Forensic Science International: Genetics Supplement Series 3 (2011) e439–e440

Contents lists available at ScienceDirect

Forensic Science International: Genetics Supplement Series

journal homepage: www.elsevier.com/locate/FSIGSS

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

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ARTICLE INFO

Article history:
Received 26 August 2011
Accepted 15 September 2011

Keywords:
Forensic entomology
Calliphoridae
COI
ITS2

ABSTRACT

Insects, in particular Calliphoridae species have a very important role in decomposition process and are the earliest insects to infest a corpse. An accurate morphological identification is essential but very difficult or even sometimes impossible to do. So, molecular identification provides a rapid and reliable method that can be done in all development stages. The potential of mitochondrial cytochrome oxidase subunit I (COI) is very well established. But in some species this gene is not effective. In this work, we used the ribosomal internal transcribed spacer 2 (ITS2) to complement COI data, demonstrating ITS2 effectiveness in insects' identification. Blowflies of the family Calliphoridae (*Calliphora vicina*, *Calliphora vomitoria*, *Lucilia caesar* and *Lucilia sericata*) were collected in Portugal. COI fragments permitted correct specimens identification using BLAST search for all blowflies, except for *L. caesar* because of the high similarity with *Lucilia illustris*. For these species, we used ITS2 sequences for species determination. This genetic marker analysis facilitated the differentiation of these two species.

Our results indicate that it would be of great importance to increase the sequences collection to prevent incorrect identification and reinforce results validity.

1. Introduction

Forensic entomology applies knowledge about insects and other arthropods to forensic and legal concerns. Necrophagous insects are useful in helping to answer questions relating to estimation of post-mortem interval (PMI), post-mortem transfer, neglect or abuse of living persons 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].

Thus, the first step is accurate species identification, traditionally done by morphological characters. Although, morphological identification can be complicated due to the similarities among species, even sometimes in adult specimens, what makes identification very difficult or even sometimes impossible [3]. Therefore, molecular identification provides a rapid, precise and reliably method that can be done 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 aim of this study was to use a nuclear gene, the ribosomal internal transcribed spacer 2 (ITS2), to complement COI data and also demonstrate ITS2 effectiveness for identification purposes of forensically important Calliphoridae.

2. Material and methods

Adult specimens were collected from 3 locations in Portugal (Serra da Estrela, Oeiras and Monsanto), using mammalian corpse-baited traps, e.g. exposed dead piglet and genets, animals sharing high degree of homology with human species. Samples identified using morphological keys to adult Calliphoridae [9,10] revealed eleven specimens of *Calliphora vicina*, eight *Calliphora vomitoria*, ten *Lucilia caesar* and one *Lucilia sericata*.

Two legs of each specimen were collected for DNA extraction using E.Z.N.A.® Insect DNA Isolation kit (Omega Bio-Tek) with overnight incubation step.

A 658 bp fragment of COI gene was amplified and sequenced with primers pair LCO1490/HCO2198 using authors previously described PCR methodology [11]. ITS2 fragment was also amplified and sequenced using primers and methodology previously described by Song et al. [12]. Amplification products were analyzed by electrophoresis in 2% agarose gels and purified with Sure Clean

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doi:10.1016/j.fsigss.2011.09.081

(Bioline) according to manufacturer's instructions. All samples were sequenced by Macrogen Inc.To ensure correct identification of *L. caesar* a third PCR was done. A 304 bp fragment of mitochondrial COI gene (another region downstream located) was amplified using primers pair previously described [13]. PCR products were digested with endonuclease *Dde* I (previously shown to differentiate between species under study) and restriction patterns analyzed in 2% agarose gel electrophoresis.

All sequences were analyzed with Sequencher® v4.0.5 (Genes Codes Corp.) and BioEdit® Sequence Alignment Editor v7.0.5.3 software. Automatic alignment was performed using ClustalX v2.0.12 to reveal interspecific differences. All sequences were matched and identified by GenBank Basic Local Alignment Search Tool (BLAST).

3. Results and discussion

The 658 pb COI gene amplicon was successfully amplified and sequenced from all 30 Calliphoridae individuals. This DNA fragment sequence allowed correct specimens identification using BLAST search for all blowflies, except for *L. caesar*. In this species, polymorphism of chosen COI fragment was not sufficient to distinguish it from *L. illustris*, a very morphologically similar species and extremely difficult to differentiate even at adult stage [10].

Comparing both species sequences it became obvious their similarity, evidenced by the fact that in all *L. caesar* BLAST searches *L. illustris* was always one of the first two hits together with *L. caesar*. The two even had the same maximum scoring segment pair (MSP) of 99%. Only one individual obtained MSP of 100%.

The *L. caesar* ITS2 sequences, used to compliment COI data, were compared to GenBank database and 100% MSP was obtained. This demonstrates the suitability of ITS2 gene to identify *L. caesar* species. However, the second hit of the search still remained to be *L. illustris* with 99% MSP.

For this reason, and just to ensure ITS2 results, a second COI region was amplified to digest PCR amplification products with *Dde* I. This restriction enzyme may allow the differentiation of these two species as referred by Malgorn and Coquoz [13]. The obtained restriction patterns clearly show that these specimens, already morphologically identified as *L. caesar*, really belong to this species.

4. Conclusions

Only *L. caesar* specimens were not suitably identified using the chosen COI sequence for Diptera identification. In this work we demonstrated the utility of another genetic marker for entomologic species identification – the ITS2. This gene is suitable to correctly identify these species, although the sequences of both are so similar that the second hit always had a very high MSP.

To overcome this situation it would be helpful to use other genetic markers more effective in these species identification. We suggest taking other aspects into account: first, the fact that there is an uncertainty in the morphological identification previously made of the specimens included in database, leaving it unclear whether the pair species/sequence is correct; second, despite the great similarity between the *L. caesar* and *L. illustris* sequences these results indicate that it would be of great importance to increase the sequences collection to prevent incorrect identification and reinforce results validity.

Conflict of interest

None.

Funding source

This work was financially supported by the Portuguese Foundation for Science and Technology, through the grant PTDC/SAU-ESA/101228/2008.

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