Original Article

Molecular Typing and Phylogenetic Analysis of Some Species Belonging to Phlebotomus (Larroussius) and Phlebotomus (Adlerius) Subgenera (Diptera: Psychodidae) from Two Locations in Iran

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Abstract

Background: Haematophagous females of some phlebotomine sandflies are the only natural vectors of Leishmania species, the causative agents of leishmaniasis in many parts of the tropics and subtropics, including Iran. We report the presence of Phlebotomus (Larroussius) major and Phlebotomus (Adlerius) halepensis in Tonekabon (Mazanderan Province) and Phlebotomus (Larroussius) tobbi in Pakdasht (Tehran Province). It is the first report of these species, known as potential vectors of zoonotic visceral leishmaniasis in Iran, are identified in these areas.

Methods: In 2006-2007 individual wild-caught sandflies were characterized by both morphological features and sequence analysis of their mitochondrial genes (Cytochrome b). The analyses were based on a fragment of 494 bp at the 3’ end of the Cyt b gene (Cyt b 3’ fragment) and a fragment of 382 bp CB3 at the 5’ end of the Cyt b gene (Cyt b 5’ fragment). We also analysed the Cyt b Long fragment, which is located on the last 717 bp of the Cyt b gene, followed by 20 bp of intergenic spacer and the transfer RNA ser(TCN) gene.

Results: Twenty-seven P. halepensis and four P. major from Dohezar, Tonekabon, Mazanderan province and 8 P. tobbi from Pakdasht, Tehran Province were identified by morphological and molecular characters. Cyt b 5’ and Cyt b 3’ fragment sequences were obtained from 15 and 9 flies, respectively. Cyt b long fragment sequences were obtained from 8 out of 27 P. halepensis.

Conclusion: Parsimony analyses (using heuristic searches) of the DNA sequences of Cyt b always showed monophyletic clades of subgenera and each species did form a monophyletic group.

Keywords: Mitochondrial Cytochrome b, Phlebotomus (Larroussius) major, Phlebotomus (Larroussius) tobbi, Phlebotomus (Adlerius) halepensis, Iran

Introduction

Visceral leishmaniasis is a deadly disease caused by parasitic protozoa belonging to genus Leishmania, transmitted to humans through the biting of infected female sandflies. Three species of Leishmania including L. donovani and L. infantum from the old world and L. chagasi from the new world are known to give rise to the visceral form of leishmaniasis. The disease is endemic in Iran and the etiological agent is known to be L. infantum, which mainly affects children, with majority of cases from primary foci in Northwestern and Southern of the country (Mohbali et al. 2005). Sandflies of the subgenera Larroussius and Adlerius belonging to genus Phlebotomus are known as primary vectors of zoonotic visceral leishmaniasis (ZVL) in Iran (Nadim et al. 1978, 1992, Parvizi et al. 2008)

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and also *P. (Paraphlebotomus) alexandri* recently reported as the vectors ZVL in Iran (Azizi et al. 2006).

Females of the subgenus *Adlerius* and some females of subgenus *Larroussius* cannot be differentiated based on morphological features. Recently, the females of the two subgenera were identified using sequence analysis of Cytochrome b gene (*Cyt b*) (Killick-Kendrick 1990, 1999, Esseghir et al. 1997, 2000, Parvizi and Amirkhani 2008).

The objective of this report was to identify and type molecularly some species belonging to *P. larroussius* and *P. adlerius* subgenera from two locations in Iran. This paper reports the presence of sandflies of subgenera *Adlerius* and *Larroussius* in Caspian Sea littoral and Pakdasht area. We could differentiate the female specimens of subgenera *Adlerius* and *Larroussius* species using analysis of *Cyt b* gene. It is noteworthy that up to present no record of any sandfly species from Caspian Sea littoral was available.

**Materials and Methods**

The study area included two villages of Meyan Kooh and Imamzadeh Ghasem in Dohezar area, (about 35 km west of Tonekaboon), Mazanderan Province (Caspian littoral) and two villages of Geshlagh and Mamazand in Pakdasht area (about 30 km south of Tehran City, Tehran Province. Sandflies were collected by aspirators and sticky papers (A4 papers soaked in castor oil) from inside and outside of animal shelters, and miniature CDC light traps (Sudia and Chamberland 1962), placed overnight in animal shelters. All collected sandflies first were processed to remove oil then stored at -20º C until used. The sandflies were identified based on morphological features of heads and last abdominal segments to the extent possible according to the keys described by Nadim and Javadian (1976) and Lewis (1982) and then thorax and abdomen of sandfly were individually subjected to DNA extraction as described by Parvizi et al. (2003).

Three pairs of primers designed by Parvizi and Ready (2006) were used to amplify the *Cyt b* gene. CB1-SE (forward) and CB3-R3A (reverse) were used to amplify a more 5’ fragment of 439 bp (CB1 fragment), CB3-FC (forward) and N1N-FA (reverse) amplified an overlapping 3’ fragment of 499 bp (CB3 fragment) and CB1-SE (forward) and CB-R06 (reverse) amplified the *Cyt b* long fragment as one piece of 717 bp length. The PCR condition and reagents for all amplifications were according to Parvizi and Ready (2006) except for the *Cyt b* long fragment in which the annealing was performed in one stage at 48 ºC.

PCR products were directly sequenced in both directions to identify sandflies haplotypes associated with individual female and male sandflies. All haplotypes were identified to species by phylogenetic analysis. DNA sequences were edited and aligned using *Sequencher* 3.1.1 software (Gene Codes Corporation). Multiple alignments of new DNA sequences and GenBank sequences were made using *PAUP* 4.0b8 software (Swofford 2002) for phylogenetic analysis.

**Results**

A total of 43 female and male sand flies from 4 different collection areas were studied, from which three phlebotomine species were morphologically identified.

**Tonekabon**

*Phlebotomus major* (4) and *P. halepensis* (28) were the only prevalent species in Tonekabon. The identity of 27 sand flies, all from Dohezar area, including 21 males and 6 females were determined as *Phlebotomus (Adlerius) halepensis* sandflies based on both morphological features and sequencing data of *Cyt b* gene.

Sequence analysis of *Cyt b* 5’ fragment obtained from 15 sandflies showed that 8
(53.3%) were haplotype IRN279, 2 (13.3%) haplotype IRN277, 2 (13.3%) haplotype IRN 282, 2 (13.3%) haplotype IRN285 and 2 the unique haplotypes IRN293 and IRN294 (Table 1). Genetic distances between haplotypes were as low as 0.000262 – 0.000581. All the 7 (there is only 4) Cyt b 3’ sequences fell in the same haplotypes, named as IRN277 (Table 1).

Cyt b Long fragment sequences were obtained from 8 out of 27 P. halepensis with the five (62.5%) revealing haplotype IRN279, 2(25%) haplotype IRN381 and 1 (12.5%) the unique haplotype IRN279 (Table 1). Genetic distances between haplotypes were as low, 0.00139 – 0.00278.

Four Phlebotomus (Larroussius) major (2 male, 2 female) were identified by morphological and molecular characters from Dohezar area of Tonekabon in Mazanderan Province.

Cyt b 5´ sequences were obtained from 3 out of 4 flies. Two (66.7%) were haplotype IRN385, and one was a unique haplotype (IRN 287) (Table 1). The genetic distance between haplotypes was as low as 0.01597.

Cyt b 3´ sequences were obtained from 3 out of 4 flies, 2 were haplotype IRN385, and 1 was haplotype (IRN287) (Table 1). The genetic distance between haplotypes was as low, as 0.01597.

Cyt b Long fragment sequences were obtained from all four P. major. Two (50%) were haplotype IRN385, and two (50%) were haplotype (IRN287) (Table 1). The genetic distance between haplotypes as low as 0.02234.

**Pakdasht**

The majority of sandflies of Pakdasht were P. papatasi and some species belong to Paraphlebotomus and Sergentomyia. Sequencing data obtained from Cytochrome b gene offemale specimen revealed that the only prevalent species of subgenera Larroussius was P. tobbi.

Based on both morphological and molecular features all the 8 male sandflies collected from Pakdasht (5 from Mamazand region using sticky papers and 3 from Gheshlagh using CDC light traps) were identified as Phlebotomus (Larroussius) tobbi.

No Cyt b 5´ fragment sequences was obtained from P. tobbi specimens as the products of PCR amplification were too weak to be sequenced. Cyt b 3´ fragment sequences were obtained from 7 out of 8 P. tobbi (Table 1). Two (28.6%) were haplotype IRN 334, 2 (28.6%) haplotype IRN335, 2(28.6%) haplotype IRN 338 and 1 was the unique haplotype IRN336. Genetic distances between haplotypes were as low as 0.00319-0.01597. Cyt b long fragment sequence was obtained only from one P. tobbi specimen (IRN413). However, the sequence was too short to be analysed (Table 1).

Nucleotide sequence data reported in this paper are available in GenBank, EMBL and DDBJ databases under accession numbers from HQ391905 to HQ391913.

**Table 1.** All DNA haplotypes of Cyt b of subgenera Larroussius /Adlerius species identified in two locations in Iran (I.H = inside house, Ash= animal shelter, S.P= sticky paper, CDC= CDC miniature light traps)

| Provinces  | location              | habitat | trap type | Cyt b haplotype         |
|------------|-----------------------|---------|-----------|-------------------------|
|            |                       |         |           | CB1-SE CB3R3A | CB3FC NINFA | CB1SE CB-R06 | specimen N. | sex |
| **P. tobbi** |                       |         |           |             |             |             |             |     |
| Tehran     | Pakdasht-Gheshlagh    | Ash     | S.P       | Not done     | IRN334      | Not done    | IRN334      | M    |
|            |                       | Ash     | S.P       | Not done     | IRN335      | Not done    | IRN335      | M    |
|            |                       | Ash     | S.P       | Not done     | IRN336      | Not done    | IRN336      | M    |
| Location          | Species         | Type       | Sample ID | Result       | Other ID   | Sex |
|-------------------|-----------------|------------|-----------|--------------|------------|-----|
| Pakdasht-Mamazand | Ash S.P         | Not done   | IRN335    | Not done     | IRN337     | M   |
| Pakdasht-Mamazand | Ash CDC         | Not done   | IRN338    | Not done     | IRN338     | M   |
| Pakdasht-Mamazand | Ash CDC         | Not done   | IRN334    | Not done     | IRN340     | M   |
| Pakdasht-Mamazand | Ash CDC         | Not done   | weak band | IRN413       | IRN413     | M   |

**P. major**

| Location          | Species         | Type       | Sample ID | Result       | Other ID   | Sex |
|-------------------|-----------------|------------|-----------|--------------|------------|-----|
| Ash               | IRN287          | Not done   | IRN287    | Not done     | IRN287     | M   |
| Ash               | IRN291          | Not done   | IRN287    | Not done     | IRN291     | M   |
| Ash               | IRN282          | Not done   | IRN282    | Not done     | IRN282     | M   |
| Ash               | IRN282          | Not done   | IRN282    | Not done     | IRN282     | M   |
| Ash               | weak band       | IRN277     | Not done  | IRN283       | M          |
| Ash               | IRN282          | Not done   | IRN282    | Not done     | IRN282     | M   |
| Ash               | IRN282          | Not done   | IRN282    | Not done     | IRN282     | M   |

**P. halepensis**

| Location          | Species         | Type       | Sample ID | Result       | Other ID   | Sex |
|-------------------|-----------------|------------|-----------|--------------|------------|-----|
| Ash               | IRN277          | Not done   | IRN277    | Not done     | IRN277     | M   |
| Ash               | IRN294          | Not done   | IRN294    | Not done     | IRN294     | M   |
| Ash               | bad sequence    | IRN277     | Not done  | IRN295       | M          |
| Ash               | weak band       | IRN279     | Not done  | IRN296       | M          |
| Ash               | Not done        | IRN380     | IRN380    | F            |
| Ash               | Not done        | IRN380     | IRN381    | F            |
| Ash               | Not done        | IRN381     | IRN382    | F            |
| Ash               | Not done        | IRN381     | IRN388    | F            |
| Ash               | Not done        | IRN381     | weak band | IRN389       | F          |
| Ash               | Not done        | IRN381     | weak band | IRN389       | F          |
| Ash               | Not done        | IRN381     | IRN392    | F            |
| Ash               | Not done        | IRN381     | weak band | IRN393       | F          |
| Ash               | Not done        | IRN381     | IRN394    | M            |
| Ash               | Not done        | IRN383     | IRN383    | F            |
| Ash               | Not done        | IRN383     | IRN387    | F            |

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Table 1. Countinued…
Fig. 1. Unrooted consensus phylogenetic tree for DNA sequences of Cyt b Long (718 nucleotides) of *Phlebotomus* (Adleri) / *Phlebotomus* (Larroussius), produced by branch and bound parsimony search using PAUP*
Fig. 2. Unrooted phylogenetic tree for DNA sequences of Cyt b 3’ end (last 316 nucleotides) of *Phlebotomus (Adlerius)/Phlebotomus (Larroussius)*, produced by branch and bound parsimony search using PAUP*.
Fig. 3. Input data matrix of variant nucleotides for PAUP* analysis of Phlebotomus (Adlerius) / Phlebotomus (Larroussius) species: 316 base pairs of Cyt b 3'.

| Single or composite DNA sequence from each specimen | Nucleotide position |
|---------------------------------------------------|--------------------|
| IRN287haplo CB3                                  | 1111111111111111111 |
| IRN385haplo R06                                  | 112222233344455566666 |
| IRN293haplo CB1                                  | 77777777777789800000 |
| IRN234haplo CB3                                  | 1539258514703691247801347893756702534154124567012459129012581 |

Fig. 4. Input data matrix of variant nucleotides for PAUP* analysis of Phlebotomus (Adlerius) / Phlebotomus (Larroussius) species: 382 base pairs of Cyt b 5'.
Discussion

Using branch and bound parsimony searches with equal character weighting, PAUP* analysis for DNA sequences of Cyt b 3’ fragment (last 316 nucleotides) of 8 haplotypes of Phlebotomus (Adlerius)/Phlebotomus (Larroussius) produced one parsimonious tree rooted using default outgroup (tree length= 145; 69 characters were parsimony-uninformative; number of parsimony-informative characters= 47) (Fig. 1, 2). The haplotypes of each species formed monophyletic clades, but it was not possible to compare regions because each species was collected in unique locations.

Using branch and bound parsimony searches with equal character weighting, PAUP* analysis for DNA sequences of Cyt b 5’ fragment (383 nucleotides) of 10 haplotypes of Phlebotomus (Adlerius)/Phlebotomus (Larroussius) produced one most parsimonious tree (tree length= 188; 60 characters were parsimony-uninformative; number of parsimony-informative characters= 78) (Fig. 3, 4). P. major and P. halepensis were monophyletic, with two unknown species located between them. It was not possible to compare regions because each species was collected in unique locations.

Using branch and bound parsimony searches with equal character weighting, PAUP* analysis for DNA sequences of Cyt b Long fragment (last 718 nucleotides) of 18 haplotypes of Phlebotomus (Adlerius)/Phlebotomus (Larroussius) produced 1 most parsimonious tree rooted using default outgroup (tree length= 343; 116 characters were parsimony-uninformative; number of parsimony-informative characters= 144). Phlebotomus major and P. tobbi were monophyletic, and both unknown species (i.e. not identified by morphology) were monophyletic with P. halepensis, not with a species of Larroussius. The longer sequence gave more phylogenetic information.

Nucleotide haplotypes within Adlerius/Larroussius species differed pairwise by < 0.1%, but absolute genetic distances were greater between some species, e.g. 0.13413-0.14093 between P. tobbi and P. halepensis, and 0.12141-0.12828 between P. halepensis and P. major.

For subgenus Adlerius (single species, P. halepensis), fixed diagnostic polymorphisms occurred at amino acid positions 97, 101, 173 and 239 of the Cyt b Long fragment and at amino acid positions 39, 72 and 105 of the Cyt b 3’ fragment.

For subgenus Larroussius, fixed diagnostic polymorphisms occurred at amino acid positions 99, 106 and 238 of the Cyt b Long fragment and at amino acid positions 74 and 104 of the Cyt b 3’ fragment.

Aransay et al. (1999) showed that the 18S rRNA gene was a useful marker for inferring phylogenetic relationships within the subfamily Phlebotominae, finding a clade containing the subgenera Euphlebotomus, Adlerius and Larroussius, a second clade with Paraphlebotomus and Phlebotomus, and a third clade with Sergentomyia and American Lutzomyia species. Depaquit et al. (2000) used ITS2 rDNA gene sequences and found a clade with Paraphlebotomus and Phlebotomus. ITS2 rDNA sequences were also monophyletic for subgenus Larroussius (Muccio et al. 2000).

In our study, the DNA sequences of Cyt b were shown to be good markers for finding clades of genera and subgenera. Esseghir et al. (2000) characterized Cyt b for species of the same subgenera as we studied, but they had to give different weights to the nucleotides in 1st, 2nd and 3rd base positions of codons to get phylogenetic results. This was not the aim of our work, which was finding diagnostic markers for the species. The important phylogenetic result for us was to show that these species were monophyletic.
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