Forensic Science International 275 (2017) 171–177

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Technical Note

Validating sonication as a DNA extraction method for use with carrion flies

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ARTICLE INFO

Article history:
Received 25 October 2016
Received in revised form 27 January 2017
Accepted 15 February 2017
Available online 20 March 2017

Keywords:
Sarcophagidae
Calliphoridae
Phylogenetics
Non-destructive DNA isolation
Forensic entomology

ABSTRACT

Entomological evidence can be critical in establishing a postmortem interval estimate. DNA-based species identification can be an extremely valuable tool for forensic entomology. The problem of processing samples in a consistent, cost-effective manner that retains the morphological attributes of the specimen for vouchering has led us to investigate sonication as a primary means of non-destructive DNA extraction from carrion flies. We analyze the efficacy of this technique and compare it to an established DNA extraction technique — the Qiagen DNeasy tissue kit. Our results indicate that sonication produces a significant reduction in the sequence length and lower PHRED quality scores when compared to sequences using DNA obtained using the DNeasy kit, but species identification and phylogenetic inferences between sonication and DNeasy extractions are equivalent.

1. Introduction

Carrion-feeding Diptera, mainly from the families Calliphoridae (blow flies) and Sarcophagidae (flesh flies), are increasingly important in medico-legal death investigations as a means to determine the postmortem interval estimation (PMI; [1]). Investigations of carrion-feeding Diptera are not only relevant for human decedents [2], but also for the illegal use of natural resources [3], and cases of animal or human neglect [4,5]. With the increased focus on these flies, there is an increased need to identify the (mainly) immature larval specimens that are recovered from bodies. Traditionally, trained entomologists carry out species identification of larvae using either the limited larval keys or by rearing the immature larvae to adulthood so that adult morphological character keys (e.g., Ref. [6]) can be used in the identification of the specimens. These means of specimen identification can take several weeks as larvae are reared to adulthood, and even then, identifications may not be specific enough to be useful [7,8]. Identification using morphological keys can be difficult and some characters used in keys may not be well visible, remain intact, or require previous training. These problems have led to the development of molecular techniques, specifically using DNA-based techniques [e.g.,9&13], to identify specimens with improved speed and accuracy.

Processing larvae from carrion potentially results in a large number of specimens, so that the best possible PMI can be obtained [14]. If at all possible, the means of analyzing the specimen should be non-destructive, so that the specimen can be vouchered for future reference [11], or as evidence at trial. Some techniques have been developed that submerge the specimen in a lysis buffer, without any grinding or other tissue disruption, followed by removal of the intact specimen and DNA purification from the remaining buffer [15–17]. We sought to remove even the need for buffers from the extraction process, simplifying and reducing the cost even further. To this end, we investigated sonication as a method of DNA extraction. Sonication was recently tested as a method of non-destructive DNA isolation for the dipteran families Simuliidae (blackflies; [18]) and Culicidae (mosquitos; [19]), and has also been used for bacterial DNA extraction [20].

Sonication involves the use of ultrasound wavelengths for various purposes, usually to penetrate a medium and measure the reflection signature (bats [21]; sonography of fetuses in wombs [22]) or supply focused energy (ultrasonic lithotripsy of gallstones; [23]). Ultrasound is a mechanical vibration (cyclic sound pressure) that occurs above the range of human hearing, roughly starting between 16–20 kHz [24]. The medical community has utilized ultrasound technology historically [22] and as such there is a larger amount of literature that reviews the uses of ultrasound within that context [21]. Most medical use of ultrasound involves

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http://dx.doi.org/10.1016/j.forsciint.2017.02.014
0379-0738/

application of the technology through an aerial medium. However, if ultrasound is applied through an aqueous medium normally therapeutic levels become destructive to DNA [24]. Aqueous ultrasound application damages DNA by either cavitations or mechanical/thermal degradation [24]. Damage to DNA includes the rupturing of the single and double nucleotide strands involved in the DNA helix, the breaking of hydrogen bonds, and/or the disruption of base pairing [24]. Still, the results of aqueous ultrasound application are impressive and fast; shearing DNA macromolecules to less than 2000 bp fragments in under two minutes and rendering 100 bp or smaller fragments within five minutes [24].

The benefits of using sonication to recover DNA are potentially significant. DNA breakdown can be started and stopped nearly instantly by turning off the sonicator and is repeatable if more DNA is desired. The DNA recovery process does not require the addition of chemical compounds, thus resulting in almost no expendable reagent costs. Sonication significantly decreases the time spent extracting DNA; reducing all procedural waiting (lysis steps, etc.) with a protocol that takes less than five minutes to complete and little to no damage to the overall structure of the specimen [18].

Daubert [25] and Kumho [26] have emphasized that error rates for techniques used in a forensic setting must be known, testable, peer-reviewed, and accepted by the scientific community using the technique. Because of this, it is essential that we understand the reliability of the sonication extraction technique compared to a more traditional DNA extraction technique. For our "traditional technique" we used Qiagen's DNeasy blood and tissue kit (Qiagen Group, catalogue number 69506).

We compared the DNA extracted by sonication to that extracted using DNeasy by performing both methods on (separate) single legs from a total of 19 specimens representing 16 different species of flesh flies (family Sarcophagidae) and blow flies (family Calliphoridae). We used each DNA extraction as the template for a PCR reaction, in which we amplified a portion of the 3′ end of the mitochondrially encoded gene cytochrome oxidase subunit I (COI), followed by sequence analysis of each PCR product. To compare the two DNA sources, we: (1) compared the overall sequence quality, as measured by the accumulated chromatogram PHRED quality-scores [27–29]; (2) compared the recovered (untrimmed and trimmed; see Section 2.4 for how these are defined) sequence length; (3) compared the sequences themselves; (4) included all sequences obtained using both extraction procedures in a single phylogenetic analysis. We did not consider the question of whether sonication preserves morphological structure in flies, because Hunter et al. [18] had already done so to our satisfaction.

2. Materials and methods

2.1. Specimens

Specimens were collected using a carrion bait net trap [30] or from malaise trapping carried out in a residential neighborhood backyard. Specimens from each sampling location were bulk frozen together for several months (usually longer than three, but sometimes as long as a year) until they were sorted. Specimens were thawed, identified to species [6] and pinned after removing three legs from the left side of the specimen. Thereafter, the legs were placed in individual 1.5 ml tubes and stored at −20 °C until DNA extraction.

2.2. DNA extraction

Each specimen underwent two DNA extractions. First, a single leg from a specimen was used for sonication (described below). Second, another leg from the same specimen was subjected to DNA extraction using Qiagen's DNeasy kit (Qiagen Group, catalogue number 69506), following manufacturer's protocols.

Our sonication method comprised the following steps:

1. Specimen legs were placed in 50 μl of DNA grade ultra-pure water (Fisher Scientific: Fair Lawn, New Jersey 07410; Lot number: 075078; CAS number: 7732-18-5) in 200 μl tubes. Specimen legs were completely submerged in the water to ensure proper aqueous sonication.
2. Tubes containing a specimen leg were placed into a sonicator bath (Branson model number 1510R-MT; Branson Ultrasonics Corp., Eagle Road, Danbury, CT, 06813, USA) and maintained below the sonicator bath water level manually for the sonication event. The sonicator aqueous solution was an ice bath of de-ionized water and chipped ice from standard ice machines in roughly a 3 to 1 ratio. Specimen sonication lasted 1–2 min, but no longer to prevent DNA damage and retain suitable DNA fragment sizes [24]. The 1510R-MT sonicator produces ultrasound at a frequency of 42 kHz ±6%, which falls within the range set forth by Elsner and Lindblad [24] for fragmenting DNA in an aqueous solution.
3. Mix tube either using a vortexer or flicking the tube by hand for about 30 s.
4. Using a sterile pipette, removed specimen leg from water (prevents specimen decomposition).
5. The sonicated DNA was placed at 2 °C for storage and subsequent use.

We repeated this procedure up to three separate times with the same specimen to extract more DNA if needed. The average time for the whole sonication technique was about five minutes, and multiple sonications could be performed simultaneously by using strip tubes (SnapStrip PCT Tube, ISCBioExpress, Cat# T-3035-1).

2.3. PCR and nucleotide sequencing

Polymerase chain reaction (PCR) was used to amplify the region of the mitochondrial DNA (mtDNA) COI gene that corresponds to positions 1800-3000 in the *Drosophila yakuba* mitochondrial genome (Genbank accession number NC_001322 [31]). Amplification of the desired mtDNA COI locus was achieved using Rapid Cyclers (Idaho Technologies) as outlined previously [11]. PCR products were visually assessed for minimum DNA concentration with agarose gel electrophoresis using the semi-quantitative ladder &x3D5;X174 DNA/*Hae* III (Promega, Madison, WI) If the concentration was at least 8 ng/μl, PCR products were then sent for purification and automated Sanger-sequencing at the High-Throughput Genomics Unit (WTC East, Suite 6002211 Elliott Avenue Seattle, WA 98121).

2.4. Trimming nucleotide sequences

In automated Sanger-sequencing, the sequence fragments are labeled with dyes that fluoresce when a laser attached to a diode passes over them. Computer analysis creates a profile (i.e., trace) where the intensities of the nucleotides are measured [28]. The program PHRED measures the accuracy of the base selected (i.e., called) in the presence of imperfections of the sequencing (e.g., unreacted dye-primer or terminator, migration of short fragments, etc.). PHRED quality scores are an accepted way to determine the accuracy of the base called, with a PHRED score of 20 signifying that the base called is 99% accurate [27,29]).

The untrimmed length of the sequence was defined as the total length of bases called by the sequencing platform, regardless of quality. Trimmed sequence length was defined by the following systematic process in an attempt to remain unbiased in the