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Investigation of natural infection of Phlebotomine (Diptera: Psychodidae) by *Leishmania* in Tunisian endemic regions

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ABSTRACT

Leishmaniases are caused by protozoan parasites of the genus *Leishmania* transmitted by females blood-feeding phlebotomine insects (Diptera: Psychodidae). In Tunisia, cutaneous and visceral leishmaniases are of public health concern. In Tunisia, 17 species of phlebotomine sand flies are described. Here we investigate natural infection in Tunisian mixed foci regions of leishmaniases. We trap female sandflies during the *Leishmania* transmission season in the country's central-eastern and northern parts. We investigate Leishmania infection using PCR-RFLP targeting the ITS1 ribosomal DNA, followed by enzymatic digestion with *Hae*III; then, we identify sand flies using molecular methodologies. We confirm the presence of *Phlebotomus papatasi* and *Phlebotomus perniciosus* infected by *L. major* and *L. infantum* parasites in Tunisia.

1. Introduction

Leishmaniases, caused by protozoan parasites of the *Leishmania* genus (Kinetoplastida, Trypanosomatidae), represent a significant health problem. They are neglected tropical diseases with a strong link with poverty (Alvar et al., 2006). Leishmaniases present clinical forms ranging from self-curing cutaneous leishmaniasis (CL) to fatal visceral leishmaniasis (VL) (Desjeux, 2001). They are transmitted by phlebotomine sand flies (Killick-Kendrick, 1990).

Three clinico-epidemiological CL forms and the visceral form of leishmaniasis occur in Tunisia. In Tunisia, 17 phlebotomine sand fly species are identified; they belong to the *Phlebotomus* and *Sergentomyia* genera (Croset et al., 1978; Depaquit et al., 1998). Among them, 6 species are proven or potential vectors of *Leishmania*. The zoonotic CL (ZCL) due to *L. major* is of public health concern in the Center and the South of the country but has recently spread to the northern territories (Aoun et al., 2012) (Haouas et al., 2012). The proven vector of *L. major* is *Phlebotomus papatasi*, which is present all over Tunisia (Chelbi

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et al., 2009) (Ben Ismail et al., 1987) (Ben-Ismail et al., 1987; Ghrab et al., 2006). The anthroponotic cutaneous Leishmaniasis, or chronic CL (CCL), is caused in Tunisia by *L. tropica,* first described in 2005 in a microfocus of Tataouine in the South-eastern part of the country (Rioux et al., 1986). It is transmitted by *P. sergenti*, mainly found in the central and southern regions (Tabbabi et al., 2011). The sporadic CL (SCL) is caused by dermotropic variants of *L. infantum* (zymodeme MON-24) that prevails in northern Tunisia, and sand flies belonging to the *Larroussius* subgenus are incriminated as a vector (Haouas et al., 2012). The visceral form is caused by *L. infantum,* with *P. perniciosus* as the proven vector (Ghrab et al., 2006).

Climate change would affect Leishmaniases' incidence and phlebotomine dispersal (Kholoud et al., 2018). Likewise, ZCL caused by *L. major* has recently spread over its traditional foci to central and southern parts of the country. Whereas CCL caused by *L. tropica* is no longer restricted to South-eastern Tunisia (Haouas et al., 2012). Such extension over historical foci has also been recorded in Morocco (Kholoud et al., 2018) (Kholoud et al., 2020) (Guernaoui et al., 2020). -Such expansion in territories has favored the emergence of CL mixed foci of *L. tropica* and *L. major*. Besides, *P. perniciosus*, a vector of visceral *L. infantum* MON-1, is also present in the North and the South of the country (Ghrab et al., 2006; Guerbouj et al., 2007). To gain insight into the activity of the epidemiological cycle of *Leishmania. spp* in endemic leishmaniasis foci, we have ascertained the *Leishmania* infection rate of the collected sand flies and re-investigated the phlebotomine fauna diversity in these foci.

2. Materials and methods

2.1. Study area and sample collection

Male and female sand fly samples were collected from previous work (Ayari et al., 2016). A set of 188 female sand flies collected in various habitats from six governorates (Beja, Zaghouan, Kairouan, Monastir, Sousse, and Mahdia) in the central, eastern, and northern parts of Tunisia, belonging to the sub-humid, semi-arid and arid bioclimatic stages were selected for the study. They were trapped with CDC miniature light traps (John W. Hock, USA) and sticky traps during the summer season of 2010 and 2012. The traps were placed in animal shelters, outside and inside houses. All specimens have been preserved in 95% ethanol or/and in nitrogen liquid.

Sex identification was able using morphological tools and based on external and internal characters of the head and genitalia (according to the keys of Lewis (Lewis, 1974; Lewis and Hitchocock Jr., 1968) and Artemiev (Artemiev, 1991)).

2.2. DNA extraction, PCR amplification, and Leishmania detection

Genomic DNA from the female specimens was extracted from the thorax and the anterior abdomen, as described by Cornel and Collins (1996) (Cornel and Collins, 1996).

2.2.1. Real-time PCR

The detection and quantification of *Leishmania* spp. DNA was realized by the real-time PCR (qPCR) technique. It consists of amplifying the conserved regions of the kinetoplast minicircle DNA (kDNA) gene. It is considered one of the most sensitive PCR approaches (Martin-Ezquerra et al., 2009). Each amplification of 5 μL of genomic extracted DNA was performed in duplicate. Eightfold dilution series of DNA from promastigotes (MHOM/ES/04/BCN-61, *L. infantum* MON-1) was used as calibrators (serial dilution from 105 parasites/ml to 0.01 parasite/ml), allowing plotting of a standard curve. Samples with Ct lower than 36 were considered potentially positives and retained for further analyses.

2.2.2. PCR-RFLP

The ribosomal internal transcribed spacer 1 (ITS1) amplification was performed using LITSR and L5.8S primers (Esseghir et al., 1997). Amplification was performed in a final volume of 50 μL containing 5 μL of DNA added to 5 μL of 10 × BioLabs Buffer, 4 mM MgCl2, 10 mM dNTP, 5 U/μL of TaqBio Labs, and 10 μM of each primer. The cycling was: 95 °C for 120 s, then 30 cycles of 95 °C for 20 s, 54 °C for 30 s, and 72 °C for 60 s. As a control, DNA extracted from reference strains of *Leishmania* was used as positive controls. Following amplification, PCR products (17 μL) were digested with 1 μL *HaeIII* enzyme without prior purification and visualized after electrophoresis on a 1% agarose ethidium bromide-stained gel.

2.3. PCR amplification for sand fly identification

We choose to identify sand flies with molecular methods due to the difficulties in identification via morphological criteria. Sandflies share several morphological characters, and their identification can be mistaken. Polymerase chain reactions (PCR) were carried out to amplify into the mitochondrial DNA 336 bp of 3′ end of cytochrome b gene along with 67 bp of complete tRNA for serine, 22 bp interval sequences (including stop codons), and 124 bp of 3′ end of NADH1 gene. Using 50 ng of genomic DNA in 25 μL PCR reaction containing 10 pmol of each primers CB3-PDR(5′GGTA(C/T)(A/T)TTGCCTCGA(T/A)TTCG(T/A)TATGA- 3′) and N1NPDR (5′-CA(T/C)ATTCAACC(A/T)GAATGATA- 3′) (Esseghir et al., 1997), 200 μmol dNTPs, 1 U Taq DNA polymerase (product number: D1806, SIGMA-ALDRICH, USA), 2.5 μL 10× buffer. The amplification cycle was: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of (denaturation at 94 °C for 60 s, annealing at 46 °C for 60 s and extension at 72 °C for 90 s), and a final extension at 72 °C for 10 min. The PCR products were analyzed after electrophoresis on 1% agarose gel stained with ethidium bromide.

2.4. Sequence analyses and sand fly phylogenetic tree construction

According to the supplier's instructions, amplicons were purified using the QIAquick PCR purification kit (QIAGEN). Sequencing was performed using a Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer, Applied Biosystems, Foster City, CA) and an ABI373 Automated DNA Sequencer. Sequences were edited using Clustal-X version 1.81 software (Larkin et al., 2007). Sequences were matched against NCBI using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Genetic diversity and identification of sand fly samples were performed using the MEGA6 software (Tamura et al., 2007) against reference sequences. The tree was constructed following the UPGMA (Sneath and Sokal, 1962) distance analyses using the Jukes-Cantor model. All bootstrap support values are based on 1000 replicates.

3. Results

3.1. Leishmania infection identification

Forty-three samples with Ct lower than 36 were retained for *Leishmania* species identification. Amplification of ITS1 detected *Leishmania's* DNA presence in 17 out of the 43 sand fly specimens analyzed (Table S1). The Enzymatic digestion of the ITS1-PCR product with the restriction enzyme *HaeIII* allowed identifying two *Leishmania* species, *L. infantum* (10) and *L. major* (7). Most of the positive phlebotomes for *Leishmania* DNA are from Zaghouan and Kairouan. Infected specimens are *P. perniciosus*, *P. perfiliewi* carrying *L. infantum* DNA, and *P. papatasi* carrying *L. major*. Surprisingly, *L. infantum* DNA was detected in one *P. papatasi* specimen. Finally, *P. longicuspis* was infected with *L. major*.

3.2. Molecular sandflies identification

The selected female specimens display the expected amplicon of 560 bp in size. The specimens were captured in the 14 stations of the North, East, and Center of the country. Five samples could not be sequenced. The obtained sequences present homology higher than 98% for six sand fly species that belong to the *Phlebotomus* (*P. papatasi, P. perniciosus, P. perfiliewi, P. longicuspis,* and *P. chabaudi*) and *Sergentomyia* (*S. minuta*) genera. All the sequences were deposited into the Gene Bank database (http://www.ncbi.nlm.nih.gov/) under the accession number MW305396 to MW305433.

An equal number of phlebotomes was collected using sticky (22) or CDC traps (21). In our survey, *Phlebotomus chabaudi* was identified only in sticky traps, *P. longicuspis* and *P. perfiliewi* were identified solely in CDC light traps (Fig. 1A). Two-thirds (29) were caught in animal shelters, the others from outside and inside houses (Fig. 1A).

A cladistic analysis was performed to identify sand fly specimens against reference sequences collected from NCBI. The tree's topology discloses two clades with high bootstrap values (Fig. 2). From our samples, *Phlebotomus* and *Sergentomyia* genera were separated. Six specimens from North and East of Tunisia gathered together within the *Sergentomyia* clade. The elven specimens belong to the *Phlebotomus* subgenus represented by a single species in Tunisia *P. papatasi*. These specimens were collected in various areas distributed over four bioclimatic stages: semi-arid medium, arid upper, and semi-arid lower. For the subgenus *Paraphlebotomus* represented in Tunisia by four species, only two *P. chabaudi* specimens were captured. In our tree, species belonging to the *Larroussius* subgenus are individualized with high Bootstrap values (98% to 100%). Thirteen specimens cluster with *P. perniciosus* reference sequences, 3 with *P. perfiliewi*, and 1 with *P. longicuspis* (Fig. 2).

4. Discussion

This study reported the molecular identification of phlebotomes trapped in the North, East, and Center of Tunisia, targeting a portion of mitochondrial DNA: cytochrome b, PCR-sequencing technique. Mitochondrial DNA (mtDNA) genes are commonly employed in population genetics studies (Mahamdallie et al., 2011) (Ready, 2013) (Ebrahimi et al., 2016). They have been widely used for population genetics analyses of the New World leishmaniasis vector sandfly, *Lutzomyia longipalpis* (Coutinho-Abreu et al., 2008), to highlight molecular variation within Phlebotomine species from a wide geographical range (Pesson et al., 2004) (Yahia et al., 2004) (Perrotey et al., 2005) (Depaquit, 2014). As already reported (Carta et al., 2020), a predominance of species belonging to the subgenus *Larroussius* (*P. perniciosus*, *P. perfiliewi*, *P. longicuspis*) is attracted to CDC light traps that might be due to the phototropisms of these species.

The majority of specimens are captured around animal shelters, while there are fewer in and around houses (Fig. 1B), that sign a preference of sand fly for animal baits and the environmental condition, temperature, and humidity, also the presence of food source and blood for coprophagous larvae (Ximenes et al., 1999) (Feliciangeli, 2004).

A majority of samples carrying *Leishmania* DNA originates from Zaghouan and Kairouan governorates (Fig. 3), an endemic focus for Cutaneous and Visceral Leishmaniases. Most of the infected sandflies were captured between August and September, which corresponds to the highest transmission period. Interestingly, most of the positive females were not found engorged by blood, highlighting their sustained infection and points to their role as a vector of leishmaniasis (Ghrab et al., 2006).

The specimens of *P. perniciosus* carrying Leishmania DNA were trapped in Kairouan and Zaghouan. *Phlebotomus perniciosus* is the primary vector of *L. infantum* MON-24, which causes human visceral leishmaniasis. This species is widespread in the North, extending towards the country's center (Benallal et al., 2017). The molecular analysis revealed molecular variant *P. perniciosus* specimen (Highlighted in Fig. 2). This molecular variant corresponds to an atypical *P. perniciosus* (PNA), previously confused

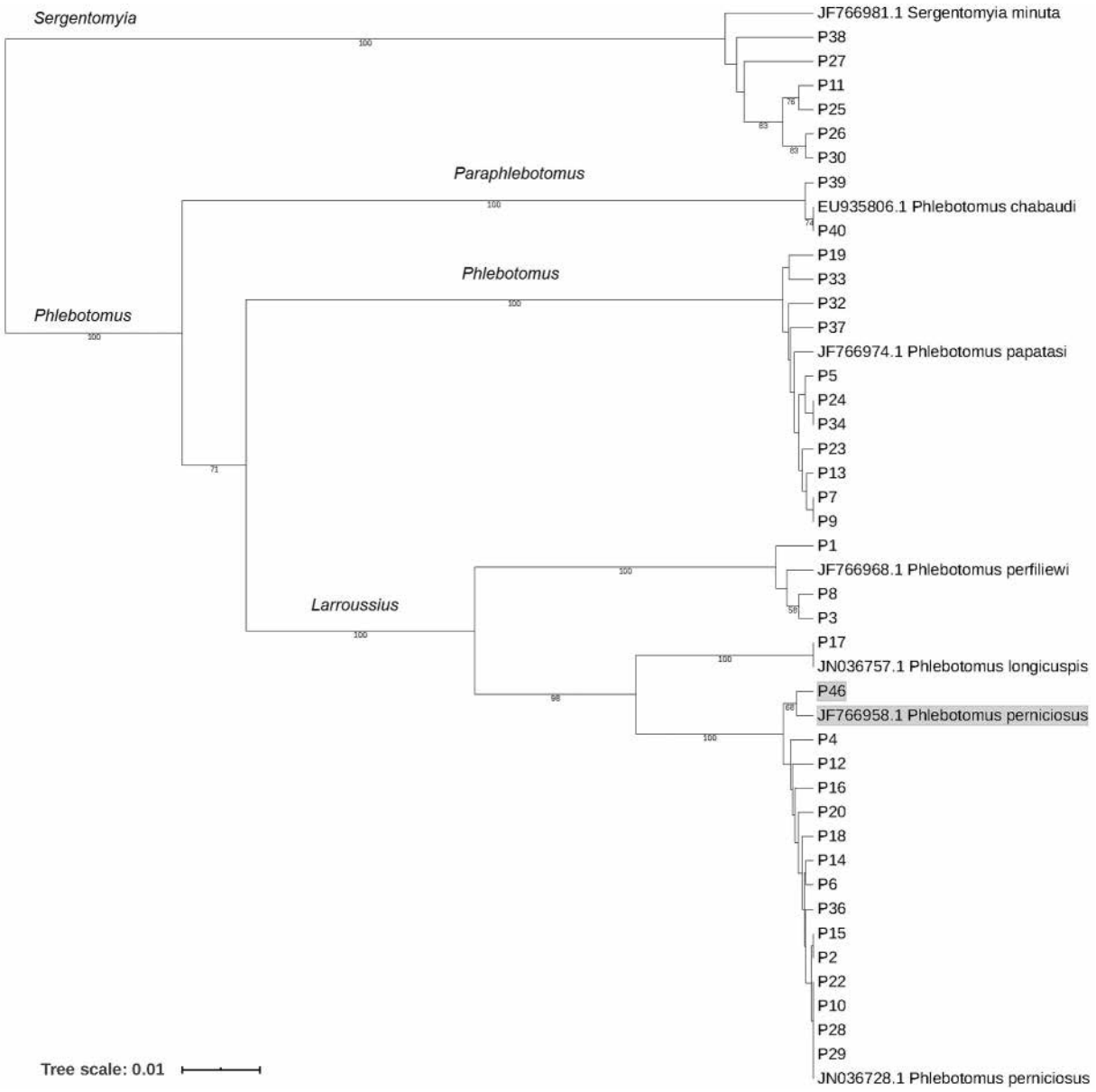

Fig. 1. Sandflies Phylogenetic tree based on UPGMA distance analyses using the Jukes–Cantor Model. Values on branches indicate bootstrap values.

with *P. longicuspis,* mainly present in the semi-arid and arid bioclimatic regions. Therefore, the question arises of the vectorial capacity of the atypical form of *P. perniciosus* in the southern regions (Benallal et al., 2017). The distribution of the atypical form of *P. perniciosus* in the south can be attributed to adaptation to these arid conditions. This is currently under investigation (data not published), and more specimens are presently analyzed in Tunisia. *Phlebotomus perfiliewi*, a known vector of *L. infantum* responsible for SCL (Izri and Belazzoug, 1993), was sampled in the north's historical foci (Beja and Zaghouan, belonging to the sub-humid bioclimatic stage). In Kairouan (arid bioclimatic stage), we found *P. papatasi*, *P. longicuspis,* and unidentified sand fly specimens carrying *L. major* DNA and *P. perniciosus* with *L. infantum* DNA. This region is a hyper-endemic focus of ZCL due to *L. major* and is also affected by VL due to *L. infantum* (Pousse et al., 1995) (Aissi et al., 2015). From Monastir (semi-arid bioclimatic), an endemic region for LCZ (Haouas et al., 2012), only one *L. infantum* infection was reported in *P. perniciosus*. Surprisingly, In Zaghouan, *L. infantum* DNA was detected in an engorged *P. papatasi* specimen. Such observation, although rare, has already been reported in Greece (Aransay et al., 1933-1938), Iran (Yavar et al., 2013), and recently in Italy (Latrofa et al., 2018). However, *L. infantum* does complete its developmental life cycle in *P. papatasi* as it is eliminated after blood digestion and defecation (Pimenta et al., 1994).

The specificity of the transmission cycle in these regions relies on the compatibility and the frequency of encounters between the reservoirs carrying the parasite and the sand fly vectors favored by the overlapping ecological niches (Esseghir et al., 1997).

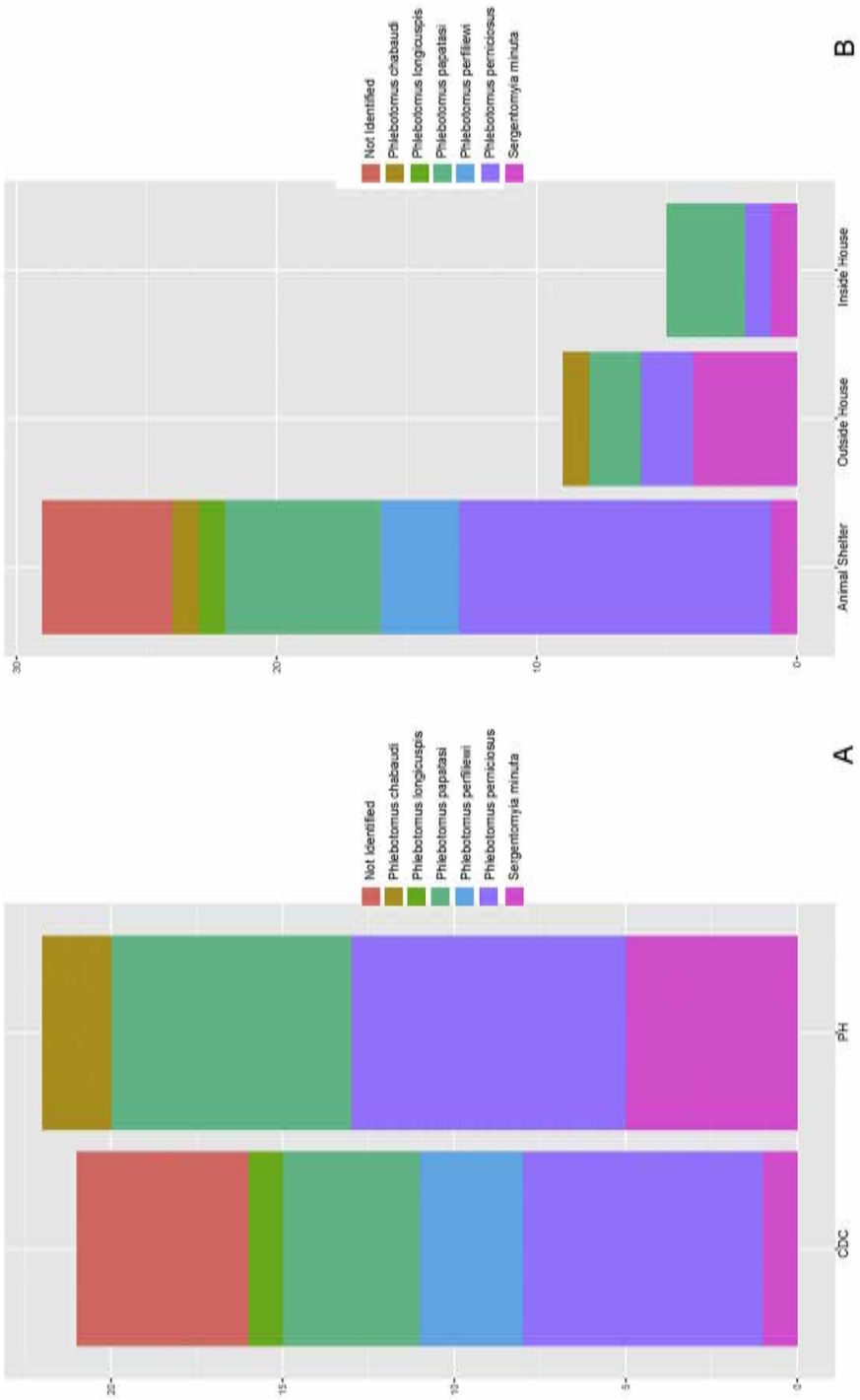

Fig. 2. Sand flies sampling according to the trap type (A) and location (B).

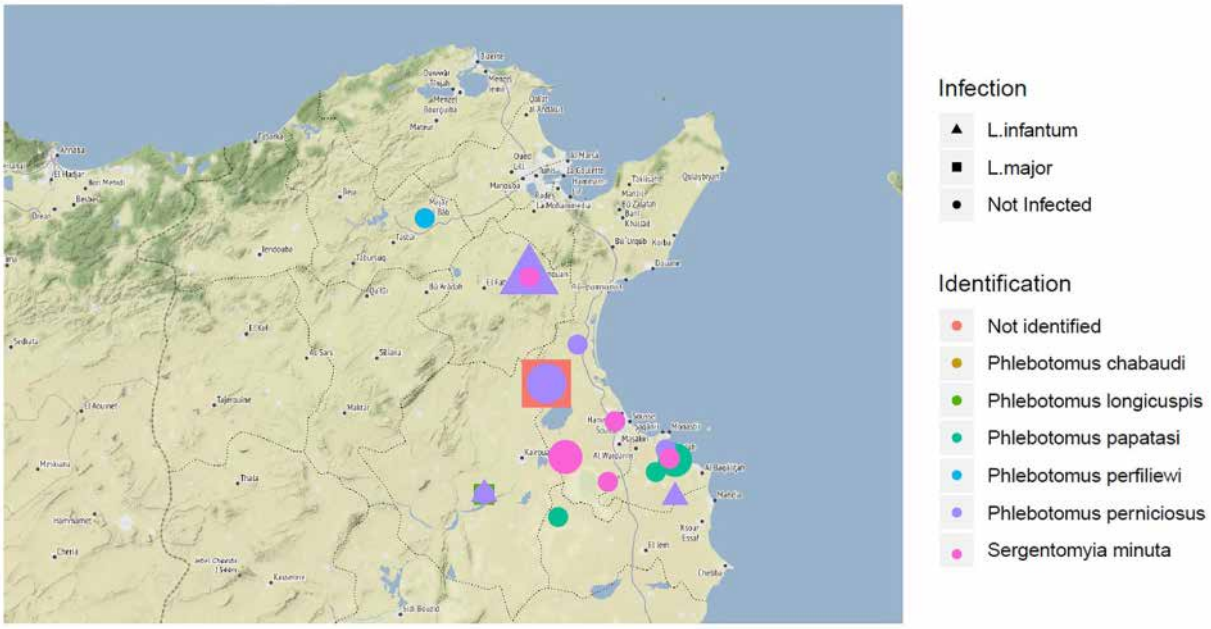

Fig. 3. Geographical distribution and natural *Leishmania* infection of captured sand flies.

Moreover, the environmental modifications due to climate change and human intervention have probably induced an increase in the reservoir and vector population densities and a change in their geographic location (Moo-Llanes et al., 2013). This study provides an updated distribution map of phlebotomine species and validates cyt b for Tunisian sand fly molecular identification. Although molecular analysis is very effective, issues have been raised. In our study, from the initial sampling, a reduced number of individuals were analyzed; this is firstly due to the selection performed based on the Real-time PCR results and then to the molecular steps, which can also reduce the number of successfully analyzed samples. For this, special attention should be paid to the sampling and pre-sequencing steps to optimize molecular identification.

Author contributions

Conceptualization, Melek Chaouch and Souha Ben Abderrazak; Data curation, Melek Chaouch and Amal Chaabane; Formal analysis, Chiraz Ayari; Funding acquisition, Souha Ben Abderrazak; Investigation, Melek Chaouch, Chiraz Ayari, Souad Ben Othman, Jomaa Chemkhi and Souha Ben Abderrazak; Methodology, Melek Chaouch, Amal Chaabane, Chiraz Ayari, Jomaa Chemkhi and Souha Ben Abderrazak; Project administration, Souha Ben Abderrazak; Supervision, Souha Ben Abderrazak; Validation, Melek Chaouch and Souha Ben Abderrazak; Visualization, Melek Chaouch; Writing – original draft, Melek Chaouch and Souha Ben Abderrazak; Writing – review & editing, Melek Chaouch, Denis Sereno and Souha Ben Abderrazak.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parepi.2021.e00212.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

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