The hidden biodiversity of the blowfly *Chrysomya megacephala* revealed by the *Cytochrome b* gene

R Kavitha¹,², VL Low², MS Azirun³, CD Chen³, FMS Ahmad⁴, N Shanti¹, AH Zaibunnisa¹ and ZMY Farida¹,⁵

¹Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), Shah Alam, 40450 Selangor, Malaysia
²Tropical Infectious Diseases Research and Education Centre (TIDREC), University of Malaya, 50603 Kuala Lumpur, Malaysia.
³Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
⁴Department of Parasitology and Entomology, Universiti Kebangsaan Malaysia Medical Centre, Bandar Tun Razak, 56000, Cheras, Kuala Lumpur, Malaysia.
⁵Integrative Pharmacogenomics Institute, Universiti Teknologi MARA, Puncak Alam Campus, Selangor, Malaysia.

*E-mail: kavith0855@uitm.edu.my*

**Abstract.** Insects or arthropods collected from a human deceased during crime scene investigation often revealed information related to the body of the deceased or the crime scene. Morphologically identical immature larvae or maggots have been identified using DNA identification methods. However, a big challenge to the DNA identification method is the presence of morphologically identical but genetically distinct taxa. The genetic diversity of *Chrysomya megacephala* flies from Malaysia will be revealed in this study. A total of 74 *Chrysomya megacephala* from Peninsular Malaysia were subjected to phylogenetic and haplotype analyses based on the *Cytochrome b* (*Cyt b*) gene. *Chrysomya megacephala* from Kuala Lumpur and Selangor are more diverse genetically compared to those from Perak, Johor and Pahang. The *Cyt b* gene revealed three distinct genetic clades of *Chrysomya megacephala*, one clade is for populations from Selangor and Kuala Lumpur, whereas the other two clades consisted of specimens from all five studied populations that is Perak, Pahang, Selangor, Kuala Lumpur and Johor. Detection of hidden lineages of *Chrysomya megacephala* based on the *Cyt b* gene may offer some clues for forensic entomological investigation in the country.

1. **Introduction**

Forensic entomology uses blowfly species during legal investigation to estimate post-mortem interval (PMI) [1]. PMI estimation based on entomological evidence is strongly dependent on accurate species identification. The morphological identification of blowflies is usually hampered by species similarities [2] and in certain cases, the specimens need to be reared until adulthood for accurate identification [3]. DNA technique has been recognised as a valuable tool to identify the immature stages of Calliphoridae, however, occurrence of cryptic taxa- morphologically identical, but genetically distinct, may differ in their behavior, development rate or other biological factors that can cause an error in PMI estimation [4]. If blowfly species identification is incorrect, it will affect the PMI estimation [5]. Previous studies reported that *C. megacephala* was the ordinary blowfly recovered from human carcasses [6; 7; 8; 9; 10]. Discriminations
of fly species based on heuristic thresholds and divergence of DNA sequences, have been the common approach to identify novel taxa [4]. The effectiveness of the mitochondrial genes in discriminating forensically important insects had been well proven [11; 12]. In this aspect, mitochondrial genes have been favorably used in characterizing the population genetic structure in several Chrysomya species [13; 14; 15].

While the Cytochrome oxidase I (COI) gene is commonly used for phylogenetic studies [16; 17; 11; 18; 19; 20], Cyt b gene was also found to be powerful for identifying the geographical origins or sources of the species [14; 15]. Herein we aim to infer the genetic lineages and haplotype dispersal patterns of C. megacephala using Cyt b gene, for the first time from its native range of SouthEast Asia, namely Malaysia. Biological data on local flies are more beneficial in PMI estimation compared to data from other countries with different environments and fauna characteristics [21].

2. Materials and Methods

2.1 Sampling Sites

A total of 74 Malaysian C. megacephala specimens were used, comprising 14 individuals from Perak (northern region), 25 individuals from Pahang (eastern region), 14 individuals from Selangor (central region), 12 individuals from Kuala Lumpur (central region) and nine individuals from Johor (southern region). Fish meat was used as bait to trap the flies as described by d’ Almeida [22]. Field-collected adult blowflies were identified according to taxonomic keys [5; 23]. The specimens were preserved in 70% ethanol prior to DNA analysis. Voucher specimens are kept in Universiti Teknologi MARA (UiTM), Shah Alam, Selangor.

2.2 DNA Extraction, Amplification and Sequencing of Cytochrome b Gene

Genomic DNA of C. megacephala was extracted from the legs of individual specimens using the i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology Inc., Seongnam, South Korea). The mitochondrial Cyt b gene fragment was amplified using primer pairs from [24] for forward primer CBI-SE2: 5’-TAT GTA CTA CCA TGA GGA CAA ATA TC-3’) and [25] for reverse primer 5’- ATT TCA CGC TCA TTA ACT-3’. The PCR cycles included an initial denaturation of 94°C for 3 min, followed by five cycles of 94°C for 30s (denaturation), 40°C for 30s (annealing) and 72°C for 90 s (extension), and 30 cycles at 94°C for 30s (denaturation), 44°C for 30s (annealing) and 72°C for 90s (extension) and a final extension at 72°C for 10 min. Purified samples were used for DNA sequencing in forward and reverse directions.

2.3 Sequence Analyses

All nucleotide sequences were edited using ChromasPro 1.5 (Technelysium Pty Ltd, Brisbane, Qld, Australia) and BioEdit 7.0.9.0. [26]. Representative haplotype sequences of the Cyt b gene were deposited in the NCBI GenBank under the accession numbers KR336662-KR336666. Four phylogenetic analyses were performed: bayesian inference (BI) using MrBayes 3.1.2 [27]; maximum likelihood (ML) using Treefinder Version October 2008 [28]; neighbour-joining (NJ) and maximum parsimony (MP) using PAUP 4.0b10. The horn fly, Haematobia irritans (FJ025715) was used as an outgroup in this study. The haplotype networks of Ch. megacephala were analysed using TCS 1.13® [29].

3. Results and discussion

Four phylogenetic analyses yielded phylogenetic trees with the same topology but with different posterior probability/bootstrap support values. Hence, only the BI tree is presented (Figure 1). The Cyt b tree disclosed three genetic lineages of C. megacephala. Lineage A, the basal clade supported by 70% (BI), 60% (ML), 63% (MP) and 72% (NJ) posterior probability/bootstrap values, was limited to flies that originated from Selangor and Kuala Lumpur. Lineage B, with 93% (BI), 72% (ML), 63% (MP) and 72% (NJ) posterior probability/bootstrap values, comprised flies from all studied populations as did lineage C, supported with
78% (BI), 72% (ML) and 62% (NJ) posterior probability/bootstrap values. Similarly, haplotype network analysis showed three distinct haplotype lineages among Malaysian populations (Figure 2). An intersection of the three genetically divergent groups of *C. megacephala* was noticed. However, the flies from the central region, Kuala Lumpur and Selangor populations (also known as Klang Valley), comprising three different lineages, suggested that the flies are genetically diverse in comparison with the flies from Pahang, Perak and Johor.

**Figure 1.** Bayesian inference phylogeny tree of *Chrysomya* taxa based on Cyt b sequences. [Bayesian inference (BI)/maximum likelihood (ML)/maximum parsimony (MP)/neighbour-joining (NJ)] posterior probability/bootstrap values are shown on the branches.
Precisely identifying blowfly species is a fundamental step in forensic entomology. Traditionally, blowfly species were identified based on morphological identification using taxonomic keys which required specific skills and far-reaching experience. Then, the applications of DNA method for blowfly identification were introduced in [18]. The COI gene was prominent for blowfly species identification [30; 31; 32; 33] followed by nuclear internal transcribed spacers (ITS) [34] and mitochondrial rRNA genes [35]. The first Malaysian forensic entomology case of Nevin in 1950, described that C. megacephala larvae were found in a dead woman body and the larvae were used in the estimation of PMI as stated by Reid [36] and until today C. megacephala is still the predominant blowfly that been found on human deceased in Malaysia [10].
The phylogeographic resolution of Malaysian *C. megacephala* needs more improvement. The selection of genetic markers is important to determine the genetic lineages and haplotype dispersal pattern of flies. Maternal inheritance of mitochondrial DNA with little recombination and a high mutation rate [37] makes the *Cyt b* gene a preferable phylogenetic marker. Based on previous findings, low genetic diversity of Malaysian *C. megacephala* was observed in the *COI* gene while no genetic variation was found in the *Cytochrome oxidase II (COII)* [13], indicating that these genes are well conserved and of limited value in determining phylogeographic information for *C. megacephala* in Malaysia. Although *COI* is useful in identification of the Calliphoridae flies [38; 39; 40], it has limited value in determining their population or geographic origin [41]. Although *C. megacephala* is known as an Asian fly, it has also been present in the USA since 1980. However, the genetic diversity estimated from the *C. megacephala* from Florida was lower compared to published data on *C. megacephala* from Malaysia, maybe reflecting the genetic effect of being introduced to a new geographical region [42].

Previous analysis using *Cyt b* gene not only provided insights into the population structure but also elicited inferences concerning population history [43]. *Cyt b* is a useful marker for recognizing the geographical origins of infestations of some fly species for those without the resources for whole genome sequencing [14; 44]. Markedly, this study also showed an overlapping of three different genetically divergent groups of *C. megacephala* population in Malaysia. The phylogenetic analysis herein revealed that *C. megacephala* flies from Kuala Lumpur and Selangor are high in genetic diversity in contrast to those from Pahang, Perak and Johor. This could be supported by the fact that the transport networks between Kuala Lumpur and Selangor states are well developed. It makes the migration of flies widely distributed due to natural dispersal, gene flow, mating system and mode of reproduction [45] and transportation through roads [13].

4. **Conclusion**

Better knowledge of *Chrysomya* genetic lineages would assist in crime scene investigation by providing information on the origin or sources of the fly. The genetic sequences obtained from this study may provide a useful database in future usage for forensic entomology investigation. Further work with more Calliphoridae species from different states of Malaysia should be conducted to accumulate forensic entomological evidence in Malaysia. More advanced development in molecular research will raise the value of Calliphoridae fly species in criminal investigation.

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