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Comparative Analysis of Four DNA Preparation Kits for *Quercus falcata, palustris,* and *rubra* DNA Extractions and Subsequent DNA Sequencing

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Introduction

In 2011, of the 8.7 million species of life suspected to populate the globe only 1.2 million had been catalogued and it was estimated that 1,200 years would pass before the remainder were processed, allowing some to go extinct before discovery (Mora, Tittensor, Adl, Simpson, & Worm, 2011). Molecular analysis via DNA barcoding, may provide the answer to this problem. DNA barcoding is organism "discrimination through the analysis of a small [standard] segment of the genome" and comparison against a database for the purpose of classification (DeWalt, 2011; Hebert, Cywinska, Ball, & DeWaard, 2003; Hollingsworth, 2007). According to Hebert & Gregory, "DNA barcoding will accelerate the pace of species discovery by allowing taxonomists to rapidly sort specimens... by highlighting divergent taxa that may represent new species… and by making species identifications more easily available" (2005)

While DNA barcoding has many potential benefits, it faces some potential setbacks. Unfortunately, DNA extraction, an integral part of barcoding, does not always yield a pure, usable product. Organic materials, such as enzymes (i.e. DNAse), polysaccharides, proteinases, or polyphenols, can often be retained in samples and act as inhibitors to later processes (Peist et al., 2001). One group of organisms susceptible to this problem, are members of the *Quercus* genus. Oak trees have been shown to have high levels of polysaccharides and polyphenolics. (A Toader et al., 2009; Moctezuma et al., 2014; Salminen et al., 2004; Zhang, Cai, Duan, Reeves, & He, 2015) The high levels of these organic particles have made it particularly difficult to obtain clean DNA extractions via commercial plant DNA preparation kits, ultimately resulting in poor downstream products. (A Toader et al., 2009; Barta et al., 2017; Csaikl et al., 1998; Echevarría-Zomeño et al., 2012).

A previous study by Johnson and Trott (2017), focused on DNA Barcoding in the *Quercus* species, found that extractions using the *Powerplant pro® DNA isolation Kit* did not produce adequate PCR or DNA sequencing results. Johnson theorized that polyphenol contamination was the likely cause of malperformance. This current experiment is a continuation of Johnson and Trott's study and seeks to find the DNA extraction method best suited to purify DNA from *Quercus falcata, palustris, velutino,* and *rubra* **(Figure 1),** all species previously sampled. In addition, we hope to pinpoint the cause of the malperformance. We tested four DNA extraction kits (*methods*) and measured the efficiency of each kit by evaluating their sequences' average percent indistinguishable nucleotide (N) instances, number of high quality electropherograms (clean non-overlapping signal), and PCR-gel-electrophoresis results. The kit producing the highest number of usable sequences with low percent N instances and PCR results with single bands of correct relative mobility was deemed most effective.

Southern Red Oak (09) (*Quercus falcata*)
Last Collected: 4/7/17
Location: 35° 02.974' N, 85° 02.903' W, Altitude: 792 ft (Collegedale, Tennessee)

Northern red oak (06) (*Quercus rubra*)
Last Collected: 7/26/16
Location: 35° 03.402' N, 85° 02.834' W, Altitude: 792 ft (Collegedale, Tennessee)

Pin Oak (22) (*Quercus palustris*)
Last Collected: 4/16/17
Location: 35° 02.716' N, 85° 03.206' W, Altitude: 867 ft (Collegedale, Tennessee)

Black oak (10) (*Quercus velutina*)
Last Collected: 8/1/16
Location: 35° 03.008' N, 85° 03.343' W, (Collegedale Tennessee)

Figure 1. Examples of the four species of Oak examined in this study. All samples were collected between July 2016 and April 2017 by Mckenzie Johnson. Samples were stored in 50 ml microcentrifuge tubes at -80° C until processing for DNA extraction.

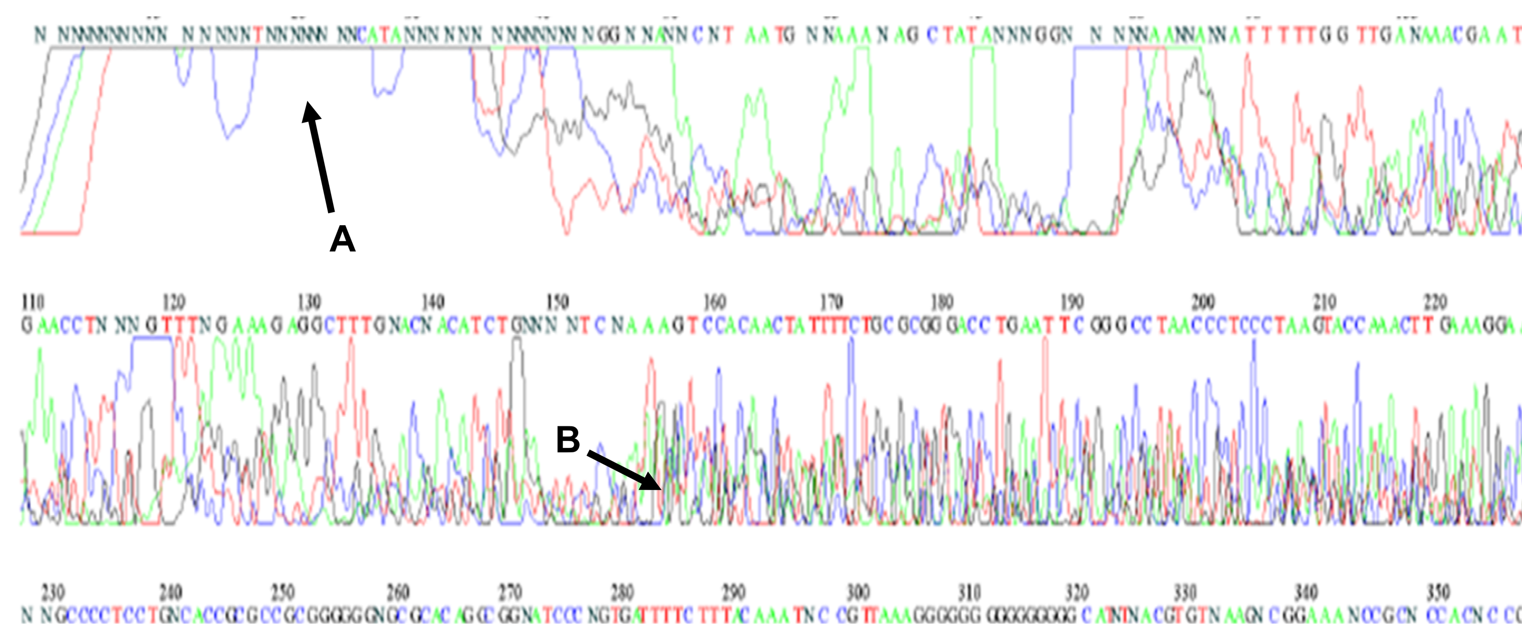

Figure 2. Electropherogram of *Purelink®* extract sample 15.3. **(A)** this is an N instance, it represents a place were the computer analyzing the DNA sequence could not determine the correct nucleotide. **(B)** An example of signal noise (multiple peaks overlapping)

Material and Methods

Sample Collection

Leaf collection was carried out as described in Johnson and Trott (2017), summarized below **(Figure 1).** Sample leaf material was collected locally (Southern Adventist University) on two separate occasions from three oak species during the 2016-2017 academic year: Northern Red Oak, Southern Red Oak, and Pin Oak. Each species was identified by its unique morphological features (shape, leaf length, etc.). After collection, leaves were washed with osmosis water and stored at -80°C in 50ml centrifuge tubes.

DNA Extraction

DNA extraction and purification was carried out according to the following protocols (15 samples per kit):

GenElute® Plant Genomic DNA Miniprep Kit,
Geneaid® Genomic DNA Mini Kit (Plant),
PureLink® Plant Total DNA Purification Kit, **(Figure 3)**
MagMAX® Plant DNA Kit.

All four kits followed five basic DNA isolation steps:
(1) Grind and homogenize plant tissue under liquid nitrogen,
(2) Lyse plant sample cells, and filter out unneeded plant tissue
(3) Bind DNA to a retention apparatus (spin column or magnetic beads),
(4) Wash DNA
(5) Elute DNA.

Figure 3. *PureLink®* Plant Total DNA Purification Kit (example)

PCR Analysis

PCR amplification was carried out according to the procedure detailed in McKenzie and Trott (2017) with modifications. PCR reactions were optimized for 0.5 µl of the template DNA as indicated by single PCR products after agarose electrophoresis. PCR reactions were initially performed for rbcL, ycf1, and matK primer sets (CBOL Plant Working Group1 et al., 2009; Wenpan et al., 2015). After initial trials, rbcL was shown to produce the best results and thus was solely used for the remainder of the experiment. The PCR amplification for all experiments were performed in a mixture containing 2.5 µl of 0.2 µM of forward and reverse primers, 25 µl Q5 High-Fidelity 2X Master Mix (New England Biolabs - M0492S) in Standard Buffer, and 2 µl of DNA nucleotides. The final volume was adjusted to 50 µl using distilled water.
The targeted gene for the DNA barcoding was amplified using BIO-RAD C1000 Touch™ Thermal Cycler following the PCR profiles for the selected primer set. The rbcL primers' PCR cycling profile began with 30 seconds at 98°C, followed by 35 cycles of 10 seconds at 98°C , 30 seconds at 55°C (CBOL plant working group, 2009), and 30 seconds at 72°C, and finally it ended with 2 minutes at 72°C. PCR intensity of band and position in relation to size standard were recorded **(Figure 4).**

Spectrophotometric Analysis

To assess purity of DNA extract, we performed spectral analysis using a Thermo Scientific Multiskan GO spectrophotometer. 5ul of each sample extract was diluted with 200ul of tris HCL (pH ~7.5) or TAE buffer and then used for analysis. tris HCL (pH 7.5) or TAE buffer was used as a blank. Absorbances were recorded at wavelengths of 230nm, 260nm, and 280nm. Ratios of 260/230 and 260/280 were calculated and compared to standard purity values (260/230 value: 2.0-2.2; 260/280 value: ~1.8)

Electrophoresis

PCR products were analyzed on 1% (w/v) agarose gels in TAE. Products were visualized by ethidium bromide staining or UViewTM 6x loading dye and imaged with an UVP EC3 imaging system. rbcL primed PCR reactions which produced single clear bands of about 500-600 bp in length (Hollingsworth, Graham, & Little, 2011) were sent off for DNA sequencing by Macrogen (www.macrogenusa.com). DNA concentration was estimated and reported by comparing the brightness of sample bands to standard bands (1 kb fast ladder) of known concentration using ImageJ image analysis software. rbcL forward and reverse primers were used for the sequencing reactions.

Electropherogram Quality Analysis

DNA electropherograms were visually inspected for amount of background noise (overlapping signals) and then examined for instances of indistinguishable nucleotides. The percentage of DNA sequence which comprised indistinguishable nucleotides (%N) was recorded **(Figure 2, Table 1)**. To analyze this data, we arbitrarily set a threshold value of 10 % N for the sequences. Values above 10% are considered to indicate low quality sequences and values below 10% indicate high quality sequences.

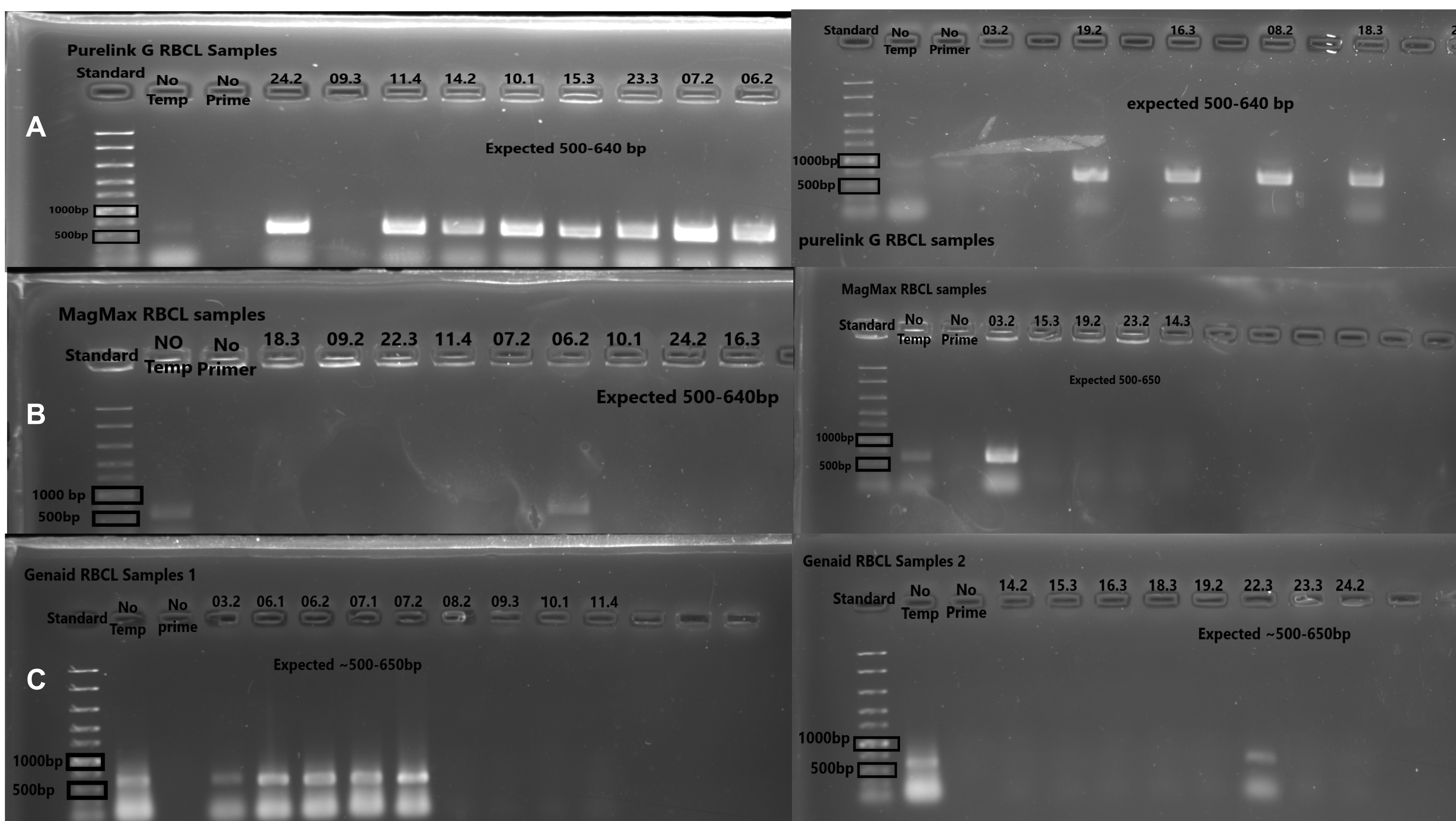

Figure 4. RBCL primed PCR results of DNA extraction Kits
(A) *PureLink® Plant Total DNA Purification Kit,*
(B), *MagMAX™ Plant DNA Kit*
(C) *Geneaid Genomic DNA Mini Kit*

Results

PureLink® Plant Total DNA Kit

- Spectrophotometric data. **(Table 2)**
- PCR was successful for 12 of 15 sample extracts **(Figure 4, Table 1)**
- All 15 PCR products were sequenced, however all samples returned very noisy electropherograms **(Figure 4)**
- None of the resultant DNA sequences met the 10% N instance threshold we set for quality assessment. **(Table 1)**
- Only one sample, 15.3, met the 10% N instance threshold after correction to eliminate free nucleotide interference as implied by the article *interpreting electropherograms* (Delaware Biological Institute, 2013) **(Table 1)**.

MagMAX™ Plant DNA Kit

- Spectrophotometric data. **(Table 2)**
- Only 2 of the 15 samples extracted produce acceptable PCR product. **(Figure 4, Table 1)**
- Retrials) produced similar results *(data not shown)*

Geneaid Genomic DNA Mini (Plant)

- 4 of the 15 extracts tested, produced acceptable PCR product. **(Table 1)**
- The majority of species that were successful for this kit were Northern Red Oak. **(Table 1)**

Table 2. Spectrophotometric analysis of DNA

	Ave. Ratios of Absorbance	
Wavelengths (nm:nm)	260:230	260:280
Purelink	0.94	1.59
MAgMax	0.95	1.45

260:230 represents the DNA to polyphenol ratio (standard purity value 2.0+)

260:280 represents the DNA to protein ratio (standard purity value 1.8+)

Table 1. PCR and DNA Sequencing Results

Sample ID #	Species of Sample	Successful PCR (P/M/G)	PureLink Electropherogram Data		
			% N instances	Single Corrected % N instances*	Double Corrected % N instances*
03.2[B]	N. Red oak (Quercus rubra)	-/+/+	42%	34%	N/A
06.2	N. Red oak	+/+/+	44%	31%	24%
07.2	N. Red oak	+/-/+	27%	15%	N/A
08.2	S. red oak (Quercus falcata)	+/-/-	45%	24%	N/A
09.3[B]	S. red oak	-/-/-	45%	35%	N/A
10.1	Black oak (Quercus velutina)	+/-/-	32%	22%	N/A
11.4	Hybrid	+/-/-	67%	60%	N/A
14.2	S. Red oak	+/-/-	53%	40%	N/A
15.3[A]	Black Oak	+/-/-	13%	6%	N/A
16.3	S. Red oak	+/-/-	42%	31%	29%
18.3	N. red oak	+/-/-	20%	17%	N/A
19.2	Hybrid	+/-/-	23%	15%	N/A
22.3[B]	Pin oak (Quercus palustris)	-/-/+	33%	19%	N/A
23.3	Pin oak	+/-/-	22%	18%	15%
24.2	Pin oak	+/-/-	53%	44%	N/A

% N = percentage of DNA Sequence which contained indistinguishable nucleotides

***** Corrected values of % N were calculated using a section of the DNA sequence which started from the 1st(single) or 2nd(double) triplet of distinguishable nucleotides in the original sequence.

A = sample 15.3 met threshold acceptable value we set when sequenced

B= these samples gave poor PCR product

P = PureLink® Plant Total DNA Purification Kit**; M =** MagMAX™ Plant DNA Kit**; G =** Geneaid Genomic DNA Mini Kit

Conclusions and Future Research

- *Purelink* has been the most consistent for generating appropriate PCR products. However, that product has not been shown to be viable for downstream DNA sequencing.
- The *MagMAX Plant DNA* and *Geneaid Genomic DNA Mini* Kits do not produce PCR results as consistently. PCR products were not used for sequencing reactions.
- It should be noted that there were faint traces of bands in some PCR reactions. This could indicate that an increased number of PCR cycles is needed to increase the yield of those samples.
- General success of the Northern Red Oak species extracts from both the *MagMax* and *Genaid* kits, despite overall poor performance in sequencing reactions.
- Future work is focused on optimizing the PCR output of the *Purelink* Kit so better DNA sequencing may be achieved.
- Additionally, it may be fruitful to further investigate how species, or age of sample affects the quality and efficiency of DNA extraction and sequencing.

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