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Molecular Identification of Synanthropic Flies in Malaysia

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ABSTRACT

This study was conducted to determine the presence of filth feeding and breeding flies at markets in Serdang, Malaysia. A total of 1,158 flies were collected during the study period and 14 species were successfully sequenced and identified. The identified species were belonged to three main families, Calliphoridae (*Chrysomya rufifacies, Chrysomya megacephala, Hemipyrellia ligurries and Lucilia cuprina*), Muscidae (*Musca domestica, Musca ventrosa, Ophyra chalcogaster, Ophyra spinigera and Synthesiomyia nudiseta*) and Sarcophagadae (*Sarcophaga dux, Sarcophaga peregrina, Sarcophaga ruficornis, Sarcophaga taenionota and Sarcophaga kempi*). Intraspecific variation ranged from 0.00% to 1.05%, and interspecific variation occurred between 5.82% and 13.82%. The phylogenetic tree revealed that all species formed distinct monophyletic clades. In addition, the COI gene sequence of *Musca ventrosa* and *Ophyra chalcogaster* was reported first time ever in Malaysia.

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INTRODUCTION

Filth flies or non-biting synanthropic flies comprise the domestic and coprophilic flies of families such as Muscidae, Sarcophagidae and Calliphoridae. These flies are annoying, contagious scavengers, ecologically associated with humans (Harwood *et al.*, 1989; Forster *et al.*, 2009) and significant in connection with public health. They are largely linked to sanitation practices and their population increases in both urban and rural areas when sanitary practices are not properly followed (Greenberg, 1973). These flies can detect compounds like carbon dioxide and ammonia emitted from decomposing matters and faeces with the help of sensory cells on their appendages (Tan *et al.*, 1997). An easy approach to animal manure, trash, human excrement, and other decaying materials ensures that they are charged with disease causing organisms on their mouthparts, body hairs and the sticky pads of their feet, stomach, faeces and vomit (Graczyk *et al.*, 1999). In addition, their access to food items, cutlery, kitchens, and human beings allows them to transmit disease-causing agents (Graczyk *et al.*, 2001; Getachew *et al.*, 2007).

In Malaysia, 6938 cases of food poisoning were reported in 2006 (MOH 2006), which increase by a 100% rise in 2007 (MOH 2007). In 2009, cholera, dysentery, typhoid and Hepatitis A were reported as food and waterborne diseases and 62.47 cases per 100,000 population were observed in 2008 and 36.17 in 2009 (MOH 2009 and 2010). The main reason behind all the incidence were insanitary food handling procedures which accounted for more than 50% of the poisoning episodes (MOH, 2007). Synanthropic flies have been reported in cafeterias, food courts, abattoirs and garbage spots (Nazni *et al.*, 2007; Nurita *et al.*, 2007; Nurita *et al.*, 2008; Chin *et al.*, 2009; Nurita and Abu Hassan 2013). Studies in Malaysia showed that insects, such as *Musca domestica*, *Musca sorbens* and *Chrysomya megacephala* are capable enough to carry rotavirus, *Burkholderia pseudomallei*, *Klebsiella oxytoca*, *Klebsiella pneumonia*, *Aeromonas hydrophila* and *Salmonella* sp, which caused diarrhoea, melioidosis, nosocomial infections, pneumonia, gastroenteritis and typhoid, respectively (Sohadi, 1988; Tan *et al.*, 1997; Suleiman *et al.*, 2000).

Presently, the identification of fly species is carried out via molecular analysis, such as mitochondrial DNA (mtDNA) and nuclear DNA markers. The procedure is quick, requires small tissue samples and can be done using fresh or preserved samples, and even from puparial cases

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(Waugh, 2007; Mazzanti *et al.,* 2010; Aly and Wen, 2013). Whereas mtDNA is preferred at large because it can easily be extracted from small or degraded samples. One of the mtDNA encoded genes is *cytochrome c oxidase subunit I* (*COI*) gene, which can be sequenced rapidly for identification purposes. Furthermore, various dipteran species have been identified using this technique (Harvey *et al.,* 2003; Boehme *et al.,* 2010; Sperling *et al.,* 1994; Wallman and Donnellan, 2001; Wells and Sperling, 2001; Boehme *et al.,* 2012; Jordaens *et al.,* 2013). To date, COI is well known for "DNA barcoding" and a 658-bp-long fragment has been suggested to be used for standardised species identification (Remigio and Hebert, 2003). The long *COI* marker (1,237 bp), however, is recommended due to its greater reliability and safety (Aly and Wen, 2013). The filth flies were identified in Malaysia by a few investigators collected from different locations (Lee *et al.,* 2004; Tan *et al.,* 2010; Chong *et al.,* 2014; Kavitha *et al.,* 2013). Our study particularly focused on the occurance of flies of public health importance in urban areas.

In Malaysia, many people go to fresh markets to buy their food and groceries. The presence of these flies in markets may contribute to food poisoning if improper handling of food is not practiced. Therefore, this study was aimed to identify fly species, those are potentially contigenious, in wet markets which may cause serious food-borne disease. Simultaneously, the data could alos helpful to create a database for future investigators to identify the flies on molecular basis.

MATERIALS AND METHOD

Sampling:

The fly specimens were collected from urban areas in District Serdang. The sampling locations were Pasar Borong, Pasar Cina, Pasar Bukit Serdang and Pasar Taman Seri Serdang. Five sampling spots have been selected and labelled as (A) centre, (B) shops, (C) vacant area, (D) garbage area and (E) food stalls. One day old chicken liver (200 g) was used as bait in plastic containers (8×10×12 inch) with a small entrance (3×4 inch). These containers were exposed to specimens from 0900 to 1200. Three replications were carried out. The collected insects were then transferred to the Laboratory of Insect Pathology at Department of Plant Protection, Universiti Putra Malaysia. Initial morphological identification was carried out according to Tumrasvin *et al.* (1979), Greenberg and Kunich (2002), and Sukontason *et al.* (2010). After grouping, the specimens were kept at -20˚C for further experiment.

DNA extraction:

The G-spin™ Total Kit (Intron, Korea) was used to extract the DNA from insect specimen according to the manufacturer's protocol. The insect specimen was surface-sterilized with 70% ethanol and two legs from each specimen were used for the extraction of total DNA. The DNA samples were kept at -20˚C until further experiments.

PCR amplification and sequencing:

The *COI* barcoding region was amplified by using primers, forward TY-J-1460: 5'-TACAATTTATCGCCTAAACTTCAGCC-3' and reverse primer C1-N-2800: 5'-CATTTCAAGCTGTGTAAGCATC-3' (Sperling *et al.,* 1994). PCR reaction (50 µl) containing 100 ng of DNA template, 1 unit of Taq polymerase, 1× PCR buffer (Bioron, Germany), 200 µM of each dNTP (Fermentas, USA) and 0.5 µM of each primer was prepared. The thermal cycling programme consisted of an initial denaturation step at 94˚C for 5 min, followed by 35 cycles of denaturation step at 94˚C for 1 min, an annealing step at 46˚C for 1 min 30s and an extension step at 72˚C for 2 min. The final elongation step was 72˚C for 5 min. The PCR products were detected on 1% agarose gel and were gel-purified using QIAquick® Gel Extraction Kit (Qiagen, Germany). The purified PCR products were then sent to 1st BASE laboratories Sdn Bhd. for sequencing.

Data analysis:

The quality of the sequencing results for both forward and reverse primers was checked and discrepancies were edited using a Sequence Scanner V1.0. The identification of each sequence was matched with the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI). The alignment, best model test, inter-intraspecific genetic divergence and construction of phylogenetic tree (ML) with 1000 bootstrap replications were performed using MEGA 6 (Tamura *et al.,* 2013). *Anastrepha ludens* (HQ_677058) was used as the out-group.

Results:

A total of 1,158 flies were collected during the study period. Fourteen species (Table 1) were identified belonging to three families; Calliphoridae, Muscidae and Sarcophagidae. The number of specimens analysed from each species is shown in Table 1. The *COI* gene fragment ranging from 476-1,250 bp from all specimens was successfully sequenced in this study. Sharp peaks were observed in the electrophoregrams and no stop codon was found between the sequences, which indicates that no co-amplification of nuclear pseudogenes occurred. The blast results in NCBI showed 99-100% similarity at the species level. All sequences were submitted to Genbank database under the accession numbers as given in Table 1.

Table 1: Species and number of specimens analysed during the study. Accession numbers of sequences submitted in Genbank are also given.

Species	No. of analysed specimens	Accession number
Calliphoridae		
Chrysomya rufifacies	5	KC_855273-74, KF_562105, KJ_496779-80
Chrysomya megacephala	10	KC_855270-72,KC_855286, KF_562106,KJ_496781-85
Hemipyrellia ligurries	5	KC_855275,KF_562113, KJ_496772-74
Lucilia cuprina	4	KF_562103,KJ_496769-71
Muscidae		
Musca domestica	5	KC_855277,KF_562113, KJ_496775-77
Musca ventrosa	2	KF_562112, KJ_496778
Ophyra chalcogaster	3	KC_855281,KF_562114, KJ_496788
Ophyra spinigera	3	KC_855280,KJ_496786-87
Synthesiomyia nudiseta	2	KF_562117, KJ_496790
Sarcophagadae		
Sarcophaga dux	7	KC_855284, KF_562109, KJ_496796-800
Sarcophaga peregrina	7	KC_855282-83, KF_562108,KJ_496791-94
Sarcophaga ruficornis	3	KF_562110, KJ_496801-02
Sarcophaga taenionota	3	KC_855285, KF_562107, KJ_496795
Sarcophaga kempi	2	KF_562111, KJ_496803

All sequences were successfully aligned for phylogenetic and sequence divergence analyses, and no insertion or deletion was observed within the sequences. This region of mtDNA was observed to have a strong AT bias (69.54%), a characteristic of insect mitochondrial DNA (Crozier and Crozier, 1993), where the nucleotide compositions were A (30.85%), T (38.69%), C (14.69%), and G (15.77%). The data revealed 372 variable positions, 316 of which were parsimoniously informative. For construction of the phylogenetic tree, the best model test was performed in MEGA6 (Tamura *et al.,* 2013). which suggested the General Time Reversible model with invariable site distribution (+*I*) on the basis of the lowest Bayesian Information Criterion (BIC=13626.899). The model showed unequal base frequencies; A = 0.308, T = 0.387, C = 0.147, G = 0.158; and the estimated proportion of invariable sites (*I*) was 0.64.

In Table 2, the intraspecific variation of species was only noted for seven fly species, namely *Chrysomya rufifacies* (0.32%), *Chrysomya megacephala* (0.57%), *Hemipyrellia ligurries* (0.40%), *Lucilia cuprina* (0.16%), *Sarcophaga dux* (0.89%), *Sarcophaga peregrina* (1.05%) and *Sarcophaga ruficornis* (0.81%). The intraspecific variation was less than 1% except *Sarcophaga peregrina* which is generally accepted at the species level (Zehner *et al.,* 2004; Wallman and Donnellan, 2001).

Among the species of the family Calliphoridae, the highest interspecific variation was found to be 9.86% between *Chrysomya rufifacies* and *Hemipyrellia ligurries,* and the lowest difference was between *Lucilia cuprina* and *Hemipyrellia ligurries* (5.82%) (Table 3). In the family Muscidae, the highest and the lowest interspecific variations were 12.44% and 7.35%, respectively. The most different species were *Musca domestica* and *Synthesiomyia nudiseta* whereas the closest species were *Ophyra spinigera* and *Ophyra chalcogaster*. The results showed the highest difference between *Sarcophaga peregrina* and *Sarcophaga kempi* (9.45%) whereas the least different species were *Sarcophaga taenionota* and *Sarcophaga dux* (6.10%). The comparison between the investigated flies and the out-group revealed that the maximum difference was from *Lucilia cuprina* (15.28%) and the minimum difference was from *Hemipyrellia ligurries* (13.98%).

A neighbour joining (NJ) tree was constructed (Figure 1) with the maximum likelihood model and 1000 bootstrap replications. All flies were rightly assigned into 14 species and yielded three distinct and congeneric groups (Calliphoridae, Muscidae, and Sarcophagidae) with monophyletic separation in the NJ tree. The neighbour joining tree revealed four groups – two distinct groups, one from Calliphoridae and one from Sarcophagidae, and two groups from family Muscidae. The bootstrap percentage value for the monophyly of *Chrysomya rufifacies*, *Lucilia cuprina*, *Sarcophaga ruficornis*, *Ophyra spinigera* and *Musca domestica* was 100%. The other species showed a bootstrap percentage ranging from 46-99%. Some species, i.e. *Chrysomya megacephala*, *Hemipyrellia ligurries*, *Sarcophaga peregrina* and *Sarcophaga dux*, produced different bootstrap percentages within the same group.

Discussion:

This study was conducted to identify dipteran flies of health importance from urban area and to test the reliability of the *COI* barcodes to distinguish between different species. *COI* gene analysis of filth flies in Malaysia has been conducted by a few researchers. Lee *et al.,* (2004) reported the flies pre-collected by the hospital and police department, Tan *et al.* (2010) reported the Sarcophagidae flies whilst

Chong *et al.*, (2014) reported the *Chrysomya megacephala* in Malaysia. The study particularly focused on the occurance of flies of public health importance in urban area. It shows that flies from three main families, namely Calliphoridae, Muscidae and Sarcophagidae comprised of members such as *Chrysomya rufifacies*, *Chrysomya megacephala*, *Hemipyrellia ligurries* and *Lucilia cuprina*, *Musca domestica*, *Musca ventrosa*, *Ophyra chalcogaster*, *Ophyra spinigera* and *Synthesiomyia nudiseta*, *Sarcophaga dux*, *Sarcophaga peregrina*, *Sarcophaga ruficornis*, *Sarcophaga taenionota* and *Sarcophaga kempi* were frequently present in fresh markets. These flies are active vector of disease causing pathogens such as mysiasis, dysentery, diarrhoea, cholera, salmonellosis and many other diseases like tuberculosis, trachoma and skin infections (WHO, 1991; Goddard, 1996; Olsen *et al*., 2001; Banjo *et al*., 2005).

Table 2: Intraspecific divergence in between the sequences of species.

Family	Species	Maximum variation within species (%)	Number of variation
Calliphoridae	*Chrysomya rufifacies*	0.32	4
	Chrysomya megacephala	0.57	7
	Hemipyrellia ligurries	0.40	5
	Lucilia cuprina	0.16	2
Muscidae	*Musca domestica*	0.00	0
	Musca ventrosa	0.08	1
	Ophyra spinigera	0.08	1
	Ophyra chalcogaster	0.08	1
	Synthesiomyia nudiseta	0.08	1
Sarcophagidae	*Sarcophaga dux*	0.89	11
	Sarcophaga peregrina	1.13	14
	Sarcophaga ruficornis	0.81	10
	Sarcophaga taenionota	0.00	0
	Sarcophaga kempi	0.00	0

Table 3: Pairwise divergence between species. Upper right panel: nucleotide divergence in percentage, lower left panel: absolute nucleotide differences.

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Chrysomya rufifacies*		6.71	9.782	9.863	10.11	11.16	10.19	11.24	11.64	11.8	10.27	10.27	10.75	11.8	14.07
2	*Chrysomya megacephala*	83.00		8.327	8.569	9.943	10.75	10.43	10.99	12.29	11.96	10.67	10.43	10.51	11.08	15.04
3	*Lucilia cuprina*	121.00	103.00		5.821	10.91	10.99	9.297	9.297	11.24	10.91	9.62	9.943	9.943	11.4	15.28
4	*Hemipyrellia ligurries*	122.00	106.00	72.00		11.4	11.32	9.782	9.62	10.83	11.72	10.11	10.59	10.67	11.32	13.99
5	*Musca domestica*	125.00	123.00	135.00	141.00		8.892	10.43	11.16	12.45	13.26	12.21	12.05	12.37	13.18	14.71
6	*Musca ventrosa*	138.00	133.00	136.00	140.00	110.00		11.24	11.72	12.37	13.42	12.93	12.69	12.77	13.18	14.15
7	*Ophyra spinigera*	126.00	129.00	115.00	121.00	129.00	139.00		7.357	9.782	12.37	11.16	10.59	11.72	12.05	14.07
8	*Ophyra chalcogaster*	139.00	136.00	115.00	119.00	138.00	145.00	91.00		9.539	11.88	11.32	11.64	11.24	11.88	13.26
9	*Synthesiomyia nudiseta*	144.00	152.00	139.00	134.00	154.00	153.00	121.00	118.00		13.82	12.69	12.93	12.05	12.61	14.47
10	*Sarcophaga peregrina*	146.00	148.00	135.00	145.00	164.00	166.00	153.00	147.00	171.00		6.71	7.599	7.68	9.458	14.31
11	*Sarcophaga taenionota*	127.00	132.00	119.00	125.00	151.00	160.00	138.00	140.00	157.00	83.00		6.063	6.952	8.973	14.47
12	*Sarcophaga dux*	127.00	129.00	123.00	131.00	149.00	157.00	131.00	144.00	160.00	94.00	75.00		7.842	8.488	14.55
13	*Sarcophaga ruficornis*	133.00	130.00	123.00	132.00	153.00	158.00	145.00	139.00	149.00	95.00	86.00	97.00		8.488	14.31
14	*Sarcophaga kempi*	146.00	137.00	141.00	140.00	163.00	163.00	149.00	147.00	156.00	117.00	111.00	105.00	105.00		14.87
15	*Anastrepha ludens*	174.00	186.00	189.00	173.00	182.00	175.00	174.00	164.00	179.00	177.00	179.00	180.00	177.00	184.00	

The decision for choosing the molecular marker is crucial in investigating the genetic variation within or between different species. It is important to use a molecular marker that evolves at a proper rate for the identification of the population composition (Avise, 2000). Mitochondrial cytochrome oxidase I is a suitable molecular marker due to its relatively high degree of genetic variation in this region (Sperling *et al.*, 1994; Benecke and Wells, 2001). However, there are two risks that should be considered in using the mtDNA marker: First, the infection rate of the endosymbiotic bacteria, Wolbachia, in insects is 15-75%, which can potentially influence the mtDNA variation of insects at the intra or interspecific level, especially in very closely related species (Werren and Windsor, 2000; Hurst and Jiggins, 2005; Whitworth *et al.*, 2007; Charlat *et al.*, 2009). Second, the amplification of the NUMTs (nuclear mitochondrial DNA) may lead to a bias pattern of mitochondrial diversity and be potentially misrepresented (Bensasson *et al.*, 2001; Charlat *et al.*, 2009). In this study the legs for DNA extraction were used to avoid contamination. The analysed mtDNA fragment showed no insertion or deletion as reported in the previous literature (Hebert *et al.*, 2003; Ward *et al.*, 2005; Meiklejohn *et al.*, 2011). The results of the study confirm that there is no issue of NUMTs.

Aly (2014) and Tan *et al.* (2010) reported that the long fragment of *COI* gene gave greater completeness of monophyletic separation and was more in accordance with their taxonomic classification compared to the short fragment of *COI* gene. We analysed the long fragment of *COI* gene of the flies collected from urban fresh markets, which generated very clear and distinct interspecific

variations between the species. These comprised a strong adenine thymine bias, which is a characteristic of insect mtDNA (Nelson *et al.,* 2007; Meiklejohn *et al.,* 2011).

Identification through *COI* barcodes has some threshold percentages for species separation. The *COI* standard threshold should be applied with great care, and specific thresholds for each group must be established (Hebert *et al.,* 2003). It was also suggested that the intraspecific variation should not exceed more than 3% and the least percentage of interspecific variation should be more than 3% (Hebert *et al.,* 2003; Hebert *et al.,* 2004a; Hebert *et al.,* 2004b; Amendt *et al.,* 2011). The same threshold was used for Sarcophagidae identification by Meiklejohn *et al.,* (2011). In this study, the highest intraspecific variation was (1.05%) found in *Sarcophaga* and the least interspecific variation (12.44%) was between *Musca domestica* and *Synthesiomyia nudiseta*. These data could be obtained conveniently using neighbour joining analysis. This is the most commonly used method for handling DNA barcode data, constructing an appropriate model, as well as building a proper tree. The barcoding region carries sufficient information to distinguish between all the species examined due to high bootstrap support and all species are reciprocally monophyletic, which is standard for species distinction (Wells *et al.,* 2007).

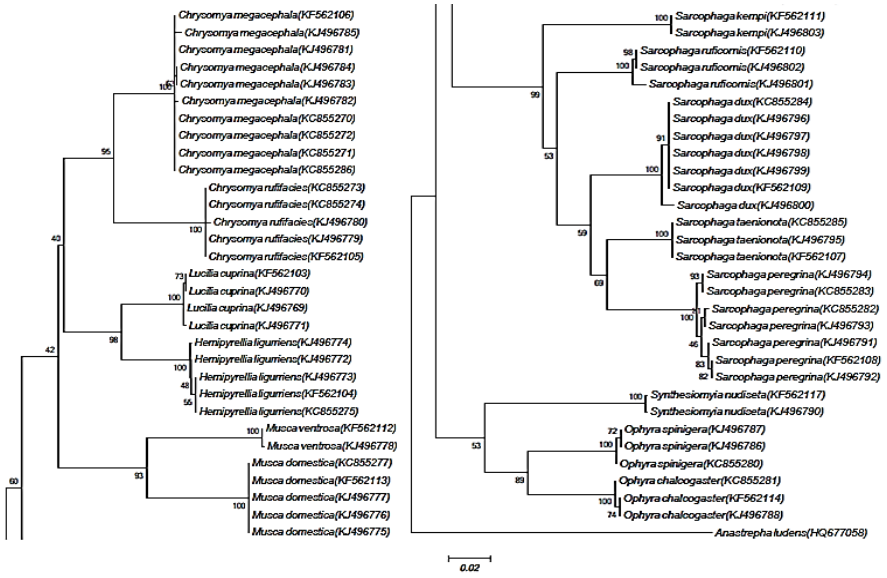

Fig. 1: The neighbour joining tree (ML) constructed for 34 individuals from 14 species. *Chrysomya rufifacies* (5), *Chrysomya megacephala* (10), *Hemipyrellia ligurries* (5*), Lucilia cuprina* (4), *Musca domestica* (5), *Musca ventrosa* (2), *Ophyra chalcogaster* (3), *Ophyra spinigera* (3), *Synthesiomyia nudiseta* (2), *Sarcophaga dux* (7), *Sarcophaga peregrina* (7), *Sarcophaga ruficornis* (3), *Sarcophaga taenionota* (3), *Sarcophaga kempi* (2). *Anastrepha ludens* was used as out group.

Conclusion:

The presence of filth flies in the study areas is a matter of great concern as these flies are potential carrier of different disease-causing agents. To date, the *COI* gene of *Musca ventrosa* and *Ophyra chalcogaster* reported first time in Malaysia. Future work on different dimensions of these flies should be carried out in Malaysia. Furthermore, this study also revealed that the contiginous flies are distributed in different fresh markets which can potentially be disperse in the surrounding, where they are not only become nuisance but threat to public health. Finally, this study may gain the attraction of people and authorities to enforce the proper sanitation practices in public places.

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