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RESEARCH ARTICLE

DNA extraction and barcode identification of development stages of forensically important flies in the Czech Republic

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ABSTRACT

Several methods of DNA extraction, coupled with 'DNA barcoding' species identification, were compared using specimens from early developmental stages of forensically important flies from the *Calliphoridae* and *Sarcophagidae* families. DNA was extracted at three immature stages – eggs, the first instar larvae, and empty pupal cases (puparia) – using four different extraction methods, namely, one simple 'homemade' extraction buffer protocol and three commercial kits. The extraction conditions, including the amount of proteinase K and incubation times, were optimized. The simple extraction buffer method was successful for half of the eggs and for the first instar larval samples. The DNA Lego Kit and DEP-25 DNA Extraction Kit were useful for DNA extractions from the first instar larvae samples, and the DNA Lego Kit was also successful regarding the extraction from eggs. The QIAamp DNA mini kit was the most effective; the extraction was successful with regard to all sample types – eggs, larvae, and pupari.

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Forensic science; forensic entomology; species identification; molecular markers; cytochrome oxidase subunit I

Introduction

Forensic entomology primarily addresses the estimation of colonization intervals ('post mortem intervals') using insects collected from a corpse (Tomberlin et al. 2011). Knowledge of the succession of insects on a dead body makes it possible to estimate the time of death. The identification of adult specimens, which have distinctive morphological characteristics, is not usually problematic, with the exception of the females of *Sarcophaga s. lato* (Byrd & Castner 2010, Tan et al. 2010; Meiklejohn et al. 2011; Jordaens et al. 2013). However, the identification of early developmental stages and puparia is often difficult (Zhu et al. 2013). In such cases, it is a standard procedure to collect eggs and first instar larvae and rear them to the adult stage (Amendt et al. 2007). However, this is time-consuming and delays investigations. Moreover, in many instances, the rearing is impossible, for example, when only dead insect specimens are collected, or when the specimens have been stored in preservative liquid.

Other problematic samples include empty puparia, as they are often incomplete and are therefore difficult to determine (Amendt et al. 2010; Meiklejohn et al. 2013). In such cases, species identification based on genetic markers can be of vital importance, and, to do so, it is essential to select the appropriate method for DNA extraction from various types of material (Linacre et al. 2011). Numerous methods and commercial kits for DNA isolation are currently available, but it is always advisable to verify their suitability and ease of use prior to implementation in the daily laboratory routine. A comparison of the relative time-efficiency and cost-effectiveness of DNA extraction methods is also appropriate, since a relatively huge amount of material is processed.

The importance of the use of molecular markers (suitable parts of nuclear and/or mitochondrial DNA) in forensic biology is increasing (Schilthuizen et al. 2011; Rolo et al. 2013). With regard to forensic insect species identification, a system of 'DNA barcoding' has been adopted, which is based on a segment of approximately 650 bp of a mitochondrial gene encoding cytochrome oxidase subunit I (Smith 1986; Hajibabaei et al. 2007); this has been proved useful as a species identification tool (Hebert et al. 2003; Hajibabaei et al. 2006; Nelson et al. 2007). Other applications of DNA analysis in forensic entomology include the identification of insect viscera contents and population genetic structure of forensically important insects (Wells & Stevens 2008).

The main goal of the present study was to test several methods of DNA extraction, and to verify the use of an appropriate method for the identification of eggs, first instar larvae, and empty puparia in selected forensically important flies, which is the essential first step in species identification using DNA barcoding. We used samples from the most common species found in the Czech Republic that are also widespread across the whole of Europe and that already have a record in the BOLD database. The samples were freshly collected from freely exposed dead animal bodies, and no significant quality differences between specimens were expected.

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Materials and methods

Specimens

Two sets of 16 samples of each developmental stage (eggs, the first instar larvae, and an empty puparia) were used. Eggs and larvae from the first set belonged to *Lucilia ampullacea* Villeneuve, 1922 (Diptera: *Calliphoridae*), and the empty puparia belonged to *Sarcophaga* (*Liopygia*) *argyrostoma* (Robineau-Desvoidy, 1830) (Diptera: *Sarcophagidae*). The second sample set contained eggs and larvae of *Protophormia terraenovae* (Robineau-Desvoidy, 1830) (Diptera: *Calliphoridae*) and the empty puparia of *Lucilia sericata* (Meigen, 1826) (Diptera: *Calliphoridae*). Samples of immature stages were reared from wild-caught females at the Institute of Criminalistics Prague, Czech Republic; they were stored in 98% ethanol with no contact with any other chemicals, and were kept in a freezer at −20 °C.

DNA extraction

Four DNA isolation procedures were used for the DNA extraction from three types of specimens. Three of the procedures used different commercial kits, and one represented a simple procedure using home-made extraction buffer. Whole specimens (30 mg of tissue) were used for all isolations. Prior to beginning the isolation, each sample was left to air-dry on filter paper to eliminate the ethanol. They were then directly manually homogenized in test tubes, using a plastic pestle provided with the microfuge tube in the correct buffer, according to the selected method.

1. Method: Simple extraction buffer protocol (Půža et al. 2015)
   A mixture for one sample contained: 17.7 µl ddH_2O, 2 µl 1 × PCR buffer with $MgCl_2$, 0.2 µl 1% Tween 20, and 0.1 µl proteinase K 100 mg/ml.
   A mixture with a homogenized sample was left in a freezer (−20 °C) for 20 min, and was then incubated at 56 °C for 90 min and then at 95 °C for 8 min.
2. Method: DEP-25 DNA Extraction Kit (Top-Bio, Praha, Czech Republic)
   The DEP-25 DNA Extraction Kit is a two-component reagent kit for the extraction of genomic DNA of various origins, and allows rapid two-step processing of samples.
3. Method: DNA Lego Kit (Top-Bio, Czech Republic)
   The column DNA Lego Kit is a universal modular system for isolation of DNA from various sources. The method is based on the capacity of silica surfaces to bind DNA in the presence of chaotropic agents.
4. Method: QIAamp DNA Mini Kit (QIAGEN, Manchester, UK)
   The manufacturer's modified protocol for tissue was used. The volume of proteinase K was increased by 5 µl per sample for puparia, and the samples were incubated overnight (12 hours) on a rocking platform. With regard to the other samples, the amount of proteinase K recommended in the instructions was used, and the length of incubation was 4 hours.

PCR amplification

PCR amplification of the standard DNA barcoding marker of each of the DNA extractions was conducted with a set of two universal primers LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Folmer et al. 1994). The PCR reaction mix for one sample contained 7.125 µl ddH_20, 1.25 µl 10× buffer, 0.5 µl BSA, 1 µl dNTP, and 0.75 µl of each primer, as well as 0.125 µl Taq Unis DNA polymerase (Top-Bio, CR). The mixture was vortexed and then 1 µl of the extracted DNA (not quantified) was added, after which the samples were loaded into a XP-Cycler 48/96/384 G (Bioer Technology, Zhejiang, China). The following protocol was applied: 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 47 °C for 35 s, and 72 °C for 45 s; 72 °C for 2 min; at 4 °C to finish. The PCR products were checked using standard agarose gel electrophoresis (in TAE buffer), and were stained with ethidium bromide.

Sequencing

Prior to sequencing, the amplicons were enzymatically purified with Exo I and Fast AP mixture. The purified products were directly sequenced with the forward primer (LCO) by SEQme (SEQme Ltd., Dobris, Czech Republic). The chromatograms were manually edited with Bioedit v.7 (Hall 1999) and confirmed as being dipteran DNA, using the GenBank BLASTn search and species identification module of the BOLD database.

Results

The outcomes of the PCR amplification are summarized in Table 1 and Figure 1. The simple extraction buffer protocol (1) was successful for half of the eggs and for the samples of the first instar larvae, but was unsuccessful for the empty puparia. The DEP-25 DNA Extraction Kit (2) extracted DNA from the first instar larvae, but not from the eggs or empty puparia. The Lego DNA kit (3) was successful with regard to the eggs and the first instar larvae, but not for the empty

Table 1. Summary of the identification.

	Egg				First instar larvae				Puparium			
DEP-25	+/+	+/+	−/+	−/−	+/+	+/+	+/+	+/+	−/−	−/−	−/−	−/−
Lego	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	−/−	−/−	−/−	−/−
Buffer	−/−	−/+	+/+	+/+	−/−	−/+	+/+	+/+	−/−	−/−	−/−	−/−
QIAgen	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/−

Two sets of species were used for every method and developmental stage, those sets were signed with + and −, depending on results of identification. Plus sign means that identification was successful, minus sign means failed identification (either no PCR product or problems with sequencing). First set combined eggs and larvae of Lucilia ampullacea (Diptera: Calliphoridae) and empty puparia of Sarcophaga (Liopygia) argyrostoma (Robineau-Desvoidy, 1830), second set contained eggs and larvae of Protophormia terraenovae (Diptera: Calliphoridae) and empty puparia of Lucilia sericata (Diptera: Calliphoridae).

puparia, even when an extra 5 µl of proteinase K was added to the extraction mixture. The QIAamp DNA Mini Kit (4) was successful for all sample types, but it was necessary to increase the amount of proteinase K and extend incubation time, preferably overnight, for the empty puparia. The sequences obtained by sequencing the PCR products from the samples were identified using the BOLD Species Identification Engine, with an almost 100% match.

The cost-effectiveness of all methods and the isolation times were markedly different. The cheapest was the simple extraction buffer protocol developed by Půža et al. (2015), with a price of one euro for 100 samples. This was followed by the DEP-25 DNA Extraction Kit, which cost 11 euros for 100 samples. These two methods represent simple extraction procedures that contain up to five steps only. DNA isolation with the remaining two methods is more expensive, as well as more time-consuming (Figure 2). The DNA Lego Kit costs 50 euros for 100 samples, and isolation using the QIAamp DNA Mini Kit costs 370 euros for 100 samples (Figure 3). These protocols are more time-consuming than previous methods, but the resulting isolated samples are cleaner. The latter method was also the only one that was successful with empty puparia, but the extraction takes far longer, and is also more expensive, particularly with the increased consumption of proteinase K.

Discussion

This is the first study that has focused on DNA extraction from different developmental stages of forensically important flies in the Czech Republic. Four DNA extraction methods were tested – the simple extraction buffer protocol and three commercial kits, two of which were manufactured in the Czech Republic. The simple extraction buffer mixture is effective for very fresh samples, but is probably not suitable for material stored for a longer time. The advantages of this method are its simplicity, short time requirements, and low cost. However, it is not highly reliable, and when it fails, the

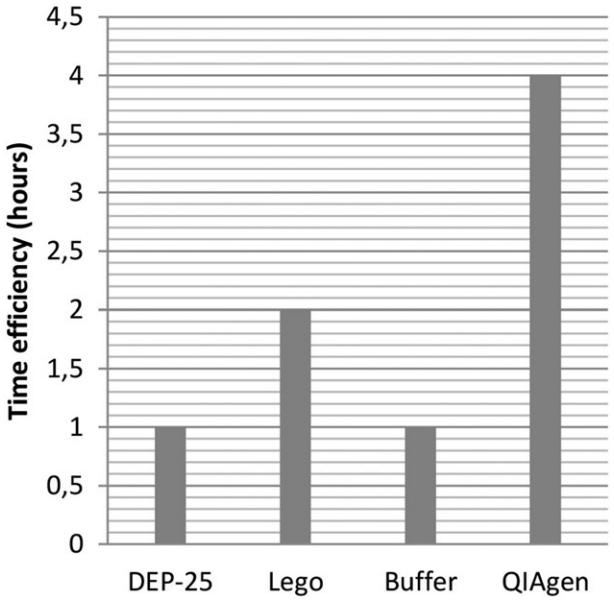

Figure 2. Extraction methods time efficiency (hours).

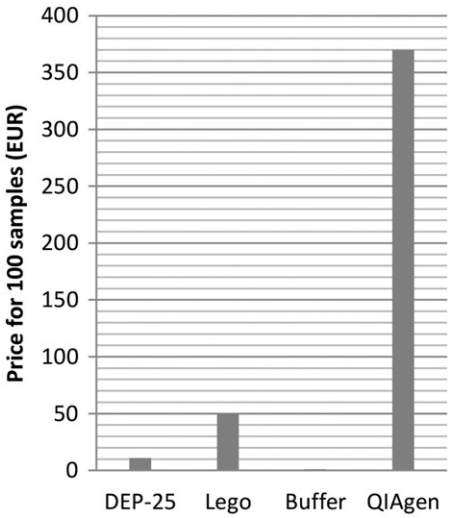

Figure 3. Extraction methods cost efficiency (EUR).

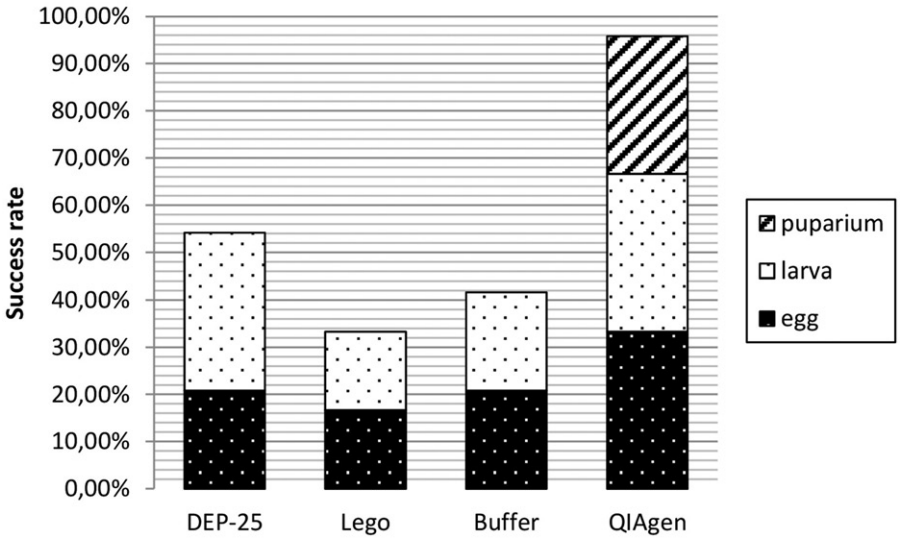

Figure 1. Extraction methods success rate, shaded parts designate different starting sample types.

material is effectively lost. Extraction from empty puparia was problematic with both Czech-produced kits (the DNA Lego Kit and the DEP-25 DNA Extraction Kit), which is in contrast with the successful extraction of DNA from the first instar larvae samples using these methods. These kits are therefore suitable for simple, DNA-rich samples. The most expensive QIAamp DNA Mini Kit performed the best of all the tested methods, although it did not yield good results with regard to DNA extraction from empty puparia when using the conditions recommended in the manufacturer's protocol. We therefore increased the amount of proteinase K to 5 µl per sample and extended the incubation time to 12 hours. It was also necessary to homogenize the samples. The QIAamp DNA Mini Kit can be recommended for forensic genetic analyses, which allows for only a single attempt. The disadvantage of this kit is the longer incubation time, although it still has the advantage of having the ability to extract DNA from empty puparia. In some cases, empty puparial samples were affected by fungal contamination, which we detected by comparing the obtained DNA sequences with DNA databases. This problem could be solved by using more specific PCR primers.

In conclusion, the choice of DNA extraction methods depends on the amount and quality of the sampled material. If only a few specimens are available, the QIAamp DNA Mini Kit appears to be the most suitable method, while less laborious and more rapid methods can be used if more surplus material is available. If a cheap and fast method for multiple samples is required, the simple extraction buffer protocol or the DEP-25 method, which worked well in the majority of cases, may also be used.

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