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Intraspecific variability of *Phlebotomus sergenti*, a major vector of *Leishmania tropica*

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Ph.D. thesis

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Outline of the thesis

This thesis consists of 5 parts. The first one introduces the topic of the thesis in a broader context of current knowledge and puts emphasis on the importance of presented problem. It further presents a review of methods, deployed in the thesis, in context of sand fly research. In part 2 the objectives of the thesis are outlined. Part 3 states the results of the thesis in a form of 4 publications, two of them are published, one is accepted and one is submitted to a peer-reviewed journal. Part 4 draws conclusions from the presented results. All references cited in the thesis are listed in part 5.

I hereby declare that all the work presented in this thesis was done by myself or in collaboration with co-authors of the presented papers and only using the cited literature.

Prague, 7.8. 2008 Mgr. Vít Dvořák

I declare that most data presented in this thesis are results of Vít Dvořák. Vít has substantially contributed to the experimental work as well as the writing of the manuscripts.

Prague, 7.8. 2008 Prof. RNDr. Petr Volf, CSc.

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1. Introduction

1.1 *Phlebotomus sergenti*, a medically important sand fly

Phlebotomine sand flies (Psychodidae: Diptera) are important biting pests and vectors of several viruses causing sand fly fever, bacterium *Bartonella bacilliformis* causing bartonellosis and most importantly, several species of protozoan genus *Leishmania*, causative agents of leishmaniases. *Phlebotomus sergenti*, Parrot, 1917 is the most important proven vector of *Leishmania tropica*, a causative agent of anthroponotic cutaneous leishmaniasis in the Old World (Jacobson, 2003).

Cutaneous leishmaniasis is endemic in 82 countries and some 10 million people suffer from it, with about 1 million of new cases occuring each year in an increasing trend (WHO, 2007). *Le. tropica* was isolated from *P. sergenti* in cutaneous leishmaniasis foci in Saudi Arabia (Al-Zahrani et al., 1988), Morocco (Guilvard et al., 1991) Ethiopia (Gebre-Michael et al., 2004) and Israel (Schnur et al., 2004; Svobodova et al., 2006). *P. sergenti* was proposed to be most probable vector incriminated in the transmission of *Le. tropica* in other foci, including Afghanistan (Killick-Kendrick et al., 1995), Syria (Tayeh et al., 1997) and Turkey (Volf et al., 2002) and its ability to transmit *Le. tropica* to BALB/c mice and golden hamsters (*Mesocricetus auratus*) was experimentally demonstrated by Svobodova and Votypka (2003). In the opposite direction, succesfull infections of *P. sergenti* by feeding on *Le. tropica* infected black rats (*Rattus rattus*) were performed under laboratory conditions by Svobodova et al. (2003).

Phlebotomus sergenti showed a high specifity for *Le. tropica* strains under laboratory conditions while it was proved to be refractory to *Le. major* and *Le. donovani* under the same conditions (Kamhawi et al., 2000). On the ground of these experiments and results of Killick-Kendrick et al. (1995), *P. sergenti* is regarded as a specific vector of *Le. tropica* (Kamhawi, 2006; Volf and Myskova, 2007) playing a major role in transmission of this parasite in urban foci (Jacobson, 2003). There are, however, sylvatic foci of *Le. tropica* where other sand fly species may serve as vectors. In Kenya, where *P. sergenti* is not present, *Le. tropica* is transmitted by *P. guggisbergi*, (Lawyer et al., 1991), as well as by *P. aculeatus* (Johnson et al., 1993). In Israel, *P. arabicus* was shown to replace vectorial role of *P. sergenti* in rural focus (Svobodová et al., 2006). Another *Adlerius* species, *P. halepensis*, was earlier demonstrated as highly susceptible

to *Le. tropica* under laboratory conditions (Sádlová et al., 2003). Nevertheless, *P. sergenti* clearly remains the most important sand fly species to consider when investigating the transmission of *Le. tropica*.

The rather confusing history of *P. sergenti* as a species is reviewed in details by Depaquit et al. (2002). The original description of a male from Algeria is based on a morphology of pyriform style with terminal and subterminal spine (Parrot, 1917). Female was described from Portugal, together with a redescription of a male (Franca, 1918), both of them later being redescribed again on material from Syria, Palestine and Mesopotamia, pointing out more accurate internal morphological characters in females (Adler and Theodor, 1929). Later, two subspecies were established within *P. sergenti s. l.* when Perfiliev (1963) described *P. sergenti similis* from the Caucasus, Crimea and southern Ukraine. Detailed morphological analysis considered *P. similis* as a distinct species (Depaquit et al., 1998) and this finding was further corroborated by molecular marker (ITS2) which indicated *P. similis* as a sister species of *P. jacuzieli* (Depaquit et al., 2002).

One of very interesting traits of *P. sergenti* is its very broad range of distribution, stretching from Canary Island and Madeira over Portugal, Spain, Maghreb, Sicily, Turkey, Egypt, Middle East up to Pakistan and India in west-east direction, from southern France to Kenya in north-south direction (Depaquit et al., 2002). In Italy, its distribution is restricted to Sicily (D´Urso et al., 2004) but authors point out a proximity of Morocco with abundant population of *P. sergenti* as well as several active foci of cutaneous leishmaniasis as a possible risk factor. Also in Portugal it is a rare species but it was documented that it has been colonising area where a decrease of abundance of *P. ariasi* was observed at the same time, presumably due to increasing aridness in this region (Afonso et al., 2005). In Morocco, *P. sergenti* is a proven vector of cutaneous leishmaniasis (Al Zahrani et al., 1988) and is widely distributed in arid and semiarid areas, showing a large ecological plasticity (Guernaoui et al., 2005). In Tunisia it is a dominant sand fly species in the south-east foci of *Leishmania killicki* (Ghrab et al., 2006), although the validity of this *Leshmania* species is questionable due to high similarity with *Le. tropica*. In Egypt, although traditionally restricted to regions north of Cairo, it was also detected in the southern Nile River valley (Hanafi et al., 2001) Authors speculate that it could spread throughout Nile River valley and warn against a potential of *Le. tropica* import into Egypt and its establishment due to the presence of its vector, *P. sergenti*. It is also abundant in Israel where it was detected as a vector of

Le. tropica in the Kfar Adumin focus (Schnur et al., 2004) and Tiberias focus (Svobodová et al., 2006) as well as in the Palestinian West Bank (Sawalha et al., 2003). In Jordan, while generally far less numerous than *P. papatasi*, *P. sergenti* was a dominant species found in the suburbs of Amman and Jarash and it was collected from both domestic and rural habitats, including areas where *Le. tropica* was detected (Kamhawi et al., 1995). In Syria, *P. sergenti*, although present, also appears to be very minor in comparison with *P. papatasi* (Ismail and Pesson, 1992) although more recent data are scarce.

In Turkey, a very abundant presence of *P. sergenti* and its incrimination in transmission of *Le. tropica* is well documented from southern and south-eastern Turkey, namely Sanliurfa (Volf et al., 2002), Hatay (Yaman and Ozbel, 2004) and Konya (Yaman and Dik, 2006). Turkey is a country where both *P. sergenti* and *P. similis* are present, but regarded as allopatric, *P. similis* occuring only west of Taurus and Antitaurus and *P. sergenti* in the east regions (Depaquit et al., 2002). However, both species were recently reported to occur sympatrically at several locailities in the province of Konya and this fact was also confirmed by sequencing internal transcribed spacer 2 (ITS2) (Simsek et al., 2007). If this finding was correct, it would be a first report of natural sympatric occurence of these two species.

Concerning the islands in eastern Mediterranean sea, *P. sergenti* is only present in Cyprus (Depaquit et al., 2001). Within samples included in RFLP analysis of sand flies from Cyprus and Greece (Aransay et al., 1999), only specimens originating from Cyprus represent *P. sergenti*, while specimens from Crete assigned as *P. sergenti* are in fact *P. similis* (Depaquit et al., 2001). Further eastwards, *P. sergenti* also has a wide distribution in Iran, where it again is incriminated in transmission of *Le. tropica* (Yaghoobi-Ershadi et al., 2003). A detailed morphological and sequencing analysis of specimens assigned as *P. sergenti s. l.* suggests that in Iran, both *P. sergenti* and *P. similis* are present (Moin-Vaziri et al., 2007).

As apparent from the review above, *P. sergenti* covers a broad area. Within such a large range of distribution, a notable intraspecific variability may be expected. This variability was indeed clearly demonstrated by a sequencing analysis of the ITS2 (Depaquit *et al.*, 2002) which included population of *Phlebotomus sergenti* from 11 different countries: Portugal, Spain, Morocco, Italy, Cyprus, Egypt, Israel, Lebanon, Syria, Turkey and Pakistan. According to the results of this study, two branches can be identified: one is related to the north-eastern Mediterranean area (Cyprus, Lebanon,

Syria, Turkey and Pakistan,), while the other occurs more in South and West (Morocco, Sicily, Egypt, Israel). These branches are in accordance with postulated migration routes of *P. sergenti* along the southern edge Thetys sea at the Miocene time and its dispersal within the current range of distribution. On the other hand, *P. similis* is hypothetized to follow a northern way of migration along Paratethys sea, colonising Azerbaijan, Russia, Ukraine, Romania, Greece, Turkey, Albania, countries of former Yugoslavia and Malta.

According to Depaquit *et al.* (2002), groups belonging to these two different branches of *P. sergenti* may differ in ecology and host preferences. It was noted that the sand fly has a more widespread distribution than the parasite (Depaquit et al., 1998), therefore, different populations may possibly also differ in vectorial capacity. It seems judicious to consider the potential existence of cyrptic species. This concept was further enhanced by a study of regional genetic differentiation of *P. sergenti* in three Moroccan foci of cutaneous leishmaniasis which revealed three mtDNA lineages, possibly markers of regionally distributed cryptic species (Yahia et al., 2004).

If cryptic species within *P. sergenti* were proven, it would certainly have very important implications in epidemiology as well as in experimental studies. It was demonstrated in a very impressive manner that erythemas produced by the bites of different populations of New World sand fly *Lutzomyia longipalpis* differ significantly in clinical manifestation (Warburg et al., 1994). Since then, a huge effort was made and many different approaches were deployed in resolving the question of cryptic species within *L. longipalpis*, the number of these species, their distribution and epidemiological significance for leishmaniases transmission. Part of this massive effort which concerns approaches relevant for the studies presented in this thesis is discussed later in more details.

In spite of a growing amount of convincing evidence (reviewed by Bauer et al., 2007) a dispute over a cryptic species complex versus a highly polymorphic single species is not yet resolved and differing opinion will doubtlesly continue concerning the criteria for a formal taxonomic recognision of these cryptic species. Nevertheless, it is clear that the understanding of intraspecific variability and population sub-structure of *L. longipalpis s.l.* is necessary for a proper reflection of eco-epidemiological consequences of American visceral leishmanias (Lainson and Rangel, 2005; Maingon et al., 2007).

In a very similar manner, a full understanding of genetic diversity, population differentiation and intraspecific variability is highly desirable to explain fully a role of *P. sergenti* in the transmission of cutaneous leishmanias. A contribution to this effort is presented in this thesis.

1.2 Sequencing analyses of sand flies

The massive development of molecular techniques in the last decades brought new methods to complement classical approaches based mainly on detailed and sometimes dubious morphological analyses. As the availability and affordability of PCR amplification and sequencing facilites increased rapidly, it offered a to perform sequencing analyses of both nuclear and mitochondrial markers on an almost routine basis.

Within nuclear markers, nuclear ribosomal DNA was largely utilised. In insect, as in others eukaryotes, the ribosomal DNA is composed of tandemly repeated units separated from each other by intergenetic spacers, known as nontranscribed spacers (NTS). Each unit contains the coding genes for the 18S, 5.8S and 28S ribosomal RNA, external transcribed spacer (ETS) and two internal transcribed spacers 1 (ITS1) and 2 (ITS2) (reviewed in Hillis and Dixon, 1991). The coding regions of 18S, 5.8S and 28S are highly conserved and therefore serve often for higher level phylogenies construction, while the noncoding ITS regions are widely variable and thus popular for phylogenies of closely related species, differentiation of populations and studies on divergence within and between species (reviewed in Thanwisai et al., 2006). Apart from different molecular markers based on nuclear ribosomal DNA sequences, two homologues of *Drosophilla* behavioral genes, *period* and *cacophony*, were characterised in sand flies (Peixoto et al., 2001) and also proved to be very useful in several studies.

Mitochondrial (mt) DNA has a maternal, nonrecombining fashion of inheritance and usually relatively fast rate of sequence divergence. As the lack of recombination allows to reflect the evolutionary history even more accuratelly (Avise, 1994), mtDNA markers were also used repeatedly in sand flies phylogenetic and taxonomic studies. Different fragments of mtDNA, containing various portions of cytochrome b (cyt b) gene, cytochrome c oxidase (COI) gene, ND4 gene or NADH gene were utilised, often in combination with nuclear molecular markers like ITS2.

Both nuclear and mitochondrial molecular markers have been recently extensively deployed in numerous sequencing studies addressing taxonomy and molecular phylogeny of sand flies at different taxonomical levels as well as specific issues concerning possible existence of complexes of cryptic species in both Old and New World sand flies. To emphasize the large portion of work done as well as to illustrate some similar traits in the research, important sequencing analyses perfomed on genera *Phlebotomus* and *Lutzomyia* are summarized separatelly.

1.2.1 Sequencing analyses of sand flies – Old World

As a first example, haplotypes of eight phlebotomine species were characterized by sequencing a mtDNA fragment of cyt b to NADH1 (Esseghir et al., 1997). Little geographical genetic diversity was found within *P. papatasi* samples, a finding to be later confirmed by others several times (Hamarsheh et al., 2006; Depaquit et al., 2008). Authors assume that this sand fly suffered a population bottleneck late in the Pleistocene and then radiated out from the Eastern Mediterranean area. For subgenera *Phlebotomus* and *Larroussius*, the mtDNA molecular clock was calibrated with a pairwise nucleotide sequence divergence rate of 1.0-2.5% per million years. A phylogenetic analysis of *Larroussius* species was presented as well as a presence of presumably isolated populations in eastern and western Mediterranen regions, in contrast with the uniformity of *P. papatasi* populations.

A following robust study, which used a combination of mitochondrial (cyt b) and nuclear (elongation alpha factor gene) marker presented a detailed analysis of subgenus *Larroussius* speciation (Esseghir et al., 2000). It was proposed that the allopatric speciation mode took place in all *Larroussius* species complexes with the exception of *P. perniciosus*, *P. langeroni* and *P. longicuspis* in Spain and Maghreb region. While both cyt b- and EF-α-inferred trees resolved the basal clades in a similar fashion, placing *P. neglectus* and *P. ariasi* to the base, they are not congruent for the derived *P. perniciosus* complex. By considering the two different gene phylogenies, the regional geology and palaeoecology as well as current ecological biogeography, authors assumed that most of the *Larroussius* subgenus speciation was not dependent on tectonic vicariance events, but rather on progressive aridification in late Miocene-Pliocene. The results of this profound study do not offer support for co-cladogenesis of

individual *Larroussius* species and the isoenzyme strains of *Leishmania infantum*, to which they serve as vectors.

The monophyly and relationships among the members of *Larroussius* subgenus was also addressed by a study which used ITS2 rDNA sequences (Di Muccio et al., 2000). This monophyly was supported, placing *P. ariasi* as a sister species to the rest of the subgenus. The trees estimated from ITS2 sequencing analysis also suggested that *P. perniciosus* and *P. longicuspis* are distinct species, despite only slight morphological differences. Finally, this study confirmed the ability of ITS2 sequence to identify recently derived phylogenetic relationships.

The somehow troublesome and morphologically hardly distinguishable species of *P. perniciosus* complex from western Mediterranean regions were addressed by a study which combined the use of detailed morphology, isoenzymes and a sequence analysis of cyt b mtDNA fragment (Pesson et al., 2004). Mitochondrial introgression was demonstrated and three lineages occuring sympatrically in Morocco were established: *P. perniciosus*, *P. longicuspis s.s.* and a new species sibling to *P. longicuspis*. Authors discuss the taxonomic significance and formal recognision of this new sibling species. A similar combined study on *P. perniciosus* from Northern Spain and France, using three polymorphic isoenzyme loci together with cyt b sequencing analysis discuss possibly different amounts of gene introgression when two distinctive populations met in this area after a period of isolation during the Pleistocene Ice Ages (Perrotey et al., 2005). This means that mitochondrial markers can provide a suitable resolution to reconstruct fairly detailed traits of sand fly speciation and distribution in time and space.

The ITS2 rDNA also proved to be useful in a phylogenetic study of subgenus *Paraphlebotomus* (Depaquit et al., 2000). Nine species of this subgenus were analyzed together with two outgroup species, *P. papatasi* and *P. duboscqi*. Three clades were formed by the sequence analysis, one containing *P. mongolensis* and *P. andrejevi*, second with *P. saevus* and *P. mireillae* and third harboring *P. sergenti*, *P. similis*, *P. jacusieli* and *P. kazeruni*, while *P. alexandri* appears to be a sister species to all the other species of the subgenus. On the base of assumption that the origin of *Paraphlebotomus* is in Africa, authors postulated a hypothesis about four routes of dispersal from the centre located in the Middle-east.

A following intraspecific study on *P. sergenti* of twelve populations from ten different countries using again ITS2 revealed a heterogenity in this molecular marker

(Depaquit et al., 2002). Two clades were constituted within *P. sergenti*: one related to the north-eastern Mediterranean area (Cyprus, Pakistan, Syria and Turkey), while the other to South and West (Egypt, Morocco, Israel). Such a division correlates with postulated migration routes of *P. sergenti* westwards along Thetys Sea at the Miocene time. Authors also discussed a possibility of different vectorial capacities of the members of these two clades and arised a question of cryptic species. When regional intraspecific variability of *P. sergenti* was examined in three foci of cutaneous leishmaniasis in Morocco using a fragment of cyt b mtDNA, three primary mitochondrial lineages were identified, with a markedly regional distribution (Yahia et al., 2004).

Recently, a study on mtDNA sequences containing a mtDNA marker containing a partial sequence of cyt B gene and NADH1 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., 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. As *P. sergenti* in this study is considered „*sensu lato*“, a part of the variability observed can be probably atributed to *P. similis*, which has never been recorded in Iran before.

A comparative molecular study deploying mitochondrial ND4 gene was also performed on three species of the subgenus *Transphlebotomus*: *P. mascittii*, *P. canaanicus* and *P. economidesi* (Depaquit et al., 2005). These are the species that are morphologically not easy to distinguish, however, they were clearly differentiated by the molecular marker. Several specimens of each species were analyzed, showing a complete identity in the sequence. Similar topologies were obtained by several methods, using *P. papatasi* as an outgroup, showing *P. mascittii* and *P. economidesi* as sister species and *P. canaanicus* as their sister species (Depaquit et al., 2005).

Earlier findings about a little differentiation of *P. papatasi* populations were confirmed in a study combining the analysis of cyt b mtDNA and the sequening analysis of wsp gene of symbiotic and maternally inherited endosymbiotic *Wolbachia pipientis* in sand flies from villages and rodent burrows in Iran (Parvizi et al., 2003). The distributions of the haplotypes of these two maternally inherited genes were analysed and they indicated the absence not only of sympatric cryptic species but also

of any long-term differentiation of lineages in different habitats. A single lineage of cytochrome b haplotypes was found, and both sexes in all populations had a high infection rate of the same A-group strain of *Wolbachia*.

This presumable low variability within *P. papatasi* together with a need for better epidemiological understanding and subsequent control strategies against this widely distributed specific vector of *Leishmania major* motivated a large-scale investigation of genetic structure within and among distant Mediterranean populations by cyt b mtDNA (Hamarsheh et al., 2007). Both colony-reared and wild specimens from 25 populations of 10 countries of Meditarranean and Middle East were studied. The pattern of sequence variation did not support the existence of a species complex, haplotypes clustered by geographical origin and demonstrated a certain degree of genetic differentiation. This differentiation seems to be associated with latitude to some extent, but not to climatic conditions, as was decribed earlier in *Lutzomyia whitmani* (Ready et al., 1997). Subsequent intraspecific study on *P. papatasi* using a combination of nuclear (ITS2) and mitochondrial (ND4 mtDNA) marker demonstrated again a surprising homogenity of *P. papatasi* within its huge range of distribution (Depaquit et al., 2008). In this study of even larger scope, 26 populations from 18 countries, stretching from Spain and Morocco up to Iran and India, were characterized. In a cladistic analysis of subgenus *Phlebotomus* based on the sequencing data, *P. papatasi* is a monophyletic species whose sister species is *P. bergeroti*. These two are sister species of *P. duboscqi*. The phylogenetic position of *P. salehi* needs to be clarified by other molecular markers.

On the intraspecific level, the limited number of variations of the selected markers in *P. papatasi* is in contrast with their variability observed in other two widespread species, *L. longipalpis* (Soto et al., 2001) and *P. sergenti* (Depaquit et al., 2002). Surprisingly, there is a higher variation within ITS2 than ND4, while generally mitochondrial markers are expected to evolve more rapidly than nuclear DNA. In terms of epidemiological consequences of this low variation within *P. papatasi*, it can be expected that it has a similar vectorial capacity of *Leishmania major* throughout its distribution area.

Apart from above described studies which focused on phlebotomine subgenera or groups of related species, a basic study revealing phylogenetic relationships of phlebotomine sand flies inferred from small subunit nuclear ribosomal RNA (SSU) gene was carried out (Aransay et al., 2000). SSU nuclear gene is suitable to infer

phylogenetic histories of more basal lineages due to the slow evolving rate. It is also benefitical that this slow rate permits the design of rather universal primers which can be used for groups of species not previously examined. In this study of a large scale, fifteen phlebotomine species and three genera were studied with the aim to determine a concordance of the inferred relationships with previously adopted system based on morphological characters. For each represented *Phlebotomus* species, several specimens from different locailites were sequenced to determine a possible, although not expected intraspecific variability in this highly conserved marker. When consensus sequences of these species were aligned with fifteen non-psychodid dipterans and other insects, a maximum parsimony analysis confirmed a monophyly of phlebotomine clade with a good bootstrap support. Within the phlebotomines, SSU proved to resolve subgeneric relationships but falied to resolve intergeneric as well as intraspecific relationships.

18S rRNA gene was also deployed in an early study on sand flies from Greece and Cyprus, which were typed by restriction fragment length polymorphism (RFLP) performed on this gene after amplification by PCR (Aransay et al., 1999). Seven different sand fly species occuring in the studied areas of Cyprus and Greece, namely *Phlebotomus papatasi*, *P. alexandri*, *P. sergenti*, *P. neglectus*, *P. tobbi*, *P. perfiliewi* and *P. simici* were typed by RFLP. Since none of the restriction enzymes tested gave a patterns that would be species-specific on their own, authors had to apply a double digestion by two restriction enzymes, *HpaII* and *RsaI*. Still they obtained identical patterns for the species of subgenus *Larroussius* (*P. neglectus*, *P. tobbi*, *P. perfiliewi*). It may be attributed to this lower level of resolution that RFLP did not become a method of choice for sand fly typing and was not deployed in any further studies until recently, when it was applied on sand flies from an endemic focus of leishmaniasis in Argentina (Baroso et al., 2007).

1.2.2 Sequencing analyses of sand flies – New World

First study addressing relationships among species groups within the genus *Lutzomyia* was performed using a part of mitochondrial cytochrome b (cyt b) gene in combination with allozyme analysis of nine enzyme loci (Torgerson et al., 2003). Twenty species of *Lutzomyia* and *Brumptomyia* species were examined by this combined effort, with results largely supporting the morphologic groups, established earlier. According to the authors, cytochrome b seems to be a suitable marker to resolve

relationships at the generic level, while the allozyme variation gives adequate resolution at the subgeneric level only. In an attempt to further update and specify the classification of *Lutzomyia* sand flies based on morphology, which in many cases suffered from the absence or difficult interpretation of morphological characters as well as from intraspecific polymorphism and presence of cryptic species, a profound study based on analysis of 12S and 28S ribosomal DNA sequences was carried out (Beati et al., 2004). Phylogenies of 32 species of phlebotomine sand flies belonging to seven sub-genera and and two species groups were inferred. Although the study was based on a limited number of taxa, the resulting phylogenies provide an initial phylogenetic backbone for further reconstruction of relationships within the genus *Lutzomyia*.

Apart from the fundamental basic taxonomical study by Beati et al.(2004), sequencing analyses of both nuclear and mitochondrial genes of New World sand flies were largely incriminated in resolving intraspecific and subgeneric relationships, mainly in the investigation of presumable complexes of cryptic species within genus *Lutzomyia*. In this aspect, a prominent role plays a long-lasting controversy wheather *Lutzomyia longipalpis* represents a complex or a single species (reviewed by Bauer et al., 2007). The concept of a species complex was established by a finding that there is a high level of genetic divergence among laboratory colonies of *L. longipalpis* of different geographical origin and sterility was detected in male progeny when intercolony crosses were performed (Lanzaro et al., 1993).

While earlier studies addressed the question of *L. longipalpis* species status mainly by morphological observations and isoenzyme electrophoresis, Lanzaro et al. (1999) showed, that there is a high variation in sequence of gene for maxadilan, salivary a peptide, in *L. longipalpis* from different regions. Further on, the species complex concept was supported by the results analysis of nucleotide variation in the ND4 mitochodnrial gene, which revealed deep genetic divisions between four clades from northern South America, Brazil, Central America and isolated population from Colombia (Soto et al., 2001). Authors, who reffered to *L. longipalpis* as „morphospecies“, also discussed possibilities of gene flow between distant localities and concludes that relatively modest geographic and climatic barriers can limit or even prevent the gene flow between different populations.

Further studies to favor the *L. longipalpis* species complex concept were based on analyses of nucleotide variation in the *period* gene, a gene controlling behavioral rhytms in *Drosophila*. This gene was studied in sand flies in order to provide a new

marker for phylogenetic studies and it was revealed that it has significantly lower evolutionary rate than in *Drosophila* (Mazzoni et al., 2002). Two consecutive studies of this marker performed on different populations from Brazil revealed high nucleotide variation and genetic distances and brought first actual molecular evidence of cryptic species distributed in Brazil sympatrically (Bauzer et al., 2002 a,b). Variation found in another gene homologous to *cacophony* gene of *Drosophila* later also supported the idea of two cryptic species occuring sympatrically in Brazil in a similar fashion (Bottecchia et al., 2004). These studies represent a nice example of beneficial use of molecular markers developed originally for a different insect species.

As mtDNA has proven useful in many molecular phylogenetic studies, a sequence from region I of the mitochondrial cytochrome c oxidase (COI) was also found to be informative for clarifying *Lutzomyia longipalpis* taxonomy at the species level as it was deployed in a study comparing twelve different populations from South and Central America (Arrivilaga et al., 2002). This study revealed that while there was only a minor sequence variation within each population, the haplotypes varried significantly between the populaions, suggesting that sequence polymorphism at the COI locus provides an excellent marker for the study of phylogenetic relationships among populations. It demostrated the existence of four clades among the studied *L. longipalpis* populations: Laran, Brazilian, cis-Andean and trans-Andean. Interestingly, the COI mtDNA data do not support existence of two sympatric species in Brazil. Authors suggested to consider these four populations being cryptic species and assumed that the four clades probably diverged as a result of vicariance events that occured during late Pliocene and Pleistocene.

Arrivilaga et al. (2002) proposed and discussed two possible historical scenarios, based on the biogeography and historical geology in Central and South America. According to first, described as dispersal hypothesis, the current populations may have derived from an ancestral Amazonian gene pool and the dispersal would have occured after the final uplift of the East Andean Cordillera. Cladogenesis, resulting in the existence of described four clades, would be a result of local environmental adaptations, isolation and local extinctions. Second scenario, described as a forrest refugia hypothesis, takes in account a possibility of climatic fluctuations that caused a geographical fragmentation and separation of the current clades. Authors finally concluded that three of the obtained clades clearly represent phylogenetic species, while the Andean clade harbors two internal clades, probably as a result of consequent periods

of isolation, dispersal and lack of gene flow due to vicariant microevents. They pointed out that this concept is supported by the post-mating isolation of these populations, as was demonstrated earlier by laboratory crosses (Lanzaro et al., 1993). The results of COI mtDNA analysis are further discussed in details mainly in the respect of two sympatrically occuring Andean clades. Results are compared with the analysis of seven isozyme loci of same populations. Authors favor the use of mtDNA against the analyses based on allozymes (Arrivilaga et al., 2003).

Nevertheless, not all studies based on mtDNA data stand in support for species complex concept. When variation in mitochondrial cytochrome b (cyt b), a widely used marker for phylogenetic studies, was examined in six locations representing a geographic transect across eastern Brazil, sequence divergence found was not considered to be sufficient to indicate cryptic species, although the authors found a signifficant differentiation betwen northern and southern populations (Hodgkinson et al., 2003).

Apart from the *Lutzomyia longipalpis* issue, *L. whitmani* was proposed as another possible candidate for a complex of two cryptic species based on the two phylogenetic lineages: a form from eastern Amazonia, which transmits *Leishmania shawi* but is not noticeably synantropic, and a form from northeastern Brazil, which plays a role in *Leishmania braziliensis* transmission (Rangel et al., 1996). Later, a third mtDNA lineage was added by Ready et al. (1997) from drier interior of Brazil. However, a thorough phylogenetic analysis of 31 cytochrome b haplotypes from the rainforest regions of Amazonia suggested that instead of two or three cryptic species with discountinuous occurence, there is rather a continous variation between *L. whitmani* populations (Ishikawa et al., 1999). Apparently, only lineages of *L. whitmani* sympatric with *L. intermedia*, a closely related and morphologically similar species, are involved in *Le. braziliensis* transmission. Therefore, it was suggested that genes controlling aspects of vectorial capacity could be passing from one species to the other. Mitochondrial introgression was reported between *L. intermedia* and both *L. neivai* and *L. whitmani* (Marcondes et al., 1997), as it was also reported in *Phlebotomus longicuspis* with introgressed cytochrome b sequences of *P. perniciosus* (Pesson et al., 2004) as well as *Lutzomyia youngi* with introgressed cytochrome b sequences of *L. townsendi* (Testa et al., 2002). However, study on *period* gene showed that nuclear introgression may also occur between *L. intermedia* and *L. whitmani* (Mazzoni et al., 2006). Since this gene is involved in speciation and might be therefore less prone to

introgression that other genes, authors speculated that much higher level of gene flow might occur between these species. Nevertheless, a definite status of *L. whitmani s. l.* remains to be further resolved (da Costa et al., 2007).

It was mentioned in previous chapter that RFLP did not become a method of choice for sand fly typing. In the New World sand flies, it was deployed on sand flies from an endemic focus of leishmaniasis in Argentina (Baroso et al., 2007). Put together with the results of a morphological investigation of the flies, the resultant DNA fragment patterns were sufficient to identify most of the sandflies caught as *Lutzomyia neivai*. A single digestion of the 18S-rRNA gene sequences with AfaI or HapII appeared sufficient and useful for the identification of Lu. neivai from the north of Salta province, and for several other Lutzomyia species. Still, two species, *L. cortelezzii* and *L. sallesi*, which were relatively rare, could only be identified morphologically.

1.3 Microsatellites and their use in sand flies

Microsatellites, or tandem simple sequence repeats (SSR), are by definition repetitions of 2 - 6 nucleotide motifs, typical by a high degree of polymorphism, which have been found so far in the genomes of all analysed organisms, often at frequencies much higher than would be predicted on the ground of base composition. They are found both in coding and non-coding regions. The evolutionary process leading to the length variability at microsatellite loci does not follow a simple mutation model or single-stepwise model. Two mechanisms of microsatellite variations were proposed instead: replication slippage and recombination. Although initially considered to be evolutionary neutral, evidence grows of their functional significance in several processes such as chromatin oranization, regulation of DNA methabolic processes or regulation of gene activity at different levels. Nevertheless, microsatellite markers became very popular choice for numerous applications including genome mapping, ecological studies, forensic studies or conservation management of biological resources (reviewed by Jarne and Lagoda, 1996; Chambers and MacAvoy, 2000; Schlötterer 2000; Li et al., 2002).

Given their large applicability, there is an extraordinary demand for suitable microsatellite markers for various groups of organisms. Unfortunately, the major drawback of microsatellites is that they often need to be isolated de novo from species examined for the first time. Although the presence of highly conserved flanking regions

was reported for several microsatellite loci in various groups of organisms, allowing cross-amplification of these markers from considerably diverged taxons (reviewed by Zane et al., 2002), generally a cross-species transfer of nuclear microsatellite markers is not always possible. It was demonstrated that not only abundance of microsatellites generally, but also cross-species transferability of these markers is unevenly distributed across taxa. The potential for successful cross-species transfer appears higher in species with long generation times and mixed or outcrossing breeding systems (Barbará et al., 2007). Concerning arthropods, it was pointed out that microsatellites are relatively scarse in Lepidopteran genomes (Zhang, 2004), as well as in *Ixodes scapularis* and *Aedes aegypti* (Fagerberg et al., 2001). When sequences of 23 insect species (Diptera, Lepidoptera, Hymenoptera) originating from whole genomes as well as partial genomic libraries were compared, it was demonstrated that there are strong differences in the abundance of microsatellites among species and a variable proportion of them cluster into sequence families based on similarities in their flanking region. In some species, this phenomenon, together with a relatively low abundance of microsatellites, makes the isolation of reliable microsatellite markers particularly difficult (Meglécz et al., 2007).

It transpires from these findings that when microsatellite markers are considered to be deployed, a possible need for *de novo* isolation can arise in species where no unique microsatellite markers were characterized yet. This task can be considerably involving in terms of effort and time. Traditionally, microsatellite loci are isolated from partial genomic libraries of the species of interest. This approach can be extremely tedious and inefficient for species with low microsatellite frequencies. Therefore, several alternative strategies were proposed to reduce the time invested and to increase the yield, mainly by different enrichment steps (reviewed by Zane et al., 2002).

Despite the described limitations, microsatellite markers became a marker of choice for countless studies, including those adressing the intraspecific variability, microgeographic structure and diagnosis of sibling species in most vectors of parasitic diseases, such as mosquitoes of all three major medically important genera, *Anopheles*, *Culex* and *Aedes* (Lehman et al., 1997; Ravel et al., 2002; Keyghobadi et al., 2004), tsetse flies (Krafsur and Endsley, 2002) or triatomine bugs (Anderson et al., 2002).

First panel of phlebotomine microsatellite markers was isolated from *Lutzomyia whitmani* in order to address a question of sibling species within this New World sand fly (Day and Ready, 1999). In total, fourteen AAT-class repeats were specifically isolated from a phagemid library. All of them had a non-coding structure, both perfect

(uninterrupted run) and imperfect (interrupted run) type was presented in a ratio of 1.8 : 1. The markers were isolated without enrichment because relatively long flanking sequences were sought and it was shown before (Hammond et al., 1998) that enrichment can have the disadvantage of selective PCR amplification of a small number of loci and of smaller fragments, containing flanking sequences too short for a comparative analysis. In the same study, relative abundances of microsatellites were studied in four sand fly species, namely *Lutzomyia longipalpis*, *L. whitmani*, *Phlebotomus papatasi* and *P. langeroni* by dotblot hybridization. It demonstrated much variation in the relative abundance of four repeat classes: $(CA)_{15}$, $(GA)_{15}$, $(ATT)_{10}$ and $(TC)_{15}$. However, there were no significant differences in microsatellite abundances of males and females in *P. papatasi* and *L. longipalpis*, where both sexes were examined. In spite of a relativelly high number of microsatellite markers isolated for *Lutzomyia whitmani* and presented in this paper, these were not used in any later study up to the present.

First Old World sand fly species, for which microsatellites were isolated, was *Phlebotomus perniciosus* (Aransay et al., 2001). This species inhabits much of the western part of Medditerranean basin and serves as a vector of *Leishmania infantum*, a causative agent of visceral leishmaniasis of humans and the reservoir hosts, mainly dogs. Two main mtDNA lineages were described to occur: one in northwest Africa, Malta and Italy, second in Iberia (Esseghir et al., 1997, 2000). However, mtDNA characterization failed to resolve the population structure of the Iberian lineage. For this purpose, four AGC- and seven AGG-class microsatellite loci were characterised, out of these five markers being polymorphic. Modified protocol with the biotin-avidin enrichment was used.

The isolation of these microsatellite markers offered a useful tool which eventually enabled to resolve the populaton differentiation of *P. perniciosus* in Spain (Aransay et al., 2003). Allelic variation at these trinucleotide loci was investigated at thirteen Spanish populations of *P. perniciosus*. The results provide strong evidence for population subdivision into two distinct regional subpopulations, one including eight populations from southern Spain, the other accomodating five populations from the northeast. While there was a very little genetic differentiation within each regional population, on the contrary, a very great genetic differentiation was observed inter-regionally. The northeastern population exhibits a reduced number of microsatellite alleles, indicating its derivation as a peripheral isolate following the postulated dispersal

of this species from a southern refuge after the Ice Age 8000 – 12000 years ago. Results also suggest that the two regional groups of populations remain isolated, while there are no significant permanent barriers to gene flow in spite of the species´ contiguous distribution. The authors discussed the possible reproductive incompatibility of the two populations and point out that maternally inherited *Wolbachia* strain was isolated from a laboratory-reared *P. perniciosus* (Ono et al., 2001). They also speculated about the epidemiological significance of the regional populations in terms of ability to disperse rapidly into northwest Europe, if climate warming provides suitable environmental conditions.

Dinucleotide microsatellite loci were characterised for *Lutzomyia longipalpis* (Watts et al., 2002). Eleven polymorphic loci that produced consistent allelic patterns were isolated. Streptavidin-coated magnetic beads and biotin-labelled oligonucleotide were used for the enrichment. The dinucleotide loci in *L. longipalpis* were more polymorphic than previously described sand fly microsatellite loci. Microsatellite variability was tested on colony-reared sand flies and despite expected prevalent inbreeding, there was a surprising and unexpected variability (Watts et al., 2002).

As in case of *P. perniciosus* described above, the panel of microsatellite markers for *L. longipalpis* provided another useful approach to address much controversial and long-lasting issue: the probable, yet not fully accepted existence of sibling species within *L. longipalpis* complex. Therefore, five microsatellite loci were applied to test the hypothesis that this sand fly occurs as two sympatric cryptic species in Sobral, Brazil (Maingon et al., 2003). This was previously predicted by male sex pheromone chemotypes isolated from field specimens collected at the same site (Hamilton et al., 1999). Two phenotypically different populations overlapping the sampling site were genotyped by the microsatellite loci. Samples with the same sex pheromone showed either no significant or low genetic differentiation, while highly significant genetic differentiation was shown between samples posessing different pheromones. Little gene flow was indicated between these two sympatric populations, all these findings gathering further support for the understanding of *L. longipalpis* as a complex of cryptic species (Maingon et al., 2003)

Another analysis of alelle frequencies distribution using same five microsatellite loci was conducted on *L. longipalpis* specimens from 11 populations originating in Venezuela and Brazil (Watts et al., 2005). Two other species, *L. pseudolongipalpis* and *L. cruzi* were also included in this analysis and the results were put in context with the

production of male sex pheromones. High genetic differentiation was found between the samples. At the same site, genetic differentiation was mostly not significant, however, sympatric populations from Sobral, which differ in the production of male sex pheromone, showed genetic distance as great as many of the comparisons between the populations separated by considerable geographical distance. The analysis showed *L. pseudolongipalpis* and *L. cruzi* are well separated species. A phenotypically and genetically different population cluster formed by sand flies producing a distinct sex pheromone cembrene was indentified in northern Brazil, supporting again the idea of *L. longipalpis* species complex.

Last phlebotomine sand fly, subjected to a succesfull and published microsatellite study so far is *Phlebotomus papatasi*, for which a panel of five di- and trinucleotide microsatellite markers was developed in an attempt to provide a better understanding of its population structure (Hammarsheh et al., 2006). Similar protocol with streptavidin-coated magnetic beads and biotin-labelled oligonucleotide was used for the enrichment. Polymorphism was evaluated by amplifying the microsatellite sequences of fifteen different *P. papatasi* populations from six countries, spanning from Italy eastwards to Cyprus, Turkey, Syria, Egypt and Palestine. All newly isolated microsatellite markers failed to amplify with *P. perniciosus* and *P. longicuspis*, illustrating a very limited potential of cross-species transfer of nuclear microsatellite markers to sand fly species of different subgenera.

1.4 RAPD analysis of sand flies

Random-amplified polymorphic DNA (RAPD) represents another powerful tool for identification of species and strains and the estimation of genetic variability between isolates (Williams *et al.*, 1990). The technique amplifies random fragments of genomic DNA by polymerase chain reaction (PCR) using single primers of arbitrary nucleotide sequences. The amplified products provide a random representation of both coding and non-coding regions across the whole genome, spanning a large number of loci and thus allowing the simultaneous examination of many genetic markers (Lynch and Milligan, 1994). It is considered to be a fast, easy, inexpensive and yet very informative method (Diakou & Dovas, 2001). While showing a sensitivity to DNA contaminations, it gives reproducible patterns even if the template DNA is subjected to a partial degradation (Neto et al., 1997). The main disadvantage of this method appears to be sonmetime

questionable reproducibility, however, a carefull optimisation of the protocol can overcome this limitation (Behura, 2006).

Although primarily designed to construct genetic maps, its potential to produce diagnostic markers for species indentification as well as to perform intra- and interspecific molecular taxonomy and phylogeny analyses was quickly recognised. Therefore, it was adopted promptly to analyse cryptic species complexes and genetic structure of populations in various groups of insects, including those incriminated as vectors of parasitic diseases, such as mosquitoes (Wilkerson et al., 1993; Ayres et al., 2003), black flies (Duncan et al., 2004), triatomine bugs (Ramirez et al., 2005; Barbosa et al., 2006) or midges (Sebastiani et al., 2001).

In the sand flies, RAPD was mostly deployed in New World species studies. First, this method was succesfully used to discriminate between two closely related and morphologically similar species, *Lutzomyia youngi* and *L. spinacrassa* (Adamson *et al.*, 1993). It also distinguished succesfully between four different laboratory populations of *L. longipalpis* from Brazil, Colombia and Costa Rica (Dias *et al.*, 1998). The authors interpretate the results of this analysis as accumulating further evidence that this taxon in fact represents a complex of cryptic species. However, of the decamer primers tested, only one generated a polymorphic fragment, all other tested primers produced only non-polymorphic band. This may be attributed to relatively low number of cycles during the PCR amplification. Nevertheless, the polymorphic primer was isolated, cloned into a vector and sequenced. It proved that the polymorphy was due to an inverted repeat which was present only in one of the four laboratory-reared populations. The apparent inefficacy of RAPD in the identification of the genetic differences between the studied populations could also be attributed to the limited number of loci included in the study.

On the contrary, results of a recent RAPD study of seven wild populations of *L. longipalpis* from north-eastern region of Brazil argue against a concept of cryptic species complex (de Queiroz Balbino et al., 2006). In this work, 24 different RAPD loci were examined in a total number of 140 specimens representing a longitudinal transect approximately 1300 km long. The level of genetic variation obtained is compatible with other insect species and is regarged by the authors as compatible with those found between the members of a single species. Authors concluded that the results of this RAPD analysis favored a concept of *L. longipalpis* as a single species, at least in the region of north-eastern Brazil. Yet this conclusion is questioned by Bauzer et al. (2007) who point out that inspection of the data indicates a few loci with very large and fixed

differences in some comparisons. This suggests that a different conclusion might be reached if a more detailed analysis was carried out on populations where evidence for a species complex was previously found by other molecular markers.

Another species targeted by two RAPD studies was *Lutzomyia whitmani*, a major vector of human American cutaneous leishmaniasis in Brazil. This sand fly species represents another possible complex of cryptic species, as was suggested on the base of geographical behavior variations (Lainson, 1988) and further supported by four distinct mitochondrial lineages (Ready et al., 1997), as well as subsequent detailed morphometric study (Dias et al., 1999). To further investigate the genetic variability of this species, four populations of *L. whitmani* from Brazilian foci of cutaneous leishmaniasis were studied by RAPD and their genetic variability was evaluated (Margonari et al., 2004a). Four used primers detected interpopulational differentiation, however, additional study based on RAPD data (Margonari et al., 2004b) pointed out the evidence of a genetic flow between some previously established lineages. The results were in partial accordance with those derived from a previous morphometric survey (Dias et al., 1999).

RAPD technique was also deployed to evaluate a possible variation among *Lutzomyia intermedia*, a species with a wide distribution in the Atlantic forest regions of Brazil, which is suspected to serve as a vector for *Leishmania braziliensis*. The presence of *L. intermedia* in different habitats in the area of its distribution led to the hypothesis that the population might be in fact genetically heterogeneous and this diversity might be associated with different transmission cycles occuring in each of the habitats: domestic, extra-domestic and sylvatic. This would certainly have major implications on the epidemiological characteristics of cutaneous leishmaniasis in the endemic areas (Meneses et al., 2002). Therefore, micro-geographical variation was tested among three male populations of *L. intermedia* (domestic, extra-domestic and sylvatic) from the same endemic area of cutaneous leishmaniasis in Brazil (Meneses et al., 2005). For the RAPD analysis, six decamer primers were chosen. One of the primers did not generate a polymorphic band pattern, however, it was used as a detector of possible exogenous DNA contamination, as it produced multiple bands in the presence of DNA originating from *L. longipalpis* and/or *L. migonei*. Although the authors did not discuss the possibility further, this primer would actually serve very nicely as a diagnostic marker for *L. intermedia*. Phenetic analysis of RAPD data obtained by the remaining five polymorphic primers showed the presence of two principal clusters, one corresponding

to domestic plus extra-domestic habitat, second to sylvatic habitat. Unique genotypes were observed in each population, sylvatic population being the most polymorphic and showing the largest number of genotypes as well as a low similarity between them. However, a gene flow between the populations was detected and estimated to be at least 50 individuals per generation. Apart from RAPD, two other methods were used, namely multi-locus enzyme electrophoresis (MLEE) and single strand conformation polymorphism (SSCP). Interestingly, it was not possible to diagnose any heterogenity among *L. intermedia* populations by MLEE, suggesting the existence of a single genetic group. This was also in a perfect accordance with the results obtained by SSCP, which revealed a low level of intrapopulational polymorphism and the presence of same predominant haplotype in all three studied populations. Further on, same findings were previously obtained when the local structure of *L. longipalpis* populations from Colombia (Munstermann et al., 1998) and Venezuela (Marquez et al., 2001) was studied by isoenzyme analysis. Although the authors do not imply this, the disagreement between results obtained by different molecular markers might suggest thar RAPD analysis offers a higher resolution on a micro-geographical scale. Another explanation, given by authors, may be that the genetic diversity given by RAPD is associated with a low level of population structure due to a number of migrants which ensure the homogenity of the populations.

As RAPD proved to be a useful tool for genetic differentiation assesment of Latin American sand flies, another study was carried on *L. intermedia*, comparing the genetic structure of two populations from two ecologic regions in Brazil (de Sousa Rocha et al., 2007). These two regions are separated spatially by a distance of approximately 50 km and also show differences in biogeography and eco-epidemiology of cutaneous leishmaniasis which is present in both areas. Similar arrangement of RAPD analysis was used, again with six decamer primers. The RAPD data showed that the number of polymorphic bands did not vary significantly according to the geographical origin of populations or microhabitat. There was also no RAPD marker that would be specific for any of the studied populations. The degree of genetic structure between the two localities was low and indicated a recent geographic separation. Estimated gene flow between microhabitats were higher than between sand fly populations and there was a tendency of individuals collected in the same habitat to cluster together. Authors discussed a possibility of specific parasite-sand fly vector interactions and distinct transmission patterns of cutaneous leishmniasis as a degree of

genetic diversity of *Leishmania braziliensis* isolates collected from the two localities reflected a same pattern in *Lutzomyia intermedia* (Cupolillo et al., 2003). They further explained that the moderate level of genetic structuring observed in *Lutzomyia intermedia* populations in different habitats (intradomestic and peridomestic) in one locality, a rural area, probably reflects extension of the domestic transmission cycle in this area. On the other hand, the higher level of genetic structuring of populations from the second locality, a periurban area, may reflect independence between the domestic and peridomestic cycle. The study of de Souza et al. (2007) and their its conclusions demonstrated that important eco-epidemiological implications could be drawn from a RAPD analysis conducted in the leishmaniasis foci.

Despite a large and fruitfull deployment of RAPD technique in the New World sand flies, similar applications on the Old World sandflies are surprisingly scarse. RAPD was first demonstrated to distinguish between sand fly species occuring sympatrically in Spain and belonging to three subgenera: *Phlebotomus, Paraphlebotomus and Larroussius* (Martin-Sanchez *et al.*, 1995). A total number of eighteen primers was used with a length ranging untypically between 10-34 nucleotides. The method was readily able to distingush between all sand fly specimens but *P. perniciosus* and *P. longicuspis*, with authors questioning the taxonomical status and validity of the latter. The comparison between the RAPD diagnostic profiles of laboratory-reared and wild sand flies revealed that differences were slight in *P. perniciosus* and high in *P. papatasi*. This is interesting in the light of later findings that present *P. papatasi* as genetically rather homogeneous (Hamarsheh et al., 2007; Depaquit et al., 2008). The individual variability of wild *P. perniciosus* and *P. sergenti* was also observed. Similar study was later published, comparing again genetic variation within and between five *Phlebotomus* species sympatric in southern Spain by RAPD and giving similar results (Martin-Sanchez *et al.*, 2000).The authors concluded that the existence of high degree of polymorphism within wild *P. perniciosus* could indicate either the existence of sibling species or could be related to morphological polymorphism. Later the results of mainly mtDNA analyses really revealed existence of sibling species within the *P. perniciosus* complex (Pesson et al., 2004).

RAPD was also used to develop species-specific diagnostic marker to distinguish *P. papatasi* and *P. duboscqi*, two morphologically similar sand fly species transmitting zoonotic cutaneous leishmaniasis (Mukhopadhyay *et al.*, 2000). These two species of subgenus *Phlebotomus* were regarded as evolutionary very close and

succesfull hybridization between them was performed (Ghosh et al., 1999). This study illustrated that RAPD, when causiously optimised, represents fast, simple and also reproducible methodnot only for both intra- and interspecific studies on sand flies in different contexts, but it can also serve very well as a discriminative tool for diagnostic purposes (Behura, 2006).

1.5 Cross-mating studies in sand flies

A constitution of a reproductive barrier between populations is considered as one of main features of speciation process. The nature if this barrier can involve several mechanisms, from a simple morphological incompatibility of two emerging species to different levels of various postmating pre- and postzygotic isolations (reviewed by Coyne and Orr, 1998). However, sexual isolation is often asymmetric, i. e. species or populations show strong isolation in only one direction of the hybridization. This appears to be a common phenomenon in many animals, including *Drosophilla* (Kaneshiro, 1980) and it usually indicates a close relationship of hybridizing species. This assymetry eventually dissappears as the sexual isolation becomes complete in both directions. As is described here, this is also true in sand flies. It is necessary to bear this fact in mind when judging the results of cross-mating studies.

Several attempts were made to conduct cross-mating studies of phlebotomine sand flies, both intraspecific and interspecific. In the Old World sand flies, much work was done on species of subgenus *Phlebotomus*. This subgenus includes three medically important species, which are either proven or suspected vectors of cutaneous leishmaniasis and sandfly fever: *Phlebotomus papatasi*, *P. bergeroti* and *P. duboscqi*. These three species are morphologically similar and their geographical distribution overlaps (Ghosh et al., 1999). *Phlebotomus papatasi* has a very wide distribution in the Mediterranean, Middle East and Indian subcontinent, *P. bergeroti* extends from northern part of Africa to Arabian Penninsula, Iran and Afghanistan and *P. duboscqi* occurs south of Sahara, in western and central Africa (Lewis, 1982).

Several cross-mating studies were conducted in order to define the systematic relationships of these three species. While attempted hybridization between *P. papatasi* and *P. duboscqi* was reported to be unsuccesfull (Madulo-Leblond et al., 1991), viable interspecific hybrid offspring were obtained when *P. bergeroti* females mated with *P. papatasi* males. The morphology and behaviour of these hybrids was described as

intermediate between the two parental species (Fryauff and Hanafi 1991). However, viable offsprings were obtained only in the described parental combination. Further on, a sucessful hybridization was performed between *P. papatasi* and *P. duboscqi* (Ghosh et al., 1999). Again, only *P. papatasi* males with *P. duboscqi* females produced F1 progeny, no hybrid progeny was ever obtained from the cross female *papatasi* with male *duboscqi*, although several tries were attempted. No F2 or backcross progeny were obtained, either. In the same study, three *P. papatasi* strains from distant areas were succesfully crossed with normal progeny numbers, this suggesting that there are no signs of speciation within *P. papatasi*. The isozyme patterns of these strains also demonstrated low variability. This finding is in accord with results of several studies on intraspecific variability of *P. papatasi*, performed later mainly by sequencing analysis of different genes (Hamarsheh et al., 2007, Depaquit et al., 2008). Although possible under experimental conditions, no crosses between *P. papatasi* and *P. duboscqi* have been found in nature so far (Depaquit et al., 2008).

Morphological differentiation between *P. papatasi* and *P. bergeroti* had been discussed before (Lane and Fritz, 1986) and several morphometric characters were later proposed to distinguish between *P. duboscqi* and *P. bergeroti* in the area of their sympatry in Ethiopia (Gebre-Michael and Medhin, 1997). However, it was RAPD method which proved an ability to distinguish between *P. papatasi* and *P. duboscqi* by giving a species-specific diagnostic decamer primer suitable for rapid and trustful identification of these two closely related and morphologicaly similar sand fly species (Mukhopadhyay et al., 2000).

The main object of cross-mating studies of New World sand flies was *Lutzomyia longipalpis*, a main vector of *Leishmania chagasi* and hence of visceral leishmaniasis in Latin America. A growing amount of evidence suggests that *L. longipalpis* represents a complex of sibling New World species within a very large area of distribution, although this issue remains still unresolved fully (reviewed by Uribe 1999, Bauzer et al., 2007). The basis of the *L. longipalpis* complex research was laid by a cross-mating study by Ward et al. (1983), which demonstrated that there are at least two sexually isolated forms of *L. longipalpis*. Insemination failure was found between two forms from allopatric and sympatric sites as well as between allopatric populations with similar tergal spot patterns, showing that the spot morphology could not be used as a species-specific marker. Further on, cross-mating between populations with different male sex pheromones was unsuccesful (Ward *et al.* 1988). The concept of a species complex was

established by a finding that there is a high level of genetic divergence among laboratory colonies of *L. longipalpis* of different geographical origin and sterility was detected in male progeny when intercolony crosses were performed (Lanzaro et al., 1993). These findings, in combination with others obtained by a number of approaches described partly in previous chapters suggest an existence of pre-zygotic reproductive barriers within this complex (Maingon *et al.*, 2003). The result on taxonomic level is so far a formal description of *L. pseudolongipalpis* as the first cryptic species of the complex (Arrivillaga and Feliciangeli, 2001).

2. Objectives

The subject of this thesis is the study of intraspecific variability of *Phlebotomus sergenti*, the most important proven vector of *Leishmania tropica*, a causative agent of anthroponothic cutaneous leishmaniasis in the Old World (Jacobson, 2003).

In part of the study I took an advantage of that time presence of two laboratory colonies of *P. sergenti* maintained by that time in our laboratory: colony originating from Turkey was established from females collected in 1998 in Sanliurfa, South-East Anatolia, colony originating from Israel was derived from females collected in Amnun, Northern Israel in 2001. Populations of *P. sergenti* from Turkey and Israel belong to two different clades established by Depaquit et al. (2002).

Most of wild specimens were collected during two field studies I participated at: in Tiberias, Israel in 2005 and Adana, Turkey in 2006. Other wild-caught sand flies were obtained due to a collaboration with our co-workers.

The main objectives of this thesis were as follows:

- to hybridise males and females of *P. sergenti* from two colonies of different geographical origin (Turkey and Israel) to examine a possible reproductive barrier among hypothetized sibling species
- to compare these two laboratory colonies by RAPD analysis and compare the results with those obtained by geometric morphometry analysis performed by a collaborating laboratory
- to test RAPD as a method of choice for the comparison of sand flies from different foci of leishmaniasis
- to test cross-applicability of microsatellite markers developed so far for phlebotomine sand flies and if not applicable, design a unique panel of species-specific microsatellite markers for *P. sergenti*
- to compare wild specimens of *P. sergenti* from different populations within the broad range of distribution by several molecular markers: RAPD, sequencing of ITS2 (nuclear marker) and sequencing of cytochrome b (mitochondrial marker). Compare obtained results with the results of geometric morphometric analysis performed by a collaborating laboratory

3. Results

Vít Dvořák, A. Murat Aytekin, Bulent Alten, Soňa Škařupová, Jan Votýpka and Petr Volf. 2005. **Intraspecific variability of *Phlebotomus sergenti* Parrot, 1917 (Diptera: Psychodidae)**. Journal of Vector Ecology 31 (2), 229-238.

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A comparison of the intraspecific variability of *Phlebotomus sergenti* Parrot, 1917 (Diptera: Psychodidae)

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ABSTRACT: *Phlebotomus sergenti* populations from different areas of the Mediterranean basin are known to exhibit high intraspecific variability. Previous studies of ITS2 revealed the presence of two branches that may represent sibling species. To corroborate this finding by other tools, two colonies of *P. sergenti* originating from Turkey and Israel, each belonging to a different ITS2 branch, were compared by three different methods: geometric morphometric analysis of wing shape, RAPD (random amplified polymorphic DNA), and cross-mating study. For geometric morphometric analysis, two-dimensional Cartesian coordinates of 16 landmarks from the wings were digitized and analyzed. Significant shape differences were found between colonies but not between sexes within each colony. RAPD results formed two distinctive clades corresponding to the origin of the colony but also showed heterogenity among members of both colonies. In cross-mating studies, viable hybrid F1 and F2 progeny were obtained when both Turkish males/Israeli females and Israeli males/Turkish females were crossed. F1 progeny was included in RAPD analysis and these hybrids formed a distinctive clade with an intermediate position between the two parental clades. No significant differences were found in egg production of crossed sand flies. The cross-mating study showed that there is no reproductive barrier between *P. sergenti* from different geographical areas. On the other hand, RAPD and geometric morphometric analysis revealed a significant difference between colonies and confirmed the suitability of previous ITS2 analysis for discrimination among sand fly populations. Further development of molecular markers should resolve a possible existence of sibling species within *Phlebotomus sergenti*. ***Journal of Vector Ecology*** **31 (2): 229-238. 2006.**

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

INTRODUCTION

As the main vector of *Leishmania tropica*, *Phlebotomus sergenti* Parrot, 1917 is a sand fly of great medical importance. Originally described from Algeria in 1917, this species has a broad range of distribution which covers areas of the southern Mediterranean (Morocco, Algeria, Tunisia), the northern Mediterranean (Portugal, Spain, Sicily), Middle East, Arabia, Afghanistan, Pakistan, and northern parts of India. Because of the broad distribution, we can expect some degree of intraspecific variability.

Intraspecific variability of the internal transcribed spacer 2 (ITS2) was studied by Depaquit et al. (2002) with 12 populations of *Phlebotomus sergenti* from ten different countries. According to this study, two branches can be identified: 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, and Israel). These branches are in accordance with postulated migration routes of *P. sergenti* along the Thetys Sea during the Miocene era. According to Depaquit et al. (2002), groups belonging to these two different branches seem to differ in ecology, host preferences, and possibly also in vectorial capacity. It is judicious to consider the potential existence of sibling species as was demonstrated by means of molecular biology in the case of *Lutzomyia longipalpis* (Diptera: Psychodidae), where different cryptic species were established (Arrivillaga et al. 2002, Maingon et al. 2003). If sibling species within *P. sergenti* were proven, it would have important implications in epidemiology as well as in experimental studies. Because we have maintained two laboratory colonies originating from areas belonging to two different above mentioned branches, we were able to study the intraspecific variability of this taxon by three different methods: RAPD, geometric morphometrics, and a cross-mating study.

Random-amplified polymorphic DNA (RAPD) represents a powerful tool for identification of species and strains and the estimation of genetic variability between isolates (Williams et al. 1990). The technique amplifies random fragments of genomic DNA by polymerase chain reaction (PCR) using single primers of arbitrary nucleotide sequences. It is considered to be a fast, easy, inexpensive, and yet very informative method (Diakou and Dovas 2001). In the New World sand flies, RAPD was succesfully used to discriminate between two closely related species, *Lutzomyia youngi* and *L. spinacrassa* (Adamson et al. 1993). It distinguished succesfully between different laboratory populations of *L. longipalpis* complex (Dias et al. 1998) and distinct biogeographical populations of *L. whitmanni* complex (Margonari et al. 2004). Only two studies using RAPD were performed on the Old World sandflies. RAPD was succesfully used to compare genetic variation within and between five *Phlebotomus* species sympatric in southern Spain (Martin-Sanchez et al. 2000) and to develop species-specific diagnostic profiles of *Phlebotomus papatasi* and *P. duboscqi* (Mukhopadhyay et al. 2000). Here we present

RAPD as a useful method for discrimination of different laboratory colonies of *Phlebotomus sergenti* and a powerful tool for studies of intraspecific variability in this species.

In spite of the growing number of molecular methods, morphological approaches are also valid. Among these, geometric morphometrics represents an important new paradigm for the statistical study of "shape" and "size" in biology and other fields of science (Rohlf and Marcus 1993). The landmark-based geometric morphometrics has gained significant support among entomologists (Alibert et al. 2001). Unlike analytical approaches, the geometric one is aimed at a comparison of the shapes themselves (Pavlinov[3]). The method is based on capturing the two- or three-dimensional Cartesian coordinates of landmarks which are the homologous points among the structures that have been previously assigned the same names (Bookstein 1991). Differences among individual configurations of landmarks can be translated to several mathematical functions which fit the differences (Alibert et al. 2001). The Procrustes distance (the square root of the sum of squared differences) can then be used as a metric for comparing shapes (Rohlf 1999). Together with warps, Principal Components Analysis (PCA) of Procrustes residuals, consensus shape of wing-triangulation (average positioning of the landmarks in a set of specimens, *eg*. a species with their artificial triangular connections), and UPGMA (unweighted pair-group method using arithmetic averages) phenograms can provide an excellent combination of techniques for the two purposes of such studies: first to *detect* and then to *describe* the differences among taxa (Lockwood et al. 2002). Secondly, the size measure can be visualized in terms of the centroid size, which is uncorrelated with shape in the absence of allometry (Zelditch et al. 2004).

Several cross-mating studies have been performed within both Old and New World sandflies. The main object of these studies was *Lutzomyia longipalpis*, a possible complex of sibling New World species (reviewed by Uribe 1999). Cross-mating between populations with different male sex pheromones was unsuccesful (Ward et al. 1988). This, in combination with other approaches, suggests an existence of pre-zygotic reproductive barriers within this complex (Maingon et al. 2003). In the Old World sand flies, cross-mating studies were performed between *Phlebotomus papatasi* and *P. duboscqi* (Madulo-Leblond et al. 1991, Ghosh et al. 1999) and between *P. bergeroti* and *P. papatasi* (Fryauff and Hanafi 1991). The aim of our study was to hybridize males and females of *P. sergenti* from colonies derived from two different geographical regions (Turkey and Israel) to examine a possible reproductive barrier among suspected sibling species and compare the results with those of morphometry and RAPD analysis.

[3]Pavlinov, I.Y. 2001. Geometric morphometrics, a new analytical approach to comparision of digitized images. Information Technology in Biodiversity Resarch. Abstracts of the 2nd International Symposium. St. Petersburg. 41–90.

MATERIALS AND METHODS

Sand fly colonies

Two colonies of *P. sergenti* were maintained at Charles University, Czech Republic. The colony originating from Turkey (TK) was established from gravid females collected in 1998 in Urfa, southeast Anatolia. The colony originating from Israel (IS) was derived from females collected in Amnun, northern Israel in 2001.

Both colonies were maintained under the same conditions at 26±1° C, 90% RH and 14/10 L:D photoperiod. Adults had constant access to cotton wool soaked with 50% honey, sugar, and a water source. Once a week, females were fed on anaesthetized mouse (ketamin 150 mg/kg, xylazin 15 mg/kg). The blood-fed females were transfered into separate cages and after defecation they were moved to a plaster-lined breeding pot to lay eggs. Larvae were maintained on a diet of mixed mold, rabbit feces, and rabbit chow that was aged for three weeks, air dried, and finely ground.

RAPD analysis

DNA was extracted from individual males and unfed females with the High Pure PCR Template Preparation Kit (Roche, France). Only the thorax was used; the digestive tract, heads, wings, and legs of flies were dissected out prior to DNA extraction to minimalize possible contamination. Five males and five females were taken from each colony. We also included F1 progeny obtained from the cross-mating study into the RAPD analysis (five males and three females descended from a Turkish female/Israeli male crossing).

To prevent possible contamination by symbionts, both colonies were previously screened for presence of *Wolbachia* using the general *wsp* primers 81F and 691R (Braig et al. 1998, Zhou et al. 1998) as described elsewhere (Benlarbi and Ready 2003). *P. papatasi* from Urfa, Turkey, was used as a positive control. No *Wolbachia* symbionts were found in any *P. sergenti*.

For RAPD analysis, 60 decamer random primers were tested (OPA 1-20, OPD 1-20, OPF 1-20, by Operon Technologies Inc, U.S.A.). Twelve of these primers were found suitable: OPA3, OPA5, OPA9, OPA10, OPA11, OPA20, OPD5, OPD8, OPD13, OPF14, OPF19, and OPF20. The PCR reaction was optimized (number of cycles, thermic profile of reaction, Mg^{2+} concentration) in order to obtain informative and reproducible RAPD patterns. The volume of each reaction was 25 µl. The reaction mixture was prepared as follows: 12.5 µl of Master Mix (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0,001% Tween 20, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 2,5 U Taq purple DNA polymerase, by Top-Bio, Czech Republic), 1.5 µl $MgCl_2$ (1.5 mM), 2 µl primer (10 pmol), and 8 µl dH_2O. RAPD reactions were performed by a PTC-200 thermocycler (MJ Research Inc, U.S.A.) 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 (Serva) gel in TAE (40 mM Tris acetate/1 mM EDTA) at 80 V for 3 h and stained with ethidium bromide. Ten µl of each product was loaded on the gel, and ethidium bromide was added into the gel prior to the separation. The electrophoretograms were captured with a digital camera using the SkyPro program (Software Bisque). Bands were transformed into a binary matrix data where presence or absence of a band was codified as 1 or 0, respectively. All detected bands were included in the analysis. Genetic distances of samples were computed from Nei-Li´s coefficient of similarity (Nei and Li 1979). Phylogenetic trees were constructed by the unweighted pair-grouping analysis (UPGMA) (Sneath and Sokal 1973). The robustness of trees was assessed by bootstrap analysis. PC program FreeTree (Hampl et al. 2001) was used for computations of genetic distances and construction of trees.

Geometric morphometric analysis

For morphometric analysis, 100 specimens were randomly taken from each of the two colonies. All the specimens were screened for the presence of known ecto- and endo-parasites to shield the morphometric data from possible traumatic variations (Mayr and Ashlock 1991, Aytekin et al. 2002). The wings were removed from each specimen by forceps and stained for proper vision of veins using the following procedure: the wings were kept in 5% KOH for 20 min to clear hairs, washed in 95% ethanol, and then washed in distilled water. The wings were transformed to methylene blue for 20 min, re-washed by distilled water and ethanol, soaked in xylene for 5 min, and mounted in entellane on labelled slides. All slides were photographed using a Leica MZ-7.5 stereoscopic zoom dissection microscope with a DC-300 digital camera system, digitized, coded, and archived. Some of the 2,000 specimens were eliminated because of problems during their preparation and 81 were used for the morphometric analysis (15 males and 66 females). Three females of the IS population were also removed as they caused a Pinocchio effect, which is a large change concentrated only at one landmark. All specimens were scored by the same person (A.M.A.). In order to reduce the measurement error, all specimens were digitized twice. The second session of measurement was conducted after the specimens had been removed and replaced under the microscope in order to take positioning error into account (Arnqvist and Mårtensson 1998, Alibert et al. 2001).

Two-dimensional Cartesian coordinates of 16 landmarks (Figure 1) were digitized by tps-DIG1.40 software (Rohlf 2004a). The landmark configurations obtained were then scaled, translated, and rotated against the consensus configuration by GLS Procrustes superimposition method (Bookstein 1991, Rohlf and Marcus 1993, Dryden and Mardia 1998) and used in Morphologika® (O'Higgins and Jones 1999) 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 front wings as an estimator with the nonparametric Kruskal-Wallis test (Zelditch et al. 2004).

Cross-mating analysis

For the cross-mating experiments, single pupae from both colonies were placed into individual vials to secure virgin individuals. Virgin females of a given colony were grouped with virgin males from the other colony (TK male / IS female, IS male / TK female) in an approximate 1:1 ratio of sexes and allowed to feed on mice. Blood-fed females were places individually into oviposition vials and ovipositing females, eggs, and emerging adults were counted. Adult F1 hybrids were used for F2 brother-sister mating, which was performed in the same fashion.

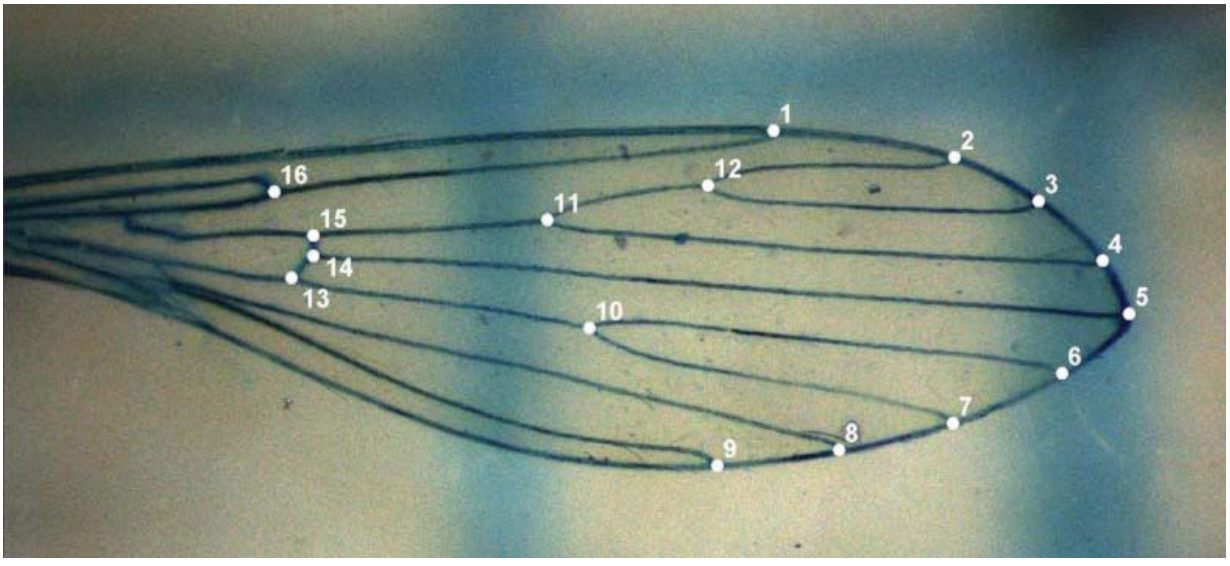

Figure 1. Location of the 16 landmarks on the wing of *Phlebotomus sergenti*.

Special attention was paid to possible differences in egg production of IS females as the results of preliminary experiments suggested that their oviposition might be affected by mating with TK males. The number of eggs in batches of IS females inseminated by TK males was compared with the number of eggs in batches of IS females inseminated by IS males (T-test, Statistica©). Care was taken to perform the mating and oviposition under the same conditions.

RESULTS

RAPD analysis

PCR conditions produced a reproducible banding pattern for each primer used for the analysis. The reproducibility was tested for several primers, and aside from minor variations in band intensity, there were no variations in banding pattern for any primer tested.

A total of 149 fragments was scored. Of these, 21 were monomorphic (shared by all individuals) and 128 (86%) polymorphic. The size of amplified products ranged from 200-1500 bp. The UPGMA analysis of the RAPD data revealed that members of each colony formed a distinct subgroup. A similar grouping pattern was also acquired by the neighbor-joining method (data not shown). There was no significant grouping pattern based on sex in any of the two groups formed. However, there was a considerably high level of variability within each subgroup with Israeli sand flies showing higher levels of such variability. When F1 progeny obtained from the cross-mating study were included in the analysis, it formed a distinct subgroup with an intermediate position between the Turkish and Israeli subgroup (Figure 2).

Geometric morphometric analysis

When the four taxa were analyzed (TK-female, TK-male, IS-female, and IS-male) in terms of relative warps, the TK and IS populations showed significant differences where there were small changes among sexes of each population. The most significant differences were determined in the shape of the basal part for TK populations and mid and apical of the IS populations when the mean shape used as reference. The intra-variation of the sexes was not determined to be as high as inter-regional differences. The results showed that the TK populations are typically different by having a thinner wing than those of IS populations when the generalized least-square superimposition for the landmark configurations are rendered to wire-frames superimposed on the reference configuration (Figure 3 A-D).

The PCA of the wing shape produced a similar grouping pattern. The SAHN clustering showed no significant difference among sexes. But when an UPGMA phenogram was conducted for each sex, distinct groups (TK and IS) in both females (Figure 4) and males (Figure 5) clustered perfectly, which indicated that there was a distinct group pattern in terms of shape morphometry among Turkish and Israel taxa. The same clustering was also observed for data obtained from the centroid sizes. The size differences among the populations were significant and in a linear gradient (Figure 6) (Kruskal-Wallis Test: $H = 38.64$ at $P < 0.001$).

Cross-mating analysis

In the first experiment, successful mating, insemination, and viable hybrid F1 and F2 offsprings were obtained from both combinations of parents (TK male/IS female and vice versa). Of 25 TK females grouped with IS males, 17 females oviposited a total number of 542 eggs. F1 progeny descended from these eggs was a total of 310 sand flies. Of these, 90 females were used for production of F2 progeny and they produced a total number of 712 F2 generation sand flies. Of 46 IS females grouped with TK males, 20 females oviposited a total number of 460 eggs. F1 progeny descended from these eggs was 25 sand flies in total. Of these, 12 females were used to produce a total number of 171 F2 generation sand flies.

In the second experiment the difference in egg production was tested between combinations of TK male/IS female and IS male/IS female. The total number of 103 IS females was mixed with TK males. Of these, 78 females fed on mice and 50 oviposited. These females produced batches with total number of 2,052 eggs, an average of 41 eggs per female. Of 97 IS females maintained under the same conditions and mixed with IS males, 71 females blood fed and 46 oviposited. Their egg production was 2,242, corresponding to an average of 48 eggs per female. Although IS females mixed with TK males produced a lower number of eggs, the difference was non-significant (T-test: $T = 1.22$, $P = 0.2$).

DISCUSSION

ITS2 sequencing by Depaquit et al. (2002) revealed that populations of *P. sergenti* from the Mediterranean basin exhibit intraspecific variability (Depaquit et al. 2002). The aim of our study was to corroborate this finding and study the differences between populations of *P. sergenti* from different branches postulated by Depaquit et al. (2002) using three different approaches: geometric morphometric analysis of wing shape, RAPD analysis, and cross-mating study.

RAPD was able to clearly distinguish between members of the Turkish and Israeli colonies. When 15 different arbitrary primers were deployed, both UPGMA analysis and neighbor-joining method analysis of RAPD-PCR revealed the existence of two distinct groups according to colony origin. All members of one colony fell into the same group. Moreover, there was no significant grouping pattern based on sex in any of these two groups. RAPD analysis also revealed a considerable variability within each of the two colonies. The patterns of bands obtained from RAPD-PCR were very complex and showed intracolonial differences, with the Israeli colony showing a higher degree of this variability. As we succesfully obtained F1 progeny from the cross-mating study, these progeny were included in the RAPD analysis. Interestingly, these samples formed a distinct group with position intermediate between the Turkish and Israeli subgroups.

As the RAPD patterns obtained were stable and reproducible, we believe that RAPD-PCR reflects a prevailing

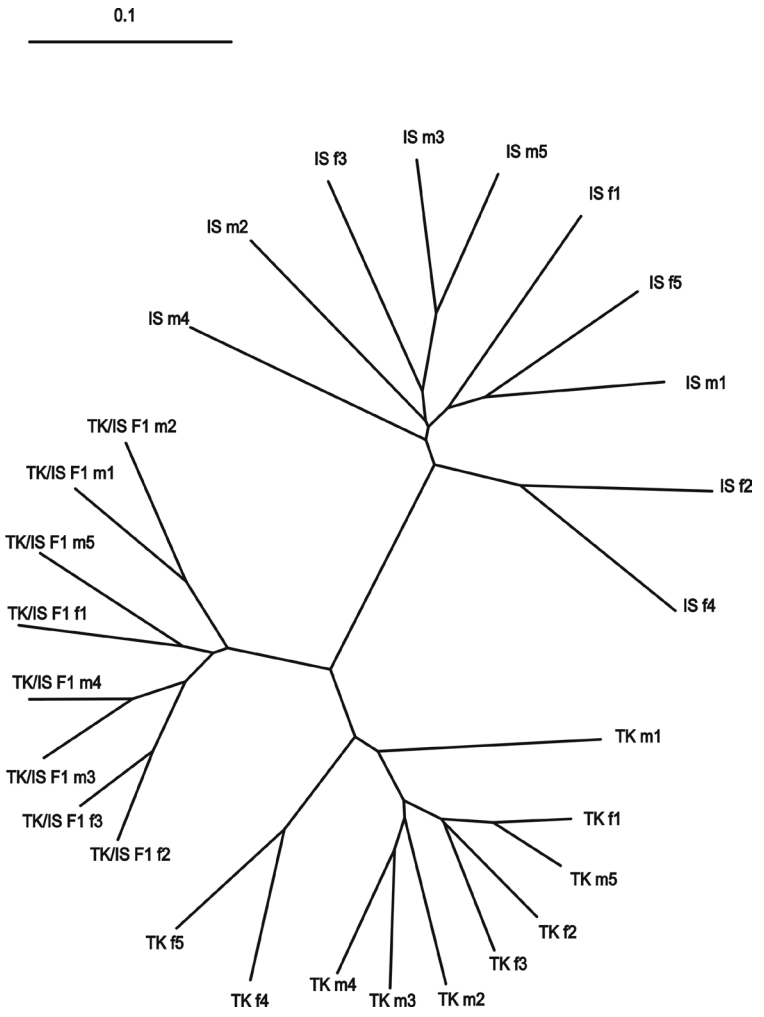

Figure 2. Unrooted UPGMA tree based on RAPD analysis of 28 individuals: 10 Turkey (5 males, 5 females), 10 Israel (5 males, 5 females), 8 F1 progeny (T♀/I♂, 5 males, 3 females). (TK-Turkey, IS–Israel, m-male, f-female).

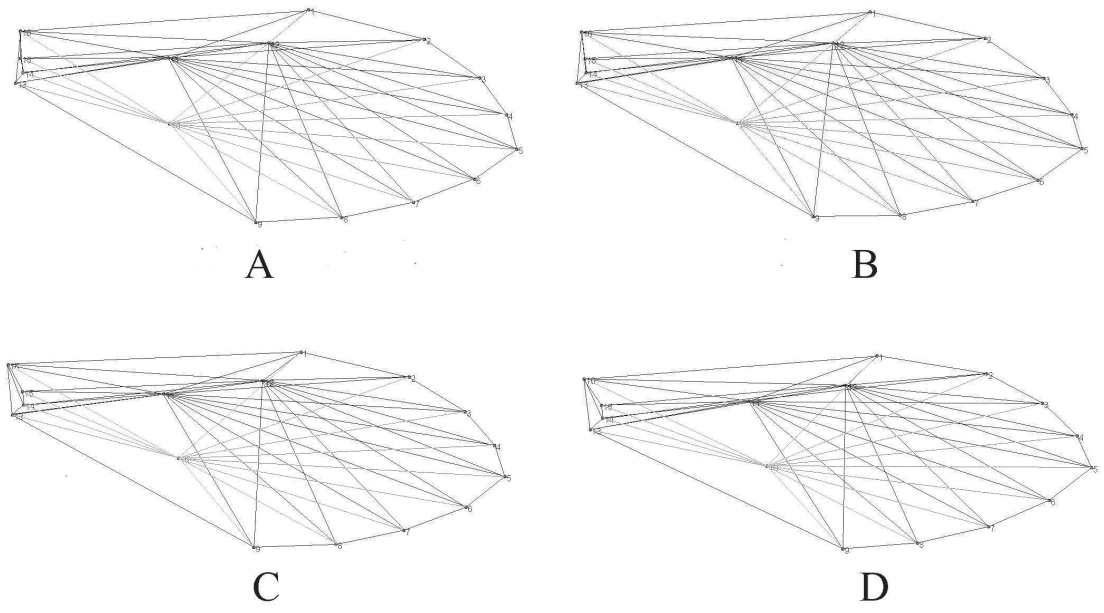

Figure 3. Results of the generalized least-square superimposition for the landmark configurations of *Phlebotomus sergenti* rendered to wire-frames of different populations superimposed on the reference configuration using affine generalized resistant fit analysis. **A**: IS-Female, **B**: IS-Male, **C**: TK-Female and **D:** TK-Male.

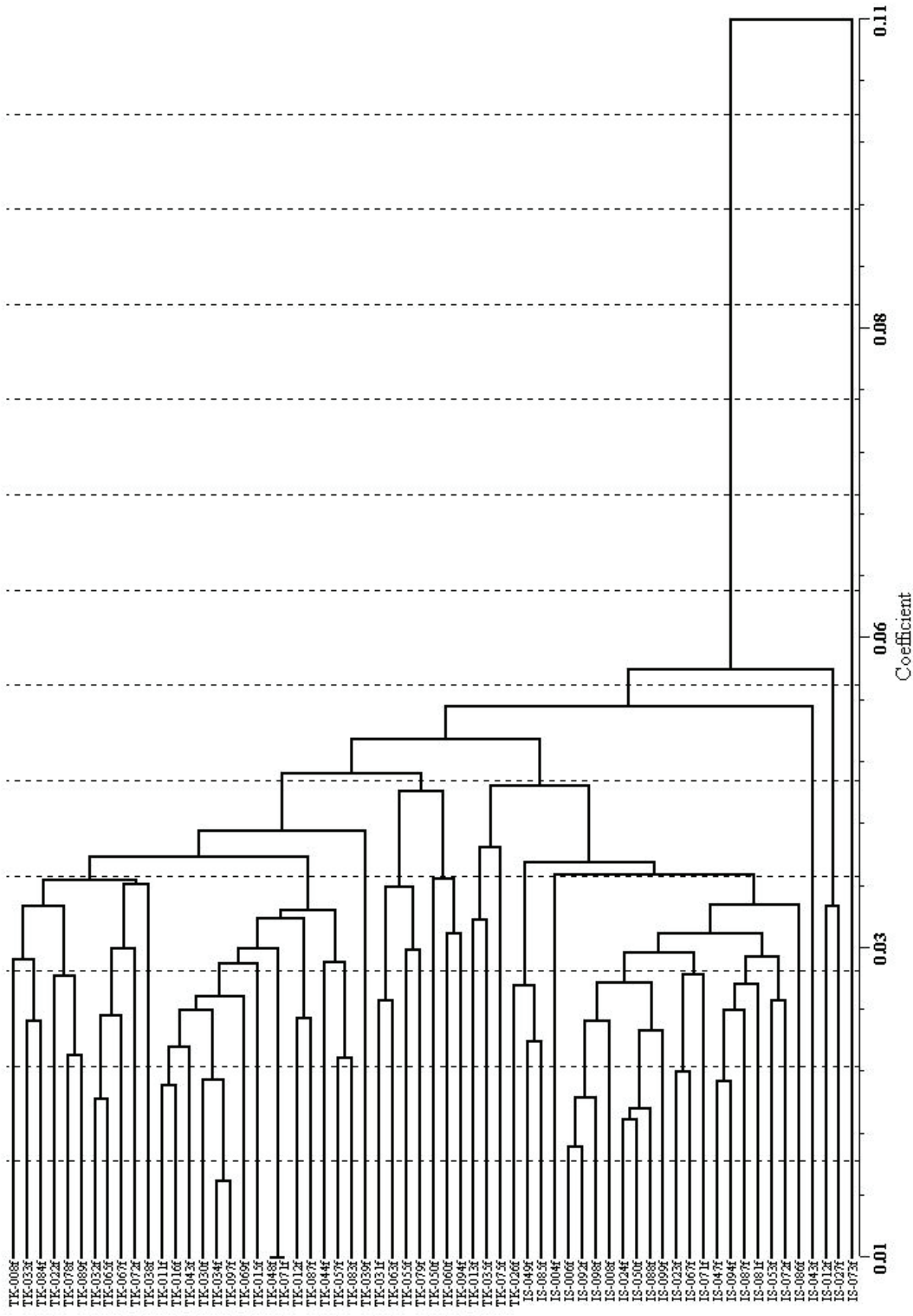

Figure 4. UPGMA phenogram showing the difference between Turkish and Israeli females (Euclid distance SAHN clustering).

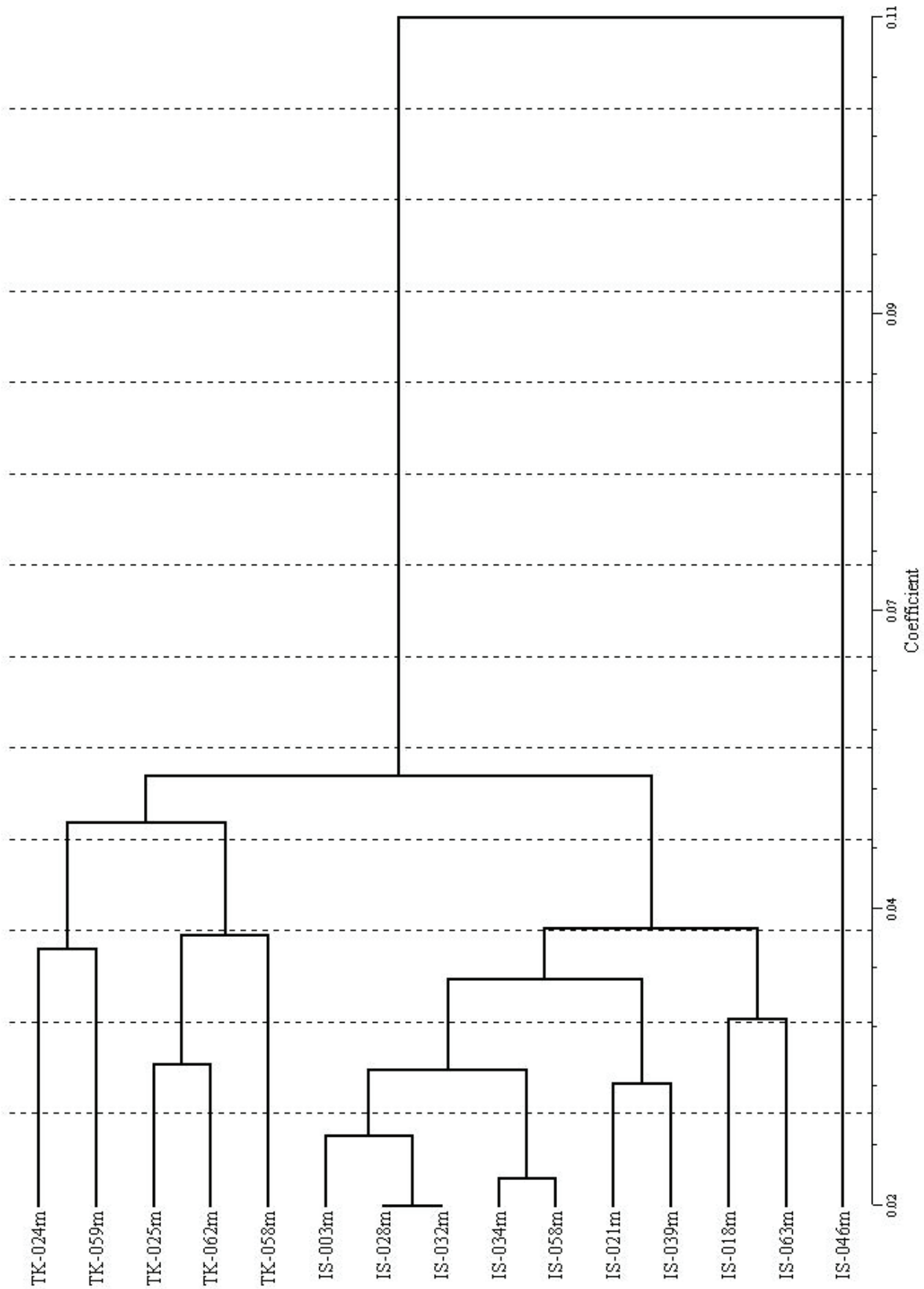

Figure 5. UPGMA phenogram showing the difference between Turkish and Israeli males (Euclid distance SAHN clustering).

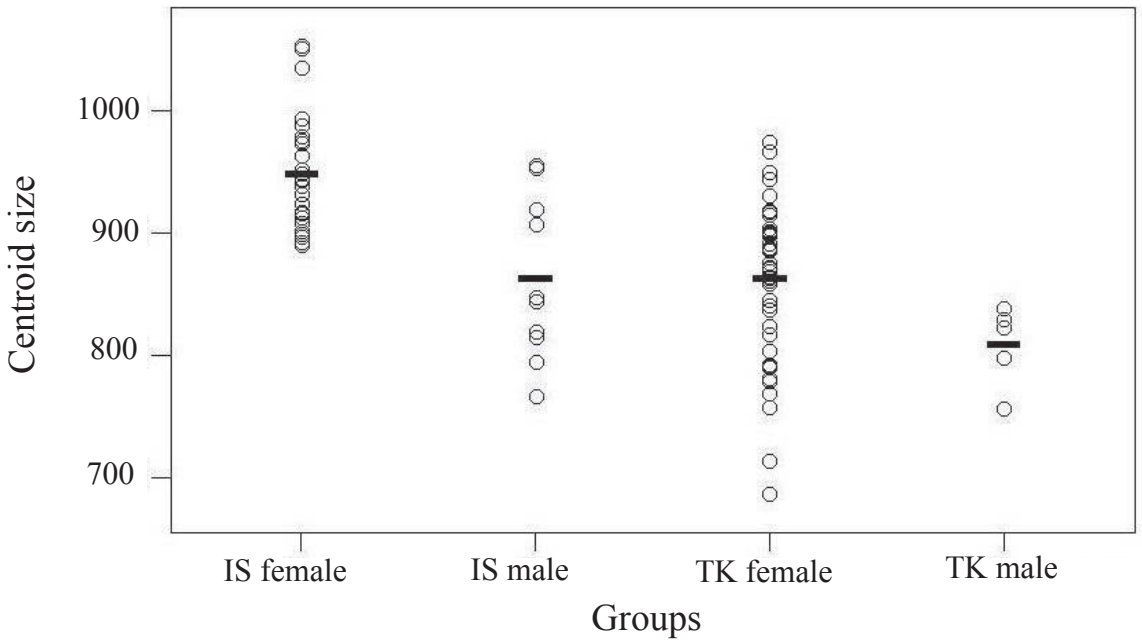

Figure 6. Plot of the individuals of the *Phlebotomus sergenti* populations examined from the data of centroid sizes from the wings. Group means are indicated by lines and dotplots by open circles.

variability in both colonies. Neither of these two colonies underwent any bottle-neck event and it is reasonable to assume that this intracolonial variability reflects a variability of original wild populations. The next step would be a comparison of field samples of *P. sergenti* from different regions of the Mediterranean basin.

Despite its limitations, RAPD analysis has been repeatedly used to evaluate intraspecific variability in sand flies. It proved to be useful in revealing heterogenity among different laboratory populations of *L. longipalpis* (Dias et al. 1998) and also different geographical populations of *L. whitmani* (Margonari et al. 2004). Both taxa are considered to be complexes of sibling species that are difficult to distinguish morphologically. The results of RAPD analysis of *L. whitmanni* sand flies originating in different regions of the distribution of this complex were in partial accordance with a previous morphometric survey from the same regions and provided additional evidence to support the existence of distinct biogeographical populations (Margonari et al. 2004). Here we present a similar kind of results for an Old World sand fly species which may also represent a complex of sibling species.

The insect wings with the least degrees of freedom are the most appropriate structures for geometric morphometric studies (Pavlinov[3]). The results obtained from the wing shape and size supported those obtained from the RAPD. Both PCA and UPGMA results showed no shape difference between sexes, but the deformation of the wing shape among the Israel and Turkish populations is significant. The means of the centroid sizes are also supported by the same scheme obtained from the configuration of the landmarks. The sizes are also significantly different in both colonies. There is a larger wing in IS females than in TK females, while in male TK populations, wing size is more typically thinner in general. The main shape deformation is generally concentrated on the tip of the wings and the basal part. These are kinematically more reflected by the different physical conditions that had the same effects on both sexes.

Cross-mating studies within the Old World sand flies of subgenus *Phlebotomus* yielded various results. Attempted hybridization between *Phlebotomus papatasi* and *P. duboscqi* was first reported to be unsuccesful (Madulo-Leblond et al. 1991). Later, however, female *P. duboscqi* inseminated by male *P. papatasi* did produce viable interspecific hybrid adults (Ghosh et al. 1999). Interspecific hybrid offsprings were also obtained when *P. bergeroti* females were inseminated by *P. papatasi* males, described as intermediate between the parent species in morphology and behavior. Male hybrids of F1 progeny were fertile (Fryauff and Hanafi 1991).

Our cross-mating study demonstrates that crossing is possible between *P. sergenti* specimens from Turkey and Israel. We observed successful mating and insemination and obtained viable hybrid F1 and F2 offspring from both Turkish male/Israeli female and Israeli male/Turkish female combinations. We tested possible differences in egg production and batches descended from Turkish male/Israeli female were compared with egg production from Israeli male/Israeli female mating. Although preliminary results showed a reduction of egg production in females from Israel inseminated by males from Turkish colony, this effect was not statistically significant. According to our results, there is no reproductive barrier among sand flies from Turkish and Israeli colonies. The examples of *P. duboscqi*/*P. papatasi* (Madulo-Leblond et al. 1991, Ghosh et al. 1999) and *P. bergeroti*/*P. papatasi* (Fryauff and Hanafi 1991) crossings show, however, that reproductive isolation may be

incomplete even among closely related, but well established species. If we consider *P. sergenti* populations from two branches postulated by Depaquit et al. (2002) being in the state of speciation or representing sibling species within *P. sergenti* complex, we may expect an incomplete reproductive barrier.

The results of RAPD analysis and geometric morphometric analysis of wing shape of *P. sergenti* from Turkey and Israel corroborated the ITS2 results of by Depaquit et al. (2002). In light of these findings, it seems judicious to extend the application of different approaches to studies of intraspecific variability of different geographic populations of the Mediterranean basin to elucidate the taxonomic status of these populations and reveal a possible existence of sibling species among *P. sergenti.* A panel of microsatellite markers is currently being developed for this purpose and the application of these markers should resolve relationships and taxonomic status of these populations.

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Distinct Transmission Cycles of *Leishmania tropica* in 2 Adjacent Foci, Northern Israel

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Transmission of *Leishmania tropica* was studied in 2 adjacent foci in Israel where vector populations differ. Only *Phlebotomus sergenti* was found infected with *L. tropica* in the southern focus; *P. arabicus* was the main vector in the northern focus. Rock hyraxes (*Procavia capensis*) were incriminated as reservoir hosts in both foci. *L. tropica* strains from the northern focus isolated from sand flies, cutaneous leishmaniasis cases, and rock hyraxes were antigenically similar to *L. major*, and strains from the southern focus were typically *L. tropica*. Laboratory studies showed that *P. arabicus* is a competent vector of *L. tropica*, and *P. sergenti* is essentially refractory to *L. tropica* from the northern focus. Susceptibility of *P. arabicus* may be mediated by O glycoproteins on the luminal surface of its midgut. The 2 foci differ with respect to parasites and vectors, but increasing peridomestic rock hyrax populations are probably responsible for emergence of cutaneous leishmaniasis in both foci.

Leishmaniases are parasitic diseases with a wide range of clinical symptoms and currently threaten 350 million persons in 88 countries (*1*). In Israel and its vicinity, *Leishmania major* and *L. tropica* cause cutaneous leishmaniasis (CL), and *L. infantum* can result in visceral leishmaniasis (*2*). Until recently, relatively little information was available on the epidemiology of CL caused by *L. tropica* in this region. Outbreaks were not investigated, and cases were usually grouped together with CL cases caused by *L. major* (*3*). However, in recent years, new foci of CL caused by *L. tropica* are emerging in different parts of the country, such as the Galilee region of northern Israel and the Judean Desert east of Jerusalem that warrant thorough investigations (*4*,*5*). Clinically, lesions caused by *L. tropica* last longer and are more difficult to treat than those caused by *L. major* (*6*). Although *L. tropica* can be anthroponotic, foci in Israel appear to be zoonotic, with rock hyraxes (*Procavia capensis*) serving as probable reservoir hosts (*4*).

Leishmania development in sand flies is facilitated by interaction with midgut molecules of the vector. Laboratory studies showed that sand flies are composed of 2 groups. Species such as *Phlebotomus* (*Phlebotomus*) *papatasi*, the vector of *L. major* and *P.* (*Paraphlebotomus*) *sergenti*, the main vector of *L. tropica*, show specificity for *Leishmania* they transmit in nature (*7*,*8*). Conversely, species such as *Lutzomyia longipalpis*, the vector of *L. infantum* in South America, and many others are permissive and support development of several *Leishmania* spp. (*8*,*9*).

Studies performed with *L. major* and *P. papatasi* showed that attachment in the midgut is mediated by the major surface glycoconjugate of promastigotes, lipophosphoglycan (LPG), which interacts with *Pp*Galec, a galactose-binding molecule in the midgut of *P. papatasi* (*10*). However, the mechanism of attachment may be redundant, and another molecule on the promastigote flagellum may be involved (*11*).

Recently, the susceptibility of phlebotomine sand flies to *Leishmania* parasites was shown to correlate with O-linked glycoproteins in sand fly midgut (P. Volf, unpub. data). The permissive species have O-glycosylated epitopes on the luminal midgut surface, which may serve as binding sites for lectinlike components found on the surface of parasites (*12*,*13*). We compare midgut glycosylation patterns of 2 sand fly species, *P.* (*Adlerius*) *arabicus*

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and *P*. (*Paraphlebotomus*) *sergenti* that transmit *L*. *tropica* in 2 adjacent foci in the Galilee region of northern Israel.

L. *tropica* is genetically heterogeneous, and strains are readily distinguishable by antigenic, biochemical, and molecular techniques (*14–16*). We report findings of extensive studies in 2 adjacent CL foci that demonstrate conclusively that both vector species and parasite strains from the northern focus are different from those in the southern focus, a mere 10 km away (Figure 1).

Materials and Methods

Study Area

Studies were conducted in 2 adjacent foci in the Galilee region of northern Israel (Figure 1). The northern focus comprises several villages situated on generally south-facing slopes ≈5 km north of Lake Kinneret in the eastern lower Galilee of northern Israel (32°55′N, 35°36′W). The area investigated encompasses the villages of Amnun (at sea level), Karkom (100–150 m above sea level), and Korazim (150 m above sea level), which have ≈1,200 inhabitants living in ≈300 single-family houses surrounded by gardens and built on basalt rock. Many boulders from the cleared land have been piled into large heaps separating individual plots and surrounding the villages. These boulder mounds are inhabited by numerous rock hyraxes (*P*. *capensis*).

The southern focus includes the city of Tiberias (32°47′N, 35°32′W; population = 38,952). Studies were conducted in the outskirts of urban neighborhoods, where boulder mounds were inhabited by large populations of rock hyraxes. These neighborhoods are built on north- to northeast-facing slopes.

Collection, Dissection, and Identification of Sand Flies

Sand flies were trapped by using CDC light traps (John W. Hock, Gainesville, FL, USA) in September 2002 and 2004. Dead flies were stored in 70% alcohol and identified by using several keys (*17–19*). Live female flies were immobilized on ice, rinsed briefly in 96% ethanol, and dissected in 0.9% sterile saline. Guts were microscopically examined for parasites. Heads and genitalia were used for identification. Guts containing promastigotes were aseptically placed in glass vials (2.5 mL) containing blood agar made from defibrinated rabbit blood overlaid with a 1:1 mixture of RPMI 1640 medium and Schneider *Drosophila* cell culture medium supplemented with 10% fetal calf serum (Sigma, Saint Louis, MO, USA, and Gibco-BRL, Gaithersburg, MD, USA), 10,000 IU penicillin (Biotika, L'upca, Slovakia), 100 μg/mL amikacin (Bristol-Myers Squibb, Princeton, NJ, USA), and 1,500 μg/mL 5-fluorocytosine (Sigma). Some data on *Leishmania* isolates from the northern focus were obtained from our previously published results.

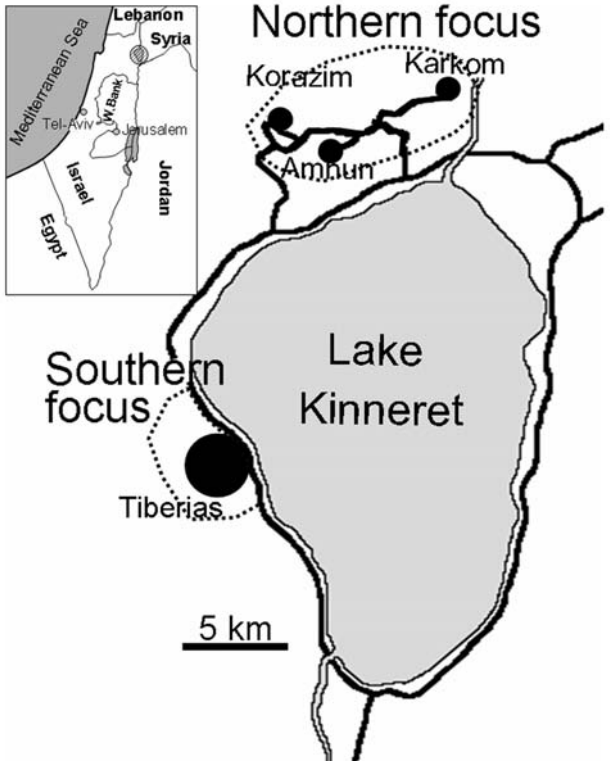

Figure 1. *Leishmania tropica* foci near Lake Kinneret in the Galilee region of Israel. Inset shows the location of the foci (circle). W. Bank, West Bank.

Collection of Animals

Rock hyraxes were trapped by using raccoon traps (http://www.havahart.com) baited with fresh leaves and anesthetized with ketamine (10 mg/kg given intramuscularly). Samples of blood and skin were obtained for parasite culture and blotted onto filter paper for PCR analysis. Animals were released at the site of capture. Skin biopsy specimens were homogenized and placed in blood agar culture medium in flat tubes (Nunclon; Nunc Nalgene International, Rochester, NY, USA). Rats (*Rattus rattus*) were trapped by using steel mesh traps (Tomahawk Live Trap Co., Tomahawk, WI, USA) placed in sewers and rock crevices. Spiny mice (*Acomys cahirinus*) were captured by using Sherman traps (H.B. Sherman Traps, Tallahassee, FL, USA). Rodents were anaesthetized with ketamine/xylazine (150 mg/kg and 15 mg/kg, respectively, given intraperitoneally). Blood from the tip of the tail was blotted on filter paper. Ear biopsy specimens were treated as described for hyraxes. Cultures were checked at 4–7-day intervals for 1 month.

DNA Extraction

DNA from wild-caught sand flies kept frozen or preserved in 100% ethanol was extracted as previously described (*20*). DNA from filter paper disks was extracted by using the phenol-chloroform method (*21*).

Detection and Identification of *Leishmania* infections by PCR

The ribosomal internal transcribed spacer region 1 (ITS1) was amplified with *Leishmania*-specific primers. ITS1 PCR products showing a *Leishmania*-specific band on agarose gels were digested with *Hae*III for species identification (*22*). Restriction fragments were subjected to electrophoresis on agarose gels and compared with DNA of *L. infantum* (Li-L699), *L. major* (Lm-L777), and *L. tropica* (Lt-L590).

Antigenic Characterization of Parasite Isolates

Initial screening of isolates was performed by using gel diffusion of glycoconjugates secreted into culture media (excreted factor) and several antileishmanial serum samples (*23*). *Leishmania*-specific monoclonal antibodies (MAbs) were used in indirect immunofluorescent antibody (IFA) assays to determine surface antigenic characteristics of parasites (*14*). Briefly, promastigotes from primary cultures of new isolates and controls of *L. infantum* (Li-L699), *L. major* (Lm-L777), and *L. tropica* (Lt-L590) were placed in wells of fluorescent antibody slides (Bellco Glass Inc., Vineland, NJ, USA), dried, and fixed in cold acetone. Slides were blocked with 5% fetal bovine serum in phosphate-buffered saline (PBS) for 1 hour at room temperature. Mouse MAbs specific for *L. major* (T1), *L. tropica* (T11, T14, and T15), *L. tropica/L. major* (T3), and *L. infantum/L. donovani* (D2) were applied for 1 hour at 37°C. Goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate was applied for 40 minutes at 37°C in the dark. The preparations were washed 3 times with PBS plus 5% Tween 20 between incubations. Slides were mounted in 3% DABCO (Sigma) in PBS/glycerol and viewed with an Axiovert microscope (Zeiss, Göttingen, Germany).

Experimental Infection of Sand Flies

Laboratory colonies of *P. sergenti* and *P. arabicus* were established from gravid females caught in the northern focus. The colonies were maintained at 23°C–25°C, 100% humidity, and 14:10 light:dark photoperiod. Adults had access to cotton wool soaked in 50% honey. Females were allowed to feed twice a week on mice anaesthetized with a ketamine/xylazine mixture (150 mg/kg and 15 mg/kg). Fed females were placed in plaster of paris–lined oviposition containers, and larvae were maintained on a decaying rabbit feces/rabbit chow mixture (*24*). Sand flies were infected by membrane feeding on heat-inactivated rabbit blood containing 5×10^5 promastigotes/mL. Fed females were maintained at 23°C and dissected on day 9 after feeding, when infections were mature. Guts were checked microscopically for *Leishmania* promastigotes. Infection intensity was scored as light (<50 promastigotes/gut), moderate (50–500 promastigotes/gut), and heavy (>500 promastigotes/gut). *L. tropica* strains from the northern (IARA/IL/2001/L810, Amnunfly1) and southern (MHOM/IL/2001/L-836, Tiberias) foci were used for comparing susceptibility of sand flies to local strains. Promastigotes from the same culture and sand flies from the same batch were used in individual experiments. For every combination, the experiment was repeated twice. Statistical tests were performed by using Statgraphics version 4.2 software (StatPoint, Englewood Cliffs, NJ, USA).

Glycosylation of Sand Fly Midguts

Midguts were dissected from 5- to 10-day-old *P. sergenti* and *P. arabicus* females. Midgut proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels under reducing conditions in a Mini-Protean III apparatus (Bio-Rad Laboratories, Hercules, CA, USA) at 200 V. Gels were stained with Coomassie brilliant blue R-250 or transferred to nitrocellulose membranes by using a Semiphor unit (Hoefer Scientific Instruments, San Francisco, CA, USA). Western blotting was performed for 90 minutes at 1.5 mA/cm^2. Membranes were incubated with 20 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween (TBS-Tw) with 5% bovine serum albumin for 2 hours and then with *Helix pomatia* agglutinin (HPA) biotinylated lectin, which recognizes N-acetyl-D-galactosamine (GalNAc), a typical carbohydrate in O-glycans. In the control groups, HPA reactions were competitively inhibited by preincubation with 250 mmol/L GalNAc for 30 minutes. After repeated washing in TBS-Tw, blots were incubated for 1 hour with streptavidin peroxidase in TBS-Tw. The peroxidase reaction was developed with the substrate 4-chloro-1-naphthol. All chemicals for lectin blotting were obtained from Sigma.

Random Amplified Polymorphic DNA Analysis

Twenty wild-caught sand flies morphologically identified as *P. sergenti*, 10 from the northern focus and 10 from the southern focus, were included in the analysis. Two flies from Tulek, Turkey, were included as an outgroup. DNA from thoraxes was extracted by using the High Pure PCR template preparation kit (Roche, Paris, France). Five decamer random primers (OPD5, OPE4, OPI1, OPI14, and OPI18; Operon Technologies Inc, Alameda, CA, USA) were used. The reaction mixture contained 12.5 μL master mixture (75 mmol/L Tris-HCl, pH 8.8, 20 mmol/L $(NH_4)_2SO_4$, 0.001% Tween 20, 800 μM deoxynucleotide

triphosphate mixture), 2.5 U *Taq* polymerase, 1.5 mmol/L $MgCl_2$, 2 μL primer (10 pmol), and 8 μL double-distilled water in a final volume of 25 μL. Random amplified polymorphic DNA (RAPD) reactions were performed in a PTC-200 thermocycler (MJ Research Inc., Waltham, MA, USA) and subjected to 45 amplification cycles. PCR products were separated by electrophoresis on a 2% agarose gel in Tris-acetate EDTA buffer at 80 V for 3 hours and stained with ethidium bromide.

ITS2 Sequencing

DNA samples for RAPD analysis were used for ITS2 sequencing. One specimen from each study area was included as previously described (*25*).

Results

Sand Fly Species

A total of 1,491 sand flies (7 species, 4 subgenera) from the northern focus and 876 sand flies (7 species, 4 subgenera) from the southern focus were identified. Phlebotomine fauna in the southern focus were relatively species poor with *P.* (*Paraphlebotomus*) *sergenti* comprising >90% of the flies. The most striking difference in the species composition between the foci was the absence of *P.* (*Adlerius*) *arabicus* and *P.* (*Adlerius*) *simici* from the southern focus, both of which were prominent species in the south-facing slopes of the northern focus (Table 1) (*26*).

Leishmania Infections in Sand Flies

To detect infections and obtain parasite isolates, sand fly females were dissected in sterile saline and guts were examined microscopically. Four (6.6%) of 61 *P. arabicus* and 1 (0.8%) of 125 *P. sergenti* from the northern focus had promastigotes in their guts. Infection intensity in *P. sergenti* from the northern focus was low, but all *P. arabicus* had heavy, mature infections. A total of 213 flies from the southern focus were dissected; 196 were *P. sergenti*, and 19 (9.7%) had promastigotes. Eleven of these females had heavy infections, and 8 had moderate-to-light infections. All infected females were caught at 1 sublocality in the southern focus, where the local infection rate was 19.6%. Promastigote cultures were established from 4 *P. arabicus* and 1 *P. sergenti* captured in the northern focus and from 18 *P. sergenti* females captured in the southern focus (Table 2). None of the other sand fly species were infected.

Sand flies that were not dissected fresh were kept frozen and were subjected to ITS1 PCR for detection of *Leishmania*. Nine (18%) of 50 *P. sergenti* females from the southern focus were positive for *Leishmania* ribosomal DNA. *Hae*III digestion of the ITS1 PCR products confirmed that all *P. sergenti* had *L. tropica* (Figure 2).

Identification of Infections in Mammals

Rodents collected in the northern focus were tested for *L. tropica* infection by ITS1 PCR. Dried blood and skin samples from 28 rats (*R. rattus*) and 46 spiny mice (*A. cahirinus)* were negative for *Leishmania* DNA. Eight of 73 rock hyraxes from the northern focus and 6 of 46 rock hyraxes from the southern focus were positive for *L. tropica* DNA by ITS-1 amplification and reverse-line blotting using sequence-specific probes (data not shown). Of the positive animals, 11 were adults (9 females and 2 males) and 1 was a juvenile male. Parasites from 1 rock hyrax captured in the northern focus were cultured and identified by ITS1 PCR and digestion with *Hae*III (Figure 3).

Antigenic Characterization of *Leishmania* Isolates

IFA assays with species-specific MAbs were used to characterize different isolates. *L. tropica* isolates from the northern focus were antigenically distinct from all other isolates, including those from the southern focus (Table 3).

Susceptibility of *P. arabicus* and *P. sergenti* to *L. tropica*

In laboratory experiments, *L. tropica* parasites from the northern focus infected only *P. arabicus*, and parasites from the southern focus infected both *P. arabicus* and *P. sergenti*. Susceptibility of *P. arabicus* for infection with *L. tropica* strains from both northern and southern foci was high (94% and 97%, respectively). In contrast, *P. sergenti* was not permissive for *L. tropica* strains from the northern focus (1 of 64 flies). Susceptibility of *P. sergenti* for

Table 1. *Phlebotomus* sand fly species in the Galilee foci, northern Israel*

	Northern focus		Southern focus	
Species	No. (%) females	No. (%) males	No. (%) females	No. (%) males
P. (*Adlerius*) *arabicus*	62 (15)	234 (22)	–	–
P. (*Adlerius*) *simici*	35 (9)	118 (11)	–	–
P. (*Paraphlebotomus*) *sergenti*	131 (32)	317 (29)	267 (91)	532 (92)
P. (*Laroussius*) *tobbi*	167 (40)	337 (31)	11 (4)	23 (4)
P. (*Laroussius*) *syriacus*	–	12 (1)	8 (3)	16 (2)
P. (*Laroussius*) *perfiliewi*	10 (2)	31 (3)	1 (<1)	2 (<1)
P. (*Phlebotomus*) *papatasi*	9 (2)	28 (3)	5 (2)	7 (1)
Total	414	1,077	292	580

*Species comprising <1% of the fauna (1 *P.* [*Paraphlebotomus*] *alexandri* and 3 *P.* [*Adlerius*] *halepensis*) found in the southern focus were not included.

Table 2. *Leishmania tropica* infection rates among *Phlebotomus* sand flies and rock hyraxes in the Galilee foci, northern Israel*

Focus	Sand flies, rate (%)		Rock hyraxes, rate (%)
	P. arabicus	*P. sergenti*	
Northern	4/61 (7)†	1/125 (1)†	8/73 (11)†
Southern	Species not found	19/196 (10)†	6/46 (13)‡

*Values are no. infected/no. tested (%). Sand fly infection rates were based on parasite isolation. Rock hyrax infection rates were determined by PCR and 1 isolate.
†*Leishmania* species confirmed by internal transcribed spacer region 1 PCR and restriction fragment length polymorphism (Figures 2 and 3).
‡*Leishmania* species confirmed by reverse line blot (data not shown).

infection with *L. tropica* from the southern focus strain was lower (66%) than that of *P. arabicus* (Figure 4).

Glycosylation of Luminal Midgut Proteins

Incubation of *P. sergenti* midgut lysates with HPA showed no reaction, indicating a lack of O-glycosylated proteins (Figure 5). In contrast, an abundant glycoprotein (37–43 kDa) was strongly labeled by HPA in *P. arabicus* midgut lysates. Controls of *P. arabicus* midgut lysates incubated with HPA blocked by preincubation with GalNAc showed no reaction, which confirmed the specificity of the lectin reactions in experimental blots (Figure 5). Labeling of midguts with fluorescein-conjugated HPA confirmed the presence of GalNAc-containing glycoproteins in the midguts of *P. arabicus*. Intensity of labeling in *P. sergenti* midguts was weaker, which reflected a nonspecific background reaction (Figure 5).

Comparison of *P. sergenti* Populations by RAPD and ITS2 Sequencing

Flies from both foci shared the same banding pattern and differed from Turkish *P. sergenti* (Figure 6). ITS 2 sequences of *P. sergenti* from both foci were identical with each other and nearly identical (99%) with the ITS 2 sequence of a *P. sergenti* specimen from the West Bank (GenBank accession no. AF462325) (data not shown).

Discussion

We have identified 2 emerging foci of CL in which rock hyraxes serve as reservoir hosts of the causative agent *L. tropica*. Despite their geographic proximity, the 2 foci show fundamental differences with regard to transmission cycles. Parasites and vector species in the southern focus are typical of most Asian zoonotic *L. tropica* foci, but the northern focus is characterized by antigenically distinct parasites that are transmitted by a newly incriminated sand fly vector.

L. tropica is widely distributed in eastern and northern Africa, the Middle East, and large parts of Asia. A recent study using 21 microsatellite loci showed that *L. tropica* is a genetically heterogeneous species composed of >80 genotypes. The genetic makeup of this complex suggests a probable African origin, with isolates from the northern focus more related to African isolates than to other strains from the Middle East (*16*).

The major surface molecule of *Leishmania* promastigotes is LPG, which has been shown to mediate attachment of parasites to the midgut of the sand fly (*8*). LPG of *L. tropica* from the northern focus is characterized by abundant terminal β-galactose residues on side chains. Conversely, β-galactose residues on LPG side chains of other *L. tropica* isolates are mostly capped with glucose (*27*). Differences in sugar moieties may have a role in infection of *P. sergenti* (Figure 4). Although β-galactose residues are present in *L. major* LPG, strains of *L. tropica* from the northern focus were not infective to *P. papatasi*, the natural vector of *L. major* (M. Svobodova, unpub. data) (*4*).

P. sergenti is probably a species complex, and its component populations show several molecular and morphologic differences (*25*). RAPD-PCR is a powerful tool for estimating genetic variability and was successfully used to compare genetic variation within and between 5 sympatric *Phlebotomus* species in Spain (*28*). Using the same primer sets, we did not find any differences between *P. sergenti* flies from the 2 foci (Figure 6). We deduce that populations from both foci are probably freely interbreeding.

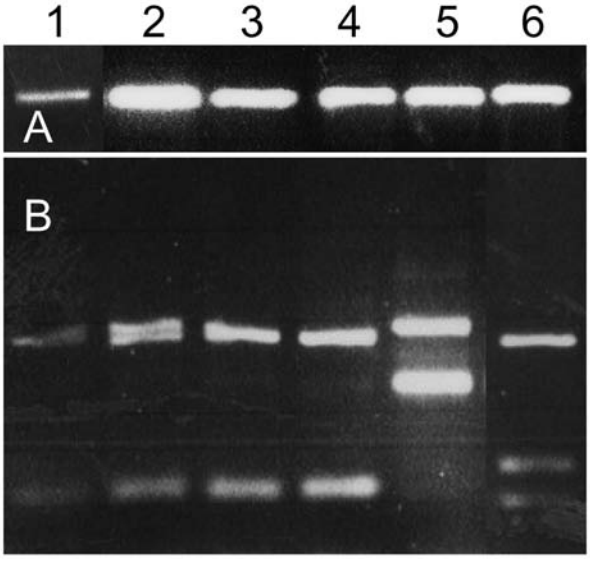

Figure 2. A) PCR of *Leishmania* internal transcribed spacer region 1 (ITS1) of naturally infected *Phlebotomus sergenti* sand flies and cultured *Leishmania* spp. controls. B) *Hae*III digestion of restriction fragment length polymorphisms of ITS1 PCR products shown in A. Lane 1, *P. sergenti* female 1; lane 2, *P. sergenti* female 2; lane 3, *P. sergenti* female 3; lane 4, *L. tropica* (Lt-L590); lane 5, *L. major* (Lm-L777); lane 6, *L. infantum* (Li-L699).

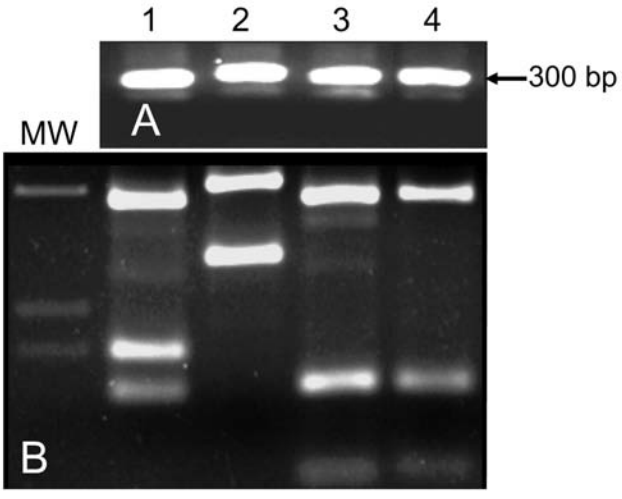

Figure 3. A) PCR of *Leishmania* internal transcribed spacer region 1 (ITS1) of cultured *Leishmania* promastigotes isolated from rock hyrax. B) *Hae*III digestion of restriction fragment length polymorphisms of ITS1 PCR products shown in A. Lane MW, molecular mass marker; lane 1, *L. infantum* (Li-L699); lane 2, *L. major* (Lm-L777); lane 3, *L. tropica* (Lt-L590); lane 4, rock hyrax.

P. sergenti is of Palaearctic origin; flies migrated into North Africa during the Miocene era (*29*). Thus, *L. tropica* and *P. sergenti* apparently originated in different continents and their geographic overlap probably arose at a later time. *P. sergenti*, *P.* (*Larroussius*) *guggisbergi*, *P.* (*Paraphlebotomus*) *saevus*, and perhaps *P. arabicus* are vectors in Africa (*30*,*31*). Since *L. tropica* variants from both foci develop in *P. arabicus*, but only the variant from the southern focus completes development in *P. sergenti*, we postulate that *L. tropica* was initially transmitted by *P. arabicus* or another permissive vector such as *P.* (*Adlerius*) *halepensis* (*9*). The more common transmission cycle is a later adaptation to *P. sergenti*, a dominant, widely distributed phlebotomine species.

Refractoriness of *P. sergenti* to variants of *L. tropica* from the northern focus is probably due to the lack of HPA-binding proteins on the luminal surface of midgut epithelium. HPA-binding epitopes are present in permissive vectors such as *P. arabicus* (Figure 4), *P. halepensis* (P. Volf, unpub. data), and *Lu. longipalpis* (*32*). These findings support infections with multiple species of *Leishmania* (*9*,*33*).

The absence of *P. arabicus* from the north-facing slopes of the southern foci contrasts dramatically with its predominance in the south-facing slopes of the northern focus. Although a satisfactory explanation for this fact is lacking, such phenomena are not unusual. For example, species richness of insects was much higher in the drier and warmer south-facing slopes of a narrow canyon (100–400 m wide) in Mount Carmel, Israel, than in the north-facing slope of the same canyon (*34*). *P. arabicus* is

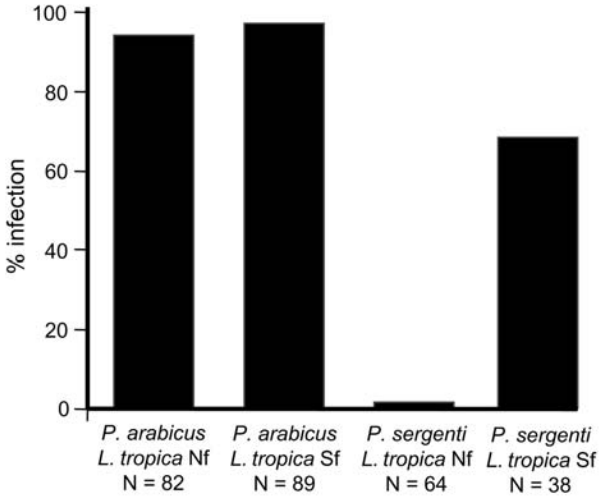

Figure 4. Artificial infection of laboratory-reared *Phlebotomus arabicus* and *P. sergenti* with *Leishmania tropica* isolates from 2 foci in Galilee, Israel. Note the high susceptibility of *P. arabicus* for both strains and refractoriness of *P. sergenti* for the northern strain. Nf, northern focus; Sf, southern focus.

Table 3. Characterization of *Leishmania tropica* isolates from the Galilee foci, northern Israel*

Focus/ source	Monoclonal antibody specificity			Excreted factor serotype
	L. major T1	*L. major*/*L. tropica* T3	*L. tropica* T11	
Northern				
Phlebotomus arabicus	5+	5+	±	A4
Girl with CL†	4+	5+	–	A4
Rock hyrax	5+	5+	±	A4
Southern				
P. sergenti	–	4+	3+	A9B2
Man with CL†	–	2+	4+	A9B2
Reference strains				
L. major	5+	5+	–	A1
L. tropica	±	3+	3+	A9

*Characterization was performed by using excreted factor serotyping (*23*) and species-specific monoclonal antibodies (*14*). CL, cutaneous leishmaniasis. Values indicate relative intensity of fluorescence under UV light. *L. tropica* isolates from the northern focus were antigenically similar to *L. major* and distinct from other *L. tropica* strains.
†Specimens were isolated by skin scraping for diagnostic purposes at the Department of Dermatology at Hadassah Hospital, Jerusalem.

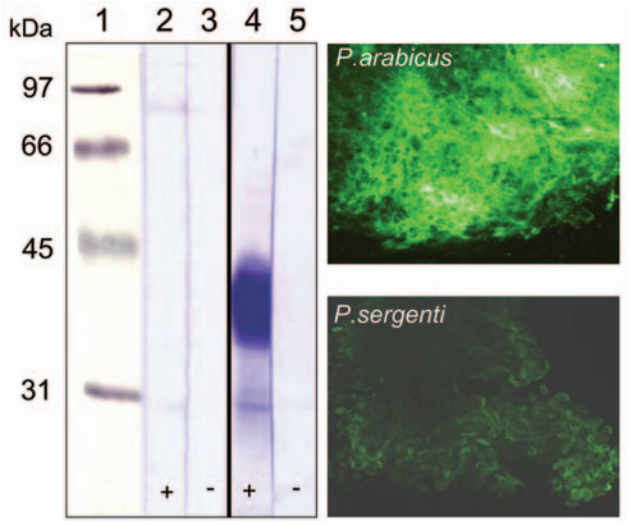

Figure 5. Left, female sand fly midgut lysates separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Blots were incubated with biotinylated *Helix pomatia* agglutinin (HPA) that detects O-glycosylated proteins. Lane 1, molecular mass markers; lanes 2 and 3, *Phlebotomus sergenti*; lanes 4 and 5, *P. arabicus*; +, preincubation of lectin with 250 mmol/L N-acetyl-D-galactosamine; -, no preincubation. Right, reactions of fluorescein-conjugated HPA with *P. arabicus* and *P. sergenti* midgut cells.

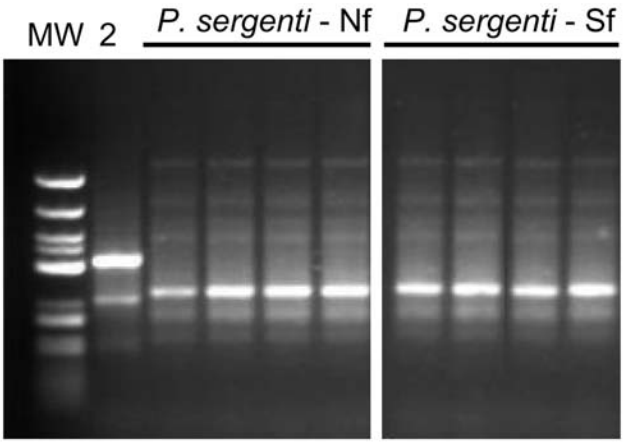

Figure 6. Random amplified polymorphic DNA PCR banding patterns of *Phlebotomus sergenti* from 2 foci in Galilee, Israel. The PCR was performed with primer OPI 1. Lane MW, molecular mass marker; lane 2, *P. sergenti* from Turkey. Shown are 4 flies from the northern focus (Nf) and 4 flies from the southern focus (Sf).

widely distributed in Africa and the Arabian peninsula (*17*), and the Galilee focus forms the northern limit of its distribution. Since *P. arabicus* originates in warmer regions, finding it in warmer, drier, south-facing slopes and not in cooler, shadier north-facing slopes of the hills in Galilee is not surprising (Table 1).

Rock hyraxes in both foci were found infected with *L. tropica*, and 1 isolate was obtained from an adult male in the northern focus. Although rock hyraxes were suspected reservoir hosts of *L. tropica* in Africa (*35*,*36*) and have been previously implicated in the northern focus (*4*), this is the first report of a rock hyrax isolate that was identified as *L. tropica* and shown to be identical to those obtained from humans and sand flies in the same focus (Table 3).

Rock hyrax populations in many parts of Israel are expanding rapidly and encroaching upon human habitation. They were extremely common in both foci studied, as well as in other *L. tropica* foci in the region (D. Meir and A. Warburg, unpub. data; [*4*,*5*]). In the Galilee foci, rock hyraxes inhabit crevices within boulder mounds that were created when land was cleared for the construction of houses. These artificial caves also afford suitable breeding sites for sand flies. Rock hyraxes are susceptible to *L. tropica*, and infected rock hyraxes are infective to feeding *P. arabicus* and *P. sergenti*. Sand flies are attracted to rock hyraxes and prefer feeding on their snouts (Figure 7) (*37*). This behavior makes them suitable as vectors because *L. tropica* is usually found in the skin above the nose (R.W. Ashford, unpub. data). Furthermore, as gregarious diurnal mammals, sleeping rock hyraxes are a readily available blood source for night-questing phlebotomine females. Lastly, rock hyraxes live for 9–10 years in the wild (*38*) and constitute an efficient parasite reservoir for infecting sand flies that emerge in the spring after their winter diapause (*39*). These facts indicate that that burgeoning, peridomestic rock hyrax populations are the primary cause of the emergence of CL caused by *L. tropica* in the region studied (*39*,*40*).

Figure 7. Rock hyrax (*Procavia capensis*). Sand flies are attracted to these animals and prefer feeding on their snouts.

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

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An intraspecific study of *Phlebotomus sergenti* was performed on populations from Turkey, Syria, Israel and Usbekistan 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 sout-easern Anatolia, which suggests a gene flow between populations separated spatially by Amanos mountains, a mountain range of a considerable heigth. The results of ITS2 rDNA sequencing complied with the previously published intraspecific division of *P. sergenti* into two branches, north-eastern and south-western. However, mtDNA haplotypes formed three lineages with specimens from Turkey and Israel sharing a common clade. Previously postulated hypothesis about a complex of sibling species within *P. sergenti* is therefore questionable. Cytochrome B seems to be more discriminative marker for intraspecific variability assesment.

Key words: *Phlebotomus sergenti*, RAPD, intraspecific variability, sibling species, geometric morphometrics, mtDNA

INTRODUCTION

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 Southern and Northern Mediterranean, Middle East, Arabia, Afghanistan, Pakistan, and northern parts of India, being wider than distribution of *L. tropica* (Depaquit et al., 2002). Therefore, a question arose about populations´ degree of intraspecific variability and vectorial capacity of different *P. sergenti* populations. Internal transcribed spacer 2 (ITS2) of twelve populations of *Phlebotomus sergenti* from 10 different countries was sequenced and two principal branches were identified: one related to the north-eastern Mediterranean area

(Cyprus, Pakistan, Syria and Turkey), while the other to South and West (Egypt, Morocco, Israel). Such a division correlates with postulated migration routes of *P. sergenti* along Thetys Sea at the Miocene time (Depaquit et al., 2002). If sibling species within *P. sergenti* were proven it may have important consequences in 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 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 focuses on comparison of specimens from the wild. 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 into the analysis. RAPD analysis was followed by a geometric morphometric study. On a larger scale, specimens from Turkey, Israel, Syria and Usbekistan were compared. Three molecular methods were used for the comparison: RAPD, ITS2 sequencing (nuclear marker), and cytochrome B sequencing (mitochondrial marker).

MATERIAL AND METHODS

Sand flies

Phlebotomus sergenti were captured using CDC miniature light traps at different localities in Turkey, Israel, Syria and Usbekistan (Table 1). After 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: one originating from Sanli Urfa, Turkey, 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

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

RAPD analysis

Out of 60 decamer random primers previously tested (Operon Technologies Inc., USA), seven were used for the RAPD analysis: OPE4, OPE 11, OPE15, OPE16, OPI1, OPI14, 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 tranformed 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) (Sneath and Sokal, 1973) which identifies topological relationships on the base 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 construction of trees.

Sequencing

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. The obtained sequences have been deposited in GenBank Database.

Phylogenetic analyses

Obtained DNA sequence data were compared with those in the GenBank database using 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 (Thompson et al., 1997) and the resulting alignment was manually edited using BioEdit (Hall, 1999). The alignment is available from the corresponding author upon request. The phylogenetic trees were constructed using maximum parsimony, maximum likelihood (PhyML; Guindon and Gascuel, 2003), and Bayesian methods. The maximum parsimony, and

maximum likelihood trees were constructed in PAUP* 4.0b10 (Swofford, 2002) 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 (Posada and Crandall, 1998). The trees were bootstrapped with 1000 (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 (Ronquist and Huelsenbeck, 2003). 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 millions and the trees were sampled every 100^{th} 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 south-eastern 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 4 semilandmarks were digitized by TPSDIG2.05 (Rohlf, 2005a) 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 GLS Procrustes superimposition method (Zollikofer and Marcia, 2005) by 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 nonparametric Kruskal-Wallis test (Zelditch et al., 2004). 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 in 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 1200 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, second

containing all field samples from Israel plus the Israeli colony specimen (Fig. 1). A similar grouping pattern was also obtained by the neighbor-joining method (data not shown).

The data obtained from the geometric morphometrics show 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 (Fig. 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 close relationship between some specimens (MANOVA Axis 1: Lambda = 0.0000, chisq = 265.9410, df = 70, $p < 0.001$; Axis 2: Lambda = 0.0000, chisq = 182.7844, df = 54, $p < 0.001$; Axis 3: Lambda = 0.0001, chisq = 114.9432, df = 40, $p < 0.001$; Axis 4: Lambda = 0.0131, chisq = 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, also the males have clearly smaller wings in all populations (Kruskall-Wallis Test: H = 18,47; df = 7; $p < 0.01$).

Same seven RAPD primers were used to analyse eleven field samples from localities in following countries: Turkey, Israel, Syria, and Usbekistan (for details see Tab. 1). Similar pattern of clades was obtained; specimens from each country formed a unique clade. Specimens from Usbekistan, originating from one locality, formed the most homogeneous clade with almost identical band patterns (Fig. 3). Three remaining clades, which contained specimens from different localities, exhibited a certain degree of variability.

ITS2 sequencing

The size of amplified fragment 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 exluded 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* (**AF462333**) and *P. jacusieli* (**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 laboratory colony originating from the same locality), three specimens from Syria (two specimens from Aleppo and one specimen from Raqah) and two specimens from Usbekistan (locality Chodak). Sequences were identical for specimens sampled from the same locality. According to phyML analysis (Fig. 4) the clustering is generally in agreement with the previous findings, which postulated two clades: north-eastern and south-western. The specimens from Usbekistan 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; however, 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, i.e. occured only in one specimen. The alignment is available upon request to the corresponding author. In total, 22 specimens from Turkey, Israel, Syria and Usbekistan were sequenced. For the tree construction, cytochrome B gene sequences of *Phlebotomus ariasi* (**AF161195**) and *P. tobbi* (**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 (Fig. 5). Three specimens from Usbekistan 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 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 haplotype. The variability of Turkish haplotypes can be atributed mainly to A/G substitution at position 84 and C/T substitution at position 168. Apart from these two polymorphic sites, the haplotypes within the clade differed in only single substitutions.

DISCUSSION

Study of *Phlebotomus sergenti* populations from different areas of distribution demonstrated the informational potential of both molecular and morphometric methods deployed to asses 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 Amanos mountain range. This range runs roughly parallel to the Gulf of Iskenderun and divides the coastal region of Cilicia from inland provinces of Turkey and Syria. Reaching a maximum height of 2240 m, it is open by several passes which descent to an altitude of approximately 700 meters 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 obtained results of RAPD analysis we may conclude that Amanos mountains do not represent a sufficient barrier for sand fly dispersion 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 Usbekistan were also included into RAPD analysis. While Syria has an intermediate geographical position between Turkey and Israel, specimens from Usbekistan represent a very distant population. Again, the samples from each country formed a distinct clade.

RAPD marker had been proposed to distinguish *P. papatasi* and *P. duboscqi*, two closely related, morphologically similar vectors of cutaneous leishmaniasis (Mudhopadhyay et al., 2000). In the respect of results of presented 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 Depaquite 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 intraspecific level, it revealed a notable variability of *P. sergenti* (Depaquit et al., 2002), while there was a significant homogenity 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, north-eastern and south-western.

Mitochondrial genes are also very popular and useful molecular markers as the lack of recombination allows to reflect the evolutionary history even more accuratelly (Avise, 1994). Mitochondrial cytochrome B (cyt B) genes were succesfully 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., 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 Usbekistan formed three lineages. All specimens from Usbekistan clustered in one clade, two being identical; this is not surprising as they all originate from one locality. Syrian specimen 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 atributed 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 concept of *P. sergenti* species complex postulated by Depaquit et al. (2002). This result of cytochrome b sequences analysis is further supported by recent finding, 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).

Combination of several approaches and the use of different markers give 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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Table 1: Origin of specimens

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 Usbekistan with bootstrap values.

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

Figure 5: PhyML tree inffered from cytochrome B sequences of specimens from Turkey, Syria, Israel and Usbekistan with bootstrap values.

6. Table(s)

Country	Province	Locality	Latitude	Longitude	Method of capture
Turkey	Adana	Zerdali	37°24´15´´ N	35°37´50´´ E	CDC trap
		Guneri	37°26´44´´ N	35°46´51´´ E	CDC trap
	Iskenderun	Karagac	36°34´01´´ N	36°08´46´´ E	CDC trap
		Konacik	36°12´15´´ N	35°15´36´´ E	CDC trap
		Tulek	36°21´49´´ N	35°51´15´´ E	CDC trap
	Nigde	Nigde	38° 35'59"N	35°44'56"E	CDC trap
	Antakya	Delicay	36°35´18´´ N	36°27´20´´ E	CDC trap
	Osmaniye	Imamoglu	37°56´16´´ N	36°64´07´´ E	CDC trap
	Kahramanmaras	Nurhak	37°96´57´´ N	37°43´95´´ E	CDC trap
		Yesilova	37°44´93´´ N	36°32´49´´ E	CDC trap
	Sanli Urfa	Sanli Urfa	37°09´04´´ N	38°47´34´´ E	colony
Israel		Amnun	32°54´ N	35°34´ E	CDC trap, colony
		Tiberias	32°47´ N	35°31´ E	CDC trap
		Kfar Adumin	31°49´ N	35°20´ E	CDC trap
Syria		Aleppo	36°12´10´´ N	36°09´31´´ E	manual aspirator
		Raqah	35°57´00´´ N	39°01´00´´ E	manual aspirator
Usbekistan		Chodak	40°56´ N	70°46´ E	manual aspirator

5. Figure(s)

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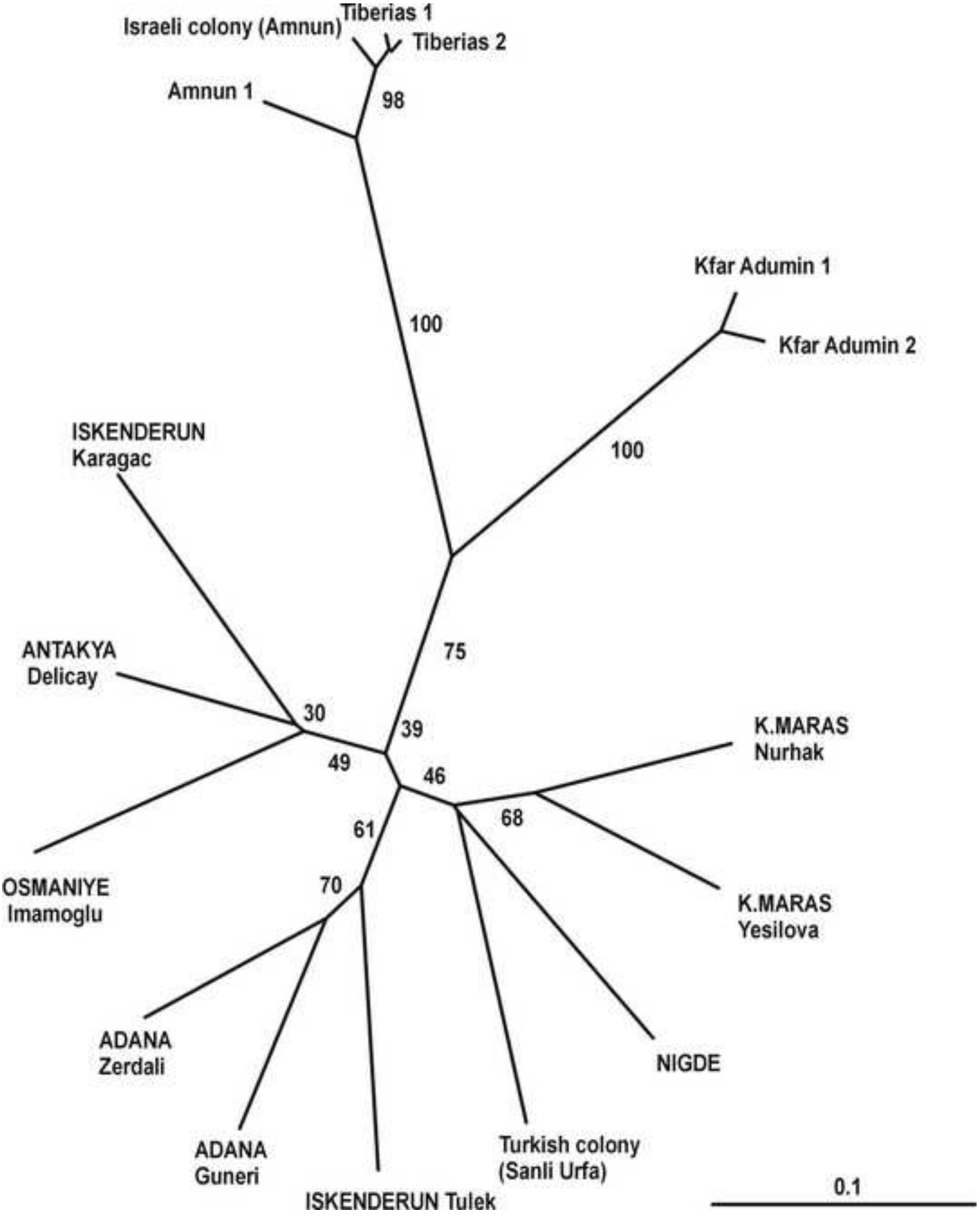

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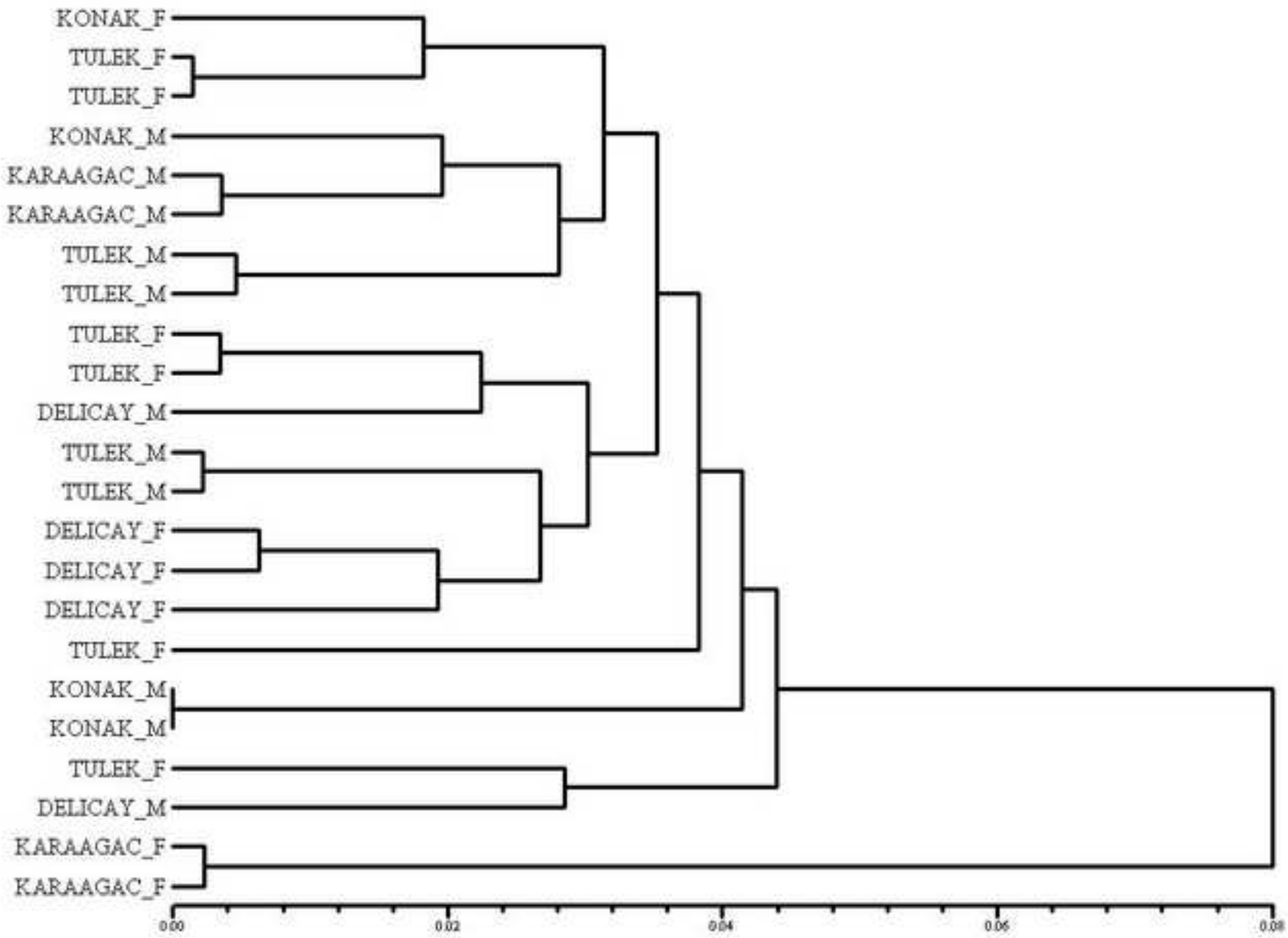

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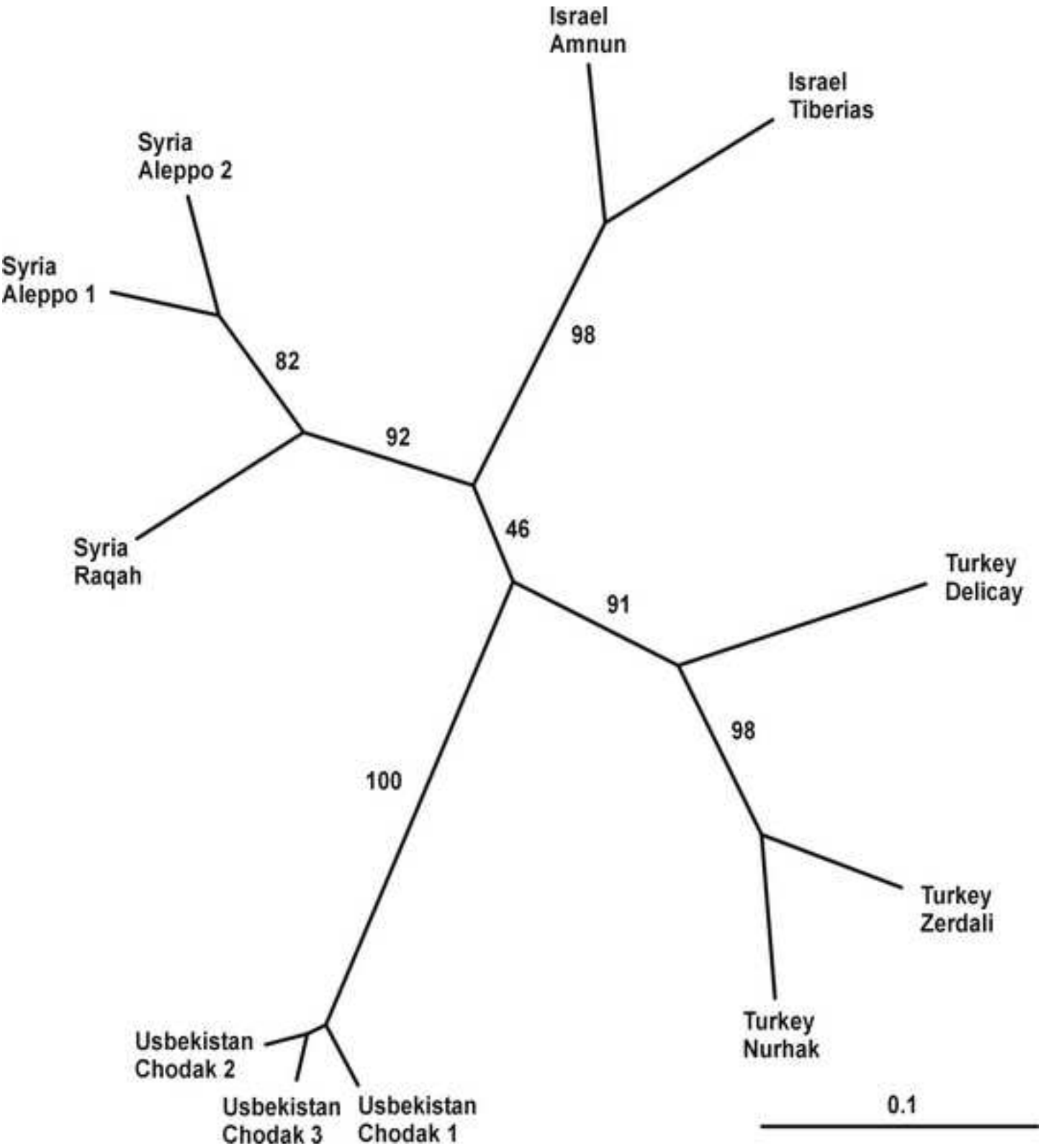

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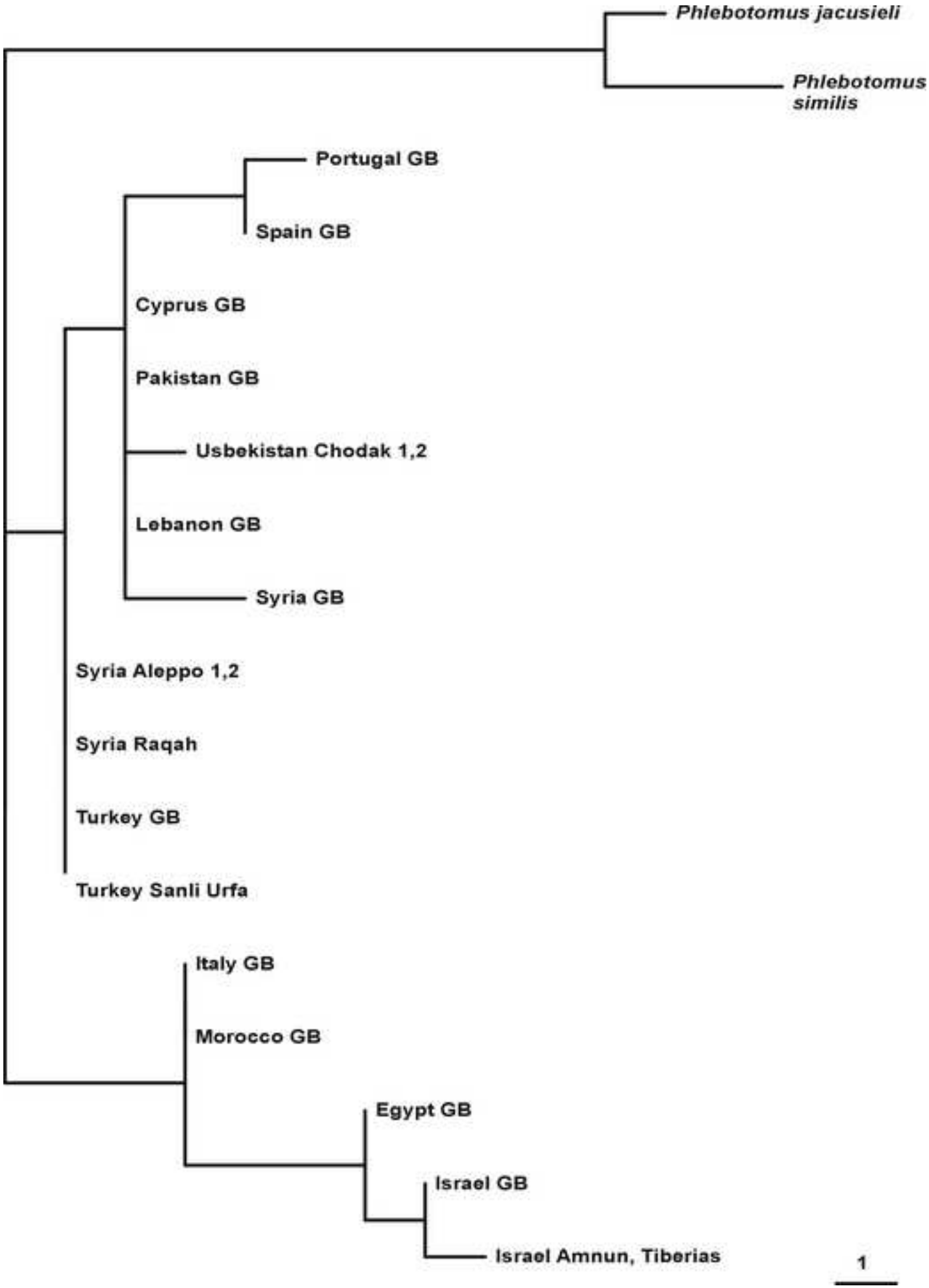

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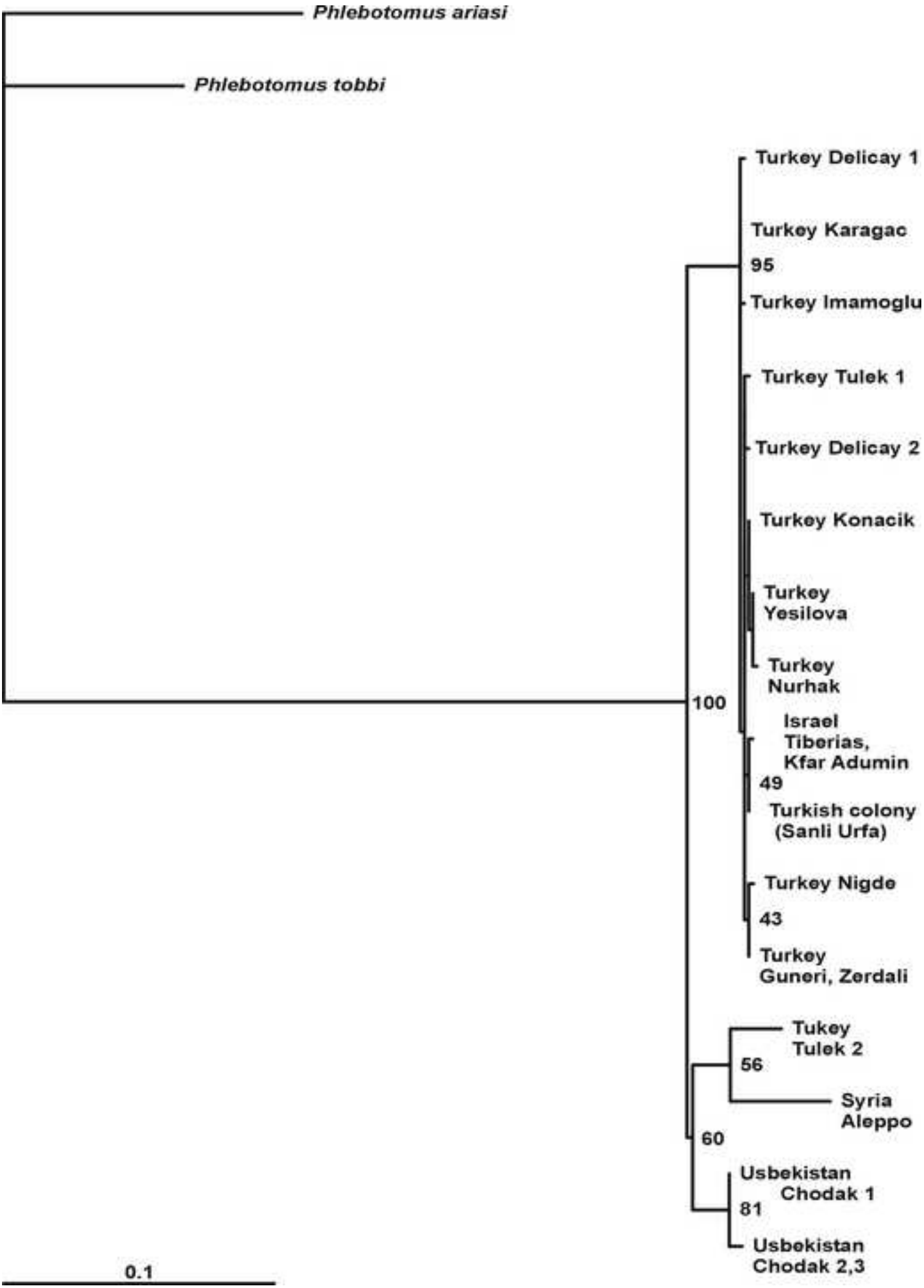

Cutaneous leishmaniasis caused by *Leishmania infantum* transmitted by *Phlebotomus tobbi*

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Nucleotide sequence data reported in this paper are available in DDBJ/EMBL/GenBank databases under Accession Nos. EU545236 – EU545255.

Abstract

Transmission of cutaneous leishmaniasis (CL) caused by *Leishmania infantum* was studied in South Anatolia, Turkey. Small, non-ulcerating lesions prevailed and patients were negative in rK39 tests for antibody detection for human visceral leishmaniasis (VL). The most abundant sand fly species, *Phlebotomus tobbi*, was found positive for *Leishmania* promastigotes with a prevalence of 1.4% (13 out of 898 dissected females). The isolated strains were identical with those obtained from patients with CL and were typed as *L. infantum*. Phylogenetic analysis revealed similarity to MON-188 and a clear difference from the MON-1 clade. Bloodmeal identification showed that *P. tobbi* feeds preferentially on cattle and humans. This finding, the high number of CL patients and relative scarcity of dogs in the focus, suggests that the transmission cycle could be anthroponotic.

Keywords: Cutaneous leishmaniasis; Sand fly; Transmission; Epidemiology; Anthroponosis; *Leishmania infantum*; *Phlebotomus tobbi*

1. Introduction

Leishmaniases are diseases with a wide spectrum of clinical forms, from relatively mild cutaneous lesions to life-threatening visceral diseases. Their causative agents, protozoans of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), are transmitted by phlebotomine sand flies (Diptera: Psychodidae).

In Turkey, visceral leishmaniasis (VL) is caused by *Leishmania infantum* and affects mainly children (Ozcel et al., 1999; Ok et al., 2002; Tamir et al., 2006). Cutaneous leishmaniasis (CL) is endemic in several regions of Turkey. It has been attributed almost exclusively to *Leishmania tropica* although sporadic occurrence of *Leishmania major* has been also reported (Akman et al., 2000). The biggest outbreak with over 11,000 reported cases occurred recently in Sanliurfa (Ok et al., 2002) and the parasite has been typed as *L. tropica* (Gramiccia et al., 1991; Waki et al., 2007). However, several other foci exist including Cukurova, a part of the Mediterranean region with Adana being the capital. Since 1985, thousands of new CL cases have emerged there and it has been suggested that the causative agent is *L. tropica* (Ok et al., 2002; Uzun et al., 1999). In parallel, vectors of *Leishmania* have been investigated in CL foci in Turkey (Volf et al., 2000; Simsek et al., 2007) but infected sand flies were never found.

We performed a study on humans and sand flies in the CL focus in Cukurova region where, according to the local health centers, hundreds of human cases continue to occur every year. Surprisingly, the causative agent of human CL was identified as *L. infantum*. We also found 13 *Phlebotomus tobbi* infected with *L. infantum*, and demonstrated that human isolates of *L. infantum* from the study area are identical to isolates from *P. tobbi*.

2. Materials and Methods

2.1. Study area

The study was performed in two adjacent areas in the North part of Cukurova region, south Anatolia, during 2005–2007. South Anatolia is bound by the West Taurus Mountains, the mountain ranges of Taurus and Anti-Taurus to the North, and the Amanos Mountains to the East (Fig. 1). The Western part of the focus comprises five villages approximately 55 km northeast of Adana city with an altitude of 150–280 m above sea level: Tepecikören (37°21’46”N, 35°37’40”E), Zerdali, Camili, Aydın and Otluk. The local centre of the Eastern part of the focus is Boyali (37°17’56”N, 36°21’33”E) situated 30 km northeast of Osmaniye city and surrounded by several other villages (Alibozlu, Bayandirli, Cercioglu, Oluklu, Pirsuntanli, Karakuyu). The altitudes range from 205–320 m above sea level. Most of the area in both parts of the focus is fertile (“mollisol” soil) and used for

agricultural activities, but young *Pinus* (pine-tree) and *Abies* (fir-tree) forests are also cultivated. Citrus orchards and cotton fields are more common in the Eastern part. The mean annual precipitation is 636.8 mm (West) and 761.3 mm (East) with 66% relative humidity and the mean annual temperature is 18.7°C (West) and 19.6°C (East). Residents live in single-family houses built from briquette, adobe, stone and cement, surrounded by gardens with henhouses and sheep or cattle sheds. Treatment of patients with CL is provided in regional health centers (Kozan in the West and Duzici in the East) by multiple weekly injections of Glucantime (meglumine antimoniate) for at least 2 months.

2.2. Sand fly collection, dissection and identification

Sand flies were collected in September 2005, 2006 and 2007, mostly using Centers for Disease Control (CDC) light traps (John W. Hock, USA) placed inside the houses, animal shelters and in house yards. A small proportion of sand flies inside the houses and shelters was collected by hand aspirators. Dead females and all males were stored in 70% ethanol for morphological identification. In males of *Phlebotomus* cf. *syriacus*, the thorax was used for DNA extraction and molecular identification based on sequencing of internal transcribed spacer 2 (ITS2), 18S rRNA, and cytochrome *b* (*cyt b*) genes (see below).

Live sand fly females were immobilized on ice, rinsed briefly in 96% ethanol, washed and dissected in 0.9% sterile saline. The head and genitalia were used for identification and the gut was examined microscopically for the presence of promastigotes. In blood-fed females the gut with the rest of the blood meal was smeared on a piece of filter paper (Whatman 3; Whatman, Brentford, U.K.), allowed to dry and than stored at –20°C until use.

Upon microscopical detection of *Leishmania* infection, the material from the slide was divided into two parts. The first was inoculated into glass vials (2.5 ml) containing SNB-9 blood agar made from defibrinated rabbit blood and overlayed with 1:1 RPMI 1640/Schneider insect medium, supplemented with 10% FCS (Sigma or Gibco), 2% sterile human urine, 10,000 IU penicillin (Biotika), 100 µg/ml amikacin (Bristol-Myers Squibb), and 1,500 µg/ml 5 fluorocytosine (Sigma). The second was soaked on a piece of filter paper (Whatman 3) for DNA extraction and *Leishmania* identification as a backup in case of unsuccessful cultivation.

2.3. Sampling from patients

Patient sampling was performed in cooperation with local medical doctors. Tissue samples were obtained from lesions by insulin syringe aspiration and immediately inoculated into 2.5 ml glass vials with SNB-9 (as above). In addition, blood plasma was taken from 26 patients with well developed lesions to detect the presence of antibodies against causative agents of VL by DiaMed-IT

LEISH (DiaMed, Switzerland) immuno-chromatographic dipstick test using the recombinant antigen K39. Two sera of visceral leishmaniasis-positive patients kindly provided by J.-C. Dujardin were used as positive controls.

2.4. DNA extraction

Extraction of total DNA from the material preserved in ethanol (sand fly thorax), filter papers (midguts of blood-fed or infected females), and from successfully established cultures (exponential phase of growth) was performed using a DNA extraction kit (Roche, France) according to the manufacturer's instruction. For the analysis of sand fly blood meals, the total DNA from the filter papers was extracted using standard proteinase k digestion (20 mg/ml; 55°C overnight). The lysate was extracted with Tris-buffered phenol (pH 8), followed by extraction with phenol–chloroform. Nucleic acid from the aqueous phase was precipitated with ethanol and resuspended in 100 µl of redistilled water.

2.5. Leishmania typing

PCR-Restriction Fragment Length Polymorphisms (PCR-RFLP) was performed with LITSR and L5.8S primers (El Tai et al., 2000) to obtain the ITS1 region. The PCR reactions were performed in 50 µl total volumes of reaction mix (Combi PPP Master Mix, Top-Bio, Czech Republic) using the following conditions: initial denaturation at 94°C for 2 min followed by 32 amplification cycles (94°C for 20 s, 53°C for 30 s, 72°C for 1 min); and 72°C for 6 min. The PCR products were restricted by *Hae*III enzyme for 1 h in 60°C and visualized by electrophoresis with 2% Metaphor agarose gel (Schönian et al., 2003). The following reference strains were used: *L. infantum* (MCAN/IL/1999/LRC-L760, MHOM/TR/2000/OG-VL, MHOM/TR/2003/Adana(dot), *L. tropica* (MHOM/SU/1974/K27) and *L. major* (MHOM/IL/1967/LRC-L137 Jericho II).

PCR-based multilocus sequence typing (MLST) was used to distinguish between *L. infantum* and *L. donovani* and for more accurate identification of *Leishmania* strains (Zemanová et al., 2007). We focused on sequence diversity of five enzymes: isocitrate dehydrogenase (*icd*), cytosolic malic enzyme (*me*), mannose phosphate isomerase (*mpi*), glucose-6-phosphate dehydrogenase (*gpi*) and fumarate hydratase (*fh*). Our isolate from a patient from the Western part of the focus (MHOM/TR/2005/CUK1) was compared with one sand fly isolate from the Western part (ITOB/TR/2005/CUK2) and one isolate from the Eastern part (ITOB/TR/2007/CUK10). In addition, a viscerotropic isolate MHOM/TR/2000/OG-VL originating from Hatay, South Anatolia, was sequenced. PCR products of expected size were purified from the gel using QIAquick gel extraction kits (Qiagen, Germany) according to the manufacturer's instructions and sequenced directly on an automated DNA sequencer (310 Genetic Analyzer; ABI Prism) using the BigDye 3.1

kit (Applied Biosystems, USA). The obtained sequences were deposited in GenBank under the following Accession Nos.: **EU545236 – EU545255**. Partial sequences were assembled manually using Seq-Man (DNAStar). Maximum likelihood (ML), maximum parsimony (MP) and neighbor-joining (NJ) were calculated by PhyML.win32 (Guindon and Gascuel) and PAUP* 4.0b10 (Swofford) phylogenetic software.

2.6. Blood-meal identification

In 2005 and 2006, blood meals were identified using the modified vertebrate-universal specific primers cytB1-F (5′-CCA TCC AAC ATY TCA DCA TGA TGA AA-3′) and cytB2-R (5′-GCH CCT CAG AAT GAT ATT TGK CCT CA-3′) (Kocher et al., 1983; Malmqist et al., 2004) to amplify a 305 bp segment of the *cyt b* gene from host mtDNA. PCR was performed in a 25 µl vol. using a reaction mix. PCR products were checked on a 1.5% agarose gel; amplified fragments were isolated and sequenced as described above. Sequence analyses were performed using DNAStar software; sequences were compared with sequences deposited in the GenBank database using standard nucleotide BLAST searches.

2.7. Identification of P. syriacus

The identification of captured specimens of *P. syriacus* was based on sequencing of a 450 bp variable region of 18S rRNA and complete sequences of ITS2 and *cyt b*. A variable region of the 18S rRNA gene (Forward primer 5′-GTGCGGGTTTTGCATTACTT-3′, Reverse primer 5′-AACATCCTTGGCAAATGTT-3′), ITS2 (Depaquit et al., 2002) and *cyt b* (Forward primer 5′TATGTACTACCATGAGGACAAATATC -3′, Reverse primer 5′-GCTATTACTCCYCCTAACTTRTT-3′, modified from (Esseghir and Ready, 2000)) were amplified from 10 randomly selected specimens of *P.* cf. *syriacus*. Six specimens from Israel (*P. syriacus*) and four from Italy (*Phlebotomus neglectus*) were used for comparison. PCR was performed in a 50 µl vol. using a reaction mix with the following cycling profile for SSU: initial denaturation at 94°C for 2 min followed by 25 amplification cycles (94°C for 30 s, 50°C for 30 s, 72°C for 1 min). PCR product was sequenced as described above. Obtained sequences were aligned and compared by ML and MP methods calculated by PhyML and PAUP with known sequences of *P. neglectus* (GenBank accession numbers **AJ244367-74**, **AF161188-91**, and **AF205524**) and *P. syriacus* (GenBank accession numbers **AJ244375-6**) and with sequences obtained from control specimens originating from Israel (*P. syriacus*) and Italy (*P. neglectus*).

3. Results

3.1. Phlebotomine fauna

In total, seven sand fly species were identified (five in the Western part and four in the Eastern part of the focus), in both parts the most abundant being *P. tobbi* (Table 1). Interestingly, *Phleboomus papatasi* and *Phlebotomus perfiliewi* were found only in the Western part while *Phlebotomus simici* was present only in the Eastern part of the focus. For *P.* cf. *syriacus* the morphological examination and measurements of male genitalia did not result in identification as the specimens were of intermediate phenotype between *P. syriacus* and *P. neglectus* (length of coxite: average 0.37, continuous range 0.32–0.43 mm; length of style: average 0.17, continuous range 0.15–0.21 mm). Phylogenetic and statistical analysis of 18S rRNA, ITS2 and *cyt b* genes revealed that all studied samples from Cukurova were significantly closer to *P. syriacus* (genetic p-distance means were 0.00, 0.01 and 1.15, respectively) than to *P. neglectus* (p-distance means were 0.01, 5.45 and 3.55, respectively).

3.2. Leishmania-infected flies

Out of 1,130 females dissected, 13 (1.1%) had promastigotes in their gut (two out of 551, six out of 228, and five out of 351 dissected females in 2005–2007, respectively). Nine (one in 2005, five in 2006, and three in 2007) of these infections were mature with parasites localized on the stomodeal valve; however in one case the midgut contained fresh blood from the subsequent blood meal. In four (one in 2005 and 2006, and two in 2007) blood-fed females *Leishmania* parasites were found within the blood-meal. Isolates were obtained from 11 females, two from the Western (in 2005) and nine from the Eastern (five in 2006 and four in 2007) part of the focus. All infected females belonged to *P. tobbi*. *Leishmania* prevalence in *P. tobbi* was 0.7%, 2.8%, and 1.9% in 2005–2007, respectively.

3.3. Human isolates

We obtained inocula from 128 people with putative CL (93 and 35 from Western and Eastern parts, respectively). In six cultures, promastigotes appeared in subsequent microscopical controls; however, only one isolate resulted in a thriving culture. In total, 26 patients with well-developed lesions were tested by dipstick test; however no positive result was obtained. Lesions occurring in patients within the study area are presented on Fig. 2. In most cases, lesions were non-ulcerating and relatively small (1–2 cm in diameter). Questioning the patients revealed that lesions lasted for at least 2 years and approximately one-third of patients had more than one lesion. In a few cases, ulceration or spontaneous healing was observed (Fig. 2).

3.4. Parasite typing

PCR-RFLP of ITS1 was successfully used for typing of a human isolate, 11 isolates from sand flies, and two DNA samples from promastigote-positive sand fly females. All samples showed the same pattern on electrophoresis and belonged to the *L. donovani* /*L. infantum* group (Fig. 3). The MLST assay determined parasites as *L. infantum* and sequences of five analyzed genes of all three strains (CUK1, CUK2 and CUK10) were identical. Comparison with other *L. infantum* strains typed by Zemanová et al., (2007) and Turkish isolate MHOM/TR/2000/OG-VL typed by us revealed highest similarity of CUK's strains to *L. infantum* strain MHOM/IT/93/ISS800 (MON 188) (Fig. 4).

3.5. Blood-meal identification

In 2005 and 2006, out of 779 dissected females of five sand fly species, 267 individuals (34%) were engorged with blood. Sequencing of the amplified part of the *cyt b* gene was found to be a very sensitive method with an efficiency level of almost 85% and the source of the blood meal was determined in 220 specimens. Five mammalian and one avian species were identified as hosts: cattle, human, goat, mouse (*Mus* sp.), vole (*Microtus* sp.), and chicken. The identity of blood-meals of four sand fly species is presented in Table 2. In four females of *P. tobbi,* mixed blood-meals of human-cattle (three specimens) and vole-cattle (one specimen) were observed. The major blood sources for *P. tobbi* were cattle (70%) and humans (10%). Furthermore, in four *Leishmania*-infected females of *P. tobbi* (one with fresh and three with extensively digested blood) human blood was detected.

4. Discussion

The etiological agent of CL in the Cukurova region was identified as *L. infantum*. Although we have attempted to isolate *Leishmania* from several dozens of patients, only in six samples were promastigotes observed in cultivation medium and one isolate has been established in vitro. *Leishmania infantum* is notoriously difficult to cultivate from cutaneous lesions (Gramiccia et al., 1991). This parasite species has been incriminated for the first time as a causative agent of human CL in France (Rioux et al., 1980). Since then, several other countries in the Mediterranean were added to the list (del Giudice et al., 1998; Serin et al., 2005). Long-term research carried out in the Abruzzi region of Italy showed an intensive CL focus, with thousands of recorded cases caused by dermotropic *L. infantum* (Corradetti, 1952; Gramiccia et al., 1987; Maroli et al., 1987). Several studies from different areas evaluating the rK39 dipstick test confirmed high sensitivity and

specificity of this method for the visceral form of leishmaniosis (Chappuis et al., 2006). Our data obtained by DiaMed-IT LEISH dipstick test clearly demonstrated the non-visceral form of leishmaniasis in tested patients from both parts of the study area.

Molecular methods revealed that isolates from humans and sand flies were identical. Moreover, identical parasites were isolated from *P. tobbi* females in the Eastern part and Western parts of the study area. Our findings suggest that both parts of study area, despite being up to 65 km apart, represent one focus with the same circulating parasite. Phylogenetic analysis based on MLST showed that this *L. infantum* strain is unambiguously distinct from the MON-1 zymodeme as well as from a viscerotropic isolate of *L. infantum* from South-East Turkey. Among accessible isolates typed using the MLST method, our strain is close to MON188, the *L. infantum* strain isolated by Gramiccia (Pratlong et al., 2003) in Sicily from an HIV-positive patient with visceral leishmaniasis.

Phlebotomus (Larroussius) tobbi was clearly shown to be a vector of CL in the Cukurova focus. Prevalence of infected females was almost 1.5%; out of 898 dissected *P. tobbi* females 13 were positive for *L. infantum* promastigotes. The relatively high *L. infantum* prevalence in *P. tobbi* coincides with widespread human infection throughout the region.

We have found quantitative and qualitative differences in the phlebotomine fauna in both studied sites of the current focus. Although the sites are less than 65 km apart, *P. papatasi* and *P. perfiliewi* are absent in the Eastern part of the focus while *P. simici* is exclusively present here. General climatic conditions seem to be similar in both parts of the focus; however, local microclimatic differences probably occur. Differences in sand fly fauna composition in close *Leishmania* foci of CL including involvement of different vectors are known in other Mediterranean regions (Svobodová et al., 2006).

Despite the differences in phlebotomine fauna composition, *P. tobbi* is the most dominant species in both parts of the focus. Being a member of the *Larrousius* sub-genus, *P. tobbi* is a suspected vector of *L. infantum*. The only previous isolation of *L. infantum* from this species has been reported from Cyprus. However, human *L. infantum* is rare in Cyprus; only visceral cases are reported and the infected sand fly specimen was caught near an infected dog (Léger et al., 2000).

Blood meal analysis assigned the close relationship of local *P. tobbi* populations with large ruminants, mainly with cattle. However, human blood was the second major blood source and 10% of females had fed on humans. In addition, human blood was detected in four females infected by *L. infantum*. We speculate that the population of *P. tobbi* in Çukurova region is more anthropophilic than populations from other areas. In Azerbaijan and Cyprus, *P. tobbi* were not attracted to humans (Gasanzade et al., 1990; Léger et al., 2000) and in Albania none of the blood-engorged *P. tobbi* females had fed on humans (Velo et al., 2005). The anthropophily of *P. tobbi* populations in the Cukurova region might be caused by local housing conditions, such as close cohabitation of humans

with domestic animals and sleeping outdoors. Leishmaniasis caused by *L. infantum* is usually a zoonosis with dogs being reservoir hosts. Surprisingly, within our study we didn't find any dog blood in engorged sand fly females, which corresponds with low numbers of dogs in both sites. Relatively high prevalence of CL in local people allows us to speculate about another transmission cycle and an anthroponotic cycle cannot be excluded.

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Fig. 1. Map of western and eastern parts of the study area.

Fig. 2. Lesions occurring in patients in both parts of the study area: A) Active protuberant lesions with ulceration on left shin of 16 year old girl; B) flat lesion about 2 years old on left shin of 43 year old woman; C) atypical large lesion on right check and temple of 14 year old girl; D-E) lesions from patients culture-positive for *Leishmania* promastigotes on left thigh of 11 year old boy and on forehead of 5 year old boy; F) typical lesions without ulceration on left calf of 54 year old woman; G-H) healing of expanded lesion in 10 year young girl, during and 2 years after treatment.

Fig. 3. PCR-RFLP of ITS1 fragments of DNA digested with *Hae*III. M – 100 bp DNA Ladder Plus (Fermentas); Lm – *Leishmania major* (MHOM/IL/1967/LRC-L137 Jericho II); Lt – *Leishmania tropica* (MHOM/SU/1974/K27); Li 1 – *Leishmania infantum* (MCAN/IL/1999/LRC-L760); Li 2 – *Leishmania infantum* (MHOM/TR/2004/CL); Li 3 – *Leishmania infantum* (MHOM/TR/2000/OG-VL); S1 – (MHOM/TR/2005/CUK1), S2 – (ITOB/TR/2005/CUK2), S3 – (ITOB/TR/2007/CUK10).

Fig. 4. Phylogenetic analysis of available strains belonging to the *Leishmania donovani* complex based on sequences of the *me*, *mpi*, *g6pdh*, *icd* and *fh* genes. The maximum likelihood (ML) tree (GTR model) was constructed from a concatenated dataset (7,614 characters, 161 parsimony-informative) of 32 strains. Numbers above branches represent bootstrap support (ML/maximum parsimony). Strains considered to be *L. donovani sensu stricto* are marked by *. Strains in bold were sequenced by us.

419 **Table1.** Phlebotomine sand flies in the study area.

Species	Western part (2005)		Eastern part (2006–2007)	
	n (%) females	*n* (%) males	*n* (%) females	*n* (%) males
Phlebotomus (Larroussius) tobbi	428 (77.7)	604 (86.4)	470 (81.2)	572 (81.5)
Phlebotomus (Larroussius) syriacus	3 (0.5)	16 (3.3)	24 (4.1)	52 (7.4)
Phlebotomus (Larroussius) perfiliewi	3 (0.5)	6 (0.9)	0	0
P. (Larroussius) sp. (unidentified)	28 (5.1)	0	16 (2.8)	0
Phlebotomus (Phlebotomus) papatasi	85 (15.4)	67 (9.6)	0	0
Phelebotomus (Paraphlebotomus) sergenti	4 (0.7)	6 (0.9)	67 (11.6)	71(10.1)
Phlebotomus (Adlerius) simici	0	0	2 (0.3)	7 (1.0)
Total	551	699	579	702

420

421

Table 2. Identification of sand fly blood-source in 2005–2006.

Sand fly host	**TOB**	**PAP**	**LAR**	**SER**
Cattle	159 (70.7%)	11	6	0
Human	23[a] (10.2%)	8	0	0
Chicken	5 (2.2%)	2	2	1
Goat	1 (0.4%)	0	0	0
Mouse	1 (0.4%)	0	0	0
Vole	1[b] (0.4%)	0	0	0
Not identified	35 (15.6%)	8	3	1
Total	**225**	**29**	**11**	**2**

TOB – *Phlebotomus tobbi*; PAP – *Phlebotomus papatasi*; LAR – *Phlebotomus* sp. (*Larrousius*); SER – *Phlebotomus sergenti*

[a]include three double meals: cattle–human and [b]one double meal: cattle–vole

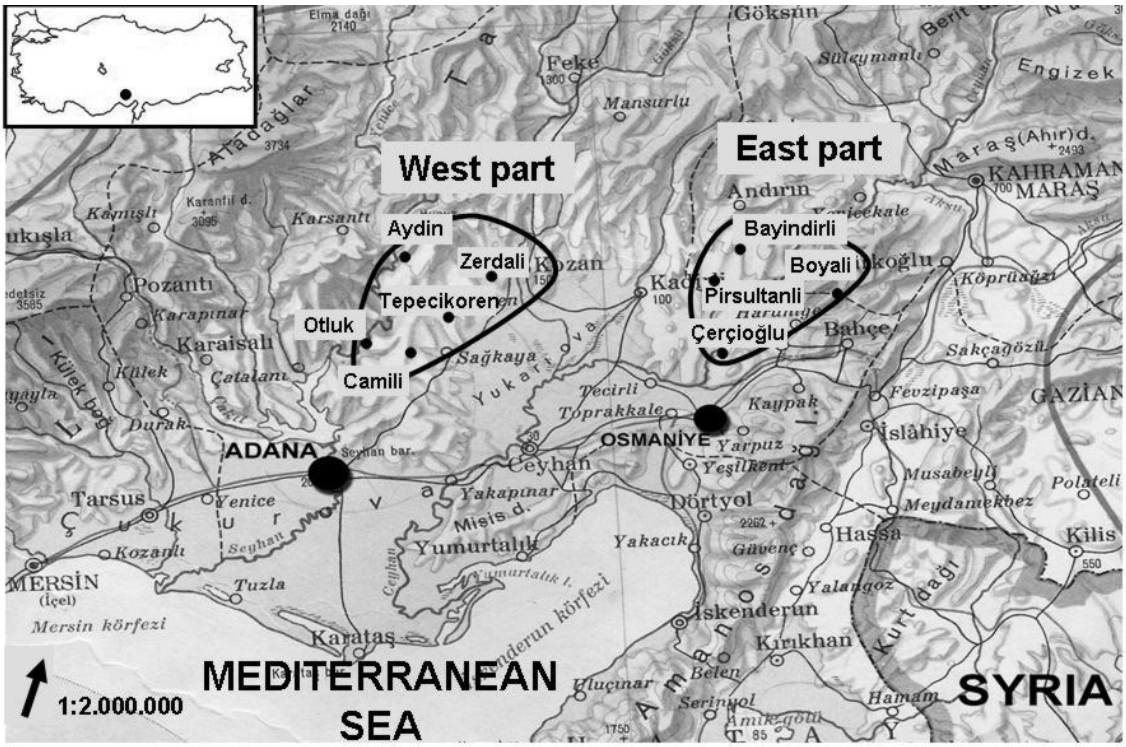
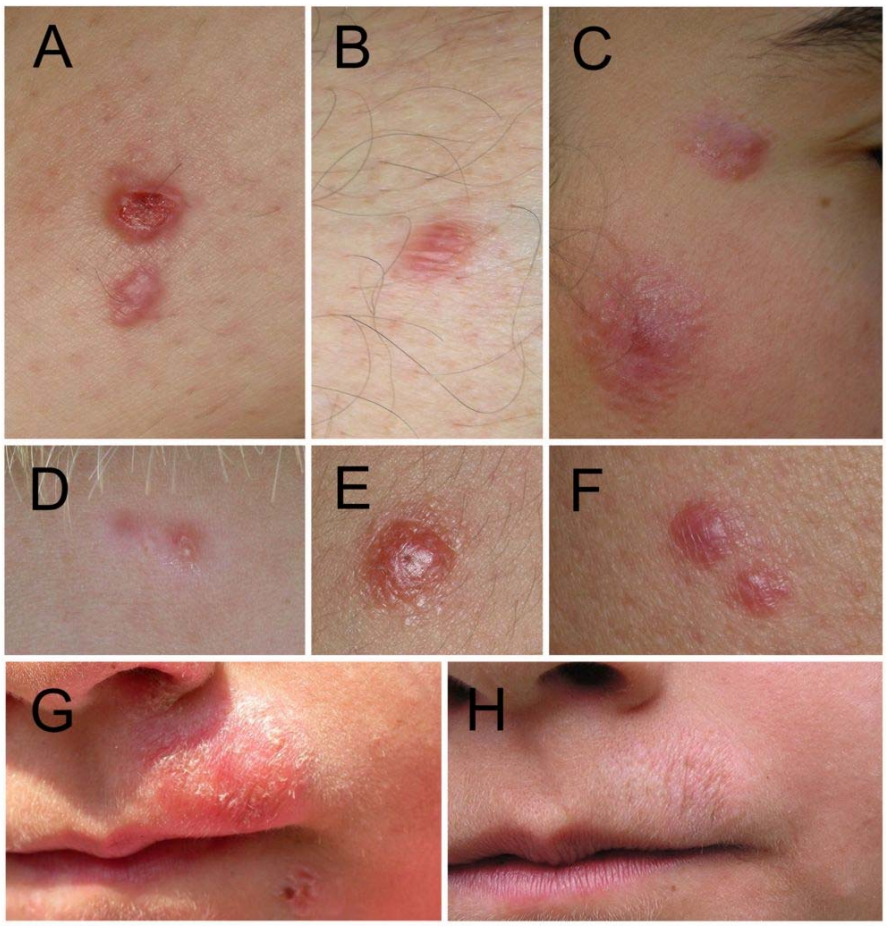
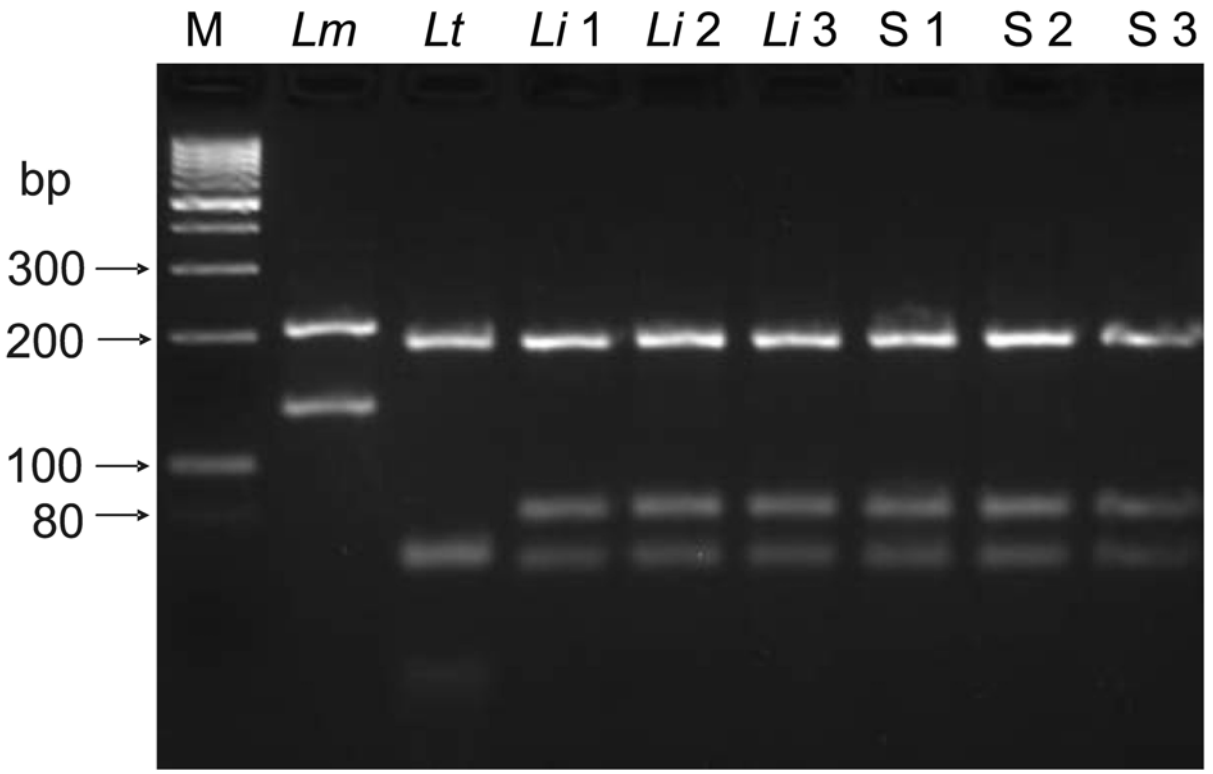

West part
East part
Aydin
Zerdali
Tepecikoren
Otluk
Camili
Bayindirli
Boyali
Pirsultanli
Çerçioğlu
ADANA
OSMANİYE
MERSİN
MEDITERRANEAN SEA
SYRIA
1:2.000.000

Fig.1


A
B
C
D
E
F
G
H

443 Fig.2

444

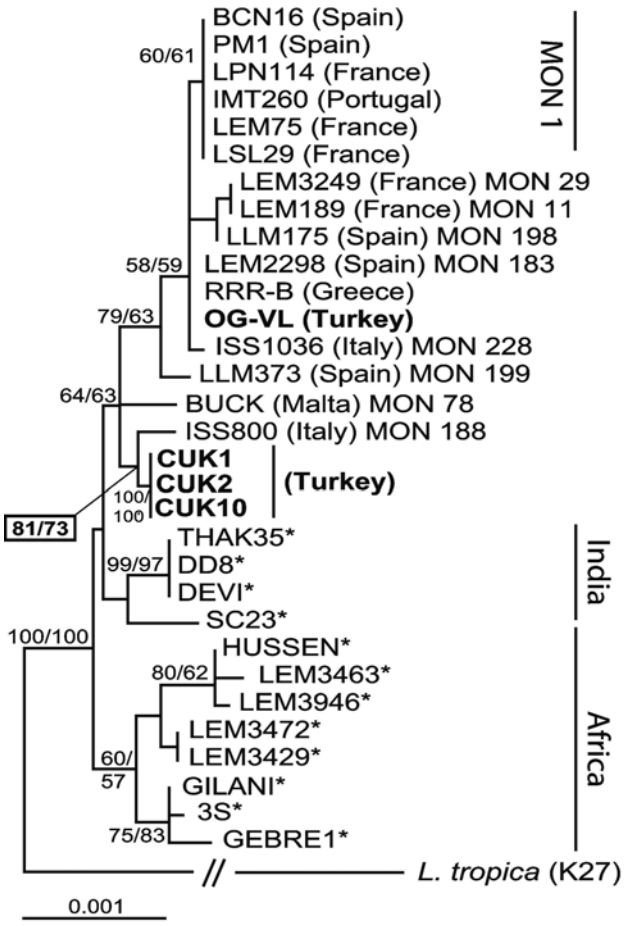


445

446

447 Fig.3

448


BCN16 (Spain)
PM1 (Spain)
LPN114 (France)
IMT260 (Portugal)
LEM75 (France)
LSL29 (France)
MON 1
LEM3249 (France) MON 29
LEM189 (France) MON 11
LLM175 (Spain) MON 198
LEM2298 (Spain) MON 183
RRR-B (Greece)
OG-VL (Turkey)
ISS1036 (Italy) MON 228
LLM373 (Spain) MON 199
BUCK (Malta) MON 78
ISS800 (Italy) MON 188
CUK1
CUK2
CUK10
(Turkey)
THAK35*
DD8*
DEVI*
SC23*
India
HUSSEN*
LEM3463*
LEM3946*
LEM3472*
LEM3429*
GILANI*
3S*
GEBRE1*
Africa
L. tropica (K27)
60/61
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100/100
80/62
60/57
75/83
0.001

Fig.4

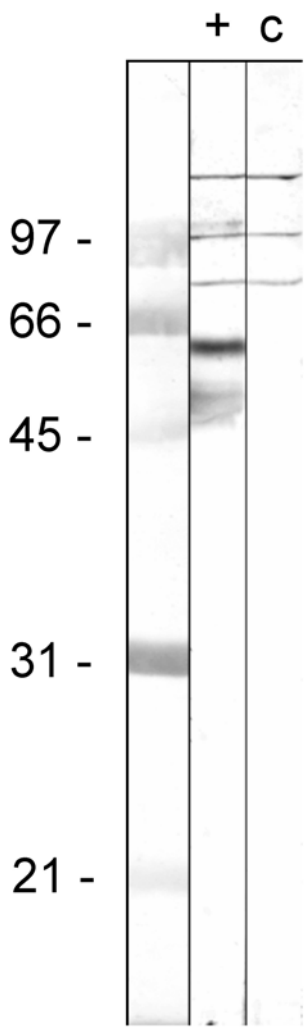

Fig.6

4. Conclusions

4.1 Cross-mating study of two laboratory colonies

Our study demonstrated succesful crossing between *P. sergenti* laboratory-reared specimens from colonies originating from Turkey and Israel. Succesful mating and insemination was observed and viable hybrid F1 and F2 offsprings were obtained from both Turkish male/Israeli female and Israeli male/Turkish female combinations. Possible differences in egg production were tested and batches descended from Turkish male / Israeli female were compared with egg production from Israeli male/Israeli female mating. There was no statistically significant difference. As these results demonstrate no reproductive barrier among sand flies from Turkish and Israeli colonies, we may speculate that there is none between the populations in the wild either.

When considering *P. sergenti* being in the state of speciation or representing sibling species within *P. sergenti* complex and in respect of other cross-mating studies performed earlier with other sand fly species, an incomplete reproductive barrier may be expected.

4.2 Comparison of two laboratory colonies by RAPD analysis

RAPD analysis was able to distinguish clearly between members of Turkish and Israeli colony. In an analysis using 15 decamer primers both UPGMA analysis and neighbor-joining method analysis revealed the existence of two distinct groups according to colony origin. All specimens of one colony fell into the same clade. Moreover, there was no significant grouping pattern based on sex in any of these two clades.

As F1 progeny was succesfully obtained from the cross-mating study, these progeny were included in the RAPD analysis. Interestingly, these samples formed a distinct group with position intermediate between Turkish and Israeli subgroup, not only sharing a portion of bands with each of the parental colonies but also exhibiting several unique new loci.

The results of RAPD analysis were in accord with geometric morphometric analysis of wing shape of *P. sergenti* from Turkey and Israel performed by a collaborating laboratory, demonstrating that both molecular biology and morphological approaches are complementary.

4.3 Comparison of *P. sergenti* populations in two leishmania foci by RAPD and ITS2 sequencing

Transmission of *Leishmania tropica* was studied in two very close emerging foci in northern Israel where the vector sand fly species differs. While in one focus leishmaniasis is transmitted in a usual fashion by *P. sergenti*, in the other focus *P. arabicus* serves as a vector. As *P. sergenti* is also present in this second focus, it was desirable to compare the specimens from both foci by several approaches.

Ten specimens of *P. sergenti* from Amnun focus were compared with ten specimens from the Tiberias focus using RAPD. Five different arbitrary primers were used. Specimens from both foci shared identical banding pattern and clearly differed from Turkish *P. sergenti* used as outgroup. The ITS 2 sequences of *P. sergenti* individuals from Tiberias and Amnun, respectively, were also identical. They were also almost completely identical (99 %) with the ITS 2 sequence of *P. sergenti* specimen of previously published sequence from West Bank, Palestine with exception of variable nucleotides at two positions. Both RAPD and ITS2 sequencing suggest that no differences occur between populations of *P. sergenti* from Amnun and Tiberias region. The different vectorial capacity of *P. sergenti* in Amnun focus was explained by different surface epitopes of local *Le. tropica* strains. These non-typical *Le. tropica* strains posessing lipophosphoglycan termnated by galactose were not able to develop in *P. sergenti* but were transmitted by *P. arabicus*, broadly permissive vector with O-glycosylation epitopes on midgut surface.

4.4 Microsatellite markers of *P. sergenti*

In the first step, a cross-applicability of microsatellite markers previously isolated for other sand fly species was tested. Five microsatellite markers originally designed for *L. longipalpis*, eleven for *P. perniciosus* and five for *P. papatasi* were subjected to PCR amplification under a range of conditions. None of the markers proved to be applicable on *P. sergenti*. Mostly, there was no PCR product at all, in some cases *P. perniciosus* markers produced unspecific products, but never in a reproducible manner.

Therefore, a development of a panel of species-specific microsatellite markers for *P. sergenti* started, using a similar protocol with enrichment step used for the isolation of microsatellites from *L. longipalpis* (Watts et al., 2002). Due to several

unexpected constrains, only two polymorphic microsatellite loci were characterized so far, maiking the results not publishable yet.

4.5 Comparison of *P. sergenti* populations by different molecular markers

In order to asses intraspecific variability of wild populations of *P. sergenti*, three different molecular methods were deployed: RAPD, sequencing of ITS2 (nuclear marker) and sequencing of cytochrome b (mitochondrial marker). Using these methods, specimens originating from populations from Turkey, Israel, Syria, and Usbekistan were analysed.

As a first step, specimens from Turkey and Israel were analysed by RAPD to proof the findings obtained from laboratory colonies, as decribed above. The UPGMA analysis as well as neighbor-joining analysis of these data revealed a same grouping for wild-caught specimens; sand flies from each country formed their own clade, one containing all field samples originating from Turkey as well as a specimen colony of Turkish origin, second containing all field samples from Israel plus the Israeli colony specimen. Interestingly, there was no distinct grouping within the Turkish clade, although the localities are separated by geographical distance and also by Amanos mountain range, which divides the coastal region of Cilicia from inland provinces of Turkey and Syria. It reaches heights well over 2000 m, however, passes which descent to an altitude of about 700 meters open it at several places. Sand flies are generally considered to be poor fliers, not traveling from their breeding and resting sites (Killick-Kendrick, 1990), however, the obtained results of RAPD analysis suggest that Amanos mountains do not represent a sufficient barrier for sand fly dispersion and the passes play a role of transitional gaps which allow a gene flow between the populations.

To get a broader context, specimens from Syria and Usbekistan were also included together with specimens from Israel and Turkey into RAPD analysis, performed by same seven RAPD primers. While Syria has an intermediate geographical position between Turkey and Israel, specimens from Usbekistan represent a very distant population. Similar pattern of clades was obtained; specimens from each country formed a unique clade.

RAPD marker had been proposed to distinguish *P. papatasi* and *P. duboscqi*, two closely related, morphologically similar vectors of cutaneous leishmaniasis

(Mudhopadhyay et al., 2000). In the respect of results of presented 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.

Our results of ITS2 rDNA sequencing corroborated the previously published intraspecific division of *P. sergenti* into two branches, north-eastern and south-western. The specimens from Usbekistan fall within the north-eastern clade, close to the samples from Pakistan, Cyprus and Lebanon. Syrian samples and Turkish samples also cluster in this clade, while Israeli samples fall within the second, south-western clade.

Sequencing analysis of cyt b mtDNA revealed that haplotypes from Turkey, Israel, Syria, and Usbekistan formed three lineages. All specimens from Usbekistan clustered in one clade, Syrian specimen clustered together with one specimen from Turkey. All remaining specimens from Turkey formed a joint clade with samples from Israel. Israeli samples were all identical, although originating from two distant localities. The haplotypes within the clade differed only in a few substitutions. As mitochondrial markers are considered to reflect the evolutionary history and phylogenetic relationships with a high accuracy, these similarities between cytochrome b sequences of specimens from Turkey and Israel, shade doubt on concept of *P. sergenti* species complex.

4.6 General conclusions of *P. sergenti* taxonomy

The results of ITS2 sequencing analysis and RAPD analysis together with geometric morphometrics demonstrated a division of *P. sergenti* populations into two clades, being in accord with previously postulated two cryptic species within *P. sergenti* (Depaquit et al., 2002). However, succesfull mating was demonstrated among sand flies from Turkish and Israeli colonies and we can conclude that there is no reproductive barrier established between individuals belonging to different ITS2 clades In addition, the sequencing analysis of cyt b put specimens from two presumably different cryptic species together in one clade. It was also shown that *P. sergenti* from different clades have similar susceptibility to *Le. tropica* (Svobodová, unpublished). These findings favor the idea of *P. sergenti* as one polymorphic species rather than a complex of two well established cryptic species.

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