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Molecular Identification and Composition of Cyclorrhaphan Flies Associated with Cafeterias

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Abstract.- Cafeterias are routinely visited by people to fulfil their daily feeding and drinking requirements. Unnoticed visitors, such as cyclorrhaphan flies, are also present in these places which can be a source of food poisoning and disease spread. These flies were collected from garbage piles, kitchen and vacant sites of two cafeterias (Cafeteria Serumpun and Cafeteria Agro-bio) within Universiti Putra Malaysia and one cafeteria (Cafeteria Old-flat) outside the university. A total of 1,037 fly specimens were collected and identified belonging to Calliphoridae, Muscidae and Sarcophagadae. The *COI* and *COII* gene sequences and phylogenetic results revealed five species of cyclorrhaphan flies, namely *Chrysomya megacephala, Lucilia cuprina, Musca domestica, Ophyra spinigera and Sarcophaga dux*. The highest number of flies was found at Cafeteria Serumpun (44%), followed by Cafeteria Old-flat (36%) and Cafeteria Agro-bio (20%). The most populated sampling site was kitchen and the abundant species was *C. megacephala* (92.66%). Analysis of data showed significant difference between individuals of different species at different cafeterias and sampling sites.

Key words: Cyclorrhaphan flies, *COI* gene, *COII* gene, Phylogenetic study, Cafeteria.

INTRODUCTION

Flies are annoying and commonly associated with human surrounding such as food courts, wet markets, village sundry shops and sanitary landfill (Nurita *et al*., 2007, 2008; Chaiwong *et al*., 2012; Nurita and Abu Hassan, 2013; Khoso *et al.,* 2015). They are known as cyclorrhaphan flies and capable of carrying disease of public health importance (Harwood and James, 1989; Gabre and Abouzied, 2003; Forster *et al*., 2009). The appendages of these flies contain sensory cells which help them to detect decomposing organic materials (Tan *et al.,* 1997). The easy access of these flies to animal manure, trash, human excrement, and other decaying materials has exposed them to disease causing organisms which often attach to their mouthparts, body hairs and the sticky pads of their feet, stomach, faeces and vomit (Graczyk *et al*., 1999).

Previous studies had shown that these flies are involved in transmission of pathogens such as helminths and protozoan parasites (Getachew *et al*., 2007). The members of genus *Chrysomya*, *Sarcophaga* and *Musca* are reported to carry the eggs of *Ascaris lumbricoides*, *Trichuris trichiura* and *Necator americanus* (Sulaiman *et al.,* 1988; Fetenea and Workub, 2009). The disease causing bacteria and viruses, such as *Shigella dysenteriae* and *Escherichia coli* (Butler *et al*., 2010), *Aeromonas hydrophila* and *Pseudomonas aeruginosa* (Sukontason *et al.,* 2007), poliovirus, coxsackie virus, entero-viruses (Gregorio *et al*., 1972; Greenberg, 1973), H5N1 virus (Kyoko *et al*., 2006) and Bovine papillomavirus (Finlay *et al*., 2009) are also carried by these flies.

Beside public health importance these flies are important in forensic entomology as they are attracted to carrion, decaying flesh, human garbage and able to breed in decomposing materials (Robinson, 2005). They are helpful in the estimation of Post Mortem Interval (PMI). The morphological based approach in fly identification has become a major challenge to researchers, particularly during the immature stages (Harvey *et al*., 2003; Zehner *et*

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al., 2004; Waugh, 2007). The technicians have to collect the larvae from the crime sites and rear the insects until adult stage for identification (Mazzanti *et al.*, 2010; Aly and Wen, 2013).

Molecular techniques in fly identification are popular as they provide a more precise, rapid and reliable results than morphological based identification (Marigorn and Coquoz, 1999). The commonly used molecular markers for species identification are the mitochondrial DNA genes, such as *cytochrome c oxidase subunit I* (*COI*) (Park *et al*., 2009), *cytochrome c oxidase subunit II* (*COII*) (Caterino *et al*., 2000), *Cytochrome b*, ND5, 12S and 16S (Low *et al*., 2014). To date, *COI* and *COII* genes are the well-known genes for DNA barcoding for species identification (Tan *et al.*, 2010). Both genes can be sequenced rapidly and easily, and provide accurate identification of insects (Mazzanti *et al.*, 2010; Boehme *et al.*, 2012; Jordaens *et al.*, 2013).

Majority of the students and staffs of Universiti Putra Malaysia take their meals at the food courts within or outside the campus. They are at high risk as they may be exposed to pathogens that may be carried by cyclorrhaphan flies. In Malaysia, different fly species have been reported capable of carrying food borne pathogens (Tan *et al.*, 1997; Sulaiman *et al.*, 1988). The presence of these flies may contribute to food poisoning if proper handling of food is not practiced. This study was conducted to investigate the occurrence of cyclorrhaphan flies at the cafeterias within and near the campus of Universiti Putra Malaysia. The data provided in this study could be helpful to provide a database for other researchers to identify the flies on molecular basis.

MATERIALS AND METHODS

Insect sampling

The flies were collected from three different food courts in Seri Serdang, Selangor, Malaysia. The food courts were Cafeteria Serumpun and Cafeteria Agro-bio (within campus) and Cafeteria old-flat (outside campus). The study was conducted from January 2014 to December 2014; the samplings were carried out at 3 different sampling sites; garbage, kitchen and vacant area near cafeterias, with 3 replications. The cyclorrhaphan flies were attracted using decayed chicken liver (200g) and sticky traps. The bait was left overnight at room temperature in order for it to decay. The decayed chicken livers were placed in aquariums with dimension 10 cm x 10 cm x 7 cm and used as traps for the flies. After every 30 min intervals within a period of three hours, the aquariums were replaced. Small plastic containers (7 cm in height and 3 cm in diameter) were used to collect individual fly specimens trapped in each aquarium. The insects were shifted to the Laboratory of Insect Pathology, Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia for identification.

Morphological identification

The flies were grouped based on their morphological characteristics which were observed under a dissecting microscope (Leica Zoom 2000, USA). The identification was carried out based on the morphological keys by Carvalho and Mello-Patiu (2008), Whitworth (2010) and Sukontason *et al.* (2014).

Molecular identification

DNA extraction

The G-spin™ Total Kit (Intron, Korea) was used to extract the DNA from individual insect specimens according to the manufacturer's protocol. The insect specimens were 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.

Polymerase chain reaction (PCR)

The amplified mtDNA region includes the *cytochrome oxidase I* and *II* genes (*COI* and *COII genes*). 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 and 2 mM of each forward and reverse primers (amplification of *COI* gene using primers TY-J-1460:
5'-TACAATTTATCGCCTAAACTTCAGCC-'3
and C1-N-2800:
5'-CATTTCAAGCTGTGTAAGCAT C-'3,
whereas *COII* gene using primers C1-J-2495:

5'-CAGCTACTTTATGAG CTTTAGG-'3
and TK-N-3775: 5'-GAG
ACCATTACTTGCTTTCAGTCATCT-'3)
(Sperling *et al.*, 1994). 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 and 48 ˚C for 1 min 30s for *COI* and *COII* genes, respectively, 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 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 Centre 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. The population study of the composition of cyclorrhaphan flies was carried out using the Statistical Software version 9.2 (SAS). The raw data was transformed with the natural log ($\log_{10}(X+1)$ in order to normalize the data.

RESULTS

Chicken liver and sticky traps have been used for trapping the fly specimens at Cafeteria Serumpun, Cafeteria Old-flat and Cafeteria Agro-bio. A total of 1,037 flies were collected during the study period. These flies were trapped by chicken liver and identified based on their morphological and molecular characteristics. Those flies, which were trapped on the sticky traps, were damage during collection for identification. Thus, specimens collected by using sticky traps were not included in the data analysis. Among the total flies collected, 5 species of cyclorrhaphan flies were identified; namely *Chrysomya megacephala, Lucilia cuprina, Musca domestica, Ophyra spinigera* and *Sarcophaga dux*, belonging to three main families; Calliphoridae, Muscidae and Sarcophagidae. *Chrysomya megacephala* and *L. cuprina* are members of the family Calliphoridae which have a sharp bent M-shaped wing vein. *Chrysomya megacephala* has a bright green metallic body with transparent wings (Fig. 1A) while *L. cuprina* has a metallic bronze with greenish sheen body colour (Fig. 1B). The males of *C. megacephala* have big red eyes with no gap in between the eyes as compared to those of the females (Fig. 1A). *L. cuprina* have brownish red eyes and transparent wings with noticeable wing veins (Fig. 1B). *Musca domestica*, a member of Muscidae, has a grey thorax with four dark longitudinal lines on it. They have brownish red eyes and transparent wings with a tinge of yellow at the base of the wings where it joins to the thorax (Fig. 1C). *Ophyra spinigera* has a shiny black body with transparent wings. The wing veins are slightly yellow (Fig. 1D). Both *M. domestica* and *O. spinigera* have slightly bent M-shaped wing vein. *Sarcophaga dux* was the only species of Sarcophagidae found during the sampling activities (Fig. 1E). They have greyish yellow thorax with dark longitudinal stripes on it. *Sarcophaga dux* has a significant checked pattern on their abdomen. They have large compound eyes, antennae and a sponging mouthpart with prominent palps. The morphological characteristics were found to be closely related to those previous studies by Carvalho and Mello-Patiu (2008), Whitworth (2010) and Sukontason *et al*. (2014).

The highest number of flies were collected from Cafeteria Serumpun (458) followed by Cafeteria Old-flat (371) and Cafeteria Agro-bio (208). Most of the flies were found near the kitchen (355), followed by vacant site (343) and garbage site (339). The most prominent and dominating species was *C. megacephala* (92.7%) which were collected from all cafeterias and sampling sites. The percentage of other species was very low as compared to *C. megacephala,* in which the percentage of *L. cuprina, M. domestica, O. spinigera and S. dux* was 3.76%, 3.20%, 0.19% and 0.19%, respectively. The number of *L. cuprina*

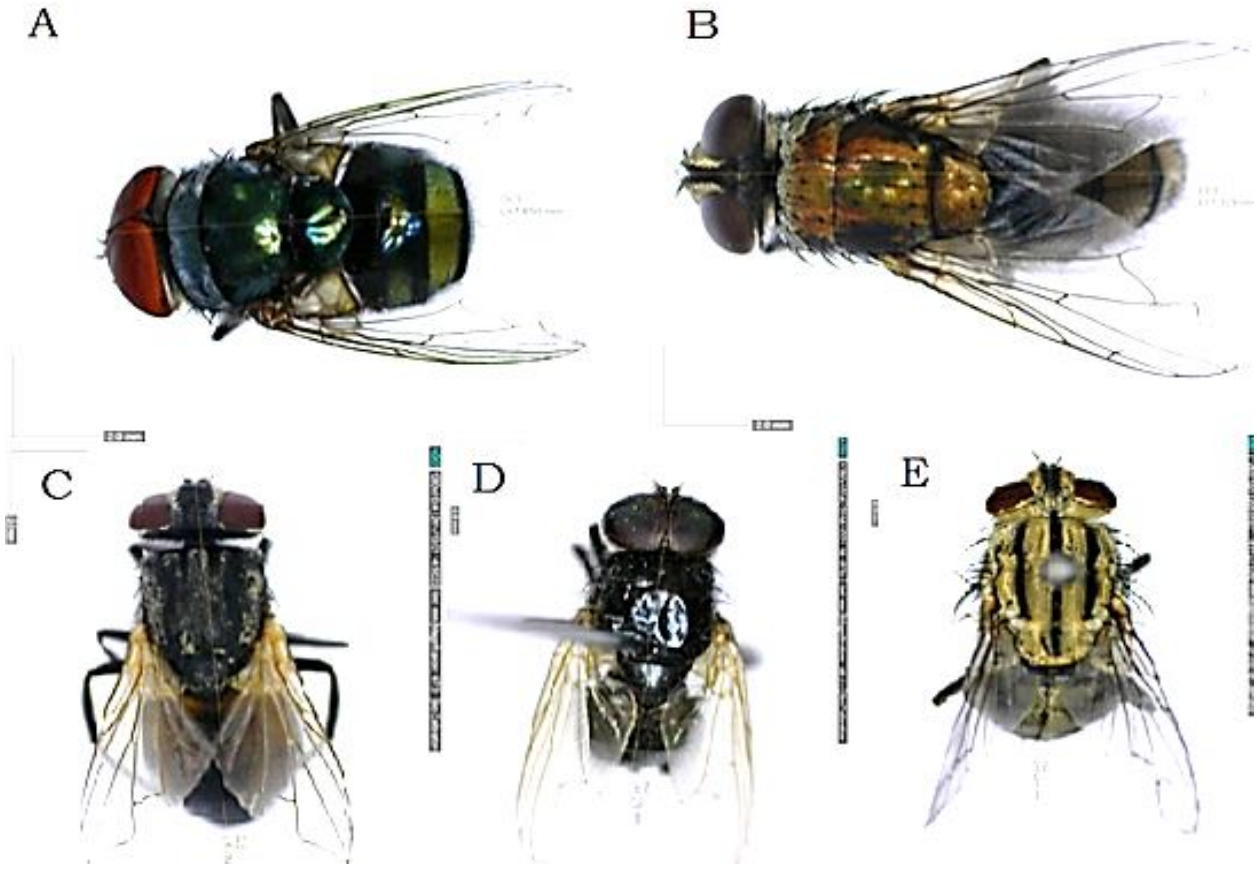

Fig. 1. Morphological appearance of cyclorrhaphan flies collected from cafeterias. (A) *Chrysomya megacephala* (B) *Lucilia cuprina* (C) *Musca domestica* (D) *Ophyra spinigera* and (E) *Sarcophaga dux.*

collected was higher than *O. spinigera* in Cafeteria Old-flat and *S. dux* in both Cafeteria Old-flat and Agro-bio. Among the sampling sites, *C. megacephala* was found higher in mean number at Cafeteria Serumpun (1.71±0.25) than Cafeteria Agro-bio (1.30±0.09) at kitchen only. A significant mean number of *L. cuprina* was observed at the garbage site of Cafeteria Old-flat (0.86±0.22) compared to Cafeteria Serumpun (0.30±0.00) and Cafeteria Agro-bio (0.10±0.17). No significant difference was found for *M. domestica*, *O. spinigera* and *S. dux* at any sampling site (Table I).

For molecular identification, the DNA sample of each species was successfully extracted. The *COI* (1,300 bp, Fig. 2A) and *COII* (630 bp, Fig. 2B) genes of different fly species were successfully amplified and sequenced. Sharp peaks were observed in the electrophoregrams and no stop codon was found between the sequences, indicating no co-amplification of nuclear pseudogenes occurred. The blast results in NCBI showed 98-100% similarity at the species level. All sequences were submitted to GenBank database under the accession numbers mentioned in Figure 4. The *COI* and *COII* sequences were aligned to perform the phylogenetic analysis. 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, 72% and 69.1% for *COI* and *COII* respectively. The nucleotide compositions were A (33.5%), T (38.5%), C (14.0%), and G (14.0%) for *COI*, and A (31.0%), T (38.1%), C (14.8%), and G (16.1%) for *COII* (Fig. 3). In order to find out the most suitable model to construct the phylogenetic tree, Find Best-fit Substitution Model (ML) was carried out showing the best model to be used was GTR + G (GTR

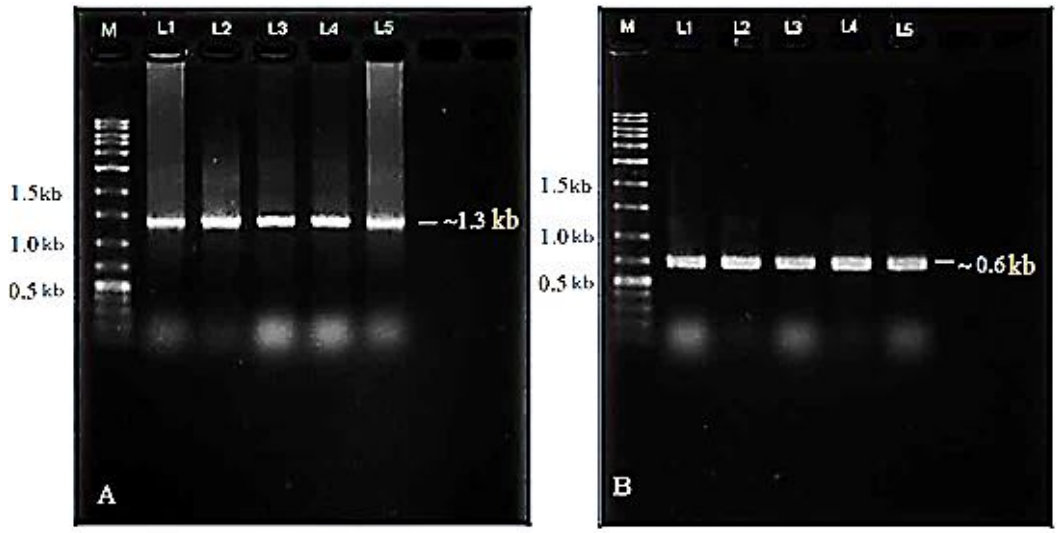

Fig. 2. Amplification of the *COI* and *COII* genes of the cyclorrhaphan fly species collected from cafeterias. (a) *COI* gene, (b) *COII* gene. M represents the 1kb marker. L1-L5 represents *Chrysomya megacephala, Lucilia cuprina, Musca domestica*, *Ophyra spinigera* and *Sarcophaga dux* respectively.

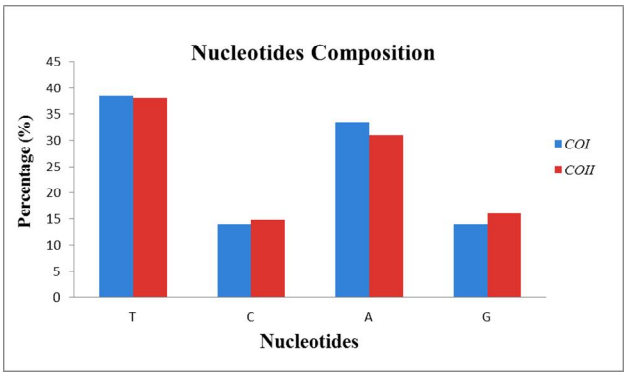

Fig. 3. Average nucleotide percentage of *COI* and *COII* genes. Thymine (T), Cytosine C), Adenine (A) and Guanine (G).

represents General Time Reversible while G represents Gamma distributed). The data revealed 265 variable positions in *COI* nucleotide sequences with 121 variable positions were parsimoniously informative in *COI* genes. Of the 205 variable positions found in *COII* nucleotide sequences, 43 were of parsimoniously informative in *COII* genes.

In Table II the highest interspecific variation in *COI* sequence was found to be 10.4% between *C. megacephala* and *S. dux* whereas the lowest difference was 8.4% between *C. megacephala* and *L. cuprina*. Among the *COII* nucleotide sequences, the highest difference observed was 6.6% between *O. spinigera* and *S. dux* whereas the lowest difference (4.7%) was found between *M. domestica* and *S. dux*. A neighbour joining (NJ) tree was constructed with the maximum likelihood model and 1000 bootstrap replications. NJ analysis was conducted to determine the relationship between the analysed species (Fig. 4). All the species were monophyletic and showed same pattern for *COI* and *COII* genes. The bootstrap percentage values for *COI* and *COII* gene were 24-52% and 33-92%, respectively.

DISCUSSION

This study was aimed to conduct a survey on the presence of cyclorrhaphan fly species at different cafeterias within and outside Universiti Putra Malaysia. The highest number of *C. megacephala* was collected from all the collecting sites which is in agreement with the previous studies (Gabre and AbouZied, 2003; Lertthamnongtham *et al.*, 2003; Ngoen-klan *et al.*, 2011; Chaiwong *et al.*, 2012; Khoso *et al.*, 2015). But, in some studies *M. domestica* was reported the most abundant fly species in many places (Winpisinger *et al.*, 2005; Goulson *et al.*, 2005; Nurita *et al.*, 2008; Nurita and Abu Hassan, 2013; Adenusi and Adewoga, 2012). This may be due to the selection of the collection

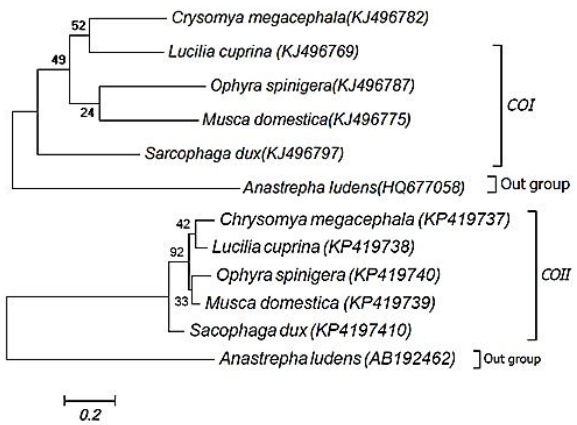

Fig. 4. Neighbour joining phylogenetic trees. *COI* and *COII* phylogenetic tree constructed with 1,000 bootstraps.

sites and the kind of bait used in their studies. Nurita *et al*. (2008) and Nurita and Abu Hassan (2013) used sticky paper bait for collecting flies from the cafeterias, food courts, slaughterhouses and sundry shops. According to some researchers, blow flies (Family: Calliphoridae) are more attracted to carrion, soggy, bloody or soiled hair, fur, or wool (Shah *et al*., 2006; Nurita and Abu Hassan, 2013; Ngoen-klan *et al*., 2011; Chaiwong *et al.*, 2012) and use these resources as the platform for egg laying and protein sources for the maturation of eggs (Mariluis *et al.*, 2010). The chicken liver bait used in the present study had attracted mostly the Calliphoridae flies and proven this family of flies are more prone to carrion bait. The sticky traps were not included in the trapping procedure in the present study due to difficulty in the collection of intact specimens for morphological and molecular identification.

Lucilia cuprina, being the second most abundant fly species, was also observed to have preference towards urban habitats (Brundage *et al*., 2011). *Musca domestica*, the common housefly which is the third most abundant fly species can be found in these cafeterias since they are known to feed on human garbage (Robinson, 2005), which can be found easily around the cafeterias. *Ophyra spinigera* were found only in Cafeteria Serumpun and Cafeteria Agro-bio while *S. dux* was only found in Cafeteria Serumpun. Both cafeterias are located within the campus and there are farms located nearby the sampling sites. Cafeteria Agro-bio is located approximately 200 meters away from an experimental animal farm while Cafeteria Serumpun is located in a university hostel which is opposite to an experimental farm cultivated with different crops. Flesh flies prefer to stay in farms because they breed on animal excrements while *O. spinigera* are parasites to the larvae of flesh flies (Farkas *et al*., 1998; Hogsette *et al*., 2002). It is not surprised to have both species found in the same vicinity. Both flies are not common pests in urban locality (Khoso *et al*., 2015).

Members of Calliphoridae, Muscidae and Sarcophagidae are cyclorrhaphan flies (Greenberg, 1973; Olsen, 1998) and their occurrence in cafeterias would be related to human inhabitants and their activities. Among the three cafeterias, Cafeteria Serumpun has the largest compound and longest operation hour from 0800 until 2200. It is located in the heart of the university hostels and serves a lot of university students and staffs during the operation hour. Cafeteria Agro-bio is located inside a faculty which is an isolated premises of the university located approximately one kilometer away from the main campus. It operates from 0800 to 1600 and the visitors are limited to students and members of the faculty. Cafeteria Old-flat is located 100 meters away from the university and surrounded by a residential area. Its operational hour is between 1000 and 1500, and it serves people from university and outside university. The waste produced by these cafeterias would be directly proportional to the number of visitors at the cafeterias, which in turn has attracted many cyclorrhaphan flies to hunt for food.

The presence of flies can be linked to the sanitation practices in an area (Nurita *et al*., 2007). They can be found abundantly in unsanitary conditions regardless of where this condition exists. The garbage area is located approximately 100 meter away from all the cafeterias. At Cafeteria Serumpun and Cafeteria Agro-bio, the garbage are properly wrapped and placed inside the garbage bins while waste food and rubbish were exposed near the surrounding of Cafeteria Old-flat. Among the sampling sites, Cafeteria Old-flat has recorded more fly specimens at garbage sites compared with Cafeteria Serumpun and Cafeteria Agri-bio. Chances

Table I.- The mean number (± S.E) of cyclorraphan flies collected from all the cafeterias at different sampling sites.

Sites	Cafeteria	*Chrysomya megacephala*	*Lucilia cuprina*	*Musca domestica*	*Ophyra spinigera*	*Sarcophaga dux*
Garbage	Cafeteria Serumpun	1.44^{a}±0.07 (81)	0.30^{b}±0.00 (3)	0.10^{a}±0.17 (1)	-	-
	Cafeteria Old-flat	1.67^{a}±0.10 (141)	0.86^{a}±0.22 (21)	0.43^{a}±0.38 (7)	-	-
	Cafeteria Agro-bio	1.43^{a}±0.16 (81)	0.10^{b}±0.17 (1)	0.30^{a}±0.00 (3)	-	-
Vacant	Cafeteria Serumpun	1.76^{a}±0.26 (188)	0.10^{a}±0.17 (1)	0.20^{a}±0.17 (2)	0.00^{a}±0.00 (0)	-
	Cafeteria Old-flat	1.42^{a}±0.28 (89)	0.20^{a}±0.17 (2)	0.10^{a}±0.17 (1)	0.00^{a}±0.00 (0)	-
	Cafeteria Agro-bio	1.29^{a}±0.09 (56)	0.26^{a}±0.24 (3)	0.00^{a}±0.00 (0)	0.10^{a}±0.17 (1)	-
Kitchen	Cafeteria Serumpun	1.71^{a}±0.26 (170)	0.26^{a}±0.24 (3)	0.36^{a}±0.40 (6)	0.16^{a}±0.28 (1)	0.10^{a}±0.17 (2)
	Cafeteria Old-flat	1.52^{ab}±0.05 (97)	0.36^{a}±0.31 (5)	0.46^{a}±0.40 (8)	0.00^{a}±0.00 (0)	0.00^{a}±0.00 (0)
	Cafeteria Agro-bio	1.30^{b}±0.09 (56)	0.10^{a}±0.17 (1)	0.36^{a}±0.32 (5)	0.00^{a}±0.00 (0)	0.00^{a}±0.00 (0)

Means followed by the same letters within a column are not significantly different ($P \leq 0.05$) according to Tukey's test analysis.

Table II.- Pairwise divergence between species. Upper: nucleotide divergence in %, lower: absolute nucleotide differences.

No	Species	*COI* gene						*COII* gene					
		1	2	3	4	5	6	1	2	3	4	5	6
1	*Chrysomya megacephala*		8.4	9.5	9.5	10.4	10.2		5.3	5.2	6.4	5.5	9.0
2	*Lucilia cuprina*	82.0		9.7	8.8	9.3	10.7	33.0		4.7	6.0	5.2	8.8
3	*Musca domestica*	104.0	115.0		9.6	10.2	10.3	34.0	28.0		5.8	4.8	9.1
4	*Ophyra spinigera*	101.0	92.0	110.0		9.4	10.1	56.0	53.0	44.0		6.6	9.1
5	*Sarcophaga dux*	108.0	105.0	128.0	111.0		11.0	38.0	35.0	30.0	56.0		9.0
6	*Anastrepha ludens*	144.0	154.0	150.0	140.0	143.0		168.0	171.0	168.0	171.0	171.0	

for a visitor to be exposed to the disease pathogens transmitted by cyclorrhaphan flies are higher if the waste from cafeteria is not managed well and the surrounding is not ensured of its cleanliness. According to Keiding (1986), by improving the environmental sanitation and hygiene, the population density of chclorrhaphan flies can be reduced. Among the three cafeterias, Cafeteria Agro-bio showed the least number of flies caught in the kitchen and vacant areas. Most of the stalls in this cafeteria cater from other restaurants and less handling of fresh food compared to other cafeterias. Therefore, in general, Cafeteria Agro-bio has the least number of flies at all sampling sites.

The *COI* and *COII* genes have further confirmed the identity of the fly species collected from the cafeterias. The nucleotide sequences of both genes comprised of a strong adenine-thymine bias, which is a characteristic of insect mtDNA (Nelson *et al.,* 2007; Meiklejohn *et al.,* 2011). The lowest interspecific variation was 4.7%, which is in agreement with the findings reported by Hebert *et al.* (2003), Hebert *et al.* (2004a,b) and Amendt *et al.* (2011). The *COI* and *COII* gene sequences carry sufficient information to distinguish between all the species examined due to high bootstrap support and all species are reciprocally monophyletic, which is a standard for species distinction (Wells *et al.,* 2007). There was no insertion or deletion found in the analysed sequences as reported in the previous literature (Hebert *et al*., 2003; Ward *et al*., 2005; Meiklejohn *et al*., 2011), which confirms no issue of nuclear mitochondrial DNA (NUMTs) in the experiment. Otherwise, amplification of NUMTs may lead to a bias pattern of mitochondrial diversity and be potentially misrepresented (Bensasson *et al.,* 2001; Charlat *et al*., 2009).

CONCLUSIONS

Cyclorrhaphan flies are not only a nuisance but they bring harm and damage to the health of people who comes in contact with them. The data obtained from this study could be useful in the improvement of cleanliness in the cafeterias as well as to manage food properly. Species identified in this study have been reported as vectors of various pathogens. They are notorious for harbouring whipworm and giant roundworm that cause severe damage to intestine, and vectors of human diseases, such as poliovirus, coxsackievirus, and Bovine papillomavirus which cause polio, foot and mouth disease and cancer, respectively. Cyclorrhaphan flies have been identified presence in the cafeterias located within or outside the university. The cleanliness of the cafeterias should be top-notch to reduce the fly population and at the same time to reduce the risk of transmission of diseases to the students and staff members.

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