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MITOCHONDRIAL COX-1 BARCODING FOR THE IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF *Sarcophaga (Liosarcophaga) dux* RECORDED FROM JEDDAH CITY OF SAUDI ARABIA

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ABSTRACT

This study was aimed to identify and phylogenetically analyze the sarcophagid flies from Jeddah (Saudi Arabia) on the basis of mitochondrial cytochrome-C-oxidase subunit-1 (COX-1) gene. Five flesh fly samples were collected from different locations of Jeddah region. All the samples were first morphologically identified as *Sarcophaga dux* and confirmed on the basis of COX-1 gene amplification and sequencing techniques using universal primers LCO1490 and HCO2198. For phylogenetic analysis, 5 sequences were checked by COX-1 service on barcode of life data system (BOLD) database. To determine the phylogenetic relationships and genetic variability between 5 collected and BOLD samples, large Neighbor-joining tree comprising of the collected samples and all the available *S. dux* BOLD samples were constructed. The 32 most related BOLD sequences were used to construct maximum likelihood (ML) and Neighbor-joining trees. The phylogenetic analysis of COX-1 gene sequences revealed significant match with *S. dux* sequences from BOLD database: GQ254446 (Australia), MG780139 (India), AY879255 (China), JN869983 (Malaysia) and KC249664 (Egypt). This is the first report of the flesh fly species recorded in the Western regions of Saudi Arabia. Results indicated that the genetic diversity and phylogenetic relationship analysis of the collected *S. dux* samples have an important role in understanding the evolutionary processes of *Sarcophaga* cryptic species in Western regions of Saudi Arabia.

Keywords: Genetic diversity, mitochondrial COX-1 gene, phylogenetic analysis, *Sarcophaga dux*

INTRODUCTION

Flesh flies (*Sarcophaga* species) are the most important insects in forensic and medical entomology due to their scavenging myiasis effects. These fly species have widespread distribution and diversity (Al-Ghamdi *et al*., 2015). Several species under genus *Sarcophaga* are mechanical vectors of several pathogens such as *Escherichia coli* (Migula), *Shigella dysenteriae* (Shiga), *Streptococcus* spp., *Salmonella* spp., tapeworms, and even the virus responsible for polio (Smith and Whitman, 1992) and their maggots cause myiasis in humans and animals. *Sarcophaga dux* has been recorded

from southern Europe (Richet *et al.*, 2011); most of the Asian countries such as Saudi Arabia (Al-Misned *et al.*, 2001), Malaysia (Tan *et al*., 2010), Thailand, India (Miroslav *et al.*, 2019), some African countries especially Egypt (Khalaf, 2004) and from China, Australia and USA (Sugiyama *et al.*, 1988). These fly species can colonize and break down the dead bodies. Besides, these have been found responsible for causing myiasis in humans and animals (Kaufmann, 1996).

The genus *Sarcophaga* is most diverse having 133 sub-genera and 790 species (Richet *et al.*, 2011). The previous taxonomic studies used only male genitalia to morphologically identify *Sarcophaga* species (Carvalho and Mello-Patiu, 2008). However, Zhang *et al.* (2014) have reported that females of some *Sarcophaga* species can be identified by their external morphological characters such as the colour of female's gena, postgena, and spots in tergites and sternites. Further, the shape of female sternites is considered a useful character in differentiating some *Sarcophaga* species through the presence or absence of fusion of the sternites 6, 7 and 8 (Vairo *et al.*, 2015). The morphological identification methods have some limitations and difficulties in many phases of life of these flies, especially in larval specimens the lack of documented thermo-biological profiles of these insects (Meiklejohn *et al.*, 2011). Therefore, the adults of sarcophagid flies are used in the identification of these flies at family level. Nonetheless, the species-level identification of *Sarcophaga* flesh flies is complicated and needs accurate examination of morphological variations of bristle placement and length, hair coloration, body pigmentation and genital structure of adults (Zhang *et al.*, 2014).

The recent studies on insect taxonomy and phylogenetic analysis suggest the molecular techniques of DNA barcoding is more convenient and useful in the identification of various genera and species (Wells *et al.*, 2001; Zehner *et al.*, 2004). Accordingly, these DNA markers can be used in both immature and adult insects, and are considered quick and accurate for genus- and species-level identification (Mazutti *et al.*, 2006; Saigusa *et al.*, 2009). These approaches utilize some regions of DNA like mitochondrial cytochrome-C-oxidase subunit-1 (COX-1), mitochondrial NADH dehydrogenase subunit-4 (ND-4) and ribosomal DNA internal transcribed spacer-2 (ITS-2) genes. These are most efficient genetic markers used in DNA barcoding of a range of taxa including gastropods, insects, birds and fishes (Seixas *et al.*, 2013; Sharma *et al.*, 2015; Abd Algalil and Zambare, 2016). Hebert *et al.* (2003) reported that these main DNA barcode of a short gene fragment can be sequenced in diverse sample sets to generate comparable sequences that enable the distinction of species from each other. In present study, the morphological identification methods and phylogenetic analysis of mitochondrial COX-I gene sequences were utilized to identify *Sarcophaga* species and to detect their phylogenetic relationships with GenBank archived sequences, thereby get further insight into the epidemiology and distribution of these insects.

MATERIALS AND METHODS

Flesh fly samples collection and morphological identification

Flesh fly samples were collected from various sites in Jeddah, Saudi Arabia during the year 2018. Adult flesh fly specimens were collected by using final flight trap. The 3 days old beef liver was used as bait trap to attract the adult flies. The live adult flies were brought to the laboratory and prior to their sorting were kept in a freezer at -4°C for 30 min. The adult flesh fly samples were morphologically identified using compound and stereo-microscopes (OMAX 40x-2000x compound binocular microscope with 1.3MP digital camera, California, USA; and AmScope stereomicroscope with 640x480 pixel digital camera, California, USA, respectively). The last abdominal segments of both male and female adults were separated from the rest of body with the help of a sharp blade and kept in 10% KOH solution for 2-3 days. The genitalia were observed under a stereomicroscope to

identify flesh fly genus and species, based on male genitalia and other external characters of adult fly using morphological identification models (Peris *et al.*, 1999; Abd Algalil and Zambare, 2016).

DNA extraction

Five adult flesh fly samples were used for total DNA extraction. The genomic DNA extraction and purification was performed from one or two legs of these flies as per Kress and Erickson (2012) and also in accordance with our previous optimized protocol for DNA extraction from the whole mosquito and fly (Abdella *et al*., 2018). Each adult flesh fly was homogenized in 50 µL of insect lysis buffer (16.5 g GuSCN, 12 mL 0.5M EDTA pH 8.0, 6 mL 1 M tris-HCl pH 8.0, 1 mL triton X-100, 10 mL tween-20 and final volume made 200 mL by double distilled water). All the five homogenate samples were incubated in a water bath for 45 min at 58ºC with quick addition of 5 µL proteinase-K for each homogenate during incubation. These tubes were incubated in ice for 30 min before centrifugation at 10000 rpm for 10 min. The clear supernatant in each tube was transferred into a fresh 1.5 mL Eppendorf tube. The precipitation and purification of DNA from supernatant was performed following the modified protocol of Abdella *et al.* (2018), using 100% ethanol and silica gel spin column, respectively. The purified DNA samples, stuck on the silica gel of column, was washed twice by adding 500 µL 70% ethanol to each spin column and then centrifuged these columns for 2 min at 10000 rpm after each wash. Finally, the columns were transferred to new 1.5 mL Eppendorf tubes and DNA solubilized by adding 50 µL double distilled water to the spin column. To collect the purified DNA, these tubes were centrifuged for 1 min at 9000 rpm. Five solubilized DNA samples were preserved at -24ºC until molecular analysis.

Mitochondrial COX-1 gene sequencing

In present study, the COX-1 gene was examined for sequence polymorphism among the collected five flesh fly samples. Five total DNA extract samples were used as templates to amplify ~ 670 bp fragments. The primers, used to amplify barcoding COX-1 region, composed of DNA primers pair, LCOI490 (forward): (5´-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (reverse): (5´-TAAACTTCAGGGTGACCAAAAAATCA-3') [Guo *et al.*, 2018]. PCR amplification reaction was performed in 25 µL PCR reaction mixture containing 10-30 ng template DNA, 0.1 mM dNTPs, 1.24 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 1X reaction buffer, 5 pmol of each forward and reverse primers, and sufficient nuclease-free water to make a volume of 25 µL (Invitrogen, CergyPontoise, France). The amplification of approximately 670 bp fragment was preceded by 1 min initial denaturation at 96ºC and subsequent five cycles of amplification were performed using the following cycle conditions: 94ºC for 40 sec (denaturation), 45ºC for 40 sec (annealing), and 72ºC for 1 min (extension); followed by 32 cycles of denaturing at 94ºC for 30 sec., annealing at 53ºC for 40 sec., extension at 72ºC for 1 min.; and final extension step by holding the reaction at 72ºC for 10 min. The PCR amplification products were confirmed by running on 1.5% agarose gel, stained with ethidium bromide, and visualized in a gel imaging system. PCR amplification products showing positive clear bands in agarose gel were purified using a nucleic acid gel extraction kit (Omega Bio-tek, 400 Pinnacle Way, USA) and subjected to the commercial sequencing (Macrogen, Beotkkot-ro, Geumcheon-gu, Seoul, Korea) using the PCR primers in both directions.

Sequence analysis and species identification

To confirm the morphological identity, the obtained sequences of COX-1 gene were compared with *Sarcophaga* species in GenBank using BLAST software program (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*). A phylogenetic tree was constructed by using MEGA-X software (Kumar *et al.*, 2018), based on the UPGMA algorithm within the Tamura-Nei genetic distance model, to determine phylogenetic relationships and genetic variability between the collected samples and the GenBank *Sarcophaga* species sequences. Besides, the 5 obtained sequences of COX-1 gene were checked by BOLD identification system (IDS) for COX-1 service on the barcode of life data system (*https://www.boldsystems.org/index.php*). Each sequence was considered as a query to detect the best match with reference sequences on BOLD database. The sequences and samples obtained from

BOLD were assembled and aligned using the statistical program R with msa package (R Development Core Team, 2011; Bodenhofer *et al.,* 2015) and the Clustal-W algorithm for the alignment.

Phylogenetic analysis

To determine the phylogenetic relationships and genetic variability amongst the collected samples and BOLD samples, a large Neighbor-joining (NJ) tree was constructed. This tree compared the collected samples and all the available BOLD samples including 172 *Sarcophaga dux*, 2 *S. aegyptica*, and 2 *S. harpax* sequences. Afterwards 32 samples of the most related BOLD sequences along with our samples were used to construct the maximum likelihood (ML) tree under Kimura (K80) model and NJ tree under the Tamura-Nei genetic distance model. All the trees were constructed using SeqinR (Charif and Lobry, 2007), ape version 5.3 (Paradis and Schliep, 2019) and Phangorn version 2.5.5 (Schliep *et al.*, 2017) packages in the statistical program R. The trees were bootstrapped with 100 replications and were rooted with *Angiometopa falleni* (GenBank accession number JQ686222) as out-group.

RESULTS AND DISCUSSION

Morphological identification of Sarcophaga dux

Flesh flies of *Sarcophaga* species are globally widely distributed insects, especially in tropical and subtropical regions. Recent taxonomic reports reveal the identification of 22 species of genus *Sarcophaga* (Sukontason *et al.,* 2014; Pekbey, 2020). These flies under genus *Sarcophaga* have forensic and medical importance as they colonize dead and living tissues of humans and animals (Al-Ghamdi *et al.*, 2015; Alikhan *et al.,* 2018). The taxonomic status and distribution patterns of *Sarcophaga* flies are the first steps in the surveillance and control of these flesh flies. As per the morphological identification model of Abd Algalil and Zambare (2016), the morphological features of *S. dux* adult flies are generally similar to the other flies of Sarcophagidae with grayish to black silvery colour. The eyes are large without hairs, frons narrower in male and wider in female. Normally the thorax has three dark blackish longitudinal stripes, which reach upto hind thorax. The abdomen of this adult fly is big in size and chequered with a silvery-grey pattern. Long hairs are on 1st and 2nd sternites and short hairs on 3rd and 4th sternites. The 5th sternite is 'V' shaped and abdominal tergite bears setae. The female anal sternite is round in shape with marginal bristles and has long hairs on dorsal surface (Fig. 1D). The 8 sternites, 1 anal sternite and 7 tergites form the female genital organs. Peris *et al.* (1999), Lehrer (2006) and Abd Algalil and Zambare (2016) reported that the adult *S. dux* male genitalia have brown/red colour with 2-3 segments. Normally, it is multifaceted and possesses unique distinct features for fly species classification. The penis is big and complex; its 1st and 2nd sternites possess long hairs, while 3rd and 4th sternites are small haired. The 5th sternite is V-shaped resembling the configuration with small lateral bristles and arms of this 5th sternite possess extended hair alongside the tips (Fig. 1A). Further, the internal forceps of these male genitalia are piercing and somewhat curled towards exterior. The outer forceps are egg-shaped having hairs on distal portion (Fig. 1B). The anterior paramere are sharp at ending, while posterior paramere are curled and broad in center an, have 2 or 3 hairs (Fig. 1B). The paraphallus of adult males is firm and sclerotized styli of glans possess comb-like configuration and petite (Fig. 1C). The morphological study demonstrated that all the collected samples from Jeddah (Saudi Arabia) shared high morphological similarity with *S. dux.* The morphological identification of *Sarcophaga* species, distributed in Saudi Arabia, has earlier been reported (Al-Misned *et al*., 2001; Al-Ghamdi *et al.*, 2015). The morphological characters of male terminalia are considered the main diagnostic features in the identification of Sarcophagidae flies and the male morphological features of our samples were similar to *S. dux* reported by Sukontason *et al.* (2010), Chakraborty *et al*. (2014) and Abd Algalil and Zambare (2017).

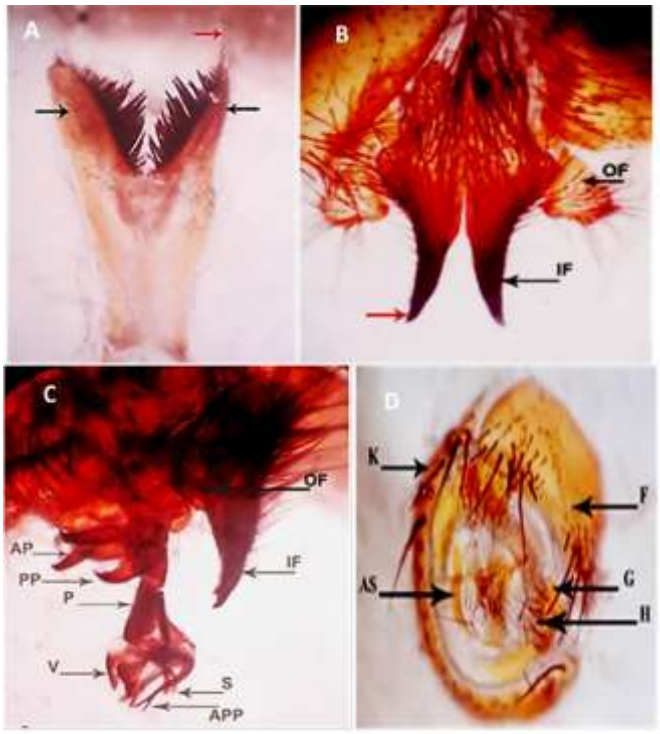

Fig. 1: Morphological microphotographs of *Sarcophaga dux* adult male and female genitalia exposing: A) Dorsal view of V-shaped 5th sternite shown two arms (black arrows) **with lateral short bristles and one or more long hair terminally on the tips of these arms** (red arrow)**. B) Dorsal view of male genitalia shown inner and outer forceps** (IF: inner forceps and OF: Outer forceps)**. Inner forceps almost straight, pointed and slightly curved at end and small notch** (red arrow)**, while the outer forceps are nearly oval with hairs on distal end. C) Lateral view of male genitalia showing the penis structure with inner and outer forceps** (IF: inner forceps, OF: Outer forceps, AP: Anterior paramere PP: posterior paramere, P: paraphallus, V: ventralia, APP: apical plate of paraphallus and S: Styli of glans)**. D) Micrograph of female genitalia showing anal sternite (**AS**), 6th sternite (**F**), 7th sternite** (G)**, 8th sternite** (H) **and 7th tergite** (K)**.**

COX-1 gene amplification and sequencing

In the present study, five extracted DNA samples were used for the amplification of ~670 bp fragment of COX-1 barcoding region. All the flesh fly specimens had amplified ~670 bp bands of COX-1 target gene which is a specific molecular marker of *Sarcophaga* species (Samerjai *et al.*, 2020). These amplified DNA fragments were purified from agarose gel and re-amplified before sequencing. The obtained sequences were AT-rich with an average of 66.9% AT content for all codes. This morphological method does not distinguish *Sarcophaga* flies among the cryptic species. So, the identification of insects based on DNA molecular diagnosis assays, especially PCR-based techniques and DNA sequencing technology, may provide a much viable information on insect taxonomic differentiation and may help in assessing the genetic diversity and phylogeny between the cryptic species (Karanovic *et al.*, 2016). In this approach the use of a specific DNA molecular marker as mitochondrial DNA (mtDNA) region of gene encoding COX-I has been shown to be appropriate tool for species identification and phylogenetic analysis of a range of taxa (Sharma *et al.*, 2015, Samerjai *et al.*, 2020). Therefore, the subsequent object of present study was to use mitochondrial COX-I gene sequences for taxonomic identification and phylogenetic analysis.

COX-1 gene sequence blast and species identification

The blast analysis of gene sequences confirmed the morphological identity, as all the 5 sequences showed similarity with mitochondrial COX-1 barcode sequences of *Sarcophaga* (*Liosarcophaga*) *dux* isolates in NCBI database with 88-98% identity (Table 1). Also, some collected COX-1 gene sequences had lower matching results (84-86%) or did not match GenBank with *Sarcophaga* species like KU746579.1 (*S. redux*), KU746581.1 (*S. tibialis*), JX987058.1 (*S. tibialis*), KX161493.1 (*S. tibialis*), JQ582060.1 (*S. teretirostris*), JN964981.1 (*S. misera*), KY859997.1 (*S. aratrix*), GQ223317.1 (*S. triplasia*) and JX861475.1 (*S. harpax*) of mitochondrial COX-1 gene for cytochrome-c oxidase subunit-1. The homology relationships between the 5 examined specimens and 24 selected GenBank samples were divided into some main clusters with sister branches depending on the similarity between these samples (Fig. 2). The GenBank *S. dux* specimens shared high similarity and grouped in one main cluster in the tree. The samples E18-Insect-AB3 and I18-Insect-AB5 clustered in one group with high similarity with *S. dux* specimens; while the other three

Table 1: Blast similarity results of five collected flesh flies from Jeddah with 24 samples *Sarcophaga* species selected from the GenBank

GenBank Accession No.	*Sarcophaga* Species	COX-1 gene length (bp)	Country	A18-Insect-AB1			C18-Insect-AB2			E18-Insect-AB3			G18-Insect-AB4			I18-Insect-AB5		
				QC (%)	E-V	Id (%)	QC (%)	E-V	Id (%)	QC (%)	E-V	Id (%)	QC (%)	E-V	Id (%)	QC (%)	E-V	Id (%)
KC249713.1	*S. dux*	855	Egypt	83	0.0	89	66	4e-160	87	90	0.0	98	75	1e-158	98	91	0.0	97
JX187399.1	*S. dux*	2206	Malaysia	83	0.0	89	66	4e-160	87	90	0.0	98	75	1e-158	98	91	0.0	97
EF405938.1	*S. dux*	2305	Malaysia	83	0.0	89	66	4e-160	87	90	0.0	98	75	1e-158	98	91	0.0	97
KY001837.1	*S. dux*	1278	China	83	0.0	89	66	4e-160	87	90	0.0	97	75	1e-158	98	91	0.0	97
JX430021.1	*S. dux*	632	India	78	0.0	89	66	2e-156	86	85	0.0	97	78	6e-157	98	86	0.0	97
JN964821.1	*S. dux*	603	Australia	77	0.0	89	--	--	--	82	0.0	98	--	--	--	83	0.0	98
KP407085.1	*S. dux*	1007	Malaysia	83	0.0	89	66	2e-157	87	90	0.0	97	68	8e-156	98	91	0.0	97
KR150142.1	*S. dux*	658	China	82	0.0	88	66	4e-160	87	88	0.0	97	75	1e-158	98	91	0.0	97
KU746579.1	*S. redux*	658	Denmark	81	3e-177	83	66	4e-141	80	88	0.0	84	--	--	--	91	0.0	84
KU746581.1	*S. tibialis*	658	Denmark	82	6e-177	82	--	--	--	--	--	--	--	--	--	--	--	--
JQ582060.1	*S. teretirostris*	1535	Belgium	83	6e-177	80	--	--	--	90	0.0	83	--	--	--	91	0.0	83
JX987058.1	*S. tibialis*	658	Spain	82	1e-173	82	63	5e-139	80	88	0.0	82	--	--	--	90	0.0	82
GQ223317.1	*S. triplasia*	2264	USA	85	2e-170	80	--	--	--	--	--	--	--	--	--	--	--	--
JN964981.1	*S. misera*	657	Australia	82	6e-171	81	66	1e-141	80	88	0.0	81	75	2e-143	81	90	0.0	82
KY859997.1	*S. aratrix*	676	Portugal	83	6e-171	82	--	--	--	89	0.0	82	--	--	--	91	0.0	82
KX161493.1	*S. tibialis*	677	Spain	80	6e-171	83	--	--	--	87	0.0	82	--	--	--	89	0.0	82
KT347335.1	*S. dux*	670	China	83	0.0	89	66	4e-160	87	90	0.0	97	75	5e-158	98	90	0.0	97
KJ496796.1	*S. dux*	1237	Malaysia	83	0.0	89	66	4e-159	87	90	0.0	97	77	6e-157	98	91	0.0	97
JX861475.1	*S. harpax*	1539	Korea	83	0.0	80	66	2e-156	80	90	0.0	84	77	6e-157	84	91	0.0	82
KF562109.1	*S. dux*	1250	Malaysia	83	0.0	89	66	4e-159	87	90	0.0	97	75	6e-157	98	91	0.0	97
KC855284.1	*S. dux*	709	Malaysia	83	0.0	89	66	4e-159	87	90	0.0	97	68	6e-157	98	91	0.0	97
KJ496800.1	*S. dux*	1237	Malaysia	83	0.0	89	66	2e-158	87	90	0.0	97	68	1e-158	98	91	0.0	97
KR021150.1	*S. dux*	658	India	82	0.0	89	66	2e-157	87	88	0.0	98	66	8e-156	98	90	0.0	97
KJ461745.1	*S. dux*	658	China	82	0.0	89	66	4e-160	87	88	0.0	98	68	1e-158	98	90	0.0	97

QC: Query cover, E-V: E-Value, and Id: Identity

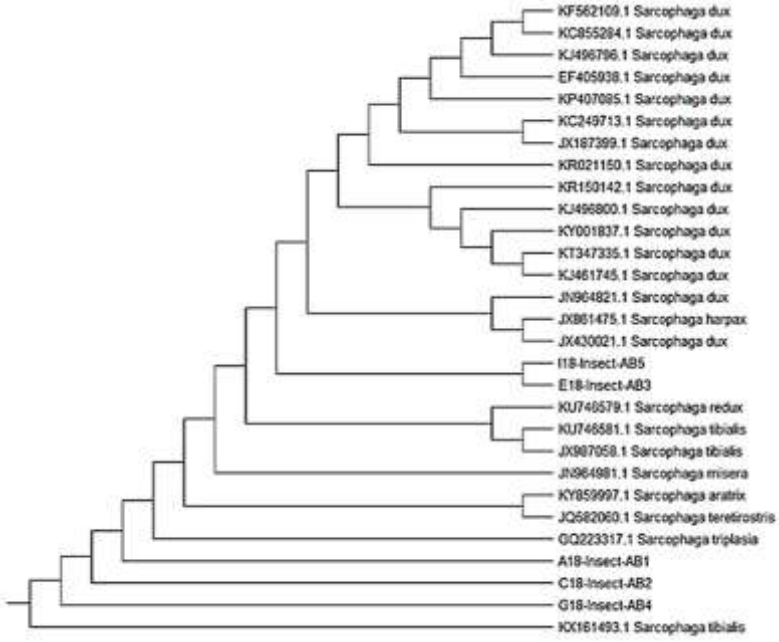

Fig. 2: Phylogenetic relationships between five *Sarcophaga dux* samples collected from Jeddah and 24 *Sarcophaga* species samples selected from GenBank, based on the UPGMA method under the Tamura-Nei genetic distance model. Our samples highlighted by yellow colour

samples A18-Insect-AB1 C18-Insect-AB2 and G18-Insect-AB4 had some sequence diversity from other specimen. Aly *et al.* (2013) studied several populations of four *Sarcophaga* species, and opined that the populations of various *Sarcophaga* species were clearly separated into 3 genetic clades. Anderson *et al.* (2019) reported that the phylogenetic analysis of COX-I gene sequences of some *Sarcophaga* species samples isolated from Portugal and Brazil showed *S. dux* samples to be more related to *S. portschinskyi* (isolated from China). Also, blast analysis showed that the

specimen G18 had a close relationship with GenBank sample KX161493.1 (*S. tibialis*) which was identified in Spain. The other 4 samples I18, C18, E18 and A18 showed close relationship with GenBank samples JN964821.1 (*S. dux* – Australia), JX430021.1 (*S. dux* – India), KJ461745.1 (*S. dux* – China) and KU746579.1 (*S. redux* – Denmark). The *Sarcophaga* species barcoding misidentifications from GenBank data and the absence of conspecific sequences are considered. Also, our obtained blast results were comparable with those found in other barcoding studies (Nelson *et al.*, 2007; Meiklejohn *et al.*, 2011). To confirm our blast results, a second data set was build using BOLD sequences. A large Neighbor-joining tree was constructed using all the available information of genus *Sarcophaga* obtained from BOLD database. This tree comprised the 5 collected samples, 172 *S. dux*, 2 *S. aegyptica*, and 2 *S. harpax* sequences. This phylogeny indicated that among all *S. dux* BOLD specimens located in different countries, our five flesh fly specimens exhibited high relationship with several *S. dux* specimens.

Phylogenetic analysis

The phylogenetic analysis was done in two steps. First, Neighbor-joining (NJ) tree under Tamura-Nei genetic distance model was constructed based on COX-1 sequences of collected flesh fly samples and all the available BOLD samples (Fig. 3). The obtained tree showed that our samples had high similarity with all *S. dux* samples in BOLD database. In second step of phylogenetic analysis, both NJ and maximum likelihood analysis used to expose the relationship between our collected samples and 32 *S. dux* samples with a high similarity relationship to our samples selected from BOLD database. Depending upon the results of previous NJ analysis, the samples showing high similarity relationship with our samples were selected on the basis of similarity values and clustering distribution of these selected samples in the main NJ tree (Fig. 3). So, 32 samples were found most similar to our samples and selected for designing a new NJ phylogenetic tree. Both NJ and ML analyses of 32 BOLD samples and our samples revealed that all the sequences were branched as main clusters in these trees (Fig. 4 and 5). These trees were bootstrapped with 100

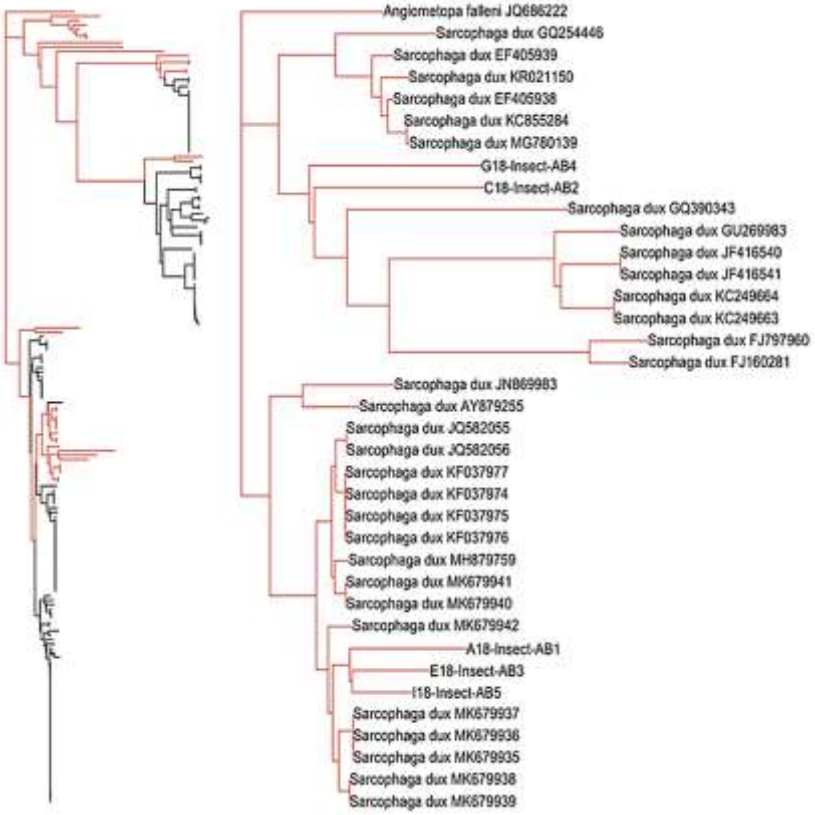

Fig. 3: The new neighbor-joining consensus tree constructed by using mitochondrial COX-1 sequences of 5 collected samples and 32 selected BOLD *Sarcophaga dux* samples of high similarity relation-ship with our samples. Diagram on the left side expose the main NJ tree and the red lines clear the samples which choose for construct the new NJ tree. *Angiometopa falleni* (GenBank accession No. JQ686222) was taken as outgroup. Evolutionary distance divergence scale bar is 0.01.

replications and rooted with *Angiometopa falleni* (GenBank accession No. JQ686222) as out-group. In addition, the NJ and ML analyses based on COX-1 barcode sequences of *S. dux* yielded similar tree topologies.

In NJ phylogenetic tree (Fig. 4), our *S. dux* samples *viz*., A18-Insect-AB1, E18-Insect-AB3, I18-Insect-AB5, C18-Insect-AB2 and G18-Insect-AB2 showed acceptable bootstrap support of 62-100% with four Indian samples (MG780139, FJ797960, FJ160281 and GQ390343), four Chinese samples (GU269983, AY879255, JF416540 and JF416541), one Australian sample (GQ254446), one Malaysian sample (JN869983) and two Egyptian samples (KC249664 and KC249663). But in ML phylogenetic tree (Fig. 5), our *S. dux* samples showed acceptable bootstrap support of 56-100% with the same samples used in NJ analysis. Although three of our samples (A18-Insect-AB1, E18-Insect-AB3 and I18-Insect-AB5) clustered together with Indian sample (MG780139), the other two samples (C18-Insect-AB2 and G18-Insect-AB2) clustered with three Indian samples (FJ797960, FJ160281, and GQ390343), four Chinese samples (GU269983, AY879255, JF416540 and JF416541), one Australian sample (GQ254446), one Malaysian sample (JN869983) and two Egyptian samples (KC249664 and KC249663).

The genetic distances and similarity are very important parameters (Samerjai *et al*. 2020). So, ML tree under Kimura (K80) and NJ tree under Tamura-Nei genetic distance models were used to find the similarity relationship between our collected samples and *S. dux* samples selected from BOLD database. The present results revealed that all the sequences were branched as outgroups in these trees bootstrapped with 100 replications. In NJ and ML phylogenetic trees, our *S. dux* samples showed acceptable bootstrap support of 62-100% with four India samples, four China samples, one Australia sample, one Malaysia sample, and two Egypt samples. To date, there are many DNA barcoding studies related to DNA based identification and phylogenetic relationships of Sarcophagid flies (Meiklejohn *et al.*, 2011; Stamper *et al.*, 2013).

The mitochondrial COX-1 barcode is potential genetic markers that can be used in conjunction with morphology-based tools for accurate species identification and phylogenetic analysis of sarcophagids. Samerjai *et al.* (2020) found that the combined COI-COII genes gave very

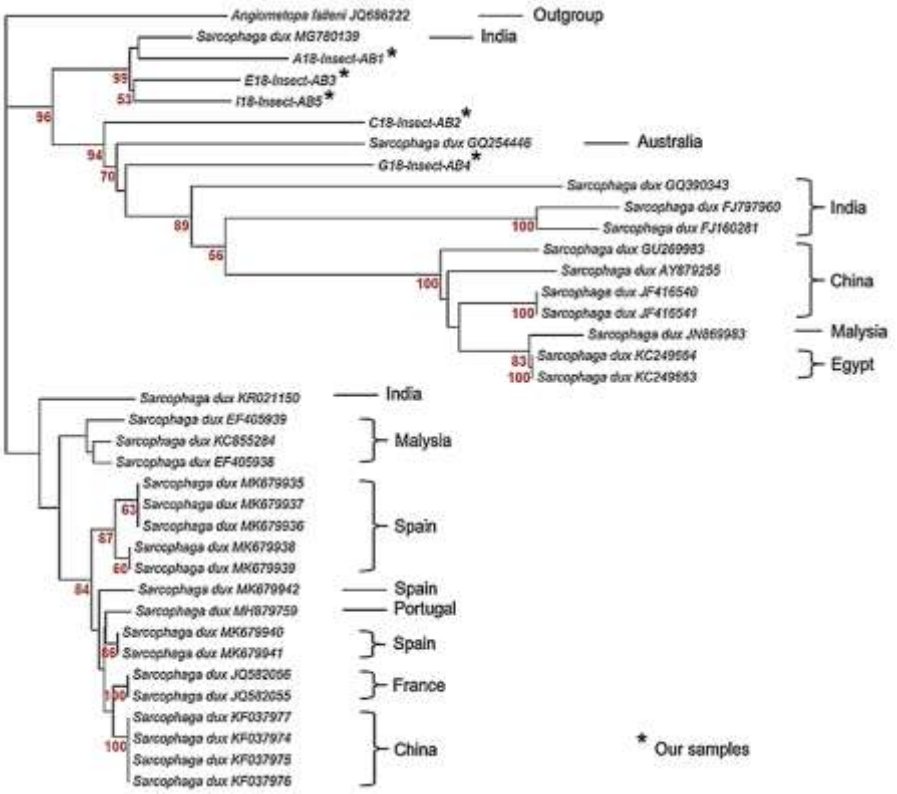

Fig. 4: Neighbor-joining (NJ) tree based on the mitochondrial COX-1 sequences of our five samples and 32 selected BOLD *Sarcophaga dux* samples. The species name, voucher code and accession No. are given in the specimen label. Bootstrap values are shown at each node. *Angiometopa falleni* (GenBank accession No. JQ686222) was taken as outgroup. Evolutionary distance divergence scale bar is 0.01.

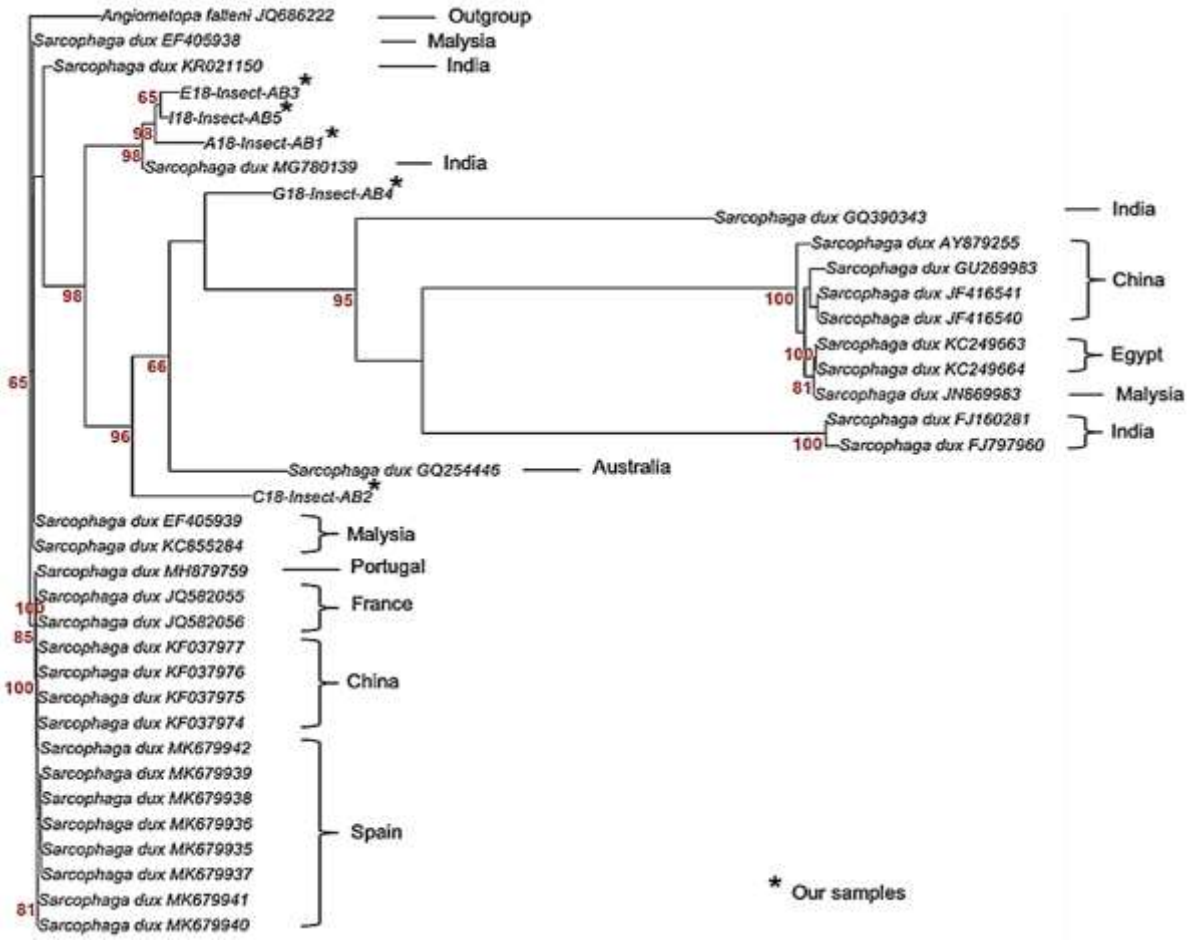

Fig. 5: Maximum likelihood tree (ML) based on the mitochondrial COX-1 sequences of our five samples and 32 selected BOLD *Sarcophaga dux* samples. Species name, voucher code, and accession No. are given in the specimen label. Bootstrap values are shown at each node. *Angiometopa falleni* (GenBank accession No. JQ686222) was taken as out-group. Evolutionary distance divergence scale bar is 0.01.

high percentage of successful species identification. Sharma *et al.* (2015) reported that the achieved COX-1 sequence analysis covering several flesh fly species can serve as a reliable reference database for further forensic entomological research in the areas where these species are present.

Conclusions: The present study revealed that the mitochondrial COX-1 gene sequence analysis is one of the viable tools for identification and phylogenetic analysis of *Sarcophaga* flesh flies. This marker has important role in understanding the evolutionary processes of *Sarcophaga* cryptic species in the western regions of Saudi Arabia. These flesh fly populations of *Sarcophaga dux* might have been introduced into Jeddah (Saudi Arabia) by the pilgrims coming from India and other East Asian countries. Further, these flesh fly species may be introduced with the imported meat and livestock (cattle and buffalo) from Australia.

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