J, Thorgaard GH, Yao J (2010) Characterization of the rainbow trout transcriptome using Sanger and 454-pyrosequencing approaches. BMC Genomics 11: 564 doi: 10.1186/1471-2164-11-564 PMID: 20942912

44. Hellsten U, Harland RM, Gilchrist MJ, Hendrix D, Jurka J, et al. (2010) The genome of the western clawed frog Xenopus tropicalis, Science 328: 633–636 doi: 10.1126/science.1183670 PMID: 20431018

45. Zhao F, Yan C, Wang X, Yang Y, Wang GY, et al. (2014) Comprehensive transcriptome profiling and functional analysis of the frog (Bombina maxima) immune system. DNA Res 21: 1–13. doi: 10.1093/dnares/dst035 PMID: 23942912

46. Sargent JR, Tocher DR, Bell JG (2002) The Lipids. In: Halver JE, Hardy RW, editors, Fish Nutrition, 3rd ed. San Diego: Academic Press; pp. 181–257.

47. Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fisheries Sci 11: 107–184

48. Bell MV, Tocher DR (2009) Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: General pathways and new directions. In: Arts MT, Brett MT, Kainz M, editors, Lipids in aquatic ecosystems. Heidelberg: Springer; pp. 211–236.

49. Somridhivej B, Wang S, Sha ZX, Liu H, Jonas Q, et al. (2008) Characterization, polymorphism assessment, and database construction for microsatellites from BAC end sequences of channel catfish (Ictalurus punctatus): A resource for integration of linkage and physical maps. Aquaculture 275: 76–80.
50. Edwardsa YJK, Elgara G, Clarka MS, Bishop MJ (1998) The identification and characterization of microsatellites in the compact genome of the Japanese pufferfish, *Fugu rubripes*: perspectives in functional and comparative genomic analyses. J Mol Biol 278: 843–854 PMID: 9614946

51. Saarinen EV, Austin JD (2010) When technology meets conservation: increased microsatellite marker production using 454 genome sequencing on the endangered okaloosa darter (*Etheostoma okaloosae*). J Hered 101: 784–788 doi: 10.1093/jhered/esq080 PMID: 20624755

52. Luo W, Nie Z, Zhan F, Wei J, Wang WM, et al. (2012) Rapid development of microsatellite markers for the endangered fish *Schizothorax biddulphi* (Günther) using next generation sequencing and cross-Species amplification. Int J Mol Sci 13: 4946–14955

53. Gao X, Han J, Lu Z, Li Y, He C (2012) Characterization of the spotted seal *Phoca largha* transcriptome using Illumina paired-end sequencing and development of SSR markers. Comp Biochem Phys D 7: 277–284

54. Bouck A, Vision T (2007) The molecular ecologist’s guide to expressed sequence tags. Mol Ecol 16: 907–924 PMID: 17305850

55. Thangaraj M, Lipton AP, Jone L, Gopalakrishnan A (2012) Genetic diversity of three spotted seahorse, *Hippocampus trimaculatus* (Leach, 1814) in India using four microsatellite loci. Notulae Scientia Biologicae 4: 7–13

56. Zhang L, Yan HF, Wu W, Yu H, Ge XJ (2013) Comparative transcriptome analysis and marker development of two closely related Primrose species (*Primula poissonii* and *Primula wilsonii*). BMC Genomics 14:329 doi: 10.1186/1471-2164-14-329 PMID: 23672467

57. Vincent ACJ, Sadler LM (1995) Faithful pair bonds in wild seahorses, *Hippocampus whitei*. Anim Behav 50: 1557–1569