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Molecular Genetic Variations of *Culex pipiens* Complex (Diptera: Culicidae) of Egypt

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Abstract: Mosquitoes in the *Culex (Cx.) pipiens* complex are important disease vectors with global distribution. *Culex pipiens* is the main vector of Filarial parasite and Rift Valley Fever virus through outbreaks in Egypt. *Culex pipiens* coexists in more than biotypes; autogenous, anautogenous. From the epidemiological view, such biotypes have variable ability for transmission the pathogens. The taxonomy status of *Cx. pipiens* complex was confused for long time in Egypt so this study aimed to explore the molecular genetic variations of these biotypes. Mosquito samples were collected from eight different geographical regions. The second internal transcribed spacer region of the ribosomal DNA (ITS2-rDNA) has been amplified as a genotype marker using polymerase chain reaction (PCR) and amplicons directly sequenced. The lengths of ITS2-rDNAvaried in size from 340 base in North Coast population (Autogenous biotype) to 375 base in Giza population (Anautogenous biotype). The point mutation was 30.1% transitions and 69.9% Inversions. Suhag population (Upper Egypt) recorded the highest rate of mutation 12.7%, while North Coast and Qaliubiya were the lowest; 6.8% and 4.8% respectively. The genetic distance fluctuated between 0.05 and 0.12. The phylogenetic tree showed that North Coast was in a clade with Qalubiya and Suez, on the other hand, Suhag and Giza were segregated in two other clades. Our findings agree with the hypothesis that there are three biotypes of *Cx. pipiens* complex coexist in Egypt.

Key words: *Culex pipiens* Complex · ITS2-rdna · Genotyping · Egypt

INTRODUCTION

Culex (Cx.) pipiens complex (Diptera: Culicidae) is a common pest in urban and suburban areas [1, 2]. Mosquitoes of the *Cx. pipiens* complex are the primary vectors for diseases that present a world-wide distribution, such as West Nile encephalitis [3, 4] St Louis encephalitis [5] Rift Valley fever [6] and Japanese encephalitis [7]. The complex demonstrates an array of behavioral, morphological and physiological characters that vary climatically from temprate to tropical regions. The *Cx. pipiens* complex are *Cx. pipiens (p.) pipiens* (Linneaus) and *Cx. p. quinquefasciatus* (fatigans). *Culex p. pipiens* includes two forms *pipiens* and *molestus. Cx. p. pipiens* is anautogenous, eurogamous and heterodynamous diapasusing, the molestus is autogenous, stenogmous and homodynamous (Nondiapausing). *Cx. p. pipiens* and *Cx. p. quinquefasciatus* are separate species which evolved in tropical regions and hybridize in non-indigenous areas [8].

Culex pipiens occupies temperate regions and has holoarctic distribution [9]. In the temperate zone (Northern European latitudes) *molestus* and *pipiens* populations occupy different habitats (Underground and aboveground). In this zone both forms are strongly isolated from one another. The degree of isolation between the two forms decreases in the southern of Europe, the two forms live in sympatry in surface habitats which promotes hybridization between the two species [10, 11]. As a result, populations with intermediate biological characteristics have been described in the hybrid zones. The two species are hybrid and occur in North America, Argentina, Madagascar and North Africa [12-16]. In the subtropical regions, *Cx. p. pipiens* and *Cx. p. molestus* larvae are abundant together in open natural reservoirs. Both forms are identified mainly by the biological features of the female (Autogeny vs.

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nonautogeny). The hybrids have intermediate shapes. It is difficult to identify in the field [11].

Culex pipiens is widespread in Egypt, where both autogenous and anautogenous biotypes coexist [17-27]. *Culex pipiens* has been found naturally infected with West Nile virus and Rift Valley Fever virus in Egypt [28-31]. The importance of *Cx. pipiens* as primary vector of periodic *Bancroftian* Filariasis in Egypt requires the rapid and accurate identification of the two biotypes [24].

The taxonomy of the *Cx. pipiens* complex is difficult to interpret. The morphology of larvae and adults of these species within these complexes are similar or identical however they show considerable differences in ecological and physiological features, including food preferences; their epidemiological significance is also different. Since members of the complex present several similarities in terms of both genetics and morphology [32], the difficulty in the identification of various members of the *Cx. pipiens* complex within the limits of traditional taxonomy stimulated the development of their molecular genetic diagnostics [11, 33-37].

One of the basic molecular genetic markers used to study species composition of mosquitoes is the second internal transcribed spacer (ITS2) of nuclear ribosomal ribonucleic acid (rRNA) genes. Ribosomal RNA genes are represented in the genome as a set of tandem repeats. Each transcription unit consists of the genes encoding three ribosomal RNAs(18 S,5.8 S and 28 S) separated by internal transcribed spacers (ITS1 and ITS2).The coding 28S gene sequence is followed by an external transcribed spacer. The rRNA genes are conserved regions and the internal transcribed spacers are variable and useful for comparing species. It is known that the structure of ITS2 varies among different mosquito species [10, 38]. Our objective is designing a rapid and cost effective PCR- based method in which each biotype of *Cx. pipiens* produces an amplification product of distinctive size.

MATERIALS AND METHOD

Sample Collection: Mosquito larvae of *Cx. pipiens* were collected from various larval habitats of eight different geographical areas of Egypt. Anautogenous *Cx. pipiens* larvae were collected from a drainage canal in Suez, El Moasasa (Cairo), Mariutia (Giza), Kashish, (Qaliubiya), Suhag and Fayioum governorates. Autogenous *Cx. pipiens* larvae were collected from the North Coast. Larvae were raised in a walk-in insectary under optimum conditions of 27 ± 2°C and fed on fish food till pupation. Emerged adults were allowed to mate for 24-48 hours. Anautogenous female mosquitoes were offered a blood meal followed by sucrose solution, while autogenous mosquitoes were fed on sucrose solution only. The *Cx. pipiens* 4th larval instar (L_4) were used for identification using the taxonomic keys [21, 24, 39].

Isolation and Extraction of Genomic DNA: Ten samples represented each geographical area were used individually to isolate and extract the genomic DNA using Wizard purification kit. The concentration of genomic DNA was measured using UV spectrophotometer at two wave lengths 260 and 280 nm and the mean value was taken. The ITS2-rDNA has been used successfully to distinguish the complex species of mosquitoes. We chose two primers CP1-P1A (Forword GTGGATCCTGTGA ACTGCAGGACACATG) and Cp-p1B (Reverse GTGTCGACATGCTTAAATTTAGGGGGTA) to amplify the ITS2-rDNA region in different populations of *Cx. pipiens*.

PCR: The following reagents were used; 1 µl of DNA template (50-100 ng/µl),1 µl of foreword primer (20 picomole/µl), 1 µl of reverse primer (20 picomole/ul), 12.5 µl of thermo -cycler master mix solution (Master cycler gradient 2x (T5A promega). The total volume is 25 µl using autoclaved distilled deionized water completed. The PCR program was performed as following; group 1was 3 min at 94 °C, group 2 was 1 min at 94°C, 2 min at 60 °C, 2 min at 72°C and group 3 was 1 min at 72°C, then park at 4°C.The gel electrophoresis was performed using 5µl of amplified products analyzed on 2% agarose /TAE gel containing ethidium bromide(5µg/l). The size of the amplified fragment was estimated using DNA marker (Promega).

RESULTS

Identification of mosquitoes is essential for controlling the species that are vectors of humans and animal diseases. Using the CP-P1A and CP-P1B on individuals from all eight populations of *Cx. pipiens* produced the typical species-specified bands. A symmetric amplification has been straight forward over the ITS2 region of all individuals from different popualtions of *Cx. pipiens*. Only the North Coast population is representing the autogeny biotype of *Cx. pipiens*, other populations represented anautogenous biotype. The amplified fragments size of the ITS2-rDNA are shown in Fig. 1 and 2. The amplified fragments represented seven different anautogenous populations of

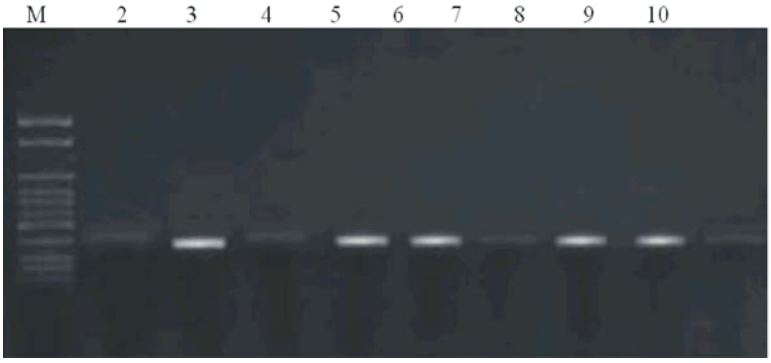

Fig. 1: PCR amplifications of ITS2 – rDNA of *Cx. pipiens* populations from different geographical regions of Egypt. Lane 1: DNA marker. Lanes: 2,3 and 4: Cairo population. Lane 5: Suez population. Lanes 6,7 and 8: North Coast population. Lane 9 and 10: Giza population.

Fig. 2: PCR amplifications of ITS2 –rDNA of eight *Cx. pipiens* populations from different geographical regions of Egypt. Lane 1: DNA marker. Lanes 2: Cairo (Moasasa) population, lane 3: North Coast population, lane 4: Giza population. Lane 5: Qalubiya population, lane 6: Kashish population, lane 7: Suhag population and lane 8: Fayuom population.

Table 1: Point mutations recorded in *Cx. pipiens* complex of Egypt

	Transitions		Inversions						
Locality	A-G	C-T	A-C	C-G	A-T	G-T	Total	Length of ITS2	Mutation %
Suhag	8	3	17	11	3	5	47	370	12.7%
Giza	2	2	7	7	7	3	28	375	7.4%
Suez	4	8	4	6	5	4	31	374	8.2%
Qalubiya	4	2	6	2	1	2	17	348	4.8%
North Coast	4	7	7	1	1	3	23	340	6.8%
	22	22	41	27	17	17	146	1804	
	-------30.1--------		---------- 69.9 % ----------						

Cx. pipiens; Moasasa (Cairo), Suez, Mariutiya (Giza), Qaliubiya, Kashish, Suhag and Faiyuom generated approximately 450 bp. There was no large difference between the fragments size of North Coast population (Autogenous) and the other seven anautogenous populations of *Cx. pipiens*.

Five populations only could be sequenced. Direct sequence of ITS2 of *Cx. pipiens* populations revealed high levels of polymorphisms. The lengths of ITS2 ranged from 340 base in North Coast population to 375 base in Giza population. The alignment of ITS2 contained 8% of base substitutions. The variations occurred over 146 sites. The variation in the ITS2 was mainly point mutation 30.1% transitions and 69.9% Inversions (Table 1). Suhag population recorded the highest rate of mutation 12.7%, while North Coast and Qaliubiya was the lowest 6.8% and 4.8% respectively.

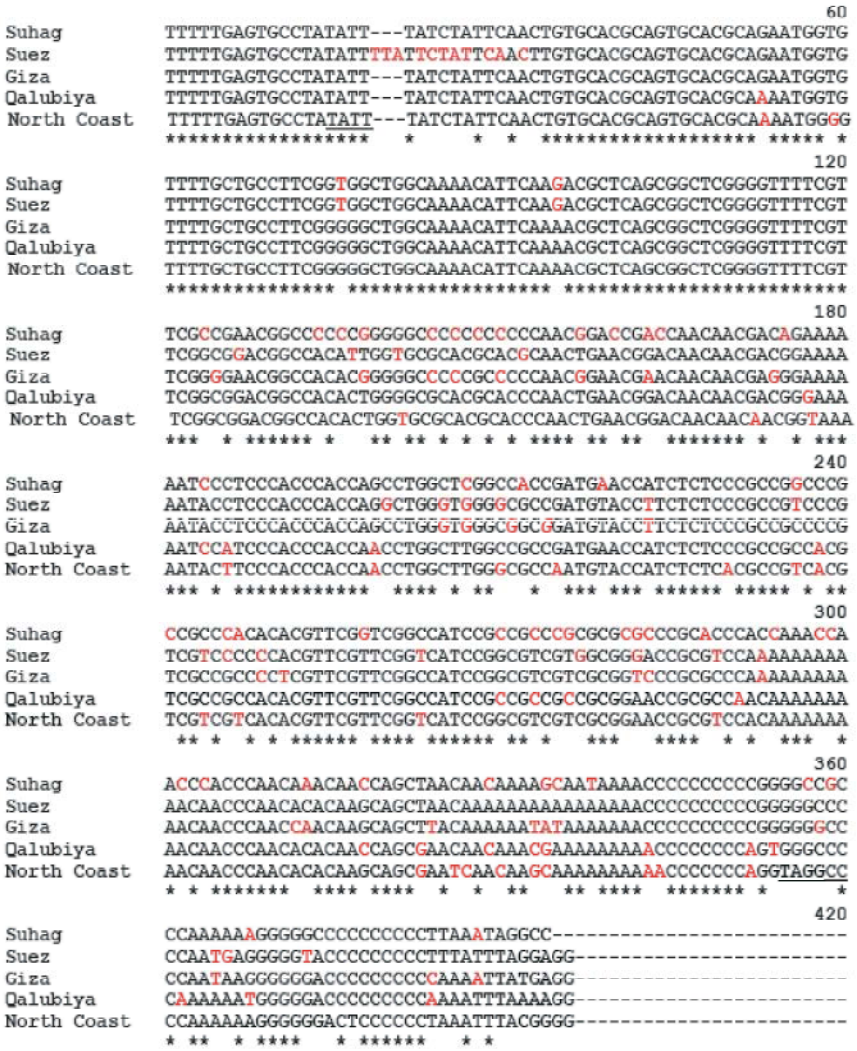

Fig. 3: Sequence alignment of ITS2 and flanking 5.8S and 28S coding of rDNA regions of *Cx.pipiens* complex

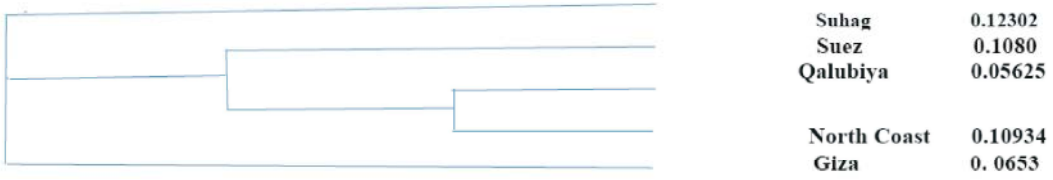

Fig. 4: Phylogenetic Tree of *Cx. pipiens* complex of Egypt

Table 2: Similarity rate of tested Egyptian *Cx. pipiens* complex

	Cx. p. pipiens		*Cx. p. molests*		*Cx.queinquefasciatus*	
Locality	*Similarity %*	*Accession no.*	*Similarity %*	*Accession no*	*Similarity%*	*Accession no*
Suhag	81	EF539854.1	83	KX866004.1	81	FJ416055.1
Giza	84	DQ41110.1	84	AJ850085.1	82	FJ416058.1
Suez	87	KU175324.1	87	KU495652.1	86	GU562872.1
Qalubiya	87	EF539854.1	88	KX866004.1	87	FJ416055.1
North Coast	91	EF539854.1	91	KX866004.1	89	DQ341113.1

North Coast population (Autogenous biotype) on latitude 31°N recorded the highest similarity rate 91% for both *Cx.p. molestus* (accession no. KX866004.1), *Cx. pipiens* (Accession no.EF539854.1) and 89% to *Cx. quinquefasciatus* (Accession no. DQ341113.1 in Genbank). On the other hand; other populations (Anautogenous biotypes) recorded lower similarity i.e Suhag population (Latitude 26° N) showed similarity 81% to *Cx. p. pipiens* (Accession no. EF539854.1), *Cx. quinquefasciatus* (Accession no.FJ416055.1) and 83% to *Cx.p. molestus* (Accession no. KX866004.1) In Genbank.

The above alignment was used in Clustal Omega phylogenetic analysis to investigate relationships among populations of the *Cx. pipiens* complex of Egypt (Fig. 3). The genetic distance fluctuated between 0.056 and 0.12. The phylogenetic tree (Fig. 4) showed that there was some concordance among the *Cx. pipiens* populations. North Coast was in a clade with Qalubiya and Suez. Suhag and Giza were segregated in two other clades.

DISCUSSION

Culex pipiens is highly plastic species which is widely distributed in the holarctic regions and cooler parts of Africa. *Culex p. molestus* Forskål was originally described as a distinct species in Egypt 1775. The previous studies demonstrated that Egyptian *Cx. p. pipiens* is not differentiated into genetically isolated demes being rather homogenous panmictic population based on great morphological and biological similarities in separate *Cx. p. pipiens* micropopulations [12, 18, 20]. Therefore, *Cx. p. molestus* was unacceptable as either a species or subspecies in the previous studies. *Cx. pipiens* in Egypt is no more than a single behaviorally and physiologically variable species [24].

The southern limit of the temperate zone of the *Cx. pipiens* complex mosquitoes extend to North Africa [12]. The structure of the copulatory structures of eleven populations from different isolated geographical regions of Egypt showed similarity to both the temperate and tropical zones and confirmed the criteria that the Egyptian *Cx. pipiens* mosquitoes are predominantly Mediterranean in form. The degree of similarity between the two forms decreases for instance, Egyptian populations of *Cx. pipiens* are relatively homogenous [27]. In this study, mosquito samples of *Cx. pipiens* were collected between the two latitudes 26°N and 31°N. Direct sequencing of the ITS2-rDNA amplicons revealed that both autogenous and anautogenous biotypes of *Cx. pipiens* have different fragment lengths. The differences fluctuated between 8 to 35 base pair. High polymorphism was recorded among the sequences of *Cx. pipiens* populations. The autogenous biotype of North Coast was less 8 to 35 base pair in length than anautogenous biotype populations. The rate of point mutation was directly proportional to the temperature and inversely proportional to the latitude. The highest rate of point mutation 12.1% was recorded in Suhag population at latitude 26° N, South of Egypt. This population (Anautogenous biotype) showed similarity 81% to *Cx. p. pipiens* (Accession no.EF539854.1), *Cx. quinquefasciatus* (Accession no.FJ416055.1) and 83% to *Cx.p. molestus* (Accession no. KX866004.1) in the Genbank. North Coast population (Autogenous biotype) at latitude 31 N° recorded the highest similarity rate (91%) for both *Cx. p. molestus* (accession no. KX866004.1), *Cx. p. pipiens* (Accession no. EF539854.1) and 89% to *Cx. quinquefasciatus* (Accession no. DQ341113.1 in Genbank).

The genetic distance among the five *Cx. pipiens* populations in this study fluctuated between 0.056 and 0.12. The previous studies concluded that the mean genetic distance was 0.013 among Egyptian *Cx. pipiens* populations and also characters of genetic differentiation in the forms were equivalent to the European *molestus* and *pipiens*. The Egyptian (North African) populations are more closely related to the Italian (European *molestus*) than the Italian (European *pipiens*). The mean genetic distance between them being 0.023 versus 0.056, respectively [22]. The phylogenetic tree of five populations in this study included three clades; the first clade was Suhag population, the second clade included North Coast, Qalubiya and Suez populations. The third clade was Giza population.

Accordingly, Harbach and others have insisted that the autogenous status of *Cx. pipiens* does not satisfy the definition of subspecies advocated by Mayer [40], Gad and Hassan [41, 42] Mosquito species in the *Cx. pipiens* complex are highly conserved morphologically but marked differences in potential vectorial capacity. Identification of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* relied on expressed ecological characteristics, including autogeny, host preference and stenogamy [43]. Autogenous *Cx. p. pipiens* may be less efficient vector of *Wuchereria bancrofti* in endemic areas of Egypt if compared to anautogenous counterparts [44]. Mainly anthropophilic populations of *Cx. pipiens* were recorded in Egypt [45]. Asgharian *et al*. [46] revealed that the

geographical distribution is the prominent factor in shaping population structure and specifying pattern of genomic selection. Multiple adaptive events involving genes implicated with autogenous and diapause. About 5-20% of the genes (including histone genes and almost half of annotated pathways were undergone selective sweeps in each population of *Cx. pipiens*.

CONCLUSION

Culex pipiens is widespread in Egypt, where autogenous, anautogenous and hybrid biotypes coexist. The importance of *Cx. pipiens* as primary vector of periodic *Bancroftian* filariasis is in Egypt requires the rapid and accurate identification of the three biotypes. One of the basic molecular genetic markers used to study complex species of mosquitoes is the second internal transcribed spacer (ITS2) of nuclear ribosomal ribonucleic acid (rDNA) genes as genotyping marker. Our finding revealed that there was genetic variability among the three biotypes; autogenous, anautogenous and hybrid of *Cx. pipiens*. The three biotypes appeared to relatively homogenous.

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