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## RESEARCH ARTICLE

### GENETIC DIVERSITY OF *PHLEBOTOMUS PAPATASI* POPULATIONS INFERRED FROM ITS2 rDNA IN TWO DIFFERENT GEOGRAPHICAL REGIONS IN EGYPT

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#### ABSTRACT

*Phlebotomus papatasi* is the potential vector of zoonotic cutaneous leishmaniasis in the north eastern parts of Egypt, specifically in Rafah, North Sinai governorate. The present study characterized DNA sequence of Internal Transcribed Spacer 2 (ITS-2) from two *Phlebotomus papatasi* strains collected from two geographically distinct localities in Egypt; Rafah, North Sinai Governorate, and El Agamy, Alexandria Governorate. DNA was extracted from morphologically identified *P. papatasi* specimens collected from Alexandria, and Sinai. The ITS2 regions amplified and their sequences submitted into GenBank and analyzed by comparison with other entries. The percentage of identity among studied sandflies was 93%. Phylogeny analysis of amplified sequences revealed a high similarity rate with *P. papatasi* from coastal Mediterranean countries (97%). Data in the present study suggested that the phylogenetic analysis could not be completely resolved, which actually makes the use of current ITS-2 sequences of little value for phylogenetic interpretation.

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## INTRODUCTION

More than 800 sand flies species have been described, however, only some serve as vectors and transmit zoonotic and human diseases such as leishmaniasis (Munstermann, 2004). In Egypt phlebotomine sandflies are important vectors of pathogen causing disease phlebotomus fever virus and three species of *Leishmania*: *L. major*, *L. tropica*, and *L. donovani*, the causative agents of zoonotic cutaneous leishmaniasis (ZCL), anthroponotic cutaneous leishmaniasis (ACL) and visceral leishmaniasis (VL), respectively. *P. papatasi* is the main proven vector of *Leishmania major* in the Old World, North of the Sahara where zoonotic cutaneous leishmaniasis is established in reservoir hosts. Cutaneous and visceral leishmaniasis have both been reported in Egypt. The cutaneous form has been primarily identified in northern Sinai (Mansour et al., 1987) and was attributed to *Leishmania major*, according to isoenzyme analysis (Mansour et al., 1989). Visceral leishmaniasis caused by *L. infantum* has been found near Alexandria, in El Agamy (Awadalla et al., 1987). *Phlebotomus papatasi* has been reported to comprise more than 94% of the sandfly population in North Sinai, Egypt (Hanafi et al., 2007). *P. papatasi* is the proven vector of cutaneous leishmaniasis and

The *Leishmania major* is the etiological agent associated (Wahba et al., 1990). The study of the genetic structure of phlebotomine sandflies in Egypt is a key factor for vector incrimination to provide insights on unknown detailed of the transmission cycle of leishmaniasis in Egypt. *P. papatasi* occur in an important inter face between the Palaearctic and Afrotropical Zoogeographic regions reflecting the possibility for genetic differentiation of this potential vector among both foci. *P. papatasi* has been reported in lower and Upper Egypt (Lane 1986) and more than 94% of the sandfly population are located in North Sinai, Egypt (Hanafi et al., 2007; Shehata et al., 1995). The identification of Phlebotomines and flies to the species level was based only on morphological local Keys (Lane, 1986). However, the morphological identification requires considerable operator skills and taxonomic expertise. Sometimes, the morphological identification may be even impaired, as in the case of severely damaged specimens or when mounting is not appropriately performed. Molecular techniques, such as random amplified polymorphic DNA or polymerase chain reaction (PCR)-coupled with approaches on of DNA sequences of ribosomal (e.g. internal transcribed spacer 2, ITS-2) or mitochondrial markers (e.g. cyt-b gene) proved to be useful for studying taxonomy and populations' genetic makeup of *Phlebotomus* spp. (Aransay et al., 2000; Depaquit et al., 2000, 2008b; Di Muccio et al., 2000, Pesson et al., 2004). Previous studies also showed a low mutation rate

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within *P. papatasi* populations from different biotopes in Iran (Parvizi et al., 2003) as well as in populations from different countries (Esseghiret et al., 1997). Hamarshah et al. (2007) by sequencing cytochrome b mtDNA emphasized avariability within Mediterranean populations of *P. papatasi* without prove the existence of a species complex. The aim of this study is to determine the genetic structure of *P. papatasi* within the Alexandria, and Sinai regions using ITS-2 and *Cyt-b* as markers. Determining the genetic structures of these populations will fill the gap in knowledge of leishmaniasis vectors in both endemic taking the advantage of using molecular markers as a useful tool to study genetics at the intra-population level.

## MATERIALS AND METHODS

### Sandflies collection and processing

Adult sandflies were collected by CDC light traps (John W. Hock, Gainesville, FL) from two geographic localities locations in Egypt; El-Agamy, Alexandria Governorate (31° 6 8 N, 29° 46 32 E) and Rafah, North Sinai governorate (31° 17 7 N, 34° 12 36 E). Both sandflies strains were collected from El Agamy and Rafah since June 2010, and August 2010, respectively. Both locations are located on the northern coast of the country. Al Agamy is an area of new housing development on a narrow limestone strip between the Mediterranean and a large brackish lake, there is very little vegetation around the patients' houses other than small fig orchards. But Rafah is a remote location on the Egyptian border with Palestine and is inhabited principally by Bedouins. Field collections were carried out during June, 2010 in Al-Agmy, and in August, 2010 from Rafah, during the main season of activity of adult sand flies in Egypt. All collected specimens were stored in 70% ethyl alcohol for morphological identification using the key of Lane, 1986.

Collected females were transported in 70% ethyl alcohol to the laboratory at the Research and Training Center for Vectors of Diseases at Ain Shams University. All collected females sandflies were morphologically identified based on morphological characters of the head and abdominal terminalia as a preliminary confirmation, then all dissected females was kept in 70% ethyl alcohol for further experiment.

### DNA Extraction

A total of 480 females' *P. papatasi* collected from Sinai and Alexandria. The samples were dried before DNA extraction. A sample of 30 adult females' *P. papatasi* was homogenized in a small mortar with a homogenizer. DNA extraction was performed using Gene JET™ Genomic DNA Purification Kit (Fermentas, K7021) in accordance with the manufacturer's instructions.

### DNA Amplification and Sequence Analysis

The amplification of genomic DNA was performed in 25 µl volume using 2 µL of extracted DNA solution and 50 pmol of each of the primers. For ITS2 primer sequences were: Forward primer (C1a): 5'-CCT GGT TAG TTT CTTTTC CTC

CGC T-3', and reverse primer (JTS3): 5'-CGC AGC TAA CTGTGT GAA ATC-3' (Depaquetet et al., 2000). The PCR mix contained (final concentration) 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% Triton X 100, 200 µM deoxynucleotide triphosphate each base and 2.5 units of Taq polymerase. After an initial denaturation at 95 C for 5 min, amplification was performed with 40 cycles of denaturation (95 C, 1 min), annealing (55 C, 1 min) and polymerization (72 C, 2 min), followed by a final extension at 72 C for 10 min. Amplicons of ITS2 was analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide. PCR products were visualized by ultraviolet transilluminator, and photographed using a digital camera (Canon, Power Shot A 460, 5.0 Mega Pixel).

Direct sequencing in both directions was performed by Sanger's method using the primers used for DNA amplification. Sequence alignment was performed using the *MUSCLE* routine included in the MEGA v.6 software (Tamura et al., 2007). Consequently, a maximum-likelihood analysis was performed, with the Kimura-2 parameters model and using uniform rates among sites for phylogenetic analysis of ITS2, among *Phlebotomus papatasi* from several countries. Gaps were treated as missing data.

## RESULTS

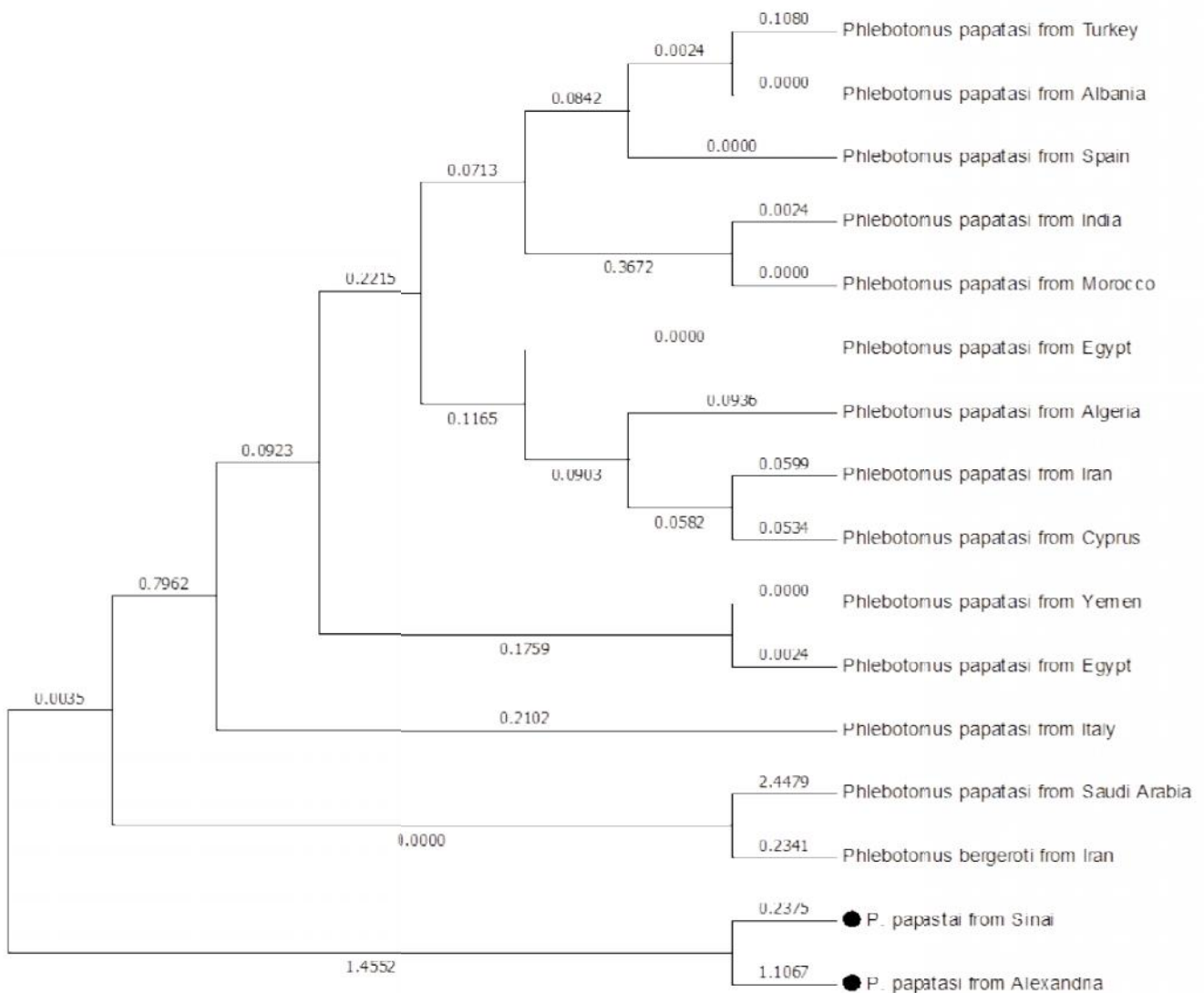
All collected Sandflies were *P. papatasi*. PCR product of about 454 bp at 3' end of the ITS2 region was detected. All amplicons generated sequence data for the entire region between primers for ITS-2 are available in GenBank under accession numbers KP773296, and KP773297 for females *P. papatasi* collected in Alexandria. KP756625 and KP773295 accessions numbers are available in GenBank for those ones collected in Sinai. The size of fragments was geographically independent, being 455 bp in females from Alexandria and 454 bp in females from Sinai. Seventeen ITS2 sequences of *P. papatasi* species from seventeen different countries were selected for the analysis (Algeria, Egypt, Tunisia, Greece, Cyprus, Montenegro, Albania, Israel, Italy, Lebanon, Spain, Iran, Morocco, Yemen, India, Turkey and Saudi Arabia). The multi-alignment of ITS2 sequences among these countries and *P. papatasi* collected from Sinai, and Alexandria showed maximum identity 95% to 97% of those collected from Sinai, compared to 94% to 95% in female sandflies from Alexandria (Table 1).

### Sequence Analysis of *P. papatasi* from El Agamy, Alexandria Governorate

Statistical analysis of ITS2 sequence alignments for *P. Papatasi* females' from Alexandria revealed that the sequence was 565 characters long. These characters included 230 variable sites, 126 of which were parsimony informative, 104 were parsimony uninformative, and 268 were conserved sites. The sequences were adenosine and thymine (AT) –rich (69.2%). Multi-alignment of two isolates *P. papatasi* females from Alexandria when compared with sequences for sand flies from various countries showed 95% identity. Fig. 1. displayed the phylogenetic tree obtained using representative ITS2 sequences for *P. papatasi* from both two isolates of females's *P. papatasi*.

**Table 1. Identity of ITS2 in the two *P. papatasi* populations and the others from seventeen countries**

Country	Query length in bp	Accession numbers	Female <i>P. papatasi</i> (Sinai strain)	Female <i>P. papatasi</i> (Alex. Strain)
Algeria	430	EF408802.1	97%	95%
Egypt	429	EF408786.1	97%	95%
Tunisia	428	EF408795.1	97%	95%
Greece	428	EF408785.1	97%	95%
Cyprus	428	EF408784.1	97%	95%
Israel	428	EF408789.1	97%	95%
Montenegro	429	EF408800.1	96%	95%
Albania	429	EF408799.1	96%	95%
Italy	431	EF408791.1	96%	95%
Lebanon	429	EF408792.1	96%	95%
Spain	429	EF408788.1	96%	95%
Iran	431	EF408801.1	96%	95%
Morocco	431	EF408794.1	96%	95%
Yemen	433	EF408798.1	96%	95%
India	434	EF408797.1	95%	94%
Turkey	432	EF408796.1	95%	94%
Saudi Arabia	434	EF408783.1	95%	94%



**Fig. 1. Molecular Phylogenetic analysis by Maximum Likelihood method of ITS2 sequence of *P. papatasi* from Alexandria, Sinai. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 422 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Scale bar 0.02**

The sequences analysis suggested that these females were closely related with no marked genetic difference between them. However, the ITS2 sequences from Alexandria closely related to the group from Morocco group from Morocco which has similar climatic conditions. It was common to observe that sequences of ITS2 from Alexandria was closely related to an unknown *P. papatasi* isolate from Egypt which is from unknown regions. Also, the mean genetic distance among *P. papatasi* flies from Alexandria and others from other countries ranged between 0.08 to 0.10.

### Sequence Analysis of *P. papatasi* collected from Rafah, North Sinai Governorate

Alignment of ITS2 sequences for *P. papatasi* from Sinai consisted of 533 characters long, including 355 characters as conserved sites, whereas 147 were variable characters from which 29 were parsimony informative. A moderate similarity rate among one isolate of *P. papatasi* from Sinai and other countries was observed, ranging from 66 to 81%. Conversely, a high identity rate (88 to 94 %) was observed *P. Papatasi* females. Phylogenetic relationships of ITS2 among *P. papatasi* were inferred by maximum-likelihood. The tree diverged into two lineages; one lineage included *P. papatasi* from Egypt, Yemen, and Saudi Arabia. Moreover, *P. papatasi* from Sinai and Jordan formed as sister group. The second lineage formed a sister groups of *P. papatasi* from Morocco, Spain, Italy, Albania, Algeria and Lebanon (Fig. 1).

## DISCUSSION

The use of rDNA ITS2 as a molecular marker has many advantages, such as a high mutation rate, easy to amplify, multiple target sites, predefined marker systems, and known PCR primers. (Depaquit et al., 2000; Di Muccio et al., 2000; Depaquit et al., 2002). This marker system has the disadvantage of it requires previous information about the species composition, since this spacer evolved diverge rapidly, however, it is usually possible to find diagnostic differences among even the most closely related species (Collins et al., 2000). The limited number of variations within, like *P. papatasi*, this species does not exist among populations of other sand fly species (Depaquit et al., 2002). The length of the ITS2 sequence was 455 bp in *P. papatasi* from both Sinai and Alexandria governorates, Egypt. However, these findings were a relatively different with data reported by Hamarsheh et al. (2007b). Indeed, these authors stated that the ITS2 fragment length ranged from 340 to 512 bp in ten *P. papatasi* populations from various Mediterranean countries, including Egypt. ITS2 sequences for *P. papatasi* from Alexandria and Sinai are AT rich (69.24%), as commonly observed for *P. papatasi* from other regions in the world (Simmons and Weller 2001; Parvizi and Ready 2006; Depaquit et al., 2002; 2008). Alignment of ITS2 sequences (88.21%) relative to the presently studied *P. papatasi* populations indicated a genetic diversity of such sequences as well as intra-species specific variations in multiple copies of the rDNA. Studies of *P. papatasi* from Mediterranean countries, including Egypt, revealed as well a high similarity in ITS2 alignments (Hamarsheh et al., 2007 b). Reflecting the similarity among ITS2 sequences of *P. papatasi* from these countries, and

indicated that they are closely related and have a common ancestor. The total length of the multi-alignment for *P. papatasi* from Alexandria and *P. papatasi* from 17 countries was 475 bp with maximum similarity 94 to 96%. Our data confirmed the informative potential of ITS2 at the intra-specific and inter-specific level for *P. papatasi*. Informative potential at the intra-specific level was reported for *P. sergenti* that is spread widely in the Old World (Depaquit et al., 2002a). Contrastingly, no intra-specific variability was observed among populations of *P. perniciosus* from Italy and Morocco (Di Muccio et al., 2000). This is true as well for populations of *P. perfiliewi* from Italy and Greece (Latrofa et al., 2011) and for *P. sergenti* from Cyprus and Pakistan (Depaquit et al., 2000). High ITS2 sequence similarity was reported for *P. sergenti* and *P. similis* sand flies (Depaquit et al., 2002a).

In other groups of insects, ITS2 sequences were informative at the intra-specific level for Rhipicephaline ticks (Barker, 1998) and South American mosquitoes (Marrelli et al., 1999; Malafronte et al., 1999). In Europe, considerable variation in ITS2 was described for six sibling species in the *Anopheles maculipennis* complex (Proft et al., 1999). More recently, in Sri Lanka, the use of ITS2 was appropriate to characterize *Anopheles barbirostris* (Gajapathy et al., 2014). In the present study, a minor differentiation was observed between *P. papatasi* populations from different climatic conditions. This is actually expected since *P. papatasi* has a large and extended distribution between the 20th and 45th latitudes and can be found in southern Europe, Asia, and Africa. One would expect such a widespread taxa to exhibit some genetic variation over its distribution range. However, previous studies dealing with the genetic diversity of *P. papatasi* have revealed little to no genetic variation within this populations of species (Esseghir et al., 1997; Parvizi and Ready 2006; Depaquit et al., 2008b). The similarity of ITS2 sequences with Morocco in case of *P. papatasi* from Alexandria and with Jordan in case of *P. papatasi* from Sinai, reflected close geographical diversity either between Morocco and Alexandria or Sinai and Jordan. The present data reported the first study identifying the ITS2 (rDNA) in the Egyptian *P. papatasi*, the major vector of cutaneous leishmaniasis (CL) in Egypt, from two geographically isolated regions Sinai and Alexandria, Egypt. Polymorphism observed in ITS2 sequences for *P. papatasi* is mainly due to: insertion; detected mutations and changes in short tandem repeat sequences (Hamarsheh et al., 2007a). Data in the present study suggested that the phylogenetic analysis could not be completely resolved, which actually makes the use of current ITS2 sequences of little value for phylogenetic interpretation. Therefore, these sequences do not constitute a suitable marker for inferring phylogenetic and population genetic relationships across *P. papatasi*. Consequently, other genetic markers are needed for this purpose. However, the study did not reveal the isolation between distant populations, and gene flow seemed to be continuous along geographically separated populations.

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