



Human autosomal DNA and X chromosome STR profiles obtained from *Chrysomya albiceps* (Diptera: Calliphoridae) larvae used as a biological trace

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ABSTRACT. The use of insects to answer questions in criminal investigations, as well as a combination of forensic genetic techniques to obtain human DNA from the organisms, especially necrophagous dipterians, have gained ground in recent decades among researchers and professionals in this area. The objective of our study was to evaluate and compare two methods of human DNA extraction,

commonly used for forensic samples, to obtain human autosomal DNA and X chromosome short tandem repeat profiles from the digestive tract of *Chrysomya albiceps* (Diptera: Calliphoridae) larvae. Immature specimens were collected from corpses at the Institute of Forensic Medicine of Pernambuco and raised in bovine ground meat to allow stabilization of the colony. Groups of larvae in the third instar were provided with bovine ground meat plus human blood for 48 h, dissected, and then subjected to DNA extraction. DNA was extracted using two methods: a DNA IQ™ kit and a phenol-chloroform method. Genomic DNA was amplified using AmpF/STR® Identifiler® Plus PCR and Argus-X-12® kits, and samples were sequenced to determine if the two extraction techniques generated reliable profiles that were compatible with a reference sample. The existence of comparable profiles from both techniques demonstrates the usefulness of dipteran larvae for obtaining human DNA from corpses, which can be further used to correlate genetic profiles in a crime scene when other traces are not available. However, several variables still require revision; thus, the technique should be further investigated for its validity, security, and, in particular, its reproducibility.

Key words: Forensic genetics; Forensic entomology; Dipterians; Short tandem repeats; Uniparental markers

INTRODUCTION

Forensic entomology, the study of insects associated with forensic procedures, aims to gather information and traces that can assist an investigation, demonstrating the importance of both science and legal processes (Thyssen, 2011). Among the various uses of insects in investigations, entomogenetics combines forensic genetic techniques and the versatility of entomology.

Among the various techniques currently used for individualization of people in a forensic context, DNA is notable, especially in crime scenes where various types of biological materials are commonly found, often in minute quantities, masked, mixed, degraded, contaminated, and decomposing (Ziętkiewicz et al., 2012). In the case of crime scenes, all traces left by the perpetrator or the victim may be essential for clarification of the facts, regardless of the type, quality, or quantity of material.

Wells et al. (2001) identified a number of situations in which food content analysis of the larval gut may be used in forensic investigations. Human DNA was found in blood from the abdomen of mosquitoes up to 26 h after ingestion, demonstrating that DNA isolated from mosquitoes is qualitatively and quantitatively sufficient for DNA typing (Kreike and Kampfer, 1999).

Human DNA isolated and amplified from pubic lice blood can be helpful in an investigation aimed at identifying individuals involved in certain cases of sexual violence because of transmission of insects from the perpetrator to the victim during the act (Campobasso et al., 2005). Human mitochondrial DNA has also been obtained from beetle larvae recovered from bones exposed to the environment for several months (Linville et al., 2004).

Recently, the assessment of cases and simulation studies based on the analysis of short tandem repeats (STR) in human DNA extracted from the intestinal contents of fly larvae has shown that flies can provide molecular evidence for identifying offenders and victims (Kester et al., 2010).

In Brazil, few studies involving entomology together with genetics have been conducted so far for forensic purposes. The current research aims to contribute to the advancement of these studies, enabling the use of new tools to increase the degree of accuracy of techniques used during criminal investigations. The union of entomology with forensic genetics can bring greater effectiveness to evidence generated during criminal investigations. However, many gaps remain to be filled, and data generated in the academic environment should be applied to instances responsible in the conduct of the criminal process.

The State of Pernambuco in Brazil has a high rate of homicides and crimes, especially in its capital, Recife, and many of these have no apparent solution. High crime, associated with a lack of empirical studies in the state, underscores the importance of studying, in depth, a new tool that can support investigations conducted by the local scientific police.

This study aims to help fill some of these gaps and also support the research data previously generated, which could help to improve methods for the analysis and validation of evidence in criminal cases.

MATERIAL AND METHODS

Dipterians with immature bodies were collected from corpses at the Forensic Medicine Institute Antonio Persivo Cunha of Pernambuco (IMLAPC/PE) in order to establish a colony of flies for the experiment. The material was collected with the help of histological tweezers, packed in 70-mL pots, and sent to the Forensic Entomology Laboratory at the Federal Rural University of Pernambuco (LEF/UFRPE) for colony creation. A sample of reference material was collected and stored in 70% alcohol for later evaluation of larval instars and taxonomy.

Immature larvae were brought to the laboratory alive in 250-mL plastic pots containing sawdust as puparian substrate and bovine ground meat for immature nutrition at a ratio of 1:1 g/larvae. The pots were covered with fabric pieces and maintained at room temperature, with an average of 28°C ($\pm 2^\circ\text{C}$), to simulate temperature development in place of oviposition. The maximum and minimum temperatures and relative humidity were measured daily using a thermohygrometer. When adults emerged, they were assembled with the aid of pin number 29, dried in an oven at 50°C for 24 h, and subjected to identification to species and family levels in accordance with an entomological key by Carvalho and Mello-Patiu (2008).

The emerging adults were maintained in cages (20 x 20 x 50 cm; length x width x height), containing a fabric sleeve for colony maintenance. The subjects were fed with two solutions. First, an aqueous solution containing sugar and water (1 g/10 mL) and second with powdered milk and powdered yeast (2 g/2 g in 5 mL water). In addition, bovine ground meat was used as a substrate for female oviposition. The whole setting was maintained at room temperature, around 28°C ($\pm 2^\circ\text{C}$), which was monitored daily with the aid of a thermohygrometer. Following oviposition in the bovine ground meat, the substrate was transferred to plastic pots containing sawdust to allow larval development to be completed. Following development, second-generation individuals were used in the experiments.

For simulation purposes, prior to feeding on bovine ground meat without any sources of human DNA, larvae were provided with bovine ground meat plus human blood. Blood

material from a female person was used for analysis of autosomal DNA and X chromosome STR loci, at a ratio of 20 g bovine ground meat to 1 mL fresh human blood for each group of 20 larvae. The specimens were selected at the third-instar larvae stage and were allowed to feed on the diet for 48 h.

The dissection of immature larvae was carried out following the recommendations by Kondakci et al. (2009). Immature larvae were removed from the substrate and placed in 70% alcohol for 5 min to remove impurities that may be on the surface of the individual. Subsequently, the dissection was carried out on an exemplary glass slide by extracting the cephalic part and the entire cephalopharyngeal skeleton using histological scalpel and tweezers. The remainder of the larva was squeezed and extracted viscera were placed individually into 1.5-mL microtubes containing 1 mL distilled water. After dissection, the samples were stored at 4°C. Each larva corresponds to a single sample being tested.

DNA was extracted from dissected material using both the DNA IQ™ kit (Promega, Madison, WI, USA) and an organic extraction protocol using phenol-chloroform. For the reference sample, which consisted of a swab of oral mucosa taken from the same source as the human blood samples, DNA was extracted using only the DNA IQ™ kit (Promega). Reference samples are always rich in DNA; therefore, there was no need to compare extraction techniques for these samples.

DNA extraction using the DNA IQ™ kit (Promega) was undertaken following the manufacturer protocol, which is commonly used in the routine procedures of the Expertise and Research Laboratory for Forensic Genetics of the Social Defense Secretariat of Pernambuco (LPPGF/SDS-PE). Organic extraction was performed based on the protocol described by DiZinno et al. (2002) using phenol, chloroform, and isoamyl alcohol, and filtering with Microcon™.

Autosomal DNA was quantified using the Quantifiler® Duo DNA quantification kit (Applied Biosystems, Foster City, CA, USA) and a real-time PCR 7500 system. The quality of DNA was also assessed for a number of samples using a Nanodrop® ND-1000.

Extracted DNA was amplified using a commercial multiplex kit for the study of autosomal STRs, the AmpF/STR® Identifier® Plus PCR kit (Applied Biosystems), following the manufacturer protocol.

This kit generates profiles for 15 autosomal loci and amelogenin STRs to enable sexual differentiation (D8S1179, D21S11, D7S820, CSF1P0, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, VWA, TPOX, D18S51, D5S818, FGA, and Amelogenin). In order to obtain the X chromosome STR loci, we used the Argus-X-12® kit (Qiagen GmbH, Hilden, Germany), which has 12 markers and amelogenin (DXS7132, DXS7423, DXS8378, DXS10074, DXS10079, DXS10101, DXS10103, DXS10134, DXS10135, DXS10146, DXS10148, and HPRTB). Fragments were analyzed by capillary electrophoresis using a DNA genetic analyzer (sequencer), the ABI Prism 3500 model, following the manufacturer protocol. All cited material is commonly used by the Forensic Genetics Laboratories worldwide.

Methodology success indicators were used to verify the quantity and quality of DNA obtained by amplification of nuclear DNA regions for forensic analysis. The results were analyzed using GeneMapper® ID-X software (Applied Biosystems), to score the alleles in electropherograms produced by the sequencer. The alleles generated in the electropherograms were passed through the statistical analysis software Patcan V.1.2 (Riancho and Zarrabeitia, 2003) to perform comparison of trace samples. The statistical analysis was performed as described by Buckleton et al. (2005).

RESULTS

Insects collected from corpses at the IMLAPC/PE and sent to the LEF/UFRPE were monitored until stabilization of the colonies and identification of specimens. Among the species collected, we selected *Chrysomya albiceps* (Wiedmann, 1819; Diptera: Calliphoridae) as a model.

Groups of 20 third-instar larvae of *C. albiceps*, left in bovine ground meat and human blood for a period of 48 h to enable stability of the colony, were dissected and used to test two DNA extraction methods. This period of 48 h was selected to ensure higher levels of larval activity with the same diet: larvae were not killed prior to dissection and when viewed following dissection, the larval gut was an intense red color.

Larval dissection was based on the methodology described by Kondakci et al. (2009), which was used throughout the larva visceral content. We adopted this technique for simplicity and reproducibility, so that it can be used by forensic professionals and academics. Since many criminal experts working with forensic genetics in Brazil do not have specific knowledge of entomology, this technique is more accessible than the gut larval dissection itself.

Following dissection of the larvae, DNA extraction was carried out. Groups of ten larvae were subjected to two extraction methods commonly used in a forensic context: the DNA IQ™ kit (Promega) and an organic extraction with phenol-chloroform.

The two extraction techniques were successful in obtaining human autosomal DNA from the larvae that was compatible with a reference sample, generating full profiles that matched the reference buccal swab mouth sample (Figure 1). Our results show complete profiles of human STRs and this only occurs for a short period during degradation of the material, typically within 48 h. This means that, within the first 48 h of death, full-DNA profiles can be obtained from larvae.

In regards to the DNA IQ™ kit, although it is more expensive, it has low toxicity and a relative sensitivity due to magnetic particles made of silica that can attract and deliver the DNA contained in the sample. It is also used for small DNA samples, such as the subungual ones.

The quality of DNA samples in the current study was assessed using a Nanodrop® ND-1000 and the quantity of DNA was measured using real-time PCR. The results obtained using the Nanodrop® showed large amounts of inhibitory substances in all cases (Table 1). These substances are probably derived from lipids and other elements contained in the gut of the larvae.

All samples were also quantified by real-time PCR using the Quantifiler® Duo DNA Quantification kit (Applied Biosystems). This kit is highly specific for human DNA and detects degraded and single chain samples. Real-time PCR revealed very low DNA amounts (and in most cases was not able to provide quantification) for the samples analyzed (Table 2).

Although the vast majority of samples quantified using real-time PCR had indeterminate results, we were able to obtain full-autosomal STRs using sequencing. The results of genotyping demonstrated that for larvae of *C. albiceps* used in the experiment with the AmpFℓSTR® Identifiler® Plus kit (Applied Biosystems) the results were reliable. The AmpFℓSTR® Identifiler® kit has been successfully used in other studies that also analyzed human DNA extracted from insects and achieved a good level of amplification (Wang et al., 2009; Li et al., 2011; de Lourdes Chávez-Briones et al., 2013).

Statistical analysis, using the rate for the population of the State of Pernambuco, showed that the profiles obtained by analyzing the intestinal contents of the larvae of *C. albiceps* were 100% compatible with the reference sample taken through the buccal mucosa swab.

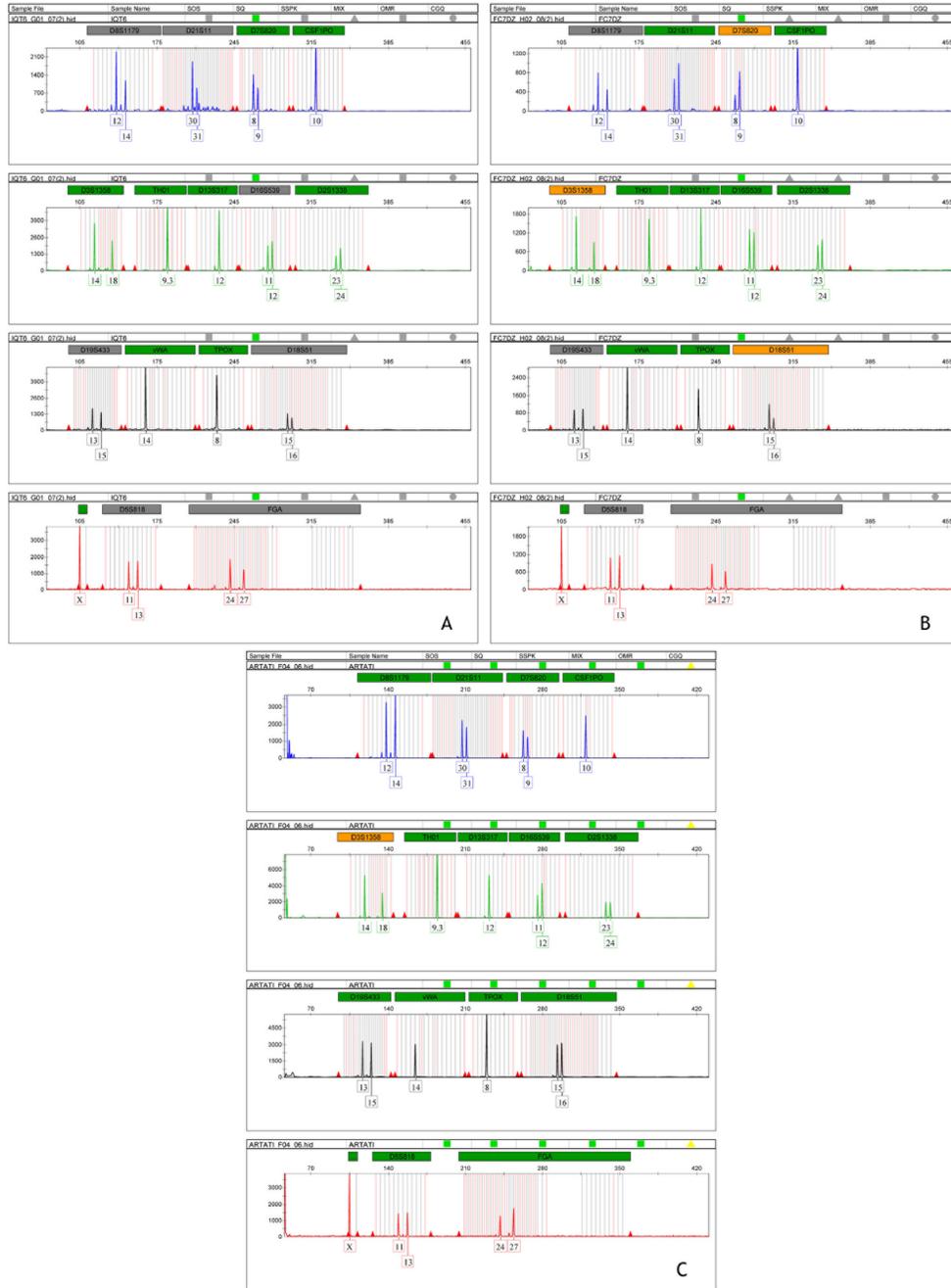


Figure 1. Comparison of autosomal DNA and X chromosome short tandem repeat (STR) profiles from (A) human DNA from *Chrysomya albiceps* larvae extracted using a DNA IQ™ kit (Promega); (B) human DNA from *C. albiceps* larvae extracted using a phenol-chloroform method; and (C) human DNA obtained from an oral buccal swab used as a reference sample.

Table 1. Quality of human DNA samples extracted from the gut contents of *Chrysomya albiceps* assessed using a Nanodrop® ND-1000.

Sample	230 nm	260 nm	280 nm	260/230	260/280
IQT1	129.2	20.45	11.7	0.15	1.74
IQT2	81.8	17.6	9.6	0.21	1.83
IQT3	470.35	23.3	13.1	0.04	1.77
IQT4	220.45	19.5	10.6	0.08	1.83
IQT5	258.15	20.55	10.9	0.07	1.88
*FC6DZ	-	-	-	-	-
*FC7DZ	-	-	-	-	-
FC8DZ	397.2	531.65	317.15	1.33	1.67
FC9DZ	167.2	246.9	153.2	1.47	1.61
FC10DZ	233.65	339.5	204.65	1.45	1.65

*Denotes samples not assessed due to low quantities of material.

Table 2. Quantity of human DNA in samples extracted from the gut contents of *Chrysomya albiceps* determined using real-time PCR with a Quantifiler® Duo DNA Quantification kit (Applied Biosystems).

Sample	DNA Quantity (ng/μL)
IQT1	Undetermined
IQT2	0.0010
IQT3	Undetermined
IQT4	Undetermined
IQT5	Undetermined
FC6DZ	0.0030
FC7DZ	Undetermined
FC8DZ	Undetermined
FC9DZ	Undetermined
FC10DZ	Undetermined

Comparison of the profiles obtained using the two DNA extraction methods showed major peaks for the majority of the loci amplified using DNA extracted with the commercial kit (Figure 2). This shows that, although more expensive, this kit may be used for samples with very limited effectiveness.

With respect to obtaining loci related to the X chromosome, the samples generated full, reliable profiles, which were compatible with the reference sample (Figure 3).

DISCUSSION

We selected *C. albiceps* (Wiedmann, 1819; Diptera: Calliphoridae) as a model because of its great importance for forensic science in Brazil, based on its wide distribution and abundance in the Brazilian territory in the colonization of bodies. These factors make this species one of the most important to study and for application as a tool for investigations involving forensic entomology. Despite being an exotic species in Brazil, *C. albiceps* began its expansion across the country in the 1970s (Guimarães et al., 1978) and today it is the most commonly found species in studies of wildlife inventories, simulation studies, and real case studies, standing out in comparison to native species (Grassberger et al., 2003; Oliveira-Costa and Mello-Patiu, 2004; Arnaldos et al., 2005; Oliveira and Vasconcelos, 2010).

Studies suggest that larvae of the third instar actively feeding on a corpse can be considered a great source of human DNA (Campobasso et al., 2005). The experimental time

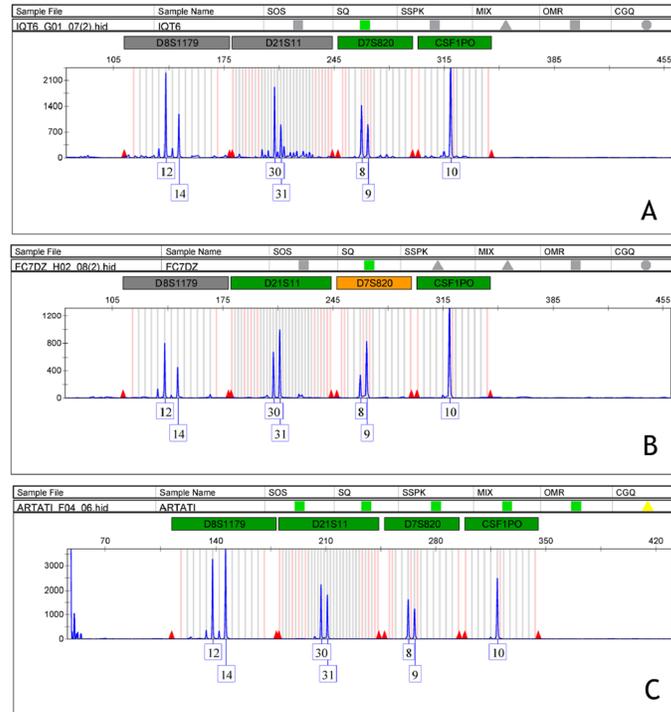


Figure 2. Comparison of peak height for loci D8S1179, D21S11, D7S820, and CSF1PO in (A) human DNA from *Chrysomya albiceps* larvae extracted using a DNA IQ™ kit (Promega); (B) human DNA from *C. albiceps* larvae extracted using a phenol-chloroform method; and (C) human DNA obtained from an oral buccal swab used as a reference sample.

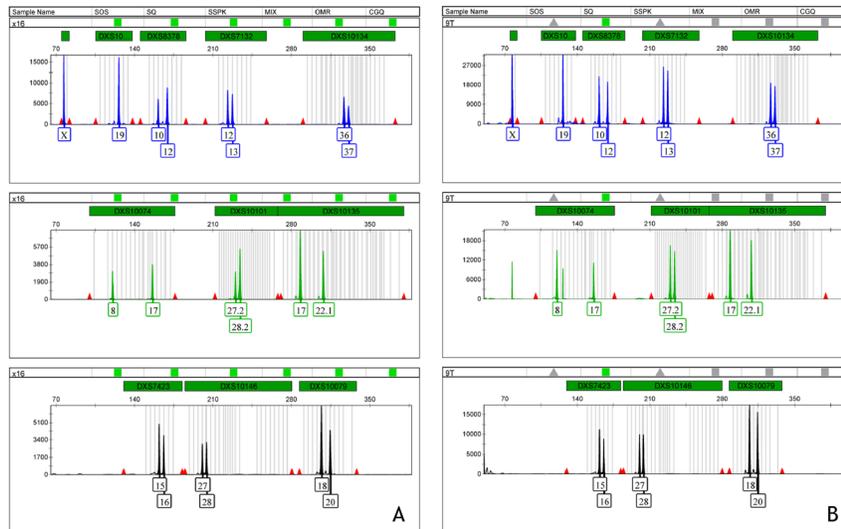


Figure 3. Comparison of X chromosome haplotypes from (A) human DNA obtained from an oral buccal swab used as a reference sample and (B) human DNA extracted from the gut contents of *Chrysomya albiceps* larvae.

used here was also selected to replicate the decomposition process, which in tropical countries is faster, thus, the larval feeding time was 48 h, enough to simulate a corpse between the gas phase and colliquative putrefaction.

The gut from the larvae of Diptera does not undergo the action of enzymes that may degrade food and consequently DNA (Hobson, 1931). For this reason, it was chosen by experts as the main source of human DNA during forensic analyses. However, while the larval gut is the best choice for DNA extraction, previous training is necessary in order to correctly dissect this region.

The literature on human DNA extracted from the larvae of muscoid flies usually yields results from mitochondrial DNA, while many autosomal STR profiles are only partially reported (Wells et al., 2001; Zehner et al., 2004; Campobasso et al., 2005; Wells and Stevens, 2008; Wang et al., 2009; Li et al., 2011). Mitochondrial DNA is used when sample degradation does not allow analysis of autosomal markers. This degradation process is also responsible for obtaining partial profiles.

The organic extraction method is considered by experts and researchers to be the most sensitive method for extracting DNA from forensic samples classified as challenging, e.g., bones. Despite its low cost and high efficiency, this technique is quite toxic, employing reagents such as phenol, chloroform, and isoamyl alcohol. Zehner et al. (2004) used the organic extraction method to obtain human DNA from larvae collected from cadavers, obtaining good results. As described by Carvalho et al. (2005), who compared the organic extraction method with commercial kits, it is the most effective method. The DNA IQ™ kit was used by Di Luise et al. (2008), who identified a high degree of variation between samples, although useful results were generated. Furthermore, de Lourdes Chavez-Briones et al. (2013) obtained a DNA profile from a crime victim through larvae and were able to compare it with the profile of the alleged father, confirming the identity of the victim.

The AmpFℓSTR® Identifiler® kit has been successfully used in other studies that also analyzed human DNA extracted from insects and achieved a good level of amplification (Wang et al., 2009; Li et al., 2011; de Lourdes Chávez-Briones et al., 2013).

The X chromosome is important for forensic analysis as it increases the probability of connecting a genetic profile to a person. With autosomal markers added to the sex chromosome, the probability of individualization is increased. In cases where autosomal markers cannot be used because of DNA degradation or other factors, generating incomplete or no profiles, the X chromosome becomes a guiding tool for the ongoing investigation.

With few exceptions, markers of the X chromosome are less powerful in the analysis of trace samples than autosomal markers (Toni et al., 2006). However, the identification of feminine characteristics in admixture with male samples, the X chromosome markers can be more efficient since the female alleles could only be fully included in the male haplotype if this were completely homozygous for loci analyzed (Szibor, 2007).

The assessment of female remains in a sex crime with a male suspect by typing the X chromosome has already been successfully carried out (Szibor et al., 2003; Szibor, 2007). The analysis of X chromosome markers from DNA samples derived from insects is still largely unexplored and only a few extraction protocols are available in the literature. The protocol used in this study for DNA extraction followed an earlier method used for autosomal STRs.

Wells and Stevens (2008) highlight the need for specific protocols aimed at recovering human DNA from insects, since any forensic expert can be, in the near future, required to perform this analysis. In order to meet this need of the professional forensic field, we designed

experiments to generate reliable protocols that pass the expert techniques required for effective human DNA analysis of samples obtained from insects to non-experts. Protocols describe all necessary steps for the success of the technique, ranging from the correct way to collect, dissect, and store samples, through the extraction and amplification to achieve the desired genetic profiles.

The results to date show that human DNA extraction from the larvae of necrophagous dipterians can generate upright profiles, as well as other forensic context samples (blood, muscles, bones). Gut content analysis of these larvae is a new method in entomology and forensic genetics, and many of the technical limitations remain unexplored. Although there are several studies that address this issue, it is known that success in the technique depends on insect development and metabolism.

The metabolism of the digestive tract of dipterans of forensic interest varies according to the family, genus, and species of the insect. In addition, climatic factors directly influence the digestion of specimens. In this way, the studies published and presented in other countries do not match the reality of the specimens found in Brazil, in particular in the north eastern part of the country where we operate (Amendt et al., 2000). The warm and humid climate, with higher temperatures throughout most of the year, causes a body to undergo rapid decomposition and, consequently, the power and digestion by necrophagous dipterans also increases (Oliveira-Costa and Mello-Patiu, 2004).

Analysis of the various extraction methods and amplification kits in the current study reveals that these types of sample cannot be treated in the same manner as those commonly used in criminal investigations. The gut contents of the larvae must first be treated by removing any substance, such as lipids and proteins, which interfere with DNA amplification.

From these experiments, it is expected to finalize and validate a protocol that details a step by step methodology that forensic professionals or scientist could follow to obtain reliable results from this type of sample and apply them to real cases. It is expected that the protocol can be followed both by experts in genetics and entomology, or by a professional with little experience in either area. Ideally, a criminal expert should be able, based on the protocols, to conduct an expert analysis in entomogenetics without specialist knowledge of either disciplines.

Currently, published protocols that could be used for DNA extraction from necrophagous insect larvae are scarce and do not offer very detailed procedures. Moreover, they do not use species and temperature conditions similar to those experienced in Brazil. Therefore, it is necessary to deepen the techniques and protocols that can be used in Brazil to better enforceability of entomogenetics, reproducibly, which is applicable to different species that have similar growing conditions, with relative safety.

Conflicts of interest

The authors declare no conflict of interest.

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