<u>**Original Article**</u>

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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(Received 5 May 2010; accepted 5 Dec 2010)

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 Packdasht, 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 egent is known to be *L. infantum,* which mainly affects children, with majority of cases from primary foci in Northwestern and Southern of the country (Mohebali 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* spiecies using analysis of Cyt b gene. It is noteworthy that up to present no record of any sandfly species from Caspian Sea littoral was avilable

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^{Tm} 3.1.1 software (Gene Codes Corporation). Multiple alignments of new DNA sequences and GenBank sequences were made using PAUP* 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 0.00262 – 0.00581. 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) (Table1). 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

Table 1. Countinued…

		Ash	S.P	Not done	IRN335	Not done	IRN337	M
		Ash	S.P	Not done	IRN338	Not done	IRN338	M
		Ash	CDC	Not done	IRN338	Not done	IRN339	M
	Pakdasht-Mamazand	Ash	CDC	Not done	IRN334	Not done	IRN340	M
		Ash	CDC	Not done	weak band	IRN413	IRN413	M
				P. major				
	Tonekabone- Meian kooh	I.H	Asp	IRN287	IRN287	Not done	IRN287	M
Mazanderan	**Tonekabone- Imamzade ghasem**	I.H	Asp	IRN291	IRN287	Not done	IRN291	M
	Tonekabone- Meian kooh	I.H	Asp	Not done	Not done	IRN385	IRN385	F
	Tonekabone- Imamzade ghasem	I.H	Asp	Not done	Not done	IRN385	IRN390	F
				P. halepensis				
	Tonekabone- Imamzade ghasem	I.H	Asp	IRN277	IRN277	Not done	IRN277	M
		I.H	Asp	IRN277	IRN277	Not done	IRN278	M
		I.H	Asp	IRN279	IRN277	Not done	IRN279	M
		I.H	Asp	IRN279	Not done	Not done	IRN280	M
		I.H	Asp	IRN282	Not done	Not done	IRN281	M
		I.H	Asp	IRN282	Not done	Not done	IRN282	M
	Tonekabone- Meian kooh	I.H	Asp	weak band	IRN277	Not done	IRN283	M
		I.H	Asp	IRN279	Not done	Not done	IRN284	M
		I.H	Asp	IRN285	Not done	Not done	IRN285	M
		I.H	Asp	IRN279	Not done	Not done	IRN286	M
		I.H	Asp	IRN279	Not done	Not done	IRN288	M
		I.H	Asp	IRN279	Not done	Not done	IRN289	M
		I.H	Asp	IRN285	Not done	Not done	IRN290	M
	Tonekabone- Imamzade ghasem	I.H	Asp	IRN279	Not done	Not done	IRN292	M
		I.H	Asp	IRN293	Not done	Not done	IRN293	M
Mazanderan		I.H	Asp	IRN294	Not done	Not done	IRN294	M
		I.H	Asp	bad sequence	IRN277	Not done	IRN295	M
		I.H	Asp	IRN279	Not done	Not done	IRN296	M
		I.H	Asp	weak band	Not done	IRN297	IRN297	M
	Tonekabone- Meian kooh	I.H	Asp	Not done	Not done	IRN380	IRN380	F
		I.H	Asp	Not done	Not done	IRN381	IRN381	F
		I.H	Asp	Not done	Not done	IRN381	IRN384	F
	Tonekabone- Imamzade ghasem	I.H	Asp	Not done	Not done	IRN381	IRN386	F
		I.H	Asp	Not done	Not done	IRN381	IRN388	F
		I.H	Asp	Not done	IRN277	weak band	IRN389	F
	Tonekabone- Meian kooh	I.H	Asp	Not done	Not done	IRN381	IRN392	F
		I.H	Asp	Not done	IRN277	weak band	IRN393	F
		I.H	Asp	Not done	Not done	IRN380	IRN394	M
		I.H	Asp	Not done	bad sequence	IRN383	IRN383	F
Mazanderan	**Tonekabone- Meian kooh**	I.H	Asp	Not done	bad sequence	weak band	IRN387	F
		I.H	Asp	Not done	Not done	Not done	IRN391	F

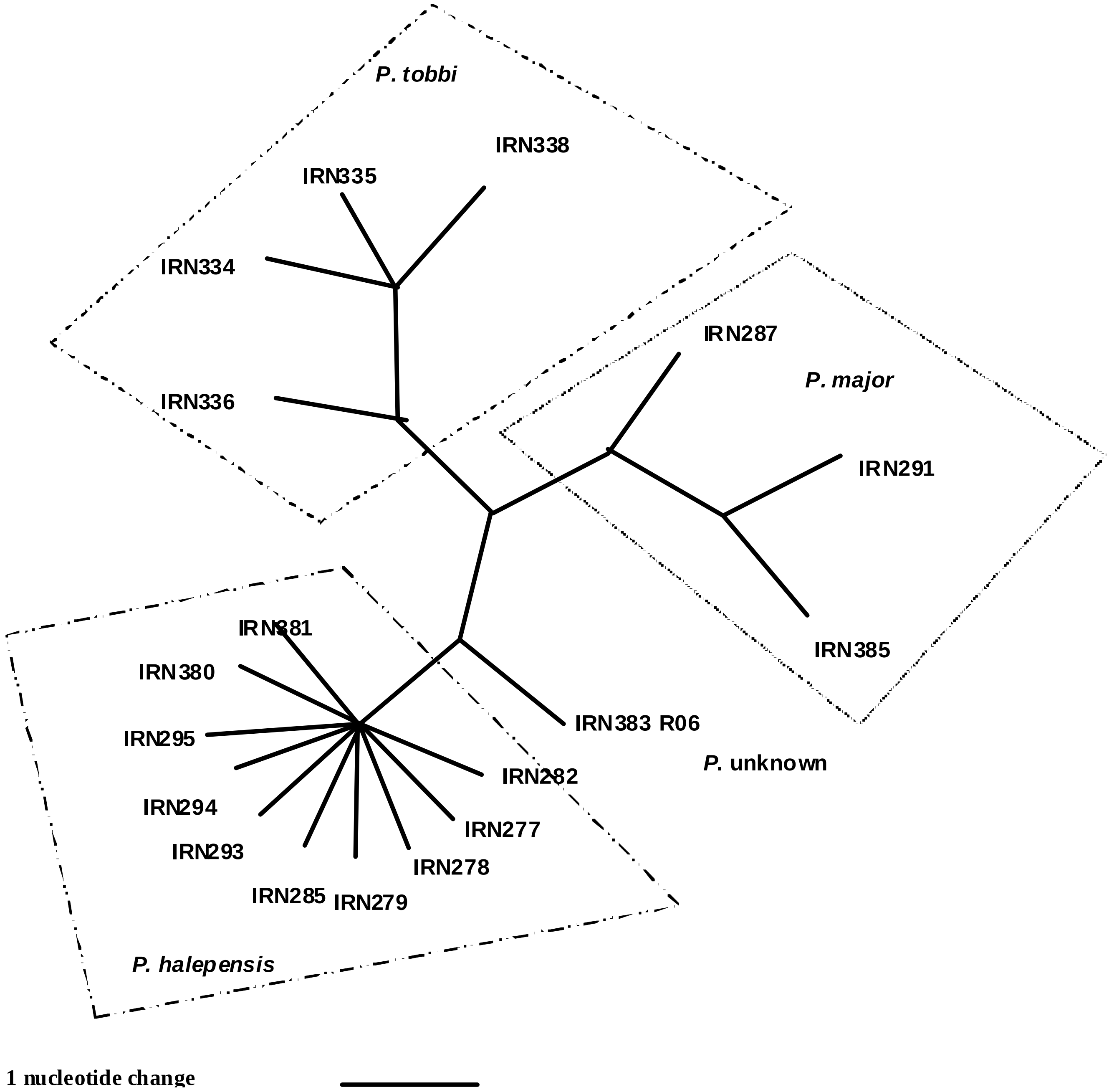

Fig. 1. Unrooted consensus phylogenetic tree for DNA sequences of Cyt b Long (718 nucleotides) of *Phlebotomus (Adlerius) / Phlebotomus (Larroussius),* produced by branch and bound parsimony search using PAUP*

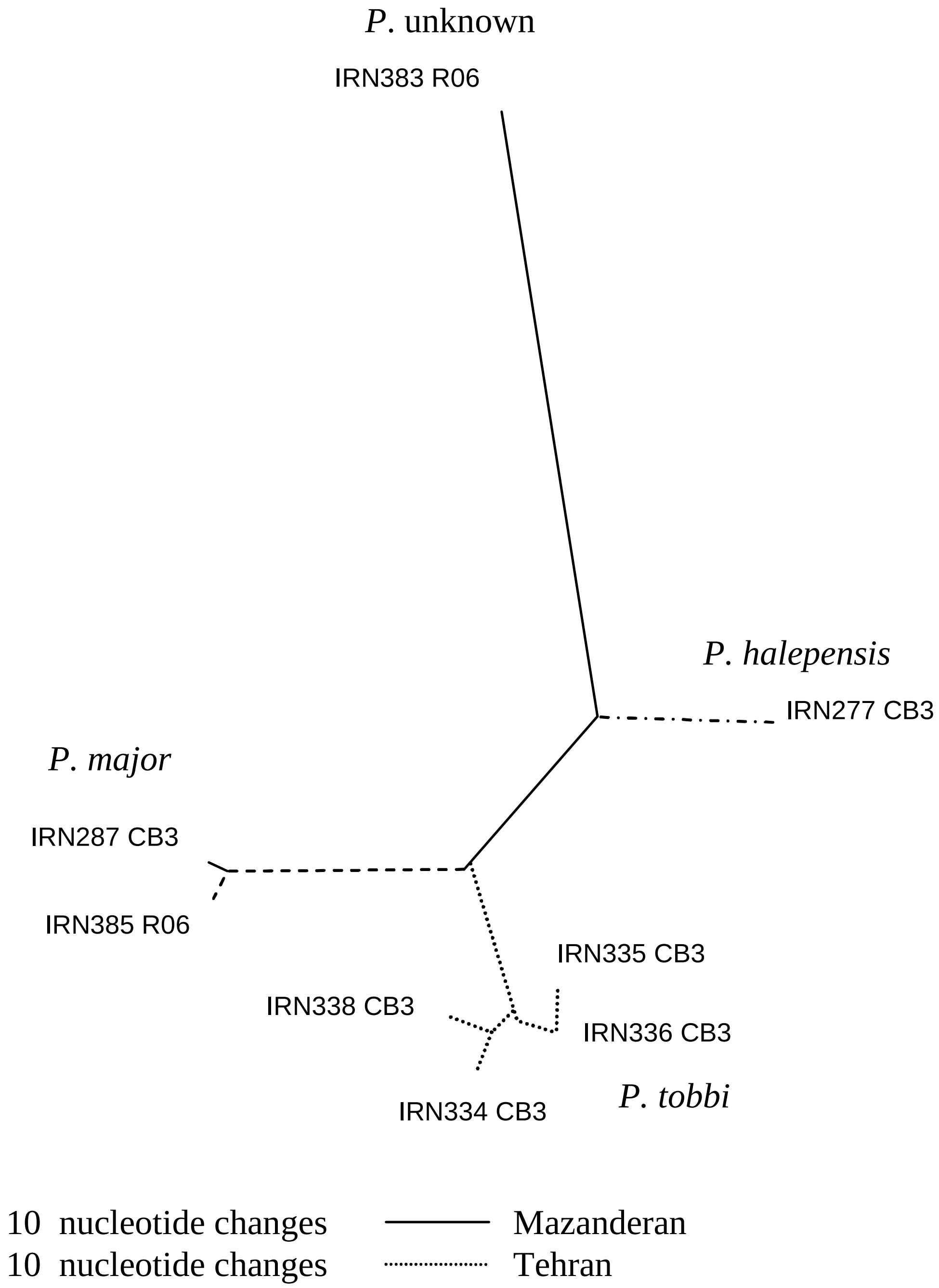

Fig. 2. Unrooted phylogenetic tree for DNA sequences of Cyt b 3' end (last 316 nucleotides) of *Phlebotomus* (*Adlerius*)/*Phlebotomus (Larroussius)*, produced by branch and bound parsimony search using PAUP*

Single or composite DNA sequence from each specimen	Nucleotide position 1111111111111111111111111111111111111222222 11222233344445666677788999990000000111222334444455666666777777889000001 1539235814703695125737909124780134789378567690234514124567012459129012581
IRN287haplo CB3	NNNATCTAACTTAAAATTGAAGAAACCTTTATACATACCCTTATTATAATTATAATAGATTTTATCAGCATAT
IRN385haplo R06	TCTACCTAACCTAAAATTGAAGAAACCTTTATACATACCTTTATTGTAATTATAATAGATTTTATCAGCATAT
IRN334haplo CB3	TTCTTCCTTTCCAAAACCGTAGTAATTAAATTACACTACATTATTACAATTATAATAGATTTTGTTAGCCCTT
IRN335haplo CB3	TTCTTCCTTTCCAAAACCGTAGTAATTAAATTACACTACATTATTACAATTATAATAGATTTTGTTAGCCCTT
IRN338haplo CB3	TTCTTCCTTTCCAAAACCGTAGTAATTAAATTACACTACATTATCACAATTATAATAGATTTTGTTAGCCCTT
IRN336haplo CB3	TTCTTCCTTTCTGAGACCGTAGTAATTAAATTACACTACATTATTACAATTATAATAGATCTTGTTAGCCCTT
IRN277haplo CB3	TTTATTAATTTTAAATTTGTTGTAATTATATCACATAATTCTCCTCTAATTATAATAGATTTTGATAGCTTTT
IRN279haplo CB3	TTTATTAATTTTAAATTTGTTGTAATTATATCACATAATTCTCCTCTAATTATAATAGATTTTGATAGCTTTT
IRN278haplo CB3	TTTATTAATTTTAAATTTGTTGTAATTATATCACATAATTCTCCTCTAATTATAATAGATTTTGATAGCTTTT
IRN295haplo CB3	NNNATTAATTTTAAATTTGTTGTAATTATATCACATAATTCTCCTCTAATTATAATAGATTTTGATAGCTTTT
IRN380haplo R06	TTTATTAATTTTAAATTTGTTGTAATTATATCACATAATTCTCCTCTAATTATAATAGATTTTGATAGCTTTT
IRN381haplo R06	TTTATTAATTTTAAATTTGTTGTAATTATATCACATAATTCTCCTCTAATTATAATAGATTTTGATAGCTTTT
IRN383haplo R06	CTTATTAACTATAGATTTATCAAGTTAATTAATATTTATTCACCTAATCAACATTATATCACCTTCTATATAA
IRN287haplo CB3	GCTTTAACAAAGTCTTTCCTATTAAAAATCTCAAATTTCTCAA
IRN385haplo R06	GCTTTAACAGAGTCTTTCCTATTAAAAATCTCAAATTTCTCAA
IRN334haplo CB3	GCTTTAACAATATCTTCCCTATATCAATATTCAAATTTTTCAA
IRN335haplo CB3	GCTTTAACAATGTCTTCCCTATATCAATATTCAAATTTTTCAA
IRN338haplo CB3	GCTTTAACAATGTCTTCCCTATATCAATATTCAAATTTTTCAA
IRN336haplo CB3	GCTTTAACAATGTCTTCCCTATATCAATATTCAAATTTTTCAA
IRN277haplo CB3	ACACTTACCACGTCTCTTCTACATAAATATTCTAATCATATAC
IRN279haplo CB3	ACACTTACCACGTCTCTTCTACATAAATATTCTAATCATATAC
IRN278haplo CB3	ACACTTACCACGTCTCTTCTACATAAATATTCTAATCATATAC
IRN295haplo CB3	ACACTTACCACGTCTCTTCTACATAAATATTCTAATCATATAC
IRN380haplo R06	ACACTTACCACGTCTCTTCTACATAAATATTCTAATCATATAC
IRN381haplo R06	ACACTTACCACGTCTCTTCTACATAAATATTCTAATCATATAC
IRN383haplo R06	AAAACGTTAATTATCCTCTATAATATTTTTAACTTAATTAATT

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 1111111111111111111111111111111111111111222222 11122223334455556666688890000001122222333333444555555566677788999000111 134925891273657890124725940136792512356034679289123467801923524369589145
IRN294haplo CB1	AAAGTACCCCACAGCTATATATAAGATGTTTAAATACTTCTTATCCTATGCACAAAATTCACCATACCTAAT
IRN291haplo CB1	TAATTATCATTCTGATATATAAAGGTTGTCCTCACACTTTTTATTATAAGCTCTAAACTTATATTATCCGTT
IRN277haplo CB1	TTAATTCTATATTAATATAAAAGAGATGTATAGCTACTTTTCTTTTTATGCACAAAATCCTTAAATCACAAT
IRN282haplo CB1	TTAATTCTATATTAATATAAAAGAGATGTATAACTACTTTTCTTTTTATGCACAAAATCCTTAAATCACAAT
IRN279haplo CB1	TTAATTCTATATTAATATAAAAGAGATGTATAGCTACTTTTCTTTTTATGCACAAAATCCTTAAATCACAAT
IRN287haplo CB1	TAATCATCATTCTAATATACAAAAGTTGTCCTCACACTTTTTATTATGAGCTCTAAACTTATATTATCCATC
IRN285haplo CB1	TTAATTCTATATTAATATAAAAGAGATGTATAGCTACTTTCCTTTTTATGCACAAAATCCTTAAATCACAAT
IRN380haplo R06	TTAATTCTATATTAATATAAAAGAGATGTATAGCTACTTTTCTTTTTATGCACAAAATCCTTAAATCACAAT
IRN383haplo R06	ACTATTTTATATTGTAGATATTGATTCCATCCAATGGCTTTATCTAAATATTGTCTTTTTATAAATTTTATC
IRN381haplo R06	TTAATTCTATATTAATATAAAAGAGATGTATAGCTACTTTCCTTTTTATGCACAAAATCCTTAAATCACAAT
IRN385haplo R06	TAATTATCATTCTGATATATAAAGGTTGTCCTCACACTTTTTATTACGAGCTCTAAACTCATATTATCCGTC
IRN293haplo CB1	???????????TTAATATAAAAGAGATGTATAGCTACTGTTCTTTTTATGCACAAAATCCTTAAATCACAAT
IRN294haplo CB1	ATAATTCAGATATCTCTACTCATAAATACACAAGATAACAAAAGCATTTTAGATTTCCGTTCTTCC
IRN291haplo CB1	TTCACTCAGACACCGCAGTGTGTAAATAGTCTTCTTATCTATAGCGCATCTGACTCTTATCTCCCC
IRN277haplo CB1	ACAACTCTGACTTTTTTGTTTATGAAAATATGTTTTATCTAAAGCTCATCAGGTATTCTACTTTCC
IRN282haplo CB1	ACAACTCTGACTTTTTTGTTTATGAAAATATGTTTTATCTAAAGCTCATCAGGTATTCTACTTCCC
IRN279haplo CB1	ACAACTCTGACTTTTTTGTTTATGAAAATATGTTTTATCTAAAGCTCATCAGGTATTCTACTTCCC
IRN287haplo CB1	TTYACTCAGACACCACAGTGTGTAAATAGTCTTCTTATCTATAGCACATCTGACTCTTCTCTTCCC
IRN285haplo CB1	ACAACTCTGACTTTTTTGTTTATGAAAATATGTTTTATCTAAAGCTCATCAGGTATTCTACTTCCC
IRN380haplo R06	ACAACTCTGACTTTTTTGTTTATGAAAATATGTTTTATCTAAAGCTCATCAGGTATTCTACTTCCC
IRN383haplo R06	ATTCTAAAATACTTATTATTAAATTTTTTAC?TTTATAATTATCTATTATAAACATCTATCTTTAT
IRN381haplo R06	ACAACTCTGACTTTTTTGTTTATGAAAATATGTTTTATCTAAAGCTCATCAGGTATTCTACTTCCC
IRN385haplo R06	TTCACTCAGACACCGCAGTGTGTAAATAGTCTTCTTATCTATAGCGCATCTGACTCTTATCTCCCC
IRN293haplo CB1	ACAACTCTGACTTTTTTGTTTATGAAAATATGTTTTATCTAAAGCTCATCAGGTATTCTACTTCCC

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 identifed 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 *Larrroussius*, 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 1^{st}, 2^{nd} and 3^{rd} 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.

Acknowledgements

We thank Dr. P.D. Ready, the leader of sandflies and leishmaniasis programme at the Natural History Museum (NHM), London, for his help, and supervising in his laboratory, in the NHM. This work was fund by the Pasteur Institute of Iran Grant 298 awarded to Dr. Parviz Parvizi. The authors declare that ther is no conflict of interests.

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