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Forensic entomology: Nuclear and mitochondrial markers for Diptera and Coleoptera identification

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ABSTRACT

Accurate identification of insect specimens is an essential step in forensic entomology. Cytochrome c oxidase subunit 1 (COI) is the most used *locus* for insects molecular identification. However, other studies were done using other genetic markers, as mitochondrial cytochrome *b* (CytB) and ribosomal second internal transcribed spacer (ITS2). Also, it was shown that COI has some limitations in this field. In this work these three markers (COI, CytB and ITS2) were used, with the aim to infer about its suitability for insects with forensic relevance identification.

DNA was extracted from insects found in carcasses of mammals with high homology with humans. COI, CytB and ITS2 regions were PCR amplified. Obtained sequences were matched in BLAST and BOLD-IDS online tools. Sequence divergences and phylogenetic analyses were performed for COI and CytB, using PAUP* v4.0b10 software. However, ITS2 analyses were not performed due to alignment problems.

Maximum Parsimony and sequence divergences data for COI and CytB allowed the distinction of insect lineages, with good support. Despite analysis problems, ITS2 proved to be suitable for insects identification.

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1. Introduction

Correct identification of insect specimens is of great importance for forensic entomology purposes. Cytochrome c oxidase subunit I is the most used *locus* for insects molecular identification, however there is no agreement about which *loci* should be used [1]. Also, in some studies, cytochrome *b* (CytB) has already been used for this purpose with successful results [2]. Furthermore, mitochondrial markers have been proven to have some limitations for insect species identification. For example, it was shown that there are high levels of overlap in inter and intraspecific distances to certain species [3]. Hence, it becomes necessary to complement insects identification with a nuclear marker [4] such as second internal transcribed spacer (ITS2).

2. Materials and methods

Fifty-two DNA samples from another study (E. Rolo et al., unpublished data) and 23 other DNA extractions, performed using commercial kits, were used. Specimens were collected from

carcasses of mammals with high homology with humans. Seventy-two samples were amplified for ITS2 [5] and 14 samples for CytB [6]. Only 21 extracted samples were amplified for COI [7], since others had already been amplified and identified as belonging to *Calliphora vicina* (5), *Calliphora vomitoria* (10), *Musca autumnalis* (12), *Helina evecta* (3), *Helina reversio* (3), *Eudasyphora cyanella* (5), *Pollenia rudis* (3), *Lucilia caesar* (5), *Delia* sp. (1) and *Hydrotaea dentipes* (5) species. Agarose gel electrophoresis was performed to confirm amplification success. Amplicons were purified and sequenced. Data was analyzed with Sequencher[®] v4.0.5 software. All sequences were matched in Blastn, and COI sequences in BOLD-IDS. Sequences were aligned using ClustalX v2.0.12 and BioEdit Sequence Alignment Editor v7.0.5.3. Filogenetic analysis (Maximum Parsimony) and nucleotide divergence (corrected – General time-reversible model (GTR) – and uncorrected – p-distance) were performed using PAUP* v4.0b10 software.

3. Results

Nineteen COI amplified samples were successfully identified (sequence identity $\geq 98\%$) as belonging to *C. vicina* (10), *L. caesar* (3), *L. caesar/illustris* (3), *Lucilia sericata* (1) and *P. rudis* (2). Other two samples were identified as *Chantaris rustica*, with 96% sequence identity, value that is below the limit to be considered as a significant identification. None of CytB amplicons achieved

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significant identification values. Among all ITS2 sequences it was only possible to use 36 in this step, because other sequences chromatograms showed multiple bases in the same position, preventing its analysis. Specimens were successfully identified, with significant identification values, as belonging to *C. vicina* (15), *C. vomitoria* (9), *L. caesar* (11) and *L. sericata* (1). For other sequences identification values were achieved below established boundary.

Cytochrome *c* oxidase subunit I analysis was performed through MP method using Cantharidae specimens as outgroup, which appeared clearly separated from other specimens. It can be distinguished 9 lineages in MP tree, almost all with high bootstrap support. Indeed, all species were grouped as reciprocally monophyletic. However, some variation was observed, since *H. evecta* and *H. reversio* (congeneric species) did not appear associated. Intraspecific divergences for COI, with both uncorrected (p-distance) and corrected (GTR analysis) distances, showed a range of values from 0% for most species to 1.1% for *Helina evecta*. Interspecific divergences showed values ranging from 4.0 to 21.2% (p-distance) and from 4.5 to 25.7% (corrected distances). For both, corrected and uncorrected distances, the lowest value was obtained between congeneric species *C. vicina* and *C. vomitoria*, and the highest value was obtained between *E. cyanella* and Cantharidae specimens. Phylogenetic analysis, for CytB, was only performed with four *C. vicina*, one *M. autumnalis* and one *Phaonia* sp., and two Cantharidae specimens as outgroup. Maximum Parsimony and sequence divergences were in accordance with results obtained for COI, and Cantharidae were clearly separated from other specimens. It was not possible to perform any phylogentic analysis for ITS2, due to sequences length variation observed mostly between distant species, which prevented an accurate alignment. Nevertheless, by aligning amplicons of conspecific specimens, it was observed that there were almost no differences within species.

4. Discussion

Through comparing results achieved for COI, CytB and ITS2 it was possible to solve some identification issues. For example, for COI, Blastn and BOLD-IDS tools gave ambiguous identifications (*L. caesar*/*L. illustris*) for 3 specimens, while for ITS2 these 3 specimens were identified as *L. caesar* with 100% of sequence identity. Also, for specimens that did not achieve significant identification values, it was possible to know its species. Intra and interspecific variations were calculated taking into account the 3% threshold value, below which a species was distinguished. COI and CytB achieved

corrected and uncorrected distance values below the threshold for intraspecific and above the threshold for interspecific variations. For COI, values obtained between congeneric species were closest to 3%, except for *Helina*, which was also seen with MP analysis.

5. Conclusion

The best results were obtained for COI, with good support. Nevertheless, it was seen that more accurate results can be obtained using more than one genetic marker. Despite not being possible to perform phylogenetic analysis for ITS2, it was possible to observe its suitability to differentiate specimens. During this work it was noted that more sequences of studied markers, from necrophagous insects, are needed in databases, in order to improve molecular identifications. Thus, with this study we also will contribute by adding some more sequences in GenBank.

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Conflict of interest

None.

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