Phlebotomus sergenti in a Cutaneous Leishmaniasis Focus in Azilal Province (High Atlas, Morocco): Molecular Detection and Genotyping of *Leishmania tropica*, and Feeding Behavior

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Abstract

Background

Phlebotomus (*Paraphlebotomus*) *sergenti* is at least one of the confirmed vectors for the transmission of cutaneous leishmaniasis caused by *Leishmania tropica* and distributed widely in Morocco. This form of leishmaniasis is considered largely as anthroponotic, although dogs were found infected with *Leishmania tropica*, suggestive of zoonosis in some rural areas.

Methodology and Findings

This survey aimed at (i) studying the presence of *Leishmania* in field caught *Phlebotomus sergenti*, (ii) investigating genetic diversity within *Leishmania tropica* and (iii) identifying the host-blood feeding preferences of *Phlebotomus sergenti*. A total of 4,407 sand flies were collected in three rural areas of Azilal province, using CDC miniature light traps. Samples collected were found to consist of 13 species: *Phlebotomus* spp. and 3 *Sergentomyia* spp. The most abundant species was *Phlebotomus sergenti*, accounting for 45.75 % of the total. 965 female *Phlebotomus sergenti* were screened for the presence of *Leishmania* by ITS1-PCR-RFLP, giving a positive rate of 5.7% (55/965), all being identified as *Leishmania tropica*. Nucleotide heterogeneity of PCR-amplified ITS1-5.8S rRNA gene-ITS2 was noted. Analyses of 31 sequences obtained segregated them into 16 haplotypes, of which 7 contain superimposed peaks at certain nucleotide positions, suggestive of heterozygosity. *Phlebotomus*

sergenti collected were found to feed on a large variety of vertebrate hosts, as determined by *Cytochrome b* sequencing of the DNA from the blood meals of 64 engorged females.

Conclusion

Our findings supported the notion that *Phlebotomus sergenti* is the primary vector of *Leishmania tropica* in this focus, and that the latter is genetically very heterogeneous. Furthermore, our results might be suggestive of a certain level of heterozygosity in *Leishmania tropica* population. This finding, as well as the feeding of the vectors on different animals are of interest for further investigation.

Author Summary

In Morocco three *Leishmania* species have been reported to cause cutaneous leishmaniasis: *Leishmania major*, *Leishmania tropica* and less frequently *Leishmania infantum*. Amongst these clinically important *Leishmania* species, *Leishmania tropica* is considered as a public health problem by the Ministry of Health in Morocco and other endemic countries. *Phlebotomus sergenti* is the known vector, which is thought to take blood meals mainly from humans, since they appear to be the sole reservoir, considering anthroponosis of the cutaneous leishmaniasis caused by *L. tropica* in many endemic areas. In the present study, we investigated by molecular tools the presence of *Leishmania* in field caught *Phlebotomus*, as well as the heterogeneity of *Leishmania tropica* in a cutaneous leishmaniasis focus in High Atlas of Morocco. Our results showed a high infection rate of *Phlebotomus sergenti*, which may be a consequence of high level of the parasite circulating in this region; and underlined the important genetic heterogeneity of *Leishmania tropica* in Morocco. Analysis of the blood meals of the vectors showed that *Phlebotomus sergenti* fed on a variety of vertebrates, including wild animals, such as rodent, monkey and bat. Whether these animals play any role in the maintenance of *Leishmania tropica* in this focus awaits further investigation.

Introduction

Leishmaniases are complex diseases of worldwide distribution caused by >20 *Leishmania* species, which are parasitic protozoa transmitted by the bite of infected female sand flies. The disease affects 98 Mediterranean and other endemic countries putting a population of 350 million people at risk of infection and causing 1.3 million new cases and 20,000 to 30,000 deaths annually [1, 2].

In Morocco, leishmaniasis is a public health problem, with 8,862 cases notified in 2010 alone. It is widely distributed from the mountains of the Riff to the peri-arid foothills of the Anti-Atlas [3, 4]. Three *Leishmania* species are responsible for cutaneous leishmaniasis (CL) in Morocco: *L. major*, *L. tropica* and *L. infantum*. CL due to *L. tropica* is endemic in the semi-arid regions and the sand fly species *Phlebotomus* (*Paraphlebotomus*) *sergenti* Parrot is considered to be the vector [5, 6]. This form of CL was described for the first time in Tanant, a rural locality in Azilal province in High Atlas [7]. Thereafter, a large rural focus has been further identified in the centre and the south of Morocco [6]. In the mid 1990s, CL caused by *L. tropica* was then reported in Taza province, in northern Morocco [8] and early in 2000, outbreaks occurred in emerging foci in the central and northern parts of the country, and *L. tropica* was also even

found in areas previously only known as *L. major* foci [9, 10]. CL due to *L. tropica* is basically anthroponotic, although the parasite was also isolated from dogs in some leishmaniasis foci in Morocco, suggesting that *L. tropica* could also be zoonotic at least in some areas [5, 11, 12].

Blood meal analysis of haematophagous insects has been informative in elucidating their natural host-feeding preference or host-feeding pattern for identifying potential reservoirs [13]. For sand flies, the blood meal sources have been identified initially by serological techniques, like ELISA [14, 15], agarose gel diffusion [16], counter immunoelectrophoresis [17], precipitin test [18, 19] and a more laborious histological technique [20]. Although all these methods have been useful in identifying vertebrate hosts for many haematophagous insects, they lack sensitivity and are time-consuming.

In the last decade, several PCR-based molecular approaches have been developed to enhance the specificity of insect blood meal identification [21, 22]. Analysis of blood meal sources of the engorged vectors has been greatly facilitated by the increasing availability of animal genomic database at the genus and species levels (NCBI, EBI. . .). Downstream applications of PCR based on primers designed from multiple alignments of the mitochondrial *Cytochrome b* gene of different vertebrate species have identified avian and mammalian blood sources in various species of sand flies. The sequencing of *Cyt b* gene, as well as the *Cyt b*-PCR-RFLP and the *Cyt b*-PCR combined with reverse line blot have been used to identify the blood meals sources of sand flies [23–25].

The vector incrimination is classically based on the dissection of freshly caught individual sand flies and subsequent culture of parasites present in the gut [26]. This method needs dissecting expertise and is time consuming, since the *Leishmania* infection rate in sand flies is usually very low [27]. In recent years, several molecular techniques have been developed to identify *Leishmania* infection in infected phlebotomine sand flies. Because of excellent sensitivity and specificity, these methods have the potential to challenge the time-consuming isoenzyme technique for timely characterization of *Leishmania* strains. They also play a prominent role in epidemiological prospective surveillance activities which are critical for planning targeted control measures of leishmaniasis [15, 28, 29].

In the present study, we used molecular tools (ITS1-PCR-RFLP) to detect and identify *Leishmania* species within naturally infected sand flies collected from three rural localities of Azilal province in Morocco. We also applied nested-PCR of *Leishmania* ITS-rDNA genes for sequencing analyses to characterize *L. tropica*. The blood meals of engorged flies were also analyzed to determine the putative animal reservoirs of *L. tropica*, using a PCR technique based on the C*yt b* gene of vertebrate mitochondrial DNA (mtDNA).

Materials and Methods

Study sites

The sand flies were collected in three neighboring rural areas in the province of Azilal, High Atlas of Morocco. The three regions are: Ait Makhlouf II: 31°01'23"N, 6°58'53"O and Guimi: 32°00'12"N, 6°55'03"O, which are located in Beni Hassan sector, and Agmeroul: 31°58'38"N, 6°51'12"O in Tabia sector. These localities are at different altitude from 679 m to 840 m, separated from one another at a distance of 5 to 11 km (Fig. 1). Vegetation is sparse and mainly dominated by cactus, jujube plant, olive, and almond trees. Agriculture remains the primary source of income and is mainly based on the production of wheat, almonds and olives. Livestock include chicken, sheep, cattle and horses and most homes have at least one dog. Houses are mainly of the traditional type, constructed with adobe. The villages are surrounded by mountains, with some groundwater sources used for irrigation.
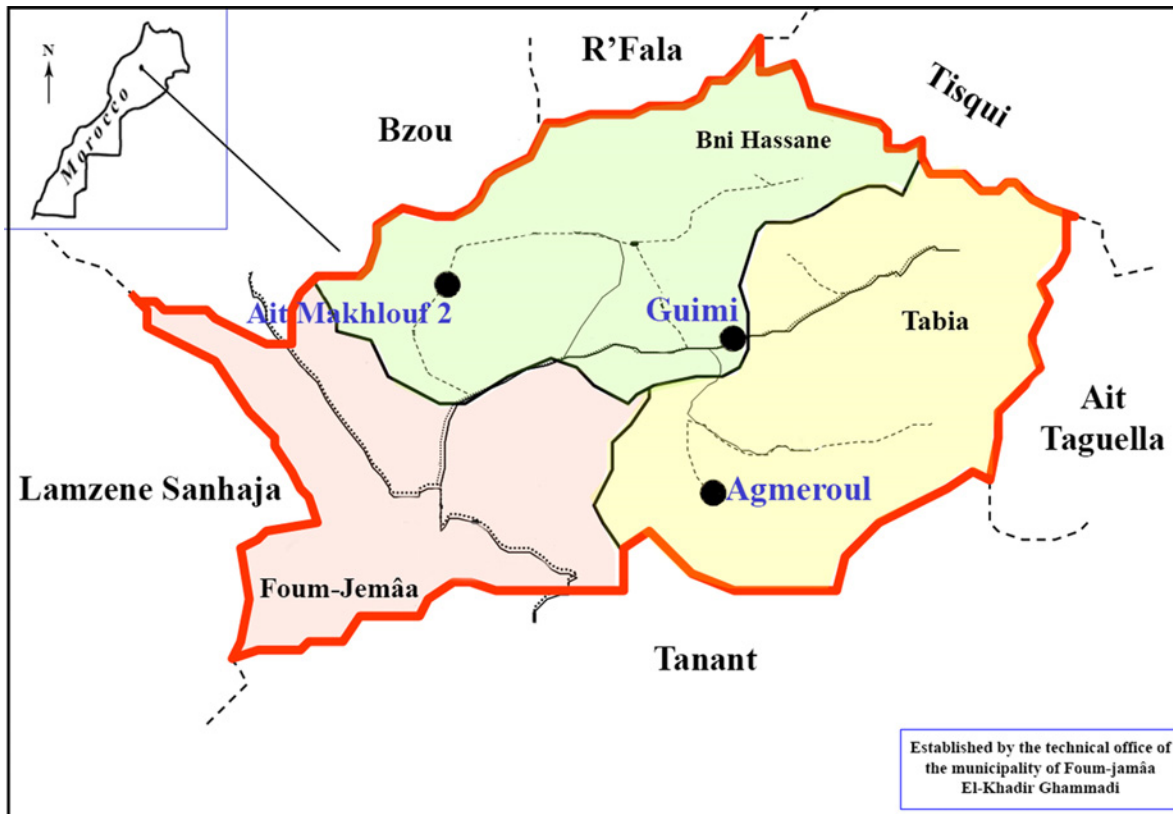
Fig 1. Location of the three sampling areas (Ait Makhlouf 2, Agmeroul and Guimi) in Azilal province.

Collection, dissection and identification of sand flies

Phlebotomine sand flies were caught during three consecutive nights from June to October 2011, using CDC light traps placed inside the houses and in domestic animal shelters, in the three rural sites. The traps placed about 1.5 meter above the ground were set before sunset and collected the next morning. The collected sand flies were then placed in 1.5 mL Eppendorf tubes, transferred to the lab in dry ice and kept at -80°C. All the sand flies were washed and dissected. The head and genitalia were used for morphological identification using Moroccan morphologic key [4]. To distinguish *P. perniciosus* and *P. longicuspis* a very closely related species, we used the morphological criteria updated by Benabdennbi *et al.* (1999) [30] and Guernaoui *et al.* (2005) [31] based on the morphological features of genitalia and the number of coxite hairs. *P. perniciosus* is characterized by 2 aedeagi forms: (i) copulatory valve with apex bifid; and (ii) copulatory valve with curved apex, the number of coxite hairs being up to 14. *P. longicuspis* has copulatory valve ending with a single and long point, and 19 or more coxite hairs.

The remainder of the body of engorged and unengorged females after dissection was stored sterilely in 1.5 mL microtube at -20°C until use.

DNA extraction

Genomic DNAs were extracted from unengorged and engorged female *P. sergenti* sand fly by using the Kit PureLink™ Genomic DNA Mini Kit and the QIAamp® DNA Blood Mini Kit, respectively, according to the manufacturer's instruction.

Molecular detection and identification of *Leishmania* species in *P. sergenti* females

For *P. sergenti* females, the leishmanial ribosomal internal transcribed spacer 1 (ITS1) region was amplified, using the primers LITSR (5′-CTGGATCATTTTCCGATG-3′) and L5.8S (5′-TGATACCACTTATCGCACTT-3′), following the protocol described by Schonian *et al.* (2003). This PCR was used to amplify a 300 to 350 bp fragment; then the ITS1-PCR products were digested with restriction endonuclease *Hae*III enzyme for *Leishmania* species identification [32]. The restriction profiles were analyzed by electrophoresis on 3% agarose gel containing ethidium bromide, and compared with *Leishmania* reference strains: *L. major* (MHOM/SU/73/5ASKH), *L. tropica* (MHOM/SU/74/K27) and *L. infantum* (MHOM/TN/80/IPT1). A 100 bp DNA size marker was used. Negative controls (containing water, without DNA) were added to each PCR run.

Further identification of *Leishmania* parasites was done using nested PCR amplification of ITS1-5.8S rRNA gene-ITS2. The primers IR1 and IR2 were used for the first PCR stage and ITS1F and ITS2R4 for the second stage, as previously described by Parvizi and Ready [33]. Negative controls were included to each PCR run. All PCR products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide. We used the standard DNA fragment 100 bp ladder as a size marker. The nested-PCR products (~460 bp including primers) were sequenced, using the primers ITS1F and ITS2R4. The sequences obtained were edited using Seq Scape, BioEdit softwares and aligned using ClustalW in MEGA6 software.

A phylogenetic tree was constructed by using the Maximum Likelihood method based on the Tamura-Nei model. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. Bootstrap replicates were performed to estimate the node reliability, and values were obtained from 1,000 randomly selected samples of the aligned sequence data. Sequences were compared with entries retrieved from GenBank. Evolutionary analyses were conducted in MEGA6.

Molecular identification of *P. sergenti* females

Morphologically identified *P. sergenti* females were confirmed by sequence analyses of PCR-amplified mitochondrial DNA fragment encompassing *Cyt b* region using PhleF (5'-AAT AAA TTA GGA GTA ATT GC-3') and PhleR (5'-GCC TCG AWT TCG WTT ATG ATA AAT T-3') primers [34]. A 500 bp fragment was PCR-amplified using the following reagents: 1X PCR buffer, 2.5 mM MgCl$_2$, 0.4 mM primers (F and R), 0.2 mM dNTPs, 4 μL DNA, made up with distilled water to a final volume of 25 μL for each reaction. The PCR runs were each initiated as follows: 94°C for 12 min, followed by 5 cycles of 94°C for 30 s; 52°C for 30 s, 72°C for 1 min. The PCR reaction was then continued for 30 cycles under the same conditions, except for the annealing step (54°C for 1 min) and a final extension at 72°C for 10 min. The PCR products were sequenced, employing the same primers used for the PCR. Sequences were processed and aligned, using the multiple alignment programs Seq Scape and BioEdit.

A phylogenetic tree was constructed by using the Neighbor-Joining method, in agreement with Kimura 2-parameter model, at uniform rate for transitions and transversions. Bootstrap replicates were performed to estimate the node reliability, and values were obtained from 1,000 randomly selected samples of the aligned sequence data. Sequences were compared with entries retrieved from GenBank. The phylogenetic tree was constructed by using the MEGA6 software.

Blood meal identification

A region of *Cyt b* gene from host mtDNA was amplified for blood meal source identification of the blood-fed female specimens. The assay is based on specific amplification and sequencing of the blood meal–derived the region of the host mtDNA *Cyt b* gene. Modified vertebrate-universal specific primers L14841 (5'-CCATCCAACATCTCAGCATGATGAAA-3') and H15149 (5'-CCCCTCAGAATGATATTTGTCCTCA-3') were used to amplify a 359 bp segment of the *Cyt b* gene [35, 36].

PCR reaction mixture contained 1x PCR Rxn Buffer, 3 mM MgCl$_2$, 300 μM deoxynucleotide triphosphates, 0.4 μM of each primer, 1 unit of *Taq* DNA polymerase, 3 μL of DNA template solution and distilled water in a final volume of 25 μL. Samples were incubated at 95°C for 5 min; followed by 36 cycles each at 95°C for 30 s, 60°C for 50 s, and 72°C for 40 s; and a final extension at 72°C for 5 min. A negative control was included for each run. Amplified products were analyzed by electrophoresis on 1% agarose gels stained with 2 mg/mL ethidium bromide and visualized under ultraviolet (UV) light. The amplified products were sequenced on both strands, using a Big Dye Terminator v3.1. Sequences were edited and aligned using Seq-Scape and BioEdit softwares.

The sequences obtained were aligned with those deposited in the GenBank database through the BLAST program for the identification of blood meal vertebrate sources. Sequences of a given pairwise alignment with the lowest E-value were selected as the most likely species of blood meal vertebrate source.

Biological diversity analysis

In order to characterize the species diversity of sand fly populations, several parameters were used: (i) species richness (S) corresponding to the number of species present in the studied habitat; (ii) relative abundance (p_i) reflecting the proportion of individuals belonging to the species *i*.

These parameters were used to calculate different ecological indexes [37, 38].

<u>Simpson's diversity index (D)</u> that quantifies the biodiversity of a habitat. It takes into account the number of species present as well as the abundance of each species. $D = \frac{1}{\sum_{i=1}^{s} p_i^2}$

<u>Shannon index (H')</u> that quantifies species diversity: $H' = -\sum_{i=1}^{s} p_i ln p_i$

<u>Pielou index (J')</u>: to calculate the equitability of the distribution of species.
$J' = H'/ln(S)$ where *H'* is the value of Shannon index and *S* is the species richness.

Results

Phlebotomine sand fly fauna

A total of 4,407 sand flies (2,007 females and 2,400 males) were collected in the three rural areas of Azilal province. Morphological analysis identified 10 *Phlebotomus* species of three subgenera as follows: *P. sergenti*, *P. kazeruni* and *P. chabaudi* of the subgenus *Paraphlebotomus*; *P. perniciosus*, *P. longicuspis*, *P. perfiliewi*, *P. ariasi*, *P. langeroni* and *P. chadlii* of the subgenus *Larroussius* and *P. papatasi* of the subgenus *Phlebotomus*. Three *Sergentomyia* species were also identified: *Sergentomyia minuta*, *S. antennata* and *S. fallax*.

The predominant species was *P. sergenti;* with a relative abundance of 47.32% of the sand flies belonging to the genus *Phlebotomus* and 45.75% of the total sample.

P. perniciosus and *P. longicuspis* are the next most abundant species, representing 26.82% and 14.23% of the sample respectively (Table 1). July had the highest sand fly abundance, whereas the greatest specific richness (S) was found in September, with the occurrence of

Table 1. Species diversity, abundance and relative frequency.

Species	June Abundance F	June Abundance M	June Frequency (%)	July Abundance F	July Abundance M	July Frequency (%)	September Abundance F	September Abundance M	September Frequency (%)	October Abundance F	October Abundance M	October Frequency (%)	Total Abundance F	Total Abundance M	Total Frequency (%)
P. sergenti	338	314	40.1	404	572	52.7	139	112	35.01	84	53	64.62	965	1051	45.75
P. perniciosus	162	408	35.06	94	309	21.76	99	76	24.41	19	15	16.04	374	808	26.82
P. longicuspis	169	63	14.27	185	103	15.55	53	30	11.58	14	10	11.32	421	206	14.23
P. papatasi	57	68	7.69	31	28	3.19	46	44	12.55	7	6	6.13	141	146	6.51
P. perfiliewi	5	-	0.31	-	-	-	-	-	-	-	-	-	5	-	0.11
P. ariasi	2	-	0.12	-	-	-	1	-	0.14	-	-	-	3	-	0.07
P. chabaudi	1	-	0.06	1	-	0.05	1	-	0.14	-	-	-	3	-	0.07
P. kazeruni	-	-	-	-	1	0.05	-	-	-	-	-	-	-	1	0.02
P. langeroni	-	-	-	-	-	-	-	2	0.28	-	-	-	-	2	0.05
P. chadii	-	-	-	-	-	-	-	1	0.14	-	-	-	-	1	0.02
S. minuta	21	4	1.54	4	2	0.32	38	10	6.69	-	-	-	63	16	1.79
S. antennata	1	2	0.18	1	4	0.27	3	11	1.95	-	-	-	5	17	0.5
S. fallax	1	-	0.06	-	2	0.11	23	16	5.44	3	1	1.89	27	19	1.04
Unidentified	-	10	0.62	-	111	5.99	-	12	1.67	-	-	-	-	133	3.02
Total	**757**	**869**	**100**	**720**	**1132**	**100**	**403**	**314**	**100**	**127**	**85**	**100**	**2007**	**2400**	**100**

doi:10.1371/journal.pntd.0003687.t001

Table 2. Species diversity of Phlebotomine sand fly, Species richness (S), Shannon-Wiener diversity index (H) and equitability (J').

Month	NF	NM	N	S	D	H'	J'
June	757	869	1626	10	3.23	1.35	0.59
July	720	1132	1852	9	2.83	1.29	0.59
September	403	314	717	11	4.58	1.75	0.73
October	127	85	212	5	2.18	1.07	0.66

NF: Number of females. NM: Number of males. N: Number of specimens. S: Specific richness. D: Simpson diversity index. H': Shannon index. J': Pielou index.

doi:10.1371/journal.pntd.0003687.t002

eleven species against ten, nine and five species recorded in June, July and October, respectively. The three biodiversity indices were maximal in September (Table 2).

Leishmania-infected flies and parasite typing

***Leishmania* ITS1-PCR-RFLP.** A total of 965 *P. sergenti* females caught by light traps were screened for *Leishmania* infection. 687 unfed specimens collected in June and July were grouped into 35 pools, each one containing up to 20 sand flies. The remaining 278 flies were tested individually for the presence of *Leishmania* DNA using ITS1 PCR-RFLP method. *Leishmania* DNA was amplified from samples of 16 pooled *P. sergenti* and 39 individual fly specimens. Altogether, 55 samples were PCR-positive, giving an expected single band of approximately 330 bp (Table 3). Digestion of the amplicons with *Hae*III produced the RFLP-specific pattern of *L. tropica* for all the 55 positive samples. The infection rate estimated based on this PCR positivity is 5.69% overall, and 25% and 3.16% for fed and unfed female flies, respectively (Table 3).

Nested PCR of ITS1-5.8S rRNA gene-ITS2. The 55 *P. sergenti* samples positive for *Leishmania* by ITS1-PCR were also positive by the nested PCR for ITS1-5.8S rRNA gene-ITS2 providing a unique 460-bp band. Of 55 PCR products, a total of 31 were completely sequenced in both directions, and showed to correspond to *L. tropica*. Sequences alignment showed substantial heterogeneity, and phylogenetic studies segregated them into 16 *L. tropica* haplotypes (GenBank accession numbers KM454141-KM454156). The most common haplotype (KM454142) included 14 sequences, presenting 99% of identity with the Indian strain of *L. tropica* (FJ948458).

Another 16 sequences were grouped into 15 haplotypes: KM454141 and KM454143 to KM454156 highly similar (97% to 99% identity) to *L. tropica* strains from India (FJ948456, FJ948455, FJ948465, FJ948458), they differed from the common haplotype by 1 to 18 nucleotides.

Table 3. *Leishmania tropica* infection within fed and unfed *Phlebotomus sergenti* females.

Month	Fed specimens		Unfed specimens		Rate of *L. tropica* infection
	Individual	*L. tropica* infection	Pool* / Individual	*L. tropica* infection	
June	9	3	17*	7	10/338 (2.95%)
July	46	10	18*	9	19/404 (4.70%)
September	42	10	97	5	15/139 (10.79%)
October	15	5	69	6	11/84 (13.09%)
Total	112	28 (25%)	853	27 (3.16%)	55/965 (5.69%)

(* = pool)

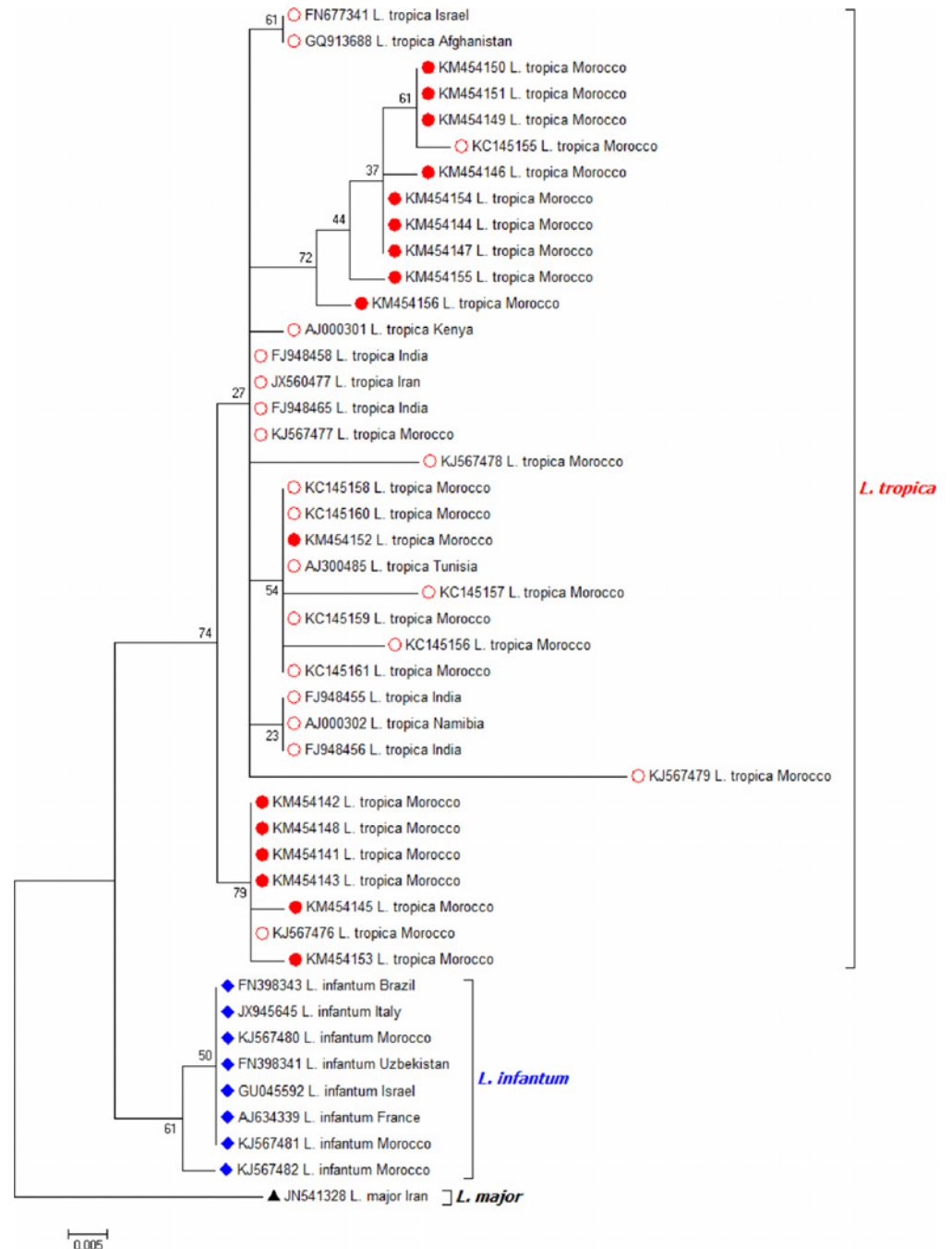doi:10.1371/journal.pntd.0003687.t003

Fig 2. Molecular phylogenetic analysis by Maximum Likelihood method of ITS rDNA sequences of different strains of *Leishmania*. (Red discs: *L. tropica* sequences).

One of the 15 haplotypes is highly similar to that identified previously in CL patients (KC145159) from an emerging focus of CL in central Morocco (Fig. 2).

In addition, the analysis of our sequences showed seven sequences with two superimposed peaks, which differ in position from one sequence to another (Fig. 3).
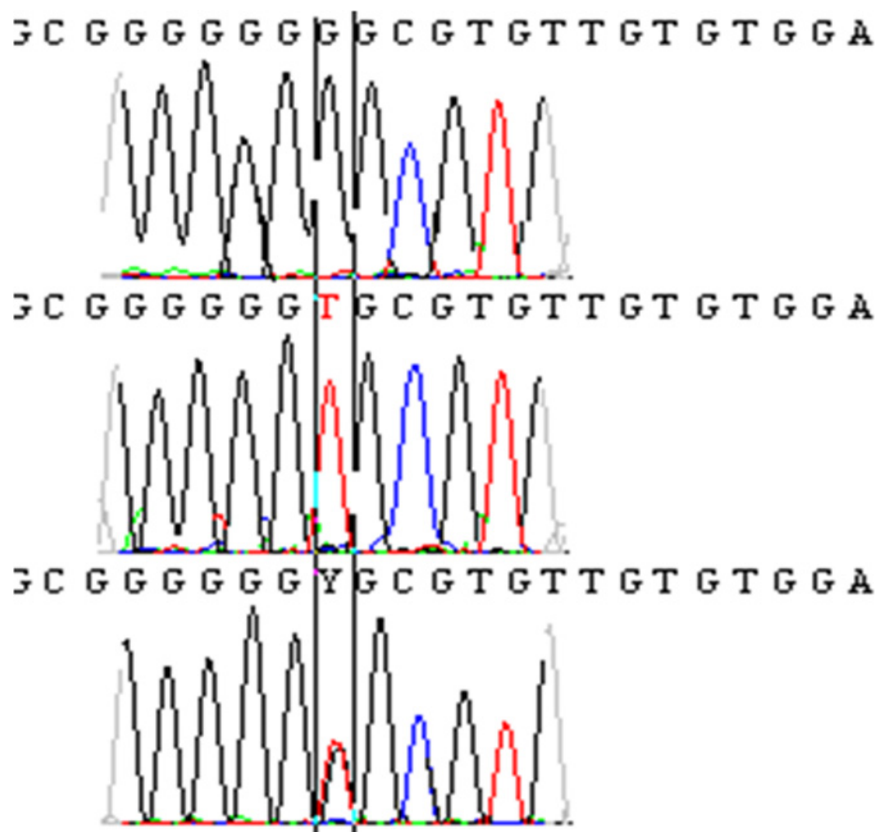
Fig 3. ITS-5.8 rDNA genes sequences with two different bases T and C, and one sequence with two superimposed peaks at the same nucleotide position.

doi:10.1371/journal.pntd.0003687.g003

All the other sand fly species identified were pooled mono-specifically and screened for *Leishmania* infection by PCR. None of these samples was PCR-positive, suggesting that *P. sergenti* is the only vector in this area.

Molecular identification of *P. sergenti* females

All the 39 *L. tropica* infected female *P. sergenti* that were identified morphologically, were validated by molecular characteristics based on the sequencing of *Cyt b* fragment. The alignment of the *Cyt b* sequences obtained confirmed that all samples corresponded to *P. sergenti*. All sequences were similar to *P. sergenti Cyt b* sequences: DQ840350, JN036763, DQ840392 and JN036764. Phylogenetic analyses grouped them in a distinct clade with other *P. sergenti* retrieved from GenBank (Fig. 4).

Sand fly blood meal identification

One hundred and twelve full or partially blood-fed females (11.6% of the total *P. sergenti* females collected) were tested for their blood feeding preferences. The *Cyt b* gene sequencing revealed that 64 *P. sergenti* fed on a variety of vertebrate hosts, including humans, chickens (*Gallus gallus*), rodents (*Myomyscus brockmani*, *Mesocritus brandti*, *Dipodomys ordii*: 83–91% of identity), birds, cattle (*Bos Taurus*), rabbit (*Oryctolagus cuniculus*), bat (*Molossus molossus* 89% of identity) and monkey (*Macaca nigra* 95% of identity) (Table 4).
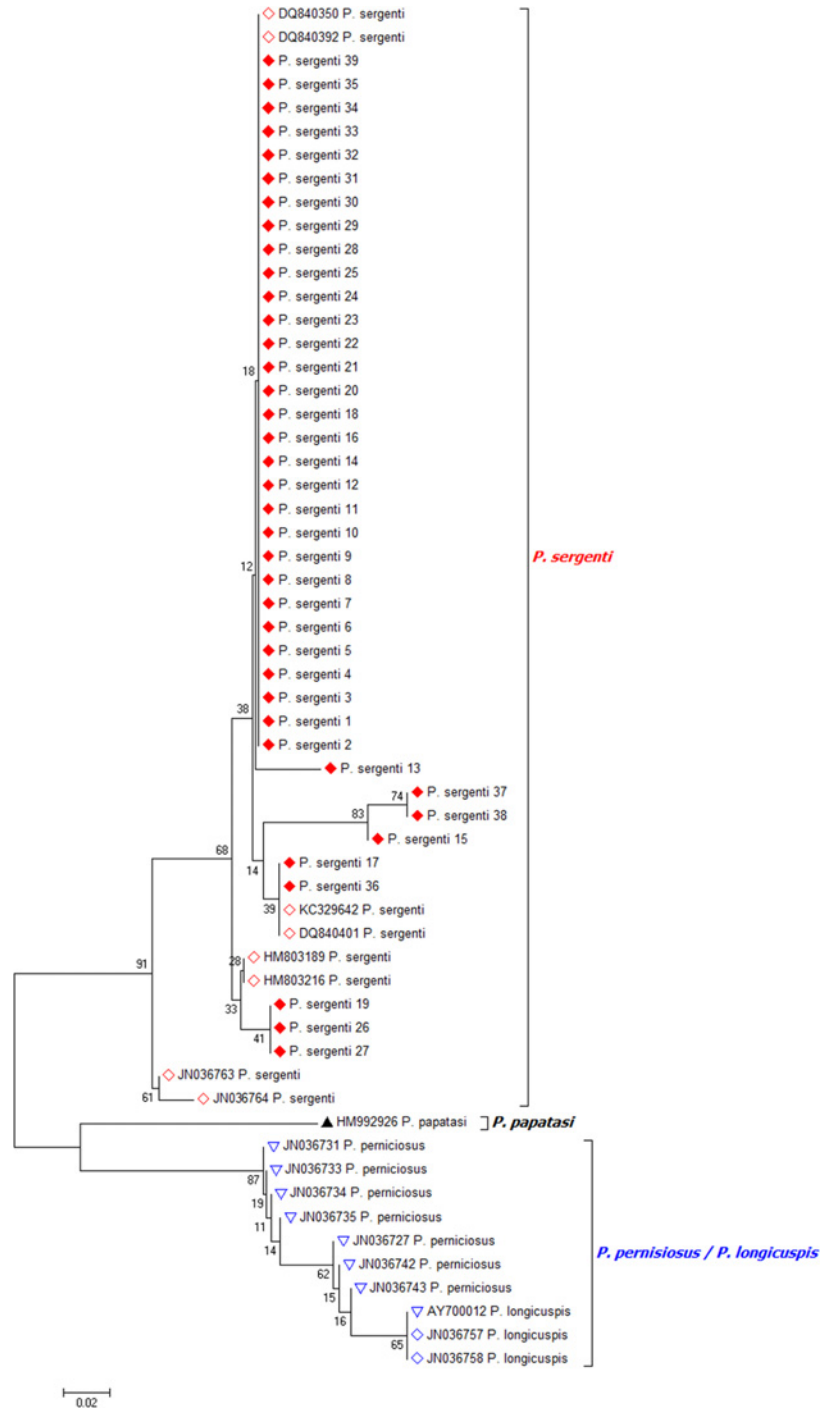
Fig 4. Phylogenetic analysis of the mitochondrial *Cytochrome b* sequences of *P. sergenti* and their homologous in GenBank *P. sergenti*, *P. perniciosus*, *P. longicuspis* and *P. papatasi*. (Red lozenges: *L. tropica* infected *P. sergenti* females).

doi:10.1371/journal.pntd.0003687.g004

Table 4. The blood meal origins of *Phlebotomus sergenti* females identified by *Cyt b* analysis.

	Blood meals source								
	Human	**Chicken**	**Rodent**	**Bird**	**Cattle**	**Rabbit**	**Monkey**	**Bat**	**Total**
Number of *P. sergenti*	41	8	7	4	1	1	1	1	**64**
Infected *P. sergenti*	10	3	1	1	-	-	-	-	**15**

Note: Nucleotide sequence data reported in this paper are available in the GenBank databases under the accession numbers: KM454141 to KM454156

doi:10.1371/journal.pntd.0003687.t004

Discussion

CL due to *L. tropica* occurs in many endemic areas, especially North Africa, Middle East, Central and South Asia. *L. tropica* has not been isolated from *P. sergenti* frequently [39, 40] as reported here. The transmission cycles of *L. tropica* vary with different geographic locations and generally have not been known to require a sylvatic reservoir. One noticeable exception is the atypical focus in Northern Israel, where *L. tropica* is transmitted by *P. (Adlerius) arabicus* with hyraxes as the reservoir, adjacent to a classical anthroponotic focus [41].

A high biodiversity of sand flies was observed in the region under study, representing 57% (13/23) of all *Phlebotomus* species hitherto described in Morocco. We have demonstrated that *P. sergenti* was the predominant vector species (45.75%) present abundantly throughout the collection period.

In agreement with previous reports, our findings showed that the rate of *L. tropica* infection is higher in fed compared with unfed females, as the latter include largely newly emerged adults, which are expected to contain no *L. tropica* before taking blood meals [42]. Our overall rate of infection with *L. tropica* is as high as 5.69%, increasing from 2.95% in June to 10.79–13.09% in September-October. These data are consistent with the previous report in the period of high transmission during fall season [43]. On the other hand, the rate of infection found in this area was much higher than the 1.44–3.66% found in other emerging foci in Morocco [44, 45].

The prevalence of infection seen in the study area may result from the increase in the circulation of *L. tropica* to a high level. Indeed, the number of CL cases has increased steadily, reaching a hundred per year since the first outbreak of CL in 2006 [46]. Our results confirm that *P. sergenti* is most likely the primary and the only vector of *L. tropica* in this region, not only due to the fact that it was the only species found infected with *L. tropica* but also because of its high abundance and infection rate. In Morocco, *L. tropica* was first detected in *P. sergenti* sand flies over 30 years ago [5] and more recently in emerging CL foci in the Centre and the North of Morocco [45, 47].

Species of the sub-genus *Larroussius* constitute more than 41% of sand flies collected in the studied area with *P. perniciosus* being the most abundant. Members of sub-genus *Larroussius* are known to be the vectors of *L. infantum*. However, PCR screening revealed no *Leishmania* infection in species belonging to this sand fly sub-genus.

Molecular methods are useful for the detection and characterization of parasites directly in field-collected samples without culturing. Previous evidence indicates that cultivation selects subpopulations of the parasites in the biological samples, including the emergence of nonpathogenic trypanosomatids [48]. In addition, direct analysis of *Leishmania* DNA in field collected samples can produce unexpected findings, including co-infections by more than one *Leishmania* species in a single subject [49]. In the present study, PCR-based amplification of the ITS1-5.8S rRNA gene-ITS2 proved to be highly sensitive for identifying *Leishmania* in sand flies to the species and strain levels [28, 47]. Analysis of ITS1-5.8S rRNA gene-ITS2 sequences from 31 *P. sergenti* specimens makes it possible to show great heterogeneity of *L. tropica*, segregating

them into 16 haplotypes and revealing their phylogenetic relatedness to Indian strains (FJ948458, FJ948456, FJ948465 and FJ948455) and one Moroccan strain isolated from CL patient (KC145159).

L. tropica is a very heterogeneous species and a high degree of intraspecific polymorphism has been described based on isoenzyme analysis and other molecular methods [50–52]. Morocco is known for being the country with the highest number of *L. tropica* zymodemes described so far: 8 zymodemes were characterized from human, dogs and the sand flies, e.g. *P. sergenti* [5, 11]. On the other hand, *L. tropica* strains from *P. sergenti* showed the largest range of zymodemes compared to isolates from the vertebrate hosts [12]. Our results underscore the high polymorphism of *L. tropica* detected in *P. sergenti*.

Furthermore, sequence analysis revealed seven sequences with two superimposed peaks at a single nucleotide position. There are at least three possible interpretations for this: (i) sequence heterogeneity of different copies in ITS1-5.8S rRNA gene-ITS2 organized as tandem repeats in the *Leishmania* genome; (ii) sequence heterogeneity of the DNAs in different individual cells, since our samples were amplified from a cell population, which can be heterogeneous in a field collected sample; (iii) these allelic base substitutions are suggestive of heterozygosity of *L. tropica*, indicating possible sexual reproduction in *L. tropica*. To confirm the last hypothesis, the two first possibilities must be ruled out by sequencing genes known to be single-copy per haploid genome and by amplifying single-copy genes from a single cell or a population grown up from a single cell. Hybrid marker profiles detected in field isolates have been considered as evidence for sexual recombination in *Leishmania* [53–55], even if the principal reproductive mechanism in *Leishmania* is asexual via clonal reproduction. Rogers *et al.* (2014) used whole genome sequencing to investigate genetic variation in *L. infantum* parasites isolated from naturally infected sand flies. They showed, for the first time, that variation in these parasites arose following a single cross between two diverse strains and subsequent recombination, despite mainly clonal reproduction in the parasite population. Their results are the most direct evidence of sexual recombination in a natural population of *Leishmania* [56].

Although cutaneous leishmaniasis caused by *L. tropica* is usually considered an anthroponotic infection [57], zoonotic transmission was reported in Jordan, the Palestinian Authority and Israel [58, 59]. In the present rural CL focus, zoonotic transmission may occur, as the human cases of CL are sporadic and the major vector species feeds not exclusively on humans. *P. sergenti* was found in high densities inside houses and animal shelters, which indicates a very close contact between this species and humans and domestic animals. The results of the blood meal analyses showed the important relationship between *P. sergenti* populations and humans, since 41 *P. sergenti* (64%) fed on humans. Chicken constituted the second highest source of blood taken by this vector (12.5%), however chickens are not susceptible to *Leishmania* infection, because of some physiological characteristics, including their body temperature of 41.0°C. Furthermore, infected sand flies may eliminate *Leishmania* parasite, when they take a second blood meal from chickens or birds [60, 61].

Among 7 *P. sergenti* females feeding on rodents, one specimen was found to be infected by *L. tropica*, which make rodents a suspected reservoir of this *Leishmania* species. Indeed, several rodent species are assumed to have a role in transmission of *L. tropica* [58, 59, 62, 63]. *P. sergenti* was found also to feed on wild animals such as monkeys and bats suggestive of their potential role as a reservoir for further investigation.

Conclusion

In the present study, we demonstrated a high infection rate of *L. tropica* in a single *Phlebotomus* species, that predominantly, but not exclusively, feeds on humans. This confirms the status of

P. sergenti as the primary vector of *L. tropica* in this focus. Our results highlighted the high diversity of *L. tropica* in Morocco. A zoonotic transmission of *L. tropica* in this region is further suggested from the blood meal analysis of the vector. The potential for an animal reservoir host for this species awaits further exploration.

Acknowledgments

Author Contributions

Conceived and designed the experiments: ALI HN ML. Performed the experiments: MA NES RNC. Analyzed the data: MA ML. Contributed reagents/materials/analysis tools: MA RNC ML. Wrote the paper: MA MR ML.

References

1. WHO. Leishmaniasis. http://wwwwhoint/mediacentre/factsheets/fs375/en/. 2014;Fact sheet N°375, Updated January 2014.

2. WHO. Control of the leishmaniases. Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases. Geneva (Switzerland): World Health Organ Tech Rep Ser. 2010;(949):xii-xiii, 1–186, back cover.

3. Moroccan Ministry of Health. État d'avancement des programmes de lutte contre les maladies parasitaires. Direction de l'épidémiologie et de lutte contre les maladies, Morocco. 1999.

4. Moroccan Ministry of Health. Lutte Contre les Leishmanioses, Guide des Activités. Direction de l'Epidémiologie et de Lutte Contre les Maladies, Rabat, Morocco. 2010.

5. Guilvard E, Rioux JA, Gallego M, Pratlong F, Mahjour J, Martinez-Ortega E, et al. *Leishmania tropica* au Maroc. III. Rôle de *Phlebotomus sergenti*. A propos de 89 isolats. Annales de Parasitologie Humaine et Comparée. 1991; 66:96–9.

6. Pratlong F, Rioux JA, Dereure J, Mahjour J, Gallego M, Guilvard E, et al. [*Leishmania tropica* in Morocco. IV—Intrafocal enzyme diversity]. Ann Parasitol Hum Comp. 1991; 66(3):100–4. PMID: 1776781

7. Marty P, Le Fichoux Y, Pratlong F, Rioux JA, Rostain G, Lacour JP. Cutaneous leishmaniasis due to *Leishmania tropica* in a young Moroccan child observed in Nice, France. Trans R Soc Trop Med Hyg. 1989; 83(4):510. PMID: 2617601

8. Guessous-Idrissi N, Chiheb S, Hamdani A, Riyad M, Bichichi M, Hamdani S, et al. Cutaneous leishmaniasis: an emerging epidemic focus of Leishmania tropica in north Morocco. Trans R Soc Trop Med Hyg. 1997; 91(6):660–3. PMID: 9509172

9. Moroccan Ministry of Health. État d'avancement des programmes de lutte contre les maladies parasitaires. Direction de l'épidémiologie et de lutte contre les maladies, Morocco. 2001.

10. Rhajaoui M, Nasereddin A, Fellah H, Azmi K, Amarir F, Al-Jawabreh A, et al. New clinico-epidemiologic profile of cutaneous leishmaniasis, Morocco. Emerg Infect Dis. 2007; 13(9):1358–60. doi: 10.3201/eid1309.060946 PMID: 18252108

11. Lemrani M, Nejjar R, Pratlong F. A new *Leishmania tropica* zymodeme—causative agent of canine visceral leishmaniasis in northern Morocco. Ann Trop Med Parasitol. 2002; 96(6):637–8. PMID: 12396327

12. Dereure J, Rioux JA, Gallego M, Perieres J, Pratlong F, Mahjour J, et al. *Leishmania tropica* in Morocco: infection in dogs. Trans R Soc Trop Med Hyg. 1991; 85(5):595. PMID: 1780983

13. Haouas N, Pesson B, Boudabous R, Dedet JP, Babba H, Ravel C. Development of a molecular tool for the identification of *Leishmania* reservoir hosts by blood meal analysis in the insect vectors. Am J Trop Med Hyg. 2007; 77(6):1054–9. PMID: 18165521

14. Gomez B, Sanchez E, Feliciangeli MD. Man-vector contact of phlebotomine sand flies (Diptera: Psychodidae) in north-central Venezuela as assessed by blood meal identification using dot-ELISA. J Am Mosq Control Assoc. 1998; 14(1):28–32. PMID: 9599320

15. Rossi E, Bongiorno G, Ciolli E, Di Muccio T, Scalone A, Gramiccia M, et al. Seasonal phenology, host-blood feeding preferences and natural *Leishmania* infection of *Phlebotomus perniciosus* (Diptera,

Psychodidae) in a high-endemic focus of canine leishmaniasis in Rome province, Italy. Acta Trop. 2008; 105(2):158–65. PMID: 18035329

16. Srinivasan R, Panicker KN. Identification of bloodmeals of phlebotomine sandflies using the agarose gel diffusion method. Southeast Asian J Trop Med Public Health. 1992; 23(3):486–8. PMID: 1488704

17. Morsy TA, Aboul Ela RG, Abdelmawla MM, el Gozamy BM. Counter immunoelectrophoresis, a modified technique for the identification of blood meals of sandflies collected from Qualyobia Governorate, Egypt. J Egypt Soc Parasitol. 1993; 23(1):109–32. PMID: 8482858

18. Tesh RB, Chaniotis BN, Aronson MD, Johnson KM. Natural host preferences of Panamanian phlebotomine sandflies as determined by precipitin test. Am J Trop Med Hyg. 1971; 20(1):150–6. PMID: 5567741

19. Afonso MM, Gomes AC, Meneses CR, Rangel EF. Studies on the feeding habits of Lutzomyia (N.) intermedia (Diptera, Psychodidae), vector of cutaneous leishmaniasis in Brazil. Cad Saude Publica. 2005; 21(6):1816–20. PMID: 16410867

20. Guzman H, Walters LL, Tesh RB. Histologic detection of multiple blood meals in *Phlebotomus duboscqi* (Diptera: Psychodidae). J Med Entomol. 1994; 31(6):890–7. PMID: 7815403

21. Meece JK, Reynolds CE, Stockwell PJ, Jenson TA, Christensen JE, Reed KD. Identification of mosquito bloodmeal source by terminal restriction fragment length polymorphism profile analysis of the cytochrome B gene. J Med Entomol. 2005; 42(4):657–67. PMID: 16119557

22. Kent RJ. Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies. Mol Ecol Resour. 2009; 9(1):4–18. doi: 10.1111/j.1755-0998.2008.02469.x PMID: 21564560

23. Oshaghi MA, Ravasan NM, Javadian EA, Mohebali M, Hajjaran H, Zare Z, et al. Vector incrimination of sand flies in the most important visceral leishmaniasis focus in Iran. Am J Trop Med Hyg. 2009; 81-(4):572–7. doi: 10.4269/ajtmh.2009.08-0469 PMID: 19815868

24. Abbasi I, Cunio R, Warburg A. Identification of blood meals imbibed by phlebotomine sand flies using cytochrome b PCR and reverse line blotting. Vector borne and zoonotic diseases. 2009; 9(1):79–86. doi: 10.1089/vbz.2008.0064 PMID: 18973440

25. Svobodova M, Alten B, Zidkova L, Dvorak V, Hlavackova J, Myskova J, et al. Cutaneous leishmaniasis caused by Leishmania infantum transmitted by Phlebotomus tobbi. International journal for parasitology. 2009; 39(2):251–6. doi: 10.1016/j.ijpara.2008.06.016 PMID: 18761342

26. Rioux JA, Lanotte G, Petter F, Dereure J, Akalay O, Pratlong F, et al. Les leishmanioses cutanées du bassin méditerranéen occidental: de l'identification enzymatique à l'analyse éco-épidémiologique. L'exemple de trois "foyers ", tunisien, marocain et français. *Leishmania* Taxonomie et phylogenèse Applications éco-épidémiologiques. (CollintCNRS/INSERM) IMEE, Montpellier. 1986; 1986:365–95.

27. Sharma U, Singh S. Insect vectors of *Leishmania*: distribution, physiology and their control. J Vector Borne Dis. 2008; 45(4):255–72. PMID: 19248652

28. Parvizi P, Mauricio I, Aransay AM, Miles MA, Ready PD. First detection of *Leishmania major* in peridomestic *Phlebotomus papatasi* from Isfahan province, Iran: comparison of nested PCR of nuclear ITS ribosomal DNA and semi-nested PCR of minicircle kinetoplast DNA. Acta Trop. 2005; 93(1):75–83. PMID: 15589800

29. Kato H, Uezato H, Katakura K, Calvopina M, Marco JD, Barroso PA, et al. Detection and identification of *Leishmania* species within naturally infected sand flies in the andean areas of ecuador by a polymerase chain reaction. Am J Trop Med Hyg. 2005; 72(1):87–93. PMID: 15728872

30. Benabdennbi I, Pesson B, Cadi-Soussi M, Morillas Marquez F. Morphological and isoenzymatic differentiation of sympatric populations of *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (Diptera: Psychodidae) in northern Morocco. J Med Entomol. 1999; 36(1):116–20. PMID: 10071503

31. Guernaoui S, Pesson B, Boumezzough A, Pichon G. Distribution of phlebotomine sandflies, of the subgenus *Larroussius*, in Morocco. Med Vet Entomol. 2005; 19(1):111–5. PMID: 15752186

32. Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, et al. PCR diagnosis and characterization of Leishmania in local and imported clinical samples. Diagnostic microbiology and infectious disease. 2003; 47(1):349–58. PMID: 12967749

33. Parvizi P, Ready PD. Nested PCRs and sequencing of nuclear ITS-rDNA fragments detect three *Leishmania* species of gerbils in sandflies from Iranian foci of zoonotic cutaneous leishmaniasis. Trop Med Int Health. 2008; 13(9):1159–71. doi: 10.1111/j.1365-3156.2008.02121.x PMID: 18631311

34. Latrofa MS, Dantas-Torres F, Weigl S, Tarallo VD, Parisi A, Traversa D, et al. Multilocus molecular and phylogenetic analysis of phlebotomine sand flies (Diptera: Psychodidae) from southern Italy. Acta Trop. 2011; 119(2–3):91–8. doi: 10.1016/j.actatropica.2011.05.014 PMID: 21679682

35. Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, et al. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci U S A. 1989; 86(16):6196–200. PMID: 2762322

36. Boakye DA, Tang J, Truc P, Merriweather A, Unnasch TR. Identification of bloodmeals in haematophagous Diptera by cytochrome B heteroduplex analysis. Med Vet Entomol. 1999; 13(3):282–7. PMID: 10514054

37. Tabbabi A, Ghrab J, Aoun K, Ready PD, Bouratbine A. Habitats of the sandfly vectors of *Leishmania tropica* and *L. major* in a mixed focus of cutaneous leishmaniasis in southeast Tunisia. Acta Trop. 2011; 119(2–3):131–7. doi: 10.1016/j.actatropica.2011.05.014 PMID: 21679682

38. Blondel J. Biogéographie et écologie. Paris: Masson. 1979:190. doi: 10.1371/journal.pone.0049440 PMID: 23152908

39. al-Zahrani MA, Peters W, Evans DA, Chin C, Smith V, Lane RP. Phlebotomus sergenti, a vector of Leishmania tropica in Saudi Arabia. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1988; 82(3):416. PMID: 3232175

40. Oshaghi MA, Rasolian M, Shirzadi MR, Mohtarami F, Doosti S. First report on isolation of Leishmania tropica from sandflies of a classical urban Cutaneous leishmaniasis focus in southern Iran. Exp Parasitol. 2010; 126(4):445–50. doi: 10.1016/j.exppara.2010.05.020 PMID: 20570590

41. Svobodova M, Votypka J, Peckova J, Dvorak V, Nasereddin A, Baneth G, et al. Distinct transmission cycles of *Leishmania tropica* in 2 adjacent foci, Northern Israel. Emerg Infect Dis. 2006; 12(12):1860–8. PMID: 17326936

42. Tiwary P, Kumar D, Mishra M, Singh RP, Rai M, Sundar S. Seasonal Variation in the Prevalence of Sand Flies Infected with Leishmania donovani. Plos One. 2013; 8(4).

43. Jimenez M, Gonzalez E, Iriso A, Marco E, Alegret A, Fuster F, et al. Detection of Leishmania infantum and identification of blood meals in Phlebotomus perniciosus from a focus of human leishmaniasis in Madrid, Spain. Parasitol Res. 2013; 112(7):2453–9. doi: 10.1007/s00436-013-3406-3 PMID: 23535889

44. Es-Sette N, Ajaoud M, Bichaud L, Hamdi S, Mellouki F, Charrel RN, et al. Phlebotomus sergenti a common vector of Leishmania tropica and Toscana virus in Morocco. J Vector Borne Dis. 2014; 51-(2):86–90. PMID: 24947214

45. Es-Sette N, Ajaoud M, Laamrani-Idrissi A, Mellouki F, Lemrani M. Molecular detection and identification of Leishmania infection in naturally infected sand flies in a focus of cutaneous leishmaniasis in northern Morocco. Parasit Vectors. 2014; 7:305. doi: 10.1186/1756-3305-7-305 PMID: 24990497

46. Arroub H, Hamdi S, Ajaoud M, Habbari K, Lemrani M. Epidemiologic study and molecular detection Of Leishmania and sand fly species responsible of cutaneous leishmaniasis in Foum Jamaa (Azilal, Atlas of Morocco). Acta Tropica. 2013; 127(1):1–5. doi: 10.1016/j.actatropica.2013.03.005 PMID: 23524126

47. Ajaoud M, Es-sette N, Hamdi S, El-Idrissi AL, Riyad M, Lemrani M. Detection and molecular typing of Leishmania tropica from Phlebotomus sergenti and lesions of cutaneous leishmaniasis in an emerging focus of Morocco. Parasites & vectors. 2013; 6:217. doi: 10.1186/1756-3305-6-217

48. Singh N, Chikara S, Sundar S. SOLiD sequencing of genomes of clinical isolates of Leishmania donovani from India confirm leptomonas co-infection and raise some key questions. PLoS One. 2013; 8(2): e55738. doi: 10.1371/journal.pone.0055738 PMID: 23418454

49. Toz SO, Culha G, Zeyrek FY, Ertabaklar H, Alkan MZ, Vardarli AT, et al. A real-time ITS1-PCR based method in the diagnosis and species identification of Leishmania parasite from human and dog clinical samples in Turkey. PLoS Negl Trop Dis. 2013; 7(5):e2205. doi: 10.1371/journal.pntd.0002205 PMID: 23675543

50. Azmi K, Schnur L, Schonian G, Nasereddin A, Pratlong F, El Baidouri F, et al. Genetic, serological and biochemical characterization of Leishmania tropica from foci in northern Palestine and discovery of zymodeme MON-307. Parasit Vectors. 2012; 5:121. doi: 10.1186/1756-3305-5-121 PMID: 22709680

51. Le Blancq SM, Peters W. *Leishmania* in the Old World: 2. Heterogeneity among *L. tropica* zymodemes. Trans R Soc Trop Med Hyg. 1986; 80(1):113–9. PMID: 3726972

52. Schonian G, Schnur L, el Fari M, Oskam L, Kolesnikov AA, Sokolowska-Kohler W, et al. Genetic heterogeneity in the species Leishmania tropica revealed by different PCR-based methods. Trans R Soc Trop Med Hyg. 2001; 95(2):217–24. PMID: 11355565

53. Kelly JM, Law JM, Chapman CJ, Van Eys GJ, Evans DA. Evidence of genetic recombination in Leishmania. Mol Biochem Parasitol. 1991; 46(2):253–63. PMID: 1656255

54. Nolder D, Roncal N, Davies CR, Llanos-Cuentas A, Miles MA. Multiple hybrid genotypes of Leishmania (viannia) in a focus of mucocutaneous Leishmaniasis. Am J Trop Med Hyg. 2007; 76(3):573–8. PMID: 17360886

55. Odiwuor S, De Doncker S, Maes I, Dujardin JC, Van der Auwera G. Natural Leishmania donovani/ Leishmania aethiopica hybrids identified from Ethiopia. Infect Genet Evol. 2011; 11(8):2113–8. doi: 10.1016/j.meegid.2011.04.026 PMID: 21558020

56. Rogers MB, Downing T, Smith BA, Imamura H, Sanders M, Svobodova M, et al. Genomic confirmation of hybridisation and recent inbreeding in a vector-isolated Leishmania population. PLoS Genet. 2014; 10(1):e1004092. doi: 10.1371/journal.pgen.1004092 PMID: 24453988

57. Ashford RW. The leishmaniases as emerging and reemerging zoonoses. Int J Parasitol. 2000; 30(12–-13):1269–81. PMID: 11428341

58. Jacobson RL, Eisenberger CL, Svobodova M, Baneth G, Sztern J, Carvalho J, et al. Outbreak of cutaneous leishmaniasis in northern Israel. J Infect Dis. 2003; 188(7):1065–73. PMID: 14513429

59. Talmi-Frank D, Jaffe CL, Nasereddin A, Warburg A, King R, Svobodova M, et al. *Leishmania tropica* in rock hyraxes (*Procavia capensis*) in a focus of human cutaneous leishmaniasis. Am J Trop Med Hyg. 2010; 82(5):814–8. doi: 10.4269/ajtmh.2010.09-0513 PMID: 20439960

60. Zilberstein D, Shapira M. The role of pH and temperature in the development of Leishmania parasites. Annu Rev Microbiol. 1994; 48:449–70. PMID: 7826014

61. Alexander B, de Carvalho RL, McCallum H, Pereira MH. Role of the domestic chicken (Gallus gallus) in the epidemiology of urban visceral leishmaniasis in Brazil. Emerg Infect Dis. 2002; 8(12):1480–5. PMID: 12498667

62. Boubidi SC, Benallal K, Boudrissa A, Bouiba L, Bouchareb B, Garni R, et al. Phlebotomus sergenti (Parrot, 1917) identified as Leishmania killicki host in Ghardaia, south Algeria. Microbes and infection / Institut Pasteur. 2011; 13(7):691–6. doi: 10.1016/j.micinf.2011.02.008 PMID: 21382502

63. Bousslimi N, Ben-Ayed S, Ben-Abda I, Aoun K, Bouratbine A. Natural infection of North African gundi (Ctenodactylus gundi) by Leishmania tropica in the focus of cutaneous leishmaniasis, Southeast Tunisia. The American journal of tropical medicine and hygiene. 2012; 86(6):962–5. doi: 10.4269/ajtmh.2012.11-0572 PMID: 22665601