

ITS 2 sequences heterogeneity in *Phlebotomus sergenti* and *Phlebotomus similis* (Diptera, Psychodidae): possible consequences in their ability to transmit *Leishmania tropica*

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Abstract

An intraspecific study on *Phlebotomus sergenti*, the main and only proven vector of *Leishmania tropica* among the members of the subgenus *Paraphlebotomus* was performed. The internal transcribed spacer 2 (ITS2) sequences of 12 populations from 10 countries (Cyprus, Egypt, Italy, Lebanon, Morocco, Pakistan, Portugal, Spain, Syria, and Turkey) were compared. Samples also included three species closely related to *P. sergenti*: *Phlebotomus similis* (three populations from Greece and Malta), *Phlebotomus jacusieli* and *Phlebotomus kazeruni*. Our results confirm the validity of the taxa morphologically characterised, and imply the revision of their distribution areas, which are explained through biogeographical events. At the Miocene time, a migration route, north of the Paratethys sea would have been followed by *P. similis* to colonise the north of the Caucasus, Crimea, Balkans including Greece and its islands, and western Turkey. *Phlebotomus sergenti* would have followed an Asiatic dispersion as well as a western migration route south of the Tethys sea to colonise North Africa and western Europe. This hypothesis seems to be well supported by high degree of variation observed in the present study, which is not related to colonisation or to intra-population variation. Two groups can be individualised, one oriental and one western in connection with ecology, host preferences and distribution of *L. tropica*. We hypothesise that they could be correlated with differences in vectorial capacities. © 2002 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Phlebotomus sergenti*; *Phlebotomus similis*; Internal transcribed spacer 2; rDNA; Vector; *Leishmania*; Biogeography

1. Introduction

Phlebotomus sergenti Parrot, 1917 is the only proven vector of *Leishmania tropica* (Wright, 1903) within the subgenus *Paraphlebotomus* (Al-Zahrani et al., 1988; Guillard et al., 1991). It has a more widespread distribution than the parasite (Depaquit et al., 1998). The presence of this sandfly in *L. tropica* free areas and the varied prevalence of anthroponotic cutaneous leishmaniasis in endemic areas suggest the possible existence of closely related sandfly species (cryptic species) with different vectorial capacities.

The history of the species since its first description is confused. The male was described from the Constantine area (Algeria), based on having a pyriform style with a terminal and a sub-terminal spine (Parrot, 1917). A description of a female caught in copula in Portugal (França, 1918) was based only on external characters, an approach considered useless for the diagnosis of species. The same authors redescribed the male at the same time and provided an original drawing in which the aspect of the style is in agreement neither with the text, nor with the original description. Does it belong to the same species?

Adler and Theodor (1929) redescribed the male and female from specimens belonging to Parrot's collection and from their own collections from Palestine, Syria and

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Mesopotamia, without taking into account that the latest specimens did not come from 'the type locality'. Similar to Parrot (1917), they noticed in the male a globulous style with two slightly shifted terminal spines. In the description of the female they stressed the presence of internal morphological characters such as the pharyngeal armature of long teeth and the reservoir of the spermatheca formed by five to six rings, which were not observed in the Algerian specimens.

Perfiliev (1963, in Lewis, 1982), in the description of *Phlebotomus sergenti similis* from the Caucasus, Crimea and southern Ukraine, and later (Perfiliev, 1968), stressed that the style of this subspecies has a sub-apical spine on a tubercle inserted slightly under the apical one. He reported that in *Phlebotomus sergenti sergenti*, the two spines are generally at the same level, and concluded that the presence of a sub-apical spine as it appeared in Parrot (1917) should not be considered as characteristic. For him, the differentiation between the two sub-species was based on the form of the basal process and length of the lateral lobe. After Artemiev and Neronov (1984) and Depaquit et al. (1998), we consider *P. sergenti* and *P. similis* as representing two distinct species.

Regarding their vectorial capacities, *P. sergenti* is a proven vector of *L. tropica* in Saudi Arabia (Al-Zahrani et al., 1988) and in Morocco (Guilvard et al., 1991). Except in eastern Africa and in Namibia, *P. sergenti* is the most probable vector in all foci of anthroponotic cutaneous leishmaniasis, where it is always found as a dominant species (Killick-Kendrick, 1990). Concerning *P. similis*, its role (considered by Garifallou et al. (1984) as *P. sergenti*) in the transmission of *L. tropica* was evoked in the Greek Ionian Islands but has never been proven.

The present molecular study concerns four species of the sub-genus *Paraphlebotomus* Theodor. Focussing on the type-species *P. sergenti* Parrot, and including material from various origins, it constitutes the first contribution to the knowledge of molecular variation within the main vector of *L. tropica*. The use of rDNA, has many advantages including high mutation rhythm (e.g. internal transcribed spacer 2, (ITS2)), speed and ease of use, multiple target sites, predefined marker systems (e.g. ITS2), known polymerase chain reaction (PCR) primers, as well as a small knowledge base for sandflies (Depaquit et al., 2000 and Di Muccio et al., 2000). This multicopy gene involves homogenisation processes usually called molecular drive (Dover, 1982).

Phlebotomine sandflies constitute a good model for biogeography because larvae live in the soil and imagoes fly on very short distances. Variation observed in sequences results from biogeographical or vicariant events, which explain the choice of distance and phylogeny as analysis methods.

2. Materials and methods

Our sample included 12 populations of *P. sergenti* from

10 countries. In order to study the inter-population differences, we also included three closely related species (Depaquit et al., 2000): *P. similis* Perfiliev (from three locations), *Phlebotomus jacusieli* Theodor and *Phlebotomus kazeruni* Theodor and Mesghali (Table 1).

To appreciate the existence of intra-population variation, a pilot study was carried out on Turkish specimens caught in two distinct localities: Adana, located at the foot of the Eastern slope of the Taurus, and Sanliurfa, 300 km eastwards, a well known focus of cutaneous leishmaniasis caused by *L. tropica* (Volf et al., 2002). From the latter location, wild flies as well as colonised flies were compared (Table 1).

The majority of the studied specimens were caught with CDC miniature light traps, but some flies were caught on oily papers or came from laboratory colonies (Table 1).

Phlebotomine sandflies were dried before DNA extraction. The head and genitalia of a single sandfly were cut off in a drop of ethanol then cleared in boiling Marc-André solution and mounted between slide and cover slide for identification (these slides are available on request from the senior author). Genomic DNA was extracted from the thorax, wings, legs and abdomen. Body parts were crushed and incubated at 60°C in a solution containing cetyltrimethylammonium bromide, following the method used by Depaquit et al. (2000). RNase (0.5 units) was added to the aqueous phase, which was then incubated at 37°C for 30 min. to remove RNA. Total genomic DNA was precipitated by the addition of isopropanol. After centrifugation, the pellet was washed, dried, and resuspended in 50 µl of water. PCR was performed in a 50 µl volume using 5 µl of extracted DNA solution and 50 pmol of each of the two primers used in a previous study (Depaquit et al., 2000). Primer sequences are 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* polymerase (Eurobio). Initial denaturation at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 30 s, 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.

PCR products were both direction sequenced directly by Qiagen using the universal C1a and JTS3 primers used for DNA amplification, or cloned into pUC 18 vectors using the 'sure clone ligation kit' (Amersham Pharmacia) following the manufacturer's instructions before sequencing three times. Identical sequences (one direction only) were always obtained within individuals, except one Moroccan specimen which needed a fourth sequencing because of a doubtful nucleotide.

Sequence alignment was performed using the MUST package (Philippe, 1993). Maximum parsimony, bootstrap and distance analysis using Neighbour-Joining (Saitou and

Table 1
Specimens sequenced

Species	Origin (country and area or locality)		Method and year of catching	Number of studied specimens ^a	Sequencing strategy ^a	GenBank accession number
<i>Phlebotomus sergenti</i>	Cyprus	Paphos	CDC miniature light trap, (1993)	Two males	C + DS	AF462323
	Egypt	South Sinai	Colony, (1997) ^b	Two males	C	AF462329
	Israel	West bank	CDC miniature light trap, (1999)	One male; one female	DS	AF462325
	Italy	Sicily	Oily paper, (1999)	One male	C	AF462330
	Lebanon	Qaa	CDC miniature light trap, (1996)	Two males	C	AF462326
	Morocco	Rif	CDC miniature light trap, (1998)	Three males	C + DS	AF462331
	Pakistan	Pringabad	Colony, (1996) ^c	Two males; one female	C	AF218323
	Portugal	Mertola	CDC miniature light trap, (1996)	Two males	C	AF462327
	Spain	Granada	CDC miniature light trap, (1994)	Three males	C	AF462324
	Syria	Kassab	CDC miniature light trap, (1995)	Two males	C + DS	AF462328
	Turkey	Cukurova	Oily paper, (1997)	Two males	DS	AF462332
	Turkey	Sanliurfa	Colony, (1999) ^d	Three males	C + DS	AF462332
	Turkey	Sanliurfa	CDC miniature light trap, (1999)	Two males; one female	DS	AF462332
	<i>Phlebotomus similis</i>	Greece	Crete	CDC miniature light trap, (1991)	Three males	DS
Greece		Macedonia	CDC miniature light trap, (1994)	Two males	C + DS	AF218324
Malta		Gozo	CDC miniature light trap, (1989)	Two males	C	AF462334
<i>Phlebotomus jacusieli</i>	Lebanon	Nachgura	CDC miniature light trap, (1996)	One male	C	AF218317
<i>Phlebotomus kazeruni</i>	Egypt	Sinai	Colony, (1997) ^e	Two males	C	AF218318

^a DS, direct sequencing of the PCR product; C, cloning before sequencing of the insert.

^b Fifth generation.

^c Killick-Kendrick et al. (1995).

^d Eighth generation.

^e Hanafi et al. (1999).

Nei, 1987) were performed using Phylogenetic Analysis Using Parsimony (PAUP*) 4.0 beta 8 version software (D.L. Swofford, PAUP*. (* and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts, 1998) on the complete dataset, as well as on this dataset, from which were excluded sites 281–314 to account for a variable poly (AT) insertion (or deletion) within *P. sergenti*. Gaps were treated as a fifth base, even in the complete dataset. *Phlebotomus kazeruni* was used as outgroup, following the results obtained by Depaquit et al. (2000).

3. Results

The size of amplified fragments was species dependent, and varied between 480 and 516 nucleotides from which 274–310 nucleotides were attributed to ITS2. The alignment was 547 sites long including 112 variable sites, of which 45 were parsimony uninformative and 67 were parsimony informative (Fig. 1). The region was AT rich, with the proportion of AT greater than 70% (70.83–73.84% for the complete amplified fragment, and 80.73–84.28% for ITS2 only).

Sequences were identical for specimens sampled from the same location. Topologies obtained from a maximum parsimony analysis carried out on the complete dataset (547 nucleotides) are given in Fig. 2. *Phlebotomus sergenti* and *P. similis* are both monophyletic and strongly supported by bootstrap values of 92 and 87%, respectively.

Phlebotomus similis is the sister species of *P. jacusieli*, confirming the results of Depaquit et al. (2000). Bootstrap support is moderate (71%), but their grouping is supported by an AA indel at sites 192–193 and two nucleotide substitutions at sites 240–241 (Fig. 1).

The grouping of *P. similis* from Greek Macedonia, Crete and Gozo (Malta) is supported by two A indel changes at sites 461–462. Additional indels (189–191 and 225) group populations from Gozo with those from Macedonia. The observed clusterings within *P. sergenti* are consistent for populations from Israel, Egypt, Sicily and Morocco, independently of the analytical methods used (maximum parsimony or neighbour-joining). This clade has 100% bootstrap support using parsimony (Fig. 2). Populations from Sicily and Morocco appear as sister populations (bootstrap value 100%). Both taxa have a long poly(AT) insertion for sites 289–296. They also share a common poly(AT) stretch (sites 297–306), one indel site (386) and two substitutions (391 and 442). Populations from Egypt and Israel appear as sister populations with strong bootstrap support (98%).

According to maximum parsimony analysis (Fig. 2), the phylogenetic position (paraphyletic or monophyletic) of populations from Spain and Portugal is not resolved, as evidenced by the lack of bootstrap support. In the neighbour-joining analysis, they appear as monophyletic, and as the sister group of populations from Israel, Egypt, Sicily and

Morocco. Their sequences are identical except for Portuguese flies which have a shorter poly(AT) pattern (two nucleotides) than the Spanish specimens (positions 307 and 308), and a G nucleotide at site 360, that is probably autapomorphic (position 360).

The Lebanese population appears as sister group of the previous ones, independently of the analytical method used. This position is well supported by bootstrap values (98%).

The sequences obtained from Cypriot, Pakistani, Syrian, and Turkish flies were the shortest: 480 bp (Fig. 1) because they include very few poly(AT) patterns. They constitute the most basal populations within *P. sergenti*. The Turkish populations shared a plesiomorphic site (387). Two additional changes (substitutions) at sites 85 and 327 distinguish the Syrian population.

The observed clusterings are supported in part by the insertion of 13–47 nucleotides in poly (AT) patterns, including positions 269–316 (Fig. 1). To measure the impact of these poly(AT) patterns, a separate analysis using the same methodology was undertaken with sites 281–314 excluded. The new dataset (513 sites) includes 78 variable sites, of which 41 were parsimony informative and 37 parsimony uninformative. The topology of trees obtained following both maximum parsimony (Fig. 3) and neighbour-joining analyses is similar. These new analyses confirm the monophyly of *P. sergenti* and *P. similis*, which are supported by bootstrap values of 100 and 91%, respectively.

Phlebotomus similis is again placed as the sister species of *P. jacusieli*. Within *P. similis*, the populations from Greek Macedonia and Malta are the most closely related, with the population from Crete as their sister group.

In comparison with the results from complete database (Fig. 2), the clustering within *P. sergenti* are consistent for populations from Israel, Egypt, Sicily and Morocco (Fig. 3). However, the phylogenetic position of populations from Spain and Portugal is very different. They appear as the sister group of populations from Pakistan, Cyprus, Lebanon, Syria, and Turkey (Fig. 3), not as the sister group of populations from Israel, Egypt, Sicily and Morocco as previously emphasised (Fig. 2). This clustering is supported by a bootstrap value of only 50%. Accordingly, the position of populations from Spain and Portugal, as sister-group of those from Israel, Egypt, Sicily and Morocco, is only supported by the poly(AT) indel events.

The two *P. sergenti* groups are not correlated to microsatellite region only. A study not taking into account this part of the gene, gave a similar bifurcating tree. However, the phylogenetic status of the AT microsatellite is of the highest interest. It resolves relationships into the western populations of *P. sergenti*. The more western the populations, the longer is the AT microsatellite, which could be correlated to evolutionary processes. Further studies seem necessary to explain why Iberic specimens have a shorter AT microsatellite than African and Sicilian species, which do not permit to resolve their phylogenetic position.

		1	1111111112	2222222223	3333333334	4444444445		
		1234567890	1234567890	1234567890	1234567890	1234567890		
P. sergenti	Pakistan	CGCAGCTAAC	TGTGTGAAT	CGTGTGAAT	CGCAGCACCA	TGAACATCGA	50	
	Israel	-----	-----	-----	-----	-----	50	
	Cyprus	-----	-----	-----	-----	-----	50	
	Spain	-----	-----	-----	-----	-----	50	
	Lebanon	-----	-----	-----	-----	-----	50	
	Portugal	-----	-----	-----	-----	-----	50	
	Syria	-----	-----	-----	-----	-----	50	
	Egypt	-----	-----	-----	-----	-----	50	
	Sicily	-----	-----	-----	-----	-----	50	
	Morocco	-----	-----	-----	-----	-----	50	
P. similis	Turkey	-----	-----	-----	-----	-----	50	
	Crete	-----	-----	-----	-----	-----	50	
	Malta	-----	-----	-----	-----	-----	50	
P. kazeruni	Greece	-----	-----	-----	-----	-----	50	
	Egypt	-----	-----	-----	-----	-----	50	
P. jacusielii	Lebanon	-----	-----	-----	-----	-----	50	
5555555556 6666666667 7777777778 8888888889 9999999990 1234567890 1234567890 1234567890 1234567890 1234567890								
P. sergenti	Pakistan	CATTTGAAC	CGATATGCG	GTCCTAGCA	AGTITTAAC	TTGTTTAAAC	100	
	Israel	-----	-----	-----	-----	-----	100	
	Cyprus	-----	-----	-----	-----	-----	100	
	Spain	-----	-----	-----	-----	-----	100	
	Lebanon	-----	-----	-----	-----	-----	100	
	Portugal	-----	-----	-----	-----	-----	100	
	Syria	-----	-----	-----	-----	-----	100	
	Egypt	-----	-----	-----	-----	-----	100	
	Sicily	-----	-----	-----	-----	-----	100	
	Morocco	-----	-----	-----	-----	-----	100	
P. similis	Turkey	-----	-----	-----	-----	-----	100	
	Crete	-----	-----	-----	-----	-----	100	
	Malta	-----	-----	-----	-----	-----	100	
P. kazeruni	Greece	-----	-----	-----	-----	-----	100	
	Egypt	-----	-----	-----	-----	-----	100	
P. jacusielii	Lebanon	-----	-----	-----	-----	-----	100	
1111111111 1111111111 1111111111 1111111111 1111111111 0000000001 1111111112 2222222223 3333333334 4444444445 1234567890 1234567890 1234567890 1234567890 1234567890								
P. sergenti	Pakistan	TGCATGGACC	AGCTATGTT	GAGTATCGTA	AATATTAAC	AATGAAATT	150	
	Israel	-----	-----	-----	-----	-----	150	
	Cyprus	-----	-----	-----	-----	-----	150	
	Spain	-----	-----	-----	-----	-----	150	
	Lebanon	-----	-----	-----	-----	-----	150	
	Portugal	-----	-----	-----	-----	-----	150	
	Syria	-----	-----	-----	-----	-----	150	
	Egypt	-----	-----	-----	-----	-----	150	
	Sicily	-----	-----	-----	-----	-----	150	
	Morocco	-----	-----	-----	-----	-----	150	
P. similis	Turkey	-----	-----	-----	-----	-----	150	
	Crete	-----	-----	-----	-----	-----	150	
	Malta	-----	-----	-----	-----	-----	150	
P. kazeruni	Greece	-----	-----	-----	-----	-----	150	
	Egypt	-----	-----	-----	-----	-----	150	
P. jacusielii	Lebanon	-----	-----	-----	-----	-----	150	
1111111111 1111111111 1111111111 1111111111 1111111112 5555555556 6666666667 7777777778 8888888889 9999999990 1234567890 1234567890 1234567890 1234567890 1234567890								
P. sergenti	Pakistan	GTITTTTTT	***CTT*CT	ATTTCCTAT	AGAAAA**	***GAAACA	188	
	Israel	-----	-----	-----	-----	-----	192	
	Cyprus	-----	-----	-----	-----	-----	188	
	Spain	-----	-----	-----	-----	-----	189	
	Lebanon	-----	-----	-----	-----	-----	188	
	Portugal	-----	-----	-----	-----	-----	189	
	Syria	-----	-----	-----	-----	-----	188	
	Egypt	-----	-----	-----	-----	-----	189	
	Sicily	-----	-----	-----	-----	-----	189	
	Morocco	-----	-----	-----	-----	-----	189	
P. similis	Turkey	-----	-----	-----	-----	-----	188	
	Crete	-----	-----	-----	-----	-----	191	
	Malta	-----	-----	-----	-----	-----	194	
P. kazeruni	Greece	-----	-----	-----	-----	-----	196	
	Egypt	-----	-----	-----	-----	-----	189	
P. jacusielii	Lebanon	-----	-----	-----	-----	-----	191	
2222222222 2222222222 2222222222 2222222222 2222222222 0000000001 1111111112 2222222223 3333333334 4444444445 1234567890 1234567890 1234567890 1234567890 1234567890								
P. sergenti	Pakistan	TGGAGTAT	GAATTTTTT	TT***CAG	CTCTAATAT	GTATTAA**G	233	
	Israel	-----	-----	-----	-----	-----	236	
	Cyprus	-----	-----	-----	-----	-----	233	
	Spain	-----	-----	-----	-----	-----	234	
	Lebanon	-----	-----	-----	-----	-----	233	
	Portugal	-----	-----	-----	-----	-----	234	
	Syria	-----	-----	-----	-----	-----	233	
	Egypt	-----	-----	-----	-----	-----	236	
	Sicily	-----	-----	-----	-----	-----	234	
	Morocco	-----	-----	-----	-----	-----	234	
P. similis	Turkey	-----	-----	-----	-----	-----	237	
	Crete	-----	-----	-----	-----	-----	233	
	Malta	-----	-----	-----	-----	-----	243	
P. kazeruni	Greece	-----	-----	-----	-----	-----	243	
	Egypt	-----	-----	-----	-----	-----	236	
P. jacusielii	Lebanon	-----	-----	-----	-----	-----	237	
2222222222 2222222222 2222222222 2222222222 2222222222 5555555556 6666666667 7777777778 8888888889 9999999990 1234567890 1234567890 1234567890 1234567890 1234567890								
P. sergenti	Pakistan	TATATTGGA	TGACCAAA	ATATATATAT	*****	*****	263	
	Israel	-----	-----	-----	-----	-----	266	
	Cyprus	-----	-----	-----	-----	-----	263	
	Spain	-----	-----	-----	-----	-----	264	
	Lebanon	-----	-----	-----	-----	-----	263	
	Portugal	-----	-----	-----	-----	-----	264	
	Syria	-----	-----	-----	-----	-----	263	
	Egypt	-----	-----	-----	-----	-----	270	
	Sicily	-----	-----	-----	-----	-----	276	
	Morocco	-----	-----	-----	-----	-----	284	
P. similis	Turkey	-----	-----	-----	-----	-----	263	
	Crete	-----	-----	-----	-----	-----	267	
	Malta	-----	-----	-----	-----	-----	273	
P. kazeruni	Greece	-----	-----	-----	-----	-----	273	
	Egypt	-----	-----	-----	-----	-----	266	
P. jacusielii	Lebanon	-----	-----	-----	-----	-----	267	

Fig. 1. ITS2 sequence alignment (from positions 131 to 472, written in bold print) with flanking 5.8 and 28S rDNA (written in thin print). Identities are denoted by dashes and gaps by asterisks. Sequences from Turkish flies are identical and include two populations from Sanliurfa (wild and colony) and another from Cukurova.

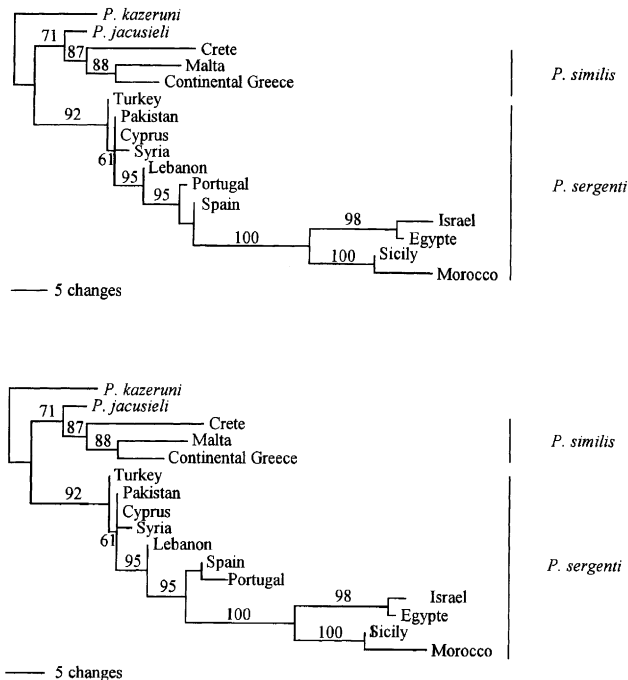


Fig. 2. Two most parsimonious phylograms (length 134) obtained after a Branch and Bound search of PAUP* performed on the complete database (547 nucleotides). *Phlebotomus kazeruni* is used as outgroup. CI = 0.88; RI = 0.91. Bootstrap values (1000 replications) are indicated on the branches.

4. Discussion

The ITS2 is known to be informative at the intraspecific level for Rhipicephaline ticks (Barker, 1998) or South American mosquitoes (Marrelli et al., 1999; Malafronte et al., 1999). In Europe, considerable variation in ITS2 was described for six sibling species from the *Anopheles maculipennis* complex (Proft et al., 1999). However, in the taxonomy of Phlebotomine sandflies, ITS2 was used in the study of two subgenera of *Phlebotomus*. In the subgenus *Larrousius* (Di Muccio et al., 2000), ITS2 contained no intraspecific variability among seven populations of *P. perniciosus* (from Italy and Morocco) as well as in populations of *P. perfiliewi* from Italy and Greece. In the subgenus *Paraphlebotomus*, a similar observation was made for two populations of *P. sergenti* from Cyprus and Pakistan (Depaquit et al., 2000). In the study reported here, two or three (exceptionally one) specimens sequenced from each population had no intra-population variability (identical sequences). Moreover, in Turkey, where we had access to three populations (two wild and one colony; Table 1), the sequences obtained from all the specimens were identical. Based on these results within populations, we can assume recency of common descent and hence origin for populations geographically distant more than 300 km.

Results presented here further demonstrate the informational potential of ITS2 at the intraspecific level for a Phlebotomine species that is widespread in the Old World. At

the present time, only one molecular study concerning Phlebotomine sandflies, carried out on *Lutzomyia longipalpis* emphasised intraspecific variation (Uribe Soto et al., 2001). The other ones focussed on interspecific and/or intergeneric variation (Esseghir et al., 1997, 2000; Aransay et al., 1999, 2000; Martin-Sanchez et al., 2000).

Phlebotomus sergenti and *P. similis* are demonstrably monophyletic on the basis of several molecular synapomorphies. This result confirms a study previously carried out using morphological and morphometric approaches for examining the species status of these two taxa (Depaquit et al., 1998). These two species are allopatric at the present time. The revised distribution of *P. similis* (Fig. 4) includes Azerbaijan, Russia, Ukraine, Romania, ex-Yugoslavia, Albania, Greece, Turkey and Malta. Turkey is the only country in which both species are present, but *P. similis* should occur only west of Taurus and Antitaurus and *P. sergenti* only to the east (Fig. 4). In Greece all of the previous continental or insular records of *P. sergenti* are actually *P. similis*. The same is true for Malta (Gozo), where *P. similis* is present, not *P. sergenti*, as reported by Léger et al. (1991). Indeed, the presence of *P. similis* is difficult to explain on this last island because *P. sergenti* (not *P. similis*) occurs in Sicily, which implies two different establishments. Should a human importation of *P. similis* be considered in Malta? The significant variability in ITS2

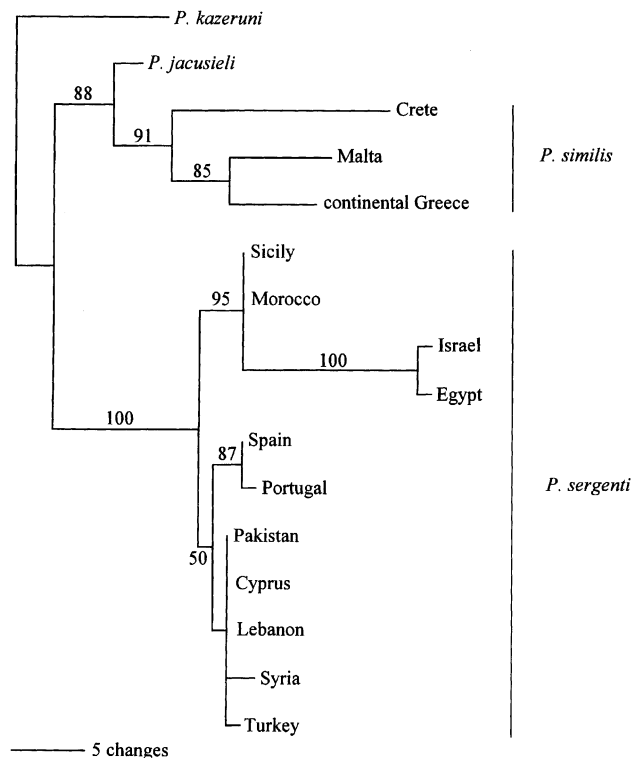


Fig. 3. Most parsimonious phylogram (length 90) obtained after a Branch and Bound search of PAUP* performed on the database with sites 281–314 excluded. *Phlebotomus kazeruni* is used as outgroup. CI = 0.92; RI = 0.93. Bootstrap values (1000 replications) are indicated on the branches.

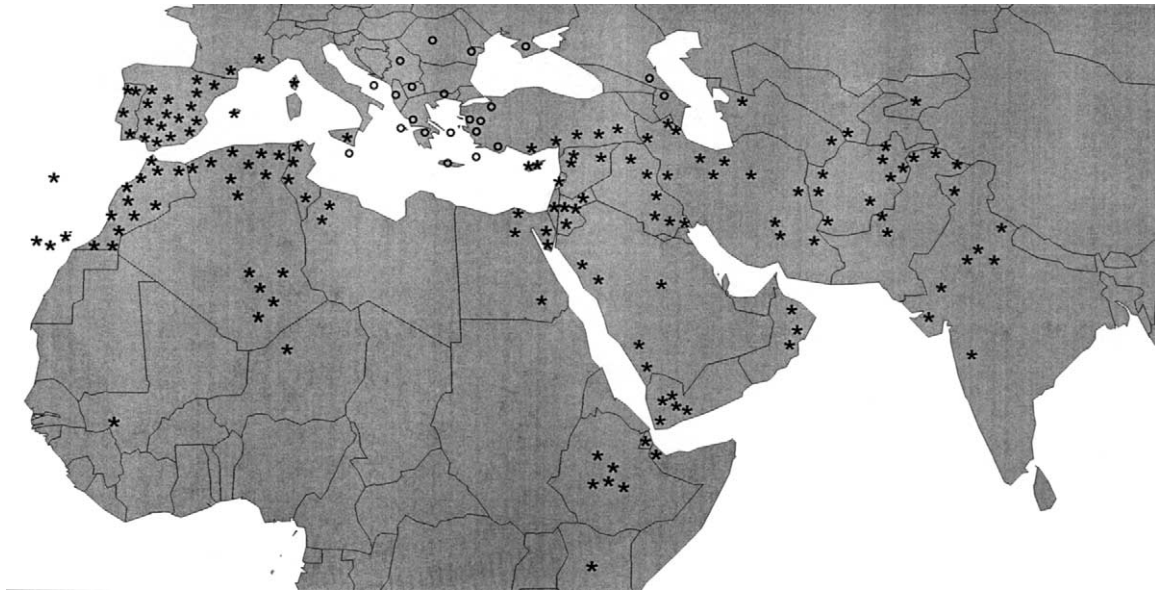


Fig. 4. Distribution of *P. sergenti* (★) and *P. similis* (○).

sequence among populations from Crete, continental Greece and Malta would suggest that this is not a recent importation by human transport.

Based on the groupings observed in the present study, and on their present distributions, *P. similis* and *P. sergenti* would have followed generalised tracks at the Miocene time (Fig. 5).

From the middle-eastern centre of dispersion of *Paraphlebotomus* (Depaquit et al., 2000), a migration route circumventing the Paratethys Sea by the north could have been followed by *P. similis* (or its ancestor), and explains today its distribution in the north of the Caucasus, Crimea, Balkans, Greece and its islands (by routes then open in the Mesogea), and Turkey where its eastern extension have

been blocked by the orogenesis of the Taurus and Antitaurus mountains (15–20 millions years ago).

If we look for intraspecific *P. sergenti* variations, two branches are individualised. One is related to the north-eastern Mediterranean area (Cyprus, Pakistan, Syria and Turkey), while the other is South and West of the first one (Egypt, Morocco, Sicily, Spain and Portugal). The Lebanese fly inserts two additional AT, which places it on the tree (Fig. 2) in an intermediate position, in agreement with the settlement corridor role of this area in Miocene. From the middle-eastern centre of dispersion, and according to its vast distribution area, *P. sergenti* would have followed a western migration route along the southern edge of the Tethys Sea via Lebanon, Egypt and North Africa. From Morocco, it

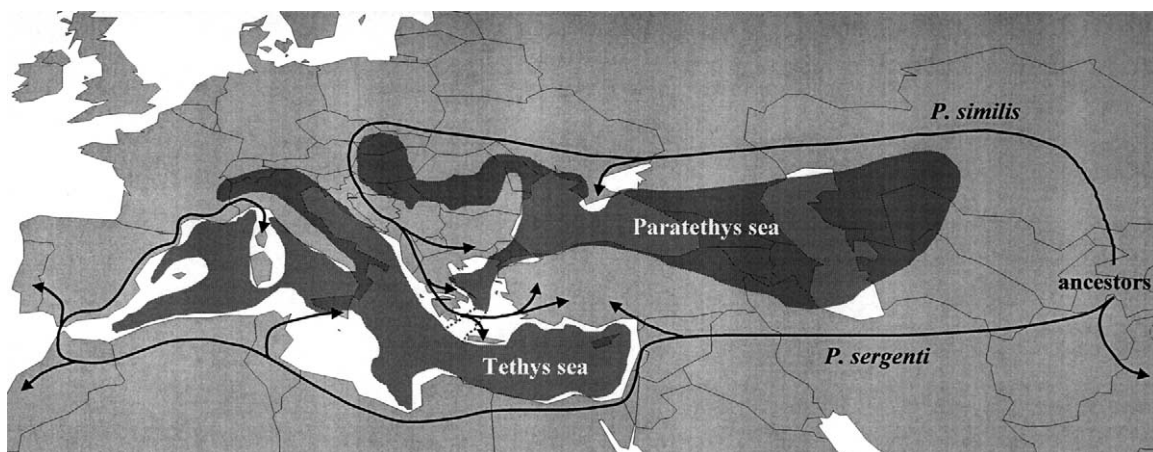


Fig. 5. Hypothesis of settlement in the Old World by *P. sergenti* and *P. similis* from a centre of dispersion located in Asia. *Phlebotomus similis* would have followed a northern way of migration, circumventing the Paratethys sea, to inhabit in due course the north-east of the Mediterranean basin. *Phlebotomus sergenti* colonised Asia and would have followed a route along the southern edge of the Tethys sea to North Africa then to the west of Europe using the Betic bridge.

would have colonised the Iberian peninsula probably before the opening of the Strait of Gibraltar, through the Betic bridge (5 million years ago), then south of France and Corsica. Its presence in Sicily could be explained (taking into account the great homology of sequences from specimens from Sicily and Morocco), by recent connections between these two areas at the time of the Tethys drying, Messinian, 6 millions years ago. In the East, there are no or little variation between the sequences of flies from populations of Cyprus, Syria, Pakistan and Turkey (where *P. sergenti* and *P. similis* seem to be separated by Taurus and Antitaurus), which constitute a homogeneous unit. Inside this Eastern group, we deduce that *P. sergenti* which settled in Cyprus came from the east, probably following the Cape Andreas during Pleistocene time, an hypothesis that we suggested recently (Depaquit et al., 2001).

In light of our results, it seems judicious to consider the variation of these molecular characters as intraspecific. In another study carried out on populations of *L. longipalpis*, Uribe Soto et al. (2001), emphasising molecular variations in the mitochondrial ND4 gene, concluded the potential existence of sibling species. Further studies like hybridisation will be necessary before sibling species can be firmly characterised in *P. sergenti*. However, independently of this taxonomic interpretation, the existence of these two populations can be correlated to the distribution and the dynamics of the foci of cutaneous leishmaniasis caused by *L. tropica* over the Old World. The most active of them are all located in the eastern part of the distribution of the parasite: Sanliurfa in Turkey (Volf et al., 2002), Aleppo in Syria (Tayeh et al., 1997), Mazar-e-Sharif and Kabul in Afghanistan (Jalili et al., 1998; Hewitt et al., 1998), Timargara in Pakistan (Rowland et al., 1999) and the traditional but not well documented foci of Central Asia and India. In the western part of the *P. sergenti* distribution area, the foci are sporadic, less active with lower human prevalences than in the eastern foci. In Morocco, the particular epidemiological situation has been revised by Rioux (2001).

These differences could be correlated with the fact that the vector does not have the same capacity to transmit the parasite everywhere. It seems, however, that *P. sergenti* is highly susceptible to *L. tropica* in the east (Killick-Kendrick et al., 1995) as well as in the west where 10–20% sandfly infection rates were found in a particular Moroccan focus (Rioux, 2001). Recently, the vectorial competence of a Jordanian strain of *P. sergenti* was experimentally shown (Kamhawi et al., 2000). It would be interesting to reproduce this work on *P. sergenti* populations from different geographical origins and on *P. similis* too. Other hypotheses could relate to ecological data differing from populations, such as biotope, relative abundance or host preferences.

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