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# Reference gene selection for quantitative real-time RT-PCR normalization in *Clarias magur* at different larval developmental stages

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ABSTRACT

Reference genes employed for normalizing quantitative PCR data are important for the accurate analysis of gene expression. To date, no reference genes have been screened for developmental gene expression studies in *Clarias magur*. In the present study, three commonly used and constitutively expressed genes viz. beta actin (β-actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor-alpha 1 (EFa1) were examined for their efficacy as internal control to avoid any variation during qRT-PCR expression analysis at different developmental stages of *C. magur*. All the selected housekeeping genes showed a variable level of mRNA expression during the developmental stages of *C. magur*. Using three independent statistical algorithms (delta-CT, BestKeeper and NormFinder), β-actin and GAPDH were identified as the suitable genes at different developmental stages. However, comprehensive gene stability evaluation denoted β-actin to be the most stable gene for carrying any gene expression studies. The present results, recommend β-actin as the optimal housekeeping gene for qRT-PCR analysis during different developmental stages of *C. magur*.

**Key words**: β-actin, *Clarias magur*, Larval development stages, qRT-PCR, Reference gene

Gene expression analysis provides a broad understanding of the biological processes in which the genes involved in the complex regulatory networks of the physiological processes or those implicated in disease development are identified. Quantitative real-time polymerase chain reaction (qRT-PCR) is one of the sensible, most precise, specific, easy to operate and widely applied techniques to examine the candidate genes expression (Gimeno *et al.* 2014*,* Imai *et al.* 2014). In addition to this, not only it is a preferable technique for quantification of gene expression, but also considered to be a 'gold standard' for validating novel techniques on gene expression (Rajeevan *et al.* 2001). qRT-PCR is often used as a valuable tool to investigate the expression of target genes, but that needs to be normalized against a reference gene, that should be highly expressed under the different designed experimental procedures (Szabo *et al.* 2004).

Ideal reference genes (housekeeping genes) should have a stable expression in all the cells or tissues in consideration irrespective of tissue type, stage of development, disease state or experimental condition (Dheda *et al.* 2005 and Bonefeld *et al.* 2008). These housekeeping genes perform the important functions required for the normal growth of cells and for the basic metabolic pathways regulation (Butte *et al.* 2001 and Eisenberg *et al.* 2003).

A key prerequisite for studying the relative change in mRNA expression of a specific target gene is the identification of specific housekeeping gene (HKG) that should be expressed in abundance with minimal variability. The most accepted method for normalizing expression levels of target gene is to correlate the mRNA levels of the gene of interest to the internal control gene (Andersen *et al.* 2004). Beta actin (β-actin) is a basic housekeeping gene commonly used in fish (Jorgensen *et al.* 2006). Until recently, the reference genes engaged in basic cellular processes such as Ubiquitin, 18S rRNA, b-tubulin, β-actin and glyceraldehyde-3-phosphate dehydrogenase were employed as endogenous controls for analysis of gene expression as they were assumed to have an even expression. But many reports reveal that the mRNA levels of these genes also varies markedly under different experimental circumstances (Thellin *et al.* 1999*,* Suzuki *et al.* 2000*,* Lee *et al.* 2002*,* Czechowski *et al.* 2005) and are therefore sometimes unsuitable for gene expression studies. Hence, a stable housekeeping gene needs to be identified and validated so as to improve the efficiency, reliability and accuracy of RT-qPCR test.

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In the present study, different developmental stages of *Clarias magur,* a catfish of Indian origin were taken to validate the most suitable housekeeping gene. The freshwater air-breathing teleost *C. magur* is designated as a neotype of *C. batrachus* (Ng and Kottelat 2008). The name *C. batrachus* to *C. magur* of the Indian origin catfish has also been supported through Mitochondrial (COI) analysis (Devassy *et al.* 2016). In this study, three reference genes were used as candidates to normalize RT-qPCR data in *C. magur* at different life stages and these genes represented the different gene families or functional classes. These are Beta actin (β-actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Elongation factor-alpha 1 (EFa1). Three software packages, delta CT method (Silver *et al.* 2006), Bestkeeper (Pfaffl *et al.* 2004) and NormFinder (Andersen *et al.* 2004), were employed to assess the stability of expression of the three housekeeping genes.

## MATERIALS AND METHODS

*Fish stocks and sample collection:* The experiment was conducted at Kakinada, Andhra Pradesh, a sub-centre of ICAR-Central Institute of Fisheries Education, Mumbai. For conducting different ontogenetic studies, two gravid pairs of male and female *C. magur* were taken and injected with 0.2 and 0.4 ml/kg body weight of ovatide at evening and kept overnight in a tank and next morning, female was striped to collect the eggs. The males were sacrificed to collect testis and were mashed in pestle and mortar. The chopped testes were applied with 0.9% NaCl and immediately mixed with eggs in the bowl. The eggs were then transported to the hatching tanks and *Eichhornia crassipes* roots were used to take out the eggs from bowl to tanks due to their adhesive nature. The roots were placed in hatching tanks till the spawn comes out from the eggs and later, taken out with attached white coloured unfertilized eggs. The water quality parameters like dissolved oxygen (5.2–7 mg/ml), water depth (15.5 cm), water flow etc. were maintained to make the fertilization successful. After fertilization, the samplings at 0 dph (day post hatch), 3 dph, 4 dph, 5 dph, 6 dph, 7 dph, 9 dph, 11 dph, 13 dph, 16 dph, 19 dph, 22 dph, 26 dph, 30 dph and 34 dph respectively was done. The first day after hatching was considered as day 0 dph. At each time point, 10 larvae were collected and stored in RNA later (Qiagen, Germany).

*RNA extraction and cDNA synthesis:* Total RNA was extracted from all the samples using Trizol™ reagent (Invitrogen, USA). The extracted RNA was treated with DNase I (Thermo Scientific, USA) as per manufacturer's instructions supplied with the kit to remove the genomic DNA. The ratio of absorbance at 260 nm and 280 nm by Nanodrop spectrophotometer (Thermo Scientific, USA) was used to determine the purity of DNAse treated RNA. The first strand cDNA was synthesized from DNase-treated RNA using RevertAid$^{TM}$ First Strand cDNA Synthesis kit (Thermo Scientific, USA) primed with Oligo dT primer as per the manufacturer's instructions.

*Primer design and standard curves:* Primers for qRT-PCR were designed based on the sequences of *C. batrachus* (neo-type of *C. magur*) β-actin, GAPDH, and EFa1 available in GenBank. All primers were designed by Gene Runner software (v. 4.0.9.62) and synthesized by BioInnovation Company, Hyderabad (Table 1). The predicted qRT-PCR amplification product sizes were 100 to 200 bp in length. Standard RT-PCR was used for checking the specificity and efficiency of primer sets before performing any qRT-PCR analysis. The amplified fragments would generate a single band of the predicted size by 2% agarose gel electrophoresis, if the designed primers were good. In addition, the melting curve would contain only a single peak. The correlation coefficient ($R^2$) was determined and the value above 0.98 was accepted. The value of the $R^2$ was based on the slopes of the standard curves generated by the dilution (10-fold) series of gut cDNA sample from adult fish.

*Quantitative real time PCR:* The mRNA expression of all the three HKGs at basal level in above-mentioned samples of *C. magur* was carried out in LightCycler® Real-time PCR detection system (Roche, USA). The 25 µl reaction mix volume was prepared to contain 12.5 µl of Maxima™ SYBR Green qPCR master mix (Thermo Scientific, USA), 1 µl of (0.3 pM) each primer, 1 µl (20 ng) of cDNA and 9.5 µl nuclease free water. Then, each sample was distributed into two wells, each having the final volume of 10 µl. The default thermal profile was used for PCR amplification and it consisted of initial denaturation at 95°C–10 min, followed by 40 cycles of denaturation at

Table 1. Reference genes, primer sets and different parameters for RT-qPCR analysis and the efficiency of primers used for amplification of different genes for expression analysis study

| Gene | Abbreviation | Accession number | Primer forward | Primer reverse | Size (bp) | Tm (°C) | PCR efficiencies (%) |
|---|---|---|---|---|---|---|---|
| Beta-actin | β-actin | EU527190 | TGCCCCAGAGGAGC ACCCTG | GACCAGAGGCGTACA GGGACAGC | 152 | 60 | 99 |
| Elongation factor 1-alpha | EFa1 | AB916539 | CCCTGCTGCCGTTG CTTTCG | CCGCTGGCATTTCCCT CCTTG | 123 | 58 | 96 |
| Glyceraldehyde 3-phosphate dehydrogenase | GAPDH | KC414932 | CAGGTTGTTAGGGTT GCTCGTGG | TGGAAGGCAGCACGG GTCAC | 187 | 56 | 98 |

95°C/20 sec and annealing at 60°C (β-actin), 58°C (GAPDH) or 56°C (EFal) for 30 sec and extension at 72°C/30s respectively. The different melting peaks of the three reference candidate genes were observed. The relative mRNA expression data were analyzed using the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001).

*Ethics statement:* The care and treatment of animals used in this study were in accordance with the guidelines of the CPCSEA (Committee for Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), Govt of India on care and use of animals in scientific research.

*Data analysis:* Statistical analysis for the difference in expression levels of β-actin, GAPDH and EFa1 was carried out by one-way ANOVA using SPSS 22.0 software (SPSS Inc., USA). The level of significance was determined at $P<0.05$. To find out the stability of selected reference genes, different statistical algorithms were employed to analyze the set of data, i.e., Delta ct method, BestKeeper (version 1) and NormFinder (version 0.953) were selected. The most suitable HKG was then evaluated according to a comprehensive gene stability method. These three different statistical tools were used, as any single program was non-reliable and insufficient to select the best HKG for normalization of the qPCR datasets.

## RESULTS AND DISCUSSIONS

*qRT-PCR amplification of primers:* Gene expression studies are one of the important tools for studying the physiological effects at molecular level. qPCR is widely employed method for accurate analysis of gene expression due to its high precision and sensitivity (Ye *et al.* 2010). It also provides a simultaneous quantification of genes in different samples. But, there are different internal factors which may influence the extent of gene expression and leads to false interpretation of results (Dheda *et al.* 2005). Thus, keeping that in mind, the selection of appropriate HKGs for expression analysis of any gene is necessary in order to reduce the experimental errors.

In the present study, three most commonly known reference genes (β-actin, EFa1 and GAPDH) were investigated, which are used as internal controls in a number of mRNA expression studies. The relative mRNA expression of selected HKGs was measured by qRT-PCR during different larval stages of *C. magur* from fertilized egg upto 34 dph (days post hatch). The melting peaks of the three selected HKGs are shown in Fig. 1. The cycle threshold (Ct) values of all the three candidate reference genes for the different samples under study were used to compare the extent of expression as well as variation among themselves and across the different samples (Fig. 2). The analysis of datasets revealed a wide range of expression differences between genes. It is clearly shown from the figure that beta-actin exhibited very less variation across the samples compared to other reference genes. As is evident from the raw Ct values of the present study that a variable expression profile of mRNA during developmental stages,

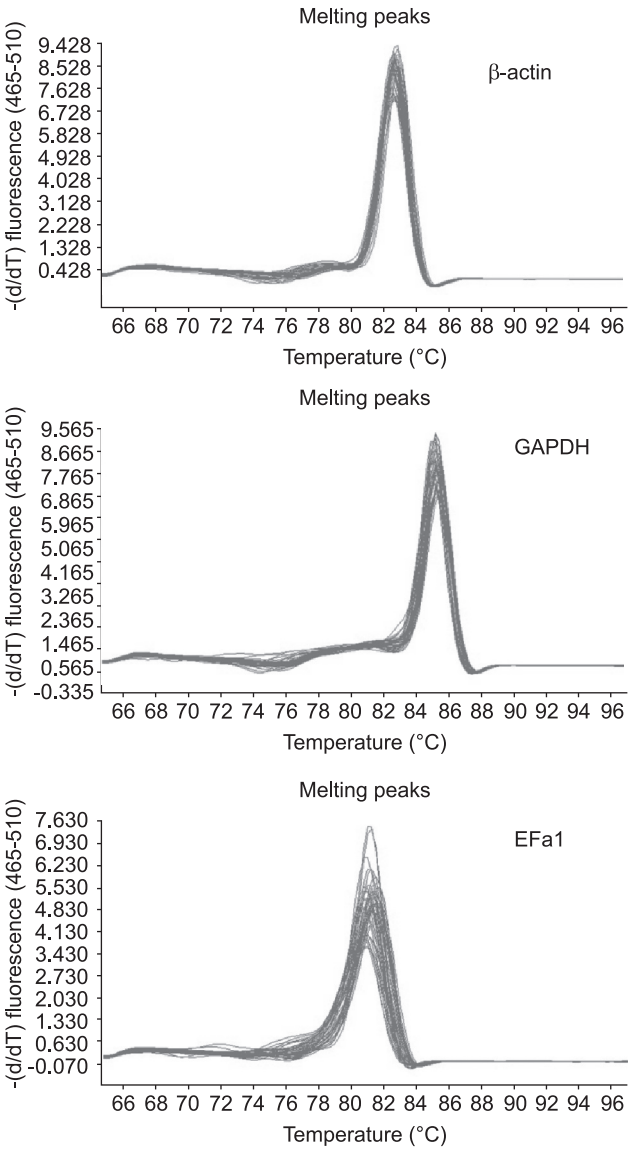


Fig. 1. Melting peaks of the three selected reference genes during different developmental stages of *Clarias magur*.

has been observed in all the three reference genes. Hence, it is apparent that a direct statistical analysis of raw CT values is not suitable to select the best HKG for normalization of the qPCR datasets (Suzuki *et al.* 2000, Czechowski *et al.* 2005). Therefore, three different statistical algorithms were employed to analyze the set of data, namely, delta-Ct method (Silver *et al.* 2006), NormFinder (Anderson *et al.* 2004) and BestKeeper (Pfaffl *et al.* 2004), in addition to comprehensive gene stability method for identification of stable reference genes.

*Expression stability analysis of HKGs by different statistical algorithms:* Although the analysis based on Ct values reveals the variation among the reference genes, but that is very less and difficult to conclude the final stability. Thus, identification of most stable HKG gene for normalizing gene expression based on different statistical algorithms is necessary. Delta Ct method, BestKeeper, NormFinder and Comprehensive gene stability method

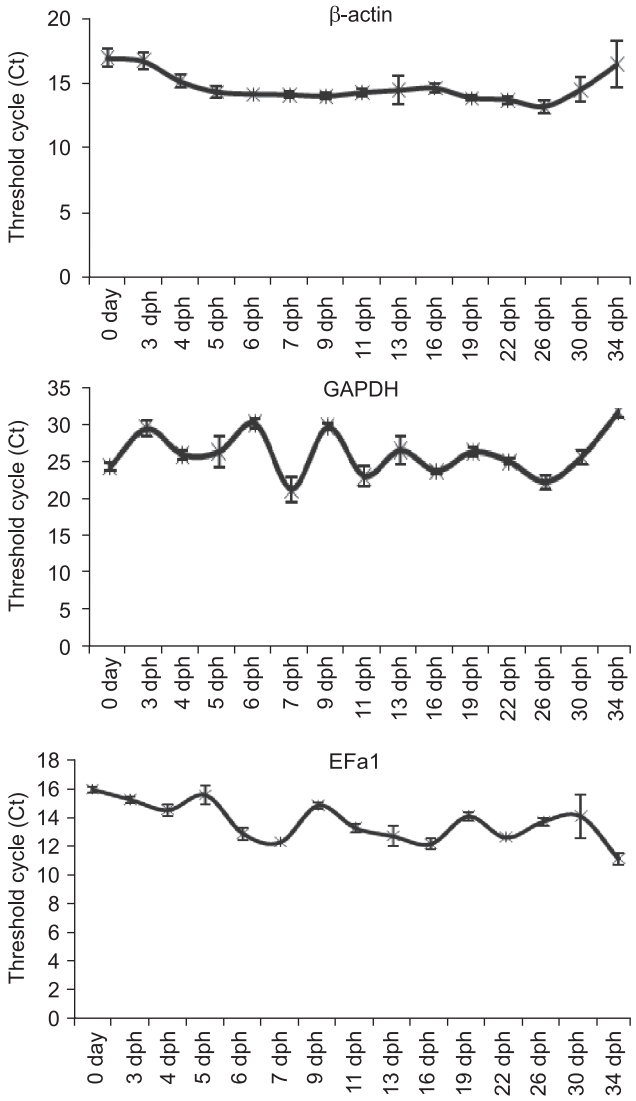


Fig. 2. Expression profiles of the three potential reference genes during different developmental stages of *Clarias magur*. Data is shown from 0 day until 34 dph as average Ct values±SEM where n = 3.

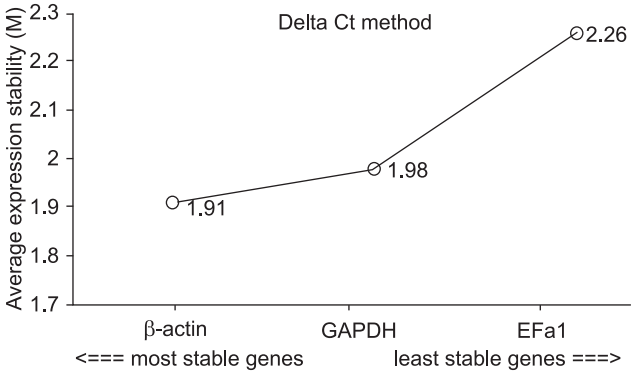


Fig. 3. Evaluation of candidate reference genes using Delta Ct method analysis in different developmental stages of *Clarias magur*.

Table 2. Statistical analysis of housekeeping genes by BestKeeper software

| | β-actin | GAPDH | EFa1 |
|---|---|---|---|
| n | 15 | 15 | 15 |
| geo Mean [CP] | 14.59 | 24.57 | 13.73 |
| AR Mean [CP] | 14.67 | 24.62 | 13.81 |
| min [CP] | 12.73 | 22.21 | 10.96 |
| max [CP] | 18.44 | 29.04 | 16.38 |
| std dev [+/- CP] | 1.33 | 1.23 | 1.3 |
| CV [% CP] | 9.06 | 5.01 | 9.42 |
| min [x-fold] | –3.64 | –5.15 | –6.84 |
| max [x-fold] | 14.42 | 22.18 | 6.27 |
| std dev [+/- x-fold] | 2.51 | 2.35 | 2.46 |

were used to analyze the expression of three genes for stability.

The comparative delta ct method revealed that most stable gene possesses the least average stability value. The current study showed the lowest stability value (1.91) for β-actin by the comparative delta Ct method (Fig. 3) and thus found to be most stable reference gene for normalization.

The stability analysis of all the three candidate reference genes used in the study was computed by BestKeeper

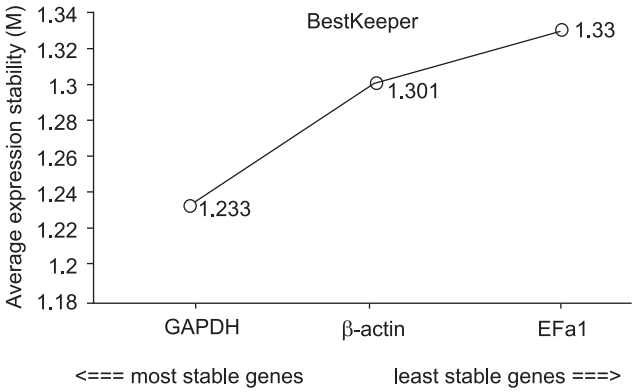


Fig. 4. Evaluation of candidate reference genes using BestKeeper software analysis in different developmental stages of *Clarias magur*.

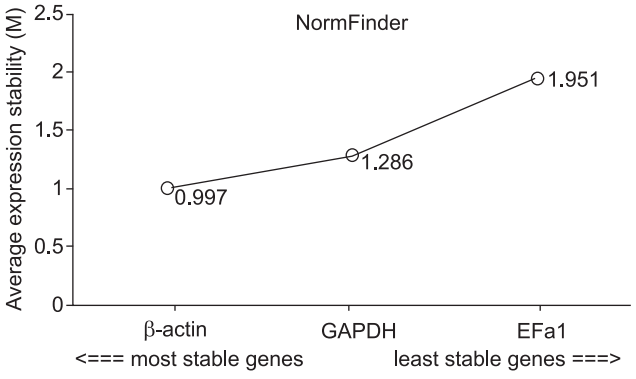


Fig. 5. Evaluation of candidate reference genes using NormFinder software analysis in different developmental stages of *Clarias magur*.

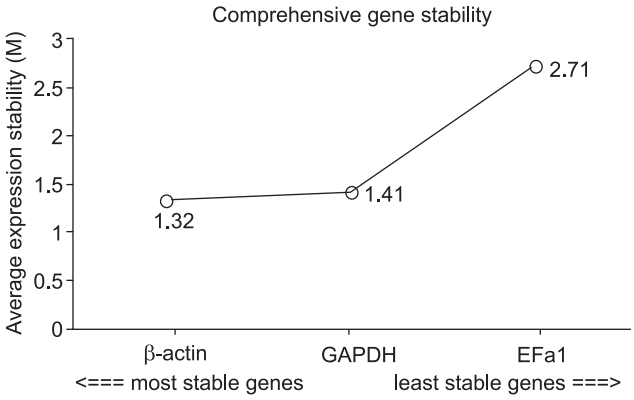


Fig. 6. Evaluation of candidate reference genes using comprehensive gene stability analysis in different developmental stages of *Clarias magur*.

algorithm. In the present study, a variation in the results has been observed and the statistical data of HKGs by BestKeeper algorithm showed least standard deviation for GAPDH gene (Table 2). Hence, through this statistical tool, GAPDH gene was the most stable gene with lowest stability value (Fig. 4). Although, GAPDH was observed to be more stable than β-actin, but the difference among the stability values of two genes was very less.

The stability of selected three reference genes was again analyzed by using the NormFinder algorithm (Anderson *et al.* 2004). NormFinder calculates the stability value of each tested candidate gene based on their minimum intra-group and inter-group variation. Thus, the genes which showed low values were considered to be the most stable and the genes which represented high values were ranked as least stable. Based on this approach, in the present study, ß-actin was most stable (average stability value, 0.997) among the three reference genes, where as EFa1 was identified to be the least stable gene (average stability value, 1.951) (Fig. 5).

The results of the statistical algorithms revealed that beta-actin is the most stable gene for normalization of gene expression by qRT-PCR, except the BestKeeper software which portrayed GAPDH to be most stable with a non-significant variation from β-actin value. Thus, the average stability of the three HKGs was finally elucidated by the comprehensive gene stability method. The variable average values of the stability were observed, with the gene possessing lower values represented to be most stable. In the present study, β-actin was reported to be most stable (average stability value, 1.32) among the three reference genes, whereas EFa1 was observed to be least stable gene (average stability value, 2.71) (Fig. 6).

Thus, the above results suggested β-actin as the most stable gene among the three HKGs to be used for the validation of gene expression by qRT-PCR analysis during different larval developmental stages of the *C. magur*. These results corroborate with the study conducted on Asian seabass (*Lates calcarifer*) during ontogenesis and in tissues of healthy and infected fishes where, EF1A and β-actin were found to be the genes with highest stability and least variation across the developmental stages (Paria *et al.* 2016). Hence β-actin is considered to be the most suitable reference gene for carrying out any gene expression related to physiological aspect during the ontogenic developmental stages in *C. magur*. The results were in agreement with Huang *et al.* (2009) which reported β-actin to be the most frequently used HK gene. The β-actin has been included as a reference gene in adult *C. magur* in many studies (Bhat *et al*. 2015*,* Bhat *et al.* 2016a*,* Bhat *et al.* 2016b) and present results also consider the particular gene to be used as an internal control for mRNA expression studies of ontogenetic development.

In conclusion, β-actin was selected as the reference gene for conducting the gene expression studies during ontogenic development of *C. magur*. Although considerable variations in expression levels of reference genes analyzed in different developmental stages were detected, we suggest β-actin as the most stable reference gene for normalization models. This report will contribute significantly to studies of qPCR analysis in the *C. magur* as well as in other species belonging to the order of Siluriformes, which includes the economically important species.

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