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Evaluation of candidate reference genes for QPCR during ontogenesis and of immune-relevant tissues of European seabass (*Dicentrarchus labrax*)

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ABSTRACT

The expression level of mRNA can vary significantly in different experimental conditions, such as stress, infection, developmental stage or tissue. Suitable reference genes are expected to exhibit constant expression levels. However no single gene is constitutively expressed in all cell types and under all experimental conditions. It has become clear that expression stability of the intended reference gene has to be examined before each experiment. For expression studies using quantitative real-time PCR (qPCR) at least two reference genes have to be applied. So far expression studies in the European seabass (*Dicentrarchus labrax*) as well as in the Gilthead seabream (*Sparus aurata*) have been performed with only one reference gene (*S18*, *Ef-1 alpha* or *Gapdh*). Though significant variations showed up in other teleost species such as the Atlantic halibut and the zebrafish affirming the need for proper normalization strategies, the present study aims at identifying suitable reference genes among nine candidates [glyceraldehyde-phosphate-dehydrogenase (*Gapdh*), *β-actin* (two regions of *β-actin*), 40S ribosomal protein S30 (*Fau*), ribosomal protein L13 a (*L13a*), β2-tubulin (*Tubb2*) and tyrosine 3 monooxygenase/tryptophan 5-*monooxygenase* activation protein (*Tyr*)] for expression analysis of 8 developmental stages and a tissue panel (spleen, liver, kidney and brain) with samples infected with Nodavirus and *Vibrio anguillarum* in *D. labrax*. Besides the analysis of raw Ct-values, the gene expression stability was determined using two different software applications BestKeeper and NormFinder. According to both algorithms the best two reference genes for an appropriate normalization approach during *D. labrax* development are *Ef-1 alpha* and *L13a* whereas in the tissue panel *Fau* and *L13a* are recommended for qPCR normalization.

1. Introduction

The European seabass (*Dicentrarchus labrax*) was the first non-salmonid marine fish species to be commercially cultured in Europe. Today *D. labrax* is along with the gilthead seabream (*Sparus aurata*) one of the most extensively aquacultured fish species in the Mediterranean and as the economic pressure on producers is steadily increasing the improvement of aquaculture techniques is significant. The increase of molecular and physiological knowledge will support the understanding of various pathways and phenotypic traits such as growth, health and flesh quality. Gene expression studies are essentially contributing to identify transcripts involved in molecular pathways. The main tools for quantification of gene expression are microarrays and quantitative real-time (qPCR). In human disease diagnostic, particularly qPCR is used (Bustin et al., 2005). The straightforwardness of qPCR, the specificity and the sensitivity, as well as the potential for high throughput have made real-time RT-PCR the benchmark technology for the detection and/or comparison of RNA levels. However the precision of qPCR results depends on several features such as RNA integrity of starting material, enzyme and primer performance, reference genes and method for chosen data analysis (Pfaffl, 2001; Bustin, 2005; Huggett et al., 2005). The molecular basis of severe malformation in fish development in particular under aquaculture conditions [e.g. insulin-like growth factor-I and myostatin, (Patruno et al., 2008)], and of fish immune response [e.g. hepcidin (Bao et al., 2005; Cuesta et al., 2008), metalloproteinase 9 (Chadzinska et al., 2008)] have been approached in several fish species by qPCR experiments. The most common method used for detecting differential expressed genes is the relative quantification by qPCR based on the expression ratio of a target gene vs. a reference gene (e.g. Dheda et al., 2004; Radonic et al., 2004). As reference genes a number of housekeeping genes have been suggested, since they are known to be

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transcribed at a moderately constant level. It is also assumed that those genes are expressed independent of any experimental conditions and tissue types. However no universal reference gene exists although a large number of studies already proposed various reference genes (Bustin, 2005; Dheda et al., 2005). Consequently it is recommended to perform individual studies for each species and for each tissue to be studied in order to determine a most appropriate set of reference genes (Bustin, 2005). In marine organism housekeeping genes were evaluated for tissues and experimental treatments of several fish species like the three-spined stickleback (*Gasterosteus aculeatus)* (Hibbeler et al., 2008). Furthermore evaluation of reference genes during larval development is of importance as the interest of fish research in gene expression during ontogenesis is increasing. For different developmental stages recently candidate reference genes were identified for the Atlantic halibut (Fernandes et al., 2008) as well as for the Atlantic cod (Saele et al., 2009) both species of commercial interest in the North Atlantic.

The aim of the present study was to identify optimal reference genes that could be used for expression analysis for 8 different developmental stages covering significant developmental time points of seabass development and for four different tissues (brain, head kidney, liver and spleen) infected with *Vibrio anguillarum*, for four tissues infected with nodavirus and for four control tissues. The eight selected reference genes (Table 1) are 'classical' housekeeping genes used in *D. labrax* studies as well as in other qPCR studies (e.g. Sepulcre et al., 2007; Faliex et al., 2008; Poisa-Beiro et al., 2008). The selection consists of genes known to be relatively stable expressed in other organisms [elongation factor 1 alpha (*Ef1-α*) and ribosomal protein S18 (*S18*), (Schmittgen and Zakrajsek, 2000; Frost et al., 2003; Sepulcre et al., 2007)], genes frequently used as reference genes [glyceraldehyde-phosphate-dehydrogenase (*Gapdh*) and two regions of *β-actin* (Franch et al., 2006; Poisa-Beiro et al., 2008)]. In addition genes previously evaluated as reference genes in other fish species were selected [40S ribosomal protein S30 (*Fau*), ribosomal protein L13 a (*L13a*), β2-tubulin (*Tubb2*), tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation protein (TYR) (Tang et al., 2007; Fernandes et al., 2008)].

2. Materials and methods

2.1. Tissue sample collection

Embryonic and larval samples used for library construction were obtained from the aquaculture facility at the Department of Aquaculture at the Institute of Marine Biology and Genetics, Crete. Eight different stages of European seabass were collected: (1) morula, (2) 1/2 epibolic, (3) neurula, appearance of embryo stage (4) before hatching, (5) mouth opening, (6) first feeding, (7) flexion and (8) fins. The determination of the stages was done after Divanach (1985). For tissue sampling in total 20 different samples were used in the present study including two infections, one with Nodavirus strain 475-9/99 isolated from diseased seabass [from the Instituto Zooprofilattico Sperimental delle Venezie (Italy)] and one with *Vibrio anguillarum* strain R-82 (serogroup 01) [from the University of Santiago (Spain)] were performed with seabass juveniles (100 g mean mass). The 20 different tissue samples comprised six different samples of spleen (4 and 24 h post-infection with *V. anguillarum*, with Nodavirus and control samples), six different samples of liver (4 and 24 h post-infection with *V. anguillarum*, with Nodavirus and control samples), four different samples of kidney (4 and 24 h post-infection with Nodavirus and control samples) and four different samples of brain (4 and 24 h post-infection with Nodavirus and control samples). Nodavirus infections were performed via intramuscularly injection of 100 µL of Nodavirus suspension in Minimum Essential Medium (MEM) (5.9×10^6 $TCID_{50}$ mL^{-1}) and placed at 25 °C. Mock-infected control fish were injected with medium alone and maintained under the same experimental conditions. Three fish from each experimental and control groups were sampled 4 and 24 h post-infection. Animals were sacrificed by anesthetic (MS-222) overdoses and dissected. For *V. anguillarum* infection fish were injected intraperitoneally (i.p) with 1 ml of phosphate-buffered saline (PBS) alone or containing either 2×10^6 live or 10^8 formalin-killed *V. anguillarum* R82 cells. Half of the fish was moribund 24 h post-infection and all of them died by 48 h post-infection. All collected samples were frozen immediately in liquid nitrogen and stored at −80 °C prior to RNA extraction.

Table 1
Candidate genes evaluated for reference genes.

Abbr.	Gene name	Function
S18	Ribosomal protein S18	Structural component of the small 40S subunit
β-actin1/2	β-actin	Cytoskeletal structural protein
Ef-1	Elongation factor-1 alpha	Factor for protein translation
Fau	40S ribosomal protein	Structural component of the small 40S subunit
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme
L13a	Ribosomal protein L13a	Structural component of the large 60S subunit
Tubb2	β2 tubulin	Major constituent of microtubules
Tyr	Tyrosine 3-monooxygenase	Signal transduction

Table 2
Accession number, primers and Tm of reference genes used in the present study.

Accession no.	Gene	Sequence (5′–3′)		Tm used
AJ537421	*β-actin 1*	FOR	TCGGTCGCCCCAGGCATC	57 °C
		REV	GGTGATGACCTGTCCGTC	
AJ537421	*β-actin 2*	FOR	GTGCGTGACATCAAGGAGAA	57 °C
		REV	GCTGGAAGGTGGACAGAGAG	
FM004681	*Fau*	FOR	GACACCCAAGGTTGACAAGCAG	57 °C
		REV	GGCATTGAAGCACTTAGGAGTTG	
DT044539	*L13a*	FOR	TCTGGAGGACTGTCAGGGGCATGC	57 °C
		REV	AGACGCACAATCTTGAGAGCAG	
FM003484	*Tubb2*	FOR	GCCTCAGGTGGCAAATATGT	57 °C
		REV	CCTCAGTGTAGTGACCCTTG	
AM973424	*Tyr*	FOR	TCTGCCAGGACGTGCTGAACC	57 °C
		REV	TCCACCGTATCCTTCTTTGAGTC	
FM019753	*Ef-1α*	FOR	AGATGACCACGAGTCTCTGC	57 °C
		REV	CTTGGGTGGGTCGTTCTTG	
AM490061	*S18*	FOR	AGGGTGTCGGCAGACGTTAC	57 °C
		REV	TTTCTGCCTGTTGAGGAACC	
AY863148	*Gapdh*	FOR	GTGCCAGCCAGAACATCAT	57 °C
		REV	TGTCGTCATATTTGGCGGGTTTC	

2.2. RNA extraction and cDNA synthesis

Disruption of the samples was performed in liquid nitrogen using mortar and pestle and the lysate was homogenized via passing it through a 20 gauge (0.9 mm) needle attached to a sterile plastic syringe for 5 times. The following extraction procedure was executed using RNeasy® Plus Mini Kit by QIAGEN GmbH (Valencia, USA) according to manufacturer's instructions. RNA concentrations were determined by the $A_{260/280}$ ratio using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington USA) and the quality was assessed on a 1% ethidium bromide-agarose gel. Absorbance ratios between 1.90 and 2.15 indicated pure RNA samples. For cDNA synthesis 1 µg of total RNA from each sample was reverse

Table 3
Used amounts of primer pairs are indicated for each candidate reference gene.

Gene abbreviation	*Ef-1*	*β-actin 1*	*β-actin 2*	*S18*	*Tyr*	*Gapdh*	*L13a*	*Fau*	*Tubb2*
FOR [µl]	0.4	0.4	0.05	0.2	0.2	0.4	0.1	0.04	0.4
REV [µl]	0.4	0.4	0.05	0.2	0.2	0.4	0.1	0.04	0.4