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Genome-Wide Identification of Novel microRNAs from Genome Sequences Using Computational Approach in the Mudskipper (*Boleophthalmus pectinirostris*)[1]

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Abstract—MicroRNAs (miRNAs), approximately 22 nucleotides (nt) long, are small, non-coding RNA molecules with important regulatory functions in gene expression. They are mostly conserved among the organisms and this conservation makes them a good source for the identification of novel miRNAs by computational genomic homology. The miRNA repertoire of a major aquaculture species, *Boleophthalmus pectinirostris*, has been unknown until recently. Currently, the *B. pectinirostris* whole-genome sequences have been completed, making it more convenient for us to focus on computational prediction for novel miRNA homologs. Following a range of strict filtering criteria, a total of 62 potential miRNAs were identified for the first time; they belong to 39 different miRNA families. All these miRNAs were observed in the stem portion of the stable stem–loop structures. The minimum free energy (MFE) of the predicted miRNAs ranged from −21.6 to −62.7 kcal/mol with an average of −39.2 kcal/mol. The A + U ranged from 32.5 to 69.1% with an average value of 52.2%. The phylogenetic analysis of predicted miRNAs revealed that miR-23a-3p, miR-184-3p, miR-214-5p, and miR-338-3p from *B. pectinirostris* are evolutionary highly conserved showing more similarity with other fish species. To verify the predicted miRNAs, selected miRNAs representing 16 of the 39 families were confirmed by stem–loop RT-PCR, indicating that the computational approach that we used to identify the miRNAs is a highly efficient and affordable alternative method. Taken together, these findings provide a reference point for further research on miRNAs identification in fish species, meanwhile, our study also will be useful for further insight into biological functions of miRNAs and improved understanding of genome in *B. pectinirostris*.

Keywords: microRNAs, *Boleophthalmus pectinirostris*, computational prediction, phylogenetic analysis

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INTRODUCTION

MicroRNAs (miRNAs) are a class of endogenous non-coding small RNAs averaging ~22 nucleotides (nt) and widely found in unicellular and multicellular organisms that act as regulators of gene expression at post-transcriptional level by either perfect complementary binding, for mRNA degradation, or imperfect binding at 3'-UTR region, to inhibit translation [1, 2]. The huge impact of miRNAs on the control of biological function is evident in computational studies, which show that miRNAs may control up to 30% of all protein-coding genes in animals [3–5]. These miRNAs are generally transcribed as long, primary miRNA (pri-miRNA) transcripts by RNA polymerase II and matured through two sequential processing events [6]. In the nucleus, the pri-miRNAs are first cleaved by the RNase III enzyme Drosha into ~70-nt precursors (pre-miRNAs) with an imperfect stem–loop structure [7]. Pre-miRNAs are then exported to the cytoplasm and further processed by another RNase III enzyme Dicer into an miRNA/miRNA* duplex [8]. One strand of the RNA duplex, the miRNA, is incorporated stably into the RNA-induced silencing complex (RISC), while the other strand, the miRNA*, is degraded [9]. The RISC loaded with miRNAs targets mRNAs causing the cleavage of target genes. It has been evidenced that miRNAs are involved in a variety of biological processes, such as development, cell proliferation and death, apoptosis, fat metabolism, and cell differentiation [10–14].

Considering the importance of miRNAs in gene regulation, currently, several approaches have been applied for miRNA investigation, including the classic cloning method [15–18], deep sequencing method [19–22], and computational approaches [23–29]. However, cloning and deep sequencing are not highly efficient approaches to find miRNAs. Compared to the

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experimental approaches, computational approaches have also been proved to be faster, more affordable, and more effective to identify novel miRNAs. Since a large number of known mature miRNAs are evolutionarily conserved in different animal and plant species, computational approaches to miRNA discovery are conveniently applied to species that have complete genomic information [30–33]. For instance, about half of pre-miRNA/miRNA pairs have been identified solely by computational approaches in miRBase [34–38]. Although miRNAs, as important regulators of gene expression, have been studied extensively in many eukaryotes, no miRNAs have been discovered in *Boleophthalmus pectinirostris* recently.

B. pectinirostris is the largest group of amphibious teleost fishes that are uniquely adapted to live on mudflats. Currently, this commercially important aquaculture species living along coastal area in China is regarded as a good model organism. This species is an exceptional model among fishes for their amphibious behavior and numerous physiological and morphological specializations adapted for amphibious life [39, 40]. Fortunately, the genome of *B. pectinirostris* has recently been sequenced, which provides an opportunity for us to identify its novel miRNAs [41]. In this study, we performed a computational analysis and a total of 62 potential *B. pectinirostris* miRNAs belonging to 39 miRNA families were identified. Then, some of the selected predicted *B. pectinirostris* miRNAs were confirmed by stem–loop RT-PCR. These findings will not only help to discover novel miRNAs in *B. pectinirostris*, but also will be useful for our understanding towards their possible regulatory roles in physiology, growth, and development of *B. pectinirostris*. Further, the novel identified miRNAs also provide potential molecular tools for gaining insights into the genetic changes underlying the terrestrial adaptations of *B. pectinirostris*.

RESULTS AND DISCUSSION

Identification of Putative miRNAs

Most mature miRNAs are evolutionarily conserved from species to species within the animal kingdom, which provides a powerful approach to predict the existence of new miRNA homologs or orthologs in other animal species. By computational prediction, a total of 62 miRNA genes were identified in the genome of *B. pectinirostris* firstly. These identified miRNAs belong to 39 miRNA different families (Table 1 and Fig. 1). Let-7 had four members; miRNA-1, miRNA-9, miRNA-133, miRNA-135, and miRNA-181 had three members each, of which miRNA-1 family included miR-1a-3p, miR-206-5p, and miR-206-3p; other miRNA families had one or two members. Note that five miRNA sequences, that is miRNA-462, miR-734, miRNA-2187, miRNA-7147, and miRNA-7552, in *B. pectinirostris* were found only in the fish species (data not shown) suggesting these miRNAs might be Pisces-specific small RNA molecules. In our study, 57 identified *B. pectinirostris* miRNA sequences perfectly (100%) matched their reference miRNAs while the rest 5 differed from the reference miRNAs by one base (Table 2). The results are in agreement with the formerly published work by other groups, where the mature sequences have a difference of <4 nucleotides [42, 43]. The finding suggests that mature *B. pectinirostris* miRNA sequences are less divergent from lower spine animal miRNAs. The length of mature miRNAs in *B. pectinirostris* varied from 21 to 23 nt. The majority of miRNAs had lengths of approximately 21 and 22 nt, accounting for 30.6 and 50.0% of miRNAs, respectively (Fig. 2). The mature miRNAs were parts of the arms of the stem–loop hairpin structure: 28 were located at the 3 arm and 34 were located at the 5 arm of the miRNA precursor sequences (data are not shown). In addition, 5 pairs of miRNA/miRNA* were located at the same precursors. Precursor length of these identified *B. pectinirostris* miRNA varied in the range of 61–167 nt with an average length of 93 nt. The majority of pre-miRNAs had lengths between 60 and 100 nt (Fig. 3), consistently with the lengths of most previously predicted animal pre-miRNAs [31, 44]. The A+U contents of the identified pre-miRNA sequences in *B. pectinirostris* ranged from 32.5 to 69.1% with an average of 52.2%, which perfectly meets the criteria of the miRNAs prediction [45]. The minimum free energy (MFE) is an important parameter in determining the stability of the perfect or near-perfect secondary hairpin structure of pre-miRNAs; a lower MFE value generally indicates a more stable secondary structure. Here, these newly identified *B. pectinirostris* miRNA precursors had MFE ranging from −21.6 to −62.7 kcal/mol with an average of −39.2 kcal/mol. Since other RNAs, such as mRNA, rRNA, and tRNA, may also form similar hairpin structures, we used the minimal fold energy index (MFEI) to distinguish other RNAs or RNA fragments. In the present prediction, the novel identified *B. pectinirostris* pre-miRNAs had MFEI values ranging from 0.51 to 1.25, with an average of about 0.90; this is significantly higher than that for tRNAs (0.64), rRNAs (0.59), and mRNAs (0.62–0.66), suggesting that most of the predicted miRNA could be present as real mature miRNAs in *B. pectinirostris* (Fig. 4).

Conservation and Phylogenetic Analysis of Identified miRNAs

Animal miRNAs are highly conserved among distantly related species and even between vertebrates and invertebrates, including primary and mature miRNA. Comparison of the precursor sequences of the predicted miRNAs with other members in the same family showed that most predicted miRNAs had a high degree of sequence similarity with related molecules.

Table 1. The novel identified 62 miRNAs in *B. pectinirostris*

miRNAs name	Source miRNA homologues	Gene source	Predicted mature sequence(5'-3')	NM, nt	Loc	Strand	LP, nt	A + U, %	MFE	MFEI
let-7-5p	bmo-let-7-5p	scaffold 116	UGAGGUAGUAGGUUGUAUAGU	0	5'	Minus	94	58.51	–42.9	1.10
let-7a-5p	aca-let-7a-5p	scaffold 116	UGAGGUAGUAGGUUGUAUAGUU	0	5'	Minus	158	56.96	–62.7	0.92
let-7a-3p	aca-let-7a-3p	scaffold 125	CUAUACAGUCUACUGUCUUUC	0	3'	Plus	110	56.36	–43.9	0.91
let-7e-5p	aca-let-7e-5p	scaffold 116	UGACCUAGUAGAUUGAAUAGUU	0	5'	Minus	149	53.68	–51.1	0.74
miR-1a-3p	aca-miR-1a-3p	scaffold 125	UGGAAUGUAAAGAAGUGUGUAU	0	3'	Plus	81	54.32	–40.5	1.09
miR-7-5p	aca-miR-7-5p	scaffold 10	UGGAAGACUAGUGAUUUUGUUGU	0	5'	Plus	71	56.34	–22.7	0.73
miR-9a-5p	bmo-miR-9a-5p	scaffold 1	UCUUUGGUUAUCUAGCUGUAUGA	0	5'	Minus	135	32.59	–46.7	0.51
miR-9a-2-3p	ssa-miR-9a-2-3p	scaffold 1	UAAAGCUAGAUAACCGAAAGUA	0	3'	Minus	104	60.58	–45.0	1.10
miR-9a-3-3p	ssa-miR-9a-3-3p	scaffold 10	UAAAGCUAGAGAACCGAAAGUA	0	3'	Minus	84	61.90	–39.8	1.24
miR-10a-5p	aca-miR-10a-5p	scaffold 15	UACCCUGUAGAUCCGAAUUUGUG	0	5'	Minus	85	57.65	–36.2	1.01
miR-10d-5p	dre-miR-10d-5p	scaffold 129	UACCCUGUAGAACCGAAUGUGUG	0	5'	Plus	85	48.24	–42.2	0.96
miR-18a-5p	ssa-miR-18a-5p	scaffold 144	UAAGGUGCAUCUAGUGUAGUUA	0	5'	Minus	143	53.85	–52.5	0.80
miR-20b-5p	ssa-miR-20b-5p	scaffold 144	CAAAGUGCUCACAGUGCAGGUA	0	5'	Minus	83	54.22	–27.7	0.73
miR-22a-3p	ssa-miR-22a-3p	scaffold 175	AAGCUGCCAGCUGAAGAACUGC	1	3'	Plus	88	46.59	–48.9	1.04
miR-22b-3p	ssa-miR-22b-3p	scaffold 199	AAGCUGCCAGUUGAAGAGCUGU	0	3'	Plus	86	52.33	–36.3	0.89
miR-23a-3p	aca-miR-23a-3p	scaffold 112	AUCACAUUGCCAGGGAUUUCC	0	3'	Minus	92	56.52	–47.1	1.18
miR-26a-5p	ssa-miR-26a-5p	scaffold 125	UUCAAGUAAUCCAGGAUAGGCU	0	5'	Minus	92	51.09	–50.6	1.12
miR-26b-5p	ssa-miR-26b-5p	scaffold 13	UUCAAGUAAUCCAGGAUAGGUU	0	5'	Minus	69	50.72	–30.3	0.89
miR-27c-3p	dre-miR-27c-3p	scaffold 112	UUCACAGUGGUUAAGUUCUGC	0	3'	Minus	117	41.88	–59	0.87
miR-29a-3p	aca-miR-29a-3p	scaffold 147	UAGCACCAUUUGAAAUCGGUU	0	3'	Plus	61	63.93	–23.0	1.05
miR-33-5p	aca-miR-33-5p	scaffold 112	GUGCAUUGUAGUUGCAUUGCA	0	5'	Plus	86	43.02	–43.7	0.89
miR-126-5p	aca-miR-126-5p	scaffold 20	CAUUAUUACUUUUGGUACGCG	0	5'	Plus	87	51.72	–34.6	0.82
miR-128-1-5p	ssa-miR-128-1-5p	scaffold 113	CGGGGCCGGGACGCUGUCUGAGA	0	5'	Minus	66	40.91	–28.5	0.73
miR-128-3p	aca-miR-128-3p	scaffold113	UCACAGUGAACCGGUCUCUUU	0	3'	Minus	82	40.24	–35.4	0.72
miR-133	bfl-miR-133	scaffold 125	UGGUCCCCUUCAACCAGCUGUA	0	3'	Plus	80	55.00	–31.5	0.88
miR-133a	aca-miR-133a	scaffold 1	UUUGGUCCCCUUCAACCAGCUA	1	3'	Minus	82	43.90	–29.4	0.64
miR-133a-5p	ssa-miR-133a-5p	scaffold 125	AGCUGGUAAAAUGGAACCAAA	0	5'	Plus	163	49.08	–53.3	0.64
miR-135a-3p	ssa-miR-135a-3p	scaffold 179	UAUAGGGAUGGAAGCCAUGCA	0	3'	Plus	99	45.45	–50.4	0.93
miR-135a-5p	ssa-miR-135a-5p	scaffold 179	UAUGGCUUUUUAUUCCUAUCUGA	0	5'	Plus	80	48.75	–44.8	1.09
miR-135b-5p	dre-miR-135b-5p	scaffold 179	UAUGGCUUUUUAUUCCUAUCUG	0	5'	Plus	78	50.00	–41.5	1.06
miR-137-5p	ssa-miR-137-5p	scaffold 112	ACGGGUAUUCUUGGGUUGAUAA	0	5'	Minus	86	50.00	–31.9	0.74
miR-137b-3p	aca-miR-137b-3p	scaffold 112	UUAUUGCUUGAGAAUACGCGUAG	0	3'	Minus	135	48.15	–46.8	0.67

Table 1. (Contd.)

miRNAs name	Source miRNA homologues	Gene source	Predicted mature sequence(5'-3')	NM, nt	Loc	Strand	LP, nt	A + U, %	MFE	MFEI
miR-143-5p	aca-miR-143-5p	scaffold 172	GGUGCAGUGCUGCAUCUCUGG	0	5'	Plus	69	46.38	–25.5	0.69
miR-143-3p	aca-miR-143-3p	scaffold 172	UGAGAUGAAGCACUGUAGCUC	0	3'	Plus	93	44.09	–40.1	0.77
miR-153b-3p	dre-miR-153b-3p	scaffold 18	UUGCAUAGUCACAAAAAUGAGC	0	3'	Plus	84	54.76	–37.8	0.99
miR-181a	aca-miR-181a	scaffold 10	AACAUUCAACGCUGUCGGUGAGU	0	5'	Plus	90	46.67	–32.5	0.68
miR-181a-5-3p	ssa-miR-181a-5-3p	scaffold 10	ACCAUCGACCGUUGACUGUGCC	0	3'	Plus	73	52.05	–24.3	0.69
miR-181b-3p	ssa-miR-181b-3p	scaffold 10	CUCACUGAACAAUGAGUGCAA	0	3'	Plus	81	50.62	–28.8	0.72
miR-184-3p	aca-miR-184-3p	scaffold 12	UGGACGGAGAACUGAUAAGGG**C**	1	3'	Minus	87	49.43	–33.3	0.76
miR-190a-5p	aca-miR-190a-5p	scaffold 12	UGAUAUGUUUGAUAUAUUAGGUU	0	5'	Minus	68	69.12	–21.6	1.03
miR-199a-5p	aca-miR-199a-5p	scaffold 16	CCCAGUGUUCAGACUACCUGU	0	5'	Minus	79	50.63	–31.4	0.81
miR-203-3p	aca-miR-203-3p	scaffold 17	GUGAAAUGUUUAGGACCACUUG	0	3'	Plus	77	62.34	–24.7	0.85
miR-203b-5p	dre-miR-203b-5p	scaffold 17	AGUGGUUCUCAACAGUUCAACA	0	5'	Plus	167	61.68	–43.0	0.67
miR-205a	aca-miR-205a	scaffold 158	UCCUUCAUUCCACCGGAGUCUG	0	5'	Plus	75	57.33	–37.5	1.17
miR-206-5p	ssa-miR-206-5p	scaffold 1	ACAUGCUUCCUUAUAUCCCCA	0	5'	Minus	97	54.64	–44.0	1.00
miR-206-3p	aca-miR-206-3p	scaffold 1	UGGAAUGUAAGGAAGUGUGUG	0	3'	Minus	82	53.66	–42.7	1.12
miR-214-5p	aca-miR-214-5p	scaffold 16	UGCCUGUCUACACUUGCUGUGC	0	5'	Minus	85	43.53	–57.1	1.19
miR-338-3p	aca-miR-338-3p	scaffold 11	UCCAGCAUCAGUGAUUUUGUU	0	3'	Plus	86	53.49	–34.2	0.86
miR-375-3p	aca-miR-375-3p	scaffold 18	UUUGUUCGUUCGGCUCGCGUUA	0	3'	Minus	86	51.16	–38.1	0.91
miR-459-5p	ssa-miR-459-5p	scaffold 195	UCAGUAACACAGAUUCAUCCUGU	0	5'	Minus	77	50.65	–31.8	0.84
miR-462a-5p	ssa-miR-462a-5p	scaffold 179	UAACGGAACCCAUAAUGCAGCUG	0	5'	Plus	73	47.95	–31.6	0.83
miR-489-5p	aca-miR-489-5p	scaffold 190	UGGUCGUAUGUAUGACGUCAUU	0	5'	Plus	109	48.62	–50.9	0.91
miR-489-3p	aca-miR-489-3p	scaffold 190	GUGACAUCAUAUGUACGGCUGC	0	3'	Plus	94	51.06	–42.6	0.93
miR-722-3p	ssa-miR-722-3p	scaffold 11	UUUGCAGAAACGUUUCAGAUUU	0	3'	Minus	91	62.64	–42.5	1.25
miR-723-3p	ssa-miR-723-3p	scaffold 20	AGACAUCAGAUAAAUCUGUGCU	0	3'	Plus	86	51.16	–47.6	1.13
miR-734-5p	ssa-miR-734-5p	scaffold 18	GAACUAUUCUGCAACAUUUGUU	0	5'	Plus	84	61.90	–26.5	0.83
miR-1788-3p	aca-miR-1788-3p	scaffold 18	CAGGCAGCUAAAGCAAGUCUG	0	3'	Minus	84	51.19	–46.3	1.13
miR-2187-3p	ssa-miR-2187-3p	scaffold 15	UUACAGGCUAUGCUAAUCUAUGC	0	3'	Minus	84	57.14	–38.3	1.06
miR-2187-5p	dre-miR-2187-5p	scaffold 2	UUAAUUAGUAUAGCCUGUUUUA	0	5'	Plus	124	62.10	–50.7	1.08
miR-7132b-5p	ssa-miR-7132b-5p	scaffold 101	GACUUGGUCAAAGCUCCUCAG**U**	1	5'	Minus	102	55.88	–30.8	0.68
miR-7147	dre-miR-7147	scaffold 187	UGUACCAUGCUGGUAGCCAGU	0	5'	Minus	82	41.46	–38.7	0.81
miR-7552a-5p	ssa-miR-7552a-5p	scaffold 2	UUACAAUUAAAGGAUAUUUCU**C**	1	5'	Minus	87	59.52	–40.0	1.14

Abbreviations: NM, number of mismatch; LP, length of precursor; Loc, location; MFE, minimal folding free energy, kcal/mol; MFEI, minimal folding free energy index; bold letters represent mismatched bases.

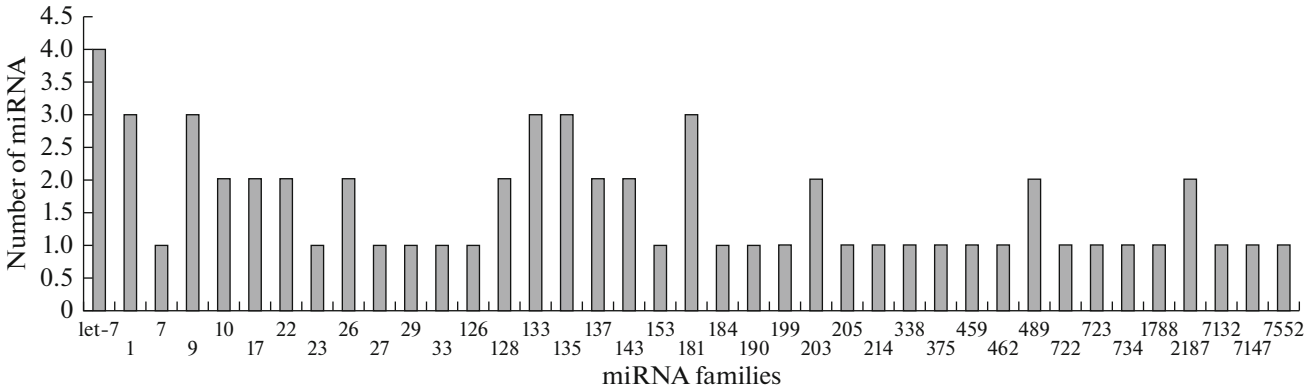

Fig. 1. The distribution of identified miRNAs in different miRNA families.

In present study, four pre-miRNAs from four different families (miR-23a-3p, miR-184-3p, miR-214-5p, and miR-338-3p) of *B. pectinirostris* were randomly selected to analyze the conservation with others animals, respectively. The novel identified four miRNA families in *B. pectinirostris* showed high mature miRNAs conservation across other animal species from invertebrate to vertebrate (Fig. 5). Mature miRNAs were more conserved than respective opposite miRNA sites and pre-miRNA sequences. The opposite miRNAs may not be so highly conserved because, although miRNAs and its opposite miRNAs are complementary, their base pairing is not perfect. This conservation made them strong candidates for miRNAs in *B. pectinirostris* and suggested their similar physiological functions. The conservation of mature miRNAs and pre-miRNAs provides the chance to investigate their evolutionary relationships. Based on the pre-miRNA sequence comparisons, the evolutionary relationships of *B. pectinirostris* miRNAs with other members from the same families were analyzed using the MEGA6.0. As follows from the phylogenetic trees, in different families, the evolutionary relationships of *B. pectinirostris* miRNAs with other species were different; for example, in miR-184 family, bpe-miRNA-184 and tni-miR-184 were on the same branch, while in miR-214 family, bpe-miR-124 had close relationship with fru-miR-124, and in miR-338 family, bpe-miR-338 was closely related to ola-miR-338-1/-2 (data are not shown). Also, it could be seen that different miRNA members in the same family often displayed evolutionary relatedness. For example, bpe-miR-23 and others six fish miRNAs were clustered into a large group. These results suggest that different miRNAs might evolve at different rates not only within the same animal species, but also in different ones.

Experimental Verification of Identified miRNAs

To further validate the expression of the identified miRNAs in *B. pectinirostris*, stem–loop RT-PCR based assay was carried out using total RNA isolated from muscle tissue, an effective and widely-used method for detecting miRNAs. The 16 miRNAs, including let-7-5p, miR-1a-3p, miR-7-5p, miR-9a-5p, miR-20b-5p, miR-33-5p, miR-128-3p, miR-133, miR-184-3p, miR-206-5p, miR-338-3p, miR-375-3p, miR-462a-5p, miR-734-5p, miR-1788-3p, and miR-7174 were chosen randomly from the predicted miRNAs and were subjected to experimental studies. The results showed that all 16 miRNAs were successfully expressed (Fig. 6). Of these, miR-206-5p and miR-7174 showed relatively high expression level suggesting that they play a very important regulatory role in growth and development of muscle in *B. pectinirostris*; meanwhile miR-7 displayed lower expression levels and other miRNAs had moderate levels in muscle tissue. These results suggest that the miRNAs have diverse expression patterns during muscle developmental stages. Also, the results are in agreement with the results of previous studies on animals. Previous studies have shown that some miRNAs, such as miRNA-1, miRNA-133, miRNA-181, and miRNA-206, are highly expressed in skeletal muscle in animals

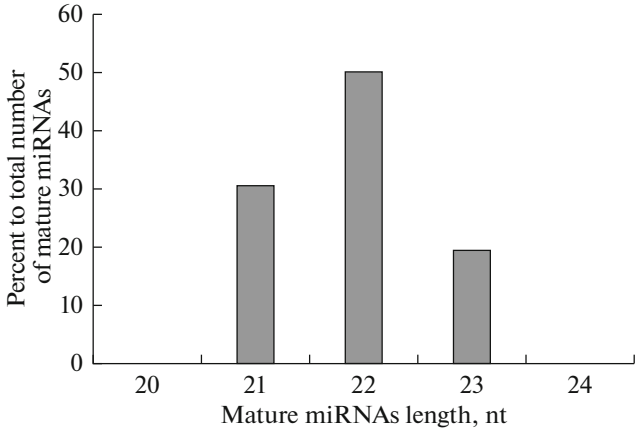

Fig. 2. Size distribution pattern of the mature miRNA lengths.

Table 2. List of potential targets of the identified miRNAs in *B. pectinirostris*

miRNA	Targeted protein	Putative target function	Gene ID
let-7a-3p	Vasa-like protein	Transcription factor	KC633182
let-7a-5p	Beta-actin	Development	KC622028
	Melatonin receptor 1a1.4	Transcription factor	KC622029
	Melatonin receptor 1a1.7	Transcription factor	KC622030
miR-7-5p	Melatonin receptor 1a1.7	Transcription factor	KC622030
miR-9a-5p	Cytochrome P450 family 19 subfamily A polypeptide 1	Metabolism	KC633183
	Melatonin receptor 1c	Transcription factor	KC622032
miR-22a-3p	Melatonin receptor 1c	Transcription factor	KC622032
miR-22b-3p	Cytochrome P450 family 19 subfamily A polypeptide 1	Metabolism	KC633183
	Melatonin receptor 1c	Transcription factor	KC622032
miR-26a-5p	Melatonin receptor 1c	Transcription factor	KC622032
miR-27c-3p	Hepcidin-2	Immunoregulation	KM924543
miR-33-5p	Cytochrome P450 family 19 subfamily A polypeptide 1	Metabolism	KC633183
miR-128-1-5p	Melatonin receptor 1a1.4	Transcription factor	KC622029
	Hepcidin-2	Immunoregulation	KM924543
miR-133a-5p	Melatonin receptor 1a1.4	Transcription factor	KC622029
miR-133a	Growth differentiation factor 9	Development	KC633181
miR-135a-3p	Beta-actin	Development	KC622028
	Melatonin receptor 1c	Transcription factor	KC622032
miR-137-5p	Vasa-like protein	Transcription factor	KC633182
miR-143-5p	Melatonin receptor 1a1.4	Transcription factor	KC622029
	Melatonin receptor 1c	Transcription factor	KC622032
miR-153b-3p	Melatonin receptor 1b	Transcription factor	KC622031
miR-199a-5p	Vasa-like protein	Transcription factor	KC633182
	Melatonin receptor 1b	Transcription factor	KC622031
miR-214-5p	Vasa-like protein	Transcription factor	KC633182
miR-338-3p	Hepcidin-1	Immunoregulation	KM924542
miR-375-3p	Arylalkylamine N-acetyltransferase-2	Metabolism	KC876050
	Beta-actin	Development	KC622028
miR-462a-5p	Beta-actin	Development	KC622028
miR-489-5p	Vasa-like protein	Transcription factor	KC633182
miR-722-3p	Thyrotropin receptor	Transcription factor	KC686694

and play a notably important role in skeletal muscle development by regulating their target genes [46, 47]. All these findings considerably broaden the scope of miRNA functions in aquatic organism. Our study also demonstrated a high accuracy rate for the computational miRNAs identification in *B. pectinirostris* genome.

Prediction of Potential Targets for Identified miRNAs

In animal genomes, miRNA targets are difficult to predict because targets of miRNAs generally display only partial complementarity to the mature miRNA sequence [48, 49], in contrast to plants, where miRNAs can bind with almost perfect complementarity to their targets [50, 51]. Computational and experimental evidence has also shown the existence of miRNA binding sites in protein-coding regions or 5'-UTRs, and not 3'-UTRs, in most cases. We tested the 62 identified *B. pectinirostris* miRNAs against the GenBank mRNA database of *B. pectinirostris* using the RNA hybrid software according to the criteria described in the Materials and Methods section. Due to the limited information about the *B. pectinirostris* mRNAs, only 12 targets were identified for the 23 *B. pectinirostris* miRNAs (Table 2) and no targets were

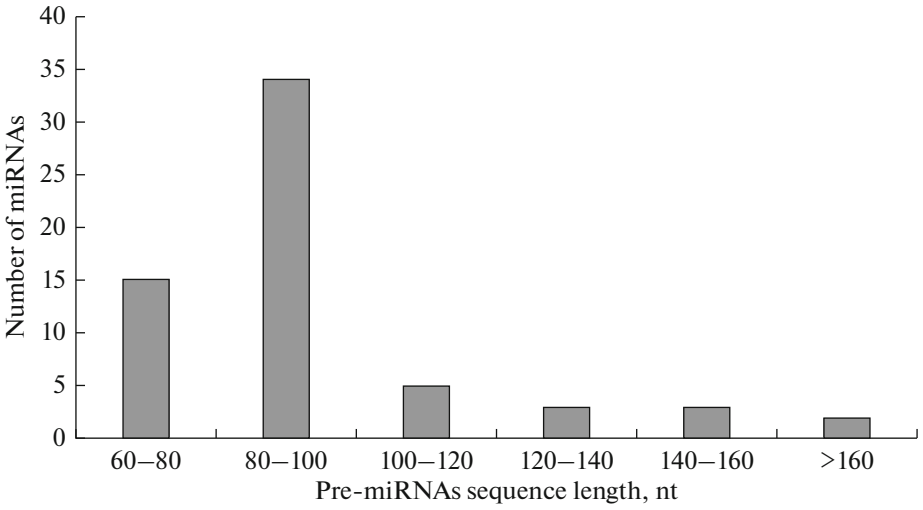

Fig. 3. Size distribution pattern of the pre-miRNA lengths.

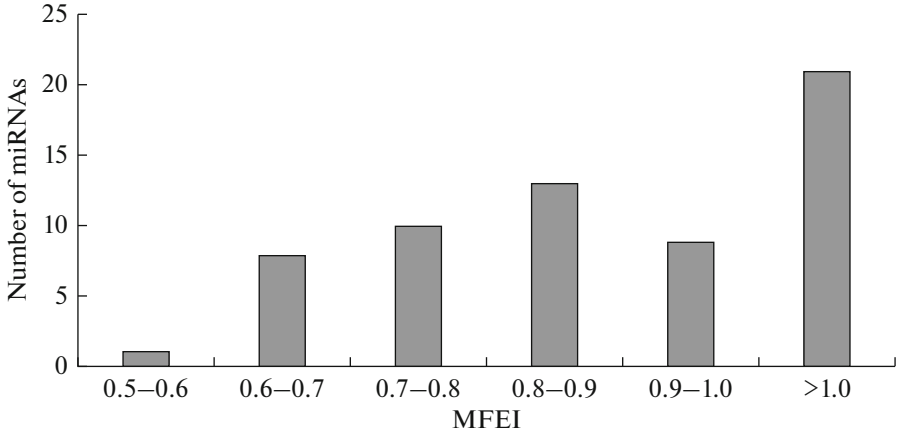

Fig. 4. Distribution of MFEI of the identified pre-miRNAs.

found in others 39 miRNAs. Some predicted targets for *B. pectinirostris* miRNAs indicated that a single miRNA might regulate several target genes or same target genes were regulated by multiple miRNAs. Particularly, melatonin receptor 1a (KC622029), a transcription factor, can be targeted by four miRNAs, including let-7a-5p, miR-128-1-5p, miR-133a-5p, and miR-143-5p. Many experimental and/or computational approaches have demonstrated that miRNAs target many transcription factors [52, 53]. This study resulted in majority of the targets being classified as transcription factors in *B. pectinirostris* and regulated by 14 miRNAs, respectively. Some miRNAs target genes were also involved in growth and development. One of the miRNAs encoded the regulator of the process of muscle growth. Let-7a-5p, miR-135a-3p, miR-375-3p, and miR-462a-5p targeted beta-actin, which is an essential regulatory muscle protein. miR-133a targeted growth differentiation factor 9 (GDF9). Prior studies indicated that miR-133 was an important regulatory factor that might help to regulate muscle terminal differentiation by targeting different growth factors [54, 55]. The proteins involved in the metabolic processes are also reported as a class targeted by miRNAs [56, 57]. In our study, miR-9a-5p, miR-22b-3p, and miR-33-5p also targeted cytochrome P450 family 19 subfamily A polypeptide 1 (CYP19A1). Immunity is a complex biological system in multicellular organisms to defend themselves from invading pathogens. The fine tuning of the innate immune response by miRNAs is a concept now supported by a rapidly growing body of evidence [58]. Two examples from this class are that miR-338-3p targeted hepcidin-1, while miR-27c-3p and miR-128-1-5p targeted hepcidin-2. These miRNAs seem to be involved in the regulation of immunity of *B. pectinirostris*. Further investigation is necessary to validate identified miRNA targets.

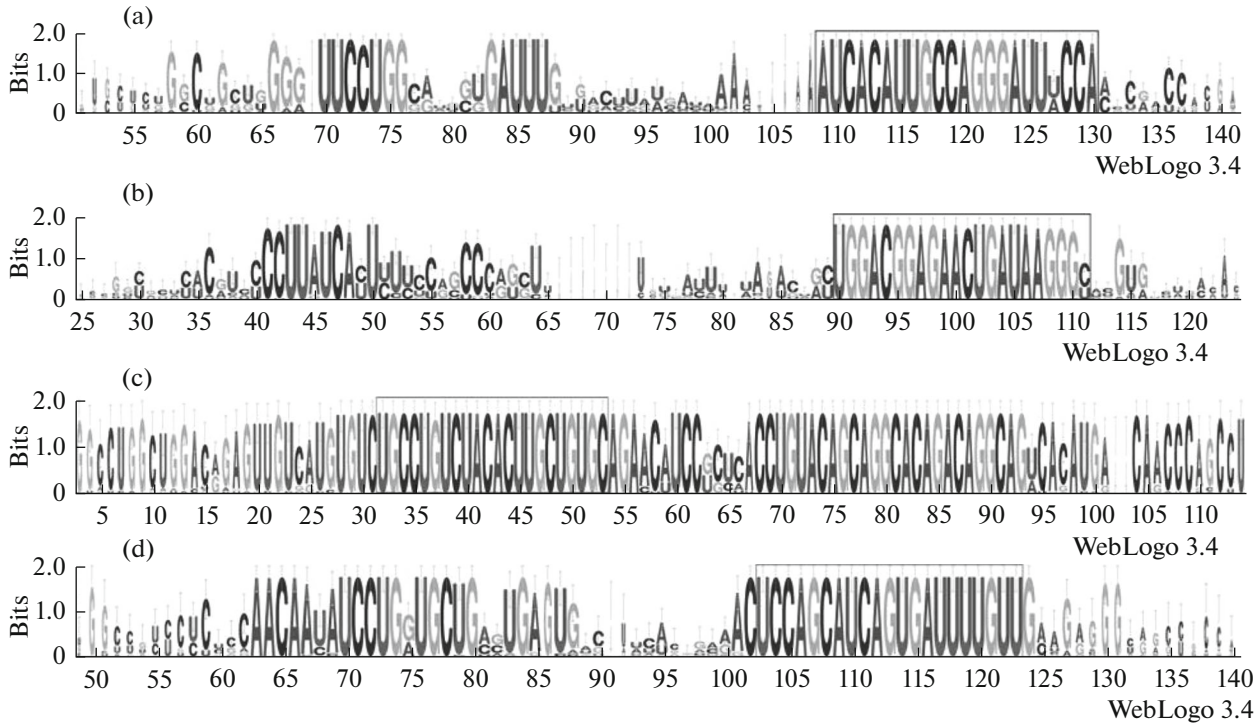

Fig. 5. Sequence logo showing mature conserved nucleotide composition of pre-miRNAs sequences using Weblogo; (a) miR-23 family, (b) miR-184 family, (c) miR-214 family, and (d) miR-338 family.

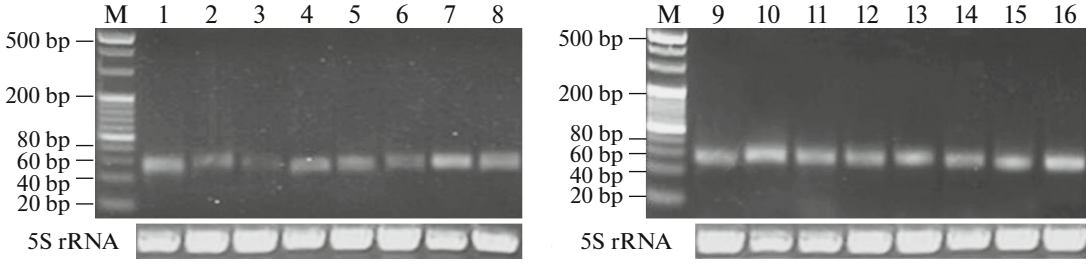

Fig. 6. Experimental validation of expression of identified *B. pectinirostris* miRNAs by stem–loop RT-PCR; M: 20 bp ladder marker; 1, let-7-5p; 2, miR-1a-3p; 3, miR-7-5p; 4, miR-9a-5p; 5, miR-20b-5p; 6, miR-33-5p; 7, miR-128-3p; 8, miR-133; 9, miR-184-3p; 10, miR-206-5p; 11, miR-338-3p; 12, miR-375-3p; 13, miR-462a-5p; 14, miR-734-5p; 15, miR-1788-3p; and 16, miR-7174.

EXPERIMENTAL

Availability of databases. In order to identify novel miRNAs in *B. pectinirostris*, a total of 8695 animal mature miRNA sequences were obtained from the publicly available miRNA database miRBase, version 21.0 (http://www.mirbase.org/). After removing repeated sequences, remaining miRNAs were used for searching *B. pectinirostris* miRNAs. The genomic sequences of *B. pectinirostris* were obtained from NCBI database (http://www.ncbi.nlm.nih.gov/genome/?term = *Boleophthalmus+pectinirostris*).

Computational prediction of miRNAs. The alignment tool BLAST version 2.2.27 was used to identify the potentially conserved miRNAs and was downloaded from the NCBI website. BLASTN parameter settings were as follows: an expect value cut-off of 10; the window size 7; a low-complexity sequence filter; number of descriptions and alignments were 1000. All BLAST results were saved and used for further analysis. The prediction process is shown in Fig. 7. Only sequences that fit the following criteria were designated as potential miRNAs in *B. pectinirostris*: (1) there were not more than three nucleotides substituted between the query miRNA sequence and the BLASTn result sequence; (2) the predicted pre-miRNA folded into a perfect or near-perfect stem–loop hairpin secondary structure; (3) the potential mature miRNA length should be 18–26 nt and located in one arm of

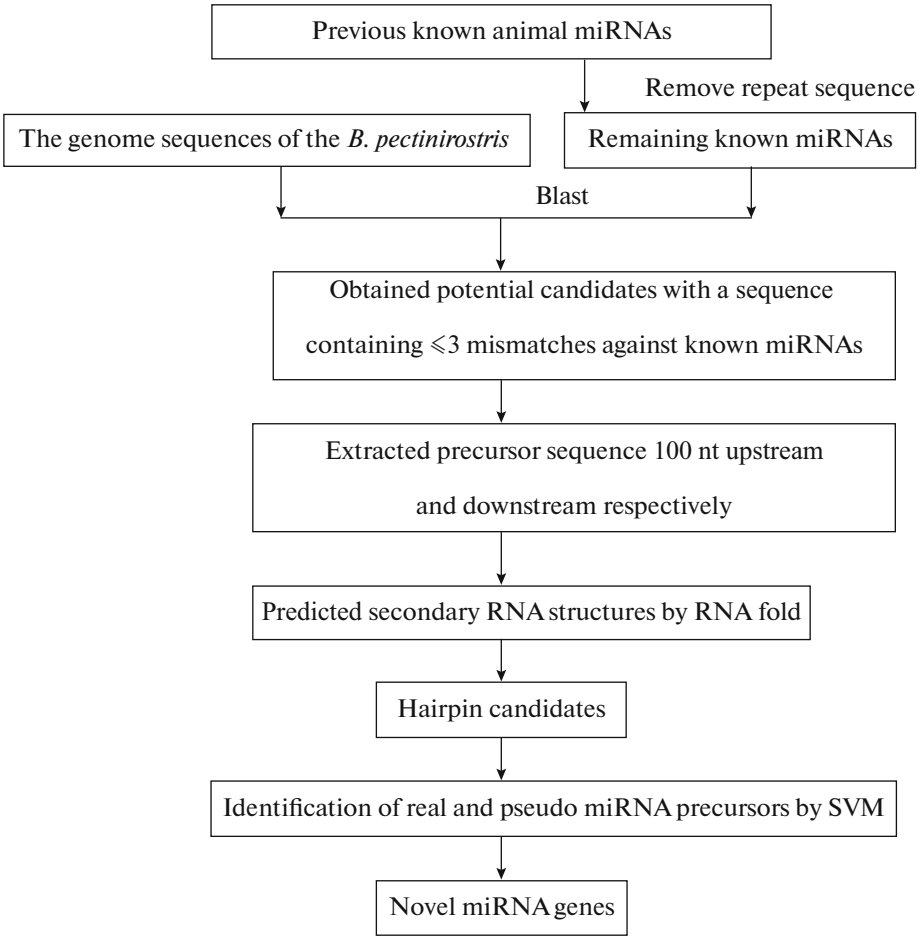

Fig. 7. Flowchart of the *B. pectinirostris* miRNA gene prediction procedure.

the hairpin, (4) no loops or breaks were allowed in the miRNA/miRNA* duplex; (5) A + U content should be 30–75%, and (6) the potential miRNA precursor sequence should have a high negative MFE and a high MFE index (MFEI) value. Besides, predicted miRNA precursor sequences were submitted to a program called SVM that was improved by our lab using the 'Support Vector Machine' model; the program identifies pre-miRNA-like hairpin sequences classifying them as real or pseudo-pre-miRNA. The model has been successfully applied to identify pre-miRNAs and mature miRNAs [59, 60]. Finally, all predicted miRNAs were further analyzed for the presence of antisense miRNAs and some possible false sequences of pre-miRNAs were to be deleted manually.

Stem–loop RT-PCR assay. To verify computational predictions, 16 miRNAs were randomly selected from the predicted miRNAs by the stem–loop RT-PCR experimental method. Small RNAs from the fish muscle tissue sample were 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 [61–63]. The stem–loop RT primers and gene specific primers are listed in Table 3. One hundred nanogram 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 final elongation step at 72°C for 7 min. The PCR products were separated through 2.5% (w/v) agarose gel. Expression of 5S rRNA gene was performed for cDNA normalization. The DNA fragments were directly subcloned into pMD18-T vector (Takara) and sequenced.

Conservation and phylogenetic analysis of identified miRNAs. Because most animal miRNAs and their precursor sequences have derived from the same gene families, they are strongly conserved and have high sequence identity, even between distantly related species. The evolutionary relationships between the precursor sequences of *B. pectinirostris* miRNAs and their counterparts in the same family from other animal species were analyzed by MEGA 6.0 (http://www.megasoftware.net/mega.php) [64]. Maximum

Table 3. Primers used in this study for stem–loop RT-PCR assay

miRNA	Primer	Sequence (5'-3')
let-7-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTATA
	Forward	CGGTGGAATGTAAAGAAGT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-1a-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATACAC
	Forward	GCGGACATACTTCTTTACAT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-7-5p	RT	GGCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAACA
	Forward	CGCGTAGCTTATCAGACTGAT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-9a-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATCA
	Forward	AGGCGTCTCACTACCTTGTC
	Reverse	GTGCAGGGTCCGAGGT
miRNA-20b-5p	RT	GGCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACCTG
	Forward	GCCGTTTGGTCCCCTTCAAC
	Reverse	GTGCAGGGTCCGAGGT
miRNA-33-5p	RT	GCCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCAAT
	Forward	CCGGCATTTGGTTCCATTTT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-128-3p	RT	GGCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAGAG
	Forward	GCGAGACTTTTCCCCTTGTCT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-133	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACAGC
	Forward	GCAGAATCTGGATGAACT
	Reverse	GTGCAGGGTCCGAGGT
miR-184-3p	RT	ATCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCCTT
	Forward	CGCGGTGATTTTGTTGTTT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-206-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGGGA
	Forward	GGCCTACAATCTTTGAAAAGTTT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-338-3p	RT	GCCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCAAA
	Forward	CGCGGTGATTTTGTTGTTT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-375-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACGC
	Forward	CGCGGTGATTTTGTTGTTT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-462a-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG
	Forward	CGCGGTGATTTTGTTGTTT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-734-5p	RT	GGCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACAAA
	Forward	CGCGGTGATTTTGTTGTTT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-1788-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGACT
	Forward	CGCGGTGATTTTGTTGTTT
	Reverse	GTGCAGGGTCCGAGGT
miRNA-7174	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTGGC
	Forward	CGCGGTGATTTTGTTGTTT
	Reverse	GTGCAGGGTCCGAGGT
5S rRNA	Forward	TCTGCCATCGTACGCCGCACACT
	Reverse	CTTCTAGAGCGCGAGGCGGCCGACATGT

likelihood trees were constructed using the Tamura–Nei distance model with 1000 bootstrap replicates to illustrate the evolutionary relationships among the members of the family.

Target prediction for identified miRNAs. The mRNA database of the 11was fish species downloaded from NCBI database. Target identification is essential for understanding the biological functions of miRNAs. Potential targets of the identified miRNAs were predicted using RNAhybrid program [65]. Criteria used were: (1) a perfect Watson–Crick match between miRNA and target at 2–8 positions in the 5' end of miRNA; (2) one G:U pair in the seed was allowable; (3) the size of max internal loop and bulge loop was 3; (4) *P*-value cutoff of 0.05 and target duplex free energy $\Delta G \leq -20$ kcal/mol.

CONCLUSIONS

Although miRNAs have been extensively studied in recent years, little is known about miRNAs in *B. pectinirostris*, the most economically important fishes in the world. In our study, 62 mature miRNAs belonging to 39 families were identified for the first time by searching the *B. pectinirostris* genome database using computational approach. We confirmed the expression of 18 of these predicted miRNAs in muscle tissue in vivo by using stem–loop RT-PCR experimental method. Because only 12 mRNAs of *B. pectinirostris* were annotated in NCBI database, 23 miRNA predict target genes in our present study. To our knowledge, this is the first time that miRNAs and their characterization were described in *B. pectinirostris*. Our results enriched the *B. pectinirostris* miRNA database and provided useful information for investigating biological functions of miRNAs in *B. pectinirostris*. These results will also assist miRNA studies in related three species, that as *Scartelaos histophorus*, *Periophthalmodon schlosseri,* and *Periophthalmus magnuspinnatus*.

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