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Why is the molecular identification of the forensically important blowfly species *Lucilia caesar* and *L. illustris* (family Calliphoridae) so problematic?

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ABSTRACT

Species of the fly genus *Lucilia* are commonly used in forensic investigations to estimate the postmortem interval (PMI). Two close-related species *Lucilia caesar* and *L. illustris* are difficult to identify. Previous studies showed that the mitochondrial cytochrome *c* oxidase subunit I (COI) marker could be used to identify many *Lucilia* species. However, mixed results were obtained for *L. caesar* and *L. illustris* due to some European specimens showing identical haplotypes. Here, we investigated 58 new European male specimens of *L. illustris* and *L. caesar* whose morphological identifications were checked and for which COI fragments were sequenced. In addition, two other mitochondrial (cytochrome *c* oxidase subunit II and 16S) and two nuclear (internal transcribed spacer 2 and 28S ribosomal RNA) markers were obtained for a subset of these samples. For each marker, genetic divergence within each species was in the same range as between species, confirming the close relationship between both species. Moreover, for each of the gene fragments, both species shared at least one haplotype/genotype. Hence, none of the molecular markers tested could be used, alone or in combination, to discriminate between *L. illustris* and *L. caesar*.

1. Introduction

For a forensic entomologist, identifying a necrophagous insect specimen is an important early step in an investigation because the immature stages can be used to estimate the postmortem interval (PMI) [1,2]. Blowflies (family Calliphoridae) are often found on dead bodies shortly after death. These species differ in their developmental times and have therefore a high potential for the accurate estimation of the PMI. Yet, a reliable PMI estimate relies on a correct identification of each of the species that is found on a corpse.

The blowfly genus *Lucilia* Robineau-Desvoidy (commonly known as greenbottle flies or greenbottles) includes many common species of forensic [3], veterinary [4,5] and medicinal importance [6]. Unfortunately, several species are difficult to identify using morphological characters, especially larvae and puparia that have to be collected for the estimation of the PMI [7,8]. Genetic analyses of *Lucilia* are mostly based on the mitochondrial cytochrome *c* oxidase subunit I (COI) marker, and are able to discriminate among most species [9–12]. However, in some closely related species, identification is less straightforward.

This is the case in the morphologically highly similar and recently diverged species *Lucilia caesar* (Linné, 1758) and *L. illustris* (Meigen, 1826) [13,14] for which a few diagnostic morphological characters have been proposed, i.e. the number of setae on the arista, the colour of the legs, the shape of the tergit VI for females and the shape of the hypopygium for males [3,14,15]. However, only the latter character seemed reliable to differentiate between both species [14] and dried female specimens and immature stages cannot be accurately identified by morphology. Although several studies found substantial differentiation between *L. caesar* and *L. illustris* using diverse fragments of the COI coding gene (Germany: 1.17% in a 511 bp fragment [13], France: 2%, 304 bp [16], France: 2.9%, 137 bp [17], China: 2.6%, 272 bp [18]), other studies including specimens from a larger geographic area and analysing COI fragments from 200 to 1573 bp (complete COI) could not differentiate between both species and found an overlap between intra- and interspecific distances [19–22]. Some studies even found haplotypes that were shared by both species [20,21] or that were only slightly divergent (0.2–0.3%) [19,22]. These low interspecific distances consistently comprised specimens from Europe (two *L. illustris*, AJ551445 from the UK, EU418574 from France; two *L. caesar*, AJ417703 and DQ453488 from the UK [19,20,22] and three haplotypes from Germany [21]). However, it is hardly possible to synthetize the results of these previous studies

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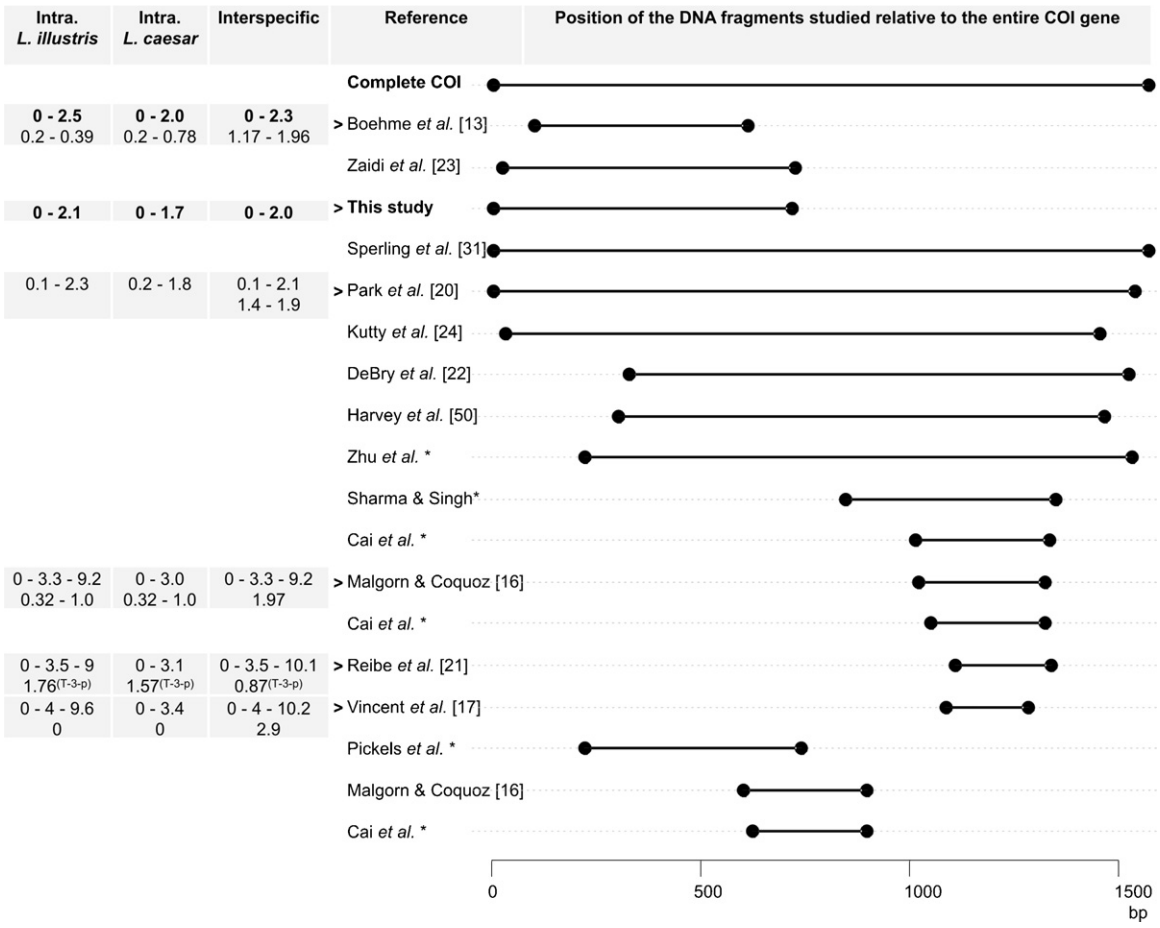

Fig. 1. Overview of studies reporting intra- and interspecific distance at the mitochondrial cytochrome *c* oxidase subunit I (COI) gene to identify *L. illustris* and/or *L. caesar*. Intra- and interspecific distances are given for DNA fragments used to differentiate between *L. caesar* and *L. illustris*. *Top row*: range of *p*-distances calculated for all available homologous sequences currently available from GenBank. For fragments of the 3′ end of COI, three values are given: 1st: minimum, 2nd: maximum without and 3rd: with GenBank sequences of Sharma et al. (unpublished). *Bottom row*: range (or single value whenever only one sequence was available) as cited in the publication. Substitution model is *p*-distance except when (T-3-p) specifies the Tamura-3-parameter model. Values in bold refer to comparisons including sequences from this study. Citations with an asterisk refer to unpublished sequences from GenBank.

since they generally included a few specimens of each species from geographically restricted areas, and used different COI fragments (see Fig. 1) for which authors applied various models of nucleotide substitution to calculate sequence divergence [23,24]. Therefore, increasing the number of homologous COI sequences from well identified European samples from a large area, and a re-evaluation of the existing data would allow a proper evaluation of the applicability of the COI gene to differentiate between *L. illustris* and *L. caesar*.

Besides the COI marker, three other genes have been used in other studies to evaluate the molecular differentiation between *L. illustris* and *L. caesar*. The nuclear internal transcribed spacer 2 (ITS-2) [25,26] and the 28S ribosomal RNA (28S) [27,28] showed sequence divergence lower than 1%. Yet, using a 250 bp fragment of the mitochondrial 16S ribosomal gene, Xinghua et al. [29] found a mean sequence divergence of 2.26% among five *Lucilia* species with a surprising 3.19% sequence divergence between two *L. caesar* and one *L. illustris* specimens from China. This remarkable result deserves confirmation, not only because of the very high sequence divergence but also because the 16S fragment would be the first molecular marker that allows to unambiguously differentiate *L. caesar* from *L. illustris*.

In this paper, we extended the mitochondrial (COI, COII, 16S) and nuclear (ITS-2, 28S) sequence datasets of *L. caesar* and *L. illustris* with specimens from Europe and re-evaluated the genetic relationships between both species.

2. Materials and methods

2.1. Specimens

Lucilia specimens were collected in Europe during forensic investigations, from laboratory breeding stocks or by net sweeping in the field. All specimens were stored in 95% EtOH except those collected by the National Institute of Criminalistics and Criminology (NICC, Brussels, Belgium) that were stored as dry-pinned material (Table S1). Initial morphological identifications [14] were performed by several identifiers and then confirmed by a single entomologist (YB). One to three legs were removed and used for DNA extraction. The remainder of each specimen was retained as a voucher at the NICC (Table S1).

2.2. DNA extraction, PCR and sequencing

Genomic DNA from individual legs was extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). After adding proteinase K, samples were incubated overnight at 56 °C. A fragment of 721 bp from the 5′ -end of the COI gene, including the standard barcode region classically used for species identification [30], was amplified using primer pair TY-J-1460 and C1-N-2191 [11,31]. Four other markers were sequenced for a restricted set of samples representing the different COI haplotypes (Table S1). Fragments of the mitochondrial 16S ribosomal RNA (16S) and cytochrome *c* oxidase subunit II (COII) genes, and of the nuclear internal transcribed spacer 2 (ITS-2) and fragment D1–D2 of the 28S ribosomal RNA (28S) were amplified using primer pairs 16Sf.dip/16Sr.dip [32], C2-J-3138/TK-N-3775 [11,33,34], ITS2F.dip/ITS2 [25] and D1F/D2R [28,35,36], respectively. For all markers, each 30 μl PCR reaction was prepared using 1× PCR buffer, 0.2 mM dNTPs, 0.4 μM of each primer, 2.0 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Platinum, Invitrogen), 2–4 μl DNA extract and enough mQ-H_2O to complete the total volume of 30 μl. The thermal cycler program consisted of an initial denaturation step of 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature and 45 s at 72 °C; with a final extension of 7 min at