Use of the CO I Gene as a Species Indicator for Forensically Important Flies: A Forensic Entomology Laboratory Exercise

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***Abstract*:** Forensic entomologists utilize insects (particularly flies) to establish the time interval between death and body discovery. This important piece of information may answer questions as to the circumstances of the individual's death and insects are now routinely utilized and recognized as being important forensic indicators. Of extreme importance is the correct identification of the fly species associated with the body, as misidentifications will cause inaccurate time of death estimates. Traditional fly identification methods rely on recognizing distinct physical traits each fly species may possess; however, this has given way to molecular techniques (i.e. DNA sequences) that are quicker and more reliable. Unfortunately no DNA sequence information exists on the forensically important fly species in the western U.S. although a molecular database has been advocated by a number of forensic entomologists. This laboratory experience allows students to develop general molecular based skills they will need later in their careers while simultaneously contributing data that will be used to create a molecular database containing DNA sequences for forensically important California flies.

Keywords: forensic entomology, undergraduate biotechnology laboratory, molecular techniques

Introduction

Medicocriminal entomology examines the utility of insect evidence in solving crimes, most often violent crimes including homicides. One of the most crucial pieces of information is determining the time elapsed since death, or postmortem interval (PMI). Under most circumstances, this can be determined by a medical examiner as bodies generally are found within 48 hrs and indicators such as rigor mortis, algor mortis, or livor mortis can be used to determine PMI. However, bodies may often lie undiscovered for many days or months and those methods become less reliable. It has long been known that insects associated with remains display PMI-dependent processes, which includes the development of insect species whose larvae consume dead tissue. Thus, a PMI estimate can be based on the oldest developmental stage of an immature insect found on a body. Due to their size, numbers, and ubiquity, flies (i.e. Muscidae, Sarcophagidae, and Calliphoridae) are most noticeable and commonly found insects when a body is discovered. The most limiting factor in using flies as a PMI indicator is proper species identification as different fly species differ in terms of growth rate and arrival time on a body. Typically, identification is done through morphological examination of important setae (hairs) found on the body. This is a tedious, and often impossible prospect as many of these setae break off. More recently, forensic entomologists have advocated using DNA sequences and it appears that the cytochrome oxidase (CO) I gene in the mitochondria is excellent in discriminating species and would allow non-entomologists to easily identify flies based on their DNA sequences (Malgorn and Coquoz 1999; Sperling et al. 1994; Wells and Sperling 1999). Although some work has been performed on a few common species found in the eastern U.S., no work has been attempted on flies found in the western U.S.

The Department of Biological Sciences at SJSU now offers a course in forensic entomology (Entomology 106) that serves undergraduate biology majors and criminal justice administration majors with a concentration in biology forensics. This course examines the science and methodology used to collect, analyze, and present information regarding insects and other arthropods that are important in legal investigations. The majority of the laboratory component is devoted to the collection and identification of carrion feeding flies in the family Calliphoridae because they are the most important forensic indicators in medicocriminal cases. Students begin the semester by trapping, identifying, and curating flies, followed by instruction in molecular based methods including: DNA extraction/purification, PCR, electrophoresis, and sequencing. Students then obtain the DNA sequences and are introduced to

bioinformatics programs that allow fly for identification.

The expected outcomes of this exercise are twofold. First, students will acquire a working knowledge of basic molecular techniques. Although, students enrolled in this course may not work specifically in biotechnology, they will at least have been exposed to various molecular techniques. The students most interested in this course generally are animal or ecological biology majors who rarely are afforded the opportunity to combine ecological study with molecular techniques. Second, the data students generate in this activity is extremely important to forensic entomologists as it will initiate an expandable database of calliphorid diversity in California.

Article III. Materials and Methods

Field Collection: Students constructed their own fly traps to collect flies for their study. Traps consisted of two 2L plastic soft drink bottles in which the capped bottle served as a lid and the bottom half of the bottle was removed. This open-ended bottle was fitted snugly over a second, uncapped bottle. The bottom portion of the second bottle was spray painted with black paint and five small holes were cut at the base of the bottle (Fig. 1). Raw meat was placed inside the holes where the meat quickly rotted. This bait attracted flies that entered through the holes, and subsequently passed the painted portion of the trap towards the top where they were collected. Flies were then preserved in 95% ethanol with data (date and location) recorded by the student. Although this method was successful in obtaining large numbers of flies, the more enthusiastic students collected flies on carrion such as dead birds and mammals. In order for students to obtain a passing grade for this portion of the exercise they must have a collection with complete data of at least 50 flies in two different Calliphorid genera, and one representative each from the families Sarcophagidae, Fannidae, and Muscidae.

DNA extraction: A quick, inexpensive method was developed for extracting fly DNA. The right mid and hind leg of each fly were carefully removed with forceps and placed in a 1.5ml microfuge tube. The legs were washed for 10m in distilled water and

FIG 1. Fly trap constructed by students to collect specimens.

repeated twice more. After the final rinse, as much of the water was removed as possible, and the legs were ground with a plastic pestle with 4ul of proteinase K (20mg/ml). When the legs were sufficiently pulverized, 50 ul of a 5% Chelex® 100 (BioRad) solution was added to the tube and incubated overnight at 56^{O}C. The DNA template was then heated for 10 min at 100^{O}C then stored at -4^{O}C until use.

PCR/sequencing: A small region of the COI gene was amplified using the following primers: 5'-CAG CTA CTT TAT GAG CTT TAG G-3' (forward) and 5'-CAT TTC AAG CTG TGT AAG CAT C-3' (reverse). PCR conditions were as described in Wallman and Donnellan (2001). PCR cycling was performed using a Perkin Elmer 9600 thermal cycler. After an initial incubation period at 95^{O}C for 10min, 40 cycles were run (94^{O}C for 30 sec, 52^{O}C for 1 min, and 72^{O}C for 1 min), followed by one cycle at 72^{O}C for and a hold cycle at 4^{O}C. PCR products were visualized via electrophoresis (1% agarose gel in TAE) and successfully amplified reactions were purified using a DNA Clean & Concentrator kit (Zymo Research). PCR products were then sequenced (one way only) using an ABIPrism® Big Dye Terminator sequencing kit and an ABI 373 (Applied Biosystems) sequencer.

Sequence Analysis: Chromatograms were examined by students, and the CO I gene region amplified was identified and imported into a Vector NTI database. Students were then able to

align their sequences and develop cladograms. Sequences were then compared with previously published sequences to verify their unknown species.

Article IV. Results and Discussion

A relatively short region (approximately 300bp) of the distal portion of the CO I gene was amplified by the students and corresponds to base pair numbers 1082-1380 found in Sperling *et al*. (1994). By comparing their unknown fly specimens with previously published sequences of known fly species via sequence alignment, students were quickly able to confirm their unknown specimens. For example, the sequence of the very common *Phaenicia serricata* from diverse geographic areas was remarkably conserved. In fact specimens AY842612 from Australia (Wallman *et al*. 2005) and AJ417717 from Hawaii (Stevens et al. 2002) were 99% similar when sequences were aligned and compared with specimens that students collected (Figure 2).

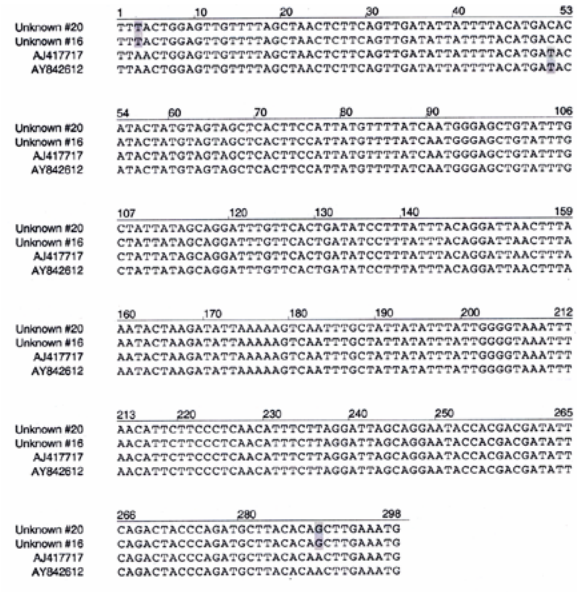

FIG 2. CO I gene sequence alignment. Note only three base pair differences (locations 3, 51, and 279) between published sequences of known fly species from two different geographical areas (Hawaii [AJ417717] and Australia [AY842612]), and two student samples of the same species.

During the first semester the course was taught, over 10 calliphorid species were collected. Of these, at least three are new collection records in the San Francisco Bay Area, and we have collected enough data to confirm that one species of fly not thought to be important as a forensic indicator is one of the most commonly found species on human corpses found in the field. In succeeding semesters it is hoped that the different families of forensically important flies (i.e. Muscidae and Sarcophagidae) will be examined. Moreover, a number of colleagues have expressed interest in this project and are now submitting samples from across the globe.

Student assessment was performed by administrating midterms on material discussed in class and laboratory practical examinations on equipment, and skills evaluation such as casting gels, pipetting, and sterile technique. This exercise also served as an introduction to bioinformatics.

Article V. Acknowledgements

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Article VI. References

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Call for Applications -- John Carlock Award

This Award was established to encourage biologists in the early stages of their professional careers to become involved with and excited by the profession of biology teaching. To this end, the Award provides partial support for graduate students in the field of Biology to attend the Fall Meeting of ACUBE.

Guidelines: The applicant must be actively pursuing graduate work in Biology. He/she must have the support of an active member of ACUBE. The Award will help defray the cost of attending the Fall meeting of ACUBE.

The recipient of the Award will receive a certificate or plaque that will be presented at the annual banquet; and the Executive Secretary will provide the recipient with letters that might be useful in furthering her/his career in teaching. The recipient is expected to submit a brief report on how he/she benefited by attendance at the meeting. This report will be published in *Bioscene*.

Application: Applications, in the form of a letter, can be submitted anytime during the year. The application