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*Cytochrom*e *b* marker reveals three distinct genetic lineages of the oriental latrine fly *Chrysomya megacephala* (Diptera: Calliphoridae) in Malaysia

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Abstract

This study investigated the hidden genetic lineages in the oriental latrine fly *Chrysomya megacephala* (Fabricius) across four states (i.e., Johore, Pahang, Perak and Selangor) and a federal territory (i.e., Kuala Lumpur) in Malaysia using *Cytochrome b* (*Cyt b*) genetic marker. The *Cyt b* phylogenetic tree and haplotype network revealed three distinct genetic lineages of *Ch. megacephala*. Lineage A, the basal clade was restricted to flies that originated from Kuala Lumpur and Selangor, while Lineages B and C, comprised of flies from all studied populations. An overlap of the three genetically divergent groups of *Ch. megacephala* was observed. However, the flies from both Kuala Lumpur and Selangor populations consisted of three different lineages, indicating that they are genetically diverse compared to those from Pahang, Perak and Johore.

Keywords: Forensic Entomology, Calliphoridae, Mitochondrial DNA, Cryptic Lineage.
Word Count: 126 words

1.0 Introduction

Forensic entomology concerns the use of certain arthropods, especially local fly species, for legal investigation and post-mortem interval (PMI) estimation (Kavitha et al. 2014). Previous reviews of forensic cases have reported that *Ch. megacephala* was the commonest fly recovered from human cadavers during crime scene investigation in Malaysia. Their ability to survive and compete successfully in the human cadaver environment accounted for the predominance of *Chrysomya* species (Lee et al. 1984, 1989, 2004; Hamid et al. 2003, Kavitha et al. 2013).

Knowledge of genetic variability and population structure of forensically important flies shall provide a strong basis in facilitating crime scene investigation. In this aspect, mitochondrial genes have been favorably used in characterizing the population genetic structure in a number of *Chrysomya* species (Ready et al. 2009, Wardhana et al. 2012, Chong et al. 2014). Among the studied genetic markers, *Cyt b* gene was found to be powerful for identifying the geographical origins/sources of the species (Ready et al. 2009, Wardhana et al. 2012). Herein we aim to infer the genetic lineages and haplotype dispersal pattern of *Ch. megacephala* using *Cyt b* gene, for the first time from its native range of South East Asia, namely Malaysia.

2.0 Materials and Methods

2.1 Sampling Sites

A total of 74 Malaysian *Ch. 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 et al. 1996. The specimens were preserved in 70% ethanol prior to DNA analysis. Voucher specimens are maintained in Universiti Teknologi MARA (UiTM), Shah Alam, Selangor.

2.2 DNA Extraction, Amplification and Sequencing of *Cytochrome b* Gene

Genomic DNA of *Ch. 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 in a final volume of 50µL containing 5µL 10X buffer, 2.5 mM of each dNTP, 10 pmol of each forward and reverse primer, 25µL ExPrime Taq Master Mix (GENETBIO Inc., Daejeon,South Korea) and 25-50ng of genomic DNA. The primers for *Cyt b* amplification were adopted from Esseghir et al. (2000) for forward primer CBI-SE2: 5'-TAT GTA CTA CCA TGA GGA CAA ATA TC-3') and Hall et al. (2001) for reverse primer 5'- ATT TCA CGC TCA TTA ACT-3'. The PCR conditions included an initial denaturation of 94°C for 3 min, followed by five cycles of 94°C for 30 s (denaturation), 40°C for 30 s (annealing) and 72°C for 90 s (extension), and 30 cycles at 94°C for 30 s (denaturation), 44°C for 30 s (annealing) and 72°C for 90 s (extension) and a final extension at 72°C for 10 min. The amplified products were purified using MEGAquick-spin PCR & Agarose Gel DNA Extraction System (iNtRON Biotechnology, Inc., Seongnam, South Korea). All samples were then sent to a commercial company for DNA sequencing in both directions.

2.3 Sequence Analyses

All nucleotide sequences were analysed and edited using ChromasPro 1.5 (Technelysium Pty Ltd, Brisbane, Qld, Australia) and BioEdit 7.0.9.0. (Hall, 1999). 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 (Huelsenbeck and Ronquist, 2001); maximum likelihood (ML) using Treefinder Version October 2008 (Jobb et al. 2004); neighbour-joining (NJ) and maximum parsimony (MP) using PAUP 4.0b10. Horn fly, *Haematobia irritans* (FJ025715) was used as outgroup in this study. The haplotype networks of *Ch. megacephala* were analysed using TCS 1.13® (Clement et al. 2000) to calculate the minimum number of mutational steps by which the sequences could be joined with >95% confidence.

3.0 Results

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 (Fig. 1). The *Cyt b* tree revealed three genetic lineages of *Ch. megacephala* (Fig. 1). Lineage A, the basal clade supported by 70% (BI), 60% (ML), 63% (MP) and 72% (NJ) posterior probability/bootstrap values, was restricted to flies that originated from Kuala Lumpur and Selangor. 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 (Fig. 2). An overlap of the three genetically divergent groups of *Ch. megacephala* was observed. However, the flies from the central region, Kuala Lumpur and Selangor populations (also known as Klang Valley), consisted of three different lineages, indicating that they are genetically diverse compared to those from Pahang, Perak and Johor.

4.0 Discussion

The current study is aimed to improve the phylogeographic resolution of Malaysian *Ch. megacephala* using *Cyt b* gene. The selection of genetic markers is of great importance to determine the genetic lineages and haplotype dispersal pattern of flies. Maternal inheritance of mitochondrial DNA with little recombination and a high mutation rate (Avise, 2000) makes the *Cyt b* gene a preferable phylogenetic marker. Based on previous findings, low genetic diversity of Malaysian *Ch. megacephala* was observed in the *cytochrome oxidase* I (COI) gene while no genetic variation was found in the *cytochrome oxidase* II (COII),

(Chong et al. 2014), indicating that these genes are well conserved and of limited value in determining phylogeographic information for *Ch. megacephala* in Malaysia. Previous analysis using *Cyt b* gene not only provided insights into the population structure but also elicited inferences concerning population history (Hodgkinson et al. 2003).

Cyt b is a powerful genetic marker for population genetic study. *Cyt b* is a useful marker for identifying the geographical origins of infestations of some fly species for those without the resources for whole genome sequencing (Ready et al. 2009, 2014). Markedly, this study also showed an overlapping of three different genetically divergent groups of *Ch. megacephala* population in Malaysia. The phylogenetic analysis herein revealed that *Ch. megacephala* from Kuala Lumpur and Selangor are genetically diverse compared to those from Pahang, Perak and Johor. This could be in support by the fact that the transport networks between these two states are very well developed. It makes the migration of flies widely distributed due to natural dispersal and transportation through roads.

Better knowledge of *Chrysomya* genetic lineages would assist in crime scene investigation by providing information on the origin or sources of the fly species because it is well known that *Ch. megacephala* is the predominant blowfly encountered from human remains in South East Asia (Kavitha et al. 2014). Future work with more Calliphoridae species from different states of Malaysia should be conducted. With continued research, the use of Calliphoridae fly species in forensic entomology will increase their value as tools in criminal investigation.

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References Cited

1. **Avise, J.C. 2000.** Phylogeography: The history and formation of species. Harvard University Press, Cambridge.

2. **Chong, Y.V., Chua, T.H. and Song, B.K. 2014.** Genetic variations of *Chrysomya megacephala* populations in Malaysia (Diptera: Calliphoridae). Adv Entomol. 2(1):49-56.

3. **Clement, M., Posada, D. and Crandall, K.A. 2000.** TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9:1657-1659.

4. **d' Almeida, J.M., José, R. and Salviano, B. 1996.** Feeding preference of the larvae of *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) and *Ravinia belforti* (Pardo e Fonseca) (Diptera: Sarcophagidae) concerning different diets. Memórias do Instituto Oswaldo Cruz. 91:137-138.

5. **Esseghir, S., Ready, P.D. and Ben-Ismail, R. 2000.** Speciation of *Phlebotomus* sandflies of the subgenus *Larroussius* coincided with the late Miocene-Pliocene aridification of the Mediterranean subregion. Biol. J. Linn. Soc. 70(2):189-219.

6. **Hall, T.A. 1999.** BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

7. **Hall, M.J.R., Edge, W., Testa, J.M., Adams, Z.J.O. and Ready, P.D. 2001.** Old World screwworm fly, *Chrysomya bezziana*, occurs as two geographical races. Med. Vet. Entomol. 15:393-402.

8. **Hamid, N.A., Omar, B., Marwi, M.A., Mohd. Salleh, A.F., Mansar, H., Siew, S.F. and Moktar, N.A. 2003.** A review of forensic specimens sent to forensic entomology laboratory Universiti Kebangsaan Malaysia for the year 2001, Trop. Biomed. 21:27–31.

9. **Hodgkinson, V.H., Birungi, J., Quintana, M., Deitze, R. and Munstermann, L.E. 2003.** Mitochondrial *cytochrome b* variation in populations of the visceral leishmaniasis vector *Lutzomyia longipalpis* across Eastern Brazil. Am. J. Trop. Med. Hyg. 69(4):386-392.

10. **Huelsenbeck, J.P. and Ronquist, F. 2001.** MRBAYES: Bayesian interence of phylogenetic trees. Bioinformatics. 17(8): 754-755.

11. **Jobb, G., von Haeseler, A. and Strimmer, K. 2004.** TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol. Biol. 4:18.

12. **Kavitha, R., Nazni, W.A., Tian Chye, T., Han Lim, L. and Sofian-Azirun, M. 2013.** Review of forensically important entomological specimens collected from human cadavers in Malaysia (2005-2010). J Forensic Leg Med. 20:480-482.

13. **Kavitha, R., Tian Chye, T., Jeffery,J. and Sofian-Azirun, M. 2014.** Forensic entomology in Malaysia: knowledge and practices. Asian Biomed 8(5):603-607.

14. **Lee, H.L. 1989.** Recovery of forensically important entomological specimens from human cadavers in Malaysia: an update. Malays J Pathol. 11:33-36.

15. **Lee, H.L., Abdullah, A.A. and Cheong, W.H. 1984.** The use of fly larvae from human corpses in determining the time of death: a review and some technical considerations. Journal of Medical and Health Laboratory Technology of Malaysia.9:15-17.

16. **Lee, H.L., Krishnasamy, M., Abdullah, A.G. and Jeffery, J. 2004.** Review of forensically important entomological specimens in the period of 1972-2002. Trop Biomed. 21:69-75.

17. **Ready, P.D., Testa, J.M., Wardhana, A.H., Al-Izzi, M., Khalaj, M. and Hall, M.J.R. 2009.** Phylogeography and recent emergence of the Old World screwworm fly, *Chrysomya bezziana*, based on mitochondrial and nuclear gene sequences. Med. Vet. Entomol. 23:43-50.

18. **Ready, P.D., Wardhana, A.H., Adams, Z.J., Sotiraki,S.and Hall, M.J. 2014.** Improved method for screening mitochondrial *cytochrome b* markers to identify regional populations of the Old World screwworm fly and other myiasis agents. Acta Trop. 138:42-48

19. **Wardhana, A.H., Hall, M.J., Mahamdallie, S.S., Muharsini, S., Cameron, M.M., and Ready, P.D. 2012.** Phylogenetics of the Old World screwworm fly and its significance for planning control and monitoring invasions in Asia. Int J Parasitol.42:729-738.

Declaration of Conflicting Interests

The author(s) declare no conflicts of interest with respect to the authorship and/or publication of this article.

Figure Captions

Fig.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. Bar indicates substitutions per site.

Fig.2. Statistical parsimony networks for *Cyt b* haplotypes of *Chrysomya megacephala* in Malaysia. Lines represent parsimonious connections between haplotypes with probabilities of >95%, with each representing one mutational step. Small circles indicate missing haplotypes. Relative sizes of squares and ovals indicate haplotype frequency.

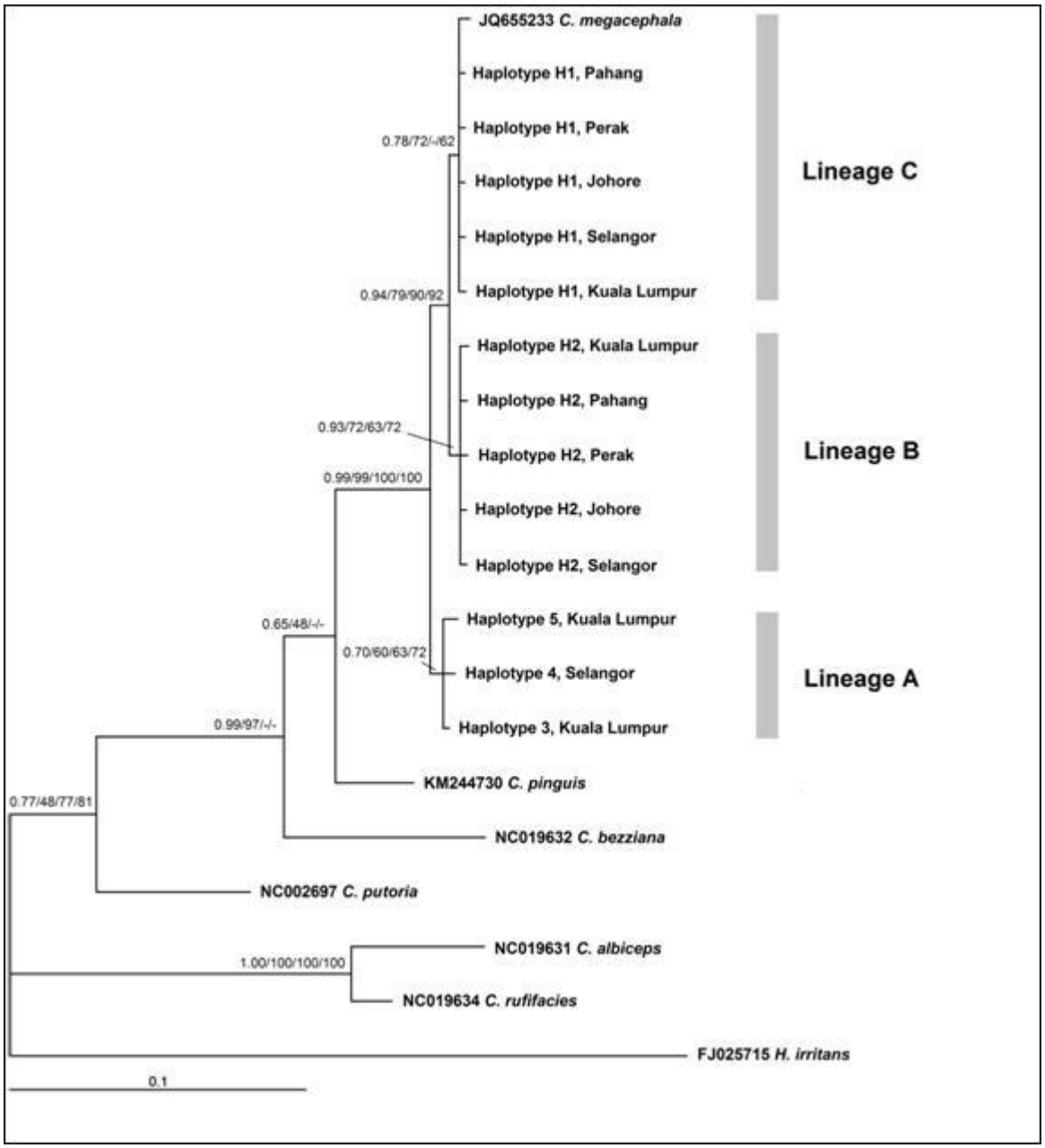
JQ655233 C. megacephala
Haplotype H1, Pahang
Haplotype H1, Perak
Haplotype H1, Johore
Haplotype H1, Selangor
Haplotype H1, Kuala Lumpur
Haplotype H2, Kuala Lumpur
Haplotype H2, Pahang
Haplotype H2, Perak
Haplotype H2, Johore
Haplotype H2, Selangor
Haplotype 5, Kuala Lumpur
Haplotype 4, Selangor
Haplotype 3, Kuala Lumpur
KM244730 C. pinguis
NC019632 C. bezziana
NC002697 C. putoria
NC019631 C. albiceps
NC019634 C. rufifacies
FJ025715 H. irritans
Lineage C
Lineage B
Lineage A
0.78/72/-/62
0.94/79/90/92
0.93/72/63/72
0.99/99/100/100
0.65/48/-/-
0.70/60/63/72
0.99/97/-/-
0.77/48/77/81
1.00/100/100/100
0.1

Fig. 1

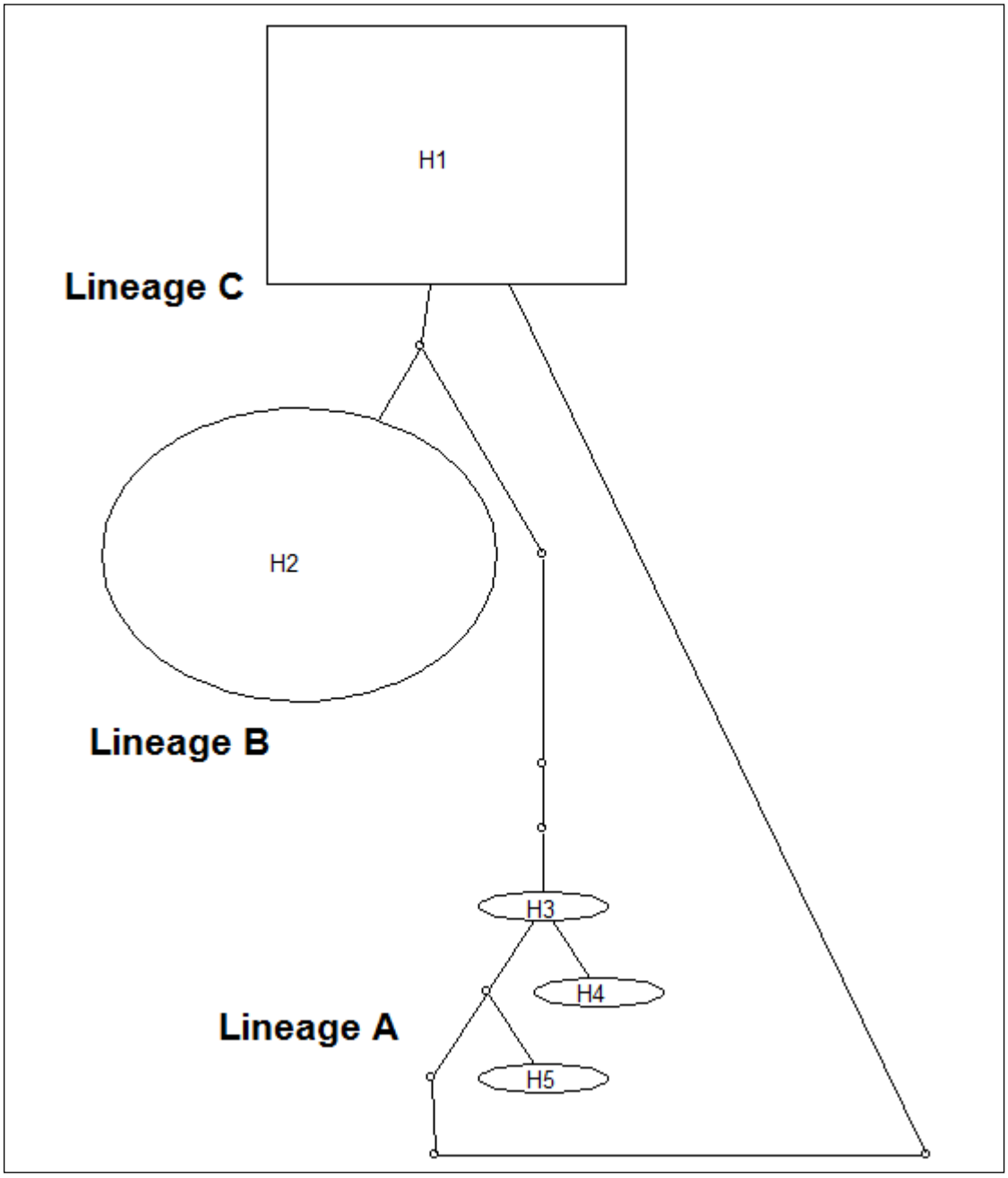

Fig. 2