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Development and Testing of Environmental DNA (eDNA) Protocols for the Endangered James Spinymussel (*Pleurobema collina*)

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The results of the study suggest that subsequent work should focus on overcoming abiotic inhibitory compounds in the water column and examining biomass across a set of locales with few individuals to determine the lower limits of detectability for this protocol. These findings suggest continued development of this approach, as it is broadly applicable to activities of the Virginia Department of Transportation beyond this sole cryptic and endangered species. The study recommends that a second phase of research be conducted to refine the eDNA methods developed in this study in order to increase their implementation potential as a survey tool.

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FINAL REPORT

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ABSTRACT

Molecular genetic techniques provide tools that may be used to locate, monitor, and survey cryptic aquatic species. This study developed genetic markers useful in determining if the James Spinymussel (*Pleurobema collina*), an endangered species, can be detected solely by sampling stream water.

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INTRODUCTION

Choosing the appropriate methodology for identifying the presence and abundance of species is difficult if the taxon is rare or cryptic in appearance. For freshwater mussels, survey approaches are commonly challenged by small, isolated populations within restricted geographic ranges (e.g., Smith et al., 2001; Strayer et al., 1996). Despite these challenges, appropriate sampling protocols are necessary to identify the presence of cryptic species, particularly in the area of proposed or ongoing impact—if the species is a listed threatened or endangered, the Virginia Department of Transportation (VDOT) faces challenges in both planning and execution of maintenance and development programs. As a result, field sampling approaches should be applied that maximize the probability of correctly identifying the presence of these cryptic taxa while at the same time allowing for a probabilistic estimation of sampling error rates (e.g., not detecting the taxon even though it is present—a Type II statistical error). This work outlines the development of molecular genetic techniques for identifying the presence and abundance of the James Spinymussel (*Pleurobema collina*; Unionidae), an endangered freshwater mussel endemic to the upper James and Dan river basins in Virginia, West Virginia, and North Carolina. Adults can grow as large as 3 inches, have prominent growth rings, and occasionally have spines on each value. The molecular techniques developed herein are designed to augment conventional physical surveys that result in a positive detection of an individual or population, and if possible replace physical surveys when a negative detection is found. The first step in this process requires the development of species-specific markers and a determination of in situ water collection and DNA amplification can be used to estimate relative standing biomass.

The James Spinymussel is a freshwater mussel endemic to the lotic aquatic habitats in non-tidal streams of the James River basin in Virginia (Hove, 1994). Ongoing landscape modification, sedimentation from upland sources, and competition from invasive species such as the Asian Clam (*Corbicula fluminea*) have increased habitat fragmentation (Petty, 2005). While Hove (1990) reported that seven common fishes may serve as hosts for the Spinymussel, the abundance of these Cyprinidae (minnow) species has not changed dramatically suggesting that the decline is not due to host availability (Petty, 2005). Over the last couple of decades, these impacts have resulted in a loss of over 90% of the species native range, resulting in listing under

the Endangered Species Act. At present, the Virginia Department of Game and Inland Fisheries (VDGIF) has implemented an extensive conservation program designed to locate populations and conserve critical habitat.

Environmental DNA (eDNA) is a non-invasive means of detecting the presence of rare, endangered, or invasive species by isolating discrete pieces of nuclear (nDNA) and mitochondrial DNA (mtDNA) from the water column. Minute particles of tissue, either excreted or shed from individuals in situ, is used as raw template for DNA extraction and subsequent amplification using species specific genetic markers. Over the past decade, an international coalition of researchers has developed targeted DNA sequences with sufficient diversity to be used as species-specific markers. These "DNA barcodes" have been used to classify taxa ranging from nematodes to elephants (Eggert et al., 2002; Floyd et al., 2002) and have recently been applied to both species identification and monitoring of presence/absence in aquatic habitats. Example applications include identifying invasive species such as the silver carp in the Mississippi drainage (Hickcox, 2011) and the American Bullfrog across Spain (Ficetola et al., 2008) and identifying the presence of cryptic species such as the Rocky Mountain Tailed Frog and the Idaho Giant Salamander (Goldberg et al., 2011).

Given the regulatory constraints on VDOT activities relative to bridge and road maintenance in proximity to endangered aquatic species, the addition of molecular genetic approaches to the existing protocols may have several implications for ongoing monitoring programs and management policies. First, given the cryptic nature of these organisms, the current detection probabilities for this species range from 12% to 20% (Esposito, 2015; VDGIF, 2015) for mark and recapture of individual mussels. At the site level, physical detection is almost guaranteed for large populations, though the ability to detect an individual is highly variable for locales with only few individuals. Molecular techniques based upon water sampling may help to augment these rates thereby increasing the confidence in where populations exist relative to road projects. Second, the life history of these organisms make sampling efficiency temporally variable as the organisms are more accessible during certain times of the year. Despite their variable position within the substrate throughout the year however, they are continually in contact with the water column with the potential for providing assayable DNA samples independent of substrate position. Third, molecular approaches are very amenable to high throughput. Evaluation of many locations can be assayed first using an eDNA approach thereby potentially reducing the number of areas and regions requiring physical field surveys. In this manner, eDNA approaches serve to create additional efficiencies in existing sampling protocols by allowing field technicians to prioritize the locations they sample.

PURPOSE AND SCOPE

The purpose of this study was to develop and field test the use of eDNA approaches to detect the presence and absence of the James Spinymussel within native stream reaches in the upper James River basin. The scope of this study included development of de novo molecular genetic markers that will differentiate this species from all other organisms that coexist in native streams. Next, using both laboratory and field experiments, the utility of quantitative polymerase

chain reaction (qPCR) in estimating relative standing biomass from the amount of DNA template found in the water column was explored, facilitating not only the identification of where individuals may occur but also the relative abundance of Spinymussel along a stream reach.

METHODS

Study Area and Sampling

The endangered James Spinymussel is currently found in the upper James River drainage and within restricted sections of the Dan River and Mayo River systems. For marker development, a VDGIF biologist provided tissue from dead individuals for analysis. Field collections were taken from stream reaches whose local densities are known to VDGIF biologists from previous surveys (Figure 1). Exact locations are not reported given this species' status as endangered.

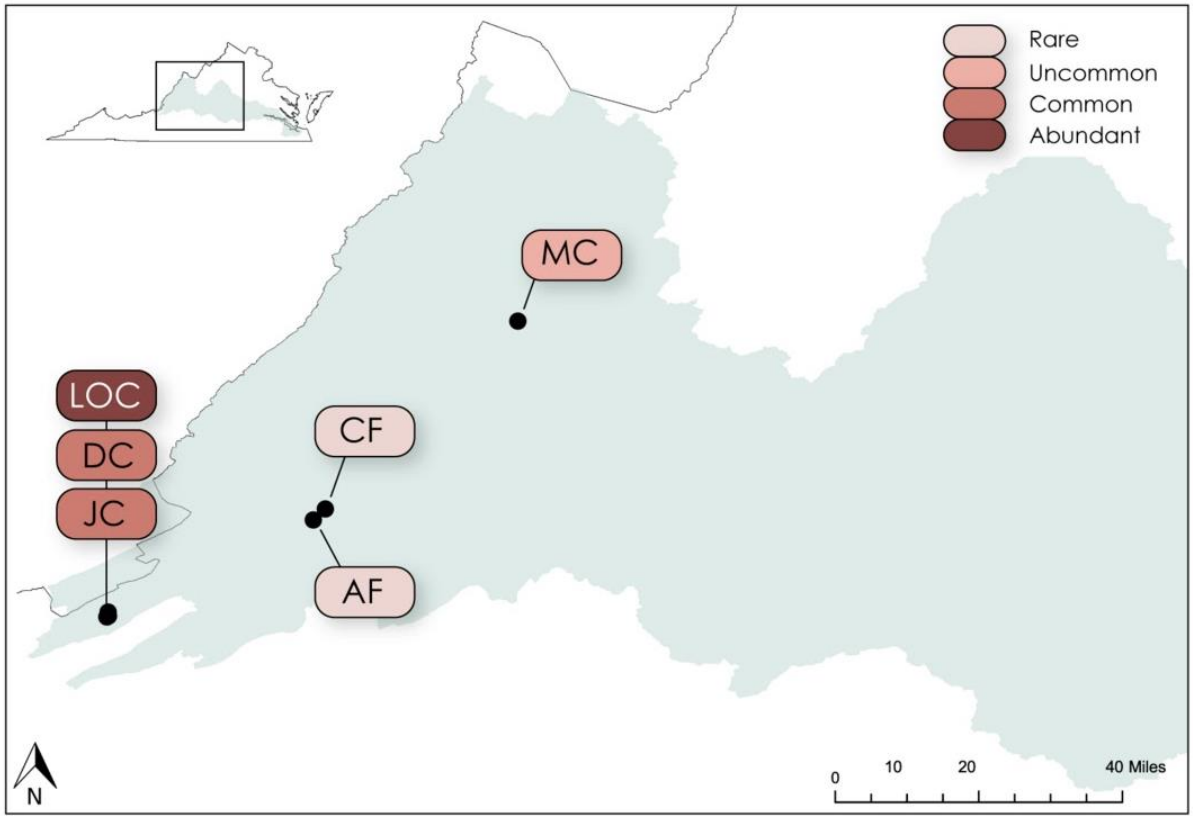

Figure 1. Sampling Region and Locales for James Spinymussel (*Pleurobema collina*). Tags denote individual stream reaches whose local densities were estimated and ranked by Virginia Department of Game and Inland Fisheries biologists. Exact locations are not reported given this species' status as endangered.

Preliminary water samples were collected in June 2016 from DC and MC to aid in initial protocol development. The main sampling was conducted in July 2017 after validation of the approach and development of species specific genetic markers (as discussed later. At each site (Figure 1), four separate samples were collected. An additional set of four samples was collected

from the Pamunkey River and served as a negative control as *P. collina* is not known to inhabit the stream water in that drainage. At all sites, samples in 1-L volume increments were collected in sterile Nalgene bottles in accordance with the procedures of Laramie et al. (2015). Stream characteristics such as flow rate, dissolved oxygen, pH, temperature, and turbidity were also measured at each site to determine if site-specific features may either inhibit or reduce the efficiency of DNA amplification (Jane et al., 2014). Samples were then filtered through 0.45-micron nitrocellulose filters and stored in 100% ethanol at -20°C for subsequent DNA extraction. Initial sampling for this study focused on taking collections during the reproductive period (Hove and Neves, 1994) to maximize the potential for detection.

Marker Development and Genetic Assays

Marker development was initiated using tissue from seven individuals provided by a VDGIF biologist. DNA was extracted and purified from these samples using the DNEasy Tissue Kit (Qiagen, Natick, Massachusetts) following standard tissue protocols (e.g., Denier et al., 2015). All samples were kept at -20°C in DNAse/RNAse-free distilled water. Genetic markers for genomic regions commonly used in eDNA or DNA Barcoding projects were taken from the literature. Primers were developed targeting the genetic regions surrounding the nuclear gene ITS-1 (*RNA internal transcribed spacer #1*) and the three mitochondrial genes ND1 (*NADH dehydrogenase-1*), CO-1 (*Cytochrome oxidase-1*), and CYT-B (*cytochrome-B*). These primers were designed considering genetic variability in both the target species *P. collina* and other mussel taxa that are thought to occur in the same general region. DNA sequences were either produced from tissue directly or retrieved from online repositories (e.g., GenBank Accessions) for the target species and the potentially co-occurring species *Alasmidonta undulata, Elliptio complanata, E. producta, E. fisheriana, E. angustata, Fusconaia masoni, Lasmigona subvirdis, Pyganodon cataracta, Strophitus undulatus,* and *Utterbackia imbecillis.* One potentially co-occurring taxon, *E. lanceolata,* had no published sequences, and the researchers were unable to obtain tissue to test—though the extent of potential spatial overlap between *E. lanceolata* and *P. collina* is rather small, so false positives were not anticipated to arise in subsequent analyses. Primers were developed in silico using NCBI Blast and verified in vitro using qPCR. The genomic region that had the most species specificity was selected, and primer design was optimized to meet project goals.

Verification of species specific primers was determined by Sanger sequencing of amplified bands and comparing to known (both derived in this study and available from publications) genetic sequences of the target and potentially co-occurring taxa. Sequencing amplifications were performed using normal PCR under the following conditions: 92°C, 2 min; 92°C, 40 seconds; 40°C, 40 seconds; and 72°C, 90 seconds for five replicates. Then, cycling 25 repetitions of 92°C, 40 seconds; 50°C, 40 seconds; and 72°C, 90 seconds were conducted, followed by a final elongation phase at 72°C for 10 minutes. All PCR products were purified with ExoSAP-IT (Affymetrix). All sequencing was performed by Nevada Genomics. Sequenced products were then compared and aligned to known species standards using BIOEDIT.

Biomass Prediction

An estimate of the relative biomass of individuals along a stream reach was estimated using qPCR. This technology allows the density of DNA fragments to be tracked during the polymerase amplification process. More initial DNA content results in a more rapid increase of amplified products than less initial DNA template. Given known standing biomass estimated from field surveys, qPCR can provide a standardized curve for estimation of local population biomass in non-surveyed areas. Amplification was conducted on a CFX96 Touch Real-Time PCR Detection System (BIO-RAD, Hercules, California) using the following settings: SYBR green only, Denaturation at 98°C for 3 minutes, 40 amplification cycles of 98°C for 15 seconds, 53°C for 30 seconds, plate read at 65°C, with curve analysis set to instrument default. All sample runs contained a negative control (e.g., a sample of stream water without *P. collina* template DNA from the Pamunkey River drainage) to detect any potential contamination.

An initial standardized curve was created from a serial dilution in the laboratory starting using a reference template of known concentration. A set of serial dilutions with 10 ng/µL, 1 ng/µL, 0.1 ng/µL, 0.01 ng/µL, 0.001 ng/µL, and 0.0001 ng/µL was created and amplified. The number of cycles necessary to reach a standardized threshold in amplified product, denoted by C_q, was recorded. The fit of this log-normal relationship provides both an internal check on the efficiency of laboratory protocols and a metric by which sample DNA concentration can be connected to local population density.

Inhibition of PCR

Inhibitors present in environmental samples can affect the reaction efficiency of qPCR by binding to nucleic acids, changing their chemical properties, or reducing the specificity of the primers (Abbaszadegan et al., 1993; John, 1992; Opel et al., 2010). For water samples, the most likely inhibitors present are dissolved or solid organic compounds such as fulmic acids, humic acids, humic material, metal ions, and polyphenol (Abbaszadegan et al., 1993; Ijzerman et al., 1997). The effect of these inhibitors can be reduced either by diluting the sample or identifying and removing the specific inhibitors.

Inhibition can be quantified by comparing the expected C_q value (e.g., DNA concentration) to the measured C_q value of a water sample spiked with template DNA at a known concentration. If the spiked sample has a higher C_q value by one or more cycles than expected, it is considered inhibited (Gibson et al., 2012). Since abiotic inhibition is a characteristic of the entire locale, any locales with known populations that fail to yield PCR products were subsequently examined for potential inhibition.

Detection Probability

Detection probability for the eDNA protocol developed herein was estimated as the fraction of all samples collected at a locale that had positive identified of *P. collina* DNA. Locales contained four to seven water samples, each of which was further subdivided into two

subsamples during DNA extraction. This stratified sampling facilitated the estimation of detection probability as a function of collected water volume and a function of sampling density at locales with known population size.

RESULTS

Study Area and Sampling

Water samples were collected from each locale (Table 1), and each sample was subdivided into replicates for DNA extraction and subsequent qPCR amplification. Sampled locales represented a broad range in flow rate, temperature, pH, and dissolved oxygen. Historical census estimates were provided by a VDGIF biologist, though predation and ongoing demographic changes at these locales necessitated the use of rank population sizes as a more realistic estimate of biomass when eDNA samples were collected.

Table 1. Site-Specific Features of Stream Sampling Intensity and Locale

Locale	N	N_{qPCR}	Turbidity	Flow	Temp.	pH	DO_1	DO_2	Census	Rank
JC	6	14	3.6	1.8	16.1	5.6	94.1	9.3	428	4
LOC	4	12	7.2	0.6	18.1	5.8	93.6	8.9	1125	6
DC	4	12	6.3	0.9	19.0	5.4	89.5	8.3	430	3
AF	4	12	0.03	7.7	17.5	7.0	94.0	9.0	5	1
CF	4	12	0	11.4	17.9	6.9	94.8	9.0	4	2
MC	7	17	1.8	0.5	18.6	7.1	101.8	9.5	173[a]	5

Exact locations are not reported given this species' status as endangered. The number of samples (N) was subdivided into multiple qPCR amplifications (N_{qPCR}). For each location, estimates of turbidity (nephelometric turbidity units, NTU); stream flow (flow in m^3/s); water temperature (in °C); pH; and dissolved oxygen (DO_1 as percent and DO_2 as mg/L). Historical census counts were provided for each locale along with an estimate of current rank population size. Population census data were provided by the Virginia Department of Game and Inland Fisheries and reflect collections taken from 2010 through 2013. Population ranks relate to best estimates for current (2017) local population sizes.

[a] This site was augmented with hatchery grown individuals between the last physical survey and the sampling for this study. At present, it is unknown what the real census size may have been.

Marker Development and Genetic Assays

The four genomic regions, ITS-1 (*RNA internal transcribed spacer #1*), ND1 (*NADH dehydrogenase-1*), CO-1 (*Cytochrome oxidase-1*), and CYT-B (*cytochrome-B*), were assayed for target and co-occurring species. The genetic region surrounding the ND-1 (*NADH dehydrogenase-1*) gene was selected as the most promising for development of species-specific markers, given the amount of nucleotide divergence between *P. collina* and all other potentially co-occurring taxa. Several candidate primers were designed to minimize the potential for false positive PCR results for *P. collina* while at the same time produced the most uniform qPCR profile. Across 14 different primer combinations (Appendix, Table A1), the primer sequences ND1_pcbr1 (forward) 5'-GCGTAGCATTCTTTACCCTTCT-3' and ND1_pcbr1 (reverse) 5'-GAGCGTCTGCTAATGGTTGT-3' were found to be the most efficient at this task and were used in all subsequent analyses.

Biomass Prediction

Biomass prediction was estimated in a two-step process. First, DNA template samples of known concentration (10ng/µL) were serial diluted six fold. Second, each sample was run through the qPCR reaction time. For each reaction, the C_q value (a measure of how long it takes to reach a threshold concentration) was recorded. DNA template concentration was linearly related to C_q as $C_q = 21.041 + -3.433 \log(\text{DNA Concentration})$ (Regression $F_{1,4}=553.2$, $R^2 = 0.991$, $P=1.97e^{-5}$; Figure 2).

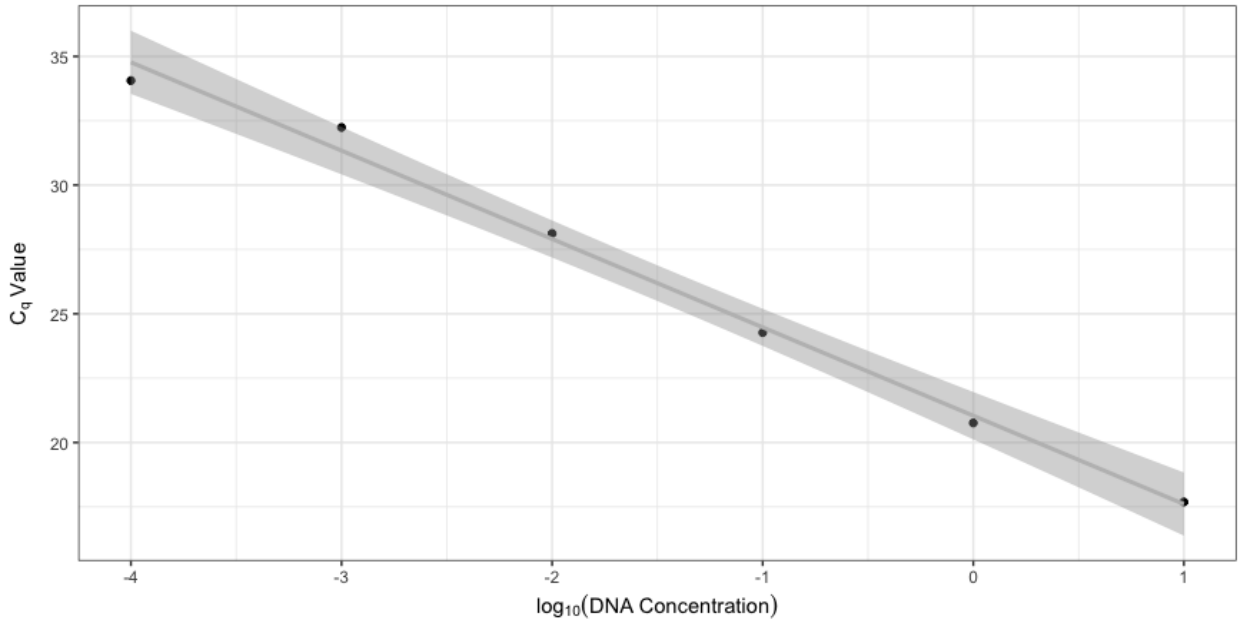

Figure 2. Serial Dilution of *P. collina* DNA Across Six Orders of Magnitude in DNA Concentration (from 10 ng/µL to 0.0001 ng/µL). The dilution series is represented as a log10 of the template concentration as a fitted linear model (fitted line) with 95% confidence interval on the model (in gray shading).

One-half of the sites collected in July 2017 did not yield assayable DNA for *P. collina* (Figure 3). Two of these sites, AF and CF, were reported to have extremely small population sizes ($N_{AF}=5$, $N_{CF}=4$ individuals) during the last physical census. It may be that five individuals in a stream are below the limit of detection for qPCR products using the parameters developed in this protocol. All negative sites were examined for the presence of qPCR-inhibiting compounds, as discussed later. Replicate runs of qPCR samples from the three sites with product (DC, JC, and MC) yielded Cq values commensurate with rank population size (Figure 3). These sampling locales had DNA concentrations ranging from $4.92e^{-5}$ng/µL to $1.87e^{-4}$ ng/µL (Table 2). More up-to-date census data would be necessary to estimate the extent to which sampled DNA concentrations can predict at-site census size beyond a relative ranking.

Table 2. Estimated DNA Concentration in Samples Collected From Locales Where VDGIF Biologists Have Conducted Physical Surveys of Population Size

Locale	Rank Density	C_q	DNA Concentration
DC	Low	35.90	$4.92e^{-5}$
JC	Medium	35.23	$7.68e^{-5}$
MC	High	33.89	$1.87e^{-4}$

VDGIF = Virginia Department of Game and Inland Fisheries; exact locations are not reported given this species' status as endangered.

Quantitative polymerase chain reaction provided threshold template density (C_q) from which standing DNA concentrations (ng/µL) were estimated from the empirical curve shown in Figure 2.

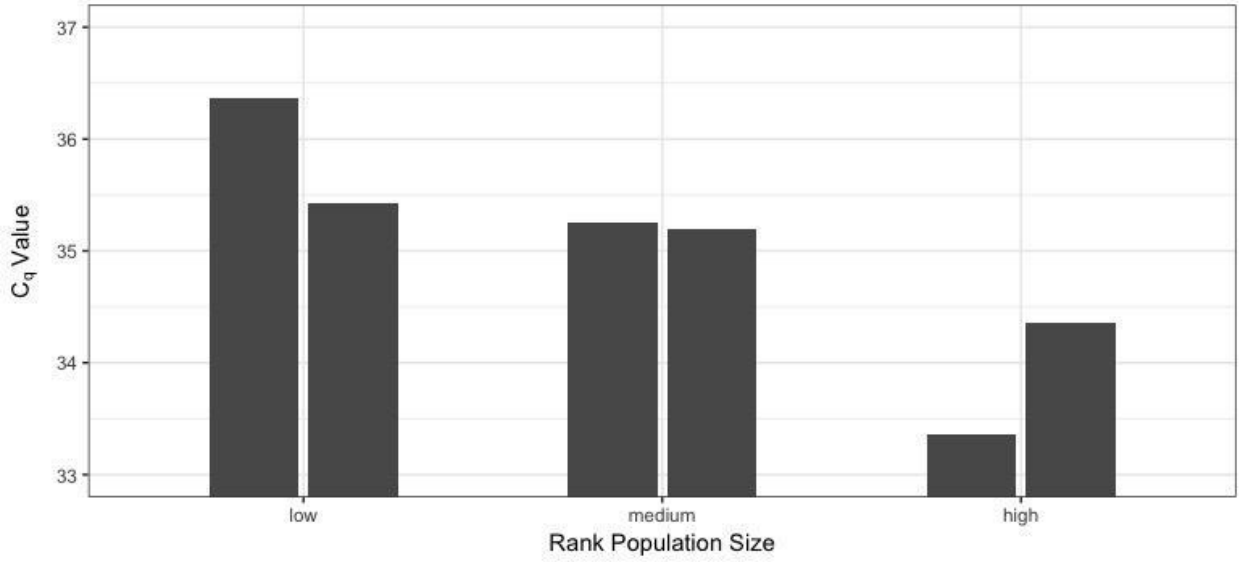

Figure 3. Starting Template DNA Concentrations Sampled From Stream Reaches of Known Population Density

Inhibition of PCR

Given that some locales known to have *P. collina* on site and did not produce any assayable PCR products, the extent to which abiotic factors may be inhibiting PCR reactions and preventing the identification of target species DNA were investigated. To identify the presence of inhibition, the samples were spiked with a known concentration of DNA and then the analyses were rerun (Gibson et al., 2012). Samples with spiked DNA that continued to yield no genetic markers were classified as inhibited (e.g., it could not be determined from these samples if there were target sequences as the entire reaction was inhibited by abiotic compounds in the water). Every spiked sample from locale LOC failed to amplify, indicating strong inhibition of the reaction. A dilution test was employed to determine if reducing the concentration of environmental inhibitors might increase the efficiency. However, these dilutions did not recover qPCR signal. For the conditions existing at the LOC locale, dilution is not an effective method for reducing qPCR inhibitors, and at this time it cannot be determined if this method would or would not amplify target DNA templates as no PCR products are created with those environmental samples. Spiking of samples from locales AF and CF did not reveal the presence of inhibition—the lack of product from these locales is thought to be related to the miniscule population size.

Detection Probability

Overall, the researchers were able to identify positive genetic signatures of the target species in 50% of the locales, though one of the sites exhibited PCR inhibition. For locales DC, JC, and MC that did produce results, the fraction of samples at each site identifying *P. collina* increased with the amount of post-processing to which each sample was subjected (Figure 4). From initial amplifications, the fraction of samples at a single locale that had positive *P. collina* products ranged from 17% to 29%. Subsequent processing of samples by gel extraction and ultimately Sanger sequencing increased detectability, resulting in 64% of the samples yielding *P. collina* positive genetic markers.

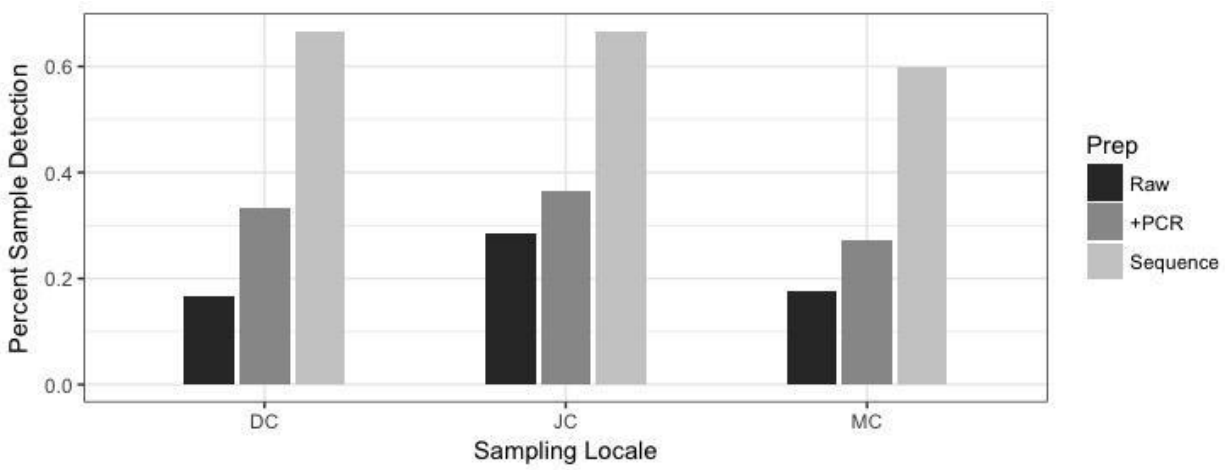

Figure 4. Detection Probabilities Among Replicate Water Samples at Each Locale As a Function of Sample Preparation. Exact locations are not reported given this species' status as endangered.

Samples subjected to Sanger sequencing did show some amplification of bands that aligned with *Elliptio complanata*. While sequences of *E. complanata* were amplified using these primers, these same products were distinguishable from *P. collina* by examination of their qPCR profiles. This suggests that subsequent work using the primers designed herein should pay close attention to qPCR thermal profiles and when bands are amplified that do not match the expectation for *P. collina*, these samples should be sequenced for positive verification.

DISCUSSION

The results show that molecular genetic approaches can be used to identify this species and that this protocol has potential to be used for ongoing monitoring of cryptic aquatic taxa. Detection probabilities for this work is site based (with replication), not individual based (as is often the case for mark-and-recapture data). While accurate population census data were not available at the time of this study, estimation using relative population density showed that the rank order of DNA concentration was identical to that of estimated population size. While beyond the scope of this particular research, the data collected herein suggested that eDNA protocols may have the potential for replacing more labor-intensive physical sampling.

The protocol developed herein showed the ability to detect *P. collina* from samples collected onsite. Findings of non-detect in some samples highlight the next set of factors needed to be addressed prior to full implementation of this work: inhibition and the limits of detectability for very small populations. While beyond the scope of this study's objectives, overcoming qPCR inhibition attributable to local conditions has not been shown in other examples to be too onerous a task and may be approached in several ways. Under the approach outlined in this study, samples from inhibited locales may be assayed for the presence of dissolved or solid organic compounds such as fulmic acids, humic acids, humic material, metal ions, and polyphenol (Abbaszadegan et al., 1993; Ijzerman et al., 1997). Once identified, the proper protocol for removing these compounds from samples may be integrated into the DNA extraction protocol (Shrader et al., 2010). Another approach would be to examine the effect that using alternative DNA extraction protocols have on removing inhibiting compounds. The extraction protocol used (Qiagen) is based on a silica purification protocol. While this is the most commonly used protocol, other approaches relying on detergents and other means may be more effective at removing compounds that may interfere with the polymerase reaction. Another approach may be one that does not rely initially on the polymerase reaction for template amplification. Previous work in the by the researchers on genotyping individual pollen grains used non-polymerase DNA replication approaches to increase target DNA concentrations. Here, all DNA in the samples would be replicated to increase initial template density followed by qPCR of specific marker regions. When combined with dilution tests, this last approach may be the most efficient as it would require knowledge of which specific compounds were inhibiting all PCR reactions.

The next issue to address should be the limits of detectability. Two of the populations in this project (AF and CF) did not produce positive qPCR products for any of the replicate samples. These populations were estimated during the 2010-2013 collection season to have as few as four individuals. Unpublished census work by VDGIF biologists have more recently verified that there are individuals at this location, though the last two physical sampling trips did not yield any positive identifications. The interpretation of the census data in this study and the molecular approaches take a conservative stance with the assumption that individuals are still onsite, which is why they were counted as failed identifications in the detection probabilities. Absence of evidence is not evidence of absence. However, the most important next step will be to test the limits of detection for eDNA protocols. In this work, 4 L of water was sampled for each locale, and this may be an insufficient volume to yield enough DNA template if there are populations whose size are in the single digits. For endangered species, the presence of even one individual is of utmost concern and subsequent work should be focused on methodological approaches that increase the specificity of the approach toward these lower limits. Potential avenues to pursue if this research is to continue may include increasing the volume of water sampled at a location. Increased volumes of water may yield sufficient template DNA. Another approach, more common in forensic DNA protocols, may be to use non-PCR-based DNA template enrichment protocols prior to qPCR. A detection probability is a site-wide feature and only one sample yielding a positive result triggers subsequent actions.

In addition to inhibition and limits of detectability, future efforts may be best served in determining the spatial extent by which template DNA may be detected. Regulatory constraints dictate a physical distance within which endangered species presence cause concern for road and

bridge activities. The objectives of this study were to show that eDNA can both detect the presence of and rank the relative biomass of *P. collina* DNA in the water column. Before this protocol can be implemented as either augmentation or replacement of other methodologies, additional study is required.

SUMMARY OF FINDINGS

- *By the use of derived and published repositories, species-specific genetic markers were developed that can be used to differentiate P. collina from other congener taxa using a qPCR protocol.*

- *With regard to detection probability, false negative results occurred when either the number of individuals was sufficiently low such that there was not enough DNA template or samples contained inhibitory compounds.* The presence of inhibition can be identified by spiking negative samples with known concentrations of DNA and may be mitigated by dilution (though not in the case of LOC herein). However, PCR inhibition does not indicate the presence of the target species, only that the protocol cannot reveal the presence of DNA if it is within the sample.

- *Standardized serial dilutions of known concentrations provided a tool through which local biomass could be estimated*. Results using rank population size as a surrogate for accurate census numbers were consistent with expectations. With the addition of more accurate census data, qPCR can provide increasingly accurate estimates of relative biomass. Examining habitat suitability and deploying physical sampling in these regions may be the most accurate method for determining if the populations are within a distance that mitigates activities.

CONCLUSIONS

- *Results showed that molecular genetic approaches may augment physical sampling protocols that result in the positive identification of cryptic aquatic species and perhaps replace existing physical sampling protocols that result in a non-detection.* Detection probabilities for individual sample locations using eDNA were consistent with what would be expected using physical survey measures.

- *Although the eDNA method applied in this study was able to determine that there was P. collina DNA in the water, it was not able to specify the distance upstream at which this tissue was being released relative to the sampling location.* Understanding the transport distance of DNA may provide more accurate estimates of proximity and should be augmented by physical surveys.

- *Results demonstrated that DNA concentrations can be used to estimate census population* size. A more precise evaluation of how closely DNA concentrations can be used to estimate

census population size could be determined with more accurate census data (i.e., surveying and evaluating more locales).

- *Findings demonstrated the limits of detectability using the eDNA protocols developed in this study.* Two of the populations in this study that were thought to have as few as four individuals at the time of last census were not detected (i.e., did not produce any positive qPCR products); however, it is unknown whether standard survey protocols would have detected these individuals. Because the presence of even a single individual at a locale is of importance, subsequent work could focus on understanding the lower limits of detection in natural populations by comparing the limits of detection of eDNA protocols to survey protocols outlined in the *Freshwater Mussel Guidelines for Virginia* (U.S. Fish and Wildlife Service and the Virginia Department of Game and Inland Fisheries, 2015). The sampling of 4 L of water in this study may have been insufficient to capture enough template DNA in concentrations sufficient for qPCR analysis.

RECOMMENDATIONS

1. *VDOT's Environmental Division should meet with the Virginia Department of Game and Inland Fisheries and the U.S. Fish and Wildlife Service to discuss the implementation potential for the eDNA approach.* Specifically, a detection limit for eDNA protocols should be determined that would allow a non-detection of the James Spinymussel to eliminate the need for VDOT to conduct physical surveys in that area.

2. *VDOT's Environmental Division and the Virginia Transportation Research Council should propose a second phase of this research on the development of eDNA protocols for detection of the James Spinymussel as a priority for FY19 research funding at the fall 2017 meeting of the Virginia Transportation Research Council's Environmental Research Advisory Committee.* The Phase II study should focus on refining the eDNA methods developed in this study in order to increase their implementation potential as a more sensitive stream survey tool for VDOT's use.

BENEFITS AND IMPLEMENTATION

Benefits

Because of the listing of the James Spinymussel as an endangered species, several surveys per year are required when VDOT activities may be in the vicinity of an individual or population. This species can be difficult to survey given its cryptic nature; thus substantial VDOT resources are required in the form of labor and other associated surveying costs. With further refinements of the methods developed in this study, eDNA sampling protocols have the potential to replace the need for physical surveys when a negative detection is found.

With regard to the implementation of Recommendation 1, discussions with the relevant regulatory agencies will determine the minimum allowable threshold for detectability that would negate the need for physical surveys by VDOT's Environmental Division. This will ultimately increase the surveying efficiencies and decrease costs for VDOT.

With regard to the implementation of Recommendation 2, conducting additional research to refine the eDNA methods developed in this study will provide VDOT a tool that will increase the efficiency and lower costs of monitoring the James Spinymussel. Subsequent research will include specific information on cost savings from the implementation of eDNA protocols.

Implementation

With regard to Recommendation 1, VDOT's Environmental Division will contact the Virginia Department of Game and Inland Fisheries and the U.S. Fish and Wildlife Service to schedule a meeting to discuss the implementation potential for the eDNA approach. The meeting will be held by January 19, 2018.

With regard to Recommendation 2, VDOT's Environmental Division and the Virginia Transportation Research Council endorsed this topic as a research priority at the October 30, 2017, fall meeting of the Environmental Research Advisory Committee. The aforementioned meeting with the regulatory agencies will help determine the decision with regard to funding additional research before FY19.

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APPENDIX

PRIMER SEQUENCES

Table A1. Tested ND1 (*NADH dehydrogenase-1*) sequences for both forward and reverse primers selected for full evaluation. The primers pair ND1_pcbr1 (denoted in bold) were found to be the most effective at identifying James Spinymussel samples.

Primer ID	Direction	Primer Sequence (5' - 3')
ND1Fq_1	Forward	CAATTCGATCAATTAATGCC
ND1Rq_1	Reverse	TTTCTGCTAAAATAACAAC
ND1Fq_2	Forward	GTTGTTATTTTAGCAGAAA
ND1Rq_2	Reverse	CAGAGACTAATTCTGACT
ND1Fq_3	Forward	TTAGCAGACGCTCTAAAGC
ND1Rq_3	Reverse	AATAAACGGTAAATAGTTCG
ND1Fq_4	Forward	CGAACTATTTACCGTTTATT
ND1Rq_4	Reverse	TGTATAGACGGTTAAAGAAG
ND1Fq_d1	Forward	AGCCATAGCCCARACCATCT
ND1Rq_d1	Reverse	AATGRCTAATGGTGCGCMGA
ND1Fq_d2	Forward	CGAGCCATAGCCCWRACCA
ND1Rq_d2	Reverse	ATGRCTAATGGTGCGCMGA
ND1Fq_d3	Forward	GAGCCATAGCCCWRACCATCT
ND1Rq_d3	Reverse	TGRCTAATGGTGCGCMGAG
ND1Fq_d4	Forward	CTCKGCGCACCATTAGYCA
ND1Rq_d4	Reverse	TTCGATGTTGAACMCAGAGAC
ND1Fq_d5	Forward	CKGCGCACCATTAGYCATTA
ND1Rq_d5	Reverse	TTCGATGTTGAACMCAGAGA
ND1Fq_d6	Forward	CKGCGCACCATTAGYCATTA
ND1Rq_d6	Reverse	TTCGATGTTGAAYCMCAGAGA
ND1_Gen2_F	Forward	ACCCTTCTAGAACGCAAAGC
ND1_Gen2_R	Reverse	TCTGCTAATGGTTGTGGGATTC
ND1_Gen3_F	Forward	TGCGCACCATTAGCCATTA
ND1_Gen3_R	Reverse	TTCGATGTTGAACCCAGAGAC
ND1_Gen4_F	Forward	CCATTTGACTTTGCTGAAGGAG
ND1_Gen4_R	Reverse	GCCATGAATAGGAAGGCAAAG
ND1_pcbr1	**Forward**	**GCGTAGCATTCTTTACCCTTCT**
ND1_pcbr2	**Reverse**	**GAGCGTCTGCTAATGGTTGT**