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Full Length Research Paper

Mitochondrial cytochrome b variation in populations of the cutaneous leishmaniasis vector *Phlebotomus papatasi* across eastern Tunisia

Boudabous Raja[1]*, Jaouadi K.[1], Haouas N.[1], Mezhoud H.[1], Bdira S.[2], Amor S.[2] and Babba Hamouda[1]

[1]Laboratoire de Parasitologie- Mycologie code 99UR/08-05, Faculté de Pharmacie, Département de Biologie Clinique B, 1 Rue Avicenne, 5000 Monastir Tunisia, University of Monastir-Tunisia.
[2]Service d'Hygiène de Monastir Tunisia.

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Cutaneous leishmaniasis remains a serious health problem in Tunisia. It is the most extensive and prevalent form with thousands of cases reported each year since the start of a large epidemic in 1982, with 96% of cases were recorded in central and southern regions of the country. In Tunisia, *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae) is the main vector of cutaneous leishmaniasis caused by *Leishmania major*. *P. papatasi* was collected from peridomestic animal shelters and around the houses in several provinces in eastern Tunisia. Tunisian *P. papatasi* population was used in other reports as one of the countries for investigation from one exemplar, so it does not reflect the real structure of the country. Indeed, it is important to understand the population differentiation of sand fly vectors because it can show where potential vectors can invade from, and how quickly they can be controlled after local measures is applied. In this study, we characterized the Tunisian *P. papatasi* population by using mitochondrial cytochrome b (Cyt b) of sand flies as gene marker and situate it among the Mediterranean population. Our results indicate a low level of genetic variability among Tunisian populations of *P. papatasi* and we do not see any "isolation by distance" over a large geographical scale among Tunisian provinces.

Key words: *Phlebotomus papatasi,* cytochrome b, mtDNA, Tunisia, population differentiation, sandflies.

INTRODUCTION

Cutaneous leishmaniasis caused by *Leishmania major* Yakimoff and Schokhor (Kinetoplastida: Trypanosomatidae) is prevalent in arid and semiarid zones of northern Africa, the Middle East and central Asia (Ashford, 1996). The distribution of *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae) extends beyond that of *L. major* and its reservoir hosts to southern Europe and eastern regions of the Indian subcontinent, and throughout its range this sand fly is often abundant in domestic and peridomestic biotopes far from the colonies of its gerbil reservoir hosts (Feliciangeli, 2004). In Tunisia, investigations in the zoonotic cutaneous leishmaniasis foci in the arid region showed that *P. papatasi* is the proven vector of *L. major* (Rioux et al., 1986) and *Meriones shawi* Duvernoy is the main reservoir host (Rioux et al., 1982). *P. papatasi* was recorded in Tunisia in 1912 (Langéron, 1912) and it is considered a ubiquitous species (Rioux et al., 1986b). It is well adapted to the conditions of arid climate (Rioux et al., 1986) and its density increases with the aridity (Croset et al., 1978). Cutaneous leishmaniasis remains a serious healthy problem in Tunisia. It is the most extensive and prevalent form with thousands of cases reported each year since the start of a large epidemic in 1982 ((Ben Rachid and Ben Ismail, 2001; DSSB, 2001). Indeed, the Tunisian Ministry of Health (MMH) reported 48914 cases of cutaneous leishmaniasis caused by *L. major* between 1982 to 1994. Among them, 96% of these cases were recorded in central and southern regions of

*Corresponding author. E-mail: hamouda.babba@gnet.tn. Tel: +216 73 448 666. Fax: + 216 73 461 830.

the country (Ben Ismail et al., 1987). The eastern cost of Tunisia is located close to the zoonotic cutaneous leishmaniasis focus of Kairouan. Thus, human movements between these two cities could facilitate the disease transmission. It is important to understand the population differentiation of sand fly vectors because it can show where potential vectors can invade from and how quickly after local control measures. Our first investigation of the population differentiation of *P. papatasi* in Tunisia was to compare populations from the center of Tunisia with natural *P. papatasi* populations from the Mediterranean basin (Syria, Turkey, Cyprus, Jordan, Israel, Palestine, Egypt, Italy and Morocco); for which a maternally-inherited gene marker was used: mitochondrial cytochrome b (Cyt b) of sand flies.

In this study, a larger fragment of sandfly Cyt b was used to see if there is any "isolation by distance" over a larger geographical scale among Tunisian provinces. The mitochondrial genome is one of the best studied of all types of DNA. It has been shown to be a good source of accessible genetic variation, and analysis of mitochondrial DNA (mtDNA) variation has been used to understand evolutionary biology both at the intraspecific and interspecific levels (Avise, 1994). Their rapid rates of evolution, clonal inheritance, and lack of recombination have made it a valuable tool for phylogeographic studies and a source of markers for species and geographic populations. This makes it a clear choice for providing markers for identifying Tunisian sand flies. In this study, a fragment of sand fly cyt b was used to do the molecular characterization of Tunisian *P. papatasi* population, situate it among the Mediterranean population and to see if there is any “isolation by distance” over a larger geographical scale among Tunisian provinces; because, the other reports included Tunisian *P. papatasi* population as one of countries for investigation from one exemplar, so it does not reflect the real structure of the country.

MATERIALS AND METHODS

Sand flies

We analyzed *P. papatasi* sand flies (n = 90) from three localities of eastern cost of Tunisia (Figure 1a). We used data of *P. papatasi* sand flies from 25 field and colony populations originating from 10 countries (Syria, Turkey, Cyprus, Jordan, Israel, Palestine, Egypt, Italy, Tunisia and Morocco) (Figure 1b) (Hamarsheh et al., 2007). Some populations like those from Palestine, Israel and Egypt were selected based on previous isoenzyme studies (Kassem et al., 1990) that suggested the presence of genetic polymorphism. Tunisian collections were carried out from 1 September to 30 October 2005 during the main season of activity of adult sand flies in Tunisia. Sand flies were collected on sticky papers and Centers for Disease Control (CDC); miniature light traps were set overnight to sample sand flies in domestic animal shelters.

All sand flies were identified by their species-specific Cyt b sequences. Most were also identified based on morphological characters of the head and abdominal terminalia which were slide mounted in Berlese fluid following dissection with sterilized forceps and micro-needles.

PCR amplification of sandfly Cyt b

Genomic DNA was extracted from the thorax, wings, legs and abdomen of individual sand flies using the QIAmp DNA Mini Kit (Qiagen, Germany). For Cyt b CB3FC (forward) (5'-3' CA(C/T)ATT CAACC(A/T)GAATGATA) was used with N1N-FA (reverse) (5'-3'GGTA(C/T)(A/T)TTGCCTCGA(A/T)TTCG(A/T)TATGA) (Esseghir et al., 1997) to amplify a 3’ fragment of 545 bp which contains the 3’end of Cyt b gene (Ready et al., 1997). 1-1.5 µl of extracted DNA was added to each 50 µl of polymerase chain reaction (PCR) mix, which contained Invitrogen buffer with-out 2 mM $MgCl_2$, 100 µM of each dNTP and 40 pmol of each primer. 1.5 units of Taq DNA polymerase (Invitrogen) were always used. PCR consisted of 5 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 30 s and extension at 72°C for 1 min followed by 35 similar cycles but with annealing at 44°C and a final extension at 72°C for 10 min, all on a PTC-100™ programmable thermal controller cycler (MJ Research INC). PCR products were purified before analysis using the (Promega Wizard®SV gel and PCR clean-up system according to the supplier’s specifications. This step was necessary; it removed bands that interfered with interpretation of results. Samples were stored at -20°C.

Direct sequencing of PCR products

The Genetic Molecular Human Laboratory (LGMH), Medicine Faculty of Sfax-Tunisia performed automated sequencing of variants. Sequence data were aligned using the program Sequencher 4.6 Demo.

Data analysis

Sequences (442 bp without primers) were manually edited and aligned using the online version of Multalin (Corpet, 1988) (http://multalin.toulouse.inra.fr/multalin). Gaps were treated as missing data.

Phylogenetic analysis

Sequence alignment is performed using the ClustalW routine included in the MEGA version 3.1 software (Kumar et al., 2004) and checked by eye. Maximum likelihood-ratio test analyses are performed using MEGA 3.1 software. Statistics file substitution model: HKY85Gamma, shape parameter: 97.761 transition/ transversion ratio: 9.024, number of categories: 4, proportion of invariant: 0.159. Tree style: radial (by tree Dyn).

RESULTS

Sequence analysis

In all cases, PCR amplification produced one band of approximately 500 bp (including primers). All sequences are available in GenBank under accession numbers JN686468 to JN686488. The sequence analysis generated 7 haplotypes defined by 4 polymorphic sites which were parsimony informative. Not more than one different nucleotides was identified at each segregating site indicating minimal homoplasy. Two of the substitutions were A → G transitions, one was C → T transition and one was G insertion (Table 2). The most

Figure 1a. Original and extensive focus of zoonotic cutaneous leishmaniasis caused by *L. major* in Tunisia.

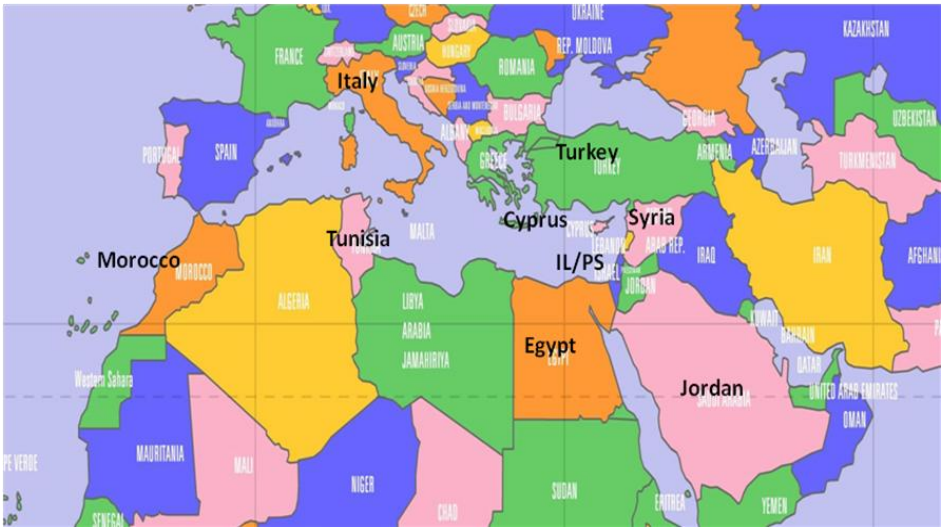

Figure 1b. Approximate geographical origins of *Phlebotomus papatasi* populations used for mtDNA analysis (IL/PS: Israel/Palestine).

Table 1. Haplotype frequencies in Tunisian *P. papatasi* population.

Locality	Code	Haplotypes (Hap)						
		Hap 1 SOS35	Hap 2 *DQ381827	Hap 3 *DQ381815	Hap 4 Pap40	Hap 5 Pap52	Hap 6 Mah54	Hap7 Mah50
Monastir (n = 30)	MON	0	20	8	1	1	0	0
Mahdia (n = 30)	Mah	11	6	11	0	0	1	1
Sousse (n = 30)	SOS	30	0	0	0	0	0	0

*Hamarsheh et al. (2007).

Table 2. Unique CB3 haplotypes found for *Phlebotomus papatasi* in this study and those by Hamarsheh et al. (2007). Alignment of variant nucleotide position for 442-bp mitochondrial cytochrome b gene. Only the polymorphic positions are shown; spaces represent identity with respect to the first reference sequence.

Parameter	Variant character position																																				
442- character CB3 haplotype	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4
	0	0	0	1	2	6	6	7	8	9	9	9	0	0	1	1	1	7	1	2	3	4	7	8	2	2	8	9	9	0	0	0	0	1	2	3	
	1	3	8	7	3	0	9	7	9	0	3	6	2	5	2	4	5	5	9	8	7	6	0	8	3	7	3	3	6	2	3	5	6	4	5	2	
	C	T	A	G	T	A	C	T	G	A	T	A	C	A	-	A	A	G	T	T	T	A	C	A	G	G	T	A	C	A	A	A	A	A	-	T	
Hap1															G																						
Hap2																															G						
Hap3																																					
Hap4															G																G						
Hap5															G																G						
Hap6															G																						
Hap7								C																													
DQ381815																															G						
DQ381816					C									G																							
DQ381817														G											A												
DQ381818														G																							
DQ381819				A										G		G										A		C				C	T				
DQ381820																				A																	
DQ381821											C						A	C																			
DQ381822																				C							A									
DQ381823											C						A	C																			
DQ381824											C							C																			
DQ381825						G					C	G						C		T	T																
DQ381826																			C																		
DQ381827																															G						

Table 2. Contd.

DQ381828									C	C							
DQ381829				T							T	G					
DQ381830				T				G			T					G	C
DQ381831				T							T						
DQ381832													T	G			
DQ381833				A	A	G											
DQ381834	A	C	T												C		
DQ381835							T							G			

frequent haplotypes were haps 1 and 2. Other shared haplotype (hap 3) was less frequent. Private haplotypes were found in individuals from two localities of Tunisia (haps 4, 5, 6 and 7) (Table 1). Sequences were compared by BLAST analysis with the last 440 bp of the mtDNA (cyt b) fragment (21 haplotypes) of widely separated collections of *P. papatasi* available in the GenBank databases (accession number DQ381815 to DQ381835) (Figure 2). Pair wise comparison between sequences from Tunisia obtained in this study and those available in GenBank were carried out and nucleotide differences (D) were calculated. This revealed a nucleotide variation ranging from 0.2 to 2% (Table 3). Haplotype 2 and hap 3 correspond to GenBank number sequence DQ381827 and DQ381815 respectively (Hamarsheh et al., 2007). For Tunisian haplotypes, except (haps 2 and 3), all the other five haplotypes were the found for the first time.

Most of the haplotypes obtained in this study revealed low nucleotide variation with those from Syria (0.23 to 0.45%) and high nucleotide difference (1.14 to 1.82%) with those from Egypt, Israel and Italy. Except some haplotypes from Palestine, Egypt and Turkey (GenBank: DQ381818, DQ381820, DQ381826, DQ381827 and DQ381835) present a medium nucleotide variability ranged between 0.23 to 0.68% (Table 3).

Phylogenetic analysis

The relationships among all haplotypes were revealed by maximum likelihood-ratio analysis. The haplotypes fall into five divergent groups; A, B, C, D and F (Figure 2), forming a well organized branching pattern. The topology of the network does not coincide with the geographical location of the populations (Table 3). Within each major clade, internal and terminal nodes can be interpreted as old and recently haplotypes respectively (Posad and Crandall, 2001). The position of the DQ381815, Haps 1, 3 and 6 haplotypes reflected an internal positioning within the network, indicating it to be the most ancestral haplotypes followed by more distantly related haplotypes. Tunisian haplotypes (Haps 2, 5 and 4) were the old haplotypes of haplotypes from Syria, Turkey and Palestine (Figure 2).

DISCUSSION

The mitochondrial genome is maternally inherited, non-recombining and highly polymorphic in insects (Avis, 1994) including sand flies (Esseghir et al., 1997). It is therefore not surprising that the cyt b sequences are good markers for characterizing *P. papatasi* populations. One or more haplotypes predominate at each geographical location with the rarest differing by single nucleotide exchanges. Differences in haplotypes shared between populations indicate that some haplotypes are more frequent than others. It has been postulated that older haplotypes have higher frequencies in the population because they have a greater probability of producing younger ones as mutational derivatives with lower frequencies (Crandall and Templeton, 1993; Smouse, 1998). It means Haps 1, 2 and 3 seems to be the older haplotypes of *P. papatasi* Tunisian populations. The tendency of some haplotypes having low, medium and high nucleotide differences or their separation into five groups (by network analysis) provides evidence of a past fragmentation event. This segregation suggests that *P. papatasi* populations from widely separated areas are substantially genetically differentiated as evidenced by differences in the frequencies of their most common haplotypes. Previous studies showed that H02 (hap 2 in this study) was derived from the widespread IRN33 (hap 3 in this study)

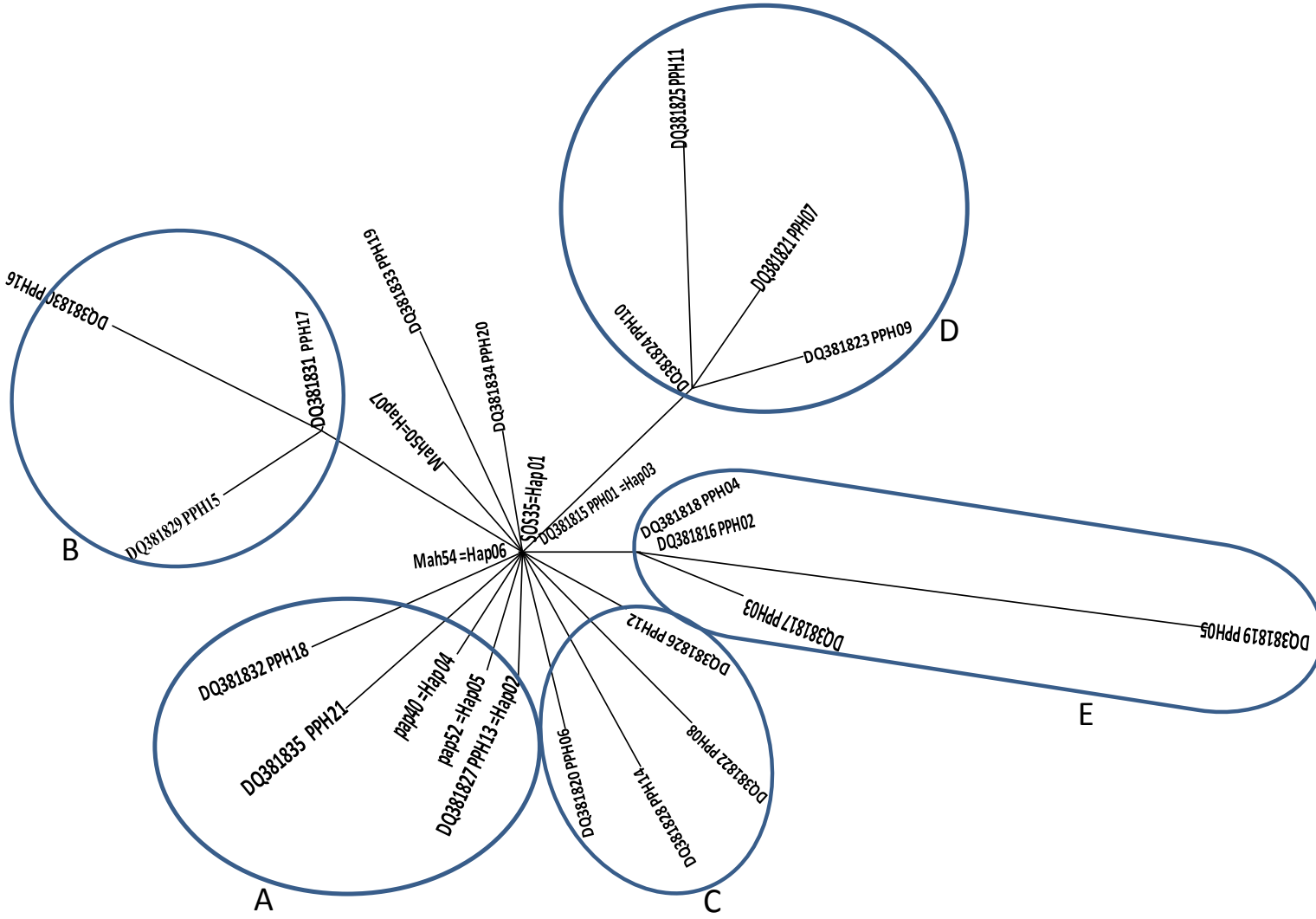

Fig. 2: Radial tree obtained by Maximum likelihood analysis for Tunisian and 21 other Mediterranean cyt b sequences of *P. papatasi*.

(Esseghir et al., 1997; Parvizi et al., 2003). It was frequent and shared between almost all populations from Syria, Turkey, Cyprus, Palestine and Tunisia, but sequence haplotype DQ381815 (hap 3 in this study) was found in some individuals from Tunisia and Cyprus (Hamarsheh et al., 2007). The haplotype H01 (hap 3 in this study) was reported to be ancestral for other haplotypes (Hamarsheh et al., 2007). The same haplotype was reported as ancestral for Iranian haplotypes. This seems to be the ancestral ones for Tunisian haplotypes as it has low nucleotide differences with all haplotypes (Table 2). By the maximum likelihood analysis of the haplotypes indicates that Haps 1 and 3 are probably the most ancestral haplotypes from which the other haplotypes which differ from Haps 1 and 3 by two or more mutational steps have evolved in radial manner (Figure 2). We found the same topology obtained by Hamarsheh et al. (2007) by median-joining network analysis.

No clear phylogeographical pattern was observed for the populations because shared haplotypes were present at the external nodes or at different nodes. These DNA fragments, particulary cytb have been regarded as good genetic markers to assess the relationships between closely related populations of *P. papatasi* in the Mediterranean region (Hamarsheh et al., 2007). Accordingly, our results indicate a low level of genetic variability among Tunisian populations of *P. papatasi* which is in line with a recent phylogenetic study reporting a genetic homogeneity among 26 populations of *P. papatasi* from 18 countries (Depaquit et al., 2008). Individual sand flies rarely migrate for more than 1000 m (Alexander, 1987; killick-kendrick, 1990). Therefore, genetic differences were expected between widely separated *P. papatasi* populations. Previous experiments carried by Kassem et al. (1993) and Ghosh et al. (1999) using isoenzyme electrophoresis of sand flies from Egypt

Table 3. Geographical origins and nucleotide differences between haplotypes of *P. papatasi* populations.

Parameter			Origine of haplotypes	Hap1	Hap2	Hap3	Hap4	Hap5	Hap6	Hap7
DQ381815	*IRN033 **PPH01	Hap 3	Syria/Tunisia	0.23	0.23	0	0.45	0.45	0.45	0.23
DQ381816			Egypt	0.68	0.68	0.45	0.91	0.91	0.91	0.91
DQ381817			Egypt	0.68	0.68	0.45	0.91	0.91	0.91	0.91
DQ381818	*H08		Egypt	0.45	0.45	0.23	0.68	0.68	0.45	0.45
DQ381819			Egypt	1.82	1.82	1.59	2.05	2.05	2.05	1.82
DQ381820			Egypt	0.23	0.45	0.23	0.45	0.45	0.23	0.23
DQ381821			Israel	0.91	0.91	0.68	1.14	1.14	1.14	0.68
DQ381822			Israel	0.68	0.68	0.45	0.91	0.91	0.68	0.68
DQ381823			Israel	1.14	1.14	0.91	1.36	1.36	1.36	1.14
DQ381824			Palestine	0.68	0.68	0.45	0.91	0.91	0.91	0.68
DQ381825			Palestine	0.45	0.45	0.91	0.68	0.68	0.68	0.45
DQ381826	*H09		Palestine	0.45	0.45	0.23	0.68	0.68	0.68	0.45
DQ381827	*H02 **PPH13	Hap 2	Palestine/Turkey	0.45	0.00	0.23	0.23	0.23	0.68	0.45
DQ381828			Jordan	0.68	0.68	0.45	0.91	0.91	0.91	0.68
DQ381829			Italy	0.91	0.91	0.68	1.14	1.14	1.14	0.91
DQ381830			Italy	1.36	1.36	1.14	1.36	1.36	1.36	1.36
DQ381831			Italy	0.68	0.68	0.45	0.91	0.91	0.91	0.68
DQ381832			Syria	0.45	0.45	0.23	0.68	0.68	0.68	0.45
DQ381833			Syria	0.68	0.68	0.45	0.91	0.91	0.91	0.68
DQ381834			Turkey	1.14	1.14	0.91	1.36	1.36	1.36	1.14
DQ381835			Turkey	0.68	0.23	0.45	0.45	0.45	0.91	0.68

**Hamarsheh et al. (2007); *Esseghir et al. (1997) and Parvizi et al. (2003).

and Jordan valley documented the presence of polymorphisms at 14 of 25 loci in Egyptian strains and 7 of 20 loci in the Jordan valley strains. Our study and those carried out by others indicate that mtDNA can be used successfully to characterize *P. papatasi* populations.

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