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ORIGINAL ARTICLE

Computational identification and characterization of miRNAs and their target genes from five cyprinidae fishes

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KEYWORDS

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Cyprinidae fishes;
Target gene;
Functions

Abstract MicroRNAs (miRNAs) are a kind of small single-strand RNA molecules with lengths of 18–25 nt, which do not encode any proteins. They play an essential role in gene expression regulation by binding to their target genes, leading to translational repression or transcript degradation. In this study, 23 miRNAs were predicted from five cyprinidae fishes by using a bioinformatics-based gene search based on blasting ESTs and GSS in NCBI, of which 21 miRNA genes have not been previously reported. To prove their validity, five of the computationally predicted miRNAs were verified by RTPCR, their transcripts were successfully detected, and, 46 potential target genes for these miRNAs were predicted, most target genes encode transcription factors, they are involved in signal transduction, metabolism and development processes.

1. Introduction

MicroRNAs (miRNAs) are endogenous small non-coding RNAs with lengths ranging from ~18 to 25 nucleotides in size, which negatively regulate gene expression by directing mRNA cleavage or interfering with translation (Ambros, 2004; Bartel, 2004; Kloosterman and Plasterk, 2006; Sun and Lai, 2013). In the nucleus, these small miRNA molecules exist as independent transcription units, which are transcribed into long primary transcripts (pri-miRNAs) by RNA polymerase II, and then cleaved to long self-complementary miRNA precursors (pre-miRNAs) (Almeida et al., 2011; Gu et al., 2012). Then, pre-miRNA is exported to the cytosol and cut into a short double-stranded RNA by the Dicer nuclease (Bartel, 2004; Song et al., 2012). Finally, the single-stranded mature miRNA is then selectively loaded into the RNA-induced silencing complex (RISC) that contains Argonaute family proteins where it regulates targets by either cleaving target mRNAs or repressing the translation process (Chua et al., 2009; Friedlander et al., 2014; Graves and Zeng, 2012;

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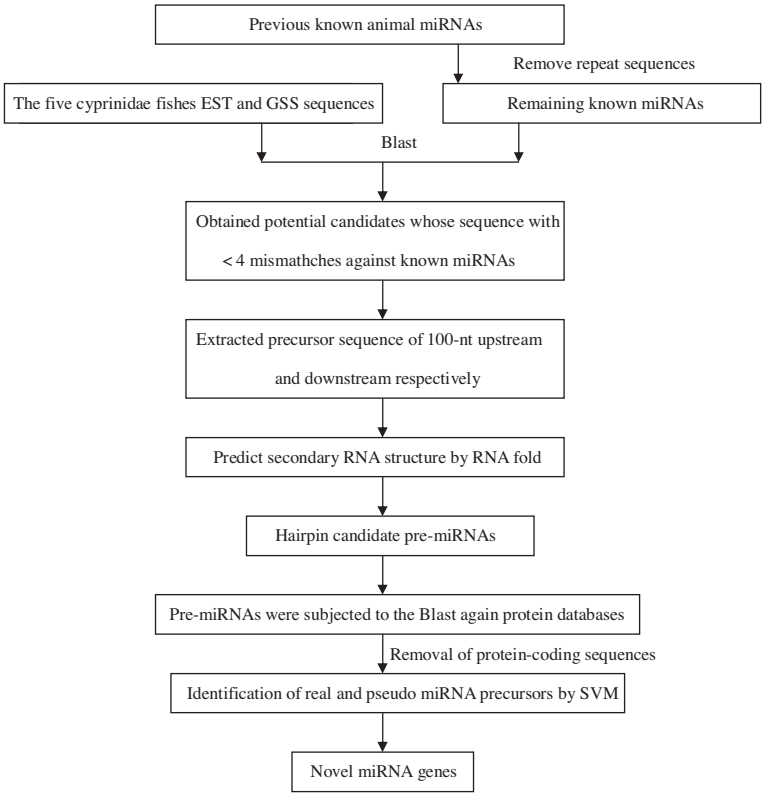

Figure 1 Procedure for prediction of the potential miRNAs from five cyprinidae fishes.

Havens et al., 2012). Many studies have demonstrated that miRNAs have multiple roles in animal diverse biological processes, including organ development, cell proliferation and division, pathological processes, fat metabolism, hormone secretion, embryogenesis, neural development, apoptosis and so on (Bartel, 2009; Bhaskaran and Mohan, 2013; Kloosterman and Plasterk, 2006; Ladomery et al., 2011; Lucas and Raikhel, 2013; Naqvi et al., 2012). It is estimated that miRNAs as the key regulators comprise 1–5% of animal genes and regulate up to 30% of genes (Friedman et al., 2009; Hendrickson et al., 2009; John et al., 2004).

Cyprinidae fishes are an important aquaculture species around the world, for example *Cyprinus carpio*, *Carassius auratus*, and the four domestic fish (*Mylopharyngodon piceus*, *Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix*, *Aristichthys nobilis*), which occupies a prominent position in the world of freshwater aquaculture and serves as a major source of animal protein for millions of people especially in China and several other East-Asia countries (Gui and Zhou, 2010; Liao et al., 2007). Fish represent approximately half of all vertebrate species. Although thousands of miRNA genes have been reported in mammals, insects, worms, plants, and viruses, but research on cyprinidae fish miRNAs was seldom reported. According to the latest miRNA Registry Database (http://www.mirbase.org/; released on 21 June, 2014), there are only nine fish miRNA in repository until now. miRNAs identified in fish have been limited to *C. carpio*, *Danio rerio*, *Hippoglossus hippoglossus*, *Fugu rubripes*, *Ictalurus punctatus*, *Oryzias latipes*, *Tetraodon nigroviridis*, *Salmo salar* and *Paralichthys olivaceus*. miRNAs can be identified through the cloning method, high-throughput sequencing method and computational approaches (Baev et al., 2009; de Souza Gomes et al., 2013; Qi et al., 2014; Wang et al., 2013; Wu et al., 2010). Compared to the experimental methods, computational approaches based on highly conserved miRNA in animals and plants have been proved to be faster, more affordable and more effective (Chaudhuri and Chatterjee, 2007; Hou et al., 2008; Li et al., 2010). Some predicted miRNA based computational approaches cannot be detected by direct cloning, particularly those miRNAs which were in low abundance, but computational approaches apply not only to the species with complete genomic information but also to those whose complete genome sequences are unavailable but have rich expressed sequence tag (EST) sequences and Genomic Survey Sequences (GSS).

In this study, we used all reported animal miRNAs deposited in the miRNA database (miRBase) to blast search the five cyprinidae fishes miRNAs homologs in the ESTs and GSSs from the NCBI GenBank database, which are *C. carpio*, *M. anguillicaudatus*, *C. auratus*, *M. amblycephala* and *C. alburnus*, respectively. A total of 23 potential miRNAs was predicted and their characteristics were investigated. The 21miRNAs were newly discovered in five different cyprinidae fishes. Five miRNA were validated by Stem-loop RT-PCR. In addition, 46 potential targets for the predicted miRNAs were identified. This research will provide useful information for miRNA research in cyprinidae fishes and other aquaculture species, and for future elucidation of regulatory roles of miRNAs in growth, organ development, metabolism, and other biological processes.

Table 1 The 23 newly identified miRNAs from five cyprinidae fishes.

miRNAs name	miRNA homologs	Gene source	Mature sequence (5′ to 3′)	Side	NM (nt)	Strand	LP (nt)	A + U (%)	MFEs
ccr-miR-6732	hsa-miR-6732	JZ508372(EST)	CAGAAGGUGGCAGGCUGGCC	3′	3	Minus	84	38.1	−38.9
ccr-miR-430a	ccr-miR-430	HR561547(GSS)	UAAGUGCUAUUUGUUGGGGUAG	3′	0	Plus	80	57.5	−25.9
ccr-miR-430b	dre-miR-430b	HN151353(GSS)	AAAGUGCUAUCAAGUUGGGGUAA	3′	1	Minus	78	61.5	−25.1
ccr-miR-430c-3p	dre-miR-430c-3p	HR561547(GSS)	UAAGUGCUUCUCUUUGGGGUAG	3′	0	Plus	92	64.1	−35.6
ccr-miR-365	ccr-miR-365	HR561450(GSS)	AAACUUUUGGGGGCAGAUUA	3′	4	Plus	119	68.9	−21.6
ccr-miR-2783	bmo-miR-2783	HR551227(GSS)	UAAUCGAGGGUGUGGGUGUGGGA	3′	4	Plus	96	60.4	−30.2
cau-miR-3198	hsa-miR-3198	GE468290(EST)	UUGGAUUCCUGGGGAAUGGAGA	5′	1	Minus	92	43.4	−34.7
cau-miR-1814b	bta-miR-1814b	FG394205(EST)	CUAUUGUUUAGUUUUGUUUU	3′	3	Plus	129	67.4	−16.3
cau-miR-2742	bmo-miR-2742	FG394388(EST)	UGUUCAUUGGAUUAGUGUU	5′	1	Minus	89	53.9	−17.7
cau-miR-149	bta-miR-149	AM403731(GSS)	UCUGGCUCCGUGUCUUCAGCUUU	3′	4	Minus	136	45.6	−56.4
man-miR-4037	hsa-miR-4703	GAAD01011012(GSS)	CGGACAACGAUGGCAAUCAG	5′	3	Plus	100	52.0	−31.0
man-miR-6751-3p	hsa-miR-6751-3p	GAAD01001061(GSS)	GCUGAGCCUCUCUCUCUGCUC	3′	3	Minus	72	52.3	−17.6
man-miR-7847-3p	hsa-miR-7847-3p	GAAD01010155(GSS)	CGUGGAGGUCGAGGAGGAGGC	3′	1	Minus	148	39.2	−58.9
man-miR-142-3p	ccr-miR-142-3p	GAAD01009678(GSS)	GUAGUGUUUCCUACUUUAUGG	3′	0	Minus	92	55.4	−39.9
man- miR-2452	bta-miR-2452	GAAD01009359(GSS)	CAGCAGUUUGUUUUCCUUUUUU	3′	3	Plus	150	57.3	−34.3
man-miR-1603	bta-miR-1603	GAAD01002573(GSS)	CUGGUUUGUUUUGUGUUUAU	3′	2	Minus	108	65.7	−17.6
man-miR-2487	bta-miR-2487	GAAD01000444(GSS)	CUCUAAGGGCUGGGCCGGUCGG	3′	0	Minus	125	46.4	−46.5
mam-miR-10a-5p	dre-miR-10a-5p	FJ746716(GSS)	AUACCCUGAGAUCCGGAUUUGU	5′	3	Minus	148	60.1	−56.1
mam-miR-10a-3p	hsa-miR-10a-3p	FJ746716(GSS)	CAAAUUCGUAUCUAGGGGAGUA	3′	1	Plus	110	60.0	−40.5
mam-miR-2369	bta-miR-2369	GQ903705(GSS)	UUAGGUUGUGGGUUUUUCUAG	3′	4	Minus	78	57.7	−17.1
cal-miR-4483	hsa-miR-4483	FJ875089(GSS)	GGGGUGGUCUGUUGUUUC	5′	1	Plus	160	46.8	−31.8
cal-miR-6852	hsa-miR-6852	GU218201(GSS)	UUUCCUCUGUUCCUCAGC	5′	1	Minus	87	52.9	−17.2
cal-miR-5600-3p	cin-miR-5600-3p	KF111429(GSS)	UGUGGAAUGUUUUGUUGUGCUU	3′	4	Plus	136	54.4	−32.9

NM, number of mismatch; LP, length of precursor; MFEs, minimal folding free energy (kcal/mol).

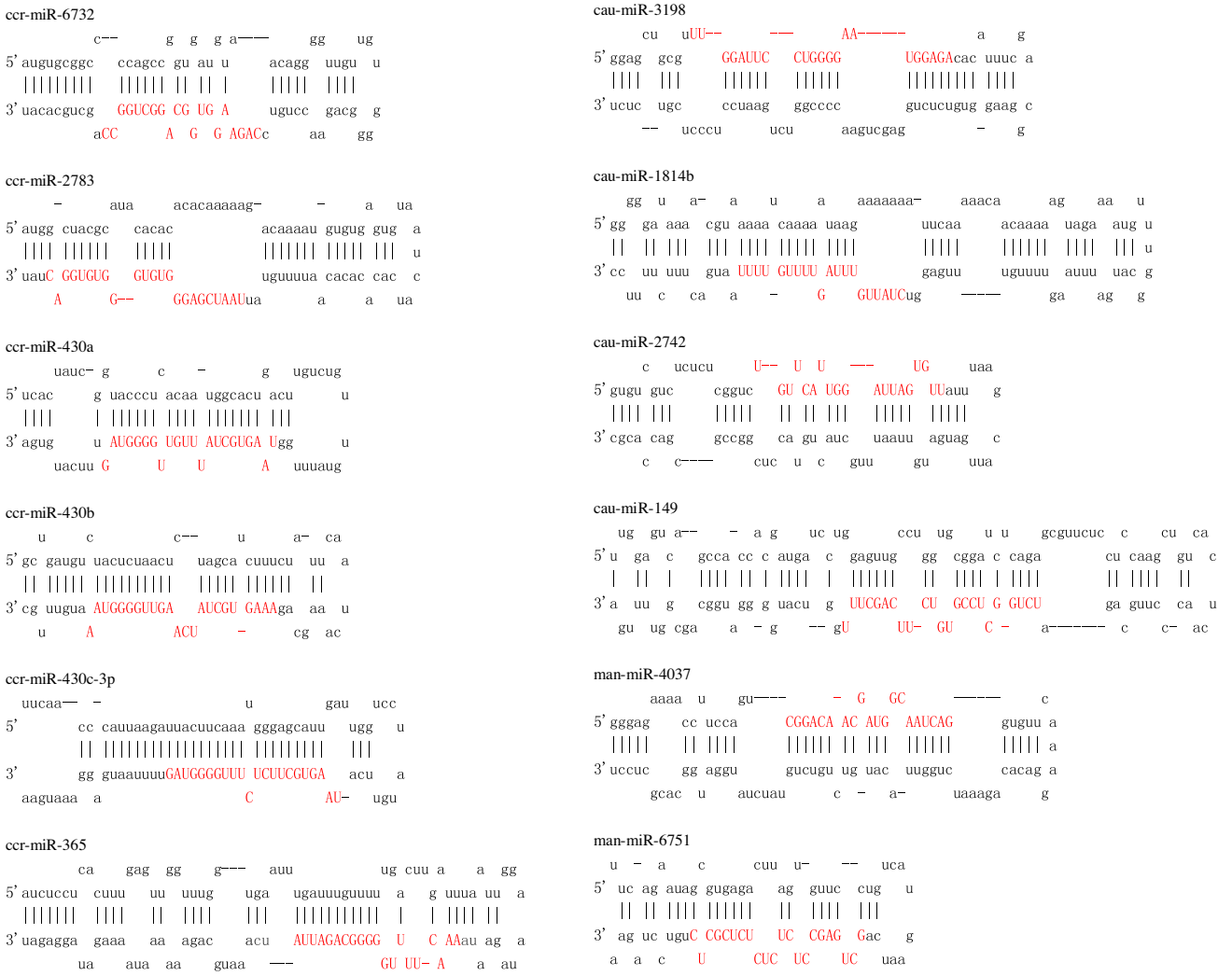

Figure 2 Predicted stem-loop structures of newly identified precursor miRNAs from five cyprinidae fishes. The mature miRNAs were labeled with red capital letters.

2. Materials and methods

2.1. miRNA reference sets

All known miRNA sequences in various animal species including fishes were obtained from miRBase (http://www.mirbase.org/). To avoid the overlapping miRNAs, the repeat sequences of miRNAs within the above species were removed. The remaining sequences were used as a reference of miRNA. The EST, GSS, and mRNA sequences of five cyprinidae fishes were obtained from the NCBI, which were used for miRNA prediction. The EST sequences of *C. carpio*, *M. anguillicaudatus*, and *C. auratus* were 50769, 22174 and 13937, respectively; The GSS sequences of *C. carpio*, *M. anguillicaudatus*, *C. auratus*, *M. amblycephala*, and *C. alburnus* were 72932, 12268, 4621, 809 and 236, respectively.

2.2. Computational prediction of miRNAs

Comparative software BLAST tool was downloaded from NCBI. BLASTN parameters were the same as those described in previous papers (Huang et al., 2010). Procedure of search for potential miRNAs was shown in Fig. 1. Five criteria used to distinguish miRNAs and pre-miRNAs from other kinds of RNAs were as follows: (1) predicted mature miRNAs were allowed to have only 0–4 nucleotide mismatches in sequence with all previously known animal mature miRNAs; (2) pre-miRNA sequence can fold into an appropriate hairpin secondary structure that contains the ~22 nt mature miRNA sequence within one arm of the hairpin structure; (3) miRNA precursors with secondary structures had higher negative minimal free energies (MFEs) and minimal free energy index (MFEIs) than other different types of RNAs by RNAfold prediction software; (4) miRNA had 30–70% contents of A + U by SVM (support vector machine) (Xu et al., 2008); and (5) no loop or break in miRNA sequences was allowed. If the sequence met all these criteria, it will be considered as a miRNA.

2.3. Stem-loop RT-PCR assay

To verify computational predictions, five miRNAs were randomly selected from the novel predicted miRNA by the stem-loop RT-PCR experiment method. Small RNA from

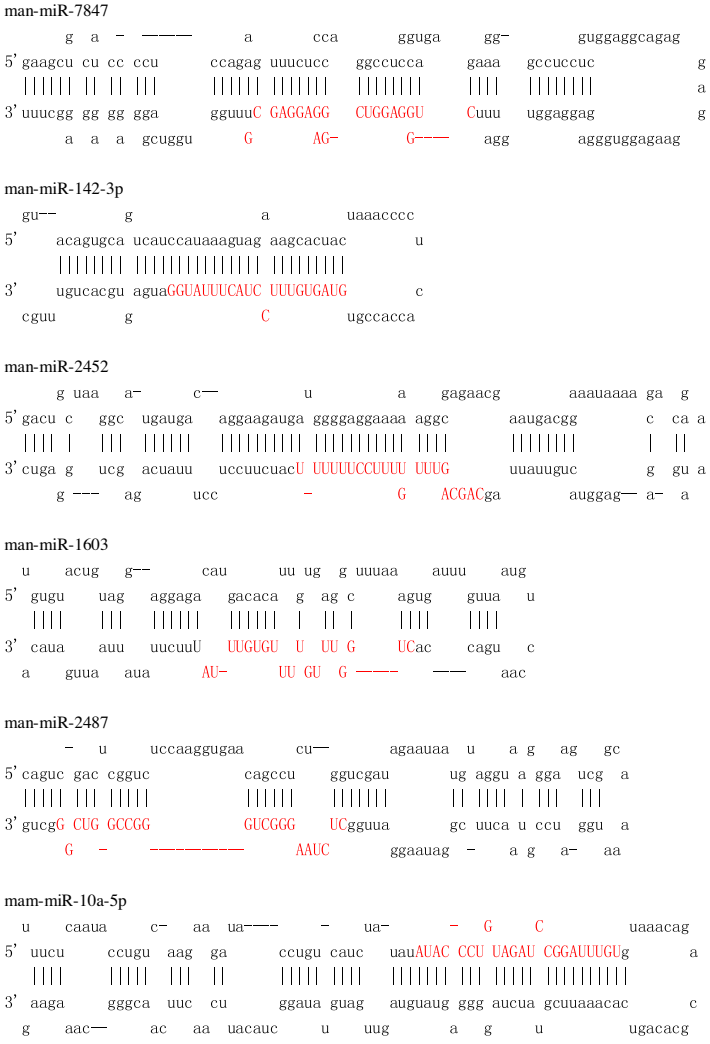

Fig. 2 (*continued*)

the fish mixed tissues (skeletal muscle, brain, liver, and spleen) was extracted using an RNeasy Mini Kit (Qiagen), according to the supplier's protocol. The cDNAs were synthesized from small RNAs using miRNA specific stem-loop RT primers according to criteria described previously (Chen et al., 2005; Mohammadi-Yeganeh et al., 2013; Varkonyi-Gasic and Hellens, 2011). The stem–loop RT primers and gene specific primers were listed in Table S1. 100 ng cDNA was used as template for the PCR. The PCR was programed as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 25 s and a final elongation step at 72 °C for 7 min. The PCR products were separated through 2.5% (w/v) agarose gel. DNA fragments were directly subcloned into PMD18-T vector (Takara) and sequenced.

2.4. Phylogenetic analysis of the new miRNAs

Due to the conservation of miRNAs and their precursors, the precursor sequences of the novel and the known miRNAs in the same family were aligned by Clustal W, and then the maximum likelihood trees were constructed with MEGA 5.0, the neighbor-joining method with default bootstrap values was set, the phylogenetic tree illustrated the evolutionary relationships with other members of the same family (Larkin et al., 2007; Tamura et al., 2004, 2011). The results were saved.

2.5. Prediction of miRNA targets and their functions

It has been reported that the target genes of miRNAs could be predicted according to their complementarity with mature miRNA sequences (Carre et al., 2013; Grimson, 2010). In the present study, the target genes are predicted with the web-based computational software RNA hybrid program (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) according to its operation manual (Rehmsmeier et al., 2004). The parameters were described as follows: P value cutoff of 0.05, target duplex free energy $\triangle G \leqslant -24$ kcal/mol. The criteria for the target gene identification were as follows: (1) four or fewer mismatched nucleotides at complementary sites between miRNA sequences and potential mRNA targets; (2) one mismatch allowed between position 2nd and 12th, but not at nucleotide positions 10th or 11th; (3) less than three additional mismatches between nucleotide positions 12–23, but no more than two continuous mismatches within this region.

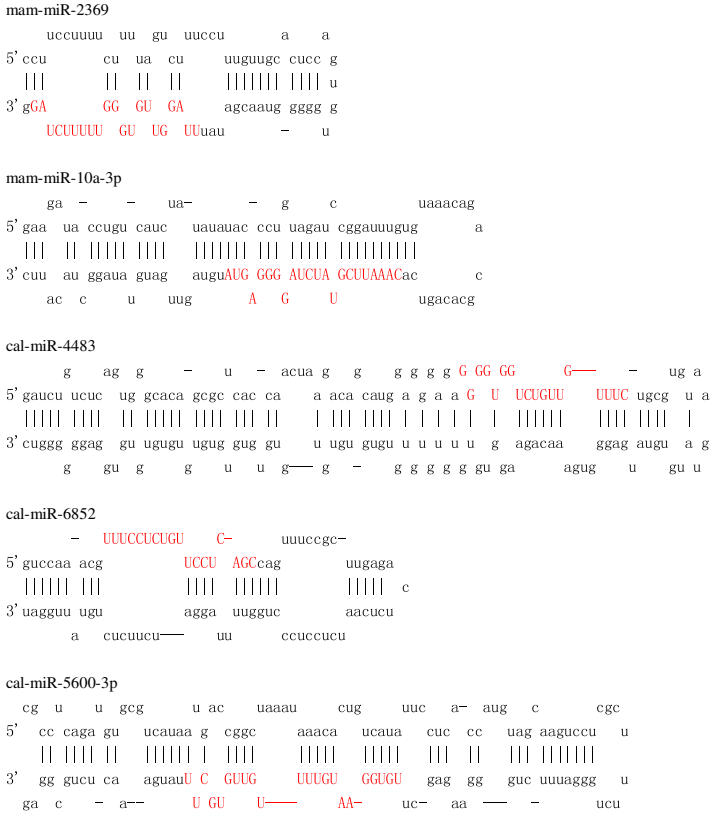

Fig. 2 (*continued*)

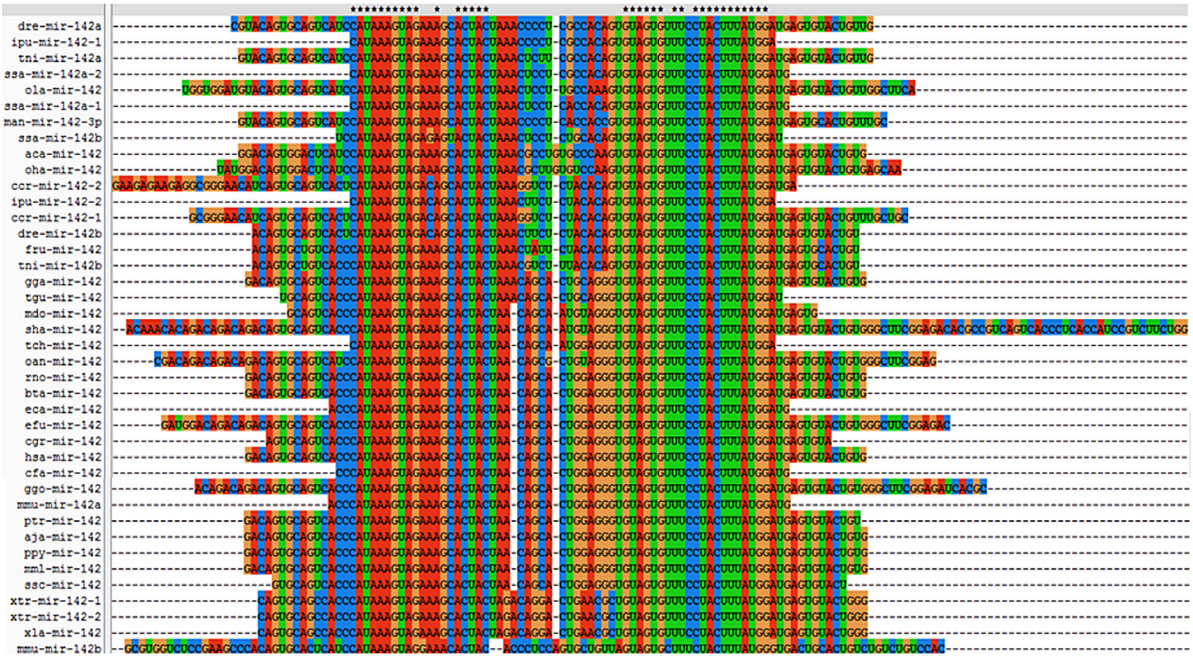

Figure 3 Multiple sequence alignment analysis of pre-miR-142 (man-miR-142) family. Abbreviations: mmu, *Mus musculus;* has, *Homo sapiens;* rno, *Rattus norvegicus;* gga, *Gallus gallus;* dre, *Danio rerio;* fru, *Fugu rubripes;* tni, *Tetraodon nigroviridis;* xtr, *Xenopus tropicalis;* bta, *Bos Taurus;* mdo, *Monodelphis domestica;* oan, *Ornithorhynchus anatinus;* mml, *Macaca mulatta;* cfa, *Canis familiaris;* xla, *Xenopus laevis;* ptr, *Pan troglodytes;* eca, *Equus caballus;* ssc, *Sus scrofa;* tgu, *Taeniopygia guttata;* ppy, *Pongo pygmaeus;* aca, *Anolis carolinensis;* ola, *Oryzias latipes;* sha, *Sarcophilus harrisii;* cgr, *Cricetulus griseus;* ggo, *Gorilla gorilla;* ccr, *Cyprinus carpio;* aja, *Artibeus jamaicensis;* ipu, *Ictalurus punctatus;* ssa, *Salmo salar;* efu, *Eptesicus fuscus;* tch, *Tupaia chinensis;* oha, *Ophiophagus Hannah;* man, *Misgurnus anguillicaudatus*. Asterisks indicate conserved region.

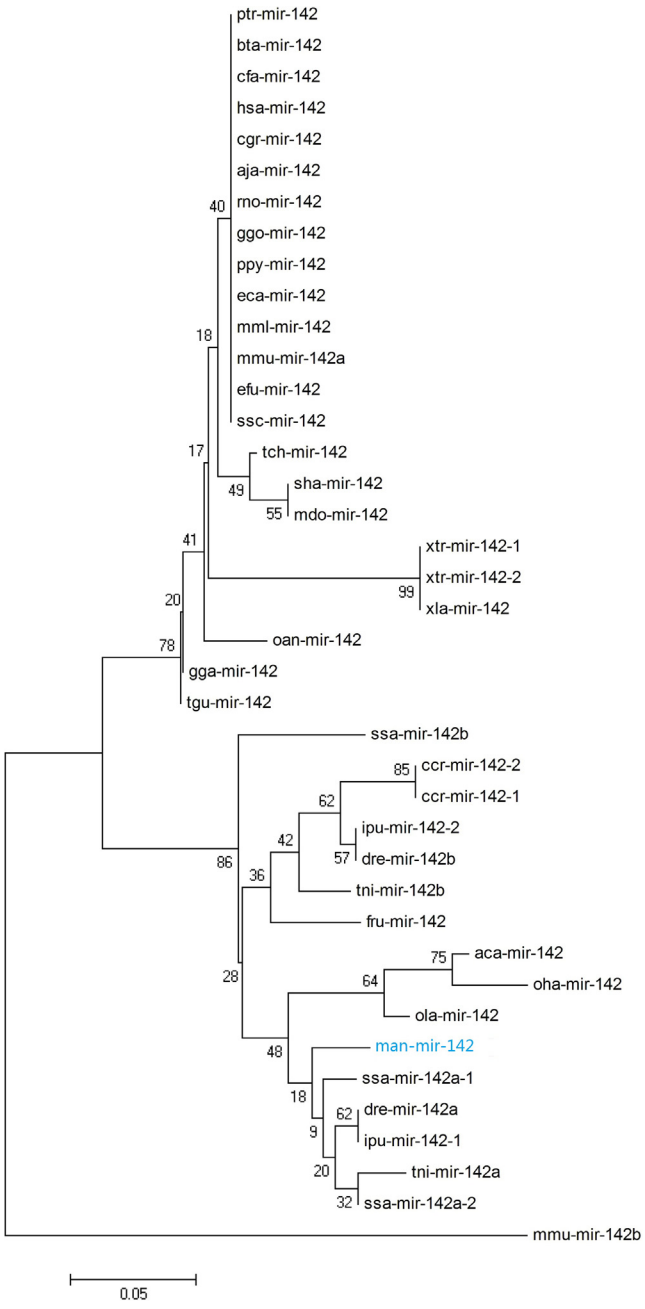

Figure 4 Phylogenetic tree for the newly identified pre-miRNA from *M. anguillicaudatus*. Maximum likelihood method was used, the new identified man-miR-142 is shown in blue letters.

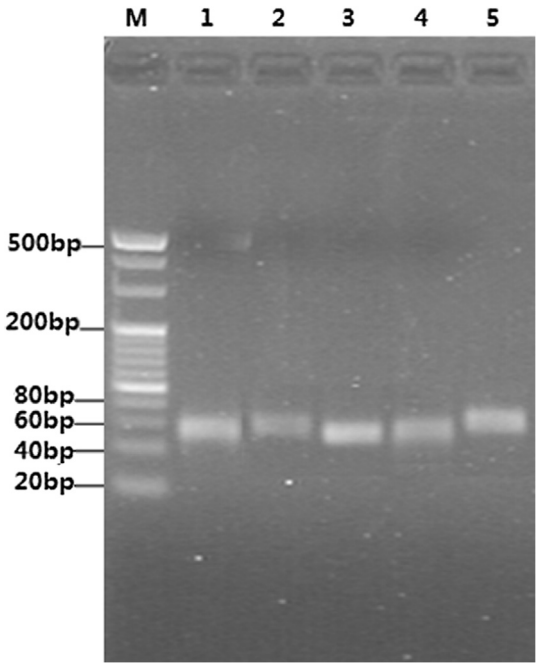

Figure 5 Experimental validation of predicted fish miRNAs. M: 20 bp ladder maker; 1, ccr-miR-430b; 2, cau-miR-3198; 3, man-miR-142-3p; 4, mam-miR-10a-5p; 5, cal-miR-4483.

3. Results and discussion

3.1. Identification of miRNAs

Sequence and structure homologies are the main theory behind the computer-based approach for miRNAs prediction. In this study, the similarity searches for miRNAs in the EST and GSS sequences yielded 62 matches, which were used for secondary structure prediction properties by RNA fold software prediction. Finally, some possible false sequences of pre-miRNAs were further eliminated by manual inspection. This resulted in 23 potential miRNAs. Amongs 6 miRNAs were identified in *C. carpio*, 4 miRNAs were identified in *C. auratus*, 7 miRNAs were identified in *M. anguillicaudatu*, 3 miRNAs were identified in *M. amblycephala*, and the rest 3miRNAs were identified in *C. alburnus* (Table 1). These newly predicted miRNAs were all first time reported except miRNA-365 and miRNA-430 family were previously identified (Yan et al., 2012; Zhu et al., 2012). Predicated miRNAs found belong to 21 miRNA families and every miRNA family has only one member, but miRNA-430 family has three members and miRNA-10a family has two members. The length of the predicted miRNAs was in the range from 72 nt to160 nt, with an average of 109 nt. These sequences folded into a typical stem-loop structure, having the mature miRNA on the 5′ arm end, or alternatively on the 3′ arm end (Fig. 2). The hairpin loop secondary structures had a minimum folding free energy ranging from −58.9 kcal/mol to −16.3 kcal/mol. The new predicted miRNAs were also evaluated for A + U content, and results showed that the A + U contents ranged from 38.1% to 68.9% in miRNA precursors, which was consistent with previous studies on other animal (Ambros et al., 2003; Gong et al., 2010; Zhang et al., 2006; Zhou and Liu, 2010). These results showed that these predicted fishes miRNAs meet these strict screening criteria.

3.2. Conserved study and phylogenetic analyses

miRNAs always showed a conserved nature among the living organisms. Our study was based on the use of the pre-miRNAs rather than mature sequences in homology search. The conservation of mature miRNAs and their precursors provides the chance to investigate their evolutionary relationships. We chose one conserved pre-miRNA sequence from miRNA-142 family which was aligned by Clstw soft in the miRBase database. Results showed that pre-miRNA sequence from different

species have same conserved sequence in 5′arm and 3′arms (Fig. 3). These data suggest that miRNAs may present a conserved organization pattern among animals in very early evolution. Furthermore, the one big miRNA family miRNA-142 was selected for phylogenetic analyses. The phylogenetic tree analysis among the members of this family illustrated the evolutionary relationships of *M. anguillicaudatus* miRNA which is more closed to the *S. salar*, *D. rerio*, *I. punctatus* and *T. nigroviridis* species (Fig. 4).

3.3. Experimental verification of predicted miRNAs

The efficiency of the computational strategy was tested by biological experiments to validate the predicted miRNA genes. A total of 5 miRNAs were selected at random, of which miRNAs from different fishes: ccr-miR-430b, cau-miR-3198, man-miR-142-3p, mam-miR-10a-5p and cal-miR-4483 were subjected to the stem-loop RT-PCR for validation studies. The transcripts of 5 miRNA genes were successfully detected, demonstrating the expression of mature miRNAs (Fig. 5).

3.4. Prediction of potential targets of miRNAs

Researches have confirmed that miRNAs are mainly complementary to their target mRNAs in animals, which is different from miRNAs binding their targets by complete or nearly complete complementarity in plants (Martinez-Sanchez and Murphy, 2013; Trakooljul et al., 2010; Wang et al., 2013).

Table 2 Potential targets of the new identified miRNAs.

miRNA	Targeted protein	Target function	Targeted genes
ccr-miR-6732	Toll-like receptor 2	Signal transduction	FJ858800
	Putative delta-6 fatty acyl desaturase	Metabolism	AF309557
	HMG box transcription factor Sox9b	Transcription factor	AY874424
	ATP synthase	Metabolism	AB023582
ccr-miR-430a	G protein-coupled receptor kinase	Signal transduction	AB119261
	NILT1 leukocyte receptor	Signal transduction	AJ811994
	Retinol dehydrogenase 8	Metabolism	AB439579
ccr-miR-430b	Na + /glucose cotransporter	Metabolism	JN867793
	Dsx and mab-3 related transcription factor 1–1	Transcription factor	KF713504
ccr-miR-430c-3p	TNF receptor-associated factor 6 b	Transcription factor	HM535645
	Insulin-like growth factor binding protein 2	Transcription factor	FJ009001
ccr-miR-365	Rhesus blood group-associated glycoprotein C	Development	KF051940
	Cytochrome P450 aromatase	Metabolism	EU499382
ccr-miR-2783	Matrix metalloproteinase 2	Metabolism	KC414857
	Pre-B cell enhancing factor	Transcription factor	AB027712
cau-miR-3198	Mx3 protein	Development	AY303812
	Progesterone receptor 1	Signal transduction	AB904788
cau-miR-1814b	Nucleotide-binding oligomerization domain-2	Signal transduction	JX965185
	Interferon regulatory factor 1	Transcription factor	EF174419
cau-miR-2742	Prominin-like protein	Signal transduction	DQ233501
	Glucose-6-phosphate isomerase	Metabolism	JQ713841
cau-miR-149	Transmembrane protein 173	Metabolism	JF970229
	Transcription factor 7-like 1a	Transcription factor	FJ231713
	Putative MYB25-like protein	Transcription factor	KF373239
man-miR-4037	Sodium glucose cotransporter 1	Metabolism	DQ285635
man-miR-6751-3p	Vitellogenin 1	Development	KF733650
man-miR-7847-3p	Elongation factor 1-alpha	Transcription factor	KF733649
	Doublesex and mab-3 related protein	Transcription factor	AB531495
	Glutamate dehydrogenase	Metabolism	JF694443
man-miR-142-3p	Vitellogenin 6	Development	KF733655
	Elongation factor 1-alpha	Transcription factor	KF733649
man-miR-2452	Transferrin	Metabolism	JX292093
	Myostatin	Development	EF551059
man-miR-1603	HMG box transcription factor Sox8b	Transcription factor	GU166140
	Forkhead box L2	Transcription factor	AB531497
man-miR-2487	Sodium/potassium ATPase	Metabolism	FJ982782
	Estrogen receptor alpha	Signal transduction	EF530590
mam-miR-10a-5p	MHC class I alpha chain	Development	JF921124
mam-miR-10a-3p	Spermatogenesis-associated protein 4	Development	JQ898682
mam-miR-2369	Isolate LZ06 peroxisome proliferator	Transcription factor	HM140628
	Selenium-dependent glutathione peroxidase	Metabolism	KF378714
	Cardiac muscle troponin T isoform 2	Metabolism	KC556827
	Toll-like receptor 3	Signal transduction	DQ986365
cal-miR-4483	Lipoprotein lipase	Metabolism	KC166231
cal-miR-6852	Myosin heavy chain b	Metabolism	JX402919
cal-miR-5600-3p	Myogenic differentiation antigen MyoD	Transcription factor	KC782835

Therefore, identification of the miRNA targets is an important step in understanding the miRNA regulatory function and gene regulation networks in five cyprinidae fishes. The predicted targets for the identified miRNAs are shown in Table 2. A total of 46 target genes are predicted, of which 15 are from *C. carpio* miRNAs, 9 are from *C. auratus* miRNAs, 13 are from *M. anguillicaudatus* miRNAs, 6 are from *M. amblycephala* miRNAs, and the rest 3 are from *C. alburnus* miRNAs. Our prediction of target genes for the five fish miRNAs discovered that more than one gene was regulated by individual miRNA, but only one gene targeted by miRNA was predicted individually in *C. alburnus*. The reason is the limited information on the *C. alburnus* mRNA transcripts in NCBI gene bank. Many experimental and/or computational approaches have documented that most of the miRNAs largely target transcription factors, signal transduction factors and development (Bartel, 2009; Friedman et al., 2009; Shibata et al., 2011). This study resulted in majority of the targets being classified as transcription factors in five cyprinidae fishes. For example, cau-miR-149 targeted MYB25-like protein, man-miR-7847-3p targeted Doublesex and mab-3 related protein and ccr-miR-430c-3p Insulin-like growth factor binding protein were found in this class. In addition, another important class of the predicted targets was various kinds of enzymes such as Glucose-6-phosphate isomerase, Lipoprotein lipase and ATP synthase, which might participate in various metabolic pathways.

4. Conclusions

The computational approaches for identifying miRNAs and their targets play an important role in understanding gene regulation. In this study, we applied this strategy to identify 23 miRNAs in five cyprinidae fishes by searching both ESTs and GSS databases. Five random predicted miRNAs were validated by RT-PCR. These fish miRNAs potentially target 46 mRNAs, which can act as transcription factors, and metabolism, development, and signal transduction. These findings will be helpful to elucidate their functions and processing of miRNAs from these fishes. The predicted miRNA targets reported in the present study are also required for validation in future studies. We believe that more miRNAs will be discovered from cyprinidae fishes in future, with updated knowledge about miRNAs from fish species and availability of more complete fish genome sequences.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.sjbs.2015.05.007.

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