Detection of the invasive crayfish *Orconectes rusticus* using environmental DNA from aquatic systems in the Upper Peninsula of Michigan

BIOS 569: Practicum in Field Biology

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Abstract

Invasive species are considered a major problem to all ecosystems and to indigenous species throughout the world through destruction of natural habitat and through competition with and predation on native species. New methods to detect these invasive species have recently been developed and may possibly aid in the prevention of these invasive species infesting new ecosystems. One omnivorous invasive species being monitored by biologists, *Orconectes rusticus*, is responsible for the decline of other native crayfish and vegetation located in the Upper Peninsula of Michigan. This work attempted to elucidate the presence of the *O. rusticus* using environmental DNA detection methods in the laboratory and in lakes shown previously to have low densities and high densities of the invasive crayfish. Results of this work show that it is possible to detect *O. rusticus* in laboratory studies after 72h using eDNA. However, this work found that eDNA was not able to detect the rusty crayfish from water samples in the field.

Introduction

According to the Global Invasive Species Programme, biological invasions by non-native species represent one of the leading threats to native ecosystems and biodiversity (Wittenberg *et al.* 2001). Invasive species presence

in a given environment contributes to destruction of agriculture, forestry, and fishery, as well as human health (Wittenberg *et al.* 2001). *Orconectes rusticus*, the rusty crayfish, is one such invasive species that is causing extensive damage to its non-native range (Wittenberg *et al.* 2001). Unlike most non-native species in the United States, the rusty crayfish originated within U.S. boarders in the Ohio River Basin, Kentucky, and Tennessee region (Benson and Kratz 2006); they were introduced to the Great Lake region in the 1970s, likely through live bait disposal by anglers (Capelli and Magnuson 1983).

With a putative evolutionary advantage of a rapid dispersal rate, the rusty crayfish are capable of contaminating an entire lake ecosystem with one fertilized female (Boersma *et al.* 2006). Other biological advantages of this species include a larger body mass, increased aggression, and high metabolic rate (Olsen *et al.* 1991). Rusty crayfish hold a competitive advantage over native crayfish species such as *Orconectes virilis* and *Orconectes propinquus*, and given time will likely extirpate these indigenous crayfish (Olsen *et al.* 1991). The presence of the rusty crayfish within a lake environment also poses immediate risks to aquatic plant diversity and abundance due to the high metabolic rate of the organism (Klocker and Strayer 2004).

According to Lodge *et al.* (2006), impact of invasive species is poorly quantified due to lack of pre-invasion data. However, new methods of detecting

and monitoring invasive species are being developed. In a newly released study, Ficetola *et al.* (2008) were able to detect the presence of the American bullfrog, *Rana catesbeiana*, which is responsible for the decline in native amphibians throughout the world, through the use of environmental DNA (eDNA). This study is exceedingly important because invasive species can be remarkably difficult to detect at low densities or in difficult to sample environments. The Ficatola et al. (2008) study is first study to use eDNA collected from fresh water to ascertain invasive species presence or absence.

Therefore, the important question addressed in this investigation is whether or not we can detect the rust crayfish at low densities, i.e. before a dominating infestation occurs using an eDNA detection approach. The goals of this project were to complete laboratory and field based eDNA detection experiments to elucidate the detection limits and potential for using eDNA detection for rusty crayfish. The laboratory experiments utilized *Orconectes propinquis* in isolated "bucket" experiments to determine if an eDNA approach would be possible in the field. The next phase of this project took place on Brown Lake and Big Lake located on the University of Notre Dame's Environmental Research Center on the Upper Peninsula of Michigan and Northern Wisconsin. Here, the eDNA techniques optimized in the laboratory were tested in field-based scenarios. The specific hypothesis being tested is that mitochondrial DNA in

water samples will be substantial enough to detect the presence of *Oronectes rusticus* in both Big Lake and Brown Lake.

Materials and Methods

Laboratory investigations

Indigenous crayfish, *Orconectes propinquus,* were caught by hand, snorkeling off the Northeast shore of Tenderfoot Lake on the UNDERC property. The use of a native crayfish species as opposed to the invasive rusty crayfish in the bucket tests was observed because it is illegal to intentionally move the invasive species into unaffected areas. For the initial experiments in bucket, five buckets were filled with ~30 liters of tap water. Two buckets were made to mimic a high-density population of crayfish by placing ten *O. propinquus* in each. In another two buckets, which mimicked a low-density population, a single adult *O. propinquus* was placed in each bucket. The last bucket was held as a control and contained no crayfish. Five liters of water were collected from each bucket at the end of a 24-hour period and again at 72 hours from the beginning of the experiment. Water samples were then filtered through 0.45 micron filter paper using a using a vacuum filter apparatus and the resulting filter paper was stored at -20ºC until DNA extraction could be performed.

Once filtered, samples underwent total genomic DNA extraction using an UltraClean™ Water DNA Isolation Kit. Sterile techniques were performed at all stages to avoid cross contamination of DNA and manufacturer's recommendations were followed throughout the procedure. The filter paper with trapped microorganisms and eDNA from the water samples was placed in water bead tubes containing small glass beads and bead solution to cause the cells to lyse. WD1, a solution containing detergent to aid in the break down of cell walls, was then added. The tube containing solution WD1, the filter paper, and glass beads were placed on a vortex for approximately 20 minutes to ensure that all cells in the solution were lysed and the filter paper was sufficiently dissolved by the silica beads. The broken pieces of filter paper and glass beads were then removed from the solution through centrifuging the mixture for one minute. In order to extract other unnecessary cellular debris and protein, solution WD2 was combined with the supernatant and a cooling treatment was administered at 4ºC for five minutes. The result of this treatment yielded unpurified genomic DNA.

The DNA was then placed in a spin filter tube with a high salt solution to purify. Once spun in the centrifuge, the sodium solution caused the DNA to be held on the filter membrane. Another solution containing 50% ethanol was then added and the solution was placed in a centrifuge to wash impurities away while keeping the DNA stuck inside the filter. Lastly, distilled water is placed in the filter tube to release the DNA from the filter membrane and spun through using a

microcentrifuge. The combination of distilled water and purified DNA was then left at the bottom of the tube.

The final DNA extract was then concentrated using a combination of ice-cold 100% ethanol and 5M NaCl. Once 6ml ethanol and 300 µl NaCl was added, the solution was centrifuged for 20 minutes and poured into a waste container in one fluid motion. This treatment caused the DNA to precipitate out of solution and cling to the bottom of the tube. DNA was then dried to remove residual ethanol using a drying oven set at approximately 45ºC. Once DNA had been sufficiently dried, a process taking three to five hours depending on the amount of liquid left in the tube after concentrating the DNA, 200 µl of distilled water was added.

The resulting DNA extracts were then used to amplify a short segment of the mitochondrial genome of the crayfish using Polymerase Chain Reaction (PCR). The purpose of PCR was to amplify species-specific regions of DNA by utilization of specific primers that act as a starting point for the whole PCR process. The specific primers used for this experiment were designed to only amplify either *O. propinquus* or *O. rusticus*. The master mix for each PCR reaction is composed of 2.5 microliters buffer, 2.5 microliters magnesium solution, 0.5 microliters forward PCR primer (10mM each; species specific) from within the mitochondrial cytochrome c oxidase subunit I (COI) gene, 0.5

microliters reverse primer (10mM each; species specific) from within cytochrome c oxidase subunit I (COI) gene, 0.5 microiters dNTPs, 0.15 microliters Taq polymerase, and 17.35 microliters of water.

PCR was performed in conjunction with the following PCR thermal program, each reaction underwent an initial denaturation in 95ºC for three minutes. After amplification, samples were refrigerated at 4ºC until use in electrophoresis.

Visualization of PCR reactions was performed on a 1% agarose gel to determine presence or absence of the crayfish. The agarose gel was prepared by combining 0.6 grams of agarose with 60 mls of SB (sodium hydroxide-boric acid) buffer. The solution was heated to boiling to melt the agarose and mixed throughout the heating as necessary. Following heating, 1.1 µl of ethidium bromide, which serves to intercolate between the double strands of DNA and fluoresce under UV light, was then added. The solution was then mixed before being poured into a gel mold. This solution was allowed to solidify for approximately 30 minutes before electrophoresis occurred.

Field investigations

Fieldwork began at Brown Lake, the low-density rusty crayfish lake in this study, during the second research week. Three sites were chosen by their physical

properties, one site with vegetation, one site with a sandy bottom, and one site with cobble. Two liters of water were sampled from three locations around the lake, and one site was a location were rusty crayfish have been historically collected by baited trap. No other sites on Brown had success trapping the rusty crayfish in previous years. Samples were taken with sterile one liter Nalgene bottles. The bottles were taken to desired site and remained capped until a depth of approximately 0.5 to 1 meter and capped again at the same depth. Water samples underwent the same filtration and DNA extraction methods in accordance with the UltraClean™ Water DNA Isolation Kit as previously described.

Fieldwork on Big Lake, the high-density rusty crayfish lake in this study, began during the second research week and continued into the third and final week. During the first day of sample extraction, two liters of water were taken from each site. These samples were extracted in the same manner as those taken from Brown Lake. During the final research week an additional two ~20 liter samples were taken. Again, samples underwent the same filtration and DNA extraction protocol in accordance with the UltraClean™ Water DNA Isolation Kit.

Results

Laboratory analyses

Controlled analysis within the bucket tests at 24 hours resulted in a negative gel with no rusty crayfish DNA detected in both high-density, both low-density, and control buckets. However, a positive gel was obtained after rusty crayfish presence in water for 72 hours in both high-density and low-density buckets (Figure 1). The control bucket again yielded negative results.

Field analyses

All site samples taken from Brown Lake had negative results and no rusty crayfish DNA was detected during electrophoresis. Big Lake samples from site 6, site 9, site 10, and site 23 also did not exhibit rusty crayfish DNA in the gel (Figure 2). A second PCR was performed on the DNA extracted from site 1 and site 2 with two microliters of DNA instead of one. Both attempts at PCR in site 1 and site 2, including the PCR with an additional microliter of DNA, yielded a negative gel with no rusty crayfish DNA detected (Figure 3).

Discussion

Based on the data collected and the resulting negative gels, I must reject the hypothesis that environmental DNA in water samples will be substantial enough to detect the presence of *O. rusticus* in both Big Lake and Brown Lake. I

had expected, with the abundance of the rusty crayfish within Big Lake, positive results from the samples extracted, however the opposite occurred as no DNA could be detected within the high-density lake. During the second day of collecting water samples, site 1 and site 2 had been chosen specifically for high densities of rusty crayfish and still no *O. rusticus* DNA had been detected. Even in Brown Lake, history suggested that rusty crayfish DNA could possibly be found due to the successful trapping of two rusty crayfish in 2008 at site 30. However, with the unsuccessful detection of the rusty crayfish DNA, it is possible that the concentration of rusty crayfish at Brown Lake was not substantial enough.

Possible reasons why DNA was not successfully collected at these sites might involve the relatively small volume of water collected. Although ~20 liters of water were taken from site 1 and site 2, a larger volume of water may be necessary in order to collect a more substantial amount of DNA for PCR amplification. Another reason DNA extraction may not have been successful would be because the samples had been collected in a relatively short period of time. It is possible that the crayfish may not slough off enough DNA during particular stages in their life cycles. A longer and more consistent sampling period may yield more results with a wider range of rusty crayfish life stages. Also, methods for extracting and amplifying DNA within a field environment had previously only been utilized with vertebrates in aquatic environments. This study was solely aimed at attempting to discover if invertebrates, more

specifically crustaceans, could successfully be detected. I believe the field methods, including keeping a container capped until desired depth, were appropriate for this study. I believe water collected from the bottom of the lakes would contain high densities of viable rusty crayfish DNA and that DNA located on the surface of the lakes would be more likely to be degraded by UV rays; however further studies must be completed on the stability of environmental DNA.

The UltraClean™ Water DNA Isolation Kit has had successful DNA extractions of eDNA of other invasive aquatic species, for example the lake sturgeon (Mahon unpublished data). Therefore, the DNA isolation kit has a history of success through the detection of aquatic vertebrates (Figure 4).

Although this study did not yield positive results when attempting to detect the invasive rusty crayfish, studies of this nature are extremely important in the protection of ecosystems around the world. This method of DNA detection could also be important in the search for threatened or endangered species presence without invasive measures being taken. Invasive species have a dramatic effect on the ecosystem by hindering the other native species present in the environment. The invasive species, which have evolved different characteristics than the other indigenous species, are able to out compete, overeat vegetation, and affect the natural order of the food chain. Therefore, even a small invasive organism can collapse an ecosystem. Being able to detect these invasive

animals before infestation will be a breakthrough in the protection of indigenous species and ecosystems everywhere.

Figures

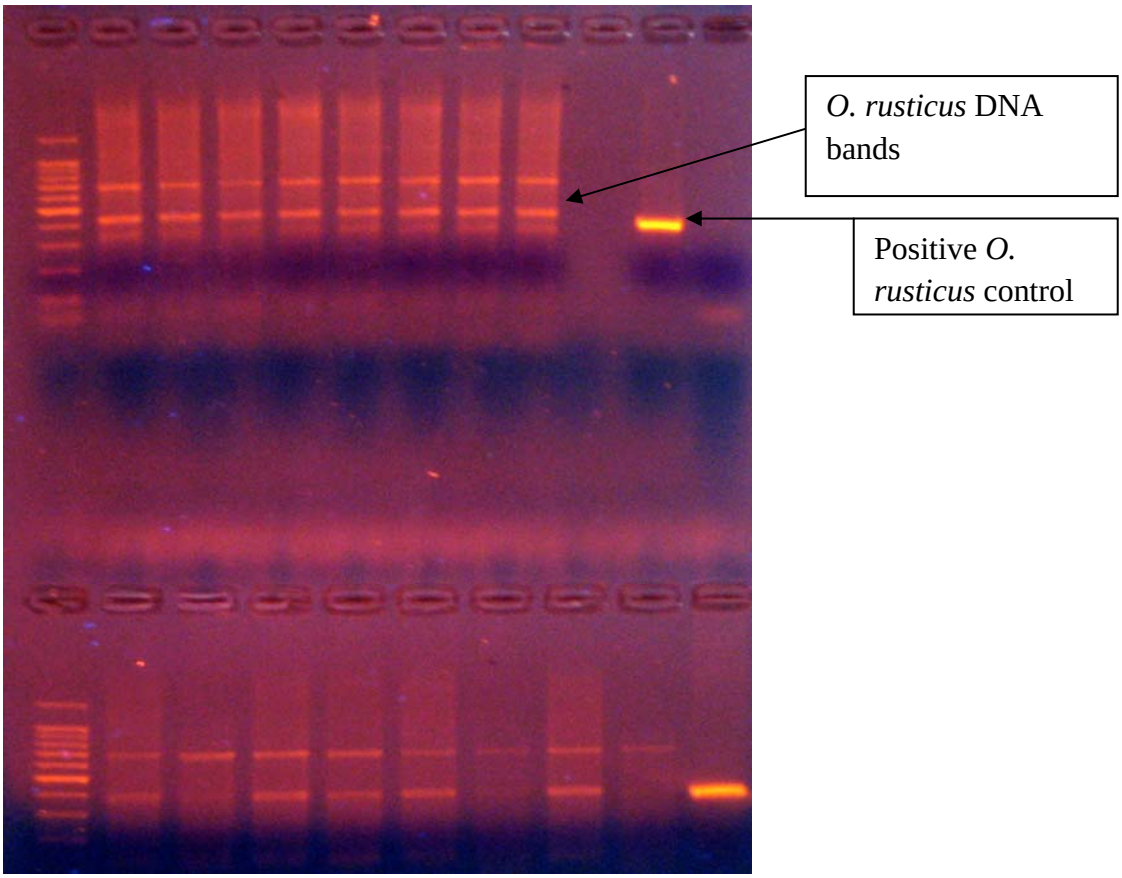

Fig. 1. This figure shows the results of a 1% agarose gel for the bucket tests completed in the laboratory. The arrows indicate that the specific DNA for the *O. rusticus* was detected in all bucket test samples except for the control. Other bands located on the left hand side show DNA molecular weight ladder.

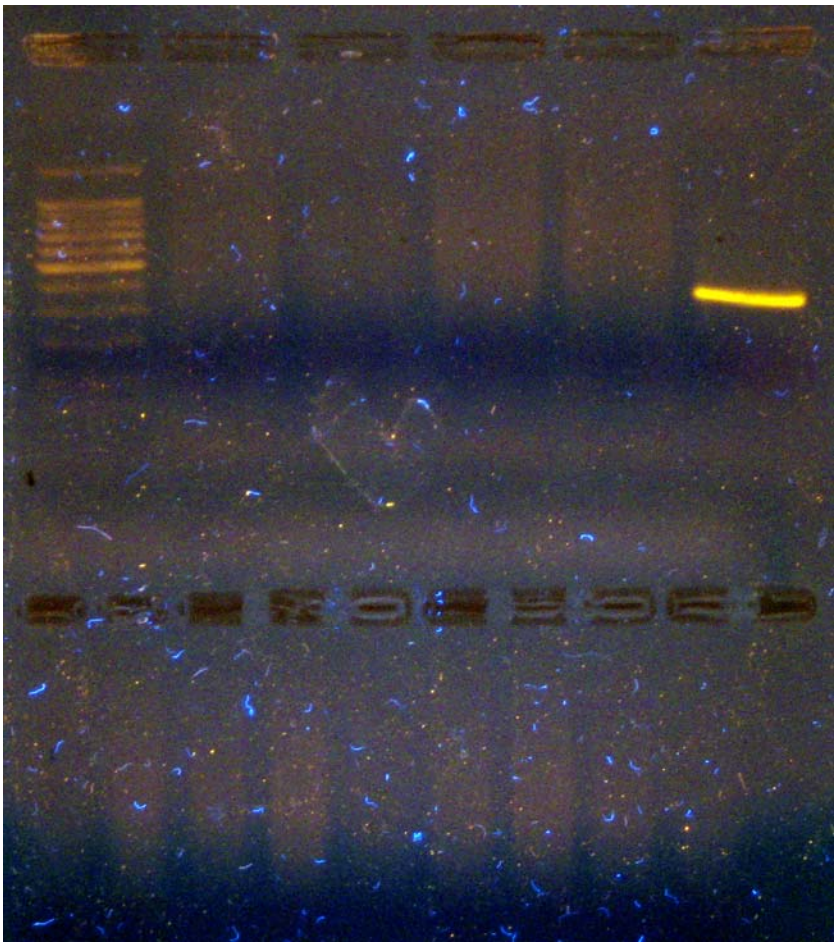

Fig. 2. This 1% agarose gel displaying samples from Big Lake show that *Orconectes rusticus* DNA was not found. The large band located toward the upper right hand side of the gel is the positive control, while the other bands located on the left hand side show DNA molecular weight ladder.

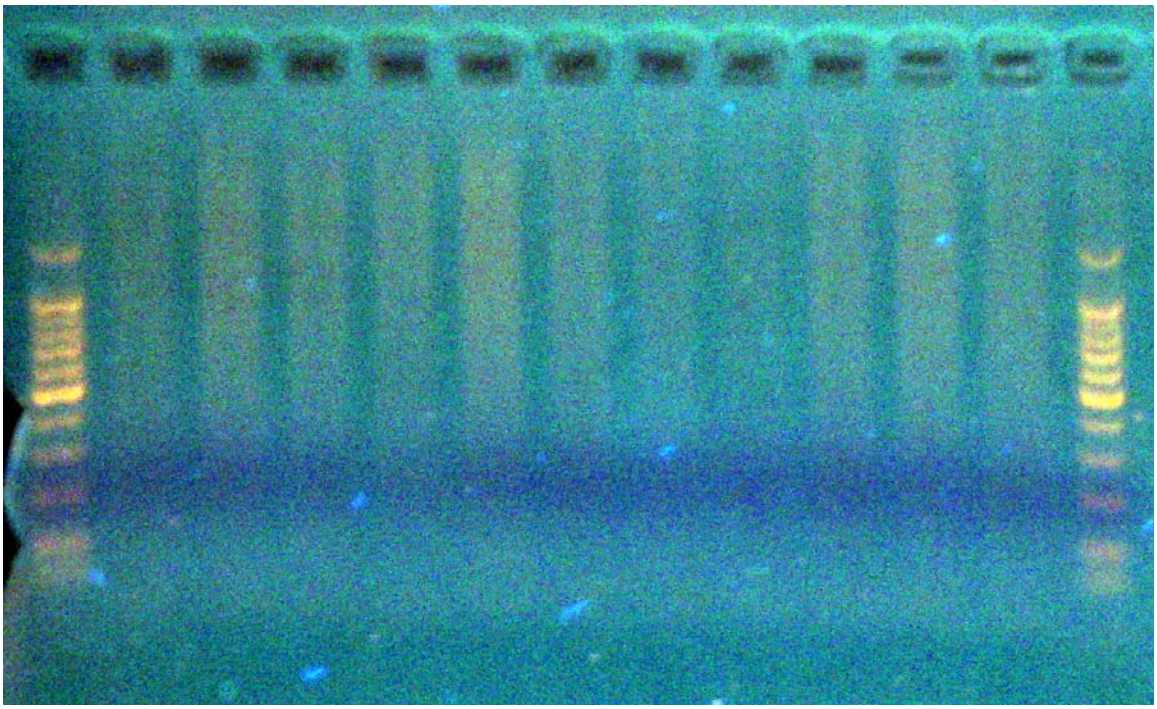

Fig. 3. This 1% agarose gel displays the PCR results from sample sites 1 and 2 in Big Lake. This PCR reaction was modified to contain two microliters of DNA from each site. Bands located on the far right and far left are DNA ladder.

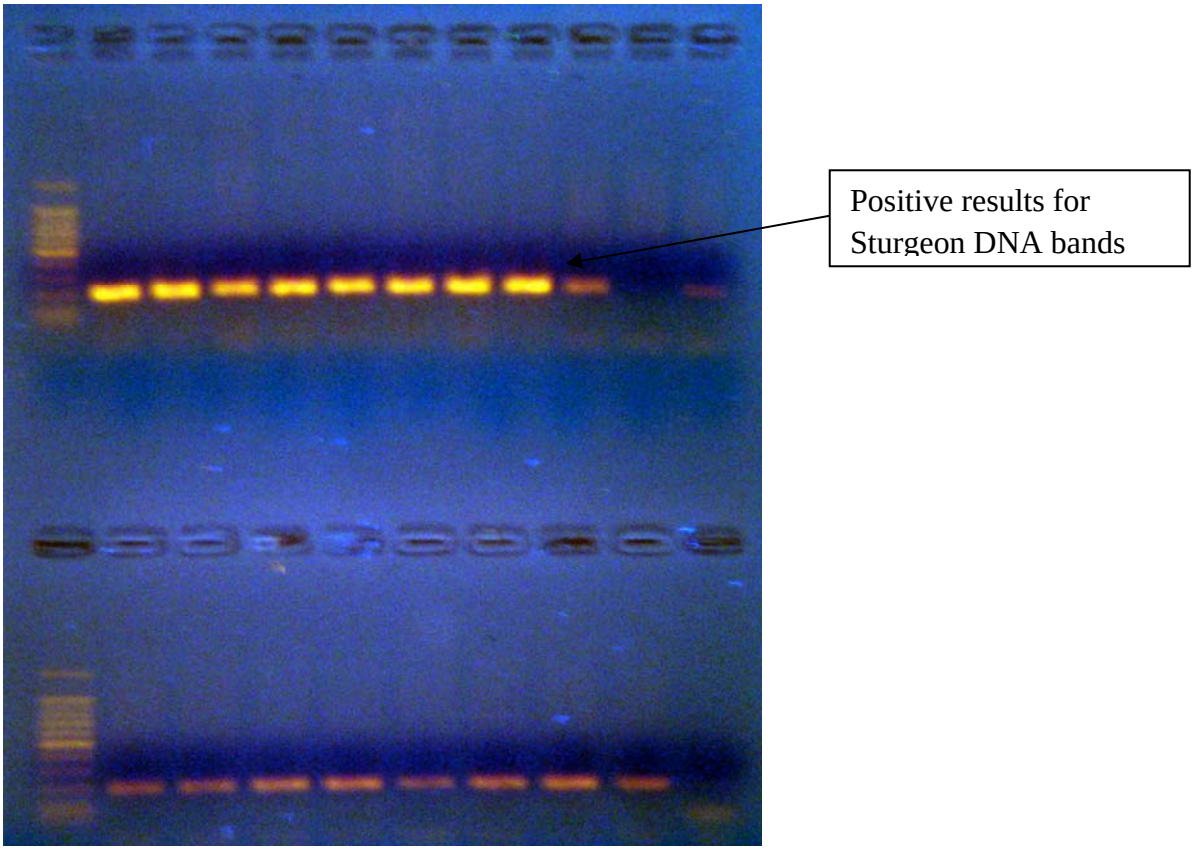

Fig. 4. This gel shows positive results for a water samples collected to find sturgeon DNA. The arrow shows the sturgeon DNA bands. Gel is the result of aquatic samples taken in a similar manner within the field. DNA extraction and PCR was performed in accordance with an identical UltraClean™ Water DNA Isolation Kit.

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Literature Cited

Benson B.J., and T.K. Kratz. 2006. Long-term dynamics of lakes in the landscape: long-term ecological research on North Temperate Lakes. Oxford University Press US.

Boersma, P. D., S. H. Reichard, and A.N. Van Buren. 2006. Invasive species in the Pacific Northwest. University of Washington Press.

Capelli, G.M., and J.J Magnuson. 1983. Morphoedaphic and biogeographic analysis of crayfish distribution in northern Wisconsin. Journal of Crustacean Biology 3:548-564.

Ficetola, F. G., C. Miaud, F. Pompanon, and P. Taberlet. 2008. Species detection using Environmental DNA from water samples. The Royal Society 4:423-425.

Klocker, C. K., and D.L. Strayer. 2004. Interactions among an invasive crayfish (*Orconectes rusticus*), and a native crayfish (*Orconectes limosus*), and native bivalves (Sphaeriidae and Unionidae). Northweatern Naturalist 11(2):167-178.

Olsen, T.M., D.M. Lodge, G.M. Capelli, and R. J. Houlihan. 1991. Mechanisms of impact of an introduced crayfish (*Orconectes rusticus*) on littoral

congeners snails, and macrophytes. Canadian Journal of Fisheries and Aquatic Sciences 48:1853-1861.

Wittenberg, R., Cock, M., GISP. 2001. Invasive alien species: a toolkit of best prevention and management practices. CAB International.