

## Original Article

# Morphological and Genotypic Variations among the Species of the Subgenus *Adlerius* (Diptera: Psychodidae, *Phlebotomus*) in Iran

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## Abstract

**Background:** Female sand flies of subgenus *Adlerius* are considered as probable vectors of visceral leishmaniasis in Iran. The objective of this study was to determine the morphological and genotypic variations in the populations of this subgenus in the country.

**Methods:** Sand flies collected using sticky traps from 17 provinces during 2008–2010. The morphometric measurements were conducted with an Ocular Micrometer. Data was analyzed by SPSS. The *Cytb* gene was used to estimate population genetic diversity and identify the female specimens. UPGMA phenetic tree was used for DNA haplotypes of *Cytb* gene.

**Results:** Six species of subgenus *Adlerius* identified from which one species, *P. (Adlerius) kabulensis*, is new record. The identification key is provided for males. Results revealed the molecular systematic in the species of subgenus *Adlerius* and determine the relationship of three females of *P. comatus*, *P. balcanicus* and *P. halepensis*.

**Conclusion:** The positions of three females and the males in the UPGMA tree are correct and the similarities among them confirm our results. The branches of each species are not genetically distinct which justify the overlapping morphological characters among them. Molecular sequencing of *Cytb*-mtDNA haplotypes can be used for female identification for different species of subgenus *Adlerius* in Iran.

**Keywords:** Phlebotomine sand flies, subgenus *Adlerius*, *P. comatus*, *P. kabulensis*, Mitochondrial DNA Cytochrome b gene

## Introduction

Zoonotic Visceral Leishmaniasis (ZVL) is a potentially fatal disease in human, primarily in children. It is caused by *Leishmania infantum* in which dogs, foxes, and jackals are the main reservoir hosts. Four species

of the subgenus *Larrousius* and one species of subgenus *Paraphlebotomus* are considered as probable vector species in Iran (Yaghoobi-Ershadi 2012). The disease is endemic in many rural communities of 7 out of 31 prov-

inces and also sporadically reported from other regions of the country (Yaghoobi-Ershadi 2012, Zahraei-Ramazani et al. 2013).

The subgenus *Adlerius* Nitzulescu includes about 20 species (Seccombe et al. 1993) in which some are suspected or proven vectors of ZVL. In the old world these include:

*Phlebotomus longiductus* in China, Kazakhstan, Kyrgyzstan, Ukraine, and Uzbekistan. *P. halepensis* ranges in Syrian Arab Republic, Azerbaijan and Georgia (WHO 2010). It should be noted that *P. (Adlerius) arabicus* has also reported as the vector of *L. tropica* in Palestinian Territories (Svobodova et al. 2006a, 2006b).

The subgenus *Adlerius* is almost entirely Palaearctic (Seyedi-Rashti and Nadim 1992) in various Eurasian countries and is thought to have originated from central Asia where biodiversity is most abundant (Léger and Pesson 1987). Females from about 20 species of the subgenus *Adlerius* can transmit *Leishmania major* (Sadlova et al. 2003, Volf and Myskova 2007), *L. tropica* (Jacobson et al. 2003, Sadlova et al. 2003, Svobodova et al. 2006a, Volf and Myskova 2007), *L. donovani* (Zhang and Leng 1997, WHO 2010) and *L. infantum* (Killick-Kendrick 1999, Volf and Myskova 2007, Giorgobiani et al. 2012). There is no published morphological key for species identification that are generally recognized on the basis of male morphology (Lewis 1982, Seyedi-Rashti and Nadim 1992).

These sand flies are found in diverse natural microhabitats, especially in mountainous areas, such as: cracks, crevices and around the nests and gatherings of the visceral leishmaniasis reservoirs (Artemiev 1973). Most are also found indoors (Theodor and Mesghali 1964). Because of the short sunny season in the mountains and highland provinces, the collection time of the sand flies is too short.

Prior to this study, the systematic (morphological and molecular) and molecular studies of some medically significant arthro-

pods such as *Aedes*, *Anopheles*, *Culex*, mites, and sand flies have been studied by molecular methods. The high sensitivity and accuracy of the molecular methods related to DNA in terms of morphological identification of species are well established. These are undetectable such as females of the subgenus *Adlerius* is important (Munsterman and Conn 1997, Artemiev 1980, Artemiev and Neronov 1984).

According to previous studies, the *Cytb*-mtDNA is broadly used for sand flies systematics (Esseghir et al. 2000, Parvizi and Ready 2006). The studies on genetic variation and molecular systematics of the subgenus *Adlerius* sand flies present evidence of ecological differences between the group.

Access to the genetic variation and molecular systematics of the subgenus *Adlerius*, in addition to assisting the systematic delineation and their classification may offer evidence to interpret and explain the ecological differences among the species. Species knowledge, abundance, and distribution of males and females, and changes in their population are useful for all research programs in control of leishmaniasis.

The objective of this study was to determine the morphological and genotypic variations among the species of the subgenus *Adlerius* in their distributional areas of Iran.

## Materials and Methods

### Study area

Iran is an arid land of 1.6 million Km<sup>2</sup>, extending north to Armenia, Azerbaijan, Turkmenistan, and the Caspian Sea, east to Afghanistan and Pakistan, south to the Persian Gulf and Sea of Oman, and bordered by Turkey and Iraq on the west. Mountains span the nation in the shape of a large V. Between these ranges lies a high plateau where flowing water from the mountains disappears into desert sand.

The Caspian Sea littoral zone comprises the northern slopes of the Alborz Mountains and the Caspian plain. This is a narrow strip of land, forest covered, with Mediterranean climate, with the average temperature ranging between 10 and 35 °C and the average relative humidity between 70 and 100%.

The Central plateau, situated between the Alborz and Zagros ranges, are very mountainous in the northwest where the ranges originate and becomes a lower desert in the east. The climate is dry with average temperature between 0 and 40 °C, with hot, dry summers and cold, snow-laden winters. Several rivers originate on the southern slopes of the Alborz Mountains.

The Persian Gulf littoral and the Khuzestan plain, to the south of the foothills of the Zagros Mountains, are characterized as a tropical climate (Fig. 1). The average temperature ranges between 12 and 50 °C. The average relative humidity ranges between 40% and 80%.

The extent and distribution of the subgenus *Adlerius* sand flies collection was conducted from 40° north to 29° south latitude and 44° western to 62° eastern longitude respectively (Statistical culture of Iran, 1990, personal communication).

A detailed study of distribution of the subgenus *Adlerius* species was performed in rural areas of 17 of 31 provinces including [(1) Azarbaijan-e-Sharqi, (2) Azarbaijan-e-Gharbi, (3) Ardabil, (4) Kordestan, (5) Ghom, (6) Ilam, (7) Lorestan, (8) Khuzestan, (9) Bushehr, (10) Fars, (11) Sistan Va Baluchestan, (12) Kerman, (13) Kohkiluyeh va Boyer Ahmad, (14) Chahar Mahal-o-Bakhtiari, (15) Esfahan, (16) Markazi, and (17) Khorasan-e-Razavi] (Fig. 1).

### Sand flies collection

The sand flies were collected using 200 sticky paper traps (consisted of white sheets 15x21 cm, coated with castor oil) placed in different habitats and various biotypes. This

included areas outdoors, such as gardens, mountain caves, animal shelters, wall cracks, burrows, tree holes, under stones, and rocks in 3–4 villages suitable for sand fly breeding. These were located in the plains and mountains areas within each province and sampling was conducted twice each year (June and September) from 2008–2010. Traps were placed before sunset and collected the next morning before the sunrise. Sand flies were preserved in 96 % ethyl alcohol glass containers and were kept at 4 °C in the fridge. The number of the trap sample, date, and location was recorded on the glass containers (Moin-Vaziri et al. 2007).

### Mounting and identification

For species identification, sand flies were mounted in Puri's medium, which was manufactured in our leishmaniasis laboratory in Iran and identified after 24 h (Yaghoobi-Ershadi and Javadian 1997) using the keys of Theodor and Mesghali (1964), Artemiev (1973), and Lewis (1982). Morphological characters used for male identification of the subgenus *Adlerius* included antennal segments, shape of the antennae and ascoid, number of hairs on coxite, length of style on ventral process, length of filament, length of pump, and sub-terminal tubercle of the aedeagus (Lewis 1982).

### Morphometric study

The eight organs were measured for our morphometrical study included: (1) length of the third segment of antennae, (2) length of style, (3) length of epipharynx, (4) length of coxite, (5) width of style, (6) length of surstyle, (7) length of aedeagus and (8) number of hairs on coxite. The morphometric measurements were conducted with an ocular micrometer and Olympus Microscope (ch-2) (Fig. 2). Magnification (40X).

### Statistical analysis

Statistical analyses were used to determine

if morphometric characters among the mounted specimens in each species were similar with the species found in different geographical areas. We used SPSS-Version 17.0, and evaluated for differences with the T-test, ANOVA, and Kruskal-Wallis test with an a priori level of significance set at  $P < 0.05$ . If statistical tests were significantly different, the post Hoc-Bonferroni test was used. When the result of the Kruskal-Wallis test was significantly different, the Mann-Whitney test was used for comparing the results.

### Molecular study

Sections of the abdomen, wings, and legs of the sand flies used for DNA extraction were stored in 1.5 ml sterile microtubes containing 96 % ethanol. DNA from 120 specimens were extracted, of which 50 were used for mtDNA-PCR having 16 sequences (Table 1). The washed, dried body of each sand fly was frozen then defrosted with liquid nitrogen and homogenised and ground with a heavy cylindrical metal and shaking vigorously. The content was washed with 300 ml lysis buffer [10 mM tris, 25 mM EDTA (pH= 8), 1 % SDS, 25 mM NaCl, 2% Triton $\times$ -100] and boiled for 10 minutes. This was followed by extraction with a Phenol-Chloroform method. After the ethanol precipitation, DNA was dissolved in 15  $\mu$ l distilled water and stored at -20 °C.

A 25  $\mu$ l PCR reaction mixture consisted of 12.5  $\mu$ l Premix 2x[10x PCR buffer, MgCl<sup>2</sup>, 10 Mm dNTPs and DNA Taq polymerase], 1  $\mu$ l of CB3-PDR forward primer [5'-CA (Y= T/C) ATTCAACC (W= A/T) GAATGATA-3'], 1  $\mu$ l of NIN-PDR Reverse primer [5'-GGTA (Y= C/T) (W= A/T) TTG CCTC GA (W =T/A) TTCG (T/A) TATGA-3'], 5.5  $\mu$ l ddH<sub>2</sub>O and 5 $\mu$ l of sand fly genomic DNA were used for amplifying of 550 bp. The PCR amplification was carried out with the following thermal profile using a Gene Amp® PCR System 2700 thermal cycle (AB Applied Biosystems): 6' min. for

initial denaturation at 95 °C, 35 cycles of denaturation at 94 °C for 45 sec., annealing at 55 °C for 1 min., extension at 72 °C for 1 min.

### Sequence alignment and analysis

In this study the chromatogram of the DNA sequencing samples were edited by using BioEdit software. The program Clustal W (Thompson et al. 1997) was used to study the alignment sequences and similarity scores. Our sequences were compared with the sequences available in GenBank by using Blast available on, [www.ncbi.nlm.gov/](http://www.ncbi.nlm.gov/). The Blast 2 sequences were used to find multiple local alignments and detect the best homologous between the female and the male specimens. DNA haplotypes of *Cytb*-mtDNA gene were used to build an UPGMA tree. We measured the eight different morphological characters in all specimens of each *Adlerius* species which captured by sticky traps from outdoors of the provinces, and then the similarity scores (%) have been identified by sequence comparing alignment pairwise. Therefore, if the more similarity score and less E-value were identified between the pairwise comparison, it showed that they are same species. Also, the haplotypes have been confirmed by the polymorphic sites.

## Results

### Composition, frequency and distribution of the *Adlerius* species

A total of 9,319 sand flies were collected, of which 3,847 (41.3%) and 5,472 (58.7%) were from the genus *Phlebotomus* and *Sergentomyia*, respectively.

From the total number of sand flies of the genus *Phlebotomus*, 167 (1.8%) were from the subgenus *Adlerius* [26 (15.57%) were female and 141(84.43%) were male]. Of the males, *Phlebotomus (Adlerius) brevis* 8 (5.67%), *P. (Adl.) halepensis* 66 (46.81%), *P. (Adl.) longiductus* 30 (21.28%), *P. (Adl.) balcanicus* 23 (16.31%), *P. (Adl.) kabulensis*

13 (9.22%) and *P. (Adl.) comatus* 1(0.71%). The *P. (Adl.) kabulensis* Artemiev, 1978 is a new record from Iran. In case of *P. (Adl.) comatus* which was reported by the authors as a new record by morphological characters from the country (Zahraei-Ramazani et al. 2013) reconfirmed by the molecular studies (Fig. 3).

We updated the morphological key for the male subgenus *Adlerius* species of Iran as follows:

- (1) One ascoid on antennal segments 9–15. Antenna 8 with two ascoids..... (2).  
 -One ascoid on antennal segments 9–15. Antenna 8 with one ascoid. Antenna 6 and 7 with one ascoid or with one long and one short ..... (6).  
 (2) Coxite with 14–27 hairs in group, rarely 29 ..... (3).  
 -Coxite with 29–115 hairs in group, rarely 27. Part of 27–85 hairs of group on distal half of coxite ..... (4).  
 (3) Whole hair-group on basal half of coxite. Tubercle of aedeagus 6–16 µm from tip .....  
 ..... *P. turanicus* (Akhoundi et al. 2011).  
 -Part of hair-group on distal half of coxite. Tubercle of aedeagus 19–28 µm from tip .....  
 ..... *P. brevis* (Theodor and Mesghali 1964).  
 (4) Aedeagus with rectangular subterminal notch ..... *P. halepensis* (Adler 1946).  
 -Aedeagus with normal obtuse-angle subterminal notch ..... (5).  
 (5) Coxite with 27–50 group-hairs .....  
 .. *P. kabulensis* (Probably *P. cf. kabulensis*).  
 -Coxite with 50–85 group-hairs. Sperm tubes are long (1200–1700 µm) .....  
 ..... *P. longiductus* (Theodor and Mesghali 1964) (Also *P.cf. longiductus*).  
 (6) Coxite with 90–220 group-hairs. Ventral process of style long (About 20 µm ..... (7).  
 -Coxite with 30–85 group-hairs. Ventral process of style long or short .....  
 ..... *P. salangensis* (Akhoundi et al. 2011).  
 (7) Coxite very wide, whole group of 125–

200 hairs on its basal half .....  
 ..... *P. comatus* (Zahraei et al. 2013).  
 -Coxite narrow, part of hair-group on its distal half ..... *P. balcanicus* (Theodor and Mesghali 1964) (Also *P.cf. balcanicus*).

The male and female sand flies of subgenus *Adlerius* were collected from 12 provinces and the male were collected from 11 out of 17 provinces. The males were not collected from Markazi, Bushehr, Kerman, Kordestan, Ghom, and Kohkiluyeh va Boyer Ahmad. The maximum number collection was from Ardabil (52 specimens) and the minimum number was from Khuzestan and Azerbaijan-e-Gharbi (1 specimen) Provinces.

#### Statistical analysis of eight morphological characters of the males of subgenus *Adlerius* sand flies

The subgenus *Adlerius* specimens collected from some provinces were very few in number. So for the statistical comparison of the 8 measureable morphological characters of the specimens of *P. halepensis*, we sorted them according to the four geographical regions (North-West, North-East, South-West and South-East). There was no significant difference between the 8 characters in the specimens. The *P. halepensis* specimens collected from three provinces (Ardabil, Azerbaijan-e-Sharqi and Esfahan) showed significant difference between the length of style and length of aedeagus.

The statistical analysis of eight morphological characters of 30 males of *P. balcanicus* specimens confirmed that there is no significant difference between the characters in the specimens of Ardabil and Azerbaijan-e-Sharqi.

There is a significant difference between the lengths of the coxite of the *P. kabulensis* specimens in the three provinces (Esfahan, Lorestan and Ilam). The Post Hoc-Bonferroni test shows that the length of coxite of *P. ka-*

*bulensis* specimens in Lorestan Province is significantly more than Ilam. The statistical analysis between the 7 specimens of *P. brevis* in Chahar Mahal-o-Bakhtiari and 1 specimen in Azarbaijan-e-Gharbi did not confirm any significant difference.

The statistical analysis of 30 *P. longiductus* specimens in four geographical regions (North-West, North-East, South-West and South-East) confirmed that there are significant difference value between the length of surstyle, the length of aedeagus, the number of hairs on coxite, the length of coxite, and the length of styles ( $P < 0.05$ )

### Molecular studies on the subgenus *Adlerius* species

The PCR experiments amplified a fragment of 550 bp of the mitochondrial genome and it was successfully achieved for 6 species of the subgenus *Adlerius* (Table 1, 2). These were registered on JX885982 to JX885998 Accession numbers in the GenBank.

In the pair wise comparison of the specimens, we focused on the genetic similarities of the specimens among each other and determined the level of such similarities of male and female specimens and identified the females among 6 male species.

The pair wise comparison in AZV8 female specimens with nucleotides of six male species showed that the maximum similarity, i.e. 98% is found in *P. comatus*. In total, 528 nucleotides were compared with these 2 sequences which show 8 (1.5%) polymorphic sites and it is confirmed that they are 2 haplotypes (Table 3).

The pair wise comparison in males of the *P. balcanicus* species with nucleotides of the three female specimens shows that there are minimum 98% and maximum 99% similarities among the male and the SHC11 female specimen. It shows 22 (4.3%) polymorphic

sites and it confirms that there are 4 haplotypes (Table 3).

The nucleotides comparison of the 5 males of the *P. halepensis* specimens with the CKHE1342 female specimen shows that there are minimum 90.1% and maximum 92.6% similarities between them. The polymorphic sites is 46 (10%) and the sequence comparison revealed that there are 6 haplotypes. Also for the two males of *P. brevis*, it shows 99% similarities with 2 (0.4%) polymorphic sites and 2 haplotypes between these two sequenced specimens (Table 3).

### *Cytb*-mtDNA Sequences results of the subgenus *Adlerius* species

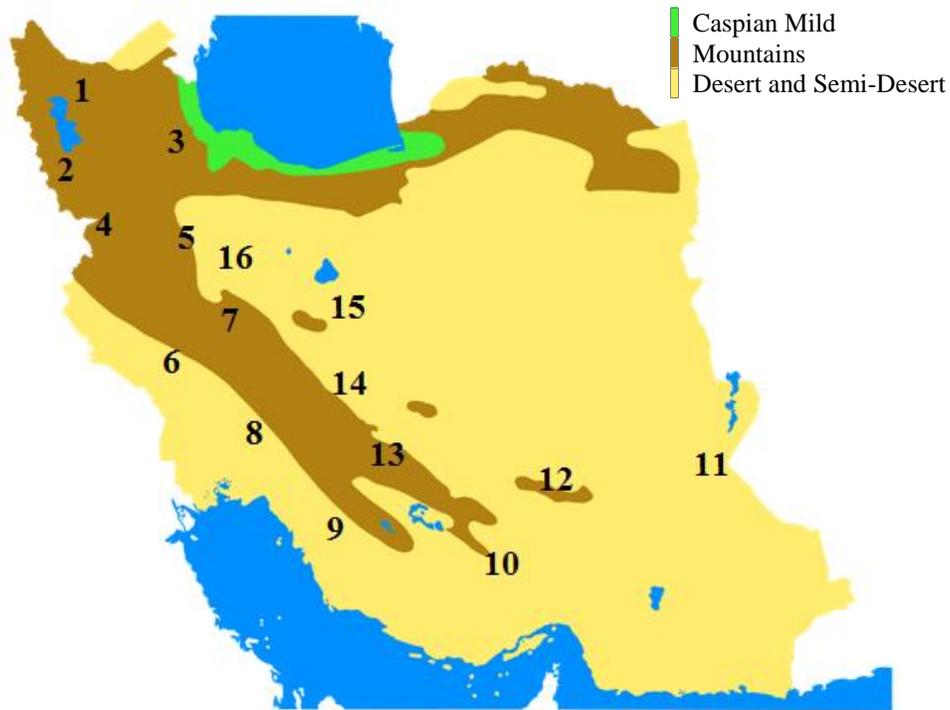
The sequence result of the length 550 bp of *Cytb* gene shows that 5 male species: *P. halepensis*, *P. brevis*, *P. kabulensis*, *P. comatus* and *P. balcanicus* are in the areas of our study. Also two male specimens: *P. cf. balcanicus* and *P. cf. longiductus* which are close to *P. balcanicus* and *P. longiductus* are on neighbor-joining tree (Fig. 4). The specimens of *P. halepensis* and *P. brevis* are in independent and different branches in the tree, but other species are close together in one branch. *P. comatus* and the female AZV8 specimen are in the same branch, which shows that they are the same species. Also the branch of female SHC11 specimen is near the branch of AZV4 and AZV5, which shows that females of this species are close to these specimens. The branches of two *P. balcanicus* specimens i.e. Ash10 and AZV11 are close together and *P. kabulensis* is in a different branch. The position in the phenetic tree of the 3 females species and the male species are correct and the similarities among the males and females confirm our above results (Fig. 4).

**Table 1.** Number of sand flies used in molecular studies

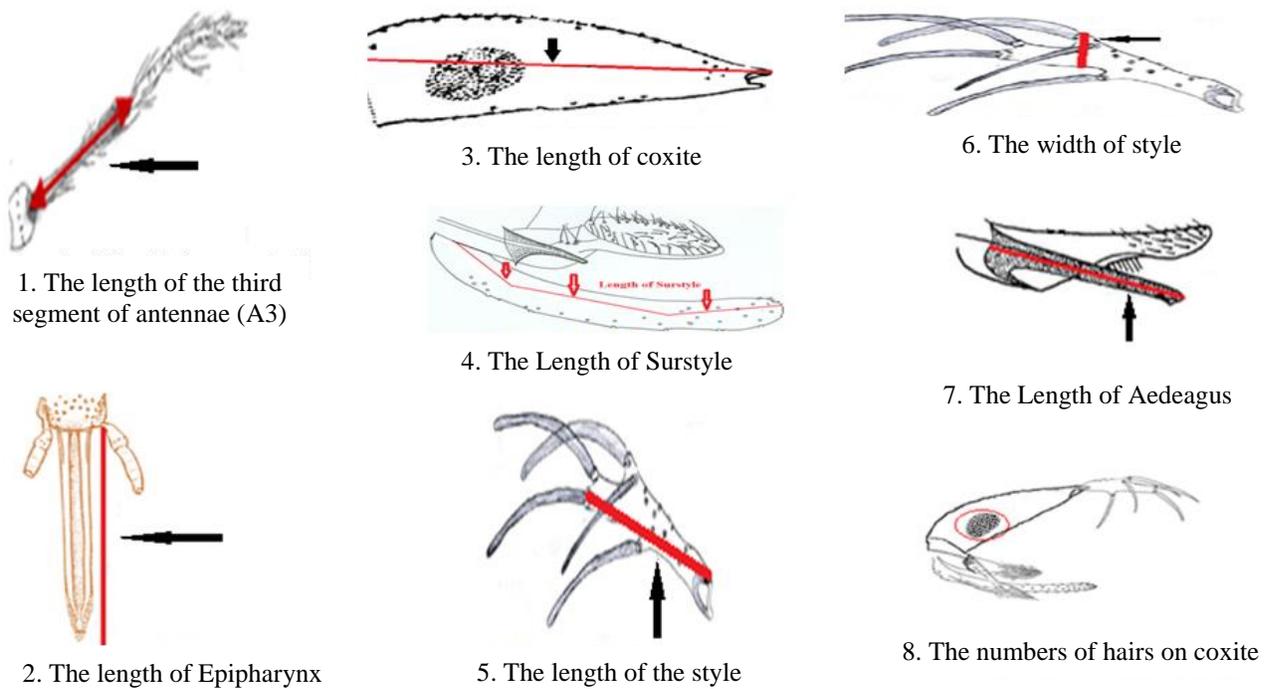
| NO.          | Species                     | Number of specimens that DNA has been extracted | Number of specimens that have been mtDNA-PCR | Number of mtDNA-PCR that have been sequenced |
|--------------|-----------------------------|---|--|--|
| 1            | <i>P. halepensis</i>        | 55  | 22   | 5  |
| 2            | <i>P. longiductus</i>       | 23  | 5  | 1  |
| 3            | <i>P. kabulensis</i>        | 12  | 5  | 1  |
| 4            | <i>P. balcanicus</i>        | 13  | 10   | 3  |
| 5            | <i>P. brevis</i>            | 8   | 3  | 2  |
| 6            | <i>P. comatus</i>           | 1   | 1  | 1  |
| 7            | <i>P. (Adlerius)</i> Female | 8   | 4  | 3  |
| <b>Total</b> |                             | 120   | 50   | 16   |

**Table 2.** Profile of sequenced species based on *Cytb* gene in the subgenus *Adlerius*

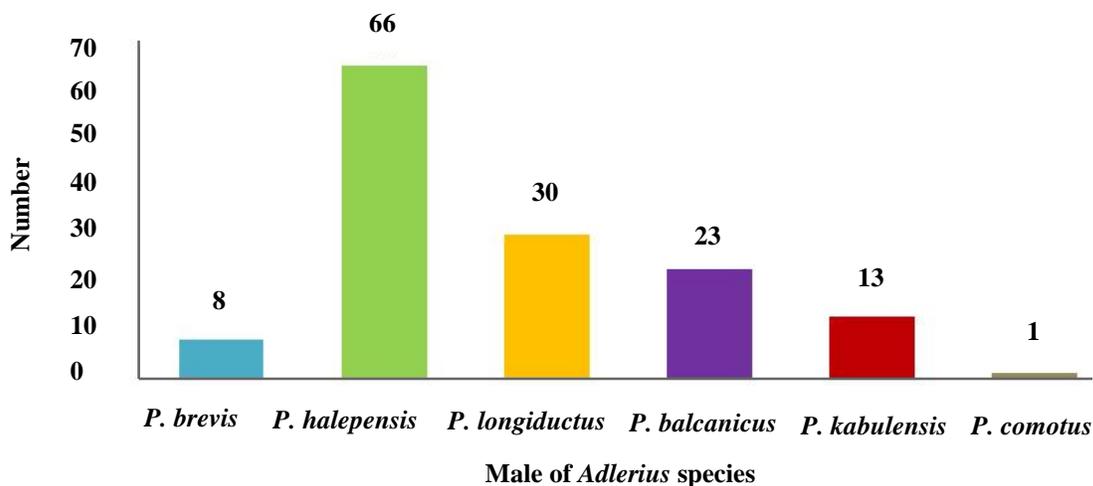
| Species                  | No. | Code no. | Sex    | Collecting area     | Township      | Province                 | GenBank accession no. |
|--------------------------|-----|----------|--------|---------------------|---------------|--------------------------|-----------------------|
| <i>P. halepensis</i>     | 1   | ARM754   | male   | Niaz village        | Meshkin-shahr | Ardabil                  | JX885982              |
|                          | 2   | AZV2     | male   | Ahar-Varzaghan road | Varzaghan     | Azarbaijan-e-Sharqi      | JX885984              |
|                          | 3   | EK77     | male   | Konjan              | Natanz        | Esfahan                  | JX885991              |
|                          | 4   | FJ103    | male   | mountainous region  | Jahrom        | Fars                     | JX885992              |
|                          | 5   | MSH458   | male   | Ghortapeh           | Meshkin-Shahr | Ardabil                  | JX885995              |
| <i>P. balcanicus</i>     | 1   | ASH10    | male   | Sharbian            | Sarsb         | Azarbaijan-e-Sharqi      | JX885983              |
|                          | 2   | AZV11    | male   | Ahar-Varzaghan road | Varzaghan     | Azarbaijan-e-Sharqi      | JX885989              |
|                          | 3   | AZV5     | male   | Ahar-Varzaghan road | Varzaghan     | Azarbaijan-e-Sharqi      | JX885986              |
| <i>P. longiductus</i>    | 1   | AZV4     | male   | mountainous region  | Tabriz        | Azarbaijan-e-Sharqi      | JX885985              |
|                          | 2   | FJ476    | male   | mountainous region  | Jahrom        | Fars                     | JX885993              |
| <i>P. kabulensis</i>     | 1   | LP7      | male   | mountainous road    | Poldokhtar    | Lorestan                 | Not clear             |
|                          | 2   | LPM5     | male   | mountainous road    | Poldokhtar    | Lorestan                 | JX885994              |
| <i>P. brevis</i>         | 1   | SHC7     | male   | Chelgerd            | Koohrang      | Chahar Mahal-o-Bakhtiari | JX885996              |
|                          | 2   | SHC22    | male   | Chelgerd            | Koohrang      | Chahar Mahal-o-Bakhtiari | JX885998              |
| <i>P. Adlerius</i> group | 1   | ARM911   | famele | Niaz village        | Meshkin-shahr | Ardabil                  | Not clear             |
|                          | 2   | AZV8     | famele | Ahar-Varzaghan road | Varzaghan     | Azarbaijan-e-Sharqi      | JX885987              |
|                          | 3   | CKHE1342 | famele | Irandedegan         | Khash         | Sistan va Baluchestan    | JX885990              |
|                          | 4   | SHC11    | famele | Chelgerd            | Koohrang      | Chahar Mahal-o-Bakhtiari | JX885997              |
| <i>P. comatus</i>        | 1   | AZV10    | male   | Ahar-Varzaghan road | Varzaghan     | Azarbaijan-e-Sharqi      | JX885988              |



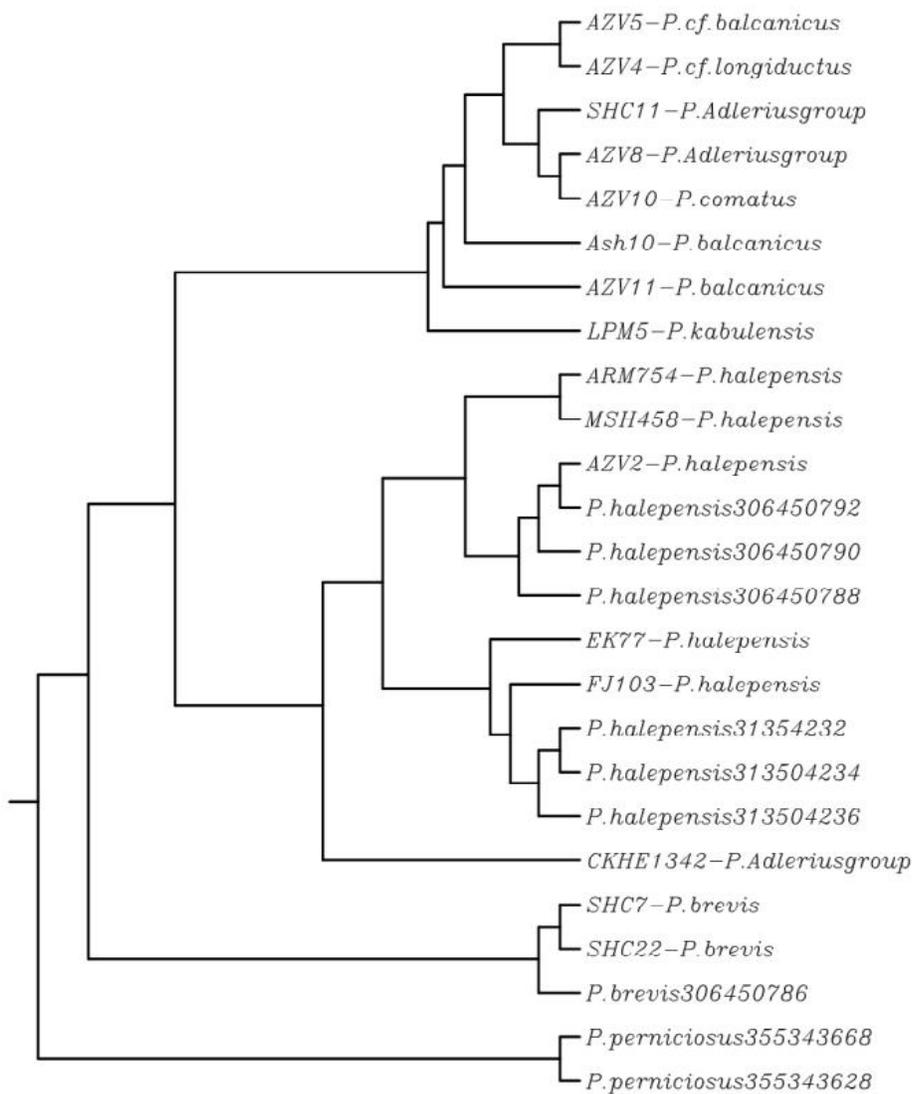
**Fig. 1.** Provinces where *Adlerius* species were collected.  
 (<http://commons.wikimedia.org/wiki/File%3AMy-iran-climate-map-simplified.png>)



**Fig. 2.** Organs measured in morphometrical studies of the subgenus *Adlerius* in Iran



**Fig. 3.** Number of males of subgenus *Adlerius* species collected from 17 provinces



**Fig. 4.** Neighbor-joining tree for DNA haplotypes of *Cytb*-mtDNA of the subgenus *Adlerius* sand flies species

**Table3.** Comparison of *Cytb* gene nucleotide sequences in the subgenus *Adlerius* species

| Species                   | Number of sequence | Identity of sequence (%) | Number of nucleotides compared | Number of haplotype | Polymorphic site (%) |
|---------------------------|--------------------|--------------------------|--------------------------------|---------------------|----------------------|
| <i>P. comatus</i>         | 2                  | 98                       | 528                            | 2                   | 1.5                  |
| <i>P. balcanicus</i>      | 4                  | 98-99                    | 516                            | 4                   | 4.3                  |
| <i>P. halepensis</i>      | 6                  | 90.1-92.6                | 458                            | 6                   | 10                   |
| <i>P. brevis</i>          | 2                  | 99                       | 478                            | 2                   | 0.4                  |
| <i>P. kabulensis</i>      | 1                  | -                        | -                              | 1                   | -                    |
| <i>P. cf. longiductus</i> | 1                  | -                        | -                              | 1                   | -                    |

## Discussion

*Phlebotomine* sand flies of the subgenus *Adlerius* transmit *Leishmania* parasites in various Eurasian countries. But, the species involved have seldom been identified because despite the taxonomic efforts of Artemiev (1980) and others. The morphological characteristics do not distinguish between the females of *Adlerius* species that are generally recognized on the basis of male morphology (Lewis 1982, Sadlova et al. 2003).

The detection of *P. comatus* (Zahraei-Ramazani et al. 2013) and *P. kabulensis* during the present study has assisted to revise the morphological key. The access to new molecular techniques and genetic information about these probable vectors (Artemiev 1973, Ardehali et al. 1995) and their systematics, may aid in resolving the problem of identification in morphological similarity, as well. We provide evidence to interpret and explain their morphology and ecology in detail.

The systematics of the *Phlebotomine* sand flies due to the nature of their diagnostic characteristics is highly variable. The latest revision of the genus *Phlebotomus* sand flies in Old World is included: (1) *Adlerius*, (2) *Anaphlebotomus*, (3) *Euphlebotomus*, (4) *Kasaulius*, (5) *Transphlebotomus*, (6) *Synphlebotomus*, (7) *Phlebotomus*, (8) *Larroussius*, (9) *Paraphlebotomus* (Rispaill and Leger 1998). Secombe et al. (1993) believed that the genus *Phlebotomus* had few morphological deficiencies. According to the morphological characteristics they are monophyletic.

The UPGMA is one of the most popular methods in ecology for the classification of sampling units on the basis of their pairwise similarities in relevant descriptor variables (such as species composition) (Legendre and Legendre 1998). In bioinformatics, UPGMA is used for the creation of phenetic trees (phenograms). In our molecular analysis, all of the *Cytb* gene sequences confirm the monophyletic of the subgenus *Adlerius* and the morphological identification of these six species are confirmed by the reliable morphological identification keys.

The sequence analysis of *P. halepensis* populations showed 2 lineages in different geographical regions. The haplotypes are 6 in this species. According to our morphological, statistical, and molecular findings, three specimens: AZV2, MSH458 and ARM754 which were collected from the North-West of Iran, fall in one branch along with three *P. halepensis* of GenBank. Arm754 and MSH458 were captured from Ardabil and AZV2 was captured from Azerbaijan-e-Sharqi, two different areas. In the statistical analysis, the length of the style in *P. halepensis* specimens in Ardabil Province was significantly different from that of Esfahan and Azerbaijan-e-Sharqi. AZV2 is in a different branch but is closer to MSH458 and ARM754 specimens. FJ103 and EK77 are in one branch too. In the statistical analysis, the mean of length of style of EK77 is more than that of MSH458, ARM754 and AZV2. So in the

UPGMA tree it shows that they are close to the FJ103 specimen in Fars Province.

The FJ103 and the three *P. halepensis* specimens from the GenBank found in the north of Iran are closer or these specimens in two different areas and are near to their phenetic characters. Some geographical event such as desert winds in the central plateau (Fig. 1) causes movement and mixing in its population. The pairwise comparison in males of *P. halepensis* specimens with nucleotides of CKHE1342-female shows that there are minimum 90.1 % and maximum 92.6 % similarity among them. In statistical analysis, the mean of the length of labrum, surstyle, and aedeagus of the *P. halepensis* specimens in the south-east (CKHE1342) of Iran are different from the specimens of other geographical regions. However, CKHE1342-female is in different branch because of the geographical distance between these specimens. In this study all the *P. halepensis* specimens are on different branch and are in a larger group. It also seems that this species has been separated from another species of *Adlerius* group many years ago than the other *Adlerius* species. According to the statistical analysis and molecular findings, further studies are required to resolve the taxonomic status of this species.

The present study on molecular analysis of *P. brevis* population shows that one lineage with two haplotypes for *Cytb*. The *P. brevis* (Theodor and Mesghali 1964) was firstly found in Iran. The SHC7 and SHC22 were captured from Chahar Mahal-o-Bakhtiari Province. Statistical analysis did not show any difference between them and the specimens from Azerbaijan-e-Gharbi. Along with them, another species i.e 306450786 was submitted to the GenBank from Iran which is in one independent branch. It seems that *P. brevis* is not a common ancestor of the other *Adlerius* but diverged early and shares one common ancestor with other species.

The second group of the phylogeny tree including LPM5, AZV11, ASh10, AZV5, AZV4, AZV10 and two females specimens: SHC11 and AZV8 show that *Cytb* gene does not have sufficient resolution for them and indicates the morphological character overlapping. The length of the branches in this group is short, which indicates that the changes among them are low. This can also be said that the specimens of each species are not far genetically.

In statistical analysis by using t-test, we did not find any difference between the specimens of *P. balcanicus* in Ardabil and Azerbaijan-e-Sharqi Provinces. In the molecular study, the pairwise comparison in three males of this species with nucleotides of SHC11-female specimen shows that there are minimum 98 % and maximum 99 % similarities among themselves. Also, it is confirmed that there are 4 haplotypes among the four sequenced specimens. In the present study, AZV4 and AZV5 have been identified by using both morphological and molecular methods. However, in our UPGMA tree, we found that they are different between *P. balcanicus* specimens and they are in an independent branch but closer to *P. balcanicus* branches. The pairwise distance between them is zero. It is clear that the taxonomy of these species should be revised. The results of the molecular investigations indicate that according to the concept of the species they (AZV5 and AZV4) cannot be considered as a separate species. Because the concept of a species is based on the reproductive isolation, while the existence of the intermediate forms and the same mitochondrial gene sequences in this study indicates that there is no reproductive isolation between AZV5 morphotypes and AZV4 morphotypes. Also, we cannot assume these two specimens as subspecies because two subspecies cannot be found as sympatric. So, this indicates that these species e.g *P. longiductus* and *P. balcanicus* in Iran, including their populations

have morphological and genetic differences. The similarity between AZSH10 and AZV11 is 97% and E-value between them is 0.0. Further, in the statistical analysis, no difference was found between them. The branches of the four haplotypes of *P. balcanicus* show that genetically they are not far. The similarity and E-value between the SHC11 and AZV5 are 99 % and 0.0 respectively. So in the UPGMA tree they are close together.

The pairwise comparison of the nucleotides of *Cytb*-mtDNA gene in AZV8 female specimen with nucleotides of six male species shows that the maximum similarity i.e 98% is found in AZV10. Results of the molecular studies reveal that there are 2 haplotypes. These two specimens are in the same branch in the UPGMA tree. The pairwise distance between them is 0.0. So, these evidences confirm our results as both AZV8 and AZV10 are *P. comatus*.

In the UPGMA tree, *P. kabulensis* is seen as independent branch. The length of the branch in this group compared with other branches is long. This indicates that many changes have occurred during the course of evolution of this species. Also this species seems a common ancestor for the other species in this group of the UPGMA tree.

The location and the number of hairs on coxite is the most important morphological characteristic feature for identifying and separating one *Adlerius* species from the other. At times it is difficult for the researchers as the numbers and locations of the hairs of different species are the same and overlap among themselves. In *P. kabulensis*, the hairs on coxite are 27–50 and in *P. longiductus* the hairs on coxite are 50–85. If we collect a specimen which has 50 hairs on its coxite, we cannot identify that it belongs to which one of the afore noted two species. Also for *P. balcanicus* and *P. longiductus*, we encountered the same difficulty. In this case, the researchers use ‘cf’. The ‘cf’ is the abbreviation of the Latin word ‘confers’ means

“to compare” or “to consult”. It is mainly used in academic writings to indicate a reference to a contrasting finding or viewpoint (it is often used instead of “but see”). It can also appear occasionally in binomial nomenclature by placing before the species name to indicate that the species is not confirmed (Strunk and White 1979). To arrive at a result, we use *P. cf. longiductus* or *P. cf. balcanicus*. In our study, these species have been confirmed by using both morphological and molecular methods. But in our phenetic tree it is found that they are different from *P. balcanicus* and *P. longiductus*. But in an independent group they are close to *P. balcanicus* branch.

Obviously, detailed studies for detection of genetic markers to identify the females of six *Adlerius* species are essential. Also, further studies on morphology and genetic diversity of the populations of the subgenus *Adlerius* will be of particular importance.

## Conclusions

*Phlebotomus (Adlerius) kabulensis* is new record for the Iran. The positions of three female and males of subgenus *Adlerius* in the UPGMA tree are correct and the similarities among them confirm our results. The branches of each species are not genetically distinct that justify the overlapping morphological characters among them. Molecular sequencing of *Cytb*-mtDNA haplotypes can be used for female identification of different species of subgenus *Adlerius* in Iran.

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