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Determination of reference microRNAs for relative quantification in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Relative quantification is the strategy of choice for processing real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) data in microRNA (miRNA) expression studies. Normalization of relative quantification data is performed by comparison to reference genes. In teleost species, such as grass carp (*Ctenopharyngodon idella*), the determination of reference miRNAs and the optimal numbers of these that should be used has not been widely studied. In the present study, the stability of seven miRNAs (*miR-126-3p*, *miR-101a*, *miR-451*, *miR-22a*, *miR-146*, *miR-142a-5p* and *miR-192*) was investigated by RT-qPCR in different tissues and in different development stages of grass carp. Stability values were calculated with geNorm, NormFinder, BestKeeper and Delta CT algorithms. The results showed that tissue type is an important variability factor for miRNA expression stability. All seven miRNAs had good stability values and, therefore, could be used as reference miRNAs. When all tissues and developmental stages were considered, *miR-101a* was the most stable miRNA. When each tissue type was considered separately, the most stable miRNAs were *126-3p* in blood and liver, *101a* in the gills, *192* in the kidney, *451* in the intestine and *22a* in the brain, head kidney, spleen, heart, muscle, skin and fin. *126-3p* was the most stable reference miRNA gene during developmental stages 1–5, while *22a* was the most stable during developmental stages 6–18. Overall, this study provides valuable information about the reference miRNAs that can be used to perform appropriate normalizations when undertaking relative quantification in RT-qPCR studies of grass carp.

1. Introduction

MicroRNAs (miRNAs) are ~22 nucleotide RNAs that regulate gene expression through translational repression and/or transcript cleavage [1] in organisms as diverse as viruses, unicellular algae, plants, worms, flies, fish and mammals [2,3]. miRNAs are essential for vertebrate development and are likely to be involved in differentiation and/or maintenance of tissue and cell identity. The current set of miRNAs is predicted to regulate several thousands of target mRNAs, which may include up to 30% of all protein-coding genes [4]. Therefore, there is strong interest in analysing the roles of miRNAs in development, disease and other cellular processes. miRNAs negatively regulate gene expression through two major mechanisms: translational repression and mRNA cleavage, which depend on the extent of complementarity between the miRNA and its mRNA target [5]. Since miRNAs seem to regulate gene expression by a mechanism of 'fine-tuning', the study of the participation of miRNAs and their targets in specific physiological or pathological experimental situations depends heavily on a reliable and accurate technique for measuring miRNA expression levels [6].

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) is an accurate and sensitive technique for performing gene expression analysis. The use of reference genes as internal controls for the measurement of target gene expression variation is the preferred method for normalizing RT-qPCR data because this approach captures all non-biological variation [7]. The selection of the reference gene(s) to use is not trivial and previous studies have demonstrated that a single universal reference gene is unlikely to exist and perform well for all tissue types or for all physiological, pathological and experimental situations [8,9].

Abbreviations: EF1a, elongation factor 1a; GAPDH, glycer-aldehyde-3-phosphate dehydrogenase; miRNA, microRNA; RT-qPCR, real-time quantitative reverse transcription polymerase chain reaction.

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Originally, normalization strategies were performed using only one reference gene; however, this idea has now evolved to include different normalization approximations, such as using the global mean normalization method or the robust multiple reference gene normalization approach where more than one reference gene is used [10].

To date, several genes, including *18S rRNA, β-actin, elongation factor 1a (EF1a) and glycer-aldehyde-3-phosphate dehydrogenase (GAPDH)*, have been used widely as candidate reference genes for gene expression studies in grass carp (*Ctenopharyngodon idella*) [11,12]. In miRNA expression studies, the most common reference genes used are ribosomal RNAs, such as *18S rRNA* [13], and small nuclear RNAs like *U6 snRNA* [14]. However, the use of miRNAs as reference genes is not widely used at present, although it is very important that the references used have the same nature as the study subjects. The reference genes used should have the same length as the molecules of interest in order to assure the same efficiency during RNA isolation and reverse transcription [15]. In this sense, only a few studies have explored the stability of some miRNAs in human, rat and porcine tissues [6,10,16,17]. To date, no one report has deeply analysed the expression stability of miRNAs to be used as references in teleost studies.

The aim of this present work was to identify suitable reference gene(s) and minimize the risk of co-regulation artifacts in grass carp. The expression of seven candidate reference miRNA genes (*101a*, *126-3p*, *146*, *192*, *22a*, *451* and *142a-5p*) were examined in 18 developmental stages (unfertilized eggs; 0 h post-fertilization; embryos at the 16-cell stage; morula stage; gastrula stage; eye sac-appearance stage; caudal fin-appearance stage; muscular effect stage; heart beating stage; mental stages; and at 1, 2, 3, 4, 5, 6, 7 and 10 days post-hatching.) and 12 tissues (blood, brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin) from control fish and fish challenged with bacteria. Furthermore, the consistency of the best-scoring reference gene was tested by four statistical approaches (geNorm, BestKeeper, NormFinder and Delta CT algorithms).

2. Materials and methods

2.1. Sample collection

Grass carp with an average weight of 50 g were cultured individually in Wujiang National Farm of Chinese Four Family Carps (Jiangsu Province, China). Animals were raised at 28 °C in 400-L aerated tanks for one week before the experiment and fed twice daily (in the morning and late in the afternoon) at a ratio of 5% of total biomass. Embryos and fries were also obtained from the Wujiang National Farm of Chinese Four Family Carps, and reared in a hatching trough with constant pool water flow at 21 ± 1 °C. On day 3 post-hatching, the fry could swim steadily and were fed with freshwater rotifers captured from the pool.

Twelve tissue samples were collected from three grass carp (blood, brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin), while embryos and early larvae were sampled at 18 different developmental stages (10 specimens from each stage). These stages were: unfertilized eggs; 0 h post-fertilization; embryos at the 16-cell stage; morula stage; gastrula stage; eye sac-appearance stage; caudal fin-appearance stage; muscular effect stage; heart beating stage; mental stages; and at 1, 2, 3, 4, 5, 6, 7 and 10 days post-hatching.

For the bacterial challenge, 12 fish were intrapleurally injected with formalin-killed *Aeromonas hydrophila* S2 (obtained from the Aquatic Pathogen Collection Centre of the Ministry of Agriculture, China) at a dose of 7.0×10^6 cells suspended in 100 μl PBS per fish; 12 control fish were similarly injected with 100 μl sterile PBS per fish. Three fish were sampled at 4 h, 1 day, 3 days and 7 days post-injection, respectively. Blood, brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin were collected from each fish. All samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until use.

Two groups were maintained in two aquariums and intraperitoneally injected with *A. hydrophila* AH10 (Aquatic Pathogen Collection Centre of Ministry of Agriculture, China) at a dose of 7.0×10^6 cells suspended in 100 μl PBS per fish. All the fish were observed every 4 h for any mortality and collecting samples until the termination of the experiment at 240 h post-challenge. Grass carp that died in the first 72 h post-challenge were classified as susceptible group (SG), while the animals that survived over 240 h post-challenge were considered as resistant group (RG).

2.2. Selection of candidate reference miRNAs

The candidate reference genes selected for evaluation included *miR-148*, *miR-192*, *miR-451*, *miR-126-3p*, *miR-101a*, *miR-142a-5p*, *miR-146b* and *miR-22a*. These miRNA candidate genes were selected due to their relative high quantities in miRNA profiles of grass carp that were generated during Illumina deep sequencing (data not shown). *18S rRNA* and *β-actin* mRNAs were selected to be evaluated as reference genes due to their wide use in the literature. This selection process was performed after considering stability values reported in previous studies [11,12].

Table 1
Sequence information for 14 selected candidate reference genes.

Gene symbol	Primer sequence (5′–3′)	Amplification efficiency
miR-148	TCAGTGCATTACAGAACTTTGT	87.5
miR-192	ATGACCTATGAATTGACAGCC	95.4
miR-451	AAACCGTTACCATTACTGAGTT	95.7
miR-let-7a	TGAGGTAGTAGGTTGTATAGTT	96.0
miR-126-3p	CTCGTACCGTGAGTAATAATGC	96.3
miR-101a	TACAGTACTGTGATAACTGAAG	96.6
miR-142a-5p	CATAAAGTAGACAGCACTACT	99.9
miR-146b	TGAGAACTGAATTCCAAGGGTG	104.8
miR-22a	AAGCTGCCAGCTGAAGAACTGT	105.0
miR-217	TACTGCATCAGGAACTGATTGG	97.8
miR-142a-3p	CGTGTAGTGTTTCCTACTTTATGGA	96.0
miR-23a	ATCACATTGCCAGGGATTTCCA	96.5
B-actin (β-actin)	Forward: CCTTCTTGGGTATGGAATCTTG Reverse: AGAGTATTTACGCTCAGGTGGG	95.8
***18S rRNA* (18S ribosomal RNA)**	Forward: GGACACGGAAAGGATTGACAG Reverse: CGGAGTCTCGTTCGTTATCGG	98.2