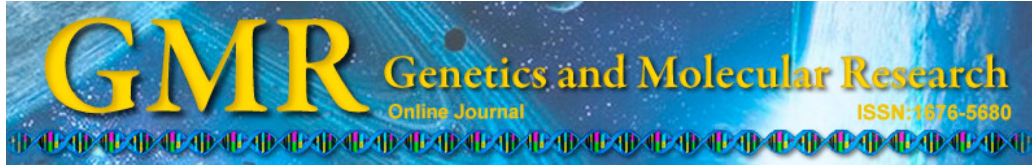

Identification of conserved microRNAs and their target genes in Nile tilapia (*Oreochromis niloticus*) by bioinformatic analysis

X.H. Li, J.S. Wu, L.H. Tang and D. Hu

School of Geography and Biological Information,
Nanjing University of Posts and Telecommunications,
Nanjing, Jiangsu, China

Corresponding author: X.H. Li
E-mail: lixh@njupt.edu.cn

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ABSTRACT. MicroRNAs (miRNAs) are a class of non-coding RNAs that play important roles in posttranscriptional regulation of target genes. miRNAs are involved in multiple biological processes by degrading targeted mRNAs or repressing mRNA translation in various organisms. Their conserved nature in various organisms makes them a good source of new miRNA discovery using comparative genomic approaches. In the present study, conserved Nile tilapia (*Oreochromis niloticus*) miRNAs were identified using a bioinformatic strategy based on expressed sequence tag and genome survey sequence databases. A total of 21 new miRNAs were detected and were found to belong to 17 families. Using mature miRNA sequences as queries, potential targets for tilapia miRNAs were predicted using a local BLAST program and the miRanda software. Target proteins identified using miRanda and BLAST analyses included transcription factors and molecules important in metabolism, transportation, immunity, stress-related activity, growth, and development. These miRNAs and their targets in tilapia may increase the understanding of the role of

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miRNAs in regulating the growth and development of tilapia.

Key words: Bioinformatic analysis; *Oreochromis niloticus*; MicroRNAs; Target genes

INTRODUCTION

MicroRNAs (miRNAs) are a growing family of endogenous, non-protein-coding sequences that are 20-25 nucleotides (nt) long. They are hairpin-derived RNAs that repress the expression of target genes, typically by binding to the 3'-untranslated region (UTR) of mRNA (Chen and Rajewsky, 2007). More than 60% protein-coding genes are computationally predicted to be targets based on base-pairing between the 3'-UTR and the 5'-seed region of the miRNA (Friedman et al., 2009). Numerous studies have demonstrated that miRNAs have important roles in biological functions, such as cell proliferation, differentiation, growth, development, carcinogenesis, responses to viral infection, and immune and stress responses in various organisms (Bartel, 2004; Blenkiron and Miska, 2007; Urbich et al., 2008).

In recent years, an increasing number of miRNAs have been identified and deposited in the major miRNA databases. Although hundreds of miRNA have been identified, only a very small number of fish miRNAs have been discovered and functionally identified. Several approaches are used to identify new miRNAs in fish. Direct cloning is a classic method that has enabled the identification of miRNAs in zebrafish (Kloosterman et al., 2006) and rainbow trout (Ramachandra et al., 2008). However, it is difficult to clone low-abundance miRNAs because all miRNAs have similar secondary hairpin structures and many of these structures are evolutionarily conserved (Ambros et al., 2003). Currently, high-throughput sequencing and computational methods have been commonly used to identify new miRNAs in fish. Using the small RNA deep sequencing protocol with bioinformatic analysis, known or new miRNAs were identified in zebrafish (Soares et al., 2009), medaka (Li et al., 2010), Atlantic halibut (Bizuayehu et al., 2013), commom carp (Zhu et al., 2012), and catfish (Xu et al., 2013). However, computational strategies based on expressed sequence tags (EST) and genome survey sequences (GSS), has been successful for identifying new miRNAs (mature and precursor) in fish (Barozai, 2012).

Nile tilapia (*Oreochromis niloticus*) is an economically important species in freshwater aquaculture. With its fast growth rate and ability to adapt to a wide range of culture conditions, tilapia is one of the most widely farmed fish in the world. Because miRNA is involved in growth and resistance to environmental stresses, several studies have attempted to identify miRNAs related to muscle growth, regulation of osmotic stress, and salt tolerance (Huang et al., 2012; Yan et al., 2013a). The full-miRNA transcriptome in muscle tissue revealed significant differences in miRNA expression between fast-growing and control strains of tilapia (Huang et al., 2012). miR-206, a muscle-specific miRNA, regulates the growth of teleost tilapia (*O. niloticus*) by regulating insulin-like growth factor 1 gene expression (Yan et al., 2013a). MyoD is a helix-loop-helix protein that regulates myogenesis, and miR-203b expression is negatively correlated with MyoD expression in tilapia (Yan et al., 2013b). In addition, miR-429 and miR-30c were found to regulate osmotic stress transcription factor 1 and salt tolerance in tilapia, respectively (Yan et al., 2012). Therefore, miRNAs play very important roles in biological processes in tilapia. However, the sequences of tilapia miRNAs, particularly precursor sequences, have not been thoroughly examined.

In this study, all known fish miRNAs from *Danio rerio*, *Takifugu rubripes*, *Oryzias*

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latipes, *Tetraodon nigroviridis*, and *Cyprinus carpio* in mirBase were used to search for conserved tilapia miRNA homologs in the EST and GSS databases. A total of 21 potential miRNAs were identified and the target sequences of these miRNAs were predicted. The results will provide information for miRNA studies of tilapia and other fish species.

MATERIAL AND METHODS

Databases of miRNAs, EST, GSS, and mRNA sequences and software

To search for potential miRNAs, a total of 675 previously identified miRNAs and their precursor sequences from 5 fish, including *D. rerio*, *T. rubripes*, *O. latipes*, *T. nigroviridis*, and *C. carpio* were obtained from miRBase (Release 19.0, September 2012; http://microrna.sanger.ac.uk). These miRNAs were defined as a reference set of miRNA sequences. To avoid the identification of overlapping miRNAs, repeated sequences were removed and the remaining sequences were used as an miRNA reference. The *O. niloticus* EST, GSS, and mRNA databases were obtained from the NCBI GenBank nucleotide databases. The comparative BLAST 2.2.28 software was downloaded from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

Prediction of potential miRNAs and their precursors

All non-redundant mature miRNA sequences were used as BLAST queries against the Nile tilapia EST and GSS databases. The initial candidate tilapia miRNAs with 0-3 mismatches in all known fish mature miRNA sequences were predicted using the local BLAST 2.2.28. Additionally, 80 bp of bilateral flanking sequences from the candidate miRNAs in the ESTs and GSS were extracted and their secondary structures were predicted using the web-based Mfold 3.1 software (http://www.bioinfo.rpi.edu/applications/mfold/) (Zuker, 2003). A sequence was considered to be a candidate miRNA precursor if the hairpin structure satisfied the criteria described by Fu et al. (2011): 1) the minimum free energy (ΔG) is ≤ -15 kcal/mol; 2) the stem region includes at least 80% mature miRNA; 3) the number of allowed errors in 1 bulge is ≤18 bp; 4) the hairpin is >53 bp long; 5) the loop region is <22 bp; and 6) the number of mismatches between the miRNA and the anti-stem sequence is ≤6 bp.

Prediction of miRNA targets

The *O. niloticus* miRNA targets were predicted using the miRanda program, which is publicly available (http://www.microrna.org/microrna/home.do). The parameters of miRanda were set as follows: the score was set to ≥100 and the free energy was set to ≤ -20 kcal/mol. In addition, another strategy was also used to predict potential target sequences of miRNAs. First, mature miRNAs identified in the present study were used to search for antisense hits in the reference RNA sequences (refseq_rna) of the tilapia (taxid: 8128). Subsequently, to predict target sequences, mRNA sequences exhibiting perfect or near perfect complementarity with corresponding miRNAs were analyzed using RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid), a miRNA target detection software program. The criteria for target prediction allowed up to 2 mismatches in the seed sequence, with 14 as a minimum number of paired-up bases in a heteroduplex and -25 as a maximum folding energy for a heteroduplex (kcal/mol).

RESULTS AND DISCUSSION

Identification of potential miRNAs in *O. niloticus*

Because most mature miRNAs are evolutionarily conserved between species, a comparative genetic strategy provides a powerful approach for predicting the existence of new miRNAs in other species. In this study, a total of 21 potential miRNAs were identified after homology searching using all previously identified fish miRNAs against the *O. niloticus* databases of 120,962 ESTs and 69,200 GSS. Thirteen miRNAs were identified in the EST database and 8 in the GSS database (Table 1). The miRNAs belonged to 17 families, including oni-let-7, mir-1, -22, -27, -31, -34, -133, -144, -153, -222, -301, and -429. In the let-7 family, 4 miRNAs were identified, followed by miR-182 and -199 with 2 miRNAs in each. The other 15 miRNA families had only 1 member each. Oni-let-7a-5 and oni-let-7e were found in the same EST (GR657356).

The lengths of the newly identified mature miRNAs varied from 20-24 nt, and were typically 22 nucleotides in length, except miR-31 (21 nt), miR-144 (20 nt), miR-199-3 (23 nt), and miR-222 (24 nt). Their precursor miRNA lengths ranged from 66-122 nt. The location of mature miRNA sequences in the identified miRNAs also showed diversity, with sequences of let-7-i, miR-133, miR-182-a, miR-182-b, miR199-3a-2, miR-199-1, miR-204, miR-222, miR-34, miR-27-a, miR-301-c, and miR-31 located at the 5'-end of the pre-miRNAs and all others at the 3'-end. There was a larger number of mature miRNAs located at the 5'-end of the pre-miRNA than at the 3'-end (13:8). The A+U content of the predicted pre-miRNAs ranged from 35.6 to 59.3%. Minimal folding-free energy (ΔG) of miRNA precursors varied from 23.30-46.70 kcal/mol. The minimum folding-free energy index is a useful criterion for distinguishing miRNAs from other types of coding or non-coding RNAs. In our study, minimum folding-free energy index of the pre-miRNAs averaged 0.81, which is significantly higher than that of tRNAs (0.64), rRNAs (0.59), and mRNAs (0.62-0.66) (Zhang et al., 2006).

Prediction of miRNA targets

miRNAs are involved in a diverse range of biological processes such as posttranscriptional regulation of target genes. In plants, miRNAs bind perfectly or near-perfectly to their targets and many of miRNAs have relatively few targets. In contrast to plant miRNAs, animal miRNAs bind to target mRNAs through imperfect complementary sequences that are typically located at the 3'-UTRs, leading to translational repression or transcript degradation. Complementarity to the core region (positions 1-10) of an miRNA is often sufficient for effective regulation in animals. Therefore, 1 miRNA can affect transcript and protein levels of hundreds of targets in animals (Ambros, 2004). Because short-complementary sequence stretches may be interrupted by gaps or mismatches, bioinformatic prediction of miRNA targets in animals is more complicated than in plants. Thus, 2 bioinformatic approaches were used to predict miRNA targets in this study.

Using the identified miRNA sequences as BLAST queries, 8 target genes for 5 miRNAs (including let-7b, mir-1, mir-153b, mir-182, and mir-429) were identified in the

Table 1. Characterization of the novel identified *Oreochromis niloticus* miRNAs.

New miRNAs	Reference miRNA	miRNA sequences (5'-3')	Gene ID	Gene source	Location	NM (nt)	LM (nt)	LP (nt)	A+U (%)	ΔG (kcal/mol)	MFEI	OE
oni-let-7a-6	dre let-7a	UGAGGUAGUAGGUUGUAUAGUU	GR610978	EST	3'	0	22	106	42.45	46.70	1.04	Whole brain
oni-let-7a-5	dre let-7a	UGAGGUAGUAGGUUGUAUAGUU	GR657356	EST	3'	0	22	122	51.63	32.60	0.52	Kidney
oni-let-7e	dre let-7e	UGAGGUAGUAGAUUGAAUAGUU	GR657356	EST	3'	0	22	99	39.39	28.80	0.74	Kidney
oni-let-7i	dre let-7i	UGAGGUAGUAGUUUGUGCUGUU	GR613051	EST	5'	0	22	80	51.25	32.10	0.78	Whole brain
oni-mir-1-2	dre-mir-1	UGGAAUGUAAAGAAGUAUGUAU	GR692253	EST	3'	0	22	85	36.50	23.60	0.76	Spleen
oni-mir-22a-1	dre-mir-22	AAGCUGCCAGCUGAAGAACUGU	FQ256693	GSS	3'	0	22	86	48.84	42.40	1.01	
oni-mir-27a	dre-mir-27	UUCACAGUGGCUAAGUUCCGCU	FQ245400	GSS	5'	0	22	85	50.59	25.40	0.59	
oni-mir-31	dre-mir-31	GGCAAGAUGUUGGCAUAGCUG	GS262704	GSS	5'	0	21	66	43.94	28.80	0.99	
oni-mir-34	dre-mir-34	UGGCAGUGUCUUAGCUGGUUGU	GR642687	EST	5'	0	22	98	47.96	38.60	0.82	Gill
oni-mir-133	dre-mir-133	UUUGGUCCCCUUCAACCAGCUG	GR659092	EST	5'	0	22	92	43.50	36.90	0.92	Kidney
oni-mir-144	dre-mir-144	UACAGUAUAGAUGAUGUACU	GR598832	EST	3'	0	20	73	35.60	29.00	1.12	Retina and retinal pigment epithelium
oni-mir-153b	dre-mir-153	UUGCAUAGUCACAAAAAUGAGC	FQ288338	GSS	3'	0	22	84	45.20	35.60	0.94	
oni-mir-182a	dre-mir-182	UUUGGCAAUGGUAGAACUCACA	GR603755	EST	5'	0	22	91	51.60	36.90	0.79	Retina and retinal pigment epithelium
oni-mir-182b	dre-mir-182	UUUGGCAAUGGUAGAACUCACA	GR599203	EST	5'	1	22	87	52.90	33.60	0.73	Retina and retinal pigment epithelium
oni-mir-199-3	dre-mir-199	CCCAGUGUUCAGACUACCUGUUC	GR620624	EST	5'	0	23	86	59.30	41.40	0.81	Embryo 0-4 days post-fertilization
oni-mir-193a-2	dre-mir-193	AACUGGCCUACAAAGUCCCAGU	FQ261999	GSS	5'	0	22	71	52.11	24.40	0.66	
oni-mir-199-1	dre-mir-199	CCCAGUGUUCAGACUACCUGUUC	FQ298175	GSS	5'	0	22	88	46.59	29.80	0.73	
oni-mir-204	dre-mir-204	UUCCCUUCGUCAUCCUAUGCCU	GR684267	EST	5'	1	22	81	53.09	28.90	0.67	Skin
oni-mir-222	dre-mir-222	AGCUACAUCUGGCUACUGGGUCUC	FQ295729	GSS	5'	0	24	79	49.37	23.30	0.60	
oni-mir-301c	dre-mir-301	CAGUGCAAUAGUAUUGUCAUAG	FQ303294	GSS	5'	0	22	79	40.51	31.10	0.97	
oni-mir-429	dre-mir-429	UAAUACUGUCUGGUAAUGCCGU	GR648701	EST	3'	0	22	80	38.75	25.90	0.84	Gill

NM = number of mismatch; LM = length of mature miRNAs; LP = length of precursor; ΔG = folding-free energies; MFEIs = minimal folding-free energy indexes; OE = organ of expression.

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tilapia mRNA database (Table 2). Target genes and miRNAs showed a high level of sequence complementarity. mir-1 was found to target the tropomyosin alpha-4 chain, stromal interaction molecule 1, and dolichol-phosphate mannosyltransferase. Tropomyosin alpha-4 chain plays a central role, in association with the troponin complex, in the calcium-dependent regulation of vertebrate striated muscle contraction (Crabos et al., 1991). mir-1 expression in the muscle modulates cardiac and skeletal muscle proliferation and differentiation in zebrafish (Mishima et al., 2009) and humans (Koutsoulidou et al., 2011). Moreover, Nile tilapia mir-1 expression has been observed in skeletal muscle (Huang et al., 2012). This suggests that mir-1 plays important roles in modulating muscle contraction in tilapia.

Table 2. Potential targets of the miRNAs in Nile tilapia by BLAST.

miRNA	Targeted protein	Target function	Target genes ID
oni-let-7b	relA-associated inhibitor-like	Regulation of apoptosis and transcription	XM_003459126
oni-mir-1	Tropomyosin alpha-4 chain-like	Muscle contraction	XM_003439846
	Stromal interaction molecule 1-like	Ion transport	XM_003455939
	Dolichol-phosphate mannosyltransferase-like	Metabolism	XM_003456960
oni-mir-153b	Apelin receptor B-like	Immunity response	XM_003444311
	Long-chain fatty acid transport protein 4-like	Fatty acid transport	XM_003444353
oni-mir-182	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	ATP binding	XM_003445257
oni-mir-429	E3 ubiquitin-protein ligase RNF180-like	Protein modification	XM_003440070

BLAST only identifies target mRNAs with perfect or near-perfect complementary. In order to identify target genes with partial complementary, we used the miRanda software to predict other target genes of tilapia miRNAs. miRanda is one of the earliest-developed large-scale target prediction algorithms for vertebrates and is often used to predict miRNA target genes in fish (Chen et al., 2005). Because the 3'-UTRs of many predicted mRNAs could not be identified, they were not used in the miRanda prediction. A total of 24 targets for the 15 tilapia miRNAs were predicted in the Nile tilapia mRNA sequence (Table 3). According to the information provided by NCBI, the identified miRNA targets were classified as transcription factors or molecules important in metabolism, signaling, transport, immunity, stress responses, reproduction related, growth and development, and osmoregulation. These groups, except for reproduction-related molecules, have previously been identified as targets of miRNAs by experimental and/or computational approaches in animals (Barozai, 2012; Zhu et al., 2012). In addition, an miRNA can be complementary to multiple targets; a target can be complementary to several miRNAs correspondingly according to the prediction results. For example, 6 sequences were detected as targets of let-7e, including the cytochrome P450 1C1 protein, the heat-shock protein 70, type I estrogen receptor, prolactin, 11-beta hydroxysteroid dehydrogenase type 2, and thrombospondin-1a. Additionally, oni-let-7a, oni-let-7e, and oni-mir-133 were all predicted to be regulatory miRNAs of the heat-shock protein 70 gene. These results suggest that an miRNA may have multiple different mRNA targets, and a target may similarly be targeted by multiple miRNAs in Nile tilapia. Similar findings were reported by many groups in different animal species (Rajewsky, 2006). Gaining insight into the Nile tilapia miRNA targets will increase the understanding of the range of miRNA expression regulation and more coherently describe the functional importance of miRNAs.

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Table 3. Potential targets of the miRNAs in Nile tilapia by miRanda program.

New miRNAs	Target gene ID	Gene name	Position	Function	Energy	Score
oni-let-7a	EU816596	Heat-shock protein 70	2080 to 2101	Stress responses	21.31	112
	AY725227	Liver hepcidin propeptide	430 to 454	Immunity	22.30	110
	FJ455498	Thrombospondin-1a	4173 to 4197	Stress responses	21.02	103
	EU827279	3beta-hydroxysteroid dehydrogenase type I	1883 to 1903	Metabolism	20.32	105
oni-let-7e	HQ829969	Cytochrome P450 1C1 protein	2135 to 2161	Metabolism	-21.15	113
	EU816596	Heat-shock protein 70	2080 to 2101	Stress responses	-21.00	118
	U75604	Type I estrogen receptor	3728 to 3749	Reproduction	-22.68	119
	M27010	Prolactin	870 to 893	Osmoregulation	-20.20	109
	AY190043	11-beta hydroxysteroid dehydrogenase type 2	2145 to 2172	Metabolism	-20.48	105
	FJ455498	Thrombospondin-1a	4173 to 4197	Stress responses	-21.14	108
oni-let-7i	GQ214535	Inducible cAMP early repressor	484 to 508	Transcription factor	-22.53	101
oni-mir-1-2	AF247822	Decorin	1959 to 1983	Fibril formation	-20.78	114
oni-mir-133	EU816596	Heat-shock protein 70	1853 to 1880	Stress responses	-23.06	102
	HQ829968	Cytochrome P450 1B1 protein	1918 to 1941	Metabolism	-22.09	100
	FJ455498	Thrombospondin-1a	4350 to 4371	Stress responses	-23.48	103
oni-mir-153b	U75605	Type II estrogen receptor	4066 to 4090	Reproduction	-21.28	100
oni-mir-199-3	AF503208	Glutamine synthetase	1676 to 1696	Metabolism	-23.95	108
oni-mir-199-1	AY725227	Liver hepcidin propeptide	397 to 421	Immunity	-21.57	107
	U75604	Type I estrogen receptor	4169 to 4190	Reproduction	-20.16	107
oni-mir-222	JN860432	Type I estrogen receptor	4404 to 4429	Reproduction	-23.56	115
	U75604	11-beta hydroxysteroid dehydrogenase type 2	1143 to 1165	Metabolism	-25.46	100
	AY190043	Orphan nuclear receptor Dax-1	1721 to 1745	Development	-27.37	123
oni-mir-22a-1	HQ829968	Cytochrome P450 1B1 protein	2024 to 2047	Metabolism	-20.09	101
	AY149606	DM-related transcriptional factor	1588 to 1606	Transcription factor	-24.08	102
oni-mir-34	AY428948	R-spondin-1 precursor	1270 to 1288	Sensory transduction	-20.58	104
	FJ411252	ATP-binding cassette subfamily G member 4 transporter protein	2563 to 2584	Transport	-21.26	100
oni-mir-27a	M26916	Growth-hormone	807 to 831	Growth and development	-21.27	102
oni-mir-301c	FJ914655	Glucose transporter type 1 4.5-kb transcript	4225 to 4251	Transport	-20.52	111
	AB182646	tilDmc1	1449 to 1470	DNA repair	-22.86	128
oni-mir-31	AY330215	MRE-binding transcription factor-1S	1367 to 1390	Transcription factor	-21.55	106
	AB075952	Elongation factor 1a	1463 to 1485	Transcription factor	-25.72	110
oni-mir-429	JN860434	Fibroblast growth factor 20b	1154 to 1177	Growth factor	-21.50	102
	FJ455498	Thrombospondin-1a	3879 to 3904	Stress responses	-20.94	103

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