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

Patricia Gijón-Robles[1], Naima Abattouy[2], Gemma Merino-Espinosa[1], Nora El Khalfaoui[3], Francisco Morillas-Marquez[1], Victoriano Corpas-López[1], Jaaouani Noureddine[4], Victoriano Díaz-Sáez[1], Myriam Riyad[3], and Joaquina Martin-Sanchez[1]

[1]University of Granada
[2]Higher Institute of Nursing and Health Techniques
[3]University Hassan II of Casablanca
[4]Ministere de la Santé, Settat

March 5, 2021

**Abstract**

Anthroponotic cutaneous leishmaniasis (ACL) due to Leishmania tropica is spreading to new areas. 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 within an ACL endemic area (El Borouj) and another undamaged (Sidi Hajjaj). Statistically significant differences were detected between P. sergenti densities with a higher density of P. sergenti in the endemic town (p[?] 0.032). A different main P. sergenti mitochondrial lineage was evidenced in each one of the 2 localities, and for the first time, the lineage of P. sergenti specimens that are acting as a vector of L. tropica has been identified. Bioclimatic differences were detected between both localities. In conclusion, between an ACL endemic locality and another ACL free there are differences in both the density of P. sergenti and the mitochondrial lineage 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 ACL undamaged character could be the absence of P. sergenti Lineage IV, which seems to prefer warmer and drier climates.

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

**Running title:** Factors for ACL emergence

Patricia Gijón-Robles[1,¥], Naima Abattouy[2], Gemma Merino-Espinosa[1], Nora El Khalfaoui[3], Francisco Morillas-Márquez[1], Victoriano Corpas-López[1], Noureddine Jaaouani[4]Victoriano Díaz-Sáez[1], Myriam Riyad[3], Joaquina Martín-Sánchez[1*]

**Author affiliations:**

[1]Department of Parasitology, Faculty of Pharmacy, University of Granada, Spain. [2]Higher Institute of Nursing and Health Techniques, Laâyoune, Morocco.

[3]Laboratory of Cellular and Molecular Pathology / Research Team on Immunopathology of Infectious and Systemic Diseases, Hassan II University of Casablanca, Morocco.

[4]Délégation du Ministère de la Santé, province de Settat, Morocco.

[¥]PhD student of the Doctoral programme in Pharmacy, University of Granada.

[*]**Author for correspondence:** Joaquina Martín-Sánchez, Department of Parasitology, Faculty of Pharmacy, Campus Universitario de Cartuja, Granada, Spain*joaquina@ugr.es*, +34 958 242094

**Data available on request from the authors:** the data that support the findings of this study are available from the corresponding author upon reasonable request.

### Summary

Anthroponotic cutaneous leishmaniasis (ACL) due to *Leishmania tropica* is spreading to new areas. 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 within an ACL endemic area (El Borouj) and another undamaged (Sidi Hajjaj). Statistically significant differences were detected between*P. sergenti* densities with a higher density of *P. sergenti*in the endemic town (p[?] 0.032). A different main *P. sergenti*mitochondrial lineage was evidenced in each one of the 2 localities, and for the first time, the lineage of *P. sergenti* specimens that are acting as a vector of *L. tropica* has been identified. Bioclimatic differences were detected between both localities. In conclusion, between an ACL endemic locality and another ACL free there are differences in both the density of *P. sergenti* and the mitochondrial lineage 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 ACL undamaged character could be the absence of *P. sergenti* Lineage IV, which seems to prefer warmer and drier climates.

### Keywords

*Leishmania tropica, Phlebotomus sergenti,* geographical expansion of ACL, comparison of endemic and non-endemic areas, genetic differences, vector density.

### 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 *P. arabicus* has been demonstrated in a focus in northern Israel (Svobodova *et al.,* 2006), and *P. similis* is considered a probable vector on the island of Crete (Ntais *et al.,* 2014). In Morocco, *P. sergenti* exhibits a wide ecological plasticity although it is believed to have a marked preference for semi-arid habitats (Boussaa *et al.,* 2009), therefore, increased vector surveillance is essential to prevent and control CL outbreaks. In emerging ACL Moroccan foci*, P. sergenti* density varies from 4 to 16 specimens/m$^2$(Ramaoui *et al.,* 2008) with 12.8% to 76.7% of relative abundance (Boussaa *et al.,* 2009).

The World Health Organization included Morocco as one of the 12 high-burden countries for CL (WHO, 2016). There are three endemic*Leishmania* species in Morocco: *L. major, L. tropica,* (both dermotropic) and *L. infantum* (mainly viscerotropic)*. Leishmania tropica* has the widest geographic distribution (Ministry of Health, Morocco, 2016; Mouttaki *et al.,* 2014) and 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 establishing as an endemic area (Amarir *et al.,* 2015; Gijon-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 ACL emergence and expansion 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 El Borouj focus (Gijon-Robles et al. in 2018).

On the other hand, the presence of four mitochondrial (mt) lineages within *P. sergenti* has been previously reported (Yahia et al. 2004; Baron et al. 2008; Merino-Espinosa *et al.,* 2016) and three of them are present in Morocco. 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 aim was to analyse the density and genetic characteristics of *P. sergenti* populations in two Moroccan localities, one endemic and another free of ACL.

**Material and methods**

### 2.1. Study area

El Borouj (coordinates 07o36'W-32o29'N) and Sidi Hajjaj (07o24'W-33o06'43"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, 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 21x29.5 cm sheets of papers covered in castor oil, 9-17 were set the same day in adult sand fly resting places (holes in house walls and on other nearby walls) and left for four days. The captured sand flies were stored in 70% alcohol. Male and female specimens were separated and morphologically identified using taxonomic keys (Rioux and Golvan, 1969; Rioux *et al.,* 1978; Leger *et al.,* 1983; El Sawaf *et al.,* 1989; Gil Collado *et al.,*1989; Benabdennbi *et al.,* 1999; Berchi *et al.,* 2007; Saez*et al.,* 2018). The specimens were placed in Marc Andre solution and heated to boiling point, and finally mounted on slides under a coverslip using Berlese solution. The genitalia of *P. sergenti*specimens was individually removed and mounted on slides under a coverslip for morphological identification whereas the rest of the body was stored at -20oC 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 (Martin-Sanchez *et al.,* 2000). A commercially available kit was used (RealPure kit from REAL (Ref. RBMEG01), according to the manufacturer instructions. Each sandfly 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).

For Restriction fragment length polymorphism (RFLP), digestion of the 550-bp mtDNA Cyt b fragment was carried out 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 (10X). 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 both localities, El Borouj and 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 min at 95 °C, then 36 cycles of 30 s at 95 °C and 60 s at 60 °C. The number of parasites in every qPCR reaction was calculated through the interpolation of the cycle threshold (Ct) value in a standard curve.

### 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 as a dependent variable the locality 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).

## 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 5,674 sand fly specimens, 2,215 females (39,0%) and 3,459 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 *Phlebotomus* and 4*Sergentomyia* ) and differences in density, relative abundance and frequency were detected between them and both 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 made with CDC traps (Table 1).

Sand fly density was higher in El Borouj and significant differences were found in the June peridomiciliary captures ($p < 0.0001$) and in the October intradomiciliary captures ($p = 0.066$). *P. sergenti* density was higher in El Borouj as well and statistically significant differences were detected in peridomiciliary captures in June ($p = 0.007$), intra domiciliary captures in October ($p = 0.007$) and global intradomiciliary ($p = 0.032$) and peridomiciliary ($p = 0.022$) (Table 1). Regarding difference between capture periods, *P. sergenti*intradomiciliary density was significantly higher in June in both cities, El Borouj (p=0.012) and Sidi Hajjaj (p= 0.020).

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

### 3.2. *Leishmania* infection rate in the vector

In El Borouj, 1 out of 112 (0.9%) female *P. sergenti* tested for*Leishmania* infection was positive for *L. tropica* using a multiplex qPCR technique. Parasite load was 7 parasites/µg DNA. The female was collected in a house with ACL cases.

*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 female *P. sergenti* from El Borouj in which *L. tropica*DNA was detected was identified as a lineage IV specimen. In addition, 4 positive females for *L. tropica* from captures made in 2014 (Gijón-Robles *et al.,* 2018) were also identified as lineage IV.

### 3.4. Bioclimatic differences between El Borouj and Sidi Hajjaj

Bioclimatic data were collected from 199 georeferenced points, 185 in El Borouj – 185 houses with ACL cases - and 14 in Sidi Hajjaj. Table 4 shows the average values, 95% confidence interval of the average, and minimum and maximum values of each of the bioclimatic variables in El Borouj and Sidi Hajjaj for which differences were detected. Statistically significant differences were detected at precipitation seasonality (BIO3, p=0.009) 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 the months of September and October, isothermality 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 (Rioux *et al.,*1986; Ramaoui *et al.,* 2008; Boussaa *et al.,* 2009). 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. *P. 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 undamaged locality of Sidi Hajjaj were made using CDC light traps and sticky papers.

*P. sergenti* density in the ACL free locality was lower than that of the endemic locality, both peridomiciliary 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 as males were more abundant in the sticky papers. In both localities, the female *P. sergenti* density was higher in the intradomiciliary June captures and all gonotrophic cycle categories, 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 (Rioux *et al.,* 1986; Ramaoui *et al.,*2008; Barón *et al.,* 2013) 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 from endemic to non-endemic cities raises the possibility of emerging foci in areas where *P. sergenti*populations are well established. Kholoud et al. in 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 main *P. sergenti* mitochondrial lineage in El Borouj is Lineage IV (97.6%) while the remaining 2.4% belongs to lineage II. In contrast, lineage II was the most abundant in Sidi Hajjaj (80%) followed by 20% lineage I specimens. Therefore, a different main *P. sergenti* mitochondrial lineage has been highlighted in each one of the 2 localities under study. *Phlebotomus sergenti* is characterised by high genetic diversity and classified in at least twenty haplotypes in four mitochondrial lineages (Yahia *et al.,* 2004; Barón *et al.,*2008).

In El Borouj, *L. tropica* DNA was detected in 5 out of 184 (2.7%) female *P. sergenti* (Gijón-Robles *et al.,* 2018) in captures made throughout 2014 (4+/72: 5.6% and up to 18,000 parasites/µg DNA; data not shown) and 2015 (1+/112: 0.9%). The five positive *L. tropica* females belonged to the most prevalent *P. sergenti*lineage in El Borouj, lineage IV. This is the first time that the mitochondrial lineage of a *P. sergenti* specimen that is acting as a vector for *L. tropica* has been identified. A local increase in the abundance of this *P. sergenti* lineage that seem transmits*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. in 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 leading geographical expansion into the rest of Europe. Similarly, there are bioclimatic differences between El Borouj and Sidi Hajjaj (Table 4) that could explain the over representation of *P. sergenti* lineage IV in El Borouj which is warmer and drier, and its absence in Sidi Hajjaj.

## 5. Conclusion

The density and genetic background of the *L. tropica*vector, *P. sergenti* , seem to play a pivotal role in the prevalence of ACL. Between these two localities, endemic and undamaged, there are differences in both the vector density and its main mitochondrial lineage that 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 justify the absence of ACL cases could be the absence of *P. sergenti* lineage IV, which seems to prefer warmer and drier climates.

### Acknowledgements

This study was funded by the University of Granada (Centro de Iniciativas de Cooperación al Desarrollo, CICODE, 2013).

**Conflict of Interest Statement**

We declare no competing interests.

**References**

Ajaoud, M., Es-sette, N., Hamdi, S., Laamrani El-Idrissi, A., Riyad, M. & Lemrani, M. (2013). Detection and molecular typing of*Leishmania tropica* from *Phlebotomus sergenti* and lesions of cutaneous leishmaniasis in an emerging focus of Morocco. Parasite & Vectors 6, 217. https://doi.org/10.1186/1756-3305-6-217

Al-Zahrani, M.A., Peters, W., Evans, P.A., Ching-Chin, I., Smith, V. & Lane, R.P. (1988). *Phlebotomus sergenti* , a vector of*Leishmania tropica* in Saudi Arabia. Transactions of the Royal Society of Tropical Medicine and Hygiene 82 (3), 416.

Amarir, F., Sebti, F., Fellah, H., Pratlong, F., Dedet, J.P., El Mansouri, B., Hmamouch, A., Delouane, B., Habbari, K., Sadak, A., Abassi, I. & Rhajaoui, M. (2015). Epidemiological characteristics of a new focus of cutaneous leishmaniasis caused by *Leishmania tropica*in Settat, Morocco. Acta Tropica 150, 116-121. https://doi.org/10.1016/j.actatropica.2015.07.020

Baghad, B., Razanapinaritra, R., Maksouri, H., El Bouri, H., Outlioua, A., Fellah, H., Lemrani, M., Akarid, K., Martin-Sanchez, J., Chiheb, S. & Riyad, M. (2020). Possible introduction of *Leishmania tropica*to urban areas determined by epidemiological and clinical profiles of patients with cutaneous leishmaniasis in Casablanca (Morocco). Parasite Epidemiology and Control 9, e00129. https://doi.org/10.1016/j.parepi.2019.e00129

Barón, S., Martín-Sánchez, J., Gállego, M., Morales-Yuste, M., Boussaa, S. & Morillas-Márquez, F. (2008). Intraspecific variability (rDNA ITS and mtDNA Cyt b) of *Phlebotomus sergenti* in Spain and Morocco. Acta Tropica 107, 259-267. https://doi.org/10.1016/j.actatropica.2008.07.003

Barón, S.D., Morillas-Márquez, F., Morales-Yuste, M., Díaz-Sáez, V., Gállego, M., Molina, R. & Martín-Sánchez, J. (2013). Predicting the risk of an endemic focus of *Leishmania tropica* becoming established in South-Western Europe through the presence of its main vector, *Phlebotomus sergenti* Parrot, 1917. Parasitology 140, 1413-1421. https://doi.org/10.1017/S0031182013000942

Benabdennbi, I., Pesson, B., Cadi-Soussi, M. & Morillas-Márquez, F. (1999). Morphological and isoenzymatic differentiation of sympatric populations of *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (Diptera: Psychodidae) in northern Morocco. Journal of Medical Entomology 36, 116–120. https://doi.org/10.1093/jmedent/36.1.116

Berchi, S., Bounamous, A., Louadi, K. & Pesson, B. (2007). Différenciation morphologique de deux espèces sympatriques: *Phlebotomus perniciosus* Newstead 1911 et *Phlebotomus longicuspis* Nitzulescu 1930 (Diptera: Psychodidae). Annales de la Société Entomologique de France (n.s.) 43, 201–203.

Boussaa, S., Pesson, B. & Boumezzough, A. (2009). Faunistic study of the sandflies (Diptera: Psychodidae) in an emerging focus of cutaneous leishmaniasis in Al Haouz province, Morocco. Annals of Tropical Medicine and Parasitology 103, 73-83. https://doi.org/10.1179/136485909x384910

El Sawaf, B.M., Mansour, N.S., El Said, S.M., Daba, S., Youssef, F.G., Kenawy, M.A &. Beier, J.C. (1989). Feeding patterns of*Phlebotomus papatasi* and *Phlebotomus langeroni* (Diptera: Psychodidae) in El Agamy, Egypt. Journal of Medical Entomology 26 (5), 497-498.

Esseghir, S., Ready, P.D., Killick-Kendrick, R. & Ben-Ismail, R. (1997). Mitochondrial haplotypes and geographical vicariance of*Phlebotomus* vectors of *Leishmania major.* Insect Molecular Biology 6, 211-225.

Gil Collado, J., Morillas Márquez, F. & Sanchís Marín, M.C. (1989). Los flebotomos en España. Revista de Sanidad e Higiene Pública 63, l5-34.

Gijón-Robles P, Abattouy N, Merino-Espinosa G, El Khalfaoui N, Morillas-Márquez F, Corpas-López V, Porcel-Rodríguez L, Jaaouani N, Díaz-Sáez V, Riyad M. & Martín-Sánchez J. (2018). Risk factors for the expansion of cutaneous leishmaniasis by *Leishmania tropica* : Possible implications for control programmes. Transboundary and Emerging Diseases 65 (6), 1615-1626. https://doi.org/10.1111/tbed.12914

Guilvard, E., Rioux, J.A., Gállego, M., Pratlong, F., Mahjour, J., Martínez-Ortega, E., Dereure, J., Saddiki, A., Martini, A., 1991.*Leishmania tropica* au Maroc. III. Ròle vecteur de*Phlebotomus sergenti* . Annales de Parasitologie Humaine et Comparée 66, 96-99.

Kholoud K, Bounoua L, Sereno D, El Hidan M. & Messouli M. (2020). Emerging and Re-Emerging Leishmaniases in the Mediterranean Area: What Can Be Learned from a Retrospective Review Analysis of the Situation in Morocco during 1990 to 2010? Microorganisms 8 (10), E1511. https://doi.org/10.3390/microorganisms8101511

Léger, N., Pesson, B., Madulo-Leblond, G. & Abonnenc, E. (1983). [Differentiation of females of the subgenus Larroussius Nitzulescu 1931 (Diptera-Phlebotomidae) of the Mediterranean region]. Annales de Parasitologie Humaine et Comparée 58 (6), 611-23.

Martín-Sánchez, J., Gramiccia, M., Pesson, B. & Morillas-Márquez, F. (2000). Genetic polymorphism in sympatric species of the genus*Phlebotomus,* with special reference to *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (Diptera, Phlebotomidae). Parasite 7, 247-254. https://doi.org/10.1051/parasite/2000074247

Merino-Espinosa, G., Corpas-López, V., Callejón-Fernández, R., Porcel-Rodríguez, L., Díaz-Sáez, V., Gállego, M., Ballart, C., Molina, R., Jiménez, M., Morillas-Márquez, F. & Martín-Sánchez, J. (2016). Differential ecological traits of two *Phlebotomus sergenti*mitochondrial lineages in southwestern Europe and their epidemiological implications. Tropical Medicine and International Health 21 (5), 630-41. https://doi.org/10.1111/tmi.12686

Merino-Espinosa G, Rodríguez-Granger J, Morillas-Márquez F, Tercedor J, Corpas-López V, Chiheb S, Alcalde-Alonso M, Azaña-Defez JM, Riyad M, Díaz-Sáez V. & Martín-Sánchez J. (2018). Comparison of PCR-based methods for the diagnosis of cutaneous leishmaniasis in two different epidemiological scenarios: Spain and Morocco. Journal of the European Academy of Dermatology and Venereology 32 (11), 1999-2003. https://doi.org/10.1111/jdv.15034

Ministry of Health, Morocco (2016). *Santé en chiffres 2015.*Ministry of Health, Morocco, 168pp.

Mouttaki, T., Morales-Yuste, M., Merino-Espinosa, G., Chiheb, S., Fellah, H., Martín-Sánchez, J. & Riyad, M. (2014). Molecular diagnosis of cutaneous leishmaniasis and identification of the causative*Leishmania* species in Morocco by using three PCR-based assays. Parasite & Vectors 4, **7,** 420. https://doi.org/10.1186/1756-3305-7-420

Ntais, P., Christodoulou, V., Tsirigotakis, N., Dokianakis, E., Dedet, J.P., Pratlong, F. & Antoniou, M. (2014). Will the introduction of*Leishmania tropica* MON-58, in the island of Crete, lead to the settlement and spread of this rare zymodeme? Acta Tropica 132, 125-130. https://doi.org/10.1016/j.actatropica.2014.01.003

Pratlong, F., Dereure, J., Ravel, C., Lami, P., Balard, Y., Serres, G., Lanotte, G., Rioux, J.A. & Dedet, J.P. (2009). Geographical distribution and epidemiological features of Old World cutaneous leishmaniasis foci, based on the isoenzyme analysis of 1048 strains. Tropical Medicine and International Health 14 (9) 1071–1085. https://doi.org/10.1111/j.1365-3156

Ramaoui, K., Guernaoui, S. & Boumezzough, A. (2008). Entomological and epidemiological study of a new focus of cutaneous leishmaniasis in Morocco. Parasitology Research 103, 859-63. https://doi.org/10.1007/s00463-008-1068-3

Rioux, J.A. & Golvan, Y.J. (1969). Épidémiologie des leishmanioses dans le sud de la France. *Institut National de la Santé et de la Recherche Médicale.* París (16e) 288-32-84.

Rioux, J.A., Lanotte, G., Petter, F., Dereure, J., Akalay, O., Pratlong, F., Velez, I.D., Fikri, N.D., Maazoun, R., Denial, N., Jarry, D.M., Zahaf, A., Ashford, R.W., Cadi-Soussi, M., Killick-Kendrick, R., Benmansour, N., Moreno, G., Périères, J., Guilvard, E., Zribi, M., Kennou, M.F., Rispail, P., Knechtli, R. &

Serres, E. (1986). 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. In*Leishmania . Taxonomie et Phylogenèse. Applications Eco-épidémiologiques* (ed. by JA Rioux), pp. 365-395. CNRS/INSERM/OMS,*Institut Méditerranéen d'Etudes Epidémiologiques et Ecologiques, Montpellier* .

Rioux, J.A.; Perieres, J.; Killick-Kendrick, R. Maistre, M. & Bayar, N. (1978). Confirmation de l´existence en Tunisie de *Sergentomyia antennata* (Newstead, 1912) (Diptera-Psychodidae). Annales de Parasitologie Humaine et Comparée 53, 4, 431-435.

Sáez, V.D., Morillas-Márquez, F., Merino-Espinosa, G., Corpas-López, V., Morales-Yuste, M., Pesson, B., Barón-López, S., Lucientes-Curdi, J. & Martín-Sánchez, J. (2018). *Phlebotomus langeroni* Nitzulescu (Diptera, Psychodidae) a new vector for *Leishmania infantum* in Europe. Parasitology Research 117, 1105–1113. https://doi.org/10.1007/s00436-018-5788-8

Schnur, L.F., Nasereddin, A., Eisenberger, C.L., Jaffe, C.L., El Fari, M., Azmi, K., Anders, G., Killick-Kendrick, M., Killick-Kendrick, R., Dedet, J.P., Pratlong, F., Kanaan, M., Grossman, T., Jacobson, R.L., Schonian G. & Warburg, A. (2004). Multifarious characterization of*Leishmania tropica* from a judean desert focus, exposing intraspecific diversity and incriminating *Phlebotomus sergenti* as its vector. American Journal of Tropical Medicine and Hygiene 70, 364-372.

Svobodova, M., Votypka, J., Peckova, J., Dvorak, V., Nasereddin, A., Baneth, G., Sztern, J., Kravchenko, V., Orr, A., Meir, D., Schnur, L.F., Volf, P. & Warburg, A. (2006). Distinct transmission cycles of *Leishmania tropica* in 2 adjacent foci, Northern Israel. Emerging Infectious Diseases 12, 1860-8. https://doi.org/10.3201/eid1212.060497

WHO (2016). Leishmaniasis in hight-burden countries: an epidemiological update based on data reported in 2014. Weekly Epidemiology Records 91 (22), 287-296.

Yahia, H., Ready, P.D., Hamdani, A., Testa, J.M. & Guessous-Idrissi, N. (2004). Regional genetic differentiation of *Phlebotomus sergenti*in three Moroccan foci of cutaneous leishmaniasis caused by*Leishmania tropica.* Parasite 11, 189-199.

### Hosted file

`Table 1.pdf` available at https://authorea.com/users/399677/articles/512158-understanding-the-factors-that-determine-the-emergence-of-anthroponotic-cutaneous-leishmaniasis-due-to-leishmania-tropica-comparison-of-the-density-and-mitochondrial-lineage-of-phlebotomus-sergenti-between-endemic-and-free-areas-in-morocco

### Hosted file

`Table 2.pdf` available at https://authorea.com/users/399677/articles/512158-understanding-the-factors-that-determine-the-emergence-of-anthroponotic-cutaneous-leishmaniasis-due-to-leishmania-tropica-comparison-of-the-density-and-mitochondrial-lineage-of-phlebotomus-sergenti-between-endemic-and-free-areas-in-morocco

### Hosted file

`Table 3.pdf` available at https://authorea.com/users/399677/articles/512158-understanding-the-factors-that-determine-the-emergence-of-anthroponotic-cutaneous-leishmaniasis-due-to-leishmania-tropica-comparison-of-the-density-and-mitochondrial-lineage-of-phlebotomus-sergenti-between-endemic-and-free-areas-in-morocco

### Hosted file

`Table 4.pdf` available at https://authorea.com/users/399677/articles/512158-understanding-the-factors-that-determine-the-emergence-of-anthroponotic-cutaneous-leishmaniasis-due-to-leishmania-tropica-comparison-of-the-density-and-mitochondrial-lineage-of-phlebotomus-sergenti-between-endemic-and-free-areas-in-morocco