In 2019 the Biology 100 class of Wenatchee Valley College at Omak (WVCO) worked on a DNA barcoding project with Tabitha Graves’s huckleberry research project for bear habitat in Glacier National Park, Montana. Students isolated DNA from huckleberry leaf samples from the National Park. They then ran a PCR with an rbcL primer pair to target the rubisco gene in plant chloroplasts. The PCR product was sequenced by a private company, Genewiz, and the DNA sequence was analyzed through DNA Subway. Twelve student groups, one or two students per group, isolated DNA from huckleberry leaf samples that was sequenced and analyzed. Twelve samples were determined to be of the genus Vaccinium. One of the twelve samples distinguished between the five species of huckleberry, identified the sample as Vaccinium membranaceum, and was published in GenBank. They showed that DNA barcoding can be used successfully to aid in the identification of this species of huckleberry. There were many student outcomes, including hands-on skills with the tools of DNA technology, contributing to a real-world project, and analyzing data for DNA sequence matches. This is a great lab exercise to use for AP biology classes, two-year college community biology classes, and four-year college biology classes at the 100 to 200 level.

Key Words: Bear; chloroplast DNA; Cold Springs Harbor Laboratory; DNA barcoding; DNA Subway; gel electrophoresis; gene; Glacier National Park; huckleberry; PCR; rbcL; rubisco; taxonomy; USGS Northern Rocky Mountain Science Center; Vaccinium; Wenatchee Valley College at Omak.

Introduction

Since 2014 the science department of Wenatchee Valley College at Omak (WVCO), in North Central Washington, has experimented with methods in DNA barcoding. We have followed the procedure outlined by Cold Springs Harbor Laboratory DNA Learning Center (DNALC, 2018). Our campus has a botanical garden, the WVCO Native Plant Garden, that contains plants native to our geographical area. Students can sample plant leaves and use the DNA barcoding methods to identify the species of numerous plants from the Native Plant Garden. In many cases student results were subsequently published in GenBank, the U.S. Government repository for DNA sequences. To date, our WVCO students have published in GenBank 32 plant DNA sequences and one American black bear DNA sequence.

In brief, the DNA barcoding lab consists of grinding the leaf of a green plant and placing the pulverized tissue in a microfuge tube with silica. The silica selectively binds the chloroplast DNA and separates the chloroplast DNA from other cell debris. Next, the plant DNA sample is combined with primers that select a specific DNA sequence of nucleotides that are multiplied by the polymerase chain reaction (PCR). The product of the PCR amplification is visualized with gel electrophoresis. Correctly sized DNA from PCR is then sent to Genewiz (http://www.genewiz.com/) for DNA sequencing. Finally, sequences are run in the DNA Subway (https://dnasubway.cyverse.org) collection of programs to find a match.

In 2018 WVCO’s science department began a collaborative project with Tabitha Graves, research ecologist, USGS Northern Rocky Mountain Science Center in Glacier National Park, Montana. Her work was to “understand variables influencing huckleberries to identify management options to improve resilience of bear food system” (Graves, n.d.). The value of our collaboration was to add a method for identifying species of huckleberry using DNA barcoding. Samples from Graves were identified in the field by workers who used traditional methods of taxonomic keys. Indeed, some of the samples did not come with the species identified. The goal of our work was to determine if DNA barcoding...
could be a reliable means for identifying species of huckleberries. Our students were to determine the DNA barcode for five known species of huckleberries found in Glacier National Park, Montana. The possible species were *Vaccinium membranaceum*, *V. myrtilloides*, *V. caespitosum*, *V. myrtillus*, and *V. scoparium* (Graves, n.d.).

The overall goal of DNA barcoding is to establish a database that allows the identification of an unknown organism by matching a specific sequence of its DNA to a known sequence for the corresponding known organism. This method takes the emphasis away from the taxonomic keys that often require extensive familiarity with the specific characteristics of the organism to be identified. DNA barcoding uses DNA primers to select and amplify an appropriate region of DNA using PCR. With PCR, primers are recommended for the type of organism, fish, plant, fungus, animal, etc. (DNALC, 2018). When mixed with DNA (template), isolated from the sample, PCR will generate millions of copies of the DNA sequence from the template. These copies of the DNA are then sequenced, and their order of DNA letters is determined. Isolated DNA samples are easily and cheaply sequenced by commercial companies, such as Geneiz. The DNA sequence is entered into the web application DNA Subway to determine the identity of the species of organism. DNA Subway is a collection of several applications combined in one system. DNA Subway takes the entered sequence and compares it with known sequences of plants by doing a BLASTN search of Gen-Bank. The compared matches are ranked according to the number of nucleotides that are not matching (DNALC and CyVerse, n.d.). In this project we looked for a 100% match to determine the species level of identity.

As a result of this work, one student was successful in determining the DNA barcode for a species of huckleberry named *Vaccinium membranaceum* (sample 114). We present this paper with the hope that other students will become involved with DNA barcoding in the science classroom.

### Literature Review

In 2003 Paul D. N. Hebert and colleagues proposed using a version of the universal price code (UPC), or barcode, to represent a sequence of DNA for a given species of organism. He coined the term DNA barcode. His choice to identify species of insects was the 658 bp fragment of the mitochondrial cytochrome c oxidase 1 (COI) gene. The COI gene has a faster rate of evolution than the 12 S and 16 S ribosomal genes. Thus, there is more of a chance to see variations among species that would act as unique markers to identify the species. The primer pair of LCO1490 (5’-GGTCAACAATATCAAAAGATATTG-3’) and HCO2198 (5’-TAAACTTCAGGGTGACCAAAAATCA-3’) was used in the PCR reaction that generated the 658 bp COI gene fragment (Hebert et al., 2003). His work showed that the COI gene sequences could distinguish between different species.

For fungi, Bellemain and colleagues (2010) determined that from nuclear DNA the internal transcribed spacer (ITS) region of DNA worked well for identifying fungi using the DNA barcode technique. Various primers were selected for sequencing the ITS regions. It was found that some of the primers worked better with basidiomycetes (ITS$_1$, ITS$_2$, and ITS$_3$) while others primers (ITS$_2$, ITS$_3$, and ITS$_4$) favored ascomycetes.

For land plants, the Consortium for Barcode of Life Working Group (CBOL), found that a combination of primers, called a 2-locus barcode, for more than one gene worked very well. These primers were the rbCL and matK primers (Hollingsworth et al., 2009). The primer rbCL targets the region of the ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) gene, and the matK primer targets the matruse K gene. Both genes are found in the chloroplast of plants. The rubisco gene codes for the enzyme that fixes (incorporates) carbon dioxide into sugars in the Calvin cycle of photosynthesis. The matruse K gene creates a product that is involved with editing of RNA in the chloroplasts. RbCl was selected for our work as the best primer for use with land-based plants, according to DNALC (2018).

A DNA barcode is a sequence of DNA letters or nucleotides (ATCG), with each letter represented by a different color and formatted like a grocery store barcode with vertical lines. In this way any gene can be represented as a barcode. Figure 1 shows the DNA barcode, as generated by Bio-Rad’s DNA Barcode Generator (http://biorad-ads.com/DNABarcodeWeb) for the plant species of thin leaf huckleberry.

![DNA Barcode](https://example.com/dna-barcode.png)

**Figure 1.** A DNA barcode for thin leaf huckleberry. In this case it indicates the DNA letter sequence (ATCGs) for 522 letters or base pairs. This was created by inputting the DNA sequence into Bio-Rad’s DNA Barcode Generator.

Once the DNA gene region(s) have been sequenced, the sequence is compared to published sequences using the DNA Subway website. DNA Subway is a collection of programs and databases created by Cold Springs Harbor Laboratory and Cyverse at the University of Arizona (n.d.). DNA Subway is used to identify the organism associated with the DNA barcode (DNA sequence) of a sample.

### Materials & Methods

DNA was isolated from huckleberry plants received from Graves’s huckleberry research project in Glacier National Park, Montana. The species of huckleberry plant and the location where collected (latitude and longitude) and included with the samples. Protocols from DNALC (2018) were followed with slight modifications. For example, we paid specific attention to cleaning the mortars and pestles prior to each use to prevent contamination. Each student individual or group was given a specific huckleberry leaf sample from Graves’s Glacier National Park samples. Students selected from these samples (Graves, n.d.).

**Isolation of DNA from Huckleberry Leaf**

A 1 cm$^2$ sample of huckleberry leaf was immersed in liquid nitrogen and, with a mortar and pestle, the leaf was pulverized to a fine powder. The 600 ul of lysis buffer (6 M Guanidine...
**Results**

There were 14 students in the class, and 12 samples had sufficient DNA to run a sequence and determine if the sample was from *Vaccinium*. One student had a successful DNA sequence match (sample 114) of their huckleberry DNA to a known sequence for *Vaccinium membranaceum*. Figure 2 shows the DNA gel of several huckleberry samples. Twelve samples (not all are shown) were sent to Genewiz for DNA sequencing. Figure 3 shows the BLASTN search from DNA Subway results for sample 114 between the published BLASTN *Vaccinium membranaceum* and sample 4-M13F_D01_ab1and sample 4-M13R_F02_ab1(our sample 114). Figure 4 shows the DNA Subway alignment viewer for sample 114 and one of the results from BLASTN (Ascension MG219766.1).

**PCR of the Isolated DNA**

In a clean 0.2 ml PCR tube, 23 μL of primer/loading dye mix (640 μL distilled water, 460 μL Cresol red loading dye, 20 μL of 15 pmol/μL 5’ primer, and 20 μL of 15 pmol/μL 3’ primer) was added. Primers were rbcLaF and rbcLa rev. Next, one illustra PuReTaq PCR Ready-to-Go PCR Bead (VWR 89497-132); per 25 μL: 2.5 units Taq DNA polymerase, 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM MgCl$_2$, and 200 μm of each dNTP) was added to the tube followed by 2 μL of sample huckleberry DNA. This was run in a thermocycler with the following settings.

- Initial step: 95°C for 1 minute
- Denaturing step: 35 cycles of 94°C for 15 seconds
- Annealing step: 54°C for 15 seconds
- Extending step: 72°C for 30 seconds, and then held at 4°C or stored at -20°C until ready for the PCR step.

**Gel Electrophoresis**

After the PCR step, 5 μL of huckleberry sample DNA was loaded onto a 2% agarose gel in 1× TBE (Tris-boric acid-EDTA) buffer and run for 30 minutes at 130 V. The DNA was visualized using GelGreen nucleic acid gel stain, 10,000x (Minipcr.com, RG-1550-01) added directly to the molten agarose gel, and a UV transilluminator. A DNA ladder was added to the outside lanes to confirm a DNA band size of between 550 and 600 nucleotides for the target DNA. Samples that had the appropriate size DNA band were chosen for the sequencing step.

**DNA Sequencing**

The 20 μL of selected huckleberry PCR samples were sent to Genewiz (2021) for sequencing.

**DNA Subway**

The sequenced files for the samples that were successfully sequenced by Genewiz (2021) were uploaded to DNA Subway (https://dnasubway.cyverse.org) and compared to potential matches to establish the identity (genus, species) of the student samples. When the DNA sequence was a 100% match between the DNA Subway database and our huckleberry samples, we adopted the name of the plant for the student sample and submitted the sample to GenBank (through DNA Subway) for publication of the DNA sequence.
Discussion of Results

Fourteen students isolated DNA that was sequenced, but not all students were able to distinguish between the five species of huckleberry. Eleven samples were determined to be in the genus *Vaccinium*. One student sample (sample 114) of huckleberry leaf matched 100% for the sequence of the huckleberry species *Vaccinium membranaceum* and was published in GenBank (National Center for Biotechnology Information, 2019).

For the DNA published in GenBank, the DNA Subway results confirmed that the student sample 114 (from latitude 48°28'38.93'' N, longitude 113°22'60.33'' W from Graves's huckleberry project. Figure 5 came from the huckleberry plant *Vaccinium membranaceum*. This was determined by comparing the student’s huckleberry sample DNA sequence to that of DNA Subway’s BLASTN search (Figure 3). The 507 bp final sequence (it was longer but the DNA Subway programs trim nucleotides that do not correspond to both inputted sequences) from the sample matched 100% with two candidates, accession MN 735532.1 and MG 219766.1. Both were *Vaccinium membranaceum*. This was further verified by using DNA Subway’s alignment view (Figure 4) to show that BLASTN search accession MG 219766.1 was a 100% match with the student sample 114. Additionally, DNA Subway’s PHYLIP MS Chart (Figure 7) indicates that the 114 sample is *Vaccinium membranaceum*.

We conclude that DNA barcoding is able to resolve, to the genus and species level, the huckleberry plant *Vaccinium membranaceum* using the primer pair rbcLaF and rbcLaRev. Students were unable to resolve other species of huckleberry plants, *V. myrtilliodes*, *V. caespitosum*, *V. myrtillus*, and *V. scoparium* (Graves, n.d.) with this primer pair. Unofficially, in later experiments we were successful using 2-locus barcode methods. A 2-locus barcode method simply means that two different primer pairs are used. For example, instead of using only the rbcL primer pair, one might use the MatK primers and rbcL primers. This gives more specificity in identifying the species.

The fact that 12 groups isolated DNA from huckleberry plants indicates that the experiment was successful. These groups were able to determine the genus was *Vaccinium* but were unable to get a resolution of the five species.

From working with students doing the DNA barcode methods, some things can be attributed to errors in the recovery of DNA. Early on we found that we were getting results that were inconsistent with what we were analyzing. We concluded that this was due to a residue left on the mortar and pestle. To combat this we had students thoroughly wash the mortar and pestle with soap and water prior to use.
The DNA barcoding was carried out over two to four weeks but could be consolidated to perhaps four lab sessions where session 1 is the DNA isolation, session 2 is the PCR step, session 3 is gel electrophoresis and mailing the PCR sample to Genewiz for sequencing, and session 4 is analysis of the sequences on the DNA Subway applications. (See figure 6 for a time frame).

Occasionally, if students had no match in their DNA BLASTN search, we would go to another website, BOLD Systems (http://www.boldsystems.org), and download the sequence of DNA that could be compared to the species of plant their sample came from. They would download the rbcL-determined sequences and enter this into the DNA Subway alignment tool to determine if the sequence was a match. This was done to give something to base the identity of the student sample on when the DNA Subway BLASTN search did not provide any suitable matches. Students would then compare the BOLD systems sequences to their sample and determine the plant identity.

To complete this lab as a classroom exercise with 24 students, the instructor would need to assemble basic DNA equipment. This would include two water-baths, micropipettes (2–20 μL and 100–1000 μL), microfuge, thermocycler, gel electrophoresis equipment, and UV transilluminator. Consumables would be the Ready-to-Go PCR Beads, agarose, TBE buffer, and reagents for the silica DNA isolation method (see Materials & Methods section).

Prior to introducing the DNA barcoding methods, it is recommended that students have a basic understanding of DNA, genes, and PCR. Once students have that knowledge base, then they can perform the experiment with an understanding of the process of DNA barcoding. All in all, the DNA barcoding/huckleberry project gave our students the opportunity to be involved in a real-life research project and to have the possibility that their work could be published in GenBank.

Our GenBank-published DNA sequence may be found at http://www.ncbi.nlm.nih.gov/nuccore/MN735532.1.

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