Received: 23 February 2021 | Revised: 26 May 2021 | Accepted: 2 June 2021

DOI: 10.1111/tbed.14179

Transboundary and Emerging Diseases

WILEY

ORIGINAL ARTICLE

Understanding the factors that determine the emergence of anthroponotic cutaneous leishmaniasis due to *Leishmania tropica* in Morocco: Density and mitochondrial lineage of *Phlebotomus sergenti* in endemic and free areas of leishmaniasis

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Abstract

Anthroponotic cutaneous leishmaniasis (ACL) due to *Leishmania tropica* is spreading to new areas in Morocco. Exposure to the vector, *Phlebotomus sergenti*, is the only proven risk factor. Our objective was to compare the densities and genetic characteristics of *P. sergenti* populations in two nearby localities in Morocco, one in an ACL endemic area (El Borouj) and another in a nonendemic area (Sidi Hajjaj). *P. sergenti* density was significantly higher in the endemic area than in the nonendemic town ($p = 0.032$). A different predominant *P. sergenti* mitochondrial lineage was evidenced in each one of the two localities, and for the first time, the *P. sergenti* lineage acting as a vector of *L. tropica* has been identified. Bioclimatic differences were detected between both localities. In conclusion we found differences in both the density and the mitochondrial lineage of *P. sergenti* populations that may explain the different epidemiological situation. Given that the density of *P. sergenti* in the locality without ACL cases seems sufficient to allow transmission, the main factor that would justify its nonendemic character could be the absence of *P. sergenti* Lineage IV, which seems to prefer warmer and drier climates.

KEYWORDS
anthroponotic cutaneous leishmaniasis, Leishmania tropica, mitochondrial lineage, Morocco, Phlebotomus sergenti, vector density

1 | INTRODUCTION

Leishmania tropica (Kinetoplastida: Trypanosomatidae) is the major cause of anthroponotic cutaneous Leishmaniasis (ACL) in the Middle East and some areas of North Africa (Pratlong et al., 2009) and *Phlebotomus sergenti* Parrot, 1917 (Diptera: Psychodidae) is its main vector (Guilvard et al., 1991; Schnur et al., 2004). For a long time, *P. sergenti* was considered the sole vector of *L. tropica* (Al-Zahrani et al., 1988; Guilvard et al., 1991) however, the vectorial capacity of *Phlebotomus arabicus* has been demonstrated in a focus in northern Israel (Svobodová

et al., 2006), and *Phlebotomus similis* is considered a probable vector on the island of Crete (Ntais et al., 2014). Although *P. sergenti* is believed to have a marked preference for semi-arid habitats (Boussaa et al., 2009), this species exhibits a wide ecological plasticity in Morocco making increased vector surveillance essential to prevent and control leishmaniasis outbreaks. In emerging ACL Moroccan foci, *P. sergenti* density varies from 4 to 16 specimens/m^2 (Ramaoui et al., 2008) and relative abundance ranges from 12.8% to 76.7% (Boussaa et al., 2009).

The World Health Organization included Morocco among one of the 12 high-burden countries for CL (WHO, 2016). There are three endemic *Leishmania* species in Morocco: *Leishmania major*, *L. tropica*, (both dermotropic) and *Leishmania infantum* (mainly viscerotropic). *Leishmania tropica* has the widest geographic distribution (Ministry of Health, Morocco, 2016; Mouttaki et al., 2014) but until 1989, ACL had been mainly reported in hypoendemic rural foci scattered around the sub-arid area of central Morocco. Later, ACL emerged in several northern, central and southern provinces of the country, initially as new outbreaks and then establishing endemic foci that highlighted the expansion of this *Leishmania* species (Ajaoud et al., 2013). The first CL case in Settat province (central Morocco) was detected in El Borouj locality in 2006, preceding an epidemic outbreak and then becoming endemic (Amarir et al., 2015; Gijón-Robles et al., 2018). Currently, El Borouj is the only active CL focus in the province of Settat (Ministry of Health, Morocco, 2016).

The identification of factors that determine the emergence and expansion of ACL is required to develop better interventions for this largely neglected disease. We showed that differences in the exposure to the *L. tropica* vector, reflected by differences in *P. sergenti* density in the households, was the only factor associated with CL cases in the El Borouj focus (Gijón-Robles et al., 2018).

On the other hand, three of the four mitochondrial (mt) lineages previously reported within *P. sergenti* are present in Morocco (Barón et al., 2008; Merino-Espinosa et al., 2016; Yahia et al., 2004). Phenotypic differences of biomedical importance may exist between these mitochondrial lineages, thus population genetics could help to assess the threat of the geographical expansion of ACL.

Therefore, our objective was to compare the densities and genetic characteristics of *P. sergenti* populations in two nearby localities in Morocco, one within an ACL endemic area (El Borouj) and another in a nonendemic region (Sidi Hajjaj).

2 | MATERIAL AND METHODS

2.1 | Study area

El Borouj (coordinates 07°36'W-32°29'N) and Sidi Hajjaj (07°24'W-33°06'43"N) are situated at an altitude of 410 and 547 m above sea level respectively, in the Settat province, central Morocco. Both localities are separated by only 51 km and have common rural features and an economic activity mainly based on agriculture and animal husbandry. In both towns, the population is around 20,000 inhabitants and the average growth rate is close to 2%. ACL by *L. tropica* is endemic in El Borouj whereas no cases have been reported in Sidi Hajjaj (Ministry of Health, Morocco, 2016).

2.2 | Sand fly collection and species identification

Sand flies were caught in both localities using CDC light traps inside households and sticky papers outside dwellings, from June 20 to July 10 and from September 20 to October 10, 2015. In El Borouj, houses with and without ACL cases were sampled. One to two CDC traps were set in each selected house for one night under favourable weather conditions. Sticky traps consisted of 21 × 29.5 cm sheets of papers covered in castor oil, 9–17 traps were set the same day in adult sand fly resting places (holes in house walls and other nearby walls) and left for 4 days. The captured sand flies were stored in 70% alcohol. Males and females were separated and morphologically identified using taxonomic keys (Benabdennbi et al., 1999; Berchi et al., 2007; El Sawaf et al., 1989; Gil Collado et al., 1989; Leger et al., 1983; Rioux & Golvan, 1969; Rioux et al., 1978; Sáez et al., 2018). The specimens were placed in Marc André solution and heated to boiling point, and finally mounted on slides under a coverslip using Berlese solution. The genitalia of *P. sergenti* specimens were individually removed and mounted on slides under a coverslip for morphological identification whereas the rest of the body was stored at −20°C for DNA extraction.

The gonotrophic cycle of the female sand flies was categorised as blood-fed, non-fed or gravid. Density (sand flies/trap), relative abundance (% specimens of a given species/total sand flies) and frequency (% positive sampling stations for a given species) data were estimated by species.

2.3 | Sand fly DNA extraction

Genomic DNA was extracted from the head, thorax and attached anterior abdomen of individual *P. sergenti* males and females (Martín-Sánchez et al., 2000). A commercially available kit was used [RealPure kit from REAL (Ref. RBMEG01], according to the manufacturer instructions. Each sand fly was individually placed in a sterile 1.5 mL Eppendorf tube and kept in liquid nitrogen for a few seconds to facilitate the mechanic rupture of the tissues using a pestle. The DNA was resuspended in 20 μL of bidistilled water and kept at −20°C until use.

2.4 | Mitochondrial lineage determination by mt DNA Cyt b PCR-RFLP

Polymerase chain reaction (PCR) was used to amplify a 550-bp fragment containing the 3′ end of the Cytochrome b mitochondrial gene (mt DNA Cyt b) following the methodology described by Esseghir et al. (1997).

Restriction fragment length polymorphism (RFLP) was carried out through the digestion of a 550-bp mtDNA Cyt b fragment with *Hae III* (Thermo Scientific, Germany). The reaction was performed at 37°C

for 10 minutes in a 20 μL total volume, containing 16 μL of PCR product, 2 μL of enzyme (10 U/μL) and 2 μL of standard buffer (10×). The digested samples were separated by electrophoresis in a 3% agarose gel and their sizes determined by comparison with HyperLadder V (Bioline, UK) leading to a characteristic banding pattern for each of the four mitochondrial lineages: Lineage I, two fragments (290 and 220 bp); Lineage II, two fragments (290 and 260 bp); Lineage III, three fragments (290, 140 and 110 bp) and Lineage IV, two fragments (330 and 220 bp) (Merino-Espinosa et al., 2016).

2.5 | Detection of *Leishmania tropica* DNA

The presence of *L. tropica* DNA was investigated in *P. sergenti* females captured in Sidi Hajjaj, using Granaleish Multiplex qPCR (University of Granada, Spain, Trade Mark Number 3667362/5). This PCR technique can differentiate between *L. infantum*, *L. tropica* and *L. major* and allows quantification of the parasite load (Merino-Espinosa et al., 2018). Primers F, R and the 3 Taqman probes were provided by the manufacturer. The following thermal profile was used: 10 minutes at 95°C, then 36 cycles of 30 seconds at 95°C and 60 seconds at 60°C.

2.6 | Bioclimatic differences between El Borouj and Sidi Hajjaj

In order to investigate the possible association between bioclimatic characteristics and the presence/absence of ACL in the two studied localities, a logistic regression analysis was carried out including the locality as a dependent variable and each bioclimatic variable under study as an independent variable. The bioclimatic data analysed were as follows: mean monthly average temperature (from January to December), maximum monthly average temperature (from January to December), minimum monthly average temperature (from January to December), maximum annual average temperature, minimum annual average temperature, monthly precipitation (from January to December), annual mean temperature (BIO1), mean diurnal range (mean of monthly – max temp – min temp–)(BIO2), isothermality (BIO2/BIO7) × 100 (BIO3), temperature seasonality (standard deviation × 100) (BIO4), max temperature of warmest month (BIO5), min temperature of coldest month (BIO6), temperature annual range (BIO5–BIO6) (BIO7), mean temperature of wettest quarter (BIO8), mean temperature of driest quarter (BIO9), mean temperature of warmest quarter (BIO10), mean temperature of coldest quarter (BIO11), annual precipitation (BIO12), precipitation of wettest month (BIO13), precipitation of driest month (BIO14), precipitation seasonality (coefficient of variation) (BIO15), precipitation of wettest quarter (BIO16), precipitation of driest quarter (BIO17), precipitation of warmest quarter (BIO18) and precipitation of coldest quarter (BIO19). This information was taken from the WorldClim global climate data (www.worldclim.org/) considering the average values from 2007 to 2015. Data were analysed with IBM SPSS Statistics 20.0 for Windows (IBM Corp., Armonk, NY, USA).

Average values (95% confidence interval) and minimum and maximum values of each of the bioclimatic variables from El Borouj and Sidi Hajjaj are shown as supplementary data.

3 | RESULTS

3.1 | Sand fly fauna and abundance by species

Species distribution by sex, sampling method, sampling period and site are shown in Table 1. In total 5674 sand fly specimens, 2215 females (39.0%) and 3459 males (61.0%), were collected. The mean density values in El Borouj were 14.9 sand flies/CDCtrap/night and 26.1 sand flies/m^2 whereas in Sidi Hajjaj were 8.1 sand flies/CDCtrap/night and 14.8 sand flies/m^2. Thirteen sand fly species were present (9 from *Phlebotomus* genus and 4 from *Sergentomyia* genus) and differences in density, relative abundance and frequency were observed between species and localities (Table 1). *Phlebotomus chabaudi*, *Phlebotomus alexandri*, *Phlebotomus duboscqi*, *Sergentomyia antenata* and *Sergentomyia dreyfussi* were caught in low numbers in El Borouj and were absent in Sidi Hajjaj. In both localities, *Sergentomyia minuta* was the most abundant species in the captures with adhesive traps whereas *Phlebotomus sergenti* was the most abundant species in the intra-household captures collected with CDC traps (Table 1).

Sand fly density was higher in El Borouj and significant differences were found in captures carried out peridomestically in June ($p < 0.0001$) and intradomiciliary in October ($p = 0.066$). *Phlebotomus sergenti* density was also higher in this locality and significant differences were detected in peridomiciliary captures performed in June ($p = 0.007$), intradomiciliary captures in October ($p = 0.007$), in global intradomiciliary collections ($p = 0.032$) and peridomestically ($p = 0.022$) (Table 1). Regarding differences between capture periods the intradomiciliary density of *P. sergenti* was significantly higher in June in El Borouj ($p = 0.012$) and in Sidi Hajjaj ($p = 0.020$).

Blood-fed, non-fed and gravid *P. sergenti* females were found both in intradomiciliary and peridomiciliary captures in both localities; density values were higher in El Borouj (Table 2).

3.2 | *Leishmania* infection rate in the vector

L. tropica DNA was not detected in any of the 84 female *P. sergenti* captured in Sidi Hajjaj.

3.3 | Mitochondrial lineage determination by mtDNA Cyt b PCR-RFLP

The mitochondrial lineage was identified in 81 male and female *P. sergenti*, 41 from El Borouj and 40 from Sidi Hajjaj. The results are shown in Table 3.

The females captured in El Borouj in which *L. tropica* DNA was detected in a previous study (Gijón-Robles et al., 2018) were identified as lineage IV.

TABLE 1 Comparison of species and densities of sand flies captured in El Borouj (ACL endemic) and Sidi Hajjaj (ACL free) in the two sampling periods, June and October 2015, both in intradomiciliary with CDC light traps and peridomiciliary with sticky papers

		El Borouj						Sidi Hajjaj					
		Capture intradomiciliary			Capture peridomiciliary			Capture intradomiciliary			Capture peridomiciliary		
		J	O	G	J	O	G	J	O	G	J	O	G
Number of stations		7	46	53	35	44	79	4	9	13	10	18	28
Number of traps		14 CDC traps	69 CDC traps	83 CDC traps	693.2 ST (85.9 m^2)	381.6 ST (47.3 m^2)	1074.9 ST (133.2 m^2)	8 CDC traps	11 CDC	19 CDC	133 ST (16.5 m^2)	309.8 ST (38.4 m^2)	442.8 ST (54.9 m^2)
P. sergenti	*N* (M/F)	352 (189/163)	231 (108/123)	583 (297/286)	462 (370/92)	274 (232/42)	736 (602+134)	105 (47/58)	7 (4/3)	112 (51/61)	10 (6/4)	51 (32/19)	61 (38/23)
	D	25.14	3.35**	7.02*	5.38**	5.79	5.53*	13.13	0.64**	5.89*	0.61**	1.33	1.11*
	A	42.21	57.61	47.21	27.62	15.21	21.18	82.68	25.93	72.73	45.45	6.49	7.53
	Fr	0.86	0.83	0.83	0.89	0.64	0.75	1.00	0.44	0.62	0.40	0.67	0.57
P. longicuspis	*N* (M/F)	415 (257/158)	56 (22/34)	471 (279/192)	45 (39/6)	21 (21/0)	66 (60/6)	20 (8/12)	11 (5/6)	31 (13/18)	2 (2/0)	86 (82/4)	88 (84/4)
	D	29.64	0.81	5.67	0.52	0.44***	0.50*	2.50	1.00	1.63	0.12	2.21***	1.60*
	A	49.76	13.97	38.14	2.69	1.17	1.90	15.75	40.74	20.13	9.09	10.91	10.86
	Fr	0.71	0.37	0.42	0.31	0.20	0.25	0.75	0.56	0.62	0.10	0.67	0.46
P. perniciosus	*N* (M/F)	43 (31/12)	25 (21/4)	68 (52/16)	78 (72/6)	73 (62/11)	151 (134/17)	1 (0/1)	1 (1/0)	2 (1/1)	0	91 (81/10)	91 (81/10)
	D	3.07	0.36	0.82	0.91*	1.54	1.13	0.13	0.09	0.11	0*	2.35	1.66
	A	5.16	6.23	5.51	4.66	4.05	4.35	0.79	3.70	1.30	0	11.55	11.23
	Fr	0.57	0.26	0.30	0.46	0.34	0.39	0.25	0.11	0.15	0	0.56	0.36
P. langeroni	*N* (M/F)	4 (4/0)	1 (1/0)	5 (5/0)	0	0	0	1 (1/0)	7 (6/1)	8 (7/1)	0	9 (8/1)	9 (8/1)
	D	0.29	0.01*	0.06*	0	0***	0***	0.13	0.64*	0.42*	0	0.23***	0.16***
	A	0.48	0.25	0.40	0	0	0	0.79	25.93	5.19	0	1.15	1.11
	Fr	0.29	0.02	0.06	0	0	0	0.25	0.22	0.23	0	0.33	0.21

(Continues)

TABLE 1 (Continued)

		El Borouj						Sidi Hajjaj					
		Capture intradomiciliary			Capture peridomiciliary			Capture intradomiciliary			Capture peridomiciliary		
P. papatasi	*N* (M/F)	17 (10/7)	35 (19/16)	52 (29/23)	119 (90/29)	218 (180/38)	337 (270/67)	0	0	0	0	9 (2/7)	9 (2/7)
	D	1.21*	0.51*	0.63**	1.39**	4.61	2.53**	0*	0*	0**	0**	0.23**	0.16
	A	2.04	8.73	4.21	7.11	12.10	9.70	0	0	0	0	1.15	1.11
	Fr	0.71	0.37	0.42	0.54	0.43	0.49	0	0	0	0	0.28	0.18
P. bergeroti	*N* (M/F)	0	6 (0/6)	6 (0/6)	3 (2/1)	4 (0/4)	7 (2/5)	0	0	0	0	1 (0/1)	1 (0/1)
	D	0	0.09	0.07	0.03	0.08	0.05	0	0	0	0	0.03	0.02
	A	0	1.50	0.49	0.18	0.22	0.20	0	0	0	0	0.13	0.12
	Fr	0	0.13	0.11	0.09	0.02	0.05	0	0	0	0	0.06	0.04
S. minuta	*N* (M/F)	1 (0/1)	13 (8/5)	14 (8/6)	671 (358/313)	674 (269/405)	1345 (627/718)	0	1 (1/0)	1 (1/0)	10 (3/7)	538 (318/220)	548 (321/227)
	D	0.07	0.19	0.17	7.81***	14.25	10.10	0	0.09	0.05	0.61***	14.02	9.99
	A	0.12	3.24	1.13	40.11	37.40	38.71	0	3.70	0.65	45.45	68.45	67.65
	Fr	0.14	0.22	0.21	0.89	0.89	0.87	0	0.11	0.08	0.20	0.94	0.68
S. fallax	*N* (M/F)	1 (0/1)	29 (17/12)	30 (17/13)	290 (198/92)	538 (264/274)	828 (462/366)	0	0	0	0	3 (2/1)	3 (2/1)
	D	0.07	0.42	0.36	3.38**	11.38**	6.22***	0	0	0	0**	0.08**	0.05***
	A	0.12	7.23	2.43	17.33	29.86	23.83	0	0	0	0	0.38	0.37
	Fr	0.14	0.22	0.21	0.63	0.59	0.61	0	0	0	0	0.11	0.07
Total	*N* (M/F)	834[1] (491/343)	401[2] (197/204)	1235[1, 2] (688/547)	1673[3] (1134/539)	1802 (1028/774)	3475[3] (2162/1313)	127 (56/71)	27 (17/10)	154 (73/81)	22 (11/11)	788 (525/263)	810 (536/274)
	D	59.57	5.81	14.88	19.48***	38.11	26.09	15.88	2.45	8.11	1.34***	20.48	14.77
	A	100	100	100	100	100	100	100	100	100	100	100	100
	Fr	0.86	0.89	0.89	1.00	0.95	0.97	1.00	0.67	0.77	0.60	1.00	0.86

N = number of captured sandflies, M = males, F = females, D = density (no. of captured sandflies/CDC trap/night or no. captured sandflies/area, m^2), *A* = relative abundance (representative percentage of each species with respect to total captured in a given period), *Fr* = frequency (presence of a given species in the stations sampled in each time period). J = June, O = October, G = global results. Total number of sand flies also includes: [1]1 ♂ *P. alexandri*; [2]1 ♀ *P. chabaudi*, 1 ♂ *P. duboscqi* and 3 ♂ *S. dreyfussi*; [3]5 ♂*S. antennata*. Statistical differences were detected at $^{*}p < 0.05$ level, $^{**}p < 01$, $^{***}p < 001$.

TABLE 2 Number of nonfed (U), blood-fed (B), and gravid (G) female sand flies, captured in the two sampling periods (June and October 2015) in both localities, El Borouj (ACL endemic) and Sidi Hajjaj (ACL free)

		El Borouj												Sidi Hajjaj											
		Capture intradomiciliary						Capture peridomiciliary						Capture intradomiciliary						Capture peridomiciliary					
		J			O			J			O			J			O			J			O		
Number of stations		7			46			35			44			4			9			10			18		
Number of traps		14 CDC			69 CDC			693.3 ST (85.9 m^2)			381.6 ST (47.3m^2)			8 CDC			11 CDC			133 ST (16.5 m^2)			309.8 ST (38.4 m^2)		
Species		U	B	G	U	B	G	U	B	G	U	B	G	U	B	G	U	B	G	U	B	G	U	B	G
P. sergenti	*NF*	71	25	67	44	24	55	50	18	24	13	18	11	34	9	15	0	1	2	0	1	3	2	5	12
	D	5.07	1.79	4.79	0.64	0.35	0.80	0.58	0.21	0.28	0.27	0.38	0.23	4.25	1.13	1.88	0.00	0.09	0.18	0.00	0.06	0.18	0.05	0.13	0.31
	A	20.70	7.29	19.53	21.57	11.76	26.96	9.28	3.34	4.45	1.68	2.33	1.42	47.89	12.68	21.13	0.00	10.00	20.00	0.00	9.09	27.27	0.76	1.90	4.56
P. longicuspis	*NF*	141	13	4	21	10	3	2	3	1	0	0	0	10	2	0	2	2	2	0	0	0	1	2	1
	D	10.07	0.93	0.29	0.30	0.14	0.04	0.02	0.03	0.01	0	0	0.00	1.25	0.25	0.00	0.18	0.18	0.18	0.00	0.00	0.00	0.03	0.05	0.03
	A	41.11	3.79	1.17	10.29	4.90	1.47	0.37	0.56	0.19	0	0	0.00	14.08	2.82	0.00	20.00	20.00	20.00	0.00	0.00	0.00	0.38	0.76	0.38
P. perniciosus	*NF*	7	3	2	2	1	1	4	1	1	5	2	4	0	1	0	0	0	0	0	0	0	3	4	3
	D	0.50	0.21	0.14	0.03	0.01	0.01	0.05	0.01	0.01	0.11	0.04	0.08	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.10	0.08
	A	2.04	0.87	0.58	0.10	0.05	0.05	0.74	0.19	0.19	0.65	0.26	0.52	0.00	1.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.14	1.52	1.14
P. papatasi	*NF*	0	2	5	9	5	2	18	6	5	17	13	8	0	0	0	0	0	0	0	0	0	2	0	5
	D	0	0.14	0.36	0.13	0.07	0.03	0.21	0.07	0.06	0.36	0.27	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.13
	A	0	0.58	1.46	4.41	2.45	0.98	3.34	1.11	0.93	2.20	1.68	1.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.76	0.00	1.90
S. minuta	*NF*	1	0	0	4	1	0	280	11	22	390	8	7	0	0	0	0	0	0	6	0	1	206	6	8
	D	0.07	0	0	0.06	0.01	0	3.26	0.13	0.26	8.25	0.17	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.00	0.06	5.37	0.16	0.21
	A	0.29	0	0	1.96	0.49	0	51.95	2.04	4.08	50.39	1.03	0.90	0.00	0.00	0.00	0.00	0.00	0.00	54.55	0.00	9.09	78.33	2.28	3.04
S. fallax	*NF*	1	0	0	11	1	0	86	1	5	262	4	8	0	0	0	0	0	0	0	0	0	1	0	0
	D	0.07	0	0	0.16	0.01	0	1.00	0.01	0.06	5.54	0.08	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0	0
	A	0.29	0	0	5.39	0.49	0	15.96	0.19	0.93	33.85	0.52	1.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.38	0	0
Total U, B and G females	*NF*	222[1]	43	78	98[2]	43[3]	63[4]	440	40	59[5]	691[6]	45	38	44	12	15	3[7]	3	4	6	1	4	216[8]	18[9]	29
	D	15.86	3.07	5.57	1.02	0.45	0.66	5.12	0.47	0.69	14.61	0.95	0.80	5.50	1.50	1.88	0.27	0.27	0.36	0.36	0.06	0.24	5.63	0.47	0.76
	A	64.72	12.54	22.74	48.04	21.08	30.88	81.63	7.42	10.95	89.28	5.81	4.91	61.97	16.90	21.13	30.00	30.00	40.00	54.55	9.09	36.36	82.13	6.84	11.03
Total females	*NF*	343[1]			204[2, 3, 4]			539[5]			774[6]			71			10[7]			11			263[8, 9]		
	D	24.50			2.96			6.28			16.37			8.88			0.91			0.67			6.85		
	A	100			100			100			100			100			100			100			100		

NF = number of captured females, *D* = density (no. of females/no. of CDC traps/night or no. of females/area in m^2 in case of adhesive traps), *A* = relative abundance (representative percentage of each species of the total captured specimens). J = June, O = October. Total number of females also includes: [1]1 *P. alexandri*; [2]4 *P. bergeroti* and 3 *S. dreyfussi*; [3]1 *P. bergeroti*; [4]1 *P. bergeroti* and 1 *P. chabaudi*; [5]1 *P. bergeroti*; [6]4 *P. bergeroti*; [7]1 *P. langeroni*; [8]1 *P. bergeroti*; [9]1 *P. langeroni*.

TABLE 3 Number of *Phlebotomus sergenti* specimens from El Borouj (ACL endemic) and Siddi Hajjaj (ACL free) in captures made throughout 2015 analysed by mt DNA Cytb PCR-RFLP with Hae III restriction enzyme and the mitochondrial lineage identified

Locality	Number		Mitochondrial lineage and Hae III RFLP banding pattern		
			I (290 and 220 bp)	II (290 and 260 bp)	IV (330 and 220 bp)
Sidi Hajjaj	**Male**	26	4	22	0
	Female	14	4	10	0
	Total	40	8 (20%)	32 (80%)	0 (0%)
El Borouj	**Male**	21	0	0	21
	Female	20	0	1	19
	Total	41	0 (0%)	1 (2.4%)	40 (97.6%)

TABLE 4 Average values (95% confidence interval), and minimum and maximum values of each of the bioclimatic variables in El Borouj (ACL endemic) and Sidi Hajjaj (ACL free) for which differences were detected

Bioclimatic variable	Average value (95% confidence interval), and minimum and maximum values		*p* Value by logistic regression
	Siddi Hajjaj	El Borouj	
Maximum temperature July	34.74 (34.66–34.81) 34.6–34.9 °C	36.42 (36.41–36.43) 36.3–36.5 °C	Absolute differences
Rainfall September	11.14 (10.93–11.35) 11–12 mm	Constant value of 9 mm	Absolute differences
Rainfall October	Constant value of 37 mm	Constant value of 34 mm	Absolute differences
BIO3	Constant value of 49	Constant value of 47	Absolute differences
BIO15	68.4 (68.1–68.7) 68–69	68.1 (68.1–68.2) 68–69	0.009 (OR = 0.218 IC95% 0.07–0.68).
BIO18	13.14 (12.93–13.35) 13–14 mm	Constant value of 11 mm	Absolute differences

BIO3 = isothermality; BIO15 = precipitation seasonality; BIO18 = precipitation of warmest quarter.

3.4 | Bioclimatic differences between El Borouj and Sidi Hajjaj

Bioclimatic data were collected from 199 georeferenced points, 185 houses with ACL cases and 14 houses in Sidi Hajjaj. Table 4 shows the average values (95% confidence interval), and minimum and maximum values of each of the bioclimatic variables in El Borouj and Sidi Hajjaj for which differences were detected. Significant differences were detected at precipitation seasonality (BIO15, $p = 0.009$) which was 78% lower in El Borouj [OR = 0.22 (IC95% 0.07–0.68)] whereas both localities showed absolute differences at maximum annual temperature in July, rainfall in September and October, isothermality (BIO3) and precipitation of warmest quarter (BIO18).

4 | DISCUSSION

In Morocco, ACL due to *L. tropica* is transmitted by *P. sergenti* which has a large geographic distribution probably related to the wide ecological plasticity of this vector (Boussaa et al., 2009; Ramaoui et al., 2008; Rioux et al., 1986). CL due to *L. tropica* is an emerging disease even though the geographical extension of the vector is greater than that of the parasitic protozoan, and the identification of factors for parasite expansion is essential for effective disease control. *Phlebotomus sergenti* density and genetic characteristics were investigated as determining factors for the existence of ACL transmission. Comparative intradomiciliary and peridomiciliary sand fly captures in the ACL endemic locality of El Borouj and the non-endemic locality of Sidi Hajjaj were made using CDC light traps and sticky papers.

Phlebotomus sergenti density in Sidi Hajjaj was lower than that of the endemic locality, both in peridomiciliary settings and within households. Interestingly, *P. sergenti* was the most abundant and densest species within households in Sidi Hajjaj, however it was the fourth species outdoors, after *S. minuta* and the *L. infantum* vectors, *P. perniciosus* and *P. longicuspis* (Table 1).The relative abundance of *P. sergenti* males and females varied between the trapping methods since the males were more abundant in the sticky papers. In both localities, the density of *P. sergenti* females was higher in the intradomiciliary captures carried out in June and non-fed, fed and gravid females, were found (Table 2).

Although to date no ACL cases have been diagnosed in Sidi Hajjaj, these sand fly density figures seem sufficient for the maintenance of

L. tropica transmission (Barón *et al*., 2013; Ramaoui et al., 2008; Rioux et al., 1986) and would make this locality susceptible to the establishment of an ACL transmission cycle. Over the last few decades, *L. tropica* foci have spread to several regions of Morocco including those where CL caused by *L. major* or *L. infantum* has been reported, which shows the changing geographical patterns of this species (Baghad et al., 2020). The growing mobility of humans raises the possibility of new emerging foci in areas where *P. sergenti* populations are well established. Kholoud et al. (2020) suggested that ACL dissemination in Morocco is associated to an increase in human travel and local tourism linked to economic expansion and infrastructure development as shown by the synchronized occurrence of new ACL foci with the construction of new motorways. However, the factors underlying the spatio-temporal transmission dynamics of leishmaniasis are not well understood, and the epidemiological picture is not as simple as deduced from the previous statement.

The molecular characterization of *P. sergenti* populations in both localities using the PCR-RFLP technique of the cytochrome b mitochondrial gene, has allowed us to find that the lineage IV is the main mitochondrial lineage of *P. sergenti* in El Borouj (97.6%) while the remaining 2.4% belong to lineage II. In contrast, lineage II was the most abundant in Sidi Hajjaj (80%) followed by the lineage I (20%). Therefore, a different main *P. sergenti* mitochondrial lineage was dominant in each locality under study. *Phlebotomus sergenti* is characterised by its high genetic diversity and classified with at least 20 haplotypes in four mitochondrial lineages (Barón et al., 2008; Yahia et al., 2004).

In El Borouj, *L. tropica* DNA was detected in 5 out of 184 (2.7%) *P. sergenti* females in captures made in 2014 and 2015 (Gijón-Robles et al., 2018). The five infected females belong to lineage IV, the most prevalent *P. sergenti* lineage in El Borouj. This is the first time that a mitochondrial lineage of *P. sergenti* is involved as a vector for *L. tropica*. A local increase in the abundance of this *P. sergenti* lineage that seems to transmit *L. tropica* more efficiently, could explain the emergence of ACL in El Borouj and its absence in Sidi Hajjaj.

Lineage I is over-represented in southwestern Europe (Merino-Espinosa et al., 2016) and this is the first time that its presence in Morocco is reported. No autochthonous ACL cases have been detected in the Iberian Peninsula, despite *P. sergenti* being commonly found at sufficient densities to act as a vector, and the existence of 2 mitochondrial lineages, one of them, held in common with Morocco (Lineage III) (Barón et al., 2008, 2013 ; Merino-Espinosa et al., 2016). The existence of differential ecological traits between *P. sergenti* mitochondrial lineages has been pointed out: Merino-Espinosa et al. (2016) found that Lineage I appear to have adaptive advantages represented by a wider tolerance to temperature and altitude changes that would make it better suited to lead a geographical expansion into Europe. Similarly, there are bioclimatic differences between El Borouj and Sidi Hajjaj (Table 4) that could explain the over representation of lineage IV in El Borouj, which is warmer and drier, and its absence in Sidi Hajjaj.

In general, rainfall is greater in Sidi Hajjaj than in El Borouj with a strong seasonality from July to October. In contrast, temperatures are almost two degrees higher in El Borouj throughout the year; however the differences were only significant between the maximum temperatures of July. The coefficient of isothermality reflects well these differences in temperature between both localities, being two points higher in El Borouj. Environmental humidity and temperature are two determining factors in the distribution of *P. sergenti* (Barón et al., 2013) and within this species there appear to be lineages with different ranges of tolerance. Particularly in El Borouj, the presence of water wells was independently associated with a greater *P. sergenti* density in the households (Gijón-Robles et al., 2018).

5 | CONCLUSION

The density and genetic background of the *L. tropica* vector, *P. sergenti*, seem to play a significant role in the prevalence of ACL in Morocco. Differences in both the vector density and its main mitochondrial lineage in two localities, one endemic and the other free of the disease, may explain the different epidemiological situation. Given that the *P. sergenti* density in Sidi Hajjaj seems sufficient to allow transmission, the main factor that would explain the absence of ACL cases could be the lack of *P. sergenti* lineage IV, which seems to prefer warmer and drier climates.

ACKNOWLEDGEMENT

This study was funded by the University of Granada (Centro de Iniciativas de Cooperación al Desarrollo, CICODE, 2013). Funding for open access charge: Universidad de Granada/CBVA.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Gijón-Robles, P., Abattouy, N., Merino-Espinosa, G., El Khalfaoui, N., Morillas-Márquez, F., Corpas-López, V., Jaaouani, N., Díaz-Sáez, V., Riyad, M., Martín-Sánchez, J. (2021). Understanding the factors that determine the emergence of anthroponotic cutaneous leishmaniasis due to *Leishmania tropica* in Morocco: density and mitochondrial lineage of *Phlebotomus sergenti* in endemic and free areas of leishmaniasis. *Transboundary and Emerging Diseases*. , 1–10. https://doi.org/10.1111/tbed.14179