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ORIGINAL ARTICLE

Molecular identification of blow flies recovered from human cadavers during crime scene investigations in Malaysia

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Abstract

Forensic entomology applies knowledge about insects associated with decedent in crime scene investigation. It is possible to calculate a minimum postmortem interval (PMI) by determining the age and species of the oldest blow fly larvae feeding on decedent. This study was conducted in Malaysia to identify maggot specimens collected during crime scene investigations. The usefulness of the molecular and morphological approach in species identifications was evaluated in 10 morphologically identified blow fly larvae sampled from 10 different crime scenes in Malaysia. The molecular identification method involved the sequencing of a total length of 2.2 kilo base pairs encompassing the 'barcode' fragments of the mitochondrial cytochrome oxidase I (COI), cytochrome oxidase II (COII) and t-RNA leucine genes. Phylogenetic analyses confirmed the presence of *Chrysomya megacephala, Chrysomya rufifacies* and *Chrysomya nigripes*. In addition, one unidentified blow fly species was found based on phylogenetic tree analysis.

Keywords: Forensic entomology, blowfly, molecular identification.

INTRODUCTION

Entomological evidence can be applied in criminal investigation. In particular, the calculation of larva age allows the determination of a minimum postmortem interval (PMI) which is often important information for police investigation. Species identification is needed and very important for the entomological evidence to be useful in the crime scene investigation. Thus, it is crucial to correctly identify the larva species feeding on decedent to calculate the PMI.[1] At present, a major challenge in forensic entomology is that immature larvae have to be collected, preserved and reared until emergence for identification. Calliphorine flies are one of the earliest visitors infesting a decedent with their larvae.[2] To ensure correct species identification, established molecular methods are now increasingly used in the field of forensic investigation.[3,4] Analysis of mitochondrial DNA (mtDNA) appeared to be a useful tool in species identification among the subfamilies of Calliphoridae.[5,6] mtDNA offers several advantages over nuclear DNA. The latter undergoes relatively slow mutation rates compared to mtDNA, so identification would require a much longer nucleotide sequence than is necessary with mtDNA. This makes mtDNA a better tool to determine differences in sequences of closely related species,[7] for molecular identification.

A review of the literature showed that in Malaysia, most studies on forensic entomology were based on collection of larvae from animal carcasses. This is attributed mainly to limited access to human corpses in Malaysia. Hence data on the occurrence of the larvae of forensically important blow fly in decedent is limited. This study aims to evaluate the usefulness of the molecular identification of blow fly larvae recovered from human corpses and to compare the findings with the morphological identification of blow fly larvae.

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MATERIALS AND METHODS

Specimen collection

Maggot specimens were collected from decedents during crime scene investigations. Maggot samples from 10 different crime scenes were each collected directly into two bottles containing 70% ethanol and stored at room temperature.

Processing of larvae for morphological identification

Specimens in 70% ethanol were transferred into a petri dish using forceps. Posterior segments of the maggots were cut vertically with a surgical blade. The maggots were then soaked in 10% potassium hydroxide (KOH) overnight for muscle softening purposes. On the next day, the internal organs of maggots were removed carefully to avoid damage to the taxonomically important parts, such as posterior spiracles, spines, anterior spiracles and the cephalopharyngeal skeleton. The maggots were later soaked in acetic acid for 10 minutes to neutralize the KOH. The process was then continued by soaking the specimens in ascending series of ethanol at 30%, 50%, 70% and 90% for 30 minutes in each concentration in order to dehydrate the specimen. The maggots were then soaked in absolute alcohol for 30 minutes and cleared in clove oil for 30 minutes. Finally the processed specimens were soaked in xylene for 30 minutes before being mounted on glass slides with a few drops of Canada balsam. The slides were dried in an incubator for 1-2 days. The maggots were identified under a light microscope at 100X and 400X magnification using taxonomy keys of Zumpt.[8]

Processing of larvae for molecular identification

Total DNA was prepared from maggot specimens using QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. The extracted blow fly DNA was eluted in 200µl of elution buffer and kept at -20°C. The fraction of extracted DNA was spectrophotometrically quantitated and diluted to 50ng/µl prior to PCR amplification step. The amplified mtDNA region spans a total of 2.2 kb and includes the cytochrome oxidase I and II genes (COI and COII) and t-RNA leucine gene. PCR amplification mixtures were prepared to contain the following: 100ng of template DNA, 1 unit of Taq polymerase (Promega®, USA), 1 x PCR reaction buffer (Promega®), 1.5 mM $MgCl_2$ (Promega®) and 200 µM of each dNTPs (Promega®) and 0.4 µM of each forward and reverse primers (1st Base). Amplification reactions were performed in a T1 Thermocycler (Biometra®). Three sets of primers were used in this study and were designed based on the description of Sperling *et al,*[3] (Table 1).

The PCR cycling conditions were as follow: initial denaturation 95°C for 5 min, 35 cycles of denaturation 95°C for 1 min, elongation 72°C for 2 min, final elongation 72°C for 7 min. It was found that the optimum annealing temperatures was 47.4°C for COI and t-RNA leucine, and 50.2°C for COII. The PCR products were separated electrophoretically on 1% agarose gel (Promega®) and visualized after ethidium bromide staining. PCR products were purified prior to cloning or direct sequencing using either the QIAquick® PCR Purification Kit or QIAquick® Gel Extraction Kit (QIAGEN. Purified PCR products were then cloned into the pGEM®-T Easy vector system (Promega®) to facilitate DNA sequencing procedures. Sequencing was performed using ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (version 3.1, Applied Biosystems®, Foster City). All the samples were sequenced for both forward and reverse DNA strands. Electrophoresis and detection of the sequencing reaction products was carried out in the capillary electrophoresis system ABI PRISM™ 3730xl capillary DNA sequencer with a capillary length of 80cm.

The reference sequence for previously reported blow flies recovered from cadavers in Malaysia[9] namely, *Calliphora vicina* AJ417702, *Chrysomya bezziana* AF295548, *Chrysomya megacephala*

TABLE 1. Primer sequences used to amplify overlapping segments of the mitochondrial COI, COII and t-RNA leucine genes.

Primer ID	Sequence (5'-3')
TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC
CI-N-2800	CATTTCAAGCTGTGTAAGCATC
CI-J-2495	CAGCTACTTTATGAGCTTTAGG
TK-N-3775	GAGACCATTACTTGCTTTCAGTCATCT

AF295551, *Chrysomya nigripes* GU174026, *Chrysomya pinguis* AY092759, *Chrysomya rufifacies* AF083658, *Chrysomya villeneuvi* FJ195382, *Hemipyrellia liguriens* AY097334, *Hermatia illucens* GQ465783, *Lucilia cuprina* AJ417707, *Megaselia scalaris* AF217464, *Ophyra spinigera* EU627714, *Sarcophaga ruficornis* EF405941, *Synthesiomyia nudiseta* EU627713 were retrieved from GenBank and used for the phylogenetic analysis. Sequence alignment and a neighbour-joining tree[10] were made using MEGA 4 bootstrap support derived from 1,000 replicates and values above 50% are shown in the phylogenetic tree.[11] All the sequences obtained from the 10 cases were included in the phylogenetic analysis.

RESULTS

The mitochondrial DNA (mtDNA) region sequenced in this study included the cytochrome oxidase subunit I and II genes (COI and COII) and the t-RNA leucine gene. One individual per species was sequenced over this region. The voucher deposit of all the specimens was stored at the Medical Entomology Unit, Institute for Medical Research, which is the WHO Collaborator Centre for Vectors since 1985. The details of the origin, collection date of the data samples, voucher deposit and GenBank references numbers are presented in Table 2. The sequences have been deposited in GenBank under accession numbers from JN 228993 to JN 229003.

In this study, the complete 2.2 kilobase nucleotide fragment was amplified. Phylogenetic analysis of the relationships between the blow fly maggots was shown in Fig 1. *C. megacephala* were found in six cases, *C. rufifacies* in two cases and *C. nigripes* in one case. Morphology and molecular identifications are in concordance

TABLE 2. Species identification of maggots recovered from human decedents during crime scene investigations in Malaysia based on morphological examination and phylogenetic analysis of the entire cytochrome oxidase genes sequences.

Case	Stage of maggot	Morphology Identification	Molecular Identification (Phylogeny tree)	Voucher Deposit	GenBank Number
1	3rd instar	*Hemipyrellia ligurriens*	Unidentified species	FE 019/2009	JN 228993
2	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE HKLG 15/2009	JN 228994
3	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE 04/2009	JN 228995
4	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE 06ii/2009	JN 228996
5	3rd instar	*Chrysomya rufifacies*	*Chrysomya rufifacies*	FE 06ii/2009	JN 228997
6	3rd instar	*Chrysomya rufifacies*	*Chrysomya rufifacies*	FE 10/2009	JN 228998
7	3rd instar	*Chrysomya pinguis*	*Chrysomya megacephala*	FE 20/2009	JN 228999
8	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE 022/2009	JN 229000
9	3rd instar	*Chrysomya nigripes*	*Chrysomya nigripes*	FE 03/2009	JN 229002
10	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE 02/2009	JN 229003

in eight cases (80%). In case 7, the recovered larvae were identified morphologically as *C. pinguis* but later molecularly confirmed to be *C. megacephala* by phylogenetic analysis (Table 2).

Interestingly, in case 1, the 3rd instar of maggot which identified as *H. ligurriens* morphologically but turned out to be an unidentified species of blow fly based upon phylogenetic analysis of the entire cyctochrome oxidase gene sequences (Fig 1). Result from BLAST system revealed a 94% similarity to *L. cuprina.*

DISCUSSION

Forensic entomology requires some knowledge of insect biology and this may not always be available when the evidence is being collected. A major advantage of the molecular approach of fly identification is that it is relatively insensitive to the state of preservation of the sample and it is not necessary to maintain the larvae in a living state which is necessary for morphological identification.[3]

This study is the first to examine the complete sequence of mtDNA consisting of cytochrome oxidase 1, cytochrome oxidase II and tRNA-leucine from maggot samples collected from decedent during crime scene investigations in Malaysia. From the 10 samples that have been analysed, 8 specimens have been identified as calliphorine flies. This finding concurs with other forensic studies in Malaysia which showed that the calliphorine flies, *C. megacephala* and *C. rufifacies* were the predominant species found in decedents.[9,12,13]

Several methods have been proposed for species identification from DNA sequence data.[14] Tree-based methods, which give more emphasis on finding monophyletic groups,[15]

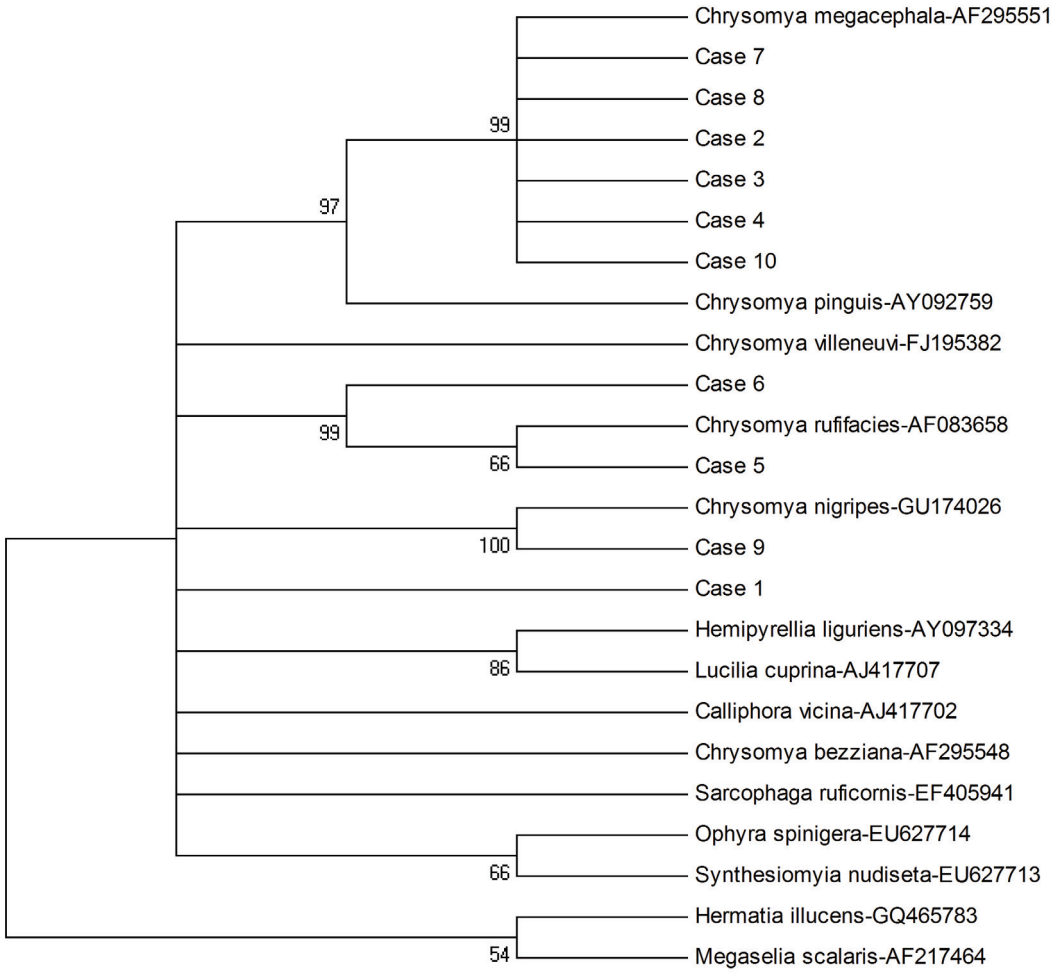

FIG. 1. The neighbour-joining tree illustrating phylogenetic relationships among blow flies recovered from crime scene investigations based on the entire cytochrome oxidase gene sequences.

have recently been shown to be particularly effective at avoiding false-positive results.[16] The phylogenetic tree also appears to be resolved at the species level and the deeper divergence among major clades. However, morphological identification for maggot sample from Case 1 which was identified as *H. ligurriens* by conventional taxonomy was determined to be otherwise. Results from BLAST system revealed the DNA sequences obtained for Case 1, 94% similarity to *L. cuprina.* There are reports that mtDNA haplotypes of a blow fly species from *H. ligurriens* are phylogenetically intermixed with those of *Lucilia cuprina* .[17,18] Well *et al.*[17] also highlighted the *Hemipyrellia* data as indicating a potential serious difficulty for DNA based species identification in the forensic context. In Taiwan, DeBry *et al*.[19] showed that *H. ligurriens* appeared to represent a problem for DNA sequence-based species identification. Since both *H. ligurriens* and *L. cuprina* belong to the subfamily Calliphorinae, hence the primers used were unable to amplify the targeted region for specific species identification. Clearly, additional samples of *L. cuprina* and *H. ligurriens* from crime scene investigation in Malaysia should be examined further to resolve this issue.

Similarly for case No. 7 in which *C. pinguis* was determined to be *C.megacephala* molecularly. Maggot of *C. pinguis* is easily distinguished from *C. megacephala* based on the shape of spines; being dome shaped in *C. pinguis*, while in *C. megacephala* they are shaped unicuspid, bicuspid and tricuspid. Since both species belongs to the sub-family Chrsomyinae; hence the COI and II region is unable to differentiate them to species level. Perhaps other regions in the genome need to be targeted in order to differentiate them.

Nevertheless, the current study successfully demonstrated that the application of mitochondrial cytochrome oxidase genes was not only useful for species identification, but also provided phylogenetic information for forensically important blow flies from several geographical areas in Malaysia. Insect evidence can prove valuable information in estimating the post mortem interval (PMI) in forensic investigation. If accurate, PMI estimation can narrow the field of suspects and aid in the identification of the decedent.[17,20] The gene sequences generated from the present study may provide a useful database for future forensic entomology investigation particularly in Malaysia since the full sequence of mitochondrial DNA that covers gene COI, COII and tRNA have been sequenced.

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