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RESEARCH ARTICLE

MITOCHONDRIAL *CYTOCHROME B* CHARACTERIZATION OF *PHLEBOTOMUS PAPATASI* POPULATIONS IN EGYPT

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ABSTRACT

Nucleotide diversity was studied using *Cyt b* (mt) DNA in different geographically *Phlebotomus papatasi* populations, but with both history of cutaneous leishmaniasis, Alexandria, and North Sinai-Egypt. Although, Egyptian P. papatasi was utilized as a part of different reports as a part of Mediterranean coat , However, it doesn't mirror the gene structure of the sandflies population in Egypt especially these reports doesn't stuck to particular regions in these reports. Sand flies (N = 842) were collected from both regions using sticky papers and CDC miniature light traps. *Cyt b* (mt) DNA was extracted from pools each of 25 females, amplified using specific primers, and sequenced. Sequence analysis revealed that *Cyt b* gene region had two haplotypes with five polymorphic sites in Egyptian *P. papatasi.* In addition, neutrality test (Tajima's D) showed no significant difference, indicating that *P. papatasi* populations from study regions were genetically homogeneous, as reported for other Mediterranean regions.

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INTRODUCTION

Phlebotomus papatasi sand flies are the primary vectors of *Leishmania major* from Morocco to the Indian subcontinent and from southern Europe to central and eastern Africa (Geraci *et al.,* 2014). In recent years, there has been growing interest in analyzing geographical variations among *Phlebotomus* populations by sequence analysis of various genes (Franco *et al*., 2010; Depaquit *et al*., 2015). Mitochondrial DNA has been shown convenient for studying molecular phylogenetics in sandflies. Its maternal inheritance, slow mutation rate and absence of recombination, particularly in the *cyt b* gene marker, make it best suited for differentiating closely related populations or populations in close geographical proximity (Esseghir *et al*., 1997, 2000; Parvizi *et al*., 2003; Hamarsheh *et al*., 2009). Therefore, knowledge of the genetic relationships and biogeography among *P. papatasi* populations would enable a better understanding of its current geographic distribution and would contribute to the design of appropriate control measures (Hamarsheh *et al*., 2009). In Egypt, leishmaniasis has become a particularly difficult health problem, both for local inhabitants and for multinational military personnel. It represents a regional 'crossroads,' and thus a potential source of infection from and to neighboring countries (Samy *et al*., 2014). Indeed, two main foci of Zoonotic cutaneous leishmaniasis are recognized in Egypt. West of Alexandria, is a popular Mediterranean summer resort El Agamy, Its beaches attract hundreds of vacationers from all over Egypt, with accompanied risk of disease transmission. In Northern Sinai, Rafah is Egypt's eastern border with the Mediterranean Gaza strip. Rafah is a heavily populated semi-desertic city, inhabited mainly by Bedouins. It harbors as well, a campus for multinational peace keeper's forces. Accordingly, we used Mt DNA *cyt b* to characterize the genetic structure of the Agamy and Rafah *P. papatasi* populations. In particular, we examined potential isolation of these geographically distant populations.

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MATERIALS AND METHODS

Sandflies

P. papatasi were collected in June 2010 from El-Agamy (Latitude 31° 6 8 N, Longitude 29° 46 32 E), Alexandria Governorate, and in August 2010 from Rafah,-Northern Sinai Governorate (Latitude 31° 17 7 N, Longitude 34° 12 36 E). Sandflies were collected on sticky papers or in CDC miniature light traps that were set overnight in domestic animal shelters *P. papatasi* females were identified based on morphological characters of the head and abdominal terminalia. Pools, each of 25 female sandflies, were kept in individual clean Eppendorf tubes for further experiments.

DNA isolation

Mitochondrial DNA was extracted from pools each of 25 adult female sandflies by the Phenol: chloroform: isoamyle alcohol method. Whole body females were homogenized with a sterile glass rod in a 1.5-mL microfuge tube containing 12 ml of CTAB buffer (1% hexadecyltrimethyl ammonium bromide, 750 mM NaCl, 50mM Tris-Cl pH 8, 10 mM EDTA pH 8), and 1.2 mg Proteinase K. The homogenate was incubated at 60° C for 3-5 hrs, and equilibrated twice; one with phenol (phenol: chloroform: iso-amyl alcohol, 25: 24 :1), then centrifuged to transfer the aqueous phase into a clean Eppendorf to add chloroform: iso-amyl alcohol (24:1). After washing the upper aqueous phase with 1/10 volume of 3 M sodium acetate (pH 5) and 2 volumes of cold absolute ethanol, homogenates were kept at – 20° C overnight then pelleted by centrifugation at 13,000g for 30 min. The pellet was washed twice with cold 70% ethanol, and dried using a speed-vac centrifuge for 8 min. DNA was eluted in 20 µl TE buffer.

PCR amplification of sandflyCyt b

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). PCR was performed in a 50 µl reaction volume containing 50 ng (mt) DNA,10×PCR buffer, and 12 pmol of each primer.*cyt b* gene was amplified using the following conditions: an initial denaturation at 95° C for 3 min; followed by 5 cycles at 94° C for 30 s, 40° C for 30 s and 72° C for 1min; 35 cycles at 94° C for 30 s, 44° C for 30 s, 72° C for 1 min; and a final extension at 72° C for 10 min. For sequencing, positive amplifications were purified with the purification kit (Jena DNA purification kits).

Sequence and phylogenetic analysis

Sequence alignment was performed using the *Clustal W*. Evolutionary analyses were conducted in MEGA7 Kumar *et al*. (2016). Determination of polymorphic sites and haplotype statistics were assessed by dnasp version 5.

RESULTS

Sequence analysis

PCR amplification produced one band of approximately 442 bp (including primers) for *P. papatasi* from Sinai, as well as from Alexandria (Fig. 1). The AT contents were 76.70% and 76% whereas the CG contents were 23.30%, 24% in female sandflies from Sinai and Alexandria, respectively. Obtained sequences were aligned with those of mtDNA available on GenBank, then trimmed using alignment trimmer http:// users-birc.au.dk/biopv/php/fabox/alignment_trimmer.php. For comparing and identifying new haplotypes, the aligned trimming sequences were analyzed, generating two haplotypes defined by five polymorphic sites which were parsimony informative, and two sites which were non-parsimony informative. Not more than one differing nucleotide was identified at each segregating site, indicating minimal homoplasy. Substitutions were a C → T transition and a G →A insertion. Obtained sequences were compared by BLAST analysis with the 442 bp long of mtDNA (cyt b), showing 23haplotypes with 421 polymorphic sites of widely separated collections of *P. papatasi* available in the GenBank databases (Pair wise comparison between sequences from Egypt and those available in GenBank were carried out. Nucleotide diversity was 0.3, and haplotype variation among these sequences was 0.991.Data of the neutrality test for *P. papastai* haplotypes were low (Tajima's D: -0.64297, *P*> 0.10), indicating an excess of low frequency polymorphisms relative to expectation, revealing population size expansion and/or purifying selection.

Fig. 1. Amplified *Cyt b*mt DNA of *P. papatasi* from Sinai (lane 2), and Alexandria (lane 3)

Phylogenetic analysis

Based on the phylogenetic tree, topologies constructed with ML (maximum likelihood) involved 26 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 431 positions in the final dataset (Fig. 2). The analysis of the tree revealed that haplotype one of *P. papatasi* from Alexandria formed a sister group with those from Turkey (DQ381835.1).However, haplotype of sandflies from Sinai appeared ancestral to *P. papatasi* from Italy (JF766973.1, JF766974.1, and JF766975.1). Also, the overall genetic distance among *P. papatasi* of the study and other populations was 0.006, indicating high similarities among *P. papatasi* from the Mediterranean coast.

DISCUSSION

The query length of *cyt b* sequence of *P. papatasi* from Alexandria and Sinai was 442 bp, being similar to *P. papatasi* from India, Iraq, Syria, Cyprus, Spain, Italy, and Morocco Esseghir *et al.* (1997). *cyt b* sequence was 476 bp long in *P. ariasi* from western Mediterranean Franco *et al.* (2010) and approximately 500 bp in *P. papatasi* from Tunisia Raja *et al.* (2012). *cyt b* was rich inAT content (greater than 75.00%) in two *Egyptian P. papatasi* populations. *P.papatasi* from Palestine, AT content of *cyt b* was 76.60% Hamarsheh *et al.* (2007) and 78.00% in *P. ariasi* and *P. perniciosus* from Algeria Franco *et al*. (2010). The low level of the CG content (23.30%, and 24.00% for sandflies from Sinai and Alexandria, respectively) reported in the present study, lies withinthe range of CG content (27.93%) reported for *P. bergeroti* in Egypt Depaquit *et al.* (2008). The advantage of using mitochondrial DNA (mtDNA) as a population genetic marker is that it has a relatively rapid rate of mutation, providing readily identified haplotypes. Within a single organism, the mitochondrial genome has multiple haploid copies (usually identical) of DNA that are maternally inherited and do not participate in recombination events. Various *cyt b* haplotypes have been identified in *P. papatasi* populations from various Mediterranean areas. Herein our study, the analysis of mt DNA of *P. papatasi* from Sinai and Alexandria included two haplotypes defined by five polymorphic sites. In Tunisian *P. papatasi* populations, there were seven haplotypes defined by four polymorphic sites Raja *et al*. (2012). Recently, *P. papatasi* collected on the border of Iran with Iraq had four unique

Fig. 2. Molecular phylogenetic analysis by the Maximum Likelihood method for Egyptian and other Mediterranean *cyt b* sequences of *P. papatasi*

haplotypes (KH417, KH419, KH421 and KH486) and a commonhaplotype of KH382, KH535 that exhibited 100 % similarity with haplotypes from Italy (GenBank accession no. HM992926 and HM992927) Ebrahimi *et al*. (2016). The choice for using *cyt b* as molecular marker in sandflies comes from its high discriminatory power, presence of distinct character classes (i.e. the three codon positions) that exhibit mutation rates reliable for phylogenetic analysis, and the fact that the gene is maternally inherited and, thus, free of recombination. Moreover, our datarevealed that the number of haplotypes was less than segregating sites, which referred to the presence of rare alleles at high frequencies compared to data from earlier studies on *P. papatasi* from Egypt that used isoenzyme electrophoresis Kassem *et al*. (1993); Ghosh *et al*. (1999). These last studies documented the presence of polymorphisms at 14 of 25 studied loci. Esseghir *et al.* (1997) determined the presence of 16 haplotypes in 27 *P. papatasi* females collected from 12 countries including India, Iraq, Syria, Cyprus, Spain, Italy, Morocco and Egypt. The papa1 haplotype from the Mediterranean basin was determined to be the ancestral sequence. However, nucleotide diversity among haplotypes was low as they were separated from each other by only one to four nucleotide substitutions. Researchers explained the homogeneity of genetic structure within the distributional range of *P. papatasi* by the absence of genetic variation in the Mediterranean basin, concluding that the Mediterranean Sea is not an effective barrier to prevent the dispersal of this species Esseghir *et al*. (1997). Also, Depaquit *et al.* (2008) identified 2 haplotypes in specimens sequenced from Egypt, Italy and Morocco per country with confirmation that *P. papatasi* probable characterized by a restricted gene flow describing phlebotomine flies as poor flyers. Similarly, no evidence for isolation by distance for *P. papatasi* populations in Iran, as gene flow seemed continuous among geographically separated populations Parviziand Ready (2006).In contrast, a study reported genetic diversity within distant *P. papatasi* populations from Palestine in relation to mtDNA *cyt b* sequences, suggesting that the main factor determining the degree of genetic diversity was latitude and not climatic conditions Hamarsheh *et al*. (2007). However, this last study inferred that the observed level of genetic differentiation was not enough to supportthe existence of a species complex. Accordingly, our results confirm genetic homogeneity within the two populations' *P. papatasi* from Egypt.

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