2012

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JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, 2012
doi: 10.1111/j.1556-4029.2012.02182.x
Available online at: onlinelibrary.wiley.com

TECHNICAL NOTE

PATHOLOGY/BIOLOGY

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ABSTRACT: Immature blow flies (Calliphoridae) are typically the first colonizers of cadavers. Identification of the early instars using traditional, morphology-based keys is difficult because of their small size, similarity, and simplicity in external morphology. Information derived from molecular genetic data would augment the accurate identification of immature flies. Nine species of blow flies commonly found in southeastern Nebraska were used to examine the utility of molecular-based keys. Polymerase chain reaction–restriction fragment length polymorphisms (PCR–RFLP) were investigated with 10 common, inexpensive, restriction enzymes from an amplicon of approximately 1500 bp spanning the mitochondrial cytochrome oxidase I gene. A simple molecular taxonomic key, comprising RFLP from the restriction enzymes HinfI and DraI, enabled the differentiation of all species used. Further development of PCR–RFLP, including more extensive and intensive examination of blow flies, would benefit forensic laboratories in the accurate identification of evidence consisting of immature blow flies.

KEYWORDS: forensic science, forensic entomology, Calliphoridae, mitochondrial DNA, cytochrome oxidase subunit I, polymerase chain reaction–restriction fragment length polymorphism, molecular taxonomic key

Entomological evidence at a crime scene can provide information on time, cause, manner, and place of death (1), specifically geographic location of death, season of death (2), sexual molestation (3), or use of drugs (4), and finally, time of death (5). Postmortem interval (PMI), the time elapsed since death, is classically calculated by medical techniques such as measurement of algor mortis, liver mortis, and rigor mortis (1). However, the onsets of these phenomena are subject to several variables and are often unpredictable (6). The analysis of blow fly species present on a corpse has been used to provide a basis to estimate the PMI following death (7).

Calliphorids (blow flies) are typically among the first colonizers of cadavers, attracted to a body often within minutes of death (7). The development of flies follows a predictable cycle, with four stages of larvae (1st, 2nd, 3rd instar, and prepupae). Larvae are typically white or cream in color, with characteristically six or more cone-shaped tubercles on the border of the last segment; this segment also contains the posterior spiracles which are the main breathing apparatus of the larvae (8). They have only a few special features useful for identification. Accurately identifying adult blow flies can be accomplished with existing keys (9), whereas correct identification of the larvae is difficult: diagnostic anatomical characters are unknown for most of the forensically important species, existing keys may require updating and their use will require specialized training in dipteran taxonomy (10). Furthermore, closely related carrion species differ in growth rate, diapause behavior, and specific habitats (10). These factors may impede correct species identification and accurate estimation of PMIs in forensic entomology, where a simple and routine method, requiring minimum technical expertise, has not been developed.

Aldrich's monograph of Sarcophagidae (11), released in 1916, paved the way for species identification in this family. The monograph suggests that male genitalia of adult flies can be used for species identification. The same technique has been used for species differentiation of Calliphoridae (6). Descriptions and keys published by Knipling for common first instars of flesh flies (12) and second instars of *Lucilia* larvae (13) and blow flies of North America (14) paved the way for species-directed work on larvae of these groups. Forensic entomologists now require a technique that enables quick and accurate identification of adults as well as larvae. DNA-based identification methods first proposed by Sperling et al. (15) have since been proposed as a solution by many (10).

A species determination diagnostic technique for routine use should reliably differentiate between species of interest at any life stage, providing the diagnostic information quickly, efficiently, and at a low cost. In areas where species identification through morphological characters is a challenge, a complementary molecular approach is attractive because of its accuracy as well as timeliness (16). Owing to their importance in forensic entomology, much of the molecular identification has been focused on Diptera (15,17–24). Most investigations of Calliphorod species have used mitochondrial cytochrome oxidase genes subunit I (COI) as this part of the genome is well known across many species and information is consistent across life stages (25,26). A number of these studies have successfully differentiated calliphorid species by polymerase chain

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*Presented at the International Symposium for Insect Molecular Science (May 2006).

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Received 9 May 2011; and in revised form 1 Sept. 2011; accepted 1 Oct. 2011.

reaction–restriction fragment length polymorphisms (PCR–RFLP) (15,18,24,27). Although PCR–RFLP has been used for the differentiation of two to three species per study, it has not been used in distinguishing between larger numbers of species.

Eleven species of blow flies of forensic importance occur in southeastern Nebraska (T. Huntington, personal communication). The secondary screwworm, *Cochliomyia macellaria* (Fabricius), is considered a valuable forensic tool as its succession and occurrence is well defined (20). Among the species that have been introduced to the United States, one species, *Chrysomya rufifacies* (Maquart), is found in Nebraska (28). Other blow flies of forensic significance in Nebraska include species in the genera *Phormia* and *Protophormia* (subfamily Chrysomyiinae, tribe Phormiini; *P. regina* Meigen and *P. terraenovae* Robineau-Desvoidy), as well as species in the genera *Calliphora* (*C. vicina* Robineau-Desvoidy and *C. vomitoria* Linnaeus) and *Cynomya* (*C. cadaverina* Robineau-Desvoidy) and the species *Lucilia illustris* (Meigen), *Lucilia sericata* (Meigen), *Lucilia coeruleiviridis* (Macquart), and *Lucilia silvarum* (Meigen) (subfamily Calliphorinae, tribe Calliphorini) (T. Huntington, personal communication).

The overall goal of our research was to develop a diagnostic tool for the routine identification of forensically important blow flies: specifically to investigate the utility of RFLP of the mitochondrial COI region for differentiating nine species of blow flies of forensic importance in southeastern Nebraska.

Methods and Materials

Fly Collection

Thirteen species were used in the study. Eight of the species, blow flies of forensic importance in southeastern Nebraska, *C. macellaria*, *L. illustris*, *L. sericata*, *C. cadaverina*, *L. silvarum*, *P. regina*, *C. vicina*, and *C. rufifacies*, were identified by experts (field collected and identified by Dr. F. Parker, Dr. S. Skoda, and Dr. J. Welsh, USDA-ARS). Field collected and identified species (T. Huntington, personal communication) of *L. coeruleiviridis* (ninth blow fly of forensic importance in southeastern Nebraska), and noncalliphorid *Ophyra* sp. (Robineau-Desvoidy) collected in the same traps as *L. coeruleiviridis* were also included. The primary screwworm, *Cochliomyia hominivorax* (Coquerel) (field collected in Mexico, identified by Dr. Whitten, 1991) a non-necrophagous calliphorid was used in the study as an internal control. *Musca domestica* (L.) and *Stomoxys calcitrans* (L.) (laboratory maintained populations, Insectary, University of Nebraska-Lincoln, 2006) were used as noncalliphorid, dipteran controls. Sixty-five specimens were investigated, five individuals of each species, for the confirmation of species and detection of intraspecific polymorphism.

All solutions and buffers required for the protocol were prepared fresh for use. Appropriate care was taken to minimize contamination in the preparation of solutions and buffers. The same solutions and buffers were used throughout the study to minimize variability in the execution of the protocol.

DNA Extraction

A single specimen of each species was washed in nanopure water for 5 min to clean the flies of external contaminants. The thorax was used for extraction, while the rest of the fly was saved for use as the voucher specimens for the study and stored at −80°C (Department of Entomology, University of Nebraska-Lincoln) in separate, labeled (specimen number, location, date collected, and method of collection) 1.5 mL microcentrifuge tubes.

All possible precautions were taken to prevent sample contamination during DNA extraction. The washed flies were dissected using separate autoclaved forceps and separate disposable scalpels. The thoracic tissue was placed in labeled, autoclaved, 1.5-mL microcentrifuge tubes and hand-ground using autoclaved plastic pestles.

Total genomic DNA was extracted using the modified cetyltrimethyl ammonium bromide extraction method described by Skoda et al. (29). DNA extraction from all species was carried out at the same time to minimize any variability in extraction chemicals, temperature conditions and overall laboratory conditions.

Five rounds of DNA extraction, containing all 13 species per round, were carried out separately. Precipitated DNA was dissolved in 25 μL of TE buffer to obtain an approximate concentration of genomic DNA of 25–50 ng/μL. DNA concentration was calculated by comparison with standard DNA (Lambda DNA 25 ng/μL; New England BioLabs, Ipswich, MA), through minigel electrophoresis.

PCR–RFLP

The complete mitochondrial COI was amplified using universal primers TY-J-1460 (15) and TL2-N-3014 (30) (Operon Biotechnologies, Inc., Germantown, MD). All reagents used in the PCR procedure were purchased from Applied BioSystems (Foster City, CA). PCRs were carried out in 25 μL volumes, on a GeneAmp PCR system 9700 or 2700 (Perkin Elmer, Branchburg, NJ). The PCR mix consisted of 2.5 μL of 10× buffer II, 1 unit AmpliTaq DNA polymerase (0.25 μL), 2.00 μL of dNTP mix (10 mM), 1.0 μL of each primer (10 pM working solution), and 3 μL $MgCl_2$ (25 mM). Autoclaved nanopure water (10.25–13.25 μL) completed the volume to 25 μL. This master mix was prepared for 20 samples. Three to six microliters of DNA from the DNA stock solution (25–50 ng/μL) of total genomic DNA was used for amplification.

Reactions consisted of a predenaturation step of 95°C for 3 min, followed by 35 cycles of 94°C (1 min), 47°C (1 min), 72°C (1.5 min) with a final extension period of 72°C for 8 min as described by Sperling et al. (15). A negative control containing no DNA template was also included for all amplifications.

Partial or complete sequences of the COI region that were available for the calliphorid species of our study were obtained from GenBank (the National Center for Biotechnology Information—NCBI) and used for conceptual digestions by NebCutter (v2.0) (New England BioLabs) software. All commercially available restriction enzymes were investigated for potential differences (the absence or presence of the enzyme recognition site, and/or differences in placement of the enzyme site) as an indication of DNA sequence differences potentially useful to differentiate species. Ten restriction enzymes were chosen as candidates for the investigation of their ability to differentiate all species in the study.

Single digestions with 10 restriction enzymes from New England BioLabs (AseI, DraI, HinfI, DdeI, RsaI, SacI, EcoRI, EcoRV, FokI, and BsrDI) were investigated for fragment length polymorphism among the species. Three microliters of the amplicon was digested in a total volume of 8 μL (1 μL of 10× restriction buffer and 0.15 μL of enzyme, with 0.1 μL of bovine albumin serum for enzymes that require it) according to manufacturer's instructions.

Separation of the digested fragments was carried out in 1% agarose gels, in 1× Tris-Borate (10.8 g Tris-base, 5.5 g Boric acid, and 4 mL 0.5 M EDTA solution, pH = 8.0). Four microliters of the digested product was mixed with 2 μL of gel loading dye and loaded into each well in a 20 well, 4 mm thick gel matrix. Three molecular ladders (1 kb, 50 bp, and 100 bp; New England

BioLabs) were used as size markers. The fragment size calculation was carried out using GelScan v5.1 (BioSciTech, Frankfurt, Germany) software. The restriction profiles of all species with each restriction enzyme were investigated; patterns of all enzymes for each species were considered a restriction profile of the species. A determination key was constructed with the minimum number of restriction digestions needed for species differentiation.

Results and Discussion

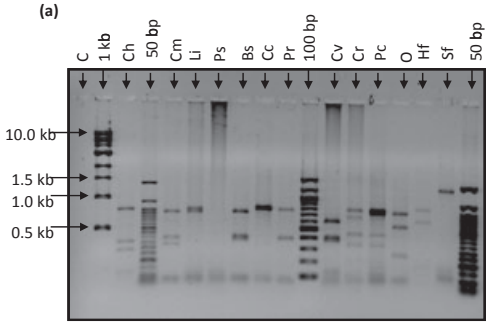
Here, we describe a molecular key, which is simple, and can be designed for any locus of choice, for routine species determination in forensic entomology. As an example, we demonstrate that it is possible to distinguish multiple blow fly species using PCR–RFLP of the COI gene. Mitochondrial DNA, especially the COI gene, has been useful in the identification of calliphorids previously (22,31). The discriminatory strength of the COI region has been highlighted for the differentiation of immature stages of certain forensic fly species in several locations including, Western Australia (23,31), southern Africa (31), Korea (32), Malaysia (33), Japan (34), and Germany (35).

A region of approximately 1500 bp overlapping the COI gene was amplified for all species. No differences were detected in the size of the fragment amplified for the individual species under optimal conditions. The *in silico* DNA constructs provided by Sequence Extractor (25) verified the general size of the amplicon generated by universal primers TY-J-1460 and TL2-N-3014. The region extended from base pair 2578–4170 of the published *C. hominivorax* (NC_002660) mitochondrial genome COI region and was 1593 bp in length including the primer regions.

The restriction fragment pattern with all 10 restriction enzymes was considered as the "restriction profile" of a species because it was unique for each species investigated. Comparisons of "restriction profiles" clearly separated the different species from each other. As expected, BsrDI did not show any variation in the restriction fragment pattern produced for all species. A combination of HinfI and DraI differentiated all species investigated in this study (Figs 1 and 2, respectively). Fragments of less than 100 bp had poor resolution and were not used for the interpretation of fragment patterns in the study. Figure 3 depicts an outline of the species differentiation based on fragment patterns generated using HinfI and DraI. Figure 4 provides a taxonomic key developed for species differentiation utilizing these two restriction enzymes. Comparison of the "restriction profile" of *Ophyra* sp. with other species used in the study showed a distinctive difference in the restriction fragment sizes and pattern, clearly excluding it as one of the calliphorids species investigated in the study.

As noted by Sperling et al. (15), enzymes for investigation of the RFLP among species were chosen based on the following criteria: restriction site differences between species (for species differentiation), the presence of restriction sites in most species for some of the enzymes (as an internal control for enzyme effectiveness), robustness, and low cost of the enzymes. As shown here, restriction analysis of a PCR amplified mitochondrial region is simple and can differentiate multiple species of calliphorids significant to forensic entomologists in southeastern Nebraska. Restriction enzyme profiling, with several single enzyme digestions for a species, could allow unambiguous identification of a panel of species quickly and efficiently. PCR–RFLP–based molecular identification keys are flexible; they can be easily tailored to a laboratory, depending on the availability of restriction enzymes, allowing molecular identification of species in laboratories that do not have access to DNA sequencing. Additionally, PCR–RFLP is a fairly routine technique; the technical expertise and equipment will already be in place in most forensic laboratories. Further, selection of enzymes requiring the same incubation temperature and buffer will enable them to be run at the same time (double digest), increasing the efficiency of the technique for running multiple samples.

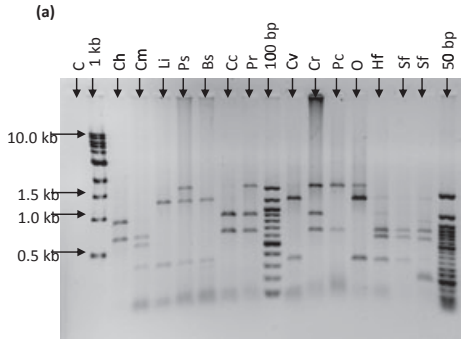
Intraspecific variation could produce alternative restriction patterns and sizes. Intraspecific variation was investigated for all restriction enzymes with all species. In general, the intraspecific variability detected was low. AseI is the only enzyme that showed alternative restriction patterns across multiple species: *C. macellaria*, *C. vicna*, *L. illustris*, *L. coeruleiviridis*, *L. silvarum*, and *S. calcitrans*. Enzymes such as DdeI, EcoRI, and EcoRV showed polymorphism in patterns generated for some species between *in silico* and *in vitro* studies for seven species of calliphorids: *C. hominivorax*, *C. macellaria*, *C. rufifacies*, *P. regina*, *C. cadaverina*, *L. illustris*, and *L. sericata*. This could be due to intraspecific



(b)

Size	Ch	Cm	Li	Ps	Bs	Cc	Pr	Cv	Cr	Pc	O	Hf	Sf
1.30													Y
1.20													
1.10													
1.00													
0.90													
0.85													
0.80	Y	Y	Y	Y	Y	Y	Y		Y	Y		Y	
0.75				Y							Y		
0.70									Y				
0.60								Y				Y	
0.50		Y									Y		
0.45			Y										
0.40	Y	Y	Y		Y		Y	Y	Y	Y			
0.35	Y												
0.30									Y	Y			
0.25											Y	Y	
0.20													

FIG. 1—*PCR–RFLP results for HinfI restriction. The mitochondrial COI region was amplified using universal primer pair TY-J-1460 and TL2-N-3014. (a) RFLP products and (b) RFLP fragment size summary for species with single digestion by HinfI. C—control; 1 kb, 50 bp, 100 bp—molecular size standards; Ch—Cochliomyia hominivorax; Cm—Cochliomyia macellaria; Li—Lucilia illustris; Ps—Lucilia sericata; Bs—Lucilia silvarum; Cc—Cynomyopsis cadaverina; Pr—Phormia regina; Cv—Calliphora vicina; Cr—Chrysomyia rufifacies; Pc—Lucilia coeruleiviridis; O—Ophyra sp.; Hf—Musca domestica; Sf—Stomoxys calcitrans. Species in (b) that are similar in restriction profiles are shaded in gray; Y designates the presence of a fragment of a particular size (in kilo bases).*



(b)

Size	Ch	Cm	Li	Ps	Bs	Cc	Pr	Cv	Cr	Pc	O	Hf	Sf
1.30			Y	Y	Y			Y			Y		
1.20													
1.10													
1.00	Y												
0.90						Y	Y		Y				
0.80													
0.70	Y	Y				Y			Y	Y			
0.65							Y						
0.60		Y								Y		Y	Y
0.50												Y	Y
0.40		Y	Y	Y				Y			Y		
0.35					Y								
0.30												Y	Y
0.25													
0.20													

FIG. 2—*PCR–RFLP results for DraI restriction. The mitochondrial COI region was amplified using universal primer pair TY-J-1460 and TL2-N-3014. (a) RFLP products and (b) RFLP fragment size summary for species with single digestion by DraI. C—control; 1 kb, 50 bp, 100 bp—molecular size standards; Ch—Cochliomyia hominivorax; Cm—Cochliomyia macellaria; Li—Lucilia illustris; Ps—Lucilia sericata; Bs—Lucilia silvarum; Cc—Cynomyopsis cadaverina; Pr—Phormia regina; Cv—Calliphora vicina; Cr—Chrysomyia rufifacies; Pc—Lucilia coeruleiviridis; O—Ophyra sp.; Hf—Musca domestica; Sf—Stomoxys calcitrans. Species in (b) that are similar in restriction profiles are shaded in gray; Y designates the presence of a fragment of a particular size (in kilo bases).*

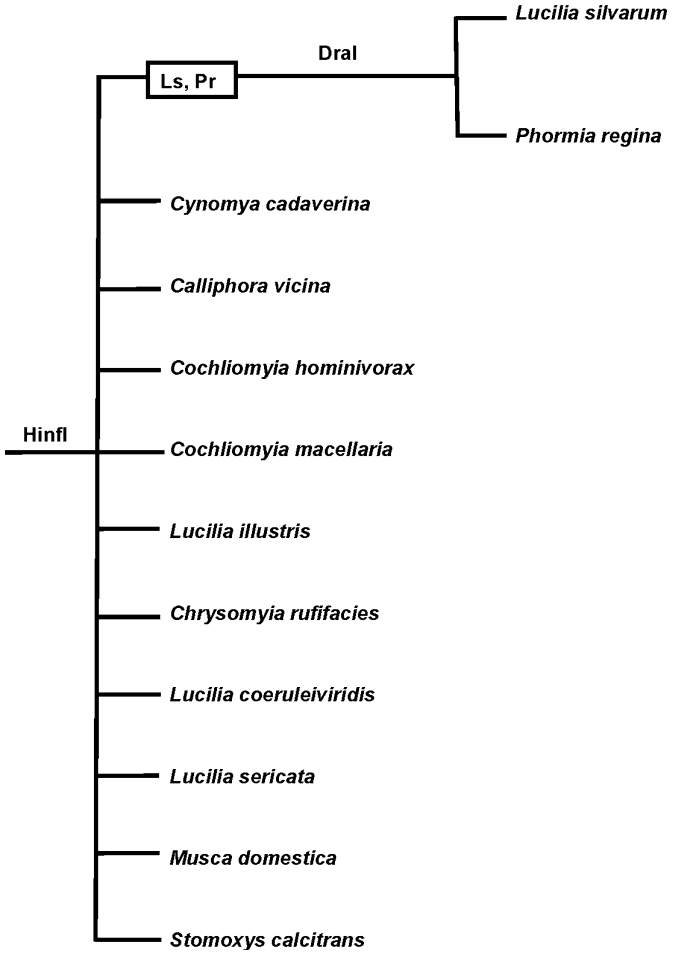


FIG. 3—*Outline of PCR–RFLP differentiation of some calliphorid species of southeastern Nebraska with DraI and HinfI restriction enzymes. The diagram does not imply phylogenetic relationships, it is a representation of species differentiation with enzymes HinfI and DraI. Musca domestica and Stomoxys calcitrans are noncalliphorid dipteran controls of the study. Cochliomyia hominivorax is an internal positive control (calliphorid).*

variations in restriction sites or errors in published sequences. Intraspecific sequence variation has been detected in COI for calliphorids previously, but the interspecific variation has been observed to be higher, enabling unambiguous species identification (26,32). Restriction site variation of mtDNA carried out by previous studies on a range of insects has shown that the within-species variation in mitochondrial DNA is small (15). Interspecific variability was more apparent with enzymes such as DraI and HinfI, which showed better fragment pattern and size (of fragments) polymorphism than the rest of the enzymes; they were also consistent in the restriction pattern generated for each species. These "robust" enzymes are good candidates for the design of a molecular key. Comparison of the results with previous studies (15,18,24) is difficult as the amplified fragments for RFLP investigation were smaller in size than the fragments generated for our study. But the *in silico* investigations are a good indication of the sizes and patterns of fragments generated by the selected enzymes.

Even though the described key is focused for species identification in southeastern Nebraska, the protocol can be adapted for other regions of the country. Expansion of the study both in Nebraska and across other geographic regions will enhance the effectiveness, the expandability, the robustness of the key by revealing the sensitivity of the method to interspecific and intraspecific variation in species. The use of more restriction enzymes will enable the adaptation of the key to identify a larger number of species. Validation of the key under field conditions, with larger sample sizes, is essential before the release of the key for routine use. Provided that molecular keys are subjected to stringent testing and validation before release for routine use, DNA-based identification keys will enable rapid identification of species for PMI estimation in death investigations. Ideally, these molecular keys will be used in conjunction with traditional morphological methods to provide two levels of identification: phenotypic and genotypic. The use of multiple approaches for species identification will increase the level of confidence associated with the use of insects as evidence in legal proceedings. In addition, when only immature stages of insects are available, the protocol described here might act as an alternative approach in cases where it is not possible to acquire the expertise for morphologically or DNA sequence–based identification.

Acknowledgments

We thank Tim Huntington (Natural Science Department, Concordia University, NE) for the identification of field

1a. PCR product size amplified by universal primer pair TY-J-1460 and TL2-N-3014 is approximately 1500 bp	**2**
1b. No PCR product for amplification with universal primer pair TY-J-1460 and TL2-N-3014	Other species
2a. PCR product digested with HinfI enzyme gives the following restriction pattern: 1.3 kb	*Stomoxys calcitrans*
2b. PCR product digested with HinfI enzyme gives the following restriction pattern: 0.8, 0.75 kb	*Lucilia sericata*
2c. PCR product digested with HinfI enzyme gives the following restriction pattern: 0.8, 0.7, 0.4, 0.3 kb	*Chrysomya rufifacies*
2d. PCR product digested with HinfI enzyme gives the following restriction pattern: 0.8, 0.6, 0.25 kb	*Musca domestica*
2e. PCR product digested with HinfI enzyme gives the following restriction pattern: 0.8,0.5, 0.4 kb	*Cochliomyia macellaria*
2f. PCR product digested with HinfI enzyme gives the following restriction pattern: 0.8, 0.4, 0.35 kb	*Cochliomyia hominivorax*
2g. PCR product digested with HinfI enzyme gives the following restriction pattern: 0.8, 0.4, 0.3 kb	*Lucilia coeruleiviridis*
2h. PCR product digested with HinfI enzyme gives the following restriction pattern: 0.8, 0.4 kb	**3**
2i. PCR product digested with HinfI enzyme gives a 0.8 kb fragment	*Cynomya cadaverina*
2j. PCR product digested with HinfI enzyme gives the following restriction pattern: 0.6, 0.4 kb	*Calliphora vicna*
2k. PCR product digested with HinfI enzyme gives none of the above fragment patterns	Other species
3a. PCR product digested with DraI enzyme gives the following restriction pattern:1.3, 0.35 kb	*Lucilia silvarum*
3b. PCR product digested with DraI enzyme gives the following restriction pattern: 0.9, 0.65 kb	*Phormia*

FIG. 4—*Molecular taxonomic key for eight calliphorids of forensic importance in southeastern Nebraska based on PCR–RFLP of the COI sequences of 11 species. Restriction enzymes HinfI and DraI were used for species differentiation. Cochliomyia hominivorax was used as a positive control, while Musca domestica and Stomoxys calcitrans were included as out groups.*

collected specimens, Dr. Dennis R. Berkebile (USDA-ARS, Lincoln, NE) for laboratory maintained samples of *Musca domestica* and *Stomoxys calcitrans.* Dr. Berkebile and Dr. David Carter (University of Nebraska-Lincoln, NE) provided valuable suggestions in review of this manuscript.

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