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Validation of Housekeeping Genes for Gene Expression Analysis in Iwagaki Oyster (*Crassostrea nippona*) Under Salinity Stress by Quantitative Real-Time PCR

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Abstract Hypo-salinity can reduce the immunological reaction in *Crassostrea nippona*, even lead to massive mortality. It is important to understand the molecular mechanism of oyster defense system, while quantitative real-time PCR can be employed in the study. However, the accuracy of quantitative real-time PCR relies on the use of suitable reference genes. In this study, the expression stability of 14 candidate reference genes including traditional housekeeping genes EF1A, TUB, TUA, GAPDH, RO21, as well as new candidate reference genes RPL5, RPL8, RPS27, RPL14, RPL4, CO3, RPS8, RPS4, CYTB in different tissues of *C. nippona* under salinity stress has been validated by quantitative real-time PCR. Ribosomal protein genes selected through expression analysis of transcriptome data from *C. nippona* generally were more stable than traditional reference genes. According to the geNorm analysis, RPL4 and RPS4 could be used as internal controls for studying gene expression in *C. nippona* with real-time PCR under salinity stress.

Key words *Crassostrea nippona*; reference gene; hypo-salinity stress; ribosomal protein genes

1 Introduction

Quantitative real-time PCR (qRT-PCR) has been widely used to measure gene expression because of its high sensitivity, flexibility, and reproducibility (Heid *et al.*, 1996). The variations caused by differences in samples, RNA extraction, efficiency of enzyme and transcriptional activity can influence the experimental accuracy (Mackay *et al.*, 2002). For accurate and reliable analysis of target gene expression, normalization of qRT-PCR data with suitable internal reference gene(s) is required (Radonić *et al.*, 2004). An ideal reference gene should express at stable level in all tissues, regardless of the experimental conditions or treatments (Vandesompele *et al.*, 2002; Radonić *et al.*, 2004). Commonly used reference genes usually are those involved in basic cellular processes, such as the components of cytoskeleton, glycolytic pathway, protein folding and degradation (Eisenberg and Levanon, 2003). However, evidences show that transcription levels of housekeeping genes vary considerably in different tissues and under variable conditions (Greer *et al.*, 2010). Therefore, selecting multiple stably expressed reference genes, other than the commonly used housekeeping genes, is important for the accurate normalization of gene expression levels.

Crassostrea nippona is an important aquaculture species for it is edible during summer when other oysters are not available (Itoh *et al.*, 2004). *C. nippona* naturally benthic in shallow water along the coast of the seas of East Asia (Boudry *et al.*, 2003). It is more sensitive to the salinity change of ambient seawater compared with euryhaline oysters, such as *C. gigas*, which live in intertidal zones (Zhang *et al.*, 2016). In the natural habitat, salinity declines with tidal cycles, rainfall and with drainage from the adjacent terrestrial sites (Drouin *et al.*, 1985; Philippart *et al.*, 2011). Hypo-salinity induces the immunological activity of oysters, such as the overexpression of heat shock proteins (HSPs) and phenoloxidase (Gagnaire *et al.*, 2006; Kuchel *et al.*, 2010; Li *et al.*, 2016), and even lead to massive mortality (Meng *et al.*, 2011).

In this study, we compared the performance of 14 candidate reference genes (consisting of 5 commonly used housekeeping genes of animals, and 9 new candidate reference genes detected from *C. nippona* transcriptome) in order to identify the most stable internal controls for normalization of real-time PCR data in *C. nippona* under salinity stress.

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2 Materials and Methods

2.1 Biological Materials

2.1.1 Unstressed samples (group A)

Adult *Crassostrea nippona* were cultured in the fish farm of Rongcheng, Shandong Province, China. Tissues from the mantle (M), visceral mass (V), adductor muscle (A) and gill (S) were collected from 12 healthy oysters. They were immediately placed into liquid nitrogen to freeze and then stored at −80℃ for the subsequent analysis.

2.1.2 Salinity stressed samples (group B)

The experimental *C. nippona* were maintained in 70 L tanks containing aerated sand-filtered seawater (salinity: 30) for one week prior to experimentation. Then they were randomly divided into 3 groups under hypo-salinity stress in seawater with 30 (S3), 20 (S2), and 10 (S1) for one week (3 pools with 9 individuals each). The low salinity water was prepared by diluting sea water with tap water. Gill tissues of the experimental group were collected from 27 oysters under variant salinity stresses. They were immediately placed into liquid nitrogen to freeze and then stored at −80℃ for subsequent analysis.

2.2 RNA Isolation, Library Construction and Sequencing of *C. nippona*

Total RNA was extracted from the samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The purity and integrity of total RNA was determined using a Nanodrop 2000 spectrophotometer (Thermo, USA) and an Agilent 2100 BioAnalyzer (Agilent Technologies, USA). The RNA was pooled proportionally from three oysters in each experimental group (group A: M, V, A, S; group B: S1, S2, S3).

The mRNA was enriched by Oligo(dT) beads and then fragmented. The cDNA was synthesized with random hexamers *via* mRNA fragments as templates. The cDNA fragments were purified and resolved with EB buffer for end repair, single-nucleotide adenine (A) addition and adaptor connections. After PCR amplification, the 150 bp library was then sequenced via Illumina Hiseq™ 4000.

2.3 *De novo* Assembly, Reads Mapping and Gene Clustering

Clean reads were obtained by removing 'dirty' reads containing adapter sequences, sequences with more than 10% unknown bases, and low-quality reads containing more than 40% of low quality (Q-value < 10) bases. Clean reads from four tissues were pooled together to assemble a comprehensive reference transcriptome by Trinity v2.8.4 (Grabherr *et al*., 2011). Then clean reads from each sample were mapped against the reference transcriptome using alignment tool Bowtie2 (Langmead and Salzberg, 2012) by default parameters. RSEM v1.3.1 (Li and Dewey, 2011) was used to quantify the mapped reads. The gene abundances were calculated and normalized to the number of reads per kb per million reads (RPKM) (Mortazavi *et al*., 2008). Gene clustering was performed with RPKM in group A and group B using kmeans command of the R statistical software.

2.4 Primer Design and Real-Time qRT-PCR Assays

The primers were designed using Primer Premier 5 software (http://www.premierbiosoft.com/). A series of 5-fold of 5 dilutions of cDNA were made to determine the gene specific PCR amplification efficiency for each primer pair using the following equation: $\text{Efficiency}(\%) = 10^{(-1/\text{slope})} \times 100\%$.

The purified RNA samples were reversely transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China), following the manufacturer's protocol. Real-time qPCR was performed with Roche480 instrument and software, while QuantiNova™ SYBR Green PCR Kit (Qiagen) was employed for RT-PCR. The PCR mixture contained 0.5 μL diluted cDNA, 5 μL 2× SYBR Green PCR Master Mix, 0.7 μL forward (reverse) primers, and 3.1 μL distilled water in a final volume of 10 μL. Cycling conditions were 95℃ for 2 min, followed by 40 cycles of 95℃ for 5 s and 60℃ for 10 s and then a melt curve stage after the cycling stage. The melting curve and agarose gel electrophoresis for all the genes demonstrated single peaks and bands, confirming gene-specific amplification. Real-time PCR was performed in triplicate for each sample. A no-template control was analyzed in parallel for each gene.

2.5 Statistical Analysis

Following PCR data collection, geNorm (Vandesompele *et al*., 2002) was used to rank the expression stability of reference genes. Briefly, the geNorm program is based on pairwise comparisons and stepwise exclusion of candidate genes according to their expression stability measure (M) values. In general, the lower the M value, the higher the gene expression stability. The program recommends $M < 1.5$ to identify sets of reference genes with stable expression. The pairwise variation (GeNorm V: $n/(n+1)$ is used to determine the number of genes required for reliable normalization. A threshold value of 0.15 was reported by Vandesompele *et al*. (2002).

3 Results

3.1 Selection of New Candidate Reference Genes

We identified 5 distinct clusters representing a variety of gene expression patterns in different tissues (group A) and under different salinity stresses (group B) respectively. Genes in cluster 3 (1491/37451 unigenes) of group A and in cluster 1 (1954/33157 unigenes) of group B showed the more stable and higher expression trends (Fig.1). A total of 1472 unigenes are presented in both groups (Fig.2). Among those common unigenes, nine unigenes with high and stable expression trends were selected to be new candidate reference genes (Table 1). In addition, five commonly used reference genes, RO21, EF1A, TUA, TUB and GAPDH, were selected as candidate reference genes.

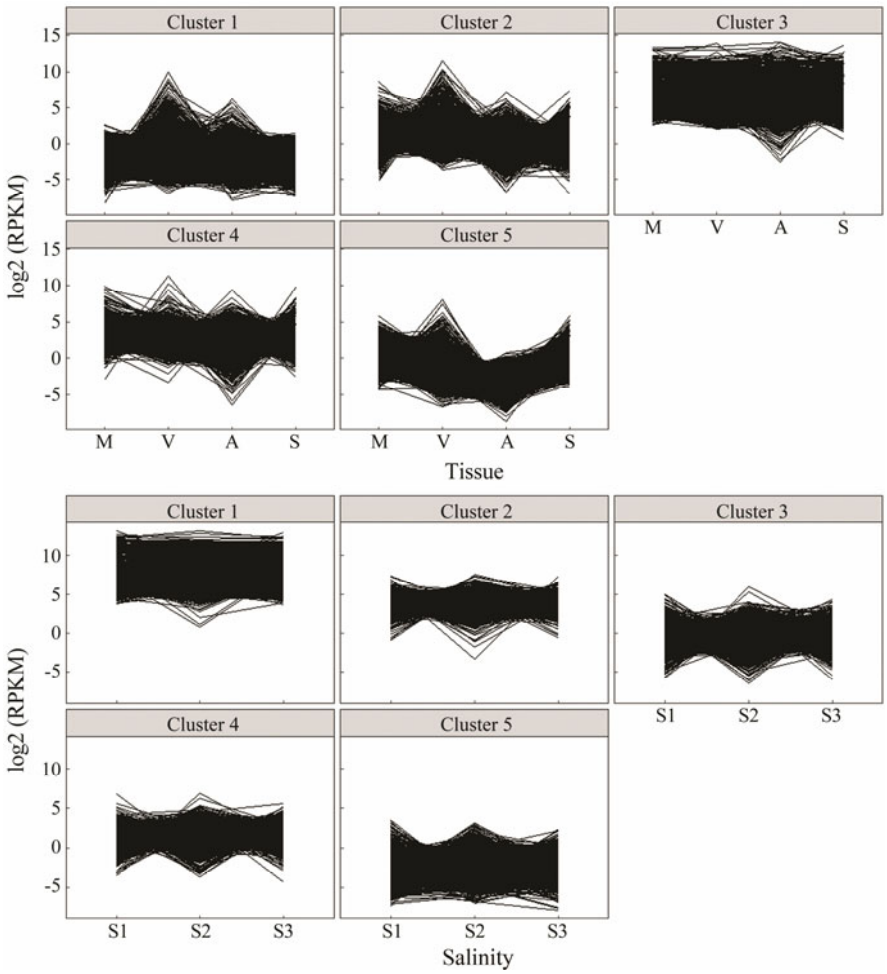

Fig.1 Clusters of genes in different tissues of *C. nippona* and under different salinity stresses. Different tissues include mantle (M), visceral mass (V), adductor muscle (A) and gill (S). Different salinity stresses include 30 (S3), 20 (S2), 10 (S1).

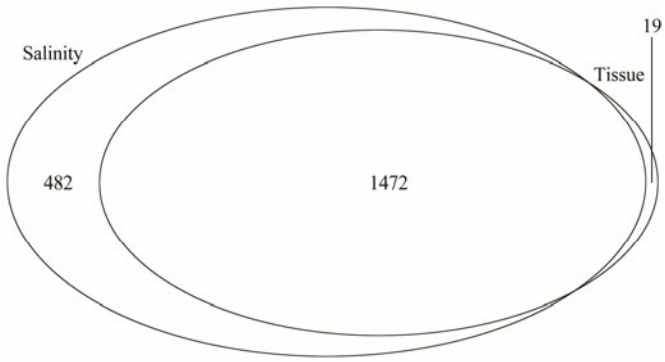

Fig.2 Venn diagram of genes in cluster 3 of different tissues and cluster 1 under different salinity stresses.

Table 1 Candidate reference genes and their primer sequences used for real-time PCR

Gene name	Abbreviation	Primer sequence (5'–3')	Product size (bp)
60S ribosomal protein L5	RPL5	F: CTGACCCATCACCAAAAGC R: GGTAGGCGACCTTCTTCTGC	120
60S ribosomal protein L8-like isoform X1	RPL8	F: CCAAAGTCAGAGGAGTAGCC R: CTGGCAGCAATAAGACCGAC	133
Ribosomal protein S27-1	RPS27	F: GCGTGGCTGAAAACTGTGGT R: TTGGAGGAGGAGAAAAGAAC	116
60S ribosomal protein L14	RPL14	F: TTTCCTGGACGCAGACCTCG R: GGACCCTGCTCTGGTGTATC	98
60S ribosomal protein L4	RPL4	F: AGCGATGGCAGAGCAGATAG R: AGAGGTGGACGAATGTTTGC	99

(*to be continued*)

(*continued*)

Gene name	Abbreviation	Primer sequence (5'–3')	Product size (bp)
Cytochrome c oxidase subunit III	CO3	F: CTATTAGTGGCATCTTCAGC R: ACAGACAGCCCCAAAGTAAC	107
40S ribosomal protein S8	RPS8	F: GATAAATGGCACAAGAGGAG R: AGACGAAGGGCTCTGTATTT	164
40S ribosomal protein S4	RPS4	F: AGGGACGCTTCACAGTTCAC R: GGGTGGTGATGTAGGGAACG	108
Cytochrome b	CYTB	F: AATAAACTCCACGGGCGAC R: ATTATTCGGCAGATGAGCAG	150
Elongation factor 1-alpha	EF1A	F: CTGGATGGCACGGAGATAAC R: GACGAAGAGGTAAGTCAGTTGG	162
Heterogeneous nuclear ribonucleoprotein A2/B1	RO21	F: TAGATTGGGCTGACCCTGTG R: CTGATGGTGGTTTGGCAAGT	273
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	F: CTACAGGGTGCTTCACTACT R: GATGTTCTGGTCTTTGGAGT	146
α-tubulin	TUA	F: CGAGGCTATCTACGACATCTGC R: GCCAGAGGGAAGTGGATACG	155
β-tubulin	TUB	F: CCTTAGCCCAGTTGTTTCCAG R: GCCAGAGGGAAGTGGATACG	192

3.2 Real-Time PCR Amplification of Candidate Housekeeping Genes

All the primer pairs amplified single PCR product with expected size, and the specificity of amplicon was confirmed by the single peak of the melt curve and the sequencing analysis. PCR efficiencies of primers ranged from 94%–106%. The mean *C*t values of reference genes ranged from 22.02–29.36.

3.3 Expression Stability of Candidate Housekeeping Genes

RPS4 (M=0.52), EF1A (M=0.52) and RPL4 (M=0.59) were the most stable genes in different tissues, while RPS8 (M=2.40), CO3 (M=2.02) and CYTB (M=1.7) were the least stable. RPL4 and RPS4 (M=0.21) were the most stable genes in gill tissue under salinity stress, while the most common used internal controls TUB (M=1.36), RO21 (M=1.05), TUA (M=0.97), EF1A (M=0.79) and GAPDH (M=0.67) appeared less stable (Fig.3).

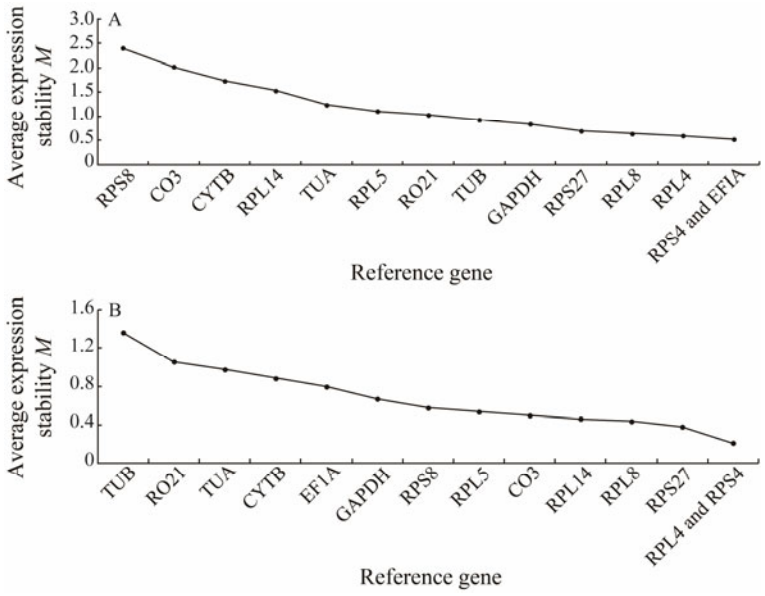

Fig.3 Expression stability of reference genes calculated by GeNorm in different tissues (A) and under different salinity stresses (B).

3.4 Optimum Number of Housekeeping Genes

The *V*2/3 value was 0.12 in different tissues, while it was 0.15 in gill tissue under salinity stress (Fig.4). This suggests that RPL4 and RPS4 can be chosen as reference genes to study the gene expression levels in *C. nippona* under salinity stress.

4 Discussion

This is the first study to analyze the stability of potential reference genes selected from transcriptome dataset of *C. nippona* under different salinity stresses. The candidate reference genes detected from transcriptome datasets are almost ribosomal protein genes and mitochondrial respiratory chain protein genes (CO3 and CYTB). The less

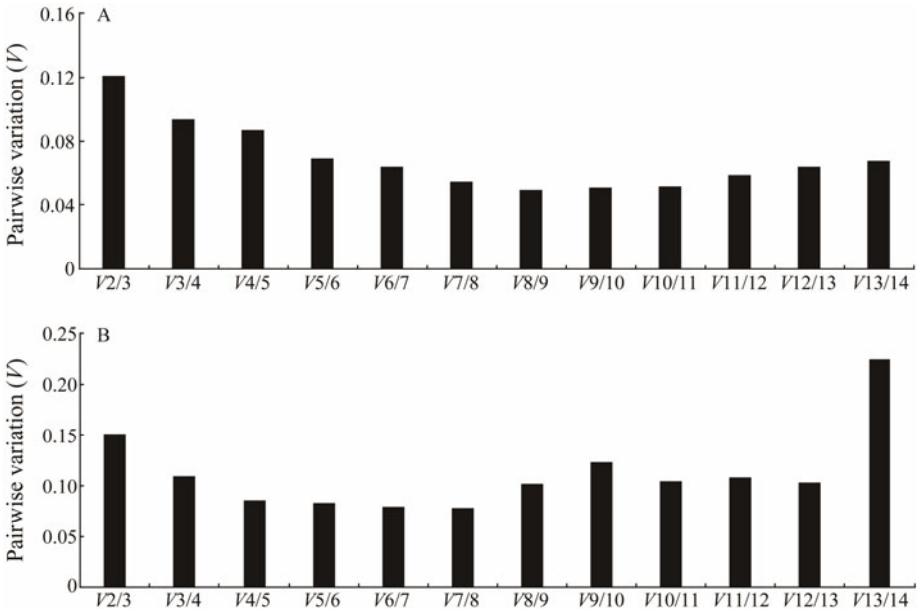

Fig.4 Determination of the optimal number of reference genes required for accurate normalization in different tissues (A) and under different salinity stresses (B), based on pairwise variation (Vn/n+1) between reference genes using GeNorm analysis.

stable expression of respiratory chain genes can be explained by the energy demand of the organism under different salinity conditions (Ellison and Burton, 2008). Ribosomal protein genes generally performed better than common housekeeping genes.

The ribosome, as a catalyst for protein synthesis, is universal and essential for all organisms. A mammalian ribosome has 79 ribosomal proteins, one more than a yeast ribosome encoded by 137 genes, of which L is for large subunit and S is for small subunit (Warner, 1999). Ribosome protein genes are considered good reference genes because of their participation in all types of cells for the synthesis of new ribosomes (Hisao *et al*., 2001). Several recently published reports have validated that ribosomal protein genes showed high stability in diverse abiotic and biotic conditions, indicating that they may become another source of reference genes (Shakeel *et al.*, 2018). They have been widely used as internal genes in both human and other animals, as well as in plants and algae (Barsalobres-Cavallari *et al.*, 2009; Rosic *et al.*, 2011; Liu *et al.*, 2012). RPL4 and RPS4 are the most stably expressed genes in different tissues under salinity stress in the current study. RPS4 was demonstrated to be the best housekeeping genes in the alga *Symbiodinium* under thermal stress (Rosic *et al.*, 2011).

EF1A, TUB, TUA, GAPDH and RO21 are commonly used as internal controls for qRT-PCR in oysters (Boutet *et al.*, 2004; Gonzalez *et al.*, 2007; Meistertzheim *et al.*, 2007). EF1A is a member of the G-protein family, which plays a key role in protein translation (Browne and Proud, 2002). Although EF1A has stable expression levels in the gill tissue under variant salinity stresses, it has less stable expression compared among each tissue, implying that it is not suitable to be a reference gene under salinity stress. The heterodimeric protein α, β-tubulin assembles the microtubule in a head-to-tail arrangement (McKean *et al.*, 2001). GAPDH functions in nuclear RNA export, DNA replication, DNA repair, exocytotic membrane fusion, cytoskeletal organization and phosphotransferase activity (Tristan *et al.*, 2011); RO21 is heterogeneous nuclear ribonucleo-protein (hnRNP) and plays a significant role in the regulation of mRNA-related processes (Siomi and Dreyfuss, 1997). None of these five commonly used housekeeping genes showed high stability under salinity stress, suggesting that they were unsuitable as internal controls in this situation.

The unstable expression levels of commonly used housekeeping genes mean that there is no 'one-size-fits-all' gene that can be used for the normalization of gene expression data under all conditions (Barber *et al.*, 2005). Vandesompele *et al.* (2002) have proposed the use of the mean expression level of several genes for normalization. Pairwise variation in the A and B groups were both below the cut-off value of 0.15, showing that the use of RPL4 and RPS4 as reference genes is sufficient in gene expression studies in *C. nippona* under salinity stress, irrespective of different tissues.

In conclusion, our data suggest that the novel genes detected from transcriptome data performed better than commonly used reference genes of *C. nippona*. The results of the present study will facilitate sensitive and accurate quantification of gene expression in *C. nippona*, which could also be extrapolated to related oyster species.

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