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The distribution of the *Phlebotomus major* complex (Diptera: Psychodidae) in Turkey

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ABSTRACT

The taxonomic status and distribution of the morphologically similar members of the *Phlebotomus major* complex in Turkey are unclear. To examine the utility of traditional morphological characters and molecular markers, sand flies were sampled from 90 localities in eleven different provinces covering a wide geographical range throughout Turkey. The morphometric variability was analysed using multivariate analyses of twelve characters, while mitochondrial cytochrome b (Cyt b) and nuclear elongation factor 1α (EF 1-α) genes were used for molecular discrimination. Three distinct monophyletic lineages were identified based on the phylogenetic analysis of the combined data set of mitochondrial and nuclear gene regions, which were also supported by parsimony haplotype network analysis and AMOVA of Cyt b. The first lineage is restricted to south eastern Turkey and represents the species *Phlebotomus syriacus*, the second is present mostly in the westernmost and the easternmost localities and represents *P. neglectus*, and the third member of this complex is distributed across the mid-northern and mid-southern regions. None of the studied morphological characters were found to be sufficient to discriminate between these three members of the *P. major* s.l. complex; however their presence sympatrically in several localities supports their status as species rather than inter-population variability.

1. Introduction

Members of the subgenus *Larroussius* Nitzulescu, 1931 are the only proven vectors in the Mediterranean region of *Leishmania infantum* Nicolle (Protozoa, Trypanosomatidae), the causative agent of visceral and cutaneous leishmaniasis in humans and canines (Killick-Kendrick, 1990; Svobodová et al., 2009). Four species complexes are included in this subgenus, namely *Phlebotomus perniciosus*, *Phlebotomus perfiliewi*, *Phlebotomus ariasi* and *Phlebotomus major*, which are considerably sympatric in their range of distribution from South West Asia to Northern Africa and Southern Europe (Esseghir et al., 2000; Pesson et al., 2004; Mahamdallie et al., 2010). Among these complexes, *P. major* s.l., which is also referred as the "major group" (Léger and Pesson, 1987), comprises morphologically similar species whose taxonomic status, geographic distribution and vectorial importance have been confusing. *P. major* Annandale, 1910, the type species of the *Larroussius* subgenus, is the first species described in this "major group". Since its first description from the Himalayas in India, *P. major* has long been regarded as a single widespread species forming different races or varieties with different ecological aspects and vectorial competence for *L. infantum* throughout its range, as it was recorded in India, South West Asia, Dalmatia, Italy and Crete (Adler and Theodor, 1931). But, comprehensive examination of the morphological characters recorded for adult specimens of different biogeographical origins (Perfil'ev, 1966; Lewis, 1982; Artemiev and Neronov, 1984; Léger and Pesson, 1987; Killick-Kendrick et al., 1991; Haddad, 1998; Perrotey, 1998) led to the recognition of *P. major* as a species complex. The controversial taxonomic history of the *P. major* complex is well reviewed by Badakhshan et al. (2011). According to the current taxonomic status of its members, the "major group" comprises six species: *P. major* (India, Nepal and Pakistan), *P. wui* (China), *P. notus* (Afghanistan), *P. wenyoni* (Iran, Iraq), *P. syriacus* (Southwest Asia, Caucasia) and *P. neglectus* (South Europe and Crimea).

Although most of the members of this species complex have allopatric distribution, *P. neglectus* and *P. syriacus* are thought to coexist in the Middle East. According to the repartition map for the *Larroussius* species given by Léger and Depaquit (2002), *P. neglectus* mainly occurs in the western Mediterranean countries and is distributed across the western and mid northern parts of Turkey. On the other hand, the distribution of *P. syriacus* is limited to the eastern countries including southern Turkey; these two species are suggested to be found sympatrically only in the south eastern Anatolia near Syrian border.

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Previous records from Croatia (Bosnic et al., 2006), Italy (Maroli et al., 2002), Greece (Ivovic et al., 2007), Montenegro (Ivovic et al., 2003), Hungary (Farkas et al., 2011), Albania (Velo et al., 2005), Israel (Orshan et al., 2010; Müller et al., 2011) and Palestine (Amro et al., 2009) confirmed the distribution borders of *P. neglectus* and *P. syriacus* suggested by Léger and Depaquit (2002). However, considerable uncertainty remains about the distribution in Turkey, as studies conducted in different locations demonstrated that these two species do not show a clear distribution pattern and can simultaneously coexist in the same area (Alptekin et al., 1999; Volf et al., 2002; Cicek et al., 2005; Yaman and Ozbel, 2004; Svobodová et al., 2009). In addition, the actual number of members of the "major group" and their taxonomic status in Turkey is still unclear. In spite of the fact that the species status of *P. syriacus* has already been confirmed and *P. major* is believed to be an Indian species, *P. major syriacus* (Daldal et al., 1998) and *P. major* (Dogan et al., 2006) were still recorded as probable vector species in surveys concerning human and canine leishmaniasis in different parts of Turkey. Together with these inconsistent data, as seen in several vector species complexes, the difficulty of specific identification due to the blurriness of morphological characters further complicates an exact delineation of the distribution of "major group" members in Turkey.

This present study analyses whether the mitochondrial cytochrome b (Cyt b) and nuclear elongation factor 1-alpha (EF 1-α) gene regions can be used for species identification of the "major group" members collected in different parts of Turkey. In order to elucidate their taxonomic status and distribution, we also evaluated the morphological characters previously given by several keys as important to identify these closely related species.

2. Materials and methods

2.1. Study area

Sand flies were collected during three consecutive sand fly seasons (2006–2009) at 86 localities in nine distinct provinces, selected based on previous faunistic data that suggested the presence of members of the "major group" (Fig. 1 and Table 1). In addition to newly collected sand flies, *P. major* s.l. specimens from Canakkale and Kars Turkish provinces provided by Prof. Yusuf Ozbel were included in the morphological and molecular analyses.

2.2. Sample collection

Adult sand flies were collected mostly from animal shelters using Centres for Disease Control (CDC, Atlanta, USA) light traps or from resting sites near animal enclosures with castor oil coated sticky traps. Specimens were stored in 96% ethanol at −40 °C until morphological and molecular examination. Head and genitalia were dissected and slide-mounted for identification, according to the keys of Theodor (1958), Artemiev (1980), and Lewis (1982). For further morphometric analyses, wings were also dissected, stained and slide-mounted following the method of Belen et al. (2004).

2.3. Morphological discrimination of members of the 'major group'

The discrimination of females belonging to the "major group" is mainly based on the length of the common spermathecal duct and the ratio of the pharyngeal armature. Since measurement of these characters often requires special preparation, both morphological and molecular analyzes were conducted using males. Specimens were observed using a light microscope (Leica DMR) with a camera system (Leica DC500). The number of coxite hairs and twelve different characters were measured using Leica QWin software (Supplementary Table 1) for each specimen.

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Morphological data of the males collected from different localities were analysed using Canonical Discriminant Analysis (CDA), which allowed the comparison of the selected 12 characters simultaneously. Mahalonobis distances derived from CDA were used in the Unweighted Pair-Group Method with Arithmetic Average (UPGMA) cluster analysis. Analysis of variance (ANOVA) was used to test the statistical significance of differences observed between the numbers of coxite hairs of specimens from different origins. To assign the taxonomic status of the specimens analysed, each individual was considered as one operational taxonomic unit (OTU) and the values obtained for these OTUs were compared with those suggested for the discrimination of members of the "major group". All statistical analyses were conducted using Statistica7 software.

2.4. DNA extraction, PCR amplification and sequencing

DNA was extracted from each specimen's thorax morphologically identified as *P. major* s.l., using the Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) following the manufacturer's instructions and kept at−20 °C until use.

PCR amplifications of both Cyt b and EF 1-α gene regions were performed in 50 μl reaction volumes with 1.25 U Bioron Super Hot Taq polymerase and 1 mM $MgCl_2$.

The mitochondrial Cyt b gene region (∼550 bp; 3′ end) was amplified using the CB1-SE/CB-R06 primer pairs (Esseghir et al., 2000). The temperature profile was as follows: an initial denaturation step at 94 °C for two min; 10 cycles of 94 °C for 30 s, 40 °C for 30 s, and 72 °C for 30 s; 25 cycles of 94 °C for 30, 48 °C for 30 s, and 72 °C for 30 s; and a final extension of 72 °C for 3 min.

Amplification of the ∼450 bp fragment of the EF 1-α gene region was conducted using EF-F09/EF-R12 primer pairs (Parvizi and Assmar, 2007) under the following conditions: an initial denaturation step at 94 °C for 5 min; 10 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min; 25 cycles of 94 °C for 30, 56 °C for 30 s, and 72 °C for 1 s; and a final extension of 72 °C for 3 min.

The amplification products were purified using a QIAquick PCR Purification Kit (Qiagen). Three μl of template DNA was used for the cycle sequencing reaction using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (v 2.0). PCR products were directly sequenced in both directions using the primers used for DNA amplification. Resolution of the sequences was carried out using the ABI 310 Genetic Analyzer System (PE Applied Biosystems).

2.5. Sequence analysis

Sequences were aligned and edited using Clustal X v.2.0.11 software. DnaSP v.5 software was used to identify the unique haplotypes and genotypes. Neighbour Joining (NJ) and Maximum Likelihood (ML) were conducted using PHYLIP 3.69 (http://evolution.genetics.washington.edu/phylip). The distance matrixes for NJ analysis were computed under the assumptions of Kimura's two parameter model. Bayesian analysis was performed using MrBayes 3.2 with 10 million generations and the first 25% samples were discarded as burn-in. Modeltest 0.1.1 was used to estimate the best fit evolutionary model for both ML and Bayesian analyses.

To clarify the taxonomic position of the specimens studied, *P. neglectus* from Greece and Italy, *P. syriacus* from Israel and *P. major* from Iran were used as reference sequences. The analyses of the differentiation, gene flow and polymorphism between the different

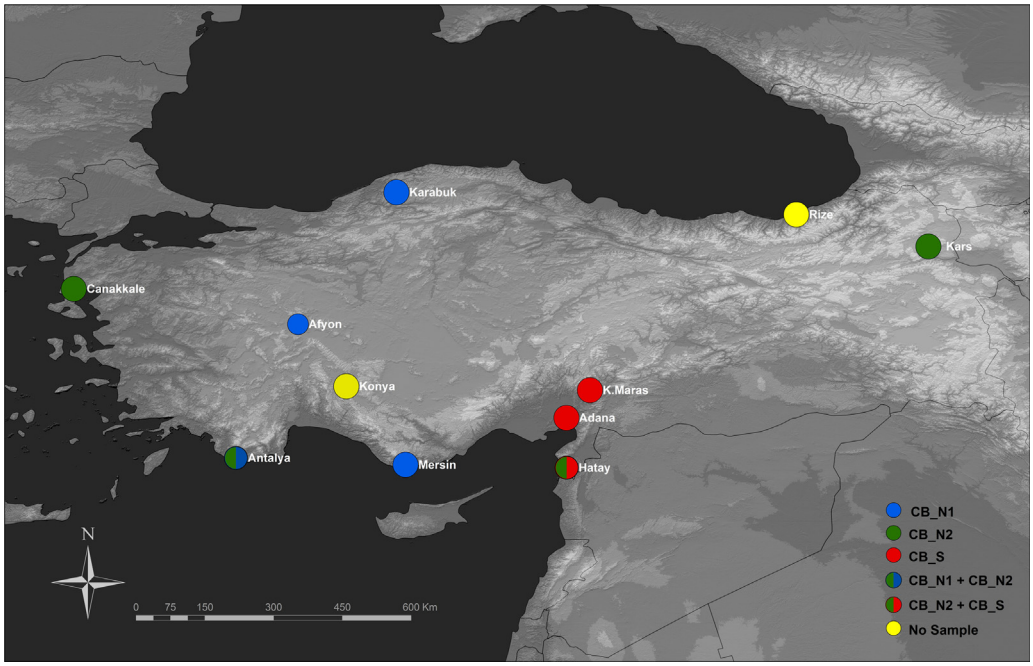

Fig. 1. Map indicating the sand fly collection sites and distribution of three lineages of *P. major* s.l. in Turkey.

Table 1
P. major s.l. sampling information and number of haplotypes/genotypes used for phylogenetic analyzes. *N*: Total number of *P. major* s.l. specimens collected, f: Haplotype/genotype frequency.

Province (*N*)	Locality	Latitude	Longitude	Altitude (m)	Cyt_b haplotypes (f)	EF_ α genotypes (f)
	Celemli	36°50′39″	35°38′50″	162		
	Gedikli	37°30′10″	35°51′40″	381		
	Tepecikoren	37°21′49″	35°37′38″	150		
	Aydin	37°24′30″	35°35′48″	279		
	Zerdali	37°24′15″	35°37′50″	275		
	Otluk	37°18′02″	35°31′03″	239		
Adana (75)	Dutlupinar	37°01′36″	36°01′02″	214	Cytb_7 (7), 8, 9, 10	EF_7 (7), 8(2), 9
	Tuysuz	37°01′45″	36°04′20″	271		
	K. Tuysuz	37°02′46″	36°05′33″	78		
	Duzici	37°15′52″	36°28′26″	521		
	Kizyusuflu	37°19′52″	36°12′35″	374		
	Sofular	37°22′57″	36°14′25″	316		
	Karakutuk	37°24′37″	36°07′14″	217		
Hatay (7)	Sebenoba	36°02′46″	36°01′20″	328	Cytb_12,14, 15, 16, 17	EF_10, 11, 12,19
K. Maras (11)	Cuhadarli	37°34′16″	36°28′44′	1058	Cytb_11 (2), 12, 13	EF_11 (3)
	Toprakkkale	38°20′31″	44°28′45″	1277		
Kars (32)	Karabag	40°23′29″	43°6′48″	1565	Cytb_4 (3), 5, 23, 24, 25, 26, 27, 28	EF_5 (7), 6
	Kotek	38°19′49″	44°32′40″	1351		
	Yenimahalle	40°8′26″	43°7′1″	1385		
Canakkale (58)	Ilyasfaki	39°46′32″	26°56′49″	351	Cytb_4 (5), 5 (4), 6 (1)	EF_1 (9), 4
	Iscehisar	38°51′54″	30°45′19″	1110		
Afyon (12)	Seydiler	38°53′17″	30°50′04″	1042	Cytb_1 (8), 2, 3	EF_1 (7), 2, 3 (2)
	Yaylabag	38°58′36″	30°32′19″	1081		
	Cariklar	36°07′44″	32°52′23″	45		
Mersin (67)	Kasdislen	36°06′14″	32°51′49″	30	Cytb_30 (7), 31, 32(2)	EF_1(7), 16, 17 (2)
	Koprubasi	36°06′54″	32°50′07″	36		
	Saricavus	36°08′07″	32°50′33″	42		
	Yaylacik	41°11′03″	32°36′03″	311		
Karabuk (167)	Belen	40°57′14″	32°28′04″	1081	Cytb_1 (4), 3, 18, 19, 20, 21, 22	EF_4 (6), 13 (3), 14
	Nebioglu	41°27′22″	32°41′06″	320		
	Tayyip	41°29′54″	32°39′24″	578		
Antalya (78)	Belenli	36°12′0″	29°41′54″	471	Cytb_1 (6), 29 (2)	EF_1 (6), 18 (2)
	Gokceoren	36°14′17″	29°32′17″	833		

lineages observed after the phylogenetic analyses were also evaluated using the Arlequin v.3.1.1 and DnaSP v.5 software packages. Parsimony networks were constructed using TCS v1.21 with a 95% connection limit between haplotypes/genotypes.

3. Results

3.1. Sand fly species sampled

A total of 10 542 sand fly specimens belonging to twelve species of the genus *Phlebotomus* and three of the genus *Sergentomyia* were collected (Faunistic data will be published separately). *P. major* s.l. specimens were collected from all of the provinces investigated except from Konya and Rize, and were found to represent 3.95% of the total catch. The geographical locations of the specimens collected and analysed are shown in Table 1.

3.2. Morphological analyses

Multivariate analysis was performed on the data set comprising twelve character measurements from 200 males, as specimens with any missing morphological character were not included in subsequent analyses. CDA revealed that these characters were highly correlated with the first (0.62) and second (0.85) canonical roots, and resulted in three separated groups, although a slight overlap was observed (Wilks' Lambda: 0.01536, $p < 0.0001$) (data not shown). UPGMA also showed the same grouping pattern: specimens originating from Kars province composed the first group, specimens from Adana, Antalya, Hatay, K. Maras and Mersin composed the second and specimens from Afyon, Canakkale and Karabuk composed the third group (Supplementary Fig. 1).

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ANOVA for the number of coxite hairs showed a significant difference between specimens collected from different provinces [$F(8,388) = 85.416$, $p = 0.0000$]. Post hoc comparisons using the Tukey HSD test indicated that the number of coxite hairs of the Kars and K. Maras specimens was significantly higher than the rest of the specimens. In addition, specimens from Antalya and Mersin were found to have the lowest number of hairs compared with those originating from other provinces.

Although the mean values obtained for each of these characters were found to be statistically sufficient for discriminating between these three groups, individual values of genital characters did not result in clear separation, since most of the OTUs were either of intermediate phenotype between the members of the "major group" or out of the discriminative range suggested by Theodor (1958), Lewis (1982), and Artemiev and Neronov (1984) in their keys (Supplementary Table 1). It was also not possible to make an alternative classification, as none of the studied characters were stable enough for clear definition of any character state coding.

3.3. Comparative analyses of mtDNA Cyt b sequences

PCR amplification of the ~550 bp 3′ end of the mtDNA Cyt b gene region was successful for 77 *P. major* s.l. specimens, and 32 unique haplotypes were identified by pairwise-distance analysis with a haplotype diversity (Hd) of 0.9187 (Table 1). The total number of polymorphic sites was 71, with 48 being parsimony informative, occurring in more than one haplotype (GenBank accession numbers: KF134667-KF134698).

Based on the phylogenetic tree topologies constructed with ML and Bayesian analyses, 32 haplotypes and the reference sequences of *P. major* s.l. were situated in three well-supported main lineages with *P. ariasi* being the outgroup: the CB_S lineage contained all the haplotypes from Adana, Hatay (except for haplotype Cytb_17) and K. Maras populations with *P. syriacus* sequences from Israel (P.syr_Is); the CB_N1 lineage contained haplotypes from Afyon, Antalya (except for haplotype Cytb_29), Karabuk and Mersin populations with two *P. major* sequences from Iran (P.maj_Ir1 and P.maj_Ir2); and CB_N2 contained all of the haplotypes from Canakkale and Kars populations along with haplotypes Cytb_17 (Hatay) and Cytb_29 (Antalya). One Iranian *P. major* sequence (P.maj_Ir3) and *P. neglectus* sequences from Greece (P.neg_Gr1 and P.neg_Gr2) and Italy (P.neg_It1, P.neg_It2, P.neg_It3 and P.neg_It4) were also incorporated into the CB_N2 lineage. The NJ tree topology was found to be the similar with the previous ones (Fig. 2).

Analysis of molecular variance (AMOVA) of the 32 Cyt b haplotypes also supported the phylogenetic structuring. Variation among lineages was found to be significantly (75.61%, $\Phi_{CT} = 0.46603$, $p < 0.001$) higher than that among populations within these lineages (11.37%, $\Phi_{SC} = 0.86976$, $p < 0.0001$) or within populations (13.02%, $\Phi_{ST} = 0.75610$, $p < 0.0001$), indicating a prolonged isolation and negligible gene exchange among these three lineages.

Parsimony network analysis sorted the 32 haplotypes into three independent networks that correspond to the three lineages recovered in the phylogenetic analysis. Cytb_1, the most frequent haplotype (23.4%) of the evaluated sequences, was also the ancestral haplotype of Network1 (=CB_N1 lineage) and distributed in Afyon, Antalya, and Karabuk populations. The oldest haplotype of Network 2 (=CB_N2 lineage) was Cytb_4, which was the second most common haplotype (10.4%) observed among all the sequences and shared among the westernmost (Canakkale) and the easternmost (Kars) populations. Cytb_7 was found to be the third frequent haplotype, which made up 9.1% of the sequences and was the ancestral haplotype of Network 3 (=CB_S lineage) with a distribution limited to the Adana, K. Maras and Hatay provinces. TCS placed the reference sequences of *P. neglectus* and *P. syriacus* in the same networks as the phylogenetic reconstructions. However, 21 mutational steps were required to connect P.maj_Ir1 and P.maj_Ir2 to Network 1 and nine mutational steps to connect P.maj_Ir3 to Network 2 and (Fig. 2). Although the separation of the P.maj_Ir1 and P.maj_Ir2 haplotypes from the CB_N1 lineage was apparent, with strong bootstrap values and a high posterior probability, any of the phylogenetic reconstructions supported the separation of P.maj_Ir3 from the CB_N2 lineage.

Alignment of the 71 polymorphic sites for the 32 Cyt b haplotypes revealed that fixed polymorphisms occurred at 10 nucleotide positions (9, 51, 159, 198, 201, 202, 237, 273, 425, and 484) for the CB_N1 lineage, at five positions (27, 60, 72, 300, and 303) for the CB_N2 lineage, and nine positions (75, 142, 148, 162, 180, 294, 300, 396, and 433) for the CB_S lineage. Including the reference sequences of *P. neglectus*, *P. major* and *P. syriacus* increased the polymorphic sites to 124, but decreased the number of fixed polymorphisms diagnostic for the CB_N1, CB_N2, and CB_S lineages to five, two and eight, respectively (Supplementary Table 2).

3.4. Comparative analyses of nuclear EF-1α sequences

The EF-1α fragment was sequenced for 73 *P. major* s.l. specimens, and the alignment of 426 nucleotides was used for further analyses. The DnaSPhase algorithm implemented in DnaSP v.5 was used to resolve ambiguous positions determined in some heterozygous specimens, but the positions of these specimens in the phylogenetic lineages did not change when the alternative alleles were used. A total of 19 genotypes were determined with a genotype diversity (Gd) of 0.8215 (Table 1). There were a total of 19 variable sites detected, and 15 of them were parsimony informative. No stop codons were observed in the 426 bp fragments (GenBank accession numbers: KF134699-KF134717).

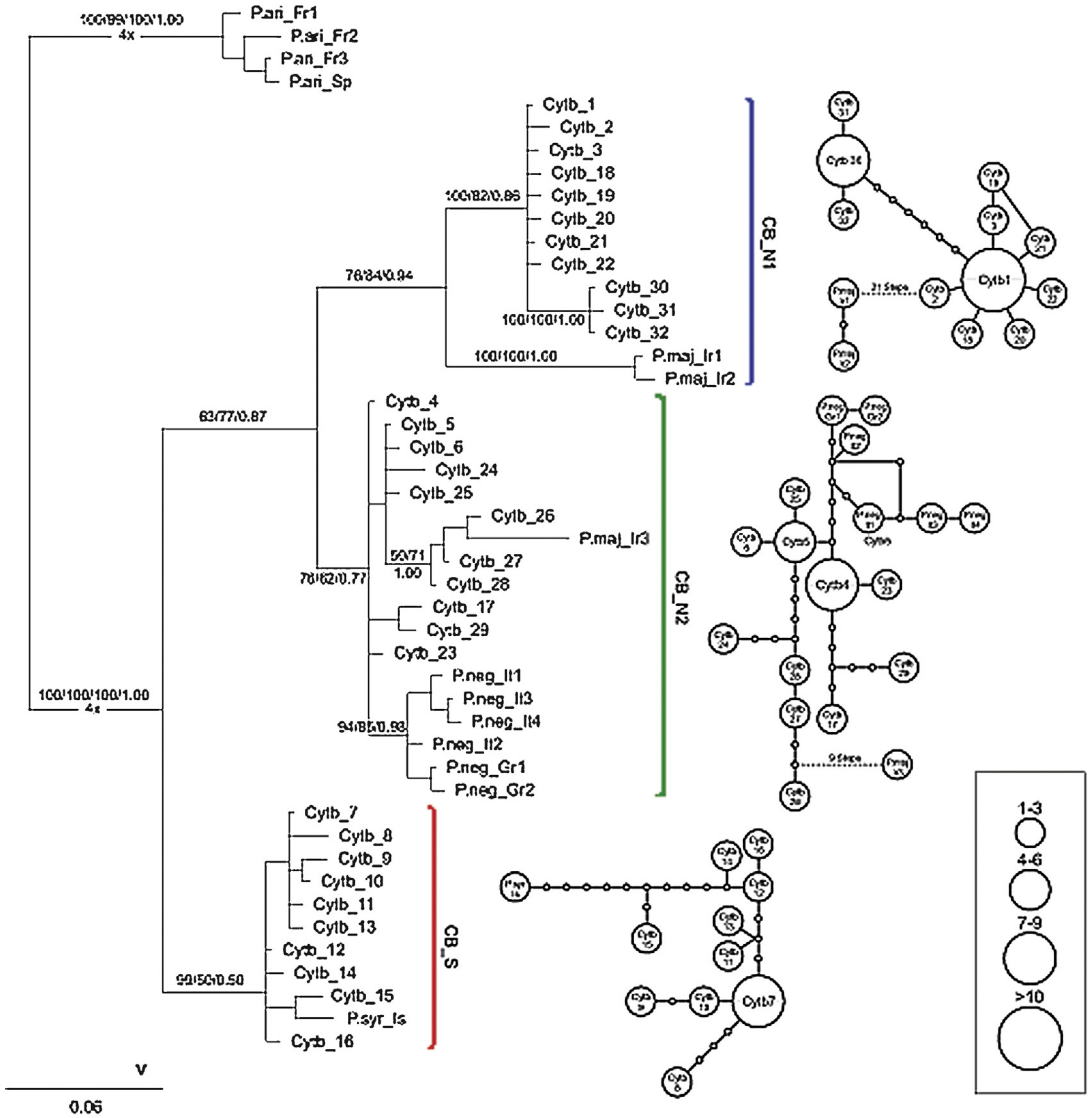

Fig. 2. Bayesian tree with corresponding haplotype networks obtained for each of three lineages of *P. major* s.l. specimens based on mt Cytb. Branch support: NJ/ML/Bayesian inference. The best fit evolutionary model for ML and Bayesian inference was HKY + G based on the AIC test of jModeltest. Codes and GenBank accession numbers for reference sequences and out group species – P.neg_It1,2,3,4: *P. neglectus* (Italy, JF76694, JF766962, JF766959, JF766960); P.maj_Gr1,2: *P. neglectus* (Greece, AF161188, AF161189); P.maj_Ir1,2,3: (Iran, GQ169334, GQ169335, GQ169337); P. syr_Is: *P. syriacus* (Israel). In networks, circles are coded by haplotype name. Haplotypes are sized according to relative abundance, and missing haplotypes are denoted by the smallest circles.

While phylogenetic analyses of the Cyt b gene region led to a well resolved tree with moderate to well supported branch support, the analysis of nuclear EF-1α of *P. major* s.l specimens produced a poorly resolved phylogeny. Using *P. ariasi* as an outgroup, ML analysis placed 19 EF-1α genotypes and reference sequences into two groups supported by low bootstrap values: the EF_S lineage contained all genotypes originating from Adana, Hatay (except from EF_19) and K. Maras; the EF_N group mostly contained the remaining genotypes studied along with *P. neglectus* sequences from Italy and *P. major* sequences from Iran. The trees constructed with NJ and BI were similar, but one genotype from Antalya (EF_18) and one from Hatay (EF_19) were separated from these two main groups, and most of the genotypes originating from Kars plus *P. major* sequences from Iran were placed as a sister group with low branch supports. The low resolution of the separation of the main lineages was also reflected in the lack of any diagnostic polymorphisms; only one fixed polymorphism at nucleotide position 222 was detected for the EF_S group when the reference sequences of *P. major* s.l. were excluded from the alignment. Statistical parsimony analysis identified a single network for the 19 EF-1 α genotypes evaluated and the reference sequences of *P. major* s.l. Representing 39.7% of the sequences, the ancestral genotype of the network was EF_1, which was shared among Afyon, Antalya, Canakkale and Mersin populations and one *P. neglectus* sequence from Italy (P.neg_It1) (Supplementary Fig. 2).

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Although the resolution of the nuclear gene phylogeny was found to be low, when the two independent data sets (Cyt b and EF-α) were concatenated, the resulting genealogical trees were in concordance with those obtained with the mitochondrial Cyt b: all phylogenetic analyses of the combined data set revealed three well supported clades that correspond to the CB_S, CB_N1 and CB_N2 mitochondrial lineages (data not shown). The morphological grouping pattern was found to be not concordant with these three groups: finding specimens that belong to similar morphogroups in distinct mitochondrial and/or concatenated lineages may

be an indicator of morphological character convergence and high morphological intra- and inter-population variability.

4. Discussion

In this work we present the first comparative morphological and molecular analysis of the *P. major* complex and its distribution in Turkey and, to our knowledge, this is the first detailed report that questions the phylogenetic relationships between some members of this complex. Cryptic speciation has been reported especially for the New World sand fly species (Soto et al., 2001; Arrivillaga et al., 2003). Correctly identifying members within species complexes is profoundly important since it is the first step in effective vector control programmes. In addition, specific diagnosis provides useful insights into distribution, diversity, species limits, ecology and vector–pathogen interactions.

Although multivariate analysis of adult morphological characters has been previously reported as a useful tool for discriminating the members of the several complexes of isomorphic sand fly species (Añez et al., 1997; Cazorla and Acosta, 2003; Ilango, 2010), we could not define the taxonomic status of the *P. major* s.l. specimens collected from different parts of Turkey using this method. Based on the analysis of the 12 male characters, specimens were found to cluster in three well-defined groups with regard to their origin. However, we failed to make a species-specific identification of the specimens present in these groups, as none of the values obtained for male genital characters was in concordance with those proposed in several keys (Theodor, 1958; Lewis, 1982; Artemiev and Neronov, 1984) for discriminating the members of this species complex. An alternative classification using the other characters we evaluated was also not possible, since we observed a marked overlap between the maximum and minimum values of characters among the three morphological groups. Our results agree with those of Badakhshan et al., 2011, who found two different morphotypes of *P. major* s.l from endemic and non-endemic foci of visceral leishmaniasis in Iran. Although significant differences were obtained by measuring 11 morphological characters, the authors were not able to make a robust identification, and the taxonomic status of these two morphotypes remained unresolved. The utility of morphological data in sand fly taxonomy is unquestionable, but when dealing with isomorphic species, previously proposed characters may be insufficient for clear distinction. In addition, body size variation under different environmental conditions has been well documented for several sand fly and mosquito species (Belen et al., 2004; Prudhomme et al., 2012; Demirci et al., 2012). Hence, selectively neutral morphological characters and/or characters under sexual selection pressure leading to reproductive isolation, as described by Ilango (2010) for the *Phlebotomus argentipes* Annandale and Brunette 1908 complex, would be advisable for the recognition of members of this complex.

In order to clarify the taxonomic status of the *P. major* s.l. specimens originating from diverse geographic origins, we used mitochondrial and nuclear DNA variation as complementary tools. Although the mitochondrial Cyt b phylogeny indicated a clear divergence in *P. major* s.l., nuclear EF-1α phylogeny was found to be poorly resolved. Unlike nuclear DNA, mitochondrial DNA is maternally inherited, non-recombining and has a faster rate of mutation (Avise, 1994). Thus, the low divergence observed in the EF-1α phylogeny may indicate that the differentiation is recent and restricted to the mitochondrial Cyt b, as reported previously for the *Lutzomyia longipalpis* Lutz and Neiva, 1912 complex (Arrivillaga et al., 2003) and *Anopheles marajoara* Galvão and Damasceno, 1942 (McKeon et al., 2010). Parvizi and Assmar (2007) showed that EF-1α was a phylogenetically informative marker for some Iranian sand fly species including those belonged to *Larroussius* subgenus but failed to distinguish morphologically similar *Phlebotomus caucasicus* s.l females. The results obtained from the phylogenetic analysis of the mtCyt b and the corresponding haplotype network indicated that *P. major* s.l. is represented by three distinct groups in Turkey. Parsimony network based approaches have been widely used to define species boundaries, especially where cryptic speciation is possible and independent networks can be interpreted as different species (Depaquit et al., 2008; Franco et al., 2010). According to this criterion, three species of the *P. major* complex are distributed in Turkey. The CB_S lineage, which should be considered to represent *Phlebotomus syriacus*, contains populations (Adana, Hatay, and K. Maras) from near the species type locality and includes the reference sequences of *P. syriacus* from Israel. The CB_N2 lineage is represented by the Kars and Canakkale populations and includes the reference sequences of *P. neglectus* from Italy and Greece and one reference sequence of *P. major* from Iran. On the basis of a 95% connection limit, TCS placed the *P. neglectus* sequences in the same network that corresponds to the CB_N2 lineage, but rejected the inclusion of one *P. major* sequence (P.maj_Ir3). Therefore, CB_N2 would be more appropriately assigned as *Phlebotomus neglectus s.s.* Represented by most of the populations (Afyon, Antalya, Karabuk and Mersin) under evaluation, the status of the CB_N1 lineage is not yet resolved; although this lineage is more closely related to two *P. major* sequences (P.maj_Ir1 and P.maj_Ir2) from Iran, TCS again excluded these Iranian haplotypes from the network. In addition, as reported by Badakhshan et al. (2011), most of the *P. major* specimens from Iran are not representatives of *P. major* s. s. The CB_N1 lineage is probably a different cryptic species of the *P. major* complex, but we suggest waiting for a formal description and assignment of a scientific name until this discrepancy is resolved.

The AMOVA results and number of fixed polymorphisms detected between the three distinct mtDNA lineages provide additional support for restricted gene flow among them. In addition, pairwise divergence between the three groups ranged from 4.5% to 5.6% (24.6–30.4 nucleotide differences) and is comparable with those observed among the members of some sand fly species complexes (Testa et al., 2002; Pesson et al., 2004; Boudabous et al., 2009). Further, incorporation of one haplotype (Cytb_17) from the Hatay population and one (Cytb_29) from Antalya into the CB_N2 indicated the sympatry of CB_N2 and CB_S in Hatay; and CB_N1 and CB_N2 lineages in Antalya. Finding these lineages to be sympatric also supports our idea that each of these lineages can be inferred as separate species rather than subspecies.

It is difficult to define the distribution limits of the members of the *P. major* complex at this early stage, but considering the sampling localities, *P. syriacus* (CB_S) appears to be restricted to south eastern Turkey while *P. neglectus* (CB_N2) is distributed mostly in the westernmost and the easternmost localities. These latter two species are also present sympatrically near the Syrian border, which is partially in agreement with Léger and Depaquit (2002) proposal and compare favourably with those of Volf et al. (2002), who indicated the peculiar position of the Southeast Anatolian populations and reported *P. neglectus* near the type locality of *P. syriacus*. Unlike previous reports (Daldal et al., 1998; Léger and Depaquit, 2002; Cicek et al., 2005), a different member of this complex (CB_N1) is distributed across the mid-northern and mid-southern parts of Turkey. On the south-western cost of Turkey, this different species and *P. neglectus* are present sympatrically. Divergence times, past demographic histories and the driving force on the separation of these groups are of interest, and we believe that together with our current data set, ongoing samplings in different parts of Turkey will contribute to efforts to clarify the distribution patterns of these closely related species.

Although the members of *P. major* s.l. have not been confirmed as vectors of *L. infantum* in Turkey, the importance of this species complex in the transmission of Mediterranean Visceral and Canine

Leishmaniasis has been confirmed (Léger et al., 1988; Ivovic et al., 2004; Amro et al., 2009). In addition, we have recently reported *P. major* s.l. as being responsible for the transmission of the Sand fly Fever Turkey Virus (Ergunay et al., 2012). Taking into account the above considerations, we suggest a combined approach using morphological characters to assign to a species complex and molecular markers for species-specific identification, especially when multiple members of this complex are found in sympatry.

Conflict of interest

None declared.

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