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Technical note

A combined protocol for identification of maggots of forensic interest

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ABSTRACT

In Forensic Entomology the estimation of the age of insects is used for the estimation of the minimum post-mortem interval. As insect development is temperature dependent and species specific, a correct species identification is therefore fundamental. In the majority of cases the molecular identification is based on a destructive approach.
In this paper a working protocol for molecular identification of fly larvae without affecting the anatomical characters used for morphological identification is presented.
The suggested technique allows the preservation of the larval exoskeleton and of the unused soft tissues in the same vial allowing a repetition of both the morphological and molecular identification and reducing the risk of loss of the evidence. This method also allows the possibility of measuring the size of the specimens before their morphological and biomolecular characterization.
In order to demonstrate that this technique can be applied on maggots of a large spectrum of dimensions it has been tested and validated using larvae of different size from ~1.7–1.3 cm [*Calliphora vomitoria* and *Lucilia sericata* (Diptera: Calliphoridae)] to ~10–6.5 mm [*Musca domestica* (Diptera: Muscidae) and *Megaselia scalaris* (Diptera: Phoridae)]. The importance of a unique identifier and of a complete database with all the specimen information (origin, sample size, identification, etc.) is also discussed.

1. Introduction

In Forensic Entomology the estimation of the age of insects is used for the estimation of the minimum post-mortem interval (mPMI) [1,2]. Insect development rate is mainly temperature dependent and species specific [3,4], so correct species identification is fundamental for any other consideration [5].

Species identification is generally performed by two different approaches: the classic morphological identification, and the more recent molecular approach based on sequencing and comparison of specific mitochondrial or nuclear nucleotide regions [e.g. 6,7]. Several genes have been used for insect identification, but the most commonly used is the mitochondrial gene coding for the cytochrome c oxidase I (COI), the main subunit of the cytochrome c oxidase complex [8]. Despite the continuous update of the public molecular banks the number of sequences available for different genes and species is still incomplete [6,9]. For this reason in some cases the possibility to verify or confirm the molecular identification using a morphological approach is essential. In the majority of cases the molecular identification is based on a destructive approach (for exceptions see [10,11,12,13]) and, for this reason, depending on the legal system, it requires a specific authorization of the authority in charge of the case, especially when only a few larvae or insect fragments are available.

In Forensic Entomology, and in Forensic Sciences in general, the correct collection and preparation of the samples is crucial before any further analysis. The preparation of the biological samples depends on the nature of the samples (e.g. body fluids, human tissues, insects, plants, microbes) and on the tests that have to be performed on them.

In Forensic Entomology preparation of adult insects mainly follows three different pathways derived from the standard entomological preparation and storage methods: dry preparation, storage in preservative solutions, and microslide mounting [14]. These preparation/storage methods allow an easy view of the characters used for species identification, despite genital extraction being sometimes required.

Immature stages of flies, because of their structure and the paucity of external characters, require in general a more complicated preparation to emphasize the systematic characters.

The visualization of the systematic characters of fly larvae requires that their diaphanisation is performed, after fixation, using chemicals able to macerate (e.g. NaOH, KOH) or to make the opaque soft tissues transparent (e.g.: methyl salicylate) [15] without affecting the sclerotized structure like spiracles, cephaloskeleton and exoskeleton where diagnostic spines are present.

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The NaOH/KOH dissolution of the tissues is a destructive method to the soft tissues of the organism that are the source of DNA used for molecular identification.

In order to dissolve the opaque tissues using NaOH/KOH, while preserving the sclerotized parts, two methods can be applied: 1) perforation of the cuticle with a pin to facilitate the NaOH/KOH diffusion into the larval tissues (Fig. 1a) and 2) the dissection of the larva in segments (Fig. 1b) which, after permanence in the solution, will be individually mounted on slides, convex slides for the pseudocephalon region, flat slides for the other parts [16]. In the first approach the specimen maintains its integrity but in case of an incomplete break up/dissolution of the soft tissues, the observation of some details can be difficult. In the second case the sample is divided in different fragments with a better diaphanisation of the soft tissue.

The normal procedure for DNA extraction requires the grinding of the sample to facilitate the DNA transfer from cells to the extraction buffer. However this causes the destruction of the sclerotized characters, which are useful for a morphological identification of the samples.

An alternative method that combines the need for preservation of both the sclerotized and soft tissues is presented here. In addition, the suggested protocol allows the preservation of the specimen's integrity and a complete dissolution of the soft tissues.

In this paper we demonstrate in a systematic way that a molecular identification can be performed several times on the same specimen (larva) without affecting the anatomical characters used for morphological identification.

The suggested technique allows the preservation of the larval exoskeleton and of the unused tissues in the same vial. The storage of them in ethanol, as preservative solution, will allow the repetition of both the molecular and morphological analyses and will reduce the risk of loss of the evidence.

In order to demonstrate that this technique can be applied on maggots of a large spectrum of dimensions it has been tested using larvae of different size from ~1.7–1.3 cm [*Calliphora vomitoria* and *Lucilia sericata* (Diptera: Calliphoridae)] to ~10–6.5 mm [*Musca domestica* (Diptera: Muscidae) and *Megaselia scalaris* (Diptera: Phoridae)].

2. Materials and methods

In order to verify and validate the proposed method and in order to identify the minimum amount of larval soft tissue that can be easily used for molecular identification in a molecular biology laboratory, the following steps were followed. All the dissections, extractions and amplification were performed in triplicate.

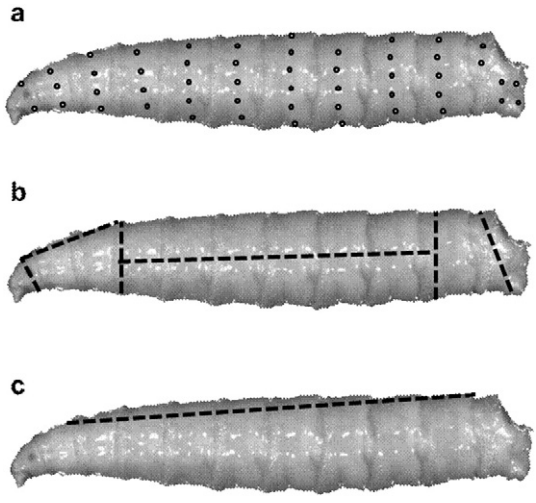

Fig. 1. Example of maggot preparation for morphological examination. a) Perforation of the cuticle with a pin; b) cuts suggested by K. Szpila (*in litteris*). This method is particularly useful for detailed morphological analyses; c) a single cut on the dorsal part of the larva is suggested. Larvae are submerged in NaOH/KOH after being cut or pinned.

2.1. Larval weighting

Thirty specimens of each species were randomly selected and, after hot water fixation for 1 min [17], weighed before and after cuticle removal in a Precisa125A SCS Digital Analytical Balance scale. Data are presented as average ± standard deviation. All statistical analyses were performed using the IBM SPSS 22.0 software (IBM® SPSS® Statistics, Armonk, NY).

2.2. Sample preparation

Post-feeding third instar larvae were collected in triplicate for each of the four forensically important fly species used for this experiment. *C. vomitoria*, *L. sericata*, *M. domestica* larvae were purchased from a fishing tackle shop in Huddersfield (West Yorkshire, UK) and stored in a refrigerator at 4 °C in covered glass jars until use. The *M. scalaris* larvae used were derived from a population which has been bred in the author's laboratory since 2011. Morphological identification was performed using the appropriate identification keys [16,18,19,20] both on the larvae and on the adults which emerged after breeding the immature stages at 25 °C in 12:12 LD condition.

Before any further preparation, larvae were fixed in hot water as described by Adams and Hall [17], both for the morphological and the molecular identification.

A longitudinal cut is performed on the dorsal part of the larva using a pin, a scalpel or corneal scissors (Fig. 1c). The two cuticle edges are stretched apart with fine point tip tweezers and the soft tissues are raised from the cuticle except for the anterior and the posterior regions. Using a scalpel the central part of the body can easily be removed from the two extremities and preserved in ethanol or immediately used for DNA extraction. The remaining cuticle with the two extremities where the soft tissues are still present can be diaphanised in a NaOH/KOH solution. After diaphanisation the specimens can be temporarily mounted between two microscopy slides in a glycerol drop or in a permanent way after dehydration through 70, 80, 90 and 99.5% ethanol in Euparal.

The cuticle and the soft tissues not used for DNA extraction can be stored together in a vial in a > 95% ethanol solution. This will allow the repetition of both the morphological and molecular identification in case of discussion about the accuracy of the species identification (Fig. 2).

After skin removal, soft tissues were cut using a surgical scalpel under a Leica M60 stereo microscope (Wetzlar, Germany). Soft tissues were progressively halved until the smallest fragment was obtained with a conventional surgical scalpel. The smallest obtained soft tissue larval fraction was 1/1024 for *C. vomitoria*, 1/512 for *L. sericata*, 1/128 for *M. domestica* and 1/64 for *M. scalaris*.

2.3. DNA extraction and quantification

DNA was extracted from the entire larvae and from any larval fraction of each species using the QIAamp DNA Mini Kit (QIAGEN, Redwood City, CA, USA). The manufacture protocol was partially modified in order to increase the quality of the reaction by adding 4 μl of RNAse A (4 mg/ml) after the enzymatic digestion by Proteinase K (100 μg/ml) which took three hours (PROMEGA, Madison, Wisconsin, USA). Samples were left at room temperature for 5 min and incubated at 37 °C for 30 min. RNAse A was then inactivated at 70 °C for 10 min. AL Buffer was added and successive steps were undertaken according the manufacturer's instructions. The extracted DNA, eluted in 400 μl of nuclease-free water, was quantified via Nano Drop™ 2000/2000 C (Thermo Scientific, Waltham, Massachusetts, USA).