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A N I N T E R N A T I O N A L J O U R N A L

Using DNA barcodes to identify forensically important species of Diptera in Espírito Santo State, Brazil

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ABSTRACT

Accurate insect specimen identification is usually a crucial first step in a forensic entomological analysis. It is traditionally done by morphological classification using identification keys. However, due to sensibility limitations in the identification of animal species based only on their morphology, new methods have been developed, including species identification by DNA barcodes. The objective of this study was to identify forensically important species of Diptera in Espirito Santo state using DNA barcodes. For this, adult flies were collected in Espirito Santo, Southeast Region of Brazil. After DNA extraction, COI gene was amplified and sequenced. All sequences were matched to BOLD platform and alternatively to GenBank MegaBLAST. As result, 281 adult flies were collected and identified morphologically. From these, 36% of samples were classified as Calliphoridae, 34% of Muscidae and 30% of Sarcophagidae. Approximately 10% of all collected samples were analyzes by DNA. It was possible to identify only 35.7% of tested samples, probably due to lack of samples deposited in databases. Therefore, more efforts should be made to deposit a greater variety of dipterous in databases to allow the use of this technique in forensic routine, especially in BOLD.

Key-words: Entomology, COI, BOLD, Forensic, Flies

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INTRODUCTION

Forensic entomology is the study of insects and other arthropods to elucidate legal issues (Smith 1986; Catts and Goff 1992; Joseph et al., 2011). In this context, the major groups of forensic relevant insects are represented by Diptera and Coleoptera orders (Pujol-Luz and Arantes 2008), which are commonly known as flies and beetles, respectively. Among flies, blowflies and flesh flies have been recorded as arriving at dead bodies within minutes of their exposure. Thus, they can be used to determine post-mortem interval (PMI) (Anderson 1997).

Accurate insect specimen identification is usually a crucial first step in a forensic entomological analysis. It is traditionally done by morphological characters, using identification keys (Oliva 2012; Yang et al., 2014). Although morphological identification can be complicated and sometimes impossible due to similarities among species, especially in immature stages or even in adult specimens (Harvey et al., 2003).

Due to problems with identification of animal species based only on their morphology, new methods have been developed, including species identification by DNA. One such method corresponds to DNA barcodes. This system corresponds to the Barcode of Life Project, which proposes a single universal system with the goal of identifying all organisms at species level, using specific DNA regions sequences (Hebert et al., 2003). The Barcode of Life Project started in 2003, in Canada, (http://www.barcoding.si.edu) and was promoted in 2004 through the Consortium for Barcoding of Life (Miller 2007). From this project is being created one global database (Barcode of Life Data Systems - BOLD - http://www.boldsystems.org) containing sequences from different species: animals, plants, fungi and other organisms (Ratnasingham and Hebert 2007). Using this database, it would be possible to identify any type of biological sample by comparing its DNA sequence with the sequences deposited in the database. BOLD database has currently over three million samples deposited encompassing about 23 thousand species of Diptera (data from January, 2016). For animal species identification (including insects), the mitochondrial Cytochrome c Oxidase subunit I (COI) region is proposed as DNA barcode since it is a powerful tool for accurate identification of animal species across various taxa (Hebert et al., 2003). An advantage of this system for forensic entomology is that it can provide a rapid, precise and reliably method that can be done at all developmental stages of flies (Sperling et al., 1994).

Although, for the practical use in forensic entomology, it is essential to study the local fauna for each geographical location for at least two reasons: first, it might be necessary to repeat many experiments at new locations because reference data developed in one place are not applicable to a death investigation at a different place; second, geographic population genetic structure might make it possible to infer the postmortem relocation of a corpse if the insects therein show a non local genotype (Wells and Stevens 2008). In Brazil, the number of known Diptera species is approximately 8,700 and the main families of forensic interest are Calliphoridae, Muscidae e Sarcophagidae (Rafael et al., 2012). However, little is known about forensic species of Diptera in each region of Brazil (Biavat et al., 2010; Luiz et al., 2012; Oliveira and Vasconcelos 2010; Souza et al., 1997).

Thus, our aim with this study was to identify forensically important species of Diptera in Espírito Santo state, Brazil, using DNA barcodes.

MATERIAL AND METHODS

Study area and collection of specimens

Adults flies were collected in four locations of Espírito Santo state, Southeast Region of Brazil (coordinates: Point A: 20°45'45.20"S/41°32'7.09"O; Point B: 20°45'33.10"S/41°31'0.58"O; Point C: 20°45'34.21"S/41°30'3.76"O; Point D: 20°45'7.65"S/41°29'18.62"O, latitude and longitude, respectively). Beef mincemeat rotten within traps made of plastic bottles was used to attract flies. The specimens collected were sacrificed at low temperature (-20º C) and preserved in microtubes with 90% ethanol and then refrigerated.

Morphologic Identification

Adult flies were identified in a stereomicroscope using a morphological identification key (Carvalho and Mello-Patiu 2008). Based on the frequency of occurrence, approximately 10% of the specimens collected were intended to molecular biology procedures.

DNA extraction

DNA was extracted from a small piece of flies' thorax muscle using Chelex 5% (Harvey et al., 2003). Then, DNA was quantified using Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) before PCR amplification.

Amplification and sequencing

A 658 bp fragment of COI gene was PCR amplified using universal primers pair LCO1490/HCO2198, described by CBOL (*Consortium for the Barcoding of Life*). PCR master mix was carried out using: 9 µL ultrapure water, 1.25 µL of 10X Buffer PCR (Invitrogen Life Technologies), 2.5 mM $MgCl_2$ (Invitrogen Life Technologies®), 50 mM dNTPs, 100 nM of each primer, 1 Unit of Platinum Taq DNA polymerase (Invitrogen Life Technologies) in a final volume of 11.25 µL of mix for each sample. 1 µL of DNA (20 ng/µL) was added in a final volume of 12.25 µL.

Samples were then loaded onto Veriti thermocycler (Life Technologies) with initial denaturation step at 94° C for 1 min followed by 5 cycles of denaturation at 94° C for 30 s, annealing of primers at 45° C for 40 s and extension at 72° C for 1 min. Then, samples were submitted to 35 cycles of denaturation at 94° C for 30 s, annealing of primers at 51° C for 40 s and extension at 72° C for 1 min. A final cycle extension of 10 min at 72° C was included, followed by 4º C.

Amplified DNA was confirmed by electrophoresis on 1.5% agarose gel stained with ethidium bromide. PCR product was purified using the GFX PCR and Gel Band Purification kit (GE Healthcare) and sequenced using BigDye Terminator v3.1, according to the manufacturer's protocol. Electrophoresis was performed on ABI 3500 and electropherograms were visualized in software BioEdit Sequence Alignment Editor v7.2.0 (Hall 1999).

Sample analysis

All sequences were matched to BOLD platform (*Barcode of Life Data system*) using option "Species Level Barcode Records" and alternatively, when the sequence was not identified by BOLD, GenBank MegaBLAST (Basic Local Alignment Search Tool) was used. For comparison in BOLD, were used at least 500 bp of COI gene sequenced and a minimum of 99% of similarity was adopted to consider sample identified. Furthermore, each sample was considered identified only when an

identical specie name had been assigned by both morphological and molecular methods.

RESULTS AND DISCUSSION

Morphological identification

A total of 281 adult flies were collected at four geographical points and identified morphologically according to characteristics observed in the identification key (Carvalho and Mello-Patiu 2008). From these, 36% of samples were classified as Calliphoridae, 34% of Muscidae and 30% of Sarcophagidae. Samples were classified to specie level with exception to Sarcophagidae, which were classified only to family level. In Figure1 is shown the percentage of each species collected. The distribution of collected species at each point was not uniform, indicating that differences may occur in the fauna and frequency of flies from one point to another, even in short distances.

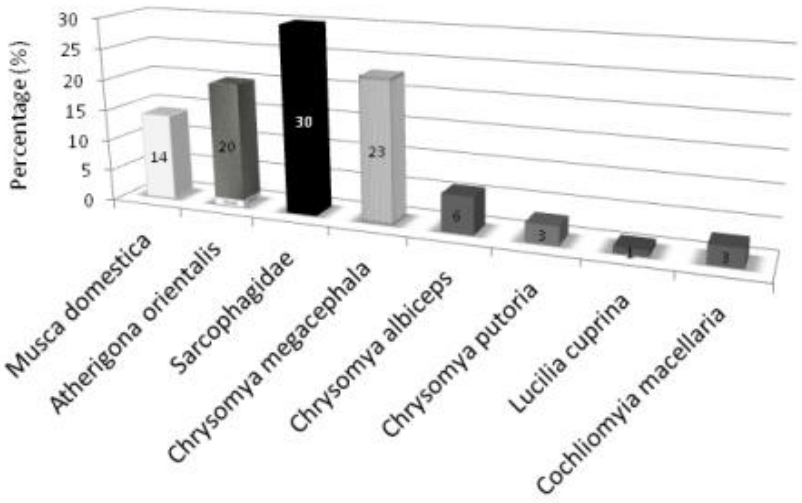

Figure 1 - Percentage of each species collected. Results are based on morphological identification.

Molecular identification

Approximately 10% (28) of all collected samples (281) were analyzed by DNA barcodes. The species morphologically identified as *Cochliomyia macellaria*, *Lucilia cuprina* and *Chrysomya putoria* were not selected for molecular analysis because they were less than 5% of the total representation.

Thereby, nine specimens morphologically classified as Sarcophagidae, nine specimens classified as Chrysomya sp. (family Calliphoridae), seven specimens classified as *Atherigona orientalis* (family Muscidae) and three specimens classified as *Musca domestica* (family Muscidae) proceeded to molecular identification. DNA could be extracted, amplified and sequenced from all samples. Of these, one sample was correctly identified as *Atherigona orientalis*, one *Chrysomya albiceps*, three *Chrysomya megacephala*, three *Musca domestica*, one *Peckia chrysostoma* and one *Oxysarcodexia thornax* (based on morphology and confirmed by DNA barcode) (Table 1).

Table 1- Result of analysis after morphological and molecular identification using DNA barcode of 28 samples from this study. Sample was considered identified if >99% of similarity were retrieved and morphological and molecular identification had a match. Universal primers pair LCO1490/HCO2198 was used.

Sample ID	Morphological identification	Similarity (%)	Database	Molecular identification using DNA barcode
1	*Atherigona orientalis*	100	BOLD	*A. orientalis vs.* Tachinidae
2	*Atherigona orientalis*	93	GenBank	*Tricharaea brevicornis*
3	*Atherigona orientalis*	93	GenBank	*Tricharaea brevicornis*
4	*Atherigona orientalis*	100	BOLD	*Bos taurus*
5	*Atherigona orientalis*	99.6	BOLD	*A. orientalis vs.* Tachinidae
6	*Atherigona orientalis*	100	BOLD	*Bos taurus*
7	*Atherigona orientalis*	100*	BOLD	*Atherigona orientalis*
8	*Chrysomya albiceps*	100	BOLD	*Bos taurus*
9	*Chrysomya albiceps*	87	GenBank	*Chrysomya albiceps*
10	*Chrysomya albiceps*	100	BOLD	*Bos taurus*
11	*Chrysomya albiceps*	100*	BOLD	*Chrysomya albiceps*
12	*Chrysomya albiceps*	100	BOLD	*Bos taurus*
13	*Chrysomya albiceps*	100	BOLD	*Bos taurus*
14	*Chrysomya megacephala*	100*	BOLD	*Chrysomya megacephala*
15	*Chrysomya megacephala*	100*	BOLD	*Chrysomya megacephala*
16	*Chrysomya megacephala*	100*	BOLD	*Chrysomya megacephala*
17	*Musca domestica*	100*	BOLD	*Musca domestica*
18	*Musca domestica*	99.15*	BOLD	*Musca domestica*
19	*Musca domestica*	99.8*	BOLD	*Musca domestica*
20	Sarcophagidae	98.22	BOLD	*Ravinia lherminieri*
21	Sarcophagidae	100*	BOLD	*Peckia chrysostoma*
22	Sarcophagidae	97.84	GenBank	*Ravinia lherminieri*
23	Sarcophagidae	99.65*	BOLD	*Oxysarcodexia thornax*
24	Sarcophagidae	96	GenBank	*Oxysarcodexia fluminensis*
25	Sarcophagidae	94	GenBank	*Oxysarcodexia fluminensis*
26	Sarcophagidae	93	GenBank	*Oxysarcodexia fluminensis*
27	Sarcophagidae	93	GenBank	*Oxysarcodexia fluminensis*
28	Sarcophagidae	100	BOLD	*Bos taurus*

*sample considered identified

Regarding the use of universal primers, matches of >99% of similarity were retrieved for only 10/28 (35.7%) samples using BOLD database (Figure 2). Low levels of identification using BOLD were also described by Klippel et al. (2015) when studying roadkilled animals. Using COI gene it was possible to identify 62.16% of samples, however, the percentage varied from about 87.5% when studying non-volant mammals to 0% in reptiles. Either, Chesalin et al. (2012), when studying rock oysters, did not identified any sample using BOLD. It shows that some taxonomic groups are, to date, better represented in BOLD than others.

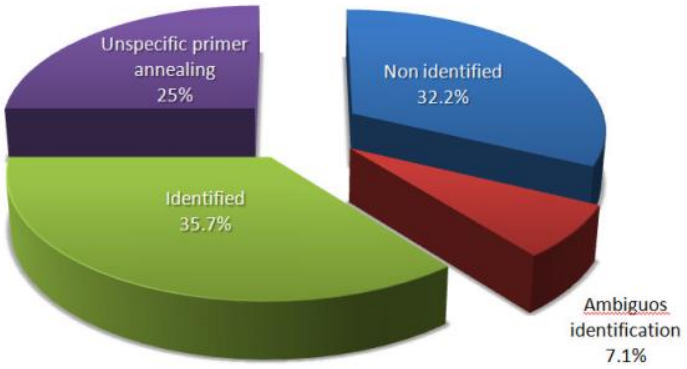

Figure 2 – General result of this work in percentage.

Ambiguous identifications were obtained for two specimens of *Atherigona orientalis* (7.1%). For both, more than 99% of similarity was found for *Atherigona orientalis* and family Tachinidae, showing a problem in BOLD database. In this case, samples were not considered identified. Sonet et al. (2013) also described ambiguous identification for 25 queries (6 species) of Dipteras from Belgium and France using best matches of >99% of similarity and Special Level Barcode Records dataset of BOLD.

Other 9/28 (32.2%) samples generated high quality sequences but less than 99% of similarity were obtained. Misidentification can occur if the database contains a limited set of species, or if species deposited are from a different geographic area, because of geographic population structuring or eventual local hybrids (Stevens et al., 2002). Our results demonstrate that DNA barcode has the potential to be used but more efforts are necessary to deposit more sequences in databases, especially in BOLD, since intraspecific variation and geographic substructuring is very important for forensic entomology (Sonet et al., 2012; Wells and Willians 2007).

From all samples analyzed, Sarcophagidae family specimens were the most problematic samples with only 22% of tested samples identified in database (medium of 40% in other families). This is probably due to the difficulty in identifying, at species level, flies from this family. Also, family Sarcophagidae has 7,152 specimens deposited in BOLD but only five specimens are from Brazil. Family Calliphoridae has 10,908 specimens in BOLD and 84 specimens from Brazil. Family Muscidae (including *Atherigona orientalis* and *Musca domestica*) has 42,689 specimens in BOLD but only 31 specimens from Brazil (data from November, 2015).

Other 7/28 (25%) samples were incorrectly identified as *Bos taurus*. In those cases, DNA from beef used to attract flies was amplified, instead of flies, demonstrating the problem of using universal primers for forensic entomology. Similar results were described by Lee and Lee (2012).

Samples identified in this study corroborates with results from other authors who demonstrated the occurrence of these species in the Atlantic Forest region (Families Calliphoridae, Muscidae and Sarcophagidae) of Southeastern Brazil (Barbosa et al., 2008; Carvalho and Linhares 2001).

In these work, 10% of samples were used to compare morphological and molecular identification. However, future studies, using more samples, are needed to certify the efficacy of this technique in a broad view.

CONCLUSIONS

It was possible to identify *Atherigona orientalis*, *Chrysomya albiceps*, *Chrysomya megacephala*, *Musca domestica*, *Peckia chrysostoma* and *Oxysarcodexia thornax* amplifying a fragment of mitochondrial COI gene and comparing the result with BOLD systems and GenBank databases. Some samples could not be identified because they showed less than 99% of similarity in databases. Also, some samples were identified as *Bos taurus* (the substrate for attracting flies) showing the problem of unspecific amplification using universal primers in forensic entomology. We can conclude that more efforts should be made to deposit a greater variety of dipterous in databases to allow the use of this technique in forensic routine.

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