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Optimal Reference Genes in Different Tissues, Gender, and Gonad of Yellow River Carp (*Cyprinus carpio* var) at Various Developmental Periods

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ABSTRACT

Yellow River carp is one of the most important breeding fish in China owing to its economic value in aquaculture, and has been extensively studied using quantitative real-time PCR. As many of the reference genes commonly used in the studies on Yellow River carp significantly vary among the species, it is important to determine their stability. To assess an appropriate housekeeping gene that is stably expressed across different tissues, gender, and gonad of Yellow River carp at various developmental stages, six reference genes (beta-2 microglobulin (*B2M*), beta-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1 alpha (*EF-1α*), 18S ribosomal RNA (*18S rRNA*), and *40S* ribosomal protein (*40S*)) were evaluated using descriptive analysis and three software programs (geNorm, BestKeeper, and NormFinder), along with RNA-seq statistics. The results showed that *40S* is the best candidate gene for normalization in 2-year-old adult Yellow River carps; *18S rRNA* and *ACTB* are the best candidate genes for normalization in juvenile carps; and *GAPDH* and *40S* are the best candidate genes for normalization in carp gonad at different developmental stages. These findings were in accordance with the RNA-seq transcripts pertaining to the expression level and stability of these reference genes. Furthermore, *EF-1α* and *40S* had the most stable expression levels in all the tissues of the juvenile and adult carps. Thus, the results of the present study could be valuable for further investigation and better molecular genetic analyses of optimal reference genes in Yellow River carps.

Article Information

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Authors' Contributions

WZ, YJ and ZC designed the study. WZ, YJ, XJ, TL and RZ isolated RNA. WZ, YJ, XJ, TL and RZ designed the primers and performed RT-PCR and qPCR. WZ, YJ and XJ analyzed the data. WZ, YJ, QD and ZC wrote the article.

Key words

Quantitative real-time PCR, reference genes, Yellow River Carp, stability

INTRODUCTION

The common carp, *Cyprinus carpio*, is one of the most important cyprinid species and accounts for 10% of the global freshwater aquaculture production (Peng *et al*., 2014). The Yellow River carp (*Cyprinus carpio* var.) is a member of this group, with stable species traits, fast growth, and high survival rates. Numerous studies on carps have provided additional insights into the molecular genetic mechanism, with quantitative real-time PCR (qPCR) being widely used. A search on PubMed and SpringerLink databases using the key terms "*Cyprinus carpio*" and "quantitative real-time PCR" revealed ~2500 publications and ~26,127 papers in the recent 5 years, which have reported extensive data on the physiology, development, immunology, disease resistance, and selective breeding of carps. The qPCR has been frequently applied in these studies and appears to be more accurate and sensitive for gene expression analysis, when compared with traditional methods such as reverse transcription PCR (RT-PCR) (Zhan *et al*., 2016) or northern blotting (Bustin, 2000). However, the qPCR is mostly based on a good housekeeping gene as reference for RNA variability or instrumental errors to a certain degree. An ideal reference gene should exhibit stable expression level in all the samples irrespective of the specimens, tissues, developmental or differential stages, and different experimental treatments.

While there is no universal housekeeping gene, those commonly used considerably vary in the same species along with different tissues and conditions (Glare *et al*., 2002; Dang and Sun, 2011). Several researchers have characterized the housekeeping genes in some species and used viable strategies to check their stability. For instance, based on the Excel-based software programs, NormFinder (Andersen *et al*., 2004), BestKeeper (Pfaffl *et al*., 2004), and geNorm (Vandesompele *et al*., 2002), *EF-1α* and *RPL7* were determined to be the best reference genes in Atlantic halibut (Øvergård *et al*., 2010). In addition, RNA Sequencing (RNA-seq) and Digital Gene Expression Profiling (DGE) could also provide important reliable data on the expression of genes, including the

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housekeeping genes, at the transcriptional level (Zhang *et al*., 2013). A survey of 100 studies on carps in which qPCR was employed showed beta-actin (*ACTB*) as the most common housekeeping gene (n=32), followed by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (n=18), 18S ribosomal RNA (*18S rRNA*) (n=11), elongation factor 1 alpha (*EF-1α*) (n=8), beta-2 microglobulin (*B2M*) (n=2), *40S* ribosomal protein (*40S*) (n=1), and other/unlisted (n=28) housekeeping genes. Housekeeping genes are a class of genes expressed in all the cells, and their products are essential for the maintenance of necessary life activities. These genes are used in several methods such as northern blotting, ribonuclease protection assays, or RT-PCR to study gene expression and gain additional insight into the complex biological processes or cellular functions. In the present study, the six housekeeping genes, *B2M*, *ACTB*, *EF-1α*, *GAPDH*, *18S rRNA*, and *40S*, in different tissues, gender, and gonads of Yellow River carps at various developmental stages, including undifferentiated larvae (40–55 day after hatch (dph)), 1-year-old juveniles, and 2-year-old adult carps, were investigated. Based on the analysis of the qPCR data using geNorm, BestKeeper, and NormFinder, along with RNA-seq statistics, the expression levels of the six reference genes were evaluated and the most stable and suitable reference gene was identified. The results obtained could be valuable for further research on reference genes and provide useful information on gene expression analysis along with relative quantification in Yellow River carps.

MATERIALS AND METHODS

Animals

Yellow River carps were obtained from Henan Academy of Fishery Science (Henan, P. R. China), and maintained at the Genetic Laboratory (Henan Normal University, Xinxiang, P. R. China) in through-flow water tanks at 25±2°C under natural photoperiod for an initial acclimation period. The fish were fed with artemia nauplii twice a day, and no fish died during the experiment. The juveniles (1 year old) and adults (2 years old) were randomly selected and grouped into five fish per pool. A total of 14 tissues, including heart, liver, kidney, forebrain, hindbrain, gonad, foregut, hindgut, scale, fin, muscle, eye, spleen, and gill, were collected under aseptic conditions. The gonads of 40, 45, 50, and 55 dph larvae were removed under stereomicroscope (Olympus SZ61).

RNA extraction and RT-PCR

The total RNA was extracted from all the samples using RNA extraction kit and RNAiso reagent (TaKaRa, Japan). Approximately 20 mg of each tissue were homogenized using mortar containing liquid nitrogen, and an aliquot of each extract was used for spectrophotometry to determine the quality and concentration of RNA. The RNA sample with an $OD_{260/280}$ ratio of 1.9–2.2, an $OD_{260/230}$ ratio of ≥2, and a 28S:18S ratio of approximately 2:1 was considered satisfactory and used in this study. For the expression profile analysis, cDNA was synthesized using PrimeScript Reverse Transcriptase (TaKaRa, Japan) with 1 µg of total RNA, according to the manufacturer's instructions.

Primer design

The reference genes *B2M*, *ACTB*, and *EF-1α*, which have been commonly used in numerous homologous species studies, were chosen in the present study and specific primers were designed in our laboratory to obtain a qPCR product of approximately 120–300 bp in length and avoid hairpin and cross dimer. In addition, *GAPDH* and *18S rRNA* had been stably expressed in a comparative transcriptomics study of carp gonads in our laboratory (unpublished data), and the primer for *40S* was acquired from Pionnier *et al.* (2014). The details of the six reference genes, including names, cellular functions, primer sequences, and GenBank accession numbers are listed (Table I). The specificity of the primers was verified by RT-PCR product sequencing, and PCR amplification was performed as follows: 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min.

qPCR

qPCR was performed using quantitative real-time PCR detection system (LightCycler 96® Roche) with SYBR Green fluorescent label. The reaction mixture comprised the tissue cDNA as the template, 2× Ultra SYBR Mixture (TaKaRa, Japan), and 0.2 µl of each primer to a final reaction volume of 10 µl. Each assay was performed in triplicate and included a negative control. The reaction parameters were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. For each sample, a dissociation step was performed to identify a single specific melting temperature for each primer set. The amplification temperature was gradually increased from 65°C to 95°C at a rate of 0.2 s.

Data analysis

The data generated by LightCycler® 96 Instrument were collected using LightCycler® 96 software. The Cq value is defined as the fractional cycle number at which the fluorescence passes the fixed threshold and is

Table I.- Primers used for qRT-PCR analysis.

Gene	Cellular functions	Primer sequence	Product size	Correlation coefficient (R^2)	GenBank accession numbers
B2M	Major histocompatibility complex	TCCCAAGATTCAGGTGTA	120	0.993	AM690441
		TCTCGCCATCCTTCAGC			
18S rRNA	18S ribosomal RNA	GAGTATGGTTGCAAAGCTGAAAC	129	0.998	FJ710826
		AATCTGTCAATCCTTTCCGTGTCC			
ACTB	Cytoskeleton	GATGATGAAATTGCCGCACTG	130	0.999	M24113
		ACCAACCATGACACCCTGATGT			
EF1 α	Translation	CTTCGTCCCAATTTCTG	109	0.995	AF485331
		ACCGTTAGCATTACCCT			
GAPDH	Glycolysis enzyme	CCGTTCATGCTATCACAGCTACACA	310	0.998	AJ870982
		GTGGATACCACCTGGTCCTCTG			
40S	40S ribosomal protein S11	CCGTGGGTGACATCGTTACA	117	0.997	AB012087
		TCAGGACATTGAACCTCACTGTCT			

inversely correlated to the amount of template present in the reaction (Livak and Schmittgen, 2001; Pfall, 2001). To assess the most stably expressed candidate reference gene, the data were analyzed using SPSS 13.0 descriptive analysis to calculate the mean and standard deviation (SD), followed by geNorm, BestKeeper, and NormFinder, which evaluated stability considering gene variation. A lower M value corresponded to a more stable expression of the gene.

RESULTS

PCR efficiency analysis

All the primers were evaluated through standard curves for specificity using serially diluted cDNA (100%, 50%, 20%, 10%, 5%, 2%, and 1%) as template in qPCR. The correlation coefficients (R^2) were all >0.99 (Table I) and the melting curve appeared as a single peak. In addition, each cloned gene of the Yellow River carp presented a single band of expected size in 1% agarose gel electrophoresis.

Each reference gene in the 112 samples (three replicates per sample) produced 336 Cq values. Descriptive analysis indicated that *18S rRNA* had the highest expression, appearing more than five cycles earlier than the other genes. In contrast, *B2M* showed the lowest expression level and largest variation with highest SD (Table II). As shown in the animation, the distribution of the Cq values of each reference gene in any case was normal.

Table II.- Descriptive statistics of the reference genes Cq values (n=316).

Gene	Mean	SD	Min. Cq	Max. Cq
B2M	27.717	1.529	241.21	31.48
18S rRNA	15.5734	0.82	14	25.71
ACTB	20.5659	1.005	18.23	27.06
EF-1α	21.71	1.201	18.38	27.37
GAPDH	21.3916	0.998	18.28	22.96
40S	22.648	1.011	19.99	25.68

Expression of housekeeping genes in adult carps

Examination of the RNA levels of the six reference genes in various tissues of the adult carps revealed minor changes in the mean Cq values and general expression levels. Higher Cq values ranging from 13.84 to 30.79 indicated higher expression, and *18S rRNA* presented the highest mRNA level, followed by *ACTB*, *GAPDH*, *EF-1α*, *40S*, and *B2M*. Individual analysis of the genes showed that *18S rRNA* had the least variation, whereas *B2M* had the highest variation. Considering individual expression within the tissues, the mRNA levels of all the genes, except *GAPDH* and *18S rRNA*, were generally low in the muscles, whereas most of the genes were variably expressed in the fin. With respect to gender, although minor variations could be translated to individual difference, higher expressions of *ACTB*, *EF-1α,* and *18S rRNA* were noted in the fin of female carps, when compared with those in the fin of male carps (Fig. 1).

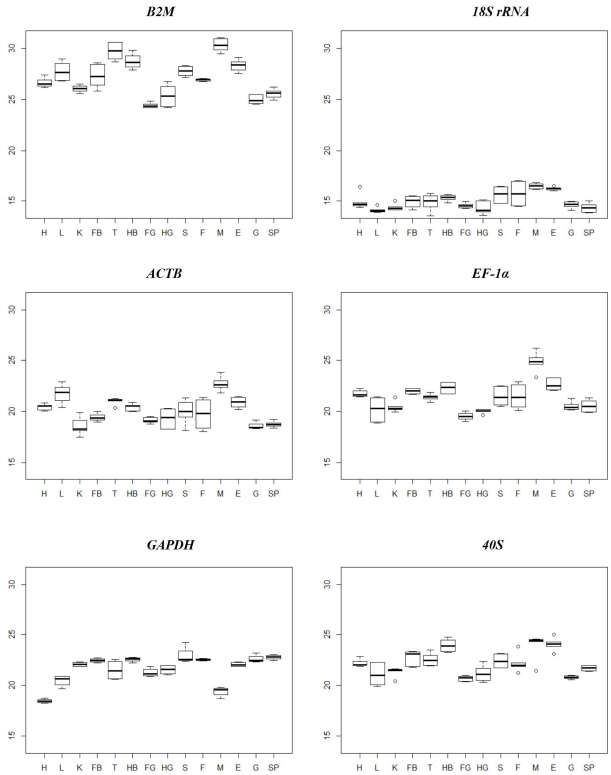

Fig. 1. Boxplots of the Cq values in various organs of adult Yellow River Carps for each of the six potential reference genes.
Data is shown as average Cq values±SD (n = 6). Abbreviations: H, heart; L, liver; K, kidney; FB, forebrain; T, gonad; HB, hindbrain; FG, foregut; HG, hindgut; S, spleen; F, fin; M, muscle; E, eye; G, gills; SP, spleen.

Analysis of the expression data of the selected genes using NormFinder, BestKeeper, and geNorm showed that *40S* had the highest stability, whereas *B2M* and *GAPDH* had the lowest stability. The geNorm software ranked *EF-1α* and *40S* as the best candidate genes, the BestKeeper software indicated *18S rRNA* and *40S* as the best candidate genes, and the NormFinder software proposed *40S* as the best candidate gene, followed by *18S rRNA* and *EF-1α* (Table III). Therefore, *40S* can be recommended as the best candidate gene in 2-year-old adult Yellow River carps.

Expression of housekeeping genes in juvenile carps

The difference in the distribution of the reference genes in juvenile carps was significant to some degree. As shown in Figure 2, *18S rRNA* exhibited the highest mRNA level, followed by *ACTB*, *GAPDH*, *EF-1α*, *40S*, and *B2M*. Furthermore, the transcripts of all the selected genes presented discrepancy between gender, with lower expression level of *40S* in the gonad and *B2M*, *ACTB*, *EF-1α*, *18S rRNA*, and *GAPDH* in the kidney of male juvenile carps, when compared with those in female juvenile carps (difference in average Cq = 6.2).

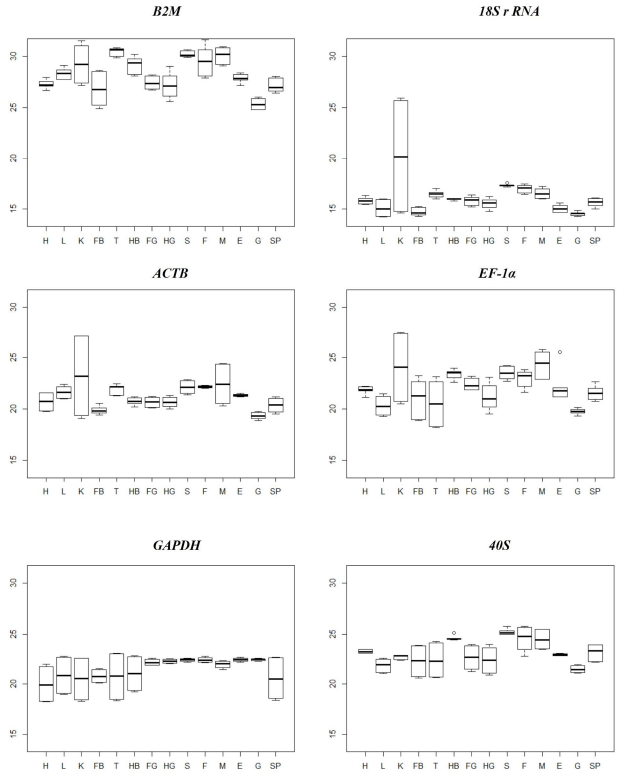

Fig. 2. Boxplots of the Cq values in various organs of juvenile Yellow River carps for each of the six potential reference genes.
For other details, see legend of Figure 1.

18S rRNA and *ACTB* were listed as the most stably expressed genes by geNorm and BestKeeper, whereas *GAPDH* was identified as the most unstably expressed genes by geNorm and NormFinder. The rankings of the genes by geNorm were as follows: *18S rRNA*> *ACTB* > *EF-1α* > *B2M* >*40S* > *GAPDH*. The rankings of the genes by BestKeeper were as follows: *18S rRNA* > *ACTB* >*40S* > *GAPDH* > *EF-1α* > *B2M*. However, NormFinder presented some disagreement in the rankings: *EF-1α* >*B2M* > *ACTB* >*40S* >*18S rRNA* > *GAPDH*. Furthermore, the BestKeeper software presented the highest inter-gene correlation for *18S rRNA*/*ACTB* (r = 0.829), indicating that these two genes might be co-regulated (Table III). Therefore, *18S rRNA* and *ACTB* can be recommended for normalization in juvenile Yellow

River carps.

Table III.- Stability rankings of reference genes obtained with different determination methods.

Samples	Rank	1	2	3	4	5	6
Adult	G	*40S/EF-1α* (0.818)		*18S rRNA* (0.892)	*ACTB* (1.031)	*B2M* (1.138)	*GAPDH* (1.494)
	B	*40S* (4.672)	*18S rRNA* (4.880)	*GAPDH* (5.015)	*EF-1α* (5.286)	*B2M* (5.517)	*ACTB* (5.8671)
	N	*40S* (0.018)	*18S rRNA* (0.032)	*EF-1α* (0.032)	*B2M* (0.038)	*ACTB* (0.051)	*GAPDH* (0.094)
Juvenile	G	*18S r/ACTB* (1.174)		*EF-1α* (1.369)	*B2M* (1.440)	*40S* (1.532)	*GAPDH* (1.750)
	B	*18S rRNA* (4.966)	*ACTB* (5.086)	*40S* (5.507)	*GAPDH* (6.205)	*EF-1α* (6.463)	*B2M* (6.785)
	N	*EF-1α* (0.039)	*B2M* (0.040)	*ACTB* (0.043)	*40S* (0.052)	*18S rRNA* (0.074)	*GAPDH* (0.090)
Development	G	*40S/GAPDH* (1.074)		*18S rRNA* (1.270)	*EF-1α* (1.459)	*ACTB* (1.850)	*B2M* (2.432)
	B	*GAPDH* (5.062)	*40S* (5.808)	*B2M* (5.925)	*ACTB* (8.375)	*EF-1α* (9.888)	*18S rRNA* (11.640)
	N	*ACTB* (0.042)	*40S* (0.050)	*GAPDH* (0.053)	*EF-1α* (0.081)	*B2M* (0.082)	*18S rRNA* (0.096)
Entire	G	*EF-1α/40S* (1.079)		*B2M* (1.240)	*ACTB* (1.302)	*18S rRNA* (1.364)	*GAPDH* (1.650)
	B	*40S* (5.316)	*B2M* (5.554)	*GAPDH* (5.584)	*ACTB* (5.911)	*EF-1α* (6.185)	*18S rRNA* (6.4990)
	N	*EF-1α* (0.035)	*GAPDH* (0.038)	*40S* (0.038)	*ACTB* (0.047)	*18S rRNA* (0.058)	*B2M* (0.095)

Stability values obtained are shown in parenthesis for each candidate reference gene by NormFinder (N), geNorm (G), BestKeeper (B). The genes are ranked from most stable (1) to least stable (2).

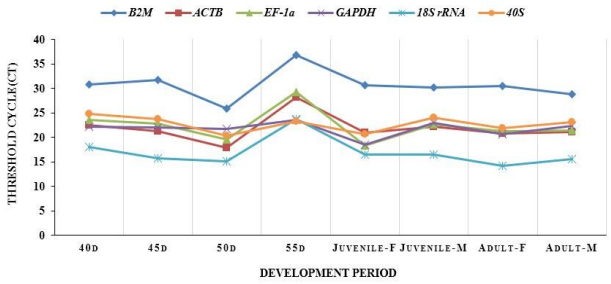

Fig. 3. Expression levels of housekeeping genes at timed gonads from 40–55 dph larvae, juveniles and adult carps.

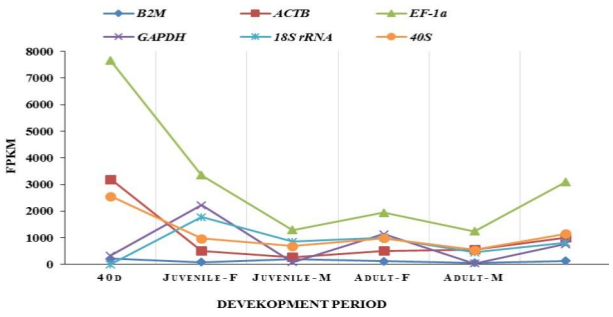

Fig. 4. Expression levels of housekeeping genes in RNA-seq of carp gonads.

Expression of housekeeping genes in the gonad of carps

The expression levels of the six reference genes were assessed in the gonad of carps at three different developmental stages, including undifferentiated period (40–55 dph larvae), juvenile stage, and adult stage. As shown in Figure 3, the Cq values ranged from 14 to 36, with *18S rRNA* showing a relatively high abundance (Cq = 14.21–18.02), *GAPDH* and *40S* presenting a stable trend (Cq = ~22), and *B2M* exhibiting the lowest expression. In addition, there was an obviously significant decrease in the expression of *B2M*, *ACTB*, *EF-1α*, and *18S rRNA* in the gonad of 55 dph larvae.

Analysis of the stability of the housekeeping gene expression at different developmental stages using BestKeeper, geNorm, and NormFinder showed some concurrence. The geNorm and BestKeeper software programs ranked *GAPDH* and *40S* as the best candidate genes, followed by *ACTB* and *B2M*. However, NormFinder proposed *ACTB* as the best gene, followed by *40S* and *GAPDH*. Nevertheless, *40S* and *GAPDH* were more regulated than *ACTB* (Fig. 3), implying that geNorm and BestKeeper showed more reliable results. Therefore, *GAPDH* and *40S* can be recommended as the best candidate genes in the gonad of Yellow River carps at different developmental stages.

Stability of expression of housekeeping genes carps

Analysis of the qPCR data of the entire sample of carps using geNorm, NormFinder, and BestKeeper software programs presented deviation in the rankings of the six genes. The geNorm software showed *40S* and *EF-1α* as the most stable gene and *GAPDH* as the least stable gene; NormFinder presented *EF-1α* as the best gene, followed by *40S* and *GAPDH*; and BestKeeper listed *40S* as the best candidate gene, followed by *B2M* and *GAPDH* (Table III). Furthermore, although *GAPDH*, with a Cq value of approximately 21, was found to be expressed at a moderate level across all the tissue types, the geNorm software did not present any advantages for *GAPDH*. Based on the data of NormFinder, which considers both intra- and inter-group gene variation, and geNorm, which determines pairwise standard deviation of the Cq values, *EF-1α* and *40S* were noted to present stable expression in all the tissues of juvenile and adult carps, and hence can be recommended as the best candidate genes in Yellow River carps at all developmental stages.

DISCUSSION

BestKeeper, geNorm, and NormFinder have become the fundamental and authoritative methods for the evaluation of the stability of the housekeeping genes. The geNorm software had been used to compute the expression stability values (M) for eight genes in zebrafish (Mccurley and Callard, 2008), and four methods (the comparative delta-Ct method, BestKeeper, geNorm, and NormFinder) had been employed in another study to select the most stable reference gene in turbot (Robledo *et al*., 2014). However, a previous study on Atlantic halibut argued that NormFinder seemingly failed to identify the most stably expressed reference gene and always listed the more intermediate gene as the best candidate gene instead (Øvergård *et al*., 2010). Similarly, in the present study, NormFinder proposed *ACTB*, which was less regulated, as the best gene in the gonads of Yellow River carps at different developmental periods. Therefore, to overcome this discrepancy, we considered other indices such as mean and SD of the six housekeeping genes, along with the rankings provided by BestKeeper, geNorm, and NormFinder, to identify the optimal reference genes.

All the six reference genes analyzed in the present study were detected in the comparative transcriptomics study of *C. carpio* (unpublished data) based on five sampling series comprising the gonads of carps at different developmental stages (40–55 dph, female and male juveniles, and female and male adults), which also accurately revealed the gene read counts in the stored mRNA. The RNA-Seq was employed to calculate the FPKM (expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced), which represented the expression level of each transcript (Trapnell *et al*., 2010). As shown in Fig. 4, all the six genes were found to have a relatively stable expression level, except those in the gonad samples from 40 dph larvae. In particular, *B2M* exhibited the lowest mRNA abundance, whereas *GAPDH* and *40S* presented the least variation in the expression levels among the various gonad samples. These findings were in accordance with the results of qPCR analysis of the carp gonads at various developmental periods. Moreover, the mRNA levels of *EF-1α*, *ACTB*, and *40S* were noted to be higher in the 40 dph gonad samples, indicating the probable activation of gonadal transcription, whereas the levels were stabilized in the samples at other developmental stages. Despite the difference in data representation and several minor changes in the reference gene expressions, the statistics of RNA-seq data further supported the results obtained in this study and indicated *GAPDH* and *40S* as more optimal and reliable for normalization of qPCR of gonad samples of Yellow River carps at different developmental stages.

In a previous study on zebrafish, a panel of eight housekeeping genes was evaluated, and it was reported that *18S*, *B2M*, and *EF-1α* were the most stable during development and across all tissue types (Mccurley and Callard, 2008). These findings are in agreement with the *EF-1α* expression results obtained in the present study. Besides, the expression levels of *40S* in all the tissues of juvenile and adult carps were significantly higher. A study on *Gossypium raimondii* using five different software programs to analyze 12 housekeeping genes revealed *SAD* and *TUA11* as the stable reference genes in all the tissues (Sun *et al*., 2015). However, these two genes have seldom been employed in the research on carps. In a study by Tang *et al.* (2012), the expression stability of the housekeeping genes in *Jian* carps was evaluated by comparing the coefficient of variation, and it was found that *EF-1α* was the most suitable housekeeping gene in all the tissues of juvenile and adult *Jian* carps. These findings, along with those noted in medaka (Zhang and Hu, 2007), Atlantic salmon (Olsvik *et al*., 2005; Ingerslev *et al*., 2006), and Atlantic halibut (Øvergård *et al*., 2010), are in accordance with the results of the present study. Nevertheless, a single optimal gene for normalization of the expression levels in all the tissues of juvenile and adult carps at different developmental stages could not be determined. Moreover, considering the diversities among the Yellow River carps, further studies on the validation and characterization of the housekeeping genes are needed. The present study

emphasizes on the need and use of three methods for normalization, and recommends *40S* as the best candidate gene in 2-year-old adult Yellow River carps, and *18S rRNA* and *ACTB* as the most stable genes in juvenile Yellow River carps. Furthermore, *GAPDH* and *40S* were found to be the best candidate genes in the gonads of Yellow River carps at different developmental stages, and *EF-1α* and *40S* exhibited the most stable expression level in all the samples of juvenile and adult carps. Thus, the present study provides valuable data for qPCR-assisted studies and could help in molecular-guided analysis of *C. carpio* and other closely related species.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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