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Hsini (Cindy) Chu April 11, 2016

Contaminant removal of non-pollen material in palynologic samples for DNA barcoding

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ABSTRACT

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By Hsini (Cindy) Chu

DNA barcoding is a recently established technique that uses genetic markers to identify plant, fungal, and animal species. DNA barcoding for pollen, specifically, uses these genetic markers to identify pollen species. Barcoding will circumvent identifying pollen through visual microscopy, which will allow for rapid species-specific identification. This has broad implications since pollen is a biomarker of space and time and has functional importance in areas such as human respiratory allergies and forensic science. In certain circumstances, however, pollen samples may become contaminated with non-pollen plant material, which will not allow pollen species to be identified through the use of barcoding. This paper presents a series of trials conducted to alleviate this issue through the removal of non-pollen plant material with chemical and enzymatic reagents. Several combinations of chemical and enzyme treatments were tested on pollen and non-pollen material with the goal of removing all non-pollen DNA while maintaining the integrity of the pollen DNA. I found pre-treatment with sodium hydroxide, followed by treatment with DNase I enzyme was the best combination in achieving this goal. While this protocol showed promise, it was not consistently effective in removing non-pollen DNA. Using the foundations of this project, contaminant removal in a DNA barcoding context will require further methods development. Improving these methodologies in contaminant removal will allow for a broader range of powerful applications for DNA barcoding and pollen identification.

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INTRODUCTION

Pollen identification is a critically important technique in a wide range of scientific and technical fields. This importance is twofold: 1) pollen is a biomarker of space and time; and 2) pollen has tremendous functional importance. Pollen is an invaluable biomarker because different plant species occur in different geographic locations and bloom at different times [1–4]. Thus, pollen can be used to trace the geographic history of people and objects, which can be utilized in tracking of agricultural products and in criminal or security-related forensics [1,2,5,6]. Pollen is also used in paleontology to understand how environments change over time [7,8]. For example, sediment cores can be analyzed for different pollen species to indicate plant diversities over geological time scales. Pollen is also functionally important in human respiratory allergies [9–12]. If specific pollen species can be identified as primary allergens, then those particular pollen species can be used in targeted immunotherapies, and warning systems can be developed for patients who are sensitive to pollen from particular plant species. Pollen identification can also be used to address pollinator declines, and being able to identify pollen can help with understanding honeybee diets [13–15], building plant-pollinator networks [16,17], and understanding the effects of heterospecific pollen on plant reproduction [18–21].

Traditionally, visual microscopy has been used to identify pollen. However, visual identification can typically classify pollen only to the family or genus level due to structural ambiguities [22,23]. Additionally, visual identification relies on an expert palynologist, and such highly trained experts are rare and increasingly fewer are being trained. Moreover, thousands of individual pollen grains must be examined for reliable bulk identification, which is very time-consuming, creating a strong limiting factor. Together, these limitations have meant that many of the potentially very powerful applications of pollen identification have been underutilized. For

example, only a handful of studies have linked respiratory allergic responses to specific pollen taxa—and the existing studies have only focused on the plant genus level—because of the difficulties in identifying large numbers of pollen samples from automated aerial samplers [24].

In the last two years, however, new research has shown proof-of-concept of pollen identification with DNA barcoding [25,26], the use of genetic markers to identify species [27–30]. These markers can sequence genetic regions that are amplifiable across taxonomic kingdoms, and these regions have enough variations to differentiate between species [22]. DNA barcoding, thus, offers several potential advantages for pollen identification relative to visual microscopy. DNA barcoding can increase the taxonomic resolution of pollen identification to about 70% of plant species using a single genetic marker [22,26,31–33] and to 90% resolution rates using a multi-locus barcode [34,35]. DNA barcoding will also prevent any bottlenecks in terms of expertise, given that laboratories capable of techniques in DNA extraction and Polymerase Chain Reaction (PCR) amplification can have the capability to genetically identify many plant species. DNA barcoding methods can circumvent visual identification methods. Fewer palynologists are needed to visually identify and barcoding will allow for rapid, species-specific identification, streamlining laboratory processes. While DNA barcoding shows a great deal of promise, it also has potential limitations.

One such limitation to using DNA barcoding for pollen identification in many applications is the presence of non-pollen plant DNA in samples. We refer to such non-pollen plant DNA as “contaminant” DNA hereafter. The presence of one or more contaminating species, such as anther or leaf fragments, in pollen samples can result in incorrect DNA barcoding results. Non-pollen plant material will likely have a higher chloroplast DNA copy number relative to pollen and will favor PCR amplification of the non-pollen material,

potentially overwhelming the genetic signature of pollen. Three examples underscore how such contaminants could reduce the effectiveness of—or even preclude the use of—DNA barcoding of pollen: 1) If drug enforcement agents wanted to identify the geographic origins of a cannabis sample, the pollen that has settled onto the cannabis sample from where it was grown can be identified via DNA barcoding. There is a signal of geographic origin from the pollen; however, the pollen is surrounded by leaf material from the cannabis. This non-pollen leaf DNA must be removed before the signal from the pollen can be identified via barcoding, otherwise the cannabis signal will overpower the pollen signal. 2) Alternatively, pollen DNA barcoding can be applicable in the provenance determination of fraudulent agricultural products, such as olive oil. Italian oil commands a higher price, so there is a large industry centered around fraudulent labeling of non-Italian oil, as Italian in order to gain a higher selling price. Olive oil can be illegally labeled as “extra virgin Italian” when it is actually of a lower grade from Tunisia [36,37]. Pollen could be extremely useful to track the origin of the olive oil, but only if all the olive DNA were removed. 3) The removal of contaminants can also impact pollination biology research. If collected stigmas of plants contain pollen from various plant species, researchers can identify the pollen with DNA barcoding. However, if the stigma material is not completely removed prior to sequencing, the stigma signal will overpower the pollen signal. Given that these contaminants may have significant negative impacts across many areas of research, it is important to take contamination control measures.

Pollen’s unique structure and resistance to degradation allows for potential methods to preserve pollen’s genetic integrity, while removing other non-pollen materials. The structural durability of pollen is due in part to its exine (outer shell), which is formed primarily of the biopolymer, sporopollenin. Sporopollenin is one of the most chemically resistant biological

compounds, acting as a shield that protects pollen from many forms of biological assailants, such as strong hydrolytic acids or bases, lipid solvents, and detergents [38,39]. Research aiming to dissolve the exine has only achieved this partially using chemicals such as sulfuric acid or strong oxidizing agents [39–43], which exemplifies sporopollenin's chemical resistance against a wide range of potential reagents. The exact biochemical pathways that lead to such resistance of sporopollenin are not yet fully understood [40]. By contrast, cellulose is the predominant material in leaf cell walls. Cellulose is a linear polysaccharide and while it is also a strong fibrous material, reinforced by hydrogen bonds, cellulose fibers can be at least partially dissolved by strong hydrolytic acids, bases, and cellulase enzymes [44–46]. For example, fungi in the genera *Trichoderma* and *Aspergillus* naturally produce cellulase that help them break down and digest the cellulose fibril linkages [47–50].

In this paper, I report on a series of experiments focused on developing a method to remove non-pollen plant DNA from samples, while keeping pollen DNA intact. Given the chemical properties of pollen and leaf materials, decontamination methods can take advantage of the chemical resistance of the pollen exine, while exploiting the vulnerability of leaf cell walls. Thus, this raises the question of whether or not all plant contaminants can be eliminated in pollen samples while maintaining the integrity of targeted pollen for identification. Other questions include: what chemical can be used to accomplish this and what is the shortest possible incubation time and process to accomplish this? I assessed different decontamination methods by incubating pollen and non-pollen material in five different chemical reagents and two enzymes, including hydrolytic acids and bases. I trialed combinations of various volumes and concentrations of chemical reagents and enzymes in order to determine the best method that would remove most of the non-pollen material, while preserving the pollen DNA.

MATERIAL AND METHODS

Overview

My goal was twofold: remove any non-pollen DNA, while retaining pollen DNA. I tested a variety of chemical reagents on pollen and non-pollen plant material (specifically, purified DNA, leaf fragments, anther filaments). I tested three types of samples: 1) Non-pollen material only; 2) pollen only; and 3) leaf and pollen samples mixed in a 1:1 ratio by weight.

My protocol development consisted of two steps. First, in preliminary trials, pollen only and non-pollen only samples were initially tested with four chemicals. The most efficacious of these chemicals was then used in the second step, primary trials, that also included subsequent enzymatic treatment. The leaf and pollen mixed samples were only tested in the primary trials. After chemical/enzymatic treatments, we extracted DNA from samples, amplified the DNA using Polymerase Chain Reaction (PCR), qualitatively visualized for bands on gel electrophoreses, and sequenced the PCR products using Sanger Sequencing. I analyzed data based on BLAST database matches of PCR product to pollen inputs, as well as the Quality scores (Q-scores) of the PCR products. If non-pollen removal were successful, ideally for pollen only and mixed samples, the results would show only pollen matches with high Q-scores. By contrast, in leaf only samples, ideally there would be no sequencing results, with Q-scores close to zero.

Pollen Collection

I collected pollen and leaf samples from Emory University campus, Lullwater Preserve and Hahn Woods. I also used commercial pollen samples (Sigma-Aldrich; St. Louis, MO and PolySciences; Warrington, PA) stored at 2 °C. Collected pollen samples were stored in 2 mL

centrifuge tubes at -20 °C for six months and for long term at -80 °C. Leaf samples were dried and stored in envelopes with silica gel at room temperature.

Chemicals Used

I tested four chemicals: concentrated bleach (sodium hypochlorite) (James Austin Company; Mars, PA), commercial DNA Exitus (PanReac Applichem; Darmstadt Germany), hydrochloric acid (Macron Fine Chemicals; Center Valley, PA) and sodium hydroxide (Avantor; Center Valley, PA). I tested two enzymes: cellulase from *Aspergillus* sp. (Sigma-Aldrich; St. Louis, MO) and DNase I (Sigma-Aldrich; St. Louis, MO).

Preliminary Chemical Treatment Trials

For initial trials, I used: pollen from *Hibiscus syriacus* L. (rose of Sharon; Malvaceae), *Lagerstroemia indica* L. (crepe myrtle; Lythraceae), *Ambrosia artemisiifolia* L. (common ragweed; Asteraceae) and leaf from *Hibiscus syriacus, Magnolia grandiflora* L. (southern magnolia; Magnoliaceae). To obtain pollen only samples, the collected anthers were vortexed for 30 seconds in 1 mL of ultrapure PCR water (Bioline; Taunton, MA) for pollen removal. Under the microscope, I removed pollen grains from the anthers with sterilized tweezers and placed them into 2mL centrifuge tubes. Tweezers were sterilized by soaking in concentrated bleach for one minute and then rinsed with ethanol. The isolated pollen samples were centrifuged at 17,500 rpm for two minutes and after the supernatant was removed, 200 µL of the remaining solution was used for chemical treatments. The amount of pollen grains added to each sample was not quantified. To obtain anther only samples, under the microscope, I cut approximately a 1 mm section of the anther filament with a sterilized scalpel and placed them into 2 mL centrifuge tubes. I examined the anther section under the microscope, and removed any remaining pollen grains that were attached. To obtain leaf only samples, I cut approximately a 1 mm section of the

dried leaf. To obtain purified DNA samples, I used 5 µL of *Hibiscus syriacus* DNA from pollen that had not been chemically treated. Pollen only samples, anther only, purified DNA only, and leaf only samples were incubated with 1500 µL of either: 1 M sodium hydroxide (NaOH), DNA Exitus, or 1 M hydrochloric acid (HCl) for one minute, ten minutes, thirty minutes, or one hour. Concentrated bleach was only tested on pollen only samples at concentrations of 1%, 2%, 3%, 4%, 5.25% for one minute, ten minutes, thirty minutes, or one hour. After incubation, samples that were incubated with hydrochloric acid were neutralized with equal volumes of sodium hydroxide, and vice versa. Samples were then washed with 500 µL of PCR water five times and centrifuged at 17,500 rpm to remove all supernatant. I replicated all treatment combinations in triplicate.

Primary Chemical Treatment Trials

I used commercial pollen: *Populus deltoides* Batram ex Marshall (Salicaceae), *A. artemisiifolia* and leaf from *M. grandiflora* and *Lactuca sativa* L. (lettuce; Asteraceae) for the primary trials after determination from the preliminary trials that sodium hydroxide least affected pollen exine and caused the most degradation in leaf samples. To obtain leaf samples, dried leaf was ground into a powder using a sterilized mortar and pestle, with DNA free sand (MP Biomedical; Solon, OH) in order to enhance grinding efficacy. For leaf samples, 0.01 grams of leaf powder was weighed and placed into 2 mL micro-centrifuge tubes. For pollen samples, 0.01 grams were weighed and placed into micro-centrifuge tubes. For pollen and leaf samples, 0.01 grams of each were combined. Samples were treated with a one-hour incubation in 1500 µL of 2 M NaOH or 4 M NaOH. After the one-hour incubation, I centrifuged the samples at 17,500 rpm. I removed 750 µL of NaOH and added an equal volume (750 µL) of equimolar hydrochloric acid to neutralize. Samples were inverted three times and centrifuged to remove supernatant. Samples

were washed one time with 500 µL of PCR water. Samples were centrifuged and supernatant was removed. Then, 24 µL of cellulase was added along with 24 µL of 8 M Tris HCl buffer and incubated for one hour at 80 °C. Samples were centrifuged and cellulase removed. Then, I added 3 µL or 6 µL of DNase I enzyme, with an equal volume of Reaction buffer (Sigma-Aldrich; St. Louis, MO), and three times the volume of ultrapure PCR water. Samples were incubated at room temperature for 15 minutes. Then, an equal volume of Stop buffer (Sigma-Aldrich; St. Louis, MO) was added and samples were incubated at 70 °C.

DNA Isolation

Following chemical treatments, all samples were disrupted with the Mini-Beadbeater (BioSpec; Bartlesville, OK) for three minutes and DNA was extracted with the FastDNA SPIN Soil Kit (MP Biomedical; Solon, OH). Negative isolation controls were done to ensure that there was no contamination from airborne pollen or other plant material. Positive controls with plant and pollen material without chemical treatments were also done to ensure that DNA could be extracted and amplified.

DNA Amplification, Visualization and Sequencing

I amplified the *rbcL* gene of extracted samples via Polymerase Chain Reaction (PCR) with 0.5 µL of *rbcL*a-F [51] and 0.5 µL of *rbcL*a-R [52] primers, 12.5 µL of 1x MyTaq polymerase (Bioline; Taunton, MA), 6.5 µL of ultrapure PCR water, and 5 µL of extracted DNA. The *rbcL* gene is one of two consensus genetic markers for DNA barcoding of plants [53,54]. Samples were run on Eppendorf Nexus Gradient MasterCycler (Hamburg, Germany) with the conditions: five minutes at 95 °C, 35 cycles of 30 seconds at 95 °C, 30 seconds at 55 °C, one minute at 72 °C, and final DNA extension of ten minutes at 72 °C. Samples were run at 35 cycles.

186 The amplified reactions were run on a 1.5% Agarose gel with GelRed (Biotium; Hayward, CA) at 120 volts for 30 minutes. The amplified reactions were sent to Beckman 188 Coulter Genomics (Danvers, MA) to be Sanger sequenced using ABI 3730XL DNA sequencers (Applied Biosystems; Foster City, CA).

190 *DNA Searches using Geneious*

Sequences were aligned using the pairwise alignment function in Geneious (Biomatters; 192 Auckland, New Zealand) and trimmed to equal coverage. The trimmed forward and reverse sequences for each sample were assembled using the *de novo* function in Geneious. The Q- 194 scores for each forward and reverse sequence were recorded post trimming and before assembly. The assembled sequences were searched through GenBank BLAST (www.ncbi.nlm.nih.gov).

196 *Data Analysis*

Preliminary trials

198 I analyzed the preliminary trials based on qualitative intensity of gel electrophoresis bands (which indicate the quantity of DNA present at a particular base pair length). I classified band 200 intensities into four ordinal categories: no bands, faint bands, intermediate bands, and bright bands. In preliminary trials, there were three main explanatory variables: sodium hydroxide, 202 commercial DNA Exitus, and hydrochloric acid with different incubation times (one minute, ten minutes, thirty minutes, and one hour).

204 I analyzed the explanatory and response variables in data subsets of: Pollen Only, Leaf Only, Anther Only, and Purified DNA Only. In the Pollen Only subset, I also analyzed an 206 additional explanatory variable: the presence of bleach. Within each data subset, I analyzed the response variables using calculated proportions in Excel and an ordinal logistic regression model 208 (OLR) in the *R* statistical computing program [55].

Primary trials

I organized the primary trials into three subsets of data: Pollen Only, Leaf Only, and Leaf/Pollen. I analyzed two quantitative response variables in the primary trials. The first variable was the presence or absence of a BLAST sequence match to the GenBank database that matched the tested pollen or leaf species. The second response variable was the sequence quality using Q-scores. Q-scores are standardized measurements of confidence in sequence determinations and are numerical estimates of error probabilities for a given base pair [56–58]. I also conducted another OLR for the primary trial band intensities, using the same ordinal categories for band intensities as the preliminary trials.

To assess the proportion of species-level matches, I used a binomial-errors generalized linear model (GLM) in *R.* I tested this model only with the Leaf/Pollen data subset, because in the Pollen Only and Leaf Only subset, the sequence always matched the tested species. In *R,* the sequence results were coded as "0" for no pollen match or "1" for matching the pollen sequence. I then ran the GLM for all sequences within the Leaf/Pollen data subset.

For analyses of Q-score quality, I conducted a linear model (LM) for the Pollen Only and Leaf/Pollen subsets. To meet the assumption of normality, I first calculated the average of the forward and reverse Q-scores for each replicate. I then removed all average Q-scores of zero (one sample in Pollen Only, and twelve samples in Leaf/Pollen) and logit-transformed the remaining mean Q-scores divided by 100, since the scores vary from 1-100. Subsequent quantile-quantile plots and residual vs. fitted-value plots confirmed that the data met the assumptions of linear models.

RESULTS

Overview

I conducted a total of seven preliminary trials (n=105) and five primary trials (n=157). In the preliminary trials, I analyzed the effects of bleach, sodium hydroxide, Exitus, and hydrochloric acid on Pollen Only (n=33), Purified DNA Only (n=10), Anther Only (n=19), and Leaf Only (n=21) data subsets and 13 negative controls and 9 positive controls. In the primary trials, I analyzed the effects of sodium hydroxide (concentration and incubation time), cellulase, and DNase I on Pollen Only (n=41), Leaf Only (n=27), and Leaf/Pollen (n=62) data subsets and 11 negative controls and 16 positive controls.

Preliminary Results

In the Pollen Only subset with bleach treatments, in any of the triplicates of each combination of concentration and incubation time, there were no PCR products observed on the agarose gel. This showed that even at low concentrations, for a short incubation time, bleach was successful in removing all DNA from pollen. In the Purified DNA Only subset with the three chemical treatments (sodium hydroxide, Exitus, hydrochloric acid), there were also no bands in any triplicates of each chemical combination. This showed that all three chemicals tested were efficient at removing purified DNA.

I filtered the data to examine individual data subsets of Pollen Only, Leaf Only, and Anther Only. For each chemical, I calculated the total number of bands, for each type of intensity. I then divided each raw count by the sample size used for each chemical. This allowed me to compare each category of band intensity across all three chemicals for each of the data subsets. In the Pollen Only subset, there were similar proportions of intermediate bands across all three chemical treatments, with the hydrochloric acid treatment showing the highest proportion

of replicates having no bands (Table 1). This indicated that none of the chemicals were successful in destroying all of the pollen DNA. In the Leaf Only subset, sodium hydroxide treatments resulted in the highest proportion of replicates having no bands (Table 2). In the Anther Only subset, hydrochloric acid treatments resulted in the highest proportion of replicates having no bands (Table 3). I also conducted an ordinal logistic regression test comparing band intensity and chemicals. Overall, I found that sodium hydroxide had a significant effect on lowering band intensity ($p=0.0010$) (Table 4). I also conducted OLR tests on incubation times for the three chemicals, but found no significant effects.

Table 1. Pollen Only data subset shows similar proportions of band intensities for intermediate bands across all chemicals. Raw band counts are in parentheses.

Pollen Only	**NaOH**	**Exitus**	**HCl**
Bright bands	0	0	0.13(2)
Intermed bands	0.78(7)	0.89(8)	0.40(6)
Faint bands	0	0	0.13(2)
No bands	0.22(2)	0.11(1)	0.33(5)

Table 2. Leaf Only data subset shows the largest proportion of no bands for the sodium hydroxide treatments. Raw band counts are in parentheses.

Leaf Only	**NaOH**	**Exitus**	**HCl**
Bright bands	0	0.33(2)	0.44(4)
Intermed bands	0	0	0.22(2)
Faint bands	0.33(2)	0.67(4)	0.11(1)
No bands	0.67(4)	0	0.22(2)

Table 3. Anther Only data subset shows the largest proportion of no bands for the hydrochloric acid treatments. Raw band counts are in parentheses.

Anther Only	NaOH	Exitus	HCl
Bright bands	0.17(1)	0.50(3)	0
Intermed bands	0	0	0
Faint bands	0	0	0
No bands	0.83(5)	0.50(3)	1(7)

Table 4. Ordinal logistic regression on band intensity with all three explanatory variables shows sodium hydroxide has a significant effect in lowering band intensity (p=0.0010).

OLR	Estimate	Std. Error	Z-value	P-value
NaOH	-1.4	0.5	-2.58	0.0010
Exitus	-0.7	0.5	-1.26	0.21
HCl	-0.3	0.4	-0.62	0.53

Primary Results

In the Leaf/Pollen data subset, I found a statistically significant effect of cellulase on decreasing the sequences that matched (p=0.00083, df=46). There were was one sequence that matched with cellulase added and eleven sequences that matched without cellulase added. There was no significant effect of NaOH concentration or incubation times (Table 5). There were only two observations in which I had increased the DNase volume to 6 µL; I removed this as an explanatory variable in the binomial GLM. In Pollen Only trials, I found that cellulase, DNase volume, and NaOH incubation time/concentration did not have a significant effect on sequence quality (Q-scores) (Table 6). In the Leaf/Pollen subset, I found a marginally significant effect of sodium hydroxide incubation time on the sequence quality ((p=0.073, df=8) (Table 7). There was also a significant effect of cellulase on the band intensity (p=0.0025) (Table 8).

Table 5. Binomial GLM for Leaf/Pollen samples showed that cellulase had a significant effect on decreasing the pollen sequence match (p=0.00083, df=46).

Binomial GLM for Leaf/Pollen	**Estimate**	**Std. Error**	**Z-value**	**P-value**
Cellulase	-4.3	1.3	-3.34	0.00083
NaOH incubation time	1.1	1.3	0.84	0.40
NaOH concentration	0.7	0.9	0.75	0.45

Table 6. Linear model for Pollen Only subset showed no significant effect of explanatory variables on Q-score quality (df=25).

Linear model for Pollen Only	**Estimate**	**Std. Error**	**Z-value**	**P-value**
Cellulase	0.4	0.3	1.41	0.17
NaOH incubation time	-0.1	0.5	-0.32	0.75
NaOH concentration	-0.1	0.1	-0.52	0.61
DNase I volume	-0.03	0.1	-0.37	0.72

Table 7. Linear model for Leaf/Pollen samples show a marginally significant difference between my explanatory variables on having a difference in the Q score quality (p=0.073, df=8).

Linear model for Leaf/Pollen	**Estimate**	**Std. Error**	**Z-value**	**P-value**
Cellulase	-0.07	1.1	-0.065	0.95
NaOH incubation time	1.2	0.6	2.03	0.073
NaOH concentration	1.1	0.7	1.62	0.14

Table 8. The OLR model for all primary trial samples showed cellulase had a significant effect on band intensity (p=0.0025).

Ordinal Logistic Regression	**Estimate**	**Std. Error**	**Z-value**	**P-value**
Cellulase	-1.3	0.4	-3.02	0.0025
NaOH incubation time	0.2	0.6	0.24	0.81
NaOH concentration	0.2	0.2	1.20	0.23

DISCUSSION

Overview

The many applications of pollen DNA barcoding are far-reaching, extending into realms from forensic investigations to pollination biology. In many applications, such as provenance tracking of agricultural products, there is a strong possibility of contamination of pollen samples with non-pollen plant material, which is why it is essential to find a method to first remove all non-pollen DNA.

Preliminary Trials

The preliminary trial results showed that bleach disrupted pollen exines and removed all pollen DNA, even at a relatively low concentration of 1% and a short incubation time of one minute. In visual studies examining exine solubility from different chemicals, the exine was found to have completely dissolved after a one to two hour incubation with bleach [39]. Bleach is also known to destroy DNA via oxidative damage and cleavage of the DNA strands [59–61]. Although this result is counter to the project goal, it is a useful finding in that bleach can be used as a reagent to sterilize all lab benches and external equipment surfaces (i.e., pipettes, centrifuge tube racks, lab coats, etc.) in order to minimize cross-contamination with pollen in between experiment trials. Bleach could also be used to decontaminate lab equipment that may become contaminated with airborne pollen grains from tubes containing intact or freshly collected pollen grains that are open near lab benches, or from the environment. Thus, sterilizing lab benches and equipment with dilute bleach for at least one minute will ensure all equipment is free of pollen grains or pollen DNA fragments.

All three chemicals tested (sodium hydroxide, Exitus, and hydrochloric acid) removed all purified DNA. These results implied that if the chemicals were able to reach beyond DNA

protective barriers, such as plant cell walls or pollen exines, they would be capable of destroying all DNA present. DNA is extremely vulnerable to pH changes, and in the presence of acids and bases, DNA can denature, compromising the structural integrity of the double helix [62,63]. Although the exact chemical composition of commercial DNA Exitus is not made public, Exitus has been shown to remove DNA contaminants as effectively as 10% bleach solutions [64]. Thus, if the known contaminant in pollen samples is purified DNA fragments, then any of the chemicals I tested is potent enough in removing the unprotected DNA. If the contaminant in pollen samples is other plant material, such as anther filaments or leaf fragments, my results suggest that the best chemical for removing leaf fragments is sodium hydroxide and hydrochloric acid for removing anther filaments.

Primary Trials

Based on the statistically significant effect of sodium hydroxide treatments on lowering band intensity, sodium hydroxide was chosen as the chemical to use in primary trials with the addition of cellulase and DNase I enzymes. Research has been conducted to discover which types of reagents will best dissolve cellulose. In these studies, hydrochloric acid was found to partially dissolve cellulose and sodium hydroxide was found to cause cellulose fibers to swell [20-22]. Although hydrochloric acid does have an effect on cellulose, I chose only to focus my primary trials on using sodium hydroxide in combination with enzymes, based on the preliminary trial results. I chose to add the cellulase enzyme after samples were treated with sodium hydroxide with the assumption that the sodium hydroxide incubation will weaken the integrity of the cell walls. Cellulase will then act as a cleaver and hydrolyze the fiber linkages [65]. This will break down the cell walls, subsequently allowing the DNase enzyme access to the DNA inside of the cells [66].

Based on my primary results and raw data counts, cellulase has a statistically significant effect on the resulting sequences of leaf/pollen samples, decreasing the number of matches to the pollen species. This result is not expected and implies that cellulase could have the potential in harming the pollen DNA integrity. This could stem from the fact that pollen's intine (inner layer) is composed primarily of cellulose [67]. When pollen lands on the stigma and produces a pollen tube, the pollen will produce some cellulase and other enzymes to soften and degrade the pollen wall [49,68]. In the two species of pollen used in the primary trials, cellulase could have gotten past the exine and degraded part of the intine. The subsequent DNase I enzyme that was added then could have digested some of the pollen DNA. Furthermore, cellulase and sodium hydroxide concentration/incubation time did not have a statistically significant effect on Q-score quality of the Pollen Only subset. This shows that the pollen exine was able to stay intact despite these chemical attacks, without compromising the pollen's genetic integrity.

Caveats and Future Directions

Although my trials had limited species richness and phylogenetic diversity, the applications of this experiment can be expanded to other plant clades such as, gymnosperms or ferns in the future. The integrity of non-pollen material and pollen may vary across clades and within clades. For example, the leaves of *Magnolia grandiflora,* from the magnoliid clade, have a particularly thick and waxy cuticle (pers. obs.), which may lessen the potency of any chemical treatments that attempt to degrade the cell wall. Pollen exine chemical resistance may also vary across clades. For example, *Ambrosia artemisiifolia* pollen from the asterid clade has many spines on the exine, while *Zea mays* L. (Poaceae) pollen from the commelinid clade has particularly large pollen (pers. obs.). These structural differences may have an effect on the

integrity of the exine in the presence of chemical treatments. In future trials, various combinations of leaf and pollen from all clades should be tested.

The relatively small sample size across the preliminary and primary trials may have affected the statistical significance of certain results. For example, in the linear model of the Q-score quality for Leaf/Pollen samples, sodium hydroxide incubation had a marginal significance. If the sample size was increased, there may have been greater statistical power. Similarly, in preliminary trials, although hydrochloric acid seemed to have an effect on the absence of bands in the Anther Only subset, ultimately hydrochloric did not have a statistically significant effect on band intensity. If sample size were increased in the initial hydrochloric acid treatments, there may be a change in statistical significance.

A wider variety of non-pollen plant material could also be tested, such as stigmas or flower petals. In the primary trials, the only non-pollen material tested was leaf. Since most of this project was completed during winter and fall, there was not a chance to collect anther filaments or other plant materials for use in the primary trials. Additionally, while many combinations of chemical treatment incubation times and concentrations were tested, more combinations with a focus on cellulase should be tested. The question of whether cellulase damages the pollen DNA integrity should be further explored in trials with larger sample sizes, more pollen and leaf species, and perhaps a higher molarity of sodium hydroxide or a larger volume of cellulase. Hydrochloric should also be tested in lieu of sodium hydroxide to examine if hydrochloric can preferentially damage the leaf DNA in mixed pollen and leaf samples.

In future trials, additional genetic markers can also be used to increase taxonomic resolution of plant species identification. In this experiment, I used a single locus genetic marker

406 (*rbcL*) on the plastid genome. Future trials can include multi-locus markers (*rbcl+matK)* on the plastid genome or other genetic markers on the nuclear genome [53,54].

408 *Conclusion*

This project found that the use of sodium hydroxide in combination with DNase I 410 enzymes in pollen samples contaminated with non-pollen material is a potentially useful method in removing non-pollen materials for pollen DNA barcoding. While this is not the perfect 412 chemical combination that successfully removes all non-pollen plant DNA, the results of this project can be used as a foundation from which other chemicals can be tested. Improving 414 contaminant removal methodologies can tap into the power of pollen DNA barcoding and dramatically improve applications in pollen identifications across many fields of research.

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REFERENCES

1. Bryant VM, Jones GD. Forensic palynology: Current status of a rarely used technique in the United States of America. Forensic Sci Int. 2006;163: 183–197. doi:10.1016/j.forsciint.2005.11.021
2. Mathewes RW. Forensic palynology in Canada: an overview with emphasis on archaeology and anthropology. Forensic Sci Int. 2006;163: 198–203. doi:10.1016/j.forsciint.2006.06.069
3. Walsh KAJ, Horrocks M. Palynology: Its position in the field of forensic science. J Forensic Sci. 2008;53: 1053–1060. doi:10.1111/j.1556-4029.2008.00802.x
4. Wiltshire PEJ. Consideration of some taphonomic variables of relevance to forensic palynological investigation in the United Kingdom. Forensic Sci Int. 2006;163: 173–182. doi:10.1016/j.forsciint.2006.07.011
5. Horrocks M, Walsh K a J. Forensic palynology: Assessing the value of the evidence. Rev Palaeobot Palynol. 1998;103: 69–74. doi:10.1016/S0034-6667(98)00027-X
6. Horrocks M, Coulson S a., Walsh K a. J. Forensic Palynology : Variation in the Pollen Content of Soil on Shoes and in Shoeprints in Soil. J Forensic Sci. 1999; 119–122.
7. Heimhofer U, Hochuli PA, Burla S, Dinis JML, Weissert H. Timing of Early Cretaceous angiosperm diversification and possible links to major paleoenvironmental change. Geology. 2005;33: 141–144. doi:10.1130/G21053.1
8. Tareq SM, Tanaka N, Ohta K. Biomarker signature in tropical wetland: Lignin phenol vegetation index (LPVI) and its implications for reconstructing the paleoenvironment. Sci Total Environ. 2004;324: 91–103. doi:10.1016/j.scitotenv.2003.10.020
9. Hylander T, Larsson O, Petersson-Westin U, Eriksson M, Kumlien Georén S, Winqvist O, et al. Intralymphatic immunotherapy of pollen-induced rhinoconjunctivitis: a double-blind placebo-controlled trial. Respir Res. Respiratory Research; 2016;17: 10. doi:10.1186/s12931-016-0324-9
10. Durham SR, Walker SM, Varga EM, Jacobson MR, Brien FO, Noble W, Till SJ, Hamid QA N-AQ. Long-term clinical efficacy of grass-pollen immunotherapy. New Engl J Med. 1999;365: 781–783.
11. Nouri-Aria KT, Wachholz P a, Francis JN, Jacobson MR, Walker SM, Wilcock LK, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. J Immunol. 2004;172: 3252–9. doi:10.4049/jimmunol.172.5.3252
12. Durham SR, Ying S, Varney VA, Jacobson MR, Sudderick RM, Mackay IS, et al. Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-gamma. J Allergy Clin Immunol. 1996;97: 1356–65. doi:S0091-6749(96)70205-1 [pii] ET - 1996/06/01
13. Haydak M. Honey Bee Nutrition1. Annu Rev Entomol. 1970.15:143-156
14. Pernal SF, Currie RW. Pollen quality of fresh and 1-year-old single pollen diets for

worker honey bees (*Apis mellifera* L.). Apidologie. 2000;31: 387–409. doi:10.1051/apido:2000130

15. Mattila HR, Otis GW. Influence of pollen diet in spring on development of honey bee (Hymenoptera: Apidae) colonies. J Econ Entomol. 2006;99: 604–613. doi:10.1603/0022-0493-99.3.604
16. Lopezaraiza-Mikel ME, Hayes RB, Whalley MR, Memmott J. The impact of an alien plant on a native plant-pollinator network: An experimental approach. Ecol Lett. 2007;10: 539–550. doi:10.1111/j.1461-0248.2007.01055.x
17. Olesen JM, Jordano P. Geographic Patterns in Plant-Pollinator Mutualistic Networks Authors. Ecology. 2002;83: 2416–2424.
18. Briggs H, Dobbs EK, Brosi BJ. Heterospecific pollen deposition in *Delphinium barbeyi* : linking stigmatic pollen loads to reproductive output in the field. 2015; 341–347. doi:10.1093/aob/mcv175
19. Morales CL, Traveset A. Interspecific Pollen Transfer: Magnitude, Prevalence and Consequences for Plant Fitness. CRC Crit Rev Plant Sci. 2008;27: 221–238. doi:10.1080/07352680802205631
20. Mitchell RJ, Irwin RE, Flanagan RJ, Karron JD. Ecology and evolution of plant-pollinator interactions. Ann Bot. 2009;103: 1355–1363. doi:10.1093/aob/mcp122
21. Ashman TL, Arceo-Gómez G. Toward a predictive understanding of the fitness costs of heterospecific pollen receipt and its importance in co-flowering communities. Am J Bot. 2013;100: 1061–1070. doi:10.3732/ajb.1200496
22. Bell KL, Burgess KS, Okamoto KC, Aranda R, Brosi BJ. Review and future prospects for DNA barcoding methods in forensic palynology. Forensic Sci Int Genet. Elsevier Ireland Ltd; 2016;21: 110–116. doi:10.1016/j.fsigen.2015.12.010
23. Holt KA, Bennett KD. Principles and methods for automated palynology. New Phytol. 2014;203: 735–742. doi:10.1111/nph.12848
24. Darrow LA, Hess J, Rogers CA, Tolbert PE, Klein M, Sarnat SE. Ambient pollen concentrations and emergency department visits for asthma and wheeze. J Allergy Clin Immunol. Elsevier Ltd; 2012;130: 630–638.e4. doi:10.1016/j.jaci.2012.06.020
25. Hebert PDN, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. Proc Biol Sci. 2003;270: 313–21. doi:10.1098/rspb.2002.2218
26. CBOL Plant Working Group, Hollingsworth PM, Forrest LL, Spouge JL, Hajibabaei M, Ratnasingham S, et al. A DNA barcode for land plants. Proc Natl Acad Sci U S A. 2009;106: 12794–7. doi:10.1073/pnas.0905845106
27. Galimberti A, De Mattia F, Bruni I, Scaccabarozzi D, Sandionigi A, Barbuto M, et al. A DNA barcoding approach to characterize pollen collected by honeybees. PLoS One. 2014;9. doi:10.1371/journal.pone.0109363
28. Hawkins J, de Vere N, Griffith A, Ford CR, Allainguillaume J, Hegarty MJ, et al. Using DNA Metabarcoding to Identify the Floral Composition of Honey: A New Tool for Investigating Honey Bee Foraging Preferences. PLoS One. Public Library of Science;

2015;10: e0134735. doi:10.1371/journal.pone.0134735
29. Kraaijeveld K, de Weger LA, Ventayol Garc??a M, Buermans H, Frank J, Hiemstra PS, et al. Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. Mol Ecol Resour. 2015;15: 8–16. doi:10.1111/1755-0998.12288
30. Richardson RT, Lin C-H, Quijia JO, Riusech NS, Goodell K, Johnson RM. Rank-based characterization of pollen assemblages collected by honey bees using a multi-locus metabarcoding approach. Appl Plant Sci. Botanical Society of America; 2015;3. doi:10.3732/apps.1500043
31. Ferri G, Corradini B, Ferrari F, Santunione AL, Palazzoli F, Alu' M. Forensic botany II, DNA barcode for land plants: Which markers after the international agreement? Forensic Sci Int Genet. Elsevier Ireland Ltd; 2015;15: 131–136. doi:10.1016/j.fsigen.2014.10.005
32. Spooner DM. Dna barcoding will frequently fail in complicated groups: An example in wild potatoes. Am J Bot. 2009;96: 1177–1189. doi:10.3732/ajb.0800246
33. Seberg O, Petersen G. How many loci does it take to DNA barcode a crocus? PLoS One. 2009;4: 2–7. doi:10.1371/journal.pone.0004598
34. Kress WJ, Erickson DL, Jones FA, Swenson NG, Perez R, Sanjur O, et al. Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. Proc Natl Acad Sci U S A. 2009;106: 18621–6. doi:10.1073/pnas.0909820106
35. Burgess KS, Fazekas AJ, Kesanakurti PR, Graham SW, Husband BC, Newmaster SG, et al. Discriminating plant species in a local temperate flora using the rbcL+matK DNA barcode. Methods Ecol Evol. 2011;2: 333–340. doi:10.1111/j.2041-210X.2011.00092.x
36. Woolfe M, Primrose S. Food forensics: Using DNA technology to combat misdescription and fraud. Trends Biotechnol. 2004;22: 222–226. doi:10.1016/j.tibtech.2004.03.010
37. Vossen P. Olive oil: History, production, and characteristics of the world's classic oils. HortScience. 2007;42: 1093–1100.
38. Brooks J, Shaw G. Sporopollenin: A review of its chemistry, palaeochemistry and geochemistry. Grana. 1978;17: 91–97. doi:10.1080/00173137809428858
39. Southworth D. Solubility of pollen exines. Am J Bot. 1974;61: 36–44. doi:10.2307/2441242
40. Domínguez E, Mercado JA, Quesada MA, Heredia A. Pollen sporopollenin: Degradation and structural elucidation. Sex Plant Reprod. 1999;12: 171–178. doi:10.1007/s004970050189
41. Tarlyn NM, Franceschi VR, Everard JD, Loewus FA. Recovery of exine from mature pollens and spores. Plant Sci. 1993;90: 219–224. doi:10.1016/0168-9452(93)90243-S
42. Jungfermann C, Ahlers F, Grote M, Gubatz S, Steuernagel S, Thom I, et al. Solution of sporopollenin and reaggregation of a sporopollenin-like material: A new approach in the sporopollenin research. J Plant Physiol. Gustav Fischer Verlag, Jena; 1997;151: 513–519. doi:10.1016/S0176-1617(97)80224-6
43. Southworth D. Cytochemical Reactivity of Pollen Walls. J Histochem Cytochem.

1973;21: 73–80. doi:10.1177/21.1.73
44. Isogai a, Atalla RH, Service F. Dissolution of cellulose in aqueous NaOH solutions. Solutions. 1998;5: 309–319. doi:10.1023/A:1009272632367
45. Narasimha G, Sridevi A, Buddolla Viswanath, Subhosh Chandra M RRB. Nutrient effects on production of cellulolytic enzymes by Aspergillus niger.pdf. African J Biotechnol. 2006;5(5): 472–476.
46. Baldan B, Andolfo P, Navazio L, Tolomio C, Mariani P. Cellulose in algal cell wall : An "in situ" localization. Eur J Histochem. 2001;45: 51–56. doi:10.4081/1613
47. Sohail M, Siddiqi R, Ahmad A, Khan SA. Cellulase production from Aspergillus niger MS82: effect of temperature and pH. N Biotechnol. 2009;25: 437–41. doi:10.1016/j.nbt.2009.02.002
48. S.Siva Sakthi PS and MR. Optimization For Cellulase Production By Aspergillus niger Using Paddy Straw As Substrate. Int J Adv Sci Tech Res. 2011;1: 4147–4152. doi:10.5897/AJB08.689
49. Konar RN, Stanley RG. Wall-softening enzymes in the gynoecium and pollen of Hemerocallis fulva. Planta. 1969;84: 304–10. doi:10.1007/BF00396422
50. Roggen HP, Stanley RG. Cell-wall-hydrolysing enzymes in wall formation as measured by pollen-tube extension. Planta. 1969;84: 295–303. doi:10.1007/BF00396421
51. Levin RA, Wagner WL, Hoch PC, Nepokroeff M, Pires JC, Zimmer EA, et al. Family-level relationships of Onagraceae based on chloroplast rbcL and ndhF data. Am J Bot. 2003;90: 107–15. doi:10.3732/ajb.90.1.107
52. Kress WJ, Erickson DL. A Two-Locus Global DNA Barcode for Land Plants: The Coding rbcL Gene Complements the Non-Coding trnH-psbA Spacer Region. Shiu S-H, editor. PLoS One. 2007;2: e508. doi:10.1371/journal.pone.0000508
53. Hollingsworth PM, Graham SW, Little DP. Choosing and using a plant DNA barcode. PLoS One. 2011;6: e19254. doi:10.1371/journal.pone.0019254
54. Group CPW, Janzen DH. A DNA barcode for land plants. Proc Natl Acad Sci U S A. 2009;106: 12794–12797. doi:10.1177/0022002705277548
55. Christensen RHB. Regression Models for Ordinal Data [Internet]. 2015. Available: http://www.cran.r-project.org/package=ordinal/
56. Drummond AJ, A.B., Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T WA. Geneious Pro v5.4 [Internet]. 2011. Available: http://www.geneious.com/.
57. Shore S, Ashrafi E, Paul N. Hot Start 7-Deaza-dGTP Improves Sanger Dideoxy Sequencing Data of GC-Rich Targets, DNA Sequencing - Methods and Applications, Dr. Anjana Munshi (Ed.),. In: intechopen.com [Internet]. Available: http://www.intechopen.com/books/dna- sequencing-methods-and-applications/hot-start-7-deaza-dgtp-improves-sanger-dideoxy-sequencing-data-of- gc-rich-targets
58. Ewing B, Hillier LD, Wendl MC. Base-Calling of Automated Sequencer Traces UsingPhred. I. Accuracy Assessment. Genome Res. 1998; 186–194.

doi:10.1101/gr.8.3.186
59. Kemp BM, Smith DG. Use of bleach to eliminate contaminating DNA from the surface of bones and teeth. Forensic Sci Int. 2005;154: 53–61. doi:10.1016/j.forsciint.2004.11.017
60. Whiteman M, Jenner A, Halliwell B. Hypochlorous acid-induced base modifications in isolated calf thymus DNA. Chem Res Toxicol. 1997;10: 1240–1246. doi:10.1021/tx970086i
61. Ohnishi S, Murata M, Kawanishi S. DNA damage induced by hypochlorite and hypobromite with reference to inflammation-associated carcinogenesis. Cancer Lett. 2002;178: 37–42. doi:10.1016/S0304-3835(01)00812-6
62. Ageno M, Dore E, Frontali C. The alkaline denaturation of DNA. Biophys J. 1969;9: 1281–1311. doi:10.1016/S0006-3495(69)86452-0
63. Lindahl T. Instability and decay of the primary structure of DNA. Nature. 1993;362: 709–715. doi:10.1038/362709a0
64. Arena A. Dna exitus plus ™ versus standard bleach solution for the removal of dna contaminants on work surfaces and tools. Investig Sci J. 2010;2: 20–29.
65. Harry J. Gilbert GPH. Review Article Bacterial cellulases and xylanases. 2016; 187–194. Available: http://citeseerx.ist.psu.edu/viewdoc/summary?doi=10.1.1.322.139
66. Laskowski M. Deoxyribonuclease I [Internet]. The Enzymes. Elsevier; 1971. doi:10.1016/S1874-6047(08)60372-8
67. Heslop-Harrison Y, Heslop-Harrison J. The Microfibrillar Component of the Pollen Intine : Some Structural Features. Ann Bot. 1982;50: 831–842.
68. Wang X-Y, Guo G-Q, Nie X-W, Zheng G-C. Cytochemical localization of cellulase activity in pollen mother cells of david lily during meiotic prophase I and its relation to secondary formation of plasmodesmata. Protoplasma. 1998;204: 128–138. doi:10.1007/BF01280319