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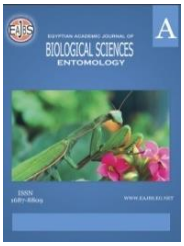

Mitochondrial DNA Identification and Ultrastructure of Spermatozoa of the Forensically Important Blowfly, *Lucilia cuprina* (Diptera: Calliphoridae) in Sharkia Governorate, Egypt.

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ABSTRACT

The blowfly, *Lucilia cuprina* (Diptera: Calliphoridae) is an important medical and forensic insect that is the main cause of ovine cutaneous myiasis. In the field of medical treatment using maggot therapy, this species could help to heal incurable infections. The morphological identification of the adult wing and Scanning Electron Microscope (SEM) of the first instar larvae was carried out and confirmed by DNA identification of the 3rd instar larvae of *L. cuprina*. The results showed a fraction of the mitochondrial cytochrome oxidase I (COI) spanning approximately 269 bp. The topology of the Neighbor-Joining (NJ), Maximum Likelihood (ML) tree indicated that *L. cuprina* species were assigned correctly to the subfamily Luciliinae, family Calliphoridae. The male reproductive system comprises two testes, a pair of vasa differentia and a pair of accessory glands, a vesicula seminalis, and an ejaculatory duct. This inquired species has the common conventional spermatozoal pattern of Calliphoridae, a single-layered acrosome at the apex, a consolidated nucleus, totally crystallized mitochondrial derivatives, and an axoneme with a 9+9+2 microtubular configuration. This investigation revealed for the first time detailed morphological and mitochondrial DNA identification, as well as the morphology of the male reproductive system and ultrastructure of spermatozoa in L. cuprina, collected from Sharkia Governorate, Egypt.

INTRODUCTION

Members of the family Calliphoridae are distributed in substantial ecological diversity all over the world, residing in a variety of environments, ranging from organic debris to degraded animal tissues (David *et al.*, 2008, Fremdt *et al.*, 2012). There are more than 1000 Calliphorid species belonging to various subfamilies such as Calliphorinae, Chrysomyinae, Melanomyiniae, and Luciliinae (Kutty et al. 2008, Vargas and Wood 2010). Because flies in the subfamily Luciliinae are diverse and heterogeneous, their taxonomic classification could be confusing (Vargas and Wood 2010). Flies belong to the family Calliphoridae (blowflies) and are frequently the first insects to appear on a body, where their larvae effectively feed and breed (Anderson and Cervenka 2001, Higley and Haskell 2010, Prado e Castro *et al.*, 2012, Bernhardt *et al.*, 2017). The developmental rates of such flies including *Lucilia* spp are frequently used to estimate the postmortem interval (PMI),

minimum interval since doom, in forensic screening at the first few weeks following doom (Smith 1986, Sandoval-Arias *et al.*, 2020).

L. cuprina is widely distributed in tropical and temperate climates in the Oriental (Kurahashi and Bunchu 2011, Yang *et al.*, 2014); Australian (Wallman 2001); Afrotropical (Lutz et al. 2018); Nearctic (Whitworth 2006 & 2010); Neotropical (Kurahashi and Kirk-Spriggs 2006, Bambaradeniya *et al.*, 2018); and North African (Egypt) regions (Aly 2014). In fact, *L. cuprina* larvae are usually applied in maggot therapy to help wound therapeutic (Sherman 2002, Paul *et al.*, 2009, Rueda *et al.*, 2010, Tantawi *et al.*, 2010, Sun *et al.*, 2014). Many forensically important species are difficult to be accurately distinguished morphologically (Boehme *et al.*, 2012). Consequently, to overcome this difficulty, gene sequence analysis is employed for its identification (Fremdt *et al.*, 2012, Aly 2014). Mitochondrial DNA is preferred in the identification over nuclear DNA because it poses several advantages, such as its easy extraction (Waugh 2007).

COI gene sequences are potent markers for the delicate identification of insect species of different taxa (Aly and Wen 2013, Jordaens et al. 2013, Sandoval-Arias et al. 2020). Numerous researches using DNA-based identification of certain forensically important blowfly samples have been recorded (Sperling *et al.*, 1994, Wells and Sperling 1999 & 2001, Benecke and Wells 2001, Wells *et al.*, 2001, Schroeder *et al.*, 2003, Ames *et al.*, 2006a & b).

Lately, the insects' sperm ultrastructure provides additional tools for taxonomic investigation (Jamieson *et al.*, 1999, Name *et al.*, 2012) and participates in our conception of relationships (Carcupino *et al.*, 1995, Name *et al.*, 2010, 2012). The spermatozoal structure variety in dipteran groups is greater than in other insect taxa. In Calliphoridae, *Chrysomya megacephala* (Name *et al.*, 2010, Sukontason *et al.*, 2011), *Calliphora vomitoria* (Dallai and Afzelius 1990), *L. cuprina, L. peruviana,* and *L. eximia*, (Name et al. 2012) were identified using spermatozoal ultrastructure. The inquired species have a common spermatozoal pattern in brachyceran flies. Such pattern constitutes a single-layered acrosome at the apex, a consolidated nucleus, mitochondrial derivatives that have completely crystallised, and an axoneme with a 9+9+2 microtubular configuration (Jamieson 1987, Jamieson *et al.*, 1999).

The study's objectives were to employ mitochondrial DNA molecular markers to confirm morphological identification, the male reproductive system description including the histological studies of testes, and spermatozoal ultrastructure of *L. cuprina* collected from Shiba village, Sharkia Governorate, Egypt.

MATERIALS AND METHODS

Insects:

L. cuprina larvae were collected from Shiba village, Sharkia Governorate, Egypt and reared at the laboratory conditions (25±2^{o}C, with a 54-73% RH and 14:10 h L: D photoperiod) at the Zoology Department, Zagazig University. Adult flies were maintained in adult rearing cages (30 x 30 x 30 cm). The adults were provided with granulated sugar, water, milk powder and small pieces of raw beef meat (as a protein source to promote oviposition). After the egg-laying on meat, each oviposition cup was placed in a plastic jar (10.5 x 7 cm) containing beef meat to feed larvae surrounded by wheat bran in a 2 L plastic box. The wheat bran served as a pupation substrate. Fifty larvae were retained in each plastic container to prevent competition till pupation. Translocation of pupae to cages for adult emergence was completed. The rearing technique was adapted according to the previously described technique with slight modifications (Khater and Geden 2018, Khater *et al.*, 2021, Khater *et al.*, 2022, Selem *et al.*, 2023).

Morphological Studies:

The third larval instars and adult flies were frozen at -20 °C for 1h (Amendt et al. 2007), then washed or cleaned with 70% ethanol and soaked overnight in a cold 10 % potassium hydroxide solution. Larvae and wings (ex-scissored from adults) were dehydrated in ascending series of alcohol concentrations (60%, 70%, 80%, 90%, 95% (2 times), 100% (2 times), equal volumes of 100% ethanol and xylol), cleared in xylol, then mounted in Canada Balsam. Specimens were examined and photographed with a stereomicroscope. The adult stage was identified using a description key (Szpila 2012). The posterior spiracle was used as the key element for larval identification (Holloway 1991).

Scanning Electron Microscope Technique:

A scanning Electron Microscope (SEM) study was performed on twenty-first instar larvae. The larvae were rinsed with distilled water many times before being fixed for 12 hours in 10% formalin. The larvae were then dehydrated in various grades of alcohol, cleaned in acetone, dried, and glued at various angles on metallic stubs. Larvae were gold-coated and scanned using the Scanning Electron Microscope (SEM) (Jeol/ EO, Version 1.0 (Instrument JSM-5500) at Al- Azhar University's regional center for Mycology and Biotechnology in Egypt.

Molecular Identification of *Lucilia cuprina:*

DNA was extracted from 50 3rd instar larvae by Gene JET Genomic DNA Purification Kit (Thermo Scientific #K0721). The larvae were grinded in liquid nitrogen, suspended in a digestion solution (180µL), 20µL of Proteinase K, and blended thoroughly using a vortex mixer. For 1- 3 hours, the sample was incubated at 56°C till the tissue was totally lysed. RNase Solution (20µL) was added, vortexed, and incubated for 10 minutes at room temperature. Following that, the lysed solution (200µL) was added, well mixed with a vortex for 15 seconds, and then 400µL of 50% ethanol was added. The prepared lysate was purified by Gene JET Genomic DNA Purification Column. The mitochondrial COI was amplified and sequenced using previously designed primers (Wells and Sperling 1999, Silva-Brandao *et al.,* 2005). PCR analysis was performed using Dream Taq Green Master Mix (2X) (Thermo Scientific #K1081). The reactions of PCR were completed in reaction volumes (25µL), containing 12.5µL Dream Taq Green PCR Master Mix (2X), Primer forward (1µL), Primer reverse (1µL), Template DNA (2µL) and nuclease-free water (8.5µL). The primer set was 5′-TACAATTTATCGCCTAAACTTCAGCC-3′, 5′-AGTAAACCAATTGCTAGTATAGC-3′. The products of PCR were visualized using EtBr dye and agarose gel electrophoresis (1.5% in 1X TBE buffer). ABI Prism Big Dye Terminator V 3.1 Sequencing Kit was used to sequence the amplicons. The amplicon sequence was aligned with the database deposited sequence by MEGA X (Kumar et al. 2018), using pairwise distances and the formation of Neighbor-Joining.

Morphology of *L. cuprina* Male Reproductive System:

To describe *L. cuprina* male reproductive system, the newly emerged adult males were fed on powdered milk; granulated sugar and water soaked in a piece of cotton for about seven days. After such a period, ten males were dissected on a dissecting plate provided with a saline solution (9.0 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, and 4.0 g sucrose /1 L). The reproductive system of the male was photographed on a slide using a Canon-Power Shot-G12 camera coupled to an Optika Stereomicroscope (Italy).

Histological Methods :

Testes taken from twenty males were directly immersed in aqueous Bouin's fixative and refrigerated for two hours at 5°C. The testes were embedded in liquid paraffin after being dehydrated in a graded series of ethanol. Hematoxylin and eosin were used to stain histological sections (Bancroft and Gamble 2007).

Spermatozoal Ultrastructure by Transmission Electron Microscope Technique:

The spermatozoa ultrastructure was investigated using a Transmission Electron Microscope (TEM). Seven-day-old males were dissected. They are fixed in 2.5% glutaraldehyde in phosphate buffer for 2-3 hours before being put in 1% osmium tetroxide. Whole testes were gradually dehydrated in an escalating sequence of alcohols and dried in acetone. Spurr's resin was used to embed specimens in a plastic block template, which was then incubated for 24 hours at 70ºC. For light microscopic analysis, semi-thin sections were stained with methylene blue and 1% azure II (1:1). The ultrathin slices were cut and stained with uranyl acetate and lead citrate before being examined and photographed using a JEOL 1200 EXIL. The TEM was performed Regional Center for Mycology and Biotechnology at the Faculty of Pharmacy's Electron Microscope Unit, Al- Azhar University, Egypt.

Data Analysis:

DNA Sequence Alignment:

The reference sequences for the previously reported blowfly were retrieved from GenBank and used for the alignment with our results, namely *L. cuprina*. Sequence alignment was made using BLASTN search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul *et al.*, 1990).

RESULTS

Morphological Identification:

Morphology of adult wings was used for the identification of *L. cuprina*. Wings were the membranous type with clear yellow basicosta and stem vein (Fig. 1A, B). The third instar larva is vermiform (Apodous) with a pointed anterior end with obvious mouth parts, a coarse posterior end, and plainly visible anal tubercles (Fig. 1C). The posterior spiracle of the third instar larva may provide an additional morphological tool in identification. The posterior spiracle is characterized by the presence of three slits (opening) surrounded by a dark, thick peritreme which has a button supporting the spiracle opening (Fig. 1D).

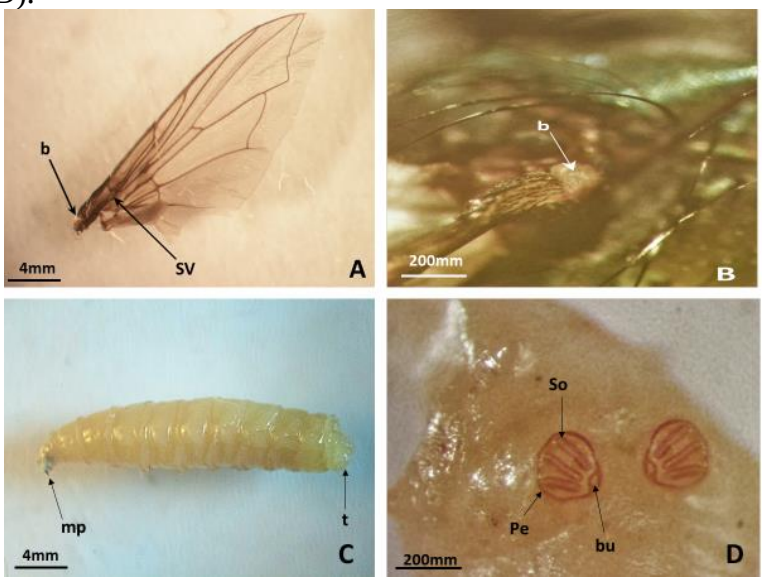

Fig. 1: Stereomicroscope photos of *L. cuprina* showing **(A)** wing with characteristic yellow basicosta (b) and stem vein (sv), **(B)** higher magnification of basicosta (b), **(C)** Third instar larva with anterior mouth parts (mp) and posterior anal tubercles (t), **(D)** posterior spiracle of third larval instar with button (bu) peritrem (pe), spiracle opening (So).

This study indicated through SEM that the first larval stage of *L. cuprina* is a vermiform type and composed of twelve body segments, a cephalic, three thoracic segments, and eight segments comprised of the abdomen (Fig. 2A). There was a ring of spines located between each larval segment (Fig. 2B). The frontal end comprised the cephalic region which is to a certain extent bilobed comprising oral groove, whereas the buccal hooks and oral cristae were not yet grown in this stage. There was a spines ring located between the cephalic zone and the foremost thoracic part (Fig. 2C). The anterior spiracle was not obvious at this stage. The posterior end was coarse, and the anal tubercles were clearly observed. The posteriors spiracle was situated at the topmost of at an elevation at the anal segment (Fig. 2D).

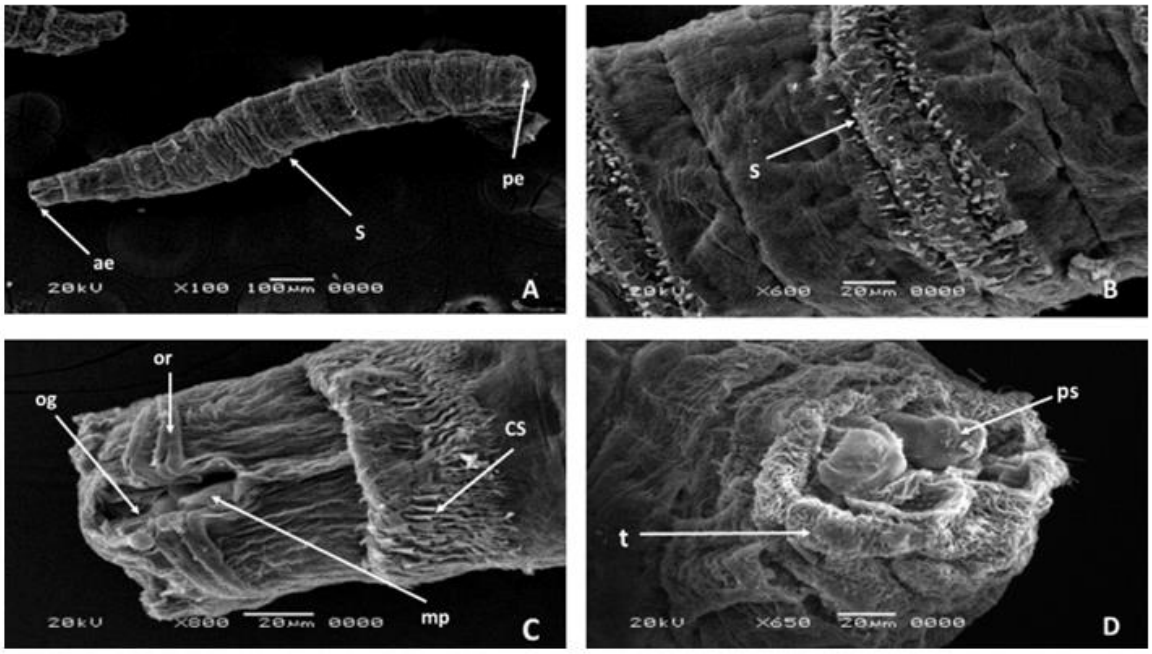

Fig. 2: Scanning electron microscope micrographs of the first larval instar of *L. cuprina* showing, **(A)** whole larval body with spines (s) located between the segments, as well as the anterior ends (ae) and posterior ends (pe). **(B)** Spines (s) between the body segments. **(C)** Ventral view in the cephalic region with oral groove (og), mouthparts (mp), cephalic spines (cs) and oral ridge (or). **(D)** Anal segment with posterior spiracle (ps) and anal tubercles (t).

Molecular Identification of *Lucilia cuprina:*

The 269-base pair (bp) fragment of the COI was amplified and visualized in agarose gel (1.5%) (Fig. 3A). Approximately 269 bp (114 A; 42 C; 35 G; 78 T) of the COI mt DNA gene was obtained for *L. cuprina*. The BLASTN (for DNA) program has been used to search the nucleotide sequence's similarity between the *L. cuprina* and DNA sequences, using GenBank reference data. The amplicon was purified and sequenced; the sequence was BLAST searched in the database without redundancy. From the sequence alignment analysis data, this sample revealed 100% symmetry by a database banked *L. cuprina* isolates. The phylogenetic similarity of the insect by the database banked isolate was conducted by MEGA 7.0. The insect displayed a 100% similarity with *L. cuprina* with accession numbers: MT023110.1, KJ496770.1, KF562103.1, KJ496771.1, MF097448.1, JN014887.1, KJ496769.1, JN604561.1, JX187387.1, and MF097452.1. The topology of the Neighbor-Joining (NJ) and Maximum Likelihood (ML) were provided (Fig. 3B). According to the phylogenetic tree, the *L. cuprina* species was assigned correctly to the subfamily Luciliinae, family Calliphoridae .

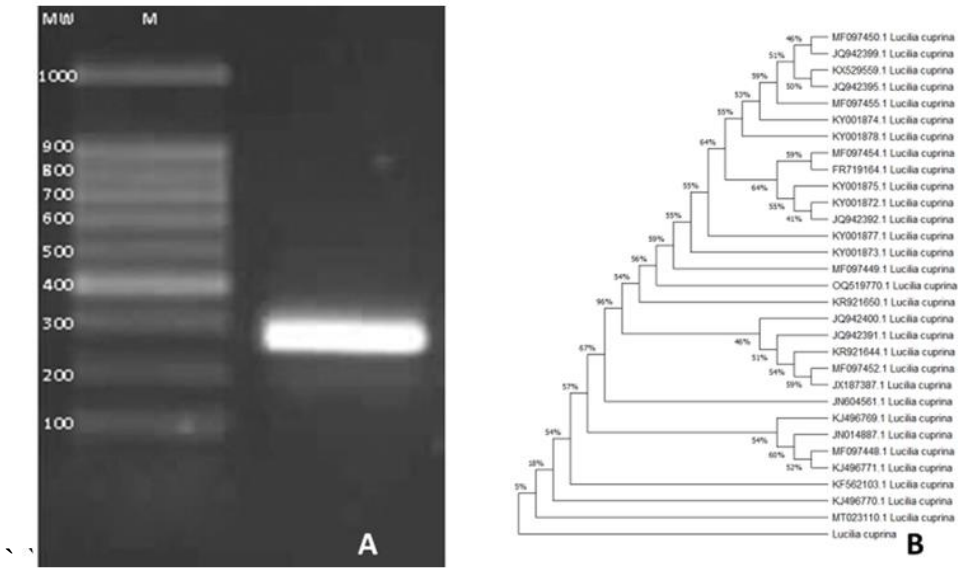

Fig. 3: (A) Agarose gel electrophoresis of primer specificity in the amplification of COI gene in *L. cuprina* (1.5% agarose gel; M, molecular genetic marker). **(B)** Molecular phylogenetic analyses of the ITS sequence of *L. cuprina* by Maximum Likelihood Model of MEGA 7.0 package.

Male Reproductive System of *Lucilia cuprina*:

The testes of a sexually mature male *L. cuprina* produced sperm and stored it in the vesicula seminalis. The male internal reproductive system comprises two testes, a pair of vas deferentia and an accessory gland, with a seminal vesicle, and an ejaculatory duct. A pair of accessory glands was instantly attached to the vesicula seminalis (Fig. 4A). The histological transverse section showed the presence of numerous follicles (Fig. 4B). The germ cells arrangement in the testes showed that such a structure was made up of particularly long follicles with numerous cysts. (Fig. 4C, D).

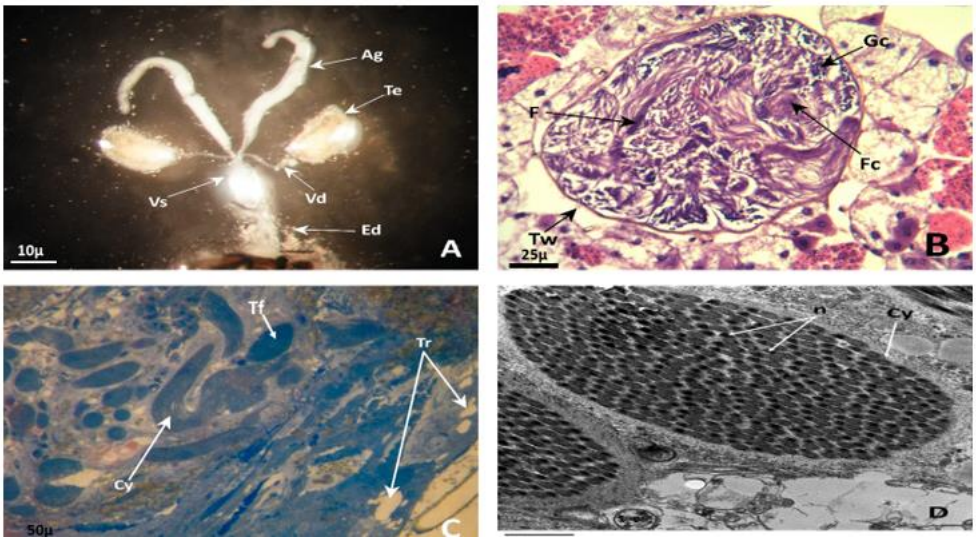

Fig. 4: Photomicrographs for male of *L. cuprina*, **(A)** stereoscopic photo of the reproductive system showing testes (Te), vas deferens (Vd), vesicula seminalis (Vs), accessory gland (Ag) and ejaculatory duct (Ed). **(B)** Normal histology of testes showing testicular wall (Tw), germinal cells (GC), follicles (F) and follicle cells (Fc). **(C)** Semithin section of testis showing testicular follicles (Tf) with cyst (cy) and trachea (Tr). **(D)** TEM section of cyst showing spermatozoa head region with nucleus (n) and cyst (cy).

The testicle is composed of an exterior wall surrounding the germ cells. A peritoneal sheath, a muscle layer, a basement membrane, and follicular epithelium make up the testicular wall. The peritoneal sheath cytoplasm is densely packed with pigmented spherical grains, giving the organ its distinctive dark yellow color. While examining via a TEM, such grains showed different sizes and electron-dense appearance. Many tracheoles were observed in the peritoneal cells. Beneath the peritoneal sheath, a characteristic muscle layer that enables the testis to fulfill its peristaltic movements was detected as an uninterrupted layer of circular muscles. Below such a muscle layer, there was a fibrous and laminated basement membrane (Fig. 5A). The germinal cells undergo mitotic divisions to form spermatogonia which become the future spermatozoa (Fig. 5B).

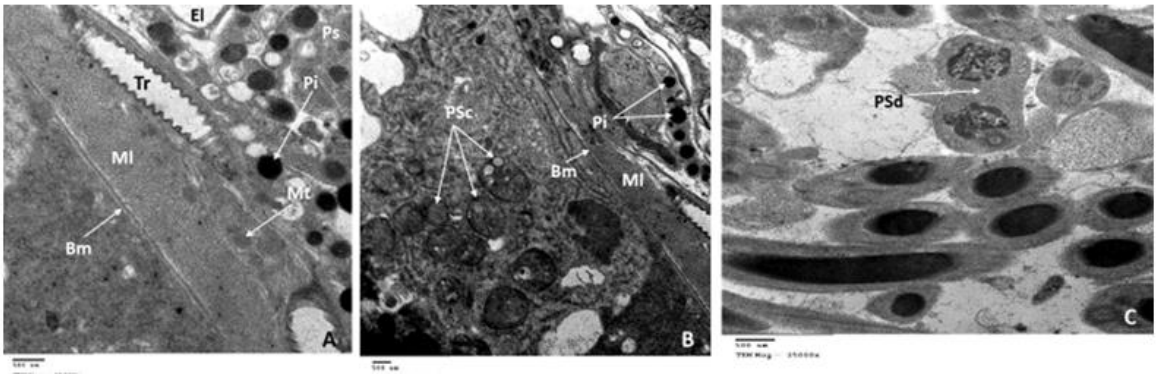

Fig. 5: (A) Wall of testes of *L. cuprina* showing, external layer (El); muscular layer (Ml); mitochondria (Mt); peritoneal sheath (Ps); pigments (Pi); basement membrane (Bm); trachea (Tr). **(B)** Primary spermatocytes (PSc). **(C)** Primary spermatocytes undergo mitotic division to form spermatozoa cells, primary spermatocyte division (PSd).

Spermatozoal Ultrastructure of *Lucilia cuprina:*

L. cuprina spermatid differentiation takes place within cysts. The spermatic cells inside each cyst are exactly aligned and at the same stage of development. The cross-section of the nucleus varied from round to oval in shape. During spermatogensis in *L. cuprina*, the nucleus was totally condensed, with many microtubules developing around it, and the accessory membranes in the cells' cytoplasm were barely visible (Fig. 6A). Cross sections demonstrated the presence of an accessory membrane next to the nuclear membrane from the early phases of nuclear condensation until the cell reached full maturity. The presence of microtubules characterizes the second region; however, they are not organized in a centriole or an axoneme. (Fig. 6B). In the third region, the axoneme, nucleus, centriolar adjunct and mitochondrial derivatives, were clearly distinguished. The centriolar adjunct is clarified as being a supportive structure, strategically located among the other organelles in cross-section (Fig. 6C). The nucleus was of reduced size in the 4^{th} region (Fig. 6D). The region of the tail is obvious by the nucleus's termination, the enlargement of the centriolar adjunct, the existence of the mitochondrial derivatives and the axoneme. The 5^{th} region was distinguished by the presence of a centriolar adjunct surrounded by mitochondrial derivatives and the axoneme was located on the dorsal side (Fig. 6E). The next regions were marked by the evanescence of the centriolar adjunct, one of the mitochondrial derivatives evanesces, and an axoneme made up of 9+9+2 microtubules (Fig. 6F).

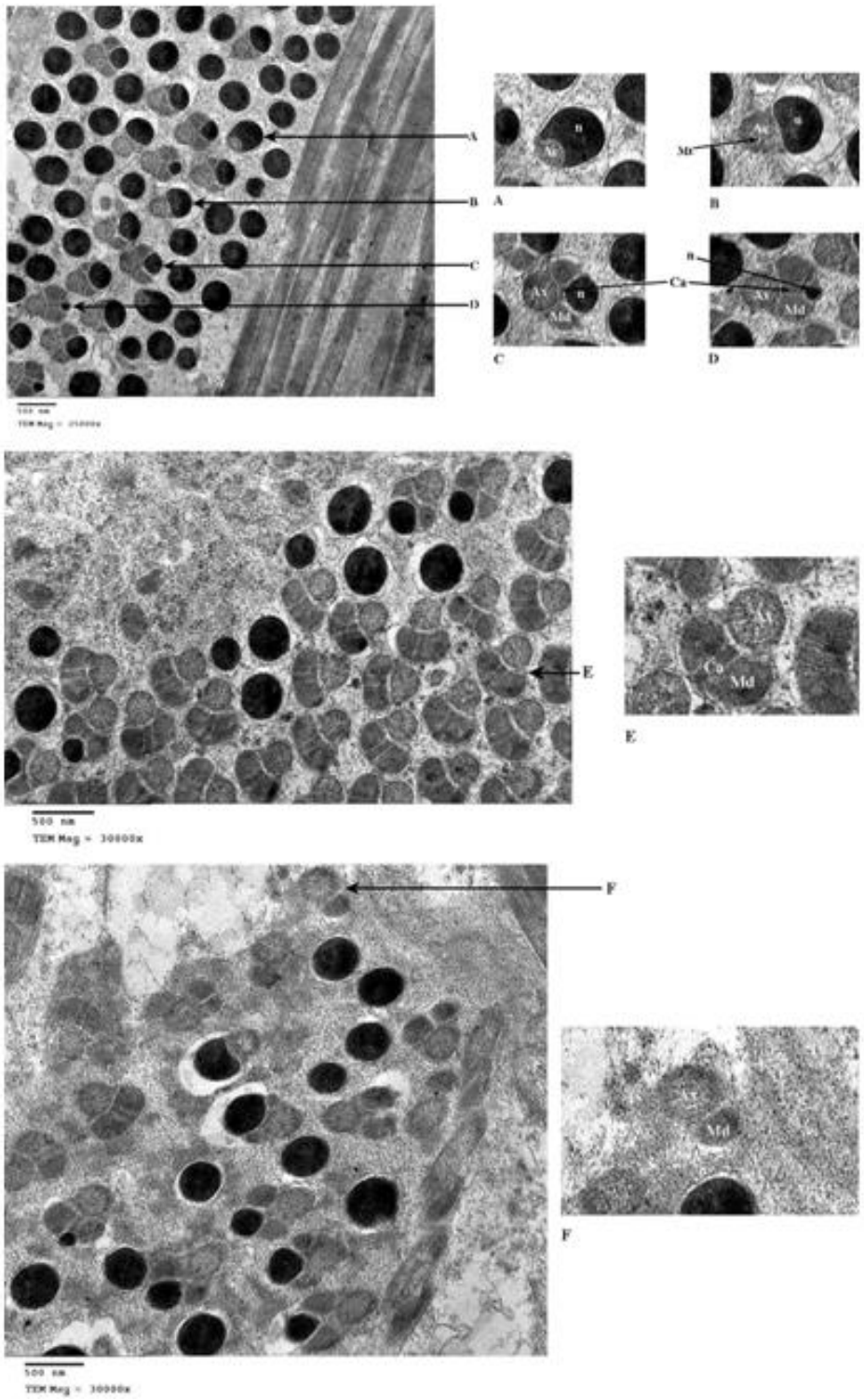

Fig. 6: (A) Photomicrographs for transverse sections of spermatozoa regions of *L. cuprina*, (A) 1st region, in cross-sections, showing the proximal region of acrosome (Ac), the surface of this organelle is in contact with the nucleus (n). **(B)** 2nd region shows the central pair of microtubules (Mt), nucleus (n) and acrosome (Ac). **(C)** 3rd region shows the complete axoneme (Ax), the two mitochondrial derivatives (Md), the nucleus (n) and the emergence of the centriolar adjunct (Ca). **(D)** 4th region, the nucleus is reduced in size. **(E)** 5th region, the nucleus disappeared, Centriolar adjunct (Ca) surrounded by mitochondrial derivatives (Md), and axoneme (Ax) located on the dorsal side. **(F)** One of the mitochondrial derivatives (Md) diminishes and the other follows the axoneme (Ax).

DISCUSSION

Lucilia cuprina has medical and forensic importance leading to ovine cutaneous myiasis. This study used mitochondrial DNA molecular markers for accurate identification and described the male reproductive system including the histological studies of testes, and spermatozoal ultrastructure of *L. cuprina* collected from Shiba village, Sharkia Governorate, Egypt.

The posterior spiracle of third larval instars provides a morphological tool in the identification of blowfly species. In the species under investigation a pair of posterior spiracles characterized by the presence of a well-developed rounded plate, peritrem, surrounded the spiracles and also the button, three straight converging openings, peritrema supported the spiracle opening and somewhat sclerotized. This finding was in accordance with Mendonça *et al.* (2014).

Similar to our findings, many authors identified the maggot with the aid of a light microscope (Oliveira *et al.,* 2007, Mendonça *et al.,* 2014), but some difficulties in raising such flies to adult stages for using the available taxonomic keys. Thus, using SEM could provide an investigative tool to aid the entomologists to recognize dipteran flies (Liu and Greenberg 1989, Mendonça *et al.,* 2010 & 2012a & b & 2013). SEM of the *L. cuprina* first instar larvae was illustrated in this study. Some morphological features are important in the identification of different dipteran genera (Greenberg and Singh 1995). Many authors described some characteristics of ultra-morphology of the same species. The buccal hooks in the mouth parts of the first larval instar are extremely short while such structures are greatly sophisticated on both the second and third instar (Sandeman *et al.,* 1987). The spines of the seventh abdominal segment were used to identify *L. cuprina* (Szpila et al. 2013). The ramifications number of spiracles is one of the greatest remarkable characteristics to differentiate between genera of Diptera and species (Costa et al. 2006). Light microscope tools reveal the existence of four to seven specular openings only at *L. cuprina* from Thailand (Sukontason *et al.,* 2010).

Alike our study, several studies relied on DNA-based identification of some forensically significant blowflies and flesh flies have been identified (Sperling et al. 1994, Zehner *et al.,* 2004, Ames *et al.,* 2006b, Rajagopal et al. 2012, Sandoval-Arias et al. 2020). Such a molecular tool could deal with various issues related to morphological concerns if it is essential to classify small fragments of insect materials or immature stages (Preativatanyou *et al.,* 2010). Mitochondrial DNA might be more easily extracted from tiny or degraded specimens than nuclear DNA (Waugh 2007). According to our knowledge, the present investigation was the first to describe the *L. cuprina* mitochondrial cytochrome oxidase I sequence collected from Shiba village, Sharkia Governorate, Egypt. The result of this investigation revealed that sequence data of 269 bp of COI genes had the potential to identify *L. cuprina*. Although the COI sequence used was somewhat short, it was precise enough to distinguish the targeted blowfly specimens. A short mitochondrial cytochrome oxidase I sequence was analyzed to distinguish among forensically crucial flies in Australia (Harvey *et al.,* 2003). The molecular analysis was in conformity with the conventional morphology-based classification demonstrating its utility. The mitochondrial genome has been widely employed for species identification, and COI was discovered to be descriptive for Chrysomyiinae identification. The Mitochondrial cytochrome oxidase I was found to be descriptive for Chrysomyiinae identification (Wells and Williams 2007). After evaluating a variety of gene areas, the COI 'barcode' region was discovered to be the most reliable or differentiating the Australian Chrysomya (Diptera: Calliphoridae) (Nelson *et al.,* 2007). The COI region was sufficient to differentiate between *Calliphora vicina* and *Calliphora vomitoria* in the UK (Ames *et al.,* 2006a). The current findings are comparable to those of

a previous study, which discovered that the 304 bp fragment of the COI gene might be used as a valuable Chrysomyiinae species identification tool for forensic entomology in Belgium-France (Desmyter and Gosselin, 2009). COI has been perfectly used in the accurate identification of animals and many blowfly species (Hebert et al. 2003 and Nelson *et al.*, 2007, 2012). The COI gene has been shown to be the main filter gene for the identification of forensically important flies (Aly 2014). The mitochondrial COI gene was used to identify the larvae and adult stages of *Calliphora*, *Chrysomya* and *Lucilia* spp. from various Lebanese places (Shayya *et al.*, 2018). Zaher (2019) elicited 735 bp. of the mitochondrial cytochrome oxidase I gene which was a valuable identification tool for *Chrysomya marginalis*. The obtained sequences from cytochrome oxidase I confirm the morphological identification of species of forensic insects, *L. cuprina* and *L. eximia* in Costa Rica (Sandoval-Arias *et al.*, 2020). COI barcodes obtained are robust enough to identify and distinguish between the cluster flies, *Pollenia rudis* and *P. vagabunda*, without ambiguity (Taleb *et al.*, 2022).

On the contrary, based on COI sequencing data, it was difficult to distinguish between *Chrysomya augur* and *C. dubia* because the two species are closely related. As a result, more sequencing is necessary to separate them (Wallman and Donnellan 2001).

In the current investigation, genetic analysis was used as a supplementary technique for the accurate identification of the investigated blowfly species, *L. cuprina*. The DNA database availability will help forensic cases by permitting the identification of immature phases (Tan *et al.*, 2009). These findings could be useful in increasing the identification quality when analyzing damaged samples or conserved larvae.

The traditional identification technique is fast and simple and is conducted in the laboratory, whereas identification of a complex species is more confounded and demands an expert taxonomist to confirm the accurate identification. DNA barcodes help to expand reference databases and allow for quick and precise identification. Contribution of DNA barcodes to the reference databases development and enable accurate and fast identification (Taleb *et al.*, 2022).

According to our knowledge, few studies have worked on the male reproductive system morphology and histology of *L. cuprina* (Name *et al.*, 2012). In Egypt, this work is the first attempt to study the male reproductive system morphology and histology of *L. cuprina*, collected from Shiba village, Sharkia Governorate. The organs of the male reproductive system in the family Calliphoridae comprised two testes, a pair of vasa differentia and a pair of accessory glands, a vesicula seminalis, and an ejaculatory duct resembling this structure noticed in Diptera species (Joly *et al.*, 2003, Name *et al.*, 2012). In Diptera, the male reproductive organs are unwell known, by few detailed comparative findings (Sinclair *et al.*, 2007). In many examined dipteran species, the testis is a sac-like structure similar to tubular follicles seen in testes of some different insect orders (Williamson 1989; Valdez 2001).

The ultrastructure of the sperm of *L. cuprina* collected from Sharkia Governorate was studied. *L. cuprina* spermatozoa structure to some extent resembled that described for *C. megacephala* (Name *et al.*, 2010), *L. peruviana, L. cuprina*, and *L. eximia* (Name et al. 2012). In the spermatozoa of the tested species, the nuclear chromatin is strongly condensed, like that of *C. megacephala* (Name et al. 2010) also, in the species of other brachyceran. (Jamieson 1987, Jamieson *et al.*, 1999). Spermatozoa are derived from germ cells at the apical end of the testes, and these stem cells undergo a synchronous mitotic phase without cellular division to produce spermatocytes. The growth of germinative cells occurs within spermatogonial cysts in *L. cuprina*, as it does in most insects (Phillips 1970). The number of spermatozoa per bundle was found to be varying in most insects. The spermatids number per bundle differs among different species of Diptera, reliant on the

spermatogonial premeiotic division number (Oguma *et al.*, 1987, Quagio-Grassiotto and Lello 1996, Cruz-Landim, 2001).

The structure of *L. cuprina* spermatozoa is comparable to the general description of insect sperms (Jamieson 1987, Jamieson *et al.*, 1999). All brachyceran spermatozoa share the monolayered acrosome state observed in spermatozoa of the investigated species (Name *et al.,* 2010). In dipteran species, the acrosome has many shapes; in some families, it is small and devoid of perforatorium and extra acrosomal layers, whereas, in others, it is an extended organelle that is partially lateral to the nucleus and contains an internal crystalline fibre. (Dallai *et al.,* 1984). The acrosome possessed the latter appearance in the investigated species but the crystalline fibre was not found. While this structure is elliptical in cross-sections, it becomes increasingly circular as the section approaches the tip. *Ceratitis capitata* (Báo *et al.,* 1989) and *Sarcophaga bullata* (Warner 1971) both have striated filaments or crystalline fibres in the acrosome. The nucleus' chromatin is extremely condensed in L. *cuprina*, as it is in other Brachyceran flies (Jamieson 1987; Jamieson *et al.*, 1999).

Two accessory membranes are hardly noticed in cross-sections in tested species of *L. cuprina* but are found in *Coelopa frigida* spermatids (Diptera: Coelopidae) and named ‘scroll-like structure’. The function of this structure is obscure (Schrankel and Schwalm 1974). The accessory membranes were described in *C. megacephala* (Name *et al.,* 2010), *L. peruviana*, *L. cuprina*, and *L. eximia* (Name *et al.,* 2012.)

Phillips (1970) describes the spermatozoa centriole as an organelle with nine microtubular triplets. The typical structure is reported by Phillips (1970) for *Drosophila melanogaster* (Dallai and Afzelius 1991), *C. capitata* (Báo and Dolder 1991), *C. megacephala* (Name *et al.,* 2010), *L. peruviana*, *L. cuprina*, and *L. eximia* (Name *et al.*, 2012) and was described in this study for *L. cuprina* in Sharkia Governorate, Egypt. On the other hand, in some insect species, this structure displays great differences. In *Dacus oleae* (Tephritidae), as in other Diptera species, an unusual configuration was noticed for this structure as the occurrence of two central microtubules in the centriole region (Dallai and Afzelius 1991). In almost all insect species, the nucleus is joined to the flagellum with a centriolar adjunct which appears as a very electron-dense structure (Jamieson *et al.,* 1999). The mitochondrial derivatives in *L. cuprina* were equal in diameter, but unequal in length; meanwhile in *Musca domestica*, such organelles are asymmetric and show variance in length, and such derivatives are entirely filled with paracrystalline material in both species (Gassner 1970, Gassner and Klemetson 1981). In two mitochondrial derivatives, one bigger than the other, near the axoneme was present primarily in *Drosophila willistoni* (Rego *et al.,* 2016).

The sperm axoneme in species of Brachycera is comparatively uniform, whilst there is a great diversity in the case of Nematocera (Dallai *et al.*, 1993). Like most Brachycera, *L. cuprina* has an axoneme with 9+9+2 microtubules configuration (Dallai *et al.*, 1993, Jamieson *et al.,* 1999), and actually, such type is similar to other insects (Dallai and Afzelius 1990). The similarity of spermatozoa ultrastructure in species under investigation to that of other brachyceran members confirmed the phylogenetic value of sperm structure.

Conclusion

We believed that the investigation revealed for the first time detailed morphological and mitochondrial DNA identification of *L. cuprina* as well as morphology of male reproductive system structure and ultrastructure of spermatozoa of this species collected from Shiba village, Sharkia Governorate, Egypt.

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