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Validation of reference genes for quantitative expression analysis by qPCR in various tissues of date mussel (*Lithophaga lithophaga*)

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Abstract: qPCR is a very popular method for identifying nucleotide sequences as a result of its sensitivity and relatively low cost and technical simplicity. Normalization is one of the most important steps in analyzing qPCR data and the use of reference genes is the most common normalization strategy. In the present study five commonly used reference genes (*18S*, *28S*, *ef1a*, *β-act*, and *α-tub*) were evaluated for their stability in the mantle, gill, foot, and pallial gland of date mussel (*L. lithophaga*) in different seasons. Four different software packages were used for evaluation: geNorm, NormFinder, BestKeeper, and RefFinder. Although these programs contain different algorithms and analytical procedures, their ranking of the candidate reference genes was similar. Of the five selected reference genes *18S*, *28S*, and *ef1a* were determined as the three most stable in different tissues and seasons. *A-tub* was evaluated as the least stable reference gene and therefore inappropriate for normalization of quantitative gene expression data. The results will help improve the accuracy of gene expression analysis in samples of date mussel and at the same time provide guidance for selection of reference genes in future qPCR studies in the species.

Key words: Mantle, gill, foot, pallial gland, endogenous control, *Lithophaga lithophaga*

1. Introduction

It has been nearly a quarter of a century since Higuchi et al. (1993) described the technique of quantitative real-time PCR (qPCR). Since then the number of research studies using the technique has grown exponentially. Today, qPCR is used for gene expression analysis, genotyping with single-nucleotide polymorphism (SNP) detection, microRNA analysis, copy number variation (CNV) analysis, and even protein detection by real-time immuno-PCR. However, although the technology is straightforward, improper analysis and interpretation of qPCR results can lead to inaccurate conclusions.

One of the most important steps in analyzing qPCR data is the normalization step. Many different strategies for normalization of qPCR data are available (Huggett et al., 2005), among which the use of reference genes is the most popular. Vandesompele et al. (2002) have shown that for accurate normalization multiple reference genes are required. Reliable reference genes are selected through a process of validation, where candidate genes are evaluated according to the stability of their expression. Various algorithms including geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004) and RefFinder (Xie et al., 2012) are now available to undertake such evaluation.

In the past few years, the number of studies validating reference genes in bivalves has increased. In mussels (*Mytilus* spp.) reference genes have been validated in various tissues (Lacroix et al., 2014), during different stages of gametogenesis (Cubero-Leon et al., 2012), and in hemocytes following bacterial infection (Moreira et al., 2014). Evaluation of reference gene stability has been carried out in oysters (*Crassostrea* spp.; Dheilly et al., 2011; Du et al., 2013; Pu et al., 2015), scallops (Feng et al., 2013; Llera-Herrera et al., 2012; Mauriz et al., 2012), and clams (Siah et al., 2008).

The date mussel (*Lithophaga lithophaga*) plays an important role in marine ecosystems. Being a rock-boring bivalve, it is among the first to inhabit bare limestone rocks and, with burrowed tunnels and holes, form the basis for settlement by endolithic, benthic, and other sessile species (Gonzalez et al., 2000). Previous studies on the date mussel have focused on morphology (Morton and Scott, 1980; Owada, 2007; Aksit and Falakali Mutaf, 2014), reproduction (Valli et al., 1986; Šimunović et al., 1990; Kefi et al., 2014), colonization patterns (Grubelić et al., 2004; Devescovi and

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Ivesa, 2008; Devescovi, 2009), and burrowing (Jaccarini et al., 1968; Kleemann, 1973; Bolognani et al., 1976; Bolognani Fantin and Bolognani, 1979; Kleemann, 1996). Only a few studies have investigated the genetics of the species (Giribet and Wheeler, 2002; Martinez-Lage et al., 2005; Vizoso et al., 2011; Nishihara et al., 2016), and none have evaluated the expression stability of reference genes.

The aim of the present research was to evaluate five reference genes, *18S ribosomal RNA* (*18S*), *28S ribosomal RNA* (*28S*), *β-actin* (*β-act*), *α-tubulin* (*α-tub*), and *elongation factor 1-alpha* (*ef1a*), to identify the most stably expressed of these genes in mantle, gill, foot, and pallial gland tissue in different seasons. Four different algorithms, geNorm, NormFinder, BestKeeper, and RefFinder, were employed for reference gene validation. This is the first study to evaluate reference genes in various tissues of date mussel and will provide useful information for future qPCR studies in this bivalve.

2. Materials and methods

2.1. Sample collection

The date mussel is a protected marine species in Slovenia (Decree on Protected Wild Animal Species, Official Gazette of RS, No. 46/2004). The collection of samples for the present study was authorized by the Slovenian Environment Agency of the Ministry of the Environment and Spatial Planning (Document No. 35601-97/2014-4).

Four adult date mussel specimens were collected on each of four occasions from breakwaters in the Gulf of Piran, Slovenia, in November 2015 and in February, April, and July 2016. On each occasion, specimens were transported in seawater to the nearby laboratory of the Piran Marine Biology Station where they were dissected. Tissue samples were taken and immediately immersed in RNAlater to inactivate RNases and preserve intact RNA for subsequent isolation.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted with the E.Z.N.A. Mollusc RNA Kit (Omega Bio-Tek, Inc., USA) following the manufacturer's instructions. Potential DNA contamination was eliminated by treating samples with DNase I. The quantity of RNA was determined with a Qubit 3.0 fluorometer and the quality was assessed by gel electrophoresis.

First-strand cDNA was reverse transcribed from 1 µg of total RNA using the SuperScript VILO cDNA synthesis kit (Invitrogen) in a volume of 20 µL, following the manufacturer's instructions.

2.3. Selection of candidate reference genes

Five commonly used bivalve reference genes were selected for gene expression analysis. The nucleotide sequences of *18S* and *28S* were obtained from the NCBI database with accession numbers AF120530 and AF120588, respectively. The rest of the nucleotide sequences were obtained from transcriptome sequencing by use of the Ion Proton System. (Sivka et al., 2018). The primers and probes were designed and synthesized as custom TaqMan gene expression assays by Thermo Fisher Scientific (Applied Biosystems, USA). Primer pair efficiency was calculated from the slope of the standard curve of five serial 10-fold dilutions using the following formula: $E = 10^{(-1/slope)}$ (Pfaffl, 2001). Primer and probe sequences, product sizes, and amplification efficiency of reference genes are reported in Table 1.

Table 1. Primer sequences, corresponding amplicon sizes, and PCR efficiency of reference genes

Gene	Primer and probe sequence	Amplicon size (bp)	Efficiency
18S	F: GGGCACCACCAGGAGTG P: CTGCGGCTTAATTTG R: GGTGAGTTTTCCCGTGTTGAGT	58	1.90
28S	F: GCCTAGGTAGGATCCCTCGTTT P: CCCCGCCGTTTAAA R: GAGACGGGCCGGTAGTG	57	1.90
β-act	F: GTACGCTAACACCGTCTTGTCT P: CTGTCGGCAATACCG R: GTGCGGTAATTTCCTTCTGCATT	80	1.91
α-tub	F: CCCACGTATTCATTTCCCATTGG P: CCCCAGTCATCTCTGC R: CTGTTCATGGTAGGCCTTCTCT	70	1.94
ef1a	F: GATTGTTGCTGCCGGTACTG P: ACCAGCTTCAAATTC R: CAGCAAAGCATGTTCTCTGGTTT	76	1.91

F, Forward primer; R, reverse primer; P, probe.

2.4. qPCR

qPCR was performed using a ViiA 7 System (Thermo Fisher Scientific, Applied Biosystems, USA). The qPCR reaction contained 1 µL of diluted cDNA (10-fold for mRNA genes and 1000-fold for rRNA genes), 5 µL of TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Applied Biosystems, USA), 0.5 µL of Custom TaqMan Gene Expression Assay with the final concentration of 900 nM per primer and probe concentration of 250 nM (Thermo Fisher Scientific, Applied Biosystems, USA), and nuclease-free water in a total volume of 10 µL. The cycling conditions included an initial step of 2 min of uracil-N glycosylase (UNG) incubation at 50 °C and 20 s of polymerase activation at 95 °C, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. All reactions were performed in three technical replicates including no template and no reverse transcriptase controls.

2.5. Data analysis

The expression stability of the five reference genes among different tissues and during different seasons was evaluated with four different programs: geNorm, NormFinder, BestKeeper, and RefFinder.

geNorm calculates the normalization factor from the geometric mean of expression in multiple reference genes (Vandesompele et al., 2002). Candidate reference genes are ranked according to their relative expression stability (M-value), where those with the lowest M-values are the most stable. Depending on the input (genomic DNA) and the heterogeneity of the sample set (e.g., different tissue samples), acceptable average M-values can range from 0.2 to 1. Candidate reference genes with an M-value larger than 1 are not stably expressed and are not used for data normalization (Hellemans and Vandesompele, 2014). With calculation of pairwise variation (V-value), geNorm also enables the determination of an optimal number of reference genes. The V-value is an indication of the difference created when using an additional reference gene for normalization. If the V-value is below 0.15, additional reference genes are not required. Analysis using geNorm was performed with the qbase+ software. Raw data (Cq values) were exported as a .txt file from the ViiA 7 System and imported into qbase+.

NormFinder is an algorithm rooted in a mathematical model of gene expression that enables estimation of the overall variation among the candidate normalization genes and the variation among sample subgroups of the sample set. A stability value, a direct measure of the variation of the estimated expression, is determined for each gene (Andersen et al., 2004). NormFinder operates as an Excel add-in and requires input data on a linear scale. Prior to analysis, raw data (Cq) were transformed via a ΔCt method into relative expression quantities.

Determination of the most stable reference genes in the Excel-based tool BestKeeper is based on pair-wise correlation analysis and the geometric mean of expression of the candidate reference genes. Expression stability among the reference genes is ranked by the standard deviation (SD) and coefficient of variance (CV). The most stably expressed genes exhibit the least variation (Pfaffl et al., 2004). For analysis with BestKeeper, raw data (Cq values) generated by the ViiA 7 System were used.

The final ranking of the expression stability of the candidate reference genes was performed with the comprehensive web-based tool RefFinder, which integrates geNorm, NormFinder, and BestKeeper with the comparative ΔCt method to compare and rank the candidate reference genes (Xie et al., 2012). Raw Cq values were used for the overall final ranking.

3. Results

3.1. Expression profile of the reference genes

The expression levels of the five reference genes were determined by qPCR in all samples (Figure 1). The mean Cq values of the genes suggested that *28S* was the most abundantly expressed with an average Cq of 15.21 (±0.46), while *α-tub* had the lowest expression, with a mean of 22.16 (±1.48). *B-act* (2.20 cycles) and *α-tub* (1.48 cycles) showed the largest variation in expression across all samples, followed by *ef1a* (0.65 cycle) and *28S* (0.46 cycle), while *18S* (0.42 cycle) exhibited the least variation.

3.2. Expression stability of the reference genes

geNorm analysis showed that across all samples, *18S* and *28S* were the most stably expressed reference genes, with an M-value of 0.250, while *β-act* (M-value: 1.387) was the least stably expressed (Figure 2). In addition, *18S* and *28S* were the most stably expressed in all investigated tissues, with M-values of 0.222 (gill), 0.225 (foot), 0.240 (mantle), and 0.259 (pallial gland). However, the least stable reference gene differed among the different tissues. *α-tub*

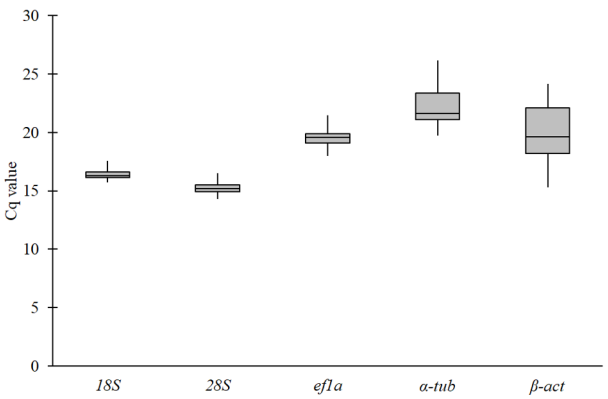


Figure 1. Distribution of Cq values of candidate reference genes in date mussel (*L. lithophaga*).

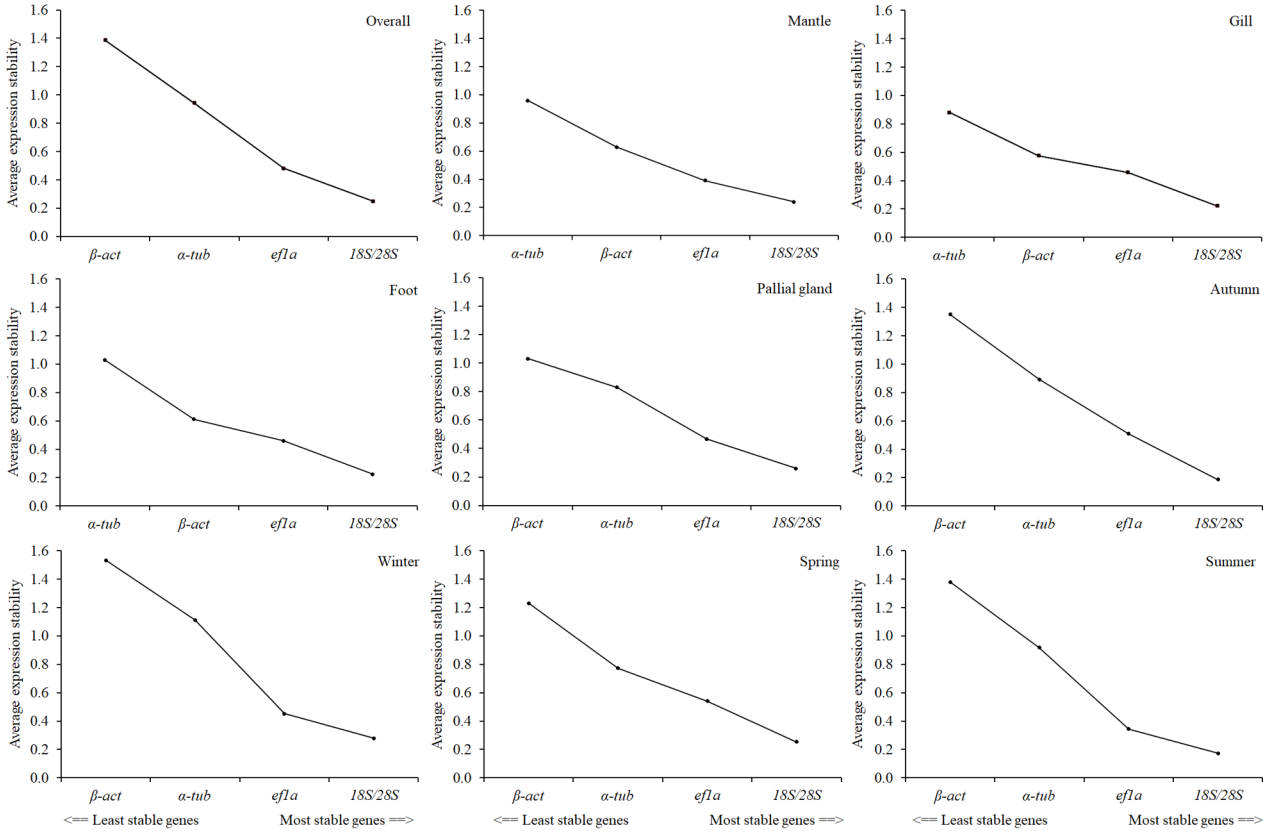


Figure 2. Average expression stability (M-value) of reference genes evaluated by geNorm.

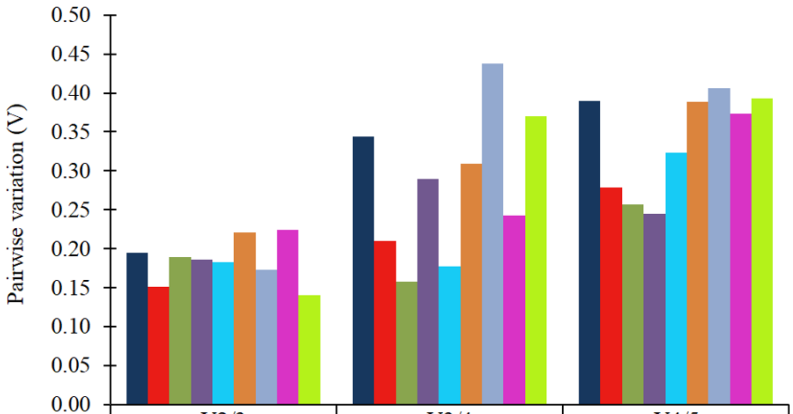
was the least stable reference gene in the mantle, foot, and gill, while in the pallial gland *β-act* had the highest M-value of 1.031 (Figure 2). The stability ranking of the reference genes was the same within each season: *18S* and *28S*; *ef1a*; *α-tub*; *β-act*.

In most samples no optimal number of reference genes could be determined from the selected reference genes because variability between normalization factors was relatively high (V-value > 0.15) (Figure 3). When no optimal number of reference genes can be determined, it is recommended that the three reference genes with the lowest M-value be employed as the use of multiple reference genes results in a more accurate normalization (Hellemans et al., 2007). Therefore, based on their M-values, *18S*, *28S*, and *ef1a* were selected as reference genes for normalization across the different tissues and seasons, though in summer V2/V3 was below a threshold of 0.15, indicating that the third reference gene (*ef1a*) was no longer necessary for normalization.

Results from analysis with NormFinder are reported in Table 2. Although selection of the most stable reference genes was based on a range of algorithms, outputs from NormFinder were similar to the features of GeNorm. Among all samples, *ef1a* and *18S* were the most stably expressed genes, with stability values of 0.181 and 0.399, respectively. In all tissues except mantle, *18S* and *28S* were the most stable reference genes. In mantle tissue, *ef1a* (0.097) and *18S* (0.211) showed the most stable expression levels, with e*f1a* being the most stable in winter, spring, and summer. Although the ranking order within the top three most stable reference genes differed between geNorm and NormFinder, *α-tub* and *β-act* were identified as the least stable genes in both analyses.

Across all samples, BestKeeper ranked *18S* as the most stably expressed gene, followed by *28S*, *ef1a*, *α-tub*, and *β-act* (Table 3). In mantle, gill, and foot tissue *18S* was the most stably expressed gene, while in the pallial gland *28S* had the steadiest expression profile. Similar results were observed across different seasons: *18S* had the lowest SD values in winter, spring, and summer, while the lowest SD of 0.38 was observed for *28S* in autumn. Overall *β-act* and *α-tub* were the least stable genes with the highest CV and SD. Results from BestKeeper were similar to those from geNorm and NormFinder analysis.

The final ranking of the candidate reference genes was generated by the RefFinder web tool. As reported in Table 4, *18S* was ranked as the first and *28S* as the second most stable gene in mantle, gill, and foot tissue. However,



	V2/3	V3/4	V4/5
overall	0.195	0.344	0.390
mantle	0.151	0.210	0.279
gill	0.189	0.158	0.257
pallial gland	0.186	0.290	0.245
foot	0.183	0.177	0.323
autumn	0.221	0.309	0.389
winter	0.173	0.438	0.406
spring	0.224	0.243	0.373
summer	0.140	0.370	0.393

Figure 3. Pairwise variation (V-value) of candidate reference genes in date mussel (*L. lithophaga*) using geNorm.

in the pallial gland *28S* ranked first and *18S* second. *18S* was also ranked as the most stable gene in winter and summer samples, while in autumn and spring *28S* and *ef1a*, respectively, were ranked as the most stably expressed genes. Across all samples, *18S*, *28S*, and *ef1a* were indicated as the most appropriate genes for normalization of qPCR data in date mussel.

4. Discussion

qPCR is a very popular method for identifying nucleotide sequences as a result of its sensitivity, relatively low cost, and technical simplicity. However, the reliability of results depends on an appropriate normalization of the qPCR data. In its early days, reference genes for normalization were selected on the assumption of stable expression of nonvalidated reference genes. Vandesompele et al. (2002) showed that use of a nonvalidated reference gene leads to erroneous normalization, ranging from 3-fold to 6-fold in 25% and 10% of cases, respectively. To produce reliable qPCR results one must also perform validation of candidate reference genes, for evaluation of which much software has been developed, with geNorm, NormFinder, and BestKeeper being the most commonly used programs.

In the present study, expression stability of five candidate reference genes was evaluated with four algorithms, geNorm, NormFinder, BestKeeper, and RefFinder, the latter of which integrates the first three packages with the use of the comparative Ct method to determine the most stable reference genes. The ranking order of reference genes was similar among the four programs, despite some slight differences, probably due to differences in the algorithms and analytical procedures. Across all samples, geNorm, BestKeeper, and RefFinder identified *18S* as the most stable reference gene, while NormFinder ranked *ef1a* as the most stable (Table S1). In gill and foot tissue, all four programs determined *18S* as the most stable reference gene, while *28S* was identified as the most suitable in the pallial gland. For mantle tissue, *28S* with geNorm, *ef1a* with NormFinder, and *18S* with BestKeeper were considered as the most reliable reference genes, though overall, RefFinder also ranked *18S* as having the most stable expression. RefFinder also ranked *18S* as the most stable gene in winter and summer samples, with *28S* and *ef1a* ranking top in autumn and spring samples, respectively. In previously reported validation studies of *Mytilus* spp., *18S*, *28S*, and *ef1a* were determined as the most stable genes in various tissues (Lacroix et al., 2014), at different stages of gametogenesis (Cubero-Leon et al., 2012), and following bacterial infection (Moreira et al., 2014). Nevertheless, in other studies these have been reported as unstable when bivalves were exposed to a range of pathogens and pollutants (Tanguy et al., 2005; Araya et al., 2008; Miao et al., 2014). In the present study results from the geNorm analysis identified no optimal

Table 2. Stability values of candidate reference genes calculated by NormFinder.

Rank	Overall	Tissue				Season			
		Pallial gland	Mantle	Gill	Foot	Autumn	Winter	Spring	Summer
1	*ef1a* 0.181	*28S* 0.135	*ef1a* 0.097	*18S* 0.187	*18S* 0.035	*28S* 0.252	*ef1a* 0.189	*ef1a* 0.243	*ef1a* 0.160
2	*18S* 0.399	*18S* 0.212	*18S* 0.211	*28S* 0.256	*28S* 0.258	*18S* 0.303	*18S* 0.373	*α-tub* 0.466	*28S* 0.449
3	*28S* 0.408	*ef1a* 0.308	*28S* 0.243	*ef1a* 0.296	*ef1a* 0.274	*ef1a* 0.376	*28S* 0.407	*18S* 0.511	*18S* 0.467
4	*α-tub* 0.867	*α-tub* 0.797	*β-act* 0.578	*β-act* 0.400	*β-act* 0.492	*α-tub* 0.779	*α-tub* 1.158	*28S* 0.549	*α-tub* 0.902
5	*β-act* 1.327	*β-act* 0.819	*α-tub* 0.955	*α-tub* 0.875	*α-tub* 1.104	*β-act* 1.258	*β-act* 1.371	*β-act* 1.283	*β-act* 1.331

Table 3. CV and SD of reference genes determined by Excel-based tool BestKeeper.

Rank	Overall	Tissue				Season			
		Pallial gland	Mantle	Gill	Foot	Autumn	Winter	Spring	Summer
1	*18S* 2.01 ± 0.33	*28S* 1.94 ± 0.30	*18S* 1.90 ± 0.31	*18S* 1.33 ± 0.22	*18S* 2.29 ± 0.38	*28S* 2.49 ± 0.38	*18S* 1.96 ± 0.32	*18S* 1.76 ± 0.29	*18S* 1.76 ± 0.29
2	*28S* 2.29 ± 0.35	*18S* 2.11 ± 0.35	*ef1a* 1.95 ± 0.38	*28S* 1.73 ± 0.26	*28S* 2.71 ± 0.42	*18S* 2.47 ± 0.41	*28S* 2.30 ± 0.35	*28S* 1.87 ± 0.29	*28S* 2.20 ± 0.34
3	*ef1a* 2.66 ± 0.52	*ef1a* 2.18 ± 0.44	*28S* 2.76 ± 0.42	*ef1a* 1.83 ± 0.37	*ef1a* 2.42 ± 0.46	*ef1a* 2.43 ± 0.48	*ef1a* 2.89 ± 0.56	*ef1a* 2.86 ± 0.57	*ef1a* 1.81 ± 0.35
4	*α-tub* 5.89 ± 1.32	*β-act* 5.00 ± 1.03	*β-act* 4.88 ± 0.86	*β-act* 2.33 ± 0.54	*β-act* 2.93 ± 0.55	*α-tub* 6.02 ± 1.37	*α-tub* 7.69 ± 1.73	*α-tub* 4.49 ± 1.00	*α-tub* 5.41 ± 1.19
5	*β-act* 9.57 ± 1.92	*α-tub* 4.97 ± 1.12	*α-tub* 5.45 ± 1.20	*α-tub* 5.17 ± 1.19	*α-tub* 5.94 ± 1.29	*β-act* 9.60 ± 1.83	*β-act* 9.85 ± 2.02	*β-act* 9.09 ± 1.85	*β-act* 9.93 ± 2.00

Table 4. Stability of candidate reference genes ranked by RefFinder.

Rank	Overall	Tissue				Season			
		Pallial gland	Mantle	Gill	Foot	Autumn	Winter	Spring	Summer
1	*18S* 1.19	*28S* 1.00	*18S* 1.19	*18S* 1.00	*18S* 1.00	*28S* 1.00	*18S* 1.41	*ef1a* 1.73	*18S* 1.57
2	*28S* 1.86	*18S* 1	*28S, ef1a* 2.06	*28S* 1.68	*28S* 1.68	*18S* 1.68	*ef1a* 1.73	*18S, 28S* 1.86	*ef1a* 1.73
3	*ef1a* 2.28	*ef1a* 1.68		*ef1a* 3.00	*ef1a* 3.00	*ef1a* 3.00	*28S* 2.06		*28S* 1.86
4	*α-tub* 4.00	*α-tub* 4.23	*β-act* 4.00	*β-act* 4.00	*β-act* 4.00	*α-tub* 4.00	*α-tub* 4.00	*α-tub* 3.36	*α-tub* 4.00
5	*β-act* 5.00	*β-act* 4.73	*α-tub* 5.00	*α-tub* 5.00	*α-tub* 5.00	*β-act* 5.00	*β-act* 5.00	*β-act* 5.00	*β-act* 5.00

number of reference genes, because the variability among sequential normalization factors was relatively high (V > 0.15). In such cases, it is recommended to use the three reference targets with the lowest M-values instead of a single target. Thus, *18S*, *28S*, and *ef1a* were determined as the top three stable reference genes by all four software

packages, suggesting they can be used for normalization of target genes in *L. lithophaga*.

In the present study *β-act* and *α-tub* were found to be the least stable reference genes. In previous studies *β-act* and *α-tub* had been used without validation for normalization in gene expression. However, validation of a large number of reference genes in different samples has indicated that expression of *β-act* and *α-tub* does vary, identifying them as inappropriate reference genes (Small et al., 2008; Cubero-Leon et al., 2012; Llera-Herrera et al., 2012; Feng et al., 2013; López-Landavery et al., 2014). Nevertheless, some studies have identified *β-act* and *α-tub* as the most stable genes (Du et al., 2013; Lacroix et al., 2014; Moreira et al., 2014). Such mixed results show the importance of undertaking appropriate reference gene validation prior to analyzing relative gene expression data.

Until now, no evaluation of reference genes has been performed in the date mussel. The present paper is the first to determine optimal reference genes for normalization of qPCR data from samples of different tissues in *L. lithophaga* and during different seasons. Among the five selected reference genes, *18S*, *28S*, and *ef1a* were evaluated as the most stable in different tissues, while *α-tub* was found to be inappropriate for normalization. The present results will help improve the accuracy of gene expression analysis in samples of date mussel and at the same time provide guidance for selection of reference genes in future qPCR studies in the species.

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Table S1. Ranking of selected reference genes by geNorm (gN), NormFinder (NF), BestKeeper (BK), and RefFinder (RF)/

Overall				Tissue															
				Pallial gland				Mantle				Gill				Foot			
gN	NF	BK	RF	gN	NF	BK	RF	gN	NF	BK	RF	gN	NF	BK	RF	gN	NF	BK	RF
18S	*ef1a*[a]	*18S*	*18S*	*18S 28S*	*28S*	*28S*	*28S*	*28S*	*ef1a*	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*
28S	*18S*	*28S*	*28S*	*ef1a*	*18S*	*18S*	*18S*	*18S*	*18S*	*ef1a*	*28S ef1a*	*28S*	*28S*	*28S*	*28S*	*28S*	*28S*	*28S*	*28S*
ef1a	*28S*	*ef1a*	*ef1a*	*α-tub*	*ef1a*	*ef1a*	*ef1a*	*ef1a*	*28S*	*28S*	*β-act*	*ef1a*	*ef1a*	*ef1a*	*ef1a*	*ef1a*	*ef1a*	*ef1a*	*ef1a*
α-tub	*α-tub*	*α-tub*	*α-tub*	*β-act*	*α-tub*	*β-act*	*α-tub*	*β-act*	*β-act*	*β-act*	*α-tub*	*β-act*	*β-act*	*β-act*	*β-act*	*β-act*	*β-act*	*β-act*	*β-act*
β-act	*β-act*	*β-act*	*β-act*		*β-act*	*α-tub*	*β-act*	*α-tub*	*α-tub*	*α-tub*		*α-tub*	*α-tub*	*α-tub*	*α-tub*	*α-tub*	*α-tub*	*α-tub*	*α-tub*
				Season															
				Autumn				Winter				Spring				Summer			
				gN	NF	BK	RF	gN	NF	BK	RF	gN	NF	BK	RF	gN	NF	BK	RF
				28S	*28S*	*28S*	*28S*	*ef1a*	*ef1a*	*18S*	*18S*	*ef1a*	*ef1a*	*18S*	*ef1a*	*ef1a*	*ef1a*	*18S*	*18S*
				18S	*18S*	*18S*	*18S*	*18S 28S*	*18S*	*28S*	*ef1a*	*18S*	*α-tub*	*28S*	*18S 28S*	*18S 28S*	*28S*	*28S*	*ef1a*
				ef1a	*ef1a*	*ef1a*	*ef1a*	α-tub	*28S*	*ef1a*	*28S*	*28S*	*18S*	*ef1a*	*α-tub*	*α-tub*	*18S*	*ef1a*	*28S*
				α-tub	*α-tub*	*α-tub*	*α-tub*	*β-act*	*α-tub*	*α-tub*	*α-tub*	*α-tub*	*28S*	*α-tub*	*β-act*	*β-act*	*α-tub*	*α-tub*	*α-tub*
				β-act	*β-act*	*β-act*	*β-act*		*β-act*	*β-act*	*β-act*	*β-act*	*β-act*	*β-act*			*β-act*	*β-act*	*β-act*

[a] *ef1a*, elongation factor 1-alpha; *α-tub*, α-tubulin; *β-act*, β-actin.