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Phlebotomus caucasicus and *Phlebotomus mongolensis* (Diptera: Psychodidae): indistinguishable by the mitochondrial *cytochrome b* gene in Iran

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Abstract

Diagnostic molecular markers for the females of *Phlebotomus* (*Paraphlebotomus*) *caucasicus* and *P. mongolensis* were sought by characterizing from individual Iranian specimens a gene fragment, namely mitochondrial *cytochrome b*, that had previously proven useful for the taxonomy of phlebotomine sandflies. Males of both species were used as reference material because their external genitalia provide the only diagnostic morphological characters. A phylogenetic analysis of the new sequences, and those previously reported for *P. grimmi*, found no support for recognizing more than one species (*P. caucasicus* s.l.) in Iran. Most of the genetic variation was geographical. An absence of lineage sorting was demonstrated, and it is proposed that any search for species-specific molecular markers for these three taxonomic species should be continued by applied biologists only if there is better evidence for associating any one of them with phenotypes important for understanding the transmission of *Leishmania* species in foci of zoonotic cutaneous leishmaniasis.

Keywords: *Phlebotomus caucasicus*, *Phlebotomus mongolensis*, *Phlebotomus grimmi*, mitochondrial *cytochrome b*, molecular markers, zoonotic cutaneous leishmaniasis, Iran

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Introduction

Haematophagous females of some phlebotomine sandflies are the only natural vectors of *Leishmania* species (Killick-Kendrick, 1990; Ready, 2008), the causative agents of leishmaniasis in many parts of the tropics and subtropics, including Iran (Nadim & Seyedi-Rashti, 1971). More than 46 sandfly species have been reported from Iran (Seccombe *et al*., 1993; Kasiri, 2000), but only a few have been incriminated as vectors of *Leishmania major* Yakimoff & Schokhor, the causative agent of rural zoonotic cutaneous leishmaniasis (ZCL) (Killick-Kendrick, 1990). Evidence includes the typing of sandfly infections and the relative abundances of sandfly species in collections from human baits and the burrows of gerbil reservoir hosts (Parvizi & Ready, 2008). The principal peridomestic vector in ZCL foci in Iran is *Phlebotomus* (*Phlebotomus*) *papatasi* (Scopoli) (Parvizi *et al*., 2005), but *Phlebotomus* (*Paraphlebotomus*) *caucasicus* Marzinowsky or species with morphologically similar females have frequently been found infected with *Leishmania*-like leptomonads in Iran (Nadim *et al*., 1968a; Nadim & Seyedi-Rashti, 1971; Javadian & Seyedi-Rashti, 1991) and other Asian countries (Killick-Kendrick, 1990).

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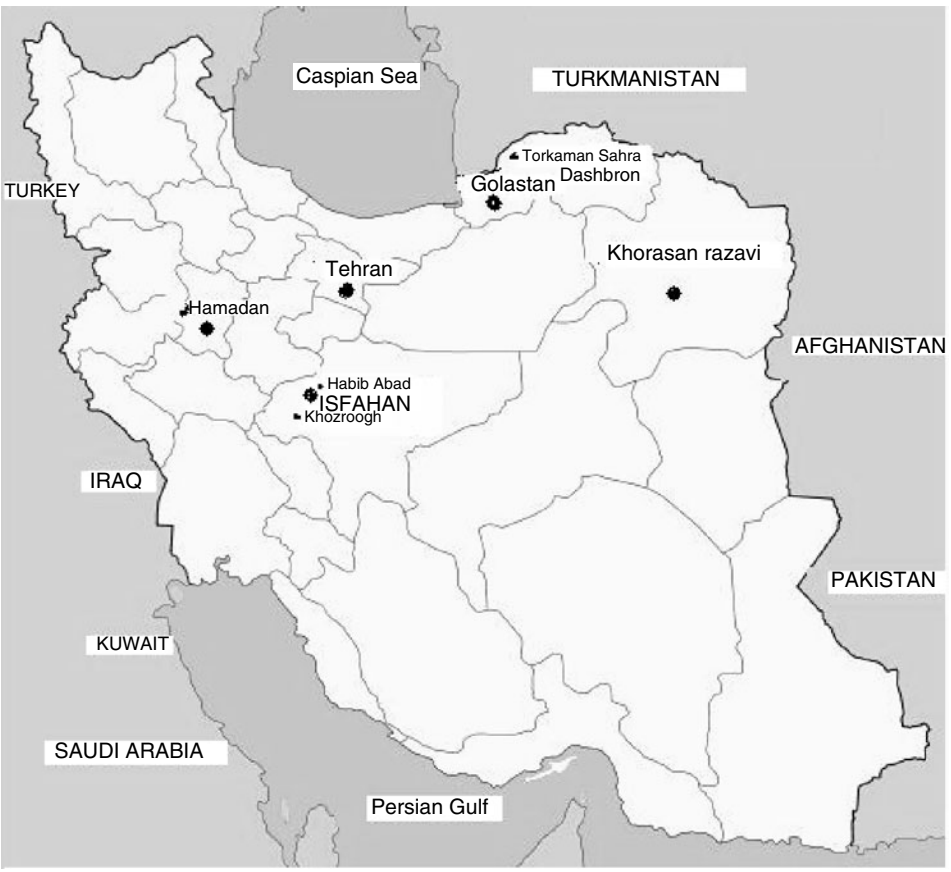

Fig. 1. Locations of Iranian provinces, cities and villages where *P. caucasicus* and *P. mongolensis* were sampled.

Sometimes, infections found in Iran have been identified to species by isoenzyme electrophoresis (Yaghoobi-Ershadi *et al*., 1995) or by phylogenetic analysis of the intergenic transcribed spacer of the nuclear ribosomal RNA gene array (ITS rDNA) of *Leishmania* (Parvizi & Ready, 2008).

Phlebotomus caucasicus and *Phlebotomus* (*Paraphlebotomus*) *mongolensis* Sinton are frequently found in the burrows of the great gerbil, *Rhombomys opimus* (Licht.), and other reservoir hosts in Iran, but the females of these sandflies are not separable morphologically (Theodor & Mesghali, 1964). This prevents a direct investigation of their roles in maintaining transmission of *L. major* (Killick-Kendrick, 1990) and other *Leishmania* species of gerbils (Parvizi & Ready, 2008).

The objective of the present study was to search for diagnostic molecular markers for the females of *P. caucausicus* and *P. mongolensis*, by characterizing a mitochondrial gene that has previously proven useful for sandfly taxonomy, namely mitochondrial *cytochrome b* (*cyt b*) (Esseghir *et al*., 2000; Testa *et al*., 2002). Males of both species were used as reference material, because their external genitalia provide the only diagnostic morphological characters (Theodor & Mesghali, 1964). Iranian populations were sampled (fig. 1) from a sympatric region in the northeast province of Golastan and, in order to assist the association of the sexes, from regions where only the more widespread *P. caucasicus* has been reported, in the northwest province of Hamadan and the central province of Isfahan (Theodor & Mesghali, 1964; Nadim *et al*., 1968).

Materials and methods

Insect samples and morphological identification

The collections were carried out in the provinces of Golastan, Hamadan and Isfahan (fig. 1) in 2001 and 2002, during the main summer season of activity of adult sandflies in Iran. Adult sandflies were collected on sticky papers placed at the entrances to gerbil burrows or in manual aspirators from resting sites during the early morning hours (Parvizi *et al*., 2003), and in Centres for Disease Control (CDC) miniature light traps (Sudia & Chamberland, 1962) set overnight in domestic animal shelters.

Following dissection with sterilized forceps and microneedles (Testa *et al*., 2002), the head and abdominal terminalia of both sexes were slide-mounted in Berlese fluid in order to view, by compound microscopy (×400), the morphological characters diagnostic for the subgenus *Paraphlebotomus* (Lewis, 1982) and most of its species found in Iran (Nadim & Javadian, 1976), including the males of *P. caucasicus* and *P. mongolensis* (Theodor & Mesghali, 1964; Perfil'ev, 1966). No attempt was made to use the morphometric and meristic characters that might distinguish the males of *P. caucasicus s.s.* and *Phlebotomus* (*Paraphlebotomus*) *grimmi* Porchinski, as partially explained by Moin-Vaziri *et al*. (2007). We treat *P. grimmi* as a senior synonym of *P. caucasicus*, as proposed by Lewis (1982). The females of *P. caucasicus* and *P. mongolensis* could not be separated morphologically based on the structure of the spermathecae

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Table 1. DNA haplotypes of *cyt b* of males of *P. mongolensis*, males of *P. caucasicus* and their morphologically indistinguishable females collected in Iran for this report. Only *cyt b* haplotype cauc08_CB3 was found in males of both species.

Sandfly species	Specimen no.	Province	Location	Habitat	Trap type	*cyt b* haplotype
Males of *P. mongolensis*	IRN343	Golastan	Turk-D	A sh	S p	cauc17_CB3
	IRN344	Golastan	Turk-D	G b	S p	cauc18_CB3
	IRN348	Golastan	Turk-D	A sh	CDC	cauc06_CB3
	IRN349	Golastan	Turk-D	A sh	S p	cauc07_CB3
	IRN351	Golastan	Turk-D	A sh	S p	cauc08_CB3
	IRN352	Golastan	Turk-D	G b	S p	cauc21_CB3
	IRN355	Golastan	Turk-D	G b	S p	cauc23_CB3
	IRN357	Golastan	Turk-D	G b	S p	cauc24_CB3
	IRN361	Golastan	Turk-D	G b	S p	cauc08_CB3
Males of *P. caucasicus*	IRN257	Hamadan	Hamadan	I h	Asp	cauc08_CB3
	IRN258	Hamadan	Hamadan	I h	Asp	cauc08_CB3
	IRN307	Isfahan	Habib Abad	A sh	CDC	cauc05_CB3
	IRN345	Golastan	Turk-D	G b	S p	cauc19_CB3
	IRN350	Golastan	Turk-D	A sh	S p	cauc20_CB3
	IRN353	Golastan	Turk-D	G b	S p	cauc22_CB3
	IRN360	Golastan	Turk-D	G b	S p	cauc25_CB3
Females of *P. mongolensis* or *P. caucasicus*	IRN242	Isfahan	Khorzoogh	G b	S p	cauc09_CB3
	IRN243	Isfahan	Khorzoogh	G b	S p	cauc16_CB3
	IRN443	Isfahan	Habib Abad	G b	S p	cauc09_CB3
	IRN444	Isfahan	Habib Abad	G b	S p	cauc07_CB3
	IRN514	Isfahan	Habib Abad	A sh	CDC	cauc09_CB3
	IRN354	Golastan	Turk-D	G b	S p	cauc06_CB3
	IRN475	Golastan	Turk-D	A sh	CDC	cauc04_CB3
	IRN578	Golastan	Turk-D	G b	S p	cauc26_CB3
	IRN580	Golastan	Turk-D	G b	S p	cauc05_CB3

A sh, animal shelter; G b, gerbil burrow; I h, inside house; S p, sticky paper; CDC, CDC miniature light trap; Asp, aspirator; Turk, Turkemen Sahara; D, Dashbron.

or the weakly developed pharyngeal armature (Theodor & Mesghali, 1964).

DNA extraction and PCR amplification of cyt b

Total DNA was extracted from the dissected thorax and attached anterior abdomen of individual sandflies using the method of Ish-Horowicz with minor modifications (Ready *et al*., 1991).

The 3′ end of *cyt b* was amplified either as one fragment of 717 base pairs (bp), by using the forward primer CB1-SE with the reverse primer CB-R06 at one annealing temperature of 48°C (Parvizi & Ready, 2006), or as two overlapping fragments if the genomic DNA was degraded, by using the primer pairs CB1-SE/CB3-R3A (Esseghir *et al*., 2000) and CB3-PDR/CB-R06 (Parvizi & Ready, 2006). General protocols for PCR and amplicon purification followed Parvizi *et al*. (2003).

Direct DNA sequencing, DNA sequence editing and alignments and phylogenetic analyses

One-hundred nanograms of each purified DNA sample was cycle-sequenced using an ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (version 2.0) and semi-automated sequencing systems AB1 377 or ABI 3730x1 (Applied Biosystems Inc.), 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).

DNA sequences were edited and aligned using Sequencher™ 3.1.1 software (Gene Codes Corporation). The multiple alignments were used to identify the limits of open reading frames (ORFs), based on deduced amino acid sequences, and analysed phylogenetically using PAUP* software (Swofford, 2002).

Results

Distribution of morphotypes

Males of *P. caucasicus* and *P. mongolensis* could be separated morphologically, based on the diagnostic characters given by Theodor & Mesghali (1964), Nadim & Javadian (1976) and Lewis (1982). The basal lobe of the coxite is large and wide, and its unrounded end has many lateral and ventral setae in *P. caucasicus*. In comparison, this basal lobe is smaller and narrower, and its rounded end has mainly terminal setae in *P. mongolensis*. The latter was distinguished from *Phlebotomus jacusieli* Theodor by its longer style (four times as long as thick) bearing a markedly elongated terminal spine.

All nine males of *P. mongolensis* were collected in the northeast province of Golastan, in association with four females, whereas the seven males of *P. caucasicus* were collected not only in Golastan province but also in the northwest province of Hamadan and with five associated females in the central province of Isfahan (table 1).

Distribution and relationships among DNA haplotypes of mitochondrial cyt b

The sequence of the last (3′) 288 bp of *cyt b* (CB3) was obtained from each of the 25 specimens listed in table 1 (GenBank accessions: FJ217389–FJ217392; GU057903–GU057914), and all these new sequences were aligned with

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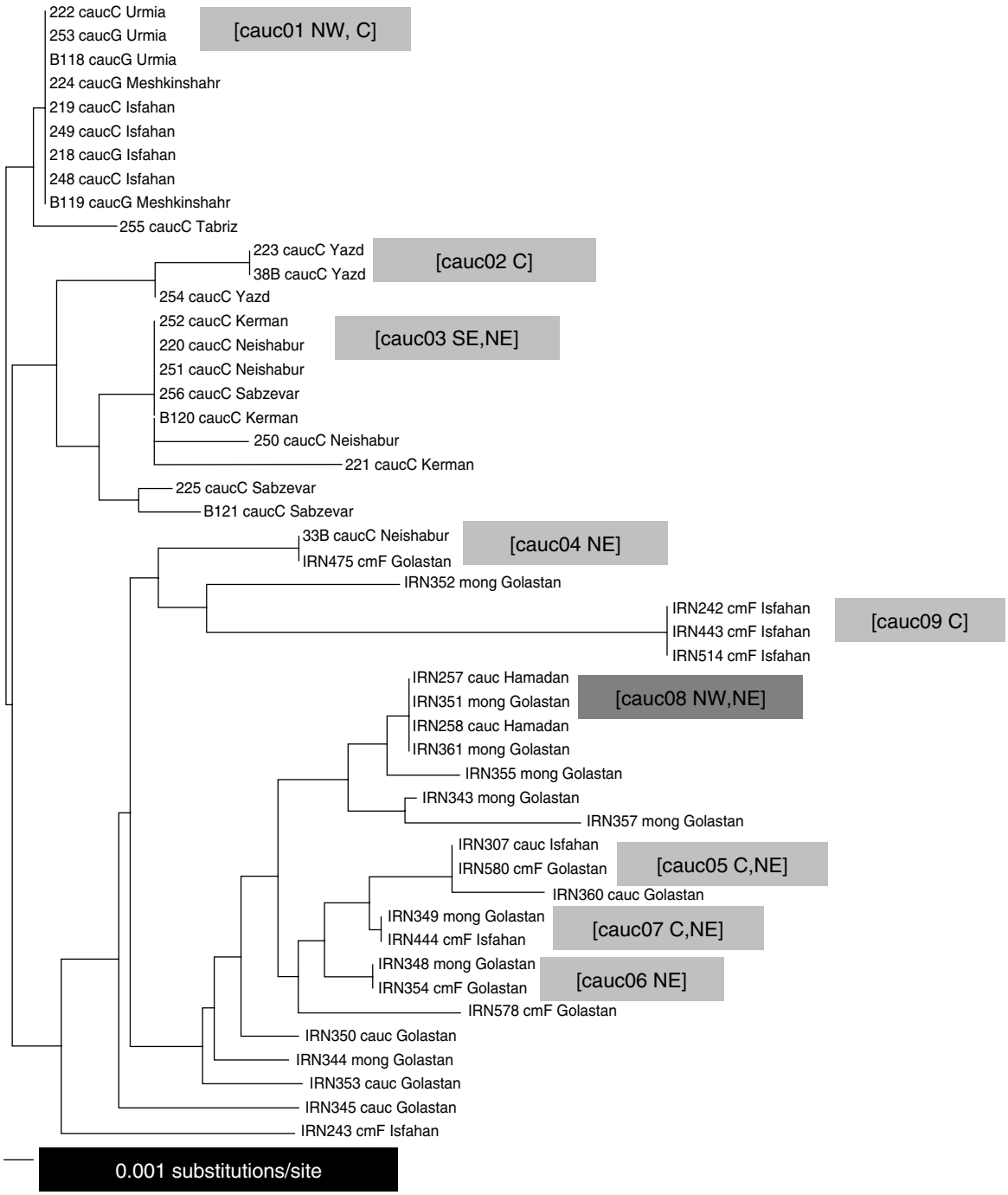

Fig. 2. Neighbour-joining tree of relationships among all short *cyt b* (CB3) sequences, including new ones (IRN) and those (other labels) published by Moin-Vaziri *et al*. (2007), based on untransformed genetic distances (*p*). Each sequence is labelled with the (alpha-) numeric specimen code, the species code (caucC, *P. caucasicus* s.s. male; caucG, *P. grimmi* male; cauc, *P. caucasicus* s.l. male; mong, *P. mongolensis* male; cmF, female of *P. caucasicus* or *P. mongolensis*, which are morphologically indistinguishable) and the Iranian province (IRN) or settlement (Moin-Vaziri *et al*., 2007) of collection. CB3 haplotype codes are given only for sequences found in two or more specimens, (e.g. cauc01 NW, C), contain abbreviations for the geographical regions (NW, northwest; C, centre; SE, southeast; NE, northeast) and the only one shared by males of *P. caucasicus* and *P. mongolensis* has darker shading.

the homologous sequences found in 23 males of *P. caucasicus* s.s. (C) or the morphotype *P. grimmi* (G) by Moin-Vaziri *et al*. (2007) (GenBank accessions: EF017349–EF017371). There were no amino acid replacements. All sequences were monophyletic with respect to an outgroup of four sequences of *Phlebotomus* (*Paraphlebotomus*) *sergenti* Parrot, as assessed

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by a maximum parsimony analysis with 1000 heuristic searches and standard defaults in PAUP* (Swofford, 2002).

The relationships among all the in-group sequences (fig. 2) were assessed by the neighbour-joining (NJ) algorithm in PAUP* (Swofford, 2002), based on uncorrected pairwise genetic distances (*p*), with random breaking of ties and, to permit comparisons with the findings of Moin-Vaziri *et al*. (2007), with a group of haplotypes from the northwest and centre of Iran designated as the out-group. A bootstrap analysis (1000 replicates) provided significant support (>70%) only for some of the terminal branches joining sequences from the same or nearby locations. The *p*-values between pairs of sequences were low (0.003–0.038), which is consistent with intra-specific variation. The *p*-values between any one of these in-group sequences and any one of the out-group sequences of *P. sergenti* was much higher (0.119–0.148).

Twenty-six haplotypes (=unique sequences) were identified (table 1, fig. 2), and their geographical distributions were restricted. Each of three haplotypes came from two or more specimens of *P. caucasicus s.l.* from populations characterized only by Moin-Vaziri *et al*. (2007): haplotype cauc01_CB3 was restricted to locations near the northwest settlements of Urmia (W Azerbaijan province) and Meshkinshahr (Ardebil province) as well as the central city of Isfahan (Isfahan province); haplotype cauc02_CB3 was restricted to the location near the central settlement of Yazd (Yazd province); and haplotype cauc03_CB3 was restricted to locations near the southeast settlement of Kerman (Kerman province) and the northeast settlements of Neishabur and Sabzevar (Khorassan-e-Razavi province). Six other haplotypes (cauc10_CB3–cauc15_CB3) were found in single specimens of *P. caucasicus s.l.* from populations characterized only by Moin-Vaziri *et al*. (2007), and their geographical locations matched those of the more abundant haplotype with which each was grouped (fig. 2), e.g. the haplotype of the specimen from the northwest settlement of Tabriz (E Azerbaijan province) grouped with haplotype cauc01_CB3.

Only the haplotype cauc04_CB3 was found both by Moin-Vaziri *et al*. (2007) and by us (fig. 2), respectively in single specimens of *P. caucasicus s.l.* or *P. mongolensis* collected near the northeast settlements of Neishabur (Khorassan-e-Razavi province) and Dashbron (Golastan province). Three of the haplotypes found only by us could possibly be species-specific: cauc05_CB3 was associated only with the male of *P. caucasicus s.l.* and, in contrast, cauc06_CB3 and cauc07_CB3 were associated only with the male of *P. mongolensis*. However, there was no suggestion of any association of each male morphotype with any one of the poorly supported branches of the NJ tree. Indeed, haplotypes cauc05_CB3, cauc06_CB3 and cauc07_CB3 were on the same major branch that contained haplotype cauc08_CB3, which contained two males of *P. caucasicus s.l.* from the northwest province of Hamadan and two males of *P. mongolensis* from the northeast province of Golastan. Further evidence against the association of *cyt b* lineages with each species comes from the mixed geographical origins of the specimens with haplotypes cauc05_CB3 and cauc07_CB3. Each had representatives from the northeast province of Golastan, as well as the central province of Isfahan from where *P. mongolensis* is unknown. Only one of the haplotypes found by us was on a long branch and represented by specimens from a single province, haplotype cauc09_CB3 from Isfahan (fig. 2), but there was no association with any male morphotype.

Eleven other haplotypes (cauc16_CB3–cauc26_CB3) were found in single specimens of *P. caucasicus* or *P. mongolensis* from populations characterized only by us, and there were no clear associations between NJ branches and geographical regions (fig. 2).

Discussion

Interest in *P. caucasicus* and related species arises mostly because they might have a role in the transmission of *L. major* in many of the widespread Asian foci of ZCL (Killick-Kendrick, 1990; Parvizi & Ready, 2008). However, these species are not often abundant in the burrows of the gerbil reservoir hosts, e.g. Parvizi *et al*. (2003), and the few typed infections of *L. major* found in them indicate only that they take blood meals from reservoirs, because no infective forms have been reported. Most of these potential vectors were described as taxonomic species, based on small differences in the setation and form of parts of the external genitalia that show much individual variation, e.g. Moin-Vaziri *et al*. (2007). Many such species of the subgenus *Paraphlebotomus* have been synonymised, including *P. grimmi* (Lewis, 1982; Seccombe *et al*., 1993), but *P. mongolensis* is still treated as a species in Iran and some nearby countries.

Mitochondrial *cyt b* sequences were obtained from males of *P. caucasicus* and *P. mongolensis* that could be separated morphologically based on the diagnostic characters given by Theodor & Mesghali (1964), Nadim & Javadian (1976) and Lewis (1982), and these were aligned with homologous sequences obtained from males of *P. caucasicus* and *P. grimmi* by Moin-Vaziri *et al*. (2007). However, this new phylogenetic analysis provided no support for considering *P. mongolensis* or *P. grimmi*, as temporarily resurrected by Moin-Vaziri *et al*. (2007), to be phylogenetic species distinct from *P. caucasicus*. Most of the genetic variation in Iran was geographical. Our results confirm the regional distributions of *cyt b* haplotypes of *P. caucasicus s.l.* in Iran, as first reported by Moin-Vaziri *et al*. (2007).

Mitochondrial *cyt b* demonstrated an absence of lineage sorting between the male morphotypes of *P. caucasicus*, *P. mongolensis* or *P. grimmi*, indicating that any biological speciation is at best incomplete. All three taxa might be good biological species showing mitochondrial introgression caused by occasional inter-breeding, as has been proposed for other sibling species of sandflies (Testa *et al*., 2002; Pesson *et al*., 2004). This could be tested by a population genetics approach using several polymorphic genes. However, where an absence of lineage sorting is demonstrated, applied biologists should consider carefully the costs of searching for species-specific molecular markers. Perhaps, these should be sought only if there is good evidence for associating specific taxa with phenotypes of epidemiological importance. There is no such evidence for *P. caucasicus* and its related species, and so there is no reason to give priority to resolving the species status of *P. mongolensis* or *P. grimmi*.

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Ethical approval: Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran.

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