Optimization of qPCR Techniques to Determine Environmental DNA Transport in Stream Systems

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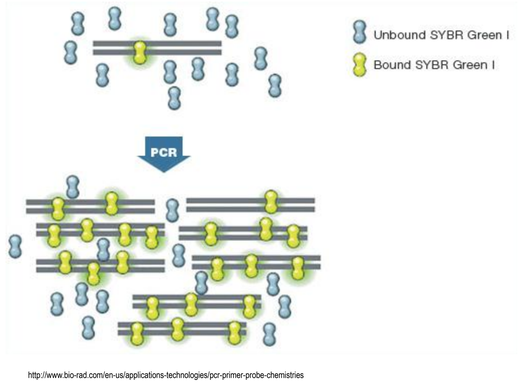
Abstract

Environmental DNA (eDNA) surveillance is an increasingly popular tool for detecting aquatic organisms that does not require visual surveys. In many studies, this process has shown to be successful at indicating the presence of specific species (Muesnier et al. 2008). PCR primers were previously developed to amplify small fragments of DNA from several species of freshwater mussels including *Margaritifera margaritifera*, *Elliptio complanata*, and *Pyganodon cataracta,* and were shown to be effective in a small stream setting (Eldridge and Borecki, pers. comm.). However, there is still a limited understanding of how eDNA moves through streams and water systems (Jane et al. 2014). Understanding how the concentration of eDNA changes over distance would provide some insight (Turner et al. 2014). Real-time quantitative PCR (qPCR) is highly sensitive (detection of 1 copy per uL is possible) and can be used to quantify the amount of eDNA present (Wilcox et al. 2013). We optimized qPCR conditions to ensure that only the target sequence was amplified, a concern when dealing with environmental samples. Optimal conditions were: 1:10 dilution of template DNA, 65˚ C annealing temperature, 1 mM $MgCl_2$ and the addition of BSA. In an outdoor flume, eDNA concentration increased over 25 meters downstream from four mussels, which may indicate that mussels release DNA in pulses. In a natural stream, eDNA was not detected 5m or more downstream from the source which could be due to the presence of natural PCR inhibitors. Applying the eDNA test to natural setting, mussel DNA was detected in water samples from one of three sites where they have not been observed by visual surveys.

How qPCR Works

In this study, we optimized and used SYBR Green real-time qPCR. SYBR Green is a dye which fluoresces when bound to double-stranded DNA. As more of the target DNA is produced through PCR, more binding occurs and thus there is an increase in fluorescence resulting in a direct relationship between the change in fluorescence and the initial quantity of the DNA template.



http://www.bio-rad.com/en-us/applications-technologies/pcr-primer-probe-chemistries

Comparison with a known standard can be used to determine the initial eDNA concentration in a sample.

Experimental Design

Four mussels were planted in a natural (small stream) or artificial (outdoor flume) setting. In all experiments, we allowed enough time for eDNA particles to equilibrate downstream and then took 1L water samples at various distances from the mussels.

Stream: Archie's Branch

Outdoor flume

Water samples were also taken at sites where mussels had not been previously detected by visual surveys. All water samples were filtered using 47mm 1.5-micron glass-fiber filters. The DNA was then extracted using the DNEasy Blood & Tissue Kit (Qiagen, Valencia, CA 91355). qPCR was conducted on a ABI 7500 Fast Real-Time PCR System using SYBR Green I and a melt curve was generated to confirm that only one fragment was amplified.

Margaritifera margaritifera, the mussel species planted for these experiments

qPCR Optimization

Prior to running the qPCR we optimized the PCR process to make sure only the mussel target sequence was amplified using the following conditions:

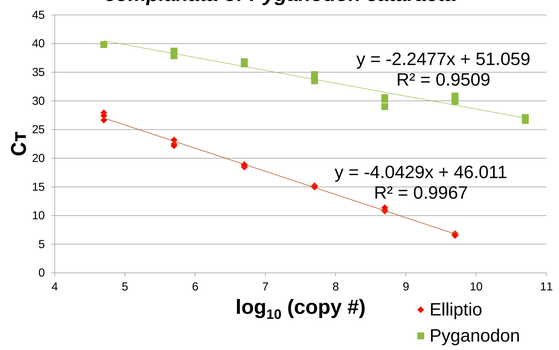
- Template DNA concentration: 1 uL template without dilution,1:10 and 1:100 dilutions
- Annealing temperature: 60-70˚ C
- $MgCl_2$ concentration: 1 mM and 1.5 mM $MgCl_2$
- BSA: present or absent

The optimal PCR conditions were determined to be: 1:10 dilution of template DNA, 65˚ C annealing temperature, 1 mM $MgCl_2$ and the presence of BSA.

qPCR using SYBR Green I (Applied Biosystems, Carlsbad, CA 92008) was further optimized by testing the intra-assay coefficient of variation (3 replicates per sample), linearity of dilution (10^0 to 10^{-6}) a melt curve and sequencing.

Results

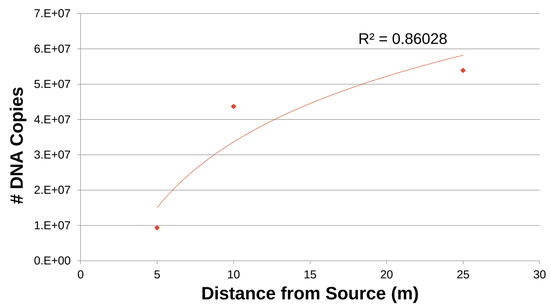
Standard Curve for EcoCOI primer and *Elliptio complanata* or *Pyganodon cataracta*



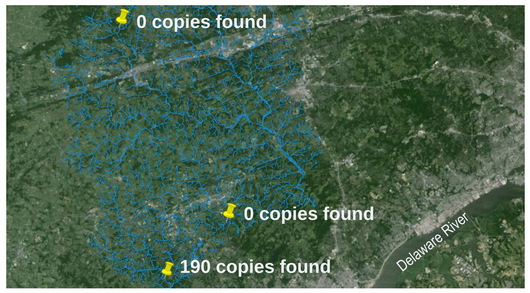
Validation test for optimization	Test Results
Coefficient of variation	1.89%
Linearity of dilution	R^2 = 0.99672 *(Elliptio)*, 0.95088 *(Pyganodon)*
Melt curve	Melt curve confirmed the presence of only one PCR product
Sequencing	DNA sequencing confirmed that the intended fragment was amplified

Using the formulas derived from the standard curves for each primer, we were able to determine the number of DNA copies for each of our unknown samples.

Change in Concentration over Distance (Flumes)



No eDNA was detected in Archie's Branch (natural setting) samples.



Mussel DNA was detected in water samples from one of three sites where they have not been observed by visual surveys

Discussion

Based on validation tests for qPCR optimization (coefficient of variation, R^2, melt curve and DNA sequencing), qPCR using the EcoCOI primers shows accurate and precise quantification of mussel DNA. We expected that the concentration of eDNA would decrease as it traveled further away from the source because of settling (Newbold et al.1982) but the flume experiment indicates that mussel eDNA does not settle quickly. One possible explanation for the concentration increase is that DNA is not released from mussels at a constant rate. Instead, DNA could be released in pulses (lots of DNA at one time) and we sampled different parts of the DNA plume downstream. Future studies should control for possible pulses of DNA release. One possible way to determine eDNA transport while eliminating the possibility of pulsed DNA is to conduct a DNA injection along with rhodamine dye and to measure DNA in the water as the dye travels downstream. The failure to detect DNA in the small stream suggests that either DNA did not travel 5m or that natural inhibitors prevented DNA amplification by PCR. Natural inhibitors include humic acids, fulvic acids, melanin, and polysaccharides which could all easily be present in the small stream where we tested. qPCR also revealed that mussel DNA was present in one site were they have not been observed by visual surveys.

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