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Comprehensive identification and profiling of Nile tilapia (*Oreochromis niloticus*) microRNAs response to *Streptococcus agalactiae* infection through high-throughput sequencing

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Abstract

MicroRNAs are a kind of small non-coding RNAs that participate in various biological processes. Deregulated microRNA expression is associated with several types of diseases. Tilapia (*Oreochromis niloticus*) is an important commercial fish species in China. To identify miRNAs and investigate immune-related miRNAs of *O. niloticus*, we applied high-throughput sequencing technology to identify and analyze miRNAs from tilapia infected with *Streptococcus agalactiae* at a timescale of 72 hours divided into six different time points. The results showed that a total of 3009 tilapia miRNAs were identified, including in 1121 miRNAs which have homologues in the currently available databases and 1878 novel miRNAs. The expression levels of 1121 tilapia miRNAs were significantly altered at 6 hour to 72 hour post-bacterial infection (pi), and these miRNAs were

therefore classified as differentially expressed tilapia miRNAs. For the 1121 differentially expressed tilapia miRNAs target 41961 genes. GO and KEGG enrichment analysis revealed that some target genes of tilapia miRNAs were grouped mainly into the categories of apoptotic process, signal pathway, and immune response. This is the first report of comprehensive identification of *O. niloticus* miRNAs being differentially regulated in spleen in normal conditions relating to *S. agalactiae* infection. This work provides an opportunity for further understanding of the molecular mechanisms of miRNA regulation in *O. niloticus* host-pathogen interactions.

Keywords: *Oreochromis niloticus*, microRNA, *Streptococcus agalactiae*, Deep sequencing, Immune response.

1. Introduction

MicroRNAs (miRNAs) are endogenous ~22 nucleotides (nt) RNAs that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression [1]. Although they escaped notice until relatively recently, miRNAs comprise one of the more abundant classes of gene regulatory molecules in development, responses to viral infection, immune and stress response in various organisms [2-6]. As the role of miRNA in all aspects of biology continues to be unraveled, the interplay between miRNAs and disease is becoming clearer. It should come of no surprise that microRNAs play a major part in the outcome of infectious diseases, since early work has implicated miRNAs as regulators of the immune response [7]. Evidence derived from profiling and functional experiments suggests that regulation of specific miRNAs during infection can either enhance the immune response or facilitate pathogen immune evasion [8]. Consequently, investigations on miRNAs of immune relevance are indispensable for understanding the host-pathogen interactions, and it remain to be seen an opportunity for therapeutic intervention for difficult-to-treat diseases [9, 10].

MicroRNAs have since been identified in all plants and animals, and are also encoded by some bacteria and viruses. In recent years, an increasing number of miRNAs have been identified and deposited in the major miRNA databases. As of 2014, over 24 thousand miRNAs have been discovered across 206 species and more miRNAs are getting cloned or in *silico* identified [7, 11]. Although hundreds ofmiRNAs have been identified, only a very small number of lower vertebrates (such as teleost) miRNAs have been discovered and functionally identified. People have been used a

classic method to cloned and identified miRNAs directly from zebrafish and rainbow[12, 13]. However, because of all miRNAs have similar secondary hairpin structures and many of these structures are evolutionarily conserved, it is difficult to clone it from low-abundance miRNAs [14]. Recent advances in next-generation sequencing have revolutionized the identification of small RNAs by profiling methods with particularly high levels of sensitivity and accuracy. The high-throughput strategies has enabled the discovery of many small RNAs (such as miRNAs, endogenous siRNAs and piwi-interacting RNA), including those with low abundance or tissue/species-specific expression patterns [15]. The SOLiD^{TM} system has emerged as a powerful tool for the large-scale identification of miRNAs and the analysis of their expression especially for those species with limited genomic information [5, 16]. These species include shrimp [17], zebrafish [18], medaka [19], Atlantic halibut [20], common crap [21], catfish [22], Japanese flounder [5], grouper [6] and Nile tilapia [14].

The identification and characterization of miRNAs involved in the immune response are now essential for the elucidation of host-pathogen interactions and immune defense and disease control mechanisms [23]. Accumulating reports have indicated that bacteria can produce miRNAs to manipulate host gene expression. On the other hand, the results are consistent with the findings of previous studies demonstrating the importance of miRNAs' effects on immune-related gene expressions in protecting against pathogens. But most information on miRNAs associated with bacterial infection comes from studies of mammalian systems. In lower vertebrates, systematic investigations on the role of miRNAs in interactions between teleost and gram positive bacteria have not yet been documented.

Nile tilapia (*Oreochromis niloticus*) is one of the most important economical fishes and widely cultured throughout the world [24]. In recent years, infectious disease caused by *Streptococcus agalactiae* has been severe, resulting in great economic loss and becoming a big obstacle to tilapia aquaculture [25]. Studies related to streptococcus virulence factors, vaccines, host immune relevant genes and investigate transcriptomic changes of *S. agalactiae*-infected tilapia have been reported [26-30]. These results showed that many immune-related genes in tilapia were up-regulated significantly after *S. agalactiae* infection [30]. MicroRNAs affect gene expression via complementary base pairing with target messenger RNAs (mRNAs). Due to their small size, multiple miRNAs can simultaneously bind to a single target mRNA, while each miRNA can potentially bind to multiple different mRNAs [31]. Thus, mRNA-miRNA interactions have an enormous impact on

the regulation of gene expression and ultimately the function of any cell. It is clear that the development and function of the immune system are highly dependent on miRNA-mediated gene regulation [32]. Considering the important role of miRNAs in mediated gene regulation, a comprehensive characterization of host miRNAs related to *S. agalactiae* is indispensable for a deep understanding of the host-bacteria interactions.

In this study, we using Nile tilapia (*Oreochromisniloticus*) as a host model, we examined bacteria and host miRNAs associated with streptococci infection by the approach of high-throughput sequencing. Meanwhile, to facilitate understanding of the functional attributes of the miRNAs, the target genes of tilapia miRNAs were predicted by in silico analysis. These data will help to uncover the complexity of immune regulatory networks mediated by tilapia miRNAs during *S. agalactiae* infection.

2. Materials and methods

2.1. Animal ethics

Experiments involving live animals were conducted in accordance with the "Regulations for the Administration of Affairs Concerning Experimental Animals" promulgated by the State Science and Technology Commission of Guangdong Province. The study was approved by the Ethics Committee of Guangdong Ocean University.

2.2. Fish

Clinically healthy juvenile tilapia (average weight of 100±10 g) were purchased from Guangdong tilapia breeding farm, China and maintained at ~28℃ in freshwater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. All tanks were supplied with flow-through aerated sand-filtered water, and a light and dark period of 12 h: 12 h was maintained. Before experiment, fish were randomly sampled for the examination of bacteria in spleen. No bacterium was detected from the examined fish. Before tissue collection, fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) [33].

2.3. Experimental infection

S. agalactiae ZQ0910, a virulent strain isolated from tilapia was used for immunostimulus [25]. The immunostimulation experiment was performed by injecting the tilapia with 0.1 ml of bacteria

esuspended in sterilized PBS with the concentration of 1×10^7 cells ml^{-1} into the abdominal cavity and the tilapia injected with 0.1 ml of sterilized phosphate buffered saline (PBS) were used as the control group. Then all processed tilapia were divided randomly into two groups and treated as before. At time points of 0 h, 6 h, 12 h, 24 h, 48 h and 72 h post-immunization (pi), fish (three at each time point) were euthanized, and spleen was collected under aseptic conditions and immediately stored in liquid nitrogen for later use. At each time point the spleen tissues of three fish were pooled together and used for subsequent small RNA sequencing.

2.4. Library construction, Solexa deep sequencing of small RNA

Small RNA isolation, library construction, and high-throughput sequencing were all carried out by Genedenovo Biothchnology Co., Ltd. (Guangzhou, China). Briefly, total RNA extraction using TRIzol reagent (life Technologies, USA) and low molecular weight RNA was purified using a DGE-Small RNA Sample Preparation Kit (Illumina, San Diego, USA) according to the manufacturer's instructions. The quantity and purity of the RNA were monitored using a NanoDrop™1000 spectrophotometer (Thermo Fisher Scientific, WI, USA) at $A_{260}/A_{280}>2.0$. After electrophoresing on 15% polyacrylamide-8 M urea gel, the small RNAs with about 30 nt were extracted, and a pair of Solexa pro-prietary adaptors as PCR primer was ligated to both 5' and 3' ends of the RNAs. After reverse transcription reaction from the RNAs, the resulting cDNA was amplified to produce sequencing libraries. The cDNA library was sequenced using the Illumina Genome Analyzer (Illumina, USA).

2.5. Analysis of sequencing reads

Initial reads obtained from Solexa sequencing were processed by removing poor quality reads, 5' adapter pollution reads, reads without 3' adapter, reads without insert fragment, reads containing poly (A) stretches, and reads less than 18nt. The clean reads were blasted against the Rfam database (http://www.sanger.ac.uk/software/Rfam) and the GenBank noncoding RNA database (http://www.blast.nvbi.nlm.nih.gov/) to annotate rRNA, tRNA, snRNA and snoRNA. The other small RNAs were mapped to the *Oreochromis niloticus* genome to perform distribution analysis and miRNA prediction using SOAP (http://www.soap.genomics.org.cn). miRNA identification was performed by comparing the sequenced small RNAs with the know microRNAs of *Oreochromis niloticus* or other animal species in miRBase v20.0. The miRNA precursors with a stem-loop structure were predicted by homologous comparison miRNA sequences to ESTs.

149 ***2.6. Real-time quantification of miRNAs by stem-loop RT-PCR***

150 Total RNAs were isolated using mirVana^{TM} miRNA Isolation Kit (Ambion, USA) according to the
151 manufacture's instruction and quantified using NanoDrop^{TM} 1000 spectrophotometer (Thermo Fisher
152 Scientific, USA).Then miRNAs were subjected to reverse transcription by specific stem-loop RT
153 primers supplied in TaqMan microRNA Assays kit (Applied Biosystems, USA). Briefly, special
154 reverse transcriptase (RT) primers with a stem-loop sequence for detection of each miRNA were
155 designed by Applied Biosystems (USA). Then 20 µl RT reaction was prepared, which consisted of 4
156 µl 5×Reverse Transcription Buffer, 3 µl 5×RT primer, 2 µl dNTPs (10mM), 1 µl RNase inhibitor (20
157 U/µl), 1 µl MultiScribe™ Reverse Transcriptase (50 U/µl), and 500 ng total RNA. Hereafter,
158 reactions were conducted in Bio-Rad Thermocycler (Bio-Rad, USA) with a program as follows,
159 16 ℃ for 30min, 42 ℃ for 30min, 85℃ for 5 min. Finally, the products of each reaction were
160 diluted 5-fold and used as template for stem-loop RT-PCR. Quantitative real-time PCR was carried
161 out in an Applied Biosystems 7500 (Applied Biosystems, Life Technologies, USA) in a 20 µl PCR
162 reaction volume containing 1 µl cDNA, 10 µl SYBR® Select Master Mix (Applied Biosystems, Life
163 Technologies, USA), 0.2 µl specific forward primer (20 µM), 0.2 µl reverse primer (20 µM) and 8.6
164 µl water. The reaction was performed at 95 ℃ for 5 min, followed by 45 cycles of 94 ℃ for 15 s,
165 65℃ for 15 s, and 72 ℃ for 15 s. U6 gene was used as reference gene to normalize all
166 experimental data. All reactions were performed in triplicate. The threshold cycle (Ct) was
167 determined using the default threshold settings, and the data were analysed using $2^{-\Delta\Delta Ct}$ program. The
168 experiment was performed independently three times.

169 ***2.7. Prediction and analysis of the target genes of miRNAs***

170 The putative 3'-untranslated regions (UTRs) of tilapia mRNAs were used for the prediction of
171 microRNA target genes with TargetScan 6.2 (http://www.targetscan.org/) [34] and miRanda
172 (http://www.microrna.org/) [35]. TargetScan was used to search for microRNA seed matches
173 (nucleotides 2-8 from the 5' end of microRNA) in 3'-UTR sequences. miRanda was used to match
174 the entire microRNA sequences. The parameters of TargetScan and microRnada were set as
175 score >50 and free energy <-20 kcal/mol respectively. The results predicted by the two algorithms
176 were combined, and the overlaps were calculated.

177 ***2.8. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.***

178 Enrichment analysis of the predicted target genes was conducted with Gene Ontology (GO)

(http://www.geneontology.org/) and KEGG pathway (http://www.genome.jp/kegg/). A heatmap chart was drawn by transforming the normalized data clustering was performed using the gplots heatmap.2 of R program.

3. Results

3.1. Overview of high-throughput sequencing data

In order to identify the miRNAs involved in bacterial infection and host immune response, tilapia were infected with *S. agalactiae* for 0 h, 6 h, 12 h, 24 h, 48 h and 72 h. Small-fragment RNA libraries representing the six time points were constructed and subjected to sequence analysis. A total of 91,469,729 raw sequences were obtained. After filtering the low-quality tags and removing adapter sequences, polyA sequences, and sequences <18 nt, 14,370,394 (98.8009% of the raw reads), 13,505,677 (98.6739% of the raw reads), 13,633,569 (98.3198% of the raw reads), 15,227,375 (98.5925% of the raw reads), 16,045,799 (98.6258% of the raw reads) and 15,150,558 (98.8467% of the raw reads) clean reads were obtained for IO-0h, IO-6h, IO-12h, IO-24h, IO-48h and IO-72h , respectively (Table 1). Around 9.3983%, 15.6529%, 11.8473%, 13.4026%, 10.242% and 13.9871% of reads matched miRNAs for IO-0h, IO-6h, IO-12h, IO-24h, IO-48h and IO-72h, respectively. 28.1289%, 29.3716%, 29.238%, 28.8337%, 30.3859% and 29.3524% matched noncoding sRNAs, (rRNA, tRNA, snRNA, snoRNA) and 62.4728%, 54.9755%, 58.9147%,57.7637%, 59.3721% and 56.6605% matched other sRNAsfor IO-0h, IO-6h, IO-12h, IO-24h, IO-48h and IO-72h, respectively (Table 2).

As small RNA with known functions are typically 20-24 nt long [36], we analyzed the length distribution of the unique sRNA sequences after removing the other RNA types. Fig. 1 shows that the majority of the unique sRNAs from the six libraries ranged from 20 to 24. The peak distribution was for sequences that were 22-nt long, followed by those that were 21, 23, 20 and 24 nt in length, which was consistent with the typical sizes of dicer processing products.

3.2. Phylogenetic conservation analysis of grouper miRNAs

To examine whether the miRNAs identified in tilapia were evolutionarily conserved across species, a key feature of miRNAs, we compared 1121 tilapia homologs of tilapia miRNAs with those from zebrafish (*Danio rerio*), pufferfish (*Takifugu rubripes*), human (*Homo sapiens*), mouse (*Mus*

musculus) (data from miRBase 20.0), and 1878 novel miRNAs have no homologues in the currently available databases, Taken together, a total of 3009 tilapia miRNAs were identified. We found 97, 72, 401 and 325 out of 1121 tilapia miRNAs have high confidence miRNA orthologs in zebrafish, pufferfish, human and mouse, respectively (Supplementary File S1). In detail, the number of tilapia-specific miRNAs and miRNAs were exclusively identified in tilapia and two kinds of fish (zebrafish and pufferfish) were 517 and 27, respectively (Fig. 2A and 2B, Supplementary File S1). While 459 miRNAs were detected in human and mouse, 72 miRNAs appeared in all five vertebrates (Fig. 2C and Fig 2D, Supplementary File S1). In additional, we compared the sequence of 72 ubiquitous miRNAs to determine their phylogenetic relationships among these five species. The results showed that 47 tilapia miRNAs shared identical sequences to pufferfish and zabrafish miRNAs, suggesting there is a closer evolutionary relationship between fish miRNAs (Fig 3E, 3F and 3H, Supplementary File S1). Moreover, 36 out of 72 miRNAs showed 100% sequence identity in all five vertebrates, and the remaining 11 and 25 miRNAs displayed 1-3 base difference compared with their homologs in three fish and in all five vertebrates, respectively (Fig 3H and 3G, Supplementary File S1). There is one noteworthy, most of the nucleotide differences occurred at the 3' end of the miRNAs, which might not affect the function of the mature miRNAs. Striking nucleotide differences that occurred in the middle regions or at the 5' end seed sequences are only observed in 2 miRNAs (mir-455 and mir-489), which account for 2.8% of the 72 miRNAs (Fig 3H, Supplementary File S1).

3.3. Differentially expressed host miRNAs induced by bacterial infection

To identify host miRNAs involved in bacterial infection, the expression profiles of the 1121 tilapia miRNAs were examined at 0h pi, 6h pi, 12h pi, 24h pi, 48h pi and 72h pi, and the amounts of miRNA were normalized with Bonferroni correction. The results showed that 1121 miRNAs expressed at all examined time points, while 69, 64, 123, 119, 98 and 68 miRNAs expressed specifically at 0h pi, 6h pi,12h pi, 24h pi, 48h pi and 72h pi, respectively (Fig. 4 , Supplementary File S2). Compared to the expression levels at 0h pi, the expression levels of 111 miRNAs at 6h pi to 72h pi were significantly (p<0.05) changed. Specifically, 17 miRNAs (including mir-17, mir-2187,mir-730,mir-7550 and mir-214) were significantly upregulated at 6h pi, 30 miRNAs (including mir-122, mir-192, mir-215, mir-5119 and mir-9100) were significantly downregulated at 6h pi, 12 miRNAs (including mir-2944, mir-17, mir-6 and mir-1711) were significantly upregulated

at 12h pi, 15miRNAs (including mir-9100, mir-187, mir-5119, mir-451 and mir-202) were significantly downregulated at 12h pi, 17 miRNAs (including mir-9100, mir-5119 and mir-1246) were significantly upregulated at 24h pi, 6 miRNAs (including mir-9100, mir-5199 and mir-1246) were significantly downregulated at 24h pi, 30 miRNAs (including mir-2187, mir-214, mir-730 and mir-7911) were significantly upregulated at 48h pi, 48 miRNAs (including mir-122, mir-192, mir-194, mir-215 and mir-5119) were significantly downregulated at 48h pi, 17 miRNAs (including mir-1711, mir-155 mir-202, mir-2187, mir-3967 and mir-6370) were significantly upregulated at 72h pi, 26 miRNAs (including mir-8159, mir-9100, mir-192, mir-194 and mir-217) were significantly downregulated at 72h pi (Fig. 5). To validate the expression patterns of the miRNAs, four miRNAs with significant expression changes were selected for stem-loop RT-PCR analysis. The results showed that all differentially expressed miRNAs were grouped together by κ-means clustering. After infecting tilapia with *S. agalactiae*, there was a clear time-dependent expression pattern of miR-17, 29, 155, 214 in the spleen, with quite different kinetic expressions. The expression level of miR-17 was up-regulated immediately and reached its peak at 6 h after infection, but miR-29 was down-regulated in the time of 0-12 h post-infection. The expression of miR-155and miR-214 in the spleen was down-regulated in the time of 0-12 h. After immunization 48 h and 72 h later**,** the expression of miR-155 and miR-214 was significantly up-regulated in the spleen (Fig. 6). For those differentially expressed miRNAs, which involved in immune response, in order to observe their expressions along with the infection process, a het map was drawn, and clustering analysis was conducted based on similar expression patterns (Fig. 7). The results showed that these differentially expressed miRNAs were grouped together by *κ*-means clustering. As the infection progressed, the expressions of the miRNAs exhibited dynamic changes and formed various patterns, including sustained upregulation/downregulation followed by downregulation/upregulation, and diphasic expression patterns.

3.4. Prediction of the target genes of tilapia miRNAs

Potential targets of the 1211 tilapia miRNAs were predicted using TargetScan[34], miRanda[35], and PicTar[37], three most frequently used prediction algorithms. Finally, a total of 41961 target genes were predicted by all three prediction programs. The 41961 putative target genes cover a wide range of functions, notably those related to immunity. Immune relevant genes include CD4 targeted by mir-1890, mir-190, mir-2584, mir-2840, mir-361, mir-372 and mir-4126, CD8 targeted by mir-106,

mir-1338, mir-137, mir-1684, mir-17, mir-20 and so on, interleukin (IL)-8 targeted by mir-1175, mir-1497, mir-2810, mir-3559, mir-6903, MHC class II targeted by mir-1271, mir-141, mir-15, mir-1582 and so on, Myeloid differentiation factor 88 targeted by mir-1175, mir-1249, mir-1252, mir-1260 and so on, NOD-like receptor C targeted by mir-1, mir-1271, mir-1331, mir-1497 and so on, TCR targeted by mir-124, mir-1277, mir-138, mir-139 and so on, complement component C1q targeted by mir-12, mir-13, mir-1338, mir-144 and so on.

3.5. Enrichment analysis of the putative target genes of tilapia miRNAs

To get an overview of the pathways in which host miRNAs were involved, the putative target genes of the differentially expressed host miRNAs were subjected to GO analysis and KEGG pathway analysis. GO enrichment analysis based on biological process showed that the 41961 predicted target genes of tilapia were clustered into 125 GO terms. The top ten enriched GO terms are associated with cellular process, single-organism process, metabolic process, biological regulation, regulation of biological process, response to stimulus, multicellular organismal process, developmental process, localization and signaling (Fig. 8). To examine the effect of the miRNAs in more detail, another GO analysis was conducted, in which the 1121 differentially expressed host miRNAs were grouped into upregulated and downregulated categories at each infection time point, and then GO analysis was performed on the target genes of the miRNAs in each category. It is showed that the numbers of target genes in all pathways changed at different time points (Fig 9). For example, at 6 h pi, the genes belonging to the GO terms of immune system process, response to stimulus, signaling, locomotion, cellular component organization or biogenesis and metabolic process were targeted by both upregulated and downregulated miRNAs; at 12 h pi, cellular component organization or biogenesis, biological regulation, biological phase and single-organism process were preferably targeted by downregulated miRNAs; at 24 h pi, relatively few targets genes were enriched into GO terms; at 48 h pi, genes involved in biological regulation, cell process, biological phase, multi-organism process, single-organism process and immune system process were the highest in number among the genes targeted by downregulated miRNAs; at 72 h pi, 579, 693 and 9 genes targeted by downregulation miRNAs were enriched into the processes of immune system process, locomotion and cell killing respectively, and 484, 560 and 15 genes targeted by upregulated miRNAs were enriched into the processes of immune system process, locomotion and cell killing respectively.

Similar to GO analysis, KEGG pathway analysis showed that the putative target genes predicted in

tilapia were grouped into 240 pathways. The top ten enriched pathways were involved in phosphatidylinositol signaling system, TGF-beta signaling pathway, spliceosome, Ubiquitin mediated proteolysis, glutamatergic synapse, neurotrophin signaling, axon guidance, focal adhesion and MAPK signaling pathway (Fig 10).

3.5. Network of putative interactions between miR-17, miR-29, miR-155, miR-214 and target genes in tilapia

GO analysis showed that the targeted genes of miR-17, miR-29, miR-155 and miR-214 are related to signal transduction and immune response. The immune relevant genes include B-cell linker protein, T-cell surface glycoprotein, interleukin, NOD-like receptor signaling, TLR4, complement C1q, and tumor necrosis factor-related protein (Supplementary File S3). The target genes were all clustered into the immunity GO term. Ten, one, two and two target genes had shared by mir-17 and mir-29, mir-17 and mir-155, mir-29 and mir-155, mir-214 and mir-155, respectively (Fig 11, Supplementary File S3).

4. Discussion

In the present study, we used a high-throughput Solexa sequencing approach to identify and investigate the expression patterns of host miRNAs in a teleost fish, *O. niloticus*, infected with *S. agalactiae*at different time points. We detected 3009 tilapia miRNAs, of which 1878 were discovered for the first time. These novel miRNAs add to the know miRNAs pools of fish. Previous studies showed that miRNAs commonly affected by various bacterial infections and involved in the control of immune response [38]. A role of miRNAs in bacterial infections was first discovered in plants where *Arabidopsis* miR-393 contributed to resistance against the extracellular pathogen *Pseudomonas syringae*, presumably by repressing auxin signaling [39]. In mammalian miRNAs were implicated in bacterial infections because of association with immunity and inflammation [40, 41]. With the gram-positive bacterial (*Streptococcus uberis*) infection, miRNAs may significantly regulate the sentinel capacity of mammary epithelial cells to mobilise the innate immune system [42]. Similar studies in lower vertebrates, *Vibrio anguillarum* infection changed a large amount of miRNAs in the *Cynoglossus semilaevis* of immune tissues [43]. Whereas *V. anguillarum* remain extracellular during infection, many bacterial pathogens actively invade host cells or become intracellular after ingestion by phagocytic immune cells such as macrophages. During their

multistage infection, intracellular pathogens extensively manipulate the signaling and gene expression cascades of the host for survival and replication [44]. *S. agalactiae* is an intensely investigated intracellular bacterial pathogen that is a major neonatal pathogen and causes severe disease in tilapia [45, 46]. Whether and how tilapia miRNAs are regulated by invasive and intracellular bacteria remained unknown. In this study, we identified 1121 tilapia miRNAs with sequence identical to that found in whole genome sequence of tilapia. Their miRNAs displayed significantly different expressions during bacterial infection, suggesting that *S. agalactiae* altered, on a large scale, the regulation of miRNA expression in tilapia. Besides, we also obtained 517 tilapia specific miRNAs via a computational pipeline, and these candidate miRNAs need further experimental confirmation.

Evolutionary conservation is one key feature of miRNAs and accumulating evidence reveals that miRNAs might be excellent phylogenetic markers [47]. Here, we showed that 1121 know tilapia miRNAs were identical in sequence to that of tilapia miRNAs with those from zebrafish, pufferfish, human and mouse. Among these, 72 miRNAs were also found to have homologs in zebrafish, pufferfish, human and mouse. Furthermore, 36 out of 72 miRNAs had 100% the same sequence with their homologs in all five species, and 25 miRNAs displayed 1-3 nt differences at the 3' end. It is suggested that a total of 61 tilapia miRNAs display identical sequences to their homologs of other four species, accounting for 85% of the miRNAs appeared in all tested species. These data indicated that miRNAs in tilapia are phylogenetically conserved with other identified fish miRNAs, which is the same as the previous research that even distantly related species shared a large number of homologous miRNAs. Some miRNAs including mir-146, mir-155, mir-125, mir-21 and let-7, are commonly affected during bacterial infection and contribute to immune responses protecting the organism against overwhelmed inflammation. For example, mir-125 is downregulated in response to LPS or bacterial pathogens and one target of mir-125 is TNF-α. In macrophages, an additional function of mir-125 may be to ensure that the TLR pathway is turned off in the absence of microbial infection, while its downregulation in response to TLR triggering may be needed for the inflammatory response [48]. In this study, we also identified mir-125 in tilapia, which shared 100% sequence with other three kinds of fish. In additional, the let-7 miRNA family is highly conserved across evolution in both sequence and function, and associated with cell differentiation and development [48]. It is also appears to be an actor of the acute innate immune response. Let-7 is

downregulated in response to *Salmonella enterica* infection. Although this pathogen affects miRNA expression in a cell type-dependent manner, it generally downregulates let-7 in both phagocytic murine macrophages and non-phagocytic Hela cells [49]. Here, we identified let-7 family members in tilapia, while it is perfectly matched with other species, it is showed that let-7 in tilapia may be have the same function with other species, especially response to the intracellular pathogens.

MicroRNAs, similar to mRNA, are expressed in a time- and tissue-specific manner, and are involved in many fundamental biological processes. So analysis of the expression profiles of tilapia miRNAs on a timescale of 72 hours revealed that dynamic changes were associated with the course of *S. agalactiae* infection, and that the expression levels of individual miRNAs varied at different time points. In our study, 1121 differentially expressed host miRNAs identified, and total 41961 target genes were predicted in tilapia. GO and KEGG pathway analysis showed that the predicted target genes are involved in diverse biological processes ranging from fundamental cellular operations to stress response. It is show that miRNAs playpivotal roles in the host system. In order to protect against intruding pathogens, organ-specific and systemic immunological host responses are both activated by the pathogen-associated molecular-pattern pathway via membrane-associated Toll-like receptors (TLR) and cytoplasmic Nod-like receptors [44]. It is worth noting that the majority of the differentially expressed host miRNAs were downregulated at early infection time (6h pi and 12h pi) and at later time (48h pi and 72h pi). All the phenomena show that the genes associated with many crucial pathogen-associated molecular-pattern pathways are probably upregulated in response to bacterial infection cause by the function of miRNAs to suppress gene expression. Among the downregulated genes at early infection time, mir-129 can trigger apoptosis by suppressing a key anti-apoptotic protein, B-cell lymphoma 2 (BCL2), and ectopic expression of miR-129 promoted apoptosis, inhibited cell proliferation and caused cell-cycle arrest in human cells [50]. In the process of bacterial infection, mature B cells play an important role in anti-inflammation. Of these downregulated miRNAs at early infection time, there is a miRNA called miR-150, is mainly expressed in the lymph nodes and spleen and is highly up-regulated during the development of mature T and B cells, overexpression of miR-150 in hematopoietic stem cells, had little effect on the formation of either mature CD8- and CD4-positive T cells or granulocytes or macrophages, but the formation of mature B cells was greatly impaired. It is indicate that downregulated miR-150 most likely upregulates mRNAs that are important for pre- and pro-B cell formation or function.

Consequently, for bacterial in infection, it is necessary to promote the B cell maturation process via downregulated miR-150 so to cleared antigens [51]. The observation in our study at early infection time of both anti-apoptosis and B cell maturation targets of miRNAs suggests that some of the tilapia miRNAs operated to promote host defense, while others may serve for bacterial infection. Aside from the differentially expressed host miRNAs were downregulated at early infection time, our study also discovered the host miRNAs were downregulated at later infection time. Among of them, it was recently reported that the levels of miR-125 is downregulated in response to LPS or bacterial pathogens [52]. In macrophages, a function of miR-125 may be to ensure that the TLR pathway is turned off in the absence of microbial infection, while its downregulation in response to TLR triggering may be needed for the inflammatory response. One target of miR-125 is TNF-α, a recently study by Rajaram *et al*. reported that the avirulent *Mycobacterium smegmatis* induces high miR-155 expression and low miR-155 expression with correspondingly low TNF production [48]. The ubiquitously expressed miR-21 has been well established as an oncogenic miRNA due to its aberrant overexpression in numerous cancers [53]. Indeed, miR-21 upregulation was reported in gastric mucosa, while treatment of human peripheral blood mononuclear cells with LPS resulted in lower PDCD4 expression, which was due to induction of the miR-21 via the adaptor MyD88 and NF-κB. Both of these processes converge to downregulated the inflammatory response [54].

Another striking feature observed with the differentially expressed tilapia miRNAs in our study is that at 24 h pi, the numbers of upregulated miRNAs increased compared to those at 6 h pi to 12 h pi. Among of these miRNAs, miR-223 is specifically expressed in neutrophils that infiltrate the infected mucosa, and possibly targets TLR4 and TLR3. When the miRNAs affected by *Helicobacter pylori* infection in human gastric mucosa, Matsushima *et al*. found that only miR-223 is significantly upregulated as compared with healthy mucosa [55]. The progression of *Actinobacillus pleuropneumonia* infection in pigs is also accompanied by a gradual increase in miR-223 expression [56]. miR-146 is found to be coordinately upregulated in immune cells in response to various bacterial pathogens including *Helicobacter pylori* [57-61], *Salmonella enterica* [49], *Mycobacterium species* [48, 62], or *Francisellatularensis* [63]. miR-146, along with miR-155 and miR-132, was initially identified in monocytic cell line challenged with the TLR4 ligand lipopolysaccharide (LPS) [64]. The current model of miR-146 function proposes a negative feedback mechanism regulating TLR signaling in response to bacterial products, thus downregulating cellular LPS sensitivity and

418 preventing excessive inflammation. Therefore the alterations in the fine-tuning of innate immune
419 responses by miRNAs may contribute to inflammatory disorders [65].

420 In our study, the tilapia miRNAs revealed that there was obvious differential expression for a
421 number of the above immune-related miRNAs. Further, qPCR confirmed differential expression
422 among the six libraries in some of the miRNAs, such as miR-17, miR-29, miR-155 and miR-214.
423 Interestingly, after infecting tilapia with *S. agalactiae*, there was a clear time-dependent expression
424 pattern of these miRNAs in the spleen, with quite different kinetic expressions. This finding could be
425 possibly associated with infection with *S. agalactiae*, which is a major cause of acute septicemia with
426 high mortality in fish. The spleen is one of the main target organs attacked by *S. agalactiae* [66, 67],
427 and it has been shown that *S. agalactiae* can survive inside macrophage, which may induce innate
428 immune response of fish to eliminate pathogenatearly stage of infection [68-70]. Interferon-γ (IFN-γ),
429 one of canonical Th1 cytokines, is critical for innate immunity against intracellular bacterial
430 infections [71], and accumulated data showed that the main functional properties ofmammal IFN-γ
431 may be conserved in teleost [72]. In mammals, intracellular bacterial infection can lead to
432 downregulation of miR-29 expression, thus removing inhibitory effects of miR-29 on IFN-γ
433 production in host [73]. In addition, miR-17was up-regulated after IFN-γ treatment in green-spotted
434 puffer fish (*Tetraodon nigroviridis*), suggesting it may play important roles in IFN-γ-mediated
435 immune response [74]. Therefore, in the spleen of infected tilapia, the down-regulated expression of
436 miR-29and up-regulated expression of miR-17 at early stage of infection may protect fish against *S.*
437 *agalactiae* intracellular infection by promoting IFN-γ-mediated immune response. In
438 mammals,miRNAs are known to be involved in lymphocyte developmentand function. In
439 miR-155-deficient mice, the weak production of interleukin-2 (IL-2), IL-4 and IFN-γby activated
440 T-cells that is observed in response to immunization, which indicated miR-155 plays a crucial role in
441 the T-cell immune response[75, 76]. miR-214 can promote T-cell activation by targeting the negative
442 regulator Pten, and up-regulation of miR-214 is dependent on CD28 costimulation [77]. miR-155 and
443 miR-214 of tilapia may possess similar function to activate T-cells, which was supporting by distinct
444 kinetic expressions of miR-214 and miR-155 in the spleen. In teleosts, the spleen functions as a
445 major secondary lymphoid organ，and acts in the antigen presentation and the initiation of
446 the adaptive immune response [78, 79]. The expression of miR-155 and miR-214 in the spleen was
447 down-regulated in the time of 0-12 h, implying a possible suppression of T-cell activation pathway in

the early period of bacterial infection [46, 80, 81]. After immunization 48 h and 72 h later**,** the expression of miR-155 and miR-214 was significantly up-regulated in the spleen, which indicated that immunization can improve the expression of miR-155 and miR-214 in the spleen and may enhance the ability of fish defending against *S. agalactiae* intracellular infection via promoting T-cell immunity.

It is currently estimated that a single miRNA can target multiple mRNA species and that a given mRNA is often targeted by multiple miRNAs, thus leading to a cell and context-specific network of miRNA: mRNA interactions [38].Transcription factors and miRNAs are both integral components of signaling pathways that maintain homeostasis and control cell function in response to external stimuli. In these pathways, miRNAs are key mediators of various modes of feedback and fee-forward regulation loops: specific transcription factors or RNA-binding proteins regulated the biogenesis of miRNAs, which in turn suppress the expression of their regulators and thus contribute to cell fate [82, 83]. In our study, we make a network drawing interactions between miR-17, miR-29, miR-155, miR-214 and the targets genes in tilapia. All of these miRNAs might participate in Natural killer cell mediated cytotoxicity, T/B cell receptor signaling pathway, NOD-like receptor signaling pathway, Toll-like receptor signaling pathway and Cytokine-cytokine receptor interaction (Fig. 11). Combined with the upregulated/downregulated expression level of miR-17, miR-29, miR-155, miR-214 during *S. agalactiae* infection (Fig. 7), we conclude that these miRNAs might play an important role in regulating tilapia immunity. In additionally, targeting of multiple genes by one miRNA, multiple tilapia miRNAs also demonstrate the ability to regulated the same target gene. For instance, miR-155 and miR-17 target some interleukin genes, which mediate the Cytokine-cytokine receptor interaction and initiate adaptive immune responses (Supplementary File S3). Furthermore, synergistic regulations among multiple miRNAs are important to understand the mechanisms of complex post-transcriptional regulations in humans. Complex diseases are affected by several miRNAs rather than a single miRNA. So, it is to be of great significance to identify miRNA synergism and thereby further determine miRNA functions at a system-wide level and investigate disease miRNA features in the miRNA-miRNA synergistic network from a new view [84]. Here, we suggested that these miRNAs share some target genes, although limitations exist in the current data, the results uncovered here are important for understanding the key roles of miRNAs in diseases, the results present here provide a new insight into the global topological properties of disease miRNAs in the comprehensive

miRNA synergistic network.

In conclusion, 1121 conserved and 517 tilapia specific miRNAs were identified in tilapia spleen tissue. The 1121 miRNAs has differently expressed at host miRNAs exhibited dynamic changes in expression during the course of bacterial infection. The targets of host miRNAs were grouped into a wide range of functional categories, in particular those associated with immune defense/evasion and T/B cell single pathways. These results suggest that in teleost, as in higher vertebrates, miRNAs prominently contribute to immune responses, protecting the organism against overwhelmed inflammation upon infection. In addition, the large amount of novel miRNAs as well as their putative target genes identified in this study will contribute to physiological or pathological situations, and will open the field to future avenues of investigation.

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Table 1 Quality of sequencing data

Sample	Raw reads	High quality	3'adapter null	Insert null	5'adapter contaminants	Smaller than 18nt	polyA	Clean reads
IO-0h	14,875,335	14,544,807	19,917	58,590	7,374	87,523	1,009	14,370,394
IO-6h	14,043,678	13,687,180	46,372	47,521	6,575	80,263	772	13,505,677
IO-12h	14,240,612	13,866,557	21,854	80,615	6,487	123,367	665	13,633,569
IO-24h	15,855,541	15,444,757	23,765	75,679	8,307	108,689	942	15,227,375
IO-48h	16,719,506	16,269,375	26,039	54,662	9,524	132,126	1,225	16,045,799
IO-72h	15,735,057	15,327,323	25,180	57,876	6,192	86,952	565	15,150,558
Total	91,469,729	89,149,999	335,270	374,943	44,459	618,920	5,178	87,933,372

Table 2 Categorization of tilapia noncoding and organellar small RNAs

Class of sRNA	IO-1h		IO-6h		IO-12h		IO-24h		IO-48h		IO-72h	
	Number of reads	Percentage (%)	Number of reads	Percentage (%)	Number of reads	Percentage (%)	Number of reads	Percentage (%)	Number of reads	Percentage (%)	Number of reads	Percentage (%)
Exon-antisense	1986	0.2495	1809	0.3483	1880	0.2772	1866	0.2837	2781	0.3432	1682	0.2707
Exon-sense	97124	12.2014	71548	13.7770	82283	12.1306	98865	15.0298	124772	15.3994	69985	11.2628
Intron-antisense	3160	0.3970	2942	0.5665	3312	0.4883	3078	0.4679	4642	0.5729	2924	0.4706
Intron-sense	24332	3.0568	21827	4.2029	21370	3.1505	24890	3.7839	32952	4.0670	21180	3.4085
Known-miRNA	**73121**	**9.1860**	**79374**	**15.2840**	**78603**	**11.5881**	**86468**	**13.1452**	**81021**	**9.9997**	**84819**	**13.6501**
Novel-miRNA	**1690**	**0.2123**	**1916**	**0.3689**	**1758**	**0.2592**	**1693**	**0.2574**	**1963**	**0.2423**	**2094**	**0.3370**
rRNA	183616	23.0672	122418	23.5724	160853	23.7139	150229	22.8384	206671	25.5075	149730	24.0964
Repeat	1136	0.1427	1108	0.2134	1173	0.1729	1206	0.1833	1509	0.1862	1161	0.1868
scRNA	177	0.0222	162	0.0312	163	0.0240	199	0.0303	188	0.0232	157	0.0253
snRNA	4006	0.5033	3214	0.6189	3788	0.5584	3868	0.5880	5208	0.6428	3324	0.5349
snoRNA	5447	0.6843	4619	0.8894	5206	0.7675	5532	0.8410	6735	0.8312	4684	0.7538
tRNA	30838	3.8741	22284	4.2909	28477	4.1982	30037	4.5663	27584	3.4044	24652	3.9673
unann	369371	46.4032	186107	35.8361	289442	42.6712	249861	37.9848	314211	38.7801	254988	41.0358
Total	796004	100	519328	100	678308	100	657792	100	810237	100	621380	100

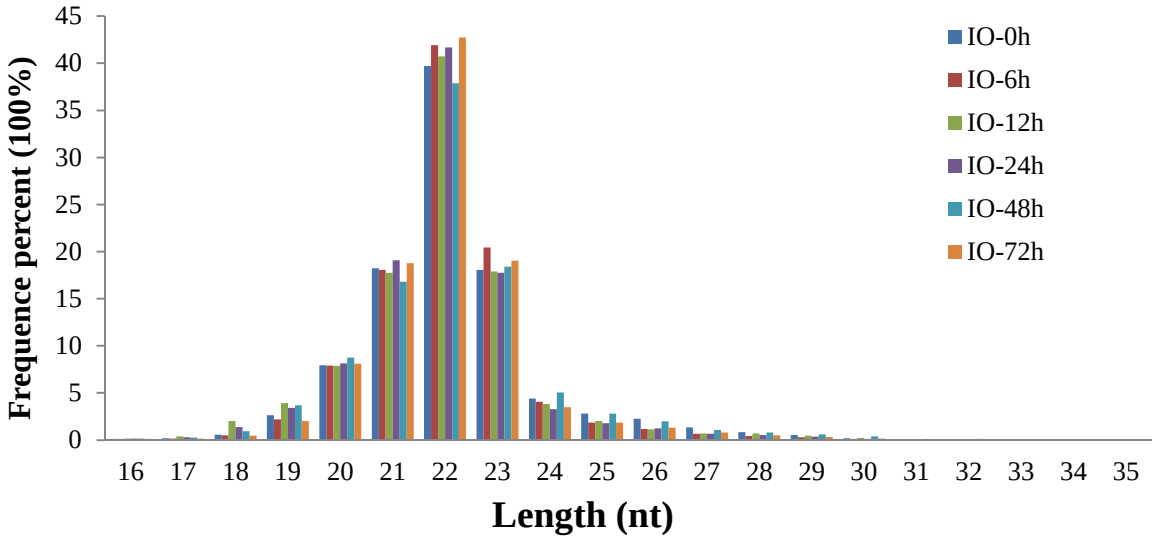

Fig. 1. Analysis of the sequencing reads of tilapia small RNAs at different times of *S. agalactiae* infection.

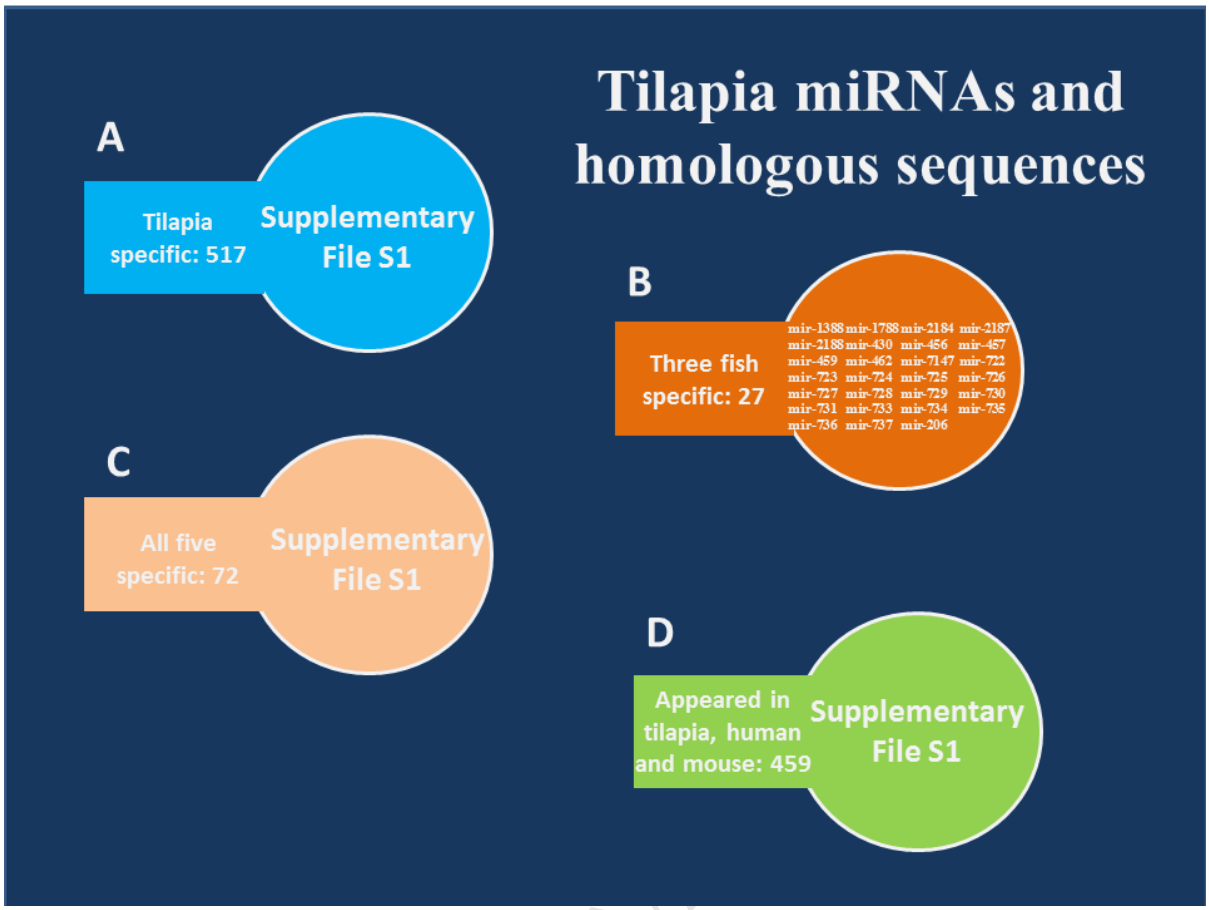

Fig. 2. Revolutionary conservation analysis of tilapia miRNAs. (A) Tilapia-specific miRNAs. (B) miRNAs merely appeared in tilapia, zebrafish and pufferfish. (C) miRNAs identified in all five species. (D) miRNAs appeared in tilapia, human and mouse.

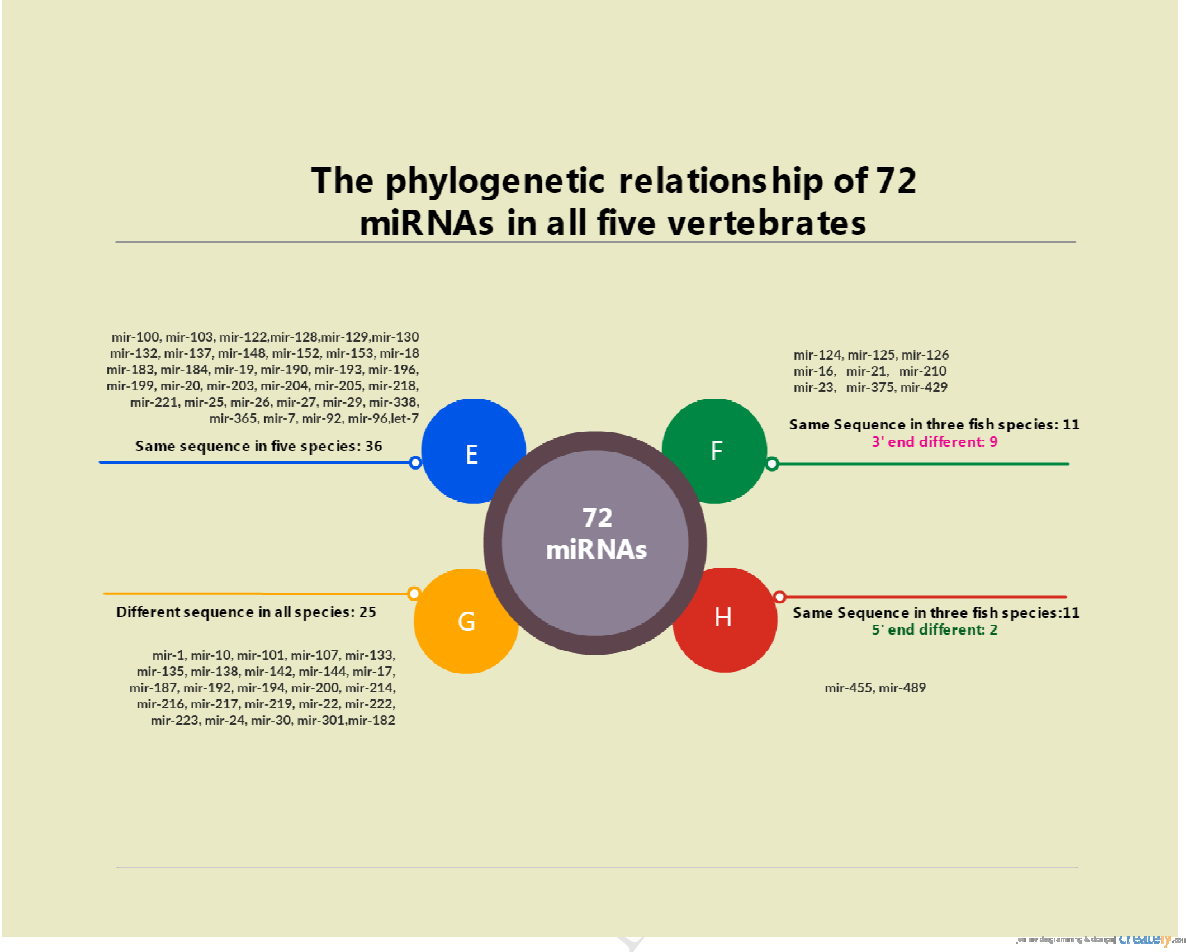

Fig. 3. Revolutionary conservation analysis of miRNAs appeared in tilapia, zebrafish and pufferfish. (E) miRNAs showed 100% identity in all five vertebrates. (F) miRNAs shared approximately same sequence, which contain 3’ end differences. (G) miRNAs with different sequences in all five species (H) miRNAs shared approximately the same sequence, which contain 5’ end or middle differences.

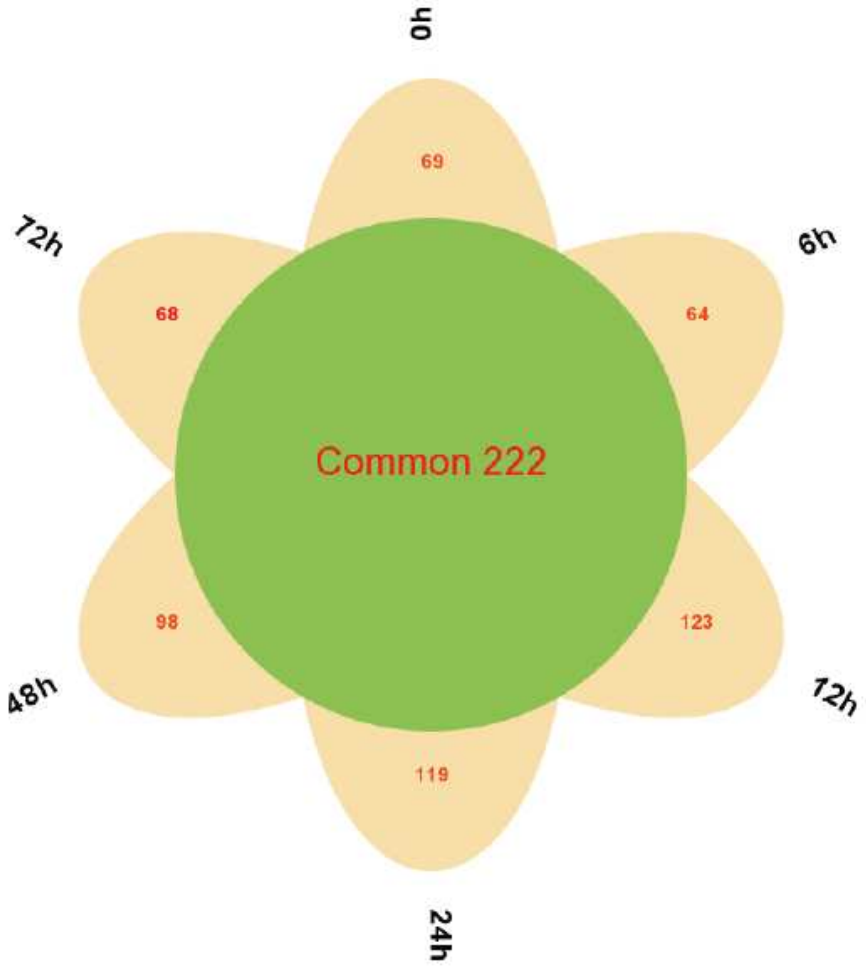

Fig. 4. Tilapia known miRNAs of common and specifically expressed at different hours (h) post-bacterial infection (pi). The part of canary yellow indicate the number of known miRNA expressed specifically, the part of green indicate the number of common known miRNA. The numbers inside the diagram indicated the numbers of miRNAs.

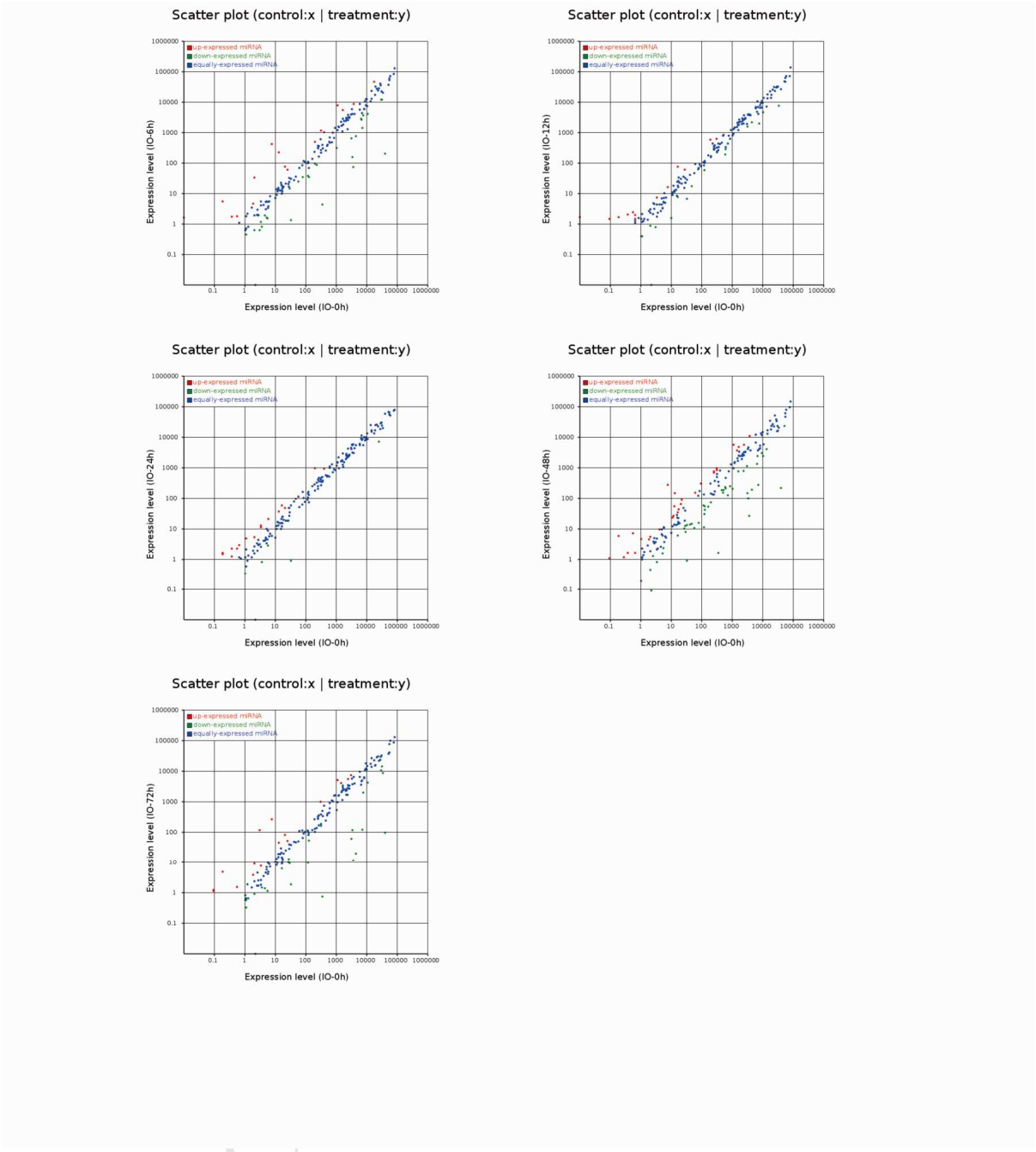

Fig. 5. Effect of *S. agalactiae* infection on the expression of tilapia miRNAs. Scatter polt the expression levels of tilapia miRNAs at 6h, 12h, 24h, 48h and 72h post-bacterial infection (pi) in comparison with that at 0h pi. Red and green spots represent miRNAs that are significantly (p<0.01) upregulated and downregulated respectively.

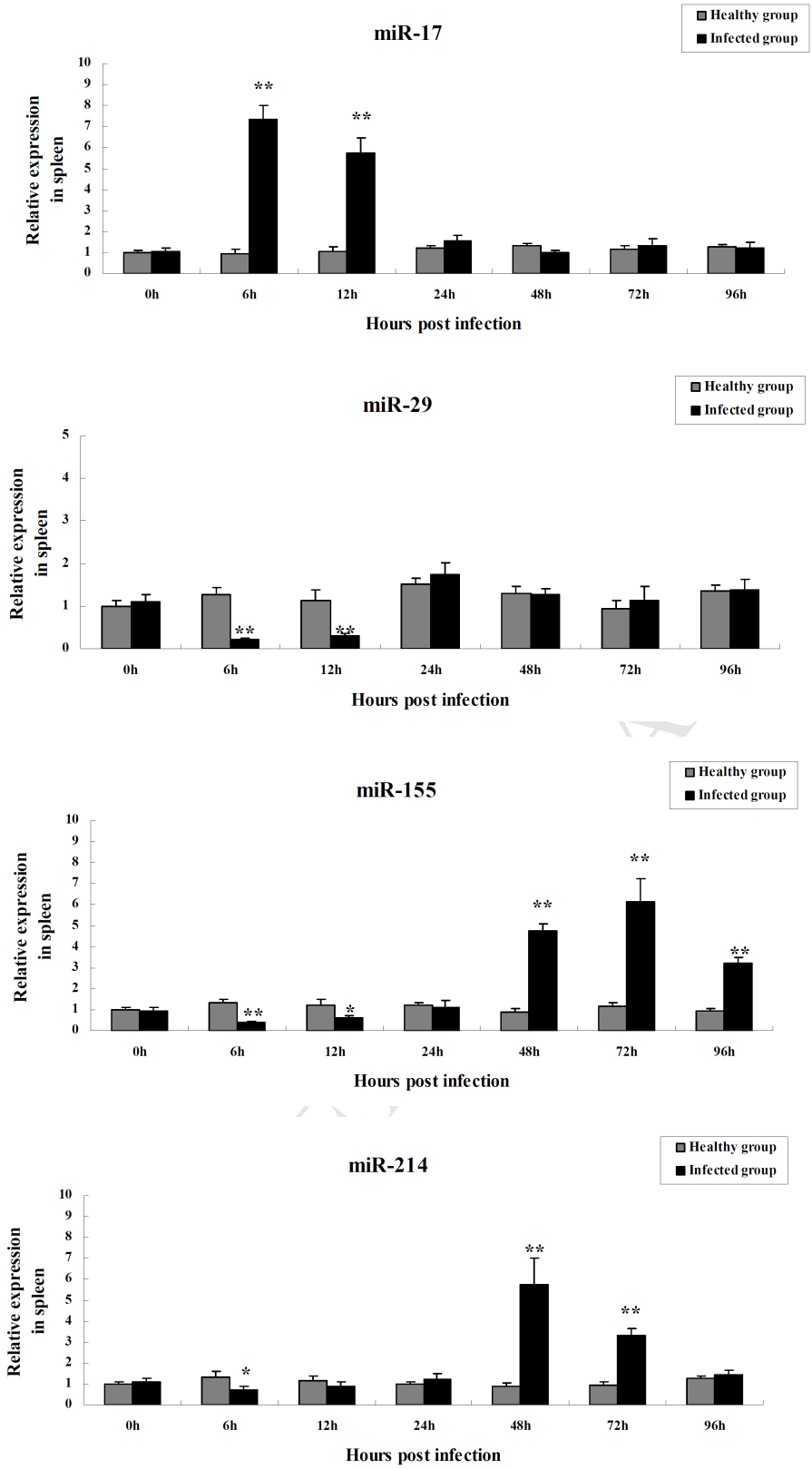

Fig. 6. Expression of four tilapia miRNAs during infection. The expression levels of four tilapia miRNAs at different hous (h) of infection were determined with stem-loop relative quantitative real-time PCR. Values are shown as menas±S.D. Significant difference was indicated by asterisks, *: 0.05>p>0.01, **: p< 0.01.

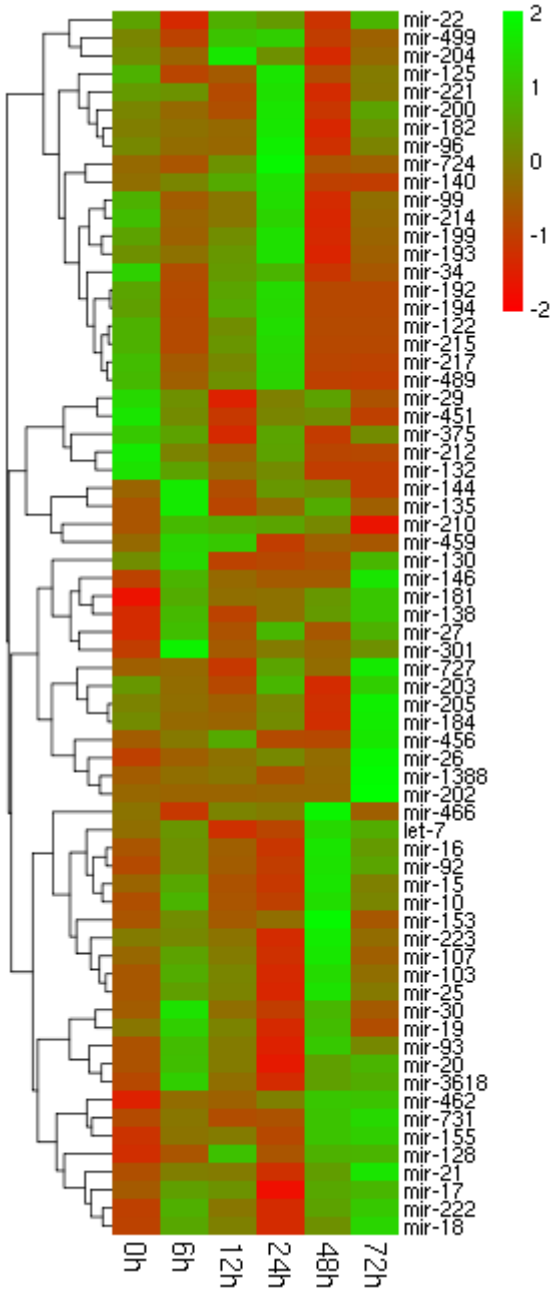

Fig. 7. Clustering of the expression patterns of 68 host miRNAs expressed differentially during bacterial infection. The expression levels of the 68 miRNAs at 0 h to 72 h post-infection (pi) are shown in different colour. Each horizontal colour bar represents one miRNA, with the name of the miRNAs indicated on the right of the bar.

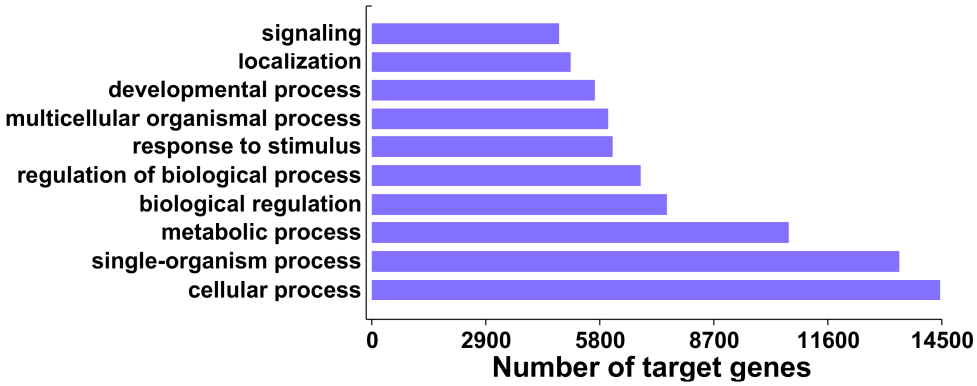

Fig. 8. Top ten enriched GO terms of biological process for the target genes of tilapia miRNAs.

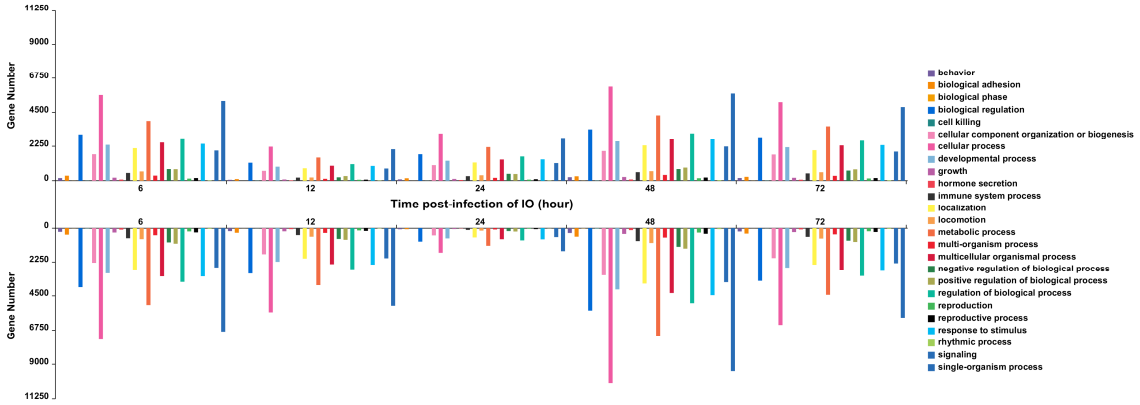

Fig. 9. The enriched GO terms of biological process for the target genes of tilapia miRNAs at different time points of bacterial infection.

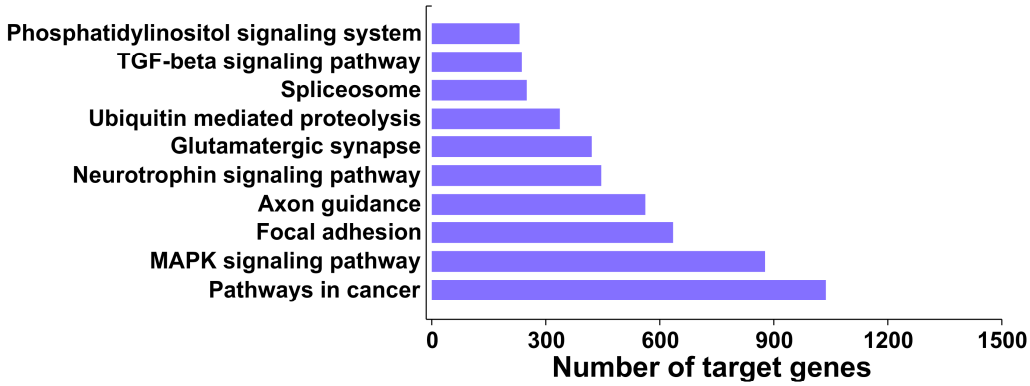

Fig. 10. Top ten abundant pathways from KEGG pathway analysis of the target genes of tilapia miRNAs.

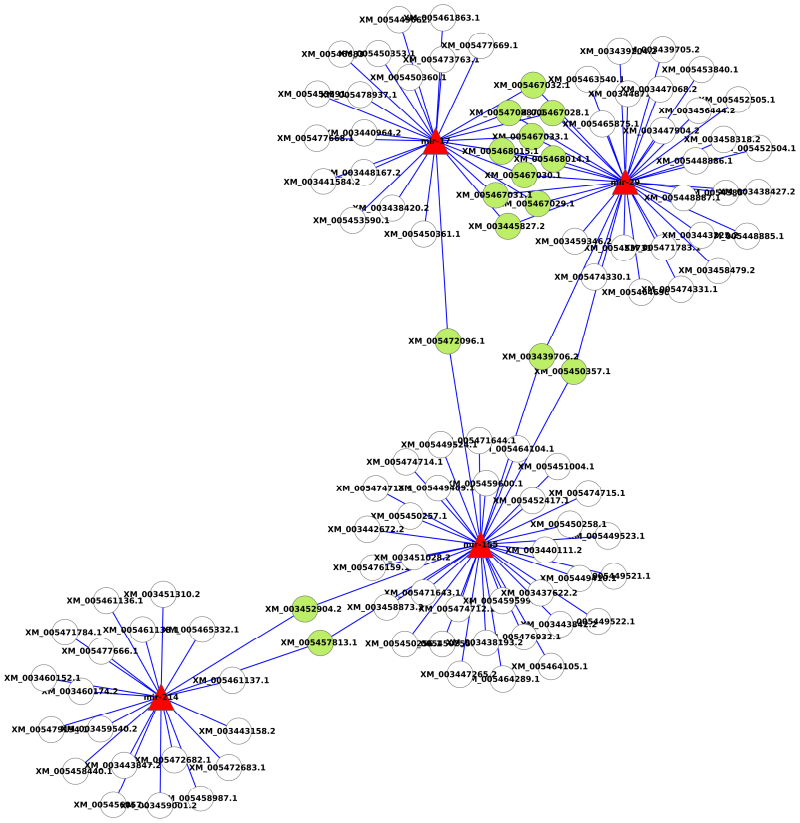

Fig. 11. A network of putative interactions between miR-17, miR-29, miR-155, miR214 and target genes in tilapia. Red indicates tilapia miRNAs, white indicates target genes known to be associated with immune response or signal transduction; green indicates targets genes are shared by miR-17, miR-29, miR-155 and miR214

Highlights

- miRNA profile of tilapia was performed by Solexa deep sequencing.
- A total of 1121 conserved and 1878 novel miRNAs were identified.
- Many miRNAs were differentially regulated during *Streptococcus agalactiae* infection in vitro.
- Target prediction indicated that a number of miRNAs involved in immunity.