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DNA typing of Calliphorids collected from human corpses in Malaysia

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Abstract. Estimation of post-mortem interval (PMI) is crucial for time of death determination. The advent of DNA-based identification techniques forensic entomology saw the beginning of a proliferation of molecular studies into forensically important Calliphoridae (Diptera). The use of DNA to characterise morphologically indistinguishable immature calliphorids was recognised as a valuable molecular tool with enormous practical utility. The local entomofauna in most cases is important for the examination of entomological evidences. The survey of the local entomofauna has become a fundamental first step in forensic entomological studies, because different geographical distributions, seasonal and environmental factors may influence the decomposition process and the occurrence of different insect species on corpses. In this study, calliphorids were collected from 13 human corpses recovered from indoors, outdoors and aquatic conditions during the post-mortem examination by pathologists from the government hospitals in Malaysia. Only two species, *Chrysomya megacephala* and *Chrysomya rufifacies* were recovered from human corpses. DNA sequencing was performed to study the mitochondrial encoded COI gene and to evaluate the suitability of the 1300 base pairs of COI fragments for identification of blow fly species collected from real crime scene. The COI gene from blow fly specimens were sequenced and deposited in GenBank to expand local databases. The sequenced COI gene was useful in identifying calliphorids retrieved from human corpses.

INTRODUCTION

Forensic investigations of human corpses at death scenes involve an estimation of the post-mortem interval (PMI) which indicates the time elapsed between death and the discovery of the body. Long term PMI estimations are best performed by calculating the age of blow fly larvae developing on the corpse (Smith, 1986; Catts & Goff, 1992; Goff, 2000; Gennard, 2007). The use of entomology to estimate post-mortem intervals (PMIs) in criminal investigations has been found to be more reliable than autopsy and as reliable as police eyewitness information (Kashyap & Pillay, 1989).

Most forensic entomological evidence is strongly dependent on accurate species identification. This is crucial first step in the investigation which can influence the overall direction of the investigation. Since the identification of immature specimens can be difficult using morphological examination, genetic analysis has been proposed to achieve this task (Sperling *et al*., 1994; Wells & Sperling, 2001). In recent years, there has been an increased interest in the use of DNA sequence data in the studies of carrion flies as an aid to accurately identifying insect species, especially in the case of immature stages (Wallman & Donnellan, 2001; Wells & Sperling, 2001).

A quick and accurate identification system is desirable in any forensic studies as well as ecology. The knowledge of local fauna is very useful in forensic investigations

because data from other regions, which may have both different environmental and faunal characteristics, may not provide a sufficient degree of accuracy (Arnaldos *et al.*, 2004). It is known that climatic factors and different environmental conditions, among others, influence the decomposition process and the presence of different species on corpses. Hence the present study examine whether the COI sequence provide sufficient resolution to identify blowflies of the genus *Chrysomya* (Diptera: Calliphoridae) found on human corpses in Malaysia.

MATERIALS AND METHODS

Specimen collection Maggot specimens were collected from 13 human corpses during crime scene investigation and 13 fly maggot specimens were available for morphological and molecular identifications. Maggot specimens were collected directly into glass universal bottle containing 70% ethanol for morphological and molecular identifications. The voucher deposits of all the specimens were stored at the Medical Entomology Unit, Institute for Medical Research, Kuala Lumpur which is the WHO Collaborating Centre for Vectors since 1985. The details of the maggot samples are presented in Table 1.

Larva processing for morphological identification The procedures followed that of Lee *et al.* (1984). Briefly, specimens in 70% ethanol were soaked in 10% potassium hydroxide (KOH) overnight. The internal organs of maggots were removed and soaked in acetic acid for 10 minutes and then in ascending series of ethanol. The maggots were then soaked in absolute alcohol, cleared in clove oil, immersed in xylene and mounted on glass slides with Canada balsam. The maggots were identified under light microscope at 100X and 400X magnification using taxonomy keys of Zumpt (1965), Mahadevan *et al.* (1980) and Omar (2002).

DNA extraction Total DNA was prepared from specimens using QIAamp DNA Mini Kit™ (QIAGEN Inc., Valencia, CA). The extracted blow fly DNA was eluted in 200ml of elution buffer and kept at -20°C for long term storage. The fraction of extracted DNA was spectrophotometrically quantitated and diluted to 50ng/ml prior to PCR amplification steps.

PCR amplification The 1300 base pairs nucleotide of the mitochondrial COI gene was amplified. 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®)

Table 1. Species identification of maggots recovered during crime scene investigation in Malaysia based on morphological examination and molecular analysis of the COI gene sequences

Case No.	Stage of maggot	Morphological Identification	Molecular Identification (Phylogeny tree)	Voucher Deposit No.	GenBank Reference Number
1	2nd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE 039i/2008	JN 571553
2	2nd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE 039ii/2008	JN 571554
3	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE 02i/2009	JN 571555
4	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE 02ii/2009	JN 571556
5	3rd instar	*Chrysomya rufifacies*	*Chrysomya rufifacies*	FE HKLG 15/2009	JN 571557
6	3rd instar	*Chrysomya rufifacies*	*Chrysomya rufifacies*	FE 08/2009	JN 571558
7	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE JB 073/2009	JN 571559
8	3rd instar	*Chrysomya rufifacies*	*Chrysomya rufifacies*	FE 03/2009	JN 571560
9	1st instar	Cannot be identified	*Chrysomya megacephala*	FE 023/2009	JN 571561
10	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE 028/2009	JN 571562
11	3rd instar	*Chrysomya rufifacies*	*Chrysomya rufifacies*	FE 038/2009	JN 571563
12	3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	FE F228/2009	JN 571564
13	3rd instar	*Chrysomya rufifacies*	*Chrysomya rufifacies*	FE F302/2009	JN 571565

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®). Sets of primers used in this study were designed based on the description of Sperling *et al.* (1994). The PCR cycling conditions were as follow: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, elongation at 72°C for 2 min, final elongation at 72°C for 7 min. The optimum annealing temperatures was 47.4°C for COI. The PCR products were separated electrophoretically on 1% agarose gel (Promega®) and visualized after ethidium bromide staining.

Purification of PCR products PCR products were purified prior to cloning or direct sequencing using the QIAquick PCR Purification Kit™ and QIAquick Gel Extraction Kit™ (QIAGEN®) according to the manufacturer's protocols. The success of PCR products purification was confirmed by agarose gel electrophoresis.

Cloning and sequencing 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.

DNA sequence alignment and phylogenetic analysis The reference sequences for previously reported blow flies recovered from cadavers in Malaysia (Lee *et al.*, 2004) namely *Calliphora vicina* AJ417702, *Chrysomya bezziana* AF295548, *Chrysomya megacephala* AF295551, *Chrysomya nigripes* GU174026, *Chrysomya pinguis* AY092759, *Chrysomya rufifacies* AF083658, *Chrysomya villeneuvi* FJ195382, *Hemipyrellia ligurriens* AY097334, *Hermetia 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 (Saitou & Nei, 1987) were made using MEGA 4™ (Tamura *et al.*, 2007) and bootstrap support derived from 1,000 replicates and values above 50% are shown. All the sequences obtained from the 13 maggot samples were included in the phylogenetic analysis.

RESULTS

In this study, a 1300 base pair region of mitochondrial DNA (mtDNA) coding for COI gene was used for identification of the forensically important species of Calliphoridae. Two blowfly species; *C. megacephala* and *C. rufifacies* were identified by morphology. Both flies were the commonest species found in the ecologically varied death scene habitats. The crime scene, where the decedents were found was classified into 3 ecotypes: 5 decedents in urban area, 7 decedents in rural area and 1 decedent in aquatic area. Male decedents were reported in 9 cases and female decedents in 4 cases.

Based on molecular phylogeny of COI gene (Figure 1), two different types of blow fly species were found, namely *C. megacephala* and *C. rufifacies* (Table 1). Speciation based on morphological and molecular phylogeny is in concordance in 12 specimens (92%). For case no. 9, morphological identification could not be done due to the presence of 1st instar larvae.

Based on COI nucleotide sequences of maggot samples collected from crime scene together with the reference sequences retrieved from GenBank, the phylogenetic tree was constructed by neighbour-joining (NJ) method and the tree were tested by 1000 bootstrap replicates (Figure 1). *Chrysomya megacephala* and *C. rufifacies* were well separated although they belonged to the same genus, implying that the COI sequence was useful for identification of these congeneric species. All of the *C. megacephala* and *C. rufifacies* isolates

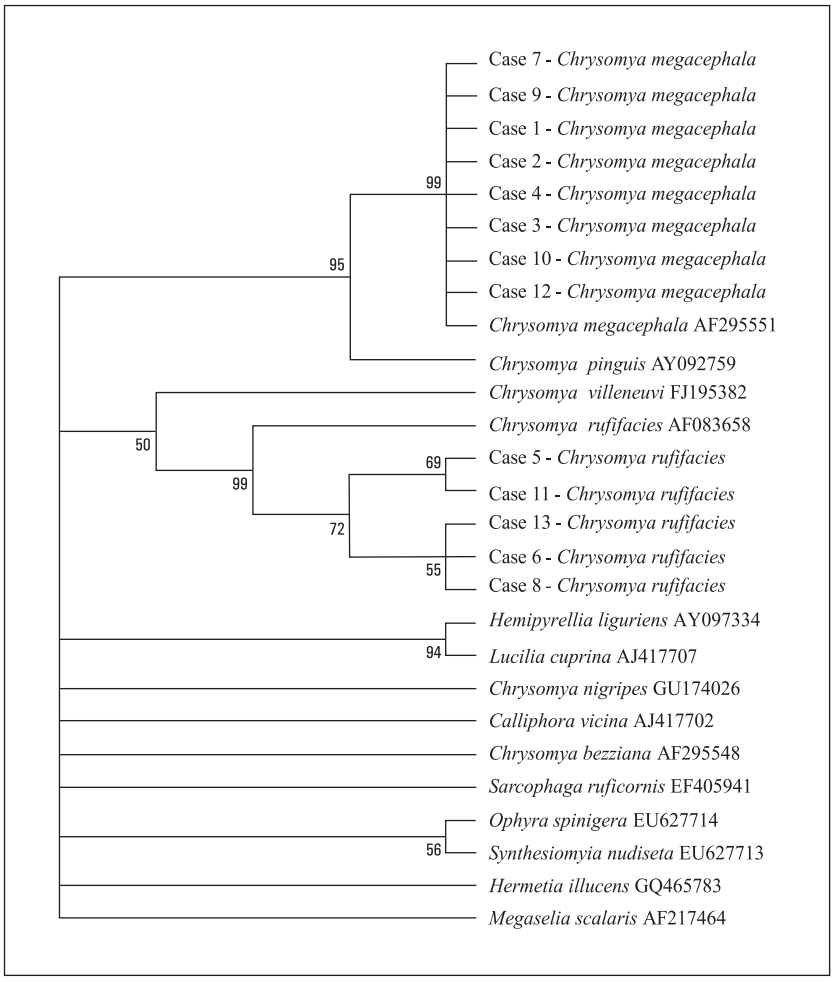

Figure 1. The neighbour-joining tree illustrating phylogenetic relationships among blow flies recovered from crime scene investigations, based on COI nucleotide sequences data with the outgroups. Numbers on branches indicate percentage of bootstrap support

formed a single cluster with branch indicating minor nucleotide variations between the same species.

DISCUSSION

The identification of blowfly species found in association with a corpse is one of the first steps undertaken by the forensic entomologist in an attempt to estimate the post-mortem interval (PMI) (Wells & LaMotte, 2001). Analysis of mitochondrial DNA (mtDNA) and particularly cytochrome oxidase 1 gene (COI) appeared to be a useful tool in species identification among the subfamilies of Calliphoridae (Harvey *et al*., 2003, 2008). This study confirms the suitability of the 1300 base pairs of COI gene for identification of two blow fly species namely *C. megacephala* and *C. rufifacies* collected from crime scene. The COI gene from blow fly specimens were sequenced and deposited in GenBank to expand local databases.

Sequence data of 1300 base pairs of COI gene have the potential to identify blow fly species and place them into the respective clusters. Genera were clearly separated and the subfamilial arrangement was in

concordance with morphological findings, with the *C. megacephala* and *C. rufifacies* were separated into two different groups. The availability of such DNA database will facilitate forensic investigation by allowing immature stages to be identified (Tan *et al.*, 2009). In the last decade, various segments of the COI region have been used to identify forensically important calliphoridae species (Harvey *et al.*, 2003, 2008; Wallman *et al.*, 2005; Wells & Williams, 2007; Wells *et al.*, 2007; Tan *et al.*, 2009). However, various issues on the use of the mtDNA fragments were raised. The dangers of relying on a single locus are illustrated by several studies (Stevens *et al.*, 2002; Wells *et al.*, 2007).

If only a small fragment of DNA is sampled, it may fail to produce an accurate representation of the total genetic variability in that gene region. This could result in misrepresentation of the intra- and interspesific divergences between closely related species, leading to an inaccurate species identification (Wells *et al.*, 2001; Roe & Sperling, 2007). Although longer fragments may minimize stochastic variation across taxa and be more likely to reflect broader patterns of nucleotide divergence (Roe & Sperling, 2007), shorter fragments have many advantages such as being quick, easy and economical because extensive application of long mtDNA segments for species identification cannot always be achieved due to constraints in time and money.

In the present study, COI gene sequencing was proven useful for the accurate identification of the Malaysian Calliphoridae and is likely to be a sensitive typing strategy for Calliphoridae associated with forensic casework, although much work still need to be done. With continued research, the use of Calliphoridae in forensic entomology will increase and their value as tools in criminal investigation will be realised. Future work with more Calliphoridae species from different states of Malaysia should be done.

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