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Genetic characterization of Asian tiger mosquito *Aedes albopictus* in Palestine

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Abstract

Aedes albopictus (= *Stegomyia albopicta*) (Skuse) (Diptera Culicidae) is an invasive mosquito species originating from the tropical and subtropical areas of South and East Asia. Since a couple of decades it has spread all over the world colonizing all continents. To uncover the routes of entry, genetic studies analysing the phylogenetic relationship of recently established and natural *Ae. albopictus* populations are of vital importance. Here we analysed the sequence variation in the mitochondrial cytochrome C oxidase subunit 1 (*COI*) and the mitochondrial NADH dehydrogenase subunit 5 (*ND5*) genes from specimens collected in Palestine in order to gain insights on genetic relation to other populations and variances within our population. The results showed the existence of three haplotypes for *COI* and *ND5* genes, with a central haplotype for *COI* and *ND5* genes and very little haplotype diversity within haplotype networks (0.186 for *COI* and 0.417 for *ND5*). The neutrality tests showed a negative value, indicating deviations from neutrality and suggesting recent population expansion. We were able to group our sequences from Palestine with *COI* and *ND5* sequences from tropical, sub-tropical and temperate areas. In conclusion, the low genetic diversity hinds to either a recent introduction or a strong genetic selection toward a specific haplotype by other factors.

Key words: *Aedes albopictus*, population genetics, phylogeny, Palestine, *COI*, *ND5*.

Introduction

The Asian tiger mosquito *Aedes albopictus* (= *Stegomyia albopicta*) (Skuse) (Diptera Culicidae) is an invasive mosquito species native to the tropical and subtropical areas of Southeast Asia (Skuse, 1894). It is characterized by an aggressive biting behaviour, which causes not only nuisance for humans and animals, but can also lead to severe localized or generalized allergic reactions. Furthermore, *Ae. albopictus* has been incriminated to transmit more than 30 viruses (Hawley, 1988; CDC, 2001). Nowadays, this mosquito is found in tropical, subtropical and temperate regions (Aguirre-Obando and Navarro-Silva, 2017), and is expected to continue to spread worldwide (Kraemer *et al*., 2015). In only few decades this species has managed to spread from its native range in South-East-Asia all over the world (Kotsakiozi *et al*., 2017), largely through the transportation of its relatively cold-hardy and long-lived eggs via the international trade (Medlock *et al*., 2015; Pichler *et al*., 2019). Since its first appearance in Europe in 1979 in Albania then in 1990 in Italy, the species has been reported from more than twenty-five European countries (Lühken *et al*., 2020), The spread of *Ae. albopictus* is of major public health concern, as this mosquito has a high potential to transmit/spread a wide range of viruses, belonging to different families such as *Flaviviridae*, *Bunyaviridae*, *Togaviridae* and *Reoviridae* (Battaglia *et al*., 2016; Aguirre-Obando and Navarro-Silva, 2017). These viruses include those causing Dengue, Chikungunya, West Nile, and the recently emerged Zika fever. Also, *Ae. albopictus* mosquitoes serve as vectors of filarial nematodes of veterinary and zoonotic significance (Cancrini *et al*., 2003).

The presence of *Ae. albopictus* in the Middle East was first reported in 2002 in Israel (Pener *et al*., 2003). In subsequent years, further dispersal of the mosquitos to the entire area including the Palestinian territories in the West Bank was reported by the Israeli Ministry of Health. Other reports showed spread in other Mediterranean countries such as Jordan, Lebanon and Syria (Haddad *et al*., 2007). According to the Palestinian Ministry of Health, newly introduced *Ae. albopictus* populations were found in a few sporadic foci in the Palestinian territories (Adawi, 2012). However, the current information does not allow conclusions regarding *Ae. albopictus* spread in Palestine. It is of great concern that, the Israeli Ministry of Health already reported the emergence of *Aedes*-borne infections in Israel only a few years after first reports of this species in Israel (Pener *et al*., 2003; Israeli Ministry of Health, 2018; Johnson *et al*., 2018).

The knowledge about the genetic diversity and the genetic structure of *Ae. albopictus* can help to understand origins and frequencies of introductions, evaluate the risk of spread of diseases and their transmission dynamics, as well as insecticide resistance, and by this, inspire the development of appropriate vector control programs. This knowledge is also required to identify loci that could be involved in environmental adaptation (Harris *et al*., 2010; Zouache *et al*., 2014; Goubert *et al*., 2016; Aguirre-Obando and Navarro-Silva, 2017). Population genetics

structure studies of native and invasive *Ae. albopictus* populations have been conducted worldwide. In these studies, different genetic markers, such as isozymes/allozymes, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), mitochondrial DNA (mtDNA) sequence haplotype, ribosomal DNA (rDNA), microsatellites (Goubert *et al*., 2016; Aguirre-Obando and Navarro-Silva, 2017; Lühken *et al*., 2020) and by analysing of the genome-wide single nucleotide polymorphisms (SNPs) (Kotsakiozi *et al*., 2017; Schmidt *et al*., 2017; Pichler *et al*., 2019), have been used.

Genetic diversity of *Ae. albopictus* has never been characterized in Palestine and many other countries around. This study was designed to determine the genetic diversity of *Ae. albopictus* based mainly on two mitochondrial genes.

Materials and methods

Sample collection

Female mosquitoes showing the typical black and white bands were collected from two areas in the north and the south of the West Bank in Palestine, representing the whole Palestinian territories: In the city of Nablus at the north (32°13'16"N 35°15'15.98"E), and the city of Hebron at the south (31°31'45.66"N 35°5'37.68"E). The collection was performed by human landing catch (HLC) method between 17:00 and 19:00 local time at warm temperatures, as the highest biting activity was noticed at days with temperatures over 28 °C (Reinhold *et al*., 2018). A total of 128 individuals were caught between May and June 2017. The mosquitoes were frozen individually in 0.2 ml tubes for further analysis. Differentiation of *Ae. albopictus* mosquitoes was achieved using morphological markers according to the identification key described by Becker *et al*. (2010). The main criterion used to differentiate *Ae. albopictus* from other mosquitoes was the long median longitudinal silvery-white stripe at the scutum, extending from the anterior margin to about the level of the wing root.

Extraction of genomic DNA

Genomic DNA was extracted from single individual mosquitoes using the DirectPCR®Lysis Reagent Cell (Peqlab). The samples were homogenized using a micropestle. For eight samples, the NucleoSpin Tissue Kit (Macherey-Nagel, Dueren, Germany) was used for genomic DNA extraction to compare the quantity of extracted DNA between the two methods. Equal quantity of DNA was extracted using either of these methods.

PCR and DNA sequencing

Amplification of the mitochondrial cytochrome C oxidase subunit 1 (*COI*) gene was done using the forward primer 5'-ATTGGATTATTAGGATTTATTG-3' and the reverse primer 5'-GCAGGAGGAAGAGTATGATATC-3' (Mousson *et al*., 2005). Reactions were performed in a volume of 25 µl, including 5.5 µl water, 12.5 µl Master Mix (QIAGEN Multiplex PCR Kit), 2 µl DNA sample, 2.5 µl from each primer. Thermocycling comprised an initial denaturation for 5 min at 95 °C followed by 35 cycles at 95 °C for 20 seconds for denaturation, annealing at 55 °C for 30 seconds, ramp to 68 °C at a rate of 1 °C every 30 seconds and extension at 70 °C for 60 seconds. Final extension was carried out at 70 °C for 10 minutes.

Amplification of the mitochondrial NADH dehydrogenase subunit 5 (*ND5*) gene was done using the forward primer 5'-TCCTTAGAATAAAATCCCGC-3' and the reverse primer 5'- GTTTCTGCTTTAGTTCATTCTTC-3' (Birungi and Munstermann, 2002). Reactions were performed in a volume of 25 µl, including 1× PCR buffer, 50 mM $MgCl_2$, 10 µM of each primer, 2 mM dNTP mix (SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase, Invitrogen), 0.4 U Taq DNA polymerase (Invitrogen, France). The thermal cycling conditions consisted of an initial denaturation at 94 °C for 2 minutes, followed by 40 cycles at 94 °C for 15 seconds for denaturation, annealing at 51 °C for 30 seconds, and extension at 68 °C for 80 seconds. Final extension comprised 5 minutes at 68 °C. The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

DNA sequencing

The amplification products from the PCR reactions components were cleaned with the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel) and subsequently Sanger-sequenced by Mix2Seq Kit, (Eurofins Genomics, Germany).

Data

For the *COI* and *ND5* genes, 21 and 10 own sequences from mosquitoes collected in Palestine were available. Obtained from public resources, 26 and 29 sequences were available for *COI* and *ND5*, representing other countries.

Bioinformatics analysis

After quality-based trimming of the Palestine sequences at both tails, multiple alignments of the *COI* and *ND5* nucleotide sequences were performed in ClustalW (Thompson *et al*., 1994). Determining the geographic origin of *Ae. albopictus* populations invading Palestine, phylogenetic relationships between *COI* and *ND5* sequences recorded in Palestine and previously published sequences were assessed by using Maximum Likelihood method based on the Tamura-Nei model (Tamura *et al*., 1993). Initial tree(s) for the heuristic searches were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. The robustness of the groupings in the Maximum Likelihood method was assessed with 1000 bootstrap resampling. The population diversity indices such as numbers of haplotype (h), haplotype diversity (Hd), nucleotide diversity (π), and the neutrality indices including (Tajima's D, Fu's and Li's F test statistic, Fu and Li's D test statistic and Fu's Fs statistic) were calculated using DnaSP 5.1001 (Librado and Rozas, 2009). Network of haplotypes of *COI* and *ND5* sequences of *Ae. albopictus* were analysed using the R-package pegas (Paradis, 2010) that implements the haplotype network algorithm presented in Bandelt *et al*. (1999).

Results

Phylogenetic relationship

The *COI* and *ND5* genes were amplified in all extracted DNA samples. Twenty one amplified *COI* products (597-bp length) and 10 amplified *ND5* products (405-bp) were successfully Sanger-sequenced and analysed. The sequences of these genes showed high identity with the same genes belonged to the *Ae. albopictus* using Basic Local Alignment Search Tool (BLAST). The sequences were deposited in GenBank database under the accession numbers MN064753-MN064773 (*COI* sequences) and MN064774- MN064783 (*ND5* sequences).

To determine the geographic origin of *Ae. albopictus* population invading Palestine, molecular phylogenetic analysis by Maximum Likelihood method based on *COI* and *ND5* sequences obtained from *Ae. albopictus* specimens in Palestine and previously published sequences in other countries were conducted. After multiple sequence alignments (figure 1), the *COI* and *ND5* sequences were clustered with *COI* and *ND5* sequences from tropical, sub-tropical and temperate areas (figure 2).

Mitochondrial DNA analysis and haplotypes

To understand the genetic diversity of *Ae. albopictus* in Palestine, mtDNA nucleotide sequences of 21 *COI* (595-bp) and nine *ND5* (334-bp; the sequence deposited under the acc. no. MN064782 was excluded from analyses because of many sequencing errors) specimens were analysed. Three haplotypes were detected each for *COI* and *ND5* genes. The main haplotypes were Hap-2 (19 out of 21 specimens, 90.5%) for *COI* and Hap-1 (7 out 9 specimens, 77.8%) for *ND5* (figure 3, table 1). The haplotype diversity was 0.186 and 0.417, while the nucleotide diversity was 0.00080 and 0.00133 for *COI* and *ND5* genes, respectively (table 2). The MJ networks constructed from haplotypes of *COI* and *ND5* sequences did not show "star-like" expansion with a major central Hap-2 and Hap-1 haplotype, respectively. The numbers of mutational steps between the major haplotype and the others ranged from two (acc. no. MN064753) to three (acc. no. MN064758) for the *COI* gene, and one mutation (acc. nos. MN064777 and MN064783) for the *ND5* gene.

Low levels of polymorphism were detected in both *COI* and *ND5* genes. Overall, there were five single nucleotide polymorphisms (SNPs) or singleton variable sites between the major core haplotype and the other haplotypes for the *COI* gene (figure 3). Similar results were obtained for the *ND5* gene with two SNPs between the major core haplotype and the other haplotypes (figure 3). In *COI* sequences, These singleton variable sites were specified on sites 915, 1391 and 1435 for MN064759 sequence and sites 939 and 963 for MN064754 sequence, while in *ND5* sequences, singleton variable sites were specified on sites 1391 and 1435 for MN064777 sequence and MN064783 sequence, respectively. The neutrality test for *COI* gene showed that Tajima's D = −1.98137 (P < 0.05), Fu's and Li's F test = −3.02181 (P < 0.05), Fu and Li's D test = −2.86511(P < 0.05) and Fs = −0.270. The neutrality test for *ND5* gene showed that Tajima's D = −1.36240, Fu's and Li's F test = −1.62607, Fu and Li's D test = −1.50507 and Fs = −1.081 (table 2).

Discussion and conclusions

These data represent the first study of the genetic variation and differentiation of *Ae. albopictus* populations invading and circulating in Palestine. In this study we focused on both *COI* and *ND5* genes which have been extensively used to assess the genetic diversity of *Ae. albopictus* populations across geographic regions (Birungi and Munstermann, 2002; Daravath *et al*., 2013; Battaglia *et al*., 2016). These markers are used widely because of their maternal inheritance, haploid status, and high rate of evolution.

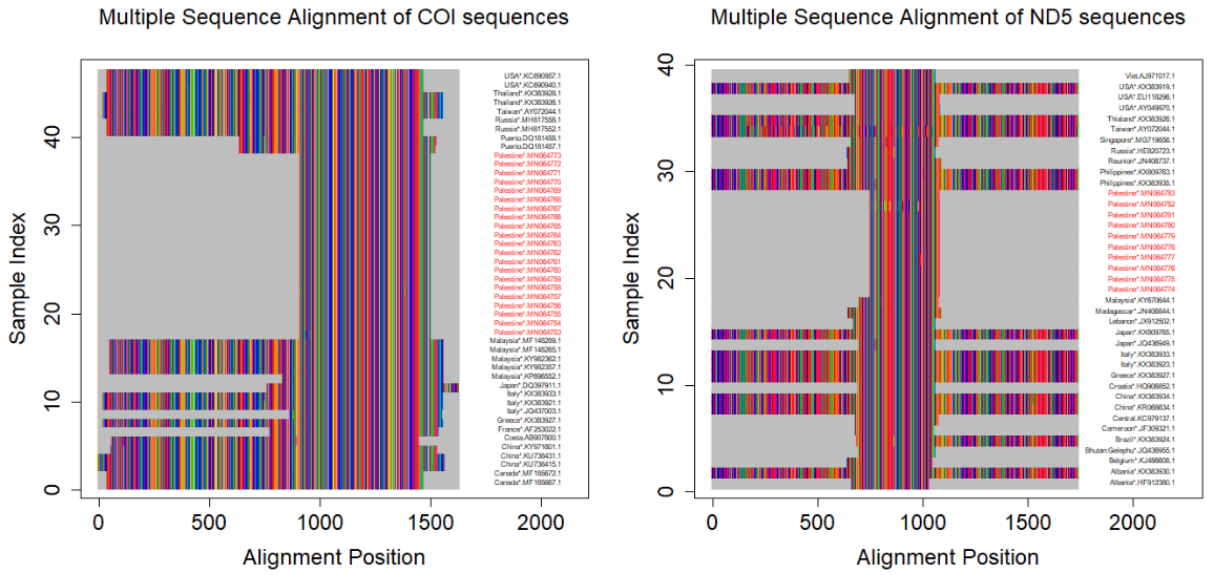

Figure 1. Multiple sequence alignments of Palestine and Non-Palestine sequences for *COI* and *ND5*, showing the available overlap of sequence positions for all samples. Colours represent nucleobases (A = red, C = green, G = yellow, T = blue). Nucleotide substitutions of haplotypes are shown in the zoom-in alignment plots in figure 3.

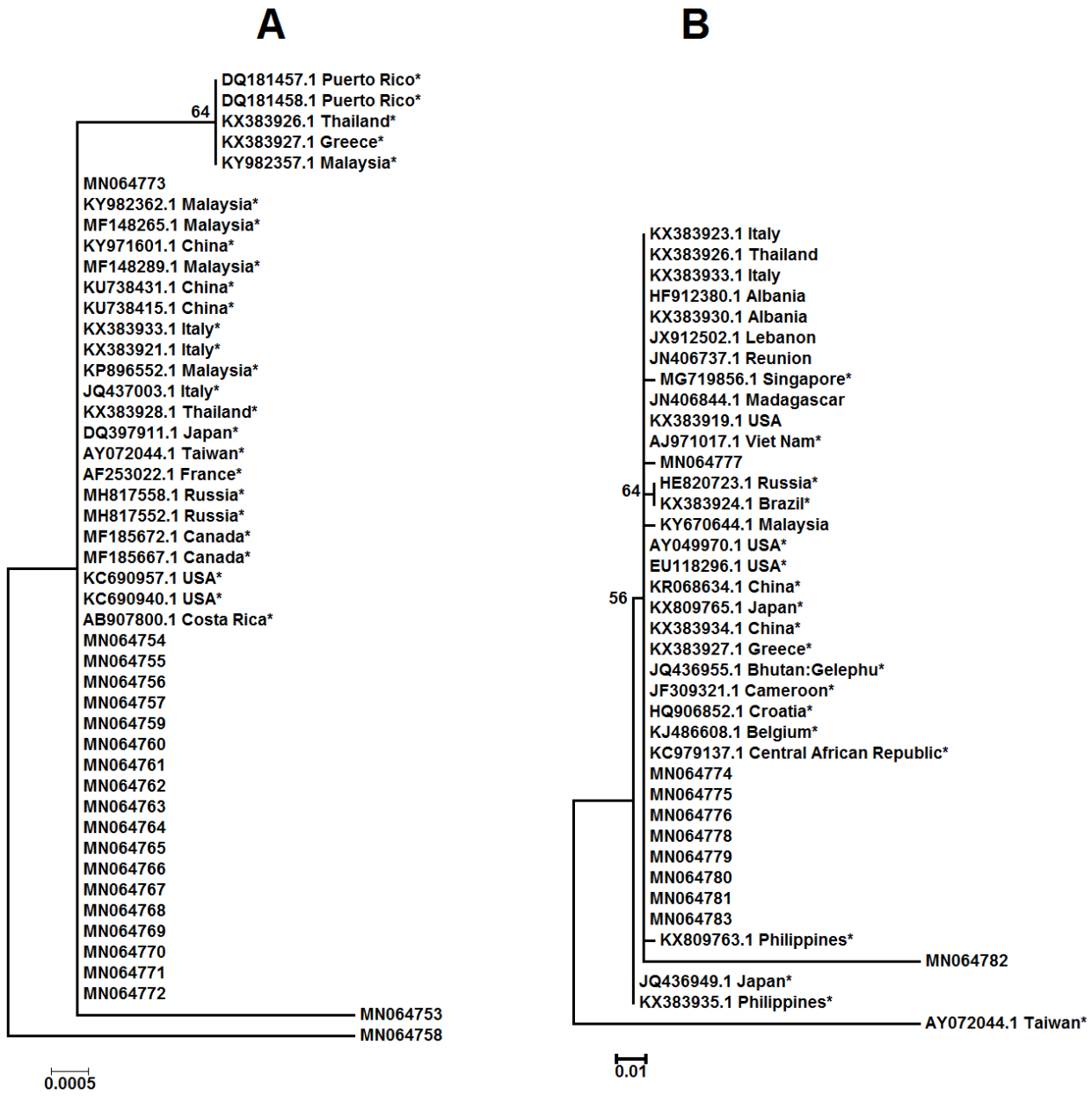

Figure 2. Molecular phylogenetic analyses by maximum likelihood method of *Ae. albopictus* based on *COI* (**A**) and *ND5* (**B**) sequences data from Palestine and other countries. Reference sequences for the *COI* and *ND5* genes of *Ae. albopictus* from different countries are denoted by asterisk.

Table 1. *Ae. albopictus* haplotype distribution for *COI* and *ND5* genes.

Haplotype	*COI* haplotype		*ND5* haplotype	
	Number of specimens	Sequence	Number of specimens	Sequence
Hap-1	1	MN064753	7	Other sequences except Hap-2 and Hap-3
Hap-2	19	Other sequences except Hap-1 and Hap-3	1	MN064777
Hap-3	1	MN064758	1	MN064783

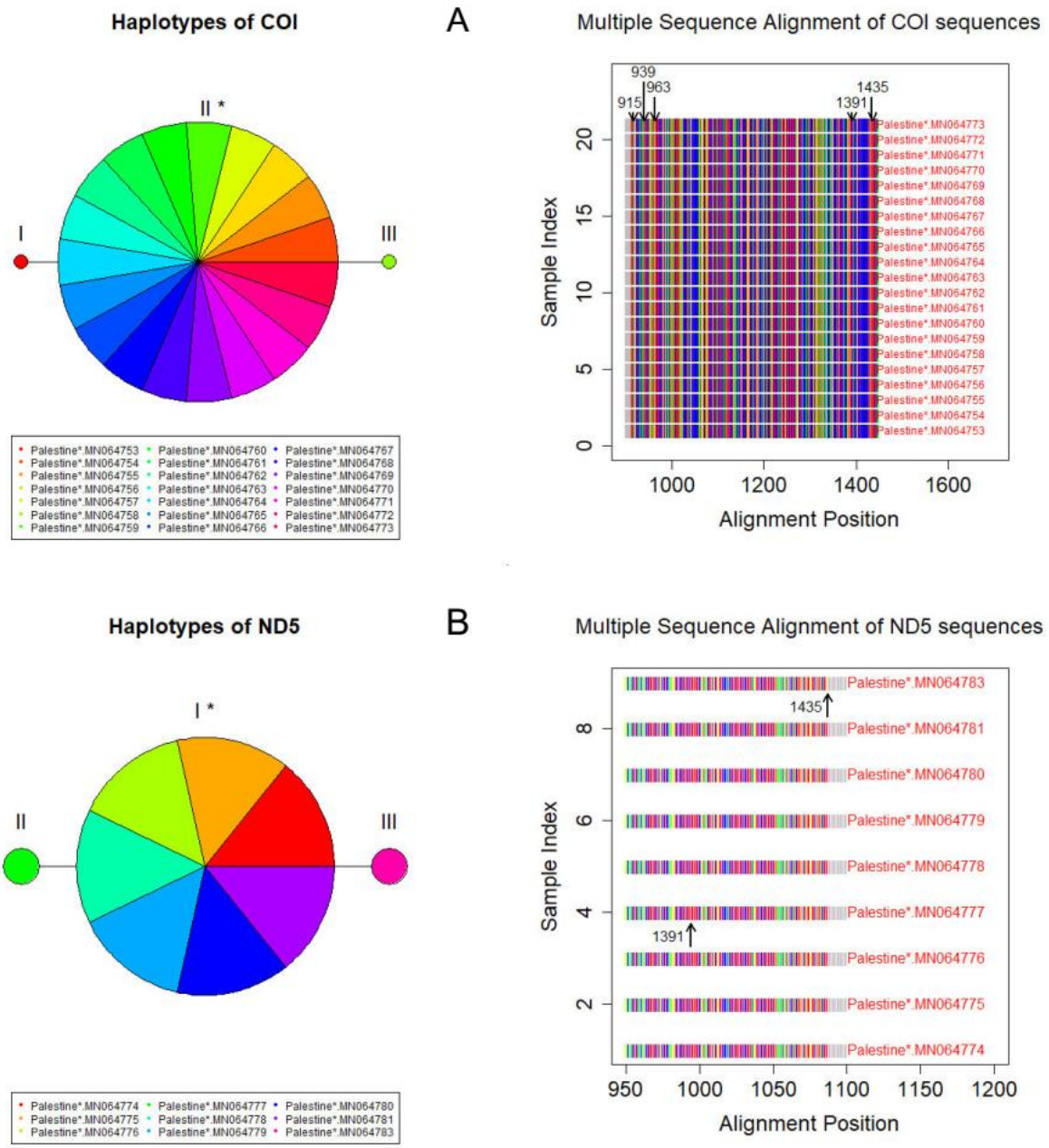

Figure 3. Median-joining network of *COI* (**A**) and *ND5* genes (**B**) of the haplotypes of *Ae. albopictus* isolates. A circle represents each haplotype. The asterisk denotes the founder haplotype. The size of circle is relative to haplotype frequency. Each colour represents one specimen. Bars indicate the number of nucleotide substitutions which differentiate the *COI* or *ND5* haplotypes from the founder haplotype. The MN064782 sequence was excluded from analyses. Arrows indicate positions of nucleotide substitutions in additional alignment plots at the right.

We found that the *Ae. albopictus COI* and *ND5* sequences from Palestine grouped with sequences from the same genes from individuals found in tropical, sub-tropical and temperate areas. However, we were not able to find apparent relation between genetic and geographic distances carried out using Maximum Likelihood cluster analysis. Results from a study analysing *COI* sequences (Kamgang *et al*., 2011) showed that Cameroonian *Ae. albopictus* were related to specimens originating from tropical rather than temperate or subtropical areas. Several reports have indicated that *Ae. albopictus* populations invading Brazil were genetically associated with tropical rather than temperate Asian populations (Birungi and Munstermann, 2002; Mousson *et al*., 2005). Birungi and Munstermann (2002) showed that one mutation step discriminates US and Brazilian populations at the *ND5* marker, and the marker later showed no variation between *Ae. albopictus* populations anywhere in the world except Brazil (Maia *et al*., 2009; Usmani-Brown *et al*., 2009). Mousson *et al.* (2005) also found evidence that Brazilian populations could belong to a separate genetic group because they formed a slightly different phylogenetic group with populations of the Indochinese peninsula according to mtDNA markers.

Table 2. Summary statistics for mtDNA gene polymorphism in *Ae. albopictus* from Palestine. Statistical significant differences ($P < 0.05$) are marked with asterisks.

Nucleotide diversity indexes	Gene	
	COI	*ND5*
Number of nucleotide sites	595	334
Number of sequence	21	9
Variable (polymorphic) sites (singleton variable sites)	5	2
Parsimony informative sites	0	0
Number of haplotypes	3	3
Haplotype (gene) diversity (Hd)	0.186	0.417
Variance of haplotype diversity	0.01215	0.03635
Nucleotide diversity (π)	0.00080	0.00133
Average number of nucleotide differences (k)	0.476	0.444
Total number of InDel sites analysed	0	0
Fu and Li's D test statistic	−2.86511*	−1.50507
Fu and Li's F test statistic	−3.02181*	−1.62607
Tajima's D	−1.98137*	−1.36240
Fu's Fs statistic	−0.270	−1.081

The level of polymorphism found in *Ae. albopictus* isolates from Palestine within *COI* and *ND5* sequences was low, with three haplotype s each for the *COI* and *ND5* genes. These results are consistent with previous studies of populations sampled in different newly invaded areas (Birungi and Munstermann, 2002; Maia *et al*., 2009; Usmani-Brown *et al*., 2009; Kamgang *et al*., 2011; Shaikevich and Talbalaghi, 2013), in which the number of haplotypes did not exceed five per country, irrespective of the mtDNA marker used (*ND5*, *COI* or *cytochrome b*). Results published previously from native areas including Japan showed a high genetic diversity with 62 *ND5* haplotypes found in 174 individuals, and 66 *COI* haplotypes in 346 individuals (Porretta *et al*., 2012; Zhong *et al*., 2013).

Analysis of the *COI* and *ND5* gene sequences indicated that the haplotype diversity was 0.186 and 0.417, and the nucleotide diversity 0.00080 and 0.00133, respectively, while the number of haplotypes was three each for the *COI* and *ND5* gene. These results suggest that the Palestinian *Ae. albopictus* haplotypes were genetically not very diverse. This assumption is supported by the analysis of the median-joining network of *COI* and *ND5* which did not show a ''star-like'' expansion networks, suggesting population expansion haplotypes from a central founder haplotype with few mutational steps. Also the overall negative values of neutrality tests such as Tajima's D test, Fu's and Li's D and F statistics, support the hypothesis of recent expansion of *Ae. albopictus* in Palestine after the introduction of founder haplotype. The *ND5* sequences had high haplotype (gene) diversity compared to the *COI* sequences, but this result may also be attributed to the low number of tested samples.

Our haplotype and nucleotide diversities correlate well with other studies describing haplotype diversities of the *Ae. albopictus* populations ranging from 0.0-0.64 in Central Africa, 0.37-0.83 in China, Singapore, Japan, Italy and the USA, 0.0-0.7 in France, Mauritius, Seychelles and Southeastern Africa, 0.282 in Croatia, Serbia and Montenegro, as well as 0.187 in Brazil, 0.749 in Venezuela and Colombia (Aguirre-Obando and Navarro-Silva, 2017). Published nucleotide diversities (π) ranged from 0.000-0.003 in Central Africa to 0.06-0.30 in China, Singapore, Japan, Italy and the USA, 0.00-0.02 in France, Mauritius, Seychelles and Southeastern Africa, 0.00358 in Venezuela and Colombia, 0.000064 in Croatia, Serbia and Montenegro as well as 0.00044 in Brazil (Kamgang *et al.*, 2011; Aguirre-Obando and Navarro-Silva, 2017). Most of the haplotype diversity (Hd) studies results were lower than 0.7 (Aguirre-Obando and Navarro-Silva, 2017). Our results revealed low levels of nucleotide diversity within *Ae. albopictus* population. This pattern may be due to severe, repeated, or long periods of population bottleneck, natural environmental conditions or events induced by humans may play a role in the reduction of effective population size. Population bottleneck caused big losses in genetic variation due to genetic drift and may reduce genetic variability and correlative effects on fitness. *Aedes* mosquitoes may experience periodic bottlenecks in population size due to changes in host abundance and breeding sites. Also, the introduction phase is usually subject to severe bottlenecks that reduce the genetic variability of founding populations to levels incompatible with an expansion phase and recent introduction of founder population (Aguirre-Obando and Navarro-Silva, 2017; Naim *et al.*, 2020). This resulted in reduced levels of genetic variation which have been observed in this current study.

These data provide useful information for future studies focusing on the evaluation of the risk of diseases transmission, insecticide resistance development and population spread and may help to development of appropriate vector control programs in Palestine and other countries in the region. Further molecular analyses are needed to obtain more information about this insect in Palestine.

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