

This is the peer reviewed version of the following article:

González E, Molina R, Aldea I, Iriso A, Tello A, Jiménez M. **Leishmania sp. detection and blood-feeding behaviour of *Sergentomyia minuta* collected in the human leishmaniasis focus of southwestern Madrid, Spain (2012-2017)**. *Transbound Emerg Dis*. 2020 May;67(3):1393-1400.

which has been published in final form at:

<https://doi.org/10.1111/tbed.13464>

1 ***Leishmania* spp. detection and blood-feeding behaviour of *Sergentomyia minuta***
2 **collected in the human leishmaniasis focus of southwestern Madrid, Spain (2012-**
3 **2017)**

4 **Molecular analysis of *Sergentomyia minuta* from Madrid, Spain**

5

6 Estela González^{1,2}, Ricardo Molina¹, Irene Aldea¹, Andrés Iriso³, Ana Tello⁴, Maribel
7 Jiménez^{1*}

8

9 ¹Laboratorio de Entomología Médica, Centro Nacional de Microbiología, Instituto de
10 Salud Carlos III, Majadahonda, Madrid, Spain

11 ²Present address: The Pirbright Institute, Woking, United Kingdom

12 ³Dirección General de Salud Pública, Consejería de Sanidad, Comunidad de Madrid,
13 Spain

14 ⁴Departamento de Biodiversidad, Ecología y Evolución, Facultad de Ciencias
15 Biológicas, Universidad Complutense de Madrid, Spain

16

17

18 * Corresponding author. Tel.: +34 91 822 3674; e-mail address: mjimenez@isciii.es (M.
19 Jiménez).

20

21

22

23

24

25 **Summary**

26 Phlebotomine sand flies are the only known vectors of *Leishmania* spp. protozoan
27 which causes leishmaniasis in 98 countries. In Spain, 11 sand fly species are
28 described, but only *Phlebotomus perniciosus* and *Phlebotomus ariasi* are proven
29 vectors of the disease. On the other hand, *Sergentomyia minuta* is one of the most
30 abundant and ubiquitous sand fly in this territory, although scarce information is
31 available about this species. Sand flies from this genus are known for their preference
32 to feed on cold-blooded animals and are traditionally involved in the transmission of
33 reptile *Leishmania*. However, studies have suggested that *Sergentomyia* sp. could be
34 implicated in the transmission of human pathogenic *Leishmania*. The present study is
35 focused on *S. minuta* sand flies collected in a human leishmaniasis focus in Madrid,
36 Spain. Sand flies were collected monthly for six successive years –2012 to 2017– in
37 the active season of these dipterans, from May to October. Molecular detection of
38 *Leishmania* spp. showed 68 positive specimens of *S. minuta* out of 377 (18%). The
39 analysis of blood meal preferences by amplification of 359 bp fragment of cytochrome
40 *b* gene revealed that blood preference of *S. minuta* is not only limited to reptiles, but
41 they also feed on mammals, including humans. Results suggest the presence of a
42 *Leishmania* spp. cycle in *S. minuta* from the studied area. Although there is no
43 evidence about its incrimination in the *L. infantum* transmission more investigation is
44 needed to elucidate the intravectorial cycle of *Leishmania* spp. in *S. minuta* sand flies,
45 their feeding behaviour and their potential contribution in *Leishmania* spp. epidemiology
46 in the country.

47 **Keywords:** *Leishmania* spp., *Sergentomyia minuta*, feeding behaviour, human
48 leishmaniasis outbreak, vector competence, Spain

49

50

51 **1. Introduction**

52 Sand flies are the only known vectors of the protozoa *Leishmania* spp. which causes
53 leishmaniasis in 98 countries (World Health Organization, 2010). More than 800
54 species of sand flies are described, but only about 100 are proven or suspected
55 vectors of the parasite (Maroli, Feliciangeli, Bichaud, Charrel, & Gradoni, 2013). In
56 Spain, the sand fly species described are *Phlebotomus ariasi*, *Phlebotomus*
57 *perniciosus*, *Phlebotomus langeroni*, *Phlebotomus papatasi*, *Phlebotomus sergenti*,
58 *Phlebotomus alexandri*, *Phlebotomus chabaudi*, *Phlebotomus mascitti*, *Phlebotomus*
59 *fortunatarum*, *Sergentomyia fallax* and *Sergentomyia minuta* (Gil Collado, Morillas,
60 Sanchís, & Alvarez, 1989; Rioux et al., 1986). From all of them, *P. perniciosus* and *P.*
61 *ariasi* are the proven vectors of *Leishmania infantum*, the only pathogenic species of
62 the parasite found in Spain (Gil Collado et al., 1989; Rioux et al., 1986). Besides, *P.*
63 *langeroni* has been recently proposed as a potential vector in a study performed in
64 Granada (southern Spain) (Sáez et al., 2018). On the other hand, although *S. minuta* is
65 one of the most abundant species in the country, limited information is available on its
66 biology. Sand flies from this genus are traditionally involved in the transmission of
67 *Sauroleishmania* species and are known for their preference to feed on cold-blooded
68 animals. However, recent studies have detected DNA from other vertebrates, including
69 humans, in blood-fed females of different species of *Sergentomyia* (Azizi, Askari,
70 Kalantari, & Moemenbellah-Fard, 2016; Berdjane-Brouk et al., 2012; Bravo-Barriga et
71 al., 2016; Karakuş et al., 2017; Maia et al., 2015; Senghor et al., 2016;
72 Siripattanapipong, Leelayoova, Ninsaeng, & Mungthin, 2018). Also, several
73 entomological studies carried out in Old World countries have shown the presence of
74 DNA from pathogenic *Leishmania* species in *Sergentomyia* genus. Hence, the
75 perception that they are only vectors of *Sauroleishmania* species is changing since
76 they could be involved in the spread of *Leishmania* species as it has been pointed out
77 in a number of countries of the Mediterranean region like Italy (Latrofa et al., 2018),
78 Portugal (Campino et al., 2013; Maia et al., 2015), Tunisia (Ayari et al., 2016; Jaouadi

79 et al., 2015) or Turkey (Özbel, Karakuş, Arserim, Kalkan, & Töz, 2016), and also in
80 other regions of Africa (Mutinga et al., 1994; Ngouateu et al., 2018; Nzelu et al., 2014)
81 and Asia (Chusri, Thammapalo, Silpapojakul, & Siriyasatien, 2014; Kanjanopas et al.,
82 2013; Mukherjee et al., 1997; Siripattanapipong et al., 2018). In Spain, only *Leishmania*
83 *tarentolae*-like DNA was reported in *S. minuta* sand flies collected in Extremadura
84 (Bravo-Barriga et al., 2016). In any case, the incrimination of a sand fly as a competent
85 vector of a certain *Leishmania* species should involve several rules and steps for its
86 confirmation (Killick-Kendrick, 1999).

87 A human leishmaniasis outbreak in southwestern Madrid region (Spain) with almost
88 800 cases reported since 2010 (Boletín Epidemiológico de la Comunidad de Madrid,
89 2017). Entomological studies have shown that the most abundant sand fly species in
90 the area are *P. perniciosus* and *S. minuta*, being the first one the only vector involved
91 in the transmission of *L. infantum* in the focus (González, Jiménez, Hernández, Martín-
92 Martín, & Molina, 2017; Tello, González Mora, Outerelo, Iriso, & Vázquez, 2015). In the
93 present study, we analysed *S. minuta* females collected in the outbreak area intending
94 to improve the information available about this species in the region by carrying out
95 *Leishmania* spp. detection and blood meal identification through molecular tools.

96 **2. Material and methods**

97 **2.1. Sand fly collection, identification and DNA extraction**

98 The entomological surveys were conducted in five municipalities from southwestern
99 Madrid region (Fuenlabrada, 40°17'00"N 3°48'00"W; Getafe, 40°18'00"N 3°43'00"W;
100 Humanes de Madrid, 40°15'14"N 3°49'40"W; Leganés, 40°19'42"N 3°45'55"W, and
101 Parla, 40°14'14"N 3°46'27"W).

102 Sand flies were monthly collected for six successive years –2012-2017– during their
103 active season, from May to October. Both sticky traps (ST) (20 X 20 cm paper sheets
104 soaked in castor oil) and CDC miniature light traps (LT) were used.

105 Sand fly identification and further DNA extraction were performed as previously
106 described (González et al., 2017). Briefly, sand flies preserved in 70% ethanol were
107 washed individually in sterile water placed in ELISA microtiter plates. After, the
108 genitalia and the head were processed for taxonomical identification. Moreover, the
109 presence of eggs (gravid status), and/or blood (total or partially engorged vs unfed) in
110 the abdomen was registered for each female. Thorax and abdomen of each sand fly
111 were used for DNA extraction using (Speedtools Tissue DNA Extraction Kit (Biotools®
112 B&M Labs S.A., Spain) according to the manufacturer's instructions. Finally, DNA
113 samples were stored at -20°C until use.

114 **2.2. Blood meal identification**

115 Blood meal identification of engorged sand flies was conducted by the amplification of
116 a fragment of 359 bp of vertebrate cytochrome *b* (*cyt b*) gene followed by sequencing
117 as described before (Jiménez et al., 2013). Universal degenerated primers were used
118 (Svobodová et al., 2009):

119 *cyt_bb1*: 5'-CCA TCM AAC ATY TCA DCA TGA TGA A-3' and

120 *cyt_bb2*: 5'-GCH CCT CAG AAT GAY ATT TGK CCT CA-3'.

121 Degenerated primer for reptiles 5'-GCH GAY ACH WVH HYH GCH TTY TCH TC-3'
122 combined with primer *cyt_bb2* was used in those samples in which universal primers
123 did not show any bands or they were too faint (Cupp et al., 2004). Bands obtained after
124 electrophoresis were purified using "Speedtools PCR Clean-Up Kit" (Biotools®, Madrid,
125 Spain). PCR products were sequenced with ABI PRISM 3730XL DNA Analyzer
126 (Applied Biosystems®, EEUU) and subsequent electropherograms were manually
127 analysed using ChromasPro program (Technelysium). Nucleotide sequences obtained
128 were examined with DNASTAR program (Lasergen v7.1®, Madison, WI). Finally,
129 homologies with the existing sequences data in GenBank® was carried out with the
130 software BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>).

131 **2.3. Molecular detection and identification of *Leishmania* spp.**

132 Detection of *Leishmania* spp. was carried out by amplification of 120 bp fragment from
133 kinetoplast DNA (kDNA) and further analysis by cysteine proteinase *b* (*cpb*) and
134 internal transcribed spacer region 1 (ITS1) PCR following protocols previously
135 described (Jiménez, González, Martín-Martín, Hernández, & Molina, 2014; Jiménez et
136 al., 2013). PCR products were separated on 1.5% agarose gel (Conda, Spain) stained
137 with “Pronasafe Nucleic Acid Staining Solution” (10 mg/ml) (Conda, Spain) and
138 visualised under UV light. ITS1 bands were excised from the gel and purified for further
139 sequencing as described in the previous section. Moreover, the resulting sequences
140 were aligned by CLUSTAL W using MEGA7 (Kumar, Stecher, & Tamura, 2016).
141 Phylogenetic tree construction was performed with *Leishmania* spp. sequences from
142 Genbank® using a Maximum Likelihood method (ML) based on the Tamura-Nei Model
143 as indicated by MEGA7 program (Kumar et al., 2016; Tamura & Nei, 1993). In this
144 analysis, the ITS1 sequence of the *L. infantum* genotype implicated in the human
145 leishmaniasis focus of Madrid (ITS-Lombardi genotype, AJ000295.1) and ITS1
146 sequence of *L. infantum* (LC028234.1) detected in *P. perniciosus* in Algarve, southern
147 Portugal (Maia et al., 2015) have been included.

148 **3. Results**

149 **3.1. Sand fly collection**

150 A total of 377 *S. minuta* females (n= 310 blood-engorged, n=34 gravid and n=33 unfed)
151 collected by both ST (n=336) and LT (n=41) in the five municipalities affected by the
152 human leishmaniasis outbreak in southwestern Madrid region were analysed. The
153 highest number of *S. minuta* females were collected in Getafe (n=148) followed by
154 Fuenlabrada (n=102), Leganés (n=78), Humanes de Madrid (n=48), and Parla (n=1).

155 **3.2. Blood meal identification**

156 Blood meal identification was performed in 310 engorged *S. minuta* females (289
157 collected by ST and 21 by LT), of which 109 were fully and recently blood-fed.
158 Amplification and further sequencing were successfully achieved in 59 females (19%).

159 Moreover, the blood meal identification was valid in 54.13% of the recently engorged
160 sand flies (53 collected by ST and 6 by LT). On the one hand, reptile blood source was
161 found in 35 samples, all captured by ST (*Tarentola mauritanica*, n=31; *Podarcis*
162 *hispanica*, n=3; *Malpolon monspessulanus*, n=1). On the other hand, four mammal
163 blood sources were detected (*Homo sapiens*, n=18; *Oryctolagus cuniculus*, n=2; *Lepus*
164 *granatensis*, n=2, and *Bos taurus*, n=1). Moreover, a mixed-blood meal corresponding
165 to *Meleagris gallopavo/Acanthodactylus erythrurus* was found in one specimen. Three
166 of the *S. minuta* fed on *T. mauritanica* and other three fed on *H. sapiens* corresponded
167 to samples captured by LT (Table 1).

168 **3.3. Molecular detection and identification of *Leishmania* spp.**

169 *Leishmania* spp. was detected in 68 specimens (n=67 blood-fed, n=1 unfed) by kDNA
170 amplification. The highest infection rate was found in Humanes de Madrid (27.1%)
171 followed by Getafe (23.6%), Leganés (18%), and Fuenlabrada (5.9%). Further analysis
172 by *cpb* gene amplification excluded infection by *L. infantum*. On the other hand, ITS1
173 amplification was positive to *Leishmania* spp. in 46 out of the 68 kDNA positive
174 females. In total, 39 complete sequences and 4 partial sequences were successfully
175 sequenced (V439, V588, V590 and V637). The ITS1 nucleotide fragments were
176 submitted to DDBJ (accession numbers: LC216356 to LC216369) and Genbank®
177 (accession numbers: MK567784 to MK567806) except for those that matched to
178 previously annotated fragments (see Table 1). The alignment analyses showed a
179 microsatellite variation in the repetition number of a poly TA at location 197. The
180 sequences segregated into two groups: 21 sequences showed 9 TA repetitions in the
181 poly TA region (position 197-215) while the other 24 sequences showed 8 TA
182 repetitions (position 197-213). Although more differences between the obtained
183 sequences were found, most of them corresponded to single nucleotide polymorphisms
184 (SNPs) (Additional file 1: Figure S1). Furthermore, BLAST analysis revealed the
185 highest identity (94-99%) with *Leishmania* sp. sequences characterised from *S. minuta*

186 collected in Portugal (LC028233.1 and LC028235.1), *Leishmania* sp. isolated from
187 lizards in China (KT990201.1, KT990199.1, KT990162.1 and KT990142.1) and *L.*
188 *tarentolae* (KU680858.1).
189 On the other hand, the phylogenetic study has located the resulting ITS1 sequences in
190 the same branch within sequences of *Leishmania* sp. from *S. minuta* captured in
191 Portugal (LC028233 and LC028235) and Spain (LC031456), ITS1 of *L. tarentolae*
192 (KU680858, LC086293) and a *Leishmania* sp. isolated from human in China
193 (HM130599). Moreover, the phylogenetic tree separates *L. infantum* from the
194 *Leishmania* sp. sequences obtained in the present study (Fig.1).
195 In addition, three blood meal sources were identified in 13 out of the 67 blood-fed
196 females positive to *Leishmania* sp., *T. mauritanica* (n=6); *H. sapiens* (n=6); *B. taurus*
197 (n=1). Only one unfed female was positive.

198 **4. Discussion**

199 The genus *Sergentomyia* is widely distributed throughout the Old World, and concretely
200 *S. minuta* is the only species present in the Iberian Peninsula. Despite being one of the
201 most abundant sand flies in the region, there are few studies about its biology and
202 vectorial competence for *Leishmania* spp. Generally, *Sergentomyia* sand flies are
203 proven vectors of reptile *Leishmania* species, and it is accepted that most of the
204 species are not anthropophilic (Lewis, 1987; Lewis & Dyce, 1988). However, this study
205 shows 18 *S. minuta* females fed on human. In this sense, Maia et al. 2015 and
206 Berdjane-Brouk et al. 2012 have also described anthropophilic behaviour in *S. minuta*
207 and *Sergentomyia darlingi* from Portugal and Mali, respectively. In Sudan, human-
208 biting behaviour was previously reported in *Sergentomyia schwetzi* and *Sergentomyia*
209 *clydei* (Hoogstraal, Dietlein, & Heyneman, 1962). Moreover, other mammal blood
210 sources were identified in engorged *S. minuta* in our study, revealing a possible
211 opportunistic feeding behaviour. This conduct has also been observed in *Sergentomyia*
212 *dentata* from Turkey (Özbel et al., 2016) and in other sand fly species as *P. perniciosus*

213 (González et al., 2017; Tarallo, Dantas Torres, Lia, & Otranto, 2010; Muñoz et al.,
214 2019). Despite the noticeable result about the *S. minuta* feeding behaviour, the rate of
215 blood meal identification was under the expectations according to studies performed in
216 sand flies from the same region (González, Jiménez, Hernández, Martín Martín, &
217 Molina, 2017), but similar to the rates showed in studies carried out in Portugal (Maia et
218 al., 2015). Such low efficiency is probably due to the scarce volume of blood present in
219 the midgut of almost 65% (n=201) of the engorged *S. minuta* collected. But it could
220 also be a consequence of the digestion progress that rapidly degrades the DNA,
221 making it more difficult to identify the origin of the ingested blood.

222 According to the *Leishmania* detection in blood-fed *S. minuta*, this study describes
223 positive specimens fed on *T. mauritanica*, *H. sapiens* and *B. taurus*. In this sense, this
224 is the first report of *Leishmania* spp. detection in *S. minuta* females fed on mammals in
225 Spain, including humans. Other studies have reported in several countries *Leishmania*
226 spp. detection in *Sergentomyia* specimens fed on mammals and their potential role in
227 the transmission of mammalian leishmaniasis (Berdjane-Brouk et al., 2012; Karakuş et
228 al., 2017; Maia et al., 2015). But the only presence of *Leishmania* DNA in a sand fly is
229 not enough for vector incrimination and other pieces of evidence are needed as the
230 feeding preference of the sand fly for human or other animal hosts, successfully
231 development of the parasite in the sand fly gut, parasites isolated from sand flies and
232 reservoirs have to be indistinguishable, and the sand fly has to be able to transmit the
233 parasite (Killick-Kendrick, 1999). On the other hand, BLAST analysis showed that ITS1
234 sequences from this study present a high similarity to *Leishmania* sp. ITS1 fragments
235 described in Portugal and Spain, also isolated in *S. minuta* (Bravo-Barriga et al., 2016;
236 Maia et al., 2015). Additionally, a high identity with *Leishmania* sp. sequences
237 described in China (B.-B. Yang et al., 2010) was revealed. Nevertheless, alignment
238 report showed that these ITS1 sequences are close to *L. tarentolae* sequences
239 annotated in the Genbank®. Moreover, the phylogenetic analysis clearly segregated the
240 *Leishmania* sp. sequences isolated from *S. minuta* from the *L. infantum* ITS1 genotype

241 identified on patients, wild reservoirs and *P. perniciosus* females from the leishmaniasis
242 outbreak of Madrid, Spain (Chicharro et al., 2013; González et al., 2017; Jiménez et al.,
243 2013).

244 The findings obtained in our work do not provide any evidence for now of human
245 pathogeny of the *Leishmania* sp. discovered in the *S. minuta* sand flies collected in the
246 leishmaniasis focus of southwestern Madrid region. However, immunocompromised
247 people could be affected by non-human pathogenic trypanosomatids as previous
248 studies have reported in our country (Jiménez, López-Vélez, Molina, Cañavate, &
249 Alvar, 1996). Even more, 17% of leishmaniasis cases in the outbreak of southwestern
250 Madrid have some degree of immunosuppression (Boletín Epidemiológico de la
251 Comunidad de Madrid, 2017). , with a maximum peak of 230.93In this context, it is also
252 important to emphasise the high density of *S. minuta* registered in this region with an
253 average of 106.8 sand flies/m² between 2012 to 2014 (González et al., 2017). Hence,
254 the high density of this sand fly species within the elevated *Leishmania* sp. infection
255 rates could suggest the existence of a well-established cycle in the region.

256 In conclusion, this study highlights several findings: i. the presence of *Leishmania* sp.
257 related to *L. tarentolae* in *S. minuta* females collected in the human leishmaniasis focus
258 of Fuenlabrada, Madrid; ii. *S. minuta* females can feed on mammals, including humans,
259 showing an opportunistic feeding behaviour and, iii: the presence of *Leishmania* sp. in
260 *S. minuta* fed on humans and other mammals. Therefore, further investigation is
261 necessary about *S. minuta* sand fly species in Spain and, more specifically, in the
262 mentioned leishmaniasis focus, to obtain more information about its biology and to
263 elucidate its possible implication in the *Leishmania* epidemiology in the country.

264 **Acknowledgements**

265 This study was partially sponsored and funded by the Dirección General de Salud
266 Pública, Consejería de Sanidad, Comunidad de Madrid; the Colegio de Veterinarios de
267 Madrid, and the Colegio de Biólogos de Madrid.

268 **Funding**

269 Estela González was supported by Dirección General de Salud Pública, Consejería de
270 Sanidad, Comunidad de Madrid. The Instituto de Salud Carlos III provided the
271 equipment and facilities.

272 **Ethics statement**

273 No ethical approval was required.

274 **Competing interests**

275 The authors declare that they have no competing interests.

276 **Supporting information**

277 Additional supporting data may be found online in the Supporting Information section at
278 the end of the article.

279 **References**

- 280 Ayari, C., Ben Othman, S., Chemkhi, J., Tabbabi, A., Fisa, R., Ben Salah, A., &
281 BenAbderrazak, S. (2016). First detection of *Leishmania major* DNA in
282 *Sergentomyia* (*Sintonius*) *clydei* (Sinton, 1928, Psychodidae: Phlebotominae),
283 from an outbreak area of cutaneous leishmaniasis in Tunisia. *Infection, Genetics*
284 *and Evolution*, 39, 241–248. <https://doi.org/10.1016/j.meegid.2015.10.030>
- 285 Azizi, K., Askari, M. B., Kalantari, M., & Moemenbellah-Fard, M. D. (2016). Molecular
286 detection of *Leishmania* parasites and host blood meal identification in wild sand
287 flies from a new endemic rural region, south of Iran. *Pathogens and Global Health*,
288 110(7–8), 303–309. <https://doi.org/10.1080/20477724.2016.1253530>
- 289 Berdjane-Brouk, Z., Koné, A. K., Djimdé, A. A., Charrel, R. N., Ravel, C., Delaunay, P.,
290 ... Izri, A. (2012). First detection of *Leishmania major* DNA in *Sergentomyia*
291 (*Spelaeomyia*) *darlingi* from cutaneous leishmaniasis foci in Mali. *PLoS ONE*, 7(1),
292 1–5. <https://doi.org/10.1371/journal.pone.0028266>
- 293 Bravo-Barriga, D., Parreira, R., Maia, C., Blanco-Ciudad, J., Afonso, M. O., Frontera,

294 E., ... Reina, D. (2016). First molecular detection of *Leishmania tarentolae*-like
295 DNA in *Sergentomyia minuta* in Spain. *Parasitology Research*, 115(3), 1339–
296 1344. <https://doi.org/10.1007/s00436-015-4887-z>

297 Campino, L., Cortes, S., Dionísio, L., Neto, L., Afonso, M. O., & Maia, C. (2013). The
298 first detection of *Leishmania major* in naturally infected *Sergentomyia minuta* in
299 Portugal. *Memorias Do Instituto Oswaldo Cruz*, 108(4), 516–518.
300 <https://doi.org/10.1590/0074-0276108042013020>

301 Chicharro, C., Llanes Acevedo, P., García, E., Nieto, J., Moreno, J., & Cruz, I. (2013).
302 Molecular typing of *leishmania infantum* isolates from a leishmaniasis outbreak in
303 Madrid, Spain, 2009 to 2012. *Eurosurveillance*, 18(30), 1–13.
304 <https://doi.org/10.2807/1560-7917.ES2013.18.30.20545>

305 Chusri, S., Thammapalo, S., Silpapojakul, K., & Siriyasatien, P. (2014). Animal
306 reservoirs and potential vectors of *Leishmania siamensis* in southern Thailand.
307 *Southeast Asian Journal of Tropical Medicine and Public Health*, 45(1), 13–19.

308 Cupp, E. W., Zhang, D., Yue, X., Cupp, M. S., Guyer, C., Sprenger, T. R., & Unnasch,
309 T. R. (2004). Identification of reptilian and amphibian blood meals from
310 mosquitoes in an eastern equine encephalomyelitis virus focus in central
311 Alabama. *The American Journal of Tropical Medicine and Hygiene*, 71(3), 272–
312 276. <https://doi.org/10.4269/ajtmh.2004.71.272>

313 Gállego Berenguer, J., Botet Fregola, J., Gállego Culleré, M., & Portús Vinyeta, M.
314 (1992). Los flebotomos de la España Peninsular e Islas Baleares. Identificación y
315 corología. Comentarios sobre los métodos de captura. In *"In memoriam" al*
316 *Profesor Doctor D.F. de P. Martínez Gómez* (pp. 580–600). Barcelona.

317 Gil Collado, J., Morillas, F., Sanchis, M. C., & Alvarez, Z. (1989). *Los flebotomos en*
318 *españa* -. *Revista de Sanidad Pública*, 63, 15-34.

319 González, E., Álvarez, A., Ruiz, S., Molina, R., & Jiménez, M. (2017). Detection of high
320 *Leishmania infantum* loads in *Phlebotomus perniciosus* captured in the
321 leishmaniasis focus of southwestern Madrid region (Spain) by real time PCR. *Acta*

322 Tropica, 171. <https://doi.org/10.1016/j.actatropica.2017.03.023>González, E.,
323 Jiménez, M., Hernández, S., Martín Martín, I., & Molina, R. (2017). Phlebotomine
324 sand fly survey in the focus of leishmaniasis in Madrid, Spain (2012-2014):
325 Seasonal dynamics, Leishmania infantum infection rates and blood meal
326 preferences. *Parasites & Vectors*, 10(368). <https://doi.org/10.1186/s13071-017->
327 2309-z

328 Hoogstraal, H., Dietlein, D. R., & Heyneman, D. (1962). Leishmaniasis in the Sudan
329 Republic: 4. Preliminary observations on man-biting sandflies
330 (Psychodidae:Phlebotomus) in certain Upper Nile endemic areas. *Transactions of*
331 *the Royal Society of Tropical Medicine and Hygiene*, 56(5), 411–422.

332 Jaouadi, K., Ghawar, W., Salem, S., Gharbi, M., Bettaieb, J., Yazidi, R., ... Ben Salah,
333 A. (2015). First report of naturally infected *Sergentomyia minuta* with *Leishmania*
334 *major* in Tunisia. *Parasites & Vectors*, 8(1), 1–3. <https://doi.org/10.1186/s13071->
335 015-1269-4

336 Jiménez, M., González, E., Martín-Martín, I., Hernández, S., & Molina, R. (2014). Could
337 wild rabbits (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the
338 focus of Madrid, Spain? *Veterinary Parasitology*, 202(3–4).
339 <https://doi.org/10.1016/j.vetpar.2014.03.027>

340 Jiménez, M. I., López-Vélez, R., Molina, R., Cañavate, C., & Alvar, J. (1996). HIV co-
341 infection with a currently non-pathogenic flagellate. *Lancet (London, England)*,
342 347(8996), 264–265. <https://doi.org/10.5555/URI:PII:S0140673696904419>

343 Jiménez, Maribel, González, E., Iriso, A., Marco, E., Alegret, A., Fúster, F., & Molina,
344 R. (2013). Detection of *Leishmania infantum* and identification of blood meals in
345 *Phlebotomus perniciosus* from a focus of human leishmaniasis in Madrid, Spain.
346 *Parasitology Research*, 112(7), 2453–2459. <https://doi.org/10.1007/s00436-013->
347 3406-3

348 Kanjanopas, K., Siripattanapipong, S., Ninsaeng, U., Hitakarun, A., Jitkaew, S.,
349 Kaewtaphaya, P., ... Leelayoova, S. (2013). *Sergentomyia* (*Neophlebotomus*)

350 gemmea, a potential vector of *Leishmania siamensis* in southern Thailand. *BMC*
351 *Infectious Diseases*, 13(1), 1. <https://doi.org/10.1186/1471-2334-13-333>

352 Karakuş, M., Pekağ Irbaş, M., Demir, S., Eren, H., Töz, S., & Özbel, Y. (2017).
353 Molecular screening of *Leishmania* spp. infection and bloodmeals in sandflies
354 from a leishmaniasis focus in southwestern Turkey. *Medical and Veterinary*
355 *Entomology*, 31(2), 224–229. <https://doi.org/10.1111/mve.12216>

356 Killick-Kendrick, R. (1999). The biology and control of Phlebotomine sand flies. *Clinics*
357 *in Dermatology*, 17(3), 279–289. [https://doi.org/10.1016/S0738-081X\(99\)00046-2](https://doi.org/10.1016/S0738-081X(99)00046-2)

358 Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics
359 Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33(7),
360 1870–1874. <https://doi.org/10.1093/molbev/msw054>

361 Latrofa, M. S., Iatta, R., Dantas-Torres, F., Annoscia, G., Gabrielli, S., Pombi, M., ...
362 Otranto, D. (2018). Detection of *Leishmania infantum* DNA in phlebotomine sand
363 flies from an area where canine leishmaniosis is endemic in southern Italy.
364 *Veterinary Parasitology*, 253(2010), 39–42.
365 <https://doi.org/10.1016/j.vetpar.2018.02.006>

366 Lewis, D. (1987). Phlebotomine sandflies (Diptera: Psychodidae) from the Oriental
367 region. *Systematic Entomology*, 12, 163–180. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-3113.1987.tb00194.x)
368 [3113.1987.tb00194.x](https://doi.org/10.1111/j.1365-3113.1987.tb00194.x)

369 Lewis, D. J., & Dyce, A. L. (1988). Taxonomy of the Australasian Phlebotominae
370 (Diptera: Psychodidae) with revision of genus *Sergentomyia* from the region.
371 *Invertebrate Systematics*, 2(6), 755–804. <https://doi.org/10.1071/IT9880755>

372 Maia, C., Parreira, R., Cristóvão, J. M., Freitas, F. B., Afonso, M. O., & Campino, L.
373 (2015). Molecular detection of *Leishmania* DNA and identification of blood meals
374 in wild caught phlebotomine sand flies (Diptera: Psychodidae) from southern
375 Portugal. *Parasites & Vectors*, 8(1), 1–10. [https://doi.org/10.1186/s13071-015-](https://doi.org/10.1186/s13071-015-0787-4)
376 [0787-4](https://doi.org/10.1186/s13071-015-0787-4)

377 Maroli, M., Feliciangeli, M. D., Bichaud, L., Charrel, R. N., & Gradoni, L. (2013).

378 Phlebotomine sandflies and the spreading of leishmaniasis and other diseases of
379 public health concern. *Medical and Veterinary Entomology*, 27(2), 123–147.
380 <https://doi.org/10.1111/j.1365-2915.2012.01034.x>

381 Mukherjee, S., Hassan, M. Q., Ghosh, A., Ghosh, K. N., Bhattacharya, A., & Adhya, S.
382 (1997). Short report: Leishmania DNA in Phlebotomus and Sergentomyia species
383 during a kala-azar epidemic. *American Journal of Tropical Medicine and Hygiene*,
384 57(4), 423–425. <https://doi.org/10.4269/ajtmh.1997.57.423>

385 Muñoz, C., Martínez-de la Puente, J., Figuerola, J., Pérez-Cutillas, P., Navarro, R.,
386 Ortuño, M., ... Berriatua, E. (2019). Molecular xenomonitoring and host
387 identification of *Leishmania* sand fly vectors in a Mediterranean periurban wildlife
388 park. In *Transboundary and Emerging Diseases*.
389 <https://doi.org/10.1111/tbed.13319>

390 Mutinga, M. J., Massamba, N. N., Basimike, M., Kamau, C. C., Amimo, F. A., Onyido,
391 A. E., ... Wachira, D. W. (1994). Cutaneous leishmaniasis in Kenya: *Sergentomyia*
392 *garhami* (Diptera Psychodidae), a possible vector of *Leishmania major* in Kitui
393 district: a new focus of the disease. *East African Medical Journal*, 71(7), 424–428.

394 Navea-Pérez, H. M., Díaz-Sáez, V., Corpas-López, V., Merino-Espinosa, G., Morillas-
395 Márquez, F., & Martín-Sánchez, J. (2015). *Leishmania infantum* in wild rodents:
396 reservoirs or just irrelevant incidental hosts? *Parasitology Research*, 114(6),
397 2363–2370. <https://doi.org/10.1007/s00436-015-4434-y>

398 Ngouateu, A. T., Kirstein, O. D., Ngouateu, O. B., Krüger, A., von Stebut, E., Maurer,
399 M., ... Dondji, B. (2018). First detection of *Leishmania donovani* in sand flies from
400 Cameroon and its epidemiological implications. *Tropical Medicine & International*
401 *Health*. <https://doi.org/10.1111/tmi.13123>

402 Nzelu, C. O., Kato, H., Pupilampu, N., Desewu, K., Odoom, S., Wilson, M. D., ...
403 Boakye, D. A. (2014). First detection of *Leishmania tropica* DNA and
404 *Trypanosoma* species in *Sergentomyia* sand flies (Diptera: Psychodidae) from an
405 outbreak area of cutaneous leishmaniasis in Ghana. *PLoS Neglected Tropical*

406 Diseases, 8(2), e2630. <https://doi.org/10.1371/journal.pntd.0002630>

407 Özbel, Y., Karakuş, M., Arserim, S. K., Kalkan, Ş. O., & Töz, S. (2016). Molecular
408 detection and identification of Leishmania spp. in naturally infected Phlebotomus
409 tobbi and Sergentomyia dentata in a focus of human and canine leishmaniasis in
410 western Turkey. *Acta Tropica*, 155, 89–94.
411 <https://doi.org/10.1016/j.actatropica.2015.12.018>

412 Rioux, J. A., Guilvard, E., Gállego, J., Moreno, G., Pratlong, F., Portús, M., ... Bastien,
413 P. (1986). Phlebotomus ariasi Tonnoir, 1921 et Phlebotomus perniciosus
414 Newstead, 1911 vecteurs du complexe Leishmania infantum. In *Leishmania*.
415 *Taxonomie et phylogénèse. Applications éco-épidémiologiques* (pp. 439–444).
416 Montpellier: IMEEE.

417 Sáez, V. D., Morillas-Márquez, F., Merino-Espinosa, G., Corpas-López, V., Morales-
418 Yuste, M., Pesson, B., ... Martín-Sánchez, J. (2018). Phlebotomus langeroni
419 Nitzulescu (Diptera, Psychodidae) a new vector for Leishmania infantum in
420 Europe. *Parasitology Research*, 117(4), 1105–1113.
421 <https://doi.org/10.1007/s00436-018-5788-8>

422 Senghor, M. W., Niang, A. A., Depaquit, J., Ferté, H., Faye, M. N., Elguero, E., ...
423 Bañuls, A. L. (2016). Transmission of Leishmania infantum in the canine
424 leishmaniasis focus of Mont-Rolland, Senegal: Ecological, parasitological and
425 molecular evidence for a possible role of Sergentomyia sand flies. *PLoS*
426 *Neglected Tropical Diseases*, 10(11), 1–17.
427 <https://doi.org/10.1371/journal.pntd.0004940>

428 Siripattanapipong, S., Leelayoova, S., Ninsaeng, U., & Mungthin, M. (2018). Detection
429 of DNA of Leishmania siamensis in Sergentomyia (Neophlebotomus) iyengari
430 (Diptera : Psychodidae) and molecular identification of blood meals of sand flies in
431 an affected area , Southern Thailand. *Journal of Medical Entomology*, 20(10), 1–7.
432 <https://doi.org/10.1093/jme/tjy069>

433 Sutherst, R. W. (2004). Global change and human vulnerability to vector-borne

434 diseases. *Clinical Microbiology Reviews*, 17(1), 136–173.
435 <https://doi.org/10.1128/CMR.17.1.136>

436 Svobodová, M., Alten, B., Zídková, L., Dvořák, V., Hlavačková, J., Myšková, J., ... Volf,
437 P. (2009). Cutaneous leishmaniasis caused by *Leishmania infantum* transmitted
438 by *Phlebotomus tobbi*. *International Journal for Parasitology*, 39(2), 251–256.
439 <https://doi.org/10.1016/j.ijpara.2008.06.016>

440 Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in
441 the control region of mitochondrial DNA in humans and chimpanzees. *Molecular*
442 *Biology and Evolution*, 10(3), 512–526.
443 <https://doi.org/10.1093/oxfordjournals.molbev.a040023>

444 Tarallo, V. D., Dantas Torres, F., Lia, R. P., & Otranto, D. (2010). Phlebotomine sand
445 fly population dynamics in a leishmaniasis endemic peri-urban area in southern
446 Italy. *Acta Tropica*, 116(3), 227–234.
447 <https://doi.org/10.1016/j.actatropica.2010.08.013>

448 Taylor, V. M., Muñoz, D. L., Cedeño, D. L., Vélez, I. D., Jones, M. A., & Robledo, S. M.
449 (2010). *Leishmania tarentolae*: Utility as an in vitro model for screening of
450 antileishmanial agents. *Experimental Parasitology*, 126(4), 471–475.
451 <https://doi.org/10.1016/j.exppara.2010.05.016>

452 Tello, A., González Mora, D., Outerelo, R., Iriso, A., & Ángeles Vázquez, M. (2015).
453 Los flebotomos del brote de leishmaniasis en el suroeste de la Comunidad de
454 Madrid (Diptera, Psychodidae, Phlebotominae). *Hist. Nat. Sec. Biol. Bol. R. Soc.*
455 *Esp. Hist. Nat. Sec. Biol*, 109(109), 57–64. Retrieved from
456 <http://bba.bioucm.es/cont/docs/518.pdf>

457 World Health Organization. (2010). *Control of Leishmaniases: Report of a meeting of*
458 *the WHO Expert Committee on the Control of Leishmaniases*. (WHO Technical
459 Report Series 949, March 2010), 22–26.

460 Yang, B.-B., Guo, X.-G., Hu, X.-S., Zhang, J.-G., Liao, L., Chen, D.-L., & Chen, J.-P.
461 (2010). Species discrimination and phylogenetic inference of 17 Chinese

462 Leishmania isolates based on internal transcribed spacer 1 (ITS1) sequences.
463 *Parasitology Research*, 107(5), 1049–1065. <https://doi.org/10.1007/s00436-010->
464 1969-9

465 Yang, B. Bin, Guo, X. G., Hu, X. S., Zhang, J. G., Liao, L., Chen, D. L., & Chen, J. P.
466 (2010). Species discrimination and phylogenetic inference of 17 Chinese
467 Leishmania isolates based on internal transcribed spacer 1 (ITS1) sequences.
468 *Parasitology Research*, 107(5), 1049–1065. <https://doi.org/10.1007/s00436-010->
469 1969-9

470 **Legends to figures**

471 **Fig. 1** Phylogenetic study of *Leishmania* ITS1 sequences. Maximum-likelihood tree
472 based on an alignment of ITS1 sequences obtained in this study and sequences
473 from Genbank® database. The phylogenetic tree has been inferred by using the
474 Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei,
475 1993). The tree with the highest log likelihood (-659.66) is shown. Initial tree(s)
476 for the heuristic search were obtained automatically by applying Neighbor-Join
477 and BioNJ algorithms to a matrix of pairwise distances estimated using the
478 Maximum Composite Likelihood (MCL) approach and then selecting the topology
479 with superior log likelihood value. The tree is drawn to scale; with branch lengths
480 measured in the number of substitutions per site (next to the branches).
481 Accession numbers of the sequences are in parentheses. *Leishmania infantum*
482 ITS1 sequence highlighted in a black square corresponds to the genotype
483 circulating in the leishmaniasis focus (ITS-Lombardi genotype). * Partial
484 sequences that matched with other complete sequences and therefore weren't
485 annotated.

486

487 **Additional file 1: Fig. S1.** Alignment of the *Leishmania* sp. ITS1 sequences isolated
488 from *Sergentomyia minuta* sand flies. The alignment was performed following the

489 CLUSTAL W algorithm using MEGA7 (Kumar et al., 2016). The black square
490 highlights the variable poly TA region at position 197.

491