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Speciation and Dispersion Hypotheses of Phlebotomine Sandflies of the subgenus *Paraphlebotomus* (Diptera:Psychodidae): The Case in Turkey

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OVERVIEW

Phlebotomine sandflies are delicate, hairy flies with long slender legs. Of the 1000 or so species, only about 70 species are thought to be involved in the transmission of disease to man. The flies are easily distinguished from other small Diptera when alive by the characteristic manner in which they hold their pointed wings above their body (like a vertical V), especially from other members of the family Psychodidae to which they belong. It is important to distinguish phlebotomine sandflies from other small biting flies known colloquially as 'sandflies' in certain parts of the world, especially midges of the genus Culicoides which abound in coastal areas of the southeastern United States, Central America and the Caribbean, and Simuliidae in Australasia. These other flies have very different biologies and medical importance from phlebotomines [1]. Phlebotomines are commonly known as *sand flies,* a reference arising from the phlebotomines-leishmaniasis associations studied extensively in the drier regions of the Mediterranean and Middle East. Unfortunately, this name is often confused with sand flies of the family Ceratopogidae, a family with very different behaviors and vector-disease associations. Second, phlebotomines in the western hemisphere have little association with sand. They are, instead, most commonly distributed in forest from southern United States to northern Argentina [2].

Knowledge of the phlebotomine biology suffers as a consequence of several characteristics: (1) They are relatively inconspicuous in size and behavior; (2) they are speciose but morphologically similar; (3) their larval stages are rarely found in the field setting, and (4) they are not readily reared [2]. Only female sandflies suck blood, and two genera contain anthropophagous species: *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Rarely, some species of the genus *Sergentomyia*, which feed principally on reptiles, will bite man but there is no evidence to suggest they are ever capable of transmitting human parasites.

Phlebotomines are mostly known as vectors of *Leishmania*, protozoan parasites which cause visceral leishmaniasis (kala-azar) and various forms of cutaneous leishmaniasis (oriental sore, espundia etc.) in man. However, they also transmit bartonellosis (Oroya fever, Carrion's disease) in northwestern

South America caused by the bacterium *Bartonella bacilliformis*, and sandfly fever virus throughout North Africa and the Middle East. Phlebotomines serve as sole vectors of leishmaniasis and other diseases in tropical and semitropical zones around the world. Only East Asia and sub-Saharan Africa are relatively free of leishmaniasis, although phlebotomine diseases are Central and South America and a broad band including the Mediterranean region and stretching across the Middle East to eastern India.

Phlebotomines transmit several viruses to man of which those causing sandfly fevers are the most important. Sandfly fever (papataci fever, three-day fever) is caused by two distinct virus serotypes (Naples and Sicilian) and results in acute febrile illness in man, lasting two to four days- and sometimes for much longer periods [3]. It is common during the summer months throughout the Mediterranean basin, the Middle East, Pakistan and parts of India and Central America. Sandflies transmit several other phleboviruses. *Phlebotomus perniciosus* transmits Toscana virus in the northern and western Mediterranean, and in the New World *Lutzomyia trapidoi* and *Lu*. *ylephiletor* transmit Chagres and Punta Toro viruses.

Bacterial infections due to *Bartonella bacilliformis* (Oroya fever, Verruga peruana) are found only in the central Andean Cordilleras of Peru, Colombia and Ecuador. The disease is endemic in valleys between 750 and 2700 m above sea level, apparently being altitudinally restricted by the ecological requirements of the vectors. Little is known of the transmission cycle but there is apparently no known animal reservoir and the vector presumably acquires the pathogen only from infected humans.

With few exceptions, leishmaniasis is acquired by the bite of a phlebotomine sand fly that has previously fed on an infected mammal. The exceptions, which are not considered further in this review, are rare venereal transmission [4], congenital transmission [5], infection by blood transfusion [6], or needle transmission by drug addicts [7]. Suggestions that transmission may be by the bite of haematophagous invertebrates other than sand flies are unsupported by convincing experimental evidence.

Leishmaniasis are diseases caused by members of the genus *Leishmania,* protozoan parasites infecting numerous species including humans, and transmitted by the bite of phlebotomine sand flies mentioned above [8]. Human leishmaniases have diverse clinical manifestations. Visceral leishmaniasis, caused by *Leishmania donovani* in the Old World and *Leishmania infantum* in both the Old and New Worlds, is the most severe form, which, if left untreated, invariably leads to death. A number of different species of *Leishmania* cause cutaneous or mucocutaneous leishmaniasis which, if not fatal, are responsible for considerable morbidity among a vast number of people from endemic foci. According to available figures, 66 Old World including Turkey and 22 New World countries are endemic for human leishmaniasis, with an estimated yearly incidence of 1.5 million cases of CL forms and o.5 million cases of VL forms [9]. Overall prevalence is 12 million people with Disability Adjusted Life Years (DALYs) burden of 860,000 for men and 1.2 million for women [8]. The disease affects the poorest countries [10]: 72 are developing countries and 13 are among the least developed. The incidence is not uniformly distributed in endemic areas; about 90% of CL cases occur in seven countries only (Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria), whereas some 90% of VL cases occur in rural and urban areas of five countries (Bangladesh, India, Nepal, Sudan and Brazil). These figures, however, must be regarded as approximate and, most probably, underestimated. Currently, it appears that global incidence of human leishmaniasis is higher than before: environmental and human behavioral factors contribute to the changing landscape of these diseases, which show a wider geographical distribution than previous known.

The basic stages of the life cycle are typical of the Nematocera, but they require a longer period for completion, up to 6 weeks or more. Phlebotomine sand flies are typically holometabolic organisms. After mating and feeding on blood, the adult females develop eggs and oviposit them singly on a crevassed substrate. In 4-10 days; in some condition in field depends on climatic factors, the hatching period takes two weeks or more. The larvae grow through four instars over a period of 4-8 weeks. After pupation, the adults emerge in 4-6 days. The adult stage is relatively short, seldom lasting more than 3 weeks, during which mating and host-seeking occur and, for the female, blood feeding, search for oviposition substrate, and oviposition [2]. Because many species serve as viral, bacterial, and protozoan vectors, the females must survive sufficiently long to take second blood meal in order to infect a second host. The immature stages of egg, larva, and pupa are described entirely by observations in a laboratory setting. Generally, phlebotomines are not strongly anthropophilic; the preferred hosts seem to be those most readily available. This opportunistic tendency makes humans an auxiliary host for sand flies that have developed a capacity for surviving in peridomestic or urban environments. Development of vector-parasite model systems has been hampered by the lack of species specificity in hosts. A single species of cutaneous leishmaniasis may have a dozen or more incriminated or suspected vectors associated with it. As a consequence, epidemiological models appropriate for one vector species-parasite pair may be irrelevant for others.

Phlebotomines can be grouped as peridomestic, with close human association, or feral, with associations independent of human activity. Peridomestic adult females are found resting on walls of human and domestic animal domiciles, away from the light and with high humidity {on the other hand, there are several studies on negative effect of humidity increasing on distribution of sand flies [11]}. During preferred times of blood feeding, usually in early evening and throughout the night, adults can be collected directly from domestic animals. Flies are found in highest densities near anticipated larval habitats, where soil and feces mix to form a conditioned, organically enriched environment. Feral environments are less readily predictable. In moist, forested areas, protected cavities in trees and buttress roots are a common refuge. Organic litter on the soil surface consisting of fallen leaves, twigs, and dead grasses also yields adult flies. Caves are a particularly rich source of flies, furnishing a humid habitat protected from the environment and common predators.

It is very important to know the dispersal capability of phlebotomine sand flies for understanding their distribution and speciation in time. Relatively little is known about the dispersal ability of phlebotomine sand flies. Adult phlebotomines are not strong fliers and therefore do not disperse far from the emergence site [2]. Mark recapture studies indicated an average flight range of <60 m, with only moving beyond 300 m. These results are typical of peridomestic species [12], although forest spcies appear to be much less active [13]. Low dispersal offers important insights into larval habitat, species distributions and diversity, speciation, and disease epidemiology. On the other hand, some studies showed their long-range movements in open habitats. They can fly up to 2.2. km over a period of a few days in open habitats [14]. They only flight at night, but in a single night can move several hundred metres in their search for a host and for subsequent resting and breeding sites [15]. Although slight air movement aids the detection of hosts along adour plumes, wind speeds of greater than 1.5 m/s inhibit flight, which ceases altogether in light winds of 4-5 m/s. In forest, sand flies such as Amazonian *Lu. umbratilis* exhibit regular vertical movements in additon to horizontal movement patterns. The flies rest in the tree buttresses during the day, migrating to the canopy in search of a host at night [16].

Sand flies are found mainly in the tropics and subtropics, with a few species penetrating into temperate regions. Although sand flies are principally found in the warm parts of the world including southern Europe, Asia, Africa, Australia, and Central and South America, their distribution extends northwards to just above latitude 50°N in south west Canada [17] and just below this latitude in northern France and Mongolia [18]. Their southernmost distribution is at about latitude 40°S, but they are absent from New Zealand and the Pacific islands [1]. Their altitudinal distribution is from below sea level (by the Dead Sea) [1] to 3300 meters above sea level in Afghanistan (*P. rupester*) [19].

Classification, General Identification and Phylogenetic Relaionships of Phlebotomine Sand Flies

The family Psychodidae, with which the phlebotomines are classified (Figure 1), is very old and maintains some of the most ancient of dipteran characters. Members of the family are distinguished by a dense covering of narrow scales on head, thorax, legs, and wing veins. Of the five psychodid subfamilies, only the Phlebotominae have piercing mouthparts capable of taking blood. The phlebotomines furthermore tend to have an elongate and more fragile structure, in contrast to a squatter and more robust appearance of the other psychodid files.

There have been few phylogenetic analyses of relationships among sand fly taxa or between sandflies and other insects [22]. For many specialists the subfamily Phlebotominae comprises six genera: ***Phlebotomus**, **Sergentomyia*** and ***Chinius*** in the Old World (OW) and ***Lutzomyia***, ***Brumptomyia*** and ***Warileya*** in the New World (NW) [20], and all the leishmania vectors are usually placed in ***Phlebotomus*** and ***Lutzomyia*** [21].

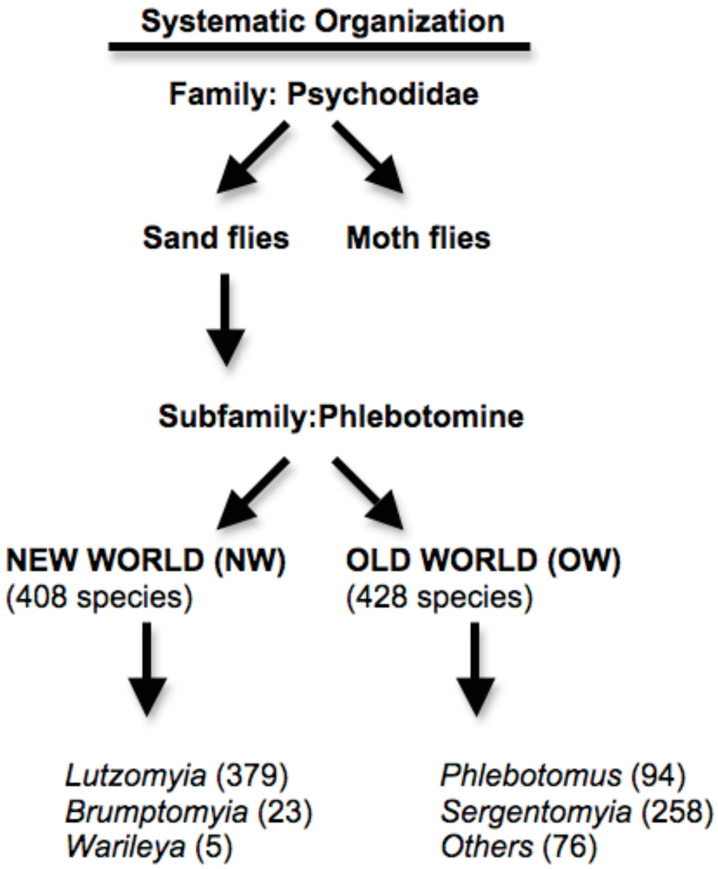

Figure 1. Systematic position of phlebotomine sandflies and their major genera [2].

However, a recent phylogenetic analysis of morphological characters led to the conclusion that the OW species should be classified in seven genera (*Chinius*, *Phlebotomus*, *Australophlebotomus*, *diophlebotomus*, *Spelaeophlebotomus*, *Sergentomyia* and *Spelaeomyia*), although the monophyly of *Sergentomyia* was questioned [23,24].

Concerning higher taxonomic levels, the family Psychodidae is classified in the rather heterogeneous infra order Psychodomorpha within the suborder Nematocera, the other dipteran suborder being Brachycera [25,26]. The Diptera and other orders of holometabolous insects are easily distinguished morphologically, but phylogenetic relationships among them have not been fully resolved [27,28]. Comparative analyses of nuclear ribosomal (r) RNA genes have been used to infer phylogenetic histories across a broad spectrum of taxa, from the basal lineages of life to relationships among closely related species and populations [29]. The small subunit (SSU) nuclear gene (16-18S rRNA) has been most studied, partly because it contains some of the slowest evolving sequences in living organisms and therefore has proven useful for examining ancient evolutionary events (Hillis & Dixon, 1991). In addition, the slow rate of change of some of these sequences permits the design of universal primers which, along with the gene's high copy number, facilitate polymerase chain reaction (PCR) amplification of SSU rDNA fragments from groups of organisms not previously investigated [30]. Several phylogenetic studies of the Holometabola have been based on SSU rDNA [31-34].

One of the most important study on phylogenetic relationship of phlebotomine sandflies was performed by Aransay et al. (2000) [22] entitled **"Phylogenetic relationship of phlebotomine sand flies inferred small subunit nuclear ribosomal DNA"**. The specific aims of this study were to determine whether or not there is concordance between the species' relationships inferred from SSU rDNA and morphological characters, to discover whether full sequence information might provide markers for geographical populations, and to relate sandflies to other holometabolous insects. In this study, relationships among seventy specimens, fifteen species and three genera of phlebotomines were inferred from the phylogenetic analysis of small subunit nuclear rDNA, obtained by the PCR amplification and cloning of almost full-length genes. Outgroups included fifteen dipterans, and single representatives of four other insect orders. The results of the study indicated that Phlebotomine sequences formed a monophyletic clade within the suborder Nematocera, with the progressively more basal sister groups of Diptera being Culicomorpha, Tipulomorpha and the suborder Brachycera. Within Phlebotominae, subgeneric relationships were resolved and the genus Phlebotomus was shown to be monophyletic, but markers for intraspecific geographical populations were not found and intergeneric relationships were not resolved. In conclusion, the five *Phlebotomus* subgenera studied (*Phlebotomus*, *Paraphlebotomus*, *Euphlebotomus*, *Adlerius* and *Larroussius*) were clustered in a monophyletic group supported by several synapomorphies (thirteen diagnostic characters out of seventeen apomorphies, 100% bootstrap value).

SUBGENUS *PARAPHLEBOTOMUS*

General identification and importance

As mentioned above the subfamily Phlebotominae comprises six genera: ***Phlebotomus, Sergentomyia*** and ***Chinius*** in the Old World (OW) and ***Lutzomyia***, ***Brumptomyia*** and ***Warileya*** in the New World (NW) [20], and all the leishmania vectors are usually placed in ***Phlebotomus*** and ***Lutzomyia*** [21]. In Old World as most important genus according to vectorial properties and capabilities of its species, *Phlebotomus* comprises 12 subgenus including *Paraphlebotomus.*

Genus *Phlebotomus* contains all man-biting sand flies, and the only vectors of pathogens to man, in the Old World. It is relatively homogeneous morphologically. Although males can be identified to subgenus, this is more difficult for females, which unfortunately is the stage most encountered in epidemiological studies. In many subgenera it is almost impossible to identify females unambiguously to species, although they can often be identified to species pair.

As identification criteria of the subgenus *Paraphlebotomus,* the pharynx of the females has large scale-like teeth with smooth margins, which appear like a network. The spermathecae usually have the terminal segment much larger than the preceding segments. The males have characteristic fleshy lobes on the inner surface of the coxite bearing tufts of long setae and the styles have only four spines. Many species are associated with rodent burrows, different species seemingly adapted to different soils, and transmit *Leishmania major* to rodents. Species of this subgenus is frequently peridomestic and transmit zoonotic and anthroponotic cutaneous leishmaniasis and visceral leishmaniasis with parasite species, *leishmania major, Leishmania infantum. Leishmania donovani* and *Leishmania tropica* in Old World.

The *Paraphlebotomus* subgenus is composed of 14 taxa [35] among which are *Phlebotomus sergenti,* the proven vectors of *Leishmania tropica* together with *Phlebotomus (Larroussius) tobbi* [38 at least in Turkey] and *Phlebotomus alexandri,* a proven vector of *Leishmania donovani* [39]. End of the 1990', the revisions of the subgenus have involved the description of two new species [40,41], the synonymization of *Phlebotomus manismortui* with *Phlebotomus alexandri* [42] and have questioned the taxonomic statute of *Phlebotomus mofidii.* At a time when the Linnean systematic and typological approaches tend to be systematically supplemented by a phylogenetic study, the scientists proposed a cladistical analysis of the Paraphlebotomus subgenus. Within this group, the few discriminating morphological characters appear to be inadequate for an acute approach and it was therefore decided to use molecular characters. However, some studies such as Depaquit et al. (2000) [35] were restricted by the limited access to live or correctly preserved specimens for exploitation by molecular biological techniques, and it was regrettably not possible to include rare species or ones that are currently difficult to collect.

Hypothesis of dispersion and speciation of the genus *Paraphlebotomus*: the case in Turkey

In this part, the studies performed by Depaquit et al. 2000 [35], Depaquit et al. 2002 [43], Şimşek et al. 2007 [44] and Aytekin et al. 2007 [45] were examined and compared to understand the hypothesis of dispersion and speciation of the genus *Paraphlebotomus.*

In first study [35], phylogenetic *Paraphlebotomus* relationships were inferred by a study basen on the sequences of ITS2, which had been sequenced in nine *Paraphlebotomus* species: *P. alexandri*, *P. andrejevi*, *P. jacusieli*, *P. kazeruni, P. mireillae, P. mongolensis, P. saevus, P. sergenti* and *P. similis* and in two out-groups species of the subgenus *Phlebotomus*: *P. papatasi* and *P. duboscqi*. According to the study, *Paraphlebotomus alexandri* appears as the sister group of all other *Paraphlebotomus* sandflies. Among the other species, three groupings are clearly highlighted: *andrejevi* and *mongolensis*; *mireillae* and *saevus*; *jacusieli*, *kazeruni*, *sergenti* and *similis*. These groupings are related to speculations about the migration of *Paraphlebotomus* from a centre of dispersion located in the Middle East sometime from the early Eocene to the late Miocene.

The phlebotomine sand flies studied in this study came from wild or colonized populations from Syria (*alexandri)*, Turkestan *(andrejevi* and *mongolensis),* Lebanon *(jacusieli),* Kenya *(mireillae),* Egypt *(kazeruni),* Oman *(saevus),* Pakistan *(sergenti),* Greece *(similis), Syria (papatasi)* and Senegal *(duboscqi).*

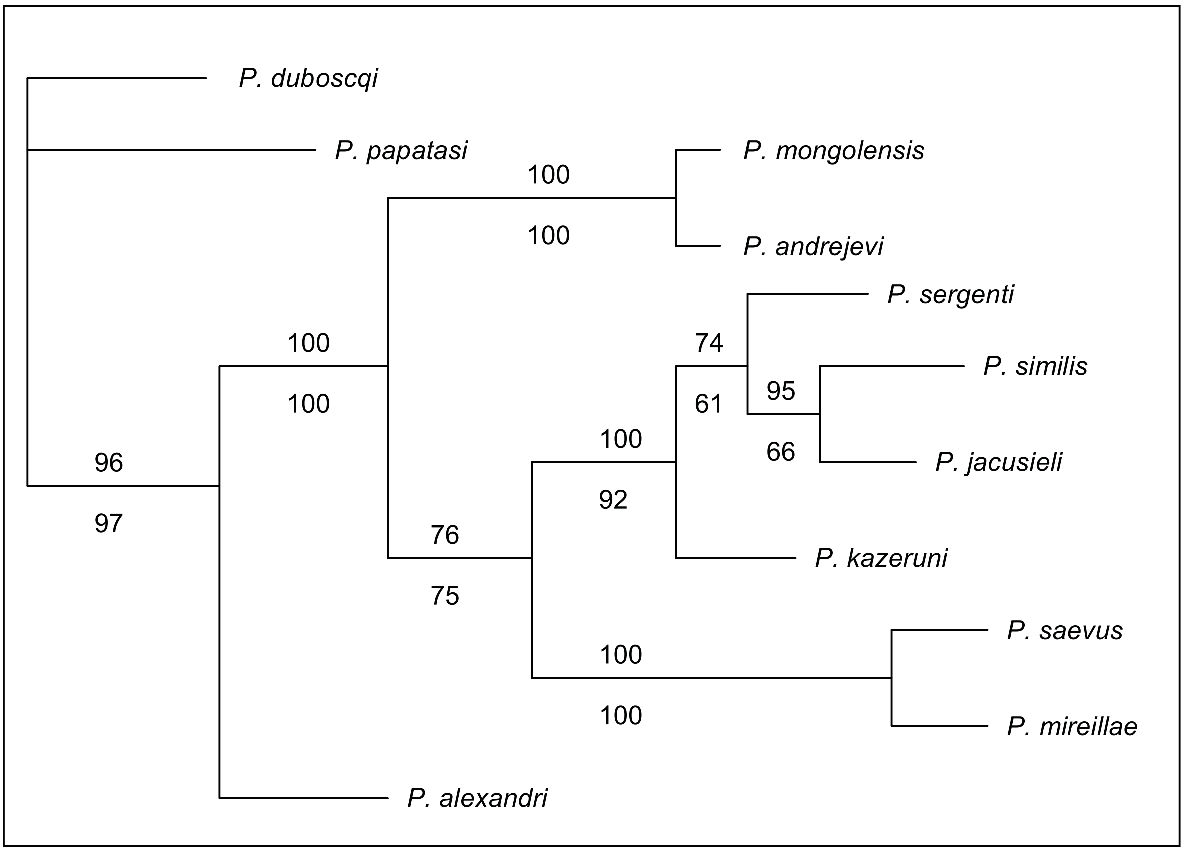

Figure 2. Maximum parsimony tree obtained by the branch and bound search option of PAUP is 425 steps long, with CI = 0.800 and RI = 0.751.All types of mutations are equally weighted. 1000 bootstrap replicates realized by PAUP program give bold and italic values mentioned on the top of the resolved branches [35].

A biogeographical approach based on the present distribution of *Paraphlebotomus* was attempted, as has been carried out previously for other subgenera of Phlebotomus [46,47] in spite of the difficulties related to the wide distributions of *P. alexandri* and *P. sergenti.*

Because *P. saevus* is distributed on both sides of the Red Sea and of the Gulf of Aden (East Africa and Arabian Peninsula), the authors assume this species arose and began its dispersion following generalized tracks [48] before the formation of the Sea, a geological event that is dated from approximately 10 to 20 millions years ago (Ma) [49]. From this assumption and the position of *P. saevus* in the cladogram, it appears that the origin of the subgenus is prior to this tectonic event, perhaps during the Eocene (33–53 Ma) or Oligocene (23.5–33 Ma). Based on current knowledge of the distribution of the species *Paraphlebotomus*, there are two palaeobiogeographic assumptions about settlement of the distribution area according to the origin from Laurasia or from Gondwana of the ancestor of the Paraphlebotomus. In fact, a centre of dispersion for the Phlebotomine sandflies, close to Iran, is suggested [46,50]. This location, situated at the contact place between Gondwana and Laurasia [51] does not enable us to support one of the two assumptions. Moreover, one centre of dispersion is not inevitably synonymous with the origin for a given group.

If we rely on the hypothesis that the origin of *Paraphlebotomus* is in Africa, the history of the group would start in the Eocene. Africa was then completely isolated from the other continents by the Tethys Sea in the north, the Atlantic Ocean in the west and the Indian Ocean in the east [51]. The ancestor of the subgenus *Paraphlebotomus* probably arose in the northeastern quarter of the continent. During this period, researchers propose that the ancestor of the current species *P. andrejevi* and *P. mongolensis* individualized from the ancestor of the other species. Because of the progressive drift of Africa towards the north, the Tethys Sea became progressively closed and, by the end of the Oligocene period, its limits were close to those of the current Mediterranean Sea [52]. From the beginning of the Miocene period (23.5 Ma), terrestrial connections with considerable biogeographic consequences were established between Africa and Asia in the area of the Middle East [51]. These generalized tracks allowed massive migrations of groups of mammals both southwards (e.g. Bovidae) and northwards (e.g. elephants). Following their vertebrate hosts, the *Paraphlebotomus* may have shared a route to Asia.

Morphologically, females of the Asiatic species *P. mongolensis* and *P. andrejevi* are indistinguishable [53], which suggests a recent speciation. This is confirmed by ITS2, of which only 11 divergent sites separate the two species. Their common ancestor, which very quickly seems to have become separated within *Paraphlebotomus*, presumably radiated as soon as the first terrestrial communication routes were established from the putative centre of dispersion close to Iran. Its multidirectional migration enabled the ancestor to occupy much of Asia, in particular towards the east (Figure 3), giving rise to the species currently present in the area: *P. mongolensis* and *P. andrejevi* (and probably *P. caucasicus*).

The East African species *P. saevus* and *P. mireillae*, which appear as the sister group of the unit containing the species *P. kazeruni, P. sergenti, P. similis* and *P. jacusieli*, would have migrated little or not at all since Miocene. A better knowledge of the distribution of *P. mireillae*, a species only recently described [40] is necessary for palaeobiogeographic hypotheses on the speciation of the African group. If it is assumed that the centre of dispersion of the phlebotomine sandflies was located in the Middle East, it seems probable that the migration with ulterior speciation by vicariance or dispersion of a common ancestor to *P. saevus* and *P. mireillae* moved towards the south. However, we cannot currently exclude a migration towards the north starting from a centre of dispersion located in Ethiopia or Kenya. To test these assumptions, it needs to be determined if *P. mireillae* occurs on the Arabic peninsula.

A common ancestor of the species *P. kazeruni*, *P. sergenti*, *P. similis* and *P. jacusieli* is assumed to have separated from the other species of the subgenus comparatively recently. Presently, we are unable to date the emergence of this ancestor before or after the collision between the African and Eurasian continents. According to the generalized tracks, it seems probable that the dispersion of these four species began somewhere in the Near- or Middle-east [54].

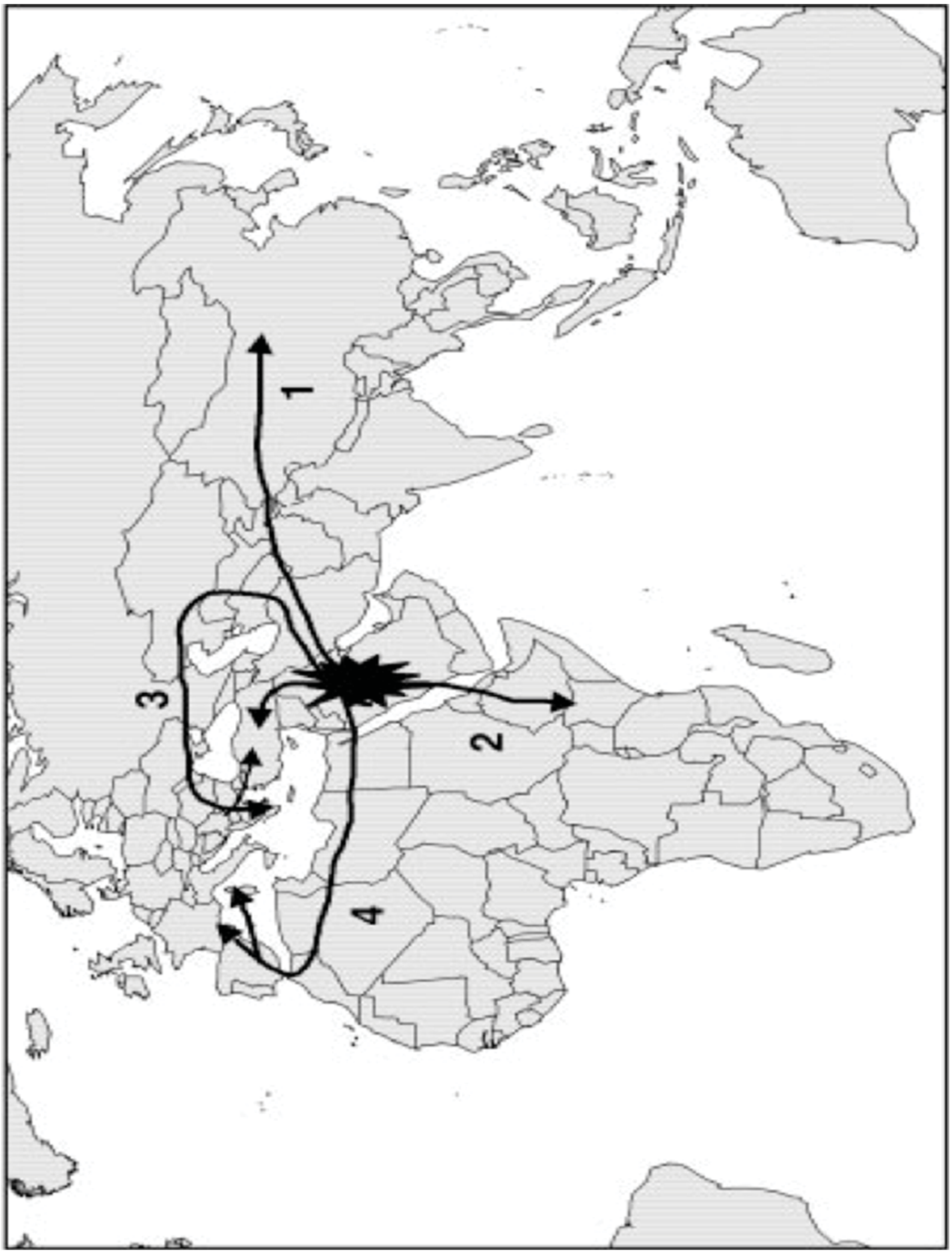

Figure 3. Map illustrating a hypothesis of settlement of the Old World by the sandflies of the subgenus Paraphlebotomus from a centre of dispersion located in the Middle-west. It is postulated the ancestor of P. andrejevi and P. mongolensis followed an eastern migration route (1), whereas P. saevus and P. mireillae followed a southern route (2). Paraphlebotomus similis, or its ancestor, would have followed the most northern way of migration (3), circumventing the Paratethys Sea, in due course to inhabit the north-east of the Mediterranean basin. Paraphlebotomus jacusieli, its vicariant, remained in the Middle-east while following route 4 and the beginning of route 1. It is assumed that P. sergenti also followed these two routes to India and skirted the southern edge of the Tethys Sea to North Africa then to the west of Europe using the Betic bridge [35].

From the Middle Eastern centre of dispersion, a northern migration route could have been followed by the common ancestor to *P. jacusieli* and *P. similis*. Another route circumventing the Paratethys Sea (of which the current Black Sea and Caspian Sea are the vestiges) by the north [54], could have been followed by *P. similis* (or its ancestor), a route undoubtedly used by sandflies of other subgenera, in particular *Adlerius* [46]. Indeed, taking into account the distribution of *P. similis* around the Black Sea and at the northeastern edge of the Mediterranean Sea [50], an initial migration using the traditional routes of the Bosphorus and of the south-Aegean islands bridge seems to be improbable. From Romania, the species could have colonized Greece and its islands (by routes then open in the Mesogea), Albania, the former Yugoslavia (western extension) and the northwestern coast of Turkey.
The western extension of its area of distribution must have been blocked by the Adriatic Sea. The orogenesis of the Alps during the Miocene period (15–20 Ma) also formed numerous barriers (Balkans in the north-west, Taurus and Antitaurus in the south, and the Caucasus in the south-east) isolating *P. similis* and limiting its extension to its present distribution.

The sister species *P. jacusieli* would have always remained south of the Paratethys Sea, thus explaining its current distribution in the Middle East. *Paraphlebotomus jacusieli* probably colonized Cyprus at the time of the draining of the Tethys Sea, about 5–6 Ma [52,54] when, from time to time, bridges connected the island to the continent. This species occurs both in continental (Macedonia) and insular (Rhodes) Greece [55]. The settlement of these areas was probably during the same period as that of Cyprus, via the southern margin of Turkey. It is interesting to note that *P. similis* and *P. jacusieli* present many morphological homologies. It seems probable that *P. kazeruni* spread along the same western route as *P. sergenti*. Its most western locality is Morocco where, from numerous collections, it is known to be rare, suggesting this is the limit of its distribution. Its absence from Europe suggests a colonization of the North Africa after the opening of the Gibraltar Straits.

Another study supporting to previous one was performed after two years by Depaquit et al. 2002 [43]. Scientists focused on *“ITS2 sequences heterogeneity in Phlebotomus sergenti and Phlebotomus similis”* in almost same geography. Cathing specimens for this study came from Cyprus, Egypt, Israel, Italy, Lebanon, Morocco, Pakistan, Portugal, Spain, Syria and Turkey for *Phlebotomus sergenti;* Greece and Malta for *Phlebotomus similis.* Both are two very important species in terms of transmission of cutaneous leishmaniasis in the genus *Paraphlebotomus.*

Phlebotomus sergenti Parrot, 1917 is the only proven vector of *Leishmania tropica* within the subgenus *Paraphlebotomus* [36,37]. It has a more widespread distribution than the parasite [41]. The presence of this sandfly in *Leishmania tropica* free areas and the varied prevalence of anthroponotic cutaneous leishmaniasis in endemic areas suggest the possible existence of closely related sandfly species (cryptic species) with different vectorial capacities.

Perfiliev [56], in the description of *Phlebotomus sergenti similis* from the Caucasus, Crimea and southern Ukraine, and later stressed that the style of this subspecies has a sub-apical spine on a tubercle inserted slightly under the apical one. He reported that in *Phlebotomus sergenti sergenti*, the two spines are generally at the same level, and concluded that the presence of a subapical spine as it appeared in Parrot (1917) [57] should not be considered as characteristic. For him, the differentiation between the two sub-species was based on the form of the basal process and length of the lateral lobe. After Artemiev and Neronov (1984) [53] and Depaquit et al. (1998) [41], authors consider *P. sergenti* and *P. similis* as representing two distinct species.

Regarding their vectorial capacities, *P. sergenti* is a proven vector of *Leishmania tropica* in Saudi Arabia and in Morocco. Except in eastern Africa and in Namibia, *P. sergenti* is the most probable vector in all foci of anthroponotic cutaneous leishmaniasis, where it is always found as a dominant species [21]. Concerning *P. similis*, its role in the transmission of Leishmania tropica was evoked in the Greek Ionian Islands but has never been proven.

According to the study performed by Depaquit et al (2002) [43], Phlebotomus sergenti and Phlebotomus similis are demonstrably monophyletic on the basis of several molecular synapomorphies. This result confirms a study previously carried out using morphological and morphometric approaches for examining the species status of these two taxa [36]. Researchers proposed that: these two species are allopatric at the present time. The revised distribution of *P. similis* (Figure 4) includes Azerbaijan, Russia, Ukraine, Romania, ex-Yugoslavia, Albania, Greece, Turkey and Malta. **Turkey is the only country in which both species are present, but *P. similis* should occur only west of Taurus and Antitaurus and *P. sergenti* only to the east** (Figure 4). In Greece all of the previous continental or insular records of *P. sergenti* are actually *P. similis*. The same is true for Malta (Gozo), *where P. similis* is present, *not P. sergenti*. The significant variability in ITS2 sequence among populations from Crete, continental Greece and Malta would suggest that this is not a recent importation by human transport.

Two of the studies on distribution of phlebotomine sand flies in Turkey performed by Şimşek et al. 2007 [44] and Aytekin et al. 2007 [45] were made very important contribution on both hypothesis of speciation and dispersal of phlebotomine sand flies and distribution of two important species, *Phlebotomus sergenti* and *Phlebotomus similis* in Old World.

First study mainly reports the sand fly fauna, species composition, and distribution in southern Anatolia, Turkey, with the aim of investigating and quantifying the possible effects of altitude and physical barriers such as Taurus and anti-Taurus Mountains on the sand fly populations in relation to the distribution of leishmaniasis. It also discusses allopatric/sympatric speciation of the two closely related species, *Phlebotomus sergenti* and *Phlebotomus similis.*

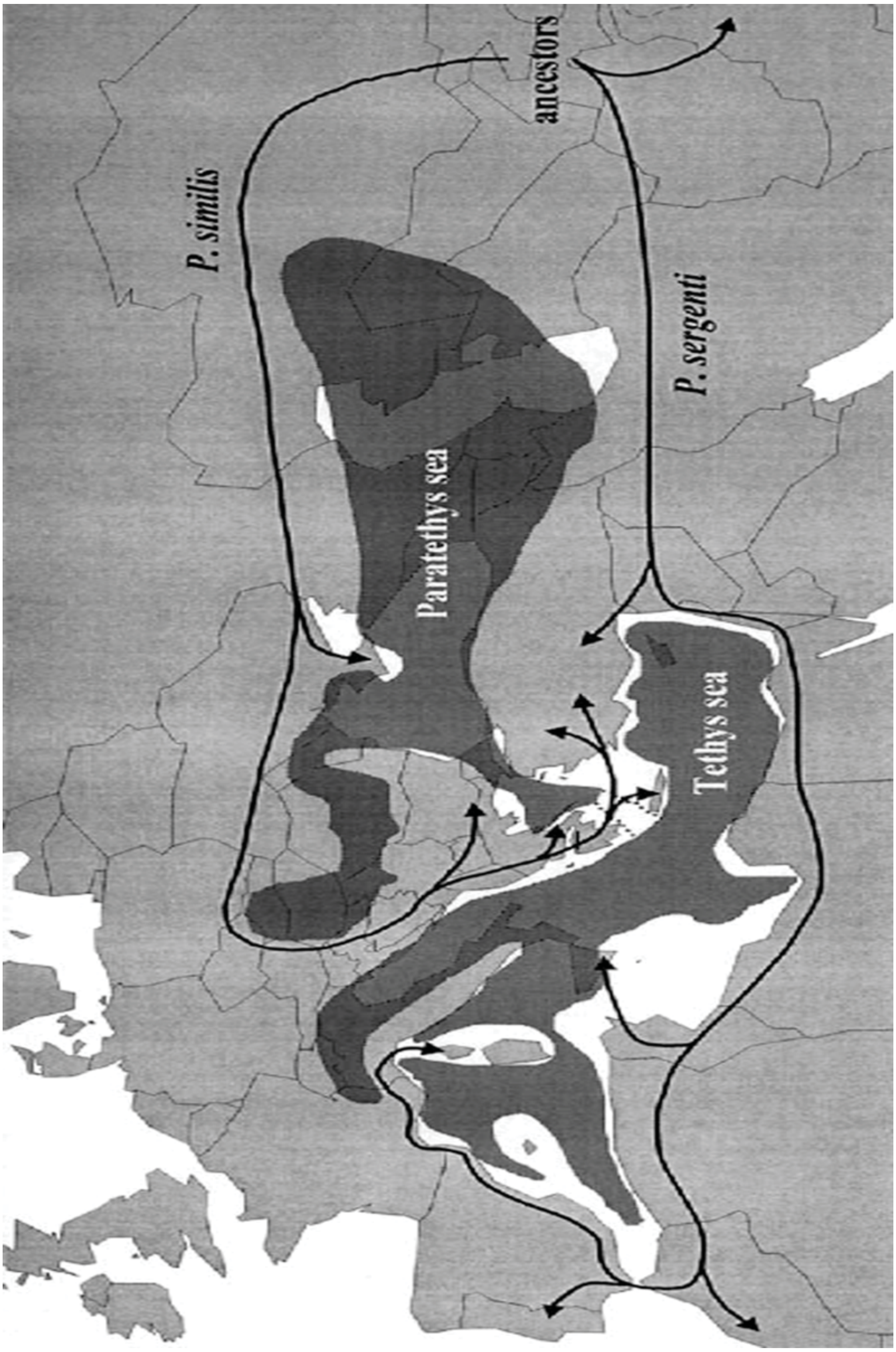

Figure 4. Distribution map of *Phlebotomus sergenti* and *Phlebotomus similis.* Hypothesis of settlement in the Old World by *Phlebotomus sergenti* and *Phlebotomus similis* from a centre of dispersion located in Asia. *Phlebotomus similis* would have followed a northern way of migration, circumventing the Paratethys sea, to inhabit in due course the north-east of the Mediterranean basin. *Phlebotomus sergenti* colonised Asia and would have followed a route along the southern edge of the Tethys sea to North Africa then to the west of Europe using the Betic bridge [43].

In this study, the two Old World genera, *Phlebotomus* and *Sergentomyia,* were both recorded in southern Anatolia in Turkey. *Phlebotomus* species predominated and comprised about 93% of the entire collection (3,172 specimens). According to the results, out of the sixteen species identified, two belonged to the genus *Sergentomyia*: *S. dentata* and *S. theodori*. The remaining fourteen species in the genus *Phlebotomus* were grouped under four subgenera including some species that are elsewhere known to act as vectors of human cutaneous leishmaniasis. Most of the *Phlebotomus* were *P. tobbi* (32.5%), but *P. papatasi*, *P. transcaucasicus, P. halepensis, P. galilaeus, P. sergenti, P. syriacus, P. neglectus, P. simici, P. alexandri, P. similis, P. jacusieli, P. perfiliewi,* and *P. brevis* were also identified. There were two associations of sand fly fauna with altitudinal gradient; the first one at relatively higher altitudes and the second one at lower altitudes. The transition between these two assemblages was within the range of 800- 1,000 m. It is likely that Adana and Hatay provinces are transitional areas between western and eastern Anatolia. Mountains do not appear to be important geographical barriers for sand fly distribution. Contrary to the results of Depaquit et al. (2002) [43], the researchers found that two of the most important vector species, *P. sergenti* and *P. similis,* were both present in the sampling sites of the Sarikoy, Beysehir, Aydinkisla, and Sille in Konya Province where located on west side of the Taurus and anti-Taurus Mountains at the range of 1,132-1,385 m altitude. Depaquit et al. (2002) hypothesized that these two species are allopatric at the present time in different countries of the Old World, including Turkey. Turkey is the only country in which both species are present, but *P. similis* is probably present only in its western part and *P. sergenti* only in the eastern part (Figure 3 and 4). To confirm if the samples collected from the west side of the West Taurus ridges do not belong to the same species, genomic DNA was extracted from each single sample collected and the ribosomal RNA ITS2 regions were sequenced. The result of molecular study confirms that the specimens are separated into two groups belonging to two different species, *P. sergenti* and *P. similis.* It indicates that *P. sergenti* is a widely distributed species throughout the study area and that these two closely related species are sympatric in Konya Province.

Aytekin et al. (2007) [45] confirmed this results using geometric morphometric analysis with same specimens collected from the study area. The wing-shape morphology of local populations of *Phlebotomus sergenti, P. papatasi, P. tobbi,* and *P. similis* were examined in both sexes by using geometric morphometrics in this study . There are three major mountain ranges that may serve as geographical barriers for species distribution in the study area and four main gaps were recognized among these barriers [44]. The researchers found no statistically important differences in wing morphology in all examined species in both sexes for all local populations. These results show that the barriers are not sufficient to stop gene flow among local populations of sand flies. The graphical depiction of PCA, CVA, and F-test confirmed our morphometric study suggesting that the difference in wing morphology between *P. similis* and *P. sergenti* indicates that these are clearly different species. These two show sympatric distribution in the Konya Plain of Anatolia.

According to these results, we can easily conclude that because of the presence of transitional gaps between the regions in suothern Anatolia, geographical variation associated with large-scale vertical and/or horizontal distribution in wing morphology does not exist among local populations of presented species. It seems likely that Hatay, Adana and Konya Regions that located on west and east sides of the Taurus Mountains are transitional areas between the west and east of Anatolia. The mountains in the study area are not important geographical barriers for sand fly distribution. Finally, the map (Figure 4 and 5) that shows distribution of both mentioned species have to re-draw according to these recent findings and the arrows that show dispersal routes of *Phelebotomus sergenti* and *similis* have to be integrated in the middle of the Anatolia.

Another interesting study was carried out by Dvorak et al. (2006) [58] to understand possible intraspecific variability of *Phelebotomus sergenti* between the populations from southeastern Anatolia, Turkey and Israel. It is very important because: 1) *Phlebotomus sergenti* populations from different areas of the Mediterranean basin are known to exhibit high intraspecific variability. Previous studies of ITS2 gene location revealed the presence of two branches that may represent sibling species, 2) one of the dispersal routes of this species pass from these two countries according to the map presented by Depaquit et al. (2000) [35], 3) ancestor of this species was located on the Middle East. To corroborate these findings by other tools, two colonies of *Phlebotomus sergenti* originating from Turkey and Israel, each belonging to a different ITS2 branch, were compared by three different methods in this study: 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.

We can conclude after these results that as the RAPD patterns obtained were stable and reproducible, we believe that RAPD-PCR reflects a prevailing 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. The results obtained from the wing shape and size supported those obtained from the RAPD. 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) [43]. 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.*

REFERENCES

1. Lane, R.P. Sandflies (Phlebotominae). Chapter Four. *Medical Insects and Arachnids.* Edited by Richard P. Lane and Rooger W. Crosskey. Chapman and Hall, ISBN 0 412 400000 6, 78-119, 1993.
2. Munstermann, L.E. Phlebotomine Sand Flies, The Psychodidae. Chapter 12. *Biology and Disease Vectors. 2nd Edition.* Edited by Marquardt, W.C., Black, W.C. IV, Freier, J.E., Hagedorn, H.H., Hemingway, J., Higgs, S., James, A.A., Kondratieff, B., Moore, C.G. Elsevier Academic Press, 141-151, 2005.
3. Tesh, R.B. The genus *Phlebovirus* and its vectors. An. Rev. of Entomol., 33, 169-181, 1988.
4. Symmers, W.S. Leishmaniasis acquired by contagion. A case of marital infection in Britain. Lancet, 7116, 127-132, 1960.
5. Eltoun, I.A., Zijlstra, E.E., Ali, M.S. Congenital kala-azar and leishmaniasis in the placenta. Am. J. Trop. Med. Hyg., 46, 57-62, 1992.
6. Bruce-Chwatt, L.J., Blood transfusion and tropical disease. Trop. Dis. Bull., 69: 825-862, 1972.
7. Desjeux, P., Alvar, J., Gradoni, L. Epidemiological analysis of 692 retrospective cases of Leishmania/HIV co-infection. Geneva: *World Health Organization,* 1-11, 1997.
8. Gramiccia, M., Gradoni, L. The leishmaniasis of southern Europe. *Emerging pests and vector-borne diseases in Europe.* Volume 1. *Ecology and control of vector-borne diseases.* Edited by: Takken, W., Knols, B.G.J. Wageningen Academic Publishers. 75-95, 2007.
9. Desjeux, P. Leishmaniasis. Public health aspects and control. Clin. Dermatol., 14, 417-423, 1996.
10. Alvar, J., Yactayo, S., Bern, C. Leishmaniasis and poverty. Trends Parasitol., 22, 552-557, 2006.
11. Belen, A. Seasonal dynamics and genetic structures of the sand fly (Diptera:Psychodidae) populations in Cukurova Region. *Hacettepe Univ., Institute of Science,* PhD Thesis, 131 p, 2009.
12. Morrison, A.C., Ferro, C., Pardo, R., Torres, M., Devlin, B., Wilson, M.L., and Tesh, R.B. Seasonal abundance of *Lutzomyia longipalpis* (Diptera:Culicidae) at an endemic focus of visceral leishmaniasis in Colombia. J. Med. Entomol., 32, 538-548, 1995.
13. Alexander, J.B. Dispersal of phlebotomine sand flies (Diptera:Psychodidae) in a Colombian coffee plantation. J. Med. Entomol, 24: 552-558, 1987.
14. Killick-Kendrick, R., Rioux, J.-A., Bailly, M., Guy, M. W., Wilkes, T. J., Guy, F. M.,Davidson, I., Knechtli, R., Ward, R. D., Guilvard, E., Perieres, J. and Dubois, H. Ecology of leishmaniasis in the south of France 20. Dispersal of Phlebotomus ariasi Tonnoir,1921 as a factor in the spread of visceral leishmaniasis in the C6vennes. Ann. de Parasitol. Hum. et compared 59: 555-572, 1984.
15. Yuval, B. and Schlein, Y. Leishmaniasis in the Jordan Valley III. Nocturnal activity of *Phlebotomus papatasi* (Diptera: Psychodidae) in relation to nutrition and ovarian development. J. Med. Entomol. 23, 411-415,1986.
16. Ready, P. D., Lainson, R., Shaw, J. J. and Ward, R. D.The ecology of *Lutzomyia umbratilis* Ward & Fraiha (Diptera: Psychodidae), the major vector to man of *Leishmania braziliensis guyanensis* in north-eastern Amazonian Brazil. Bull. Entomol. Res., 76: 21-40, 1986.
17. Young DG, Perkins PV. Phlebotomine sand flies of North America (Diptera: Psychodidae). J Amer Mosquito Cont Ass 44:263–304,1984.
18. Lewis DJ. A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). Bull Br Mus Nat Hist (Ent), 45,121–209, 1982.
19. Artemiev MM. A revision of sandflies of the subgenus *Adlerius* (Diptera, Phlebotominae, *Phlebotomus*) (in Russian). Zool Zh., 59,1177–1192, 1980.
20. Lewis, D.J., Young, D., Faichild, G.B. and Minter, D.M. Proposals for a stable classification of Phlebotomine sandflies (Diptera: Psychodidae). Syst Ent 2, 319-332, 1977.
21. Killick-Kendrick, R. Phlebotomine vectors of the leishmaniases: a review. Med Vet Entomol., 4, 1-4, 1990.

22. Aransay, A.M., Scoulica, E., Tselentis, Y., Ready, P.D. Phylogenetic relationship of phlebotomine sandflies inferred from small subunit nuclear ribosomal DNA. Insect Mol. Biol., 9,2, 157-168, 2000.
23. Rispail, P. and Ldger, N. Numerical taxonomy of Old World Phlebotominae (Diptera: Psychodidae) 1. Considerations of morphological characters in the genus Phlebotomus Rondani & Bert 1840. Mere Inst Oswaido Cruz, Rio Janeiro 93, 6, 773-785, 1998a.
24. Rispail, R and Leger, N. Numerical taxonomy of Old World Phlebotominae (Diptera: Psychodidae) 2. Restatement of classification upon subgeneric morphological characters. Mem Inst Oswaido Cruz, Rio de Janeiro 93, 6, 787-793, 1998b.
25. Hennig, W.*Insect Phylogeny*. John Wiley & Sons, Chichester, New York., 1981.
26. Wood, D.M. and Borkent, A. Phylogeny and classification of the Nematocera. In: Manual of Nearctic Diptera, 3 (McAlpine, J.F. and Wood, D.M., eds), pp. 1333-1370. Agriculture Canada, Ottawa, 1989.
27. Kristensen, N.P. *Phylogeny of extant Hexapods. In: The Insects of Australia* (CSIRO, eds), 2nd edn, pp. 125-140. Melbourne University of Press, Melbourne, 1991.
28. Oosterbroek, RF.L.S. and Courtney, G. Phylogeny of nematocerous families of Diptera (Insecta). Zool J Linn Soc 115, 267-311, 1995.
29. Hillis, D.M., Moritz, C. and Mable, B.K. *Molecular Systematics*, 2nd edn. Sinauer Associates Inc., Sunderland, Massachusetts, USA, 1996.
30. Hillis, D.M. and Dixon, M. Ribosomal DNA: molecular evolution and phyiogenetic inference. Q Rev Biol 66,411-453, 1991.
31. Carmean, D., Kimsey, L.S. and Berbee, M.L. 18S rDNA sequences and the holometabolous insects. Mol Phylogenet Evol 1,4, 270-278, 1992.
32. Miller, B.R., Crabtree, M.B. and Savage, H.M. Phylogenetic relationships of the Culicomorpha inferred from 18S and 5.8S ribosomal DNA sequences (Diptera: Nematocera). Insect Mol Biol 6,2, 105-114, 1997.
33. Whiting, EM., Carpenter, J.C., Wheeler, Q.D. and Wheeler, W.C. The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. Syst Biol 46,1, 1-68, 1997.
34. Carreno, R.A. and Barta, J.R. Small subunit ribosomal RNA genes of Tabanids and Hippoboscids (Diptera: Brachycera): evolutionary relationships and comparison with other Diptera. J Med Entomol 35,6, 1002-1006, 1998.
35. Depaquit, J., Ferte´, H., Le´ger, N., Killick-Kendrick, R., Rioux, J.A., Killick- Kendrick, M., Hanafi, H.A., Gobert, S. Molecular systematics of the Phlebotomine sandflies of the subgenus *Paraphlebotomus* (Diptera, Psychodidae, Phlebotomus) based on ITS2 rDNA sequences. Hypotheses of dispersion and speciation. Insect Mol. Biol. 9, 293–300, 2000.
36. Al-Zahrani, M.A., Peters, W., Evans, P.A., Ching-Chin, I., Smith, V., Lane, R.P. *Phlebotomus sergenti*, a vector of *Leishmania tropica* in Saudi Arabia. Trans. R. Soc. Trop. Med. Hyg. 82, 416, 1988.
37. Guilvard, E., Rioux, J.A., Gallego, M., Pratlong, F., Mahjour, J., Martinez- Ortega, E., Dereure, J., Saddiki, A., Martini, A. *Leishmania tropica* au Maroc . Ann. Parasitol. Hum. Comp. 66, 96–99, 1991.
38. Svobodova M, Alten B, Zidkova L, *et al.* Cutaneous leishmaniasis caused by *Leishmania infantum* transmitted by *Phlebotomus tobbi*. *Int J Parasitol.*, 39, 251–256, 2009.
39. Martin-Sanchez, J., Gramiccia, M., Pesson, B., Morillas-Marquez, F. Genetic polymorphism in sympatric species of the genus *Phlebotomus*, with special reference to *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (Diptera, Phlebotomidae). Parasite 7, 247–54, 2000.
40. Killick-Kendrick, R., Killick-Kendrick, M., Tang, Y. Anthroponotic cutaneous leishmaniasis in Kabul, Afghanistan : the high susceptibility of *Phlebotomus sergenti* to *Leishmania tropica.* Trans. R. Soc. Trop. Med. Hyg. 89, 477, 1995.
41. Depaquit, J., Le´ger, N., Ferte´, H. Le statut taxinomique de *Phlebotomus sergenti* parrot, 1917, vecteur de *Leishmania tropica* (Wright 1903) et *Phlebotomus similis* Perfiliev, 1963 Diptera – Psychodidae. Approches morphologique et morphome´trique. Corollaires bioge´ographiqueset e´pide´miologiques. Bull. Soc. Path. Exot. 91, 346–52, 1998.
42. Depaquit, J., Le´ger, N., Ferte´, H., Rioux, J.A., Gantier, J.C., Michaelides, A., Economides, P. Les phlebotomes de l'ı^le de Chypre. Parasite 8, 11–20, 2001.
43. Depaquit, J., Ferte´, H., Le´ger, N., Lefrane, F., Alves-Pires, C., Hanafi, H., Maroli, M., Morillas-Marquez, F., Rioux,

JA., Svobodova, M., Volf, P. ITS2 sequences heterogeneity in *Phlebotomus sergenti* and *Phlebotomus similis* (Diptera, Psychodidae): possible consequences in their ability to transmit *Leishmania tropica.* Int. J. Parasitol., 32, 1123-1131, 2002.

44. Şimşek, F.M., Alten, B., Çağlar, S.S., Ozbel, Y., Aytekin, A.M., Kaynas, S., Belen, A., Kasap Enisoz, O., Yaman, M., Rastgeldi, S. Distribution and altitudinal structuring of phlebotomine sand flies (Diptera:Psychodidae) in southern Anatolia, Turkey: their relation to human cutaneous leishmaniasis. J. Vec. Ecol., 32,2, 269-279, 2007.
45. Aytekin, A.M., Alten, B., Cağlar, S.S., Ozbel, Y., Kaynas, S., Şimşek, F.M., Erisoz Kasap, O., Belen, A. Phenotypic variation among local populations of phlebotomine sand flies (Diptera:Psychodidae) in southern Turkey. J. Vec. Ecol., 32,2, 226-234, 2007.
46. Léger, N. and Pesson, B. Sur la taxonomie et la repartition géographique de *Phlebotomus* (Adlerius) *chinensis* s.l. et de *P.* (Larroussius) *major* s.l. (Psychodidae-Diptera). Statut des espèces présentes en Grèce. Bull Soc Path Ex 80: 252–260, 1987.
47. Pesson, B., Léger, N., Madulo-Leblond, G. et al. Spéciation et vicariance chez les phlébotomes des îles grecques (reference à *Phlebotomus neglectus* et au sous-genre Larroussius). Bios 2: 233–243, 1994.
48. Humphries, C.J. and Parenti, L.R. Cladistic Biogeography.Monographies on Biogeography, Oxford, 1985.
49. Dercourt, J., Zonenshain, L., Ricou, L. et al. Présentation de 9 cartes paléogéographiques au 1/20 000 000e s'étendant de l'Atlantique au Pamir pour la période du Lias à l'actuel. Bull Soc Géol Fr 1: 637–652, 1985.
50. Depaquit, J., Perrotey, S., Lecointre, G. et al. Systématique moléculaire des Phlebotominae: étude pilote. Paraphylie du genre *Phlebotomus*. C R Acad Sci Paris (Life Sc) 321: 849–855, 1998b.
51. Buffetaut, E. and Le Loeuff, J. Les mondes disparus. Atlas de la dérive des continents. Berg International, Paris, 1998.
52. Rage, J.C. La Téthys et les dispersions transtéthysiennes par voie terrestre. Biogeographica 71, 109–126, 1995.
53. Artemiev, M.M. and Neronov, V. Distribution and ecology of sandflies of the world (genus Phlebotomus). Inst Evol Morph Anim Ecol Acad Sci USSR, Moscow, 1984.
54. Steininger, F., Rabeder, G. and Rögl, F. *Land mammal distribution in the Mediterranean Neogene: A consequence of geokinematic and climatic events.* In: Geological Evolution of the Mediterranean Basin (Stanley, J. and Wezel, F.C., eds), 559–571. Springer-Verlag, New-York, 1985.
55. Depaquit, J., Ferté, H. and Léger, N. Les phlébotomes de l'île de Rhodes (Grèce). Bull Soc Fr Parasitol 14: 28–38, 1996.
56. Perfiliev, P.P., Fauna of USSR. Diptera. Phlebotomidae (sandflies), Israel Program for Scientific Translations, Jerusalem pp. 1–363, 1968.
57. Parrot, L. Sur un nouveau phle´botome alge´rien *Phlebotomus sergenti* sp.nov.. Bull. Soc. Pathol. Exot. 10, 564–567, 1917.
58. Dvorak, V., Aytekin, A.M., Alten, B., Skarupova, S., Votypka, J., Volf, P. A comparison of intraspecific variability of *Phlebotomus sergenti* Parrot, 1917 (Diptera:Psychodidae), J.Vec.Ecol., 31,2, 229-238, 2006.