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Evaluation of reference genes for gene expression analysis in Japanese flounder (*Paralichthys olivaceus*) under temperature stress

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Abstract

Background Quantitative Real-time PCR (qRT-PCR) is a powerful technique to analyze gene expression patterns by measuring the relative abundance of mRNA transcription levels. The most crucial step in obtaining accurate results of qRT-PCR is to select suitable reference genes. Water temperature is an important factor that affects various physiological processes of fish. Presently, Japanese flounder is a commercially important marine culture species and the study of its gene expression is increasing rapidly. However, the reference genes used for Japanese flounder in previous studies, especially under temperature stress, only focused on those well-known genes widely reported in vertebrates, which might not be the proper reference genes.

Results In this study, we evaluated the suitability of eight genes including ribosomal protein L6 (*rpl6*), ribosomal protein L9 (*rpl9*), delta (4)-desaturase, sphingolipid 1 (*degs1*), cathepsin L (*ctsl*), eukaryotic translation elongation factor 1 gamma (*eef1g*), NSA2 ribosome biogenesis homolog (*nsa2*), eukaryotic translation initiation factor 3, subunit E, a (*eif3ea*), glutamine amidotransferase class 1 domain containing 1 (*gatd1*) analyzed from RNA sequencing (RNA-Seq) data and two genes including β-actin (*actb*) and 18S rRNA ribosomal RNA (*18S RNA*) selected from literature to obtain the best internal controls in qRT-PCR analysis of Japanese flounder under temperature stress. The statistical analysis methods (delta-Ct, BestKeeper, geNorm, and NormFinder) were further used to determine candidate reference gene stability. Initial results showed the suitability of eight genes from RNA-Seq data, which exhibited more stable expression levels than two commonly reported reference genes. Further analysis revealed that *gatd1* and *rpl6* were the best reference genes in Japanese flounder exposed to temperature stress.

Conclusion This study transcriptome-wide identified reference genes in different tissues of Japanese flounder exposed to temperature stress for the first time, providing a basis for gene expression research in flatfish.

Keywords Japanese flounder, Reference gene, qRT-PCR, Temperature stress, Transcriptome-wide

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Introduction

RNA sequencing (RNA-seq), northern blotting, microarrays and quantitative real-time reverse transcription PCR (qRT-PCR) are used for quantitative analysis of gene expression levels. Among these techniques, qRT-PCR is the first choice for analyzing gene expression due to its specificity, high sensitivity, accuracy and rapidity [1, 2]. The qRT-PCR determines the accumulation of amplification products by measuring fluorescence which can bind with the PCR products [3], and changes in gene expression can be monitored truly in real-time. However, the gene expression changes might be influenced by various factors, such as the template quantity and quality, enzymatic reactions and reference gene expression variation. The reference genes can reduce or remove the impact of the quantity and quality of different sample templates [4]. However, many studies still use reported reference genes, although some reported reference genes might be inappropriate in certain conditions [5]. For example, 18S rRNA ribosomal RNA (*18S RNA*) is a widely reported reference gene [6], which has been reported improperly in some species under certain circumstances [7, 8], as well as other traditional reference genes, including β-actin (*actb*) [9] and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) [10, 11]. Therefore, it is crucial to select the proper reference genes for normalization gene expression levels.

With the development of biotechnology, various methods have been used for reference gene identification, such as microarray analysis, gene expression serial analysis and RNA-seq analysis [12, 13]. Among them, RNA-seq has become the most useful tool to identify reference genes in recent years [14] and realized the identification of housekeeping genes in both model organisms and nonmodel organisms. Up to date, reference genes for specific situations have been identified from RNA-seq data in various organisms, widely including lettuce [15], gennadius [16], goat [17], scallop [9], black rockfish [14] and so on.

Japanese flounder (*Paralichthys olivaceus*) is a commercially important marine culture flatfish widely distributed in China, Korean Peninsula, Japan and so on. Temperature is one of the most prominent environmental factors, which influences the physiology, behavior and even survival of fish [18]. In the cultivation of Japanese flounder, the sustained high temperatures in summer and severe weather conditions cause high-temperature episodes frequently [19]. Compared with high-temperature stress, Japanese flounder has also been reported to be exposed to low temperatures due to natural or anthropogenic factors [20]. The studies on the gene expression of this species under temperature stress mainly via *actb* [21], *18S RNA* [22] and eukaryotic translation elongation factor 1 alpha 1 (*ef-1α*) [23] as the endogenous control for qRT-PCR. However, many studies have indicated that commonly reported reference genes might not be the most suitable under different conditions [24–27].

In this study, a total of 36 Japanese flounder transcriptome datasets under temperature stress were used to identify candidate reference genes that were more suitable for different temperature conditions. Eight candidate reference genes including ribosomal protein L6 (*rpl6*), ribosomal protein L9 (*rpl9*), delta (4)-desaturase, sphingolipid 1 (*degs1*), cathepsin L (*ctsl*), eukaryotic translation elongation factor 1 gamma (*eef1g*), NSA2 ribosome biogenesis homolog (*nsa2*), eukaryotic translation initiation factor 3, subunit E, a (*eif3ea*), glutamine amidotransferase class 1 domain containing 1 (*gatd1*), and two reported reference genes containing *18S RNA* and *actb* were further analyzed and validated by four criteria and qRT-PCR. The results revealed that candidate genes were more stable than commonly used reference genes, which could provide better reference genes for normalization analysis of qRT-PCR in Japanese flounder under temperature stress.

Materials and methods

Sample preparation

The Japanese flounder (total length: 40.5±1.5 cm; weight: 750.5 ± 67.8 g) were sampled from Nanshan market, Qingdao, Shandong Province, China. After acclimation in the laboratory environment (18℃, 30 ppt, light:dark=14:10 h) for a week, the healthy Japanese flounder were divided into three groups randomly: control group (CG), heat stress group (HSG) and cold stress group (CSG). The temperature and treatment time of this experiment were set according to the pre-experiment and our previous studies [28, 29]. The water temperature in CG was 18℃, in HSG was increased to 28℃ at the rate of 1°C/h and maintained for 8 hours, and in CSG was decreased to 8°C at the rate of 1°C/h and remained constant for 8 hours. After temperature stress, the fish were euthanized with MS-222, and then one part of the gill, liver, spleen and heart of each group were collected in liquid nitrogen and stored at −80°C, and the other part of these tissues was fixed in PFA (Boster Biological Technology, USA). Throughout the entire experiment, all the fish were in good condition and did not die.

Histological observation

After fixation 48 h in PFA reagent, the tissues were dehydrated with different methanol concentrations (30%-50%-70%-80%-90%-95%-100%) and rinsed using ethanol and xylene. Then the tissues were embedded with paraffin and sectioned into 5 μm by microtome (Leica, Germany). The slices were stained with hematoxylin and

eosin (Solarbio, China), and observed and photographed using a Nikon Eclipse TiU microscope (Nikon, Japan).

Total RNA extraction and sequencing

The total RNA of four tissues was extracted by TRIzol (Invitrogen, USA) following protocol, and the genomic DNA was removed by RNase-free DNase I (TaKaRa, China). The RNA quality was detected by 1.5% agarose gel electrophoresis, and the concentration was determined using a Nano photometer (Implen, Germany). The libraries of four tissues were constructed via NEBNext® UltraTM RNA Library Prep Kit (NEB, USA) of Illumina, and sequencing was conducted on the Illumina NovaSeq 6000. Raw reads were assessed by Fast QC, and clean reads which eliminated low-quality reads were mapped to the Japanese flounder genome (BioProject ID PRJNA73673). Transcripts per kilobase million (TPM) were used to analyze gene expression levels.

Reference genes identification from transcriptome data

The methods of selecting reference genes in Japanese flounder under temperature stress were performed following the description by Li *et al* [9]. In brief, four criteria were used to identify reference genes: (I) the expression level could be detected in four tissues under temperature stress; (II) tissues showed a low variance with standard deviation $\log_2$(TPM)<1; (III) no tissue exhibited the abnormal expression with a difference of more than two between $\log_2$(TPM) and mean $\log_2$(TPM); (IV) genes had medium to high tissues expression level with mean $\log_2$(TPM)>5. In addition, the CV value (stdev/mean) was further used to evaluate the stability of the reference gene. In our study, the CV value of candidate reference genes with mean $\log_2$(TPM)>5 and standard deviation $\log_2$(TPM)<1 was less than 0.1.

cDNA synthesis and qRT-PCR

The cDNA was synthesized using the Reverse Transcriptase M-MLV kit (TaKaRa, China) with a total 20 µl volume including 1 µg RNA, and stored at −20°C until use. In total, ten genes including eight new candidates and two commonly used reference genes were selected for qRT-PCR. The primers of these genes were designed by IDT (https://sg.idtdna.com/) and listed in Table 1. The qRT-PCR was conducted using a Light Cycler 480 (Roche, Switzerland) with 2µL cDNA (5 ng/µL), 10 µL SYBR qPCR SuperMix Plus, 7.2 µL ddH_2O, 0.4 µL forward and reverse primers. Each sample included three biological replicates, and each biological replicate had three technical replicates.

Table 1 List of candidate reference genes primer pairs and qRT-PCR efficiency

Gene name	Accession number	Primer sequence (5′–3′)	Correlation coefficient	qRT-PCR efficiency (%)
rpl6	XM_020099166.1	F: GAACCTGAATGACGCCTAC R: TCTGTCAGCTGGTACTTCT	0.995	94.09
ctsl	XM_020109356.1	F: CGAATCGTTCCAGTTCTACC R: TTCACCCTCGAAACCATAAC	0.990	97.37
rpl9	XM_020100366.2	F: GTTGTCGTCGATGTCGTATC R: GACTGCTGAGAATGGTCTTC	0.994	95.16
eef1g	XM_020082283.2	F: TGGGACAGGATGGTGTAA R: CTGTCGTTCCAAGTCATTCT	0.998	99.33
nsa2	XM_020081565.2	F: TATGCCCAGGTGACGAATA R: CCTGACTTGTTGCGTCTTT	0.992	94.84
eif3ea	XM_020091458.2	F: CGTCAGGAGTATTTGGACAC R: AAGACGCGGAAGAAGTAAAG	0.991	91.71
gatd1	XM_020079009.2	F: GGTGGATCTTCAACGGATAC R: GATCCTCCACTGTCTTTCAC	0.999	103.37
degs1	XM_020103564.1	F: CTACTCTGCCTCCTTCAAAC R: AGAACCAGCCTTCAAACTC	0.994	97.71
18S RNA	EF126037	F:GGTCTGTGATGCCCTTAGATGTC R: AGTGGGGTTCAGCGGGTTAC	0.991	99.92
actb	EU090804	F:GAGATGAAGCCCAGAGCAAGAG R: CAGCTGTGGTGGTGAAGGAGTAG	0.989	101.01

qRT-PCR data analysis

The ReFinder (https://www.heartcure.com.au/reffinder/) was further conducted to evaluate the reference genes' stabilities in our study, including four commonly used approaches: geNorm, NormFinder, Bestkeeper and delt-Ct method [30–32]. The comprehensive analysis used the geometric mean to calculate the stability of the reference genes [33].

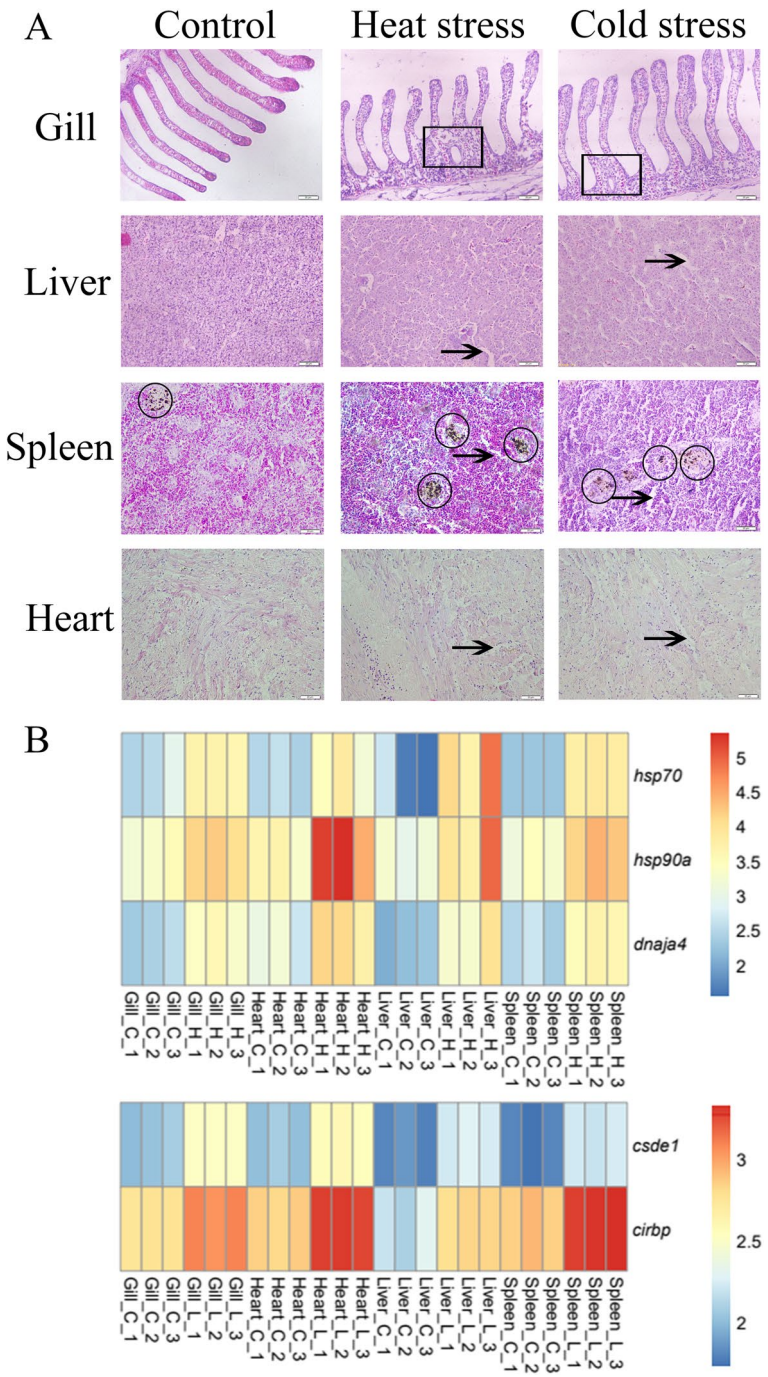

Fig. 1 Evidence of temperature stress on Japanese flounder. **A** Histopathological structures of four tissues under temperature stress. Black arrows represented tissue distance, rectangular represented gill cells fusion and circle represented melanin macrophage center. Scale bar = 20 μm. **B** Heatmap of gene expression after temperature stress. Each cell in the heatmap corresponded to an expression level which was normalized into $\log_{10}(\text{TPM}+1)$. L was low temperature stress, C was control group and H was high temperature stress. X-1, X-2 and X-3 were the three biological replications

Results

Evidence of Japanese flounder subjected to temperature stress

To determine whether the Japanese flounder were subject to temperature stress, the histological observation of four tissues and heatmap of temperature stress-related genes were proceeded. The HE staining results showed that Japanese flounder suffered temperature stress (Fig. 1A). Specifically, the gill lamellae swelled and cells fused, the liver cells interstitially enlarged, the spleen's white and the red pulps arranged irregularly and the melanin macrophage center number increased, and the cardiomyocytes sarcoplasmic distance widened after temperature stress. In addition, the heatmap further demonstrated that Japanese flounder was subjected to stress, with significant changes in the gene expression of DnaJ heat shock protein family member A4 (*dnaja4*), heat shock protein HSP70 (*hsp70*) and heat shock protein 90a (*hsp90a*) after high-temperature stress, cold shock domain containing E1 (*csde1*) and cold inducible RNA binding protein (*cirbp*) after low-temperature stress (Fig. 1B). These genes had been used to determine whether an individual was under temperature stress in many researches [34, 35]. All these results indicated that Japanese flounder was subjected to temperature stress.

Identification of reference genes from Japanese flounder transcriptome

The original data of RNA-seq had been submitted to NCBI, the accession numbers of CSG were PRJNA717098, PRJNA717103, PRJNA717106 and PRJNA717107, the accession numbers of CG were PRJNA716172, PRJNA715052, PRJNA716759 and PRJNA716811, and the accession numbers of HSG were PRJNA716735, PRJNA716837, PRJNA716860 and PRJNA717095. The candidate reference genes of Japanese flounder under temperature stress were identified from 24,419 genes in 36 RNA-seq databases. According to the four criteria described above, 11,372 (46.57%), 3,997 (13.91%), 2,319 (9.49%) and 1,016 (4.16%) genes were obtained respectively (Fig. 2). Then the CV value was further used to estimate and screen the candidate reference genes, and eight genes with the lowest CV value were finally selected (Table 2). These candidate reference genes had higher expression levels with mean $\log_2$(TPM) changed from 5.60 to 12.14, and lower CV values varied from 0.036 to 0.067.

RNA-seq analysis of candidate reference genes expression levels

To evaluate the stability of candidate (*rpl6*, *rpl9*, *eef1g*, *nsa2*, *eif3ea*, *ctsl*, *gatd1* and *degs1*) and reported (*18S RNA* and *actb*) reference genes, the $\log_2$(TPM) values

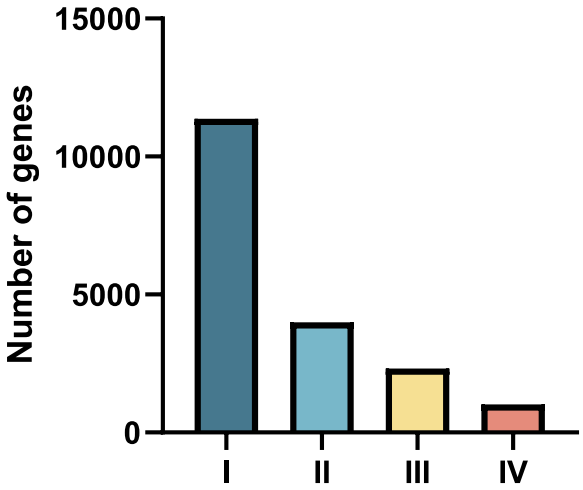

Fig. 2 Gene numbers after four criteria screening in Japanese flounder transcriptome data. (I) TPM > 0; (II) standard deviation $\log_2$(TPM) < 1; (III) $\log_2$(TPM) differed from mean $\log_2$(TPM) less than two; (IV) mean $\log_2$(TPM) > 5

from RNA-seq were used. As shown in Fig. 3, the *18S RNA* showed the highest variance with $\log_2$(TPM) ranging from 0.3 to 10.7 in Japanese flounder tissues under temperature stress, and the *actb* gene also exhibited a relatively high variance, which changed from 9.1 to 13.9. On the contrary, the variance of $\log_2$(TPM) in eight candidates identified from transcriptome was much smaller in different tissues faced with temperature stress. The *gatd1* ranged from 5.4 to 6.7, *rpl6* ranged from 11.2 to 13.0, *rpl9* ranged from 11.2 to 12.8, *eef1g* changed from 9.7 to 11.3, *degs1* ranged from 4.6 to 6.5, *ctsl* ranged from 7.2 to 10.3, *nas2* ranged from 7.5 to 9.3, and *eif3ea* ranged from 7.9 to 9.9, respectively. In a word, the candidate reference genes identified from transcriptome datasets were more stable than reported reference genes in different tissues of Japanese flounder under temperature stress.

qRT-PCR and stability analysis

The qRT-PCR was performed to further validate the candidate and reported reference genes, and the results were consistent with the transcriptome data, as evidenced by the more stable Ct values of the candidate reference genes compared to the reported reference genes. The Ct values of these reference genes in different tissues were shown in Fig. 4. The reported reference genes *18S RNA* (Ct, 12.3-29.2) and *actb* (Ct, 13.4-25.2) had a higher variation of Ct values than the candidate reference genes, including *gatd1* (Ct, 26.5-33.7), *rpl6* (Ct, 19.7-24.8), *rpl9* (Ct, 18.3-24.8), *eef1g* (Ct, 20.9-26.3), *nas2* (Ct, 26.1-32.2), *eif3ea* (Ct, 20.9-27.6), *degs1* (Ct, 27.6-32.5) and *ctsl* (Ct, 22.4-28.8). In order to determine the most optimal reference genes, four methods (geNorm, NormFinder, Bestkeeper and delt-Ct)

Table 2 Detail information on 8 selected candidate reference genes and 2 commonly used reference genes

Gene name	Description	Mean	Stdev	CV value
rpl6	ribosomal protein L6	12.14	0.46	0.037
rpl9	ribosomal protein L9	12.09	0.42	0.038
eef1g	eukaryotic translation elongation factor 1 gamma	10.39	0.37	0.036
nsa2	NSA2 ribosome biogenesis homolog	8.70	0.45	0.052
eif3ea	eukaryotic translation initiation factor 3, subunit E, a	8.69	0.40	0.046
ctsl	cathepsin L	8.62	0.47	0.040
gatd1	glutamine amidotransferase class 1 domain containing 1	6.06	0.40	0.067
degs1	delta(4)-desaturase, sphingolipid 1	5.60	0.35	0.062
18S RNA	18S rRNA ribosomal RNA	6.74	3.48	0.515
actb	β-actin	11.84	1.25	0.106

and comprehensive analysis were applied to compare the expression stability in candidate and reported reference genes (Fig. 5). According to BestKeeper method, *rpl9* and *rpl6* were identified as the best reference genes. While *rpl6* and *gatd1* were the most stable genes under the other four analysis methods (geNorm, NormFinder, delt-Ct and Comprehensive analysis). Although different statistical analysis methods had different gene stability rankings, the candidate gene selected from RNA-Seq data were much more stable than the reported reference genes. All these results illuminated that the reference genes identified from transcriptome were more conductive to the normalization of gene expression. We believed that the *rpl6* and *gatd1* genes were the most stable reference genes for Japanese flounder under temperature stress.

Discussion

qRT-PCR is a reliable gene expression analysis tool, and its application in aquaculture research has been increasing rapidly [36]. The most commonly used reference genes in fish are directly selected from the reference genes reported in mammals [14]. The *gapdh*, *18S rRNA* and *actb* are mammals' historically popular housekeeping genes for reference genes in qRT-PCR [37], but they have been found to be not the optimal choices for teleost. For example, ribosomal protein S18 gene (*rps18*) and 28S ribosomal protein S5 gene (*rps5*) are reported as the most suitable reference genes for polyploid carp [27]. In crimson snapper, ras-related protein RAB-10 (*rab10*) and prefoldin subunit 2 (*pfdn2*) have exhibited relatively stable expression in astaxanthin treatment [38]. It is well-known that inappropriate reference genes might cause normalization errors, making qRT-PCR results unreliable. Therefore, the selection of optimal reference genes is critical in specific species under different experimental or environmental conditions.

In this study, the selection criteria of reference genes were performed as described by Li *et al* [9], which were modified from the methods introduced in a previous study [12]. Interestingly, 231 and 349 candidate reference

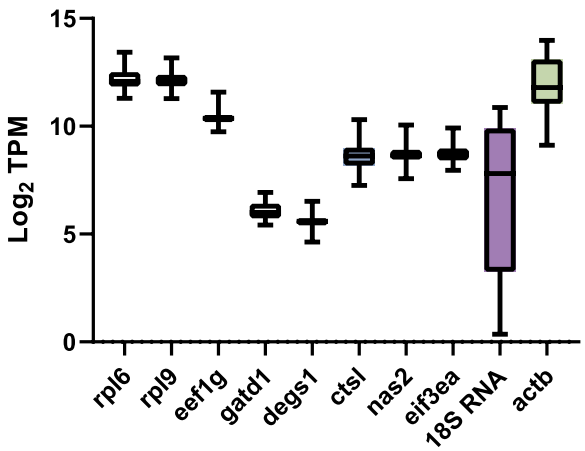

Fig. 3 Evaluation of new candidate and reported reference genes based on RNA-seq data. The boxplots showed the $\log_2$(TPM) values in the new eight candidates and two reported reference genes of Japanese flounder under temperature stress

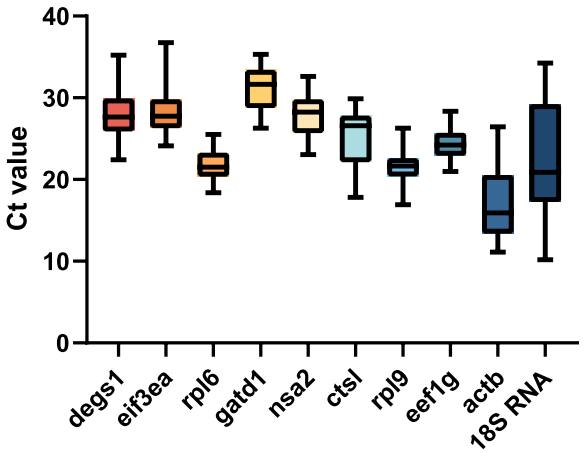

Fig. 4 Evaluation of new candidate and reported reference genes based on qRT-PCR results. The boxplots showed the Ct values in eight new candidates and two reported reference genes of Japanese flounder under temperature stress

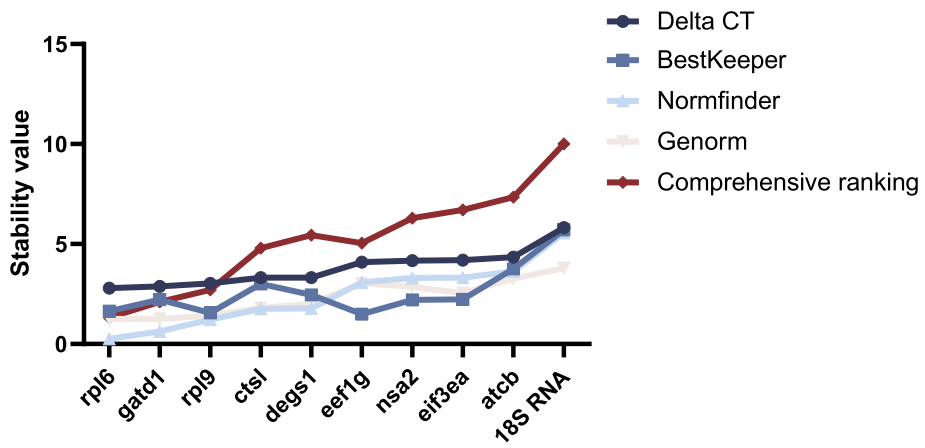

Fig. 5 The expression stability of new candidate and reported reference genes based on qRT-PCR results. The stability was evaluated by geNorm, NormFinder, Bestkeeper, delt-Ct and comprehensive ranking of the qRT-PCR results, with smaller values indicating greater stability

genes were identified in low and high temperature stress, respectively. This result was similar to other studies [31, 39], which could further indicate the importance of selecting the most optimal reference genes. Based on the overlap reference genes under low and high temperature stress, eight candidate reference genes (*gatd1*, *rpl6*, *rpl9*, *eef1g*, *nas2*, *eif3ea*, *ctsl* and *degs1*) with the lowest CV value and two traditional reference genes (*18S RNA* and *actb*) selected from previous studies [21, 22] were used for choosing the most optimum reference genes in Japanese flounder under temperature stress.

Subsequently, different algorithms geNorm, NormFinder, Bestkeeper, delt-Ct and comprehensive ranking had been further to evaluate the stability of reference genes based on qRT-PCR results. The results of all the algorithms used in this study were very similar in which the candidate reference genes from RNA-Seq data were much more stable than traditional reference genes. Glutamine amidotransferases (GATs) play a central role in catalyzing the synthesis of different aminated products, with *gatd1* being a member of GATs and ubiquitously expressed in all cells [40]. Ribosomal proteins are the components of ribosomes, which not only participate in protein folding but also have extra-ribosomal functions [41]. The *rpl6* is involved in maintaining the stability of genetics and regulating DNA damage repair [42], and *rpl9* is a component of the large subunit of the ribosome [43]. Similar to the present study, *rpl6* has been characterized by high stability in *Octopus minor* gill under acute ammonia stress [44], and *rpl6* and *rpl13* are the most optimal reference gene combination for gene expression in *Mylabris sibirica* under diverse temperatures [45]. Other genes including *eef1g* [46] and *eif3* [47] play a central role in the steps of protein biosynthesis. The gene *nas2* promotes cell growth in different cells and regulates the cell cycle, which is also related to ribosome biogenesis [48]. The *ctsl* is a cysteine endopeptidase that mainly exists in lysosomes and participates in protein degradation [49], while *degs1* is a necessary early signal in many eukaryotic organisms [50]. All these candidate reference genes are involved in protein biosynthesis and degradation, cell cycle and catalytic activity, indicating they might be suitable for reference genes. The stability of candidate genes was *rpl6*>*gatd1*>*rpl9*>*ctsl*>*eef1g*>*degs 1*>*nas2*>*eif3ea*. In our study, *rpl6* was almost the optimal single reference gene in all the algorithms, except Bestkeeper. However, many studies have reported single gene as a reference gene is unstable [51, 52]. Therefore, we strongly recommend using genes *rpl6* and *gatd1* to normalize gene expression in Japanese flounder under temperature stress.

In addition, we noticed the traditional reference genes *actb* and *18S RNA* showed high variability in all tissues in this study, illuminating that they were not recommended as the appropriate reference genes for qRT-PCR analysis of Japanese flounder under temperature stress. All these results illustrated the choices of reference genes were closely related to species and experimental conditions. Therefore, it is important to evaluate potential reference genes to ensure the selection of the best for specific environments and species before gene expression-related experiments.

Conclusion

In conclusion, our study investigated eight candidate reference genes and two traditional reference genes to determine the most reliable reference genes for normalizing gene expression in Japanese flounder under different temperature conditions. The stability of traditional

reference genes was much lower than that of identified candidate reference genes from RNA-Seq data, and we recommend using *rpl6* and *gatd1* as the best reference genes for gene expression analysis under temperature stress. These data emphasized the necessity for selecting proper reference genes in any analysis of experimental expression and provided the basis for analyzing the gene expression levels of Japanese flounder.

Abbreviations

rpl6	Ribosomal protein L6
rpl9	Ribosomal protein L9
degs1	Delta (4)-desaturase sphingolipid 1
ctsl	Cathepsin L
eef1g	Eukaryotic translation elongation factor 1 gamma
nsa2	NSA2 ribosome biogenesis homolog
eif3ea	Eukaryotic translation initiation factor 3
subunit E, a, *gatd1*	Glutamine amidotransferase class 1 domain containing 1
actb	β-Actin
18S RNA	18S rRNA ribosomal RNA
RNA-Seq	RNA sequencing
qRT-PCR	Quantitative Real-time PCR
TPM	Transcripts per million
CV	Coefficient of variation
FPKM	Fragments per kilobase of exon model per million mapped fragments

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11285-7.

Supplementary file 1.

Supplementary file 2.

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Authors' contributions
XW, SR and JC designed the experiments. PH performed the experiments and drafted and revised the manuscript. ZS conducted the statistical analysis. All the authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All the experiments in the research involving animals were conducted following relevant guidelines, including the Utilization Committee of Ningbo University and the China Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (State Science and Technology Commission of the People's Republic of China for No. 2, October 31, 1988. http://www.gov.cn/gongbao/content/2011/content_1860757.htm).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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