Forensic DNA Recovery for Insect Identification from

Specimens Prepared for Morphological Analysis

By

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ABSTRACT

Calliphoridae, known as blow flies, are among the first forensically important insects to arrive at a corpse to lay eggs. The larvae are difficult to identify due to the minute nature of the morphological characteristics used for identification. Two complementary methods for insect identification are scanning electron microscopy (SEM) and DNA barcoding. Morphological identification of insects by microscopy is the gold standard but requires considerable training and expertise, while DNA barcoding relies on standard molecular techniques. In this research, we tested the hypothesis that DNA suitable for barcode identification may be obtained from specimens prepared for environmental scanning electron microscopy (ESEM) imaging in a minimally destructive manner.

Specimens were prepared in three different drying treatments: unalters, dried, dried and imaged. For specimens that were dried and dried and imaged the larvae were placed increasing dilutions of ethanol and acetone mixture. Following the imaging process, three different DNA extraction methods: whole, pierced, and homogenized specimens. Total DNA yield from each method was measured by Qubit 4 fluorometry. The suitability of extracted DNA for DNA barcoding was tested by amplification via PCR of the cytochrome oxidase I mitochondrial gene. Identity of the PCR products was confirmed by Sanger sequencing and comparison of sequences to known references.

ESEM imaging showed that there were no visual differences between specimens that underwent drying treatments and ones that were not dried. No significant difference in DNA yield were observed drying treatments and extraction methods post extraction; however, extraction methods demonstrated significant differences in yield post amplification. Once

amplified, six of the nine PCR products resulted in usable Sanger sequencing where the sample sequences aligned with 100% identity to *Chrysomya rufifacies* in a BLAST search.

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CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

The relationship between decomposition and insects has been recognized for centuries (Gennard, D., 2012). A major contribution of entomology to forensic science is the estimation of the post-mortem interval (PMI), the time between death and discovery of the body, by analyzing known developmental rates and succession timelines of carrion insects (Matuszewski, 2021). Various adult insects and larvae feed on the carrion, contributing to decomposition stages. Calliphorids, commonly known as blow flies, are some of the first insects to arrive at a corpse to lay eggs. Their wide geographic distribution and early colonization of carrion makes Calliphorids forensically significant because they are frequently encountered during investigations (Mendonça et al., 2008; K. Sukontason et al., 2003a).

Identification of Diptera larvae, commonly known as fly larvae or maggots, requires microscopes due to the minute nature of morphological characteristics used for identification (Martoni et al., 2019). Scanning Electron Microscopy (SEM) is considered the gold standard for morphological identification for creating high-resolution images. High-resolution images are created with an SEM by scanning a sample with a beam of electrons, known as the incident electrons, which interact with the surface of the sample (Barhoum & Luisa García-Betancourt, 2018). The interaction of the incident electrons and the sample surface releases secondary electrons (Barhoum & Luisa García-Betancourt, 2018). Lastly, these secondary electrons are collected by the SEM detector to create the image (Barhoum & Luisa García-Betancourt, 2018).

DNA barcoding, using standard molecular techniques, is a complementary technique to SEM imaging. Using DNA barcoding to identify insect taxa is common, especially as DNA testing has become more available. DNA barcoding compares short DNA sequences that contain species-level genetic variability to a known reference database for accurate identification

(Chimeno et al., 2019; Kress & Erickson, 2008; Yusseff-Vanegas & Agnarsson, 2017). DNA is a fast and valuable tool for identification of larvae that lack distinguishable morphologies early in development. Mitochondrial DNA (mtDNA) is often used for species identification because it has a high copy number per cell (Sperling et al., 1994).

Coupling DNA analysis and SEM, a combination of two well established techniques, allows for a two-step identification process to increase access to forensic entomology as a tool for investigators. Once larvae are collected at a crime scene, they can be sent to entomologists for identification. An SEM could be used to take preliminary images used for initial identification. DNA could be extracted to confirm the visual identification using morphological features. The DNA results could replace the need to rear larvae collected from the crime scene into adult flies for confirmation of species identification. Accurate species identification is critical because PMI is a function of species and different fly species colonize decomposing tissues at different times (Rivers, D. B. & Dahlem, G. A., 2023). DNA analysis can confirm identification without rearing larvae to adulthood, the forensic entomologists still play a key role in identification and in interpreting the temporal significance of finding particular species at a crime scene.

Post-Mortem Interval Estimation

Post-mortem interval (PMI) is the estimated time since death which is reported in a range of the minimum and maximum time since death, denoted as PMI_{Min} and PMI_{Max} (Rivers, D. B. & Dahlem, G. A., 2023). PMI_{Max} measures from the time since the decedent was last seen until discovery, while the PMI_{Min} is determined by scientific evidence collected from the crime scene (Rivers, D. B. & Dahlem, G. A., 2023). In the first 24-48 hours after death, pathological testing of tissue samples, body fluids, and lividity can accurately be used to determine PMI_{Min} (Rivers,

D. B. & Dahlem, G. A., 2023). After 72 hours, the used of entomological evidence becomes important as pathological analysis is no longer reliable (Rivers, D. B. & Dahlem, G. A., 2023). PMI_{Min} using insects is estimated by recording the development of the larva to compare to a species specific growth model that account is most similar to the environmental conditions of the crime scene (López-Esclapez et al., 2014; Rivers, D. B. & Dahlem, G. A., 2023). Growth models remain linear despite the fluctuation of temperature throughout a 24-hour period (Rivers, D. B. & Dahlem, G. A., 2023). Species specific linear growth models is used to extrapolate the amount of time needed at average temperature needed, calculated to be accumulated degree day (ADD), to compete that stage of development (Rivers, D. B. & Dahlem, G. A., 2023). The ADD must be calculated and added cumulatively until the calculated larval development matches the development observed in the scene samples (Rivers, D. B. & Dahlem, G. A., 2023). For these calculations to be performed, it is necessary to correctly identify the larvae collected from the scene.

There has been a discussion about the accuracy of PMI calculations, including identifying sources of error and ways to calculate the confidence interval of PMI calculations. A great deal of uncertainty stems from the variability in temperature that may be found at a crime scene, including temperature fluctuations, maggot mass temperature, soil temperature (Gennard, D., 2012; Rivers, D. B. & Dahlem, G. A., 2023). These variables are compounded by the fact that the linear growth models which are used for comparison receive heat at a consistent temperature constantly (Rivers, D. B. & Dahlem, G. A., 2023). Wells and LaMotte (2020) addressed concerns about accuracy by comparing experimental conditions for reference to the recorded scene conditions to assess the significance of the difference between the conditions. If the difference of conditions is not found to be significant, a confidence interval will be created with those

conditions based on time (Wells & LaMotte, 2020). Updates on calculating confidence intervals for PMI are expected in the forthcoming edition of Wells and LaMotte's work.

Collection and Preservation of Larval Specimens

Applying correct collection and preservation methods to collected larvae is important for accurate PMI estimations (Rivers, D. B. & Dahlem, G. A., 2023). There have been numerous studies that have compared different collection and preservation methods to evaluate how different methods may affect PMI estimations to help inform standard operating procedures. The Organization of Scientific Area Committees (OSAC), administered by the National Institute of Standards and Technology (NIST), drafts and evaluates forensic science standards for various disciplines. According to OSAC standards, the best practice is to collect approximately 50 specimens, if possible, from all life stages from all sites of infestation, including visually differing species (2022). Samples from separate infestation sites should be placed in separate containers because various locations of the body may have been infested at different times by different species (OSAC 2022-N-0039). Samples should be further separated into two groups, one to be reared into adult flies, and the other to be killed and preserved. It is recommended to kill the larvae by placing them in near boiling water for 30 seconds to prevent larval shrinkage, which can affect PMI calculations (OSAC 2022-N-0039; Rivers, D. B. & Dahlem, G. A., 2023). However, using near boiling water is not feasible at every crime scene. The alternative protocol is to place live larvae directly into at least 70% ethanol (OSAC 2022-N-0039). Since Diptera larvae are soft-bodied specimens, they must be stored in a liquid medium rather than being pinned. OSAC guidelines recommend that larvae should be preserved in 70 to 80% ethanol for long- or short-term storage (2022). An analysis of larval length found that 80% ethanol was the best solution for maintaining larval length (Zaher et al., 2025).

Preparing Specimens for SEM

Preparing specimens for SEM imaging involves drying processes that evaluated prior to imaging because it can vary between different instars and species. Most studies use two solutions for drying, the first solutions are alcohol-based, which replaces the water content of the specimen (SEM Sample Preparation Techniques, 2021). The alcohol solution is followed by a second solution, known as a fixative, such as McDowell solution, potassium hydroxide (KOH), or hexamethyldisilazane (HMDS) (López-Esclapez et al., 2014). Fixative solutions cross-link the proteins on the exterior surface of the larva, known as the cuticle, to increase the strength of the sample surface (Karnovsky, 1964).

Fixative solutions like McDowell, KOH, and HMDS are toxic chemicals, prompting researchers to evaluate whether their use is essential in SEM drying process. In particular, studies have highlighted the toxicity of HMDS and cautioned researchers when drying larvae (Melzer et al., 2021). Multiple studies have been done to compare different drying solutions used on larvae for visual comparison from SEM imaging. López-Esclapez et al. (2014) concluded there was no significant difference between using McDowell solution and a series of ethanol-acetone mixtures (2014). Additionally, López-Esclapez et al. (2014) found that specimens stored in ethanol can be successfully imaged by SEM, supporting OSAC's alternative killing and preservation protocols (OSAC 2022-N-0039). Looking forward to the coupling SEM and DNA barcoding, fixatives become a key consideration. Kumar et al. (2014) found that HDMS, used for preparing thrips for SEM analysis, did not negatively impact DNA quality used for species identification (2014).

Once the drying processes are complete, a thin layer of conductive material, usually gold, is evenly applied to the sample, known as sputter coating (*Choosing the Right Coating for SEM Imaging | Au vs Pt*, 2024). The sputter coating is necessary to create a conductive surface that

will allow secondary electrons to be released and to be captured by the sensor (*Choosing the Right Coating for SEM Imaging | Au vs Pt*, 2024). Without the sputter coating, the electrons accumulate on the non-conductive surface of the sample, distorting the primary electrons released by the beam and reducing the number of secondary electrons captured by the sensor, causing distortion in the received image (*Choosing the Right Coating for SEM Imaging | Au vs Pt*, 2024).

Scanning Electron Microscopy

Scanning electron microscopy depends on electron emission to create high magnification images with a lot of detail. Electrons are emitted and pass through a column made up of electromagnetic lenses, followed by deflection coils and a final lens (Figure 1) (Mohammed & Abdullah, 2018). The electrons enter the chamber and interact with the surface of the specimen. An electron detector then captures the secondary and backscattered electrons that are emitted or reflected from the specimen's surface (Figure 1) (Mohammed & Abdullah, 2018).

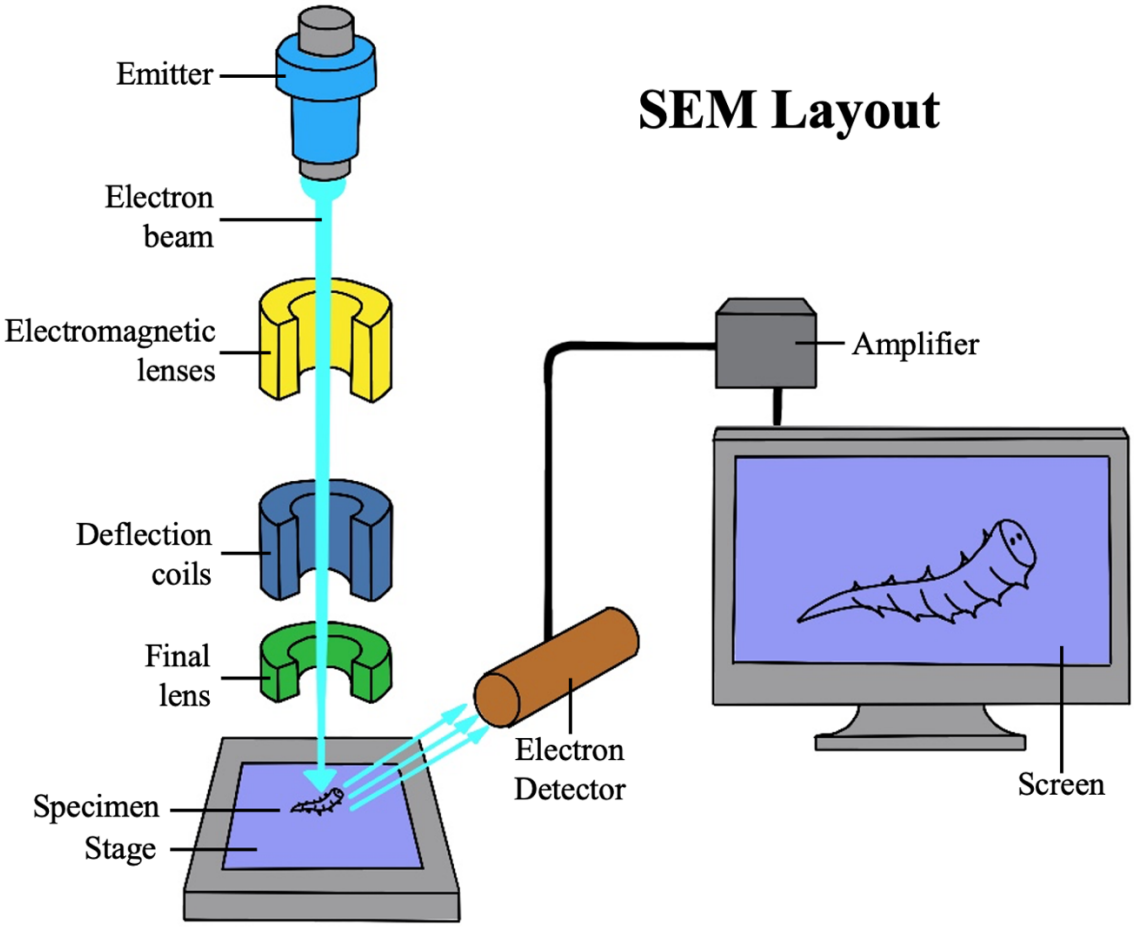

Figure 1. Schematic layout of scanning electron microscope (SEM). The electron beam is produced by the emitter and passes through a series of lenses and deflection coils to focus it onto the specimen. Electrons interacting with the specimen surface are detected and amplified to produce a magnified image on a screen (Adapted from Mohammed & Abdullah, 2018).

A traditional SEM creates a high-vacuum and high-pressure chamber which the insect is required to be dehydrated for the electron interactions to create an image (Mohammed & Abdullah, 2018; Tardi et al., 2012). Environmental scanning electron microscopes (ESEMs) uses lower vacuum within the chamber and they do not require specimens to be dried or coated because of a pressure limiting opening at the bottom of the column (Tardi et al., 2012). Electron interactions with the vapors present in the chamber of an ESEM create backscatter electrons,

which produce blurriness, noise, in the resulting images when they interfere with the path of the secondary electrons to the detector (Nedela et al., 2024). While ESEMs are not as common as SEMs, ESEMs require little to no preparation to the specimen for imaging which is useful when overserving soft-bodied specimens that are prone to physical distortion dehydration (Tardi et al., 2012).

Morphological Identification

Diptera larvae go through multiple stages of development prior to becoming an adult (Figure 2). Developmental times of *Chrysomya rufifacies* may change because of variation between different climates, with temperature and humidity fluctuating, and other species at a crime scene (Andrade-Herrera et al., 2021). Under semi-control lab conditions it has been found that it take the eggs of *Chrysomya rufifacies* around 12 hours (Figure 2) to hatch after they are deposited into feeding substrate (Andrade-Herrera et al., 2021). After hatching, the larval stage takes approximately 60 hours in total. The larval stage, which is defined by the soft body of the specimen, is divided into three instars. The larva stays in the first instar for 12 hours (Figure 2), the first instar is small; lacks tubercules along the body; has small posterior spiracles with two spiracular slits; and has single-pointed intersegmental spines (Andrade-Herrera et al., 2021; K. L. Sukontason et al., 2003). As the larva eats, it grows into the second instar and remains in this stage of development for another 12 hours (Figure 2) (Andrade-Herrera et al., 2021). The second instar is characterized by prominent mouth hooks (Figure 3A), intersegmental spines with 2-4 points (Figure 3A), visible anterior spiracles with a single row of 10 papillae (Figure 3B), tubercles on each body segment, and a button, or ecdysial scar (Figure 3F), located below the two spiracular slits (K. L. Sukontason et al., 2003). The last stage of larval development is the third instar which is in active growth for 24 hours before stopping eating 12 hours during which

the larva will find a safe place to pupate (Figure 2) (Andrade-Herrera et al., 2021). The third instar is similar to the second instar but differs by having increased body size, serrated mouth hooks, elongated tubercles, with three rows of spikes on the end (Figure 3F), around each segments (Figure 3C), tubercles surrounding the posterior spiracles (Figure 3E), and the posterior spiracles having three slits (Figure 3F) (K. L. Sukontason et al., 2003). The protruding tubules along the body and the pattern of the tubercles on the posterior end of the larvae are identifiable features of *Chrysomya rufifacies* that do not require a microscope (Gennard, D., 2012). *Chrysomya rufifacies'* protruding tubules, which are species specific and visible with the naked eye, make them easily identifiable in later developmental stages and commonly used as test species. During pupation, the exterior of the larva hardens into a pill like capsule where it will remain in such state for approximately 84 hours before emerging as an adult fly (Figure 2) (K. L. Sukontason et al., 2003).

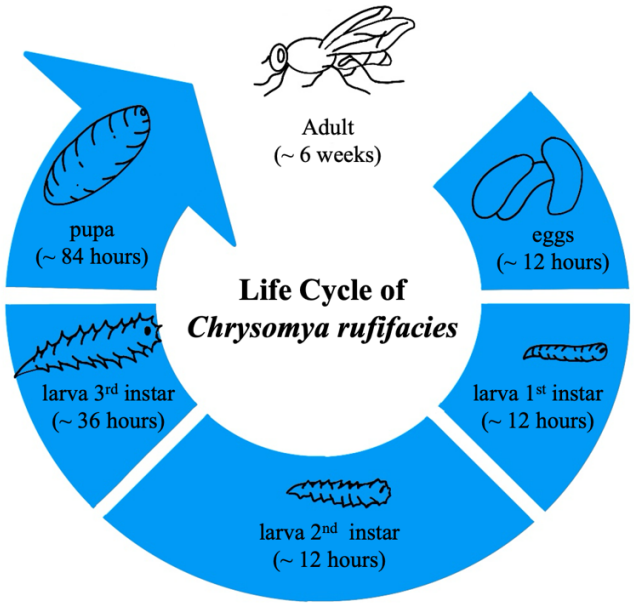

Figure 2. Illustration of life cycle of *Chrysomya rufifacies* from eggs to adult under controlled environmental conditions.

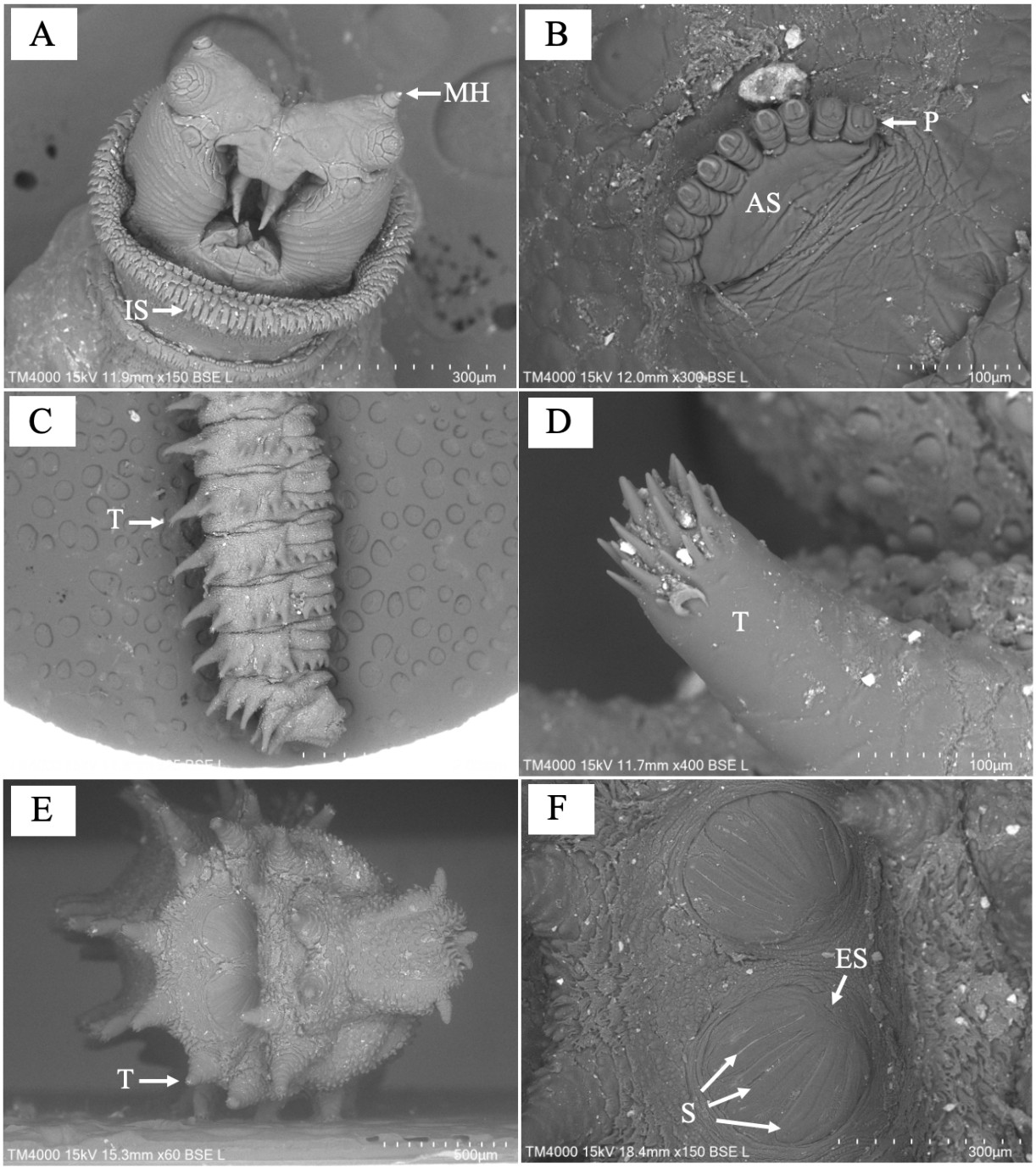

Figure 3. Morphological features used for larval identification as shown on 3rd instar *Chrysomya rufifacies using ESEM*. (A) Anterior end of larva showing prominent mouth hooks (MH) and intersegmental spines (IS) that have 2-4 points on the ends; (B) a fan-like anterior spiracle (AS) with 10 papillae along the edge of the spiracle; (C) elongated tubercles (T) encircling each segment of the larva; (D) three rows of spines present on the end of each elongated tubercle; (E)

elongated tubercle (T) surrounding the posterior spiracle; (F) two posterior spiracles with the splits (S) with an ecdysial scar (ES) below the three slits.

SEMs studies like Sukontason et al. (2003) are used by forensic entomologies to compare specimens at all stage of development to known images of *Chrysomya rufifacies* to identify the unknown specimens. These comparisons are important as many larval keys can only be used on third instar specimens, which are not always present and may be difficult to successfully rear larvae to the third instar, especially in cases with few specimen samples available. Comparison of known *Chrysomya rufifacies* is also important for excluding larval specimens from this species when preforming identifications. While the anterior and posterior spiracles described above are used to broadly identify the Calliphoridae family, surface structures like the intersegmental spines (Figure 3A), elongated tubercles (Figure 3C), especially the posterior pattern of the tubercles, and other topographical features can be used to identify larvae down to species (Szpila & Villet, 2011). However, the use of posterior spiracles images for species-level identification is a current area of research, focusing on analyzing the angle of the spiracular slits and creating computer algorithms to automate identification of larvae to species-level (Apasrawirote et al., 2022; Kang et al., 2025).

OSAC recommends some of the larvae collected from a crime scene to be used to rear into adults for morphological identification (OSAC 2022-N-0039). This suggestion is based on the fact that species-level identification of larvae is difficult and requires a considerable amount of time and training compared to identification of adult flies (Apasrawirote et al., 2022). With the use of SEM imaging studies of morphological structures across all life stages of *Chrysomya rufifacies* can be used to develop taxonomic identification keys and to characterize stage specific morphological distinctions (K. L. Sukontason et al., 2003). Using these identification keys for

different stages of development is important as an alternative to larval identification through rearing, as rearing adult flies from larvae can be unsuccessful (K. L. Sukontason et al., 2003).

DNA Barcoding

DNA barcoding uses a short sequence, between 400 and 800 base pairs, of the mitochondrial gene cytochrome oxidase I (COI) as species specific identifier (Chimeno et al., 2019; Kress & Erickson, 2008). DNA barcoding aims to use these short sequences to quickly and efficiently recognize known species with sequence comparisons to reference libraries (Kress & Erickson, 2008). The mitochondrial gene COI is used because mitochondrial genes do not recombine (Dong et al., 2021). However, mitochondrial genes are not the only genetic regions that can be used for DNA barcoding. The requirements for a genetic region to be successfully used for DNA barcoding include: significant species-level genetic variability, conserved flanking regions for universal PCR primers, and short sequence length (Kress & Erickson, 2008).

Robust libraries of reference genomes, which include forensically important species, are used to aid in the identification in wild-caught Diptera through molecular analysis (Chimeno et al., 2019; Yusseff-Vanegas & Agnarsson, 2017). Studies like Sukontason et al. (2003b) tested larvae taken from human corpses where there was no delay in testing or degradation to affect the samples negatively. There is a large amount of research using DNA for barcoding was retrieved from museum specimens that may simulate preservation and storage time of casework samples.

Coupling SEM and DNA

Pimsler et al. (2014) and Kumar et al. (2014) showed proof-of-concept for coupling SEM and DNA barcoding, illustrating that it is possible to extract DNA from specimens that have been prepared for SEM imaging. In these studies, a thin gold coating was applied to the

larvae to prepare specimens for imaging (Kumar et al., 2014; Pimsler et al., 2014). Unfortunately, the gold could not be removed from the specimen, so the insect was homogenized for DNA analysis. If specimens must be destroyed for analysis, taking a photograph before and after each step of the process is in accordance with collection protocols for comparison evidence (Ogle, R. R., JR., 2012). Once a photo is taken for comparison, the photo itself is considered evidence (Ogle, R. R., JR., 2012). By using an Environmental Scanning Electron Microscope (ESEM) instead of a standard SEM, there is no need for the specimens to be coated with gold and therefore does not need to be homogenized for DNA extraction (Friedrich et al., 2014; Tardi et al., 2012).

SEM imaging and DNA barcoding have been used separately for Diptera larvae identification. Coupling the tests could make identification of larvae and creating investigatory timelines based on entomological evidence more accessible to forensic investigations. Utilizing the collection and preservation protocols provided by OSAC, which are easily accessible to law enforcement agencies, is important for setting realistic and cost-effective expectations. DNA analysis can be completed to confirm identification and negate the need to rear larvae into their adult morphs. Entomologists can review the images to visually identify the larvae and explain the importance of finding the specimen by estimating PMI. The PMI can be used to help establish an investigative timeline and facilitate potential leads. By conducting these tests in a minimally destructive manner, the evidence remains intact and available for cross-examination by other expert witnesses if the case goes to trial. Further preservation of the specimens also allows for the defense to have their own entomologist to review the specimens later.

CHAPTER II. MATERIALS AND METHODS

Workflow Overview

This workflow outlines the major steps and methodologies followed throughout the study from larval treatments to in silico analysis.

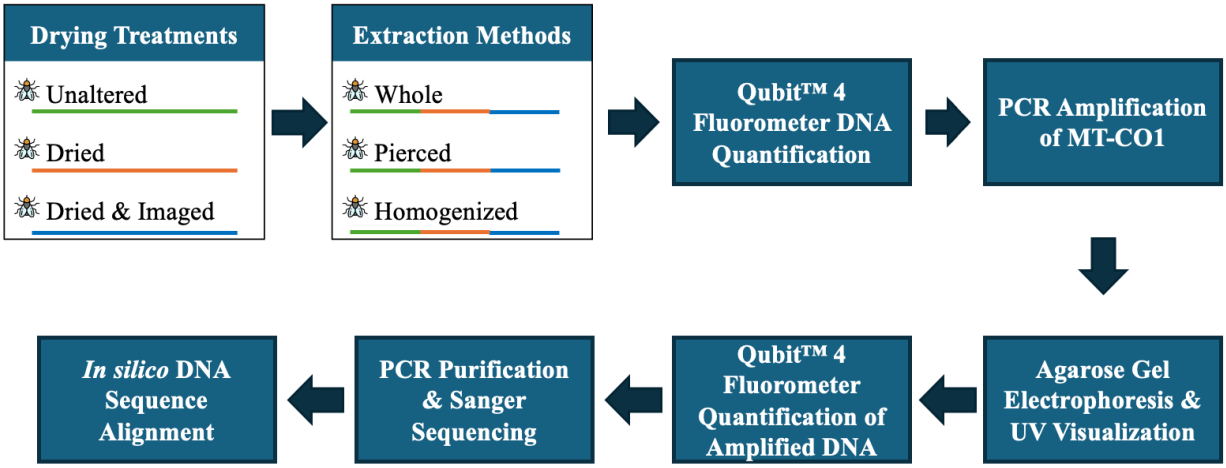

Figure 4. Overview of the experimental workflow, from specimen treatment DNA extraction to PCR amplification, sequencing, and data analysis.

Sample Collection

Third instar *Chrysomya rufifacies* larvae were obtained from a colony kept by Dr. Aaron Tarone's laboratory at Texas A&M University. The colony was fed with bovine blood meals and powdered milk. Larvae were collected using soft forceps and placed directly into 70% ethanol, with specimens stored in a 4:1 ethanol-to-larvae volume ratio. Larvae were subsequently transported to UC Davis.

Drying Method Trouble Shooting

Based on published drying protocols, three different drying assays were tested to establish the best drying protocol. Larvae were placed in fresh 70% ethanol for approximately 15 minutes. Three solutions were used, 100% ethanol, 100% acetone, and a 1:1 solution was created with 100% ethanol and 100% acetone. All three solutions were diluted down to 70%, 80%, and 90% using DI water. Individual larvae were immersed in each dilution for 10 minutes, moving from the 70% dilution up to 100%. After the final dehydration step, larvae were placed on clean glass microscope slides and air-dried for 30 minutes at room temperature.

Once the 1:1 ethanol/acetone solution was selected for the drying protocols, the amount of time in solution needed to dry the specimens was tested. During the testing of different solutions, liquid was still present when the larvae were patted with a Kimwipe® after being in solution for 10 minutes and air-dried. Larvae were placed in fresh 70% ethanol for approximately 15 minutes. Individual larvae were immersed in each dilution for 15 minutes, 30 minutes, and 1 hour, moving from the 70% dilution up to 100%. %. After the final dehydration step, larvae were placed on clean glass microscope slides and air-dried for 30 minutes at room temperature. The larvae were placed on a Kimwipe® and patted to see if any liquid was present. None of the Kimwipes® showed any liquid to be present, therefore, the minimum amount of time in solution was selected to streamline the drying process.

Sample Preparation

Three different specimen preparation methods were tested on a total of 90 specimens, with 30 specimens assigned to each method prior to DNA extraction.

1) *Unaltered Treatment:* Larvae were removed directly from 70% ethanol and gently blotted dry using a Kimwipe® to remove excess liquid. These specimens immediately proceeded to DNA extraction without further processing.
2) *Dried Treatment:* Larvae were placed in fresh 70% ethanol for approximately 15 minutes. A 1:1 solution was created with 100% ethanol and 100% acetone, which was diluted down to 70%, 80%, and 90% using DI water. Larvae were immersed in each dilution for 15 minutes, moving from the 70% dilution up to 100%. After the final dehydration step, larvae were placed on clean glass microscope slides and air-dried for 30 minutes at room temperature. Larvae were then stored in 70% ethanol for approximately 30 minutes to 1 hour prior to DNA extraction, to mimic post imaging transportation and storage in ethanol.
3) *Dried and Imaged Treatment:* Larvae underwent the same dilution and drying protocol as described in the dried treatment. After drying, specimens were mounted on a double-sided carbon tape placed on the movable microscope stage of an environmental scanning electron microscope (ESEM). Imaging was performed at two different angles, requiring the ESEM chamber to be pressurized twice Once imaging was completed, larvae were carefully removed using featherweight forceps and briefly returned to 70% ethanol for transport to the DNA extraction laboratory.

Images were taken using the Hitachi Tabletop Microscope TM4000 and associated software in Dr. Jason Bond's lab at UC Davis. A piece of double-sided carbon tape was placed on a circular metal stage. The circular stage was then screwed onto a stage where the angle could be adjusted between 0° and 90°. The combined stage was placed into the ESEM, and feather-touch forceps were used to place the larva on carbon tape. Depending on the size of the larva, one to

two could be placed on the stage at a time and the chamber was closed. Once the machine was turned on, the TM4000 Software was opened on the connected desktop computer to the user interface page, the ESEM chamber was closed and pressurized. Once the chamber had been pressurized, the software started receiving images at X25. Control knobs on the front of the ESEM could then be used to center the larva on the image receiving screen. The magnification could be increased once the larva was in the desired location for the feature to be centered. Once the magnification was deemed to be sufficient, the "auto" and "slow" buttons were selected on the computer software to make the image clearer. The brightness of the image could then be adjusted before saving the image and raw data.

DNA Extraction Treatment

In addition to the specimen preparation methods, three DNA extraction treatments were tested prior to overnight incubation at 55°C. Ten specimens were assigned to each of the three extraction treatments described below.

1) *Whole:* Larvae were left intact and incubated overnight in the lysis mixture.
2) *Poked:* A small hole in the larvae was poked using a fine insect pin on the ventral side of the anterior end of the larvae. The hole was created after the drying process was completed for the dried larvae. The hole was created after the larvae were imaged but before they were placed back in ethanol for travel.
3) *Homogenized:* Each larva was homogenized in a microcentrifuge tube with 60 µl of the lysis mixture using a plastic pestle. Once the larvae were homogenized more lysis mixture was added to bring the volume up to 200 µl.

Following overnight incubation, whole and poked specimens were removed from the microcentrifuge tubes and place in 70% ethanol, while homogenized samples were briefly centrifuged to allow tissue debris to pellet.

DNA extractions were performed using the Zymo Research Quick DNA™ Miniprep Plus Kit (Zymo Research Operations, Tuslin, CA), following the Solid Tissue Protocols. Each sample was mixed with 95 µL nuclease-free water, 95 µL Solid Tissue Buffer, and 10 µL of 20 mg/mL Proteinase K. After vortexing for 10-15 seconds, samples were incubated at 55°C for approximately 16 hours, then vortexed again for 10-15 seconds. The lysate was mixed with 400 µL Genomic Binding Buffer, vortexed, and transferred to Zymo-Spin™ IIC-XLR Column in a collection tube. After centrifugation at ≥12,000 × g for 1 minute, the flow-through and collection tube were discarded. The column was washed with 400 µL DNA Pre-Wash Buffer in a new collection tube and centrifugation at ≥12,000 × g for 1 minute. The flow through was discarded and the column was returned to the same collection tube. The column was washed with 700 µL g-DNA Wash Buffer followed by centrifugation at ≥12,000 × g for 1 minute. A final wash with 200 µL g-DNA Wash Buffer was performed, and the flow-through and tube were discarded after centrifugation. The spin column was placed in a clean microcentrifuge tube, and 50 µL DNA Elution Buffer was added to the column. Following a 5-minute incubation at room temperature, DNA was eluted by centrifugation at maximum speed for 1 minute and stored at 4°C.

DNA Extract Quantification

All extracted DNA was quantified using the Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Fresh standards and a Qubit blank were prepared for every quantification session. For each sample, 5 µl of sample was mixed 195 µl of Qubit buffer and measured using dsDNA High Sensitivity Assay. Qubit™ 4 Fluorometers detect fluorescent dyes

which bind to a specific type of molecule, determined by the assay being used (Thermo Fisher Scientific, n.d.). The dsDNA High Sensitivity Assay detects any double stranded DNA in a sample. The dsDNA High Sensitivity Assay comes with two standards, 10 µl were mixed with 190 µl of Qubit buffer into their respective tubes, which are placed into the Qubit™ 4 Fluorometer first to create a standard curve (Thermo Fisher Scientific, n.d.). The samples are then placed into the Qubit™ 4 Fluorometer where the DNA concentrations are calculated by measuring the relative fluorescence units (RFUs) and comparing the RFU values to the standards (Thermo Fisher Scientific, n.d.). The Qubit™ 4 Fluorometer was used to report DNA concentrations in nanograms per microliter.

PCR Amplification and Gel Visualization

A total of six primer pairs (Table 1) were selected from Wells and Sperling (1999) and were evaluated using AmplifX by aligning the primers against a published *Chrysomya rufifacies* mitochondrial genome (accession number NC_019634.1). The PCR amplification mixtures contained the following components: 2 µM primer, 1X Buffer, 1.5 mM of MgCl2, 2.5 mM dNTPs, and 1 unit of Taq DNA polymerase per reaction. The volumes of template DNA and nuclease-free water were adjusted so every reaction contained 2.5 ng of template DNA, based on the extraction quantification results. Cycling conditions consisted of initial denaturation of 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 1 min; annealing at 50°C for 1 min; and extension at 72°C for 1 min, and final extension at 72°C for 5 min

Table 1. Primer pairs used for PCR amplification, including forward and reverse primer labels, nucleotide sequences, and expected fragment sizes. and sequences. All primers were sourced from previously published references for DNA analysis of Class Insecta.

Primer Pair	Forward Primer Label	Forward Primer Sequence	Reverse Primer Label	Reverse Primer Sequence	Fragment Size
Primer Pair 1	TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC	C1-N-1687	CAATTTCCAAATCCTCCAATTAT	268
Primer Pair 2	C1-J-1709	ATAATTGGAGGATTTGGAAATTG	C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC	530
Primer Pair 3	C1-J-1709	ATAATTGGAGGATTTGGAAATTG	C1-N-2329	ACTGTAAATATATGATGAGCTCA	665
Primer Pair 4	C1-J-2183	CAACATTTATTTTGATTTTTTGG	C1-N-2659	GCTAATCCAGTGAATAATGG	518
Primer Pair 5	C1-J-2495	CAGCTACTTTATGAGCTTTAGG	TL2-N-3013	TCCATTACATATAATCTGCCATATTAG	562
Primer Pair 6	C2-J-3138	AGAGCCTCTCCTTTAATAGAACA	TK-N-3775	GAGACCATTACTTGCTTTCAGTCATCT	686

Preliminary testing was conducted using all the primer pairs (Table 1) on a subset of extracted samples. PCR products were visualized on 6.2 x 10 cm, 1.2% agarose gels with 15 µl of each sample and 3µl of Thermo Scientific 6X TriTrack DNA Loading Dye prior to electrophoresis, which were run for 1 hour at 55 volts. PCR products were compared to a Thermo Scientific GeneRuler DNA size standard ranging from 100 bp to 1000 bp. Fluorescence intensity was then recorded. Primer pair 1 consistently produced strong fluorescence across multiple samples.

Due to the consistency of primer pair 1, all 90 extracted samples were amplified using this primer pair following the same PCR amplification protocol. 5 µl of PCR product was diluted in 45 µl of DI water before being quantified using Qubit™ 4 Fluorometer with Qubit™ dsDNA Quantification Assay Kit and run on 12 x 13.8 cm, 1.2% agarose gels with 15 µl of each sample and 3µl of loading dye prior to electrophoresis which was run for 1 hour at 100 volts. The fluorescence of each band for all 90 samples was given a rating from 0 - not present at all; 1 - present but difficult to distinguish; 2 - present but somewhat dim; 3 - very bright.

Sequencing

One sample was picked from each of the nine drying treatment and extraction treatment combinations to be sequenced based on the concentrations post PCR. Extraction samples were amplified again using primer pair 1 and the PCR protocols above. The nine samples had to be amplified a second time because Qubit™ quantification and the gel electrophoresis consumed the first round of PCR product. Primer pair 1was used to target the beginning of the COI gene from the extracted DNA to amplify the selected 268 base pairs used to identify the specimens through DNA barcoding. The PCR products were then purified using the Qiagen MinElute PCR Purification Kit following the MinElute PCR Purification Kit using a Microcentrifuge Protocol. Five volumes of Buffer PB were added to 1 volume of PCR reaction in the original 0.2 ml PCR tubes and vortexed. A MinElute column was then placed in a 2 ml collection tube and the Buffer PB and PCR reaction dispensed in the MinElute column and centrifuged for 1 minute. The flow-through was discarded and MinElute column was placed into the same collection tube. Then 750 µl of Buffer PE was dispensed in the MinElute column and centrifuged for 1 minute. Flow-through was discarded and the column was placed into the same collection tube before being centrifuged for an additional minute. The flow-through and collection tube were discarded, and the column was placed into a clean 1.5 ml microcentrifuge tube. 10 µl of Buffer EB was dispensed onto the center of column and left at room temperature for 1 minute, then centrifuged for 1 minute. The column was discarded, and the eluted products were sent to Psomagen (Rockville, MD) for sequencing

Each PCR product was used as a template in two sequencing reactions, one reaction for each primer direction (forward and reverse). Once the sequencing results were received, the sequences were cropped to clean the ends and remove the opposite primer using 4Peaks

(Nucleobytes, 2017). The cleaned forward and reverse sequences were copied into one text file and uploaded to Galaxy (Hiltemann et al., 2024). In Galaxy the two sequences were then assembled using the cap3 tool (Huang & Madan, 1999). The output sequence was entered into BLAST as a nucleotide sequence to confirm the amplicon was identified as being from *Chrysomya rufifacies* (Altschul et al., 1990). Once the sample sequences were assembled, nine other Diptera sequences with accession numbers: NC_028413.1:1-14866, NC_026196.1:1-14882, C_041072.1:1-14885, NC_029487.1:6247-14865, KT272857.1:8-14887, NC_007102.1:1-14888, NC_002697.1:1-15004, KT272775.1:1-15050, NC_019631.1:1-15237. were retrieved from GenBank and were aligned with the amplicon fragment using the "EMBOSS needle" tool in Galaxy (Benson et al., 2017; Blankenberg et al., 2007; Rice et al., 2000). From the needle results the other Diptera sequences were trimmed to the same amplicon length. All the sample sequences and sequences from the Diptera were aligned using "ClustalW" which was then entered into the Multiple Sequence Alignment under visualizations in Galaxy and into Quicktree tool (Larkin et al., 2007; Saitou & Nei, 1987). The output from the Quicktree tool was entered into iTOL to create a Neighbor-Joining tree (Letunic & Bork, 2007). The aligned output was entered into Ident and Sim to create a matrix of percent identity (Stothard, 2004).

CHAPTER III. RESULTS

Drying Method Trouble Shooting

Six specimens were used to test each solution, ethanol, acetone, and an ethanol/acetone mixture, for a total of 18 specimens. Through this initial testing, it was observed that both the 100% ethanol and 100% acetone caused white coloration on the exterior of the larvae after 10 minutes in each dilution, moving from 70% to 100% (Figure 5A and 5B). Meanwhile, the ethanol/acetone mixture did not cause any color change to the specimen throughout the drying process (Figure 5C).

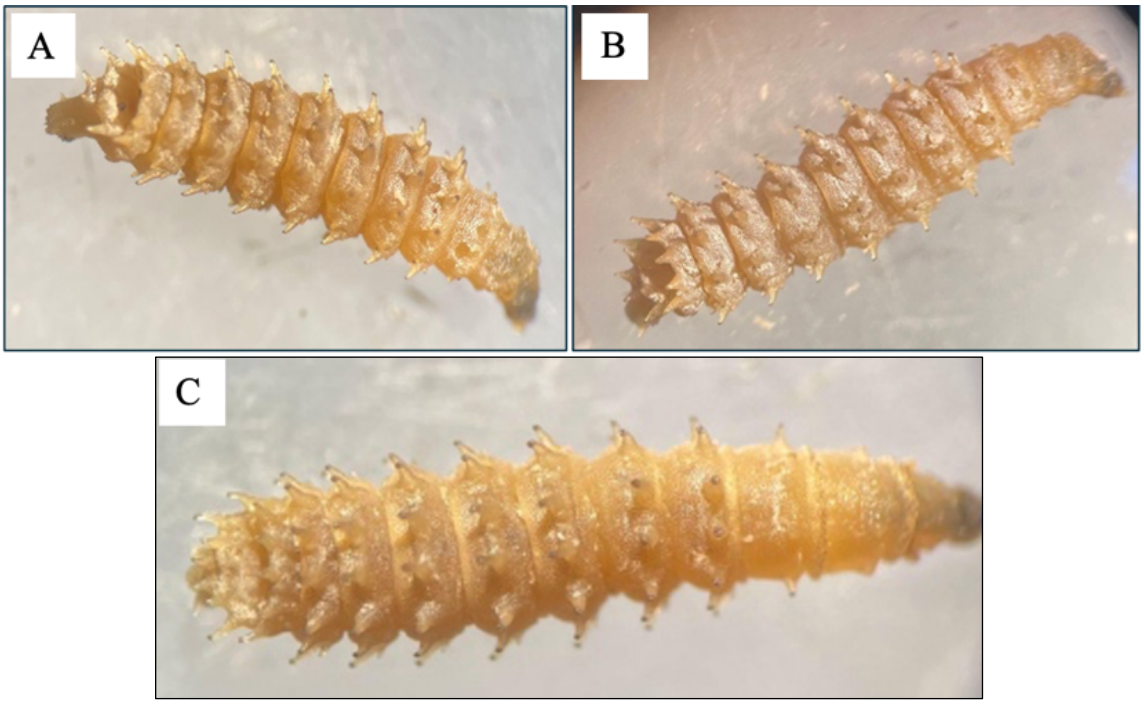

Figure 5. Effect of different solvents on larval cuticle appearance. (A) Larva soaked in dilutions of 100% ethanol; (B) larva soaked in dilutions of 100% acetone; (C) larva soaked in dilutions in a 1:1 ethanol/acetone mixture.

Once the ethanol/acetone mixture was found to be the solution to use for the drying protocols, different periods of time were tested to identify the minimum period of time

specimens needed to be kept in solution for total drying to occur. Drying was determined by patting the specimen with a Kimwipes® and observing if any liquid was found on Kimwipe® after the specimen was airdried for 30 minutes. Trace of liquid had been observed on some of the ethanol/acetone specimens in the solution tests above after the specimens had been immersed for 10 minutes. Three larvae at each time frame; 15 minutes, 30 minutes, and 1 hour, were immersed in ethanol/acetone mixtures at all dilutions for a total of 9 test specimens. All specimens were observed to have no liquid on the Kimwipe® after they were patted. It was decided to use the time interval of 15 minutes in each solution because it was the minimum amount of time needed to sufficiently dry the larvae.

Imaging

Thirty larvae were dried with the ethanol/acetone mixture, and used for imaging, and then proceeded to DNA extraction. One larva was taken directly out of ethanol, patted dry with a Kimwipe®, and imaged for comparison to dried sample since the ESEM does not require specimens to go through a drying process for imaging (Tardi et al., 2012). When visually comparing images taken of insects dried versus not dried for ESEM imaging, no notable differences were observed between two specimens. The anterior spiracles, a fan-like structure, remained intact on both specimens (Figure 6A and 6B). The topographical surface of the specimen in Figure 6A appears more pronounced; however, it appears to be unchanged compared to the texture in Figure 6B. The only difference is that the specimen in Figure 6A was dried, which likely resulted in the more pronounced textures. In addition, the posterior spiracular plates of the dried and wet specimens seen in Figures 7A and 7B were unchanged. Therefore, the drying process left the external morphologies unchanged, which means the specimens could move forward to visual identification.

The anterior and posterior spiracles remaining unchanged allowed identification of the specimens as belonging to the family Calliphoridae, while the tubercules encircling the body and posterior segment supported its identification of the larvae as *Chrysomya rufifacies* (K. L. Sukontason et al., 2003).

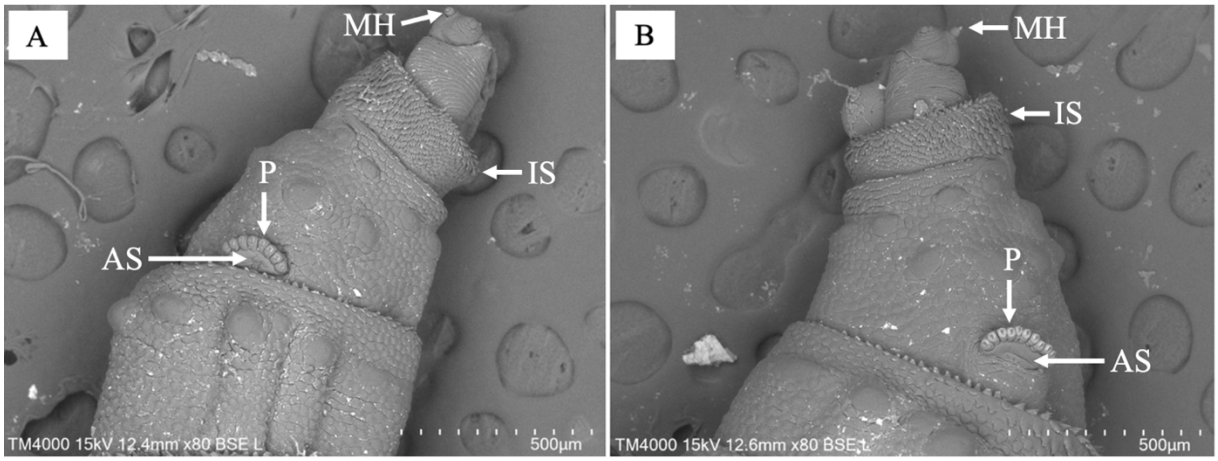

Figure 6. Comparison of the **anterior end** of *Chrysomya rufifacies* larvae using ESEM at 80x magnification with BSE detection signal in left orientation: (A) dried larva sample and (B) undried larva sample.

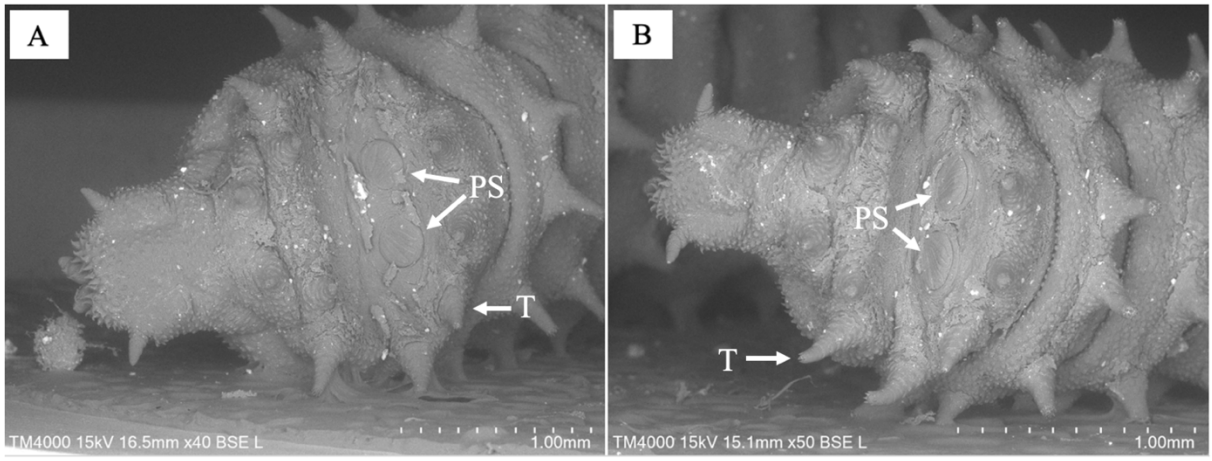

Figure 7. Comparison of the **posterior end** of *Chrysomya rufifacies* larvae using ESEM at 40x magnification with BSE detection signal in left orientation: (A) dried larva sample and (B) undried larva sample.

Extraction Method

DNA was successfully recovered and quantified under all preparations and DNA extraction methods (Table 2). An analysis of variance (ANOVA) was performed in RStudio using the Qubit™ results from the DNA extractions. Neither specimen drying treatments nor DNA extraction methods had a significant effect DNA yield when looking at the p-values on Table 3 ($p>0.05$). Since there was no significant difference in DNA yield across the drying treatments or extraction methods all extracted samples were amplified PCR.

Table 2. Total DNA Yield (ng) post DNA extraction from different specimen treatments per extraction method.

Specimen Treatment	**Extraction Method (ng)**		
	Whole	*Poked*	*Homogenized*
Untreated	93.36	83.58	136.32
Drying	100.65	108.65	122.00
ESEM	74.02	91.34	112.35

Table 3. Analysis of Variance (ANOVA) of DNA yield (ng/µL) from larval specimens post extraction using different extraction methods and specimen treatments.

Source of Variability	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F Statistic**	**p-Value**
Extraction Method	2	8.16	4.081	2.669	0.0754
Specimen Treatment	2	1.98	0.992	0.649	0.5255
Interaction	4	2.01	0.503	0.329	0.8576
Residuals	81	123.83	1.529		

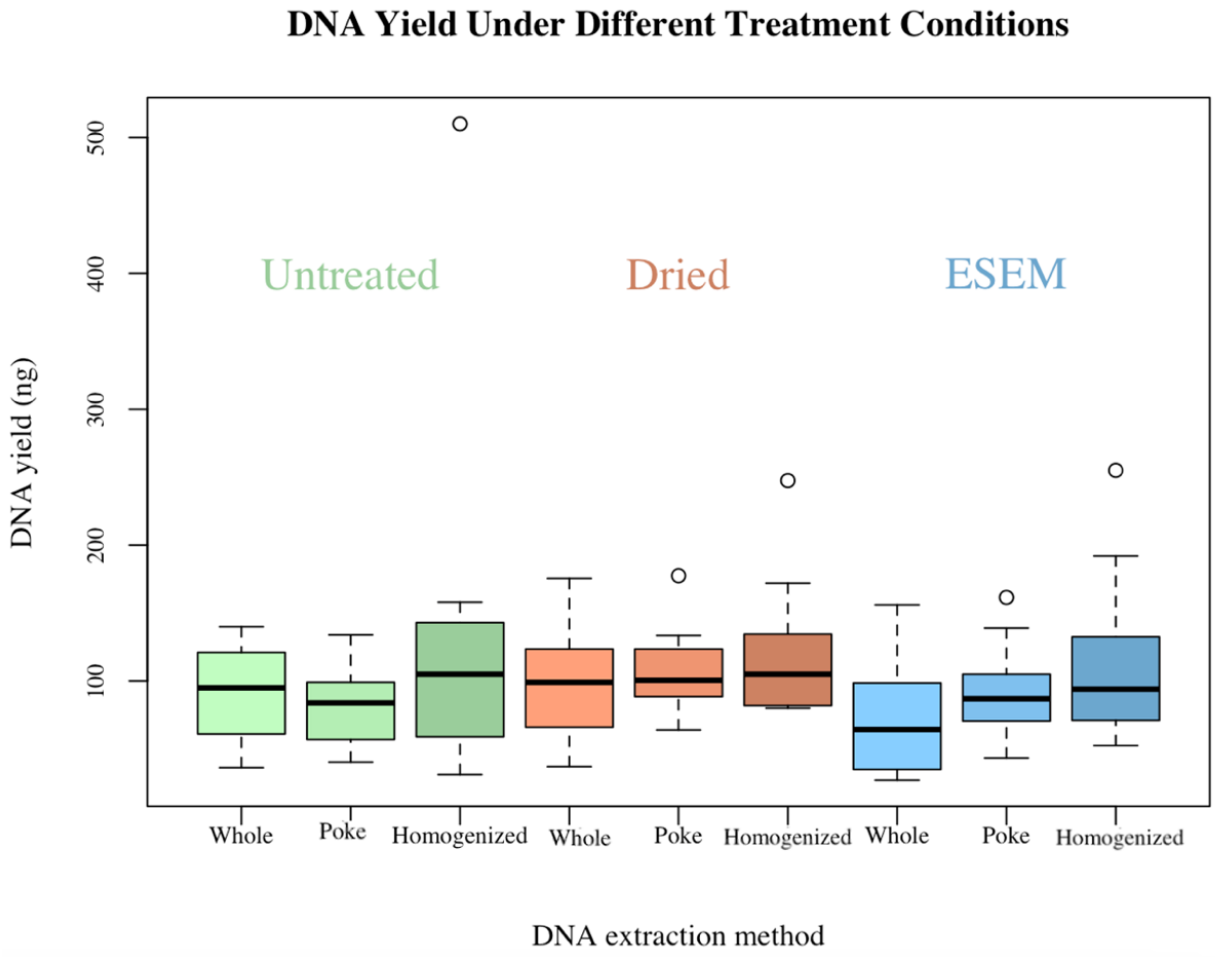

Figure 8. Box plot analysis of DNA yield (ng) per specimen post extraction under different DNA extraction methods and specimen treatments.

Preliminary Primer Pair Testing

Each of the six primer pairs was tested on a subset of extracted samples. In the Figure 9, primer pairs were tested by alternating between an unaltered homogenized sample and an amplification blank to observe the fluorescence of each fragment. Primer pair 4 did not have a visible band and the band for primer pair 6 was difficult to distinguish. Primer pairs 1, 2, 3, and 5 had easily visible bands. Primer pairs 3 and 5 had weaker bands compared to primer pairs 1 and 2, which had very apparent bands. Primer pair 1 was selected for its consistently strong

fluorescence across different samples and its coverage of the beginning of the COI gene (Figure 10).

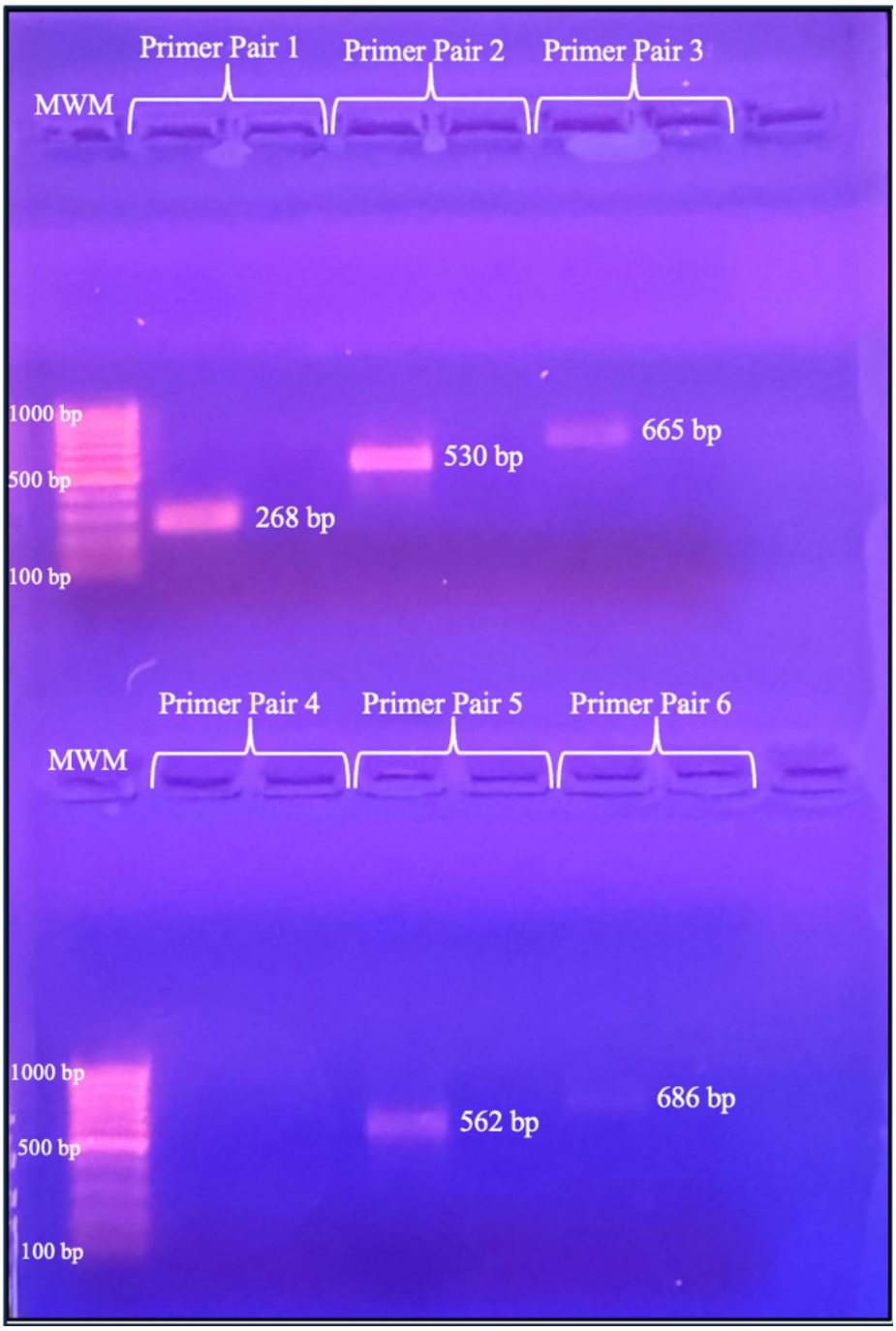

Figure 9. Agarose gels using primers for method development. 6.2 x 10 cm 1.2% agarose gel showing the amplification of multiple primer pairs alternating between the same homogenized sample and amplification blank.

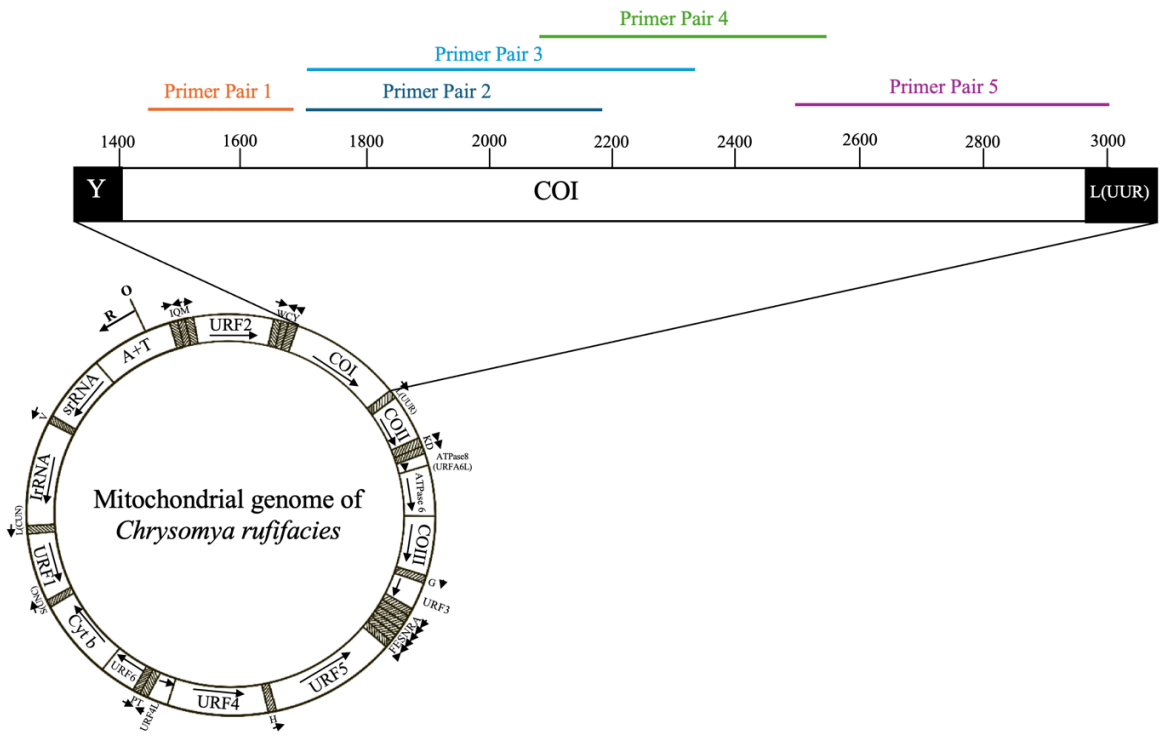

Figure 10. Chrysomya rufifacies Mitochondrial genome with cytochrome oxidase I subunit with primer pair fragments that were visible in Figure 9.

Amplification

Once primer pair 1 was selected, all 90 samples were amplified and run in a 12 x 13.8 cm, 1.2% agarose gel for one hour at 100 volts (Figure 11).

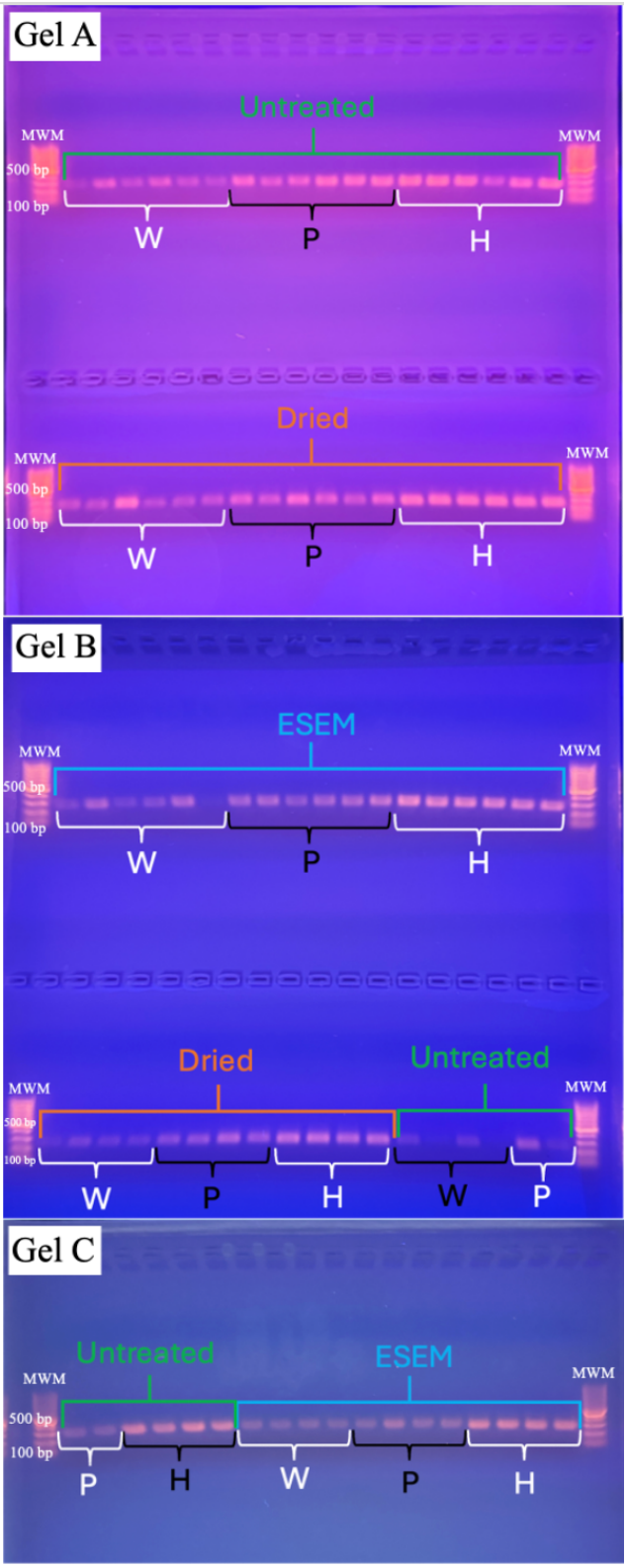

Figure 11. Agarose gels using Primer pair 1. 12 x 13.8 cm 1.2% agarose gel showing the amplification of all 90 samples. (Gel A) Row 1: Samples 7-24; Row 2: Samples 25-42. (Gel B) Row 1: Samples 43-60; Row 2: Samples 61-78. (Gel C) Row 1: Samples 79-90.

Fluorescence of each band in Figure 11 was given a 0-3 rating. The fluorescence rating along with the Qubit™ results of the PCR products were placed on a scatter plot (Figure 12) to visualize the trends of the drying and extraction treatments. The scatter plot showed that the concentration is correlated with fluorescence, higher concentrations have greater fluorescence. The whole and poked specimens appeared to make up most of the points for the 0-2 florescence scale, while the homogenized specimens were plotted majority on the 3 of the florescence scale. The scatter plot (Figure 12) was created to check the quantification values (Table 4) and trends seen during the PCR product ANOVA testing (Table 5) with the fluorescence seen on the gels (Figure 11).

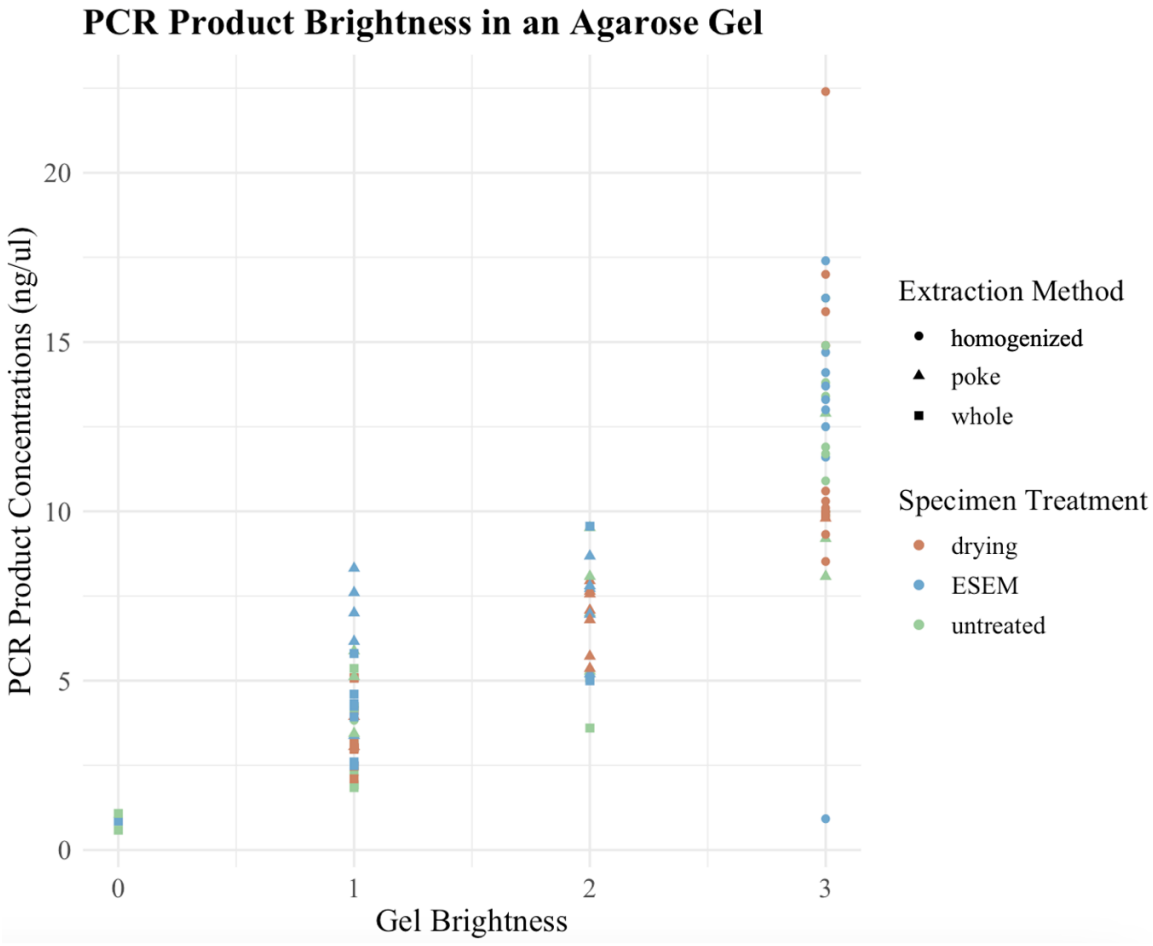

Figure 12. Scatter plot comparing fluorescence-based ratings of band intensity with PCR product concentration (ng/µl).

The PCR products were in a 1:10 dilution with DI water and quantified using Qubit ™ (Table 4). Back calculations using the dilution factor were then performed to estimate the original DNA concentrations. An ANOVA was conducted in RStudio to evaluate the effects of extraction method, specimen treatment, and their interaction on DNA concentration after PCR (Table 5). The ANOVA revealed that the extraction method was the only factor that had a significant effect on the DNA concentration ($p<0.05$). The researchers had to consider multiple hypotheses to explain the extraction method only being significant for amplification DNA yield compared to extraction DNA yield.

Table 4. Mean of amplicon concentration (ng/µL) post PCR from different specimen treatments per extraction method.

Specimen Treatment	**Extraction Method***		
	Whole	*Poked*	*Homogenized*
Untreated	2.9114	7.4500	11.6610
Drying	4.089	6.4900	12.9040
ESEM	4.3388	6.8820	12.7520
Mean	3.7797a	6.9407b	12.4390c
*Values are *highly significant; $p < 0.05$*			

Table 5. Analysis of Variance (ANOVA) of DNA concentration (ng/µL) from larval specimens post PCR using different extraction methods and specimen treatments.

Source of Variability	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F Statistic**	**p-Value**
Extraction Method	2	1152.1	576.0	65.204	$<2e^{-16}$*
Specimen Treatment	2	6.9	3.4	0.388	0.679
Interaction	4	18.6	4.7	0.527	0.716
Residuals	81	715.6	8.8		

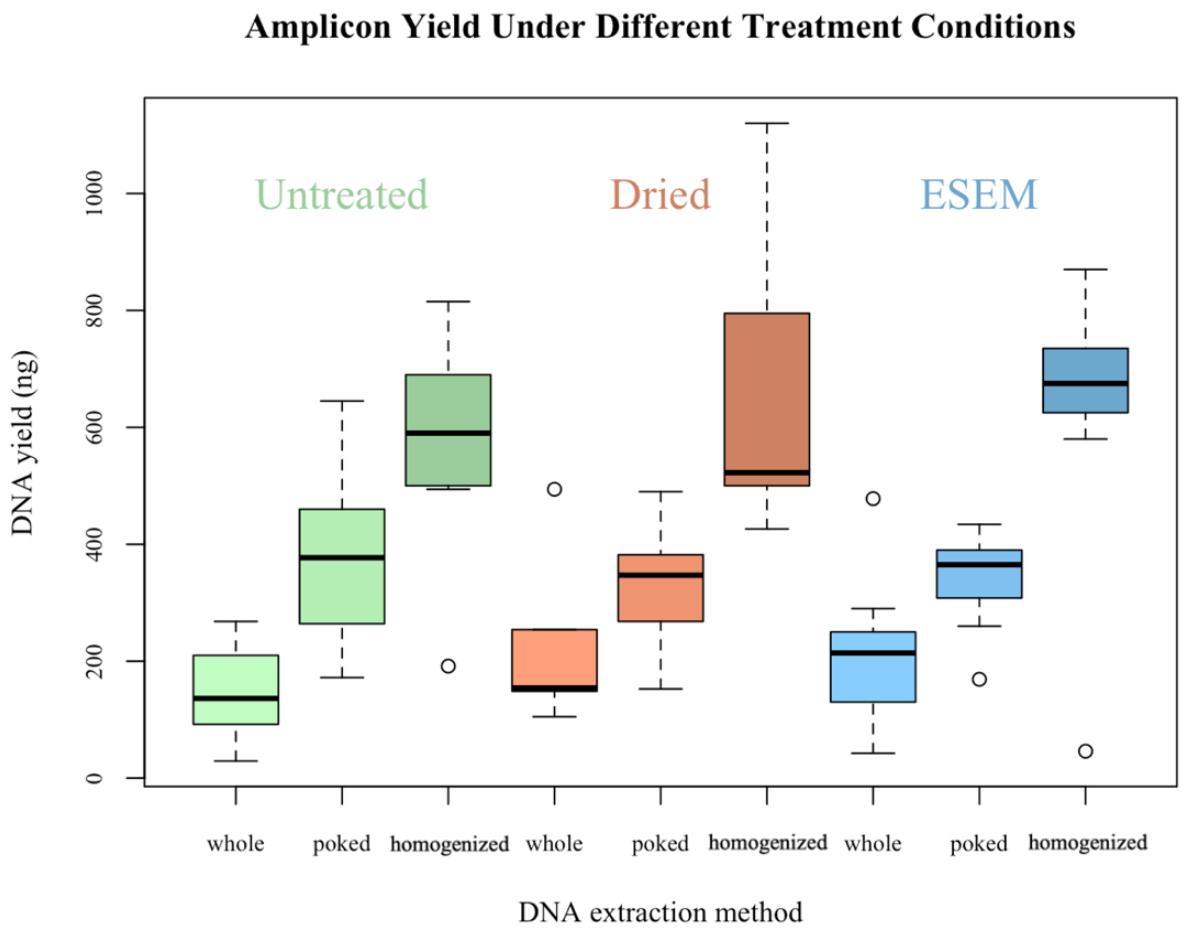

Figure 13. Box plot analysis of amplicon yield (ng) per specimen post amplification under different DNA extraction methods and specimen treatments.

Sequencing

Once the sequencing data was received, the results were evaluated for their usability. Only six out of the nine samples were able to be assembled. Of the three samples that were not usable; one specimen had been unaltered and pierced; one specimen was dried, imaged, and extracted whole; and the last specimen was dried, imaged, and homogenized. The assembled fragments were then aligned with sequences of various Diptera from the families Sarcophagidae, Calliphoridae, and Muscidae (Figure 14). The alignment was then visualized in a Neighbor-

Joining tree (Figure 8) and summarized in matrix of percent identity (Table 6). The sequence alignment (Figure 14) shows where mismatches in the base pairs occurs within the beginning fragment of the COI gene between the samples and sequences from other flies. The samples shared all the same base pairs with the known *Chrysomya rufifacies* sequence. The neighbor-joining tree (Figure 15) showed that all the samples were within the same clade on the same plane as the *Chrysomya rufifacies* sequence and showed a distinction in relationship to other flies. This distinction in relation was also visualized in the pairwise matrix of percent identity (Table 6) where green showed the highest percent similarity in sequences while red showed the lowest similarities. The samples and the known *Chrysomya rufifacies* sequence are showed 100% similarity, represented by the most the most vibrant green. Sequences with lower similarity percentages, shown in lighter shades of green, belong to other species within the genus and family of Calliphorids.

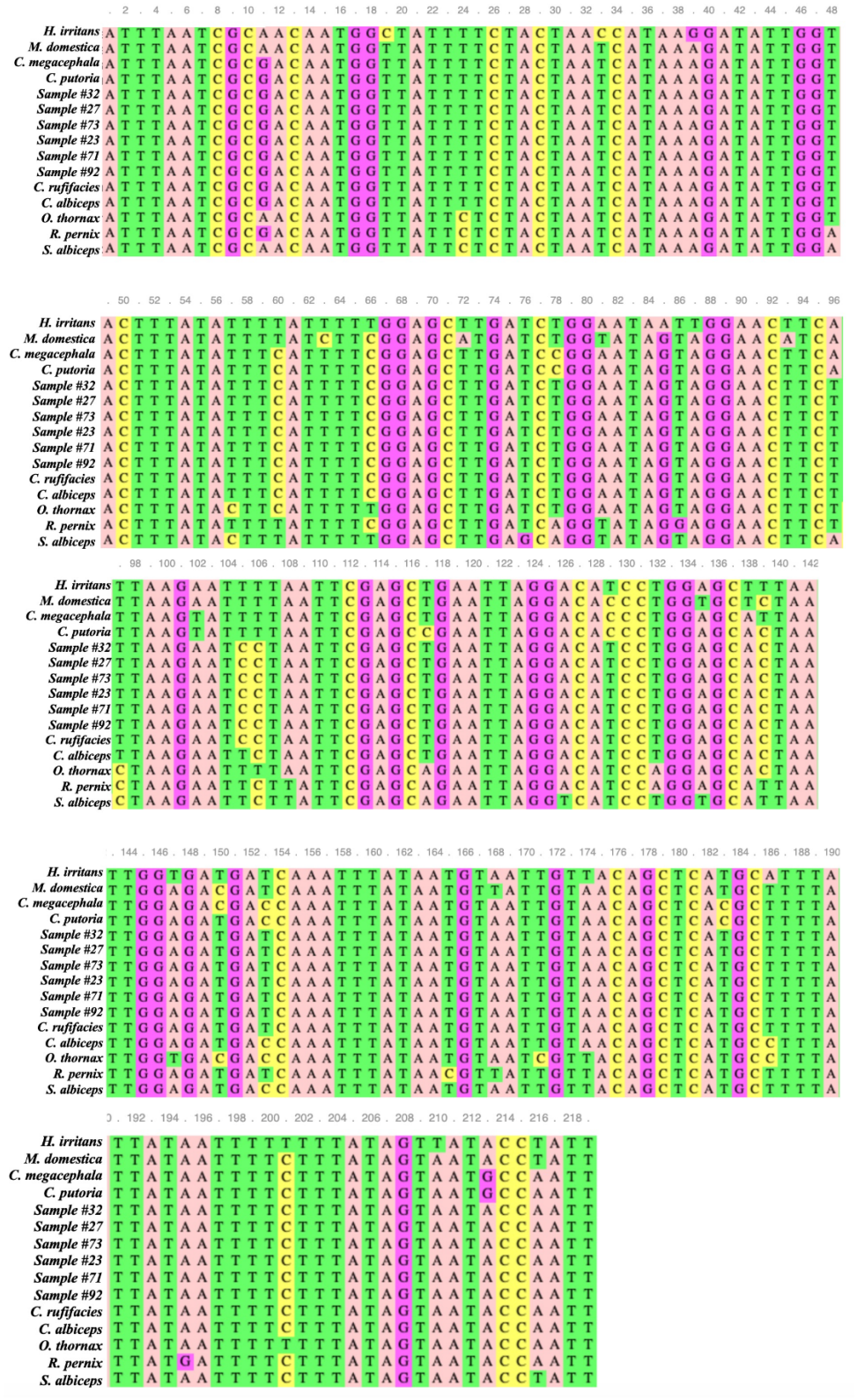

Figure 14. Multiple sequence alignment of six samples and reference sequences from other Diptera obtained from GenBank database.

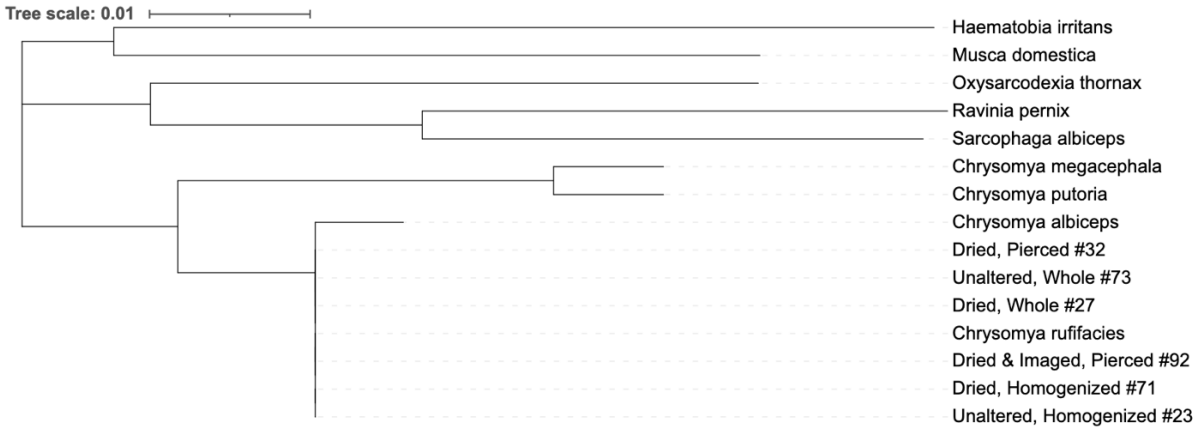

Figure 15. Neighbor-Joining tree including six samples and nine reference sequences from other Diptera. Samples were labeled according to their specimen treatment and extraction method.

Table 6. Pairwise matrix of percent identity across six samples and nine reference sequences from other Diptera. Higher percent similarity is indicated by more intense green shading, while lower similarity is shown yellow to red.

Species	*C. rufifacies*	Whole Samples (27 & 73)	Pierced Samples (32 & 92)	Homogenized Samples (23 & 71)	*Chrysomya albiceps*	*Chrysomya putoria*	*Chrysomya megacephala*	*Sarcophaga albiceps*	*Ravinia pernix*	*Oxysarcodexia thornax*	*Musca domestica*	*Haematobia irritans*
Chrysomya rufifacies	100											
Whole Samples (27 & 73)	100	100										
Pierced Samples (32 & 92)	100	100	100									
Homogenized Samples (23 & 71)	100	100	100	100								
Chrysomya albiceps	98.6	98.6	98.6	98.6	100							
Chrysomya putoria	95.4	95.4	95.4	95.4	95.8	100						
Chrysomya megacephala	94.9	94.9	94.9	94.9	95.4	98.6	100					
Sarcophaga albiceps	90.8	90.8	90.8	90.8	91.3	89.9	89.9	100				
Ravinia pernix	92.6	92.6	92.6	92.6	92.2	89.9	89.9	93.1	100			
Oxysarcodexia thornax	92.6	92.6	92.6	92.6	94.1	91.3	91.3	91.7	90.8	100		
Musca domestica	93.1	93.1	93.1	93.1	92.6	92.2	92.6	90.4	89.5	89.5	100	
Haematobia irritans	91.3	91.3	91.3	91.3	91.3	89.5	89.9	87.6	87.6	90.8	90.8	100

CHAPTER IV. DISCUSSION

Imaging

Diptera larvae are forensically important insects which are present during decomposition and are typically analyzed with microscopy. Imaging the larvae with an ESEM provides high-resolution images of morphological structures that can be used for identification. Observations across all developmental instars of *Chrysomya rufifacies* were already recorded to identify distinguishing features and morphological changes in external structures larvae. Third instar larvae have previously been cut into three portions in order to be examined with a SEM (K. L. Sukontason et al., 2003). Most of the research in this field has employed SEM rather than ESEM. In this study, ESEM was used to take images of important exterior structures. ESEMs use a lower vacuum, which allows for higher vapor pressure in the chamber, meaning that specimens do not need to be dried prior to imaging (Tardi et al., 2012). The ESEM used in this study was the instrument available to the researchers and is primarily intended for imaging insects.

The drying protocol followed in this study closely followed published protocols for traditional SEM imaging, which require specimens to be completely dry prior to imaging (K. L. Sukontason et al., 2003). It should be noted that vapors present in the chamber of the ESEM may contribute to more noise – blurriness – being present in ESEM images compared to SEM images (Tardi et al., 2012). Since we were not limited by needing larvae to be dried prior to being imaged, this allowed for a specimen taken directly from the 70% ethanol to be imaged for comparison (Tardi et al., 2012). No major differences in external morphology, specifically the posterior and anterior spiracles, was noted between the specimen directly removed from ethanol and the specimens that were dried before imaging (Figures 6 and 7). Thus, the drying method does not change the visual observation leading to identification that would be made by a forensic

entomologist. Only third instar specimens were examined because they have the most differentiating external morphology and are easily evaluated as a test species. Various larval developmental stages of *Chrysomya rufifacies* have been imaged which can be used for comparisons in the future (K. L. Sukontason et al., 2003). Although different developmental stages may require different specimen preparation methods, and such methods have been examined, further evaluation is needed to determine whether it is practical to implement these procedures for all instars (Pimsler et al., 2014). The analysis of larvae at different developmental stages should be approached with caution, as ethanol/acetone solution may have adverse effects that compromise key morphological features, potentially resulting in misidentification or failure to identify. Further research is needed to evaluate how drying solutions impact various larval stages and DNA recovery, while also considering factors such as cost and accessibility. Regardless of developmental stage, these images would likely be useful to an expert in the field assuming the images captured the accurate and relevant characteristics used for identification, especially if the images taken at the lab could be compared to known published images.

One limitation in Zaher et al.'s (2025) study is the length of time spent larvae in storage because the duration was only monitored for fifteen days. More imaging studies should include duration of specimen storage, as larvae may sit on a shelf for months before being analyzed by another expert witness for court. Further research is needed to investigate the long-term effects of ethanol storage on specimens and to determine whether maintenance is required.

DNA Extraction and Quantification

The *Chrysomya rufifacies* in this study were stored in 70% ethanol for four to five months before being dried for imaging, followed by minimally destructive DNA extraction methods typically used for museum specimens. Many studies have shown evidence of obtaining

DNA from larval museum specimens in a minimally destructive manner that have been stored in ethanol for an extended period of time (Martoni et al., 2019; Thomsen et al., 2009). Minimally destructive methods typically use overnight incubation with gentle agitation in lysis buffer for DNA extraction (Martoni et al., 2019; Thomsen et al., 2009). This minimally invasive technique involves a small disruption to the external wall of the specimen (Favret, C., 2005). These methods have shown to be successful in retrieving DNA from specimens while maintain external morphology without homogenizing the specimen.

The DNA yield for the extraction method did not show any significance between the treatments used on the samples (Table 3). However, some difference in the extracted DNA yield was expected between the treatment groups. Whereas the quantification of the amplicons using the Qubit™ 4 Fluorometer showed a significant different in extraction methods (Table 5) and increase of DNA yield within all the drying treatments and was similar across all extraction methods (Figure 13). All extraction samples and post-PCR samples were quantified using the Qubit™ 4 Fluorometer. The Qubit assay is highly specific for detecting double-stranded DNA and measures total double-stranded DNA present in a sample, not exclusively Diptera DNA. In this study, Qubit™ was used as a quality control step to confirm that DNA was consistently extracted from the larvae, regardless of the drying or extraction method.

Visual confirmation of the amplicons using agarose gels could be improved by measuring the fluorescence more accurately with gel densitometry because the fluorescence scale used above was subjective to error. Given the extraction method was found to be significant for the PCR fragment yields (Table 5) and the mean of the homogenized DNA yield being higher for the fragment yields (Table 4), it is reasonable that the bands for the homogenized samples are the most visible (Figure 11). The significance of the extraction method is reflected in Figure 12,

where there is a relatively clear distinction with the least bright being the whole larvae and the brightest being the homogenized larvae. There is overlap between extraction types within the scatterplot, however, variation within reactions and human error could account for that. Additionally, the different drying treatments were not found to be statistically significant, which could be the cause of having all the drying treatments being represented at each brightness.

There are a few hypotheses that may be the cause for the significance in extraction method when quantify the amplicons. There may have been PCR inhibitors present internally and externally on all the larvae, which could contribute to the equivalent results of DNA yield between each drying method reflected within each drying treatment (Figure 13). The larvae were fed blood meals and powdered milk. The powdered milk may contain calcium which would act as a PCR inhibitor and may be present on the larvae's surface and internally (Schrader et al., 2012). The powdered milk could have removed during the drying and purification processes; however, it is unknown when the larvae had last eaten the powder milk before being killed. In the future the powdered milk portion of the meals could be removed from the diet of the larvae prior to going through the same drying and extraction processes to remove this possibility. If inhibitors were co-extracted, could have resulted in the DNA samples used for PCR amplification would be halted or reduced by the inhibitors. Additionally, in Stein et al. (2022), it was found that minimally destructive extractions using larval insects tend to contain higher amounts of inhibitors compared to minimally destructive extractions using adult insects. Stein et al (2022) evaluated different PCR inhibitor-resistant master mixes, which should be added to this procedure in the future to increase PCR amplification. PCR inhibitor-resistant master mixes would need to be validated within this procedure but could be beneficial when working with wild-caught larvae from crime scenes.

Since Qubit™ quantification is not specific to the COI gene, it is possible DNA from the larvae's meals and other bacteria was also being quantified (Rusch et al., 2019). Therefore, 2.5 ng of larval DNA may not have been added to the PCR mixtures. If less Diptera DNA was added to the PCR mixtures this could have contributed to the amplicon quantification trends because there would have been less Diptera DNA to amplify if it was diluted down with other DNA. To improve the quantification values and to be more accurate with the amount of Diptera larvae being used in PCR mixtures further steps should be taken to understand what DNA the extraction samples contain. To quantify the amount of Diptera DNA found in an extraction would be to use qPCR and Diptera primers and use those results to add the 2.5 ng of DNA to the PCR mixtures. Similarly, one could use qPCR and primers for the bovine blood meals to identify and quantify the amount of DNA from the blood meal contained in the samples. Bacteria could also be quantified within the extracted DNA by using 16S rRNA primers (Yu et al., 2015). One drawback to running these specific quantification tests is that the instruments will only quantify what you tell them to look for. A better understanding of what is contained within the entire sample would be by completing high throughput sequencing to fully identify the contents of the samples.

DNA-barcoding

While one specimen from each of the nine specimen groupings was amplified and sent for Sanger sequencing, only six sequences were usable. One larva from each extraction group was unable to be analyzed likely due to the samples being overloaded with DNA. Therefore, we must consider how reliable the methods are for sequencing if these methods were to be used for evidence taken from a crime scene. Further validation with more replicates would be necessary to reliably extract and amplify DNA in a minimally destructive manner.

To improve the success of DNA amplification during PCR future researchers could utilize nested PCR. Nested PCR uses multiple primers that create two amplicons, one of which overlaps or is nested within the other amplicon (Mitchell, 2015). The initial PCR products are reamplified with the internal primers, making the resulting amplicon base pairs more refined (Mitchell, 2015). Nested PCR has been proven successful in increasing amplicon yield and improving identification accuracy for DNA barcoding, especially when working with preserved museum specimens (Mitchell, 2015). Incorporating nested PCR into these protocols would increase the success rates for PCR yield and improve the reliability of species-level identification from larval specimens minimally invasive extractions more reliable.

The six usable sequences were trimmed and assembled all had a 100% identity to a databased *Chrysomya rufifacies* sequence (Figure 14 and Table 6). A Neighbor-Joining tree (Figure 15) was created to identify how precisely the fragment of the COI gene could be used to identify the larvae within the family, genus, and species of Diptera. The Neighbor-Joining tree showed that the segment used resulted in a distinction being made between different Diptera families (Figure 15). Even though the fragment size used in this study was 268 base pairs, which is smaller than the typically fragment length used for DNA barcoding, the segment of DNA can still be considered useful for DNA because it contained species-level genetic variability that was used for identification (Kress & Erickson, 2008).

Having public databases of complete reference genomes available for comparison is important for identification via sequencing. To prepare for working with wild caught specimens, *Chrysomya rufifacies* samples should be collected across the United States and then sequenced. By sequencing the COI gene from specimens collected in a wild geographic region, we would be able to understand the variability that this segment of the genome has and if it would remain as a

100% match in a pairwise matrix. Yusseff and Agnarsson (2017) asserts that the COI gene does not reliably distinguish between closely related species of Calliphorids, however, *Chrysomya rufifacies* has yet to be reported as one of these species that cannot be distinguished using COI. Therefore, further investigation into the reliability of using COI to identify *Chrysomya rufifacies* should be conducted. Yusseff and Agnarsson (2017) suggests using ITS2 as a second marker along with COI identify forensically important larvae to increase the certainty of identifications of wild-caught specimens.

Overall, the larvae in this study remained identifiable at the species-level despite undergoing various specimen treatments and extraction methods. This demonstrates that, regardless of the protocol used, species identification is still achievable. Therefore, using the least minimally destructive method is preferable, as it preserves the specimen for potential repeat analyses and provides high-quality images suitable for court proceedings.

CHAPTER V. CONCLUSION

Coupling ESEM imaging and DNA barcoding for larval identification of third instar *Chrysomya rufifacies* in a minimally destructive manner is possible. This study was able to follow drying methodologies for imaging which did not affect major morphological features used for identification. It was also shown that a segment of COI could be used to identify and differentiate *Chrysomya rufifacies* from other Calliphorids and other Diptera families. ESEM images of minute morphologies can be used to identify larval specimens with extensive training and rigor from a forensic entomologist. The use of DNA barcoding could confirm species identification without the need to rear larvae to adulthood, which is especially useful when rearing is unsuccessful or when only a few larvae are available from a scene. Drying and visual identification can be performed promptly upon receiving of the larvae, allowing for the development of initial PMI estimates, which depend on the species and environmental conditions. Using visual identification maintains the rigor of skills maintained by the forensic entomologist and allows them to speak to the importance of finding larvae at a specific developmental stage at the scene. Overall DNA workflow from DNA extraction through sequencing takes time, especially if the samples cannot be sequenced in-house, making initial visual identification a necessity for time-sensitive investigations. DNA confirmation could be used in court proceedings as a confirmation of identification, while images and preserved specimens would be maintained for cross-examination.

We have demonstrated that it is possible to identify to Diptera larvae to species-level with DNA obtained from specimen that were prepared for ESEM imaging in a minimally destructive manner. Future studies should focus on creating and optimizing reliable methodologies to test the PCR amplicon yield for sequencing, including inhibitor resistant PCR master mixes, nested PCR,

and specific quantification of Diptera DNA. The methodologies should also be expanded to first and second instar *Chrysomya rufifacies* to ensure that drying and visual identification of these instars is possible. Once a reliable method has been optimized, a nationwide sample set of wild *Chrysomya rufifacies* should be created to study the variability of this segment of COI.

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