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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

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ABSTRACT: The identification of insects found on a dead body can lead to the estimation of the time of death (postmortem interval). We report an updated version of an established method based on sequence analysis of PCR products from a region of the cytochrome b oxidase subunit I mitochondrial gene of different members of the family Calliphoridae, by sequencing six European species: *Lucilia sericata* (Meigen), *Lucilia caesar* (Linné), *Lucilia illustris* (Meigen), *Calliphora vicina* (Robineau-Desvody), *Calliphora vomitoria* (Linné), *Protophormia terraenovae* (Robineau-Desvody) and one Guianese species: *Cochliomyia macellaria* (Fabricius). This technique provided clear results when applied to the larvae and we also report the identification of empty puparia.

KEYWORDS: forensic science, postmortem interval, Calliphoridae, blow flies, puparia, mitochondrial DNA, cytochrome oxydase gene, polymerase chain reaction, sequencing

A dead body is an attractive place for the colonization and development of several insects. During different stages of decomposition, specific species are found inhabiting remains (1,2). The most frequently encountered insects are blow flies. By the analysis of the species found near a murder scene, and the estimation of the ambient temperature, investigators are able to estimate the date of the death: the postmortem interval. The classical way to identify the insect species present is by morphological observation. But differences are sometimes minute, especially for the young larval stages (3). Sometimes, delays between the collection of the larvae and examination in the forensic laboratory impair the preservation of the samples. Often, immature specimens must be reared to adulthood in the laboratory in order to facilitate accurate species identification. In other cases, when the remains are old or badly decomposed, only empty puparia or puparial remnants are found. In these cases, species identification can be impossible and the estimation of the postmortem interval is less accurate.

In an attempt to identify larvae, more accurately, different methods have been developed including: studying of the alloenzymes (4), or mapping mitochondrial DNA by restriction enzyme digestions (5). These methods can be used for forensic purposes but have limitations because of the stability of the proteins and the amount of DNA required to do a restriction analysis on the mitochondrial DNA (mt DNA).

However, the mitochondrial genome is a valuable marker for the identification of different fly species (5–8). The use of PCR and sequencing techniques, applied to a region of insect mitochondrial DNA, allows the determination of species, even in relatively advanced states of degradation. Mitochondrial DNA is a small circular molecule, present in hundreds of copies surrounded by a lipidic envelope. Moreover, mt DNA mutation rates are high enough to provide differences in sequences between closely related species (9). This method has already been described for animal identification (10). A similar method has been developed for the identification of three members of the family Calliphoridae (8). The latter study was conducted on North American species of blow flies. We report the adaptation of this system to European species.

We sequenced a region of 160 to 270 bp of the cytochrome b oxidase subunit I mitochondrial gene (*COI*) from the larvae and adults of six European (*Lucilia sericata* (Meigen), *Lucilia caesar* (Linné), *Lucilia illustris* (Meigen), *Calliphora vicina* (R.-D.), *Calliphora vomitoria* (Linné), *Protophormia terraenovae* (R.-D.)) and one Guianese (*Cochliomyia macellaria* (Fabricius)) species of the family Calliphoridae. We were also able to establish the sequence for this region from empty puparia.

Materials and Methods

Samples

Adult blow flies, maggots, and puparia were collected in the vicinity of Rosny sous Bois (France) and from laboratory colonies. These blow flies were identified by Bernard Chauvet, Jean Bernard Myskoviak, and Christophe Rocheteau from the Department of Entomology (IRCGN), according to classical morphological criteria (11). *Cochliomyia macellaria* (Fabricius) collected from a Guianese case, were identified by Thierry Pasquerault from the Department of Entomology (IRCGN).

The insects were stored either at −80°C or in alcohol prior to DNA extraction. Entire insects were used in the DNA extraction process. Empty puparia were stored at room temperature, in alcohol, or dried.

DNA Extraction

Total genomic DNA was extracted according to a modification of the method reported by Harrison et al. (12) and Sperling et al.

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(8). Briefly, each insect was ground into powder using a sterile teflon pestle in liquid nitrogen in a 2 mL Eppendorf tube. The powder was digested at least 2 h under agitation at 56°C in 800 μL of filtered Lifton buffer (0.1 M Tris HCl pH 8, 0.2 M sucrose, 0.05 M EDTA, and 0.5% SDS) with 100 μg Proteinase K from Merck. After phenol/chloroform extraction, the recovered aqueous phase was precipitated with two volumes of cold ethanol, 100 mM NaCl during 2 h at −20°C. After washing with 70% ethanol, the pellet was resuspended in T10E1 (10 mM Tris HCl pH 8 1 mM EDTA).

For empty puparia, the procedure was the same, except that the digestion was done in a volume of 500 μL for 12 h, then 100 μg of proteinase K was added for a 2-h incubation. The pellet was resuspended in 50 μL of T10E1.

DNA was quantified by measuring OD at 260 nm. After DNA extraction, a small aliquot was loaded on a 0.8% agarose gel and visualized by ethidium bromide staining after electrophoresis (13).

Amplification

DNA amplification (14) was performed on a Perkin Elmer Cetus GeneAmp 9600 apparatus, using primers previously described (8). Briefly, 100 ng of genomic DNA was used to provide enough material for several sequences. PCR was carried out in the presence of 1 mM primers CO-I 2f (5′-CAG CTA CTT TAT GAG CTT TAG G-3′) and CO-I 3r (5′-CAT TTC AAG C/TTG TGT AAG CAT C-3′), 0.2 mM dNTPs from Pharmacia, and 0.5 U of AmpliTaq (Perkin Elmer Cetus) in a final volume of 50 μL. Amplification cycles were: 94°C for 30 s, 45°C for 1 min, and 72°C for 45 s. These cycles were repeated 35 times and followed by a final 10-min extension at 72°C.

Purification of Amplified DNA

The PCR product was loaded on a 1.5% agarose gel (Pharmacia). After electrophoresis, the band of 348 bp was quickly cut on a UV table with a scalpel and purified on a GenElute™ Agarose Spin column (Supelco). DNA was precipitated with ethanol and the pellet was resuspended in 20 to 30 μL of sterile water, depending on the intensity of the band. Two microliters were loaded on a 1.5% ethidium bromide agarose gel to estimate the amount of purified DNA.

DNA Sequencing

Purified PCR products were sequenced by PCR using the Silver Sequence™ DNA Sequencing System from Promega according to the recommendations of the supplier. The sequences were loaded on an 8% acrylamide denaturing gel. After electrophoresis, the gel was stained with silver nitrate. Both strands of DNA were sequenced using the primers CO-I 2f and CO-I 3r.

Sequence Analysis

The new double strand sequences were submitted to Genbank under the following access numbers: *Lucilia sericata* (Meigen); AF017425, *Lucilia caesar* (Linné); AF017424, *Lucilia illustris* (Meigen); AF022369, *Calliphora vicina* (R.-D.); AF017422, *Calliphora vomitoria* (Linné); AF017423, *Protophormia terraenovae* (R.-D.); AF017426 and *Cochliomyia macellaria* (Fabricius); AF022370.

Restriction Analysis

Fifteen microliters of the amplified products were digested in a total volume of 30 μL with 10 U of Hinf I (Boehringer Manheim). The DNA was electrophoresed on a 2% agarose gel stained with ethidium bromide. The bands were visualized on a UV table.

Results

We sequenced 160 to 270 bases on both strands of the mitochondrial cytochrome b subunit I gene from seven different species of the family Calliphoridae. To determine the standard sequence for each species, we analyzed one sample and compared it with other individuals from the same species. Figure 1 shows the comparison between different European species of the family Calliphoridae. As previously described for other members of this family (8), the sequences were different from one to another (Table 1) and diverged to an extent of 2.9 to 13.1%. The closest species were *Lucilia caesar* (Linné) and *Lucilia illustris* (Meigen) (2.9% of divergence) and *Protophormia terraenovae* (R.-D.) was the most divergent species within this family considering the standard of 137 nucleotides sequenced (from 9.5 to 13.1% of divergence).

The differences between these sequences were only substitutions. We did not observe any deletions within the sequences of the different species, as previously described for other members of the same family (8). We performed the same analysis on a flesh fly of the family Sarcophagidae and observed a deletion of 9 bp compared to the family Calliphoridae (data not shown).

For each species, we sequenced the same gene from at least two individuals. We recorded only one case with a difference among four *Lucilia sericata* (Meigen) individuals, namely a point mutation in the sequence of 164 bp (see Fig. 2). To determine the possible influence of geographic distribution, we compared our se-

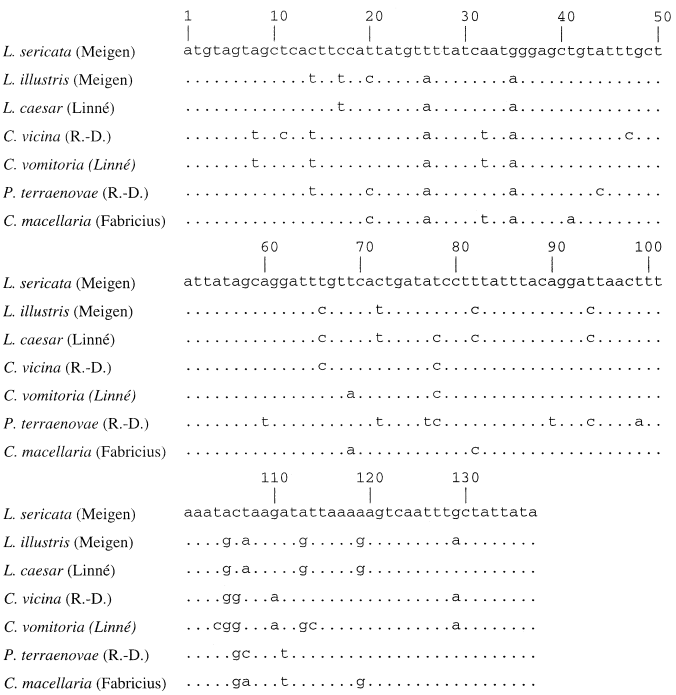

FIG. 1—*Sequence comparison, across mitochondrial COI gene, between* Lucilia sericata *(Meigen),* Lucilia illustris *(Meigen),* Lucilia caesar *(Linné),* Calliphora vicina *(R.-D.),* Calliphora vomitoria *(Linné),* Protophormia terraenovae *(R.-D.), and* Cochliomyia macellaria *(Fabricius). Dots indicate identity to sequence of* L. sericata *(Meigen). The nucleotides 1 and 137 of the sequence of* L. sericata *correspond to the nucleotides 1115 and 1251 of the sequence of* Phaenicia sericata *published by Sperling et al. (8) (Genbank accession number: L14947).*

TABLE 1—*Divergence and substitutions across the mt DNA sequences of the COI gene from different species of the Calliphoridae family. Above the diagonal: percentage of DNA divergence (number of different nucleotides/size of the sequence compared) and below the diagnonal: number of substitutions across the 137 nucleotides of Fig. 1.*

	L. sericata (Meigen)	*L. illustris* (Meigen)	*L. illustris* (Meigen)	*C. vicina* (R.-D.)	*C. vomitoria* (Linné)	*P. Terraenovae* (R.-D.)	*C. macellaria* (Fabricius)
L. sericata (Meigen)	/	10.2%	8.6%	9.5%	10.2%	10.9%	8.0%
L. illustris (Meigen)	14	/	2.9%	9.5%	10.9%	10.9%	9.5%
L. caesar (Linné)	12	4	/	9.5%	10.2%	9.5%	9.5%
C. vicina (R.-D.)	13	13	15	/	5.1%	13.1%	11.7%
C. vomitoria (Linné)	14	15	16	7	/	13.1%	10.9%
P. terraenovae (R.-D.)	15	15	16	18	19	/	10.9%
C. macellaria (Fabricius)	11	13	13	16	15	15	/

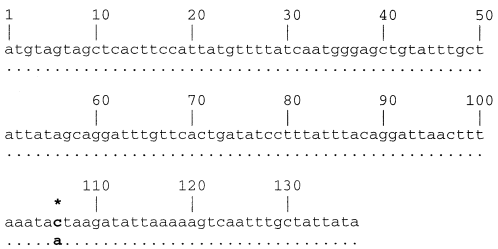

FIG. 2—*Divergences within the mt DNA sequence of the COI gene from* Lucilia sericata *(Meigen). The asterisk (∗) indicates the only divergence (substitution) observed among four different individuals.*

TABLE 2—*Different material tested for DNA extraction and COI mt DNA sequencing.*

Imago	Dead, found on the ground
Maggot	Conserved in alcohol
Pupae	Conserved in alcohol
Empty puparia	Conserved in alcohol
Empty puparia	Dry at room temperature

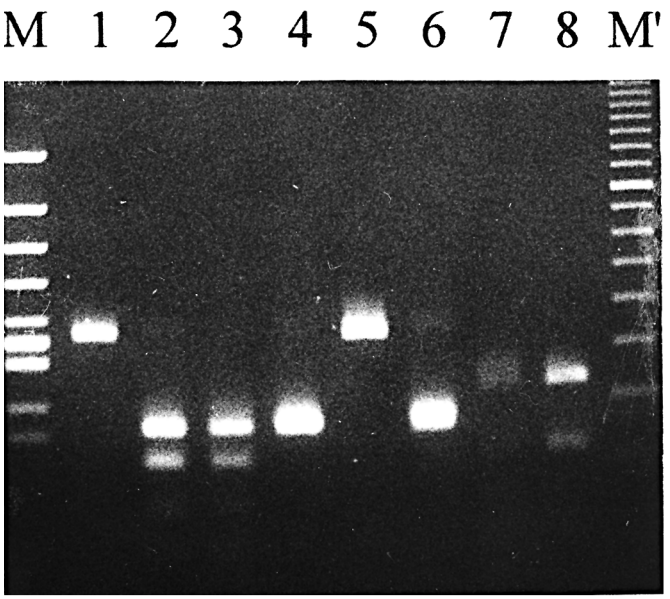

FIG. 3—*Restriction patterns obtained by digestion of the PCR product with Hinf I. M—molecular weight marker (band sizes: 1057, 770, 612, 495, 392, 345/341/335, 297, 210, 162), (1)* L. sericata *(Meigen), (2)* L. caesar *(Linné), (3)* L. illustris *(Meigen), (4)* C. vicina R.-D., *(5)* C. vomitoria *(Linné), (6)* P. terraenovae (R.-D.), *(7 and 8)* C. macellaria *(Fabricius), M′—molecular weight marker (100 bp ladder, Pharmacia).*

quence from *Lucilia sericata* (Meigen) with the corresponding sequence from *Phaenicia sericata* (8). These two genera are considered to be closely related in North America (15) and to be one in the same in Europe (16). The sequences did not show any differences, even between blow flies from different continents. We also compared the sequence of European *Lucilia illustris* to the sequence of North American *Phaenicia sericata* without finding any difference in the 201 bp sequenced. These two arguments are in favor of a low degree of intraspecific variation. We are currently confirming this point, by sequencing species from different geographic places in France.

Sequences could be established for the COI locus of blow flies at different stages of development or stored in different conditions (Table 2). Even the empty puparia provided enough DNA for several PCR reactions. Extraction from the alcohol-stored samples always produced lower amounts of DNA. Concerning the empty puparia, we noticed that dry samples generated better results (data not shown, see discussion).

Sperling et al. (8) proposed an easy way to distinguish between blow flies. They showed that restriction enzymatic digestion of the 348 bp PCR product with Hinf I led to three distinct fragment patterns for *Phaenicia sericata* (*Lucilia sericata* (Meigen)), *Lucilia illustris* (Meigen), and *Phormia regina* (Meigen). We tested this restriction polymorphism on the seven blow flies under study (see Fig. 3). We found the different expected fragment sizes for *Lucilia illustris* (Meigen) and *Lucilia sericata* (Meigen). However, Hinf I generated the same pattern of restriction for *Lucilia illustris* (Meigen) and *Lucilia caesar* (Linné), for *Calliphora vicina* R.-D. and *Protophormia terraenovae* (R.-D.), and for *Lucilia sericata* (Meigen) and *Calliphora vomitoria* (Linné). Therefore, this enzyme is not useful in distinguishing between these different members of the family Calliphoridae, even within the genus Lucilia.

Discussion

Species Identifications

Six European and one Guianese species of the family Calliphoridae were analyzed in this study. As previously described for three North American species (8), the sequence variability of a short region of the mitochondrial cytochrome oxidase b subunit I

gene is enough to provide larval identification. We analyzed the sequence of this short region for seven different calliphorid flies. Other interesting blow flies are currently being analyzed at this locus. We observed only one case of nucleotide substitution within *Lucilia sericata* (Meigen). The standard sequence of the cytochrome oxidase b subunit I gene of this species appears to be identical to the sequence of *Phaenicia sericata* (8). These blow flies were found to be genetically identical even though one was collected in France and the other in North America (15,16). Since *Lucilia caesar* (Linné) and *Lucilia illustris* (Meigen) seem to be very closely related species on a molecular level, more extensive analysis of the variation in these two species needs to be carried out to verify the hypothesis that a short sequence of the subunit I cytochrome oxidase b gene is enough to distinguish between these two blow flies species.

Our research demonstrates that restriction analysis of the 348 bp PCR product with the Hinf I enzyme is not sufficient to distinguish between calliphorid flies, even those within the genus Lucilia. We are currently analyzing the sequences to search for other restriction polymorphism, and we may have to sequence larger fragments of the subunit I cytochrome oxidase b gene.

Identification of Poorly Preserved Larvae and Empty Puparia

The aim of this work was first to distinguish, on a molecular level, members of the genus Lucilia. Typically, it is very difficult to identify immatures and puparia from this genus and entomologists must rear larvae to adulthood to facilitate accurate species identification. Mortality in rearing can further complicate this means of identification. Genetic identification methods, adapted to European species of blow flies can help European investigators since PCR technology facilitates accurate species identifications even when specimens are scarce or degraded. We clearly demonstrate that it is possible to obtain a DNA sequence from both poorly preserved blow flies and from empty puparia.

Interest of the Molecular Identification

Observation of morphology is very often the easiest and the fastest way to identify insects. Molecular techniques do not aim to supplant morphological methods, but can help the entomologists in cases where the classical approach does not give reliable results.

We employed molecular identification techniques in a murder case near a big city in France. A person had been apprehended while throwing foul-smelling bags into a bin. In these bags, police found half of a dismembered body. In the apartment of the suspect, investigators found numerous dead pupae and empty puparia. Among these insect remnants, most were easily identified; others, however, were identified only as members of the genus Lucilia. We identified the empty puparia as *Lucilia sericata* (Meigen) and this facilitated a more accurate estimation of the postmortem interval.

We describe here the genetic identification of species from the genus Lucilia. Also, we report the successful sequencing of additionnal species including *Calliphora vicina* (R.-D.), *Calliphora vomitoria* (Linné), *Protophormia terraenovae* (R.-D.), and *Cochliomyia macellaria* (Fabricius). These species are easily identified using the classical morphological approach. Since we were able to identify blow flies from empty puparia, it may be possible to identify such insects from legs or wings. This is currently being tested in the lab and may help entomologists in cases where no intact larvae or puparia are found. This technique may also be extended to other insects of forensic interest, such as beetles.

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