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Reference gene selection for microRNA expression analysis in the Pacific oyster

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Abstract

Real-time quantitative polymerase chain reaction (qRT-PCR) is a widely used technique for microRNA (miRNA) expression analysis. The accuracy and reproducibility of this technique depends on the availability of stably expressed reference genes, which is absent in molluscs. Here, based on the microRNA transcriptome data of the Pacific oyster, Crassostrea gigas, candidate reference genes were selected from the stably expressed microRNAs and the commonly used housekeeping genes (U1, U6, and 5.8S). The stability of expression was evaluated and the comprehensive ranking of the reference genes was obtained using two statistical algorithms, geNorm and NormFinder. Specific combinations of reference genes were recommended for the developmental larval samples (DLS) and adult organ samples (AOS), respectively; a combination of cgi-miR-71 and U1 was recommended for DLS and a combination of cgi-let-7, cgi-miR-87, and U1 for AOS, under normal physiological conditions. The effectiveness of the reference gene combinations was further validated by quantitative analysis of two selected miRNAs in the DLS and AOS, respectively. This work was the first systematic analysis of miRNA reference gene selection in molluscs. The reference genes identified in this study will be useful for the functional analysis of miRNA in Crassostrea gigas, and also for the miRNA reference gene selection in other molluscan species.

Keywords: *Crassostrea gigas*, MicroRNA, Real-time quantitative PCR, Reference gene

1. Introduction

MicroRNAs (miRNAs) are endogenous, single-stranded, non-coding RNAs that are involved in several biological processes such as development, proliferation, differentiation, and apoptosis (BARTEL [1], HE & HANNON [2], BRENNECKE & al. [3]). Real-time quantitative polymerase chain reaction (qRT-PCR) is widely used in miRNA expression analysis because of its low cost, high sensitivity, wide dynamic range, low template requirements, and high throughput (SHI & CHIANG [4]). A reliable qRT-PCR result should be based on a set of competent reference genes that can rectify the variations between different reactions. For miRNA qRT-PCR analysis, genes encoding small RNAs were recommended as the reference genes (PELTIER & LATHAM [5]). The most commonly used reference genes in miRNA qRT-PCR are the housekeeping genes encoding snRNAs U1 and U6, or the 5S rRNA (ABDELMOHSEN & al. [6]). However, a recent study demonstrated that the levels of the most

commonly used reference genes varied extensively and were statistically inferior to the stably expressed miRNAs (PELTIER & LATHAM [5]). To avoid introducing further error, it was essential to determine reliable reference genes in the samples of interest. To our knowledge, no verified reference gene for miRNA expression analysis was available in molluscs.

Molluscs compose a large phylum of invertebrate animals, which play important roles in evolutionary research. Several studies on reference gene selection for long mRNA quantification have been conducted in various experimental settings in molluscs (DU & al. [7]). However, information on molluscan miRNAs is limited. There are only 69 mature miRNAs in molluscs, which were reported in the miRBase (the latest released miRBase, Release 21: June 2014, WHEELER & al. [8]). Recent studies have expanded the miRNA reservoir of molluscs. In 2014, 199 immune-related miRNAs and 258 biomineralization-related miRNAs were identified by deep RNA sequencing of the Pacific oyster *Crassostrea gigas* and pearl oyster *Pinctada martensii*, respectively (ZHOU & al. [9], JIAO & al. [10]). We independently identified 100 miRNAs from the Pacific oyster genome (ZHANG & al. [11]), and determined their expression pattern by miRNA deep sequencing of larval samples in different stages of development (DLS) and adult organ samples (AOS) (XU & al. [12]). Notably, deep sequencing of miRNAs had the advantage of being high-throughput and capable of detecting novel miRNAs. However, due to complicated sample processing and data analysis, deep sequencing is considered inferior to qRT-PCR for miRNA expression analysis, and validation by qRT-PCR is recommended. Indeed, qRT-PCR was used to confirm the accuracy of the deep sequencing data in two recent articles. Because of the dearth of appropriate reference genes for miRNA quantification in molluscs, the commonly used reference gene encoding the snRNA U6 and 5.8S rRNA were chosen for evaluation (ZHOU & al. [9], JIAO & al. [10]). Therefore, it is important to identify reliable reference genes for miRNA estimation in molluscs.

Recently, the Pacific oyster has received attention for its importance in ecosystems, aquaculture, and evolutionary studies. The two miRNA deep sequencing experiments that were conducted in *C. gigas* generated a massive miRNA resource for studies on miRNA expression and reference gene selection in this mollusc. Previously, we found that the swimming stages tended to express younger miRNAs (XU & al. [12]), and therefore, we decided to study the differentially expressed miRNAs in those stages (from trochophore to umbo larvae). The differentially expressed miRNAs in different organs were also investigated. In this study, we identified suitable miRNA reference genes in the various developmental stages from the trochophore to the umbo larvae, and in different organs. The reference genes recommended in this study will be useful for validation of deep sequencing data and for functional study of miRNAs in *C. gigas* and other molluscs.

2. Material and Methods

Oyster sample collection

The Pacific oysters were collected from a cultured population in Qingdao, China. The DLS, including the trochophore, D-shape, and umbo larvae, were taken at appropriate time points. The AOS, which included the mantle, adductor muscle, gill, labial palps, and the digestive gland, were collected from three one-year-old healthy individuals. Samples were frozen in liquid nitrogen and stored at -80°C.

Identification of snRNA U1, U6, and 5.8S rRNA-encoding genes in the Pacific oyster

The widely used housekeeping genes (*U*1, *U6*, and *5.8S*) were selected as candidate reference genes (CRGs) for miRNA quantification. The housekeeping genes of *C. gigas* were identified by a homology search in the oyster genome using the Rfam database at *E* value < 1×10^{-20}. The full-length sequences of U1, U6, and 5.8S RNA-encoding genes consisted of 164,

109, and 154 nucleotides, respectively (Supplementary Table S3). Furthermore, the full-length sequence of *U6* was confirmed by direct cloning, which also verified the accuracy of the homology search. Therefore, we designed primers based on the sequences identified in the homology search. Alignment with other known *U6* sequences revealed that *U6* was highly conserved from *C. gigas* to humans (Fig.1). The sequences of the U1 and U6 snRNA-encoding genes and the 5.8S rRNA-encoding genes were submitted to the NCBI database (*U1,* KJ599655; *U6,* KJ580834; *5.8S*, KJ599654).

CRG selection from the stably expressed miRNAs

To select the miRNAs that were expressed stably in the DLS or AOS, two criteria were used (PELTIER & al. [5]): 1) the miRNA must be highly and consistently expressed in the majority of samples; 2) only one representative from a particular miRNA family should be considered. Reads per million (RPM) values were calculated and the stability of expression of each miRNA was assessed by the *CV* values determined from the miRNA sequencing data. Lower *CV* values represented higher miRNA stability. Finally, five miRNA genes in the DLS (*cgi-miR-8*, *cgi-miR-29*, *cgi-miR-71*, *cgi-miR-92*, and *cgi-miR-1992*) and AOS (*cgi-let-7*, *cgi-miR-1*, *cgi-miR-71*, *cgi-miR-87*, and *cgi-miR-1994*) each were chosen as CRGs, respectively. The RPM and *CV* values of the selected miRNAs in the DLS and AOS are listed in Supplementary Table S1 and Supplementary Table S2.

Figure 1. Alignment of the snRNA U6-encoding gene sequence from *Crassostrea gigas* (*cgU6*, KJ580834) with that from *Caenorhabditis elegans* (*ceU6*, NR_053231.1), *Drosophila melanogaster* (*deU6*, NR_002081.1), *Mus musculus* (*mmU6*, NR_003027.2), and *Homo sapiens* (*hsU6*, NR_004394.1).

Total RNA extraction and cDNA synthesis

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity of the RNA samples was assessed by agarose gel electrophoresis. The concentration of each RNA sample was measured by the Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The first-strand of cDNA was synthesised by reverse transcribing 1 μg polyadenylated total RNA in a final reaction volume of 20 μL with the One Step PrimeScript miRNA cDNA synthesis kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions.

Primer design and qRT-PCR

The sequences and primers used in this study are listed in Supplementary Table S3. The primers for amplifying *U1*, *U6* and *5.8S* were designed by Primer Premier 5. Considering that the reverse primer was fixed, the miRNA-specific primers were critical for PCR specificity. For most miRNAs, the primer sequence was the same as the miRNA gene sequence. To avoid

dimers and ensure that the Tm was around 60°C, certain miRNA-specific primers were designed to be G-rich at the 5′ end or A-rich at the 3′ end (CASTOLDI & BENES [13]).

The qRT-PCR reactions were performed in 96-well plates in the ABI 7500FAST real-time PCR system. Each PCR reaction included 2 μL of the cDNA template, 10 μL of the 2 × SYBR Green PCR Master Mix (ABI), 0.4 μM each of the forward and reverse primers, and sterile water in a 20 μL final volume. The amplification program was as follows: 2 min at 50°C, 2 min at 95°C, 40 cycles of 3 s at 95°C, and 30 s at 60°C, followed by a thermal denaturing step to generate the dissociation curves. In all the cases, PCRs were conducted in triplicates, and negative controls (cDNA was replaced with sterile water) were included to rule out DNA contamination. For each gene, gel electrophoresis (Supplementary Fig. S1) and melting curve analysis (Supplementary Fig. S2) were performed to verify the specificity of the PCR primers.

Statistical analysis

The threshold cycle (Ct) was calculated automatically using the 7500 Software v2.0.1. The mean Ct values of DLS and AOS are listed in Supplementary Table S4 and Supplementary Table S5, respectively. The qRT-PCR data of CRGs was analysed by two frequently used statistical algorithms, geNorm V3.5 (VANDESOMPELE & al. [14]) and NormFinder V20 (ANDERSEN & al. [15]) to rank the expression stability of CRGs across the experimental sets. geNorm ranked the candidate genes based on M values, where a lower M value indicated more stable expression. NormFinder calculated a stability value for each CRG. A low stability value indicated a high stability in gene expression. The expression of the target genes was analysed by using the $2^{-\Delta\Delta Ct}$ method.

3. Results

Stability in the expression of the candidate reference genes in the larval developmental stages

In the DLS, eight CRGs displayed high expression with Ct values ranging from 11.48 to 31.55 (Supplementary Table S4). The most highly expressed genes were the housekeeping genes encoding the 5.8S rRNA, and the U1 and U6 snRNAs with mean Ct values of 12.48, 13.88, and 15.33, respectively. All the other CRGs had mean Ct values larger than 20 (Supplementary Table S4). The most highly expressed miRNA candidates was *cgi-miR-71*, while *cgi-miR-29* presented the lowest level of expression with a mean Ct value 28.89.

To evaluate the stability of CRG expression in the DLS, the expression data was analysed by geNorm and NormFinder. geNorm analysis showed that *cgi-miR-71* was the most stable gene, followed by *U1*, *cgi-miR-8*, *cgi-miR-92*, *cgi-miR-29*, *cgi-miR-1992*, *5.8S*, and *U6* (Table 1). Based on the default cut-off value of $M < 1.5$ in the geNorm program, *5.8S* and *U6* were too unstable to be considered as reference genes in these developmental stages. In the DLS, geNorm determined the pairwise variation v2/3 value to be 0.124 (Fig. 2), which implies that a combination of the top two reference genes was sufficient for accurate qRT-PCR normalization and the addition of a third gene was not necessary.

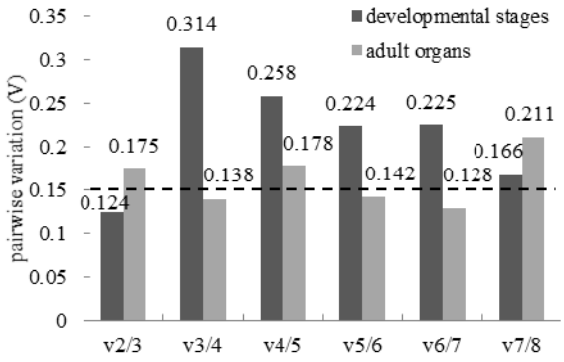

Figure 2. Pairwise variation (V) analysis of the candidate reference genes in developmental stages and adult organs. geNorm pairwise variation Vn/Vn+1 was calculated to determine the minimum number of genes required to obtain accurate results, with the default cut-off value set to 0.15.

Similar to geNorm, NormFinder ranked *cgi-miR-71* and *U1* first and second, respectively, followed by *cgi-miR-92*, *cgi-miR-8*, *cgi-miR-29*, *cgi-miR-1992*, *U6*, and *5.8S* (Table 1). The two least stable CRGs were also *U6* and *5.8S*. Considering the CRG ranks from geNorm and NormFinder and the optimal number of required genes from geNorm, the combination of *cgi-miR-71* and *U1* was recommended for accurate normalization in the DLS.

Table 1. Ranks of candidate reference genes and optimal combinations of reference genes in developmental stages and adult organs.

Rank	Developmental stages		Adult organs	
	geNorm	NormFinder	geNorm	NormFinder
1	*cgi-miR-71* (1.146)	*cgi-miR-71* (0.168)	*cgi-let-7* (0.796)	*cgi-let-7* (0.169)
2	*U1* (1.320)	*U1* (0.616)	*cgi-miR-87* (0.866)	*cgi-miR-87* (0.274)
3	*cgi-miR-8* (1.357)	*cgi-miR-92* (0.616)	*U1* (0.885)	*U1* (0.353)
4	*cgi-miR-92* (1.367)	*cgi-miR-8* (0.749)	*cgi-miR-1994*(1.003)	*cgi-miR-1994*(0.391)
5	*cgi-miR-29* (1.392)	*cgi-miR-29* (0.806)	*5.8S* (1.026)	*5.8S* (0.424)
6	*cgi-miR-1992*(1.419)	*cgi-miR-1992* (0.829)	*cgi-miR-71* (1.052)	*cgi-miR-71* (0.566)
7	*5.8S* (1.547)	*U6* (0.880)	*U6* (1.214)	*U6* (0.732)
8	*U6* (1.1.548)	*5.8S* (0.921)	*cgi-miR-1* (1.740)	*cgi-miR-1* (1.160)
Optimal combination	***cgi-miR-71+U1***	***cgi-miR-71+U1***	***cgi-let-7+ cgi-miR-87+U1***	***cgi-let-7+ cgi-miR-87+U1***

The candidate reference genes were ordered from the most to the least stable based on their stability values (in brackets) calculated by geNorm or NormFinder. Lower stability value indicated higher stability.

Stability of CRG expression in adult organ samples

In the AOS, the expression of eight CRGs ranged from 12.01 to 29 (Supplementary Table S5). The most highly expressed genes were *5.8S* and *U1*, with a mean Ct value of 13.39 and 15.48, respectively. *cgi-miR-1* exhibited the lowest expression, with the mean Ct value of 26.54.

The ranking of each candidate determined by geNorm and NormFinder were identical (Table 1). The most stably expressed gene was *cgi-let-7*, followed by *cgi-miR-87*, *U1*, *cgi-miR-1994*, *5.8S*, *cgi-miR-71*, *U6*, and *cgi-miR-1*. Except for *cgi-miR-1*, all the candidates passed the default value 1.5 set in geNorm. The v2/3 value was set higher than the cut-off value of 0.15 to determine the optimal number of genes required for accurate normalization. The v3/4 value was 0.138 (Fig. 2) indicating that the combination of the three most stable genes (*cgi-let-7*, *cgi-miR-87*, and *U1*) was sufficient for accurate qRT-PCR normalization.

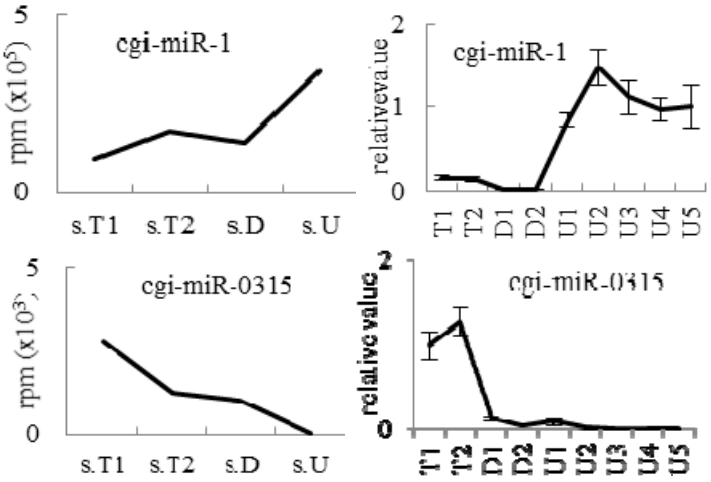

Figure 3. Comparison of the results of sequencing (left) with qRT-PCR (right) of *cgi-miR-1* and *cgi-miR-0315* in the larval developmental stage samples. Data are given as means ± SD. T: trochophore; D: D-shape; U: umbo.

Reference Gene Validation

To validate the effectiveness of the reference gene combinations recommended in this study, the expression patterns of *cgi-miR-1* and *cgi-miR-0315* were analysed using the reference combination of *cgi-miR-71* and *U1* in the DLS; the expression patterns of *cgi-miR-7* and *cgi-miR-0078* were analysed using the reference combination of *cgi-let-7*, *cgi-miR-87*, and *U1* in the AOS. The expression patterns of *cgi-miR-1*, *cgi-miR-0315*, *cgi-miR-7*, and *cgi-miR-0078* were compared between the results obtained from qRT-PCR and deep sequencing. In the DLS, the results of *cgi-miR-1* and *cgi-miR-0315* expression deduced by qRT-PCR were in agreement with the result from deep sequencing (Fig. 3). *cgi-miR-1* and *cgi-miR-0315* were highly expressed in the umbo larva and trochophore, respectively. In the AOS, the qRT-PCR results of *cgi-miR-7* and *cgi-miR-0078* expression were in accordance with the result from deep sequencing (Fig. 4). *cgi-miR-7* was highly expressed in the labial palps followed by gill, mantle, and digestive gland. *cgi-miR-0078* was highly expressed in the gill, followed by digestive gland, and labial palps, whereas the expression level in the mantle and adductor muscle remained low. The consistency of the data obtained by qRT-PCR and sequencing indicated that the reference genes recommended in this study were reliable.

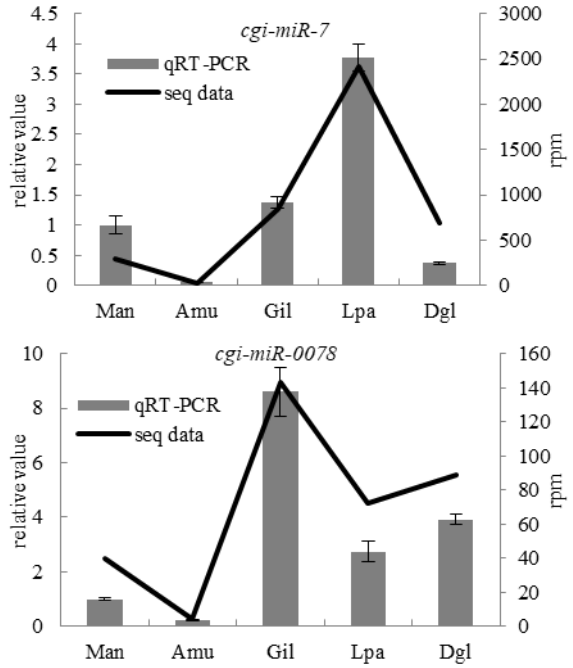

Figure 4. Comparison of qRT-PCR (left y axis, relative value) with deep sequencing data (right y axis, RPM) of *cgi-miR-7* and *cgi-miR-0078* in adult organ samples. Data are given as means ± SD. Man: mantle, Amu: adductor muscle, Gil: gill, Lpa: labial palps, Dgl: digest gland.

5. Discussion

qRT-PCR is a powerful tool used for the detection of differentially expressed miRNAs, which, however, requires competent reference genes to ensure reliability and accuracy of data analysis. For the first time, specific combinations of reference genes for the DLS and AOS were identified through the assessment of expression stability using two statistical algorithms, geNorm and NormFinder, which were the most frequently used algorithms for reference gene selection.

As the most frequently used reference genes, the housekeeping genes *U6* and *5.8S* have been used as references in the Pacific oyster and pearl oyster (ZHOU & al. [9], JIAO & al. [10]). However, their suitability as normalization factors for the corresponding samples was not evaluated. The performance of *U6* and *5.8S* were unsatisfactory in this study. In the DLS, they

ranked last and were excluded by geNorm, which suggested that expression of *U6* and *5.8S* was not stable and hence they should not be used as reference genes in the future. In the AOS, *U6* and *5.8S* ranked low, with *U6* in the seventh and *5.8S* in the fifth position. The analysis indicated that *U6* and *5.8S* were inferior to the stably expressed miRNAs as reference genes in qRT-PCR analysis. Similar results were reported in a study on the identification of reference genes in normal and cancerous human solid tissues (PELTIER & al. [5]). Despite the exclusion of *U6* and *5.8S*, *U1* was included in the reference gene combinations for both DLS and AOS. In the present study, *U1* ranked second and third in the DLS and AOS, respectively, and was included in the recommended combinations in the two sample sets. Thus, *U1* may be stably expressed under other experimental conditions such as virus infection, and was recommended to be a CRG while selecting reference genes in those samples.

Among the nine CRGs selected from miRNAs in *C. gigas, let-7* was the most important. Although *let-7* is a heterochronic gene that regulates development timing (SOKOL [16]), it was the most stably expressed miRNA in the AOS of *C. gigas*. Moreover, *let-7* was reportedly a competent reference gene in human cancer and normal tissue (PELTIER & al. [5]). Considering the conserved expression pattern and function in a wide range of animals, *let-7* may be a more reliable reference gene than the traditional *U6* or *5.8S* in adult organs. Another interesting CRG was *cgi-miR-71*, which was selected as the only candidate in both DLS and AOS. It was the most stable one in the DLS, and ranked sixth in the AOS. The function of *cgi-miR-71* may be indispensable for larval development and organ function, which, however, remains to be elucidated.

The expression of *cgi-miR-1*, *cgi-miR-0315*, *cgi-miR-7*, and *cgi-miR-0078* were consistent between qRT-PCR and deep sequencing analysis. This not only validated the reliability of the reference gene combinations, but also provided some insights into the function of these miRNAs. *miR-1* is evolutionarily conserved from oyster to human. It has pivotal roles in development and physiology of muscle tissues, including the heart (KLOOSTERMAN & PLASTERK. [17]). In *C. gigas*, it is highly expressed in the umbo larva, implying that muscle genesis might occur in this stage. The newly identified *cgi-miR-0315* had no homolog and was considered as an oyster-specific miRNA. With negligible expression in the late developmental stage (Fig. 4) and organs (Supplementary Table S2), *cgi-miR-0315* was highly expressed in the trochophores, during which the velum, one of the typical organs in molluscan larva, was formed. Thus, *cgi-miR-0315* may be involved in the development of the velum in molluscs. *miR-7* was found in most sequenced bilateria species, and its mature miRNA product is highly conserved from molluscs to humans, indicating a strong functional conservation; it was essential for the maintenance of regulatory stability under conditions of environmental flux (RUBY & al. [18]). The expression of *cgi-miR-7* was about 3.5 times higher in the labial palps than in other organs, indicating the relatively high sensitivity of the labial palps to environmental stress compared to other organs in oysters. The oyster-specific miRNA, *cgi-miR-0078*, was widely detected in organs and its function remains to be illustrated. In future, we will focus on determining the role of these oyster-specific miRNAs in the regulation of development and organ function.

6. Conclusion

In this study, we selected candidate reference genes from the stably expressed microRNAs and the commonly used housekeeping genes (*U1, U6*, and *5.8S*) using the microRNA transcriptome data of the Pacific oyster *Crassostrea gigas*. The stability of these candidates was evaluated using two statistical algorithms, geNorm and NormFinder. Thereafter, a combination of *cgi-miR-71* and *U1* for the larval developmental samples, and a combination of *cgi-let-7*, *cgi-miR-87*, and *U1* for the adult organ samples were recommended. Finally, the effectiveness of the reference gene combinations was further validated. This study will be useful for microRNA expression analysis in *C. gigas* and microRNA reference gene selection in other molluscan species.

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