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## Present situation of phlebotomine sand flies with first record of *Phlebotomus (Larrousius) neglectus* in the endemic focus of visceral leishmaniasis in northwest of Iran

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### Abstract

This study was characterized morphologically and by comparative DNA sequence analysis of a fragment of Mitochondrial *Cytochrome b* (mtDNA). A total of 1743 specimens belonging to 20 phlebotomine species were collected and identified at the species level. Individual sand flies from 33 specimens of *Phlebotomus (Larrousius) major* species complex [*P. (Larr.) major* and *P. (Larr.) neglectus*] were characterized morphologically and by comparative DNA sequence analysis of a fragment of Mitochondrial *Cytochrome b* (mtDNA). The phylogenetic analyses were performed to investigate the relationships among specimens of *P. major* species complex based on the 242 bp of *Cyt b* gene amplified with the primers CB3-FC/CB-R06. *Cytochrome b* fragment sequences were obtained from 4 out of 23 specimens of *P. major* and from 3 out of 10 specimens of *P. neglectus*. The present investigation confirmed the presence of *P. neglectus* for the first time in Iran. In this study morphological identification of specimens was confirmed by molecular characterization.

**Keywords:** *Larrousius*, phylogeny, morphology, *Cytochrome b*.

### Introduction

Phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) belonging to genera *Phlebotomus* (Old World) and *Lutzomyia* (New World) are important vectors of many human disease including leishmaniasis [1-2]. The parasitic protozoa of the genus *Leishmania* is the pathogenic agent of mammalian leishmaniasis [3-4]. Out of over 800 sand fly species that have been described to date, approximately 10% are proven or suspected vectors of bacteria (e.g., *Bartonella bacilliformis*), viruses (e.g., *Phlebovirus*, *Vesiculovirus*) as well as *Leishmania* spp. protozoa [2, 5-7]. About 30 species of sand flies are proven vectors of 21 *Leishmania* spp., which have been identified to be pathogenic to human [8, 4, 9-10]. Leishmaniasis occurring in four distinct manifestation: cutaneous, muco-cutaneous, diffuse cutaneous and visceral (kala-azar) [11].

Visceral leishmaniasis (VL) due to *Leishmania infantum* (Protozoa, Trypanosomatidae), is a zoonotic, severe and fatal disease in the Mediterranean regions and transmitted by sand fly species of the subgenus *Larrousius* [2, 12-16]. Among 27-30 species of phlebotomine sand flies of the old world subgenus *Larrousius*, at least 12 species are proven or probable vectors of leishmaniasis [2, 17-20]. Visceral leishmaniasis is endemic in about 70 countries and estimated 200 million people are at risk of disease and annual incidence of VL is about 300,000 cases and over 20,000 deaths annually [14, 21-22]. Over 90% of the world's visceral leishmaniasis burden occurs on the Indian subcontinent, Bangladesh, Nepal, Brazil, Ethiopia and Sudan [22]. Endemicity of Zoonotic Visceral Leishmaniasis (ZVL) in the Mediterranean and Middle Eastern regions has been known since 1908 [23]. Infections occur in geographically distinct foci where vectors and canids reservoir hosts are found in the presence of suitable ecological conditions for the transmission of the parasite [24].

The epidemiological studies have shown that the Mediterranean type of kala-azar (ZVL) occurs in different parts of Iran that is caused by *L. infantum* LON 49 [25-26].

There are seven endemic foci of ZVL in Iran including Fars, Bushehr, Ardabil, Azarbaijan-e-Sharqi, Lorestan, Khuzestan and Khorasan-e-Shomali Provinces. At the moment most of ZVL cases are reported from Fars and Ardabil Provinces. In other parts of the country the disease has been reported sporadically [25, 27-34].

In Iran, according to surveying on vectors of ZVL in important endemic foci of the disease, four sand flies species belonging to *Larrousius* subgenus including *Phlebotomus (Larrousius) kandelakii*, *P. (Larr.) perfiliewi transcaucasicus*, *P. (Larr.) keshishiani*, *P. (Larr.) major* and one species of *Paraphlebotomus* subgenus including *P. (Para.) alexandri* have been observed naturally infected with promastigote due *Leishmania infantum* [35-46].

Ardabil province, especially Meshkin-shahr district, is an important endemic focus of VL caused by *L. infantum* in north-west of Iran [35]. According to the previous entomological studies there are several potential vectors of kala-azar present in Meshkin-shahr district, including *P. (Larr.) kandelakii*, *P. (Larr.) perfiliewi* and *P. (Larr.) major*, but only in the first species have been found *L. infantum* infection [35, 44].

Description of the subgenus *Larrousius* created by Nitzulescu (1931). The subgenus *Larrousius* proposed by Lewis [47] and Theodor [48] respectively, on the basis of male genitalia morphology (five long spines on the style including two distal ones, long aedeagus and simple paramers). Morphological characters of females are identified by the development long extension of the *spermathecal* neck and pharyngeal armature [49-50]. Comparatively among the vectors of VL, taxonomic and phylogenetic position of the large numbers of species of the subgenus *Larrousius* is relatively recent and closed related to the subgenera *Transphlebotomus* and *Adlerius* [19-20, 51].

The subgenus *Larrousius* divided into four species complex, including *P. major* complex, *P. ariasi* complex, *P. perfiliewi* complex and *P. perniciosus* complex, which are considerably sympatric in their range of distribution from South West Asia to Northern Africa and Southern Europe [52-54]. Each of four species complexes is a natural group of morphologically

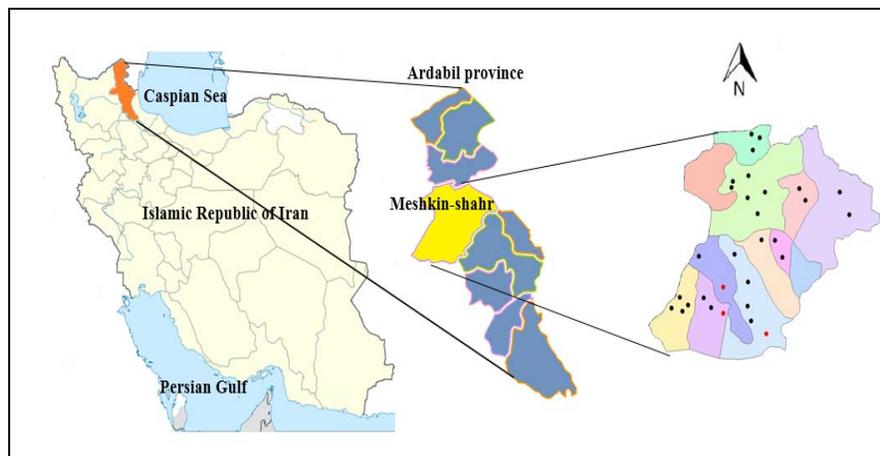
similar species or subspecies and were originally described as varieties [18]. *Phlebotomus major* complex (*P. major s.l.*) which is also referred as the “major group” [55], comprises morphologically similar species whose taxonomic status, geographic distribution and vectorial importance have been confusing. Members of the *Phlebotomus major* complex is characterized by a discrete common *spermathecal* duct in the female and a relatively long aedeagus (or in tromittent organ) in the male [55-56]. *Phlebotomus major* Annandale 1910, the type species of the *Larrousius* subgenus, is the first species described in “major group” [47, 49, 52-53, 57-68,].

This present study reports the results of entomological surveys of phlebotomine sand flies fauna and species composition in Meshkin-shahr district, northwest of Iran, with the aim of investigation on phylogenetic relationships and accurate identification of *P. major* complex in this area. For this purpose we used mitochondrial DNA fragment - Cytochrome b (Cyt b) which was known to be phylogenetically informative for *Phlebotomus* species and to be useful for dating speciation events because of its clock-like rate of nucleotide substitution [52, 69-72]. We describe and report for the first time the presence of six sand flies species especially *P. neglectus* in the most important focus of ZVL in Iran.

## Materials and Methods

### Study area

Meshkin-shahr district (48° 17' N, 38° 15' E) is located at 1890 m.a.s.l. in Ardabil province. The district occupies the northern foothills of the Sabalan Mountains, which rise to an altitude 4881m.a.s.l. Temperature varies from -27 °C in winter to 41 °C in summer. The human population was 156141 in 2012 and the principal economic activity is sheep farming (Fig 1.).



**Fig 1:** Study areas and collection sites of sand flies with location of Ardabil Province and Meshkin-shahr district in Iran.

### Sand fly collection

Sand fly collection was carried out from Jun - October 2012 (during the period of peak activity), in 31 collection locations distributed throughout the Meshkinshahr district. Sand flies were collected biweekly from indoor habitats (bedroom, toilet, bath room, sheep pen, hen house, hay loft and store room) and outdoor habitats (house yard, rodent burrow, stone and wall crevices, kennel, ruins and riverbanks) using Sticky and CDC miniature light traps. Sand flies were captured overnight with castor oil impregnated A4 papers (sticky trap). In each sites, sticky papers were placed from sunset to sunrise in various biotopes, inside and around human

dwelling and animal housing. CDC miniature light trap was installed overnight peridomestically, inside or near stable and houses. Sand fly specimens were stored in 96% ethanol at -20 °C, after killing by freezing.

### Dissection, Mounting and Morphological identification

After recording the sampling data and collection locations, sand fly specimens were washed for a few minutes, once in 5% detergent solution and then twice in sterile distilled water. Thorax and attached anterior abdomen of each specimen was stored at -20 °C in individual 1.5 ml microtubes for subsequent molecular studies. The head and

genitalia of the same individual were slide-mounted in Puri's or Berlese's medium for morphological identification at the species level, following the characters described by Theodor and Mesghali [73], Perfil'ev [49] and Lewis [47].

#### DNA extraction

DNA was extracted from the dissected thorax and attached anterior abdomen of individual sand flies using the method of Ish-Horowicz [74]. In the 1.5 ml microtubes, the thorax plus anterior abdomen of each sand fly were frozen and defrosted twice to break up tissue using a sampler tip, with grinding mix. Then SDS mix was used to denature proteins associated with the DNA, Then ice cold 8M KOAc was added to effectively remove the SDS-bound proteins from solution. Cell debris and proteins were separated from the DNA by centrifugation then the DNA in the supernatant was precipitated over night at -20 °C in 96% ethanol. Following ethanol precipitation, the DNA was dissolved in 15µl 1X TE (10mM Tris-HCl, 1mM EDTA PH = 8.0) and stored at -20 °C.

Polymerase chain reaction (PCR) for mitochondrial DNA fragment - *Cytochrome b* (*Cyt b*) were performed in a 20µl volume using 2µl 10X PCR buffer, 1.2 µl MgCl<sub>2</sub>, 0.15µl primers (forward and reverse), 2µl dNTPs, 1.5µl DNA with the reaction volume completed to 20µl by distilled water. In this study one pair primers were used: CB3-FC (forward) (5'-CA(C/T) ATTCAACC (A/T)GAATGATA-3') with CB-R06 (reverse) (5'-TATCTAATGGTTTCAAACAATTGC-3') to amplify an overlapping 3' fragment of 242 bp without primers.

Following initial denaturation at 94 °C for 3 min. PCR consisted of 35 cycles of denaturation at 94 °C for 30 sec, annealing1 at 40 °C for 30 sec, annealing2 at 44 °C for 30 sec, extension at 72 °C for 90 sec and then final extension at 72 °C for 10 min. The purification of the PCR product was made by agarose-gel fractionation and binding to Glassmilk using GeneClean II Kit, BIO 101 Inc.

#### Direct sequencing of PCR products

One hundred nano grams of each purified DNA sample was cycle-sequenced using an ABI Parsim® Big Dye™ Terminator cycle sequencing Ready Reaction Kit (version 2.0) and ABI 373/377 sequencing systems (ABI, PE Applied Biosystems), with 3.2 Pmol of the same primers that were used for PCR.

#### Aligning and phylogenetic analysis of DNA sequences

DNA sequences from both strands were aligned and edited using Sequencher Demo 4.7 and BioEdit soft wares. Multiple or pairwise sequence alignments of DNA were used with CLUSTAL W PPC: Clustalw version 1.7. Phylogenetic analyses were done using Parsimony PAUP. Relationships were inferred based on genetic distances using the Neighbor Joining (NJ) option with default settings.

#### Results

##### Sand fly fauna and species composition

In this study, 31 collection locations were determined in various geographical locations in Meshkin-shahr district. A total of 1743 specimens belonging to 20 phlebotomine species were collected during Jun to October 2012 (80% males and 20% females). Altogether 16 species and 4 subgenera belonging to the genus *Phlebotomus* and 4 species and 2 subgenera belonging to the genus *Sergentomyia* were collected (Table 1).

In present investigation, totally 33 specimens (26 males and 7 females) of *P. major* complex (*P. major* and *P. neglectus*) belonging to the subgenus *Larrousius* were identified by morphological and molecular characters (Table 2). Out of 10 specimens of *P. neglectus*, only one female specimen was identified. The highest elevation (altitude) at which *P. neglectus* specimens were captured abundantly (Number = 3, Percentage = 9.1%), was 2780 m.a.s.l. in Mueel, while the highest altitude at which *P. major* specimens were captured abundantly (Number = 8, Percentage = 24.2%), was 1551 m.a.s.l. in Urkandi.

**Table 1:** Sand fly species collected in Meshkin-shahr district, Ardabil province, 2012

No.	Species	Male No. (%)	Female No. (%)	Total	Relative population (%)
1	<i>P.(Larr.) kandelakii</i>	457 (32.8)	83 (23.8)	540	31.0
2	<i>P.(Larr.) major</i>	17 (1.2)	6 (1.7)	23	1.3
3	<i>P.(Larr.) neglectus</i>	9 (0.6)	1 (0.3)	10	0.6
4	<i>P.(Larr.) tobbi</i>	27 (1.9)	12 (3.4)	39	2.2
5	<i>P.(Larr.) perfiliewi</i>	30 (2.2)	18 (5.2)	48	2.7
6	<i>P.(Para.) sergenti</i>	138 (9.9)	34 (9.7)	172	9.9
7	<i>P.(Para.) similis</i>	12 (0.9)	0 (0.0)	12	0.7
8	<i>P.(Para.) caucasicus</i>	63 (4.5)	24 (6.9)	87	5.0
9	<i>P.(Para.) mongolensis</i>	110 (7.9)	43 (12.3)	153	8.8
10	<i>P.(Para.) jacusieli</i>	47 (3.4)	23 (6.6)	70	4.0
11	<i>P.(Para.) andrejevi</i>	4 (0.3)	1 (0.3)	5	0.3
12	<i>P.(Ad.) halepensis</i>	77 (5.5)	13 (3.7)	90	5.2
13	<i>P.(Ad.) longiductus</i>	46 (3.3)	5 (2.0)	51	3.0
14	<i>P.(Ad.) balcanicus</i>	20 (1.4)	4 (1.2)	24	1.4
15	<i>P.(Ad.) brevis</i>	7 (0.5)	0 (0.0)	7	0.4
16	<i>P.(Ph.) papatasi</i>	265 (19.0)	38 (10.9)	303	17.4
17	<i>S.(Ser.) dentata</i>	61 (4.4)	34 (9.7)	95	5.5
18	<i>S.(Ser.) sintoni</i>	0 (0.0)	2 (0.6)	2	0.1
19	<i>S.(R.) pawlowskyi</i>	2 (0.1)	7 (2.0)	9	0.5
20	<i>S.(R.) hodgsoni</i>	2 (0.1)	1 (0.3)	3	0.2
Total		1394 (100.0)	349 (100.0)	1743	100.0

**Table 2:** Prevalence of *P. major* and *P. neglectus* species identified from eight locations of Meshkin-shahr district, Ardabil province, 2012

Location	Longitude & Latitude	Altitude (m.a.s.l.)	<i>P. major</i>		<i>P. neglectus</i>	
			♂	♀	♂	♀
Ghourt tappeh	38° 21' N, 47° 37' E	1547	2	1	1	0
Ur kandi	38° 21' N, 47° 37' E	1551	6	2	0	0
Mueel	38° 17' N, 47° 42' E	2780	2	1	3	0
Mizan	38° 35' N, 47° 48' E	1390	2	2	1	0
Niaz suee	38° 23' N, 47° 25' E	1080	0	0	1	0
Agh daragh	38° 42' N, 47° 40' E	1390	3	1	2	0
Chenar	38° 43' N, 47° 39' E	1231	1	0	1	0
Alni	38° 22' N, 47° 34' E	1267	0	0	0	1
Total			17	6	9	1

### PCR amplification, aligning and phylogenetic analysis of *Cyt b* sequences of *P. major* complex

PCR amplification of *Cyt b* gene was performed successfully for 7 out of 33 specimens of *P. major* complex. Geographical origin and other information about *P. major* and *P. neglectus* captured in this study are provided in Table 3.

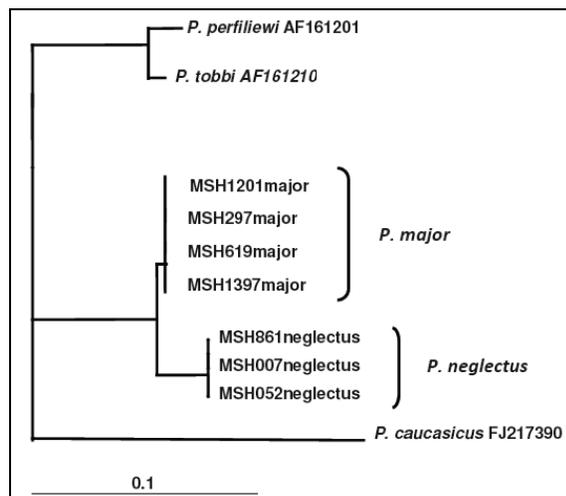
The analyses were based on the 242 bp of *Cyt b* gene amplified with the primers CB3-FC/CB-R06. *Cytochrome b* fragment sequences were obtained from 4 out of 23 specimens of *P. major* and from 3 out of 10 specimens of *P. neglectus*. All sequences studied in this investigation, are available in GenBank under accession number GQ169331 - GQ169337 (Table 3). Unique haplotype were found among 4 sequences from *P. major* as well as among 3 sequences from *P. neglectus*. Comparison of pairwise genetic similarity of seven sequences for *P. major* species complex showed 87% - 100% similarity and 2.7% genetic diversity was observed.

Phylogenetic analyses were performed to help assess the significance of the geographical variation and collecting sites among *P. major* species complex sequences. Haplotypes from the phlebotomine species, *P. caucasicus* and also *P. perfileiwi* and *P. tobbi*, a morphologically distinctive *Larroussius* species, were used as out groups (Fig. 2). The Neighbor-joining (NJ) phylogram for *Cyt b* sequences of *P. major* complex showed two lineages, and each of these had subgroups with short branches. One of the lineages had one haplotype from specimens from the same collecting site (Stone crevice), but different locations (Agh daragh, Ur kandi and Mueel) and belonging to *P. major* sequences. The second lineage had one haplotype from specimens from different collecting sites (Kennel and Rodent burrow) and different locations (Niaz suee, Alni and Ghourt tappeh) and belonging to *P. neglectus* sequences (Fig. 2).

**Table 3:** Information about collection of the *P. major* species complex in this study, Meshkin-shahr district, Ardabil province, 2012

No.	Species	Code No.	Sex	Location	Collection site	Method of capture	Type of Gene	Genbank accession number
1	<i>P. major</i>	MSH-1201	♂	Agh daragh	SC	ST	<i>Cyt b</i>	GQ169331
2	<i>P. major</i>	MSH-297	♂	Ur kandi	SC	ST	<i>Cyt b</i>	GQ169332
3	<i>P. major</i>	MSH-619	♂	Mueel	SC	ST	<i>Cyt b</i>	GQ169333
4	<i>P. major</i>	MSH-1397	♂	Mueel	SC	ST	<i>Cyt b</i>	GQ169337
5	<i>P. neglectus</i>	MSH-861	♂	Niaz suee	Kennel	ST	<i>Cyt b</i>	GQ169334
6	<i>P. neglectus</i>	MSH-007	♀	Alni	Kennel	ST	<i>Cyt b</i>	GQ169335
7	<i>P. neglectus</i>	MSH-052	♂	Ghourt tappeh	RB	ST	<i>Cyt b</i>	GQ169336

Abbreviation: SC = Stone crevice, RB = Rodent burrow, ST = Sticky trap

**Fig 2:** Neighbor-joining phylogenetic tree based on *Cyt b* (mtDNA) sequence data and generated by maximum parsimony method. The tree was rooted with *P. caucasicus*.

### Identification of *P. major* and *P. neglectus* using morphological characters

The results of our study showed that morphological identification was confirmed by molecular characterization. A total of 33 specimens of *P. major* species complex of the subgenus *Larroussius* were caught and identified into two closely related species including *P. major* and *P. neglectus*. For identification of males, the external genitalia (Aedeagus), the numbers of setae on the two inner faces of the coxites (Coxite hairs) and palpal formula were examined. Among 26 male specimens of *P. major* complex, 17 specimens belonging to *P. major* and 9 specimens belonging to *P. neglectus* (Table 2).

The male of *P. neglectus* differentiated due to long and slender aedeagus with drum-stick like tip, its margins almost parallel from near the base, coxite with less than 20 ventrally directed hairs, widely spaced and sparser and palpal formula 1, 4, 2, 3, 5 (Fig. 3). For *P. major*, male terminalia is longer than *P. neglectus* with rounded tip, coxite with 20 – 30 ventrally directed and long hairs stand more densely than *P. neglectus* and palpal formula 1, 4, (2,3), 5 (Fig. 4).

Females of *P. major* and *P. neglectus* were identified

morphologically by examining the morphology and structure of spermatheca and pharyngeal armatures. Among 7 female specimens of *P. major* complex, only one female of *P. neglectus* and 6 females of *P. major* were captured and identified (Table 2). Females of *P. neglectus* and *P. major* were displayed very similar morphologically. Pharynx with fine punctiform armature (denticle) and arranged in several rows and anterior elements have serrated margin. The armature occupied the posterior third of pharynx, but

pharyngeal armature of *P. neglectus* extending further anteriorly than in *P. major*. Spermatheca with 14 – 16 segments and spermatheca neck  $\frac{2}{3}$  the length of spermatheca body (Figs. 5 and 6).

For confirming, female of *P. neglectus* separated by comparing and accompanying of *Cyt b* sequences of males. One out of six females of *P. major* captured from the same location (Agh daragh) and subsequently confirmed by comparing of *Cyt b* sequences of males (Table 2).



A: Aedeagus

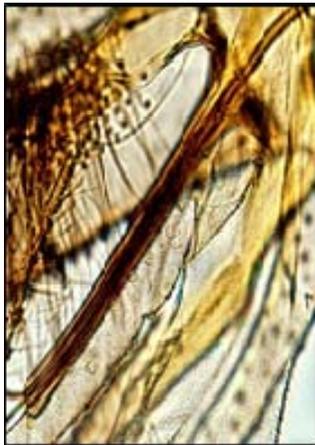


B: Coxite hairs



C: Palpal formula

Fig 3: *Phlebotomus neglectus* (Male)



A: Aedeagus



B: Coxite hairs

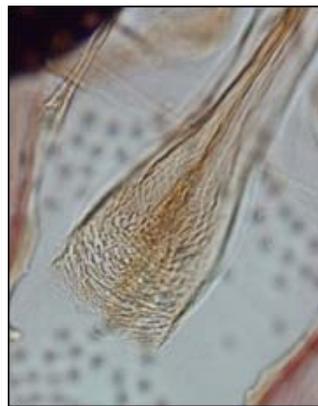


C: Palpal formula

Fig 4: *Phlebotomus major* (Male)



A: Spermatheca



B: Pharyngeal armature

Fig 5: *Phlebotomus neglectus* (Female)



A: Spermatheca



B: Pharyngeal armature

Fig 6: *Phlebotomus major* (Female)

## Discussion

Phlebotomine sand flies, the vectors of leishmaniasis, have received considerable attention in recent years due to the resurgence of leishmaniasis in some endemic areas of Iran. Visceral leishmaniasis is one of the most important parasitic disease caused by *L. infantum* and is endemic in different parts of Iran especially in Meshkin-shahr, Ardabil province [35, 75]. The fauna of phlebotomine sand flies of Meshkin-shahr district was studied for the first time by Nadim *et al.* [25, 35] and later by Rassi *et al.* [76]. In this study we present the situation of phlebotomine sand flies and comparative morphological and molecular analysis of the *P. major* complex in the endemic focus of visceral leishmaniasis, northwest of Iran.

In the present study, 16 *Phlebotomus* and 4 *Sergentomyia* species were collected and identified. For the first time, *P. neglectus*, *P. tobbi*, *P. similis*, *P. jacusieli*, *P. andrejevi* and *S. hodgsoni* were reported in Meshkin-shahr district. The current finding according the systematic and distribution *P. major* complex, established for the first time the presence of *P. neglectus* in northwest of Iran.

Although morphological characteristics are the most practical methods for species distinguishing, new molecular techniques are very useful to resolve problems of identification in the cases with morphologically similarities. Correctly identifying members within species complexes is profoundly important since it is the first step in effective vector control programs. In addition, accurate identification and discriminating of species complex provides useful insights into distribution, diversity, ecology and vector-pathogen interactions.

Access on genetic diversity and molecular systematic of *Larroussius* sand flies species not only useful to finding the taxonomic status of them, but also indicates the ecological and geographical differences of species in this subgenus.

The mode of speciation of Mediterranean *Larroussius* has recently been inferred from comparative sequences analyses not only of mtDNA but also of a nuclear gene, elongation factor alpha (EF-1 $\alpha$ ) [52, 71]. The molecular phylogenies were congruent basally, where their clades matched species complexes defined by a few genitalic characters of each sex. Esseghir *et al.* [52] have considered not only the molecular phylogenies but also the ecological niches of the *Larroussius* species and the historical biogeography and paleoecology of the Mediterranean subregions.

The phylogenetic analysis in our study indicated that the subgenus *Larroussius*, especially *P. major* complex is a phylogenetically coherent unity, as suggested by previous studies [19-20, 51-52, 77]. We have characterized *Cyt b* (mtDNA) sequences of *P. major* complex in a region of the gene without introns. Analyses of the *Cyt b* sequences in *P. major* and *P. neglectus* have identified a substantial geographical transition that separates two sympatric species.

The findings show that all of the branches of the parsimony tree had strong quantitative support and indicates the validity of many characters in inferring evolutionary relationships. The tree was also topologically similar to the parsimony tree of previous studies [52, 63, 71, 78]. The tree (Fig. 2) placed *P. perfiliewi* and *P. tobbi* as the sister groups to the assemblage consisting of the remaining *Larroussius* taxa and they show more distant relationships to *P. major* and *P. neglectus* than that suggested by morphological evidence.

Based on morphological evidence, *P. major* and *P. neglectus* are closely related species. In our study, relatively little distinction was found between *Cyt b* sequences isolated from

*P. major* and *P. neglectus*, therefore these two species were identified as monophyletic and sympatric species. On morphological characteristics the present study confirms and extends the observations of Perfil'ev [49], Lewis [47], Leger *et al.* [79] and Killick-Kendrick *et al.* [80].

The morphological identification of subgenus *Larroussius* especially *P. major* complex is based on the genital apparatus. Until 1983, the females of subgenus *Larroussius*, considered to be indistinguishable, were identified only by cohabitation (the presence of the corresponding male in the same place of capture). However, this criterion is not valid for sympatric populations. Currently, subgenus *Larroussius* are identified and separated according the structure of the basal part of the *spermathecal* ducts [80-81] or by examining the morphology of *spermathecal* body segments and pharyngeal armatures [81-82]. For males, morphological identification proposed by Theodor, Perfil'ev, Lewis and Rispaal on the basis of genital structure (aedeagus, coxite and style) [47-50]. Currently these males also can be separated basis on the numbers of setae on two inner faces of the coxites (coxite hairs). For example, differentiation between males of *P. perniciosus* and *P. longicuspis* was made by examining both the copulatory valves form and the number of coxite hairs [16, 53, 83-84]. In our study, this morphological identification was confirmed by molecular characterization. Both morphological and molecular analyses were used to correctly identify the *P. major* complex in study area.

In this investigation, the striking variety of structures in *spermathecal* segments, pharyngeal armature in females and palpal formula, coxite hairs in males provides a practical way of reliably identifying the sympatric *Larroussius* spp. which are difficult or impossible to distinguish by other means.

The *P. major* complex is reviewed by Artemiev & Neronov [17], Leger & Pesson [55] and Lewis [47]. According to the current taxonomic status of its members, the "major group" comprises six species: *P. major* (India, Nepal and Pakistan), *P. neglectus* (South Europe and Crimea), *P. syriacus* (Southwest Asia, Caucasia), *P. notus* (Afghanistan), *P. wenyoni* (Iran, Iraq) and *P. wui* (China). Most of these members have been referred to as *P. major* in the past [18].

*Phlebotomus major* was first described from the Himalayas in India, and 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 [85]. But, comprehensive examination of the morphological characters recorded for adult specimens of different biogeographical origins [17, 47, 49, 55, 80] led to the recognition of *P. major* as a species complex.

Theodor [86] believed that *P. major* occurred only in India, and the two subspecies comprising *P. major syriacus* and *P. major neglectus* existed in southern Europe and southwest Asia.

Although most of the members of this species complex have allopatric distribution, *P. neglectus* and *P. syriacus* are thought to co-exist in the Middle East. According to the repartition map for the *Larroussius* species given by Léger and Depaquit [87], *P. neglectus* mainly occurs in the western Mediterranean countries such as Albania, Turkey, Italy, Greece, Yugoslavia, Montenegro, Hungary, Dalmatia, Israel and Palestine [18, 47, 52, 71]. In some Italian territories, *P. neglectus* is suspected to play a role in human transmission and is a proven vector of *L. infantum* in Greece [79, 88].

*Phlebotomus major* is an eastern Mediterranean species which occurs from Italy to India, Nepal, Pakistan and

Thailand. In the Middle East it has been recorded from Israel and Syria and also from the Caucasus and Turkistan. The species is one of the most important and principal vectors of VL in other countries of Mediterranean basin [18, 47, 89]. This species has many geographical variants the females of which have conventionally been distinguished by differences in the pharyngeal armatures.

Ardabil province in the northwest of Iran has a general fauna like that of Transcaucasia and forms a link between Iran and south-eastern Europe [90]. In Iran, *P. major* was first reported from northern parts, mainly from the eastern parts of the Caspian littoral area [91]. Based on epidemiological evidence it was reported that *P. major* is suspected to be the main vector of VL in Iran and is found in all regions of the Iran except the southeastern parts [25, 36, 46, 73, 92-93]. The wide distribution of *P. major* in endemic and nonendemic foci of VL in Iran, and its complex taxonomic status, may be due to different populations in endemic and nonendemic foci of VL in this country.

According to morphological and morphometrical surveys (shape of the aedeagus and ventrally located hairs of coxite and pharyngeal armatures), two different morphotypes were observed among the specimens found sympatrically in the endemic and non-endemic (Borazjan and Miyandoab, respectively) foci of visceral leishmaniasis in Iran. A comparison of morphology and morphometric results from this study showed that the morphotypes that are found only in Borazjan (Bushehr province), in abundance compared with the other morphotype, were generally correlated with *P. major krimensis* or *P. neglectus*. However, the other morphotype, which included all nonendemic Miyandoab (Western-Azerbaijan) specimens and a few endemic Borazjan specimens, was closer to *P. major neglectus* or *P. neglectus*. 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 [94]. According to Artemiev and Neronov [17], *P. major neglectus* and *P. major krimensis* are synonymous with *P. neglectus*.

According the previous reports, *P. neglectus* was a subspecies of *P. major* [48-49, 73]. (or, bristle) on two inner faces of the coxites in male, but similar and indistinguishable in female [49]. *Phlebotomus neglectus* has probably often not been distinguished in the past from *P. major*, therefore it has not been reported in Iran. The utility of morphological data in sand fly taxonomy is unquestionable, but when dealing with isomorphic species, previously proposed morphological characters may be insufficient for clear distinction. In order to clarify the taxonomic status of the *P. major* complex specimens originating from diverse geographic origins, we used mitochondrial DNA variations as complementary tools.

### Conclusion

According to results of this study, *P. neglectus* and *P. major* are suggested to be found sympatrically only in Iran especially in the northwestern provinces.

Although the members of *P. neglectus s.str.* have not been confirmed as vectors of *L. infantum* in Iran, the importance of *P. major s.l.* in the transmission of Mediterranean Visceral and Canine Leishmaniasis has been confirmed [25, 36, 46, 73, 92-93].

Further entomological studies and additional data involving both morphological and molecular approaches are required to confirm our observations and to more precisely determine the phylogenetic position and taxonomic status of *P. major*

complex (especially *P. major* and *P. neglectus*) in Iran. In summary, the *Cyt b* (mtDNA) molecular data produced informative phylogeny and clear divergence within the subgenus *Larroussius* especially *P. major* complex. Our study and those carried out by others indicate that *Cyt b* can be used successfully to characterize *P. major* species complex. Also it will be necessary to analyze additional *Larroussius* taxa and data from mitochondrial and/or nuclear genes with morphological comparisons of all life stages.

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