Deanship of Graduate Studies
Al-Quds University

Genetic Analysis and Population Structure of *Phlebotomus sergenti* Sandflies; Vectors of *Leishmania tropica* in Palestine

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Genetic Analysis and Population Structure of *Phlebotomus sergenti* Sandflies; Vectors of *Leishmania tropica* in Palestine

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Jerusalem- Palestine

1445 – 2024

Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this study or any part thereof has not been submitted for a higher degree to any other university or institution.

Signature: Bushra Tahboub

Bushra Abdel Qader Tahboub Al-Amawi

Date: 19-May-2024

Dedication

To my mother and father

To my sister and brothers

To my friends

And to knowledge seekers

Bushra Tahboub Al-Amawi

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All praise be to Allah, the lord of the world.

I would like to thank everyone who contributed to this work and supported me during this time. In particular, I would like to thank my supervisor Prof. Dr. Omar Hamarsheh for all his guidance and help throughout this study.

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Abstract

Phlebotomus (Ph.) sergenti is a widespread sandfly, it is considered a vector of *leishmania tropica* in Palestine and other countries; it is the cause of cutaneous leishmaniasis. This study aimed to investigate and analyze the phylogenetic and phylogeographic structure of *Ph. sergenti* among separated populations in the West Bank; Palestine. Population analysis of these sandflies is necessary for developing models that may provide better understanding of their current geographic distribution.

The genetic population structure analysis of fifty Ph. sergenti sandflies were collected from West Bank, Palestine. This study inspected the genetic differentiation between *Ph. sergenti* populations using a 442 bp fragment of *Cyt. b* mtDNA gene. Maximum parsimony tree and median joining network were constructed depending on the identified haplotypes. Additionally, one hundred *Ph. sergenti* sequences previously deposited in the GenBank from ten countries (Iran, Pakistan, Afghanistan, Turkey, Cyprus, Greece, Syria, Lebanon, Morocco and Spain), were included in the study.

Nine haplotypes were obtained from the Palestinian populations; six of them were private haplotypes, suggesting the presence of genetic isolation. The global analysis exhibited forty-five global haplotypes and grouped according to their geographical location. Pairwise F_{ST} values ranged between 0.58533 and 0.89288, indicating a high genetic differentiation and low genetic flow.

Population genetic analysis of *Ph. sergenti* is considered crucial and may be needed to design an appropriate insecticide spraying programs and for sandfly control models, to restrict the transmission of *leishmania* parasites in the endemic areas of cutaneous leishmaniasis.

Keywords: Sandfly, *Phlebotomus sergenti*, genetic structure, mtDNA, *Cyt. b*, haplotypes.

العنوان: التحليل الوراثي لمجموعات فليبوتومس سيرجنتي من ذبابة الرمل الفاصدة؛ ناقلة طفيل الليشمانيا الجلدية في فلسطين.

إعداد: بشرى عبد القادر طهبوب الأموي

بإشراف: د. عمر يوسف حمارشة

الملخص:

تعد ذبابة الرمل *Phlebotomus sergenti*، ذبابة منتشرة على نطاق واسع، وتعتبر الناقل الأساسي لطفيل اللشمانيا الجلدية ، والتي تسبب بشكل رئيس داء الليشمانيات الجلدي. هدفت هذه الدراسة إلى فحص وتحليل البنية التطورية الجينية والجغرافية لمجموعات ذبابة الرمل *Ph. sergenti* في الضفة الغربية في فلسطين. تعد دراسة مجتمع ذبابة الرمل أمرًا ضروريًا لتطوير النماذج التي قد توفر فهمًا لتوزيعها الجغرافي الحالي بشكل أفضل. شملت الدراسة التحليلية خمسين ذبابة رمل *Ph. sergenti* تم جمعها من فلسطين. فحصت الدراسة الاختلاف الوراثي بين مجموعات *Ph. sergenti* عبر أخذ مقطع جيني من الحمض النووي الخاص بالمايتوكندريا (mtDNA) الذي يبلغ طوله 442 قاعده نيتروجينية من جين Cytochrome b.

تم إنشاء شجرة التطور الجينية باستخدام معيار الحد الأقصى (Maximum parsimony) وشبكة (Median Joining Network) اعتمادًا على الأنماط الفردية المحددة (Haplotypes). بالإضافة إلى العينات السابقة، تم تضمين مئة عينة من *Ph. sergenti* تم نشرها سابقًا في المستودع الجيني العالمي من عشر دول (إيران، باكستان، أفغانستان، تركيا، قبرص، اليونان، سوريا، لبنان، المغرب وإسبانيا) للحصول على تحليل للمجموعات على نطاق جغرافي أوسع.

وأظهرت النتائج تسعة أنماط فردية من مجموعات ذبابة الرمل *Ph. sergenti* من مناطق فلسطينية في الضفة الغربية، ستة منها كانت نماذج فردية خاصة، مما يشير إلى وجود عزلة وراثية بين مجموعات *Ph. sergenti* الفلسطينية. أما تحليل العينات العالمية فقد أظهرت خمسة وأربعين نمط فردي، تتوزع تبعًا للموقع الجغرافي. عرضت نتائج F_{ST} قيمًا عالية تراوحت بين 0.58533 و0.89288، مما يشير إلى تمايز جيني عالي وتدفق جيني منخفض بين المجموعات. كما ويعد التحليل الوراثي لمجموعات *Ph. sergenti* أمرًا بالغ الأهمية لتصميم نماذج مكافحة ذبابة الرمل، للحد من انتشار طفيليات الليشمانيا الجلدية.

<u>الكلمات المفتاحية:</u> ذبابة الرمل، *Phlebotomus sergenti* ، التحليل المجتمعي الجيني، الحمض النووي الخاص بالمايتوكندريا، cytochrome b ، الأنماط الفردية.

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Abbreviations

WHO: World Health Organization

CDC: Centers for Disease Control and Prevention

Ph.: *Phlebotomus*

L.: *leishmania*

CL: Cutaneous leishmaniasis

mtDNA: Mitochondrial DNA

Cyt b: Cytochrome b gene

EF-1α: Elongation factor - 1-alpha

ITS: Ribosomal internal transcribed spacer

ND4: NADH dehydrogenase subunit 4

COI: Cytochrome c oxidase subunit 1

µL: Microliter

Xg: Gravitational force

°C: Celsius

PCR: Polymerase Chain Reaction

g: Gram

H: Haplotype

Sec: Second

bp: Basepair

H.d: Haplotype diversity

K: Nucleotide differences

Pi: Mean nucleotide diversity

MP: Maximum Parsimony

Chapter One

1. Introduction

1.1. Phlebotomine Sandflies

Phlebotomus sandflies (Fig. 1.1) are arthropods belong to Diptera (two-winged flies), suborder Nematocera, family Psychodidae, and subfamily Phlebotominae (Cecílio et al., 2022). Phlebotomine sandflies are small flies, their sizes range between 2-3mm (Shaw & Rangel, 2018).

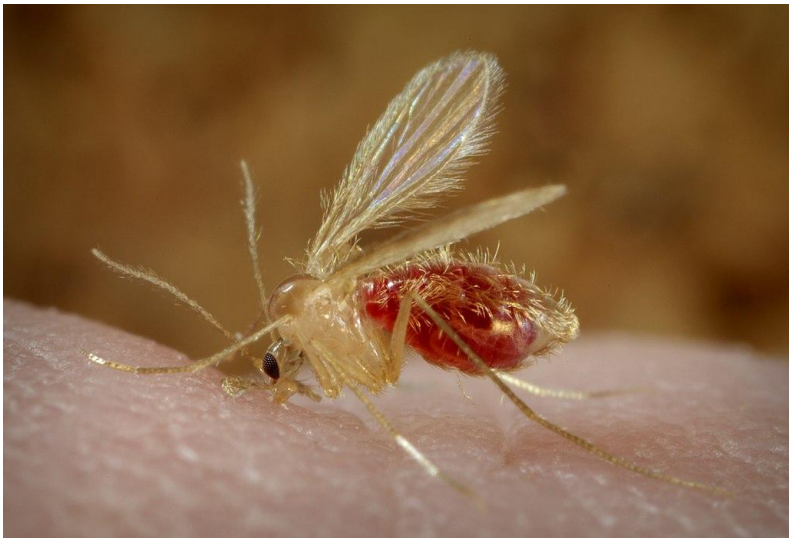

Figure 1.1 Phlebotomine sandfly having blood meal (Gathany. J. 2006. CDC.)
https://phil.cdc.gov/details.aspx?pid=10277

Around 1000 species of sandflies are known around the world in numerous regions; they are distributed between latitude 50° North and latitude 40° South (Cecílio et al., 2022; Shimabukuro et al., 2017). They also have a wide altitude from -300 meter below the sea level in Jericho, Palestine, to 3600 meters above the sea level in Iran (Akhoundi et al., 2016).

It is known that *Phlebotomus* sandflies are vectors of pathogens, they can transmit several viruses as Toscana virus in the Mediterranean region and Vesicular stomatitis virus in Central and Northern-South America (Reed, 2018). Also, they can transmit bacteria as *Bartonella bacilliformis* which cause Carrion's disease (CDC, n.d.-a; Cecílio et al., 2022) and protozoa i.e. *leishmania*.

Around 90 species of phlebotomine sandflies are known as a vector of different species of *leishmania* (WHO, 2023), which can cause different types of leishmaniasis as; cutaneous, mucocutaneous and visceral leishmaniasis (Kravchenko et al., 2004).

1.2. *Phlebotomus sergenti*

Phlebotomus sergenti (Fig 1.2) is a widespread sandfly that transmit *leishmania*; particularly *leishmania tropica* in different regions (Waitz et al., 2019), it is considered as the causative of anthroponotic cutaneous leishmaniasis (CL) in the old world i.e. Asia, Europe and Africa (Karmaoui et al., 2022).

Ph. sergenti is considered as highly anthropophilic (Kamhawi et al., 1995), as it is attracted to human blood and rely on it to afford nutrients to the development of their new eggs (Cecílio et al., 2022).

Figure 1.2 *Phlebotomus sergenti* sandfly under dissecting microscope.

1.2.1. *Ph. sergenti* Habitat and Activity

Ph. sergenti populated in rural areas and outskirts of cities; they inhabit rocky areas such as caves and rock piles, and areas with irrigated green plants and shade. *Ph. sergenti* activity varies according to the climate and the seasons; for instance their activity is present during April until November, like other sandfly species; their activity peak is concentrated from July until September and have a nocturnal activity pattern that starts after the sunset (Kravchenko et al., 2004; Waitz et al., 2019).

1.2.2. Geographical Distribution of *Ph. Sergenti*

Phlebotomus sergenti inhabit wide distribution areas in the World (Fig. 1.3), at tropical and sub-tropical zones. In Africa, *Ph. sergenti* was found in Morocco (Karmaoui et al., 2022) and in Tunisia (Chaouch et al., 2021). In the southern part of Europe, *Ph. sergenti* was found in Spain, the southern France, the Canary Island, Italy and Greece (Merino-Espinosa et al., 2016). It is also found in Asia; in Iran, Iraq, Palestine, Jordan, Syria, Turkey, Pakistan and Afghanistan (Kamhawi et al., 1995; Moin-Vaziri et al., 2007; Vaselek & Volf, 2019).

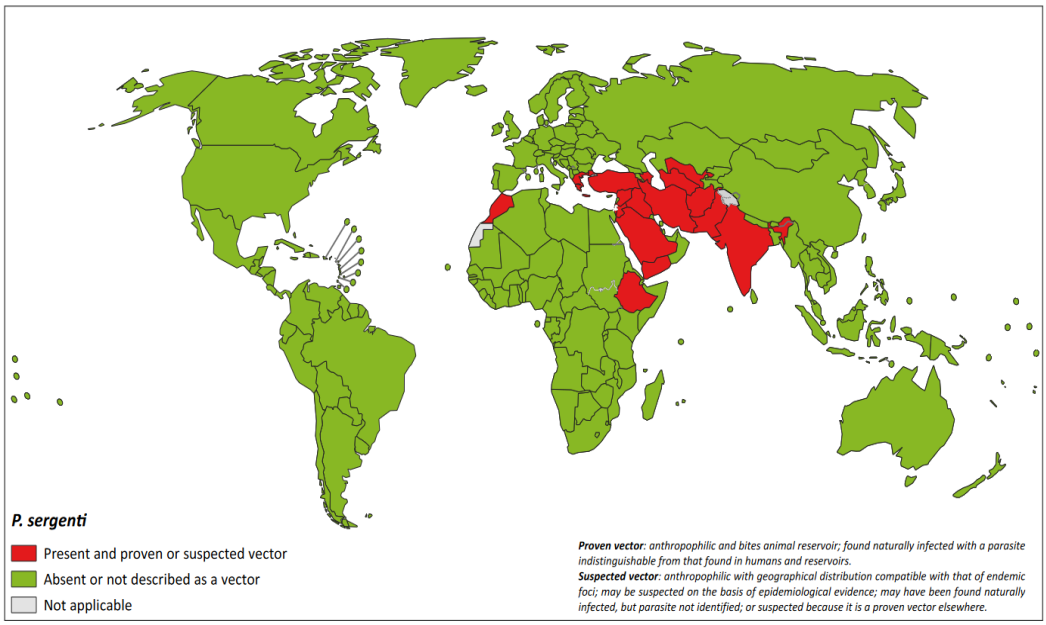

Figure 1.3 Geographical distribution of *Ph. sergenti* worldwide (WHO, Geographical distribution of leishmaniasis vectors-*Phlebotomus sergenti*, 2019)

In Palestine, an activity of *Ph. sergenti* were detected in the West Bank in the Eastern slopes of Jerusalem and the areas between Jerusalem and Jericho including Abu Dies (Moncaz et al., 2012; Waitz et al., 2019) and in the north of Palestine in the Upper Galilee to the north of Tiberias (Kravchenko et al., 2004).

1.2.3. *Ph. sergenti*: The vector of *Leishmania tropica*

Leishmaniasis is a disease caused by *leishmania* species, it is found in more than 90 countries around the world, in tropical and subtropical areas. About 0.7-1 million of new cases of cutaneous leishmaniasis occurs worldwide, and less than 100,000 new case of visceral leishmaniasis (WHO, 2023).

The transmission of *leishmania tropica* is correlated with the activity of its correspondent vector; *Ph. sergenti* sandfly. Therefore, the rate of leishmaniasis increase at the season when *Ph. sergenti* activity increase, also *leishmania* transmission risk increased right after the sunset, the same way as the nocturnal activity of other sandfly (CDC, n.d.-b; Karmaoui et al., 2022). Worth mentioning that the presence of *Ph. sergenti* sandflies in any area doesn't mean that it is infected by leishmania but provide risks for the transmission of the disease once infected from an infected host (Karmaoui et al., 2022).

Female sandfly have to feed on blood in order to develop around 70 - 90 eggs (Cecílio et al., 2022). While infected female sandfly, is having blood meal, it injects the infective stage; the metacyclic promastigotes to human skin facilitated by the sandfly saliva which is secreted during biting human skin (Waitz et al., 2019). Human phagocytic cells ingest *leishmania* parasites, and in there, promastigote stages transform into a motile rounded amastigote stage. Amastigotes infect other cells developing skin lesions in case of cutaneous leishmaniasis or involvement of visceral organs like spleen causing splenomegaly and finally visceral leishmaniasis (CDC, n.d.-b).

1.3. Population Analysis of *Ph. sergenti*

Genetic population analysis is a systemic study that explains the evolutionary change in a population, by the analysis of the genetic variation and their frequencies within and between populations overtime. This variation or heterozygosity of genetic alleles is due to evolutionary forces like random genetic drifting, mutation, selection, and migration (Amorim, 2013).

Construction of a population structure of sandflies is important to understand the relationship between the genetic variation among populations, the geographical distribution of these populations, their ability to adapt in the environmental conditions which probably enhance its ability to transmit *leishmania* parasites. On the other hand, understanding the population structure provide a better understanding of their vectorial capacity and competence, and consequently their potential epidemiological effects on leishmaniasis disease (Flanley et al., 2018). This genetic information helps to understand the dispersal of sandflies and the *leishmania* parasites they transmit, which is crucial to design control strategies to interrupt *leishmania* transmission cycle in the endemic areas.

1.3.1. Mitochondrial Genetic Markers

Mitochondrial DNA is a maternal-inherited DNA (Esseghir et al., 1997), it is very useful genetic marker; and used extensively to study the differences between insect species and populations. It was used previously to study closely related sandflies including populations of *Ph. sergenti* from Morocco (Kacem et al., 2023).

Mitochondrial DNA has many advantage features that make it a wide usable genetic marker; mtDNA does not have repair enzymes for replicated or damaged DNA; therefore it has many transition and transversion mutations (Castro Antönia & Ramon, 1998). It also has high rate of evolution (Bronstein et al., 2018), and evolve faster than nuclear DNA (Kocher et al., 1989).

Mitochondrial DNA has variable polymorphic gene patterns and haplotypes which are more preferred for species and population identification as well as phylogenetic analysis (Flanley

et al., 2018; Seddigh & Darabi, 2018). In addition, mtDNA lacks the ability of recombination which results in nucleotide sequences that reflect the evolutionary history (Hamarsheh et al., 2007). All these properties make the mtDNA ideal for the evolutionary and phylogenetic studies.

1.3.1.1. *Cytochrome b* Gene:

Mitochondrial DNA encodes several proteins, tRNA and rRNA which are needed for mitochondrial metabolism (Castro Antönia & Ramon, 1998).

Cytochrome b gene (*Cyt. b*) is a mtDNA, its size is about 1,140 nucleotides and translated into a reductase protein in the electron transport chain which is involved in the cellular respiration.

As other mtDNA genes; *Cyt. b* gene is maternally inherited and has a slow rate of silent mutations (Flanley et al., 2018), therefore it has a variable sequences which provides a phylogenetic information in the level of both intraspecific and intergeneric for population in the same geographical area or areas with long distance geography (Kocher et al., 1989; Seddigh & Darabi, 2018).

1.4. Literature Review

As aforementioned *Ph. sergenti* is the main vector of *L. tropica* and the causative agent of CL, several studies proved that *Ph. sergenti* is dominant in the foci where CL is extensively reported (Dvorak et al., 2011; Yahia et al., 2004). This medically important sandfly urged the researchers to investigate the intraspecific genetic variability and its vectorial capacity as well as its competence in transmitting *Leishmania* infection.

Genetic analysis of *Ph. sergenti* can be performed using genetic markers depending on two sources; either nuclear DNA or mtDNA (Kacem et al., 2023) analyzed the genetic diversity of *Ph. sergenti* in four locations in Morocco. They had made a comparison between mtDNA and nuclear DNA to observe which marker is better for genetic and phylogenetic diversity of *Ph. sergenti*. The used markers were *Ef-1α* as a nuclear marker and *Cyt. b* as mitochondrial marker. Their study proved that *Cyt. b* is much useful than *Ef-1α* in which *Cyt. b* provides more polymorphic sites and able to resolve *Ph. sergenti* populations in the country. The low polymorphic sites in *Ef-1α* are due to the conserved nature of this nuclear gene as explained by the authors (Kacem et al., 2023). In contrast to *Ef-1α* haplotypes where its phylogenetic tree tends to be structured in one clade and it did not show any geographical isolation, the constructed network of the obtained *Cyt. b* haplotypes distribution tended to cluster in accordance with their geographical origins. It is noted that *Ef-1α* haplotypes are separated with single mutation, this is due to the conserved nature of this nuclear gene, comparing to mtDNA (Kacem et al., 2023).

In the aspect of the genetic differentiation, *Cyt. b* displayed high F_{ST} values, indicating high genetic differentiation between *Ph. sergenti* populations, in contrast to *Ef-1α* which shows low genetic differentiation between the same populations (Kacem et al., 2023).

In another study by (Barón et al., 2008); where mtDNA *Cyt. b* and rDNA *ITS* markers were compared using samples from Spain and Morocco. They found high genetic diversity between *Ph. sergenti* populations in the neighboring countries. The ratio of *ITS* haplotypes to *Cyt. b* haplotypes was (3:7) in Spain and (3:10) in Morocco. All ITS haplotypes were belong to one lineage; however the *Cyt. b* haplotypes were grouped in four lineages. This shows that the

maternal inheritance of mtDNA provides greater genetic information of intraspecific variability than nuclear DNA.

Even though *L. tropica* is not endemic is Spain; *Ph. sergenti* is widely present in the country, (Barón et al., 2008)studied the intraspecific variability among *Ph. Sergenti* trying to control any possible migrant flow of the parasite.

The study emphasize that each geographical area has a specific haplotypes, i.e. haplotypes are much related in populations in close regions, for example Moroccan haplotypes are similar to each other, however different from the Spanish ones. Knowing that there is a shared haplotypes between both countries, since Spain and Morocco are geographically very close countries.

Cyt. b marker is extensively used in the investigation of genetic diversity in sandflies (Esseghir et al., 1997; Kacem et al., 2023). In Morocco (Yahia et al., 2004)had genetically analyzed *Ph. sergenti* sandflies and identified their haplotypes depending on the 3` end of *Cyt. b,* by using two related sequences; the first was CB3 fragment published by (Esseghir et al., 1997), however primers used to amplify this fragment did not work well on all *Ph. sergenti* samples. Therefore they designed internal primers based on the sequence of CB3 fragments obtained from previously sequenced *Ph. sergenti*. The new resulted fragment is called SER fragment. The obtained haplotypes were very useful and have great power to discriminate populations according to their geographical origins.

Other mtDNA markers other than *Cyt. b* have been used to analyze the genetic variation of sandflies. (Pavlou et al., 2022) used cytochrome c oxidase subunit 1 (*COI*), however *COI* have less sequence divergence than *Cyt. b*. Also NADH dehydrogenase subunit 4 (*ND4*) have been used in the analysis of *Ph. papatasi* populations from 18 countries, conducted by (Depaquit et al., 2008). Microsatellite markers is very useful for genetic population structure analysis of sandflies, in which microsatellites are numerously distributed in the genome and characterized by a high degree of polymorphism (Hamarsheh et al., 2009, 2018; Hamarsheh & Amro, 2011; Kacem et al., 2023)

The genetic variation of sandfly populations and their geographical dispersal is related to many factors, including geographical isolation, environmental factors; such as climate parameters, vegetation cover, soil type and consistency and many others (Flanley et al., 2018; Kacem et al., 2023).

The previous studies demonstrated that mtDNA markers are more informative and distinctive for sandfly intraspecific variability studies due to its maternally inherited and the existence of adequate sequence variation among individuals originated from different regions. In addition, haplotypes obtained from the mtDNA may provide useful information of the geographical distribution of *Ph. sergenti* sandflies

1.5. Study Significance

Little is known about genetic relationships among populations of most Phlebotomine sandflies including species of epidemiological significance like *Ph. sergenti* (Depaquit et al., 2008). Population analysis of these sandflies is necessary for developing models that may provide better understanding of their current geographic distribution. In addition, an improved knowledge of the biogeography, genetics and the behavior of these populations may help in designing appropriate control measures for the *Ph. sergenti* fly, which is important to restrict the expansion and the transmision of leishmaniasis.

1.6. Study Aims and Objectives

The main objectives of this proposed research are to collect, analyze several *Ph. sergenti* sandflies collected from the West Bank, Palestine to infer the population relationships among widely separated populations in the country. The main aims of this research include the following:

A. To collect sandflies from different regions in Palestine.
B. To genotype sandfly individuals from Palestine using mitochondrial DNA markers.
C. To carry on phylogenetic and median joining analysis using Mega11, DNASP, and Network softwares.
D. To investigate population differentiation, among *Ph. sergenti* populations locally and globally.

Chapter Two

2. Material and Methods

2.1. Samples Collection

Male *Ph. sergenti* sandflies (*n* =50) were collected from different locations of the West Bank with the collaboration with local entomologists. Collected specimens were preserved in 70 % ethanol for further processing and identification of *Ph. sergenti* sandfly males using appropriate identification keys.

Additional sequences were searched in the GeneBank (Table 2.1) which include previously deposited *Ph. sergenti* sequences from ten countries (*n* = 100); Iran(54), Afghanistan (9), Pakistan (2), Turkey(1), Cyprus (2), Greece (13), Syria(1), Lebanon(1), Morocco (14), and Spain (3) were included in the analysis, based on the targeted gene (*Cyt. b*) to provide a geographically broader genetic variation analysis (Table 2.1)

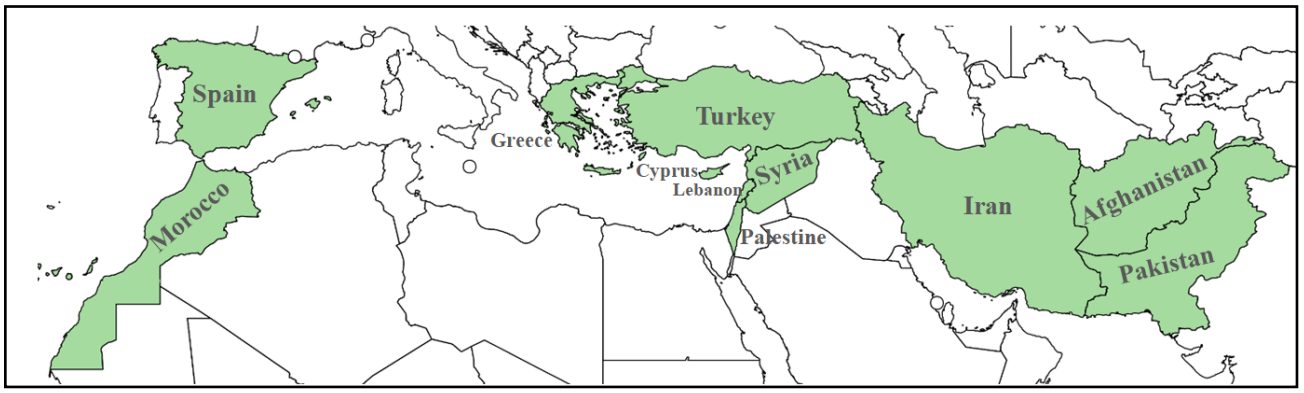

Figure 2.1 Geographical distributions of *Ph. sergenti* populations used in the study to analyze their genetic variation.

2.2. DNA Extraction

Individual *Ph. sergenti* sandflies were removed from alcohol into1.5mL microfuge tube, DNA was extracted according to the manufacturer's specifications using DNA extraction kit, (NucleoSpin®, Macherey-Nagel, Germany) with slight modification.

Each sandfly was grinded by sterilized wooden stick. The homogenized sandfly tissues were incubated over night at 65°C with 80µl T1 lysis buffer and 8µl proteinase K to facilitate the destruction of the insect tissues.

Samples were lysed by adding 80µl B3 buffer and incubated at 70°Cfor five minutes, then 80 µl absolute Ethanol was added to the lysate mixed well for five seconds, and the whole lysate volume was transferred into the binding column (Binding DNA in nucleoSpinRtissue XS) and centrifuged at 11,000X g for 1 minute.
Two washing steps were carried out with 50 µl B5 washing buffer, each step followed by centrifugation at 11,000X g, for 1 and 2 minutes respectively.

Ph. sergenti DNA were eluted into a new microfuge tube by adding 30 µl of elution buffer, centrifuged for 1 minute at max speed, and finally DNA was preserved and stored at - 20 °C. DNA concentration and purity for all sandfly samples were measured by Nanodrop (Table 2.1) (Nanodrop 1000 spectrophotometer; ThermoScientific, USA).

2.3. PCR Amplification

The 3′-end of the *Cyt. b* gene (SER fragment) was amplified by PCR using forward SERF1: (5' to 3': TACGATCAATTCCTAATAA) and reserve SER2 primers(5' to 3':ATTTACCTGCGTCTTTGT) (Yahia et al., 2004).

In a total volume of 25 µl, PCR amplification was carried out, volumes were pipetted as the following: 11 µl of PCR-ready mix (HY TAQ Ready mix, HyLabs), 1.2 µl of each primer (forward and reverse), 8.6 µl double distilled water purified for the use in PCR and 3 µl of *Ph. sergenti* DNA.

As described in (Yahia et al., 2004), the following PCR cycles were performed: denaturation at 94° C for three minutes; five cycles of denaturation at 94° C for 30 sec, annealing at 40° C for 30 sec, extension at 72° C for 90 sec; followed by 30 cycles of denaturation at 94° C for 30 sec, annealing at 42° C for 30 sec, extension at 72° C for 90 sec; and a final extension at 72 ° C for 10 minutes.

2.4. PCR Analysis and Sequencing

The PCR product was analyzed using 2% agarose gel electrophoresis, in which two grams of standard agarose were dissolved in 100 ml of 1X TAE buffer, the solution was boiled for 99 seconds, 8µl ethidium bromide was added to the gel solution before pouring it into casting tray.

PCR product was run at 120 volts for 60 minutes to separate the targeted PCR bands which visualized and captured by gel documentation system (GelDoc, Bio-Rad, USA).

All samples (50 samples from the West Bank, Palestine) were directly sequenced by using the forward and reverse primers that have been used for DNA amplification.

2.5. Phylogenetic and Phylogeographic Analysis

All sequences from West Bank, Palestine and other countries were imported to MEGA11 software (Tamura et al., 2021). The whole data set was edited and aligned using MUSCLE algorithm imbedded in MEGA software, sequences were analyzed and phylogenetic tree was constructed using Maximum Parsimony method implemented in MEGA11 software.

DnaSP6 (Rozas et al., 2017) analyze DNA polymorphism from nucleotide sequences, and apply some calculations of several measures to identify and analyze the haplotypes, their frequencies, polymorphic sites information, GC-contents, haplotype diversity and others.

Median joining calculations and analysis of the obtained haplotypes were carried out by Network 10.2.0 Software (Bandelt et al., 1999; Posada & Crandall, 2001) (http://www.fluxus-engineering.com), it was also used to generate Median Joining Network.

AQLEQUIN (Excoffi et al., 2005) software was used for genetic differentiation analysis among populations, and F_{ST} was estimated.

Table 2.1 DNA concentration (ng/µl) for Palestinian *Ph.* sergenti sandflies

Sample	DNA Concentration (ng/µl)	Sample	DNA Concentration (ng/µl)
PSE01	6	PSE26	1
PSE02	8.1	PSE27	3
PSE03	3.9	PSE28	2.5
PSE04	3	PSE29	8.7
PSE05	3.1	PSE30	1.2
PSE06	4	PSE31	4
PSE07	3.5	PSE32	5
PSE08	3.2	PSE33	5.1
PSE09	7.5	PSE34	3.4
PSE10	-	PSE35	10.6
PSE11	-	PSE36	4.4
PSE12	4.5	PSE37	1.7
PSE13	3.5	PSE38	2.2
PSE14	5.1	PSE39	3.2
PSE15	3.9	PSE40	1.2
PSE16	2.4	PSE41	1.2
PSE17	7.7	PSE42	3.4
PSE18	8.5	PSE43	3.6
PSE19	3.6	PSE44	11.2
PSE20	2.7	PSE45	2.2
PSE21	19	PSE46	3.3
PSE22	1.7	PSE47	1.1
PSE23	2.6	PSE48	2.8
PSE24	-	PSE49	-
PSE25	1.9	PSE50	-

Table 2.2 Geographical origins and GenBank accession numbers of 100 samples from different countries and included in the study.

No.	Country	Code	Accession Number	No.	Country	Code	Accession Number
1.	**Afghanistan**	AFG01	HM803187	51.	**Iran**	IRN27	DQ840372
2.		AFG02	HM803189	52.		IRN28	DQ840371
3.		AFG03	HM803190	53.		IRN29	DQ840370
4.		AFG04	HM803191	54.		IRN30	DQ840369
5.		AFG05	HM803192	55.		IRN31	DQ840368
6.		AFG06	HM803193	56.		IRN32	DQ840367
7.		AFG07	HM803197	57.		IRN33	DQ840366
8.		AFG08	HM803214	58.		IRN34	DQ840365
9.		AFG09	HM803216	59.		IRN35	DQ840364
10.	**Cyprus**	CYP01	OL376968	60.		IRN36	DQ840363
11.		CYP02	OL376927	61.		IRN37	DQ840362
12.	**Greece**	GRC01	OL376940	62.		IRN38	DQ840361
13.		GRC02	OL376939	63.		IRN39	DQ840360
14.		GRC03	OL376938	64.		IRN40	DQ840359
15.		GRC04	OL376937	65.		IRN41	DQ840358
16.		GRC05	OL376936	66.		IRN42	DQ840357
17.		GRC06	OL376935	67.		IRN43	DQ840356
18.		GRC07	OL376934	68.		IRN44	DQ840355
19.		GRC08	OL376933	69.		IRN45	DQ840354
20.		GRC09	OL376932	70.		IRN46	DQ840353
21.		GRC10	OL376931	71.		IRN47	DQ840352
22.		GRC11	OL376930	72.		IRN48	DQ840351
23.		GRC12	OL376929	73.		IRN49	DQ840350
24.		GRC13	OL376928	74.		IRN50	DQ840349
25.	**Iran**	IRN01	DQ840405	75.		IRN51	DQ840348
26.		IRN02	DQ840397	76.		IRN52	DQ840347
27.		IRN03	DQ840396	77.		IRN53	DQ840346
28.		IRN04	DQ840395	78.		IRN54	DQ840345
29.		IRN05	DQ840394	79.	**Lebanon**	LBN01	DQ840402
30.		IRN06	DQ840393	80.	**Morocco**	MAR01	DQ840404
31.		IRN07	DQ840392	81.		MAR02	OM860454
32.		IRN08	DQ840391	82.		MAR03	OM860324
33.		IRN09	DQ840390	83.		MAR04	EU980365
34.		IRN10	DQ840389	84.		MAR05	EU980364
35.		IRN11	DQ840388	85.		MAR06	OM860356
36.		IRN12	DQ840387	86.		MAR07	OM860371
37.		IRN13	DQ840386	87.		MAR08	OM860379
38.		IRN14	DQ840385	88.		MAR09	OM860389
39.		IRN15	DQ840384	89.		MAR10	OM860396
40.		IRN16	DQ840383	90.		MAR11	OM860401
41.		IRN17	DQ840382	91.		MAR12	OM860408
42.		IRN18	DQ840381	92.		MAR13	OM860417
43.		IRN19	DQ840380	93.		MAR14	OM860430
44.		IRN20	DQ840379	94.	**Pakistan**	PAK01	DQ840400
45.		IRN21	DQ840378	95.		PAK02	DQ840399
46.		IRN22	DQ840377	96.	**Turkey**	TUR01	DQ840403
47.		IRN23	DQ840376	97.	**Syria**	SYR01	DQ840401
48.		IRN24	DQ840375	98.	**Spain**	ESP01	EU980375
49.		IRN25	DQ840374	99.		ESP02	EU980381
50.		IRN26	DQ840373	100.		ESP03	EU980380

Chapter Three

3. Results

3.1. PCR and Gel Results

A 442 bp SER fragments (PCR product) was amplified from *Ph. sergenti* sandflies individual and separated on 2% agarose gel. Figure 3.1.A and 3.1.B displays bands of thirty-five representative samples of *Ph. sergenti* DNA (PSE1- PSE35).

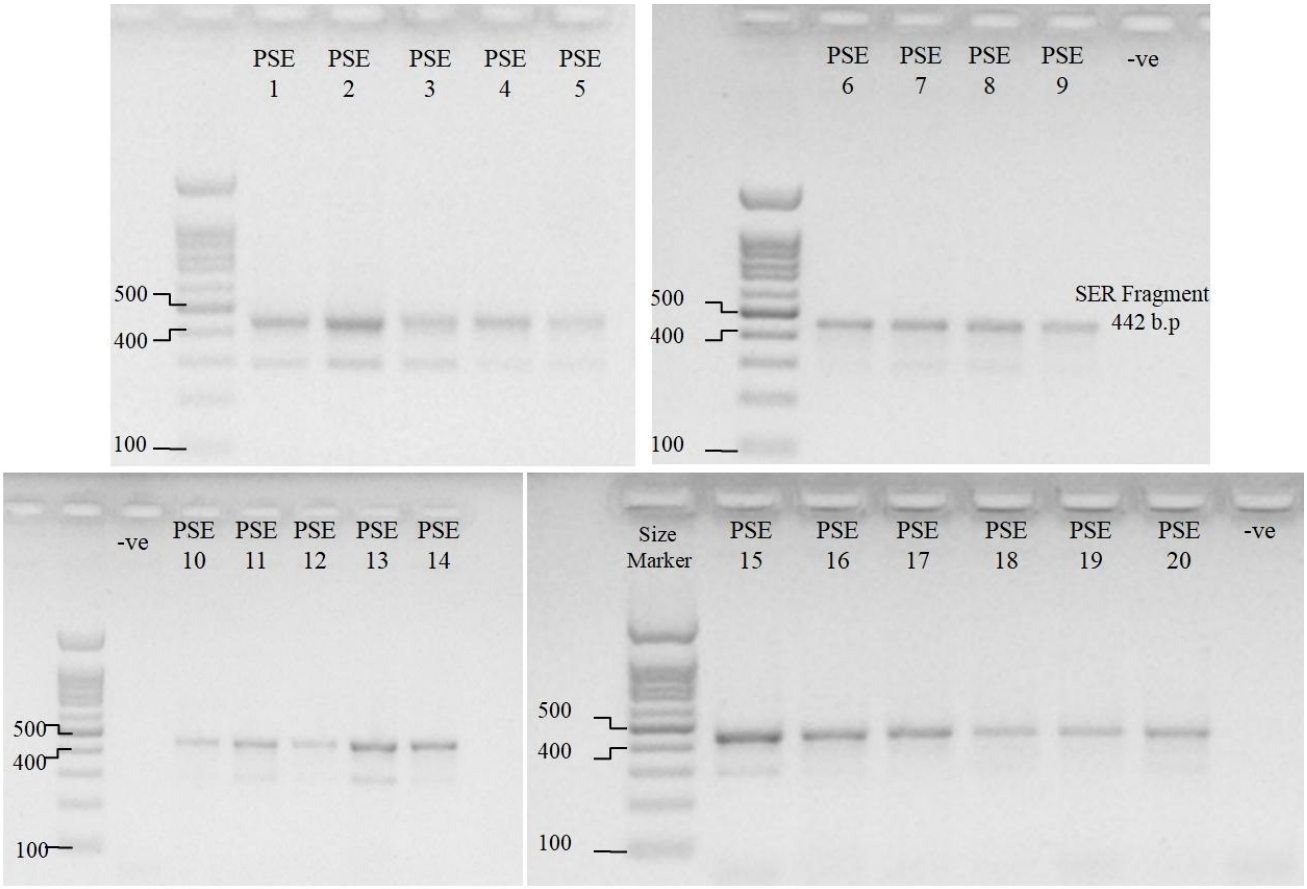

Figure 3.1.A A 442 bp SER PCR fragments amplified from *Ph. sergenti* sandflies' DNA from Palestine, the first lane is DNA size marker (100 bp), and -ve is the negative control.

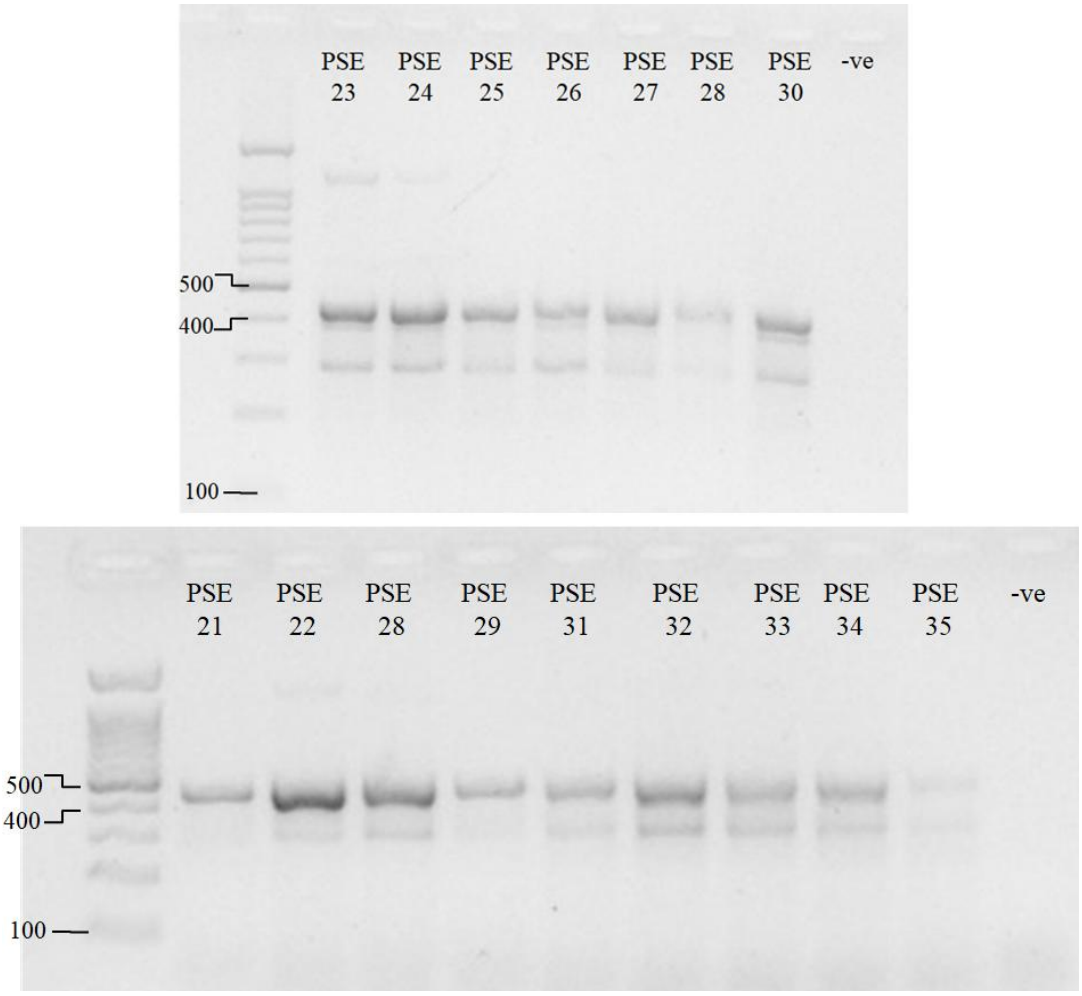

Figure 3.1.B A 442 bp SER PCR fragments amplified from *Ph. sergenti* sandflies' DNA from Palestine, the first lane is DNA size marker (100 bp), and -ve is the negative control.

3.2. Phylogenetic and Phylogeographic Analysis

3.2.1. Analysis of Samples from the West Bank, Palestine

All samples from Palestine (*n* =50) were sequenced, edited and aligned, the total SER length of the analyzed sequences ended up with 389 bp.

The whole analysis of the Palestinian sequences generated 9 haplotypes, defined by 26 polymorphic sites (Table 3.1). Five polymorphic sites were found to be parsimony informative.

Haplotype one (H_1) was the most frequent haplotype; it was shared by 40 individuals. Each of H_2 and H_3 haplotypes are represented by two samples. The remaining seven

haplotypes (H_4, 5, 6, 7, 8 and 9) are private haplotypes, where they are represented by only one individual. Haplotype nine (H_9) has the most polymorphic sites when compared to other haplotypes.

Sequence analysis of the haplotypes showed high AT and GC contents; (74.8%) and (25.2%) respectively. The transition-substitutions events (A→G, C → T) to transversion (C→A/G, T→A/G) substitutions events were 19:19.

Haplotype diversity showed a low value (H.d: 0.362), the average number of nucleotide differences (K) was 1.611, and the mean nucleotide diversity (Pi) is 0.0043.

Table 3.1 Obtained haplotypes of Palestinian *Ph. sergenti* and the alignment of variant nucleotide position for SER fragment of *Cyt. b*, only polymorphic positions are shown.

Palestine Haplotypes	Frequency	Variant Character Position																										
		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	3
		0	1	1	1	1	1	2	2	2	3	4	5	5	6	7	8	9	2	3	5	5	5	8	9	9	0
		3	3	5	6	7	8	1	3	4	6	2	3	4	6	3	4	0	0	2	0	3	9	6	5	8	7
H_1	40	T	T	T	A	A	T	C	C	T	C	T	G	G	T	C	T	C	G	G	T	C	C	T	C	C	G
H_2	2	.	.	.	.	.	.	A	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_3	2	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_4	1	.	.	A	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
H_5	1	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_6	1	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
H_7	1	.	G	.	.	C	A	A	G	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_8	1	.	.	.	.	.	.	A	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_9	1	C	.	.	.	.	.	.	.	.	.	A	A	A	C	T	C	T	A	A	C	T	T	C	T	T	A

The relationship between the haplotypes were displayed by constructing a median joining network, the connected haplotypes are very similar in their genetic sequences except of few mutation changes, the length of the connection lines is proportional to the number of mutation changes that differentiated between each connected haplotypes. The internal position of the haplotype (H_1), represented its high frequency and its multiple connections with other haplotypes in the network, indicate that (H_1) is the most ancestral haplotype. The external

haplotypes are diverged from the ancestral haplotypes and their small frequencies indicated that they are newly diverged haplotypes from (H_1) as shown in Figure 3.2.

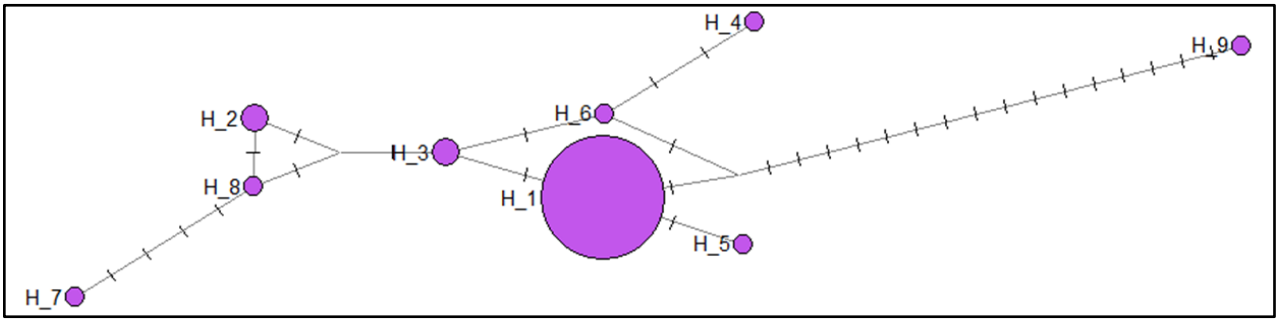

Figure 3.2 Median joining network for 9 haplotypes from Palestinian *Ph. sergenti* populations in the West Bank, the size of each circle represents the frequency of the haplotypes. Dash marks on the connected lines represent mutation changes and nucleotide substitution.

Maximum Parsimony (MP) phylogenetic analysis of the Palestinian *Ph. sergenti* haplotypes was determined using *Phlebotomus papatasi* (DQ381824) as an outgroup, supported by bootstrap analysis of 1000 iterations. The clustering of the haplotypes in MP tree is very similar to the clustering of haplotypes in the median joining network (Figure 3.3).

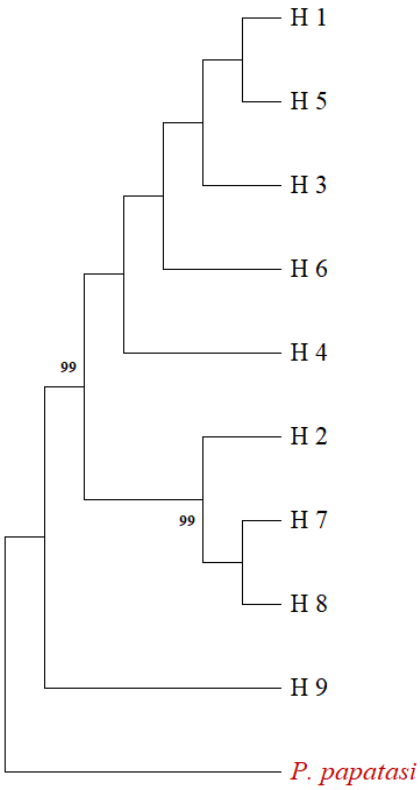

Figure3.3 Maximum parsimony analysis of Palestinian *Ph. sergenti* haplotypes was determined with *Phlebotomus papatasi* (DQ381824) as an outgroup. Bootstrap values > 90 are indicated on the branches.

3.2.2. Analysis of Global Samples

The previous Palestinian samples were compared with one hundred sequenced samples published in the GenBank from ten countries: Iran, Afghanistan, Pakistan, Turkey, Cyprus, Greece, Syria, Lebanon, Morocco and Spain. Forty-five haplotypes were identified representing 11 countries, with 87 polymorphic sites; thirty seven sites were parsimony informative.

Haplotype diversity showed high value (H.d: 0.901), the average number of nucleotide differences (K) was 8.497, and the mean nucleotide diversity (Pi) is 0.02814.

Median joining network analysis of 45 global haplotypes (Figure 3.4) shows that the most frequent haplotypes is haplotype (H_12); which is shared with the 41 of the Palestinian samples in addition to one Iranian sample (Table 3.2).

The topology of the network indicated a clustering pattern which reveals the relationship between the haplotypes. Beginning with Iranian haplotypes; they are clustered in almost three main groups. The first cluster contains H_15, 16, 17, 19 and the private haplotype H_18. The second cluster contains H_30, and two private haplotypes H_31 and 34.This group is much related to the haplotypes from its east neighboring countries; Pakistan which has the haplotype (H_38) and Afghanistan which have haplotypes (H_1, 2 and 3). The third Iranian clustering group represented by haplotypes (H_12, 14, 20, 21, 22, 23, 24, 25, 28, 29, 32 and 31) are connected to the west neighboring country; Turkey, which has only one sample represented by haplotype H_20 which also shared with other 14 Iranian haplotypes. The third Iranian group is also attached to three Palestinian haplotypes. H_13 is an Iranian private haplotype shows a very high mutation rate and it is far away from other haplotypes in the network (Figure 3.4).

The Turkish haplotype (H_20) is shared with Iranian haplotype, similarly, Syrian haplotype (H_35), Lebanese haplotype (H_35) and the two private Cyprus haplotypes (H_4 and 5) are connected with Iranian haplotypes.

Palestinian haplotypes (H_1 - 9) are also included in one group, one private haplotype (H_44) which connected to the Iranian haplotype (H_19). Haplotype 12 is shared with only one Iranian haplotype which also represented by 41 Palestinian *Ph. sergenti* individuals. The remaining Palestinian haplotypes are (H_40), (H_41) and the private haplotypes (H_39, 42 and 43).

Greek haplotypes (H_9, 10 and 11) are as well clustered together. Haplotypes (H_6 and 7) are private Spanish haplotypes, (H_37, 36 and 32) are representing the Moroccan haplotypes. H_8 is shared haplotype by Spanish and Moroccan populations. Haplotypes (H_7, 8, 37 and 38) are clustered together while (H_32 and H_6) are far from the previous cluster.

Table 3.2 Forty five obtained haplotypes of *P. sergenti* and the alignment of variant nucleotide position for SER fragment of Cyt b, only polymorphic position are shown. (Palestinian haplotypes are underlined)

Haplotypes	Frequency	Variant Character Position
		0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3
		0 0 0 0 0 1 3 3 3 3 3 3 3 4 4 4 4 4 5 5 5 5 5 6 6 6 7 7 8 8 8 8 9 0 0 1 1 1 1 2 2 2 3 3 3 3 4 4 4 5 5 5 6 6 6 6 7 8 8 9 9 9 0 0 0 1 1 1 1 1 1 2 2 2 3 3 3 3 5 6 6 9 0 0 0 2 2
		2 3 4 5 6 2 1 2 3 4 6 7 8 0 3 4 7 9 1 2 5 6 8 1 4 7 0 1 0 1 2 8 1 0 5 0 2 5 8 1 5 7 0 3 6 9 2 6 8 1 7 8 0 3 4 6 9 2 4 0 3 6 3 5 8 1 2 4 7 8 9 0 4 6 2 5 6 7 1 0 6 0 0 5 6 6 7
H_1	7	T T C T A T T T A C C A C T C G A C A G T C C A T C A T A T T C A A T C C A G T A C A C T A A G T C T A T T G T A C T C C T A T C T T A T A T C T A C T A T T A A T T A C A T
H_2	1	. . . . G . . . .
H_3	1	. . . . G . . . . T G . . . .
H_4	1	. . . . T . . . . C . . . . T . . . . T . . . . . A . . C . . . . C . . . . C . . . . . G . . .
H_5	1	. . . . T . . . . C . . . . T . . . . T G . . . . C . . . A . . . . . . C . . . . G . . .
H_6	1	. . . . G . . . T . . . . . G G . . . . G . . . . . C . . . . C . C . . C . . . . . . C . . . . T .
H_7	1	. . . . T . . . . T . A C . T . C . . . G . . . . A . . . . . C . C . . C . . . . . . A . . . . T . . . .
H_8	3	. . . . T . . . . T . A C . T . C . . . G . . . . C . C . . C . . . . . . A . . . . T . . . .
H_9	11	. . . . G A . . T G . . . . T . . . . . . A . . T G . . . . . C . . . . C . . . . C . . .
H_10	1	. . . . A . . . . . . A . . T . . . . T . . . . . . A . . T G . . . . . C . . . . C . . . . C . . .
H_11	2	. . . . G A . . T . . . . T . . . . . . A . . T G . . . . . C . . . . C . . . . C . . .
H_12	**42**	**. . . . . G . . . T . . . . C . . . . T . . . . T G . . . . C . . . . C . . . . G . . .**
H_13	1	. . T . . . C . T . T G T . T C T T . A A T T . . T T . . . . T . T C A . T A A . T . T A C T A . T . T A A . . G T A T T . T A T C C T C T C T C T T . T . . G G . C . T . A
H_14	4	. . . . T . . . . C . . . . T . . . . T . . . . C . . . . C . . . . G . . .
H_15	2	. . . . A . . T . C . . . . . . T . . . . . . A . . . G . . . . . C . . . . C . . . . C . . . .
H_16	5	. . . . A . . T . C . . . . . . T . . . . . . A . . . G . . . . . C . . . . C . . . . C . . . .
H_17	2	. . . . A . . T . C . . . . . . T . . . . . . A . . . G . . . . . C . . . . C . . . .
H_18	1	. . . . A . . T . C . . . . . . T . . . . . . A . . . G . . . . . C . . . . C . . . . C . . . . C . . . .
H_19	2	. . . . A . . T . C . . . . . . T . . . . . . A . . T . . . . . . C . . . . C . . . .
H_20	15	. . . . T . . . . C . . . . T . . . . T G . . . . C . . . . C . . . . G . . .
H_21	1	. . . . G . . . T . . . . C . . . . T . . . . T . . . . . . C . C . . . . C . . . . G . . .
H_22	1	. . . . T . . . . T . . . . C . . . . T . . . . T . . . . C . . . . C . . . . G . . .
H_23	1	. . . . T . . . . C . . . . T G . . . T . . . . C . . . . C . . . . G T . .
H_24	6	. . . . G . . . T . . . . C . . . . T . . . . T . . . . C . . . . C . . . . G . . .
H_25	1	. . . . T . . . . C . . . . T . . . . T G . . . . C . . . . C . . . . C . . . . G . . .
H_26	1	. . . . A . . T . . T . C . . . . T . . . . T G . . . . C . . . . C . . . . G . . .
H_27	1	. . . . G A . . T . . T . C . . . . T . . . . T G . . . . C . . . . C . . . . G . . .
H_28	2	. . . . T . . . . C . . . . T . . . G T G . . . . C . . . . C . . . . G . . .
H_29	1	. . . . G . . . T . . . . C . . . . T . . . . T G . . . . . C . C . . . . C . . . . G . . .
H_30	3	. . . . G . . . . G . . . .
H_31	1	. . . . A . . . .
H_32	6	. . . . G . . . T . . . . C . . . . T . . . . T G . . . . C . . . . C . . . .
H_33	1	. . . . T . . . . C . . . . T . A . . T G . . . . C . . . . C . . . . G . . .
H_34	1	. . . . G A . . . . C . . . . C . . . . .
H_35	1	. . . . A . . T . . . . C . . . . T . . . . T G . . . . C . . . . C . . . . G . . .
H_36	2	. . . . T . . . . T . A C . T . C . . . G . . T . . . . C . C . . C . . . . . . A . . . . T . . . .
H_37	4	. . . . T . . . . T . A C . T . C . . . . G . . . . C . C . . C . . . . . . A . . . . T . . . .
H_38	2	. . . . T . . . . . . A . A . . . . T . . . .
H_39	**1**	**. . . . . . A . . G . . . . G . . . T . . . . C . . . . T . . . . T G . . . . C . . . . C . . . . G . . .**
H_40	**3**	**. . . . . G . . . . G . . . T . . . . C . . . . T . . . . T G . . . . C . . . . C . . . . G . . .**
H_41	**2**	**. . . . . G . . . . G . . . T . . . . C . . . . T . . . . T . . . . C . . . . C . . . . G . . .**
H_42	**1**	**C A . G . C A . . G . . . . G . . . T . . . . C . . . . T . . . . T G . . . . C . . . . C . . . . G . . .**
H_43	**1**	**. . . . . . . G . G . . . . G . . . T . . . . C . . . . T . . . . T G . . . . C . . . . C . . . . G . . .**
H_44	**1**	**. C . . . C . . . . A . . . . . A . . T . C . . . . . C T . . . . T . A . . T . . . . . . C T . . . . C . T . . . .**
H_45	1	. . . . T . . . . T . . . . T . . . . T G . . . . C . . . . G . . .

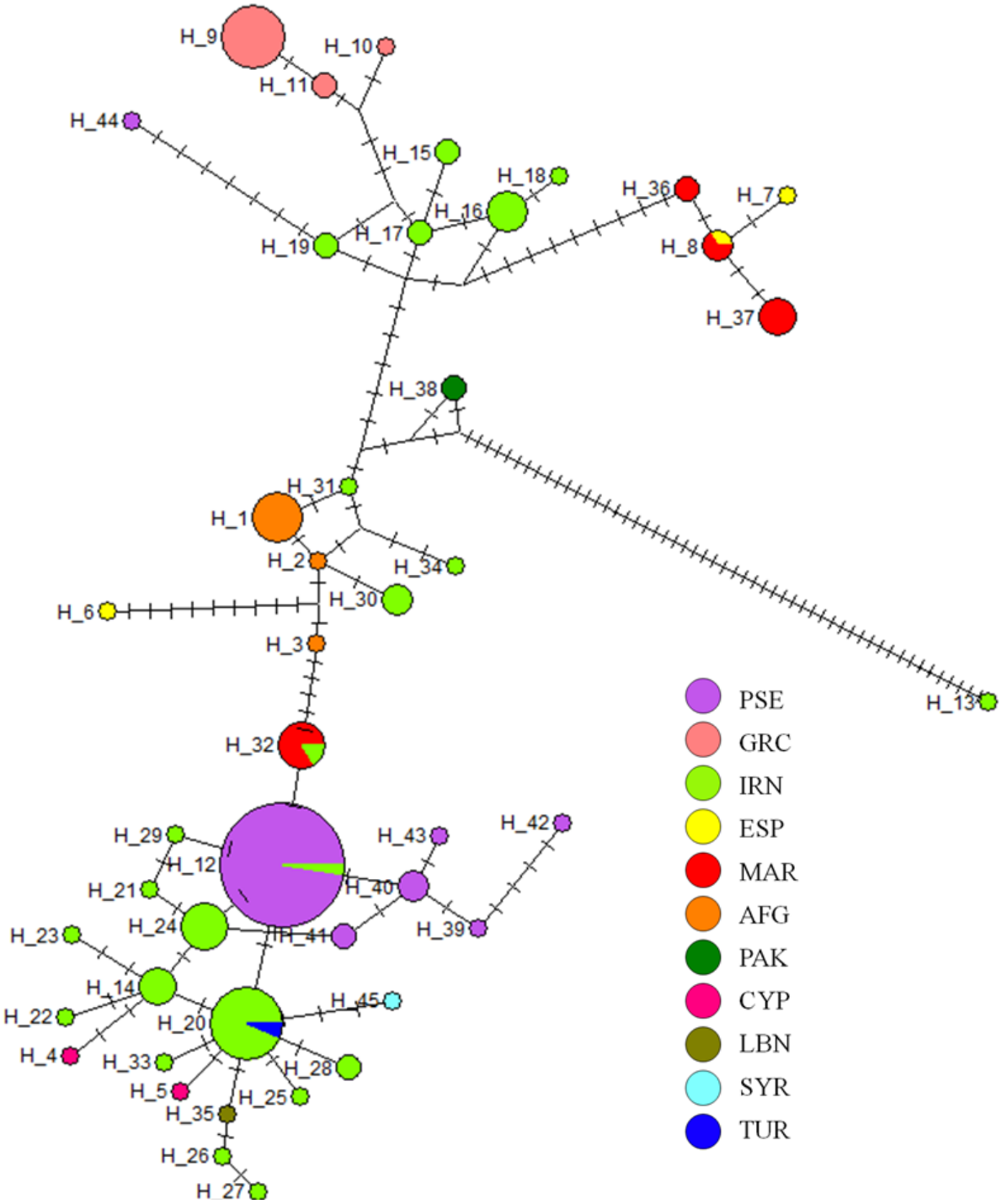

Figure 3.4 Median Joining Network for 45 haplotypes of all *Ph. sergenti* populations from different countries, the size of each circle represents the frequency of the haplotypes; each color represents a geographical region. Dash marks (/) on connection lines between haplotypes represent mutations and nucleotide substitutions. (PSE: Palestine, GRC: Greece, IRN: Iran, ESP: Spain, MAR: morocco, AFG: Afghanistan, PAK: Pakistan, CYP: Cyprus, LBN: Lebanon, SYR: Syria, TUR: Turkey)

Genetic differentiation among six population groups was identified. PSE group: (H_12, 39, 40, 41, 42 and 43), IRN group: (H_15, 16, 17, 18 and 19), IRN2 group; which include populations from Iran, Pakistan and Afghanistan: (H_1, 2, 3, 30, 31, 34 and 38), IRN3: (H_14, 20, 21, 22, 23, 24, 25, 28, 29, and 33), MAR_ESP group (H_7, 8, 36 and 37) and GRC group: (H_9, 10 and 11).

Pairwise F_{st} values (Table 3.3) indicated a significant genetic differentiation between all groups. The lowest value was 0.25238 between IRN3 and PSE, while others value between the rests of groups are relatively high; ranges between 0.58533 and 0.89288.

Table 3.3 Genetic differentiation Estimation among *Ph. sergenti* populations (groups were previously identified) based on mitochondrial *Cyt. b* gene. Values in the lower diagonal are pairwise tests for population differentiation (F_{ST}). Significance test revealed $P< 0.005$.

	PSE	IRN	IRN2	IRN3	MAR_ESP	GRC
PSE	0.00000					
IRN	0.83566	0.00000				
IRN2	0.75205	0.80518	0.00000			
IRN3	0.25238	0.89369	0.81272	0.00000		
MAR_ESP	0.78576	0.58533	0.61949	0.76835	0.00000	
GRC	0.81023	0.83350	0.83457	0.89288	0.66243	0.00000

The Maximum Parsimony analysis of the global haplotypes (45; H_1 through H_45) resulted in a phylogenetic tree with branches supported the clustering in the median joining network (Figure3.5). *Phlebotomus papatasi* (DQ381824) is the outgroup, the topology of the tree is supported by bootstrap values >70 on the branches. The distributions of haplotypes in the tree are similar to the distribution of the haplotypes in the median joining network shown in figure 3.4.

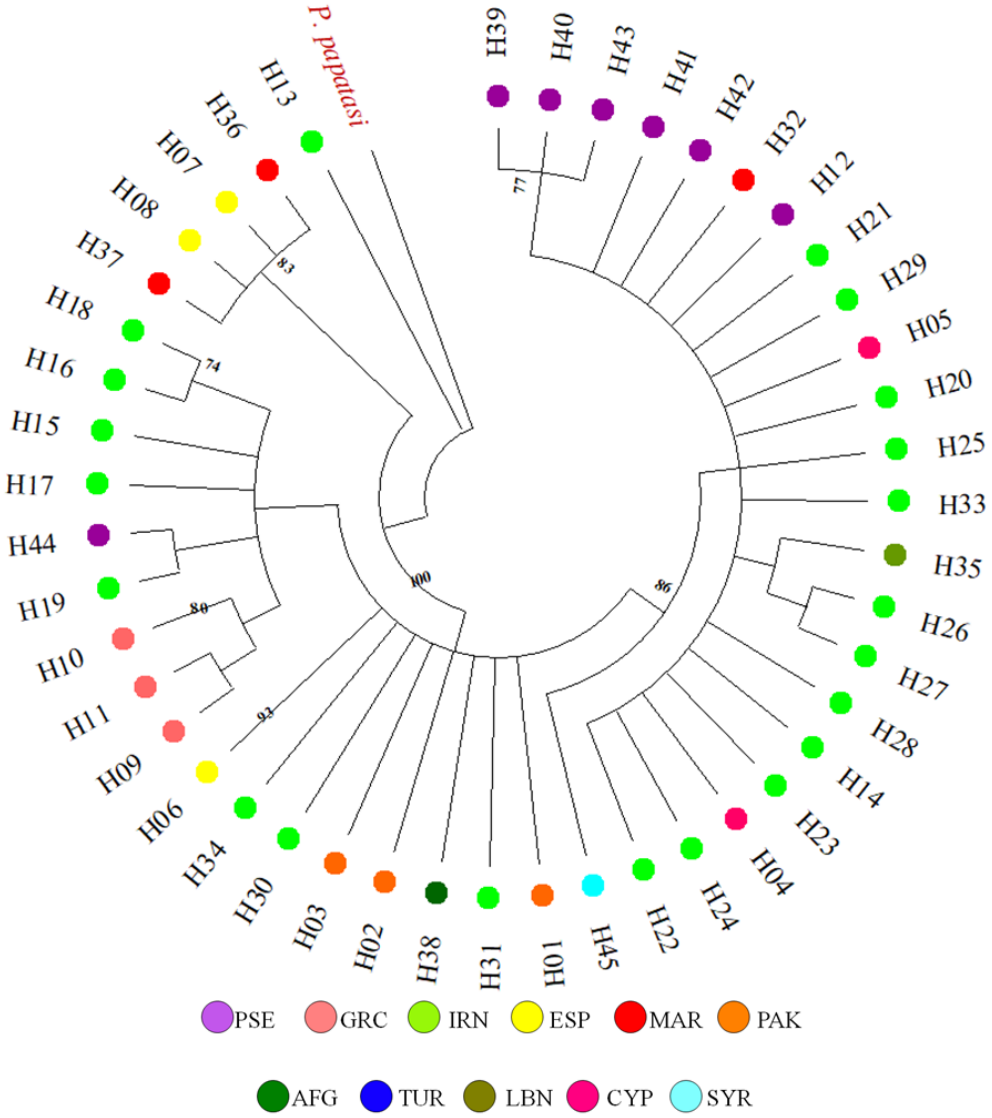

Figure 3.5 Maximum parsimony radial tree of all *Ph. sergenti* haplotypes (45 haplotypes; H_1-45), *Ph. papatasi* (DQ381824) is an outgroup. Bootstrap values>70 are indicated on the branches. The color near each haplotype represents the origin of the haplotype as described in Figure 3.4.

Chapter Four

4. Discussion

Mitochondrial DNA is very useful marker to study intraspecific and interspecific variability, as it reflects the evolutionary history due to the lack of recombination and other characteristics (Dvorak et al., 2011; Seddigh & Darabi, 2018). *Cyt. b* mtDNA gene usage has presented its efficiency and sensitivity to study the genetic differences between populations in the same species, especially in the study of sandflies genetics (Flanley et al., 2018; Hamarsheh et al., 2007; Kacem et al., 2023). The 3`- end of *Cyt. b* has a high rate of nucleotide divergence and variable intergenic repeats comparing to the full gene (Esseghir et al., 1997). The amplified fragment (SER fragment) contain 3`- end of *Cyt. b*, tRNA ser sequence, and 3' end of the *NADH1* gene (Esseghir et al., 1997).

Despite the small geographical size of Palestine, this study has proven the presence of intraspecific genetic variability in *Cyt. b* gene between *Ph. sergenti* individuals in the Palestinian population. Nine haplotypes were obtained from fifty *Ph. sergenti* samples. H_1 can be considered as the ancestral haplotype, due to several criteria; first its central position in the median joining network, which means that other peripheral haplotypes are derived from it as a result of later mutations. Its high frequency, since it is shared between 40 individuals of the sandflies, and the presence of multiple connections with other haplotypes.

Palestinian haplotypes are mainly clustered in two groups. The first consists of H_1, 3, 4, 5 and 6, and the second cluster contains H_2, 7 and 8, the groups are showed clearly in both median joining network and maximum parsimony phylogenetic tree (Figure3.2 and Figure3.3). There is only one private haplotype that is genetically far away from other haplotypes (H_9); it differs from H_1 with around 17 substitutions, this might be explained as this haplotype may

represent a newly diverged subspecies or it might be core for an establishment of new of *Ph. sergenti* population.

The analysis of Palestinian haplotypes showed a low value of both haplotype diversity and nucleotide diversity, the low values of nucleotide diversity indicate only small differences between haplotypes which is expected since the West Bank regions are geographically very close.

The generation of Palestinian haplotypes in both local and global analysis, displayed a differences in the haplotypes number and structure. In the Palestinian analysis, nine haplotypes were obtained, where in the global analysis only seven Palestinian haplotypes were obtained. This differentiation refers to the algorithm calculations of DNASP, where closely related sample were redistributed and gathered in one haplotype. For example; H_4 and H_6 gathered in one haplotype (represented by H_41), while the only sample of H_5 joined the most frequent haplotype; H_1 (H_12 in the global network).

It is known that *Ph. sergenti* in the Mediterranean Basin has a high intraspecific variability (Dvorak et al., 2011), and in this study, comparing Palestinian *Ph. sergenti* haplotypes to countries on the Mediterranean Basin; (Morocco, Spain, Greece, turkey, Cyprus, Syria and Lebanon), shows an obvious polymorphic variation between population.

Iran is considered one of the largest countries in the world, which have an expanded geographical area with different geographical features. Iranian samples in the median joining network were gathered in three groups. The same samples were analyzed by neighbor joining distance analysis (Moin-Vaziri et al., 2007), it showed three lineages with low gene flow, their grouping were related to three different geographical areas, isolated by natural barriers as desert and mountain chains.

Pakistan and Afghanistan are western neighbors to Iran, and due to this close areas, their haplotypes are also much related to one of the three Iranian haplotypes.

Spain and Morocco are also neighboring countries, and most of their haplotypes are also gathering together, it is believed that *Ph. sergenti* sandflies had migrated from Morocco to Spain

millions of years ago before the opening of the Strait of Gibraltar (Barón et al., 2008; Depaquit et al., 2002), this explains the large genetic diversity of the sandflies in Morocco; and the Spanish variants are resulted from an evolutionary events of the migrated individuals (Barón et al., 2008).

Turkish, Syrian, Lebanese and Cyprus haplotypes are originated from a very few numbers of individuals; however their haplotypes still connected to each other, the same as their geographical connection and may represented an Eastern Mediterranean group but more *Ph. sergenti* samples should be analyzed on micro geographical level in each country.

A comparison between all 45 haplotypes, displayed a high value of haplotype diversity and relatively low nucleotide diversity, this highlight a high number of closely related haplotypes, and propose that this population has undergone population expansion (Mendez-Harclerode et al., 2007; Zhang et al., 2014). The presence of private haplotypes from all countries, suggested a considerable degree of genetic isolation.

F_{ST} statistic is widely used calculation to measure the genetic differentiation between breeds that diverged from each other, and it gives a sight about migration and gene flow. F_{ST} values is considered as moderate to high differentiation if it ranges between 0.05 to 0.25, and values greater than 0.25 is very high differentiation (Hall, 2022). In this study, all F_{ST} values displayed a very high differentiation as values were all greater than 0.25 with a high significance ($P< 0.005$) between the studied regions. The minimum value was 0.25238 between Palestinian and Iranian *Ph. sergenti* haplotypes. These high F_{ST} values suggest a low gene flow between populations (Hamarsheh et al., 2007), and low gene flow between populations can lead to genetic subdivision of populations (Zhang et al., 2014). Sandflies are an old species, existed long ago (248 million years ago) (Andrade Filho & Brazil, 2003), since then, various outstanding events had occurred which had led to the emergence of numerous mutations and polymorphism, this may explain the high genetic differentiation between the sandflies populations. Generally sandflies are poor fliers; they just travel at very short distances; not more than 1000 m around their resting sites (Dvorak et al., 2011; Hamarsheh et al., 2007), this low dispersal and mobility plays a significant role in decreasing the genetic flow between populations (Hastings & Harrison, 1994).

The genetic variation between sandflies populations and their distribution returns to several factors, including bottle necks and even plate tectonic and palaeo-geological events that shaped the topography of the earth in ancient histories (Esseghir et al., 1997; Pavlou et al., 2022). Altitude has a great role in the genetic variations among sandflies, for example, in Jordan, the number of private haplotypes of *Ph. papatasi* were different between two areas that have different altitudes, one is below the sea level and the other is above the sea level (Flanley et al., 2018). Topography, soil type and composition, climate and vegetation cover also increase the genetic heterozygosity among sandfly individuals (Flanley et al., 2018; Kacem et al., 2023).

The distribution of clades in phylogenetic tree analysis using maximum parsimony showed a topology similar to the haplotypes clustering patterns in the median joining network, in both Palestinian and global analysis. This indicated the great power mtDNA as an reliable marker for population structure analysis and its usefulness to resolve very fine genetic variations among individual *Ph. sergenti* flies in a small geographical area like the Palestinian West Bank.

5. Conclusion

In this study, genetic population structure of Palestinian and global *Ph. sergenti* haplotypes had been presented. The structure displayed the presence of genetic variation in the mtDNA *Cyt. b* gene among *Ph. sergenti* populations. The obtained haplotypes from both Palestine and other countries were clustered on regional level, the Palestinian samples appeared in one clade in the global analysis reflected the high genetic variability of Palestinian *Ph. sergenti* collections and high genetic differentiation with low gene flow comparing to the global samples.

6. Recommendation

More analysis by other markers (nuclear and mitochondrial DNA) is recommended to resolve the population structure of *Ph. sergenti* and their geographical dispersion on micro-geographical level especially in the West Bank and the neighboring countries like Jordan.

For Palestinian population, the determination of exact geographical areas of *Ph. sergenti* will provide a clearer vision of their geographical distribution and their genetic isolation.

Also the determination of the presence or absence of *L. tropica* in the same area of *P. sergenti*, and studying their vectorial capacity of leishmania for every population is important to understand the epidemiology of leishmania disease and its distribution and weather this vector distribution is correlated with the severity of the disease or the number of cases every year. Population genetic analysis of vectors of infectious diseases is crucial to design an appropriate insecticide spraying programs and design probably more sandfly control models, to restrict the transmission of *leishmania* parasites among correspondent vectors and finally eradicate the disease.

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