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Original article

Selection of reference genes for quantitative real-time RT-PCR on gene expression in Golden Pompano (*Trachinotus ovatus*)

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Abstract

Golden pompano (*Trachinotus ovatus*) is an important economically fish species. In this study, with an aim to identify reliable reference genes for quantitative real-time PCR (qRT-PCR) in golden pompano, we evaluated the expression stability of eight housekeeping genes in the presence and absence of poly I:C stimulation in eight tissues. The PCR data was analyzed by geNorm and NormFinder algorithms. The results showed that the expression of all the examined genes exhibited tissue-dependent variations. When under normal physiological condition, geNorm and NormFinder identified B2M and 18S as suitable genes. When studying gene expression under conditions of poly I:C stimulation, the selection of the internal controls should be selected on a tissue basis. At 12 h stimulation, geNorm ranked Actin/UBCE, Actin/B2M, UBCE/B2M, Actin/UBCE, RPL13/B2M, UBCE/GAPDH, B2M/RPL13, and UBCE/B2M, respectively, as the most stably expressed genes in liver, spleen, kidney, gill, intestine, heart, muscle, and brain. Comparable ranking orders were produced by NormFinder. Similar results were obtained at 48 h stimulation. Taken together, these results indicate that B2M and 18S are the most stable gene across tissue types under normal physiological conditions. However, during poly I:C stimulation, no single gene or single pair of genes in the examined set of housekeeping genes can serve as a universal reference across all tissue types. If one gene is preferred, B2M, B2M, UBCE, Actin, B2M/RPL13, B2M, B2M, and RPL13 may be used in spleen, kidney, liver, gill, intestine, brain, muscle, and heart of golden pompano, respectively.

Key words: housekeeping gene, expression stability, reference gene, *Trachinotus ovatus*

Introduction

At present, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is the most widely used molecular method based on traditional PCR, which converted the polymerase chain reaction technology from qualitative to quantitative. Compared to traditional RNA quantitation techniques such as northern blot and RNase protection assay, qRT-PCR is much more sensitive and can

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detect the mRNA at a very low abundance (Heid et al. 1996, Haller et al. 2004, Ransbotyn and Reusch 2006, Yoo et al. 2009). It has become an important tool to analyze gene expression, as often used in the transcriptional regulations in aquatic science. However, it has been reported that the expression of some internal reference genes is not stable under some experimental conditions (Selvey et al. 2001, Liu et al. 2005). Many studies have shown that the expressions of the internal reference genes are affected by many external factors. No expression of an internal reference gene is always stable and the expression of the internal reference gene varies depending on the experimental conditions. Therefore, selecting the appropriate internal reference gene under some specific condition is most important (Radonic et al. 2004, Huggett et al. 2005).

Golden pompano (*Trachinotus ovatus*) belongs to family *Carangidae*, *Perciformes,* and *genus Trachinotus* (Tutman et al. 2004). It is an important economic species in China, Japan, Australia, and other countries (Zhou et al. 2014). *Trachinotus ovatus* (*T. ovatus*), as a carnivorous fish, feeds on some small fish, crustaceans, shellfish, and zooplankton (Liu and Chen 2009). In recent years, golden pompano industries developed rapidly and it became the main species of cage culture. Along with the expansion, *T. ovatus* aquaculture industries worldwide have suffered viral diseases outbreaks what caused serious economic losses (Xu et al. 2010, Su et al. 2015). Recently, qRT-PCR has become a widely used method of investigating gene expression in golden pompano. But, no study on normalization strategy has been documented.

In this report, we aim to select the most stable reference genes for accurate application in real-time quantitative PCR (qRT-PCR) analysis in the presence and absence of poly I:C stimulation in *Trachinotus ovatus*. Eight commonly used housekeeping genes, including beta- actin (Actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (18S), β-2-Microglobulin (B2M), elongation factor-1-α (EF1A), ubiquitin-conjugating enzyme E2 (UBCE), tubulin tyrosine ligase-like family member 1 (TTLL1) and ribosomal protein L13 (RPL13) were chosen to analyze the expression stability with qRT-PCR in different tissues under different experimental conditions with two independent normalization algorithms, geNorm and NormFinder, which were widely used for normalization of qRT-PCR data (Olsvik et al. 2008, Penna et al. 2011). Moreover, this is the first time to select the best internal genes after stimulating golden pompano with poly I:C and the results will provide an useful guidance of internal controls in future RT-qPCR studies in this species.

Materials and Methods

Fish

Golden pompano (*Trachinotus ovatus*) fish were purchased from a commercial fish farm in Hainan Province, China, and maintained at 26°C in aerated running seawater and were acclimatized in the laboratory for one week. Before using for any experiment, fish were randomly sampled for confirming healthe as reported previously (Zhou et al. 2015) and were euthanized by tricaine methanesulfonate (Sigma, St. Louis, MO, USA) before collection of the tissues.

poly I:C stimulation and tissues collection

Golden pompano (average weight, 13.8 g ± 0.5 g) were randomly divided into two groups, A and B, twenty fish in each group. Group A was stimulated with 100 μl of poly I:C (InvivoGen, USA, 1 mg/ml), while group B was injected with PBS. Five fish from each group were sacrificed with tricaine methanesulfonate and tissues, i.e., spleen, head kidney, liver, gill, intestine, brain, muscle, and heart), were collected under aseptic conditions at 12h and 48h post-treatment, respectively. Sample tissues were pooled together at equal amounts and frozen in liquid nitrogen. All experiments were independently performed three times.

RNA extraction and cDNA library construction

Total RNA was extracted from all collected tissues (including spleen, head kidney, liver, gill, intestine, brain, muscle and heart) and first-strand cDNA was synthesized with PrimeScript™ 1st Strand cDNA Sythesis Kit (Takara, Dalian, China) as reported previously (Liu et al. 2010).

Primer design and PCR efficiency

Eight housekeeping genes, i.e., beta-actin (Actin), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (18S), β-2-Microglobulin (B2M), Ubiquitin-conjugating enzyme E2 Elongation factor-1-α (EF1A), Ubiquitin-conjugating enzyme E2 (UBCE), Tubulin tyrosine ligase-like family, member 1 (TTLL1), and Ribosomal protein L13 (RPL13) (Table 1), were selected. The specific primers were shown in Table 2. PCR efficiency (E) and correlation coefficient (R2) were determed in the standard curves generated. The efficiency was

Table 1. The housekeeping genes used in this study.

Symbol	Name	Function	GenBank accession number
Actin	Beta actin	Cytoskeletal protein	KX987228
GAPDH	Glyceraldehyde-3-phosphate	Glycolysis enzyme	KY006114
18S	18S ribosomal RNA	Ribosome subunit	KY014076
B2M	β-2-Microglobulin	Majoy histocumpatibility complex	KX987233
EF1A	Elongation factor-1-α	Translation	KX987227
UBCE	Ubiquitin-conjugating enzyme E2	Protein degradation	KX987232
TTLL1	Tubulin tyrosine ligase-like family, member 1	Tubulintyrosine ligase-like protein	KX987231
RPL13	Ribosomal protein L13	Ribosome Protein	KX987230

Table 2. Primers and PCR amplification efficiencies.

Gene	Primer sequence(5'→3')	Product Size(bp)	PCR efficiency (%)	Correlation coefficient
Actin	CGTGCGTGACATCAAGGAGAA AAGGAAGGAAGGCTGGAAGAGG	178	98	0.998
GAPDH	GAAGGGTGGTGCCAAGAGGG AGGCAGTTGGTTGTGCAGGAA	137	96	0.998
18S	AGTTGGCATCGTTTATGGTCG GCATTCGTATTGTGCCGCTA	160	99	0.996
B2M	AAGTCAGTCCACCCAAGGTTCA GGGATTTCCATTCCGTTCTTCATG	139	95	0.993
EF1A	GTCCGTCAAGGAAATCCGTCG TTGAACTTGCAGGCAATGTGAG	174	102	0.999
UBCE	CACGATGTCCAGCGAAGTACA GACCTCCACTCGTAGATGTTGTC	270	97	0.998
TTLL1	GAGGCAAGTGGACGGTCAGTA TCACAGGAGCCACAGCTTTCA	124	103	0.994
RPL13	CCGTCTCATCGCTCCTCGTC CAATGGTGCGGGCTGTCTTT	155	99	0.994

estimated as follows: $E(\%) = (10^{-1/slope}-1) \times 100$ (Kubista et al. 2006). The E value was acceptable ranging from 90 to 110%.

Quantitative real time reverse transcription-PCR (qRT-PCR)

Quantitative real time reverse transcription-PCR (qRT-PCR) was used to analyze the stability of housekeeping genes using the SYBR® Premix DimerEraser (Perfect Real Time, Takara, Dalian, China). qRT-PCR was carried out in an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany). The procedure was performed as reported by Qiu et al. 2013. Each sample was examined in triplicate.

Data analysis

The PCR data were analyzed using geNorm (version 3.5) and NormFinder algorithms (Vandesompele et al. 2002, Andersen et al. 2004).

Results

Expression levels of selected housekeeping genes in golden pompano tissues under normal physiological conditions

The results of PCR efficiency (E) and correlation coefficient (R2) of the eight candidate reference genes, i.e., Actin, GAPDH, 18S, B2M, EF1A, UBCE,

Table S1. Ct values of the housekeeping genes expressed in the golden pompano tissues at 12 h stimulated with PBS or poly I:C.

		Spleen	Kidney	Liver	Gill	Brain	Intestine	Muscle	Heart
Actin	PBS	21.6 ± 0.6	20.4 ± 0.6	18.3 ± 0.5	21.6 ± 0.1	18.5 ± 0.1	16.3 ± 0.8	15.2 ± 0.2	23.0 ± 0.7
	Poly I:C	19.7 ± 0.8	19.8 ± 0.3	19.0 ± 0.4	21.3 ± 0.2	18.7 ± 0.1	15.9 ± 0.6	15.5 ± 0.2	18.4 ± 0.3
GAPDH	PBS	25.2 ± 0.1	22.1 ± 0.5	15.8 ± 0.7	23.8 ± 0.8	23.8 ± 0.1	16.7 ± 0.3	15.2 ± 0.2	18.6 ± 0.6
	Poly I:C	28.1 ± 0.3	20.6 ± 0.4	16.1 ± 0.3	29.0 ± 0.6	18.4 ± 0.1	18.3 ± 0.7	16.0 ± 0.1	17.1 ± 0.3
18S	PBS	16.3 ± 0.3	15.8 ± 0.3	16.1 ± 0.3	15.8 ± 0.5	16.4 ± 0.4	12.8 ± 0.6	16.6 ± 0.2	16.7 ± 0.5
	Poly I:C	14.9 ± 0.3	14.4 ± 0.3	14.9 ± 0.2	16.6 ± 0.5	16.3 ± 0.2	14.2 ± 0.6	16.3 ± 0.1	14.6 ± 0.1
B2M	PBS	23.9 ± 0.3	23.4 ± 0.4	21.5 ± 0.4	24.2 ± 0.2	24.5 ± 0.1	20.8 ± 0.8	23.5 ± 0.4	26.5 ± 0.8
	Poly I:C	21.9 ± 0.8	20.8 ± 0.6	21.7 ± 0.3	23.5 ± 0.5	20.6 ± 0.1	21.0 ± 0.5	22.3 ± 0.2	24.4 ± 0.3
EF1A	PBS	21.7 ± 0.6	20.8 ± 0.5	17.5 ± 1.2	20.7 ± 0.1	19.1 ± 0.1	16.0 ± 0.2	19.8 ± 0.6	24.4 ± 0.5
	Poly I:C	21.4 ± 1.0	17.2 ± 0.3	18.7 ± 0.2	20.3 ± 0.1	19.0 ± 0.1	17.0 ± 0.5	22.0 ± 0.6	22.6 ± 0.4
UBCE	PBS	29.9 ± 0.9	31.7 ± 0.3	27.0 ± 0.4	30.3 ± 0.2	24.6 ± 0.1	27.9 ± 0.6	27.5 ± 0.4	28.0 ± 0.8
	Poly I:C	26.3 ± 0.5	29.0 ± 0.4	27.6 ± 0.1	30.1 ± 0.2	24.3 ± 0.2	27.6 ± 0.7	28.6 ± 0.4	29.8 ± 0.2
TTLL1	PBS	33.8 ± 0.6	32.8 ± 0.3	33.4 ± 0.5	35.8 ± 0.8	27.8 ± 0.4	33.7 ± 0.3	34.7 ± 0.3	33.8 ± 0.6
	Poly I:C	30.8 ± 0.8	30.4 ± 0.8	32.8 ± 0.3	34.9 ± 0.5	27.1 ± 0.1	32.8 ± 0.5	34.0 ± 0.1	33.6 ± 0.1
RPL13	PBS	24.4 ± 0.6	21.0 ± 0.3	18.2 ± 1.1	22.0 ± 0.4	20.5 ± 0.6	18.7 ± 0.4	19.8 ± 0.6	20.2 ± 0.7
	Poly I:C	21.5 ± 0.9	18.3 ± 0.3	18.0 ± 0.1	21.7 ± 0.1	21.4 ± 0.1	18.8 ± 0.5	22.0 ± 0.3	18.3 ± 0.1

Table S2. Ct values of the housekeeping genes expressed in the golden pompano tissues at 48 h stimulated with PBS or poly I:C.

		Spleen	Kidney	Liver	Gill	Brain	Intestine	Muscle	Heart
Actin	PBS	21.6 ± 0.5	21.2 ± 0.5	18.7 ± 0.5	21.2 ± 0.6	15.8 ± 0.5	16.4 ± 0.2	18.6 ± 0.6	18.1 ± 0.2
	Poly I:C	20.7 ± 0.5	19.6 ± 0.3	19.3 ± 0.3	19.9 ± 0.5	19.9 ± 0.2	17.6 ± 0.3	17.9 ± 0.7	17.9 ± 0.3
GAPDH	PBS	30.6 ± 0.2	23.0 ± 0.3	16.4 ± 1.0	23.0 ± 0.9	17.2 ± 0.8	17.2 ± 0.3	19.6 ± 0.8	25.7 ± 0.2
	Poly I:C	30.4 ± 0.3	21.8 ± 0.6	15.4 ± 0.4	28.6 ± 0.6	18.0 ± 0.1	17.1 ± 0.5	18.8 ± 1.0	18.4 ± 0.3
18S	PBS	15.0 ± 0.3	15.1 ± 0.3	18.0 ± 0.8	15.8 ± 0.4	16.1 ± 0.5	12.5 ± 0.3	17.9 ± 0.9	17.2 ± 0.4
	Poly I:C	15.8 ± 0.3	16.0 ± 0.3	15.2 ± 0.3	15.9 ± 0.2	18.0 ± 0.1	11.5 ± 0.5	16.8 ± 0.5	16.2 ± 0.3
B2M	PBS	25.2 ± 0.3	25.0 ± 0.3	21.3 ± 0.1	25.7 ± 0.7	21.6 ± 0.3	20.4 ± 0.3	27.5 ± 0.6	23.5 ± 0.5
	Poly I:C	23.1 ± 0.3	22.9 ± 0.3	19.7 ± 0.4	21.5 ± 0.6	25.2 ± 0.1	20.7 ± 0.6	22.7 ± 0.5	21.5 ± 0.2
EF1A	PBS	23.1 ± 0.3	24.3 ± 0.9	21.9 ± 0.1	20.5 ± 0.5	18.9 ± 0.6	16.6 ± 0.2	22.0 ± 0.2	19.4 ± 0.1
	Poly I:C	21.3 ± 0.3	19.7 ± 0.4	15.5 ± 0.3	19.2 ± 0.5	20.9 ± 0.1	17.2 ± 0.7	20.1 ± 0.2	17.8 ± 0.3
UBCE	PBS	29.7 ± 0.3	31.4 ± 0.9	27.8 ± 1.1	30.8 ± 0.5	28.0 ± 1.0	29.0 ± 0.2	29.8 ± 0.2	27.2 ± 0.2
	Poly I:C	29.8 ± 0.3	30.8 ± 0.5	27.8 ± 0.1	28.6 ± 0.5	26.2 ± 0.3	29.9 ± 0.8	26.9 ± 0.3	30.7 ± 0.3
TTLL1	PBS	34.8 ± 0.1	31.2 ± 1.1	32.4 ± 0.2	35.0 ± 0.1	31.7 ± 0.5	33.1 ± 0.2	35.4 ± 0.2	34.7 ± 0.3
	Poly I:C	35.2 ± 0.4	31.0 ± 0.4	32.5 ± 0.1	35.0 ± 0.2	28.1 ± 0.1	30.8 ± 0.1	33.7 ± 0.5	34.3 ± 0.5
RPL13	PBS	24.3 ± 0.3	23.0 ± 0.6	24.3 ± 0.2	22.7 ± 0.8	17.7 ± 0.2	18.4 ± 0.3	24.7 ± 0.3	20.9 ± 0.5
	Poly I:C	22.3 ± 0.2	20.9 ± 0.3	16.4 ± 0.4	20.5 ± 0.3	21.4 ± 0.2	18.6 ± 0.5	22.3 ± 0.3	20.4 ± 0.1

TTLL1, and RPL13, were ranging from 95% to 103% and from 0.993 to 0.999 (Table 2), respectively. Under normal conditions, the Ct values of the housekeeping genes were ranging between 12.5 and 35.8 (Tables S1 and S2) in all the eight examined tissues. 18S with Ct values ranging between 12.5 and 18.0 in different tissues showed the highest levels of expression. Actin, GAPDH, EF1A, RPL13 and B2M showed comparable Ct values ranges (15.2 to 23.0, 15.2 to 30.6, 16.0 to 24.4, 17.7 to 24.7 and 20.4 to 27.5, respectively). UBCE and TTLL1 had Ct values greater than 25, with the highest Ct values being 31.7 and 35.8, respectively. For all the examined genes, significant ($p<0.05$) variations were observed in different tissues. In comparison, GAPDH showed a Ct variation of 15.4 between muscle and spleen. The smallest Ct variation was observed for 18S, which showed a maximum Ct difference of 5.5 between liver and intestine. All other genes exhibited Ct variations between 7.1 and 8.4.

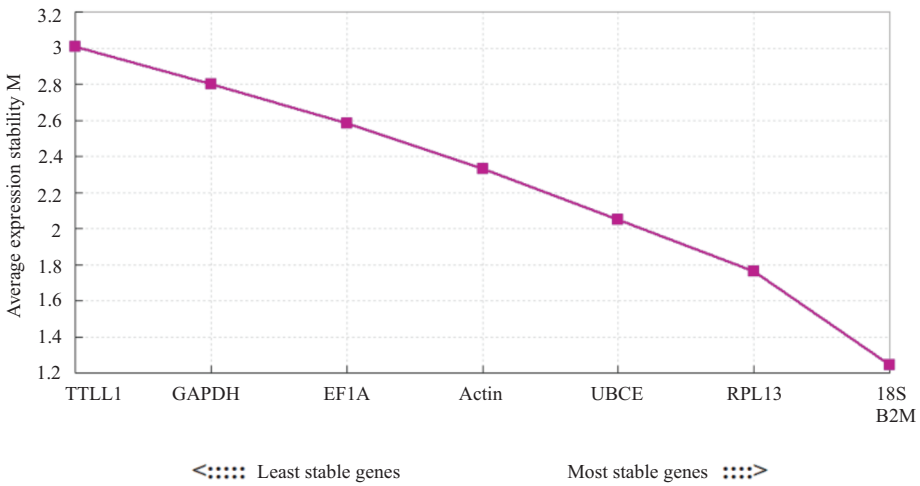

Fig. 1. GeNorm analysis of the expression of the housekeeping genes in golden pompano tissues under normal physiological condition. The expressions of the housekeeping genes in Trachinotus ovatus tissues (spleen, head kidney, liver, gill, intestine, brain, muscle, and heart) were determined by qRT-PCR and the data were analyzed with geNorm to calculate the expression stability (M) of the genes.

Table 3. Expression stability of the housekeeping genes in golden pompano tissues under normal physiological condition as calculated by NormFinder.

Ranking order	1	2	3	4	5	6	7	8
Gene	B2M	18S	RPL13	UBCE	Actin	GAPDH	EF1A	TTLL1
Syability	0.499	0.789	0.977	1.297	1.436	1.974	2.172	2.207

Expression stability of selected housekeeping genes in golden pompano tissues under normal physiological condition

geNorm and NormFinder were used to determine the expression stability of the tested genes. geNorm analysis indicated that 18S and B2M were the most stable genes across tissues types with the lowest M values in the examined tissues (Fig. 1) under normal condition. RPL13, UBCE, Actin, EF1A, GADPH and TTLL1 were ranked in a decreasing stability order. Similarly, NormFinder showed B2M as the most stable gene and TTLL1 as the least stable one (Table 3).

Expression levels of selected housekeeping genes under *poly I:C* stimulation

During poly I:C stimulation, the expression levels of the eight housekeeping genes were shown in Tables 3 and 4. 12 h after poly I:C stimulation, GAPDH showed a maximum Ct variation of 5.4 in brain (Table S1). The Ct variations observed in the expression of Actin, B2M, EF1A, UBCE, TTLL1, and RPL13 were 4.4, 3.7, 3.5, 3.4, 2.8, and 2.8, respectively. 18S showed comparable and the smallest changes under poly I:C stimulation, with Ct varying from 0.1 to 2.1. 48 h after poly I:C stimulation, Ct variation of GAPDH in heart was 7.3, being the highest one (Table S2).

Expression stability of selected housekeeping genes under poly I:C stimulation determined by geNorm and NormFinder

Analysis by geNorm

geNorm analysis indicated that the M values of all the genes in the eight examined tissues, except for that of GAPDH in spleen, gill and heart, TTLL1 in brain and RPL13 in liver, varied between 0.01 and 1.49, which were lower than the expression stability threshold (1.5) proposed by geNorm (Fig. 2, 3). 12 h after stimulation, B2M together with Actin, UBCE, RPL13, UBCE, or TTLL1 exhibited the lowest M values in spleen, kidney, intestine, brain, and muscle, so they were recognized as the most stable genes (Fig. 2A, B, E to G). In liver and gill, Actin and UBCE were identified as the most stable pairs of genes (Fig. 2C, D). In heart, UBCE/GADPH, was identified as the most stable pairs of genes (Fig. 2H). 48 h post-infection, the most stable genes in spleen, kidney, intestine, and brain were B2M/RPL13, while

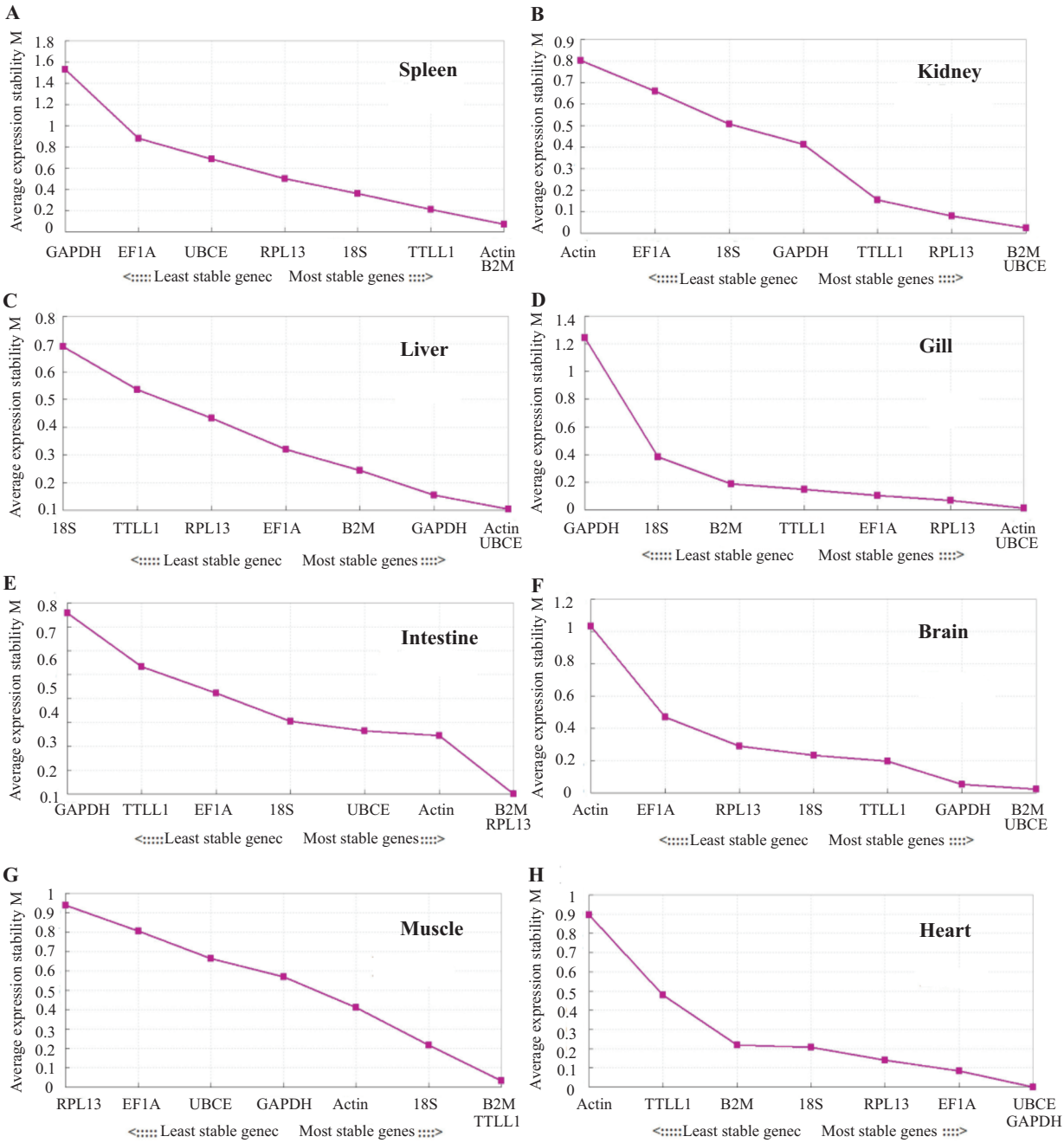

Fig. 2. Expression stability of the housekeeping genes in golden pompano tissues 12 h after poly I:C stimulation obtained with geNorm. The expression of the housekeeping genes in Trachinotus ovatus tissues 12 h after PBS or poly I:C stimulation was determined by qRT-PCR and using geNorm to calculate the expression stability (M) of each of the genes. A lower M value indicates more stable expression.

those in liver, gill, muscle, and heart were, respectively, UBCE/TTLL1, EF1A/Actin, Actin/GAPDH, and TTLL1/RPL13 (Fig. 3).

To determine the optimal number of genes required for data normalization, pair wise variation (V) between two sequential normalization factors containing an increasing number of genes was determined. The results showed that at 12 h post-treatment, the V2/3 values were lower than 0.15 in all the examined tissues (Fig. 4). At 48 h post-treatment, the V2/3 values were all lower than 0.15 for expressions in liver, kidney, spleen, heart, muscle, intestine, and brain (Fig. S1); therefore for gene expression in these tissues, two reference genes will suffice for reliable normalization. For expression in gill, the V2/3 value was 0.217, while the V3/4 value (0.132) was lower than 0.15 (Fig. S1), suggesting that the third most stable gene, i.e., RPL13, should be included in the normalization factor.

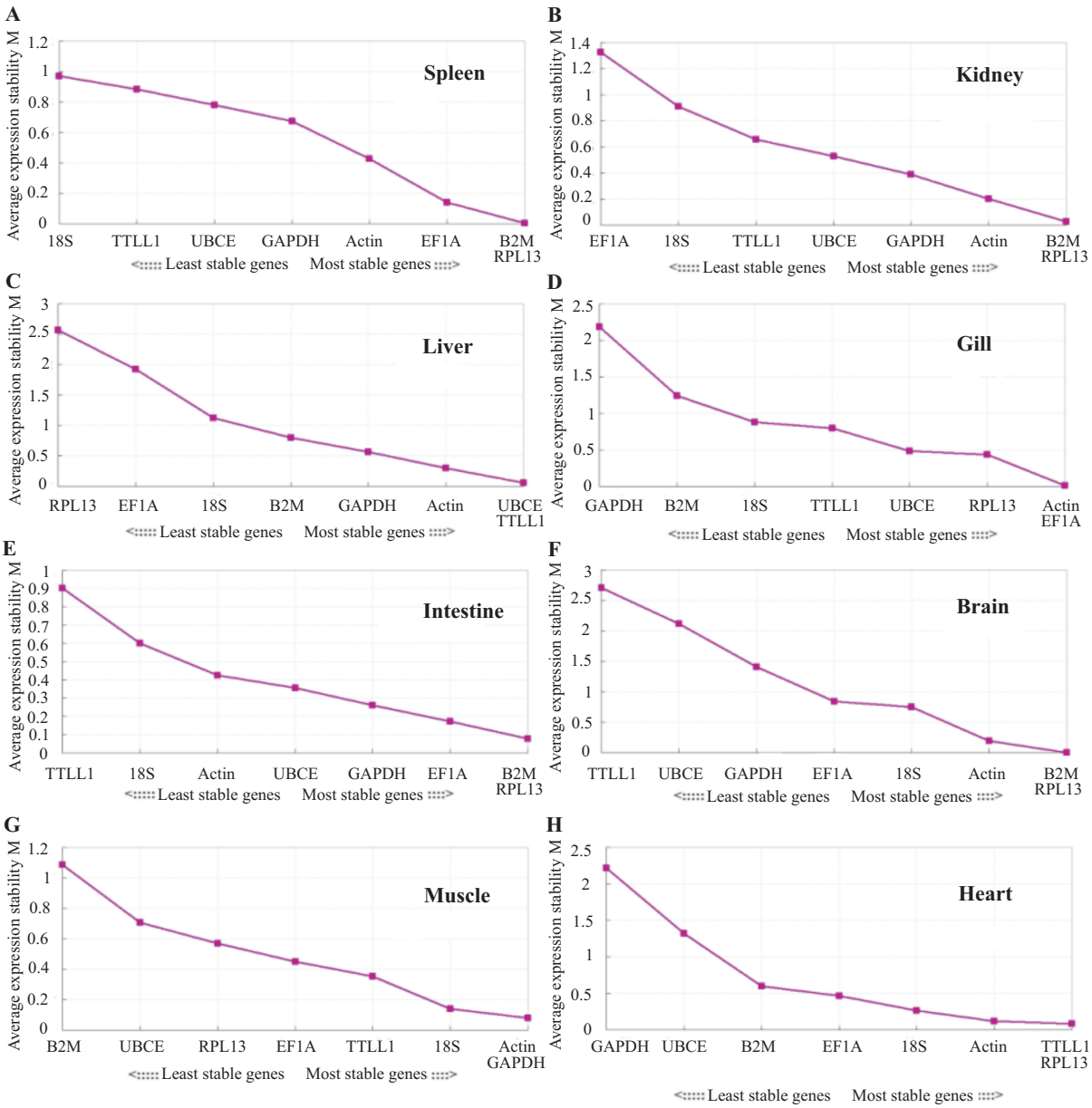

Fig. 3. Expression stability of the housekeeping genes in golden pompano tissues 48 h after poly I:C stimulation obtained with geNorm. The expression of the housekeeping genes in Trachinotus ovatus tissues 48 h after PBS or poly I:C stimulation was determined by qRT-PCR and using geNorm to calculate the expression stability (M) of each of the genes. A lower M value indicates more stable expression.

Analysis By NormFinder

12 h after stimulation, according to the expressions in spleen, kidney, gil, intestine, muscle, heart, NormFinder produced the ranking orders (Table 6) and the results were similar as obtained by geNorm, with consensus identifications of the most stable genes. For expressions in liver and brain, geNorm identified Actin/UBCE and UBCE/B2M, respectively, as the best pairs of reference genes, while NormFinder differed from geNorm by ranking B2M and 18S/TTLL1, respectively, as the best reference genes. However, NormFinder recognized B2M and 18S/TTLL1 as the second best reference genes in liver and brain, respectively. 48 h after poly I:C stimulation, for the expressions in kidney, gill, brain, heart, and intestine, the ranking orders produced by NormFinder (Table 4) was similar to those produced by geNorm, with consensus identifications of the most stable genes. The most stable genes ranked by NormFinder (Table 5) were identical to those ranked by geNorm. For expressions in spleen, liver, and muscle, geNorm identified RPL13/B2M, UBCE/TTLL1 and GAPDH/Actin, respectively, as the best pairs of reference genes, while NormFinder differed from geNorm by ranking GAPDH, B2M and EF1A/TTLL1,

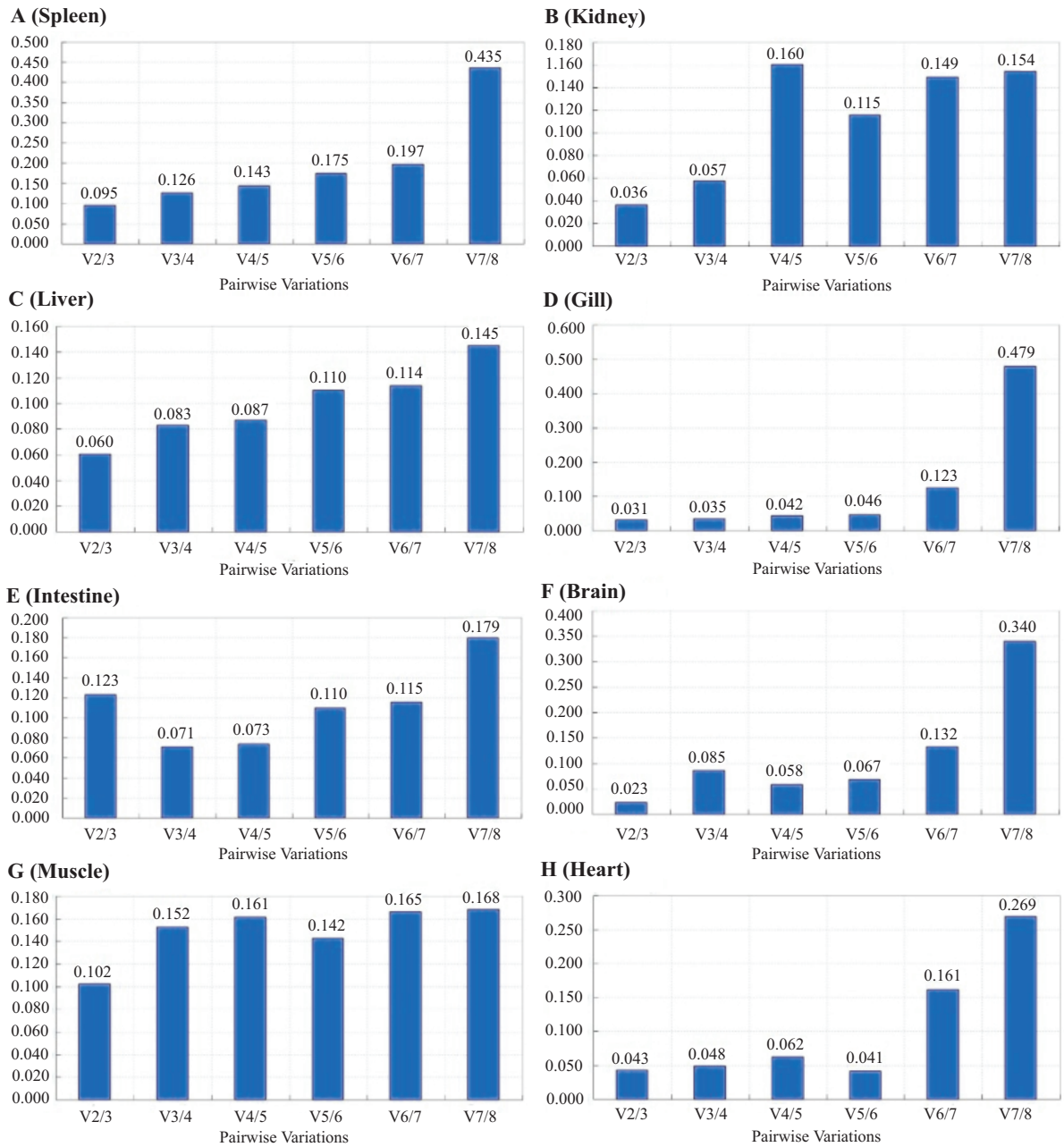

Fig. 4. The number of reference genes required for accurate normalization of gene expression 12 h after stimulation with poly I:C as determined by geNorm.

respectively, as the best reference genes. However, NormFinder recognized GAPDH, B2M and EF1A/TTLL1 as the second best reference genes in spleen, liver, and muscle, respectively.

Discussion

At present, a lot of related statistical software have been developed to select the most optimal candidate genes for qRT-PCR, such as geNorm, NormFinder, bestKeeper (Vandesompele et al. 2002, Andersen et al. 2004, Pfaffl et al. 2004), and so on. In our study, we selected eight housekeeping genes, including beta-actin (Actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (18S), β-2-Microglobulin (B2M), elongation factor-1-α (EF1A), ubiquitin-conjugating enzyme E2 (UBCE), tubulin tyrosine ligase-like family member 1 (TTLL1) and ribosomal protein L13 (RPL13), which are commonly used in many other species as internal reference genes for qRT-PCR. Analysis by geNorm and NormFinder aim to find the most stable reference genes in golden pompano. geNorm selected multiple

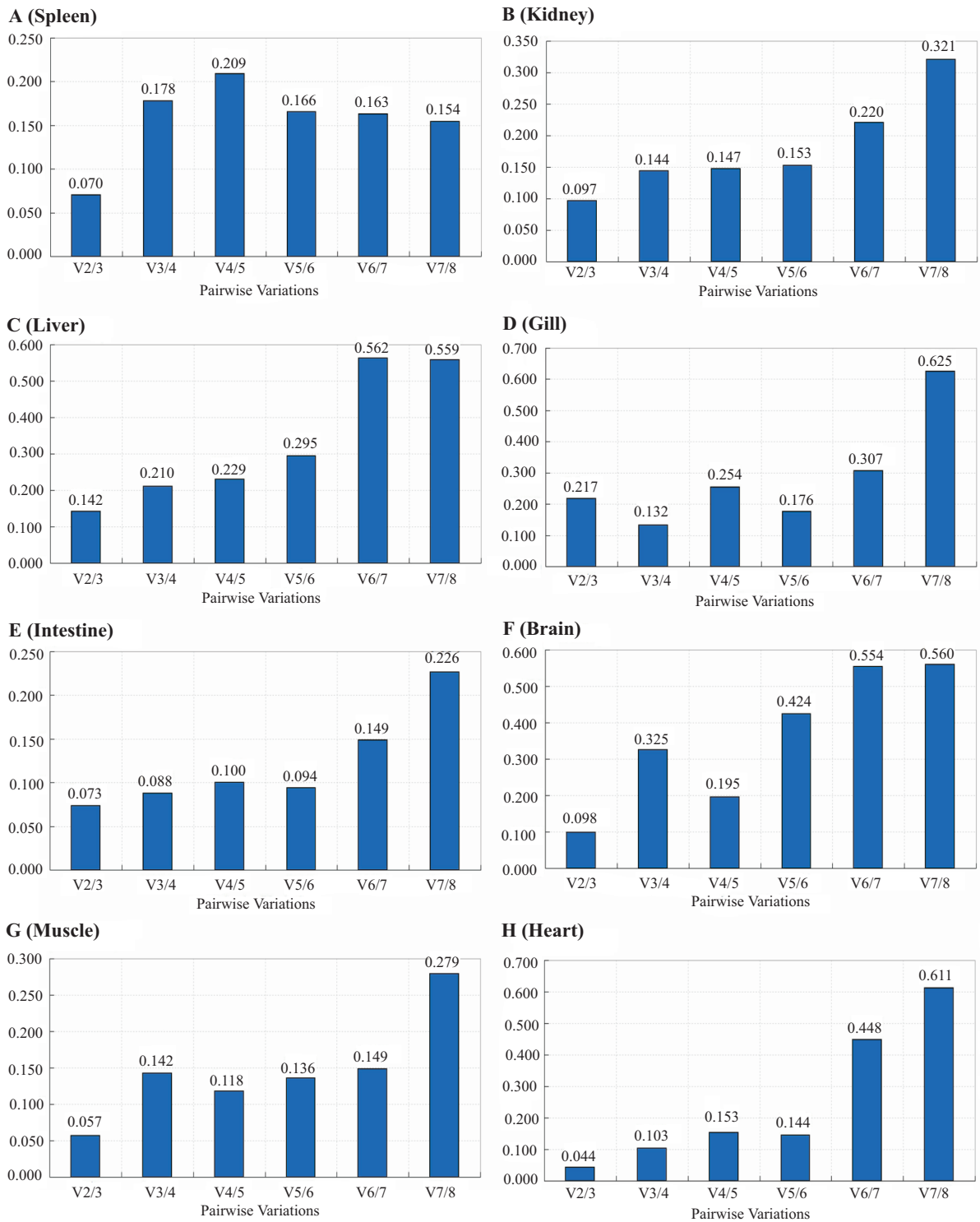

Fig. S1. The number of reference genes required for accurate normalization of gene expression 48 h stimulated with poly I:C as determined by geNorm.

internal controls to generate a normalization factor utilized in qRT-PCR analysis, while NormFinder identifies the optimal reference gene according to expression stability of the candidate genes (Andersen et al. 2004).

In this study, we found that both in the absence and presence of poly I:C stimulation, all the eight selected genes exhibited tissue-specific expression. This is similar to the housekeeping genes, i.e., ACTB, 18S, GAPDH, UBCE and B2M, which display tissue-specific expression in zebrafish (*Danio rerio*) and red drum (*Sciaenops ocellatus*) (McCurley and Callard 2008, Sun and Hu 2015). In our study, geNorm analysis showed that under normal condition, 18S

Table 4. Ranking by NormFinder of the candidate reference genes for analysis of gene expression at 12 h poly I:C stimulation. The numbers in brackets indicate stability values.

Ranking order	Spleen	Kidney	Liver	Gill	Intestine	Brain	Muscle	Heart
1	Actin (0.024)	B2M (0.062)	B2M (0.076)	Actin (0.004)	RPL13 (0.003)	TTLL1 (0.015)	B2M (0.051)	UBCE (0.002)
2	B2M (0.024)	TTLL1 (0.129)	UBCE (0.118)	UBCE (0.004)	B2M (0.003)	18S (0.015)	Actin (0.051)	PRL13 (0.002)
3	18S (0.130)	UBCE (0.163)	RPL13 (0.167)	PRL13 (0.180)	18S (0.058)	EFIA (0.231)	EFIA (0.267)	B2M (0.007)
4	EF1A (0.130)	RPL13 (0.266)	Actin (0.233)	18S (0.244)	Actin (0.285)	UBCE (0.284)	PRL13 (0.281)	18S (0.007)
5	TTLL1 (0.310)	GAPDH (0.370)	GAPDH (0.352)	EF1A (0.298)	UBCE (0.312)	B2M (0.313)	GAPDH (0.295)	EF1A (0.043)
6	RPL13 (0.722)	18S (0.416)	TTLL1 (0.352)	TTLL1 (0.426)	EF1A (0.344)	GAPDH (0.374)	UBCE (0.497)	GAPDH (0.043)
7	UBCE (1.137)	EF1A (0.817)	EF1A (0.548)	B2M (0.537)	TTLL1 (0.667)	PRL13 (0.574)	18S (0.659)	TTLL1 (0.946)
8	GAPDH (2.406)	Actin (0.841)	18S (0.793)	GAPDH (2.652)	GAPDH (0.927)	Actin (1.850)	TTLL1 (0.927)	Actin (1.432)

Table 5. Ranking by NormFinder of the candidate reference genes for analysis of gene expression at 48 h poly I:C stimulation. The numbers in brackets indicate stability values.

Ranking order	Spleen	Kidney	Liver	Gill	Intestine	Brain	Muscle	Heart
1	GAPDH (0.168)	B2M (0.096)	B2M (0.179)	Actin (0.006)	B2M (0.027)	B2M (0.007)	EF1A (0.057)	TTLL1 (0.030)
2	Actin (0.178)	Actin (0.104)	18S (0.295)	EF1A (0.006)	RPL13 (0.027)	RPL13 (0.007)	TTLL1 (0.057)	RPL13 (0.030)
3	UBCE (0.339)	GAPDH (0.105)	GAPDH (0.510)	TTLL1 (0.014)	GAPDH (0.083)	EF1A (0.007)	RPL13 (0.119)	Actin (0.031)
4	EF1A (0.537)	RPL13 (0.164)	UBCE (1.191)	18S (0.014)	EF1A (0.246)	18S (0.007)	UBCE (0.408)	B2M (0.087)
5	TTLL1 (0.619)	UBCE (0.281)	TTLL1 (1.242)	RPL13 (0.617)	18S (0.483)	Actin (0.104)	18S (0.500)	EF1A (0.087)
6	B2M (0.715)	TTLL1 (0.610)	Actin (1.590)	UBCE (0.715)	UBCE (0.497)	UBCE (2.365)	GAPDH (0.616)	18S (0.112)
7	RPL13 (0.718)	18S (1.274)	EF1A (2.163)	B2M (1.890)	Actin (0.637)	TTLL1 (3.365)	Actin (0.686)	UBCE (2.571)
8	18S (0.832)	EF1A (1.767)	RPL13 (3.064)	GAPDH (3.458)	TTLL1 (1.247)	GAPDH (4.743)	B2M (1.541)	GAPDH (3.374)

and B2M displayed the lowest M values, hence are the most stable genes. The results identified by NormFinder were in line with the prediction obtained with geNorm, thus, B2M and 18S were exhibited as the most stably housekeeping genes under normal physiological condition.

As reported in many other studies, under different experimental conditions, different tissues and different growth stage, the most stably housekeeping gene is different (Filby and Tyler 2007, Fernandes et al. 2008, Infante et al. 2008, Zhong et al. 2008, Bower and Johnston 2009, Li et al. 2010, Rverg4rd et al. 2010, Dang and Sun 2011, Lrvoll et al. 2011, Zheng and Sun 2011). For example, in red drum under conditions of bacterial infection, RPS35, ACTB, EF1A, ND1, TUBB, EF1A, ACTB and ACTB were analyzed as the ideal internal references in brain, gill, heart, intestine, kidney, liver, muscle and spleen, respectively (Sun and Hu 2015). In rock bream, similarly for most tissues, the optimum genes are different under conditions of bacterial and viral infections (Zhang et al. 2014). In our study, 12h after poly I:C stimulation,

geNorm identified Actin/B2M, B2M/UBCE, Actin/ /UBCE, Actin/UBCE, B2M/RPL13, B2M/UBCE, B2M/TTLL1 and UBCE/GAPDH as the most stable genes in spleen, kidney, liver, gill, intestine, brain, muscle and heart, respectively; while 48h after poly I:C stimulation, geNorm identified B2M/RPL13, B2M/RPL13, UBCE/TTLL1, Actin/EF1A, B2M/ /RPL13, B2M/RPL13, Actin/GAPDH and TTLL1/ RPL13, as the most stable genes in spleen, kidney, liver, gill, intestine, brain, muscle and heart, respectively. Thus, the most stable genes in spleen, kidney, liver, gill, intestine and brain were B2M, B2M, UBCE, Actin, B2M/RPL13 and B2M, respectively. In line with the results obtained with NormFinder, the most stable genes were the same as the results obtained with geNorm.

Currently, a number of methods to identify the most suitable reference genes for real time PCR study are available. To improve the accuracy of evaluation, using two or more of these methods is an effective way. So, in this study, we used geNorm and NormFinder to identify the optimal reference gene of golden pompano under normal physiological condition and after poly I:C stimulation. When under normal physiological condition, B2M and 18S may be the suitable genes. When studying gene expression under conditions of poly I:C stimulation, the selection of the internal controls should be chosen on a tissue basis. However, if one gene is preferred, B2M, B2M, UBCE, Actin, B2M/RPL13, B2M, B2M and RPL13 may be used in spleen, kidney, liver, gill, intestine, brain, muscle, and heart of golden pompano, respectively.

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