FINAL REPORT: Status and products of National Institute of Justice Research Grant, "Development of DNA-Based Identification Techniques for Forensic Entomology. Phase 2", 8/99-7/01. 99-IJ-CX-0034.

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This research program is an extension of an earlier NIJ-funded project by Dr. Felix Sperling at the University of California at Berkeley. Analysis of insect evidence is now a common tool of death investigators in this country. The most common objective is an estimation of the time of death, also called the postmortem interval (PMI), based on the age of a maggot collected from the corpse. A technical difficulty faced by a forensic entomologist is that it is often difficult or impossible to identify the species of a maggot using classical (anatomical) methods. Precise identification can be crucial to the analysis because some closely related species grow at very different rates. Therefore, taxonomic uncertainty can lead to corresponding uncertainty in an estimation of time of death. The misidentification of a specimen could produce a PMI estimate that, depending on the species and temperature, could be off by more than a week. For example, at 19 C the greenbottle fly *Lucilia cuprina* (= *Phaenicia pallescens*) requires an average of 47 days to develop from a newly laid egg to an adult, while its closest relative *L. sericata* requires an average of 84 days at the same temperature. Such samples are often killed and preserved by other investigators long before the entomologist is consulted, so it is not possible to rear the specimen to the more easy to identify adult stage.

A MITOCHONDRIAL DNA DATABASE USEFUL FOR IDENTIFYING FORENSICALLY IMPORTANT FLY SPECIES

Using NIJ funds we have developed a mitochondrial DNA database suitable for identifying forensically important fly species in any life stage. In almost all experiments the study locus was the region including the genes for cytochrome oxidase subunits one and two (COI+II). Currently we have data from 48 species (Table 1). This list includes the insect species collected as evidence in the vast majority of death investigations in the USA and Canada

and we have been using these methods in death investigation casework. These data have or soon will be deposited in the GenBank WWW database maintained by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) where they may be easily retrieved by anyone with internet access.

At this point in time we believe that the most reliable DNA-based identification of evidence samples employs phylogenetic analysis (Figures 1-6). Sequence data from the unknown (=evidence) sample is added to a data file including reference sequences from identified adult specimens. These data are then analyzed by any of the commonly used phylogenetic software packages such as PAUP (Swofford 1998). The output of such an analysis is to identify the reference sequence that is the closest relative of the unknown sample. If the investigator has enough morphological and ecological evidence to conclude that the unknown specimen is one of the species represented in the reference database then the closest relative will be the same species as the unknown specimen. Using common software to apply statistical methods (e.g., the bootstrap) allows one to objectively express how much the DNA evidence supports a "sister group" relationship between known and unknown haplotypes. If the support is too weak then additional sequence data are probably needed. The analysis of several mock and one genuine forensic specimen shown in Figure 2 best illustrate this. Short (166-340 bp) mtDNA sequences such as those that might be recovered from old and degraded samples were obtained from specimens not included in the database. Each was paired with the correct reference sequence with ≥97% bootstrap support. The reliability of this approach was further supported by a global survey of the two species *Chrysomya rufifacies* and *C. albiceps*. These species along with *C. megacephala* and *C. putoria* were introduced from the Old World into Latin America in the 1970s (Baumgartner & Greenberg 1984). *C. rufifacies* and *C.*

megacephala spread north and are now abundant in the continental USA (both were already common in Hawaii) where they are often found in a human corpse. *C. albiceps* and *C. putoria* are likely to reach this country as well. Many forensically important flies display the strong association with humans and agricultural products that promotes transport to new locations via shipping containers, etc. It is inevitable that the USA will continue to occasionally see the establishment of new carrion fly species as well as foreign mtDNA haplotypes of species that are already present. However as shown in Figure 3, flies of these two species from around the world are easily assigned to the correct lineage using mtDNA sequence data.

Even if the unknown is a species not found in the reference sequence database, phylogenetic analysis can still provide useful information for an investigator. Here is an illustration based on feeding behavior. A maggot can come to inhabit a corpse prior to death because it is a species that is a parasite (e.g., one that feeds on live flesh) or a species that feeds on feces in clothes. While I am not aware of an example in which the parasitic kind of maggot was collected as forensic evidence, there have certainly been instances in which someone was already afflicted with such maggots at the time of death (Spradbery 1994). There have been both criminal and civil cases involving maggots in human feces within clothes (unpublished observations, Goff et al. 1991). The forensic relevance of all this natural history is that some of the maggots that can be or must have been present before death can easily be mistaken for a species that almost certainly arrived after death. Obviously mistaking an antemortem species for a postmortem species could lead the investigator to think, based on the inferred age of the insect, that the victim was dead prior to the real time of death. Feeding location, such as in a dirty diaper, could be an important clue. However larvae can crawl to any location on the corpse, particularly if the body has been physically disturbed, and even become mixed with a population

of carrion feeding maggots.

The phylogeny of sarcophagid fly species shown in Figure 1 is an example in which evolutionary lineage corresponds to these forensically important behaviors. All species in the genus *Ravinia*, represented in our reference data set by *R. lherminieri*, have larvae that feed only on feces. If an unidentified larva were found to be most closely related to *R. lherminieri* the exact species would still be uncertain because we did not include many other *Ravinia* in our study. Even so, an investigator would suspect that the larva was a feces feeder and that it was therefore a poor indicator of PMI. Similarly, all members of the genus *Wohlfahrtia*, represented by *W. vigil* in Figure 1, have larvae that only eat live tissue.

Although COI+II sequence data appear to work very well for identifying these insects, there is one situation in which this was less straightforward than usual. An earlier published study examined the calliphorids *Lucilia* (=*Phaenicia*) *cuprina* and *L. sericata* using randomly amplified nuclear DNA and 12S rRNA (Stevens & Wall 1996). An analysis of our still incomplete COI+II reference data for *Lucilia* also paired a Hawaiian *L. cuprina* haplotype with that of an *L. sericata* from Canada rather than with another *L. cuprina* from Australia (Figure 4). We further explored this using a worldwide sample of both species and by also sequencing the nuclear locus 28S ribosomal RNA. As shown in Figure 5, there is a clear conflict between the phylogeny based on nuclear DNA and that based on mitochondrial DNA.

Cytochrome oxidase is still the best locus we have for identifying *L. cuprina* from Hawaii. This is supported by the extremely high bootstrap support for the Hawaiian lineage (Figure 5) and there is little doubt that an evidence specimen would be properly assigned to that lineage. The problem would have occurred if we had not gathered Hawaiian data and an investigator had attempted to i.d. a Hawaiian *L. cuprina* specimen by comparison to only non-

Hawaiian haplotypes. This would probably have led to a misidentification of the specimen as *L. sericata*, a species reported to have a very different growth rate (see introduction).

Although it is not important for forensic science purposes, we suspect that this odd genetic pattern indicates that the Hawaiian *L. cuprina* population is descended from a *L. cuprina* X *L. sericata* hybrid. This sort of event is particularly likely to take place when a species is newly introduced (such as the case with both of these species in Hawaii (Hardy 1981)) into a small and isolated region such as an island. So far we have failed to detect a similar mismatch between DNA and morphology in any other forensic fly species. However this does make clear that regional sampling, particularly of isolated populations, is a necessary step for evaluating these methods.

POPULATION SURVEYS

As described in the previous section, we use data from additional specimens whose identity is known to test the performance of our proposed DNA-based identification methods. In an effort to capture the widest range of haplotype diversity, I have assembled specimens of these taxa from as many geographical locations as possible. Table 2 lists those locations and species for which we have replicated haplotype data. Many more specimens have yet to be processed. All of these haplotypes were assigned to the correct lineage in the manner shown in Figures 2 and 5, although it should be noted that the distinct lineage of Hawaiian *Lucilia cuprina* would not have been recognized before our study (see previous section).

Another reason for collecting regional haplotype data was inspired by a paper by Byrne et al. (1995). They extracted the cuticular hydrocarbons (CH), the waxy surface coat that prevents desiccation, from *Phormia regina* collected from two locations in Washington State and

one location in Indiana. Principal component analysis of these hydrocarbons produced clusters corresponding to geographical origin. The authors believed that CH could therefore be used to infer the postmortem movement of a corpse. Although Byrne et al. did not discuss the population genetic implications of their results, they are startling. According to Byrne et al. CH profiles are genetically determined. The insects they sampled were reared in the lab so there was no direct connection to the physical environment of the collection sites. A clear implication of the Byrne et al. study is that there is either a barrier to gene flow between the study sites and/or different selection pressures on CH. What makes this paper surprising is that the two Washington locations are only about 70 km apart. There is no obvious barrier to fly movement between them making a barrier to gene flow difficult to fathom. Large carrion flies such as *P. regina* are extremely mobile and have often been observed to travel several kilometers in one day. In addition, both Washington sites represent similar habitats in that they both sit in the middle of the wheat fields that blanket the southeastern corner of the state. Differential selection on CH is therefore also difficult to fathom.

Of course the fact that these things are difficult to fathom does not mean that they are not true, and if so they have profound implications for forensic entomology. Based on the mobility of these flies, researchers have assumed that on a continental scale a given species was genetically homogeneous and very unlikely to show local variation in forensically important phenomena such as larval growth rate. The few studies that compared growth rates of the same species from different locations seemed to support this assumption, although they were limited in size (see review in Wells & LaMotte 2001). Experimental data on growth rates that were obtained in one location are routinely applied to the same species in another location. In contrast, the results of Byrne et al. indicate population differentiation on a geographic scale so

small that the flies in neighboring counties might represent separate populations.

The methods section of the Byrne et al. paper leaves open the possibility that their finding of regional differentiation is an artifact. They used laboratory colonies but did not describe the number of individuals used to found those colonies or the number of generations that had passed before the experiment was done. A lab colony of blow flies always experiences a genetic bottleneck because a single female lays 150-250 eggs at a time and only a few such females are needed each generation to maintain the population. It is possible that Byrne et al. measured individual rather than geographic variation in CH.

Because this phenomenon must be understood we have initially emphasized *P. regina* when analyzing regional variation (Table 2). Collection sites include the two Washington locations sampled by Byrne et al. We have replicated Byrne et al.'s Washington CH extractions and have sent them to a chemist for analysis. The *P. regina* specimens listed in Table 2 displayed a total of 60 haplotypes for a 731 bp section of COI plus a 284 bp region of 12S rRNA and the A+T-rich region. An exact test failed to find a non-random association between haplotype and location and there appears to be no evidence of *P. regina* mtDNA regional differentiation within the continental USA.

EFFORTS TO FIND A MORE POLYMORPHIC LOCUS

In my proposal I expressed a desire to find a more variable locus than COI+II because it was thought at that time that one could not distinguish between the species pairs *Phormia regina /Protophormia terraenovae* and *Chrysomya megacephala/Chrysomya pacifica*. One unanticipated outcome of this research was that we discovered that the *P. regina* GenBank sequence (Sperling et al. 1994) that is almost identical to out *P. terraenovae* sequence was in

fact from a misidentified *P. terraenovae* specimen! In fact the two species are easily distinguished based on DNA (Figure 2). Because *C. pacifica* does not occur in the Western Hemisphere (at least not yet) there is at present no need to develop a more variable locus for identifying forensic specimens in this country. We drew attention to this error, and a much more minor mistake in a separate fly DNA paper, in a letter to the editor published in volume 45 number 6 of the *Journal of Forensic Sciences* . This prompted an official acknowledgment of the mistakes and correction of the relevant GenBank record.

A locus that is more polymorphic than COI+II might still be useful for at least two reasons. The first is a more discriminating measure of population structure (see previous section). The other would be a more discriminating measure for kinship analysis. On several occasions I have been approached by an investigator who, within a short period of time, discovered a maggot-infested corpse at one location and maggots but no corpse at another location. Was a dead human present at the second site (see the next section for more about determining a maggot's "last meal")? If so, was it the victim that was discovered elsewhere or should investigators be looking for a second body?

One likely hypervariable locus is the mtDNA A+T-rich region. This is a non-coding section that is probably homologous to the vertebrate D-loop. Unfortunately compared to the D-loop the A+T-rich region is difficult to analyze because of the presence of tandem repetition, heteroplasmy, and length variation within and between species (Zhang & Hewitt 1997). Using our standard protocols we routinely obtain only about a 100 bp sequence from the A+T-rich region adjacent to the 12S rRNA gene. However even this short region contains a large number of polymorphic sites compared to coding region of the same length.

One of my students has been able to increase the amount of A+T-rich sequence we can

obtain from cycle sequencing to about 200 bp using a commercial PCR optimization kit. She is currently in the process of trying to obtain the entire ~1kb sequence by cycle sequencing cloned PCR product.

OTHER PCR-BASED METHODS FOR SPECIMEN IDENTIFICATION

In my proposal I described a plan to evaluate the technique of a restriction digest of PCR product (PCR-RFLP) as an inexpensive alternative to cycle sequencing for specimen identification. The strategy was to design a test or tests based on diagnostic restriction sites found in the COI+II sequence of a few individuals, and then to undertake a population survey to see if those diagnostic restriction sites were apparently fixed in the population. Once the project started I decided that this aspect of the research should receive less priority for two reasons. First, it seemed that a better first use of our population survey was the validation of the phylogenetic approach described above. The second was the publication of a methods paper describing species identification using a modified protocol for heteroduplex analysis (HDA) (Boakye et al. 1999). The technique involves the mixing of PCR product from the unknown sample with product (referred to as the "driver") from a standard known species. The mixture is denatured by heat and allowed to re-anneal, and this is then eletrophoresed on a polyacrylamide gel. Boakye et al. found that the vertebrate cytochrome b heteroduplex patterns observed on the gel could be classified by species even when an intraspecific polymorphism was observed. This is an apparent advantage over PCR-RFLP in which a point mutation can completely alter fragment size.

We evaluated the performance of this HDA approach for forensically important Sarcophagidae using a house fly driver. All of these species could be distinguished in this way

(Figure 10). Subsequently, we performed HDA analysis on 50 additional sarcophagid specimens captured at decayed meat baits in Birmingham. The reliability of this technique appeared to be confirmed. However because adult sarcophagids are so difficult to identify, I have sent the specimens to Thomas Pape, the world authority on sarcophagid taxonomy at the Swedish Natural History Museum, to confirm my identifications. He has not yet had time to respond.

OBTAINING A VERTEBRATE GENOTYPE FROM MAGGOT GUT CONTENTS

In addition to the primary focus of this research, we have taken advantage of the fact that carrion-fly larvae are a potential source of DNA for human identity testing. As described in a previous section, situations occur in which maggots are found in the absence of a corpse. For obvious reasons it would be useful to know on what (and if it was a human, whom) they were feeding. Recently I received an inquiry concerning an unanticipated application of this procedure. A forensic entomologist was accused of returning a different vial of evidence larvae than was sent to him by police. The detection of the murder victim's DNA in the disputed larvae would resolve the matter.

We have shown that in a laboratory setting a human haplotype recovered from maggot gut contents matches the haplotype obtained from the blood of the individual who donated the liver (following a transplant) we used to feed the larvae. Figure 9 shows a comparison between the same small section of a sequencing electropherogram from each sample.

We advocate the use of the dissected maggot crop as the source of such tissue rather than the destruction of the entire maggot. The crop is a food storage organ attached to the rest of the gut between the mouth and stomach. Dissection preserves taxonomically important external structures and the crop is a concentrated and relatively undegraded source of gut contents

(Figure 7). As a precaution against external contamination, we have found that the exterior surface of a maggot can be sterilized with 20% bleach without interfering with crop-content analysis (Figure 8).

Conveniently, the crop tissue that is included in the extract yields more than enough DNA to identify the larva as well. Taxon-specific primers make it easy to amplify the DNA, insect or vertebrate, of choice. In Figure 6 the insect haplotype from a crop extract is matched with that of an adult fly reared from the same piece of tissue.

This work is ongoing. We have prepared the samples needed to evaluate the effect of preservation technique on our ability to recover a crop-content genotype. The purpose is to devise optimal collection methods for scene investigators. We also have the samples needed to measure the amount of time recoverable food DNA remains in the crop after a maggot ceases to feed, and to observe the replacement of one food genotype with another following the movement of a larva to a new food source. The goal is to describe the window of time within which a maggot can be used to identify a body that is no longer present, and to how soon the historical record of a previous food source is erased following movement to a new corpse.

TRAINING OF FORENSIC SCIENCE GRADUATE STUDENTS

One graduate student (Diana Williams nee Wagner) working on this project defended her M.S.F.S thesis and is now employed as a DNA analyst with the Georgia Bureau of Investigation. She was specifically hired for the mtDNA skills and experience with the Applied Biosystems 310 than she acquired during this study. Her successor is expected to defend her thesis this coming August. A Ph.D. student working on the maggot gut-content study is approximately 9 months away from defending his dissertation.

PUBLISHED MATERIAL PRESENTING THESE DATA AND TECHNIQUES TO THE FORENSIC SCIENCE COMMUNITY

The following scientific publications have resulted from this work. The DNA sequences have all been deposited in the publicly available databank maintained by the National Center for Biotechnology Information (GenBank) (Table 1).

Refereed papers or book chapters

Wells, J.D., & F.A.H. Sperling. 1999. Molecular phylogeny of *Chrysomya albiceps* and *C. rufifacies* (Diptera: Calliphoridae). *Journal of Medical Entomology* 36:222-226.

Wells, J.D. and F.A.H. Sperling. 2000. Commentary on: Sperling, FAH, Anderson, GS, Hickey, DA. A DNA-based approach to the identification of insect species used for postmortem interval estimation. J Forensic Sci 1994;39:418-427 and on Vincent, S, Vian JM, Carlotti MP. Partial sequencing of the cytochrome oxydase b subunit gene I: a tool for the identification of European species of blow flies for postmortem interval estimation. J Forensic Sci 2000;45:820-823. *Journal of Forensic Sciences* 45:1358-1359.

Wells, J.D. and F.A.H. Sperling. 2001. DNA-based identification of forensically important Chrysomyinae (Diptera: Calliphoridae). *Forensic Science International* 120:110-115.

Benecke, M. and J.D. Wells. 2001. DNA techniques for forensic entomology. in Byrd, J. and Castner, J.L. (eds.) *Forensic Entomology: Utility of Arthropods in Legal Investigations.* CRC Press.

Wells, J.D., F.A.H. Sperling and T. Pape. 2001. DNA-based identification and molecular systematics of forensically important Sarcophagidae. *Journal of Forensic Sciences.* 46:1098-1102.

Wells, J.D., F.G. Introna, G. Di Vella, C.P. Campobasso, J. Hayes and F.A.H. Sperling. 2001. Human and insect mitochondrial DNA analysis from maggots. *Journal of Forensic Sciences* 46:685-687.

Stevens, J. R., R. Wall and J.D. Wells. in press. Paraphyly in Hawaiian hybrid blowfly

populations and the evolutionary history of anthropophilic species. *Insect Molecular Biology*

Linville, J. and J.D. Wells. submitted. A simple wash method for removing external contamination from maggots for crop content analysis. *Journal of Forensic Sciences*

Based on the amount of data in hand, we expect to submit for publication at least three additional manuscripts on this topic. Ongoing research using specimens and reagents acquired using these funds is expected to yield 2-4 manuscripts.

Thesis

Wagner, D.H. 2000. Investigation of molecular population genetic substructure in forensically important flies. M.S.F.S. thesis, University of Alabama at Birmingham

PRESENTATION OF THESE RESULTS AT SCIENTIFIC CONFERENCES

In addition to the progress reports presented annually at the NIJ grantees' workshop, members of our laboratory have regularly described the results of our NIJ sponsored research to the scientific community.

Invited seminars:

"Mitochondrial DNA based species identification for forensic and political purposes", Marshall University Forensic Sciences Department, 12 November 1999

"Mitochondrial DNA analyses for forensic and political purposes", Samford University Biology Department, 3 May 2000

"DNA-based identification techniques for forensic entomologists", Texas A&M University at College Station Entomology Department, 21 September 2000

"DNA-based identification techniques for forensic entomologists", University of Richmond Biology Department, 27 November 2000

"Forensic entomology research: recent technical advances", Virginia Commonwealth University, Department of Criminal Justice, 29 November 2000

"Forensic entomology research: recent technical advances", New York State Museum, Albany, 1 December 2000

"Molecular systematics of higher flies (Oestroidea): the utility of mitochondrial DNA for answering basic and applied questions", Virginia Commonwealth University, Department of Biology, 12 February 2001

Presentations at scientific meetings:

"DNA-based identification of forensically important Sarcophagidae", poster presented at the American Academy of Forensic Sciences annual meeting, Reno, February 2000

"An investigation of reported regional genetic variation in the forensically important blow fly, *Phormia regina*, using mitochondrial DNA" poster presented at the American Academy of Forensic Sciences annual meeting, Reno, February 2000

"DNA-based diagnostic tests for forensic entomologists", International Congress of Entomology, Iguaçu Falls, Brazil, 26 August 2000

"Human and insect mitochondrial DNA analysis from maggots", Entomological Society of America annual meeting, Montreal, 5 December 2000

"DNA-based diagnostic tests for forensic entomologists", Southeastern Branch of the Entomological Society of America annual meeting, Augusta, GA, 6 March 2001

"Species identification of sarcophagid flies using PCR-based methods", American Academy of Forensic Sciences annual meeting, Seattle, WA 23 February 2001

"Paraphyly in *Lucilia cuprina* (Diptera: Calliphoridae) mitochondrial DNA: implication for forensic entomology and evidence for an ancient hybridization event". Entomological Society of America annual meeting, San Diego, 11 December 2001

"Non-human mitochondrial DNA analysis", part of the FBI-sponsored workshop "Forensic Mitochondrial DNA Analysis: A community Forum" held in conjunction with the American Academy of Forensic Sciences annual meeting, Atlanta, 11 February 2002

"Paraphyly in *Lucilia cuprina* (Diptera: Calliphoridae) mitochondrial DNA: implication for forensic entomology and evidence for an ancient hybridization event". American Academy of Forensic Sciences annual meeting, Atlanta, 14 February 2002

"Removing external contamination of maggots with a bleach solution without inhibiting genetic analysis of maggot crop contents". American Academy of Forensic Sciences annual meeting, Atlanta, 15 February 2002

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Goff, M.L., Carbonneau, S. & Sullivan, W. 1991. Presence of fecal material in diapers as a potential source of error in estimation of the postmortem interval using arthropod development rates. J. Forensic Sci. 36:1603-1606.

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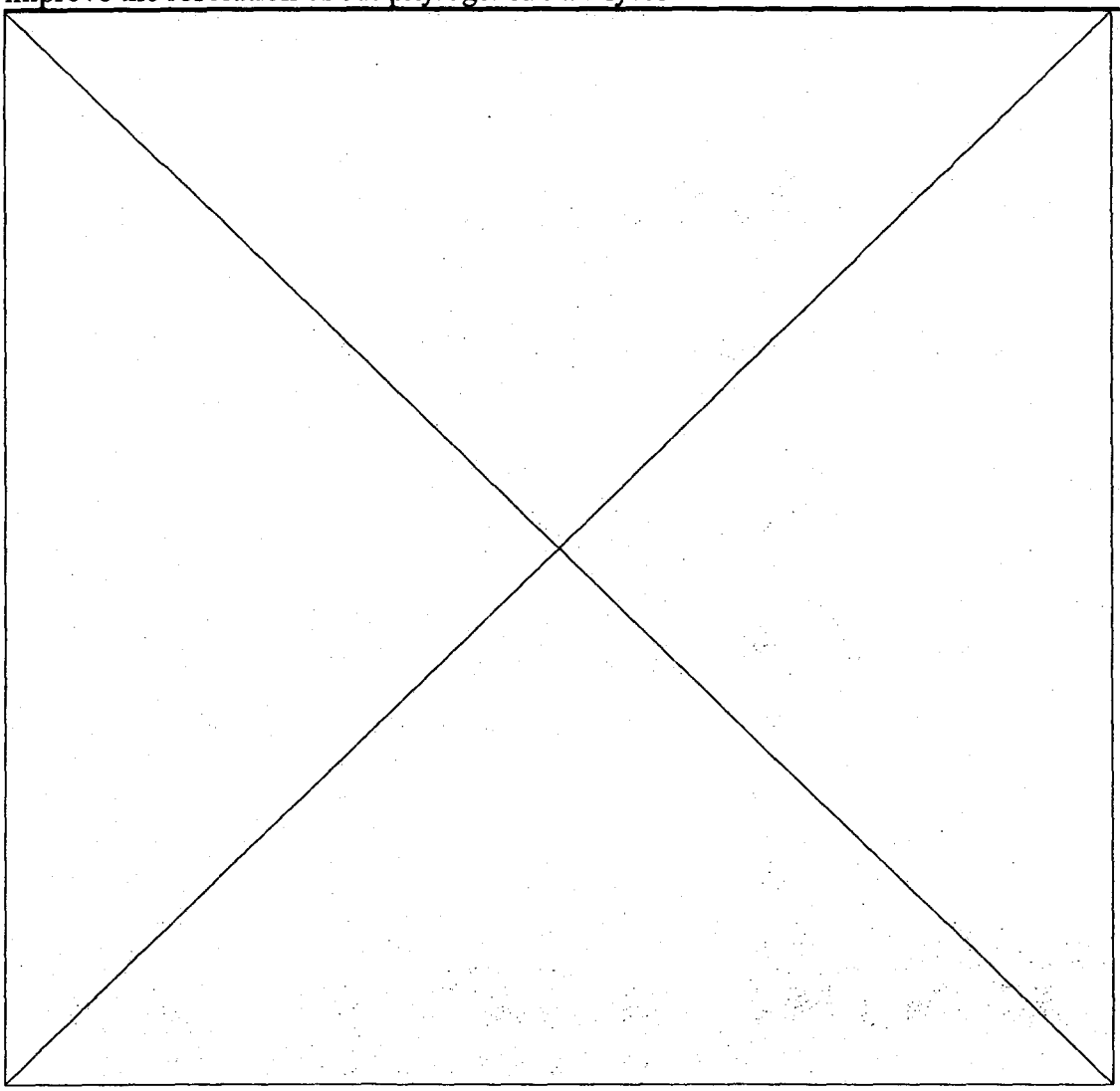
Stevens, J. & Wall. R. 1996. Species, subspecies, and hybrid populations of blowflies *Lucilia cuprina* and *L. sericata*. Proc. Roy. Soc. London B 263:1335-1341.

Swofford, D.L. 1998. PAUP, phylogenetic analysis using parsimony (and other methods). Version 4. Sinauer, Sunderland, MA.

Wells, J.D. & LaMotte, L.R. 2001. Estimating the postmortem interval. pp. 263-285 *in* Byrd, J.H. and Castner, J.L., Forensic entomology. The utility of arthropods in legal investigations. CRC Press, Boca Raton.

Zhang, D. & Hewitt, G.M. 1997. Insect mitochondrial control region: a review of its structure, evolution and usefulness in evolutionary studies. Biochem. Syst. Ecol. 25:99-120.

TABLE 1. Fly species from which we have obtained mitochondrial DNA sequence data covering the genes for cytochrome oxidase subunits one and two (COI+II) and the intervening transfer RNA leucine (tRNA-leu). Four of these species (indicated by an asterisk) are not likely to be collected as forensic evidence. They were included because the data were expected to improve the resolution of our phylogenetic analyses.



see attached table

TAXONOMIC FAMILY	SUBFAMILY	SPECIES	GENBANK ACCESSION #
Oestridae	Hypodermatinae	*Hypoderma lineatum**	AF295558
Muscidae	Muscinae	*Musca domestica*	AF259518
Sarcophagidae	Paramacronychiinae	*Brachicoma devia**	AF259517
"	"	*Wohlfahrtia vigil*	AF259516
"	Sarcophaginae	*Ravinia iherminieri*	AF259513
"	"	*Blaesoxipha plinthopyga*	AF259514
"	"	*Peckia chrysostoma*	AF259515
"	"	*Sarcophaga peregrina*	AF259509
"	"	*Sarcophaga argyrostoma*	AF259512
"	"	*Sarcophaga ruficornis*	AF259511
"	"	*Sarcophaga crassipalpis*	AF259510
"	"	*Sarcophaga africa*	AF259508
"	"	*Sarcophaga cooleyi*	AF259507
"	"	*Sarcophaga bullata*	AF259506
Calliphoridae	Chrysomyinae	*Phormia regina*	AF295550
"	"	*Protophormia terraenovae*	AF295553
"	"	*Protophormia atriceps*	AF295560
"	"	*Protocalliphora sialia**	AF295559
"	"	*Cochliomyia macellaria*	AF295555
"	"	*Compsomyiops callipes*	AF295549
"	"	*Chrysomya megacephala*	AF295551
"	"	*Chrysomya putoria*	AF295554
		Chrysomya chloropyga	not yet submitted
"	"	*Chrysomya varipes*	AF295556
"	"	*Chrysomya norrisi*	AF295552
"	"	*Chrysomya semimetalica*	AF295562
"	"	*Chrysomya bezziana*	AF295548
"	"	*Chrysomya albiceps*	AF083657
"	"	*Chrysomya rufifacies*	AF083658
"	Luciliinae	*Dyscritomyia fasciata**	AY074902
"	"	*Lucilia cuprina*	AJ417704, AJ417707
"	"	*Lucilia cluvia*	not yet submitted
"	"	*Lucilia ampullacea*	not yet submitted
"	"	*Lucilia thatuna*	not yet submitted
"	"	*Lucilia caesar*	not yet submitted
"	"	*Lucilia exima*	not yet submitted
"	"	*Lucilia mexicana*	not yet submitted
"	"	*Lucilia silvarum*	not yet submitted
"	"	*Lucilia adisoemartoi*	AY74901
"	"	*Lucilia porphyrina*	AY074900
"	Calliphorinae	*Cynomya cadaverina*	AF259505
"	"	*Cynomya mortuorum*	not yet submitted
"	"	*Eucalliphora latifrons*	AF295557
"	"	*Calliphora vicina*	not yet submitted
"	"	*Calliphora vomitoria*	not yet submitted
"	"	*Calliphora coloradensis*	not yet submitted
"	"	*Calliphora lata*	not yet submitted
"	"	*Aldrichina grahami*	not yet submitted

Table 1 99-IJ-CX-0034

TABLE 2. Geographic locations for which we now have mtDNA sequence data from multiple individuals.

SPECIES	LOCATION	NO. INDIVIDUALS
Phormia regina	Quantico, VA	10
"	Birmingham, AL	20
"	International Falls, MN	10
"	Santa Barbara, CA	10
"	White Mts., CA	10
"	Tucannon R., WA	10
"	Lyle Grove, WA	10
Cochliomyia macellaria	Baja, Mexico	5
"	Brownsville, TX	3
"	Birmingham, AL	5
"	Gainesville, FL	5
"	Miami, FL	5
Chrysomya rufifacies	Brownsville, TX	5
"	Birmingham, AL	5
"	Miami, FL	5
"	Dominica, West Indies	5
Chrysomya megacephala	Brownsville, TX	5
"	Birmingham, AL	5
"	Miami, FL	5
"	Dominica, West Indies	5
Lucilia cuprina	Honolulu, HI	5

FIGURE CAPTIONS

Figure 1. Single most parsimonious phylogeny of forensically important sarcophagid species likely to occur in Canadian or USA habitats other than pristine wilderness. This particular topology (tree shape) resulted from a heuristic search with 2000 random step-wise additions using of a 783 bp region of COI. The phylogeny is presented in the form of a phylogram in which branch length indicates the number of base substitutions separating haplotypes. Numbers on branches indicate percent bootstrap support if greater than 50% (5000 reps.). An analysis based on 2.3 kb of sequence data produced the same topology with slightly increased bootstrap support.

Figure 2. Single most parsimonious phylogeny of blow flies in the subfamily Chrysomyinae including all species likely to be collected from a human corpse in Canada or the USA. The analysis was based on on a 2.3 kb sequence from the genes for COI, COII, and tRNA-leu for most specimens. Analysis software parameters were the same as in Figure 1. Specimens marked by an asterisk were represented by 304 bp, or 166 bp in the case of *P. terraenovae*, in order to mimic very degraded forensic evidence. All mock forensic specimens were paired with the correct reference sequence with ≥97% bootstrap support. The larva paired with *Compsomyiops callipes* was an actual evidence specimen. It could not be identified based on microscopic inspection. However there were many adult *C. callipes* present at the scene.

Figure 3. A phylogenetic analysis of *Chrysomya albiceps* and *C. rufifacies* haplotypes from around the world based on 584 bp COI. The methods and reference sequences are those illustrated in Figure 2. Only the branch leading to these two species is included here.

Figure 4. Most parsimonious phylogram for forensically important flies in the genus *Lucilia*. See Figure 1 for a more complete description of the analysis. Most specimens were represented by 2.3 kb of COI+II sequence. Several specimens have not yet been completely sequenced and are represented by as little as 930 bp. Most notable is the sister group relationship between *L. cuprina* from Hawaii and *L. sericata* rather than with the *L. cuprina* from Australia. This was further explored in the analysis shown in Figure 5.

Figure 5. Maximum parsimony majority bootstrap consensus trees for a global survey of *Lucilia cuprina* and *L. sericata*. Separate phylogenies are shown for analyses based on 1542 bp of mitochondrial cytochrome oxidase subunit one (COI) and nuclear 28S ribosomal RNA (28S).

Figure 6. Consensus bootstrap tree (1000 reps.) based on maximum parsimony analysis of 439 bp insect COI sequence data. The unidentified larva also used for crop content human genotyping is paired with the adult *Cynomya cadaverina* reared from the same sample.

Figure 7. Method for dissection of a maggot crop for DNA analysis. In these photos the maggot head is to the left. Prior to these steps the exterior of the specimen was sterilized using a 20% bleach solution, and then the posterior end was cut off with the iris scissors. A. A ventral longitudinal cut is made to avoid breaking the dorsally located crop. B. The crop is teased away from the surrounding tissue. C. The crop is removed with forceps. During this procedure the instruments are frequently flamed.

Figure 8. A PCR yield gel stained with ethidium bromide. Maggots were reared on pig liver and killed by freezing. Deliberate contamination consisted of soaking a maggot for one hour in cow's blood. Wash treatments consisted of soaking a maggot overnight in H_2O, soaking overnight in 20% bleach, and treatment with Dnase following the manufacturer's protocol (Promega). Following these treatments each maggot was rinsed in 500 ul distilled H_2O, and any DNA present in that H_2O was concentrated using a Centricon column prior to PCR amplification. Cytochrome b primers that preferentially amply cow over pig DNA were used to detect DNA still present on the maggot exterior surface.

Figure 9. Electropherograms showing human HV2 sequence data from crop contents of a maggot fed on human liver (top) and directly from blood drawn from the liver donor (bottom).

Figure 10. Heteroduplex analysis of forensically important Sarcophagidae. Left to right: *Ravinia lherminieri, Sarcophaga argyrostoma, Sarcophaga crassipalpis, Sarcophaga africa, Sarcophaga cooleyi, Peckia chrysostoma, Blaesoxipha plinthopyga, Sarcophaga bullata Wohlfahrtia vigil, Sarcophaga peregrina, Sarcophaga ruficornis, Musca domestica* (the driver).