A Thesis Presented to

The Faculty of Alfred University

One Small Band: Troubleshooting a Genetic Study of Common Loons (*Gavia immer*) in Vermont

Lauren Schramm

In Partial Fulfillment of the Requirements for

Alfred University

Honors

Supervised by:

Research Advisor:

Cheryld Emmons, Ph. D.

Committee Members:

Heather Zimbler-DeLorenzo, Ph. D.

Frederic Beaudry, Ph. D.

May 2016

Division of Biology

Alfred University College of Liberal Arts and Sciences

Alfred, NY

Contents

Abstract:

The common loon (*Gavia immer*) is an iconic species for North America. Populations in Vermont reached a low of 8 nesting pairs in 1978. Today the population contains over 80 nesting pairs. The population, due to this bottleneck, seems to be at risk of inbreeding. This puts the species at greater risk for genetic problems. During the summer of 2013 samples of eggshells and membranes were collected. DNA was extracted from these, as well as from historical samples, yielding over 75 samples of DNA. A nanospectrophotometer was used to verify the purity and concentration of these samples. Using primers previously used in loon genetic research PCR reactions where done on the samples to amplify specific DNA sequences. (In a PCR reaction you make a lot of copies of one area of DNA. This section of DNA is determined using the primers you add to the reaction.) Unfortunately, there was an unidentified source of interference in these reactions. A variety of techniques were done including adjusting the PCR cycle, primers changing, the addition of an enhancer and magnesium chloride and a variety of Taq polymerases. This work will be continued at Texas State University, there different DNA extraction methods will be used. There is also potential to work with Dr. McMillan from SUNY Buffalo to analyze her genetic data for loons all over the United States. This research has management implications as in Vermont many lakes have artificial nesting rafts for loons, which promote a larger breeding population. If the loons face genetic problems the use of these rafts will need to be evaluated.

Introduction:

Common Loons

Loons (family Gaviidae) consist of 5 different species that are some of the oldest evolved birds in the world. The common loon (*Gavia immer*) is an iconic species that is often associated with the wilderness in North America (Piper 2002). Worldwide there are estimated to be 607,000 to 635,000 individuals. Populations are found in North America, with the exception of some birds that overwinter in Europe (Evers 2010). Populations in Vermont, New Hampshire, and Massachusetts have all suffered recent population declines and recolonization (Spagnuolo 2014). They serve as an important umbrella species, enabling conservation of freshwater lakes. An umbrella species is a species that when conserved allows for the conservation of other species. This is due to their large habitat requirement, and position as the apex predator in many aquatic systems (Piper 1997). An apex predator is one that is on the top of the food chain, it has no predators. While common loons have not been directly studied in terms of habitat diet interactions, black-throated loons (*Gavia arctica*) have. It was found that lakes with higher numbers and variety of fish were more desirable habitats (Jackson 2005).

Common loons require large lakes as habitats because they are more adapted as water birds than soaring birds; they have solid bones and high wing loading, and therefore, require a large strip of lake to use as a runway for takeoff (Barr 1996). In addition, their diet consists of fish, so there must be a suitable supply. This diet of fish puts them at risk for bioaccumulation of toxins in the environment and therefore they can serve as an indicator species for freshwater lakes. Bioaccumulation occurs when an organism ingests a toxin that is then stored in the body as there is no method to remove it. (Van der Oost 2003) An example of a compound that bioaccumulates is mercury. Since loons are at the top of the trophic levels, the mercury travels up

the food chain and is found in the highest level of loons. This means they can serve as an indicator species which means that their health is highly linked to the health of the ecosystem, so they can be used to predict ecosystem health.

Loons in Vermont

In Vermont, loon populations are managed by the Vermont Center for Eco-studies. In 1978 the populations crashed with a low of just 8 nesting pairs (Hanson 2000). This was due to a variety of pressures including shoreline development, mercury poisoning, led poisoning due to fishing gear, oil spills in wintering grounds, and hunting (Lindsay 1999). On their wintering grounds, loons are very vulnerable to oil spills since they undergo molting on the ocean yearly and are unable to fly at that time (Dunnet 1982). Since that time populations have grown to over 81 nesting pairs and the species has been removed from the state endangered species list (Hanson 2013). This raises the question, where are all of these birds coming from? Are they recolonizing the area or will the population suffer genetic problems in the future due to inbreeding?

Artificial nest rafts are often used in the management of these birds by the Vermont Center for Eco-studies, which increases the number of nests. These are made of floating logs with mess and nesting material in-between, and are anchored in place with cider blocks (see figure 1). Rafts are desirable by loons as they are viewed as small islands, they have the necessary space for a nest, and there is also a lack of predators (Piper 2002). The desirability of these nesting rafts has been observed in the increase of aggressive behavior of birds on lakes with the rafts compared to those on lakes without the rafts (Mager 2007). It is possible that these rafts may be doing more harm than good to the population if they are decreasing genetic diversity (Piper 2002). Populations of loons are much larger in other states and therefore the

offspring produced by these rafts are not important to the population as a whole. If the offspring have genetic problems allowing them to reproduce allows genetic issues to continue in the population. The rafts are typically placed on lakes that would not otherwise be able to support nesting. The birds that live on those lakes have lower levels of fitness, evident in that they do not engage in takeover activity as it would be pointless for them. Allowing birds with lower fitness to reproduce reduce the overall genetic health of the population.

While there is little dispersal data for Vermont, there is a considerable amount of data for the Midwest. Loons have a high rate of breeding fidelity to their territories (Piper 2000). This is increased when they have successfully hatched out chicks from those territories; 83% return rate compared to a 67% return rate in successful pairs (Piper 1997). However, according to a report by the USDA Forest Service territories, tenure averages (how long a pair continues to breed on the same lake) for both sexes is no more than 6 years (USDA Forest Service, Eastern Region 2011). This means the loon pairs will change location multiple times in their lifetime. Other research supports this notion, showing annual territorial turnover to be 20-23% (Piper 2000, Evers 2001). This could bring both birds into and out of Vermont.

A study of red-throated loons (*Gavia stellate*) found that dispersal depended highly on sex, with females averaging a dispersal distance of 37.79 km and males 2.17 km (Okill 1992). These patterns have yet to be studied in common loons. Breeding dispersal distance is usually small for birds and long term tracking of the birds is difficult due to their lifecycle. Loons lay their eggs during the spring time. The chicks are then raised during the summer time. After the summer is over, adults and young head to the ocean. This often occurs at separate times. Once the juveniles are at the ocean they will stay there for two years. The adults return after ice out

(Evers 2010). Ice out occurs in the spring when the winter ice melts off of lakes and loons can once again inhabit them.

If a territory is highly desirable, a loon may try to take it over. Lakes that are desirable have had successful nest hatching, and large amounts of good nesting sites. Good nesting sites may be small islands, marshes with hummocks, and artificial nesting rafts. Typically, males challenge males and females challenge females. In Northern Wisconsin, territory takeovers have been highly studied. While the origin of the loons that takeover is hard to determine there is a great deal of data related to where the displaced loons go. Over the six-year study, displaced loons established new territories 0.2, 0.9, 1, 2, and 5.4 km from their original territory (Piper 2000). If the data is similar in Vermont this is not a significant source of genetic exchange in the population.

The juvenile stage is likely more impactful on the genetic diversity of the population. This is the stage at which birds return to near where they were born and try to establish a breeding territory of their own. If there is a high population density they may be forced to move further away from their natal lakes. Again, only data for the Midwest is available. In Michigan and Minnesota juveniles typically returned 13- 16 km from their natal lakes, but some birds dispersed as far as 92 km (Evers 2000). In Wisconsin numbers were similar with the average dispersal distance from natal lakes being 16- 21 km (Piper 2000). This means that the birds likely are staying near where they were born, which could create genetic issues for the birds of Vermont, as the state is 24,933 km^2. However, Vermont has a low population density, unlike the Midwest so the data may not be the same for the state. In addition, the state is long and narrow so there may be enough dispersal to significantly offset any inbreeding depression.

Loons are a k-selected species (Evers 2010). This means that they have a long life span and produce very few offspring. Their generation time is much longer than say the American Robin (*Turdus migratorius*), which is an r-selected species. The effects of inbreeding make take more time to be observed. Continuous genetic monitoring would be beneficial in their management. On average common loons first breed at age 6 (Evers 2010). They produce 1-2 chicks per year if their nests do not fail. In Vermont loons produced an average of 0.72 chicks per pair per year (Hanson 2002). Chicks are preyed on by birds of prey, large fish, and snapping turtles. Chicks have also been documented being killed in territory disputes (Hanson 2002).

Genetic techniques in conservation

In general populations that are of conservation interest tend to also be small. Smaller populations are more likely to suffer the effects of inbreeding depression caused by a bottleneck. Inbreeding depression occurs when related organisms breed. This is a problem because it reduces genetic diversity and makes it more likely for an organism to have two copies of a recessive deleterious allele. A bottleneck occurs when a population goes from a much larger size to a much reduced size. This reduced population may have a different genetic makeup than the original population; leading to a non-representative set of genes in the next generation. It is called a bottleneck because it is similar to placing the diversity of organism in a population in a bottle and pouring out just a few from the bottle to start the new population. A bottleneck also makes a population more susceptible to genetic drift, genetic drift is the chance that random events will cause a genetic change in a population.

Populations can be studied at a much more accurate level using tissue samples, and events like bottlenecks can be observed in this process. Typically, for genetic studies, once the tissue samples are obtained from the animals the DNA is extracted. This can be done using a

simple kit that takes about 30 minutes to do the whole process once the cells are lysed. The DNA is then subjected to polymerase chain reactions, or PCR. A protein called taq polymerase is added to the DNA as well as primers. (The primer binds to the DNA at a target sequence and the polymerase makes copies of the DNA sequence between where the primers bind). A large number of copies are need to visualize the amplified fragments. The DNA after PCR is placed in the wells of an agarose gel (see gel images in appendix). The gel is surrounded by buffer and an electrical current is passed through it. Since DNA is negatively charged it is pulled to the positive end of the gel than larger fragments. Smaller fragments travel faster in the gel. A DNA ladder is used that has fragments of known sizes. When you compared your fragments to the gel you can tell how large they are. The number and size of fragments can be compared between individuals of the same species in order to gauge the genetic diversity of a population. Between individuals that are related the sizes of these fragments are similar in size. There are specific statistics that can be applied to determine the genetic health of these populations using the fragments (Apostol 1996).

There are two levels at which evolution occurs; microevolution and macroevolution. Macroevolution occurs when you can see physical changes in the organisms. This is caused by microevolution, which is changes at the genetic level. Predicting future genetic problems at the micro level and then using management techniques can prevent issues at the macroevolution scale. Management of populations of interest differ depending on if there is inbreeding or genetic health. If the population has a high level of genetic diversity no actions need to be taken, in addition to management plans that are already in place. However, if there is inbreeding a variety of steps can be taken. In the case of common loons nesting rafts that allow more nesting pairs than naturally can be removed. This will reduce the number of inbred chicks that are produced.

Facilitated migration can also take place. The capture of loons has become a fairly standard procedure. At night time the bird is blinded by a spotlight while another person scoops up the confused bird with a large net. In the Midwest a large number of birds are captured using this technique each year. Most of these captured birds also have a blood sample taken and then are given a unique band on their leg. Genetic analysis could be done on the blood sample and birds selected that have variability that is not seen in the area of interest. These birds could be captured and then moved.

Goals

The objective of this study was to determine if loons in the past in Vermont went through a bottleneck that will result in genetic problems in the future. During the summer of 2013 I served as the loon intern for the Vermont Center for Eco-studies. In this time I was able to collect/ obtain samples of both eggshells and membranes from 75 nests, some dating back to the 1980s. I attempted to analyze these samples using standard genetic techniques to determine the genetic diversity of the loon populations in Vermont. The process resulted in a great deal of trouble shooting in regards to the PCR process.

Methods

Samples where obtained during the summer 2013, when I was the loon intern for Vermont Center for Eco-studies. Samples of eggshells and egg membranes where collected after nests hatched out. Samples were collected from a kayak and then allowed to air dry for a few weeks. After drying samples were placed in labeled plastic bags with paper towels. Historical samples were also obtained from the loon project (table 1). DNA samples where extracted using a Qiagen DNeasy Tissue kit and then polymerase chain reactions where done. The resulting

DNA fragments where then separated by running through an electrophoresis gel. Samples where run both at Alfred University and SUNY Buffalo. Extractions where done on both membranes (when available) and eggshell fragments. A sample was combined with EDTA to see if that improved the extractions. It did not, so the standard Qiagen DNeasy Tissue kit method was used. The adaptions suggested by Trimbos (2009) did not prove to be beneficial so they were not used after the first 16 extractions. Elutions of 200 µl buffer AE were used in one addition in the final steps. After adding AW2 buffer, the spin columns where centrifuged at 13,000 rpm as that was the highest speed on the centrifuge available. All of the samples were placed in the thermo scientific nanospectrometer and ng/µl DNA as well as the 260/280 ratios where recorded.

Troubleshooting PCR

A number of techniques with regard to the PCR process were used. The primers were selected based on the band results from Dhar (1997). The loons in this genetic study where from the Midwest and Northeast. Those with the fewest number of bands were selected. In theory these would produce the clearest bands and will be the easiest to do analysis on. These primers target the nuclear DNA, as opposed to mitochondrial DNA. Mitochondrial DNA is inherited from the mother only.

At first primers Z14, A08, M18, M08, and B12 where used. Primers for cytochrome B and sex primers (P8 and P2) were also used. Dr. McMillan from SUNY Buffalo had me try using sex primers on my samples. They can differentiate between the Z and W chromosomes of the samples. I ran six of my samples along two of hers, which were extracted from loon blood.

PCR reactions take place in a controlled environment called a thermocycler. The thermocycler cycle was edited a number of times (see appendix). This was due to information from Dr. McMillan and the manufacturer of the thermocycler.

An enhancer was added to improve the quality of areas with GC repeats (Ralser 2006). This study showed the enhancer to improve the bands. The bans were clearer and easier to analyze when it was added. As suggested by the makers of the enhancer, AmpliTaq, 1 µl of enhancer was included in 25 µl reactions.

Magnesium chloride was also added to the PCR reactions. This was shown by Harris (1997) to improve the results of PCR. This was added in varying amounts from 0 µl to 3.5 µl per 25 µl reactions.

Alfred Samples

Samples were run on 1.5% agarose gel. Samples were loaded with 10 µl of sample and 5 µl of Thermo Scientific 6X DNA Loading Dye containing Bromophenol Blue, Xylene Cyanol FF (XCFF). Ladders where 100 base pairs, with 3 µl being used and 2 µl of dye. Gels where imaged using UVP Bio Digital Imaging System. Gels where stained using SYBR Safe DNA Gel Stain by Invitrogen.

SUNY Buffalo Samples

Samples were 2% gels. Samples had 3 µl of 5x orange G loading dye added to each PCR reaction and then 25 µl of that was loaded into gels, the exception being the samples that used the taq from Alfred, which were only 15 µl reactions so 3 µl of 5x orange G loading dye was still used but all of the samples where loaded into gels. Gels where imaged using a Bio Rad Gel Doc

Ez Imager. The gel with the sex primers was pre stained, using 4 µl ethidium bromide. The other gel was stained using a post stain of 200 ml buffer and 20 µl ethidium bromide on a shaking bath.

Results

While the primary literature indicated that others had issues extracting DNA from eggshells I found no issues using the Qiagen DNeasy Tissue kit. The majority of the samples contained both eggshell and membrane samples. When available membrane samples where used, as the literature suggested that this would be more favorable for extractions than using eggshells. Not all samples contained both membranes and eggshells. Most samples contained eggshells, as adults throw the membranes out of the nest often. At first I added EDTA to some samples to see if that would aid in the extraction (as suggested by Rikimaru 2009). This did not improve the elution quality and could provide interference with the PCR so the standard method of a Qiagen extraction was used.

After about the first 10 PCR reactions failed new extraction kits where purchased so that the potential for interference from an old buffer would be reduced. The reactions still failed but the quality of the extracted DNA improved.

After doing a number of PCR reactions and seeing no bands, Dr. Emmons recommended that I contact the lead authors on the paper that studied the genetic diversity of loons in the whole Northeast. They put me in touch with a researcher at SUNY Buffalo, Dr. McMillan. She suggested that I change the annealing temperature (was too low). Similar to older ovens, the temperature changes of older thermocyclers were less specific, and likely that is why Dhar (1997) got results at such low annealing temperatures. I then changed the temperature from 35^0C

to 50^0C. I also researched annealing temperatures and found out that they are dependent on the primer that you use. Using an online calculator to determine the Tm, or optimal melting temperature, I found that most of my primers should have an annealing temperature that was 30^0C, which is very low. Annealing temperatures below 50^0C typically do not work. In the base paper, they use a temperature of 30^0C which in theory should not work. It would seem in the future that different primers should be used.

The nanospectrophotometer confirmed that the concentration of the primers was ideal. In the course of this project there was a bad batch of TAE buffer made, which bonded to the nucleic acid. This was discovered as the ladders stopped showing up on gels, and when a new batch was made, they started to appear again.

Out of all the PCR reactions, only 3 bands were observed from my samples. One was observed using sample 38, primer Z14, and PCR cycle set up 2. The other band was observed using sample 46, the enhancer, 3.5 µl magnesium chloride, primer M18, and PCR cycle set up 4. Another band was observed using sample 44, primer Z14, 1.5 µL magnesium chloride, 0.5 µL dTNPs, and PCR cycle set up 7. The samples at SUNY Buffalo had negative reactions for both the samples with sex primers and Dhar primers. These were also run on gels with samples of Dr. McMillan which had positive results for the sex chromosomes but no results for the primers. These primers allow the birds to be sexed, with the females having two bands, one for the Z chromosome and one for the W chromosome (Debiak 2014). Males show one band for their two Z chromosomes. The sex primers did not yield any results on my samples. The Z14 primer did not yield any results on Dr. McMillan's samples but it did on mine.

Discussion

While Dr. McMillan's samples where properly sexed using the primers, none of the samples that I used resulted in any sexing (Samples 63, 49, 44, 23, 54, and 43). In addition, another genetic study of loons used cytochrome b as well and did obtain banding patterns (Bartolome 2011). This indicates the possibility that the samples have DNA that is not loon DNA or possible degradation of DNA over time resulting in extractions that contain either, or both fragments of DNA or single strained DNA. It could possibly be microbial DNA from the samples sitting in the nests before collection. Loons prefer to nest in wet, low lying, areas that are on the shoreline. In a lake this is the littoral zone which is rich in microscopic aquatic life. In addition, the Z14 primer did not result in any bands on Dr. McMillan's samples which were known to be loon samples and of high quality. Other papers suggested that extractions of DNA from egg samples was difficult (Bush 2005, Rikimaru 2009). The nano spectrometer readings suggested, at first, that this was not an issue. However, likely this was because the DNA extracted was not from the egg samples themselves but of the microorganisms on the samples. This would explain why occasionally there would be bands produced, as there was likely a wide variety of different species DNA extraction from the samples.

Genetics has made huge progress in terms of our understanding of populations and their genetic health. These techniques and studies are important in order to make proper management decision about populations. They can prevent changes at the macroevolution scale. The way that loons are management makes them good candidates for interventions if it was found that populations have low genetic diversity. In Vermont this management could look at assisted migration and removal of artificial nesting rafts. However, techniques for extracting DNA from nest samples need to be modified in a way to correct for microbial contamination.

Future work/ suggested studies:

As one of the last aspects of this project at Alfred I will be doing PCR on my samples using bacterial and fungal primers that Dr. Cardinale has (63f, 1387r, Ns1, and Fr1). If these have positive banding results, then the samples are contaminated. If there are negative results it does not mean that the samples are valid as they could potential be bacterial or fungal DNA that is just not targeted with the primers that I am using.

I will be continuing this research at my master's program at Texas State University. first I will try doing the extractions using a Qiagen kit again, but this time using an RNAzyme. If those samples do not produce smears or results using the sex primers, then I will move on to try the Rikimaru (2009) procedure. It is clear from my connection with Dr. McMillan that using the RAPD technique, which was used by Dhar, is not ideal. There is potential to use the primers that Dr. McMillan has developed, which uses microsatellites sequences. Microsatellites are commonly used in genetic studies. For loons the base pair differences when using microsatellite primers is very small therefore acrylamide gel has to be used.

For the analysis I will calculate diversity based on the proportion of samples that are heterozygous at the loci tested, using f-statistics. For these I will assume that over time genetic diversity has remained the same. I will also assume that diversity of loons in Vermont is the same as those in the rest of the Northeast. For the data for the loons in the rest of the Northeast I will use data from Dhar (1997). I also have obtained 50 samples of extracted loon DNA from Wisconsin that I will be able to use to play with different genetic techniques. In the event that I cannot get the primers to work, I could at least work with Dr. McMillan in analyzing the results

that she has gotten in the past from her work with loons. See her paper for the technique that has been used on those samples (McMillan 2004).

Literature cited

Apostol, B. L., Black, I. V., William, C., Reiter, P., & Miller, B. R. 1996. Population genetics with RAPD–PCR markers: the breeding structure of Aedes aegypti in Puerto Rico. *Heredity*, *76*(4).

Barr, J. F. 1996. Aspects of common loon (Gavia immer) feeding biology on its breeding ground. *Hydrobiologia*, *321*(2), 119-144.

Bartolomé, C., Maside, X., Camphuysen, K. C., Heubeck, M., & Bao, R. 2011. Multilocus population analysis of Gavia immer (Aves: Gaviidae) mtDNA reveals low genetic diversity and lack of differentiation across the species breeding range. *Organisms Diversity & Evolution*, *11*(4), 307-316.

Bush, K. L., Vinsky, M. D., Aldridge, C. L., & Paszkowski, C. A. 2005. A comparison of sample types varying in invasiveness for use in DNA sex determination in an endangered population of greater Sage-Grouse (Centrocercus uropihasianus). *Conservation Genetics*, *6*(5), 867-870.

Debiak, A. L., McCormick, D. L., Kaplan, J. D., Tischler, K. B., & Lindsay, A. R. 2014. A molecular genetic assessment of sex ratios from pre-fledged juvenile and migrating adult Common Loons (Gavia immer). *Waterbirds*, *37*(sp1), 6-15.

Dhar, A. K., Pokras, M. A., Garcia, D. K., Evers, D. C., Godron, Z. J., and Alcivar-Warren, A. 1997. Analysis of genetic diversity in common loon *Gavia immer* using RAPD and mitochondrial RFLP techniques. Molecular Ecology, 6: 581-586.

Dunnet, G. M., Crisp, D. J., Conan, G., & Bourne, W. R. P. 1982. Oil Pollution and Seabird Populations [and Discussion]. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *297*(1087), 413-427.

Evers, D., Kaplan, D. J., Reaman, S. P., Paruk, J. D. 2000. A demographic characterization of the Common Loon in the upper Great Lakes. Am. Ornithol. Union, N. Am. Loon Fund: 78-90.

Evers, David C., James D. Paruk, Judith W. Mcintyre and Jack F. Barr. 2010. Common Loon (Gavia immer), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online

Evers, D. 2001. Common Loon population studies: Continental mercury patterns and breeding territory philopatry. University of Minnesota, Ph. D. dissertation.

Kjølner, S., Såstad, S. M., Taberlet, P., & Brochmann, C. 2004. Amplified fragment length polymorphism versus random amplified polymorphic DNA markers: clonal diversity in Saxifraga cernua. *Molecular ecology*, *13*(1), 81-86.

Jackson, D. B. 2005. Environmental correlates of lake occupancy and chick survival of Black-throated Divers Gavia arctica in Scotland: Capsule Lake occupancy was related to the abundance of fish prey for adults (salmonids) and chick survival was related to type of diet, prey abundance and weather. *Bird Study*, *52*(3), 225-236.

Hanson, E. W., Rimmer, C. C., & Gobeille, J. 2000. The 2000 breeding status of Common Loons in Vermont. Unpubl. report. Vermont Institute of Natural Science, Woodstock, VT and Vermont Fish and Wildlife Department, Waterbury, VT.

Hanson, E. W., C. C. Rimmer, and J. Gobeille. 2002. The 2001 breeding status of Common Loons in Vermont. Unpubl. Rept. Vermont Inst. of Natural Science, Woodstock, Vermont.

Hanson, E. W., Rimmer, C. C., & Gobeille, J. 2013. The 2013 breeding status of Common Loons in Vermont. Unpubl. report. Vermont Institute of Natural Science, Woodstock, VT and Vermont Fish and Wildlife Department, Waterbury, VT.

Harris, S., & Jones, D. B. 1997. Optimisation of the polymerase chain reaction. British journal of biomedical science, 54(3): 166-173.

Mager Iii, J. N., Walcott, C., & Piper, W. H. 2008. Nest platforms increase aggressive behavior in common loons. *Naturwissenschaften*, *95*(2), 141-147.

Mcmillan, A. M., Bagley, M. J., & Evers, D. C. 2004. Characterization of seven polymorphic microsatellite loci in the Common Loon (Gavia immer). *Molecular Ecology Notes*, *4*(2), 297-299.

Okill, J. D. 1992. Natal dispersal and breeding site fidelity of red-throated Divers Gavia stellata in Shetland. *Ringing & Migration*, *13*(1), 57-58.

Piper, W. H., Tischler, K. B., Klich, M. 2000. Territory acquisition in loons: the importance of take-over. Animal Behavior. 59: 385- 394.

Piper, W. H., Meyer, M. W., Klich, M., Tischler, K. B., & Dolsen, A. 2002. Floating platforms increase reproductive success of common loons. *Biological Conservation*, *104*(2), 199-203.

Piper, W.H., Paruk, J.D., Evers, D.C., Meyer, M.W., Tischler, K.B., Klich, M. Hartigan, J.J., 1997. Local movements of color-marked common loons. The Journal of wildlife management, 61(4):1253-1261.

Ralser, M., Querfurth, R., Warnatz, H. J., Lehrach, H., Yaspo, M. L., & Krobitsch, S. 2006. An efficient and economic enhancer mix for PCR. Biochemical and biophysical research communications, 347(3): 747-751.

Spagnuolo, V. A. 2014. Landscape Assessment of Habitat and Population Recovery of Common Loons (Gavia immer) in Massachusetts, USA. Waterbirds, 37 (sp1): 125-132.

Rikimaru, K.,Takahashi, H. 2009. A simple and efficient method for extraction of PCR-amplifiable DNA from chicken eggshells. Animal Science, 80(2):220-3.

Trimbos, K. B., Brokeman, J., Kentie, R., Musters, C. J. M., and Snoo, G. R. May 2009. Using eggshell membranes as a DNA source for population genetic research. Ornithology 150: 915-920.

USDA Forest Service, Eastern Region. September 2011. Species Conservation Assessment for the Common Loon (*Gavia immer*) in the Upper Great Lakes.

Van der Oost, R., Beyer, J., & Vermeulen, N. P. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental toxicology and pharmacology*, *13*(2), 57-149.

Appendix

Figure 1. An example of a loon nesting raft. Source: seattle.gov.

Figure 2. A common loon family. Source: Peter Spawn.

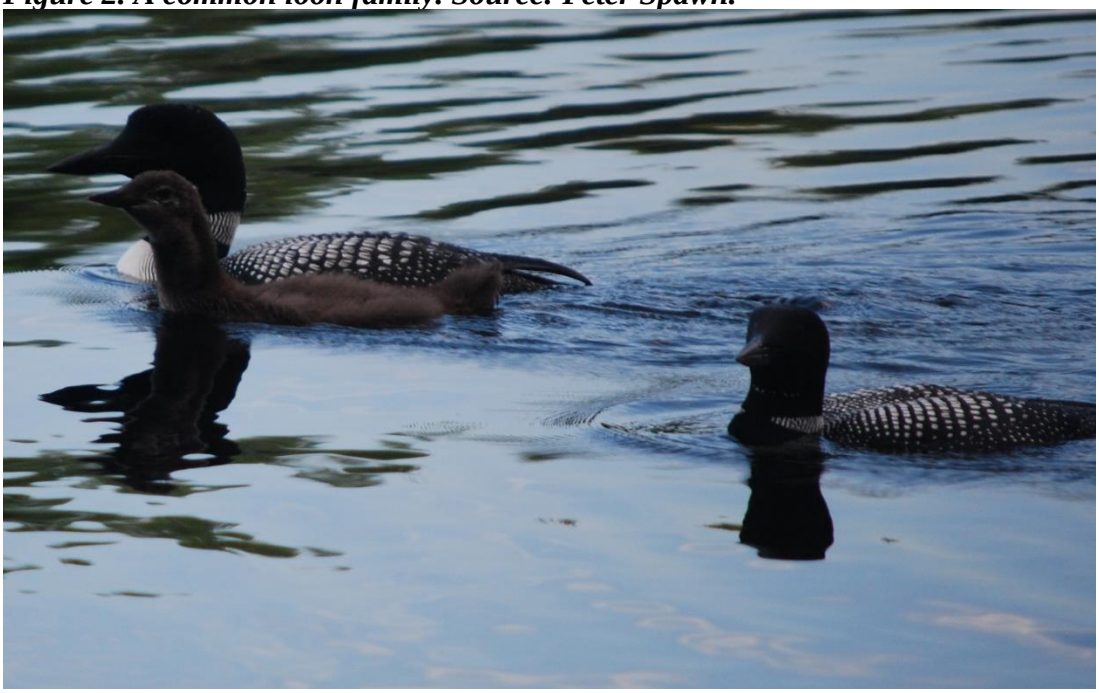


Figure 3. Vermont Center for Eco-studies graph of Vermont loon population trends

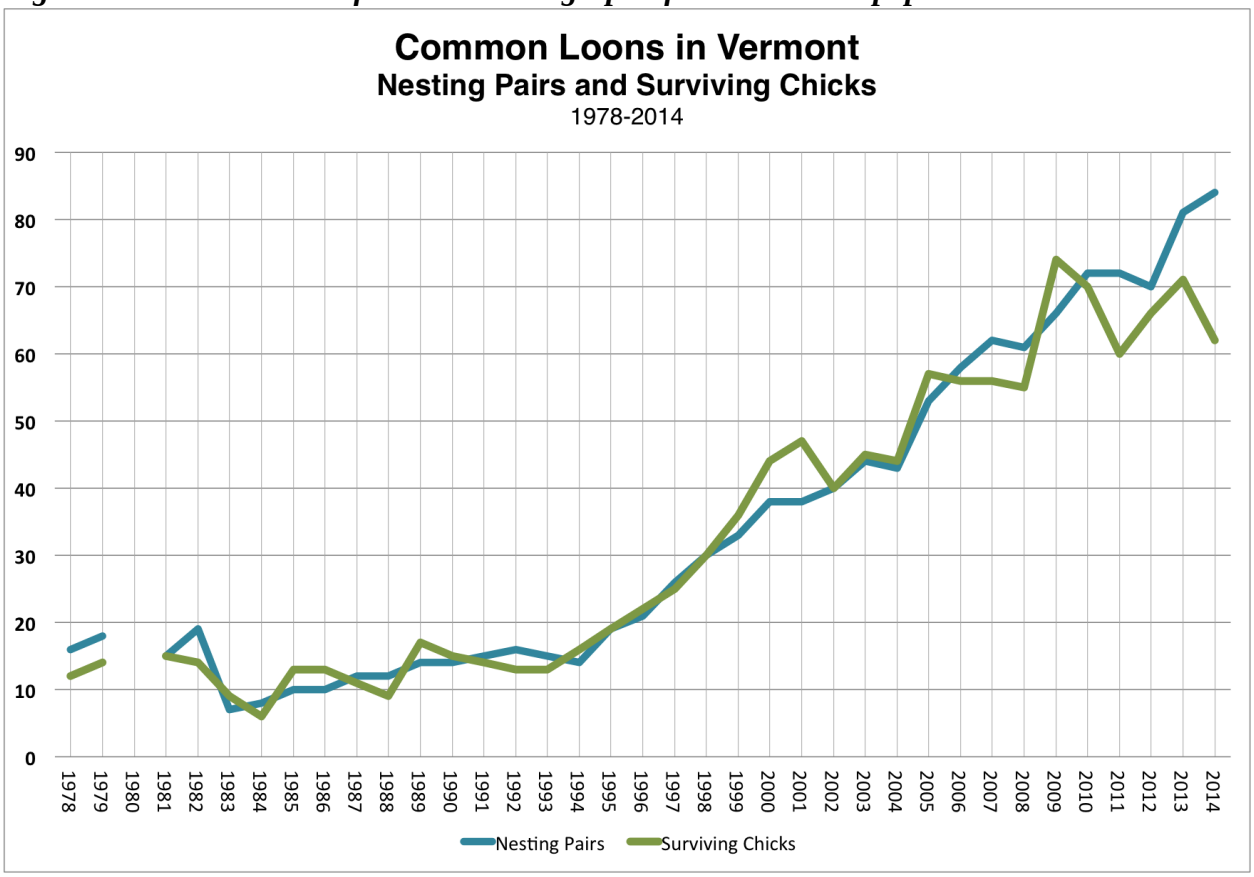


Table 1: Nest sample list from Vermont common loons
Sample Class: 1= eggshell and membrane, 2= eggshell, and 3= membrane

Pond/Lake	Number of chicks	Date collected	Sample class	Town	Date chicks first seen on	Collected by
Maidstone Lake	2	July 17, 1998	1	Maidstone, Vt	July 13, 1998	Sally Borden
Miles Pond	2	June 29, 1998	1	Concord, Vt	Unknown	Reg Gonard
Somerset Reservoir	2	July 15, 1999	1	Seasburg, Vt	July 15, 1999	Sally Borden
Green River Reservoir -NW	2	July 1999	1	Hyde Park, Vt	June 29, 1999	Sally Borden
Unknown	Unknown	Unknown	2	Unknown	Unknown	Unknown
Osmore Pond	Unknown	Unknown	1	Unknown	Unknown	Lauren Schramm
Unknown	Unknown	Unknown	1	Unknown	Unknown	Unknown
May Pond	2	June 18, 1998	2	Barton, Vt	Unknown	Sally Borden
Miles Pond	1	July 17, 2001	1	Concord, Vt	July 10, 2001	Eric Hanson
Zach Woods Pond	2	June 20, 2001	2	Hyde Park, Vt	June 13, 2001	Eric Hanson
Lower Symes Pond	1	July 20, 2001	1	Ryegate, Vt	July 4, 2001	Eric Hanson
Spectacle Pond	2	June 2000	2	Brighton, Vt	June 8, 2000	Eric Hanson
McConnell Pond	1	July 7, 2001	1	Brighton, Vt	June 19, 2001	Sally Borne
Norton Pond Island	2	June 26, 2000	1	Norton, Vt	June 26, 2000	Eric Hanson
Island Pond	Unknown	Unknown	1	Brighton, Vt	Unknown	Eric Hanson
Green River Reservoir -NW	2	July 2, 2001	1	Hyde Park, Vt	June 30, 2001	Eric Hanson
Nichols Pond	2	June 20, 2001	1	Woodbury, Vt	June 17, 2001	Eric Hanson
Norton Pond - South	2	2001	2	Norton, Vt	July 9, 2001	Eric Hanson
Newark Pond	2	July 2, 2001	2	Newark, Vt	June 27, 2001	James Gillis
May Pond	2	June 2,	1	Barton, Vt	June 20,	Eric

		2001			2001	Hanson
May Pond	2	July 2, 2000	1	Barton, Vt	June 18, 2000	James Gillis
Unknown	Unknown	Unknown	2	Unknown	Unknown	Unknown
Wolcott Pond	2	June 24, 2001	1	Wolcott, Vt	June 19, 2001	Eric Hanson
Thurman Dix Reservoir	1	July 2, 2001	1	Barre, Vt	June 25, 2001	Michael Balding
Long Pond	2	July 2, 2001	1	Westmore, Vt	June 19, 20001	James Gillis
Woodbury (Sabin) Pond	2	August 2, 2013	1	Woodbury, Vt	July 2, 2013	Lauren Schramm
Bean Pond (Sutton)	2	August 2, 2013	1	Sutton, Vt	July 17, 2013	Lauren Schramm
Flagg Pond	2	August 8, 2013	1	Wheelock, Vt	July 8, 2013	Lauren Schramm
Joe's Pond- first Pond	2	August 5, 2013	2	Danville, Vt	June 10, 2013	Lauren Schramm
Greenwood Lake	1	August 2, 2013	1	Woodbury, Vt	June 31, 2013	Lauren Schramm
Hardwick Lake	2	August 2, 2013	2	Hardwick, Vt	June 17, 2013	Lauren Schramm
Center Pond	0	August 6, 2013	1	Newark, Vt	NA	Lauren Schramm
Ricker Pond	2	August 5, 2013	1	Groton, Vt	June 22, 2013	Lauren Schramm
Lyford Pond	2	August 2, 2013	2	Walden, Vt	June 20, 2013	Lauren Schramm
Coitis Pond	1	August 2, 2013	1	Cabot, Vt	July 7, 2013	Lauren Schramm
West Daniels Pond	2	August 8, 2013	1	Glover, Vt	June 19, 2013	Lauren Schramm
Green River Reservoir - Access Bay	2	August 8, 2013	1	Hyde Park, Vt	July 8, 2013	Lauren Schramm
Long Pond	1	August 8, 2013	1	Eden, Vt	June 20, 2013	Lauren Schramm
Newark Pond	2	June 2001	1	Newark, Vt	June 27, 2001	John Warren
Metcalf Pond	2	August 8, 2013	1	Fletcher, Vt	July 6, 2013	Lauren Schramm
Great Hosmer Pond	2	August 6, 2013	2	Craftsbury, Vt	June 27, 2013	Eric Hanson
Newark Pond	2	August 7, 2013	1	Newark, Vt	June 26, 2013	Lauren Schramm
Martins Pond	2	August 5,	1	Peacham, Vt	July 10,	Lauren

		2013			2013	Schramm
Joe's Pond-Inlet	2	August 5, 2013	1	Danville, Vt	June 16, 2013	Lauren Schramm
Osmore Pond	2	July 23, 2013	1	Groton, Vt	July 6, 2013	Lauren Schramm
Norton Pond-Island	2	July 24, 2013	1	Norton, Vt	July 17, 2013	Lauren Schramm
Molly's Falls Reservoir-Island	1	July 1, 2013	1	Cabot, Vt	June 30, 2013	Lauren Schramm
Great Averill Pond - North	1	July 20, 2013	2	Norton, Vt	July 8, 2013	Eric Hanson
Mirror Lake (#10 Pond)	1	July 31, 2013	2	Calais, Vt	July 31, 2013	Lauren Schramm
Ewell Pond	2	July 1, 2013	1	Peacham, Vt	July 2, 2013	Lauren Schramm
Little Averill Pond- North	1	July 29, 2013	1	Averill, Vt	Unknown	Lauren Schramm
Spectacle Pond	1	June 26, 2013	1	Brighton, Vt	June 26, 2013	Lauren Schramm
Forest Lake	1	July 29, 2013	1	Averill, Vt	July 29, 2013	Lauren Schramm
Little Averill Pond-West	1	July 29, 2013	1	Averill, Vt	Unknown	Lauren Schramm
Foster's Pond	1	July 1, 2013	1	Peacham, Vt	July 7, 2013	Lauren Schramm
Pensioner Pond	1	June 19, 2013	1	Charleston, Vt	June 19, 2013	Lauren Schramm
Lake Elmore	1	July 31, 2013	2	Elmore, Vt	July 8, 2013	Lauren Schramm
Zach Woods Pond	2	July 1, 2013	1	Hyde Park, Vt	June 30, 2013	Lauren Schramm
Unknown	Unknown	Unknown	1	Unknown	Unknown	Lauren Schramm
Lake Elligo	1	July 12, 2013	1	Greensboro, Vt	June 18, 2013	Lauren Schramm
Molly's Falls Reservoir-North	1	June 25, 2013	1	Cabot, Vt	June 24, 2013	Lauren Schramm
Shadow Lake	1	July 24, 2013	1	Concord, Vt	June 19, 2013	Eric Hanson
Unknown	Unknown	Unknown	1	Unknown	Unknown	Unknown
Jobs Pond	1	July 6, 1998	1	Newark, Vt	Unknown	Sally Borden
Beaver Pond	2	July 4, 1998	1	Holland, Vt	Unknown	Sally Borden

Long Pond	2	June 29, 1998	1	Westmore, Vt	Unknown	Sally Borden
May Pond	1	June 18, 1998	1	Barton, Vt	Unknown	Sally Borden
Unknown	Unknown	Unknown	1	Unknown	Unknown	Unknown
Unknown	Unknown	Unknown	1	Unknown	Unknown	Unknown
Unknown	Unknown	Unknown	1	Unknown	Unknown	Unknown
Wolcott Pond	Unknown	Unknown	1	Wolcott, Vt	Unknown	Unknown
Newark Pond	2	June 19, 1998	1	Newark, Vt	Unknown	Sally Borden
Coles Pond	1	July 4, 1998	1	Walden, Vt	Unknown	Sally Borden
Spectacle Pond	1	June 14, 1998	1	Brighton, Vt	Unknown	Sally Borden
Forest Lake	2	June 12, 1998	1	Averill, Vt	Unknown	Sally Borden

Table 2: Primer Sequences used for PCR.

Primer	Sequence
B12	5’ CCTTGACGCA 3’
Z14	5’ TCGGAGGTTC 3’
A08	5’GTGACGTAGG 3’
M09	5’ GTCTTGCGGA 3’
M18	5’ CACCATCCGT 3’
P8	5’ CTCCCAAGGATGAGRAAYTG 3’
P2	5’ TCTGCATCGCTAAATCCTTT 3’

Table 3: PCR Cycles used.

Cycle number	Initial	Step 1	Step 2	Step 3	Number of cycles	Final
1	5 minutes at 92C	92C for 60 seconds	35C for 90 seconds	72C for 60 seconds	40 cycles	72C for 5 minutes
2	5 minutes at 92C	92C for 60 seconds	55C for 90 seconds	72C for 60 seconds	40 cycles	72C for 5 minutes
3	5 minutes at 95C	95C for 60 seconds	45C for 90 seconds	72C for 60 seconds	35 cycles	72C for 5 minutes
4	5 minutes at 95C	95C for 60 seconds	35C for 90 seconds	72C for 60 seconds	35 cycles	72C for 5 minutes
5	5 minutes at 95C	95C for 60 seconds	30C for 90 seconds	72C for 60 seconds	35 cycles	72C for 5 minutes
6	3 minutes at 94C	94C for 30 seconds	62C for 20 seconds	72C for 45 seconds	30 cycles	72C for 5 minutes
7	5 minutes at 94C	94C for 60 seconds	50C for 90 seconds	68C for 60 seconds	40 cycles	68C for 5 minutes

Table 1: PCR reactions

Sample	Primer	Enhancer?	$MgCl^2$?	Cycle	TAQ
EDTA extracted membrane from Long Pond Eden, VT 2013	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
EDTA extracted membrane from Long Pond Eden, VT 2013	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw shell from Long Pond Eden, VT 2013	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw shell from Long Pond Eden, VT 2013	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw membrane from Long Pond Eden, VT 2013	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw membrane from Long Pond Eden, VT 2013	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
EDTA extracted shell from Long Pond Eden, VT 2013	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
EDTA extracted shell from Long Pond Eden, VT 2013	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
EDTA	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/

extracted membrane from Long Pond Eden, VT 2013 (Repeat)					std buffer
EDTA extracted membrane from Long Pond Eden, VT 2013 (Repeat)	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw shell from Long Pond Eden, VT 2013 (Repeat)	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw shell from Long Pond Eden, VT 2013 (Repeat)	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw membrane from Long Pond Eden, VT 2013 (Repeat)	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw membrane from Long Pond Eden, VT 2013 (Repeat)	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
EDTA extracted shell from Long Pond Eden, VT 2013 (Repeat)	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
EDTA extracted shell from Long Pond Eden, VT 2013	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer

(Repeat)					
Raw shell from Long Pond Eden, VT 2013 (Repeat)	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw membrane from Long Pond Eden, VT 2013 (Repeat)	A011	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
Raw membrane from Long Pond Eden, VT 2013 (Repeat)	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
EDTA extracted shell from Long Pond Eden, VT 2013 (Repeat)	A08	No	No	Cycle set up 1	Bio-rad 2XMM w/ std buffer
2	B12	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
2	M18	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
9	M09	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
9	A08	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
2	M09	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
2	A08	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
9	B12	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
2	Z14	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
9	Z14	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
9	M18	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
17	Z14	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix

17	A08	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
17	M09	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
17	M18	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
17	B12	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
20	A08	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
20	Z14	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
20	B12	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
20	M18	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
20	M09	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
32	B12	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
32	M19	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
32	M09	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
32	Z14	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
32	A08	No	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
38	Cytochrome B Reverse	Yes	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
2	Cytochrome B Reverse	Yes	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
2	Cytochrome B Forward	Yes	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
38	Cytochrome B Forward	Yes	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
38	M09	Yes	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
38	A08	Yes	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
38	Z14	Yes	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
38	M18	Yes	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix
38	B12	Yes	No	Cycle set up 1	AmpliTaq Gold 360 Master Mix

45	B12	Yes	No	Cycle set up 2	AmpliTaq Gold 360 Master Mix
45	Z14	Yes	No	Cycle set up 2	AmpliTaq Gold 360 Master Mix
45	M18	Yes	No	Cycle set up 2	AmpliTaq Gold 360 Master Mix
45	A08	Yes	No	Cycle set up 2	AmpliTaq Gold 360 Master Mix
45	M09	Yes	No	Cycle set up 2	AmpliTaq Gold 360 Master Mix
2	B12	Yes	No	Cycle set up 3	Bio-rad 2XMM w/ std buffer
2	A08	Yes	No	Cycle set up 3	Bio-rad 2XMM w/ std buffer
2	Z14	Yes	No	Cycle set up 3	Bio-rad 2XMM w/ std buffer
2	M18	Yes	No	Cycle set up 3	Bio-rad 2XMM w/ std buffer
2	M09	Yes	No	Cycle set up 3	Bio-rad 2XMM w/ std buffer
2	A08	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
2	Z14	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
2	M09	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
2	B12	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
2	M09	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
9	A08	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
9	M09	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
9	Z14	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
9	M18	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
9	B12	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
6	A08	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
6	M09	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
6	M18	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix

6	Z14	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
6	B12	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
43	M18	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
43	Z14	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
43	M09	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
43	B12	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
43	A08	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
38	M18	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
38	Z14	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
38	B12	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
38	M09	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
38	A08	Yes	No	Cycle set up 3	AmpliTaq Gold 360 Master Mix
64	A08	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
64	M09	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
64	B12	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
64	Z14	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
64	M18	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
63	A08	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
63	M09	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
63	B12	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
63	Z14	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
63	M18	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
69	A08	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix

69	M09	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
69	B12	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
69	Z14	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
69	M18	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
70	M18	Yes	No	Cycle set up 4	Bio-rad 2XMM w/ std buffer
70	Z14	Yes	No	Cycle set up 4	Bio-rad 2XMM w/ std buffer
70	M09	Yes	No	Cycle set up 4	Bio-rad 2XMM w/ std buffer
70	B12	Yes	No	Cycle set up 4	Bio-rad 2XMM w/ std buffer
70	A08	Yes	No	Cycle set up 4	Bio-rad 2XMM w/ std buffer
73	M09	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
73	B12	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
73	A08	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
73	Z14	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
73	M18	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
53	M18	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
53	Z14	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
53	M09	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
53	B12	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
53	A08	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
23	A08	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
23	B12	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
23	M18	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
23	M09	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer

23	Z14	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
59	A08	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
59	M09	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
59	Z14	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
59	M18	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
59	B12	Yes	No	Cycle set up 5	Bio-rad 2XMM w/ std buffer
46	B12	Yes	3.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	M18	Yes	3.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	Z14	Yes	3.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	M08	Yes	3.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	A08	Yes	3.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	B12	Yes	2.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	M18	Yes	2.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	Z14	Yes	2.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	M08	Yes	2.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	A08	Yes	2.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	B12	Yes	1.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	M18	Yes	1.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	Z14	Yes	1.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	M08	Yes	1.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	A08	Yes	1.5 ul	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	B12	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	M18	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix

46	Z14	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	M08	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
46	A08	Yes	No	Cycle set up 4	AmpliTaq Gold 360 Master Mix
63	Sex primers	No	0.75 ul	Cycle set up 6	PlantinumTaq
44	Sex primers	No	0.75 ul	Cycle set up 6	PlantinumTaq
49	Sex primers	No	0.75 ul	Cycle set up 6	PlantinumTaq
23	Sex primers	No	0.75 ul	Cycle set up 6	PlantinumTaq
54	Sex primers	No	0.75 ul	Cycle set up 6	PlantinumTaq
43	Sex primers	No	0.75 ul	Cycle set up 6	PlantinumTaq
Dr. McMillan 1	Sex primers	No	0.75 ul	Cycle set up 6	PlantinumTaq
Dr. McMillan 2	Sex primers	No	0.75 ul	Cycle set up 6	PlantinumTaq
64	Z14	No	1.5 ul	Cycle set up 7	PlantinumTaq
44	Z14	No	1.5 ul	Cycle set up 7	PlantinumTaq
49	Z14	No	1.5 ul	Cycle set up 7	PlantinumTaq
23	Z14	No	1.5 ul	Cycle set up 7	PlantinumTaq
54	Z14	No	1.5 ul	Cycle set up 7	PlantinumTaq
43	Z14	No	1.5 ul	Cycle set up 7	PlantinumTaq
Dr. McMillan 1	Z14	No	1.5 ul	Cycle set up 7	PlantinumTaq
Dr. McMillan 2	Z14	No	1.5 ul	Cycle set up 7	PlantinumTaq
Dr. McMillan 1	Z14	No	0.75 uL	Cycle set up 7	AmpliTaq Gold 360 Master Mix
Dr. McMillan 2	Z14	No	0.75 uL	Cycle set up 7	AmpliTaq Gold 360 Master Mix
63	Z14	No	0.75 uL	Cycle set up 7	AmpliTaq Gold 360 Master Mix
44	Z14	No	0.75 uL	Cycle set up 7	AmpliTaq Gold 360 Master Mix
43	Z14	No	0.75 uL	Cycle set up 7	AmpliTaq Gold 360 Master Mix

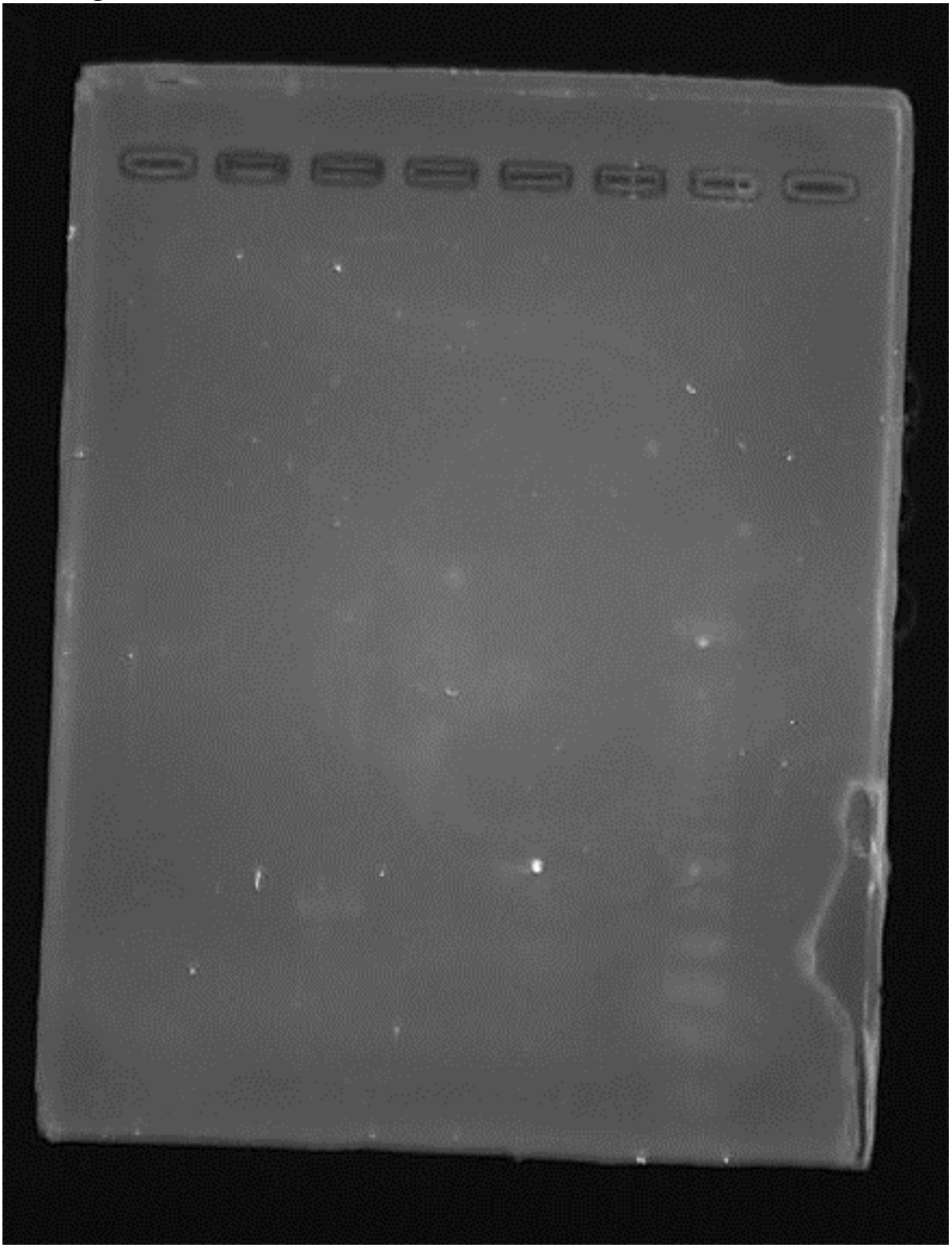
Table 3. Extraction results

Sample	Location	Year	ng/ul	260:280
15	Greenwood Lake	2013	0	0.01
56	Thurman Dix Reservoir	2001	5.8	0.71
52	Island Pond	Unknown	0.9	0.81
31	Zach Woods Pond	2001	1.7	0.94
26	Woodbury (Sabin) Pond	2013	2.6	1

58	May Pond	2001	6.5	2.02
60	Unknown 1	Unknown	1.4	1.22
36	Mirror Lake (#10)	2013	1.6	1.25
61	May Pond	2001 (June 2)	2.4	1.26
66	Unknown 5	Unknown	4	1.3
37	Great Hosmer Pond	2013	1.3	1.31
50	Lower Symes Pond	2001	12	2.17
54	Beaver Pond	1998	6.9	1.83
55	Newark Pond	1998	1.4	1.33
33	Nichols Pond	2001	1.8	1.36
6	Molly's Falls Island	2013	5.6	1.45
3	Long Pond	2013	8.9	1.48
67	Lake Elmore	2013	5.8	1.48
18	Newark Pond	2013	4.4	1.58
57	Jobs Pond	1998	3.4	1.59
34	Norton Pond Island	2000	1.6	1.64
27	Green River Reservoir	2013	2.6	1.65
72	Unknown 8	Unknown	4.8	1.65
43	Green River Reservoir NW	2001	10.5	1.69
64	Unknown 3	Unknown	3.2	1.71
49	Long Pond	1998	8.7	1.72
30	Osmore Pond	2013	1.2	1.73
63	Hardwick Lake	2013	5.1	1.74
2	Molly's Falls North	2013	23.1	1.75
44	Coles Pond	1998	7.1	1.75
59	Norton Pond South	2001	2.2	1.78
23	Zach Woods Pond	2013	2.6	1.82
53	Long Pond	2001	5.2	1.84
69	Joe's Pond- first Pond	2013	4.8	1.86
73	Osmore Pond	Unknown	9.2	1.87
17	Ewell Pond	2013	10.9	1.92
38	May Pond	2000	12.1	1.92
21	Metcalf Pond	2013	2.6	1.93
9	Little Averill West	2013	40.1	1.98
32	Wolcott Pond	2001	14.5	2
68	Unknown 6	Unknown	6.8	2.01
14	Ricker Pond	2013	2.7	2.05
48	Green River Reservoir NW	1999	1.5	2.09
70	Wolcott Pond	Unknown	39	2.11
19	Martin's Pond	2013	3.2	2.12
35	Newark Pond	2001	3.3	2.21
7	Lake Elligo	2013	3.9	2.23
16	Little Averill North	2013	2.3	2.23
20	Bean Pond (Sutton)	2013	5.2	2.2

47	May Pond	1998		2.3	2.25
71	Spectacle Pond	2000		1.7	2.29
46	Spectacle Pond	1998		2.1	2.1
45	McConnell Pond	2001		2.5	2.31
4	Pensioner Pond	2013		8.1	2.32
13	Center Pond	2013		3	2.37
10	Coitis Pond	2013		5.5	2.81
42	Somerset Reservoir	1999		7.1	2.82
12	Spectacle Pond	2013		1.2	2.91
28	West Daniels Pond	2013		2.3	2.91
22	Flagg Pond	2013		2.3	3.01
8	Foster's Pond	2013		4.5	3.2
40	Miles Pond	1998		1.1	3.44
25	Joe's Pond Inlet	2013		2.6	3.52
62	Unknown 2	Unknown		2.2	3.59
11	Shadow Lake (Concord)	2013		3.1	3.71
51	Miles Pond	2001		1.3	3.81
39	Forest Lake	1998		2.9	4.09
24	"Class 1 sample"	2013		1.8	4.37
41	Maidstone Lake		1998	1.7	5.24
65	Unknown 4	Unknown		0.9	5.54
74	May Pond		1998	2.1	5.94
5	Forest Lake		2013	2.5	12.43
29	Norton Pond Island		2013	1.9	34.07
75	Newark Pond		2001	7.9	1.73
76	Great Averill Pond-North		2013	2.1	2.1
77	Lyford Pond		2013	5.9	2.19

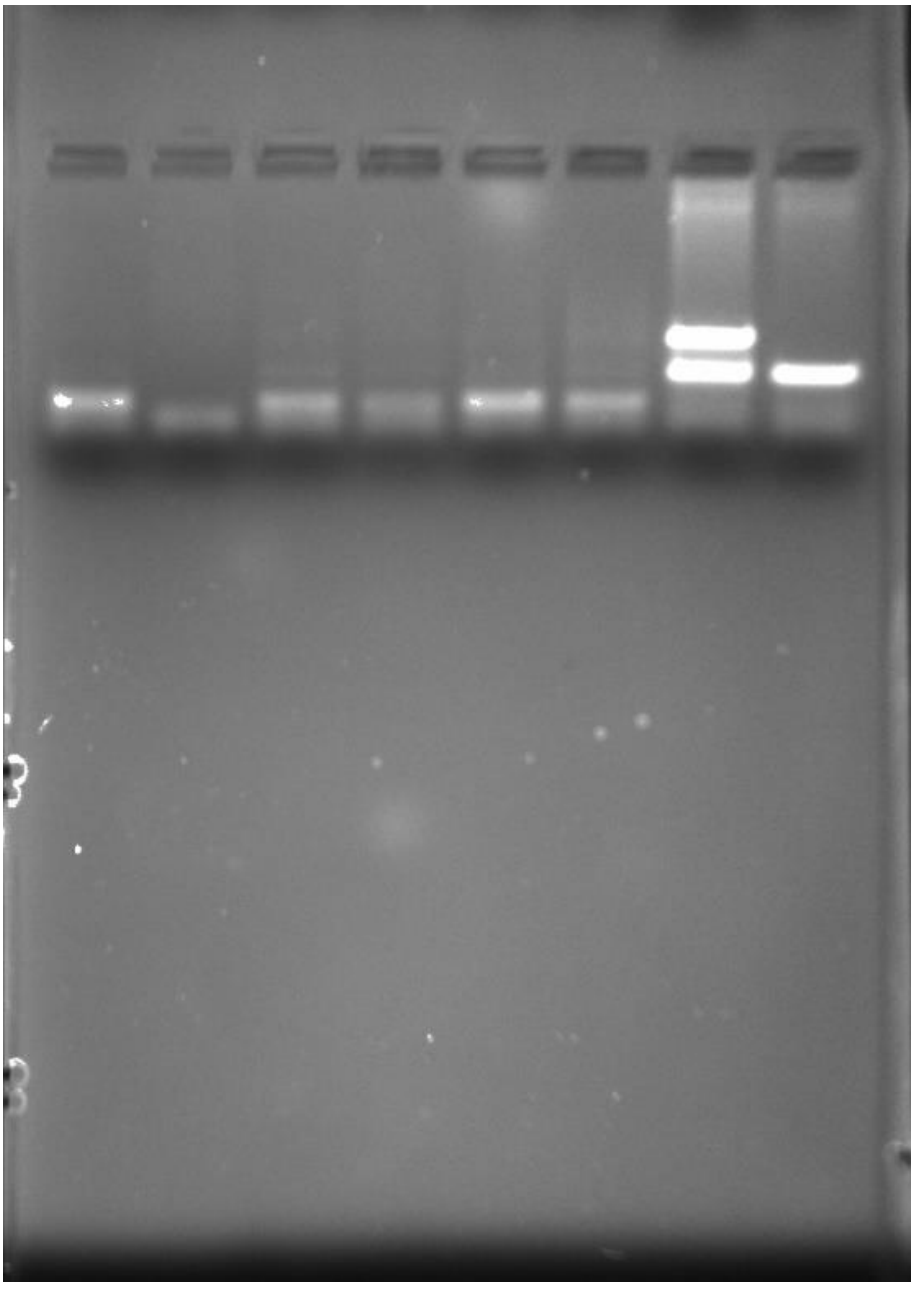
Gel Images



Gel 1. 03.01.2016

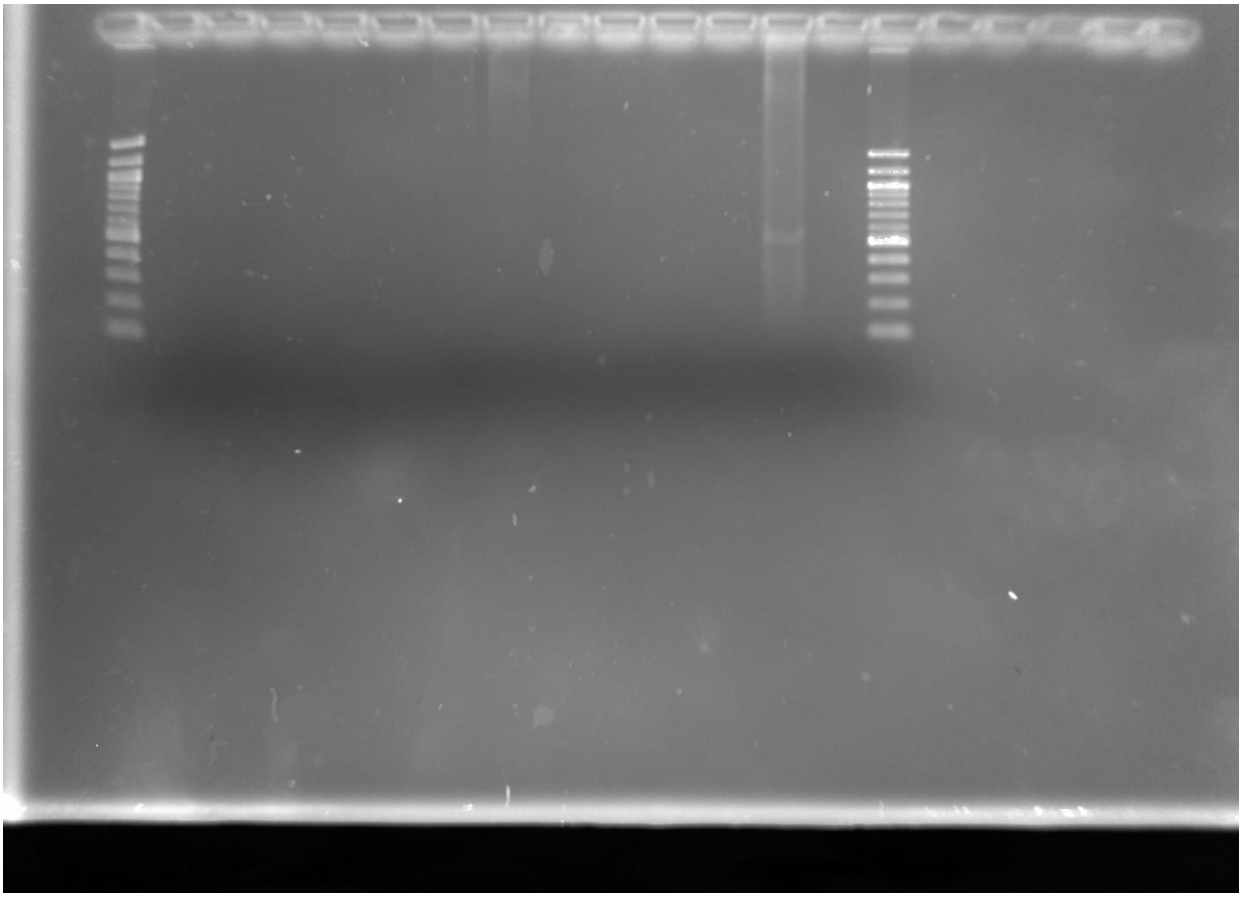
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8

38-M09	38-A08	38-Z14	38-M18	38-B12	--	Ladder	--



Gel 2. 04.10.2016

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
23-Sex	63-Sex	49-Sex	54-Sex	44-Sex	43-Sex	Dr. McMillan A- Sex	Dr. McMillan B- Sex



Gel 3. 04.10.2016

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
Ladder	Dr. McMillan A- Z14	Dr. McMillan B- Z14	63-Z14	44-Z14	43-Z14	Dr. McMillan A- Z14	Dr. McMillan B- Z14
Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15	
23-Z14	49-Z14	54-Z14	64-Z14	44-Z14	43-Z14	Ladder	