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National Institute of Justice

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Statement of the Problem

Approximating time since colonization of human remains by local fly populations is considered one of the most accurate methods for estimating post-mortem interval during medicolegal death investigations. A key requirement for estimating PMI is that the species of each specimen under consideration must be determined since colonization and developmental timelines vary between fly species. Species determinations are performed by identifying distinct morphological characteristics as indications of particular taxonomic groups. Limitations to this process include that recognizing species-specific morphologies requires specialized knowledge and expertise in the field of entomology. In addition, many immature stages lack distinguishing morphological characteristics or taxonomic reference keys, thus precluding species determination unless samples can be successfully reared into adult flies.

Molecular identification has been proposed for making species determinations of forensically important insects during medicolegal death investigations. Molecular identification involves comparing DNA sequencing data of samples against a library of known references. With respect to forensically important flies, molecular identification is advantageous over morphological examinations because the technology is accessible to non-specialists of entomology and enables analysis of immature specimens.

A common challenge in past studies is that PCR methods are limited by the diversity of target species: any PCR method, i.e., a primer set and optimized cycling conditions, will be effective for only a subset of species. Past studies utilizing a singular PCR method focus on members of a specific genus or members of related genera. These methods are unsuitable for HCIFS entomological investigations because prominent species are too diverse. Other studies utilize multiple primer sets or cycling conditions; however, these approaches are too resource-

intensive for publicly-funded laboratories. A simple PCR method for identifying species prominently encountered in casework would be advantageous to HCIFS. A prior study identified thirteen species that are relevant to HCIFS: the blow fly species *Calliphora coloradensis* Hough, *Calliphora livida* Hall, *Calliphora vicina* Robineau-Desvoidy, *Calliphora vomitoria* Linnaeus, *Chrysomya megacephala* Fabricius, *Chrysomya rufifacies* Macquart, *Cochliomyia macellaria* Fabricius, *Lucilia cuprina* Wiedemann, *Lucilia eximia* Wiedemann, *Lucilia sericata* Meigen, and *Phormia regina* Robineau-Desvoidy (Diptera: Calliphoridae); the flesh fly *Blaesoxipha plinthopyga* Wiedemann (Diptera: Sarcophagidae); and the scuttle fly *Megaselia scalaris* Loew (Diptera: Phoridae). Investigators serving southeast Texas outside of HCIFS jurisdiction would additionally benefit from a simple method for identifying these species. Such a method may also benefit investigators outside southeast Texas since most of these species are widespread across the United States.

Project Goals

The purpose of this project is to develop and validate an optimized DNA barcoding strategy for identifying blow, flesh, and scuttle flies commonly encountered in forensic entomology casework. Objectives were divided into two phases:

Phase I: Development of a DNA Barcoding Strategy for Molecular Identification of Forensically Relevant Blow and Flesh Fly Species

Preliminary data supported a DNA barcoding assay for analyzing a 561 basepair fragment from cytochrome oxidase (COI). The assay should be demonstrated using species that are relevant to medicolegal casework. These included specimens representing species that are

relevant for approximating post-mortem interval and were identified through morphological examinations by trained entomologists. In addition, a survey was proposed for building a database of barcoding sequences from regional specimens that may serve as references for future casework. Specimens would be collected from Harris County, Texas and identified by a trained entomologist.

Phase II: Validation of Insect DNA barcoding as a Function of the Harris County Institute of Forensic Science (HCIFS) Forensic Genetics Laboratory

The assay would be validated by HCIFS in accordance with the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories (FBI-QAS) and the ISO/IEC 17025 International Standards. Validation would allow the assay to be implemented by HCIFS for ongoing casework. Experiments would evaluate accuracy, sensitivity, species specificity, population studies, analysis of casework-type samples, challenging reaction conditions (degraded DNA or inhibited reactions), demonstrate methods for statistical analysis, and quality control (repeatability and reproducibility).

Phase I Accomplishments

A single primer set was found to amplify COI from various species: blow flies *Calliphora coloradensis* Hough, *Calliphora livida* Hall, *Calliphora vicina* Robineau-Desvoidy, *Calliphora vomitoria* Linnaeus, *Chrysomya megacephala* Fabricius, *Chrysomya rufifacies* Macquart, *Cochliomyia macellaria* Fabricius, *Cochliomyia minima* Shannon, *Cynomya cadaverina* Robineau-Desvoidy, *Lucilia cuprina* Wiedemann, *Lucilia eximia* Wiedemann, *Lucilia sericata* Meigen, *Lucilia illustris* Meigen, *Lucilia mexicana* Macquart, *Lucilia*

coeruleiviridis, Macquart, *Cynomya cadaverina* Robineau-Desvoidy, and *Phormia regina* Robineau-Desvoidy (Diptera: Calliphoridae); the flesh fly *Blaesoxipha plinthopyga* Wiedemann (Diptera: Sarcophagidae); and the scuttle fly *Megaselia scalaris* Loew (Diptera: Phoridae). Many of these species are prominent in HCIFS casework or jurisdictions elsewhere in the United States. We additionally tested the livestock pest *Cochliomyia hominivorax* Coquerel which was previously eradicated from North and Central America. New eradication efforts occur in response to outbreaks resulting from transplantation from South America or the West Indies; the present method may assist in the detection of *C. hominivorax* in the United States. All specimens were verified by trained entomologists. Strong amplification was achieved for each specimen while using common PCR conditions, in particular, annealing temperature and time, template concentration, primer concentration, and cycling number (Fig 1). Amplification of secondary products was not observed.

The 561 basepair COI fragment was amplified using primers (C1-J-2495: 5`-CTGCTACTTTATGAGCTTTAGG-3`; and TL2-N-3014: 5`-TCCATTGCACTAATCTGCCATATTA-3`) based on previously described sequences that were modified to fit consensus blow, flesh, and scuttle fly sequences. PCR was conducted in 50 µL reactions comprising Q5® high-fidelity mastermix (New England BioLabs), 0.1 µM of each primer, and up to 1.0 ng total DNA. The reaction conditions consisted of initial denaturation at 98°C for 30 seconds, 40 cycles at 98°C for 10 seconds, 56°C for 1 minute, and 72°C for 20 seconds, and final elongation at 72°C for 2 minutes. PCR products were visualized using the FlashGel™ electrophoresis kit (Lonza). PCR reactions were submitted to Lone Star Laboratories (Houston, Texas) for amplicon purification and bidirectional Sanger sequencing using the BigDye™ chemistry (Applied Biosystems). Chromatograms were reviewed using Sequence

Scanner Analysis Software (Applied Biosystems). The forward and reverse reads consistently terminated at the 3' end after displaying the opposite primer sequence. 3' primer sequences and nucleotide reads with Phred scores less than 20 at the 5' periphery were trimmed. Individual nucleotide reads scoring quality values less than 20 within the sequence were changed to "N" to indicate an undetermined nucleotide. Bidirectional reads were manually tiled. The expected COI fragment length based on published references was 514 basepairs after trimming primer sequences; an exception was *Megaselia scalaris* for which the expected length was 517 basepairs.

A major goal of this project was to produce a database of COI sequences from regional specimens. The database includes data for 98 specimens (Table 1). All sequences in the database are full-length. Species represented in the database include: blow flies *Calliphora vicina*, *Chrysomya megacephala*, *Chrysomya rufifacies*, *Cochliomyia macellaria*, *Cynomya cadaverina*, *Lucilia cuprina*, *Lucilia eximia*, and *Phormia regina*; flesh flies *Blaesoxipha plinthopyga*, *Ravinia stimulans* Walker, *Sarcophaga africa* Wiedemann, *Sarcophaga sarracenioides* Aldrich, and *Oxysarcodexia conclausa* Walker; the scuttle fly *Megaselia scalaris*; and the cheese fly *Piophila casei* Linnaeus. Additional specimens were collected that are members of other taxonomic groups which will require additional expertise to verify by morphologically: genus *Hydrotea*, genus *Fannia*, and Family Muscidae. The intraspecific variations ranged up to 0.35%. Interspecific variations ranged 4.9% – 29.5%. The observation that interspecific variations were much greater than intraspecific variations suggests that these groups may be differentiated. Specimens used in the study were deposited with TAMUIC as vouchers, and respective sequences were submitted to GenBank.

Sequences were compared to references from public databases through phylogenetic analysis using “NGPhylogeny.fr” which streamlined several independent processes. References included sequences produced for species which we expected to encounter as well as any closely related species. Sequences were aligned using “multiple alignment using fast fourier transform” (MAFFT). Midpoint-rooted trees were built using FastME software which combines algorithms for distance-based calculations: an initial tree is produced through neighbor-joining, and the solution is improved using nearest neighbor interchanges (NNI) and subtree pruning and regrafting (SPR). Node support was calculated by nonparametric bootstrapping with 1000 replicates; strong support was considered for nodes supported with 95% (950/1000) or greater replicates. The tree was visualized using “interactive tree of life” (ITOL) software.

Sequences were compared to published references and those produced in-house from a reference collection using phylogenetic analysis (Table 2 and Fig 2). References corresponded to species identified in this study and others that are closely related. Many species were monophyletic, i.e., sequences clustered with 95% bootstrap support and independently of all other species: *Blaesoxipha plinthopyga*, *Calliphora vicina*, *Calliphora vomitoria*, *Cochliomyia hominivorax*, *Cochliomyia minima*, *Chrysomya rufifacies*, *Cynomya cadaverina*, *Oxysarcodexia conclausa*, *Phormia regina*, *Ravinia stimulans*, *Sarcophaga africa*, and *Sarcophaga sarracenioides*.

There were several closely-related species that could not be differentiated although identification of individuals may be further supported by considering known geographic distributions. For example, *Chrysomya megacephala* clustered with *C. saffranea* Bigot and *C. pacifica* Kurahashi. However, *C. megacephala* is the only member of this group that is known to inhabit North America. Thus, individuals collected in the United States and whose sequences

align to this cluster are most likely *C. megacephala*. Similarly, *Cochliomyia macellaria* is known to have common mitochondrial haplotypes with *C. aldrichi* Del Ponte, but *C. aldrichi* is limited to the West Indies. Thus, individuals collected in the United States are most likely *C. macellaria*. Finally, *Lucilia illustris* and *L. caesar* Linnaeus have common haplotypes, but only *L. illustris* is found in North America. Thus individuals collected in the United States are most likely *L. illustris*.

There were several groups of related species that inhabit the United States and could not be differentiated. First, *Lucilia eximia* and *L. mexicana* found in Texas exhibit a haplotype that was identical to *L. coeruleiviridis* despite individuals inhabiting other regions of North America, Central America, and South America having unique haplotypes. Second, *Calliphora livida* and *C. coloradensis* were similar and could not be differentiated with significant bootstrap support; although, it is possible that this could be achieved in a future study where sampling could be expanded for these species. Finally, *Lucilia cuprina* and *L. sericata* did not exhibit reciprocal monophyly although they carry mitochondrial haplotypes which may be differentiated through phylogenetic analysis; however, identification of individual specimens is challenged by the possibility of hybridization. This would require further analysis using nuclear markers to separate hybrid individuals.

Further studies are needed for evaluating the remaining species: *Megaselia scalaris* and *Piophila casei*. Genus *Megaselia* is extremely diverse and very little information is available for members that may be very similar to *Megaselia scalaris*. *Piophila casei* sequences appear to be distinct from other GenBank entries although they are identical to recently described sequences for *P. megastigmata* McAlpine; however, this may be inconsequential for specimens collected in North America since *P. megastigmata* have only been described in Europe and Africa.

Application to medicolegal casework was demonstrated by analyzing unidentified, immature specimens from HCIFS casework. COI amplification was attempted from seventeen casework specimens. These included eight larvae, two pupae, and seven pupal exuviae. Full-length COI sequences were produced for each larva and pupa as well as one pupal exuvia (Table 3). During phylogenetic analysis, the casework specimens clustered with one of four species: *Chrysomya megacephala*, *Blaesoxipha plinthopyga*, *Phormia regina*, or *Calliphora vicina*. Bootstrap support for these clusters were greater than 99%. This was supported using a BLAST analysis which showed that the GenBank reference sequences most similar to the casework specimens belonged to these four species. BLAST was also used to compare casework specimens to individual sequences derived from regional specimens. Eight sequences from casework specimens were identical to regional specimens, and the remaining sequences had 1 – 2 nucleotide differences from a regional specimen. These observations provided support for identifying the casework specimens.

Phase II Accomplishments

A formal validation was completed for the COI barcoding method during the reporting period. Experiments were performed in accordance with the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories and ISO/IEC 17025 International Standards. Detailed reports were produced that documented findings from each experiment conducted during validation. Standard operating procedures were produced encompassing the entire process as guidance for analysts (DNA extraction, PCR purification, gel electrophoresis, and interpretation of Sanger sequencing data). Results from validation experiments are described below.

Sensitivity

COI was amplified at varied template input and analyzed by gel electrophoresis and Sanger sequencing. A single intense band (excluding the dye front and primer dimerization) should be observed during gel electrophoresis. Quality of sequencing results may be assessed by reviewing Phred scores of individual base calls, identifying primer sequences near the read termination, and reviewing the size of reads. High-quality sequencing results were produced after amplifying at least 0.5 ng Chrysomya megacephala template.

Accuracy for Verified Specimens

COI amplification and sequencing was demonstrated for species relevant to medicolegal death investigations. Full length sequences were produced from all specimens tested in this study using common PCR conditions. Similar outcomes were observed across species when evaluating quality at intermediate steps.

Specificity

COI amplification was tested using human, canine, and feline genomic DNA. The assay is not expected to be disrupted by contamination with human, canine, or feline genomic DNA.

Challenging conditions: Degradation, Inhibition, and long-term Storage

PCR inhibition, template degradation, and storage conditions may reduce COI amplification and negatively impact sequencing results. Some PCR products may contain insertions or deletions unsupported by published references: these are indicative of nuclear-encoded “COI-like” sequences and should be excluded from further analysis. Positive control

reactions are necessary for diagnosing failures due to challenging conditions versus failed reagents, processes, etc.

Population Studies

COI sequences were analyzed for forensically relevant species originating from Harris County, Texas (Table 1). COI sequences of unknown specimens may be compared to these populations to support taxonomic identifications. In particular, interspecific variations describe the expected differences between groups, and intraspecific variations describe the expected differences within groups. Published resources may support identifications of other species when their intraspecific variation is expected to be small relative to interspecific variations, e.g., ~1%.

Phylogenetic Analysis

COI sequences may be compared using "NGPhylogeny.fr" to produce phylogenetic trees (Fig 2). Many species were resolved based on COI sequences: *Blaesoxipha plinthopyga*, *Calliphora vicina*, *Calliphora vomitoria*, *Chrysomya rufifacies*, *Cochliomyia hominivorax*, *Cochliomyia minima*, *Cynomya cadaverina*, *Phormia regina*, *Ravinia stimulans*, *Sarcophaga africa*, and *Sarcophaga sarracenioides*. Identification of other species may be supported when additionally considering geographic distributions: *Cochliomyia macellaria* and *Chrysomya megacephala* are very common in Harris County, Texas while their close relatives inhabit elsewhere. Clusters showed 95% bootstrap support, i.e., bootstrap values 950 or greater out of 1000 replicate trees, were identified.

Repeatability and Reproducibility

Concordant results may be expected when the same specimens are tested by different analysts. Cross-sample contamination was not observed; this was evaluated by comparing results across analyst trials including positive controls, no-template PCR negative controls, and reagent blanks produced during DNA extraction. Results were additionally concordant with morphology-based verifications for each specimen.

Casework-type Samples and Unidentified Specimens

Taxonomic identification may be supported by comparing specimen COI sequences to references. This was demonstrated in a blinded experiment using eight specimens provided by an outside expert. The specimens were colony-bred, thus their identifications were known; however, the identifications were withheld from HCIFS staff until analysis was completed. The specimens were a combination of adult flies and larvae. For each specimen, a band was observed during gel electrophoresis at the appropriate size. Sequencing reads passed the quality assessments: the vast majority of bases showed Phred scores greater than 20; primer sequences were identified near the read termination; and the total read lengths were appropriate. The sense and antisense reads were tiled to produce the 514 base COI sequence needed for further analysis; full-length sequences were produced for all eight specimens. The eight sequences were compared to published references using phylogenetic analysis. Sequences for three specimens clustered with *Cochliomyia macellaria*. Sequences for two other specimens clustered with *Blaesoxipha plinthopyga*. Sequences for two other specimens clustered with *Lucilia cuprina* ("*Lucilia sericata*-like" haplotype). The remaining specimen clustered with *Chrysomya rufifacies*. Bootstrap values ranged 97% – 100%. The donating laboratory communicated that the

identifications proposed by this analysis were correct. This experiment demonstrated that accurate results could be obtained for specimens whose identifications were unknown by HCIFS.

In a second experiment, identifications were proposed for eleven unidentified immature specimens (as described previously; Table 3). Sequences were produced from larvae, pupae, and pupal exuvia. During phylogenetic analysis, bootstrap support values greater than 95% (950 /1000 replicates) were observed for clusters containing unknown specimens. BLAST was used to perform pairwise comparisons between sequences produced from unknown specimens and references. Sequences from unidentified specimens were identical or nearly identical to regional specimens. BLAST additionally enabled comparisons against the GenBank database which may be useful for identifying less common species. These identifications require high sequence identity, reasonable coverage, and additional support from the literature that differentiation from close relatives is appropriate.

Another purpose of these studies was to demonstrate analysis from various sample-types relevant to casework. High-quality data may be expected when processing adult legs, larvae, and pupae specimens. Producing high-quality data from pupal exuviae was possible but unlikely.

Impact for Forensic Entomology

This work is likely to impact knowledge and research in the field of entomology by demonstrating a simple DNA barcoding method for identifying a broad range of blow, flesh, and scuttle flies. The purpose of our current work is to develop and conduct internal validations for the method so that it may be applied to death investigations. This tool may also be used for academic studies of regional populations comprising species that overlap with our study. This work additionally discusses means for improving the quality and accuracy of the results.

Completion of this project will further the application of forensic entomology by HCIFS Forensic Genetics Laboratory and Medical Examiner Services. Insect identification is required for calculating the time-of-colonization of insects which assists in estimating the post-mortem interval. DNA barcoding permits identification of specimens which may not be identified using morphology alone. The tool may be useful to other organizations since the species relevant to our region are widespread in the United States.

The assay enables identification of the agricultural pest *Cochliomyia hominivorax*. Thus, this work may additionally impact local agricultural industry which could be negatively impacted in the event of an outbreak. *Cochliomyia hominivorax* has been eradicated from the United States, but outbreaks may occur due to transplantation from South America. Because Harris County includes the Port of Houston, which is the largest port on the Gulf Coast, our region may be susceptible to such an outbreak.

Professional Development, Training, and Dissemination

During previous reporting periods, we presented a webinar through the Forensic Technology Center of Excellence, presented our work at the American Academy of Forensic Science (AAFS) 2021 Annual Meeting, the 2021 NIJ Research and Development Symposium hosted by the Forensic Technology Center of Excellence, and described our work in a poster presentation at the National Association of Medical Examiners (NAME) 2021 Annual Meeting.

A manuscript was submitted for peer-review and publication describing project accomplishments: application of the assay to a wide range of species including those that are relevant to HCIFS; building of a database of COI sequences produced from regional specimens; and demonstrating analysis of unidentified casework specimens.

Validation experiments were described in a series of reports that will guide implementation of the assay for medicolegal casework at HCIFS. These include detailed analysis of each experiment completed during the validation and standard operating procedures describing step-by-step instructions.

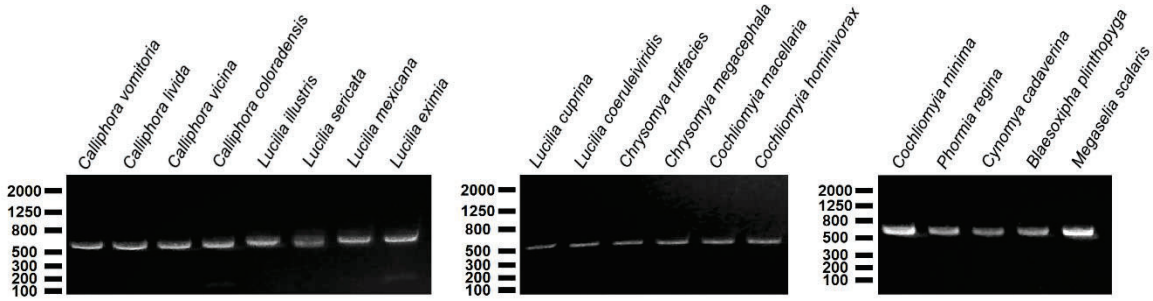

FIG 1. *Gel electrophoresis showing the amplified 561 basepair COI fragment produced from various species. Specimen vouchers: Calliphora vomitoria* XG1, *Calliphora livida* XG2, *Calliphora vicina* XG3, *Calliphora coloradensis* XG4, *Lucilia illust*ris XG5, *Lucilia sericata* XG6, *Lucilia mexicana* XG7, *Lucilia eximia* XG9, *Lucilia cuprina* XG8, *Lucilia coeruleiviridis* XG12, *Chrysomya rufifacies* XG10, *Chrysomya megacephala* XG11, *Cochliomyia macellaria* XG14, *Cochliomyia hominivorax* XG16, *Cochliomyia minima* XG15, *Phormia regina* A11, *Cynomya cadaverina* XG13, *Blaesoxipha plinthopyga* MS11, *Megaselia scalaris* SC1.

Species	Voucher	Accession No.	Species	Voucher	Accession No.
Blaesoxipha plinthopyga	MS2	ON540165	*Lucilia cuprina*	B4	ON540182
Blaesoxipha plinthopyga	MS11	ON540166	*Lucilia cuprina*	D1	ON540199
Blaesoxipha plinthopyga	MS28	ON540167	*Lucilia cuprina*	D8	ON540203
Blaesoxipha plinthopyga	MS36	ON540168	*Lucilia cuprina*	D24	ON540216
Calliphora vicina	D4	ON540200	*Lucilia cuprina*	E5	ON540217
Calliphora vicina	D5	ON540201	*Lucilia cuprina*	E8	ON540220
Calliphora vicina	D7	ON540202	*Lucilia cuprina*	E11	ON540223
Calliphora vicina	D9	ON540204	*Lucilia cuprina*	R1	ON540257
Calliphora vicina	D10	ON540205	*Lucilia cuprina*	R4	ON540260
Calliphora vicina	D11	ON540206	*Lucilia cuprina*	R6	ON540262
Calliphora vicina	D12	ON540207	*Lucilia cuprina*	R7	ON540263
Calliphora vicina	D17	ON540211	*Lucilia eximia*	B1	ON540179
Calliphora vicina	D19	ON540212	*Lucilia eximia*	B2	ON540180
Calliphora vicina	D20	ON540213	*Lucilia eximia*	B6	ON540184
Calliphora vicina	D21	ON540214	*Lucilia eximia*	B7	ON540185
Calliphora vicina	D23	ON540215	*Lucilia eximia*	B8	ON540186
Calliphora vicina	E6	ON540218	*Lucilia eximia*	B9	ON540187
Calliphora vicina	E7	ON540219	*Lucilia eximia*	C11	ON540195
Calliphora vicina	E9	ON540221	*Lucilia eximia*	C12	ON540196
Calliphora vicina	E30	ON540228	*Lucilia eximia*	D13	ON540208
Calliphora vicina	E32	ON540229	*Lucilia eximia*	D14	ON540209
Calliphora vicina	H19	ON540255	*Lucilia eximia*	D16	ON540210
Chrysomya megacephala	B5	ON540183	*Lucilia eximia*	E12	ON540224
Chrysomya megacephala	C6	ON540191	*Lucilia eximia*	E13	ON540225
Chrysomya megacephala	C8	ON540192	*Lucilia eximia*	F9	ON540230
Chrysomya megacephala	C9	ON540193	*Lucilia eximia*	H3	ON540248
Chrysomya megacephala	C10	ON540194	*Lucilia eximia*	H6	ON540249
Chrysomya megacephala	H1	ON540246	*Lucilia eximia*	H7	ON540250
Chrysomya megacephala	H2	ON540247	*Lucilia eximia*	H8	ON540251
Chrysomya megacephala	GG8	ON540238	*Lucilia eximia*	H10	ON540252
Chrysomya rufifacies	C3	ON540188	*Lucilia eximia*	R2	ON540258
Chrysomya rufifacies	C4	ON540189	*Lucilia eximia*	R3	ON540259
Chrysomya rufifacies	C5	ON540190	*Lucilia eximia*	R5	ON540261
Chrysomya rufifacies	GG2	ON540232	*Lucilia eximia*	R8	ON540264
Chrysomya rufifacies	GG3	ON540233	*Oxysarcodexia conclausa*	C14	ON540197
Chrysomya rufifacies	GG4	ON540234	*Oxysarcodexia conclausa*	C15	ON540198
Chrysomya rufifacies	GG5	ON540235	*Phormia regina*	A3	ON540171
Chrysomya rufifacies	GG6	ON540236	*Phormia regina*	A4	ON540172
Chrysomya rufifacies	GG7	ON540237	*Phormia regina*	A5	ON540173
Cochliomyia macellaria	GG9	ON540239	*Phormia regina*	A7	ON540174
Cochliomyia macellaria	GG10	ON540240	*Phormia regina*	A8	ON540175
Cochliomyia macellaria	GG11	ON540241	*Phormia regina*	A11	ON540176
Cochliomyia macellaria	GG12	ON540242	*Phormia regina*	GG14	ON540244
Cochliomyia macellaria	GG13	ON540243	*Phormia regina*	GG15	ON540245
Cynomya cadaverina	F22	ON540231	*Piophila casei*	A16	ON540177
Cynomya cadaverina	H15	ON540253	*Piophila casei*	A17	ON540178
Cynomya cadaverina	H18	ON540254	*Ravina stimulans*	E10	ON540222
Cynomya cadaverina	H20	ON540256	*Sarcophaga africa*	E23	ON540226
Lucilia cuprina	B3	ON540181	*Sarcophaga sarracenioides*	E28	ON540227

Table 1. *Sequences produced for specimens collected in Harris County, Texas.*

Species	Location	Voucher	Accession No.
Calliphora vomitoria	King County, Washington	XG1	ON540154
Calliphora livida	Alberta, Canada	XG2	ON540153
Calliphora vicina	Pierce County, Washington	XG3	ON540159
Calliphora coloradensis	San Juan County, Utah	XG4	ON540152
Lucilia illustris	Del Norte County, California	XG5	ON540161
Lucilia sericata	Pierce County, Washington	XG6	ON540163
Lucilia mexicana	Grant County, New Mexico	XG7	ON540162
Lucilia cuprina	Hereida, Costa Rica	XG8	ON540164
Lucilia eximia	Harris County, Texas	XG9	ON540169
Chrysomya rufifacies	Grant County, New Mexico	XG10	ON540158
Chrysomya megacephala	Kaw Mtn, French Guiana	XG11	ON540157
Lucilia coeruleiviridis	Putnam County, Missouri	XG12	ON540160
Cynomya cadaverina	Alberta, Canada	XG13	ON540151
Cochliomyia macellaria	Grant County, New Mexico	XG14	ON540155
Cochliomyia minima	Mayaguez, Puerto Rico	XG15	ON540156
Cochliomyia hominivorax	Panama	XG16	ON540150
Megaselia scalaris	Harris County, Texas	SC1	ON540170

Table 2. *Sequences produced for verified specimens.*

FIG 2 *(on next page). Species-level identifications were assessed using phylogenetic analysis. Bootstrap support is shown as percentage of 1000 replicates. Nodes with less than 70% bootstrap support were collapsed. The scale bar relates horizontal distance to sequence changes (5% of sequence length).*

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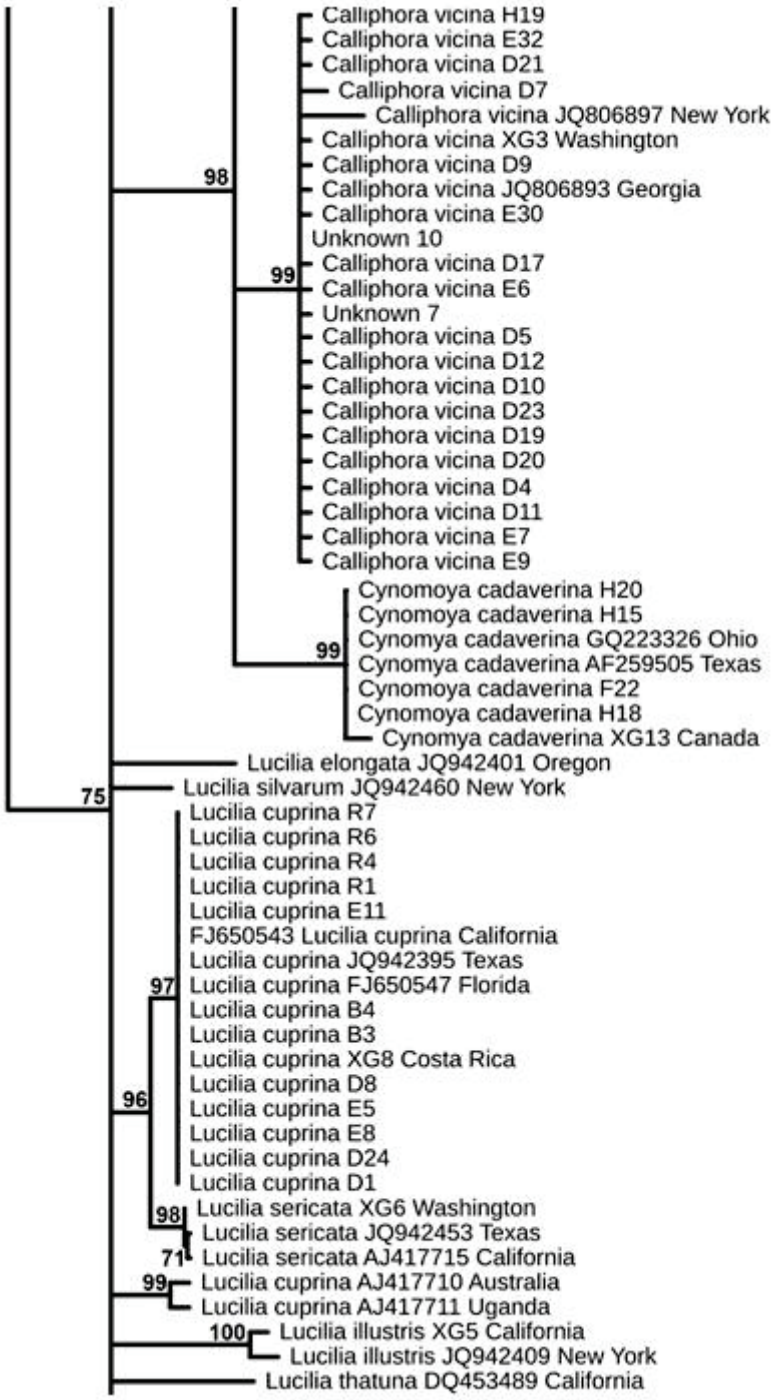

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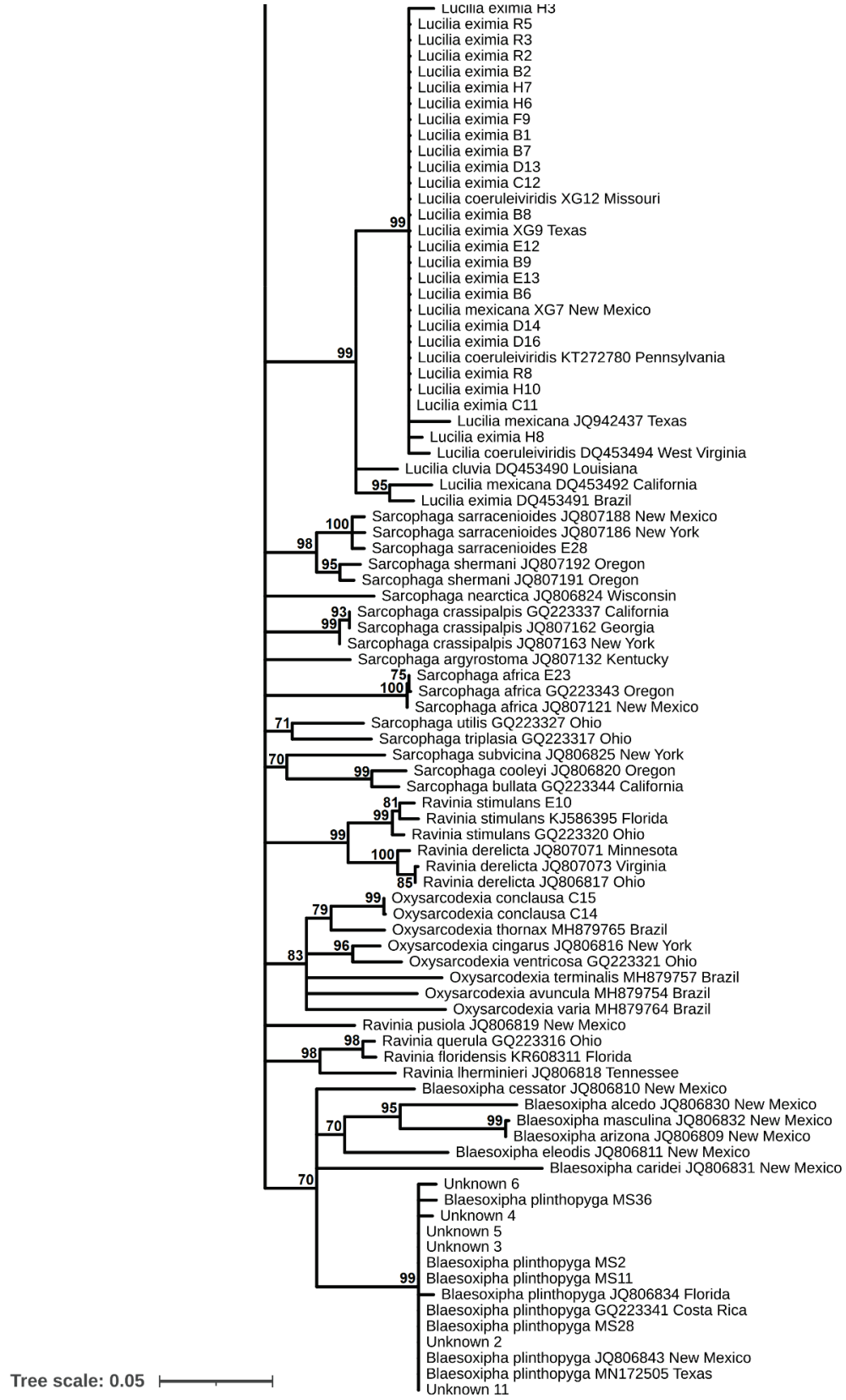

Specimen #	Life Stage	Species Cluster	Node Support (%)	Comparison to Regional Specimens
1	larva	*Chrysomya megacephala*	99	Identical sequence
2	pupa	*Blaesoxipha plinthopyga*	99	Identical sequence
3	larva	*Blaesoxipha plinthopyga*	99	Identical sequence
4	larva	*Blaesoxipha plinthopyga*	99	One nucleotide difference
5	larva	*Blaesoxipha plinthopyga*	99	Identical sequence
6	larva	*Blaesoxipha plinthopyga*	99	Two nucleotide differences
7	pupal exuvia	*Calliphora vicina*	99	Identical sequence
8	larva	*Phormia regina*	100	One nucleotide difference
9	pupa	*Phormia regina*	100	One nucleotide difference
10	larva	*Calliphora vicina*	99	Identical sequence
11	larva	*Blaesoxipha plinthopyga*	99	Identical sequence

Table 3. *Analysis of non-probative, immature specimens.*