

Plant Barcoding

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Abstract

Scientists all over the world are sequencing plant DNA for criminal, medical, and general knowledge. The goal is to obtain a leaf of a plant and, in the end, receive the scientific name. With the scientific name, further research can be conducted to find the sought-after information. With a public database of all of these sequences and correlating information, scientists can gather data more efficiently and people can help understand biodiversity (De Vere et al., 2015). Sequencing plant DNA includes laboratory techniques such as DNA extraction, Polymerase Chain Reaction (PCR), gel electrophoresis, and Sanger Sequencing. Research in our laboratory proved to have a difficult time with primers and DNA degradation, however, I was able to obtain the correct sequence for six plants found on Oklahoma State University's campus: *Chionanthus retusus* (from two separate plants), *Pseudosasa japonica*, *Dichondra occidentalis*, *Jasminum nudiflorum*, and *Spiraea thunbergia*.

Introduction

The term Plant Barcoding refers to the idea that each plant has a unique sequence, like a barcode, that can identify the plant. To get that 'barcode,' scientists use primers that search for a specific sequence in the plant's genome. Each primer set searches for a specific sequence, and often are utilizing different identification points in the plant's genome (*Primer*, n.d.). The primer is mixed with the plant DNA and other reagents during Polymerase Chain Reaction (PCR) to denature the DNA into single strands, anneal the primers, and then extend by amplifying the DNA. This allows the isolation of the target sequence for identification. Gel electrophoresis is then used to visualize the band of DNA to confirm the extraction and PCR worked. In the gel, the











fragments of DNA are separated based on size, meaning, the smaller fragments can move through the gel faster and migrate closer to the bottom of the gel. The bottom of the gel is referring to the more positive side because DNA is negatively charged, and positive attracts negative. For this research, only one band in each well with the PCR sample of DNA is desired. This band is the target sequence that can be used to identify the plant. That sample is then sent to another laboratory that uses Sanger sequencing. The laboratory then sends the sequence back, and the plant can be identified by copying the sequence into BLAST. BLAST is an online tool that 'plugs' the information in to give a genus and species of an organism which correlates to the sequence sent.





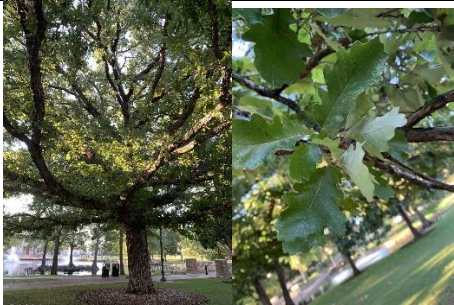



The primers used in this research were Plntits2, PlntrnL, matK, and rbcL. In the beginning, only Plntits2 and PlntrnL were utilized, until later in the study matK and rbcL were brought in. Plntits2, referred to as S, in general, looks at the internal transcribed spacer (ITS) region of the DNA. The ITS region is a specific part of the ribosome (Moorhouse-Gann et al., 2018). Its average melting temperature is 52.0° C. PlntrnL, referred to as R, examines the whole chloroplast of a plant (Taberlet et al., 2006). Its average melting temperature is 54.9° C. MatK, referred to as K, looks at proteins that act as group II intron maturases (Selvaraj et al., 2008). Its average melting temperature is 39.0° C. RbcL, referred to as L, examines RuBisCo (Tran et al., 2021). Its average melting temperature is 49.0° C.

The plants obtained in this study are all found on Oklahoma State University's campus. Images of the plants are found in table 2. The coordinates for each plant location can be found in table 1. After collecting the plant samples, DNA from the samples was extracted and used to obtain a specific sequence, targeted by the primers.

Plant	Location	Plant	Location
1	36.13174° N, 97.07943° W	12	36.11985° N, 97.07121° W
2	36.12496° N, 97.07049° W	13	36.12010° N, 97.07146° W
3	36.12496° N, 97.07049° W	14	36.12080° N, 97.07186° W
4	36.11876° N, 97.06662° W	15	36.12622° N, 97.06902° W
5	36.11912° N, 97.06706° W	16	36.12608° N, 97.07050° W
6	36.11993° N, 97.07021° W	17	36.12619° N, 97.07200° W
7	36.12019° N, 97.07036° W	18	36.12619° N, 97.07200° W
8	36.12059° N, 97.07108° W	19	36.12619° N, 97.07200° W
9	36.12056° N, 97.07085° W	20	36.12589° N, 97.07021° W
10	36.12019° N, 97.07111° W	21	36.12570° N, 97.07041° W
11	36.11981° N, 97.07108° W		

Table 1: Plant samples with their corresponding coordinates. All plants located on Oklahoma State University's campus. Plants 1-14 were collected in July 2022. Plants 15-21 were collected in April 2023.

Plant	Image of sample from campus	Plant	Image of sample from campus
1		12	
2		13	
3		14	
4		15	
5		16	

6		17	
7		18	
8		19	
9		20	




10		21	
11			

Table 2: Images of the plants found on campus taken on iPhone. Some plants have 2 corresponding images, because the details of the leaf could not be seen in the image of entire plant.

Methods

There are preparations necessary to begin the experiments. 18 plant samples were acquired from the University's campus and 3 plants samples from Dr. DeSilva's office on campus were used. The order in which the experiment was conducted will follow the instructions for each technique used.

First, the DNA from the plant samples needed to be extracted. To extract DNA, a small portion of the plant sample, roughly 1 cm, was ground to a fine powder with liquid nitrogen in 2 mL Eppendorf tubes. Next, 500 uL of DNA extraction buffer and 500 uL of phenol:chloroform:isoamyl alcohol (25:24:1) were added and mixed well. These samples were then centrifuged for 15 minutes at 12,000 rpm (rotations per minute). After centrifugation, the upper phase was removed and added to a different set of tubes, and the old tubes were discarded in the fume hood. There is an optional step to add chloroform, centrifuge, and keep only the

upper phase again, but it was not performed in our lab. The next step included adding 50 uL of 3M sodium acetate and 500 uL of cold isopropanol and mixing gently. The samples were then centrifuged at 8,000 rpm for 5 minutes, then cooled in the freezer for 15 minutes. After those 15 minutes, I poured off the supernatant and washed the pellet with 500 uL of cold 70% ethanol. Next, I poured off the wash and allowed the pellet to dry on the counter for hours. Later that day, I added 50 uL of molecular grade water (MGW) and let it sit on the bench overnight. The next morning the samples were good to keep in the freezer until needed.

Further preparations include making a working stock for the primers. I began with Plntits2 (S) and PlntrnL(R) primers, and made a working stock for both. To make a working stock of Plntits2 at a concentration of 24 mM, I added 25 uL of the forward primer (Plntits2F) and 25 uL of the reverse primer (Plntits2R) with 150 uL of MGW. These steps were repeated for the PlntrnL primer. The working stock is used in PCR. It is important to know the melting temperature for each set of primers, as that will also be utilized in PCR. Later in the experiment, I had to make working stock for primers matK (K) and rbcL (L) using the preceding steps.

Another preparation included quantifying the DNA. To quantify, I had to take my DNA extraction samples to the Core Facility to use the NanoDrop. First, the NanoDrop has to be cleaned with water to ensure there is no contamination. Then, a blank test is conducted to confirm proper calibration. For each sample, a 1.5 uL drop is placed on the sensor of the NanoDrop and recorded. Between each sample, the sensor needs to be cleaned with water. An example of a few results from quantifying can be found in figure 1. The 260/280 absorbance number is the only value to take note of. A ratio of approximately 1.8-1.9 is ideal for this value, because any less of a ratio indicates protein contamination, and any greater of a ratio indicates RNA contamination.

Sample ID	User	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos	Cursor Abs	240
4	desiva2	10/20/2022	12:38 PM	1609.76	32.195	25.076	1.28	0.67	50.00	230	37.007	11.294
5	desiva2	10/20/2022	12:40 PM	1733.65	34.673	20.331	1.71	1.87	50.00	230	18.579	4.195
6	desiva2	10/20/2022	12:44 PM	2996.23	69.965	31.658	1.89	2.17	50.00	230	27.597	4.639
7	desiva2	10/20/2022	12:46 PM	1538.02	30.760	17.427	1.77	2.04	50.00	230	15.060	9.039
8	desiva2	10/20/2022	12:47 PM	1475.17	29.503	17.446	1.69	1.84	50.00	230	16.053	2.664
9	desiva2	10/20/2022	12:48 PM	1347.98	26.960	16.254	1.66	2.00	50.00	230	13.492	1.993
10	desiva2	10/20/2022	12:50 PM	1503.59	30.072	17.756	1.69	1.92	50.00	230	15.638	3.578
11	desiva2	10/20/2022	12:52 PM	1916.32	38.326	21.600	1.77	2.01	50.00	230	19.110	3.912
11	desiva2	10/20/2022	12:53 PM	2018.95	40.377	22.122	1.83	1.77	50.00	230	22.807	4.368
13	desiva2	10/20/2022	12:54 PM	1698.16	33.963	19.305	1.76	2.17	50.00	230	15.687	3.169
14	desiva2	10/20/2022	12:56 PM	1288.88	25.778	15.043	1.71	2.22	50.00	230	11.638	3.062

Figure 1: Results of quantifying the extracted plant DNA. Plants 4-14 have their results displayed; plant 12 was mistakenly labeled as '11' but the label was not corrected in this image.

Extracting the DNA, making a working stock of the primers, and quantifying the DNA only has to happen in the beginning of the experiments and if new samples need to be obtained.

Polymerase Chain Reaction (PCR) is the next step in determining what plant was obtained. The first step in PCR is making the master mix. The amount of each solution that goes into the master mix depends on how much is needed to make, and in this instance, I will explain the amount of master mix for 1 sample. This master mix consists of 2.5 uL of 10x buffer, 0.5 uL of MgCl₂, 0.5 uL of dNTPs, 0.25 uL of Taq, 0.5 uL of the working stock of primers, and 18.75 uL of molecular grade water, for a total of 23 uL. PCR tubes were labeled with the primer letter (S, R, K, or L) and the plant number (1-21). There were also negative control tubes, labeled with primer letter and negative (-) sign. 23 uL of master mix was added to a newly labeled PCR tube (or set of tubes), along with 2 uL of DNA or MGW. The 2 uL of DNA was added to the tubes with plant numbers. The DNA of the plant matched the plant number (plant 1's DNA was added

to the PCR tube labeled with the number 1, etc). The 2uL of MGW was added to PCR tubes that did not include DNA, and had the negative sign. Once the PCR tubes were labeled and contained the 25 uL of total solution, they were added to the PCR machine, or the thermal cycler. The thermal cycler could be programmed so the same file could be used each time. This is where the melting temperature of the primers comes in. As explained previously, the PCR machine is set up to anneal, denature, and extend. The annealing temperature for these experiments was 92° C, the denaturing temperature corresponded to the primer melting temperature, and the extending temperature was 72° C. Each run lasted roughly an hour and a half, and then the samples were placed in the freezer until I ran a gel in gel electrophoresis.

Gel electrophoresis allows for visualization of the DNA band that should have been produced from the previous techniques. First, the gel needs to be made, then heated to be dissolved, cooled so it does not melt the plastic mold, and then solidified in the mold. To make a 1.5% agarose gel, add 1.5 g of agarose and 98.5 mL of buffer for a total of 100 mL. Mix by swirling and microwave in 45 second intervals until the solution is clear and there are no visible solutes. Allow to cool on a stir plate until the flask can be handled with bare hands. Once cooled enough, the solution can be poured into the assembled mold. The mold consists of the unit, tray, and comb with the appropriate number of teeth. Allow gel to solidify. I could now add my PCR samples into the wells of the gel. To do so, I added drops of dye onto parafilm, and mixed a sample from the PCR with each dot of dye. When the sample was mixed, I loaded that into the corresponding well. The amount of PCR and dye did change throughout the experiment, but I never added more than 15 uL into each well, and the amount of dye was half of the amount of sample. When all wells were loaded, the unit was covered with the lid and the leads were plugged in. The machine ran at approximately 80 volts until the samples were visibly roughly $\frac{3}{4}$

of the way down the gel. After the gel ran, it was removed to be visualized. There is a UV light machine in the laboratory, but the imager itself was located in the Core Facility.

If the gel looked good, meaning the appropriate wells had one solid band, it could be prepared for sequencing. If the gel had any streaking, multiple bands, no bands, or bands in the inappropriate lane, then the process would have to start over.

The next step would be to prepare to send a sample to be sequenced. The sample can be made from the gel or the PCR sample. To send from the gel, the band would be cut from the rest of the gel, and I would follow the gel extraction protocol. This process is recommended if there is not enough PCR sample to take from. To prepare from the PCR sample, add ExoSAP-It and put through the thermal cycler. Both routes also need to be quantified and diluted to 15 ng/uL. The samples for sequencing would then be placed in a refrigerator in the Core Facility, and would later be picked up by those who sequence it.

When completed, the sequence results were sent to me via email, and I could look at the sequence via the UGENE application. An example of the sequence results on UGENE can be found in figure 2. To get the results, I would copy the entire sequence, put in in FASTA format so it was easier to copy and paste, delete the first 55 base pairs due to primer binding, and delete any base pairs found after the reverse complement primer sequence (usually less than 5 base pairs). This sequence could then be copied and pasted into BLAST, a website that takes a sequence and reads it. I would filter it to only look at plant results. When looking at the results, the most important numbers are the query cover, percent identity, and E value. The query cover and percent identity should both be 100%, or as close as possible. Query coverage is the amount of my sequence that aligns with the sequence on BLAST, and percent identity is the amount of my sequence that aligns and matches the correct sequence. The expect (E) value should be 0,

because this describes the background noise. A value of 0 means the match is more significant. An example of BLAST results can be found in figure 3. Table 3 has the resulting values for each plant with a sequence result.

The PCR and gel electrophoresis steps were repeated frequently for this research. I obtained samples for plants 1-14 and performed multiple rounds for each sample. For plant 1, I began with PCR and gel, and when that gave no results, I conducted a gradient PCR. This utilizes different amounts of MgCl₂ and annealing temperatures. After finding the ideal amount of MgCl₂ and temperature, as stated in the PCR procedure, I conducted PCR and made a new gel again. I continued to get no results in the wells with DNA, but I did have bands in my negative control wells. I decided to make a new master mix and repeat, which yielded no results. I moved on to plants 2 and 3 and decided to revisit plant 1 later. After a round of PCR and gel, there were no results. I decided to check my DNA for those plants, so I added the DNA samples with dye into the gel, and there were no bands visible. This means I had no DNA left. I moved on to plants 4-7, and one round of PCR and gel produced good bands for plants 5 and 6, which we sent for sequencing. At this point, there was discussion of trying new primers on the plants that produced no results, so I moved on to plants 8-11. 2 rounds of PCR and gel did not produce any results. After the first round, I decided to conduct a touchdown PCR, which allows the annealing temperature to gradually decrease at each cycle to target non-specific binding. This still did not help, and again there were no results. I decided to wait for the new primers to tackle this set of plants again. After moving on to plants 12-14, one round still produced no results. I then rehydrated my primers and made new working stock and new master mix with the new primers that were delivered. The new primer sets (K and L) had very different annealing temperatures, therefore I conducted PCR separately unlike before. This means that instead of running a PCR

for (i.e.) plants 8-11 with both S and R primers (8R, 8S, 9R, 9S, etc), I now would run the primers separate (i.e.) 8-11K and 8-11L. The new set of primers allowed results for plant 13 with both primers and plant 14 with the new L primer.

I then went back to plants 1-7 and conducted PCR and gel with the new primers, and no results were produced. I was then wondering if I was the problem, so the graduate student, Blake, made his own samples with my DNA and his reagents. I loaded those samples in a gel along with a few of mine, and there were no results. The next assumption was that perhaps the DNA was now degraded. 8 months has passed since the collection of plants and extraction of their DNA, and as described in this procedure, the DNA was frequently thawed and refroze. I loaded DNA and dye into a gel, and the results produced faint, streaked bands in only a few lanes. This indicated that my DNA was no longer usable for these experiments.

Next, I obtained more samples, labeled as plants 15-21. I restarted the entire process, including extraction, quantifying, PCR, and gel electrophoresis. The first round produced results for plants 18 and 19 for both primers, K and L. Unfortunately, the semester was ending, and I had no time to continue this experiment.

Results

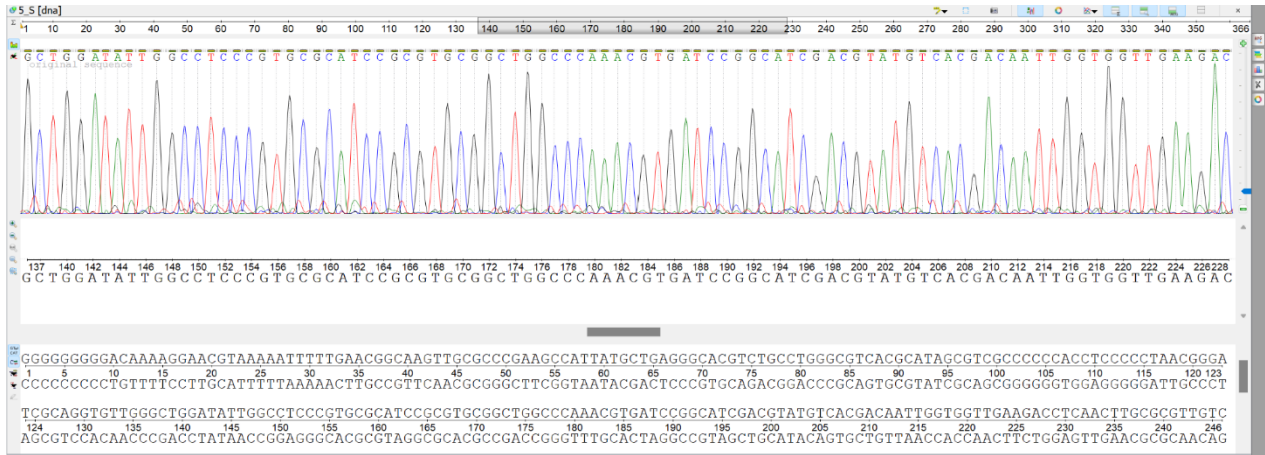


Figure 2: The sequence results from plant 5 and primer S can be seen in this image of a screenshot of the UGENE application. The colored spikes correspond to a specific nucleotide.

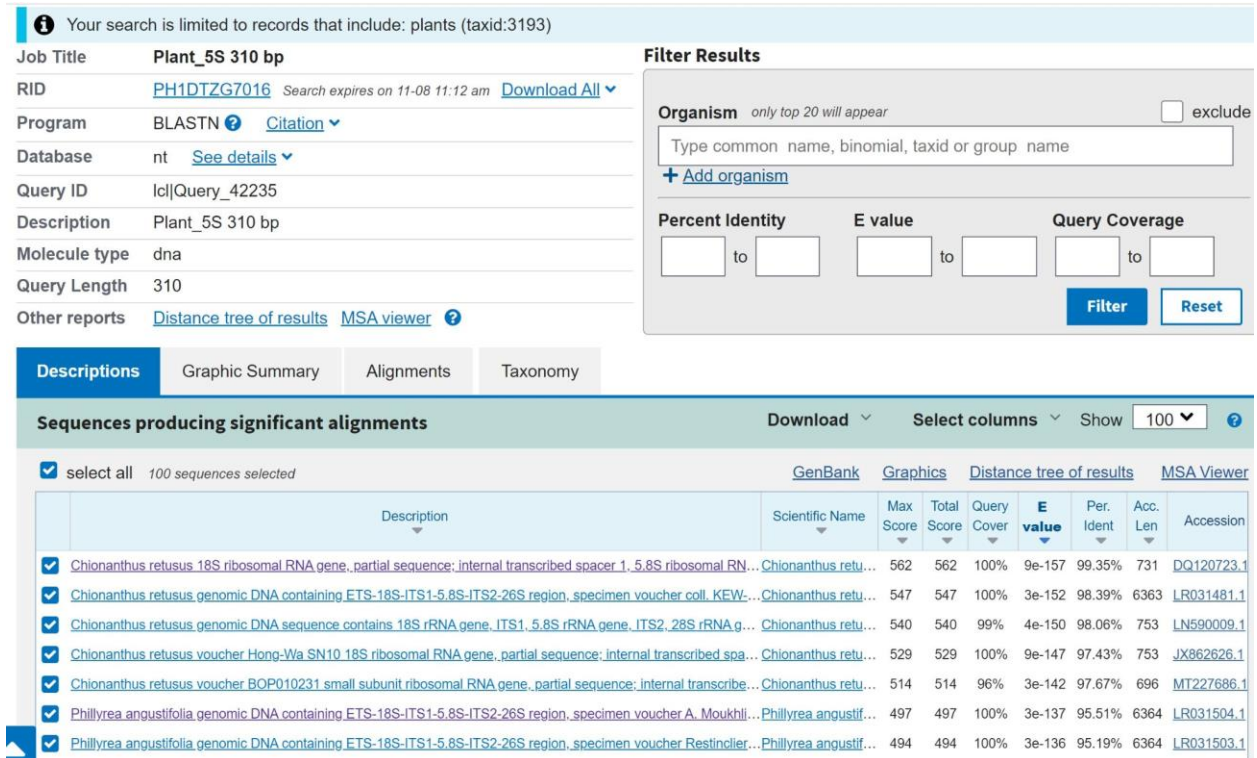










Figure 3: The BLAST results of plant 5 and primer S can be seen in this image. The highest query cover, percent identity, and lowest E value tells us the results of the unknown plant. Research is still conducted on the result to confirm the correct DNA was sent.

Plant	Result	Query Cover	Percent Identity	E value
1	n/a			
2	n/a			
3	n/a			
4	n/a			
5	<i>Chionanthus retusus</i>	100%	99.35%	9e-157
6	<i>Chionanthus retusus</i>	100%	100%	0.0
7	n/a			
8	n/a			
9	n/a			
10	n/a			
11	n/a			
12	n/a			
13	<i>Pseudosasa japonica</i>	100%	100%	0.0
14	<i>Dichondra occidentalis</i>	100%	99.43%	0.0
15	n/a			
16	n/a			
17	n/a			
18	<i>Jasminum nudiflorum</i>	100%	99.81%	0.0
19	<i>Spiraea thunbergii</i>	100%	100%	0.0
20	n/a			
21	n/a			

Table 3: This table displays the genus and species result with the corresponding plant number. Results marked 'n/a' indicated no result.

Positive Test Results Images

Plant	Image of sample from campus	Image of resulting plant
5		 <p data-bbox="776 663 1385 695"><i>(Chionanthus Retusus - Moon Nurseries, 2017)</i></p>
6		 <p data-bbox="776 974 1328 1045"><i>(Chionanthus Retusus Landscape Plants Oregon State University, n.d.)</i></p>
13		 <p data-bbox="776 1335 1157 1377"><i>(Pseudosasa Japonica, 2015)</i></p>
14		 <p data-bbox="776 1759 1271 1795"><i>(Lowe's Dichondra in 1-Pint Pot, n.d.)</i></p>

18		 <i>(Jasminum Nudiflorum North Carolina Extension Gardener Plant Toolbox, n.d.)</i>
19		 <i>(Spiraea Thunbergii "Ogon," n.d.)</i>

Table 4: This table displays the plants with results. Column one states the plant number, column 2 exhibits the image of the plant taken on campus with an iPhone, and column 3 exhibits the image of the plant results from the source credited.

Discussion

Plant 5 and plant 6 (table 4) came back with the same genus and species result - *Chionanthus retusus*. When I collected this plant, the leaves and tree looked different enough that I assumed they were different plants. This plant is known as the Chinese Fringe Tree, and was selected by Oklahoma State University in its project called "Selecting Deciduous Trees for Oklahoma" (*Chionanthus Retusus - Plant Finder, n.d. ; Selecting Deciduous Trees for Oklahoma - Oklahoma State University, 2018*). Plant 13 (table 4) is a *Pseudosasa japonica*, better known as Arrow Bamboo. This is an evergreen commonly used as hedges and screens (*Pseudosasa Japonica - Plant Finder, n.d.*). Plant 14 (table 4) is *Dichondra occidentalis*, also known as Western Ponysfoot. *Dichondra argentea*, the same genus but different species to our plant 14, is commonly used in gardening, known as Silver Falls (*Lady Bird Johnson Wildflower Center, n.d.*). It was interesting to know that the more common plant in this family was not used,

instead a different species that looks very similar was used on campus. Plant 18 and plant 19 (table 4) came back with switched results. When looking into the *Spiraea thunbergia* plant that BLAST sequenced as plant 18, it was clear that this was incorrect. I looked at the results for plant 19, and it became clear that these samples were at some point switched, but were very clearly correct for the opposite sequence. These could have been switched in the beginning when extracting DNA, when making the PCR, when preparing for sequencing, or even at the sequencing location. Plant 18 is in fact *Jasminum nudiflorum*, which is also known as Winter Jasmine, and is commonly used as a ground cover (*Jasminum Nudiflorum* - *Plant Finder*, n.d.). Plant 19, *Spiraea thunbergia*, is referred to as Spirea, and is interestingly susceptible to many diseases that the rose family is susceptible to (*Spiraea Thunbergii* - *Plant Finder*, n.d.).

There is no obvious answer as to why there were so many poor results during this research. There are a multitude of possibilities, including but not limited to, unfavorable PCR recipe, bad DNA, incorrect PCR temperatures, contaminated reagents, primer design, and the specific plants used. I believe that the first two primers were not ideal for the types of plants being collected, and by the time I attempted new primers, the DNA had gone bad. When I finally used the new primers and new plants, I did not have enough time to optimize the PCR or obtain new reagents.

The result rate is disappointing, however, there is room for improvement should this research be conducted further. First, I believe more time with newer plant DNA and the newer primers would produce more results. If I were to work on this further, I would collect all of the plants that had no results, plants 1-4, 7-12, 15-17, and 20-21, and attempt them again with the new primers. It would probably be in the best interest to conduct a new gradient PCR to optimize

the annealing temperature for the new primers. Further, I believe research into the type of plant to look for when collecting could also be beneficial.

Appendices

Chionanthus retusus / *Landscape Plants* / *Oregon State University*. (n.d.).

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Chionanthus retusus - *Plant Finder*. (n.d.).

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3

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2

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