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Selection of Appropriate Housekeeping Genes for Gene Expression Normalization in Hybrid Grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂)

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Abstract

Since lots of researches using qPCR technique have addressed gene expression in groupers, the suitable housekeeping genes are needed to obtain reliable and precise results. In this study, by using different mathematical algorithms (Genorm, normFinder, BestKeeper and comparative ΔCt method) and the comprehensive ranking software (RefFinder), the stability of seventeen housekeeping genes were assessed in eleven tissues of the hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂). Results showed that the stability of housekeeping genes were various in different tissues and the newly identified housekeeping genes were likely more stable than that of commonly used. In conclusion, the expression stability of housekeeping genes showed a tissue-specific manner, and the *conserved oligomeric Golgi complex subunit 5* (*cog5*) and *peroxisomal biogenesis factor 14* (*pex14*) could be universally used as appropriate housekeeping genes across different tissues in hybrid grouper. The present study provides the foundation for more accurate results of qPCR assay in hybrid grouper and other groupers.

Introduction

Gene expression analysis reveals the key information about the molecular mechanisms regarding physiological process and pathways in organisms (Wang et al., 2018). Quantitative real-time reverse transcription polymerase chain reaction (qPCR) has become a popular technique for gene expression research on account of its accuracy, specificity and sensitivity (Ma et al., 2019; Wang et al., 2017). Therefore, it is crucial to evaluate suitable reference genes to choose the best performing candidate in all the experimental settings to prevent the misinterpretation of qPCR output and to get an accurate gene expression profile. In relative quantification, the normalized expression of target genes of each treatment group will be compared to control group for obtaining the fold change in transcriptional level as the consequence of treatment. Due TO the ease operation and consistent results, relative quantification is much popular than absolute (Kamran et al., 2017). The reference genes are usually structure genes that indispensable to sustain the basic function of cell, and should be high stability under a series of different biological or experimental conditions (including organs, developmental stage, etc.) in theory (Kamran et al., 2017). But researches have revealed that given housekeeping genes could vary significantly and present different expression patterns in differ experimental conditions, which could seriously influence the interpretation of qPCR results (Lu et al.,

2018). For example, the reference genes β-actin (*actin*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), α-tubulin (*tuba*), elongation factor-1-α (*ef1a*) and 18S ribosomal RNA (*18s*), were showed considerable variations of expression across different cellular conditions, and ignored this phenomenon may result in the wrong normalization in qPCR assay (Wang et al., 2017; Zheng & Sun, 2011). Furthermore, the expression stability of housekeeping genes exhibited a tissue-specific way in rainbow trout (Kamran et al., 2017), *Pelteobagrus fulvidraco* (Ye et al., 2019), a freshwater silverside fish (Rojas-Hernandez et al., 2019) and other teleost fishes (Li et al., 2020). So, the selection of appropriate housekeeping genes in specific tissues is an essential procedure for the study of gene expression in fish.

The hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) is a kind of popular economic marine fish in Asia, and has a great potential in aquaculture industry due to its rapid growth and delicate taste (Yin et al., 2018). Recently, in order to promote the researches of nutrition, development, endocrine, etc. in grouper, more and more studies have used qPCR technique to explore the functional genes referred to these aspects (Shapawi et al., 2019). Nearly all of qPCR researches on groupers currently have selected *18s* or *actin* as the housekeeping genes (Wang et al., 2017). However, one research in grouper (*E. akaara*) revealed the instability of a few commonly used housekeeping genes (such as *actin* and *gapdh*), and indicated that some newly identified housekeeping genes were more stable, such as conserved oligomeric Golgi complex subunit 5 (*cog5*) and ADP ribosylation factor guanine nucleotide exchange factor 1 (*arf1*) (Wang et al., 2017). So, the selection of appropriate housekeeping genes from either commonly used and newly identified is necessary for accurately studying gene expression in hybrid grouper.

In this study, the gene expression datasets from seventeen reference genes in eleven organs of hybrid grouper were analyzed by four mathematical algorithms (including the Genorm, normFinder, BestKeeper and comparative ΔCt method) and the comprehensive ranking software (RefFinder) (Wang et al., 2017). The purpose of this research was to assess and determine the stability of candidate housekeeping genes, and explore whether the most suitable reference genes showed a tissue specific manner. So far, this study was the first verification and evaluation of the expression stability of housekeeping genes in hybrid grouper. This work would be helpful in improving the precise of qPCR assay for studying gene expression in hybrid grouper and other groupers.

Materials and methods

This study was carried out following the recommendations for the Care and Use of Laboratory Animals in China, Animal Ethical and Welfare Committee of China Experimental Animal Society. The protocol was approved by the Animal Ethical and Welfare Committee of Guangdong Ocean University (Guangdong, China), processing ID: GDOU-AEWC-20180063.

Fish culture and sample collection

The juveniles (before sex differentiation, body weight 21.31 ± 0.12g) of hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) were purchased from a local fish farm (Zhanjiang, China) and acclimatized as described before (Yin et al., 2018). After the period of acclimation and fasting for 24h, all fish were euthanized with MS-222 (100 mg/L). Eleven organs including brain, gill, head kidney, spleen, heart, liver, trunk kidney, white muscle and proximal, mid, distal intestine from six fish (pool of two fish in each replication, three replications in all) were collected, frozen in liquid nitrogen and stored at -80 °C for the next assay. All animals experimental process strictly conformed to the guidelines for the care and use of animals for scientific purposes set by the Ministry of Science and Technology, Beijing, China (No. 398, 2006).

Total RNA extraction and cDNA synthesis

The total RNA of the sampled tissues was extracted by conventional method (TRI Reagent solution, Invitrogen, Carlsbad, CA, USA). The RNA quality and quantity were evaluated by electrophoresis in 1.5 % agarose gel and NanoDrop 2000 spectrometer (Thermo Scientific, Delaware, USA). The cDNA sample of extracted total RNA (amount of substance for each sample = 800 ng) was synthesized using the methods of PrimeScript®RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's instructions.

Primers of housekeeping genes

The candidate housekeeping genes were selected from publications on gene expression in groupers from PubMed (K. Anderson et al., 2018; K. C. Anderson et al., 2018; Wang et al., 2017), including eight commonly used reference genes: *actin*, *ef1a*, *gapdh*, hypoxanthine phosphoribosyltransferase 1 (*hprt1*), ribosomal protein L8 (*rpl8*), *18s*, β-2-microglobulin (*b2m*), *tuba*, and nine newly identified reference genes: *cog5*, putative ATP-dependent RNA helicase dhx30 (*dhx30*), neuron navigator 3 (*nav3*), homeodomain-interacting protein kinase 3 (*hipk3*), probable E3 ubiquitin-protein ligase MYCBP2 (*myc2*), E3 ubiquitin-protein ligase MGRN1 (*mgr1*), TATA binding protein (*tbp*), peroxisomal biogenesis factor 14 (*pex14*) and *arf1*. Primers of these genes were shown in Table 1 and synthesized by the Shanghai Sheng gong Co. The products length and reaction efficiency value of qPCR were shown in Table 1. The specificities of all the primers were demonstrated by the single bands of expected size in agarose gel

electrophoresis and by the single-peak melting curves of the qPCR products. The standard curves were determined for each primer set by generating standard curves of Ct values from serial dilutions (1x, 10x, 100x and 1000x) of the cDNA samples and plotting the observed Ct values against the log transformed template concentration. After determining the slope of the standard curve, the homologous qPCR reaction efficiency (E) value were calculated according to the equation: $E = (10[^{-1/slope}] - 1) \times 100$ and E value in this study were between 92%-102%.

qPCR analysis

The expression level of reference genes in sampled tissues were analyzed by qPCR on LightCycler®480 II (Roche Diagnostics GmbH, USA) using 384-well plates. The qPCR mixture reaction contained the SYBR Premix Ex Taq™ (5 µL), ddH_2O (3.6 µL), forward and reverse primers (0.4 µL, 10 µmol L^{-1}) and cDNA template (1 µL, 40 ng μL^{-1}). Each sample was repeated in triplicate and the procedures of qPCR contained: 5 min at 95°C; 45 cycles of 10 s at 95°C, 10 s at 58 °C, 10 s at 72 °C.

Gene expression stability analysis

The appropriateness of the all housekeeping genes were determined by Genorm (Vandesompele et al., 2002), normFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and comparative ΔCt method (Silver et al., 2006), and ranked them with a comprehensive tool RefFinder (Xie et al., 2012). The geometric means of the comprehensive ranking order of commonly used and newly identified reference genes were calculated by comparing to each other. The generm program calculates the M value, which is the average pairwise variations of one housekeeping gene against to other genes, and the lower M value indicates the higher

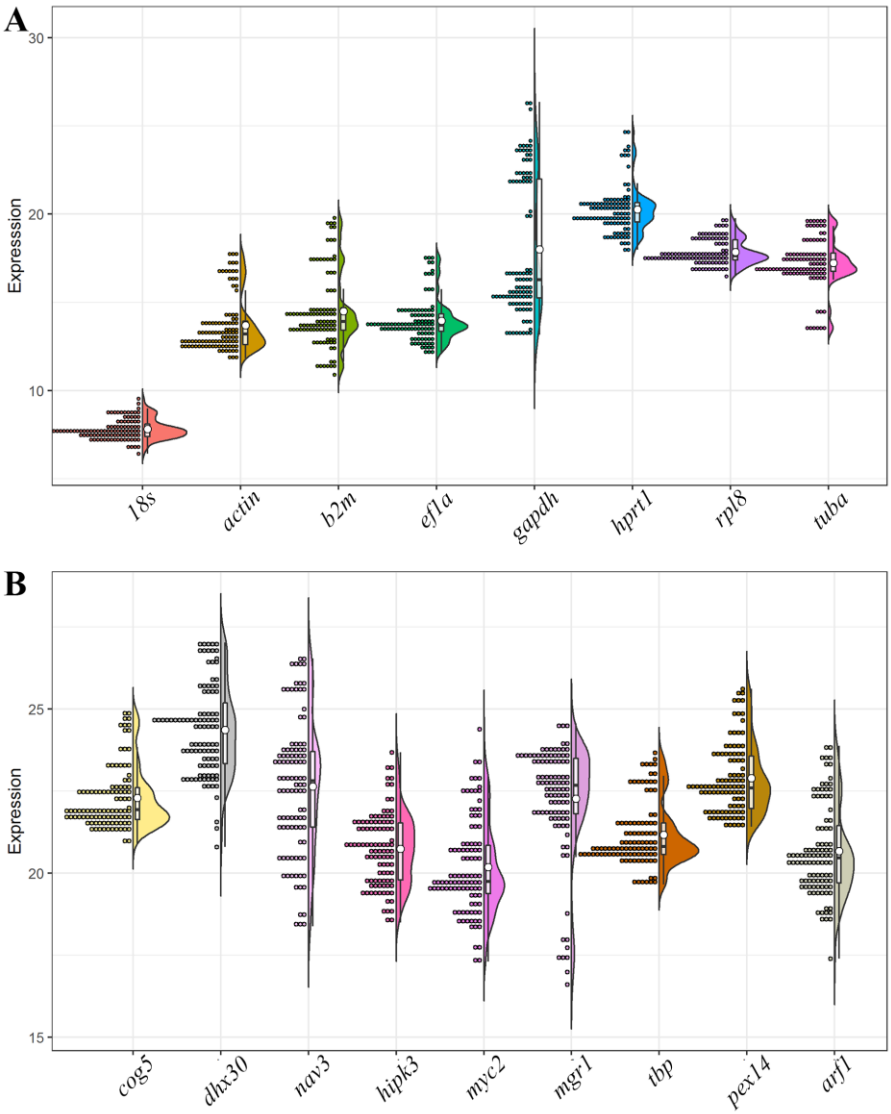

Figure 1. Expression levels of the reference genes. The expression of eight commonly used reference genes (**A**) and nine newly identified reference genes (**B**) across different tissues are showed by half violin plot (comprising with half of the box, beeswarm and violin plot). Values are Ct values. In box plot, a line across the box depicts the median; the box indicates the 25th and 75th percentiles; upper/lower whisker represents the upper/lower extreme value; the longer boxes and whiskers mean the greater variations. In beeswarm plot, the values that difference below 0.24 are shown as plots that arranged one line.

stability. In addition, when the value of Vn/n+1 in Genorm below the cutoff value (0.15), thn is the optimal number of housekeeping genes required. The normFinder analysis the variations of intra-group and inter-group, and the lower variations indicates the higher stability. The Best Keeper calculates the standard deviation (SD) of variations of each housekeeping gene, and the lower SD indicates the higher stability. The comparative ΔCt method calculates the average SD (ASD) of variations of ''pairs of genes'' in each group, and the lower ASD indicates the higher stability (Kamran et al., 2017). According to the ranking orders from above four methods, the RefFinder assigns a proper weight to each housekeeping gene and re-calculates the finally comprehensive ranking (Xie et al., 2012).

Results

Expression stability of housekeeping genes across different tissues

As shown in Figure 1, the CT values of all housekeeping genes across different tissues were ranged between 6.42 and 27.03. Meanwhile, *18s* and *tbp* had least variation (interquartile range), whereas *gapdh* had largest variations. The *cog5* and *pex14* had the least M value in Genorm (Figure 2A), the least variable value in normFinder (Figure 2B) and the least ASD in comparative ΔCt method (Figure 2C); the *18s* and *rpl8* had the least SD in BestKeeper (Figure 2D). The generm analysis displayed that the value of $V_{2/3}$ across and in different tissues were less than 0.15 (Figure 3A and Figure S1), therefore, two reference genes were required in hybrid grouper. According to RefFinder, the comprehensive rankings of housekeeping genes (unless if stated otherwise, all rankings are showed from the most to the least stable) were as follows: *cog5*, *pex14*, *tbp*, *rpl8*, *arf1*, *18s*, *hipk3*, *myc2*, *ef1a*, *hprt1*, *dhx30*, *tuba*, *actin*, *mgr1*, *b2m*, *nav3*, *gapdh* (Figure 3B). The geometric mean of the ranking order of commonly used housekeeping genes was higher than that of newly identified (9.8 *vs*. 5.4, data not shown). We next showed the results of expression stability in each tissues according to their relative distance in body, such as brain and gill, head kidney and spleen, heart and liver, trunk kidney and white muscle, proximal, mid, distal intestine. Actually, the way of group not affect our results and conclusions.

Expression stability of housekeeping genes in brain and gill

In the brain, the *cog5* and *pex14* had the least M value in Genorm; the *tbp* and *rpl8* had the least variations value in normFinder; the *cog5* and *actin* had the least SD in BestKeeper; the *tuba* and *18s* had the least ASD in comparative ΔCt method (Figure S2). The RefFinder ranked genes as follows: *tuba*, *18s*, *tbp*, *cog5*, *actin*, *rpl8*, *pex14*, *mgr1*, *ef1a*, *nav3*, *hprt1*, *myc2*, *hipk3*, *arf1*, *dhx30*, *b2m*, *gapdh* (Figure 4A). In gill, the *nav3* and *gapdh* had the least M value, the least variable value and the least ASD; the *cog5* and *nav3* had the least SD (Figure S3). The RefFinder ranked genes as follows: *nav3*, *gapdh*,

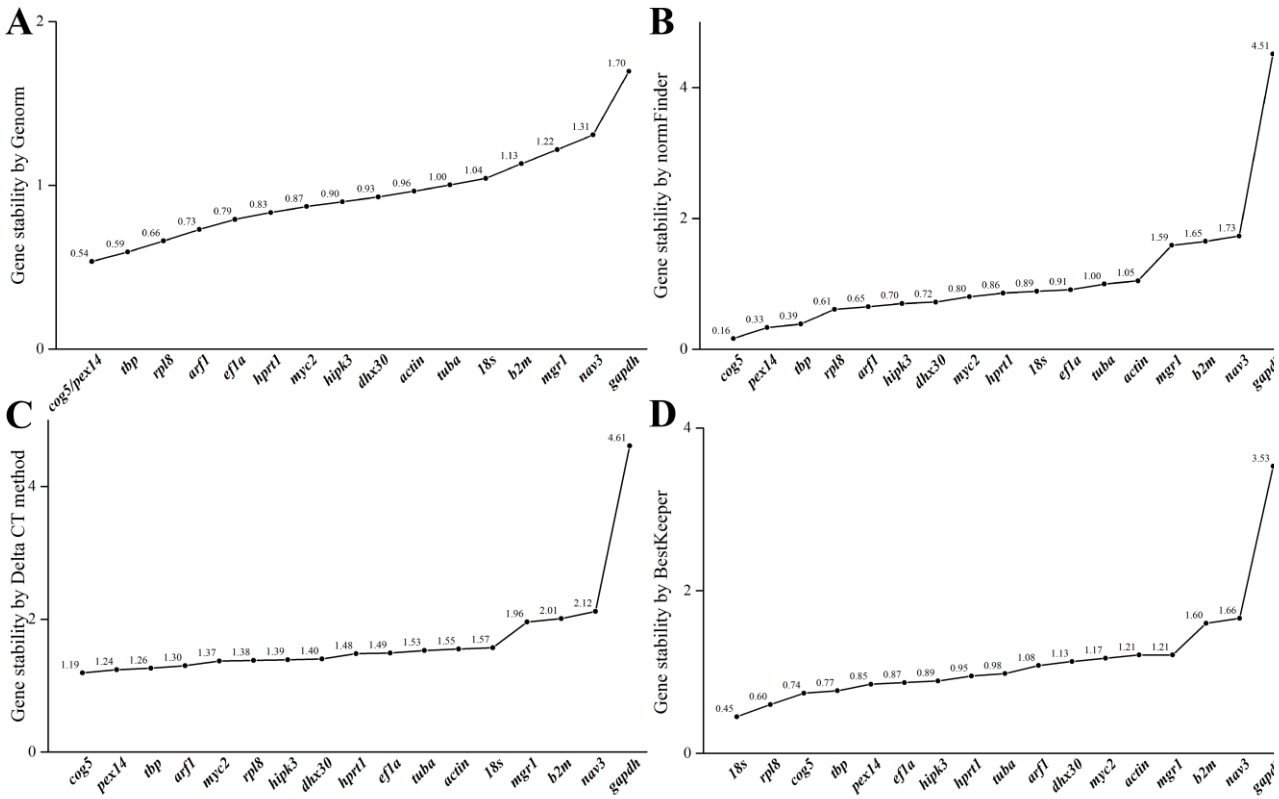

Figure 2. Determination of the expression stability of reference genes across different tissues according to Genorm (A), normFinder (B), comparative ΔCt method (C) and BestKeeper (D). The lower value means the higher stability.

cog5, *actin*, *b2m*, *tuba*, *tbp*, *rpl8*, *arf1*, *hipk3*, *18s*, *pex14*, *ef1a*, *myc2*, *dhx30*, *mgr1*, *hprt1* (Figure 4B).

Expression stability of housekeeping genes in head kidney and spleen

In head kidney, the *b2m* and *tuba* had the least M value and the least SD; the *tbp* and *cog5* had the least variable value and the least ASD (Figure S4). The RefFinder ranked genes as follows: *b2m*, *cog5*, *tuba*, *tbp*, *arf1*, *rpl8*, *ef1a*, *18s*, *myc2*, *nav3*, *pex14*, *hprt1*, *actin*, *hipk3*, *dhx30*, *mgr1*, *gapdh* (Figure 4C). In spleen, the *b2m* and *tuba* had the least M value; the *actin* and *myc2* had the least variable value and the least ASD; the *myc2* and *tuba* had the least SD (Figure S5). The RefFinder ranked genes as follows: *myc2*, *b2m*, *tuba*, *actin*, *rpl8*, *pex14*, *cog5*, *tbp*, *dhx30*, *hprt1*, *arf1*, *nav3*, *ef1a*, *18s*, *mgr1*, *hipk3*, *gapdh* (Figure 4D).

Expression stability of housekeeping genes in heart and liver

At heart, the *dhx30* and *gapdh* had the least M value, the least varblens value and the least ASD; the *rpl8* and *dhx30* had the least SD (Figure S6). The RefFinder ranked genes as follows: *dhx30*, *gapdh*, *rpl8*, *tbp*, *arf1*, *18s*, *hprt1*, *pex14*, *b2m*, *nav3*, *cog5*, *myc2*, *actin*, *tuba*, *ef1a*, *hipk3*, *mgr1* (Figure 5A). In liver, the

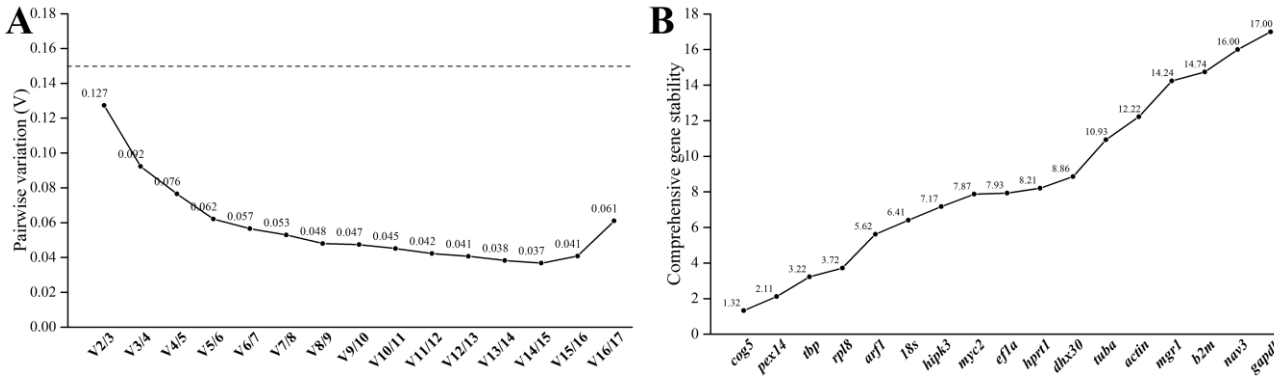

Figure 3. Determination of the optimal number of reference genes by Genorm (**A**) and the comprehensively ranking order of the stability of reference genes by RefFinder (**B**) across different tissues. Pairwise variations (V) of the candidate reference genes are calculated and the broken line indicates the Genorm cut-off value of 0.15 in **A** panel. The lower value means the higher stability in **B** panel.

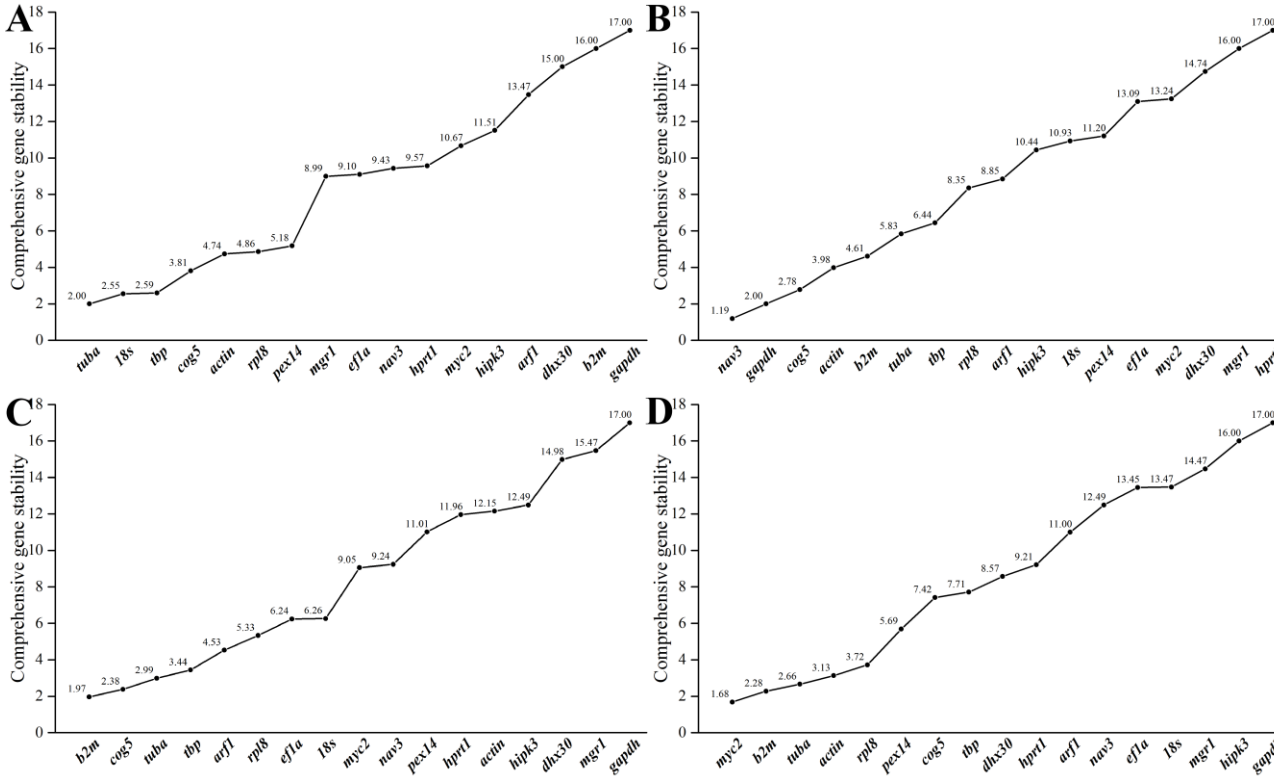

Figure 4. The comprehensively ranking order of the stability of reference genes in brain (A), gill (B), head kidney (C) and spleen (D) by RefFinder. The lower value means the higher stability.

actin and *b2m* had the least M value; the *18s* and *actin* had the least variable value and the least ASD; the *rpl8* and *gapdh* had the least SD (Figure S7). The RefFinder ranked genes as follows: *18s*, *actin*, *rpl8*, *b2m*, *pex14*, *tbp*, *arf1*, *gapdh*, *cog5*, *hipk3*, *tuba*, *nav3*, *hprt1*, *mgr1*, *ef1a*, *myc2*, *dhx30* (Figure 5B).

Expression stability of housekeeping genes in trunk kidney and white muscle

In the trunk kidney, the *actin* and *nav3* had the least M value, the *b2m* and *actin* had the least variations value; the *pex14* and *tuba* had the least SD; the *b2m* and *arf1* had the least ASD (Figure S8). The RefFinder ranked genes as follows: *b2m*, *actin*, *nav3*, *arf1*, *hipk3*, *rpl8*, *pex14*, *tuba*, *cog5*, *tbp*, *hprt1*, *myc2*, *gapdh*, *dhx30*, *mgr1*, *ef1a*, *18s* (Figure 5C). In white muscle, the *cog5* and *tbp* had the least M value, the least variable value and the least ASD; the *gapdh* and *tbp* had the least SD (Figure S9). The RefFinder ranked genes as follows: *cog5*, *tbp*, *arf1*, *18s*, *gapdh*, *actin*, *tuba*, *rpl8*, *nav3*, *pex14*, *ef1a*, *mgr1*, *dhx30*, *b2m*, *hprt1*, *hipk3*, *myc2* (Figure 5D).

Expression stability of housekeeping genes in proximal, mid, distal intestine

In proximal intestine, the *actin* and *b2m* had the least M value, the least variable value and the least ASD; the *gapdh* and *actin* had the least SD (Figure S10). The RefFinder ranked genes as follows: *actin*, *b2m*, *gapdh*, *cog5*, *18s*, *rpl8*, *ef1a*, *arf1*, *pex14*, *myc2*, *tuba*, *tbp*, *mgr1*, *hprt1*, *nav3*, *hipk3*, *dhx30* (Figure 6A). In the mid intestine, the *myc2* and *mgr1* had the least M value, the *actin* and *hprt1* had the least variations value; the *gapdh* and *nav3* had the least SD; the *actin* and *hprt1* had the least ASD (Figure S11). The RefFinder ranked genes as follows: *actin*, *myc2*, *mgr1*, *hprt1*, *gapdh*, *tbp*, *nav3*, *ef1a*, *pex14*, *cog5*, *18s*, *b2m*, *rpl8*, *hipk3*, *arf1*, *tuba*, *dhx30* (Figure 6B). In distal intestine, the *tuba* and *dhx30* had the least M value, the *gapdh* and *nav3* had the least variation value; the *gapdh* and *hprt1* had the least SD; the *gapdh* and *ef1a* had the least ASD (Figure S12). The RefFinder ranked genes as follows: *gapdh*, *hprt1*, *ef1a*, *nav3*, *pex14*, *actin*, *dhx30*, *tuba*, *rpl8*, *hipk3*, *cog5*, *tbp*, *myc2*, *mgr1*, *b2m*, *arf1*, *18s* (Figure 6C).

Discussions

As an essential part of qPCR technique, reference gene is easily ignored by taking *actin* and *18s* for the "gold-standard" (Li et al., 2020). In fish, about 50% of researches used *actin* as the only housekeeping gene, while 30% and 10% of researches used *18s* and *ef1a*, respectively (De Santis et al., 2011). Yet, these reference genes, even any single reference gene, had not been found to constantly and stably express in various fish species and tissues (Kamran et al., 2017; Mahanty et al., 2017). For instance, it was recommended to use *ef1a*, *18s* and *b2m* as housekeeping genes in the gonad of zebrafish (McCurley & Callard, 2008), whereas *rpl7* was most suitable in the gonad of Medaka (Zhang & Hu, 2007). In addition, *ef1a* showed highest stability in ovary, while *b2m* in testis of *Puntius sophore* (Mahanty et al., 2017). However, less than 40% of studies experimentally validated reference genes stability, and using of un-validated genes could introduce artefactual variances (Volland et al., 2017). For example, the qPCR results that generating from stability reference genes (*actin* or *ef1a*) and non-stability reference genes (*gapdh* or *18s*) presented significant differences in *Schizothorax prenanti* (Li et al., 2020). Accordingly, it is indispensable to assess and determine the appropriate housekeeping genes in focus tissues of the given fish.

The most popularly used mathematical algorithm software (Genorm, normFinder, BestKeeper and comparative ΔCt method) and the comprehensive

Table 1. Primers for qPCR

Target	Sequences Forward	Sequences Reverse	Fragment length (bp)	Primer efficiency (%)	References
actin	TACGAGCTGCCTGACGGACA	GGCTGTGATCTCCTTCTGC	239	96.28	Anderson *et al.*, 2018a
ef1a	TCCCACAGAAGCCCATGGTT	CCGACGGCTACGGTCTGTCT	94	94.62	Anderson *et al.*, 2018b
gapdh	CACGAAGGGCATTCTGGGATA	CATCAGGTCGCAGACACGGTT	175	98.83	Anderson *et al.*, 2018b
hprt1	GCGTGCTCAAAGGGGGTTAC	TCATTGGGATGGAACGGTCA	90	102.05	Anderson *et al.*, 2018b
rpl8	CGTCAGGAAACTACGCCACA	TTTCTTGGAGCCTGAGGGGA	83	98.22	Anderson *et al.*, 2018a
18s	AGCAACTTTAGTATACGCTATTG	CCTGAGAAACGGCTACCACATC	221	94.69	Anderson *et al.*, 2018a
b2m	GGACAGTATGGCAAAGACAAC	GCTTGGTCAGATGGAAGTG	156	99.25	Anderson *et al.*, 2018a
tuba	TACGATATCTGCCGCAGGAAC	ACGAAGGGACGCAGTGATG	102	102.53	Anderson *et al.*, 2018a
cog5	ATGGAGATGGCAGTGGCTCC	GTCTGAAACAGCAGCGGCCT	243	101.81	Anderson *et al.*, 2018b
dhx30	CAGCACGGCTCTAATGAA	CCTCGTCTGGGCAAAGT	192	95.39	Wang *et al.*, 2017
nav3	AGGGAAGGAGTGGTTGAGGT	GGCTCAGCAGGTTGGAGTAG	127	92.89	Anderson *et al.*, 2018a
hipk3	CGTTACAGTGCCGAGTTT	ACAGGCGGTAATAGAGTAGAT	130	97.96	Wang *et al.*, 2017
myc2	CAGAGGTGCGTCCAAGAG	AGGTGACAGGGTAAGGGTG	115	99.71	Wang *et al.*, 2017
mgr1	TCGGCAACCTTTGATTC	CAAGTGGTGGATGGAGTG	86	92.44	Wang *et al.*, 2017
tbp	ACGTGGACGCAGACGACATC	CGGGAGGGGAGTCTGGTTCT	122	95.04	Anderson *et al.*, 2018b
pex14	TGTAGGGCCTCCTACGGTGA	GTGGCAGCCCTCGTTGTCTT	88	100.85	Anderson *et al.*, 2018b
arf1	CAGCACTTTACCGCCAATCAA	TGTAAACAGTCGAGCGAGGT	124	93.63	Anderson *et al.*, 2018a

ranking software (RefFinder), were performed to determine the stability of housekeeping genes. Same as other researches (Wang et al., 2018; Wang et al., 2017), the stability ranking order calculated by these softwares were similar in this study, and there were slight differences due to different algorithms. In addition, a single reference gene was largely performed in studies of qPCR normalization, but it was insufficient in some experimental conditions (Volland et al., 2017; Wang et al., 2018). For example, comparing to the research using two or more housekeeping genes, 25% of the research using one housekeeping gene could produce 3.0-fold errors in qPCR assay, while 10% produce 6.4-fold errors (Mo et al., 2014). As reported by other studies (Wang et al., 2017), the genorm could analysis the V value to determine the appropriate amount of housekeeping genes. In this study, the value of $V_{2/3}$ in and across all different tissues were below the threshold (0.15), which meant that two housekeeping genes were required to improve the accuracy of normalization in qPCR assay of hybrid grouper.

Among seventeen of testing, reference genes, the gene *18s* had the highest expression TCt range 7-9), while the rest displayed the lower expression (Ct larger than 13). This difference (about 4-6 Ct values) was corresponds to a difference of 10-100 folds in expression level and it might be caused by the much higher richness of rRNA (85-90% in cell) than mRNA (5% in cell), because *18s* belongs to the component of rRNA (Wang et al., 2018; Ye et al., 2010). The results of the highest expression level and least variation of *18s* meant it could be a suitable housekeeping gene for qPCR assay across different tissues. But in rainbow trout, a massive difference expression (10^4-10^5 fold) of *18s* would shield the minor variations of target genes expression, and it indicated *18s* as a housekeeping gene would reduce resolution of the qPCR experiment (Kamran et al., 2017). This discrepancy might be caused by the different activity of RNA polymerases 1/2 that controlled the synthesis of rRNA/mRNA, respectively, in each species (Kamran et al., 2017; Liman et al., 2013). Furthermore, it was unclear whether the difference of methodology were similar between quantification of rRNA and mRNA (Kamran et al., 2017), more research was needed to explore the feasibility of rRNA gene (*18s*) as a reference gene in the mRNA expression assay.

Same as Japanese flounder (Zheng & Sun, 2011) and other groupers (Krishnan et al., 2019; Wang et al., 2017), the expression pattern of reference genes existed the tissue-specific manner in hybrid grouper. For example, the results showed that *18s* had the highest stability in brain (2^{nd}; ranked from the most to the least stable) and liver (1^{st}), but ranked medium (6^{th}) across all tissues. Differently, *18s* was most stable across tissues in spotted sea bass (Wang et al., 2018), Nile tilapia (Yang et al., 2013) and zebrafish (Tang et al., 2007). Our findings partly agreed that *18s* performed as the housekeeping gene for liver in *Anguilla australis* (Setiawan & Lokman, 2010), and indicated that *18s* expression was quite different among tissues of hybrid grouper. Owing to the most of housekeeping genes belong to structure genes (described in the section "Introduction") and encode the protein that vital to the basal physiology function and metabolism, the different

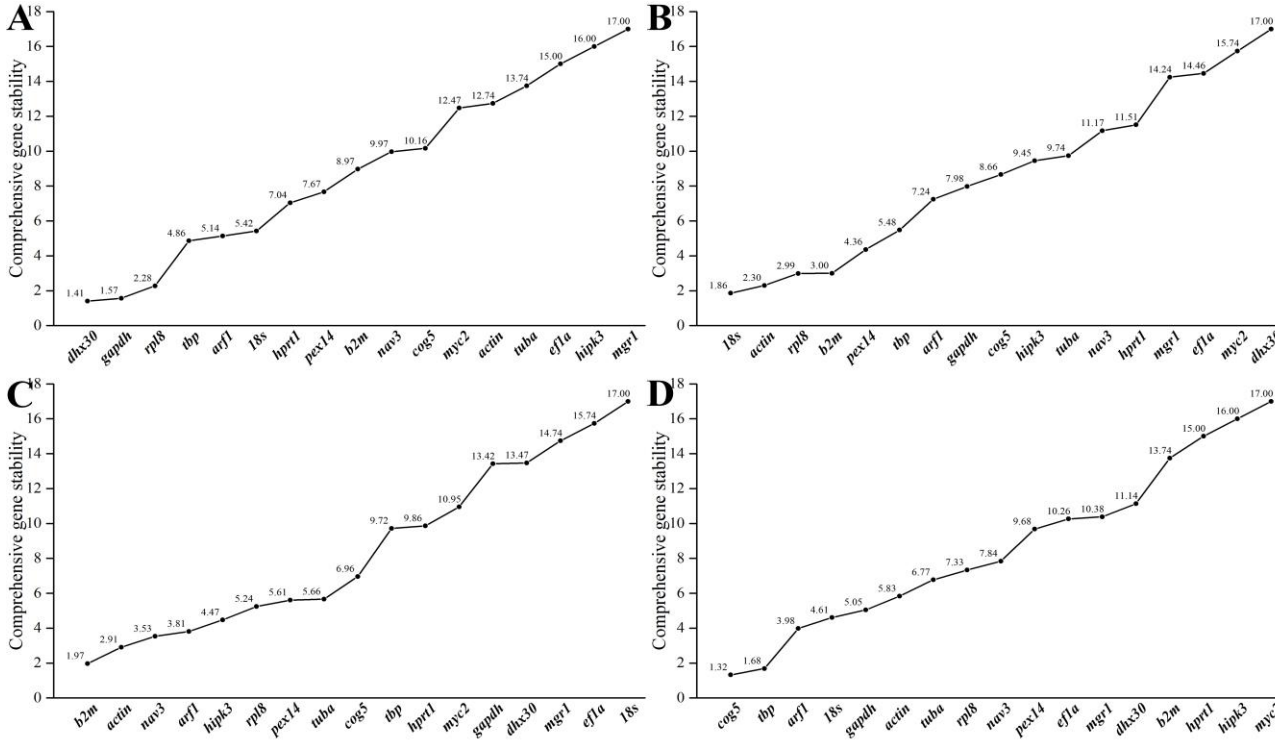

Figure 5. The comprehensively ranking order of the stability of reference genes in heart (**A**), liver (**B**), trunk kidney (**C**) and white muscle (**D**) by RefFinder. The lower value means the higher stability.

organ had shown different level of gene expression for their special performance (Jaramillo et al., 2017). This also confirmed that it is indispensable to assess and determine the appropriate housekeeping genes in focus tissue.

The expression of *actin* was very stable across tissues of rainbow trout (Kamran et al., 2017), Asian seabass (Paria et al., 2016) and *Schizothorax prenanti* (Li et al., 2020). In contrast, same as other groupers (Krishnan et al., 2019; Wang et al., 2017), this gene (ranked 13th) was not the suitable choice across all tissues in hybrid grouper. Partly agreed with the finding in Japanese flounder (Zheng & Sun, 2011), *Macrobrachium olfersii* (Jaramillo et al., 2017) and crucian carp (Mo et al., 2014) that *actin* was performed as a suitable housekeeping gene in liver (Jaramillo et al., 2017; Mo et al., 2014; Zheng & Sun, 2011), it showed the high stability in liver (2nd), trunk kidney (2nd) and proximal (1st), mid intestine (1st) in the present study. The different expression pattern of *actin* might be due to its different profiles of transcription and translation existed in different tissues, as it is the structural component of microfilaments and cytoskeleton and involves in multiple biological processes (Wang et al., 2017).

The *gapdh* is ubiquitously and constitutively expressed in all tissue/cell types (Xu et al., 2016). Same as the Japanese flounder, spotted sea bass and other groupers (Krishnan et al., 2019; Wang et al., 2018; Wang et al., 2017; Zheng & Sun, 2011), the instability of *gapdh* had been found across different tissues (17th) and in most of the tissues of hybrid grouper. But it was the most stable reference genes in distal intestine on this study, just like the *Pelteobagrus fulvidraco* (Ye et al., 2019). As the *gapdh* involves the export of RNA and replication and repair of DNA, its expression is susceptible to a lot of perturbations of cellular homeostasis and show the high instability. Besides, the highly variable expression of *actin* and *gapdh* might be caused by cross-amplification of the putative paralogous gene because of the higher instability of *gapdh1* and *actin1* compared to *gapdh2* and *actin2*. (Altmann et al., 2015; Infante et al., 2008).

In zebrafish, *b2m* showed the highest stability among the tested housekeeping genes in all tissues (McCurley & Callard, 2008). Although *b2m* (ranked 15th) was regarded as the unstable one across all tissues, it showed the high stability in the head kidney (1st), spleen (2nd), liver (4th), trunk kidney (1st) and proximal intestine (2nd) in the present study. These organs, mainly belongs to immune organs, showed stability of *b2m* might be caused by its role in against bacterial infection. Because, the expression instability of *b2m* is induced by infection, and *b2m* involves in the process of antigen binding and presentation via the major histocompatibility complex. It also explained the limitation of *b2m* as a reference gene in all tested tissues of *E. akaara* (Wang et al., 2017), turbot (Dang & Sun, 2011) and flounder (Zheng & Sun, 2011) after bacterial infection.

Through various functions in transduction of cell signaling and formation of cytoskeleton, etc., the *ef1a* was involved in the cell growth and proliferation. The

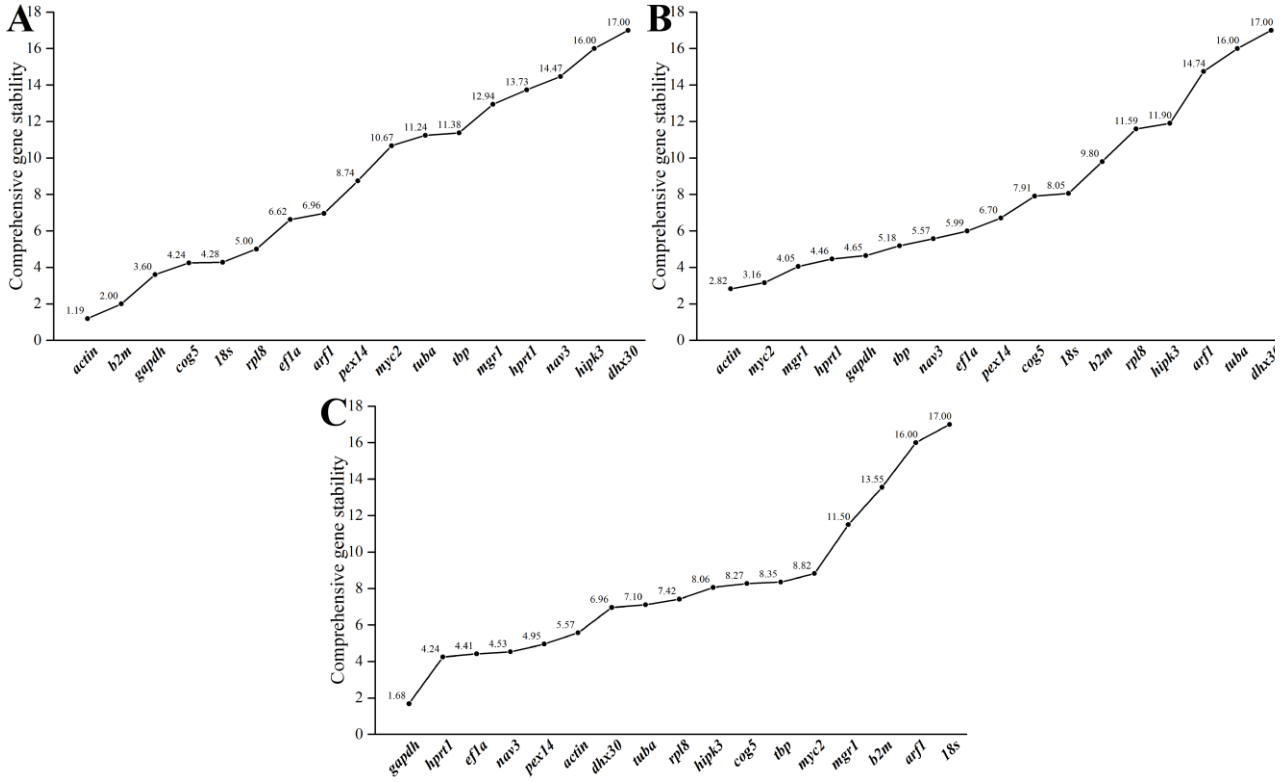

Figure 6. The comprehensively ranking order of the stability of reference genes in proximal (A), mid (B) and distal intestine (C) by RefFinder. The lower value means the higher stability.

ef1a presented high stability across tissues of seven-band grouper (Krishnan et al., 2019), *Salmo salar* (Olsvik et al., 2005), Asian seabass (Paria et al., 2016) and *Macrobrachium rosenbergii* (Krishna et al., 2015). But we found that *ef1a*, as the medial or least stable genes in zebrafish (Xu et al., 2016), was medial stable across different tissues (9th) and in most of the tissues of hybrid grouper. The HPRT1, as a key enzyme in purine nucleotides metabolism, is the basic structure of RNA and DNA (Kamran et al., 2017). Different with Atlantic salmon that the *hprt1* showed the high stability (Kortner et al., 2011), this gene showed the medial or least stability across different tissues and in most of tissues of hybrid grouper. Although the *tuba* had been regarded as a housekeeping gene in amount of studies as its stability at various of experimental conditions (Purohit et al., 2016), we found it was unstable across different tissues (12th) and in most of tissues of hybrid grouper.

The *rpl8*, belongs to the ribosomal proteins, ubiquitously expresses in all kinds of cells and tissues (Altmann et al., 2015). Actually, the ribosome is a complex piece of molecular machinery, the oldest one to have evolved in biological systems. The level of expression of *rpl8* genes showed considerable variations in most of tissues on this study and others (Panicz, 2016). Bur as the only stable and commonly used reference gene (4th) across different tissues in present study, it also expressed steady across all tissues of *Basilichthys microlepidotus* (Rojas-Hernandez et al., 2019), zebrafish (Xu et al., 2016) and *Macrobrachium olfersii* (Jaramillo et al., 2017).

As a novel identified housekeeping gene in groupers, the *cog5* also showed the high stability across all tissues in Pacific oyster (Dheilly et al., 2011), giant grouper (K. C. Anderson et al., 2018), *E. akaara* (Wang et al., 2017) and hybrid grouper (1st, this study). In addition, previous studies used the *cog5* as a reference gene in gonadal tissue of brown-marbled grouper and hypothalamus tissue of *E. akaara* (Palma et al., 2019; Qiu et al., 2018). The highly stable expression of *cog5* might be on account of its character of the conserved oligomeric Golgi complex subunit (Oka et al., 2005). The PEX14 promotes the introduction and movement of peroxisomal proteins by working as the membrane anchor for microtubules to achieve its multifunction (Bharti et al., 2011). In addition, it was a suitable internal reference antibody for the assay of immunoblotting, immunohistochemistry and immunofluorescence in most kinds of cell, organs and species (Grant et al., 2013). In fish, different with the giant grouper and *E. akaara* (K. C. Anderson et al., 2018; Wang et al., 2017), the *pex14* was a suitable reference gene (2nd) across all tissues in hybrid grouper. Because of the litter application of *pex14* as a reference gene in literature, more researches are needed to explore its feasibility.

The other researchers revealed that the *arf1* was a high stability housekeeping gene across all tissues in orange-spotted grouper and *E. akaara* (K. Anderson et al., 2018; Wang et al., 2017), but it wasn't a stable housekeeping gene (5th) in the present study. The TATA-box binding protein TAP is known to be a general transcription initiation factor. In poultry, the reference gene *tbp* showed the high stability in pig (Xianghong et al., 2011), and was the recommended reference gene for analyzing the muscle and liver of chicken (Bages et al., 2015). In fish, *tbp* was an appropriate housekeeping in qPCR studies on the effects of estrogens in fathead minnow (Filby & Tyler, 2007), the effects on temperature and reproductive development in Atlantic salmon (Anderson & Elizur, 2012). Same as the giant grouper (K. C. Anderson et al., 2018), the *tbp* showed the comparatively high stability (3rd) across all tissues of hybrid grouper. But *tbp* was the most unstable across all tissues in zebrafish (McCurley & Callard, 2008). The discrepancy might be caused by the different experimental conditions or species. The stable expression of *tbp* (2nd) in muscle was consistent with that it showed the high stability in gonad of minnow (Mahanty et al., 2017) and in muscle of *Pelteobagrus fulvidraco* (Ye et al., 2019). In addition, the *tbp* showed the highest stability in the adult Atlantic salmon for normalization of target genes, but the it showed the relatively lower stability (3rd) in juvenile (Anderson & Elizur, 2012). This phenomenon confirmed that the given housekeeping genes might vary significantly in different experimental conditions and it was necessary to assess and determine the appropriate housekeeping genes according to the actual experimental situation.

The stability ranking order of housekeeping genes from the most to the least across tissues from previous researches were: *ef1a*> *actin*> *gapdh* in seven-band grouper (Krishnan et al., 2019), *ef1a*> *hprt1*> *actin* in Atlantic halibut (Øvergård et al., 2010), *actin*> *b2m*> *gapdh* in turbot (Robledo et al., 2014), *18s*> *ef1a*> *actin*> *gapdh*> *tuba*> *b2m* in half-smooth tongue sole (Liu et al., 2014), *18s*> *ef1a*> *actin* in *Anguilla australis* (Setiawan & Lokman, 2010), *tbp*> *ef1a*> *hprt1*> *tuba* in Atlantic salmon (Anderson & Elizur, 2012), *rpl8*> *18s*> *actin*> *gapdh* in tench (Panicz, 2016). These comprehensive orders were roughly same as the ranking results of the present study, *cog5*> *pex14*> *tbp*> *rpl8*> *arf1*> *18s*> *hipk3*> *myc2*> *ef1a*> *hprt1*> *dhx30*> *tuba*> *actin*> *mgr1*> *b2m*> *nav3*> *gapdh*. A lot of genes were expressed stable in the present study, but we suggested that the *cog5* and *pex14* were used as housekeeping genes across all tissues of hybrid grouper.

Some of the previous studies showed that the commonly used housekeeping genes (*actin*, *ef1a*, *gapdh*, *hprt1*, *18s*, *b2m* and *tuba*) were ranked lower in the stability ranking order than the newly identified reference genes (Manoli et al., 2012; Migocka & Papierniak, 2010; Wang et al., 2017; Xu et al., 2016). The instability of commonly used reference genes under different experimental conditions might be caused by the strong influence of oestrogen in fish on the expression of these genes (Filby & Tyler, 2007; Xu et al., 2016). We also found some of the newly identified reference genes were more stable than that of

commonly used across different tissues. These results confirmed that some of the newly identified housekeeping genes were generally stable in kinds of experimental conditions, and should be assessed and determined together with the that of commonly used (Migocka & Papierniak, 2010).

There were some limitations and future directions. First, after using the mathematical algorithm software, many of housekeeping genes showing the instability of expression were eliminated, and the genes with top of stability were suggested for the next analysis. But the most stable genes could not be "good" housekeeping genes for quantification of target genes if the absolute effects of the contaminants from samples or operations were major (Kamran et al., 2017). So, any reference gene that showed the highest stability from software analyses should also be applied carefully in actual. Then, the newly identified reference genes must be tested more before its usage in trial because of the litter application of these genes in literatures. Last, as the expression stability of housekeeping genes could be significantly different in differing experimental conditions, the results of this study from normal condition of health hybrid grouper juveniles limited its extensive application.

Conclusions

To date, this study was the first work to verify and evaluate the expression stability of housekeeping genes from samples of different tissues in this commercially important fish, hybrid grouper. The results indicated that: 1) the most stable housekeeping genes were *tuba*/*18s* in the brain, *nav3*/*gapdh* in gill, *b2m*/*cog5* in head kidney, *myc2*/*b2m* in spleen, *dhx30*/*gapdh* in heart, *18s*/*actin* in the liver, *b2m*/*actin* in trunk kidney, *cog5*/*tbp* in white muscle, *actin*/*b2m* in proximal intestine, *actin*/*myc2* in mid intestine, *gapdh*/*hprt1* in distal intestine. All of the tested housekeeping genes exhibited a tissue-specific manner in transcriptional level; 2) the *cog5* and *pex14* were the most stable housekeeping genes across all tissues, while the *gapdh* was the worst one; 3) some of the newly identified housekeeping genes might be more stable than that of commonly used. Hence, same as the proposition of previous studies, we suggest that the appropriate housekeeping genes need to be assessed and determined in experimental conditions before the actual qPCR assay.

Ethical Statement

All animals experimental process strictly conformed to the guidelines for the care and use of animals for scientific purposes set by the Ministry of Science and Technology, Beijing, China (No. 398, 2006).

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Author Contribution

The authors' responsibilities were as follows: Junhui Liang: Methodology, Validation, Formal analysis, Investigation, Data Curation, and Writing-Original Draft; Jia Xu: Methodology, Validation, Formal analysis, Investigation, Data Curation, and Writing-Original Draft; Shiwei Xie: Conceptualization, Resources, Writing-Review & Editing, and Visualization; Junming Cao: Conceptualization, Project administration, Funding acquisition; Beiping Tan: Conceptualization, Writing-Review & Editing, Project administration, and Funding acquisition. All authors read and approved the final manuscript.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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