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Molecular identification of Malaysian *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) using life stage specific mitochondrial DNA

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Abstract. DNA identification of blow fly species can be a very useful tool in forensic entomology. One of the potential benefits that mitochondrial DNA (mtDNA) has offered in the field of forensic entomology is species determination. Conventional identification methods have limitations for sibling and closely related species of blow fly and stage and quality of the specimen used. This could be overcome by DNA-based identification methods using mitochondrial DNA which does not demand intact or undamaged specimens. Mitochondrial DNA is usually isolated from whole blow fly and legs. Alternate sources for mitochondrial DNA isolation namely, egg, larva, puparium and empty puparium were explored in this study. The sequence of DNA obtained for each sample for every life cycle stage was 100% identical for a particular species, indicating that the egg, 1st instar, 2nd instar, 3rd instar, pupa, empty puparium and adult from the same species and obtained from same generation will exhibit similar DNA sequences. The present study also highlighted the usefulness of collecting all life cycle stages of blow fly during crime scene investigation with proper preservation and subsequent molecular analysis. Molecular identification provides a strong basis for species identification and will prove an invaluable contribution to forensic entomology as an investigative tool in Malaysia.

INTRODUCTION

Chrysomya megacephala (Fabricius) and *Chrysomya rufifacies* (Macquart) are forensically important blow fly species in many parts of the world including Malaysia. Larvae of both species have been reported to be associated with human corpses (Lee *et al*., 2004; Sukontason *et al*., 2007). The correct identification of maggot specimen is a critical prerequisite in the estimation of post-mortem interval (PMI) using insects, but this may be difficult using the traditional morphological-based approach (Prins, 1982; Wallman, 2001). If a species determination is incorrect, the estimated post-mortem interval and other inferences will be invalid and inaccurate (Byrd & Castner, 2001).

In recent years, mitochondrial DNA (mtDNA) typing has become established as a powerful tool for forensic investigations. Sperling *et al*. (1994) were the first to demonstrate how mtDNA sequence data from (easy to identify) adult specimens of forensically important flies could be used to identify immature forms of the same species. The eggs or larvae of many forensically important Dipteran species are particularly difficult to distinguish morphologically (Benecke & Seifert, 1999) and an incorrect or uncertain identification can seriously harm or impede an investigation. This is because adult arrival times, egg duration and larval growth rates can vary dramatically between species. Proper species identification is usually an essential first step in the use of

entomological evidence in legal investigation.

Furthermore, DNA identification can also be used to identify blow fly species rapidly which can also be used by personnel who are non-entomologists for maggots found during crime scene investigations. The sequences of DNA that have been obtained can be documented for future reference. This study aims to provide a comprehensive set of DNA sequences, across several mitochondrial genes, for two calliphorid species that colonise human corpses in Malaysia.

MATERIALS AND METHODS

Blow fly colony

Adult *C. megacephala* and *C. rufifacies* blow flies were used in this study, as adult morphological characters allowed more accurate identification to species level than larval characters. Specimens were identified using taxonomic keys and characters described by Zumpt (1965), Cheong *et al.* (1971) and Omar (2002).

Chrysomya megacephala and *C. rufifacies* were successfully colonized in the insectarium at the Medical Entomology Unit, Institute for Medical Research (IMR) Kuala Lumpur. Laboratory colonies were maintained at about 28ºC and R.H. of 80%. The procedures of fly rearing used in this study followed IMR Standard Operating Procedures (SOP), 2000.

Precautions were taken to start colonies with an adequate number of clean specimens and maintaining them with very limited levels of mortality. The aluminium fly cages (size 36x36x36 cm) were covered with fine material for ventilation and prevention of other small insects from entering the cage to oviposit. The lid was sealed tightly with adhesive tape to prevent the larvae from crawling out. Fresh cow liver was replaced daily in the cage until third instar maggots developed into prepupae, the non-feeding period.

Sample treatment

Part of the eggs laid was used for molecular analysis, whereas the rest were allowed to hatch and grow. As the larvae grew, through the 3 instars (1st instar, 2nd instar and 3rd instar), about half of each instar were removed for DNA profiling. When the larvae pupated, 5 pupae were taken and introduced into glass jars which were covered and tightly sealed with netting until the emergence of adults. Pupae were separated into different jars to ensure the empty puparium obtained after eclosion corresponded to the specific fly species. The jars were placed into a rearing cage and the adults and the empty puparium were collected. The complete development from egg to adult took approximately 1 week.

Samples from each life stages of egg, larva, pupa, empty puparium and adult were collected. The egg and immature were killed by placing them in hot water (≈90ºC) for 3 min to fix their protein and prevent darkening of the specimens in ethanol. They were then preserved in 70% ethanol. The adults were killed by exposure to -20ºC for 10 minutes and preserved in 70% ethanol. Specimens were refrigerated at -4ºC till used for DNA extraction.

Morphological identification

The 3rd instar maggot specimens were processed by the following standard procedures (Lee *et al.*, 1984). Larvae were first cleared by soaking in 10% KOH solution for 24 hours. After rinsing in distilled water, they were transferred to 10% acetic acid and left for 30 min. After this neutralising process, the larvae were soaked in distilled water and the last segment was partially cut transversely to facilitate removal of internal organs. The last segment which contain the posterior spiracles, an important taxonomic feature, was completely detached to be mounted separately. The treated specimens were then dehydrated in ascending series of ethanol, rinsed briefly with xylene, mounted on glass slide with Canada balsam and dried at 30ºC overnight. Identification of larvae was accomplished by studying the anterior and posterior spiracles, the cephalopharyngeal skeleton and other taxonomic features according to Zumpt (1965), Mahadevan *et al.* (1980) and Omar (2002).

Molecular Identification of Immature Stages

DNA Extraction

Samples were prepared for DNA extraction by using the method described by Sperling *et al*. (1994) with slight modifications. Samples were soaked in distilled water for 10 minutes prior to DNA extraction. Samples were then placed in 1.5 ml microfuge tubes immersed briefly in liquid nitrogen and then ground into powder using sterile plastic pestles. Total genomic DNA was then extracted using QIAamp® DNA Mini Tissue Kit (Qiagen™, Germany) according to the manufacturer's protocol. After overnight incubation in ATL buffer, the samples were treated with RNASE A. At the end of the extraction process, the DNA was eluted in 200µl of elution buffer and kept at -20ºC. The fraction of extracted DNA was spectrophotometrically quantitated and diluted to 50 ng/µl prior to PCR amplification.

Voucher deposit for all the specimens was stored at the Medical Entomology Unit, Institute for Medical Research, which is the WHO Collaborating Centre for Vectors since 1985.

PCR Amplification

PCR amplification mixtures were prepared to contain the following: 100 ng template DNA, 1 unit of Taq DNA polymerase (Promega™, USA), 1 x PCR reaction buffer (Promega™), 1.5 mM MgCl2 (Promega™), 200 µM of each dNTPs (Promega™) and 0.4 µM of each forward and reverse primer and ddH2O to a final volume of 50 µl. Amplification reactions were performed in T1 Thermocycler® (Biometra™) thermal cycler.

Three sets of primers were designed based on those of Sperling *et al.* (1994) as shown in Table 1. The PCR cycling conditions were as follow: initial denaturation at 95ºC for 5 min, 35 cycles of denaturation at 95ºC for 1 min, extension at 72ºC for 2 min and final extension at 72ºC for 7 min.

The annealing temperature was 47.4ºC for partial CO1gene (C1-J-2495 & C1-N-2800) and t-RNA gene (TY-J-1460 & C1-N-2800) and 50.2ºC for CO11 gene (C1-J-2495 & TK-N-3775) which was used to amplify fragments of 2.3 kb length (2303-2306 base pairs plus primers). 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 and direct sequencing using the QIAquick® PCR Purification Kit and QIAquick® Gel Extraction Kit (Qiagen™), according to the manufacturer's protocols. The successes of PCR product purification were 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™, Forster City, CA, USA, according to the manufacturer's recommendations. All samples were sequenced for both forward and reverse DNA strands using forward and reverse primers according to Sperling *et al.* (1994).

Table 1. Primer sequences used to amplify overlapping segments of the mitochondrial COI, COII and t-RNA genes (Sperling *et al.*, 1994)

Primer ID	Sequence (5' – 3')
TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC
C1-N-2800	CATTTCAAGCTGTGTAAGCATC
C1-J-2495	CAGCTACTTTATGAGCTTTAGG
TK-N-3775	GAGACCATTACTTGCTTTCAGTCATCT

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 80 cm.

DNA Sequence Alignment and Phylogenetic Analysis

DNA sequence chromatograms were read and discrepancies between forward and reverse sequences were resolved using the Chromas® software version 2.33 (http://www.technelysium.com.au/chromas.html). The DNA sequences obtained were aligned using ClustalW® alignment analysis from BioEdit® Version 7.0.9. and a Neighbour-Joining tree (Saitou & Nei, 1987) were made using MEGA 4™ (Tamura *et al*., 2007), bootstrap support derived from 1000 replicates and values >50% are shown in the phylogenetic trees.

RESULTS

The results obtained for *C. megacephala* samples are shown in Table 2. Only COI gene was successfully amplified for all life stages i.e. egg, 1st instar, 2nd instar, 3rd instar, pupa, empty puparium and adult. For COII gene, only 1st instar, pupa, empty puparium and adult of *C. megacephala* were successfully amplified, while only the egg has not been successfully ampli fied for t-RNA gene.

Similar results were obtained for *C. rufifacies* (Table 3). For COI, each life stage of *C. rufifacies* was successfully amplified while for COII gene, only the sample of 2nd instar maggot and adult was successfully amplified. The gene t-RNA was also successfully amplified for all life stages except 1st instar maggot. The full sequences of COI, COII and t-RNA genes for *C. megacephala* and *C. rufifacies* have been deposited in GenBank under accession numbers: JN571566 and JN571567.

The molecular analysis of all life cycle stages of blow fly species showed a 100% concordance with the morphological identification of the adult blow fly. Based on the phylogenetic tree, species of the maggot samples were identified.

Phylogenetic tree analysis

The constructed phylogenetic trees are based on COI, COII and t-RNA nucleotide sequences. The reference sequence for the previously reported blow fly species found in Malaysia according to Lee *et al.* (2004) namely *Calliphora vicina* AJ417702, *Chrysomya bezziana* AF295548, *C. megacephala* AF295551, *Chrysomya nigripes* GU174026, *Chrysomya pinguis* AY092759, *C. 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. The phylogenetic tree was constructed by neighbour-joining (NJ) method implemented in the MEGA_ version 3.1 and the tree were tested by 1000 bootstrap replicates as shown in Figure 1 and Figure 2.

Table 2. Molecular analysis of *Chrysomya megacephala*

Sample	Size of Sample	Mitochondrial DNA		
		COI	COII	t-RNA
Egg	5	√	N.A.	N.A.
1st instar	7	√	√	√
2nd instar	8	√	N.A.	√
3rd instar	3	√	N.A.	√
Pupae	2	√	√	√
Empty puparium	2	√	√	√
Adult	2	√	√	√

√ = molecular analysis was successful; N.A.= Not Available

Table 3. Analysis of *Chrysomya rufifacies*

Samples	Quantity of Samples	Mitochondrial DNA		
		COI	COII	t-RNA
Eggs	7	√	N.A.	√
1st instar	7	√	N.A.	N.A.
2nd instar	7	√	√	√
3rd instar	3	√	N.A.	√
Pupae	2	√	N.A.	√
Empty puparium	2	√	N.A.	√
Adult	2	√	√	√

√ = molecular analysis was successful; N.A. = Not Available

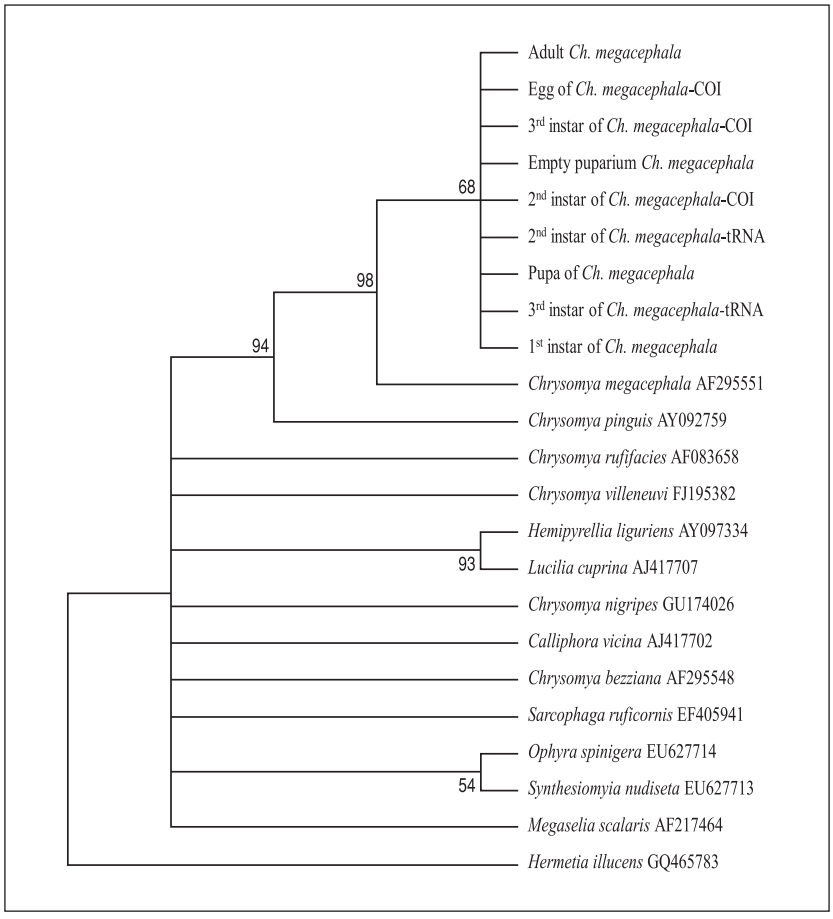

Figure 1. Neighbour-joining tree illustrating phylogenetic relationships for *Chrysomya megacephala* based on COI, COII and t-RNA nucleotide sequences data. Numbers on branches indicate percentage of bootstrap support

Figure 1 shows the phylogenetic tree for *C. megacephala*. It involved the sequencing of a total length of 2200-base pairs encompassing the 'barcode' fragments of the mitochondrial cytochrome oxidase I (COI), cytochrome oxidase II (COII) and

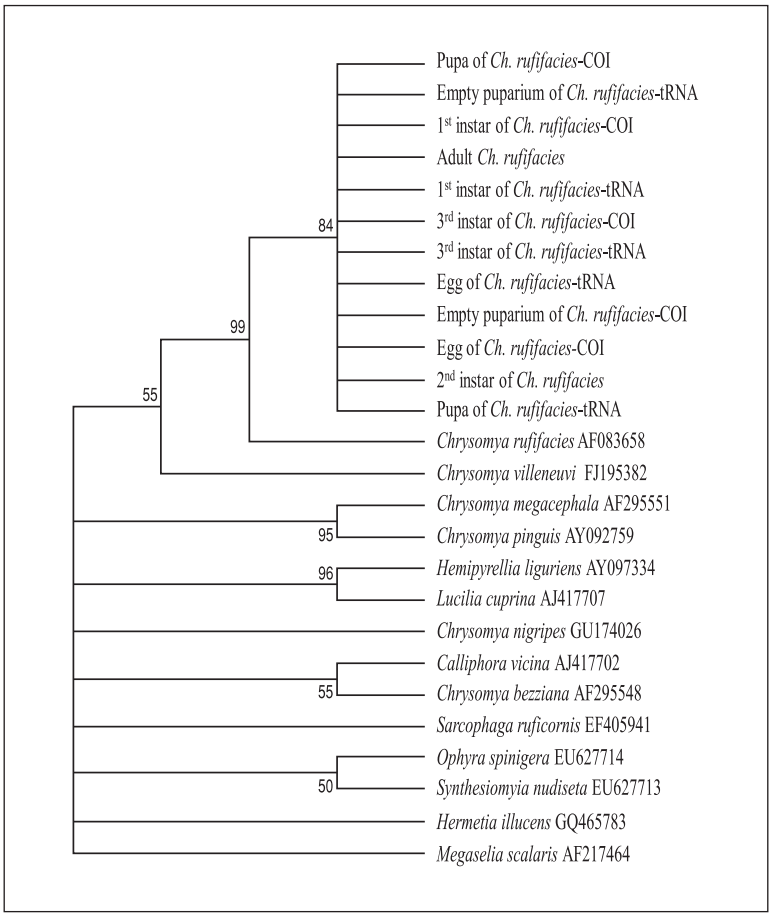

Figure 2. Neighbour-joining tree illustrating phylogenetic relationships for *Chrysomya rufifacies* based on COI, COII and t-RNA nucleotide sequences data. Numbers on branches indicate percentage of bootstrap support

t-RNA genes for 1st instar, pupa, empty puparium and adult of *C. megacephala*. Besides that, the phylogenetic tree also included the sequencing of a 330- base pairs 'barcode' fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene for egg, 2nd instar and 3rd instar of *C. megacephala* together with the sequences of 1300-base pairs for t-RNA for 2nd instar and 3rd instar of *C. megacephala*. Phylogenetic analysis also confirmed the presence of *C. megacephala*, for samples collected from each life stages of the blow flies. Each of the blow fly species was clearly separated from *C. megacephala*, and one isolate was also clustered together. *Chrysomya rufifacies*, *C. pinguis*, *C. bezziana*, *C. villeneuvi*, *C. nigripes*, *S. ruficornis*, *H. liguriens*, *C. vicina*, *L. cuprina*, *O. spinigera*, *S. nudiseta*, *H. illucens* and *M. scalaris* were used as the outgroups of the phylogenetic tree.

According to the tree, all of the stated blow flies clustered together showing no significant differentiation between DNA sequences obtained from different mitochondrial gene regions. *Chrysomya megacephala*, *C. rufifacies*, *C. nigripes*, *C. pinguis*, *C. bezziana* and *C. villeneuvi* could be well separated although they belonged to the same genus, implying that either the COI, COII and t-RNA sequences were useful for identification of these congeneric species. All of the samples of *C. megacephala* isolates formed a single cluster with branches indicating minor nucleotide variations between the same species.

Figure 2 shows the phylogenetic tree for *C. rufifacies*. The tree was reconstructed based on the sequencing of a total length of 2200-base pairs encompassing the 'barcode' fragments of the mitochondrial cytochrome oxidase I (COI), cytochrome oxidase II (COII) and t-RNA genes for 2nd instar and adult of *C. rufifacies* and sequencing of 330-base pairs 'barcode' fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene for egg, 1st instar, 3rd instar, pupa and empty puparium of *C. rufifacies*. The phylogenetic tree also includes the sequencing of 1300-base pairs 'barcode' fragment of the mitochondrial t-RNA leucine (t-RNA) gene for 1st instar, 3rd instar, pupa and empty puparium of *C. rufifacies*. Phylogenetic analysis also confirmed the presence of *C. rufifacies*. Each life stage sample of *C. rufifacies* were clearly separated from *C. megacephala*, *C. pinguis*, *C. bezziana*, *C. villeneuvi*, *C. nigripes*, *S. ruficornis*, *H. liguriens*, *C. vicina*, *L. cuprina*, *O. spinigera*, *S. nudiseta*, *H. illucens* and *M. scalaris* and 1 isolate of *C. rufifacies* was clustered together. *Chrysomya megacephala*, *C. pinguis*, *C. bezziana*, *C. villeneuvi*, *C. nigripes*, *S. ruficornis*, *H. liguriens*, *C. vicina*, *L. cuprina*, *O. spinigera*, *S. nudiseta*, *H. illucens* and *M. scalaris* were used as the outgroups of the phylogenetic tree.

All the samples collected from each life stages of blow fly *C. rufifacies* isolates formed a single cluster with branches indicating minor nucleotide variations between the same species. The nucleotide sequences alignment that have been obtained for the maggot samples either in the form of egg, 1st instar, 2nd instar, 3rd instar, pupa, empty puparium or adult showed the same sequences of nucleotide for the same species of blow flies.

DISCUSSION

Forensic entomology is a very important aspect of law enforcement. With the voluminous information that can be gathered, investigators can determine more accurately the time of death, location, how long a body has been in a specific area, if it has been moved and other important factors (Hall, 2008). As this branch of entomology progresses, it will become a key facet in crime investigations due to its usefulness. In this study, the species identification of blow fly immature stages like egg, larva, puparium, empty puparium and adult of *C. megacephala* and *C. rufifacies* were highlighted.

The mitochondrial DNA region sequenced in this study included the cytochrome oxidase subunit I and II genes (COI and COII) and the t-RNA gene. Identification of blow flies collected in a corpse is an initial and mandatory step when using flies as entomological evidence in forensic investigations. There are a number of identification keys to mature larvae of forensically important blow flies. However, morphological identification of blow fly larvae is not easy because only about 2% of the fly species known to science have been described in their immature stages (Smith, 1989). Having identified the species, the next step is to estimate their age, which will indicate the minimum post-mortem interval. It is important to note that ageing larvae or other stages will not determine the actual time of death but rather only estimation of the time of death.

After the DNA sequences were obtained, the phylogenetic tree was constructed to determine the species of the maggots sample based on the DNA sequences. The phylogenetic tree representing the mitochondrial genetic structure of blow fly *C. megacephala* and *C. rufifacies* based upon COI, COII and t-RNA genes sequences. Therefore two phylogenetic trees have been constructed for *C. megacephala* and *C. rufifacies*. Neighbour-joining results showed that the maggot samples from same blow fly formed one clade. In general, members of the same species were closer to each other than to members of other subfamilies. The phylogenetic tree included not only the *C. megacephala* and *C. rufifacies* sequences but also sequences from specimens worldwide. It was not possible to distinguish between blow fly originating from Malaysia and those from other countries by a BLAST search or by simple alignment.

The present study also highlighted the usefulness of collecting all life cycle stages of blow fly during crime scene investigation with proper preservation and subsequent molecular analysis. Ethanol is recommended as a preservation solution because of its ability to denature nucleases and dehydrate specimens (Dessauer *et al*., 1996). Smith *et al.* (1987) and Dillon *et al.* (1996) also confirmed that ethanol is a useful preservative for DNA. Preservation of entomological evidence becomes even more important if the DNA analysis of the maggot crop is to be attempted because some preservation methods suitable for storing maggots for microscopic examination may not be suitable for keeping DNA intact. Colder temperatures are often used as a preservation strategy as they help to reduce or eliminate bacterial growth and enzymatic activity. Keeping maggots frozen at a temperature below refrigeration (4°C) will greatly improve the preservation of DNA in the maggot crop. Maggots held at a cooler environment helped physical preservation as well as DNA preservation (Linville *et al*., 2004).

After the samples of DNA were amplified and when alignment made to the DNA sequences, the arrangement of DNA sequence obtained from every stage of life cycle is similar. This shows that the egg, 1st instar, 2nd instar, 3rd instar, pupa, empty puparium and adult from the same species and obtained from same generation will show the same sequence of DNA. The sequences of DNA which are obtained from this study will be used as reference DNA sequences.

Using molecular analysis, the entomological evidence that has been obtained from the crime scene for the immature stages of the blow fly species can be recognized easily and in a shorter period. This saves the time for rearing the immature stages of the blow fly to the 3rd instar or adult stage for morphological identification using conventional taxonomy.

In Malaysia, *C. megacephala* and *C. rufifacies* are the predominant blow fly species associated with human corpses (Lee *et al*., 2004). Therefore species identification can be done using DNA sequences for these two blow flies species using its various life cycle stages available at the crime scene.

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