REGIONAL GENETIC DIFFERENTIATION OF *PHLEBOTOMUS SERGENTI* IN THREE MOROCCAN FOCI OF CUTANEOUS LEISHMANIASIS CAUSED BY *LEISHMANIA TROPICA*

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Summary:

Phlebotomus sergenti was identified morphologically in samples from three Moroccan foci of leishmaniasis caused by *Leishmania tropica* in the provinces of Azilal, Essaouira and Taza. Three primary mitochondrial DNA lineages were identified, and they could be markers for regionally distributed cryptic species. Greater mitochondrial diversity in Azilal indicated that this central province could have been the origin of dispersal of *P. sergenti* or the zone of secondary contact. All except one of the 21 mitochondrial haplotypes showed a marked regional distribution, and this indicates that vector control would not always be followed by rapid, long-distance reinvasion. Only mitochondrial haplotype SER18 was a putative marker for long-distance dispersal, for which there is no evidence of human assistance.

KEY WORDS : *Leishmania tropica*, *Phlebotomus sergenti*, mitochondrial lineages, host-parasite co-associations, phylogeography.

Résumé : DIFFÉRENTIATION GÉNÉTIQUE RÉGIONALE DE *PHLEBOTOMUS SERGENTI* DANS TROIS FOYERS MAROCAINS DE *LEISHMANIA TROPICA*

Phlebotomus sergenti est identifié morphologiquement sur des spécimens originaires de trois foyers marocains à *Leishmania tropica* : Azilal, Essaouira et Taza. Trois lignées mitochondriales sont identifiées et pourraient être marqueurs d'espèces cryptiques avec une distribution régionale spécifique. La grande diversité mitochondriale trouvée à Azilal ferait de cette région l'origine de dispersion de *P. sergenti* ou la zone de contact secondaire des lignées vicariantes. Les distributions régionales des 21 haplotypes mitochondriaux, à l'exception de SER18, possible marqueur d'une dispersion anthropogène, suggère que le contrôle vectoriel pourrait être efficace dans plusieurs localités, vu la lenteur et la faible portée de la recolonisation.

MOTS CLÉS : *Leishmania tropica*, *Phlebotomus sergenti*, lignées mitochondriales, co-association hôte-parasite, phylogéographie.

INTRODUCTION

In Morocco, three species of *Leishmania* (Kinetoplastida: Trypanosomatidae) are the causative agents of human leishmaniasis transmitted by haematophagous female sandflies (Diptera: Psychodidae). *Leishmania major* Yakimoff & Schokhor, 1914 is transmitted by *Phlebotomus* (*Phlebotomus*) *papatasi* (Scopoli, 1786) and is associated with zoonotic cutaneous leishmaniasis (CL) in the arid regions along the northern edge of the Sahara desert (Rioux *et al.*, 1986). *Leishmania infantum* Nicolle is transmitted by *Phlebotomus* (*Larroussius*) species and causes cutaneous and visceral disease in humans and domestic dogs in the North and Centre-South regions of the country (Guessous-Idrissi *et al.*, 1997b). Lastly, *Leishmania tro-*

pica Wright, 1913 was first associated with human CL in the Centre-South region, in Azilal and Essaouira provinces (Pratlong *et al.*, 1991), where it is transmitted by *Phlebotomus* (*Paraphlebotomus*) *sergenti* Parrot, 1917 (Guilvard *et al.*, 1991). In 1995, another focus of CL caused by *L. tropica* was discovered in urban and peri-urban areas of the province of Taza, in northeast Morocco, and *P. sergenti* was suggested as a local vector (Guessous-Idrissi *et al.*, 1997a, 1999). However, no natural infections were discovered in sandflies (Hamdani, 2001).

Two hypotheses are most likely to explain the emergence of CL in the province of Taza, and these are not mutually exclusive. Firstly, it could have resulted from the immigration of infected people from the Centre-South region, because transmission of *L. tropica* is often reported to be anthroponotic (Pratlong *et al.*, 1991; Desjeux, 2001; Volf *et al.*, 2002) and the same zymodeme (MON-102) predominates in human infections in both regions (Pratlong *et al.*, 1991; Bichichi *et al.*, 1999; Guessous-Idrissi *et al.*, 1999; Riyad *et al.*, 2001). Secondly, there might have been a local increase in the abundance of a genotype, race or cryptic species of *P. sergenti* that transmits *L. tropica* more efficiently, as a result of vector-parasite co-adaptation or an improved "encounter filter" (Combes, 2001) resulting from ecological adaptations.

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The current report investigates the possibility that variation in *P. sergenti* helps to explain the changing pattern of CL transmission in Morocco. Populations of *P. sergenti* were sampled in the three foci of CL associated with *L. tropica* (Azilal, Essaouira and Taza) and from a non-endemic province (Khemisset), and individual adult sandflies were characterized morphologically and by the comparative sequence analysis of a fragment of mitochondrial DNA (mtDNA).

MATERIALS AND METHODS

STUDY AREAS

Essaouira province lies by the Atlantic coast in the Centre-South region. 59 CL cases were reported for 1992-2001 (Table I). Sandflies were collected from two locations in the commune of Smimou (31° 12' N, 9° 42' W; altitude 237 m a.s.l.) and six km away in the rural locality of Ikhemissen, all within the CL focus reported by Pratlong *et al.* (1991). The bioclimate is semi-arid (Emberger, 1930).

Azilal province is located on the western lower slopes of the Middle Atlas mountains in the Centre-South region. 200 CL cases were reported for 1992-2001 (Table I). Sandflies were sampled from two locations in the commune of Tanante (31° 52' N, 6° 57' W; altitude 889 m a.s.l.), and 18 km away in the rural village of Ait Faska in the commune of Ait Majdane (31° 49' N, 6° 57' W; altitude 770 m a.s.l.). All locations lie within the CL focus reported by Pratlong *et al.* (1991) and are near the border of the semi-arid/sub-humid bioclimate zones (Emberger, 1930).

Taza province is located on the northeastern lower slopes of the Middle Atlas mountains. 301 CL cases were reported for 1992-2001 (Table I). Sandflies were collected in the provincial capital "Taza ville" (34° 13' N, 4° 1' W; altitude 598 m a.s.l.), within three suburban neighbourhoods (Hay el Qods, Magoussa and Bouhjar, including the ruined city walls at the escarpment edge) and in the urban neighbourhoods of Bab Tete and Abattoirs, all in the CL focus reported by Guessous-

Idrissi *et al.* (1997a, 1999). Other samples were collected in the rural administrative region of Tahla, forty km west of Taza ville, in Hay Nehda (34° 26' N, 4° 25' W; altitude 572 m a.s.l.) and in the small town of Zrada. The bioclimate is semi-arid (Emberger, 1930).

Khemisset province lies in northwest Morocco between the coastal plain and the Middle Atlas mountains. Unlike the previous provinces, it is known to be endemic for canine leishmaniasis caused by *L. infantum* and not CL. Six CL cases were reported for 1992-2001 (Table I), but all are considered by the Ministry of Health to be imported from other Moroccan foci. Sandflies were sampled from a single village in the rural district of Ait Tiber in the commune of Ait Siberne (33° 49' N, 6° 4' W; altitude 409 m a.s.l.) and in nearby caves of the valley Oued Beht. The bioclimate is sub-humid/semi-arid (Emberger, 1930).

COLLECTION METHODS FOR SANDFLIES

Manual aspirators and torches were used to capture sandflies resting on the walls and ceilings of living rooms and bedrooms of houses. Most captures were made during daylight hours, in the morning or evening. Sandflies were released into gauze-topped cartons at 5-10 min. intervals.

CDC miniature light traps (Hauschers Machine Works, New Jersey, USA) were suspended from hooks in the walls of living rooms and bedrooms of houses, or occasionally on outside walls. They were started at dusk and stopped by the householders at dawn. After local transport in a cool box, sandflies were immobilized in a freezer before being stored in liquid nitrogen or in 80 % ethanol (– 20° C).

MORPHOLOGICAL IDENTIFICATION OF SANDFLIES

Sandflies were identified in Casablanca, based on external and internal morphological characters of the head and genitalia (Depaquit *et al.*, 1998; Hamdani, 2001), which were slide-mounted in Berlese fluid following dissection with sterilized forceps and syringe needles. The thorax and attached anterior abdomen of individual flies were stored at – 20° C. Morphometric and meristic characters were measured in London,

Year	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	Totals
Province											
Essaouira	N/A	N/A	N/A	8	2	N/A	9	12	28	N/A	59
Azilal	15	18	6	3	17	1	25	26	15	74	200
Khemisset	4	1	N/A	N/A	N/A	N/A	N/A	N/A	1	N/A	6
Taza	N/A	2	1	26	115	25	20	35	28	49	301
Totals	19	21	7	37	134	26	54	73	72	123	566

Table I. – Numbers of human cases of CL (probable aetiological agent *L. tropica*, except in Khemisset where *L. infantum* predominates) recorded from each of the prospected Moroccan provinces, 1992-2001 (Source: Bulletin d'Épidémiologie de la Santé. Ministère de la Santé, Maroc, 1999; 2000; 2001). N/A = data not available.

using a Zeiss compound microscope with a Kontron Videoplan Image Analysis System. Statistical analyses (Bailey, 1968) were performed using Statistica software (version 5.0., StatSoft Inc., 1995).

DNA ISOLATION, PCR AMPLIFICATION, SEQUENCING AND COMPARATIVE SEQUENCE ANALYSIS OF SANDFLY MTDNA

In London, genomic DNA was extracted from the cryopreserved thorax and attached anterior abdomen of individual sandflies (Esseghir *et al.*, 1997), and then the polymerase chain reaction (PCR) was used to amplify one of two overlapping fragments containing the 3' end of the mitochondrial gene Cytochrome b (Cyt b). DNA extraction was carried out according to Testa *et al.* (2002) to reduce the risk of PCR carryover. Initially, the CB3 fragment (545 base pairs (bp) including primers) was amplified with the primer pair CB3-FC/N1N-FA (Esseghir *et al.*, 1997), but this did not work well for all *P. sergenti*. Therefore, internal primers were designed, based on an alignment of our CB3 sequences of *P. sergenti* and those of other *Phlebotomus* species in GenBank (Esseghir *et al.*, 1997, 2000). The SER fragment (442 bp including primers) was amplified using the forward primer SER-F1 (5' to 3' TACGATCAATTCCTAATAA, ending on base 8 in the sequence alignment of Esseghir *et al.* (1997: 217)) together with the reverse primer SER-R2 (5' to 3' ATT-TACCTGCGTCTTTGT, ending on base 422 in the sequence alignment of Esseghir *et al.* (1997: 218)).

A 50 µl PCR reaction mixture consisted of 1x Promega buffer, 1.5 mM MgCl₂, 60 µM dNTP, 0.5 µM each of the two primers and 1.5 units of Taq DNA polymerase (Promega). DNA amplification was performed in a

Techne Genius thermal cycler with the following amplification cycles: denaturation at 94° C for three minutes; five cycles of denaturation at 94° C for 30 s, annealing at 40° C for 30 s, extension at 72° C for 90 s; followed by 30 cycles of denaturation at 94° C for 30 s, annealing at 42° C for 30 s, extension at 72° C for 90 sec; and a final extension at 72° C for 10 minutes. The size and quantity of the amplified DNA fragments were determined by fractionation in 1.5 % agarose gels along with DNA standards (Promega PCR Markers G316A). The DNA in each excised gel fragment was purified with glassmilk (GeneClean II Kit, BIO 101 Inc) and each strand directly sequenced using one of the PCR primers (3.2 pmoles) and the ABI Prism®, Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (version 2.0, PE Applied Biosystems). The extension products were purified by ethanol precipitation. Sequences were resolved using the ABI 373A or 377 system, all according to ABI protocols (PE Applied Biosystems), then edited and aligned with database sequences using Sequencher software (Gene Codes Corp.) to identify haplotypes (= unique sequences) and to permit their phylogenetic analysis using PAUP* software (Swofford, 2002).

RESULTS

MORPHOLOGICAL IDENTIFICATION OF *P. SERGENTI*

All males (Table II) were identified as the species *P. sergenti*, rather than *P. similis*, based on some of the diagnostic characters given by Depaquit *et al.* (1998). The mean length and mean

Diagnostic Characters	<i>P. sergenti</i> (Depaquit <i>et al.</i> , 1998)	<i>P. similis</i> (Depaquit <i>et al.</i> , 1998)	<i>P. sergenti</i> (Morocco)
Males (M)	n = 116	n = 75	n = 31
Length of basal lobe (BL) of coxite	Mean = 44.00 Std dev = 6.29	Mean = 49.00 Std dev = 5.57	Mean = 40.60 Std dev = 4.50, range = 30.40-49.10
Width of BL of coxite	Mean = 14.70 Std dev = 2.25	Mean = 25.00 Std dev = 2.67	Mean = 15.65 Std dev = 2.10, range = 10.70-19.90
Length/Width of BL of coxite	Mean = 3.02 Std dev = 0.57	Mean = 1.98 Std dev = 0.21	Mean = 2.60 Std dev = 0.40, range = 2.00-3.40
Number of setae on BL of coxite	Mean = 18.61 Std dev = 3.36	Mean = 30.00 Std dev = 3.10	Mean = 15.19 Std dev = 2.72, range = 11.00-22.00
Females (F)	n = 59	n = 49	n = 31
Height of pharyngeal armature	Mean = 46.00 Std dev = 9.70	Mean = 55.00 Std dev = 6.40	Mean = 50.30 Std dev = 5.30, range = 37.70-59.70
Number of annuli on spermatheca	Mean = 4.53 Std dev = 0.58	Mean = 5.47 Std dev = 0.90	Mean = 5.20 Std dev = 0.60, range = 4.00-6.00
Lateral pharyngeal teeth	Absent	Present	Absent

Table II. – Descriptive statistics for morphometric and meristic characters of males and females of *P. sergenti* from Morocco compared with the data reported by Depaquit *et al.* (1998).

width of the basal lobe (BL) of the coxite were statistically shorter (Student's *t* test, $P < 0.001$) than those reported for *P. similis*, but not those reported for *P. sergenti*. The mean of the ratio BL length/width was intermediate in value compared with the specimens of Depaquit *et al.* (1998), and statistically this character distinguished the current specimens from *P. sergenti* and *P. similis* (Student's *t* test, $P < 0.001$). The mean number of setae on the BL was statistically less (Student's *t* test, $P < 0.001$) than that reported for both species (Table II).

For females (Table II), the means of the length of the pharyngeal armature and the number of spermathecal annuli were intermediate between those given for *P. sergenti* and *P. similis* by Depaquit *et al.* (1998), and statistically these characters distinguished the current specimens from both species (pharyngeal armature) or from *P. sergenti* (spermathecal annuli) (Student's *t* test, $P < 0.001$). However, the absence of lateral pharyngeal teeth unambiguously identified all Moroccan specimens as *P. sergenti*.

RELATIVE DENSITIES OF *P. SERGENTI* IN DIFFERENT MOROCCAN REGIONS AND HABITATS

Both sexes were aspirated from the inside walls of houses in rural and suburban locations in all provinces (Table III). Relative densities varied within each region, but *P. sergenti* was no less abundant and widespread in the province of Taza than it was in the Centre-South region. Males predominated (100/137 *P. sergenti*), and

48.6 % of the females contained fresh blood meals or blood meal remains with developing eggs.

Both sexes of *P. sergenti* were also collected in CDC miniature light traps inside and outside houses in all provinces, and once again this species was no less abundant and widespread in the province of Taza than it was in the Centre-South region (Table IV). Relative densities varied between locations, but they were higher for rural or suburban locations compared with urban ones and, in Khemisset, were higher for caves compared with a nearby rural village. In contrast to the aspirator collections, females predominated in the light traps (105/153 *P. sergenti*), and again many of the females (44.8 %) contained fresh blood meals or blood meal remains with developing eggs.

DIVERSITY AND PHYLOGENETIC RELATIONSHIPS OF MTDNA HAPLOTYPES OF *P. SERGENTI* BY REGION AND HABITAT

Twenty-one haplotypes were identified (Fig. 1) from 83 males and females of Moroccan *P. sergenti* (Table V). A phylogenetic analysis indicated that the mtDNA haplotypes formed three well-supported lineages (Fig. 2), which were named after the region in which they were first discovered and predominated. Ten fixed polymorphisms, underlined in Figure 1, were diagnostic for the CS mtDNA lineage of Centre-South Morocco, three were diagnostic for the NW mtDNA lineage of northwest Morocco, and eight were diagnostic for the TZ mtDNA lineage of the province of

Sandfly populations sampled by PROVINCE and district	Capture effort (No. of capturers × visits × houses)	Total number of males captured	Total number of females captured (eng./gr.*)	Number of males captured per person- hour of aspiration	Number of females captured per person- hour of aspiration	Number of both sexes captured per person- hour of aspiration
ESSAOUIRA (R) Ssmiou-Ikhmissen (1)	$1 \times 1 \times 3 = 3$	3	6 (4)	$3 \times (4/3) = 4.0$	$6 \times (4/3) = 8.0$	12.0
AZILAL (R) Tanante (2)	$(1 \times 1 \times 1) +$ $(1 \times 2 \times 2) = 5$	23	8 (5)	$23 \times (4/5) = 18.4$	$8 \times (4/5) = 6.4$	24.8
KHEMISSSET (R) Ait Siberne (3)	$1 \times 1 \times 1 = 1$	0	1 (1)	0.0	$1 \times (4/1) = 4.0$	4.0
TAZA (S) Ville-suburban (4)	$2 \times 1 \times 1 = 2$	8	1 (1)	$8 \times (4/2) = 16.0$	$1 \times (4/2) = 2.0$	18.0
TAZA (S) Tahla (5)	$1 \times 1 \times 2 = 2$	36	14 (0)	$36 \times (4/2) = 72.0$	$14 \times (4/2) = 28.0$	100.0
TAZA (R) Zrarda (6)	$2 \times 1 \times 4 = 8$	30	7 (7)	$30 \times (4/8) = 15.0$	$7 \times (4/8) = 3.5$	18.5
TOTALS [Means]	21	100	37 (18) [†]	[20.9]	[8.7]	[29.6]

Table III. - Numbers and relative densities of *P. sergenti* collected with manual aspirators inside suburban (S) or rural (R) houses in different regions of Moroccan provinces, 1999-2000. (1) Aspiration for 15 min. by one person in one visit to three houses; (2) Aspiration for 15 min. by one person in one visit to one house, and by one person in two visits to two houses; (3) Aspiration for 15 min. by one person in one visit to one house; (4) Aspiration for 15 min. by two persons in one visit to one house; (5) Aspiration for 15 min. by one person in one visit to two houses; (6) Aspiration for 15 min. by two persons in one visit to four houses. [†] = 48.6 %. * eng. = engorged female, or gr. = gravid female.

Sandfly populations sampled by PROVINCE and district	Capture effort (No. sites/ No. visits)	Number of trap-nights	Total number of males captured	Total number of females captured (eng./gr.*)	Number of males captured per trap-night	Number of females captured per trap-night	Number of both sexes captured per trap-night
ESSAOUIRA (R) Smimou-Ikhmissen	2/1	3 × 1 = 3	5	16 (5)	1.7	5.3	7.0
ESSAOUIRA (S) Smimou-Cs	2/1	3 × 1 = 3	4	3 (0)	1.3	1.0	2.3
AZILAL (R) Ait Faska	1/1	2 × 1 = 2	4	7 (6)	2.0	3.5	5.5
KHEMISSET (R) Ait Siberne	1/1	1 × 1 = 1	1	0 (0)	1.0	0.0	1.0
KHEMISSET (R) Grottes (caves)	1/1	1 × 1 = 1	4	5 (1)	4.0	5.0	9.0
TAZA (U) Ville-urban	6/2	6 × 1 = 6	15	28 (15)	2.5	4.7	7.2
TAZA (S) Ville-suburban	2/2	3 × 1 = 3	15	46 (20)	5.0	15.3	20.3
TOTALS [Means]	15/9	19	48	105 (47) [†]	[2.5]	[5.0]	[7.5]

Table IV. – Numbers and relative densities of *P. sergenti* collected in CDC miniature light traps placed on the inside or outside walls of houses in rural (R), suburban (S) or urban (U) locations in different regions of Moroccan provinces, 1999-2000. [†] = 44.8 %. * eng. = engorged female, or gr. = gravid female.

3' end of Cytochrome b gene			tRNA ser	3' end NADH1
Primary lineage		111111111122222222	33	333
(sub-lin) Provinces*		1111245556667777789901355788990123356	33	779
Haplotype		470369332564790168921878947524030137892	23	132

CS				
SER01	ES	TAAATTGAACCCCTACGCAGTTCAATTGTACTTTTCCTCT	GC	CCT
SER02	ES AZ	TAAATTGAGCCCTACGCGGTTCAATTGTACTTTTCCTCT	AC	CCT
NW				
SER03	KH	TAAACCGAACCCCATGCGGTTTAACCACTACTCTCTCT	GC	TCT
SER04	KH	TAAACCGAACCCCATACGGTTTAACCACTACTCTCTCT	GC	TCT
SER05	KH	TAAACCGAACCCCATGCGGTTTAACCACTACTCTCTCT	GC	TAT
SER06	KH	TAAACCGAATCTCATGCGGTTTAGCCACTACTCTCTCT	GC	TCT
SER07	AZ	TAAACCGAATCTCAGCGGTTTAACCACTACCTCTCTCT	GC	TCT
SER08	AZ	CAAACCGAATCTCAGCGGTTTAACCACTACCTCTCTCT	GC	TCT
TZ (CAA)				
SER09	TZ	TGGACcGTACCCTACGTAACCTaaccATATCCTCTCATC	AC	CTC
SER10	TZ	TGGACcGTACCCTACGTAACCTaaccATATCCTCTCAT	AC	CTC
TZ (CGA)				
SER11	TZ	TGGACcGTACCCTACGTAACCTgaCCATATCCTCTCAT	AC	CTC
SER12	TZ	TGGACcATACCCTACGTAACCTgaCCATATCCTCTCAT	AT	CTC
SER13	TZ	TGGACcGTACCCTACGTAACCTgaCCATGTCTCTCAT	AC	CTC
SER14	TZ	TGGACcGTATCCTACGTAACCTgaCCATATCCTCTCAT	AC	CTC
SER15	TZ	TAGGCcGTACCCTACGTAACCTgaCCATATCCTCTCAT	AC	CTC
SER16	TZ	TGGACcGTACCCTGCGTAACCTgaCCATATCCTCTCAT	AC	CTC
SER17	TZ	TGGACcGTACCCTGCATAACCTgaCCATATCCTCTCAT	AC	CTC
TZ (TAG)				
SER18	TZ ES AZ	TGGACtGTACCCTACGTAACCTagCCATATCCTCTCAT	AC	CTC
SER19	TZ	TGGACtGTACCCTACGTAACCTagCCATATCCTCTCGT	AC	CTC
SER20	TZ	TGGACtGTACCCTACGTAACCTagCCATATCCTCTTAT	AC	CTC
SER21	ES AZ	TAGACtGTACCCTACGTAACCTagCCATATCCTCTCAT	AC	CTC
Base position in codon		333333133313323132333113333313331332333		
Synonymous change (s)		ssssssNssssssNsNsNssssNssssssNssssssNss		
Or non-synonymous (N)				

Fig. 1. – Alignment of nucleotide characters at the polymorphic sites in the SER haplotypes of mitochondrial Cytochrome b of Moroccan *P. sergenti*. Fixed polymorphisms diagnostic for each primary lineage (CS, NW, TZ) are underlined. Fixed polymorphisms diagnostic for each sub-lineage (sub-lin) of TZ (CAA, CGA, TAG) are in lowercase. * The provinces are Essaouira (ES), Azilal (AZ), Khemisset (KH) and Taza (TZ).

LINEAGE		CS		NW					TZ CAA		TZ CGA							TZ TAG					
SER	haplotype	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	TOTALS
ESSAOUIRA	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F	M+F=T
Smi-Ikhmissen	0+1																		0+5			1+0	1+6=7
Smimou-CS	1+1	1+0																	0+1				2+2=4
SUBTOTAL		1+2	1+0																0+6			1+0	3+8=11
AZILAL																							
Ait Faska		1+5																					1+5=6
Tanante		1+0						4+0	1+0										1+0			0+1	7+1=8
SUBTOTAL		2+5						4+0	1+0										1+0			0+1	8+6=14
KHEMISSSET																							
Ait Siberne				0+1		1+0																	1+1=2
Grottes				2+1	1+0	0+1	1+2																4+4=8
SUBTOTAL				2+2	1+0	1+1	1+2																5+5=10
TAZA																							
Ville-urban										0+1		1+2							6+3		1+0		8+6=14
Ville-suburb										1+0		3+2	0+1	1+0	0+1		2+0	0+1	3+14				10+19=29
Tahla											1+0	0+1				0+1							1+2=3
Trada																			1+0	1+0			2+0=2
SUBTOTAL										1+1	1+0	4+5	0+1	1+0	0+1	0+1	2+0	0+1	10+17	1+0	1+0		21+27=48
TOTALS	M+F	1+2	3+5	2+2	1+0	1+1	1+2	4+0	1+0	1+1	1+0	4+5	0+1	1+0	0+1	0+1	2+0	0+1	11+23	1+0	1+0	1+1	37+46
=T		=3	=8	=4	=1	=2	=3	=4	=1	=2	=1	=9	=1	=1	=1	=1	=2	=1	=34	=1	=1	=2	=83

Table V. – Numbers of males (M)+females (F) and totals (=T) of *P. sergenti* with each SER haplotype of mitochondrial Cytochrome b, from populations collected in different PROVINCES and locations in Morocco, 1999-2000.

Fig. 2 – Neighbor joining (NJ) phylogram relating the SER haplotypes of all three primary lineages of *P. sergenti* collected in the four Moroccan provinces (e.g. SER01 ES is haplotype 01 and it was found only in the province of Essaouira). Branches supported by bootstrap values > 50 % are marked. Haplotype codes are explained in the legend to Figure 1.

Taza. Haplotypes of the CS mtDNA lineage were found only in the provinces of Essaouira and Azilal, and haplotypes of the NW mtDNA lineage were found only in the provinces of Khemisset and nearby Azilal. In contrast, haplotypes of the TZ mtDNA lineage were found not only in the province of Taza but also in the Centre-South region (Table V). Pairwise genetic distances were greater between haplotypes from different primary mtDNA lineages (3.8-4.5 %) than between haplotypes from the same primary mtDNA lineage (0.2-1.3 %).

Most of the mtDNA haplotypes were grouped in the TZ lineage (13/21) and these formed three sub-lineages (CAA, CGA and TAG), named after the fixed nucleotide polymorphisms diagnostic for each, which are shown in lowercase in Figure 1. A phylogenetic analysis of only the TZ haplotypes indicated that the CGA and TAG sub-lineages were derived from one of the two haplotypes of the CAA sub-lineage (Fig. 3). The relative frequencies of haplotypes were similar in all habitats. The abundant haplotypes were found in rural, suburban or urban environments, and both inside

Fig. 3 – Unrooted phylogram relating the SER haplotypes of the TZ primary lineage of *P. sergenti* collected in the Centre-South region as well as in the province of Taza. The phylogram is the result of a parsimony analysis with a branch and bound search; branches supported by bootstrap values > 50 % are marked. Haplotype codes are explained in the legend to Figure 1.

and outside houses (Table V). For example, haplotypes SER11 (CGA sub-lineage of TZ) and SER18 (TAG sub-lineage of TZ) were frequent in light traps placed inside or outside houses in the province of Taza, and SER18 was also frequent inside and outside houses in the province of Essaouira.

REGIONAL DISTRIBUTION OF MTDNA HAPLOTYPES AND LINEAGES OF *P. SERGENTI* IN MOROCCO

It is clear from Table V that mtDNA haplotypes, as well as their lineages, have a markedly regional distribution.

For example, both haplotypes of the CS mtDNA lineage were found only in the Centre-South region but, whereas haplotype SER02 occurred in both provinces, haplotype SER01 occurred only in Essaouira. Similarly, four haplotypes of the NW mtDNA lineage were found in the province of Khemisset and two haplotypes of the same lineage were found in the province of Azilal, but not one of these six haplotypes was found in both provinces. The haplotypes of the TZ mtDNA lineage also showed regionality, with all nine haplotypes of the CAA and CGA sub-lineages occurring only in the

(a)

Province	Lineage	CS Observed	CS Expected	NW Observed	NW Expected	TZ CRA Observed	TZ CRA Expected	TZ TAG Observed	TZ TAG Expected	Totals
ES		4	1.46	0	1.99	0	2.52	7	5.04	11
AZ		7	1.86	5	2.53	0	3.20	2	6.41	14
KH		0	1.32	10	1.81	0	2.29	0	4.58	10
TZ		0	6.36	0	8.67	19	10.99	29	21.97	48
Totals		11	11.00	15	15.00	19	19.00	38	38.00	83

(b)

Province	Lineage	CS Observed	CS Expected	NW Observed	NW Expected	TZ CRA Observed	TZ CRA Expected	TZ TAG Observed	TZ TAG Expected	Totals
ES + AZ + KH		11	5	15	6	0	8	9	16	35
TZ		0	6	0	9	19	11	29	22	48
Totals		11	11	15	15	19	19	38	38	83

Table VI. – a) Observed and expected numbers of *P. sergenti* from each Moroccan province with haplotypes in each mitochondrial Cytochrome b lineage, compiled from Table III and with lineages TZ CAA and TZ CGA combined as TZ CRA; b) the same data amalgamated so that expected numbers (rounded to the nearest integer) are not less than 5, in order to permit Chi-squared analysis (Bailey, 1968). The proportion of haplotypes in each lineage was significantly different for Taza province compared with the other provinces ($P < 0.001$; Chi-squared = 54.7 for three degrees of freedom).

province of Taza, in which two out of four haplotypes (SER19, SER20) of the TAG sub-lineage were also restricted (Table V). Of the other two haplotypes of the TAG sub-lineage, SER21 also had a regional distribution, being found only in the two provinces of the Centre-South, whilst SER18 was unique, because it was widespread not only in the province of Taza but also in the Centre-South region.

The regional distribution of the haplotypes is analysed in contingency tables (Table VI). Table VIa shows that the haplotypes of the CS lineage were over-represented in the provinces of Essaouira and Azilal but under-represented in the other provinces, whereas the haplotypes of the NW lineage were over-represented in the provinces of Khemisset and Azilal, and haplotypes of the TZ CRA amalgamated lineage were over-represented in the province of Taza. Only the haplotypes of the TZ TAG sub-lineage had observed frequencies in each province approaching the expected. The proportion of haplotypes in each lineage was significantly different for the province of Taza compared with the other provinces (Table VIb; Chi-squared = 54.7, 3 degrees of freedom, $P < 0.001$).

DISCUSSION

TAXONOMY AND SPECIATION OF *P. SERGENTI*

In the current study, all specimens were morphologically closer to *P. sergenti* than to *P. similis*, although there were some significant variations from the morphometric and meristic values reported

by Depaquit *et al.* (1998). However, these authors did note that there is an important overlap in many characters for these two species. The Moroccan specimens were identified as *P. sergenti*, based on the mean length and width of the basal lobe of the male coxite and the absence of lateral pharyngeal teeth in the female (Depaquit *et al.* 1998). The distinctive values for some characters of Moroccan specimens could be an artefact arising from pooling more than one cryptic species.

A phylogenetic analysis of nuclear ribosomal DNA (Depaquit *et al.*, 2002) had confirmed the monophyly of *P. sergenti* and that *P. similis* is not its sister species. The focus of the current report was on geographical variation within *P. sergenti*. Cytoplasmic mtDNA is usually maternally inherited and does not recombine, making it a good source of markers not only for identifying sympatric species and geographically isolated races but also for tracing their dispersal (Avice, 1994; Esseghir *et al.*, 2000). Three primary mtDNA lineages of *P. sergenti* were found in Morocco, and they showed a markedly regional distribution. The genetic distances were as great as those between members of other *Phlebotomus* species complexes (Esseghir *et al.*, 1997, 2000), and this could indicate that the Moroccan lineages represent incipient species. Assuming individual haplotypes are selectively neutral, then the greater mtDNA lineage diversity in the centrally-located Azilal suggests that this province is either the origin of dispersal of the mtDNA lineages or the region of their secondary contact after allopatric divergence. This can be resolved only by more comprehensive sampling and analysis.

EPIDEMIOLOGICAL CONCLUSIONS

The large geographical range of *P. sergenti* extends beyond that of CL and this led Depaquit *et al.* (1998) to suggest the presence of a species complex. Members of species complexes can exhibit polymorphism in their host preferences and resting sites (Ready *et al.*, 1998). This can affect their vectorial roles and must be considered for control strategies.

Four conclusions of epidemiological significance can be drawn from the present findings. First, there was no evidence that mtDNA haplotypes and lineages are markers for cryptic species or genetic lineages of *P. sergenti* that have different susceptibilities to *L. tropica* strains or strain-specific "encounter compatibilities" (Combes, 2001). In other words (Ready, 2000), there is no suggestion that physiological co-adaptations or ecological adaptations, respectively, are driving co-evolution or co-association of vectors and parasites. One testable hypothesis is that the haplotype SER18 could be a marker for the vector of the widespread *L. tropica* strain (MON102) that most frequently infects humans in Morocco. Only haplotype SER18 and its sub-lineage (TZ TAG) were found in all three Moroccan foci of CL, but the following evidence suggests that neither is a marker for a genetic lineage of *P. sergenti* uniquely associated with the transmission to humans of MON102: all common haplotypes of *P. sergenti*, not just SER18, were abundant in the same environments in different provinces (altitudes 200-700m a.s.l.; semi-arid/sub-humid bioclimate zones; and man-made peridomestic habitats); SER18 was not more abundant in urban locations of Taza than in suburban locations unlike CL; and, all lineages showed endophily, which is typical of this species in many parts of its range, including Syria and Afghanistan (Desjeux, 2001).

The second conclusion concerns co-speciation between sandfly vector and parasite, in the strict sense of co-cladogenesis (Ready, 2000). The available results do not permit us to test whether or not incipient speciation of the vectors has been driving that of the parasites. It is true that both *P. sergenti* and *L. tropica* have shown greater diversity in the Centre-South region than in the province of Taza, but this could be a false co-association because parasites have been isolated from sandflies only in the Centre-South. There, diverse strains of *L. tropica* were isolated from the vectors, rather than from human cases, and most isolates from *P. sergenti* were not of the strain (zymodeme MON102) predominating in humans (Guilvard *et al.*, 1991; Pratlong *et al.*, 1991). Therefore, the diversity of the parasites in sandflies is not obviously driving the diversity of parasites infecting humans.

Almost all the Moroccan mtDNA haplotypes and lineages showed marked regionality, and so the third conclusion of epidemiological significance is that much

dispersal of *P. sergenti* is natural, not assisted by human activities. When this is so, vector control (e.g. by using insecticides to reduce biting rates) should not be followed by rapid, long-distance reinvasion. Only haplotype SER18 could be a marker for long-distance dispersal that might be human-assisted.

The fourth epidemiological conclusion does not relate to mtDNA markers. The relative abundances of males and females of *P. sergenti* varied with the trapping method and the location of the collections, with males predominating among the flies aspirated from the inside walls of houses. However, regardless of the trapping method (aspirators or light traps), many of the females had taken a bloodmeal in the previous 48 hours but had not fully matured their eggs. These observations are consistent with males resting indoors so that they could mate with host-seeking or engorged females, which later went outdoors to oviposit. If this is correct, then the spraying of residual insecticides in houses could be an effective method of intervention.

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