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Molecular Investigation of the Population Differentiation of *Phlebotomus papatasi,* Important Vector of *Leishmania major,* in Different Habitats and Regions of Iran

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ABSTRACT

***Phlebotomus papatasi* is an important vector of *L. major* in Iran. *P. papatasi* was collected from peridomestic animal shelters, inside and around the houses and also the nearby burrows of the gerbil reservoir hosts, *Rhombomys opimus*, in several provinces in Iran. Mitochondrial Cytochrome b (Cyt b) of sandflies, which is a maternally-inherited gene marker, was used to see if there is any "isolation by distance" over a large geographical scale among Iranian provinces. The analyses were based on the last 717 bp of the Cyt b gene followed by 20 bp of intergenic spacer and the transfer tRNA ser (TCN) gene, i.e. the 737 bp fragment (without primers) amplified with the primers CB1-SE and CB-R06. Cyt b Long fragment sequences were obtained from 149 out of 177 specimens of *P. papatasi* and 49 haplotypes were identified. Based on the Cyt b Long fragment examined, *P. papatasi* showed only recent divergence in Iran, because the genetic distances between haplotypes were small. However, some evidence for isolation by distance was found. First, all the haplotypes from Iran did not belong to a single network, whereas most from Golestan province (Iran) did belong to a single network. Second, there were some abundant haplotypes that were found only in one province.** *Iran. Biomed. J. 10 (2): 69-77, 2006*

Keywords: Mitochondrial cytochrome b (Cyt b), Iranian sandflies, *Phlebotomus papatasi*, Isolation by distance

INTRODUCTION

The bloodsucking adult females of *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae) are important vectors of *Leishmania major* Yakimoff and Schokhor (Kinetoplastida: Trypanosomatidae) in Iran [1-4]. The distribution of *P. papatasi* extends beyond that of *L. major* and its reservoir hosts to southern Europe and eastern regions of the Indian subcontinent, and throughout its range this sandfly is often abundant in domestic and peridomestic biotopes far from the colonies of its gerbil reservoir hosts [5, 6]. *P. papatasi* is also the vector of arboviruses in many countries including Iran [7, 8].

It is important to understand the population differentiation of sandfly vectors because it can show where potential vectors can invade from, and how quickly after local control measures. Our first investigation of the population differentiation of *P. papatasi* in Iran was to compare peridomestic populations on the edges of villages with populations in nearby gerbil colonies in Isfahan province (Iran), for which two maternally-inherited gene markers were used: mitochondrial Cytochrome b (Cyt b) of sandflies and a surface protein gene (*wsp*) of their endosymbiotic *Wolbachia* bacteria [6]. In this study, a larger fragment of sandfly Cyt b was used to see if there is any "isolation by distance" over a larger geographical scale among Iranian provinces.

The mitochondrial genome is one of the best studied of all types of DNA [9]. It has been shown to be a good source of accessible genetic variation, and analysis of mitochondrial DNA (mtDNA) variation has been used to understand evolutionary biology both at the intraspecific and interspecific levels [10]. Its rapid rate of evolution, clonal inheritance, and lack of recombination have made it a valuable tool for phylogeographic studies and a

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source of markers for species and geographic populations. This makes it a clear choice for providing markers for identifying Iranian sandflies. Metazoan mtDNA occurs as a double stranded, circular molecule ranging in size from approximately 14 kb in some nematodes [11] to around 40 kb in the scallop *Placopecten magellanicus* [12]. Among the insects, the first mitochondrial genomes were obtained by cloning and sequencing, e.g. for *Drosophila yakuba* [13] and *Apis mellifera* [14], and then by using PCR, e.g. for *Anopheles quadrimaculatus* [15] and *Anopheles gambiae* [16]. These data show polymorphisms for total size, gene order, restriction sites, and nucleotide sequences of specific loci, and all have been informative. Each copy of mtDNA contains a control region, known in insects as the (A-T)-rich region, which contains the replication origin [16, 17]. Among insects, the location and orientation of the genes encoding proteins and ribosomal RNA are the same, although those of some tRNA vary [16].

Some of the proteins of the inner mitochondrial membrane contain haem prosthetic groups, which give characteristic visible absorption spectra, and are called the cytochromes [9]. One of these is Cyt b, which is usually useful for the molecular systematics of insect species in related genera but not always for the phylogenetics of higher taxa [18]. The last (or 3') 700 bp of Cyt b of sandflies has been successfully used for several phylogeographic studies of Old World species [6, 19-21]. This region was also shown to be useful for dating speciation events because of its clock-like rate of nucleotide substitution [19, 20], but it should be used together with nuclear markers because mtDNA can pass among species by introgressive hybridization [20, 22]. The mtDNA is multicopy in somatic cells [9], and a single insect (or even one of its cells) can contain more than one sequence variant, or haplotype. This heteroplasmy is a potential problem for using mtDNA as a marker. We have tried to avoid this problem by directly sequencing PCR products, which should usually produce sequences only of the predominant haplotype in each sandfly.

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 the main summer season of activity of adult sandflies in Iran. Sandflies were collected on sticky papers and funnel traps were placed at the entrances to gerbil burrows, Centers For Disease Control (CDC) miniature light traps were set overnight to sample sandflies in domestic animal shelters, and aspirators were used to collect adult sandflies from their resting sites during the early morning hours [4]. All sandflies were identified by their species-specific Cyt b sequences. Most were also identified based on morphological characters of the head and abdominal terminalia, which were slide-mounted in Berlese fluid following dissection with sterilized forceps and micro-needles [4].

PCR amplification of sandfly Cyt b. For Cyt b, three pairs of primers were used. In the published report [6]: 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 (this report) the same part of the gene was amplified as one piece (Cyt b Long fragment), by using the primer CB1-SE (forward) (5´TATGT ACTACCCTGAGGACAAATATC3´) with a new reverse primer CB-R06 (5´TATCTAATGGTTTC AAAACAATTGC 3´), with one annealing temperature of 48°C. Sandfly DNA (1.5 µl) was used for PCR amplification of each Cyt b fragment.

Direct sequencing of PCR products. Each purified DNA sample (100 ng) 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 [20].

Aligning and phylogenetic analysis of DNA sequences. DNA sequences were edited and aligned using Sequencher^{Tm} 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. Phylogenetic Analysis Using Parsimony (PAUP*) software was used for phylogenetic analysis [23].

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RESULTS

Diversity and distribution of Cyt b Long fragment haplotypes. 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.

Males (n = 31) and females (n = 146) of *P. papatasi* were identified by morphological and molecular characters (Table 1). Out of 177 specimens, 4 were from Bushehr province (Ahram), 48 were from Golestan province (Turkemen Sahara), 114 were from Isfahan province (Isfahan, Iran), and 11 were from Tehran province (Pakdasht and Varamin).

Cyt b Long fragment sequences were obtained from 149 out of 177 specimens of *P. papatasi* and 49 haplotypes were identified (Table 1). Only haplotype IRN02 was found in all four provinces. Only three other haplotypes were found in more than one province: haplotype IRN491 in Isfahan, Tehran and Golestan; haplotype IRN420 in Isfahan and Tehran; and haplotype IRN434 in Isfahan and Golestan. The other 45 haplotypes which were "private haplotypes" were found only in a single province: 30 in Isfahan (out of 34 haplotypes), 13 in Golestan (out of 16 haplotypes), 2 in Tehran (out of 5 haplotypes) and 0 in Bushehr (out of 1 haplotype).

Out of 45 haplotypes found in a single province, only three were "abundant", because they were found in more than two flies and in 10% or more of all flies sequenced. These three haplotypes were IRN06 from Isfahan (found in 18 flies out of 114 sequenced), and both IRN584 (found in 4 flies out of 41 sequenced) and IRN591 (found in 6 flies out of 41 sequenced) from Golestan (Table 1). These "abundant" haplotypes could be proof of "isolation by distance", because they were found only in one province. Most of the haplotypes found in a single province were isolated only once ("unique" haplotypes, 37 out of 45) or twice (5 out of 45).

Phylogenetic analysis of DNA haplotypes of the Cyt b Long fragment of Iranian P. papatasi. Forty-two of the haplotypes were complete (737 bp) and could be used for distance analysis (Fig. 1). The Neighbor-joining (NJ) phylogram showed two primary branches, and each of these had subgroups with relatively long branches. One of the primary branches had only haplotypes from flies from Golestan province (Branches ▬▬). The second primary branch had haplotypes from flies from all four provinces, but some of the subgroups had haplotypes only from one province: Isfahan province (Branches shown ———) or Tehran province (Branches shown - - - - - - -). The pairwise genetic distances between haplotypes were low, 0.00136-0.00866 or 1-6 bp. The highest pairwise genetic distance was between IRN542 (a female from an animal shelter in Khorzoogh, Isfahan province) and IRN569 (a female from a gerbil burrow near Dashbron, Golestan province). However, there was no evidence for some Cyt b lineages being found mostly in different habitats-see habitat symbol codes in Figure 1.

Sequences with such small genetic distances are best analysed by distance methods [24], but we wanted to use parsimony to find haplotype networks. Among the 38 variant characters, only 11 were phylogenetically informative in maximum parsimony analyses (Characters 55, 152, 205, 265, 321, 379, 407, 451, 514, 541, 619; Table 2.). However, it was not possible to complete over three days even an heuristic search with all 42 haplotypes. Therefore, some haplotypes were removed in order to find sets of haplotypes that were monophyletic and could be analysed by heuristic searches.

The sets of haplotypes for separate parsimony analysis were suggested by the NJ phylogram (Fig. 1). One set contained the widespread haplotype IRN02 (found in all four provinces), all three haplotypes found only in Tehran province, and 18 out of the 30 haplotypes found only in Isfahan province (Fig. 1). A heuristic search with equal character weighting and Tree Bisection Replacement (TBR) branch swapping gave a single most parsimonious tree (Fig. 2), i.e. a single monophyletic network of haplotypes. This network can be explained as a radiation of short branches (recent evolution from a common ancestor). The three longer branches grouped haplotypes IRN418, IRN419 and IRN420 from Tehran (Animal shelter, Pakdasht), haplotypes IRN505, IRN525 and IRN549 from Isfahan (Animal shelters and gerbil burrows, Khorzoogh and Habib Abad villages), and haplotypes IRN425, IRN522, IRN528, IRN535 and the second most abundant haplotype IRN06 from Isfahan (Animal shelters and gerbil burrows) (Fig. 2). Adding other haplotypes to this first set (even just IRN430 and IRN496) prevented the heuristic search from finding a solution overnight, and so we conclude that the other haplotypes from Isfahan and Golestan provinces are probably not monophyletic with the haplotypes in the first set.

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Table 1. No. of *P. papatasi* with each haplotype of the Cyt b Long fragment.

Province /Town	Site/Population (Dates / nights)	No. of flies screened	No. of flies with each haplotype of Cyt b Long fragment													Not done, or bad sequences	Total no. of haplotypes
			IRN02	IRN06	IRN491	IRN591	IRN584	IRN430	IRN420	IRN425	IRN434	IRN516	IRN571	IRN605	Unique Haplotypes		
BUSHEHR Tangestan	Ahram, Ash., CDC (21-22. 8. 2002)	4	4														1
	Turk-Inch, G.B., S.P., (01-02. 9. 2002)	3	1												1	1	2
GOLESTAN Gonbad	Turk-Dash, G.B., S.P., (02-03. 9. 2002)	22	4		3	3	3				1		1		3	4	9
	Turk-Dash, Ash, CDC (02-03. 9. 2002)	23	3		6	3	1						1	2	5	2	11
	Habib Abad, G.B., S.P., (24-25. 8. 2001)	33	9	8	2				1	1					8	4	13
ISFAHAN Isfahan	Habib Abad, Ash., CDC (24-25. 8. 2001)	37	20	4				2		1	1	1			8		14
	Khorzoogh, G.B., S.P., (22-24. 8. 2001)	8														8	
	Khorzoogh, Ash., CDC (22-24. 8. 2001)	36	12	6	1							1			10	6	14
TEHRAN Varamin	Abardejh, G.B., F.T., (11-12. 9. 2002)	6	2		1											3	2
TEHRAN Pakdasht	Gheshlagh, Ash., CDC (25-26. 8. 2002)	5			1				2						2		4
	Total	177	55	18	14	6	4	2	3	2	2	2	2	2	37	28	49

G.B., Gerbill burrow; Ash, Animal shelter; S.P., Sticky paper; CDC (Centers for Disease Control), CDC miniature light traps; F.T., Funnel trap; Turk, Turkemen Sahara; Inch, Inchebron; Dash, Dashbron

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Fig. 1. Neighbor-joining phylogenetic tree for DNA haplotypes of Cyt b Long fragment of Iranian *P. papatasi*, produced using PAUP* (Swofford, 2002).

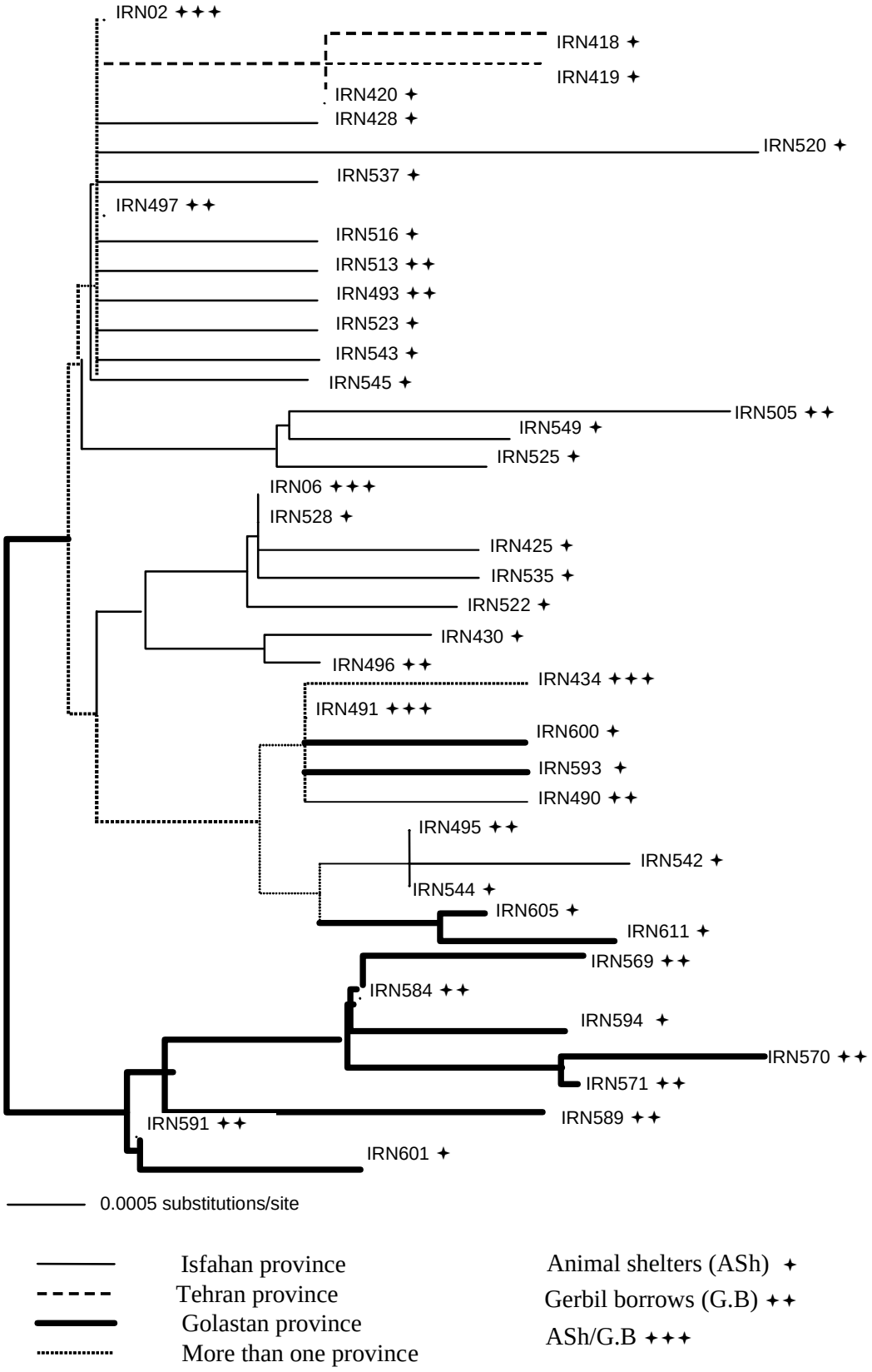

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Table 2. Alignment of all variant nucleotide positions for Cyt b Long haplotypes (IRNXXX) of mitochondrial Cyt b of *P. papatasi*.

737- character CB-R06 haplotypes (IRN code of first Iranian specimen)	No. of Iranian specimens with each haplotype	Variant character position 11111222223333344444455555666666 3456722355016781245704555801245700134771529476235254461359751487846117169790	Iranian provinces with each haplotype
IRN02	55	CAATAACAGCCGGTGCCATTCAAAATTTAGATAGTATT	All four provinces
IRN491	14	CAATAACAGCCGGTGCCATTCAAAATTTAAATAGTATT	Golestan, Isfahan and Tehran
IRN420	3	CAATAACAGCCGGTGCCATTCAAAATTTAGATAGCATT	Isfahan and Tehran
IRN434	2	CAATAACAGTCGGTGCCATTCAAAATTTAAATAGTATT	Isfahan and Golestan
IRN06	18	CAATAACAGCCGGTGCCATCCAAAATTTAGATAGTATT	Isfahan
IRN425	2	CAATAACAGCCGGTGCCATCCAAAATTTAGATAGTATC	
IRN430	2	CAATAACAGCCGATGCCATCCAAAATTTAGATAGTATT	
IRN516	2	CAATAACAGCCGGCGCCATTCAAAATTTAGATAGTATT	
IRN428	1	CAATGACAGCCGGTGCCATTCAAAATTTAGATAGTATT	
IRN438	1	CAATAACAGCCGGTGCTATTCAAAATTTAGA ? ? ? ? ? ? ?	
IRN490	1	CAATAACAGCCGGTGCTATTCAAAATTTAAATAGTATT	
IRN493	1	CAATAACAGCCGGTGCCATTCAAAATCTAGATAGTATT	
IRN495	1	CAATAACAGCCGGTGCCATCCAAAATTTAAATAGTATT	
IRN496	1	CAATAACAGCCGATGCCATTCAAAATTTAGATAGTATT	
IRN497	1	CGATAACAGCCGGTGCCATTCAAAATTTAGATAGTATT	
IRN505	1	CAGTAACAGCCGGTGTCATTCGAAATTTAGATAGTATT	
IRN513	1	CAATAACAGCCGGTGCCATTCAAAATTTAGATAGTAAT	
IRN520	1	CAATAACAGCCGGTGCCATTCAAGATTTAGATGGTGTT	
IRN522	1	?AATAACAACCGGTGCCATCCAAAATTTAGATAGTATT	
IRN523	1	CAATAACAGCCGGTGCCACTCAAAATTTAGATAGTATT	
IRN525	1	?AGTAACAACCGGTGCCATTCAAAATTTAGATAGTATT	
IRN528	1	TAATAACAGCCGGTGCCATCCAAAATTTAGATAGTATT	
IRN535	1	??ATAACAGCCAGTGCCATCCAAAATTTAGATAGTATT	
IRN537	1	?AATAACAGCCGGTGCCATTCAAAGTTTAGATAGTATT	
IRN542	1	?AATAACAGCCGGTGCCATCCAAAATTTGAATAGTATT	
IRN543	1	?AACAACAGCCGGTGCCATTCAAAATTTAGATAGTATT	
IRN544	1	?AATAACAGCCGGTGCCATCCAAAATTTAAATAGTATT	
IRN545	1	?AATAACAGCCGGTGCCATTCAGAATTTAGATAGTATT	
IRN546	1	?AATAACAGCCGGTGCCATTCAAAATTTAGAA ? ? ? ? ? ?	
IRN549	1	??GTAATAGCCGGTGCCATTCAAAATTTAGATAGTATT	
IRN591	6	CAATAACAGCCGGTGCCATTCAAAATTCAGATAGTATT	Golestan
IRN584	4	CAATAACAGCCGGTGCCATTTAAAATTCAGATAGTATT	
IRN571	2	CAATAACAGCTGGTGCCATTTAAAATTCAGATAGTATT	
IRn605	2	CAATAACAGCCGGTGCCATTCAAAATTCAAATAGTATT	
IRN569	1	CAATAACAGCCGGTGCCGTTTAAAATTCAGATAGTATT	
IRN570	1	CAATAACAACTGGTGCCATTTAAAATTCAGATAGTATT	
IRN589	1	CAATAACAACCGGTGCCATTCAAAATTCAGATAATATT	
IRN593	1	CAATAACAGCCGGTACCATTCAAAATTTAAATAGTATT	
IRN594	1	CAATAACAGCCGGTGCCATTTAGAATTCAGATAGTATT	
IRN600	1	CAATAGCAGCCGGTGCCATTCAAAATTTAAATAGTATT	
IRN601	1	CAATAACAGCCGGTGCCATTCAAAACTCAGATAGTATT	
IRN611	1	CAATAACAGCCGGTGCCATCCAAAATTCAAATAGTATT	
IRN418	1	CAATAACAGCCGGTGCCATTCAAAATTTAGGTAGCATT	Tehran
IRN419	1	CAATAACGGCCGGTGCCATTCAAAATTTAGATAGCATT	

The second set of haplotypes for separate parsimony analysis contained all the haplotypes found in Golestan province (Turkemen Sahara) except for the widespread haplotype IRN02. A heuristic search with equal character weighting and TBR branch swapping gave a single most parsimonious tree (Fig. 3), i.e. a single monophyletic network of haplotypes. This was congruent with the NJ phylogram. Adding other haplotypes to this first set (even just IRN02) prevented the heuristic search from finding a solution overnight, and so we conclude that the other haplotypes from other provinces are probably not monophyletic with the haplotypes from Golestan.

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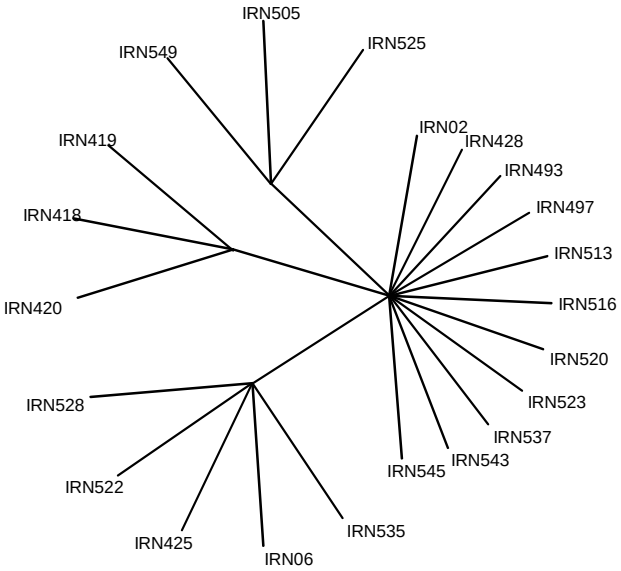

Fig. 2. Single most parsimonious unrooted cladogram from an heuristic search (PAUP* [Swofford, 2002]) of most DNA haplotypes of Cyt b Long fragment from the Iranian provinces of Bushehr, Tehran and Isfahan (not Golestan).

DISCUSSION

Phylogenetic analyses were performed to investigate the pattern of the geographical variation among haplotypes. Based on the Cyt b Long fragment examined, *P. papatasi* showed only recent divergence in Iran, because the genetic distances between haplotypes were small. However, some evidence for isolation by distance was found. First, all the haplotypes from Iran could not be shown to belong to a single network, whereas most from Golestan province did belong to a single network (Fig. 3.). Second, there were some abundant haplotypes that were found only in one province. Only one haplotype was abundant and widespread in Iran. IRN02 was found in all four provinces, and this suggests there may has been secondary contact among some Cyt b lineages that diverged in geographical isolation, by vicariance [10, 19]. These data show that most haplotypes from the provinces of Isfahan, Bushehr and Tehran belonged to a second single network (Fig. 2.), different from the one from Golestan province.

In our previous report [6], CB1 haplotype IRN06 was not found in the two northern Iranian provinces of Hamadan and Tehran, and this suggested that Cyt b might be a marker for Iranian populations of *P. papatasi* isolated by distance. Isolation by distance has occurred "recently" (in the last 135,000 years), as reported by Esseghir *et al*. [19]. They analysed *P. papatasi* collected from many countries within its geographical range. They showed that the widespread CB3 haplotype H01 (= IRN33) was at the centre of a single evolutionary network, in which it diverged from haplotype H08 (=IRN02/ IRN06) by just one nucleotide. Also, the network was geographical, centred on the Eastern Mediterranean, and so the predominance of CB3 haplotype IRN02 in Iran was not a surprise because it was previously found in Saudi Arabia and in the Jordan Valley [6]. Yahia *et al*. [25] studied *P. sergenti* in Morocco and found that most lineages of this vector of *L. tropica* were restricted to different regions. They concluded that vector control would not always be followed by rapid long-distance reinvasion. Now, the same conclusion can be made for *P. papatasi* in Iran, and this favours vector control.

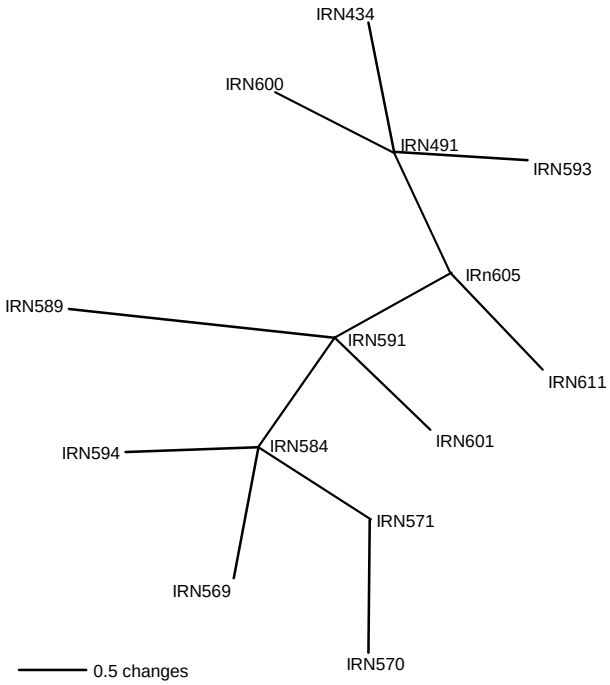

Fig. 3. Single most parsimonious unrooted phylogram from a heuristic search (PAUP* [Swofford, 2002]) of all DNA haplotypes of Cyt b Long fragment (except widespread haplotype IRN02) from the Iranian province of Golestan.

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