# *COI* DNA Barcoding of Forensically Relevant Insects Collected during Decomposition in Huntsville, TX, USA

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## INTRODUCTION

- Forensic entomology is the application of insects to civil and criminal law, commonly associated with death investigations. Time of death estimations can employ methods such as insect succession. Insect colonization changes throughout decomposition, with colonization patterns depending on location and time of year.
- Accurate species identifications are crucial for successional data to be reliable. Morphological identifications can be challenging due to the entomologist's experience, the availability of identification keys, and insect life stages. An alternative approach for insect identification is DNA barcoding.
- The traditional barcoding marker for insects is a 658 bp region of cytochrome c oxidase subunit I (*COI*) [1]. While DNA sequences remain similar within a species, geographical variations can arise as single nucleotide polymorphisms (SNPs).
- Degraded DNA can be encountered when using museum specimens, especially after years from the initial collection [2,3]. Specimens are not typically preserved for DNA analysis, leading to fragmented DNA. Shorter fragments of DNA can be targeted to increase the success of recovering sequences [2,3].
- This study aims to increase the available sequences of forensically relevant insects collected from decomposing cadavers in Huntsville, TX (**Fig. 1**). Due to these specimens being stored in a museum, primers were designed to target smaller segments overcome DNA degradation.

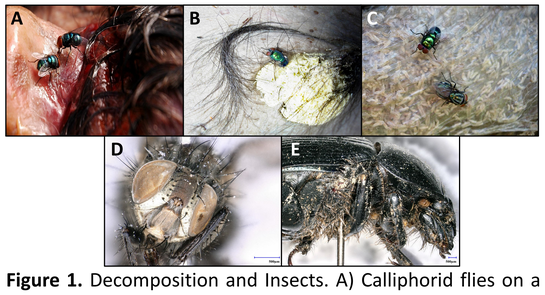


**Figure 1.** Decomposition and Insects. A) Calliphorid flies on a body. B) Calliphorid fly and eggs. C) Diptera with maggots. D) *Ravinia derelicta* (anterior). E) *Geotrupes blackburnii* (lateral).

## MATERIALS & METHODS

**Insect Collection**

Insects were collected at the Southeast Texas Applied Forensic Science (STAFS) Facility, successional matrices were constructed, and specimens were deposited at the Sam Houston State Natural History Collections. A representative specimen from several Diptera and Coleoptera species were used for DNA barcoding. Specimens were photographed on a Keyence VHX-5000 microscope prior to DNA extraction.

**DNA Extraction and Quantification**

DNA was extracted with DNeasy® Blood & Tissue Kit (QIAGEN) Insect protocol. Samples were initially cleaned with 20% bleach followed by a di$H_2O$ wash. Initial extractions were performed on a hind leg of Diptera specimens with a 30 µL elution. Coleoptera and select Diptera underwent a modified overnight lysis protocol. DNA concentration was determined using Qubit™ 1X dsDNA High Sensitivity Assay (ThermoFisher) using 2 µL of extract.

## RESULTS & DISCUSSION

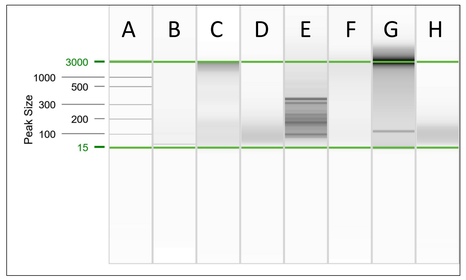


**Figure 2.** Degradation of DNA. Extracts of recent (R) and museum (M) samples are shown. A) Size marker, B) *P. regina*-M, C) *P. regina-R,* D) *L. mexicana*-M, E) *L. mexicana*-R, F) *D. gibbosum*-M, G) *D. gibbosum*-R. H) *F. femoralis*-M, was only successfully sequenced with Mini-COI primers.

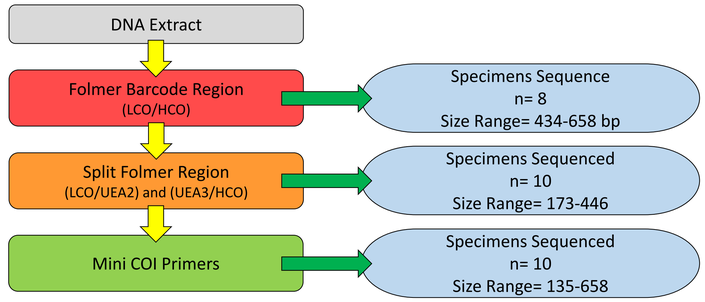


**Figure 3.** Successful sequences recovered. Three DNA barcoding iterations were attempted to overcome degraded DNA.

**Table 1.** Primers designed for Mini-COI segments. Primers sequences contain the M13-tag (*italics*) and specific sequences (**bold**). $T_a$ indicates annealing temperature.

| Region | Primer Name | Direction | Primer Sequence (5'-3') | Diptera $T_a$ (°C) | Coleoptera $T_a$ (°C) |
|---|---|---|---|---|---|
| Mini-COI-1 | LCO1490 | Forward | *TGTAAAACGACGGCCAGT***GGTCAACAAATCATAAAGATATTGG** | 50 | 50 |
| | COI-1R-RD | Reverse | *CAGGAAACAGCTATGACC***RACTARTCAATTTCCAAATCCTCC** | | |
| Mini-COI-2 | COI-2F | Forward | *TGTAAAACGACGGCCAGT***TGTAATTGTWACAGCTCATGC** | 58 | 58 |
| | COI-2R | Reverse | *CAGGAAACAGCTATGACC***ACAGTTCAWCCTGTTCCAGC** | | |
| Mini- COI-3 | UEA3RD | Forward | *TGTAAAACGACGGCCAGT***TATRGCWTTTCCWCGAATRAATAA** | 53 | 53 |
| | COI-3R | Reverse | *CAGGAAACAGCTATGACC***ACWGCTCCTAAAATWGAAGA** | | |
| Mini-COI-4 | COI-4F | Forward | *TGTAAAACGACGGCCAGT***ATYGCYCATGGAGGAKCTTC** | 56 | 56 |
| | COI-4R | Reverse | *CAGGAAACAGCTATGACC***AGTTAAWAGTATWGTAATWGCTCCWGC** | | |
| Mini-COI-5 | COI-5F | Forward | *TGTAAAACGACGGCCAG***TYCGAATACCTTTATTTGTTTGATC** | 55 | 53 |
| | HCO2198 | Reverse | *CAGGAAACAGCTATGACC***TAAACTTCAGGGTGACCAAAAAATCA** | | |

**Table 2.** Novel polymorphisms for COI sequences. Samples highlighted in green indicate the recovery of a new sequence for that species. Samples in red indicate a morphological misidentification resolved through DNA barcoding.

| Morphological ID | COI Region | Length | BLASTn Result | % Identity | Novel Polymorphisms |
|---|---|---|---|---|---|
| *Cochliomyia macellaria* | LCO/HCO | 556 | *C. macellaria* | 99.82 | 1808C |
| *Fannia femoralis* | MiniCOI-5 | 145 | *Fanniidae sp.* | 99.31 | 631G |
| *Fannia oregonensis* | MiniCOI2-3 | 280 | *Fannia pusio* | 100 | New sequence |
| *Milichiella hendeli* | MiniCOI-1 | 176 | *Milichiidae sp.* | 99.43 | 84G |
| *Polietes hirticrura* | MiniCOI-4 | 181 | *Muscidae sp.* | 98.33 | 547Y, 548Y, 559A |
| *Muscina fulvacrura* | MiniCOI | 520 | *Emmesomyia sp.* | 99.42 | 287C, 449T, 451A |
| *Phaonia protuberans* | LCO/UEA2 | 261 | *Ravinia derelicta* | 98.47 | 107G,108DEL,115G,129W |
| *Gymnodia humilis* | LCO/UEA2 | 208 | *G. delecta* | 100 | |
| *Geotrupes blackburnii* | UEA3/HCO | 409 | *Geotrupes semiopacus* | 97.31 | New Sequence |
| *Onthophagus tuberculifrons* | MiniCOI-3-4 | 311 | *Cleptocaccobius convexifrons* | 89.35 | New Sequence |
| *Onthophagus hecate* | LCO/UEA2 | 328 | *O. hecate* | 99.09 | 99A, 132T, 211C |
| | UEA3/HCO | 173 | *O. hecate* | 97.69 | 414A, 489T, 495A, 499C |
| *Onthophagus medorensis* | MiniCOI | 658 | *Onthophagus orpheus* | 93 | New Sequence |
| *Necrobia rufipes* | UEA3/HCO | 249 | *N. rufipes* | 99.2 | 417A, 497T |
| *Omorgus monachus* | LCO/UEA2 | 329 | *O. monachus* | 99.7 | 28A |
| *Trox plicatus* | MiniCOI-3 | 135 | *Trox scaber* | 90.37 | New Sequence |
| *Trox variolatus* | UEA3/HCO | 321 | *T. variolatus* | 95.95 | 259T, 265G, 334A, 340T, 355A, 364C, 388A, 407C, 418T, 424C, 514T, 535G, 557T |
| *Necrophila americana* | MiniCOI | 619 | *N. americana* | 99.68 | 469A, 596Y |
| *Deltochilum gibbosum* | LCO/UEA2 | 285 | *Dichotomius satanas* | 93.68 | New Sequence |

- DNA recovered from insects 12-14 years after collection was degraded (**Fig. 2**). Degraded DNA can lead to unsuccessful DNA barcoding when using larger amplicons, such as the Folmer region.
- Targeting smaller amplicons, for example splitting the barcoding region into two or five segments can provide sequencing results for samples that had previously failed (**Fig. 3**).
- Primers designed for forensically relevant Diptera and Coleoptera (**Table 1**) can be used to produce smaller sequences capable of identifying specimens to the species level and resolving morphological identifications (**Table 2**).
- Sequences for commonly collected insects (e.g., *Calliphora livida, Phormia regina,* and *Lucilia sp.*) resulted in 100% identity.
- Specimens that were sequenced resulted in a variety of sequence lengths that showed new polymorphisms (**Table 2**).

## MATERIALS & METHODS

**Initial PCR and Sequencing**

Initial PCR was performed using Type-it® Microsatellite PCR Kit (QIAGEN) with LCO1490 and HCO2198 primers [1] at an annealing temperature of 50°C and 3.75 µL DNA. PCR products were purified with QIAquick® PCR Purification (QIAGEN) with a 30 µL elution. BigDye™ Terminator 3.1 (ThermoFisher) was used for sequencing following manufacturer's protocol. A second round of PCR was performed to amplify two segments of the barcoding region with the following primers: LCO1490 with a modified UEA2 and HCO2198 with a modified UEA3 primer [1,4]. Samples that met a 3 ng DNA input threshold were sequenced with Terminator 3.1. Sample degradation was assessed for select samples using the QIAxcel® High Sensitivity Kit (QIAGEN) following manufacturer's protocol.

**Primer Design**

NCBI Genbank was searched for COI sequences greater than 650 nucleotides with the top 20 results selected. Primer design was done using Geneious Prime, which uses Primer3 [5]. Sequences were aligned, and areas of high similarity were targeted for design with amplicons of 250 bp or less. Gradient PCR for the mini-COI primers (**Table 1**) was performed to determine annealing temperatures, further optimized through sequencing results.

**BigDye™ Direct PCR and Sequencing**

Sequencing of the mini-COI barcoding regions was done using BigDye™ Direct Kit (ThermoFisher) with modifications to the PCR annealing temperatures on a Veriti™ Fast Thermal Cyler. Four nanograms of DNA were targeted, and 3.5 µL DNA extract used when the target DNA input could not be met. BigDye Xterminator™ (ThermoFisher) was used for sequencing clean up prior to capillary electrophoresis on a 3500 Genetic Analyzer (Applied Biosystems).

**Sequence Analysis**

Sequence data was viewed, assembled, and edited on Geneious R7. Sequences were saved as FASTA files and uploaded to BLASTn to determine a species identification and novel polymorphisms.

## CONCLUSIONS

- *COI* DNA barcoding is a valuable tool to provide species identifications, necessary for forensic entomologists.
- Targeting smaller segments can help recover sequences that were previously unsuccessful with larger sequencing regions.
- Novel polymorphisms and sequences were recovered for forensically relevant insects in Huntsville, TX.
- Reverse primer for Mini-COI-1 has been redesigned to solve primer dimer issues.
- Future work will consist of mini-COI sequencing for various other specimens.

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