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Molecular Identification and Species Richness of Flies (Diptera) and Their Associated Bovidae Hosts at Cattle Farms in Selangor, Malaysia

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ABSTRACT

Flies (Diptera) play a significant role in the ecosystem as pollinators and decomposers, and they are also important vermin and disease vectors. Studies on the dipteran species are still lacking in Malaysia; therefore, the dipteran species' biology, morphology, distribution, and abundance are necessary. The objectives of this study were to identify dipteran species using a molecular approach, determine flies' Bovidae hosts, and investigate the diversity of the fly's species at three different cattle farms purposively selected in Selangor, Malaysia. The fly species were identified using cytochrome oxidase subunit I (*COI*) (*Haematopota javana*, *Tabanus rubidus*, *Tabanus fontinalis*, *Iranihindia martellata*, *Musca domestica*, and *Chrysomya megacephala*), while another six species only up to genus level (*Haematopota* sp. 1, *Musca* sp. 1, *Asilus* sp., *Metopia* sp., *Anasillomos* sp., and *Ommatius* sp.). In addition, two species of hosts: *Bos indicus* and *Bos taurus,* were proven to be the associated host species for the dipteran species based on molecular data of cytochrome b (*cytb*). However, there were no significant differences between farms in species diversity and richness ($F = 1.262$, $df = 2$, $p = 0.2459 > 0.05$). Interestingly,

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the two most dominant dipteran genera collected from the cattle farms were *Musca* and *Chrysomya*. At the same time, its abundance may have been influenced by the structure of the cattle cage flooring, which serves as a breeding site and food source. These findings contribute to fundamental epidemiological data in developing control strategies for dipteran species and are of great economic and health importance to livestock production in Malaysia.

Keywords: Blood-sucking insect, *COI*, *cytb*, DNA barcode, fly, host, livestock, Malaysia

INTRODUCTION

Members of the dipteran species (Diptera) are commonly referred to as true flies or two-winged flies and comprise black flies, fruit flies, horse flies, house flies, midges, and mosquitoes. It is among the most diverse insect orders, with an estimation of 120,000 to 150,000 species (Brown, 2001; Colless & McAlpine, 1991; Courtney et al., 2017; Schumann, 1992). The diversity of the dipteran group is evident not only in their species richness but also in their diverse habits and habitats, as well as their significant effect on agriculture, forestry, animal, and human health (Courtney et al., 2017; Skevington & Dang, 2002; Ssymank et al., 2008).

Diptera is one of the most important insect orders in terms of their interaction with humans, especially in spreading diseases and causing agricultural losses (Courtney et al., 2017; Marshall, 2012; Pape, 2009). However, Diptera's benefits to the ecosystem are also significant, albeit less understood. For example, flies contribute to plants' pollination, biological control of pests, and degrade dung, carrion, and other organic matter (Marshall, 2012; Skevington & Dang, 2002). Previous researchers like Marshall (2012) and Pape (2009) have emphasised the study of this diverse group of insects on their impact on humans and their active role in ecosystem functions.

As a primary group in diverse ecosystems and environments, these pestiferous insects significantly impact animal and human health, agriculture, and forensic sciences (Singh & Bharti, 2000). Their close association with humans has led them to be recognised as important vermin and disease vectors, and some flies are responsible for a multitude of illnesses and deaths inflicted by humans worldwide. However, flies also play beneficial roles as key components in many diverse ecosystems (Skevington & Dang, 2002). It is worth noting that studies and reports on Diptera are still lacking in Malaysia compared to other parts of the world, where substantial studies have been documented on the biology, morphology, distribution, and abundance of the dipteran species (Gerhardt & Hribar, 2019; Marshall, 2012).

Morphological identification is a traditional taxonomic tool where a species is identified based on the morphological characters and by referring to the description of the identification keys. Morphological identification is still the preferred method for research as it requires little technical equipment, is easy to implement in the

field, and is relatively cheap even when large numbers of individuals need to be identified. However, the limitations in using morphology-based identification include being tedious, time-consuming, and difficult to distinguish cryptic species. In addition, the dwindling number of skilled and experienced taxonomists globally has triggered critical problems in identifying species based on morphology alone (Renaud et al., 2012).

Molecular identification methods are faster and have increased sensitivity; thus, they are now being advocated for identification. Accurate identification of dipteran species in livestock farms is very important for determining their role in disease transmission and planning effective vector control and management strategies. Although dipteran species identification based on morphological features is economical, easy to perform, and requires no complicated equipment, this methodology requires the skills of experienced taxonomists. In addition, the specimens need to have clear external morphological characteristics, involving proper specimen preparation. Misidentification would adversely affect the efficacy of vector control and influence the control and preventive measures in disease transmission. Therefore, molecular-based identification can be used to solve identification ambiguities in morphologically similar species, as well as in species lacking important morphological traits or specimens in immature life stages.

DNA barcoding is a molecular method that uses a short fragment of the nucleotide of a specific gene for accurate species identification. DNA barcoding has gained wide attention and plays an important role in precise species identification and in revealing genetic diversity in the presence of any biotypes, haplotypes, and/or genotypes, with the power to resolve taxonomic ambiguities at the species level and within species complexes. The barcoding region of mitochondrial cytochrome oxidase subunit I (*COI*) has been shown to effectively discriminate species in a range of dipterans including tabanids (Changbunjong et al., 2018; Cywinska et al., 2010; Morita et al., 2016). *COI* is a mitochondrial gene commonly used to support morphological identification by amplifying a region of the gene using a set of universal primers during polymerase chain reaction (PCR) (Banarjee et al., 2015). Advances in molecular techniques for blood meal analysis and barcoding using PCR-based assays and direct sequencing of the cytochrome b gene (*cytb*) have permitted the identification of hosts with a higher degree of accuracy compared to previous serological techniques (Alcaide et al., 2009; Molaei et al., 2008; Townzen et al., 2008).

One of the significant problems in livestock farms all over Malaysia is the fly menace as disease vectors for human and animal health reported on several stable and horse fly species vectoring *Trypanosoma evansi* that lead to the trypanosomiasis outbreak in Malaysian ruminants such as buffalo, cattle, and deer that cause severe losses in body weight and milk production (Erwanas et al., 2015). Documented literature

on Dipterans in local livestock farms is still considerably sparse despite this group's significance and economic importance as pests and disease vectors. Current studies on host identification of Dipteran species in Malaysia have various technical and related constraints. Therefore, the objectives of this study were to molecularly identify the fly species at several cattle farms in Selangor, Malaysia: to determine the mammalian host for the fly's species molecularly and to determine the flies' abundance and richness from three different farms with different structures of cages flooring.

METHODS

Sampling Sites

This study was conducted on three different livestock farms in Selangor: Ladang Bangi (2°55'44.6"N 101°46'31.6"E), Ladang Rasa (3°29'32.4"N 101°37'31.6"E), and Ladang 16, UPM (3°0'26"N101°42'16"E). The livestock farms in Selangor were sampled as the model farms to determine the richness of dipteran species in Peninsular Malaysia. The selection of the cattle farm in this study was purposively made based on the building of the cage structure and the surrounding area. For Ladang Bangi, the cattle cage had ground flooring and was adjacent to the fragmented forest ecosystem and a river. Meanwhile, for Ladang Rasa, the cattle cages were partially covered with ground and concrete flooring, while the farm was adjacent to the fragmented forest ecosystem. As for Ladang 16, UPM, the cage was built from concrete flooring, and the farm was in the open area of the UPM campus and connected to the main road.

Sampling Methods

Passive Sampling. Six baited traps were placed randomly in the cages in each of three selected livestock farms in Selangor from May 2019 to August 2019. The bait trap is a useful method to study the fly populations. A mixture of chicken liver and cow urine was used as bait for each trap. Four visits were made to each livestock farm during the sampling period. Six traps were left randomly for a week in the field. Afterward, the fly specimens were collected in a bottle containing 100% alcohol for wet preservation and taken back to the laboratory for identification and molecular analysis. The specimens were sorted based on external morphology and stored in vials containing 100% alcohol, each vial with a label indicating sampling locality and collection date of the specimens.

Active Sampling. Flies were collected from the bodies of their host cattle in the cage areas using a sweeping net. During every visit, the cattle were kept inside the cowshed on all the farms throughout the sampling period. The samples were collected in a bottle containing 100% alcohol and immediately brought back to the laboratory for sorting based on external morphology. The specimens were stored in 100% alcohol for wet preservation vials, with labels indicating locality and collection date. Before molecular analysis, these specimens were stored in the freezer at -20°C.

Laboratory Works

Morphological Identification of Dipteran Species. The specimens collected were examined using a stereo microscope (Zeiss Stemi DV4, Germany). Several taxonomic keys and species descriptions were utilised to identify the specimens (Al-Talafha et al., 2017a, 2017b; Kurahashi et al., 1997; Nihei & de Carvalho, 2009), which were mainly based on external morphology characteristics (eyes, antennae, wing venation, thorax, and abdominal pattern and colour, as well as body length). Images of the specimens were taken using a camera (Canon EOS1000D, Japan). Molecular identification was then utilised to reconfirm the morphological-based identification.

DNA Barcoding Analysis. The *COI* region was PCR-amplified to identify the flies at the species level to support the morphological identification of the flies. The five main processes of molecular methods of DNA barcoding performed were DNA extraction, polymerase chain reaction (PCR), purification of PCR products, DNA sequencing, and data analysis.

DNA Extraction. DNA from individual fly specimens per species or morphospecies was extracted from the whole body using the NucleoSpin® DNA Insect (Machery-Nagel, Germany) extraction kit. The samples were soaked in ATL buffer and proteinase K for lyses, according to the manufacturer's instructions (Halim et al., 2017, 2018), and stored at -4 °C for further molecular work.

Polymerase Chain Reaction (PCR) and DNA Purification. DNA extracted from different fly samples was amplified using the GeneAmp PCR System 2400 (Perkin Elmer, USA) and cytochrome oxidase subunit I (*COI*) mitochondrial DNA based on Hebert et al. (2003) and Shariff et al. (2014) profiles. The amplification product of forwarding primer, COI- LCO1490 5'GGT CAA CAA ATC ATA AAG ATA TTG G 3' and reverse primer COI- HCO22198 5'TAA ACT TCA GGG TGA CCA AAA AAT CA 3' was 750 bp, in reference to Folmer et al. (1994). The PCR parameter for amplification of *COI* gene consisted of pre-heating 94 °C (60 sec), initial denaturation, 94 °C (60 sec) for 5 cycles, cooling 45 °C (45 sec), extension 72 °C (90 sec), denaturation 94 °C (45 sec) for 30 cycles, cooling 52.5 °C (75 sec), extension, 72 °C (75 sec), final extension, 72 °C (300 sec), and storage/holding, 4 °C (∞).

The same DNA extracted from the flies was used for host identification. PCR with universal animal primers targeting the cytochrome b gene (*cytb*) was used to detect the flies' host. The primers used for host identification of dipteran species were according to Kocher et al. (1989). Primers 5'-3' *cytb* L14841 5' AAAAAGCTTCCATCCAACATCTCAGC ATGATAA 3' and H15149 5'AAACTGCAGCCCCTCAGAATGATAT TTGTCCTCA 3' produced an amplicon of around 500 bp. The PCR parameters consisted of pre-heating 95 °C (180 sec), initial denaturation 95 °C (15 sec), cooling 51.8 °C (30 sec), extension 72 °C (10 sec), denaturation 95 °C (15 sec) for 30 cycles,

cooling 51.8 °C (30 sec), extension 72 °C (10 sec), final extension 72 °C (600 sec), and storage at 4 °C (∞). PCR products were purified using GF-I PCRCLEAN-UP Kit (Vivantis, United Kingdom) to remove excess dNTP and buffer. The purification procedure was conducted based on the manufacturer's instructions. The PCR products were electrophoresed for 30 minutes at 90V and 1.5% agarose gel and photographed under UV light. Images were captured with a gel imager AlphaImager HP (Alpha Innotech, USA).

DNA Sequencing Analysis and Sequences Alignment

PCR products with clear bands after electrophoresis along with the forward and reverse primers were sent for sequencing at Apical Scientific Sdn. Bhd. (Seri Kembangan, Malaysia). DNA sequences were edited using BioEdit Sequence Alignment Editor (BioEdit v7.0.5) to obtain accurate sequences. The edited sequences were then used as the input in the Basic Local Alignment Search Tool (BLAST) for molecular identification. The species separation presented was based on the Neighbour-joining (NJ) analysis using Phylogenetic Analysis Using Parsimony* (PAUP* v4.0b10). The NJ tree was constructed based on the Kimura-2 parameter (K2P) model and bootstrap with 1,000 replications.

Species Abundance and Composition

The total number of individuals and species collected from all localities was recorded to determine the species composition and abundance and is expressed as a percentage. Parameters of the diversity indices were calculated using Shannon-Weiner Index (*H'*), Margalef Index (*D*), and Evenness Index (*E*). One-way analysis of variance (ANOVA) was conducted to measure the significance of diversity between farms. Paleontological statistics software for education and data analysis (PAST 4.03) (Hammer et al., 2001) was used to analyse the diversity data.

RESULTS

Morphological Identification of Dipteran Species

Four dipteran species were successfully identified morphologically at the species level - *Haematopota javana*, *Tabanus rubidus*, *Tabanus fontinalis*, and *Musca domestica*. Meanwhile, nine species were successfully identified up to the genus level: *Sarcophaga* sp., *Iranihindia* sp., *Haematopota* sp. 1, *Musca* sp. 1 *Chrysomya* sp., *Asilus* sp., *Metopia* sp., *Anasillomos* sp., and *Ommatius* sp. Finally, all the species and morphospecies were subjected to molecular barcoding to re-confirm the species identification (Table 1, Figure 1).

Molecular Identification of the Dipteran Species

A representative from each species and morphospecies underwent the DNA barcoding process. The BLAST analysis indicated a high similarity percentage (>97%) for *Chrysomya megacephala* (99.86%), *Tabanus fontinalis* (99.85%),

Table 1
Species composition, abundance, and richness of the collected samples from three farms

Species	Number of individuals collected		
	Ladang Bangi	Ladang Rasa	Ladang UPM
Anasillomos sp.	2	0	0
Asilus sp.	1	0	0
Chrysomya megacephala	0	0	40
Haematopota javana,	0	3	0
Haematopota sp. 1.	1	0	0
Iranihindia martellata	0	0	0
Metopia sp.	0	4	0
Musca domestica	0	14	21
Musca sp. 1	0	0	9
Ommatius sp.	5	0	0
Sarcophaga sp.	4	0	0
Tabanus rubidus	1	3	0
Tabanus fontinalis	0	1	0
Number of individuals	16	25	70
Number of species (N)	6	5	3
Margalef Index (*D*)	1.895	1.259	0.4708
Evenness Index (*E*)	0.8004	0.6819	0.8574
Shannon-Weiner Index (*H'*)	1.569	1.227	0.9447

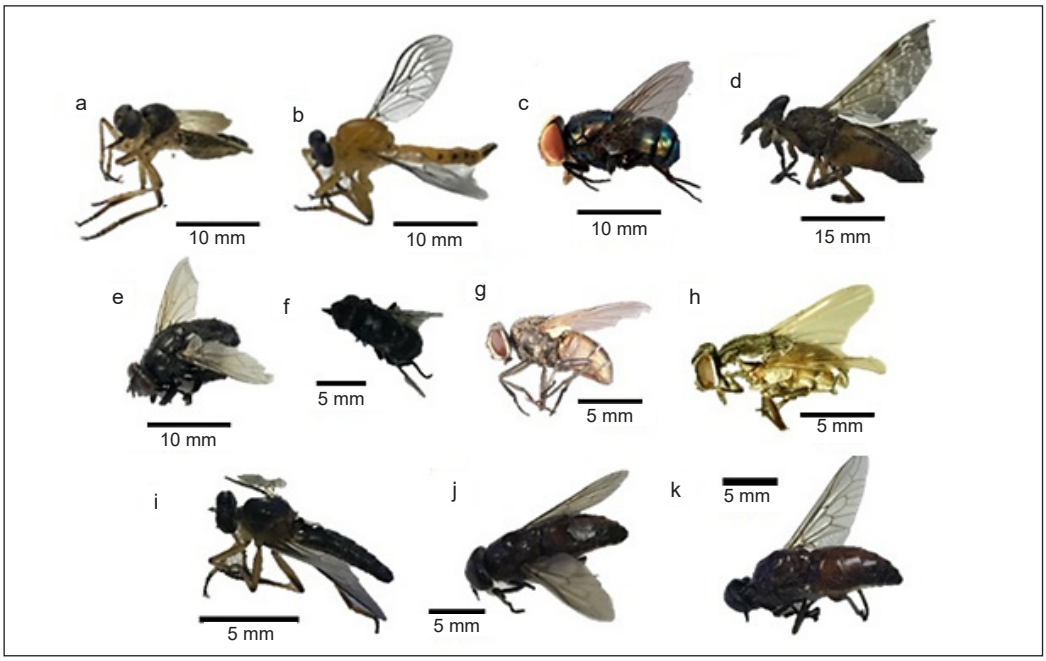

Figure 1. Dipteran species successfully collected and used for molecular analysis in this study: a. *Anasillomos* sp.; b. *Asilus* sp.; c. *Chrysomya megacephala*; d. *Haematopota javana*; e. *Iranihindia martellata*; f. *Metopia* sp.; g. *Musca domestica*; h. *Musca* sp. 1; i. *Ommatius* sp.; j. *Tabanus fontinalis*; k. *Tabanus rubidus*

Tabanus rubidus (99.09%), *Iranihindia martellata* (99.29%), *Haematopota javana,* (98.59%), *Haematopota* sp. (97.72%), *Musca domestica* (99.57%), but low similarity percentage (<97%) for *Musca* sp. (94.63%), *Anasillomos* sp. (93.02%), *Ommatius* sp. (89.97%), *Metopia* sp. (90.0%), and *Asilus* sp. (85.11%) (Table 2). Species separation was visualised in the NJ tree (Figure 2).

Identification of the Associated Mammalian Host Species

Two host mammal species were successfully detected from the DNA of the dipteran samples collected from four localities: *Bos indicus* and *B. taurus*. Based on the BLAST results, *B. indicus* and *B. taurus* showed a high similarity percentage (>98%) in all species, except 98.33% in *B. taurus* (Table 2). However, several species were not successful PCR-amplified, while others had contamination.

Species Composition and Abundance

A total of 111 dipterans were successfully collected from three farms and four livestock localities in Selangor, Malaysia, and comprised nine genera in five families. The dipteran flies collected from all study sites consisted of five families: Muscidae, Tabanidae, Asilidae, Sarcophagidae, and Calliphoridae. The dipteran genera collected in this study were *Musca* (40%), *Chrysomya* (37%), *Ommatius* and *Sarcophaga* (4%), *Haematopota, Tabanus*, *Metopia* and *Iranihindia* (3%), *Anasillomos* (2%), and *Asilus* (1%). The genus *Musca* recorded the highest abundance, with 44 individuals or 40% of the overall Diptera collected, followed by *Chrysomya* with 40 individuals (37%), *Ommatius* and *Sarcophaga*, with five individuals (4%), respectively. The genus *Asilus* recorded the lowest abundance, with only one individual or 1% of the overall Diptera collected (Figure 3). Ladang Bangi presented the highest Shannon-Weiner Index (*H'*) and Margalef Index (*D*), followed by Ladang Rasa, and lastly, Ladang UPM, with 1.569 (1.895), 1.227 (1.259), and 0.9447 (0.4708), respectively. One-way ANOVA with ($F = 1.262$, $df = 2$, $p = 0.2459 > 0.05$) showed no significant difference between farms in species diversity and species richness.

DISCUSSION

The various dipteran families of Muscidae, Sarcophagidae, Asilidae, Calliphoridae, and Tabanidae, were sampled in the field to gather information on the species richness and abundance. Although previous studies have been conducted on these families, they focused more on the diversity of a specific family, genus, and species in Malaysia (Khofar et al., 2019; Phasuk et al., 2011; Ya'cob et al., 2020). Three farms were purposively selected in this study as the sampling sites in Selangor to serve as model farms to represent other parts of Peninsular Malaysia. This assumption is based on earlier study findings and the rationale that the dipteran species are randomly distributed throughout Peninsular Malaysia, mainly along the altitudinal gradient (Ya'cob et al., 2016).

Table 2
Dipteran species and their host species were identified by using molecular data, the similarity percentage, and the accession number

Sample code	Locality	Fly species (from the Genbank)	Similarity percentage (BLAST)	Fly species (Accession no.)	Host species (from the Genbank)	Similarity percentage (BLAST)	Host species (Accession no.)
SBJ 1	West Malaysia: Selangor: Ladang Bangi	*Haematopota javana*	97.72% (e-value = 0.0)	*Haematopota* sp. 1 (MZ769394)	*Bos indicus*	99.38% (e-value = 0.0)	*Bos indicus* (MZ851350)
SBJ 3		*Ommatius* sp.	89.97% (e-value = 0.0)	*Ommatius* sp. (MZ769388)	-	-	-
SBJ 4		*Tabanus rubidus*	99.09% (e-value = 0.0)	*Tabanus rubidus* (MZ769389)	*Bos indicus*	99.56 % (e-value = 0.0)	*Bos indicus* (MZ851349)
SBJ 5		*Anasillomos* sp.	93.02% (e-value = 0.0)	*Anasillomos* sp. (MZ769390)	-	-	-
SBJ 6		*Asilus sericeus*	85.11% (e-value = 0.0)	*Asilus* sp. (MZ769391)	-	-	-
SBJ 7		*Iranihindia martellata*	99.29% (e-value = 0.0)	*Iranihindia martellata* (MZ769392)	*Bos taurus*	98.33% (e-value = 0.0)	*Bos taurus* (MZ851356)
SR 1	West Malaysia: Selangor, Ladang Rasa	*Haematopota javana*	98.59% (e-value = 8e-176)	*Haematopota javana* (MZ769393)	*Bos indicus*	99.79% (e-value = 0.0)	*Bos indicus* (MZ851351)
SR 4		*Metopia campestris*	90.00% (e-value = 0.0)	*Metopia* sp. 1 (MZ769384)	*Bos indicus*	99.79% (e-value = 0.0)	*Bos indicus* (MZ851352)
SR 3		*Musca domestica*	99.57% (e-value = 0.0)	*Musca domestica* (MZ769386)	*Bos indicus*	98.35% (e-value = 0.0)	*Bos indicus* (MZ851353)
SR 5		*Tabanus fontinalis*	99.85% (e-value = 0.0)	*Tabanus fontinalis* (MZ769395)	*Bos indicus*	99.79 %. (e-value = 0.0)	*Bos indicus* (MZ851354)
SR2		*Metopia campestris*	90.00% 8996 (e-value = 0.0)	*Metopia* sp. (MZ769385)	-	-	-
L1	West Malaysia: Selangor: Ladang UPM	*Chrysomya megacephala*	99.86% (e-value = 0.0)	*Chrysomya megacephala* (MZ769396)	*Bos indicus*	98.96% (e-value = 0.0)	*Bos indicus* (MZ851355)
L2		*Musca convexifrons*	94.63% (e-value = 0.0)	*Musca* sp. 1 (MZ769387)	-	-	

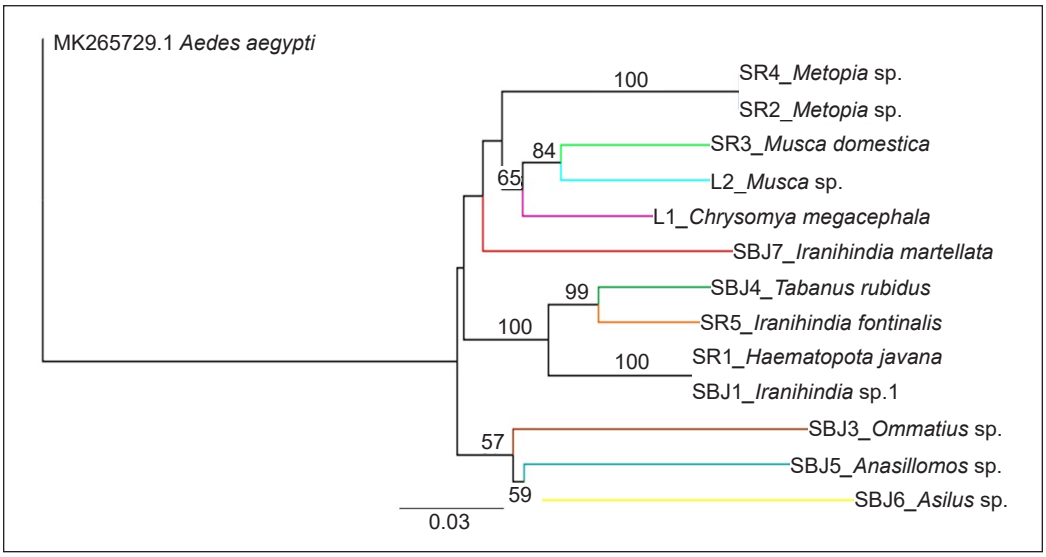

Figure 2. Neighbor-joining tree implemented for all the barcoded flies' species with 1,000 bootstrap replications

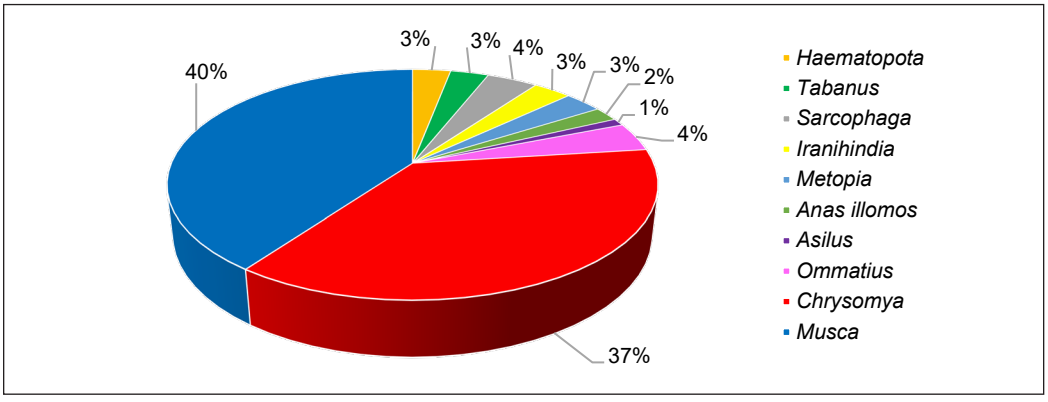

Figure 3. Composition of dipteran species according to genera collected from three cattle farms in Selangor

Identification of species based on morphological characteristics is a conventional method in the insect identification process. However, the lack of morphological characteristics has limited their efficiency and accuracy (Tahir et al., 2018). Based on the available keys and taxonomic references, only four species could be identified up to the species level. In contrast, some morphospecies of the genus *Haematopota* sp. 1, *Ommatius* sp., *Anasillomos* sp., *Asilus* sp., and *Metopia* sp. could not be identified at the species level due to a lack of available taxonomic keys. Therefore, the problematic species were barcoded for precise identification due to ambiguities in species identification.

The barcoding analysis was conducted using the *COI* marker, which is a highly useful molecular tool that can discern cryptic, closely related, and morphologically similar species (Clark et al., 2005; Della Torre et al., 2002) and has been successfully utilised for species identification in various organisms (Rivera & Currie, 2009). In addition, DNA barcoding is also very valuable in species reconfirmation during the immature stages of insects (Barrett & Hebert, 2005). All the sequences obtained in this study showed more than 84% similarity to the Genbank sequences. In 13 sequences, 7/13 sequences showed low species detection (84-97%), while 6/13 sequences were considerably high (>97% similarity). According to Morinière et al. (2019), the sequences that showed more than 82% similarity to Genbank could be confirmed as genus level, while >97% similarity with Genbank would prove that the species in question was the right species. Failure to identify up to the species level by molecular approach might be due to the DNA quality. However, we thought this would not be the major issue because the 5–10ng/µl of DNA concentration in insect species can be amplified successfully. The newly presented *COI* sequences not yet available in the Genbank may cause a lower percentage with the Genbank similarity (Molaei et al., 2008).

Two dipteran genera, i.e., *Musca* and *Chrysomya* sp., were the most abundant in the cattle farms, and our results confirmed those of Al-Shaibani and Al-Mahdi (2014), who reported that *Musca* was the most abundant genus in Yemen animal farms and functioned as the main decomposer of cattle dung (Hussein et al., 2017) since various species under the genus *Musca* were ubiquitous, and highly adaptive to different ecosystems, even under extreme environmental conditions. However, this housefly species is a great nuisance and pest to humans and livestock, causing reduced livestock productivity as a disease carrier and vector of many pathogens, with an economic loss estimated at billions of dollars annually (Khamesipour et al., 2018; Taylor et al., 2012).

The blowfly or oriental latrine fly *Chrysomya megacephala*, which belongs to the family Calliphoridae, has significant importance in environmental health as a decomposer of faeces and decaying carrion, as well as in forensic entomology (Mullens, 2009; Sharanya & Zuha, 2019). For this reason, the species may breed prolifically on cattle farms and in human settlements. In addition, both the housefly and the blowfly species had been reported to have the potential to carry pathogenic bacteria and were abundant in human settlements in Northeast Thailand (Chaiwong et al., 2014).

Ladang Bangi farm showed the highest number of fly diversity, while Ladang UPM farm showed the lowest. The fly species diversity and richness appeared to be associated with the conditions of the farm, such as the structure of the dairy building and the type of cage flooring (Lysyk & Axtell, 1986). Our results indicated that high infestations of *Musca* spp. and *Chrysomya* sp. were recorded in Ladang UPM, mainly

due to the sanitation status or manure management around the cages, where both groups of fly species utilised cattle dung as their breeding sites (Khan et al., 2012). The sanitation status refers to the type of cage flooring, whether concrete, half concrete, or ground flooring, as a medium or surface which affects the decomposition rate of the manure or its natural disposal in the environment.

The high presence of *Chrysomya* sp. (besides *Musca* spp.) on Ladang UPM farm was due to the behaviour of this species which sought cattle dung as its preferred food source, as well as its breeding site (Wang et al., 2018). Infestation of fly larvae in live animals can cause myiasis, leading to tissue necrosis in cattle (Ferraz et al., 2010), which was not recorded in our study area. Therefore, we surmised that the Ladang UPM farm might need further judicious sanitary management, such as disposing of the cattle dung twice during the peak breeding period of the flies, together with a single or combined application of pesticide to overcome the infestation problem (Issa, 2019). However, both *Musca* spp. and *Chrysomya* sp. were absent at Ladang Bangi, likely because the earthen flooring of the cages was not a conducive breeding site for the flies, besides the lack of food source, i.e., the faeces, which had dried out and dissipated into the farmlands. Ladang Rasa farm recorded the presence of *Musca* sp., but not *Chrysomya* sp., most likely due to the combined structure of the ground and cement flooring of the cages, favouring *Musca* sp. infestation (Issa, 2019; Lysyk & Axtell, 1986). Comparing the three farms, the Ladang Bangi farm provided the highest number of fly species diversity, followed by Ladang Rasa and Ladang UPM, respectively. Both farms (Ladang Bangi and Rasa) had a high diversity of fly species probably because both were adjacent to the fragmented forest, which could provide suitable niches for different fly species. The Ladang Bangi farm recorded the highest fly diversity, most likely because of its proximity to a river, as well as the fragmented forest, and thus, could provide more microhabitats for a wider variety of fly species and their hosts (Brockerhoff et al., 2017). Ladang UPM farm presented the lowest fly species diversity and richness, probably due to the farm being on open ground and adjacent to the main road, thus, subjected to anthropogenic factors which could affect the breeding sites and food sources of the various fly species (Papanastasis et al., 2017).

The depredation of blood-sucking and myiasis-producing flies is detrimental to the productivity and profitability of animal husbandry worldwide (Gerhardt & Hribar, 2019). In this study, *Tabanus* sp. from the family Tabanidae was present on the cattle farm but did not feed on human blood. However, there were records of several species under the genus *Tabanus*, namely *Tabanus bromius* and *Tabanus distinguendus,* that sucked human blood, causing human granulocytic anaplasmosis (HGA) by transmitting the pathogen *Anaplasma phagocytophilum* (Werszko et al., 2019). Their attack can lead to

weight loss and reduced milk production in livestock, and they can transmit several disease pathogens, including protozoa, bacteria, and viruses (Baldacchino et al., 2014; Foil, 1989; Mullens, 2009).

Furthermore, *Haematopota javana* was also found in the cattle farms in this study, but the family richness of Tabanidae in this study was considered low compared to a previous study by Phasuk et al. (2011). Only three species of tabanids were collected in our study compared to 10 species by Phasuk et al. (2011). However, our findings were not directly comparable because of differences in methodology and study duration (i.e., their sampling method utilised malaise traps for 18 months versus our sampling method used baited traps and sweeping nets for three months). Furthermore, the abundance of tabanids is also significantly higher during the wet season in Thailand, but this seasonal variation was not apparent during our sampling period in the field.

Notably, the black fly species *Simulium* sp. (family Simuliidae) was not found in this study, although it has been recorded as a vector that transmits pathogens in livestock farms. According to Adler et al. (2010), this species is prevalent in the stream area and causes the parasitic disease known as onchocerciasis or river blindness in livestock and humans. Four sarcophagids (also known as flesh flies) species, i.e., *Sarcophaga* sp., *Metopia* sp. 1, *Metopia* sp. 2, and *Iranihindia martellata,* were reported in this study. Most sarcophagid flies can cause myiasis (invasion of tissues and organs in humans and animals by the larvae of the saprophagous flies). These larvae feed on the host tissues and body fluids or ingest food as parasites in the skin, many body parts, and other soft tissues of humans (Hall et al., 2016).

Anasillomos sp., *Asilus* sp., and *Ommatius* sp. are classified under Asilidae, also known as the robber fly. The asilid adults are predators capable of taking on larger prey such as dragonflies, but the selected prey size varies among the species. The type of prey, whether stationary, crawling, or flying, is also species-specific among the robber flies. Their mouthparts contain a stout proboscis that the adult uses to inject paralysing venom into the prey during hunting. Asilids are not blood-feeders, but their bites can cause pain to humans when confronted or disturbed (Newton, 2006).

Eight sequences under six dipteran species were detected carrying the DNA of the host cattle, as confirmed through PCR and DNA sequencing. The positive and negative controls supported the results in each PCR process (Banasik et al., 2016). The main idea was to detect the small amount of DNA of the host species in the flies' DNA using the targeted animal species primers. Identification of the host species was obtained with >98% match for *B. indicus*, except for 98.33% similarity with the Genbank for *B. taurus*. In the host preference analysis using *cytb*, *B. taurus* and *B. indicus* were successfully detected in the DNA of the fly species. Both cattle species are important for meat and dairy products and have higher productivity from

the breeding process with selective local and pure breeds (International Atomic Energy Agency [IAEA], 2009). Furthermore, DNA of *B. taurus* was detected in the sucking flies, namely *H. javana*, *T. rubidus*, and *T. fontinalis*, which might act as potential vectors of pathogens causing Surra disease or trypanosomiasis in cattle, as reported in India (Veer, 1999) and in Malaysia (Erwanas et al., 2015).

Using the molecular approach, several flies in this study were associated with the cattle species, namely *Sarcophaga* sp., *Iranihindia martellata*, *Musca domestica*, and *Musca* sp. 1. These novel findings were proven using molecular approaches to detecting the DNA of the cattle species (either *B. taurus* or *B. indicus*) from the DNA of the dipteran species. The detected fly species were categorised under Sarcophagidae and Muscidae, which play a significant role as flesh flies and mechanical vectors of pathogens. Furthermore, the DNA of the cattle species was found in the bodies of the flies that had visited the carrion of the cattle species (Patton, 1922; Sukontason et al., 2014; Tan et al., 2010).

Identifying meal with DNA barcoding using the *cytb* gene was essential to determine the insect vector's host range and host preference. Although this can also be achieved using previous serological techniques (Alcaide et al., 2009), the PCR-based method enabled the identification of hosts up to the species level with a much higher degree of accurate identification, indicating >95% similarity with Genbank (Molaei et al., 2008; Townzen et al., 2008). Species confirmation of the flies and their associated hosts is important in studying pathogen transmission and determining potential biological and mechanical vectors of infectious pathogens.

Bovidae hosts could not be detected in several fly samples via PCR amplification, probably due to the lack of cattle DNA inside the dipteran samples and human contamination. The DNA of the potential Bovidae host can be amplified even in very low quantities and by designing specific Bovidae primers (Lee et al., 2015). The utilisation of gBlock primers is necessary to avoid human contamination (Boessenkool et al., 2012).

The effectiveness of DNA sequence detection in barcode analysis was highlighted by Ernieenor et al. (2015) in ticks and by Slama et al. (2015) in their blood meal analysis of the blood-sucking *Culicoides* (Diptera: Ceratopogonidae). Furthermore, the pathogens carried by the dipteran species can also be detected by using different pairs of primers targeting the pathogens, as reported by Hemmatinezhad et al. (2015).

CONCLUSION

The new barcodes of 11 dipteran species successfully identified in this study were deposited in Genbank, as well as eight sequences for two cattle species (*Bos taurus* and *B. indicus*), which acted as hosts of the dipteran flies. The species identification of the dipteran parasites and their hosts was confirmed molecularly and simultaneously by utilising the DNA of

the fly species. The most abundant genera collected were *Musca* and *Chrysomya*, the main decomposer for cattle dung. No significant differences were reported for the fly species composition and species richness between buildings and cage structures. It might be due to the anthropogenic elements of the farm locations as they were near the main public roads, human settlements, and fragmented forests. Even though some of the abundant fly populations (such as the family Tabanidae) did not transmit any pathogens, they were closely associated with the cattle by feeding on dung and played a role as biting livestock pests, leading to stunted growth and reduced milk production. Therefore, this study provides crucial insight into the relationship between parasitic fly species and their Bovidae hosts for future pest management within Malaysia's growing livestock production industry.

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