Phlebotomus (Adlerius) kabulensis (Diptera: Psychodidae) a new record sand fly species from Iran: Morphological and molecular aspects

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ABSTRACT

Objective: To represent a new geographical record, Phlebotomus (Adlerius) kabulensis (P. kabulensis), which is suspected to be a potential vector of visceral leishmaniasis. Methods: For the first time, P. kabulensis specimens were collected using the sticky paper traps method in outdoor places in mountainous areas with vegetation coverage of three provinces in Iran. Identification of males was based on ecological, morphological, morphometric and molecular (mtDNA cytochrome b gene sequences) criteria. Generally, males have two ascoids on the 8th antennal segment and one ascoid on segments 9th to 15th, aedeagus with normal obtuse-angled sub-terminal notch and coxite with 27–50 groups of hairs on the distal half of its body. Results: Morphometric measurement revealed that P. kabulensis specimens were the same as compared with seven other morphological characters in three provinces of the country but lengths of the coxite were significantly different. The PCR result of the cytochrome b (Cyt b)-mtDNA fragment shows 550-bp length, with its special nucleotide arrangement. The male and female of P. kabulensis were newly discovered members of the subgenus Adlerius from Iran. Initial DNA analysis indicated how distinct this species is. Conclusions: The results show that the P. kabulensis female will be identified by comparing with other Adlerius female groups regarding its morphometric characters and molecular sequencing.

1. Introduction

Phlebotomine sand fly’s species recorded in Iran belong to the genera, Phlebotomus Rondani and Berté 1840, and Sergentomyia Franca and Parrot 1920[1]. The genus Phlebotomus has 12 subgenera[2]. Among them, the species of subgenus Adlerius Nitzulescu, in 1931 were reported to be the vectors of leishmaniasis and since then, a few studies have been carried out on this subgenus in Iran. Recently, eight species belonging to this subgenus: (i) Phlebotomus (Adlerius) balearicus Theodor, 1958; (ii) P. (Adl.) brevic Theodor and Mesghali, 1964; (iii) P. (Adl.) comatus Artemiev 1978; (iv) P. (Adl.) halepensis Theodor, 1958; (v) P. (Adl.) kabulensis Artemiev, 1978; (vi) P. (Adl.) longiductus Parrot, 1928; (vii) P. (Adl.) tarsicus Artemiev, 1974 and (viii) P. (Adl.) salangensis Artemiev, 1978 have been reported in Iran[3-5]. Phlebotomus chinensis Newstead, 1916 is the type-species of this subgenus[6] (Artemiev, 1980), the vector of Leishmania infantum (L. infantum) in China[7]. The Phlebotomine sand flies of the subgenus Adlerius involved have seldom been identified, because despite the taxonomic efforts of Artemiev (1980) and others, the morphological characters have not clearly been distinguished between the females of about 20 Adlerius species[8]. In addition, identification of male Adlerius by morphological means replace was difficult by remains.

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a difficult task. They differed slightly because some morphological characters were closely related, for instance the location and number of hairs on coxite are the most important morphological characteristic features in identifying and distinguishing Adlerius species from others. At times, it is important to identify the number and location of hairs on the body of different species of sand flies to check how similar or different they are. According to morphological keys, total number of coxite hairs in P. kabulensis are between 27–50 and in P. longiductus the hairs on coxite are 50–85; therefore, when a specimen collected has 50 hairs on its coxite, it is difficult to say which of the two species it belongs to. Also, the antennae of sand flies is the first morphological character; in preparing morphological slides careful handling and care are needed during collection and mounting because of their minute and delicate body parts. The antennae usually break and disappear. Without the antennae, species identification becomes difficult or impossible. Also, the females of the subgenus Adlerius do not have morphological keys for species identification and the inclusion of its species can be justified on the basis of characters of the males’ morphology[9,10]. Because morphological characters do not distinguish between species of the Adlerius subgenus, it is important to thoroughly revise the identification key based on the morphological characters. Additionally, access to modern molecular techniques and genetic information regarding these vectors and detailed classification of their systematic positions, it seems far too ambitious to state that genetic information will help to elucidate the biological, morphological, ecological and effective leishmaniasis control methods. In this study, we compared the morphological and morphometric characteristics of P. kabulensis male specimens and also extracted the sequence of cytochrome b (Cyt b) [Mitochondrial DNA (mtDNA)] gene of P. kabulensis sand fly in order to identify and distinguish females of this species in subsequent studies. Also, we compared the 68 sequences with that of species in the GenBank. This gene is normally 69 inherited maternally and had been used to relate geographical populations of many species of 70 Larroussius in the Mediterranean sub-region[11,12]. The aim of this study was to detect P. kabulensis from other Adlerus species regarding its morphometric characters and molecular sequencing.

2. Materials and methods

2.1. Study area

Sticky paper traps were used for collecting P. kabulensis specimens in mountainous areas with vegetation coverage (e.g., gardens, mountainous caves, reservoir hosts, trees, river side and stones) in outdoor places of Ilam province of Iran between 5 p.m. and 7 a.m. (33°38′14″ N, 46°25′21″ E and altitude: 1 381 m above sea level), Lorestan (33.4871° N, 48.3538° E and altitude: 4 050 m above sea level) and Esfahan (32°39′25″ N, 51°40′39″ E and altitude: 1 571 m above sea level) provinces. In Esfahan province, sand flies were collected in August 2016. In Lorestan and Ilam provinces, July 2016 was the time of collection.

2.2. Monitoring sand flies

Sand fly specimens were preserved in glass containers with 96% ethanol. Sand flies were washed twice with distilled water. The head as well as the 4th and the 5th ends alongside the genitalia of the male sand fly were mounted separately on every slide and two drops of puri’s medium were added and the cover slip was gently put on the specimen[10]. These slides of male sand flies were prepared for morphological identification and the morphometric measurements using standard keys[10,13-15]. Eight characters were measured on the 13 P. kabulensis specimens in order to know if there was any difference between these specimens in the three provinces. The morphometric measurements were done using ocular micrometer and Olympus Microscope (ch-2).

2.3. Statistical analysis

Begin sentence with difference of morphological measurements were carried out with ANOVA and Kruskal–Wallis test with a priori level of significance set at P<0.05. Given that statistical tests were significantly different, post Hoc-Bonferroni test was used[16]. For the molecular description and identification of P. kabulensis females by pair-wise alignment comparison in subsequent studies, the authors used DNA sequence of the males for the cyt-b-mtDNA gene. The remaining sections of the abdomen, wings and legs of the sand fly were preserved in 96% ethanol in 1.5 mL sterile micro tubes for DNA extraction. The sequences were compared with sequences available in GenBank using BLAST available on http://blast.ncbi.nlm.nih.gov.

3. Results

In total of 46 specimens of P. kabulensis were collected, of which 14 specimens were from Esfahan, 15 from Lorestan and 17 were from Ilam provinces. The main morphological characters of subgenus Adlerius were: the coxite has no lobe, the style bears five spines, and the paramere was not truncated and carries no ventral process. The most aedeagus form has a sub-terminal minute fin-like barb. The female’s spermatheca was incompletely segmented. In addition, the morphological description of male P. kabulensis was; presence of one ascoid on antennal segments 9–15, antenna 8 with two ascoids, aedeagus with normal obtuse-angled sub-
terminal notch, a group of 27–50 hairs on coxite with dark body, sub terminal tooth in aedeagus is 12 μm, coxite is moderately wide, distal border of the hairy spot is about 0.50% of the coxite and genital filament/genital pump was seven (Figures 1 and 2). Morphometric measurements among the specimens of *P. kabulensis* in the three provinces showed there was no significant difference between (i) the length of A3, (ii) the length of epipharynx, the length of surstyles, (iv) the length of styles, (v) the width of styles, (vi) the length of aedeagus, and (vii) the number of hairs on coxite (Table 1). Therefore, this indicates the *P. kabulensis* specimens in the three provinces were within the same population or there was adequate gene flow to keep the groups similar. But there was a significant difference between the lengths of the coxite of the specimens from the three provinces (*P* = 0.045). Post Hoc-Bonferroni test shows that the length of coxite of *P. kabulensis* specimens in Lorestan province was significantly more than that from Ilam province (Table 2). This showed that they were not morphologically similar judging from the length of the coxite. In the conduct of the molecular experiment, DNA was extracted from 12 specimens of *P. kabulensis* sand fly from which five were used for mtDNA-PCR. PCR product of a male specimen was sent to the Department of Genetics, Faculty of Health Tehran University of Medical Sciences (TUMS) for sequencing. In this study, PCR experiments for *Cyt b* gene, primers CB3-PDR and N1N-PDR were used. These primers amplified a fragment of 550 bp of the mitochondrial genome of Males specimens which were captured from mountainous areas with vegetation within Poldokhtar Township in Lorestan province. This was the first time the sequenced result of *P. kabulensis* has been submitted to the GenBank from Iran (ACCESSION NO: JX885994). The comparison alignment of the 528 nucleotides with the specimens in the GenBank using NCBI-BLAST software showed that was 87% similarity with *P. (Adl.) chinensis* with the accession number: HM747243.1 and E-value 2e-174 (Figure 3). The *P. kabulensis* specimens have been deposited in the corresponding author’s medical entomology laboratory in Tehran University of Medical Sciences, Iran.

### Table 1

Samples used in morphometric study of *P. (Adl.) kabulensis* specimens in Iran.

<table>
<thead>
<tr>
<th>Molecular no.</th>
<th>A3</th>
<th>Labrum</th>
<th>Coxite</th>
<th>Surstyle</th>
<th>Style</th>
<th>Aedeagus</th>
<th>Width of style (μm)</th>
<th>Number of setae</th>
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<tr>
<td>EN87</td>
<td>36</td>
<td>26</td>
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<td>37</td>
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<td>36</td>
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<td>16</td>
<td>3</td>
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### Table 2

Post Hoc-Bonferroni test results showing comparison of the differences of the length of coxite of *P. kabulensis* specimens.

<table>
<thead>
<tr>
<th>Province</th>
<th>Length of coxite (μm)</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esfahan</td>
<td>2.000 ±1.713 91*</td>
<td>-7.896–2.729 9</td>
</tr>
<tr>
<td>Lorestan</td>
<td>2.583 ±1.851 24a</td>
<td>-2.919–6.919 1</td>
</tr>
<tr>
<td>Ilam</td>
<td>2.000 ±1.713 91*</td>
<td>0.092–9.073 8</td>
</tr>
</tbody>
</table>

*a* Data are expressed as Mean±SEM. Data with the same small letter (superscript) are not statistically different, while those with different letters are statistically different (*P*<0.05).

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Figure 1. Male *P. (Adl.) kabulensis* Artemiev, 1978. (1A) Genital filament, genital pump and surstyle; (1B) Clypeus, epipharynx (labellum, maxilla) and palp; (1C) Dense group of 33 hairs on coxite; (1D) Length and width of aedeagus and paramere.

Figure 2. Drawing of coxite and dense group of hairs in *P. (Adl.) kabulensis* Artemiev, 1978.
The Phlebotomus genus entails all known medically important species in the Old World, including incriminated vectors of mammalian Leishmania species and of sand fly fever serogroup of arboviruses[17,18]. This genus included 12 subgenera and Adlerius Nitzulescu (1931) is one of the important subgenus in the Old World as it contains the species of leishmaniasis vectors[7]. It is a Central Asian species and it is possible that a number of Central Asiatic species have entered Afghanistan and Iran from central Asia. Artemiev (1978) revised the subgenus Adlerius species of Afghanistan. He captured P. (Adl.) kabulensis Artemiev (1978) which is found in dwellings and is rather thermophilic and hydropilic. Since this species belongs to the subgenus Adlerius; he said, perhaps it can transfer visceral leishmaniasis agents like other species of this subgenus. But so far this has not been reported. Generally, there are five factors that all must be satisfied for vector incrimination: finding parasite in wild specimens, transmission from host to vector (in the laboratory), transmission from vector to vertebrate (Lab.), correlation of vector presence with disease outbreak, and laboratory confirmation that life cycle of parasite can be maintained in the purported vector. Although finding the parasite in collected specimens from the wild does not necessarily indicate that the species is a vector. Before the present study, known sand fly species of the subgenus Adlerius fauna from Iran were four species and this has been confirmed by senior sand fly specialists[1,10].

4. Discussion

The Phlebotomus genus entails all known medically important species in the Old World, including incriminated vectors of mammalian Leishmania species and of sand fly fever serogroup of arboviruses[17,18]. This genus included 12 subgenera and Adlerius Nitzulescu (1931) is one of the important subgenus in the Old World as it contains the species of leishmaniasis vectors[7]. It is a Central Asian species and it is possible that a number of Central Asiatic species have entered Afghanistan and Iran from central Asia. Artemiev (1978) revised the subgenus Adlerius species of Afghanistan. He captured P. (Adl.) kabulensis Artemiev (1978) which is found in dwellings and is rather thermophilic and hydropilic. Since this species belongs to the subgenus Adlerius; he said, perhaps it can transfer visceral leishmaniasis agents like other species of this subgenus. But so far this has not been reported. Generally, there are five factors that all must be satisfied for vector incrimination: finding parasite in wild specimens, transmission from host to vector (in the laboratory), transmission from vector to vertebrate (Lab.), correlation of vector presence with disease outbreak, and laboratory confirmation that life cycle of parasite can be maintained in the purported vector. Although finding the parasite in collected specimens from the wild does not necessarily indicate that the species is a vector. Before the present study, known sand fly species of the subgenus Adlerius fauna from Iran were four species and this has been confirmed by senior sand fly specialists[1,10].

Currently, there are eight species of the subgenus Adlerius in Iran. So, it can be mentioned that through further field studies, one can discover a greater number of new records or even newer species due to the large area of the country. During the conduct of this study across three provinces (Esfahan, Lorestan and Ilam) in Iran, P. kabulensis was collected in outdoor places situated far from human dwellings. These three provinces are located on the visceral leishmaniasis areas of the foothills to high altitude of the Zagros mountain range in the country and this geographical climate is similar to the dwellings of this species in Afghanistan. However, previous field surveys have given evidence of the subgenus’s anthropophilic nature which is usual in human houses, cattle sheds and gardens[14], but the researchers collected all of them outside human dwellings. The collections were made by sticky paper traps but testing by other methods like CDC light traps may be more efficiency for this species. The P. kabulensis has not been naturally and/or experimentally proven as vector of leishmaniasis; however, it is important to note that its occurrence alongside known and proven vectors in L. infantum endemic regions enhances the risk of transmission. Nevertheless, the P. kabulensis of the subgenus Adlerius, which includes the potential vectors of Mediterranean kala-azar, suggests that the role of this species should be given serious attention in Iran. In this country, P. kabulensis is very uncommon and even the few that are present show little considerable variation in morphological characters. In this study, P. kabulensis species of sand flies were collected at low densities and in small numbers together with other local vectors; therefore, presently, there is little information available regarding its biology and ecology. Ecologically, the successful completion of the life cycle of L. infantum depends entirely on the number of efficient vector specimens. Based on the observed densities in nature, P. kabulensis cannot restrict L. infantum life cycle to itself. Notwithstanding, we cannot rule out the possibility that P. kabulensis plays a role in the transmission of L. infantum, as in cases of canine leishmaniasis. Sand fly taxonomy in most cases is based on morphometric means and is either measurable or countable characters. In this present study, length of the 3rd segment of antennae, length of style, length of epipharynx, length of coxite, width of style, length of substyle, length of aedeagus and number of hairs on coxite, eight taxonomic characters were illustrated in order to describe the morphology of the P. kabulensis. Some taxonomic characters of P. kabulensis from Iran have shown considerable morphological similarities such as the sub-terminal tooth in aedeagus, Filament/Pump and the hairs on coxite, having been compared with the published data of this species from Afghanistan[14]. The mean and standard deviation of eight morphometric characters of the species aided the researchers in the field of ecological study and control of the vectors. DNA sequences alone can be used for species identification. Previous studies have been showed that the PCR technique is an appropriate tool for detecting sand flies species from others[19-21]. P. kabulensis was studied and identified by morphological, morphometric and molecular characterization. The present study suggests a sand fly survey in Iran. Initial DNA analysis has shown how distinct this species is. More entomological intensive studies are needed in order to discover its distribution and abundance as well as further molecular comparisons to effectively control this species in Iran. This is because of its similarity with the female
specimen of Adlerius group sand flies in morphological characters and the morphological identification of the females of P. kabulensis sand fly is impossible too. So, by extracting and sequencing the DNA of the males of P. kabulensis sand fly, we can identify the female of this species by comparing their sequences in the future studies. It is feasible that the populations share vector competence. The natural or experimental infection of the P. kabulensis with Leishmania parasites thus seems attractive for future testing.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

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