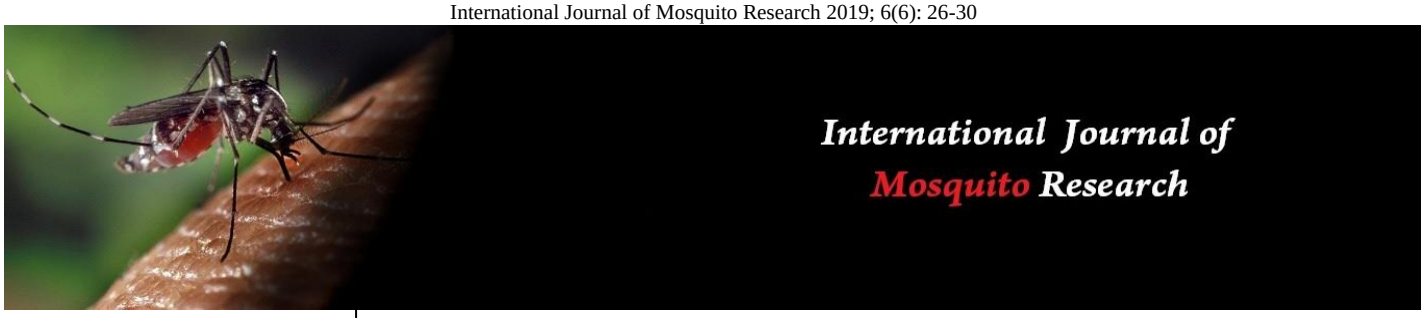

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Molecular characterization of mosquito in eastern Tripoli, Libya for species *Culex pipiens*, *molestus* and hybrids

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Abstract
Our study provides a molecular tool for identification of both *Culex pipiens* forms and their hybrids at Souq Al-jum'aa municipality east of Tripoli district / Libya. Notably, the two forms are morphologically indistinguishable but differ extensively in behavior and physiology and have different epidemiologic importance. Mosquito samples were collected during 2016 from 10 sites within six months; molecular analysis preceded using primers designed for the flanking region of microsatellite CQ11 to identify the two forms of *Culex pipiens*: and molestus compared to their hybrids. The genetic analysis of collected samples indicated that *Culex pipiens* species had the highest distribution among the other species at percentage of 51% followed by *Culex molestus* at 35%, and 14% for the Hybrids. This molecular evidence was confirmative tool to establish the presence of both forms of *Cx. pipiens* and distinguish them between the hybrids in Souq Al-jum'aa / Tripoli district.

Keywords: Souq Al-jum'aa, *Culex pipiens,* form *molestus,* form *pipiens,* hybrid, microsatellite CQ11, Libya

1. Introduction
With all the mosquito species currently recognized worldwide, only a small number responsible for the biological transmission of arbor viruses [1, 2]. Mosquito of *Cx. pipiens* are the vectors of bird - hosted arbor viruses cause outbreak in the world such as sindbis virus (SINV), West Nile Virus (WNV), Usutu virus (USUV) and Rift valley fever virus (RVFV); this species have shown to cause notable biting nuisance to humans and animals, but not definitely known to transmit diseases in Libya [3],

The species *Cx. pipiens* consist of complexes or forms, complexes are genetically distinct from each other, yet these closely related species inherit different biological characteristics such as feeding patterns, breeding preferences and vector competence, they are widely distributed mosquito species [6, 7]. Members of *Cx. pipiens* complex include *Cx. quinquefasciatus* Say 1823, *Cx. pipiens* pallens Coquillet 1898, *Cx. australicus* Dobrotworsky & Drummond 1953, *Cx. pipiens* Linnaeus 1758, comprising two morphologically identical biotypes (forms): *Culex pipiens f. pipiens and Culex pipiens f. molestus* [2, 8]. They exhibit ecophysiological differences, which include autogeny, reproductive diapause, stenogamy, feeding behavior and vector potential [9, 10], hybrids between the two biotype have an intermediate host preference, which makes them epidemiological importance as a bridge vectors for WNV from birds to mammals [11]. The presence of hybrids may increase the risk of WNV outbreak in human population, WNV infection has been investigated in 14 countries in the Eastern Mediterranean region including Libya [12].

The potential public health importance of hybrids as human disease vector has led researches to accurately identify *Cx. pipiens* biotype and their hybrids, moreover, Identifying *Cx. pipiens* complexes or forms by traditional morphological methods are time consuming, difficult, often limited to adult males and the availability of taxonomic medical entomology expertise, therefore DNA based methods are essential to replace other methods; it's quick, reliable and will fulfill the gap in the knowledge of mosquito identification and assessment of vector statue, which will aid in the control of medically important pathogens [13].

For this purpose this study conducted to identify members of the *Cx. pipiens* forms present in Souq Al-Juma Municipality in eastern Tripoli district, Libya based on a molecular identification.

2. Materials and Methods

2.1 Study Area

This study was carried out at semi urban area Souq Al -jum'aa municipality - East of Tripoli / Libya, (32.8894 °N, 13.2419 °E) (Fig 1), with an area approximately 45.17 km^2 and population 259133 inhabitants. The ten localities were selected by using the simple random sampling.

2.2 Mosquito collections

During the year 2016 in period from June to December, mosquitoes were collected once a fortnight, traps were made to collect mosquito from all potential breeding sites in ten identified spots. Adult mosquitoes were trapped in open areas away from building and lights, at height of about 1.5 meters above ground level by using CDC Miniature Black Light (UV). The traps were set in the selected locations after sunset and collected next day before sunrise, or by rearing the immature stages to adults‟ emergence which were collected from aquatic habitats by using standard mosquito larval dipper. Adult mosquitoes that were collected were killed by chilling in deep freeze and preserved in 1.5 eppendorf tube for further PCR analysis. Specimens labeled and sent to Parasitology and Vector Borne diseases laboratory (NCDC) for sorting, identification and molecular analysis (PCR).

2.3 Morphological identification

Adult mosquito's specimens were counted and identified to species based on diagnostic characters such as body parts and three caudal abdominal segments of male, Mosquito adults were identified to species level using Stereomicroscope with objectives W- PI 10x, 23 and an identification software; offline keys Mos Key Tool: an interactive identification key for mosquitoes of Euro-Mediterranean [14].

2.4 Molecular identification

2.4.1 Extraction of genomic DNA

Genomic DNA was extracted from whole adult female mosquitoes, 146 adult mosquito specimens were extracted using simple and affordable Chelex-based technique protocol [15], Isolated DNA from each sample was stored at -20 °C.

2.4.2 DNA Amplification by Polymerase Chain Reaction (PCR)

The CQ11 polymorphic microsatellite marker of *Culex pipiens* complex was used to distinguish between form pipiens and form molestus. The amplification of the CQ11 microsatellite was carried out using sets of primers CQ11F2, molCQ11R and pipCQ11R [12, 13]. The PCR reactions were performed in 25 µl of reaction mix, according to the following protocol: (2.5 µl of 1X CoralLoad PCR Buffer, 0.5 µl volume each of forward and reverse primer, 0.5µM magnesium chloride, 0.125 µl of 200 µM $dNTP_s$, 1.5 µl (0.15 mg/mL) of bovine serum albumin (BSA), 1µl of template DNA, and the remaining volume with ddH20).

The PCR conditions used for PCR amplification were as follows: one cycle at 94 °C for 5 min, followed by 35 cycles at denaturation 94 °C for 30 sec, annealing at 58°C for 30 seconds, extension at 72°C for 30 sec; and a final extension at 72°C for 5 min. The amplified DNA was loaded onto an agarose gel (2%) with the 100-bp ladder loading marker (Bio-Rad, Richmond, Calif., USA), stained with GelRed™ Nucleic Acid Gel Stain (Biotium., Hayward, Calif., USA), and visualized on a MultiDoc -It™ Imaging System Benchtop UV transilluminator (230v; Cambridge, UK). The *pipiens* and *molestus* forms presented a PCR product of 200 bp and 250 bp, respectively. Hybrids exhibited both amplicons (200 bp/250 bp).

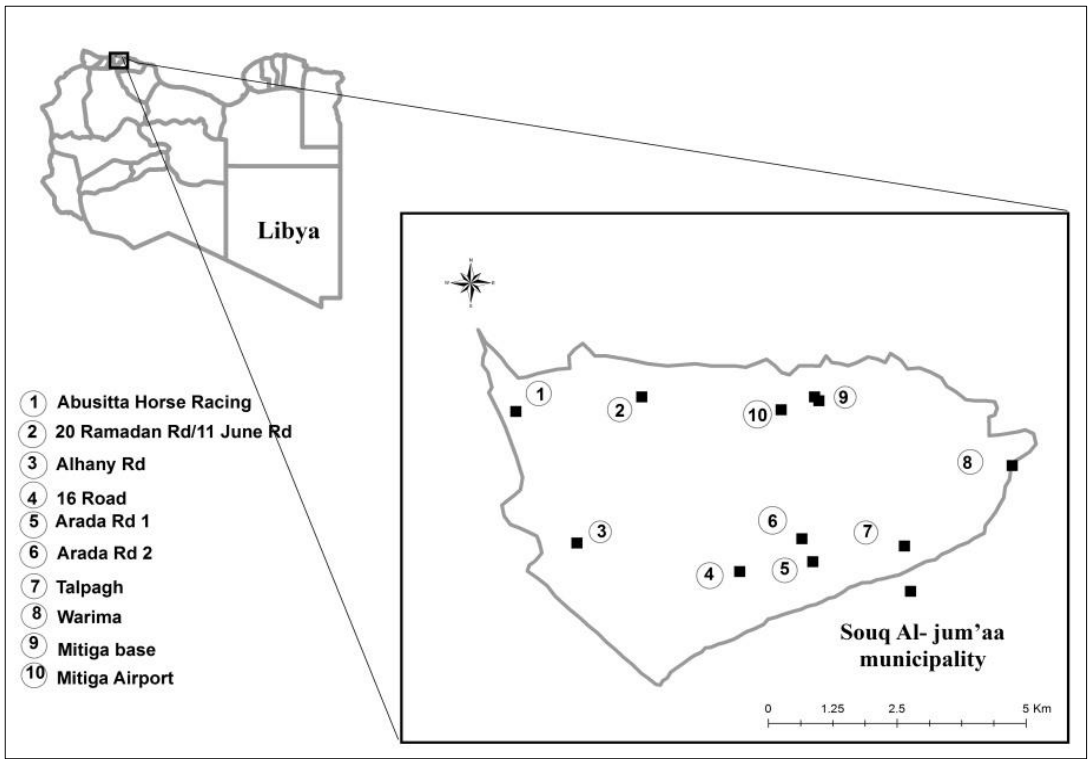

Fig 1: Map of Study area at Souq Al Jumaa municipality.

3. Results

A total of 146 adult mosquitoes were collected from 10 sites in Souq Al-Jum'aa Municipality- Eastern Tripoli, Libya (Table 1); *Cx. pipiens* were characterized by PCR assay and frequencies of different forms were represented in Table 1. Genetic analysis of PCR product by agarose gel electrophoresis with the species – specific primers was indicating that two species of *Culex*: *Cx. pipiens*, and *molestus* were found as well as their hybrids (Fig 2). From the total samples tested about 51% of adults were homozygous for the 200 bp fragments, 35% were homozygous for the 250 bp fragments identifying the *molestus* form and the remaining 14% corresponded to hybrids (Fig 3).

Table 1: Frequency (%) of *Cx. pipiens* complex identification by PCR assay in Souq Al-jum'aa Municipality/ Tripoli.

Site	Breeding site (Ground)	(%) Pipiens form	(%) Molestus form	(%) Hybrids Pipiens / Molestus
Arada Rd 1	Above	14.1	7	2.6
Arada Rd 2	Above	7.1	5.3	2.5
16 Road	Above	1.7	0	0
Alhany Rd	Above	2.6	3.5	1
20 Ramadan Rd/11 June Rd	Above	3.5	1	0
Mitiga International Airport	Above	1.7	1	0
Mitiga base	Above	3.5	1.7	1.7
Talpagh	Above	8.8	8	3.5
Warima	Above	7	4	1.7
Abusitta Horse Racing	Above	1	3.5	1
Total		51	35	14

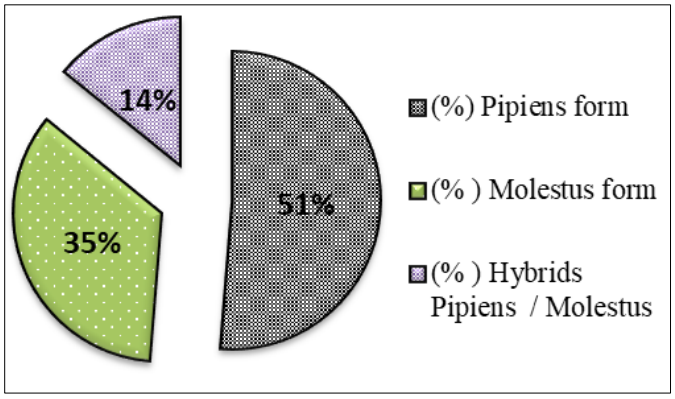

Fig 2: The percentage of distribution of *Cx. pipiens* complex in Souq Al- Jum'aa Municipality / Tripoli detected by PCR technique

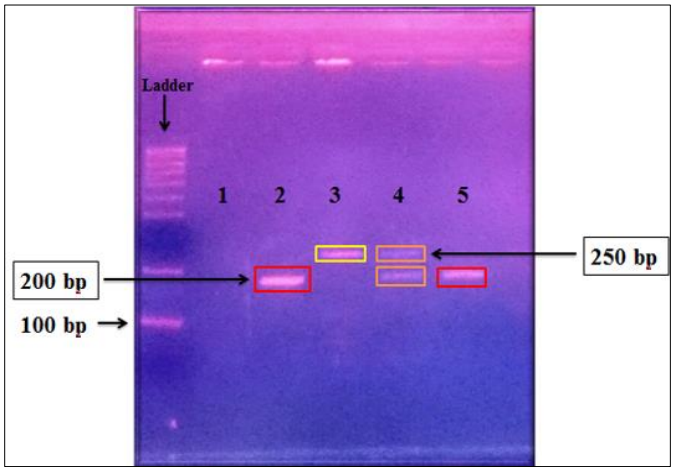

Fig 3: 2% Agarose gel electrophoresis of PCR product from the flanking region of the CQ 11 microsatellite of Cx. pipiens, fragments amplified with pipCQ11R, molCQ11R, and CQ11F2 specific primers. The lane M= Marker (size standard 100-bp ladder), lane 1= Negative control, 2 = *Cx. pipiens f. pipiens*, lane 2 = *Cx. pipiens f. molestus*, lane 3 = *Cx. p. pipiens/* molestus hybrids, lane 4 = Positive control

4. Discussion and Conclusions

The results investigated genetic characterization of the *Culex pipiens* population by screening the 146 *Culex pipiens* adult mosquitoes with CQ11 microsatellite; were it demonstrats the presence of two *Culex pipiens* forms (*pipiens* and *molestus*) and their hybrids at 10 breeding sites; they showed variances at 10 localities with high proportion of 51% of *Cx. pipiens f. pipiens*, followed by 35% of the form *Cx. pipiens f. molestus*, but not reported at 16 Road site, the present study also revealed that *Cx. p. pipiens/ molestus* hybrids were found with 14%. However, hybrids weren't reported at 16 Road, 20 Ramadan Rd/ 11 June Rd, Mitiga International Airport. The *Cx. pipiens* and their hybrids that were found in the study were vastly different in site studied, despite that there were no huge differences in the habitat, for all sites studied had plants (Olive tree and palm) animals (Camels, Chickens, Rabbits, Sheep's), birds (Pigeons, ducks and house sparrow).

This suggests that there is difference in the diversity and composition of mosquitoes across different land used area types. The present study provided the first molecular evidence for the presence of *Culex pipiens* forms (*pipiens* and *molestus*) and their hybrids at Souq Al-Juma Municipality of Libya, In fact it was the first attempt for PCR detection of medically important mosquitoes *Cx. pipiens* forms and their hybrids*;* these mosquito species are considered of great epidemiological importance, They play a significant role in transmitting pathogens such as West Nile virus, from birds (Amplification hosts) to humans, because of their opportunistic feeding behavior on avian and mammalian hosts [11]. *Culex pipiens* is the most widespread mosquito vector that occurs throughout temperate latitudes. It was first mentioned in Libya by [16]. Zavattari 1934, however, the epidemiology of mosquito borne Diseases (MBD) related to *culex pipiens* remains poorly studied, much of the published literature detailing MBD reviewed limited surveillance including WNV, RVF, SINV [3, 17]. In addition, the current situation such as mobility of people, animals, and goods; availability of effective drugs; quality of public health services; the ongoing conflict war and political instability that would lead to break down in public health care and poor vector control measures, also the limited setting resources are a concern risk of an outbreak with mosquito borne Diseases (MBD). Moreover in response to global warming, Mosquito borne diseases may re

-emerge and expansion of mosquito ranges, were non endemic areas are at risk if source of infections were available, such that patterns of transmission of MBD are likely to change in coming decades [18].

The *Cx. pipiens* complex (or forms) are indistinguishable morphologically, can be separated by behavioral and physiological characteristics or by molecular analysis [19, 20]. It is crucial to correctly identify different field-collected mosquito species which is paramount to the planning of targeted vector control strategies to mitigate disease transmission. However, morphological techniques rely on the quality of the specimen and overwhelmingly unavailable taxonomic expertise, Species identification by using DNA-based method; have now largely replaced other methods of species determination for such complexes [21]. The present study revealed that *Cx. pipiens* and natural hybridization between *Cx. pipiens* and *Cx. molestus* in Souq Al-Juma Municipality, the occurrence of *Cx. pipiens* and hybrids in different sites were similarly observed in other studies in Tunisia [9] Morocco [8], Algeria [22, 6] Chicago and New York (USA) [23], several European countries, i.e. Portugal [24], the Netherlands [25] and Italy [26].

In contrast, the (re) – emerge of mosquito Borne Diseases and climate change influence on mosquito population; accreting the expansion of mosquito populations; facilitating the introduce of new species in Eastern Mediterranean region including Libya; that could be associated with future threats of an amplification of mosquito-borne diseases such as malaria, yellow fever, Chikungunya virus, West Nile virus, Rift valley fever virus, Usutu virus, sindbis virus, Zika virus and others across Eastern Mediterranean region [4,5].

In conclusion, molecular characterization of mosquitoes collected in Souq Al-Juma Municipality revealed the presence of *Cx. pipiens* and their hybrid, further studies are needed to conduct a nationwide Surveillance on *Cx. pipiens* Complex to understanding vector distributions and the dynamics of pathogen transmission; this is an important step towards a realistic risk assessment for mosquito-borne virus transmission to humans in Libya.

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