Genomics 110 (2018) 414–422

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Original Article

Identification and comparative analysis of the miRNA expression profiles from four tissues of *Micropterus salmoides* using deep sequencing

Wangbao Gong[a], Yong Huang[b,*], Jun Xie[a,c,**], Guangjun Wang[a,c], Deguang Yu[a,c], Kai Zhang[a,c], Zhifei Li[a,c], Ermeng Yu[a,c], Jingjing Tian[a,c], Yun Zhu[a,c]

[a] Key Laboratory of Tropical & Subtropical Fishery Resource Application & Cultivation, Ministry of Agriculture, Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510380, China
[b] College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471003, China
[c] Guangdong Ecological Remediation of Aquaculture Pollution Research Center, Guangzhou 510380, China

ARTICLE INFO

Keywords:
Micropterus salmoides
miRNA
Deep sequencing
Expression

ABSTRACT

In the present study, four small RNA libraries were constructed from an *M. salmoides* population and sequenced using deep sequencing technology. A total of 9,888,822; 8,519,365; 20,566,198; and 15,762,254 raw reads representing 666,097; 755,711; 978,923; and 840,175 unique sequences were obtained from the spleen, liver, kidney, and muscle libraries, respectively. As a result, 509 known miRNAs belonging to 143 families and 1157 novel miRNAs were identified. The miRNAs displayed diverse expression levels among the four libraries, among which most of the known miRNAs were expressed at higher levels than the novel miRNAs. Furthermore, stem-loop qRT-PCR was applied to validate and profile the expression of the differentially expressed miRNAs in the four different tissues, which revealed that some miRNAs showed tissue specific expression. The identification of miRNAs in *M. salmoides* will provide new information and enhance our understanding of the functions of miRNAs in regulating biological processes.

1. Introduction

MicroRNAs (miRNAs) are ~22 nt, endogenous, noncoding small RNAs that regulate gene expression at the posttranscriptional level by binding to the target 3′ untranslated region (UTR) of mRNA, coding region, or 5′ end the non-coding region [1,2]. In animals, most miRNAs are transcribed from noncoding regions of the genome and are initially transcribed by RNA polymerase II into pri-miRNAs and then processed in the nucleus into pre-miRNAs with hairpin stem-loop structures. Finally, pre-miRNAs are exported to the cytoplasm, where they are processed into mature miRNAs [3]. Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC), leading to decreased mRNA stability or translational inhibition [4]. It is estimated that 1–3% of the genome encodes miRNAs, and over 30% of protein-coding genes are believed to be regulated by these small RNAs [5,6]. The regulation of gene expression by miRNAs can be very complex, because a single miRNA can regulate hundreds of target genes, and a single gene can be targeted by multiple miRNAs simultaneously [7]. Many studies have demonstrated that miRNAs play important roles in biological processes, including cell proliferation, differentiation, apoptosis, organ developmental processes, pathogenesis, metabolic control, and antiviral defense [8–11]. Since the first miRNA (lin-4) was discovered in *Caenorhabditis elegans* [12], thousands of miRNAs have been discovered by experimental, bioinformatic, and/or deep sequencing approaches in a wide range of organisms, including animals, plants, and viruses [13,14] (http://www.mirbase.org/).

The largemouth bass (*M. salmoides*), belonging to the family Micropterus of the order Perciformes, is an ecologically and economically important member of a diverse array of ecosystems across North America, from small ponds and streams to large rivers and lakes [15,16]. Since *M. salmoides* was firstly introduced into Guangdong province in 1983, this freshwater fish has been increasingly cultured in China. Its advantages for aquaculture, such as fast growth rate, wide temperature tolerance, good meat quality, and ability to adapt to a relatively wide range of environmental conditions, have meant that *M. salmoides* has been extensively cultured throughout China, and its production reached 351,772 tons in 2014 (Ministry of Agriculture in China, 2015).

* Corresponding author.
** Corresponding author at: Key Laboratory of Tropical & Subtropical Fishery Resource Application & Cultivation, Ministry of Agriculture, Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510380, China.
E-mail address: huangyong1979111@163.com (Y. Huang).

https://doi.org/10.1016/j.ygeno.2018.09.017
Received 13 February 2018; Received in revised form 21 September 2018; Accepted 27 September 2018
Available online 01 October 2018

Currently, miRNAs have been described for several fish species, providing insights into their possible mechanisms of sexual development, evolution, skeletal muscle development, and growth [17–20]. Nonetheless, to the best of our knowledge, no studies have reported the identification of miRNAs and their expression profiles in *M. salmoides*. In this study, four small RNA libraries were constructed from the spleen, liver, muscle, and kidney of *M. salmoides* and sequenced using deep sequencing technology. Bioinformatic analysis was performed to identify known and novel miRNAs in *M. salmoides*, and to study their different expression levels. Subsequently, stem-loop qRT-PCR was used to validate differentially expressed miRNAs in *M. salmoides* from different tissues. Our study provides the basis for further studies to understand the functions of miRNAs in the regulation of gene expression in *M. salmoides*.

2. Results and discussion

2.1. Small RNA sequencing analysis

In this study, four small RNA libraries from the spleen, liver, kidney, and muscle were sequenced using the Genome Analyzer II and the Illumina Cluster Station (Illumina Inc., USA). In total, 9,888,822; 8,519,365; 20,566,198; and 15,762,254 raw reads representing 666,097; 755,711; 978,923; and 840,175 unique sequences were obtained from the spleen, liver, kidney, and muscle libraries, respectively. The remaining reads were further filtered and retained for analysis if the read length was > 17 bp or < 27 bp. Based on this criterion, we analyzed the length distribution and found that among the unique size distribution pattern, most of the reads were distributed between 18 and 26 nt, a typical size range for Dicer-derived products (Fig. 1). However, there was a slight difference in the length distribution among the different small RNA libraries. In the kidney library, small RNAs of 21 nt represented the highest number of reads, while in the muscle library, 18 nt small RNAs had the highest number of reads. In the liver library, the most abundant small RNA length was 22 nt, followed by 20, 21, and 23 nt. The 23 nt sequences were the most abundant in the spleen library, followed by the 22 nt class. This result was consistent with previous studies in other species, such as cattle [21], goat [22], chickens [23], African clawed frog [24], and silkworm [25]. After removing the reads using the 3ADT (3′ adapters) &length filter and also junk reads, RNA family (Rfam) sequences (rRNA, tRNA, snRNA, snoRNA, and other Rfam RNAs) (Fig. 2), and Repbase sequences, a total of 245,051; 245,709; 514,819; and 241,210 mappable small RNA sequences were obtained from the four libraries, respectively and retained for miRNA analysis (Table 1).

2.2. Identification of known and novel miRNAs

To investigate known miRNAs in *M. salmoides*, the mappable sequences were aligned to all mature animal miRNA and miRNA precursor sequences in the miRBase datasets (http://www.mirbase.org/; Release 21). Consequently, 509 known miRNAs were identified in *M. salmoides* (Table S1), covering 143 miRNA families (Table S2). The most abundant miRNA families were let-7 and mir-10 (both 15 members) followed by mir-15 (14 members), mir-17 (13 members), mir-221 (11 members), and mir-130 (10 members). The remaining miRNA families contained one to nine members. Among the 509 identified miRNAs, 369, 330, 437, and 383 were only expressed in the spleen, liver, kidney, and muscle, respectively (Table S3), indicating these tissue-biased miRNAs that may play critical regulatory roles in different tissues of *M. salmoides*. Moreover, 273 (53.6%) miRNAs were co-expressed in all four libraries (Table S4). The miRNAs that are present in at least one major ancient clade of animals are referred as conserved miRNAs [26]. In our study, the conserved miRNAs was observed in comparison with 46 animal species (Fig. 3) with the most miRNA members being homologous to sequences from *Salmo salar* (355), followed by *Danio rerio* (290), *Ictalurus punctatus* (237), *Homo sapiens* (221), *Mus musculus* (207) and only one member was identified in *Oryctolagus cuniculus* and *Macropus eugenii*, respectively. This suggested the conserved nature of mature miRNAs among living organisms.

In deep sequencing, the relative expression level of a unique miRNA can be measured by the frequency of its read count. According to this principle, we found the miRNAs had a very wide range of expression levels, which varied from thousands or tens of thousands of reads to 1 read (Table S5), and the expression abundance of each miRNA varied. Among them, aca-miR-100 was both the most abundant in the spleen (539,182 reads) and the kidney (397,336); ssa-miR-122-5p_R + 1 was both the most abundant in the liver (798,643 reads); and gga-let-7a-5p was both the most abundant in muscle (708,227 reads), indicating that these miRNAs are highly expressed and function as negative regulators of gene expression in the physiological process of *M. salmoides*. The spleen and kidney are regarded as major immune organs in fish [27]. A previous study also reported that miR-100 is associated with the immune response in teleost fish [28]. MiR-122 was one of the first reported examples of a tissue-specific miRNA in mammals and is highly conserved across different species, from humans to *C. elegans*, which suggests that it is likely to have important biological functions [29]. Thus, our results are similar to those of other studies. Tsai et al. reported that miR-122 is a tumor suppressor miRNA, which negatively regulates *ADAM17*, resulting in the repression of hepatocellular carcinoma angiogenesis and metastasis [30]. Moreover, studies showed that

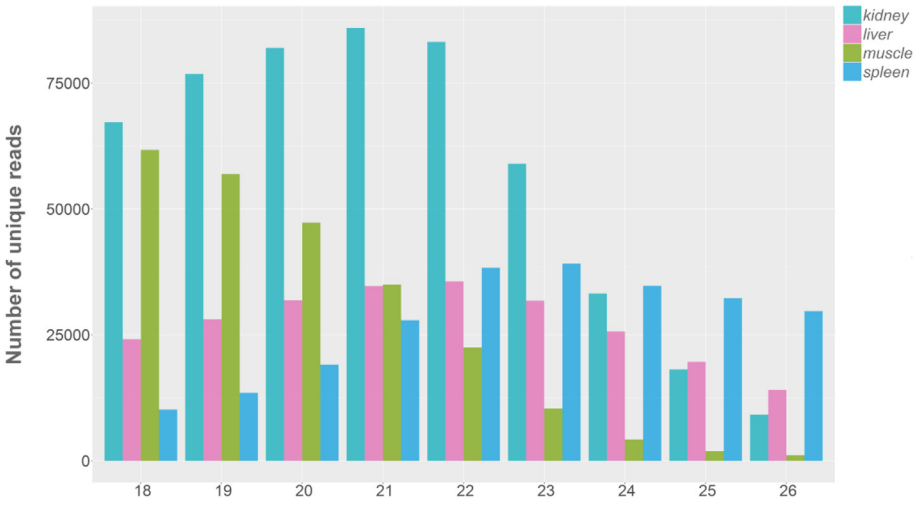

Fig. 1. Sequence length distribution of unique small RNA reads in four libraries from *M. salmoides*.

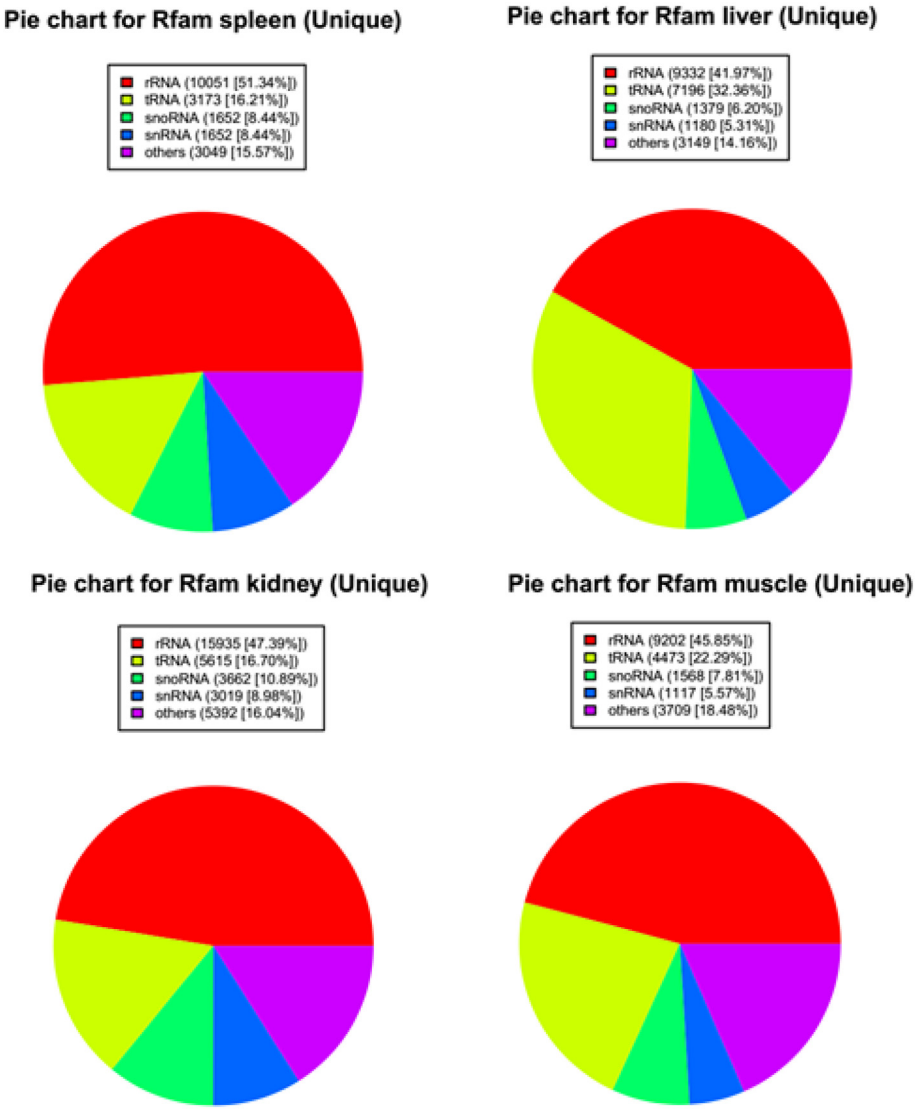

Fig. 2. Unique Small RNA reads were BLAST searched against the Rfam database of non-coding RNAs to annotate rRNA, tRNA, snoRNA, snRNA and others RNAs.

downregulation of miR-122 could be used as a biomarker for hepatic cancer [31]. In fish, miR-122 was also found to be highly specifically expressed in the liver of zebrafish [32] and blunt snout bream [33]. Many previous studies on let-7 in fish species demonstrated that this miRNA show the highest expression in muscle [20,34], and plays a key role in regulating differentiation and proliferation of myoblasts [35,36]. These data were in agreement with other studies of miRNAs, showing that let-7a ranked among the highest expressed miRNAs in muscle compared with that in other tissues [37,38]. By contrast, some miRNAs displayed low expression abundance, such as dps-miR-92b_L-1_1ss10AT, ccr-miR-457b_L + 1R-1_1ss17CT, gga-miR-222b-5p_R-7, and aca-miR-19a-3p_R + 1 had < 1 read in the spleen, liver, kidney, and muscle, respectively. It is hypothesized that these miRNAs may be expressed at low levels in certain tissue development stages or under specific conditions.

Sequencing reads that did not match any of the known miRNAs were further analyzed to identify novel miRNAs. This analysis showed 1157 novel miRNA were identified from the four libraries as having the typical miRNA stem-loop secondary structure, which forms the Dicer enzyme cleavage site (Table S5). Among these novel miRNAs, only 29 were found in all four libraries (Table 2). These novel miRNAs had expression levels represented by from 0.24–52,512 reads and most

Table 1
Analysis of small RNA sequences from the spleen, liver, kidney, and muscle libraries of *M. salmoides*.

Type	Spleen tissue				Liver tissue				Kidney tissue				Muscle tissue			
	Total	%	unique	%	Total	%	unique	%	Total	%	unique	%	Total	%	unique	%
Raw reads	9,888,822	100	666,097	100	8,519,365	100	755,711	100	20,566,198	100	978,923	100	15,762,254	100	840,175	100
3ADT&length filter	3,474,978	35.14	398,861	59.88	6,070,221	71.25	486,295	64.35	1,568,302	7.63	424,310	43.34	8,512,975	54.01	577,059	68.68
Junk reads	12.723	0.13	2540	0.38	3279	0.04	1418	0.19	55,414	0.27	5880	0.60	6094	0.04	1619	0.19
Rfam	219,045	2.22	19,577	2.94	321,433	3.77	22,236	2.97	680,574	3.31	33,623	3.43	583,775	3.70	20,069	2.39
Repeats	175	0.00	104	0.02	407	0.00	68	0.01	986	0.00	396	0.04	2455	0.02	326	0.04
Mappable reads	6,181,946	62.51	245,051	36.79	2,124,094	24.93	245,709	32.51	18,261,072	88.79	514,819	52.59	6,657,477	42.24	241,210	28.71

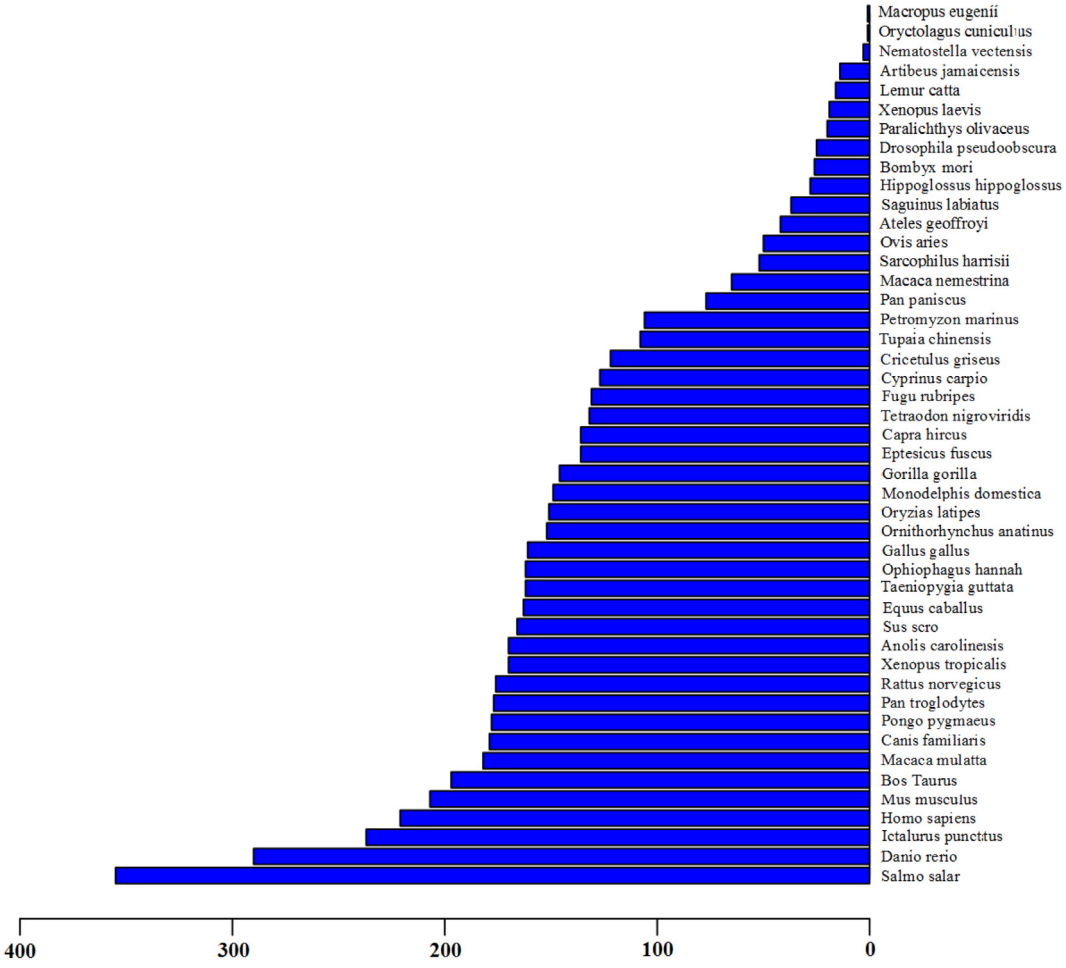

Fig. 3. Conservation profiles of identified miRNAs, values on the x-axis indicate the number of conserved miRNAs between *M. salmoides* and the queried species.

Table 2
The 29 co-expressed novel identified miRNAs from the spleen, liver, kidney and muscle libraries of *M. salmoides*.

miRNA index	miRNA name	miRNA sequence	Spleen (Read counts)	Liver (Read counts)	Kidney (Read counts)	Muscle (Read counts)
1	PC-5p-11116_111	GAGGGATGATGAGAGACA	61.55	22.18	46.13	167.45
2	PC-3p-91564_5	CTGGCTGAACTCCTGTGATGTC	2.10	2.46	3.40	10.30
3	PC-5p-49172_14	TGGACAAACTGAAGGCAGAGC	7.69	276.02	12.14	113.35
4	PC-3p-36089_23	CTGTGGAAGTTTGAGACC	9.09	241.52	8.50	54.10
5	PC-5p-123515_3	ACATGCTTGTCTCTCAGTC	2.10	7.39	0.49	12.88
6	PC-5p-155352_3	ATCAGGGACTAGGAGGTTT	0.70	2.46	1.46	5.15
7	PC-3p-222960_2	AAGGCTCTGTCTGATCACCAC	1.40	41.90	2.91	10.30
8	PC-5p-43985_17	TCTACTCTGCACATCATGAGG	6.30	4.93	8.74	5.15
9	PC-5p-66038_8	AACAGGCCACCCATCTCCATC	8.39	76.40	8.26	46.37
10	PC-3p-40311_19	AACAGGCCACCCATCTCCATCT	15.39	125.69	18.45	33.49
11	PC-5p-119311_3	CAACCTGACTGCCTTCGC	0.70	2.46	1.21	7.73
12	PC-3p-602340_1	TCAGATGGTAGAGTCAGAATTT	2.10	2.46	0.97	2.58
13	PC-3p-874078_1	TCAGTTTTTATTGCCCTCCTCACT	1.40	2.46	1.94	2.58
14	PC-5p-83069_6	CGGTTCTTGTGAATGACTTCTT	2.10	29.57	1.21	6.44
15	PC-3p-160660_2	TTGAGACCTTCTGCAGAATCC	0.70	9.86	0.49	7.73
16	PC-3p-243504_2	AAGCACAGCGCCTGCACCCCATGC	2.80	71.47	1.46	20.61
17	PC-5p-339351_1	TTCCAATCAGTCGATGCATCCTATGT	0.70	4.93	0.49	2.58
18	PC-5p-1611_1057	TGAGGTCCTCGGATCGGCCC	7149.20	13,690.30	6803.03	52,512.11
19	PC-3p-1635_1038	CCTCGGATCGGCCCCGCC	1795.52	3511.91	1846.50	13,584.00
20	PC-5p-89217_5	CAACAAGGACGTCTTCAGG	0.70	59.15	1.94	15.46
21	PC-3p-171518_2	CCGTTTTTGTAGGAGAGGC	0.70	4.93	2.19	5.15
22	PC-5p-172062_2	GCTAGTGGGAGCTAAATGC	0.70	2.46	0.49	7.73
23	PC-3p-79692_6	ATGAGTGAACTATCTGTAAAG	12.59	24.65	14.33	12.88
24	PC-5p-143416_3	AAGTCCTGGATGTTCCTC	2.10	34.50	1.21	12.88
25	PC-3p-246131_2	CACAGCCACCTCTGTCACCAT	2.10	17.25	0.97	2.58
26	PC-5p-87155_5	GATCGGAGTTGGCCGGAGA	2.80	22.18	2.43	46.37
27	PC-3p-13700_87	TCACAACAGTGACCAATCCTCT	15.39	86.26	18.94	10.30
28	PC-3p-137697_3	TTCTCTTCATGTCGGGGGC	0.70	2.46	0.97	2.58
29	PC-5p-236548_2	GCGGAACTCTGCTCTTTAAGC	0.70	7.39	1.21	2.58

novel miRNAs reads were < 1000. Interestingly, the novel miRNA PC-5p-891_1763 showed the highest expressed compared with the identified some conserved miRNAs from four libraries with spleen (7149 reads), liver (13,690 reads), kidney (6803 reads) and muscle (52,512 reads), suggesting that this novel miRNA might have a specific role in various tissues, or during the developmental stages of *M. salmoides*. To the best of our knowledge, this is the first report of *M. salmoides* miRNAs. Previous reports have indicated that known miRNAs are often widespread, highly expressed, and most of them have more than one family member, while novel miRNAs are often species specific and weakly expressed [39]. In this respect, our results were in accordance with previous studies. These novel miRNAs exhibited much lower expression levels than the reads of known miRNAs. Notably, the novel miRNAs are either 3′-derived sequences or 5′-derived sequences. The predicted length of the identified hairpin precursors ranged from 50 to 167 nt, which is similar to those previously identified in other fish [40–43]. The G + C contents of these novel miRNAs ranged from 20.6% to 72.8%, with an average of 47.3%. The minimum free energy (MFE) of these novel pre-miRNAs ranged from −72.6 to −15.0 kcal/mol with an average of −31.9%.

2.3. Differential expression analysis of identified miRNA

Differentially expressed miRNAs provide clues to molecular events related to their physiological significance in different tissues [44]. These differentially expressed miRNAs spanned a wide broad range of expression levels, which varied from several reads to several hundred thousand reads in the four libraries. In our present study, we normalized the reads density measurement and used P-value < 0.001 and the absolute value of $\log_2$ratio fold-change > 1.0 as a threshold to judge the statistical significance of miRNA expression, and then compared the differential expression of these identified miRNAs between the liver and spleen, kidney, and spleen, and muscle and spleen, respectively. We found that 171 out of 256 miRNAs detected between the liver and spleen were significantly differentially expressed, with 93 miRNAs upregulated and 78 miRNAs downregulated in liver (Fig. 4A and Table S6). Similarly, 134 out of 270 miRNAs between the kidney and spleen were significantly differentially expressed, with 49 miRNAs upregulated and 85 miRNAs downregulated in the kidney (Fig. 4B and Table S7). When comparing the differential expression of miRNAs between the muscle and spleen, we found that 256 out of 295 miRNAs were significantly differentially expressed, with 128 miRNAs upregulated and remaining half miRNAs downregulated in muscle (Fig. 4C and Table S8). Similarly, 83 miRNAs were significantly upregulated, while 74 miRNAs were significantly downregulated in the liver compared with that in the kidney (Fig. 4D and Table S9). Notably, the most differentially expressed miRNA was ola-miR-206_R + 1, which had a 15.76-fold increase in muscle compared with its level in the spleen of *M. salmoides*. Studies have confirmed that miR-206 is a muscle-specific miRNA that promotes skeletal muscle development and differentiation by regulating connexin43 expression [45,46]. Although the physiological functions of these differentially expressed miRNAs are not known, their expression patterns indicate that they are also likely to play a role in organ growth and development in *M. salmoides*. Further experiments are needed to elucidate their roles in this process.

2.4. Confirmation of differentially expressed miRNAs using stem-loop qRT-PCR

Information about the differential expression of an miRNA is useful to understand its functions [47,48]. To verify the existence and expression patterns of the miRNAs from the sequencing data, several miRNAs with different expression patterns from the deep sequencing results were randomly selected for stem-loop qRT-PCR analysis in eight different tissues from *M. salmoides*, including spleen, liver, kidney, muscle, heart, gonad, intestine, and gill. These miRNAs included the following: Six known miRNAs (aca-miR-18a-5p_R-1, aca-miR-126-5p, fru-miR-458, ccr-miR-722_L-2R + 3, cgr-miR-1260, hhi-miR-7641_L-1R + 1) and four novel miRNAs (PC-3p-36089_23, PC-5p-83069_6, PC-5p-89217_5 and PC-3p-13700_87). Although a few of the known and novel miRNAs were detected with relatively low read numbers by deep sequencing, in the present study, all these miRNAs could be detected using qRT-PCR. Generally, the qRT-PCR analyses showed similar results for these miRNAs to deep sequencing (Fig. 5). These data also provided evidence that deep sequencing is a more sensitive and reliable method to identify differentially expressed miRNAs in *M. salmoides*.

For example, aca-miR-18a-5p_R-1 was expressed most abundantly in the kidney followed by the spleen and liver, and exhibited lower levels of expression in the heart, gill, muscle, gonad, and intestine. Aca-miR-126-5p was highly expressed in the liver and kidney, moderately in the heart, spleen and muscle, and showed the least expression in the gill, gonad, and intestine. Similarity, fru-miR-458 was predominantly expressed in the kidney, moderately expressed in the spleen, and weakly expressed in the other six tissues. Intriguingly, ccr-miR-722_L-2R + 3 and cgr-miR-1260 showed tissue specific expression, in that they were strongly expressed at their highest levels in the liver and muscle, respectively, and exhibited very low expression in remaining tissues. Hhi-miR-7641_L-1R + 1 displayed the most abundant expression in the muscle, followed by the liver, and showed moderate expression in the kidney, spleen, and heart. However, these novel miRNAs were expressed at relatively low levels in the tissues of *M. salmoides*, which consistent with the miRNA profile by sequencing, and suggested that novel miRNAs are usually weakly expressed, while conserved miRNAs are highly expressed. Remarkably, all four novel miRNAs, including PC-3p-36089_23, PC-5p-83069_6, PC-5p-89217_5, and PC-3p-13700_87, were expressed at relatively high levels in the liver, indicating that they may be involved in many fundamental functions in *M. salmoides*. Our expression data provide the basis for further research to understand the regulatory roles of miRNAs in the physiological functions of different tissues in fish.

3. Conclusions

This study represents the first characterization of the *M. salmoides* miRNA transcriptome from four organs/tissues. A total of 1666 miRNAs were identified in *M. salmoides*, including 509 known miRNAs and 1157 novel miRNAs. Some miRNAs are expressed with significantly higher or lower abundance in the spleen, liver, kidney, and muscle, respectively. Our results also showed that differential expression analysis of miRNAs in the four tissues is possible using the deep sequencing strategy and stem-loop qRT-PCR to confirm the expression data. This study provides a starting point for future studies aimed at understanding the roles of miRNAs in major physiological process, such as growth, development and immunity in Perciformes. Moreover, this *M. salmoides* miRNA repertoire also provides a novel resource for advanced genomic research in fish species.

4. Materials and methods

4.1. Fish and sample preparation

Three *M. salmoides* individuals at six months old with an average weight of 500 g were selected and purchased from a fish farm (Guangzhou, Guangdong, China). The fish were reared in a recirculating aquaculture system in a large water tank containing 300 L freshwater at 25 °C and fed twice daily with pelleted feed. After 1 week of acclimation, the fish were anaesthetized in well-aerated water using a lethal dose (100 mg/L) of tricaine methanesulfonate (MS-222) before tissue collection. The fish were then put on ice for tissues dissection. The samples of the same tissue from three *M. salmoides* were collected and pooled, and then frozen immediately in liquid nitrogen before extraction of total RNA. All experimental procedures were approved by

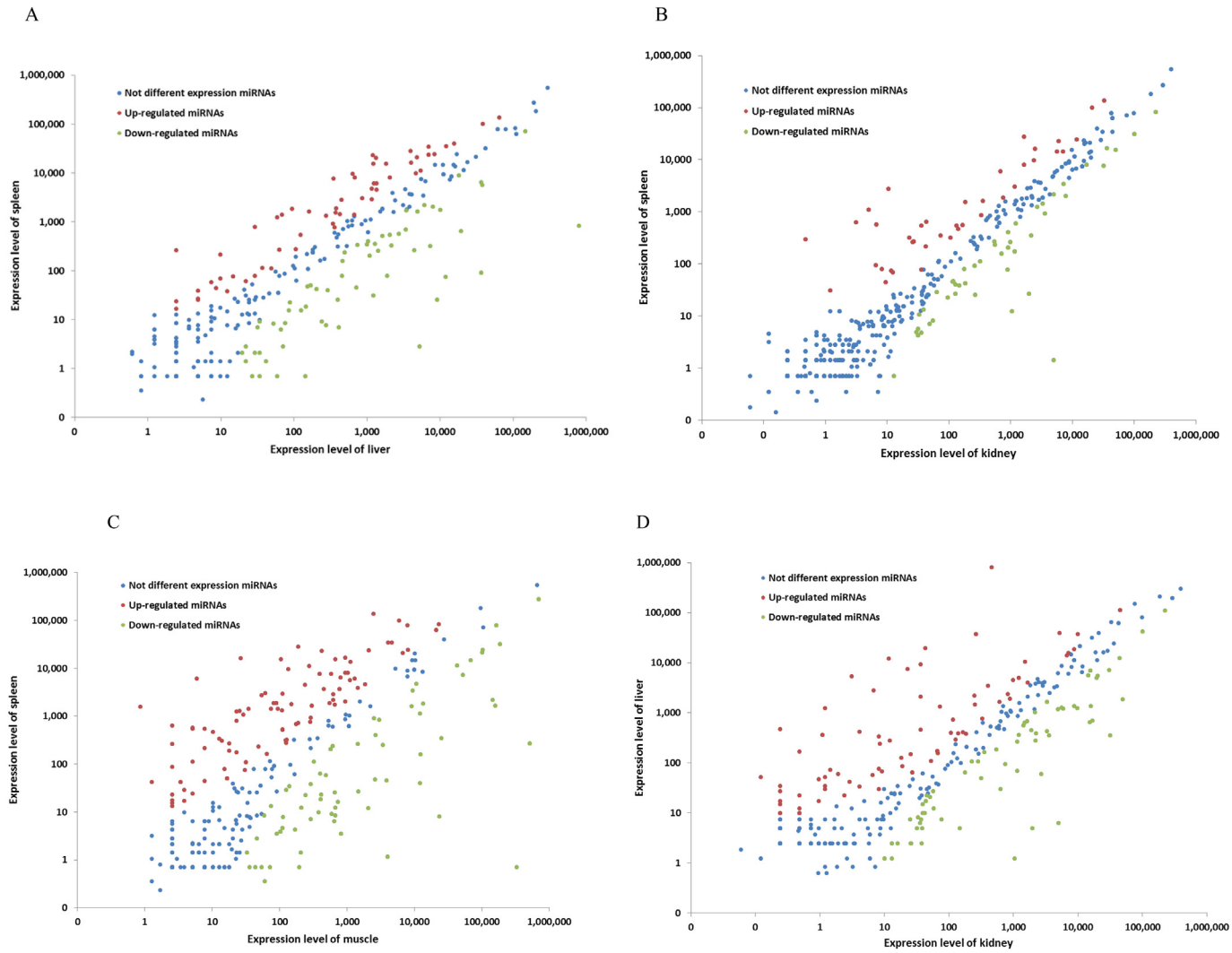

Fig. 4. Comparison of expression patterns of identified miRNAs in *M. salmoides* between the liver and spleen (A), kidney and spleen (B), muscle and spleen (C), and kidney and liver (D).

the Ethics Committee of the Animal Laboratory of Henan University of Science and Technology. All efforts were made to minimize the fish's suffering.

4.2. Construction of small RNA library and deep sequencing

Total RNA was isolated separately using TRIzol reagent (Takara, China) according to manufacturer instructions. The quality and quantity of each RNA sample was assessed using a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, Santa Clara, CA, USA). Only RNA samples with a RNA integrity number (RIN) value > 8.0 and a 28S/18S ratio between 1.8 and 2.2 were used to construct the small RNA libraries. Approximately one microgram of total RNA of each sample was used with TruSeq Small RNA Sample Prep Kits (Illumina, USA). The small RNA fraction was isolated using polyacrylamide gel electrophoresis and ligated to proprietary adaptors (Illumina, USA). Short RNAs were then reverse-transcribed into cDNA using RT-PCR. Four small RNA libraries were constructed for *M. salmoides* and sent to LC-Sciences (Hangzhou, China) for deep sequencing using an Illumina Hiseq2500 platform.

4.3. Small RNA bioinformatic analysis

The raw reads were subjected to the Illumina pipeline filter, and then the dataset was further processed using an in-house program, ACGT101-miR (LC Sciences, USA) to remove adapter dimers, junk, and low complexity data, the remaining 18 to 26 nt reads were regarded as clean reads and were used for further analysis. The clean reads were analyzed by BLAST software searching against the zebrafish genome [49–53]. The matched sequences were blasted against the Rfam (ftp.sanger.ac.uk/pub/databases/Rfam) and Repbase (http://www.girinst.org/) databases to remove mRNA, rRNA, tRNA, snRNA, snoRNA, other noncoding RNAs, and repeat sequences. The remaining reads were then mapped to miRBase 21.0 using BLAST to identify known animal miRNAs. Sequencing reads that did not match any known miRNA were further analyzed to identify novel miRNAs. Novel candidate miRNAs were identified by prediction of their secondary structures using the Mfold program (http://mfold.rna albany.edu/?q=mfold/RNA-Folding-Form). The naming system of the miRNAs identified in this study is as follows: the miRNA name comprises the first known miR name in a cluster, an underscore, and a matching annotation: L-n means that the miRNA_seq (detected) is n bases less than the known rep_miRSeq on the left side; R-n means that the miRNA_seq (detected) is n bases less than the known rep_miRSeq on the right side; L + n means that the miRNA_seq (detected) is n bases more than the known rep_miRSeq on the left side; R + n means that the miRNA_seq (detected) is n bases more than the known rep_miRSeq on the right side; 2ss5TC13TA means two substitutions in (ss), which are T to C at position 5 and T to A at position

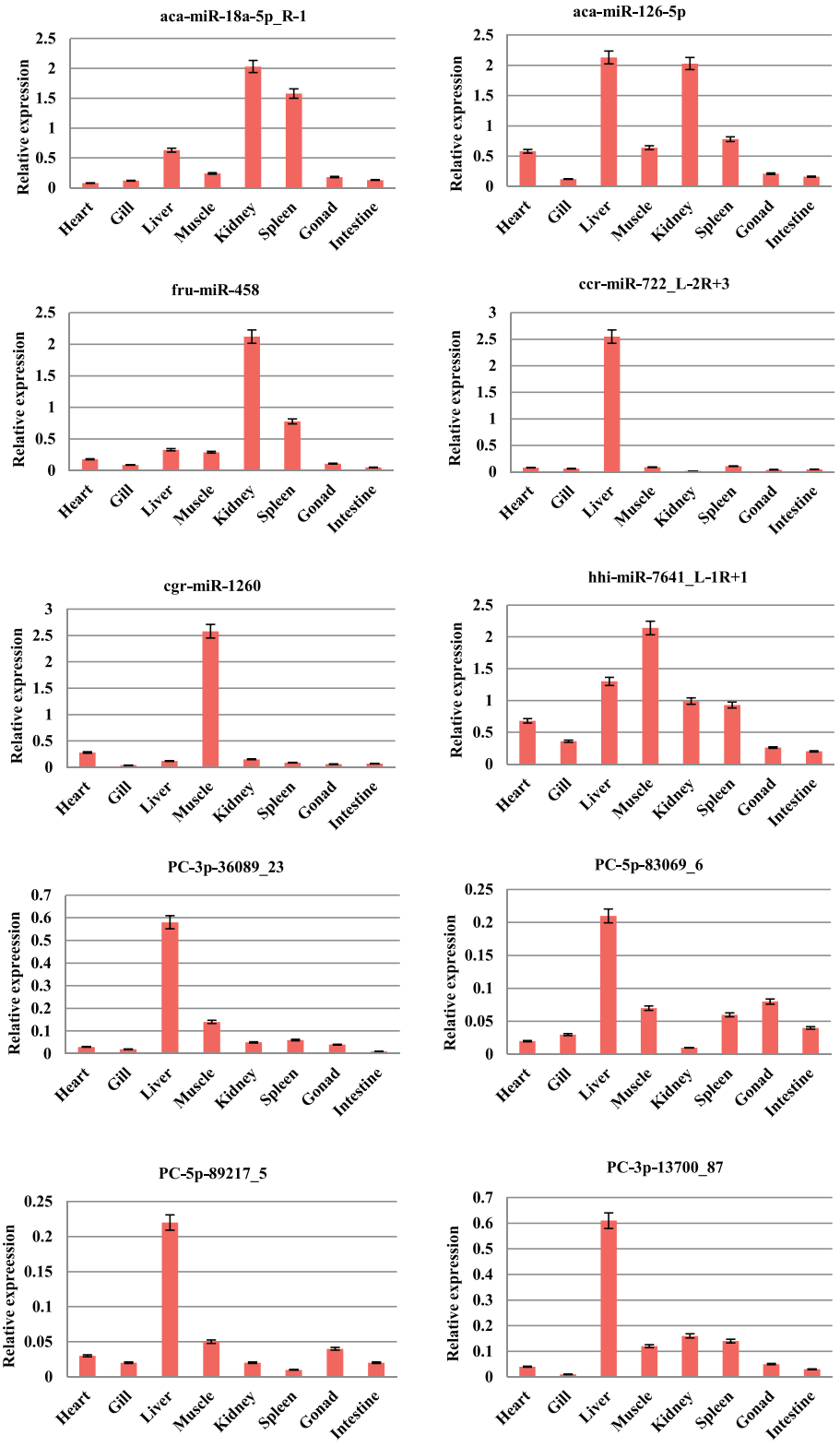

Fig. 5. Stem-loop qRT-PCR validation and expression analysis of known and novel miRNAs in *M. salmoides*. Each reaction was performed in triplicate, and the expressions of miRNAs were normalized to the level of 18S rRNA.

13. If there is no matching annotation, the miRNA_seq (detected) is exactly the same as known rep_miRSEq. Newly discovered 5′/3′ sequences were annotated as p5/p3 to distinguish them from the reported 5′/3′ sequences. All sequencing reads were deposited in the Short Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra/), which are retrievable under the accession numbers SRR5980136, SRR5980138, SRR5980137 and SRR5947192.

4.4. Analysis of differentially expressed miRNAs

To compare the miRNA expression levels in four libraries to identify differentially expressed miRNAs, miRNA expression in each library was

normalized. The following procedures were used to analyze differential expression: (1) the expression levels of the miRNA was normalized as transcripts per million. Normalized expression (NE) = Actual miRNA count/total count of clean reads × 10^6; (2) After normalizing the miRNA read count, the $\log_2$fold-change was calculated from the NE data. Meanwhile, a P-value was also calculated using the following formula: When an absolute value of $|\log_2 \text{ratio}| > 1$ and a threshold of the P-value < 0.001, the miRNA was regarded as differentially expressed. The P-value formula was:

$$p(x \mid y) = \left(\frac{N_2}{N_1}\right) \frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}} \frac{C(y \le y_{min} \mid x) = \sum_{y=0}^{y \le y_{min}} p(y \mid x)}{D(y \ge y_{max} \mid x) = \sum_{y3y_{max}}^{\infty} p(y \mid x)}$$

where N_1 and x represent the total count of clean reads and normalized expression, respectively, for a given miRNA in the peak lactation small RNA library. N_2 and y represent the total count of clean reads and normalized expression respectively, for a given miRNA in the late lactation small RNA library.

4.5. Validation of miRNA expression by stem-loop qRT-PCR

Total RNA was extracted from same population as described above. Then, two micrograms of total RNA from each sample were reverse-transcribed into cDNA using miRNA specific stem-loop RT primers, according to previously described criteria [54,55]. The resulting cDNA was diluted ten times with sterile water. qPCR was then performed to profile the expression levels of miRNAs in the four tissues using the MyiQ2 Two Color Real-time PCR Detection System (Bio-Rad, USA). The conditions used for real-time PCR were: one cycle of 94 °C for 2 min; followed by 45 cycles at 95 °C for 10 s, 56 °C for 30 s; and a final 70 cycles at 60 °C for 30 s. All reactions were run in triplicate for each sample. 18SrRNA was selected as the reference gene for normalization. Relative expression levels were quantified by the $2^{-\Delta\Delta Ct}$ method. All primers for stem-loop qRT-PCR are listed in Table S10.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant number 31302201), Pearl River S&T Nova Program of Guangzhou (grant number 2014J2200088), and the National key Technology R & D Program of China (grant number 2012BAD25B04).

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2018.09.017.

References

[1] G. Obernosterer, P.J. Leuschner, M. Alenius, J. Martinez, Post-transcriptional regulation of microRNA expression, RNA 12 (2006) 1161–1167.
[2] W. Filipowicz, S.N. Bhattacharyya, N. Sonenberg, Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9 (2008) 102–114.
[3] M.R. Fabian, N. Sonenberg, W. Filipowicz, Regulation of mRNA translation and stability by microRNAs, Annu. Rev. Biochem. 79 (2010) 351–379.
[4] O. Flores, E.M. Kennedy, R.L. Skalsky, B.R. Cullen, Differential RISC association of endogenous human microRNAs predicts their inhibitory potential, Nucleic Acids Res. 42 (2014) 4629–4639.
[5] V.N. Kim, J.W. Nam, Genomics of microRNA, Trends Genet. 22 (2006) 165–173.
[6] V.N. Kim, J. Han, M.C. Siomi, Biogenesis of small RNAs in animals, Nat. Rev. Mol. Cell Biol. 10 (2009) 126–139.
[7] F. Liu, W. Peng, Z. Li, W. Li, L. Li, J. Pan, S. Zhang, Y. Miao, S. Chen, S. Su, Next-generation small RNA sequencing for microRNAs profiling in Apis mellifera: comparison between nurses and foragers, Insect Mol. Biol. 21 (2012) 297–303.
[8] Y. Huang, X.J. Shen, Q. Zou, S.P. Wang, S.M. Tang, G.Z. Zhang, Biological functions of microRNAs: a review, J. Physiol. Biochem. 67 (2011) 129–139.
[9] X. Gao, R. Tyagi, V. Magrini, A. Ly, D.P. Jasmer, M. Mitreva, Compartmentalization of functions and predicted miRNA regulation among contiguous regions of the nematode intestine, RNA Biol. (2016) 1–18.
[10] X. Lin, M.H. Tsai, A. Shumilov, R. Poirey, H. Bannert, J.M. Middeldorp, R. Feederle, H.J. Delecluse, The Epstein-Barr virus BART miRNA cluster of the M81 strain modulates multiple functions in primary B cells, PLoS Pathog. 11 (2015) e1005344.
[11] R.W. Carthew, P. Agbu, R. Giri, MicroRNA function in Drosophila melanogaster, Semin. Cell Dev. Biol. 65 (2017) 29–37.
[12] R.C. Lee, R.L. Feinbaum, V. Ambros, The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14, Cell 75 (1993) 843–854.
[13] J. Zhang, Y. Liu, X. Zhang, J. Pan, Z. Nie, W. Zhang, W. Yu, J. Chen, L. Liu, J. Li, Y. Zhang, J. Guo, W. Wu, H. Zhu, Z. Lv, The identification of microRNAs in the whitespotted bamboo shark (*Chiloscyllium plagiosum*) liver by Illumina sequencing, Gene 527 (2013) 259–265.
[14] M.E. Herkenhoff, A.C. Oliveira, P.G. Nachtigall, J.M. Costa, V.F. Campos, A.W.S. Hilsdorf, D. Pinhal, Fishing into the MicroRNA Transcriptome, Front. Genet. 9 (2018) 88.
[15] S. Li, H. Liu, J. Bai, X. Zhu, Transcriptome assembly and identification of genes and SNPs associated with growth traits in largemouth bass (*Micropterus salmoides*), Genetica 145 (2017) 175–187.
[16] T. Taguchi, Y. Miura, D. Krueger, S. Sugiura, Utilizing stomach content and faecal DNA analysis techniques to assess the feeding behaviour of largemouth bass *Micropterus salmoides* and bluegill *Lepomis macrochirus*, J. Fish Biol. 84 (2014) 1271–1288.
[17] T.T. Bizuayehu, J. Babiak, B. Norberg, J.M. Fernandes, S.D. Johansen, I. Babiak, Sex-biased miRNA expression in Atlantic halibut (*Hippoglossus hippoglossus*) brain and gonads, Sex. Dev. 6 (2012) 257–266.
[18] X. Chen, Q. Li, J. Wang, X. Guo, X. Jiang, Z. Ren, C. Weng, G. Sun, X. Wang, Y. Liu, L. Ma, J.Y. Chen, K. Zen, J. Zhang, C.Y. Zhang, Identification and characterization of novel amphioxus microRNAs by Solexa sequencing, Genome Biol. 10 (2009) R78.
[19] X. Yan, L. Ding, Y. Li, X. Zhang, Y. Liang, X. Sun, C.B. Teng, Identification and profiling of microRNAs from skeletal muscle of the common carp, PLoS One 7 (2012) e30925.
[20] S. Yi, Z.X. Gao, H. Zhao, C. Zeng, W. Luo, B. Chen, W.M. Wang, Identification and characterization of microRNAs involved in growth of blunt snout bream (*Megalobrama amblycephala*) by Solexa sequencing, BMC Genomics 14 (2013) 754.
[21] J. Sun, B. Zhang, X. Lan, C. Zhang, C. Lei, H. Chen, Comparative transcriptome analysis reveals significant differences in MicroRNA expression and their target genes between adipose and muscular tissues in cattle, PLoS One 9 (2014) e102142.
[22] Z. Ji, G. Wang, Z. Xie, C. Zhang, J. Wang, Identification and characterization of microRNA in the dairy goat (*Capra hircus*) mammary gland by Solexa deep-sequencing technology, Mol. Biol. Rep. 39 (2012) 9361–9371.
[23] G.R. Sun, M. Li, G.X. Li, Y.D. Tian, R.L. Han, X.T. Kang, Identification and abundance of miRNA in chicken hypothalamus tissue determined by Solexa sequencing, Genet. Mol. Res. 11 (2012) 4682–4694.
[24] S. Ambady, Z. Wu, T. Dominko, Identification of novel microRNAs in *Xenopus laevis* metaphase II arrested eggs, Genesis 50 (2012) 286–299.
[25] X. Yu, Q. Zhou, Y. Cai, Q. Luo, H. Lin, S. Hu, J. Yu, A discovery of novel microRNAs in the silkworm (*Bombyx mori*) genome, Genomics 94 (2009) 438–444.
[26] Y.H. Loh, S.V. Yi, J.T. Streelman, Evolution of microRNAs and the diversification of species, Genome Biol. Evol. 3 (2011) 55–65.
[27] G. Li, Y. Zhao, L. Wen, Z. Liu, F. Yan, C. Gao, Identification and characterization of microRNAs in the spleen of common carp immune organ, J. Cell. Biochem. 115 (2014) 1768–1778.
[28] R. Andreassen, B. Hoyheim, miRNAs associated with immune response in teleost fish, Dev. Comp. Immunol. 75 (2017) 77–85.
[29] C. Jopling, Liver-specific microRNA-122: Biogenesis and function, RNA Biol. 9 (2012) 137–142.
[30] W.C. Tsai, P.W. Hsu, T.C. Lai, G.Y. Chau, C.W. Lin, C.M. Chen, C.D. Lin, Y.L. Liao, J.L. Wang, Y.P. Chau, M.T. Hsu, M. Hsiao, H.D. Huang, A.P. Tsou, MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma, Hepatology 49 (2009) 1571–1582.
[31] H. Kutay, S. Bai, J. Datta, T. Motiwala, I. Pogribny, W. Frankel, S.T. Jacob, K. Ghoshal, Downregulation of miR-122 in the rodent and human hepatocellular carcinomas, J. Cell. Biochem. 99 (2006) 671–678.
[32] E. Wienholds, W.P. Kloosterman, E. Miska, E. Alvarez-Saavedra, E. Berezikov, E. de Bruijn, H.R. Horvitz, S. Kauppinen, R.H. Plasterk, MicroRNA expression in zebrafish embryonic development, Science 309 (2005) 310–311.
[33] D. Zhang, K. Lu, Z. Dong, G. Jiang, W. Xu, W. Liu, The effect of exposure to a high-fat diet on microRNA expression in the liver of blunt snout bream (*Megalobrama amblycephala*), PLoS One 9 (2014) e96132.
[34] W. Chi, C. Tong, X. Gan, S. He, Characterization and comparative profiling of MiRNA transcriptomes in bighead carp and silver carp, PLoS One 6 (2011) e23549.
[35] W.H. Townley-Tilson, T.E. Callis, D. Wang, MicroRNAs 1, 133, and 206: critical factors of skeletal and cardiac muscle development, function, and disease, Int. J. Biochem. Cell Biol. 42 (2010) 1252–1255.
[36] J.J. McCarthy, K.A. Esser, MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy, J. Appl. Physiol. 102 (2007) 306–313.
[37] T.G. McDaneld, T.P. Smith, M.E. Doumit, J.R. Miles, L.L. Coutinho, T.S. Sonstegard, L.K. Matukumalli, D.J. Nonneman, R.T. Wiedmann, MicroRNA transcriptome profiles during swine skeletal muscle development, BMC Genomics 10 (2009) 77.
[38] M. Nielsen, J.H. Hansen, J. Hedegaard, R.O. Nielsen, F. Panitz, C. Bendixen, B. Thomsen, MicroRNA identity and abundance in porcine skeletal muscles determined by deep sequencing, Anim. Genet. 41 (2010) 159–168.
[39] J.T. Cuperus, N. Fahlgren, J.C. Carrington, Evolution and functional diversification

of MIRNA genes, Plant Cell 23 (2011) 431–442.
[40] Y. Huang, W. Gong, H. Ren, J. Xiong, X. Gao, X. Sun, Identification of the conserved and novel microRNAs by deep sequencing and prediction of their targets in *Topmouth culter*, Gene 626 (2017) 298–304.
[41] X. Hong, J. Qin, R. Chen, L. Yuan, J. Zha, Z. Wang, Identification and characterization of novel and conserved microRNAs in several tissues of the Chinese rare minnow (*Gobiocypris rarus*) based on illumina deep sequencing technology, BMC Genomics 17 (2016) 283.
[42] W. Gong, Y. Huang, J. Xie, G. Wang, D. Yu, X. Sun, Genome-wide identification and characterization of conserved and novel microRNAs in grass carp (*Ctenopharyngodon idella*) by deep sequencing, Comput. Biol. Chem. 68 (2017) 92–100.
[43] B. Yan, Z.H. Wang, C.D. Zhu, J.T. Guo, J.L. Zhao, MicroRNA repertoire for functional genome research in tilapia identified by deep sequencing, Mol. Biol. Rep. 41 (2014) 4953–4963.
[44] S.E. Linsen, E. de Wit, G. Janssens, S. Heater, L. Chapman, R.K. Parkin, B. Fritz, S.K. Wyman, E. de Bruijn, E.E. Voest, S. Kuersten, M. Tewari, E. Cuppen, Limitations and possibilities of small RNA digital gene expression profiling, Nat. Methods 6 (2009) 474–476.
[45] H.K. Kim, Y.S. Lee, U. Sivaprasad, A. Malhotra, A. Dutta, Muscle-specific microRNA miR-206 promotes muscle differentiation, J. Cell Biol. 174 (2006) 677–687.
[46] C. Anderson, H. Catoe, R. Werner, MIR-206 regulates connexin43 expression during skeletal muscle development, Nucleic Acids Res. 34 (2006) 5863–5871.
[47] I. Lee, S.S. Ajay, H. Chen, A. Maruyama, N. Wang, M.G. McInnis, B.D. Athey, Discriminating single-base difference miRNA expressions using microarray Probe Design Guru (ProDeG), Nucleic Acids Res. 36 (2008) e27.
[48] A.M. Reddy, Y. Zheng, G. Jagadeeswaran, S.L. Macmil, W.B. Graham, B.A. Roe, U. Desilva, W. Zhang, R. Sunkar, Cloning, characterization and expression analysis of porcine microRNAs, BMC Genomics 10 (2009) 65.
[49] W. Tang, S. Wan, Z. Yang, A.E. Teschendorff, Q. Zou, Tumor origin detection with tissue-specific miRNA and DNA methylation markers, Bioinformatics 34 (2018) 398–406.
[50] X. Zeng, L. Liu, L. Lu, Q. Zou, Prediction of potential disease-associated microRNAs using structural perturbation method, Bioinformatics 34 (2018) 2425–2432.
[51] Q. Zou, Y. Mao, L. Hu, Y. Wu, Z. Ji, miRClassify: an advanced web server for miRNA family classification and annotation, Comput. Biol. Med. 45 (2014) 157–160.
[52] W. Tang, Z. Liao, Q. Zou, Which statistical significance test best detects oncomiRNAs in cancer tissues? An exploratory analysis, Oncotarget 7 (2016) 85613–85623.
[53] L. Wei, M. Liao, Y. Gao, R. Ji, Z. He, Q. Zou, Improved and promising identification of human MicroRNAs by incorporating a high-quality negative set, IEEE/ACM Trans. Comput. Biol. Bioinform. 11 (2014) 192–201.
[54] J. Marcial-Quino, S. Gomez-Manzo, F. Fierro, A. Vanoye-Carlo, Y. Rufino-Gonzalez, E. Sierra-Palacios, A. Castillo-Villanueva, R.A. Castillo-Rodriguez, E. Rodriguez-Bustamante, R. Arreguin-Espinosa, H. Reyes-Vivas, Stem-loop RT-qPCR as an efficient tool for the detection and quantification of small RNAs in *Giardia lamblia*, Genes (Basel) 7 (2016).
[55] Y. Huang, J.H. Cheng, F.N. Luo, H. Pan, X.J. Sun, L.Y. Diao, X.J. Qin, Genome-wide identification and characterization of microRNA genes and their targets in large yellow croaker (*Larimichthys crocea*), Gene 576 (2016) 261–267.