The sandflies (Diptera: Psychodidae, Phlebotominae) in military camps in northern Afghanistan (2007–2009), as identified by morphology and DNA ‘barcoding’

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As part of a continuous, standardized programme of monitoring the *Leishmania* vectors in German military camps in northern Afghanistan between 2007 and 2009, a detailed taxonomic analysis of the endemic sandfly fauna, as sampled using light and odour-baited traps, was conducted. Of the 10 sandfly species that were recorded, six may serve as enzootic and/or zoonanthroponotic vectors of parasites causing human leishmaniasis.

The use of a simple DNA-‘barcoding’ technique based on the mitochondrial *cyt b* gene, to identify the collected sandflies to species level, revealed (1) a clear discrimination between the potential vector species, (2) clustering of species within most subgenera, and (3) particularly high heterogeneity within the subgenus *Paraphlebotomus* (*Phlebotomus alexandri* being grouped with *Ph. papatasi* rather than with other *Paraphlebotomus* species). The data also indicate a high level of genetic heterogeneity within the subgenus *Sergentomyia* but close similarity between *Sergentomyia sintoni* and *Sergentomyia murgabiensis*. The morphological similarity of many medically important sandflies can make species identification difficult, if not impossible. The new DNA-barcoding techniques may provide powerful discriminatory tools in the future.

Sandflies of the genus *Phlebotomus* (Diptera: Psychodidae) can serve as obligate vectors of protozoan *Leishmania* parasites (Kinetoplastida: Trypanosomatidae), some of which are the causative agents of human leishmaniasis. In Central Asia, *Phlebotomus* (*Phlebotomus*) *papatasi* is widely distributed and confirmed to be the primary vector of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis (zCL). Although the major ‘reservoir’ host of *Le. major* in this region is the great gerbil, *Rhombomys opimus* (Rodentia:

Muridae), other rodents can be infected with the parasite (Nadim *et al.*, 1979). Since the deployment of international military forces to Iraq and, subsequently, to Afghanistan, hundreds of the soldiers involved have developed zCL (Coleman *et al.*, 2006; Faulde *et al.*, 2006). Appropriate preventive schemes based on monitoring and control need to include sandfly trapping, assessments of the prevalences of infection in local sandflies, reduction in the numbers of potential breeding sites for the vector species, and personal protection against sandfly bites (Faulde *et al.*, 2009).

The identification of vector species is essential for a good understanding of the

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epidemiology of any vector-borne disease. Until the last few years, the most recent information on the sandfly fauna of Afghanistan and its taxonomy dated back to the 1970s, when 44 species — half assigned to the genus *Sergentomyia* and half to the genus *Phlebotomus* — were recorded (Artemiev, 1981; Seccombe *et al.*, 1993). The genus *Sergentomyia* comprises predominantly non-anthropophilic species, some of which are known to feed on reptiles or birds. All of the primary or secondary vectors of parasites causing human leishmaniasis in the Old World [i.e. zCL, anthroponotic CL (aCL; caused by *Le. tropica*) and visceral leishmaniasis (VL; caused by *Le. donovani* s.l.) — all of which occur, at varying levels of endemicity, in different parts of Afghanistan] are *Phlebotomus* species. Apart from *Ph. papatasi*, the only other proven vector of parasites causing human leishmaniasis in Afghanistan is *Ph. sergenti*, which transmits *Le. tropica* (Artemiev, 1978). Several other *Phlebotomus* species have, however, been reported as vectors in neighbouring countries or other Old-World regions (e.g. some *Phlebotomus* species in the subgenus *Laroussius* are the vectors of *Le. infantum* in the Mediterranean basin; Seccombe *et al.*, 1993). Unfortunately, several sandfly taxa (such as '*Ph. caucasicus/andrejevi/mongolensis*') have so far only been identified as vectors of *Le. major*, *Le. tropica* and/or *Le. donovani* s.l. on the basis of untyped promastigotes found in wild-caught adult females.

In a recent analysis of 8442 sandflies caught in Balkh province in northern Afghanistan, at least 2.5% of the female *Ph. papatasi* were found infected with *Le. major* (Faulde *et al.*, 2008b) whereas the other sandfly species (*Ph. caucasicus* and *Se. sintoni*) were never found infected. Balkh not only has very high densities of the great gerbil (the highest known) but also high densities of *Ph. papatasi*, the most important vector of *Le. major* (Faulde *et al.*, 2008a). In contrast, the mountainous surroundings of Feyzabad, in the north-eastern province of Badakhshan province, do not favour the

same vector–reservoir combination, with great gerbils not detected in this area until 2009 (M. Faulde, unpubl. obs.).

The study of the bionomics and vector potential of sandflies is hampered by the nocturnal activity, cryptic life-cycles (with the sub-adult stages often in animal burrows), small size and general morphological similarity of the insects. In the initial study of wild-caught Old-World sandflies, *Phlebotomus* species are usually separated from *Sergentomyia* (Coleman *et al.*, 2009), and the female sandflies from the males (the males being easily recognised by their terminalia). If based, as usual, on morphology, any further identification of the sandflies requires the insects to be mounted on slides, with the time-consuming micro-dissection of the female spermathecae and head capsule (cibarium and pharynx) and the male terminalia, genital pump and head capsule (cibarium) often necessary to make an accurate identification to species level. In many cases, the antennae and palpi (which are easily lost or damaged during capture) also have to be examined.

Over the last couple of decades, molecular methods have been increasingly employed to explore the taxonomy and phylogeny of many insects, including sandflies. A prerequisite for the development of molecular taxonomy is a reference collection of specimens that have been correctly identified to species (usually morphologically) and from which DNA can be extracted. Even then, the development of molecular identification tools can be hindered by intraspecific and geographical genetic variation. So far, large numbers of sandflies from the Mediterranean region (Aransay *et al.*, 1999; Di Muccio *et al.*, 2000; Hamarshah *et al.*, 2007), the Middle East (Moin-Vaziri *et al.*, 2007a, b; Parvizi *et al.*, 2010), Africa (Depaquit *et al.*, 2008), Madagascar (Depaquit *et al.*, 2002, 2007) and Latin America (Ready *et al.*, 1997; Uribe Soto *et al.*, 2001) have been studied using DNA-based tools. In Afghanistan, however, only two specimens of *Ph. papatasi* have been investigated using such tools (Esseghir *et al.*, 1997).

FIG. 1. Study area in northern Afghanistan/southern Uzbekistan. Collection sites are marked by asterisks.

One of the most recently employed tools in molecular systematics is DNA ‘barcoding’, which uses standardized, well-conserved, short sequences of mitochondrial DNA for the identification of unknown specimens. Although *cytochrome c oxidase I* (*COI*) is the gene most frequently investigated (Hebert *et al.*, 2003; Boudabous *et al.*, 2009), the *cytochrome b* (*cyt b*) gene has also been used (Hajibabaei *et al.*, 2007), and the *cyt b* gene of several sandfly species has already been characterised (Esseghir *et al.*, 1997; Ready *et al.*, 1997; Parvizi *et al.*, 2003; Hamarshah *et al.*, 2007; Moin-Vaziri *et al.*, 2007a, b).

The aim of the present study was to assess the usefulness of *cyt-b*-based DNA barcoding for the identification of the vector and non-vector sandflies that live in and around German military camps in northern Afghanistan and in nearby southern Uzbekistan.

MATERIALS AND METHODS

Study Area

Sandflies were collected in and around German military camps in northern

Afghanistan and nearby southern Uzbekistan between 2007 and 2009 (see Figure 1 and Table 1). These camps were located, at altitudes of 400–1800 m, north of the Hindukush Mountains (except the site near Kabul) and south of the Turanian Basin. They included the semi-arid grasslands of the Turkistan plains in the east, the ephemeral desert of the northern foothills of the Marmal Mountains, and the mountains of Badakhshan in the west (Breckle, 2007). The Uzbek city of Termez lies on the banks of the Amu-Darya River, which marks the international border between Afghanistan and Uzbekistan. Sandfly collections were made between May and September, when the activity of adult sandflies peaks. At this time of the year, maximum daily temperatures normally reach 30–40°C and do not drop below 20°C at night, and there are occasional sandstorms but virtually no rainfall.

Field Collections of Sandflies

Adult sandflies were usually collected using CDC miniature light traps (Model 512; John W. Hock, Gainesville, FL) but some

TABLE 1. *The morphologically identified specimens and their origins*

Province	Site	Altitude (m)	Co-ordinates	Date	No. of female/male specimens morphologically identified as:										Total no. of sandflies
					<i>P.p.</i>	<i>P.a.</i>	<i>P.s.</i>	<i>P.c.*</i>	<i>A.t.</i>	<i>L.k.</i>	<i>S.m.</i>	<i>S.g.</i>	<i>S.c.</i>	<i>G.d.</i>	
Badakhshan	Feyzabad	1160	37°7'N, 70°31'E	Aug 2007	1/0	45/10	5/1		0/1	1/0		4/0		16/4	88
Kondoz	Kondoz	430	36°42'N, 68°53'E	Aug 2008			4/0								4
				Jul-Sep 2009	0/8	0/3	0/5		0/7						23
				May 2007	10/10	1/0	0/1				3/0		3/0		28
Balkh	Mazar-e Sharif	375	36°43'N, 67°14'E	Sep 2008	6/0						5/0		3/0		14
				May-Jun 2009	10/18	0/1					5/4	0/1		1/0	40
				Aug-Sep 2009	0/127	0/5	0/15	0/1							148
				Aug 2007	2/0										2
Badghis	Ghowrmach	640-690	35°44'N, 63°47'E	May-Jun 2009	32/13			2/5	4/12		6/2		0/1		77
				Sep-Nov 2009	0/2			0/1	0/1						4
Kabul	Kabul	1780	34°32'N, 69°18'E	May 2009	3/4			24/4	6/6						47
Surxondarya Uzbekistan	Termez	310	37°17'N, 67°20'E	2009	1/1			1/0	1/0		1/0				5
				Jun 2009	13/10						1/0	4/2	1/0		31
All investigated	All investigated				78/193	46/19	9/22	27/11	11/27	1/0	21/6	8/3	7/1	17/4	511

A.t., *Phlebotomus (Aldlerius) turanicus*; *G.d.*, *Sergentomyia (Grassomyia) dreyfussi turkestanica*; *L.k.*, *Ph. (Laroussius) keshishiani*; *P.a.*, *Ph. (Paraphlebotomus) alexandri*; *P.c.*, *Ph. (Para.) caucasicus*; *P.p.*, *Ph. papatasi*; *P.s.*, *Ph. (Para.) sergenti*; *S.c.*, *Se. (Simoniuss) clydei*; *S.g.*, *Se. (Paratomyia) grekovi*; *S.m.*, *Sergentomyia murgabensis*; *F.* females; *M.* males.

*Female *Ph. caucasicus* are indistinguishable, morphologically, from female *Ph. andrejevi* and *Ph. mongolensis* (Artemiev, 1978).

additional collections were made using the BG-Sentinel mosquito traps (BioGents, Regensburg, Germany) baited with BG-lure (BioGents), which produces an odour resembling that of human skin. The traps were run overnight from 17.00 to 07.00 hours, mostly on the periphery of each camp. The CDC traps were hung at a height of about 1 m, attached to a tripod. After each collection night, any sandflies caught were put in a freezer, to kill them, and then either preserved in absolute ethanol (about 50% of each collection) or dried.

Morphotaxonomy

Single specimens were left in 70% ethanol for about 5 min, and then the distal parts of the abdomen (for the examination of the genitalia) and the entire head (for the examination of the cibarium and pharynx) were removed with fine forceps. The heads and distal parts of the abdomens were cleared by incubation in 1 M KOH until the dark-reddish eye pigment had mostly faded (about 30 min). The remaining body parts were kept in absolute ethanol, for subsequent DNA extraction. After being cleared, the genitalia and heads were transferred onto a slide, slightly squashed under a coverslip, and then immediately examined and photographed under a light microscope with an attached digital camera. Each specimen was thus documented photographically. Each was identified morphologically, usually to species, using the keys provided by Artemiev (1978).

DNA Extraction and Analysis

Genomic DNA was extracted, from the unmounted parts (thorax and proximal parts of the abdomen) of each sandfly of interest, by homogenization, lysis and Chelex treatment, as described, for blackflies, by Dumas *et al.* (1998) and Krueger and Hennings (2006). This DNA was used as the template in a PCR that targeted a fragment, of approximately 550 bp, that included the mitochondrial *cyt b* gene, a

transfer-RNA gene and part of the *ND1* gene (Ready *et al.*, 1997). The amplicons produced using DNA from representatives of each sandfly species collected in Afghanistan (and reference specimens of species from Kosovo; see Table 2) were purified with the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and commercially sequenced by direct sequencing [either by Eurofins (Ebersberg, Germany) or SeqLab (Göttingen, Germany)]. The respective sequences were deposited in GenBank under accession numbers HM803186–217 and HQ204193–94 (Table 2). These sequences and several reference sequences that were already available from GenBank (see Figures 2 and 3) were aligned with ClustalW2 (Larkin *et al.*, 2007). The alignment files were uploaded into the PAUP 4.0b10 software package (Swofford, 2002), which was then used to produce taxon-identity trees. As the goal was not phylogenetic estimation, a simple neighbour-joining algorithm was generally considered sufficient for tree construction. A maximum-parsimony analysis was, however, used for an intraspecific analysis of *Ph. sergenti*.

RESULTS

Overall, 286 male and 225 female sandflies were identified morphologically. In total, six species of the genus *Phlebotomus* (*Ph. alexandri*, *Ph. caucasicus*, *Ph. keshishiani*, *Ph. papatasi*, *Ph. sergenti* and *Ph. turanicus*) and four species of *Sergentomyia* (*Se. clydei*, *Se. dreyfussi turkestanica*, *Se. grekovi* and *Se. murgabiensis*) were identified (Table 1). *Phlebotomus papatasi* was the most widespread of the 10 species collected, and was also the most abundant species at Kondoz, Mazar-e Sharif (MeS) and Termez.

Phlebotomus alexandri, *Ph. sergenti* and, most notably, *Se. dreyfussi turkestanica* showed a relatively limited geographical distribution, being found only in one (*Se. dreyfussi turkestanica*) or two of the sampled camps. All three of these species were found

in the eastern subalpine semidesert of Feyzabad, with *Ph. alexandri* and *Ph. sergenti* also being collected in Kondozi (desert–woodland mosaic). *Phlebotomus caucasicus* was most abundant in the eastern hills of Ghowrmach (semi-arid *Pistacia vera* woodland), rather scarce in MeS (ephemeral desert) and Kondozi, and not collected in Feyzabad or Termez (peri-urban lowland).

In terms of annual population fluctuations, *Ph. turanicus* was found to be quite numerous in 2009 (when it represented

23.5% of the *Phlebotomus* collected in MeS and 30.0% of those collected in Feyzabad) compared with 2007 (when it represented only 1.6% of the *Phlebotomus* collected in Feyzabad) [or compared with the collections made in 2006 by Faulde *et al.* (2008b), when it represented 0% of the *Phlebotomus* collected in MeS].

Twenty-seven specimens representing all 10 species collected in Afghanistan (plus one male of an unidentified *Sergentomyia* species, presumably of the subgenus

TABLE 2. Collection details of DNA specimens

Species	Specimen code(s) and (gender)	Locality	Date	Accession number(s)
TEST SAMPLES FROM AFGHANISTAN				
<i>Phlebotomus (Paraphlebotomus) alexandri</i>	01 and 04 (♀♀)	Feyzabad	Aug 2007	HM803186, HM803188
<i>Ph. (Para.) caucasicus</i> *	31 and 32 (♀♀)	Ghowrmach	May 2009	HM803204, HM803206
	40 and 41 (♂♂)	Kondozi	2009	HM803213, HM803215
<i>Ph. papatasi</i>	08d (♀)	Feyzabad	Aug 2007	HM803194
	44 (♂)	Ghowrmach	May 2009	HM803211
	45 (♀)	Kondozi	Jun 2009	HM803210
	48 (♀)	Mazar-e Sharif	Jun 2009	HM803212
<i>Ph. (Para.) sergenti</i>	03, 06, 08, 08a, 08c and 13 (♀♀)	Feyzabad	Aug 2007	HM803187, HM803189, HM803191–3, HM803197
	07, 42 and 43 (♂♂)	Kondozi	2007, Aug 2009	HM803190, HM803214, HM803216
<i>Ph. (Adlerius) turanicus</i>	29 (♀)	MeS	May 2009	HM803195
<i>Ph. (Adlerius) sp.</i>	46 (♀)	Kabul	2009	HM803196
<i>Ph. (Laroussius) keshishiani</i>	10 (♀)	Feyzabad	Aug 2007	HQ204193
<i>Sergentomyia murgabiensis</i>	17 (♀)	Kondozi	2007	HM803201
<i>Se. (Parrotomyia) sp.</i>	24 (♂)	Kondozi	Aug 2007	HM803203
<i>Se. (Grassomyia) dreyfussi turkestanica</i>	15 (♀)	Feyzabad	Aug 2007	HM803200
	50 (♀)	Kondozi	May 2009	HM803199
<i>Se. (Sintonius) clydei</i>	20 (♀)	Kondozi	2007	HQ204194
<i>Se. (Parrotomyia) grekovi</i>	19 (♀)	Feyzabad	Aug 2007	HM803202
REFERENCE SAMPLES FROM KOSOVO				
<i>Ph. (Laroussius) neglectus</i>	35 (♂)	Prizren	Aug 2009	HM803208
<i>Ph. (Laroussius) perfiliewi</i>	38 (♀)	Prizren	Aug 2009	HM803217
<i>Se. (Sergentomyia) minuta</i>	36 and 39 (♂♂)	Prizren	Aug 2009	HM803209, HM803205

*Female *Ph. caucasicus* are indistinguishable from female *Ph. andrejevi* and *Ph. mongolensis*, both morphologically (Artemiev, 1978) and in terms of their *cyt b* sequences (Parvizi *et al.*, 2010).

The resulting trees (Figures 2 and 3) show clear discrimination between the potential vector species collected in northern Afghanistan, most significantly between the known vector *Ph. papatasi* and potential or suspected vectors such as *Ph. sergenti* and '*Ph. caucasicus/mongolensis*' [according to Parvizi *et al.* (2010), *Ph. caucasicus* and *Ph. mongolensis* are indistinguishable in terms of their *cyt b* sequences] and any other (non-vector) sympatric sandfly species.

The results also indicate that *Ph. (Paraphlebotomus) alexandri* is, in terms of its *cyt b*

FIG. 3. An unrooted (neighbour-joining) tree of Afghan *Sergentomyia* taxa, based on comparison of 270-bp sequences of the mitochondrial *cyt B* gene. The specimens included in the present study are indicated by bold text (see Table 2 for details). Bootstrap values of >50% are shown at the corresponding branches.

sequence, more similar to species in the subgenus *Phlebotomus* than to other species in the subgenus *Paraphlebotomus* (Fig. 2). Likewise, two species assigned to the subgenus *Sergentomyia* — *Se. (Se.) murgabiensis/sintoni* and *Se. (Se.) minuta* — showed less genetic similarity to each other than to other subgenera of *Sergentomyia* (Fig. 3).

The distance matrix for all the sequenced specimens (with data from other studies excluded) is shown in Table 3. Generally, the level of intrasubgeneric genetic differentiation lay between 0 and 15% while the level of intersubgeneric genetic differentiation exceeded 15% (reaching up to 27%). However, as

well as the two exceptions mentioned above (*Ph. alexandri* and the subgenus *Sergentomyia*), sandflies in the subgenus *Adlerius* only differed by 13%–17% from the subgenera *Phlebotomus* and *Laroussius*, whereas subgeneric distances within the genus *Sergentomyia* lay between 12% and 20%.

Because no specimens of *Ph. sergenti* from Afghanistan have been included in previous DNA studies, a more detailed comparison of about 430 bp of the *cyt b* genes of 19 specimens of this species was conducted. Ten published *Ph. sergenti* sequences (mainly from neighbouring Iran and Pakistan; Moin-Vaziri *et al.*, 2007a), nine

FIG. 4. A neighbour-joining tree of Afghan *Phlebotomus sergenti*, based on comparison of 434-bp sequences of the mitochondrial *cyt B* gene. The specimens included in the present study are indicated by bold text (see Table 2 for details). The tree is rooted with the two *Paraphlebotomus* species: *Ph. caucasicus* and *Ph. alexandri*. The lineage abbreviations NCS, NE and SW refer to the nomenclature of Moin-Vaziri *et al.* (2007a). Bootstrap values of >50% are shown above the corresponding branches (the values below branches are from simultaneous maximum-parsimony analysis).

sequences of *Ph. sergenti* caught in the present study, one *Ph. caucasicus* sequence and one *Ph. alexandri* sequence (serving as an outgroup) were aligned and phylogenetically analysed (Fig. 4). The estimated neighbour-joining and maximum-parsimony trees resolved three clusters, one of which (tentatively named the NE lineage) contained all the Afghan specimens. The Afghan specimens resemble Iranian morphotype B in terms of the shape of the style (with one sub-terminal spine) and the width of the basal lobe of the coxite (data not shown).

DISCUSSION

Accurate vector identification is an essential factor in the effective surveillance and control of any vector-borne disease. Sandfly-transmitted *Le. major* is a significant public-health threat in northern Afghanistan, causing rural

zCL, and both anthroponotic CL and visceral leishmaniasis may also occur in the country, albeit at lower prevalences (Leslie *et al.*, 2006; Faulde *et al.*, 2008a). The present results indicate that *Ph. papatasi* (the primary vector of *Le. major*) is widely distributed in northern Afghanistan and particularly abundant in MeS and Kondo, which are both foci of endemic zCL (Faulde *et al.*, 2008a).

Phlebotomus sergenti is a typical element of urban or peri-urban highland environments and is the main vector of the *Le. tropica* that causes urban aCL in Kabul (Omar *et al.*, 1969; Killick-Kendrick *et al.*, 1995) and probably, in recent years, also in urban Feyzabad (Reithinger *et al.*, 2004). In the present study, *Ph. sergenti* was also detected in Kondo, where aCL has occurred only sporadically in recent years (Faulde *et al.*, 2008a). The results of the detailed molecular analysis of Afghan, Iranian and Pakistani *Ph. sergenti* (Fig. 4) indicate that the distribution of this species extends from

Iran (Moin-Vaziri *et al.*, 2007a) into northern Afghanistan. The Afghan *Ph. sergenti* specimens that were investigated clearly cluster with the Iranian 'NE' lineage that probably originated in the Iranian province of Khorassan (which shares a border with Herat province, in north-western Afghanistan, and southern Turkmenistan). It remains unclear to what extent morphological and molecular characters truly define the subspecies of *Ph. sergenti*. Morphologically, the male specimens of *Ph. sergenti* caught in northern Afghanistan resembled morphotype B (Moin-Vaziri *et al.*, 2007a), probably indicating that they were *Ph. sergenti similis*, whereas most specimens in the Iranian 'NE' lineage are of morphotype A and therefore *Ph. sergenti sergenti*.

Artemiev (1981) reported 44 sandfly species and subspecies from Afghanistan, with some members of the subgenus *Adlerius* endemic, but had very few data for remote areas such as Feyzabad, or Ghowrmach. Although Artemiev (1981) listed six species from the greater Feyzabad area, two of these (*Ph. longiductus* and *Se. sodgiana*) were not collected (from Feyzabad or anywhere else) in the present study, and the *Ph. papatasi*, *Ph. turanicus* and *Se. dreyfussi turkestanica* collected in Feyzabad during the present study are apparently the first records of these species for this location.

Although *Ph. (Adlerius) turanicus* (apparently the only *Adlerius* species collected in the present study) is normally considered xerophilic, in 2009 it was collected in the slightly cooler subalpine setting of Feyzabad as well as in the hot semi-desert of Ghowrmach and MeS. As all 27 male *Adlerius* collected during the present study were identified as *Ph. (A.) turanicus*, it was assumed that the single *Adlerius* female (collected from MeS) that was used for sequencing belonged to this species [although morphologically indistinguishable from female *Ph. (A.) kabulensis*].

Sergentomyia (Grassomyia) dreyfussi turkestanica was previously only known from a few places in northern Afghanistan (Kondoz

and central-eastern Afghanistan (Artemiev, 1976, 1981). The collection of this species in Feyzabad (present study) appears to be an unusual occurrence, since this species is described as thermo- and polyhygrophilic (Artemiev, 1983), two characters that also define *Ph. papatasi* (Artemiev, 1983), which was collected only very rarely in Feyzabad.

Factors such as micro-habitat differences (e.g. vicinity of the lizard-harbours walls that favour *Sergentomyia* species), trap type (e.g. light *v.* sticky), geographical region and seasonality can greatly alter the species composition and sex ratios of collections of *Phlebotomus* and *Sergentomyia* (Yuval *et al.*, 1988; Kamhawi *et al.*, 1995; Wheeler *et al.*, 1996; Burkett *et al.*, 2007; Hanafi *et al.*, 2007; Fahmy *et al.*, 2009; Kasap *et al.*, 2009; Kasili *et al.*, 2010). It is important to remember, therefore, that the sex ratios and species compositions reported here only represent trap-related samples and do not necessarily reflect the natural abundances of each gender and species.

In the past, Afghan sandflies have been poorly studied using molecular techniques. The simple barcoding method employed in the present study appeared to be generally adequate for their identification to species level. The *cyt b* gene was chosen merely because it had been used by other researchers in the investigation of various sandfly taxa, and so there was already a useful dataset of *cyt b* sequences available in GenBank (Esseghir *et al.*, 1997; Ready *et al.*, 1997; Parvizi *et al.*, 2003; Hamarsheh *et al.*, 2007; Moin-Vaziri *et al.*, 2007a, b). As well as permitting the successful and unambiguous typing of most of the sandfly specimens collected in the camps, the present results also allowed for further taxonomic hypotheses. Though not a phylogenetic analysis, the genetic-distance calculations indicated that the subgenus *Paraphlebotomus* is paraphyletic, confirming the observations made by Depaquit *et al.* (1998, 2000) in an investigation of internal-transcribed-spacer-2 (ITS2) ribosomal DNA. In a taxon-identity tree produced in a recent comparison of

ITS2 sequences that included the subgenera *Laroussius* and *Anaphlebotomus*, *Ph. alexandri* was placed, with high bootstrap support, at the base of the subgenus *Phlebotomus* as a sister clade of *Paraphlebotomus* (unpubl. obs.), indicating a similar relationship to that seen with the *cyt b* sequences (present study).

Another example of possible paraphyly occurs in the subgenus *Sergentomyia*, where the two species *Se. (Se.) sintoni/murgabiensis* (see discussion below) and *Se. (Se.) minuta* do not cluster together. This result was clearly foreshadowed by Seccombe *et al.* (1993) who stated: 'However, *Sergentomyia* is clearly a genus of practical convenience: its larger subgenera (... *Sergentomyia* ...) are not well-defined ...'.

There is a taxonomic inconsistency in the literature concerning the use of the names *Se. (Se.) sintoni* and *Se. (Se.) murgabiensis* (plus subspecies). According to Artemiev (1978) and Seccombe *et al.* (1993), *Se. murgabiensis* is a valid species, with *Se. (Se.) murgabiensis sintoni* as one of three valid subspecies. Recently, however, Parvizi and Amirkhani (2008), for unspecified reasons, re-used the name *Se. sintoni* for specimens from Iran. In the present study, an Afghan specimen of '*Se. murgabiensis*' was found to share 97%–99% nucleotide identity with Iranian *Se. sintoni* in its *cyt b* gene, indicating possible conspecificity. If the synonymy of *Se. sintoni* with *Se. murgabiensis* is proven in the future, *Se. sintoni* will become a junior synonym. This taxonomic problem merits further attention, since both *Se. murgabiensis* (as '*Se. arpaklensis*') and *Se. sintoni* seem to play a role in the enzootic cycles of *Le. major* and other *Leishmania* species (Seccombe *et al.*, 1993; Parvizi and Amirkhani, 2008).

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