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Identification and characterization of novel type of RNAs, circRNAs in crucian carp Carassius auratus gibelio

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ABSTRACT

Circular RNAs (circRNAs) with regulatory potency activity was identified from varieties of species. Crucian carp (*Carassius auratus* gibelio) is one of the most freshwater aquaculture species in China. Every year, huge economic damage to the farming was caused by the virus and bacterial infection. Until now, there is any information about circRNA reported from the Crucian carp. In this study, the expression pattern of circRNA in Crucian carp was investigated with transcriptomic analysis. The results showed that only 37 circRNAs were identified from the Crucian carp, and these circRNAs biogenesis was formed with canonical GU-AG splicing mechanism with unevenly distributed on the chromosomes. Wherein, most of the circRNAs were derived from the sense overlapping strategy. Reverse transcript PCR and Sanger sequencing data indicated that these circRNAs were existed authenticity in Crucian carp. The bioinformatics analysis indicated that circRNAs identified from the Crucian carp with potential miRNA sponge regulate the expression level of mRNAs. GO annotation and KEGG pathway analysis of these circRNAs showed that more than 20% circRNAs were related with catalytic activity and binding in the category of molecular function, and these circRNAs were enriched in 9 signaling pathways, such as, Wnt signaling pathway, MAPK signaling pathway, Ubiquitin mediated proteolysis et al. 220 mRNAs would be regulated by the circRNAs via miRNAs mediation. These target mRNAs were further analyzed with functional annotation and KEGG analysis. GO annotation analysis showed that several genes were related with function of nucleotide binding, transcription regulatory activity. KEGG pathway analysis showed that 5 genes were enriched in the pathway of Endocytosis. The circRNA-miRNA-mRNA regulation network indicated that one miRNA can link one or more circRNA and one or more mRNA. Overall, these results will not only help us to further understand the novel RNA transcripts in Crucian carp, but also provide the novel clue to investigate the interaction between host and pathogens from this novel circRNA molecule.

1. Introduction

Circular RNA (circRNA) is a novel type of non-coding RNA, which is widespread and diversely in natural species with huge regulatory potency [1]. CircRNA can be used as the miRNA sponge to regulate the expression of mRNAs; circRNA has the ability to translate into small proteins with the internal ribosome entry site (IRES); circRNA can interact with RNA binding proteins to form the RNA-protein complex (RPC) to regulate the transcription of linear parent genes [1]. CircRNAs were not only found in the eukaryotes, but also identified from viruses [2–4]. Similar to the linear RNA, such as mRNA, the expression level of circRNA were also changed after virus infection [5–8]. Besides, many

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circRNAs with dysregulation was associated with pathogenesis of cancers [9–12].

Crucian carp (*Carassius auratus* gibelio) is one of the most freshwater aquaculture species in China [13,14]. However, with the development of the intensive culture and lack of the efficiency prevention strategy, viral infection is one of the most serious damage to the aquaculture industry, which brings heavy economic damage to the farmer [[13,15,16]]. Although it is considered as the lower vertebrate, but it has strong immunity system, such as, innate and adaptive immune systems. In the invasion of viruses and other pathogens infection, innate immunity mainly contributed to defending against them [17–20]. In the past several years, the function of many genes, miRNA and genes of Crucian carp were uncovered [21–23], however, this type of non-coding RNA, circRNAs has no reported on them in Crucian carp. Comprehensively understanding the expression patterns and responding to the pathogens invasion of circRNAs will contribute to know the interaction between Crucian carp and pathogens.

In this study, the expressions of circRNAs were investigated with transcriptomic analysis of kidneys of crucian carp to elucidate this style of novel RNAs in Crucian carp. These results will help us to further understand the transcripts in Crucian carp, and provide the novel clue to investigate the interaction between host and pathogens from this novel circRNA molecule.

2. Materials and methods

2.1. Sample preparation

Crucian carp (mean body weight 30–35 g) were collected from a farm in Yancheng, Jiangsu Province, China. Six tissues (heart, kidney, spleen, liver, intestine, gill and muscle) from three healthy fishes were separated and extracted the total RNAs for *de novo* transcriptome sequencing. These *de novo* sequencing data was used as the reference data (SRR8293179) for further circRNA sequencing analysis.

2.2. RNA extraction and circRNA sequencing

Total RNAs were extracted using the mirVana miRNA Isolation Kit (Ambion) following the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The samples with RNA Integrity Number (RIN) ≥ 7 were subjected to the subsequent analysis. The libraries were constructed using TruSeq Stranded Total RNA with Ribo-Zero Gold according to the manufacturer's instructions. Then these libraries were sequenced on the Illumina sequencing platform (HiSeqTM 2500 or other platform) and 150 bp/125bp paired-end reads were generated in ShangHai Oebiotech Co. (Shanghai, China). The sequencing data was deposited into the NCBI database with accession number (SRR8293177).

2.3. Validation of circRNA with reverse transcription PCR

To validate the correction of circRNAs identified from the Crucian carp, several circRNAs were randomly selected from the sequencing data and validated with reverse transcription PCR using the divergent primers and convergent primers (Supplementary Table 1). The divergent primers flanking the back-splice site and convergent primers across the junction site were designed according to candidate circRNAs obtained from the sequencing data. The total RNAs were extracted from the kindey of healthy Crucian carp. The obtained cDNA with the random primer was used as the temple to clone the junction site and circular sequence of each circRNA. The procedure of PCR was carried out according to the normal regulation. The PCR production was separated with 1% agarose gel electrophoresis. The purified PCR production of the junction site and circular sequence of each circRNA was further validated with Sanger sequencing.

2.4. Prediction of circRNA encoded by crucian carp

After quality control, the clean reads were obtained and aligned with the Crucian carp transcriptomic data by Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml). All of the unmapped reads were extracted out using back-splice algorithm. The candidate circRNAs were verified with a software developed by OE.

2.5. Prediction of targets (miRNAs) of circRNAs and miRNA targets of mRNAs

CircRNA can sponge miRNA to regulate the expression of mRNA. Therefore, the circRNAs targets were predicted using miRanda software with energy score less than -30 kCal/Mol. The 3' UTR of the mRNAs were extracted and the binding sites of the miRNAs were predicted with miRanda software with energy score less than -30 kCal/Mol [24].

2.6. GO annotation and KEGG pathway analysis

WEGO (Web Gene Ontology Annotation Plot) was applied to analyze the GO annotation of the genes (http://wego.genomics.org.cn/). KEGG analysis was carried out on the online software (https://david-d.ncifcrf.gov/).

2.7. Construction of circRNA-miRNA-mRNA network

It reported that circRNA mainly regulated the gene expression level by miRNA mediation. Here, the circRNA-miRNA-mRNA network was constructed with the above targets prediction results using cytoskope sftware. The selected target of circRNAs and miRNAs were following the max energy < -30 kCal/Mol [25].

3. Results

3.1. Identification of circRNAs from the crucian carp

CircRNA-sequencing was applied to investigate the circRNA expression pattern in Crucian carp. The CIRI software was used to predict the circRNAs with the sequencing data. Only 37 circRNAs were identified from kidney of Crucian carp (Table 1). The length distribution showed that 29 circRNAs were mainly at 201–1000 nt, the length of one circRNA was distributed at 1801–1900 nt, and the length of 7 circRNA was more than 2000 nt (Fig. 1A). Due to unavailable of the genome of *Carassius auratus* gibelio, all of the identified circRNAs were mapped onto the genome/scaffold of *Carassius auratus* gibelio in our lab data. CircRNA distribution on chromosome/scaffold was analyzed and the results showed that more than 2 circRNAs were distributed on the chromosome/scaffold 1, 3, 5, 6, 9, 10, 11, 13, 14, 20 and 21. Only one circRNA was distributed on the chromosome/scaffold 2, 12, 15, 16, 17, 18, 19, 24 and 25. No circRNA was found on the other chromosomes/scaffold (Fig. 1B). It indicated that the length of these circRNAs was mainly between 200 and 600 nucleotides, and were not evenly distributed on the chromosomes/scaffold.

3.2. Analysis of circRNA structure

The identified circRNAs were aligned with gene element to explore the distribution of circRNAs on the genome/scaffold of *Carassius auratus* gibelio. According to the distribution of circRNAs on the genome, the circRNAs were divided into five types: exonic circRNA, intronic circRNA, antisense circRNA, sense overlapping circRNA and intergenic circRNA (Fig. 2A). However, the identified circRNAs were mainly classified into three types (Fig. 2B): exonic circRNA (3), intergenic circRNA (1) and sense overlapping circRNA (33). It indicated that most of the circRNAs were derived from the sense overlapping strategy. The splicing sites of these circRNAs were analyzed and the results showed

Table 1
Identification of circRNAs from the identified from the Crucian carp with circRNA sequencing.

id	circRNA_chr	circRNA_length	Type	best-transcript	product
circRNA_01	Chr1	616	sense-overlapping	XM_021477329.1	nuclear factor 1 X-type-like, transcript variant X1
circRNA_02	Chr1	532	exonic	XM_021477329.1	nuclear factor 1 X-type-like, transcript variant X1
circRNA_03	Chr10	414	sense-overlapping	XM_001923512.6	ADP-ribosylation factor-like 15b, transcript variant X5
circRNA_04	Chr10	2322	sense-overlapping	XM_021479191.1	Down syndrome cell adhesion molecule a, transcript variant X1
circRNA_05	Chr11	336	sense-overlapping	XM_005172780.4	zinc finger protein 740a, transcript variant X1
circRNA_06	Chr11	420	sense-overlapping	XM_005172780.4	zinc finger protein 740a, transcript variant X1
circRNA_07	Chr11	6558	sense-overlapping	XM_005172823.4	transducin (beta)-like 1 X-linked receptor 1a, transcript variant X1
circRNA_08	Chr12	323	sense-overlapping	NM_131259.1	transcription factor 7-like 2 (T-cell specific, HMG-box)
circRNA_09	Chr13	412	sense-overlapping	NM_213234.1	sprouty-related, EVH1 domain containing 2b
circRNA_10	Chr13	250	sense-overlapping	XM_009307103.3	golgi brefeldin A resistant guanine nucleotide exchange factor 1, transcript variant X1
circRNA_11	Chr13	394	sense-overlapping	XM_021480581.1	c13h10orf11 homolog (H. sapiens), transcript variant X2
circRNA_12	Chr14	430	sense-overlapping	NM_001002180.1	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme B
circRNA_13	Chr14	550	sense-overlapping	XM_009291090.3	casein kinase 1, alpha 1, transcript variant X4
circRNA_14	Chr15	80233	sense-overlapping	XM_005157800.4	si:ch211-250k18.8
circRNA_15	Chr16	255	sense-overlapping	XM_009292711.3	SEC16 homolog A, endoplasmic reticulum export factor, transcript variant X1
circRNA_16	Chr17	261	sense-overlapping	XM_009293118.3	QKI, KH domain containing, RNA binding a, transcript variant X1
circRNA_17	Chr18	407	exonic	NM_001317766.1	RGD motif, leucine rich repeats, tropomodulin domain and proline-rich containing
circRNA_18	Chr19	532	sense-overlapping	NM_201492.2	tropomyosin 3, transcript variant 2
circRNA_19	Chr2	549	exonic	XM_005171260.4	insulin receptor a, transcript variant X1
circRNA_20	Chr20	475	sense-overlapping	XM_001923318.7	cyclin-dependent kinase 19, transcript variant X1
circRNA_21	Chr20	936	sense-overlapping	XM_021468374.1	influenza virus NS1A binding protein a, transcript variant X1
circRNA_22	Chr21	418	sense-overlapping	XM_009295410.3	Rho GTPase activating protein 32b, transcript variant X1
circRNA_23	Chr21	303	sense-overlapping	NM_200581.1	Alport syndrome, mental retardation, midface hypoplasia and elliptocytosis chromosomal region gene 1 homolog (human)
circRNA_24	Chr24	15873	sense-overlapping	XM_005162711.4	acyl-CoA thioesterase 9, tandem duplicate 1, transcript variant X1
circRNA_25	Chr25	19758	sense-overlapping	NM_001256177.1	cytochrome P450, family 2, subfamily X, polypeptide 7
circRNA_26	Chr3	15645	sense-overlapping	NM_131117.3	homeobox B3a
circRNA_27	Chr3	1809	intergenic		
circRNA_28	Chr3	704	sense-overlapping	NM_181601.4	actin, beta 2
circRNA_29	Chr5	504	sense-overlapping	NM_199863.1	ubiquitin-conjugating enzyme E2G 1a (UBC7 homolog, yeast)
circRNA_30	Chr5	269	sense-overlapping	NM_001083123.1	androgen receptor
circRNA_31	Chr5	306	sense-overlapping	XM_005165313.4	Rho-related BTB domain containing 2a, transcript variant X2
circRNA_32	Chr6	309	sense-overlapping	XM_001921794.7	lysine (K)-specific demethylase 4A, genome duplicate a, transcript variant X1
circRNA_33	Chr6	3279	sense-overlapping	XM_021477112.1	zinc finger protein 644b, transcript variant X1
circRNA_34	Chr9	563	sense-overlapping	NM_001079683.1	armadillo repeat containing 8
circRNA_35	Chr9	721	sense-overlapping	NM_001109865.1	Kruppel-like factor 12b
circRNA_36	Chr9	309	sense-overlapping	NM_001080673.1	dachshund d
circRNA_37	Chr9	251	sense-overlapping	XM_002667521.6	serine threonine kinase 39

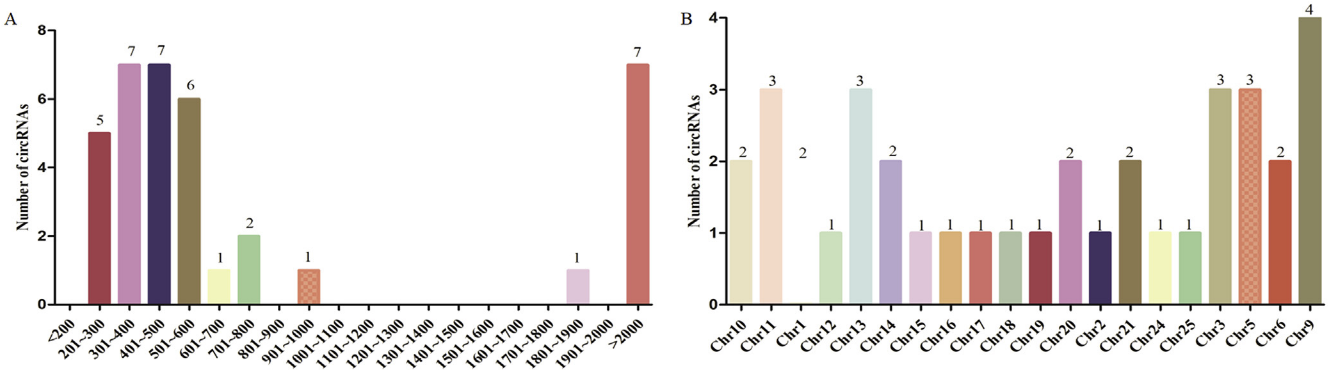

Fig. 1. The length and chromosome distribution of circRNAs identified from the Crucian carp. (A) The length distribution of circRNAs. (B) The chromosome distribution of circRNAs.

that 37 circRNAs contained the canonical GU-AG splicing sites (Fig. 2C). It indicated that circRNAs derived from the genome/scaffold of Crucian carp were following the canonical GU-AG splicing mechanism.

3.3. Validation of circRNA with reverse transcript PCR using divergent primers

To understand the authenticity of identified circRNA from the Crucian carp, 5 circRNAs selected from the 37 circRNAs were validated with reverse transcript PCR using divergent primers and convergent primers of each circRNA (Fig. 3A and B). The PCR production was further validated with Sanger sequencing. The results showed that junction cites and circular sequences of each circRNAs obtained from PCR were consistent with the transcriptomic data (Fig. 4).

3.4. GO annotation and KEGG pathway analysis of parental genes related to these circRNAs

To known the potential function of these circRNAs, the GO annotation and KEGG pathway were analyzed with the parental genes. The results showed that more than 20% circRNAs were related with the

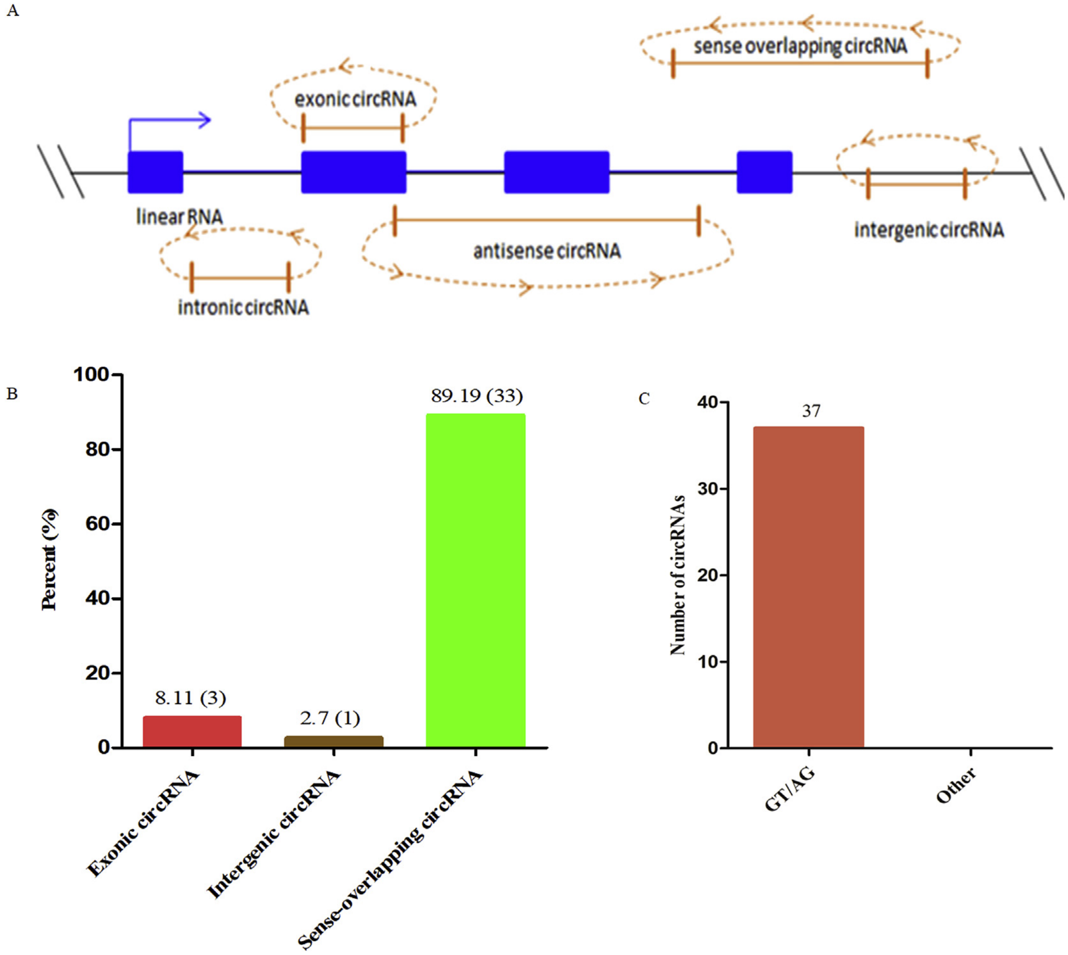

Fig. 2. The category and backsplcing signal analysis ofcircRNA identified from the Crucian carp. (A) CircRNA divided into five categories. (B) Identified circRNAs divided into three types. (C) The backsplcing signal analysis of circRNAs identified from the Crucian carp.

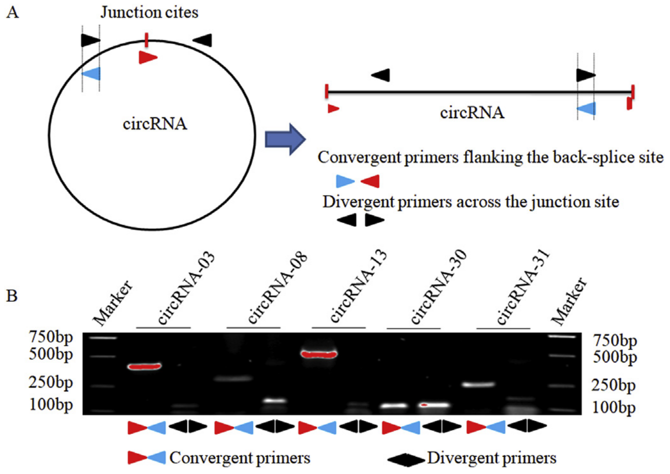

Fig. 3. Validation of circRNAs identified from the Crucian carp. (A) The divergent primers and convergent primers were designed for circRNA validation. (B) The PCR products were detected with 1% agarose gel electrophoresis.

function of catalytic activity and binding in the category of molecular function (Fig. 5A). KEGG analysis showed that these circRNAs were enriched in 9 signaling pathways, such as, Wnt signaling pathway, MAPK signaling pathway, Ubiquitin mediated proteolysis et al. (Fig. 5B and Table 2). These results indicated that circRNAs indentified from the kideny of Crucian carp may be associated with important biological processes.

3.5. Target miRNAs and mRNAs prediction

CircRNA can sponge miRNAs and regulate the expression level of mRNAs. Through the interaction prediction between circRNA and miRNA will help to know the function of circRNA and regulation mechanism in Crucian carp. Generally, one circRNA contains one or numerous miRNA binding sites, therefore, miranda software was applied to predicted the target miRNA genes. The results showed that total 520 miRNAs were sponged by the 37 circRNAs, wherein, circRNA-1, circRNA-14 and circRNA-288 contained 514, 518 and 288 miRNA binding sites (Supplementary Table 2). It indicated that one circRNAs can sponge more than one miRNAs by complementary base pairing.

According to the regulation of miRNA on the expression of mRNA, miranda software was applied to predicted the target mRNA genes. The results showed that only 96 miRNAs had 220 mRNA binding sites (Supplementary Table 3). It indicated that one miRNA can regulated one or more mRNA expression levels.

3.6. GO annotation and KEGG pathway analysis of target mRNAs

From the target prediction results, 220 mRNAs would be regulated by the circRNAs via miRNAs mediation. Therefore, these mRNAs were further analyzed to uncover the potential roles. GO annotation results showed that several genes were related with function of nucleotide binding, transcription regulatory activity (Fig. 6A). KEGG pathway analysis showed that 5 genes were enriched in the pathway of Endocytosis (Fig. 6B).

3.7. CircRNA-miRNA-mRNA regulation network construction

Based on the prediction results, the circRNA-miRNA-mRNA regulation network was constructed with cytoskope software. The network showed that the function of circRNA was dependent on the regulation of miRNA on mRNA. One miRNA can link one or more circRNA and one or more mRNA (Fig. 7).

4. Discussion

More and more novel circRNAs were reported from numerous

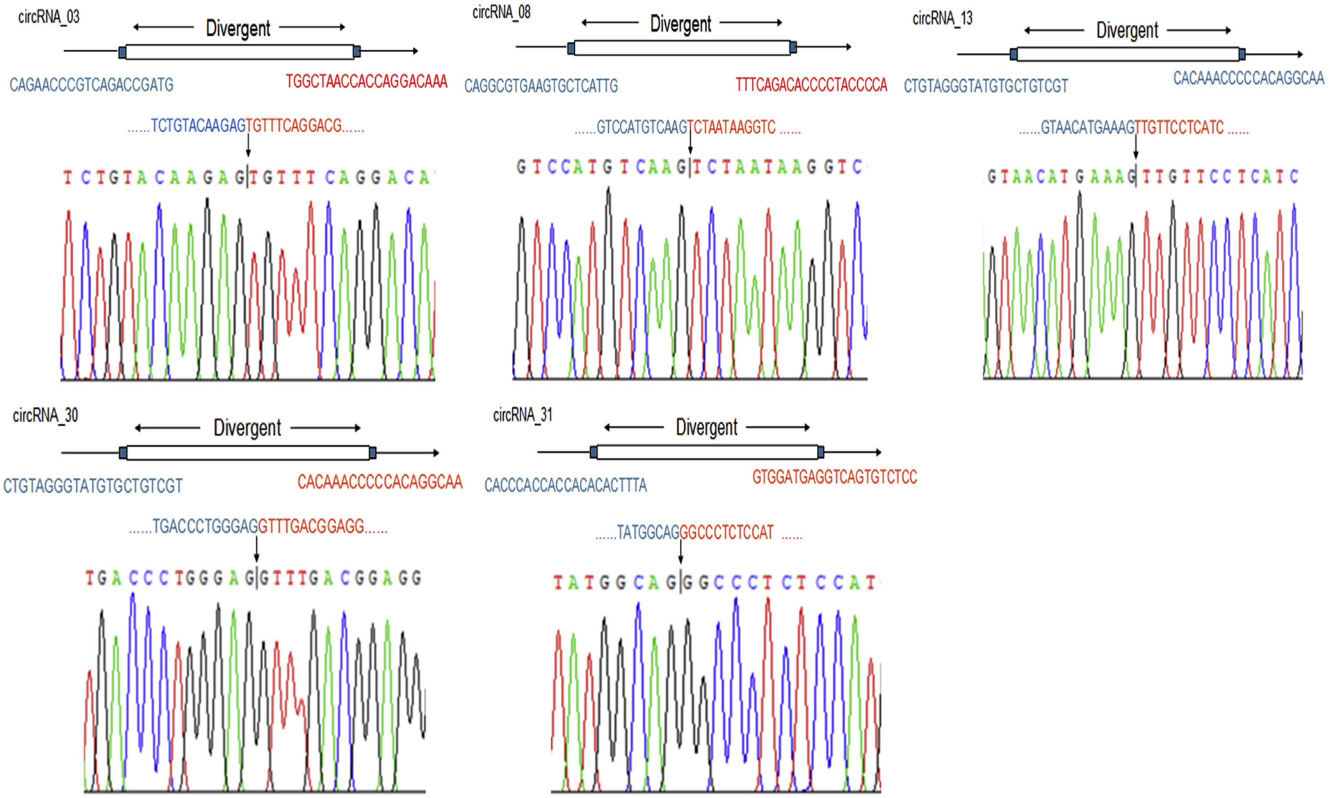

Fig. 4. The Sanger sequencing of 5 circRNAs junction cites with reverse transcript PCR using divergent primers.

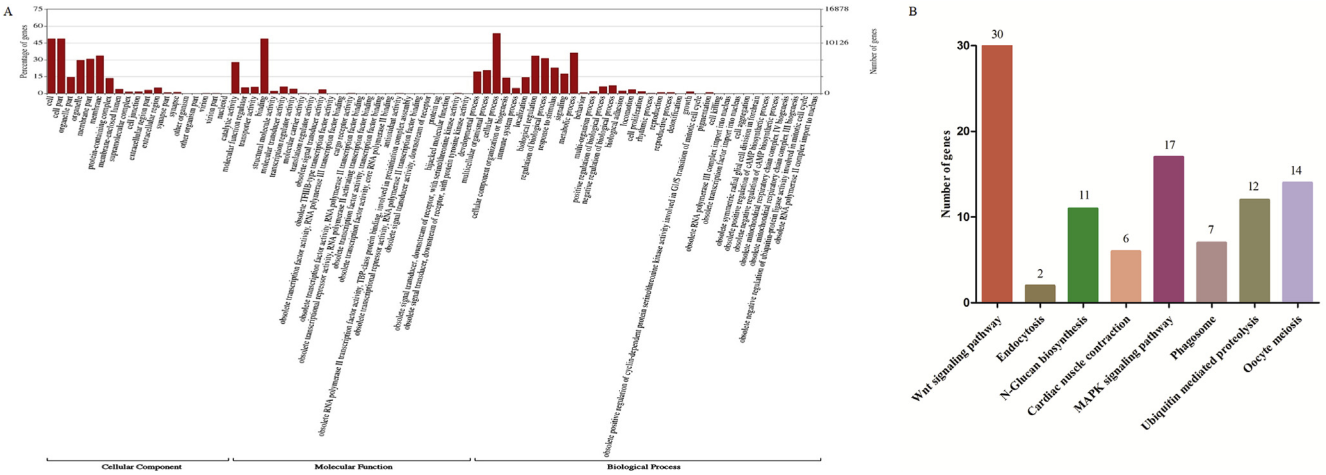

Fig. 5. GO and KEGG analysis of circRNAs identified from the identified from the Crucian carp. (A) GO analysis of circRNAs according to the values in the enrichment score under the theme of BP, CC and MF. (B) KEGG analysis of circRNAs with parental genes.

Table 2
KEGG pathways of circRNAs.

pathway_description	GO terms
Wnt signaling pathway|Adherens junction|Melanogenesis	30
Endocytosis	2
N-Glycan biosynthesis	11
Cardiac muscle contraction|Adrenergic signaling in cardiomyocytes	6
MAPK signaling pathway|FoxO signaling pathway|mTOR signaling pathway |Adherens junction|Insulin signaling pathway	17
Phagosome|Apoptosis|Focal adhesion|Adherens junction|Tight junction |Regulation of actin cytoskeleton|Salmonella infection	7
Protein processing in endoplasmic reticulum|Ubiquitin mediated proteolysis	7
Oocyte meiosis	14
Ubiquitin mediated proteolysis	5

species with varieties of functions, which were involved in verities of biological processes, such as, the development of various diseases [26,27]. In this study, we firstly identified 37 circRNAs from the kidney of Crucian carp, which is the lower vertebrate in natural. The finding of circRNAs in this species will provide novel information about the genome of Crucian carp, and these circRNAs could be developed into the new targets for prevention of pathogens infection.

CircRNAs were produced in the process of RNA transcription with the back splicing mechanism or other formation mechanism [1,28]. Through the splicing sites of these circRNAs analysis, we concluded that all of them were produced as the linear RNAs splicing with the GU/AG sites, which is similar with the circRNAs identified from the other species [8,29]. Furthermore, these circRNAs were not evenly distributed on the chromosomes, which indicated that some circRNAs derived from the transcription of RNAs may happen on the hotspot of the sequences. The biogenesises of circRNAs were derived from five categories, and identified circRNAs were derived from the exonic, intronic, antisense, sense overlapping and intergenic. Most of identified circRNAs belong to the sense overlapping circRNA. This result indicated that most of the circRNAs were derived from the sense overlapping strategy with unknown mechanism, which is different from circRNAs from other species.

Numerous circRNAs were identified from varieties species and some of circRNAs functions were resolved. However, there is still any

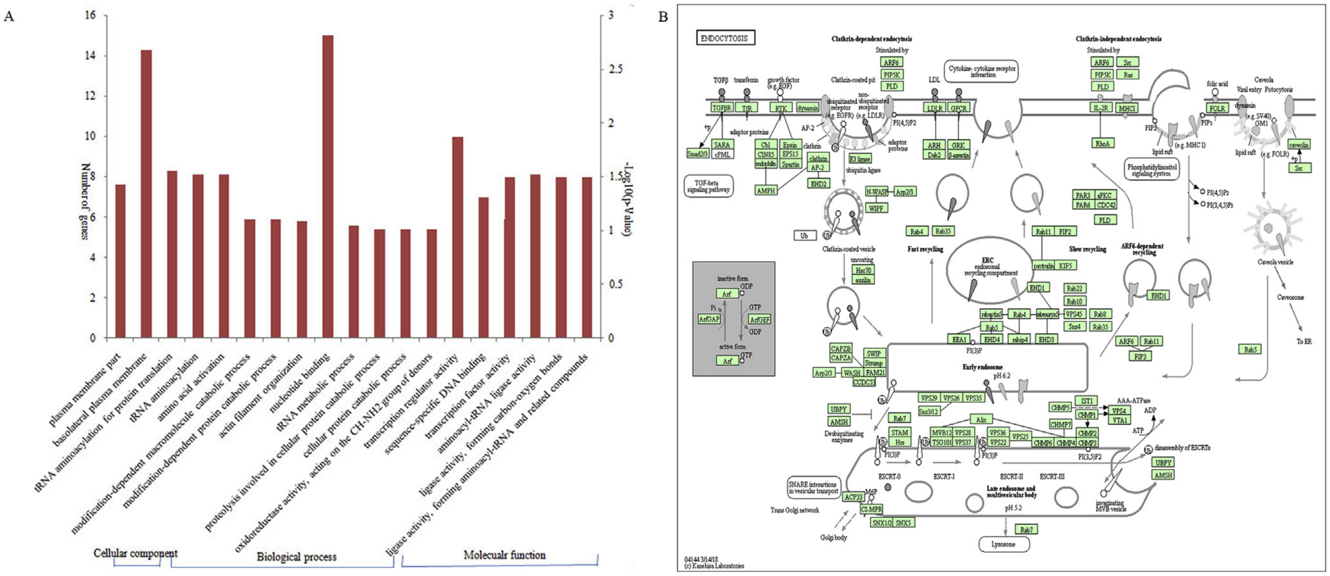

Fig. 6. GO and KEGG analysis of the target mRNAs of miRNAs sponged by circRNA. (A) GO analysis of circRNAs according to the values in the enrichment score under the theme of BP, CC and MF. (B) Endocytosis pathway.

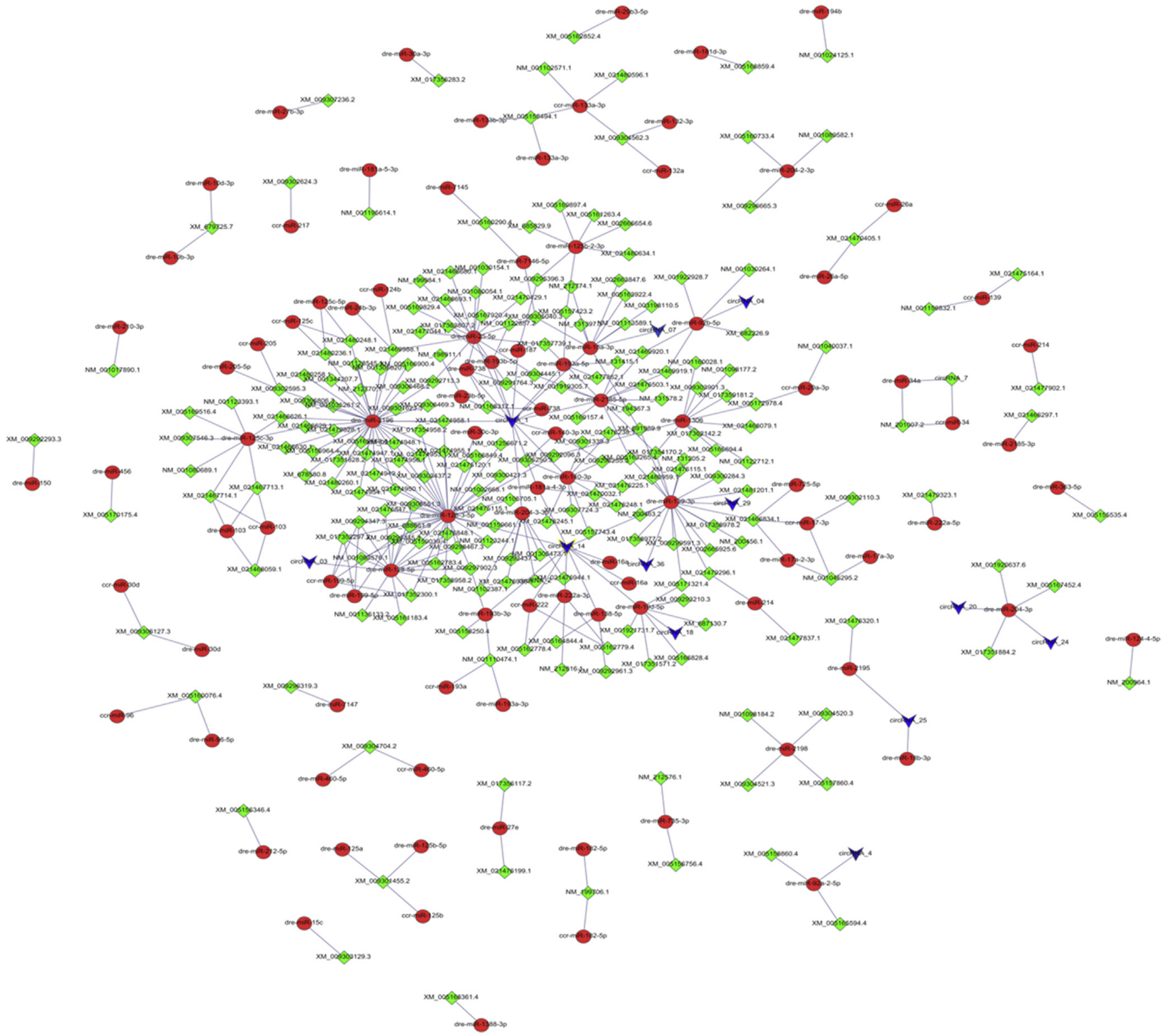

Fig. 7. The network of circRNA-miRNA-mRNA. Red represents miRNA; Blue represents circRNA; Green represents mRNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

information report about circRNAs in Crucian carp. The parental genes of circRNAs generation were derived and further analyzed the potential roles of these circRNAs. GO analysis showed that more than 20% circRNAs were related with the function of catalytic activity and binding function. KEGG analysis showed that these circRNAs were enriched in 9 signaling pathways, such as, Wnt signaling pathway, MAPK signaling pathway, Ubiquitin mediated proteolysis et al. These results indicated that circRNAs identified from the kideny of Crucian carp mey be associated with important biological processes.

It was reported that circRNAs as the endogenous competitive RNA to sequester and competitively suppress miRNA activity [30]. Using the target prediction software, 520 miRNAs was predicted as the targets of 37 circRNAs, wherein, 96 miRNA has 220 mRNA targets. Therefore, the potential roles of 220 mRNAs were applied to analyze with GO and KEGG analysis to better understand the regulatory potency in Crucian carp. Several genes were related with function of nucleotide binding and transcription regulatory activities were enriched and 5 genes were enriched in the pathway of Endocytosis. These results were similar to the finding of the potential roles of identified circRNAs from other species.

In order to explore the role of these circRNAs in Crucian carp, the circRNA-miRNA-mRNA network was constructed according to the miRNA-binding sites of circRNA using miRanda algorithms [7,8,25]. Furthermore, the circRNA-miRNA-mRNA network was constructed according to the miRNA-binding sites of circRNA using miRanda algorithms. The network showed that the function of circRNA was dependent on the regulation of miRNA on mRNA. One miRNA can link one or more circRNA and one or more mRNA. Collectively, these results suggested that circRNAs identified from the Crucian carp may have the similar regulatory mechanism to medidate the gene expression level in many biological processes. These results provided the novel clue to investigate the interaction between host and pathogens from this novel circRNA molecule.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.070.

Conflicts of interest

The authors declare that they have no conflict of interest.

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