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

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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 (Adleriuss) halepensis (0.27%), Ph. (Larroussius) kandeladii (0.10%), Ph. (Lar.) neglectus (0.91%), Ph. (Lar.) perfliewi (53.88%), Ph. (Lar.) tobbi (43.45%), Ph. (Paraphlebotomus) sergenti (0.82%), Ph. (Phlebotomus) papataci (0.10%), Sergentomyia (Parrotomyia) baghdadis (0.27%), Se. (Sintonius) clydei (0.05%) and Se. (Sin.) tibieriadis (0.10%). The species Ph. halepensis, Ph. neglectus, Ph. perfliewi, Se. baghdadis, Se. clydei and Se. tibieriadis 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 classification 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 comprises 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 Larroussi 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. wenyon [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. sergentii 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 Zanjan 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 200–500mm 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.
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), Seyed 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 Larroussius. 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): eudominat (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 ($D_c$)=

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

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. halensis, Ph. neglectus, Ph. perfiliewi, Se. baghdadis, Se. clydei and Se. tiberiadi 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. kendelakii, Ph. neglectus, Ph. perfiliewi and Ph. tobbi) were distinguished mostly by using the morphology of parameral sheath (aedagus) 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. kendelakii (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.

![Map of Iran highlighting Guilan Province and its 16 counties, including Rudbar County, surveyed in 2015](http://jad.tums.ac.ir)

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

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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          |
| Klayah                  | 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          |
| Parch                   | 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 |
|-----------------------------|-------|---------|----------------|-------|---|---------------------|
| Ph. (Adl.) halepensis       | 6     | 1       | 6              | 6     | 0.27 | subrecedent         |
| Ph. (Adl.) sp.              | -     | 1       | -              | 1     | 0.05 | subrecedent         |
| Ph. (Lar.) kandelakii       | -     | 2       | -              | 2     | 0.10 | subrecedent         |
| Ph. (Lar.) neglectus        | 18    | 2       | 9              | 20    | 0.91 | subrecedent         |
| Ph. (Lar.) perfiliewi       | 360   | 818     | 0.44           | 1178  | 53.88| Eudominat           |
| Ph. (Lar.) tobbi            | 496   | 454     | 1.09           | 950   | 43.45| Eudominat           |
| Ph. (Par.) sergenti         | 10    | 8       | 1.25           | 18    | 0.82 | subrecedent         |
| Ph. (Phl.) papatasi         | 1     | 1       | 1              | 2     | 0.10 | subrecedent         |
| Se. (Par.) baghdadis        | 4     | 2       | 2              | 6     | 0.27 | subrecedent         |
| Se. (Sin.) clydei           | 1     | -       | -              | 1     | 0.05 | subrecedent         |
| Se. (Sin.) tiberiadi        | -     | 2       | -              | 2     | 0.10 | subrecedent         |
| **Total**                   | 896   | 1290    | 0.69           | 2186  | 100  | -                   |

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

| Species                     | Locality     |
|-----------------------------|--------------|
| Ph. (Adl.) halepensis       | Rudbar       |
| Ph. (Adl.) sp.              | Parch        |
| Ph. (Lar.) kandelakii       | Lafandsara   |
| Ph. (Lar.) neglectus        | Khaskool     |
| Ph. (Lar.) perfiliewi       | Klayah       |
| Ph. (Lar.) tobbi            | Rudabad      |
| Ph. (Par.) sergenti         | Harkian      |
| Ph. (Phl.) papatasi         | Siahroodposhteh |
| Se. (Par.) baghdadis        | Upper Harzavil |
| Se. (Sin.) clydei           | Lower Harzavil |
| Se. (Sin.) tiberiadi        | Halaj (Loshan) |

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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 |                 |
| Ph. (Adl.) sp.           | -  | - | -  | 1 (100) | 1 (0.07) |
| Ph. (Lar.) kandelakii    | -  | - | -  | 2 (100) | 2 (0.16) |
| Ph. (Lar.) neglectus     | 1 (50.0) | - | -  | 1 (50.0) | 2 (0.16) |
| Ph. (Lar.) perfiliewi    | 653 (79.8) | 48 (5.9) | 97 (11.9) | 20 (2.4) | 818 (63.41) |
| Ph. (Lar.) tobbi         | 262 (57.7) | 67 (14.8) | 94 (20.7) | 31 (6.8) | 454 (35.19) |
| Ph. (Par.) sergenti      | 5 (62.5) | 1 (12.5) | -  | 2 (25.0) | 8 (0.62) |
| Ph. (Phl.) papatasi      | -  | - | -  | 1 (100) | 1 (0.07) |
| Se. (Par.) baghdadis     | 2 (100) | - | -  | -  | 2 (0.16) |
| Se. (Sin.) tiberiadi     | 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                  | Abdominal appearance (%) | Total (%) |
|--------------------------|--------------------------|-----------|
|                          | Light Trap | Sticky Trap | Hand Catch |          |
|                          | Male | Female | Male | Female | Male | Female |               |
| Ph. (Adl.) halepensis    | 6 (2.6) | 437 (20.6) | 217 (10.0) | 18 (0.82) |
| Ph. (Adl.) sp.           | 1 (0.5) | 2 (1.0)  | 7 (0.3)  | 18 (0.82) |
| Ph. (Lar.) kandelakii    | 2 (0.10) | 1 (0.5)  | 2 (0.10) | 18 (0.82) |
| Ph. (Lar.) neglectus     | 2 (0.10) | 2 (0.10) | 2 (0.10) | 18 (0.82) |
| Ph. (Lar.) perfiliewi    | 56 (2.6) | 354 (16.7) | 818 (38.8) | 18 (0.82) |
| Ph. (Lar.) tobbi         | 354 (16.7) | 18 (0.82) | 18 (0.82) | 18 (0.82) |
| Ph. (Par.) sergenti      | 31 (1.5) | 354 (16.7) | 818 (38.8) | 18 (0.82) |
| Ph. (Phl.) papatasi      | 31 (1.5) | 354 (16.7) | 818 (38.8) | 18 (0.82) |
| Se. (Par.) baghdadis     | 31 (1.5) | 354 (16.7) | 818 (38.8) | 18 (0.82) |
| Se. (Sin.) tiberiadi     | 31 (1.5) | 354 (16.7) | 818 (38.8) | 18 (0.82) |
| Total                    | 680 (35.6) | 1232 (64.4) | 197 (91.2) | 19 (8.8) |

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)=\(\sqrt{1+\text{number of sand flies/number of traps}}\times100\)]

| Species                  | Sticky Trap | Light Trap |
|--------------------------|-------------|------------|
|                          | Dn | Dm2 | Ds |               |
| Ph. (Adl.) halepensis    | -  | -  | 0.42 |
| Ph. (Adl.) sp.           | -  | -  | 0.07 |
| Ph. (Lar.) kandelakii    | 0.002 | 0.09 | 1.09 | 0.07 |
| Ph. (Lar.) neglectus     | 0.025 | 0.81 | 1.87 | 0.78 |
| Ph. (Lar.) perfiliewi    | 0.411 | 13.06 | 6.48 | 70.71 |
| Ph. (Lar.) tobbi         | 0.160 | 5.08 | 4.12 | 62.85 |
| Ph. (Par.) sergenti      | 0.008 | 0.27 | 1.34 | 1.07 |
| Ph. (Phl.) papatasi      | 0.002 | 0.09 | 1.09 | 0.07 |
| Se. (Par.) baghdadis     | 0.005 | 0.18 | 1.22 | 0.28 |
| Se. (Sin.) clydei        | -  | -  | 0.07 |
| Se. (Sin.) tiberiadi     | -  | -  | 0.14 |
| Mean total               | 0.610 | 19.60 | 7.78 | 136.57 |
Fig. 2. The parameral sheaths of the subgenus *Larroussius* 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 *Larroussius* 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 *Larroussius* 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. kandekalii (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 Palearctic 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 Larroussius 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 Larroussius (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. halepenesis 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 Larroussius 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 Larroussius 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 Larroussius 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-Rashiti 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 ascod 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 Larroussius sympathetically 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 Larroussius were easily differentiated using the parameral sheeth (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. perfilievi (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 ascod 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.

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