Mitochondrial DNA characterization of *Sergentomyia sintoni* populations and finding mammalian *Leishmania* infections in this sandfly by using ITS-rDNA gene

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Summary

Sergentomyia sintoni is the natural vector of *Sauroleishmania* species of lizards. This sandfly is abundance in and around the burrows of great gerbils. *S. sintoni* was collected from peridomestic animal shelters, inside and around houses and also from the nearby burrows of the gerbil reservoir hosts, *Rhombomys opimus*, in several provinces of Iran. Mitochondrial Cytochrome b (Cyt b) of sandflies, which is a maternally-inherited gene marker was used to characterize different haplotypes and populations of this sandfly. The analyses were based on the last 717 bp of the Cyt b gene followed by 20 bp of intergenic spacer and the transfer RNA ser (TCN) gene, i.e. the 737 bp fragment (without primers) amplified with the primers CB1-SE and CB-R06. The ITS-rDNA gene was also used to find *Leishmania* infections in *S. sintoni*. Cyt b 5´ fragment sequences were obtained from 22 *S. sintoni*, Cyt b 3´ fragment sequences were also obtained from 22 and Cyt b Long fragment sequences were obtained from 19 *S. sintoni*. By using nested PCR of ITS-rDNA gene, at least two species of *L. major* and *L. gerbilli s.l.* were found in *S. sintoni*. It needs further studies for considering the vectorial role of *S. sintoni* in ZCL foci, it's important role in maintaining *L. major* infections in the great gerbil and transmitting *Leishmania* species to people and among the reservoir hosts.

Key words: Cytochrome b, ITS-rDNA, *Sergentomyia sintoni*

Introduction

Zoonotic cutaneous leishmaniasis (ZCL) is a group of arthropod borne parasitic diseases of humans and other mammals caused by infections with protozoan haemoflagellates of the genus *Leishmania* Ross in the family Trypanosomatidae, order Kinetoplastida (Molyneux and Ashford, 1983).

All species in the genus alternate between mammalian hosts and their vectors, blood-feeding females of phlebotomine sandflies (Insects, Diptera, Psychodidae, subfamily Phlebotominae) - tiny sand-coloured flies that breed in forested areas, caves, or the burrows of small rodents (Lewis, 1974). Wild and domesticated mammals act as reservoirs of zoonotic infections and humans can be the sources of anthroponotic infections (Killick-Kendrick, 1990; Lane, 1993).

According to the most widely accepted concept (Lewis *et al*., 1977), there are six genera of sandflies: *Lutzomyia* França, *Brumptomyia* França and Parrot and *Warileya* Hertig in the New World and *Phlebotomus* Rondani and Berté, *Sergentomyia* França and Parrot and *Chinius* Leng in the Old World (Leng, 1987). Species and subspecies of *Phlebotomus* and *Lutzomyia* are the only proven vectors of *Leishmania*, although man-biting flies occur in other genera (Lane, 1993).

In general, the subgenera of *Phlebotomus* are better defined, though many species of *Sergentomyia* cannot be placed, whether because of aberrant features, unusual combinations of features, or inadequate information (Seccombe *et al*., 1993). Most species of *Sergentomyia* feed on reptiles and birds and some feed on

mammals. In many species infection with trypanosomatids was found other than *Leishmania*.

The first important objective of this report is to understand the phylogeography of mitochondrial Cytochrome b (Cyt b) of *Sergentomyia* (*Sergentomyia*) *sintoni* population differentiation because it can show where potential vectors can invade and how quickly, after local control measures (Parvizi and Ready, 2006). In particular, we wished to investigate the possibility that different lineages (or even cryptic species) of *S.* (*Se.*) *sintoni* might predominate in villages and in the burrows of the local reservoir host of *L. major*, the gerbil *Rhombomys opimus*.

Secondly, it is believed that *S.* (*Se.*) *sintoni* is the natural vector of *Sauroleishmania* species of lizards (Seccombe *et al*., 1993; Seyedi-Rashti *et al*., 1994) but finding *Leishmania* species of mammals on this sandfly by using nested PCR protocols (Parvizi *et al*., 2005), which amplified two regions of the ribosomal RNA amplicon of *Leishmania* (ITS1-5.8S rRNA gene, and a microsatellite DNA region of ITS2), can show the roles of this sandfly in maintaining *L. major* infections in the great gerbil and transmitting *Leishmania* species to people and among the reservoir hosts.

Previously, we had developed a nested PCR to amplify and directly sequence the complete ITS-rDNA region as one fragment, and then successfully applied this diagnostic test to identify genetic strains of *L. major* infecting Iranian sandflies (Parvizi *et al*., 2005). For the current investigation, nested PCR was made more sensitive by targeting two fragments of this ITS-rDNA region.

Leishmaniasis is usually a zoonosis, involving mammalian reservoirs other than man, since in many cases man is simply an accidental “dead end” host. However, there are a few examples where there is no reservoir host, and the parasites are found only in man (anthroponotic leishmaniasis) and in the insect vector. Female sandflies become infected by ingesting blood from infected reservoir hosts or from infected people. It is important to understand the natural transmission cycles for two reasons: first, enzootic cycles can pose an unknown threat in remote areas when these are being developed and secondly, it is possible that transmission is maintained by several sandfly species of which only one transmits to man (Killick-Kendrick and Ward, 1981; Killick-Kendrick, 1990).

Molecular epidemiology has become an essential tool to define the elements of a transmission cycle, and to identify the possible sources of infection. The methods and concepts of evolutionary genetics (population genetics and phylogenetic analysis) are essential for understanding the origin and the predicable properties of the genetic polymorphism of the pathogens (Esseghir *et al*., 1997, 2000; Parvizi *et al*., 2003). In the case of the host, genetic susceptibility to infectious disease has been demonstrated in several models. This susceptibility, may not be the same for different strains of the same *Leishmania* species. Finally, different populations of the same species of sandflies could differ in their transmissibility potential, and also different sandfly species for the same species of *Leishmania* could have different impact on strain virulence (Parvizi and Ready, 2006).

Materials and Methods

Collection and identification of sandflies

The collections were carried out from 24 July to 27 August 2001 and from 21 August to 12 September 2002 during summer, the main season of activity of adult sandflies in Iran. Sandflies were collected on sticky papers and funnel traps placed at the entrances of gerbil burrows (Parvizi *et al*., 2003), CDC (Centers for Disease Control) miniature light traps (Sudia and Chamberland, 1962) were set overnight to capture sandflies from domestic animal shelters, and aspirators were used to collect adult sandflies from their resting sites during the early morning hours (Parvizi and Ready, 2006).

All sandflies were identified by their species-specific Cyt b sequences. Most were also identified based on morphological characteristics of the head and abdominal terminalia, which were slide-mounted in Berlese fluid following dissection with sterilized forceps and micro-needles (Parvizi

et al., 2003, 2005).

Extraction of DNA from sandflies for PCR

Total DNA was extracted from the dissected thorax and attached anterior abdomen of individual sandflies using the method of Ish-Horowicz with minor modifications (Lewis, 1982; Ready *et al*., 1991). In the 1.5 ml microfuge tube, the thorax plus anterior abdomen of each sandfly were frozen and defrosted twice to break up tissues and lyses cells. They were then ground to break up the tissue using a sealed Pasteur pipette. SDS was used to denature proteins associated with the DNA, then ice cold 8M KOAc was added to effectively remove the SDS-bound proteins from solution. Cell debris and proteins were separated from the DNA by centrifugation, afterwards, the DNA in the supernatant was precipitated overnight at -20°C in 70% ethanol. Following ethanol precipitation, the DNA was dissolved in 15 µl 1 × TE (10 mM Tris-HCL, 1 mM EDTA, pH = 8.0), to give a concentration of 5-10 ng/µl, and stored at -20°C.

PCR amplification of sandfly Cytochrome b

For Cyt b three pairs of primers were used. CB1-SE (forward) was used with CB3-R3A (reverse) to amplify a more 5' fragment of 439 bp without primers (CB1 fragment), and CB3-FC (forward) was used with N1N-FA (reverse) to amplify an overlapping 3' fragment of 499 bp without primers (CB3 fragment). The last 717 bp of Cyt b was amplified by these two primer sets, and later the same part of the gene was amplified as one piece (Cyt b Long fragment), by using the primer CB1-SE (forward) with a reverse primer CB-R06, with one annealing temperature of 48°C. 1.5 µl of sandfly DNA was used for PCR amplification of each Cyt b fragment (Parvizi and Ready, 2006).

PCR amplification of sandfly Leishmania infections of ITS-rDNA

For nested PCR of ITS-rDNA of *Leishmania* species, the first-stage PCR used the forward primer IR1 at 3' end of SSU rRNA gene with the reverse primer IR2 at 5' end of LSU rRNA gene (Cupolillo *et al*., 1995), and the second-stage PCR used the nested forward primer ITS1F overlapping the 3' end of SSU rRNA gene and ITS1 with the nested reverse primer ITS2R4 at 5' end of ITS2. For nested primers for amplifying a microsatellite DNA region of ITS2, the first-stage PCR was retained and the nested PCR was again carried out in a separate tube, but the latter was performed with forward primer ITSmF1 with fluorescent marker dye, near 5' end of ITS2 and reverse primer within ITS2.

Direct sequencing of PCR products

One hundred ng of each purified DNA sample was cycle-sequenced using an ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (version 2.0) and AB1 373/377 sequencing systems (ABI, PE Applied Biosystems), with 3.2 pmol of the same primers that were used for PCR, except for primer CB1-SE which was replaced by CB1 (Esseghir *et al*., 2000).

Aligning and phylogenetic analysis of DNA sequences

DNA sequences were edited and aligned using Sequencher™ 3.1.1 software (Gene Codes Corporation). Multiple alignments of DNA sequences, and deduced amino acid sequences to check the end of Cyt b, were made using ClustalwPPC: Clustal W 1.7 with default parameters (Higgins *et al*., 1997). Phylogenetic Analysis Using Parsimony (PAUP*) software was used for phylogenetic analysis (Swofford, 2002).

Results

Diversity and distribution of mitochondrial haplotypes of *S.* (*Se.*) *sintoni*

The analyses were based on up to 494 bp (without primers) at the 3´ end of the Cyt b gene (Cyt b 3´ fragment), up to 382 bp (without primers) more towards the 5´ end of the Cyt b gene (Cyt b 5´ fragment) and up to 721 bp (without primers) of the 3' end of the Cyt b gene (Cyt b Long fragment).

Thirty-six *S. sintoni* (9 males and 27 females) were identified by morphological

and molecular characteristics. Seventeen flies (1 male and 16 females) were from Habib Abad, Isfahan province: 2 females captured in and around domestic animal shelters using CDC traps and the others from gerbil burrows using sticky papers. Five flies (2 males and 3 females) were from Turkemen Sahara: 2 females and one male from Fadavi (gerbil burrow), one female from Inchebron (animal shelter) and one male from Dashbron (gerbil burrow). Eight flies (4 males and 4 females) were from Bushehr province: 2 males from Bushehr city (inside house), 4 females from Ahram (3 from gerbil burrows and 1 from an animal shelter) and 2 males from Ahram (1 from gerbil burrow and 1 from an animal shelter). Six flies (2 males and 4 females) were from Tehran province: 1 male from Mamazand, Pakdasht, Tehran (animal shelter), 1 female and 1 male from Gheshlagh, Pakdasht, Tehran (yard wall close to animal shelter) and 3 females from Aberdej, Varamin, Tehran (gerbil burrow) (Table 1).

Cyt b 5´ fragment sequences were obtained from 22 out of 36 *S. sintoni*. Seven (31.8%) were haplotype IRN426, and 15 were unique haplotypes. Genetic distances between haplotypes were low, 0.00262-0.03292.

Cyt b 3´ fragment sequences were also obtained from 22 of the flies. Four (19%)

Table 1: All DNA haplotypes of Cyt b of *Sergentomyia sintoni* identified in Iran

Specimen N.	Sex	Provinces	Location	Habitat	Trap type	Cyt b haplotype		
						CB1-SE CB3-R3A	CB3-FC NIN-FA	CB1-SE CB-R06
IRN224	F	Isfahan	Habib Abad	G.B.	S.P.	W.P	W.P	Not done
IRN255	F	Isfahan	Habib Abad	G.B.	S.P.	IRN255	IRN255	W.P
IRN256	F	Isfahan	Habib Abad	G.B.	S.P.	IRN255	W.P	Not done
IRN437	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	W.P
IRN440	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN440
IRN441	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN441
IRN442	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	B.S.
IRN445	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN445
IRN446	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN446
IRN447	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN447
IRN448	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN448
IRN453	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN453
IRN450	M	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN450
IRN498	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN498
IRN509	F	Isfahan	Habib Abad	G.B.	S.P.	Not done	Not done	IRN509
IRN426	F	Isfahan	Habib Abad	Ash	CDC	Not done	Not done	IRN426
IRN427	F	Isfahan	Habib Abad	Ash	CDC	Not done	Not done	IRN427
IRN455	F	Golastan	Turk-F	G.B.	S.P.	Not done	Not done	IRN455
IRN457	F	Golastan	Turk-F	G.B.	S.P.	Not done	Not done	W.P
IRN461	M	Golastan	Turk-F	G.B.	S.P.	Not done	Not done	Not done
IRN465	F	Golastan	Turk-I	Ash	CDC	Not done	Not done	IRN465
IRN346	M	Golastan	Turk-D	G.B.	S.P.	Not done	IRN346	W.P
IRN331	M	Bushehr	Bushehr	I.H.	S.P.	W.P	W.P	Not done
IRN332	M	Bushehr	Bushehr	I.H.	S.P.	W.P	W.P	Not done
IRN395	F	Bushehr	Ahram	G.B.	S.P.	W.P	W.P	IRN395
IRN396	M	Bushehr	Ahram	G.B.	S.P.	Not done	Not done	IRN396
IRN397	F	Bushehr	Ahram	G.B.	S.P.	Not done	Not done	IRN397
IRN398	F	Bushehr	Ahram	G.B.	S.P.	Not done	Not done	IRN398
IRN399	F	Bushehr	Ahram	Ash	CDC	Not done	Not done	IRN399
IRN402	M	Bushehr	Ahram	Ash	CDC	Not done	Not done	IRN402
IRN341	M	Tehran	Pakdasht	Ash	CDC	W.P	W.P	Not done
IRN415	F	Tehran	Pakdasht	Y.W.	S.P.	Not done	Not done	IRN415
IRN416	M	Tehran	Pakdasht	Y.W.	S.P.	Not done	Not done	B.S.
IRN481	F	Tehran	Varamin	G.B.	F.T.	Not done	Not done	IRN481
IRN482	F	Tehran	Varamin	G.B.	F.T.	Not done	Not done	Not done
IRN484	F	Tehran	Varamin	G.B.	F.T.	Not done	Not done	IRN484

G.B. = gerbill burrow; Ash = animal shelter; S.P. = sticky paper; CDC = CDC miniature light traps; Turk = Turkemen Sahara; F = Fadavi; I = Inchebron; D = Dashbron; F.T. = funnel trap; B.S. = bad sequences; W.P = weak PCR; I.H. = inside house; Y.W. = yard wall

Single or composite DNA sequence from each specimen	Nucleotide position 1111111111111111111111111111112222222222222222222222222222222222223333333333333333333 1111122233344555556666688990001222233334555566667777889990011112222222334445556667788899999900000012222233344455 1034685891470902568012472814036212470369212341239256814369581457034678928147369589144560125689123470012582470392 4	Other sequence of same haplotype
anteIRN463 R06	CATATTTTCTTTTTTAAATTACACATTTTAACCAACTTACCTGCAACATTATACCCATTTTATACAATAGACTTTTTTAGTTATATTTTAGCTCAACCCGAGTATTAATATTT	-
anteIRN466 R06	?ATATTTTCTTTTTTAAATTACACATTTTAACCAACTTACCTGCAACATTATACCCATTTTATACAATAGACTTTTTTAGTTATATTTTAGCTCAACCCGAGTATTAATATTT	-
anteIRN469 R06	CATATTTTCTTTTTTAAATTACACATTTTAACCAACTTACCTGCAACATTATACCCATTTTATACAATAGACTTTTTTAGTTATATTTTAGCTCAACCCGAGTATTAATATTT	-
anteIRN470 R06	CATATTTTCTTTTTTAAATTACACATTTTAACCAACTTACCTGCAACATTATACCCATTTTATACAATAGATTTTTTTAGTTATATTTTAGCTCAACCCGAGTATTAATATTT	-
clydIRN403 R06	TAAATTTCTATAACCTGATTACTTATATTTCTCTCTTTACAAACTGTTCCTTACCAATCCTATATCGTTCATTATTCTAATCTCATAACTATATAGTTCATCTTCCTATACCT	-
clydIRN405 R06	?AAACTTCTATAACCTAATTGCTTATATTTCTCTCTTTACAAACTGTTCCTTACCAATCTTATATCGTTCATTATTCTAATCTCATAACTATATAGTTCATCTTCCTATACCT	-
clydIRN406 R06	TAAACTTCTATAACCTAATTACTTATATTTCTCTCTTTATAAACTGTTCCTTACCAATCTTATATCGTTCATTATTCTAATCTCATAACTATATAGTTCATCTTCCTACACCT	-
clydIRN411 R06	??AACTTCTATAACCTAATTACTTATATTTCTCTCTTTACAAACTGTTCCTTACCAATCTTATATCGTTCATTATTCTAATCTTATAACTATATAGTTCATCTTCCTATACCT	-
dentIRN460 R06	TATATAACCTTTTTTAAATTATACGTTTCATTTAATTCATTGTAGTATTATATTTAATTTATATTATAGATTTTTTTAGTTATATTTTAACTCTACCCAAGCATTAACTTTT	IRN472,IRN478
dentIRN462 R06	TATATAACCTTCTTTAAATTATACATTTCATTTAATTCATTGTAGTATTACATTTAATTTATACTATAGATTTTTTTAGTTATATTTTAACTCTACCCAAGCATTAACTTTT	-
dentIRN467 R06	?ATATAACCTTTTTTAAATTATACATTTCATTTAATTCATTGTAGTATTATATTTAACTTATATTATAGATTTTTTCAGTTATACTTTAACTCTACCCAAGTATTAACTTTT	IRN480
sintIRN395 RO6	??????TTCCTTTTTAGATTATACACTATACTTAATTCGCCTGCAACACTATATTGTTTAATATTACAGCTTTTTTTAGTTATGTCTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN397 R06	????????CCTTTTTAAATTATACACTATACTTAATTCACTTGCAACACTATATTTGTCTAATATTACAGCTTTTTTTAGTTATGTCTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN398 R06	TATATTTTCCTTTTTAAATTATACACTATACTTAATTCACTTGCAACACTATATTTATTTAATATTACAGCTTTTTTTAGTTATGTCTTAACTTAACCCAAGCATTAATTTTT	-
sintIRN402 R06	?ATATTTTCCTTTTTAAATTATACACTATACTTAATTCACTTGCAACACTATATTTGTTTAATATTACAGCTTTTTTTAGTTATGTCTTAACTTAACCCAAGTATTAGTTTTT	-
sintIRN426 R06	TATATTTTCCTTTTTAAATTATACGCTATACTTAATTCACTTGCAACACTATATTTATCTAATATTACAGCTTTTTTTCGTTATGTTTTAACTTAACCCAAGTATTAATTTTT	IRN440
sintIRN427 R06	TATATTTTCCTTTTTAAATTATACGCTATACTTAATTCACTTGCAACACTATATTTATCTAATATTACAGCTTTTTTTCGTTATGTTTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN441 R06	TATATTTTCCTTTTTAAATTATACGCTATACTTAATTCACTTGCAACACTATATTTATCTAATATTACAGTTTTTCTTCGTTATGTTTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN445 R06	TATATTTTCCTTTTTAAATTATACGCTATACTTAATTCACTTGCAACACTATATTTATCTAATATTACAGCTTTTTTTCATTATGTTTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN446 R06	TATATTTTCCTTTTTAAATTATACGCTATACTTAATTCACTTGCAACACTATATTTATCTAATATTACAGCTTTTTTTCGTTATGTTTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN447 R06	TATATTTTCCTTTTTAAATTATACGCTATACTTAATTCACTTGCAACACTATATTTATCTAATATTACAGCTTTTTTTCGTTATGTTTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN448 R06	TATATTTTCCTTTTTAAATTATACGCTATACTTAATTCACTTGCAACACTATATTTATCTAATATTATAGCTTTTTTTCGTTATGTTTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN450 R06	TATATTTTCCTTTTTAAATTATACGCTATACTTAATTCACTTGCAACACTATATTTATCTAATATTACAGCTTTTTTTCGTTATGTTTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN455 R06	TATATTTTCCTTTTTAAATTATACACCATACTTAATTCACTTGCAACACTATATTTGTTTAACATTACAGATTTTTTTAGTTATATCTTAACTCTACCCAAGYATTAGYATTT	-
sintIRN465 R06	?????TTTCCTTTTTAAATTACACACCATACTTAACTCACTTGCAACACTATATTTGTTTAACATTACAGATTTTTTTAGTTATATTTTAACTCAACCCAAGTATTAATTTTT	-
sintIRN481 R06	TATATTTTCCTTTTTAAGTTATACGCTATACTTAATTCACTTGCAACACTATATTTATCTAATATTACAGCTTTTCTTCGTTATGTTTTAACTTAACCCAAGTATTAATTTTT	-
sintIRN484 R06	TATATTTTCCTTTTTAGATTATACACCATACTTAATTCACTTGCAACACCATATTTGTCTAACATTATAGATTTTTTTAGTTATATCTTAACTCAACCCAAGTATTAATTTTT	-
sintIRN509 R06	??TATTTTCCTTTTTAAATTATACACCATACTTAATTCACTTGCAACACCATATTTGTCTAACATTATAGATTTTTTTAATTATATCTTAACTCAACCCAAGTATTAATTTTT	-
tibeIRN342 R06	TATCATATCTAAACTAAAACTTATGTATTAATTACCCTACTTGCTACTTCACTTTAAATCATCTTTACTCATCCCCCCTGCCATATTACAATTCTGTATAGCCTTTAACATTC	IRN347
tibeIRN358 CB1CB3	TGTAATATCTAAACTAAAACTTATGTACTAATTACCCCACTCGCTACTTCACTTCAAATCATCTTTACTCATCCCCCCTGCCATATTACAATTCTGTATAGCCTTTAACATTC	-
tibeIRN458 R06	??TCATATCTAAACTAAAACTTATGTATTAATTACCCCACTTGCTACTTCACTTTAAATCATCTTTACTCATCCCCCCTGCCATATTACAATTCTGTATAGCCTTTAACATTC	-
tibeIRN459 R06	TATCATATCTAAACTAAAACTTATGTATTAATTACCCCACTTGCTACTTCACTTTAAATCATCTTTACTCATCCCCCCTGCCATATTACAATTCTGTATAGCCTTTAACATTC	-

Fig. 1: Input data matrix of variant nucleotides for PAUP* analysis of Cyt b Long (717 bp) for all *Sergentomyia* species

Single or composite DNA sequence from each specimen	Nucleotide position 3333333333444444444444444444444444444444455555555555555555555555555555566666666666666666666666666667777777 5666777889000001222233344455566667888999990001111122223344555567788888889000111222233445555577778889990011111 8178069251356795145703956814703495145013463590134706795817134641601234679214703925691739012350346269017790234 5	Other sequence of same haplotype
anteIRN463 R06	ATTTACGATATTTTATATCTGAAGTAAATTACTTATTTGACTCTGTTATATTTATTATTGACAATTTATTCTTACAAAGCTTTACCTTTGGCTATATATGATTATATAAAT	-
anteIRN466 R06	ATTTACGATATTTTATATCTGAAGTAAATTACTTATTTGACTCTGTTATATTTATTATTGACAATTTATTCTTACAAAGCTTTACCTTTGTCTATATATGATTATATAAAT	-
anteIRN469 R06	ATTTACGATATTTTATATCTGAAGTAAATTACTTATTTGACTCTGTTATATTTATTATTGACAATTTATTCTTACAAAACTTTACCTTTGTCTATATATGATTATATAAAT	-
anteIRN470 R06	ATTTACGATATTTTATATCTGAAGTAAATTACTTATTTGACTCTGTTATATTTATTATTGACAATTTATTCTTACAAAACTTTACCTTTGTCTATATATGATTATATAAAT	-
clydIRN403 R06	AATCTTTTTACTTTACTCCTAATACTAAATCTTAATCTATTATAAATGTGTTCTCCTTATAAAATCTGTTATATACATTCCTACAACTACACTTCATTATCATTACTCAC	-
clydIRN405 R06	AATCTTTTTACTTTACTCCTAATACTAAATCTTAATCTATTATAAATGTGTTCTCTTTATAAAATCTGTTATATACATTCCTACAACTACACTTCATTATCATTACTCAC	-
clydIRN406 R06	AATCTTTTTACTTTACTCCTAATACTAAATCTTAATCTATTATAAATGTGTTCTCCTTATAAAATCTGTTATATACATTCCTACAACTACACTTCATTATCATTACTCAC	-
clydIRN411 R06	AATCTTTTTACTTTACTCCTAATACTAAATCTTAATTTATTATAAATGTGTTCTCCTTATAAAATCTGTTATATACATTCCTACAACTACACTTCATTATCATTACTCAC	-
dentIRN460 R06	ATTTAATTTATTTCTTATCTTATATAGATTACCTACTTGCCTCTGTTATATTTATTTTGTCTATTTACTCACACAACATCTTACCTCCGTCTACATATAATTATATAAAT	-
dentIRN462 R06	ATTTAACTTATTTCTTATCTTATACAGATTACCTACTTGCCTCTGTTATATTTATTTTGTCTATTTACTCACACAACATTTTACTTCCGTCTACATATAATTATATAAAT	-
dentIRN467 R06	ATTTAATTTATTTCTTATCTCATATAGGTTACCTACTTGCCTCTGTTATATTTATTTTGTCTATTTACTCACACAACATCTTACCTCCGTCTACATATAATTATATAAAT	-
sintIRN395 R06	ATCTAACCTATTTCATATCTTATATATATTACTTATTTGCCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTCACTCTCGTCTAT????????????????	-
sintIRN397 R06	ATCTAACTTATTTCATATCTTATATATGTTACTTATTTGCCCCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTCACTTTCGTCTATATACAATTATATAAAT	-
sintIRN398 R06	ATCTAACTTATTTCATATCTTATATATATTACTTATTTGCCTCTGTTGTACTTATTATATCTATCTGTTCATATACCATCTTACTTTCGTCTATATACAATTATATAAAT	-
sintIRN402 R06	ATCTAACCTATTTCATATCTTATATACATTACTTATTTGCCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTCACTTTCGTCTATATACAATTATATAAAT	-
sintIRN426 R06	ATCTAACTTATTCCATATCTTATATATATTACTTATTTGCCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTCACTTTCGTCTATATACAATTATATAAAT	-
sintIRN427 R06	ATCTAACTTATTCCATATCTTATATATATTACTTATTTGCCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTCACTTTCGTCTATATACAATTATATTAAT	-
sintIRN441 R06	ATCTAACTCATTCCATATCTTATATATATTACTTATTTGCCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTCACTTTCGTCTATATACAATTATATAAAT	-
sintIRN445 R06	ATCTAACTTATTCCATATCTTATATATATTACTTATTTRCCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTCACTTTCGTCTATATACAATTATATAAAT	-
sintIRN446 R06	ATCTAACTTATTCCATATCTTATATATATTACTTATTTACCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTTACTTTCGTCTATATACAATTATATAAAT	-
sintIRN447 R06	ATCTAACTTATTCCATATCTTATATATATTACTTATTTACCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTTACTTTCGTCTATATACAATTATATAAGT	-
sintIRN448 R06	ATCTAACTTATTCCATATCTTATATATATTACTTATTTGCCTCTGTTGTACTTATTATATCTGTTTGTTCATATACCATCTCACTTTCGTCTATATACAATTATATAAAT	-
sintIRN450 R06	ATCTAACTTATTCCATATCTTATATATATTACTTATTTGCCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTTACTTTCGTCTATATACAATTATATAAAT	-
sintIRN455 R06	ATCTAATTTGTTTCATATCTTATATATATCACTTATTTGCCTCTGTTGTACTTATTATATCTATTTGCTCATATACCATCTTACCTTCGTCTATATATAATTATATAAAT	-
sintIRN465 R06	GTCTAATTTGTTTCATATCTTATATATATCACTTATTTGCCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTTACCTTCGTCTATATATAATTATATAAAT	-
sintIRN481 R06	ATCTAACTTATTCCATATCTTATATATATTACTCATTTGCCTCTGTTGTACTTATTATATCTATTTGTTCATATACCATCTCACTTTCGTCTATATACAATTATATAAAT	-
sintIRN484 R06	ATCTAACTTGTTTCGTATCTTATATATATTACTTATTTGCCTCTGTTGTACTTATTACATCTATTTGTTCATATACCATCTTACCTTCATCTATATATAATTATATAAAT	-
sintIRN509 R06	ATCTAACTTGTTTCGTATCTTATATATATTACTTATTTGCCTCTGTTGTACTTATTACATCTATTTGTTCATATACCATCTTACCTTCGTCTATATATAATTACATAAAT	-
tibeIRN342 R06	ATTTATTCTACTCCAATCTAATTATAAAATTTAATTTCTTCTCTATAGAACACTTTATATTAAATTTGCTACTAGCAGACTTATATTTGTCTATAATAAACTACTATTCT	-
tibeIRN358 CB1CB3	ATTTATTCTACTCCAATCTAATTATAAAATTTAATTTCTTCTCTATAGAACACTTTATATTAAATTTGCTACTAGCAGACTTATATTTGTCTATAATAAACTACTATTCT	-
tibeIRN458 R06	ATTTATTCTACTCCAATCTAATTATAAAATTTAATTTCTTCTCTATAGAACACTTTATATTAAATTTGCTACTAGCAGACTTGTATTTGTCTATAATAAACTACTATTCT	-
tibeIRN459 R06	ATTTATTCTACTCCAATCTAATTATAAAATTTAATTTCTTCTCTATAGAACACTTTATATTAAATTTGCTACTAGCAGACTTATATTTGTCTATAATAAACTACTATTCT	-

Fig. 1: continued

were haplotype IRN426, 2 (9.5%) were haplotype IRN396 and 16 were unique haplotypes. Genetic distances between haplotypes were low, 0.00319-0.018609.

Cyt b Long fragment sequences were obtained from 19 out of 36 *S. sintoni*. Two (10.5%) were haplotype IRN426, and 17 were unique haplotypes (Table 1; Figs. 1 and 2). Genetic distances between haplotypes were low, 0.00139-0.17759.

Fixed diagnostic polymorphisms for specimens obtained from Isfahan province occurred at nucleotide positions 127, 247 and 385 of the Cyt b Long fragment (Fig. 1) and at nucleotide positions 305 and 313 of the Cyt b 3´ fragment, for specimens from Bushehr province at nucleotide positions 322, 337 and 382 of the Cyt b Long fragment, and at nucleotide positions 49, 94, 174 and 235 of the Cyt b 3´ fragment, for specimens from Golastan province at nucleotide positions 127, 343 and 379 of the Cyt b Long fragment and at nucleotide position 180 of the Cyt b 3´ fragment, and for specimens from Tehran province at nucleotide positions 409, 475 and 650 of the Cyt b Long fragment (Fig. 1) and at nucleotide position 73 of the Cyt b 3´ fragment. Nucleotide sequence data of *S. sintoni* reported in this paper are available in GenBank, EMBL and DDBJ databases under accession numbers from EU159490 to EU159507.

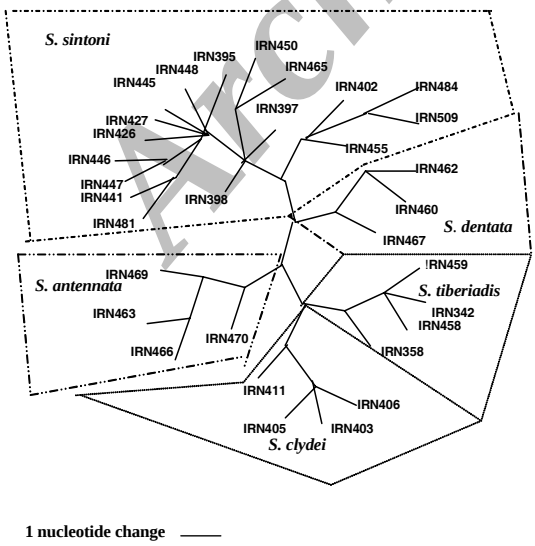

Fig. 2: Unrooted consensus phylogenetic tree for DNA sequences of Cyt b Long fragment (715 nucleotides) of *Sergentomyia* species, produced by heuristic parsimony search using PAUP*

Leishmania infections of *S.* (*Se.*) *sintoni* identified by ITS-rDNA haplotypes and sizing the DNA haplotypes of the ITS2 microsatellite

By using nested PCR of ITS-rDNA, at least two species of *Leishmania* were found in *S. sintoni*. Haplotypes of *L. major* were found in *S. sintoni* from Isfahan (not sampled in Golastan). Infections of *L. major* in single specimens of the three other sandfly species were all of the common haplotype for each ITS-rDNA fragment, and all came from Isfahan province.

L. gerbilli was never certainly found, because all infections except one were detected as the microsatellite fragment, which may have the same sequence in *L. gerbilli* and related species. These infections of *L. gerbilli s.l.* were found in Isfahan in six *S. sintoni*.

Discussion

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

In our study, the DNA sequences of Cyt b were good 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 which was not the aim of our work, it was to find diagnostic markers for species. The important phylogenetic result for us was to show that this species was monophyletic.

Many sandflies from the Isfahan focus and other Iranian foci of rural ZCL have

been dissected and found to be infected with leptomonads: *P. papatasi*, species of the subgenus *Paraphlebotomus* (including *P. caucasicus*, *P. mongolensis*), *P.* (*Sy.*) *ansarii*, *S. sintoni* and other often unidentified *Sergentomyia* species (Nadim *et al*., 1968b; Nadim and Seyedi-Rashti, 1971; Nasseri and Modabber, 1977; Javadian *et al*., 1980). Usually the authors did not report how they distinguished mammalian *Leishmania* from lizard *Sauroleishmania* or parasites of other genera.

In countries of north of Iran, much transmission of *L. major*, *L. turanica* and *L. gerbilli* is believed to occur in or near the burrows of the great gerbil *R. opimus* (Strelkova *et al*., 2001). These burrows provide refuges for many sandfly species as well as for mammals and reptiles, making them a high risk habitat for the transmission of parasites to mammals and lizards (Perfil'ev, 1966). In the ex-U.S.S.R., the distribution of *L. turanica* in rodents coincides with that of the sandflies *P. papatasi*, *P. caucasicus*, *P. mongolensis* and *P. andrejevi* (Strelkova *et al*., 1990), but there are no reports of *L. turanica* or *L. gerbilli* being isolated from sandflies and unambiguously typed. However, *P. caucasicus* or a related species was judged to be a proven vector of an undescribed *Leishmania* species in the ex-U.S.S.R. (Killick-Kendrick, 1990; Strelkova *et al*., 2001), and parasites from the same habitat were later described as *L. turanica* (Strelkova *et al*., 1990). In China, only *L. turanica* (not *L. gerbilli*) was isolated and typed isoenzymically from the following sandflies: *P. caucasicus*, *P. mongolensis*, *P. andrejevi* and *P. major wui* (Guan Li-Ren *et al*., 1986, 1995).

More than 46 species of sandflies have been reported in Iran (Seccombe *et al*., 1993), but only a minority are common in ZCL foci and bite gerbils and/or people.

In the current report, *L. major* was identified in the ZCL foci in Isfahan province in *S. sintoni*; infections of *L. gerbilli s.l.* were also found in Isfahan in *S. sintoni*. *L. major* previously identified firmly from Iranian sandflies, in single females of *P. papatasi*, *P. caucasicus* and *P. mongolensis* from the Isfahan focus of rural ZCL (Yaghoobi-Ershadi *et al*., 1995, 1996), but many natural leptomonad infections were found by dissection and microscopy and most assumed to be *L. major*, both in Isfahan (Yaghoobi-Ershadi and Javadian, 1996; Yaghoobi-Ershadi and Akhavan, 1999) and in Golastan provinces (or Turkemen Sahara) (Nadim *et al*., 1968a; Seyedi-Rashti *et al*., 1994) without distinguishing *Leishmania* species.

All of the sandfly species found naturally infected in Iran might be vectors of one or more of the gerbil parasites, but the detection of *Leishmania* DNA in a sandfly does not prove that it is a vector. For example, our PCR assays did not distinguish between infective-stage metacyclic parasites and non-infective stages of *Leishmania* promastigotes.

Based on detecting parasite DNA in sandflies, we can only conclude that *S. sintoni* is common in Iranian ZCL foci. It sometimes acquires one or more gerbil parasites by feeding on reservoir hosts or lizards, which are often great gerbils in the regions sampled.

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References

Aransay, AM; Scoulica, E and Tselentis, Y (2000). Detection and identification of *Leishmania* DNA within naturally infected sandflies by semi-nested PCR on minicircle kinetoplastic DNA. Appl. Environ. Microbiol., 66: 1933-1938.

Cupolillo, E; Grimaldi, JrG; Momen, H and Beverly, SM (1995). Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of *Leishmania*. Mol. Biochem. Parasitol., 73: 145-155.

Depaquit, J; Ferte, H; Leger, N; Killik-Kendrick, R; Rioux, JA; Killick-Kendrick, M; Hanafi, HA and Gobert, S (2000). Molecular systematics of the phlebotomine sandflies of the subgenus *Paraphlebotomus* (Diptera, Psychodidae, *Phlebotomus*) based on ITS2

rDNA sequences. Hypotheses of dispersion and speciation. Insect Mol. Biol., 9: 293-300.

Esseghir, S; Ready, PD; Killick-Kendrick, R and Ben-Ismail, R (1997). Mitochondrial haplotypes and phylogeography of *Phlebotomus* vectors of *Leishmania major*. Insect Mol. Biol., 6: 211-225.

Esseghir, S; Ready, PD and Ben-Ismail, R (2000). Speciation of *Phlebotomus* sandflies of the subgenus *Larroussius* coincided with the late Miocene-Pliocene aridification of the Mediterranean subregion. Biol. J. Linn. Soc., 70: 189-219.

Guan, Li-Ren; Xu, Yong-Xiang and Li Bao-Shan, Dong Jiang (1986). The role of *Phlebotomus alexandri* Sinton, 1928 in the transmission of kala-azar. Bull. World Health Organ., 64: 107-114.

Guan, Li-Ren; Yang, Yuan-Qing; Qu, Jing-Qi and Shen, Wei-Xia (1995). Discovery and study of *Leishmania turanica* for the first time in China. Bull. World Health Organ., 73: 667-672.

Higgins, DG; Thomson, JD and Gibson, TJ (1997). Clustal W Multiple Sequence Alignment Program, Version 1.7.: www.sgi.com/chembio/resources/clustalw/

Javadian, E; Seyedi-Rashti, MA and Rouhbakhsh Khaleghdoust, A (1980). Status of leishmaniasis in Iran. WHO travelling seminar on leishmaniasis control, Moscow. P: 50.

Killick-Kendrick, R (1990). Phlebotomine vectors of the leishmaniasis: a review. Med. Vet. Entomol., 4: 1-24.

Killick-Kendrick, R and Ward, RD (1981). Ecology of the *Leishmania*. Parasitology. 82: 143-152.

Lane, RP (1993). Sandflies (Phlebotominae). In: Lane, RP and Crosskey, RW (Eds.), *Medical insects and arachids*. (1st. Edn.), London, Chapman and Hall. PP: 78-119.

Leng, YJ (1987). A preliminary survey of phlebotomine sandflies in limestone caves of Sichuan and Guizhou provinces, sought-vest China, and description and discussion of a primitive new genus *Chinius*. Ann. Trop. Med. Parasitol., 81: 311-317.

Lewis, DJ (1974). The biology of phlebotomidae in relation to leishmaniasis. Ann. Rev. Ent., 19: 363-385.

Lewis, DJ (1982). A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). Bull. Brit. Mus. Nat. Hist. (Ent.), 45: 121-209.

Lewis, DJ; Young, DG; Fairchild, GB and Minter, DM (1977). Proposals for a stable classification of the phlebotomine sandflies (Diptera: Psychodidae). Syst. Ent. Res., 2: 435-442.

Molyneux, DH and Ashford, RW (1983). The biology of the *Trypanosoma* and *Leishmania*, parasites of man and domestic animals. New York, Taylor and Francis, Inc., P: 294.

Muccio, TD; Marinucci, M; Frusteri, L; Maroli, M; Pesson, B and Gramiccia, M (2000). Phylogenetic analysis of *Phlebotomus* species belonging to the subgenus *Larroussius* (Diptera, Psychodidae) by ITS2 rDNA sequences. Insect Biochem. Mol. Biol., 30: 387-393.

Nadim, A and Seyedi-Rashti, MA (1971). A brief review of the epidemiology of various types of leishmaniasis in Iran. Acta Med. Iran. 14: 99-106.

Nadim, A; Seyedi-Rashti, MA and Mesgali, A (1968a). Epidemiology of cutaneous leishmaniasis in Turkemen Sahara Iran. J. Trop. Med. Hyg., 71: 238-239.

Nadim, A; Seyedi-Rashti, MA and Mesgali, A (1968b). On the nature of leptomonads found in *Sergentomyia sintoni* in Khorassan, Iran and their relation to lizard leishmaniasis. J. Trop. Med. Hyg., 71: 240.

Nasseri, M and Modabber, F (1977). Generalized infection and lack of delay hypersensitivity in BALB/c mice infection with *Leishmania major*. Presented at 8th Pahlavi medical Congress, Shiraz, Iran.

Parvizi, P; Benlarbi, M and Ready, PD (2003). Mitochondrial and *Wolbachia* markers for the sandfly *Phlebotomus papatasi*: little population differentiation between peridomestic sites and gerbil burrows in Isfahan province, Iran. Med. Vet. Entomol., 17: 351-362.

Parvizi, P; Mauricio, I; Aransay, AM; Miles, MA and Ready, PD (2005). First detection of *Leishmania major* in peridomestic Iranian sandflies: comparison of nested PCR of nuclear ITS ribosomal DNA and semi-nested PCR of minicircle kinetoplast DNA. Acta Trop., 93: 75-83.

Parvizi, P and Ready, PD (2006). Molecular investigation of the population differentiation of *Phlebotomus papatasi*, important vector of *Leishmania major* in different habitats and regions of Iran. Iranian Biomed. J., 10: 69-77.

Perfil'ev, PP (1966). Fauna of the U.S.S.R. Diptera. Academy of Sciences of U.S.S.R., Zoological Institute. New Series, No.93, III: 382 (Translated from Russian by Israel Program for Scientific Translations, Jerusalem, 1968).

Ready, PD; Lainson, R; Shaw, JJ and Souza, AA (1991). DNA probes for distinguishing *Psychodopygus wellcomei* from *Psychodopygus complexus* (Diptera:

Psychodidae). Mem. Inst. Oswaldo Cruz. 86: 41-49.

Seccombe, AK; Ready, PD and Huddleston, LM (1993). A catalogue of Old World phlebotomine sandflies (Diptera: Psychodidae, Phlebotominae). Occas. Pap. Syst. Ent., 8: 1-57.

Seyedi-Rashti, MA; Agh-Atabay, MD and Mohebali, M (1994). Natural promastigote infection of *Sergentomyia sintoni*, its seasonal variation and reservoir host in Turkemen-Sahara, Iran. Iranian J. Pub. Health. 23: 41-50.

Strelkova, MV; Eliseev, LN; Ponirovsky, EN; Dergacheva, TI and Evans, DA (2001). Mixed leishmanial infections in *Rhombomys opimus*: a key to the persistence of *Leishmania major* from one transmission season to the next. Ann. Trop. Med. Parasit., 95: 811-819.

Strelkova, MV; Shurkhal, AV; Kellina, OI; Eliseev, LN; Evans, DA; Peters, W; Chapman, CJ; Leblancq, SM and Van Eys, GJJM (1990). A new species of *Leishmania* isolated from the great gerbil *Rhombomys opimus*. Parasitology. 101: 327-335.

Sudia, WD and Chamberland, RW (1962). Battery operated light trap, an improved model. Mosq. News. 22: 126-129.

Swofford, DL (2002). PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods) version 4.0. Sinauer Associates, Sunderland, Massachusetts.

Yaghoobi-Ershadi, MR and Akhavan, AA (1999). Entomological survey of sandflies (Diptera: Psychodidae) in a new focus of zoonotic cutaneous leishmaniasis in Iran. Acta Trop., 73: 321-326.

Yaghoobi-Ershadi, MR; Akhavan, AA and Mohebali, M (1996). *Meriones libycus* and *Rhombomys opimus* (Rodentia: Gerbillidae) are the main reservoir hosts in a new focus of zoonotic cutaneaous leishmaniasis in Iran. Trans. R. Soc. Trop. Med. Hyg., 90: 503-504.

Yaghoobi-Ershadi, MR and Javadian, E (1996). Seasonal variation of *Leishmania major* infection rates in sandflies from rodent burrows in Isfahan province. Iranian Med. Vet. Entomol., 10: 181-184.

Yaghoobi-Ershadi, MR; Javadian, E and Tahvildare-Bidruni, GH (1995). *Leishmania major* MON-26 isolated from naturally infected *Phlebotomus papatasi* (Diptera: Psychodidae) in Isfahan Province, Iran. Acta Trop., 59: 279-282.