

The first detection of *Leishmania major* in naturally infected *Sergentomyia minuta* in Portugal

Lenea Campino^{1,2/+}, Sofia Cortes^{1,3}, Lídia Dionísio⁴, Luís Neto⁴, Maria Odete Afonso^{1,5}, Carla Maia^{1,3,6}

¹Grupo Leishmanioses ³Centro de Malária e Outras Doenças Tropicais ⁵Unidade de Parasitologia e Microbiologia Médicas, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal ²Departamento de Ciências Biomédicas e Medicina

⁴Departamento de Biologia e Bioengenharia, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Faro, Portugal

⁶Faculdade de Medicina Veterinária, Universidade Lusófona de Humanidades e Tecnologias, Lisboa, Portugal

Phlebotomine sandflies of the genus Sergentomyia are widely distributed throughout the Old World. It has been suggested that Sergentomyia spp are involved in the transmission of Leishmania in India and Africa, whereas Phlebotomus spp are thought to be the sole vectors of Leishmania in the Old World. In this study, Leishmania major DNA was detected in one Sergentomyia minuta specimen that was collected in the southern region of Portugal. This study challenges the dogma that Leishmania is exclusively transmitted by species of the genus Phlebotomus in the Old World.

Key words: *Leishmania major* - *Sergentomyia minuta* - vector

Leishmaniasis are parasitic diseases caused by protozoans of the genus *Leishmania*. These parasites, which infect various wild and domestic mammals, are transmitted by the bite of phlebotomine sandflies. Species of the genus *Sergentomyia* are widely distributed throughout the Old World and are known to feed on reptiles as well as other vertebrates, including humans (Bates 2007, Berdjane-Brouk et al. 2012). Although these sandflies are considered vectors of lizard *Sauroleishmania*, it has been recently suggested that certain species of the genus *Sergentomyia* are involved in the transmission of *Leishmania infantum* among dogs in Senegal (Senghor et al. 2011) and *Leishmania major* DNA was detected in *Sergentomyia darlingi* in a cutaneous leishmaniasis (CL) focus in Mali (Berdjane-Brouk et al. 2012). Earlier, Mukherjee et al. (1997) detected *Leishmania donovani* DNA in *Sergentomyia* spp in India.

In Portugal, 213 parasite strains have been isolated from sandflies and human and canine leishmaniasis cases have been identified as *L. infantum*, with *Phlebotomus perniciosus* and *Phlebotomus ariasi* as the proven vectors (Campino et al. 2006). However, *L. major*/*L. infantum* hybrids have also been isolated from four autochthonous human leishmaniasis cases, two of which were described elsewhere (Ravel et al. 2006). The identification of these multiple hybrids led to the hypothesis that *L. major* circulates in the country, most likely in in-

fect sandflies. The risk of introduction of new *Leishmania* species in Portugal from travellers or immigrants from North Africa and the Indian subcontinent is a real concern, especially in the southern part of the country (Algarve region). Portuguese military missions in the Middle East pose an additional introduction risk.

An entomological surveillance study of phlebotomine species was performed during the last five years. During *Leishmania* transmission season, sandflies were captured by CDC miniature light traps and identified using entomological keys. Subsequently, kinetoplastid DNA-polymerase chain reaction (PCR) using primers Uni21 and Lmj4 (Anders et al. 2002) and nuclear ribosomal internal transcribed spacer (ITS)-1 PCR analyses were used to screen female sandflies for *Leishmania* infection (Maia et al. 2009). After ITS1-PCR, *HaeIII* digestion was performed on the positive PCR products to differentiate *Leishmania* species (Schonian et al. 2003).

L. major DNA was detected in one *Sergentomyia minuta* specimen (sample sm3), which was collected in a rural area of the municipality of Albufeira, Algarve region. Furthermore, a characteristic *L. major* PCR product of 650 base pairs (bp) was obtained using the Uni21 and Lmj4 kinetoplastid primers as determined by agarose gel electrophoresis. After PCR amplification and *HaeIII* digestion of the product, restriction fragments characteristic of *L. major* (203 bp and 132 bp) were observed. The amplified fragment obtained by ITS1-PCR was sequenced, aligned and compared with ITS-1 *Leishmania* sequences that were available in the GenBank database. A neighbour-joining tree (Figure) was constructed using SplitsTree4 (Huson & Bryant 2006). The *L. major* sequence from sample sm3 had a high level of identity with *L. major* sequences from strains recovered in the Middle East and Northern Africa (Egypt, accession FJ460456.1, Iran, accessions FN677357.1, AY550178.1, AY260965.1, Israel, accessions EU326229.1, DQ300195.1 and Tunisia, accession FN677342.1).

The foci of CL caused by *L. major* occur in 14 countries of the World Health Organization-Eastern Mediterranean Region (EMR), extending from Morocco to

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+ Corresponding author: campino@ihmt.unl.pt

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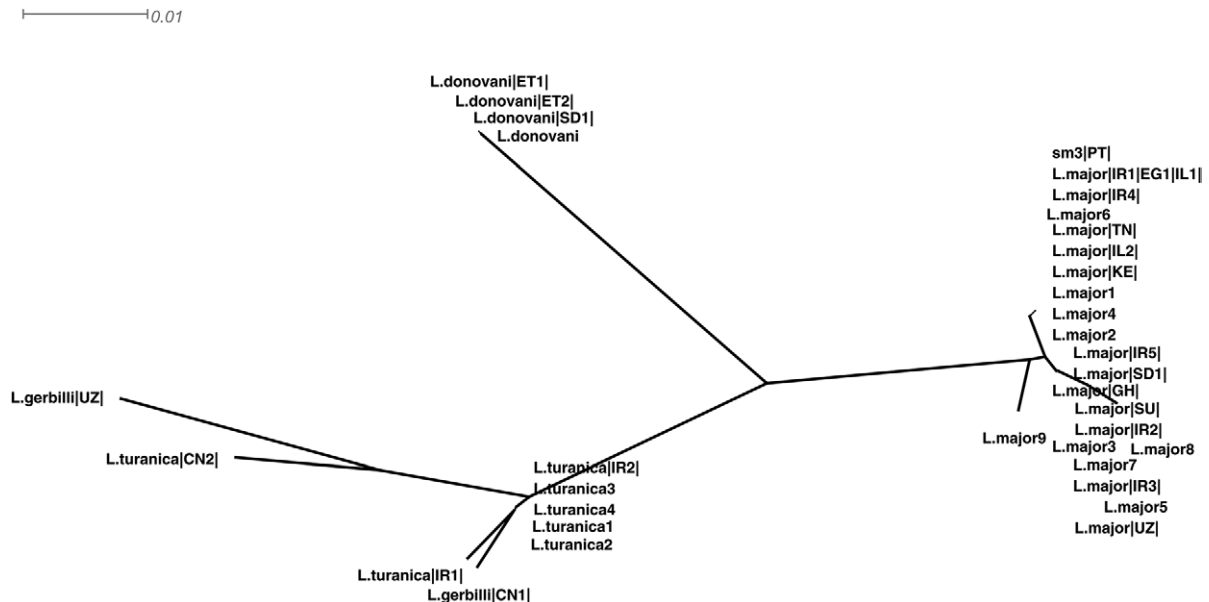
Afghanistan (Postigo 2010). Although approximately 100,000 new CL cases were reported in the EMR in 2008, these were rarely caused by *L. infantum*. In contrast, *L. infantum* is the causative agent of CL in Western Europe. To date, no autochthonous human CL due to *L. major* has been identified in Portugal. However, unlike visceral leishmaniasis, the cutaneous clinical form is not under compulsory notification in the country and the misdiagnosis of cutaneous cases is frequent. Until now, the parasites were identified in only six CL cases: four strains were identified as zymodeme MON-1 (3) and MON-29 (1) (Campino et al. 2006) based on isoenzyme typing and two were identified by molecular typing. All of these cases were identified as *L. infantum*. It is imperative to know the causative agent, as the outcome and treatment may differ for *L. infantum* and *L. major* infections.

To our knowledge, this study presents the first detection of *L. major* DNA in a sandfly from the *Sergentomyia* genus in Europe. This finding challenges the dogma that *Leishmania* is exclusively transmitted by species of the genus *Phlebotomus* in the Old World and that *Sergentomyia* is exclusively a *Sauroleishmania* vector. Unfortunately, little prior work has been conducted regarding the development of *Leishmania* sp. in *Sergentomyia* sandflies. To determine the possible role of *S. minuta* in the transmission of *L. major*, it should be demonstrated that the species feeds on humans and that it supports the complete development of the parasite in natural condi-

tions after the infectious blood meal has been digested.

L. major typically uses rodents as reservoir hosts and Jaouadi et al. (2013) have found *Mus musculus* DNA in *S. minuta*. These findings have prompted us to continue to examine *Leishmania* infection in rodents, namely in *M. musculus*, as it is common and widely distributed in the country. Few studies in rodents have been performed by our research group, although *Leishmania* infection has not been detected. However, Charrel et al. (2006) found Toscana virus, a virus responsible for human meningitis and encephalitis in Mediterranean countries, in *S. minuta* (Charrel et al. 2006); thus, the blood meal preferences of this species should also be studied to better understand if *S. minuta* can play a role in the transmission of human pathogens or vector borne diseases.

Further surveillance with extensive and systematic epidemiological surveys on *Leishmania* hosts and vectors are crucial given that increased migration, military deployments and other travel increase the risk of the introduction and spread of *Leishmania* species to non-endemic regions. Furthermore, the “new” parasites could be transmitted by sandfly species that are normally considered non-permissive to *Leishmania* infection. Eco-epidemiological and phylogenetic studies should be performed to clarify the introduction or evolution of *L. major* and potential reservoirs and vectors in Portugal. In addition, the national health services should be encouraged to improve CL diagnosis and parasite identification.



Neighbour-joining unrooted tree built up from internal transcribed spacer (ITS)-1 sequence data of 195 characters from 37 sequences using Kimura-2P model with equal rates variation. Tree generated by SplitsTree4. Sequences used for these analysis (species code and accessions) were as follows: *Leishmania major* [IR1],[IR2],[IR3],[IR4],[IR5] (FN677357.1, AY550178.1, AY283793.1, AY260965.1, EF653269.1), *L. major* [EG1] (FJ460456.1), *L. major* [IL1],[IL2] (EU326229.1, DQ300195.1), *L. major* [TN] (FN677342.1), *L. major* [UZ] (FN677357.1), *L. major* [SU] (AJ000310.1), *L. major* [KE] (AJ300482.1), *L. major* [GH] (DQ295825.1), *L. major* [SD] (AJ300481.1), *L. major* 1, 2, 3, 4, 5, 6, 7, 8, 9 (FJ753394.1, FJ753393.1, FJ753392.1, FJ753391.1, FJ831924.1, FJ753395.1, EF413075.1, GQ402544.1, GQ402543.1), *Leishmania turanica* [IR1],[IR2] (EU395712.1, JN860744.1), *L. turanica* [CN2] (HQ830350.1), *L. turanica* 1, 2, 3, 4 (AJ272380.1, AJ272379.1, AJ272378.1, HM130607.1), *Leishmania gerbilli* [CN1] (HQ830351.1), *L. gerbilli* [UZ] (AJ300486.1), *Leishmania donovani* [ET],[ET2] (FN182209.1, FN182207.1), *L. donovani* [SD] (FN677362.1), *L. donovani* (AJ249620.1), sm3, Portuguese ITS-1 sequence. CN: China; EG: Egypt; ET: Ethiopia; GH: Ghana; IL: Israel; IR: Iran; KE: Kenya; PT: Portugal; SD: Sudan; SU: ex-URSS; TN: Tunisia; UZ: Uzbekistan.

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