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Cytochrome b as a useful tool for the identification of blowflies of forensic interest (Diptera, Calliphoridae)

Maite GilArriortua [a,b], Marta I. Salona Bordas [a], Laura M. Cainé [c,d], Fátima Pinheiro [c], Marian M. de Pancorbo [b,*]

[a] Dpto. de Zoología y Biología Celular Animal, Facultad de Ciencia y Tecnología, Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Barrio Sarriena, 48940 Bilbao, Spain

[b] BIOMICs Research Group, Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Centro de Investigación Lascaray Ikergunea, Avda. Miguel de Unamuno 3, 01006 Vitoria-Gasteiz, Spain

[c] Delegação do Norte do Instituto Nacional de Medicina Legal, Jardim Carrilho Videira, 4050-167 Porto, Portugal

[d] Faculdade de Medicina da Universidade do Porto, Alameda Prof. Hernâni Monteiro, 4200-316 Porto, Portugal

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ABSTRACT

In Forensic Entomology the fast and accurate identification of insects collected at the scene of events is essential if errors are to be avoided in estimating infestation times of interest and determining the post-mortem interval (PMI). Traditional identification based on morphological characteristics can be complicated due to physical similarities between different species, especially at immature stages. Genetic analysis provides a fast and reliable identification method. In this paper, molecular analysis is focused on a fragment of 307 bp of the mitochondrial DNA region that codes for cytochrome b (cyt b). Six species belonging three genera of Calliphoridae of forensic interest (*Calliphora vicina*, *Calliphora vomitoria*, *Lucilia sericata*, *Lucilia caesar*, *Lucilia ampullacea*, *Chrysomya albiceps*) were collected and identified. These are the most common species of cadaveric entomofauna on the Atlantic seaboard of the Iberian Peninsula. The results show the robustness of the cyt b locus as a diagnostic tool, since its nucleotide variability enables reliable distinctions to be drawn between species. This study also contributes new consense sequences which may be of interest for correct species identification.

1. Introduction

Forensic Entomology studies the structure and dynamics of the populations of insects and other communities of arthropods to assist in legal problems of a judicial nature (abuse, mistreatment in dependent persons, murders, etc.) and in financial matters, associated with infestations and plagues affecting stored byproducts, furniture and buildings [1,2]. The most significant application of this area of forensic science is in criminal investigations, where it is used to estimate the post-mortem interval (PMI) [3,4]. In that sense, the critical prerequisite for the accurate practice of Forensic Entomology is the proper identification of the collected specimens. Moreover, we have to consider that blowflies can provide information not only about the circumstances of events but also on the seasonal and environmental conditions [5,6], since specific composition vary according to the biogeographical characteristics of each habitat [7,8].

Conventional entomological identification is based on external morphological features, based on a comparative technique that requires the selection and preservation of reference specimens and complex taxonomic keys. This task can sometimes be highly difficult, time-consuming and may even prove impossible due to the loss of morphological features in damaged specimens or to physical similarities that make difficult to identify taxonomic features. In particular, immature specimens may need to be raised until the emergence of the adult before they can be correctly identified which takes time and entails no guarantees unless the specimens are alive when they reach the specialist [9,10].

When morphological methods are uncertain and judicial errors may result, species have to be identified by alternative procedures, among which molecular techniques play a significant role [11]. Mitochondrial DNA (mtDNA) is most widely used, since it provides the largest number of copies [12] and has a higher mutation rate than nuclear DNA. These features facilitate the analysis of specimens in poor conditions, increase the possibility of generating species-specific markers, provide information for taxonomic and phylogenetic investigations [13] and even permit the rapid

* Corresponding author at: University of the Basque Country (UPV/EHU), "Lascaray" Research Center, Avda. Miguel de Unamuno, 3, 01006 Vitoria-Gasteiz, Spain. Tel.: +34 945 01 4528; fax: +34 945 01 4458.
E-mail addresses: marianpancorbo@gmail.com, marian.mdepancorbo@ehu.es (M.M. de Pancorbo).

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generation of different sequences between subspecies [14]. Most of the studies that deal with species identification have used mtDNA markers such as cytochrome oxidase subunits I & II (*COI* & *COII*) or 16S rRNA [15]. Currently, the *COI* gene is the standard locus for invertebrate identification; however, recently, this genetic marker has shown limitations in its ability to identify closely related species [16]. Forensic laboratories perform usually species identification based on the analysis of cytochrome b (cyt b), although to date it has been used almost exclusively to discriminate vertebrate species [17,18].

This work proposes the analysis of a specific region of cyt b gene (307 bp), widely used and established in the forensic genetic field to identify vertebrates [17,18], as an alternative or complement when the traditional genes show difficulties to discriminate closely related species. This paper seeks to show the capabilities and robustness of the mitochondrial cyt b gene for fast, accurate species-specific identification for Diptera of forensic importance. An added value is the characterisation for the first time of this specific region (307 bp) of cyt b genetic marker for five of the six blowfly species reported.

2. Materials and methods

2.1. Samples

One hundred and eighty five maggots were collected from 38 autopsies on human cadavers at the Northern Delegation of the National Institute of Legal Medicine in Porto (Portugal). They were identified morphologically using taxonomic keys [19–21], preserved in ethanol and kept at −80 °C up to the time of DNA extraction [22].

2.2. DNA extraction

The specimens were washed with a 20% bleach solution to eliminate external contaminants [23]. DNA was extracted using either the DNeasy Tissue Kit (QIAGEN, Valencia, CA, Spain) or the BioRobot EZ1 workstation using an EZ1 ADN Forensic Card (QIAGEN, Valencia, CA, Spain) [22].

2.3. PCR amplification

A 307 bp region of the mitochondrial cyt b gene was amplified via PCR in an iCycler thermocycler (BioRad, Madrid, CA, Spain) using the L14816 and H15173 primer pair for PCR [17], using approximately 10 ng of DNA in a final volume of 25 μl: 0.2 mM dNTP (Bioline, Berlin, Germany), 2.5 mM $MgCl_2$ (Bioline, Berlin, Germany), 0.2 μM of each primer, 1.6×10^{-4} mg/μl BSA (Bioline, Berlin, Germany), 2 units of Taq polymerase (Biotaq, Bioline, Berlin, Germany) and 1× Buffer (pH: 8.8, Bioline, Berlin, Germany), under the following PCR conditions: 95 °C for 11 min, 35 cycles 94 °C → 30 s, 50 °C → 45 s and 72 °C → 45 s, followed by holding at 4 °C.

2.4. Sequencing analysis

PCR products were purified using a Millipore Montage TM SEQ 96 sequencing reaction clean-up kit (Millipore, Madrid, CA, Spain) and sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Unincorporated terminators were cleaned with a BigDye X-TERMINATOR purification kit (Applied Biosystems, Foster City, CA, USA). Electrophoretic separation and sequencing reaction product detection were handled by an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

2.5. Statistical analysis

The forward and reverse sequences obtained for each specimen were edited with the aid of the software ChromasPro V. 1.41 (Technelysium Pty Ltd.) and ClustalX 1.83 [24], the latter being used for alignment together with BLAST [25]. Consensus sequences were obtained with Sea View 4.0 [26], which was also used for interspecific analysis and phylogenetic tree viewing, while intraspecific variation was analysed with Arlequin V. 2000 [27].

Additionally, a 310 bp region within *COI*, which is a standard locus employed for insects identification, was used to compare the discrimination capability of both regions. We used 187 sequences recorded in the GenBank database. The analysed individuals belonged to the six species of Calliphoridae referred in this paper (*C. vicina* (35), *C. vomitoria* (15), *L. sericata* (58), *L. caesar* (46), *L. ampullacea* (14) and *Ch. albiceps* (15)).

3. Results and discussion

This study characterised a 307 bp region of the cyt b gene satisfactorily in 185 individuals, collected from human cadavers. In total, 6 different species of forensic interest, belonging to the subfamilies Calliphorinae, Luciliinae and Chrysomyinae, were collected and identified morphologically: *Calliphora vicina* Robineau-Desvoidy, 1830 (61 specimens), *Calliphora vomitoria* Linnaeus, 1758 (41), *Chrysomya albiceps* (Wiedemann, 1819) (65), *Lucilia sericata* (Meigen, 1826) (12), *Lucilia caesar* (Linnaeus, 1758) (1), *Lucilia ampullacea* Villeneuve, 1922 (5). All the species belong to the family Calliphoridae, which are pioneers in cadaver colonisation and decomposition processes since they are primary colonisers of great forensic interest [28,29].

The species of Calliphoridae collected reflect the diversity of this family, as described in previous studies conducted in the Iberian Peninsula [30–32]. The only exception is the presence of *L. ampullacea*, which was not reported in previous studies developed in the Upper Aragon [33] or Madrid regions [34]. Given its geographical distribution, *L. ampullacea* seems to prefer temperate weather with a maritime influence, where temperatures are kept milder through the heat-regulating effect of the sea, being its southern limit associated to the Atlantic range [35,36].

All sequences were deposited in the GenBank under the following accession numbers: JF705985–JF706169.

3.1. Analysis of intraspecific variability

Intraspecific analysis was conducted on the basis of the information obtained from the cyt b sequences of the specimens analysed. After removed both primers, forward and reverse, the remaining DNA sequences of 307 bp length were used in all further studies. As expected, the analysis of this region of mtDNA was observed to have a strong AT bias (73%) (Table 1), which is characteristic of insect mitochondrial DNA, including Diptera (mean A = 30%, T = 43%, C = 12%, G = 15%) [37,38].

The cyt b sequences of *C. vicina* (*N* = 61) contained the largest number of polymorphic bases (7) which determine eleven different haplotypes with an average locus diversity of

Table 1
Intraspecific characteristics of the cyt b sequences analysed.

	No.	Nucleotide composition (%)				No. of haplotype	Gene diversity	Nucleotide diversity	Mean no. of pairwise differences	No. of polymorphic sites
		C	T	A	G					
Ch. albiceps	65	12.70	42.68	29.96	14.66	2	0.06 ± 0.04	0.000197 ± 0.000479	0.060577 ± 0.132753	1
C. vicina	61	11.41	42.66	30.60	15.33	11	0.54 ± 0.08	0.002860 ± 0.002256	0.878142 ± 0.624539	7
C. vomitoria	41	11.97	42.10	30.95	14.98	4	0.47 ± 0.07	0.001426 ± 0.001450	0.437805 ± 0.400643	2
L. sericata	12	10.42	44.63	30.59	14.36	2	0.17 ± 0.13	0.000543 ± 0.000881	0.166667 ± 0.240121	1
L. caesar	1	11.07	43.97	30.29	14.66	1	NCE[a]	NCE[a]	NCE[a]	NCE[a]
L. ampullacea	5	11.40	44.63	29.38	14.59	2	NCE[a]	NCE[a]	NCE[a]	NCE[a]

[a] NCE no statistical estimates are drawn up.