Reliability of egg rafts electron micrographs for confirming the taxonomic status of *Culex pipiens* mosquitoes collected from Al-Ahsa, eastern Saudi Arabia

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Abstract. Mosquitoes are undesirable arthropods transmitting many diseases not only in Saudi Arabia but also worldwide. Identifying mosquito species relied for long time on both larval and adult characters whilst little or no attention was given to eggs. Electron microscopic studies of mosquito eggs are important as it is not only characterizing the external morphology of the eggs never seen by stereoscopic microscopes but also facilitates mosquito species identification. Accordingly, morphology and morphometric of *Culex pipiens* eggs collected from Al-Ahsa oasis, eastern Saudi Arabia were examined by scanning electron microscopy (SEM) for the first time in Saudi Arabia in the present work. Mosquito egg rafts were collected from breeding sites in Al-Ahsa by using of special long aquatic net. A portion of the rafts was reared for identification whilst the other portion was preserved in glutaraldehyde and prepared for SEM examination. Eggs appeared to be conical in shape with two ends, the anterior one that is represented with the micropyle is more tapered than the posterior end. The morphometrics gave many characteristics for the eggs such as length, width, proportion of length/width and so on. Eggs morphology and morphometrics were then compared to that of other *Culex* eggs. Our findings using SEM of the eggshell confirmed that the present mosquito species is *Cx. pipiens*. Scanning electron micrographs of any mosquito species eggs are valuable in correlating its fine structure that cannot be easily seen by light microscope and can assist in species separation. Thus, identifying medically important mosquito species is crucial in both mosquito and disease control.

INTRODUCTION

Mosquitoes are the most famous vectors of many diseases such as dengue (Khan et al., 2008), malaria (Abdoon and Alsharani, 2003) and Rift valley fever (Al-Hazmi et al., 2003). The previous diseases are the most prevalent diseases harbored by mosquitoes in Saudi Arabia whilst filaria is uncommon in Saudi Arabia, three cases have been reported during a period of 20 years (1981-2001) from the areas adjoining the Red Sea, in particularly the South-Western region (Haleem et al., 2002). Identifying species of such medical importance is crucial in both disease and mosquito control. Mosquito surveys in Saudi Arabia pointed out the presence of 26 mosquito species (Shaalan et al., 2017). Results of such surveys are sometimes contradictory as well as the recording of the species complex in particular *Cx. pipiens* and *Cx. univittatus* mosquitoes among Saudi Arabia mosquitoes (Harbach, 1985; Al-Khreji, 2005). *Culex pipiens* (including two forms *pipiens* and *molestus*) and *Cx. quinquefasciatus* are the most prevalent mosquitoes in temperate and tropical regions respectively (Dehghan et al., 2011; Shaikevich et al., 2016).
The contradictory findings of some mosquito surveys in Saudi Arabia alerting for utilizing other reliable techniques such as electron microscopy for accurate mosquito life stages identification in particular egg stage. It could be said that one advantage of utilizing scanning electron microscopes over more recent and accurate DNA based methods in insect identification and classification is that it provides researchers with information on some obscure structures that definitely neither seen by light microscopes nor isolated by DNA extraction method. Literatures revealed that such structures are helpful in mosquitos’ eggs identification. Linley (1989a&b), Linley and Clark (1989) and Linley and Craig (1994) mentioned the morphology, development and physiology of some aedine mosquito eggs. Significant morphological differences among four Ochlerotatus mosquitoes (Oc. albifasciatus, Oc. fluviatilis, Oc. scapularis and Oc. taeniorhynchus) were found when their morphological characters were analyzed by Scanning Electron Microscope (Santos, 2013). Both morphology and morphometric variations of Anopheles fluviatilis (Sehrawat, 2014), An. quadrimaculatus (Linley et al., 1993), An. nuneztovari (Linley et al., 1996), An. gambiae complex (Lounibos et al., 1999) have been mentioned. Recently, Mello et al. (2014&2017a&b) have studied morphology of eggs of some Coquillettidia and Psorophora mosquitoes respectively. Similarly, Linley and Chadee (1991) and Alencar et al. (2003) have mentioned the structure of the Haemagogus mosquito. Eggs structure of Cx. pipiens have been studied by Chadee and Haeger (1986) and Sahlen (1990) whilst egg morphometrics are used to differentiate Cx. quinquefasciatus from Cx. tritaeniorhynchus (Suman et al., 2008).

Mello et al. (2017a&b) have mentioned and summarized the benefits of scanning electron microscopic (SEM) investigations of mosquito’s eggs. Where it has facilitated more detailed descriptions of mosquito egg morphology compared to light microscopes (LM) and correlating these fine and obscure structures with mosquito species discrimination. Consequently, it will facilitate the direct identification of mosquito species rather than depending on laboratory rearing to more identifiable stages which is usually time consuming, costly, and risky particularly if the mosquito is a disease vector. Furthermore, egg characters produced by SEM can also be used for phylogenetic analyses and as a tool for the characterization of possible species complexes and for comparison between species (Pacheo et al., 2012; Sarmento et al., 2014). For instance, Soliman et al. (2014) distinguished the two forms of the Egyptian Aedes (Ochlerotatus) caspius species complex by their eggs ultrastructure. Eggs of Ae. aegypti and Ae. albopictus were differentiated by using morphological measurements produced by the scanning electron microscopy (SEM) (Suman et al., 2011). Sallum and Flores (2004) mentioned that ultrastructure of eggs of two morphologically similar species, An. costai and An. mediopunctatus, are distinct and reliable compared to similarity in adult, larval and pupal stages. Similarly, Cx. tritaeniorhynchus summorosus has been confirmed as separate species by Airi and Kaur (2015). Linley and Chadee (1990) used SEM of Psorophora ferox eggs to differentiate some populations. The ultrastructure of Ps. ferox populations from Florida (USA) and Trinidad were found clearly different in both the number and shape of the external chorionic tubercles in each chorionic cell. Likely, Mello et al. (2017b) compared eggs from different Ps. ferox populations. The Brazilian population was different from both Florida (USA) and Arena (Trinidad) populations. Populations differed considerably in tubercles morphology, external chorionic reticulum, micropylar collar, and micropyle. Likely, Suman et al. (2009) suggested that ecological variation may have influenced morphometrics of the egg of four strains of Cx. quinquefasciatus from different geographical areas of India. This phenomenon is also observed in eggs of Anopheles mosquitoes’ whereas Almeida et al. (2014) have concluded that eggs of An. darlingi were polymorphic and that some morphological patterns were regional.
A recent study has revealed that egg morphometrics are as reliable as PCR assays in differentiating sibling mosquito species. Tyagi et al. (2016) provided the first evidence on the efficacy of morphometrics produced by EM in identifying sibling species of the malaria vector *An. culicifacies* in addition to PCR assays. Results also implied the dissimilarity in eggs morphology of sibling mosquito species and possibility of separation by using electron microscopy.

Hence and based on the aforementioned information, the present study was designated to employ the scanning electron microscopic (SEM) studies of mosquito eggs in identifying and confirming status of *Culex pipiens* mosquitoes prevail in Al-Ahsaa, eastern Saudi Arabia.

**MATERIALS AND METHODS**

**Mosquito eggs samples**

Al-Ahsa is the largest oasis in the world with its 2.5 million date palms and located about 60 km inland from the coast of the arabian gulf, in the eastern Saudi Arabia. It has a dry, tropical climate, with long very hot summer (five-month) and a relatively cold winter. It has many springs and copious reserves of underground water which allowed the development of such large number of date palms.

Mosquito eggs were collected from breeding sites around Al-Ahsa with special aquatic net. Eggs were kept in the water from the breeding sites inside plastic jars and transformed to the laboratory then divided into two portions. First one left in the water from the breeding sites in the plastic jars and kept inside the insectary for hatching and rearing to 4th instar larval stage. Hatched larvae were identified by using keys of Harbach (1985) and AL-Ahmad et al. (2011). The second portion was preserved in gluteraldehyde for scanning electron microscopic examinations (SEM).

**Scanning Electron Microscopic Studies**

For SEM studies, the egg rafts were prepared as the method descibed by Suman et al. (2009) and Mello et al. (2017a&b) but with slight modification. Eggs were fixed in 2.5% glutaraldehyde then in 1% osmium tetroxide, both with 0.1 M sodium cacodylate buffer at 7.2 pH. Eggs were washed in buffer then dehydrated in an increasing ethanol series, and dried using super-dry CO2 in a Balzers device. Finally, eggs were mounted on metal supports, coated with gold, and examined using a JEOL JSM/6390LV scanning electron microscope (JEOL, Ltd., Akishima, Tokyo, Japan) at 100-6,500× magnification. Measurements were made directly on the images obtained. The measured attributes included egg length and width, micropyle diameter, corolla diameter, micropyle disc diameter, micropyle mound diameter and tubercle diameter. Both description and terminology of egg morphology in the present work were adopted from Harbach and Knight (1978).

**RESULTS**

Eggs are black in color, elongate and conical in shape (Cr) with tapered posterior end (P) exposed to the atmosphere and round anterior end (A) in direct contact with the water surface (Fig. 1). The chorion consists of two very distinct layers, thicker inner one always referred to as the endochorion (EN) and outer layer always referred to as the exochorion (EC) (Fig. 2A).

The posterior end of the egg is devoid of the exochorion and hosts a slightly circular depression the micropyle (M) (Fig. 2A) that could be either empty or filled with either water droplets or lipid material according to Hinton (1968). The micropyle is surrounded by a collar (MC) followed by exochorionic tubercles (T) with diverse shapes (rectangular, pentagonal, hexagonal and octagonal) and assist in the adhesion of the eggs together within the egg raft. These tubercles are supported by chorionic bridges (Cb) (Fig. 2B). The majority of the exochorionic cells have ornamentation with an octagonal or hexagonal appearance, but sometimes this was pentagonal or rectangular (Fig. 2B).

The micropylar apparatus is located at the posterior end of the egg. It is consisting of a continuous micropylar collar or corolla...
Figure 1. Micrograph showing external morphology of egg raft of *Culex pipiens*. Anterior end of entire egg (A), posterior end (P), conical structure (Cr).

Figure 2. Micrograph showing micropyle (M), located in the posterior end of the egg. A) Showing empty micropyle (M) and filled micropyle (M**) with drop of water. The exo-, and endochorionic membranes (EC, EN). B) Showing micropyle (M), collar (Mc), exochorionic network bridges (head arrows, Cb) and flattened tubercles with variable angles (4-8).

(Mc) with an irregular surface (Fig. 3). In the center of the micropyle a micropylar disc (Md) is found (Fig. 3A) and possess a 3 lobed micropylar mound (Mmd) in its center (Fig. 3B). Tubercles of various sizes (small, medium, and large) are radially arranged in longitudinal rows around the micropylar apparatus (Fig. 3A&B) and directed from the micropylar disc downwards.

The exochorion is a thin porous layer with small and large, rounded or polygonal pores situated around the outside of the tubercles and connecting them forming a mesh or web like layer, but more often sheet and occupied with two distinct layers outer and inner and in between them there were an empty space (Fig. 3B). Tubercles size is seen decreasing in a descending order (Fig. 4). Tubercles of
Figure 3. Micrograph showing the posterior end of the egg. A) Showing the micropylar apparatus of the posterior end; micropyle mound (Mmd), micropylar disk (Md), exochorionic sheet (EC) collar (C) and tubercles (T). Non porous between tubercles rows (black arrows) and porous between wheel like tubercles (white arrows). B) Lateral view showing trilobite micropyle mound (black head arrow) and large, medium and small sizes of chorionic tubercles (Tl, Tm, Ts respectively); ornamentation of the outer chorionic reticulum (OCR) showing the outer and inner layers of the exochorion (OEC, IEC) and endochorionic sheet (EN); arrangement of pores of the reticulum either in a line (LP) or in a wheel (WP).

Figure 4. Showing the descending order of tubercles sizes (arrow).

the conical-shaped region are almost structurally differing than the tubercles of micropylar region, which is polygonal in anterior region (Fig. 5) whereas completely different in the middle and the posterior region. In the dorsomedian region, tubercles supported with chorionic network with different wheel pore sizes as large-, medium- or small-sized tubercles wheels are present (Fig. 5).

Additionally, comparing measurements of the present eggs shown in Table 1 to those of other Culex eggs such Cx. pipiens, Cx. quinquefasciatus and Cx. tritaeniorhynchus summerosus, it reveals that present eggs belongs to the former mosquito species. This is coincidence with the identification of larvae that hatched from the second portion of the eggs that collected from the same breeding place and reared inside the insectary to the larval stage.
Table 1. Attributes of the present *Culex* species eggs

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Mean ± SE</th>
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<tr>
<td>Egg length</td>
<td>341.27 ± 9.7</td>
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<tr>
<td>Egg width</td>
<td>122.86 ± 0.63</td>
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<tr>
<td>Ratio (egg length/width)</td>
<td>2.78 ± 0.12</td>
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<tr>
<td>Micropylar diameter</td>
<td>7.77 ± 1.66</td>
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<tr>
<td>Corolla Diameter</td>
<td>29 ± 0.1</td>
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<tr>
<td>Micropylar Disc diameter</td>
<td>21.21 ± 0.03</td>
</tr>
<tr>
<td>Micropylar mound diameter</td>
<td>13.93 ± 1.22</td>
</tr>
<tr>
<td>Micropylar Tl diameter</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>Micropylar Tl height</td>
<td>1.51 ± 0.02</td>
</tr>
<tr>
<td>Micropylar Tm diameter</td>
<td>1.65 ± 0.05</td>
</tr>
<tr>
<td>Micropylar Tm height</td>
<td>1.05 ± 0.04</td>
</tr>
<tr>
<td>Micropylar Ts diameter</td>
<td>1 ± 0.01</td>
</tr>
<tr>
<td>Micropylar Ts height</td>
<td>0.80 ± 0.09</td>
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</table>

**DISCUSSION**

Several SEM studies investigating morphology of mosquito eggs surface have shown significant influence in mosquito species identification (Linley, 1989b; Lounibos et al., 1999; Choochote et al., 2001; Alencar et al., 2003 & 2005; Suman et al., 2008; Santos, 2013). This is due to SEM complements traditional classification because invisible structures by light microscopes could be important for morphological characterization of mosquito eggs. Such structures are formed by desiccation on the egg surface and are characteristic for each mosquito species. Exochorion ornamentation of mosquito eggs is an excellent and reliable character for making comparisons and differentiating species among mosquitoes (Alencar et al., 2003 & 2005; Sehrawat, 2014; Soumare & Ndiaye, 2005). It has been suggested that the function of these ornamentations are adaptive structures that covering and protecting the embryo, preventing it from desiccation and regulating embryonic gaseous exchange (Soumare and Ndiaye, 2005).

The discrimination of Culicidae species relies on the exochorion ornamentation that shows significant differences (Alencar et al., 2003 & 2005; Suman et al., 2008; Santos-Mallet et al., 2009 & 2010) whilst the micropylar apparatus was the most characteristic feature for species confirmation in *Anopheles* (Lounibos et al., 1999; Rodriguez et al., 2002) and *Culex* (Suman et al., 2008; Airi & Kaur, 2015) mosquitoes.

Morphologically, description and dimensions of the present eggs are found to be similar to the description of *Cx. pipiens* eggs by Sahlen (1990) who mentioned that
eggs are conical in shape and slightly curved in their long axis. The whole egg surface was ornamented with polygonal outer chorionic cells (OCC), containing large central, medium and small peripheral tubercles (OCTs), except the micropylar apparatus region (Fig. 3B). These tubercles acting as protective and adhesive structures (Suman et al., 2008) and providing support to the egg shell from sudden forces exerted by either the water waves and/or embryonic movement (Sahlen, 1990) in egg rafts of Culex mosquitoes. Such egg rafts play an important role in egg flotation on the water surfaces (Soumare and Ndiaye, 2005). The adhesion occurs by the interlocking of the larger tubercles on the surface of one egg between the smaller ones on the surface of the opposite egg and therefore provide necessary stability in the egg-rafts (Sahlen, 1990). In Aedes mosquitoes, tubercles contribute to the adhesion of the eggs to the substrate (Santos, 2013).

The dimensions and densities of tubercles found on the present eggs were different to some extent from that of Cx. quinquefasciatus. Data in table (1) showing that all attributes of the present Culex eggs are smaller than that of Cx. quinquefasciatus (Suman et al., 2008) except for micropylar mound (Mmd) diameter (13.94 and 13.30 μm respectively) and medium and small micropylar tubercles diameter (1.65, 1.0 and 1.13, 0.74 μm respectively). Such larger diameters particularly micropylar mound of the present eggs compared to Cx. quinquefasciatus could be due to its trilobite structure and implying in the same time to another species of the Cx. pipiens complex rather than Cx. quinquefasciatus. Additionally, the height of the tubercles ranged from 0.80 to 1.51 μm which is nearly similar to height of Cx. pipiens tubercles that measured 0.90-1.60 μm (Sahlen, 1990).

Although all morphological characters and morphometrics of the present eggs are looking alike that of Cx. pipiens and different from Cx. quinquefasciatus, Cx. tritaeniorhynchus and Cx. summorous eggs, the very distinctive tri-lobed micropylar mound which have been shown for the first time in the present eggs highlighting the importance of this finding and the reliability of SEM in differentiating such Cx. pipiens mosquito complex. The micropylar mound appeared as either flat not protruding outwards as in Cx. quinquis fasciatus and Cx. tritaeniorhynchus (Suman et al., 2008) or evaginated outwards to form a conical structure as in Cx. summorous eggs (Airi and Kaur, 2015). Additionally, the half of the egg rafts that separated and left to hatch and develop to 4th instar larval stage was identified as Cx. pipiens according to keys of Harbach (1985) and AL-Ahmad et al. (2011). Finally, present eggs were collected from breeding sites characterized by stagnant water that are supplied with algae and subjected to sun rays which are characteristic to breeding sites of Cx. pipiens in Saudi Arabia as mentioned by AL Ashry et al. (2018).

According to all the previously mentioned information it could be said that the present eggs are belonging to Cx. pipiens mosquitoes and the tri-lobed micropylar mound has been shown for the first time in the present work.

CONCLUSION

Complete knowledge of the egg morphology of different mosquito species through SEM is not only useful in correlating its fine structure with the invisible ones examined under ordinary microscope, but also can assist in species differentiation. Such SEM studies may allow for the emergence of Pictorial keys for mosquito eggs that should be helpful in studies involving eggs recovered from ovitraps or soil samples. Our findings using SEM of the eggshell tubercles and the characteristic tri-lobed micropyle mound seen for the first time indicated that the present mosquito species is Cx. pipiens.

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Harbach, R.E. (1985). Pictorial keys to the genera of mosquitoes, subgenera of *Culex* and the species of *Culex (Culex)* occurring in southwestern Asia and Egypt, with a note on the subgeneric placement of *Culex deserticola* (Diptera: *Culicidae*).


