Molecular detection of *Leishmania* DNA and identification of blood meals in wild caught phlebotomine sand flies (Diptera: Psychodidae) from southern Portugal

Carla Maia¹*, Ricardo Parreira¹,², José Manuel Cristóvão¹, Ferdinando Bernardino Freitas¹, Maria Odete Afonso¹ and Lenea Campino¹,³

**Abstract**

**Background:** Zoonotic visceral leishmaniasis caused by *Leishmania infantum* which is transmitted by phlebotomine sand flies (Diptera, Psychodidae) is endemic in the Mediterranean basin. The main objectives of this study were to (i) detect *Leishmania* DNA and (ii) identify blood meal sources in wild caught female sand flies in the zoonotic leishmaniasis region of Algarve, Portugal/Southwestern Europe.

**Methods:** Phlebotomine sand flies were collected using CDC miniature light traps and sticky papers. Sand flies were identified morphologically and tested for *Leishmania* sp. by PCR using ITS-1 as the target sequence. The source of blood meal of the engorged females was determined using the *cyt-b* sequence.

**Results:** Out of the 4,971 (2,584 males and 2,387 females) collected sand flies, *Leishmania* DNA was detected by PCR in three females (0.13%), specifically in two specimens identified on the basis of morphological features as *Sergentomyia minuta* and one as *Phlebotomus perniciosus*. Haematic preferences, as defined by the analysis of *cyt-b* DNA amplified from the blood-meals detected in the engorged female specimens, showed that *P. perniciosus* fed on a wide range of domestic animals while human and lizard DNA was detected in engorged *S. minuta*.

**Conclusions:** The anthropophilic behavior of *S. minuta* together with the detection of *Leishmania* DNA highlights the need to determine the role played by this species in the transmission of *Leishmania* parasites to humans. In addition, on-going surveillance on *Leishmania* vectors is crucial as the increased migration and travelling flow elevate the risk of introduction and spread of infections by *Leishmania* species which are non-endemic.

**Keywords:** Blood-meal, *Leishmania*, Phlebotomine sand flies, *Phlebotomus perniciosus*, *Sergentomyia minuta*, Portugal, Southwestern Europe

**Background**

Leishmaniasis caused by *Leishmania infantum* is the only tropical vector-borne disease that has been endemic in southern Europe for decades [1]. Most of the reported cases are due to zoonotic visceral leishmaniasis (VL), the most dangerous form of *Leishmania* infection, being lethal when untreated. Dogs are considered the major host for these parasites, and the main reservoir for human infections. In nature, the pathogen transmission occurs via the infective bite of phlebotomine sand flies (Diptera, Psychodidae), for both humans and dogs.

In Portugal, as in other countries in the south of Europe, VL was initially described as a pediatric disease but from the end of the 1980s onwards, the number of cases in children has decreased with a concomitant increase of infection in adults, commonly associated with HIV/AIDS [2]. In the last ten years (2005–2014), 119 new cases of human VL (17 in immunocompetent adults, 36 in children and 66 in immunocompromised patients) and 16 cutaneous...
leishmaniasis cases were diagnosed at the Leishmaniasis Laboratory at the Institute of Hygiene and Tropical Medicine. *Leishmania infantum* zymodeme MON-1 is the most common aetiological agent of autochthonous human and canine leishmaniasis cases [3] and *Phlebotomus perniciosus* and *Phlebotomus ariasi* have been confirmed as proven vectors [4].

As data regarding *Leishmania* infection rate and blood meal sources of phlebotomine sand flies in Portugal is still too scarce, this study was implemented so as to allow the (i) detection of *Leishmania* DNA and (ii) identification of blood meal sources in wild caught female sand flies in Algarve, Portugal/Southwestern Europe.

**Methods**

**Study area**

Algarve, located in southern Portugal, has an area of 5,412 Km2 with an estimated number of permanent inhabitants approximating 450,000 [5], which triplicates during summer months. Figs (*Ficus carica*), almonds (*Prunus amygdalus*), oranges (*Citrus sinensis*), carobs (*Ceratonia siliqua*), strawberries trees (*Arbutus unedo*) and cork oaks (*Quercus suber*), are the most common crops in the region [6]. Algarve has a Mediterranean climate with warm weather (annual average temperature of 18°C) and low rainfall almost all year round (annual average of 500 mm). Summer (June-September) is the driest and warmest season with average monthly temperatures between 16° and 28-30°C (www.ipma.pt).

**Collection and identification of sand flies**

Between May to October from 2011 to 2013, CDC light traps and sticky oil papers were set up in 11 sampling points during three consecutive days per month. Collection places included domestic, peri-domestic and sylvatic environments. In most of the studied biotopes, in addition to humans and dogs, the major vertebrates visible within a 50 m radius of the collection spots were livestock, horses, pigs, rabbits and poultry. Collected sand flies were stored in 70% ethanol for further analysis. A total of 4,971 sand flies (2,584 males and 2,387 females) were collected and identified morphologically. Phlebotomine specimens of both genders were identified by their morphological characteristics to the species level, according to Pires [7]. Female identification was done by microscopic observation of the spermatheca, after dissection and mounting of the three last abdominal segments in Marc-André solution, while males were identified by direct stereomicroscopic observation of the genitalia. In addition, for each female, the presence of eggs (gravid status), and/or blood (engorged: total or partial vs. unfed) in the abdomen was recorded (Table 1).

**DNA extraction, PCR amplification and DNA sequencing**

For each female sand fly, the remainder of the body (minus genitalia) was used as the source of DNA, extracted using the Citogene Cell and Tissue kit (Citomed, Portugal) following the manufacturer's instructions, with the exception that the maceration of the insect's tissues was carried out with a piston pellet, and the final elution volume was 30 μl.

The PCR amplification of the internal transcribed spacer 1 (ITS-1) of the ribosomal operon of *Leishmania* was performed using the LITSR and L5.8S primers generating amplicons with 300–350 bp [8]. A positive control containing *L. infantum* DNA (MHOM/PT/88/IMT151) and a negative control without DNA template were included. To identify the origin of the blood meal of engorged females, the modified vertebrate-universal specific primers (cytB1-F and cytB2-R) were used to amplify a 350 bp segment of the host mitochondrial *cytochrome b* gene (*cyt-b*) [9]. PCR amplifications were performed in a 25 μl final volume containing 12.5 μl of NZYTaq 2× Green Master Mix (Nzytech, Portugal), 1 μl of each primer (10 pmol) and 2 μl of template DNA. The cycling profile used for the amplification of ITS-1 sequences included an initial denaturation step at 95°C for 2 min, followed by 32 repeats of 95°C-20 sec, 53°C-30 sec, 72°C-1 min followed by a final extension step at 72°C-6 min, while the preparation of *cyt-b* PCR products was carried out starting from 95°C for 5 min, followed by 40 cycles of 94°C-1 min, 55°C-1 min, 72°C-1 min followed by 72°C-7 min. Both amplicons were visualized under UV illumination after their resolution by conventional electrophoresis on 1.5% agarose gels stained with Greensafe premium® (Nzytech, Portugal), using a 100 bp DNA ladder as a molecular weight marker. PCR products were purified with a High Pure PCR Product Purification Kit (Roche® Mannheim, Germany) according to the manufacturer's instructions. Subsequently, purified products were sent to LIGHTrun™ Sequencing Service (GATC-biotech, Germany) for direct sequencing by Sanger's method with the same primers used for DNA amplification.

**DNA sequence analyses**

The identity of the feeding host (species level), carried out on the basis of the analysis of the obtained *cyt-b* sequences, was determined according to the closest BLASTn match (identity ≥ 99%) to a homologous sequence deposited at GenBank. The sequences obtained in the course of this work were deposited at DNA Data Bank of Japan (DDBJ) (http://www.DDBJ.nig.ac.jp).

Restriction profile was obtained by virtual digestion for ITS-1 sequence by using the Restriction Mapper (version 3 available online at http://www.restrictionmapper.org/).
Phylogenetic relationships were inferred from ITS-1 nucleotide sequence alignments produced with the MAFFT multiple alignment program using a combination of the Q-INS-i and E-INS-i alignment options [10]. Phylogenetic tree construction was carried out using a Maximum Likelihood (ML) approach, and the Kimura’s 2-P (K2P) evolutionary model, also assuming Γ distributed substitution rates among sites, as indicated by Mega6 [11] and as defined by the Akaike information criterion. Alternatively, an empirically defined model (GTR + Γ + I) was also used. The topological robustness of the obtained trees was assessed by bootstrapping, using 1000 resampling of the original alignment data. The final trees were manipulated for display using FigTree v.1.2.2. (available at http://tree.bio.ed.ac.uk/software/figtree/). NeighborNet networks (NNn) were constructed using the same distance matrix using Splits Tree4 software [12]; software available at http://www.splitstree.org/). Mean genetic distance values were calculated with the K2P formula, using Mega6 [11].

Results
Morphological identification of sand flies
S. minuta was the most prevalent species totaling a number of 3,208 specimens (64.53%), followed by P. perniciosus with 1,542 specimens (31.02%). Phlebotomus sergenti (174; 3.50%), P. ariasi (46; 0.93%) and one P. papapasi female (0.02%) were also collected. Eighty five females (2 P. ariasi, 1 P. papapasi, 32 P. perniciosus, 1 P. sergenti and 49 S. minuta) were gravid.

Leishmania DNA detection, sequencing, and phylogenetic inference analysis
Leishmania DNA was detected in three apparently unfed females (0.13%) identified as P. perniciosus (n = 1) and in S. minuta (n = 2). The three positive females were collected in peridomestic biotopes (i.e. P. perniciosus was collected in a horse stable, and S. minuta were collected in a cattle pen and close to a kennel, respectively). The three ITS-1 obtained sequences were submitted to DDBJ (DDBJ accession numbers: LC028233 to LC028235). PCR product obtained from P. perniciosus had a similar size as L. infantum control while the PCRs products from both S. minuta were slightly bigger (data not shown). Furthermore, a HaeIII restriction profile characteristic of L. infantum (184 bp, 72 bp and 55 bp) was obtained after virtual digestion of the ITS-1 sequence obtained from the positive DNA control as well as from P. perniciosus. Finally, sequence homology searches using BLASTn (megablast search option) revealed >99% identity with L. infantum, L. chagasi or L. donovani (E-values = e−154), and a sequence coverage >94%. Curiously, however, species assignment to the ITS-1 sequences amplified from S. minuta could not be carried out on the basis of nucleotide sequence homology search results. In this case the 15 best matches obtained with BLASTn (megablast) revealed >93% sequence identity (>95% sequence coverage and E-values < e−122) with only Leishmania sequences of Chinese origin referred to as Leishmania sp. [13], indicating relatively low identity with any sequence references already deposited in the sequence databases. Virtual HaeIII restriction profiles of the ITS-1 sequences amplified from S. minuta (strains 5277 and 3400) were characterized by three DNA fragments (<193 bp, 89 bp, <54 bp), which were found to be similar, though not identical, to the virtual HaeIII profiles determined for the Chinese Leishmania sp. sequences (<210 bp, 87 bp, <43 bp) mentioned above.

Definition of the species status of the obtained ITS-1 sequences was further pursued on the basis of phylogenetic analyses, along with others directly downloaded from the public database, and used as references (Table 2). The use of the suggested evolutionary model (K2P + Γ) or a more robust one (GTR + Γ + I), empirically defined by the user, resorted in phylogenetic trees with identical topologies as that shown in Figure 1 (data not shown).

One of the sequences obtained in this study (strain 1704), amplified from P. perniciosus, was found to segregate in a large monophyletic cluster that included L. infantum, L. donovani, L. archibaldi and L. chagasi (Figure 1), characterized by low genetic variability (average genetic distance of 0.2%). On the other hand, the remainder two ITS-1 sequences (strains 5277 and 3400),
| Species* | Strain/isolate/haplotype | Origin/host | Accession number |
|----------|--------------------------|-------------|-----------------|
| Leishmania donovani | MHOM/KE/83/NLB189 | Kenya/Human | AJ634374 |
| Leishmania donovani | MHOM/SD/93/95 | Sudan/Human | AJ634372 |
| Leishmania donovani | MHOM/LK/2002/L60c | Sri Lanka/Human | AM901447 |
| Leishmania donovani | MHOM/LK/2002/L60b | Sri Lanka/Human | AM901448 |
| Leishmania archibaldi | MHOM/SD/93/GE | Sudan/Human | AJ634357 |
| Leishmania archibaldi | MHOM/SD/97/LEM3429 | Sudan/Human | AJ634358 |
| Leishmania archibaldi | MHOM/SD/97/LEM3463 | Sudan/Human | AJ634359 |
| Leishmania donovani | MHOM/SU/84/LEM0946 | Soviet Union/Human | HGS12918 |
| Leishmania donovani | MCan/MA/2002/AD3 | Morocco/Canine | AM901453 |
| Leishmania donovani | MHOM/Q/1981/SUKAR2 | Iraq/Human | AM901452 |
| Leishmania donovani | MHOM/IN/1983/CHANDIGARH | India/Human | AM901449 |
| Leishmania infantum | MCan/UZ/2007/LRC-L1309 | Uzbekistan/Canine | FN398341 |
| Leishmania infantum | MHOM/BR/2007/JFF BM | Brazil/Human | FN398343 |
| Leishmania infantum | MHOM/T/93/55800 | Italy/Human | AJ634354 |
| Leishmania infantum | MHOM/PT/00/MT260 | Portugal/Human | AJ634344 |
| Leishmania infantum | MHOM/MT/85/BUCK | Malta/Human | AJ634350 |
| Leishmania infantum | MHOM/SD/93/452BM | Sudan/Human | AJ634371 |
| Leishmania chagasi | MHOM/BR/85/M9702 | Brazil/Human | AJO00306 |
| Leishmania chagasi | MHOM/PA/79/WR317 | Panama/Human | AJO00305 |
| Leishmania tropica | MHOM/L/01/LRC-L838 | Israel/Human | FN677341 |
| Leishmania tropica | MHOM/EG/90/LPN65 | Egypt/Human | HGS12927 |
| Leishmania tropica | MHOM/PS/01/15L590 | Palestine*/Human | FN677345 |
| Leishmania tropica | MHOM/YE/86/LEM1015 | Yemen/Human | HGS12919 |
| Leishmania tropica | MHOM/TN/88/TAT3 | Tunisia/Human | AJO00485 |
| Leishmania tropica | IHAM/GH/2007/KLE-18 | Ghana/Sergentomyia hamrini | AB787190 |
| Leishmania aethiopica | MHOM/ER/2009/7457 | Eritrea/Human | FN252411 |
| Leishmania aethiopica | MHOM/KE/71/KP5-H2 | Kenya/Human | HGS12908 |
| Leishmania tuzanica | KD85001 | Uzbekistan/Rhombomys opimus | AJ272378 |
| Leishmania tuzanica | K3 | Kazakhstan/Rhombomys opimus | AJ272382 |
| Leishmania gerbilli | MRHO/UZ/87/KO-87555 | Uzbekistan/Rhombomys opimus | AJS00486 |
| Leishmania major | MTAT/KE/1NL089A | Kenya/NID | AJS00482 |
| Leishmania major | MHOM/UZ/02/17h | Uzbekistan/Human | FN677357 |
| Leishmania major | MHOM/BF/2004/REN04-8 | Burkina Faso/Human | HGS12963 |
| Leishmania major | MHOM/JO/90/JH39 | Jordan/Human | HGS12945 |
| Leishmania major | MHOM/TN/97/LPN162 | Tunisia/Human | FN677342 |
| Leishmania major | MHOM/DZ/89/UPA228 | Algeria/Human | HGS12924 |
| Leishmania mexicana | MHOM/PE/02/LH2312 | Peru/Human | HGS12965 |
| Leishmania mexicana | MHOM/EC/90/LM | Ecuador/Human | HGS12934 |
| Leishmania amazonensis | MHOM/BR/73/M2269 | Brazil/Human | DQ182536 |
| Leishmania amazonensis | IFLA/BF/67/PH8 | Brazil/NID | AF339753 |
| Leishmania braziliensis | MHOM/PE/2003/LH2920 | Peru/Human | FN398337 |
| Leishmania braziliensis | MHOM/BR/00/LTB300 | Brazil/Human | FN398338 |
| Leishmania peruviana | MHOM/PE/2006/LH3667 | Peru/Human | FN398340 |
| Leishmania peruviana | MHOM/PE/1990/MB86 | Peru/Human | FN398339 |
amplified from *S. minuta*, were found to locate in a bootstrap-supported (99%) assemblage of multiple reference sequences of Chinese origin, merely defined as *Leishmania* sp. [13], and that included a multitude of *Leishmania* sequences from human and canine origin, with an average genetic distance of 2.6% (ranging from 0% to 8.0%), indicating considerably higher genetic variability than that associated with the *L. infantum*/*L. donovani*/*L. archibaldi*/*L. chagasi* cluster. Similar conclusions were achieved when, instead of assuming a strict tree-like evolution, the phylogenetic relationships between ITS-1 sequences were represented as a NNn (Figure 2).

Vertebrate DNA detection in female sand flies

A total of 78 engorged female sand flies (3 *P. ariasi*, 49 *P. perniciosus*, 1 *P. sergenti* and 25 *S. minuta*) were tested to determine the vertebrate host source of the blood meal. A positive PCR amplification result was obtained for 43 of the collected specimens. After DNA sequencing of the amplified partial *cyt-b* sequences, the origin of 30 (69.77%) blood-meals was identified (Table 3) on the basis of the closest sequence matches, as defined by BLASTn sequence homology searches (>99% identity with deposited at the GenBank/EMBL/DDBJ public databases).

Discussion

Phlebotomine sand flies are distributed in all countries around the Mediterranean basin, turning both human populations and domestic animals living in these areas into potential targets to sand fly-borne diseases such as leishmaniasis. Therefore, knowledge on the host preferences of sand flies under natural conditions is essential not only to understand their vectorial role, but also as a means to identify potential reservoir hosts. In this work, we detected *Leishmania* DNA and evaluated blood meal sources of fed females sand flies captured in southern Portugal, where zoonotic leishmaniasis is known to be endemic [2].

Similarly to what has been observed by others [14-19] the blood meal analysis of the engorged *P. perniciosus* revealed that this species fed on a broad variety of vertebrate hosts (i.e. horses, cattle, sheep, pigs, rabbits and chickens) highlighting its opportunistic feeding behaviour. Interestingly, no dog or human blood was detected in blood-fed *P. perniciosus*, despite the fact that it has been clearly defined as a proven vector of *L. infantum* in the Algarve region [19-21]. The apparent absence of *P. perniciosus* feeding on dogs and humans might indicate that in the sampled biotopes, neither of them were the main blood sources for this sand fly species due to the presence of other larger sized vertebrates (e.g. horses) and/or present in greater numbers (i.e. chicken, rabbits), making them easier targets.

In addition, *Leishmania infantum* DNA was detected in one unfed *P. perniciosus* specimen. Assignment of species status for the 1704 ITS-1 sequence could not be clearly carried out solely based on phylogenetic tree analysis due to the low genetic variability of the ITS-1 sequences that define the *L. donovani* complex [22] (that

### Table 2 Nucleotide reference sequences used in this work (Continued)

| Leishmania species | Reference | Country/Species | Accession Number |
|--------------------|-----------|----------------|-----------------|
| *Leishmania* sp.   | MHOM/BR/2002/NMT-RBC013 | Brazil/Human | FN39831 |
| *Leishmania* sp.   | MHOM/BR/2002/NMT-RBC013 | Brazil/Human | FN39832 |
| *Leishmania* panamensis | Isolate 18, clone 4 | ND/Human | FJ948442 |
| *Leishmania* sp.   | MHOM/CN/80/X801 | P.R.China/Human | HQ830357 |
| *Leishmania* sp.   | MHOM/CN/89/G55 | P.R.China/Human | HQ830360 |
| *Leishmania* sp.   | MHOM/CN/90/SC10H2 | P.R.China/Human | HQ830352 |
| *Leishmania* sp.   | MHOM/CN/86/SC6 | P.R.China/Human | HQ830356 |
| *Leishmania* sp.   | MHOM/CN/90/SC10H2 | P.R.China/Human | HM130601 |
| *Leishmania* sp.   | MCAN/CN/60/G51 | P.R.China/Canine | HM130600 |
| *Leishmania* sp.   | MHOM/G56/CHN/SCgq | P.R.China/Human | HM130599 |
| *Leishmania* sp.   | MCAN/CN/86/SC9 | P.R.China/Canine | HQ830359 |
| *Leishmania* sp.   | MHOM/CN/83/G52 | P.R.China/Human | HM130603 |
| *Leishmania* sp.   | MHOM/G55/CHN/SCH2g | P.R.China/Human | HM130602 |
| *Leishmania* sp.   | MHOM/SC11/CHN/SCgq | P.R.China/Human | HM130606 |
| *Leishmania* sp.   | MHOM/CN/84/JS1 | P.R.China/Human | HM130605 |
| *Leishmania* sp.   | MHOM/CN/84/SD1 | P.R.China/Human | HM130604 |
| *Leishmania* sp.   | MHOM/CN/89/G56 | P.R.China/Human | HQ830355 |
| *Leishmania* sp.   | MHOM/CN/90/SC11 | P.R.China/Human | HQ830361 |

*Species as defined by the depositors; Israel: Occupied Palestinian Territories; P. R. China: People’s Republic of China; ND: not defined.*
Figure 1 Maximum likelihood phylogenetic tree (midpoint rooted) of *Leishmania* ITS-1 sequences amplified from phlebotomine sand flies collected in Portugal. The percentages of significant (≥ 77%) bootstrap values of 1000 resamplings of the original data are indicated at specific branch-nodes. The size bar indicates 0.02 substitutions per site.
include *L. infantum*, *L. donovani*, *L. archibaldi* and *L. chagasi*, precluding a clear resolution of this genetic cluster, as previously observed [13]. Nevertheless, the ITS-1 amplicon size and virtual *Hae*III restriction profile obtained for the 1704 sequence amplified from *P. perniciosus* were compatible with it corresponding to *L. infantum*, and reinforces the maintenance of this sand fly species as vector of *L. infantum* in southern Portugal [19–21].

Sand flies of the *Sergentomyia* genus, which is widely distributed throughout the Old World, are proven vectors of reptile *Leishmania* species [23]. It is generally accepted that most of *Sergentomyia* species are not anthropophilic, and as a consequence cannot transmit either *Leishmania* or any other pathogens to humans. However, in the present study, apart from detecting *Tarentola mauritanica* (a reptile widely distributed around the Mediterranean area [23,24]) DNA in one engorged *S. minuta*, human DNA was also amplified in four specimens corroborating that at least some *Sergentomyia* species disclose sporadic/opportunistic anthropophilic feeding-behaviour [25,26]. Furthermore, *Leishmania* sp. DNA was detected in two unfed *S. minuta* females, which unambiguously allocated with references within a cluster of Chinese *Leishmania* sp. previously isolated from humans and canine leishmaniasis cases [13]. While phylogenetic tree reconstruction and NNn analyses showed that the two ITS-1 sequences amplified from *S. minuta* (strains 5277 and 3400) clearly segregated away from all the others in a genetically consistent assemblage of *Leishmania* strains, in this case species assignment was limited by the unavailability of well characterized reference strains. However, despite the inability to clearly define the species of origin of the obtained sequences using phylogenetic analyses, the detection of *Leishmania* DNA phylogenetically related to those considered pathogenic to humans and dogs in China [13] was somewhat unexpected.

According to Yang et al. [13], the above mentioned *Leishmania* strains of Chinese origin belonged to an undefined species, that was found to be genetic divergent from any of the known New and Old World *Leishmania*,

---

**Figure 2** NeighborNet network constructed with SplitsTree software employing the matrix of genetic distances (corrected with the K2P formula) between individual *Leishmania* ITS-1 sequences amplified from phlebotomine sand flies collected in Portugal, and reference sequences.
| Sand fly host   | P. ariasi | P. perniciosus | S. minuta | Blast identity for the blood meal | DDBJ accession no. |
|----------------|-----------|----------------|-----------|-----------------------------------|-------------------|
| Horse (Equus caballus) | 0         | 12             | 0         | 99-100%                           | AB985687 AB985693-97 AB985699 AB985703 AB985708 AB985711 AB985714 |
| Chicken (Gallus gallus) | 0         | 5              | 0         | 99-100%                           | AB985704 AB985705 AB985710 AB985713 AB985715 |
| Human (Homo sapiens) | 0         | 0              | 4         | 99-100%                           | AB985688 AB985689 AB985698 AB985712 |
| Rabbit (Oryctolagus cuniculus) | 0         | 3              | 0         | 99%                              | AB985690 AB985700 AB985709 |
| Pig (Sus scrofa) | 0         | 2              | 0         | 99                                | AB985707 AB985716 |
| Cattle (Bos taurus) | 1         | 1              | 0         | 99                                | AB985702 AB985706 |
| Sheep (Ovis aries) | 0         | 1              | 0         | 99                                | AB985701 |
| Lizard (Tarentola mauritanica) | 0         | 0              | 1         | 99                                | AB985692 |
| **Total**       | 1         | 24             | 5         |                                   |                   |
on the basis of ITS-1 sequence analysis. Similar results were obtained when kinetoplast cytochrome oxidase II (COII; [27]) or CYT-b coding sequences [28] amplified from these same strains were analysed. Interestingly, both phylogenetic inference reconstruction studies revealed that the Chinese *Leishmania* sp. isolates were most closely related to the lizard-infecting *L. tarentolae*. Unfortunately, in the present study it was not possible to evaluate if the two *Leishmania* sp. detected in *S. minuta* were genetic related to this reptile *Leishmania* species, as no ITS-1 sequences of *L. tarentolae* have yet been deposited in DNA sequence databases for public access. On the other hand, exhaustion of the DNA extracts on which the analysis presented in this report was based ruled out any possibility of generating *cyt-b* and/or *coll* sequence data. Nevertheless, and taking into account the results obtained with *cyt-b/coll* [27,28], in the near future it will be important to analyse more of these *Leishmania* parasites obtained from both vertebrate (including reptiles) and invertebrate infected hosts for assessment of the parasite species as well as to determine their clinical significance, and estimate the potential risk their endemic establishment in Portugal/Europe. Ideally, should the laboratory settings allow it, further genetic analysis-based studies should be supported, as much as possible, by sequence datasets combining information from multiple genetic loci, so as to tentatively increase the phylogenetic signal, and achieve a better resolution of the observed genetic clusters, including the *L. donovani* complex [29].

Based upon literature reviews, a consideration of the role of *Sergentomyia* in the circulation of mammalian leishmaniasis becomes apparent as *Leishmania* DNA has been identified in several species. These include the molecular detection of *L. major* in *S. sintoni* in Iran [30], *S. garnhami* in Kenya [31], *S. darlingi* in Mali [25], and *S. minuta* in Portugal [32]. Furthermore, *L. donovani* has been detected in *S. babu* in India [33], *L. infantum* in *S. dubia*, *S. magna* and *S. schwetzi* in Senegal [34], and *L. siamensis* in *S. gemmea* in Thailand [35]. Finally, more recently, *L. tropica* has been found in *S. ingrami* and *S. hamoni* in Ghana [26]. Nevertheless, PCR positivity alone should not be used for incrimination of *Sergentomyia* sand flies as *Leishmania* vectors since the detection of DNA does not give any information about the parasites’ viability or its presence as virulent metacyclic promastigotes [36,37]. In fact, and although *L. infantum* DNA had been detected in *S. schwetzi* from Senegal [34], the refractoriness of this African species to some *Leishmania* species infecting humans (including *L. donovani*, *L. infantum* and *L. major*) has also been recently demonstrated [38]. In any case, the refractoriness of this particular *Sergentomyia* species does not necessarily extend to the whole of the genus. In this line of reasoning, the competence and permissiveness of the different species from *Phlebotomus* spp. to different Old World *Leishmania* has also been observed [39].

As *L. major* DNA had previously been detected in one *S. minuta* captured in the same region [32], together with the detection in this study of both human and *Leishmania* sp. DNA in this species, it would be important to determine if *S. minuta* fulfills the criteria that support its incrimination as vector for this parasite, and that include (i) the isolation of metacyclic promastigotes from the digestive tubes of field-collected specimens; and (ii) the experimental demonstration of its capacity to transmit Old World *Leishmania* species with medical and veterinarian importance as a result of blood-feeding on mammals.

**Conclusion**

The apparent anthropophilic behavior of *S. minuta* together with the detection of *Leishmania* sp. DNA highlight the need to determine the role played by this sand fly species in the transmission of pathogenic *Leishmania* to humans. In addition, our data confirms that *P. perniciosus* is an opportunistic feeder and suggest that is responsible for the maintenance of *L. infantum* in southern Portugal. Altogether, the obtained results reinforce the need for ongoing surveillance with systematic epidemiologic surveys on *Leishmania* vectors so as to investigate the transmission, distribution and spread of infections by *Leishmania* species.

**Competing interests**

The authors declare that they have no competing interests.

**Authors contributions**

CM planned the study, collected samples, performed DNA extraction and wrote the manuscript; RP performed DNA sequence analysis and revised the manuscript; JMC performed molecular analyses; FF collected samples and performed DNA extraction; MIA performed morphological identification and revised the manuscript; LC supervised the study and reviewed the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

This work was supported by EU grant FP7-261504 EDENext, and is catalogued by the EDENext Steering Committee as EDENext306 (http://www.edenext.eu). The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission. We would like to thank to all the inhabitants that allowed the placement of the CDC and sticky traps in their properties. CM (SFRH/BPD/44082/2008) holds a scholarship from Fundação para a Ciência e a Tecnologia, Ministério da Educação e Ciência, Portugal. The work of C. Maia was done under the frame of EurNegVec COST Action TD1303. Publication of the CVBD10 thematic series has been sponsored by Bayer Healthcare – Animal Health division.

**Author details**

1. Unidade de Parasitologia Médica, Global Health and Tropical Medicine (GHTM), Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa (UNL), Lisbon, Portugal. 2. Unidade de Microbiologia Médica, GHTM, IHMT-UNL, Lisbon, Portugal. 3. Departamento de Ciências Biomédicas e Medicina, Universidade do Algarve, Faro, Portugal.

Received: 15 January 2015 Accepted: 6 March 2015

Published online: 23 March 2015
References
1. Dujardin J, Campino L, Cañavate C, Dedet J, Gradoni L, Almeida G, et al. Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. Emerg Infect Dis. 2008;14:1013–8.
2. Campino L, Maia C. Epidemiology of leishmaniasis in Portugal. Acta Med Port. 2010;23:59–64.
3. Campino L, Pratlong F, Abrahao P, Rovira I, Santos-Gomes G, Alves-Pires C, et al. Leishmaniasis in Portugal: enzyme polymorphism of Leishmania infantum based on the identification of 213 strains. Trop Med Int Health. 2006;11:1708–14.
4. Pires C. Les phlébotomes du Portugal I- Infestation naturelle de Phlebotomus ariasi Tonnoir, 1921 et Phlebotomus perniciosus Newstead, 1911, par Leishmania dans le foyer zoonotique de Arábia (Portugal). Ann Parasitol Humaine Comparée. 1984;59:521–4.
5. Censos. Resultados Preliminares 2011. Instituto Nacional de Estatística. Available from http://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_indicadores&indOcorrCod=0005889&setTab=tab0. Accessed 12 Jan 2015.
6. Franco J. Zonas fitogeográficas predominantes de Portugal Continental. Anais ISA. 1994;44:39–56.
7. Pires C. Contribuuição para o conhecimento da distribuição e biocologia dos flebótomos em Portugal (Diptera: Psychodidae). B Soc Port Cien Nat. 1979;19:197–210.
8. Schonian G, Nazereddin A, Dinse N, Schweynoch C, Shallig H, Presber W, et al. PCR diagnosis and characterization of Leishmania in local and imported clinical samples. Diagn Microbiol Infect Dis. 2003;47:349–58.
9. Srivadovida M, Allen B, Zdilova L, Drorov V, Hlavackova I, Mysková J. Cutaneous leishmaniasis caused by Leishmania infantum transmitted Phlebotomus tobbi. Int J Trop Parasitol. 2008;39:251–6.
10. Koth K, Toh H. Recent developments in the MAFFT multiple sequence alignment program. Brief Bioform. 2008;9:286–98.
11. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30:2725–9.
12. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 2006;23:254–67.
13. Yang BB, Guo XG, Hu XS, Zhang JG, Liao L, Chen DL, et al. Species discrimination and phylogenetic inference of 17 Chinese Leishmania isolates based on internal transcribed spacer 1 ITS (ITS1) sequences. Parasitol Res. 2010;107:1949–65.
14. Bongiorno G, Habluetzel A, Khoury C, Maroli M. Host preferences of phlebotomine sand flies (Diptera: Psychodidae) from an outbreak area of cutaneous leishmaniasis in Kitui District: a new species of Sergentomyia (Spelaeomyia) Leishmania infantum. Parasit Vectors. 2013;6:32.
15. Bongiorno G, Habluetzel A, Khoury C, Maroli M. Host preferences of phlebotomine sand flies (Diptera: Psychodidae) in a high-endemic area of cutaneous leishmaniasis in Torres Novas municipality, central region, Portugal. Acta Parasitol. 2013;58:333–41.
16. Parvizi P, Amirkhani A. Mitochondrial DNA characterization of Phlebotomus argentipes from an outbreak area of cat scratch disease caused by Leishmania infantum in Iran. Parasitol Res. 2006;99:190–4.
17. Alves-Pires C, Campino L, Janz JG, Almeida G, Afonso MO, Maia C. First detection of Leishmania major DNA in Sergentomyia (Spelaeomyia) from cutaneous leishmaniasis foci in Mali. PLoS One. 2012;7:e28266.
18. Alves-Pires C, Campino L, Janz JG, Afonso MO, Os flebótomos de Portugal. XIV. Os vetores de leishmanioses no foco zoonótico do sotavento Algarvio. Acta Parasitol Port. 2008;14:509–14.
19. Branco S, Alves-Pires C, Maia C, Cortes S, Dionísio L, Neto L, et al. Molecular detection of Leishmania infantum in naturally infected Phlebotomus perniciosus from Algarve region, Portugal. J Vector Borne Dis. 2009;46:268–72.