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Quality Evaluation of Reference Gene Expression on Different Tissues in Adults of Tropical Gar *Atractosteus tropicus*

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LDJM and CAFQ presented the concept. CAA-G, BCF, LDJM and CAFQ suggested the methodology. LDJM, GGAA, CSAV and ACCC curated data. ACCC, GGAA, VMC, LDJM, ESPM, CAA-G, BCF, CSAV and CAFQ wrote the manuscript. CAFQ, CAA-G and BCF supervised the study. CAFQ, ACCC, GGAA, ESPM, BCF, CSAV and CAA-G did formal analysis. CAA-G acquired funds for the study.

Key words
***Atractosteus tropicus*, Reference gene, Gene expression, Stability, Relative expression**

ABSTRACT

Tropical gar (*Atractosteus tropicus*) is an ancestral subtropical fish species in southeastern Mexico, which has great potential as a model species for physiological, biomedical and genomic studies. The quantification of gene expression through RT-qPCR is one of the most commonly used techniques, due to its precision, sensitivity and high performance, particularly in gene expression to compare between cells, tissues and organs; as well as different populations, stages of development, metabolism, among other conditions. This study analyzed the stability and normalization of six commonly used reference genes such as alpha elongation factor (*ef1-α*), beta-actin (*actb*), 18S ribosomal RNA (*18s rrna*), beta-2-microglobulin (*b2m*), tubulin alpha (*α-tub*) and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) in the different tissues of the intestine, muscle, gill, stomach, brain and liver in adult males of *A. tropicus* from reared in captivity through three BestKeeper, geNorm and NormFinder algorithms. Based on our results we can conclude that in the three BestKeeper, NormFinder and geNorm algorithms, the most stable genes are *ef1*, followed by *18s rrna* and *actb* where the gene stability will depend on specific tissue to analyze in tropical gar *A. tropicus* adults.

INTRODUCTION

Tropical gar (*Atractosteus tropicus*) is a tropical fish, characterized by being a very important carnivorous species in southeastern Mexico (Márquez-Couturier *et al*., 2006). In the last 30 years, several studies have been carried out with the species, from the aspects of reproduction, larviculture, digestive physiology, feeding and nutrition. This has allowed advances in *A. tropicus* aquaculture techniques. Nutrition and energy requirements studies let maximizing growth in less time by improving small-scale culture to seek profitability (Lee, 2002).

In this sense, *A. tropicus* is considered an ancestral species since it has kept its physiognomy intact by not suffering genetic duplication that separated them from the diversification of the Teleostean lineage 320-350 million years ago (Braasch *et al*., 2016). That is why the interest of carrying out molecular biology works to understand and expand the knowledge of the different physiological and metabolic processes of the species.

The quantification of gene expression through RT-qPCR is one of the most commonly used techniques, due to its precision, sensitivity and high performance. There are two methods: absolute and relative quantification

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(Schmittgen and Livak, 2008). The absolute quantification is determined by defining the exact number of copies by extrapolating the value from a standard or calibration curve while the relative quantification is not necessary a calibration curve, changes in the expression of a gene in response to a treatment are analyzed, comparing and relativizing another untreated control and normalizing with an endogenous control or reference gene whose expression does not change in response to any kind of treatment or condition (Wan *et al*., 2010). A reference gene must be reliable to conduct a good relative expression study (Dundas and Ling, 2012). Ideal reference genes must be stably expressed in various cells, tissues and organs; different populations; different stages of development; different stages of the cell cycle; and different treatment circumstances (Li *et al*., 2019). Currently, for the analysis of the stability of the reference gene there are different algorithms such as BestKeeper (Pfaffl *et al*., 2004), geNorm (Vandesompele *et al*., 2002) and NormFinder (Andersen *et al*., 2004). These are tools using different algorithms where BestKeeper uses peer correlation analysis of each internal gene with an optimal normalization factor, NormFinder adjusts the data to a mathematical model, which allows to compare intra and intergroup variation and the calculation of expression stability and finally genNorm uses comparisons between pairs and the geometric average of the Cq values to establish the most stable genes (Wang *et al*., 2012).

Currently, the alpha elongation factor (*ef1-α*), beta-actin (*actb*), 18S ribosomal RNA (*18s rrna*), beta 2 microglobulin (*b2m*), tubulin alpha (*α-tub*) and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) genes are considered the most stable and used as reference genes. The *ef1-α* gene encodes a critical protein that acts in the formation of the cytoskeleton of cells (Ingerslev *et al*., 2006); actb gene encodes an essential protein of the cytoskeleton that is an essential component of cells to maintain the necessary activities of life and the performance of an important role in cell secretion, phagocytosis, migration, cytoplasmic transmission and cytoplasmic segregation and recombination (Guo *et al*., 2013). The *18s rrna* gene is an essential component of eukaryotic cells and part of RNA ribosomes (Wang *et al*., 2018). The *b2m* gene encodes a protein found on the surface of virtually all body cells and is released by cells into the blood, especially B cells (Winchester *et al*., 2003; McCurley and Callard, 2008). The *α-tub* gene encodes a type of tubulin that exists as a dimer in the cell with tubulin and is involved in important physiological functions such as cell division and differentiation, substance transport, and signal transduction (Fortes *et al*., 2016). Finally, the *gapdh* gene encodes a protein that acts in glycolysis for energy creation (Nicholls *et al*., 2012). These reference genes have been studied in marine fish such as *Paralichthys olivaceus* (Zheng and Sun, 2011), *Danio rerio* (Casadei *et al*., 2011; McCurley and Callard, 2008), *Gasterosteus aculeatus* (Hibbeler *et al*., 2008), *Solea senegalensis*, and *Hippoglossus hippoglossus* (Infante *et al*., 2008; Øvergård *et al*., 2010), *Salmo salar* (Kortner *et al*., 2011), *Gadus morhua* (Olsvik *et al*., 2008) and in freshwater fish such as *Siniperca chuatsi* (Zhou *et al*., 2010) *Cyprinus carpio* (Tang *et al*., 2012) *Oreochromis niloticus* (Yang *et al*., 2013) *Ctenopharyngodon idella* (Su *et al*., 2011) and *Oncorhynchus mykiss* (Salem *et al*., 2015). The objective of the study was to determine the best reference genes in the expression in different tissues in *A. tropicus*, knowing the most stable gene is of vital importance for future relative expression studies in the areas of physiology, biomedicine and genomics in this species.

MATERIALS AND METHODS

Fish

A total of 20 male adults *A. tropicus* were obtained. Individuals (550-580 g and from 30 to 35 cm total length) from captivity at the facilities of the Tropical Aquaculture Laboratory of the DACBiol-UJAT. The organisms were kept in polyethylene tanks measuring 1.94 m in diameter and 0.70 m in height. The diet provided was based on balanced feed for trout and contained 46% protein and 16% lipids with pellet sizes ranging from 5.5 to 9.0 mm (El Pedregal® Silver Cup, Toluca, Mexico).

Sampling, total RNA extraction and cDNA synthesis

Subsequently, individuals of *A. tropicus* were euthanized by thermal shock (-4 ºC) according to the methodology of Matthews and Varga (2012) and dissected to obtain six tissues: intestine, muscle, gill, stomach, brain and liver. Extraction of RNA was performed from tissues pooled per replicate using the Trizol Reagent (Invitrogen, Carlsbad, USA) obtaining an integrity value of 9.7. cDNA was synthesized using one microgram of RNA and random primer with an iScriptTM Select 170-8896 cDNA synthesis kit (Bio-Rad, Hercules, California, USA) following the manufacturer's instructions.

Quantitative polymerase chain reaction (qPCR)

Resulting cDNA from adult tissues were diluted in 200 µL-distilled water. The qPCR reactions were performed in a 96-well CFX96 Real-Time System Thermal Cycle (Model C1000, California, USA) thermocycler. The reaction mixture included 10 µl of Eva Green master mix, 2-µl cDNA and 0.2 µM of each primer. The specific primers used in this analysis are given alfa elongation factor (*ef1-α*), beta-actina (*actb*)

(from sequences of *Atractosteus tropicus*), 18S ribosomal RNA (*18s rrna*) (from sequences of *Lepisosteus osseus*), beta-2-microglobulin (*b2m*), Tubulin alpha (*α-tub*) and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) (from sequences of *Lepisosteus oculatus*) (Table I). The thermal program included 2 min at 95 °C, followed by 38 cycles at 95 °C for 10 s, 60 °C for 30 s and extension at 70° C for 5 s. All reactions were performed in duplicate. A standard curve for each pair of primers was generated to estimate amplification efficiencies based on known amounts of cDNA (four serial dilutions corresponding to cDNA transcribed from 100 to 0.1 ng of total RNA). In the melting curve analysis, it was determined that the melting temperature peak varied between 81.5 and 83 °C, which corresponded to the product obtained by these primers. In addition, the absence of primer dimers and nonspecificities was confirmed.

Stability analysis of candidate reference genes

The original cycle thresholds (cq values) of the six candidate reference genes were obtained from Bio-Rad CFX-96 Manager, and the data was sorted by Excel to assess differences in the expression levels of six candidate reference genes. Subsequently, the stability analysis of the candidate reference genes was carried out using three software packages, including BestKeeper v1 (https://www.gene-quantification.de/bestkeeper.html#download), geNorm integrated in qBasePlus (https://www.gene-quantification.de/hkg.html#genorm) and NormFinder v20 (https://www.moma.dk/normfinder-software/). The results of the three software packages were compared and analyzed to determine which gene is the most appropriate reference gene according to the methodology of Li *et al*. (2019).

RESULTS

Analysis of specificity and reliability of qPCR primers

The primers for qPCR of the six candidate reference genes elongation factor alpha (*ef1-α*), beta-actin (*actb*), 18S ribosomal RNA (*18s rrna*), beta-2-microglobulin (*b2m*), Tubulin alpha (*α-tub*) and glyceraldehyde 3- phosphate dehydrogenase (*gapdh*) were subjected to PCR analysis specific ordinary before qPCR. The results showed that the six reference genes produced a single band (Fig. 1). The melting curves of the six candidate reference genes are all single peaks, indicating that the primers' specificity is right, and no primer-dimers are present (Fig. 2). Besides, standard curves were performed to calculate amplification efficiency (E) and correlation coefficients (R^2) according to the Cq values of each candidate reference gene amplified by qPCR using the *A. tropicus* brain cDNA (Fig. 3).

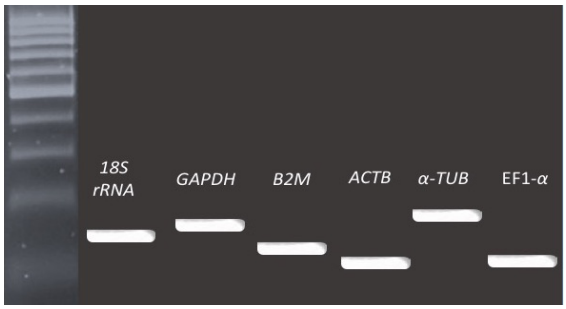

Fig. 1. Agarose gel with PCR products of six candidate reference genes of *Atractosteus tropicus*. six candidate reference genes of *Atractosteus tropicus* for qPCR. (A) alfa elongation factor (*ef1-α*), (B) beta-actin (*actb*), (C) 18S ribosomal RNA (*18s rrna*), (D) beta-2-microglobulin (*b2m*), (E) Tubulin alpha (*α- tub*) and (F) Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*).

Table I. Primer sequences and amplification parameters of six candidate reference genes of *Atractosteus tropicus* used in qPCR analysis.

Gene	Primer sequences (5´–3)	Product size (bp)	Amplification efficiency (%)	R^2	GenBank accession numbers
18s rrna	F: GGTAACGGGGAATCAGGGTT R: TCCAATTACAGGGCCTCGAA	156	100.18	0.9974	AF188369.1
gapdh	F: GGAATCAACGGATTTGGCCG R: TCACCTCCCCATGAAAACGG	163	97.40	0.9972	XM_006642348.2
b2m	F: TTTACCTGGACTGGGGGCTA R: GCGAGGCGCCATAAATCAAC	139	94.23	0.9924	XM_015346206.1
actb	F: GAGCTATGAGCTGCCTGATGG R: GTGGTCTCATGAATGCCACAGG	119	97.10	0.9956	KT351351.1
α-tub	F: TCAGCCTCTTTTTGTCAGGCT R: GCATGTGATGAGCAAAGACCA	181	94.35	0.9977	XM_015359451.1
ef1-α	F: CCTGCAGGACGTCTACAAGATCG R: GACCTCAGTGGTCACGTTGGA	120	99.82	0.9891	KT351350.1

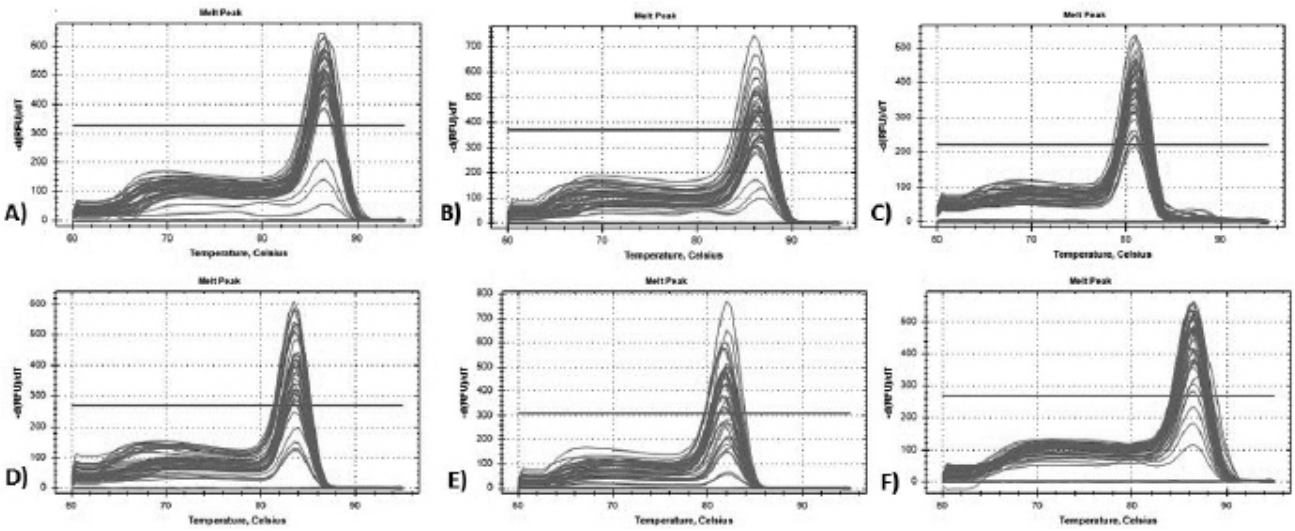

Fig. 2. Melting curve analyses of six candidate reference genes of *Atractosteus tropicus* for qPCR. (A) alfa elongation factor (*ef1-α*), (B) beta-actin (*actb*), (C) 18S ribosomal RNA (*18s rrna*), (D) beta-2-microglobulin (*b2m*), (E) Tubulin alpha (*α- tub*) and (F) Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*).

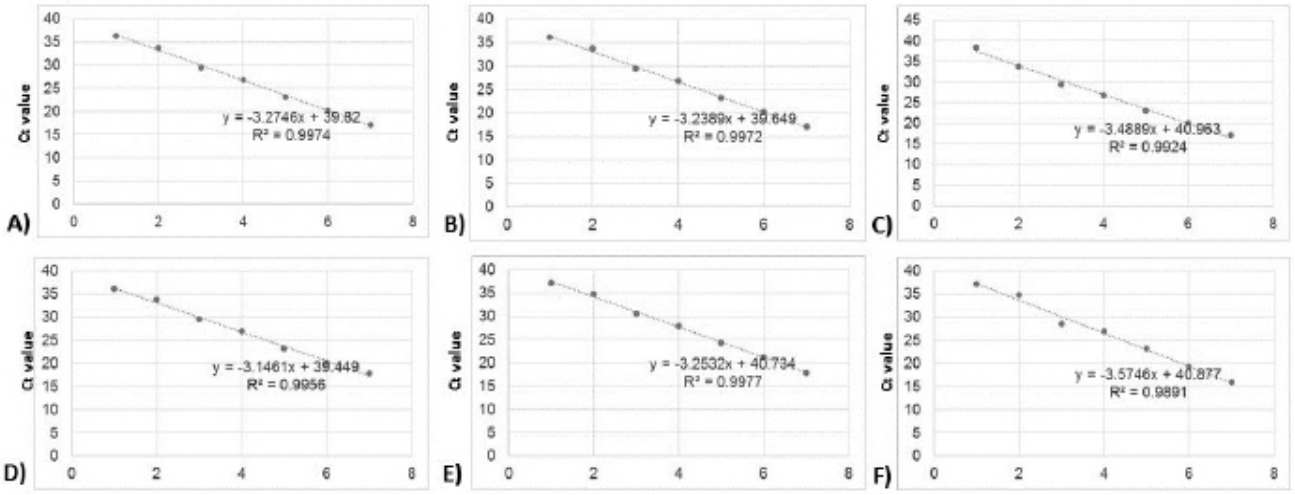

Fig. 3. Standard curves for the determination of efficiency (E) and correlation coefficients (R2) of six candidate reference genes of *Atractosteus tropicus* for qPCR. (A) alfa elongation factor (*ef1- α*), (B) beta-actin (*actb*), (C) 18S ribosomal RNA (*18s rrna*), (D) beta-2-microglobulin (*b2m*), (E) Tubulin alpha (*α- tub*) and (F) Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*).

BestKeeper gene analysis

The stability analysis shows BestKeeper SD and CV values of the six genes of references in the different tissues analyzed (Table II). Likewise, the results obtained in this study are shown in order of the most stable at least stable *ef1-α*, *actb*, *18s rrna*, *b2m*, *α-tub* and *gapdh* in the intestine, *ef1-α*, *actb*, *α-tub*, *b2m*, *18s rrna* and *gapdh* in the case of muscle, *actb*, *ef1-α*, *18s rrna*, *gapdh*, *b2m* and *α-tub* in gill, *18s rrna*, *ef1-α*, *b2m*, *actb*, *gapdh* and *α-tub* in stomach, *ef1*, *18s rrna*, *actb*, *b2m*, *α-tub* and *gapdh* in brain and finally *ef1-α*, *b2m*, *gapdh*, *actb*, *18s rrna* and *α-tub* liver.

Normfinder gene analysis

According to the results of the NormFinder analysis, they show both the stability value and the standard error of each candidate gene in the different tissues analyzed in adults of *A. tropicus* (Table III). Likewise, the results obtained in this study show that the most stable genes are *18s rrna*, *b2m* and *actb* in the case of intestine, *ef1-α*, *b2m* and

Table II. BestKeeper analysis results of six candidate reference genes of *Atractosteus tropicus*.

	Intestine			Muscle			Gill			Stomach			Brain			Liver		
Gen name	**SD (±cq)**	**CV (%cq)**	**r**	**SD (±cq)**	**CV (%cq)**	**r**	**SD (±cq)**	**CV (%cq)**	**r**	**SD (±cq)**	**CV (%cq)**	**r**	**SD (±cq)**	**CV (%cq)**	**r**	**SD (±cq)**	**CV (%cq)**	**r**
18s rrna	0.12	0.53	0.99	1.25	4.82	0.84	0.40	1.71	0.54	0.03	0.13	0.96	0.14	0.57	0.93	0.31	1.56	1.00
gapdh	3.66	14.78	1.00	1.00	3.60	0.47	0.43	1.91	0.09	0.47	1.74	0.79	0.35	1.70	0.77	0.23	0.21	0.77
b2m	0.38	1.34	0.83	0.35	1.02	0.77	0.48	2.01	1.00	0.22	0.99	0.44	0.25	0.93	0,81	0.21	0.93	0.68
actb	0.12	0.36	0.98	0.10	0.31	0.81	0.01	0.26	0.82	0.41	1.47	1.00	0.20	0.86	0.74	0.29	1.12	1.00
α- tub	0.54	1.99	0.81	0.12	0.44	1.00	0.61	2.45	0.72	0.78	3.24	0.09	0.29	1.46	0.90	0.42	1.64	0.99
ef1-α	0.10	0.49	0.53	0.01	0.002	0.62	0.08	0.27	0.51	0.10	0.55	1.00	0.13	0.49	1.00	0.12	0.66	0.78

SD, Standard; CV, coefficient of variance, r, coefficient of correlation.

Table III. NormFinder analysis results of six candidate reference genes of *Atractosteus tropicus*.

Gen name	Intestine		Muscle		Gill		Stomach		Brain		Liver	
	Stability value	**Standard error**	**Stability value**	**Standard error**	**Stability value**	**Standard error**	**Stability value**	**Standard error**	**Stability value**	**Standard error**	**Stability value**	**Standard error**
18s rrna	0.004	0.0055	0.067	0.0060	0.706	0.0052	0.085	0.0042	0.689	0.0145	0.233	0.0086
gapdh	0.971	0.0086	1.545	0.0086	0.117	0.0040	0.613	0.0048	0.048	0.0067	0.011	0.0045
b2m	0.004	0.0065	0.014	0.0058	0.117	0.0042	0.504	0.0088	0.290	0.0157	0.014	0.0048
actb	0.152	0.0089	2.190	0.0080	0.753	0.0068	0.085	0.0043	0.336	0.0092	0.026	0.0080
α- tub	0.164	0.0293	0.163	0.0094	0.929	0.0059	1.421	0.0065	0.050	0.0086	0.051	0.0172
ef1-α	1.368	0.0178	0.014	0.0056	0.706	0.0172	0.085	0.0043	0.046	0.0055	0.266	.00290

18s rrna in muscle, *gapdh*, *b2m*, *18s rrna* in gill, *18s rrna*, *actb* and *ef1-α* in stomach ef1-α, *gapdh* and *α-tub* in brain and finally *gapdh*, *b2m* and *actb* in liver.

geNorm gene analysis

The results of the geNorm analysis of each candidate reference gene in the different tissues analyzed in adults of *A. tropicus* show that the M values of the six candidate reference genes are all less than 1.5, indicating that the seven genes are suitable genes of reference (Fig. 4). The stability of the expression of the seven candidate reference genes in both is classified in descending order according to the principle that the lower the M value, the better the stability of the gene expression: *18s rrna*, *α-tub*, *actb*, *b2m*, *ef1-α*, and gapdh in intestine, *ef1-α*, *actb*, *18s rrna*, *b2m*, *gapdh*, *α-tub* in the case of muscle, *actb*, *ef1-α*, *18s rrna*, *gapdh*, *α-tub* and *b2m* in gill, *18s rrna*, *ef1-α*, *gapdh*, α-tub, *b2m* and *actb* in stomach, *ef1-α*, *gapdh*, *actb*, *b2m*, *18s rrna* and tuba in brain and finally *18s rrna*, *ef1-α*, *gapdh*, *actb*, *α-tub* and *b2m* in liver.

DISCUSSION

The qPCR is an effective method based on the polymerase chain reaction (PCR), which can amplify and quantify DNA molecules or specific complementary DNA (cDNA). This method allows us to access reliable data and precise information on the genetic expression of the cells under study, and frequently it is combined with a retro-transcription reaction (RT-qPCR). This method has become a more suitable choice for performing a rapid and quantitative examination for specific gene expression (Wang and Zhang, 2012). Nevertheless, selecting a suitable reference gene is the precondition for analysis of the relative expression of a target gene in quantitative real-time PCR (Wang *et al*., 2018).

Considering the above-mentioned, the most important characteristic of any reference gene candidate is the stability of its expression, regardless of in which tissues, developmental restrictions or physiological states are expressed (Yang *et al*., 2013). Likewise, Schaeck *et al*. (2016) suggest using at least two reference genes for normalization and that these genes are previously validated using a larger set of reference genes (n=10) to identify the most stable genes for each tissue/ cell type and each experimental condition. In this sense, algorithms such as BestKeeper, NormFinder and GenNorm (Wang *et al*., 2012) are used to evaluate the stability of these genes. Thanks to these algorithms, the stability of the reference gene candidate can be identified and evaluated for the

correct normalization being therefore, an essential tool for an adequate selection. However, due to the characteristics of each of these platforms, it is necessary to select what gathers the requirements of the research work (De Spiegelaere *et al*., 2015).

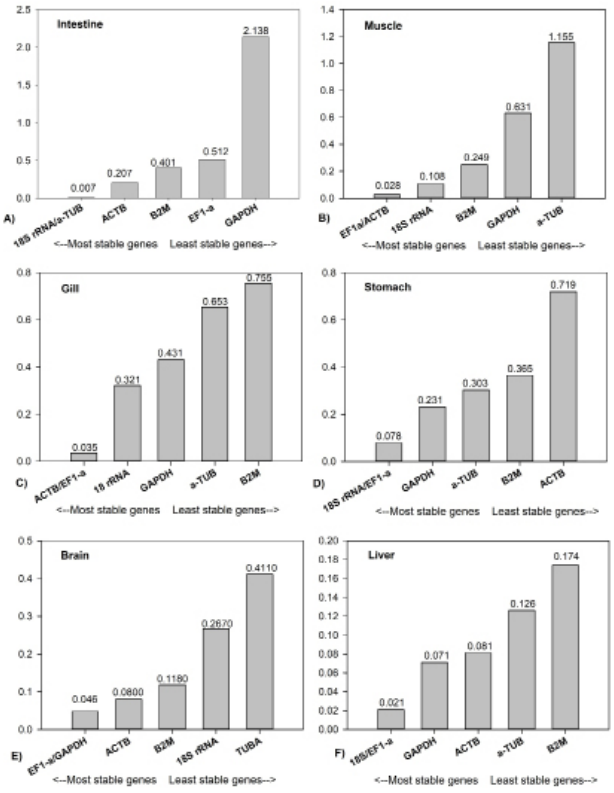

Fig. 4. geNorm analysis results of six candidate reference genes of *Atractosteus tropicus.*

Regarding our results, in the case of intestine and stomach the most stable gene was *18s rrna*, which correspond with the work done in species such as Nile tilapia (*Oreochromis niloticus*), carp (*Ctenopharyngodon Idella*), Asian sea bass (*Lateolabrax maculatus*) and American catfish (*Ictalurus punctatus*) (Yang *et al*., 2013; Su *et al*., 2011; Wang *et al*., 2018; Small *et al*., 2008), presenting good stability in other tissues such as heart, liver, muscle, spleen, skin and kidney without being affected by any treatment.

It is important to mention that *18s rrna* is considered an appropriate gene in embryogenesis in Zebra fish (*Danio rerio*), fathead minnow (*Pimephales promelas*), Channel catfish (*Ictalurus punctatus*) and Atlantic salmon (*Salmo salar*) (McCurley and Callard, 2008; Filby and Tyler, 2007; Small *et al*., 2008; Jorgensen *et al*., 2006; Kortner *et al*., 2011). Additionally, *18S rRNA* gene has been detected in ancestral species such as longnose gar (*Lepissoteus osseus*), green sturgeon (*Acipenser medirostris*) in muscle and in bowfin (*Amia calva*) in fin (Krieger and Fuerst, 2002). Studies on the expression of growth hormone in larvae of alligator gar (*Atractosteus spatula*) used the *18s rrna* gene to normalize the relative expression (Cahu *et al*., 2004; Revol *et al*. 2005; Panserat and Kaushik, 2010). It is important to highlight that for *A. tropicus* this gene can be used for nutrigenomic studies with tissues such as the intestine and stomach since it has good stability.

In the case of muscle, brain and liver, the most stable gene was *ef1-α* for *A. tropicus* adults. These results are consistent with those reported in species such as common carp (*Cyprinus carpio*), reporting greater stability in the liver, brain, hypothalamus, heart and kidneys (Tang *et al*., 2012), in *O. niloticus* (Yang *et al*., 2013) in muscle and heart. Similarly, *ef1-α* has been reported in common lancelet (*Branchiostoma lanceolatum*), Senegal sole (*Solea senegalensis*), Atlantic halibut (*Hippoglossus hippoglossus*), grass carp (*Ctenopharyngodon idella*), Korean rockfish (*Sebastes schlegeli*) (Carlos *et al*., 2008; Infante *et al*., 2008; Tang *et al*., 2012; Wang and Zhang, 2012; Liman *et al*., 2013), showing high stability in the different tissues analyzed in these species. On the other hand, *ef1-α* would be the ideal reference gene in studies involving the expression of genes involved in metabolism and neurofunctional (Diotel *et al*., 2010; Zheng *et al*., 2013), as well as in rainbow trout (*Oncorhynchus mykiss*) when analyzing the gene expression in liver and muscle tissues using different diets (Kolditz *et al*., 2008). Finally, for *A. tropicus*, EF1-α was an excellent reference gene for analysis of relative gene expression of lipogenic genes during the initial ontogeny for *A. tropicus* (Jiménez-Martínez *et al*., 2019) and in juveniles feed with diets supplemented with different concentrations of β-glucans (Nieves-Rodríguez *et al*., 2018).

In gills, *actb* gene was highly stable in adults of *A. tropicus*, which are agreed with species such as Turbot (*Scophthalmus maximus*) (Dang and Sun, 2011) and bastard halibut (*Paralichthys olivaceus*) (Zheng and Sun, 2011), been the best reference gene not only for gill, also for liver, spleen, kidney, heart muscle, brain and intestine. Likewise, *actb* is considering the best reference gene in different tissues in teleosts (Deloffre *et al*., 2012). It is important to point-out that gill tissue is the first line of defense against pathogens suspended in the aquatic environment, therefore, cells need to be fastly replicated (Hibbeler *et al*., 2008). Finaly, *ACTB* has been used as reference gene for toxicology, osmorrgulation and hypoxia studies (Wong *et al*., 2001; Lin *et al*., 2004; Bridle *et al*., 2006).

In the case of *ef1-α* and *b2m* they were the least stable

genes in our study, however in bastard halibut (*Paralichthys olivaceus*) stands out, in which it mentions that *α-tub* is the best reference gene for spleen, heart, muscle and gill tissues (Zheng and Sun, 2011). While *b2m* alone in the geNorm program was one of the most stable in branchia *A. tropicus* similar to those occurring in larval development and tissues in the zebrafish *Danio rerio* under treatments such as different chemicals (McCurley and Callard, 2008).

Based on BestKeeper, NormFinder, and geNorm analyses, the most stable genes for *A. tropicus* were *ef1-α*, followed by *18s rrna* and *actb*, so they are adequate as reference genes and can be used individually; however, it is advisable to use any of them to quantify the relative gene expression in *A. tropicus*.

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Statement of conflict of interest

The authors have declared no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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