RESEARCH ARTICLE

Identification of housekeeping genes as references for quantitative real time RT-PCR analysis in *Misgurnus anguillicaudatus*

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Abstract

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) is a well-known method to quantify gene expression through comparing with the reference genes. Usually, housekeeping genes were set as the references, as for their stable expression in varies conditions. Here, we try to evaluate some such genes to identify suitable housekeeping genes as references for qRT-PCR analysis of gene expression in *Misgurnus anguillicaudatus*. This study evaluated the expression of four commonly used housekeeping genes, i.e. b-actin (*ACTB*), elongation factor 1 alpha (*EF-1a*), glyceraldehyde-3-phosphate (*GAPDH*), and 18S ribosomal RNA (*18S rRNA*), in gender difference, effects of tissue type, different developmental stages, chemical treatment of embryos/larvae with commonly used vehicles for administration and agents that represent known environmental toxicant. Rank ordering of expression stability was done using geNorm, NormFinder and BestKeeper algorithms. Results suggested that in the qRT-PCR test, in all experimental conditions, *EF-1a* could be selected as reference gene when analyzing a target gene. For study of different development stages, *ACTB* could be a candidate as reference gene. For studies associated with different gender and tissue types, *EF-1a* would be better target as reference gene. Meanwhile, in toxicant treatment, expression of *EF-1a* seems more stable than others and could be considered as reference gene. This study could provides a useful guidline that can be expected to aid *Misgurnus anguillicaudatus* researchers in their initial choice of housekeeping genes for future studies and enable more accurate normalization of gene expression data.

Keywords: *Misgurnus anguillicaudatus*; Housekeeping gene; Quantitative real time RT-PCR; geNorm; NormFinder; BestKeeper

Introduction

Gene expression analysis is widely studied using northern-blotting, ribonuclease protection assays, comparative RT-PCR and qRT-PCR where qRT-PCR is becoming more popular in various studies, which has features, like fast readout, high sensitivity, reproducibility, and the potential for high throughput as well as accurate quantification (Huggett et al., 2005; Nygard et al., 2007). However, in order to accurate quantify the gene expression, reference genes would be vital, which should be stably expressed and unaffected with gender, age, nutrient, varies treatment with drugs and so on. So, normalization of the qRT-PCR data with reference genes would avoid many problems caused by errors, including the amount of starting samples, the quality of RNA, the efficiencies of revers transcription and PCR itself (Bustin et al., 2000).

As we know, housekeeping genes are referred to constitutive genes involved into cellular basic function, which are required for the maintenance of basic cellular function. Usually, housekeeping genes are constitutively expressed under normal and patho-physiological conditions. So, housekeeping genes are frequently selected as the reference genes for qTR-PCR analysis. It would be misinterpret the qRT-PCR results if inappropriate reference genes were involved into the quantitative analysis (McCurley et al., 2008). Theoretically, an ideal housekeeping gene should not be

regulated or influenced in all samples from different subject, difference tissues, during all developmental stages, and under difference experimental treatment conditions (Radonic et al., 2004). However, some recent literatures suggested that the some widely used housekeeping genes, eg. *b-actin*, *glyceraldehyde-3-phosphate dehydrogenase*, *ribosomal proteins, and ribosomal RNAs*, expressed at different transcription levels according to different tissue/cell type, development, or experimental design (Schmittgen et al., 2000; Dhedar et al., 2004; Silve et al., 2006; Gilsbach et al., 2006). So, perfect reference gene selection would be crucial to accurate profiling of gene expression.

Misgurnus anguillicaudatus (Cypriniformes; Cobitidae) is one kind of fresh water teleost, widely distributed along the eastern coasts of the Asian continent and emerging aquaculture species since 1990s. However, no perfect reference gene used in quantitative gene analysis was identified and evaluated for scientific research of *Misgurnus anguillicaudatus*. Based on earlier gene expression studies in *halibut* (Infante et al., 2008; Fernandes et al., 2008), *Japanese flounder* (Zhong et al., 2008), *medaka* (*Oryzias latipes*) (Zhang et al., 2007) and *Atlantic salmon* (*Salmo salar*) (Jørgensen et al., 2006; Olsvik et al., 2005), this study set out to assess different housekeeping genes in *Misgurnus anguillicaudatus*, including *ACTB*, *EF-1a*, *GAPDH* and *18S rRNA*. Here, we evaluated gene expressions for all the four housekeeping genes in different groups based on gender, tissue type, developmental stages and chemical treatment of embryos/larvae. Data were evaluated by three methods, geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and

BestKeeper (Pfaffl et al., 2004). We discuss differences in results, taking into account different approaches to identify the most stably expressed reference gene. Aim of the present study is to identify reference genes that can be used for expression analysis of *Misgurnus anguillicaudatus*. Results of this study are intended to guide *Misgurnus anguillicaudatus* researchers with initial selection of a reference gene, but underline the importance of accurate housekeeping gene validation for each new experimental paradigm.

Materials and methods

Fish husbandry and samples collection

Adult *Misgurnus anguillicaudatus* were collected from wetlands in the old course of the Yellow River, Yanjin County (Henan, China). Females and males were induced to mate by intramuscular injection of human chorionic gonadotropin hormone, and fertilized eggs were allowed to develop until use. Animal maintenance and handling procedures followed the recommendations of the Association of Animal Behaviour (ASAB, 2003) and national regulations. For developmental expression analysis, embryos/larvae were collected after timed intervals: 8, 9, 14, 24, 48, 96, 240, 360, and 480 hours post-fertilization (hpf), then quick-frozen on dry ice, stored at −80°C until needed for RNA extraction. For treatment expression analysis, embryos/larvae were left untreated until 24 hpf and then exposed to $CdCl_2$ (Cd^{2+}, 2 mg/l and 0.05 mg/l), Fluoroglycofen (F, 80 mg/l and 20 mg/l), Imidaclopri (I, 2 mg/l and 0.05 mg/l)

dissolved in dH_2O. Embryos were collected at 96 hpf. Then they all quick-frozen on dry ice, and stored at −80°C until needed for RNA extraction. Tissues (brain, fore-intestine, hind-intestine, liver, kidney, heart, muscle, gonads, tail fin) from adult (males and females) were collected, quick-frozen on dry ice, and stored at −80°C until needed for RNA extraction.

RNA extraction and reverse transcription

For total RNA extraction, approximately 25 mg of mature adult tissues and embryos/larvae were homogenized using a glass tissue homogenizer, via Trizol reagent (RNA Extraction Kit, Invitrogen, CA, USA) according to the manufacturer's protocol, and DNA contamination was removed by DNase I (Roche, Indianapolis, IN) treatment. Assessment of RNA quality was performed by agarose-gel electrophoresis (1%) and photographed. An aliquot of each extract was used for spectrophotometry to determine RNA quality and concentration. RNA with a 260/280 ratio between 1.95 – 2.2 and a 260/230 ratio > 1 and < 3 was considered satisfactory and was used in this study. Each RNA extract was assayed in triplicate and an average value was determined. RNA was stored at -80°C. cDNA synthesis was carried out with Prime Script reverse transcriptase (Takara, Japan), following the recommendation of the manufacturer, and using 1 μg of total RNA isolated as described above. A minimum of two RT reactions were performed for each biological replicate for technical replicate comparison. cDNA was stored at -20°C until use.

Selection of candidate reference genes and Primer design

Four housekeeping genes were selected from commonly used reference genes (table 1). The primer pairs for candidate reference genes were designed with Primer Premier 5.0 software based on the partial sequences of *Misgurnus anguillicaudatus*. All primers were synthesized by BGI Sequencing. All primer sets spanned an exon-exon junction to avoid errors due to contaminating genomic DNA. The correlation coefficient (R^2) were determined based on the slops of the standard curves generated using serial 10-fold (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) dilutions of sample cDNA and an R^2 value above 0.98 was accepted. The specificities of the PCR products were also examined by agarose gel electrophoresis.

qRT-PCR and data analysis

qRT-PCR was performed on an LightCycler® 96 (Roche, Basel, Switzerland) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China). Samples contained 5 μl SYBR Premix buffer, 0.5 μl diluted cDNA template (using 200 ng of genomic DNA as template in qRT-PCR reaction), 0.4 μl each of the primers, and 4.1 μl sterilized ddH_2O for a final volume of 10 μl. Cycling parameters were as follows: an initial step of 30 s at 94°C, followed by 40 cycles at 94°C for 15 s and 60°C for 45 s. qRT-PCR was performed in triplicate on each sample for a minimum of 54 data sets generated

for each sample/gene combination (3 biological replicates × 2 RT reactions × 3 PCR runs × 3 reactions per PCR run). The expression levels of the candidate reference genes were determined by the cycle threshold (C_t) value. The stability of the four candidate reference genes were evaluated by three independent statistical applications: geNorm, NormFinder and BestKeeper. Three programs were used as none of these are accepted methods on their own for evaluation and ranking of reference genes, thus by using three independent methods a more reliable result was expected.

Results

Primer specificity analysis

ACTB, *EF-1a*, *GAPDH* and *18S rRNA* were selected for analysis based on the previous publications (Schmittgen et al., 2000; Dhedar et al., 2004; Silve et al., 2006; Gilsbach et al., 2006; Paria et al., 2016; Ma et al., 2013; Robledo et al., 2014). Gene names, gene symbol, functions and GeneBank accession numbers are listed in table 1. The sequences, corresponding amplicon sizes and the R^2 of primers were listed in table 2. All primer pairs generated specific amplicons with expected size and no visible primer dimers could be detected in agarose 1% gel electrophoresis (figure 1).

Developmental stages expression levels of candidate reference genes

Expression levels in different developmental stages, represented as mean C_t values, are shown in figure 2. For all the four housekeeping genes, Ct values were ranged from 15.23 to 30.37 with *18S rRNA* the highest expressed gene, having Ct values from 15.23 to 19.93 and *GAPDH* was the lowest expressed gene, with Ct varying 24.30 to 30.37 whereas *ACTB* showed highly variable expression levels (Ct 20.60 – 28.75) and *EF-1a* at a moderate level (Ct 19.88 – 25.22).

Expression levels in different gender and different tissues

qRT-PCR results, on all four genes showed high diversity in different tissues in both male and female (figure 3). We found that *18S rRNA* was highly expressed, whereas *GAPDH* was expressed at low levels. The most pronounced variation (15.07 Ct) was in *GAPDH*, when expression was compared between heart and tail fin in male. In addition, some genes shows variable expression in same tissue between male and femle, eg. the expression of *GAPDH* in liver was significantly higher in female than in male.

Housekeeping genes expression in embryos/larvae treated with toxicant

The effect of toxicant on expression of housekeeping genes in embryos/larvae was shown in figure 4. All four genes tested revealed differences in expression levels across treatment groups. *18S rRNA* was highly expressed (average Ct value $C_t < 16$),

whereas *GAPDH* was expressed at low levels (average C_t value> 19.63). The most pronounced variation (2.34 Ct) for *18S rRNA* was noticed in embryos/larvae when treated with CdCl2 2 mg/l and 0.05 mg/l. The overall variability in expression levels of the four genes following treatment of embryos/larvae was less than that seen when the same mRNAs were measured in different tissue types of adult fish or during the course of development (compare figure 4 with figure 2 and figure 3). It is noteworthy here that all Ct values had low SD, signifying low sample-to-sample biological and technical variation within a given experimental condition.

Expression stability of candidate reference genes

Since all the four housekeeping genes appeared large scale range of expression levels in different developmental stages, different tissue source, chemical treatment in embryos/larvae and gender differences, statistical methods need to apply to optimize the analysis to determine which one might be the best reference gene. The expression stability can be evaluation by computational methods, namely geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004).

The software program geNorm calculates and compares gene expression stability by measure M of all candidate genes. The most stable reference gene has the lowest M value, whereas the least stable has the highest M value. As shown in table 3, we analyzed data from three experimental sets. In different developmental stages, *ACTB*

(M = 1.182) was showed most stable expression and *18S rRNA* (M = 1.459) had the least stable expression. Similarly chemical treatment in embryos/larvae, *ACTB* (M = 0.317) had the most stable expressions, but *EF-1a* (M = 0.386) showed the highest variation. Between male/female and different tissue source, although significant sex differences in expressed levels of certain genes and tissue types, the order of gene stability by geNorm analysis varied little when male and female were plotted separately (data not shown). *EF-1a* (M = 2.670) can thereby be recommended as the best candidate reference gene, while *GAPDH* (M = 4.857) was the least stably expressed.

Normfinder selects the best reference gene of a set of candidates by a model-based approach. Moreover, inter-group and intra-group variations are estimated and combined to give a stability value and the gene with the lowest value is selected as the most stable. Figure 5 shows the results from Normfinder analysis. This analysis method identified that *ACTB* (M = 0.018) was the most stable reference gene in different developmental stages. In tissues from adult (males), *EF-1a* (M = 0.035) had the most stable expression and was the ideal reference gene. In tissues from adult (female), *ACTB* (M = 0.039) had the most stable expression and was the ideal reference gene. For chemical treatment samples, *ACTB* (M = 0.007) was the most appropriate for use as a reference gene. The results obtained by NormFinder showed slightly differed from those obtained by geNorm. In toxicant treatment, the geNorm and NormFinder programs showed opposite results for *18S rRNA*. These differences between the two methods were expected given that the programs are based on distinct

statistical algorithms.

BestKeeper evaluates gene expression stability on two variables: standard deviation (SD) and coefficient of variance (CV) (Pfaffl et al., 2004). And the lowest variation represent highest stability. In different developmental stages, *18S rRNA* was the most stable reference gene, while *ACTB* had the most unstable expression (table 4). In the different tissues of adult male, *ACTB* was ranked at the top position for the most stable gene, whereas GAPDH was considered to be the least stable. In different tissues of adult female, *EF-1a* was ranked at the top position for the most stable gene, whereas *GAPDH* was considered as most unstable one. In the chemical treatment to embryos/larvae all the reference genes showed high stabilities, *EF-1a* was ranked at the top position for the most stable gene, whereas *18S rRNA* was considered as most unstable reference gene. For the above, BestKeeper showed different results to that of geNorm and NormFinder, which may be due to distinct statistical algorithms used by these three methods.

A summary of the rankings generated by the three softwares was shown in table 5. It reflects the varying expression between the four genes selected. But through the results of stability analysis by geNorm, BestKeeper and NormFinder, the most stable reference gene was identical, so was the least stable gene. In different developmental stages, we find that *ACTB* generally listed as a good candidate and *18S rRNA* is at the bottom having the lowest ranking. In different tissues in adult (male and female), *EF-1a* and *ACTB* generally listed as good candidates, and *GAPDH* at the bottom having the lowest ranking. In toxicant treatment in embryos/larvae *ACTB* is generally

listed as good candidate, and *18S rRNA* at the bottom having the lowest ranking. The gene with the least variability across all the conditions assessed in this study was *EF-1a.*

Discussion

qRT-PCR is an important tool for measuring gene expression (Morga et al., 2010). However, this method requires an appropriate reference gene usually selected from housekeeping genes for data normalization to integrate experimental variations. Theoretically, an ideal reference gene should not be regulated or influenced in all samples from different subjects, difference tissues, during all delopmental stages, and under difference experimental conditions (Radonic et al., 2004). Accumulating evidences show that some of the widely used reference genes, such as *18S rRNA* and *GAPDH* may have a huge difference in expression level under varies conditions (Long et al., 2010; Tang et al., 2007; Mascia et al., 2010). Considering many of the classical reference genes have proven unreliable (Dheda et al., 2005; Glare et al., 2002; Murphy et al., 2002; Oliveriera et al., 1999; Selvey et al., 2001; Tricarico et al., 2002; Zhong et al., 1999), choosing suitable housekeeping genes as references for qRT-PCR analysis is crucial to accurate profiling of gene expression. In this study, we evaluated the expression levels of four housekeeping genes using qRT-PCR analysis in *Misgurnus anguillicaudatus* and tried to identify the best one suitable in different conditions, developmental stages, chemical treatment in embryos/ larvae, gender

differences and varies tissue types.

To choose a better reference gene, the first step is to consider the C_t value of candidate genes. Therefore, it is recommended to select a target gene with a suitable C_t value as an internal reference gene (Cruz et al., 2009; Filby et al., 2007; Frost et al., 2003). Due to the lack of introns (Mishra et al., 1997), high expression level (Hildahl et al., 2007), and as the mRNA fraction of total RNA is not always represented properly by the rRNAs expression level (Solanas et al., 2001), markers of rRNA, therefore, *18S* or *28S rRNA* might be suboptimal as normalization factors in many settings (Huggett et al., 2005; Solanas et al., 2001; Thellin et al., 1999). Also, in our study, *18S rRNA* is not an optimal candidate for normalization of low copy mRNA target genes.

In our study, three statistical software geNorm, BestKeeper and NormFinder, were involved into the qRT-PCR data analysis, each of which had its own calculation algorithms. Different calculation algorithms might supply different results (Glare et al., 2002), to determine the most stably expressed gene (Hendriks-Balk et al., 2007). NormFinder takes into account both intra-group and inter-group gene variation to evaluate its stability (Andersen et al., 2004). Both BestKeeper and geNorm uses a pair-wise comparison approach, and are highly dependent on the assumption that none of the genes being analyzed are co-regulated (Vandesompele et al., 2002; Pfaffl et al., 2004). GeNorm determines the pairwise standard deviation of Cq values of all genes, and then excludes the one with the lowest stability, repeating the process until only two genes remain, which are then considered the most stable ones. BestKeeper ranks

the genes according to the standard deviation (SD) of their Cqs, but the output includes more information, for example the coefficient of variation (CV), which was proposed as a validation method for the results offered by NormFinder and GeNorm (Caradec et al., 2010). Robledo et al. results suggest that for research purposes, NormFinder implement the best approaches for reference gene selection (Robledo et al., 2014). However, as different analytical software give different ranking order of housekeeping genes, it is difficult to take into account the results obtained using single software. Three programs were used as none of these are accepted methods on their own for evaluation and ranking of reference genes, thus by using three independent methods a more reliable result was expected (Zhang et al., 2007; Chang et al., 2012; Bower et al., 2009; Olsvik et al., 2008; Urbatzka et al., 2013). We use different algorithms to analyze the variation in the expression of reference genes, which could result in different recommendations for the most suitable reference gene. When analyzing the data in different developmental stages, tissue type in adult (male and female) and effect of toxicant treatment in embryos/larvae by NormFinder, geNorm, and BestKeeper, the software programs deviated in the ranking of the four candidate genes (table 5). Similar observations have been made by other investigators in the study of other fish such as *zebrafish* (Tang et al., 2007) and *fathead minnows* (Frost et al., 2003), *GAPDH* was the most unstable under all experimental conditions tested in our study and should excluded from the referrer candidates. Considering all experimental conditions, *EF-1a* has the top behavior as a referrer candidate, followed by *ACTB* and *18S rRNA*, whereas the *GAPDH* remains at the bottom. Meanwhile,

expression of *ACTB* appears the most stable in different developmental stages, followed by *GAPDH* and *EF-1a*, and *18S rRNA*. Expression of *EF-1a* appears the most stable in different tissue in both male and female groups, followed by *ACTB* and *18S rRNA*, and *GAPDH*. Additionally, expression of *ACTB* in toxicant treatment samples have the lowest change when comparing with the normal samples, which much better than the following ones, *EF-1a* and *GAPDH*, and *18S rRNA*.

Conclusion

To date, this was the first report determining reference genes for normalization of qRT-PCR data from samples of varies developmental stages, affection by chemical treatment in embryos/ larvae, different gender and tissue types in *Misgurnus anguillicaudatus*. The results of this study indicate that all the tested housekeeping genes of *Misgurnus anguillicaudatus* were found to have some degree of variability under the conditions tested but the most suitable genes could be identified as referrer in different conditions. In summary, in all experimental conditions, *EF-1a* should be selected as reference gene when analyzing a target gene. For study of different development stages, *ACTB* could be a candidate as reference gene. For studies associated with different gender and/or tissue types, *EF-1a* would be better target as reference gene. Meanwhile, in toxicant treatment, expression of *EF-1a* seems more stable than others and could be considered as referrer gene to study expression level of other genes. This study provides a useful guideline for researchers in field of *Misgurnus anguillicaudatus* to select sutable housekeeping genes in different conditions which would provide a relative accurate gene expression data.

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Figure Captions

Figure 1. PCR amplification of housekeeping genes in *Misgurnus anguillicaudatus* liver. Lane 1: *18S rRNA*; Lane 2: *EF-1a* ; Lane 3: *ACTB*; Lane 4: *GAPDH*.

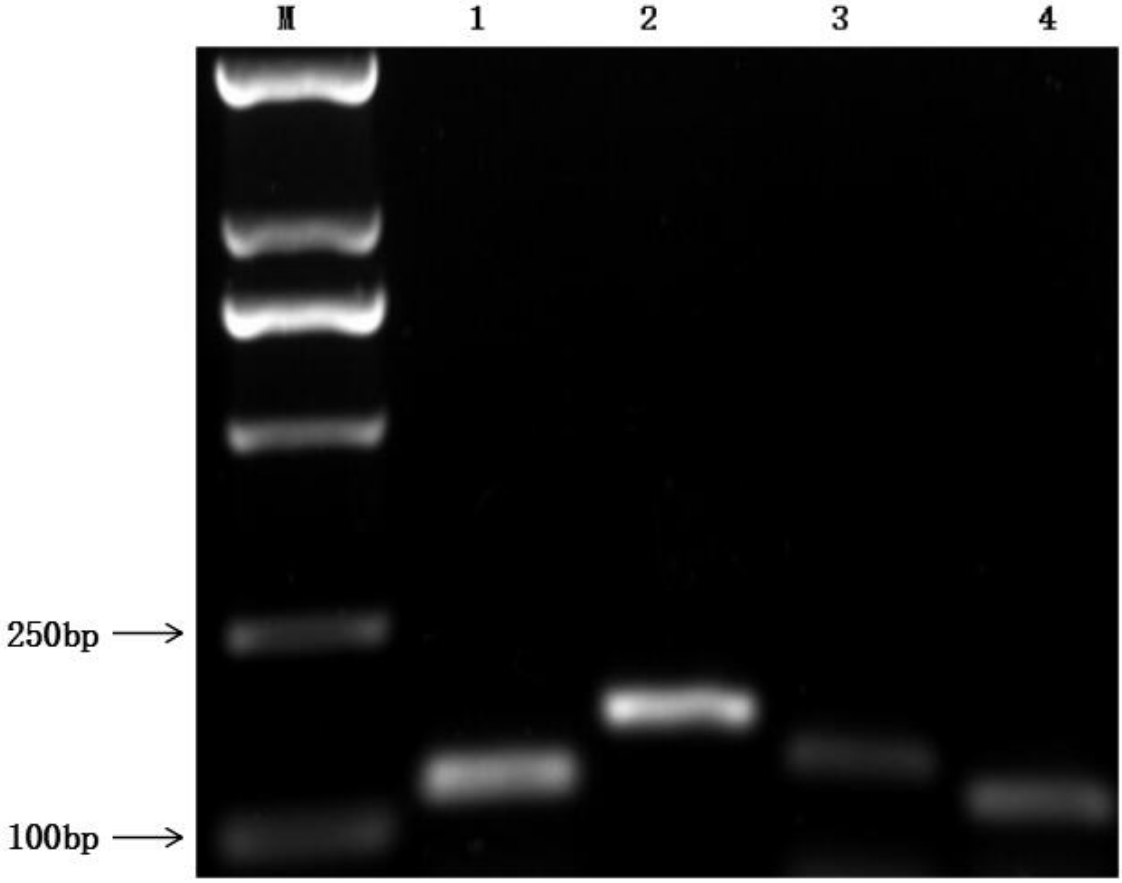

Figure 2. The Ct values (mean + SD) of housekeeping genes expressed in different developmental stages of *Misgurnus anguillicaudatus*: 8, 9, 14, 24, 48, 96, 240, 360, and 480 hours post-fertilization.

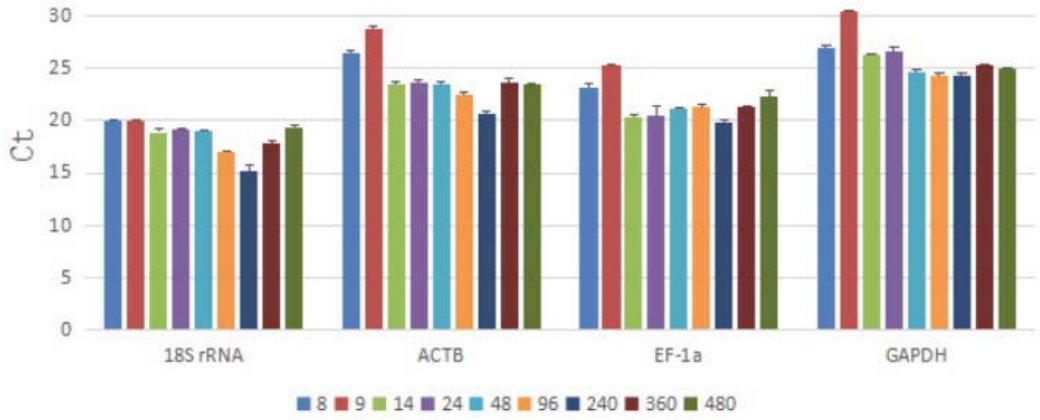

Figure 3. Expression levels of four candidate reference genes in different tissues of adult male (M) and female (F). Values are given as Ct values (mean + SD).

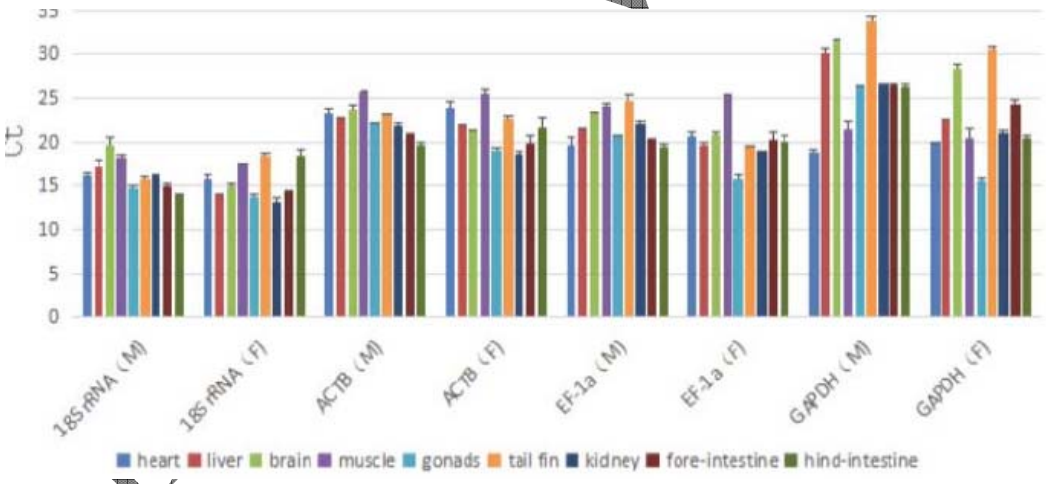

Figure 4. Housekeeping gene expression levels following toxicant treatment from 24 – 96 hpf. Values are given as Ct values (mean + SD). $Cd^{2+}1$ represents exposed to $CdCl_2$ (0.05 mg/l), $Cd^{2+}2$ represents exposed to $CdCl_2$ (2 mg/l). F1 represents exposed to Fluoroglycofen (20 mg/l), F2 represents exposed to Fluoroglycofen (80 mg/l). I1 represents exposed to Imidaclopri (0.05 mg/l), and I2 represents exposed to Imidaclopri (2 mg/l).

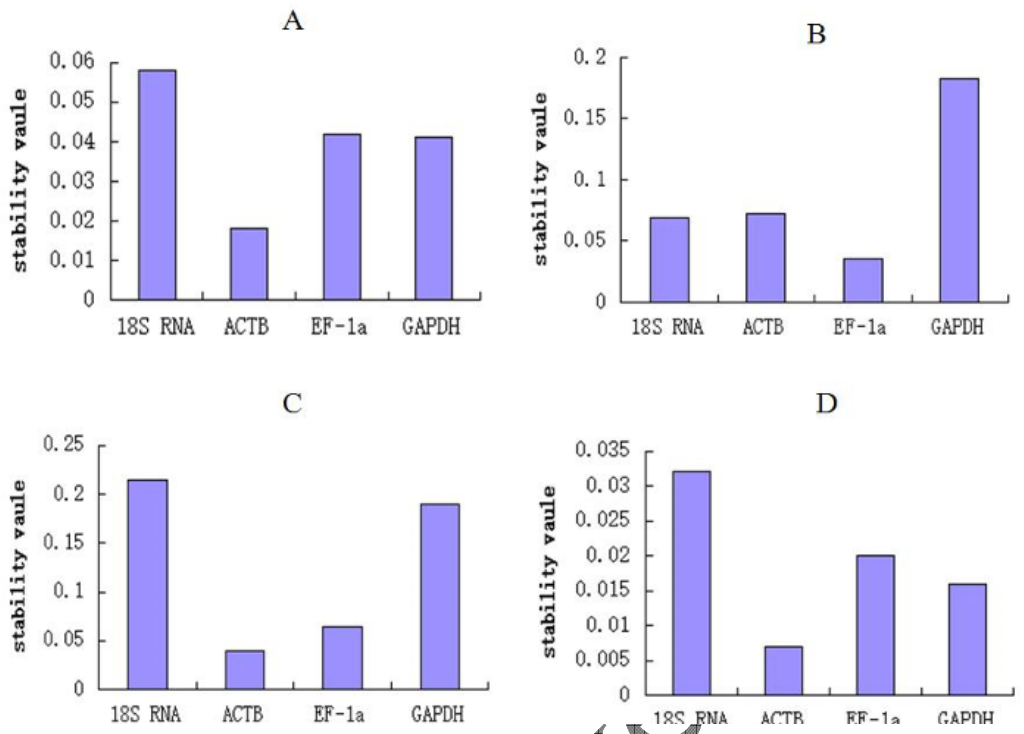

Figure 5. Expression stability values of the candidate reference genes calculated by NormFinder. Expression stability values (M) of the four candidate reference genes are shown for different larvae developmental stages (A), different tissues of adult male (B), different tissues of adult female (C) and toxicant treatment (D).

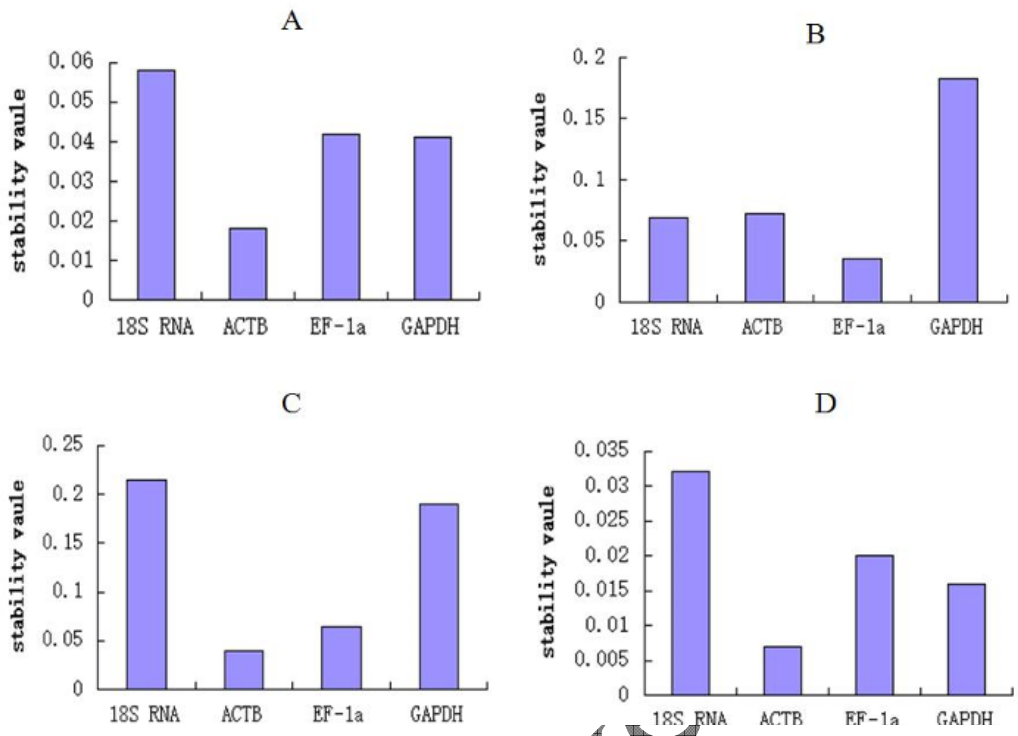
A
stability vaule
0.06
0.05
0.04
0.03
0.02
0.01
0
18S RNA
ACTB
EF-1a
GAPDH
B
stability vaule
0.2
0.15
0.1
0.05
0
18S RNA
ACTB
EF-1a
GAPDH
C
stability vaule
0.25
0.2
0.15
0.1
0.05
0
18S RNA
ACTB
EF-1a
GAPDH
D
stability vaule
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
18S RNA
ACTB
EF-1a
GAPDH

Table 1. Genes selected for expression analysis

Gene symbol	Gene name	Function	Accession
18S rRNA	18s ribosomal RNA	Ribosome subunit	EU120032
ACTB	Beta-actin	Cytoskeletal protein	AB200265
EF-1a	Elongation factor 1 alpha	Protein synthesis	KF733649
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis enzyme	AB200264

Table 2. Primers used for RT-PCR analysis in the present study

Gene	Primer sequence (5'→3')	Product size (bp)	Correlation coefficient (R^2)
18S rRNA	F) GAGTATGGTTGCAAAGCTGAAAC R) AATCTGTCAATCCTTTCCGTGTCC	129	0.99
ACTB	F) AGAGAGAAATTGTCCGTGAC R) GCCAATGGTGATGACCTGT	140	0.99
EF-1a	F) GTTAAGTCCGTTGAGATGCAC R) GATGATGACCTGAGCATTGAAG	174	0.99
GAPDH	F) GCAAAGTCATTCCTGAGC R) GTCATCATACTTGGCGGGTTTC	119	0.99

All the primers are designed with Primer 5.0 software and synthesized by BGI Sequencing. F: forward primer, R: reverse primer.

Table 3. Average expression stability values (M) of four candidate reference genes as calculated by geNorm

Gene	Stability			
	developement	tissues	toxicant	total
	(n=9)	(n=18)	(n=6)	(n=33)
18S rRNA	1.459	2.744	0.326	2.263
ACTB	1.182	2.701	0.317	2.174
EF-1a	1.230	2.670	0.386	2.174
GAPDH	1.227	4.857	0.360	3.676

Stability data for reference gene expression were determined with the geNorm applications. The stability values are inversely correlated to gene expression stability. The most and least stable reference genes are shaded blue and yellow, respectively.

Table 4. Descriptive statistics of four candidate genes based on their cycle threshold

values analyzed by BestKeeper

CP: equivalent terminology for C_t;min (CP) and max (CP): the extreme values of CP;

		18S rRNA	ACTB	EF-1a	GAPDH
Developmental stages n=9	Geo mean [CP]	18.40	23.89	21.65	25.89
	Min [CP	15.23	20.60	19.88	24.30
	Max [CP]	19.93	28.75	25.22	30.37
	Std dev [±CP]	1.21	1.60	1.24	1.41
	CV [% CP]	6.55	6.66	5.72	5.44
	Coeff. of corr.[r]	0.842	0.992	0.906	0.899
Different tissues of male n=9	Geo mean [CP]	16.30	22.50	21.69	26.50
	Min [CP	14.00	19.70	19.37	18.87
	Max [CP]	19.63	25.79	24.79	33.94
	Std dev [±CP]	1.40	1.30	1.63	3.37
	CV [% CP]	8.56	5.74	7.50	12.53
	Coeff. of corr.[r]	0.749	0.560	0.882	0.694
Different tissues of female n=9	Geo mean [CP]	15.49	21.52	19.97	22.18
	Min [CP	13.19	18.65	15.90	15.62
	Max [CP]	18.46	25.54	25.24	30.54
	Std dev [±CP]	1.68	1.71	1.45	3.45
	CV [% CP]	10.80	7.89	7.23	15.28
	Coeff. of corr.[r]	0.777	0.780	0.754	0.688
toxicant treatment n=6	Geo mean [CP]	13.67	19.65	17.58	20.26
	Min [CP	13.01	18.83	16.99	19.63
	Max [CP]	15.35	21.01	18.75	21.66
	Std dev [±CP]	0.60	0.54	0.50	0.59
	CV [% CP]	4.38	2.76	2.82	2.89
	Coeff. of corr.[r]	0.986	0.972	0.935	0.953

std dev [±CP]: standard deviation of the CP; CV [%CP]: the coefficient of variance expressed as a percentage of the CP level; coeff. of corr. [r]: pairwise correlation coefficient.

Table 5. Ranking output of the four reference genes according to their expression

GENE	Rank														
	developement			Tissues（M）			Tissues（F）			toxicant			total		
	G	N	B	G	N	B	G	N	B	G	N	B	G	N	B
18S rRNA	4	4	1	2	2	3	2	4	3	2	4	4	3	3	3
ACTB	1	1	4	3	3	1	1	1	2	1	1	2	2	2	2
EF-1a	3	3	2	1	1	2	3	2	1	4	3	1	1	1	1
GAPDH	2	2	3	4	4	4	4	3	4	3	2	3	4	4	4
stability															

The rankings are calculated by NormFinder (N), geNorm (G), BestKeeper (B). A ranking of 1 is considered the best candidate, while 4 is listed as the worst.