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Morphology does not allow differentiating the species of the *Phlebotomus perniciosus* complex: Molecular characterization and investigation of their natural infection by *Leishmania infantum* in Morocco

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Abstract

Morphological and DNA-based complemented approaches were applied for characterization of sympatric populations of *Phlebotomus longicuspis* and *Phlebotomus perniciosus* in Morocco. Both sand fly species are generally recorded in sympatry in North Africa but on few occasions have been molecularly characterized. The diagnostic confusion of these species has led to errors in their geographical distribution and probably, in the assignment of their role in the transmission of *L. infantum*. Sand flies were caught inside households in El Borouj, central Morocco, in 2014–2015. For female sand flies, detection of *L. infantum* natural infection and blood meal identification were carried out. According to morphological identification, *Phlebotomus longicuspis* s.l. (34.7%) was the second most abundant *Phlebotomus* species after *P. sergenti*, followed by atypical *Phlebotomus perniciosus* (7.1%); 11.6% of the male specimens of *P. longicuspis* s.l. were identified as *P. longicuspis* LCx according to the number of coxite setae. The density of *Larroussius* species was very high (31 *Larroussius*/light trap/night) in the peripheral neighbourhood of Oulad Bouchair ($p = 0.001$) where the first case of cutaneous leishmaniasis due to *Leishmania infantum* was detected in 2017. Phylogenetic trees based on three independent genes highlighted three well-supported clusters within *P. perniciosus* complex that could be interpreted as corresponding to *P. perniciosus*, *P. longicuspis* s.s. and an undescribed species, all coexisting in sympatry. Some females with typical morphology of *P. longicuspis* were genetically homologous to *P. perniciosus*. The taxa cannot be differentiated by morphological methods but characterized by a distinctive genetic lineage for which the synapomorphic characters are described. *Leishmania infantum* was detected in females of all clusters with a low parasite load. Population genetics will help to assess the threat of the geographical spread

of *L. infantum* in Morocco by determining the density, abundance and vector role of the species of the *P. perniciosus* complex identified correctly.

KEYWORDS
diagnostic confusion, genetic lineages, *Leishmania infantum* vectors, *Phlebotomus perniciosus* complex, sympatric populations, synapomorphic characters

1 | INTRODUCTION

Cutaneous leishmaniasis (CL) is a parasitic disease that is endemic in more than 70 countries, with an incidence of 1.2 million cases per year. The World Health Organization included four Maghrebi countries (Algeria, Tunisia, Libya and Morocco) among the countries with a high CL burden in its latest report (WHO, 2021). The sand fly fauna is very diverse in these countries and many species are incriminated in the transmission of the three endemic *Leishmania* species: *Leishmania major*, *L. tropica*, (both dermotropic) and *Leishmania infantum*. The latter is a causative agent of visceral, cutaneous and mucosal human leishmaniasis as well as canine leishmaniasis. Haematophagous females of subgenus *Larroussius* Nitzulescu, 1931 such as *Phlebotomus perniciosus* Newstead, 1911, *Phlebotomus perfiliewi* Parrot, 1930, *Phlebotomus ariasi* Tonnoir, 1921 and *Phlebotomus langeroni* Nitzulescu, 1930 are *L. infantum* proven vectors whereas *Phlebotomus longicuspis* Nitzulescu, 1930 is a suspected vector (Benallal et al., 2022).

Phlebotomus perniciosus is widely distributed in the western Mediterranean basin from subhumid to arid bioclimate zones being the main vector of *L. infantum* (Alcover et al., 2014; Ballart et al., 2014; Benabdennbi et al., 1999; Martín-Sánchez et al., 1994). *Phlebotomus longicuspis* is only present in North Africa where it is most frequent in the semi-arid, arid and peri-arid bioclimatic zones (Benallal et al., 2022; Martín-Sánchez et al., 2000; Pesson et al., 2004). In the Maghreb region, both species are generally found in sympatry from the subhumid to Sahara bioclimatic zones (Benallal et al., 2022). Morphological examinations of *P. perniciosus* males revealed the existence of two morphotypes: a typical form with bifid copulatory valves and an atypical form with single tipped copulatory valves sharply curved at their apex; some specimens can show mixed features as well (Benabdennbi et al., 1999; Martín-Sánchez et al., 2000; Morillas Márquez et al., 1991; Pesson et al., 2004). Atypical specimens have been misidentified as *P. longicuspis* until morphological diagnosis had been resolved by isoenzyme and genetic studies (Martín-Sánchez et al., 2000; Pesson et al., 2004). This misinterpretation has likely led to mistakes in the geographical distribution of these species and their involvement in the transmission of *L. infantum* in the area (Guernaoui et al., 2005; Martín-Sánchez et al., 2000).

Furthermore, isoenzyme and mitochondrial DNA analysis of *P. longicuspis* specimens from Morocco have revealed that this species does not have the genetic characteristics of a single, reproductively isolated species, but two sibling species, named *P. longicuspis* s.s. and *P. longicuspis* LCx (Boussaa et al., 2008; Pesson et al., 2004).

Impacts

- Diagnostic confusion has led to errors in the geographical distribution and vector role of two species.
- We describe how to recognize them correctly.
- This will help to assess the threat of the geographical spread of *Leishmania infantum* in North Africa.

Phlebotomus perniciosus and *P. longicuspis* females are differentiated morphologically through the examination of the dilatation in the distal part of the spermathecal ducts, finding a relatively thick- or thin-walled bulb respectively. No morphological variation and intermediate aspects have been found (Berchi et al., 2007; Guernaoui et al., 2005; Léger et al., 1983). Therefore, the females of two forms of *P. perniciosus* and those of two *P. longicuspis* sibling species are considered, respectively, morphologically indistinguishable.

Atypical *P. perniciosus* and *P. longicuspis* s.l. are widespread in Morocco, Tunisia and Algeria whereas typical *P. perniciosus* is limited to the north of these countries (Benallal et al., 2017; Boudabous et al., 2012; Zarrouk et al., 2016). Depaquit et al. in 2005 recorded for the first time the presence of *P. longicuspis* s.l. south of the Sahara. The distribution of *P. longicuspis* LCx is unknown (Zarrouk et al., 2016). According to these authors, the typical form of *P. perniciosus* and *P. longicuspis* s.l. would be involved in *L. infantum* transmission whereas the epidemiological role of the atypical *P. perniciosus* and *P. longicuspis* LCx must be put into question.

Given the possibility of phenotypic differences of biomedical importance between these taxa, more extensive characterization of their populations could help assess their vectorial capacity. Mitochondrial DNA fragments such as cytochrome oxidase I (COI) and cytochrome *b* (Cyt*b*) are efficient markers to distinguish the two *P. perniciosus* morphotypes and the closely related *P. longicuspis* s.l. (Boudabous et al., 2012). We also selected a nuclear marker, the internal transcribed spacer 2 region (ITS2) of nuclear ribosomal DNA. Therefore, our objective was to characterize sympatric populations of the species of the *P. perniciosus* complex in an area of Morocco and investigate their natural infection by *L. infantum*.

2 | MATERIALS AND METHODS

2.1 | Study area

El Borouj (coordinates 07°36′W–32°29′N) is situated at an altitude of 410 m above sea level in the Settat province, central Morocco. The population is around 20,000 inhabitants and the average growth rate is close to 2%. Anthroponotic cutaneous leishmaniasis (ACL) by *L. tropica* is endemic in El Borouj while no cases of human visceral or cutaneous leishmaniasis due to *L. infantum* have been described (Ministry of Health, Morocco, 2016). In 2017 we detected a case of cutaneous leishmaniasis due to *L. infantum* in the Oulad Bouchair neighbourhood using a Granaleish Multiplex qPCR (University of Granada, Spain, Trade Mark Number 3667362/5).

2.2 | Ethical approval statement

The project was approved by the Ethics Committee of the University of Granada and by the Moroccan Ministry of Health. The study and its procedures were explained to the mayor and heads of local healthcare centre and households in El Borouj. Written consent was obtained from the families who agreed to let us place the traps in their homes.

2.3 | Sand fly collection and morphological identification of the species

Sand flies were caught using CDC light traps inside households in 2014–2015. Houses with and without ACL cases distributed in nine neighbourhoods were sampled. CDC traps were set in each selected house (one to two per house) for one night under favourable weather conditions. Males and females were separated and morphologically identified using taxonomic keys (Gijón-Robles et al., 2018). For molecular studies, the genitalia of *Larroussius* 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 number of setae on the two inner faces of the coxites was used to differentiate *P. longicuspis* LCx (LCx, 19–22 setae) and *P. longicuspis* s.s (LC, 21–31 setae) whereas male copulatory valves were used to differentiate atypical and typical form *P. perniciosus* specimens, both with 10–16 coxite setae (PN) (Pesson et al., 2004).

Density (sand flies/trap/night), abundance (% specimens of a given species/total sand flies) and frequency (% positive sampling stations for a given species) data were calculated. Mean density values, 95% confidence interval (CI 95%) of the mean density, and minimum and maximum values of density in the sampled households were calculated for the peripheral neighbourhood of Oulad Bouchair compared to the other neighbourhoods in El Borouj. Statistical significance (*p* value) and odds ratio were determined by logistic regression. Software package IBM SPSS Statistics version 21.0 was used for the statistical analysis.

2.4 | Sand fly DNA extraction

Genomic DNA was extracted from the head, thorax and attached anterior abdomen of individual *Larroussius* 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 mechanical rupture of the tissues using a pestle. The DNA was resuspended in 20 µL bidistilled water and kept at −20°C until use.

2.5 | PCR amplification for molecular characterization of *Phlebotomus perniciosus* complex species

Polymerase chain reaction (PCR) was used to amplify: (1) a fragment of 418–433 bp from the nuclear ribosomal DNA internal transcribed spacer 2 region (ITS2) using C1a (5′CCTGGTTAGTTTCTTTTCCTCCGCT3′) and JTS3 primers (5′CGCAGCTAACTGTGTGAAATC3′) under the following thermal profile: 94°C for 5 min; 35 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min; 72°C for 10 min (Depaquit et al., 2000); (2) a 550 bp fragment from the mitochondrial DNA (mtDNA) Cyt*b* gene using forward primer CB3-PDR (5′CA(T/C)ATTCAACC(A/T)GAATGATA3′) and the reverse primer N1N-PDR [5′GGTA(C/T)(A/T)TTGCCTCGA(T/A)TTCG(T/A)TATGA3′] under the following thermal profile: 94°C for 3 min; 5 cycles of 94°C for 30 s, 40°C for 30 s and 72°C for 1.5 min; 35 cycles of 94°C for 30 s, 44°C for 30 s and 72°C for 1.5 min; 72°C for 10 min (Ready et al., 1997); (3) a fragment of 700 bp from the mtDNA COI gene using LepF (5′ATTCAACCAATCATAAAGATATTGG3′) and LepR (5′TAAACTTCTGGATGTCCAAAAAATCA3′) primers (Hajibabaei et al., 2006) under the following thermal profile: 94°C for 5 min; 5 cycles of 94°C for 30 s, 45°C for 90 s and 72°C for 1 min; 35 cycles of 94°C for 30 s, 51°C for 1.5 min and 72°C for 1 min; 72°C for 10 min. The reaction was carried out in a final reaction volume of 25 µL, containing 2 µL genomic DNA, 100 µM dNTPs, 1 µM primers, 2.5 µL 10× PCR buffer, 2 mM $MgCl_2$ and 1.25 U Taq DNA polymerase as reported elsewhere (Barón et al., 2008).

2.6 | Sequencing and comparative sequence analysis

Amplified PCR products were eluted from agarose gel using Real Clean Spin kit (Zymoclean™ Gel DNA Recovery Kit, Cat No D4007), according to the manufacturer's protocol. Direct cycle sequencing of the PCR product was performed in both directions by an automated sequencer (3130XL from Applied Biosystems) using the primers used for DNA amplification.

Sequences were aligned using the multiple alignment program CLUSTALX 2.0 and manually adjusted. Analysis was performed using PHYLIP version 3.69 (http://evolution.genetics.washington.edu/phylip)

and MEGAX (https://www.megasoftware.net/) for each gene separately and for concatenate sequences. Three different methods were used: maximum likelihood (ML), maximum parsimony (MP) and distance matrix analysis with both neighbour-joining (NJ) and unweighted pair group method using an arithmetic average (UPGMA) method of clustering. 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. Genetic distances between the sequences were measured using the p-distance (gamma) model (MEGAX). GenBank accession numbers: OQ244377 to OQ444413, OQ305911 to OQ305921 and OQ826592 to OQ826627.

TABLE 1 Sand flies captured intradomiciliary with CDC light traps in 96 households from El Borouj, Morocco sampled throughout two sampling periods, June–July 2015 and September–October, 2014–2015.

		Intradomiciliary captures		
		September/October	June/July	Total
	N households	96	7	96
Species	*N* traps	165	14	179
Phlebotomus longicuspis s.l. males				
Phlebotomus longicuspis s.s.	*N* (m)	159	254	413
	D	0.96	18.14	2.31
	*A**	77.94	98.83	89.59
	F	0.17	0.57	0.17
Phlebotomus longicuspis LCx	*N* (m/f)	45 (45/0)	3 (3/0)	48 (48/0)
	D	0.27	0.21	0.27
	*A**	22.06	1.17	10.41
	F	0.07	0.29	0.08
Phlebotomus longicuspis s.l.	*N* (m/f)	357 (204/153)	415 (257/158)	772 (461/311)
	D	2.16	29.64	4.31
	A	25.63	49.76	34.67
	F	0.42	0.71	0.42
Phlebotomus perniciosus	*N* (m/f)	116 (90/26)	43 (31/12)	159 (121/38)
	D	0.70	3.07	0.89
	A	8.33	5.16	7.14
	F	0.27	0.57	0.27
Phlebotomus langeroni	*N* (m/f)	2 (2/0)	4 (4/0)	6 (6/0)
	D	0.01	0.29	0.03
	A	0.14	0.48	0.27
	F	0.02	0.29	0.04
Total *Larroussius*	*N* (m/f)	475 (296/179)	462 (292/170)	937 (588/349)
	D	2.87	33.00	5.23
	A	34.10	55.40	42.07
	F	0.46	0.71	0.46
Other sand fly species	*N* (m/f)	918 (371/547)	372 (199/173)	1290 (570/720)
	D	5.57	26.57	7.21
	A	65.90	44.60	57.93
	F	0.87	0.86	0.87
Total	*N* (m/f)	1393 (667/726)	834 (491/343)	2227 (1158/1069)
	D	8.44	59.57	12.44
	A	100	100	100
	F	0.87	0.86	0.87

Abbreviations: *A*, relative abundance; *A**, relative abundance with respect to *P. longicuspis* s.l. males; *D*, density (sand flies per CDC trap and night); f, number of females captured; *F*, frequency; m, number of males captured; *N*, number.

2.7 | Leishmania infantum DNA detection

Parasite detection was carried out with a *L. infantum*-specific PCR-ELISA (Martín-Sánchez et al., 2002) and Granaleish Multiplex qPCR (University of Granada, Spain, Trade Mark Number 3667362/5) that can differentiate between *L. infantum*, *L. tropica* and *L. major* (Merino-Espinosa et al., 2018). The number of parasites in every qPCR reaction was calculated through the interpolation of the cycle threshold (Ct) value in a standard curve. DNA obtained from a male and from 1000 *Leishmania* promastigotes (*L. infantum* MHOM/ES/08/DP532; *Leishmania tropica* MHOM/MA/88/LEM1314 and *Leishmania major* MHOM/MA/81/LEM265) were used as negative and positive controls, respectively.

2.8 | Blood meal identification

A PCR-RFLP method using universal primers complementary to the conserved region of the mtDNA Cyt*b* gene in vertebrates was applied for the identification of the meal source in sandflies; selective restriction enzyme cleavage of a short variable region of 359 bp with HaeIII and HinfI enzymes was performed (González et al., 2015). DNA samples from potential hosts of different taxa were included as reference controls. When necessary, sequencing and comparative sequence analysis were also used to identify the hosts.

3 | RESULTS

3.1 | Abundance and density of *Larroussius* species

Overall 2227 sand flies were intradomiciliary captured of which 1158 were male specimens (52.0%) and 1069 females (48.0%). *Phlebotomus sergenti* was the most abundant species captured (47.42%). Table 1 shows the densities of the *Larroussius* species captured (42.07% abundance). *Phlebotomus longicuspis* s.l. (34.67%) was the most abundant *Larroussius* species followed by *P. perniciosus* (7.14%) whereas only six *P. langeroni* male specimens were captured (0.27%) and no *P. ariasi* were found. A positive correlation was detected between *P. perniciosus* and *P. longicuspis* s.l. sand fly densities detected in the 96 sampled households (Pearson correlation = 0.927, $p < 0.001$). Most male *P. perniciosus* specimens had atypical copulatory valves, as typical bifurcated penile valves were only found in only one specimen. 10.41% (48/461) *P. longicuspis* s.l. males were identified as LCx according to the number of coxite setae. The average number of setae on the two inner faces of the coxites was 26.48 [CI 95% 25.36–27.60] for *P. longicuspis* s.s., 20.75 [CI 95% 20.30–21.20] for LCx and 13.83 [CI 95% 13.18–14.48] for *P. perniciosus*. The density of *P. perniciosus* and *P. longicuspis* in the peripheral neighbourhood of Oulad Bouchair was significantly higher than in the other neighbourhoods sampled (Laamarcha, Bkakcha, Oulad Youssef, Oulad Ghanem, Mellalia, Douar Hliba, Chbarat and Douar El Kayd) (Table 2).

TABLE 2 Density of sand fly species (sand flies per CDC trap and night) from the subgenus *Larroussius* in the peripheral neighbourhood of Oulad Bouchair compared to the other neighbourhoods (Laamarcha, Bkakcha, Oulad Youssef, Oulad Ghanem, Mellalia, Douar Hliba, Chbarat and Douar El Kayd) from El Borouj, Morocco: Mean density values, confidence interval at 95% (CI 95%) of the mean density and minimum and maximum values of density in the sampled households.

		September/October		June/July		Total	
		Oulad Bouchair	Other	Oulad Bouchair	Other	Oulad Bouchair	Other
Sand fly species		*N* = 11	N = 85	*N* = 2	*N* = 5	*N* = 11	*N* = 85
Phlebotomus perniciosus	Mean density [CI 95%] Minimum/maximum values	3.77 [0–10.22] 0/32.5	0.27 [0.11–0.44] 0/4.5	9 [0–97.94] 2/16	0.70 [0–2.06] 0/2.5	4.58 [0–10.27] 0/32.5	0.30 [0.13–0.46] 0/4.5
	p value OR [CI 95%]	0.042 1.792 [1.021–3.142]		0.339		0.002 1.883 [1.103–3.215]	
Phlebotomus longicuspis	Mean density [CI 95%] Minimum/maximum values	12.23 [0–27.66] 0/78	0.67 [0.32–1.01] 0/11	101 [0–1231.85] 12/190	1.1 [0–2.77] 0/3	25.88 [0–58.26] 0/190	0.69 [0.36–1.02] 0/11
	p value OR [CI 95%]	0.003 1.468 [1.142–1.886]		0.995		0.001 1.503 [1.187–1.904]	
Phlebotomus langeroni	Mean density [CI 95%] Minimum/maximum values	0.05 [0–0.15] 0/0.5	0.01 [0–0.02] 0/0.5	1 [0–7.35] 0.5/1.5	0	0.19 [0–0.45] 0–1.5	0.01 [0–0.02] 0/0.5
	p value OR [CI 95%]	0.999		0.999		0.999	
Larroussius subgenus	Mean density [CI 95%] Minimum/maximum values	16.05 [0–37.73] 0/110.5	0.95 [0.54–1.36] 0/11.5	111 [0–1337.15] 14.5/207.5	1.80 [0–3.84] 0/3	30.65 [0–67.37] 0/207.5	0.99 [0.60–1.39] 0/11.5
	p value OR [CI 95%]	0.002 1.392 [1.130–1.714]		0.996		0.001 1.418 [1.164–1.728]	

Abbreviations: *N*, number of households sampled; OR, odds ratio determined by logistic regression; *p*, probability.

A high *P. longicuspis* s.l. density was found in one of the houses of Oulad Bouchair neighbourhood, both in September (66.7 sand flies/CDC trap from which 39.7 *P. longicuspis* s.s. and 11.3 LCx males) and June (190 sand flies/CDC trap from which 116.5 *P. longicuspis* s.s. and 0 LCx males). Density of *P. perniciosus* was 29 in September and 13 sand flies/CDC trap in June with a large predominance of males. A CL case due to *L. infantum* was diagnosed in 2017 in this house.

3.2 | Sequence analysis of the *P. perniciosus* complex

A total of 29 *P. perniciosus* complex sand flies from the Oulad Bouchair neighbourhood (two atypical *P. perniciosus* males, two *P. perniciosus* females, five *P. longicuspis* s.s. males, seven *P. longicuspis* LCx males and 13 *P. longicuspis* s.l. females) were analysed and compared to the sequences of 10 sand flies captured in Spain: *P. perniciosus* (three females and three typical bifurcate males), one *P. langeroni*, one *P. ariasi*, one *P. sergenti* and one *P. papatasi*. The fragment length used for analysis was 430 bp for Cyt*b*, 269 bp for COI and 322 for ITS2. The topologies of the obtained phylogenetic trees were quite congruent, regardless of the reconstruction method used. The phylogenetic tree obtained with Cyt*b* sequences highlighted three clusters within the *P. perniciosus* complex. The first cluster is supported by a bootstrap value of 92%; this cluster is subdivided into two subclusters, the first one comprised of only Spanish *P. perniciosus* specimens, both male and female, and the second one comprised a mix of Moroccan *P. perniciosus* (females and atypical males) and morphologically identified *P. longicuspis* females. The second cluster is composed of *P. longicuspis* females, *P. longicuspis* s.s. males and *P. longicuspis* LCx males. For this cluster the bootstrap value was 91%. The third cluster is only composed of two morphologically identified *P. longicuspis* LCx males and supported by a bootstrap value of 79.9% (Figure 1). A similar topology including the same three clusters containing the same individuals was found through ITS2 analysis (Figure 2) and concatenate trees but supported by lower bootstrap values (Appendix S1); for all three trees, cluster 1 and 2 are sister groups.

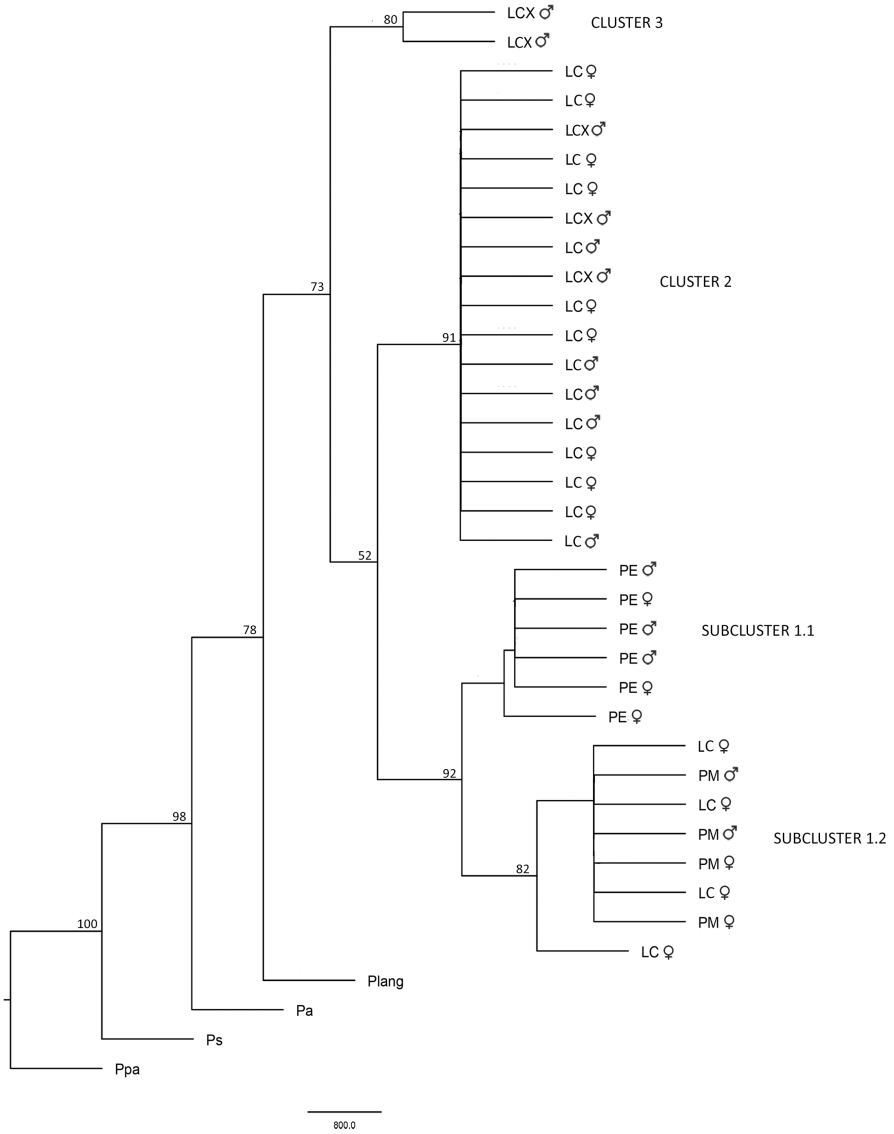

FIGURE 1 Maximum parsimony tree based on Cyt*b* sequences from individuals in which the three genes were analysed. *Phlebotomus papatasi* is used as the outgroup. Based on morphology, PE is typical *Phlebotomus perniciosus* from Spain, PM is atypical *Phlebotomus perniciosus* from El Borouj, Morocco, both males with 10–16 coxite setae; LC is *Phlebotomus longicuspis* (males with 21–31 coxite setae) and LCX is *Phlebotomus longicuspis* LCx (males with 19–22 coxite setae).

COI analysis adding more *Larroussius* species showed clusters 2 and 3 as sister groups. (Figure 3 and Appendices S2 and S3). Genetic distances within clusters and between clusters ranged from 0 to 0.019 and 0.045 to 0.072, respectively whereas *P. langeroni* displayed genetic distances values ranging from 0.060 to 0.079 when compared to clusters, and pairwise distance between *P. ariasi* and *P. chadlii* was 0.038 (Appendix S4).

The comparative sequence analyses allowed the identification of eight Cyt*b*, 10 COI and 28 ITS2 haplotypes. The fixed differences that characterize each cluster are shown in Table 3.

Additionally, we have analysed the Cyt*b* sequence of another 16 female specimens, 15 morphologically identified as *P. longicuspis* and 1 *P. perniciosus*, and we have compared all the sequences generated in this study with the Cyt*b* sequences published by Boudabous et al. (2012): *P. perniciosus* and *P. longicuspis* in that study are associated with cluster 1.2 and cluster 2, respectively; none of the *P. longicuspis* sequences from Tunisia could be associated with group 3. The phylogenetic relationships among the 16 haplotypes of the *P. perniciosus* complex are shown in Figure 4 and Appendices S5–S7.

3.3 | Leishmania infantum DNA detection

Leishmania infantum was detected in one of nine females morphologically classified as *P. perniciosus* and 7 of 56 females identified as *P. longicuspis* s.l. based on morphology. The female *P. perniciosus* found infected showed 100% homology with other *P. perniciosus* specimens in the subcluster 1.2 of the tree of Figure 1. Five *P. longicuspis* females that were found infected showed 100% homology with the Cyt*b* haplotype of cluster 2 from Figure 1. The sixth infected *P. longicuspis* female differed in two to three bases from the two male specimens included in cluster 3 of Figure 1, showing as a new Cyt*b* haplotype in Figure 4. The seventh infected *P. longicuspis* female belonged to subcluster 1.2. Parasite loads were low in all of them (<10 parasites/sand fly).

3.4 | Analysis of sand fly blood meals

Sixteen engorged females morphologically classified as *P. perniciosus* (six individuals) and *P. longicuspis* s.l. (10 individuals) were tested to

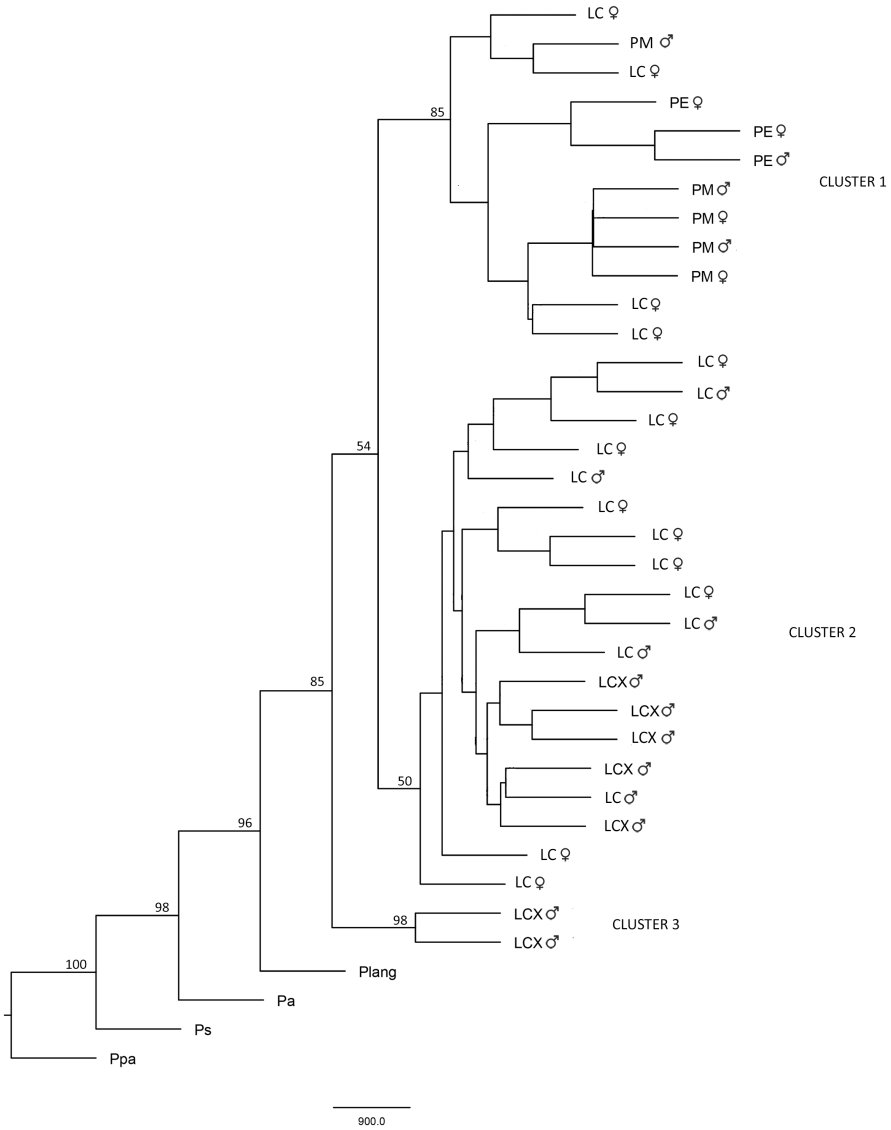

FIGURE 2 Maximum parsimony tree based on ITS2 sequences from individuals in which the three genes were analysed. *Phlebotomus papatasi* is used as the outgroup. Based on morphology, PE is typical *Phlebotomus perniciosus* from Spain, PM is atypical *Phlebotomus perniciosus* from El Borouj, Morocco, both males with 10–16 coxite setae; LC is *Phlebotomus longicuspis* (males with 21–31 coxite setae) and LCx is *Phlebotomus longicuspis* LCx (males with 19–22 coxite setae).

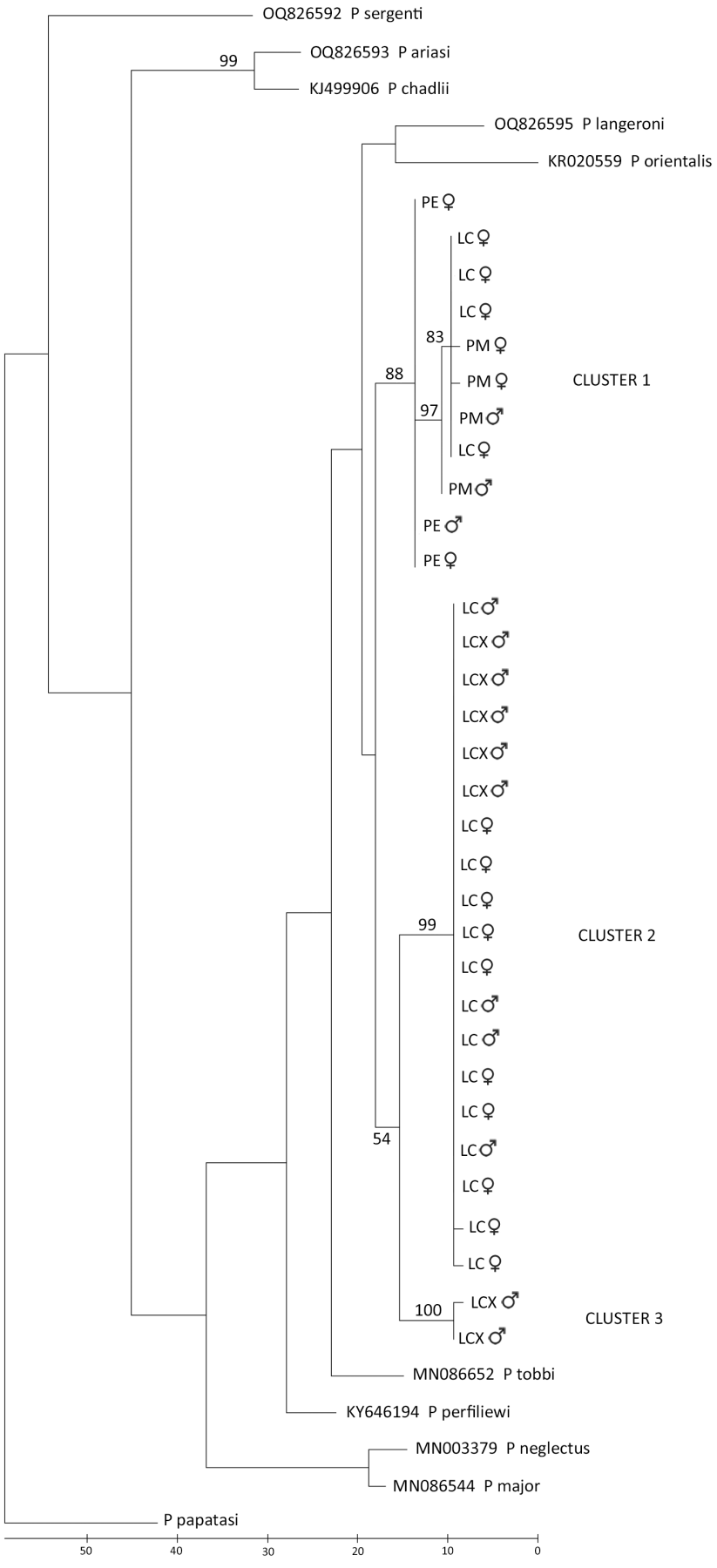

FIGURE 3 Maximum parsimony tree based on COI sequences from individuals in which the three genes were analysed adding more *Larroussius* species. *Phlebotomus papatasi* is used as the outgroup. Based on morphology, PE is typical *Phlebotomus perniciosus* from Spain, PM is atypical *Phlebotomus perniciosus* from El Borouj, Morocco, both males with 10–16 coxite setae; LC is *Phlebotomus longicuspis* (males with 21–31 coxite setae) and LCX is *Phlebotomus longicuspis* LCx (males with 19–22 coxite setae).

TABLE 3 Haplotypes and fixed differences that characterize each of the three phylogenetic clusters of the *P. perniciosus* complex according to the three genes analysed.

Clusters	Subclusters	Cyt*b* (55 sand fly specimens)	COI (35 sand fly specimens)	ITS2 (35 sand fly specimens)
Cluster 1 equivalent to *Phlebotomus perniciosus*	1.1	52T, 72C, 162T, 278T, 361T	126T, 139A, 172A, 201T, 207A	– (one haplotype) 207T, 228A
		21T, 177A (three haplotypes)		96C, 97A, 207A, 270A, 293A, 300A, 319C (three haplotypes)
	1.2	– (three haplotypes)	123C, 181T (four haplotypes)	74T, 100C, 102C, 169A, 207T, 228A (five haplotypes)
Cluster 2 equivalent to *Phlebotomus longicuspis* s.s.	21T, 48C, 139G, 165T, 246C, 277A, 282C, 303C (three haplotypes)		78A, 111G (three haplotypes)	- (18 haplotypes)
Cluster 3 equivalent to an unnamed species	9G, 21C, 99A, 165A, 279A, 285C (three haplotypes)		99G, 136T, 160C, 204C (two haplotypes)	49C, 96T, 97C, 104T, 160A, 166A, 167C, 168A, 202T, 204T, 247G, 255T, 266A (two haplotypes)
Total haplotypes	12		10	28

Note: Underlined nucleotides are synapomorphic characters diagnostics of the three main lineages after comparing all the Cyt*b* sequences of the specimens studied with the Cyt*b* sequences published by Boudabous et al. (2012) and Depaquit et al. (2005) (16 Cyt*b* haplotypes compared), and the data provided by Pesson et al. (2004) for which sequences are not published.

determine the vertebrate host source of the blood meal. The origin of blood meals was identified as: *Homo sapiens* for three *P. perniciosus* and four morphological *P. longicuspis* from subcluster 1 (1), cluster 2 (2) and cluster 3 (1) from Figure 4; *Homo sapiens* + *Felis catus* for one *P. perniciosus* and two morphological *P. longicuspis* from cluster 2; *Felis catus*, *Bos taurus* and *Equus asinus* for, respectively, 2, 1 and 1 morphological *P. longicuspis* from cluster 2; and *Ovis aries* for 2 *P. perniciosus* (subcluster 1.2).

4 | DISCUSSION

Phlebotomus sergenti was the most abundant and densest species within households in the recently endemic focus of ACL in El Borouj (Gijón-Robles et al., 2018) followed by the *Larroussius* species morphologically identified as *P. longicuspis* s.l. (35%) and *P. perniciosus* (7%). *Phlebotomus ariasi* is found only in western Europe and the western Maghreb of North Africa, where it is most abundant in the humid and subhumid bioclimatic zones, often in oak forests at higher altitudes (Ballart et al., 2014; Franco et al., 2010; Mahamdallie et al., 2011; Rioux, 1995; Zarrouk et al., 2016) as opposed to the semi-arid or arid character of the sampled areas of El Borouj. *Phlebotomus langeroni* is found from Lebanon to Spain (Esseghir et al., 2000; Haddad et al., 2003; Sáez et al., 2018) and is poorly represented in the inhabited studied area where only six males were captured. The abundance of the species of the *P. perniciosus* complex was especially relevant in the peripheral neighbourhood of Oulad Bouchair where the average density reached values of 31 sand flies/light trap/night.

About 130 human visceral leishmaniasis cases are annually reported on average in Morocco, most of them in the north of the country but some sporadic cases are recorded in the central and southern areas (Ministry of Health, Morocco, 2016; Mouttaki et al., 2014). However, this number does not reflect the true infection rate by *L. infantum* since 2.5%–5% HIV-infected patients, depending on the diagnostic technique used, have been found to be asymptomatic carriers of the parasite (Echchakery et al., 2018). In addition, CL due to *L. infantum* has been considered to have a sporadic occurrence so far, nevertheless its distribution area is not well defined and it has been frequently found in *L. tropica* foci; recently, a wide extension of CL due to *L. infantum* from the north to the southern areas, has been reported (El Mazini et al., 2021). Natural infection by the parasite has been detected by PCR in pools of morphologically identified *P. longicuspis* (Es-Sette et al., 2014) and *P. perniciosus* (Mhaidi et al., 2018). Both sand fly species are sympatric in almost all the Moroccan studied areas (El Mazini et al., 2021) but on few occasions have these species been molecularly characterized.

The abundance of these vector species in the neighbourhood of Oulad Bouchair might explain the CL case due to *L. infantum* diagnosed in 2017. The molecular characterization of sand fly specimens of *P. perniciosus* complex from this neighbourhood, using both mitochondrial and nuclear markers with a high mutation rate and different

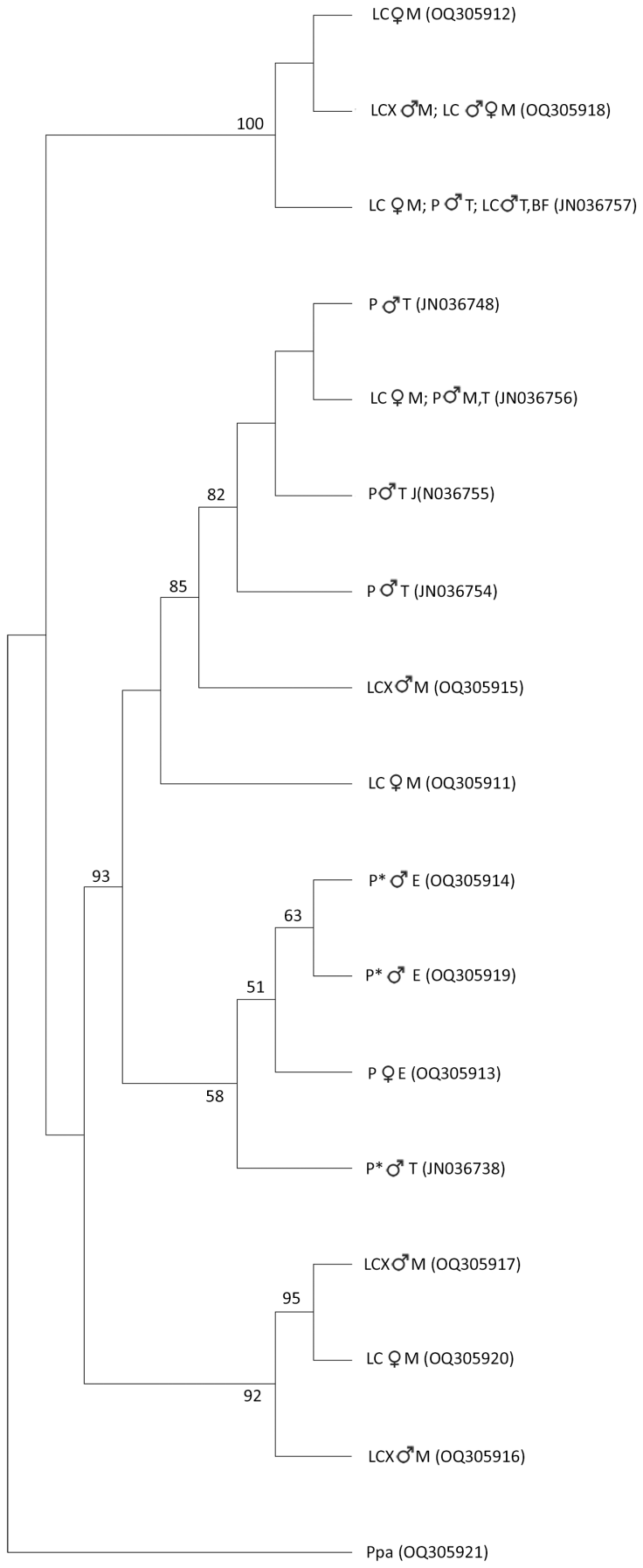

FIGURE 4 Maximum parsimony tree based on the sequences of 16 Cyt*b* haplotypes derived from this study and those published by Boudabous et al., 2012. *Phlebotomus papatasi* is used as the outgroup. Based on morphology, P is *Phlebotomus perniciosus* (P* is typical male and P is atypical *Phlebotomus perniciosus* male); LC is *Phlebotomus longicuspis* and LCX is *Phlebotomus longicuspis* LCx male (males with 19–22 coxite setae); E is Spain, M is Morocco (El Borouj); T is Tunisia and BF is Burkina Faso.

inheritance mechanisms, revealed a striking haplotype diversity with the three genes but particularly with ITS2. All trees were congruent in showing three clusters that could be interpreted as corresponding to *P. perniciosus* (cluster 1), *P. longicuspis* s.s. (cluster 2) and *P. longicuspis* unnamed species (cluster 3), all coexisting in sympatry. However, the molecular observations were not fully consistent with the morphology: subcluster 1.2. corresponding to Moroccan *P. perniciosus* also includes morphological female *P. longicuspis* that are not simple cases of mitochondrial introgression as shown by the ITS2 tree; cluster 2 includes morphological females and both males, *P. longicuspis s.s* (21–31 coxite setae) and *P. longicuspis* LCx (19–22 coxite setae). All these clusters appear defined by a variety of fixed differences in the three genes studied. Only cluster 2 does not present fixed differences in the ITS2 gene supporting the existence of recent recombination events. Cluster 3 of which we have been able to detect males and females, seem to represent an additional speciation phenomenon and could correspond with an unnamed species (Pesson et al., 2004) although based on all the figures, it cannot be referred to them as sister species. The number of setae on the coxite would not allow the identification of males of this species and it would be necessary to carry out the molecular characterization. COI genetic distances between the three clusters support their consideration as different species.

Genome-wide studies through random amplified polymorphic DNA included under the species *P. perniciosus* the morphological heterogeneity of the aedeagus of *P. perniciosus* males, from the typical bifurcated to simple and curved tips, and the intermediate forms (Martín-Sánchez et al., 2000; Morillas Márquez et al., 1991). In contrast, *P. longicuspis* showed a high degree of genetic polymorphism that seemed to indicate the existence of past introgressive hybridizations and cryptic species (Martín-Sánchez et al., 2000), later confirmed by isoenzymes and Cyt*b* sequence analyses (Pesson et al., 2004) and currently in the present study. These taxa appear to be reproductively isolated biological species even within local populations. In addition to the consequences at the epidemiological level, due to the morphological assignment to the species *P. longicuspis* of specimens that are genetically *P. perniciosus*, there could be phenotypic differences of biomedical importance between these taxa, one of the most notable being the vectorial character. We have detected *L. infantum* in females of cluster 1.2 (both, *P. perniciosus* and females with *P. longicuspis* morphology but *P. perniciosus* genetics), cluster 2 (*P. longicuspis* s.s) and cluster 3 (unnamed species). None of the infected females found had a parasite load greater than 10 parasites/sand fly, while 25% typical *P. perniciosus* females that were infected in endemic areas had very high infection rates with more than 10,000 parasites/sand fly (Díaz-Sáez et al., 2022) highlighting typical *P. perniciosus* as the main vector of *L. infantum*.

Female sand flies need to take a blood meal for the protein supplementation essential for egg production, and similar to most vectors, take multiple blood meals during their lifespan. Most available sand fly studies indicate that most vector species are rather generalists than specialists regarding their host range (Gijón-Robles et al., 2018; González et al., 2015; Guy et al., 1984). In this work, humans, cats and other species were identified as blood meal hosts of female sand flies indicating opportunistic behaviour showing that they have fed on available hosts within the dwellings.

Population genetics will help to assess the threat of the geographical spread of *L. infantum* in Morocco by determining the density, abundance and vector role of the species of the *P. perniciosus* complex identified correctly. Although the three genes tested are efficient markers to differentiate the taxa involved, we highlighted the use of Cyt*b* which has been more intensively studied and has proven capable of defining more precisely its synapomorphic characters. Furthermore, Cyt*b* is known to be phylogenetically informative for *Phlebotomus* species and useful for dating speciation events because of its clock-like rate of nucleotide substitution (Esseghir et al., 1997, 2000).

5 | CONCLUSION

Our study of a sympatric population of these *Larroussius* using three different genes evidence that some females with typical morphology of *P. longicuspis* are genetically homologous to *P. perniciosus*; these females and also those with typical morphology of *P. perniciosus* were naturally infected by *L.infantum*. Moreover, it shows the existence of *P. longicuspis* s.s. and one undescribed morphologically indistinguishable species, both found naturally infected by *L. infantum*. These taxa cannot be differentiated by morphological methods but characterized by a distinctive genetic lineage for which synapomorphic diagnostic characters are described.

AUTHOR CONTRIBUTIONS

JMS, VDS and MR conceived and designed the research. PGR, NA and MR were involved in acquisition of local data. PGR, VDS, NA, JMS and FMM conducted captures and morphological identification. PGR, MGM, ECL, GME and JMS analysed the data and conducted phylogenetic analyses. JMS, PGR and VCL wrote the manuscript. JMS, VCL, VDS, MR and FMM revised the manuscript critically. All authors read and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

We (all 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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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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