Screening for Suitable Reference Genes for Quantitative Real-Time PCR in *Heterosigma akashiwo* (Raphidophyceae)

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Abstract

The raphidophyte *Heterosigma akashiwo* is a globally distributed harmful alga that has been associated with fish kills in coastal waters. To understand the mechanisms of *H. akashiwo* bloom formation, gene expression analysis is often required. In this study, we assessed ten of the previously reported algal candidate genes (*rpL17-2, rpL23, cox2, cal, tua, tub, ef1, 18S, gapdh, and mdh*) for their suitability as reference genes in this species. We used qRT-PCR to quantify the expression levels of these genes in *H. akashiwo* grown under different temperatures, light intensities, nutrient concentrations, and time points over a diel cycle. The expression stability of these genes was evaluated using geNorm and NormFinder algorithms. Although none of these genes exhibited invariable expression levels, *cal, tub, rpL17-2* and *rpL23* expression levels were the most stable across the different conditions tested. For further validation, these selected genes were used to normalize the expression levels of ribulose-1, 5-bisphosphate carboxylase/oxygenase large unite (*HrbcL*) over a diel cycle. Results showed that the expression of *HrbcL* normalized against each of these reference genes was the highest at midday and lowest at midnight, similar to the diel patterns typically documented for this gene in algae. While the validated reference genes will be useful for future gene expression studies on *H. akashiwo*, we expect that the procedure used in this study may be helpful to future efforts to screen reference genes for other algae.

Introduction

Harmful algal blooms (HABs) cause significant damages to marine ecosystems, local economies and human health [1–3]. *Heterosigma akashiwo* (Hada) Hada ex Y. Hada et Chihara is a HAB species within the class Raphidophyceae. This species is distributed worldwide, and is an eurythermal and euryhaline organism [4, 5]. *H. akashiwo* is notorious because its blooms have caused massive mortality of cultured finfish, but the mechanisms for ichthyotoxicity are not
well resolved and remain controversial [6–9]. To understand how *H. akashiwo* forms blooms [10–13] and how the bloom kill fish, information on the molecular machinery or biochemical processes regulating growth and metabolism in this species is of great importance. Determining gene expression patterns under different environmental conditions is essential toward gaining such understanding.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is one of the most frequently-used technologies to study the expression patterns of genes, because it offers high sensitivity, specificity, reproducibility and accuracy [14, 15]. However, several variables associated with RNA samples may influence the accuracy of gene expression analysis, such as variations in RNA quantity and quality, enzymatic efficiency of reverse transcription and PCR amplification [16]. One common way to correct the biases caused by these factors is to normalize gene expression data against some properly established reference genes (housekeeping genes) [17–19]. Generally, an ideal reference gene should be expressed at stable levels in different tissues, under different treatments, or under different environmental conditions. Many genes have been used as reference genes, such as 18S ribosomal RNA (18S), glyceraldehyde-3-phosphate dehydrogenase (gapdh) and elongation factor 1α (ef1), simply because they have been shown to be appropriate in some model organisms. But, they have subsequently been found not to be always expressed at stable levels under different conditions in different species [20, 21]. Therefore, it is necessary to establish the suitability of the reference gene(s) for different species or specific types of conditions before use in gene expression studies. Until now, many studies have been carried out using or selecting reference genes in animals and plants [22–24] as well as algae (S1 Table). Yet no comprehensive screening for reference genes for *H. akashiwo* has been reported.

In this study, the stabilities in expression of ten candidate genes (Table 1), α-tubulin (tua), β-tubulin (tub), cytochrome c oxidase subunit II (cox2), 60S ribosomal protein L17-2 (rpL17-2), 60S ribosomal protein L23 (rpL23), calmodulin (cal), malate dehydrogenase (mdh), 18S, gapdh and ef1 in *H. akashiwo* were analyzed under four different experimental conditions. As a way to further validate the suitability of the most promising candidate genes identified, the two top-ranked reference genes for diel cycle studies were used to normalize ribulose large subunit (*HrbcL*) expression levels throughout a diel cycle. A set of four genes was found to be the most suitable as reference genes for all the four conditions we tested, while each condition had its own specific set of top-ranked reference genes.

**Materials and Methods**

**Heterosigma akashiwo** culture and treatment

*Heterosigma akashiwo* were maintained in a glass bottle with f/2 medium (without added silicate) prepared with 0.22 μm-filtered and autoclaved seawater (salinity 30 PSU). Stock cultures were kept at 20°C under a 14: 10 h light: dark cycle with an average photon flux of 100 ± 10 μE.m⁻².s⁻¹. For all experiments, cell concentrations were determined using a Sedgwick-Rafter chamber under the microscope.

For experiments, each treatment group was set up in triplicate. Control group was cultured in normal f/2 medium under the temperature and light conditions as described above. Temperature treatment included exposure to 10°C, 20°C and 30°C, respectively for 24 h. Light treatment consisted of exposure to 200 (high light), 100 (normal light) and 50 μE.m⁻².s⁻¹ (low light), respectively for 96 h. For nitrogen (N) and phosphorus (P) stress treatment, the cultures were treated as previously described [25] with minor modification. Briefly, the pre-treatment stock culture in f/2 medium was inoculated to low nutrient media that were either stoichiometrically low in N (181.5 μM NaNO₃ and 36.3 μM NaH₂PO₄.H₂O, with 5: 1 of N: P) or low in P
(883 μM NaNO₃ and 7.06 μM NaH₂PO₄.H₂O, with 125: 1 of N: P) conditions. When these cultures were growing in the exponential phase, they were re-inoculated into fresh batches of their corresponding media. Cell concentration was monitored daily as described above, and when cultures were exhibiting exponential growth again, samples were collected. In addition, to examine the diel patterns of gene expression, the control group cultures were sampled, in which sampling started at 6 h (T6) after the onset of light period (T0), and 12 (T12), 18 (T18), 24 (T24), and 30 (T30) h after T0, throughout a 24-h diel cycle. Samples were collected by centrifugation at 3000 × rpm for 5 min at 4°C. The cell pellets were suspended in 1 mL TRIzol reagent (Invitrogen), and stored at -80°C until RNA extraction within a month.

### Total RNA extraction and cDNA synthesis

Total RNA was extracted as recently reported [26] and potential genomic DNA contamination was eliminated by incubating the RNA samples with RQ1 DNase (Promega). RNA was further purified by RNeasy Mini kit (Qiagen). RNA purity and concentration were measured by NanoDrop spectrophotometer (Theromo, Germany), and RNA integrity was evaluated by agarose gel electrophoresis.

The first-strand cDNA was synthesized from 300 ng of total RNA in a total volume of 20 μL using the ImProm-Ⅱ reverse transcriptase (Promega) with random hexamer primer. The RNA template was first incubated with 0.5 μg of the primer at 70°C for 5 minutes, and then at 4°C for 5 minutes. Next, 4 μL ImProm-Ⅱ 5 × reaction buffer, 4 μL MgCl₂ (25 mM), 1 μL dNTP mix (10 mM each dNTP) and 1 μL ImProm-Ⅱ reverse transcriptase were added and the mixture was incubated at 25°C for 5 minutes and then 42°C for 1 h. The reaction was terminated by

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| Gene symbol | Primer sequences (5’—3’) Forward/Reverse | Amplicon length (bp) |
|-------------|------------------------------------------|----------------------|
| rpL17-2     | TACACGATCAAGGAGCAGAACC | 93                   |
|             | GCCCTGTGGGCCACCTCCTGCA |                      |
| rpL23       | GTGTTGGGCGCACCCTTCTCA | 118                  |
|             | GCCCTGATTTTCACCTTGCTG |                      |
| cox2        | CAACTGACCACTCGACTCTG   | 130                  |
|             | GGTGTGGGCCACCTCCTGCA  |                      |
| cal         | GACATGATGGAAGGTCGTA    | 113                  |
|             | GCCCTTCTGCTGCTGATAAC  |                      |
| tua         | CTCTCTTCTGCTGCTGATA   | 174                  |
|             | TGCTGTCTGCCTGCTGATA   |                      |
| tub         | CCTGAGACCACGACGATGCA  | 123                  |
|             | TCAGCTCTCTCTGCTGATA   |                      |
| ef1         | ACGCGGTGTTAGGAACTGCA  | 105                  |
|             | CAGGTTGACATGGAACCTGCA |                      |
| 18S         | TGAGGATTGATTGATTGAG   | 133                  |
|             | CCACTTCTTCTGCTGATA    |                      |
| gapdh       | TACTCGAGATGAGCTTGTG   | 96                   |
|             | GGAGGTTGGTTGAGGAA     |                      |
| mdh         | CCGTGGATGAGGAGGAGTA   | 115                  |
|             | CGGTTGACATGGAACCTGCA  |                      |
| HrbcL       | ACCAACAACGTGATGAGGCC | 182                  |
|             | CGTATGCGATGATGCGAGA   |                      |

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doi:10.1371/journal.pone.0132183.t001
incubating it at 70°C for 15 minutes. The cDNAs were diluted 1:50 with nuclease-free water before use in subsequent experiments.

Selection of candidate reference genes for study

A group of genes that have been used as reference genes in various algae were summarized in S1 Table. Based on the availability of the sequences from H. akashiwo, we selected eight (tua, tub, 18S, gapdh, rpl23, cal, ef1, and mdh) from this list and added two others (cox2 and rpl17-2) that are functionally related to cox1 and rpl19 in the list to achieve a set of ten candidate genes. The nucleotide sequences of tua (AY729829), tub (AY729817), 18S (JX026930), cox2 (GQ222228), and gapdh (AF319449), were available from GenBank database, and the others were identified from transcriptome data of H. akashiwo [27].

Primer design and evaluation

The primers for qRT-PCR were designed using the Primer Premier 5.0 software and had estimated melt temperature of 57–60°C and amplicon lengths of 80–180 bp (Table 1). To check the specificity of the primers, regular PCR were run and all PCR products were examined on agarose gel for size, and then purified, cloned, and sequenced. For each primer, the PCR amplification efficiency (E) was calculated following $E = \left[ 10^{-1/\text{slope}} \right] \times 100\%$, in which the slope were obtained from the standard curve generated from a serial dilutions of pooled cDNAs [28]. From the same dilution series correlation coefficient ($R^2$) was also calculated.

qRT-PCR

Using the cDNAs as the templates, qRT-PCR was conducted using the CFX96 Real-Time System (Bio-Rad, USA). Each PCR reaction was carried out in a total volume of 12 μL containing 6 μL of 2 × iQ SYBR Green supermix (Bio-Rad, USA), 375 nM of each primer, and 5 μL of 1:50 diluted cDNA. The PCR program was composed of a denaturation step of 3 min at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C for 32 s. In each run, negative controls were set up with ddH2O and RNA as templates, respectively. Each reaction had three technical replicates. At the end, to confirm primer specificity, all the PCR products were subjected to melting curve analysis.

Gene stability analysis using geNorm and NormFinder

The geNorm [29] and NormFinder [30] software packages were employed to assess the stability of the expression levels of the candidate reference genes under the different experimental conditions. geNorm produces a stability measure (M) and by stepwise exclusion of the genes with the lowest stability creates a ranking of the tested genes (the lower the M value, the more stable the expression of the gene). The number of genes required for normalization of target gene expression also was estimated, and the normalization factor was calculated. NormFinder is a another program used to evaluate the candidate reference genes in given experimental design; it takes into consideration the intra- and inter-group variations and combines these results to estimate a reference gene stability value for each gene, avoiding influence caused by co-regulated candidate genes.

Relative quantification of HrbcL gene expression

Ribulose 1, 5-bisphosphate carboxylase/oxygenase (rbc) is the key enzyme catalyzing CO₂ fixation and is thus essential for photosynthetic organism [31]. Diel regulation of rbc transcription has been well documented in many photosynthetic organisms from cyanobacteria, algae,
to higher plants [32]. Therefore, we attempted to characterize the diel expression patterns of *H. akashiwo rbc* large subunit (*HrbcL*) using the best reference genes (*rpL23* and *rpL17-2*) identified under diel cycle in the present study, as a way to further validate the selected reference genes. The sequence of *HrbcL* (EU168191) was obtained from GenBank, and primers were designed (Table 1). Relative expression levels of *HrbcL* were calculated by dividing the raw expression value of *HrbcL* for each sample by the normalization factor generated by geNorm.

**Statistical analyses**

All statistical analyses were performed with SPSS (version 16.0). Variations in the Ct values between and within treatments as well as in the relative expression of *HrbcL* in *H. akashiwo* were analyzed using one-way ANOVA, and the level of significance was defined at *P* < 0.05.

**Results**

**RNA quality, primer specificity and amplification efficiencies**

RNA samples prepared from *H. akashiwo* all displayed good quality, with A260/A280 ratios ranging from 1.9 to 2.2, and A260/A230 ranging from 1.9 to 2.1. RNA integrity was confirmed on agarose gel electrophoresis, which showed two discrete bands, one (more abundant) 28S rRNA and another 18S rRNA. Specificities of primers were confirmed by the presence of a single band with the expected size on agarose gel electrophoresis (S1 Fig), the presence of a single peak in melting curve analysis after qRT-PCR (S2 Fig), and sequencing results (S1 File). No product was detected in negative control (ddH2O or RNA as template), indicating that there was no gDNA contamination in the RNA extracts, and the qRT-PCR results were thus reliable. The PCR efficiency (E) of the ten candidate reference genes and *Hrbc* ranged from 90.1%–101%, and the correlation coefficient (*R*^2^) ranged from 0.991 to 0.999, which were within the commonly reported range of qRT-PCR.

**Expression profiling of candidate reference genes**

In order to investigate the relative expression levels of the ten candidate reference genes in *H. akashiwo*, the Ct values of these genes were calculated (S2 Table). The Ct median value of reference genes varied from 10.51 to 29.91, and most of the values were between 25.08 and 26.87 across all the samples (Fig 1). The 18S gene showed the highest expression level with Ct value ranging from 8.33 to 13.32 in different samples, while *mdh* exhibiting the lowest expression level with Ct value ranging from 27.47 to 32.72. Based on the comparative ranges of Ct values, the smallest gene expression variation seemed to occur in *tua*, while *gapdh* seemed to be the most variable. However, a simple comparison of the raw Ct value is not sufficient to determine expression stability of the candidate reference genes; therefore, further analyses using geNorm and NormFinder software were conducted to provide more accurate results.

**Expression stability of candidate reference genes**

Average expression stability values (M) were obtained using geNorm, and all candidate genes were ranked based on the M values (Fig 2). All ten genes investigated in this study showed M values below the threshold value 1.5, indicating that the expression levels of these genes were relatively stable under all the conditions we examined. By comparison, as shown in Fig 2, the most stable genes were *cal* and *tub* under temperature treatment, 18S and *tub* under light treatment, *rpL17-2* and *rpL23* in the diel cycle samples, *cal* and *rpL17-2* under nutrient treatment and all treatments combined (M = 0.488) (Total). We found that *gapdh* was the least stably
expressed under temperature treatment, light treatment, time points over the diel cycle, and total (M = 1.219), and ef1 was the least stably expressed gene under nutrient treatment. In addition, our ANONA analysis showed that the variations in the gapdh and ef1 Ct values were attributable to experimental treatments rather than inconsistencies between replicates, because the between group variance was much higher than the within group variance (S3 Table).

The geNorm software was also used to determine the optimal number of references genes required for accurate normalization, according to the pairwise variation (Vn/Vn+1) value. Vandesompele et al. (2002) proposed 0.15 as a Vn/Vn+1 threshold value, below which the inclusion of an additional control gene is not required [29]. For individual experimental treatment, V2/V3 value was 0.056, 0.117, 0.086 and 0.119 in nutrient, light, temperature and diel time point treatments, respectively (Fig 3). These results indicated that the inclusion of a third gene would not have significant effect for any of the four treatments, so the two most stable reference genes were sufficient for accurate normalization of gene expression under these conditions. When all the samples were considered together, V2/V3, V3/V4 and V4/V5 value were 0.201, 0.167 and 0.145, respectively. Combined with the result of stability ranking, the results showed that the four most stable reference genes, cal, rpL17-2, tub and rpL23, would be sufficient for accurate gene expression normalization for H. akashiwo under any combination of the four conditions.

For an independent assessment, the expression stabilities of reference genes were also ranked by NormFinder (Table 2). The most stable genes were cal for light and nutrient treatments, rpL17-2 for temperature treatment, and rpL17-2 and rpL23 for diel cycle. When all the conditions were considered together, the same five most stable genes (cal, rpL17-2, tub, rpL23, and cox2) were identified by NormFinder as by geNorm, although cox2 was ranked first by NormFinder and fifth by geNorm. NormFinder identified gapdh and ef1 as the most unstable gene across all the conditions, which was consistent with the result from the geNorm.

Fig 1. Ct values of candidate reference genes across conditions tested. Boxes show the median values (central lines), Q1 (lower outline) and Q3 (upper outline), and whiskers. The whiskers are set at 1.5 times IQR (interquartile range) above Q3 and 1.5 times IQR below the Q1. If the Maximum or Minimum values are outside this range, they are shown as outliers (*).

doi:10.1371/journal.pone.0132183.g001
Diel expression pattern of \textit{HrbcL} and validation of the reference genes

To evaluate the usefulness of the selected reference genes, we compared the expression pattern of \textit{rbcL} in \textit{H. akashiwo} (\textit{HrbcL}) under diel cycle (sampled every six hours) using the most stable (\textit{rpL17-2} and \textit{rpL23}) and the least stable genes (\textit{gapdh} and \textit{mdh}) in the diel cycle among our ten candidate reference genes. When the most stable genes (\textit{rpL17-2} and \textit{rpL23}) were used in combination for normalization, the expression levels of \textit{HrbcL} decreased rapidly between six hours after the onset of light (T6) and two hours before the end of the light period (T12), reaching the lowest value at four hours after the onset of the dark period (T18), and increased thereafter (Fig 4). A similar expression pattern was observed when either \textit{rpL17-1} or \textit{rpL23} was used alone for normalization. When the least stable genes (\textit{gapdh} and \textit{mdh}) were employed together,
Fig 3. Determination of the optimal number of reference genes for normalizing gene expression (qRT-PCR) data. Pair-wise variation ($V_n/V_{n+1}$, where $n$ represents number of genes) was analyzed between the normalization factors ($N_{F_n}$ and $N_{F_{n+1}}$) by geNorm software against all different conditions examined (nutrient, temperature, light, timing in the diel cycle and all these factors combined).

doi:10.1371/journal.pone.0132183.g003

Table 2. Expression stability values of the candidate reference genes calculated by NormFinder.

| Temperature | Light | Nutrient | Diel | Total |
|-------------|-------|----------|------|-------|
| Ranking     | SV    | Ranking  | SV   | Ranking | SV  | Ranking | SV  | Ranking | SV  | Ranking | SV  |
| rpL17-2     | 0.247 | cal      | 0.130| cal    | 0.096| rpL23   | 0.207| cal     | 0.353|
| cox2        | 0.266 | rpL23    | 0.176| rpL23  | 0.103| rpL17-2 | 0.230| tub     | 0.360|
| mdh         | 0.292 | 18S      | 0.226| tub    | 0.111| cox2    | 0.274| cal     | 0.361|
| cal         | 0.300 | tub      | 0.234| mdh    | 0.142| cal     | 0.316| rpL17-2 | 0.370|
| ef1         | 0.319 | ef1      | 0.285| gapdh  | 0.145| tua     | 0.405| rpL23   | 0.473|
| 18S         | 0.374 | tua      | 0.289| rpL17-2| 0.149| ef1     | 0.570| 18S     | 0.481|
| tub         | 0.375 | mdh      | 0.302| 18S    | 0.167| tub     | 0.576| mdh     | 0.489|
| rpL23       | 0.386 | cox2     | 0.335| cox2   | 0.209| 18S     | 0.588| tua     | 0.580|
| tua         | 0.390 | rpL17-2  | 0.340| tua    | 0.234| mdh     | 0.848| ef1     | 0.689|
| gapdh       | 0.553 | gapdh    | 0.367| ef1    | 0.408| gapdh   | 1.741| gapdh   | 1.020|

Ranking indicates the genes stability from the most stable to the least stable.
SV represents stability value.

doi:10.1371/journal.pone.0132183.t002
the normalized expression levels of *HrbcL* were not significantly different between time points T12 and T18, and entirely different expression patterns were obtained when either one of these two least stable genes was used for normalization (Fig 4).

**Discussion**

Research on *H. akashiwo* has uncovered its life cycle and ecological characteristics, such as diel vertical migration [33], benthic stage [11], and strong ability to tolerate sudden salinity decrease [12], which may be linked to its ecological success. However, molecular mechanisms underlying these features [34] are poorly understood and need to be investigated. The qRT-PCR technology has been extensively used in the study of marine phytoplankton to understand transcriptional responses to physical stressors [35, 36], nutrient starvation [37], and diel cycle [26, 38, 39], and to detect the activity and modulation of many metabolic processes [39, 40]. As the accuracy of gene expression data is highly dependent on the selection of suitable reference genes for normalizing gene expression against experimental variations, most of those studies used reference genes for the normalization. However, only a small fraction of those reference genes has been systematically evaluated to ensure they are suitable for the species under investigation.

In this paper, we conducted careful evaluation on expression stability of ten candidate reference genes, eight of which have been used in previous studies on various organisms. We chose to investigate the effects of temperature, light, and nutrient conditions because they are the most common environmental variable influencing marine phytoplankton. Diel cycle was also examined because sampling for molecular analysis, particularly on research cruises, often occurs at different times of the day. Two widely used analysis programs, geNorm and NormFinder, were used to ensure reliability of gene expression stability assessment. Due to their distinct algorithms, slight differences were observed when the rankings of the candidate reference genes from these two programs were compared. For example, under temperature variations, *rpL17-2* was ranked to be the most stable by NormFinder, whereas *cal* and *tub* were ranked as
the most suitable candidate reference genes by geNorm (Fig 2, Table 2). Corresponding different results have also been reported and discussed in many previous studies [22, 23, 41]. However, both analysis programs produced the same top five most stable genes: \( cal, rpl17-2, \) \( tub, \) \( rpl23, \) and \( cox2. \) Therefore, these five can be considered the “short list” of reference genes for common transcriptional studies in \( H. \) akashiwo. Based on the below-threshold pairwise variation, \( V_2/V_3 \) value (0.145) and the high frequencies at which these genes were ranked favorable across the four separated sets of conditions, \( cal, rpl17-2, tub \) and \( rpl23 \) are particularly preferable for use under different environmental stress conditions or diel cycle of \( H. \) akashiwo. Picking common top-ranked genes from multiple analysis programs should give high confidence about the selection of the reference genes.

Even for specific environmental conditions or treatments, the picking common-gene approach can be also helpful for identifying best reference genes available. For temperature treatment, for instance, geNorm identified \( cal \) and \( tub \) as the most stable genes, followed by \( mdh, 18S, \) and others (Fig 2), whereas NormFinder ranked \( rpl17-2 \) as the best reference gene, followed by \( cox2, mdh, cal, \) and others (Table 2). Based on the ranking orders, \( cal \) and \( mdh \) would be the best choices among the ten examined presently for temperature effect studies. This result agrees with the previous studies on \( cal \) and \( mdh \) genes in algae. The calmodulin gene has been identified as a stable reference gene in the dinoflagellate \( Symbiodinium \) sp. under temperature stress [35], while \( mdh \) was shown to be relatively stable in the dinoflagellate \( Prorocentrum minimum \) across many experimental conditions (heat shock, toxic chemical exposures and different life stages) [42].

By the same way, we found \( cal \) and \( tub \) were sufficiently stable to be used for normalizing gene expression under light and nutrient treatments. The beta-tubulin gene has also been selected as reference gene in studies of gene expression in the chlorophyte macroalga \( Ulva linza \) [43] and the diatom \( Pseudo-nitzschia multisiriata \) [44].

For diel cycle samples, both geNorm and NormFinder identified \( rpl23 \) and \( rpl17-2 \) as the ideal reference genes, and the pairwise variation \( V_2/V_3 \) of 0.119 indicated that these two ribosomal protein genes could be used in combination for normalization (Fig 3). Recently, various ribosomal proteins genes have frequently been identified as suitable reference genes in algae and other organisms. \( rps30 \) (ribosomal protein small subunit 30S) gene has been selected as the most stable gene among twelve candidate reference genes compared throughout a diel cycle [38]. \( rpl19 \) (60S ribosomal protein L19) and \( rpl23 \) were validated as reference genes in the chlorophytes \( Chlamydomonas \) sp. (for freezing condition) [45] and \( Volvox carteri \) (for different cell types) [46], respectively.

The GAPDH gene (\( gapdh \)) has been considered to be a suitable reference gene for quantifying gene expression in many algal species under different conditions, such as \( Prorocentrum donghaiense \) under diel cycle [26], \( Alexandrium catenella \) in P-limited conditions [47] and \( Chlamydomonas \) sp. under different light treatment [48]. However, in our study, \( gapdh \) was ranked as one of the least stable genes under different treatments and in all samples combined, indicating that it is not suitable as a reference gene for \( H. \) akashiwo under our experimental conditions. In agreement of our result, the mRNA and protein levels of \( gapdh \) have been reported to be regulated by light in other algae [38, 49]. The variability of \( gapdh \) expression level also has been increasingly recognized for other types of organisms [29, 50, 51].

Like \( gapdh, 18S \) is commonly used as a reference gene [52], and it has been used to design probes for quantifying the abundance of \( H. \) akashiwo in field samples [53]. In the present study we found that \( 18S \) was a moderately stable gene across all conditions we examined, although it appeared to be a suitable reference gene under light treatment (Fig 2). Compared with other genes (mRNA transcripts) the Ct median value of \( 18S \) was much lower (Fig 1), indicating that the abundance of \( 18S \) transcript was much higher (~1000 folds that of \( cox2 \) and ~700,000 folds
that of mdh). The high abundance of 18S compared to mRNA transcripts (target genes) makes it difficult to reliably subtract the background baseline value in qRT-PCR data analysis [29, 54]. In addition, 18S rRNA content may be affected by nutrient stress and vary over the diel cycle [25, 55]. Therefore, we recommend that 18S not be selected as a reference gene for qRT-PCR in H. akashiwo.

Applying the candidate reference genes to normalizing a well-characterized target gene would provide further validation for the reference genes. We chose to assess the expression profile of rbcl in H. akashiwo (HrbcL) under a diel cycle because rbcl mRNA abundance is known to exhibit strong diel rhythm in many algal species [32]. We observed a similar diel rhythm in HrbcL expression whether the most stable genes reference genes (rpL23 and rpL17-2) for diel cycle were used individually or in combination (Fig 4) to the diel pattern reported for many algae [32, 56], i.e. expression level being the highest around the middle of the light period and lowest in the middle of the dark period. Many previous reports also have shown that the expression patterns of target genes showed similar trends when either single or combined most stable reference genes were used [28, 57]. This further verifies that rpL23 and rpL17-2 are suitable reference genes for H. akashiwo gene expression studies under diel (light dark) cycle. When the expression levels of HrbcL were normalized with the least stable genes (gapdh, mdh) for diel cycle, either singly or in combination, different diel patterns of HrbcL expression were observed, either by comparison to each other, to previously reported patterns, or to patterns when rpL23 and rpL17-2 were used for normalization (Fig 4). Clearly, gapdh and mdh are not suitable reference genes for gene expression studies in H. akashiwo under diel cycle. These results demonstrate that the use of reference genes without validation risks misinterpretation of results.

**Conclusion**

To our knowledge, this work is the first study to evaluate candidate reference genes for gene expression analysis in H. akashiwo under different environmental conditions (temperature, nutrient and light) and different time points over the diel cycle. After careful assessment using qRT-PCR combined with statistical analysis based on geNorm and NormFinder, our results show that cal and tub are good reference genes for gene expression studies under different light and nutrient conditions, rpL17-2 and rpL23 for diel cycle studies, cal and mdh for varying temperature conditions. Our results also lead us to conclude that if used in combination cal, tub, rpL17-2 and rpL23 are suitable reference genes for gene expression analysis under all the four different experimental conditions we examined, and because these conditions represent the most common environmental or sampling factors, they may be applicable to most field studies. The identification of the suitable reference genes in this study will facilitate future studies on gene expression in H. akashiwo to improve our understanding on the molecular mechanisms of bloom formation.

**Supporting Information**

S1 Table. Summary of reference genes that had been tested or used in algal research.

(DOC)

S2 Table. Ct values of candidate reference genes examined in this study.

(XLS)

S3 Table. Effects of different treatments on the Ct value of two least stable genes (gapdh and ef1).

(DOC)
S1 Fig. Agarose gel electrophoresis results of ten candidate reference genes and *HrbcL* PCR products. (TIF)

S2 Fig. Melting curves of ten candidate reference genes and *HrbcL*. (TIF)

S1 File. Sequence results of ten candidate reference genes and *HrbcL*. (FASTA)

**Author Contributions**

Conceived and designed the experiments: NJ SL L. Li. Performed the experiments: NJ L. Lin. Analyzed the data: NJ SL. Contributed reagents/materials/analysis tools: SL L. Li. Wrote the paper: NJ SL.

**References**

1. Anderson DM, Gilbert PM, Burkholder JM. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries. 2002; 25: 704–726.

2. Anderson DM. Approaches to monitoring, control and management of harmful algal blooms (HABs). Ocean Coast Manage. 2009; 52: 342–347.

3. Hoagland P, Scatasta S. The economic effects of harmful algal blooms. In: Granéli E, Turner JT, editors. Ecology of harmful algae. Berlin: Springer; 2006. pp. 391–402.

4. Smayda TJ. Ecophysiology and bloom dynamics of *Heterosigma akashiwo* (Raphidophyceae). In: Anderson DM, Cembella AD, Hallegraef GM, editors. Physiological Ecology of Harmful Algal Blooms. Berlin: Springer; 1998. pp. 113–131.

5. Honjo T. Overview on bloom dynamics and physiological ecology of *Heterosigma akashiwo*. In: Smayda TJ, Shimizu Y, editors. Toxic Phytoplankton Blooms in the Sea. New York: Elsevier; 1993. pp. 33–41.

6. Chang FH, Anderson C, Boustead NC. First record of a *Heterosigma* (Raphidophyceae) bloom with associated mortality of cage-reared salmon in Big Glory Bay, New Zealand. New Zealand J Mar Fresh. 1990; 24: 461–469.

7. Khan S, Arakawa O, Onoue Y. Neurotoxins in a toxic red tide of *Heterosigma akashiwo* (Raphidophyceae) in Kagoshima Bay, Japan. Aquac Res. 1997; 28: 9–14.

8. Twiner MJ, Dixon SJ, Trick CG. Toxic effects of *Heterosigma akashiwo* do not appear to be mediated by hydrogen peroxide. Limnol Oceanogr. 2001; 46: 1400–1405.

9. Twiner MJ, Chidiac P, Dixon SJ, Trick CG. Extracellular organic compounds from the ichthyotoxic red tide alga *Heterosigma akashiwo* elevate cytosolic calcium and induce apoptosis in Sf9 cells. Harmful Algae. 2005; 4: 789–800.

10. Harvey EL, Menden-Deuer S. Predator-induced fleeing behaviors in phytoplankton: a new mechanism for harmful algal bloom formation? PLoS One. 2012; 7: e46438. doi:10.1371/journal.pone.0046438 PMID: 23029518

11. Tobin ED, Grünbaum D, Patterson J, Cattolico RA. Behavioral and physiological changes during benthic-pelagic transition in the harmful alga, *Heterosigma akashiwo*: potential for rapid bloom formation. PLoS One. 2013; 8: e76663. doi: 10.1371/journal.pone.0076663 PMID: 24124586

12. Strom SL, Harvey EL, Fredrickson KA, Menden-Deuer S. Broad salinity tolerance as a refuge from predation in the harmful raphidophyte alga *Heterosigma akashiwo*. J Phycol. 2013; 49: 20–31.

13. Harvey EL, Menden-Deuer S. Avoidance, movement, and mortality: The interactions between a protistan grazer and *Heterosigma akashiwo*, a harmful algal bloom species. Limnol Oceanogr. 2011; 56: 371–378.

14. Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. Biotechniques. 2005; 39: 75–85. PMID: 16060372

15. Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR—a perspective. J Mol Endocrinol. 2005; 34: 597–601. PMID: 15956331
16. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol. 2002; 29: 23–39. PMID: 12200227
17. Dundas J, Ling M. Reference genes for measuring mRNA expression. Theor Biosci. 2012; 131: 215–223.
18. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 2005; 6: 279–284. PMID: 15815687
19. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29: e45. PMID: 11328886
20. Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. Biotechniques. 2000; 29: 332–337. PMID: 10948434
21. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 2005; 139: 5–17. PMID: 16166256
22. Liu D, Shi L, Han C, Yu J, Li D, Zhang Y. Validation of reference genes for gene expression studies in virus-infected Nicotiana benthamiana using quantitative real-time PCR. PLoS One. 2012; 7: e46451. doi: 10.1371/journal.pone.0046451 PMID: 23029521
23. Gimeno J, Eattock N, Van Deynze A, Blumwald E. Selection and validation of reference genes for expression analysis in switchgrass (Panicum virgatum) using quantitative real-time RT-PCR. PLoS One. 2014; 9: e91474. doi: 10.1371/journal.pone.0091474 PMID: 24621568
24. Yang C, Pan H, Liu Y. Zhou X. Selection of reference genes for expression analysis using quantitative real-time PCR in the pea aphid, Acyrthosiphon pisum (Harris) (Hemiptera, Aphidiae). PLoS One. 2014; 9: e110454. doi: 10.1371/journal.pone.0110454 PMID: 25423476
25. Main CR, Doll C, Bianco C, Greenfield DI, Coyne KJ. Effects of growth phase, diel cycle and macronutrient stress on the quantification of Heterosigma akashiwo using qPCR and SHA. Harmful Algae. 2014; 37: 92–99.
26. Shi X, Zhang H, Lin S. Tandem repeats, high copy number and remarkable diel expression rhythm of form II RuBisCO in Procracentrum donghaiense (dinophyceae). PLoS One. 2013; 8: e71232. doi: 10.1371/journal.pone.0071232 PMID: 23976999
27. Keeling PJ, Burki F, Wilcox HM, Allam B, Allen EE, Amaral-Zettler LA, et al. The Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP): illuminating the functional diversity of eukaryotic life in the oceans through transcriptome sequencing. PLoS Biol. 2014; 12: e1001889. doi: 10.1371/journal.pbio.1001889 PMID: 24959919
28. Zhu J, Zhang L, Li W, Han S, Yang W, Qi L. Reference gene selection for quantitative real-time PCR normalization in Caragana intermedia under different abiotic stress conditions. PLoS One. 2013; 8: e53196. doi: 10.1371/journal.pone.0053196 PMID: 23301042
29. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002; 3: Research0034. PMID:12184808
30. Andersen CL, Jensen JL, Ørtoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004; 64: 5245–5250. PMID: 15289330
31. Portis AR Jr. Regulation of ribulose 1, 5-bisphosphate carboxylase/oxygenase activity. Annu Rev Plant Biol. 1992; 43: 415–437.
32. Paul JH, Kang JB, Tabita FR. Diel Patterns of Regulation of rbcL Transcription in a Cyanobacterium and a Prymnesiophyte. Mar Biotechnol. 2000; 2: 429–436. PMID: 11246409
33. Watanabe M, Kohata K, Kunugi M. Phosphate accumulation and metabolism by Heterosigma akashiwo (Raphidophyceae) during diel vertical migration in a stratified microcosm. J Phycol. 1998; 24: 22–28.
34. Coyne KJ. Nitrate Reductase (NR1) sequence and expression in the Harmful Alga Heterosigma Akashiwo (Raphidophyceae). J Phycol. 2010; 46: 135–142.
35. Rosic NN, Perrine M, Rodriguez-Lanetty M, Hoegh-Guldberg O. Validation of housekeeping genes for gene expression studies in Symbiodinium exposed to thermal and light stress. Mar Biotechnol. 2011; 13: 355–365. doi: 10.1007/s10126-010-9308-9 PMID: 20668900
36. Davis AK, Hildebrand M, Palenik B. Gene expression induced by copper stress in the diatom Thalassiosira pseudonana. Eukaryot Cell. 2006; 5: 1157–1168. PMID: 16835459
37. Stuart RK, Dupont CL, Johnson DA, Paulsen IT, Palenik B. Coastal strains of marine Synechococcus species exhibit increased tolerance to copper shock and a distinctive transcriptional response relative to those of open-ocean strains. Appl Environ Microbiol. 2009; 75: 5047–5057.
38. Siaut M, Heijde M, Mangogna M, Montsant A, Coesel S, Allen A, et al. Molecular toolbox for studying diatom biology in Phaeodactylum tricornutum. Gene. 2007; 406: 23–35. PMID: 17658702
39. Bender SJ, Parker MS, Armbrust EV. Coupled effects of light and nitrogen source on the urea cycle and nitrogen metabolism over a diel cycle in the marine diatom Thalassiosira pseudonana. Protist. 2012; 163: 232–251. doi: 10.1016/j.protis.2011.07.008 PMID: 21873112
40. Moseley JL, Chang C-W, Grossman AR. Genome-based approaches to understanding phosphorus deprivation responses and PSR1 control in Chlamydomonas reinhardtii. Eukaryot Cell. 2006; 5: 26–44. PMID: 16400166
41. Yan X, Dong X, Zhang W, Yin H, Xiao H, Chen P, et al. Reference Gene Selection for Quantitative Real-Time PCR Normalization in Reaumuria soongorica. PLoS One. 2014; 9: e104124. doi: 10.1371/journal.pone.0104124 PMID: 25117551
42. Guo R, Ki JS. Evaluation and validation of internal control genes for studying gene expression in the dinoflagellate Prorocentrum minimum using real-time PCR. Eur J Protistol. 2012; 48: 199–206. doi: 10.1016/j.ejop.2011.11.001 PMID: 22209541
43. Dong M, Zhang X, Chi X, Mou S, Xu J, Xu D, et al. The validity of a reference gene is highly dependent on the experimental conditions in green alga Ulva linza. Curr Genet. 2012; 58: 13–20. doi: 10.1007/s00294-011-0361-3 PMID: 22205301
44. Adelfi MG, Borra M, Sanges R, Montresor M, Fontana A, Ferrante MI. Selection and validation of reference genes for qPCR analysis in the pennate diatoms Pseudo-nitzschia multiserialta and P. arenysenica. J Exp Mar Biol Ecol. 2014; 451: 74–81.
45. Liu C, Wu G, Huang X, Liu S, Cong B. Validation of housekeeping genes for gene expression studies in an ice alga Chlamydomonas during freezing acclimation. Extremophiles. 2012; 16: 419–425. doi: 10.1007/s00792-012-0441-4 PMID: 22527038
46. Kianianmomeni A, Hallmann A. Validation of reference genes for quantitative gene expression studies in Volvox carteri using real-time RT-PCR. Mol Biol Rep. 2013; 40: 6691–6699. doi: 10.1007/s11033-013-2784-z PMID: 24057254
47. Zhang C, Lin S, Huang L, Wang L, Li M, Liu S. Suppression subtraction hybridization analysis revealed regulation of some cell cycle and toxin genes in Alexandrium catenella by phosphate limitation. Harmful Algae. 2014; 39: 26–39.
48. Mou S, Zhang X, Miao J, Zheng Z, Xu D, Ye N. Reference genes for gene expression normalization in Chlamydomonas sp. ICE-L by quantitative real-time RT-PCR. J Plant Biochem Biot. 2014; 1: 1–7.
49. Fagan T, Morse D, Hastings JW. Circadian synthesis of a nuclear-encoded chloroplast glyceraldehyde-3-phosphate dehydrogenase in the dinoflagellate Gonyaulax polyedra is translationally controlled. Biochemistry. 1999; 38: 7689–7695. PMID: 10387008
50. Wang GP, Xu CS. Reference gene selection for real-time RT-PCR in eight kinds of rat regenerating hepatic cells. Mol Biotechnol. 2010; 46: 49–57. doi: 10.1007/s12033-010-9274-5 PMID: 20339955
51. Gu C, Chen S, Liu Z, Shan H, Luo H, Guan Z, et al. Reference gene selection for quantitative real-time PCR in Chrysanthemum subjected to biotic and abiotic stress. Mol Biotechnol. 2011; 49: 192–197. doi: 10.1007/s12033-011-9394-6 PMID: 21416201
52. Chung CC, Hwang S-PL, Chang J. Nitric oxide as a signaling factor to upregulate the death-specific protein in a marine diatom, Skeletonema costatum, during blockage of electron flow in photosynthesis. Appl Environ Microb. 2008; 74: 6521–6527.
53. Blanco EP, Hagström J, Salomon PS, Granéli E. Detection of Heterosigma akashiwo (Hada) using specific RNA probes: Variability of RNA content with environmental conditions. Harmful Algae. 2013; 24: 80–88.
54. Boldt L, Yellowlees D, Leggat W. Measuring Symbiodinium sp. gene expression patterns with quantitative real-time PCR. Proceedings of the 11th ICRS, 7–11 July 2009, Ft. Lauderdale, Florida, pp. 118–122.
55. Vrede T, Dobberfuhl DR, Kooijman S, Elser JJ. Fundamental connections among organism C: N: P stoichiometry, macromolecular composition, and growth. Ecology. 2004; 85: 1217–1229.
56. Pichard SL, Campbell L, Kang JB, Tabita FR, Paul JH. Regulation of ribulose bisphosphate carboxylase gene expression in natural phytoplankton communities. I. Diel rhythms. Mar Ecol Prog Ser. 1996; 139: 257–265.
57. Lee JM, Roche JR, Donaghy DJ, Thrush A, Sathish P. Validation of reference genes for quantitative RT-PCR studies of gene expression in perennial ryegrass (Lolium perenne L.). BMC Mol Biol. 2010; 11: 8–21. doi: 10.1186/1471-2199-11-8 PMID: 20989196