

## Selection of Reliable Reference Genes for Quantitative Real-Time PCR in Golden-Line Barbell (*Sinocyclocheilus grahami*) During Juvenile and Adult Stages

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Received: 24.04.2017 / Accepted: 03.05.2017 / Published online: 05.05.2017

### Abstract:

In order to obtain reliable results of gene expression, appropriate internal control gene(s) were required for normalization before real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Here, we selected eight candidate housekeeping genes as the potential reference genes in normalizing qRT-PCR data in ten tissues of the juvenile and adult stages of a naturally tetraploid cyprinid fish *Sinocyclocheilus grahami*. Before qRT-PCR, appropriate primers were designed and compared for distinguishing different copies in some duplicate genes. Candidate reference genes were evaluated for their stability using online software RefFinder, which is integrated by four normal software of the comparative delta-C<sub>T</sub>, BestKeeper, NormFinder and GeNorm. According to the rankings produced by these analyses, Eef2, ACTB and G6PD were the most stable reference genes as internal controls for qRT-PCR in juvenile, while B2M, Eef2 and RPL17 were shown to be most appropriate in adult fish. All the analyses show that GAPDH was the least stable expression gene which would be unsuitable as reference gene at both stages. This is the first report about reference gene selection in *Sinocyclocheilus* and we think that is beneficial for the future gene expression studies in golden-line barbel.

**Keywords:** Quantitative real-time PCR; Reference gene; Tetraploid; *Sinocyclocheilus grahami*

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## Introduction

Quantitative real time PCR (qRT-PCR) is an efficient molecular technique that used to study gene expression due to the advantages of high sensitivity, specificity, reproducibility and broad dynamic range (Tricarico et al., 2002; Bustin et al., 2005; Kubista et al., 2006). In order to obtain the results of gene expression reliable and with biological significance, suitable reference genes need to be selected in different experimental conditions. The ideal reference gene should present a relative stable mRNA expression pattern and also should be minimally regulated under different treatments (Nicot et al., 2005; Araya et al., 2008; de Boer et al., 2009).

These most commonly used reference genes included 18S ribosomal RNA(18S), cytoskeletal protein (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Suzukietal.,2000). However, some studies have indicated that the expression of these genes can vary at different development stages or external treatments (Bustin, 2002; McCurley & Callard, 2008; Kortner et al., 2011).

Consequently, other housekeeping genes, such as tubulins, albumins, cyclophilin, micro-globulins, ribosomal proteins, ubiquitin (UBQ), although less common used it might be, have also been described and recommended to be reference genes in specific experimental treatment (Thellin et al., 1999; Morga et al., 2010; Zheng & Sun, 2011). Recent studies showed that most of the reference genes could also change the expression levels under internal or external factors, so there was no universal internal control gene that can be applied for normalization of gene expression under all conditions (Peters et al., 2007; Mitter et al., 2009). What we can do and should do was screening relative most stable reference genes to keep the results of gene expression with higher reliability.

Dianchi golden-line barbel (*Sinocyclocheilus grahami*) is a freshwater cyprinid fish that endemic in the Dianchi Lake basin in Yunnan province of China (Chu & Gui, 1989). It has now been categorized as a second-class, state protected animal due to a dramatic population decrease by human activities (Pan et al., 2009). In the recent decade, the artificial breeding of this fish had been succeeded (Yang et al., 2007) and many other following studies were also conducted, such as the aquaculture management and conservation (Pan et al., 2010; Pan et al., 2011), the whole-genome sequencing (Yang et al., 2016) and etc. But up to now, gene expression research on this fish is still blank. For better understanding the genes expression pattern of some interesting genes, qRT-PCR was an effective technique that can be used at different developmental stages of this fish. To achieve this goal, reliable internal control genes have to be chosen for standardizing the RNA input.

In this study, eight candidate reference genes were used for preferential choose in qRT-PCR at two stages (juvenile and adult) in this study: tubulin beta-4b(Tubb4b), beta-actin(ACTB), glyceraldehyde-3-phosphate dehydrogenase(GADPH), eukaryotic translation elongation factor 2 (Eef2),beta-2microglobulin(B2M), glucose-6-phosphate dehydrogenase(G6PD), ribosomal protein L17 (RPL17), hypoxanthine phosphor ribosyl transferase1 (Hprt1)

(Table 1). One user-friendly web-based comprehensive online tool, RefFinder (Xie et al., 2012), which integrates four frequently used methods namely, the comparative delta-C<sub>T</sub> method (Silver et al., 2006), BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), GeNorm (Vandesompele et al., 2002) were used to compare and rank the stability of tested candidate reference genes at juvenile and adult stages of *S. grahami*.

## Materials and Methods

### Fish husbandry and sample preparation

The juvenile and adult fish were obtained from the endangered fish conservation center, Kunming Institute of Zoology, Chinese Academy of Sciences. Juveniles were random selected from the same lineage (from the same father and mother fish) of 5 months old after in vitro fertilization. During larval and juvenile stage, fry were fed twice daily to satiate with egg yolk and fairy shrimps respectively. Adults were collected from the pool that came from the same generation after artificial propagation and fed twice daily to apparentsatiation with a commercial diet (protein 40%, lipid 18%). Twelve juvenile and eight adult fish (four male and four female) were using for sample preparation. The fresh intestine, liver, muscle, brain, heart, kidney, eye, gill, skin and spleen were frozen in liquid nitrogen and then extracted total RNA immediately.

### Total RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using the TRIzol<sup>®</sup> reagent (Takara, Japan) according to the manufacturer's recommendation. To eliminate residual genomic DNA in the preparation, RNA samples were treated with the RNase-Free DNase I (Qiagen). The quality of total RNA was examined by agarose gel electrophoresis and quantified using UV-Vis spectrophotometer (NANODROP 2000c; Thermo Scientific, USA). For qRT-PCR, 0.5 µg of total RNA was used for reverse transcription with a PrimeScript<sup>®</sup> RT reagent Kit with gDNA

**Table 1:** List of candidate reference genes evaluated in this study.

Gene symbol	Gene name	Function
Tubb4b	Tubulin beta-4B	Major constituent of microtubules
ACTB	Beta-actin	Cytoskeletal structural protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme
Eef2	Eukaryotic translation elongation factor 2	Translation elongation factor
B2M	Beta-2 microglobulin	Small subunit of the MHC1
G6PD	Glucose-6-phosphate dehydrogenase	Pentose phosphate metabolism
RPL17	Ribosomal protein L17	Structural component of the large 60S ribosomal subunit
Hprt1	Hypoxanthine	Purine metabolism

Eraser (Perfect Real Time; Takara) in a final reaction volume of 20  $\mu$ L.

### Copy number of candidate genes identification and primers selection

The copy number of eight candidate references genes was identified through gene annotation of *S. grahami*. CDS sequences were downloaded from the National Center for Biotechnology Information (NCBI) database and BLAST was used to find gene identities with zebrafish. As *S. grahami* is a tetraploid fish, generally, it has two copies corresponding to the single copy gene in zebrafish. For genes which had relatively low similarity among two CDS sequences (exist of deletion or insertion), we used different primers to distinguish them. For those genes which had high similarity between two copies (just exist mutation or SNP) and could not distinguish by primers, we used a pair of primers that suit for combining both of sequences. For one copy gene, we used a pair of primers. Oligonucleotide primers were designed using Primer Premier 5.0 software (PREMIER Biosoft International, CA, USA). All primers were designed to cross at least one intron/exon border that containing both donor and acceptor sites for avoiding amplification of any contaminating genomic DNA. Polymerase chain reaction (PCR) was conducted using cDNA as template before qRT-PCR reaction. Products of PCR were checked by agarose gel electrophoresis to confirm a single band without non-specific bands or false-positive products, and then using DNA sequencing to check whether the target products were correct or not. All primer pairs were used in gradient reactions for determining their optimal annealing temperatures.

### Three-step real-time PCR

After screening, the right primer for every gene was conducted to qRT-PCR reactions. A total of 20  $\mu$ L volume was performed which containing 160 nM of each primer, 5  $\mu$ L of diluted cDNA template ( $\approx 2$  ng/ $\mu$ L) and 2  $\times$  SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Tli RNaseH Plus; TaKaRa) on the LightCycler 480 (Roche). Each PCR reaction was assayed in triplicate and a no template control

(NTC) was included. The three-step qRT-PCR program included an enzyme activation step at 95°C (5 min) and 45 cycles of 95°C (10 s), 57°C (10 s) and 72°C (10 s). Gene expression levels were recorded as  $C_T$  values that corresponding to the number of cycles at which the fluorescence signal can be detected above a threshold value that was arbitrarily set to 0.3. Five-point standard curves of a 5-fold dilution series (1:1-1:625) from pooled cDNA were used for PCR efficiency calculation. The PCR efficiency (E) was given by the equation  $E=(10^{-1/m}-1) \times 100$ , where m was the slope of line argression model fitted overlog-transformed data of the input cDNA concentrations versus  $C_T$  values (Radonić et al., 2004).

### Statistical Analysis

Each sample was run in triplicate and the mean  $C_T$  value was taken as an individual data setting for all statistical analyses including juvenile and adult stages respectively. The  $C_T$  values were analyzed using the comparative delta- $C_T$  method, BestKeeper, NormFinder, GeNorm. The level of statistically significant difference was set at  $P<0.05$ .

### Results

#### The basic information of eight candidate genes and primers selection

By bioinformatic searching and BLAST to the eight candidate genes, we found *Tubb4b*, *ACTB*, *GAPDH*, *G6PD*, *RPL17* and *Hprt1* have two copies while *Eef2* and *B2M* only have one single copy in *S. grahami* (Table 2). For *Tubb4b* and *G6PD*, their two copy sequences have low similarity, so we design different primers to amplify each sequence separately. Electrophoresis results shown that only the high identified copy (BLAST with zebrafish) could get a single band at the expected length, while the other low identified copy amplified either non-specific bands or false-positive bands even though from the copy specific primers (Figures 1A and 1B). Sequencing results also showed single peak and correct base sequence alignment corresponding to the high identity copy. Gene of *ACTB*, *GAPDH*, *RPL17* and *Hprt1*,

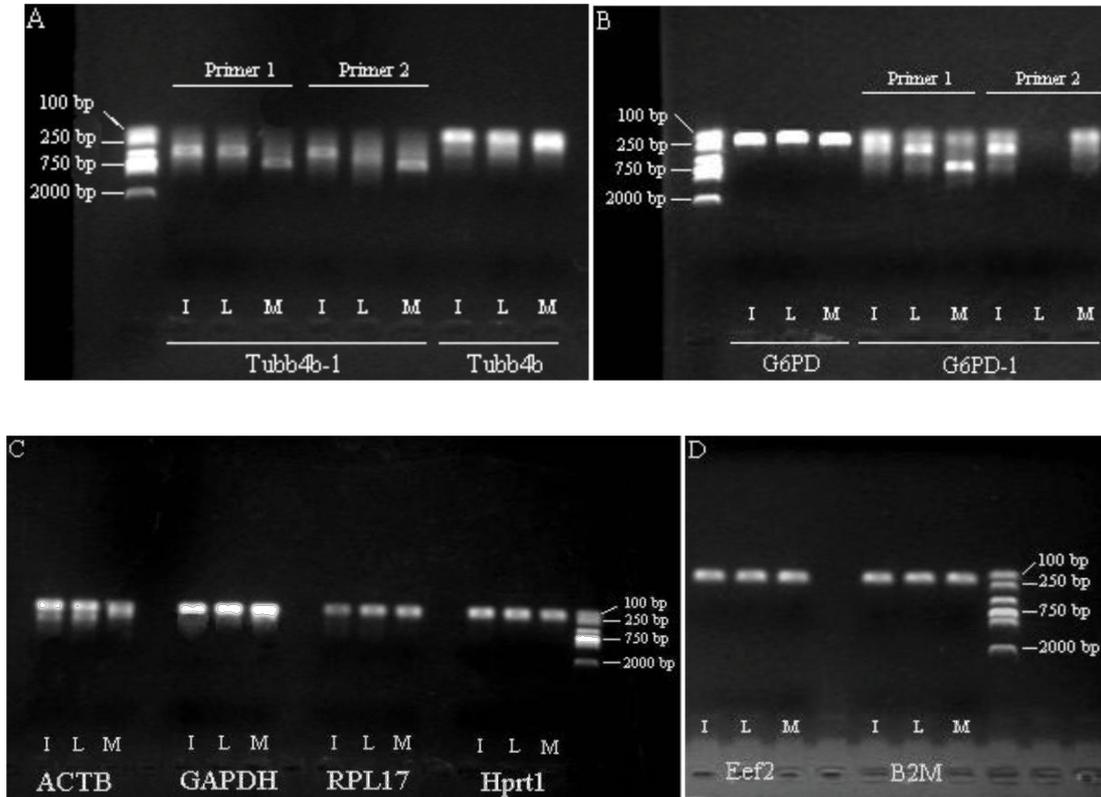
**Table 2:** The number of copy in eight candidate genes and their identity to the corresponding genes in zebrafish.

Gene symbol	Max score	Total score	Query cover	Identities	Genbank accession number of <i>Sinocyclocheilus grahami</i>	Genbank accession number of <i>Danio rerio</i>
<i>Tubb4b</i>	1849	1849	100%	93%	XM_016277458.1	BC062827.1
<i>Tubb4b-1</i>	1267	1267	99%	85%	XM_016275187.1	BC062827.1
<i>ACTB</i>	1807	1807	100%	96%	XM_016249717.1	BC063950.1
<i>ACTB-1</i>	1801	1801	100%	95%	XM_016279613.1	BC063950.1
<i>GAPDH</i>	1362	1362	100%	92%	XM_016287463.1	BC083506.1
<i>GAPDH-1</i>	1330	1330	100%	91%	XM_016232196.1	BC083506.1
<i>Eef2</i>	665	665	100%	91%	XM_016234651.1	BC063965.1
<i>B2M</i>	272	272	100%	81%	XM_016244768.1	BC162420.1
<i>G6PD</i>	2117	2117	100%	91%	XM_016509184.1	XM_694076.7
<i>G6PD-1</i>	654	999	49%	91%	XM_016293468.1	XM_694076.7
<i>RPL17</i>	488	488	100%	93%	XM_016266630.1	BC055097.1
<i>RPL17-1</i>	477	477	100%	92%	XM_016265528.1	BC055097.1
<i>Hprt1</i>	826	826	100%	91%	XM_016293151.1	BC071311.1
<i>Hprt1-1</i>	787	787	100%	90%	XM_016245006.1	BC071311.1

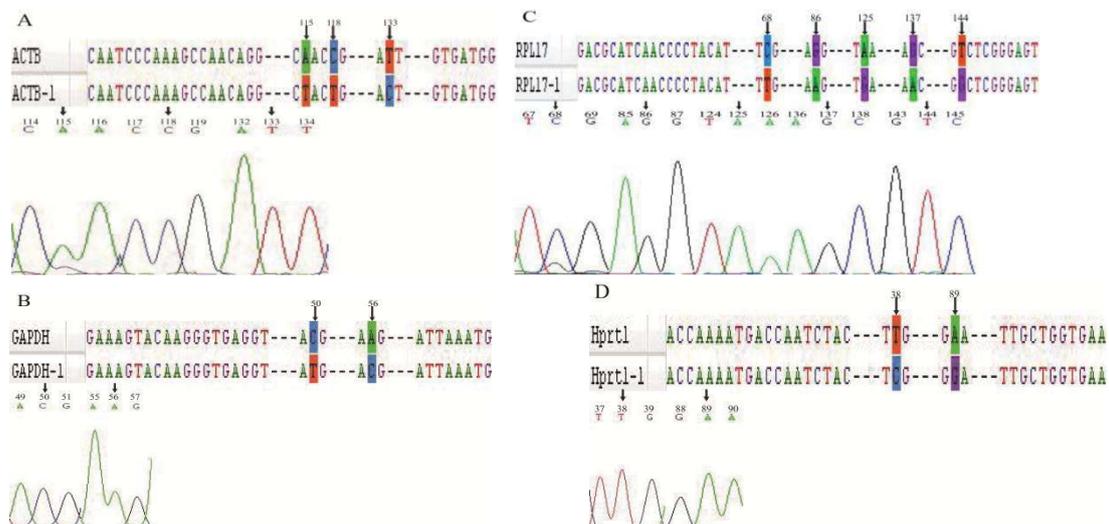
which have high similarities of their two copies, sequences were amplified using a pair of primer that suit for both of them.

Electrophoresis results showed that only one single band was presented (Figure1C) and sequencing results revealed similar phenomena that only the high identified copy has been sequenced

for all the base sites were identical to this copy and without any hybrid site (Figure 2). All the above results indicated that there might be only one copy maintained normal function of these important housekeeping genes although the genome sequencing isolated and annotated two different copies. A pair of primer was



**Figure 1(A-D):** Electrophoresis results of two low similarity copy sequences of Tubb4b (A), G6PD (B), high similarity copy sequences of ACTB, GAPDH, RPL17 and Hprt1 (C) and single copy genes of Eef2 and B2M (D). Two pair of primers (Primer 1 & 2) was designed for the low similarity (here were Tubb4b-1 and G6PD-1) and one pair of primer for Tubb4b and G6PD. The cDNA templates using for PCR came from three different tissues: intestine (I), liver (L) and muscle (M).



**Figure 2:** The SNP sites of amplified fragment in high similarity copy sequences of ACTB (A), GAPDH (B), RPL17 (C) and Hprt1 (D) and their sequencing results. Arrowheads denote SNP sites.

used to amplify part of CDS sequence of Eef2 and B2M, and both the electrophoresis (Figure 1D) and sequencing results verified a single band with correct sequence.

### Real time PCR amplification of candidate reference genes

The primer sequences, amplicon size and the optimal annealing temperatures of eight candidate gene were shown in Table 3. Expression level of each gene in different tissues of juvenile and adult fish was shown in Figure 3. All the standard deviation of CT value between replicates was less than 1. Standard curves exhibited correlation coefficient (R<sup>2</sup>) higher than 0.99 and PCR efficiencies ranged from 95% to 100% (Table 3). The melting curves for all genes demonstrated a single peak confirming gene-specific amplification.

### Stability of reference genes at juvenile stage

Eef2, ACTB and G6PD were the most stable reference genes at juvenile stage according to the comprehensive ranking orders from RefFinder (Figure 4A). For separate software analysis, except

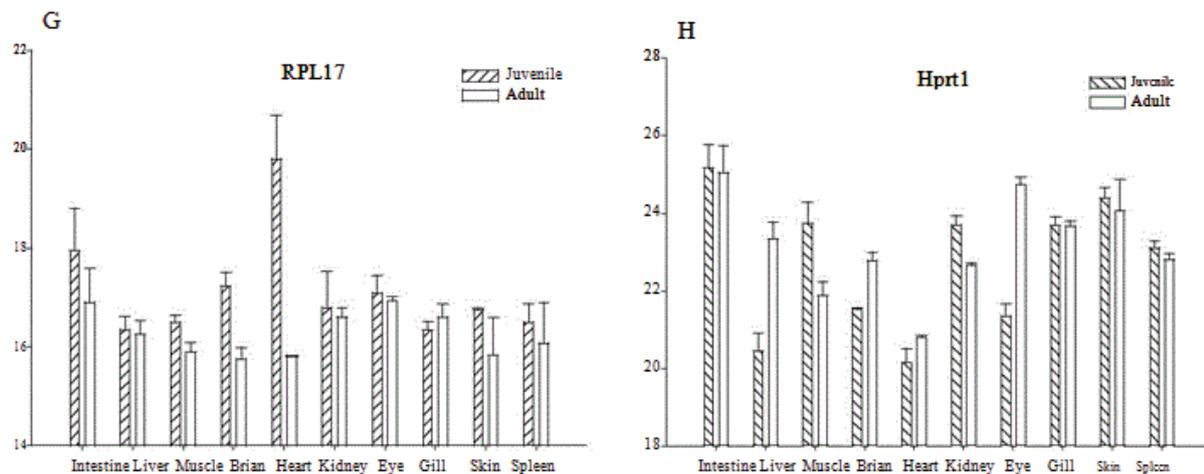
BestKeeper, these three genes also perform the most suitable ones but their rankings have some differences: the comparative delta-Ct method and NormFinder (Eef2>ACTB>G6PD) and GeNorm (Actb|G6PD>Eef2) (Table 4). GAPDH was the least stable reference gene no matter using any methods.

### Stability of reference genes at adult stage

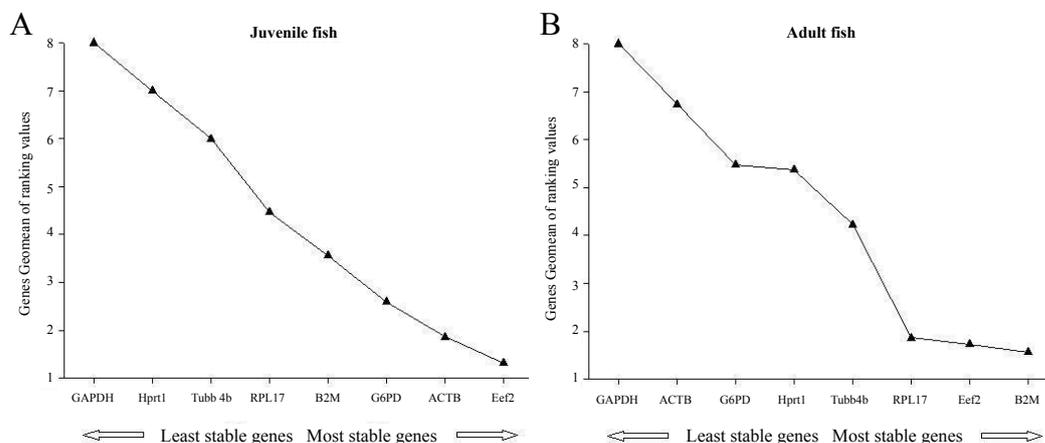
B2M, Eef2 and RPL17 were the most stable reference genes at adult stage according to the comprehensive ranking orders from RefFinder (Figure 4B). For separate software analysis, all four methods showed that these three genes also perform the most suitable ones but their rankings were different: the comparative delta-Ct method (Eef2>B2M>RPL17), BestKeeper (B2M>RPL17>Eef2), NormFinder (Eef2>RPL17>B2M) and GeNorm (B2M|RPL17>Eef2) (Table 4). The similar results of the least stability of GAPDH were found at adult compare to juvenile stage.

### Discussion

In previous studies about reference gene selection in fishes,



**Figure 3:** Expression levels of eight candidate genes in ten different tissues during juvenile and adult stages of *S. graham*.



**Figure 4:** Expression stability ranking orders of the eight candidate reference genes calculated by the Geomean method of RefFinder. A lower Geomean ranking values indicates more stable expression among different tissues.

**Table 3:** The final gene copy and corresponding primers that chosen for qRT-PCR reaction and their PCR amplification efficiencies

Gene symbol	5'-3' primer sequence		Amplicon size (bp)	Annealing temp. (°C)	RT-PCR efficiency (%)	Correlation coefficient
Tubb4b	Forward	GACCCAACAGGCAGTTACCA	120	57	96.2	0.994
	Reverse	GGGCTCCAAATCCACCAG				
ACTB	Forward	CAATCCCAAAGCCAACAG	144	57	99.1	0.999
	Reverse	CCATCACCGGAATCCATC				
GAPDH	Forward	GAAAGTACAAGGGTGAGGT	100	57	95.5	0.991
	Reverse	CATTTAATGTTGGCTGGAT				
Eef2	Forward	CCCCATCTTCAAGGTGTT	116	57	99.5	0.999
	Reverse	GCTTGCCTTCTTATCCT				
B2M	Forward	CATCACTTTTGCCTGT	113	57	99.4	0.999
	Reverse	GGGTGTTCTCCTTCCAT				
G6PD	Forward	AATCCTCTTTCGCTAAC	158	57	97.3	0.998
	Reverse	TATCCTGTTCCAGCCTCT				
RPL17	Forward	GACGCATCAACCCCTACA	153	57	96.9	0.999
	Reverse	ACTCCCGAGACATAAGCT				
Hprt1	Forward	ACCAAATGACCAATCTACA	170	57	96.2	0.999
	Reverse	CCTCTTACCAGCAAAC				

**Table 4:** Ranking orders of the eight candidate genes at juvenile and adult *S. graham*.

Developmental Stages	Rank	Delta Ct		BestKeeper		NormFinder		GeNorm	
		Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
Juvenile	1	Eef2	1.4	Eef2	0.33	Eef2	0.45	Actb G6PD	0.58
	2	ACTB	1.48	B2M	0.57	ACTB	0.7		
	3	G6PD	1.51	ACTB	0.58	G6PD	0.8	Eef2	0.84
	4	RPL17	1.74	RPL17	0.72	RPL17	1.1	B2M	1.04
	5	B2M	1.74	G6PD	0.82	B2M	1.15	RPL17	1.13
	6	Tubb4b	1.97	Tubb4b	1.39	Tubb4b	1.58	Tubb4b	1.31
	7	Hprt1	2.12	Hprt1	1.49	Hprt1	1.66	Hprt1	1.5
	8	GAPDH	2.95	GAPDH	1.93	GAPDH	2.76	GAPDH	1.86
Adult	1	Eef2	1.45	B2M	0.33	Eef2	0.25	B2M RPL17	0.7
	2	B2M	1.58	RPL17	0.4	RPL17	0.8		
	3	RPL17	1.61	Eef2	0.8	B2M	0.84	Eef2	0.86
	4	Tubb4b	1.69	Hprt1	0.99	Tubb4b	1.04	Tubb4b	1.11
	5	G6PD	1.79	Tubb4b	1.15	Hprt1	1.12	G6PD	1.23
	6	Hprt1	1.83	G6PD	1.19	G6PD	1.22	ACTB	1.31
	7	ACTB	1.92	ACTB	1.61	ACTB	1.47	Hprt1	1.4
	8	GAPDH	3.44	GAPDH	2.44	GAPDH	3.3	GAPDH	1.91

most objective species were diploids, such as zebrafish (McCurley & Callard, 2008), rice field eel (Hu et al., 2014) and Nile tilapia (Wang et al., 2015) and few studies concerned about polyploidy. For most diploids fishes, these common used reference genes were only presenting one copy, so the gene expression analyses using qRT-PCR was straightforward. Even if the reference genes study on the tetraploid *Xenopus laevis* by Šindelka et al. (2006), they did not take any different gene copies into account after genome duplication. In our study, we use a strategy to design two pairs of primers in distinguishing different copies that with relative low similarity (BLAST to zebrafish), such as Tubb4b and G6PD; and to design the same pair of primers in amplifying both the copies

to these relative high similarity genes, such as ACTB, GAPDH, RPL17 and Hprt1. All the results from PCR indicated that there might be only one copy maintained normal function of these housekeeping duplicated genes although the genome sequencing isolated and annotated two different copies. Additionally and interestingly, all these copies being sequenced were these high similarity ones when BLAST to zebrafish. Although the mechanisms of how to retain gene function and which copy is preferred after gene duplication was beyond the aim of this study, at least one pair of primer for each gene was finally verified and picked out for the following qRT-PCR reactions.

Generally, the typical reference genes are those expressed

highly and stably in most cells under certain experimental conditions (Janssens et al., 2004). Integrating several applications to assess the applicability of different reference genes from alternative methods can make the result more reliably. Previous studies had demonstrated that reference genes could be different under different conditions, in different tissues and during different developmental stages (McCurley & Callard, 2008; Van Hiel et al., 2009; Zheng & Sun, 2011). In this study, ranking stabilities of eight candidate genes exhibited various degrees of expression among ten tissues during juvenile and adult stages no matter which methods were used for evaluation. Similar observations were found in other fish species, such as channel catfish (Small et al., 2008), Atlantic halibut (Fernandes et al., 2008) and fathead minnow (Filby & Tyler, 2007).

In present study, the most stable reference genes were Eef2, ACTB and G6PD at juvenile stage while Eef2, B2M and RPL17 at adult stage, which had stability values below 1.0 using BestKeeper, NormFinder and GeNorm. Summarizing the results from four programs by RefFinder, Eef2 was the most stable gene in different tissues at both juvenile and adult stages. Eef2 is a translation elongation factor that promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome (Kaul et al., 2011). In the reference gene selection study of Atlantic halibut, Eef2 presented low stability under different development stages or experimental conditions (Fernandes et al., 2008; Hagen et al., 2009). But based on our data, Eef2 was evaluated as the most stable expression gene in both stages of *S. grahami* and the mean  $C_T$  values were also presented minimum changes ( $15.25 \pm 0.33$  and  $15.95 \pm 0.21$  at juvenile and adult stages, respectively). ACTB is an essential gene in cellular shape and anchorage where transmembrane glycoproteins link fibronectin in the extracellular matrix with actin microfilaments on the cytoplasmic side of the membrane and often using as a common reference gene in many experiments (Glare et al., 2002, Dundas & Ling, 2012). According to this study in *S. grahami*, ACTB can also be used as one of the reference genes due to higher stability in juvenile. However, ACTB is not suitable for using as reference gene due to high variation in different tissues at adult fish. Other studies involve in zebrafish and channel catfish have a similar result to rejected ACTB as a reference gene, especially in adult fish (McCurley & Callard, 2008; Small et al., 2008). Although many studies used G6PD as one of candidate genes in the process of selecting reliable reference genes as we do, few results recommended it because of its high variation (Hibbeler et al., 2008; Aursnes et al., 2011). Calculating by GeNorm, ACTB combine with G6PD were recommended as the most stable reference genes to juvenile while not to adult. In Japanese flounder and zebrafish, B2M was known to be one of the most stable genes across different tissue types (McCurley & Callard, 2008; Zheng & Sun, 2011), which was similar to our study in adult fish. RPL17 was listed as a best reference gene when exploring the expression levels of six genes in various Atlantic halibut tissues, both in non-injected fish and in mock- and NNV-injected fish (Overgard et al., 2010). In this study of adult fish, the gene expression of RPL17 among different tissues is also relative stability.

GAPDH contributes to diverse cellular functions such as nuclear RNA export, DNA replication, DNA repair, exocytotic membrane fusion, cytoskeletal organization and phosphor transferase/kinase reactions (Sirover, 1999). Compared to other species, GAPDH was identified less frequently as reference genes for qRT-PCR in some published studies on fish (Olsvik et al., 2005; Tang et al., 2007; Olsvik et al., 2008; Zheng & Sun, 2011). The similar result also found in the fish of our study, GAPDH exhibited variation seriously among different tissues in both development stages using four different methods, which suggested it might be the least suitable reference gene in *S. grahami*. As far as we know, this is the first report about the reference gene selection in Sinocyclocheilus fishes. The species in this genus were served as an emerging cavefish model (Yang et al., 2016) that it can be expected more and more researches were conducting to this group of fishes in the future. As a natural group of tetraploid fishes, designing appropriate primers for distinguishing different copies was the very fundamental step before the qRT-PCR analyses. The strategy we used in this study may provide some reference to the coming researches. Screening eight candidate reference genes in *S. grahami* indicated that Eef2, ACTB and G6PD to be the most stable reference genes and could be used as the suitable internal controls in juvenile, and Eef2, B2M and RPL17 were considered to be the most reliable reference genes for accurate qRT-PCR in adult fish.

## Conflict of Interests

The authors declare no conflict of interest.

## Acknowledgements

We thank the members at the endangered fish conservation center, Kunming Institute of Zoology and the colleagues in the State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology for their help to conduct the experiment. This study was supported by the Basic Research Program of Yunnan Province (2016FA044 to Yang, J.X., 2012FB183 to Pan, X.F.); the Yunnan Biodiversity Protection Program, a major program of the Chinese Academy of Sciences (Y206B51181); CAS "Light of West China" program to Wang, X.A. and Pan, X.F.

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