

## **OCCURRENCE OF SYNANTHROPIC FLIES IN TASEK BERA RAMSAR SITE, PAHANG**

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### **ABSTRACT**

A study of the occurrence and species distribution of synanthropic flies species at Tasek Bera Ramsar Site was carried out during the Biodiversity Inventory Program from 10<sup>th</sup> to 12<sup>th</sup> May 2014 and 14<sup>th</sup> to 16<sup>th</sup> August 2014. A total of 716 synanthropic flies were collected and grouped according to their morphological characteristics. Molecular identification with partial mitochondrial *cytochrome oxidase I* (COI) gene sequences confirmed the identity of the flies, namely *Chrysomya megacephala*, *Chrysomya ruffifacies*, *Hemipyrellia ligurriens*, *Hypopygiopsis infumata*, *Lucilia cuprina*, *Atherigona orientalis*, and *Sarcophaga dux* and one unidentified fly. These flies belong to three main families of synanthropic flies (Sarcophagidae, Calliphoridae and Muscidae) and they are important in forensic studies and veterinary entomology. The most abundant family was Calliphoridae (73.2%), followed by Muscidae (21.1%) and Sarcophagidae (5.7%). The highest number of species and individuals captured came from the family Calliphoridae with *Chrysomya megacephala* as the most prevalent species at 52.7 %.

**Keywords:** Tasek Bera Ramsar Site, synanthropic flies, COI gene.

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## INTRODUCTION

Flies (Dipteran) are insects that are important in agriculture, medical and forensic study. Flies that are ecologically associated with human activities are known as synanthropic flies. Examples are house flies, blow flies and flesh flies that have been recognised as unpleasant and disturbing creatures to human. They exploit food and habitat from human activities, and are capable of transmitting human pathogen such as viruses, fungi, bacteria, and parasites in different regions of the world (Banjo *et al.*, 2005). Synanthropic flies have been identified as vectors of protozoan parasites such as *Cryptosporidium parvum* and *Giardia lamblia* that cause diarrheal disease worldwide (Conn *et al.*, 2007), carrier of bacteria such as *Escherichia coli* (Butler *et al.*, 2010), *Aeromonas hydrophila* and *Pseudomonas aeruginosa* (Sukontason *et al.*, 2007), and viral pathogen such as *Bovine papillomavirus* (Finlay *et al.*, 2009). Some species of synanthropic flies also play an important role in forensic entomology. In Malaysia, many researches were conducted to focus on the fly species that are medically and forensically important (Heo *et al.*, 2007; Azwandi & Abu Hassan, 2009). They use the information derived from either the succession of arthropods on human corpses, animal carcasses or the temperature-dependent development of insects (primarily flies) to estimate the time elapsed since death, or postmortem interval (PMI). Calliphoridae, Muscidae, and Sarcophagidae are among the synanthropic flies that are valuable entomological evidence. Since synanthropic flies are proven nuisance and significant both as disease carrier and entomological evidence, species identification is a key step in estimating the PMI from entomological evidence as well as for the management of flies in public access.

Information of synanthropic flies in forest reserve of Malaysia is limited. Occurrence of forest synanthropic flies has been reported by Heo *et al.* (2008) and Goh *et al.* (2013). Heo *et al.* (2008) reported three synanthropic flies species from Bukit Pelindung Recreational Forest in Kuantan, Pahang, while Goh *et al.* (2013) reported seven synanthropic flies species from Bukit Cincin Forest in Pahang. Tasek Bera Ramsar Site is the largest freshwater lake in Malaysia which is located in the southeastern of Pahang state. It received her international recognition as a Ramsar site in Malaysia during the Ramsar Convention of Wetlands on 10<sup>th</sup> November 1994 with the aim to maintain the ecological features of wetlands and in addition to allow the resources utilisation in a sustainable manner (Wetland International Malaysia, 2016). To date, the fauna and flora which have been identified in Tasek Bera including 67 species of mammals, 230 species of birds, 40 species of herpetofauna, 92 species of spiders, more than 94 species of fish and 374 species of plants (Lau *et al.*,

2011). The information on the diversity of synanthropic flies in Tasek Bera Ramsar Site is lacking. Thus, the aim of this study was to generate a baseline data on the synanthropic flies in Tasek Bera Ramsar Site.

## MATERIALS AND METHODS

### Sampling location

The sampling activities were carried out at Tasek Bera Ramsar Site during the 2014 Biodiversity Inventory Program conducted by the Department of Wildlife and National Parks Peninsular Malaysia from 10<sup>th</sup> to 12<sup>th</sup> May 2014 and 14<sup>th</sup> to 16<sup>th</sup> August 2014. Specimens were collected from the base camp and the forest trails leading to the base camp. It is a unique and remote wetland surrounded by lowland dry dipterocarp forests and peat swamp forests which support a diverse local flora and fauna.

### Insect sampling

Samplings were conducted with custom made traps using mixture of chicken, fish and prawn as bait weighing approximately 200 grams, which was kept overnight at room temperature to allow it to become rotten. The traps were exposed for 6 hours started from 8 am until 2 pm. The distance between traps was standardised at 50 meters. The specimens collected were kept in ethyl acetate and then transferred to the Laboratory of Insect Pathology in the Department of Plant Protection, Universiti Putra Malaysia. Initial morphological identification was carried out under a dissecting microscope using the pictorial keys of Dodge (1953), Scott and Borom (1962), Byrd and Castner (2009) and Wallman (2001), and taxonomy keys of Byrd and Castner (2009), Couri *et al.* (2006), de Carvalho and Mello-Patiu (2008), Sukontason *et al.* (2010) and Moophayak *et al.* (2011). The specimens were kept at -20°C for molecular identification.

### Molecular identification

The insect specimens were surface-sterilised with 70% ethanol and two legs from each specimen were cut using a surgical blade and placed in a 1.5 ml microcentrifuge tube. The sample was grinded with 1 ml of lysis buffer, 100 µl of 10% SDS and 20 µl of proteinase K (500 mg/mL) and then incubated overnight at 55°C. The phenol and chloroform extraction was carried out, followed by washing steps with absolute ethanol and 70% ethanol. The DNA template was resuspended in 30 µl of dH<sub>2</sub>O and kept at -20°C. Partial cytochrome oxidase I (COI) gene region was amplified by using forward primer TY-J-1460: 5'-

TACAATTTATCGCCTAAACTTCAGCC -3' and reverse primer C1-N-2800: 5'-CATTTC AAGCTGTGTAAGCATC -3' (Sperling *et al.*, 1994). PCR was performed in 50  $\mu$ l reaction mixture containing 36.7  $\mu$ l of distilled water, 5.0  $\mu$ l of reaction buffer, 1.0  $\mu$ l of 0.2 mM dNTPs, 3.0  $\mu$ l of 10 mM forward primer, 3.0  $\mu$ l of 10 mM reverse primer, 0.3  $\mu$ l of 0.1 unit/ $\mu$ l *Taq* DNA polymerase and 1.0  $\mu$ l of DNA from each sample. 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 (Tan *et al.*, 2010). The final elongation step was performed at 72°C for 5 min. PCR products were detected on 1% agarose gel. PCR products with expected size were gel-purified using QIAquick<sup>®</sup> Gel Extraction Kit (Qiagen, Germany). Samples were sent to 1<sup>st</sup> BASE sequencing company for sequencing service. 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, nucleotide composition, best model test, inter-intraspecific genetic divergence and construction of phylogenetic tree with 1000 bootstrap replications were performed using MEGA 6 (Tamura *et al.*, 2013). *Anastrepha ludens* (HM538311) was used as the out-group.

### Frequency and dominance of a species

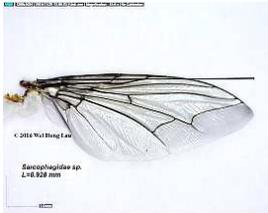
The relative frequency of synanthropic flies was calculated by taking the total number of flies in a given species and dividing it by the total number of flies collected and multiplying by 100. The dominance of the synanthropic flies were calculated according to the equation of Oliveira and Vasconcelos (2010). Dominance was calculated as  $D = [\text{abundance of the family } i / \text{total abundance of the specimens}] \times 100$ . When  $D \geq 5\%$  = dominant family;  $2.5\% \leq D < 5\%$  = accessory family, when  $D < 2.5\%$  = occasional family. The species were grouped as low ( $N \leq 50$ ), Intermediate ( $50 < N \leq 100$ ) and high ( $N > 100$ ).

## RESULT

A total of 716 flies were collected during the 2014 Biodiversity Inventory Program and identified based on their morphological appearance including body color, characteristic of wing venation, and body markings. These flies were identified belonging to three main synanthropic fly families, namely Muscidae, Calliphoridae, and Sarcophagidae (Table 1). The Calliphoridae flies, also known as blow flies, are medium-sized to large robust flies with a mean body length

7.51±0.38 mm. They have metallic thorax and abdomen. The Muscidae flies are smaller in size (5.73±0.01 mm) with greyish yellow thorax and abdomen. The only species of Sarcophagidae fly collected from Tasek Bera Ramsar Site has the largest body length (11.28±0.17 mm) with dark grey body, three longitudinal black strips on thorax and checkered abdomen.

**Table 1** Morphological appearances of flies captured at Tasek Bera Ramsar Site

Family	Calliphoridae	Sarcophagidae	Muscidae
Specimen			
Colour	Blue mettalic	Greyish	Greyish yellow
Strips	Do not have strips	Three black strips on thorax	2 to 4 black strips on thorax
Vein M	Sharply bent	Sharply bent	Not sharply bent
			

Among the flies collected, seven species of flies were identified up to species level. Examples are *Chrysomya megacephala*, *Chrysomya rufifacies*, *Hemipyrellia ligurriens*, *Hypopygiopsis infumata*, *Lucilia cuprina*, *Atherigona orientalis* and *Sarcophaga dux* (Table 2, Figure 1). One species of Calliphoridae fly (unidentified sp.) was identified only up to the family level. The most abundant family was Calliphoridae (73.3%), followed by Muscidae (21.1%) and Sarcophagidae (5.7%) (Table 3). Family Calliphoridae had the highest number of individuals captured and highest number of species. *Chrysomya megacephala* was the most prevalent species (52.7%) in the sampling sites, followed by *Atherigona orientalis*, *C. rufifacies*, *S. dux*, *H. ligurriens*, *L. cuprina*, unidentified sp. and *H. infumata*. *Chrysomya megacephala* and *A. orientalis* showed high abundance while *C. rufifacies* showed intermediate abundance in the sampling sites. Other species were low in abundance. For dominance assessment, *C. megacephala*, *C. rufifacies*, *A. orientalis* and *S. dux* were identified as the dominant species while *H. ligurriens* was accessory species. *Hypopygiopsis infumata*, *L. cuprina* and unidentified sp. were occasional species.



*Chrysomya megacephala*



*Chrysomya rufifacies*



*Lucilia cuprina*



*Hypopygiopsis infumata*



*Sarcophaga dux*



*Atherigona orientalis*



*Hemipyrellia ligurriens*



Unidentified sp.

**Figure 1** Flies collected from Tasek Bera Ramsar Site, Pahang.

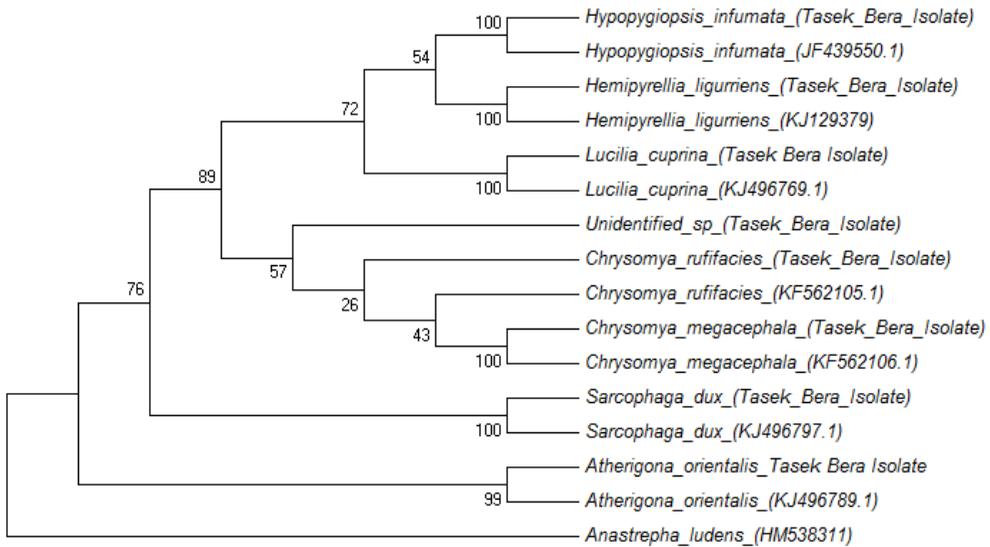
**Table 2** Flies collected from Tasek Bera Ramsar Site, Pahang

Family	Morphological identification	Molecular identification
Calliphoridae	<i>Chrysomya megacephala</i>	<i>Chrysomya megacephala</i>
	<i>Chrysomya rufifacies</i>	<i>Chrysomya rufifacies</i>
	<i>Hemipyrellia ligurriens</i>	<i>Hemipyrellia ligurriens</i>
	<i>Hypopygiopsis</i> sp.	<i>Hypopygiopsis infumata</i>
	<i>Lucilia cuprina</i>	<i>Lucilia cuprina</i>
	Unidentified sp.	Unidentified sp.
Muscidae	<i>Atherigona</i> sp.	<i>Atherigona orientalis</i>
Sarcophagidae	<i>Sarcophaga</i> sp.	<i>Sarcophaga dux</i>

**Table 3** Species abundance and dominance

Family	Species	Number of individual	Relative frequency (%)	Abundance	Dominancy
Calliphoridae	<i>C. megacephala</i>	377	52.7	High	Dominant
	<i>C. rufifacies</i>	83	11.6	Intermediate	Dominant
	<i>H. ligurriens</i>	31	4.3	Low	Accessory
	<i>H. infumata</i>	10	1.4	Low	Occasional
	<i>L. cuprina</i>	12	1.7	Low	Occasional
	Unidentified sp.	11	1.5	Low	Occasional
Muscidae	<i>A. orientalis</i>	151	21.1	High	Dominant
Sarcophagidae	<i>S. dux</i>	<u>41</u>	<u>5.7</u>	Low	Dominat

The COI gene sequence of the Tasek Bera flies have a characteristic of insect mitochondrial DNA. This region of mtDNA was observed to have a strong AT bias (69.8%), where the nucleotide compositions were A (30.5%), T (39.3%), C (15.5%), and G (14.6%). All sequences revealed 445 variable positions with 300 of which were parsimoniously informative. The General Time Reversible model with Gamma site distribution (+G) on the basis of the lowest Bayesian Information Criterion (BIC=3959.91) was suggested the best model test for the construction of phylogenetic tree for the Tasek Bera flies. This model showed unequal base frequencies; A = 0.307, T = 0.399, G= 0.140, C= 0.154; and the estimated proportion of gamma sites (*G*) was 0.161. The bootstrap percentage value for *Hypopygiopsis infumata*, *Hemipyrellia ligurriens*, *Lucilia cuprina*, *Chrysomya megacephala*, and *Sarcophaga dux* are 100% (Figure 2). The other species showed a bootstrap percentage less than 100%.



**Figure 2** The neighbour joining tree (ML) constructed for the flies collected from Tasek Bera, Ramsar Site, Pahang. *Hypopygiopsis infumata* (2), *Hemipyrellia ligurriens* (2), *Lucilia cuprina* (2), Unidentified sp (1), *Chrysomya rufifacies* (2), *Chrysomya megacephala* (2), *Sarcophaga dux* (2), *Atherigona orientalis* (2) and *Anastrepha ludens* was used as out group.

Within the Calliphoridae family, the highest difference was detected between *H. infumata* and the unidentified species (11.05%) while the lowest difference was found to be between *C. rufifacies* and *H. ligurriens* (4.84%) (Table 4). The unidentified species which was categorised under the Calliphoridae family is closest to *H. ligurriens* (6.54%) while having the highest difference when compared with *H. infumata* (11.05%). The comparison between the investigated flies and the out- group (*A. ludens*) revealed that the maximum difference was from *H. infumata* (10.29%) and the minimum difference was from *C. rufifacies* (8.73%).

**Table 4** Pairwise divergence between species. Upper right panel represents the nucleotide divergence (in %) while the lower left panel represents the absolute nucleotide differences.

No. Species	1	2	3	4	5	6	7	8	9
1. <i>Lucilia cuprina</i>		7.30	10.71	10.18	5.67	10.02	10.12	8.38	9.81
2. <i>Chrysomya rufifacies</i>	57		7.87	7.64	4.84	7.77	8.66	7.24	8.73
3. <i>Chrysomya megacephala</i>	118	61		10.49	7.00	10.79	10.08	7.84	9.94
4. <i>Hypopygiopsis infumata</i>	100	63	122		5.57	11.05	10.25	8.65	10.29
5. <i>Hemipyrellia ligurriens</i>	31	26	52	32		6.54	8.42	7.90	8.82
6. Unidentified sp	121	62	125	135	49		10.17	8.46	9.67
7. <i>Atherigona orientalis</i>	111	93	116	121	74	110		9.20	9.94
8. <i>Sarcophaga dux</i>	62	50	59	73	59	68	86		9.72
9. <i>Anastrepha ludens</i> (HM538311)	109	83	106	111	87	105	104	92	

## DISCUSSION

This investigation was the first field research attempt carried out to determine the synanthropic flies at Tasek Bera Ramsar Site. Throughout the sampling activities conducted at the site, a total of 8 species of synanthropic flies were recorded. Some species identified in this investigation were also reported from different forests in Malaysia. *Chrysomya megacephala*, *Sarcophaga* sp. and *M. domestica* were reported in Bukit Pelindung Recreational Forest by Heo *et al.* (2008) while *C. megacephala*, *C. rufifacies* and *H. ligurriens* were reported to be present in the forest near Ulu Gombak, Selangor by Nazmi *et al.* (2011). *Chrysomya megacephala*, *C. rufifacies* and *A. orientalis* were found to be present in the Selangor forest as reported by Azwandi *et al.* (2013). A total of 6 different species from the Calliphoridae family were collected from the Tasek Bera Ramsar Site with *C. megacephala* as the predominant fly. Goh *et al.* (2013), Bunchu *et al.* (2012) and Moophayak *et al.* (2011) also reported findings of having *C. megacephala* as the predominant fly in forests. *Chrysomya megacephala* was also reported as the most abundant fly species in local fresh market (Khosso *et al.*, 2015a) and cafeterias (Khosso *et al.*, 2015b). *Musca domestica*, is another synanthropic fly that is normally present in most areas such as cafeterias (Tahir *et al.*, 2007) and animal farms (Albarrak, 2009). However, throughout the sampling carried out, *M. domestica* was not captured even though these flies can be seen hovering around the garbage in the kitchen. This may happen due to food and environmental preferences whereby this species are more attracted to garbage areas because of the presence of more organic matter and a higher moisture level compared to the baits set up at the sampling sites. Among the flies collected, a Calliphoridae species,

*Hypopygiopsis infumata* is the first record in Malaysia. This species have been identified in the forest located at northern Thailand as reported by Bunchu *et al.* (2012) and Moophayak *et al.* (2011). The larvae of another Calliphoridae species, *H. violacea* was presence in the oil palm plantation in Malaysia (Firdaus *et al.*, 2010).

From this study, it was observed that the Calliphoridae species captured which were *C. megacephala*, *C. rufifacies*, *H. ligurriens*, *L. cuprina*, *H. infumata* have a sharply bend wing vein M. The adults of *Chrysomya megacephala* are bright metallic green with black margins on the second and third abdominal segments and have a size of over 9.5 mm long. This fly has a pair of large and prominent shade of red eyes that almost touch each other. The area below the eyes is slightly yellowish-orange. These findings were in agreement with Siriwattanarungsee *et al.* (2005). The sex of *C. megacephala* can be differentiated according to their eyes whereby the males have eyes that are close together while the females further apart (Shiao & Yeh, 2008). *Chrysomya rufifacies* was observed to have a shiny metallic blue-green colour with a body length of 6-12 mm, a pale genal dilation, and a vestiture of the anterior thoracic spiracle that is pale in colour. These observations on *C. rufifacies* were in accordance as mentioned by Whitworth (2010). Both *C. megacephala* and *C. rufifacies* are flies of forensic importance recorded in Malaysia (Hamid *et al.*, 2003; Lee *et al.*, 2004).

*Hemipyrellia ligurriens*, another forensically important blow fly species in Thailand and Malaysia was identified based on the morphological characteristics as described by Bunchu *et al.* (2012). It was observed that *H. ligurriens* has a metallic copper green abdomen, with greyish white pollinose on the anterior part of the thorax and has gena covered with black hairs. The adult of *H. ligurriens* was about 12 mm in length. Another important species of blow flies found in this study is *L. cuprina*, a facultative ectoparasite of warm blood vertebrates (Stevens & Wall, 1996). *Lucilia cuprina* has a metallic bronze-green dorsal thorax and abdomen, and metallic green femoral joint in the first pair of legs and bare squamae (Williams & Villet, 2014). *Lucilia cuprina* regularly colonises carcasses in human habitation and is also known as a highly synanthropic fly species (Chaiwong *et al.*, 2012). *Hypopygiopsis infumata* is larger in size, approximately 5-20 mm in length, with a metallic blue appearance. The role of *H. infumata* in forensic evidence and as a disease transmitter is still unknown as not much findings on these species were reported. A yellow fly labelled as an unidentified sp., was collected in this study and was grouped under the Calliphoridae family based on the preliminary morphological identification.

It is still pending for further morphological and molecular identification due to lacking of reference specimen and genes for identification.

*Atherigona orientalis*, a member of Muscidae, is normally referred as the pepper fruit fly or tomato fruit fly. It was observed to be a small, greyish yellow fly with a long face and a large antennal flagellomere as described by Pont and Magpayo (1995). These flies are considered as secondary pests or trash flies as they lay eggs on ripen or rotten fruits. Despite being a fruit pest, *A. orientalis* also showed a marked necrophagous behaviour, confirming their importance in forensic entomology (Uribe-M. *et al.*, 2010). *Sarcophaga dux* is the only Sarcophagidae species found in this study. It has a greyish thorax with dark longitudinal stripes on it. It is also observed that *S. dux* has checkering on the abdomen as well as large compound eyes, antennae and a sponging mouthpart with prominent palps. The morphological description by Sukontason *et al.* (2010) used to describe *S. dux* was in accordance with the morphological characteristics of *S. dux* sampled. *Sarcophaga dux* has been reported as a forensically important flesh fly in Thailand (Bänziger & Pape, 2004).

Most of the fly species recorded in this study have been reported to play an important role in forensic and medical studies. However, there is limited information for *H. infumata* and the unidentified sp. Lee *et al.* (2004) reported the identification of several forensically important specimens collected from cases involving humans, which includes the families of Calliphoridae, Sarcophagidae, and Muscidae. Heo *et al.* (2008) recorded the presence of *C. megacephala* and *C. rufifacies* on floating carcass. Other Calliphoridae species reported to be involved in forensic cases are *Lucilia*, *Hemipyrellia* and *Calliphora* (Lee *et al.*, 2004). *Aeromonas hydrophila*, *Enterobacter agglomerans*, *Klebsilla oxytoca*, and *Escherichia coli* are among the pathogenic bacteria associated with *C. megacephala* in Malaysia and Thailand (Sulaiman *et al.*, 2000; Sukontason *et al.*, 2007). A study conducted by Fetene and Worku (2009) showed that non-biting cyclorrhaphan flies including *C. rufifacies* and *L. cuprina* carried *Ascaris lumbricoides*, *Trichuris trichiura*, *Hymenolepis nana*, *Taenia* spp., *Strongyloides stercoralis*, *Entamoeba histolytica*, *Entamoeba coli*, *Cryptosporidium* spp. and *Giardia lamblia*. The forensic importance of *A. orientalis* has been established based on the occurrence of adults and immature stages on or in the animal carrion (Vasconcelos & Araujo, 2012). This species is known for its role as a vector of various pathogens (de Oliveira *et al.*, 2002). *Sarcophaga dux* species is of medical importance as a myiasis-producing agent (Kaufmann, 1996) as well as forensic importance as it is known to colonise decomposing human remains (Cherix *et al.*, 2012).

Among the species of the family Calliphoridae, the highest interspecific variation was found to be 11.05% between *H. infumata* and the unidentified species, and the lowest difference was between *C. ruffifacies* and *H. ligurriens* (4.84%). There are threshold percentages to be followed for species separation identified through COI barcodes. According to Hebert *et al.* (2003), specific thresholds for different group species should be established and the COI standard threshold should be applied with great care. The intraspecific variation should not exceed more than 3% while the interspecific variation having the least percentage should be more than 3% (Hebert *et al.*, 2003; Hebert *et al.*, 2004a; Hebert *et al.*, 2004b; Amendt *et al.*, 2011).

## CONCLUSION

This study illustrates the diversity of synanthropic flies collected at Tasek Bera Ramsar Site. Flies such as *C. megacephala*, *C. ruffifacies*, *H. ligurriens*, *L. cuprina*, *A. orientalis* and *S. dux* are cosmopolitan flies as they can be found in various sampling areas including urbanised area. These species have been recorded as important synanthropic flies as they are closely associated with human activities. *Hypopygiopsis infumata* and the unidentified sp. are species that can only be found in the forest area. Identification of synanthropic flies using only morphological features is proven to be challenging due to limited taxonomic keys available. Current morphological identification is hindered by the high level of resemblance in the external characteristics. Molecular identification with the COI gene is helpful because it can facilitate and complement the morphology-based species identification. Data from this study may be useful for the study of the importance of synanthropic flies as forensic evidence and disease transmitter around forest reserve areas. Further investigation is required as there are limited studies on the forensically and medically important synanthropic flies in forest reserve areas in Malaysia.

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