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Identification and characterization of circular RNAs in zebrafish

Yudong Shen[1], Xianwu Guo[2] and Weimin Wang[1]

1 College of Fisheries, Key Lab of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education/Key Lab of Freshwater Animal Breeding, Ministry of Agriculture, Huazhong Agricultural University, Wuhan, Hubei, China
2 Lab of Biotecnología Genómica, Centro de Biotecnología Genómica, Instituto de Politécnico Nacional, Reynosa, Tamaulipas, Mexico

Correspondence
W. Wang, College of Fisheries, Key Lab of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education/Key Lab of Freshwater Animal Breeding, Ministry of Agriculture, Huazhong Agricultural University, Wuhan, Hubei 430070, China
Fax: +86-27-87282114
Tel: +86-27-87282113
E-mail: wangwm@mail.hzau.edu.cn

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Circular RNA (circRNA), a class of RNAs with circular structure, has received little attention until recently, when some new features and functions were discovered. In the present study, we sequenced circRNAs in zebrafish (*Danio rerio*) and identified 3868 circRNAs using three algorithms (find_circ, CIRI, segemehl). The analysis of microRNA target sites on circRNAs shows that some circRNAs may function as miRNA sponges. Furthermore, we identified the existence of reverse complementary sequences in the flanking regions of only 25 (2.64%) exonic circRNAs, indicating that the mechanism of zebrafish exonic circRNA biogenesis might be different from that in mammals. Moreover, 1122 (29%) zebrafish circRNA sequences showed homology with human, mouse and coelacanth circRNAs.

Keywords: circRNAs biogenesis; homology; zebrafish

Circular RNAs (circRNAs) are a class of special RNAs that covalently forms a closed loop in eukaryotes [1–4]. circRNAs were confirmed to be more stable than linear RNAs and to be able to survive under conditions of RNase R treatment [5]. It is assumed that circRNAs are a distinct class of endogenous noncoding RNAs. However, some studies have shown that a few circRNAs have the potential to translate into proteins by inserting an internal ribosome entry site [6,7]. Wang and Wang [6] reported that circRNAs can direct protein synthesis as mRNA [6].

With the technological breakthroughs in high-throughput deep sequencing, large numbers of circRNAs have been found recently in humans [8–10], mice [8,10], nematodes [10–12] and coelacanths [13]. Using a bioinformatics algorithm to analyze available genome-wide RNA-seq data, Ye *et al.* [14] identified circRNAs in the model species of dicot and monocot plants, *Oryza sativa* and *Arabidopsis thaliana*, respectively, and reported that the biogenesis mechanisms of circRNAs from plants could be different from those in animals. To date, three biogenesis mechanisms of circRNAs [15–17] have been proposed suggesting that the circRNAs originating from exons (exonic circRNA), introns (intronic circRNA) and the intergenic region were cyclized *via* back-splicing. Recently, Li *et al.* [18] reported the exon-intron circular RNA (EIciRNA) and suggested that EIciRNAs predominantly localized in the nucleus.

CircRNAs showed their specifically expressed patterns in different tissues, cell types or developmental stages [9,10]. Although the circRNAs were regarded as a small percentage of total RNAs, some of their

Abbreviations
circRNA, circular RNA; miRNA, microRNA.

expression quantities were ten-fold higher than their linear transcripts in human cells [15,19].

In fish, Nitsche *et al.* [13] had already analyzed the circRNAs of coelacanths and observed that 'aberrant' isoforms of circRNAs were much more abundant in coelacanths than in zebrafish and humans based on the transcriptome of muscle data. Nevertheless, the basic circRNA data for zebrafish are still unavailable. As a result of approximately 70% genes being orthologous between zebrafish and humans, the zebrafish, as a model organism, has made an important contribution to the study of human diseases. In addition, many zebrafish studies also uncovered the common characters of vertebrate biology and development [20]. In the present study, we attempted to identify the circRNAs from extensive tissues in zebrafish, as well as subsequently characterize the zebrafish circRNAs, which could provide a basis for further studies of the functions of fish circRNAs.

Materials and methods

Zebrafish (*Danio rerio*) were supplied by China Zebrafish Resource Center. Three male and three female individuals were randomly selected from a population of zebrafish AB strain. To ensure good a representation of zebrafish circRNAs, different tissues were sampled, including the brain, eyes, heart, liver, spleen, kidney, intestines, skin, muscle, gill, ovary and testis, and then total RNAs were isolated from the respective tissues. After assessing the quality and quantity of the RNAs, twelve types of qualified RNAs were mixed equally according to their concentration. Those total RNAs were used as templates for subsequent reverse transcription.

To obtain abundant circRNAs, we used a Cloudseq CircRNA Enrichment Kit (Cloudseq Biotech lnc., Shanghai, China) and RNase R to treat the leftovers after removing rRNA from the total RNA. First, circRNAs are randomly fragmented, followed by reverse transcription with random primers, sequencing and retrieval of data using a HiSeq4000 system (Illumina, Inc., San Diego, CA, USA). The software TOPHAT2 [21] was used to map the sequence data to zebrafish genome (danRer10). Three bioinformatics analytic methods, Find_circ [10], CIRI [22] and segemehl [13], were used for circRNA identification with default parameters.

The default parameters of Find_circ version 1 were:

```
find_circ.py unmapped_anchors.sam -G danRer10_Base.fa -p projectid -s out/sites.log > out/sites.bed 2> out/sites.readsgrep circ out/sites.bed | sum.py -2,3 | scorethresh.py -16 1 | scorethresh.py -15 2 | scorethresh.py -14 2 | scorethresh.py 7 2 | scorethresh.py 8,9 35 | scorethresh.py -17 100000 > out/circ_candidates.bed
```

The default parameters of CIRI version 1.2 were:

```
CIRI_v1.2.pl -I input.sam -O output_circRNAs.txt -F danRer10_Base.fa -P -A danRer10_transcriptome.gtf
```

The default parameters of segemehl 0.2.0-418 were:

```
segemehl.x -d danRer10_Base.fa -i segemehl_danRer10_Base.idx -q sequence_R1.fastq -p sequence_R2.fastq -t 24 > output.sam
samtools view -bS output.sam | samtools sort -o - deleteme | samtools view -h - > output_mapped.sam
testrealign.x -d danRer10_Base.fa -q output_mapped.sam -n
cat splicesites.bed | grep C:P >circular.bed
```

CircRNAs were divided into five types: exonic circRNA, intronic circRNA, sense overlapping circRNA, antisense circRNA and intergenic circRNA [10,23]. In the intersection of results derived from the three methods, five circRNAs were selected randomly from each type of circRNAs (25 circRNAs in total) to validate the reliability of circRNAs by experiments. In addition, the total RNAs of six individual zebrafish were used to obtain cDNA in accordance with the manufacturer's instructions using a PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time; Takara Bio Inc., Otsu, Japan). The cDNA was used as template for PCR and quantitative RT-PCR. Two methods were used to verify circRNAs. Sanger-sequencing [14] was used to check the target fragment after the PCR product were purified, aiming to show the results intuitively. The PCR parameters were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, an appropriate annealing temperature (according to the T_m of primers) for 35 s and 72 °C for 30 s, and then 72 °C for 10 min. The quantitative RT-PCR helps to monitor the specific amplification *via* a dissociation curve and this method has general applicability. The quantitative RT-PCR reaction [9] performed comprised: pre-denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 60 s. After the amplified reaction, the temperature was adjusted to 95 °C for 10 s, 60 °C for 60 s and 95 °C for 15 s, with slow heating of the reaction system at 0.05 °C per second ramp rate until the temperature reached 99 °C to obtain the dissociation curve.

For the circRNAs in the intersection identified by the three methods, MIRANDA, version 3.3a [24] and TARGETSCAN, version 6.0 [25] were used to predict the microRNA (miRNA) sites in circRNAs with the default parameters (default parameters of MIRANDA, version 3.3a: miranda mirna.fa target.fa -out output.txt; default parameters of TARGETSCAN, version 6.0: targetscan_60.pl miRNA_family_info.txt targetscan_format_INPUT.txt targetscan_60_output.txt targetscan_60_context_scores.pl miRNA_mature_info.txt targetscan_format_INPUT.txt targetscan_60_output.txt targetscan_60_context_scores_output.txt) and to select five miRNA binding sites with the highest binding force. The five miRNAs were considered as an element that could respond to the target circRNAs with high probability. The number of miRNA sites in circRNA helps to identify the circRNAs that may act as a miRNA sponge. The flanking sequences of three types circRNAs (exonic circRNAs, sense overlapping circRNAs and antisense circRNAs) that contain the nearest

intron sequences were analyzed by BLAST (e-VALUE: $< 1e^{-5}$) [26] to determine whether there were reverse complementary sequences and *Alu* elements. Furthermore, each of the flanking sequences of intronic circRNAs and intergenic circRNAs was also analyzed in the range of 200 nculeotides. The circRNA homologs among zebrafish, humans, mice and coelacanths were analysed using BLAST (*e*-value: $< 1e^{-5}$) [26]. The data on human, mouse and coelacanth circRNAs were downloaded from circBase (http://www.circbase.org).

Results

Zebrafish circRNAs distribution and their validation by experimentation

The Illumina sequencing reads reach 21.32 Gb in total and all raw reads have been submitted to the NCBI SRA database (SRR4302022). Through the data analysis, we obtained a total of 3868 circRNAs, of which 176 circRNAs (4.6%) were commonly identified by all the three algorithms (find_circ, CIRI and segemehl) and 382 (9.9%) were identified by two of the three algorithms. The other circRNAs were only determined by one algorithm (Fig. 1A). After comparison with the genome sequence of zebrafish, five types of circRNAs [10,23] (exonic, intronic, sense overlapping, antisense and intergenic circRNAs) were found, comprising 24.48%, 19.62%, 39.30%, 3.18% and 13.42%, respectively (Fig. 2).

In the intersection of results derived from the three methods, total 25 circRNAs, randomly taking five samples from each type, were used to test the veracity of zebrafish circRNAs and the results showed that 21 circRNAs were confirmed (Fig. 1B,C; Fig. S1 and S2; Table S1). Among them, five from exonic circRNAs and five from intronic circRNAs were confirmed completely.

The target miRNAs of circRNAs

Of 176 circRNAs with high reliability, their potential target miRNAs were analyzed and five miRNA sites with the highest binding force were identified (Table S2). The dre-miR-2193 could target 29 circRNAs

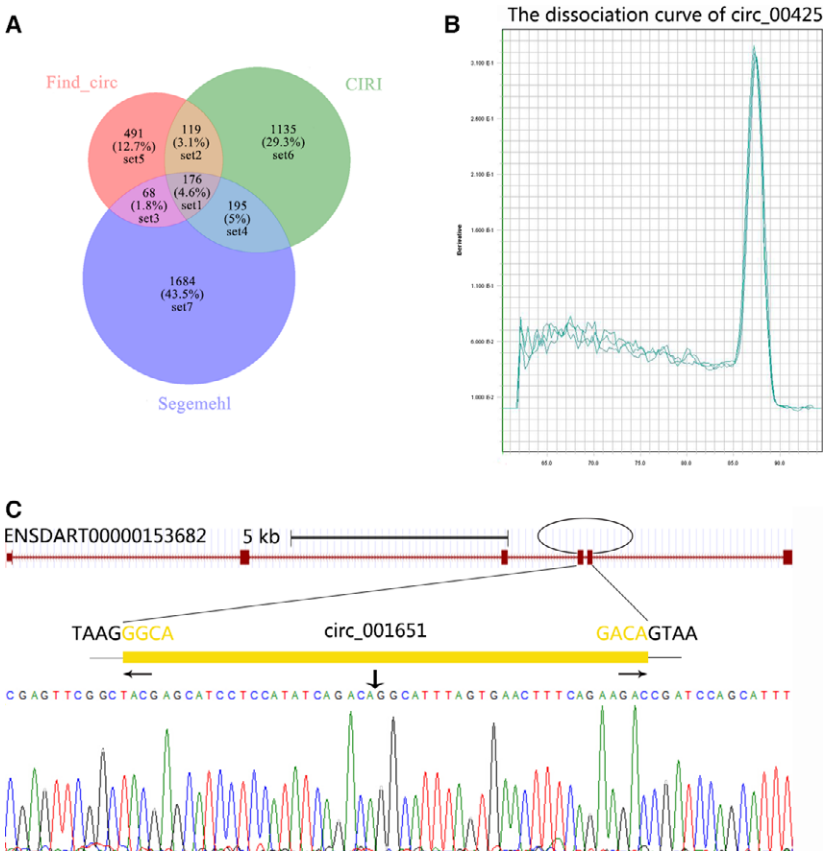

Fig. 1. The identification and confirmation of zebrafish circRNAs. (A) The circRNAs were identified by three algorithms (find_circ, CIRI and segemehl). (B) A dissociation curve example showing that the junction sites of circ_00425 was extended by qRT-PCR. (C) A Sanger sequencing example showing that circ_001651 was derived through exon back-splicing from ENSDART00000153682.

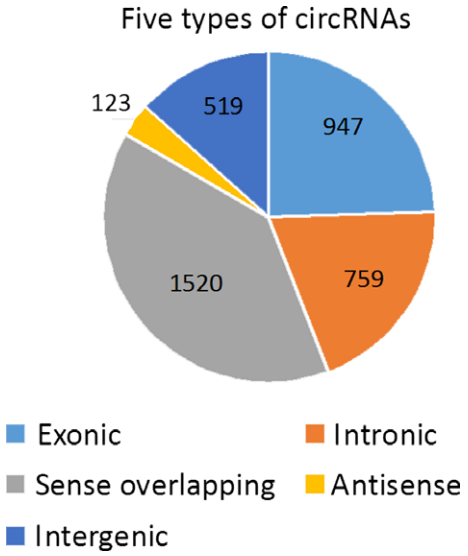

Fig. 2. The percentage of five types of circRNAs.

(Fig. 3). Furthermore, 19 circRNAs shared the common dre-miR-2196 target sites and dre-miR-737-5p, dre-miR-92b-5p and dre-miR-9-5p also interacted with 13 circRNAs. Most circRNAs only have one or two sites for the same miRNA target. Focusing on the highest binding force on miRNA, circ_000207, circ_000348, circ_000423, circ_000425, circ_000479, circ_001175, circ_002081, circ_002167 and circ_002177 contain 21, 28, 37, 76, 25, 62, 112, 52 and 18 target sites for dre-miR-2193, respectively. This implied that these circRNAs could function as a dre-miR-2193 sponge. The network of circRNAs involved in the interaction with dre-miR-2193 is shown in Fig. 3.

The flanking intron sequence of circRNAs

The flanking intron sequences of circRNAs were analyzed. Unexpectedly, in a total of 3868 circRNAs, reverse complementary sequences were identified for only 214 circRNAs. Furthermore, many different ways of pairing were found. However, the number of ways of pairing did not indicate the relationship with the number of reads of circRNAs. By checking the origin of the 214 circRNAs, there were 25 exonic circRNAs, 32 sense overlapping circRNAs, 101 intron circRNAs and 56 intergenic circRNAs. This meant that only 2.64% (25/947) zebrafish exonic circRNAs matched the conditions of the Intron-Pairing-Driven Circularization model.

The homology of zebrafish circRNAs

Comparing zebrafish circRNAs with those of humans and other vertebrates, the results showed that 1326 (34.3%) zebrafish circRNAs were homologous with humans, 1240 (32.1%) were homologous with mice and 1221 (31.6%) were homologous with latimeria chalum. However, the majority (62.4%) of zebrafish circRNAs showed no homology with the three species. In total, 1122 zebrafish circRNAs (29.0%) showed homology with all three species (Fig. 4).

Discussion

Obtainment and identification of zebrafish circRNAs

Nitsche *et al.* [13] preliminarily analyzed zebrafish circRNAs using the segemehl algorithm based on the data from zebrafish muscle transcriptome (ERR145647). They searched splice junctions to infer the number of circRNAs and found 7850 splice junctions supported by at least three split reads [13]. It should be noted that, because of the procedure of mRNA purification before transcriptome sequencing, the RNAs need to be enriched with poly-A tail, and the use of transcriptome data to analyze circRNAs may lose some of the circRNA information. In the present study, according to the tissue specificity of circRNAs, circRNAs derived from brain, eyes, heart, liver, spleen, kidney, intestines, skin, muscle, gill, ovary and testis were enriched to establish a zebrafish database, which should be more extensively representative for zebrafish circRNAs. Our sample treatments with RNase R and the circRNA Enrichment Kit, sequenced data of up to 21.3 Gb in total and the test for veracity of circRNA performed by RT-PCR indicates that the results are reliable. In total, 3868 zebrafish circRNAs were found and 176 of these were commonly identified. Considering the large differences shown among the methods with respect to identifying circRNAs when analyzing HeLa cell transcriptome data [22], we combined the advantages of three algorithms to search the possible circRNAs [27,28]. Indeed, the number of identified circRNAs revealed a big difference between humans, mice and zebrafish. The principal reasons could be the distinct parameters used for identification, the different methods employed or the species features of circRNAs.

The flanking intron sequence of zebrafish circRNAs

Back-splicing formation by downstream donor and upstream acceptor could be promoted by the reverse complement sequences across their flanking introns in circRNA biogenesis [1,29]. In *Caenorhabditis elegans*,

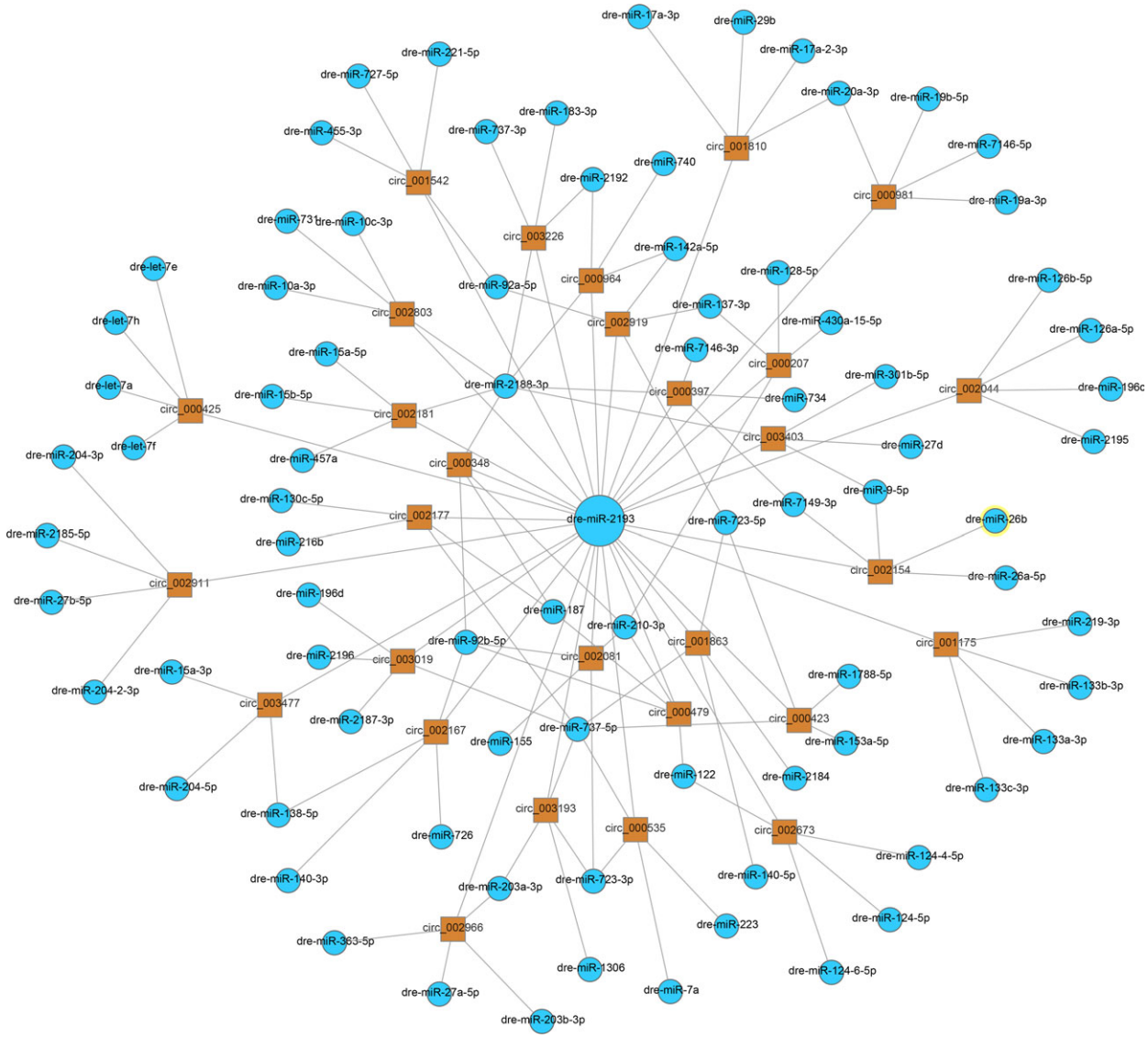

Fig. 3. The network graph of 29 circRNAs that could be able to interact with dre-miR-2193.

96.33% of circRNA flanking sequences possessed reverse complementary sequences [12]. In humans, 88% of circRNAs had *Alu* repeats in their flanking introns [11], implying that some repeat sequences such as *Alu* elements are important factors in circRNA formation [30]. circHIPK3, a circRNA from humans, showed no circularization when one or both *Alu* elements were deleted. This suggests that the two inverted repeat elements were indispensable [23]. In zebrafish, 25 circRNAs, all of which were exonic circRNAs, had the reverse complementary sequences, although none of them had *Alu* elements. Thus, only 2.64% (25/947) of zebrafish exonic circRNAs matched the conditions of the Intron-Pairing-Driven Circularization model, which was used to explain the circRNAs biogenesis in mammals. Ye *et al.* [14] noted that the intronic sequences bracketing circRNAs of *O. sativa* (6.2%) and *A. thaliana* (0.3%), which contain repetitive sequences, were in a very low proportion and that the biogenesis of plant circRNAs could use mechanism(s) different from those proposed for animals. On the other hand, exonic circRNAs of *Drosphila melanogaster* were also found to lack flanking intronic pairing sequences [1,31]. In addition, the RNA binding protein Mbl in *Drosophila* could promote circularization [16]. Thus, our analysis suggests that some zebrafish exonic circRNAs might cyclize by different mechanisms. The biogenesis of circRNAs was distinct in a variety of species.

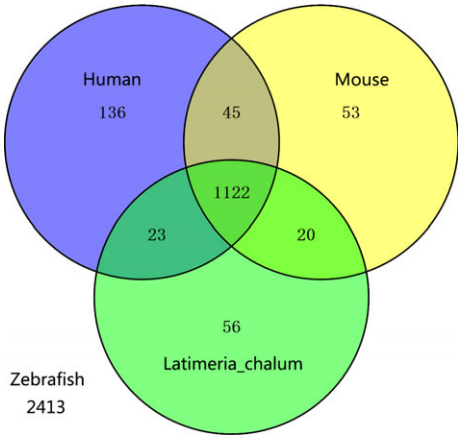

Fig. 4. Zebrafish circRNAs shared homology among human, mouse and latimeria chalum.

The target miRNAs of circRNAs

CircRNAs showed an ability to be acts as an antagonist as miRNA sponges [32]. Human CDR1as had confirmed its regulatory potency in zebrafish midbrain development [10]. The circRNA generated from the *Sry* gene also proved to be a sponge for miR-138 [33]. Wang *et al.* [34] showed that a heart-related circRNA (*HRCR*) acts as an endogenous miR-223 sponge. Recently, circHIPK3 was reported to sponge multiple miRNAs [23]. In the present study, circ_000207, circ_000348, circ_000423, circ_000425, circ_000479, circ_001175, circ_002081, circ_002167 and circ_002177 showed dre-miR-2193 target sites. This implied that the nine circRNAs could function as a dre-miR-2193 sponge. By contrast, many studies report that the majority of circRNAs are unable to function as miRNA sponges [12,35]. A recent study suggested that circRNAs might be able to function as RBP sponges [16,36]. Whether circRNAs functioning as miRNA sponges is a universal characteristic of circRNA still remains to be clarified. All of the results described above indicate that circRNAs could have diverse functions in cells.

The homology of zebrafish circRNAs

Most of the circRNAs contained highly conserved sequences among different taxonomic species [15,19], whereas a few circRNAs were not conserved throughout their evolution [30,37]. Homologous analysis for circRNAs is helpful when aiming to understand their common characteristics. Guo *et al.* [35] annotated 635 mouse circRNAs, 20% of which were orthologous to human circRNAs. We checked the mouse circRNAs in circBase and showed that, out of a total 1903 mouse circRNAs, 1236 mouse circRNAs (65%) were homologous to human circRNAs. In zebrafish, a considerable part of zebrafish circRNAs is highly conserved (Fig. 4). Interestingly, the eight circRNAs (circ_000207, circ_000348, circ_000423, circ_000425, circ_000479, circ_002081, circ_002167 and circ_002177), which might function as a dre-miR-2193 sponge, were also conservative among humans, mice and latimeria chalum, implying that they could have similar functions in vertebrates.

The RNase R used in this experiment was considered as an enzyme that preserved circRNAs but digested linear RNAs [5]. Some of the circRNAs were still not confirmed to be intolerant to RNase R enzyme in *C. elegans* [12]. In addition, the loop portions of lariat RNAs remained fully intact after RNase R enzyme treatment [5]. Although loop portions of lariat RNAs with unique a 2′–5′ linked loop structure were different from circRNAs, it was still difficult to distinguish the loop portions of lariat RNAs from circRNAs. This means that the circRNAs of zebrafish obtained in the present study maybe contain some lariat RNAs and some of the circRNAs could have been eliminated by RNase R.

In summary, 3868 zebrafish circRNAs have been identified. In total, 176 circRNAs were commonly identified with all the three algorithms and the 84% (21/25) credibility of 176 circRNAs was confirmed by experiment. Through prediction for target miRNAs, analyses for flanking intron sequences and conservation of zebrafish circRNAs, we found that some circRNAs had the potential to function as a dre-miR-2193 sponge and the biogenesis of zebrafish exonic circRNAs might not cyclize *via* the model of Intron-Pairing-Driven Circularization. In total, 1122 zebrafish circRNAs showed conservative properties. The results reported in the present study lay a solid foundation for further studies of zebrafish circRNAs.

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Author contributions

WW and YS designed experiments; WW provided funds; YS performed experiments and analysis; YS wrote the manuscript; XG made manuscript revisions.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. The Sanger-sequencing results of 11 circRNAs.

Fig. S2. The qRT-PCR dissociation curve of eight circRNAs.

Table S1. Divergent primers for validation of candidate circRNAs.

Table S2. miRNA binding sites prediction of set1 circRNAs.