

Forensic entomology: molecular identification of blowfly species (Diptera: Calliphoridae) in Portugal



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Introduction

Forensic entomology applies knowledge about insects and other arthropods to forensic and legal issues. Necrophagous insects help to answer questions relating to estimation of post-mortem interval (PMI), post-mortem transfer, neglect or abuse of living people and presence of drugs or poisons [1]. Blowflies have the greatest role in the early decomposition process, being Calliphoridae adults usually one of the earliest sarcosaphagous insects to infest a corpse [2]. Forensic entomology demands a fast, accurate and precise identification of the insects collected.

The first step is accurate **morphological identification**, traditionally done by morphological characters. However, morphological identification can be complicated due to the similarities among species, even sometimes in adult specimens, what makes identification very difficult or even impossible [3].

Molecular identification provides a rapid, precise and reliably method that can be performed at all developmental stages [4,5].

The potential of the gene for subunit I of the mitochondrial encoded cytochrome oxidase (**COI**) has been shown and very well established for species determination in previous studies [3,6]. However, in some cases this gene is not effective enough to differentiate two close species [7,8].

The **goal** of this study was to use the ribosomal internal transcribed spacer 2 (**ITS2**) to complement COI data and also demonstrate the ITS2 effectiveness for identification purpose of forensically important Calliphoridae.

Material and Methods

Specimens collection:

- Adult specimens were collected from 3 sites (Serra da Estrela, Oeiras and Monsanto) in Portugal;
- Mammalian corpse-baited traps, e.g. exposed dead piglet and genets;

Morphological identification:

- Specimens identified using morphological keys to adult Calliphoridae [9,10]:
 - 11 *Calliphora vicina*;
 - 8 *C. vomitoria*;
 - 10 *Lucilia caesar*;
 - 1 *L. sericata*.

DNA was extracted from two legs using E.Z.N.A.® Insect DNA Isolation kit (Omega Bio-Tek), following manufacturer's protocol, with an overnight incubation step.

Three PCRs were done:

- COI** - 658 bp amplicon using the primer pair LCO1490 and HCO2198 using authors previously described PCR methodology [11];
 - COI** - 304 bp product amplified using primers pair previously described [12];
 - ITS2** - amplification using primers and methodology previously described [13].
- Amplification products were analyzed by **electrophoresis** using 2% agarose gels and purified with Sure Clean (Bioline), according to manufacturer's specifications.
 - Sequenced** by Macrogen Inc.
 - PCR products from 304 pb COI amplicon were **digested with endonuclease Dde I** and restriction patterns were analyzed using 2% agarose gel electrophoresis.

DNA Sequences:

- Analysis** was done with **Sequencher®** v4.0.5 (Genes Codes Corp.) and **BioEdit®** Sequence Alignment Editor v7.0.5.3 software;
- Alignment** was performed using **ClustalX** v2.0.12 to find interspecific differences;
- Match and identification by BLAST.

Results and Discussion

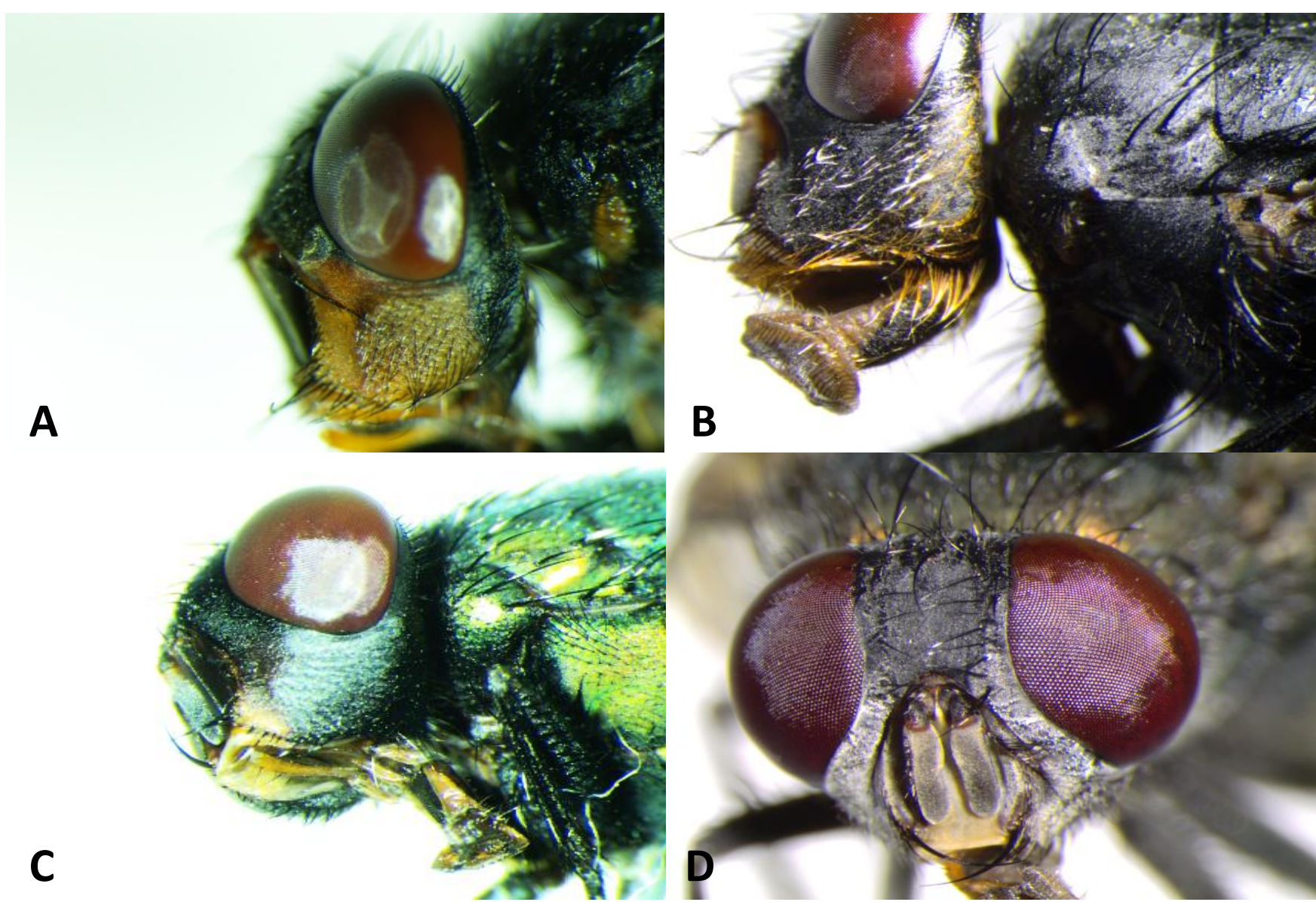


Figure 1 – Specimens of the 4 Calliphoridae species collected and used for molecular analysis. A- *Calliphora vicina*; B- *C. vomitoria*; C- *Lucilia sericata* and D- *L. caesar*.

A 658 pb amplicon of mitochondrial COI gene was successfully sequenced from all 30 specimens, allowing correct identification using BLAST search for only 20 blowflies of the family Calliphoridae: 11 specimens of *Calliphora vicina*, 8 *C. vomitoria* and 1 *Lucilia sericata* (Figure 1).

For the remaining 10 individuals, previously morphologically identified as *L. caesar*, this COI region was not sufficient to distinguish it from *L. illustris*, a very morphologically similar species and extremely difficult to differentiate even at adult stage [10].

In BLAST search *L. illustris* was always one of the first two hits together with *L. caesar* (figure 2) and the two species even had the same maximum scoring segment pair (MSP) of 99%. Only one individual obtained MSP of 100%.

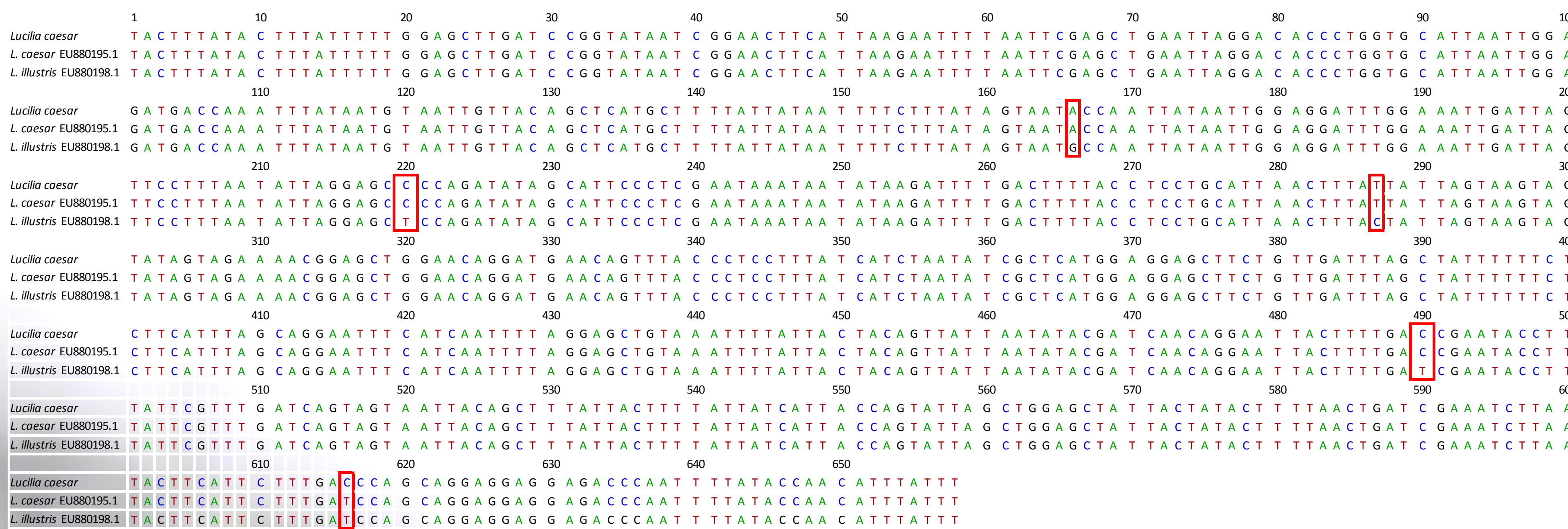


Figure 2 – Difference in bases between the consensus COI sequences obtained from *L. caesar* specimen and the first two hits in BLAST search.

To complement COI data and to prove ITS2 effectiveness, ITS2 sequences were compared to GenBank database and 100% MSP was obtained. However, the second hit still remained to be *L. illustris* with 99% MSP.

Just to ensure ITS2 results, a second COI region was amplified in order to digest PCR amplification products with *Dde I* (figure 3). Results clearly show that these specimens belongs to *L. caesar* species, as expected.

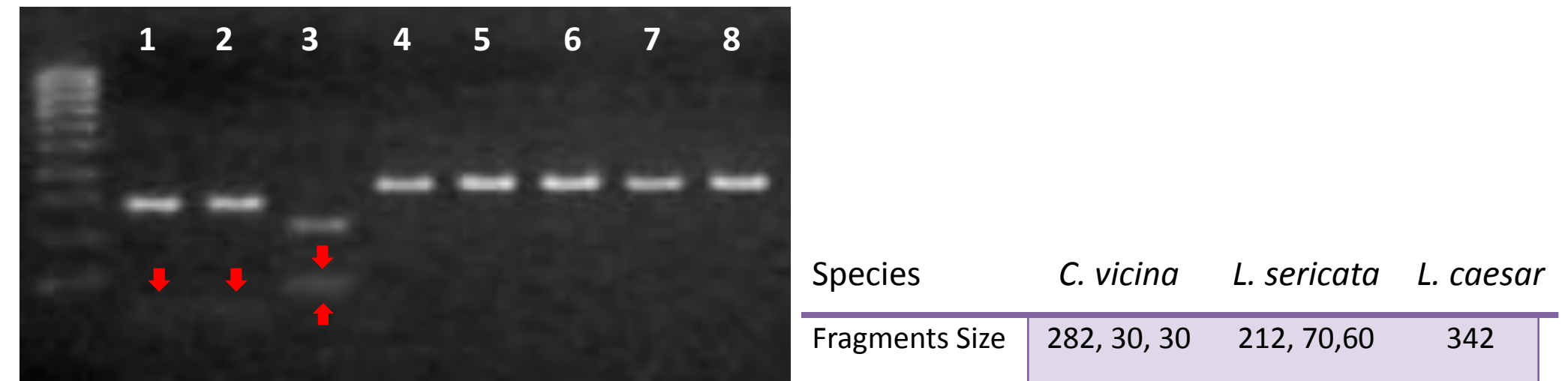


Figure 3 – Restriction patterns obtained with *Dde I* and fragments size of *C. vicina* (1,2), *L. sericata* (3) and *L. caesar* (4-8).

Conclusions

Only *L. caesar* specimens were not suitably identified using the chosen COI sequence. ITS2 is suitable to correctly identify this species, although DNA sequences are so similar with *L. illustris* that this one obtained very high MSP too. We must take into account two aspects when using BLAST search: a) there is an uncertainty in the morphological identification previously made of included specimens in database, leaving it unclear whether species/sequence pair is correct and b) these results indicate that it would be of great importance to increase the sequences collection to prevent incorrect identification and reinforce results validity.

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