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DNA barcoding identifies all immature life stages of a forensically important flesh fly (Diptera: Sarcophagidae)

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Abstract

Carrion-breeding insects, such as flesh flies (Diptera: Sarcophagidae), can be used as evidence in forensic investigations. Despite their considerable forensic potential, their use has been limited because morphological species identification, at any life stage, is very challenging. This study investigated whether DNA could be extracted and cytochrome oxidase subunit I (COI) barcode sequences obtained for molecular identification of each immature life stage of the forensically important Australian flesh fly, Sarcophaga (Sarcorohdendorfia) impatiens (Walker). Genomic DNA extracts were prepared from all larval instars and puparia. Amplification of the barcoding region was successful from all extracts, but puparia amplicons were weak. All sequences were identified as S. impatiens with 99.95% confidence using the Barcoding of Life Database (BOLD). Importantly, crop removal was necessary to eliminate PCR inhibition for specimens from late second and early third instars. Similar results are expected for immatures of other carrion-breeding species, enhancing the use of evidence from immature flies in forensic investigations.

Keywords

all, identifies, sarcophagidae, diptera, fly, flesh, important, barcoding, forensically, dna, stages, life, immature, CMMB

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DNA barcoding identifies all immature life stages of a forensically important flesh fly (Diptera: Sarcophagidae)

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ABSTRACT:

Carrion-breeding insects, such as flesh flies (Diptera: Sarcophagidae), can be used as evidence in forensic investigations. Despite their considerable forensic potential, use has been limited because morphological species identification, at any life stage, is very challenging. This study investigated whether DNA could be extracted and COI barcode sequences obtained for molecular identification of each immature life stage of the forensically important Australian flesh fly, *Sarcophaga* (*Sarcorohdendorfia*) *impatiens* (Walker). Genomic DNA extracts were prepared from all larval instars and puparia. Amplification of the barcoding region was successful from all extracts, but puparia amplicons were weak. All sequences were identified as *S. impatiens* with 99.95% confidence using the Barcoding of Life Database (BOLD). Importantly, crop removal was necessary to eliminate PCR inhibition for specimens from late second and early third instars. Similar results are expected for immatures of other carrion-breeding species, enhancing the use of evidence from immature flies in forensic investigations.

KEYWORDS: forensic science, forensic entomology, mitochondrial DNA, DNA barcoding, species identification, immatures, Diptera, Sarcophagidae

Insect specimens collected at crime scenes can be used to estimate the minimum post mortem interval (PMI), season of death, presence of toxins or corpse transportation (1). For accuracy, forensic entomologists, where possible, utilise evidence from initial corpse colonisers, which include carrion-breeding species of blow flies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae) (2). Flesh flies can provide precise PMI estimations as they are viviparous (lay live larvae), producing immatures that are ready to start feeding immediately on the corpse and contribute to its decomposition. Despite the considerable forensic potential of sarcophagids, their use to date in forensic investigations has been limited in comparison to calliphorids, as accurate species-level morphological identification at any life stage is very difficult (1-4).

By contrast, family-level identification of flesh flies is relatively straightforward. Adults generally share the common features of grey-black longitudinal stripes on the thorax, a heavily bristled body and a tessellated abdomen (5). Larvae have their posterior spiracles in a deep cavity on the last abdominal segment. Adult species-level identification is difficult even for taxonomic experts, because it requires close examination of subtle morphological variation (6). Identification of immature specimens to the species-level is even more challenging, as there are fewer characters to draw upon, and larval and pupal descriptions for most carrion-breeding species are incomplete. Sarcophagids collected from crime scenes are generally therefore reared to adults to assist with taxonomic identifications, however this is not always possible (2, 5, 6).

Recent studies have shown that the identification of adult flesh flies can be achieved using molecular methods. Mitochondrial genes have been a focus, including cytochrome oxidase subunit I (COI) and NADH dehydrogenase subunit 4 (ND4) (7, 8). Additionally, the 658 bp

barcoding region of COI has been shown to effectively distinguish between Australian Sarcophagidae (9), along with the flesh fly *Sarcophaga* (*Liopygia*) *argyrostoma* (10), blow flies of eastern Australia (11), butterflies (Lepidoptera) (12), blackflies (Diptera: Simuliidae) (13), mayflies (Ephemeroptera) (14) and tachinids (Diptera: Tachinidae) (15).

Despite the success of barcoding, it is important to consider the coamplification of nuclear mitochondrial psuedogenes (NUMTs), non-functional copies of mitochondrial genes found in the nucleus (16, 17). The inclusion of NUMT sequences in barcoding studies can overestimate the number of species resolved. To minimise the chance of obtaining NUMTs and allow for accurate evaluation of the barcoding approach, it has been suggested that researchers should extract only mitochondrial DNA and use taxon-specific primers for amplifications (18, 19).

Taking into account the success of DNA barcoding for the recognition of adult Australian sarcophagids, this study investigated whether barcoding sequences could be obtained from immature flesh flies of all life stages (first-, second- and third-instar larvae, along with puparia). If possible, this technique could be used by forensic entomologists to make accurate species identifications of flesh flies in cases where the immature evidence is incomplete, has been killed prior to rearing, or only the puparia remain.

Materials and Methods

Species and culture rearing

A laboratory culture of the forensically important flesh fly species, *Sarcophaga* (*Sarcorohdendorfia*) *impatiens* (Walker) (Mt Keira, NSW, Australia), was used to obtain experimental immatures. This endemic species was chosen as it is a carrion-breeder, is

frequently collected from crime scenes and has larvae that cannot be identified morphologically. Gravid females laid larvae on sheep's liver; the larvae were subsequently separated into plastic weigh boats in replicates of 50. Each replicate was provided with sufficient liver for growth and placed in a rearing container on a bed of wheaten chaff. To obtain first-instar larvae, immatures were allowed to develop at 25^{o}C for 12 h, after which they were killed in boiling water. This is the standard approach for killing maggots (20), and also assists with the removal of residual feeding substrate from the skin. The larvae were then stored in absolute ethanol to preserve their DNA for molecular analysis (20). The posterior spiracular slits of each immature were examined using a stereomicroscope (Leica MZ7$_5$; Leica Microsystems, Australia) to determine the specific developmental stage (first, second or third instar). This procedure was repeated for larvae aged 24, 48, 72, 96, and 120 h, until pupation occurred at 144 h. Upon pupation, pupae were placed into transparent containers and monitored closely until emergence. Immediately following emergence, intact puparia were collected and placed into open Petri dishes.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from the immature specimens using a previously published protocol (16). Triplicate extractions were performed for each time point for whole *S. impatiens* larvae and for whole puparia. These extractions were carried out immediately following emergence, and after both one and two weeks. As some puparia were missing the puparial cap and lining following adult emergence, any remnants of these were removed for standardisation of the samples prior to extraction. Excess ethanol was evaporated from the tissues and they were subsequently ground with a pestle in a sterile 1.5 mL tube. Ground immature tissue was incubated with an extraction buffer, which consisted of 50 mM Tris–HCl (pH 8), 20 mM ethylenediaminetetraacetic acid (EDTA) (pH 8), 400 mM NaCl, 1.0%

sodium dodecyl sulphate (SDS) (pH 8) and 0.2 mg proteinase K (added just prior to extraction). The final volume of the extraction buffer was proportional to tissue size and ranged from 200-1000 µL. Importantly, to assist with complete tissue digestion, the final SDS and proteinase K concentrations reported here were higher than those given by Sunnucks and Hales (16). Each extraction was incubated overnight at room temperature, after which the liquid was decanted into a sterile 1.5 mL tube and sufficient 5 M NaCl added to give a final NaCl concentration of 1.1 M. Upon mixing, the reactions were incubated at room temperature for 10 min to precipitate any undigested proteins. The extractions were then centrifuged for 5 min at 16,100 x *g* (Eppendorf 5415D microcentrifuge; Crown Scientific, Australia) and the resulting supernatants removed and placed into fresh 0.6 mL screw-cap tubes, with an equal volume of absolute ethanol (95%). The mixtures were then incubated at room temperature for 15 min, and spun at 13,400 x *g* for 15 min. The supernatants were removed and the DNA pellets washed with 200 µL of ice-cold 70% ethanol and spun at 13,400 x *g* for 15 min. The ethanol wash was drained and the DNA pellets were air dried. Each DNA pellet was resuspended in 50 µL of fresh TE [1 mM Tris–HCl (pH 8), 0.1 mM EDTA] solution and stored at 4^{o}C. The 658 bp barcode region of COI was amplified and sequenced with barcoding primers specific to Diptera, following the protocols described in Meiklejohn *et al.* (2011) (9).

DNA sequence analysis

Sequence electropherograms were verified and edited using ChromasPro Version 1.33 (Technelysium Ltd., Australia; available online at www.technelysium.com.au/ChromasPro .html). Consensus sequences were submitted to the Barcoding of Life Database (BOLD; available online at www.boldsystems.org) for identification. Each sequence was uploaded into both BOLD and GenBank (accession numbers JN231257 – JN231282). Sequences from

all immatures were aligned to an adult *S. impatiens* reference sequence using BioEdit Sequence Alignment Editor Version 7.0.5.3 (21), to establish any nucleotide substitution between individuals and immature life stages.

Results and Discussion

COI amplification

We examined whether DNA extracted from various immature stages of *S. impatiens* could be used to amplify the COI barcoding region using the primers specific to Diptera. Larval immatures from 12, 24, 96 and 120 h developmental time points strongly amplified the 658 bp region (Figure 1a). This result correlates with studies by Cainé *et al.* (2006) who extracted DNA from 141 maggots and successfully amplified both 305 and 519 bp portions of COI for the identification of numerous blow fly species (22).

Interestingly, the barcode region was unable to be consistently amplified from 48 and 72 h larval extracts, which represent late second and early third instars, respectively (Figure 1a). Unpublished studies have shown that *S. impatiens* larvae show exponential growth at 25^{o}C at these time points, with extended crops having contents that are clearly visible. Campobasso *et al.* (2005) indicated that, throughout different stages of larval development, the crop may become more prominent and its contents could interfere with successful extractions of larval DNA (23). Some published studies recommend removal of the crop prior to larval DNA extraction at any larval stage (24). This was not performed initially, in order to establish whether dissection of this structure was mandatory for amplification of the barcoding region.

As a consequence of the above, the crop was subsequently dissected from three larvae from both the 48 and 72 h time points. Eye surgery scissors were used to make a lateral incision

along the mid-dorsal region of the anterior of each larva. With fine forceps, the incision was then carefully widened and the entire crop pulled out and cut off at its neck. Larvae from which the crop had been successfully dissected were used as tissue in additional DNA extractions. The barcode region was successfully amplified from five out of six of these extracts in which the crop was removed (Figure 1b). This indicated that the presence of the crop in the extraction tissue had prevented the barcode fragment from amplifying. This was also confirmed through an inhibition PCR experiment, in which three separate reactions were set up, each containing different sources of DNA. The sources included either 'failed' 48 or 72 h larval DNA or 'positive' larval DNA, which had previously amplified COI. In tubes one and two, either the 'failed' 48 or 72 h extract was combined with the 'positive' larval DNA in equal volumes. The third tube was a positive control and contained only the 'positive' larval DNA. In the presence of this 'failed' DNA, the 'positive' larval DNA source failed to amplify the 658 bp fragment (Figure 1b). Investigations to identify the specific crop inhibitor were not undertaken, as the objective was to establish an effective approach for DNA extraction, allowing for amplification at these problematic time points. Interestingly, no issues with larval DNA amplification were documented by Cainé *et al.* (2006) (22). As problems in larval extraction were only encountered in this study with late second- and early third-instar larvae, the success by Cainé *et al.* (2006) may be linked to the age of the immatures that they used, which they did not specify. The crop may not have been entirely dissected from the extract that failed to amplify (22).

Whole larvae were used as the tissue for DNA extractions for all stages. In casework, it would always be prudent to instead use only a portion of the larva for DNA extraction, leaving most of the cuticular spines, the cephaloskeleton and the anterior and posterior spiracles intact to assist with identification (25).

Puparial extracts from all time points also successfully amplified the barcode region, however the resultant amplicons were weak (Figure 1b). This result indicated that any possible pupal DNA degradation was not significant enough to prevent amplification in these extracts. Similarly, Mazzanti *et al.* (2010), showed that a 931 bp region of COI-COII was able to be amplified for 77.4% of dipteran specimens <5 years old (26). However, none of the specimens >5 years old amplified the complete region, which was most likely a result of DNA degradation. However, studies by Dhananjeyan *et al.* (2010) on *Aedes* mosquitos showed that ITS markers were unable to be amplified from pupal exuviae collected one to nine days post emergence (27). These inconsistent results for gene amplification between different pupal exuviae of different families of flies could be linked to the targeted gene region (nuclear or mitochondrial genes), but also the chemical composition of exuviae. It has been documented that insect exuviae contain mostly ash, lipids, proteins, metal ions and chitin, with the absence of substantial nucleic acids necessary for DNA-based species identification (27-32). Considering that the puparial cap and lining were removed in our study prior to extraction, and these are thought to be DNA-rich (27), the production of weak or no amplicons for all extracts was expected. It is important to note that the cap and lining were removed for standardisation, as not all puparia remained intact after adult emergence, which is also common in specimens collected in the field (32).

COI sequencing

COI barcoding amplicons from larval and puparial extractions were sequenced successfully, with edited sequences producing full-length consensus sequences 658 bp long. Interestingly, sequence data obtained from the two-week puparial amplicons showed some base ambiguities and decreased signal. This is likely to be due to the sequencing template being

limited in quantity. To verify species identity, all immature sequences were searched against the COI animal database available online at BOLD (www.boldsystems.org). The BOLD-ID output indicated 100% confidence that all immature sequences belonged to the genus *Sarcophaga*, and at least 99.95% confidence that the sequences were *S. impatiens*. This high sequence similarity was also seen after all immature sequences were aligned to an adult sequence, obtained from sequencing the entire mitochondrial genome of *S. impatiens* (Nelson *et al.* unpublished). Some nucleotide substitutions were seen between sequences and immature life stages, but none changed the amino acid sequence of the COI protein upon translation – all substitutions were transitions and were restricted mostly to the third codon position. Importantly, all sequences, even those obtained from weak products, were verified as *bona fide* mitochondrial sequences: signal background in electropherograms was minimal; unexpected insertions or deletions that cause frameshifts or stop codons did not occur. From this we conclude that *S. impatiens* lacks easily identifiable NUMTs.

Conclusions

The results of this study indicate that immatures of *S. impatiens*, either larvae or puparia, are an adequate tissue source for molecular species-level identification using DNA barcodes. This straightforward approach eliminates the need for difficult morphological identifications by taxonomic experts, and is likely to show similar results if applied to other flesh flies or blow flies. It is important to note that this study was completed using intact larvae and puparia. Although immature specimens collected from crime scenes are sometimes incomplete, it is nonetheless suggested that similar successful identifications would be obtained even if smaller tissue amounts were used in initial extractions. For success in future studies, we suggest that the crop be dissected out prior to larval extractions, especially for specimens in the late second and third instars of development. The ability to obtain an

accurate species-level identification of an immature specimen may be vital in a forensic investigation for estimating the minimum PMI, identifying corpse transportation and determining season of death. Given the results of this study, it is hoped that morphologically unidentifiable immature specimens may have increased potential in criminal cases.

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