2020 Initial Findings Report: Environmental DNA Detection of Chinese Pond Mussels (*Sinanodonta woodiana*) at the NJCF Huey Property Aquaculture Ponds in Franklin Township, Hunterdon County, New Jersey

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Abstract

After the discovery of invasive Chinese pond mussel (CPM) in derelict aquaculture ponds located in Franklin Township, Hunterdon County, New Jersey, the New Jersey Conservation Foundation conducted eradication efforts within the ponds. In order to verify the eradication of this invasive species, the Natural Resource eDNA lab at Rutgers University developed an qPCR assay to detect the presence of CPM within the treated ponds and downstream creek. Initial findings strongly suggest that a population of Chinese pond mussel still exist within ponds W_4 and W_5. Further specificity testing of the developed assay will lend increased confidence that these detections are from Chinese pond mussel and not a closely related native species of freshwater mussel with an identical genetic sequence along the ITS2 region chosen for the qPCR assay. We suggest that a next-step is visual confirmation of reproducing Chinese pond mussels in these ponds before treatment. We also suggest that a broader eDNA-based survey of New Jersey rivers is on order to determine the extent that Chinese pond mussels may have dispersed out of the NJCF property ponds, or 'hitch-hiked' to other streams and rivers through movement of larvae (parasitic on common New Jersey fish) via human actions (e.g., dumping of unused bait).

Introduction

Chinese Pond Mussel

Chinese Pond Mussel, *Sinanodonta woodiana* Lea 1834, is a species of freshwater mussel native to Eastern Asia (Kraszewski and Zdanowski, 2007). Invasive populations have been established in a number of countries in Europe, the Caribbean, and Southern Asia (Donrovich et al. 2017). A small population of Chinese pond mussel (CPM) was initially discovered in 2010 in derelict aquaculture ponds located in Franklin Township, Hunterdon County, New Jersey. The identity of these specimens was confirmed by researchers at the North Carolina State Museum of Natural Sciences Research Laboratory via genetic sequencing along the COI region, representing the first discovery of a CPM population in the United States (Bogan et al. 2011). CPM were likely introduced into the ponds as larvae, during an obligatory parasitic stage (glochidium) (Beran 2008; Colomba et al. 2013) in conjunction with the bighead carp (*Hypophthalmichys nobilis*) that were farmed in the ponds before it was purchased by the New Jersey Conservation Foundation (NJCF). After the CPM were identified, NJCF enacted eradication measures using EarthTec QZ, a copper-based algaecide in summer 2019. To confirm the eradication of CPM, the NJCF sought to use environmental DNA detection methods to determine if the CPM were successfully eradicated from the ponds.

Environmental DNA

Environmental DNA (eDNA) is shed genetic material into the environment by living things in a variety of forms (skin cells, waste products, reproductive secretions). eDNA can be used to detect even rare or elusive species through the use of the quantitative polymerase chain reaction (qPCR) to amplify and detect target genetic material, in order to determine the presence or absence of the species in an environmental sample. The sensitivity of eDNA has enabled reliable and efficient detection of target species when other survey methods have failed and has been widely deployed across aquatic and terrestrial systems (Beng and Corlett 2020).

In an aquatic system, eDNA samples are often collected by concentrating cells and/ or genetic material by filtering water through a membrane and then extracting the genetic material from the filtered sample. The genetic material represents a snapshot of the organisms present in the system, and through the use of a targeted qPCR assay, detections of minute quantities of genetic material from a targeted species of interest can be amplified and detected. A qPCR assay utilizes a set of primers to amplify the genetic region of choice (amplicon) and a probe labeled with a fluorescent reporter dye to allow for the detection and quantification of the target amplicon. The region of choice needs be genetically conserved within species to allow the assay to detect individuals of that species no matter where from within their native range they reside, but not so conserved as to be shared by individuals of non-target species. A successful assay is able to detect the desired genetic region from any individual within the target species, but does not yield false positive results in the presence of genetic material from off-target species.

The objectives of this study were to determine: 1) if there is any evidence of a population of Chinese pond mussel in the ponds at the NJCF Huey site, 2) the extent of any existing population on the property, and 3) if there is any eDNA evidence that a population has been established in the creek leading downstream of the aquaculture ponds.

Materials and Methods

Sampling Location

Sampling occurred within the aquaculture ponds and nearby creek on the New Jersey Conservation Foundation's Huey Property (**Figure 1**) in Franklin Township, Hunterdon County, NJ (40°32'49.3"N, 74°54'58.1"W). Six ponds (W_B, W_4-W_8), the outflow drain from pond W_8 (O), and the creek (C) that exits the property were sampled for eDNA. Surface water samples (S_1 and S_2) were taken near the east and west ends of each pond, in a small outflow drain that leads to several other small ponds, and from the creek just before it flows beneath Allens Corner Road on the southern edge of the property. Grab bottle samples (B) were taken near the center of each of the five largest ponds (W_4-W_8). In order to more intensively sample each of the five largest ponds, input water source to the system, and outflow stream, the decision was made to forgo sampling the three smaller, square ponds between ponds W_B and W_4. This decision maximized the number of replicates at these focal locations and allowed for sampling at different depths (with surface and grab bottle methods) during each site visit to maximize the chance of detection of CPM.

Figure 1: Map of NJCF Huey Property. eDNA sampling occurred in the five aquaculture ponds (W_4-W_8), upstream "beaver pond" (W_B), outflow drain (Outflow), and nearby creek as it exits the property (Creek), labeled in yellow. Surface samples (S_1 and S_2) and grab bottle (B) sample locations are labeled in white. Satellite imagery: Google Earth.

Field Sampling

Surface samples were filtered *in situ*, at a depth of 5-10 cm using filter assemblies housing a 5 µm pore, polyethersulfone (PES) filter membrane with a short piece of sterile tubing mounted downwards (Smith-Root, Inc.). The filter assembly was attached to a 7 meter-long piece of silicon tubing that was mounted to a 3.5 meter-long collapsible aluminum pole. A field peristaltic pump (Pegasus Alexis, Proactive Environmental Products) was used to pump approximately 0.5-1 liter of water through the filter assembly and tubing, or until the filter clogged with debris. The volume of pumped water was measured with a large graduated cylinder. If the filter clogged prior to filtering 0.5 liters of water, an additional filter assembly was used to continue filtering the sample at a given site. The sampling pole was rinsed with D.I. water between each pond and was thoroughly rinsed with chlorinated tap water and D.I. water between sampling events. The filter assemblies were purchased pre-sterilized and individually packaged. A fresh glove was used to handle and attach the filter assembly to the sampling pole for each sample to prevent cross contamination.

Grab bottle samples were taken with a separate, 3.5 meter-long collapsible aluminum grab sampler (Grab Sampler II, Wheaton, Inc.) equipped with a 1 liter plastic screw top bottle. The sampling bottles were purchased sterile and were rinsed out on site several times prior to acquiring a sample using pond water from each pond. Between sampling events the bottles

were soaked for >5 minutes in chlorinated tap water and then triple rinsed with D.I. water and stored in sealed buckets cleaned with a 10% bleach solution, a tap water rinse, and a triple D.I. water rinse until they were used on site. The grab end of the pole and mechanism were rinsed thoroughly with chlorinated tap water and D.I. water between sampling events. Between each sample the sampling pole was rinsed on site with D.I. water and vigorously swirled around in pond water away from the grab sample location. The sampling bottle was submerged 0.5-1 meter below the surface (taking care not to disturb bottom sediment) and filled with approximately 1 liter of pond water. The bottle was then removed from the pole and the filter assembly tube was placed into the bottle with a fresh glove and the sample was filtered immediately after collection using the peristaltic pump, again measuring the volume of water filtered. One negative control was taken at the conclusion of each sampling event by filling one of the bottle storage buckets with D.I. water and filtering >1 liter of water while submerging the end of the sampling pole in the D.I. water.

After each sample was filtered, a pair of flame-sterilized forceps was used to remove the filter membrane. The filter membranes were folded and stored in sterile, 1.5 ml tubes filled with 1 ml of 100% Ethanol. Samples were brought back to the lab and stored at room temperature until extraction.

General Lab Protocols

All sample extractions, Polymerase Chain Reaction (PCR), and Quantitative Polymerase Chain Reaction (qPCR) setup occurs in a clean lab free of PCR products. Additionally, all qPCR setup occurs in a secondary, positive pressure chamber equipped with a UV sterilization unit. Any PCR products generated are handled in a separate lab, with its own lab instruments. All lab benches are cleaned with 10% bleach and D.I. water prior to sample handling, and all lab surfaces are cleaned on a regular basis with 10% bleach to maintain a low-DNA environment. Nitrile gloves are worn whenever handling sample tubes and are changed at regular intervals between steps. All sample tubes and pipette tips are purchased sterile and kept in a sterile environment prior to use. The use of negative controls at various stages of sample processing allows for the detection of cross-contamination.

DNA Extraction

The ethanol used to preserve each sample filter was evaporated off using a vacuum centrifuge (approx. 2 hours at 45°C). Sample filters were extracted with a DNeasy Blood and Tissue Extraction Kit (Qiagen, Inc.) along with at least one negative control per extraction batch to rule out cross contamination. Following extraction, the samples were stored at -20°C until the qPCR run.

qPCR Assay Development

We began by exploring modification of an existing eDNA assay for CPM published by Clusa et al. (2017) that was based on the COI (Cytochrome oxidase I) genetic region. However, we found through exploration of publicly available sequences collected from NCBI GenBank (ncbi.nlm.nih.gov/genbank) that the of Chinese pond mussel contained too much intraspecific genetic variation at COI for the creation of a qPCR assay that would reliably detect CPM eDNA.

Thus we explored internal transcribed spacer (ITS) 1 and 2, and found that ITS2 was chosen as the region with a usable, well-conserved sub-region for the creation of a qPCR assay.

In addition to GenBank sequences, mantle tissue from the three CPM samples collected from the New Jersey aquaculture ponds were solicited from Dr. Arthur E. Bogan at the North Carolina Museum of Natural Sciences. Additionally, CPM tissue samples were solicited from Dr. Malgorzata Ozgo at the Kazimierz Wielki University, Poland, where CPM are also invasive. These tissue samples were extracted with a DNeasy Blood and Tissue Extraction Kit (Qiagen, Inc.) and amplified using a custom set of PCR primers along the internal transcribed spacer 2 (ITS2) region. The PCR products from this reaction were visualized in a 1% agarose gel to optimize the PCR annealing temperature and to verify amplicon size. A subset of the PCR product was cleaned with an Exo-SAP-IT enzymatic clean-up step (Applied Biosystems, Inc.), and sent out for Sanger sequencing (GeneWiz, South Plainfield, NJ). The resulting sequences were aligned with the sequences available within GenBank to identify a consensus sub-region (Geneious Prime, Geneious, Inc.). A BLAST (Basic Local Alignment Search Tool, NIH.gov) search revealed that the consensus sub-region was well conserved even among other mussels, so a molecular beacon assay was chosen to ensure a high degree of assay specificity.

The consensus sub-region was used to design a primer set and molecular beacon probe using OligoArchitect Online (Sigma-Aldrich, Inc.). A BLAST search was performed using the resulting 107 base pair (bp) amplicon (the region between the forward and reverse primers to be amplified) to test *in silico* for any known off-target amplification of the assay. The assay includes a forward primer (CPM_MB_F: 5'-GCGAACGCTCCATAATTC-3'), reverse primer (CPM_MB_R: 5'-GGTCGAAAATGGTCAGAAA-3', both synthesized as custom oligonucleotide sequences, HPLC purified, ThermoFisher Scientific, Inc.) and a molecular beacon probe (CPM_MB_P: 5'-CGCGATCATCGTCAGGCCCGAGAGAGATCGCG-3', with a FAM reporter dye and BHQ1 quencher, Sigma-Aldrich, Inc.).

qPCR Assay

qPCR reactions were set up in a positive-pressure, qPCR hood (AirClean 600 PCR Workstation, AirClean Systems, Inc.) with UV sterilization between qPCR runs. Each 96-well plate included a five-fold serial dilution of extracted CPM genomic DNA (~0.6 ng down to ~6 fg), a negative (no template DNA) control, and 25 samples, each run with three technical replicates.

qPCR was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems, Inc.) in 20 µl reaction volumes with 10 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Inc.), 1 µl of each primer, and 0.5 µl of probe (10 µM), 5.5 µl of nuclease-free water, and 2 µl of DNA. After optimizing the assay by testing a range of annealing temperatures (50 – 60°C), the following PCR cycling conditions were used to analyze field samples: 96°C for 10 minutes and then 50 cycles of: 96°C for 15 seconds, 59.2°C for 45 seconds, and 72°C for 45 seconds, with a plate read during the annealing step of each cycle. Following the plate run, there was a 4°C hold to allow for the preservation of any amplified product for Sanger sequencing, if desired.

A subset of field positive results was sequenced to confirm assay specificity along the 107 bp amplicon. The PCR products from this qPCR reaction were cleaned with an Exo-SAP-IT enzymatic clean-up step and sent out for Sanger sequencing. The resulting sequences were

aligned with the sequences available within GenBank to determine if the PCR product was an exact match to the desired amplicon.

A subset of samples, and any samples in which environmental PCR inhibitors were suspected to be present, were treated with an inhibitor removal spin-column kit (Zymo Research, Inc.), and rerun in triplicate to reduce the chance of false negatives.

Results

Field Sampling

Sampling was conducted weekly from 8/11/20 - 9/9/20. There were six site visits over this 5 week period (an equipment malfunction precluded the collection of all field samples on 8/31/20, so sampling continued the following morning). In total, 105 unique samples were collected, including 73 surface water samples, 25 grab bottle samples, and 5 negative control samples (one per week). The presence of stinging insects prevented sampling of the outflow of pond W_8 on 8/18/20. The mean volume of water filtered for surface samples was 880 ml (range: 300 – 1300 ml). For grab bottle samples, the mean volume filtered was 713 ml (range: 180 – 1100 ml).

qPCR Assay and Sample Results

The developed qPCR assay demonstrated consistent detection of serial dilutions of CPM DNA ranging from 0.6 ng down to 60 fg of DNA (100% of technical replicates) and was often able to detect CPM DNA down to 6 fg (66% of technical replicates).

To date, 71/105 unique field samples have been extracted and analyzed with the qPCR assay (**Table 1**). These samples represent all five weeks of sampling across all sites with the exception of pond W_B on 8/18/20 and 8/25/20 (in progress). Across field samples, there was detectable amplification of the target DNA region for 8 of the 71 samples. Of the 8 samples that tested positive for the target region, three ultimately had 3/3 technical replicates test positive, two had 2/3 technical replicates test positive, and three had 1/3 technical replicates test positive. The C_t values for these positive hits ranged from 39.4 to 45.5 cycles (approximately 5.63 fg and 0.918 fg of target DNA, respectively). The efficiency across all qPCR sample runs was 85-95%., mean R^2 =0.982. All field, extraction, and qPCR plate negative controls resulted in no amplification of the reporter dye above the threshold indicating no contamination issues in the field or the lab.

The eight samples that resulted in positive results for the target region were collected from ponds W_4 and W_5, including grab bottle and surface samples collected from both ends of the pond on 8/11/20 through 8/31/20 (**Figure 2**). Within pond W_5, only surface samples taken from the east end (S_1) of the pond on 8/25/20 and 8/31/20 tested positive. No other samples have tested positive to date.

The first two positive results (highest and lowest DNA concentration to date) were sent for Sanger sequencing. The resulting sequences were a 100% match along the entire 107 bp amplicon to the CPM ITS2 sub-region, with no polymorphisms.

A subset of six field samples were treated with the inhibitor removal step (three of which had only one of three technical replicates initially test positive). Only one of these samples had amplification on a second qPCR run, with 3/3 technical replicates testing positive.

Location	8/11/20	8/18/20	8/25/20	8/31/20	9/1/20	9/9/20
WB	Negative (0/1)	In Progress	In Progress	Negative (0/1)	N/S	Negative (0/1)
W4	Positive (1/3)	Positive (1/2)	Positive (1/2)	Positive (3/3)	N/S	Negative (0/2)
W5	Negative (0/2)	Negative (0/2)	Positive (1/2)	Positive (1/3)	N/S	Negative (0/2)
W6	Negative (0/2)	Negative (0/2)	Negative (0/1)	N/S	Negative (0/2)	Negative (0/2)
W7	Negative (0/2)	Negative (0/3)	Negative (0/2)	N/S	Negative (0/2)	Negative (0/2)
W8	Negative (0/2)	Negative (0/2)	Negative (0/2)	N/S	Negative (0/2)	Negative (0/2)
Outflow	Negative (0/1)	N/S	Negative (0/2)	N/S	Negative (0/1)	Negative (0/1)
Creek	Negative (0/1)	Negative (0/1)	Negative (0/1)	N/S	Negative (0/1)	Negative (0/1)
Negative Control	Negative (0/1)	Negative (0/1)	Negative (0/1)	N/S	Negative (0/1)	Negative (0/1)

Table 1: Results of eDNA samples across each sampling location and sampling date. "Negative" indicates all samples failed to amplify the target region and "Positive" indicates at least one sample tested positive from that sampling location. The number in parenthesis indicates the number of samples that tested positive out of the number of samples tested. Color indicates the number of positive technical replicates in the sample with the most positive results (green: 0/3, yellow: 1/3, orange: 2/3, red: 3/3). "N/S" indicates no sample was taken. "In Progress" indicates a sample is yet to be processed.

Figure 2: Map depicting eDNA sampling results. The sampling locations: five aquaculture ponds (W_4-W_8), upstream "beaver pond" (W_B), outflow drain, and nearby creek as it exits the property are labeled in white. Surface samples (S_1 and S_2) and grab bottle (B) sample locations are labeled to indicate the number of positive technical replicates in the sample with the most positive results (green: 0/3, yellow: 1/3, orange: 2/3, red: 3/3). Satellite imagery: Google Earth.

Discussion

Based on the results of the qPCR assay, it appears that Chinese pond mussel may still be present in at least ponds W_4 and W_5. The negative controls employed during this study demonstrate that there was no detectable contamination in the field, lab, or during qPCR analysis.

While not all qPCR runs for a given sample resulted in 3/3 technical replicates testing positive for the target amplicon, this is expected due to the low concentrations of DNA in some samples or the presence of environmental PCR inhibitors (e.g., humic acids and tannins). The presence of environmental inhibitors causing false negatives in eDNA samples is always a possibility, but of the subset of six samples treated with an inhibitor removal step, only one sample yielded an increase in the number of technical replicates testing positive. In some instances there can be a loss of DNA during inhibitor removal, and so it was deemed not appropriate to treat all samples with the inhibitor removal step to maximize the chances of detection of the target region.

Given the resulting exact match of the PCR product sequence to the target amplicon from the sample with the highest C_t value (and theoretical lowest quantity of DNA), even samples exhibiting only 1/3 positive technical replicates should be considered as strong evidence of a low concentration of the target sequence in a field sample, and not a false positive. While we can estimate the quantity of DNA in a given water sample, the relationship between DNA concentration and the biomass of a given species present in an aquatic system is not yet well understood and is dynamic in nature due to life history and behavioral considerations (Lacoursiere-Roussel et al. 2016). Given that most of the samples within this survey were taken from surface water and that care was taken to not disturb the bottom sediment during grab bottle sampling, this suggests that the signal being detected is not from dead or decaying tissue in the system from the 2019 eradication efforts.

The fact that not all samples collected on a given sampling day within a pond tested positive for CPM eDNA underscores the importance of taking multiple water samples per pond. While no outflow or creek samples have yielded positive results to date, the eDNA signal from a very small population may prove below the detection capability of our eDNA assay due to dilution of CPM material as it moves through the system. There is the concerning evidence of CPM shells found in the Wickecheoke Creek downstream of the aquaculture ponds (Bogan et al. 2011) prior to the eradication effort. The frequent use of the ponds by recreational anglers also represents a risk of accidental introduction of CPM to surrounding waterways. Further eDNA sampling, in conjunction with traditional sampling methods of the ponds and the downstream area is suggested, especially in areas which may present ideal habitat for CPM, to determine whether there is a small localized population in ponds W_4 and W_5, or if CPM have spread more widely.

In order to more conclusively demonstrate that the eDNA detected amplicon is in fact from Chinese pond mussel, further specificity testing should be carried out against other bivalve species that could be co-occurring in these ponds. We performed *in silico* specificity testing by performing a BLAST search against all publicly available genetic sequences with no other exact matches to the 107 bp amplicon used in this qPCR assay, with the exception of other CPM ITS2 sequences. The closest matching sequence available is from another Chinese

mussel species, *Anondonta arcaeformis*, which has two base pair polymorphisms and an insertion along the amplicon.

We also compared the target amplicon against the *single* ITS2 region sequence available within GenBank for another mussel species that would be expected in NJ waters, Eastern Floater (*Pyganodon cataracta*). The ITS2 sequence of this species has a 9 bp difference along the target region, and was ruled out by the Sanger sequencing of our amplified qPCR product. By utilizing a molecular beacon probe, we believe our assay is highly specific to the target CPM amplicon, and coupled with sequencing of the resulting PCR product of positive samples, we are safely able to rule out a species with even one base pair polymorphism. Unfortunately, GenBank does not contain sequences along the ITS2 region for the other 15+ freshwater mussel species that may be found in New Jersey, so there is a non-zero possibility that another mussel found in New Jersey has an identical sequence. To this end we have solicited an additional set of tissue samples which will be sequenced along the ITS2 region and tested *in vitro* using the CPM qPCR assay to ensure specificity.

Conclusion

We developed a novel qPCR assay that is a promising basis for broad-based CPM eDNA surveys in New Jersey. Through deploying this eDNA tool, we provide strong evidence for the persistence of Chinese pond mussel in ponds W_4 and W_5 at the Huey site. We suggest further testing of our developed CPM assay on extracted DNA of other New Jersey mussel species to further raise our confidence that the assay is species-specific, and thus that our results for the NJCF site reflect the presence of CPM. We suggest traditional sampling take place to confirm the extent of any potential CPM population present in the pond and further eDNA sampling be conducted to measure the extent of any potential spread of CPM.

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