DNA Barcoding: Differentiation Among Three Species of Polytrichopsida

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Abstract

This investigation attempts to differentiate among three species of Polytrichopsida, namely *Polytrichum formosum*, *Polytrichum strictum* and *Polytrichum longisetum,* in terms of their genetic sequences. This applies the molecular tool of DNA barcoding which identifies species using a specific gene in the organism's DNA. The gene of focus in this experiment is the chloroplast gene *rbcl,* or the ribulose-bisphosphate carboxylase gene, and is known for its good discriminating capacity. We collected four populations each of *formosum* and *strictum* and one population of *longisetum* and extracted their DNA for polymerase chain reaction (PCR) and sequencing. *P. formosum* and *P. strictum* were successfully differentiated from each other according to the differences found in their DNA sequences; however, *P. longisetum* was found to be very closely related to *P. formosum.* It was also determined that all three species of Polytrichum diverged from the same common ancestor.

Introduction

Bryophyta is a phylum that represents the earliest extant lineages of land plants and consists of a number of classes, including Polytrichopsida, and comprises of a diverse number of species (Liu, Yan, Cao & Ge, 2010). To this day, there have been 10,000 species known and classified. as well as approximately 700 genera and between 110 to 120 families. Bryophyta is considered to be the third most diverse group of land plants after angiosperms and ferns (De Luna, Newton & Mishler, 2003). They play a crucial role in many ecosystems ranging from the tundra to the tropical rain forest and are responsible for capturing water and nutrients, providing shelter for microfauna and aid in seedling succession and regeneration (De Luna, 2003). Bryophytes are an important historical group of plants that allows understanding of the transition of terrestrial life. There have been many taxonomic, ecological and evolutionary studies conducted in this field, yet there are still many ambiguities regarding some mosses and liverworts (Liu et al, 2010). Hence, the focus of this research investigates the differentiation among species of Polytrichopsida to gain a better understanding of their classification.

The class Polytrichopsida contains vast number of species with diverse morphological characteristics. Members of this class may vary in height, ranging from very short, such as *Pogonatum pensilvanicum,* to very tall, as seen in *Dawsonia superba,* which can reach a height of 50cm (Ellis, 2000). Species of this class are acrocarpous and are widely distributed, especially in habitats that are acidic with nutrient-poor soils (Ellis, 2000). The gametophyte of these mosses have a well developed central stand consisting of hydroids, which are water conducting cells, and leptoids, which are sugar conducting cells. Every leaf contains a midrib and unique vertical photosynthetic lamellae which are only found in species of Polytrichopsida. Also, since this species usually resides on exposed substrate, leaf tips may be coloured or possess hairpoints to prevent UV damage. The sporangium is also characterized by a hairy calyptra which aids in desiccation prevention. Nematodontous peristome teeth, which exhibit evenly thickened walls that are comprised of whole dead cells, are present on the sporangium (Glime, 2007). These teeth are attached to an epiphgram, which develops from the columella, and cause the peristome teeth of these mosses to display less movement than hygroscopic teeth (Ellis, 2000).

Polytrichum, a genus of mosses in the class Polytrichopsida, is known to cover a wide geographical region. There are approximately 70 species known belonging to the genus *Polytrichum,* and all species exhibit distinguished morphological characteristics and habitat conditions. For example, some species have dark, sharply-shaped leaves

while others have light, bluntly-shaped leaves, and some species prefer fairly moist habitats while others prefer drier habitats. This was the primary reason for conducting this study: To explain these distinguished features and how they are related to each other in the class Polytrichopsida.

Three species of *Polytrichum* will be investigated, namely *Polytrichum formosum*, *Polytrichum strictum* and *Polytrichum longisetum.* Differentiation among the species will be analyzed by applying the method of DNA barcoding. DNA barcoding is a taxonomic method used in a variety of fields ranging from the food industry to forensic sciences (Liu et al, 2010). It is heavily reliant on variable, short standardized regions of an unknown organism's mitochondrial or chloroplast genome, enabling more accurate species identification than morphology-based identification systems. which are inherent with limitations. A DNA barcode is similar to a UPC code in that each species has a specific DNA sequence pattern which is unique to that group (Correri, 2012). This technique uses polymerase chain reaction (PCR) to amplify a specific region of a gene. In plants, the chloroplast gene region that is most commonly used in barcoding is Rubisco large subunit, or *rbcL*, which is an important catalyst in the carbon fixation process. Its frequent use is due to the fact that *rbcL* is easily amplified in PCR and shows high genetic variability between species, which are two important characteristics that make a barcode ideal (Liu et al, 2010). It is also known to be a very successful coding region in its ability to accurately discriminate species (Liu et al, 2010). Hence, we will attempt to apply DNA barcoding using the rbcL primer to investigate the DNA differences among our chosen three species of *Polytrichum*.

1 Materials and Methods

1.1 Sampling site and collection

Four populations each of *Polytrichum strictum* and *Polytrichum formosum* were collected from Camosun Bog, and one population of *Polytrichum longisetum* was collected from Trout Lake by Steve Joya. Each site in Camosun Bog was very moist, fairly shady and consisted of numerous species of *Sphagnum* growing within and around *Polytrichum.* All samples were placed into plastic bags containing silica gel, which acted as a desiccant to absorb and hold water vapor from the collected samples.

1.2 Barcoding Region

The barcoding region *rbcL* was chosen for this study. The primers used to amplify the region were *rbcL*-a_f and *rbcL*-a_r, where f stands for forward primer and r stands for reverse primer. The forward primer contained the sequence ATGTCACCACAAACAGAGACTAAAGC (Kress & Erickson, 2007), whereas the reverse primer contained the sequence CTTCTGCTACAAATAAGAATC (Kress & Erickson, 2007).

1.3 Extraction

Pestles were used to grind up leaves from each sample in sterilized mortars, and a small amount of sand was added to each mortar. Each sample was placed into the corresponding labelled tubes (1S, 1F, 3S, 3F, 4S, 4F, 5S, 5F, A and L) for a total of 10 reactions. The numbers of the labels represented the population site where they were found, with S representing *Polytrichum strictum*, F representing *Polytrichum formosum*, A representing *Atrichum* and L representing *Polytrichum longisetum*.The first two steps were based on the CTAB Extraction (Whitton, 2006), where a 50 ml total volume of CTAB Buffer (2X) was made using 33 ml of water, 5 ml of 1M tris (pH 8.0), 2 ml of 0.25 EDTA (pH 8.0), 4.09 g of NaCl and 2 ml of 10% CTAB. The solution was then aliquoted into ten 50 ml tubes. 700 ul of buffer and 1.4 ul of beta-mercaptoethanol were added to each tube in the fume hood. The remaining procedures followed the Solitis Lab CTAB DNA Extraction Protocol (2002).

The samples were then incubated at 65 degrees C for an hour and 30 min. 500 ul of 24:1 chloroform to

isoamyl alcohol was added, and the tubes were centrifuged for 15 min at maximum speed. The aqueous phase was then pipetted off and placed into newly labelled eppendorf tubes: 1S', 1F', 3S' 3F', 4S', 4F', 5S' 5F', A' and L'. 44 ul of 7.5 M ammonium acetate and 297 ul of isopropanol were added to the first four tubes, whereas 48 ul of ammonium acetate and 324 ul of isopropanol were added to the last 6 tubes. The tubes were mixed and placed into the freezer for 15 minutes. Next, the tubes were centrifuged for 3 minutes at the highest speed, and the liquid was pipetted out of the tubes. 700 ul of cold 70% ethanol was added to each tube where they were then mixed and placed into the centrifuge for 1 minute. The liquid was removed once again, and the process was repeated using 95% ethanol. These procedures were conducted so that ethanol could induce a structural change in DNA molecules to cause them to aggregate and precipitate out of the solution, thus "cleaning" the DNA samples from any proteins, small nucleic acids and salts needed to be removed with the supernatant after centrifugation (Davidson, 2012). The tubes were placed upside down on paper towel to allow the DNA pellets to dry. 30 ul of water was used to resuspend the samples overnight.

1.4 PCR, Gel and PCR Cleanup

To make a 100 uM stock of each primer, 27.5 nmoles of forward primer was diluted with 275 ul of water and 29.5 nmoles of reverse primer was diluted with 295 ul of water. 20 ul of each 100 uM primer stock was then diluted with 80 ul of water. The master mix consisted of 62.5 ul of 10X PCR reaction buffer, 25 ul of 50 mM magnesium sulfate, 12.5 ul of 10 mM dNTP, 12.5 ul of 20 uM forward primer, 12.5 ul of 20 uM reverse primer, 2.5 ul of Taq DNA polymerase (5U/ul) and 472.5 ul of water. Taq was added last. Master mix was kept on ice. Twelve 200ul PCR tubes were labelled (C, C+, 1S, 1F, 3S, 3F, 4S, 4F, 5S, 5F, A, L). C refers to the negative control without template DNA and C+ refers to the positive control with *Polytrichum juniperinum* template DNA. Enough master mix for 12.5 reactions was made (0.5 added for error), meaning 48 ul was added to each tube. Samples were placed into the PCR machine for 30 seconds at 94 degrees C, 30 seconds at 38 degrees C and 1 min and 30 seconds at 72 degrees C. 35 cycles were performed. Samples were stored at -20 degrees C.

After PCR was complete, the DNA was ready to be run on a gel: The gel was poured and given time to set. A comb was used to create wells in the gel. The gel was then submerged into 1% 1X TAE buffer. 3 ul of loading dye and 5 ul of PCR product were pipetted into the corresponding well. The gel rack, which was connected to a voltage meter, used 80 V to run the gel for 30 min. The order of the gel was as follows: Gene DireX 100bp DNA ladder RTU, C, C+, A, L, 1S, 1F, 3S, 3F, 4S, 4F, 5S, 5F. The gel was washed in EtBr for 5 minutes several times and then viewed under VWR Scientific UV illuminator to visualize the orange glow of the bands.

PCR cleanup was then performed using the QIAquick PCR Purification Kit which purifies up to 10 ug of PCR product. In each 1.5mL tube, 45 uL of the reaction solutions and 225 uL of Buffer PB were added to yield a 5:1 Buffer PB: PCR reaction mix. 10uL of 3M sodium acetate at pH 5.0 were added to convert the colour of the solution from light violet back to yellow. The mixture in each tube was transferred to 2 mL collection tubes and QlAquick column, then centrifuged for thirty to sixty seconds. The flow-through solution was discarded. 750 uL of Buffer PE was added to the columns to wash the samples, and centrifugation was performed again for thirty to sixty seconds. The samples were centrifuged a second time for 1 minute to remove residual wash buffer. The QlAquick columns were then placed into 1.5 mL microcentrifuged tubes, and 30 uL of water was added to the center of the QlAquick membrane to elute the DNA. The samples were centrifuged again for 1 minute.

1.5 Sequencing Preparation

A NanoDrop, which is a micro-volume spectrophotometer, was used to measure the amount of DNA from the PCR product prior to making the solutions for sequencing. Approximately 40 ng/ul is normally a sufficient amount of DNA to use.

The sequencing solution is comprised of four components to give a total of 10 uL: Big dye mix, PCR product, primers (forward and reverse) and H2O. The PCR product volumes used in this procedure were calculated according to the nanodrop results and the amount of forward and reverse primers used were calculated according the concentration of the primers.

The sequencing profile was as follows: 96.0 □C for denaturation, 45 □C for the annealing temperature (primer-specific) and 60 □C for the extension temperature, where the polymer starts to work.

The DNA products were washed in Sephadex G-50 solution with bead diameter of 20-80 um. Sephadex is a gel filtration media comprised of macroscopic beads synthetically made from polysaccharide, dextran. The chains will crosslink to form a three dimensional network. For this procedure, Sephadex was pipetted into a column which formed a matrix on the column membrane to catch unwanted particles from our DNA samples. The remaining solution that passed through into the 1.5 mL collection tubes was then submitted for sequencing.

2 Results

2.1 Gel Information

DNA from the three species of *Polytrichum* represented by the white bands in the gel (Figure 1) ran for half an hour at a voltage between 78V - 80V. The DNA samples migrated in a vertical fashion from the top of the gel to the bottom. There were a total of thirteen lanes on the gel. The first lane (from the left) is the Gene DireX 100bp DNA ladder comprised of DNA fragments that ranged from 100 - 1,500 base pairs, and its molecular weight ranged from 40 - 72.5 ng/5uL bottom to top on the gel (genedireX). The second lane consists of the no-template control (labelled C), thus no bands were visualized. The third lane contained the DNA template of *Polytrichum juniperinum,* the positive control (C+), which displayed a thick band. Lane 4 contains *Atrichum* (A) which did not show up on the gel. Lane 5 was comprised of Polytrichum longisetum DNA template that was visualized as a white band. Lanes 6 and 7 consisted of *Polytrichum strictum* and *Polytrichum formosum,* respectively, from site 1. Lanes 8 and 9 contained *P. strictum* and *P. formosum* from site 3. Lanes 10 and 11 were comprised of *P. strictum* and *P. formosum* found in site 4, and lanes 12 and 13 were *P. strictum* and *P. formosum* from site 5. All white bands were visible at around 700 to 800 base pairs and approximately 27.5 to 40ng/5uL.

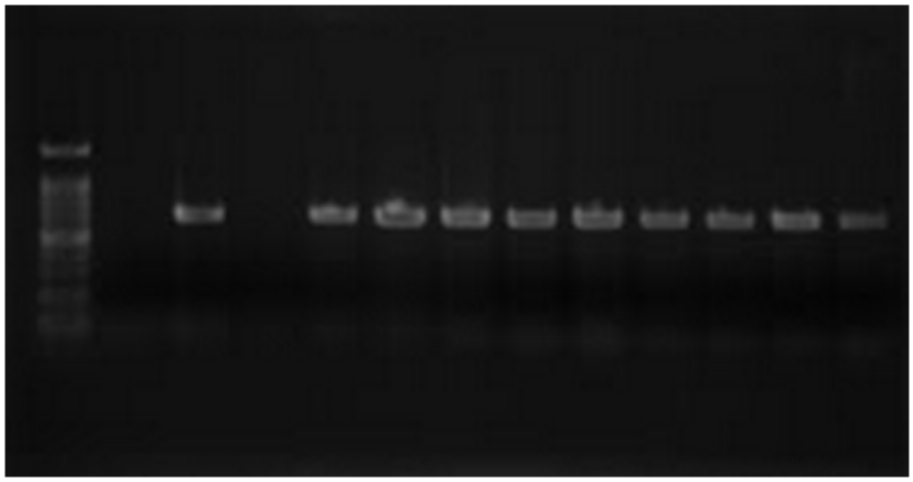

Figure 1a: Thirteen-lane gel of DNA bands of *Polytrichum* specimens from four different populations. Order of bands from left to right: Gene DireX 100bp DNA ladder RTU, C, C+, A, L, 1S, 1F, 3S, 3F, 4S, 4F, 5S, 5F.

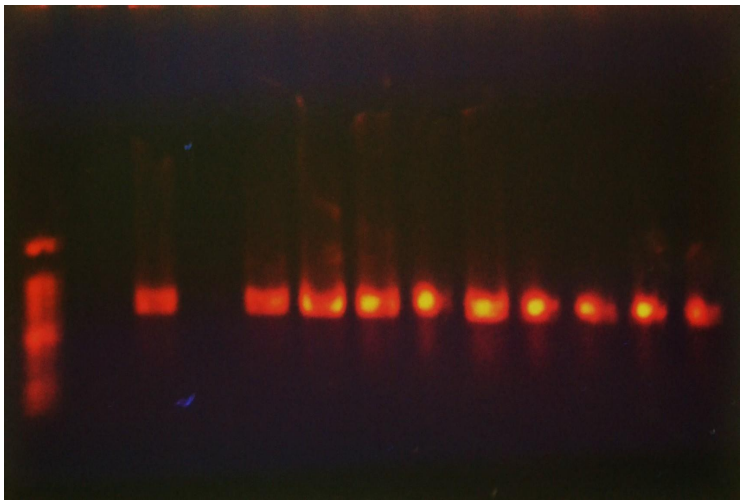

Figure 1b: DNA bands of *Polytrichum* species. This was viewed using a UV Illuminator after washing in EtBr for five minutes.

2.2 Chromatograms:

The selected barcoding region, *rbcL,* was amplified and then sequenced to determine the nucleotide sequence of each specimen. The DNA sequences are displayed as a four- colour chromatogram comprised of peaks of various heights. Each peak represents the signal intensity for a particular base. The peaks were colour-coded in order to determine which peak corresponds to which base: Green = A, Red = T, Black = G, Blue = C. Most chromatograms for the species in their respective population were fairly clean. A clean sequence is defined by its clear and evenly-spaced peaks each with only one colour. It should also have low noise levels (baseline) within each peak that represents a reliable identification of the nucleotide base in the sequence as those seen in figures 2a and 2b. Each chromatogram was also analyzed manually again to detect for any errors generated from the computer program itself used for sequencing. The gel loses its resolution as it progresses where the peaks start to broaden and shift, making it difficult to identify the bases accurately. Most of these low resolution peaks of the chromatograms appeared near the beginning and end of the sequence, displaying large overlapping peaks that were too difficult to predict the possible nucleotide present.

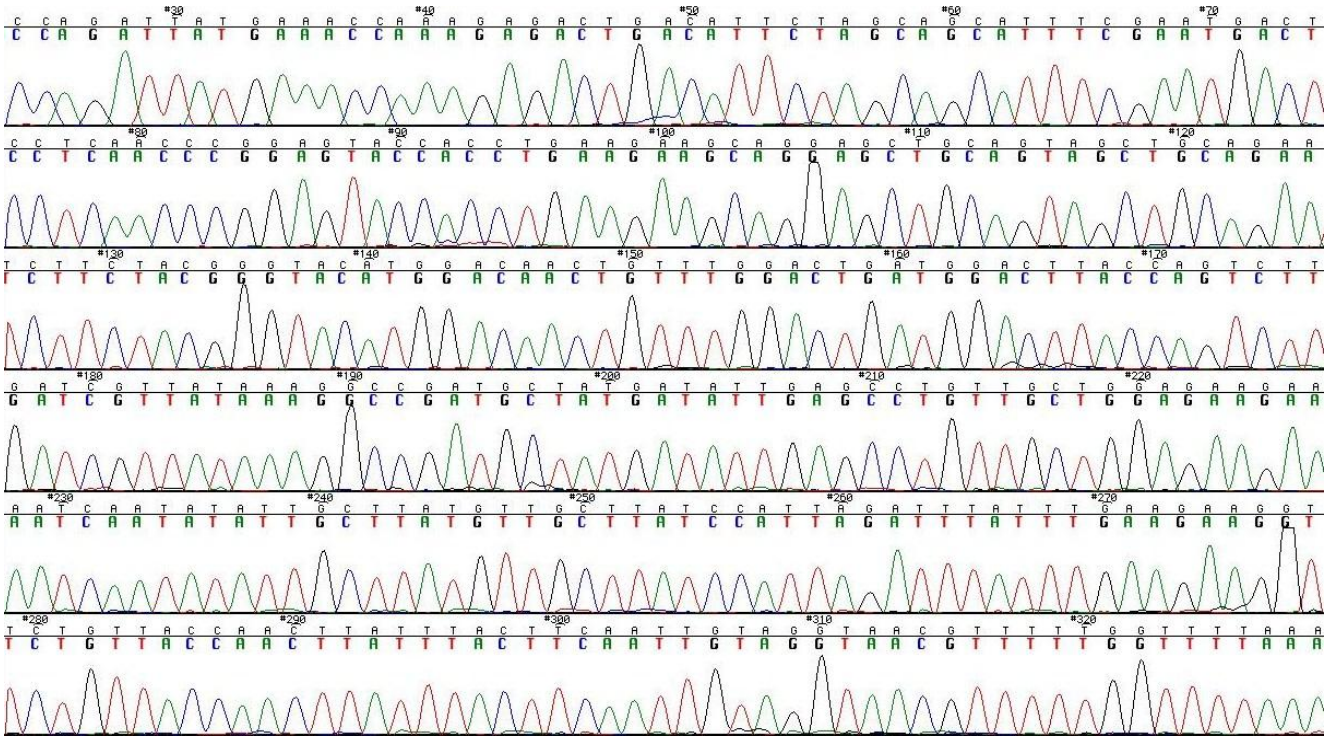

Figure 2a: Chromatogram of *strictum* from population one (1S).. This figure displays a visual representation of a portion of the DNA. Green = A, Red = T, Black = G, Blue = C.

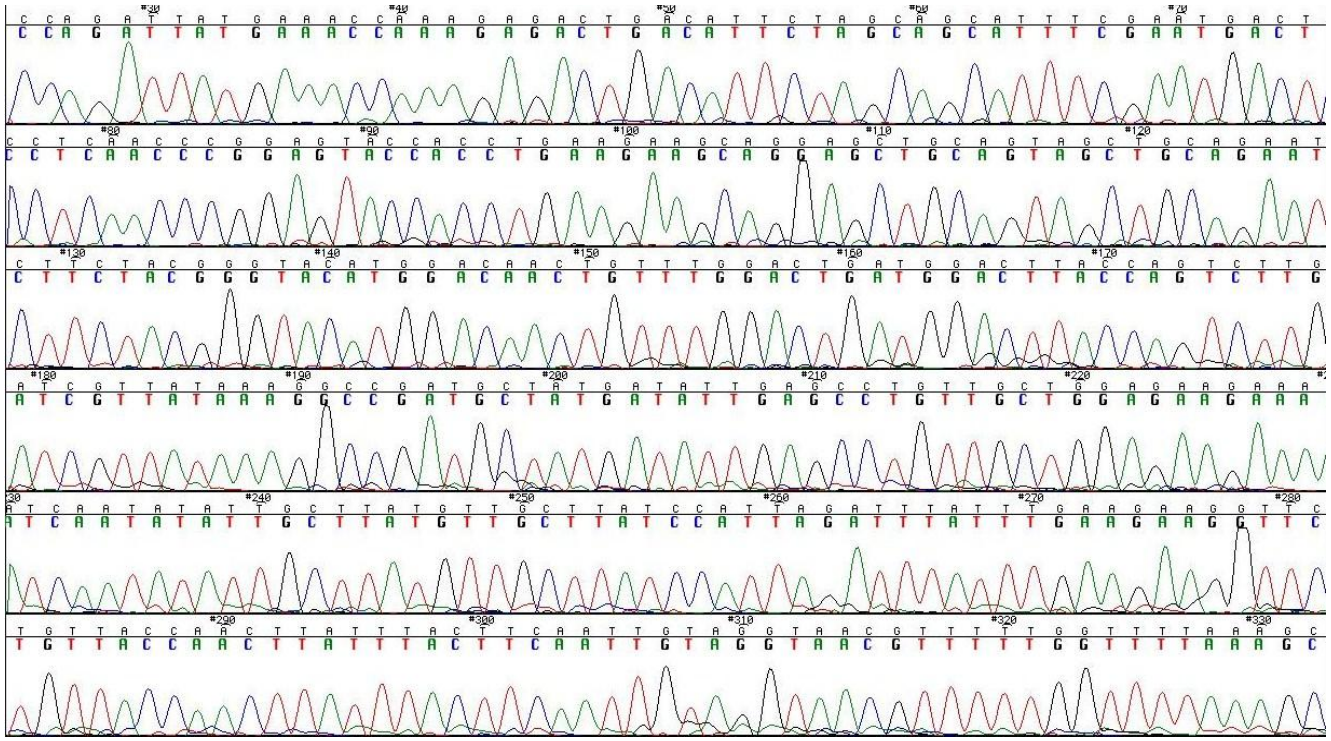

Figure 2b: Chromatogram of *strictum* from population five (5S).
This figure illustrates the peaks of a portion of the DNA. The peaks are colour-coded the same way as in figure 3.

2.3 DNA Sequences/Alignments and Phylogram Information:

The identified nucleotide bases from the chromatograms were arranged into an orderly sequence of nucleotide bases and sequences of the different species, and their respective populations were aligned (Figure 3) to identify any similarity or differences in the nucleotide bases among the species. Each sequence was put into a consensus sequence and any mismatches were detected. The black dots under the consensus sequence represent a mismatch. A total of three mismatches are displayed in Figure 3. The first mismatch appeared at the 187th nucleotide base. All *P. formosum* and *P. longisetum* populations exhibited Adenine (A) at the location and all *P. strictum* population exhibited Guanine (G). The only difference was seen in the third population of *P. strictum* (3S), which displayed an Adenine at the region. The second mismatch occurred at base 221, where all *P. formosum* populations had Cytosine (C) while all *P. strictum* populations had Tyrosine (T). Again, the third population of *strictum* was an exception, showing a C instead of a T. The third mismatch occurred at the 250th base, where all *P. formosum* populations had a T while all *P. strictum* populations had an A, except for 3S which had a T.

A phylogram (Figure 4) was constructed to depict the evolutionary history of the species *formosum, strictum* and *longisetum*. A phylogram is a type of phylogenetic tree where the length of each branch represents the number of evolutionary events that took place. In this tree, the evolutionary event that it illustrates was the number of nucleotide substitutions that took place. The number of changes were measured using the scale bar that indicated 0.5 changes. As supported by the nucleotide sequences results, all *strictum* populations were grouped as one species while all populations of *formosum* were grouped as one species, with the exception of the third population of strictum (3S) which were grouped with *formosum*. Also, *longisetum* was grouped together with *formosum.*

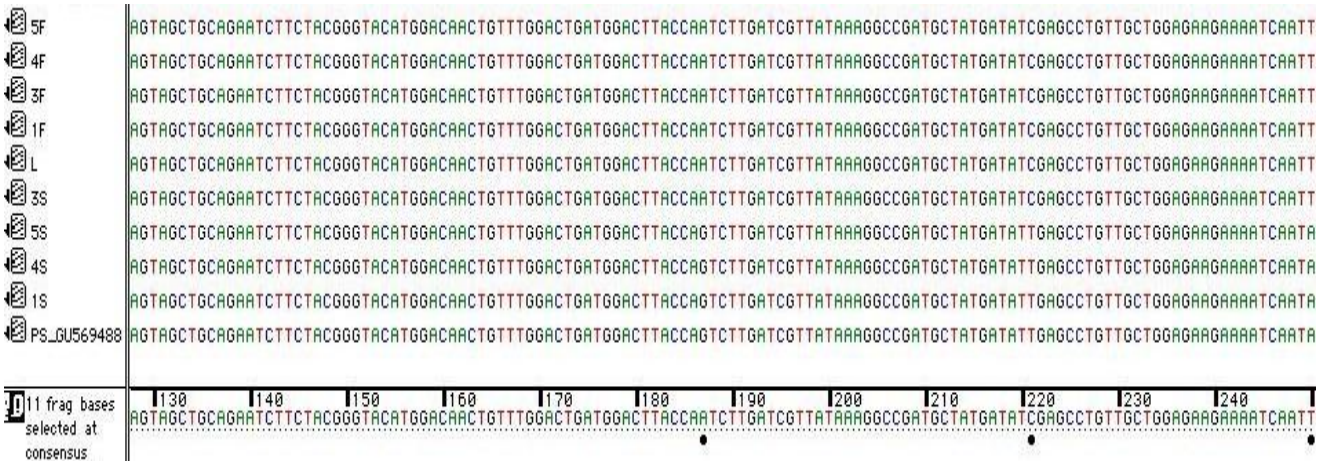

Figure 3: DNA alignments. This figure shows a portion of the DNA sequences from each population. The black dots represent mismatches, or areas where bases do not match up between populations. The first mismatch shows that all populations of *formosum* and *longisetum*, as well as the third population of *strictum* (3S) have the same base, "A", wheres all populations of *strictum*, except for 3S, have the same base, "G". The second and third mismatches show the same pattern: All populations of *formosum* and *longisetum*, as well as 3S, have the same base, whereas all populations of *strictum*, except 3S, have the same base.

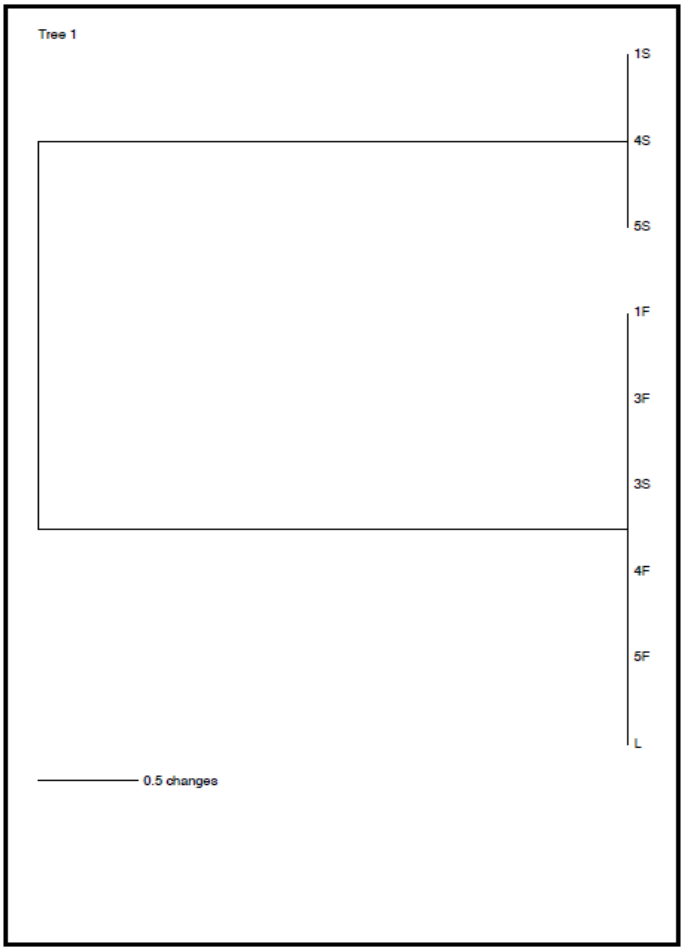

Figure 4: Phylogram for species of *formosum, strictum, and longisetum.* This figure represents the evolutionary history between the three species in the form of a phylogram, which is a type of phylogenetic tree. The length of each horizontal branch illustrates the evolutionary change that took place. The amount of change usually corresponds to the number of nucleotides that have been substituted. The amount of change can be measured using the small horizontal line, representing 0.5 changes, near the bottom of the graph.

Discussion:

The goal of this investigation was to answer the question of how three species (*P. formosum, P. strictum and P. longisetum*) within the class Polytrichopsida differed such that they were classifs as separate species. Even though they belong to the same class and genus *Polytrichum*, each plant continues to demonstrate very different characteristics. *Polytrichum formosum*, for instance, grows to be approximately 4 to 12 cm in height with long, narrow and dark green leaves that may sometimes display a reddish-coloured leaf tip (Memset dedicated servers). Its leaves also project from the main stem in a spiral arrangement (Memset dedicated servers). They are usually found in deciduous woodlands in lowlands on mildly acidic, drier soil (Hale, 2010). *Polytrichum strictum* on the other hand is found in areas with deep peat deposits and on very wet heaths (Hale, 2010). They are identified by their unbranched, yellow-green shoots and narrow, spearhead-shaped leaves (Hale, 2010). It is also known for its role as a nurse-plant where it can act as a seed trap to keep more artificial seeds than bare peats thus aiding in the

restoration of peatlands and restore areas that frequently experience frost and harsh microclimatic conditions (Groweneveld, Masse & Rochefort). The third species investigated was *Polytrichum longisetum*. This species exhibits dark green shoots and consists of stiff, straight leaves that have toothed margin. They grow to be approximately 1.5 to 10 cm tall. When mature, its calyptra is partly covered (Hale, 2010). Hence we are interested in how these species differ genetically and result in the expression of such distinct phenotypes.

The experiment began by performing extractions of the DNA samples and running a gel. The objective of running a gel is to determine if PCR was successful in amplifying the *rbcL* region. In order to tell if the gel was successful, a negative control was included to aid in the determination of any defective results, and a positive control, containing *P. juniperinum* template, was added to serve as proof that the experimental lanes produced positive results. Hence, there should be no band in the negative control lane, the experimental lanes should have fairly bright bands with no smear marks and the bands should be the same size. Taking these criteria into account, the gel displayed successful results, with no band in the negative control and very strong bands for all of the populations except for *Atrichum*. This may have been due to a couple factors: There were not enough leaves ground up during the extraction, so the amount of DNA was too small to detect, or the DNA was lost during part of the experiment. It was most likely due to the first speculation that there was not enough DNA. This is because the tube with the ground up *Atrichum* leaves was mostly brown, meaning that there was likely more soil in the tube than leaves. This was not detrimental to the experiment due to the fact that *Atrichum* was used as an extra sample in case the other samples did not yield successful results.

The DNA sequences from figure 3 showed that *formosum*, *longisetum*, and the third population of *strictum* have identical DNA sequences, and all of the populations of *strictum* except for 3S are identical. Further investigation of 3S was done in attempts to determine why 3S differed from the other populations of *strictum*. In fact, it turned out that a couple small shoots of *formosum* were found in the collection bag of 3S, hence leaves from those *formosum* shoots must have been used in the extraction instead of the leaves from *strictum.* This explains why the DNA of 3S was identical to the DNA of *formosum*. The sequences for each species analyzed showed a total of 6 mismatches among the sequences. Thus, *rbcL* was able to successfully differentiate *formosum* from *strictum*. These were the differences in their genome that distinguish them from each other, and perhaps the differences are linked to the varied morphological characteristics observed among the species; however, such a conclusion would have to be followed up with further research where specific genes in the plants are analyzed to determine their relation to a specific function of a structure.

Even though *rbcL* was successful in differentiating *formosum* from *strictum*, it was unsuccessful in differentiating *longisetum* from *formosum*. This does not mean that the two species are molecularly identical due to the fact that approximately 500 bases were analyzed, which only makes up less than 1% of the chloroplast genome (Sugiura, 1992). This most likely indicates that the *rbcL* barcode region is unable to discriminate between species that are very closely related (Gielly & Taberlet, 1994), which may explain why *P. longisetum* strongly resembles *P. formosum* in terms of appearance and size (Hale, 2010). Another possible reason why longisetum was unable to be distinguished from formosum could be due to errors, such as pipetting or contamination, that arose in the PCR and sequencing solutions. This may have led to the low resolution and noise observed in the chromatograms making it difficult to correctly identify certain nucleotide bases in the sequences. Also, the sequencing computer program could have possibly generated inaccurate base readings, such as interpreting peaks from noise as true peaks.

The phylogram from figure 5 presents the DNA sequence results in the form of a phylogenetic tree, which allows for an analysis of evolutionary relationships among species to be made, as well as the amount of changes that occurred over time. It shows how *formosum* and *strictum* diverged from the same common ancestor and *longisetum* is very closely related to *formosum*.

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