Intraspecific variability of natural populations of *Phlebotomus sergenti*, the main vector of *Leishmania tropica*

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ABSTRACT: An intraspecific study of *Phlebotomus sergenti* was performed on populations from Turkey, Syria, Israel, and Uzbekistan by four different approaches: geometric morphometrics, RAPD analysis, internal transcribed spacer 2 (ITS2) sequencing (nuclear marker), and cytochrome B sequencing (mitochondrial marker). In RAPD analysis, distinct clades were formed in accordance with the geographical origin of the specimens. There was no distinct grouping according to place of origin within the Turkish samples from various localities in south-eastern Anatolia, which suggests a gene flow between populations separated spatially by the Amanos mountains, a mountain range of a considerable altitude. The results of ITS2 rDNA sequencing complied with the previously published intraspecific division of *P. sergenti* into two branches, northeastern and southwestern. However, mtDNA haplotypes formed three lineages with specimens from Turkey and Israel, sharing a common clade. A previously postulated hypothesis about a complex of sibling species within *P. sergenti* is therefore questionable. Cytochrome B seems to be a more discriminative marker for intraspecific variability assessment. *Journal of Vector Ecology* 36 (Supplement 1): S49-S57. 2011.

Keyword Index: *Phlebotomus sergenti*, RAPD, intraspecific variability, sibling species, geometric morphometrics.

INTRODUCTION

The phlebotomine sand fly *Phlebotomus sergenti* Parrot, 1917 is a main vector of *Leishmania tropica*, a causative agent of cutaneous leishmaniasis. It is usually a dominant sand fly species in foci of anthroponotic cutaneous leishmaniasis in the Old World (Killick-Kendrick 1990, Jacobson 2003, Schnur et al. 2004), although in some specific areas other sand flies can play a role in *Leishmania tropica* transmission (Svobodova et al. 2006). Yet, *Phlebotomus sergenti* is a species of great medical importance.

The geographical distribution of *P. sergenti* is very broad and includes areas of both the southern and northern Mediterranean, Middle East, Arabia, Afghanistan, Pakistan, and northern parts of India, being wider than the distribution of *L. tropica* (Depaquit et al. 2002). Therefore, a question arose about the degrees of intraspecific variability and vectorial capacity of different *P. sergenti* populations. Internal transcribed spacer 2 (ITS2) of twelve populations of *Phlebotomus sergenti* from ten different countries was sequenced and two principal branches were identified: one related to the northeastern Mediterranean area (Cyprus, Pakistan, Syria, and Turkey), while the other to South and West (Egypt, Morocco, and Israel). Such a division correlates with postulated migration routes of *P. sergenti* along the Thetys Sea at the Miocene time (Depaquit et al. 2002). If sibling species within *P. sergenti* were demonstrated it may have important consequences for the epidemiology of *L. tropica* as well as control strategies against this sand fly species.

Recently, laboratory colonies of *P. sergenti* originating from Turkey and Israel, areas belonging to the two different branches mentioned above, were studied by random-amplified polymorphic DNA (RAPD), geometric morphometrics, and cross-mating experiments. RAPD and geometric morphometrics clearly discriminated between laboratory colonies. However, in a cross-mating study, a viable hybrid F1 and F2 progeny was obtained, suggesting that there is not a reproduction barrier between these two *P. sergenti* branches (Dvorak et al. 2006).

The present study compares sand flies acquired from different wild populations. The sand flies were collected in Turkey and Israel at the sites from which the previously tested colonies originated. As considerable intrapopulation variation in mitochondrial haplotypes of *P. sergenti* from close foci of *L. tropica* was reported (Yahia et al. 2004), specimens from other localities in south-east Turkey were also included in the analysis. RAPD analysis was followed by a geometric morphometric study. On a larger scale, specimens from Turkey, Israel, Syria, and Uzbekistan were compared. Three molecular methods were used for the comparison: RAPD, ITS2 sequencing (nuclear marker), and cytochrome B gene sequencing (mitochondrial marker).

MATERIALS AND METHODS

Sand flies

Phlebotomus sergenti were captured using CDC miniature light traps at different localities in Turkey, Israel, Syria, and Uzbekistan. After morphological identification, individual sand flies were stored in 95% ethanol for further analysis. In addition, specimens from two laboratory colonies of *P. sergenti* maintained at Charles University in Prague were included: the first originating from Sanli Urfa, Turkey, and the second from Amnun, Israel (for more details about colonies, see Dvorak et al. 2006). Both colonies

were routinely maintained as described elsewhere (Benkova and Volf 2007).

DNA extraction and RAPD analysis

DNA was extracted from individual sand flies using the High Pure PCR Template Preparation Kit (Roche, France), according to the manufacturer´s guidelines. Only the thorax was used; the head and abdomen were used for species determination. The wings and legs of flies were dissected out prior to DNA isolation.

Out of 60 decamer random primers previously tested (Operon Technologies Inc., U.S.A.), seven were used for the RAPD analysis: OPE4, OPE 11, OPE15, OPE16, OPI1, OPI14, and OPO11. RAPD reactions (25 µl) were performed by a PTC-200 thermocycler (MJ Research Inc, USA) and subjected to 45 amplification cycles. The temperature profile was 94° C for 1 min, 35° C for 2 min, and 72° C for 3 min. An initial denaturation step of 94° C for 4 min and a final extension step of 72° C for 10 min were added.

After PCR amplification, the reaction products were separated on 1.5% agarose gel stained with ethidium bromide. Obtained bands were transformed into a binary matrix data where presence or absence of a band was codified as 1 or 0, respectively. Genetic distances of samples were computed from Nei-Li´s coefficient of similarity (Nei and Li 1979). Phenograms were constructed by the unweighted pair-grouping analysis (UPGMA) which identifies topological relationships on the basis of similarity and constructs the phylogenetic tree in a stepwise manner. The robustness of trees was assessed by bootstrap analysis. PC program FreeTree (Hampl et al. 2001) was used for computations of genetic distances and the construction of trees.

Sequencing and phylogenetic analyses

Templates for direct sequencing were amplified by PCR in a 50-µl volume. For ITS2 amplification, primers and PCR conditions previously published (Depaquit et al. 2000) were used. For cytochrome B amplification, a pair of primers was designed by Primer3 (v. 0.4.0) (Rozen et al. 2000): VD-F (5´-TATGTACTACCATGAGGACAAATATC-3´) and VD-R (5´-TAAAAGGGGCTTCAACTGGA-3´). Thermal cycling was denaturation at 94° C for 5 min followed by 35 cycles of 94° C for 30 sec, 55° C for 1 min, and 72° C for 1 min. The amplification was completed by elongation at 72° C for 10 min. The reaction mixture and thermocycler were the same as in RAPD amplification described above.

PCR products were sequenced in both directions using the same primers as for the DNA amplification on 3100 Avant Genetic Analyser (Applied Biosystems, USA). All PCR products were cleaned by QIAquick PCR Purrification Kit (Qiangen, Germany) prior to the sequencing.

Obtained DNA sequence data were compared with those in the GenBank database using the BLAST algorithm. A data set containing sequences of three sand fly species, including the new sequences of *Phlebotomus sergenti*, was created. The sequences were aligned using ClustalX 1.81 and the resulting alignment was manually edited by BioEdit. The alignment is available from the corresponding author upon request. The phylogenetic trees were constructed using maximum parsimony, maximum likelihood (PhyML), and Bayesian methods. The maximum parsimony, and maximum likelihood trees were constructed in PAUP* 4.0b10 by ten replicates of heuristic search. The starting tree was obtained by the stepwise addition procedure with a random order of taxa addition and swapped using the tree bisection-reconnection (TBR) algorithm. The models of nucleotide substitution for maximum likelihood and distance analyses were chosen by hierarchical nested likelihood ratio tests implemented in Modeltest 3.06. The trees were bootstrapped with 1,000 (maximum parsimony and distance method) or 500 (maximum likelihood) replicates, each with ten replicates of random taxa addition with TBR branch swapping. Bayesian analysis was performed using MrBayes 3.1.1. Base frequencies, rates for six different types of substitution, proportion of invariant sites, and shape parameter of the gamma correction for the rate heterogeneity with four discrete categories, were allowed to vary. The covarion model was used to allow the rate heterogeneity along the tree. The number of generations of Markov chain Monte Carlo was five million and the trees were sampled every 100th generation. The first 12,500 trees were discarded as burn-in.

Geometric morphometric analysis

For geometric morphometrics, 23 specimens were randomly taken from each of the colonies collected from Tülek, Konak, Karaagac, and Delicay localities in southeastern Anatolia. All the specimens were screened for the presence of known ectoparasites to shield the morphometric data from possible traumatic variations (Aytekin et al. 2007a). The wings were processed as described earlier (Dvorak et al. 2006) and photographed using a Leica MZ-7.5 stereoscopic zoom dissection microscope with a DC-300 digital camera system then coded and archived. Two-dimensional Cartesian coordinates of 16 landmarks and four semi-landmarks were digitized by TPSDIG2.05 (Rohlf 2005) software after the procedures of standard utilization by TPS-Util (Rohlf 2007). The landmark configurations obtained were then scaled, translated, and rotated against the consensus configuration by the GLS Procrustes superimposition method using the software Morphologika2® (O'Higgins and Jones 2006) to perform Principal Components Analysis (PCA) and to calculate centroid sizes. The principal components were later used for SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering method) clustering to obtain an UPGMA phenogram by Ntsys-Pc2.1® (Rohlf 2000). Euclid distance was preferred for the pooled interval data to obtain the similarity matrix. The size morphometry of the taxa were investigated by using the centroid sizes of the wings as estimator with the nonparametric Kruskal-Wallis test. Multivariate regression of the first two PC scores on an independent variable (ln centroid size) was also carried out by Morphologika2®. As the number of independent variables exceeds the number of specimens within each

group, we decided to perform a Canonical Variate Analysis of landmark data by using the PCA axis scores of our specimens instead of using partial warp scores in the analysis by IMP CVAGEN6n (Zelditch et al. 2006). No analogous systems were used during the whole procedure to keep the digital errors to a minimum.

RESULTS

RAPD analysis and geometric morphometrics

Seven decamer random primers were used for the RAPD analysis of wild specimens of *P. sergenti* from eight localities in Turkey and three localities in Israel. Primers amplified a total number of 105 fragments, 19 being monomorphic and 86 (82%) polymorphic (shared only by a proportion of specimens). The size of the fragments ranged from 100 to 1,200 bp. The band pattern given by amplification with each primer was reproducible and stable. The UPGMA analysis of these data revealed a position of two clades: one containing all field samples originating from Turkey as well as a specimen colony of Turkish origin, the second containing all field samples from Israel plus the Israeli colony specimen (Figure 1). A similar grouping pattern was also obtained by the neighbor-joining method (data not shown).

The data obtained from the geometric morphometrics show a pattern similar to the one obtained by molecular techniques. Only the Karaagac population showed a distinct pattern through the negative extreme of the first principal component. The same pattern was also observed in the UPGMA clustering of the females of Karaagac (Figure 2). In general, it is not possible to observe a differentiation among populations although the CANOVAR graphs show four distinct groups. These are mainly based on sexual differences and possibly a close relationship between some specimens (MANOVA Axis 1: Lambda = 0.0000, chi square = 265.9410, df = 70, p < 0.001; Axis 2: Lambda = 0.0000, chi square = 182.7844, df = 54, p < 0.001; Axis 3: Lambda = 0.0001, chi square = 114.9432, df = 40, p < 0.001; Axis 4: Lambda = 0.0131, chi square = 56.4022, df = 28, p< 0.01). The regression analysis also showed that there is no correlation between size and shape. The centroid sizes of the populations showed some differences as females of Delicay have bigger wings than the rest of populations, and the males have clearly smaller wings in all populations (Kruskall-Wallis Test: H = 18,47; df = 7; p < 0.01).

The same seven RAPD primers were used to analyze eleven field samples from localities in following countries: Turkey, Israel, Syria, and Uzbekistan. A similar pattern of clades was obtained; specimens from each country formed a unique clade. Specimens from Uzbekistan, originating from one locality, formed the most homogeneous clade with almost identical band patterns (Figure 3). Three remaining clades, which contained specimens from different localities, exhibited a certain degree of variability.

ITS2 sequencing

The size of amplified fragments was similar for all sequenced specimens; the alignment was 539 sites long including 68 (12.6 %) variable sites. The poly (AT) region at position 290–306 was excluded from the analysis. The alignment is available upon request to the corresponding author. Eleven sequences of *P. sergenti* ITS2 previously published (Depaquit et al. 2002) were included in the analysis. For the tree construction, ITS2 sequences of *Phlebotomus similis* (accession number AF462333) and *P. jacusieli* (accession number AF218317) were chosen as outgroup species.

We sequenced the following *P. sergenti* specimens: two specimens from Israel (localities Amnum and Tiberias), three specimens from Turkey (two specimens from Sanli Urfa plus one specimen from a laboratory colony originating from the same locality), three specimens from Syria (two specimens from Aleppo and one specimen from Raqah), and two specimens from Uzbekistan (locality Chodak). Sequences were identical for specimens sampled from the same locality. According to phyML analysis (Figure 4), the clustering is generally in agreement with the previous findings which postulated two clades: northeastern and southwestern. The specimens from Uzbekistan fall within the oriental clade, close to the samples from Pakistan, Cyprus, and Lebanon. Syrian samples from Aleppo and Raqah were identical but differed from a previously sequenced specimen from Kassab, a different locality in Syria, in two positions: C/T substitution at position 85 and G/A substitution at position 273. Israeli samples from two different localities were identical as well, but they also differed from a previously sequenced specimen from West Bank in one position, C/A substitution at position 341.

Cytochrome B sequencing

In all specimens, PCR amplification produced a single band. The alignment was 625 sites long including 152 (24%) variable sites. Of these, 51 were uninformative, occurring in only in one specimen. The alignment is available upon request to the corresponding author. In total, 22 specimens from Turkey, Israel, Syria and Uzbekistan were sequenced. For the tree construction, cytochrome B gene sequences of *Phlebotomus ariasi* (accession number AF161195) and *P. tobbi* (accesion numer AF161210) were chosen as outgroups as there were no sequences of cytochrome B available for other members of *Paraphlebotomus* subgenus.

Sequencing data analysis revealed that mtDNA haplotypes formed three lineages (Figure 5). Three specimens from Uzbekistan formed a clade where two specimens were identical and differed from a third in two C/A substitutions and one T/A substitution. One specimen from Turkey formed a clade together with a Syrian specimen, sharing three unique C/T substitutions. All remaining samples formed a third clade, which consists of all sequenced samples from Israel and the remaining 12 specimens from Turkey (11 specimens from the wild populations and one from a laboratory colony). Five samples from Israel, originating from two localities, Tiberias and Kfar Adumin, shared identical haplotypes. The variability of Turkish haplotypes can be attributed mainly to A/G substitution at position 84 and C/T substitution at position 168. Apart from these two
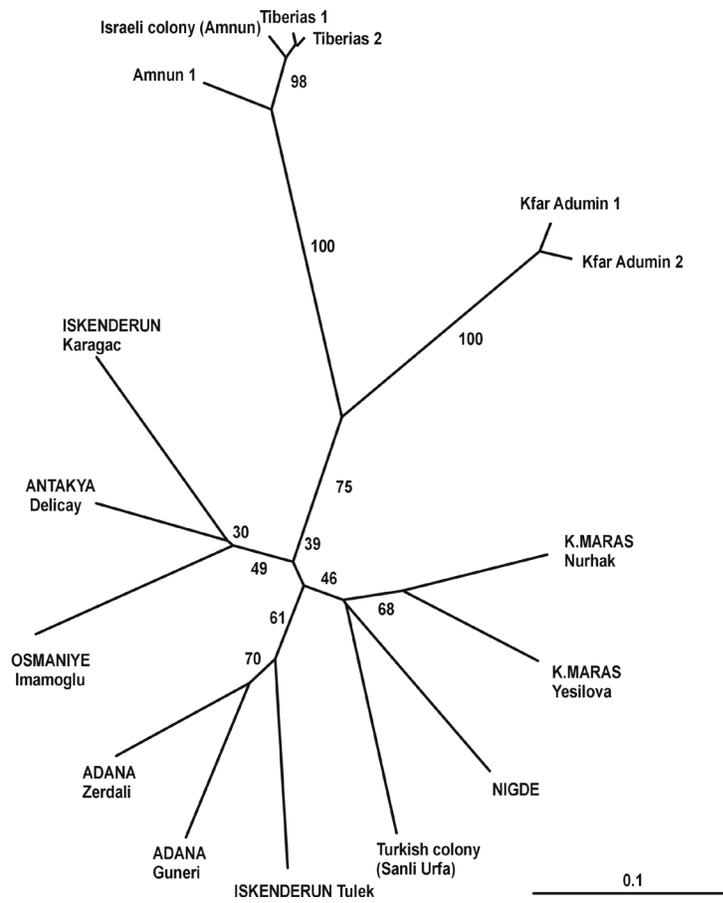
Figure 1. Unrooted UPGMA dendrogram generated from the results of RAPD analysis of Turkish and Israeli specimens with bootstrap values.
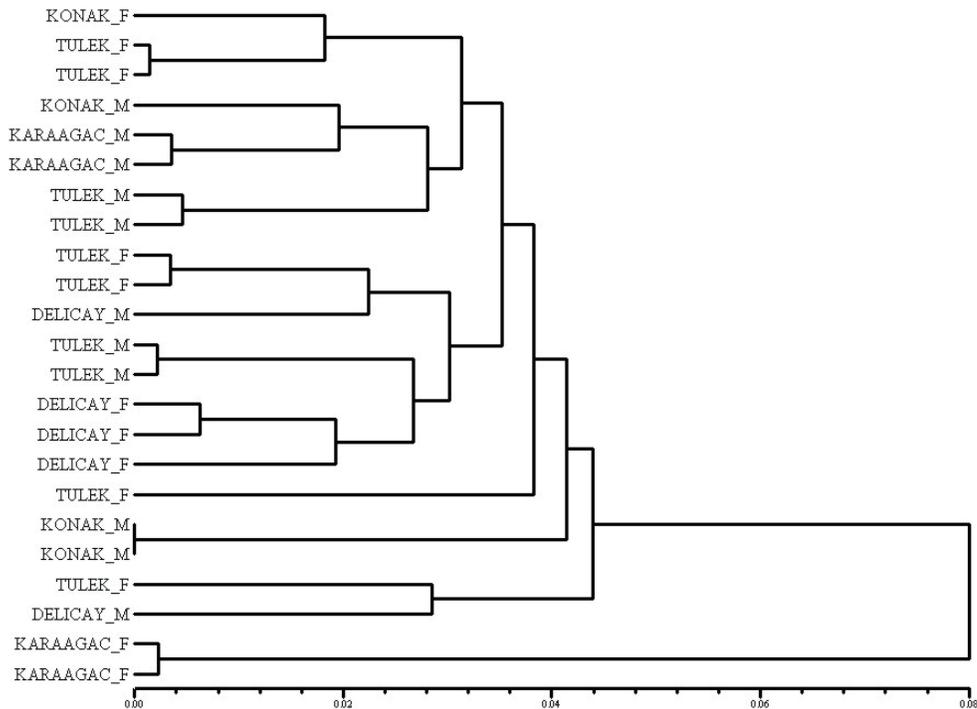
Figure 2. UPGMA phenogram based on the geometric morphometric analysis of Turkish specimens from different localities (Euclid distance SAHN clustering).
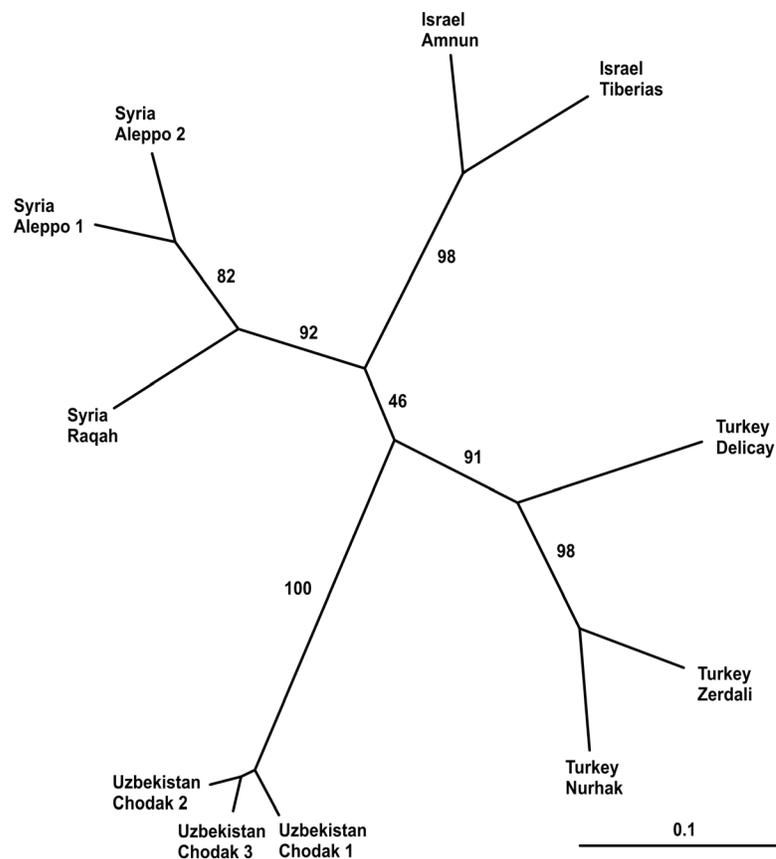
Figure 3. Unrooted UPGMA dendrogram generated from the results of RAPD analysis of specimens from Turkey, Syria, Israel and Uzbekistan with bootstrap values.

polymorphic sites, the haplotypes within the clade differed in only single substitutions.

DISCUSSION

The study of *Phlebotomus sergenti* populations from different areas of distribution demonstrated the informational potential of both molecular and morphometric methods deployed to assess the intraspecific variability of this important cutaneous leishmaniasis vector. Previously, we have shown by both RAPD and geometric morphometrics that two laboratory colonies originating from Turkey and Israel form two clearly distinguishable groups (Dvorak et al. 2006). In the present study, a same grouping was observed for wild-caught specimens; sand flies from each country formed their own clade. Similar grouping obtained by RAPD and geometric morphometrics suggests that these two methods are complementary. There was no distinct grouping within the Turkish clade, although the localities are separated not only by geographical distance but also by the Amanos mountain range. This range runs

roughly parallel to the Gulf of Iskenderun and divides the coastal region of Cilicia from the inland provinces of Turkey and Syria. Reaching a maximum height of 2,240 m, it is open at several passes that descent to an altitude of approximately 700 m above sea level. Sand flies are generally considered to be poor fliers, not traveling from their breeding and resting sites (Killick-Kendrick 1990). However, *P. papatasi* was reported to disperse in the open desert when carried by air currents (Perfiliev 1968). According to the results of RAPD analysis, we may conclude that the Amanos mountains do not represent a sufficient barrier for sand fly dispersal as the passes play a role of transitional gaps. This is in accord with previously published results based on geometric morphometrics (Aytekin et al. 2007b).

To get a broader perspective, specimens from Syria and Uzbekistan were also included in the RAPD analysis. While Syria has an intermediate geographical position between Turkey and Israel, specimens from Uzbekistan represent a very distant population. Again, the samples from each country formed a distinct clade.
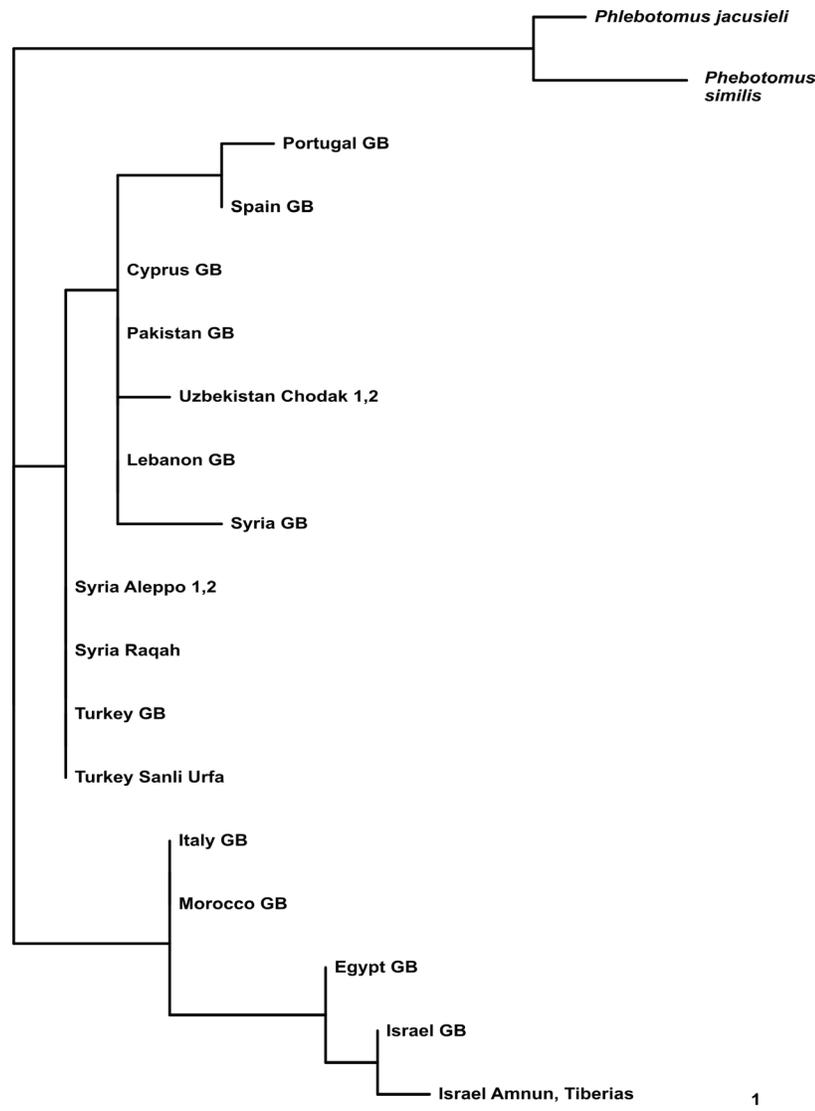
RAPD marker had been proposed to distinguish

Figure 4. Neighbor-joining tree inferred from rDNA ITS2 data. Sequences obtained from GenBank are marked "GB".

between *P. papatasi* and *P. duboscqi*, two closely related, morphologically similar vectors of cutaneous leishmaniasis (Mukhopadhyay et al. 2000). Based on the results of RAPD analysis, we could not find such a marker which would have appropriate discriminatory power to distinguish clearly and reproducibly *P. sergenti* specimens from two hypothetical sibling species, proposed by Depaquit et al. (2002).

ITS2 rDNA sequencing is widely used in numerous studies of molecular evolution and phylogeny of various organisms, including dipterans (Nirmala et al. 2001). In Old World phlebotomine sand flies, it was succesfully deployed in phylogenetic analyses of subgenus *Larroussius* (Di Muccio et al. 2000) and *Paraphlebotomus* (Depaquit et al. 2000). On an intraspecific level, it revealed a notable variability of *P. sergenti* (Depaquit et al. 2002), while there was a significant homogeneity in diverse populations of *P. papatasi* (Depaquit et al. 2008). Our results of ITS2 rDNA sequencing corroborated the previously published intraspecific division of *P. sergenti* into two branches,

northeastern and southwestern.

Mitochondrial genes are also very popular and are useful molecular markers, as the lack of recombination reflects the evolutionary history even more accurately (Avise 1994). Mitochondrial cytochrome B (cyt B) genes were successfully used to evaluate intraspecific variability of *P. papatasi* populations (Esseghir et al. 1997, Hamarsheh et al. 2007), as well as the New World phlebotomine sand flies including *Lutzomyia longipalpis* (Hodgkinson et al. 2003, Torgerson et al. 2003). When regional intraspecific variability of *P. sergenti* was examined in three foci of cutaneous leishmaniasis in Morocco, three primary mitochondrial lineages were identified, with a markedly regional distribution (Yahia et al. 2004). Recently, a study on mtDNA sequences containing a partial sequence of cytochrome B gene was performed with specimens of *P. sergenti* from geographically separated regions of Iran, together with several samples from Greece, Morocco, Lebanon, Turkey, Pakistan, and Syria (Moin-Vaziri et al.
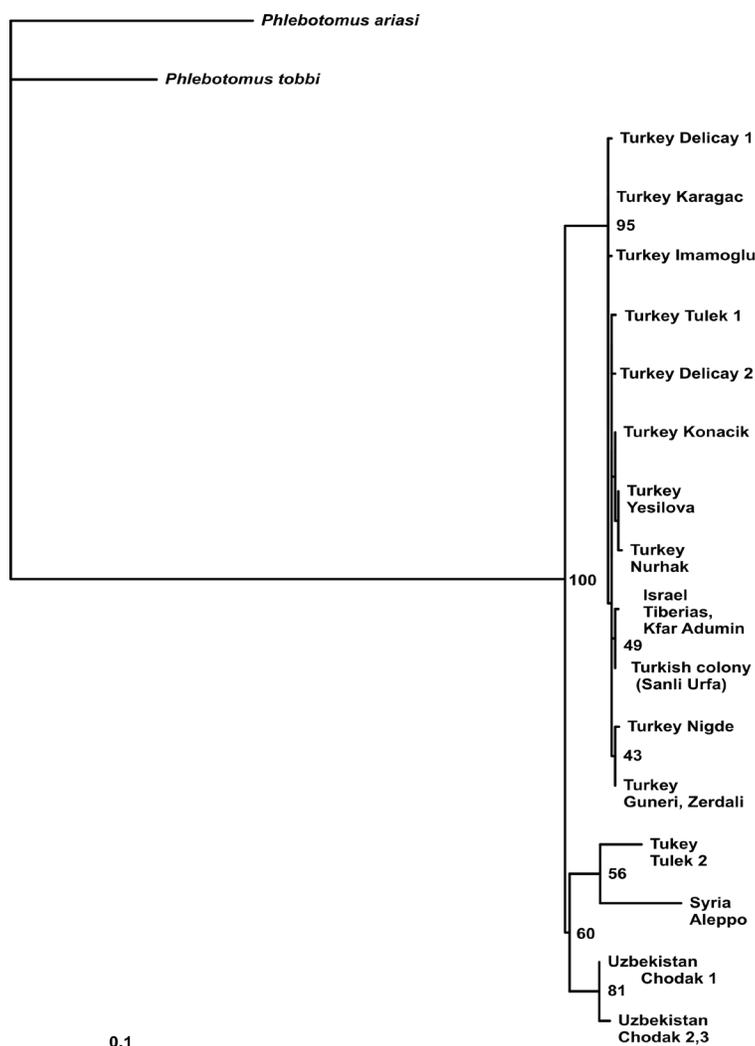
Figure 5. PhyML tree inffered from cytochrome B sequences of specimens from Turkey, Syria, Israel and Uzbekistan with bootstrap values.

2007). Sequence analysis revealed a 6-7% genetic distance within the Iranian populations and among the specimens of other countries and constituted three main groups. However, three morphotypes identified according to male terminalia were not consistent with these genotype groups.

In our study, mtDNA haplotypes from Turkey, Israel, Syria, and Uzbekistan formed three lineages. All specimens from Uzbekistan clustered in one clade, two being identical; this is not surprising as they all originate from one locality. Syrian specimens clustered together with one specimen from Tulek, Turkey. All remaining specimens from Turkey formed a clade with samples from Israel. Israeli samples were all identical, although originating from two distant localities. Most of the variability of Turkish haplotypes can be attributed to two substitutions. Apart from these two polymorphic sites, the haplotypes within the clade differed only in single substitutions. As mitochondrial markers are considered to reflect the evolutionary history and phylogenetic relationships with a high accuracy, currently found similarities between cytochrome B sequences of specimens from Turkey and Israel put in doubt the concept of a *P. sergenti* species complex postulated by Depaquit et

al. (2002). This result of cytochrome b sequences analysis is further supported by recent findings, that males and females originating from Turkey and Israel mated readily and produced viable and fertile progeny under experimental laboratory conditions (Dvorak et. al. 2006).

A combination of several approaches and the use of different markers gives a more appropriate and relevant idea about the intraspecific variability of *P. sergenti*. Results presented here, together with cytochrome B sequencing analysis of specimens from Morocco (Yahia et al. 2004), favor this marker as more discriminative than ITS2. Nevertheless, further analysis with more molecular markers is worth testing to resolve the taxonomy status of *P. sergenti* and closely related species of subgenus *Paraphlebotomus*.

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