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DNA Extraction and Microsatellite Amplification of Daphnia pulicaria Resting Eggs: Analysis of Allele Frequencies Through Time

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DNA Extraction and Microsatellite Amplification of *Daphnia pulicaria* Resting Eggs: Analysis of Allele Frequencies Through Time

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Abstract

A paleoecological approach allows for the study of genetic change in populations over longer periods of time than would be possible if one were sampling populations from year to year. *Daphnia* and other cladoceran zooplankton are amenable to this type of study because they produce diapausing embryos (ephippial eggs) when they sexually reproduce, and these resting eggs can remain viable for decades to centuries in lake sediments. This study uses paleoecological methods as well as a new methodology for ephippial DNA extraction and amplification to assay for genetic variation in ephippial eggs obtained from sediments of varying ages from Square Lake (Washington County, MN). Using this new methodology was successful and it was possible to extract 22 ephippial embryos and test 12 different loci for each one. Starting in 1981, Square Lake was annually stocked with rainbow trout, a selective predator on *Daphnia pulicaria*, by the Minnesota Department of Natural Resources (MDNR). Eggs extracted from the sediment cores of this lake were screened for 15 loci and genotyped by capillary electrophoresis and of those, 12 loci amplified and 11 were polymorphic. Genetic analyses of the ephippial eggs deposited before and after the advent of the trout stocking program indicates that the pre-1981 (1930-1977) individuals have higher allelic diversity than the those assayed from recently deposited sediments (2015 dredge sample). In addition, there were relatively few alleles in common between the resting eggs assayed from the two time periods. It is possible to use this methodology and resulting data at Hamline University to conduct further research on the topic of *Daphnia pulicaria* population genetics as it produces data with resolution and also replicability.

Introduction

Studying the evolution of populations can be challenging given that evolution is a gradual and often slow process, and it is difficult to obtain data over enough generations through time to observe evolutionary change. However, by using a paleoecological approach, researchers can examine evolutionary changes in populations of organisms with dormant life stages over many decades or even centuries (Frisch et. al, 2014).

Freshwater crustaceans in the order Cladocera are examples of species that are amenable to this approach (Jeppesen et. al, 2001; Frisch et. al, 2014). Animals in these species are able to reproduce both asexually and sexually (Fig. 1). Asexual reproduction (via parthenogenesis) is the usual mode of reproduction by cladocerans, but individuals periodically switch to sexual reproduction in response to environmental cues such as changes in food availability or increased population density (Carvalho & Hughes, 1983, Kleiven et. al, 1992). Offspring that are the products of sexual reproduction enter into a dormant state in early embryonic development. The dormant embryos ("resting eggs") are held within a protective case called an ephippium, and when the ephippia are released by the mothers, many of them fall to the lake bottom. The diapausing embryos in the ephippia that sink to the sediment may complete their development and hatch if signaled by environmental cues (Frisch et. al, 2014, Bergue et. al, 2015) or the embryos may not receive the cues to hatch and the ephippia may become buried in sediment and become part of the "egg bank" (Weider et. al, 1997). The rates of sedimentation in a given lake or pond will affect how long it takes for an ephippium to become buried and no longer sensitive to hatching cues (Radzikowski et. al, 2016)

The resting eggs that remain in dormancy, become buried, and fail to hatch will not contribute to the genetic makeup of the active population in the lake. However, those resting eggs that are deposited over time produce a record in the sediments that can be sampled using paleoecological techniques (coring) to evaluate how populations have evolved over many decades (Binford, 1990) rather than over just several years if sampling the populations in the water column (Frisch et. al, 2014). Observing genetic changes in populations through time can reveal information about changing environmental conditions such as temperature, nutrient loading, changes in size of habitat or food web dynamics (Haag et. al, 2010, Frisch et. al, 2014, Frisch et. al, 2017) . Understanding evolution of populations through time and analyzing the alleles that have been selected for is important for understanding the changes in both biotic and abiotic factors and the impact they have on populations and the population adaptations through time (Forest et. al., 2007; Frisch et. al, 2014).

Microsatellites are tandem repeats made up of two- or three-base pair sequences. The regions that contain microsatellites are prone to sequence errors during recombination. However, these are not corrected as they are generally not located in the coding region, and therefore not coding for proteins. These can provide information on the genetic components of evolutionary processes (Schierwater et. al, 1994). Microsatellite markers are useful for population genetics studies because they typically have high allelic variation within and between populations, thus increasing the probability of distinguishing between populations and detecting changes over time (Selkoe & Toonen, 2006). Also, microsatellites are neutral markers (in the non-coding regions of

the genome) and do not affect the fitness of the organism. This provides an opportunity to understand gene flow and to analyze the degree of connection among contemporary populations (Haag et. al, 2010) or populations through time (Frisch et al., 2014). Although these markers are not in coding regions of the genome, they may be linked to genes that influence phenotype and subject to selection. So in some cases it is possible to correlate microsatellite marker genotypes with changes in the environment (e.g., increased phosphorus loading to an ecosystem, Frisch et al., 2014). The markers can be used to assess gene flow to determine the extent to which current populations (spatial) or past populations (temporal) of organisms are connected (Haag et al, 2010, Frisch et. al, 2014).

This study uses DNA microsatellite markers to characterize the genetic composition through time of the *Daphnia pulicaria* population from Square Lake (Washington County, MN) using ephippial eggs obtained from different depths (ages) in sediment cores that were collected in 2015. Square Lake is an oligotrophic to mesotrophic lake that has become increasingly mesotrophic over the last 30-40 years. This eutrophication trend has been attributed to the annual stocking of rainbow trout (Hembre, 2018) by the Minnesota Department of Natural Resources (MDNR) that began in 1981. Rainbow trout are zooplanktivorous fish that preferentially prey on large-bodied zooplankton (e.g., the large-bodied grazer *D. pulicaria*). This predation then causes a top-down cascade that results in less grazing pressure on algae, higher levels of algae biomass, and decreased water clarity (Carpenter et al., 1985; Hembre, 2018).

A paleoecological study was initiated in 2015 to complement the monitoring study that evaluated the effect of trout stocking on Square Lake's zooplankton community and water quality (Hembre, 2018). Sediment cores from Square Lake and a reference lake, Big Carnelian Lake, were obtained and sediment samples from various depths in the cores (from recently deposited sediments to sediments deposited several hundred years ago) were examined to determine the abundances and sizes of remains from two species of *Daphnia* (the larger-bodied *D. pulicaria* and the smaller-bodied *D. mendotae*) found in these lakes. Consistent with our expectations (collaborative research among Mike Gilray, Leif Hembre, and I) that predation by trout would select for smaller body size in *D. pulicaria*, the size of the exoskeletal and ephippial remains of *D. pulicaria* in the sediments decreased significantly after 1981 (year that the stocking program began) in the Square Lake core, but not in the core from the reference lake (Gilray, 2018).

We observed a phenotypic change in the size of the exoskeletal and ephippial remains of *D. pulicaria* over time in Square Lake, but it is not known whether this change in body size was a result of phenotypic plasticity or genetic change in the population. Investigating whether the genetic composition of the *D. pulicaria* population in Square Lake has changed since the initiation of the trout stocking program (in 1981) was the impetus for this study. In a similar study (Frisch et. al, 2014) the genetic makeup of a *D. pulicaria* population in South Center Lake (Chisago County, MN) was assessed using microsatellite markers, and temporal structuring over more than 200 years was documented. Frisch et al. (2014) also discovered significant correlations between the shifts in the genetic structure of the population and changes in land use and human population size in the lake's watershed. Here, it may be possible that the initiation of

trout stocking in Square Lake in 1981 promoted a change in the genetic structure of the lake's *D. pulicaria* population that could be reflected in the microsatellite data.

This study aims 1) to test a methodology for DNA extraction from ephippial eggs obtained from sediments across wide range of ages and 2) to test for consistency of amplification of microsatellite markers, and 3) to genotype individuals from before and after 1981 (first year of rainbow trout stocking in Square Lake) to evaluate differences in the genetic composition of these temporal populations. Specifically, the research question that I investigated was: Is there evidence that the *Daphnia pulicaria* population has changed through time?

Methods

Study Site

Square Lake (Washington County, Minnesota: 45°09.40' N; 92°48.26' W) is a relatively deep lake (maximum depth = 20.7 m, mean depth = 9 m) that has a surface area of 81.9 ha and a volume of 6.95 x 10^6 m^3 (Hembre, 2018). The lake is oligotrophic/mesotrophic and is ranked in the top 1% for secchi disk transparency in the *North Central Hardwood Forest* ecoregion of Minnesota (Johnson, 2017). However, monitoring data from the Minnesota Pollution Control Agency (MPCA) database show that Square Lake's secchi disk transparency has decreased by 2.5 m since the 1970s when the average summer secchi transparency depth was 7.5 m (Hembre, 2018).

Sediment Core Collection and Processing

Three sediment cores were collected from Square Lake on 8 July, 2015 from the deepest part of the lake using a piston corer fitted with a 7-cm diameter polycarbonate tube. One of the cores was extruded in the field with a portable extruder (Glaser & Griffith, 2007). The top 15 cm of the core was extruded into 0.5 cm sections, and depths greater than 15 cm were extruded in 1 cm sections. The intact cores and samples from the extruded core were brought to the University of Minnesota's *LacCore* facility for various analyses.

Samples from the field-extruded core were processed for loss on ignition (LOI) analyses at the LacCore laboratory and prepared for radiometric dating (Pb^{210}). LOI analyses were done to determine the dry mass and composition (i.e. organic matter, carbonates, non-carbonate inorganic matter) of the sediments (Dean, 1974). Changes in sediment composition can correlate with environmental changes in the lake's history (e. g., land clearing during European settlement in the mid-1800s). Pb^{210} dating (using freeze-dried subsamples from the field-extruded core) was conducted at the Minnesota Science Museum's St. Croix Watershed Research Station. Pb^{210} is an isotope with a half-life of 20 years. Therefore, it is useful for providing relatively precise estimates of the ages of sediments deposited in the past 100-200 years.

One of the two intact cores was split for imaging and archival at the University of Minnesota *LacCore* lab. The other intact core was extruded in the lab in the same manner as the field extruded core, with 0.5 cm increments to a core depth of 15 cm, and 1 cm increments thereafter.

The lab-extruded core samples and leftover samples from the field-extruded core were transferred to Hamline University and stored in the dark at 10°C. These core samples were then subsampled to examine various zooplankton remains. Exoskeletal remains (post-abdominal claws of *Daphnia* and head shield of *Bosmina*) and *Daphnia* ephippia (open and intact) were enumerated and measured for the paleoecological study (Gilray, 2018), and intact *Daphnia* ephippia were extracted from the sediment samples for use for this genetic study.

Ephippia Extraction

Extruded core samples from a variety of depths were subsampled (1-2.5 cm^3 of sediment) to obtain ephippia. To remove fine sediments, samples were washed through a 80 μm mesh screen using filtered lake water. The remaining sediment was divided into sterile 15 mL centrifuge tubes filled with a 30% sucrose solution, and the tubes were centrifuged at 2500 rpm for 2 min to separate the lipid-rich ephippia from the sediments (Caceres, 1998). Both the supernatant and the pellet were then carefully examined using a stereomicroscope to search for ephippia. Intact ephippia (closed cases with visible embyros) were extracted with jeweler's forceps, measured along the ephippium's dorsal ridge (Fig. 2b), and stored at 10°C in filtered lake water in 15 mL centrifuge tubes blacked-out with aluminum foil until they could be used for DNA extraction. Ephippia that were not intact (i.e., open cases) were extracted, identified to species, and measured before disposing of them (Fig 2c) to add more data to the paleoecological study's data set.

Dissection of ephippia and DNA extraction from resting eggs

Intact ephippia were dissected using jeweler's forceps under a stereomicroscope to separate the resting eggs (embryos) from the ephippial case. A total of 22 individual embryos were obtained for this study. Ephippia were obtained from two sources: a surface sediment sample collected with an Ekman dredge in 2015, and the sediment core samples (also collected in 2015). Embryos obtained from the dredge sample (n=10) are estimated to be from 2015, while embryos obtained from the cores samples (n=12) were from multiple sediment depths with ^{210}Pb dates older than 1981 (the year that rainbow trout were first stocked in Square Lake). The specific sediment ages and sample sizes for embryos predating 1981 are as follows: 1977 (n=5), 1963 (n=2), 1954 (n=2), 1940 (n=2) and 1930 (n=1).

A modified HotSHOT protocol (Montero-Pau et al. 2008) was used to extract DNA from the resting eggs. Individual eggs were placed into a 0.2 mL tube with an alkaline lysis buffer (NaOH 25mM, disodium EDTA 0.2mM, pH 8.0) using a pipette. The sterile pipette tip was then used to crush the egg against the tube to release the embryo. The tubes with released embryos were incubated for 30 minutes at 95℃, and then cooled on ice for 3-4 minutes. Once cooled, a neutralizing solution (Tris-HCl 40mM, pH 5.0) was added to each tube. The tubes were then vortexed and spun using a Spout Mini Centrifuge. Samples were stored at -20℃ until PCR reactions could be conducted.

DNA microsatellite PCR reactions

After the HotSHOT protocol, 2 **µ**L of extracted DNA for each embryo reaction was used in polymerase chain reactions (PCR) for each of the 15 microsatellite loci assayed (Dp38, Dp43, Dp90, Dp162, Dp173, Dp283, Dp291, Dp311, Dp369, Dp375, Dp376, Dp433, Dp437, Dp446, Dp461 (Colbourne et. al, 2004, Frisch et. al, 2014)). For microsatellite primer sequences and PCR settings used see Table 1. Each microsatellite locus was amplified in single 24 **µ**L reactions using the thermocycler conditions recommended by the *Daphnia Genomics Consortium (http://wfleabase.org/).* Amplification of the microsatellite markers was tested by gel electrophoresis in 1% agarose gel. Gels were stained with ethidium bromide and visualized in a transilluminator. Due to amplification problems, one locus (Dp375) was removed from further analysis.

For genotyping, PCR reactions were run using fluorescent primers for all microsatellite loci except Dp375 (removed after initial amplification issues in trial gel electrophoresis). DNA concentrations for the PCR reactions were tested using a NanoDrop™, and PCR products were diluted 2:8 to obtain a 10 ng/**µ**L concentration. Slight variations to the dilutions (1.5:8.5, 2.5:7.5, and 1:9) were also done to allow us to assess the optimal dilutions for the best fragment analysis results.

Fragment analysis was conducted in 2017 on 15 embryos with markers Dp38, Dp43, Dp433, Dp446 and Dp461. For this study in 2018, more individuals were tested, however to check the

reliability of the methodology used, 8 of the original 15 embryos were tested again with the same 5 markers listed above in addition to 9 new markers (Dp90, Dp162, Dp173 Dp283, Dp291, Dp311, Dp369, Dp376, Dp437). The replicates used the same DNA, loci, procedure and equipment. All diluted PCR products from the reactions were sent to the University of Minnesota Genomics Center for capillary electrophoresis using an ABI 3730xl platform. The size standard ladder ROX-400HD was used for capillary electrophoresis based on the fragment size range expected for these genetic markers (Frisch et. al, 2014).

Genotyping

Data files received from the University of Minnesota's Genomics Center (in early September 2018) were analyzed using *Geneious*, a bioinformatics software program. Each individual file was analyzed for stutter peaks and alignment of the ladder. Once all stutter peaks were removed, *Geneious* fit the ladder to the file data and displayed sizes of the ladder and all peaks (Figure 3). Allele peaks were then identified for each individual embryo tested for each microsatellite locus individuals were scored as heterozygous or homozygous. All peaks seen that were smaller than 100 base pairs were eliminated from analysis as all alleles were expected to be between 100-400 base pairs (Frisch et. al, 2014). This genotyping also enabled each microsatellite locus to be characterized as monomorphic or polymorphic based on the sample size analyzed.

Characterization of the genetic composition of the population

Genetic diversity was determined by the number of alleles per locus (per temporal population), expected heterozygosity (H_e) and observed heterozygosity (H_O). Expected heterozygosity was calculated using the Hardy-Weinberg equation adapted for number of alleles present in each population (Nei, 1978). Comparison between the expected and observed heterozygosity can determine if the populations have higher or lower general heterozygosity than would be expected and might be explained by ecological reasons. Private alleles (alleles found in one population and not others) were documented between temporal populations examined and recorded as non bolded numbers. Alleles that were not private (located in both temporal populations) were bolded for better visualization (Table 3). Allelic diversity was tested between the two temporal populations using an independent sample t-test.

Results

Pb210 Dating

Lead-210 dating provided ages of Square Lake sediment cores down to the core depth of 38 cm dated to be 1845 (Fig. 4). At this depth, the ^{210}Pb isotope was depleted and could no longer provide information about the age of the sediments. However, the ages of depths beyond this threshold were able to be approximated by extrapolation using the sedimentation rates around the threshold depths. Using that approach, the age of the sediment at the base of the core (60 cm) was estimated to be from 1660. An important date for this study is the year that the rainbow trout

stocking program began (in 1981). That date corresponds to sediment core depth of 11 cm (Fig. 4).

Gel electrophoresis

Agarose gel electrophoresis used to test for amplification of PCR products (using the non-fluorescent primers) showed amplification for all microsatellite loci except Dp375 (Fig. 5). Because PCR products for this locus did not amplify, they were removed from further testing. Band sizes for alleles for the various loci were between 100 and 400 base pairs as was expected based on findings of other studies. Test DNA 1 had the largest amplification throughout all primers tested as can be clearly seen by the bright band on the left of each marker area (Fig 5). The other two DNA test samples used produced faint, less fluorescent bands, but bands could still be recognized in these lanes and therefore were determined to have amplified.

Dilutions to PCR Products

All dilutions of PCR products sent to the University of Minnesota Genomic Center for fragment analysis worked properly and no specific dilution worked better than the rest. The standard 2:8 dilution was chosen and used for all remaining fragment analysis plates as it was in the best range to accommodate the DNA concentrations of the PCR products that were being tested.

Fragment Analysis of Capillary Electrophoresis Analyses (using Geneious software)

After files were edited for stutter peaks to fit the size standard ladder, each file was analyzed to identify allele peaks. Individuals were determined to be heterozygous or homozygous based on the peaks detected. Homozygotes were detected with one singular allele peak with high intensity (Fig. 6a), while heterozygotes were detected with two distinct allele peaks with the same relative intensity (Fig. 6b). It was occasionally difficult to decipher allele peaks from noise peaks in the fragment analysis files. When more than two peaks were observed, peaks were identified as true alleles based on their relative intensity. Lower intensity peaks were considered to be noise for these ambiguous files. Other cases in which the files were difficult to interpret were when two peaks of similar intensity were seen but the peaks were many base pairs apart (Fig. 6c). These individuals were scored as heterozygotes even though it is possible that one of the peaks is not real. Finally, some files contained no peaks other than the ladder. These individuals were determined to have not amplified correctly and no data was recorded for that individual and locus combination (Fig. 6d). Dp311 and Dp437 did not amplify correctly and did not produce any allele peaks for any of the individuals. One other issue with interpreting the fragment analysis files was for cases with allele peaks that were one base pair apart. These files were re-examined and a single allele size between the two was recorded (i.e 188 and 189 were decided to both be 188 for a specific locus). This was done as tandem repeats could not be less than 2 base pairs as and the one base pair difference could be explained as *Taq* DNA polymerase has been shown to occasionally add one base pair to the sequence during amplification (Huang et al, 1992).

Genetic characterization of the D. pulicaria *population in the egg bank*

Once data was interpreted using the rules above, genetic data was categorized by locus and age of the sediment. Alleles for each depth tested were listed and an expected heterozygosity for each depth was calculated (Table 2). 11 of the 12 loci tested using the fluorescent primers and capillary electrophoresis had multiple alleles (were polymorphic), while only a single allele was detected for one locus (Dp376). These data were also sorted into two time categories: pre-1981 and post-1981 to examine population genetic differences between the pre-trout era and the years that rainbow trout were stocked in the lake. The post-1981 data are from ephippial eggs from the dredge sample of surface sediments and the pre-1981 data are from ephippial eggs from sediment core samples that predate 1981 (age of sediments from which samples tested were 1977, 1963, 1954, 1940 and 1930). Expected and actual heterozygosity were calculated and recorded (Table 3). Observed heterozygosity was almost always lower than expected heterozygosity (Table 2 and 3). Private alleles were identified between the temporal populations for each locus. The private alleles are observed in the categorized pre- vs post-1981 data as well as seen between temporal populations between 2015, 1977, 1963, 1954, 1940 and 1930. Private alleles were more common than shared alleles between pre- and post-1981 temporal populations (Table 3). Dp38 and Dp43 had no shared alleles and the other loci found only 1-3 shared alleles between the pre- and post-1981 populations, the rest being private alleles. Allelic diversity was found to be significantly higher in the older sediments than in the current dredge sample sediment (Fig. 7) ($t=2.59$, $df=11$, $p = 0.024$).

Surprisingly and frustratingly, amplification of the PCR products was poorer when using fluorescent primers compared to the non-fluorescent primers that were used. Gels run using the non-fluorescent primers consistently produced strong and bright bands for the microsatellite PCR products, but conventional electrophoresis does not provide the necessary precision to distinguish allele sizes that are sometimes only a few base pairs apart. For the capillary electrophoresis analysis using the fluorescent primers, only 5 out of the 12 loci tested gave interpretable results for the majority of the individuals assayed. For the other 7 loci, the success rates for genotyping ranged between 5-45%.

Discussion

The central aims of this study were achieved in that 1) viable *Daphnia* ephippial embryos were isolated from sediments from a range of depth (ages) from the Square Lake egg bank, 2) DNA was successfully extracted from the embryos, 3) individuals were successfully genotyped for 12 microsatellite markers, and 4) the allelic composition of samples of the pre-1981 population was found to significantly differ from that of the population assessed from a sample of recent sediments. Additionally, the data from the fragment analysis found polymorphism in 11 out of the 12 loci analyzed by fragment analysis, despite the relatively small sample sizes for several of the loci (Tables 2 and 3). Because the one locus that was found to be monomorphic (Dp376) only had one viable data point, it is still quite possible that this locus could in fact also be polymorphic once more individuals are tested.

Interestingly, the majority of the alleles recorded were different between pre-and post-1981 sediments (Table 3). Allele frequencies for alleles unique to each temporal population are low. A possible explanation for such differences in alleles between time periods is genetic drift. Alleles with low frequencies in the earlier populations would likely be lost due to random genetic drift (Lacy, 1987). New alleles observed in the cohort of embryos from the dredge sample of surface sediments could arise from mutation or also could be a result of reemergence of ephippia from older sediments from past generations.

Allelic diversity was also significantly greater in the older sediments (Table 3, Fig. 7). This could be due to the pre-1981 sediment samples originating over a larger span of time compared to the sample from the surface sediments. The sediments in the pre-1981 temporal population came from sediments from 1977, 1963, 1954, 1940, and 1930. This nearly 50 year range is a longer span of time than the possible 10 year span for the dredge sample obtained in 2015.

Here, observed heterozygosity was almost always lower than expected heterozygosity (Table 2 and 3). Low observed heterozygosity could be the result of inbreeding or also homozygous individuals mating with each other. Clone competition has been seen to be the driving evolutionary force for a *Daphnia* population (Hembre & Megard, 2006). This competing force could decrease the types of homozygous individuals so one homozygous would be more likely to mate with a homozygote with the same alleles. However, sexual reproduction is infrequent in *Daphnia* populations and only occurs under environmental stress (i.e low food availability, high population densities and short photoperiods) (Kleiven et. al, 1992) so it is more likely inbreeding

would cause the relatively low observed heterozygosity. Another explanation of the relatively low observed heterozygosity could originate from using microsatellite markers. Expected heterozygosity increases as the number of alleles increases and because the majority of the markers used are multiallelic, the expected heterozygosity increases as a function of the marker system we used (Lowe, 2004) and therefore not necessarily as a characteristic of the actual population.

One problem that arose in the genetic analyses was the poor amplification and resolution for some loci when the fluorescent primers were used. Specifically, five loci (Dp38, Dp43, Dp433, Dp446 and Dp461) amplified better than the other seven (Table 3). For these five, all of the individuals assayed were able to be genotyped. The other seven were spotty, and sometimes only a few individuals had files with allele peaks that were detectable. One explanation that could account for this is that the first five fluorescent primers were ordered from a different supplier than the last seven. The primer sequences were double checked and verified, so the vendor difference could be the reason for the differences in amplification. For future research, the seven problematic primers should be reordered from a different supplier and the analyses for those loci should be redone for the samples analyzed in this study (and used for any future work).

Also, due to the relatively small sample size in this study, it was difficult to confidently differentiate real allele peaks from noise peaks. When an allele appears in more than one individual, it is more probable that that allele peak is real. More individuals would provide a better set of data to compare peaks to determine if a peak is likely real or not.

Finally, it was very challenging to find intact ephippia in the deep sediments that predate 1981. Although it was easy to find ephippia in the sediments, often they were damaged and not viable or empty cases. Moving forward, more emphasis and time must be spent looking through these deep sediment samples for more intact ephippia to be able to conduct this methodology on. Once there is a large enough sample of ephippia, more genetic fragment analysis can be conducted, creating a larger sample group to analyze for trends.

Recommendations for methodological improvements for future research

The methodology used here is time consuming and labor intensive. Testing multiple individuals for multiple loci takes a lot of time, energy and materials. A way to cut time, costs and supplies needed would be to take advantage of multiplexing. Multiplexing is a form of PCR in which not just one, but multiple primer pairs can be mixed in the PCR reaction mixture to amplify more than one target sequence at a time (Oliveira and Lencastre, 2002, Zhang et. al, 2005). This would substantially decrease the number of individual PCR reactions that would need to be done and significantly decrease the time it takes to prepare each individual set of PCR reactions. This would allow more time to genotype more individuals at the same cost.

Also, the ambiguity of interpreting some of the data files introduces some doubt into the accuracy of the data. Although some of the data files are clear-cut and easily interpreted, others are noisier and hard to interpret. A way to make the analysis process faster and less ambiguous

would be to test a larger sample size of individuals to see which alleles appear in more than one individual and across more temporal populations. Once the common allele sizes are found, it will be easier to interpret which allele peaks are noise or contamination compared to a real allele peak. To do this, it would be ideal to process more sediment in sediment cores to find more intact and viable ephippia to use for fragment analysis.

Future research to better understand evolutionary changes in the Square Lake Daphnia *population and beyond*

To better understand how the *Daphnia pulicaria* population has changed over time (particularly since 1981 when trout were first stocked in the lake) more ephippial eggs from core depths between 1981 and the current sediments need to be analyzed for these genetic markers. This would allow data from the post-1981 era to be analyzed over a similar period of time as the pre-1981 sediments have been (about 50 years). Examining two periods of time that include data from similar spans of time would help to determine if the allelic variation seen in pre-1981 sediments is truly greater than post-1981 sediments.

Once the above suggestions are accomplished for genetically analyzing the Square Lake egg bank, the same processes could be done for the sediment cores from Big Carnelian Lake. Zooplankton remains (ephippia and exoskeletal fragments) from the Square Lake cores were compared to those in the Big Carnelian Lake in the paleoecological study that was the subject of Michael Gilray's honors thesis (Gilray, 2018) because Big Carnelian served as a reference

ecosystem that has never been stocked with trout and is comparable to Square Lake morphometrically and with respect to its trophic state. Therefore, it would be possible to examine if similar allelic changes have occured in that lake, despite not having had the food web alteration that Square Lake has had with the stocking of rainbow trout. Analyzing the population structure of the two lakes through time (Frisch et al., 2014) would enable researchers to see if Big Carnelian Lake *D. pulicaria* population would have experienced less genetic differentiation through time than the Square Lake population due to the lack of food web alterations in the lake.

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Table 1: Primer sequences for DNA microsatellite loci and PCR thermocycler settings. All PCR cycles started with 2 min at 95℃ and had the same denaturing and extending settings (95℃ for 45 s and 72℃ for 60 s respectively). Annealing temperatures varied based on the primers used, but were always set for 30 s. All three stages were repeated 39 times. Products were held at 10℃ after PCR was completed, before being used for gel amplification or tested for DNA concentration. Primer sequences and PCR settings were determined as recommended by *Daphnia Genomics Consortium (http://wfleabase.org/).*

Marker Name	F Sequence	R sequence	PCR Settings Used (℃)	PCR Optimal Settings (℃)	PCR Max (℃)	PCR Min (℃)
DP38	CTTGCGCTTCGTGAGTTCTG	TGCGCGTTATACAGTTTTGATG	45.5	45.5	65	45.5
DP43	TTGGGCGCCGAGAATGT	GGCCGGGCAAAACACAC	45.5	N/A	N/A	N/A
DP90	ATTCCACGGAAAACGAAAAA	CAAAACTGGTTGAGGGGAAA	50	50	50	45
DP162	CGAATCCGTTCGTCAAAAGC	AAGCGACGATGAAGCATTCC	50	50	61	46
DP173	GTCTACACGCCGATTTTCGC	GCGCTTGTTTACCGGTTTGA	50	50	61	46
DP283	CGACTCTGCTCGAACGTGTG	CTGAGGCAGGAAATGATCCG	59	59	65	45
DP291	GAAGAATTCGGTCGGTGTGG	TCGAAACCGTCTCGTCTCGT	48.2	48.2	65	45
DP311	TCCACCTCCTTCCTCACCAA	GCGCGGCAGTGAAATAAATC	53	53	58	46
DP369	GTCGACTGTCACACCCCCAT	AACGGGCACTTACCAGACGA	46.5	46.4	61	45
DP375	TCATTCCGGCAGGAAAAATG	ATGCGATCACAGCCAGGAGT	47.1	N/A	N/A	N/A
DP376	TTACGCCTGGTCTTGGGTTG	AACCTCAACCGCTGTAGCCA	56.7	56.7	64.6	45
DP433	GACACTCTCCACGCCTGCTT	ACCAAGGCGAGAGGTTTTCC	46.5	46.5	64.6	45

DP437	CCCCTCTTGTTCTTC CGCTT	CTTTGATTTCCGTCTC CCCC	56.7	56.7	61.8	45
DP446	TGCTGGATTGGAATT CGCTT	TTGTGGTGAAATAAGG GCGG	48.2	48.2	65	45.5
DP461	AGAGTGGGCGAACG AACAAA	CGGAGAAAACACGGA CGAAG	50	50.5	64.6	46.5

Table 2. Allele frequency results from fragment analysis (using *Geneious* software) of capillary electrophoresis files. Data below is categorized by locusand by the age of the sediment from which ephippial embryos were extracted. The number of alleles, base pair (bp) sizes of alleles, and allele frequencies for samples from different years and for the different loci are recorded. Sample size (n) is the number of embryos assayed from the sediment depths. H_e is the expected heterozygosity calculated using the Hardy-Weinberg equation and H_o is the observed heterozygosity in the samples.

Sediment Core depth	Age (Pb210)	n	Loci	# of alleles	Alleles (bp)	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	H_o	H_e
1cm	2015	10	DP38	3	150,237,239	0.1	0.55	0.35				0.1	0.565
12cm	1977	5	DP38	3	143, 157, 183	0.2	0.6	0.2				0	0.56
14cm	1963	2	DP38	1	157	1						0	0
15cm	1954	2	DP38	1	236	1						0	0
17cm	1940	2	DP38	2	133,157	0.5	0.5					0	0.5
18cm	1930	1	DP38	1	236	1						0	0
1cm	2015	10	DP43	6	120,126,128, 130,332, 335	0.1	0.15	0.35	0.1	0.05	0.25	0.2	0.77
12cm	1977	2	DP43	6	184, 188, 316, 328, 370, 372	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.82
14cm	1963	2	DP43	3	268,270, 326	0.25	0.25	0.5				0.5	0.625
15cm	1954	2	DP43	2	262,294	0.5	0.5					0	0.5
17cm	1940	2	DP43	2	165,167	0.25	0.75					0.5	0.375

18cm	1930	1	DP43	2	248,250	0.5	0.5					1	0.5
1cm	2015	10	DP433	4	138,186,188, 198	0.05	0.2	0.7	0.05			0.3	0.465
12cm	1977	5	DP433	4	116,157, 183, 188	0.2	0.2	0.2	0.4			0	0.72
14cm	1963	2	DP433	4	131,157, 186,188	0.25	0.25	0.25	0.25			1	0.75
15cm	1954	2	DP433	2	118,278	0.5	0.5					0	0.5
17cm	1940	2	DP433	2	188,239	0.5	0.5					0	0.5
18cm	1930	1	DP433	1	188	1						0	0
1cm	2015	10	DP446	3	272, 277, 294	0.35	0.55	0.1				0.1	0.565
12cm	1977	3	DP446	3	168,267,279	0.333	0.333	0.333				0	0.667
14cm	1963	2	DP446	2	224, 295	0.5	0.5					0	0.5
15cm	1954	1	DP446	1	295	1						0	0
17cm	1940	1	DP446	1	240	1						0	0
18cm	1930	1	DP446	2	275,277	0.5	0.5					1	0.5
17cm	1940	1	DP376	1	242	1						0	0
1cm	2015	2	DP90	2	189,194	0.5	0.5					0	0.5
12cm	1977	3	DP90	2	195,197	0.333	0.667					0	0.444
14cm	1963	1	DP90	2	184,187	0.5	0.5					1	0.5
15cm	1954	1	DP90	1	189	1						0	0
18cm	1930	1	DP90	1	189	1						0	0

1cm	2015	2	DP162	2	190, 198	0.5	0.5					0	0.5
12cm	1977	2	DP162	2	198,200	0.5	0.5					0	0.5
18cm	1930	1	DP162	1	189	1						0	0
1cm	2015	4	DP173	4	181,193,197,199	0.25	0.25	0.25	0.25			0	0.75
12cm	1977	2	DP173	2	195,197	0.5	0.5					0	0.5
14cm	1963	1	DP173	2	184,187	0.5	0.5					0	0.5
15cm	1954	1	DP173	1	184	1						0	0
1cm	2015	9	DP461	3	184, 186,188	0.389	0.111	0.5				0.333	0.586
12cm	1977	5	DP461	3	139, 157, 194	0.1	0.7	0.2				0.2	0.46
14cm	1963	2	DP461	3	157,184,186	0.5	0.25	0.25				0.5	0.625
15cm	1954	2	DP461	3	157,186 ,188	0.5	0.25	0.25				0.5	0.625
17cm	1940	2	DP461	2	148,157	0.25	0.75					0.5	0.375
18cm	1930	1	DP461	2	182,186	0.5	0.5					1	0.5
1cm	2015	5	DP369	5	107,116,184, 187,190	0.1	0.3	0.2	0.3	0.1		0.4	0.76
12cm	1977	1	DP369	1	116	1						0	0
14cm	1963	2	DP369	4	116,118, 120,122	0.25	0.25	0.25	0.25			1	0.75
17cm	1940	1	DP369	1	188	1						0	0
18cm	1930	1	DP369	1	188	1						0	0
1cm	2015	4	DP291	3	125,184, 188	0.333	0.333	0.333				0	0.667
14cm	1963	2	DP291	2	186,188	0.75	0.25					0.5	0.375

15cm	1954	2	DP291	2	118,186	0.5	0.5					0	0.5
18cm	1930	1	DP291	1	237	1						0	0
12cm	1977	1	DP283	1	192	1						0	0
14cm	1963	1	DP283	1	239	1						0	0
15cm	1954	1	DP283	1	190	1						0	0
18cm	1930	1	DP283	1	186	1						0	0

Table 3. Allele frequency results from fragment analysis (using *Geneious* software) comparing genetic makeup of population in recently deposited surface sediments to that of ephippial embryos from core samples from sediments deposited before the advent of the rainbow trout stocking program (before 1981). Data are categorized by locus and categorical age of the sediment from which ephippial embryos were extracted. The pre-1981 category includes sediments from 1977, 1963, 1954, 1940 and 1930, and the post-1981 ephippial embryos were obtained from a sediment dredge sample of surface sediments that was taken in 2015. Depths listed for the sediment core samples is the bottom depth of the core sample. The number of alleles (A#), base pair (bp) sizes of alleles, and allele frequencies for samples from different time periods and for the different loci are recorded. Sample size (n) is the number of embryos assayed from the sediment depths. H_e is the expected heterozygosity calculated using the Hardy-Weinberg equation and H_o is the observed heterozygosity in the samples. Loci are considered monomorphic if only one allele was found for that locus and polymorphic if more than one allele was found. Bolded allele sizes are alleles observed in both temporal populations.

Loci	Depth	Age (Pb210)	n	#A	Alleles (bp)	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	He	Ho	Polymorphism
DP38	1cm	2015	10	3	150,237,239	0.1	0.55	0.35													0.565	0.1	Polymorphic
DP38	>12cm	pre-1981	12	5	133,143,157, 183,236	0.083	0.083	0.500	0.083	0.25											0.6667	0	Polymorphic
DP43	1cm	2015	10	6	120,126,128, 130,332, 335	0.100	0.150	0.350	0.100	0.050	0.250										0.770	0.200	Polymorphic
DP43	>12cm	pre-1981	12	15	165,167,184,188, 248,250,262, 268,270,294, 316,326,328,370, 372	0.042	0.125	0.083	0.083	0.042	0.042	0.083	0.042	0.042	0.083	0.083	0.083	0.083	0.042	0.042	1	0.333	Polymorphic
DP433	1cm	2015	10	4	138,**186,188**, 198	0.050	0.200	0.700	0.050												0.465	0.300	Polymorphic
DP433	>12cm	pre-1981	12	9	116,118,131, 157,183,**186, 188**,239,278	0.083	0.083	0.042	0.125	0.083	0.042	0.375	0.083	0.083							0.806	0.167	Polymorphic
DP446	1cm	2015	10	3	272, **277**, 294	0.350	0.550	0.100													0.565	0.100	Polymorphic
DP446	>12cm	pre-1981	8	8	168,224,240, 267,275,**277**, 279,295	0.125	0.125	0.125	0.125	0.063	0.063	0.125	0.250								0.852	0.125	Polymorphic
DP90	1cm	2015	2	2	**189**, 194	0.500	0.500														0.500	0	Polymorphic
DP90	>12cm	pre-1981	6	5	184,187,**189**, 195,197	0.083	0.083	0.333	0.167	0.333											0.736	0.167	Polymorphic
DP162	1cm	2015	2	2	190, **198**	0.500	0.500														0.500	0	Polymorphic
DP162	>12cm	pre-1981	3	3	189, **198**, 200	0.333	0.333	0.333													0.667	0	Polymorphic
DP173	1cm	2015	4	4	181, 193, **197**, 199	0.250	0.250	0.250	0.250												0.750	0	Polymorphic
DP173	>12cm	pre-1981	4	4	184,187, 195, **197**	0.375	0.125	0.250	0.250												0.719	0.250	Polymorphic
DP461	1cm	2015	9	3	**184,186,188**	0.389	0.111	0.500													0.586	0.333	Polymorphic
DP461	>12cm	pre-1981	12	8	139,148,157, 182,**184, 186,188**,194	0.042	0.042	0.583	0.042	0.042	0.125	0.042	0.083								0.628	0.417	Polymorphic
DP369	1cm	2015	5	5	107,**116**,184, 187,190	0.100	0.300	0.200	0.300	0.100											0.760	0.400	Polymorphic
DP369	>12cm	pre-1981	5	5	**116,**118,120, 122,188	0.300	0.100	0.100	0.100	0.400											0.720	0.400	Polymorphic
DP291	1cm	2015	4	3	125,184, **188**	0.333	0.333	0.333													0.667	0	Polymorphic
DP291	>12cm	pre-1981	5	4	118,186, **188,**237	0.200	0.500	0.100	0.200												0.660	0.200	Polymorphic
DP283	1cm	2015	0	0																			N/A
DP283	>12cm	pre-1981	4	4	186,190,192,239	0.250	0.250	0.250	0.250												0.750	0	Polymorphic
DP376	1cm	2015	0	0																			N/A
DP376	>12cm	pre-1981	1	1	242	1.000																0	Monomorphic

Figure 1. *Daphnia* life cycle. The asexual component of the life cycle (parthenogenesis) is shown in the inner circle of the diagram with a mature female creating parthenogenetic daughters. Parthenogenetic sons can also be created (as pictured below) when females are switching to sexual reproduction. Sexual females then produce haploid eggs (within a protective case called an ephippium). Those eggs may then be fertilized by a male to produce a diploid zygote. The zygotes with the ephippium then develop into diapausing embryos and are released from the mother when she molts. The ephippia then are dispersed and may fall to the sediments and become part of the "egg bank". The diapausing embryos (resting eggs) within the ephippia can subsequently hatch as parthenogenetic females after receiving hatching cues and enter the water column (Ebert, 2005).

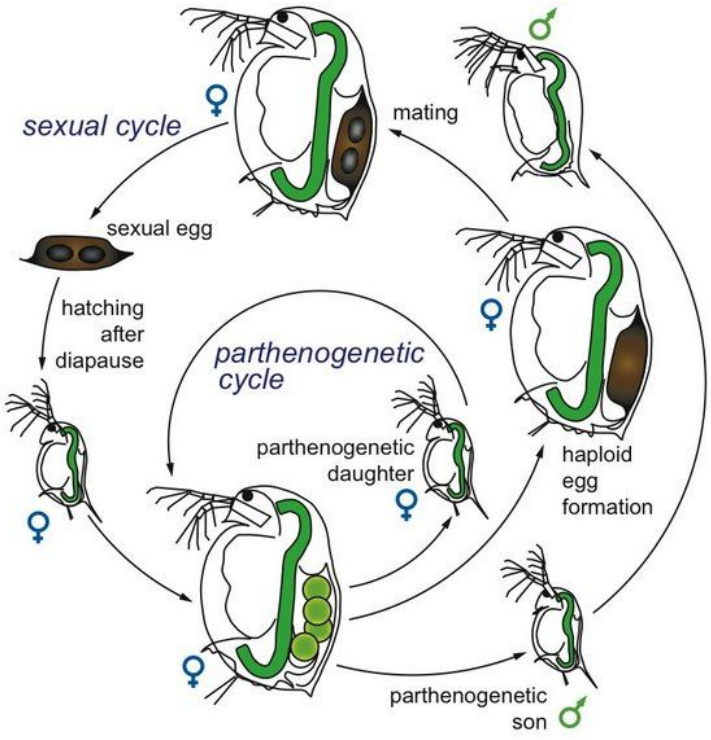

Figure 2: Ephippia extracted from sediment. A. *Daphnia pulicaria* ephippium*:* identified based on small spikes on the dorsal ridge, B. *Daphnia mendotae:* smaller-bodied species that produces ephippia with smooth dorsal ridge, and C. Ephippia identified as open (left), partially intact with one egg (top) or intact with 2 eggs (right).

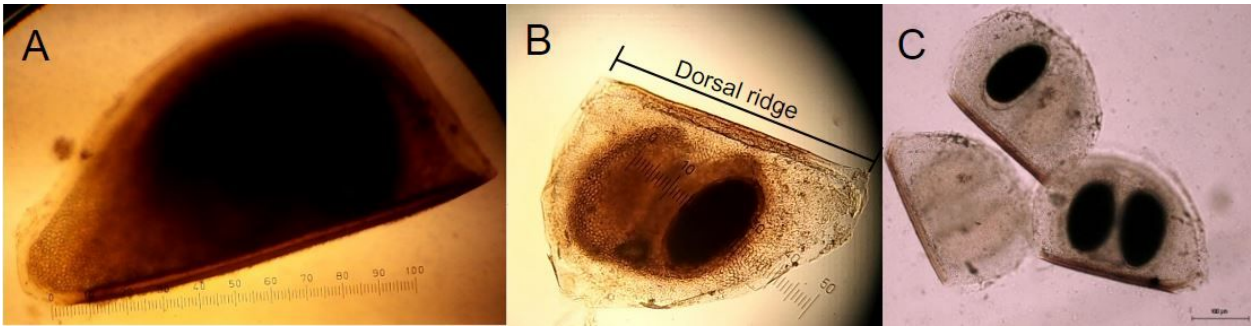

Figure 3. Example of process for fragment analysis file editing using *Geneious*. Each fragment analysis file was first cleansed of stutter peaks, short peaks labeled with arrows (top panel). Once all stutter peaks were deleted, *Geneious* would fit the size standard ladder (red peaks on lower panel). This enabled and the *Daphnia* allele peaks (blue peaks) to be identified.

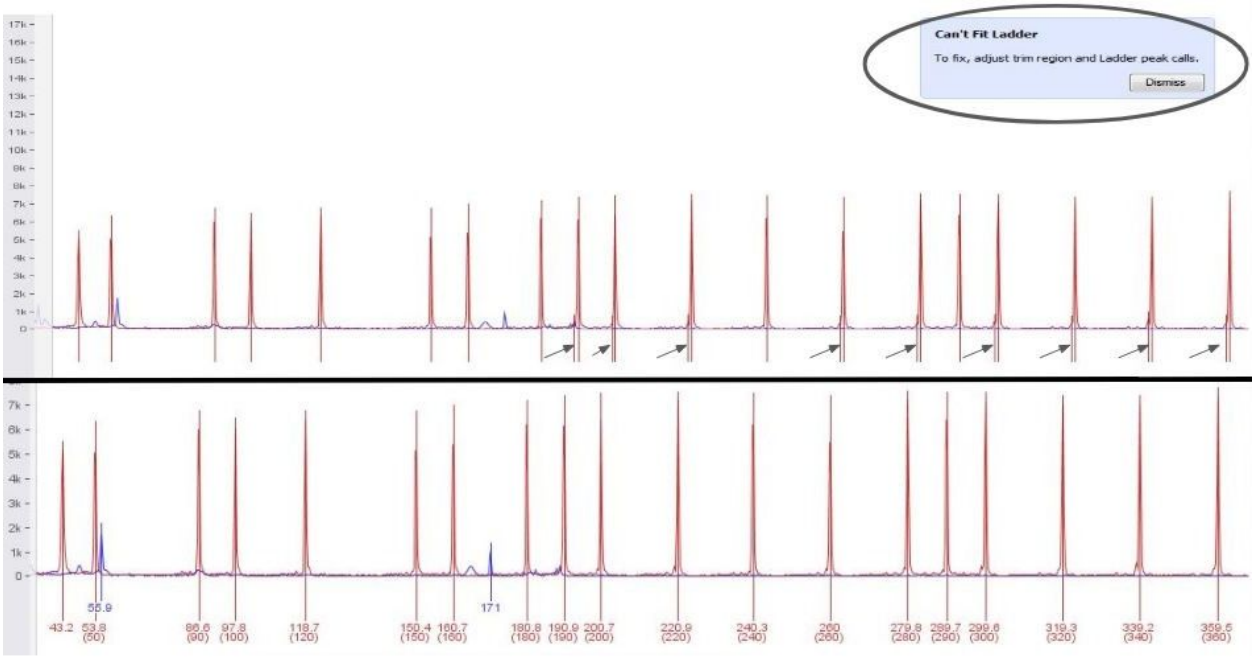

Figure 4. Lead-210 dating results for Square Lake. Top panel shows the depths at which ^{210}Pb was supported in the sediment. The bottom panel shows the age of the sediments at each depth throughout the core. Error bars are ±1 standard error.

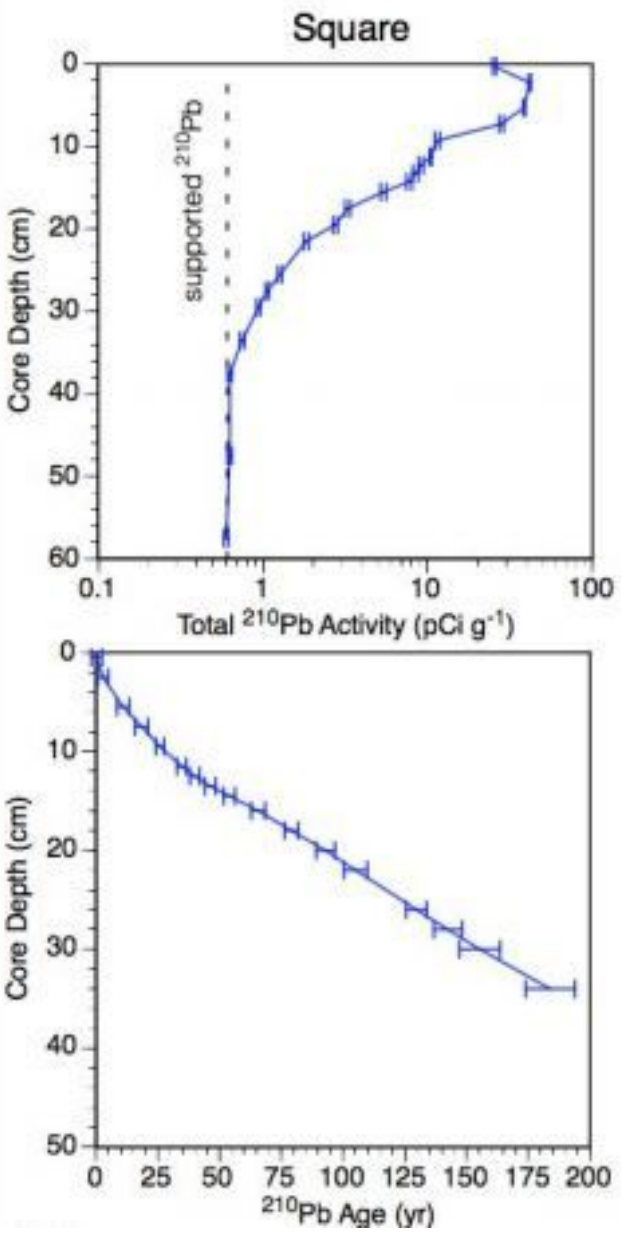

Figure 5. Example of results from gel electrophoresis screening of *Daphnia* microsatellite primers. The image below shows the base pair sizes for the size standard ladder (Bioline Hyperladder) to the left of the gel. The ladder itself is used in the gel twice for ease of band interpretation (in the far left-hand lane and in the middle of the gel). Each microsatellite marker occupied 4 wells of the plate (3 DNA samples from 3 different ephippial embryos and 1 H_2O control located on the right end of each set of 4 wells). 2 of the 3 DNA samples did not amplify to produce strong bands for some of the markers, but faint bands are seen in all 3 lanes for each locus except for Dp375. Although not all 3 DNA samples amplified as well as the first (left lanes), amplification was still seen and the markers were considered to have amplified embryo DNA. All markers except for Dp375 continued to be used.

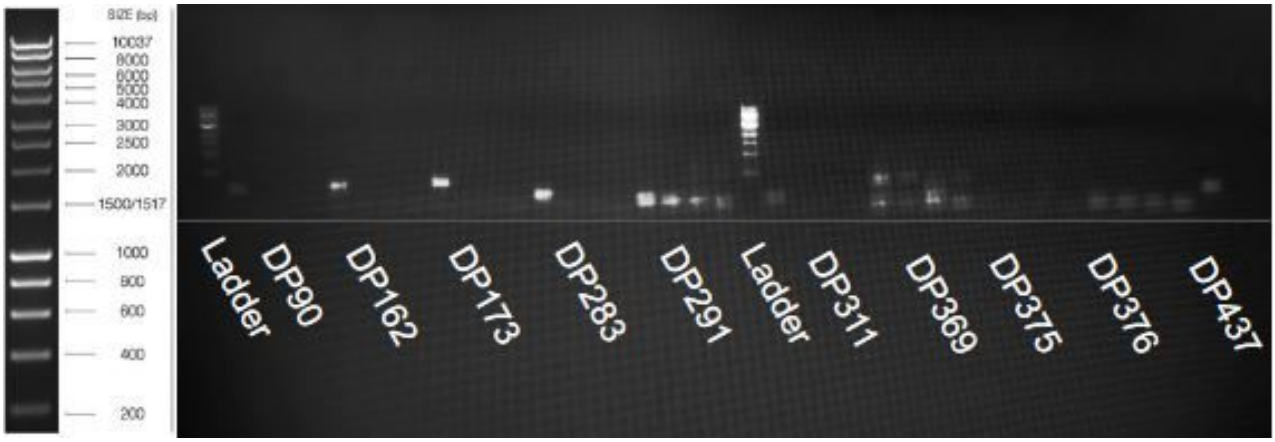

Figure 6. Examples of fragment analysis files displayed in *Geneious* software. Panel A shows a clear example of a homozygote (single large blue peak) and panel B shows a clear example of a heterozygote (2 large blue peaks). Panel C shows a case with an individual with 2 allele peaks many bp apart from each other. This individual was scored as a heterozygote, despite the large difference in allele sizes, because the peaks were the same intensity and it is hard to know which peak is real and which is noise without more individuals to compare. Panel D shows a file with no allele peaks present, meaning that the marker was not amplified.

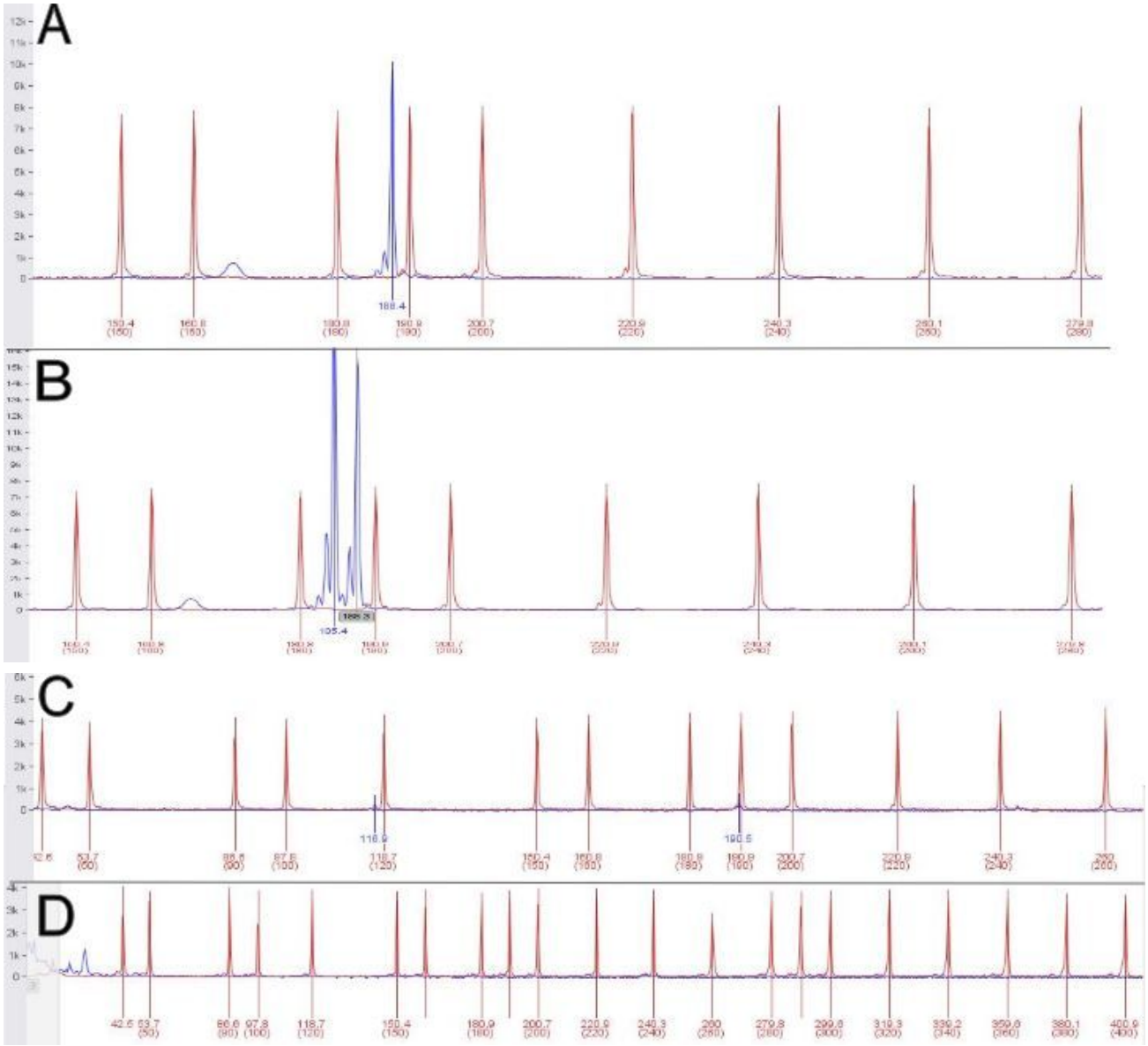

Figure 7. Plot of means of the allelic richness of both the pre-trout (before 1981) and Modern (after 1981) ephippial populations. Allelic diversity is significantly greater in the pre-trout ephippial population (t=2.59, df=11, p = 0.024).