Developing Microsatellite Markers for *Galearis rotundifolia* (the Roundleaf Orchid)

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Abstract

There is a human-caused global biodiversity crisis imperiling both the natural and human world. Plant species are particularly vulnerable, and must be preserved. Genetic diversity is important to species' health and survival, so genetic tools should be developed to assess it. Microsatellite markers, which are regions of DNA where a few base pairs repeat in a pattern, are a tool which can assess genetic diversity as well as other parameters helpful to conservation. They can also help guide *ex situ* conservation efforts. *Galearis rotundifolia* is an orchid species found throughout northern North America, and this project aims at developing microsatellite markers for this species. Leaf samples were collected from the Wagner Natural Area, and their DNA was extracted, fragmented, and amplified according to the FIASCO method. Fragments likely to contain microsatellites were pulled down using FIASCO, and next generation sequencing was done on these fragments to find microsatellites. Primer 3 was used to find primers for the longest dinucleotide repeat microsatellites, and these primers were tested in the same PCR conditions, with an annealing temperature of 56°C. 13 primer pairs were successful at amplifying *G. rotundifolia* DNA. 3 Primer PCR was done with the DNA of 3 individuals, amplifying 11 of the microsatellites that had been successfully amplified in the first round of PCR. This DNA was sent for fragment analysis to test which of the microstatellites were polymorphic. 5 microsatellites showed evidence of being polymorphic. The primer pairs developed for these 5 microsatellites, as well as primer pairs developed by future students, can be used to assess genetic diversity and other parameters, and inform and guide conservation.

Introduction

There is currently a global biodiversity crisis of immense magnitude. Approximately one million species faced extinction risk in 2019 (Diaz et al. 2019) due to human caused destruction of the environment, and such destruction has only increased since then (Sponsel 2024). Humans are using more resources than the earth can regenerate (Raven 2023), and this is leading to the earth not being able to use its regenerative capacity to sustain other species. This is causing us to enter Earth's sixth mass extinction event.

The review by Amy Ando (2022) explains that most of the degradation towards the environment is being done by the overconsumption of a small number of wealthy individuals and by the unsustainable production practices of large scale industries.

The United Nations have found that there are five main drivers to biodiversity loss. Invasive species and diseases are increasingly being introduced to ecosystems as global trade increases. Much habitat is being lost or degraded as agriculture, industry, and human settlements expand into natural land. Climate change, which is from fossil fuel use and the degradation of natural carbon stores such as wetlands, is also a major threat to global biodiversity. Pollution, including plastic pollution and the overuse of pesticides, is also a major cause of biodiversity loss. Additionally, over hunting and over harvesting species in the wilderness also leads to ecosystem degradation.

Bar-On et al. (2018) have done a census on the biomass of the earth, and how this biomass is distributed amongst different taxa, from viruses to archaea to fungi to livestock. They estimate that about 82% of Earth's biomass is composed of plant life. And plants are particularly threatened by the biodiversity crisis. Marco et al. (2019) discuss how around 16 thousand plant species were headed towards extinction that year due to human impacts. In 2022, the IUCN projected that 40% of plants, or over 25 000 species, were threatened with extinction.

Conservation of species is of course very important to the health of ecosystems. There are two factors to conservation, the number of individuals of a given species in an ecosystem, and the genetic diversity of the species.

Nonić and Šijačić-Nikolić (2018) explain that genetic diversity is the diversity in the alleles that a population has. These alleles can be different versions of coding genes, or different versions of the regulatory elements of a gene, or different versions in the sequences of the non-coding part of the DNA. If a population has lots of different alleles and the rarer alleles are still fairly common, then that population has a lot of genetic diversity.

Maintaining genetic diversity is important for species.

Vinceti et al. (2013) show that populations with greater genetic diversity can adapt better to changes in the environment. They looked at the genetic diversity of the tree species *Prunus africana,* and how the range of the species is expected to shift due to climate change. They used this knowledge to select populations of conservation priority. The sampled populations all over the range of the species. They used 13 microsatellites, both nuclear and mitochondrial, to assess the genetic diversity of populations. They found between 6 and 18 populations that should be a conservation priority, due to the fact that conserving these populations would conserve the most genetic diversity, which would allow the species to adapt better to the changes caused by climate change. Different populations that are less genetically connected should be managed as separate units because they are likely adapted to different environments. They concluded that the populations with the most genetic diversity were the most important to conserve, because these populations could adapt better to changing conditions caused by climate change.

Szczecińska et al. (2016) showed how populations in a species with more genetic diversity are more fit. And as populations decline, genetic diversity declines and so does fitness. They looked at 29 populations of the endangered flower *Pulsatilla patens* in Central Europe, and assessed genetic diversity and other parameters using 6 microsatellites. They found three genetic groups, two in the Baltic region and one in the Southern region, which all had low genetic diversity and very low levels of heterozygosity due to historic and ongoing bottlenecks. Smaller populations had less genetic diversity. Populations were genetically distinct from each other, even if they were geographically close. This indicates that there has been habitat fragmentation going on, and the genetic connectivity of the populations is low. They also found that the populations that were less fit had less genetic diversity, and the populations with higher diversity were more fit.

Reed and Frankham (2003) looked at the data from 34 different papers and found that there is a correlation between genetic diversity and fitness for many different species.

Therefore it is very important to develop tools that can help us assess genetic diversity in species and populations.

The review by Azhar et al. (2018) describes many different molecular markers and how to use them. It looks at many different papers which use, develop, study or review different molecular markers. It describes microsatellites as regions of DNA that have a few base pairs repeating again and again in a pattern. There are many alleles of each microsatellite, and these alleles each have a different number of repeats. The number of times the pattern repeats determines what allele of the microsatellite a chromosome has.

Researchers look at microsatellites to assess the genetic diversity of a population (Coates et al. 2015; Islam et al. 2023; Jacquemyn et al. 2010; Vinceti et al. 2013), which correlates with species health (Reed & Frankham 2003; Szczecińska et al. 2016) and adaptability (Vinceti et al. 2013). Microsatellites can detect inbreeding or tell us about connectivity (Coates et al. 2015; Islam et al. 2023; Jacquemyn et al. 2010; Vinceti et al. 2013; Wang 2020), habitat fragmentation

(Szczecińska et al. 2016; Jacquemyn et al. 2010) or whether populations are adapted to different environments. (Coates et al. 2015; Wang 2020).

Coates et al. (2015) used 12 microsatellites to look at the genetic diversity and the genetic connectivity of extinct and extant populations of the endangered shrub *Banksia brownii.* They found that there were three distinct genetic groups in three different regions, that were not genetically connected to each other and were likely adapted to different environments and therefore should be kept separate in conservation efforts. The three different groups had different genetic diversity levels. Much of the genetic diversity has been lost in the species due to the pathogen that is causing the species to decline.

Islam et al. (2023) used 17 start codon targeted primers and 15 microsatellite primers to look at the genetic diversity, genetic structure, gene flow, and inbreeding estimates of *Trillium govanianum*, a plant in the Himalayas which faces many stressors. They found that there was low genetic diversity, and two main genetic clusters which were further divided. Genetic diversity has decreased since the last study, indicating habitat fragmentation and an ongoing bottleneck. There was also lots of inbreeding, indicated by low heterozygosity. Individuals from different populations should be planted in other populations to increase genetic diversity. Seed banks would also improve conservation efforts. And of course the habitat fragmentation and destruction must be stopped. They found that the microsatellite markers and the start codon targetted markers gave the same results.

Jacquemyn et al. (2010) looked at the European herb *Cirsium acaule* using seven microsatellites. They compared the genetic diversity, connectivity, and population sizes of populations in 2002 versus 2010. They used the heterozygosity of the microsatellites versus allelic richness to predict whether there have been bottlenecks. They found that populations that were smaller had experienced greater bottlenecks and were less genetically diverse. There was low genetic connectivity amongst the populations, indicating that there is likely habitat fragmentation and the populations are more susceptible to genetic drift.

Wang (2020) used 16 microsatellites to assess the genetic diversity statistics, inbreeding, and genetic differentiation among populations of *Paeonia decomposita*, an endangered woody flowering plant in China. They found 3 genetically distinct groups with geographical barriers to gene flow between them. Therefore, three different *ex situ* breeding groups should be made. There is habitat fragmentation, which calls for conservation areas that connect different populations. There is less genetic diversity in this species compared to other similar species, and the genetic diversity is higher in areas with less human disturbance, indicating that human disturbance is decreasing the genetic diversity of the species.

Knowing about the genetic diversity of a species also helps with *ex situ* conservation programs, which are very helpful to the conservation of species. Two major ways of *ex situ* conservation are the creation and maintenance of seed banks, and the translocation of individuals from one population to another.

In their review article, Wambugu et al. (2023) discuss the importance of seed banks for the preservation of a species. Seed banks are collections of living seeds whose parents or ancestors came from wild or agricultural plants. They can supply seeds from a diverse group of species for ecosystem restoration efforts, which is more sustainable than sourcing seeds from the wild. They also store lots of knowledge about the seeds and plants they have, and the source populations of these seeds. There are over 1750 seed banks in the world, but many of these banks need to be given resources to scale up their work.

The review talks about the importance of genetic diversity within seed banks. Seed banks need to store seeds that have a lot of intraspecific diversity. These seeds need to be from a variety of populations adapted to different environments, and the seeds from the same environment need to also be diverse. This diversity helps seeds be more successful at repopulating ecosystems in the wild, and it also helps repopulated populations be more resilient against climate change. Seed bulking is a way to create many seeds from a few seeds, and requires successive generations of growing plants and storing their seeds. Care needs to be taken that the genetic makeup and genetic diversity of plants are maintained during seed bulking.

Therefore, it is important to assess the genetic diversity and structure of populations that seeds in banks come from. It is important to assess the genetic diversity of the seeds themselves. And it is important to constantly monitor the genetic diversity of plants being used in seed bulking to ensure that no genetic drift is happening and no diversity is being lost.

Translocation is another way of improving the survival of a species. This is a practice where individuals from one population or from a breeding area are planted in a population that is declining or extirpated.

Genetic diversity is important for the initial survival and overall establishment of translocated plants. In his 2013 review, Anders Forsman looked at 18 experiments that assessed the effects of genetic diversity on the establishment of translocated populations of a variety of animal and plant species. He saw that in 17 of the experiments, more genetically diverse translocated groups were more successful at establishing themselves and/or their offspring. This effect was more pronounced in experiments done in the field. Schafer et al. (2020) looked at the survival of translocated populations of 8 struggling plant species. They found that clusters of more genetically diverse individuals (with different mothers) had more surviving plants than clusters of less genetically diverse individuals (with the same mothers).

The Wagner Natural Area is a forest and wetland ecosystem near Edmonton. Its latitude is 53.57019 and its longitude is -113.8275. (2 Markers) It is made of marl ponds fed by underground springs, which are surrounded by forests with trees such as spruce, aspen, birch, tamarack and willow. There are many species in this area, including rare carnivorous plants, and 16 of the 24 orchid species found in Alberta (Alberta Parks 2023). This is where the samples of Galearis rotundifolia, the orchid species studied in this project, came from.

Galearis rotundifolia, or the Roundleaf Orchid, is a terrestrial orchid (North American Orchid Conservation Center) found throughout northern North America, stretching from the North United States, through Canada, and even in Alaska. They have white or pink flowers with purple spots, and they have a round leaf at the base. They grow in alkaline wetlands. While these flowers are not at risk of extinction, for now, in the northern parts of their range, they are at risk in the United States, with many American populations having been extirpated (North American Orchid Conservation Center).

The goal of this project is to develop microsatellite primers for use in *Galearis rotundifolia.* The aim is to find different microsatellites in the plant's genome and create primers with which they can be amplified. With these primers, future researchers will be able to amplify microsatellite alleles in plants and use the alleles they find to assess genetic diversity and other things, which will help inform *in situ* and *ex situ* conservation efforts. If there are more alleles in a population for example, then they will know that there's more genetic diversity in that population.

Methods

Previous Work On the Project

There were many steps in the project done before I joined. The student before me went to the Wagner Natural Area to collect G. rotundifolia leaf tissue. DNA was extracted from this tissue, fragmented, and ligated with adapters so that the fragments all had the same start and end sequences that were reverse complements. The fragments of DNA that had dinucleotide repeats were pulled down. This was done by using DNA probes attached to streptavidin-coated beads. These probes were dinucleotide repeats (AG) and therefore attached to dinucleotide repeat-containing fragments. When the metal beads on the probes were pulled down with a magnet, the fragments were pulled down as well. The fragments were sent to the University of Alberta so that they could do next generation sequencing on them. The sequences were uploaded to the software Geneious and analyzed there. Anna Munezero made a list of the microsatellites found in the Geneious.

The exact details of these procedures were the same as the procedures done by Lina Lim in her 2022 study of the orchid *Cypripedium passerinum*, except the organism used was *G. rotudnifolia*. All the steps and protocols used by Lina in *C. passerinium* were also used on *G. rotundifolia* in this project. The difference is that instead of taking the PCR amplified copies of DNA fragments pulled down by FIASCO, and then adding them into a bacteria library and sequencing the DNA in that library, these fragment copies were directly sequenced by the University of Alberta Faculty of Medicine core facility, using High Throughput DNA sequencing with the Illumina platform.

And this is where I came into the project.

Part 1: Finding Primer Pairs that Work at an Annealing Temperature of 56ºC

The microsatellites on Anne's list were analyzed. The 25 longest dinucleotide repeat sequences on the list were selected for further study. For each of these microsatellites, the number of the micosatellite on the spreadsheet and its corresponding ID on Geneious were written down. The ID was looked up on Geneious and the microsatellite and the sequences around it were copied and pasted onto a document. The sequences were copied and pasted into Primer 3 to create primers for them. Primer 3 is a software that creates primers for Polymerase Chain Reaction (PCR). The default settings on the website were used. The default settings look for primers that have optimal binding temperatures for PCR between 57°C and 63°C according to Primer 3. The Primer 3 optimal PCR binding temperatures are actually a couple of degrees higher than the actual optimal binding temperatures though.

For each microsatellite that had primers, there was one main primer pair and usually between 1-5 alternate primer pairs. All the primer pairs, their sequences, and their Primer 3 annealing temperatures were written down on a spreadsheet. The primer pairs were named according to the number of the microsatellite they amplified and their number given on Primer 3. 21 main primer pairs were found, but primer pairs 3400 and 3473 were the same, so there were actually only 20. These main primer pairs were used to try to amplify the 21 microsatellites.

PCR - a reaction which copies DNA - was done using each of the main primer pairs, using an annealing temperature of 56°C. This annealing temperature was used because all the optimal annealing temperatures for all the primer pairs were a little bit higher than that. The optimal binding temperatures given by Primer 3 are a bit high, so using a PCR annealing temperature that is a little bit lower than the Primer 3 annealing temperatures should give successful PCR reactions.

Primer pairs 3473, 535, 1959, 3212, 82, 3400, 3359, 2678, 3517, and 3230 all used the same

PCR preparation process. When doing PCR with these primer pairs, the template *G. rotundifolia* DNA used was 15 ng/ul. Because all these PCR reactions had the same concentration of template DNA, the steps used to set up the PCR reactions were the same.

For these primer pairs, the steps for setting up each pair's PCR reactions were as follows: The primer stock is 100 moles/L. 1/10 dilutions of each primer were made by pipetting 5 uL of primer stock into 45 uL of sterlized water. Then a master mix was made that had 22.5 uL of Ready Taq (which has the Taq polymerase, ddNTPs, and other reaction components), 2.25 uL of the forwards primer, 2.25 uL of the reverse primer, and 10.58 uL of sterilized water. Enough master mix was made for 2.25 reactions though only two reactions were made for each primer pair. The first reaction was a control reaction, where there wasn't any DNA template. This was to ensure that there was no DNA contaminating the reaction components that could be amplified by the primer pair. The control reaction had 16.7 uL of master mix and 3.3 uL of sterilized water. The second reaction made for a given primer pair was obviously the sample reaction, which tested whether the primer pair could actually amplify G. rotundifolia DNA in the given PCR conditions. This sample reaction had 16.7 uL of master mix and 3.3 uL of template *G. rotundifolia* DNA.

For primer pairs 616, 1678, 2169, 2295, 22, 2246, 2036, 3624, 2036, 3624, 1249, 717, 222, and 2321 all the PCR reactions were set up in the same way for each pair, but differently from the last group of primer pairs. These primer pairs were tested using template G. rotundifolia DNA that had a concentration of 22.65 ng/uL, and since the template DNA was more concentrated, the concentrations of other reaction components had to be changed. First the primer dilutions were made, like with the other reactions. The primer stock was still 100 moles/L and the dilutions were made in the same way as described in the last paragraph. For the master mixes, 22.5 uL of Ready Taq, 2.25 uL of forwards primers, 2.25 uL of reverse primers, and 13.5 uL of sterilized water were added. In the control PCR reaction 2 uL of water and 18 uL of master mix were added. In the sample PCR reaction 2 uL of G. rotundifolia DNA and 18 uL of master mix were added.

When setting up the PCR reactions, all the pipetting was done with filtered tips to stop contamination. All the work was also done in the laminar flow hood to stop contamination. All the ingredients were kept on ice when they weren't being used.

The PCR was all done in the following protocol: 30 seconds at 95°C for initial melting; 30 rounds of: 95°C for 15 seconds, 56°C for 15 seconds, 68°C for twenty seconds; finally, three minutes of 68°C for final extension.

After the PCR of each primer pair was done, the PCR products were run on mini agarose gels to determine whether the PCR of each primer pair was successful.

First, a ~1.5% agarose-buffer mix had to be made. Approximately 4.75 g of Froggarose agarose powder was mixed with about 300 mL of 0.5X TBE buffer. That was put in a bottle and microwaved in 10-15 second increments until the agarose was completely dissolved and the liquid in the bottle was clear and bubbling. Making one agarose-buffer mix would last a few weeks, and a lot of gels would be able to be poured from one mix. The mix did have to be made a few different times though.

Before pouring an agarose gel, the agarose in the bottle had to be melted in 10-15 second increments in the microwave until it was clear and bubbling. 50 mL of the melted agarose was poured into a flask and 2.5 uL of Red Safe was added. The flask was swirled around to mix the liquids. After mixing, the solution was poured into a mini gel tray and left to become solid. When the gel was solid, it was oriented in the proper way and 0.5X TBE was poured into the gel box so that the buffer covered the gel but wasn't too much higher than the gel. 5 uL of DNA ladder was put in the first well of the mini gel and the rest of the wells had 10 uL of either no-DNA control or DNA-added sample PCR products. The gels were run at around 85 volts until the loading dye was between halfway and two thirds of the way to the bottom of the gel.

A UV illuminated photo was taken of each gel.

Each day PCR was done with two primer pairs and within the same week the gel electrophoresis of those primer pairs was done. After all the primer pairs were tested, two full-size agarose gels were run, with 125 mL of agarose and 6.26 uL of Red Safe, also run at around 85 volts. On these gels was added the control and sample reactions of all the primer pairs that worked at the annealing temperature of 56^{o}C. So the first lane of each gel was 5 uL of a DNA ladder. On the second lane would be 10 uL of a control PCR reaction and the third lane would be 10 uL of the corresponding sample reaction, the fourth lane would have 10 uL of a control reaction for another microsatellite, the fifth lane would have 10 uL of the PCR products of the corresponding sample reaction, and so on.

The PCR products were stored in the -20^{o}C fridge when they weren't being used. All the other solutions were also kept in this fridge, except the Red Safe which was stored in the 4^{o}C fridge and the original primer stocks which were stored in the -80^{o}C fridge.

Part 2: Extracting More DNA

The next part of the project involved extracting DNA from more *G. rotundifolia* individuals. 16 leaf discs were taken from each plant. The plants used were the following individuals: 01, G15, 27, G2, G3, G4, G6, G7, G8, G9, G10, G11, G12, G13, and G16. Each leaf disc was cut into 6 to 8 pieces. The leaf tissue from each individual plant was kept away from the leaf tissue of the other plants. So there was a cutting board with a piece of parafilm on it, and the leaf discs from one plant was put on it. A razor-sharp blade that had been dipped in 70% ethanol was used to cut the discs. Then another piece of parafilm was put on the cutting board, the blade was dipped in the ethanol again, and leaf discs from a different plant were cut.

The Quick DNA^{TM} Plant/Seed Miniprep Kit from Zymo Research was used and the instructions given there were followed. Some steps were changed though. Steps 1-11 of the instruction manual were done in double, with half the leaf tissue of one plant being used in one extraction of said plant and half the leaf tissue of the same plant being used in another extraction of said plant. So for example, for the BashingBead step, there were two BashingBead Tubes for each plant, each which had half the leaf tissue in them and 750 uL of buffer. In step 2 the leaf tissue was for bashed 20 minutes. In step 10, 50 uL of Elution Buffer was used for each Zymo-SpinTM IICR Column. In step 12, both extractions for each plant were combined into one. So the 50 uL of eluted DNA from the first microcentrifuge tube of one plant, and the 50 uL of eluted DNA from the second microcentrifuge tube of the same plant, would both be put onto the same Zymo-SpinTM III-HRC Spin Filter and filtered into the same microcentrifuge tube.

For individuals 01 and 27, a bit more elution buffer was added into their tubes so that the final concentration of the DNA would be 25 ng/L.

Part 3: PCR for Fragment Analysis

Individuals 01, G15, and 27 had DNA that was the most concentrated. So these three individuals were used as template DNA for the next round of PCR reactions. We wanted to know if there were multiple alleles of each microsatellite in the population. In order to do this, the DNA of multiple *G. rotundifolia* individuals needed to be amplified to see if there were multiple alleles in the amplification products. PCR was done with the 11 primer pairs that successfully produced products at a an annealing temperature of 56^{o}C.

Enough of each master mix was made for 4.5 reactions, since four PCR reactions were needed for each primer pair. In one master mix there was 45 uL of 2X Taq Mix, which has Taq polymerase, dNTPs and other reaction components but no loading dye. There was no loading dye because loading dye would mess with future fragment analysis steps. There was also 1.8 uL of 20 uM forwards primer, and 1.8 uL of 20 uM reverse primer. The forwards primer had an M13 sequence at its beginning and then the rest of the sequence was normal. Also added was 1.35 mL of 20 uM fluorescently-tagged M13 primer, which was just the M13 sequence with a fluorescent tag. Finally, 31.05 uL of sterilized water was added.

The way these PCR reactions worked is as follows: At first, the forwards and reverse primers, complementary to the microsatellite flanking sequences, attached to the DNA and created fragments of DNA. These fragments were complementary to the M13 primer because of the M13 sequence on the first forwards primer. Then the M13 primer bound to and amplified the DNA fragments, which created many fragments that had fluorescent labels on them because of the labelled M13 primers that were incorporated into them. This made fragment analysis easier.

In parallel to the master mixes being prepared, the PCR tubes were also prepared. There were 11 strips of tubes, with 4 tubes in each strip. One strip for each microsatellite. For each strip, there was 2 uL of sterilized water in the first tube, 2 uL of DNA from individual 01 in the second tube, 2 uL of DNA from individual G15 in the third tube, and 2 uL of DNA from individual 27 in the fourth tube. 18 uL of the strip’s corresponding master mix was added into all the tubes of the strip. So all the tubes in each strip had the same master mix with the same primers.

As always, when setting up our PCR reactions filtered tips were used for all the pipetting and the work was done in the laminar flow hood.

The PCR protocol was as follows: 1 minute at 95°C for initial melting. Thirty repeats of: 95°C for 30 seconds for melting, 56°C for 30 seconds for annealing, 68°C for 45 seconds for extension. 8 repeats of: 95°C for 30 seconds for melting, 53°C for 30 seconds for annealing, 68°C for 45 seconds for extension. 68°C for 5 minutes for a final extension.

The first cycle of repeats in the PCR protocol was when the forwards and reverse primers that were complementary to the DNA were incorporated. The second round of repeats was when the fluorescently tagged M13 primer was incorporated.

The PCR products were stored in the -20°C fridge as all PCR products are.

The next step was doing gel electrophoresis of some of the PCR products to see if the PCR reactions actually amplified the target microsatellites and didn’t amplify anything that wasn’t supposed to be amplified.

A full-size gel box was used for the experiment. 125 mL of melted agarose was poured into a 125 mL Erlenmyer flask and 6.25 uL of Red Safe was added. It was swirled together and then poured onto the tray so that it could dry. Once it was solid, it was oriented onto the gel box and 0.5X TBE buffer was poured in.

While the gel was solidifying, the PCR reactions for microsatellites 3359, 535, 1678, and 1249 were taken out of the -20°C fridge, thawed, and mixed. 10 uL of the PCR products from each tube was put into a corresponding microcentrifuge tube. So 10 uL from the control reaction for the first microsatellite was put into its own tube, 10 uL of the 01 sample reaction of the first microsatellite into its own tube, and so on. 2 uL of 6X loading dye was added into each tube and the two liquids were mixed together.

On the first gel lane 5uL of a DNA ladder was loaded. Then on the next lane 10 uL of the control reaction for microsatellite 3359 was loaded. The next well had 10 uL of individual 01's microsaellite 3359 amplification. The next lane had individual G15's microsatellite 3395 PCR products. The next lane had individual 27's microsatellite 3395 PRC products. The next four lanes had the PCR products of microsatellite 535, in the same order as the last 4 lanes. The next four lanes had the reactions for microsatellite 1678, and the next four had the reactions for microsatellite 1249.

The gels were run at around 82 volts until the loading dye reached about halfway down the gel. A UV-illuminated photo was taken of the gel.

Two more gels were made in the same way as this gel that was just made. The gels were loaded the same way, with the DNA ladder in the first well, and then the control, 01, G15, and 27 PCR products in that order for each microsatellite. The second gel had the PCR products of microsatellites 3624, 616, 2169, and 2246, in the order listed. The third gel had the PCR products of microsatellites 2295, 3212, and 3400, in the order listed.

Part 4: Fragment Analysis

It had to be tested whether the microsatellites the primer pairs amplified were polymorphic in the population. That's why PCR was done with all our working primer pairs with the DNA of three different *G. rotundifolia* individuals. Now the PCR products needed to be prepared for fragment analysis.

There were 44 PCR reaction products. For each reaction product, a 1/10 dilution was made. A fully concentrated solution and a 1/10 diluted solution of each reaction was sent to the University of Alberta Faculty of Medicine core facility because we didn’t know whether the peaks detected by the fragment analysis would be too high in the fully concentrated solution. The dilution of each PCR reaction was made by putting 2 uL of PCR products into 18 uL of sterilized water.

2 uL of each diluted or undiluted solution was transferred into its own well in the fragment analysis plate. The plate was set up like a grid, and each reaction product had its own place on the grid.

The master mixes of the internal ladder and the Hi-Di Formamide were prepared. The internal ladder was basically a DNA ladder that was put into every PCR product to show what size the fragments made by the PCR was. The appliedbiosystems GeneScan 600 LIZ ladder was used. Hi-Di Formamide was added, which helps the DNA fragments stay suspended during capillary electrophoresis. (Fisher Scientific). 45 uL of 600 Liz ladder and 720 uL of Hi-Di was mixed. This was enough for 90 reactions, though only needed 88 reactions were needed.

18 uL of master mix was pipetted into each well. Then a plastic seal was put over the tray so that all the wells would be covered and the samples were sent to the University of Alberta so that they could do fragment analysis on them.

A multichannel pipette was used to do all the pipetting here, and it had eight different ends to allow pipetting from and into eight things at a time.

Part 5: Concentrating the Extracted DNA

The Zymo DNA Clean and Concentrator kit was used to try to concentrate the *G. rotundifolia* DNA that had been extracted but wasn't up to standard. Tthe instructions given in the kit were followed.

Results

Part 1: Finding Primer Pairs that Work at and Annealing Temperature of 56°C

Primer pairs were generated for the following microsatellites: 82, 22, 222, 3359, 535, 1678, 3230, 3517, 1249, 1959, 2036, 2678, 3624, 616, 717, 2169, 2246, 2295, 2321, 3212, 3400, 3473 (Table 2). According to Primer 3, the optimal annealing temperatures for all of these primers was between 56.33°C and 59.45°C. The actual best annealing temperature is usually a degree or two below the optimal temperature given by Primer 3.

Out of the 21 primer pairs tested, 13 were successful at amplifying *G. rotundifolia* DNA at the PCR conditions used. These 13 primer pairs all were able to amplify *G. rotundifolia* DNA well at an annealing temperature of 56°C. The successful primers pairs were as follows: 22, 3359, 535, 1678, 1249, 3624, 616, 2169, 2246, 2259, 3212, 3400, and 3473. Primer pair 3400 and 3473 had the exact same sequence so we considered both those pairs as one single primer pair. So we had 12 primer pairs that worked at the PCR conditions given (Table 1).

The next step was to test if these primers were polymoprphic in the population. Doing that required testing DNA from three individuals and doing PCR with the DNA of these three individuals using 11 of the successful primer pairs, and then doing fragment analysis with the PCR products to see what alleles of each microsatellite each individual had.

Part 2: Extracting DNA from 3 *G. rotundifolia* Individuals for Fragment Analysis

DNA was extracted from *G. rotundifolia* individuals 01, G15, and 27.

For individual 01, the DNA extracted had a concentration of 30.3 ng/uL. This is a high concentration. The A260/A280 ratio was 1.82. This is above a ratio of 1.6, so the DNA is pure and doesn't have proteins or other molecules in it. An A260/A280 ratio of 1.8 or higher is ideal, meaning that the DNA from this individual is really pure (Lucena-Aguilar et al. 2016). The A260/A230 ratio was 1.198, which is fine because this ratio, which estimates the presence of certain organic compounds such as phenol, isn't relevant to the work currently being done (DeNovix 2019). For individual G15, the concentration of the DNA extracted was 25.95 ng/uL. This is a high concentration as well. The A260/A280 ratio was 1.808. This is definitely above 1.6 and even above 1.8, so the DNA is very pure. The A260/A230 ratio was 0.886. For individual 27, the concentration was 31.4ng/uL, which was the most concentrated DNA so far. The A260/A280 ratio was 1.784. This isn't quite 1.8, but it's very close to 1.8, so this DNA can still be considered very pure. The A260/A230 ratio was 1.192 (Table 4).

The extracted DNA of individuals 01 and 27 were diluted with elution buffer so that their concentrations would be 25 ng/uL. This is still very concentrated DNA, and now all three individuals' DNA is about the same concentration, which makes the next round of PCR much easier (Table 4).

Part 3: Three-Primer PCR for Fragment Analysis

So 3 primer PCR was done using the DNA of the three individuals and 11 of the working primer pairs, as described in the Methods section. The majority of the PCR reactions worked as hoped for. For microsatellites 3359, 535, 1678, 1249, 3246, 696, 2295, and 3400, there was no DNA contaminating the reaction components and all three *G. rotundifolia* individuals' DNA was adequately amplified, hopefully with the fluorescently tagged primers incorporated into them (Figure 3).

For the 3 primer PCR reactions of microsatellites 2246 and 3212, there were faint bands in the control lanes of the agarose gel. This means that there might have been some contamination of DNA in the other reaction components, and perhaps the DNA amplified in the sample reactions is not from *G. rotundifolia*. However, the faint bands seen could be a result of improperly loading the gel so the DNA from another lane got into these lanes. The three sample reactions of each of these two microsatellites were successful at amplifying DNA and hopefully successful at incorporating the fluorescent tags into the amplified fragments (Figure 3).

In microsatellite 2169 there was no amplification in the reaction that used DNA from individual 27, but there was also no contamination of DNA into the reaction components and the other two individuals had their DNA amplified. So when fragment analysis is done of the fragments made of microsatellite 2169, there will only be microsatellites from two individuals to look at (Figure 3).

Part 4: Fragment Analysis

So the fluorescently tagged microsatellite copies were sent to the University of Alberta for fragment analysis, to see how many alleles of each microsatellite were amongst the three individuals. Five microsatellites had multiple alleles in the three individuals. These were microsatellites 616, 1249, 1678, 2246, and 3624. These microsatellites are definitely polymorphic in the species. Other microsatellites might be polymorphic as well but this can't be said for sure since multiple alleles weren't seen amongst the three individuals tested. (FIgure 4; Table 3).

Part 5: Extracting more DNA from more Individuals and Concentrating It

So DNA was extracted from individuals G2 to G4, G6 to G13, and G16. The DNA from these extractions were much less concentrated than the DNA from the three individuals used for further testing. The concentrations of the DNA ranged from 8.450 ng/uL to 17.1 ng/uL. Individuals G3, G4 and G8 had A260/A280 ratios below 1.6. Individuals G6 and G16 had A260/A280 ratios between 1.6 and 1.7. And individuals G2, G7, G9, G10, G11, G12 and G13 had ratios over 1.7. The A60/A230 ratios ranged from 0.239 to 0.637 (Table 5). (Lucena-Aguilar et al. 2016).

An attempt was made to concentrate and further purify the DNA but it did not work very well, which is no-one's fault, the kit used just falsely advertised itself. After trying to concentrate this DNA, for half the individuals the DNA ended up being less concentrated than it was before, and the A260/A280 ratio became weird for all the individuals. After the concentrator kit was used, the concentrations of the DNA ranged from 3.5 to 18.95 ng/uL and the A260/A280 ratios ranged from 1.951 to 33.71. Except for individuals G2 and G4, which had negative A260/A280 ratios (Table 5).

Discussion

The methods used in this project to develop microsatellite markers are similar to the methods used by other researchers.

For example, Du et al. (2018) used FIASCO to create microsatellite primers for the *Thalictrum smithii* plant which is found in China. They collected fresh leaves and extracted the DNA, used *Mse*I to cut the DNA into fragments, attached *Mse*I adapters to the fragments, and used biotinylated AC and AG probes and magnetic beads to pull down fragments containing microsatellites. They amplified the fragments and added them to a pGEM-T vector and put the vector in DH5α cells. They sequenced clones with inserts and found 77 microsatellites. They selected 39 microsatellites, made primers for them, and did PCR using the DNA of 119 individuals. At the end they found 13 polymorphic microsatellites, which they used to assess the genetic diversity and Hardy-Weinberg equilibrium of three wild populations.

Another student before this project also used FIASCO to develop microsatellite markers for another orchid species, *Cypripedium passerinum*. Lina Lim also selected leaf tissue from plants in the Wagner Natural Area. She extracted the DNA and did digestion with *Mse*I and added *Mse*I AFLP adapters, after which she amplified the DNA and used AT, AC, and AAG biotinylated probes and streptavidin beads to pull down fragments containing microsatellites. She amplified these fragments and put the fragments into vectors which she transformed into *E.coli*. Other students did colony PCR and Sanger sequenced the products. They found 158 microsatellites, which they made primers for. They used these primers to do PCR with *Cypripedium passerinum* DNA, to see which of the primers worked and which of the microsatellites were polymorphic.

This current experiment with *G. rotundifolia* also used the FIASCO method. However, instead of transforming the microsatellite-enriched fragments into bacteria, the fragments were amplified and directly sequenced using next generation sequencing. In Lina's project, she didn't do the step of fragment analysis of the microsatellites that could be amplified in the PCR conditions used. The project with *G. rotundifolia* was able to save some time by doing next generation sequencing, and polymorphism was better assessed using fragment analysis.

Future students working on this *G. rotundifolia* project can do many different things in order to move the project forwards.

First of all, remember that the research so far only worked with 21 of the longest dinucleotide repeat microsatellites found in the microsatellite enriched fragments that were sequenced. There were many more microsatellites that were not investigated. Future researchers could find primers for some of the microsatellites that primers have not yet been made for, and they could test for whether the primers they found worked at the same PCR conditions or not, and whether the microsatellites they looked at were polymorphic in the population or not.

Another very important thing that future researchers should do would be to look at the microsatellites this project was not able to amplify with the primer pairs and PCR conditions used. Remember that there were 8 primer pairs that did not work well at amplifying their microsatellites in the PCR conditions used. An important next step would then be to figure out what PCR conditions, including what annealing temperature, these 8 primer pairs work well in. If all 8 primer pairs work in the same conditions, that would be good because it would mean that they could be put in the same multiplex reaction. If some of them amplify their products at certain conditions and some of them amplify their products at certain other conditions, that would be okay as well since researchers could always do more than one multiplex reaction.

Speaking of multiplex reactions, the 5 primer pairs found that amplify polymorphic microsatellites in the same conditions should be put into a multiplex PCR reaction. A multiplex PCR reaction is a PCR reaction in which multiple primer pairs are added at once and multiple microsatellites are amplified at once. The primer pairs all have fluorescent tags on them and each primer pair is a different colour. Therefore, each microsatellite gets a differently coloured fluorescent tag added onto it and when the PCR products are run on a capillary gel, one can see which alleles are present for which microsatellites by looking at the colours of the bands. This allows for many microsatellites to be assessed from a single individual in one quick step. Since the five primer pairs found so far in this project all work under the exact same PCR conditions, they will likely all work in the same multiplex reaction. Future students should figure out what conditions a multiplex PCR reaction using these primer pairs would require.

Primer 3 found alternate primers for most of the 21 microsatellites it found primers for. Students who look at the 8 primer pairs that didn't work in the PCR conditions used in this project so far might find that some of the primer pairs don't work in the PCR conditions they use either. If that is the case, then they can perhaps use the alternate primers that Primer 3 found and they can see if those primers work well at the PCR conditions that they are using. If a microsatellite cannot be amplified using the main primer pairs found by Primer 3, then the alternate primer pairs could perhaps amplify that microsatellite.

This research looked at the DNA of three individuals when doing fragment analysis to determine whether the amplified microsatellites were polymorphic. And while this is definitely enough to affirm the polymorphicness of microsatellite loci, there could be microsatellites that are polymorphic in the population that just happen to not be polymorphic amongst the three individuals tested. There were 7 microsatellite loci that were not polymorphic amongst the three individuals tested. However, by testing more *G. rotundifolia* individuals future students might find that some of these 7 microsatellites are in fact polymorphic in the populations. So future students could do PCR and fragment analysis with these microsatellites on more *G. rotundifolia* individuals to see if some of the microsatellites could be polymorphic.

Ultimately, it's ideal to have as many microsatellite markers as possible that have primer pairs that work well at amplifying them. And those primer pairs should hopefully work in multiplex PCR reactions, which would save time and resources for researchers using these microsatellite markers. Most of the papers I read used 15 to 25 microsatellite markers to do their analyses, so having 10-20 more microsatellite markers for this species would be ideal.

Scientists could use the microsatellite markers developed by me, past students, and future students for a number of purposes.

First of all, they could use the microsatellites to assess the genetic diversity of wild populations of *G. rotundifolia*. Assessing genetic diversity will let them know about how healthy the populations are, because reduced genetic diversity leads to reduced fitness in populations. (Reed & Frankham 2003; Szczecińska et al. 2016). It also leads to reduced adaptability to climate change and other disturbances (Vinceti et al. 2013). Learning about the genetic health of different populations would help them find populations that might need help in order to survive.

Using the microsatellites developed, along with knowledge about the geography and environment of *G. rotundifolia* habitats, researchers could assess the whether there has been habitat fragmentation in the ecosystem that has lead to a lack of connectivity (Islam et al. 2023; Jacquemyn et al. 2010; Szczecińska et al. 2016; Wang 2020). If there are no geographical barriers or major environmental differences between populations, and yet their genetics are distinct, this indicates that there has been habitat fragmentation. This is destructive to the health of populations because the lack of gene flow causes increased genetic drift within each population. Protected areas that connect different populations would be a way to combat the habitat fragmentation faced by not only *G. rotundifolia* but all the other species that share the ecosystem.

There are cases where a lack of genetic connectivity is due to different populations having natural geographic barriers between them and also being in different environments (Coates et al. 2015; Wang 2020). If different populations of *G. rotundifolia* are not connected due to facing appreciably different abiotic and biotic conditions, then it is best to keep these populations separate in conservation efforts, to reduce outbreeding depression and keep each ecotype’s adaptations. The microsatellites found in this project can help assess whether different populations in different environments share their genes in natural conditions or if they do not.

Researchers could also use the microsatellites developed to detect inbreeding within a population (Islam et al. 2023), which would tell them about which populations need to have more diversity put into them. Inbreeding of course causes a lot of problems for individuals and species. Adding seeds or plants from different, more diverse populations would help introduce new genes and new ancestry to populations.

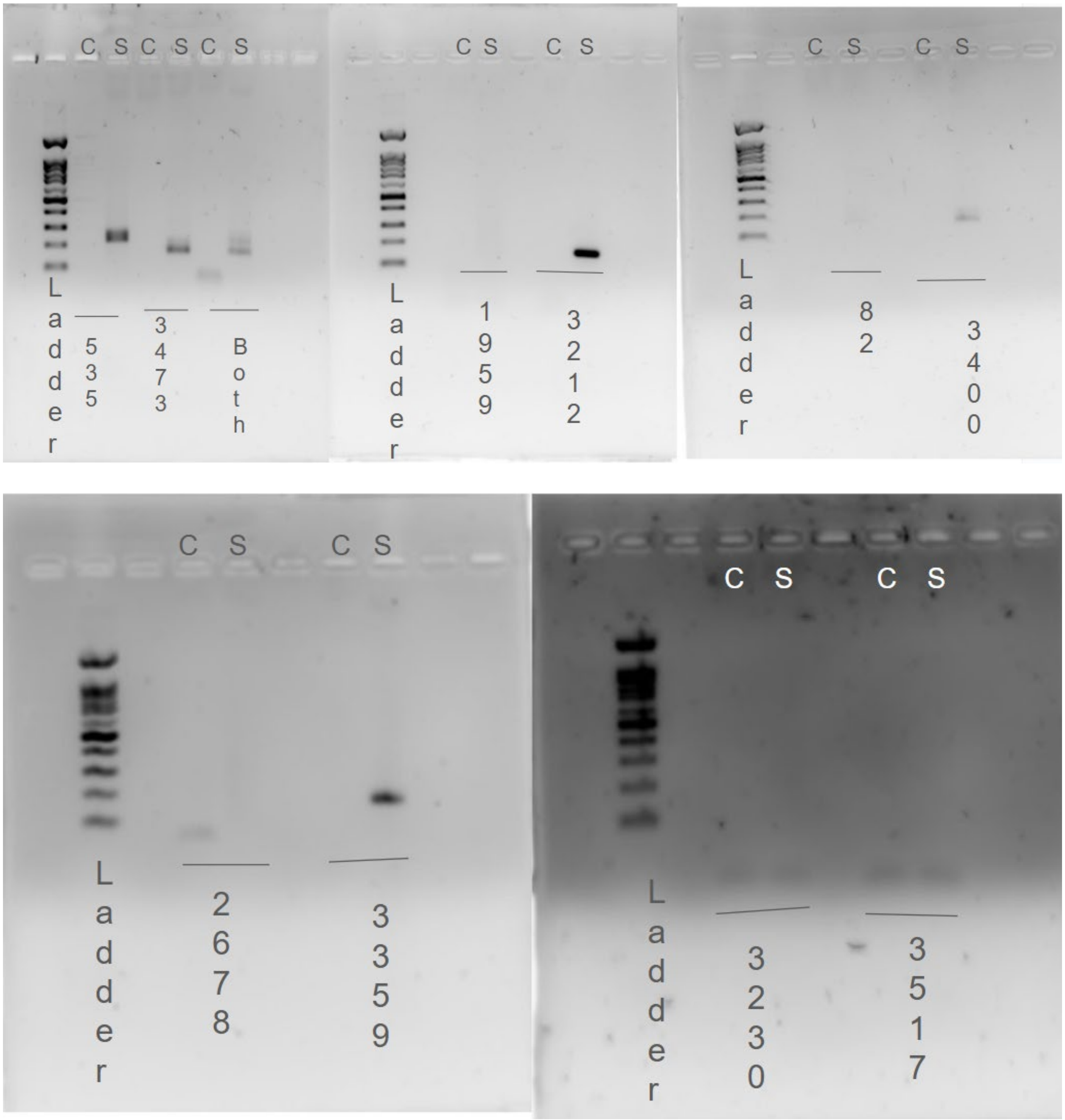
A way to introduce new genetic diversity into *G. rotundifolia* populations that have less diversity, more inbreeding, and/or bottlenecks, is to do translocations, which bring plants from other populations into the struggling population. Both Anders (2013) and Schafer et al. (2020) discuss how increased genetic diversity amongst translocated individuals increases the chances of the translocation being successful. Using the microsatellites developed in this project, researchers could figure out which populations are more genetically diverse, and they could use these populations as sources for translocations. They could also directly assess the genetic diversity of plant groups that are candidates for translocation.

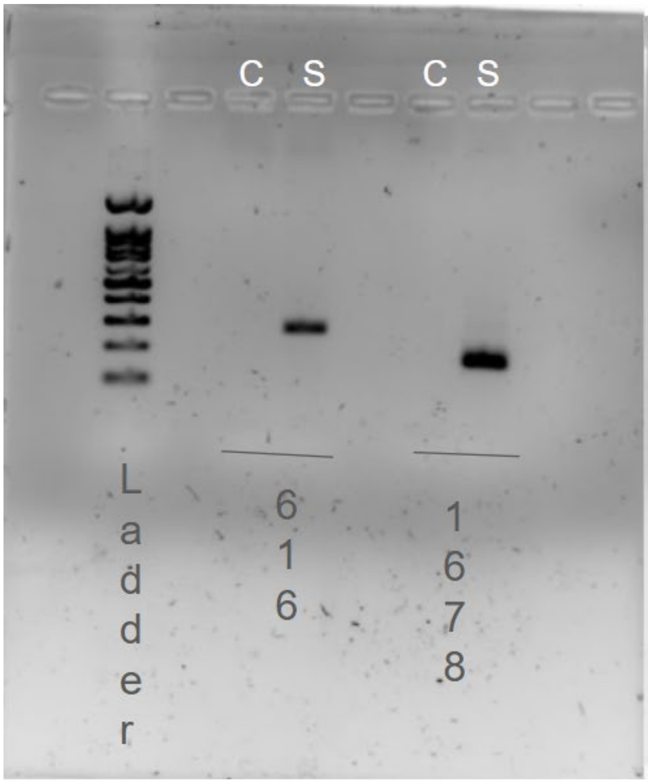
Another important way to help populations in the wild is to use the seeds and knowledge stored and grown in seed banks to restore ecosystems, as explained by Wambugu et al. (2023). Seeds in seed banks need to have a high genetic diversity in order to safeguard the genetic diversity of a species and the genetic diversity of any populations fed by the seed bank. Therefore, the microsatellites developed here will be able to help people who work in seed banks find diverse populations of *G. rotundifolia* that could feed their seed banks. The microsatellites could also tell people which populations are different ecotypes, so that seeds from every ecotype could be stored in the seed banks. Finally, during seed bulking, the process of growing new seeds, the diversity of the plants needs to be maintained. The microsatellites this project finds could help asses the genetic diversity of plants and seeds grown during seed bulking.

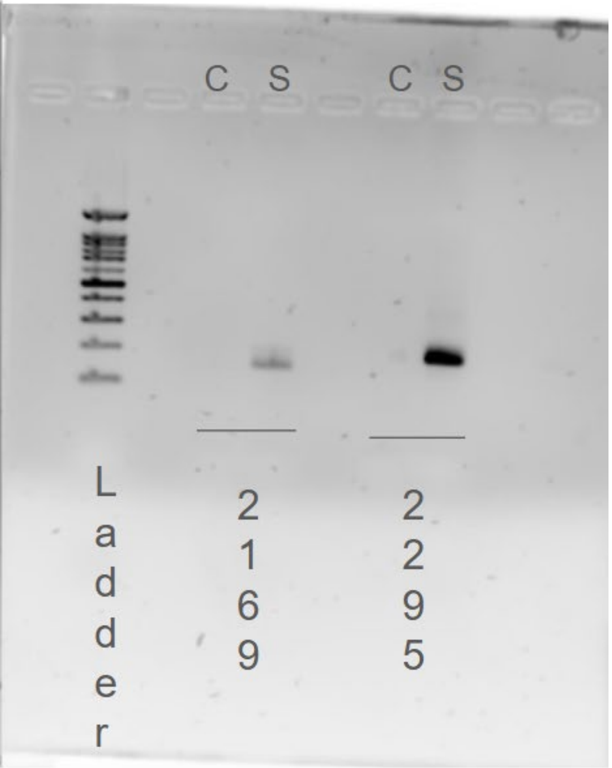
There are many ways in which microsatellites can help in the protection and preservation of plants, and therefore the polymorphic microsatellite markers for which primers were developed here can be used to inform and guide the conservation of *G. rotundifolia* in many ways. Future research should seek to increase the amount of polymorphic microsatellites there are primers for.

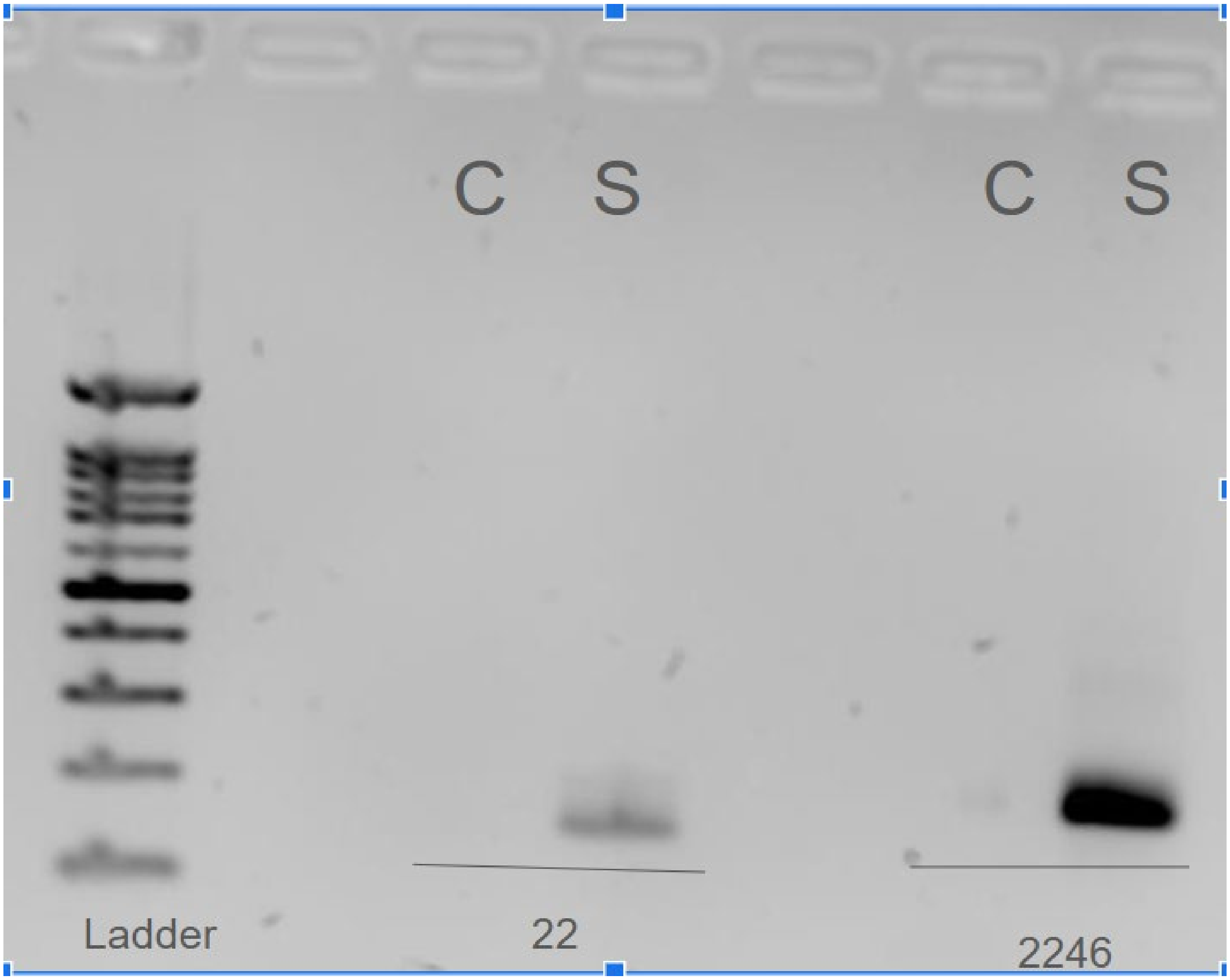
Figures

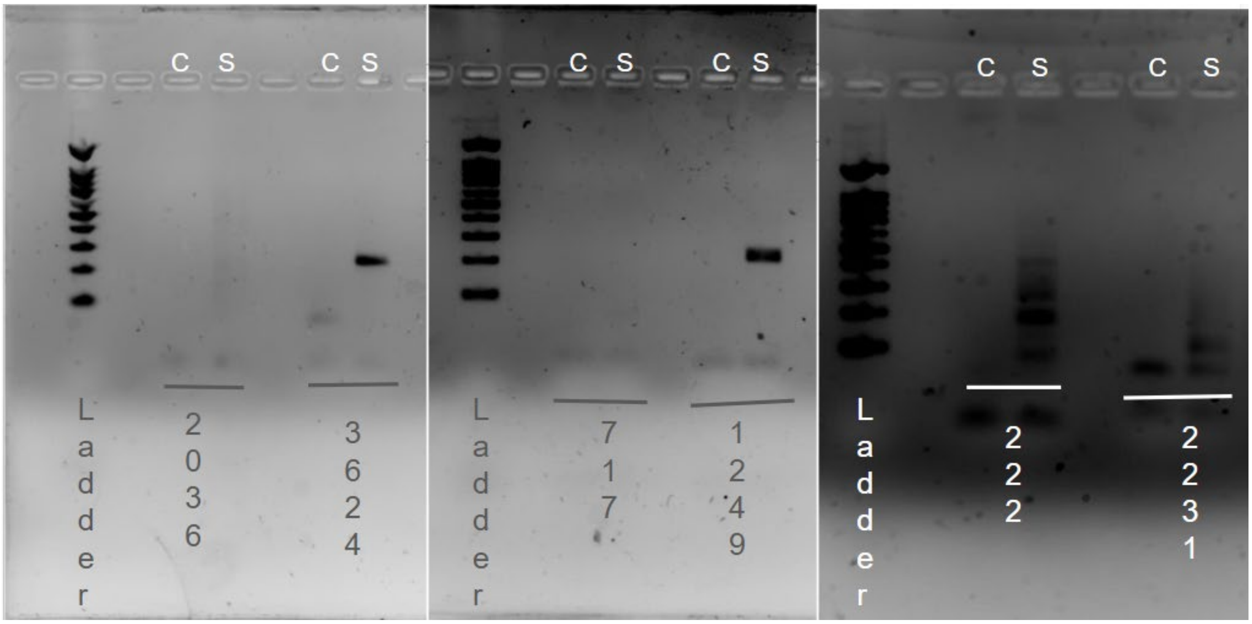
Figure 1: Agarose gels of the 21 PCR Reactions Done at an Annealing Temperature of 56^{o}C.




C
S
C
S
Ladder
616
1678

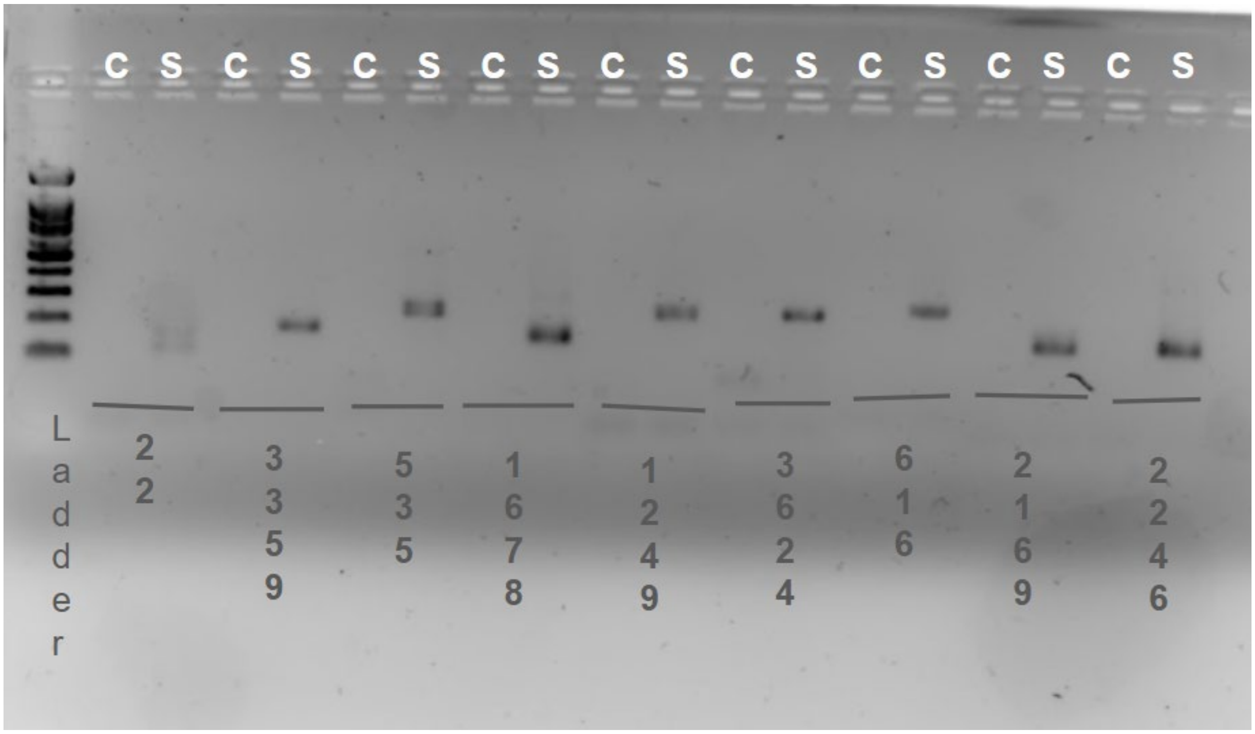

C
S
C
S
Ladder
2169
2295

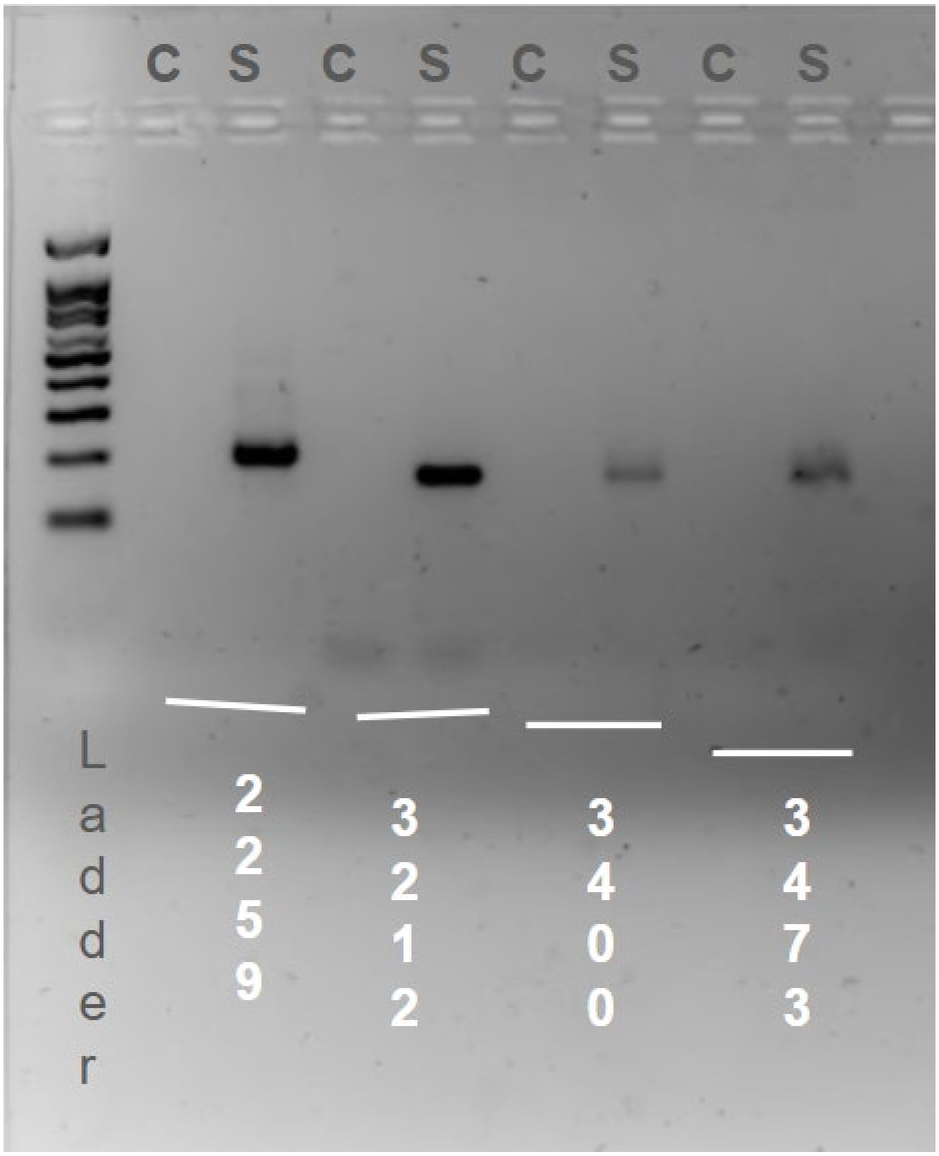




There were 21 microsatellites which Primer 3 created primers for. The main primer pairs for these microsatellites were used in PCR reactions done at an annealing temperature of 56°C to see if the primer pairs worked in these conditions. The products of these reactions were run on agarose gels, and these are the results. The primer pairs used in the reaction are labelled at the bottoms of the gels and whether a lane contains the products of a control reaction or a sample reaction is labelled on the top. Primer pair 22 has two bands in its sample lane, indicating either that there were two alleles of the microsatellite in the template DNA or that there was nonspecific priming. There are no bands in my control lanes, indicating no contamination of DNA in the reaction components, and the sample lanes that have bands indicate that the PCR was successful with that primer pair. Faint bands all the way at the bottom are just primer dimers and don't indicate anything.

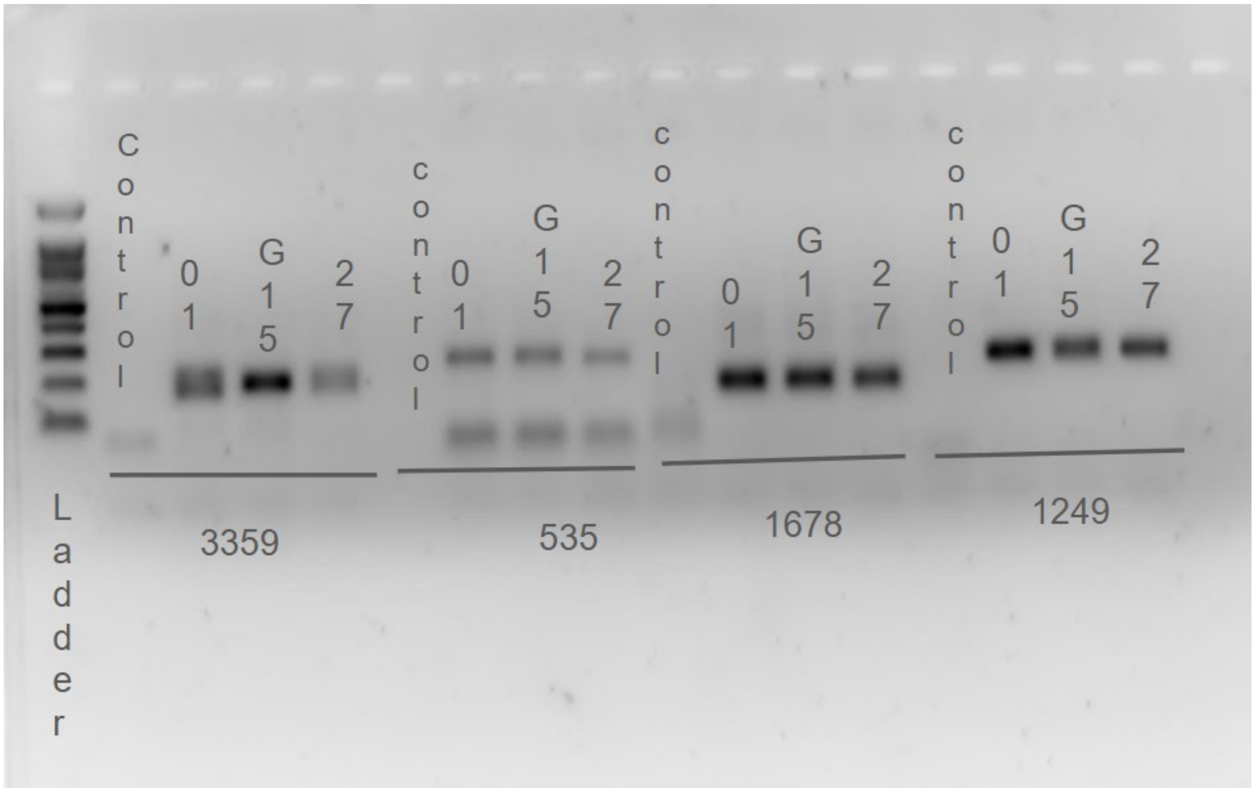
Figure 2: All the Sucessful Primer Pairs that Worked at an Annealing Temperature of 56°C

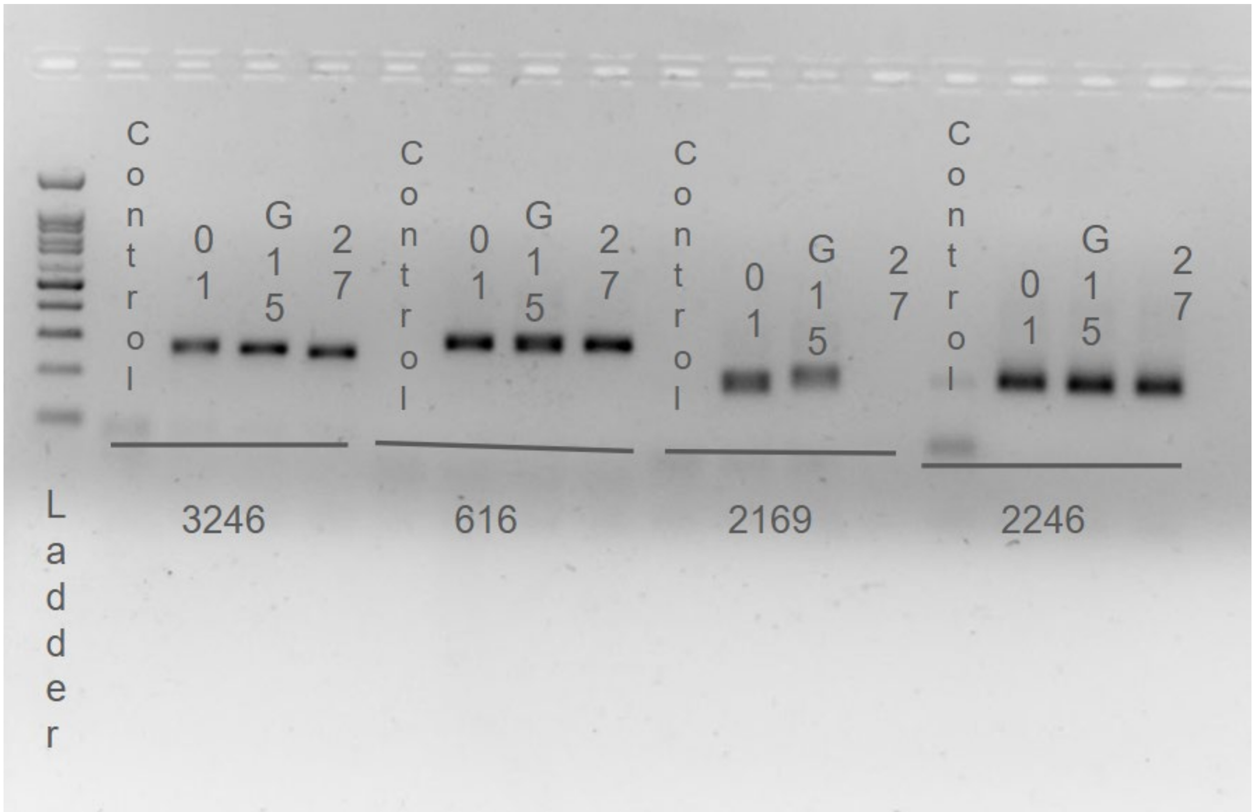


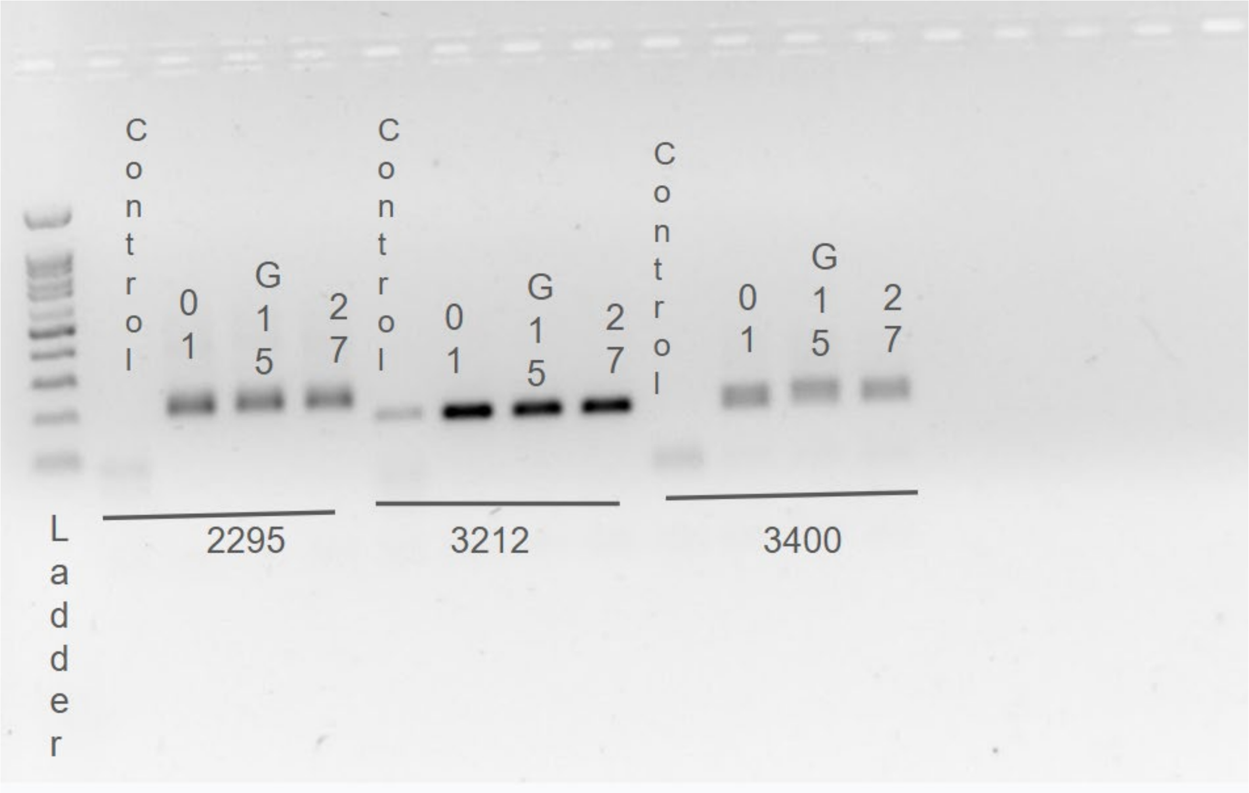


These are the agarose gels of all the PCR reactions in which the primer pairs successfully amplified *G. rotundifolia* DNA in the PCR conditions used and an annealing temperature of 56ºC. The primer pairs used are labelld on the bottom and whether a lane has the products of a control reaction or a sample reaction are labelled on top. The control lanes all have no bands, so there was no contamination of DNA in our reaction components. The sample lanes all have bands, meaning that all the primer pairs successfully amplified DNA. Any faint bands at the bottom are just dimerized primers.

Figure 3: Agarose Gel Electrophoresis of the Products of 3 Primer PCR With M13 Primers Using DNA from 3 Individuals.

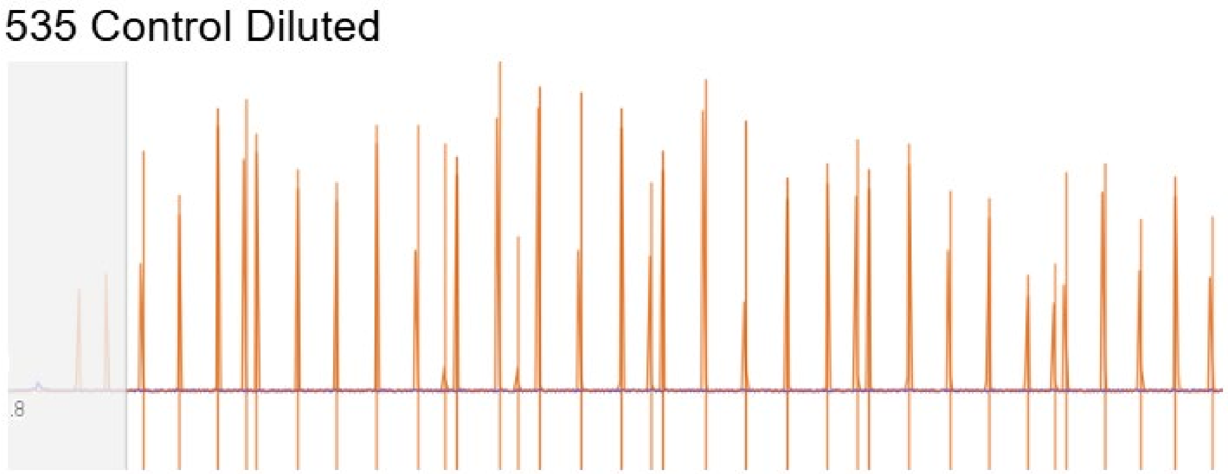






PCR was done on the 11 microsatellites that were able to be amplified at annealing temperatures of 56°C. This PCR used 3 primers, a normal reverse primer, a forwards primer that had both a sequence that attached to the microsatellite flanking region and an M13 sequence, and a forwards primer that was only an M13 sequence with a fluorescent probe. This was done for the sake of incorporating the fluorescent probe into the microsatellites so that fragment analysis could be done. There were four PCR reactions for each microstatellite, a no DNA control, and three reactions with DNA from individuals 01, G15, and 27 respectively. The DNA ladders are in the first lane of each gel. The microsatellite that is being amplified is labelled under the bands, and the DNA source is labelled above the bands. None of the microsatellites being amplified had bands in their control lane, except for microsatellites 3212 and 2246. This means that the PCR reactions for 3212 and 2246 might perhaps have contamination of non-*G. rotundifolia* DNA in their PCR components but all the other microsatellies' reaction components definitely do not have contamination. All the sample lanes produced bands except for micrsoatellite 2169 individual 27, meaning that the microsatellites in all the sample reactions were amplified except for 2169-27.

Figure 4: The Fragment Analysis Results of the 11 Microsatellites Amplified from the Genomes of Three Individuals



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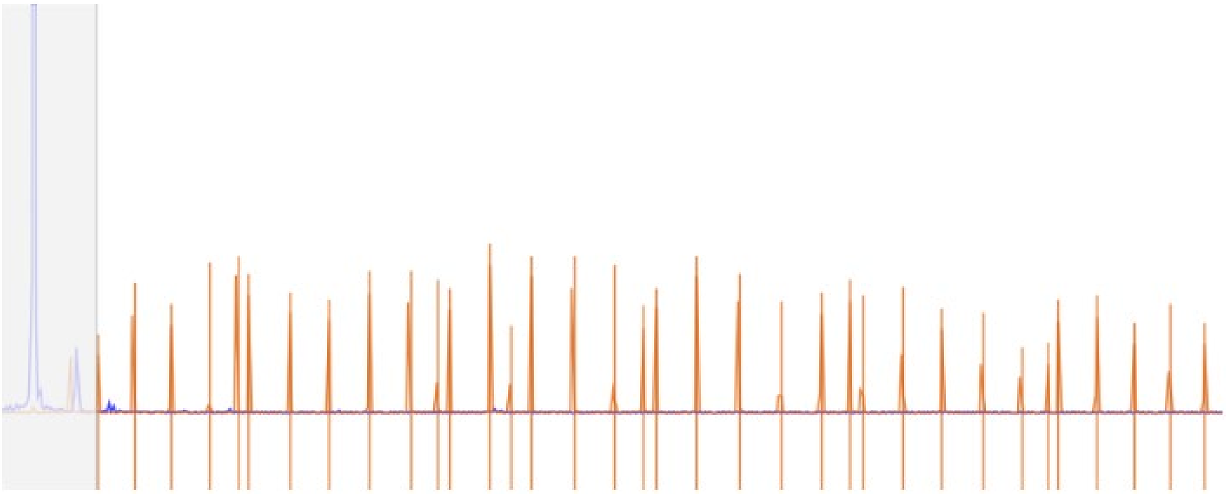

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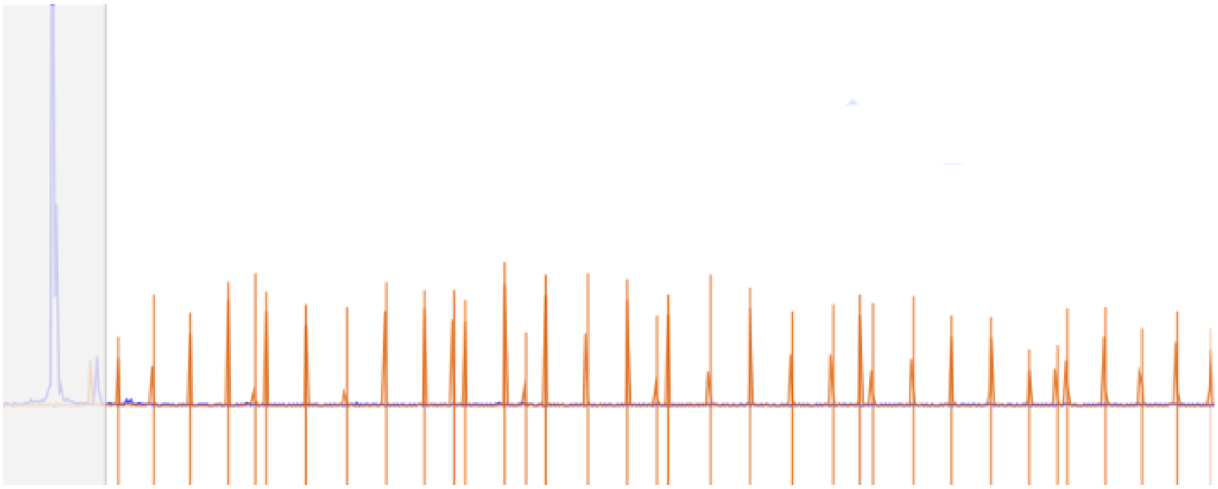
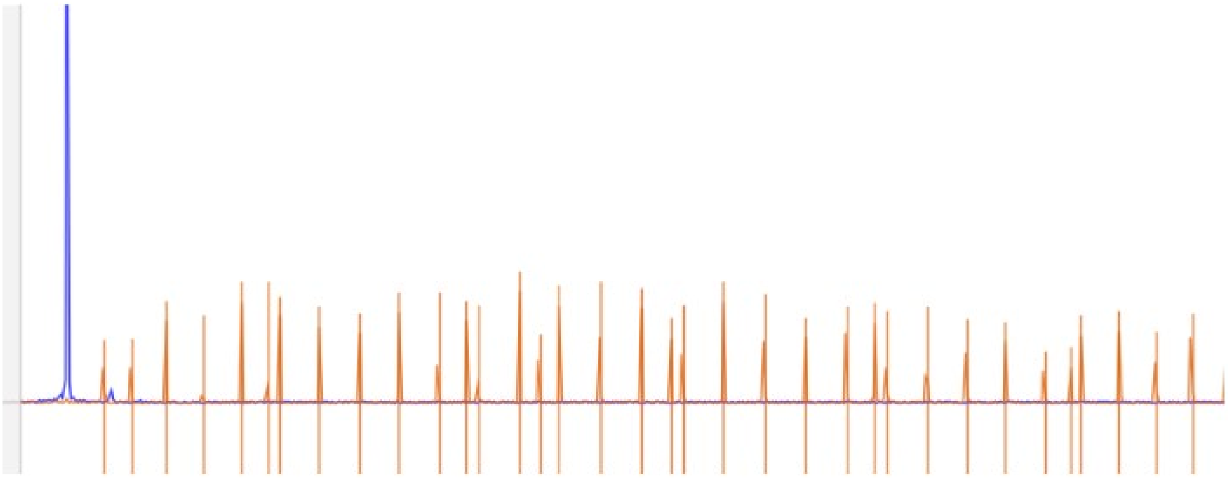
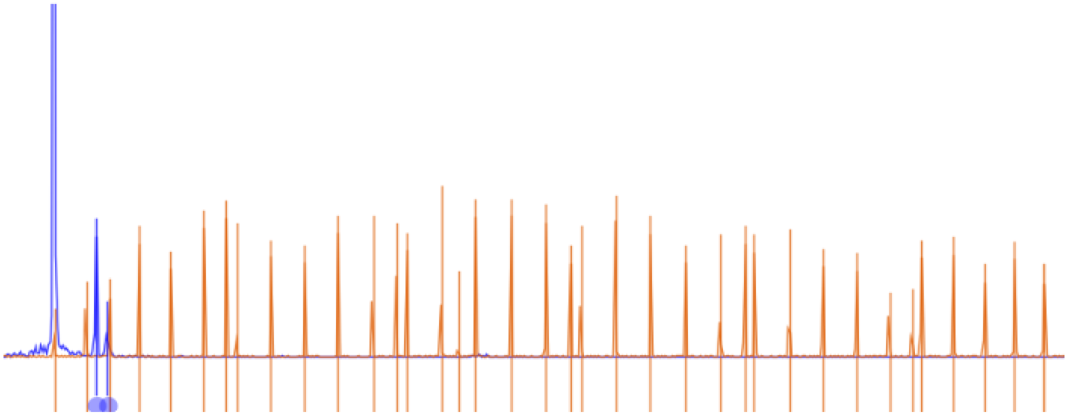
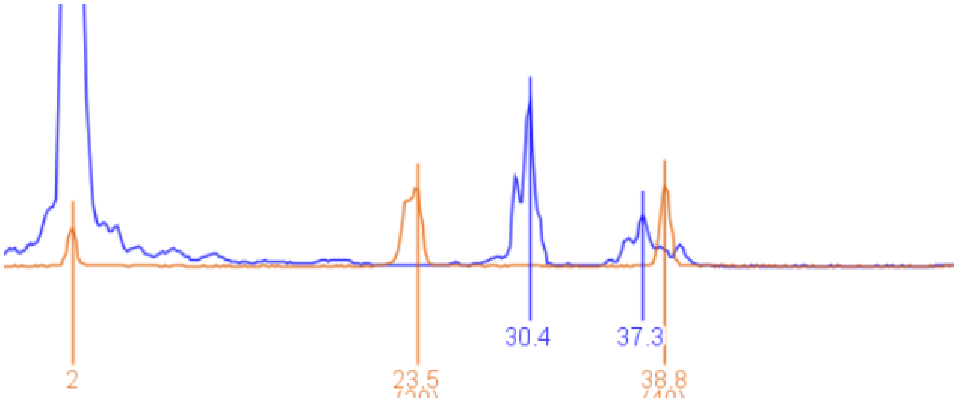


535 27 Diluted



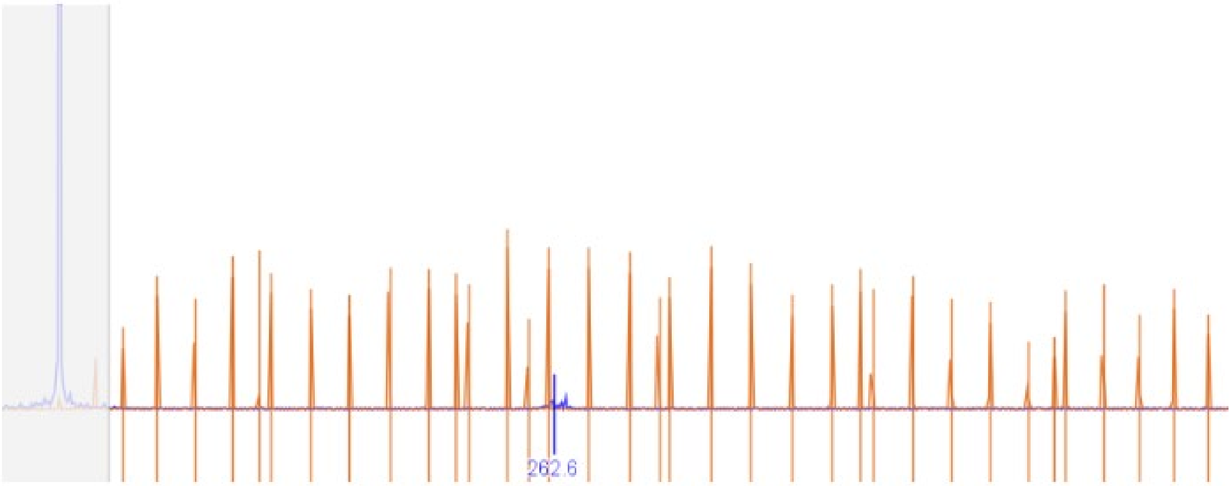
616 Control Diluted





The peaks are very close to the beginning so they’re just primer products and not microsatellite alleles.

616 01 Diluted



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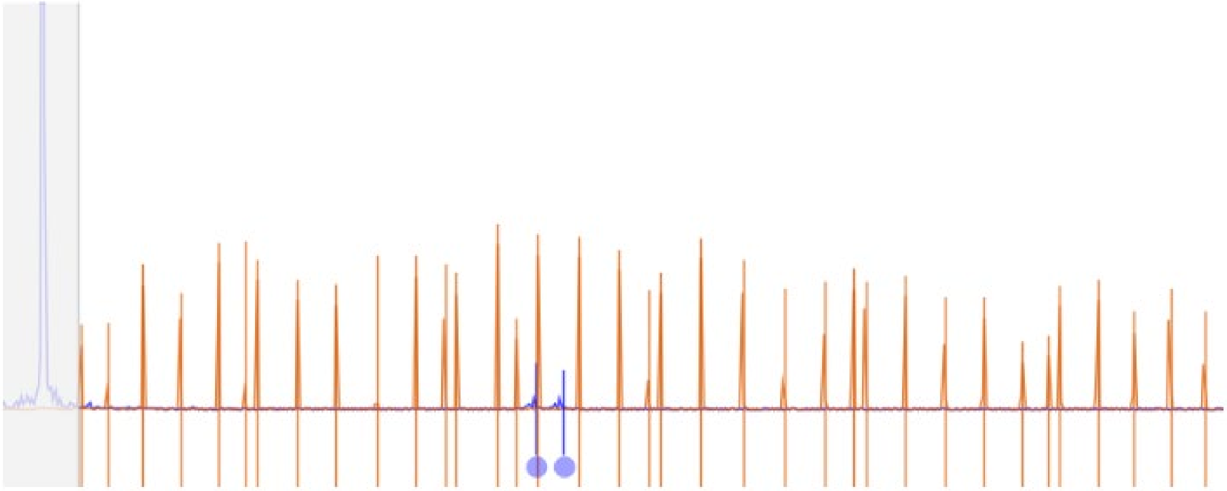

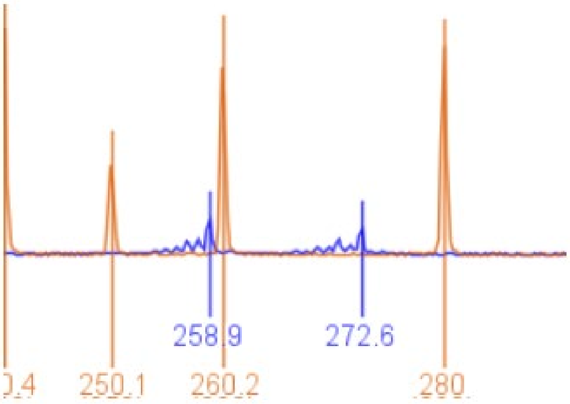

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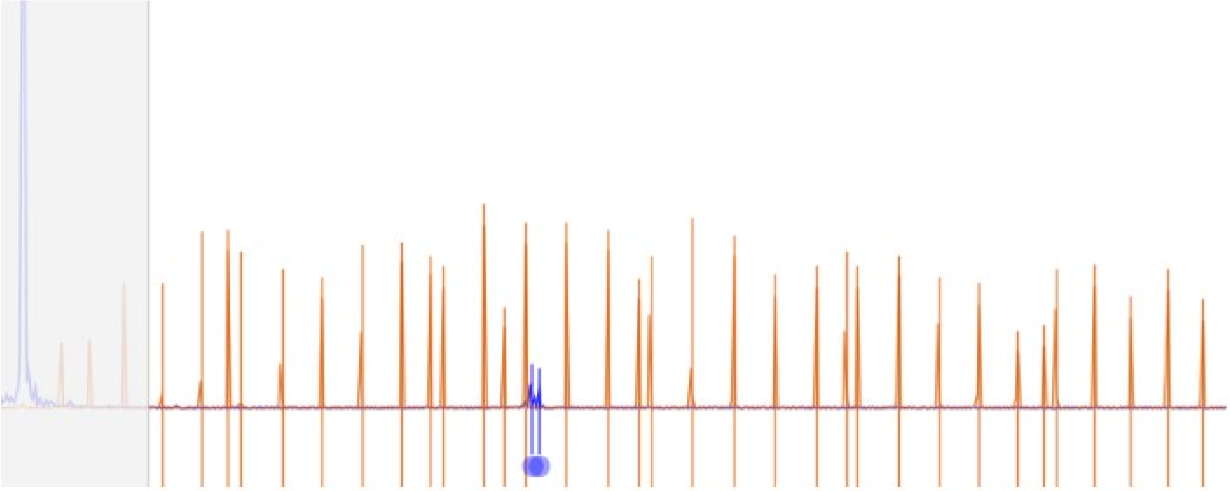

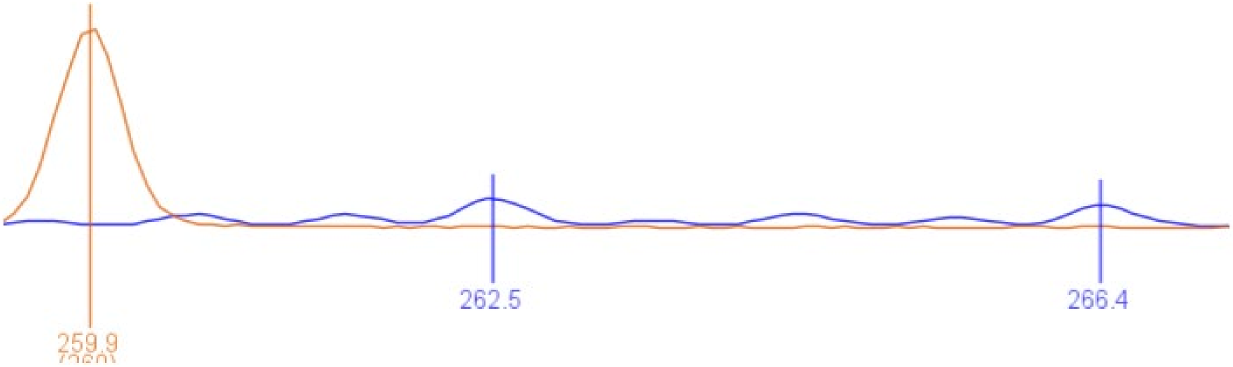

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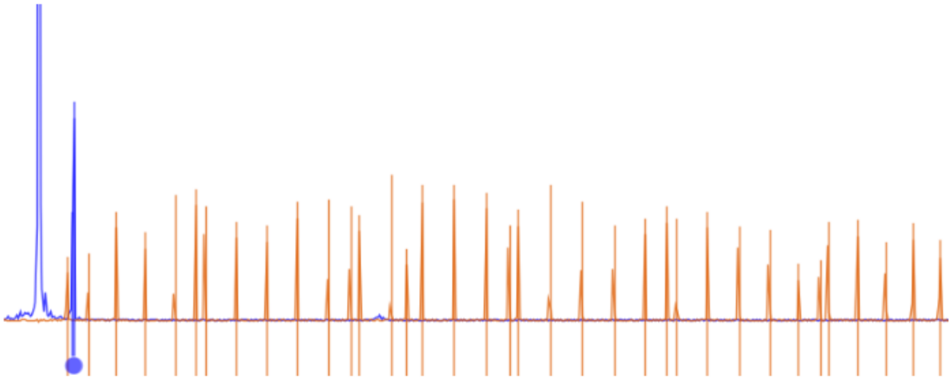
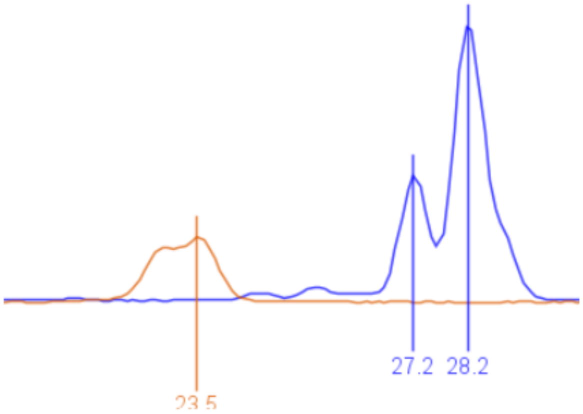
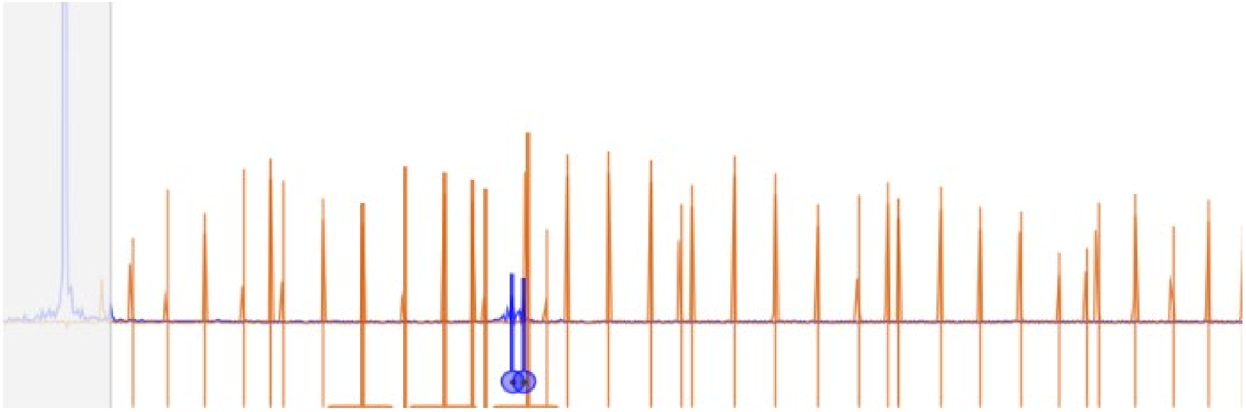
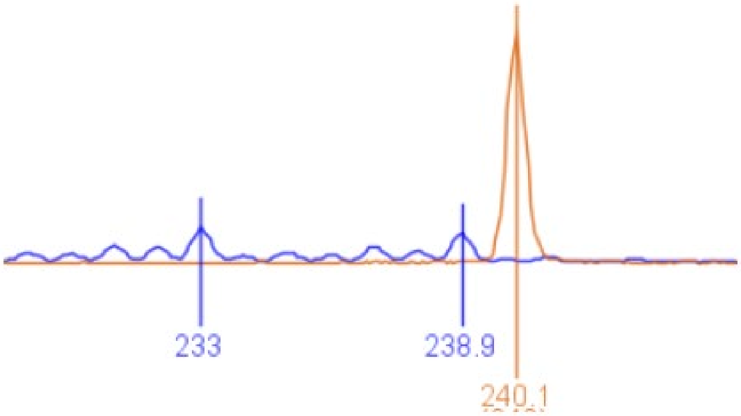




The peaks are very close to the beginning so they’re just primer products and not microsatellite alleles.

1249 01 Diluted





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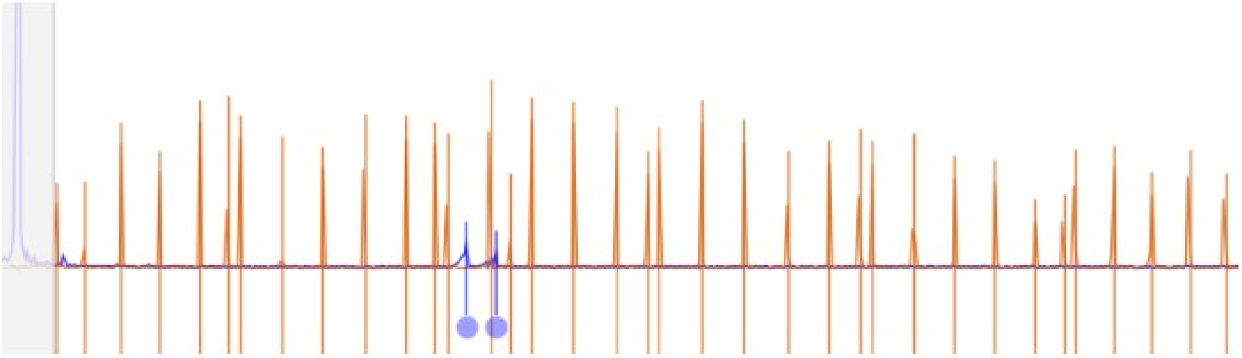

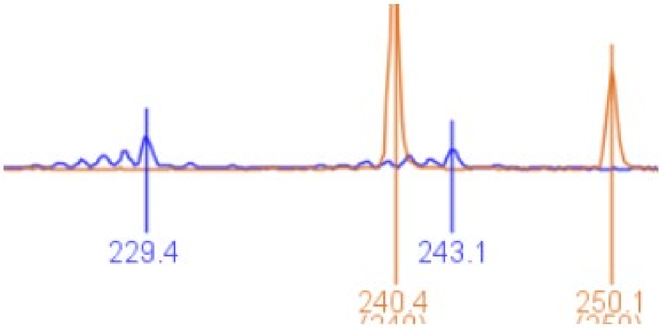

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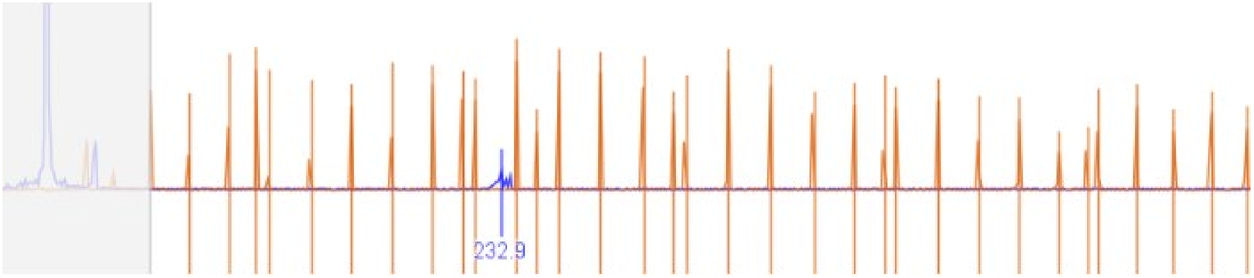
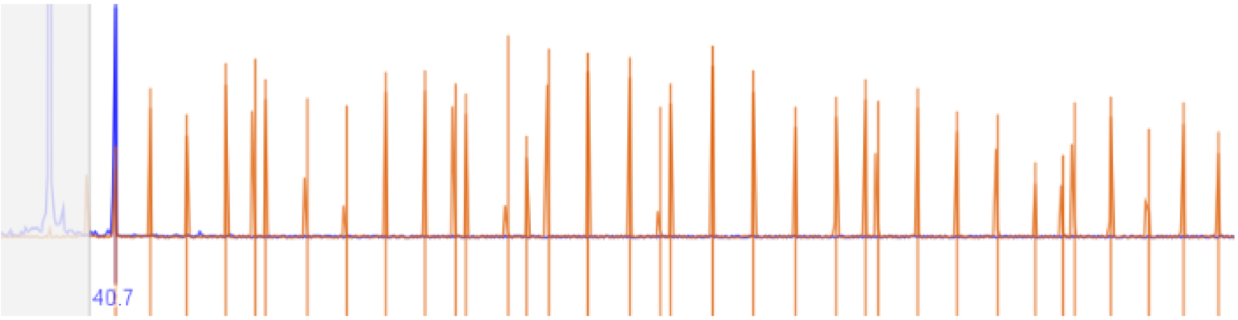
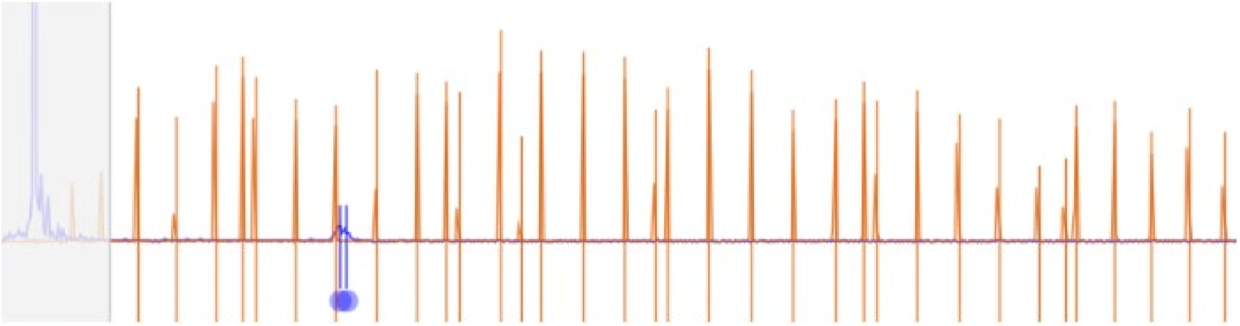


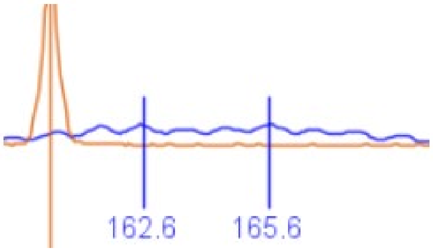
1678 Control Diluted



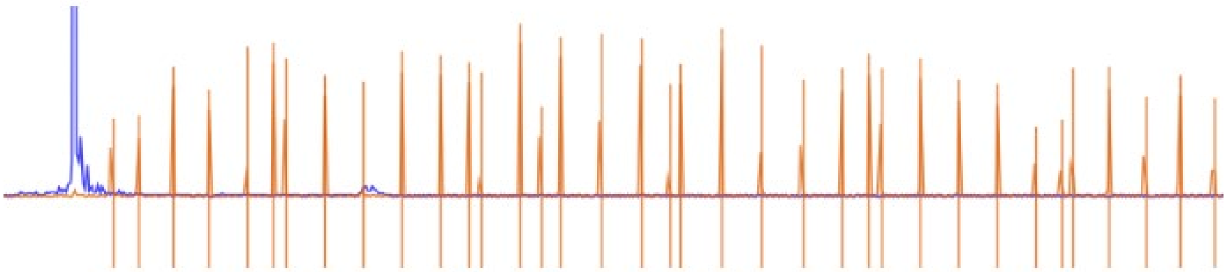
The peaks are very close to the beginning so they’re just primer products and not microsatellite alleles.

1678 01 Diluted

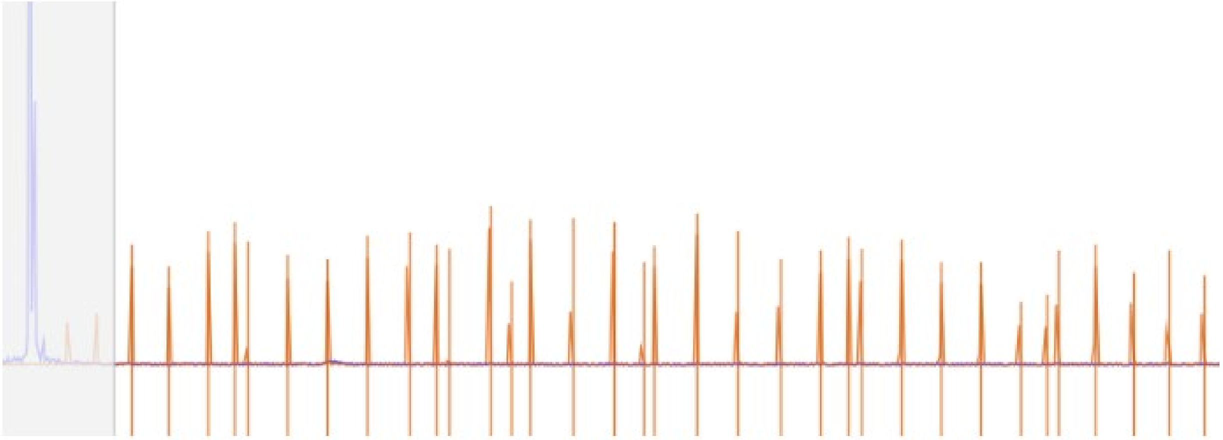




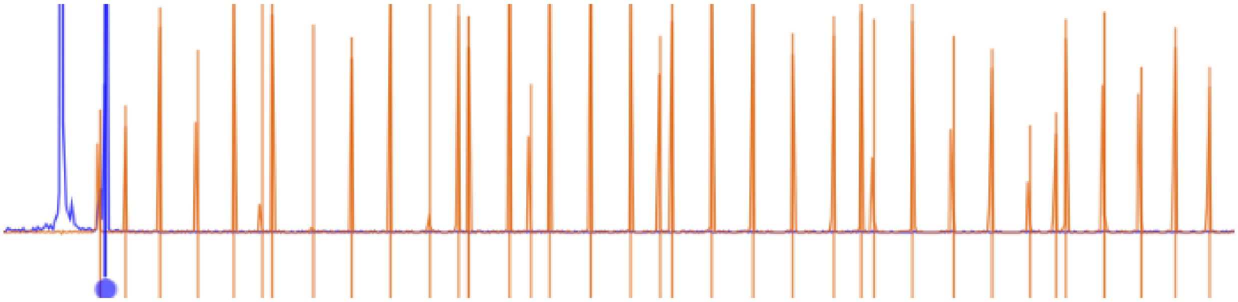
1678 15 Diluted

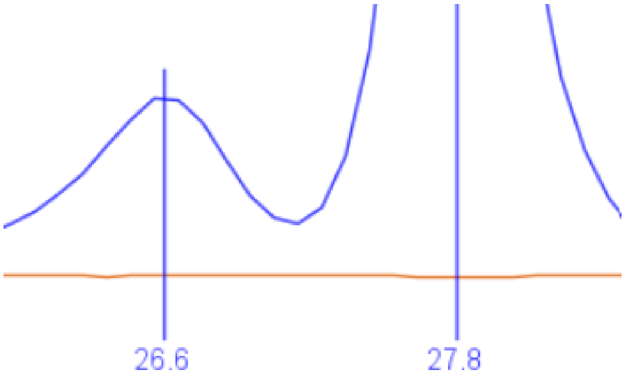


1678 27 Diluted



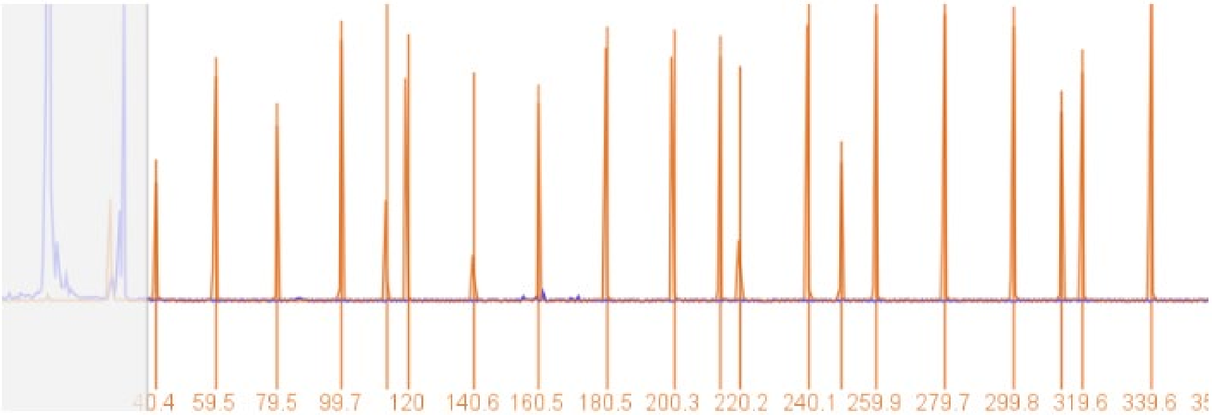
2169 Control Diluted



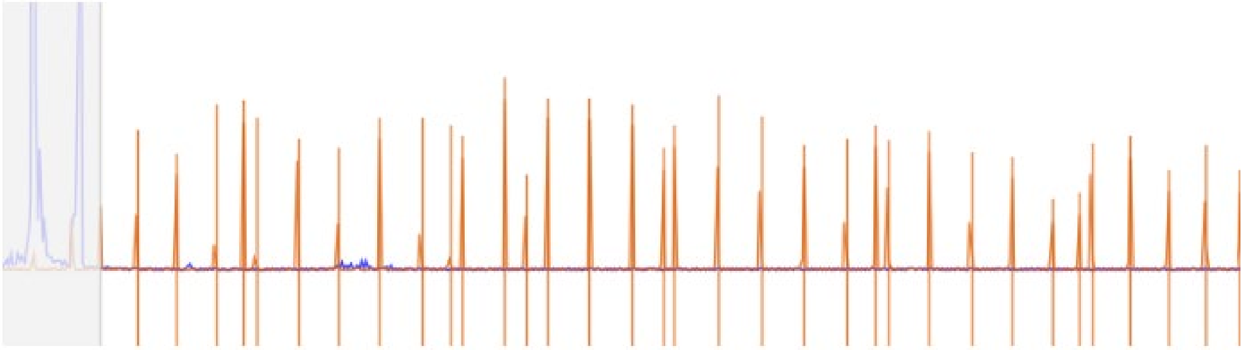


The peaks are very close to the beginning so they're just primer products and not microsatellite alleles.

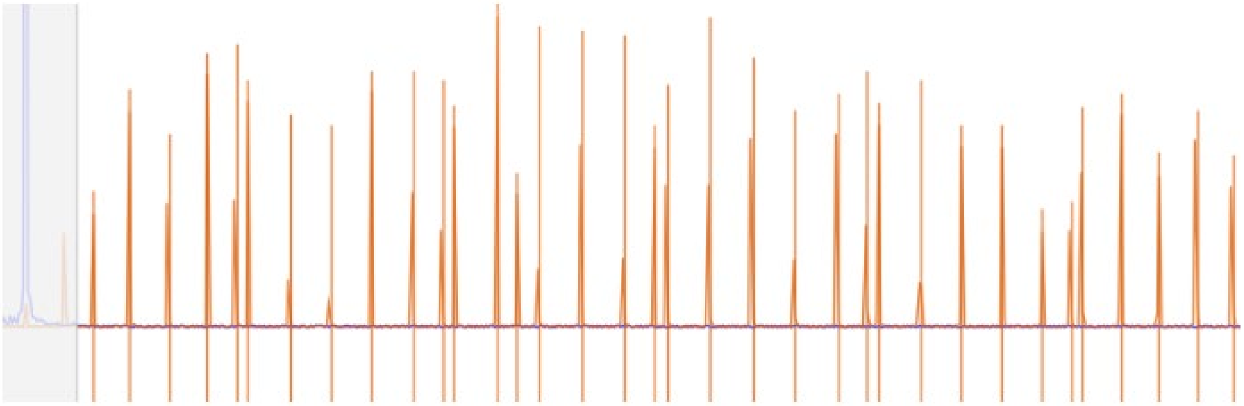
2169 01 Undiluted



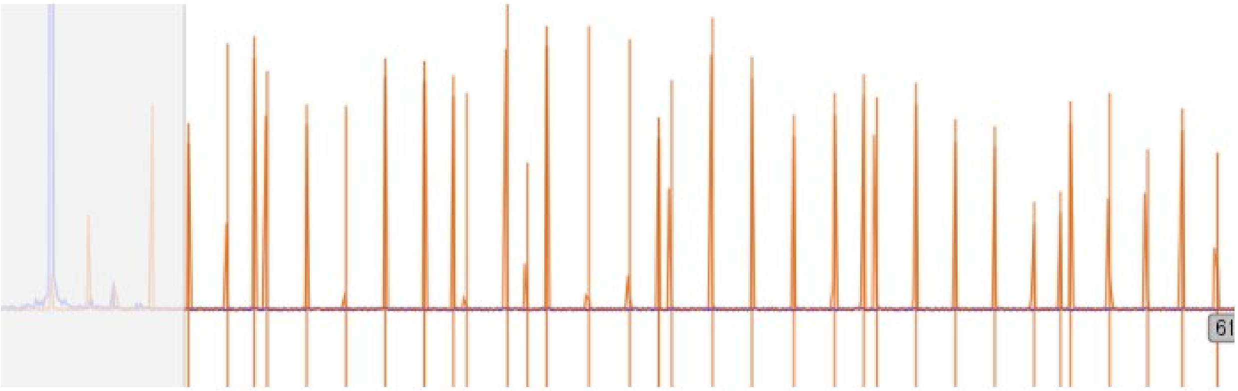
2169 15 Undiluted



2169 27 Undiluted



2246 Control Undiluted



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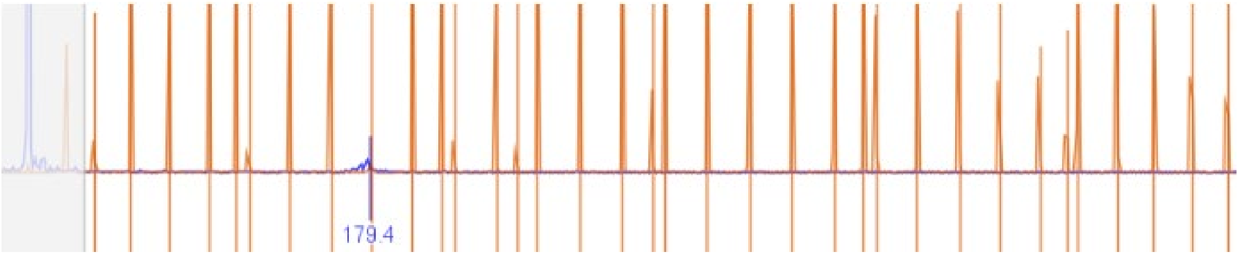

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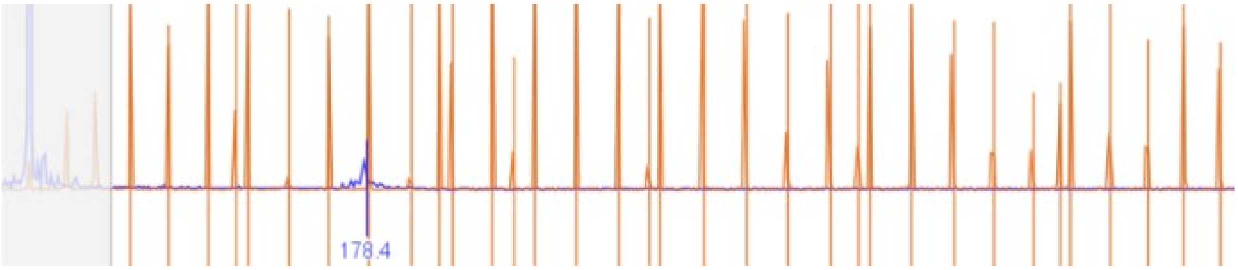

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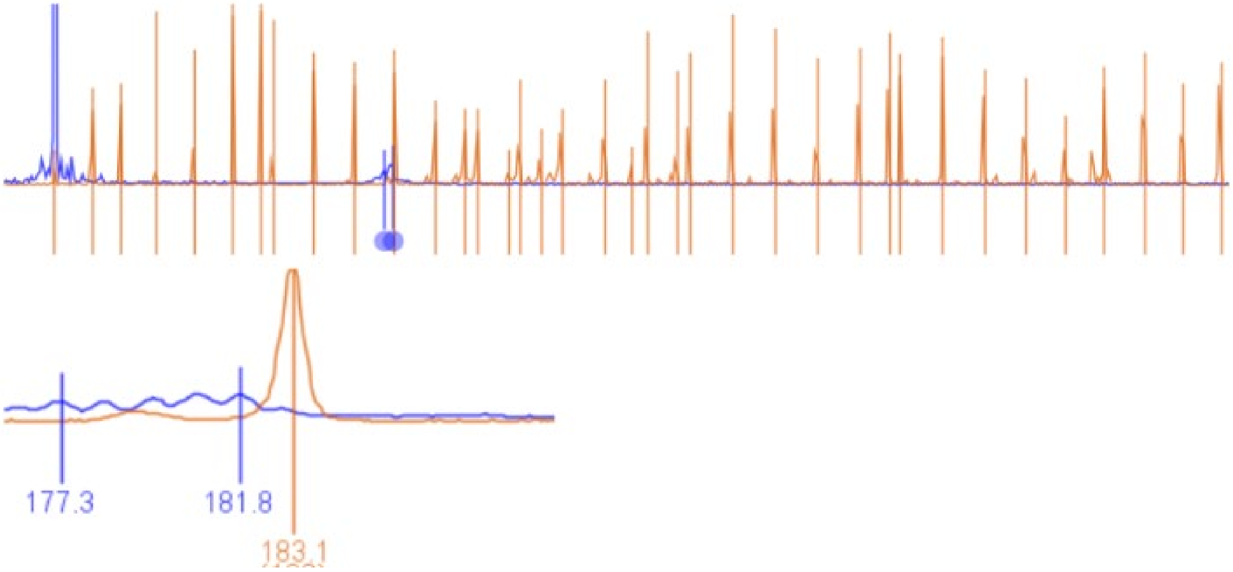

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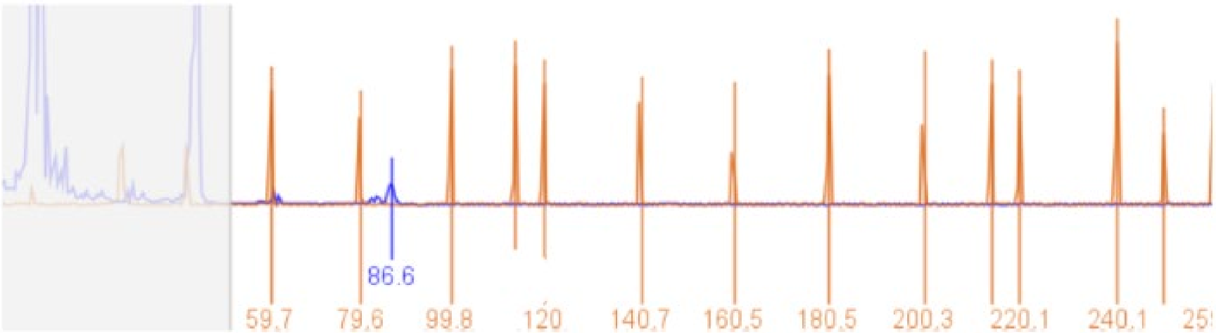

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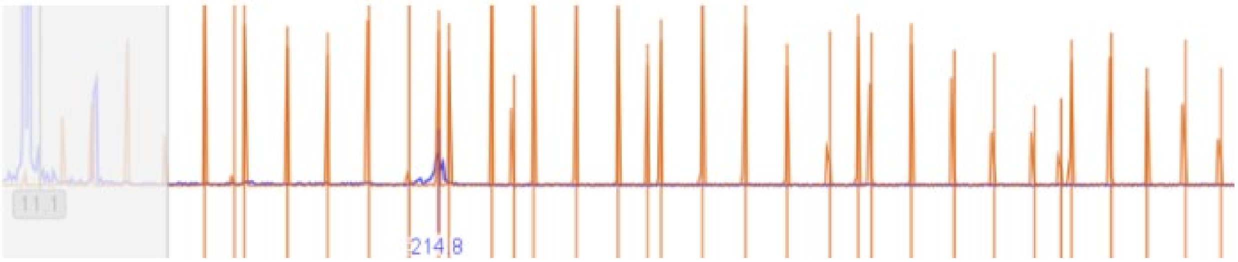

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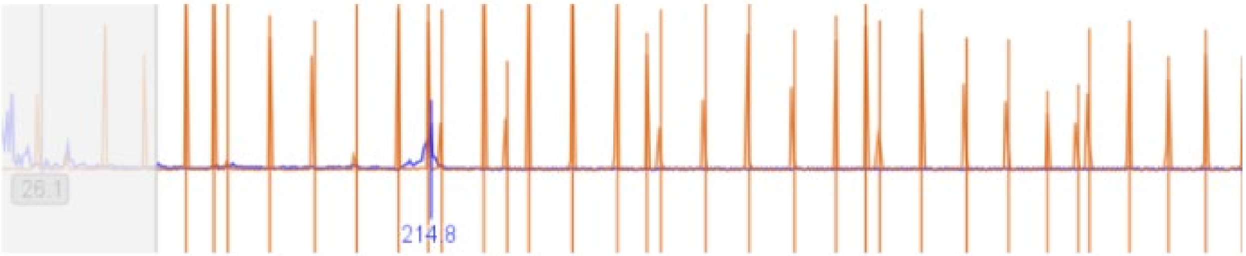

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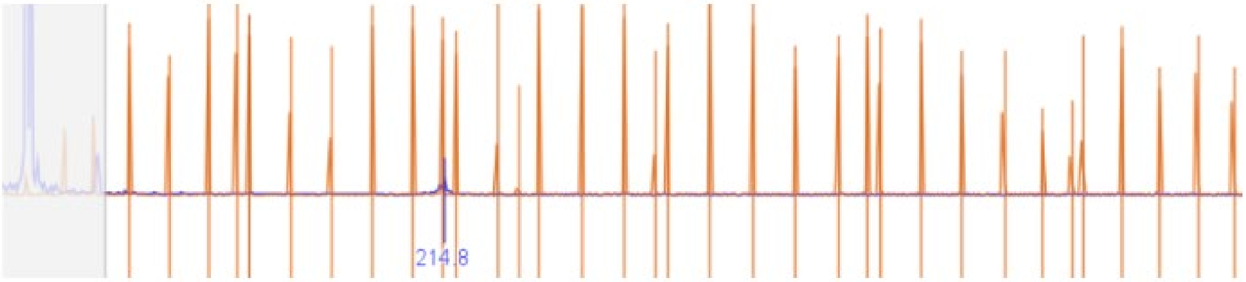
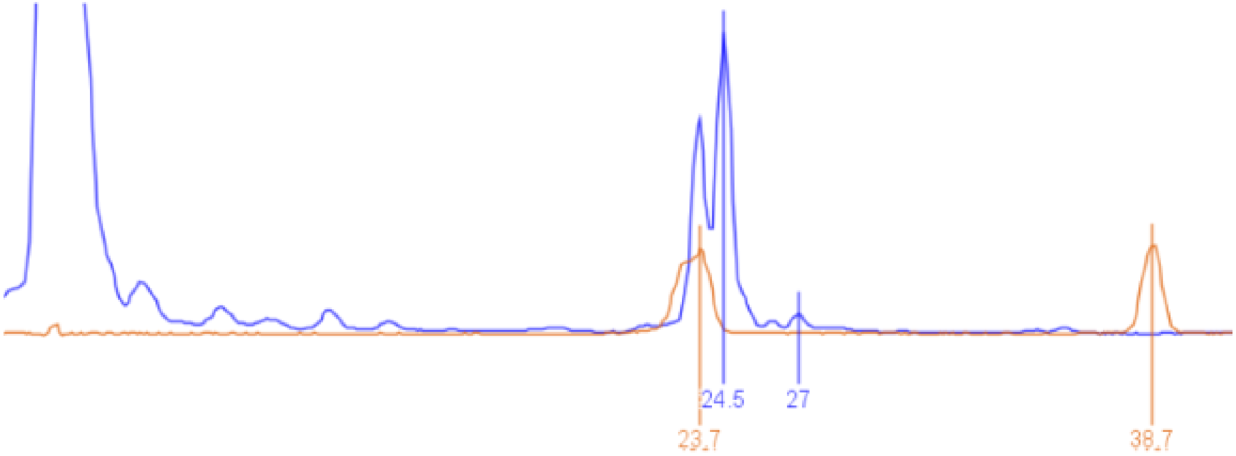
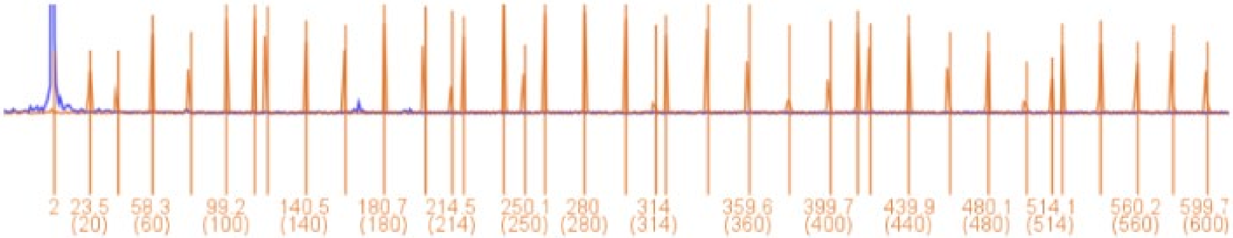


3212 Control Undiluted



The peaks are very close to the beginning so they're just primer products and not microsatellite alleles.

3212 01 Undiluted



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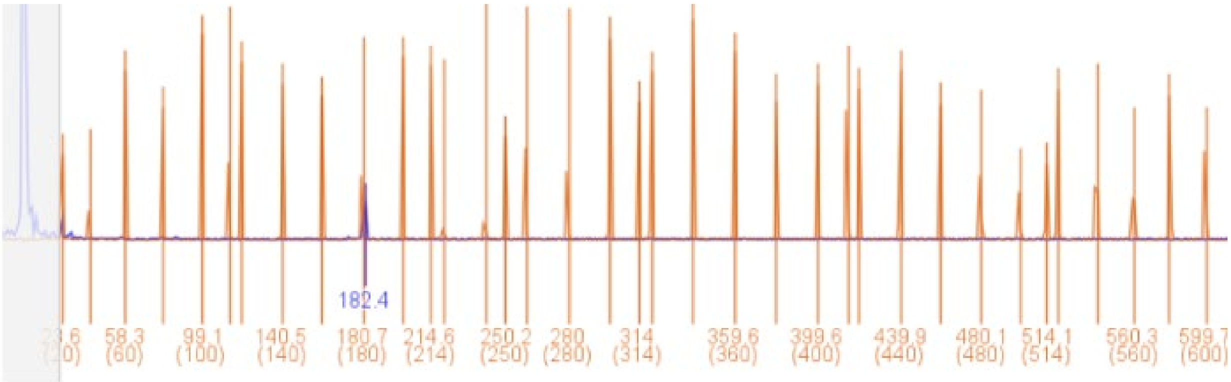

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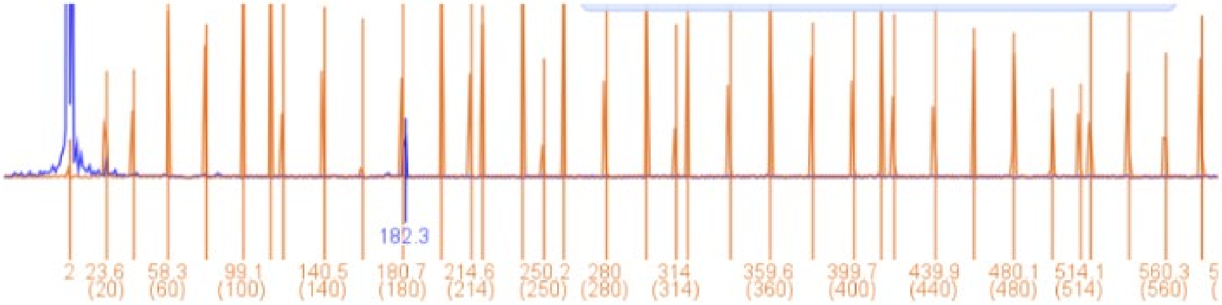

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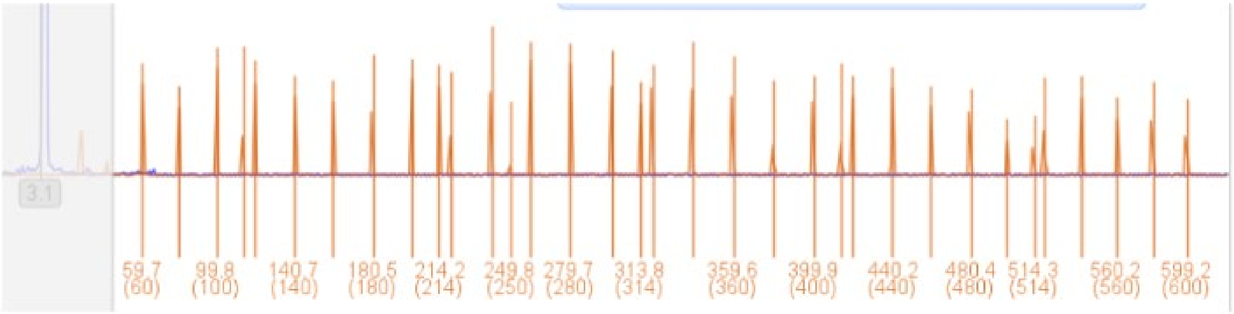

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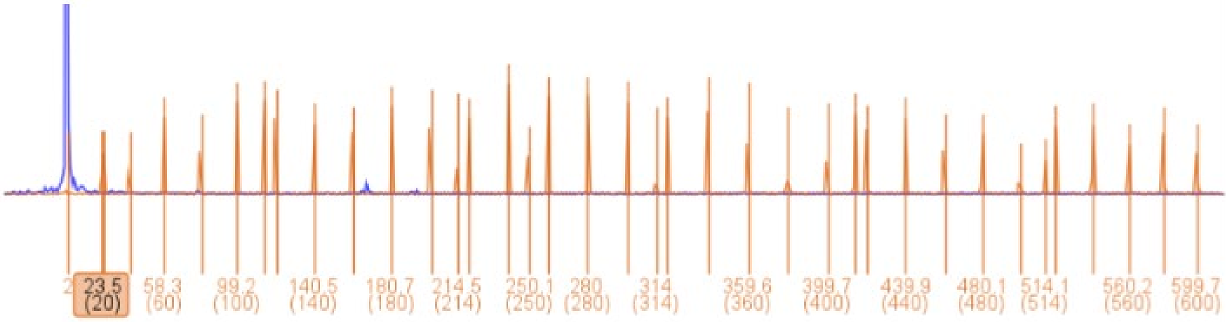

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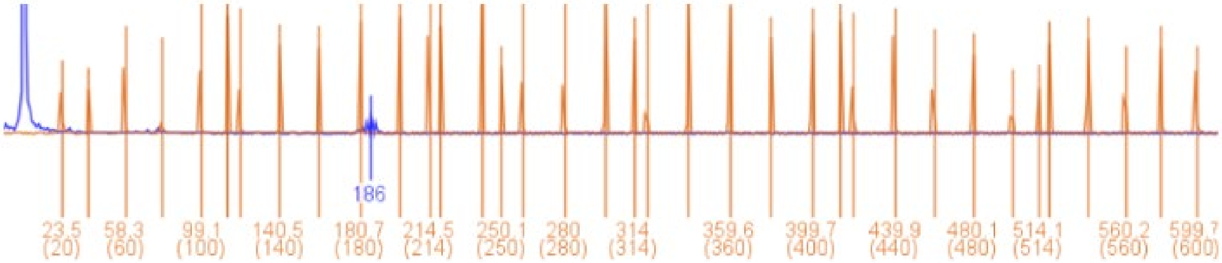

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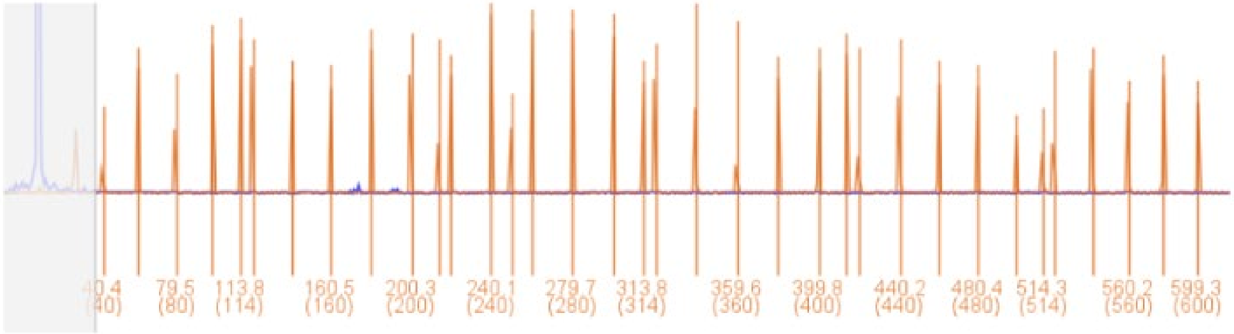
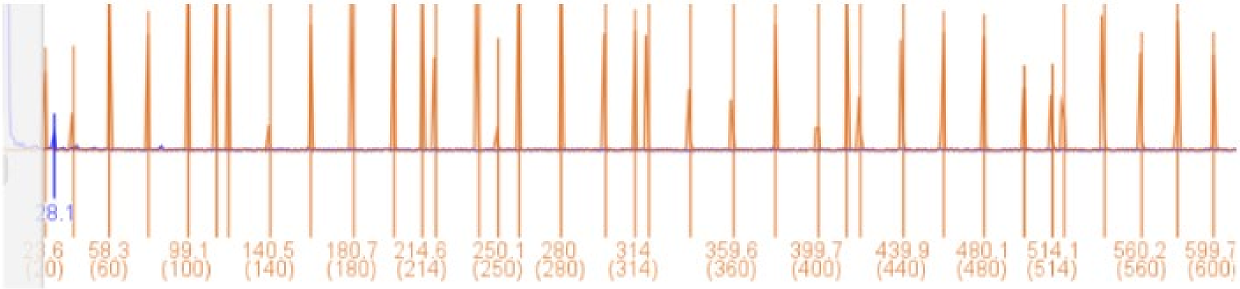
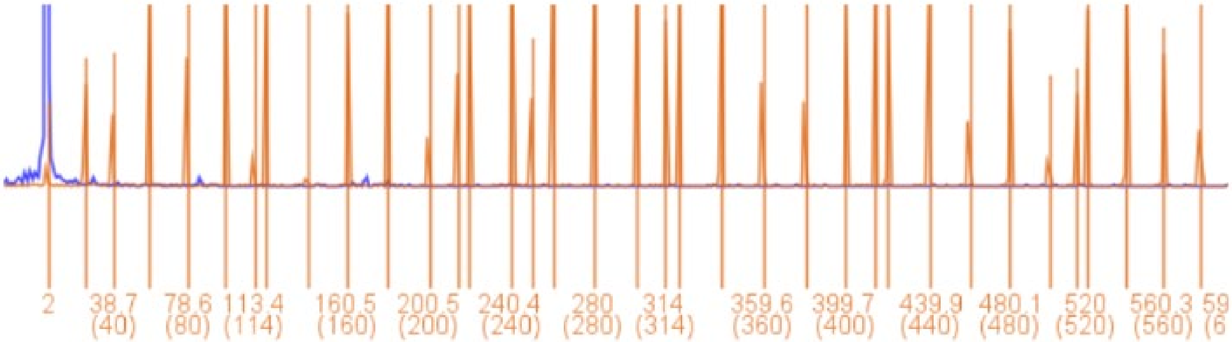


3400 Control Undiluted



3400 01 Undiluted



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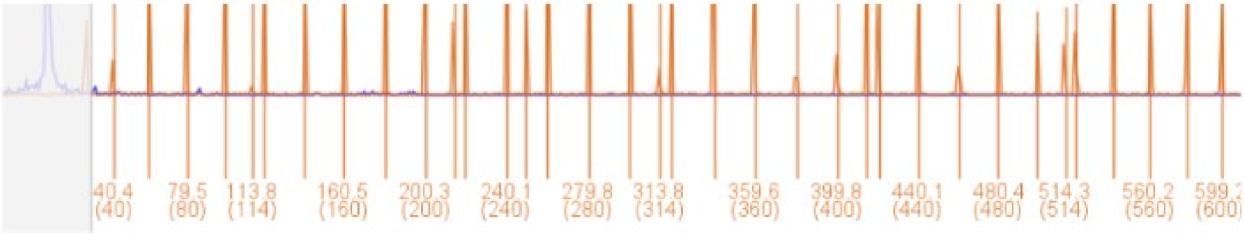

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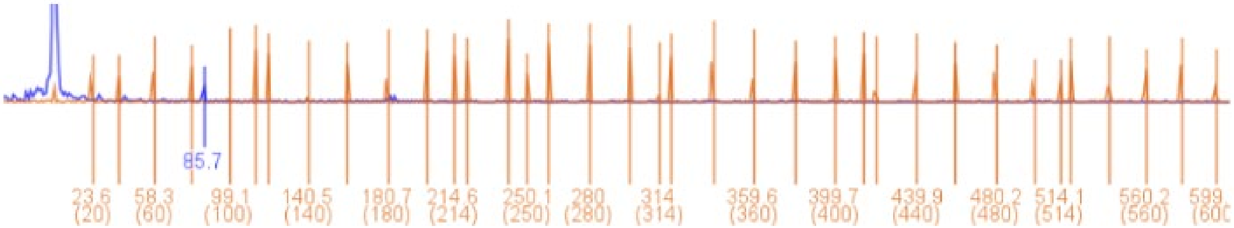
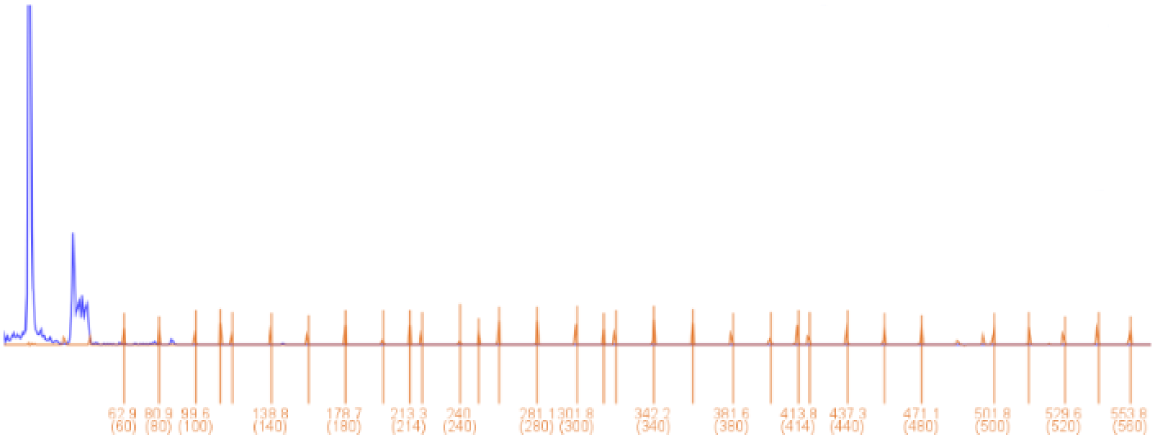
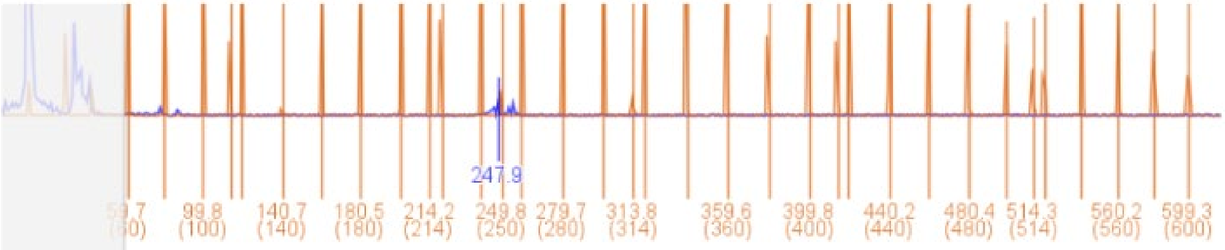


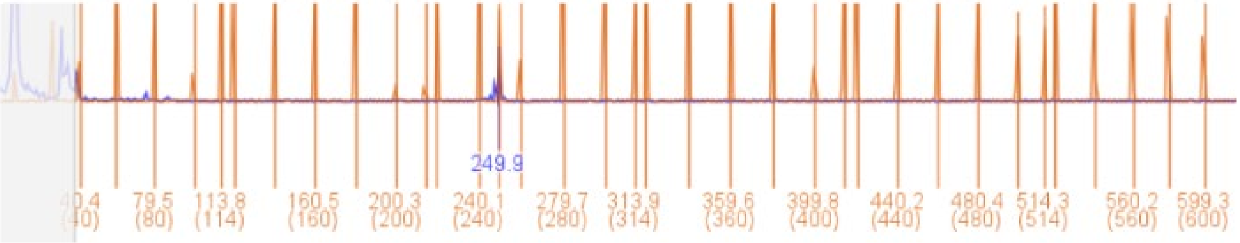
3624 Control Undiluted



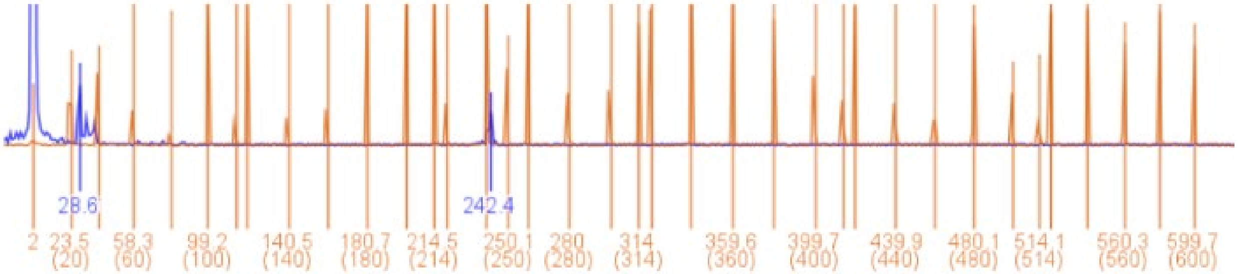
3624 01 Undiluted



3624 15 Undiluted



3624 27 Undiluted



11 of the microsatellites that were successfully amplified by their primer pairs at an annealing

temperature of 56ºC were amplified once again, with the genomes of three different individuals - 01, G15, and 27 - and fluorescent tags were incorporated into them. These PCR products had fragment analysis done to them. The orange peaks in the diagrams are the fragments of the DNA ladder, Liz 600. This figure shows the results of the fragments analysis of samples where the PCR products were diluted 1/10 because the 1/10 dilutions showed better results. The blue peaks are the fragments of the microsatellite alleles. The microsatellite amplified, the individual whose DNA was amplified (and the no DNA controls), and whether the samples were diluted are labelled above each diagram. Some diagrams show the overall fragment analysis and also zoom in to show the microsatellite alleles.

Tables

Table 1: The Primer Pairs Tested at an Annealing Temperature of 56ºC. These are the 25 primer pairs that were PCR amplified at an annealing temperature of 56ºC. The Primer Name is what the forwards or reverse primers were labelled as. The Sequence is the order of base pairs that make up the primer. The Optimal Annealing Temperature is the annealing temperature given by Primer 3. The Microsatellite Motif is the sequence of the microsatellite that the primer amplifies. The Annealing Temperature Used During PCR is the temperature that the annealing step of PCR was done in. Pass/Fail indicates whether the primer was successful at amplifying *G. rotundifolia* DNA in the PCR conditions used. The primer pairs in the red background were unsuccessful and the primer pairs in the green background were successful.

Primer Name	Sequence	Optimal Annealing Temperature	Microsatellite Motif	Product Size (Base Pairs)	Annealing Temperature Used During PCR	Pass/Fail
Galrot 82F	TGAAGGGCTCACAACGGTAT	59.02	(CT)25	225	56	Fail
Galrot 82R	GCGAGTTCTTACG	58.52				

	TGTGTGT					
Galrot 22F	GTAAGGTAGTGCTCAGGATCGA	59.05	(AG)5GG(AG)22	150		Fail
Galrot 22R	TGACCCATCACCATCAAATGT	57.55				Fail
Galrot 222F	CCTACGATTTGTACGCTCCAG	58.54	(TC)22	223		
Galrot 222R	GTGGACCACTAGCCTCATGA	58.8				
Galrot 3359F	CCACACAGAACATGAACCGG	59.13	(AG)27	171	56	Pass
Galrot 3359R	GTCACAGCATCGTCACCATC	59				
Galrot 535F	TCCACGGTCCCATTATCACC	59.17	(AG)25	264	56	Pass
Galrot 535R	TGGGAGAAATTGAATCTGGCG	58.63				
Galrot 1678F	GCTTTCCGAATCTATGCTCCG	59.2	(CT)25	190		Pass
Galrot 1678R	GGACCGTACACCTTCTTCCT	58.74				Pass
Galrot 3230F	CATGGTGCACAACAATCCCA	59.03	(AG)25	172		Fail
Galrot 3230R	GCTAAGGGGAGT	58.13				

	TGAGGAGA					
Galrot 3517F	TTCCCCAATGTAATCGGCCA	59.37	(AG)24	214		Fail
Galrot 3517R	TCCGTTGTGAGCGTAGTCAT	59.11				
Galrot 1249F	CGAAGGGCCTATCAGCAAAA	58.25	(AG)23	220		Pass
Galrot 1249R	TTCTAGGGAGGGGAGAGACG	59.45				Pass
Galrot 1959F	GCATGATAGGGGCGTTGAAG	59.05	(AG)23	240	56	Fail
Galrot 1959R	ACAGCAAGTTCCAATGTGACA	58.35				
Galrot 2036F	AATGGGGAGAATTGAGGGG	56.33	(TC)23	234		Fail
Galrot 2036R	CGTGAGGAGTTGTCGAGGAT	59.19				
Galrot 2678F	AGAGGGAGAGAGAGACTGGG	59.07	(AG)23	202	56	Fail
Galrot 2678R	ACTCAGCACCCCGACAAATA	59.02				
Galrot 3624F	AACCCACTCACCACTAGTCG	59.03	(GA)23	232		Pass
Galrot 3624R	CCCTACTATAGCACTTGGGGT	58.31				Pass

Galrot 616F	AGTTACCTCATTGACTGACTCCA	58.84	(AG)22	246		Pass
Galrot 616R	TCCTTCTCGCAATGACCTCA	58.73				Pass
Galrot 717F	TTTGCAGATGGGGCTTTTCA	58.29	(GA)22	242		Fail
Galrot 717R	GAGTCCACAATCGCATCACA	58.27				Fail
Galrot 2169F	CTTCTCCACCTCCTCCGATC	58.96	(CT)22	153		Pass
Galrot 2169R	AGGCTGCAGTGATGGTGATA	58.79				Pass
Galrot 2246F	CCCCAACATTCTTGAACCCA	57.99	(GA)22	202		Pass
Galrot 2246R	CATTTGCCCTAAGTGTCGCA	58.84				Pass
Galrot 2295F	GCTTCAAGGGATTCTCGAGC	58.71	(AG)22	229		Pass
Galrot 2295R	GAGAGGGAAGAACAGCTGAGA	58.82				Pass
Galrot 2321F	GAGGAGGAGAGGTATTGGGC	58.95	(AG)22	256		
Galrot 2321R	ATATACACGAGTGCCCGACA	58.6				
Galrot 3212F	GATGCAACGGATT	59.33	(TC)22	166	56	Pass

	CATGGGG					
Galrot 3212R	AGCTCAGATGCCTCGAGTAC	58.97				
Galrot 3400F	TTCTTCTCCTCCTCCTCCGA	59	(CT)22	161	56	Pass
Galrot 3400R	AGAGGGAGGCTGCAGTAATG	59.16				
Galrot 3473F	TTCTTCTCCTCCTCCTCCGA	59	(CT)22	161	56	Pass
Galrot 3473R	AGAGGGAGGCTGCAGTAATG	59.16	(CT)22	161	56	Pass

Table 2: All the Primer Pairs Given by Primer 3. This is a list of all the primer pairs that Primer 3 gave. The number in the Primer Name column describes the microsatellite that is amplified by the primer, the letter describes whether it's a forwards or reverse primer, and the word or number after the dash shows if the primer is one of the main primers generated or whether it is one of the alternate primers generated, and if so which alternate primer it is. The Sequence describes the order of base pairs within the primer. And the Optimal Temperature describes the optimal annealing temperature given by Primer 3.

Primer Name	Sequence	Optimal Temperature
82F - Main	TGAAGGGCTCACAACGGTAT	59.02
82B - Main	GCGAGTTCTTACGTGTGTGT	58.52
22F - Main	GTAAGGTAGTGCTCAGGATCGA	59.05

22B - Main	TGACCCATCACCATCAAATGT	57.55
222F - Main	CCTACGATTTGTACGCTCCAG	58.54
222B - Main	GTGGACCACTAGCCTCATGA	58.8
3359F - Main	CCACACAGAACATGAACCGG	59.13
3359B - Main	GTCACAGCATCGTCACCATC	59
535F - Main	TCCACGGTCCCATTATCACC	59.17
535B - Main	TGGGAGAAATTGAATCTGGCG	58.63
1678F - Main	GCTTTCCGAATCTATGCTCCG	59.2
1678B - Main	GGACCGTACACCTTCTTCCT	58.74
3230F - Main	CATGGTGCACAACAATCCCA	59.03
3230B - Main	GCTAAGGGGAGTTGAGGAGA	58.13
3517F - Main	TTCCCCAATGTAATCGGCCA	59.37
3517B - Main	TCCGTTGTGAGCGTAGTCAT	59.11
1249F - Main	CGAAGGGCCTATCAGCAAAA	58.25
1249B - Main	TTCTAGGGAGGGGAGAGACG	59.45
1959F - Main	GCATGATAGGGGCGTTGAAG	59.05
1959B - Main	ACAGCAAGTTCCAATGTGACA	58.35
2678F - Main	AGAGGGAGAGAGAGACTGGG	59.07
2678B - Main	ACTCAGCACCCCGACAAATA	59.02
3624F - Main	AACCCACTCACCACTAGTCG	59.03
3624B - Main	CCCTACTATAGCACTTGGGGT	58.31
616F - Main	GCGCTGATCCGAGAGAAAGA	59.9
616B - Main	TCCTTCTCGCAATGACCTCA	58.73
717F - Main	TTTGCAGATGGGGCTTTTCA	58.29
717B - Main	GAGTCCACAATCGCATCACA	58.27
2169F- Main	CTTCTCCACCTCCTCCGATC	58.96
2169B - Main	AGGCTGCAGTGATGGTGATA	58.79
2246F - Main	CCCCAACATTCTTGAACCCA	57.99
2246B - Main	CATTTGCCCTAAGTGTCGCA	58.84

2295F - Main	GCTTCAAGGGATTCTCGAGC	58.71
2295B - Main	GAGAGGGAAGAACAGCTGAGA	58.82
2321F - Main	GAGGAGGAGAGGTATTGGGC	58.95
2321B - Main	ATATACACGAGTGCCCGACA	58.6
3212F - Main	GATGCAACGGATTCATGGGG	59.33
3212B - Main	AGCTCAGATGCCTCGAGTAC	58.97
3400F - Main	TTCTTCTCCTCCTCCTCCGA	59
3400B - Main	AGAGGGAGGCTGCAGTAATG	59.16
3473F - Main	TTCTTCTCCTCCTCCTCCGA	59
3473B - Main	AGAGGGAGGCTGCAGTAATG	59.16
82F - 1	GGGCTCACAACGGTATTAGG	58.05
82B - 1	CGAGTTCTTACGTGTGTGTATGT	58.78
82F - 2	GGCTCACAACGGTATTAGGAAC	59.07
82B - 2	TCTTACGTGTGTGTATGTGTGA	57.43
82F - 3	GGCTAAATCCCAAAGTTCCCT	57.9
82B - 3	TGCTATGAGATAATGTGATTGCG	57.15
22F - 1	GGTAGTGCTCAGGATCGATGA	59.04
22B - 1	TGTTACACTCAACCTACCCCT	58.02
22F - 2	GTGCTCAGGATCGATGAAAGA	57.83
22B - 2	GTTACACTCAACCTACCCCTAGT	58.91
222F - 1	TGGAGCCTACGATTTGTACG	57.43
222B - 2	TGAGAGGGGTGACTAGGAGA	58.32
3359F - 1	CACAGAACATGAACCGGACA	58.12
3359B - 1	ACAGCATCGTCACCATCAAC	58.56
3359F - 2	TCCCAAAAGTTCCACACAGAA	57.63
3359B - 2	CATCGTCACCATCAACTGCT	58.27
3359F - 3	ACAGAACATGAACCGGACAAAT	58.51
3359B - 3	GAAAATGGAGGAGCGTCACA	58.19

3359F - 4	CCTGAGTAACCGGACAAATC	57.4
3359B - 4	CGTCACCATCAACTGCTAGTG	59
535F - 1	TCGATCTCCTTTTCCACGGT	58.74
525B - 1	GATCTTCGTCTTCAGCCGC	58.71
525F - 2	TCCTTTTCCACGGTCCCATT	59.23
525B - 2	CGACATTCATCCCGGCTCG	60.95
525F - 3	ATCTCCTTTTCCACGGTCCC	59.38
525B - 3	GACGGTCCCAGTGTGCAC	60.67
535F - 4	CCTTTTCCACGGTCCCATTATC	59.05
525B - 4	GAGCATCGCCACGGATCG	61.03
1678F - 1	GTGCACCGAAAATCCCGTG	59.79
1678B - 1	ACCGTACACCTTCTTCCTCAT	58.46
1678F - 2	CAAGAGCTTTCCGAATCTATGCT	58.87
1678B - 2	ATTTCTTTTCTTCCGCCGCC	59.47
1678F - 3	TGGAGAAAACTTACCGTCAACAC	59.13
1678B - 3	CCTCATTTCTTTTCTTCCGCC	58.92
1678F - 4	ACGAAATGGAGAAAACTTACCGT	58.62
1678B - 4	CCGTACACCTTCTTCCTCATTTC	59.07
3230F - 1	AGGGGTTCTGTATAGGTGGC	58.49
3230B - 1	GGGGAGTTGAGGAGAAGACT	58.05
3230F - 2	GGTGCACAACAATCCCATCT	58.45
3230B - 2	GGTGTAAGCTAAGGGGAGTTG	58.01
3230F - 3	GCACAACAATCCCATCTCCT	57.87
3230B - 3	GTAAGCTAAGGGGAGTTGAGG	59.16
3230F - 4	CAAGCCAGAGAAGCATAAGAGT	58.13
3230B - 4	TTCTCTCTCTATCTCATCAAGCG	57.31
3517F - 1	CCCAATGTAATCGGCCACAA	58.53
3517B - 1	GCATCCGTTGTGAGCGTAG	59
3517F - 2	CAAACTCTCTTCAACACCCCA	58.07

3517B - 2	ATGTGCATCCGTTGTGAGC	58.83
3517F - 3	ACTCTCTTCAACACCCCAAAG	57.79
3517B - 3	CGTTGTGAGCGTAGTCATAGT	57.87
3517F - 4	TCCTTTCCCCAATGTAATCGG	57.71
3517B - 4	TGTGAGCGTAGTCATAGTAGTGA	58.49
1249F - 1	GTCTGAGACGGAACCAAAGG	58.2
1249B - 1	GGATGCCCTTACACTACTCTTG	58.2
1249F - 2	GAGACGGAACCAAAGGCAC	58.76
1249B - 2	GACGCCCCTGCCAAGAAG	60.75
1249F - 3	AGGTCAAATCAAATCGCTCGT	58.3
1249B - 3	GAGAGACGCCCCTGCCAA	61.71
1959F - 1	TGCGGTCGATCTCTTCCTAG	58.69
1959B - 1	AGAGGTAGTCGTTGCAGATTCA	59.17
1959F - 2	GGTCGATCTCTTCCTAGCGT	58.69
1959B - 2	GAAGAGGTAGTCGTTGCAGAT	57.49
1959F - 3	TGATAGGGGCGTTGAAGGAG	59.17
1959B - 3	ACTCCAAGGACAACAAGAAGAC	58.19
1959F - 4	CTAGCTGCGGTCGATCTCTT	59.33
1959B - 4	AGCAAGTTCCAATGTGACAATCT	58.85
2678F - 1	GGGAGAGAGAGACTGGGTGA	59.67
2678B - 1	CAGCACCCCGACAAATATGC	59.62
2678F - 2	TGGGTGAGAAAGTGAGAGAGT	58.04
2678B - 2	CGACAAATATGCGGAAGGGC	59.69
2678F - 3	AAGGTAGAGTGCAGGAGAGA	57.09
2678B - 3	AACATACTCAGCACCCCGAC	59.75
2678F - 4	GAGAGAGAGACTGGGTGAGAAA	58.57
2678B - 4	GGTTCGTTCTTTTCACACAAACA	58.52
616F - 1	GCTGATCCGAGAGAAAGAGAGA	59.05
616B - 1	GAGCTCCTTCTCGCAATGAC	58.71

616F - 2	GGCGCTGATCCGAGAGAA	59.19
616B - 2	AGAAGAGGAAGAGGGTTGCA	58.27
616F - 3	GGTCAATTCAAATCGCTCGTAA	57.41
616B - 3	TTCTAGGGAGGGGAGAGACG	59.45
616F - 4	AGTCAAGAGTAGTGTAAGGGCA	58.49
616B - 4	CTTCTCGCAATGACCTCAAAT	58.99
717F - 1	AGTTTTGCAGATGGGGCTTT	58.28
717B - 1	GTCCACAATCGCATCACATCT	58.72
717F - 2	CTTTGTGGACTGAAGAAGGGG	58.49
717B - 2	TCAGAGTCCACAATCGCATC	57.7
717F - 3	TGCAGATGGGGCTTTTCATAT	57.68
717B - 3	ACAATCAGAGTCCACAATCGC	58.65
717F - 4	AGATAGTTTTGCAGATGGGGC	58.34
717B - 4	GCTTATGCCCGATACATTTCCA	58.85
2169F - 1	GACCCCTCGCCGTGTATTAT	59.32
2169B - 1	GGTGCTTTGCCATTGATCGA	59.19
2169F - 2	ATGCCCTTCTTCTCCACCTC	59.08
2169B - 2	GATGTCGGTTGCAGCAAGAG	59.55
2169F - 3	CTTCTTCTCCACCTCCTCCG	59.18
2169B - 3	AATTCTGCCGGAGTTGAGCT	59.67
2169F - 4	CACCTCCTCCGATCCATCAC	59.61
2169B - 4	GGAGTTGAGCTGATGTCGGT	59.75
2295F - 1	CTGTCAGGATTCAGGAGCAA	57.22
2295B - 1	ACCGCAAACTCTCTCTCTCT	58.08
2295F - 2	AGAGAGAGAGAGTTTGCGGT	58.08
2295B - 2	TGAAGGAGAAGGTTTGGTGTC	57.79
2295F - 3	GGATTCTCGAGCCCATCAAA	57.67
2295B - 3	TGTAGAGAGGGAAGAACAGCTG	59.17
2295F - 4	TGAGAGTTATGGCCGTTGAAG	58.03

2295B - 4	AGAAAACCGCAAACTCTCTCTC	58.61
2321F - 1	TTTCAAGGAGGAGGCCGAAA	59.23
2321B - 1	TCAAACGCGACATTACCCG	58.55
2321F - 2	GTATTGGGCGGCGGTTTTC	59.86
2321B - 2	CCGCACCCAGACGAAATTTT	59.4
2321F - 3	GTTTTCAAGGAGGAGGCCG	58.44
2321B - 3	CACATATATACACGAGTGCCCG	58.76
2321F - 4	GCGGTTTTCAAGGAGGAGG	58.44
2321B - 4	ATTACCCGGCCTGCGATG	59.89
3212F - 1	GCACAAGTTATCCGGAAGCA	58.55
3212B - 1	CTCGCATGGCCATCTTCTTC	59.06
3212F - 2	CCGGAAGCAAATTACAGAGGG	58.98
3212B - 2	CAGATGCCTCGAGTACCCTC	59.33
3212F - 3	GGGGACAGCTTGAAAATGTGA	58.76
3212B - 3	GCTTCTTCTTGATGACGCCA	58.56
3212F - 4	TCGAAGATGCAACGGATTCA	57.62
3212B - 4	GACGCCATTCATGAGATCCC	58.48
3400F - 1	GACCCCTCGCCGTGTATTAT	59.32
3400B - 1	GGTGCTTTGCCATTGATCGA	59.19
3400F - 2	CTCCTCCTCCGATCCATCAC	59.03
3400B - 2	GATGTCGGTTGCAGCAAGAG	59.55
3400F - 3	ATGCCCTTCTTCTCCTCCTC	58.49
3400B - 3	GGAGGCTGCAGTAATGGTGA	59.75
3400F - 4	CCCTTCTTCTCCTCCTCCTC	58.21
3400B - 4	CAAGAGGGAGGCTGCAGTA	58.71
3473F - 1	GACCCCTCGCCGTGTATTAT	59.32
3473B - 1	GGTGCTTTGCCATTGATCGA	59.19
3473F - 2	CTCCTCCTCCGATCCATCAC	59.03
3473B - 2	GATGTCGGTTGCAGCAAGAG	59.55

3473F - 3	ATGCCCTTCTTCTCCTCCTC	58.49
3473B - 3	GGAGGCTGCAGTAATGGTGA	59.75
3463F - 4	CCCTTCTTCTCCTCCTCCTC	58.21
3473B - 4	CAAGAGGGAGGCTGCAGTA	58.71

Table 3: The Alleles Found through Fragment Analysis. There were 11 primer pairs that were successful at amplifying *G. rotundifolia* DNA in a PCR reaction with an annealing temperature of 56ºC. The microsatellites they amplified were amplified again in 3 primer PCR where they had fluorescent tags added to them, using the templates of 3 *G. rotundifolia* indviduals. Fragment analysis was done with these PCR products. These are the alleles for each microsatellite found by the fragment analysis. The first column labels what microsatellite was looked at. The second column shows the number of alleles found for that microsatellite. And the third column shows the size of those alleles.

Microsatellite Name	**Number of Alleles**	**Size of the Alleles**
616	4	262.5; 258.9; 272.6; 266.4
1249	4	233; 238.9; 229.4; 243.6
1678	2	162.6, 165.6
2246	4	179.4; 178.4; 177.3; 181.8
3624	3	247.9; 249.9; 242.4

Table 4: Results of Extraction of DNA from Individuals 01, G15, and 27. DNA from *G. rotudifolia* individuals 01, G15, and 27 was extracted for the purpose of use in fragment analysis. The first column gives the label for the individual, the second column is the concentration of the DNA after extraction, the third column is the concentration of the DNA after slight dilution, the fourth column gives the A260/A280 ratio, and the fifth column gives the A260/A230 ratio. The DNA extracted had good concentrations, and the A260/A280 ratios show us that the DNA is pure.

Individual	Initial Concentration	Final Concentration	A260/A280	A260/A230
01	30.3 ng/uL	25 ng/uL	1.82	1.198
G15	25.95 ng/uL	25.95 ng/uL	1.808	0.886
27	31.4 ng/uL	25 ng/uL	1.794	1.192

Table 5: Results of DNA Extraction and Concentration. DNA from 12 *G. rotundifolia* individuals was extracted. The initial concentrations of the DNA after extraction, the initial A260/A280 ratios after extraction, and the initial A260/A230 ratios are given in columns 2, 4, and 6 respectively. The Zymo DNA Clean and Concentrator kit was used to attempt to concentrate and purify the DNA. The concentrations, A260/A280, and A260/A230s ratio after this step are given in columns 3, 5, and 7 respectively. Half of the individuals' DNA was more concentrated after the concentration step, while the other half of the individuals had their DNA become less concentrated than it was after initial extraction. After the concentration step, the A260/A280 ratios and the A260/A230 ratios became very weird so unfortunately this DNA can't be used.

Individual	Initial Concentration	Final Concentratiion	Initial A260/A280	Final A260/A280	Initial A260/A230	Final A260/A230
G2	11.350 ng/uL	9.7500 ng/uL	1.733	-9.750	0.257	-3.482

G3	13.550 ng/uL	18.350 ng/uL	1.576	4.318	0.577	-22.94
G4	8.450 ng/uL	3.5000 ng/uL	1.565	-0.986	0.327	-2.414
G6	17.100 ng/uL	11.800 ng/uL	1.660	33.71	0.637	-16.86
G7	13.400 ng/uL	10.300 ng/uL	1.707	7.923	0.276	-12.12
G8	16.100 ng/uL	14.350 ng/uL	1.548	7.553	0.363	10.63
G9	15.350 ng/uL	13.200 ng/uL	1.872	11.00	0.337	-15.53
G10	15.400 ng/uL	18.950 ng/uL	1.913	4.797	0.306	-4.407
G11	8.500 ng/uL	14.050 ng/uL	1.910	1,951	0.230	-3.230
G12	13.35o ng/uL	14.450 ng/uL	1.816	7.811	0.367	-72.25
G13	13.350 ng.uL	16.900 ng/uL	1.792	7.511	0.377	7.191
G16	12.250 ng/uL	17.200 ng/uL	1.667	4.914	0.376	-5.831

References

Alberta Parks. (2023, June 9). *Wagner Natural Area Information and Facilities* [Government Service]. Alberta Parks. https://www.albertaparks.ca/parks/central/wagner-na/

Amy W. Ando. (2022). Equity and Cost-Effectiveness in Valuation and Action Planning to Preserve Biodiversity. *Environmental and Resource Economics*, *83*, 999–1015. https://doi-org.ezproxy.macewan.ca/10.1007/s10640-022-00674-1

Anders Forsman. (2023). Effects of genotypic and phenotypic variation on establishment are

important for conservation, invasion, and infection biology. *Biological Sciences*, *111*(1), 302–307. https://doi-org.ezproxy.macewan.ca/10.1073/pnas.1317745111

Anna Fleck. (2023). *40 Percent of Assessed Plants Are on the Red List* (Energy and Environment) [Graph]. Statista. https://www.statista.com/chart/30045/number-of-species-assessed-on-red-list/#:~:text=40%20percent%20of%20assessed%20plant,are%20on%20the%20red%20list.

Applied Biosystems™ Hi-Di™ Formamide. (n.d.). [Online Store]. Fisher Scientific. Retrieved April 23, 2025, from https://www.fishersci.ca/shop/products/hi-di-formamide-5/4440753

Azhar, M., Nawaz, M., Shahid, M., Dogan, Y., Comertpay, G., Yildiz, M., Hatipoglu, R., Ahmad,

F., Asaleh, A., Labhane, N., Ozkan, H., Gyuhwa, C., & Baloch, F. (2017). DNA molecular markers in plant breeding: Current status and recent advancements in genomic selection and genome editing. *Biotechnology & Biotechnological Equipment*, *32*(2), 261–285.

https://doi.org/10.1080/13102818.2017.1400401

Bin-Bin Du, Ya Zhan, Jiao-Kun Li, Ying-Zhuo Chen, & Lu-Lu Tang. (2018). Development and

characterization of microsatellite loci for the endemic Thalictrum smithii (Ranunculaceae). *Applications in Plant Sciences*, *6*(8). https://doi-org.ezproxy.macewan.ca/10.1002/aps3.1176

Coates, D., McArthur, S., & Byrne, M. (2015). Significant genetic diversity loss following pathogen driven population extinction in the rare endemic Banksia brownii (Proteaceae). *Biological Conservation*, *192*, 353–360. https://doi.org/10.1016/j.biocon.2015.10.013

David H. Reed & Richard Frankham. (2003). Correlation between Fitness and Genetic Diversity. *Conservation Biology*, *17*(1), 230–237. https://doi.org/10.1046/j.1523-1739.2003.01236.x

Deborah Schäfer, Hugo Vincent, Markus Fischer, & Anne Kempel. (2020). The importance of

genetic diversity for the translocation of eight threatened plant species into the wild. *Global Ecology and Conservation*, *24*. https://doi.org/10.1016/j.gecco.2020.e01240

Diaz, S., Settele, J., Brondizio, E., Agard, J., Ngo, H., Arneth, A., Patricia, B., Brauman, K., Butchart, S., Chan, K., Garibaldi, L., Kazuhito, I., Liu, J., Subramanian, S., Midgley, G., Miloslavich, P., Molnar, Z., Obura, D., Pfaff, A., … Zayas, C. (2019). Pervasive human-driven decline of life on Earth points to the need for transformative change. *Science*, *366*(6471). https://doi.org/10.1126/science.aax3100

Gema Lucena-Aguilar, Ana María Sánchez-López, Cristina Barberán-Aceituno, José Antonio

Carrillo-Ávila, José Antonio López-Guerrero, & Rocío Aguilar-Quesada. (2016). DNA Source Selection for Downstream Applications Based on DNA Quality Indicators Analysis. *Biopreservation and Biobanking*, *14*(4), 264–279.

https://doi.org/10.1089/bio.2015.0064

Islam, S., Mangral, Z., Lubna, T., Bhat, B., Tantray, W., Ahmad, R., Khuroo, A., & Dar, T. (2023).

Conservation genetics of endangered Trillium govanianum Wall. Ex D. Don – A pharmaceutically prized medicinal plant from the Himalaya and implications for species recovery. *Gene*, *888*. https://doi.org/10.1016/j.gene.2023.147748

Jacquemyn, H., Roldan-Ruiz, I., & Honnay, O. (2010). Evidence for demographic bottlenecks

and limited gene flow leading to low genetic diversity in a rare thistle. *Conservation Genetics*, *9*, 1979–1987. https://doi-org.ezproxy.macewan.ca/10.1007/s10592-010-0089-5

Lina Lim. (2022). *Developing Microsatellite Markers for Cypripedium passerinum (Sparrow's*

Egg Lady's Slipper) (Biological Sciences - Student Works) [Undergraduate Thesis, MacEwan University]. Research Online at Macewan. https://hdl.handle.net/20.500.14078/2711

Marina Nonić & Mirjana Šijačić-Nikolić. (2015). Genetic Diversity: Sources, Threats, and Conservation. In *Life on Land* (pp. 1–15). Springer Cham. https://link.springer.com/referenceworkentry/10.1007/978-3-319-71065-5_53-1

Moreno Di Marco, Tom D. Harwood, Andrew J. Hoskins, Chris Ware, Samantha L. L. Hill, & Simon Ferrier. (2019). Projecting impacts of global climate and land-use scenarios on plant biodiversity using compositional-turnover modelling. *Global Change Biology*, *25*(8), 2763–2778. https://doi.org/10.1111/gcb.14663

North American Orchid Conservation Center. (n.d.). *Galearis rotundifolia* [Non-Governmental Organization]. North American Orchid Conservation Center. Retrieved April 21, 2024, from https://goorchids.northamericanorchidcenter.org/species/galearis/rotundifolia/

Peterson W. Wambugu, Desterio O. Nyamongo, & Desterio O. Nyamongo. (2023). Role of Seed Banks in Supporting Ecosystem and Biodiversity Conservation and Restoration. *Diversity*, *15*(8). https://doi.org/10.3390/d15080896

Raven, P., Ouyang, Z., Smih, P., & Wackernagel, M. (2023). The Shenzhen Congress and plant conservation: What have we accomplished in the 6 years since? *Journal of Systematics and Evolution*, *61*(6), 949–956. https://doi-org.ezproxy.macewan.ca/10.1111/jse.13030

Sponsel, L. (2024). Human Impact on Biodiversity, Overview. *Encyclopedia of Biodiversity*, *3*, 430–448. https://doi.org/10.1016/B978-0-12-822562-2.00010-4

Szczecińska, M., Sramko, G., Woloz, K., & Sawicki, J. (2016). Genetic Diversity and Population Structure of the Rare and Endangered Plant Species Pulsatilla patens (L.) Mill in East Central Europe. PLoS ONE, 11(3). https://doi.org/10.1371/journal.pone.0151730

TN 130 Purity Ratios Explained. (2019, March 9). [Company Website]. DeNovix. https://www.denovix.com/tn-130-purity-ratios-explained/#:~:text=Generally%20acceptable%20260%2F230%20ratios,contamination%20with%20the%20aforementioned%20compounds

United Nations Environment Programme. (2023, September 5). Five drivers of the nature crisis. United Nations Environment Programme. https://www.unep.org/news-and-stories/story/five-drivers-nature-crisi

Vinceti, B., Loo, J., Gaisberget, H., Zonneveld, M., Schueler, S., Konrad, H., Kadu, C., & Geburek, T. (2013). Conservation Priorities for Prunus africana Defined with the Aid of Spatial Analysis of Genetic Data and Climatic Variables. PLoS ONE, 8(3). https://doi.org/10.1371/journal.pone.0059987

Wagner Natural Area. (2024). *About Us* [Non Governmental Organization]. Wagner Natural Area. https://wagnerfen.ca/about-us/

Wagner Natural Area on the map, Spruce Grove, Canada. (n.d.). [Educational]. 2 Markers. Retrieved April 23, 2025, from https://vg.2markers.com/577530

Wang, R., Xia, Y., Shen, Z., Wang, Y., Zhou, X., Xian, M., & Yang, Y. (2023). Genetic diversity analysis and potential suitable habitat of Chuanminshen violaceum for climate change. *Ecological Informatics*, *77*. https://doi-org.ezproxy.macewan.ca/10.1016/j.ecoinf.2023.102209

Yinon M. Bar-On, Rob Phillips, & Ron Milo. (2018). The biomass distribution on Earth. *Proceedings of the National Academy of Sciences*, *115*(25), 6506–6511. https://doi.org/10.1073/pnas.1711842115