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Research article

Species Abundance and Identification of Forensically Important Flies of Saudi Arabia by DNA Barcoding

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Abstract

Because they may demonstrate characteristics of the environment where a body has been laying prior to the discovery, flies are insects of forensic interest. We investigated the fly abundance and the effect of location in Kingdom of Saudi Arabia on fly species diversity that attack decomposing human and animal remains. Using baited traps deployed in each location, we collected 3,697 flies of seven species belonging to three families. *Chrysomya albiceps* Wiedmann represented 60.86% of the collected flies, whereas *Musca domestica* L. represented 25.8%; the other species made up $<6\%$ each. To facilitate species identification by DNA barcoding, we sequenced a 710-bp "Folmer region" of cytochrome oxidase subunit 1 (COI) gene for 22 samples from collection sites distributed through entire Saudi Arabia. The COI sequences from *Musca albina* Wiedmann, *Musca lucidula* Loew, *Musca calleva* Walker, *Musca sorbens* Wiedmann, and *Physiphora alceae* Preyssler were obtained for the first time. This primary study indicates that even when Folmer primers were widely used in DNA barcoding, the Folmer's region is not adequate when discriminating between *Musca* species, and sequencing the whole COI or other genes is required for forensic purpose.

Key words: DNA barcoding, *Musca*, *Physiphora*, *Chrysomya*, Saudi Arabia

Flies, beetles, and many other arthropods have been reported to attract to dead bodies (Kim et al. 2014). Species identification is necessary for precise estimation of the postmortem interval (PMI) using insect evidence, with subsequent examination of thermobiological profiles to determine age (Catts 1992, Catts and Goff 1992, Amendt et al. 2004). A fly community in a given geographic area comprises mainly synanthropic, myiasis causing, and forensically important flies, which are associated with decomposing remains and serve as physical evidence in the death investigations (Smith 1986). Some fly species, such as *Lucilia cuprina*, *Lucilia sericata*, *Chrysomya albiceps* Wiedmann, and *Calliphora vicina*, can be placed in more than one of these mentioned categories, as they can infest living tissue and breed on carrion, feces, or decaying matter.

Each biogeographic zone has specific climatic, seasonal, and developmental requirements of certain fly species. Thus, there is great variability in the fly species involved in the sequential colonization of animal remains and their times of arrival with respect to geographic location (Tabor et al. 2005). Geographical region and the season will affect species composition and insect succession on a cadaver (Anderson 2001, Watson and Carlton 2003, Grassberger and Frank 2004). Therefore, data collected for a particular region should be used with caution when determining time of death in another region. The pattern of insect colonization is affected by the ecology of the area and the degree of sun exposure (Smith 1986, Erzinclioglu 1996). In Saudi Arabia, Setyaningrum and Al Dhafer (2014) identified 34 species of Calliphoridae. Al-Ghamdi et al. (2015) reported flies from four forensically important families—Calliphoridae, Muscidae, Ulidiidae, and Fanniidae.

Morphological identification cannot differentiate between morphologically similar species (Pape 1996). Furthermore, for a correct species identification and an estimation of PMIs in forensic entomology, many factors should be in consideration, such as that closely related species may have different diapause behaviors, different growth rates, and different habitats (Samarakoon et al. 2012). DNA-based techniques can be used with different insect samples

either dead, preserved, or live samples, without further rearing (Wells et al. 2007, Boehme et al. 2012, Aly and Wen 2013). These DNA-based methods can be carried out on any life cycle stage (Aly and Wen 2013). Mitochondrial DNA (mtDNA) offers advantages over nuclear DNA because it undergoes faster mutation rates (Boehme et al. 2012) and occurs in several copies within the cell (Waugh 2007). Hebert et al. (2003) pioneered the use of cytochrome oxidase subunit 1 (COI) for taxonomical assignment of animal specimens, for which they coined the term DNA barcoding. Sequences of COI have been proven useful in the identification of forensic fly species in several localities, including Australia (Harvey et al. 2003), Japan (Saigusa et al. 2005), Germany (Reibe et al. 2009), Korea (Park et al. 2009), Malaysia (Kavitha et al. 2012), China, and Egypt (Aly and Wen 2013).

Insects form the majority of the carrion invertebrate fauna. And, successional forensic entomology is based on studying the sequence of which fly species appear on the cadaver. The main goals of this project were to develop DNA barcoding as a fundament for a database of forensic insects in Saudi Arabia and to determine the abundance of forensically relevant fly species in Saudi Arabia.

Materials and Methods

Insect Collection and Morphological Identification

Samples included in this study were collected between November and December, 2014, from decaying sheep carcasses (air exposed) in different cities of Saudi Arabia (Dammam, Tabuk, Qasim, Arar, Medina, Jazan, and Riyadh). Each of the four pitfall traps (10 cm in diameter) were filled with a solution of water, soap, and salts, and then was placed around each carcass. To collect flies, traps were kept in place for 4 h, then flies were collected and preserved in 70% ethanol and labeled with the date and time of collection. The flies were identified morphologically to the lowest possible taxonomic level using dichotomous and pictorial taxonomic keys by Dr. Ashraf M.A. Mashaly, using the key described by Bei-Bienko and Steyskal (1988), Pont (1991), and Watson and Dallwitz (2003), and confirmed by Prof. Magdi El-Hawagry, Entomology Department, Faculty of Science, Cairo University, Egypt.

DNA Extraction and PCR

Following the "salting out" protocol of Sunnucks and Hales (1996), DNA was extracted from individual flies. Then using autoclaved plastic pestle, the fly was homogenized in 2 µl of a 20 mg/ml proteinase K and 300 µl TNES (400 mM NaCl, 50 mM Tris-HCl, 20 mM EDTA, 0.5% SDS, pH 7.5). In all, 85 µl of 5 M NaCl was added to the mix, which was incubated at 37°C for 3 h, and the sample was mixed. For 10 min, the mixture was centrifuged at 14,000 rpm. DNA was precipitated with 400 µl ethanol by gentle inversion of the tubes and the supernatant was transferred to a new reaction tube. The mixture was kept at −8 °C for 1 h prior to centrifugation at 14,000 rpm for 15 min. Alcohol was decanted and DNA pellet was washed with 70% ethanol, dried, and dissolved in 50 µl sterile water. A 710-bp fragment of the mitochondrial cytochrome oxidase subunit 1 (COI) gene was amplified using universal primers from the COI: LCO1490 (ggtcaacaaatcataaagatattgg) and HC02198 (taaacttcagggtgaccaaaaaatca; Folmer et al. 1994). The PCR mixture contained PCR buffer with 2.5 mM $MgCl_2$, 0.15 mM dNTPs, 0.3 mM of each primer, and 0.25 U Taq polymerase (Nippon Genetics, Europe GmbH) in a total volume of 25 µl. PCR was performed using the following protocol: 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 45 °C for 45 s, 72 °C for 45 s; and a final extension step for 5 min after the final cycle at 72 °C. Negative controls were included in all amplifications.

Purification and Electrophoretic Analysis of PCR Products

By adding isopropanol to a final concentration of 80% for 20 min at room temperature, centrifuging for 20 min at 15,000 rpm, washing with 70% ethanol, drying in vacuum, and dissolving in 12 µl bidistilled water, PCR products were purified. In electrophoresis, 1 µl was loaded on 1.7% agarose gel; DNA was separated at 10 V/cm for 60 min in 40 mM Tris-acetate buffer (pH 8.0) with 20 mM EDTA and visualized by staining with 0.5 µg/ml ethidium bromide.

Sequencing and Phylogenetic Analysis

In all, 5 µl of purified PCR product containing 50 ng/µl of sample mixed with 5 µl of 5 µM primer was sent for sequencing by Macrogene Europe. The chromatograms were edited using BioEdit; the DNA sequences were aligned using the Clustal W tool incorporated into MEGA 6 software, and phylogenetic and molecular evolutionary analyses were conducted using MEGA 6. The phylogenetic analyses were conducted in MEGA 6 (Tamura et al. 2013) using neighbor-joining (N-J) methods (Saitou and Nei 1987). For the N-J method, the pairwise deletion option was selected, which eliminated positions containing alignment gaps and missing data in pairwise sequence comparisons only (i.e., not in the multiple alignment). The N-J tree was drawn to scale with branch lengths in the same units as the evolutionary distances used. The evolutionary distances were computed using Kimura 2-parameter method and are expressed in terms of the number of base substitutions per site. Bootstrap tests were conducted with 500 replicates to determine the support for individual nodes.

Results

Fly specimens used in this work were obtained from different regions in Saudi Arabia (Dammam, Tabuk, Qasim, Arar, Medina, Jazan, and Riyadh; Table 1). Sequence results approved the morphological identification where the collected flies belong to three Dipteran families of seven species. The following fly species were collected from carcasses—one fly species belonging to family Calliphoridae, namely, *Chrysomya albiceps*; five fly species from family Muscidae, namely, *Musca albina* Wiedmann, *Musca calleva* Walker, *Musca domestica* L., *Musca lucidula* Loew, and *Musca sorbens* Wiedmann; and one species from family Ulidiidae, namely, *Physiphora alceae* Preyssler. Geographical information and ecological description of the study sites are described in Table 1. Muscidae were the only flies collected from all cities. Ulidiidae were collected only from Riyadh, whereas Calliphorid flies were collected from all cities except Medina (Table 2). From a total of 3,697 flies which were recorded, *Ch. albiceps* was the most abundant species, and it represented 60.86%, followed by *M. domestica* (25.8%), whereas *M. lucidula* was the least abundant species (0.62%; Table 3).

In the present study, a 710 bp of COI gene was sequenced, where all the 22 samples representative of the collected specimens from different area were sequenced successfully (Table 4). The previous sequences were identified using NCBI BLAST and then aligned with other 12 sequences of other flies from GenBank to confirm molecular identification and enable phylogenetic analysis (Fig 1). We excluded all the bootstrap values <70% and all *Musca* sp. were separated from *Ch. albiceps* and *Ph. alecea*, which were also separated in a supported clade (99%). However, *Ch. albicpes* and *Ph. alecea* were too close and their clade was not supported.

Table 1. Ecological description of study sites in Saudi Arabia

Study site	Geographical information	Ecological description
Riyadh	24° 44′36.54″ N, 46° 33′45.12″ E Elevation: 612 m.a.s.l	• Agricultural area • With many palm trees and grasses.
Jizan	17° 8′19.52″ N, 42° 37′52.56″ E, Elevation: 19 m.a.s.l	• Agricultural area with grasses. • Near a sheep market.
Tabuk	28° 28′31.40″ N, 36° 35′9.77″ E, Elevation: 768 m.a.s.l	• Agricultural area with grasses. • Near a sheep market.
Arar	30° 59′48.59″ N,41° 2′31.99″ E,Elevation: 555 m.a.s.l	• Urban area. • Near a sheep market.
Al-Qassim	26° 9′20.13″ N, 44° 20′5.01″ E, Elevation: 714 m.a.s.l	• Agricultural area. • Near a sheep farm.
Medina	24° 25′7.56″ N, 39° 28′59.56″ E, Elevation: 610 m.a.s.l	• Agricultural area. • Near a sheep farm.
Dammam	26° 13′57.42″ N, 50° 11′17.08″ E, Elevation: 12 m.a.s.l	• Agricultural area. • Near a sheep market.

Table 2. Distribution and abundance of fly species along different regions of Saudi Arabia

City	Family	Species	No. of flies
Riyadh	Calliphoridae	*Chrysomya albiceps* Wiedmann, 1819	89
	Muscidae	*Musca calleva* Walker, 1849	36
		Musca domestica L., 1758	193
	Ulidiidae	*Physiphora alceae* Preyssler, 1791	27
Dammam	Calliphoridae	*Chrysomya albiceps* Wiedmann, 1819	62
	Muscidae	*Musca calleva* Walker, 1849	23
		Musca domestica L., 1758	22
Al-Qassim	Calliphoridae	*Chrysomya albiceps* Wiedmann, 1819	93
	Muscidae	*Musca calleva* Walker, 1849	31
		Musca domestica L., 1758	94
Arar	Calliphoridae	*Chrysomya albiceps* Wiedmann, 1819	803
	Muscidae	*Musca calleva* Walker, 1849	35
		Musca domestica L., 1758	221
Jizan	Calliphoridae	*Chrysomya albiceps* Wiedmann, 1819	556
	Muscidae	*Musca albina* Wiedmann, 1830	204
		Musca domestica L., 1758	323
		Musca sorbens Wiedmann, 1830	65
Medina	Muscidae	*Musca calleva* Walker, 1849	49
		Musca domestica L., 1758	83
Tabuk	Calliphoridae	*Chrysomya albiceps* Wiedmann, 1819	647
	Muscidae	*Musca domestica* L., 1758	18
		Musca lucidula, Loew, 1856	23

Table 3. Species composition and relative abundance of collected fly species

Family	Species	Total no.	Percentage (%)
Calliphoridae	*Chrysomya albiceps*	2,250	60.86
Muscidae	*Musca albina*	204	5.52
	Musca calleva	174	4.71
	Musca domestica	954	25.8
	Musca lucidula,	23	0.62
	Musca sorbens	65	1.76
Ulidiidae	*Physiphora alceae*	27	0.73
		3,697	100%

There is only one record of *Ph. alecea* sequence in the GenBank, which did not cover our sequenced part. Also, we calculated the evolutionary divergence between species (Table 5); the divergence between different genus like *Musca* and *Chrysomaya* is >10%, whereas within the same genus is <5%. The differences between species varied from 1 to 3%. By comparing the obtained sequences with those in GenBank (Table 4), we found that the barcoding confirms the morphological identification of *Ch. albiceps* and *M. domestica*. In addition, we did not find any records for *M. albina*, *M. lucidula*, and *M. calleva*; thus, we sequenced many individuals for this study. Although the other species such as *M. sorbens* and *Ph. alceae* have only one record of partial COI sequence, which is not covered in our sequenced part so far, they are not published yet in any journal. All sequences that were obtained from this study were submitted to the GenBank NCBI under the accession numbers KU578289–KU578310.

Discussion

Many insect families are associated with forensic ecology; however, only families Calliphoridae, Sarcophidae, Muscidae, and Fanniidae are the most important for minimum PMI estimation (Aspoas 1994, Anderson and Van Laerhoven 1996). There are about 86,000 fly

Table 4. Basic Local Alignment Search Tool (BLAST) search

Sample code	Species	Region	Closest match	Accession no.	Identity
T1	*Musca domestica* L., 1758	Tabuk	*Musca domestica*	KP713680.1	99%
T2	*Musca lucidula*, 1856		*Musca domestica*	KP713680.1	100%
T3	*Chrysomya albiceps* (Wiedmann, 1819)		*Chrysomya albiceps*	KJ193726.1	99%
M1	*Musca domestica* L., 1758	Medina	*Musca domestica*	KM200723.1	99%
M2	*Musca calleva* Walker, 1849		*Musca larvipara*	EU627700.1	99%
J1	*Musca domestica* L., 1758	Jizan	*Musca domestica*	JX438043.1	99%
J2	*Musca sorbens* Wiedmann, 1830		*Musca domestica*	KM571920.1	97%
J4	*Chrysomya albiceps* (Wiedmann, 1819)		*Chrysomya albiceps*	KJ193726.1	99%
J5	*Musca albina* (Wiedmann, 1830)		*Musca domestica*	KP713680.1	100%
A1	*Musca domestica* L., 1758	Arar	*Musca domestica*	KM200723.1	99%
A2	*Musca calleva* Walker, 1849		*Musca domestica*	KP713680.1	99%
A3	*Chrysomya albiceps* (Wiedmann, 1819)		*Chrysomya albiceps*	KM434340.1	100%
D1	*Musca domestica* L., 1758	Dammam	*Musca domestica*	KM571920.1	100%
D2	*Chrysomya albiceps* (Wiedmann, 1819)		*Chrysomya albiceps*	KM434340.1	100%
D3	*Musca calleva* Walker, 1849		*Musca domestica*	KP713680.1	100%
R1	*Physiphora alceae* Preyssler, 1791	Riyadh	*Chrysomya albiceps*	KJ193726.1	99%
R2	*Musca domestica* L., 1758		*Musca domestica*	KP713680.1	100%
R3	*Chrysomya albiceps* (Wiedmann, 1819)		*Chrysomya albiceps*	KM407601.1	99%
R4	*Musca calleva* Walker, 1849		*Musca domestica*	KP713680.1	99%
Q1	*Musca domestica* L., 1758	Qassim	*Musca domestica*	KP713680.1	99%
Q2	*Musca calleva* Walker, 1849		*Musca domestica*	KM200723.1	99%
Q3	*Chrysomya albiceps* (Wiedmann, 1819)		*Chrysomya albiceps*	KM434340.1	100%

T: Tabuk; M: Medina; J: Jazan; A: Arar; D: Dammam; R: Riyadh; Q: Qasim.

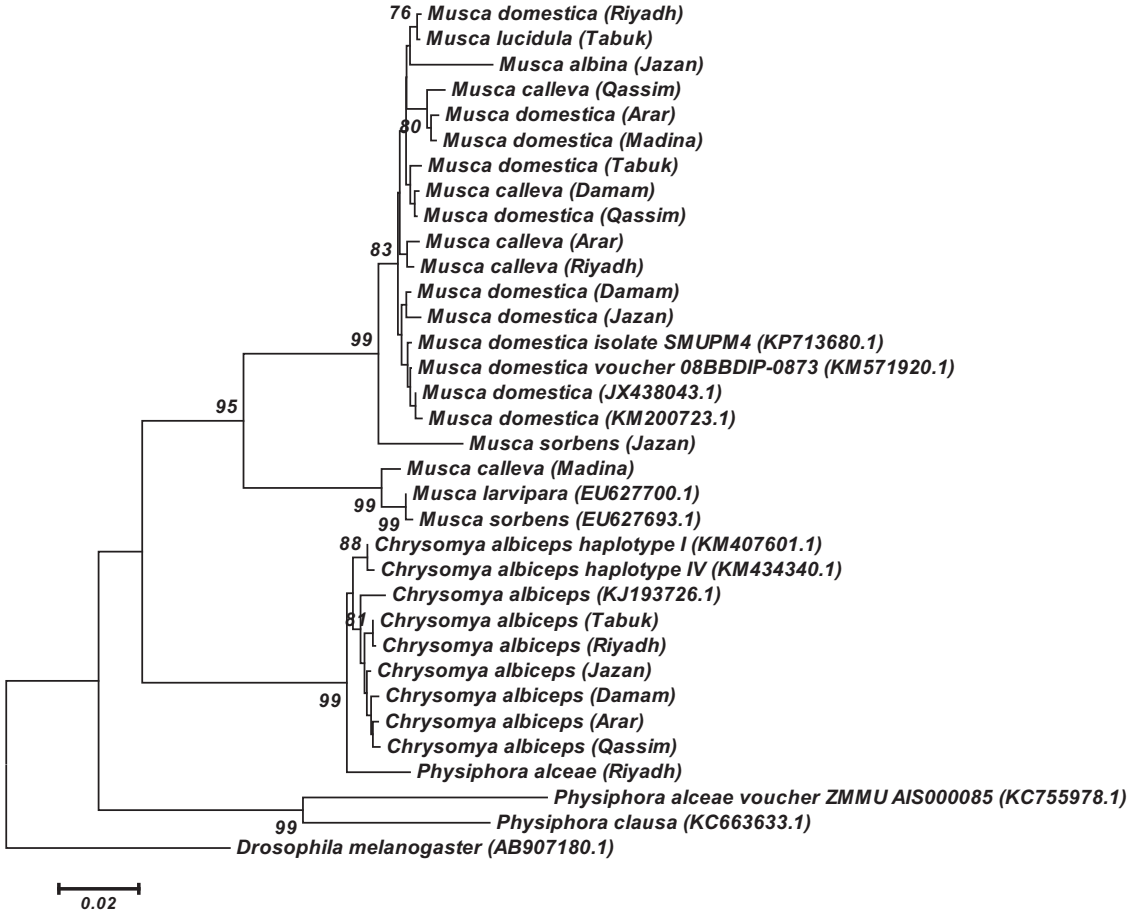

Fig. 1. Evolutionary relationships of taxa. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 0.53929092 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 34 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair. There was a total of 676 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.

Table 5. Estimates of evolutionary divergence between sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Chrysomya albiceps (Tabuk)																																		
Musca sorbens (Jazan)	0.114																																	
Physiphora alceae (Riyadh)	0.000	0.114																																
Chrysomya albiceps (Arar)	0.002	0.116	0.002																															
Chrysomya albiceps (Damam)	0.002	0.116	0.002	0.000																														
Chrysomya albiceps (Jazan)	0.000	0.114	0.000	0.002	0.002																													
Chrysomya albiceps (Qassim)	0.002	0.116	0.002	0.000	0.000	0.002																												
Chrysomya albiceps (Riyadh)	0.000	0.114	0.000	0.002	0.002	0.000	0.002																											
Musca albina (Jazan)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104																										
Musca calleva (Arar)	0.102	0.027	0.102	0.104	0.104	0.102	0.104	0.102	0.004																									
Musca calleva (Damam)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104	0.000	0.004																								
Musca calleva (Madina)	0.116	0.093	0.116	0.118	0.118	0.116	0.118	0.116	0.077	0.077	0.077																							
Musca calleva (Qassim)	0.105	0.030	0.105	0.107	0.107	0.105	0.107	0.105	0.004	0.007	0.004	0.080																						
Musca calleva (Riyadh)	0.102	0.025	0.102	0.104	0.104	0.102	0.104	0.102	0.002	0.002	0.002	0.075	0.005																					
Musca domestica (Arar)	0.105	0.030	0.105	0.107	0.107	0.105	0.107	0.105	0.004	0.007	0.004	0.080	0.000	0.005																				
Musca domestica (Damam)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104	0.000	0.004	0.000	0.077	0.004	0.002	0.004																			
Musca domestica (Jazan)	0.105	0.029	0.105	0.107	0.107	0.105	0.107	0.105	0.002	0.005	0.002	0.079	0.005	0.004	0.005	0.002																		
Musca domestica (Madina)	0.105	0.030	0.105	0.107	0.107	0.105	0.107	0.105	0.004	0.007	0.004	0.080	0.000	0.005	0.000	0.004	0.005																	
Musca domestica (Qassim)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104	0.000	0.004	0.000	0.077	0.004	0.002	0.004	0.000	0.002	0.004																
Musca domestica (Riyadh)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104	0.000	0.004	0.000	0.077	0.004	0.002	0.004	0.000	0.002	0.004	0.000															
Musca domestica (Tabuk)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104	0.000	0.004	0.000	0.077	0.004	0.002	0.004	0.000	0.002	0.004	0.000	0.000														
Musca lucidula (Tabuk)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104	0.000	0.004	0.000	0.077	0.004	0.002	0.004	0.000	0.002	0.004	0.000	0.000	0.000													
Drosophila melanogaster (AB907180.1)	0.155	0.159	0.155	0.157	0.157	0.155	0.157	0.155	0.143	0.146	0.143	0.138	0.145	0.145	0.145	0.143	0.145	0.145	0.143	0.143	0.143	0.143												
Musca larvipara (EU627700.1)	0.118	0.095	0.118	0.120	0.120	0.118	0.120	0.118	0.079	0.079	0.079	0.002	0.082	0.077	0.082	0.079	0.080	0.082	0.079	0.079	0.079	0.079	0.139											
Musca domestica (JX438043.1)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104	0.000	0.004	0.000	0.077	0.004	0.002	0.004	0.000	0.002	0.004	0.000	0.000	0.000	0.000	0.143	0.079										
Chrysomya albiceps (KJ193726.1)	0.000	0.114	0.000	0.002	0.002	0.000	0.002	0.000	0.104	0.102	0.104	0.116	0.105	0.102	0.105	0.104	0.105	0.105	0.104	0.104	0.104	0.104	0.155	0.118	0.104									
Musca domestica (KM200723.1)	0.105	0.029	0.105	0.107	0.107	0.105	0.107	0.105	0.002	0.005	0.002	0.079	0.002	0.004	0.002	0.002	0.004	0.002	0.002	0.002	0.002	0.002	0.145	0.080	0.002	0.105								
Chrysomya albiceps haplotype I (KM407601.1)	0.000	0.114	0.000	0.002	0.002	0.000	0.002	0.000	0.104	0.102	0.104	0.116	0.105	0.102	0.105	0.104	0.105	0.105	0.104	0.104	0.104	0.104	0.155	0.118	0.104	0.000	0.105							

(continued)

Table 5. Continued

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Chrysomya albiceps haplotype IV (KM434340.1)	0.002	0.116	0.002	0.000	0.000	0.002	0.000	0.002	0.105	0.104	0.105	0.118	0.107	0.104	0.107	0.105	0.107	0.107	0.105	0.105	0.105	0.105	0.157	0.120	0.105	0.002	0.107	0.002						
Musca domestica voucher 08BBDIP-0873 (KM571920.1)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104	0.000	0.004	0.000	0.077	0.004	0.002	0.004	0.000	0.002	0.004	0.000	0.000	0.000	0.000	0.143	0.079	0.000	0.104	0.002	0.104	0.105					
Musca domestica isolate SMUPM4 (KP713680.1)	0.104	0.027	0.104	0.105	0.105	0.104	0.105	0.104	0.000	0.004	0.000	0.077	0.004	0.002	0.004	0.000	0.002	0.004	0.000	0.000	0.000	0.000	0.143	0.079	0.000	0.104	0.002	0.104	0.105	0.000				
Musca sorbens (EU627693.1)	0.120	0.096	0.120	0.121	0.121	0.120	0.121	0.120	0.080	0.080	0.080	0.004	0.084	0.079	0.084	0.080	0.082	0.084	0.080	0.080	0.080	0.080	0.141	0.002	0.080	0.120	0.082	0.120	0.121	0.080	0.080			
Physiphora alceae voucher ZMMU AIS000085 (KC755978.1)	0.141	0.186	0.141	0.143	0.143	0.141	0.143	0.141	0.171	0.171	0.171	0.136	0.175	0.171	0.175	0.171	0.173	0.175	0.171	0.171	0.171	0.171	0.173	0.134	0.171	0.141	0.173	0.141	0.143	0.171	0.171	0.136		
Physiphora clausa (KC663633.1)	0.134	0.163	0.134	0.136	0.136	0.134	0.136	0.134	0.154	0.152	0.154	0.136	0.155	0.152	0.155	0.154	0.155	0.155	0.154	0.154	0.154	0.154	0.157	0.138	0.154	0.134	0.155	0.134	0.136	0.154	0.154	0.139	0.102	

The number of base differences per site from between sequences are shown. The analysis involved 34 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 560 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6

species described worldwide (Castner 2009). In our study, Calliphorid flies were collected from all cities except Medina. Al Ahmadi and Salem (1999) reported that the Calliphoridae in Saudi Arabia are common components of the insect fauna and widely distributed throughout the country.

In Saudi Arabia, Al-Ghamdi et al. (2015) and Mashaly (2016) found that some flies like *M. domestica* and *M. sorbens* feed on the carrion. Adult identification of Muscidae like other Diptera is based on chaetotaxy, wing venation, and the structure of genitalia (Huckett 1965). The identification based on morphological characters is not easy for nonexperts and is time-consuming, and the identification of adult Muscidae is complicated because of sexual dimorphism and the morphological identity of females of some species. Hwang and Turner (2005) stated that Ulidiid flies can be associated with carrion in large numbers.

In the perspective of DNA barcoding, the family Muscidae has been little studied, and there are only few published studies involving the COI gene in the flies which have used the sequence data to perform phylogenetic analyses (Kutty et al 2008). Also, DNA barcoding is used to compare haplotype diversity between and among populations (Marquez et al. 2007), and to determine the necrophagous species in forensic entomology (Cai et al. 2005). The neighbor-joining method used to construct the phylogenetic tree was useful, and we used the recommended parameters: Kimura 2-Parameter distance model with pairwise deletion of missing data and inclusion of all substitutions, and this model has been most commonly used in the barcoding literature and was employed to facilitate comparison across studies (Srivathsan and Meier 2012).

And like other studies, we generate the barcoding profile using the Folmer primer pair (Folmer et al 1994); these primers are most universally used in the DNA barcoding of Metazoa including insect (Virgilio et al 2010). The barcoding of the collected species with the universal primers did enable us to identify *Musca* sp. from *Ch. albicpes* and *Ph. alecea*, whereas the evolutionary divergence was >10%. It was obvious on the N-J tree (Fig. 1) that each of the mentioned species was located in very strong supported clade. *Chrysomya albicpes* from this study were identical to those from the previous studies, and the divergence between and among them was <1%, confirming the morphological identification with the barcoding. Also, the case is same for *M. domestica* collected from this study, where we confirmed the barcoding with the morphological identification while the divergence was very low (<1%). In case of *Ph. alecea*, from this study, it was identical to *Ch. albicpes* from Riyadh, but this does not mean that the barcoding is not an effective tool because from one side there is only one entry in the GenBank which is not covered by the whole Folmer regions which are sequenced in this study. In addition, it is not published and so far the morphological identification is not confirmed. In case of the other species from this study which have been for the first time identified on a molecular basis like *M. albina*, *M. lucidula*, and *M. calleva*, they were clustered with *M. domestica* clade, even when Folmer primers were widely used in DNA barcoding (Cai et al. 2005). This study indicates that Folmer's regions are not adequate to discriminate between *Musca* species.

Results of the present study showed that the ecological difference between studied areas did not affect the sequence of collected fly species, as sequence of individuals of the same species were almost identical even though they were from different localities with different topography and different environment. Same results were indicated by Srivathsan and Meier (2012) in the study on Australian Sarcophagidae.

In conclusion, we identified seven species belonging to three families, Calliphoridae, Muscidae, and Ulidiidae, from 3,697 flies collected. *Chrysomya albiceps* was the most abundant species, whereas *M. lucidula* was the least abundant. We obtained the sequences from *M. albina*, *M. lucidula*, and *M. calleva* for the first time. Also,

we confirm the finding of previous studies that barcoding is an effective tool to identify the insect species for any purpose, but the morphological identification is still important to generate accurate database and sequencing the whole COI is recommended, especially in case of insects which have no records in the GenBank.

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