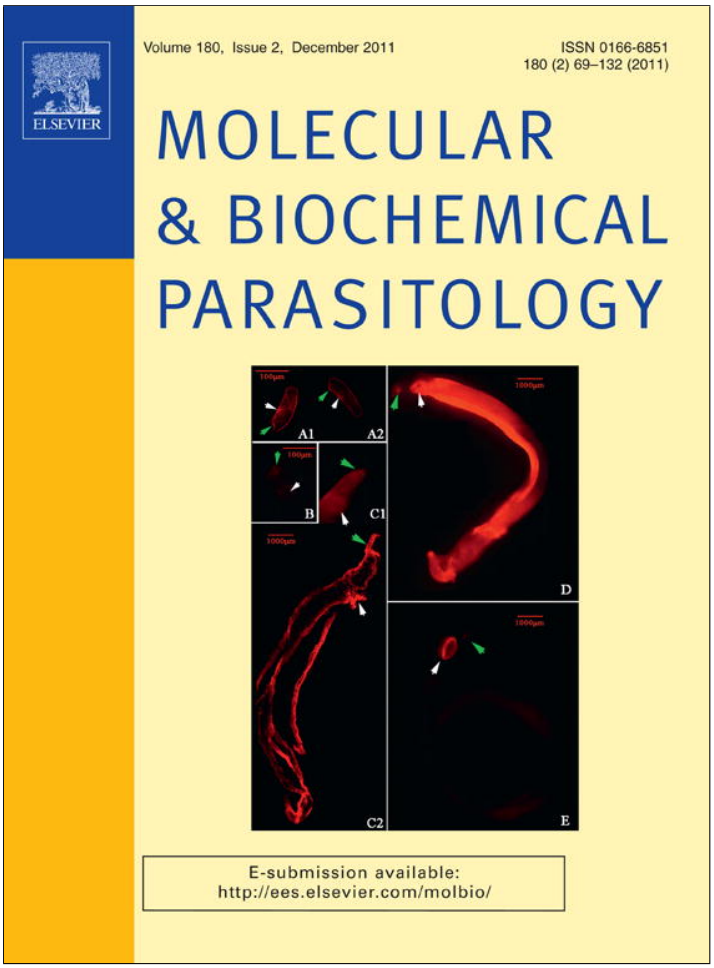

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Short communication

Spatial and temporal consistency of putative reference genes for real-time PCR in a model tapeworm

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ABSTRACT

Relative quantification of gene expression by real-time PCR relies on the use of reference genes whose expressed levels remain consistent across experimental conditions. Here we compare expression levels of commonly employed endogenous housekeeping genes against a developmental regulatory gene in the model tapeworm *Hymenolepis microstoma*, examining variation both spatially across regions of the adult worm and temporally through the course of larval metamorphosis. β-Tubulin, RNA polymerase II and 60S ribosomal subunit L28 showed the most variance among candidate reference genes when comparing changes in expression along the anteroposterior gradient of development represented by the adult body, whereas expression of 18S rDNA and cyclic AMP were highly consistent and could be used reliably for relative quantification. The transcription factor Hox4, referenced to either 18S or cAMP, showed 3-fold higher expression levels in the neck region than in more mature regions of the strobila. In contrast, variance among samples representing progressive stages of larval metamorphosis were greater for candidate reference genes than for Hox4, indicating that none of the candidates can be used reliably for quantifying relative changes in gene expression during metamorphosis.

Determining relative gene expression by quantitative real-time PCR (QPCR) relies on a reference gene that is ideally expressed at equal levels throughout ontogeny and in all regions of the body, and may thus be used to compare changes in expression levels of genes of interest (GOI) whose expression is expected to vary according to experimental conditions. Relative comparison circumvents the need for precise quantification of starting material and is especially convenient when performing experiments where sample quantities are limited. Although endogenous housekeeping genes are employed commonly for this purpose, consistency in their expression levels has been examined explicitly in few organisms. Confirmation may be particularly important when regarding animals with complex developmental sequences, as exhibited by most helminth groups. For example, Trivedi and Arasu [1] examined expression levels of housekeeping genes throughout the life cycle of the hookworm *Ancylostoma caninum*, finding considerable variability throughout ontogeny in many commonly employed genes. The anteroposterior gradient of development represented by the adult tapeworm body, together with the metamorphosis of the larvae in the intermediate host, suggest significant spatial and temporal changes in gene expression throughout tapeworm development that may involve changes in the expression levels of housekeeping, in addition to developmental regulatory and other genes. Here we examine orthologs of five highly expressed candidate reference genes in the model tapeworm *Hymenolepis microstoma* [2] together with that of a developmental regulatory transcription factor [3], comparing consistency of expression both spatially along the gradient of adult development, and temporally throughout larval metamorphosis, from oncosphere to cysticercoid.

Partial transcript sequences of 60S acidic ribosomal protein subunit L28 (GenBank no. JN030765), cyclic AMP-dependent protein kinase A (cAMP; JN030767), β-tubulin (JN030766), RNA polymerase II subunit (RNA pol2; JN030768) were identified either from SMARTer (Clontech) cDNA libraries shot-gun sequenced using Roche 454 next generation sequencing and representing adult worms, or from more recently generated draft genome sequences (see [4]) publically available from http://www.sanger.ac.uk/resources/downloads/helminths/hymenolepis-microstoma.html. Partial sequences for 18S ribosomal RNA (AJ287525) and an ortholog of the central class Hox gene Hox4 (EU817097), homologous to the *deformed* gene of *Drosophila*, were characterized previously [3,4]. Hox4 was included to provide comparison with a transcription factor exhibiting spatially and temporally restricted expression (unpub. data). Multiple sets of QPCR primers for each gene were designed using PrimerSelect (Lasergene) or Primer3Plus [5], specifying a product size of 95–105 bps and an annealing temperature of 60 °C. Specificity and efficiency of each primer combination was tested empirically by QPCR on a dilution series of cDNA from a whole adult worm and including a post-PCR high-stringency melt step to assay for

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the presence of extraneous amplicons. Standard curve analysis confirmed the efficiency of reactions in the range of 96–100% for each of the following primer combinations: 60S L28 (Hm_60S-L28-QF2:AGCAGGAAAAGGGAGGTAAAGGCG + Hm_60S-L28-QR2:GTGCGTCGATTCCCCCGGAC), β-tubulin (Hm_BTubulin-QF2: TCTTGTCTCTGCCACTATGTCAGGT + Hm_BTubulin-QR2: GCAAACGGGGGAAGGGAACCA), cyclic AMP-dependent kinase (Hm_cAMP-QF1: CGCCAAAGTGGTCAAAGGCCG + Hm_cAMP-QR1: CGCCCACCAATCCACCGCTC), RNA polymerase II (Hm_RNAPol2-QF1: CCGCCACGGCCAAAAGGGTA + Hm_RNAPol2-QR1: GTCATACGACTGGGAATAGCGTGAG), 18S rRNA (Hm_18S-QF2: TTACGGAAGGGCACCACCAGGAGT + Hm_18S-QR2: AACGGCCATGCACCACCAACC), and Hox4/*deformed* (Hm_Dfd-QF2: CCCCAAGCGCCGAGGTTCAA + Hm_Dfd-QR2:TAGCGGTGGCGGTCCAGGAA).

cDNA samples representing increasingly mature regions of the adult tapeworm body and 'stages' of larval development were prepared as follows. Stock cultures of *H. microstoma* (Nottingham strain) [2] were maintained *in vivo* using outbred conventional mice and flour beetles (*Tribolium confusum*). Fully developed, gravid tapeworms were dissected from the bile ducts of mice and washed thoroughly in saline. Two whole worms were cut into four equal regions corresponding to the scolex, neck and nascent segments (R1), immature segments (R2), mature segments (R3) and mature, senescent and gravid segments (R4), providing two independent samples of each region of the adult worm. Following dissection all samples were placed immediately on dry ice and processed immediately or stored at −80 °C for up to a week.

A larval developmental time series was produced by exposing beetles to macerated gravid tissues of *H. microstoma* for 5 h, after which they were replaced with flour (see www.olsonlab.com for more information on the culture of *H. microstoma*). The cultures were then sub-sampled on days 3, 4, 5, 6, and 7 post-exposure and ~100 larvae from each developmental time period dissected from their abdomens, pooled, frozen on dry ice and stored at −80 °C for up to a week. These time intervals correspond approximately to the five 'stages' of metamorphosis originally described by Voge [6, see also 2], although the combination of a 5 h exposure window and non-uniform growth rates results in a degree of mixing of 'stages' that becomes more pronounced throughout development.

Total RNA was extracted from the samples using an RNeasy Plus Micro Kit (Qiagen) according to manufacturer guidelines. Samples were additionally treated with TURBO DNA-free DNase (Ambion) to further reduce genomic DNA contamination, ethanol precipitated and re-suspended in nuclease free water. RNA concentration was measured with a NanoDrop-1000 spectrophotometer and quality assessed by analyzing the absorbance spectra at A260/280 and A260/230 ratios. Total RNA was further subjected to quality control, where possible, and visualized for integrity on a 2% agarose gel. For each sample, 100 ng of total RNA was used for cDNA synthesis. All template cDNAs were synthesized using a QuantiTect Reverse Transcription Kit (Qiagen). A single aliquot of each sample (100 ng total RNA) was treated identically, but without the addition of reverse transcriptase, in order to serve as a negative (RT−) control template for detection of sample contamination with genomic DNA. All QPCR reactions and dilution series were set up using a dedicated Corbett liquid handling robot to ensure consistency, and negative controls were included in each run. Twenty μl reactions were run in duplicate on a Corbett RotorGene 6000 using RotoGene SYBR-Green chemistry (Qiagen) in a two-step RT-PCR protocol including a post-run melt curve step to verify specificity of the reaction products. Cycling conditions were: 5 min at 95 °C; followed by 40 cycles of 5 s at 95 °C and 10 s at 60 °C. Real-time data were recorded and analyzed using Corbett's dedicated software. Ct values (*i.e.* number of cycles required for the fluorescent signal to cross the threshold above background) were used to compare variability in transcript levels. In order to compare Ct values between

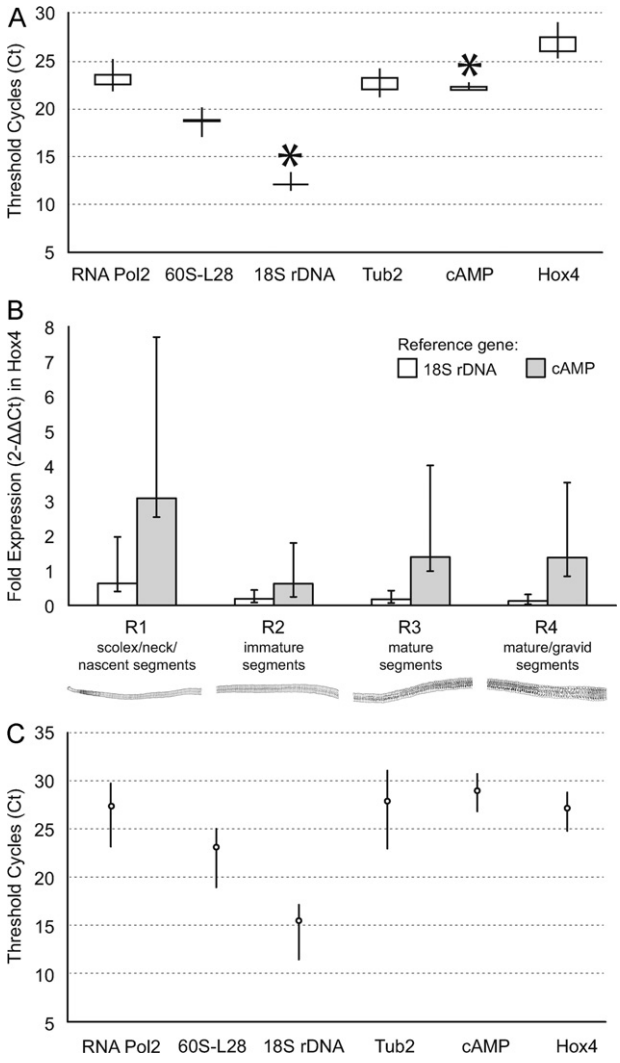

Fig. 1. Spatial and temporal variation in expression of candidate reference genes and the transcription factor Hox4 in the model tapeworm *Hymenolepis microstoma*. (A) Variance in expression across four progressively maturing regions of adult worms (see B). Boxes show average Ct values for two replica samples from duplicate runs and lines indicate max/min Ct values. 18S and cAMP showed significantly lower variances (asterisks) than either the other candidate reference genes or Hox4. (B) Fold-difference in expression of Hox4 across four progressively maturing regions of the adult worm as referenced against 18S or cAMP. Histograms show average and lines show max/min relative expression levels for Hox4. The four regions of the worm are illustrated below the histogram (as modified from [2]). Referenced against either gene, Hox4 is shown to be expressed in a gradient, with the highest levels seen in the R1 region (see text). However, as cAMP is expressed at levels more similar to Hox4, it provides a better choice of reference for detecting changes in low abundance transcripts such as transcription factors and signaling molecules. (C) Variance in expression throughout larval metamorphosis. Dots show average Ct for each gene across the five larval 'stages' and lines show max/min Ct values. Candidate reference genes show greater variance than Hox4, making them unsuitable for comparing changes in expression of GOI during level development.

different assays, threshold values were set to the same level. Variances among the four regions of adult worms and five larval time stages were calculated and *F*-tests used to test for significant differences in expression levels of adult samples. Relative expression levels of Hox4 as referenced to either 18S or cAMP and normalized to a calibrator (*i.e.* standard cDNA from a whole adult worm) were calculated by the $2^{-\Delta\Delta Ct}$ method [7].

Fig. 1A shows comparison of Ct values for all candidate reference genes and Hox4 in progressively maturing regions of the adult tapeworm. Of the candidate genes, β-tubulin, RNA pol2 and 60S-L28 showed the highest levels of variance within datasets (1.78,

1.60 and 1.13 respectively), whereas variances for 18S (0.72) and cAMP (0.25) were significantly lower. Based on a *F*-test, cAMP had a significantly smaller variance when compared to all other genes except 18S (cAMP *vs* RNApol2: *P-value* = 0.026; cAMP *vs* 60S-L28: *P-value* = 0.065; cAMP *vs* 18S: *P-value* = 0.19; cAMP *vs* Tub2: *P-value* = 0.058; and cAMP *vs* Hox4: *P-value* = 0.017).

As expected, the highest overall variability across the four regions was found in the transcription factor Hox4 (variance = 1.87). Spatial expression of Hox4 examined *via in situ* hybridization (manuscript in prep.) shows that its expression is restricted primarily to the neck region, with only small foci of expression persisting in developing proglottides. This is consistent with changes in expression levels across the four progressively mature regions of the adult body detected *via* QPCR as seen in Fig. 1B. This figure compares change in Hox4 expression as referenced against 18S and cAMP, both of which show consistency of expression across the different regions, but very different absolute expression levels (as seen in Fig. 1A). Because cAMP is less abundantly expressed than 18S, Ct values for cAMP (22.6) are more similar to those of Hox4 (26.7). cAMP is thus a better reference than 18S (cf. Ct = 12.3) for assaying relative change in genes of low abundance, such as transcription factors.

Fig. 1C shows variance in expression during larval metamorphosis, ranging from 3.9 (cAMP) to 9.9 (β-tubulin), whereas Hox4 showed a variance of only 4.2. We thus detect larger changes in the expression of 'housekeeping' genes throughout metamorphosis than in a developmental regulatory gene whose expression we have found to be restricted spatially and temporally throughout larval development. Thus the candidate genes examined here are unreliable for assaying changes in the expression levels of GOI.

With the recent availability of whole genome data for tapeworms [4], it is increasingly likely that researchers will want to assay gene expression using techniques such as QPCR or whole transcriptome profiling *via* RNA-seq [8]. Our results show that expression levels of *H. microstoma* cAMP and 18S rDNA remain consistent spatially across different regions of adult worms, but that significant temporal changes in expression are found in both endogenous 'housekeeping' and developmental regulatory genes throughout the course of larval metamorphosis. Although some variation in reference gene expression may be tolerable when assaying GOI that exhibit high levels of expression or large differences among experimental samples, it may mask changes in expression during metamorphosis of less abundantly expressed genes, such as developmentally related transcription factors and signaling molecules [4,9].

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