Original Article

ITS2-rDNA Sequence Variation of *Phlebotomus sergenti* s.l. (Dip: Psychodidae) Populations in Iran

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Abstract

Background: *Phlebotomus sergenti* s.l. is considered the most likely vector of *Leishmania tropica* in Iran. Although two morphotypes- *P. sergenti sergenti* (A) and *P. sergenti similis* (B)-have been formally described, further morphological and a molecular analysis of mitochondrial cytochrome oxidase I (mtDNA-COI) gene revealed inconsistencies and suggests that the variation between the morphotypes is intra-specific and the morphotypes might be identical species.

Methods: We examined the sequence of the ITS2-rDNA of Iranian specimens of *P. sergenti* s.l., comprising *P. cf sergenti*, *P. cf similis*, and intermediate morphotypes, together with available data in Genbank.

Results: Sequence analysis showed 5.2% variation among *P. sergenti* s.l. morphotypes. Almost half of the variation was due to the number of an AT microsatellite repeats in the center of the spacer. Nine haplotypes were found in the species constructing three main lineages corresponding to the origin of the colonies located in southwest (SW), northeast (NE), and northwest-center-southeast (NCS). Lineages NCS and NE included both typical *P. cf sergenti* and *P. cf similis* and intermediate morphotypes.

Conclusion: Phylogenetic sequence analysis revealed that, except for one Iranian sample, which was close to the European samples, other Iranian haplotypes were associated with the northeastern Mediterranean populations including Turkey, Cyprus, Syria, and Pakistan. Similar to the sequences of mtDNA COI gene, ITS2 sequences could not resolve *P. sergenti* from *P. similis* and did not support the possible existence of sibling species or subspecies within *P. sergenti* s.l..

Keywords: *Phlebotomus sergenti*, *P. similis*, *Leishmania tropica*, ITS2-rDNA, Iran

Introduction

"The Phlebotomine sand fly *Phlebotomus sergenti* s.l. Parrot, 1917 originally described from Algeria in 1917, has a broad range of distribution which covers areas of the southern Mediterranean (Morocco, Algeria, Tunisia), the western Mediterranean" (Portugal, Spain, Sicily), Middle East, Arabia, Iran, Afghanistan, Pakistan, and northern parts of India.

Flies of this species have been incriminated as the main vector of cutaneous leishmaniasis due to *Leishmania tropica* (CLT) throughout Iran and other CLT foci in the world (Nadim et al. 1971, Al-zahrani et al. 1988, Guilvard et al. 1991, Yaghoobi-Ershadi et al. 2002, Oshaghi et al. 2010). It has an extensive geographical distribution, wider than

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that of the parasite. The presence of this sand fly in *L. tropica* free areas and the differences in the transmission patterns of the CLT could be related to the existence of intraspecific variability or even cryptic vector species (Depaquit et al. 2002, Yahyia et al. 2004).

Intraspecific variability has been shown in some morphological and molecular characteristics of various populations from different countries. Depaquit et al. (2002) studied the intraspecific variability of the internal transcribed spacer 2 (ITS2) of 12 populations of *P. sergenti* s.l. from ten different countries. Accordingly, two branches could be identified: one was related to the northeastern Mediterranean area (Cyprus, Syria, and Turkey) and Pakistan, and the second related to southwestern of the first one including North Africa, Egypt, and Morocco. These branches are in accordance with postulated migration routes of *P. sergenti* s.l. along the Tethys Sea during the Miocene era. The sand flies belonging to these two different branches seem to differ in ecology, host preferences, and possibly also in vectorial capacity (Depaquit et al. 2002).

Morphological and mtDNA characters of *P. sergenti* s.l. were also studied on 28 Iranian populations and a few samples from Greece, Morocco, Lebanon, Turkey, Pakistan, and Syria (Moin-Vaziri et al. 2007). According to this study, based on the number of setae and the width of basal lobe of coxite, three morphotypes were identified as A, B and C, with some intermediate forms. Sequence analysis of mtDNA revealed a 6–7% genetic variation among the populations studied. However, there was no consistency between the morphotypes and the genotypes. Morphotype A was considered as *P. sergenti sergenti*, morphotype B, was identified as *P. sergenti similis*, and morphotype C had an elongated style in comparison with *P. sergenti sergenti.* In another molecular study on *P. sergenti* s.l. populations of Spain and Morocco using rDNA ITS2 and mtDNA Cyt*b* sequences (Baron et al. 2008), a high genetic diversity including five ribosomal and 16 mitochondrial haplotypes was found within 25 specimens. The authors suggested testing the vectorial capacity of those haplotypes.

Based on these studies, it is judicious to consider the potential existence of sibling species within this taxon. If sibling species within *P. sergenti* s.l. were proven, it would have important implications in epidemiology as well as in experimental studies. However, having found several intermediate morphotypes as well as sympatric ecological niche of morphotypes A, B and C, postulated us to test the gene flow between the sympatric morphotypes of this taxon using sequence analysis of the ITS2 region of rDNA gene. This multicopy gene involves homogeneization processes usually called molecular drive (Dover 1982) and has provided resolution in several studies at the taxonomic level for the *Larroussius* and *Paraphlebotomus* subgenera (Dover 1982, Depaquit et al. 2002). The use of ITS2 region has many advantages including, high and low mutation rhythm at interspecies and intraspecies level respectively, speed and ease of use, multiple target sites, predefined marker systems, known PCR primers, pre-existing knowledge of them in some sand fly species (Di Muccio 2000, Depaquit et al. 2002).

This study was conducted to verify the molecular variation of Iranian *P. sergenti* populations, and to compare them with available data in GenBank.

Materials and Methods

Sand fly collections and morphological identification

The geographical locations from which *P. sergenti* s.l. was sampled are shown in Fig. 1. Sand flies were collected from different provinces using sticky traps and aspirators. Specimens caught by sticky papers were

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washed in a bath of acetone before being stored. They were stored in 96% ethanol at 4 °C until morphological and molecular identification. Only male specimens were selected for the study of morphological and molecular variability since their characters tends to be more reliable.

The head and genitalia of individual male sand flies were cut off within a drop of ethanol, cleared in boiling Marc-André solution, and mounted between slide and cover slide in Berlese fluid and morphologically identified based on external and internal characters of the head and genitalia according to the known identification keys (Theodor and Mesghali 1964). Morphometric measurements were performed to determine morphotypes of specimens as explained by Moin-Vaziri et al. (2007). The body related to the specimen was stored dried in a vial at -20 °C before DNA extraction.

DNA extraction

Based on ecological conditions of the location where specimens were collected and on the morphological differences noted by means of morphometric analysis, a few specimens from each morphotype (Moin-Vaziri et al. 2007) and ecological condition were chosen for molecular investigation. Genomic DNA was extracted from the thorax, wings, legs and abdomen of either individual sand flies using the QIAmp DNA Mini Kit (Qiagen, Germany) (Depaquit et al. 2002) or ISH-Horowicz with small modification as described by Rassi et al. (2011).

PCR amplification

PCR was used to amplify a fragment of 480–516 bp containing the ITS2 of sand fly rDNA (Depaquit et al. 2002). PCR were performed in a 50 µl volume using 5 µl of extracted DNA solution and 50 pmol of each of the two primers of C1a: 5'-CCT GGT TAG TTT CTT TTC CTC CGC T-3' and JTS3 : 5'-CGC AGC TAA CTG TGT GAA ATC-3'. The PCR mix contained (final concentrations) 10 mM Tris HCl, pH 8.3, 1.5 mM MgCl2 , KCl 50 mM, Triton X 100 0.01%, 200 µM dNTP each, and 0.25 µl (1.25 units) of *Taq* DNA polymerase (Eurobio). Initial denaturation at 94 °C for 5 min was followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 62 °C for 1 min. and extension at 72 °C for 1 min with a final elongation time of 10 min at 72 °C. Amplicons were analysed by electrophoresis in 1.5% agarose gel containing ethidium bromide.

Sequencing and comparative sequence analysis

Purification of the PCR product was made by agarose-gel fractionation, using the Perfect Prep Gel Cleanup (Eppendorf, Germany). Direct sequencing of both DNA strands was performed by Qiagen (Hilden, Germany) and the Department of Parasitology (University of Valencia, Spain) using the primers used for DNA amplification. Sequences were edited and aligned to identify haplotypes (=unique sequences) by means of the CLUSTALW software package (www.ebi.ac.uk/clustalw) and manually adjusted, if necessary. They were analyzed using the Neighbor-Joining (NJ) method provided in the MUST software package (Philippe 1993). Available sequences of *P. sergenti* and *P. similis* were retrieved from GenBank and used for phylogenetic analysis (Table 1).

Results

Morphometric analysis

Morphological investigation on specimens of 28 populations of *P. sergenti* sl collected from 11 provinces of Iran revealed three main morphotypes (A, B and C) and a few intermediate forms (Moin-Vaziri et al. 2007). Morphotype A was similar to *P. sergenti* Parrot (1917). Morphotype B was related to *P. cf similis*, according to Perfiliev (1968) and morphotype C represents specimens with

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a curved basal lobe of coxite without a style as globulous as that of *P. sergenti*. In addition to the three main morphotypes, some intermediate forms were identified among the collected samples. These morphotypes were found sympatric in several provinces of the country. However, the proportion of each morphotype varied within each region. Morphotype A was the most frequent in all collection sites of the country.

Molecular analysis

The size of rDNA-ITS2 fragments amplified was about 480 bp, from which 278 nucleotides were attributed to ITS2, 130 bp to 5.8S and 72 bp to 28S genes. However, in addition to the main PCR product, there was an additional product, close to the main amplicon, in all amplification. Despite of gel purification before sequencing, these unwanted products affected the results of sequencing. By the way, we could obtain a total of 249–253 bp length, including most part (218–222 bp) of ITS2 and 31 bp of 28S, from 11 specimens comprising 5, 4, and 2 individuals of morphotype A, B and intermediate form A/C respectively. The sequences were submitted to Genbank (Accession Numbers: EF434818-EF434828). Comparative ITS2 sequence analysis of 11 *P. sergenti* s.l. individuals showed 13 (5.2%) polymorphic sites, from which 46% was due to indel (insertion/deletion) and 54% was due to substitutions. All polymorphic sites were located in ITS2 region and 28S region was identical in all specimens. The region was AT rich, with an average of 75%.

There were two variable microsatellite regions in the ITS2 aligned part of the specimens, which showed variable repeats of a poly (AT) microsatellite. In the first variable microsatellite sites, two specimens contain 9, three specimens had 8, and six other specimens comprised 7 repeats of AT (Fig. 2). Other entries from Genbank also showed variable number of AT repeats in this site. In the second polymorphic microsatellite, variations between the Iranian specimens were due to two single nucleotide insertions/deletions (indels) and one variable repeats of a poly (AT) microsatellite. Lower degrees of variation in the poly (AT) microsatellite also have been observed in non-Iranian entries (Fig. 2).

Based on the sequence alignment, nine different haplotypes (nominated as haplotype I–IX) have been identified, three haplotypes (VII–IX) in the south west (SW), two haplotypes (I and VI) in the north east (NE) and 5 haplotypes (I–V) in the north-center-south (NCS) populations (Table 1, 2, Fig. 3). A sample of haplotype I was positioned in NE lineage. These haplotypes differed in 1–11 nucleotide positions. The length of the segment sequenced varied between 249 to 253 bp. The ITS2 ribosomal haplotype I with three repeats was the most frequently haplotype among all haplotypes.

Distance analysis of the ITS2 sequences indicated three main lineages (Fig. 3), which supports correlations between geographical distributions of those populations. These lineages were so-called SW, NE, NCS lineages which included haplotypes of South-West, North-East and a mix of haplotypes extended from Northwest to the Central to the Southeast of the country, respectively. When we added the ITS2 sequence data of other *P. sergenti* and *P. similis* populations as representatives of other parts of the world for phylogenetic analysis, except for one Iranian sample which was close to the European samples, all of the Iranian haplotypes were associated with the north-eastern Mediterranean populations including Turkey, Cyprus, Syria, and also Pakistan (Fig. 4). The *P. similis* populations from Iran were associated with other *P. sergenti* populations, however, the European *P. similis* populations clustered separately. This might be due to independent accumulated mutations in distinct geographical populations resulted in separate branch

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in the tree. The phylogenetic tree deduced from combination of the ITS2 and the 381 bp of mtDNA CytB-NADH1 sequences obtained in our previous study (Moin-Vaziri et al. 2007) revealed similar tree and did not resolve *P. segenti* from *P. similis* (data are not shown).

Table 1. Details of *Phlebotomus sergenti* sand flies and their sequence data used in this study

Specimen code (No. assigned in Fig. 1)	Locality	Morphotype	Haplotype	ITS2 Gen-Bank Ac. No	CytB GenBank Ac. No	Reference
Iran-ser(A)-1	Tehran, Iran	A	I	EF434822	DQ840367	Moin-Vaziri et al. 2007
Iran-sim(B)-1	Tehran, Iran	B	V	EF434821	DQ840363	Moin-Vaziri et al. 2007
Iran-sim(B)-2	Mashhad, Iran	B	I	EF434825	DQ840345	Moin-Vaziri et al. 2007
Iran-serI(A/C)-3	Neishabur, Iran	A/C	VI	EF434820	DQ840352	Moin-Vaziri et al. 2007
Iran-serII(A/C)-3	Neishabur, Iran	A/C	I	EF434819	DQ840359	Moin-Vaziri et al. 2007
Iran-ser(A)-4	Izeh, Iran	A	VIII	EF434828	DQ840384	Moin-Vaziri et al. 2007
Iran-ser(A)-5	Bushehr, Iran	A	IX	EF434826	DQ840391	Moin-Vaziri et al. 2007
Iran-sim(B)-6	Bandar-e-Abbas, Iran	B	VII	EF434824	DQ840395	Moin-Vaziri et al. 2007
Iran-ser(A)-7	Bam, Iran	A	III	EF434818	DQ840376	Moin-Vaziri et al. 2007
Iran-sim(B)-7	Bam, Iran	B	IV	EF434827	DQ840375	Moin-Vaziri et al. 2007
Iran-ser(A)-8	Iranshahr, Iran	A	II	EF434823	DQ840397	Moin-Vaziri et al. 2007
Malta-similis*	Malta	-	-	AF462334		Depaquit et al. 2002
Greece-similis1*	Greece	-	-	AF462333	-	Depaquit et al. 2002
Greece-similis2*	Greece	-	-	AF218324	-	Depaquit et al. 2000
Portugal-sergenti*	Portugal	-	-	AF462327	-	Depaquit et al. 2002
Turkey-sergenti*	Turkey	-	-	AF462332	-	Depaquit et al. 2002
Syria-sergenti*	Syria	-	-	AF462328	-	Depaquit et al. 2002
Cyprus-sergenti*	Cyprus	-	-	AF462323	-	Depaquit et al. 2002
Pakistan-sergenti*	Pakistan	-	-	AF218323	-	Depaquit et al. 2000
Spain-sergenti1*	Spain	-	S-C	EU980387	-	Baron et al. 2008
Spain-sergenti2*	Spain	-	S-B	EU980386	EU980375	Baron et al. 2008
Spain-sergenti3*	Spain	-	S-A/M-B	EU980385	EU980375	Baron et al. 2008
Spain-sergenti4*	Spain	-	-	AF462324	-	Depaquit et al. 2002
Italy-sergenti*	Italy	-	-	AF462330	-	Depaquit et al. 2002
Morocco-sergenti1*	Morocco	-	M-C	EU980384	EU980371	Baron et al. 2008
Morocco-sergenti2*	Morocco	-	M-A	EU960382	EU980370	Baron et al. 2008
Egypt-sergenti*	Egypt	-	-	AF462329	-	Depaquit et al. 2002
Israel-sergenti*	Israel	-	-	AF462325	-	Depaquit et al. 2002
P. papatasi	Iran	-	-	EF408801	-	Depaquit et al. 2007

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Table 2. Comparison of nucleotide characters of *Phlebotomus sergenti* of Iran at polymorphic sites of the rDNA ITS2. Gap (indel) shows by –

Specimen code	11	19	23	24	50	75	76	77	78	140	146	147	148
Iran-ser(A)-8	A	T	T	A	T	T	A	T	A	G	A	-	-
Iran-ser(A)-7	A	T	T	G	T	T	A	T	A	G	A	-	-
Iran-sim(B)-7	C	T	C	G	C	T	A	-	-	G	A	-	-
Iran-sim(B)-1	A	T	T	G	T	T	A	-	-	G	A	-	-
Iran-serII(A/C)-3	A	T	T	G	T	-	-	-	-	G	A	-	-
Iran-ser(A)-1	A	T	T	G	T	-	-	-	-	G	A	-	-
Iran-sim(B)-2	A	T	T	G	T	-	-	-	-	G	A	-	-
Iran-serI(A/C)-3	A	T	T	G	T	T	A	-	-	G	C	A	T
Iran-ser(A)-4	A	C	T	G	T	-	-	-	-	G	C	A	T
Iran-ser(A)-5	A	C	T	G	T	-	-	-	-	A	C	A	T
Iran-sim(B)-6	A	T	T	T	T	-	-	-	-	A	A	-	-

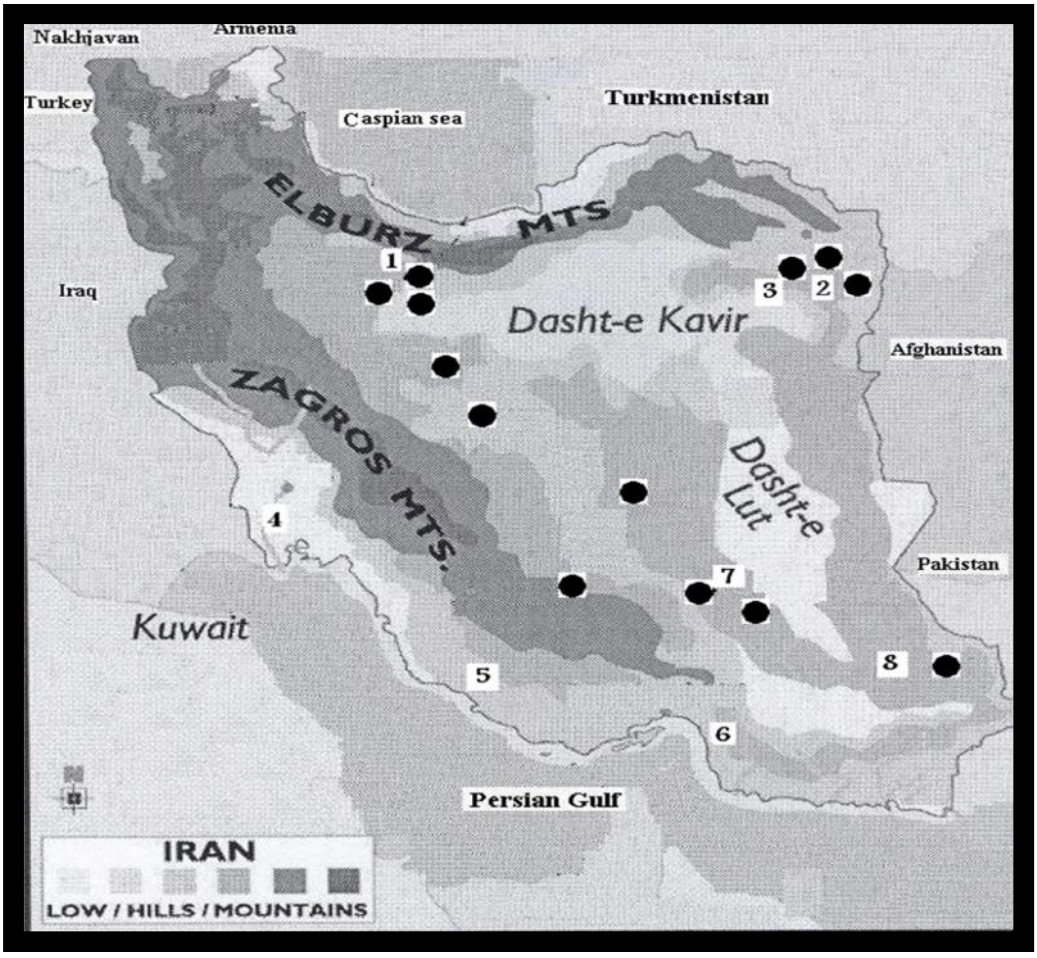

Fig. 1. Collection sites of *Phlebotomus sergenti* s.l. for this study, (1) Tehran, (2) Mashhad, (3) Neishabur, (4) Izeh, (5) Bushehr, (6) Bandar-e-Abbas, (7) Bam, (8) Iranshahr. Dark spots represent CLT endemic foci in Iran

http://jad.tums.ac.ir
Published Online: October 04, 2016

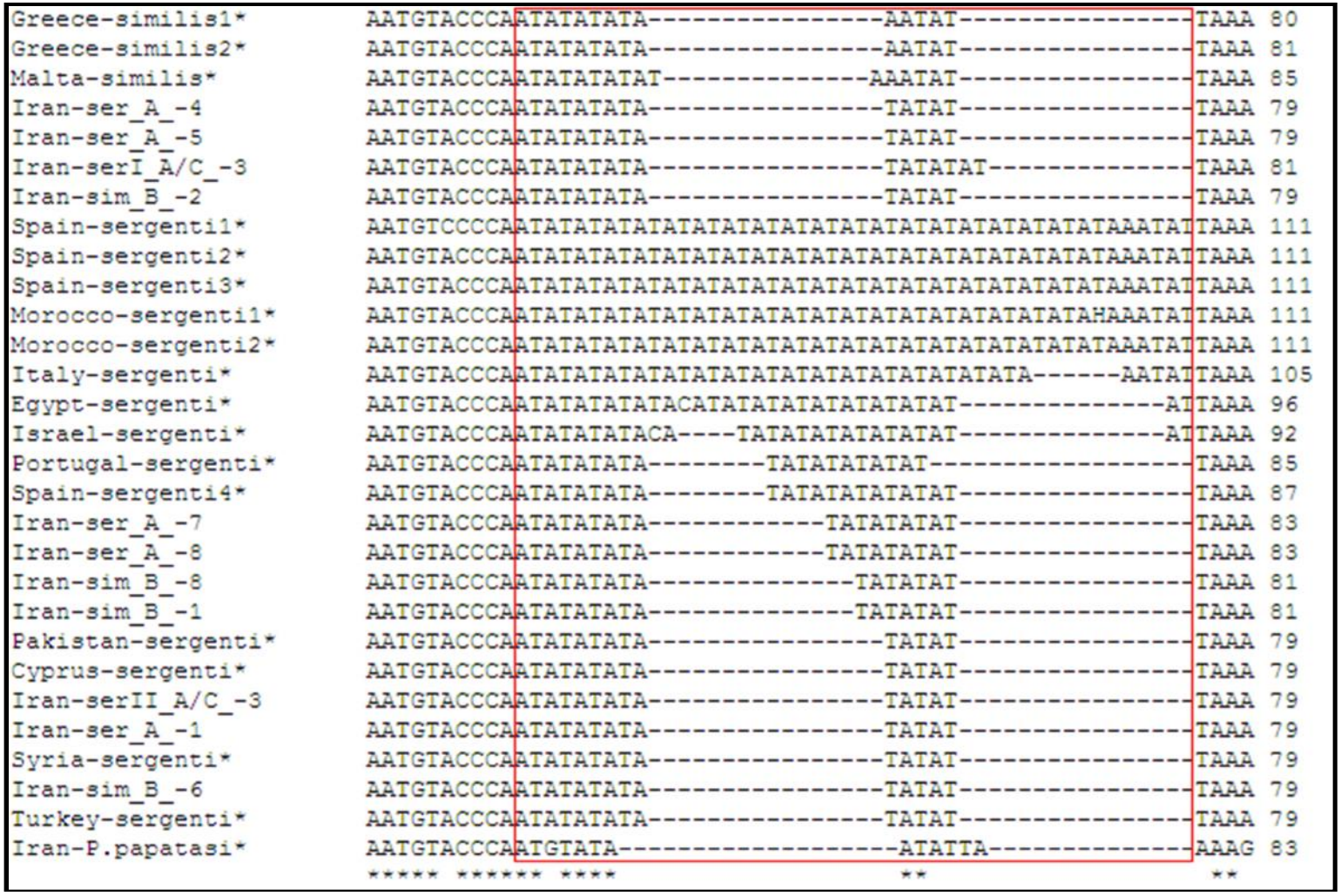

Fig. 2. Microsatellite region in ITS2-rDNA sequences of different populations of *Phlebotomus sergenti* s.l. from Iran and other parts of world retrieved from GenBank (Marked with *). A: Morphotype A, B: Morphotype B, A/C: Intermediate forms of morphotype A and C, ser, *P. sergenti*, sim, *P. similis*

http://jad.tums.ac.ir
Published Online: October 04, 2016

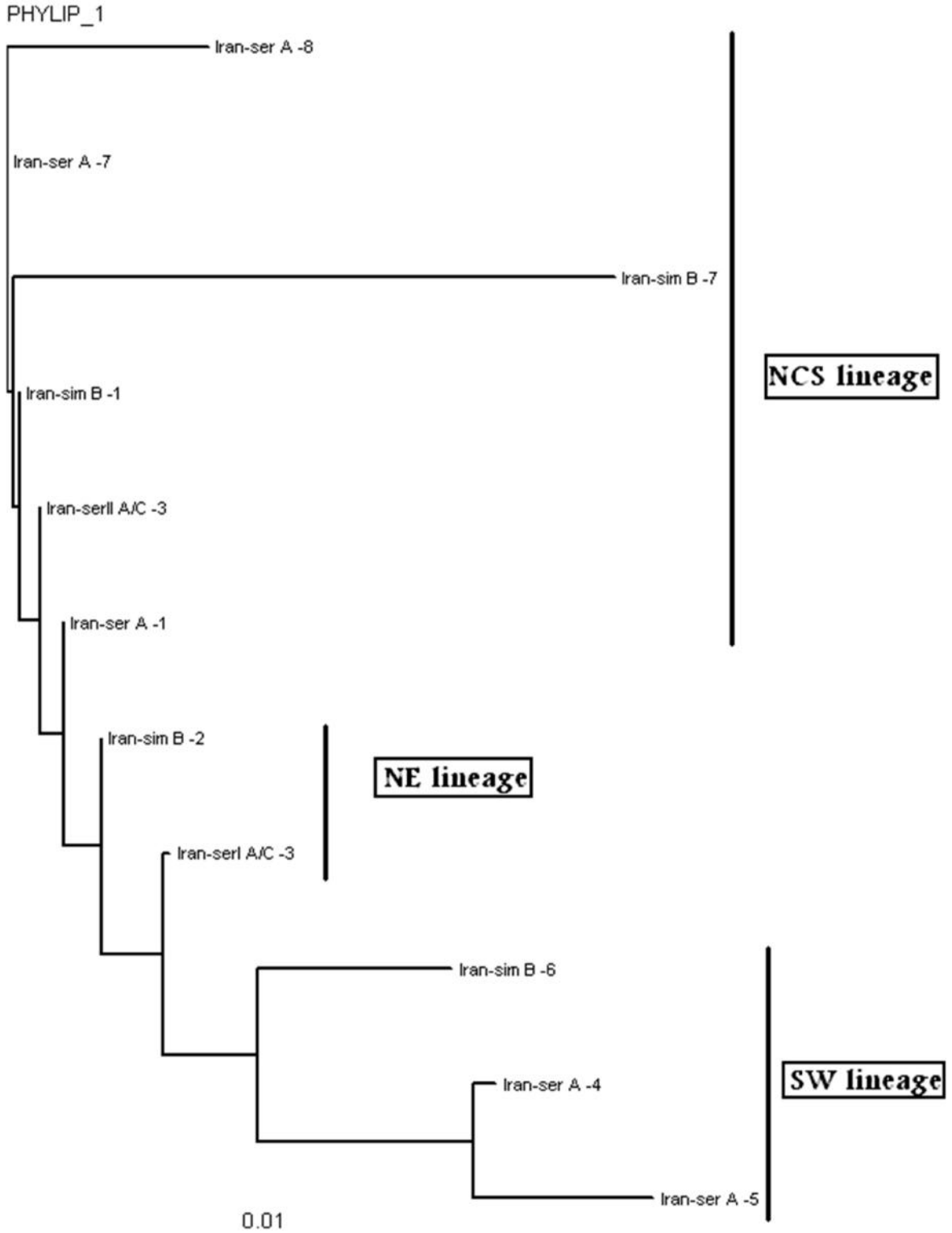

Fig. 3. Neighbor-Joining tree inferred from 253 bp of ITS2-rDNA sequences including 222bp of ITS2 and 31 bp of 28S of *Phlebotomus sergenti* s.l. populations from Iran. A, morphotype A, B, morphotype B, A/C, intermediate form of morphotype A and C, ser, *P. sergenti*, sim, *P. similis*

http://jad.tums.ac.ir
Published Online: October 04, 2016

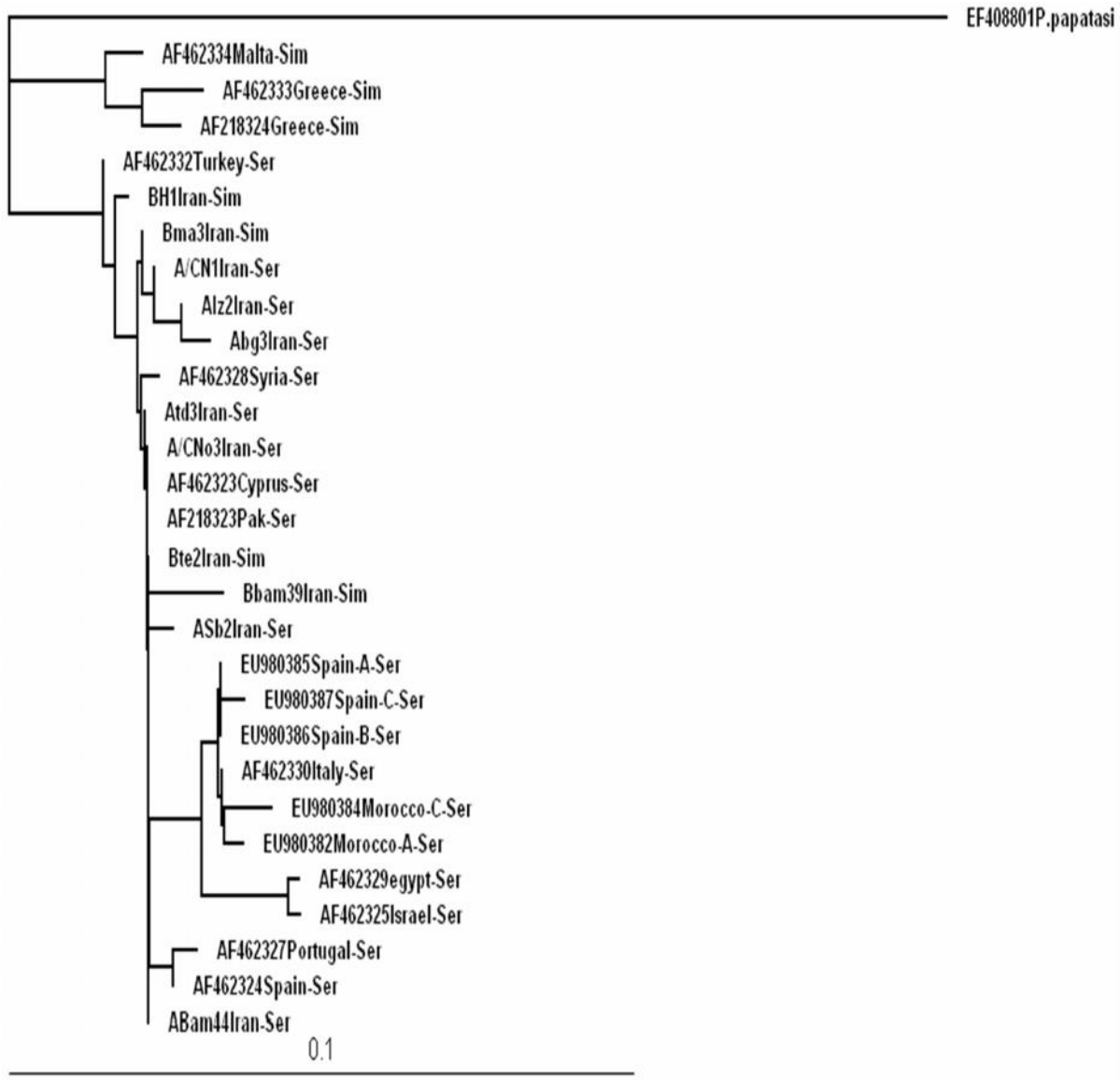

Fig. 4. Neighbor-Joining tree inferred from 253 bp of ITS2-rDNA sequences including 222 bp of ITS2 and 31 bp of 28S of *Phlebotomus sergenti* s.l. populations from Iran and other available data from Genbank originated from Europe, Africa, and Asia. A, morphotype A, B, morphotype B, A/C, intermediate form of morphotype A and C, ser, *P. sergenti*, sim, *P. similis*, *P. papatasi* (AN: EF408801) has been used as an outgroup

Discussion

Results of this study showed a high diversity between specimens of *P. sergenti* s.l. in Iran where we found nine ribosomal haplotypes. However, the rate of genetic variation (3%) within the ITS2 locus between the Iranian populations is half of the rate (6%) that was previously observed in the mitochondrial (CytB-NADH1) sequences (Moin-Vaziri et al. 2007). It seems that mtDNA sequences are more appropriate for the study of the intraspecific variability of *P. sergenti* s.l. in a more limited geographical environment.

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The high diversity observed in ITS2 is in agreement with previous studies indicating different ecological, morphological, and molecular variation among Iranian *P. sergenti* populations. Previous studies revealed at least three morphotypes (A, B and C). However, in this study like previous study, we have not found any correlation between genotypes, ecotypes, or morphotypes and the results obtained here do not support the presence of sibling species (*P. sergenti* and *P. similis*) within the taxon. However, results of this study are in conflict with a phylogenetic analysis of nuclear ribosomal DNA of Depaquit et al. (2002) that showed the monophyly both of *P. sergenti* s.l. and *P. similis* and they were not sister species. Their result confirmed a study previously carried out, using morphological and morphometric approaches for examining the status of the two species by the same investigators (Depaquit et al. 1998). Their worldwide attempt showed allopatric situation of the two taxa at that time.

According to our findings, it seems that this group (morphotypes) cannot be considered as two different species because firstly we have found many intermediate morphological and genetically forms among the specimens, which indicate possible gene flow and lack of reproductive isolation between them. Secondly, finding identical genotypes (100%) among different morphotypes that are *P. sergenti* and that *P. similis* in mtDNA (Moin-Vaziri et al. 2007) and ITS2 sequences support our morphological findings. Considering the molecular drive characteristic of ITS2-rDNA strongly indicated that these two morphotypes are not isolated reproductively. Moreover having found these two taxa sympatric in most provinces of Iran differs from taxonomic conception of subspecies. According to our data, we encountered with different morphologically populations of P. *sergenti* s.l. in Iran. In light of our results, it is too early to come to a final decision on taxonomic status of the species. More molecular, morphological, and hybridization studies between the two taxa, particularly between geographically distinct populations is necessary.

In Iran, CLT is endemic in 14 foci located in 8 provinces restricted to large and medium sized cities in different parts of the country (Yaghoobi-Ershadi 2012).We identified three ITS2 lineages of *P. sergenti* which is in agreement to the ones identified using the mtDNA loci (Moin-Vaziri et al. 2007). Lineage NE and NCS correspond to the main foci of CLT in the country which included both typical and intermediate morphotypes of *P. cf sergenti* and *P. cf similis*. In other hand SW lineage exist in clean area of CLT. These findings warrant studies to examine if CLT is due to differences in the vectorial capacity of the *P. sergenti* s.l. lineages (NE/ NCS versus SW) or other ecological and epidemiological factors are involved.

Conclusion

Phylogenetic sequence analysis revealed that most Iranian haplotypes were associated with the northeastern Mediterranean populations. Similar to the sequences of mtDNA COI gene, ITS2 sequences could not resolve *P. sergenti* from *P. similis* and did not support the possible existence of sibling species or subspecies within *P. sergenti* s.l.. More molecular studies on other genes or hybridization should be done to clarify the status of different morphotypes of mentioned species in Iran. Moreover, with regard to the importance of the epidemiology of leishmaniasis, further studies need to be performed on the possible role of these three morphotypes in the transmission of *L. tropica.*

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