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Research Article

Cloning, Expression and Stability Analysis of Candidate Reference Genes in the Glacial Relict *Hucho bleekeri* Under Immune Stress

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To find reference genes (RGs) and establish a screening method for RGs in Sichuan taimen (*Hucho bleekeri*), six candidate RGs—*β-actin*, *b2m*, *β-tubulin*, *ef1α*, *gapdh* and *hprt*—were cloned, and the stability of expression of the six candidate RGs were analysed. The six RG sequences in *H. Bleekeri* had higher similarity with the corresponding sequences in *Salmo* or *Oncorhynchus*. The RGs geomean of ranking values of gene expression in tissues of *H. bleekeri* was *ef1α* > *β-actin* > *β-tubulin* > *hprt* > *b2m* > *gapdh*. After lipopolysaccharide (LPS) treatment, the RGs geomean of ranking values of gene expression in spleen was *ef1α* > *β-actin* > *b2m* > *gapdh* > *β-tubulin* > *hprt*, in head kidney was *β-actin* > *ef1α* > *β-tubulin* > *hprt* > *gapdh* > *b2m*, in liver was *β-tubulin* > *hprt* > *β-actin* > *gapdh* > *ef1α* > *b2m*; after polyinosinic: polycytidylic acid (Poly (I:C)) treatment, the values in spleen was *ef1α* > *hprt* > *β-actin* > *b2m* > *β-tubulin* > *gapdh*, in head kidney was *β-actin* > *ef1α* > *β-tubulin* > *hprt* > *gapdh* > *b2m*, in liver was *β-tubulin* > *β-actin* > *ef1α* > *gapdh* > *b2m* > *hprt*. The results indicated that *ef1α* can be used as the most stable RG for the tissue distribution analysis of gene expression in *H. bleekeri*, and *ef1α, β-actin* and *β-tubulin* was suitable for spleen, head kidney and liver cells, respectively, under LPS and Ploy (I: C) stimulation.

Keywords: qPCR; RefFinder; Salmonidae; tissue distribution

Summary

- Six pairs of R-PCR primers were screened for the most suitable RGs in *Hucho bleekeri*, and the optimal RGs were identified under LPS and Ploy (I:C) stress in *Hucho bleekeri*.

1. Introduction

Real-time quantitative PCR (qRT-PCR) is a method for quantitatively detecting specific DNA fragments by detecting fluorescent signals in polymerase chain reactions. Owing to its sensitivity and reliability, it is widely used in mRNA abundance quantification [1–3]. Relative quantification is a qRT method that uses a known and stably expressed reference gene (RG) as a comparation to quantify the expression levels of the tested gene. Therefore, the stability of expression of the RG is particularly crucial. Most RGs are involved in cell maintenance or primary metabolism, and are widely and stably expressed in different cell types or conditions [4, 5]. *β-actin*, for example, is widely used for relative quantification detection in animals and plants. Depending on the species, developmental stage, nutritional status, and other conditions [5–7], the stability of RGs fluctuate accordingly. Therefore, it is a basic requirement to screen candidate RGs, which mainly include

beta-2-microglobulin (*b2m*) [8], elongation factor 1*α* (*ef1α*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) [9], hypoxanthine-guanine phosphoribosyltransferase (*hprt*) [10] and *β-tubulin* [11].

Many methods have been developed to screen the stability of expression of RGs. In addition to an initial evaluation of the stability of RGs based on the range of cycle threshold (Ct) results displayed by qRT-PCR, GeNorm [12], comparative ΔCt method [13], NormFinder [14], BestKeeper [15] and RefFinder [16, 17] are also used to evaluate the stability of RGs. Owing to the different algorithms, the results obtained might differ somewhat with each internal parameter stability evaluation software. ΔCt method and GeNorm can be used to screen any number of RGs in an experiment, and ultimately select two or more combinations of RGs to correct the data, making relative quantitative results more accurate [18]. NormFinder can not only compare the expression differences of candidate RGs, but also can calculate the variation between sample groups [19]. BestKeeper can not only be used to compare the stability of expression of RGs but also to analyse target genes [20]. RefFinder, which combines the ranking of each gene in the above methods and assigns appropriate weighting, calculates the geometric average of their weights to obtain the result [16].

Hucho bleekeri, a rare endemic species in China, is distributed in the upper reaches of the Yangtze River including Min River and Qingyi River, the middle and upper reaches of the Dadu, Weishui, and Taibai Rivers, tributaries of the Han River, and mountain streams in the southern part of the Qinling Mountains [21]. It is a rare fish species found in the upper reaches of the Yangtze River. Some biogeographical scholars have speculated that *H. bleekeri* is a remnant fish that invaded from the north to the south during the Quaternary glacial period and is regarded as a glacial relict [22]. In our protection and breeding process, we have found that *H. bleekeri* has obvious disease outbreak, which may involve bacteria or viruses. Therefore, to serve the protection and breeding work, we need to explore and analyze the immune model of *H. bleekeri*. Lipopolysaccharide (LPS), as the main component of Gram-negative bacterial cell membranes, is a potent inflammatory inducer [23–25], and polyinosinic-polycytidylic acid (Poly (I: C) is also an immune stimulator used to mimic viral RNA double strands [26]. These two stimulants are widely used to establish immune models in fishes [25, 27–31]. RGs are the foundation of many studies, and the expression stability of RGs also change in cases of infection [32]. Currently, research on *H. bleekeri* mainly focuses on early development [33], morphological characteristics [34], mitochondrial genome [34], transcriptome [35], and resource conservation [36]; however, molecular research on the species remains scarce. To the best of our knowledge, no reports on RGs and their screening in *H. bleekeri* exist. In this study, we screened for the stable expression of RGs in *H. bleekeri*, and evaluated suitable screening methods for candidate genes, which will provide basic information for the future research of molecular biology on this precious species.

2. Materials and Methods

2.1. Fish and Sample Preparation. H. bleekeri individuals were obtained from the Cold Water Fish Breeding Centre of the Fishery Institute of the Sichuan Academy of Agricultural Sciences. All experimental protocols were approved by the Animal Care Advisory Committee of the Sichuan Academy of Agricultural Sciences (20230308001A). Three healthy *H. bleekeri* were all anesthetized with MS222 (60 mg/L). The brain, eye, gill, skin, muscle, head kidney, middle kidney, heart, stomach, duodenum, hindgut, liver, gonads and spleen of the fish were quickly placed in liquid nitrogen and stored at −80°C till further use.

2.2. RNA Extraction. The tissues were ground in liquid nitrogen until powdered, and 30 mg (of each tissue) was dissolved in 1 mL RNAiso Plus (Takara), and RNA was extracted according to the manufacturer's instructions. A 1.2% agarose gel was used to assess RNA integrity, and a microspectrophotometer (IMPLE 50) was used to quatify RNA concentration. A reverse transcription kit (Takara, RR047A) was used to obtain cDNA according to the manufacturer's instructions.

2.3. Cloning of RGs. Based on our previous study, six RG sequences were obtained from the full-length transcriptome data of *H. bleekeri* (PRJNA639035). Primer premier 5 was used to design the cloning primers [37]. Primers used for amplification of the six RGs are listed in Table 1. In a 50 μL PCR mixture, 2 μL cDNA, 1.5 μL each primers (10 μmol/L), 25 μL 2 × Taq MAstermix (NEST), and 20 μL ddH_2O, were added. Thermocycler conditions for PCR included predenaturation 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at a different temperature for each gene (Table 1) for 30 s, and 72°C for 1 min and a final 72°C extension for 5 min.

2.4. Quantitative RT-PCR. PCR primers for *β-actin*, *b2m*, *β-tubulin*, *ef1α*, *gapdh* and *hprt* are shown in Table 1. The 20 μL qPCR mixture included 10 μL TB Green Premix Ex Taq II (Takara), 0.5 μL each of forward and reverse primers from 10 μmol/L stocks, 1 μL cDNA and 8 μL ddH_2O. The cycling conditions included 98°C for 2 min, followed by 40 cycles of 98°C for 5 s, annealing at a different temperature for each gene (Table 1) for 10 s, and 72°C for 15 s.

2.5. Exposure Experiment. After anesthesia with MS-222, we took the head kidney, spleen and liver tissues of *H. bleekeri* for primary cell culture. The tissues were cut into pieces in 1640 RPMI medium and passed through 100 μm Nylon mesh cell filter. After collecting cells into a 15 mL centrifuge tube, at 4°C 400 × g centrifuge for 10 min, we removed the supernatant and resuspended the cells in 1640 RPMI culture medium containing 15% fetal bovine serum. Cell viability was measured using trypan blue staining, and the survival rates of the cells were both above 95%. We uniformly distributed cells into a 6-well cell culture plate. Primary cells

Table 1: The primers for candidate six reference genes for *Hucho bleekeri*.

Name	Sequence	OAT (°C)	Efficiency	Usage
β-actin-F	ATGGAAGATGAAATCGCCGC	50	—	Cloning
β-actin-R	TTAGAAGCATTTGCGGTGGAC			
β-tubulin-F	ATGAGGGAAATCGTGCACATTC			
β-tubulin-R	CACTCCGTCTTTAAGTGTCC			
b2m-F	ATGAAGTCTATTCTGTCAATCGTTG			
b2m-R	TTACATATCTGCCTCCCAGG			
ef1α-F	ATGGGAAAGGAAAAGATCCACATT			
ef1α-R	TCATTTGGCTTTGGTGGCCTTA			
gapdh-F	ATGGTGAAGATTGGGATCAATG			
gapdh-R	TTACTCCTTGGAGGCCATG			
hprt-F	CTAAACGACGATACGAACATGG			
hprt-R	GCTGTCCTCTTCATGCTTTG			
β-actin-qF	AGGGAGTGATGGTTGGGAT	63	99.5%	qRT-PCR
β-actin-qR	CGTTGTAGAAGGTGTGGTGC			
b2m-qF	TCTGTCAATCGTTGTGCTTGG	63	92.6%	
b2m-qR	TCAGGGTGTTGCCTTTCCC			
β-tubulin-qF	TTACTGTCGCCGCTGTCTT	62.6	90.5%	
β-tubulin-qR	TGTTGCCGATGAAGGTGA			
ef1α-qF	TGGAGACAGCAAGAACGACC	62.6	98.1%	
ef1α-qR	ATGTGAGCGGTGTGGCAAT			
gapdh-qF	GCACAACTAACTGCCTGGCT	62.6	91.4%	
gapdh-qR	CGTCCATCTCTCCACTGCTT			
hprt-qF	CGATGATGAGCAGGGATATG	62.6	90.7%	
hprt-qR	TCATAGGAATGGAGCGGTC			

Abbreviation: OAT, optimal annealing temperatures.

were stimulated with 50 mg/mL of LPS, 100 mg/mL of Poly (I: C), and an equal volume of PBS, with three replicates for each treatment [38]. Cells were collected at 0, 12, 24 and 48 h. At each time point, the cells were subjected to centrifugation at 400 × gfor 10 min at 4°C and the supernatants were discarded. Cells were washed with PBS and then centrifugation was repeated with 4°C 400 × g centrifuge for 10 min. RNA extraction and cDNA synthesis of the obtained cells followed at the method described in 2.2, and the RT-PCR reaction system and program followed to the method described in 2.4.

2.6. Data Analysis

2.6.1. RG Sequences Analyses. The open reading frame (ORF) of the sequences was predicted using Vector NTI 11 [39]. DNAman was used to analyse the amino acid sequences [39]. ExPASy tools (https://www.expasy.org/tools) were used to predict the molecular weights and isoelectric points of the products. Transmembrane structure analysis was performed using SMART (https://smart.embl-heidelberg.de/). Multiple alignments of amino acid sequences were performed using ClustalX. A phylogenetic tree was constructed using Mega X software [39].

2.6.2. Tissue Distribution Analyses. Mean ± standard deviation was used to represent the Ct value of the candidate internal RGs. The geNorm, BestKeeper and NormFinder softwares were used to analyse the tissue expression stability for *β-actin*, *b2m*, *β-tubulin*, *ef1α*, *gapdh* and *hprt* RGs. Six primers for the RGs were screened to ensure an amplification efficiency of 90%–110%. Finally, RefFinder (https://blooge.cn/RefFinder/type=reference) was used to calculate the stability ranking of the RGs using the four methods.

3. Results

3.1. DNA Sequence Analysis of RGs. We cloned the six RGs—*β-actin* (GenBank:PP209399), *b2m* (GenBank: PP209400), *β-tubulin* (GenBank:PP209401), *ef1α* (GenBank: PP209402), *gapdh* (GenBank:PP209403) and *hprt* (GenBank:PP209404)—of *H. bleekeri*, and the amino acid sequences of RGs are shown in Figures S1–S6. The ORFs of *β-actin*, *b2m*, *β-tubulin*, *ef1α*, *gapdh* and *hprt* encode 375, 116, 444, 462, 333 and 218 amino acids, respectively. Their molecular weights are 41,890.53, 13,013.83, 49,719.27, 50,044.85, 35,930.47 and 24,766.38 Da, respectively, and their isoelectric points are 5.30, 6.46, 4.78, 9.25, 6.83 and 5.98, respectively. None of the six genes contain transmembrane structures or signalling peptides.

3.2. Sequence Alignment and Phylogenetic Analysis. Multiple sequence alignment analysis revealed that mRNA and amino acid sequences of the candidate RGs are relatively conserved (Figure S7–18). In addition, according to the NCBI amino acid sequence alignment results, mRNA and amino acid sequences of the six RGs are highly homologous between *H. bleekeri* and Atlantic salmon *Salmo salar*. Similarity of the six RGs are above 90%; *β-actin* (95.39%, 100%), *b2m* (94.60%, 94.83%), *β-tubulin* (97.60%, 99.77%), *ef1α* (97.26%, 100%), *gapdh* (96.41%, 97.90%) and *hprt*

(98.17%99.54%). The evolutionary trees revealed the relationship between *H. bleekeri* and these related species (Figure 1). The six RG sequences in *H. Bleekeri* had higher similarity with the corresponding sequences in *Salmo* or *Oncorhynchus*.

3.3. Amplification Efficiencies Analysis of RGs. Screening and program optimization of the qRT-PCR primers for the six RGs yielded the corresponding optimal annealing temperatures based on the standard curve, as shown in Figure 2 and Table 1. The amplification efficiencies of the six RGs were *β-actin* ($E = 99.5\%$, $R^2 = 0.998$), *b2m* ($E = 92.6\%$, $R^2 = 0.999$), *β-tubulin* ($E = 90.5\%$, $R^2 = 1.000$), *ef1α* ($E = 98.1\%$, $R^2 = 0.996$), *gapdh* ($E = 91.4\%$, $R^2 = 0.998$) and *hprt* ($E = 90.7\%$, $R^2 = 0.999$).

3.4. The Ct Values of Various Tissues. The Ct values for qRT-PCR are shown in Table 2. The smallest range of Ct values is *ef1α* (3.62), and the largest is *gapdh* (13.80). The lowest average Ct value is *β-actin* (19.10), and the highest is *β-tubulin* (26.40).

3.5. Stability Analyses of RGs. The results of stability analyse of RGs expression are shown in Figure 3. The average standard deviation of RGs in pairwise comparisons are used for comparative ΔCt method sorting [13] and the stability values (SVs) were 1.687 (*ef1α*), 1.878 (*β-actin*), 1.884 (*β-tubulin*), 1.952 (*hprt*), 2.151 (*b2m*) and 4.505 (*gapdh*).

In Figure 3, we demonstrated the calculation of the SV for the six RGs using geNorm; the smaller the SV, the higher the stability of expression of the RG. The SVs were 0.872 (*β-actin/ef1α*), 1.036 (*hprt*), 1.089 (*β-tubulin*), 1.262 (*b2m*), and 2.343 (*gapdh*). The SVs of the six different RGs using NormFinder is shown in Figure 3. The SVs were 0.436 (*ef1α*), 0.448 (*β-tubulin*), 1.053 (*β-actin*), 1.170 (*hprt*), 1.381 (*b2m*) and 4.420 (*gapdh*). The SVs calculated by BestKeeper were 0.663 (*β-tubulin*), 0.720 (*ef1α*), 1.149 (*β-actin*), 1.184 (*hprt*), 1.588 (*b2m*) and 3.448 (*gapdh*) (Figure 3).

RefFinder assigns appropriate weights to individual genes based on the ranking of each item, calculates the geometric average of their weights, and obtains the final overall ranking [38]. The SVs were 1.189 (*ef1α*), 2.060 (*β-actin*), 2.213 (*β-tubulin*), 3.722 (*hprt*), 5.000 (*b2m*), 6.000 (*gapdh*) (Figure 4).

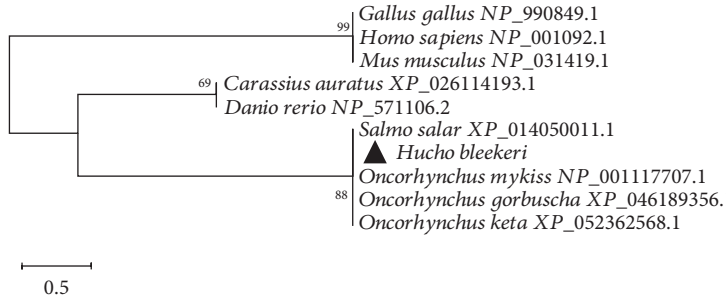
3.6. Stability of RGs Under Exposure Experiment. Under LPS and Poly (I:C) treatment, the stability ranking (SR) of the six candidate RGs are shown in Figures 5 and 6. By the ΔCt method, the SR of RGs at various time points after LPS treatment in spleen were *ef1α* (1.167), *β-actin* (1.204), 1.217(*b2m*), *β-tubulin* (1.410), 1.411(*gapdh*), *hprt* (1.880); in head kidney were 1.506 (*β-actin*), 1.534 (*hprt*), 1.590 (*β-tubulin*), 1.607 (*ef1α*), 2.013 (*b2m*), 2.960 (*gapdh*); in liver were 1.504 (*β-tubulin*), 1.590 (*hprt*), 1.790 (*β-actin*), 2.050 (*b2m*), 2.057 (*ef1α*), 2.126 (*gapdh*; the SR of RGs at various time points after Poly (I:C) treatment in spleen were 1.982 (*ef1α*), 2.023 (*hprt*), 2.375 (*β-actin*), 2.389 (*β-tubulin*), 2.670 (*b2m*), 3.790 (*gapdh*); in head kidney were 1.237 (*β-actin*), 1.257 (*hprt*), 1.364 (*ef1α*), 1.367 (*β-tubulin*), 1.877 (*b2m*), 2.472 (*gapdh*); in liver were 2.578 (*β-tubulin*), 2.779 (*β-actin*), 3.014 (*ef1α*), 3.134 (*b2m*), 3.162(*gapdh*), 7.170 (*hprt*) (Figure 5).

The SR of RGs at various time points after LPS treatment by BestKeeper in spleen were 1.022 (*β-actin*), *ef1α* (1.146), 1.351 (*gapdh*), 1.687 (*b2m*), *hprt* (1.927), 1.984 (*β-tubulin*); in head kidney were 1.432 (*gapdh*), 2.361 (*ef1α*), 2.388 (*β-tubulin*), *2.779* (*β-actin*), 2.916 (*hprt*), 3.652 (*b2m*); in liver were 2.380 (*gapdh*), 2.430 (*β-actin*), 2.256 (*ef1α*), 2.590 (*β-tubulin*), 3.145 (*hprt*), 3.199 (*b2m*); the SR of RGs at various time points after Poly (I:C) treatment in spleen were 1.463 (*gapdh*), 1.873 (*ef1α*), 2.083 (*β-tubulin*), *2.585* (*β-actin*), 2.730 (*hprt*), 3.071 (*b2m*); in head kidney were 1.242 (*gapdh*), 2.212 (*ef1α*), 1.972 (*β-tubulin*), 2.467 (*β-actin*), 2.552 (*hprt*), 3.127 (*b2m*); in liver were 1.947 (*β-actin*), 2.064 (*ef1α*), 2.164 (*gapdh*), 2.236 (*β-tubulin*), 2.770 (*b2m*), 3.929 (*hprt*) (Figure 5).

The SR of RGs at various time points after LPS treatment by NormFinder in spleen were 0.629 (*ef1α*), 0.705 (*b2m*) 0.739 (*β-actin*), 1.031 (*gapdh*), 1.093 (*β-tubulin*), 1.674 (*hprt*); in head kidney were 0.312 (*β-tubulin*), 0.671 (*β-actin*), 0.690 (*ef1α*), 0.750 (*hprt*), 1.825 (*b2m*), 2.842 (*gapdh*); in liver were 0.429 (*β-tubulin*), 0.719 (*hprt*), 1.204 (*β-actin*), 1.623 (*ef1α*), 1.707 (*b2m*), 1.815 (*gapdh*); the SR of RGs at various time points after Poly (I:C) treatment in spleen were 0.471 (*hprt*), 0.661 (*ef1α*), 1.314 (*β-tubulin*), 1.782 (*β-actin*), 2.346 (*b2m*), 3.647 (*gapdh*); in head kidney were 0.390 (*β-actin*), 0.418 (*β-tubulin*), 0.477 (*hprt*), 0.620 (*ef1α*), 1.676 (*b2m*), 1.596 (*gapdh*); in liver were 0.585 (*β-tubulin*), 0.691 (*β-actin*), 1.489 (*b2m*), 1.666 (*gapdh*), 2.125 (*ef1α*), 7.046 (*hprt*) (Figure 5).

The SR of RGs at various time points after LPS treatment by Genorm in spleen were 0.317 (*β-actin/ef1α*), 0.800 (*gapdh*), 1.035 (*b2m*), 1.132 (*β-tubulin*) 1.381 (*hprt*); in head kidney were 0.736 (*β-actin/ef1α*), 0.936 (*hprt*), 1.128 (*β-tubulin*), 1.345 (*b2m*), 1.863 (*gapdh*); in liver were 1.043 (*β-tubulin/hprt*), 1.430 (*β-actin*), 1.520 (*b2m*), 1.716 (*ef1α*), 1.853 (*gapdh*); the SR of RGs at various time points after Poly (I:C) treatment in spleen were 1.127 (*β-actin/b2m*), 1.465 (*hprt*), 1.603 (*ef1α*), 1.912 (*β-tubulin*), 2.538 (*gapdh*); in head kidney were 0.564 (*β-actin/ef1α*), 0.755 (*hprt*), 0.917 (*β-tubulin*), 1.157 (*b2m*), 1.596 (*gapdh*); in liver were 1.171 (*ef1α/β-tubulin*), 1.436 (*β-actin*), 1.670 (*gapdh*), 1.881 (*b2m*), 3.644 (*hprt*) (Figure 5).

The SR of RGs at various time points after LPS treatment by RefFinder in spleen were *ef1α* (1.189), 1.565 (*β-actin*), 3.130 (*b2m*), 3.663 (*gapdh*), 4.949 (*β-tubulin*), 5.733 (*hprt*); in head kidney were 1.682 (*β-actin*), 3.310 (*hprt*), *2.213* (*ef1α*), 2.449 (*β-tubulin*), 3.843 (*gapdh*), 5.233 (*b2m*); in liver were 1.414 (*β-tubulin*), 2.115 (*hprt*), 2.711 (*β-actin*), 3.834 (*gapdh*), 4.162 (*ef1α*), 4.681 (*b2m*); the SR of RGs at various time points after Poly (I:C) treatment in spleen were 1.189 (*ef1α*), 1.565 (*β-actin*), 3.130 (*b2m*), 3.663 (*gapdh*), 4.949 (*β-tubulin*), 5.733 (*hprt*); in head kidney were 1.414 (*β-actin*), 2.449 (*ef1α*), 2.828 (*β-tubulin*), 3.080 (*hprt*), 3.843 (*gapdh*), 5.233 (*b2m*); in liver were 1.414 (*β-tubulin*), 1.861 (*β-actin*), 2.340 (*ef1α*), 3.936 (*gapdh*), 4.162 (*b2m*), 6.000 (*hprt*) (Figure 6).



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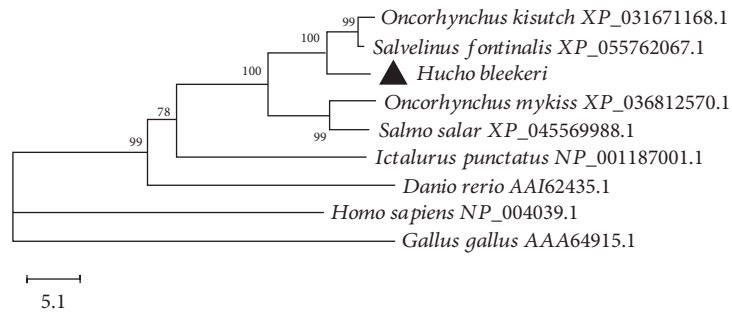

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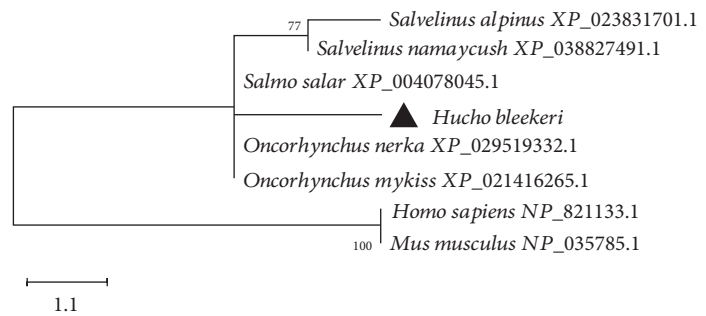

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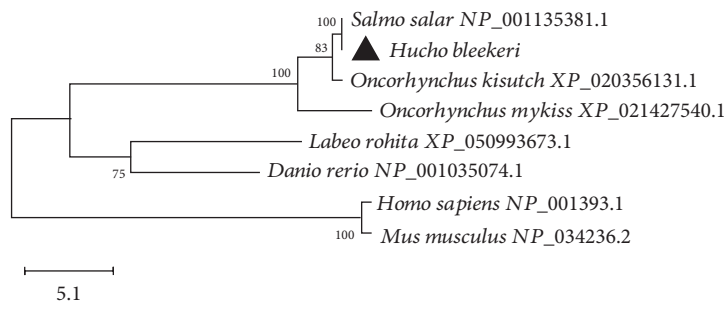

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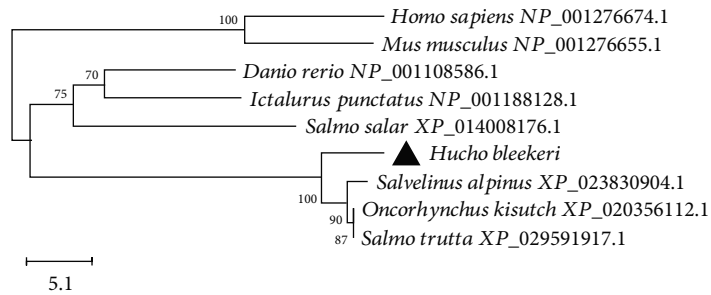
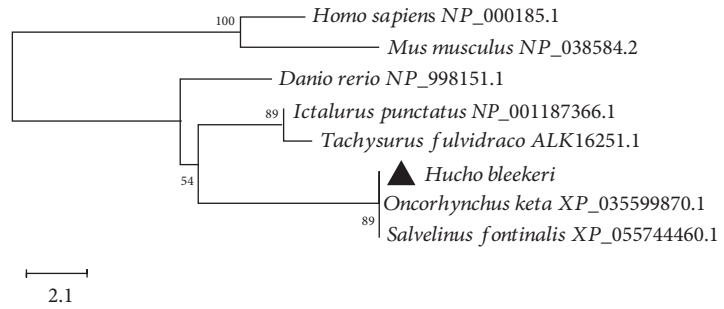


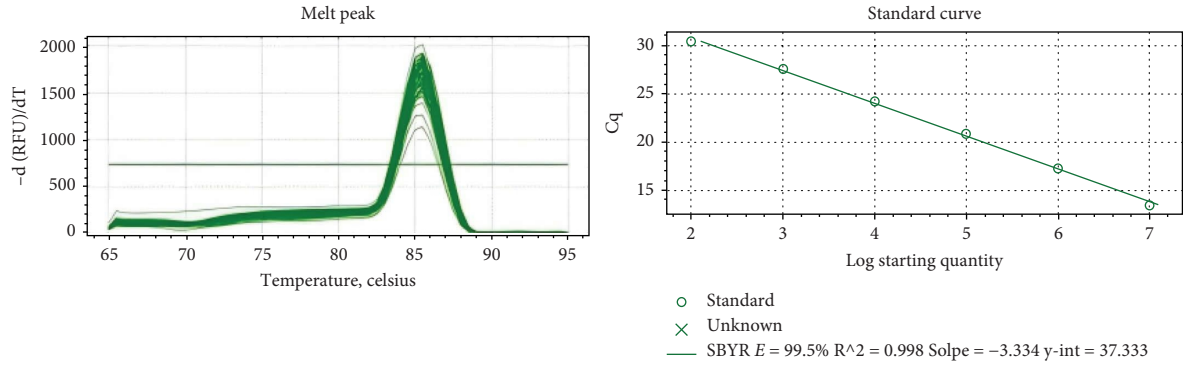
(e)

Figure 1: Continued.

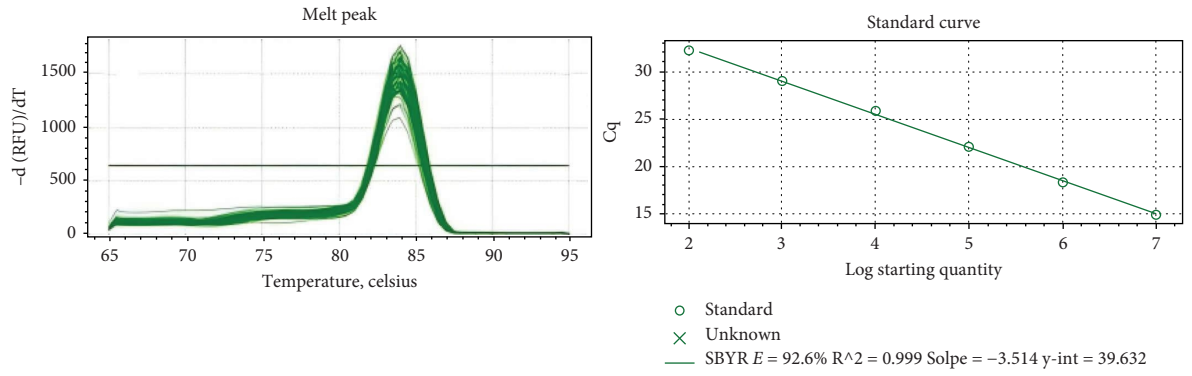


(f)

Figure 1: Phylogenetic tree of the amino acid sequences of *β-actin* (a), *b2m* (b), *β-tubulin* (c), *ef1α* (d), *gapdh* (e) and *hprt* (f). ▲ was used to highlight *Hucho bleekeri*.



(a)



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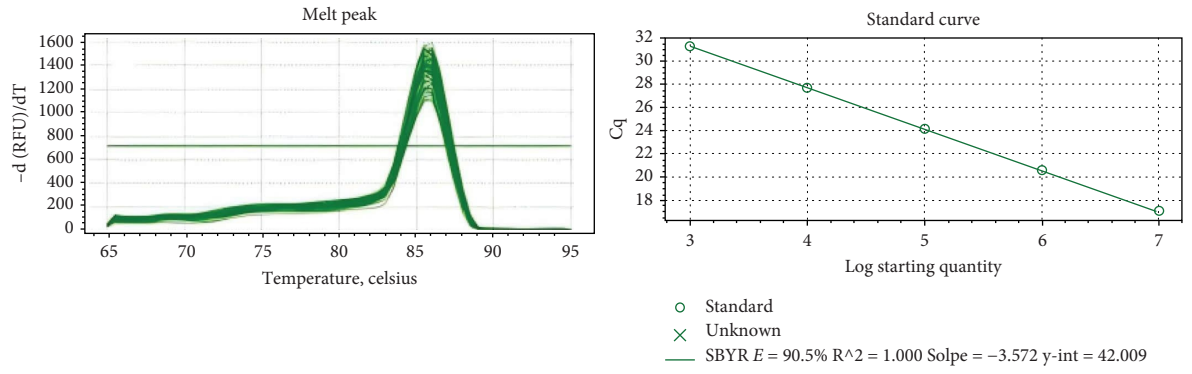

(c)

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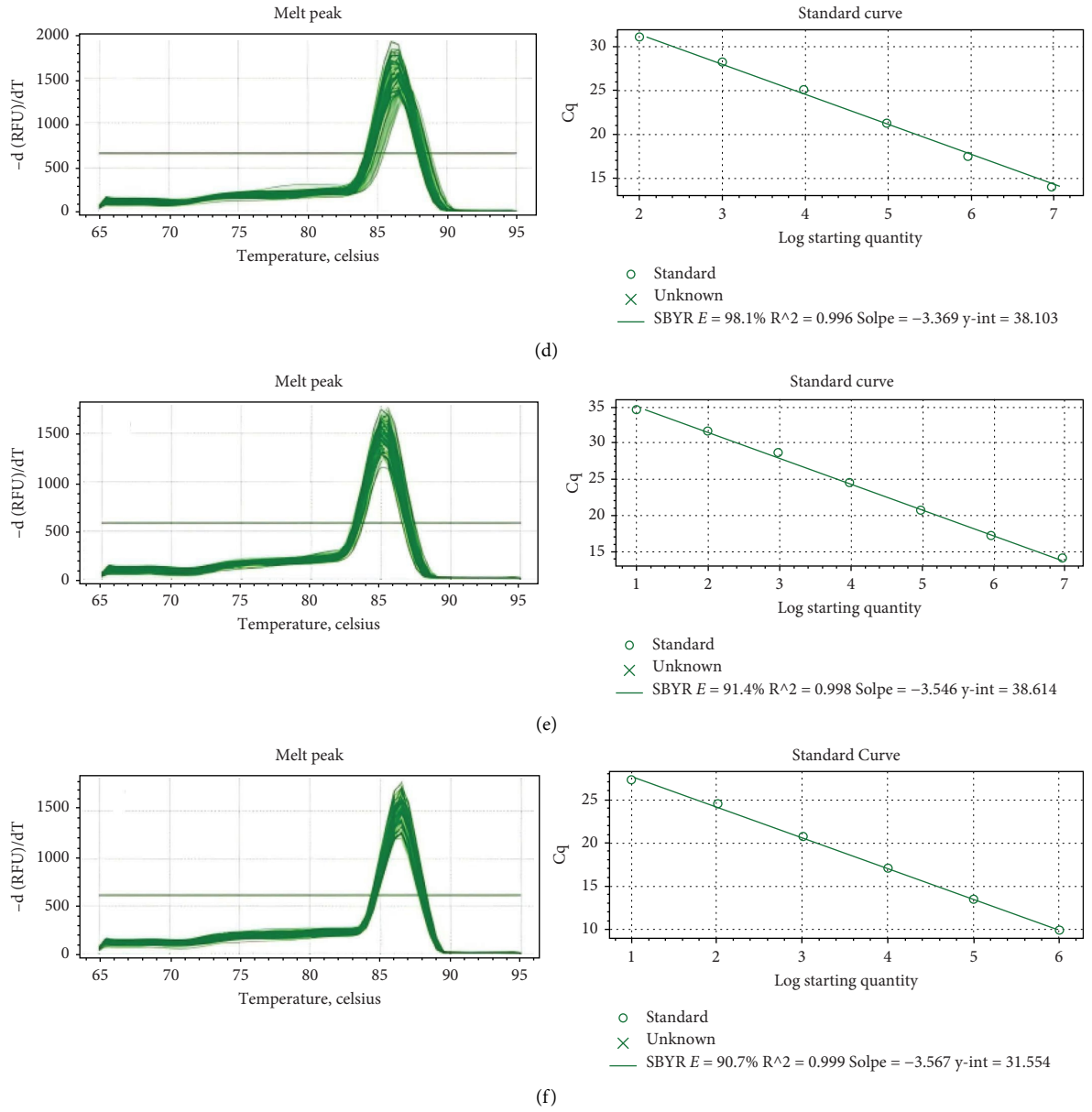

FIGURE 2: Melting curves and standard curves for six reference genes in 14 different tissues of *Hucho bleekeri*. (*β*-actin (a), *b2m* (b), *β-tubulin* (c), *ef1α* (d), *gapdh* (e) and *hprt* (f)).

4. Discussion

The number of *H. bleekeri* individuals in Sichuan Province is very limited, and coupled with their specific living conditions and adaptability, the numbers available for scientific research are even more scarce. This study explored the cloning of internal RGs and established the basis for molecular research for the species.

The *β-actin* gene is present in all eukaryotes and plays an important role in maintaining physiological activities such as cell structure, cell motility, and cell division [38]. It is extensively used as an RG for quantitative RT-PCR in fishes and other taxa [40–42]. However, it is not always the optimal RG. Owing to different species, developmental stages [7, 43, 44], tissues [45], nutritional conditions [46], stress [47], at other factors, the stability of RGs may change. Therefore, the stability of RGs under different conditions needs to be ascertained to select the most suitable one. The *b2m*, *ef1-α*, *gapdh*, *hprt* and *β-tubulin* genes were selected for screening to identify the most stably expression RGs.

Results of the amino acid sequence and evolutionary tree alignment showed that the six RGs of *H. bleekeri* are highly conserved and form a branch with family Salmonidae. The results of expression stable analysis showed that the most unstable gene of *H. bleekeri* is *gapdh*, whereas the most stable is *ef1α*. Similar results have been reported for Yangtze sturgeons *Acipenser dabryanus* [10] and striped jacket *Pseudocaranx dentex* [48]. The stability ranking of the six RGs in *H. bleekeri* is *ef1α* > *β-actin* > *β-tubulin* > *hprt* > *b2m* > *gapdh*, which aligns with the results

Table 2: Ct values of the RGs expressed in *Hucho bleekeri*.

Tissue	*β-actin*	*b2m*	*ef1α*	*β-tubulin*	*gapdh*	*hprt*
Brain	19.52 ± 0.31	24.45 ± 0.19	21.78 ± 0.26	26.23 ± 0.40	32.07 ± 0.30	25.36 ± 0.26
Head kidney	17.85 ± 0.28	20.11 ± 0.62	19.96 ± 0.03	26.71 ± 0.16	30.71 ± 0.10	24.41 ± 0.38
Middle kidney	17.64 ± 0.18	19.88 ± 0.52	19.79 ± 0.12	26.84 ± 0.33	30.48 ± 0.06	23.97 ± 0.36
Muscle	22.02 ± 0.40	24.52 ± 0.49	21.38 ± 0.28	27.66 ± 0.28	21.17 ± 0.49	27.20 ± 0.16
Heart	19.55 ± 0.17	21.30 ± 0.25	20.44 ± 0.04	25.33 ± 0.21	19.07 ± 0.04	24.82 ± 0.08
Hindgut	18.79 ± 0.27	19.76 ± 0.24	20.54 ± 0.05	27.06 ± 0.11	21.65 ± 0.34	26.32 ± 0.26
Duodenum	19.28 ± 0.18	20.17 ± 0.18	20.51 ± 0.08	26.07 ± 0.20	21.61 ± 0.09	26.53 ± 0.29
Skin	20.97 ± 0.36	24.11 ± 0.27	21.78 ± 0.26	27.84 ± 0.26	24.61 ± 1.37	29.07 ± 0.32
Spleen	17.87 ± 0.42	19.34 ± 0.34	19.51 ± 0.09	26.08 ± 0.08	27.90 ± 0.10	24.65 ± 0.39
Liver	20.92 ± 0.10	22.9 ± 0.15	20.76 ± 0.25	25.35 ± 0.40	23.83 ± 0.32	25.28 ± 0.46
Gonad	17.44 ± 0.63	21.84 ± 0.37	19.30 ± 0.28	24.86 ± 0.50	19.89 ± 0.76	23.36 ± 0.59
Eye	19.54 ± 0.69	22.76 ± 0.96	21.55 ± 0.48	26.37 ± 0.61	25.97 ± 1.02	26.53 ± 0.68
Gill	17.79 ± 0.15	19.97 ± 0.25	19.49 ± 0.18	26.31 ± 0.05	23.40 ± 0.34	25.15 ± 0.03
Stomach	18.33 ± 0.26	22.18 ± 0.11	19.73 ± 0.19	26.93 ± 0.11	22.04 ± 0.50	26.60 ± 0.40
Means	19.11 ± 0.23	21.66 ± 0.29	20.47 ± 0.14	26.40 ± 0.15	24.60 ± 0.65	25.66 ± 0.24
Range	6.41	6.57	3.62	3.89	13.80	6.95
Minimum	16.41	18.90	18.82	24.33	18.80	22.52
Maximum	22.82	25.47	22.44	28.22	32.60	29.47

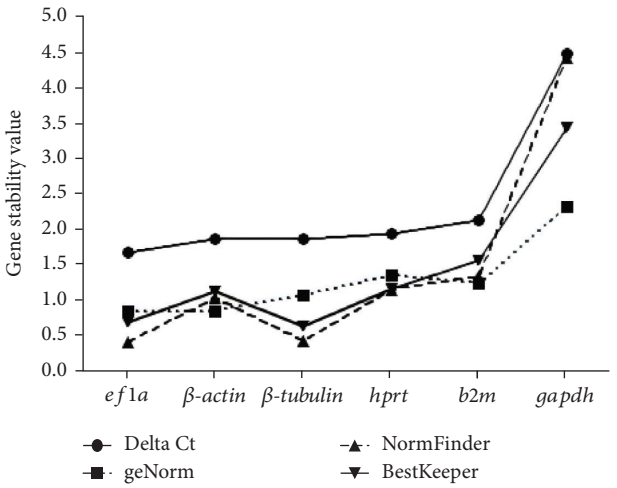

Figure 3: Expression stability of the candidate reference genes in *Hucho bleekeri* tissues as calculated by the comparative delta-Ct method, geNorm, NormFinder and BestKeeper.

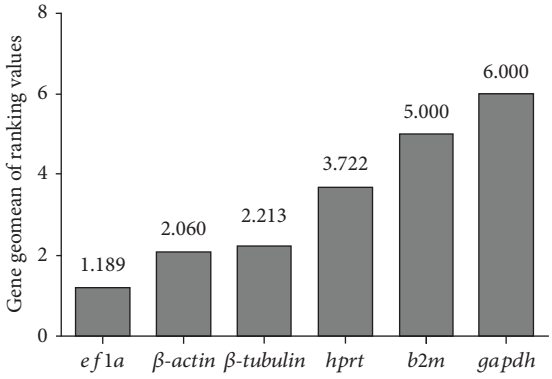

Figure 4: The six reference genes Geomean of ranking values in *Hucho bleekeri* as calculated by RefFinder.

of a previous study [49], which reported that the expression of *b2m* and *gapdh* in polyploid Common carp *Cyprinus carpio* and goldfish *Carassius auratus* tissues and fin cells is unstable. The stability ranking for *Salmo salar* (eight tissues) is *ef1αB* > *ef1αA* > *β-actin* > *gapdh* [50], and that for Grass carp *Ctenopharyngodon idella* (nine tissues) is *ef1α* > *gapdh* > *β-actin* [51]. These results indicate that the stability of RGs among different species differs, and *β-actin* is not the most stable RG under several conditions. Evidently, the stability of *ef1α* is relatively high for most fish species. In eukaryotes, the content of *ef1α* is second only to actin, accounting for 1%–2% of the total protein of normal growing cells [52]. *ef1α* is a GTP-binding protein, which can catalyse the binding of aminoacyl-transfer RNA to the ribosome [53], and plays an important role in protein translation. The *ef1α* was used as a control gene for qRT-PCR in *Branchiostoma japonicum* [54], and for RT-PCR in sex reversal and tissues in *Monopterus albus* [18]. It was also used as one of the RGs with a relatively stable expression for embryonic development and tissue distribution studies in *Danio rerio* [55]. Similar results have also been observed in *Takifugu bimaculatus* [56]. These findings are consistent with the results of the present study, which showed that *ef1α* is commonly well-suited as an internal control gene.

The *gapdh* is an important glycolytic enzyme that catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate [57]. The instability of *gapdh* is due to its extensive involvement in multiple intracellular biological activities, such as nuclear RNA output, DNA replication, and fusion nuclear phosphotransferase activity in the extracellular membrane [58]. Therefore, it may be highly sensitive to intracellular changes. The use of *gapdh* as an RG for fish qRT-PCR is controversial, but it is often tested as a candidate RG. Regarding tissue distribution, previous studies have reported that *gapdh* showed high instability as an RG [18, 55, 59, 60], which is similar to the

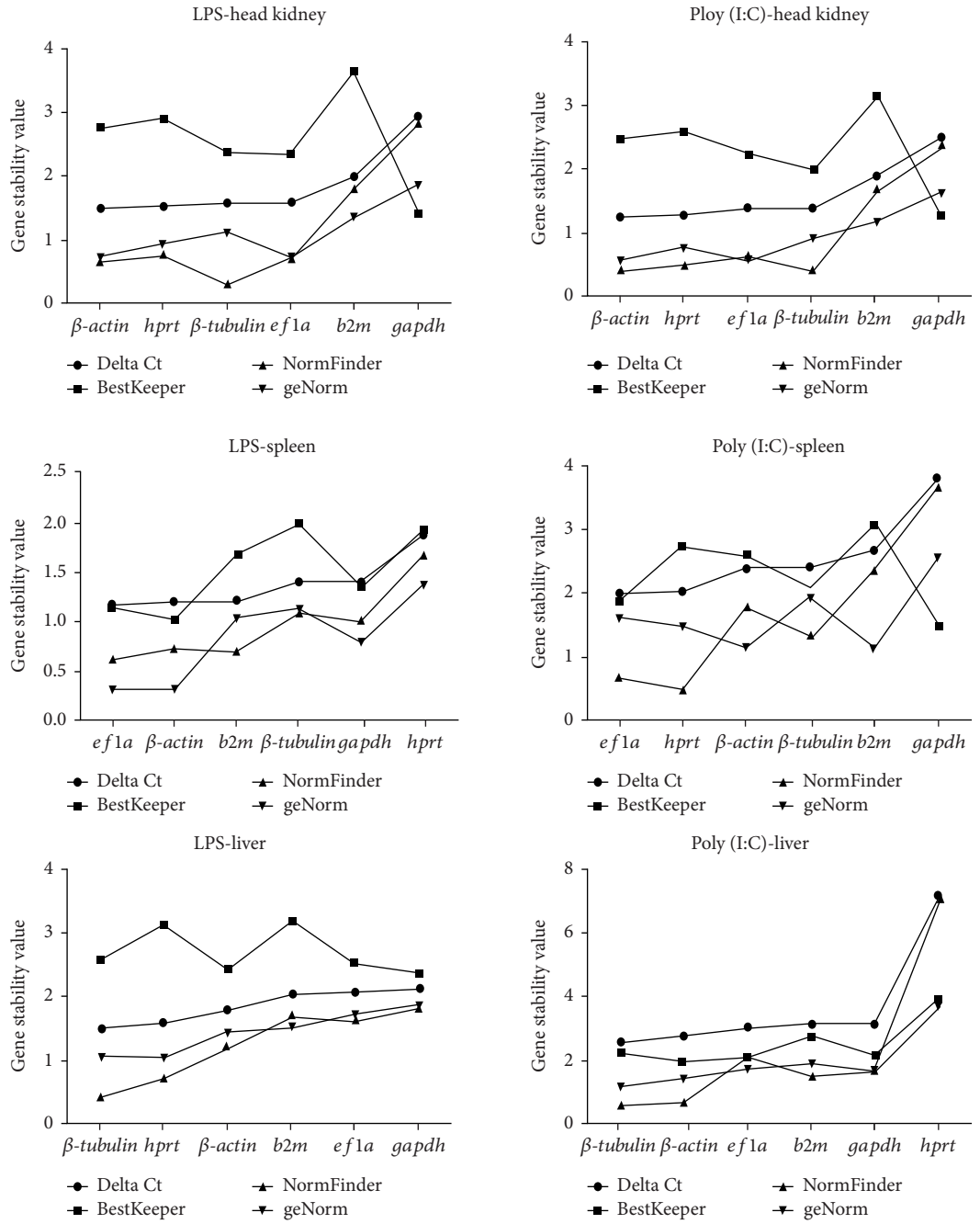

Figure 5: Expression stability of six candidate reference genes under LPS and Ploy (I:C) stress in *Hucho bleekeri* spleen, head kidney and liver cell as calculated by the comparative delta-Ct method, geNorm, NormFinder and BestKeeper.

results of the present study. However, this approach is not entirely without reason. In the present study, *gapdh* had a relatively stable expression in specific tissues under specific conditions. After a 5-d viral nervous necrosis virus infection, the expression of *gapdh* was stable in humpback grouper *Cromileptes altivelis* liver [60]; the stability of *ef1α* and *gapdh* was higher than that of others in European flat oyster (*Ostrea edulis*) haemolymph [61]. Dhar et al. [60] reported that *gapdh* is a better control gene among low-expression genes in shrimp tail muscle infected with the white spot syndrome virus. Zheng and Sun [32] suggested that *gapdh* could be used as an RG in Japanese flounder *Paralichthys olivaceus* intestine and liver after *Edwardsiella tarda* infection. Further, after *Streptococcus agalactiae* infection, *gapdh* was found to be the most suitable gene for Nile tilapia *Oreochromis niloticus* intestine and brain [45]. In addition to *gapdh*, other RGs exhibited this phenomenon; in zebrafish (*Danio rerio*) exposed to cadmium, instead of *gapdh*, the candidate gene *tubulin* showed the highest instability among all tissues selected [62]. Notably, *β-tubulin* also showed good stability in the present study, but its Ct value was slightly higher compared than that of *β-actin*.

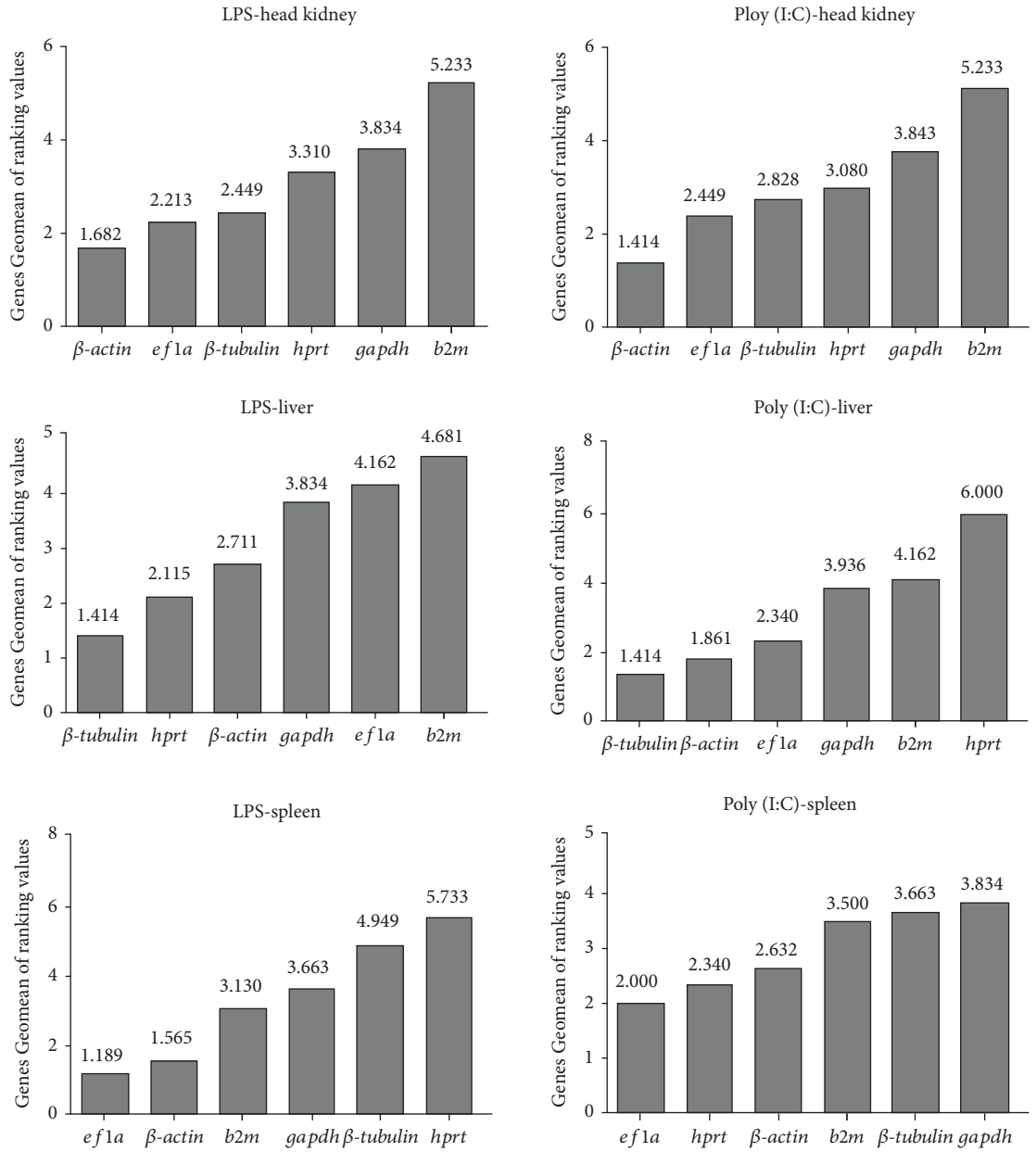

FIGURE 6: Geomean of ranking values for six reference genes under LPS and Ploy (I:C) stress in *Hucho bleekeri* spleen, head kidney and liver cell as calculated by RefFinder.

In addition, we focused further on the stability of RGs under immune stress. The expression of most RGs is not always stable, and the optimal RGs for different tissues varies [45, 62]. Luo et al. [31] demonstrated that no single gene or a pair of genes had been found to serve as a reference across all tissue types under bacterial challenge in *Paralichthys olivaceus*. The *ef1α* was the most suitable in *Oreochromis niloticus* heart and muscle after *Streptococcus iniae* or *Streptococcus agalactiae* infection [45]. In the study of *Oreochromis niloticus* [63], *b2m* and *gapdh* as a combination of genes more accurately calibrated the expression levels of head kidney lymphocytes target genes in LPS and lipoteichoic acid models. According to our results of immune stress in *H. bleekeri*, the stability ranking of the six RGs were different in the head kidney, spleen, and liver cells which treated with LPS or Poly (I: C). Our results suggest that it is necessary to screen for the stability of RGs in different tissues under immune stress.

5. Conclusions

In conclusion, we cloned six candidate RGs-*β-actin*, *b2m*, *ef1α*, *gapdh*, *hprt* and *β-tubulin* of *H. bleekeri*. Optimal amplification conditions were explored, and an optimal RG screening method was established. Based on our research on the six candidate RGs of *H. bleekeri*, we recommend *ef1α* as the most suitable for tissue distribution analysis. Under LPS and Poly (I: C) stimulation, we recommend using *ef1α* for

spleen cells, *β-actin* for head kidney cells, and *β-tubulin* for liver cells. Given the known whole-genome duplication in salmonids, future analysis of potential paralogs of the six candidate RGs will be conducted using the ongoing genome assembly of the Sichuan population of *H. bleekeri*.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization, X.W., J.L. and H.L.; formal analysis, Y.C.; investigation, Z.L., Y.Y. and Q.T.; resources, H.Y. and Y.C.; data curation, X.W.; writing–original draft preparation, X.W. and Y.C.; writing–review and editing, X.W. and H.L.; project administration, H.Y.; funding acquisition H.L. and Y.C.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information 1. Figure S1: The amino acid sequence of *β-actin* in *Hucho bleekeri*. (Single underline represents the start codon, double underline and ∗ represents the stop codon).

Supporting Information 2. Figure S2: The amino acid sequence of *b2m* in *Hucho bleekeri*. (Single underline represents the start codon, double underline and ∗ represents the stop codon).

Supporting Information 3. Figure S3: The amino acid sequence of *β-tubulin* in *Hucho bleekeri*. (Single underline represents the start codon, double underline and ∗ represents the stop codon).

Supporting Information 4. Figure S4: The amino acid sequence of *ef1α* in *Hucho bleekeri*. (Single underline represents the start codon, double underline and ∗ represents the stop codon).

Supporting Information 5. Figure S5: The amino acid sequence of *gapdh* in *Hucho bleekeri*. (Single underline represents the start codon, double underline and ∗ represents the stop codon).

Supporting Information 6. Figure S6: The amino acid sequence of *hprt* in *Hucho bleekeri*. (Single underline represents the start codon, double underline and ∗ represents the stop codon).

Supporting Information 7. Figure S7: Multiple alignment analysis of *β-actin* mRNA sequences. (*Carassius auratus* XM_026258408.1; *Danio rerio* NM_131031.2; *Gallus gallus* NM_205518.2; *Oncorhynchus gorbuscha* XM_046333400.1; *Oncorhynchus keta* XM_052506608.1; *Oncorhynchus mykiss* NM_001124235.1; *Salmo salar* XM_014194536.2).

Supporting Information 8. Figure S8: Multiple alignment analysis of *b2m* mRNA sequences. (*Ictalurus punctatus* NP_001187001.1; *Oncorhynchus kisutch* XP_031671168.1; Oncorhynchus mykiss XP_036812570.1; *Salmo salar* XP_045569988.1; *Salvelinus fontinalis* XP_055762067.1).

Supporting Information 9. Figure S9: Multiple alignment analysis of *β-tubulin* mRNAsequences. (*Chanos chanos* XM_030784286.1; *Oncorhynchus mykiss* XM_021560590.2; *Oncorhynchus nerka* XM_029663472.1; *Salmo salar* XM_004077997.4; *Salvelinus namaycush* XM_038971563.1).

Supporting Information 10. Figure S10: Multiple alignment analysis of *ef1α* mRNAsequences. (*Homo sapiens* NM_001402.6; *Mus musculus* NM_010106.2; *Oncorhynchus kisutch* XM_020500542.2; *Salmo salar* NM_001141909.1; *Oncorhynchus mykiss* XM_021571865.2; *Labeo rohita* XM_051137716.1; *Danio rerio* NM_001039985.1).

Supporting Information 11. Figure S11: Multiple alignment analysis of *gapdh* DNA sequences. (*Danio rerio* NM_001115114.1; *Ictalurus punctatus* NM_001201199.1; *Oncorhynchus kisutch* XM_020500523.2; *Salmo salar* XM_014152701.2; *Salmo trutta* XM_029736057.1; *Salvelinus alpinus* XM_023975136.1).

Supporting Information 12. Figure S12: Multiple alignment analysis of *hprt* mRNA sequences. (*Danio rerio* NM_212986.2; *Homo sapiens* NM_000194.3; *Ictalurus punctatus* NM_000194.3; *Mus musculus* NM_013556.2; *Oncorhynchus keta* XP_035599870.1; *Salvelinus fontinalis* XM_035743977.2).

Supporting Information 13. Figure S13: Multiple alignment analysis of *β-actin* amino acid sequences in *Hucho bleekeri*. (*Carassius auratus* XP_026114193.1, *Danio rerio* NP_571106.2, *Gallus gallus* NP_990849.1, *Homo sapiens*

NP_001092.1, *Mus musculus* NP_031419.1, *Oncorhynchus gorbuscha* XP_046189356.1, *Oncorhynchus keta* XP_052362568.1, *Oncorhynchus mykiss* NP_001117707.1, *Salmo salar* XP_014050011.1).

Supporting Information 14. Figure S14: Multiple alignment analysis of *b2m* amino acid sequences in *Hucho bleekeri*. (*Ictalurus punctatus* NP_001187001.1, *Oncorhynchus kisutch* XP_031671168.1, *Oncorhynchus mykiss* XP_036812570.1, *Salmo salar* XP_045569988.1, *Salvelinus fontinalis* XP_055762067.1).

Supporting Information 15. Figure S15: Multiple alignment analysis of *β-tubulin* amino acid sequences in *Hucho bleekeri*. (*Chanos chanos* XP_030640146.1, *Oncorhynchus mykiss* XP_021416265.1, *Oncorhynchus nerka* XP_029519332.1, *Salmo salar* XP_004078045.1, *Salvelinus namaycush* XP_038827491.1).

Supporting Information 16. Figure S16: Multiple alignment analysis of *ef1α* amino acid sequences in *Hucho bleekeri*. (*Homo sapiens* NP_001393.1, *Mus musculus* NP_034236.2, *Oncorhynchus kisutch* XP_020356131.1, *Salmo salar* NP_001135381.1, *Oncorhynchus mykiss* XP_021427540.1, *Labeo rohita* XP_050993673.1, *Danio rerio* NP_001035074.1).

Supporting Information 17. Figure S17: Multiple alignment analysis of *gapdh* amino acid sequences in *Hucho bleekeri*. (*Danio rerio* NP_001108586.1, *Ictalurus punctatus* NP_001188128.1, *Oncorhynchus kisutch* XP_020356112.1, *Salmo salar* XP_014008176.1, *Salmo trutta* XP_029591917.1, *Salvelinus alpinus* XP_023830904.1).

Supporting Information 18. Figure S18: Multiple alignment analysis of *hprt* amino acid sequences in *Hucho bleekeri*. (*Danio rerio* NP_998151.1, *Homo sapiens* NP_000185.1, *Ictalurus punctatus* NP_001187366.1, *Mus musculus* NP_038584.2, *Oncorhynchus keta* XP_035599870.1, *Salvelinus fontinalis* XP_055744460.1).

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