

Short Communication

An Inventory of the Sand Flies (Diptera: Psychodidae) of Rudbar County, a New Focus of Leishmaniasis in Northern Iran, with a Taxonomic Note on the Subgenus *Larroussius*

Behzad Norouzi¹; Ahmad Ali Hanafi-Bojd²; Vahideh Moin-Vaziri³; Ayoob Noorallahi⁴; *Shahyad Azari-Hamidian⁵

¹Research Center of Health and Environment, Guilan University of Medical Sciences, Rasht, Iran

²Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

³Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁴Department of Disease Control and Prevention, Health Vice-Chancellorship, Guilan University of Medical Sciences, Rasht, Iran

⁵Department of Health Education, Research Center of Health and Environment, School of Health, Guilan University of Medical Sciences, Rasht, Iran

*Corresponding author: Dr Shahyad Azari-Hamidian, E-mail: azari@gums.ac.ir

(Received 27 Jan 2020; accepted 24 Sep 2020)

Abstract

Background: Different forms of leishmaniasis are significant infectious diseases in Iran. While, Rudbar County of Guilan Province has been introduced as a new cutaneous leishmaniasis focus, there are few published data about the phlebotomine sand flies (Diptera: Psychodidae) of the province.

Methods: To study the phlebotomine fauna of Rudbar County, the sampling was performed in 12 collection sites by light traps, sticky traps and manual aspirators throughout August–December 2015. Sand flies were removed from the sticky traps, rinsed in acetone and stored in 80% ethanol along with the collections of light traps and hand catches.

Results: In total, 2186 sand flies were collected and ten species representing two genera were morphologically identified: *Phlebotomus (Adlerius) halepensis* (0.27%), *Ph. (Larroussius) kandeladii* (0.10%), *Ph. (Lar.) neglectus* (0.91%), *Ph. (Lar.) perfiliewi* (53.88%), *Ph. (Lar.) tobbi* (43.45%), *Ph. (Paraphlebotomus) sergenti* (0.82%), *Ph. (Phlebotomus) papatasi* (0.10%), *Sergentomyia (Parrotomyia) baghdadis* (0.27%), *Se. (Sintonius) clydei* (0.05%) and *Se. (Sin.) tiberiadis* (0.10%). The species *Ph. halepensis*, *Ph. neglectus*, *Ph. perfiliewi*, *Se. baghdadis*, *Se. clydei* and *Se. tiberiadis* were reported for the first time in Guilan Province. This study also verified the presence of *Ph. neglectus* (*Ph. major krimensis* as a synonym and morphotype) in Iran. Moreover, the taxonomy of the subgenus *Larroussius* of the province was discussed.

Conclusion: The prevalence of suspected or proven cutaneous and visceral leishmaniasis vectors is noteworthy. The study of ecology of sand flies and detecting the exact vectors of leishmaniasis and phlebotomine fever by molecular specific tests in Guilan Province are recommended.

Keywords: *Phlebotomus*; *Sergentomyia*; Visceral leishmaniasis; Cutaneous leishmaniasis; Sand fly fever

Introduction

By now, approximately one thousand phlebotomine species (Diptera: Psychodidae) have been described worldwide (1). Traditionally, sand flies include six genera of the Old World *Chinius*, *Phlebotomus*, *Sergentomyia* and the New World *Brumptomyia*, *Lutzomyia* and *Warileya* (2-3), however, more recent classifi-

cation has been recognized 31 genera in the subfamily (1).

Among phlebotomine sand flies, at least 98 species of *Phlebotomus* and *Lutzomyia* are proven or suspected vectors of human leishmaniasis, also sand flies play a role in the transmission of viral infections caused by

Phlebovirus (Phenuiviridae) and *Vesiculovirus* (Rhabdoviridae) and the causal agent of bartonellosis (the bacterium *Bartonella bacilliformis*) (4).

The last checklist of Iranian sand flies composes 54 species, 31 species of *Phlebotomus* and 23 species of *Sergentomyia* (5). At least 62 species of sand flies have been reported from Iran (5-12), however there are some controversial debates about the occurrence of some species and the numbers of Iranian species are mentioned from 44 to 50 by different authors (13-16).

The Old World subgenus *Larrousius* includes at least 25 species (8), in which at least 12 species are proven or suspected vectors of leishmaniasis (17). Eleven species of the subgenus have been reported from Iran by now which are as follow: *Ph. ilami* [type locality in Iran (Ilam and Mehr)], *Ph. kandelakii*, *Ph. keshishiani*, *Ph. langeroni*, *Ph. major*, *Ph. neglectus*, *Ph. notus*, *Ph. perfiliewi*, *Ph. smirnovi*, *Ph. tobbi* [type locality in Iran (Rasht) and Palestine] and *Ph. wenyoni* [type locality in Iran (Hamadan)] (5, 8, 9, 18).

There are few published documents about the sand flies of Guilan Province in the Caspian Sea littoral of northern Iran. Only four species including *Ph. kandelakii*, *Ph. papatasi*, *Ph. sergenti* and *Ph. tobbi* have been recorded in the province by now (19-20).

More than 20000 human cases of cutaneous and visceral leishmaniasis are annually reported from Iran (21). Adler et al. (19) have noted a few autochthonous cases of human leishmaniasis in Rasht, capital of Guilan Province. Golchay et al. (22) have reported three cases of cutaneous leishmaniasis (CL) in the province and introduced CL as an endemic disease in Guilan Province. Nadim et al. (23) have mentioned that Rudbar, located in eastern Guilan Province, could be considered as a less important focus of CL. Majidi-Shad et al. (24) have introduced Rudbar County of the province as a new focus of CL. During the period of 2006–2016, one to 15 autochthonous

cases of CL and/or visceral leishmaniasis (VL) (Kala-azar) have been recorded in Guilan Province yearly, especially Rudbar County (Center of Disease Control, Health Vice-Chancellorship, Guilan University of Medical Sciences and 21). Also, sand fly fever is found in the province (25-27). Moreover, lizard leishmaniasis is reported in Guilan Province (28).

There is no recent study on phlebotomines in Guilan Province. The aim of this study was to determine the phlebotomine fauna of Rudbar County during 2015.

Materials and Methods

Study area

Guilan Province in the Caspian Sea littoral of northern Iran, between Caspian Sea and Alborz Mountain range, has coastal, plain, foothill, and mountainous areas with an area of about 14700km². The province is surrounded by Mazandaran Province in the east, Ardebil Province in the west and Zanzan and Qazvin Provinces in the south. It is also bordered by Azerbaijan Republic in the north as well as Russia across Caspian Sea (Fig. 1). Guilan Province with temperate climate and relatively warm-humid summer is located between 36°33'–38°27' N latitude and 48°32'–50°36' E longitude and formally composes 16 counties. Most areas of the province with about 1000–2000mm annual rainfall have the highest precipitation in Iran and the main agricultural crop is rice. Rudbar County is located in the southern part of Guilan Province with about 200–500 mm annual rainfall and showed mountainous and less humid temperate climate similar to the Mediterranean Region (Gilan Meteorological Organization). The county has an area of 2574km² and the main agricultural crop is olive.

Specimen and data collection

The specimens of phlebotomines were collected from 12 localities of Rudbar County by means of light traps, sticky traps and manual aspirators throughout August–December 2015

(Fig. 1) (Table 1). The collection localities were mostly selected based on the previous reported cases of leishmaniasis (Department of Disease Control and Prevention, Health Vice-Chancellorship of Guilan University of Medical Sciences and 24). Two CDC light traps and 50 sticky traps were used in each locality. The light traps were suspended from ceiling in animal (sheep, cattle and poultry) shelters (near houses) from sunset to sunrise, from 18:00 PM to 06:00 AM. The electricity of traps was provided by 6-volt rechargeable batteries. Sticky papers consisted of 10x15cm white sheets coated with castor oil. Sticky traps were used in animal shelters and yards around animal shelters and houses. Also ad hoc hand catch collections were performed by manual aspirators. Phlebotomines were removed from the sticky traps and separated from other insects, rinsed in acetone and then preserved in 80% ethanol as well as the collections of light traps and hand catches. The microscope slides of all specimens were prepared using Puri's fluid. The specimens were identified using the morphological-based keys of Perfil'ev (29), Nadim and Javadian (30), Lewis (31), Seyedi Rashti and Nadim (32), Rassi and Hanafi-Bojd (33) and Zahraei-Ramazani et al. (12). Also, Absavaran et al. (18), Léger et al. (34) and Killick-Kendrick et al. (35) were consulted for differentiating the females of the subgenus *Larrousius*. Galati et al. (1) was followed for the phlebotomine name genera and subgenera abbreviations. The sex ratio (males: females) was calculated for each species. Sampling ratio (samples of sticky traps/ samples of light traps: ST/LT) was also calculated.

Species dominance structure

The dominance structure of a species is described as the percent of specimens of the species in the whole specimens. The following five dominance structure categories were used according to Tischler (36) and Heydemann (37): eudominant (ED) species (> 30%), dominant (D) (10–30%), subdominant (SD) (5–10%), recedent (R) (1–5%) and subrecedent

(SR) (< 1%).

Gonotrophic cycle stages of female sand flies

Abdominal appearance of female phlebotomines is used for determining ovary development status and follows the same stages of *Anopheles* (29). World Health Organization (38) introduced a simplified classification for abdominal appearances follows: empty or unfed (U), freshly fed (F), semi-gravid (SG) and gravid (G).

Density of sand flies

The following formulae were used to calculate the mean density of sand flies collected by sticky traps: Density (D_n)=number of specimens/number of sticky traps, Density (D_{m2})=number of specimens/m² of sticky traps (39–40). The density was corrected according to the following formula (41): Density (D_s)=

$$\sqrt{1 + \frac{\text{number of sand flies}}{\text{number of traps}} \times 100}$$

Results

Sand fly inventory

In total, 2186 sand flies (896 males, 1290 females) were collected during 8 surveys from 11 localities during August–December 2015 (Tables 2 and 3). Ten species representing two genera were found. Only 0.42% of the specimens belonged to the genus *Sergentomyia* and 99.58% to the genus *Phlebotomus* (Table 2). The species *Ph. halepensis*, *Ph. neglectus*, *Ph. perfiliewi*, *Se. baghdadis*, *Se. clydei* and *Se. tiberiadis* were found for the first time in Guilan Province.

The overall abundance of gonotrophic cycle stages of female phlebotomines based on abdominal appearance was presented in table 4. In total, 71.6% of female sand flies were unfed. The percentages of unfed female sand flies were 75 and 71.6 for *Sergentomyia* and *Phlebotomus*, respectively. The percentages were 79.8 and 57.7 for the most abundant species of *Ph. perfiliewi* and *Ph. tobbi*, respectively (Table 4).

The overall sex ratio (M: F) of sand flies was 0.69. The ratio was 1.25 and 0.69 for *Sergentomyia* and *Phlebotomus*, respectively (Table 2). Regarding the trapping method, the sex ratio of *Sergentomyia* specimens was 1.33 and 1 using light traps and sticky traps, respectively. The ratio was 0.55, 10.88 and 0.48 using light traps, sticky traps and hand catches, respectively, for *Phlebotomus* (Table 5).

General sampling ratio of phlebotomines (ST/LT) was 0.11 and the ratio was 0.28 and 0.11 for *Sergentomyia* and *Phlebotomus*, respectively. 22.2% of *Sergentomyia* specimens were collected by sticky traps and 77.8% by light traps. The percentages were 9.8 and 87.5 for *Phlebotomus*, respectively. No *Sergentomyia* specimen was collected by hand catches. Regarding the collection methods and sex of sand flies, 64.4% and 67.2% of sand flies were females when light traps and hand catches were used, respectively. However, 91.2% of collected sand flies by sticky traps were male (Table 5).

The subgenus *Larroussius* with four species and 2150 (98.4%) specimens of the total collection was eudominant. The sex ratio (M: F) was calculated 0.68 (874: 1276) for the subgenus (Table 2). The species belong to this subgenus showed the widest distribution and were collected from all localities, except for Rostamabad (Table 3). Regarding the collection methods, most of the *Larroussius* species, 87.5% (1882 specimens), were collected by light

traps and 9.8% (210) and 2.7% (58) by sticky traps and manual aspirators, respectively (Table 5). The subgenus showed the most average density (134.42) in light traps.

The species *Ph. halepensis*, *Se. clydei* and *Se. tiberiadis* were collected only by light traps and ad hoc collections by manual aspirators yielded only *Ph. perfiliewi* and *Ph. tobbi* (Tables 5 and 6). *Phlebotomus perfiliewi* and *Ph. tobbi* were the most abundant and eudominant species and showed the widest distribution in the studied areas (Tables 2 and 3). Also, both species showed the most density in sticky and light traps (Table 6).

Taxonomic note

The subgenus *Larroussius* species (*Ph. kandelakii*, *Ph. neglectus*, *Ph. perfiliewi* and *Ph. tobbi*) were distinguished mostly by using the morphology of parameral sheath (aedeagus) and coxite in the males and the base of spermathecal ducts, pharyngeal teeth (pharyngeal armature) and spermatheca segment numbers in the females (Figs. 2–5). The ranges, numbers and means of spermatheca segments were as follow: *Ph. kandelakii* (27–33, n=4, mean=28.5, SD=3, SE=1.5), *Ph. neglectus* (11, n=1), *Ph. perfiliewi* (12–20, n=20, mean=15.35, SD=1.66, SE=0.37) and *Ph. tobbi* (9–15, n=20, mean=12.26, SD=1.62, SE=0.37). This study also verified the presence of *Ph. neglectus* (*Ph. major krimensis* as a synonym and morphotype) in Iran.

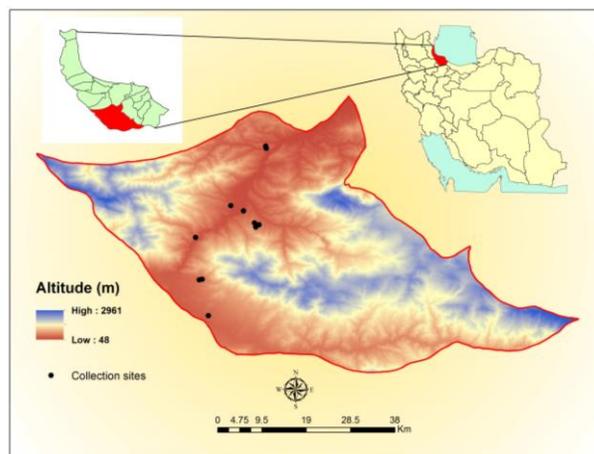


Fig. 1. Map of Iran highlighting Guilan Province and its 16 counties, including Rudbar County, surveyed in 2015

Table 1. The data collection of phlebotomines for localities in Rudbar County of Guilan Province, Iran, August–December 2015

Locality (City/ Village)	Topography	Coordinates	Altitude (m)
Rostamabad	Plain	36° 52.999' N, 49° 29.385' E	215
Khaskool	Foothill	36° 50.789' N, 49° 32.669' E	470
Lafandsara	Foothill	36° 50.522' N, 49° 32.271' E	620
Rudbar	Foothill	36° 49.314' N, 49° 25.322' E	270
Klayeh	Foothill	36° 50.992' N, 49° 32.132' E	438
Rudabad	Plain	36° 52.397' N, 49° 30.871' E	192
Harkian	Foothill	36° 59.592' N, 49° 33.491' E	149
Siahroodposhteh	Foothill	36° 59.862' N, 49° 33.432' E	269
Upper Harzavil (Manjil)	Foothill	36° 44.495' N, 49° 26.072' E	506
Lower Harzavil (Manjil)	Foothill	36° 44.837' N, 49° 25.735' E	453
Halaj (Loshan)	Foothill	36° 40.306' N, 49° 26.792' E	307
Pareh	Foothill	36° 50.800' N, 49° 32.650' E	487

Table 2. The abundance and dominance structure of phlebotomines in Rudbar County, Guilan Province, Iran, 2015

Species	Males	Females	Sex ratio (M:F)	Total	%	Dominance structure
<i>Ph. (Adl.) halepensis</i>	6	-	6	6	0.27	subrecedent
<i>Ph. (Adl.) sp.</i>	-	1	-	1	0.05	subrecedent
<i>Ph. (Lar.) kandelakii</i>	-	2	-	2	0.10	subrecedent
<i>Ph. (Lar.) neglectus</i>	18	2	9	20	0.91	subrecedent
<i>Ph. (Lar.) perfiliewi</i>	360	818	0.44	1178	53.88	Eudominat
<i>Ph. (Lar.) tobbi</i>	496	454	1.09	950	43.45	Eudominat
<i>Ph. (Par.) sergenti</i>	10	8	1.25	18	0.82	subrecedent
<i>Ph. (Phl.) papatasi</i>	1	1	1	2	0.10	subrecedent
<i>Se. (Par.) baghdadis</i>	4	2	2	6	0.27	subrecedent
<i>Se. (Sin.) clydei</i>	1	-	-	1	0.05	subrecedent
<i>Se. (Sin.) tiberiadis</i>	-	2	-	2	0.10	subrecedent
Total	896 (41.0%)	1290 (59.0%)	0.69	2186	100	-

Table 3. The distribution of phlebotomines in Rudbar County, Guilan Province, Iran, 2015

Species	Locality										
	Rudbar	Pareh	Lafandsara	Khaskool	Klayeh	Rudabad	Harkian	Siahroodposhteh	Upper Harzavil	Lower Harzavil	Halaj (Loshan)
<i>Ph. (Adl.) halepensis</i>	-	-	-	-	-	-	-	-	*	-	-
<i>Ph. (Adl.) sp.</i>	-	-	-	-	*	-	-	-	-	-	-
<i>Ph. (Lar.) kandelakii</i>	-	*	*	-	-	-	-	-	-	-	-
<i>Ph. (Lar.) neglectus</i>	-	*	-	*	*	*	-	*	*	-	-
<i>Ph. (Lar.) perfiliewi</i>	-	*	*	*	*	-	-	-	-	-	-
<i>Ph. (Lar.) tobbi</i>	*	*	*	*	*	*	*	*	*	*	*
<i>Ph. (Par.) sergenti</i>	*	-	-	-	*	*	-	-	*	-	*
<i>Ph. (Phl.) papatasi</i>	-	-	-	-	-	-	-	-	*	-	*
<i>Se. (Par.) baghdadis</i>	-	-	-	-	-	-	-	-	-	-	*
<i>Se. (Sin.) clydei</i>	-	-	-	-	-	-	-	-	-	-	*
<i>Se. (Sin.) tiberiadis</i>	-	-	-	-	-	-	-	-	-	-	*

Table 4. The abundance of gonotrophic cycle stages of female phlebotomines based on abdominal appearance in Rudbar County, Guilan Province, Iran, 2015 (*U: Unfed, F: Fed, SG: Sub-gravid, G: Gravid)

Species	Abdominal appearance (%)				Total	%
	U*	F	SG	G		
<i>Ph. (Adl.) sp.</i>	-	-	-	1 (100)	1	0.07
<i>Ph. (Lar.) kandelakii</i>	-	-	2 (100)	-	2	0.16
<i>Ph. (Lar.) neglectus</i>	1 (50.0)	-	-	1 (50.0)	2	0.16
<i>Ph. (Lar.) perfiliewi</i>	653 (79.8)	48 (5.9)	97 (11.9)	20 (2.4)	818	63.41
<i>Ph. (Lar.) tobbi</i>	262 (57.7)	67 (14.8)	94 (20.7)	31 (6.8)	454	35.19
<i>Ph. (Par.) sergenti</i>	5 (62.5)	1 (12.5)	-	2 (25.0)	8	0.62
<i>Ph. (Phl.) papatasi</i>	-	-	-	1 (100)	1	0.07
<i>Se. (Par.) baghdadis</i>	2 (100)	-	-	-	2	0.16
<i>Se. (Sin.) tiberiadis</i>	1 (50.0)	1 (50.0)	-	-	2	0.16
Total	924 (71.6)	117 (9.1)	193 (15.0)	56 (4.3)	1290	100

Table 5. The abundance of phlebotomines based on collection method in Rudbar County, Guilan Province, Iran, 2015

Species	Method of Collection (%)						Total (%)
	Light Trap		Sticky Trap		Hand Catch		
	Male	Female	Male	Female	Male	Female	
<i>Ph. (Adl.) halepensis</i>	6	-	-	-	-	-	6 (0.27)
<i>Ph. (Adl.) sp.</i>	-	1	-	-	-	-	1 (0.05)
<i>Ph. (Lar.) kandelakii</i>	-	1	-	1	-	-	2 (0.10)
<i>Ph. (Lar.) neglectus</i>	9	2	9	-	-	-	20 (0.91)
<i>Ph. (Lar.) perfiliewi</i>	217	773	130	14	13	31	1178 (53.88)
<i>Ph. (Lar.) tobbi</i>	437	443	53	3	6	8	950 (43.45)
<i>Ph. (Par.) sergenti</i>	7	8	3	-	-	-	18 (0.82)
<i>Ph. (Phl.) papatasi</i>	-	1	1	-	-	-	2 (0.10)
<i>Se. (Par.) baghdadis</i>	3	1	1	1	-	-	6 (0.27)
<i>Se. (Sin.) clydei</i>	1	-	-	-	-	-	1 (0.05)
<i>Se. (Sin.) tiberiadis</i>	-	2	-	-	-	-	2 (0.10)
Total (%)	680 (35.6)	1232 (64.4)	197 (91.2)	19 (8.8)	19 (32.8)	39 (67.2)	2186 (100)
	1912 (87.5)		216 (9.9)		58 (2.6)		

Table 6. The mean density of phlebotomines in Rudbar County, Guilan Province, Iran, 2015 [Density (Dn)=number of specimens/number of sticky traps, Density (Dm2)=number of specimens/m2 of sticky traps, corrected density (Ds)=√(1+(number of sand flies/number of traps)×100)]

Species	Sticky Trap			Light Trap
	Dn	Dm2	Ds	
<i>Ph. (Adl.) halepensis</i>	-	-	-	0.42
<i>Ph. (Adl.) sp.</i>	-	-	-	0.07
<i>Ph. (Lar.) kandelakii</i>	0.002	0.09	1.09	0.07
<i>Ph. (Lar.) neglectus</i>	0.025	0.81	1.87	0.78
<i>Ph. (Lar.) perfiliewi</i>	0.411	13.06	6.48	70.71
<i>Ph. (Lar.) tobbi</i>	0.160	5.08	4.12	62.85
<i>Ph. (Par.) sergenti</i>	0.008	0.27	1.34	1.07
<i>Ph. (Phl.) papatasi</i>	0.002	0.09	1.09	0.07
<i>Se. (Par.) baghdadis</i>	0.005	0.18	1.22	0.28
<i>Se. (Sin.) clydei</i>	-	-	-	0.07
<i>Se. (Sin.) tiberiadis</i>	-	-	-	0.14
Mean total	0.610	19.60	7.78	136.57

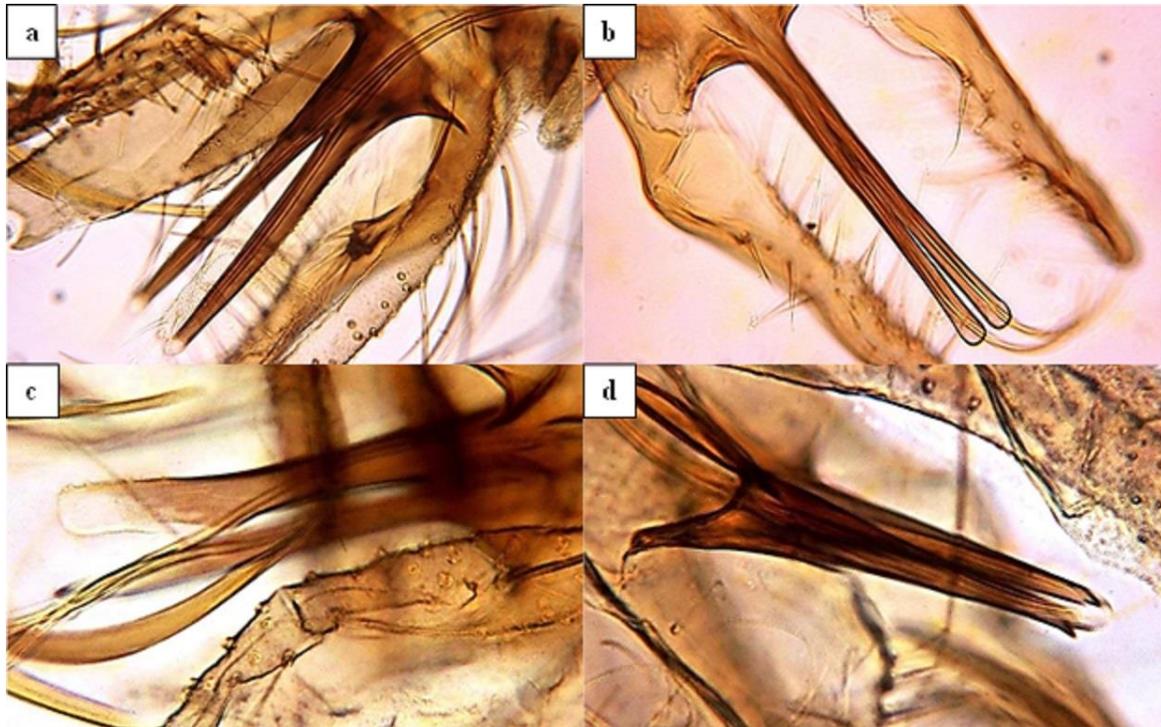


Fig. 2. The parameral sheaths of the subgenus *Larrousius* species in Rudbar County, Guilan Province, Iran, 2015, a: *Phlebotomus kandelakii*, b: *Ph. neglectus*, c: *Ph. perfiliewi*, d: *Ph. tobbi* (original photos)

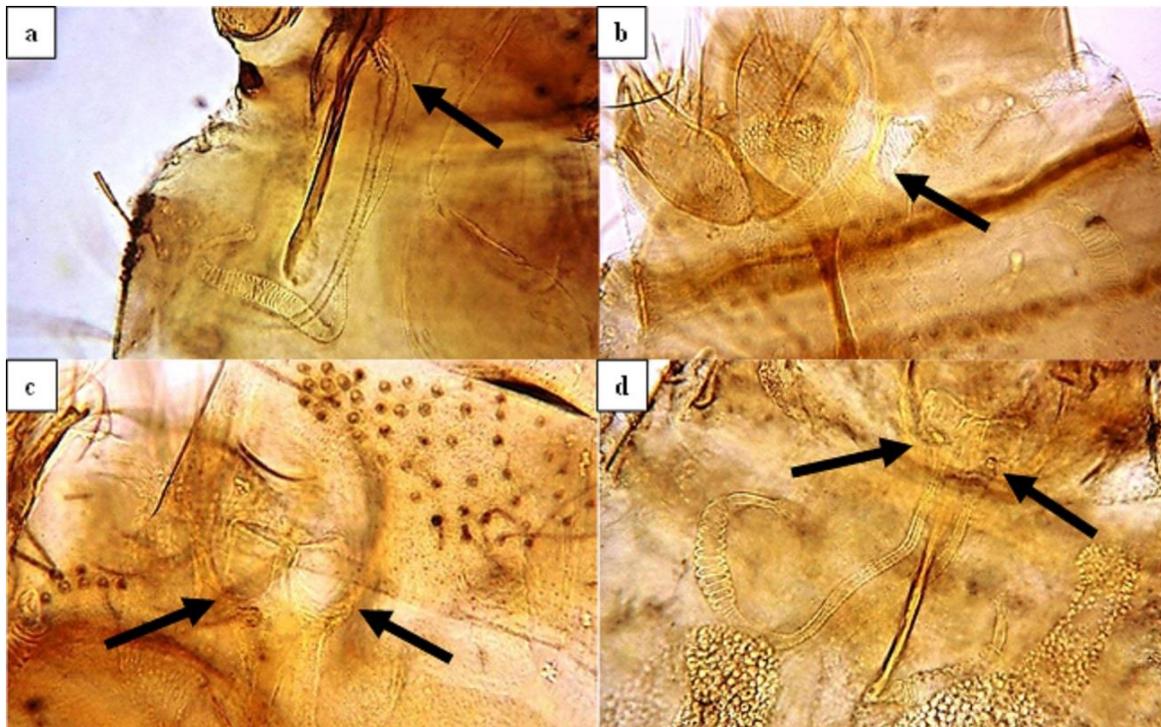


Fig. 3. The bases of spermathecal ducts (showed by arrows) and spermathecae of the subgenus *Larrousius* species in Rudbar County, Guilan Province, Iran, 2015, a: *Phlebotomus kandelakii*, b: *Ph. perfiliewi*, c and d: *Ph. tobbi* (original photos)

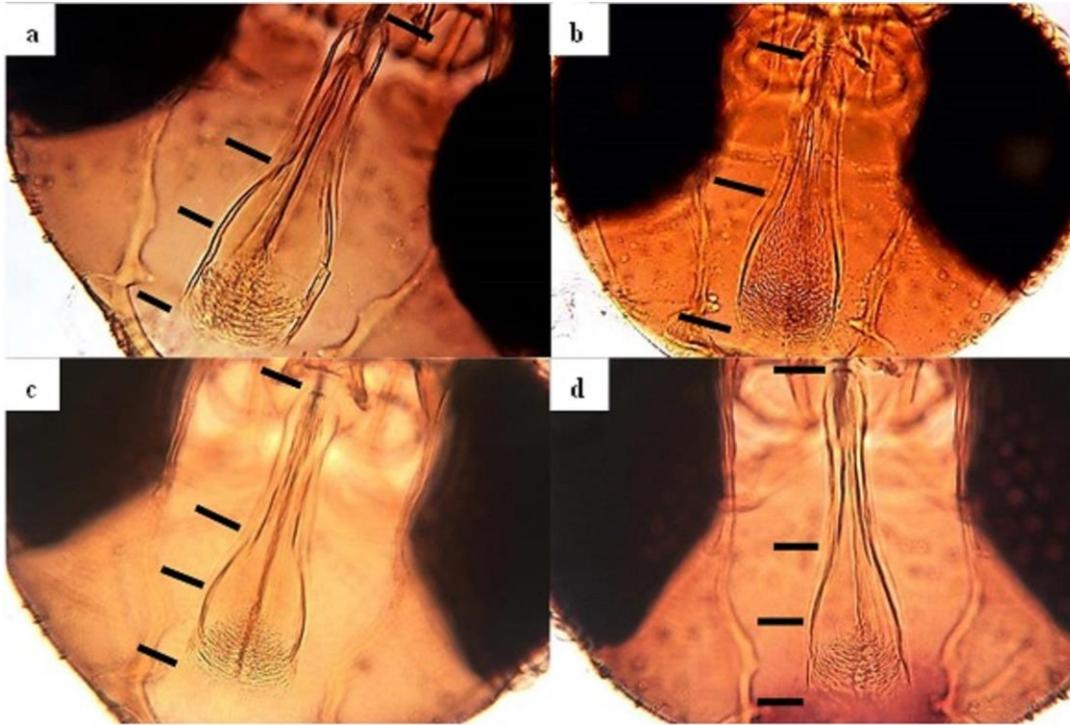


Fig. 4. The pharyngeal teeth of the subgenus *Larrousius* species in Rudbar County, Guilan Province, Iran, 2015, a: *Phlebotomus kandelakii*, b: *Ph. neglectus* (*Ph. major krimensis* morphotype), c: *Ph. perfiliewi*, d: *Ph. tobbi* (Dashes show the pharynx, the wide part of pharynx and pharyngeal teeth) (original photos)



Fig. 5. The ventrally-directed setae of coxite (showed by arrow) in *Phlebotomus neglectus* (*Ph. major krimensis* morphotype), Rudbar County, Guilan Province, Iran, 2015 (original photo)

Discussion

Sand fly inventory

During this study 10 species representing two genera were identified in which six species were new records for the sand fly fauna of Guilan Province.

Considering the role of sand flies in disease transmission, among collected phlebotomines, two species are known the vectors of CL in Iran: *Ph. sergenti* (suspected) (42-43) and *Ph. papatasi* (proven) (42, 44) and four species the suspected vectors of VL: *Ph. kandelakii* (45-46), *Ph. neglectus* (as *Ph. major* s.l.) (47-48), *Ph. perfiliewi* (45, 49) and *Ph. tobbi* (50). *Phlebotomus halepensis* is known a suspected or proven vector of VL in other countries of the western Palaearctic Region (4). In view of the occurrence of four suspected vectors of VL in Guilan Province and locating one of the most important foci of VL, Ardebil Province (51) in the west of Guilan Province, this can be very important for the health system of Guilan Province. *Sergentomyia clydei* is assumed to play a role in the transmission of lizard leishmaniasis in Iran (28, 42, 52). Also, *Ph. papatasi* is known a vector of sand fly fever (27).

In the present investigation, the sand flies of the genus *Sergentomyia* were collected in a very low prevalence (0.42%) (Table 2). Other investigations in northwestern Iran also showed the low abundance of the genus in comparison to the genus *Phlebotomus*, for example: 4.3% using just sticky traps (49) and 9.2% by means of both light traps and sticky traps (53). However, much more specimens of *Sergentomyia* (29.62%) were collected in an arid area of Iran (Qom Province) using sticky traps (13). Many *Sergentomyia* species mainly inhabit natural microhabitats, for example the burrows of animals and caves (29). In this regard, a large amount of *Sergentomyia* species have been found in rodent burrows in Iran (52-53) where were not studied in the present investigation. Also, *Sergentomyia* species usually suck blood from reptiles and *Phlebotomus* species principally from warm-blooded vertebrates (2). In the present

study, the collections were performed mostly in domesticated animal shelters and yards around those shelters and houses that is may be another reason of low abundance of *Sergentomyia* specimens. Interestingly, in this investigation, all species of *Sergentomyia* and *Ph. papatasi* were sampled only from Loshan City (Halaj) and Manjil City (Lower and Upper Harzavil) with a more arid climate than other localities (Table 3). It seems that *Ph. papatasi* mostly inhabits semi-arid areas (53) and high precipitation is a limiting factor in the distribution of the species (29). Despite the vast distribution of *Ph. papatasi* in Iran, the species is infrequent in the lowland and humid areas of Guilan Province with high precipitation (54-55). In contrast, the species of the subgenus *Larrousius* mainly inhabit regions with higher humidity and are intermediate between xerophilous and hygrophilous species (29). That might be the reason why the subgenus composed the majority of the specimens (98.4%) in the present investigation (Table 2). Another important factor is the collection method. Many species of *Sergentomyia* and the species *Ph. papatasi* and *Ph. sergenti* may not be or less attracted by artificial lights in contrast to some species of *Larrousius* (*Ph. major* s.l., *Ph. kandelakii* and *Ph. perfiliewi*) and the subgenus *Adlerius* (29). That may explain the high prevalence of phlebotomines sampled by light traps (87.5%) (Tables 5 and 6). In this investigation, the sampling ratio (ST/LT) was more in *Sergentomyia* (0.28) than *Phlebotomus* (0.11) which is similar to the previous results in northwestern Iran (53). Moreover, the majority of females were unfed (Table 4). That was also seen in the phlebotomine collection by light traps in northwestern Iran (53). In this study, the numbers of *Sergentomyia* specimens and the specimens collected by manual aspirators were a few (Table 2), however *Phlebotomus* displayed different sex ratio based on the collection method. The ratio was very lower in the collection

by light traps (0.55) than sticky traps (10.88). In a study in northwestern Iran, the major part of phlebotomines collected by light traps was female and by sticky traps was male (53). They indicated sex ratio 1.3 for *Phlebotomus* and 1.1 for *Sergentomyia*. This higher abundance of males to females may be explained by the higher collection of phlebotomines by sticky traps (2701) than light traps (1281) in that study (53).

The females of the subgenus *Adlerius* are not identifiable (10), and the species *Ph. halepensis* was identified based on the male characters in the present investigation (Tables 2 and 3). This species is the most prevalent and widespread species of the subgenus in Iran (10). Three other species of the subgenus: *Ph. balcanicus*, *Ph. brevis* and *Ph. longiductus* which are found in Ardebil Province (10) adjoining the west of Guilan Province could also be found in Guilan Province.

In the present study, the subgenus *Larrousius* and especially the species *Ph. perfiliewi* and *Ph. tobbi* were the most abundant and widespread sand flies (Tables 2–6). The previous findings displayed that *Ph. perfiliewi* and *Ph. kandelakii* were the most abundant species in northwestern Iran (45, 53). In the present investigation, *Ph. kandelakii* was found in a lower prevalence in comparison to *Ph. perfiliewi* and *Ph. tobbi* (Tables 2–6). This species had also been introduced as an infrequent species around Rasht and Anzali of Guilan Province before (8).

Taxonomic note

The major group of the subgenus *Larrousius* includes *Ph. major* (type locality in India), *Ph. neglectus* (type locality in Albania, former Yugoslavia, Italy, including *Ph. major krimensis* as a synonym, type locality in the former USSR), *Ph. notus* (type locality in Afghanistan), *Ph. syriacus* (type locality in Palestine and Syria), *Ph. wenyoni* (type locality in Iran) and probably *Ph. wui* (type locality in China) (8). There are controversial debates about the situation of *Ph. wui*. Lewis (31) considered it a subspecies of *Ph. major* as it was

originally described. Artemiev and Neronov (56), Léger and Pesson (57), Xiang and Jin (58) raised it to specific rank, but Leng et al. (59) synonymized it with *Ph. smirnovi*. On the other hand, Killick-Kendrick et al. (35) considered it outside the major group because they assumed a bell-shaped spermathecal duct for the species. Four species of the major group; *Ph. major*, *Ph. neglectus*, *Ph. notus*, and *Ph. wenyoni* have been reported in Iran by now (5, 9, 18). In view of wide distribution of *Ph. syriacus* in the Mediterranean and Caucasus regions (31), this species may be occurring in Iran.

The identification of male sand flies of *Larrousius* is usually not difficult, but some female species of the subgenus are not easily differentiated (35). The key of Nadim and Javadian (30) does not differentiate the female sand flies of *Ph. perfiliewi* and *Ph. tobbi*. The keys of Seyedi-Rashti et al. (32) and Rassi and Hanafi-Bojd (33) do not distinguish the females of *Ph. neglectus* (as *Ph. major*), *Ph. perfiliewi* and *Ph. tobbi* from each other. Morphological characters which are used to identify the female sand flies of the subgenus are: pharyngeal teeth (pharyngeal armature), spermatheca segment numbers, length of spermathecal neck, palpal and ascoid formulae, length of wings, base of spermathecal ducts (18, 29, 31, 34, 35). Though, the shape of base of spermathecal ducts is mentioned as a reliable character (34–35) that is not used in the keys to identify the female sand flies of the subgenus in Iran (30, 32, 33). In the areas where different species of *Larrousius* sympatrically occur, including the VL foci of northwestern Iran, the shape of base of spermathecal ducts has not been used to identify the females of the subgenus in many studies (18, 50). As far as the authors know only Akhoundi et al. (53) noted and used that character in their investigation.

During the present study, the male sand flies of *Larrousius* were easily differentiated using the parameral sheath (adeagus) morphology (Fig. 2). The females of *Ph. kandelakii* were easily identified based on the number of spermatheca

segments and the base of spermathecal ducts which is simple (Fig. 3a) without a common duct or lateral structure (Fig. 3b) or bell-shaped duct opening (Fig. 3c, d). The number of spermatheca segments of *Ph. kandelakii* is mentioned about 30 by Perfil'ev (29) and 30–35 by Artemiev (7) and Lewis (31). In the present investigation, the number was 27 in three specimens and 33 in one specimen. The mean number of spermatheca segments is less in *Ph. tobbi* (mean=12.26, SD=1.62, SE=0.37) than *Ph. perfiliewi* (mean=15.35, SD=1.66, SE=0.37), but because of overlap it cannot be used as an exact character of differentiation. Unfortunately, the number of females of *Ph. neglectus* was not enough in this investigation to provide range and mean for this character. Léger et al. (34) and Killick-Kendrick et al. (35) have mentioned that the base of spermathecal ducts is a very reliable character to differentiate aforementioned species (Fig. 3), although this character is not easy to see in some specimens, especially when they are not dissected or in gravid or semi-gravid specimens. That is why no photo of the base of spermathecal duct for *Ph. neglectus* is presented here. However, the character could be seen even in the microscope slides provided by Puri's medium using usual microscopes. The main feature of differentiation of females of *Larroussius* species in this investigation was the base of spermathecal duct and then other characters were checked. The pharyngeal teeth (pharyngeal armature) of the present investigation specimens for *Ph. neglectus* (Fig. 4b) are exactly similar to the subspecies *Ph. major krimensis* described by Perfil'ev (29) (Plate III, B). Perfil'ev (29, 60) mentioned that the females of *Ph. major krimensis* differ from other subspecies or species of the major group by the form of pharyngeal teeth which occupy the whole wide part of pharynx which was exactly observed in this investigation (Fig. 4b). In males, another specific character of *Ph. major krimensis* is about 20 ventrally-directed setae situated in the middle of inner surface of coxite which forms

a sparser group of setae than in other species of the major group (29, 60). This feature is also observed in the collected specimens of this study (Fig. 5). Lewis (31) considered *Ph. major krimensis* a subspecies of *Ph. major* as it was originally described by Perfil'ev (29), but Artemiev and Neronov (56) synonymized *Ph. major krimensis* with *Ph. neglectus*. On the other hand, there is a suggestion for a possible species rank (*Ph. krimensis*) for it (40). Badakhshan et al. (9) reported two morphotypes using the male terminalia characters (the shape of parameral sheath and the ventrally-directed setae of coxite) including the morphotype *Ph. major krimensis* (as a synonym of *Ph. neglectus*) and another morphotype including *Ph. neglectus* or *Ph. notus*. They reported *Ph. notus* for the first time in Iran. According to aforementioned differentiating morphological characters which observed in the present study and by Badakhshan et al. (9) and Absavaran et al. (18), the presence of *Ph. major krimensis* (as a synonym and morphotype of *Ph. neglectus*) verifies in Iran.

Other morphological characters which are used to identify the females of *Larroussius* including the length of spermathecal neck, palpal and ascoid formulae and length of wings (18, 29, 31, 34, 35) were not found to be reliable enough for the identification in the study area.

Léger et al. (34) and Killick-Kendrick et al. (35) provided the figures of the base of spermathecal duct for *Ph. tobbi* using the specimens from Syria and Greece. In the present article, the photos (Figs. 2–4) were taken from specimens collected from localities close to the type locality (Rasht of Guilan Province).

Conclusion

This is the first investigation of sand flies in Guilan Province which includes six new records for the province. This study verifies the presence of *Ph. neglectus* (*Ph. major krimensis* as a synonym and morphotype) in Iran.

More investigation on the major group using morphological and molecular data is suggested. All collected *Phlebotomus* species are suspected or proven vectors of CL and/ or VL in Iran or adjoining countries. The investigation of bionomics of suspected or proven vectors and detecting the exact vectors of leishmaniasis and three-day fever by means of molecular specific tests in Guilan Province should be the subject of future studies.

Acknowledgements

The research was supported financially by the Research Vice-Chancellorship of Guilan University of Medical Sciences (Project Number: 93062502). The authors appreciate the administrative support of Dr SM Rezvani, Mr M Mirzanejad, Mr M Moslem and Mr A Rasaei, Department of Disease Control and Prevention, Health Vice-Chancellorship of Guilan University of Medical Sciences, and the personnel of aforementioned vice-chancellorship for their cooperation in the field. The project was carried out in the Research Center of Health and Environment, School of Health, Guilan University of Medical Sciences, with the collaboration of Health Vice-Chancellorship of Guilan University of Medical Sciences.

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