THE LINEAGES OF POTAMOPYRGUS ANTIPODARUM FOUND IN MICHIGAN

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Abstract

The New Zealand Mud Snail (Potamopyrgus antipodarum) is an invasive species that can be found around the world. Its presence in Michigan has been documented for years; however, during this research it has been found that an alternate genetic family had been introduced into the rivers. This was determined by collecting samples, extracting their DNA through a DNeasy PowerSoil Pro Kit, amplifying the targeted genes (*cytochrome b* and *16S rRNA*) via PCR (Polymerase Chain Reaction), and sequencing them in order to compare those sequences to other reported sequences. This allowed verification of which variants were present in sites around Michigan. It was found that the previously reported lineage (US1) was still inhabiting rivers, but most of the samples belonged to the US2 lineage. The US2 lineage was a new discovery in the area, but it was already known to be in the Great Lakes region.

Introduction

The New Zealand Mud Snail (NZMS) is an invasive species which can be found across the world. They are obstructive to the ecosystems they invade because they are usually able to outcompete other animals such as native snails and insects. This can cause a greater effect due to the disruption of the food chain in the local ecosystem; in particular, fish who rely on those snails and insects as their primary food source have difficulty feeding on New Zealand Mudsnails because the NZMS are able to tolerate digestion and desiccation to a certain degree (Geist et al., 2022). In extreme cases, the New Zealand Mudsnails are predicted to consume half the food in some streams (*California's Invaders: New Zealand Mudsnail*, n.d.). It is difficult to manage due to its ability to reproduce clonally, and it usually lacks natural predators in areas where it invades. This combined with their ability to rapidly reproduce and outgrow native populations in just a year cause them to be a large disrupting force (Cross et al., 2010). They have been known to reach densities of over ¾ million individuals/m^2 (*California's Invaders: New Zealand Mudsnail*, n.d.). Even when there are animals such as birds or fish to consume them, these animals usually poorly digest them (Bersine et al., 2008). This can even lead to allowing the species to spread further because it will later be dropped off in a distant location potentially where it can continue reproducing. Due to the species reproducing clonally, this causes almost all of the snails in an area to be genetically identical, and this makes it possible to classify the species into genetic families. This can indicate whether new members have been introduced into an area, if they do not belong to the same family. Their ability to reproduce clonally also makes them able to colonize different areas quickly. This combined with their ability to reproduce throughout the year makes them incredibly difficult to deal with once they are allowed to enter an area (Richards 2002). They also tend to exhibit strong predator-avoidance behaviors; in lab

settings, they have been observed to display surfacing, climbing, and floating behaviors (Haddaway et al. 2014).

The first sighting of these snails was in 1987 in the Snake River Basin (Bowler 1991). Snake River Basin is located in Idaho, and they were first discovered in Michigan rivers in 2013 (Geist et al., 2022). NZMS have a size of about 5 mm and are darkly colored, so they are easy to miss if they happen to get stuck to a fisherman's boot or the underside of his boat. This species has been documented in Michigan previously (Geist et al., 2022). However, it is important to update information on invasive species in order to document their spread and help evaluate the effectiveness of countermeasures that were put in place. This can be done through DNA sequencing, which involves collecting a snail sample, extracting its DNA, amplifying the DNA into adequate quantities, and then sequence the DNA utilizing the Sanger method and a machine to analyze the data. The *16S rRNA* and *Cytochrome b* gene (*CYTB* gene) were the focus of this research, and the genes were amplified via PCR (Polymerase Chain Reaction). These targets were selected due their long history being used for this application (Reid et al., 1997). This is a process known as "DNA barcoding" in which a species can be identified using a characteristic short sequence of DNA within its genome. This removes the need to sequence most of an organism's genome, and it allows easier identification while only looking at a relatively small section of DNA. The *Cytochrome b* gene in particular needed two rounds of PCR in order to obtain usable quantities for sequencing. The two lineages discussed are US1, normally found in the western United States, and US2 which has been reported in the Great Lakes (Dybdahl & Drown, 2010). These lineages, while different on a genetic level, did not have any apparent differences in their appearance, so differentiation of the two could only be accomplished by

analyzing their DNA. This study aimed to determine the lineages of NZMS in various Michigan rivers and assess whether any new lineages were introduced.

Methods

Sample Acquisition

Samples from Grasshopper Creek, Kids Creek, Main Branch Four Mile Creek, Mitchell Creek, North Branch Boardman Ottaway, and South Branch Boardman Ottaway were collected by natural resources managers in the Northern Lower Peninsula and sent to Dr. Scott Tiegs in the Department of Biological Sciences for identification. Dr. Wendell had obtained samples from the Au Sable river. The samples were collected in vials containing ethanol in order to preserve the snail while it was in storage. A majority of the samples were collected in a vial with other snails from that location. However, a few samples were taken and stored in their own separate vials. This was to mitigate the chances of cross-contamination between snails in case they differed in genotype (although that was not expected). These vials were used first when beginning DNA extraction, and, once those were used, the other samples were also utilized.

Location	**Latitude**	**Longitude**
East Branch Au Sable	44.667863	-84.705357
Mitchell Creek	44.7334	-85.5484
Kids Creek	44.7645039	-85.6311546
North Branch Boardman Ottaway	44.69002	-85.36731
South Branch Boardman Ottaway	44.64959	-85.29153
Grasshopper Creek	44.648216	-85.475912
Main Branch Four Mile Creek	44.733434	-85.54835

Figure 1 A table showing the locations where the samples taken along with Latitude and Longitude coordinates

DNA Extraction

The samples were stored in a 4 °C refrigerator and were stored in ethanol. The first attempts of DNA extraction utilized the CTAB method (Coyne et al., 2005; Turner et al., 2014). However, it was found that it was difficult to extract sufficient quantity and quality of DNA from all samples using this method. Therefore, a DNeasy PowerSoil Pro Kit was used instead to extract the DNA, and this was found to consistently provide DNA of sufficient quantity and quality for genotyping. The procedure was modified to better accommodate the research: in the quick-start protocol instead of adding a soil sample, a whole snail was added into the tube, then using a set of forceps, partially crushed the snail's shell to assist the process. The remainder of the procedure can be followed as written. The results of the DNA extraction were verified through a Thermo Fisher Scientific Qubit dsDNA Quantification Assay following manufacturer's instructions. For each site, two samples were used to ascertain which genetic lineage they belonged to. The extracted DNA could then be prepped for PCR.

PCR

The primers used to amplify the *16S rRNA* gene were S2 Potamo (GTGGTCGAACAGACCAACCC) from Städler's study and 16S_OU_inner1F (TCGGCAAATTTTGTTTCGCCT) which was designed (2005). The primers utilized for the *CYTB* gene were CYTB_2011_F (CTTGAATGACAGCAAGAAGT) and CYTB_2011_R (GTTGCTAGGCTGCTAAGTGA) (Neiman et al., 2011). For each sample of DNA PCR was performed with the following parameters: 10 µL Invitrogen Platinum SuperFi II PCR Master Mix, 4 µL water, 2 µL 5 µM Forward Primer, 2 µL 5µM Reverse Primer, and 2 µL template

DNA. For each round of PCR it had a control with water in place of the template. PCR cycles were as given in the Invitrogen Platinum SuperFi II PCR Master Mix User Guide. When amplifying the *CYTB* gene, a second round of PCR was often needed in order to get usable quantities of DNA. Prior to the second round of PCR, the first round of PCR needed to be diluted down 1:100 with MilliQ Water and vortexed vigorously. The second round of PCR was done with 10 µL Platinum SuperFi II, 4 µL Water, 2 µL 5µM CYTB_OU_2F (ACGTTCCTCATTGCGTCCTT), 2 µL 5 µM CYTB_OU_2R (CCCTGGGAGCAGCCTTAAAA), and 2 µL diluted PCR reaction. For the nested PCR, the control from the first round was put through the PCR process again and set up another control with just water as the template. The results of the PCR were checked using a 2% Agarose (40mL tris-borate-EDTA). Before casting the gel add 4 µL SYBR Safe, and the gel used a 10-well comb with 1.0 mm teeth. The gel was allowed to harden prior to removing the comb, and 1-2 µL of PCR reaction were used for the *16S rRNA* gene and 4 µL for the *CYTB* gene in each well. In the first well, 1 kb+ GeneRuler DNA Ladder (Thermo Fisher Scientific) was used to estimate the size of the sample and the quantity of DNA that was amplified. Gel electrophoresis was conducted at a constant voltage of 170 Volts for 30 minutes. Gel electrophoresis is a process where the gel conducts electricity, and the DNA in the wells will be drawn towards the positive electrode. The larger fragments of DNA moved more slowly, so they were near the top of the gel, and the smaller fragments moved more quickly towards the bottom. The ideal amount for the DNA should be 20 ng DNA/µL, but a slightly smaller amount will still work for sequencing. The amount of DNA in each well was determined by comparing the brightness of each fragment to the standard set by the GeneRuler DNA Ladder manufacturer. The bands of DNA would glow

under a UV light, so the approximate DNA concentration was recorded, and this would verify the success of the PCR process.

DNA Sequencing

Once the DNA had been amplified, it was prepared by combining 5 µL of the PCR reaction with 2 µL ExoSAP-IT Express. Then, the solution was vortexed and incubated in the Thermocycler using the ExoSAP-IT Express program (37 °C for 4 minutes, 80 °C for 1 minute, then held at 4 °C). Using the adjusted ng DNA/µL, it was diluted down to ~10ng DNA/µL with water. The Sanger method was used for sequencing with the Applied Biosystems BigDye Terminator Cycle Sequencing Kit v1.1 (Thermo Fisher Scientific). The 1.1 kit was used due to the abnormally high A:T base pair content found in the targeted genes, and it allowed for higher quality sequencing. Each sample had one tube for the forward reaction and a second tube for the reverse reaction. In the tube 1 µL of template or enough to equate 10-15 ng DNA/µL, 2 µL primer, 1 µL BigDye Terminator RR mix v1.1, 1.5 µL 5X Ready Reaction Mix and enough water to have the total be 10 µL were added. Once all of the samples were mixed, this was then incubated according to the directions found in the BigDye Terminator v1.1 Sequencing Kit Quick Reference. Prior to sequencing, the excess ddNTPs needed to be removed, and this was accomplished by adding 45 µL SAM solution and 10 µL XTeriminator to each tube. This was then set into a plate shaker for 30 minutes and then centrifuged for 2 minutes at 1000 x g; once this was done, it was imperative not to agitate the tube or else the pellet at the bottom would be disturbed and need to be re-centrifuged. This was then loaded into the SeqStudio genetic analyzer. After the samples were taken out of the machine, the data would be looked at to ensure that the quality values were high enough. A quality value of at least 20 was the ideal when

looking at the sequences. The quality values were a measure of the certainty that the machine was making accurate base calls.

Alignment

Individual sequences were assembled into contiguous sequences, also known as contigs, using the CAP3 Sequence Assembly Program. The results were then compared to the corresponding gene for the US1 and US2 variants. The references for the US1 and US2 *CYTB* gene were GenBank accession numbers AY570216 and AY570201 respectively (Dybdahl & Drown, 2010). The references for the US1 and US2 *16s rRNA* gene were GenBank accession numbers AY955393 and AY955376 respectively (Städler 2005). These are linked to the z and t haplotype, but these are shown to be the same as the US1 and US2 haplotype (Geist et al., 2022). The sequences we collected were uploaded in JalView, a program that allows you to view and align sequences, along with the sequence of the US1 and US2 variant sequences of either the *CYTB* or the *16S rRNA* gene. These sequences were aligned using MUSCLE alignment with default settings to both of their corresponding gene's variants. The sequences were matched up to where the repeating base pairs are aligned with each other, and it allowed a determination on which variant the sequence belonged to, and therefore, the snail could be identified confidently. The snail samples were generally confirmed with alignments of both the *CYTB* and the *16S rRNA* gene to ensure the results of the first alignment were correct and to rule out mixups of samples.

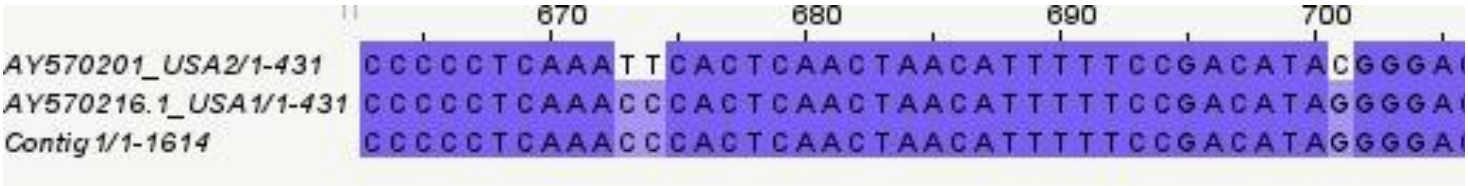

Figure 2 An example of alignment comparing a sample's CYTB gene to two reference sequences (US2 reference on top and US1 example in the middle). The sample sequence aligned more closely to the US1 sample.

Results

The samples collected from East Branch Au Sable were shown to belong to the US1 variant, while the others were aligning with the US2 variant which had not been reported in Michigan prior.

Location	**Lineage Found**
East Branch Au Sable	US1
Mitchell Creek	US2
Kids Creek	US2
North Branch Boardman Ottaway	US2
South Branch Boardman Ottaway	US2
Grasshopper Creek	US2
Main Branch Four Mile Creek	US2

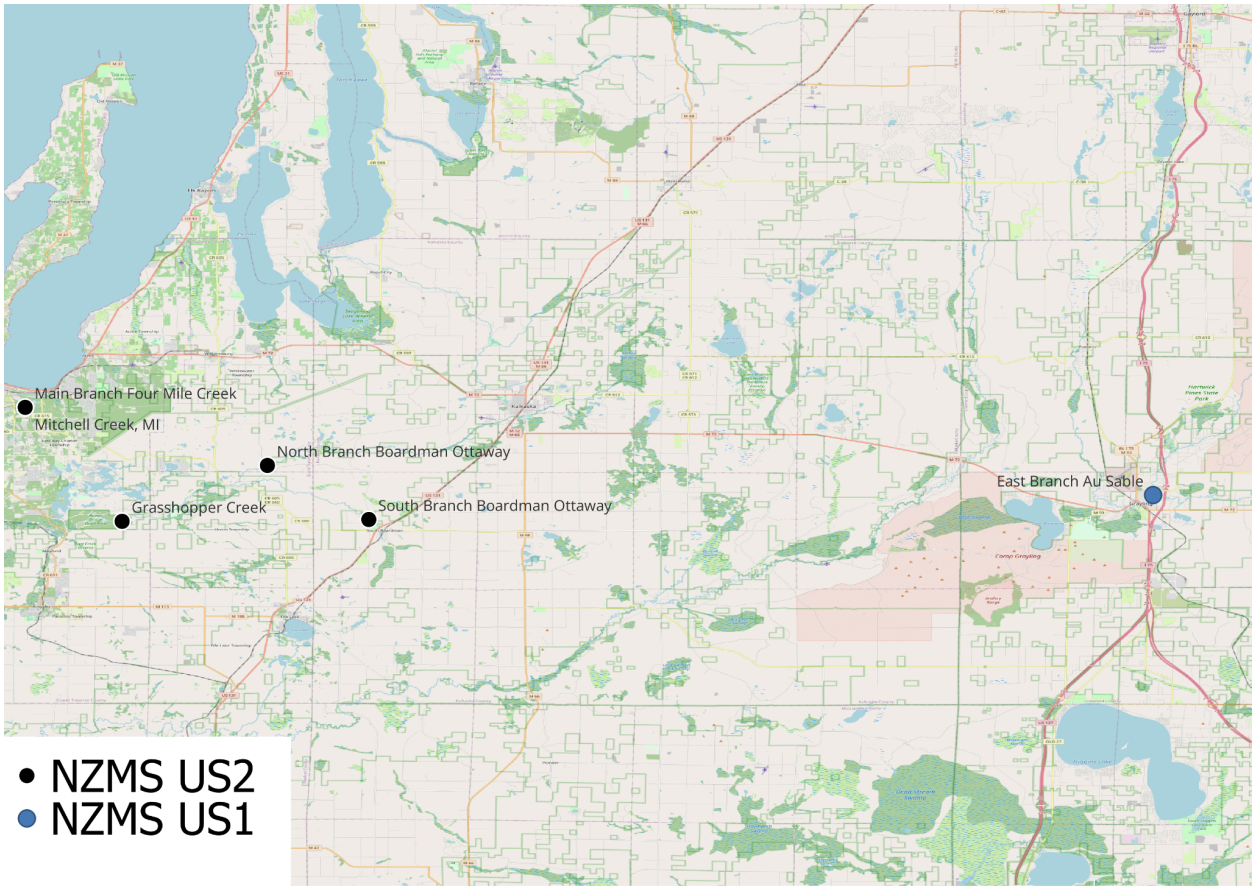

Figure 3 (A) A table showing the results of the alignment; it shows which lineages were found at which sites (B) A map showing where the samples were collected along with which gene family was found there. The sites on the eastern half of the map were found to have US2 variant snails, which likely means that they were introduced somewhere upstream and followed their way down.

The alignments of the CYTB and 16S rRNA genes would have most of the base pairs matching the other 2 sequences, and there would only be a few select spots where base pairs would be different. This meant that having high quality data was a must to ensure that the sequencer was not making incorrect calls in key locations for comparison. Otherwise, the validity of the results would have been questionable, and no conclusive statements could be made.

Discussion

The samples aligning with US2 was relatively unexpected due to the US1 variant being thought to be the only one of its kind in Michigan. The existence of the US2 variant in Michigan rivers would imply that the restrictions and guidelines were not enough to completely halt the spread. The exact point where this newer lineage was introduced is beyond the scope of this study, but a possible way that these could have been introduced is from the neighboring Lake Ontario or Lake Superior (Dybdahl & Drown, 2010). Considering the locations where the US2 samples were collected it is likely that the snails were initially introduced somewhere upstream and carried down either via the current or possibly latching onto another organism. This could also be a way that they are able to survive the colder temperatures. The New Zealand Mud Snail is unable to survive once the temperature gets to 0 °C (Moffitt & James, 2012). However, attaching to a larger fish or even being eaten by one could give it a higher chance of survival by allowing it to be moved to potentially warmer conditions or staying warmer inside the fish. In regards to the nested PCR which was required for most of the Cytochrome b gene amplifications, it was not necessary in Neiman's study, so it is thought that it could be an issue of minor differences in equipment, method, or the size of individual snails (2011).

This could have occurred due to recreational fishing which has occurred previously with the Zebra Mussel (Johnson et al., 2001). Zebra Mussels are another invasive species of invertebrates which have invaded Michigan waters, and they are also typically transported via boats and boating equipment (Johnson et al., 2001). Much can be learned when comparing these species' movement through the United States and Michigan. However, they were not transported via direct attachment to a boat's hull; rather, they were most commonly transported through the engine cooling system (Johnson et al., 2001). This type of data could prove invaluable as it could

direct policy making decisions and cleaning protocols to emphasize the checking and cleaning of areas where the New Zealand Mudsnail is likely to be attached. It would likely improve the success rate of these policies, and it would lessen the load on fishers by only needing to heavily focus cleaning on one or two components of their boat rather than the entire vehicle.

Prevention would be the best tactic when trying to deal with the New Zealand Mudsnail; their ability to rapidly reproduce asexually causes them to be extremely difficult to manage once they are allowed to settle in a new location. NZMS tend to attach themselves to boots, boats, and other objects commonly used by anglers; these are then transported to other locations where they will detach and be able to spread (*California's Invaders: New Zealand Mudsnail*, n.d.). For this reason, they are hard to completely track and adequately stop their spread. This leads to further questions of if the US2 variant may have also been transported to other states that border the Great Lakes. This is a known issue, and there are actions being taken in order to prevent the further spread of NZMS throughout Michigan. In 2016, Dr. Tiegs was awarded a $191,888 grant by the Michigan government in order to utilize fly fishers to detect New Zealand Mudsnail invasions early (*Michigan Invasive Species Grant Program*, n.d.). Other states are taking notice of these invaders. For example, California is encouraging its citizens to report sightings to the CDFW Invasive Species Program (*California's Invaders: New Zealand Mudsnail*, n.d.).

It could also be worthwhile to check surrounding rivers in Michigan to better understand how far the US2 variant had spread, and it may be interesting to survey the Au Sable river in order to determine if the US1 variant may have been carried into Lake Huron where the river's mouth is located. Another potential continued experiment could be to attempt extracting DNA from the small traces that can be found in the water that they inhabit (eDNA). eDNA has been used previously to detect aquatic macroorganisms, and it allows researchers to not only

determine their abundance, but it would allow identification of which lineages those samples had originated (Turner et al., 2014). It would allow for a more comprehensive analysis of the lineages in various lakes and streams because there is a possibility that each site had multiple lineages, but one lineage may have been more prevalent than the other(s). Utilizing eDNA would allow for the small population to show up in the results, and it could lead to newer questions regarding the species and its path through not only Michigan but the US as a whole. This would allow researchers to also determine relative abundance of the lineages which is a shortcoming of this study. The various lineages are only distinguishable when you analyze their DNA, so there is a low likelihood that the US2 variant will cause any more ecological turmoil when compared to the US1 variant. The area of concern is that there is not a viable way to control the spread of them without checking almost everything entering or leaving the water. Considering the amount of samples that came from US2, it should be a priority to keep track of boats and equipment that are moving between the Great Lakes and the inner rivers. This can most easily be done by encouraging regular cleanings of equipment and boats. The Washington Department of Fish and Wildlife has protocols and instructions for cleaning depending on whether the boat or equipment is simply being moved from one body to another or if it is being moved from known infested waters (*Preventing the spread of aquatic invasive species*, n.d.). There are studies that show that salinity could be a way to combat those that live in freshwater (Hoy et al., 2012). However, this is not feasible to do on a large scale, and it would likely result in more damage to the environment and ecosystem compared to what the snails themselves are capable of.

An alternative to dealing with the New Zealand Mudsnail directly may be to attempt to destroy their embryos when they are released. The New Zealand Mudsnails in Michigan are believed to be entirely female (Hershler et al. 2010). The New Zealand Mudsnail females are

born with embryos developing inside of them, so they are ready to reproduce as soon as they are born, and they produce 230 new females each year once reaching maturity at 3 years old (*California's Invaders: New Zealand Mudsnail*, n.d.). Killing off the mudsnails and their embryos could be attempted with chemicals which are typically used for hatchery and fish egg disinfection; however, it has been shown that using those chemicals at those high concentrations for that long will also kill rainbow trout eggs (Oplinger & Wagner, 2009). Despite these fish not being native to Michigan, it is not unreasonable to assume that similar effects could happen to other native inhabitants of Michigan's rivers especially considering the number of endangered fish which reside there. Therefore, it could be better to test alternative chemicals to try to disrupt their embryos from developing or a lower concentration could be tried to see the effects on developing mudsnails rather than trying to kill off the mature individuals.

Ignoring this invasive species is not an option due to the competition it forces upon the other invertebrates in areas that it invades. It is also shown that these snails can cause damage to plumbing infrastructure by clogging their pipes (Ponder, 1988). While this was in Australia, this is applicable here especially because some cities use the nearby rivers for access to water. This could lead to many places not having access to clean water, so it is imperative that action is taken in order to prevent the situation from reaching such a dire state.

Conclusion

The New Zealand Mud Snail has invaded many parts of the world, and it has been shown that another lineage has entered the rivers of Michigan. This has been determined by extracting DNA from samples across various rivers, amplifying the DNA through PCR, and sequencing it. An alternate method of DNA extraction was used due to lower than expected qualities, and two rounds of PCR were required for the Cytochrome b gene in order to get an adequate quantity of

DNA for sequencing. The sequenced DNA was then assembled into contigs which allowed them to be compared to reference sequences through MUSCLE alignment. It was found that the US1 and US2 lineages were found in various locations. There were many sites where the US2 lineage was identified, and this was unusual due to that lineage not being reported previously in these areas. The likely source of the newer US2 variant is from the nearby Great Lakes, and this should alert agencies to try stricten their policies and guidelines or to investigate alternate ways that the NZMS could be spreading. Otherwise, it could lead to ecological troubles and force out many of the native animals from their homes.

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