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Are Mussel Shells Environmental DNA Time Capsules? A Comparison Of Extraction Methods For Obtaining DNA From Shell Material.

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BY

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ABSTRACT

Freshwater mussels have become some of the most imperiled species in North America and widespread populations have succumbed to pollution and many other anthropogenic-related factors. With molecular techniques evolving, a recent interest in ancient DNA and museum specimens has emerged and prompted a study to test the ability of several extraction methods to isolate DNA from museum mussel specimens. The purpose of this study was to determine if four DNA extraction methods had influence on total DNA yield (ng/mg) from mussel tissue. The hinge ligaments of freshwater mussels ranging in collection date (1984-2015) were used as the source of genetic material for this study. Additionally, collection date was tested for influence on the total DNA yield. An interaction between collection year and extraction method was also explored. A total of 40 hinge ligaments were removed from dried museum shells and subjected to four different DNA extraction methods. Total DNA yield (ng/mg) from the extractions was quantified using a Qubit 3.0 Fluorometer and a Nanodrop 2000. A modified CTAB extraction method was found to be statistically higher for extracting total DNA compared to the other three methods. This suggests that chloroform-based extractions may be optimal for DNA extraction from historic museum specimens containing fragile and degraded DNA. Future research will be necessary to determine the origin of DNA from the extracted genetic material. Now, with a more optimized extraction method, the hinge ligaments from shells stored in museums can be used for extraction of host DNA and potentially eDNA released from other organisms.

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1. INTRODUCTION

Freshwater mussels are known to play an important biological role in the freshwater ecosystem specifically in nutrient cycling and biodeposition processes, which provide clean water and food for many other fauna. These bivalves are also known to aid in the removal of algae through filtering processes (Howard & Cuffey 2006; Nalepa et al., 1991; Vaughn et al., 2004;). Additionally, mussel beds provide substrate stability and a home to many other aquatic organisms. Therefore, the decline of mussel populations could be detrimental to many other freshwater fauna and could negatively impact entire freshwater ecosystems (Haag 2012; Lydeard et al., 2004).

Freshwater mussels have become some of the most imperiled species in North America and are constantly facing many anthropogenic-related hardships that affect their stability in many aquatic ecosystems (Strayer et al., 2004; Williams et al., 1993; Walker et al., 2014). Due to their sedentary and filter-feeding lifestyles, freshwater mussels are extremely sensitive to rapid environmental and climatic changes, and can perish easily during rapid habitat disturbances. Over 30 North American mussel taxa have become extinct within the last 100 years and it is estimated that approximately 65% of the remaining taxa are endangered, threatened, or vulnerable (Haag & Williams 2014). These serious deteriorations of mussel populations can be attributed to many reasons; loss of overall habitat, pollution, fish host reduction, and many other anthropogenic causes. The loss of aquatic habitats often occurs through habitat fragmentation and degradation and in many waterways a large proportion of pollution comes from many industrial and farming practices (Haag & Williams 2014; Mock et al., 2010; Makhrov et al., 2014). The reduction of fish can affect the distribution of many

mussel species, as most juvenile mussels are obligate parasites to fish during their early stages of life and thus require fish hosts to continue their life cycle (Haag & Warren 1998; Schwalb et al., 2013). The loss of these important fish hosts can drastically interrupt the life cycle of freshwater mussels. Other anthropogenic-related factors such as construction of bridges, dams and highways can affect the overall health and distribution of freshwater mussels. Because of serious deteriorations in many freshwater mussel populations, finding a live mussel during field surveys can be very difficult, particularly in cases of rare and evasive species.

However, even when these important organisms are no longer living in an aquatic ecosystem, they can provide proof of their existence through the remains of their shells. In the field, shells can be collected from dead organisms after episodes of mortality usually without implicating negative effects on the remaining living populations (Geist 2010). Mussel shells have been collected and stored in museums for many decades in efforts to describe and document species dating back to the late 1700's (Baker 1921). And sometimes shells of stored museum specimens can serve as some of the only archives of a rare or extinct animal's existence and contain valuable genetic information about a species. Before recent advancements in sequencing technologies and molecular biology, malacologists and field biologists often relied solely on morphological shell characteristics for both identification and occurrence data. These morphological characteristics included things like; size, shape, and color, as well as location found. Museum specimens were traditionally collected and stored for preserving morphological characteristics but perhaps they can also serve as potential archives of the past by storing genetic material within the shell layers like a time

capsule. With DNA sequencing technologies evolving rapidly there has been a recent interest in analyzing historical museum specimens across many biological fields (Burrell et al., 2016). However, analyses involving ancient DNA and museum specimens still remains challenging to researchers because of degradation that occurs after biological samples are not stored properly thus leading to DNA fragmentation (Dabney et al., 2013). It is expected that DNA collected from ancient samples will be fragmented and contain chemically modified bases and cross-links as well as a myriad of other contaminants (Smith et al., 2015). These modifications make it extremely difficult to amplify the DNA strand using traditional PCR (polymerase chain reaction) methods, which are routinely used to detect and amplify a DNA marker before sequencing begins (Burrell et al., 2016).

Nevertheless, previous studies have shown success in sequencing DNA from museum specimens across many biological taxa including; insects, birds, and various mammals (Besnard et al., 2014; Blaimer et al., 2016; Campbell et al., 2005; Hawkins et al., 2016; McCormack et al., 2016). Samples from museum specimens generating even as little as 1 ng/µl of DNA were found to be successful in sequencing when using Next Generation Sequencing (NGS) methods (Sproul & Maddison 2013). The shells of museum mussel specimens could potentially contain valuable genetic information about the mussel itself and its surrounding environment. The problems between amplification through PCR and ancient museum samples have led to the need for a method to extract the most DNA molecules from museum specimens which are expected to contain a high degree of DNA degradation. Therefore, the development of an optimal protocol for extracting DNA from freshwater museum mussel shells could be beneficial to

researchers interested in isolating DNA from other ancient specimens while also searching for additional environmental sources of DNA (eDNA). Answering some missing information for optimizing a DNA extraction protocol for museum-stored shells could work to help recreate entire historical ecosystems, delineate phylogenetic relationships, and reveal cryptic gene diversity hidden within the genetic information stored within the layers of the shells.

The idea of using shells to obtain additional information expanding beyond that of morphological data is not a novel one, and many studies have exemplified this. In previous studies, shells from mollusks have been found to be a source of several key elements; DNA pertaining to the host species it was collected from, data from fluctuating environmental conditions, and additionally records of DNA from the surrounding environment. In previous studies involving freshwater mussels, DNA was successfully extracted from shell material, amplified, and samples were genotyped using a cytochrome oxidase 1 (COI) mitochondrial DNA gene marker (Geist et al., 2008). Mitochondrial markers are genes that can be used for the identification of species. In another study, DNA was effectively extracted from a freshwater mussel hinge ligament and the DNA was sequenced and data was analyzed and used to make inferences on past population genetic structure (Doherty et al., 2007). In addition to providing information about the individual host itself, shells can also offer evidence about the environment the animal once lived in. In several previous studies, there has been success in using shell material to examine a range of environmental parameters. These parameters include environmental conditions such as; climate, records of vegetation, and times of nutrient influx. Shells can also provide growth estimations by

using the layers in the shells, comparable to botanists using rings in a tree, to estimate the age of an ancient specimen. Climate can be estimated by evaluating isotopic signatures using stable isotopes, such as carbon and oxygen, which are found stored within shell material. These stable isotopic patterns have been derived from ancient mollusk snail shell material in previous studies and used to reconstruct and mark fluctuations in paleoclimates and vegetation records (Prendergast et al., 2015; Yanes et al., 2009). Similar isotopes have also been used to record changes in the growth of mollusk shells during times of nutrient pollution and eutrophication (Fritts et al., 2017; Jones 1983; Jones & Quitmyer 1996; Schöne et al., 2003).

However, aside from providing genetic information about the host and records of environmental conditions, shells can additionally be used for capturing environmental DNA (eDNA) molecules, which are released from other organisms and captured in-between the layers of the porous shells. In a recent study, marine mollusk shells were positively identified as sources of eDNA by using DNA barcoding and metabarcoding approaches to explore the shells for other aquatic taxa, including microbial species' (Sarkissan et al., 2017). By using marine clams shells, this study had a success rate of only 29% for ancient shells, which they considered to be shells 60 years and older. In some cases, most or all of the shell material had to be used. They did however, have success in some extraction of DNA and also with the taxonomic identification of several species using a combination of mitochondrial DNA genomes, barcoding, and metagenomic approaches. They were also able to identify microbial communities such as a *Vibrio* species known to be pathonogenic to shellfish and were also negatively affecting the clams (Sarkissan et al., 2017). Therefore, it is known that

shell material can be used as an eDNA time capsule to lock within its layers the DNA that is released from other organisms in the environment. However, the question of which DNA extraction method is best suited for extracting fragile DNA from hinge ligament material in the most efficient way remains unanswered, and is what will be primarily addressed in this study.

The shells of freshwater mussels are formed through an accretionary growth process, making this protective outer-covering present and growing for the animal's entire lifetime. The shell is made of a protein and calcium-carbonate matrix that is secreted by the mantle and the hinge ligament is the structure that attaches the two halves of the shells and allows for movement between them (Doherty et al., 2007). Hinge ligament is also present throughout an animals' lifetime and, like shell material, could also potentially contain genetic information inside. This ligament is an elastic structure that contains several layers of lamellar and filamentous materials and is primarily comprised of keratin and proteins (Ubukata 2003). These layers are porous and can permit DNA molecules to become trapped within the layers, ultimately allowing this non-cellular ligament to be a potential source of genetic material for both host and eDNA like a time capsule (Doherty et al., 2007; Geist et al., 2008). The hinge ligament is what was used in this study as the source of genetic material. Destructive sampling is usually undesirable, particularly for rare and extinct museum specimens which can be very precious. However, the hinge ligament was chosen for this study because it is not usually taxonomically informative or used for morphological identification purposes, making it an ideal candidate for removal from historical and ancient specimens.

1.1 *Cyprogenia stegaria* Background Information

A bivalve of interest, *Cyprogenia stegaria* (Bivalvia: Unionoida) (Rafinesque, 1820), commonly known as the Fanshell mussel, is a critically endangered species that was once abundant in many rivers spanning across Kentucky, Ohio and Tennessee. This species was added to the federally endangered list in 1990 by the US Fish and Wildlife Service (USFWS) following severe population declines (USFWS, 1991). Historically, this species was endemic to the Ohio, Tennessee, and Cumberland rivers of the Mississippi River drainage, USA (Ortmann 1918, 1919). Now, however, reproducing populations of this species are extremely rare and have been limited to only three known river systems in North America including; the Green and Licking rivers of Kentucky, USA., and also the Upper Clinch River of Tennessee and Virginia, USA. (Jones & Neves 2002). There may be a few potential relict populations still residing in several rivers in Ohio, Illinois, Indiana, West Virginia, Kentucky and in Tennessee, where the presence of this species was historically documented (USFWS, 1991). *Cyprogenia stegaria* has a round shell that is greenish-yellow and covered in small bumps and lined with dark green rays (USFWS, 2016).

Cyprogenia stegaria was selected as the study species for several reasons. This mussel is a critically endangered species and therefore, any genetic information harvested from this species could help aide in restoration of their declining population. Secondly, at maturity these bivalves are also relatively large and can produce a large hinge ligament, making it easier to remove and allowing more tissue to be harvested for data collection compared to that of smaller species. Additionally, the *Cyprogenia* genus has been the subject of several genetic studies which have supplied biologists with

important information for understanding genetic relationships within and among different species in the genus (Serb 2006, Serb & Barnhart 2008). These studies explored genetic relationships utilizing molecular tools such as mitochondrial DNA (mtDNA) and microsatellite sequencing to determine if monophyletic clades exist among the same river drainages and if those clades have any correlation with morphological characteristics of shell features (Chong et al., 2016). Because of a high degree of morphological similarities among mussel groups and great ranges of variability within species, taxonomic uncertainties by morphological characteristics still pose a potential problem to conservation management (Zieritz & Aldridge 2009). Therefore, establishing a source of genetic material for both current and historic populations of freshwater mussels and additionally other sources of eDNA may aide in conservation efforts by identifying unique genetic characteristics and revealing other environmental counterparts of importance. *Cyprogenia stegaria* has become critically endangered due to many anthropogenic factors and with some populations becoming increasingly isolated, extensive conservation and propagation efforts might become necessary in the future (Campbell et al., 2005; Jones & Neves 2002). Both genetic and ecological studies should be used in combinatory efforts for devising and maintaining effective conservation strategies (Geist 2010). Harvesting genetic material from *C. stegaria* museum specimens could contribute to their conservation by revealing genetic variation and delineating phylogenetic relationships while simultaneously obtaining information about other taxa present in the same environment to accurately describe and potentially recreate historical ecosystems.

1.2 Objective of Research and Project Goals

The goal of this study was to assess the potential of using hinge ligament tissue from museum mussel shells for a DNA-based analyses using the endangered Fanshell mussel (*C. stegaria*) (Figure 1)[1]. The objective of this research project was to assess the effects of four extraction protocols on extracting total DNA from hinge ligament tissue on the total DNA yield (ng/mg) measured by fluorometric quantitation (Figure 2). The collection date (more historic vs. most recent) was also analyzed to see if there was any interaction between the time shells were collection and the total DNA derived from an extraction method. Hinge ligament tissue weight ranged between the shells chosen for collection (1.4 mg–217.1 mg), however, the total DNA obtained was standardized by the starting dry tissue weight, thus removing tissue weight as a variable in extraction success.

1.3 Outline of Hypotheses

Null Hypothesis for Extraction Method: There is no significant difference between the total DNA obtained between the four extraction methods.

Null Hypothesis for Collection Year: There is no significant difference between the total DNA obtained between collection years.

Null Hypothesis for the Interaction Effect between Extraction Method and Collection Year: There is no significant interaction between the total DNA obtained and the extraction method with collection year.

[1]All figures and tables are presented in an appendix at the end of this thesis (Appendix A and B).

2. METHODS

2.1 Sample Selection

A total of 40 shells were obtained from the Branley A. Branson Museum of Zoology, Eastern Kentucky University (Table 1). These mussels were harvested from the Licking River during several collection bouts; two historical collections in 1984-1986 and 1990-1995 and a more recent collection in 2013-2015. The hinge ligament was removed from the 40 shells and randomly assigned to an extraction protocol only after ensuring that at least one shell from the historic collections (1984-1995) and one from the more recent collection (2013-2015) was included for each of the four extraction processes.

2.2 Quality Control

Before sample preparation began, all countertops were decontaminated with a 10% bleach solution. Before any molecular techniques were implemented, all equipment was exposed to UV light for a minimum of 30 minutes. Metal tools were soaked in bleach and flame sanitized. Additionally, pipettes, pipette tips, and microcentrifuge tubes were autoclaved at 121°C before use. Filtered pipette tips were also used. Extensive efforts were taken to ensure proper handling of the samples and prevention of any potential contamination. All 40 specimens were subjected to a brief wash using deionized water and then baked at 100°C for 4 hours (Doherty et al., 2007; Pedersen et al., 2014). This step was implemented to remove any exogenous DNA present on the outside of the mussel shell and to also rid the sample of any potential bacterial or fungal contamination which may have accumulated during sample storage.

2.3 Sample Preparation: The "Breakdown Steps"

The tissue was subjected to several lysing steps before a DNA extraction method was used and these series of steps will be referred to as "Breakdown Steps" for the remainder of this paper. During the first part of this sample preparation, the tissue was attempted to be physically smashed by subjecting the hinge ligament tissue to mechanical homogenizing. This homogenizing step was completed by using two sterilized zinc-coated beads within a 1.5 ml microcentrifuge tube and then vortexing the tube on high power and high speeds for five minutes. This was repeated several times. This mechanical force did break up some of the softer hinge ligament into smaller pieces but it was not sufficient in breaking the larger and more calcified tissue pieces up.

Therefore, a second part of the "Breakdown Steps" was implemented, and all tissue samples were subjected to an additional chemical lysis step. This chemical lysis contained several steps and was initiated by a 48-hour pre-soak of the hard tissue in a solution of 0.5 M EDTA (Ethylenediaminetetraacetic acid) at room temperature accompanied by gentle shaking using an orbital shaker (Villanea et al., 2016). The addition of EDTA, a chelating agent, renders DNases inactive and thus enabling more DNA molecules to survive the extraction process and avoid being dissolved by enzymes. After this initial pre-soak in EDTA, several incubations using; proteinase K (20 mg/ml) and β-Mercaptoethanol (CAS # 60-24-2) were included, with volumes dependent on each extraction method. Proteinase K is known to cleave peptide bonds and digest proteins and was utilized in all four extractions methods. β-Mercaptoethanol was added because of its known activity in reducing disulfide bonds in proteins and by

reducing other inhibitors such as tannins. β-Mercaptoethanol is commonly used in other extraction methods such as the RNeasy Mini Kit extraction (Qiagen, USA), and was adopted for all extraction methods. An additional solvent, 10% sodium dodecyl sulfate (SDS), was used for only one extraction method per manufacture suggestion. SDS is a strong anionic detergent and can remove lipid and protein membranes. Temperatures for the overnight soaking were determined by the suggestion of each extraction method per manufacture protocols and these vary among the four methods as well as the core components of the soak. The additional chemical soak was still not sufficient in breaking down all parts of the hinge ligament and filamentous tissue into a desired fluid sample so all samples were subjected to a polyethylene microcentrifuge column. This column worked to remove and filter larger particles of ligament out from the aqueous solution and the remaining liquid, which included any genetic material, was then subjected to the four different extraction methods (See 4.3 Special Notes).

2.4 DNA Extraction

Four extraction protocols were compared at their ability and efficiency to isolate DNA from the hinge ligaments of 40 *Cyprogenia stegaria* museum specimens ranging in collection years (1984-2015). One modified Cetyltrimethylammonium bromide (CTAB) (GBiosciences, MO, USA.) extraction based upon chloroform was selected. In addition to the CTAB method, three commercially available extraction kits including DNeasy® Blood and Tissue Kit (Qiagen, Germany), GeneClean® for Ancient DNA Kit (MPBiomedicals, CA, USA.), and MagJET® Genomic DNA Kit (Thermoscientific, MA, USA.), were selected and all four extraction methods were compared. A total of ten hinge ligament tissue samples per extraction method were prepared and used.

Additionally, a sample of deionized water was used as a negative control for each extraction protocol and included all reagents used for each extraction process. This was to account for any genetic material present in the kits or any contaminant that may have been introduced during an extraction process.

2.4.1 Modified CTAB Extraction

A slightly modified CTAB and chloroform extraction process was used for the extraction of genomic DNA from hinge ligament tissue. CTAB is a classic chloroform-based extraction method and is commonly used for the extraction of DNA from many different sample types. Samples were incubated at 55°C for 48 hours to complete the "Breakdown Steps" by using 7 µl β-Mercaptoethanol and 10 µl proteinase K. 700 µl of premixed CTAB Extraction Solution (GBiosciences®) was added to the starting samples and incubated at 55°C for an additional 24 hours before the extraction process began. This additional incubation period was implemented because of success in previous studies for optimal DNA extraction by using dried freshwater mussel tissue (Inoue et al., 2013). Following the extended incubation period, the manufacture protocols were followed. Samples were incubated at 65°C for one hour and cooled to room temperature. Then 700 µl of chloroform (CAS# 67-66-3) was added and the samples were centrifuged at 10,000 g and the supernatant was isolated and precipitated with 600 µl of 100% isopropanol (CAS # 67-63-0). Samples were centrifuged again at 10,000 g and a pellet of DNA was collected and washed with 70% ethanol twice. The pellet was re-suspended in 100 µl of molecular grade water and stored at -20°C.

2.4.2 MagJET® Genomic DNA Extraction

This method was used for the isolation and purification of genomic DNA from hinge ligament tissue by utilization of magnetic bead capture methods. This is a basic bind, wash, and elution method. The magnetic beads are coated with a silica surface and this will allow for selective DNA binding under high chaotropic salt conditions, and then the DNA is later removed from the surface of the bead through the force of a magnet and under low salt conditions. These samples were incubated at 56°C for 48 hours and the "Breakdown Steps" were implemented in a solution including: 20 µl proteinase K, 200 µl Digestion Solution™, and 2 µl β-Mercaptoethanol. Then the samples were extracted following manufacture instructions under *Protocol E: Manual genomic DNA purification from up to 20 mg tissue, rodent tail, and insects*. Samples were eluted into an elution buffer of 100 µl and stored at -20°C. Three samples during the extraction processes were destroyed and later removed from the dataset. In these destroyed samples, the microcentrifuge tubes became thick with a white substance that hindered the completion of the extraction process because the magnetic beads could not move through the dense matrix. These samples were possibly destroyed through an unexpected protein denaturation reaction, however additional research will be required to identify a true explanation.

2.4.3 GeneClean® for Ancient DNA Extraction

This DNA extraction method is designed for the purification of fragmented/damaged DNA from preserved or ancient samples, making it suitable for extracting DNA from stored museum specimens. This method uses GLASSMILK™, a suspension silica matrix solution that is used to isolate and purify DNA. The

"Breakdown Steps" were implemented and samples were incubated at 37°C for 48 hours in a solution consisting of: 5 µl 0.5 EDTA, 200 µl 10% SDS, and 200 µl 20 mg/ml proteinase K at 37°C. SDS was included in this extraction method as a detergent to remove lipid membranes. Samples then followed the manual extraction protocol listed in the manufacturer instructions and a 100 µl elution step was completed using DNA free elution solution provided by the kit and samples were stored at -20°C.

2.4.4 DNeasy® Blood and Tissue DNA Extraction

This DNA extraction kit was used for the isolation of genomic DNA from hinge ligament tissue by using a spin column binding in combination with a specific buffer system. This method uses a bind, wash, and elution process of the DNA. The DNA will bind to the silica membrane under high salt conditions, however proteins and other polysaccharides will not usually bind to this column and are washed away during the alcohol wash step. The DNA can then be eluted under low salt conditions using a buffer. After the 48-hour "Breakdown Steps" were implemented at 56°C using 20 µl proteinase K and 7 µl β-Mercaptoethanol, and then manufacturer protocol was followed for the extraction method *Purification of Total DNA from Animal Tissues (Spin-Column Protocol)*. The DNA was eluted into 100 µl of Buffer AE and the samples were stored at -20°C.

2.5 Qubit Sample Preparation

Quantification of DNA was performed using a Qubit™ 3.0 Fluorometer (Life Technologies). The Qubit dsDNA HS (high sensitivity) assay kit and two standards (high and low) were used following the manufacturer protocol. For a total of 200 µl solution, 195 µl of Qubit working solution was added to 5 µl of each sample DNA. The

volume of 5 µl of sample was determined through previous studies on similar hinge ligament tissue, and additionally a midpoint for the company's recommendation on volume usage. The solution containing the DNA and Qubit working solution was incubated for 2 minutes at room temperature (25°C) and then sample DNA concentration was read using the fluorometer (Table 2). This instrument was selected because it uses a fluorescent molecule that is only reported when it is bound to target DNA, ultimately minimizing the chance to read free particulates such as RNA, proteins, and other contaminants that may still be present in the sample.

2.6 Nanodrop Sample Preparation

All samples were quantified using a Nanodrop™ 2000 spectrophotometer to evaluate purity of the DNA samples. Only 1 µl of each sample was used and the results for the 260/280 nm and 260/230 nm absorbance ratios were recorded (Table 2). The ratios of absorbance at 260 nm and 280 nm are often used to assess DNA purity. It is generally considered that a ratio of ~1.8 for 260/280 nm absorbance is a "pure" genomic DNA sample. The 260/230 ratio is also used as a secondary measure of DNA purity, and a generally accepted ratio is within the range of 2.0–2.2 (Thermoscientific, 2009). This machine does not use a florescent reporter molecule and it can measure other particulates and contaminants such as proteins and phenols present within the sample.

3. ANALYSIS AND RESULTS

3.1 Data Analysis

All statistical analyses were completed using R version 3.3.1 (R Core Team, 2016). The dataset was assessed for normality using a QQ plot and then transformed by the square root of the dataset. A factorial ANOVA was conducted to compare the effect extraction method and collection year, and the interaction between extraction method and collection year on the total DNA yield collected (Full Model). Several Tukey's Post Hoc multiple pairwise comparison tests were completed to compare means of both variables and their interaction. A customized R function was included to report only the significant pairwise comparisons for the full model ($p \leq 0.001$). Two boxplots were generated to illustrate the differences between the means for extraction method and collection year on the total DNA yield collected. An interaction plot was generated to illustrate the differences for the interaction between the two variables; extraction method and collection year.

3.2 Results

The total DNA yield (ng/mg) was measured using the Qubit and DNA quality absorbance ratios (260/280 nm and 260/230 nm) were measured using the Nanodrop. This difference in machinery can account for a few samples which were unable to be analyzed by the Qubit but the Nanodrop was still able to assign quality ratios. In these few samples the amount of total DNA may have been too low to be analyzed by the Qubit, however the remaining free particulates were able to be detected by the Nanodrop and the absorbance ratios were measured. The lowest 260/280 nm absorbance ratio was 1.16. There were several outliers with large 260/280 nm absorbance ratios

(e.g. 76.46, 8.86, 5.71), which indicate those samples may contain contaminants such as proteins. The lowest 260/230 nm absorbance ratio was 0.05 and the highest nm absorbance ratio was 1.46. Most samples contained a low 260/230 ratio, suggesting some samples may contain a high amount of contaminate such as EDTA, which has an absorbance around 230 nm.

The means for total DNA yield (ng/mg) were calculated for the non-transformed data for each of the four extraction methods and reported with the standard deviation. The GeneClean extraction produced the lowest mean when compared to the other methods (0.7420 ng/mg ± 0.09894 ng/mg). The DNeasy extraction and MagJET produced similar means to each other (2.2989 ng/mg ± 0.3017 ng/mg and 2.6955 ng/mg ± 0.4894 ng/mg, respectively). The modified CTAB extraction produced the largest mean when compared to the other three methods (47.9181 ng/mg ± 6.0087 ng/mg).

A factorial ANOVA was conducted on the influence of two variables; extraction method and collection year, and their interaction, on the total DNA obtained. Collection year included three collection bouts (1984-1986, 1990-1995, 2013-2015) and extraction method included four extraction methods (DNeasy, Modified CTAB, GeneClean, MagJET). This ANOVA revealed that all variables had a statistically significant effect on the total DNA obtained ($p \leq 0.001$) (Table 3, Figure 5). To further analysis, a Tukey's Post Hoc pairwise comparison analysis was used to determine differences in the means for the extraction method and the total DNA yield rates the modified CTAB extraction protocol was found to be significantly different than the yield obtained by the MagJET, GeneClean, and DNeasy Kit (Tukey's, $\alpha=0.05$, $p \leq 0.001$) (Figure 6, Table 4). The modified CTAB extraction method (a) was the only method found the be

statistically different than the other methods (b) in extracting total DNA (Figure 3). A Tukey's Post Hoc pairwise comparison analysis test was used to determine differences in the means for the collection year and, unsurprisingly, the total DNA yield rates from the most recent collection bout (2013-2015) were found to be significantly different than those obtained by the other two historical collections (Tukey's, $\alpha=0.05$, $p \leq 0.001$) (Figure 4, Table 5). Another Tukey's Post Hoc pairwise comparison analysis test was used and the total DNA yield rates from the all interactions which contained CTAB extraction method were significantly different than those obtained by the other interactions that did not include CTAB (Table 6). This suggests that the Modified CTAB extraction method does have an interaction with the most recent collection year (2013-2015), however it is still able to extract the most DNA from hinge ligament tissue when compared to the other three methods.

4. CONCLUSSIONS AND DISCUSSION

4.1 Learned and Discovered

The hinge ligament of freshwater mussel shells is a source of DNA and all four extraction methods were successful in extracting DNA. Despite the overall success, DNA yield rate in the individual hinge ligament samples varied within, and across, each of the four extraction methods. All negative controls had DNA concentrations too low to be read by the Qubit, suggesting that no contamination was present within the samples. The modified CTAB extraction method was found to perform the best in extracting total DNA (ng/mg) from mussel hinge ligament tissue when compared to the other three methods. These findings suggest that a CTAB extraction method and potentially other chloroform-based extractions such as phenol-chloroform, may be best suited for total genomic DNA extraction from museum and ancient specimens. The interaction between extraction method and collection year was strongest between the Modified CTAB extraction method and the 2013-2015 collection bout. This suggests that the most recent collection could recover more genetic material than the historical collections.

I hypothesize that the modified CTAB extraction method performed the best in terms of isolating total DNA yield collection in comparison to the other methods because the CTAB method did not rely on silicon binding like the other three methods did. Because DNA obtained from museum specimens is expected to contain a high degree of fragmentation, the fragments isolated from the ligament tissue were perhaps too small and unable to bind to the magnetic beads or the silicon binding membrane. Due to this inability to bind, some of the DNA fragments may have been washed away

and lost to the remainder of the extraction process. However, the modified CTAB extraction was the only extraction method that did not rely on silicon binding of the DNA fragments. This method might have worked best because the small/degraded DNA fragments were able to survive the extraction process because they were never filtered out by means of a binding column or beads.

4.2 Future Research and Goals

Now that a more optimized extraction method has been determined, future research will be necessary to identify how much of the total DNA yield is genetic material obtained from the host mussel and how much is eDNA from the surrounding environment. Because DNA from museum specimens is expected to be fragmented, traditional PCR methods are not normally successful in amplification. However, with recent molecular advancements, PCR-free target capture methods have been developed and shown to be successful in sequencing DNA by using high throughput sequencing methods, even from museum specimens with DNA of low molecular weight (Sproul & Maddison 2013). These methods will be implemented in future research projects specifically by using MyBaits®, a targeted molecular probe approach, to perform targeted gene enrichment. This targeted probe approach generally works by utilizing small starting quantities of DNA and targeting only a specific region of the DNA for enrichment through NGS. This is completed through the hybridization of target DNA using many customized and complementary biotinylated RNA baits (MYcroarray®, The Oligo Library Company™) and can enable researchers to sequence only desired portions of the DNA while disregarding other DNA which could cloud analysis. A variety of markers will be designed for *Cyprogenia stegaria* and other freshwater

mussels as well as for other aquatic organisms such as; fish, crayfish, and insects, that are anticipated to be present in the same environment as the mussels. Then the eDNA from these aquatic organisms can be extracted from the mussel hinge ligament tissue.

Despite the presence of diverse communities comprised of freshwater invertebrates, the overall genetic diversity of many mussel species is still poorly understood (Geist and Kuehn 2005). The genetic information derived from a targeted probe approach can help to alleviate this lack of genetic knowledge by identifying cryptic diversity in mussels and other aquatic organisms in the environment. These targeted approaches can provide genetic data that can be used in a variety of ways such as; recreating historical ecosystems and aiding in conservation management programs. Studies using genetic markers, such as the COI marker, have been used to establish presence of genetic diversity and additionally facilitated discovery of important haplotypes in other freshwater fauna while also establishing an important basis for conservation status (Helms et al., 2014). Identifying how ecosystems, and counterparts of ecosystems, have responded to environmental disturbances and stressors in the past, can also provide an insight on how they may respond to future environmental disruptions. The genetic information that can be derived from historical mussel shells can be used to create phylogenies showing patterns of evolutionary ecological processes for both freshwater mussels and their community counterparts. Therefore, by using genetic data to reconstruct historical ecosystems, data can be collected to predict the stability, resilience, and potential fluctuations that an ecosystem might encounter in the future and, moreover, used for management and restoration projects for current environments in need (Barak et al., 2016).

Using freshwater mussel shells as DNA reservoirs could open many opportunities for biologists expanding across many scientific fields interested in answering some of the missing genetic and evolutionary components of freshwater mussel history. Now, a more optimized DNA extraction method used for obtaining the fragile DNA from museum specimens can aide scientists in the identification process of individuals and could reveal unknown biodiversity within the aquatic community. Museums full of historical shells can be used to obtain both DNA from the individual while also searching for eDNA released from organisms in the surrounding environment. Acquiring eDNA from museum specimens could facilitate biological studies focused on the reconstruction of ancient and historical ecosystems, and this information can be applied to perfecting management strategies for current communities. This optimized method will enable scientists to use mussel shells as DNA time-capsules to obtain optimal DNA from the host specimen and other potential eDNA sources simultaneously. This genetic information that can be obtained from the hinge ligament can provide a snapshot of aquatic fauna that was present in the same ecosystem as the mussel, which will allow scientists to rebuild entire historic communities from shell material and instill a deeper knowledge in what counterparts are necessary for conservation of current aquatic ecosystems.

4.3 Special Notes

To improve upon the "Breakdown Steps" methodology of this research, some additional options for chemical and mechanical lysing may be considered. A more efficient way of grinding ligament tissue into a powdered sample by use of a sterile and DNA-free homogenizer or grinding tool may be necessary. If the tissue is unable to be

ground into a powdered sample and must be filtered through a polyethylene microcentrifuge tube, weighing the particulates is suggested for maximum accuracy when standardizing starting tissue weight into the total DNA obtained. Also, longer EDTA soaks may necessary to release more DNA molecules into the aqueous sample. Extended EDTA soaks are commonly used for the DNA extraction from hard and calcified materials such as bone and teeth (Cho et al., 2010; Higgins et al., 2014). Additionally, smaller elution volumes for each extraction method could be implemented to obtain a higher DNA concentration. Furthermore, researchers interested in already processed samples could use ethanol precipitation to re-concentrate DNA that was eluted into larger volumes.

APPENDICES

APPENDIX A:

Figures

Figure 1. Representative shells of *Cyprogenia stegaria*

Figure 2. Hinge ligament from a representative *Cyprogenia stegaria* shell

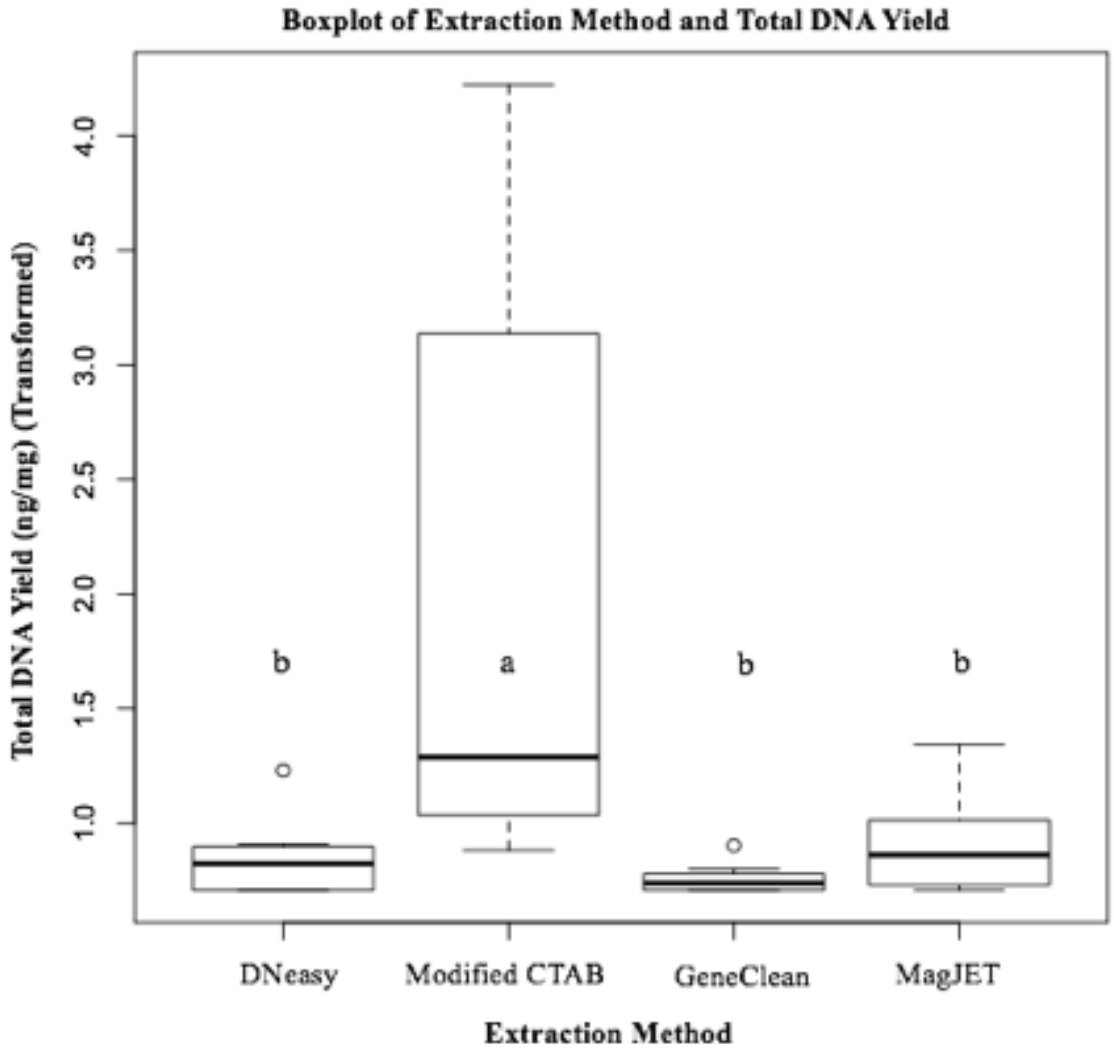

Figure 3. Boxplot comparing DNA extraction methods and total DNA yield (Factorial ANOVA). Modified CTAB extraction is the only extraction method found to be statistically different (a) from the other three methods, which are not statistically different from each other (b).

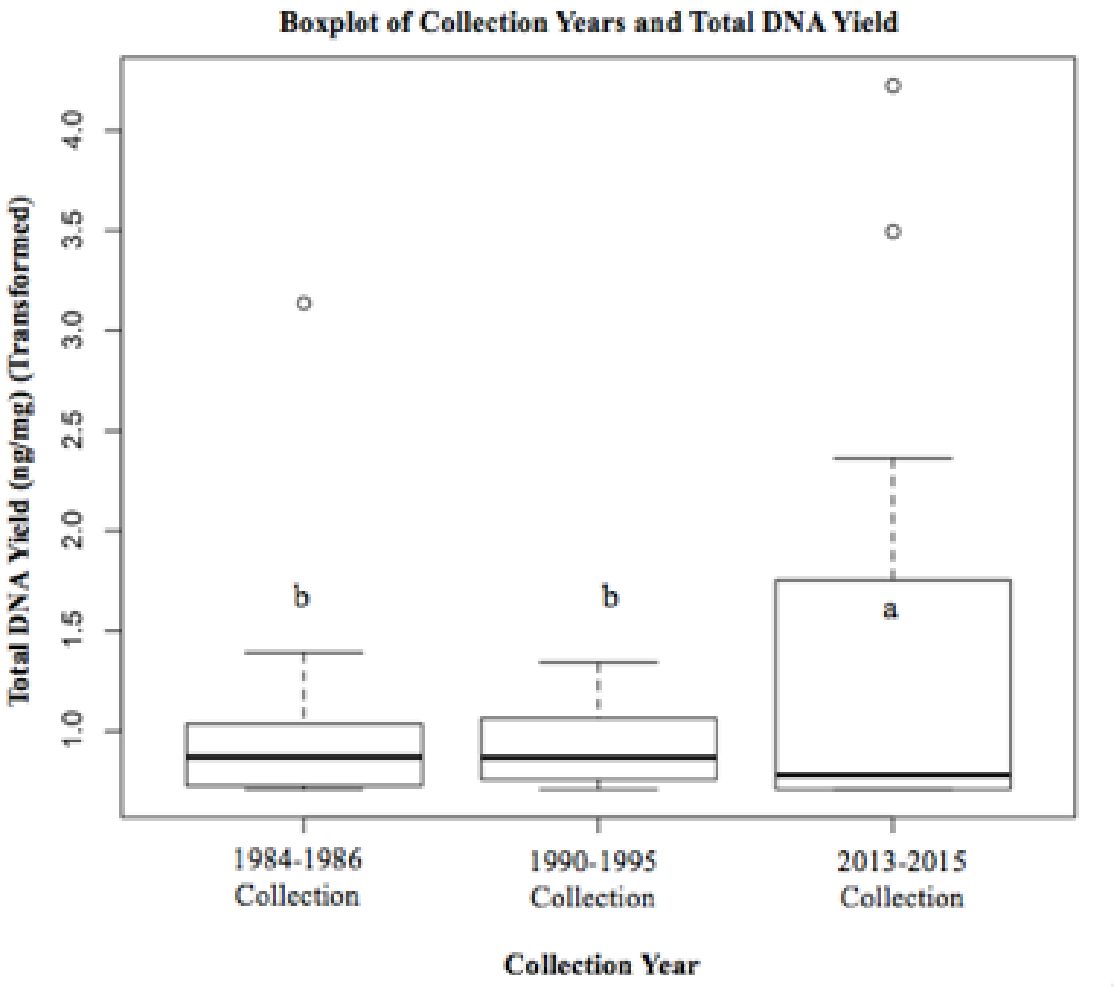

Figure 4. Boxplot comparing collection year and total DNA yield (Factorial ANOVA). The 2013-2015 recent collection is the only collection bout found to be statistically different (a) from the other two collection bouts which are not statistically different from each other (b).

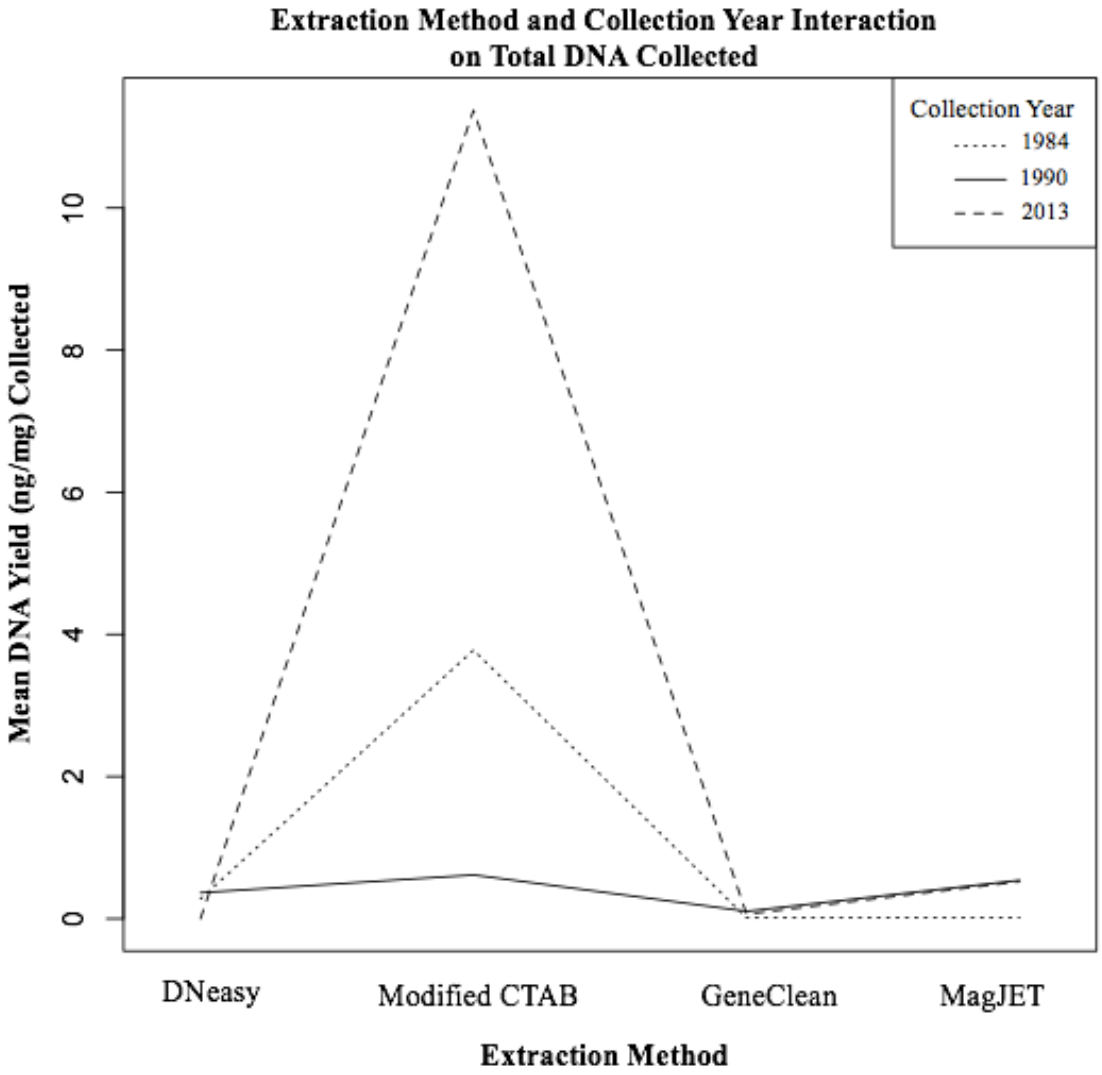

Figure 5. Interaction line graph comparing the interaction between the collection year and the extraction method on the total DNA yield. Collection year 1984: (1984-1986), collection year 1990: (1990-1995), collection year 2013: (2013-2015). The Modified CTAB extraction has a strong correlation with the most recent collection bout 2013-2015.

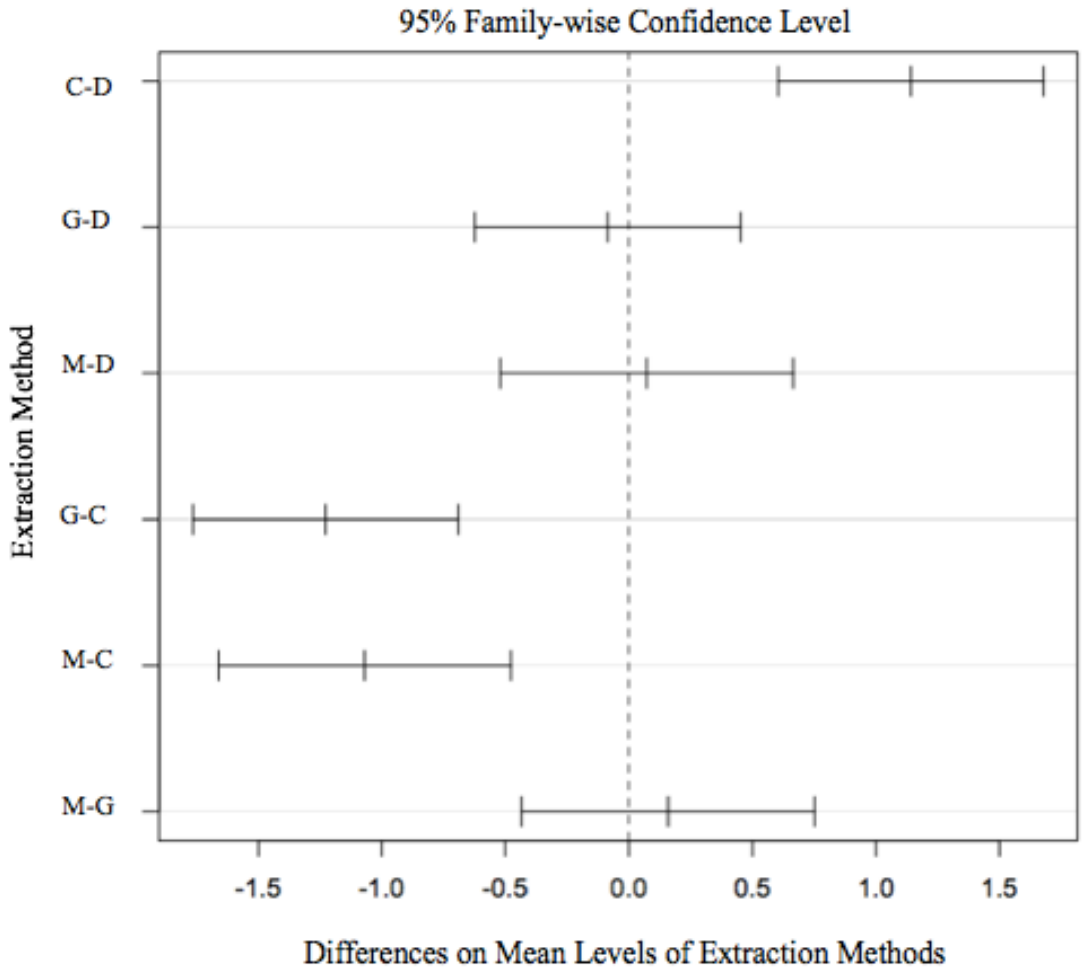

Figure 6. Tukey's Multiple Comparison of Means in extraction method using 95% Family-wise Confidence level. C (Modified CTAB), D (DNeasy), M (MagJET), G (GeneClean). The only three extractions that do not contain a 0 in the interval use Modified CTAB extraction method.

APPENDIX B:

Tables

Table 1. General Information for *Cyprogenia stegaria* Specimens

EKU #	Col Yr	County, State	Lat	Lon	Extraction
430.1	1984	Pendleton Co., KY	NA	NA	MagJET
439.1	1984	Pendleton Co., KY	NA	NA	CTAB
439.2	1984	Pendleton Co., KY	NA	NA	DNeasy
327.1	1986	Pendleton Co., KY	38.789345	-84.367856	DNeasy
327.3	1986	Pendleton Co., KY	38.789345	-84.367856	GeneClean
327.4	1986	Pendleton Co., KY	38.789345	-84.367856	CTAB
327.5	1986	Pendleton Co., KY	38.789345	-84.367856	MagJET
327.6	1986	Pendleton Co., KY	38.789345	-84.367856	DNeasy
814.1	1986	Pendleton Co., KY	38.789345	-84.367856	GeneClean
814.2	1986	Pendleton Co., KY	38.789345	-84.367856	CTAB
587.1	1990	Campbell Co., KY	38.866439	-84.45229	GeneClean
587.2	1990	Campbell Co., KY	38.866439	-84.45229	CTAB
587.3	1990	Campbell Co., KY	38.866439	-84.45229	MagJET
587.4	1990	Campbell Co., KY	38.866439	-84.45229	DNeasy
587.5	1990	Campbell Co., KY	38.866439	-84.45229	GeneClean
587.6	1990	Campbell Co., KY	38.866439	-84.45229	CTAB
587.7	1990	Campbell Co., KY	38.866439	-84.45229	MagJET
587.8	1990	Campbell Co., KY	38.866439	-84.45229	DNeasy
587.9	1990	Campbell Co., KY	38.866439	-84.45229	GeneClean
587.10	1990	Campbell Co., KY	38.866439	-84.45229	CTAB
587.11	1990	Campbell Co., KY	38.866439	-84.45229	MagJET
587.12	1990	Campbell Co., KY	38.866439	-84.45229	DNeasy
570.1	1991	Montgomery Co., KY	38.173320	-83.89549	DNeasy
570.2	1991	Montgomery Co., KY	38.173320	-83.89549	MagJET
570.3	1991	Montgomery Co., KY	38.173320	-83.89549	GeneClean
570.4	1991	Montgomery Co., KY	38.173320	-83.89549	CTAB
738.1	1995	Pendleton Co., KY	38.789345	-84.367856	GeneClean
738.2	1995	Pendleton Co., KY	38.789345	-84.367856	MagJet
2013.1	2013	Pendleton Co., KY	38.789345	-84.367856	DNeasy
2013.2	2013	Pendleton Co., KY	38.789345	-84.367856	GeneClean
2013.3	2013	Pendleton Co., KY	38.789345	-84.367856	CTAB
2013.5	2013	Pendleton Co., KY	38.789345	-84.367856	MagJET
2013.6	2013	Pendleton Co., KY	38.789345	-84.367856	DNeasy
2015.1	2015	Pendleton Co., KY	38.789345	-84.367856	GeneClean
2015.2	2015	Pendleton Co., KY	38.789345	-84.367856	CTAB
2015.3	2015	Pendleton Co., KY	38.789345	-84.367856	MagJET
2015.4	2015	Pendleton Co., KY	38.789345	-84.367856	DNeasy
2015.5	2015	Pendleton Co., KY	38.789345	-84.367856	GeneClean
2015.6	2015	Pendleton Co., KY	38.789345	-84.367856	CTAB
2015.7	2015	Pendleton Co., KY	38.789345	-84.367856	MagJET

Table 2. Total DNA yield (ng/mg) and 260/280 and 260/230 Ratios*

Sample #	Starting Tissue Weight (mg)	DNA Yield (ng/µl)	DNA Yield x 100 µl	Total DNA Yield (ng/mg)	260/280 Ratio	260/230 Ratio
327.1	101.4	0.3090	30.90	0.3047	1.69	0.13
327.3	217.1	0.0540	5.40	0.0249	1.19	1.46
327.4	163.4	2.3401	234	1.4321	1.64	0.67
327.5	72.6	0.0220	2.20	0.0303	1.31	0.21
327.6	122.0	0.3960	39.60	0.3246	1.15	0.63
430.1	201.7	0	0	0	5.71	0.19
439.1	15.0	1.4012	140	9.3333	2.43	0.15
439.2	14.7	0.0304	3.040	0.2068	2.24	0.24
570.1	31.8	0.0481	4.80	0.1509	3.05	0.10
570.2	108.8	0.0422	4.20	0.0386	2.31	0.46
570.3	91.0	0	0	0	1.99	0.46
570.4	80.4	0.728	72.8	0.9055	2.57	0.11
587.1	10.3	0.0324	3.24	0.3145	2.30	0.39
587.2	8.9	0.0248	2.48	0.2786	2.48	0.14
587.3	35.6	0.1012	10.1	0.2837	1.34	0.59
587.4	49.5	0.1113	11.1	0.2242	4.82	0.02
587.5	50.3	0.0332	3.32	0.0660	1.80	0.04
587.6	33.5	0.2942	29.4	0.8776	2.27	0.13
587.8	14.1	0.1431	14.3	1.0141	1.59	0.47
587.9	38.9	0	0	0	1.45	0.10
587.10	112.3	0.4520	45.2	0.4025	1.38	0.58
587.11	5.5	0.0716	7.16	1.3018	1.60	0.52
587.12	70.3	0.0516	5.16	0.07339	1.16	0.61
738.1	187.0	0.2650	26.50	0.1417	1.36	0.42
814.1	27.7	0	0	0	1.58	0.24
814.2	133.4	0.7601	76	0.5697	1.63	0.62
2013.1	3.5	0	0	0	76.46	0.07
2013.2	30.0	0.0320	3.20	0.1066	1.5	0.12
2013.3	11.6	2.0100	201	17.3275	2.36	0.13
2013.6	60.2	0	0	0	2.68	0.03
2015.1	198.5	0.1750	17.50	0.0881	1.32	0.49
2015.2	1.4	0.1640	16.40	11.7142	2.27	0.15
2015.3	34.4	0.2750	27.50	0.7994	1.36	0.61
2015.4	6.4	0	0	0	8.86	0.09
2015.5	14.4	0	0	0	1.82	0.04
2015.6	13.0	0.6602	66	5.0769	2.53	0.11
2015.7	14.4	0.0348	3.48	0.2416	1.39	0.49

*DNA Yield (ng/µl) measured using Qubit and 260/280 and 260/230 ratios measured using Nanodrop

Table 3. Analysis of Variance Table Reporting F-Statistics for Collection Year, Extraction Method, and Interaction (Factorial ANOVA)

	Degrees of Freedom	F-value	P-value
Collection Year	2	5.7629	8.74×10^{-3}
Extraction Method	3	16.9073	3.29×10^{-6}
Interaction	6	6.4392	3.36×10^{-4}
Residuals	25		

Table 4. Tukey Multiple Comparisons of only Significant Means for Extraction Method (95% Family-wise Confidence Level)

Extraction Method	Lower Limit	Upper Limit	P-value
CTAB-DNeasy	0.6043	1.6793	2.43×10^{-5}
GeneClean-CTAB	-1.7651	-0.6901	1.0×10^{-5}
MagJET-CTAB	-1.6613	-0.4768	2.5×10^{-4}

Table 5. Tukey Multiple Comparisons of only Significant Means for Collection Year (95% Family-wise Confidence Level)

Collection Year	Lower Limit	Upper Limit	P-value
1990-2013	0.1527	1.0053	6.44×10^{-3}

Table 6. Tukey Multiple Comparisons of only Significant Means for Interaction (95% Family-wise Confidence Level)

Interaction Type	Lower Limit	Upper Limit	P-value
2013,CTAB:1984,DNE	1.1962	3.7589	1.54×10^{-5}
2013,CTAB:1990,DNE	1.2495	3.6467	6.16×10^{-6}
2013,CTAB:2013,DNE	1.3711	3.9338	4.89×10^{-6}
2013,CTAB:1984,CTAB	0.2248	2.7876	1.18×10^{-2}
2013,CTAB:1990,CTAB	1.1131	3.5103	1.61×10^{-5}
1984,GC:2013,CTAB	-4.0764	-1.2112	3.29×10^{-5}
1990, GC:2013,CTAB	-3.7316	-1.4394	1.06×10^{-6}
2013, GC:2013,CTAB	-3.8899	-1.3272	6.25×10^{-6}
1984, MJ:2013,CTAB	-4.0745	-1.2093	3.33×10^{-5}
1990, MJ:2013,CTAB	-3.6537	-1.0910	3.13×10^{-5}
2013, MJ:2013,CTAB	-3.7916	-0.9264	1.87×10^{-4}

CTAB: Modified CTAB, DNE: DNeasy, GC: GeneClean, MG: MagJET

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