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Phlebotomus langeroni Nitzulescu (Diptera, Psychodidae) a new vector for *Leishmania infantum* in Europe

Victoriano Díaz Sáez, AQ1 1,2✉

Phone +34958243859
Email diazsaez@ugr.es

F. Morillas-Márquez, 1

G. Merino-Espinosa, 1

V. Corpas-López, 1

M. Morales-Yuste, 1

B. Pesson, ~~3~~2

S. Barón-López, 1

J. Lucientes-Curdi, ~~4~~3

J. Martín-Sánchez, 1

~~1~~ University of Granada, Granada, Spain

~~2~~1 Departament of Parasitology, Faculty of Pharmacy, University of Granada, Campus de Cartuja sn, 18071 Granada, Spain

~~3~~2 Laboratory of Parasitology, University of Strasbourg, Strasbourg, France

~~4~~3 Department of Animal Pathology, Faculty of Veterinary, University of Zaragoza, Zaragoza, Spain

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Abstract

Burrows of the wild rabbit, *Oryctolagus cuniculus*, a lagomorph that has been recently suggested as a *Leishmania infantum* reservoir, constitute an unspoilt biotope in phlebotomine studies in Europe. We hypothesize that *Phlebotomus langeroni*, a proven vector of *L. infantum* in North Africa, is associated with rabbits and may have been overlooked in Europe. Sandfly captures were carried out with CDC light traps in an *L. infantum* endemic area of southern Spain with a high density of lagomorphs and a large numbers of burrows. The stable, permanent, and highly abundant presence of *P. langeroni* was assessed. After morphological identification, this sandfly species was characterized by comparing it with *P. perniciosus* and other *P. langeroni* populations from North Africa through molecular techniques. *P. langeroni* had not been found in southern Spain to date, despite being a highly investigated area, except for this particular biotope. Its activity period turned out to begin in mid-July, ending in late October, accounting for a maximum activity during this month. This study shows that *P. langeroni* is associated with the existence of rabbit burrows and has been overlooked in Europe. *L. infantum* DNA was found in almost half of the female specimens (47.6%) captured inside a biotope where wild rabbits are infected as well.

Keywords

Phlebotomus langeroni
Leishmania infantum
Europe
Wild rabbit burrows
Oryctolagus cuniculus

Introduction

Southern Europe is considered a hypoendemic zone for leishmaniasis although an increased number of the registered cases may occur through epidemic outbreaks such as the recent one that occurred in Madrid (Spain). Different factors related to the urbanization and changes in land use have been involved in the leishmaniasis re-emergence in this area where hares and rabbits have been implicated in the transmission of *Leishmania infantum* (Molina et al. 2012; Jiménez et al. 2014).

Eight *Phlebotomus* species have been incriminated as *L. infantum* vectors in the Mediterranean subregion that includes southern Europe, northern Africa, and parts of Asia: *P. perniciosus* Newstead, 1911; *P. ariasi* Tonnoir, 1921; *P. neglectus* Tonnoir 1921; *P. kandelakii* Shchurenkova, 1929; *P. perfiliewi* Parrot, 1930; *P. langeroni* Nitzulescu, 1930; *P. tobbi* Adler and Theodor, 1930; and *P. balcanicus* Theodor, 1958 (Léger et al. 1983; Doha and Shehata, 1992; Ready, 2010; Giorgobiani et al. 2012; Antoniou et al. 2013). All these species but *P. langeroni* have been implicated in the transmission of this *Leishmania* species in southern Europe.

P. langeroni was described for the first time among male sandfly specimens captured in the north of Tunisia (Nitzulescu, 1930). Small numbers of this species were demonstrated later in North African countries (Croset et al. 1978; Ghrab et al. 2005; Es-Sette et al. 2014); however, its presence was assessed only in the northern regions of such countries, but not in the southern parts (Rioux et al. 1986; Fares et al. 2015). A female specimen was first described in El Agamy, Alexandria (El Sawaf et al. 1989). The eastern most zone where this sandfly species has been found has been in Lebanon, where a single male specimen was identified among 43,570 sandflies captured (Haddad et al. 2003).

P. langeroni is a proven vector of *Leishmania infantum* since it meets all the established criteria to incriminate a species as vector (Killick-Kendrick, 1990); essentially, it is an anthropophilic sandfly that also feeds on dogs; its spatial distribution is associated with human cases of the disease; it becomes naturally infected by *L. infantum* and transmits the disease by bite (El Sawaf et al. 1989; Doha and Shehata, 1992; Guerbouj et al. 2007; Kassem et al. 2012). This vector species acquires its highest epidemiological relevance in El Agamy, Egypt, where a noticeable abundance was found (El Sawaf et al. 1989) and some specimens were found naturally infected by *L. infantum* (Doha and Shehata, 1992; Kassem et al. 2012).

In Europe, the presence of *P. langeroni* has only been reported in a small number in central and northern regions of Spain, always in the vicinity of rabbit burrows (Martínez Ortega et al. 1992; Lucientes et al. 1994). However, this sandfly species has not been found in southern Spain to date, despite being a highly investigated area (Morillas-Márquez et al. 1983, 2010; Martínez Ortega et al. 1996; Barón et al. 2011) and the most arid region of the Iberian Peninsula. It has not been found in other European countries either (Rioux et al. 2013; Alten et al. 2016). Leishmaniasis is endemic in southeastern Spain where a low number of visceral, cutaneous, and mucosal human cases are annually reported despite the high prevalence of canine leishmaniasis (seroprevalence of 13.0% and PCR

positivities of 49.8%) (Martín-Sánchez et al. 2004, 2009; Morales-Yuste et al. 2012). In addition, *L. infantum* infection has been reported in various host species, both domestic and wild, in some cases with high figures; the prevalence rate in wild rabbits (*Oryctolagus cuniculus*) is 20.7% (Martín-Sánchez et al. 2006; Díaz-Sáez et al. 2014; Navea-Pérez et al. 2015). *P. perniciosus* is the main vector showing high densities and an infection rate of 0.43% (Martín-Sánchez et al. 1994; Barón et al. 2011). Although much less abundant, *Phlebotomus ariasi* can act as a vector in sympatric conditions (Morillas et al. 1996).

We hypothesize that *P. langeroni* might be involved in the transmission of *L. infantum* in this area. In order to show that a potential vector of *L. infantum* associated with rabbits may have been overlooked in Europe, sandfly captures were carried out in wild rabbit burrows, an unspoilt biotope where no samplings had been made before.

Materials and methods

Study area

The study was conducted on a private farmland in the province of Granada in southeastern Spain (geographical coordinates 37° 17′ 18″ N, 3° 52′ 47″ W). Extending over 1000 ha, it is located in the mesomediterranean bioclimatic level (Rivas Martínez et al. 1987), at an altitude of 750–900 m above sea level, with an annual rainfall of 500 mm, and hot and dry summers. There was a high lagomorph density—11 rabbits per hectare, with an average life span of 2.5 years—and a large number of burrows, intended for shelter and reproduction. The farmland has private hunting areas, and hunting dogs are not allowed. There were seven shepherd or guard dogs, three of which were diagnosed with canine leishmaniasis through serology and parasite culture. The livestock, mainly sheep, were put out to pasture during the day and overnight in stables. The tree cover consisted mainly of olive trees in 45% of the estate and 10% pine and Holm oak on scrubland; the remaining 45% was destined to dry land crops such as wheat and barley and irrigated crops such as alfalfa. A stream runs through the estate.

Six sampling stations were established with a capture area of 30–50 m^2, a distance between them of more than 400 m, and differences in orientation, type of irrigation, main vegetation, and the presence of dogs or domestic animals. One of the stations lacked rabbit burrows within its perimeter, but they were in its surroundings (sampling station E-5); in the remaining five stations, there were a high number of burrows (stations E-1 to E-4, and E-6).

Capture and morphological identification of the sandflies

Sandflies were collected during the months of July, September, October, and November, annually from 2008 to 2010. CDC traps were placed in the six specified sampling stations every 15 days hanging 0.5–1 m above the entrance of the rabbit burrows (Fig. 1), except for one of them that lacked burrows within its perimeter (E-5). The traps were set in the evening, and they were collected the following morning. Sticky traps were not used given that in previous tests, these were disassembled by the rabbits. The captured sandflies were removed and mounted using Berlese solution. Morphological classification was carried out in accordance with Leger et al. (1983); El Sawaf et al. (1985); Gil Collado et al. (1989), via examination with optical microscope, particularly considering the spermatheca in females and the external genitalia in males. The following morphological characteristics were used to identify *P. langeroni*: males with aedeagus finished in bevel and antennal formula of 2/3 − 11/(12), 1/12 (13) − 15; females with spermatheca with 9–14 well-defined segments, those in the middle larger than end segments, and individual ducts of uniform diameter until terminal where they widen progressively.

Fig. 1

CDC trap placed hanging 0.5–1 m above the entrance of some rabbit burrows where *Phlebotomus langeroni* was catched

Gonotrophic conditions of female sandflies were identified as engorged, non-engorged, and gravid (Wagué et al. 2016).

Some *P. langeroni* specimens that were described elsewhere (Lucientes et al. 1994), captured in the town Burgo de Ebro (Zaragoza, northern Spain) were also

included in this study in order to perform the molecular characterization of the European populations of this species.

Data analysis

Density (sandflies/trap/night), abundance (% specimens of a given species/total sandflies), and frequency (% positive sampling stations for a given species) data were estimated.

The identification of the factors associated with the presence and density of *P. langeroni* was performed through logistic regression using the statistical package IBM SPSS Statistics 20. Data was collected from each of the sampling sites via notes made on-site with a personal digital assistant (PDA) for their use as variables. A p value $\leq 0{\cdot}05$ was considered significant for association.

Sandfly DNA extraction

A portion of the body was processed individually for genomic DNA extraction as described elsewhere (Barón et al., 2008). Briefly, each sandfly portion was placed in a 1.5-ml Eppendorf tube and kept in liquid nitrogen for a few seconds to facilitate the rupture of cell membranes. A commercially available kit was used (RealPure Genomic DNA Extraction kit: REAL Durviz S. L., Valencia, Spain), according to the manufacturer instructions. The DNA was re-suspended in 20 µl of bidistilled water and kept at − 20 °C until use.

Infection of female sandflies by *L. infantum*

L. infantum DNA was detected in female sandflies of *P. perniciosus* and *P. langeroni* through the amplification of the parasite kDNA using a PCR-ELISA technique specific for *L. infantum*. It was performed following the protocol described elsewhere (Martín-Sánchez et al. 2001) using kits PCR-ELISA DIG Labeling and PCR-ELISA DIG Detection (Roche Diagnostics GmbH, Mannheim, Germany). In the PCR, primers 9 (forward): 5′-CAAAAGTCCCCACCAATCCC-3′ and 83 (reverse): 5′-AAACCCTGGTCTGGAGGCTTAG-3′ amplify a 75-bp fragment belonging to the variable region of the *L. infantum* kDNA minicircle using 3 and 5 µl of re-suspended DNA. The amplified fragment was sprinkled with digoxigenin due the use of dUTP labeled with this hapten. It was detected on a streptavidin-coated microtiter plate, through hybridization with the oligonucleotide probe specific to *L. infantum* 5′-CCA AAC AGG GCA AAA ACC-3′, labeled at the 5′ end with biotin, followed by ELISA using a peroxidase-labeled anti-digoxigenin antibody and ABTS as substrate. The results were read in a spectrophotometer at a λ of 405 nm with an OD threshold of 1. DNA obtained from a male and from 1000 *L.*

infantum promastigotes were used as negative and positive controls, respectively. The absorbance values obtained were always less than 0.1 for male sandfly DNA and more than 2.5 for the positive control.

Twenty one *P. langeroni* female specimens captured in October from all 3 years and 20 females from *P. perniciosus* from the same biotope and capture dates were analyzed using this molecular approach.

Molecular characterization of *P. langeroni* populations

PCR amplification

Polymerase chain reaction was used to amplify a fragment of approximately 500 bp containing the 3′ end of the mitochondrial DNA (mtDNA) cyt b following the methodology described by Esseghir et al. (1997). The reaction was carried out in a final reaction volume of 25 μl, containing 2 μl of genomic DNA, 100 μM of each dNTP, 1 μM of each primer, 2.5 μl of 10× reaction buffer, 2 mM $MgCl_2$, and 1.25 U Taq DNA polymerase.

Eleven *P. langeroni* specimens (7 from southern Spain and 4 from Zaragoza, northern Spain) and 7 *P. perniciosus* specimens from the same biotope in southern Spain were characterized using this molecular approach.

Sequencing and comparative sequence analysis

Amplified PCR products were eluted from agarose gel using Real Clean Spin kit (REAL Durviz S. L., Valencia, Spain). Purified PCR products were bi-directional sequenced using the primers for DNA amplification. Sequences were edited and aligned to identify haplotypes (=unique sequences) using Clustal X 1.81 software and manually adjusted, if necessary. Two published sequences from *P. langeroni*, and various sequences published of other species described in southern Spain (*P. perniciosus*, *P. ariasi*, and *P. sergenti*) obtained from GenBank have also been included in the study.

The analysis was performed using PHYLIP version 3.65 (http://evolution.genetics.washington.edu/phylip) for the construction of trees. Maximum parsimony analysis and analysis based on distance matrices (NJ and UPGMA) were carried out; for the latter, we used the F84 model of nucleotide substitution (the default method). Robustness of the internal branches was tested by bootstrap analysis from 1000 bootstrap replications using the heuristic search option and retaining groups compatible with the 50% majority rule consensus tree. *Phlebotomus papatasi* was used as outgroup.

Results

Specific richness, frequency, density, and abundance of Phlebotomine species

Six sandfly species were present in this biotope: the five species commonly found in southeastern Spain (*P. perniciosus*, *P. papatasi*, *P. ariasi*, *P. sergenti*, and *Sergentomyia minuta*) and the species *P. langeroni.*

P. perniciosus and *S. minuta* were the most frequent species, thus finding them in 100% (6/6) of the established sampling stations. *P. langeroni* was found in 5 out of 6 stations (frequency of 83.3%; no specimens were captured in E-5) followed by *P. papatasi*, *P. sergenti*, and *P. ariasi*, with frequencies of 66.7% (4/6), 50% (3/6), and 33.3% (2/6), respectively (Table 1). Throughout the 3-year sampling, 180 traps were placed in the specified stations since early July to late November. During these 3 years, no *P. langeroni* specimens were captured until mid-July or during the last sampling month, when 38 CDC traps were used. Neither was the sampling effort the same in the different stations nor in the different months and years. It was smaller in the stations E-4, E-5, and E-6 due to the smaller number or even absence of rabbit burrows, as well as in 2009 and in September. Only stations E-1 and E-3 which had a greater number of rabbit burrows were sampled each month of each one of the 3 years.

Table 1

Phlebotomine sand flies collected by CDC light traps: density, abundance and frequence fr the set of the six sampling stations (E-1 to E-6)

Station	CDC(N)	Pp	Pa	Ppa	Ps	Sm	Plan	Total	Sandflie trap/nig
E-1	44	319	4	30	0	43	158	554	12.60
E-2	32	175	0	41	4	34	62	316	9.90
E-3	46	511	13	25	6	183	70	808	17.56
E-4	8	68	0	4	2	25	11	110	13.75
E-5	5	13	0	0	0	6	0	19	3.80
E-6	7	107	0	0	0	36	6	149	21.28
Total	142	1193	17	100	12	327	307	1956	13.77
Dens	–	8.40	0.12	0.70	0.08	2.30	2.16	–	–
Abun	–	61.00	0.87	5.11	0.61	16.72	15.70	–	–
Freq	–	100%	33.3%	66.7%	50%	100%	83.3%	–	–

Pp, *Phlebotomus perniciosus*; *Pa*, *Phlebotomus ariasi*; *Ppa*, *Phlebotomus papatasi*; *Ps*, *I* *Sergentomyia minuta*; *Plan*, *Phlebotomus langeroni*; *Dens*, density; *Abun*, abundance; *Fr*

A total of 1956 sandflies (1037 male and 919 female) were captured in the 142 CDC light traps that were placed between mid-July and late October finding a mean density of 13.77 sandflies/trap/night (Table 1). The highest sandfly density was obtained in 2010, accounting for 16.84 sandflies/trap, whereas the lowest sandfly density was found in 2008, with 10.0 sandflies/trap (Table 2).

Table 2

Total number of *Phlebotomus langeroni* and sandfly specimens captured in CDC light traps year and monthly

		CDC number	Total *N* *P. langeroni*	*P. langeroni*/trap/night	Total *N* sandfly	Sandfly/
	2008	58	181	3.12	580	10.00
Year	2009	34	58	1.71	534	15.70
	2010	50	68	1.36	842	16.84
	July	56	30	0.54	567	10.13
Month	September	28	26	0.93	620	22.14
	October	58	251	4.33	769	13.26

N, number

P. perniciosus was the most abundant species (61.0%), and it also showed the highest density (8.40), followed by *S. minuta* (16.72%, 2.30) and *P. langeroni* (15.70%, 2.16) (Table 2).

The highest percentage of *P. langeroni* specimens was found in sampling station E-1 (28.52%), also showing the greatest density (3.59 Plan/trap/night) This station is the only one provided with sprinkler irrigation, and it was assessed that *P. langeroni* density is positively associated to this sort of irrigation ($p = 0.034$). Station E-5 is the only one where *P. langeroni* was not captured. This sampling station lacks rabbit burrows or rabbit population within its perimeter, which would be a necessary condition for the presence of this sandfly species.

Results shown in Table 2 indicate that the *P. langeroni* activity peak takes place in October, when more than 4 specimens/trap/night were captured; nevertheless, the maximum sandfly density was found in September. Similarly, the highest *P. langeroni* density was noticed in 2008 (3.12 *P. langeroni*/trap/night) without

correspondence with the maximum density of sandflies that was detected in 2010 (Table 2).

Infection of *P. langeroni* by *L. infantum*

Of 1 engorged and 20 non-engorged and non-gravid *P. langeroni* females checked for the presence of *L. infantum* DNA, 6 showed optical density (OD) values ≥ 2.5, in agreement with the *L. infantum* positive controls bearing DNA equivalent to 1000 parasites. Eleven specimens showed OD < 0.1, in accordance to negative controls (male *P. langeroni* specimens). Four specimens showed intermediate values with 3 μl DNA and values over the threshold (OD ≥ 1) with a higher DNA quantity (5 μl). Of five engorged and 15 non-engorged and non-gravid *P. perniciosus* females, one non-engorged was positive (OD value ≥ 2.5). These analyses were performed in triplicate, carrying out three independent experiments, obtaining similar results. As a result, *L. infantum* DNA was detected in 10 out of the 21 *P. langeroni* (47.6%) and 1 out of the 20 *P. perniciosus* female specimens (5.0%) analyzed through PCR-ELISA, thus indicating an infection by this parasite of *P. langeroni.*

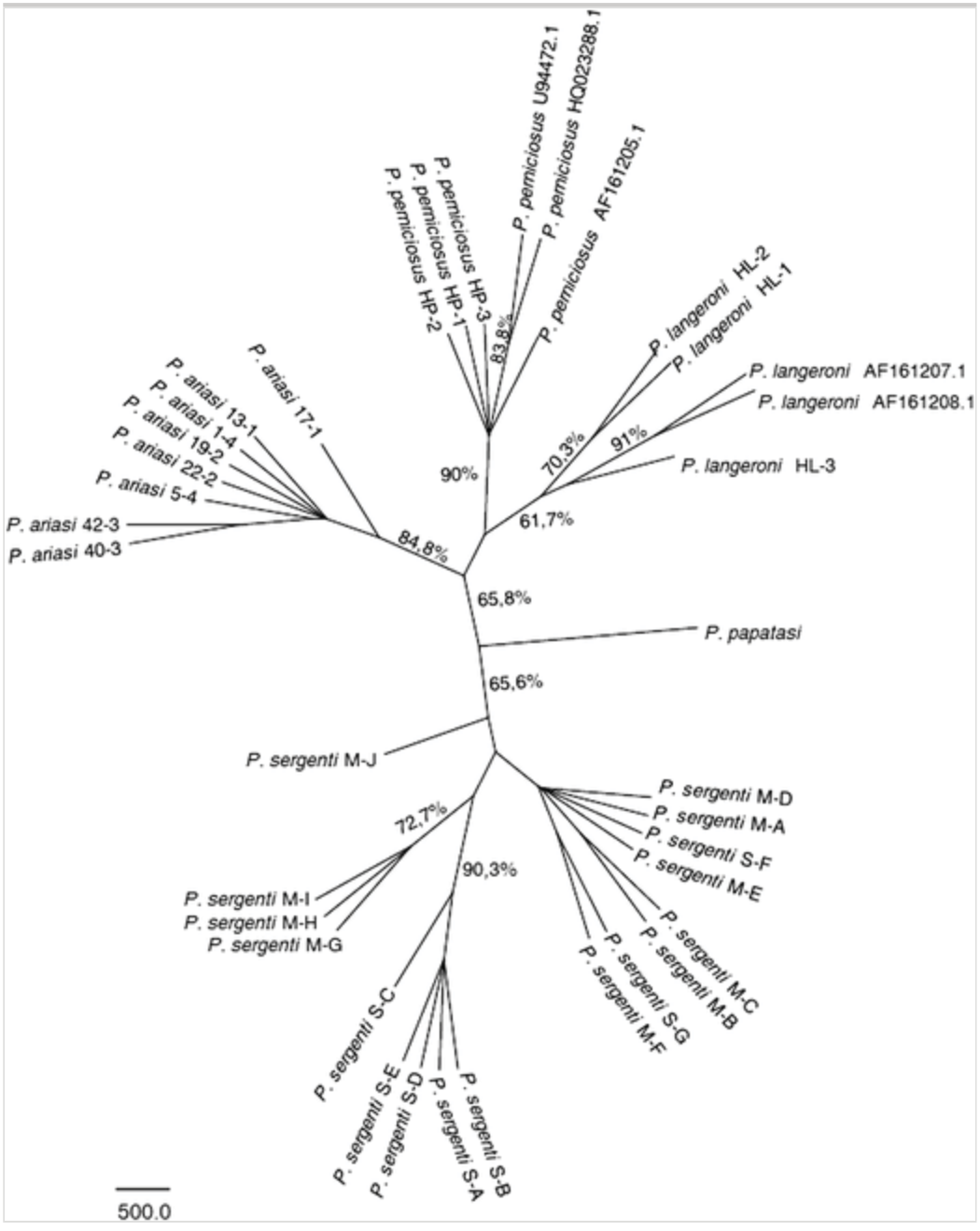
Molecular characterization of *P. langeroni*

For *P. langeroni*, the mtDNA cyt b fragment amplified has a size of 525 bp, while 546 bp for *P. perniciosus*. The comparative analysis of the 11 *P. langeroni* sequences of Spanish origin revealed a similarity of 99–100% and the existence of three haplotypes (two of them in specimens from southern and one from northern Spain) that vary in 2 to 6 bases in a 509-bp fragment (0.4–1.2%). These sequences (accession number: LT223557, LT223558, LT223559) were compared to another two published in GenBank corresponding to a specimen captured in Egypt and other specimens from Tunisia, between which three nucleotides differ. Between European and African specimens, 6 to 11 different bases were detected (similarity of 98–99%). Among 7 *P. perniciosus* specimens from the same biotope of southeastern Spain, three haplotypes were identified differing in one base (accession number: LT223560, LT223561, LT223562). The similarity between *P. perniciosus* and *P. langeroni* sequences was 94–95%; both species differ in 25–29 bp of the 510-bp fragment.

Figure 2 shows the relationship of *P. langeroni* populations between them and with other European sandfly species: *P. langeroni* specimens are integrated in the group that represents the subgenus *Larroussius* showing a clear separation from *P. perniciosus* which is constituted of specimens captured in the same biotope and other places. A closer relationship with this last species than with *P. ariasi* is observed.

Fig. 2

Maximum parsimony tree. Robustness of the internal branches was tested by bootstrap analysis. *Phlebotomus langeroni* HL-1 and HL-2 haplotypes were captured in southeastern Spain and HL-3 belongs to northern Spain. *Phlebotomus perniciosus* HP-1, HP-2, and HP-3 captured in the same biotope of *P. langeroni* HL-1 and HL-2. Sequences of *P. sergenti* and *P. ariasi* were described by Barón et al. (2008) and Franco et al. (2010), respectively, in southeastern Spain. *Phlebotomus papatasi*, captured in the same area, was used as an outgroup. Topology of UPGMA tree was similar, but bootstrap values were higher (data not shown)



Discussion

The presence of *P. langeroni* in southern Spain is surprising, in spite of the fact that it was found in small numbers in Spain several years ago (Martínez Ortega et al. 1992; Lucientes et al. 1994). As mentioned above, *P. langeroni* is considered characteristic of North Africa where it acts as a vector of *L. infantum* (Doha and Shehata, 1992; Guerbouj et al. 2007). Southeastern Spain is the most arid region of the Iberian Peninsula, and its sandfly fauna has been intensely sampled. In the last 35 years, hundreds of sampling stations have been surveyed, particularly in retaining wall holes and stables but this species has not been described to date (Rioux et al. 1986; Morillas-Márquez et al. 2010; Barón et al. 2011). Identical outcomes have been reported in other regions of Spain (Gálvez et al. 2011; Ballart et al. 2014), France, and Portugal (Rioux et al. 2013; Alten et al. 2016). Similarly, no specimens of *P. langeroni* were caught among the 99,000 sandflies collected by Alten et al. in 2016 throughout an area spanning from Portugal at west to Georgia at east. This finding leads to the hypothesis that the features of the biotope determine the presence of this vector species, in particular the presence of rabbit burrows. Several facts suggest that *P. langeroni* is not a species typical of arid regions: 1. It has not been found in the driest zones of the southern regions of North African countries (Rioux et al. 1986; Barhoumi et al. 2015; Ouanaimi et al. 2015); 2. its presence in a zone of the mesomediterranean bioclimatic level, with a pluviometry above 500 mm (Rivas Martínez et al. 1987); 3. its highest density in October, when mean temperature is markedly lower than that in summer; 4. its highest density in 2008, when maximum and minimum temperatures in October were considerably lower than those in 2009 and 2010 (temperature data obtained from Consejería de Medio Ambiente, Junta de Andalucía, Spain); and 5. the positive association between density and sprinkler irrigation.

P. langeroni activity period seems notably shorter than that of other vector species of southern Spain that are captured between April and November, which suggests that its temperature requirements are more stringent. Other authors provided evidence for the vulnerability of this species in the assessment of the drastic effect of urban development on its abundance in a former focus of visceral leishmaniasis in El Agamy, Egypt (Kassem et al. 2012).

The beveled shape of the male aedeagus and the shape of the spermatic ducts of the female without an accessory gland allow to easily distinguish *P. langeroni* from *P. perniciosus* catched in the same biotope. Even so, it is interesting to confirm this differentiation with molecular methods. Molecular characterization of Spanish *P. langeroni* population and its comparison with cyt b sequences from African specimens shows a 98–99% of similarity, thus ~~which confirms~~confirming its morphological identification and the presence of *P.*

langeroni in Spain. A clear differentiation of *P. langeroni* from the species *P. perniciosus* is noted, showing a difference in 25–29 bp of the 510-bp fragment, in agreement with a previous study (Schuh, 1999), ~~which~~that corroborates morphological discrimination. The major relationship of both species with *P. ariasi* has been previously reported by other authors (Esseguir and Ready, 2000).

The presence of three haplotypes in 11 Spanish *P. langeroni* specimens, with the addition of two African haplotypes, demonstrated the variability of this species. Mitochondrial DNA shows a mutation rate higher than nuclear DNA; and maternal inheritance and the absence of gene-pool homogenization by recombination tend to increase the geographical divergence. However, a close relationship between *P. langeroni* populations from North Africa (AF) and Spain (HL) was evidenced in this study (Fig. 2). In North Africa, *P. langeroni* has been captured in dumping sites, farmyards, and inside houses (El Sawaf et al. 1989; Guerbouj et al. 2007), which might imply a change of behavior in contrast to the populations of southern Spain, involving genetic differences (6 to 11 different bases). On the other hand, the abundance of this species in our captures (approximately 15%), similar to that reported in Tunisia (Guerbouj et al. 2007), and slightly higher than that found in Alexandria region, in northern Egypt (El Sawaf et al. 1989), where its vectorial relevance has been pointed out in a focus of human leishmaniasis, is noticeable.

The analysis of the 21 female *P. langeroni* through PCR-ELISA revealed that in 10 specimens (47.6%), *L. infantum* DNA was found. This PCR has been extensively used in epidemiological surveys in Southern Spain showing a great reliability (Martín-Sánchez et al. 2009; Morales-Yuste et al. 2012; Díaz-Sáez et al. 2014; Navea-Pérez et al. 2015). Given that this study mainly focused on non-fed female sandflies, the presence of *L. infantum* DNA in 9 non-engorged and non-gravid specimens should be attributed to the real and active presence of the parasite. The prevalence of infection in *P. perniciosus* was 5.0%. Although the high figure of *P. langeroni* infection seems unexpected, it is crucial to consider that 1. PCR techniques are more sensitive than classic techniques such as dissections, particularly those carried out on individual female sandflies (Martín-Sánchez et al. 2006; Alcover et al. 2012), and 2. *P. langeroni* inhabits a biotope where 20.7% rabbits are infected, and the parasite is located in the skin and blood of these lagomorphs, accessible sites for the sandfly (Díaz-Sáez et al. 2014). These facts indicate that *P. langeroni* plays a remarkable role in the maintenance of *L. infantum* in the wild together with *P. perniciosus*.

In this regard, it is worth to mention that wild rabbits (*O. cuniculus*) are widely distributed across the whole of southwestern, central, and northern Europe. This species is native to the Iberian Peninsula, and its presence and abundance gave

rise to the name of Spain. At particular times, this animal has been considered a pest throughout Europe, ~~which puts forward~~that reinforces the need of performing new epidemiological studies that consider these biotopes.

Conclusions

P. langeroni is associated with the existence of rabbit burrows and has been overlooked in Europe. *L. infantum* DNA was found in almost half of the female specimens (47.6%) captured inside a biotope where wild rabbits are infected as well.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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