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Phlebotomine sand flies in transmission cycles of leishmaniases in Algeria

Flebotomové v přenosových cyklech leishmanióz v Alžírsku

Ph.D. Thesis / Disertační práce

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Praha, 2024

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I declare that the submitted thesis is my own work and that I properly cited all the scientific literature used. The entire work of this thesis, as well as its substantial part, has not been submitted for the award of any other degree or diploma.

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I declare that Kamal Eddine BENALLAL substantially contributed to the experimental work in the four projects presented in his thesis and had a principal role in writing three of the six publications presented.

Prohlašuji, že se Kamal Eddine BENALLAL významně podílel na experimentální práci na čtyřech projektech, shrnutých v této dizertační práci, a je hlavním autorem textu tří publikací.

RNDr. Vít Dvořák, Ph.D.

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Abstract

Leishmaniases remain among the most neglected tropical diseases reported worldwide which are caused by flagellated protozoa belonging to the Kinetoplastida order, the Trypanosomatidae family and the *Leishmania* genus. This disease involves different actors in its transmission cycle including mammals as reservoirs and sand flies (Diptera: Psychodidae) as vectors. This PhD thesis summarises the results of four subjects including six published articles related to the fauna of sand flies, interaction between *Leishmania major-Phlebotomus papatasi-Meriones shawi*, and putative reservoir hosts such as *Gerbillus amoenus* for *L. major*. The first subject referred to the construction of databases of DNA barcodes and MALDI-TOF protein profiles from sand flies and rodents in Algeria.

These data allowed the description of a new sand fly species *Sergentomyia* (*Sergentomyia*) *imihra* n.sp and the determination of sand fly blood meal sources using the DNA typing and MALDI-TOF mass mapping peptide technique which revealed that *Ph. perfiliewi*, *Ph. perniciosus, Ph. papatasi*, *Ph. bergeroti*, and *Ph. alexandri* were opportunistic and fed on various hosts such as cattle, camel, goat, sheep, dog, chicken and human highlighting the risk of zoonotic disease transmission. The second part focuses on putative host of *L. major* in areas where the proven reservoirs *Psammomys obesus* and *M. shawi* do not occur. Results from experimental infections and xenodiagnoses demonstrated that *G. amoenus* can act as a reservoir, as it was shown to be susceptible, capable of maintaining, and transmitting parasites to naïve sand flies after six months of infection. The third topic assessed the role of asymptomatic rodents in the transmission of parasites. Findings revealed that asymptomatic *M. shawi* are involved in the dissemination of the parasite and should be considered in epidemiological modelling studies so that proper disease control strategies can be implemented. The last topic focused on the assessment of effect of Sb(III)-resistance on the fitness of *L. major*. The results revealed that although the resistant lines showed reduced metacyclogenesis in *Ph. papatasi*, still they produced mature infections and parasites recovered from sand fly guts showed enhanced antimony resistance. This suggests that *Ph. papatasi* supports the development and circulation of *L. major* Sb(III)-resistant lines in the field.

Abstract in Czech

Leishmaniózy, které patří mezi celosvětově nejvíce zanedbávaná tropická onemocnění, jsou způsobeny parazitickými prvoky z řádu Kinetoplastida, čeledi Trypanosomatidae a rodu Leishmania. Onemocnění v rámci přenosového cyklu zahrnuje několik aktérů: savčí rezervoárové hostitele a hmyzí přenašeče ze skupiny flebotomů(Diptera: Psychodidae). Tato disertační práce shrnuje výsledky čtyř cílů, shnrutých v šesti publikovaných článcích, týkajících se interakce mezi původcem onemocnění, prvkoem *Leishmania major*, hmyzím přenašečem *Phlebotomus papatasi* a rezervoárovými hostiteli *Meriones shawi* a *Gerbillus amoenus*, domnělý rezervoár hostí *G. amoenus* až *L. major*. První cíl zahrnoval konstrukci databází nukleotickových sekvencí a MALDI-TOF proteinových profile přenašečů a hostitelských hlodavců v Alžírsku. Výsledky umožnily popis nového druhu flebotoma *Sergentomyia* (*Sergentomyia*) *imihra* n.sp a určení zdrojů hostitelské krvě pomocí technik DNA sekvenování a peptidového mapování. Ty odhalily, že flebotomové druhů *Ph. perfiliewi, Ph. perniciosus* , *Ph. papatasi*, *Ph. bergeroti* a *Ph. alexandri* byli oportunní a sáli na různých hostitelích(kráva, velbloud, koza, ovce, pes, kuře a člověk), což potvrzuje riziko přenosu zoonotických onemocnění. Druhý cíl studuje domnělého hostitele *L. major* v oblastech, kde se nevyskytují prokázaní hostitelé *Psammomys obesus* a *M. shawi*. Výsledky, získané experimentálními infekcemi a xenodiagnosticými pokusy ukázaly, že *G. amoenus* by mohl hrát roli potenciálního rezervoáru, protože byl vnímavý, schopný udržet infekci a přenášet parazity na neinfikované flebotomy po 6 měsících infekce. Třetí cíl hodnotil roli asymptomatických hlodavců v přenosu parazitů. Výsledky ukázaly, že asymptomatické *M. shawi* se podílely na šíření parazitů a měly by být brány v úvahu při volení strategie kontroly onemocnění. Poslední cíl byl zaměřen na studiumrezistentních kmenů *L. major*. Výsledky ukázaly, že parazité získávají na infekčnosti a rezistenci vůči antimonu Sb(III) i přesto, že rezistentní kmeny vykazovaly nízkou zralou infekci z celkového počtu infikovaných samic, což by mohlo být považováno za potenciálně závažné z pohledu epidemiologie oneocnění.

Introduction

1. Leishmaniases

Leishmaniases, regarded as the most prevalent neglected tropical diseases (NTDs), are caused by protozoa belonging to the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) and are transmitted to humans through the bites of infected females of phlebotomine sand flies (Diptera: Psychodidae), small and blood-sucking insects with nocturnal activity [1]. So far, 1060 sand fly species have been described, 555 in the New World (538 extant and 17 fossils) and 505 in the Old World (490 extant and 15 fossils) [2]. In the Old World, 42 species are proven or suspected to transmit *Leishmania* parasites, of which 20 are involved in the transmission of *L. infantum*, 6 in the transmission of *L. donovani*, 7 in the transmission of *L. major*, 7 in the transmission of *L. tropica* and 2 in the transmission of *L. aethiopica*. Globally, each species of sand fly transmits one strain of *Leishmania* sp, except *Ph. sergenti* which can transmit *L. tropica* and *L. aethiopica*, and *Ph. alexandri* can transmit *L. donovani* and *L. infantum*. In the New World, 56 species are involved in the transmission of the 15 *Leishmania* strains reported on this continent [3]. In addition to their incrimination in the transmission of *Leishmania* parasites, various other pathogens are also transmitted such as the bacteria *Bartonella bacilliformis* recorded only in the New World and viruses belonging to the Phlebovirus genus such as the Toscana virus and Sand Fly fever virus of the Bunyaviridae family, Vesicular stomatitis, Chandipura virus, Oropouch virus and Pappataci fever virus whose occurrence is larger and reported worldwide [4], [5]. Several factors contribute to the outcome of leishmaniasis for which three main clinical manifestation forms are generally recognised: cutaneous, mucocutaneous, and visceral forms. Besides these, another form can be observed named the Post-Kala Azar Dermal (PKDL) form globally related to the immune system of the patient, age and some particular strains [6]. Cutaneous leishmaniasis (CL) affects the skin, it is the most common form of the disease and causes a skin lesion that may be painless or causes itching. It may be caused by different parasite species such as *Leishmania major*, *L. tropica*, *L. infantum, L. mexicana,* and *L. donovani*. Mucocutaneous leishmaniasis (MCL) caused by *L. braziliensis* affects both the mucus and the skin and causes disfigurement of the face and mouth. Visceral leishmaniasis (VL), also known as Kala-Azar, affects internal organs such as the liver, spleen, and bone marrow. It causes enlargement of two organs

(spleen and liver) with loss of weight and appearance of fever that can be fatal in the case of untreated patients. In the Old World, it is caused by *L. infantum* (known as *L. chagasi* in the New World) and *L. donovani*. In the mid-1980s, the first adult cases of co-infection with HIV were reported in southern Europe and have since been reported in 45 countries throughout the world and increased lethality from 4.6% to 16.6% [7], [8]. Finally, PKDL is a dermatosis generally observed in patients with a history of kala-azar which is caused by the immune system response against the latent parasites and/or the migration of the parasites from infected organs (liver, spleen, lymph nodes) to the skin; it is caused mainly by *L. donovani* [9]. Leishmaniasis is considered zoonotic if parasites are found in reservoir animal hosts such as rodents, canids, and felines; in this case, the human is considered an incidental host. Species such as *L. major*, *L. killicki,* and *L. infantum* are zoonotic. If human plays the role of the main host for parasites and no animal reservoirs are involved, the transmission is regarded as anthroponotic such as reported for *L. donovani*.

The classification of *Leishmania* species is based on intrinsic and extrinsic characteristics. The intrinsic characters regard DNA and isoenzyme profiles while the extrinsic characters regard clinical forms, epidemiological cycles (zoonotic or anthroponotic), host type, geographical distribution, the reservoir such as reptiles for *Sauroleishmania* parasites, the location of parasite replication in the guts of sand fly females, i.e. suprapylaria parasites develop in the abdomen midgut and includes *Leishmania* subgenus reported in the Old World, peripylaria parasites replicate in the hindgut and includes *Viannia* subgenus reported in the New World [10]. Recently, a new *Leishmania* subgenus called *Mundinia* has been reported based on molecular evidence for which biting midges are suspected to be potential vectors [11], [12]. *Leishmania* parasites are labelled using a universal code containing *i*) the name of the host (M for mammalian or I for insect) and three letters to design the name of the host, *ii*) the country where the parasite was isolated, *iii*) the year of isolation and *iv*) the laboratory designation [13]. The taxonomy unit corresponds to a zymodeme, it regroups several strains expressing the same electromorph or enzymatic profile of at least 20 different enzymes. The nomination of zymodeme by 'Mon' or 'Lon' corresponds to the name of the centre where the strain was typed Montpellier and London respectively [14]. Until now, 53 species of *Leishmania* have been described of which more than 20 are known to be pathogenic to humans and animals [15]. These species are responsible for different clinical outcomes (Figure 1). The complex *Leishmania donovani sl* includes *L. donovani* and *L. infantum* which causes VL and known to be the most severe form of the disease that can be fatal in the absence of treatment. *L.*

donovani is found in East Africa, the Middle East, and India whereas *L. infantum* is found in Europe and North Africa. *Leishmania tropica* causes CL is found primarily in the Middle East and North Africa. It is characterized by a small and dry lesion which heals for up to one year [16]. *Leishmania major* causes CL and is found primarily in the Middle East and North Africa. It is characterized by a wet lesion that heals between three and six months. *Leishmania braziliensis* causes MCL and is found primarily in Central and South America. *Leishmania mexicana* causes CL and is found primarily in Central and South America and *Leishmania chagasi* in the South American continent.

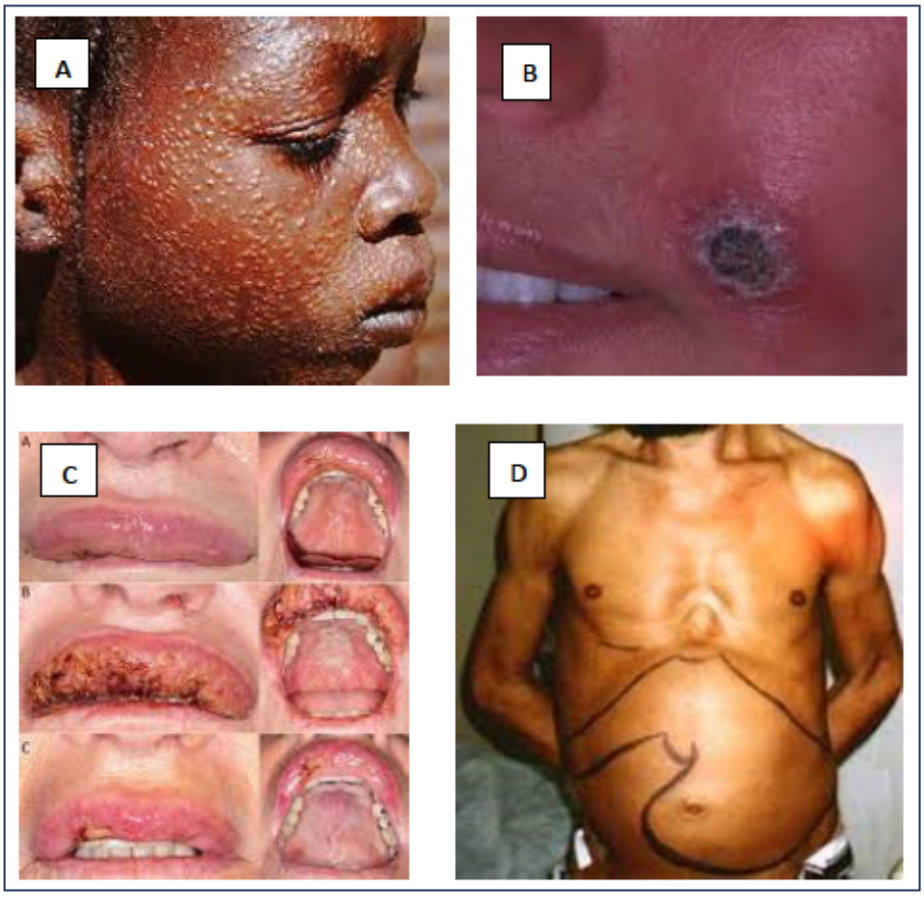


Figure 1: Different forms of leishmaniasis manifestation. A) PKDLform [6], B) CL form (https://dermnetnz.org/topics/leishmaniasis), C) MCL form [17] and D) VL form (https://www3.paho.org/hp/index.php?option=com_content&view=article&id=6420:2012-leishmaniasis-visceral&Itemid=39347&lang=en#gsc.tab=0).

1.1. Leishmaniasis in the World:

Leishmaniasis represents a major health problem in many endemic parts of the world, particularly in the tropical and subtropical regions including Asia, Africa, South America, and the Mediterranean basin (Figures 2 and 3). According to the World Health Organisation, 200 countries and territories have reported cases of leishmaniasis [18], in fact approximately 0.7

to 1.2 million cases of CL and between 0.2 and 0.4 million cases of VL occurring annually worldwide, with approximately 20.000 to 40.000 deaths per year [19]. Different clinical manifestation forms are present throughout the world and are linked to the distribution of the parasite strains, vectors, and reservoirs. Three clinical forms are reported in the Old World: CL, VL, and PKDL. In addition to the three forms previously cited, the mucocutaneous form is so far reported only in the New World [18].

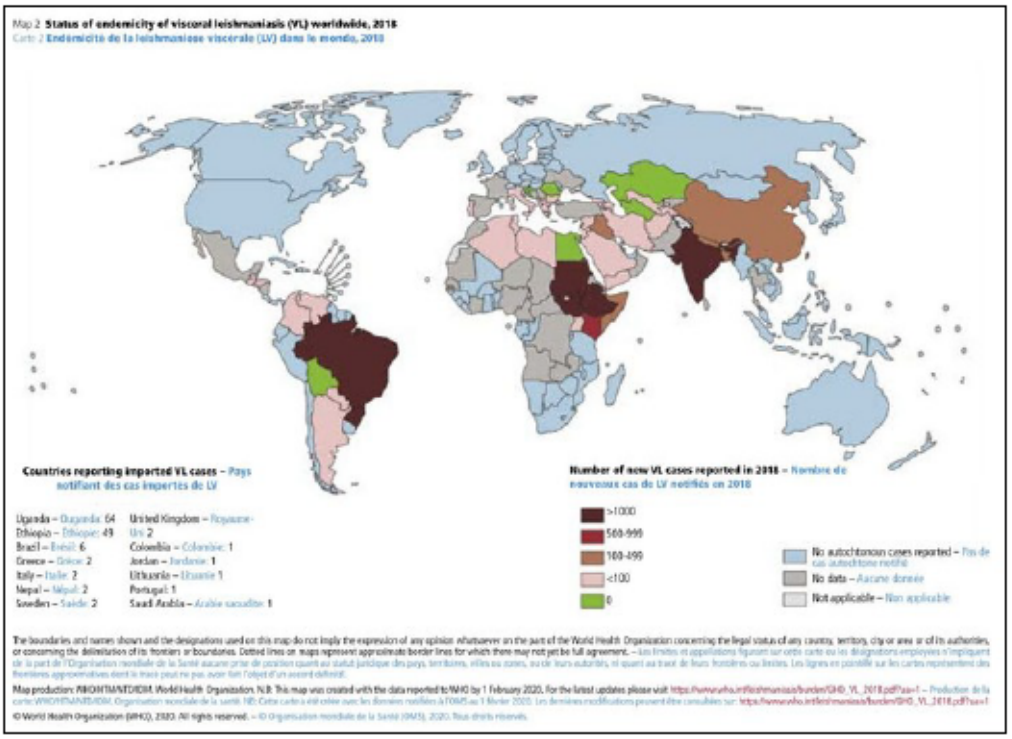


Figure 2: Distribution of cutaneous leishmaniasis in the world [18].

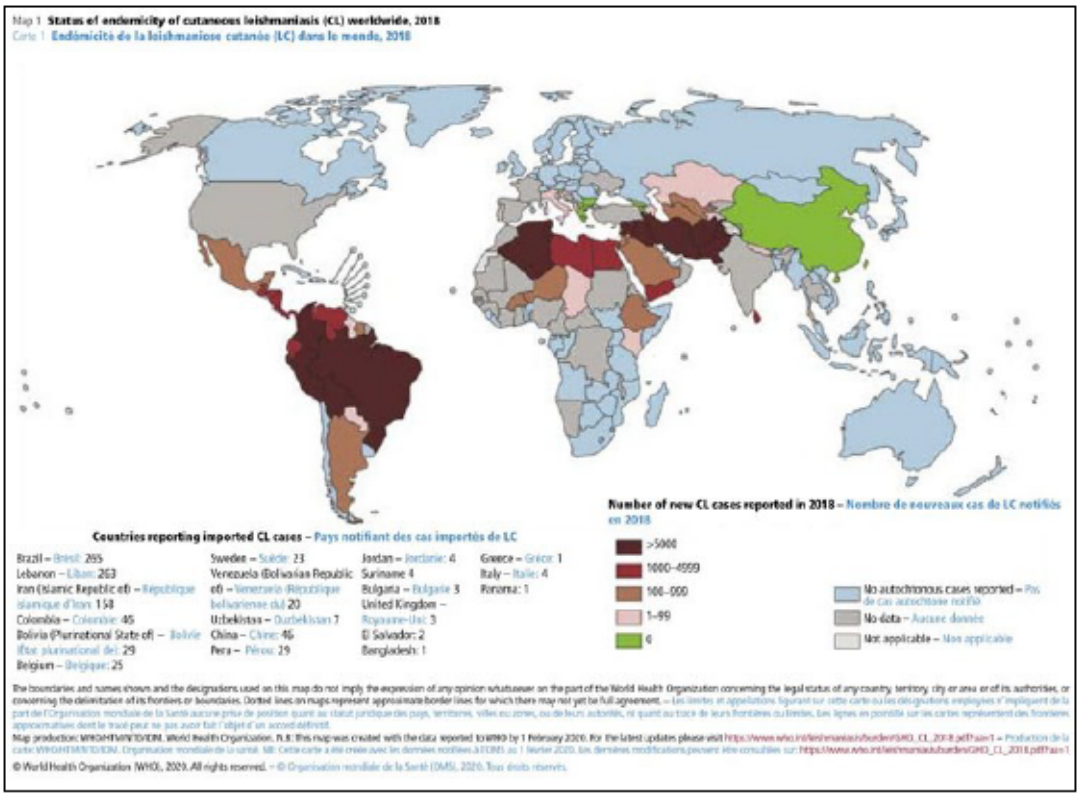


Figure 3: Distribution of visceral leishmaniasis in the world [18].

1.2 Leishmaniasis in Algeria:

According to WHO reports and classification, Algeria is considered endemic for leishmaniasis which has been a significant public health problem for many decades. The country reports mainly CL cases with approximately 6000 cases per year and is ranked second after Afghanistan in the world [20] while the number of VL remains mild compared to CL and has decreased from 115 to 34 cases per year between 2005 and 2017 [21], [22] (Figures 4 and 5). The CL has been reported in almost all Algerian provinces (45/48). So far in Algeria, five *Leishmania* parasite strains are known to be endemic and autochtonous: *L. major* Mon-25 transmitted by *Ph. Papatasi* and *L. killicki* Mon-301 transmitted by *Ph. Sergenti* both forms occur in the highland and in north of Sahara. *L.infantum*-Mon-24 transmitted by *Ph. perfiliewi* and *L. killicki* Mon-306 causing sporadic CL in northern Algeria. *L. infantum* Mon-1 transmitted by *Ph. perniciosus* and causing VL in the northern Algeria and Central Sahara [16], [23], [24], [25], [26], [27]. Recently, an atypical form of cutaneous sporotrichoid 12eishmaniasis caused by *L. Major* was reported in Algeria, previously known to occur in Saudi Arabia and Sudan [28], [29].

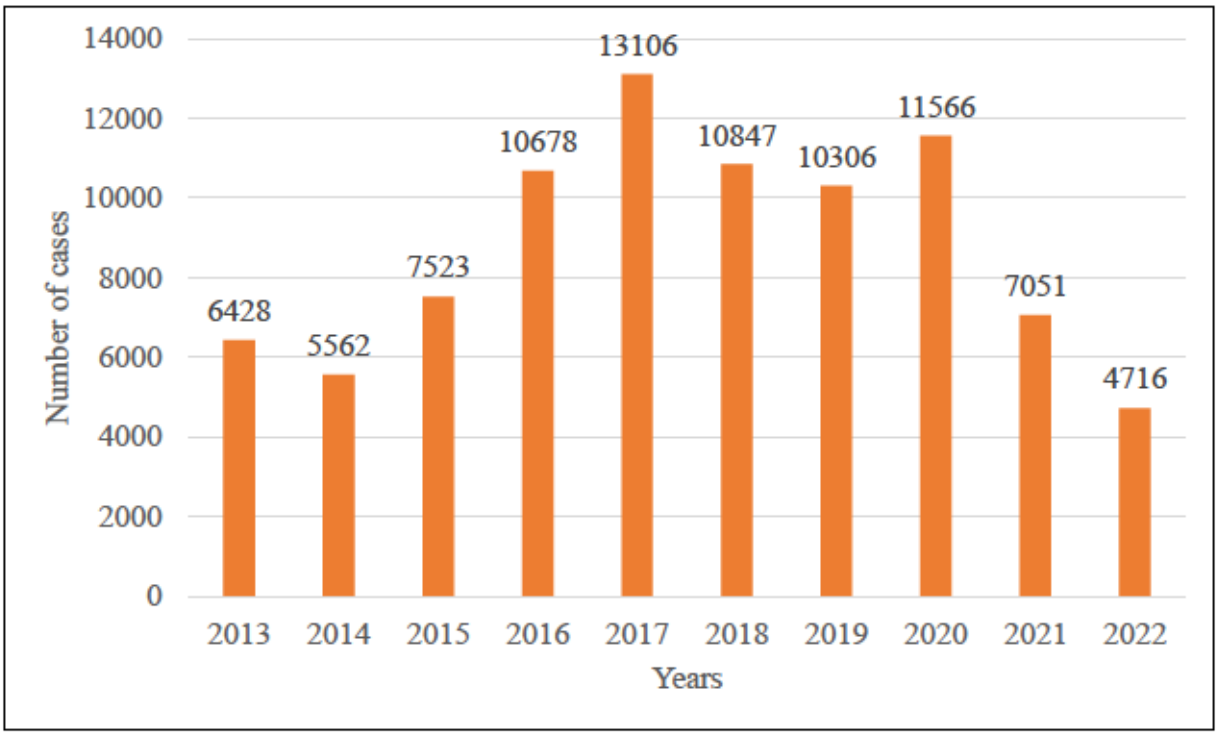


Figure 4: Cutaneous leishmaniasis cases in Algeria, 2013- 2021 (source: Algerian Ministry of Health).

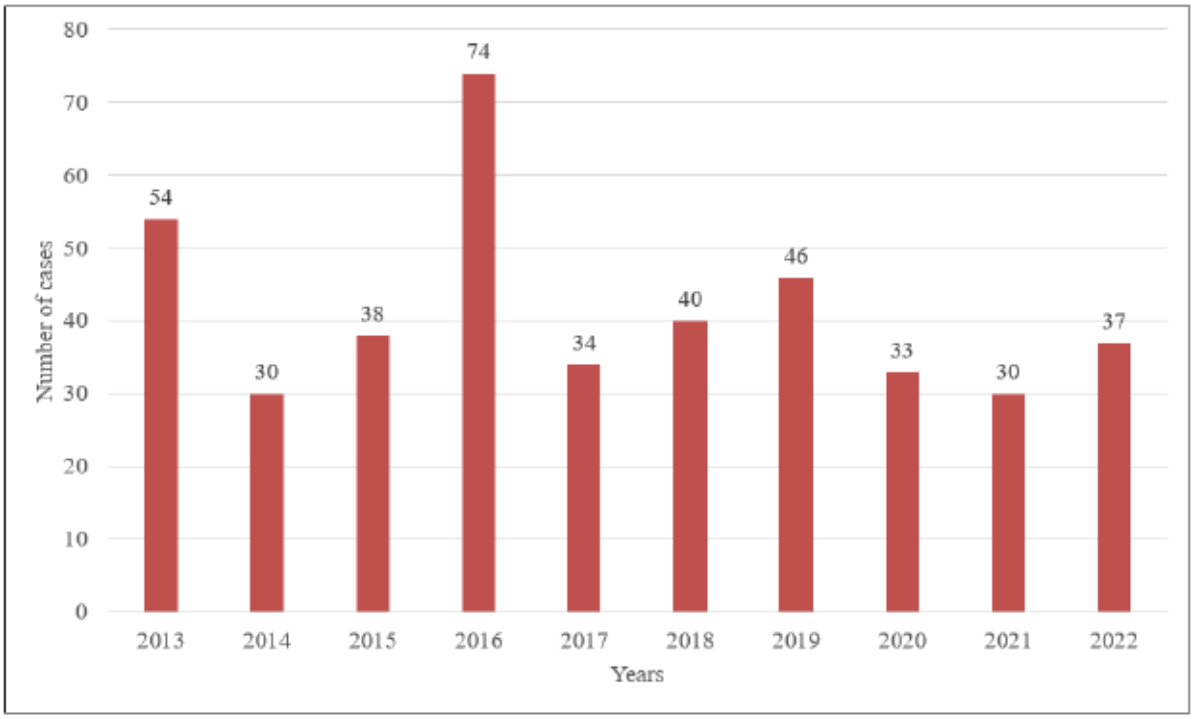


Figure 5: Visceral leishmaniasis cases in Algeria,2013- 2022 (source: Algerian Ministry of Health).

Since 2006, the Algerian government has made leishmaniasis a priority to reduce the burden of the disease by controlling its spread through various measures such as improving diagnosis and treatment, implementing vector control strategies and promoting public awareness of the disease, however, the disease remains a concern particularly in areas with poor situations and limited access to healthcare [30].

1.3. The life cycle of *Leishmania* parasites

The life cycle of *Leishmania* parasites is dixenous and involves two hosts alternating between the extracellular promastigote stage in the blood-feeding sand fly vectors and the intracellular amastigote stage in mammalian hosts including humans (Figure 6). When a female sand fly feeds on an infected host, it ingests infected macrophages with amastigotes that transform into elongated and flagellated promastigotes in the midgut. Here, the parasites must face several barriers mainly the immune system, the digestive enzymes and the peritrophic matrix (PM) that surrounds the blood meal [31], [32], [33].

In gut of sand flies, parasites undergo several transformations from immobile amastigotes to infectious metacyclic mobile promastigotes. Parasites escape from infected macrophages and transform into mobile procyclic promastigotes that multiply by binary fission, after a few days give rise to nectomonads. After breaking the PM, the elongated nectomonads attach to the intestinal epithelium to avoid being eliminated with defecation. Then nectomonads transform to short forms called leptomonads that multiply, migrate to the thoracic midgut and produce promastigote secretory gel (PSG) which plugs the anterior part of the gut. At the last stage of the development, non-motile haptomonads attach to the chitinous lining of the stomodaeal valve (SV) thus blocking food intake and facilitating injection of highly motile metacyclic infectious promastigotes into the skin of next mammalian host. Transmission of metacyclics is enhanced by damage of the SV chitin layer by chitinases released by the parasites. The disrupted SV and the blockage of cardia region by plugs formed by haptomonads, leptomonads and PSG force the sand fly female to feed repeatedly, thus increasing the transmission of the parasites [34], [35], [36].

Metacyclic promastigotes are injected into the skin together with *Leishmania*-derived exosomes, the sand fly microbiota, PSG, and salivary components which recruit mononuclear phagocytic cells such as dendritic cells, neutrophil monocytes, and especially macrophages and cause local inflammation [37], [38]. The proliferation of amastigote within the

phagolysosomes of the host cell, the rupture of the infected cells, and the infection of the new one mark the onset of the disease [39].

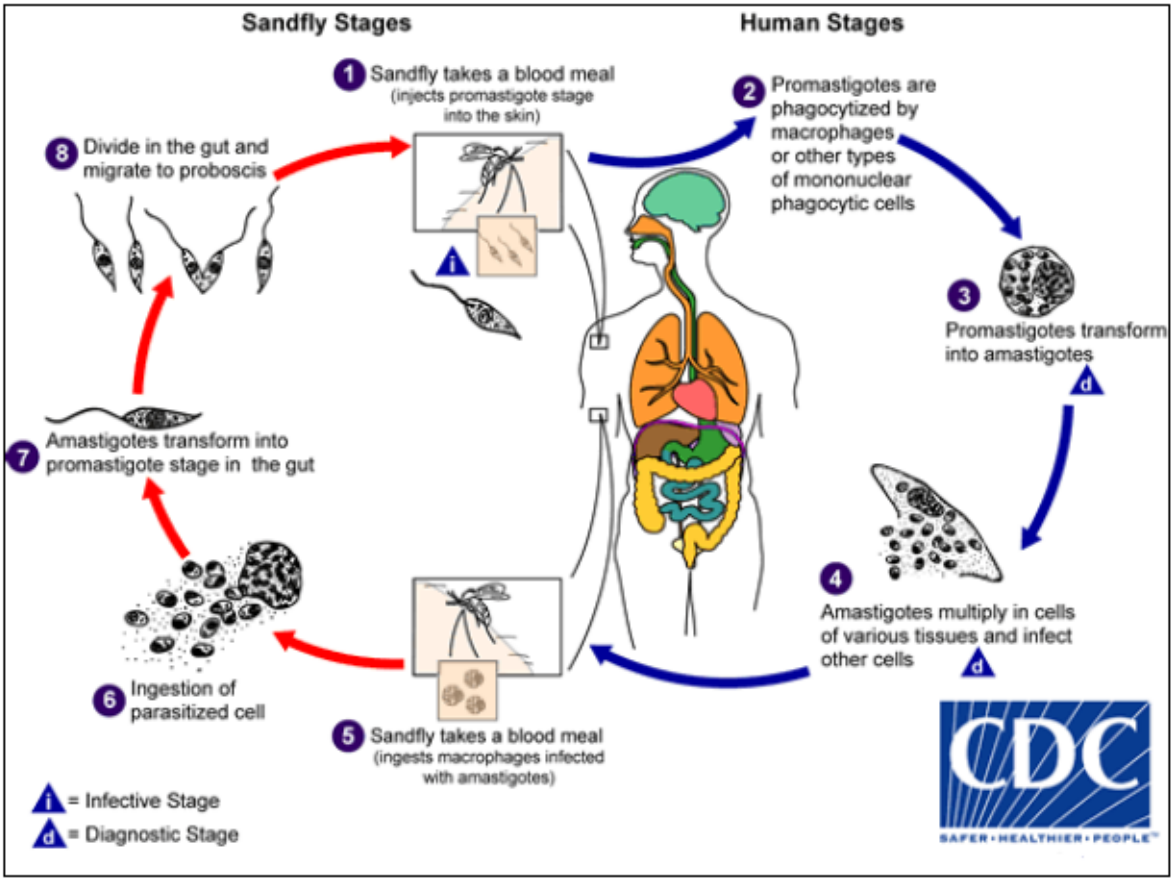


Figure 6: Life cycle of *Leishmania* sp parasites.

Taken from https://www.cdc.gov/parasites/leishmaniasis/biology.html

1.4. Drug resistance of the *Leishmania* parasites

The treatment of leishmaniasis is purely based on the use of chemotherapy. So far, very few drugs are available such as pentavalent antimonial (SbV), miltefosine (MIL), paromomycin, and amphotericin B (Amb), often known for their toxicity, side effects, and high production cost. Unfortunately, in some foci, the emergence of clinical resistant parasite strains against the few available drugs cited above, treatment failures and relapse in patients were reported [15], [40]. In Algeria, meglumine antimoniate (Glucantime®), often toxic to patients, remains the most widely used drug and first-line treatment given to patients with CL and VL [41]. Many factors participated in the emergence of these resistant parasite strains such as host genetics and immunological responses to treatment, drug quality, non-respect of therapy, and intrinsic drug susceptibility of *Leishmania* species [42], [43]. Furthermore, demographic changes, urbanisation, and the movement of infected people from endemic areas to *Leishmania*-free areas can contribute to the spread of *Leishmania* parasites and drug-resistant isolates [32]. In

addition, recent experimental infection studies demonstrated the ability of sand fly females to transmit resistant *L. infantum* and *L. major* strains [44], [45].

2. The Life Cycle of Sand Flies:

Sand flies are small insects with body sizes of 2-5 mm, typically active during dusk and dawn. They are present worldwide between latitude 40 ° S and 50 ° N and absent in the Pacific Islands, New Zealand, and Antarctica [33], [46]. Their life cycle is fully terrestrial and involves four developmental stages: eggs, larvae (four instars), pupae, and adults. Their development is influenced by environmental factors such as temperature, humidity, and soil type [47], [48]. Understanding the life cycle of sand flies is important for developing effective control measures to reduce the transmission of leishmaniasis and other diseases. Generally, sand fly females lay eggs in suitable breeding sites such as soil cracks, animal burrows or plant debris. Depending on environmental conditions and a given species, after 6-10 days, eggs hatch into first-stage worm-like larvae characterised by the presence of a pair of caudal setae on the last segment of the body. These larvae feed on organic matter and microorganisms in the soil to develop through four larval instars. At the end of the fourth stage, the larvae defecate all the nutrients and prepare to moult into an immobile pupa that undergoes metamorphosis into an adult fly. Adult sand flies emerge from the pupa after 7-10 days (Figure 7). Adults of both sexes feed on plant juices and nectar but the females usually also require blood meal to produce eggs except for some species such *Ph. bergeroti* which is autogenous and can lay the first egg batch without taking blood meal [49]. Each female can lay hundreds of eggs during its lifespan which can range from several weeks to several months in nature [46], [50].

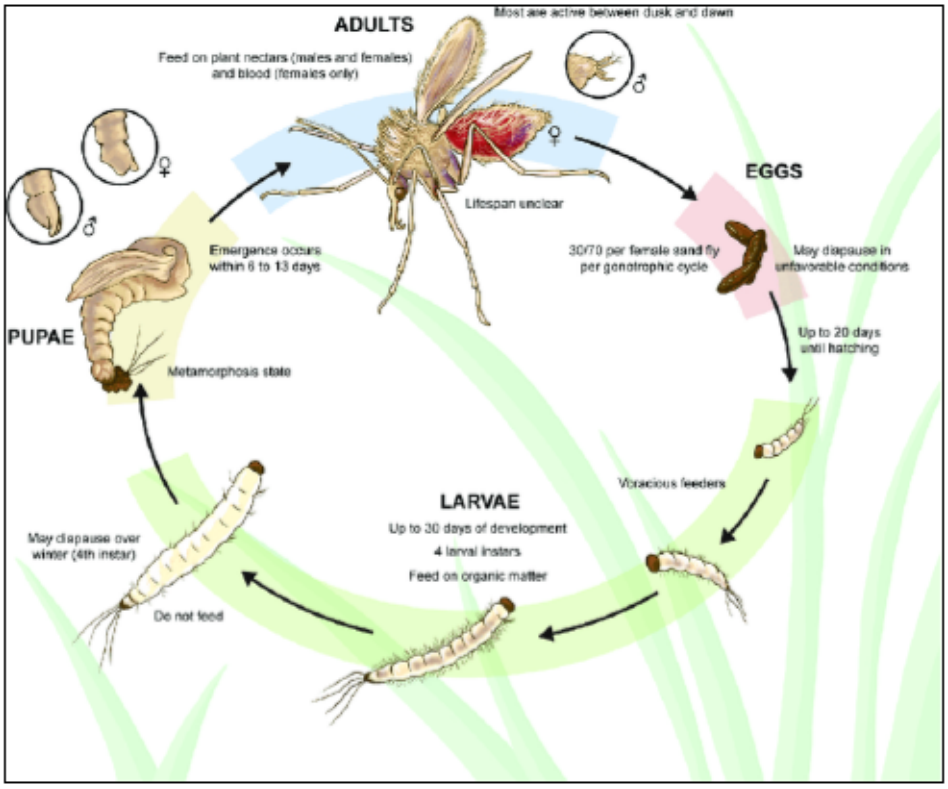


Figure 7: Sand fly lifecycle of sand fly [33].

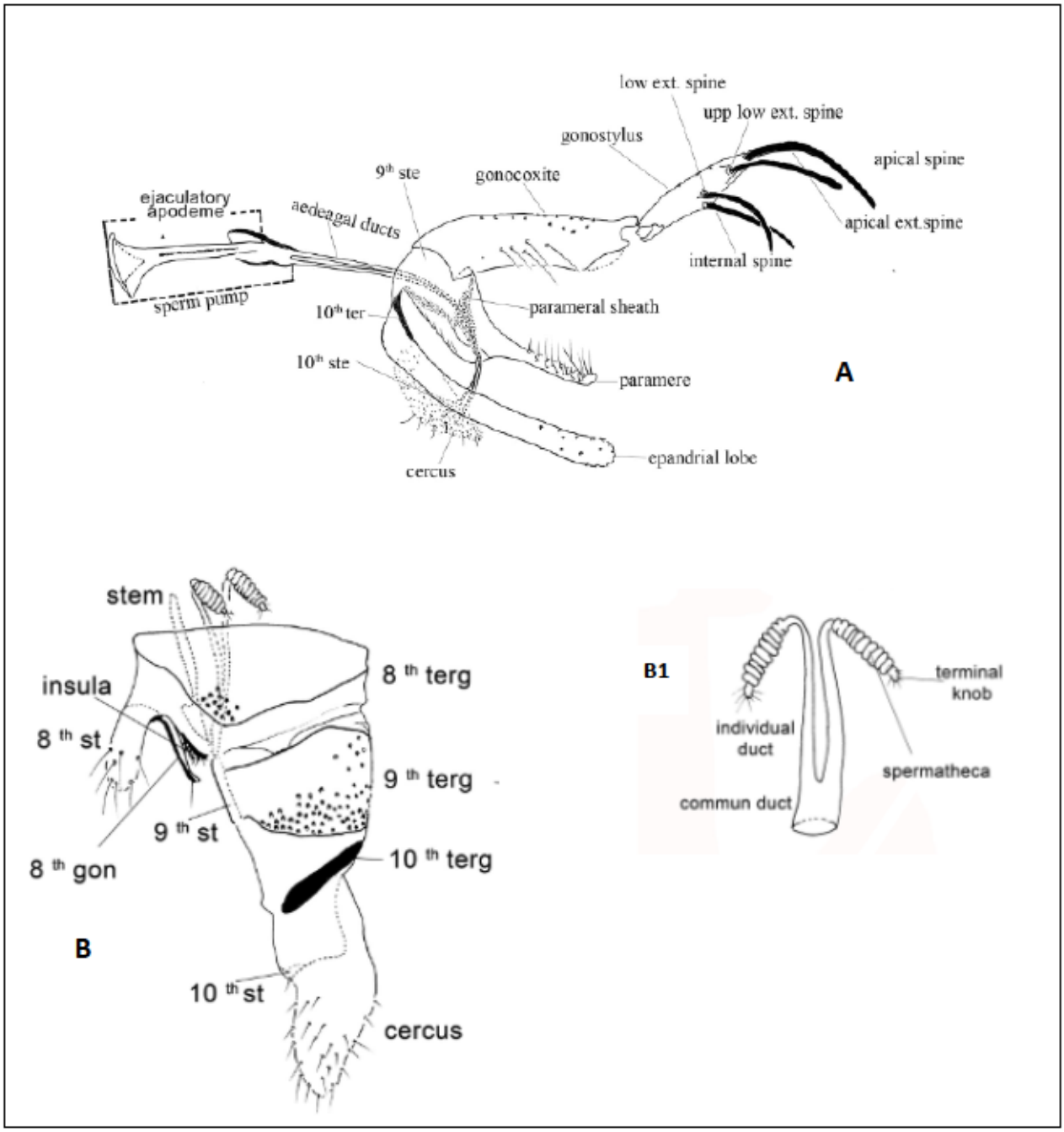
Sand flies belong to the order Diptera, suborder Nematocera, family Psychodidae, and subfamily Phlebotominae [51]. Their taxonomy is traditionally based on the analysis of morphological characters, mainly those on the head and genitalia, however, understanding of the phylogenetic relationships among the group is nowadays best revealed by assessing the genetic markers. In the Old World, sand flies are traditionally divided into 3 genera. The genus *Sergentomyia* which includes seven subgenera: *Capensomyia*, *Grassomyia*, *Neophlebotomus*, *Parrotomyia*, *Sergentomyia*, *Spelaeomyia,* and *Sintonius.* The genus *Phlebotomus* which includes 12 subgenera: *Alderius*, *Anaphlebotomus* (syn *Abonnencuis*), *Australophlebotomus*, *Euphlebotomus*, *Idiophlebotomus*, *Kasaulius*, *Larroussius*, *Paraphlebotomus*, *Phlebotomus*, *Spelaephlebotomus*, *Synphlebotomus* and *Transphlebotomus* and finally the genus *Chinius* described in 1987 [2]. Recently, *Ph. alexandri* which belonged to the *Paraphlebotomus* subgenus was raised and classified into a new subgenus *Artemievus subg.nov. Depaquit*, in addition, two other subgenera were proposed *Madaphlebotomus* and *Legeromyia* (Cruaud et al., 2021; Galati and Rodrigues, 2023). In the New World, Galati has classified sand flies based on a systematic phylogenetic analysis of morphological characters in which sand flies are divided into two tribes named Hertigiini (including two genera *Hertigia* and *Warileya*) and Phlebotomini which are subdivided into 4 subtribes and 20 genera [2].

Several species belonging to *Phlebotomus* genus are involved in the transmission of *Leishmania* parasites. *Phlebotomus papatasi* is a principal vector of *L. major* [27] in the Middle East and North Africa, although its distribution extends to South Europe and eastern regions of the Indian subcontinent beyond that of the *L. major* distribution and its reservoir. *Phlebotomus sergenti* vector of *L. tropica* and *L. killicki* in North Africa and the Middle East [24], [53] and finally *Phlebotomus perfiliewi* vector of *L. infantum* causing CL in North Africa [25]. *Phlebotomus perniciosus , Ph. ariasi* and many other species belonging to *Larroussius* subgenus are vectors of *L. infantum* in the Mediterranean basin, central and west Asia while in Indian subcontinent and East Africa *Ph. argentipes, Ph. orientalis* and *Ph. martini* are the main vector of *L. donovani* [26], [54], [55]

Until now, Algeria counts 26 sand fly species belonging to two genera: *Phlebnotomus* genus includes 5 subgenera and 14 species and *Sergentomyia* genus includes 4 subgenera and 12 species [56]. Recently, a new species has been described named *Se. imihra* n.sp which raises the sand fly fauna to 27 species (Benallal et al., under review).

2.1. Identification of sand fly species

The identification of sand flies is mainly based on the morphological decisive features differencing between the huge number of sand fly species using the characters present on the head (mainly pharynx and cibarium shapes and armatures) and genitalia (gonostyle and spermathecae shapes for male and female respectively) (Figure 8). However, the presence of sibling and cryptic species forced the taxonomists to search for other alternatives based on DNA and protein techniques.


low ext. spine
upp low ext. spine
gonostylus
apical spine
9th ste
gonocoxite
ejaculatory apodeme
aedeagal ducts
apical ext.spine
internal spine
sperm pump
10th ter
parameral sheath
10th ste
paramere
A
epandrial lobe
cercus
stem
8 th terg
insula
8 th st
9 th terg
9 th st
10 th terg
8 th gon
B
10 th st
cercus
B1
terminal knob
individual duct
spermatheca
commun duct

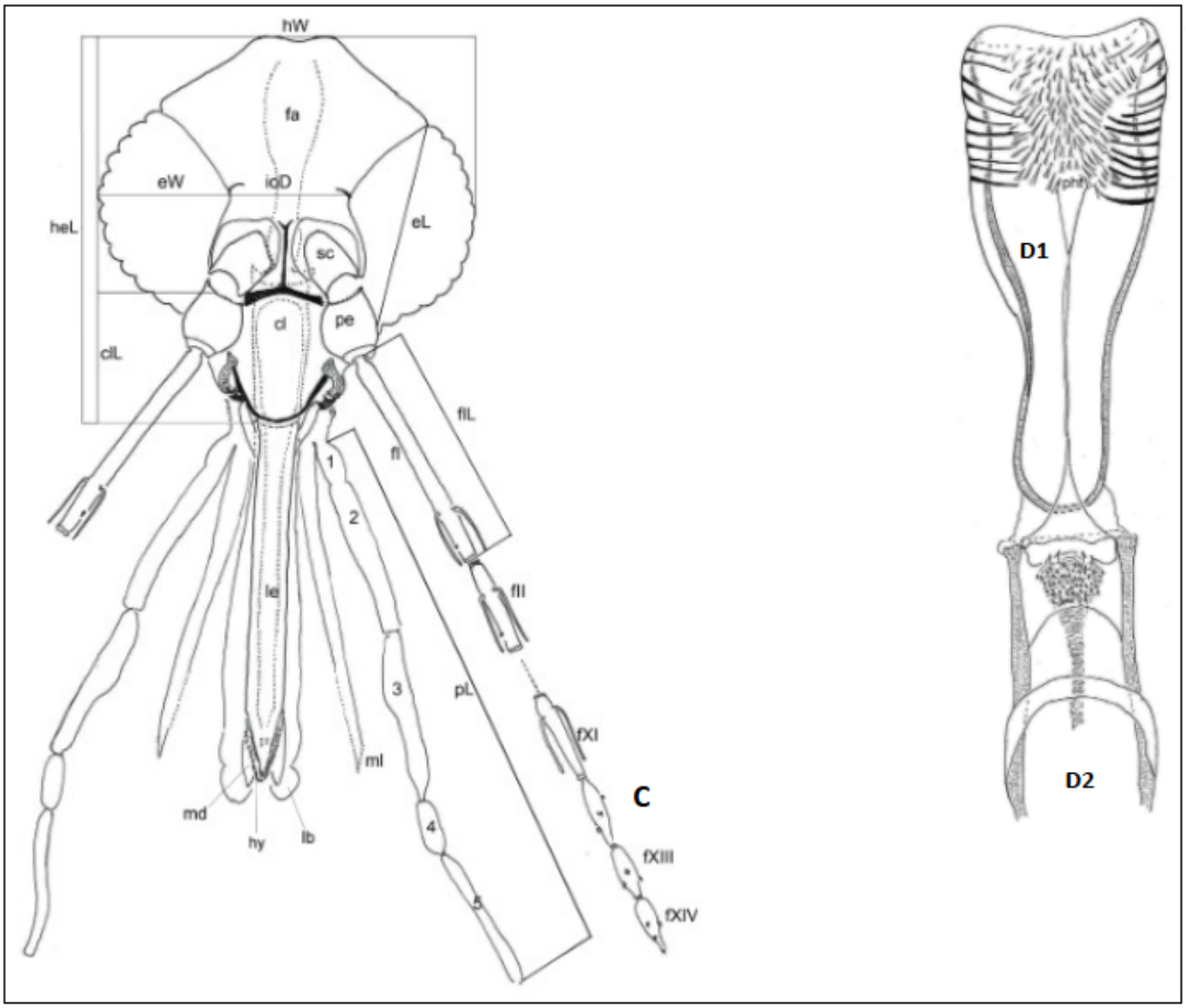


Figure 8: A) male genitalia, B) female genitalia, B1) details of spermathecae, C) details of the head, D1) pharynx, D2) cibarium[57].

2.1.1. DNA-based techniques:

Since the discovery of DNA and the revolution raised by increasing availability of routinely performed polymerase chain reaction (PCR) molecular techniques such as random amplification polymorphic DNA (RADP), microsatellite and mitochondrial markers, PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) and DNA sequencing technologies have played an important role in sand fly taxonomy, phylogeny, and phylogeographic studies [58] allowing distinction between cryptic species such as *Ph. chabaudi/ Ph. riouxi* or *Ph. perniciosus/Ph. pernicious* atypical form and *Ph. longicuspis* (Benabdennbi and Pesson, 1998; Lehrter et al., 2017; Martin-Sanchez et al., 2000; Morillas Márquez et al., 1991; Pesson et al., 2004), description of new species such as *Ph. betisi*, *Ph. creticus, Se. imihra* and readjustment of *Ph. alexandri* in a new subgenus [52], [64], [65].

2.1.2 Protein-based techniques:

The use of protein-based techniques to identify and study the diversity of sand flies was first based on the isoenzyme system which was a very laborious, time-consuming, and cost-effective technique [60]. Therefore, it was gradually abandoned in the favour of the DNA-based techniques as they were becoming more affordable and routinely applied. However, recently the protein-based techniques resurfaced with the development of another protein approach called Matrix-Assisted Laser-Desorption/Ionisation Time of Flight Mass Spectrometer (MALDI-TOF MS) based on acidic extraction of peptides and time flight of the low molecular weight proteins (up to 25 kDa). It was developed initially for the clinical identification of pathogenic bacteria then later it was successfully applied on a wide array of multicellular organisms such as fungi and yeasts due to several beneficial characteristics such as simple sample preparation, time and cost-effectiveness [66]. Growing mass of data allowed the creation of several commercial and non-commercial databanks that are available for routine microbiological assays [67]. In the field of entomology, it was first tested on the order Notoptera, suborder Mantophasmatodea using neural peptides, then the technique has proved its efficiency on Diptera order by discriminating between two siblings from the *Drosophila melanogaster* subgroup [68], [69]. Following that and by using different protocol of proteins extraction, MALDI-TOF MS was extended to study the taxonomy of other insects especially those of medical importance such as ticks, TseTse fly, fleas, *Culicoides*, mosquitoes, lice, triatomine and sand flies. This technique helped even experienced entomologists to identify damaged or immature specimens [70], [71], [72], [73], [74], [75], [76], [77], [78]. In sand flies, beside the application on adults, immature stages were also analysed by the same technique (Halada et al., 2018). For the adults, it became a method of choice especially in large-scale entomological surveys [80], [81]. Despite its limitation in some cases to distinguish between cryptic species as reported for the *Larroussius* subgenus, a machine learning approach was recently introduced to improve the quality of data analysis [82]. Furthermore, integrative approaches that combine morphological characteristics, wing morphometry, DNA and proteomic approaches were used to describe new species such as *Ph. creticus*, *Ph. anatholicus*, *Ph. killicki*, *Ph. betisi,* and the proposition of a new subgenus *Lewisius* [64], [65], [83].

Beside species identification, the mass spectrometry was used to identify the source of the host blood. Although the differentiation of host protein profiles in the bloodmeal of engorged sand fly females was shown to be achievable, in order to avoid the necessity to establish a

reference database of potential host, a modified technique of mass spectrometry namely MALDI-TOF peptide mass mapping (MALDI-TOF PMM) was successfully developed to identify the host blood in engorged sand fly females despite the small amount of the ingested blood. MALDI-TOF PMM requires proteolytic hydrolysis of the sample prior to MALDI-TOF MS reference database creation or integration. It has the advantage to analysis lower mass range 500 to 4000 Da and the discrimination up to one amino acid changes by comparing the obtained peptide sequences against the vertebrate subset of SwissProt or UniProt databases. This technique has been successfully used and identified the blood sources of sand flies trapped in field [84], [85].

3. Host reservoir of *Leishmania* sp.

By definition, a host reservoir is an ecological system in which a pathogen is maintained persistently. This reservoir has a dynamic feedback to the other hosts through pathogen transmission by the vector, contrary to an incidental host, which cannot transmit the pathogen to new hosts (Chaves et al., 2007; World Health Organization, 1984). It can be an animal that maintains zoonotic diseases or a human that maintains anthroponotic diseases. Regarding leishmaniasis, the parasite strains and their hosts and vectors shape the geographical distribution of the different forms throughout the world [86]. Dogs, cats, foxes and jackals serve as main reservoir hosts for *L. infantum* in Europe, North Africa, Asia, and South America whereas humans play the role of reservoir for *L. donovani* in the Indian subcontinent [55], [87], [88]. Humans can also serve as the reservoir for *L. tropica* in the MENA area [89], [90], [91], [92]. Besides, a wide range of rodents that represent 42% of the global mammalian population [93] are known to play a crucial role as host reservoirs of *Leishmania* parasites [94]. So far, rodents such as gerbils and jirds *Psammomys obesus* and *Meriones shawi* are known to be the main reservoir for *L. major* [95], [96]. Rock hyrax *Procavia capensis*; a small herbivorous in the order Hyracoidea; and *Ctenodactylus gundi* are regarded as potential reservoir hosts for *L. killicki* and *L. tropica* in the Middle East and North Africa [97], [98]. Recently, hedgehogs *Atelerix algirus* and *Paraechinus aethiopicus* and many other species such as *Gerbillus nanus* have also been reported naturally infected by *L. major*, *L. tropica,* and *L. infantum* but their complete implication in *Leishmania* transmission has not been completely studied [99], [100], [101]. Whether suspected species could be classified as reservoir hosts is best tested in the laboratory using experimental infections and xenodiagnoses [102], [103], [104], [105].

Objectives

Efficient disease control requires extensive knowledge of the major interactions between the principal actors involved in the maintenance and transmission of the disease in nature, especially vector-borne zoonoses for which humans are often regarded as incidental hosts. In this context, leishmaniases in Algeria are considered zoonoses involving several species of mammals and sand flies, some of which are known and others remain to be revealed in order to understand the life-cycle and transmission of the disease in this part of the world.

My Ph.D. project focused on the study of the different components (vectors, reservoirs and parasites) involved in the transmission cycle of leishmaniasis in Algeria using different experimental approaches: *i)* DNA and protein molecules to identify vector and rodent species and to construct DNA barcodes and MALDI-TOF databases, and *ii)* experimental infections of sand flies and suspected wild rodents to understand their role in the transmission and maintaining of parasites. To achieve our goals, four sub-objectives were established:

1. **Identification and construction of databases of DNA barcodes and MALDI-TOF protein profiles**. Sand flies were trapped in endemic localities of *Leishmania major, L. tropica* and *L. infantum*. Head and genitalia of sand flies were dissected and slide mounted for morphology identification while the rest of the body (thorax and abdomen) was used for DNA barcodes and MALDI-TOF protein profiling.
2. **Evaluation of host competence of putative rodent reservoirs for *L. major***. *Gerbillus amoenus* rodents were infected with culture-derived *L. major*. Infected rodents were monitored for several months and tested by xenodiagnoses for their infectiousness to *Ph. papatasi* the natural vector of *L. major*. Parasite distributions and loads were estimated post-mortem using qPCR from skin and viscera samples.
3. **Study of important transmission parameters on a natural model: *L. major* - *Phlebotomus papatasi* - *Meriones shawi***. *Meriones shawi* rodents, natural reservoir hosts of *L. major,* were infected with sand fly-derived promastigotes. Rodents were monitored for several months and tested by xenodiagnoses for their infectiousness to *Ph. papatasi* to assess the role of asymptomatic rodents in parasite transmission.
4. **Study of the drug-resistant *Leishmania major* fitness in natural vectors *Ph. papatasi***. Two lines of *Leishmania major* resistant to Sb(III) at different concentrations were generated. Females of *Ph. papatasi* were experimentally infected with parasites of both lines and the wild-type strain to evaluate the impact of sand fly digestive tracts on the fitness of the parasite lines.

Publications:

1- Phlebotomine sand flies (Diptera: Psychodidae) of the Maghreb region: A systematic review of distribution, morphology, and role in the transmission of the pathogens. **Benallal KE**, Garni R, Harrat Z, Volf P, Dvorak V. PLoS Negl Trop Dis. 2022 6;16(1):e0009952. doi: 10.1371/journal.pntd.0009952. eCollection 2022 Jan.

2- Identification of blood source preferences and *Leishmania* infection in sand flies (Diptera: Psychodidae) in north-eastern Algeria. Nacer Eddine Messahel, **Kamel Eddine Benallal,** Petr Halada, Ismail Lafri, Hemza Manseur, Ahcene Hakem, Karim Houali, Zoubir Harrat, Petr Volf, Vit Dvorak. Veterinary Parasitology: Regional Studies and Reports. Volume 31, 2022, 100729. https://doi.org/10.1016/j.vprsr.2022.100729.

3- Phlebotomine sand fly survey, blood meal source identification and description of *Sergentomyia imihra* n. sp. in the central Sahara of Algeria. Kamal Eddine Benallal, Mohammed Mefissel, Yassine Dib, Jérôme Depaquit, Daniel Kavan, Zoubir Harrat, Vit Dvořák, Petr Volf, Petr Halada (under review)

4- Infectiousness of Asymptomatic *Meriones shawi*, Reservoir Host of *Leishmania major*. 2023. Jovana Sadlova, Barbora Vojtkova, Tereza Lestinova, Tomas Becvar, Daniel Frynta, **Kamal Eddine Benallal**, Nalia Mekarnia, Zoubir Harrat and Petr Volf. Pathogens, 12, 614. https://doi.org/10.3390/pathogens12040614.

5- Host competence of Algerian *Gerbillus amoenus* for *Leishmania major.* **Kamal Eddine Benallal**, Ghania Mezai, Mohammed Mefissel, Nadia Klari, Cylia Lardjane, Ahmed-Fayez Khardine, Ihcen Kherachi, Yacine Dib, Karima Brahmi, Jovana Sadlova, Petr Volf, Zoubir Harrat. International Journal for Parasitology: Parasites and Wildlife 21 (2023) 69–73. https://doi: 10.1016/j.ijppaw.2023.04.007.

6- Effect of *Phlebotomus papatasi* on its Fitness, infectivity and antimony-resistant *Leishmania major* MON-25. Nalia Mekarnia, **Kamal Eddine Benallal**, Jovana Sádlová, Barbora Vojtková, Aurélie Mauras, Nicolas Imbert, Maryline Longhitano, Zoubir Harrat, Petr Volf ,Philippe M. LOISEAU, Sandrine Cojean. International Journal for Parasitology:Drugs and Drugs Resistance. 25(2024) 100554 https://doi.org/10.1016/j.ijpddr.2024.100554.

Phlebotomine sand flies (Diptera: Psychodidae) of the Maghreb region: A systematic review of distribution, morphology, and role in the transmission of the pathogens.

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PLOS NEGLECTED TROPICAL DISEASES

REVIEW

Phlebotomine sand flies (Diptera: Psychodidae) of the Maghreb region: A systematic review of distribution, morphology, and role in the transmission of the pathogens

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Abstract

Background

Phlebotomine sand flies (Diptera: Psychodidae) are important vectors of various human and animal pathogens such as *Bartonella bacilliformis*, *Phlebovirus*, and parasitic protozoa of the genus *Leishmania*, causative agent of leishmaniases that account among most significant vector-borne diseases. The Maghreb countries Mauritania, Morocco, Algeria, Tunisia, and Libya occupy a vast area of North Africa and belong to most affected regions by these diseases. Locally varying climatic and ecological conditions support diverse sand fly fauna that includes many proven or suspected vectors. The aim of this review is to summarize often fragmented information and to provide an updated list of sand fly species of the Maghreb region with illustration of species-specific morphological features and maps of their reported distribution.

Materials and methods

The literature search focused on scholar databases to review information on the sand fly species distribution and their role in the disease transmissions in Mauritania, Morocco, Algeria, Tunisia, and Libya, surveying sources from the period between 1900 and 2020. Reported distribution of each species was collated using Google Earth, and distribution maps were drawn using ArcGIS software. Morphological illustrations were compiled from various published sources.

Results and conclusions

In total, 32 species of the genera *Phlebotomus* (*Ph.*) and *Sergentomyia* (*Se.*) were reported in the Maghreb region (15 from Libya, 18 from Tunisia, 23 from Morocco, 24 from Algeria, and 9 from Mauritania). *Phlebotomus mariae* and *Se. africana* subsp. *asiatica* were recorded only in Morocco, *Ph. mascitti*, *Se. hirtus*, and *Se. tiberiadis* only in Algeria,

whereas *Ph. duboscqi*, *Se. dubia*, *Se. africana africana*, *Se. lesleyae*, *Se. magna*, and *Se. freetownensis* were reported only from Mauritania. Our review has updated and summarized the geographic distribution of 26 species reported so far in Morocco, Algeria, Tunisia, and Libya, excluding Mauritania from a detailed analysis due to the unavailability of accurate distribution data. In addition, morphological differences important for species identification are summarized with particular attention to closely related species such as *Ph. papatasi* and *Ph. bergeroti*, *Ph. chabaudi*, and *Ph. riouxi*, and *Se. christophersi* and *Se. clydei*.

Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are small insects with nocturnal activity, females being hematophagous and feeding on various vertebrate hosts depending on species [1,2]. They live in various habitats, some species thriving in the vicinity of human dwellings and shelters of domestic animals that provide favorable humidity and temperature conditions and breeding sites as their larval stages are terrestrials, living in microhabitats with organic material [1,2]. So far, over 950 described species were classified into several genera, approximately 100 species in both Old and New World are incriminated in transmission of various pathogens including those infecting humans: bacteria *Bartonella bacilliformis*, sand fly–borne viruses, and, most importantly, parasitic protozoa of the genus *Leishmania* [3], causative agents of human and veterinary diseases known as leishmaniases that constitute a major health problem, threatening more than 350 million people in many countries and approximately 2 million of new cases reported each year [4].

Regarded as neglected diseases, leishmaniases have a huge impact on affected countries, challenging their public health services, exacerbating poverty, decreasing workers productivities, and threatening various age groups including children [5]. In 2018, of the 200 countries and territories that reported data to WHO, 97 (49%) were considered endemic and 4 countries having previously reported cases of leishmaniasis [6]. The diseases occur in 4 main clinical forms, cutaneous leishmaniasis (CL), mucocutaneous form (MCL), visceral leishmaniasis (VL) also known as kala-azar, and post–kala-azar dermal leishmaniasis (PKDL). These clinical forms, ranging from self-healing but potentially disfiguring skin lesion to serious visceral disease that is potentially life threatening when untreated, are typically associated with particular *Leishmania* species; however, the actual clinical outcome depends on multiple factors including the immunocompetence of the patient. The annual incidence of leishmaniasis is estimated at around 0.2 to 0.4 million cases for VL and from 0.7 to 1.2 million cases for CL [7].

The Maghreb region is located between 19˚ and 37˚ N and 15W˚ and 25˚E, and it covers an area of 6,045,741 km^2 including Mauritania, Morocco, Algeria, Tunisia, and Libya. In such a vast region, different bioclimatic zones occur ranging from humid to the hyperarid (Fig 1). Leishmaniases are known as dynamic diseases influenced by various factors [8].

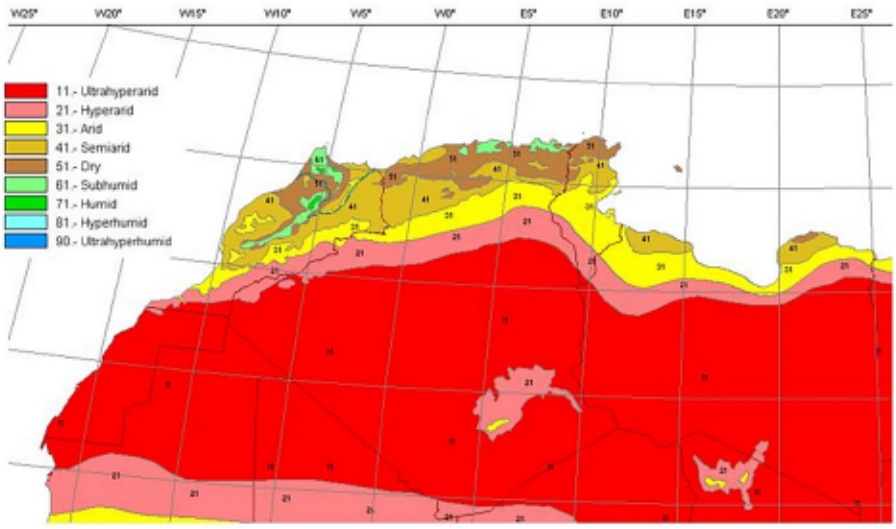


Fig 1. Bioclimatic stages of the Maghreb region.

https://doi.org/10.1371/journal.pntd.0009952.g001

Subsequently, between 2004 and 2008, 355 VL and 58,651 CL cases were reported in the Maghreb region [7]. Algeria is ranked first in CL cases and second worldwide after Afghanistan, reporting 44,050 cases, followed by Tunisia (7,631), Libya (3,540), and Morocco (3,430) cases. VL is less frequent, Morocco is ranked first in VL, reporting 152 cases, followed by Algeria with 111, Tunisia with 89 cases, and Libya with 3 cases for the same period [9]. Despite published VL and CL cases in the Maghreb region, the data are compromised by expected underreporting [5].

These high numbers were the consequence of several human and natural factors such as the looseness of the sand fly control campaigns, strong urbanization of the rural and suburban areas, and changes in land use that stimulate the expansion or migration of the reservoir hosts such as *Psammomys obesus* from its natural habitats caused by hydric stress and the decrease in food sources; all these factors led to the emergence of new foci of transmission as reported in Algeria [10,11]. Thus, synergic effects of globalization, climatic change, and various human activities allow the parasites and their vectors to spread in space and time as it was noticed in Europe [8].

Sand flies belong to the order Diptera, suborder Nematocera, family Psychodidae, and subfamily Phlebotominae. In the Old World, sand flies are traditionally divided into 7 genera, some of these comprising further subgenera. Sand fly taxonomy is traditionally based on analysis of decisive morphological characters on the head and genitalia [3,12], but the advent of molecular techniques that deploy mainly sequencing analyses of suitable genetic markers [13] provided further insights into cryptic species including challenging taxa of the Maghreb region like *Phlebotomus perniciosus* and *Ph. longicuspis* or *Ph. riouxi* and *Ph. chabaudi*. Moreover, they allowed to associate males with females, e.g., *Chinius samarensis* [14], to give an insight about vectors and nonvectors by the detection of DNA parasite or to propose evolutionary systematic [13,15,16]. In addition, next-generation sequencing (NGS) assays and statistical analysis of the subsequent reads were used to help species identification within pooled samples in several virological studies [14–16]. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a protein-based method, was introduced for rapid and cost-effective species identification of various

arthropods including sand flies upon their species-specific protein profiles [17]. These molecular approaches complement the morphological identification that is still regarded as the golden standard and the backbone of entomological surveys and vector control programs [18].

The incrimination of particular sand fly species as a vector of leishmaniasis is based on assessment of several criteria previously established [1,4,19]. As a complete evaluation of all these criteria requires a sustained field and experimental research, beside sand fly species fully proven as leishmaniasis vectors, many other species are considered as suspected vectors based on circumstantial evidence and partial fulfillment of some of these criteria [15,20]. In the Maghreb region, the omnipresence of many proven and suspected vectors of several *Leishmania* species supports transmission cycles of both CL and VL [21]. While vast knowledge of sand fly species present in Maghreb countries was accumulated during several decades of focused research including precise morphological analysis of some species, the information remain fragmented, albeit it could serve as a valuable background for advanced molecular studies. The correct and conclusive morphological identification requires accurate and updated identification keys and experienced entomologists who can provide sufficient expertise for evaluation of often minute morphological characters.

This review aimed to update the list of sand fly species so far recorded in the Maghreb region (Morocco, Algeria, Tunisia, and Libya) providing (i) their presumable geographic distribution with respect to the bioclimatic zones; (ii) their proven or suspected role in pathogen transmission in the Maghreb region (Table 1); and (iii) defining morphological characters that enable a species identification with special attention to taxonomically challenging species such as *Ph. chabaudi/Ph. riouxi*, *Ph. papatasi/Ph. bergeroti*, or *Ph. perniciosus* species complex.

Materials and methods

Literature search and data extraction

For our study, we have followed PRISMA-P protocol [22]. We consulted all available data on sand fly fauna of the Maghreb regions, namely Mauritania, Morocco, Algeria, Tunisia, and Libya. Various databases like PubMed, Google Scholar, Archive de Institut Pasteur d'Algérie (IPA), and Archive de Institut Pasteur de Tunis were searched for available data from the period 1900 to 2020. The available English and French resources published in full text, articles, reports, congress presentations, book chapters, and thesis dissertations containing information on sand flies were added, treated, and analyzed together with data available from the Laboratoire d'Eco-épidémiologie Parasitaire et Génétiques des Populations of Institut Pasteur of Algeria. The following search string was used: terms in title: [(phlebotomine OR sand flies OR sand flies) AND in all fields: (*Phlebotomus* OR *Sergentomyia*) AND in all fields: (*Phlebotomus* OR *Paraphlebotomus* OR *Larroussius* OR *Transphlebotomus* OR *Sergentomyia* OR *Parrotomyia* OR *Grassomyia* OR *Sintonius*) AND in all fields: (species name) AND in all fields: (distribution OR presence OR occurrence OR report OR spread OR disperse OR detect) AND in all fields:(Algeria OR Libya OR Tunisia OR Maghreb region OR Morocco) AND in all fields: (leishmaniasis OR "cutaneous leishmaniasis" OR "CL" OR "visceral leishmaniasis" OR "VL" "canine leishmaniasis" OR "CanL" OR "Phlebovirus" OR "Bunyaviridae") AND in all fields: ("neglected tropical diseases" OR "vector-borne diseases") AND in all fields: ("vector control")] as suggested by Dvorak and colleagues [23].

Table 1. Sand fly species their distribution and vectorial role in the Maghreb region.

Genus	Subgenus	Species	Figure		Map of distribution	Country of distribution			
			Female	Male		M	A	T	L
Phlebotomus	*Phlebotomus*	*Ph. papatasi**ǂ	4	6	2	X	X	X	X
		Ph. bergeroti	5	7	3	X	X	X	X
	Paraphlebotomus	*Ph. sergenti**ǂ	8	9	10	X	X	X	X
		Ph. alexandri	11	12	13	X	X	X	X
		Ph. kazeruni	14	15	16	X	X		
		Ph. chabaudi	18	17	19	X	X	X	X
		Ph. riouxi	21	22	20	X	X	X	X
	Larroussius	*Ph. perniciosus**ǂ	25	23	26	X	X	X	X
		Ph. perniciosus atypical	-	24	27	X	X	X	
		*Ph. longicuspis***ǂ	29	28	30	X	X	X	X
		*Ph. perfiliewi**	32	31	33	X	X	X	
		*Ph. ariasi**ǂ	35	34	36	X	X	X	X
		Ph. chadlii	37	38	39	X	X	X	
		*Ph. langeroni**	41	40	42	X	X	X	X
		Ph. mariae	-	43	44	X			
	Transphlebotomus	*Ph. mascittii*	45	46	47		X		
Sergentomyia	*Sergentomyia*	*Se. minuta*	48	49	50	X	X	X	X
		Se. fallax	51	52	53	X	X	X	X
		Se. antennata	55	54	56	X	X	X	X
		Se. cincta	57	-	58		X		
		Se. schwetzi	59	60	61	X	X	X	X
	Parrotomyia	*Se.africana* subsp. *asiatica*	63	-	62	X			
		Se. africana subsp. *eremitis*	66	65	64		X		
		Se. lewisi	68	69	67	X	X	X	X
	Grassomyia	*Se. dreyfussi*	72	71	70	X	X	X	X
	Sintonius	*Se. clydei***	75	74	73	X	X	X	X
		Se. christophersi	78	77	76	X	X	X	X
		Se. hirtus	-	80	79		X		
		Se. tiberiadis	83	82	81		X		

*Proven vector of the parasites in the Maghreb region.

**Detection of *Leishmania* DNA.

ǂDetection of *Phlebovirus* RNA.

X, occurrence.

A, Algeria; L, Libya; M, Morocco; T, Tunisia.

https://doi.org/10.1371/journal.pntd.0009952.t001

Titles relevant to the scope of this review were obtained in full text and selected for inclusion. Beside these, some data also come from direct consultations with experts and their in-house unpublished databases.

The distribution of Moroccan sand fly species was reported in [24–27], Algerian species [11,28–35], Tunisian species [36–38], and Libyan species [39–42]. For each sand fly species, geographical positions of published records (S1 Data) were first gathered in Google Earth software from sourced publications and then processed with ArcGIS Pro software version 2.5.0 for analysis and maps drawing. The bioclimatic zones classification (Fig 1) was based on the Worldwide Bioclimatic Classification System [43].

We adopted the abbreviation for the genus and subgenus according to the last suggestions of the experts during the International Symposium on Phlebotominae Sand Flies (ISOPS) 2016 meeting and the designation "Sand fly" or "Sand flies" along this review since during the same meeting, the experts did not converge to a common designation [44].

Morphological analysis

Morphological illustrations were compiled from the following publications and books: Abonnenc [2], Dedet and colleagues [33], Bailly-Choumara and colleagues [24], Rioux and colleagues [25,26,45–47], Annajar [41], Depaquit and colleagues [48], Léger and colleagues [49,50], Parrot and colleagues [51–53], Croset and colleagues [54,55], El Sawaf and colleagues [56], Chamkhi and colleagues [57], and Theodor and colleagues [58].

Results

I- Genus *Phlebotomus*

The genus *Phlebotomus* is defined by the usual absence of cibarial teeth and pigmented patch. The hind end of the abdomen tergites 2 to 6 holds many erected setae that arise from large sockets of the same size as those on the tergite. In Maghreb, the species belong to 4 subgenera: *Phlebotomus* Rondani, 1843, *Paraphlebotomus* Theodor, 1948, *Larroussius* Nitzulescu 1931, and *Transphlebotomus* Artemiev 1984. In males, the styles bear either 3 terminal and 2 subterminal, rather short spines (subgenus *Phlebotomus*) or 2 long terminal and 2 or 3 subapical spines without accessory spine (other subgenera). In females, the spermathecae have superficial striation or are annulated with basal structures in some species depending of the subgenus [2,12,59]. The main differences between the species are summarized in **Tables A and B in** S1 Text for males and females, respectively.

I-1 Subgenus *Phlebotomus* Rondani, 1843

The species belonging to this subgenus are recognized by (i) large male genitalia with 5 short spines (3 spatulated terminal spines and 2 at the mid-style position) upon a long cylindrical style; (ii) a paramere with 2 lengthy secondary appendices; (iii) short and conical aedeagus; and (iv) a small button at the base of the coxite bearing few long setae. Females possess pharynx armed with large teeth fringed with minute denticules and spermathecae with uniform shaped segments which are ended by a terminal knob. In Maghreb, 2 species of this subgenus were recorded: *Ph. papatasi* (Scopoli, 1786) and *Ph. bergeroti* (Parrot, 1934).

I-1-1 *Phlebotomus (Phlebotomus) papatasi*, Scopoli, 1786

It is one of most studied species due to its large geographical area of occurrence and its medical importance as it is the principal vector of the zoonotic cutaneous leishmaniasis (ZCL) in highland and north of Sahara caused by *Leishmania major* [60]. Except the zymodeme MON-269 from Algeria, all isolates of *L. major* in the region belong to zymodeme MON-25 [61,62]. This species also has been reported to transmit the Sicilian virus in Egypt [63] and found infected by Toscana virus (TOSV) in Morocco [64]. It has a vast distribution from northern to the southern part of the Maghreb region (Algeria, Morocco, Tunisia, and Libya) with a high abundance in the highlands and north of Sahara between 27˚/35˚ N and –4˚/58˚ E worldwide [65]. It is encountered from the humid to ultra-hyperarid bioclimatic zones (Fig 2).

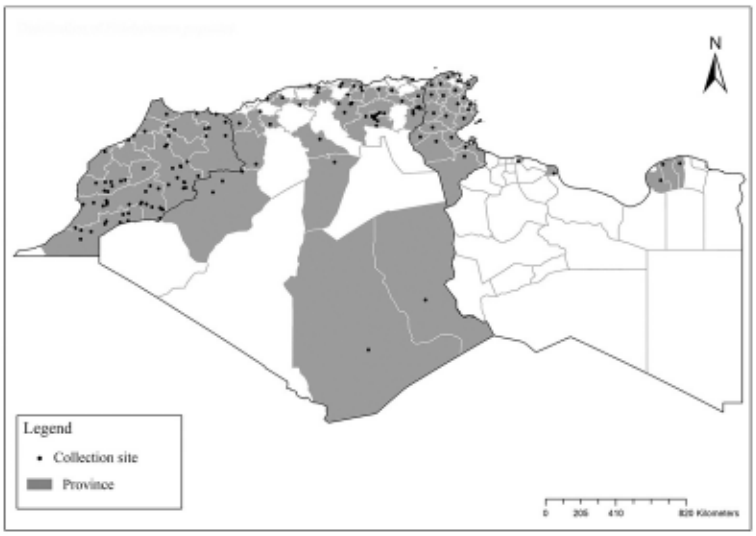


Fig 2. Distribution of *Phlebotomus papatasi*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g002

I-1-2 *Phlebotomus bergeroti* Parrot, 1934

This species was shown to transmit *L. major* under laboratory conditions [66], and it is suspected to play a role in the transmission of this parasite in Burkina Faso, Chad, Egypt, Iran, Mauritania, Oman, and Yemen on the basis of epidemiological evidence [67]. It occurs in the southern part of the Maghreb region (Mauritania, Morocco, Algeria, and Libya), and it is encountered in the hyperarid bioclimatic zone (Fig 3). In central Sahara regions of Algeria (Tamanrasset and Illizi), *Ph. bergeroti* and *Ph. papatasi* are often confused in the overlapping areas of occurrence despite the description of distinct characters between the 2 species

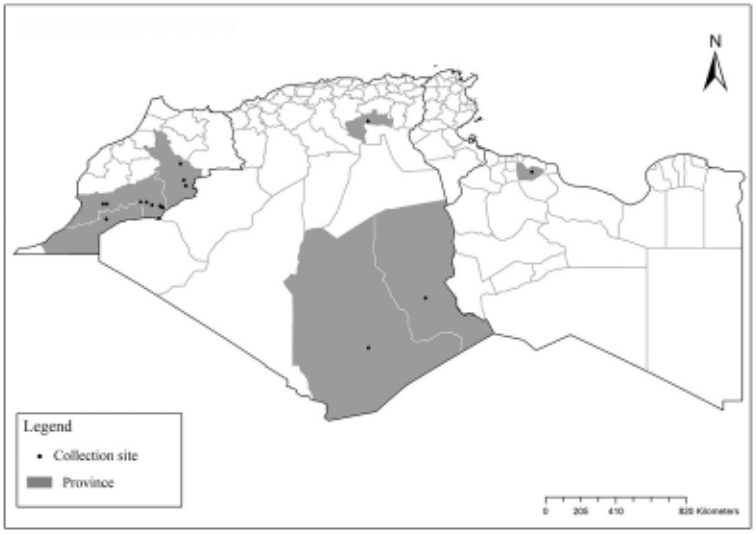


Fig 3. Distribution of *Phlebotomus bergeroti*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g003

(**Table C in** S1 Text). For the females, 2 differences are known: Posterior part of pharynx has weakly distended shape in *Ph. papatasi* (Fig 4), while it has a bottle shape in *Ph. bergeroti* (Fig 5). The ascoids length is as long as the fourth antennal segment in *Ph. bergeroti* compared

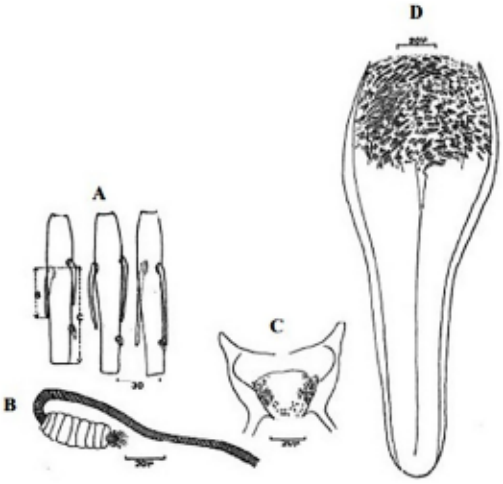


Fig 4. ***Phlebotomus papatasi*** ♀ [2]. **(A)** Fourth antenna segment. **(B)** Spermathecae. **(C)** Cibarium. **(D)** Pharynx.

https://doi.org/10.1371/journal.pntd.0009952.g004

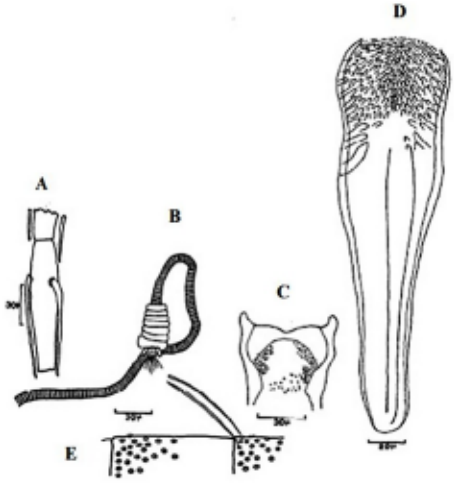


Fig 5. ***Phlebotomus bergeroti*** ♀ [2]. **(A)** Fourth antenna segment. **(B)** Spermathecae. **(C)** Cibarium. **(D)** Pharynx. **(E)** Abdomen with dressed setae.

https://doi.org/10.1371/journal.pntd.0009952.g005

to *Ph. papatasi* where the ascoids are shorter. For the males, 4 major differences were highlighted: (i) the number of setae in the basal lobe coxite is typically 6 for *Ph. bergeroti* and 10 for *Ph. papatasi*; (ii) the length between the apical and median spines of the style is longer in *Ph. papatasi* (Fig 6) compared to *Ph. bergeroti* (Fig 7); (iii) the spines of the style are sharp and spatulated in *Ph. papatasi* and *Ph. bergeroti*, respectively; and (iv) the number of the tuft hairs of the coxite is less than 10 in *Ph. bergeroti* and more than 12 in *Ph. papatasi* [2].

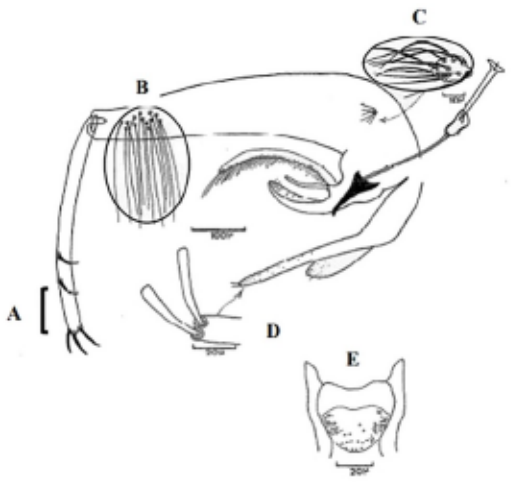


Fig 6. ***Phlebotomus papatasi*** ♂ [2]. **(A)** Spines position. **(B)** Coxite setae. **(C)** Tuft of seta. **(D)** Setae of the lateral lobe. **(E)** Cibarium.

https://doi.org/10.1371/journal.pntd.0009952.g006

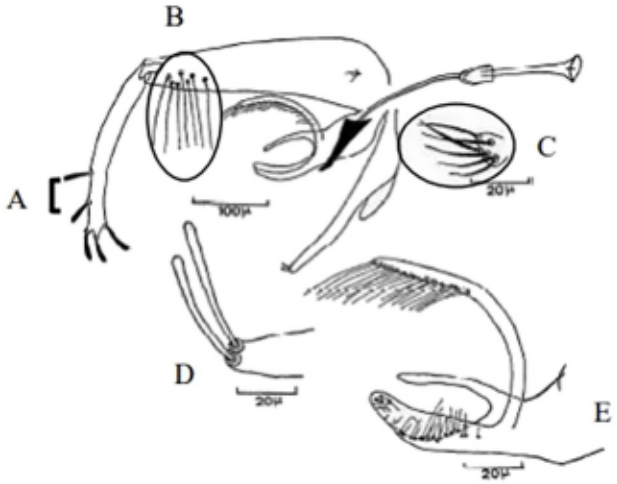


Fig 7. ***Phlebotomus bergeroti*** ♂ [2]. **(A)** Spines position. **(B)** Coxite setae. **(C)** Tuft of seta. **(D)** Setae of the lateral lobe. **(E)** Paramere.

https://doi.org/10.1371/journal.pntd.0009952.g007

I-2 Subgenus *Paraphlebotomus* Theodor, 1948

The pharynx of the females bears large, backwardly directed teeth with smooth margins, which appear as a network. The spermathecae usually have their terminal segment much larger than the other ones. Males have characteristic lobes on the inner surface of their coxite, bearing tufts of long setae, and relatively short style with 4 long spines, 2 near the tip and 2 near the base. Species of this subgenus occur mainly in the Palearctic region, some are proven or suspected to transmit leishmaniases, typically CL due to *Leishmania tropica* [67]. In Maghreb region, 5 species of the subgenus *Paraphlebotomus* were recorded:

I-2-1 *Phlebotomus sergenti* Parrot, 1917

It is the proven vector of various zymodemes of *L. tropica* complex, including *L. killicki*-MON 301 [68–71], and suspected vector of *L. killicki* MON-306, recently reported in the East of Algeria [72]. Furthermore, it was found infected with TOSV in Morocco [73]. This species is collected both inside houses and outdoors, reported in all the Maghreb regions from the north to the central Sahara, occurring in the humid and arid bioclimatic zones (Figs 8–10).

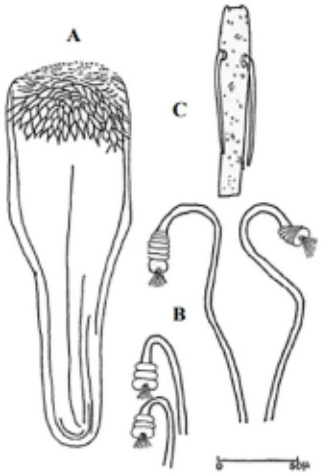


Fig 8. *Phlebotomus sergenti* ♀[2]. (A) Pharynx. **(B)** Spermathecae. **(C)** Fourth antenna segment.

https://doi.org/10.1371/journal.pntd.0009952.g008



Fig 9. *Phlebotomus sergenti* ♂ [57]. General genitalia.

https://doi.org/10.1371/journal.pntd.0009952.g009

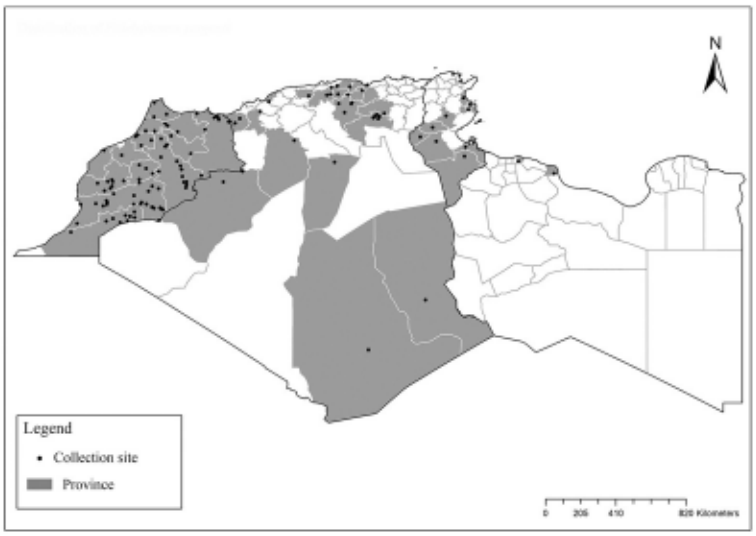


Fig 10. Distribution of *Phlebotomus sergenti*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g010

I-2-2 *Phlebotomus alexandri* Sinton, 1928

Despite it is the proven vector of VL in China [74], no vectorial role in the Maghreb region was so far reported. In Tunisia, Croset and colleagues [54] suspected its role in leishmaniasis cycle transmission due to its abundancy in dry and rocky biotopes (cavities and crevices dug in rocky cliffs) where rodents especially *Ctenodactylus gundi* and reptiles occur abound [33]. It was reported in entire Maghreb region, occurring from subhumid to arid bioclimatic zones (Figs 11–13).

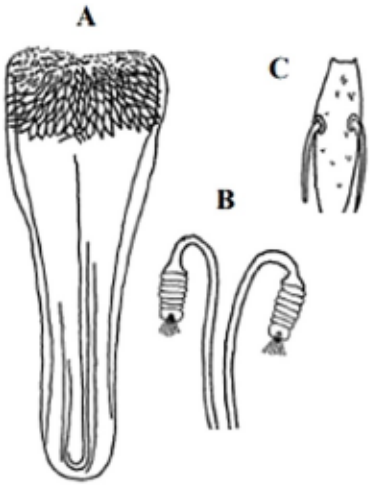


Fig 11. *Phlebotomus alexandri* ♀ [2]. (A) Pharynx. **(B)** Spermathecae. **(C)** Fourth antenna segment.

https://doi.org/10.1371/journal.pntd.0009952.g011

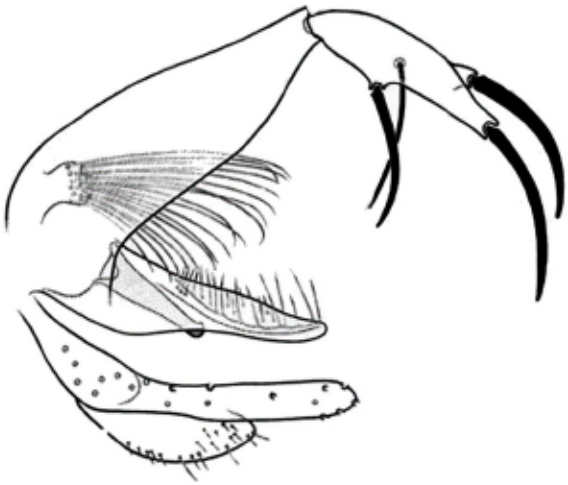


Fig 12. *Phlebotomus alexandri* ♂ [57]. General genitalia.

https://doi.org/10.1371/journal.pntd.0009952.g012

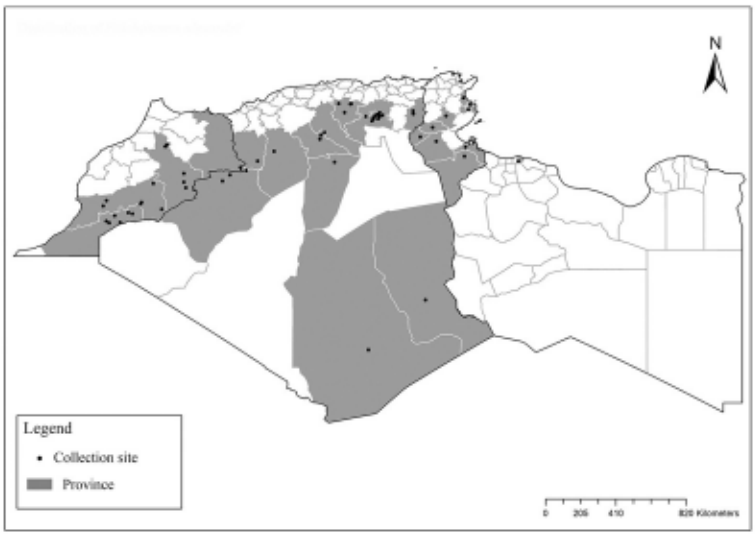


Fig 13. Distribution of *Phlebotomus alexandri*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g013

I-2-3 *Phlebotomus kazeruni* Theodor and Masghali, 1964

This species is suspected to transmit causative agents of CL in Saudi Arabia [75] and was shown to support development of *L. major* under laboratory conditions [76]. It was first reported in Morocco but remains rare in Algeria where until now, few females were reported in the north in Constantine and Jijel, also in the central Sahara in Tamanrasset [26,31,77,78]. It occurs in the subhumid and arid bioclimatic zones (Figs 14–16).

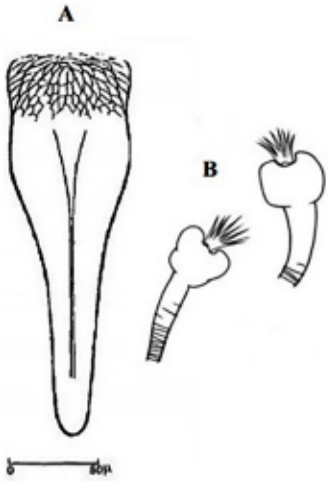


Fig 14. *Phlebotomus kazeruni* ♀ [29,61]. (A) Pharynx. **(B)** Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g014

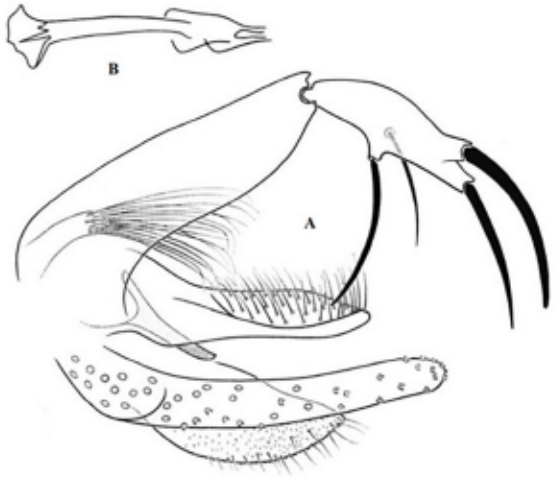


Fig 15. *Phlebotomus kazeruni* ♂ [29].

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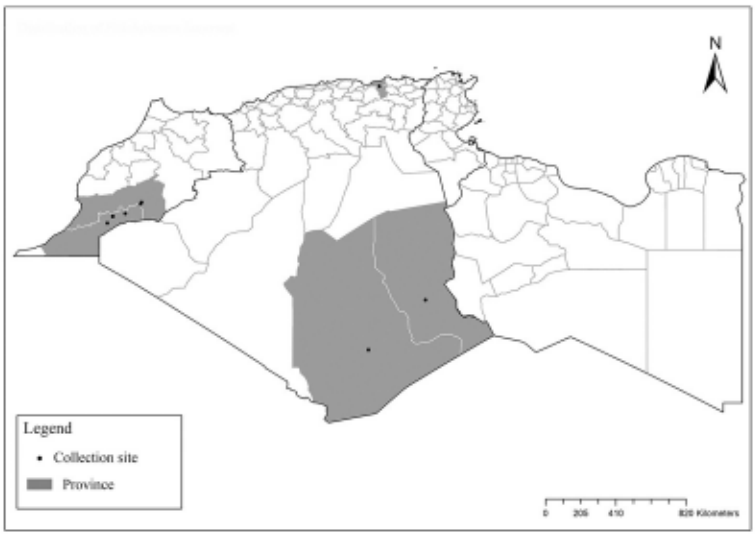


Fig 16. Distribution of *Phlebotomus kazeruni*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g016

I-2-4 *Phlebotomus chabaudi* Croset, Abonnenc and Rioux, 1970

So far not reported as a vector of any pathogen, it was described in 1970 based on a single male collected in Tunisia (Fig 17) [55]. In 1974, the first female (Fig 18) was collected in Algeria [79,80]. It occurs in the subhumid to arid bioclimatic zones (Fig 19).

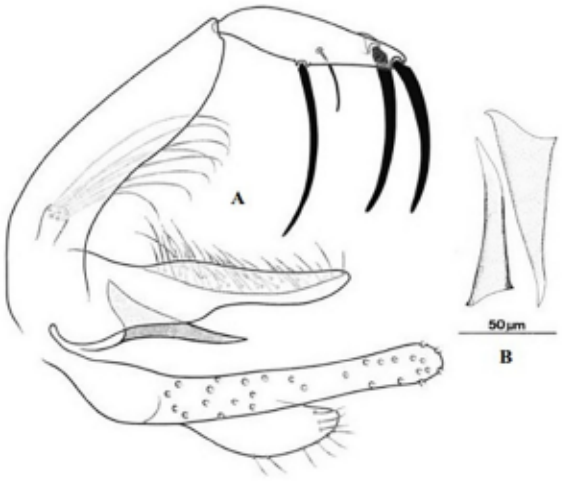


Fig 17. *Phlebotomus chabaudi* ♂ [51,58]. (A) General genitalia. **(B)** Aedeagus shape.

https://doi.org/10.1371/journal.pntd.0009952.g017

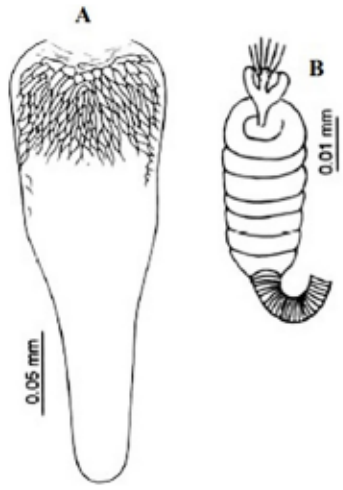


Fig 18. *Phlebotomus chabaudi* ♀ [44]. (A) Pharynx. **(B)** Spermathecae.

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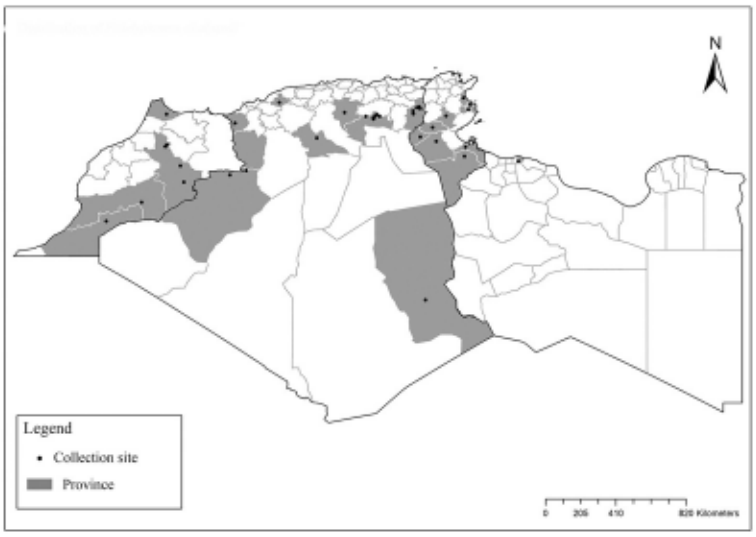


Fig 19. Distribution of *Phlebotomus chabaudi*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g019

I-2-5 *Phlebotomus riouxi* Depaquit, Léger and Killick-Kendrick, 1998

Until now, no role in the transmission of any pathogen has been reported for this species. Its known distribution in the Maghreb region is very limited, reported only in Algeria and Tunisia in the arid and hyper arid bioclimatic zones (Fig 20). It is closely related to *Ph. chabaudi*: Females are morphologically distinguished only by the absence of lateral pharyngeal teeth (Fig 21), which are present in *Ph. chabaudi* [15], and males differ by the higher number of setae at the basal coxite (Fig 22).

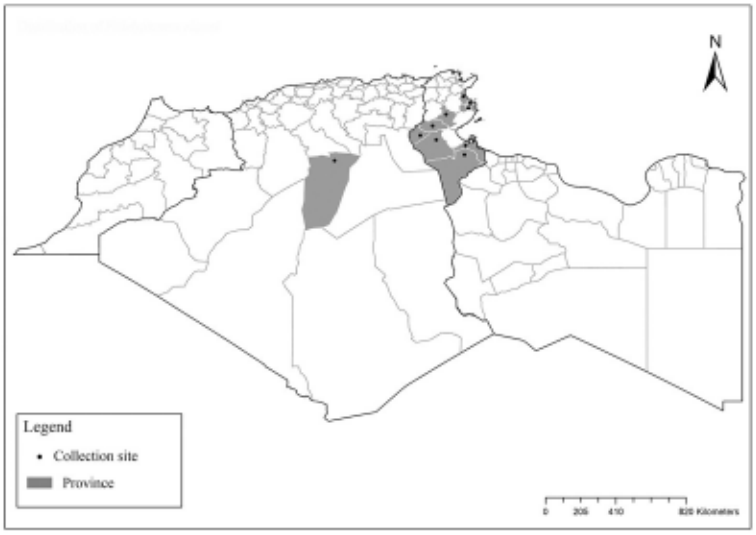


Fig 20. Distribution of *Phlebotomus riouxi*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer

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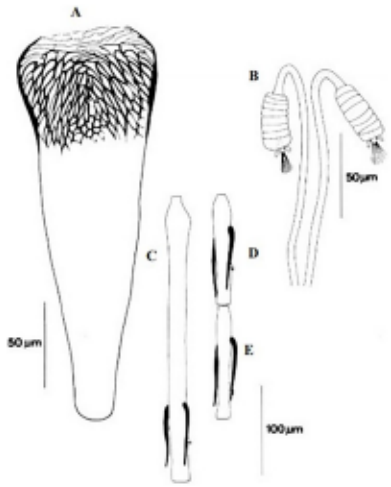


Fig 21. ***Phlebotomus riouxi*** ♀ [51]. **(A)** Pharynx. **(B)** Spermathecae. **(C)** Third antenna segment. **(D)** Fourth antenna segment. **(E)** Fifth antenna segment.

https://doi.org/10.1371/journal.pntd.0009952.g021

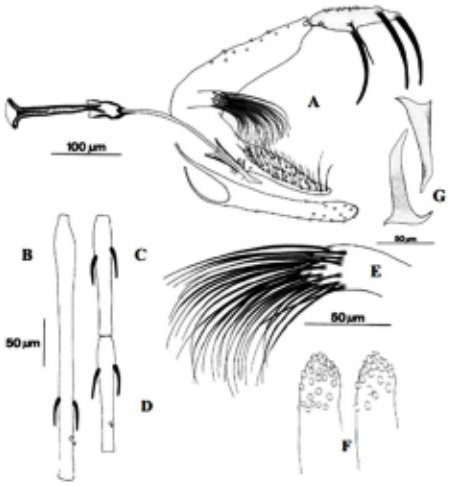


Fig 22. ***Phlebotomus riouxi*** ♂ [51]. **(A)** General genitalia. **(B–D)** Third, fourth, and fifth antenna segments. **(E)** Basal lobe of the coxite. **(F)** Internal face of the basal lobe of the coxite. **(G)** Aedeagus shape.

https://doi.org/10.1371/journal.pntd.0009952.g022

I-3 Subgenus *Larroussius* Nitzulescu 1931

This subgenus includes 7 species present in Maghreb, all either proven or suspected to transmit *Leishmania infantum*. Males of this subgenus have 5 long spines on the style (3 terminal spines and 2 at the distal position), long aedeagus with species-specific tip shape and simple paramere. In females, pharyngeal armature consists of typical punctiform teeth and segmented spermathecae bear finger-like necked head with a basal structure [59].

I-3-1 *Phlebotomus perniciosus* Newstead, 1911

This species is significant in the epidemiology of VL as well as canine leishmaniasis (CanL), being the major vector of *L. infantum* in the western part of the Mediterranean basin [5,21,81,82]. TOSV and Punique virus were isolated from this species in Tunisia [82]. Aedeagus has typically bifurcated at its tip (Fig 23). However, an atypical form of males (for long time confused with *Ph. longicuspis*) was recently reported in Morocco, Algeria, and Tunisia; its aedeagus was not typically bifurcated but rather curved at the tip (Fig 24) [30,83,84]. The females are recognized by a necked spermathecae, each spermiduct has a lateral and relatively thick-walled bulb at the base (Fig 25B). In some females, collected in Morocco and Libya, no bulb structures were observed (Fig 25A), which let to speculations that they may belong to the atypical form [24,41], but this was not confirmed yet. This species occurs mainly from the humid to arid bioclimatic zone but can also be found in the Saharan bioclimatic zones in the south area and central Sahara (Figs 26 and 27), albeit with low density compared to the northern regions [30,85].

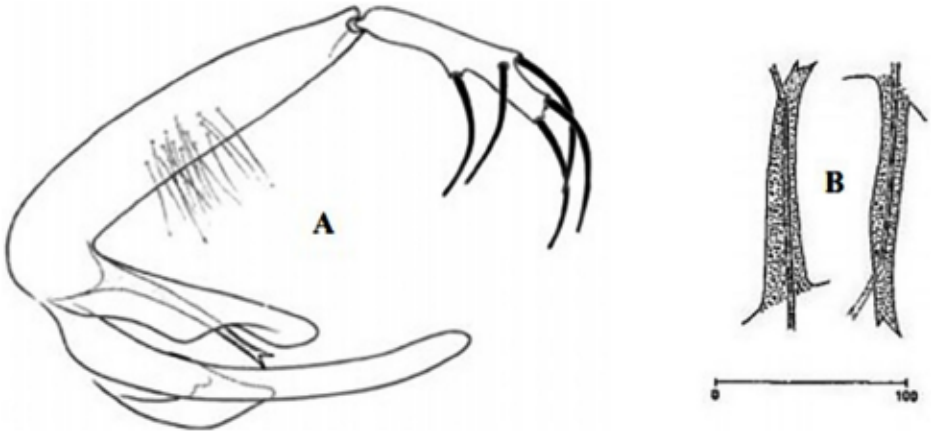


Fig 23. ***Phlebotomus perniciosus*** ♂ [36,57]. **(A)** General genitalia. **(B)** Typical aedeagus.

https://doi.org/10.1371/journal.pntd.0009952.g023



Fig 24. ***Phlebotomus perniciosus*** **atypical form** ♂ [44,57]. **(A')** General genitalia. **(B)** Aedeagus shape.

https://doi.org/10.1371/journal.pntd.0009952.g024

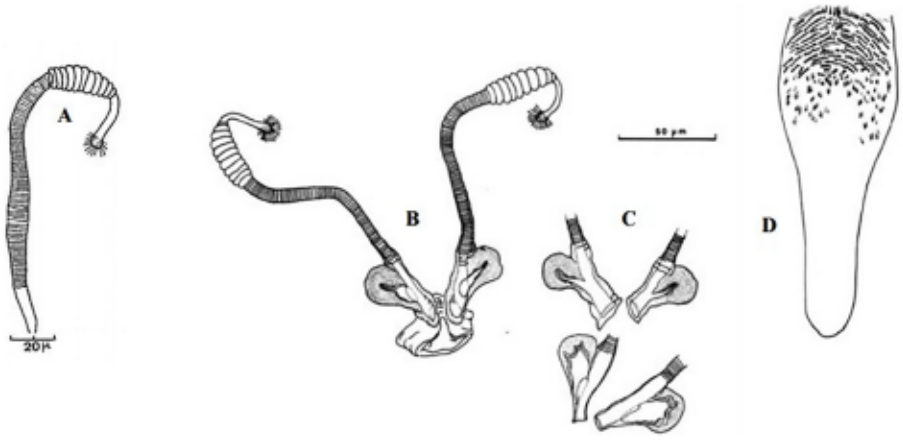


Fig 25. ***Phlebotomus perniciosus*** ♀ [27,52]. **(A)** Atypical spermathecae. **(B)** Typical spermathecae. **(C)** Reservoir at the subterminal part of the duct. **(D)** Pharynx.

https://doi.org/10.1371/journal.pntd.0009952.g025

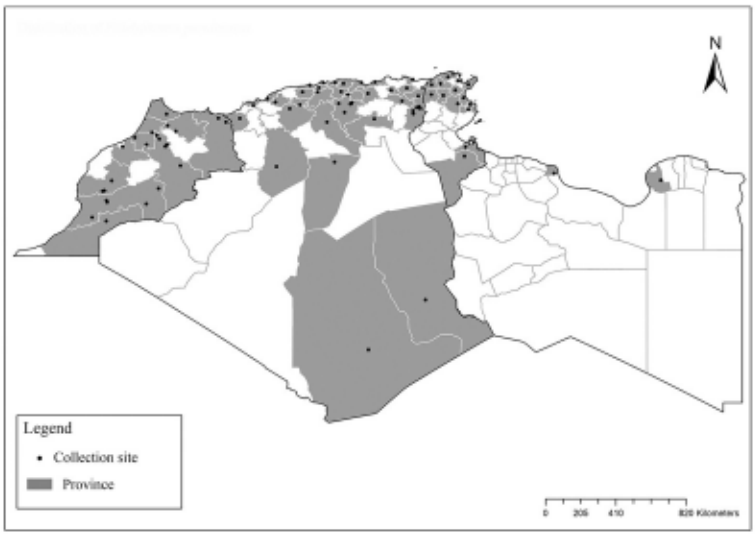


Fig 26. Distribution of *Phlebotomus perniciosus*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g026

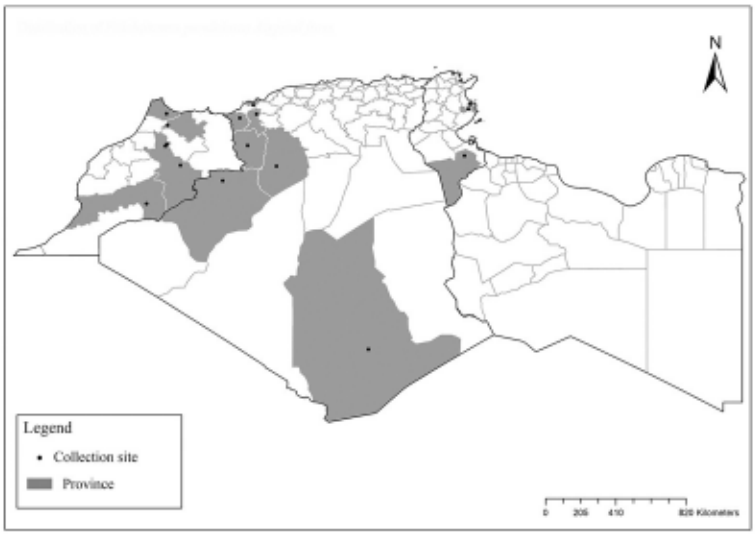


Fig 27. Distribution of typical *Phlebotomus perniciosus*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g027

I-3-2 *Phlebotomus longicuspis* Nitzulescu, 1930

Reported to be naturally infected by unidentified *Leishmania* parasites [33] and recently shown to be positive for *L. infantum* DNA by PCR screening [86], it still is a suspected vector of VL in Algeria, Morocco, and Tunisia [67]. It has been found infected also by Naples virus in Algeria [87], by Punique virus in Tunisia [82], and by TOSV in Morocco [73]. The males are recognized by a long aedeagus pointed at the tip (Fig 28) whereas the females by long-necked spermathecae similar to *Ph. perniciosus* but having relatively thin-walled bulb at the base of spermiducts (Fig 29). This species is generally recorded in sympatry with *Ph. perniciosus* from the subhumid to Sahara bioclimatic zones (Fig 30) [30].

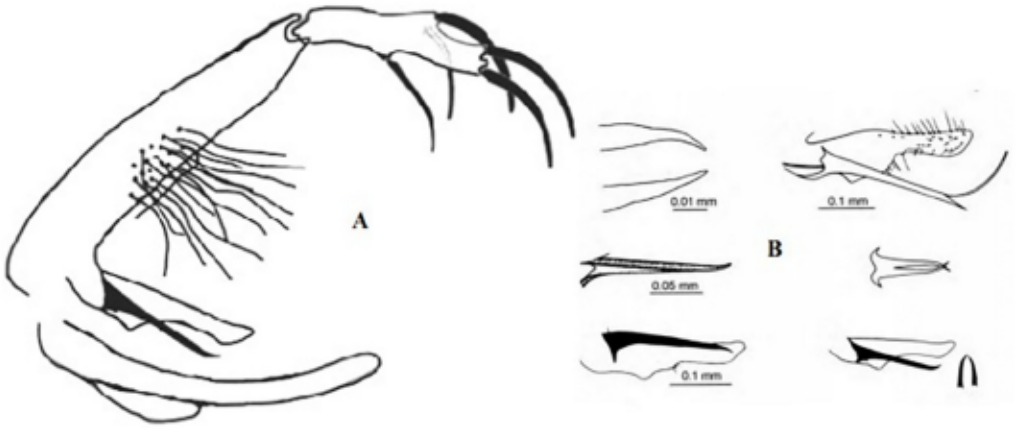


Fig 28. ***Phlebotomus longicuspis*** ♂ [44,57]. **(A)** General genitalia. **(B)** Aedeagus shapes.

https://doi.org/10.1371/journal.pntd.0009952.g028

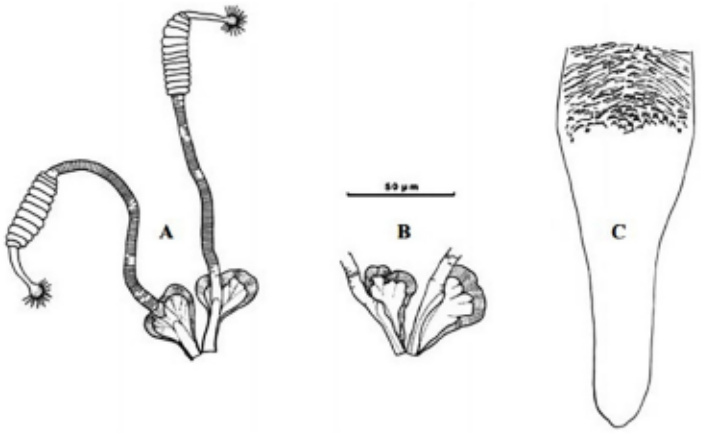


Fig 29. ***Phlebotomus longicuspis*** ♀ [52]. **(A)** Spermathecae. **(B)** Reservoir at the subterminal part of the duct. **(C)** Pharynx.

https://doi.org/10.1371/journal.pntd.0009952.g029

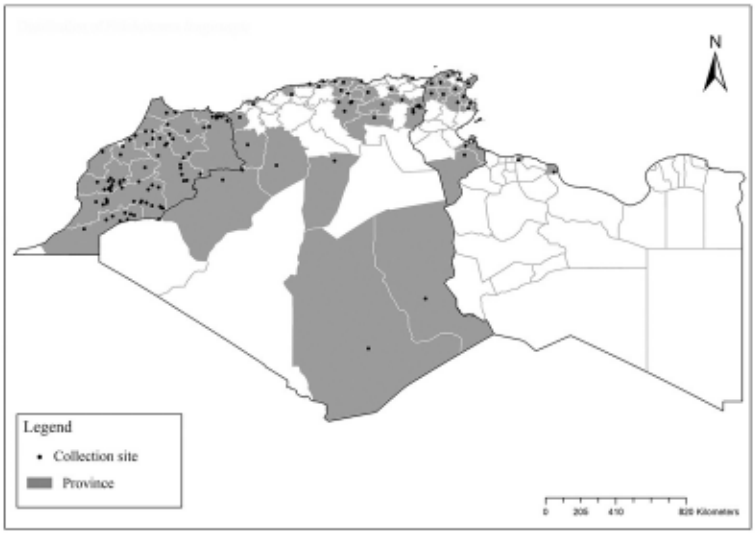


Fig 30. Distribution of ***Phlebotomus longicuspis*****.** Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g030

I-3-3 *Phlebotomus perfiliewi* Parrot, 1930

It is a proven vector of *L. infantum* in Mediterranean basin. In Algeria, this species transmits *L. infantum* Mon-24, which causes sporadic CL [88] and TOSV [89]. Depaquit and colleagues [90] showed that this species forms a complex of species that includes 3 taxa: *Ph. perfiliewi* (Parrot, 1930) *s. s.*, *Ph. galilaeus* (Theodor, 1958), and *Ph. transcaucasicus* (Perfiliev, 1937). Males (Fig 31) of these taxa differ in shape of aedeagus, but females are indistinguishable by morphology, all having large lateral and triangular pocket near the opening of each spermiducts (Fig 32). In the Maghreb region, only *Ph. perfiliewi s. s.* is present, occurring in Morocco, Algeria, and Tunisia [33,36,90,91]. It is found in subhumid, arid, and subarid regions at the latitudes up to 1,200 meters above the sea [33]. Until now, it was not reported in Libya (Fig 33).

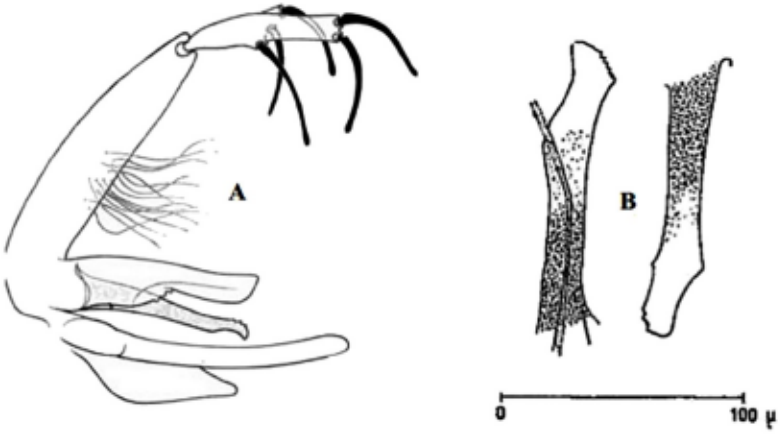


Fig 31. *Phlebotomus perfiliewi* ♂ [36,57]. (A) General genitalia. **(B)** Aedeagus shape.

https://doi.org/10.1371/journal.pntd.0009952.g031

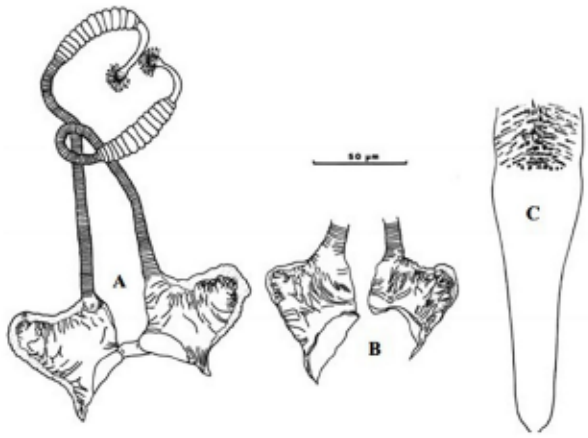


Fig 32. *Phlebotomus perfiliewi* ♀ [52]. (A) Spermathecae. **(B)** Reservoir at the subterminal part of the duct. **(C)** Pharynx.

https://doi.org/10.1371/journal.pntd.0009952.g032

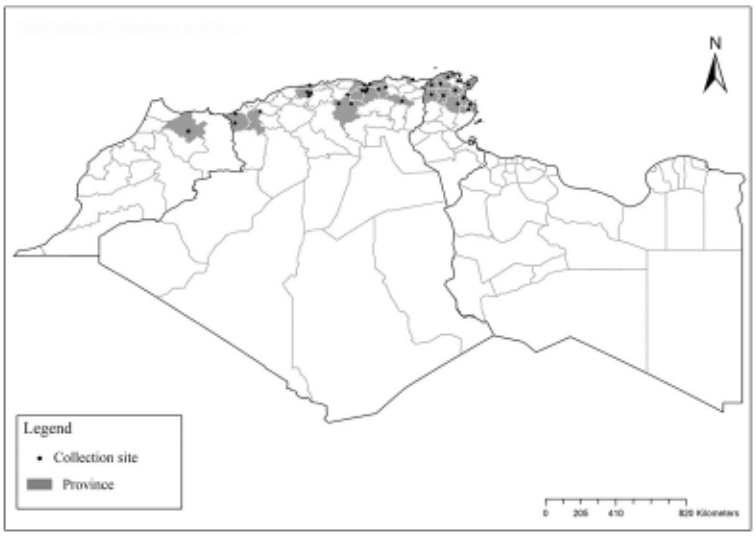


Fig 33. Distribution of *Phlebotomus perfiliewi*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g033

I-3-4 *Phlebotomus ariasi* Tonnoir, 1921

It is considered as a proven vector of *L. infantum* in western part of the European Mediterranean countries, and, rarely, it transmits the located and diffused CL [67]. Until now, a single female was found naturally infected by *L. infantum* in Morocco [92]. A recent study reported its infection by Sicilian virus in Algeria [93]. Aedeagus is relatively thick with rounded tip (Fig 34). In females, distal half of the spermiducts is swollen (Fig 35). It occurs from the humid to arid bioclimatic zones, mainly in the olive, Ilex oaks, and cedar vegetation parts. In Algeria (Great Kabylia), it was reported at 4,400 meters above sea level [27,33,36]. This species has not been reported in Libya yet (Fig 36).

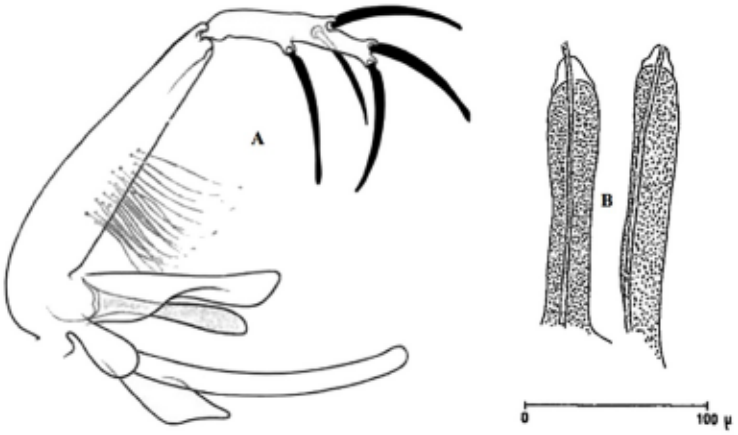


Fig 34. *Phlebotomus ariasi* ♂ [36,57]. (A) General genitalia. **(B)** Aedeagus shape.

https://doi.org/10.1371/journal.pntd.0009952.g034

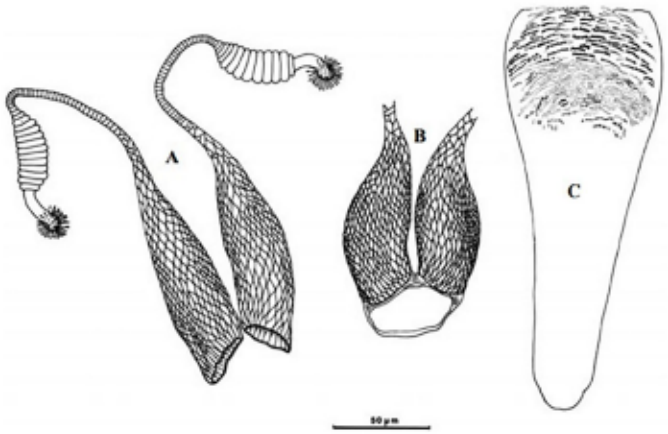


Fig 35. ***Phlebotomus ariasi*** ♀ [52]. **(A)** Spermathecae. **(B)** Reservoir at the subterminal part of the duct. **(C)** Pharynx.

https://doi.org/10.1371/journal.pntd.0009952.g035

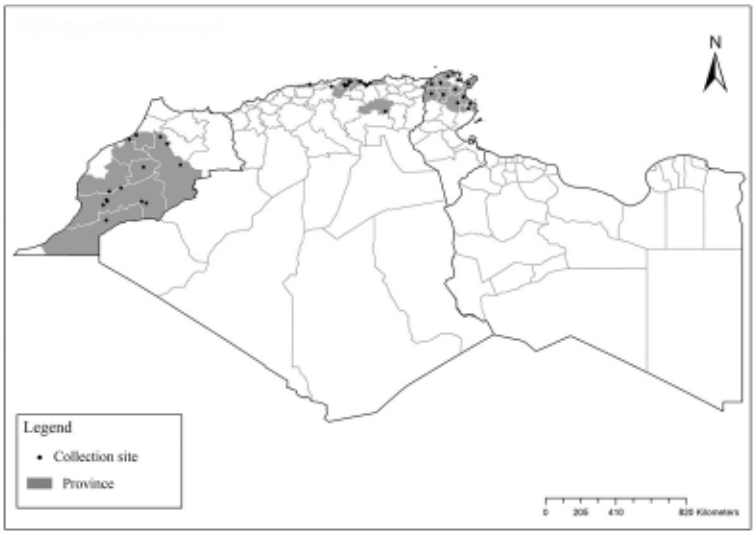


Fig 36. Distribution of ***Phlebotomus ariasi***. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g036

I-3-5 *Phlebotomus chadlii*, Rioux, Juminer et Gibily, 1966

There is no information about its involvement in *Leishmania* transmission cycles and due to its rare occurrence, it is likely not to be of epidemiological significance. Its description is based on a male from southern Tunisia in 1965 [94]; later, it was reported in Algeria in 1970 [80] then in Morocco in 1975 [95]. The first female was described in Tunisia in 2006 [57] and in 2011 in Algeria (Fig 37) [32]. So far, this species has been not recorded in Libya. Morphologically it is close to *Ph. ariasi*, but males have shorter aedeagus and higher number of coxite hairs (Fig 38). It occurs between the humid and hyperarid biodimatic zones (Fig 39).



Fig 37. *Phlebotomus chadlii* ♀ [60]. Spermathecae and the reservoir at the subterminal part of the duct.

https://doi.org/10.1371/journal.pntd.0009952.g037

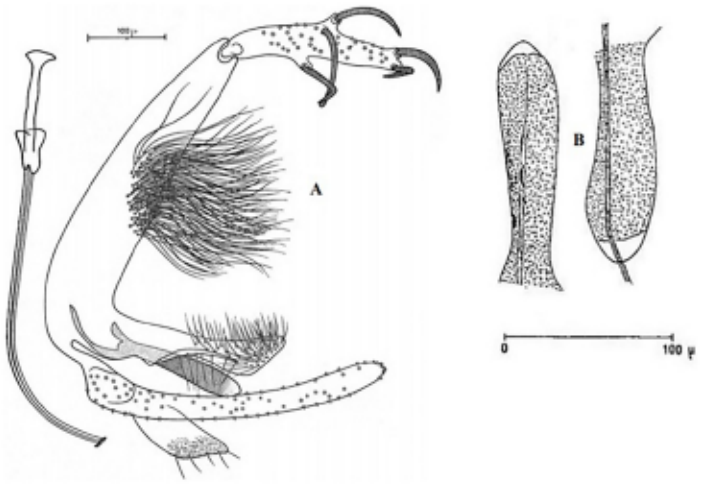


Fig 38. *Phlebotomus chadlii* ♂ [36,49]. **(A)** General genitalia. **(B)** Aedeagus shape.

https://doi.org/10.1371/journal.pntd.0009952.g038

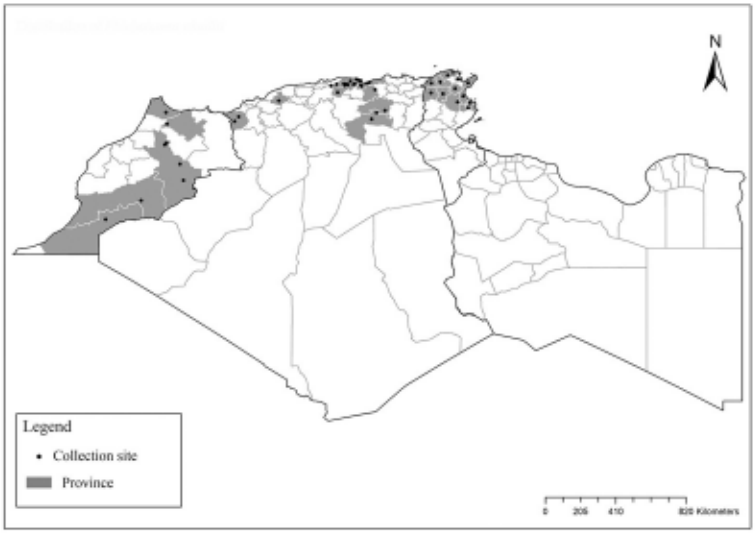


Fig 39. Distribution of *Phlebotomus chadlii*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g039

I-3-6 *Phlebotomus langeroni*, Nitzulescu, 1930

This species was found naturally infected by *L. infantum* in Tunisia [96,97] and shown to transmit *L. infantum* MON-98 under laboratory conditions [98]. The male is recognized by a long and bevel-shaped aedeagus at the tip (Fig 40). Female spermathecae have middle segments larger than the end segments and long slender neck with apical knob (Fig 41). Distal part of spermiducts is swollen but differs in shape from *Ph. ariasi*. Described first as a subspecies of *Ph. perniciosus*, based on males collected in Tunisia, then in 1930, Nitzulescu arose it to the species level [99]. In 2005, the first female specimen was also described in Tunisia [100]. It occurs from humid to arid bioclimatic zones (Fig 42).

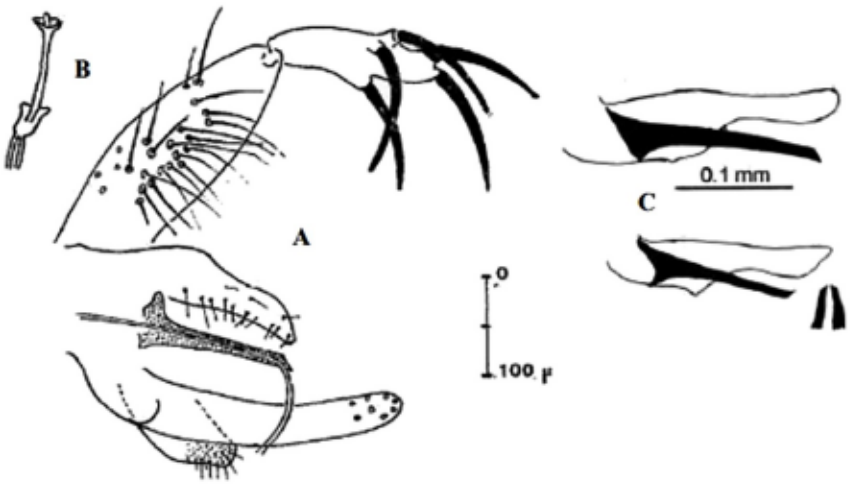


Fig 40. ***Phlebotomus langeroni*** ♂ [36,44]. **(A)** General genitalia. **(B)** Genitalia pump. **(C)** Different reported aedeagus.

https://doi.org/10.1371/journal.pntd.0009952.g040

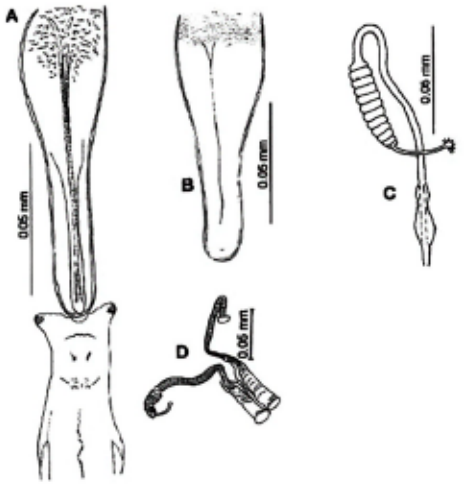


Fig 41. ***Phlebotomus langeroni*** ♀ [44]. **(A, B)** Pharynx. **(C, D)** Spermathecea and the reservoir at the subterminal part of the duct.

https://doi.org/10.1371/journal.pntd.0009952.g041

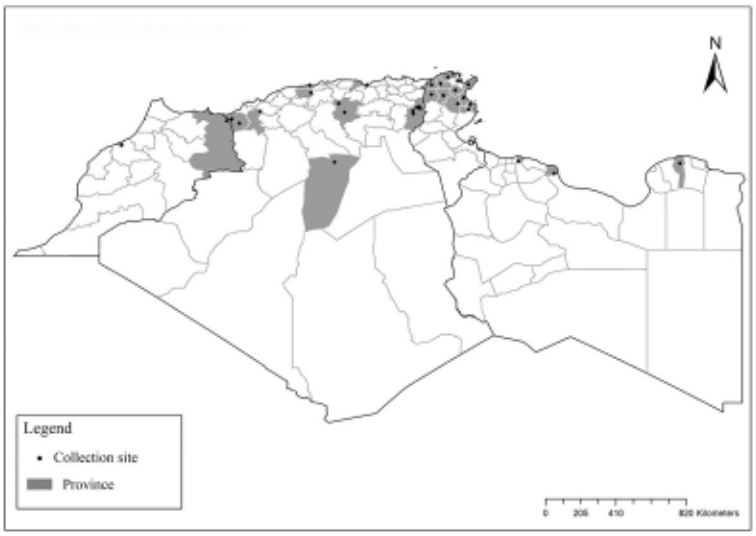


Fig 42. Distribution of *Phlebotomus langeroni*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g042

I-3-7 *Phlebotomus mariae*, Rioux, Croset, Léger et Bailly-Choumara, 1974

One of the most elusive *Larroussius* species, described based only on males and considered to be closely related with *Ph. ariasi* [101]. Aedeagus are long, narrow with subparallel edges and very discreetly swelling at the end tip (Fig 43). So far, it was recorded only in few localities of Morocco from subhumid to subarid bioclimatic zones (Fig 44). Females of this species remain unknown [25,101,102].

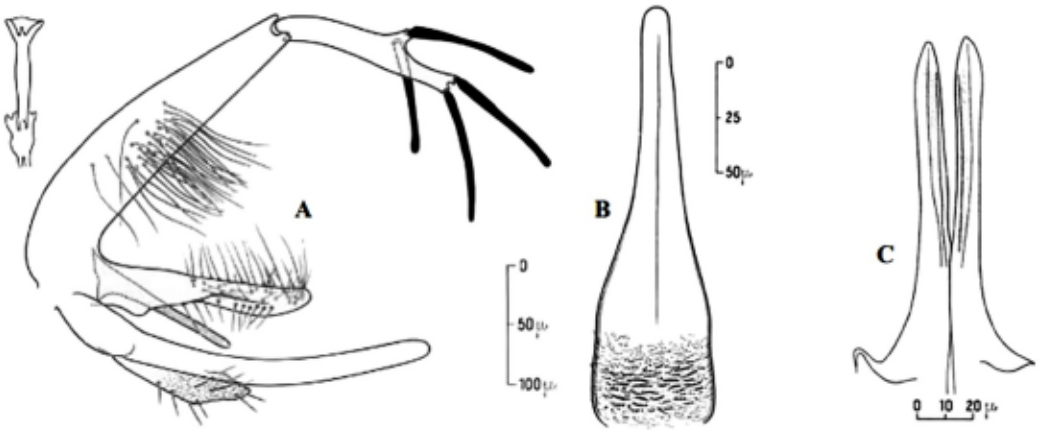


Fig 43. ***Phlebotomus mariae*** ♂ [28]. **(A)** General genitalia. **(B)** Pharynx. **(C)** Aedeagus.

https://doi.org/10.1371/journal.pntd.0009952.g043

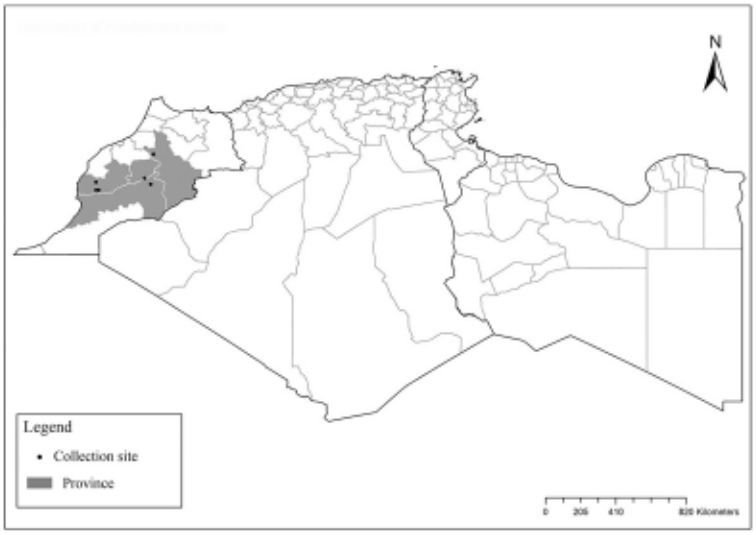


Fig 44. Distribution of *Phlebotomus mariae*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g044

I-4 Subgenus *Transphlebotomus* Artemiev 1984

The subgenus *Transphlebotomus* was established by Artemiev in 1984 based on the male genital morphology and female characters for spermathecae and pharyngeal armature [103]. The species belonging to this genus are defined by individual spermathecal ducts being broad, joining at genital opening; spermathecae are simple striated tubes, not clearly demarcated into segments, with a small head and pharyngeal teeth point forward [59]. Of 5 species so far described in the Mediterranean region, some of these notably recently [104,105], only 1 has been recorded in Maghreb region. Due to minor morphological differences between species, morphological identifications of *Transphlebotomus* should be confirmed by molecular methods.

I-4-1 *Phlebotomus mascittii*, Grassi, 1908

Suspected but so far not proven vector of *L. infantum* due to circumstantial findings of parasite DNA by PCR screening [106]. This species (Figs 45 and 46) exhibits a wide distribution in Europe that extends the usual Mediterranean regions to the north up to Austria, Germany, and Slovakia [107–109]. In the Maghreb region, its presence was recorded by a sole finding of 1 female in Tizi Ouzou, Algeria (Fig 47) in the humid bioclimatic zone [32].

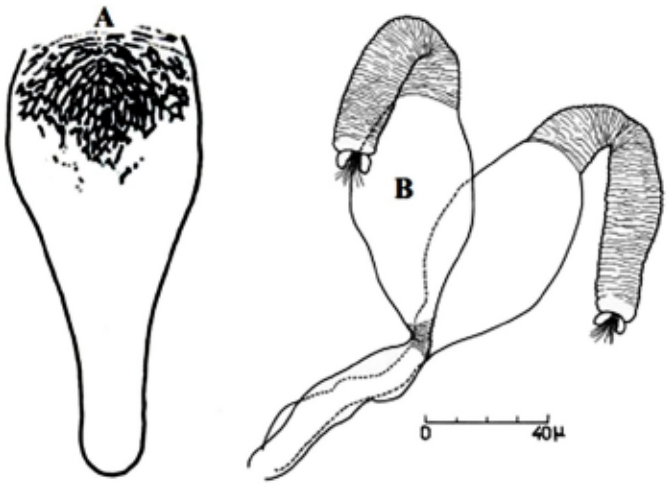


Fig 45. *Phlebotomus mascitti* ♀ [53]. A) Pharynx, B) Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g045

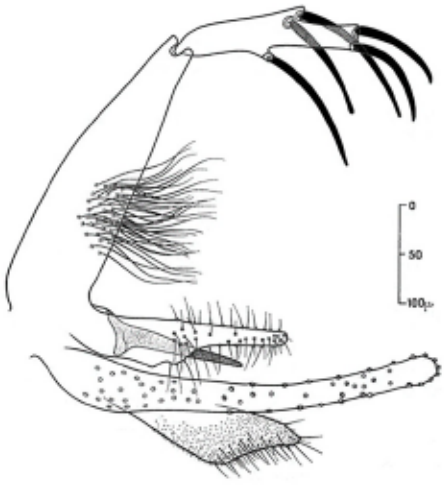


Fig 46. *Phlebotomus mascitti* ♂ [50]. General genitalia.

https://doi.org/10.1371/journal.pntd.0009952.g046

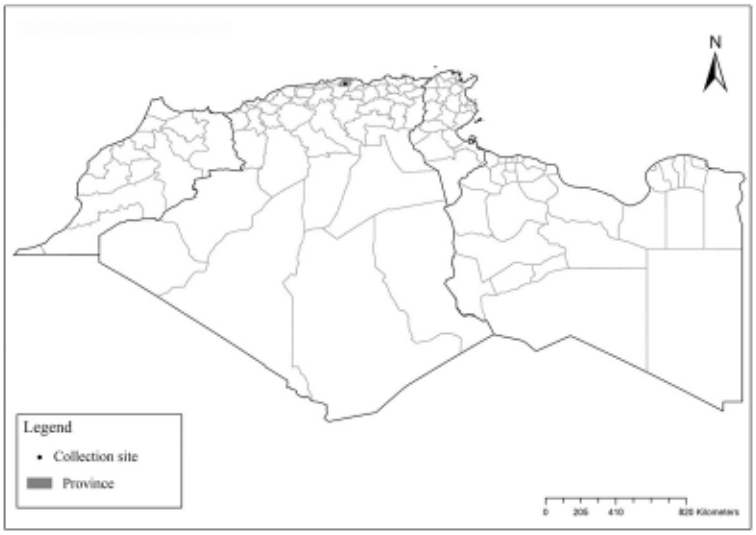


Fig 47. Distribution of *Phlebotomus mascittii*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g047

II- Genus *Sergentomyia* França and Parrot 1920

In Maghreb region, the genus *Sergentomyia* is represented by species of 4 subgenera described below (*Sergentomyia*, *Parrotomyia*, *Grassomyia*, and *Sintonius*), and the fifth subgenus *Parvidens* is present only in Mauritania. They are all defined by (i) cibarial teeth and/or denticles (more developed in females than in males and arranged in a transverse row); (ii) a pigmented patch on the dorsal wall of cibarium; and (iii) the hind end of abdomen tergites 2 to 6 bear all or most setae recumbent arising from small sockets as compared with large round sockets on first tergite. Males possess (i) a single paramere; and (ii) style with 4 terminal spines (sometime arranged as 2 terminal and 2 subterminal) and a short nondeciduous seta (accessory spine). Females spermathecae are smooth or segmented, either tubular or capsuliform [2,110]. The main differences between the species are summarized in **Tables D and E in** S1 Text for males and females, respectively.

Unlike genus *Phlebotomus*, none of the *Sergentomyia* species was conclusively proven to be a vector of *Leishmania* parasites that infect humans. However, for a number of species, including some that are also recorded in the Maghreb region, circumstantial evidence based on microscopic observations and/or PCR detection of parasite's DNA suggest that they may be also play a role in *Leishmania* transmission. Such information is therefore mentioned for respective species listed below.

II-1 Subgenus *Sergentomyia*

Females of this subgenus have tubular spermathecae with smooth walls of uniform width along their length, antennal segment 3 is shorter than segments 4 and 5 together and usually shorter than labrum. Males have a stout finger-like aedeagus with blunt tip [59]. So far, 5 species were reported in the Maghreb region.

II-1-1 *Sergentomyia minuta parroti*, Adler et Theodor, 1927

Sergentomyia minuta parroti was reported to transmit *L. tarentolae* that infects geckoes [111,112]. Females are recognized by presence of net pigmented patch upon the straight row of cibarial teeth (Fig 48). In males, accessory spine is located in the middle of the style (Fig 49).

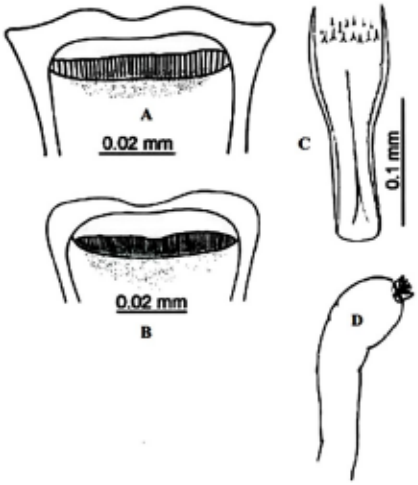


Fig 48. *Sergentomyia minuta* ♀ [44]. A) Cibarium of *Se. minuta minuta*, B) Cibarium of *Se. minuta parroti*, C) Pharynx, D) Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g048

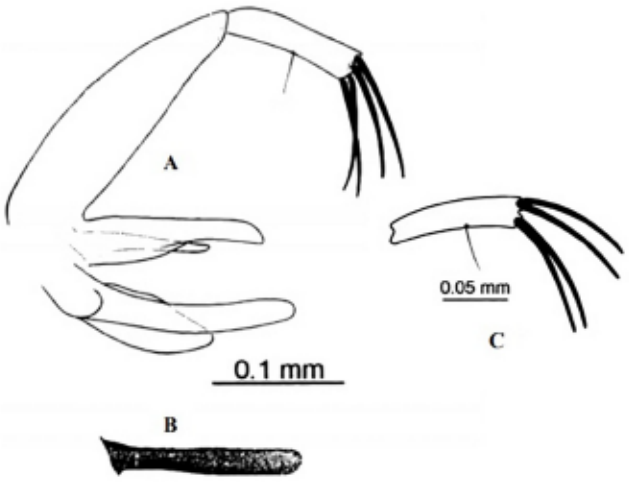


Fig 49. *Sergentomyia minuta* ♂ [44]. A) General genitalia, B) Aedeagus, C) Insertion position of nondeciduous seta.

https://doi.org/10.1371/journal.pntd.0009952.g049

It is an herpetophilic species found in the whole Maghreb region (Fig 50) from the humid to arid bioclimatic zones [2]. Two subspecies were described, *Se. minuta minuta* (Fig 48A) and *Se. minuta parroti* (Fig 48B), which differ in the number of cibarial teeth: around 40 for *Se. minuta minuta* and over 70 for *Se. minuta parroti* [111].

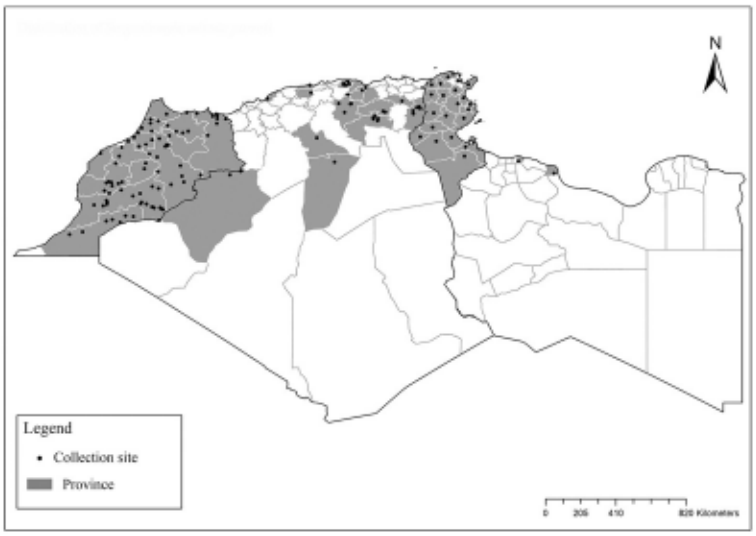


Fig 50. Distribution of *Sergentomyia minuta*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g050

II- 1–2 *Sergentomyia fallax*, Parrot, 1921

Females are recognized by strongly distended posterior heart-shaped part of pharynx and the presence of 16 to 18 sharped cibarial teeth aligned on concave shape and oval or rounded pigmented patch (Fig 51). Male style is 4 times longer than wide, with accessory spine in close to distal end (Fig 52) [33]. It occurs in the whole Maghreb region in all bioclimatic zones (Fig 53).

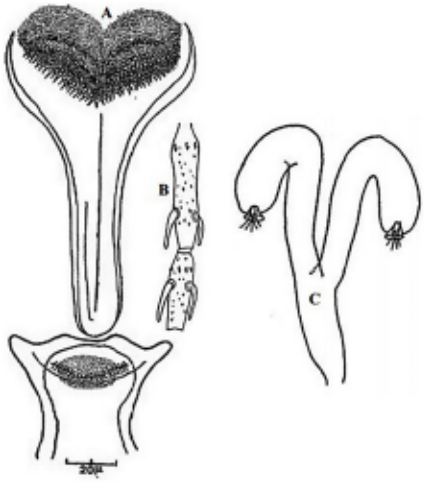


Fig 51. *Sergentomyia fallax* ♀ [2]. (A) Pharynx and Cibarium. **(B)** Third and fourth antenna segments. **(C)** Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g051

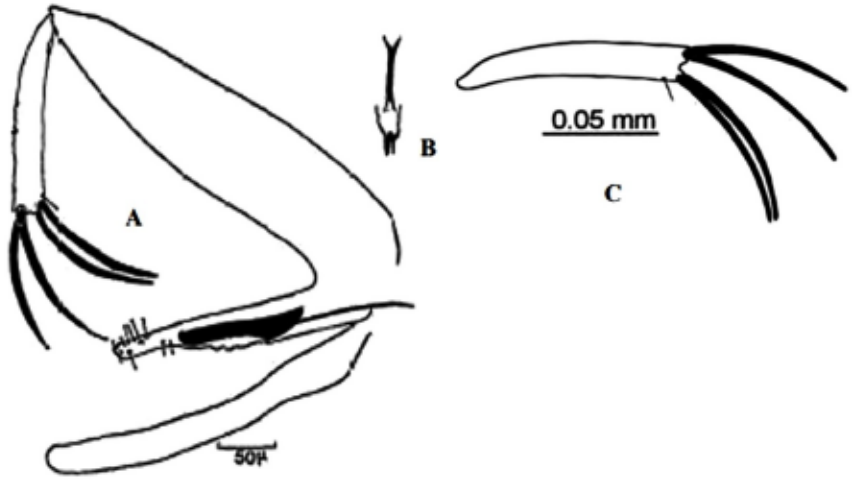


Fig 52. *Sergentomyia fallax* ♂ [44]. (A) General genitalia. **(B)** Genitalia pump. **(C)** Insertion of accessory spine.

https://doi.org/10.1371/journal.pntd.0009952.g052

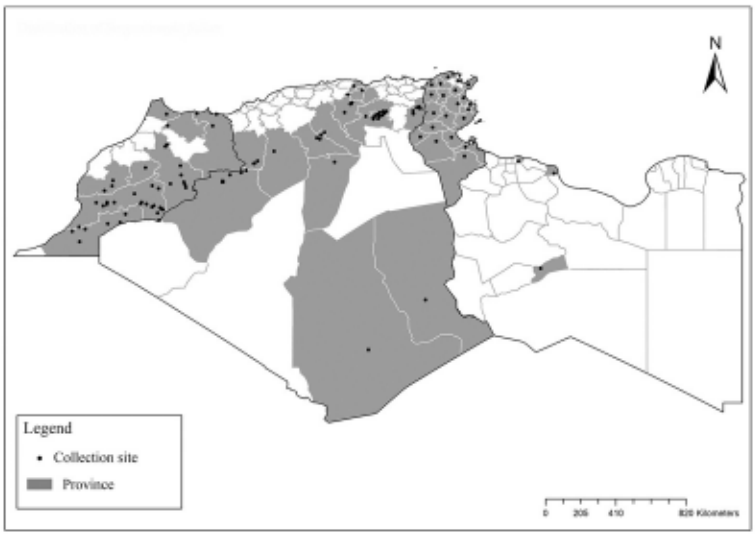


Fig 53. Distribution of *Sergentomyia fallax*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g053

II-1-3 *Sergentomyia antennata*, Newstead, 1912

This species was demonstrated to be also mammaliophilic, feeding on rodents as well as reptiles [33]. Male genitalia are similar to those of *Se. minuta*, but the accessory spine is located near the 4 spines (Fig 54). Female possesses 23 to 34 sharped cibarial teeth aligned on a concave shape and triangular pigmented patch (Fig 55). The pharynx has a distended posterior

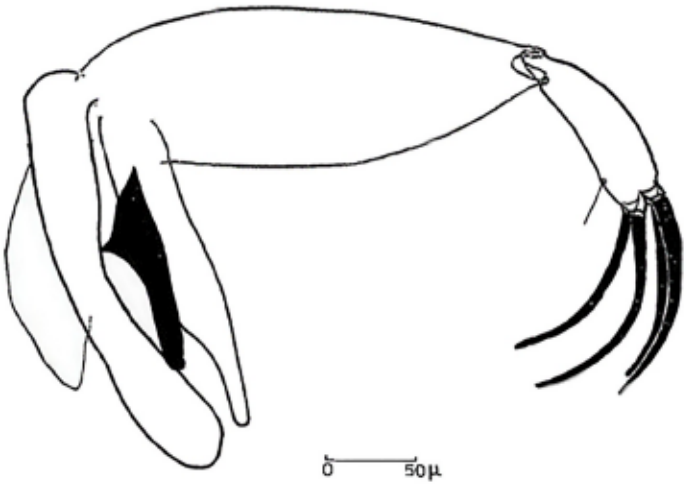


Fig 54. *Sergentomyia antennata* ♂ [44]. General genitalia.

https://doi.org/10.1371/journal.pntd.0009952.g054

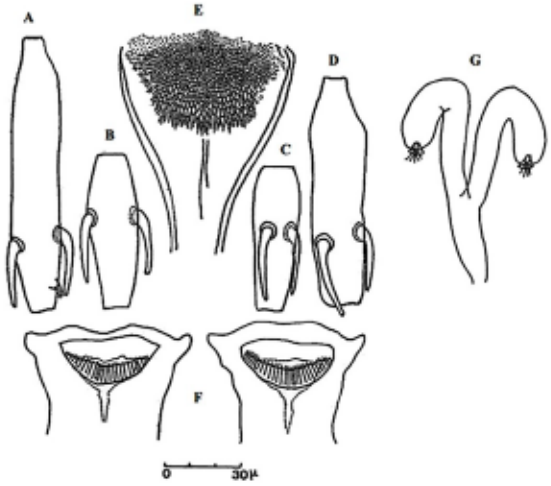


Fig 55. *Sergentomyia antennata*♀ [2]. **(A, B)** Third and fourth antenna segments. **(C, D)** Third and fourth antenna segments. **(E)** Pharynx. **(F)** 2 different aspect of the cibarium. **(G)** Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g055

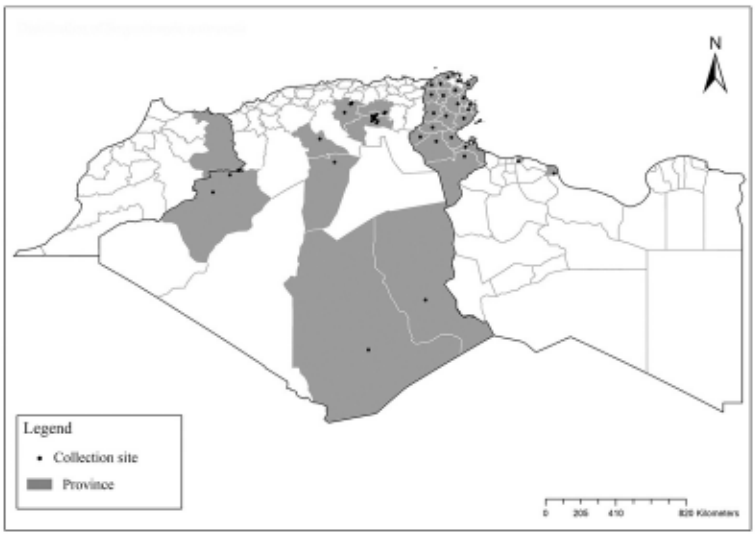


Fig 56. Distribution of *Sergentomyia antennata*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

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part heart-shaped less marked than *Se. fallax*. *Sergentomyia antennata* also occurs in the whole Maghreb region in all bioclimatic zones (Fig 56).

II-1-4 *Sergentomyia cincta* Parrot and Martin 1948

Its morphology is similar to *Se. antennata*. Males are difficult to distinguish but females have only 12 to 18 cibarial teeth (Fig 57) [2]. It is found in the hyperarid bioclimatic zone and reported only in Iherir an Oasis of Algeria (Fig 58). Lewis and Büttiker considered it as synonymous of *Se. antennata* [33].

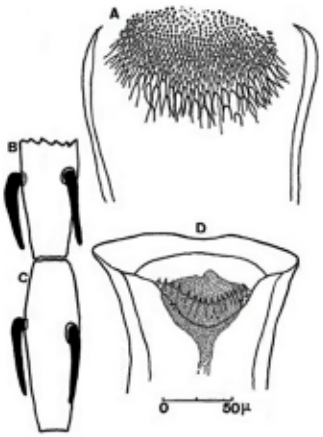


Fig 57. *Sergentomyia cincta*♀ [2]. (A) Pharynx. **(B, C)** Third and fourth antenna segments. **(D)** cibarium.

https://doi.org/10.1371/journal.pntd.0009952.g057

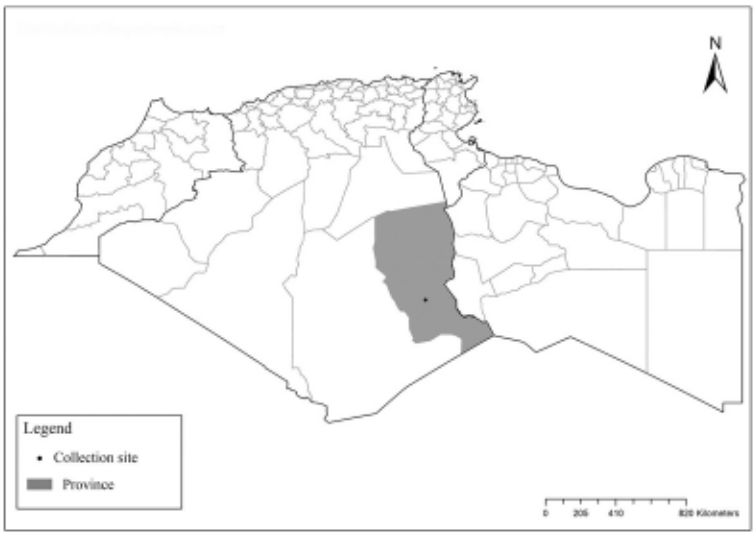


Fig 58. Distribution of *Sergentomyia cincta*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g058

II-1-5 *Sergentomyia schwetzi*, Adler, Theodor et Parrot, 1929

Typical species belongs to the Ethiopian region. Females possess 13 to 20 strong and sharped cibarial teeth aligned on concave shape with pigment patch large in middle (Fig 59). The pharynx has a narrow and strong-armed posterior part (lamp glass shaped). In males, the style holds 4 distal spines arranged in 2 well-separated groups and accessory spine on the distal 1/3. The short and sharped cibarial teeth are arranged in 2 rows, 15 to 20 and 10 to 15, respectively (Fig 60). It was reported only in Algeria and Libya [31,113,114] from subarid to hyperarid bioclimatic zones (Fig 61).

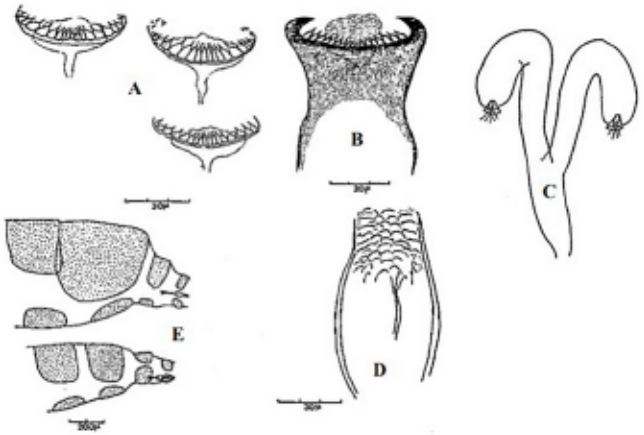


Fig 59. *Sergentomyia schwetzi* ♀ [2]. (A) Different cibarium shapes. **(B)** Pharynx. **(C)** Spermathecae. **(D)** Atypical form of the abdomen. **(E)** Typical form of the abdomen.

https://doi.org/10.1371/journal.pntd.0009952.g059

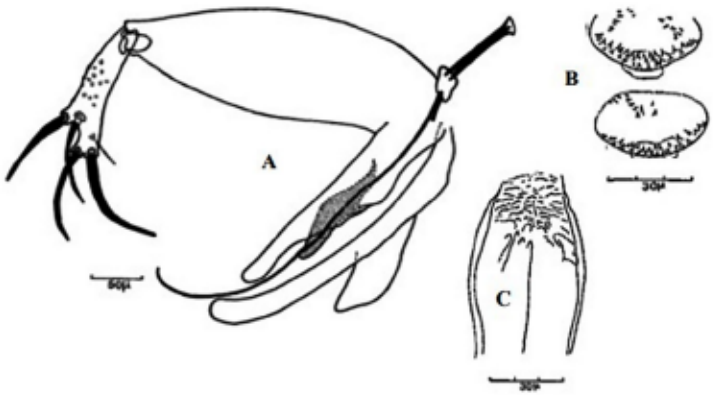


Fig 60. *Sergentomyia schwetzi* ♂ [2,44]. (A) General genitalia. **(B)** Different cibarial forms. **(C)** Pharynx.

https://doi.org/10.1371/journal.pntd.0009952.g060

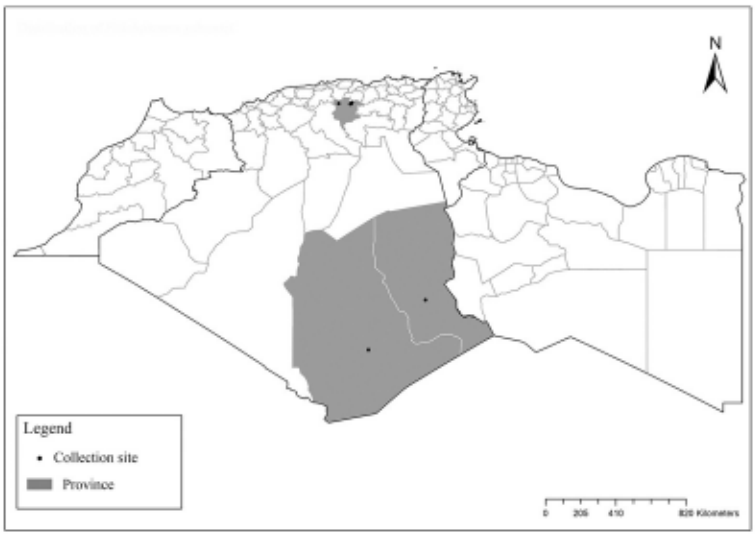


Fig 61. Distribution of *Sergentomyia schwetzi*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g061

II-2 Subgenus *Parrotomyia*

Females have cibarium with comb-like row of strong parallel teeth and pharynx narrowing posteriorly (lamp glass shape). The spermathecae have a form of smooth spherical or ellipsoid capsule. Aedeagus is long, slender, triangular, and narrowing gradually to a sharp point, and paramere is hooked [59]. Three species were reported in the Maghreb region.

II-2-1 *Sergentomyia africana* subsp. *asiatica*, Newstead, 1912

This species, also known as *Se. africana* var. *cherifiensis*, is distributed in the Ethiopian region, and only 1 female has been reported from Morocco (Fig 62) in the hyperarid bioclimatic zone

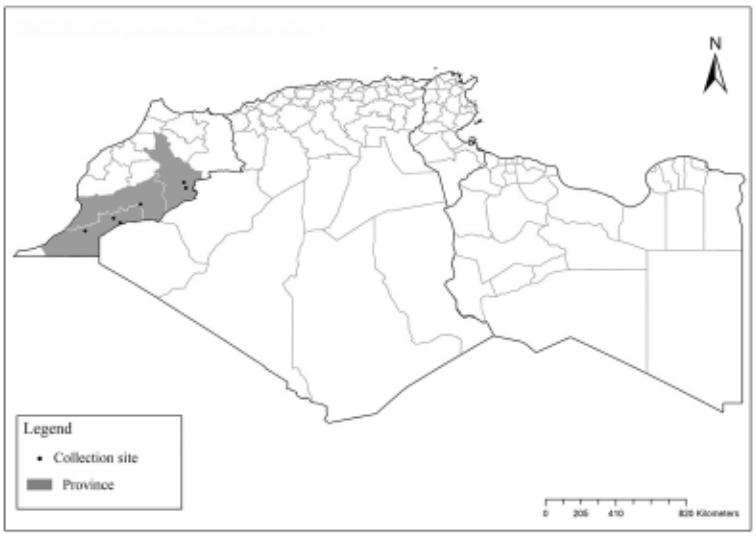


Fig 62. Distribution of *Sergentomyia africana* subsp. *asiatica*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g062

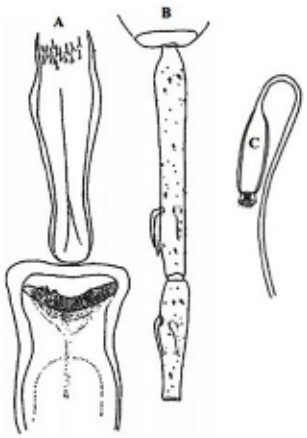


Fig 63. ***Sergentomyia africana*** **subsp** ***asiatica*** ♀ [2]. **(A)** Pharynx and cibarium. **(B)** Third and fourth antenna segments. **(C)** Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g063

[24,115]. The spermathecae have an elongated capsule shape; females have 40 to 48 cibarial teeth aligned in a palisade line, 15 punctiform denticules and headband pigmented patch, and their pharynx is long and thread- ike (Fig 63).

II-2-2 *Sergentomyia africana* subsp. *eremitis*, Parrot et De Jolinière, 1945

Another species of the Ethiopian region [2]. Until now, it was reported only in Algeria (Fig 64) in the hyperarid bioclimatic zone [52]. Male has 30 to 35 cibarial teeth and 8 to 10 denticules disposed on a concave-shape line (Fig 65). Female spermathecae have an elongated capsule shape, 60 to 65 long cibarial teeth aligned on concave-shaped line and 12 punctiform denticules with a mushroom-shaped pigmented patch (Fig 66) [52].

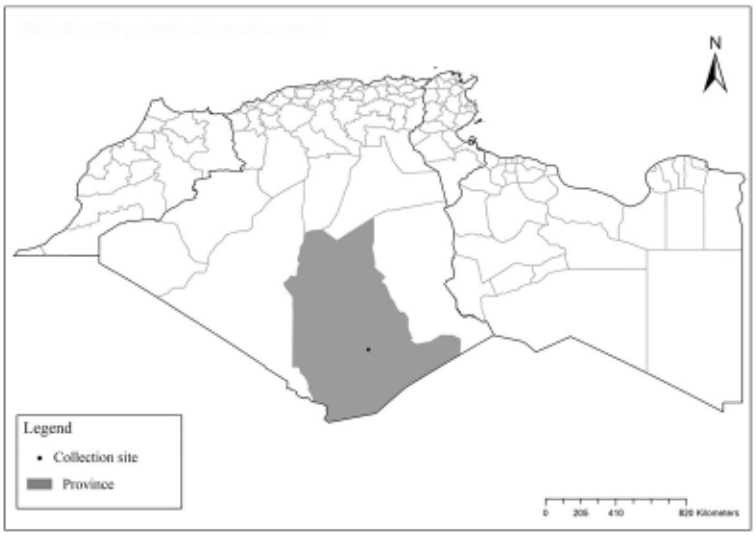


Fig 64. Distribution of ***Sergentomyia africana*** **subsp** ***eremitis***. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

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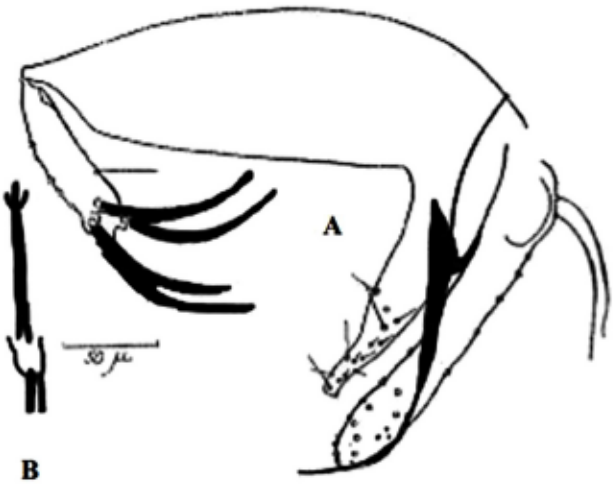


Fig 65. ***Sergentomyia africana*** **subsp** ***eremitis*** ♂ [54]. **(A)** General genitalia. **(B)** Genital pump.

https://doi.org/10.1371/journal.pntd.0009952.g065

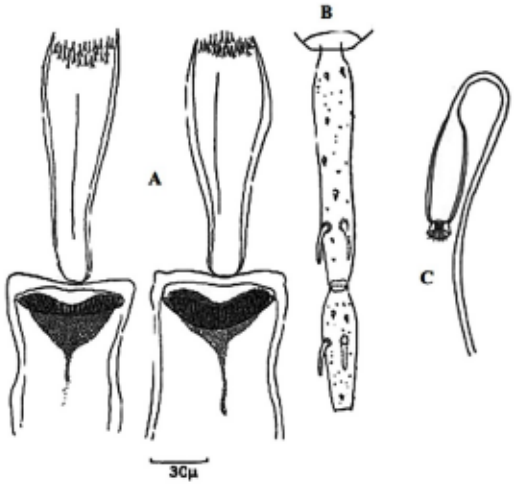


Fig 66. ***Sergentomyia africana*** **subsp** ***eremitis*** ♀ [2]. **(A)** Pharynx and cibarium. **(B)** Third and fourth antenna segments. **(C)** Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g066

II-2-3 *Sergentomyia lewisi* Parrot, 1948

This species has been reported in Morocco, Algeria, and, recently, in Tunisia [24,38,53] from subarid to hyper arid bioclimatic zones (Fig 67). The females are recognized by the spermathecae with a poppy capsule shape, 15 to 20 strong and sharped cibarial teeth aligned on concave line, 16 to 20 denticules, and mushroom-shaped pigmented patch (Fig 68). Male has 8 to 12 cibarial teeth disposed on a posterior concave line and 8 to 14 denticules (Fig 69) [2].

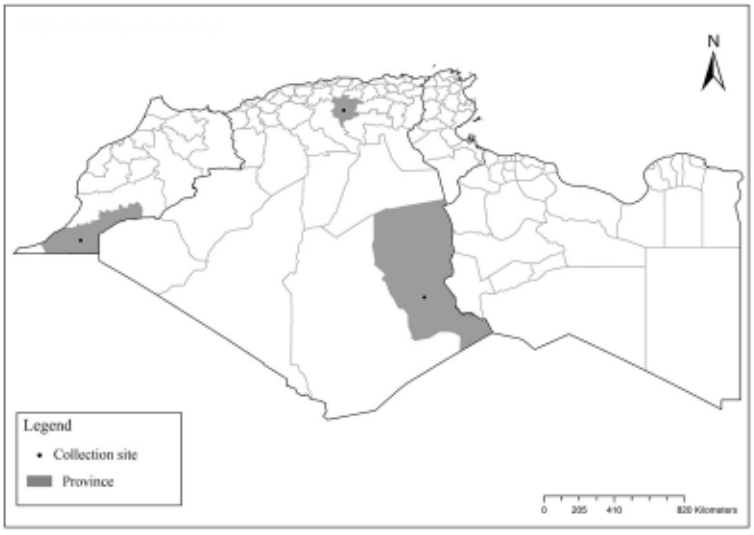


Fig 67. Distribution of ***Sergentomyia lewisi.*** Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer

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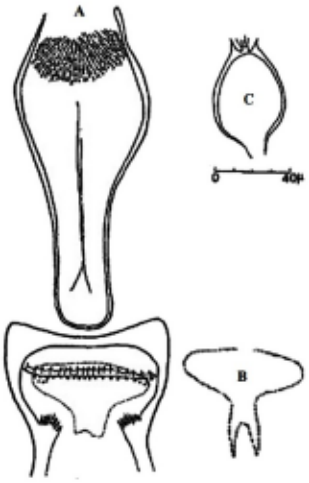


Fig 68. *Sergentomyia lewisi* ♀ [2]. (A) Pharynx and Cibarium. **(B)** Pigmentation of the patch. **(C)** Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g068

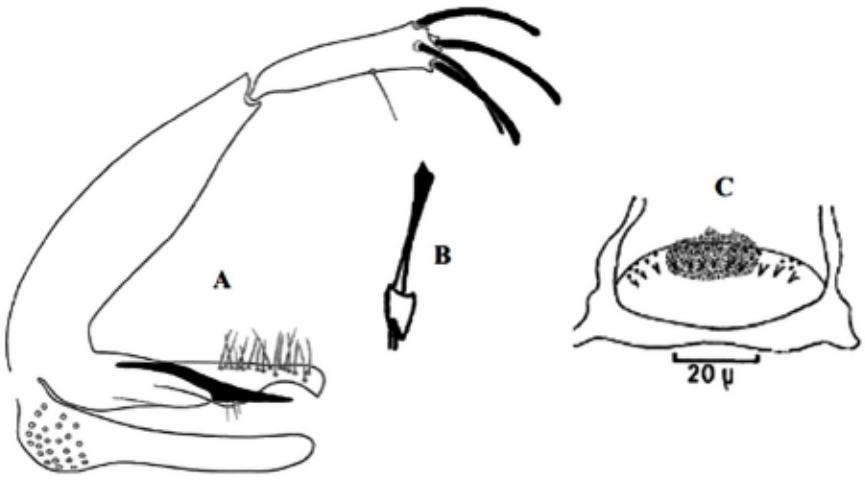


Fig 69. *Sergentomyia lewisi* ♂ [2,57]. (A) General genitalia. **(B)** Genitalia pump without pavilion. **(C)** Cibarium.

https://doi.org/10.1371/journal.pntd.0009952.g069

II-3 Subgenus *Grassomyia*

Females of this subgenus have spermathecae in form of round, sclerotized capsules with small spicules and their antenna segment 3 lacks ascoids. Females possess cibarial teeth in distinct convex row. Males have simple parameres (not bifid). Species of this subgenus are often associated with vegetation close to water sources and feed on reptiles and amphibians [59,116]. Only a single species is reported in the Maghreb region.

II-3-1 *Sergentomyia dreyfussi* Parrot, 1933

This species is reported in the whole Maghreb region (Fig 70) from arid to hyperarid bioclimatic zones [37,41,51]. Males have a style with 4 strong terminal spines, accessory spine

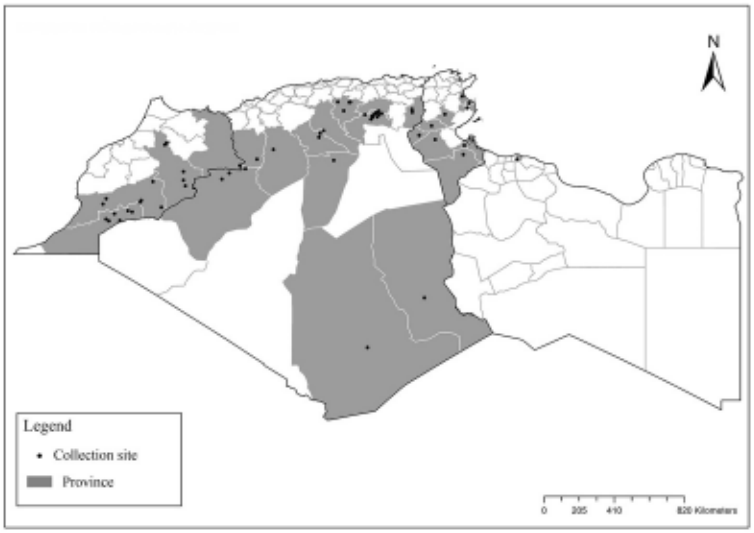


Fig 70. Distribution of *Sergentomyia dreyfussi*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

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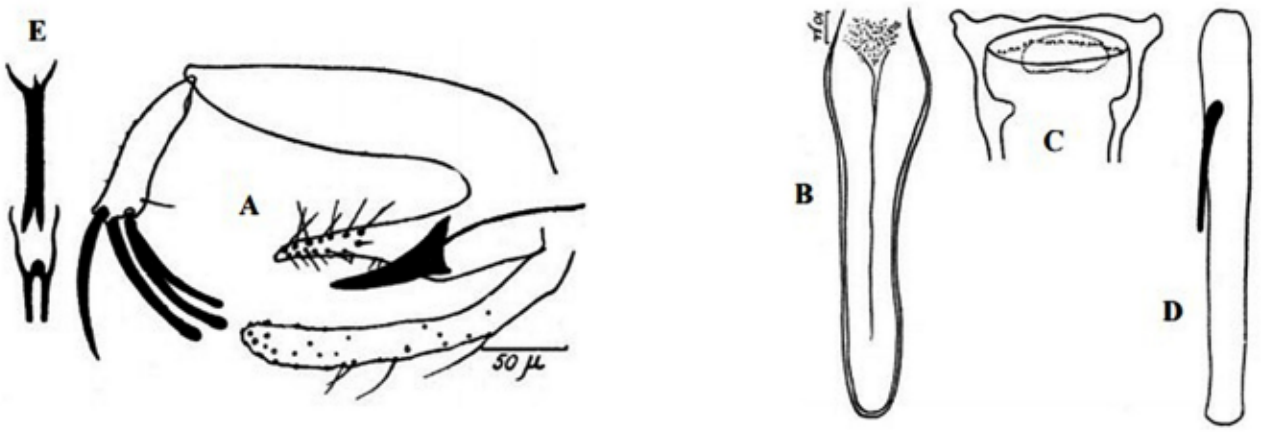


Fig 71. ***Sergentomyia dreyfussi*** ♂ [54]. **(A)** General genitalia. **(B)** Pharynx. **(C)** Cibarium. **(D)** Third antenna segment. **(E)** Genitalia pump.

https://doi.org/10.1371/journal.pntd.0009952.g071

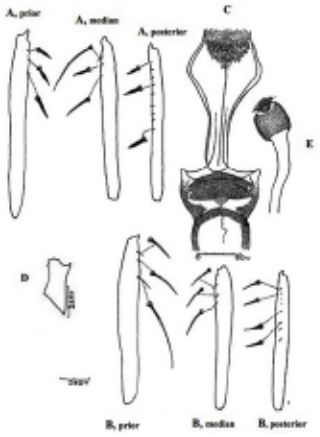


Fig 72. ***Sergentomyia dreyfussi*** ♀ [2]. **(A, B)** Femurs of Moroccan and Algerian specimens. **(C)** Pharynx and Cibarium. **(D)** Meteplsternum. **(E)** Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g072

located close to them, strong and triangular (conical) aedeagus, 20 separated cibarial teeth, and presence of spines on the femur of the first leg (Fig 71). The females possess characteristic rounded spermathecae covered by thin spicules (Fig 72) [51].

II-4 Subgenus *Sintonius*

Species with scanty erect hairs on the dorsal aspects of segments II to VI in females with segmented or crenulated spermathecae shaped similarly to subgenus *Phlebotomus*. Males have a pointed aedeagus and hooked intermediate appendage, [2,117]. Four species are reported from the Maghreb region.

II-4-1 *Sergentomyia clydei* Sinton, 1928

This species was reported to transmit *Sauroleishmania* sp. and DNA of *L. major* was recently detected in specimens from Tunisia [118]. This species is reported in the whole Maghreb region (Fig 73) from arid to hyperarid bioclimatic zones [37,41,119]. The males are recognized by the presence of 4 strong spines, 2 apical and 2 subapical, and an accessory spine on the distal 1/3, 2 to 3 strong teeth and 25 to 35 denticules. The aedeagus is short, conical, and pointed (Fig 74). This species is closely related to *Se. christophersi*, which differs only in the denticules number [119]. In the females, the spermathecae have 7 or 8 segments. The cibarium includes 2 rows of teeth, 13 long and sharped and 18 short and blunt with a diamond pigmented patch (Fig 75) [2].

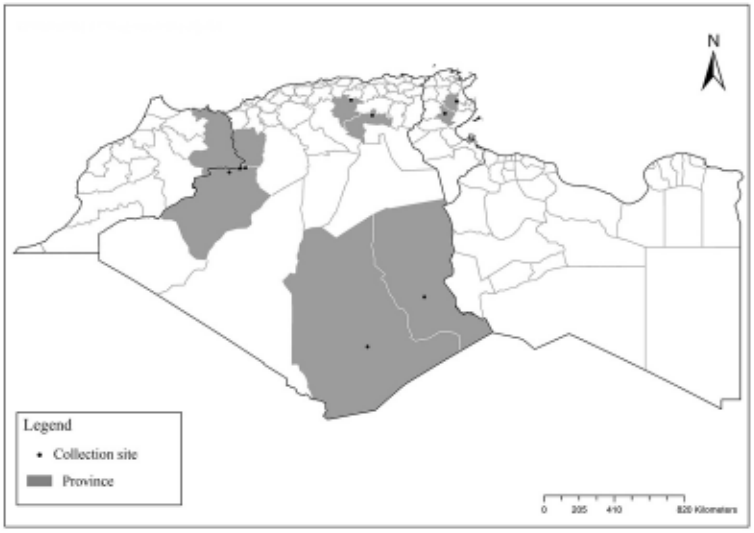


Fig 73. Distribution of *Sergentomyia clydei*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer

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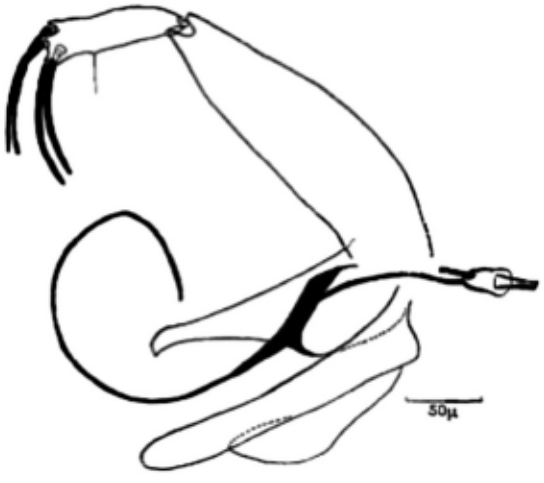


Fig 74. *Sergentomyia clydei* ♂ [2]. General genitalia.

https://doi.org/10.1371/journal.pntd.0009952.g074

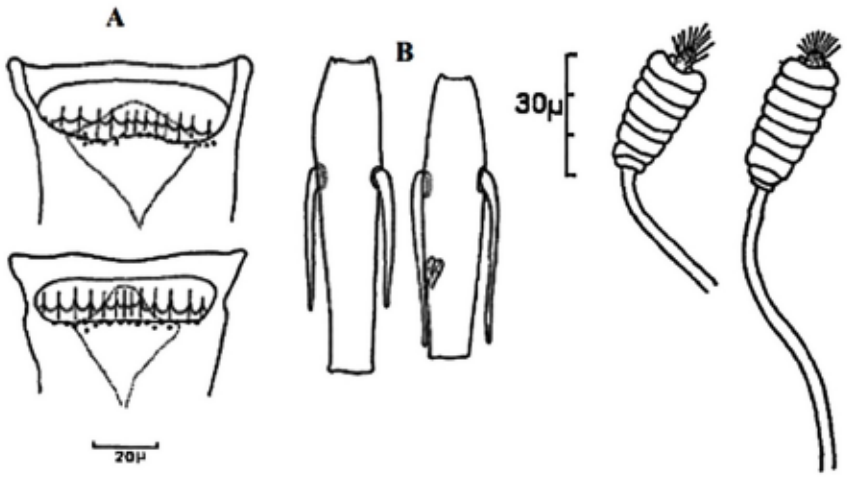


Fig 75. *Sergentomyia clydei* ♀ [2]. (A) Cibarium. **(B)** Third and fourth antenna segments. **(C)** Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g075

II-4-2 *Sergentomyia christophersi* Sinton, 1927

Originally described from Pakistan by Sinton [120], this species has been reported first in Tunisia in 1971. It occurs in all Maghreb region (Fig 76) between the arid and hyperarid bioclimatic zones [33,45,95,121]. The males are similar to *Se. clydei* except the number of denticules that equals to 05 (Fig 77). In females, the spermathecae have 8 segments, 4 to 7 sharped and pointed cibarial teeth, 4 to 5 denticules, and small triangular pigmented patch (Fig 78) [2].

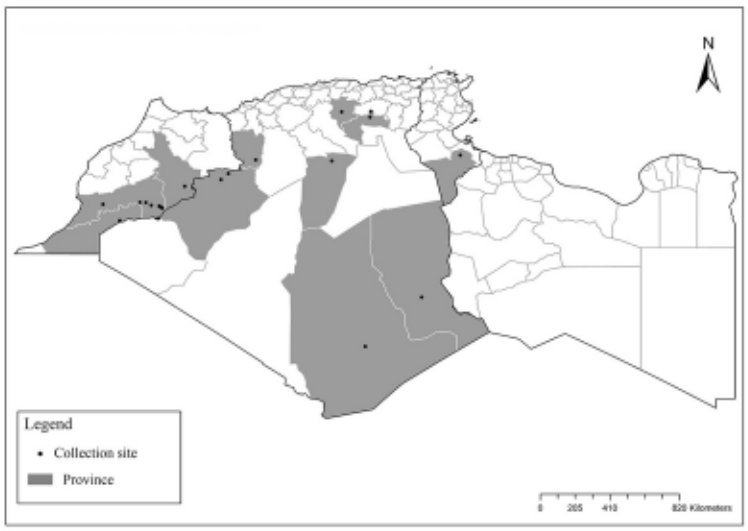


Fig 76. Distribution of *Sergentomyia christophersi*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

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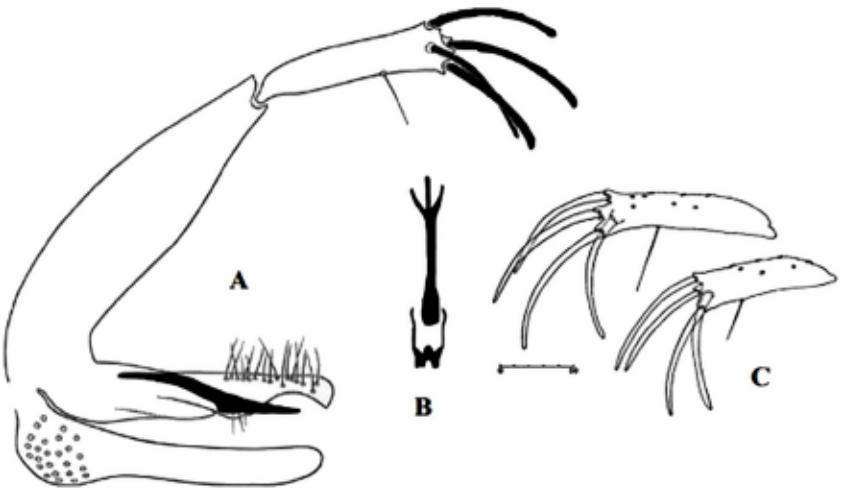


Fig 77. *Sergentomyia christophersi* ♂ [2,48]. (A) General genitalia. **(B)** Genital pump. **(C)** Insertion position of accessory spine.

https://doi.org/10.1371/journal.pntd.0009952.g077

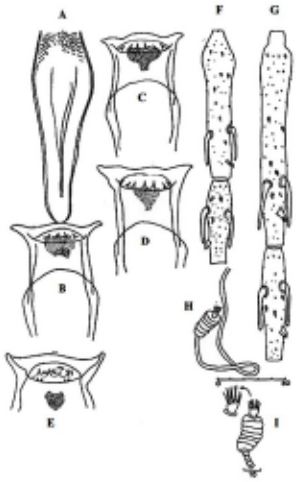


Fig 78. *Sergentomyia christophersi* ♀ [2]. (A) Pharynx. **(B–D)** Cibarium Guinean specimens. **(E)** Cibarium Tibetan specimen. **(F)** Third and fourth antenna segments, Guinean specimen. **(G)** Third and fourth antenna segments, Tibetan specimen. **(H)** Spermathecae, Tibetan specimen. **(I)** Spermathecae Guinean specimen.

https://doi.org/10.1371/journal.pntd.0009952.g078

II-4-3 *Sergentomyia hirtus* Parrot et de Jolinière, 1945

This species was described based on a single male captured in In-Amguel (Tamanrasset) in Algeria in the hyperarid bioclimatic zone (Fig 79). Its style holds 7 strong spines (3 submedians, 2 subapical, and 2 apical) and an accessory spine. The aedeagus is very short (18 μm) and subconical (Fig 80). The females remain unknown [52]. Dedet and colleagues [33] challenged the validity of the species since it was not reported from elsewhere and could be just a malformation often reported in *Sergentomyia* species.

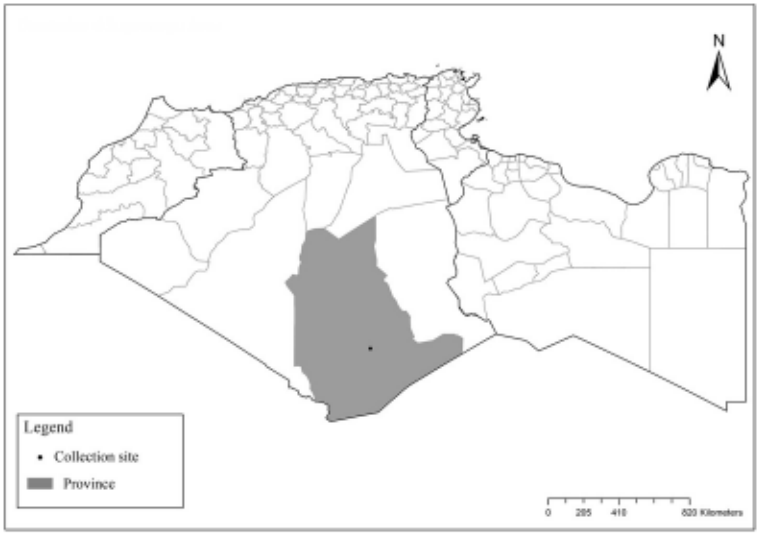


Fig 79. Distribution of *Sergentomyia hirtus*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

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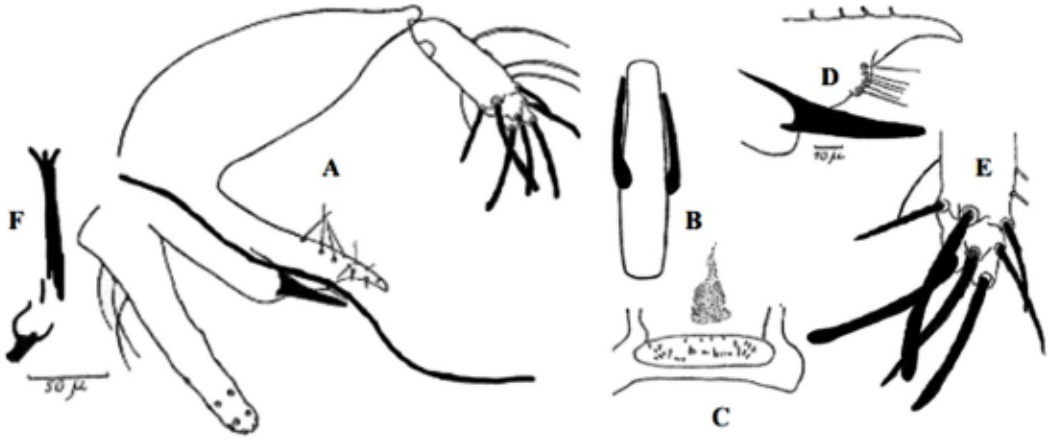


Fig 80. *Sergentomyia hirtus* ♂ [55]. (A) General genitalia. **(B)** Fourth antenna segment. **(C)** Cibarium. **(D)** Aedeagus. **(E)** Style. **(F)** Genitalia pump.

https://doi.org/10.1371/journal.pntd.0009952.g080

II-4-4 *Sergentomyia tiberiadis* Adler, Theodor, and Lourie, 1930

This species was reported only in the Central Sahara of Algeria where the hyper arid bioclimatic zone occurs (Fig 81). Previously, it was named as *Se.* near *clydei* before considering it as a new species [122]. The males have a long, slender, and pointed end aedeagus without subapical tubercle, 12 to 14 pointed cibarial teeth standing almost in straight line and about 15 punctiform denticules arranged in a zigzag pattern (Fig 82). In females, the spermathecae comprise 6 to 8 segments, narrowing toward the apex, 16 to 17 wide and pointing upward cibarial teeth arranged in a line slightly convex interiorly, and about 10 to 15 punctiform denticules arranged in zigzag row. The pharynx seems completely unarmed (Fig 83) [2,122].

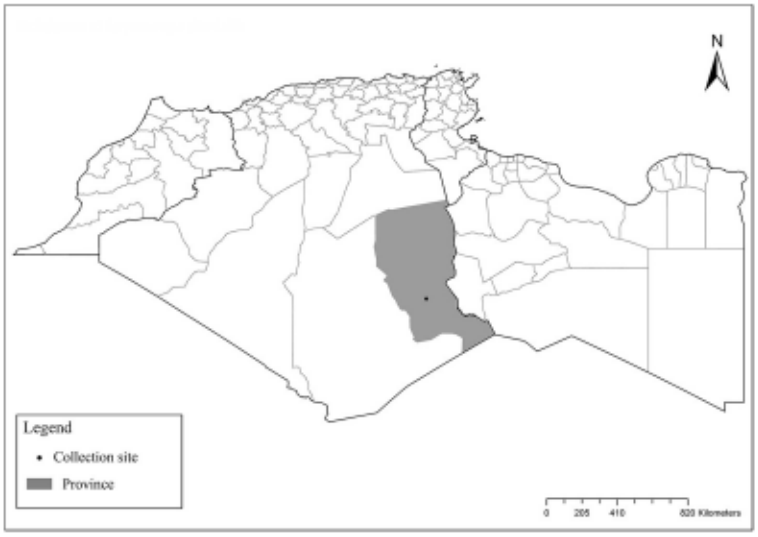


Fig 81. Distribution of *Sergentomyia tiberiadis*. Available from: https://services3.arcgis.com/W1gaXmEpGR8h1K59/arcgis/rest/services/maghreb/FeatureServer.

https://doi.org/10.1371/journal.pntd.0009952.g081

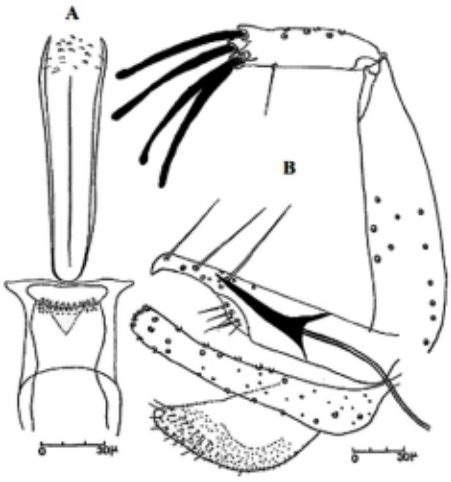


Fig 82. *Sergentomyia tiberiadis* ♂ [2]. (A) Pharynx and Cibarium. **(B)** General genitalia.

https://doi.org/10.1371/journal.pntd.0009952.g082

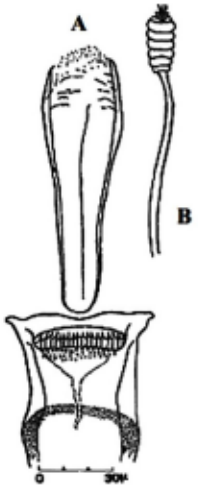


Fig 83. *Sergentomyia tiberiadis* ♀ [2]. (A) Pharynx and cibarium. **(B)** Spermathecae.

https://doi.org/10.1371/journal.pntd.0009952.g083

Discussion

Our study reviews the presence of sand fly species in Morocco, Algeria, Tunisia, and Libya countries where sand fly fauna is known to be very diverse, and many species are incriminated in the transmission of sand fly–borne pathogens as arboviruses of the *Phlebovirus* group [123] and most importantly parasitic protozoa of the genus *Leishmania*. We decided to exclude Mauritania from the analyses because accurate data regarding the distribution of sand fly species and the incidence of CL and VL are unavailable. To our best knowledge, we summarize all sand fly species recorded in the Maghreb region for the first time. We also update the information concerning their vector role in transmission of diseases, morphological characters important for species identification and their biology.

The first sand fly species reported from North Africa was *Ph. papatasi* in 1908 [124]. Since then, a plethora of studies reported presence of so far 32 sand fly species, 16 species of the genus *Phlebotomus* and 16 species of the genus *Sergentomyia*. The genus *Phlebotomus* is represented by 4 subgenera, namely *Larroussius*, *Phlebotomus*, *Paraphlebotomus*, and *Transphlebotomus* and the genus *Sergentomyia* comprises 5 subgenera, namely *Sergentomyia*, *Parrotomyia*, *Grassomyia*, *Sintonius*, and *Parvidens*, species of the last one recorded only in Mauritania. The importance of the Maghreb region for sand fly research is highlighted by the fact that within the 32 species recorded here, 13 species were first described in one of the Maghreb region countries [116]: male of *Ph. sergenti* Parrot, 1917 [125], male of *Se. fallax* Parrot, 1921, male of *Ph. perfiliewi* Parrot, 1930, male of *Ph. langeroni* Nitzulescu, 1930 [99], male and female of *Ph. longicuspis* Nitzulescu, 1930 [126], male and female of *Se. dreyfussi* Parrot, 1933, male and female of *Ph. bergeroti* Parrot, 1934, male and female of *Se. eremitis* Parrot et De Jolinière, 1945 [52], male of *Se. hirtus* Parrot et de Jolinière, 1945 [52], male of *Ph. chadlii* Rioux, Juminer et Gibily, 1966 [94], male of *Ph. chabaudi* Croset, Abonnenc, and Rioux, 1970 [55], male of *Ph. mariae* Rioux, Croset, Léger et Bailly-Choumara, 1974 [25] and finally, male and female of *Ph. riouxi* Depaquit, Léger, and Killick-kendrick, 1998 [48].

The 4 reviewed countries within the region share some ubiquitous sand fly species, namely *Ph. papatasi*, *Ph. bergeroti*, *Ph. perniciosus*, *Ph. longicuspis*, *Ph. langeroni*, *Ph. sergenti*, *Ph. alexandri*, *Ph. chabaudi*, *Se. antennata*, *Se. christophersi*, *Se. clydei*, *Se. fallax*, *Se. dreyfussi*, *Se. minuta*, and *Se. schwetzi*. Algeria and Morocco have a record of 24 and 23 species, respectively [15,27,32,127]. Several rare species were reported only in Algeria: *Ph. mascittii*, *Se. africana* subsp *eremites*, *Se. tiberiadis*, and *Se. hirtus* [32,33,52], whereas *Ph. mariae* and *Se. africana* subsp. *asiatica* were reported only from Morocco [24,25]. Tunisia is ranked at third place with 18 species so far [38]. For Libya, we are inclined to count 15 species, excluding 6 other species reported without any morphological evidence: *Ph. orientalis*, *Ph. tobbi*, *Se. bedfordi*, *Se. cineta*, *Se. adleri*, and *Se. palestiniensis* [113]. Record of these species was later considered as not sufficiently proven [41,121]. So far, the proven vectors of *Leishmania* parasite reported in Libya are *Ph. papatasi*, *Ph. perniciosus*, *Ph. langeroni*, *Ph. perfiliewi*, and *Ph. sergenti* [69]. Interestingly, the study carried out in Libya [41] provides illustration of some male specimens identified as *Larroussius* sp. that in our opinion displayed an aedeagus with curved tip characteristic of *Ph. perniciosus* atypical form; unfortunately, the author passed beside the right identification despite that the atypical forms were clearly described in Spain and Morocco since 1991 and 1998, respectively [83,128]. The studies considering the atypical forms of *Ph. perniciosus* were focused mostly on its molecular and morphological characterization and how to discriminate it from *Ph. longicuspis* [30,83,128–130]. So far, this species was reported in Morocco, Algeria, and Tunisia trapped in sympatric with *Ph. longicuspis* and *Ph. perniciosus* [30,83,84]. By contrast, relatively little is so far known about its ecology, host preferences, behavior, and vectorial capacity. These important aspects shall be addressed in future research in order to understand

better its potential in the transmission of VL. In Mauritania, 10 sand fly species were historically reported: *Ph. bergeroti*, *Ph. duboscqi*, *Se. dubia*, *Se. africana africana*, *Se. antennata*, *Se. schwetzi*, *Se. leslyae*, *Se. magna*, and *Se. freetownensis* [2,116,131]. However, as these data stem exclusively from general keys and catalogs and no results of dedicated entomological surveys are available from this country, we cannot evaluate its sand fly fauna in more details. The update of this scant knowledge is much desired as 6 of the 10 Mauritanian species (*Ph. duboscqi*, *Se. dubia*, *Se. africana africana*, *Se. lesleyae*, *Se. magna*, and *Se. freetownensis*) were not reported from any other Maghreb country.

It is important to note that the current known distributions of sand fly species within the Maghreb region are not static but rather prone to dynamic changes. The countries share the same climatic conditions and landscape environments in large borderland areas, allowing the exchange of species either naturally or via human activities such as trade (e.g., cattle manure for the soil fertilization) and movement of the populations due to the drought or political crises across the Maghreb and north of the Sahel region. These factors may contribute to a potentially rapid spread of both pathogens and their vectors. Moreover, ongoing climate change may further facilitate the adaptation of arthropods and emergence of arthropod-borne pathogens in new areas [132]; this hypothesis can be supported by the occurrence of *Anopheles gambiae*, a primary vector of malaria in Africa, in the Algerian territory at the Malian border [133], although this species was originally not expected to be able to cross the Saharan band of the Sahel region. Sand fly distribution is known to be impacted by the climatic conditions mainly temperature and humidity. Under the scenarios of a prediction model assessing the impact of potential climate change on the distribution of sand fly populations, expected rising temperatures may allow the expansion of sand fly populations into environmentally suitable areas north off the current Mediterranean distribution [134]. Based on recent climate change predictions, North Africa region is expected to become more arid by 2100 when only the coastal parts will retain their semihumid bioclimatic zone [135]. Subsequently, some species such as *Ph. papatasi*, *Ph. alexandri*, *Ph. sergenti* and *Ph. ariasi*, while gaining more new areas by their northward expansion into currently nonendemic regions of Europe, may disappear from some regions in North Africa [136]. Such scenarios allow us to expect some species from the arid part expanding further north of their current distribution, possibly contributing to enhance transmission of various pathogens.

As already described, both cutaneous and visceral leishmaniases constitute one of major public health problems in the Maghreb region: While they were historically established here, recently, these diseases show a significant rise through many local outbreaks and thousands of new human cases annually. This is partially facilitated by abundant sand fly fauna as in each country, several proven and/or suspected sand fly vectors are present: *Ph. papatasi* transmitting *L. major*, *Ph. perniciosus*, and *Ph. perfiliewi* transmitting *L. infantum*, *Ph. sergenti* transmitting *L. tropica* and *Ph. longicuspis*, which is suspected to transmit *L. infantum* [21]. It is important to note that while firm criteria for rigorous incrimination of a sand fly species as a vector were set [1], these are often difficult to meet for practical and logistical reasons. The increasing availability of molecular methods of pathogen detection in sand fly vectors lead to widespread use of these techniques in the screening of field-caught specimens for *Leishmania* detection, this including a majority of studies in Maghreb. However, such findings shall be considered with caution as a detection of *Leishmania* DNA does not prove the role of analyzed sand fly species in its transmission. Typically, species of the genus *Sergentomyia* were often suspected to be incriminated in the transmission of *Leishmania* sp. based on circumstantial evidence [114]. Thus, it is important to determine the potential vectorial role of certain species especially in the central Sahara where *Sergentomyia* species are more abundant than species of the genus *Phlebotomus* [110]. Despite it was demonstrated that *Se. schwetzi* did not support

the development of *Leishmania* parasites (including *L. major* and *L. infantum*) under laboratory conditions [137], such experimental assessment of *Sergentomyia* species remains rare and should deserve more attention in the future studies.

Several arboviruses of the order Bunyaviridae were reported from the Maghreb countries, including well-characterized TOSV, which is of most importance as it infects humans and was determined as a causative agent in a human case in Tunisia [138], Naples virus and Sicilian virus. However, despite the fact that these 3 sand fly–borne viruses known from other parts of the Mediterranean pose a potential human health risk, they remain understudied compared to leishmaniases: A recent literature review reported that of all publications on TOSV, only 8 refer to Algeria, 4 to Morocco, and 13 to Tunisia, while no data are available from Libya and Mauritania [139]. This contrasts with the expectation that these viruses may in fact be very prevalent in the region. High rates of TOSV-neutralizing antibodies reported from studies performed on humans or domestic animals in Algeria [87,93,140] and Tunisia [141,142] suggest frequent circulation of the virus in the region; in fact, some of human seroprevalence studies demonstrated percentage of human population that possess neutralizing antibodies against TOSV to be 2 to 3 times higher than in southern Europe [143]. The 3 abovementioned phleboviruses were detected in sand fly species of several subgenera in the Maghreb region as summarized in Table 1: TOSV in *Ph. papatasi* [64], *Ph. sergenti* [73], *Ph. perniciosus* [82], and *Ph. longicuspis* [73], Naples viruses in *Ph. perniciosus* and *Ph. longicuspis* [82,87], and Sicilian virus in *Ph. ariasi* [93]. This list is far from being exhaustive, and it is expected that other sand fly species, especially of the subgenus *Larroussius*, may be incriminated in circulation of these viruses [144]. Some of these viruses were detected in other sand fly species in neighboring countries, for example, Sicilian virus in *Ph. papatasi* in Egypt [63]. Sometimes, a virus is detected in pooled sand flies without their exact species identification, relying on parallel morphological identification of a subset of sand fly catch to estimate the relative abundance of each sand fly species; this was the case of TOSV detection in Algeria with 6 sand fly species known to occur in the focus [145] and in Tunisia where *Ph. perniciosus* was the most abundant species [146]. NGS and statistical analysis of the subsequent reads would enable species identification within pooled samples; however, such analysis is not always available. The diversity of phleboviruses in the Maghreb region is still not fully understood, and several novel viruses were detected or isolated from sand flies, such as Punique virus [82] and Saddaguia virus [147] detected in sand flies in Tunisia. To determine the involvement of different sand fly species in their transmission remains one of the future research tasks.

While species identification based on a detailed analysis of decisive morphological features remains a golden standard in sand fly taxonomy, the advent of molecular methods in last decades allowed it to be complemented by several other approaches. DNA-based techniques targeting various genetic markers such as Cytochrome b (Cytb), Cytochrome C oxidase subunit I (COI), and Internal Transcribed Spacer (ITS) regions allowed further insight into sand fly taxonomy, revealing the presence of *Ph. perniciosus* atypical form so long confused with *Ph. longicuspis*, a closely related species [129,130], boosting morphological identification criteria proposed for the couple *Ph. chabaudi*/*Ph. riouxi*, 2 closely related and morphologically challenging species [15,48]. In another study assessing the status of *Se. minuta* populations from different parts of the Mediterranean Basin, the analysis of COI and Cytb sequences allowed to reveal the presence of a complex genetic structure including 2 subpopulations *Se. minuta minuta* in Europe and *Se. minuta parroti* in North Africa [148]. Moreover, molecular techniques proved useful for species identification of pooled sand fly specimens in studies aiming to detect arboviruses where NGS analysis and statistical analysis of the subsequent reads was used to help species identification within pooled samples [149–151]. Recently, protein-based method of MALDI-TOF, which allows a rapid and cost-effective species identification of sand flies

[152], was successfully implemented in sand fly surveys in Algeria and Morocco [153,154]. Both DNA and protein-targeting molecular methods were successfully combined with morphological analysis in other geographical regions [155,156] and may be applied in the Maghreb countries to resolve taxonomical issues such as species status confirmation of *Se. hirtus* or *Se. africana* subsp. *eremites* and *Se. africana* subsp. *asiatica*, which were considered as synonymous [157]. In this respect, updated and detailed knowledge of the sand fly fauna as summarized in this study may be a valuable background for further studies.

In conclusion, this review summarizes the sand fly fauna occurring in the Maghreb region, compiling the distribution maps of each species based on published findings, discussing the suspected or proven role of each species in the transmission of pathogens, providing species-specific morphological features for the species identification, and highlighting the gaps to be filled regarding the vectorial status and presence of some sand fly species, pointing out a complete absence of recent data from Mauritania and fragmented knowledge of sand fly fauna in Libya, a country suffering a prolonged political unrest that disrupted healthcare system and sand fly control programs.

Key Learning Points

- Leishmaniases remain a major public health problem in the Maghreb region.
- Current gaps of knowledge highlight the necessity to establish or strengthen the entomological networks to study sand fly fauna, species distribution, and their involvement in transmission of pathogens in the Maghreb region.
- Sand fly–borne viruses and the diseases they may cause deserve more attention in this region where different phleboviruses were reported.
- There is an urgent need to fulfill the data gaps concerning leishmaniases and the sand fly vectors in Mauritania.
- Taxonomy of several sand fly species shall be addressed by integrating both morphological and molecular approaches and implementing sustained field research.

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Supporting information

S1 Data. Geographic location of sand fly species in Maghreb region.
(XLS)

S1 Text. Main differences between *Phlebotomus* and *Sergentomyia* species.
(DOCX)

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Identification of blood source preferences and *Leishmania* infection in sand flies (Diptera: Psychodidae) in north-eastern Algeria

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ABSTRACT

Leishmaniases are among the most neglected vector-borne diseases, infecting humans as well various animal hosts with clinical outcomes varying from cutaneous disorders to visceral and life-threatening disease. In Algeria, canine leishmaniasis (CanL) caused by *Leishmania infantum* is endemic mainly throughout the northern regions of the country with the Mediterranean climate that favours the occurrence of *Larroussius* sand flies, the vectors of the parasite. This study conducted in Bougaa and Kherrata, two regions located in north-eastern Algeria and endemic for CanL, focuses on: i) composition of sand fly fauna, ii) screening of *Leishmania* parasites and iii) the blood sources of engorged females. Entomological surveys were conducted between June and September 2019 using CDC light-traps in rural areas of both regions. Sand fly specimens were morphologically identified, females were screened for *Leishmania* DNA using kDNA and ITS1 primers, blood meals in engorged females were identified by peptide mass mapping (PMM)-based MALDI-TOF mass spectrometry and confirmed by DNA sequencing analysis. Overall, 1940 specimens (844 males, 1096 females) were collected, all belonging to the subgenus *Larroussius*: *Phlebotomus perniciosus*, (94.64%), *Ph. perfiliewi* (4.74%) and *Ph. longicuspis* (0.62%). No *Leishmania* DNA was detected in the evaluated pools ($n = 106$) (1096 females). PMM-based MALDI-TOF MS successfully identified a source of blood in 92% (141/154) of engorged females (135 *Ph. perniciosus* and 6 *Ph. perfiliewi*). All blood meals were taken from domestic cattle (*Bos taurus*) except for one originating from a dog (*Canis lupus familiaris*) and one from sheep (*Ovis aries*). Sequencing of host cytochrome B gene confirmed these identifications but showed lower success rate of 58% (29/50), demonstrating the high effectivity of peptide mass mapping (PMM)-based MALDI-TOF mass spectrometry for routine identification of blood meals of varying degree of digestion. Our findings represent first record of cattle and dog blood in sand flies in Algeria and striking feeding preference of local sand fly population at domestic sites of studied regions for cattle which may play an important role in parasite transmission. Further studies are needed to better understand potential contribution of cattle to ecology of sand flies and epidemiology of leishmaniasis in north-eastern Algeria.

1. Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are small nocturnal insects proven as vectors of various pathogens, including bacteria *Bartonella bacilliformis*, viruses of several genera and most importantly, parasitic protozoa of the genus *Leishmania* that cause diseases known as

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leishmaniases in human and animal hosts (Maroli et al., 2012; Ready, 2013). So far, approximately 1000 species and subspecies of sand flies were described and grouped into several genera of which three are found exclusively in the Old World namely: *Phlebotomus* Rondani & Berté, 1840, *Sergentomyia* França & Parrot, 1920 and *Chinius* Leng, 1987 (Bates et al., 2015; Munstermann, 2019). Among these genera, only species of the genus *Phlebotomus* are considered as the proven or suspected vectors of *Leishmania* spp. that infect humans and animals (Maroli et al., 2012).

Canine leishmaniasis (CanL) caused by L. *infantum* is a zoonotic parasitic disease widely distributed in the Mediterranean area where it represents a major veterinary problem and raises human health concerns (Velez et al., 2018). In Algeria, this zoonosis is believed to affect the entire national territory but with variable prevalence rate from one region to another, ranging generally between 11% and 37% (Harrat et al., 1996; Bessad et al., 2012). However, classic endemic foci of the disease are known mainly throughout the northern areas characterized by humid and sub-humid Mediterranean climates. In these bio-climate stages, high *Leishmania* infection rate was reported in dogs (Harrat and Belkaid, 2003; Ait-Oudhia et al., 2009; Adel et al., 2015; Medkour et al., 2019a). While the causative agent of CanL in northern Algeria is seldom responsible for the sporadic human cases of cutaneous leishmaniasis (CL), its ability to cause human visceral leishmaniasis (VL) which is a fatal disease if left untreated remains a high risk (Harrat et al., 1996). Data from the study of Adel et al. (2014) showed that 81.56% of all HVL cases recorded in Algeria during the period from 1998 to 2008 originated from regions located in the eastern and central North parts of the country. Unfortunately, HVL cases increased also in the extreme south of Algeria in Tamanrasset where the incidence reached 2.54 cases/100.000 inhabitants between 1997 and 2011 against 0.75 cases/100.000 inhabitants for the old focus of Grande Kabylie (Benallal et al., 2013).

In Algeria, twenty-four sand fly species were described so far, six of these within the subgenus *Larroussius*: *Ph. perniciosus* Newstead, 1911 in both typical and atypical morphological forms, *Ph. perfiliewi* Parrot, 1930, *Ph. ariasi* Tonnoir, 1921, *Ph. langeroni* Nitzulescu, 1930, *Ph. longicuspis* Nitzulescu, 1911 and *Ph. chadlii* Rioux, Juminer et Gibily, 1966 (Dedet et al., 1984; Berchi et al., 1986; Berdjane-brouk et al., 2011; Benallal et al., 2017; Benallal et al., 2022). Classical method for *Leishmania* detection which requires the dissection of freshly caught individual sand flies, isolation and culture of parasites from the sand fly gut has incriminated *Ph. perniciosus* and *Ph. perfiliewi* as the vector of L. *infantum* and *Ph. sergenti* as vector of L. *killicki* in Algeria (Izri et al., 1990; Izri and Belazzoug, 1993; Boubidi et al., 2011). However, this traditional approach is laborious, time-consuming and often not easy to perform as it requires equipment and expertise. Recent advances of PCR-based molecular techniques allowed them to complement conventional dissection methods which become scarce epidemiological studies performed in Algeria (Berdjane-Brouk et al., 2012; Mouloua et al., 2017; Bennai et al., 2018; Gherbi et al., 2020).

The application of molecular methods, especially sequencing assays targeting various genetic markers of the hosts, has further been extended to study the blood-feeding patterns of phlebotomine sand flies as a convenient alternative to serological techniques (Kent, 2009). In a single study performed so far in Algeria using PCR-sequencing targeting mitochondrial *COI* gene, an opportunistic feeding behaviour was observed in field-caught blood-fed sand flies (Bennai et al., 2018). Beside apparent advantages, most molecular techniques still face certain complications and limitations, mostly related to the rapid process of blood degradation (Hlavackova et al., 2019). Recently, a novel method, peptide mass mapping (PMM) analysis of host-specific haemoglobin peptide using MALDI-TOF mass spectrometry, was introduced as a sensitive and cost-effective tool, allowing host blood meal identification in hematophagous insects such as sand flies or mosquitoes by a fast and simple preparation protocol that provides conclusive host determination within a prolonged period post blood meal intake (Hlavackova et al., 2019).

This study aimed to assess the diversity and relative abundance of sand fly species in two localities within Kabylia region in north-eastern part of Algeria where L. *infantum* infection in dogs is endemic (Adel et al., 2015; Medkour et al., 2019a). DNA-based molecular tools were applied to detect *Leishmania* DNA among sand fly females and to confirm morphological identification of some specimens while PMM-based MALDI-TOF MS was used for the identification of blood meals in engorged sand fly females to understand their feeding behaviour and determine potential reservoir hosts. The results of this study will contribute to estimate the risk of disease transmission in the study areas.

2. Materials and methods

2.1. Sand fly collection and morphological identification

2.1.1. Ethical considerations

Verbal informed consent was obtained from head of animal shelters which were selected for sampling of sand flies.

2.1.2. Study area and sand flies trapping

This study was conducted in two villages, Bougaa (36° 19′ 57″ N, 5° 05′ 19″ E) located in the northwest of Setif wilaya (province) and Kherrata (36° 29′ 34″ N, 5° 16′ 39″ E) located in the southeast of Bejaïa wilaya (Fig. 1). Distance between the two study areas is 42.1 km. The elevation is between 650 and 1250 m above sea level (a.s.l.) for Bougaa, whereas it varies between 498 and 1896 m a.s.l in Kherrata. Both areas, with their specifically mountainous relief, support a Mediterranean sub-humid climate with an average annual rainfall exceeding 500 mm. The average annual temperature is between 14 and 15 °C, dropping to as low as 2 °C in winter and reaching up to 33 °C in summer. Relative humidity is about 77% and 47% in January and July, respectively. The main vegetation is composed by olive, fig and oaks trees as well as Aleppo pine and Cedar trees. Agriculture is dominant human activity, growing crops and raising livestock (i.e. sheep, goats, cows and poultry), thus providing suitable environment for the development of sand flies. Animal shelters are mostly found attached to or near human dwellings with an accumulation of animal waste in proximity.

Ten collection sites (five in each locality) represented by animal stables and located in rural environments were selected for this study. The fauna inside each selected animal shelter consisted mainly of 9 to 13 cows, 5 to 7 sheep and 2 to 3 goats. The presence of cats and farm dogs in or near to livestock shelters was also noted (< 3). Donkeys are found in almost all selected biotopes, although in low numbers (< 2). Sand flies were collected inside animal shelters using CDC light traps (John W. Hock Company, Gainesville, FL, USA, Centers for Disease Control) placed from the second half of June to mid-September, corresponding to the active season of sand flies. Once every 10 days, one of the 10 sites was sampled for one night using 4 CDC light traps. Traps were suspended from ceiling in animal shelters from sunset to sunrise, i.e. from 18:00 PM to 7:00 AM at height of about 1.5 m from the ground level. A total of 10 trapping nights were performed during the whole study period. All caught sand flies were pooled to obtain 30 specimens per tube and dry stored at −20 °C until further analyses.

2.1.3. Morphological identification of sand flies

The species identification of caught sand flies was done by observing the decisive morphological characters on the head (pharynx and cibarium) and genitalia (male external appendices and female spermathecae). Each sand fly was transferred to a sterilized microscopic slide and dissected using sterile micro-needles. Subsequently, the head and terminal segment of the abdomen were slide mounted in CMCP-9 mounting medium (Polysciences) and the rest of the body was stored in ethanol for molecular analyses as described later. Morphological identification was carried out using the identification keys of Abonnenc (1972) and Dedet et al. (1984).

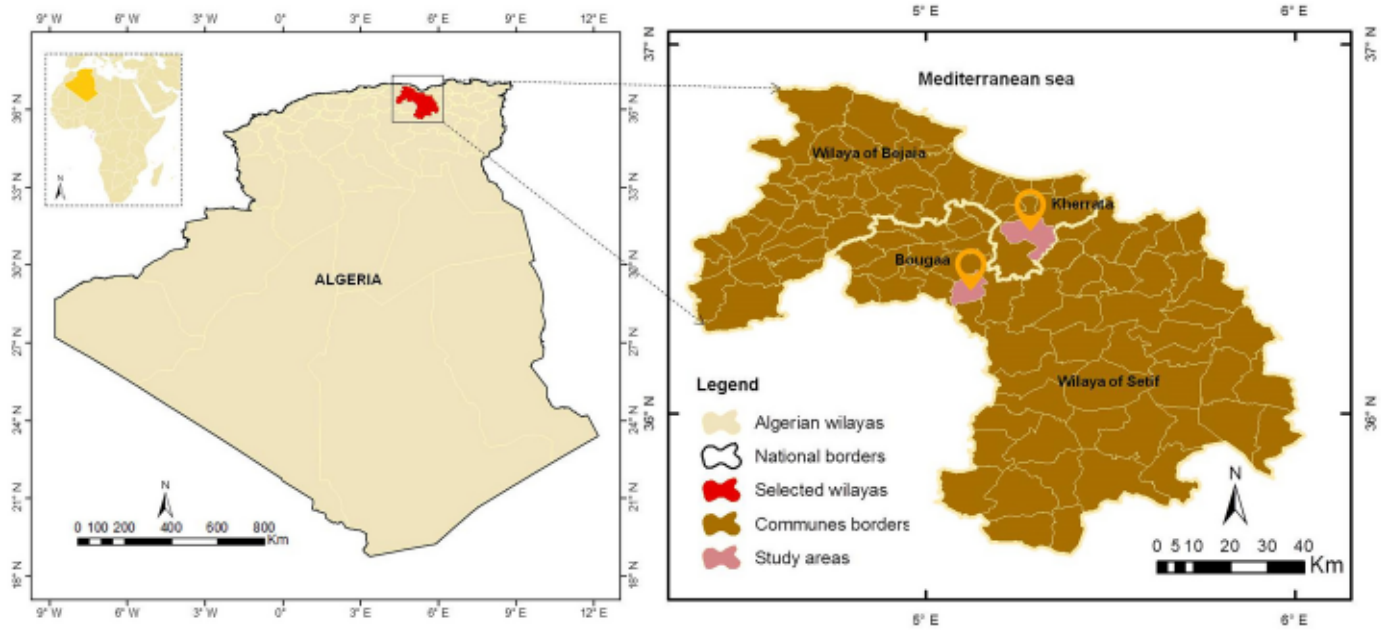


Fig. 1. Geographic locations of the phlebotomine sand fly collection sites in Setif and Bejaia. This map was created using ArcGIS 10.3.

2.2. Host blood meal identification

2.2.1. Sample preparation for PMM-based MALDI-TOF mass spectrometry

All 154 collected blood fed females (74 from Kherrata and 80 from Bougaa) were analysed individually using MALDI-TOF mass spectrometry to determine the origin of their blood meals. Before PMM analysis, engorged females were identified into species using decisive morphological characters as previously described. For specimens with inconclusive species assignment and those morphologically identified as *Phlebotomus perfiliewi*, DNA barcoding by universal LCO/HCO primers was performed according to standard protocol (Folmer et al., 1994) using DNA isolated as described below to confirm their species identity. The abdomens were air-dried and homogenized individually in 1.5 mL Eppendorf tubes utilizing a manual BioVortexer homogenizer (BioSpec) with sterile disposable pestles in 50 µL of distilled water (Merck KGaA) for 15 s. The homogenates were then shortly centrifuged at 10000*g* for 15 s and 10 µl of each supernatant were mixed in a new microtube with 10 µL of 50 mM N-ethylmorpholine acetate buffer (pH 8.1; Sigma-Aldrich) and 100 ng of sequencing grade trypsin (Promega). The resulting mixture was subsequently incubated for 30 min at 37 °C. After the digestion, 0.5 µL of the mixture was deposited on a MALDI plate in duplicate, subjected to air-drying at room temperature and later covered with 0.5 µL of MALDI matrix prepared with α-cyano-4-hydroxycinnamic acid (5 mg/mL) in a mixture solution of 50% acetonitrile and 0.1% trifluoroacetic acid (Sigma-Aldrich) (Hlavackova et al., 2019). Simultaneously, the remaining 40 µL of the abdomen homogenate were utilized as a template for DNA isolation by High Pure PCR Template Preparation Kit (Roche, Germany) following the manufacturer's protocol. The isolated DNA was used for DNA barcoding to verify species identification achieved by morphological characters and to confirm blood meal determination in chosen samples.

2.2.2. Identification of blood meal sources by PMM-based MALDI-TOF mass spectrometry

The 154 samples were measured on an Ultraflex III MALDI-TOF mass spectrometer (Bruker Daltonics) in the mass range of 700–4000 Da. To determine blood meal source, the obtained peptide mass maps were compared with in silico generated tryptic peptides from haemoglobins of all potential vertebrate hosts. For equivocally identified blood meals (19 females) with lower number of matched peptide fragments at least two peptides per sample were selected for MS/MS sequencing using LIFT technology. The MS/MS spectra were searched against a vertebrate subset of a SwissProt database using an in-house MASCOT 2.4 search engine (Matrix Science) to confirm the tentative host assignment (Supplementary Table S1).

2.2.3. Confirmation of blood meal sources by DNA sequencing analysis

In total, 50 females were chosen for confirmation of blood meal identification by DNA-based methods. Within these, 13 specimens that yielded no identification by MALDI-TOF mass spectrometry were included together with both non-bovine blood meal samples (2). In addition, 19 females which provided lower quality of mass spectra that required additional verification by MS/MS sequencing were selected. Finally, 16 specimens (8 from each locality) with conclusive identification using PMM-based peptide mapping were randomly chosen for DNA sequencing as well (*Ph. perfiliewi* and *Ph. perniciosus* females).

DNA isolated from engorged females was utilized as a template to amplify a fragment of *Cyt b* gene by universal vertebrate primers *Cyto 1* and *2* (Abbasi et al., 2009) in 50 µL reaction mixture with Emerald Amp GT PCR Master Mix (Takara). The amplicons were purified and subjected to Sanger sequencing in two directions with the primers used for DNA amplification, obtained sequences were queried in GenBank database using BLAST algorithm.

Moreover, multiplex PCR assay using species-specific primers to amplify size-specific fragments of *Cyt b* gene was done as previously published (Garros et al., 2011) to confirm identified canine and ovine blood meals. Multiplex PCR was done in 50 µL reaction mixture with Emerald Amp GT PCR Master Mix (Takara) for samples KA37 (canine blood amplified by forward primer UNIV1 and reverse primer CANIS), KA60 (ovine blood amplified by forward primer UNIV2 and reverse primer OVIS), KA23 and KA24 (bovine blood amplified by forward primer UNIV2 and reverse primer BOS). Amplicons were visualized on a 2% agarose gel alongside GeneRuler 100 bp Plus DNA ladder (Thermo Scientific) to determine their size difference.

2.3. Molecular screening for Leishmania infection in sand flies

A High Pure PCR Template Preparation Kit (Roche® Mannheim, Germany) was used for extracting DNA from the abdomen of unfed and fed females following the manufacturer's instructions. After extractions,

4 µL DNA of each sand fly female were pooled by 2 to 12 sand flies' DNA per pool based on the same sand fly species, repletion status and sampling locality.

These DNA pools were screened for *Leishmania* infection by the amplification of a 300–350 bp fragment corresponding to the internal transcribed spacer of the *Leishmania* DNA (ITS1), using the primers L5.8S (5′-TGATACCACTTATCGCACTT-3′) and LITSR (5′-CTGGATCATTTTCCGATG-3′) (Schönian et al., 2003). Moreover, presence of *Leishmania* DNA from the same sand fly DNA pools was also tested using PCR targeting the conserved sequences of the *Leishmania* kinetoplast minicircle DNA (kDNA) with the following primer pair F (5′-GTGGGGGAGGGGCGTTCTG-3′) and R (5′-AAACTGGGGGTTGGTGTAAAAT-3′) (Rodgers et al., 1990). For the former assay, the amplification was performed in a 50 µL final volume of a reaction mixture containing 25 µL of Dream Taq Master Mix, 21 µL of deionized water, 2 µL of the reverse and forward primers and 4 µL of DNA template. The optimized thermocycling conditions were as follows: 94 °C for 2 to 3 min, followed by 39 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 1 min, with a final extension step at 72 °C for 6 min. For the second assay, the amplification reaction totalled 25 µL, containing 12.5 µL of Dream Taq Master Mix, 8.5 µL of deionized water, 1 µL of each primer and finally 2 µL of DNA template under the following cyclic conditions: an initial denaturation step at 94 °C for three minutes followed by 40 cycles of 94 °C for 20 s, 61 °C for 30 s, 72 °C for 7 s and terminated with an extension step at 72 °C for 5 min. Negative (DNA-free water) and positive (*L. major* DNA) controls were included for both PCR assays. The amplified PCR products were separated and visualized on 2% agarose gel under a UV illuminator.

3. Results

3.1. Entomological data

3.1.1. Sand fly identification

In total, 1940 sand fly specimens, belonging to three sand fly species were collected and identified based on their morphological characters. All collected sand flies belonged to subgenus *Larroussius*: *Phlebotomus perniciosus*, *Ph. perfiliewi* and *Ph. longicuspis*. No atypical forms of *Ph. perniciosus* were identified. Further confirmation was obtained by using DNA barcoding for all engorged *Ph. perfiliewi* females as well as for six engorged *Ph. perniciosus* specimens, which provided inconclusive morphological characters. The obtained sequences successfully aligned with GenBank entries using BLAST algorithm (all barcoded specimens marked by asterisk in Supplementary Table S1).

3.1.2. Sand fly abundance and density

Out of 1940 caught specimens, 1188 (61.24%) were trapped in Bougaa and 752 (38.76%) in Kherrata. At both sites, the most abundant species was *Ph. perniciosus* representing 94.64% of the total individuals. *Ph. perfiliewi* was represented by 92 specimens (56 in Bougaa; 36 in Kherrata) and *Ph. longicuspis* with only 12 specimens (0.62%) (Table 1). The global phlebotomine density was found to be 48.5 sand flies/ CDC trap/night. At the species level, the density of *Ph. perniciousus* is represented by 45.9 caught specimens /trap-night while it is 2.3 and 0.3 for *Ph perfiliewi* and *Ph. longicuspis*, respectively.

In total, 844 (43.51%) specimens were males and 1096 (56.49%) were females. The F/M sex ratio was 1.29. Among the 1836 specimens of *Ph. perniciosus* captured, 1024 were females of which 588 (57.42%) were collected from Bougaa and 436 (42.58%) from Kherrata. Of 92 *Ph. perfiliewi* specimens, 68 (73.91%) were females; 48 from Bougaa and 20 from Kherrata (Table 1).

Out of 1096 female specimens, 154 (14.05%) were engorged, 74 of which (48.05%) were collected in Kherrata and 80 (51.95%) in Bougaa. *Ph. perniciosus* was the most dominant species with 148 (96%) specimens, followed by 6 (4%) specimens identified as *Ph. perfiliewi*.

3.2. Host blood meal analysis

3.2.1. Blood meal analysis by PMM-based MALDI-TOF mass spectrometry

The PMM-based approach successfully determined blood meal origin in 92% (141/154) of the engorged females (Supplementary Table S1, Fig. 2). For thirteen samples, the analysis did not provide any identification, probably because of the small volume of blood taken or high degree of blood digestion. Majority of identified females (122 of 141) yielded spectra of high quality allowing conclusive blood meal source determination. For 19 specimens giving spectra with lower number of matched peptides, the host assignment was confirmed using additional MS/MS sequencing. In all successfully identified samples, cattle (*Bos taurus*) was detected as a source of the blood meal, except two females where blood of domestic dog (*Canis lupus familiaris*) and sheep (*Ovis aries*), respectively, was identified. No mixed blood meals were observed (Supplementary Table S1).

3.2.2. Blood meal analysis by DNA sequencing and multiplex PCR

No amplification of the targeted gene fragment was obtained in 13 out of 50 females used for sequencing analysis of host *Cyt b* gene, for which MALDI-TOF MS did not provide any host identification. For 18 samples where peptide mass mapping yielded unambiguous host identification, the blood meal source was confirmed in all cases (16 bovine, 1 canine and 1 ovine) when sequences were queried in GenBank using BLAST algorithm. In remaining 19 samples where host blood meal origin was verified using MS/MS sequencing, amplification and sequencing of *Cyt b* gene was successful for 12 samples (bovine and canine). Overall success rate of cytochrome *b* sequencing assay was 58% (29/50).

For one canine, one ovine and two randomly chosen bovine samples, multiplex PCR assay using species-specific reverse primers successfully amplified PCR products of expected species-specific sizes that confirmed the host identification in these samples when separated and visualized on agarose gel.

3.3. Molecular detection of Leishmania spp. in phlebotomine sand flies

A total of 106 pools, consisting of 2–12 sand flies' DNA per pool, were molecularly assessed for *Leishmania* spp. *Leishmania* DNA was not detected in any of *Ph. perniciosus* 92 monospecific pools (fed and unfed females) either by kDNA-PCR or ITS1-PCR. Similarly, the examined 12 *Ph. perfiliewi* and 2 *Ph. longicuspis* female pools were found to be *Leishmania*-negative.

Table 1
Composition, abundance and relative frequency of sand fly species caught in Bougaa (Setif) and Kherrata (Bejaia) from June to September 2019.

Genus	Species	Study areas								
		Bougaa			Kherrata			Total		
		Male/Female	*n*	%	Male/Female	*n*	%	Male/Female	*N*	%
Phlebotomus	*Ph. perniciousus*	536/588	1124	94.61	276/436	712	94.68	812/1024	1836	94.64
	Ph. perfiliewi	08/48	56	4.71	16/20	36	4.79	24/68	92	4.74
	Ph. longicuspis	08/00	08	0.67	00/04	04	0.53	8/4	12	0.62
Total		552/636	1188	100%	292/460	752	100%	844/1096	1940	100%

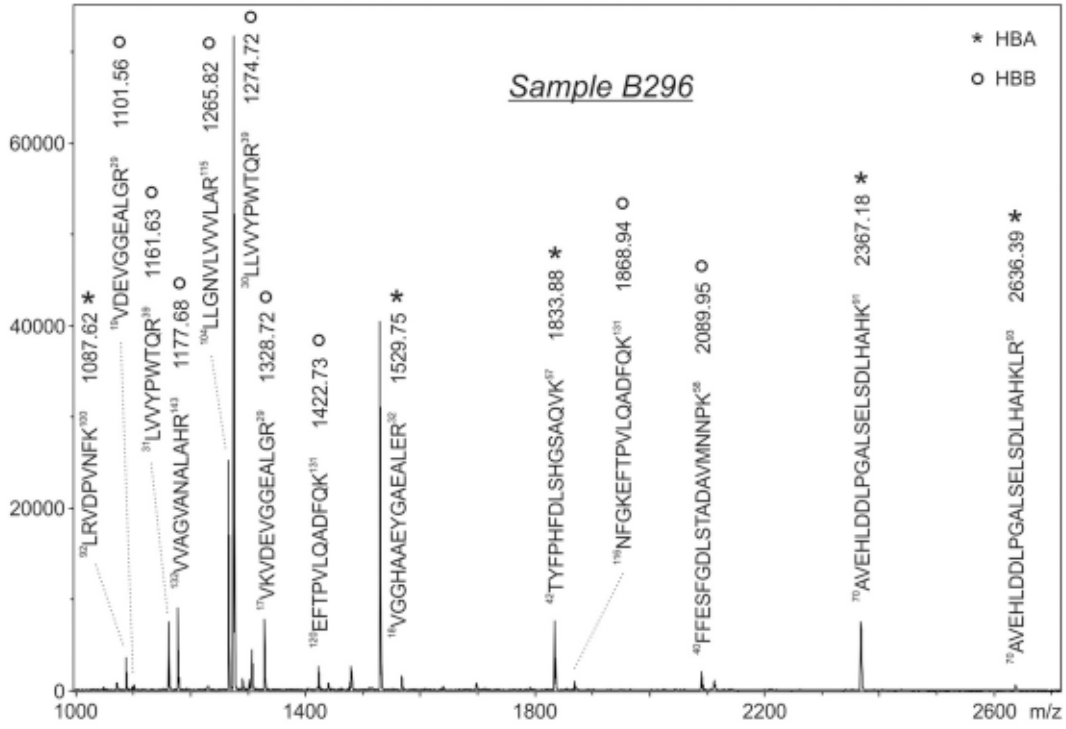


Fig. 2. Host blood meal identification by PMM-based MALDI-TOF mass spectrometry. Peptide mass map of trypsin-digested abdomen of the field-caught female B296. The assigned peaks are labelled by peptide sequences of cow haemoglobin subunit alpha (HBA; asterisks) and haemoglobin subunit beta (HBB; circles).

4. Discussion

In this study, we reported sand fly fauna in the rural areas of Bougaa and Kherrata, northeastern Algeria, where high prevalence of L. *infantum* infection has been recently confirmed in dogs (Adel et al., 2014; Medkour et al., 2019a, 2019b). Three sand fly species of the subgenus *Larroussius* were found with *Ph. perniciosus* being a dominant species. Composition of sand fly fauna in the two study areas was similar which is not surprising as the surveyed regions share similar parameters of climate, topography and land use.

High abundance of *Larroussius* species was previously reported by most studies carried out in the northern regions of Algeria, suggesting that environmental and climatic conditions in this region provide favourable conditions for members of this subgenus, while populations of other *Phlebotomus* subgenera were represented by markedly lower densities (Izri et al., 1990; Izri and Belazzoug, 1993; Harrat and Belkaid, 2003; Berdjane-Brouk et al., 2011, 2012; Allal-Ikhlef et al., 2017; Mouloua et al., 2017; Bennai et al., 2018). Our results showed a relatively low species richness rate compared to other regions of northern Algeria which share similar Mediterranean bioclimatic pattern. Indeed, in the Kabylia region, the main focus of canine and human leishmaniases due to L. *infantum*, the species richness varies between six and ten species according to the studied focus (Izri et al., 1990; Berdjane-Brouk et al., 2011; Allal-Ikhlef et al., 2017), while in Tipaza located in the north-centre, Bennai et al. (2018) have reported the presence of seven sand fly species.

Phlebotomus perniciosus was found to be the predominant species (94.64%). This finding is in accord with previous studies from northern Algeria (Izri et al., 1990; Berdjane-Brouk et al., 2011; Bennai et al., 2018). This species is widely present in the western part of the Mediterranean basin; in Algeria it occurs in all bioclimatic stages from the humid regions in the north to the Saharan edge of Tassili and Hoggar in the extreme south (Benallal et al., 2017). However, its distribution is predominantly in Tellian (northern Algeria) where the Mediterranean climate (sub-humid and semi-arid) prevails (Dedet et al., 1984). Given that our collecting sites are located within the main distribution area of this species, its predominance was unsurprising.

While climatic characteristics have a profound impact at macro-environmental scale, anthropic factors may be determining at a micro-geographical scale (Risueño et al., 2017). Trapping sites of sand flies in this study were chosen in livestock farms which constitutes another relevant factor for the greater abundance of *Ph. perniciosus* species since the accumulation of large amounts of organic matter and the presence of stable microclimates in these biotopes provide ideal breeding and resting sites (Guernaoui and Boumezzough, 2009; Branco et al., 2013). This observation was previously emphasized by several studies which highlighted an increasing risk of encountering this vector in locations where domestic animals i.e. livestock are in close proximity (Guernaoui and Boumezzough, 2009; Gálvez et al., 2010; Barón et al., 2011; Branco et al., 2013; Alcover et al., 2014; Bravo-Barriga et al., 2016). Besides the climatic and environmental conditions, highly endophilic (Benabid et al., 2017) and anthropozoophilic behaviour of this sand fly species (Rioux et al., 1970; Bravo-Barriga et al., 2016; Gherbi et al., 2020) could reflect also its important frequencies.

Other two sand fly species found, *Ph. perfiliewi* and *Ph. longicuspis* were present in relatively low numbers (4.75% and 0.6%, respectively). These results are in agreement with those found in Larbaa Nath Irathen (north-centre) where *Ph. perfiliewi* is poorly represented (Berdjane-Brouk et al., 2012) but are in contrast with those reported from Draa El Mizan (north-centre) where this species is highly abundant (Allal-Ikhlef et al., 2017; Mouloua et al., 2017). Although the same sampling methods (CDC-light traps) were used, the apparent discrepancy in results may be ascribed to differences between the studied biotopes (exclusively rural vs. more diversified), altitude (around 1000 m vs. < 400 m), hosts diversity and other environmental factors (e.g. vegetation type), which might have influenced the distribution and abundance of sand flies (Barón et al., 2011; Alcover et al., 2014; Bravo-Barriga et al., 2016). Low numbers of *Ph. longicuspis* were expected since this species occurs mainly in the semi-arid and the arid bioclimatic zones (Dedet et al., 1984; Berdjane-Brouk et al., 2012) and it was previously reported in low abundance from northern Algeria (Berdjane-Brouk et al., 2012; Allal-Ikhlef et al., 2017).

A novel method of mass spectrometry, PMM-based MALDI-TOF MS analysis targeting host specific haemoglobin peptides was recently introduced as a sensitive and cost-effective alternative that offers a fast and simple preparation protocol and enables host blood identification in a longer interval post blood meal (Hlavackova et al., 2019). In our study, this novel approach successfully revealed the origin of blood meal in 92% of analysed engorged females, showing that almost all engorged *Ph. perniciosus* and *Ph. perfiliewi* females fed exclusively on domestic cattle *B. taurus*, this being the first identification of cattle blood in engorged sand flies in Algeria. Only one *Ph. perniciosus* female was identified to feed on a dog and one on a sheep. Such rare detections of blood meals of origin other than bovine in a considerably large sample of engorged females suggest a strong preference of local sand fly populations at the studied sites for cattle as abundant and easily accessible blood source. The obtained high success rate of blood meal identification indicates a high degree of sensitivity of the method. In contrast, the success rate of identifications provided by sequencing analysis of host *Cyt b* was markedly lower, suggesting that PMM-based MALDI-TOF MS analysis of host specific haemoglobin peptides is a promising tool that may rival DNA-based techniques for blood meal identification in minuscule hematophageous insects like sand flies. When MS/MS sequencing was applied, conclusive host identification was possible even in samples where conventional DNA-based method failed due to unsuccessful PCR amplification or sequence of a low quality.

Numerous studies conducted in leishmaniasis foci in Italy (Bongiorno et al., 2003; Rossi et al., 2008), Spain (De Colmenares et al., 1995; Jiménez et al., 2013; Bravo-Barriga et al., 2016; González et al., 2017), Portugal (Branco et al., 2013; Maia et al., 2015) and Tunisia (Remadi et al., 2020) demonstrated that *Ph. perniciosus* showed an opportunistic feeding behaviour, taking a variety of blood sources, including dogs which represent the main reservoir of L. *infantum* in Algeria (Ait-Oudhia et al., 2009; Adel et al., 2010; Medkour et al., 2019a). Cattle were previously demonstrated as a blood source of *Ph. perniciosus* in Spain (De Colmenares et al., 1995) and Tunisia (Remadi et al., 2020). In contrast, Bennai et al. (2018) showed that *Ph. perniciosus* feed mainly on small ruminants and humans in Tipaza (north-centre Algeria). Here we confirmed that cattle serve as a blood source to *Ph. perniciosus* but its susceptibility to L. *infantum* was not demonstrated yet. In a previous study in Kabylia, equids were found to be infected with L. *infantum* (Medkour et al., 2019b); however, in our study, no equid blood was found in the tested engorged females, mainly due to the very low abundance of these animals.

Prevalence studies of *Leishmania* infection in phlebotomine sand flies are valuable indicators of *Leishmania* transmission intensity (Rossi et al., 2008). All three sand fly species caught in the present work are regarded as either proven (*Ph. perniciosus*, *Ph. perfiliewi*) or suspected (*Ph. longicuspis*) vectors of L. *infantum* in Algeria (Izri et al., 1990; Izri and Belazzoug, 1993; Berdjane-Brouk et al., 2012; Lafri and Bitam, 2021; Benallal et al., 2022). Nevertheless, no *Leishmania* spp. DNA was detected in any of the analysed pools despite two different markers deployed. This may reflect usually very low infection rates reported in sand fly populations even in the endemic areas, ranging from 0.4% to 0.6% in Bourkika (Tipaza) and in Larbaa Nath Irathen (Tizi-Ouzou) (Boubidi et al., 2011; Berdjane-Brouk et al., 2012; Benallal et al., 2013; Bennai et al., 2018; Gherbi et al., 2020). Moreover, infection rates of *Leishmania* spp. are usually low in *Larroussius* sand flies (Svobodová et al., 2009; Branco et al., 2013; Chargui et al., 2013; Maia et al., 2015; Barhoumi et al., 2016; Bravo-Barriga et al., 2016; Mhaidi et al., 2018). In the present work, the *Leishmania*-negative infections might also be ascribed to the presence of non-competent hosts in our sampled biotopes, which could promote a dilution effect supposed to reduce feeding of vectors on transmission-competent hosts, thereby reducing infection rates of the vectors (Keesing et al., 2006; Muñoz et al., 2019). Apparent feeding preferences of tested *Ph. perniciosus* for domestic cattle, which is not considered a proven reservoir host of *Leishmania*, support this assumption. Nonetheless, given the recent findings regarding the detection of *Leishmania* spp. within *Larroussius* sand flies engorged with cattle blood in Tunisia (Remadi et al., 2020) and the molecular identification of L. *infantum* DNA in cattle in some VL foci in China (Gao et al., 2015) and Brazil (Paixão-Marques et al., 2019). An extensive survey into the possible role of these animals in the epidemiology of leishmaniasis should be planned.

5. Conclusion

Our study showed the occurrence of three proven or suspected *Larroussius* vectors of L. *infantum* in the endemic focus of canine leishmaniasis in north-eastern Algeria. Despite all tested sand flies being screened by two molecular markers for the presence of *Leishmania* parasites, all were found negative. High abundance of *Ph. perniciosus* suggests a potential risk of L. *infantum* spread within canine and human populations. MALDI-TOF MS mass spectrometry was successfully applied to study feeding preferences of *Ph. perniciosus* and *Ph. perfiliewi* and proved more effective than conventional DNA-based sequencing analysis. It demonstrated that at studied localities, domestic cattle is a predominant source of blood for sand fly females, providing a first record of cattle blood in engorged sand flies in the country. These findings highlight the need to further study the potential role of cattle in the epidemiology of leishmaniases and sand fly ecology in Algeria. The establishment of large-scale monitoring projects on sand fly vectors to cover all areas of CanL dissemination would help mapping the areas with the high-risk of *Leishmania* transmission.

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Ethical statement

The study was carried out in accordance with Algerian legislation guidelines. In addition, sand flies were collected after obtaining a verbal consent from owners of animal shelters were CDC light traps were deposit.

Declaration of Competing Interest

None.

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Phlebotomine sand fly survey, blood meal source identification and description of *Sergentomyia imihra* n. sp. in the central Sahara of Algeria.

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Phlebotomine sand fly survey, blood meal source identification, and description of *Sergentomyia imihra* n. sp. in the central Sahara of Algeria

Kamal Eddine Benallal[1,2], Mohammed Mefissel[3], Yassine Dib[3], Jérôme Depaquit[4,5], Daniel Kavan[6], Zoubir Harrat[7], Vít Dvořák[1], Petr Volf[1] and Petr Halada[6*]

Abstract

Background Phlebotomine sand flies (Diptera: Psychodidae) are important vectors of various pathogens, mainly *Leishmania* parasites. In the Old World, the most important genus in term of pathogens transmission is the genus *Phlebotomus*, which includes many proven or suspected vectors of several *Leishmania* species, while the genus *Sergentomyia* remains so far unproven as a vector of human pathogens. Algeria is one of the most affected countries by human leishmaniasis.

Methods In the present study, an entomological survey was carried out in two provinces, Ghardaïa and Illizi, located in the north and central Sahara, respectively, where cases of human leishmaniasis are recorded. Our goal was to understand the role of the local sand fly species in the transmission of *Leishmania* parasites and to analyze their blood meal preferences. Collected sand flies were identified by a combination of morphological and molecular approaches that included DNA-barcoding and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) protein profiling. In addition, female blood meals were analyzed by peptide mass mapping using MALDI-TOF MS.

Results In total, 640 sand fly specimens belonging to *Phlebotomus* and *Sergentomyia* genera were collected in the two provinces. *Sergentomyia antennata* and *Se. fallax* were most abundant species in Ghardaïa, and *Ph. papatasi* and *Ph. alexandri* in Illizi. In addition, a new sand fly species was described in Illizi named *Sergentomyia* (*Sergentomyia*) *imihra* n. sp. Blood meal analysis of the engorged females revealed various mammalian hosts, especially goats, but also humans for *Phlebotomus papatasi* and *Ph. alexandri*, suggesting that these vector species are opportunistic feeders.

Conclusions Integrative approach that combined morphological analysis, sequencing of DNA markers, and protein profiling enabled the recognition and description of a new *Sergentomyia* species, raising the number of the Algerian sand fly fauna to 27 species. Further sand fly surveillance in the central Sahara is recommended to identify the thus-far unknown males of *Se. imihra* n. sp.

Keywords *Leishmaniasis*, *Phlebotomus*, *Sergentomyia*, Barcode, Blood meal, MALDI-TOF mass spectrometry, Algeria

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Background

Despite affecting human populations in 200 countries and territories worldwide, leishmaniases are still regarded as neglected tropical diseases. In 2020, more than 200,000 cases of cutaneous leishmaniasis (CL) and 12,000 cases of visceral leishmaniasis (VL) were reported. The Eastern Mediterranean region and Algeria constitute an eco-epidemiological "hotspot", as together they contribute 79% (162 371) of all reported CL cases [1]. In Algeria, both CL and VL are endemic [2]. Cutaneous leishmaniasis occurs in almost all provinces except those of the central Sahara [3–5]. Visceral leishmaniasis occurs mainly in northern Algeria, but recently reemerging foci in the central Sahara reported the occurrence of VL cases, with an incidence higher than the historical focus in the province of Tizi Ouzou located in the northern part of the country [6–8].

Sand fly fauna of Algeria is composed by 26 species belonging to two genera, *Phlebotomus* and *Sergentomyia* [9]. Local members of the genus *Phlebotomus* are classified into five subgenera: *Phlebotomus* Rondani, 1843, *Paraphlebotomus* Theodor, 1948, *Larroussius* Nitzulescu, 1931, *Transphlebotomus* Artemiev, 1984 recently added to the check-list due to a single record of *Phlebotomus mascittii*,, and newly established subgenus *Artemievus* Depaquit, 2021, which contains only one species, *Ph. alexandri*, previously included in *Paraphlebotomus* subgenus [10, 11]. The genus comprises the proven vectors of *Leishmania* parasites and arboviruses such as *Ph. papatasi*, *Ph. sergenti*, *Ph. perfiliewi*, and *Ph. perniciosus* [12, 13]. The *Sergentomyia* species present in this area belong to four subgenera: *Parrotomyia* Theodor 1958, *Sintonius* Nitzulescu, 1931, *Grassomyia* Theodor, 1958 (sometimes considered as a genus), and *Sergentomyia* França and Parrot 1919 [14, 15]. *Sergentomyia* species are expected to feed mainly on cold-blooded vertebrates and therefore their epidemiological role and involvement in the transmission of *Leishmania* parasites and arboviruses remain unclear [15], even though some studies reported the natural infection of some species with *Leishmania* promastigotes and Toscana virus [16, 17].

The taxonomy of sand flies is traditionally based on decisive morphological characters present on the head (cibarium and pharynx) and genitalia (spermathecae for the females, whole genitalia for males). Their assessment remains the golden standard method used to describe and identify species. However, conclusive species identification of some cryptic species that are morphologically challenging or indistinguishable requires the use of molecular methods such as DNA-based techniques to provide further resolution. Examples from the Maghreb region include *Ph. chabaudi*/*Ph. riouxi* and atypical *Ph. perniciosus*/*Ph. longicuspis* [18–20]. Besides species identification, the determination of the source of the ingested blood in hematophagous insects is an essential parameter to understanding the interaction between sand fly and hosts and their impact on the epidemiology of leishmaniasis to incriminate putative hosts as reservoirs and to design correct and efficient disease control strategies [21]. Recently, two matrix-assisted laser-desorption/ionization time of flight mass spectrometry-based methods, MALDI-TOF MS protein profiling and MALDI-TOF MS peptide mass mapping, were successfully applied in sand fly studies, showing their efficiency in (i) time and cost-effectiveness, (ii) identification and discrimination of specimens to the species level, and (iii) determination of blood meal sources even if the ingested blood load was low and/or in process of digestion [22, 23].

This study aimed to conduct an entomological survey in Ghardaïa and Illizi provinces where CL cases have been steadily increasing since 2017. To understand the role of the local sand fly species in the transmission cycles of *Leishmania*, we identified sand flies morphologically, characterized their DNA barcodes and MALDI-TOF protein profiles, determined the blood meal sources of engorged females, and screened them for *Leishmania* infection.

Methods

Sand fly collections

Ghardaïa and Illizi are two provinces located in north and central Sahara of Algeria, respectively (Fig. 1). They are characterized by a Saharan bioclimatic stage. Ghardaïa, located about 600 km from Algiers, is an oasis in a depression, surrounded by hills. The local human population lives in well-built houses of which some have

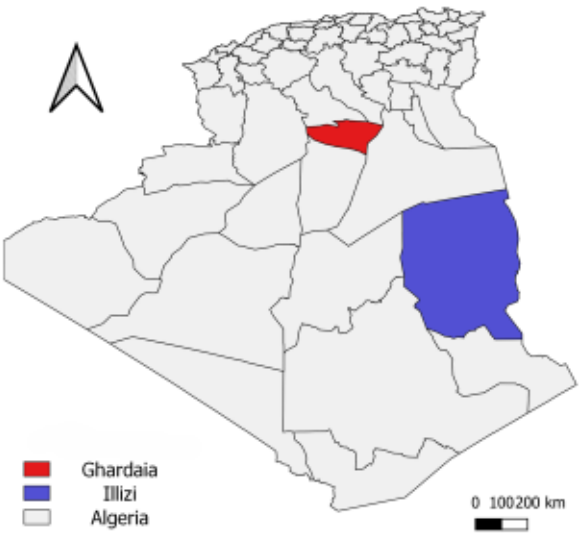


Fig. 1 Location of the sand fly study areas

gardens with animal shelters. Most of the households spend the summertime in their secondary houses located typically in the palmeries to avoid hot summer temperatures, thus coming in direct contact with rodents and sand flies, which increases the risk of parasite transmission. Illizi, located about 2000 km from the capital city of Algeria, is a mix of sandy and rocky regions where the human population lives mainly on pastoral activity by breeding dromedaries, sheep, and goats. The human habitations and animal shelters are huts built with mud and stones, thus providing favorable environmental conditions for sand fly breeding and development.

In June and October 2021, sand flies were trapped for 5 nights in each province using ten CDC miniature light traps (model 512 John W. Hock Company, Gainesville, FL, USA). In Illizi, the traps were set in human dwellings and animal shelters (sheep, goats, and camels), and in Ghardaïa, the traps were set in a zoo near animal shelters (chicken, birds, dogs, wolves, hyena), empty cages, and burrows in the hill surrounding the zoo. The traps were set at 2 m above the ground from sunset to sunrise. The collected sand flies were anesthetized with cigarette smoke and pooled by site and date of collection. The samples were conserved and stored in 70% ethanol at +4 °C until further analysis. The map of the study areas (Fig. 1) was performed using QGIS version 3.20.3-Odense [24].

Morphological identification of sand flies

Head and 2–3 terminal segments of the sand fly abdomens were dissected and slide-mounted in a drop of CMCP-10 mounting medium (Polysciences, Inc., Warrington, PA, USA) for morphological identification following the identification keys described in [9, 25, 26]. The thoraxes and the rest of the abdomens were conserved in 70% ethanol at +4 °C for molecular and MALDI-TOF MS analysis. Images of the specimens and measurements of decisive morphological characters were taken and drawn using an Olympus BX51 microscope equipped with drawing tube. The morphological description of the new species was performed according to Galati et al. [27].

DNA-based identification of sand flies

DNA was isolated from the sand fly abdomens with High Pure Template Preparation Kit (Roche, Germany) following the manufacturer's instructions. Morphological identification was confirmed by sequencing two different targets of the mitochondrial genome: cytochrome oxidase I (Cox I) or cytochrome B (Cyt B), which was used as an alternative genetic marker in the case that Cox I gene amplification failed. Polymerase chain reaction (PCR) amplification of both markers was performed in 50 µl reaction volume using LCO 1490/HCO 2198 and the Cyt B CB3-PDR/N1N-PDR primers pairs and amplification conditions previously described [28, 29]. The amplification products were first separated and visualized on 2% agarose gel, then purified using a High Pure PCR product Purification kit (Roche). Double-stranded Sanger sequencing was performed using the same amplification primers (ABI Prism BigDye terminator Cycle Sequencing Ready Reaction Kit). The data were edited in BioEdit v.7.2.5 [30] and the generated sequences of Cox I gene were deposited in GenBank database (accession numbers given in Results). Each sequence was blasted against the GenBank database for identification. The sequence was considered correct if it matched > 98% of resemblance, *E*-value equal to 0 and rate coverage equal to 100%.

Molecular screening for *Leishmania* infection in sand flies

Extracted DNA was either tested individually or pooled (2–12 sand flies of the same species and location per pool). The screening for *Leishmania* infection was done by the amplification of conserved sequences of the *Leishmania* kinetoplast minicircle DNA 13A/13B. The amplification reactions were carried out in a total volume of 25 µl containing 12.5 µl of Dream Taq Master Mix, 8.5 µl of deionized water, 1 µl of each primer, and finally 2 µl of DNA template and subjected to the following PCR conditions: an initial denaturation step at 94 °C for 3 min followed by 40 cycles of 94 °C for 20 s, 61 °C for 30 s, 72 °C for 7 s, and terminated with an extension step at 72 °C for 5 min [31]. Negative (DNA-free water) and positive (*L. major* DNA) controls were included for PCR assays. The amplified PCR products were separated and visualized on 2% agarose gel under a UV illuminator.

Data analysis

The sequences were aligned using the MUSCLE alignment algorithm. In addition to our sequenced specimens, five *Se. cincta* Cox I sequences originating from Cameroon were obtained from GenBank database (MH577067, MH577068, MH577069, MH577087, MH577094) and used in the analysis. Maximum likelihood (ML) tree was built, and the nearest-neighbor interchange tree search method was performed with 1000 bootstrap replicates under assumptions of the Tamura 3-parameter model (T92 + I), the best fit evolutionary model as defined in Mega v10.1.7 [32]. The different haplotypes were inferred using DnaSp v6.12.03 [33] and parsimony network using TCS method was generated in PopArt [34]. The obtained results were also compared by ABGD using K2P distances generated through pairwise sequence comparisons using the default settings of *P*-values (0.1–0.001) in the Automatic Barcode Gap Discovery (ABGD) web-interface program (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html), which was used to assign each barcode sequence into molecular operational taxonomic

units (MOTUs) [35]. Pairwise Kimura 2-parameters genetic distances were obtained for Cox I dataset under Mega v10.1.7 [32].

Species identification by MALDI-TOF MS protein profiling

MALDI-TOF MS protein profiling was carried out as previously described [22]. Briefly, the thoraxes were air-dried and then manually ground in 10 µl of 25% formic acid by a BioVortexer homogenizer (BioSpec, Bartlesville, USA) using disposable pestles for 15 s. After a short centrifugation of the homogenate (10,000 × g for 15 s), 2 µl of the supernatant was mixed with 2 µl of MALDI matrix and 1 µl of this solution was deposited on a MALDI target (Bruker Daltonics, Bremen, Germany) in duplicate. The MALDI matrix was freshly prepared as an aqueous 60% acetonitrile/0.3% TFA solution of sinapinic acid (30 mg/ml; Bruker Daltonics). Protein mass spectra were measured on an AutoFlex Speed MALDI-TOF spectrometer (Bruker Daltonics) within a mass range of 3–25 kDa, calibrated externally using the Bruker Protein Calibration Standard I and visualized by FlexAnalysis v.3.4 (Bruker Daltonics). Each spectrum corresponded to an accumulation of 8000 laser shots (40 × 200 laser shots from different positions of the sample spot). For cluster analysis and species identification, the spectra were processed by MALDI Biotyper v.3.1 (Bruker Daltonics) and searched against an in-house reference database comprising reference protein profiles of 35 different sand fly species including five members of the subgenus *Sergentomyia* (*Se. antennata*, *Se. dentata*, *Se. fallax*, *Se. minuta*, and *Se. schwetzi*). Log score value (LSV) > 2.0 was considered as a threshold for unambiguous species identity assignment.

Blood meal analysis by peptide mass mapping MALDI-TOF mass spectrometry

The air-dried abdomens were homogenized individually in 50 µl of ultra-pure water (Merck KGaA, Darmstadt, Germany) by BioVortexer homogenizer (BioSpec) for 15 s; 10 µl of the homogenate were then incubated with 10 µl of 50 mM N-ethylmorpholine acetate buffer (pH 8.1; Sigma-Aldrich) and 100 ng of trypsin (Promega) at 37 °C for 30 min. After the digestion, 0.5 µl of the mixture was deposited on a MALDI plate in duplicate, air-dried, and covered with 0.5 µl of MALDI matrix (aqueous 50% acetonitrile/0.1% TFA solution of α-cyano-4-hydroxycinnamic acid; 5 mg/ml; Bruker Daltonics). Simultaneously, the remaining 40 µL of the abdomen homogenates were utilized as a template for DNA isolation by High Pure PCR Template Preparation Kit (Roche, Germany) following the manufacturer's protocol. The isolated DNA was screened for presence of *Leishmania* sp. in the ingested blood. Peptide mass mapping (PMM) spectra were acquired on an Ultraflex III MALDI-TOF instrument (Bruker Daltonics) in the mass range of 700–4000 Da and calibrated externally using a PepMix II peptide standard (Bruker Daltonics). For the tentative host assignment, at least two peptides per specimen were selected for MS/MS sequencing. The obtained spectra were searched against SwissProt database subset of vertebrate hemoglobins using in-house MASCOT v.2.7 search engine (Matrix Science).

Results

Sand fly species composition

A total of 640 sand flies were collected in the two study areas; 430 specimens from Illizi (villages: Ifni, Imoussouane, Indgidad-Imihrou, and Tihoubar-Imihrou) and 210 from Ghardaïa (districts: Ghardaïa and El Atteuf). A total of 14 different species belonging to *Phlebotomus* and *Sergentomyia* genera, previously found in Algeria, were identified in our study (Table 1). El Atteuf, Ifni, and Tihoubar were the most diversified sites with ten, seven, and six different species, respectively. In Illizi, *Ph. papatasi* was the most abundant (260 specimens), followed by *Ph. alexandri* (76 specimens), while in Ghardaïa, *Se. antennata* was the most common species (78 specimens), followed by *Se. fallax* (35 specimens). Interestingly, one female specimen collected in Illizi belonged to the genus and subgenus *Sergentomyia* but showed unique morphological features that could not be attributed to any described species so far.

Sequencing analysis

PCR amplification of Cox I was successful for all studied species except for *Se. clydei*, for which the amplification attempts of the Cox I gene repeatedly failed for all analyzed specimens. Cyt B gene was successfully amplified for the unknown *Sergentomyia* female in addition to *Se. clydei*, for which Cox I amplification failed. Cox I generated sequences of 680 pb for all the species except *Se. christophersi* (661 bp) and *Se. imihra* (687 bp). After alignment and trimming of the sequences generated from the specimens belonging to the *Sergentomyia* subgenus, a ML tree was built using the Cox I marker to solve the identity of the new *Sergentomyia* specimen (Fig. 2). *Se. christophersi* (OQ194119) was used as an outgroup. The ML tree showed the presence of six distinct clusters: *Se. minuta* (OQ194093 to OQ194095), *Se. fallax* (OQ194092, OQ194101 to OQ194104, OQ194111), *Se. schwetzi* (OQ194114 to OQ194118), *Se. antennata* (OQ194091, OQ194096 to OQ194100, OQ1940105 to OQ194110, OQ194112, OQ194113), *Se. cincta* (MH577067, MH577068, MH577069, MH577087, MH577094), and *Sergentomyia* sp. (ON548470), which we further characterized and formally described as

Table 1 Sand flies from Illizi and Ghardaïa identified by morphological and molecular assessment

Province	Region	Genus	Subgenus	Species	Male	Female	Total
Illizi	Ifni (25.77069°N, 7.93326°E)	*Phlebotomus*	*Phlebotomus*	*papatasi*	2	8	10
				bergeroti	8	3	11
			Larroussius	*perniciosus*	0	1	1
			Artemievus	*alexandri*	41	27	68
		Sergentomyia	*Sergentomyia*	*antennata*	13	13	26
			Sintonius	*clydei*	4	14	18
				christophersi	6	1	7
	Indgidad-Imihrou (25.80916° N, 8.68107° E)	*Phlebotomus*	*Phlebotomus*	*papatasi*	1	12	13
			Artemievus	*alexandri*	1	2	3
		Sergentomyia	*Sintonius*	*clydei*	0	2	2
	Imoussouane (26.38665°N, 8.54841°E)	*Phlebotomus*	*Phlebotomus*	*papatasi*	51	117	168
		Sergentomyia	*Sergentomyia*	*schwetzi*	1	4	5
			Sintonius	*clydei*	6	12	18
	Tihoubar-Imihrou (25.94156°N, 8.72736°E)	*Phlebotomus*	*Phlebotomus*	*papatasi*	7	62	69
			Artemievus	*alexandri*	0	5	5
		Sergentomyia	*Sergentomyia*	*antennata*	1	0	1
				imihra n. sp.	0	1	1
			Sintonius	*clydei*	1	1	2
				christophersi	2	0	2
		Total			145	285	430
Ghardaïa	Ghardaïa (32.511310°N, 3.627452°E)	*Phlebotomus*	*Phlebotomus*	*papatasi*	1	0	1
		Sergentomyia	*Sergentomyia*	*minuta*	1	0	1
				fallax	2	0	2
			Sintonius	*clydei*	1	0	1
	El Atteuf (32.439935°N, 3.715423°E)	*Phlebotomus*	*Phlebotomus*	*papatasi*	20	7	27
			Larroussius	*perniciosus*	1	5	6
				longicuspis	4	2	6
			Paraphlebotomus	*sergenti*	30	0	30
		Sergentomyia	*Sergentomyia*	*antennata*	58	20	78
				fallax	21	12	33
				minuta	1	2	3
			Sintonius	*clydei*	12	4	16
				christophersi	5	0	5
			Grassomyia	*dreyfussi*	0	1	1
		Total			157	53	210

Sergentomyia imihra n. sp. The ABGD software using the prior intraspecific divergence threshold value of 1.29% showed the same result (Additional file 1: Fig. S1) and resolution of the species order in the phylogenetic tree (Fig. 2A). Furthermore, the haplotype analysis (Additional file 2: Fig. S2) revealed the presence of 29 different haplotypes (haplotype diversity, $Hd = 0.9911$). The TCS network showed the presence of six haplogroups: *Se. minuta*, *Se. fallax*, *Se. schwetzi*, *Se. antennata*, *Se. cincta*, and *Se. imihra* n. sp. Interestingly, the TCS tree also supported the validity of *Se. imihra* n. sp., which was branched between *Se. fallax* and *Se. antennata*, 61 substitutions discriminating it from *Se. antennata*, 70 from *Se. fallax*, 68 from *Se. cincta*, 127 from *Se. minuta*, and 138 from *Se. schwetzi*. The results (Fig. 2B) confirmed its status as a new species and not a morphological malformation often reported within the *Sergentomyia* genus. The genetic distance between *Se. imihra* n. sp. and the rest of the subgenus *Sergentomyia* species varied between 0.160 and 0.189 (Additional file 3: Table S1). Furthermore, congruent assignment of species was seen between ABGD, TCS network, and ML tree. In addition, *Se. antennata* specimens, which were grouped in a single clade in ML analysis while they were assigned to two

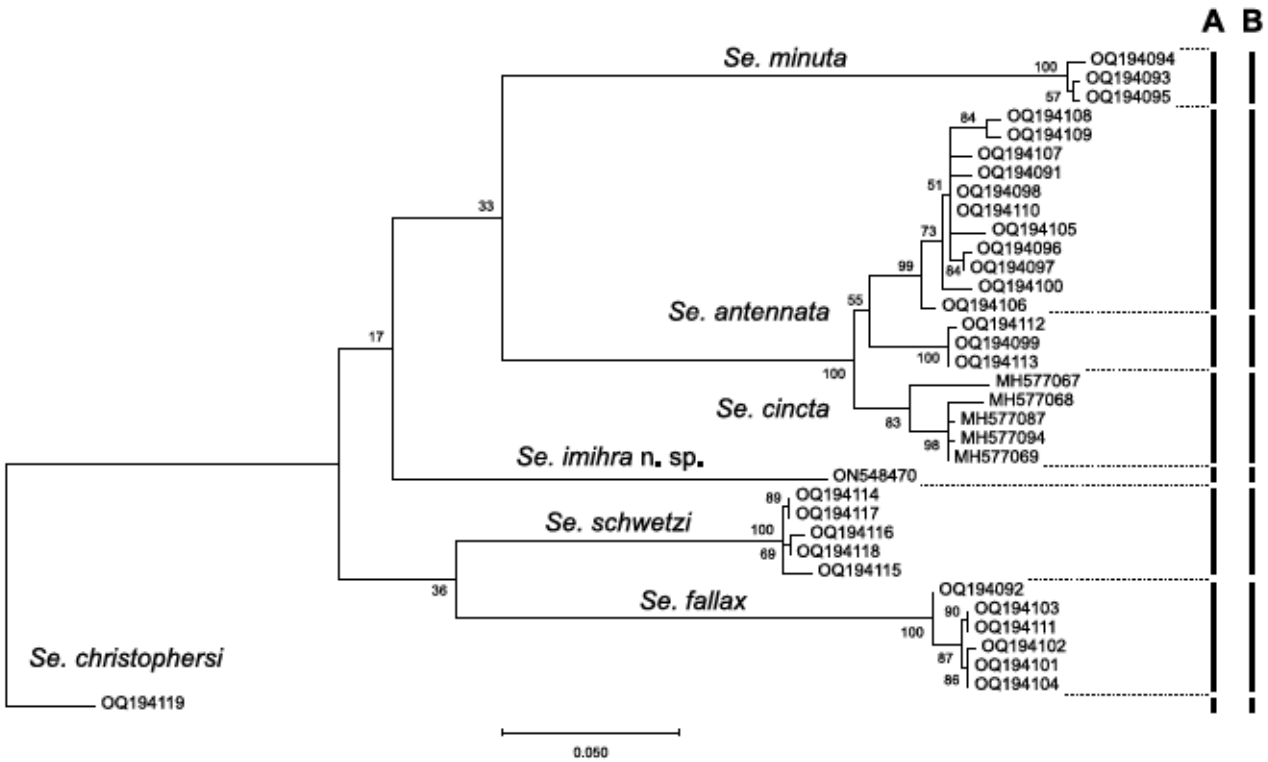


Fig. 2 Maximum likelihood tree of *Sergentomyia* Cox I gene, 1000 bootstrap replicates. **A** ABGD result, **B** TCS haplotype network

clusters by ML tree, ABGD, and TCS network, showed a genetic distance ranging from 0.037 to 0.056 between three specimens: OQ194099, OQ194113 (females trapped in Illizi), and OQ194112 (male trapped in Ghardaïa) and the rest of *Se. antennata* (Additional file 4: Table S2). The statistical analysis performed in DnaSP using *Fu and Li's* $D^*=0.20764$ ($P>0.10$) and *Fu and Li's* $F^*=0.10483$ ($P>0.10$) [36] did not show significant difference among analyzed *Se. antennata* specimens. Similarly, morphological identification and MALDI-TOF MS protein profiling did not show any difference.

Molecular screening for *Leishmania* infection in sand flies

A total of 112 females (53 form Ghardaïa, 59 from Illizi) were subjected to *Leishmania* screening; 47 were engorged and 65 specimens were unfed. The results showed that all analyzed females were negative for *Leishmania* parasites (Additional file 5: Table S3).

Species identification by MALDI-TOF MS protein profiling

Of all trapped sand flies, 110 specimens were subjected to MALDI-TOF MS analysis either for species identification (72) or blood meal origin determination (38); 29 of those 72 specimens were identified by both morphology and DNA sequencing, and 33 were assigned solely by morphology (Additional file 6: Table S4). The remaining ten specimens were not reliably identified either by molecular analysis or morphology due to missing or poorly visible decisive morphological characters or failure of PCR amplification or sequencing. Of 72 specimens analyzed by MALDI-TOF protein profiling, 70 provided high-quality spectra suitable for further evaluation. Only two specimens (DZ36, DZ38) gave spectra of poor quality, yielding no species identification. The protein profiles of some sand flies conclusively determined by DNA sequencing and/or morphologically were used to upgrade our in-house reference database [*Se. fallax* (2), *Se. antennata* (9), *Se. dreyfussi* (1), *Se. imihra* n. sp. (1), *Se. christophersi* (3), *Se. clydei* (9), *Ph. alexandri* (9), *Ph. bergeroti* (6)]. The species assignment obtained by comparison of protein profiles with the upgraded database nicely matched with the species identity of 60 specimens uncovered earlier by DNA sequencing and/or morphologically. The protein profiling further corrected the species determination of five specimens—DZ11, DZ12, DZ23, DZ29, and DZ81—and confirmed DNA sequencing result of DZ24 and DZ25, which were incorrectly determined by morphology as *Se. fallax* (Additional file 6: Table S4). Interestingly, the result of protein profiling was also congruent with the molecular identification of the novel species *Se. imihra* n. sp., which formed a separate branch that was distinct from other analyzed species of

Sergentomyia subgenus and positioned close to the clusters of *Se. antennata* and *Se. fallax* in the dendrogram (Fig. 3A). In addition, the protein profile of *Se. imihra* n. sp. was obviously different from the spectra of four other members of *Sergentomyia* subgenus showing a number of species-specific peaks (Fig. 3B). This technique further supported the proposition of including *Ph. alexandri* into the new subgenus *Artemievus* [11]. In the dendrogram, all *Ph. alexandri* specimens grouped unequivocally into a single and distant cluster from *Ph. sergenti* (Fig. 4), which belongs to *Paraphlebotomus* subgenus. The appearance of two branches for *Ph. papatasi* could be explained by the compromised spectra quality of some specimens.

Blood meal analysis by PMM-based MALDI-TOF mass spectrometry

A total of 38 blood-fed sand fly females (4 from Ghardaïa and 34 from different villages of Illizi) were analyzed individually using PMM-based MALDI-TOF mass spectrometry to determine the origin of their blood meals. Before PMM analysis, the engorged females were taxonomically classified using decisive morphological characters [9], resulting in four different species: *Ph. papatasi* (31), *Ph. alexandri* (5), *Ph. longicuspis*, (1) and *Ph. perniciosus* (1). The PMM-based MALDI-TOF MS approach successfully determined blood meal origin in 87% (33/38) of the engorged females (Additional file 7: Table S5). For five samples, the analysis did not provide any host identification, probably due to the small volume of blood taken or the advanced blood digestion. However, the majority of the analyzed females yielded spectra of high quality, allowing for conclusive blood meal source assignment. Altogether, eight different hosts were identified: camel (*Camelus dromedarius*), dog (*Canis lupus familiaris*), sheep (*Ovis aries)*, goat (*Capra hircus*), chicken (*Gallus gallus*), human (*Homo sapiens*), donkey (*Equus asinus*), and horse (*Equus caballus*). The most numerous blood meal source (Additional file 8: Table S6) was goat (13), followed by camel, dog, and human (5); sheep and donkey (2); and finally, chicken and horse (1). In all successfully identified samples, *Ph. papatasi* showed opportunistic behavior with six different hosts and one mixed blood meal (human + goat) followed by *Ph. alexandri* with five different hosts. Only one blood-fed female of *Ph. perniciosus* and one *Ph. longicuspis* were collected and analyzed, both blood meals identified as originating from a donkey.

Morphological description of *Sergentomyia imihra* n. sp.

The evaluation of the decisive morphological characters as well as findings of molecular analyses described above suggest the presence of a new species belonging to the genus *Sergentomyia* França and Parrot, 1920, and subgenus *Sergentomyia*, defined by having (i) recumbent hairs on the abdominal tergites, (ii) tubular spermathecae with smooth walls of uniform width along their length, and (iii) antennal segment 3 is shorter than segments 4 and 5 together and usually shorter than the labrum [14, 25, 37].

Family Psychodidae Newman, 1834
Genus *Sergentomyia* França and Parrot, 1920
Subgenus *Sergentomyia*
Species *Sergentomyia imihra* n. sp. Benallal, Depaquit and Dvořák, 2024

Type locality: Tihoubar-Imihrou (25°0.94365N, 8°0.72767 E) is a rocky region located in south of the province of Illizi, central Sahara, South-Eastern Algeria.

Type material: Holotype female has been deposited in the Institut Pasteur of Algeria (IPA) under identification number 472.

Representative DNA sequences: GenBank accession numbers: ON548470 (Cox I) and OQ190728 (Cyt B).

ZooBank registration: To comply with the regulations set out in Article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN) [38], details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:673E442C-ED4E-4C44-942C-41FF8BD6FF00. The LSID for the new species *Sergentomyia imihra* is urn:lsid:zoobank.org:act:C420A58D-8A10-4348-AE33-E524E2A13594.

Note: The authors of the new taxa are different from the authors of this paper: Article 50.1 and Recommendation 50A of the International Code of Zoological Nomenclature.

Etymology: The species is dedicated to the village where the specimen was trapped.

Description

Female. The counts and measurements provided below are those of the holotype labeled *Se.* 472 and deposited at IPA (Fig. 5, Additional file 9: Fig. S3) *Head*. Occiput with two narrow lines of well-individualized setae. On the line above the eyes, insertion of seta on each side. Clypeus 115 µm long with 30 setae randomly distributed. Eyes 221 µm long, 148 µm wide. Interocular sutures complete reaching the interantennal one. Flagellomeres: f1 (= AIII) = 100 µm shorter than f2 (= AIV) = 57 µm + f3 (= AV) = 58 µm, f12 (= AXIV) = 40 µm, f13 (= AXV) = 36 µm and f14 (= AXVI) = 50 µm. Ascoidal formula: 2/f1-f11 & 1/f12-f13 with short ascoids, covering three-fourths of the article. One papilla one flagellomeres 1 and 2. Absence of papilla on the third flagellomere. Palpi: p1 = 20 µm long; $_p$2 = 70 µm; $_p$3 = 112 µm; $_p$4 = 105 µm, $_p$5 = 197 µm. Palpal formula: 1, 2, 4, 3, 5. About 38

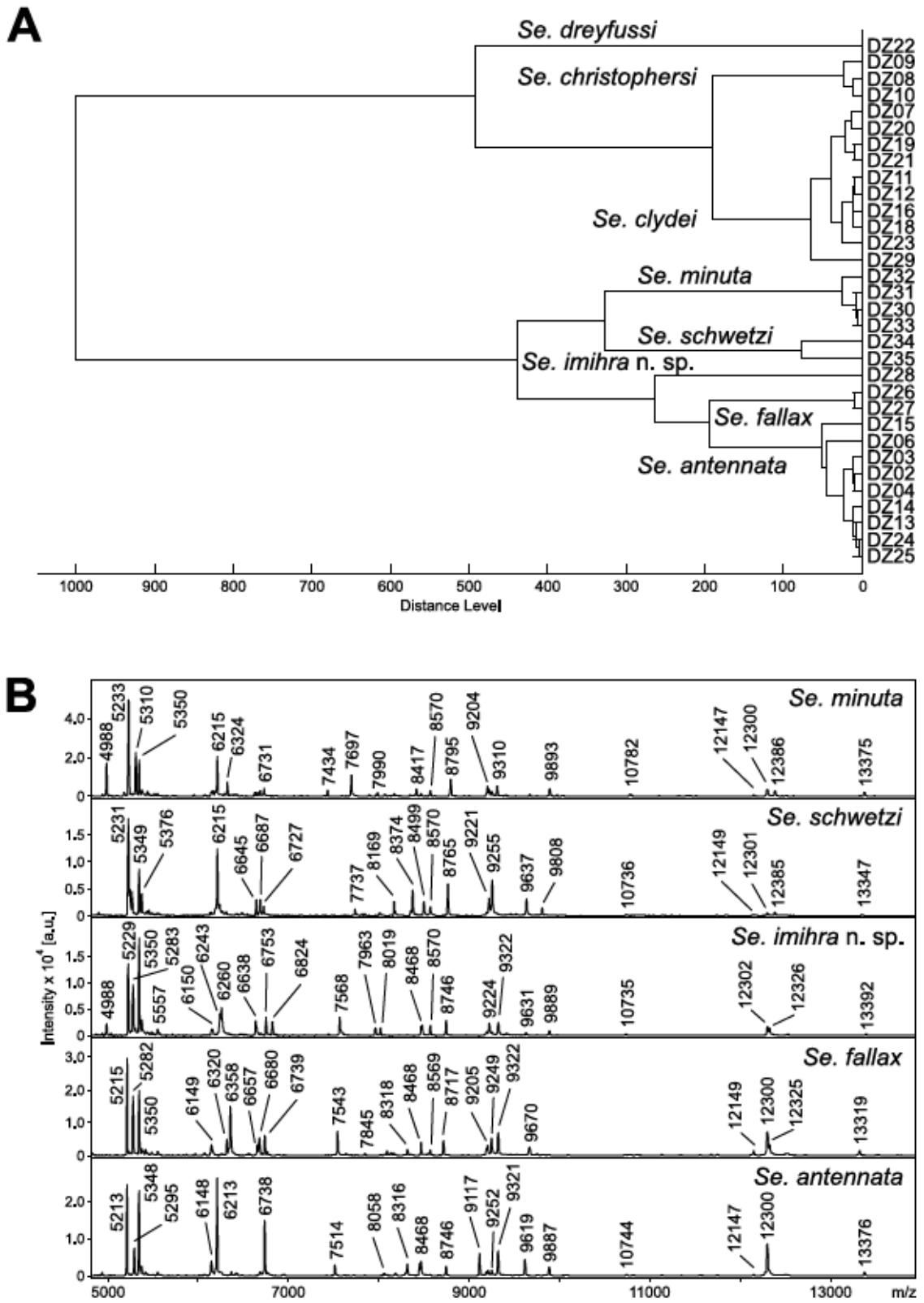


Fig. 3 **A** Dendrogram of MALDI-TOF MS protein profiles of eight *Sergentomyia* species collected in Algeria. The distances are in relative units. **B** Comparison of mass spectra of *Se. imihra* n. sp. with four other members of the *Sergentomyia* subgenus found in the Central Sahara of Algeria

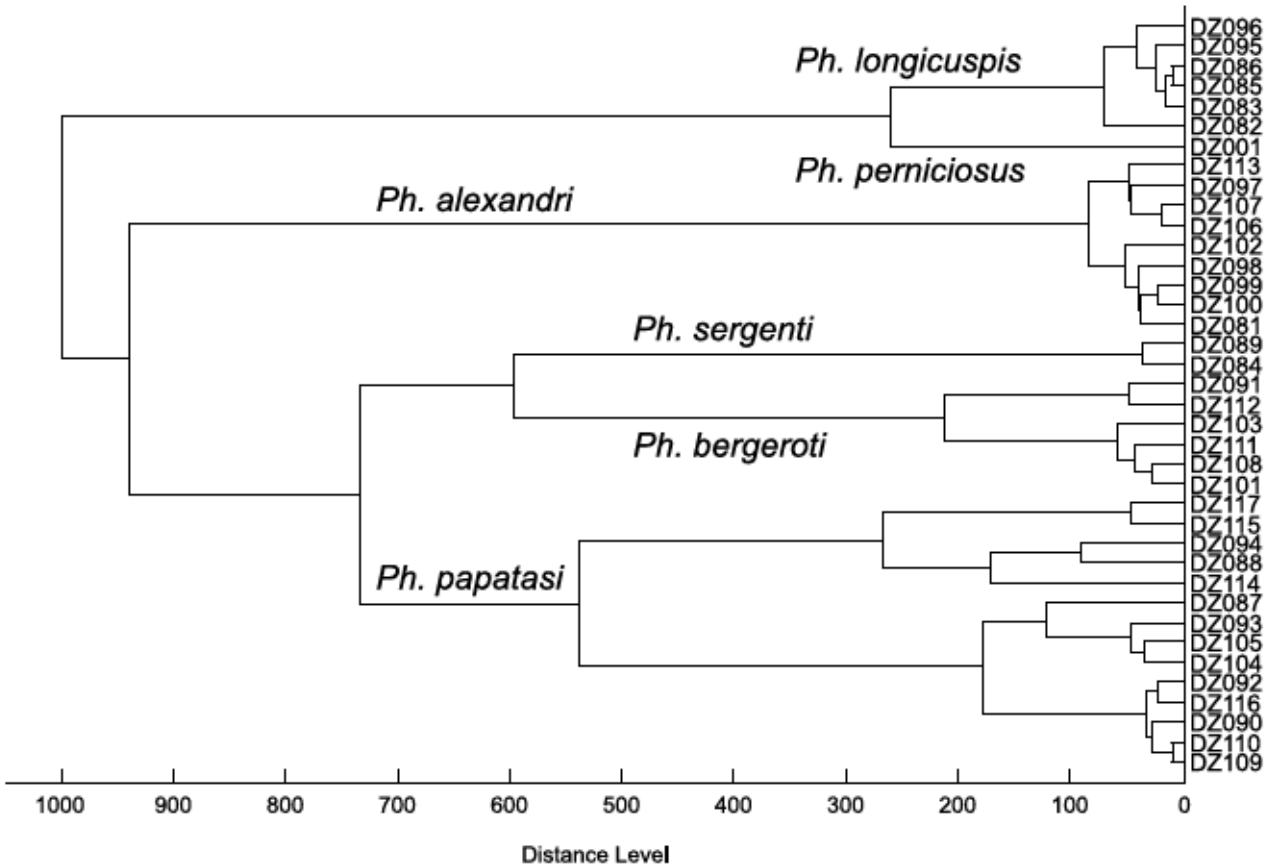


Fig. 4 Dendrogram of MALDI-TOF mass spectra of six *Phlebotomus* species collected in Algeria. The distances are shown in relative units

Newstead's sensilla are visible in the third palpal segment, part of a larger group. No Newstead's sensilla on other palpal segment. Labrum-epipharynx 160 µm long. f1/L = 0.625. Maxillary lacinia exhibiting 4 external teeth and 30 fine internal teeth. More than 50 teeth on the mandible. Hypopharynx with a dozen of wavy teeth. Cibarium is covered with a small heterogeneously pigmented sclerotised area, and one row of 16 concave sharply pointed teeth oriented backward. Pharyngeal armature is well developed, occupying the last third of the pharynx, made with large teeth oriented forward. *Thorax, wings, abdomen, and legs.* Not observed due to molecular processing. *Genitalia.* Spermathecae are tubular with smooth and thin walls of uniform width along their length, length = 95 µm, width = 17 µm.

Detailed morphological analysis of this new species provided morphological features distinguishing it from the phylogenetically closely related species *Se. cincta* described by Abonnenc and Parrot [25, 39], *Se. antennata*, and *Se. sintoni* [40] as summarized in Table 2.

Discussion

Sand fly fauna in Ghardaïa and Illizi, two Algerian provinces with emerging cases of human cutaneous leishmaniasis, was described by combined morphological and molecular approaches. In total, we recorded 14 sand fly species, 6 belonging to *Phlebotomus* genus and 8 *Sergentomyia* genus. Species composition and abundance of recorded species differed among the two studied foci. All identified species of the genus *Phlebotomus*, belonging to four different subgenera, are known to be either proven or potential vectors of several *Leishmania* species that are causative agents of human leishmaniases [41]. In our study, the PCR screening for *Leishmania* spp. was negative for the proven vectors of *Ph. papatasi*, *Ph. sergenti*, and *Ph. perniciosus* [6, 42, 43], as well as for all screened species of the genus *Sergentomyia* expected to transmit *Sauroleishmania* spp. [44]. Vector competence of *Sergentomyia* species for human parasites remains hypothetical. Thus far, performed assays deploying experimental infections disapproved this hypothesis using specimens from laboratory colonies [45, 46]. However, the vast majority

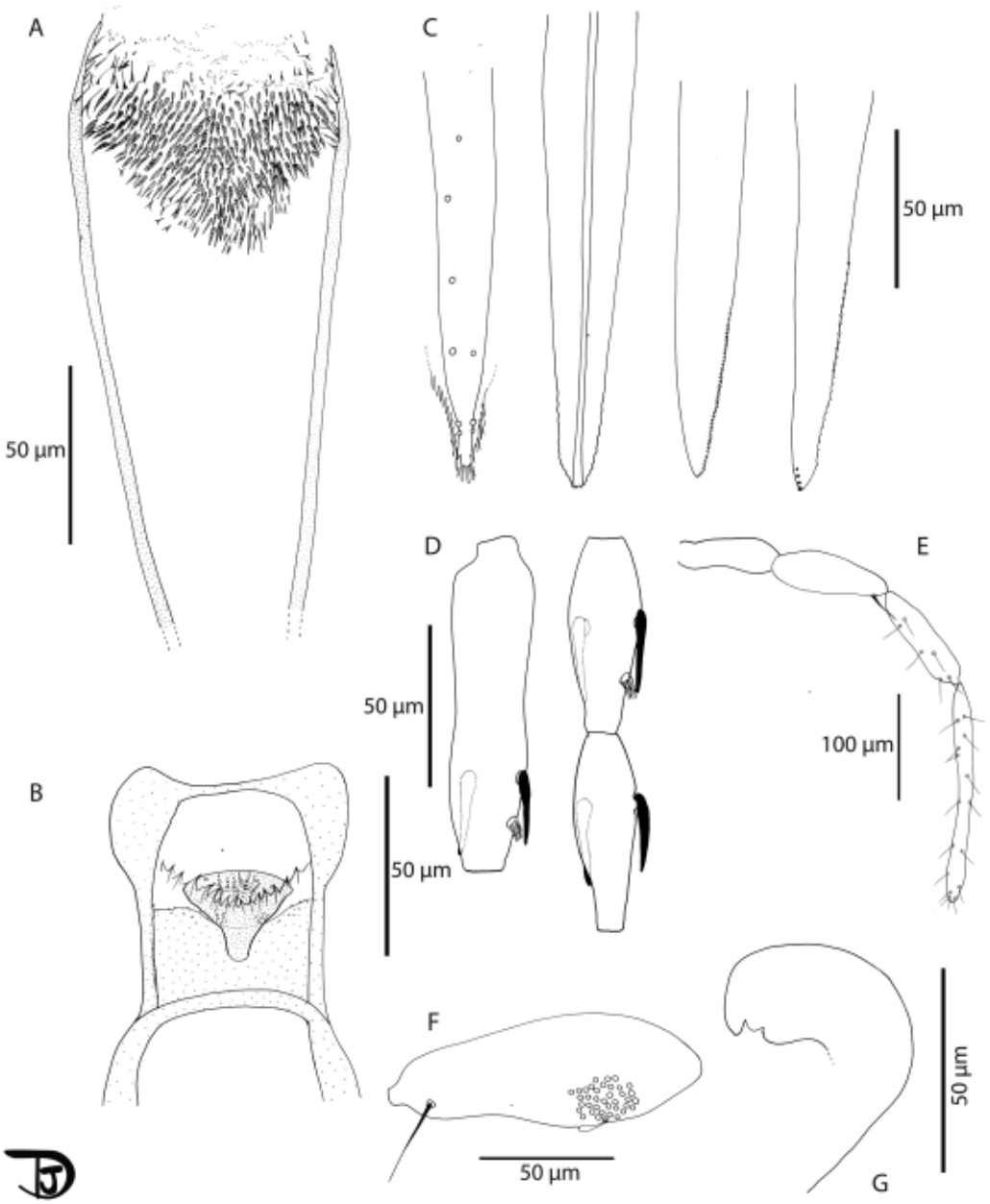


Fig. 5 Morphological description of *Sergentomyia imihra* n. sp. female. **A** Pharynx. **B** Cibarium. **C** Mouthparts (labrum, hypopharynx, mandible, and maxilla). **D** Flagellomeres 1, 2, and 3. **E** Labium: palpi. **F** Detail of the third article of the palpi. **G** Spermatheca

of species of the genus *Sergentomyia* cannot be bred in captivity, hence making the experimental testing of their vector competence impossible [47]. *Sergentomyia minuta* is suspected to play a role in the epidemiology of leishmaniasis and arboviruses in the African continent in relation to its distribution and feeding habits [16, 17].

We recorded eight species of the *Sergentomyia* genus, which is known as the most diversified among Old World sand flies, harboring 276 species [14]. In Algeria, 12 species of the genus have been recorded [9]; *Se. hirtus* is the last described species in the central Sahara [48] but its morphological features are sometimes regarded as a malformation [26]. Recent systematics of this genus recognizes seven subgenera on the basis of their spermathecal morphology: *Sergentomyia* with smooth, thin-walled, and wide spermathecae; *Sintonius* Nitzulescu, 1931; *Trouilletomyia* Depaquit and Léger, 2014, with annealed spermathecae; *Parrotomyia* Theodor, 1958, with elliptical

Table 2 Summary of morphological differences between *Se. imihra* n. sp. and the phylogenetically closely related *Sergentomyia* species

Character	*Se. imihra* n. sp. Current study	*Se. dncta* [25]	*Se cincta* [39]	*Se. antennata* [25]	*Se. sintoni* [40]
Ascoidal formula	2/f1–f11 and 1/f12–f13	*2/f1–f13*	*2/f1–f13*	*2/f1–f13*	N/I
Flagellomere	f1:100 µm	*f1:78–88 µm*	f1:105–120 µm	f1: 80–100 µm	*f1:142–152 µm*
Palpal formula	p1: 20 µm; p2: 70 µm; p3: 112 µm; p4:105 µm; p5:197 µm Total length: 504 µm	Total length: 490–510 µm	*Total length: 205 µm*	Total length: 560 µm	N/I
Cibarium	16 sharply pointed teeth and about 4–6 denticules on each side	14–18 short sharply pointed teeth and about 5–8 denticules on each side	16–20 sharply pointed teeth and about 5–6 denticules on each side	*22–34 sharply pointed, mono-morph teeth displayed on a backward concave shape, covered with a triangular pigmented patch area, including one row of 4–6 denticules on each side*	*10–14 pointed teeth, without dentides*
Pharyngeal armature	Well developed, occupying the last third of the pharynx, displaying sharp and large teeth	*Helmet-like shape, anterior pharynx about three times larger backward than forward carrying strong and tight brush-like spines*	*Heart-shaped, three times larger backward than in front*	*Large backward and displayed rounded and strong denticules*	*Heart-shaped*

Distinguishing characters are in italics. N/I, not indicated

capsule, smooth, thin, or thick-walled spermathecae; *Rondanomyia* Theodor, 1958, with smooth and wide spermathecae; *Capensomyia* Davidson, 1979, with convoluted spermathecae; and *Vattieromyia* Depaquit, Léger, and Robert, 2008. Subgenus *Grassomyia* Theodor, 1958, which exhibits round, sclerotized capsules with small spicules spermathecae, is considered by some authors as a genus [14].

Our complex effort enabled recognition and formal description of a new species *Sergentomyia (Sergentomyia) imihra* n. sp. Benallal, Depaquit and Dvořák, 2024, within the subgenus *Sergentomyia*, as suggested by presence of smooth spermathecae [14, 25]. Unique identification of the newly described species, its classification and distinction from other species of *Sergentomyia* subgenus, has been successful thanks to a combination of morphological and molecular tools as recently suggested in the description of *Phlebotomus betisi* and the new subgenus *Lewisius* [49]. Moreover, the species status of *Se. imihra* n. sp. is supported by sufficiently divergent sequences of mitochondrial Cox I and Cyt B genes recommended as molecular markers to confirm the species identification [29, 50]. Blast analysis of Cox I sequence in GenBank showed that *Se. imihra* n. sp. presented 89.25% and 88.66% of similarity with *Se. punjabensis* and *Se. inermis*, respectively, while the CytB sequence showed 91.35% and 94.61% of similarity with *Se. dubia* and *Se. sintoni*, respectively. Although the bootstrap values in the ML tree were quite low, the genetic distance, which is high between *Se. imihra* n. sp. and the rest of the analyzed species (16.8–18.9%), the clear morphological differences, and also unique and species-specific protein profile distinct from other analyzed members of the *Sergentomyia* subgenus support that the newly described sand fly species conclusively differs from the known species of the subgenus *Sergentomyia* thus far.

During the last decade, MALDI-TOF MS protein profiling has become a method of choice for conclusive, rapid, and cost-effective species identification of various arthropod vectors including sand flies [22, 23], especially suitable for field-collected specimens from large-scale entomological surveys. In the current study, this mass-spectrometry-based technique provided high-quality and reproducible spectra yielding reliable species assignment of all except two analyzed specimens, while allowing for parallel application of other complementary approaches (DNA barcoding, morphological analysis) and screening for sand fly-borne pathogens. As was recently demonstrated for *Ph. creticus* and two other sand fly species from Laos [49, 51], MALDI-TOF MS protein profiling represents a useful tool in integrative taxonomy, and in this study supported the formal description of the newly discovered species *Se. imihra* n. sp. and further elucidated taxonomical classification of other species. Albeit not always reflecting the phylogenetic relationships within the subgenera, as shown by clustering of *Ph. sergenti* and *Ph. bergeroti* groups, the method also supported the inclusion of *Ph. alexandri* into the new subgenus [11].

Blood meal analysis is one of key steps to correctly understand trophic inclinations of various hematophagous insects, their relationship with hosts, and their roles in the eco-epidemiology of the zoonotic diseases that they transmit. Therefore, a wide array of different methods including precipitin tests, enzyme-linked immunosorbent assay (ELISA), and sequencing of mitochondrial and nuclear DNA markers were deployed to study the origin of blood meal sources [52–56]. However, success of host blood identification is challenged by various factors such as the low amount of ingested blood, its advanced digestion, possible contamination of samples, and varying time, labor, and cost investments of the analysis. Recently, PMM-based MALDI-TOF mass spectrometry allowed for a significant reduction in cost and time by a simple sample preparation, and also showed its efficacy in the determination of the blood sources, even for mixed origin, advanced blood digestion, and low amount of blood [23, 57]. In our study, this technique provided a high rate of reliable identifications and even enabled the detection of mixed blood meal for one *Ph. papatasi* specimen. We conclude that this species as well as *Ph. alexandri* can be both zoophilic or anthropophilic, suggesting that the opportunistic behavior depends on the presence of different hosts and their proximity to breeding sites. The identification of several mammalian blood sources such as camel, sheep, goat, donkey, horse, and chicken in the study areas suggests potential zooprophylactic barrier reducing human–vector contact as previously reported in the northern part of Algeria, where cattle was the main blood source identified [57]. This shall be considered for potential vector control measures and transmission risk assessment.

Conclusions

This study provided new data on sand fly fauna in Ghardaïa and Illizi, regarding the species composition and the trophic relationships of hematophagous females with their animal hosts. Integrative approach that combined morphological analysis, sequencing of DNA markers, and protein profiling enabled the recognition and description of a new *Sergentomyia* species, raising the number of the Algerian sand fly fauna to 27 species. Further sand fly surveillance in the central Sahara is recommended to identify the thus-far unknown males of *Se. imihra* n. sp., to further elucidate the extent of the species presence in the central Sahara, and search for

natural infections by *Leishmania* sp. within the sand fly fauna to understand the vector involved in the parasite transmission in the foci of emerging importance.

Abbreviations

ABGD	Automatic barcode gap discovery
Cox I	Cytochrome *c* oxidase subunit 1
Cyt B	Cytochrome B
CL	Cutaneous leishmaniasis
IPA	Institut Pasteur of Algeria
K2P	Kimura two-parameter model
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
ML	Maximum likelihood
PMM	Peptide mass mapping
TFA	Trifluoroacetic acid
VL	Visceral leishmaniasis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-024-06542-9.

Additional file 1: **Figure S1.** MOTUs inferred from ABGD software.

Additional file 2: **Figure S2.** TCS haplotype network for 34 *Sergentomyia* specimens using Cox I sequences. Circle size and color indicate frequency and species of haplotypes, respectively. Haplotype numbers are written next to the corresponding circle, dashes between haplotypes indicate mutation sites.

Additional file 3: **Table S1.** Genetic distances of Cox I sequences between Algerian *Sergentomyia* groups estimated using Tamura 3-parameter model. The number of base substitutions per site from averaging over all sequence pairs between groups are shown. The rate variation among sites was modeled with a gamma distribution.

Additional file 4: **Table S2.** Genetic distances of Cox I sequences of Algerian *Sergentomyia* species estimated using Tamura 3-parameter model. The number of base substitutions per site from between sequences are shown. The rate variation among sites was modeled with a gamma distribution.

Additional file 5: **Table S3.** Sand fly species tested for *Leishmania* spp. parasites.

Additional file 6: **Table S4.** List of sand fly specimens analyzed by MALDI-TOF MS protein profiling.

Additional file 7: **Table S5.** Peptide mass mapping MALDI-TOF MS identification of blood meal origin of engorged females.

Additional file 8: **Table S6.** Result summary of blood meal analysis using PMM-MALDI-TOF mass spectrometry.

Additional file 9: **Figure S3.** Morphological features of *Sergentomyia imihra* n. sp. female **A** Pharynx **B** Cibarium **C** Flagellum.

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Author contributions
K.E.B., M.M., and Y.D. collected sand fly specimens. K.E.B., J.D., and V.D. carried out morphological identification. K.E.B., D.K., V.D., and P.H. performed molecular analyses and data evaluation. J.D. provided drawings of the new species. K.E.B., J.D., V.D., and P.H. wrote the manuscript. K.E.B., Z.H., V.D., and P.V. designed the study. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this article and its additional files. Cox I and Cyt B sequences of sand fly specimens analyzed in this study were deposited in GenBank database under accession numbers given above. Holotype female *Se. imihra* n. sp. has been deposited in the Institut Pasteur of Algeria (IPA) as described above.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Infectiousness of Asymptomatic *Meriones shawi*, Reservoir Host of *Leishmania major*

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Abstract: Leishmaniases are neglected diseases caused by protozoans of the genus *Leishmania* that threaten millions of people worldwide. Cutaneous leishmaniasis (CL) caused by *L. major* is a typical zoonosis transmitted by phlebotomine sand flies and maintained in rodent reservoirs. The female sand fly was assumed to become infected by feeding on the skin lesion of the host, and the relative contribution of asymptomatic individuals to disease transmission was unknown. In this study, we infected 32 *Meriones shawi*, North African reservoirs, with a natural dose of *L. major* obtained from the gut of infected sand flies. Skin manifestations appeared in 90% of the animals, and xenodiagnosis with the proven vector *Phlebotomus papatasi* showed transmissibility in 67% of the rodents, and 45% were repeatedly infectious to sand flies. Notably, the analysis of 113 xenodiagnostic trials with 2189 sand flies showed no significant difference in the transmissibility of animals in the asymptomatic and symptomatic periods; asymptomatic animals were infectious several weeks before the appearance of skin lesions and several months after their healing. These results clearly confirm that skin lesions are not a prerequisite for vector infection in CL and that asymptomatic animals are an essential source of *L. major* infection. These data are important for modeling the epidemiology of CL caused by *L. major*.

Keywords: *Leishmania*; *Phlebotomus*; *Meriones*; reservoir host; asymptomatic infection; xenodiagnosis

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1. Introduction

Leishmaniases are diseases caused by parasitic protozoans of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) endemic in 98 countries of 5 continents, where they threaten more than 1 billion people. There are three main forms of leishmaniasis: the most severe form is visceral leishmaniasis, which can be fatal without treatment. Mucocutaneous leishmaniasis is a disabling form producing mucosal lesions and cartilage destruction in the oronasal and pharyngeal regions. The most common form is cutaneous leishmaniasis (CL). Skin lesions heal spontaneously in most patients but can leave stigmatic scars for life. It is estimated that 600,000 to 1 million new cases of CL occur worldwide annually but only around 200,000 are reported to the WHO [1].

Cutaneous leishmaniasis caused by *L. major* is a typical zoonosis maintained in rodents. The main reservoir hosts have been shown to be the Great Gerbil *Rhombomys opimus* and jirds of the genus *Meriones* in Central and SW Asia [2]; the Fat Sand Rat *Psammomys obesus* and *Meriones* species in North Africa and the Middle East [3] and species of the genera *Arvicanthis* and *Mastomys* in Sub-Saharan Africa [4,5]. Other species of rodents and other

mammals naturally infected with *L. major* may also be involved in local transmission cycles, but their role has yet to be confirmed.

Field reports had revealed a high representation of asymptomatic individuals among rodent reservoirs infected with *L. major* [6–9]. The importance of asymptomatic hosts in the epidemiology of leishmaniasis has so far been studied and discussed in only visceral leishmaniasis caused by *L. donovani* and *L. infantum* [10,11], and mathematical modeling has suggested the importance of asymptomatic infection in the transmission of *L. donovani* [12]. Therefore, asymptomatic hosts could pose a major challenge to control programs if their infectiousness is confirmed [10]. Indeed, a meta-analysis of xenodiagnostic studies by Quinnell and Courtenay (2009) [13] confirmed that the infectiousness of dogs infected with *L. infantum* increased with the clinical severity of the disease, but since the infectiousness of asymptomatic dogs was sufficiently high, control must target both asymptomatic and symptomatic dogs.

However, the comparative contributions of asymptomatic and symptomatic hosts to the transmission of CL remain unknown. Therefore, we have established the laboratory colony of *Meriones shawi*, one of the main reservoir species in North Africa. The colony was derived from animals captured in M'Sila, the Algerian province most affected by CL [14]. Using a *Leishmania* isolate from the same locality and a proven insect vector in the area, *Phlebotomus papatasi*, we obtained a natural model for studying important factors affecting the outward transmission of *L. major*, including the infectiousness of asymptomatic animals.

2. Materials and Methods

2.1. Sand Flies, Parasites and Rodents

The colony of *P. papatasi* originating from Turkey has been maintained in the insectary of the Department of Parasitology, Charles University in Prague, under standard conditions (26 °C on 50% sucrose, humidity in the insectary 60–70% and 14 h light/10 h dark photoperiod) as described previously [15].

The human isolate *L. major* MHOM/DZ/2009/LIPA100/MON-25 from M'Sila region in Algeria was used. *Leishmania* were fluorescence-marked with m-Scarlet according to the methodology described by [16] to enable analysis of the distribution of parasites on a microscale (these analyses will be published separately). Promastigotes were cultured in M199 medium (Sigma-Aldrich, Merck, Darmstadt, Germany) containing 10% heat-inactivated fetal bovine calf serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% BME vitamins (Basal Medium Eagle, Sigma-Aldrich, Merck, Darmstadt, Germany), 2% sterile human urine and 250 µg/mL amikacin (Amikin, Bristol-Myers Squibb, New York, NY, USA).

The breeding colony of *M. shawi* was established in the LEEPGP animal facility at the Pasteur Institut of Algiers from animals originating in M'Sila, Algeria, and then bred at the animal facility of the Faculty of Science, Charles University in Prague. Animals were maintained in T4 breeding containers (Velaz, Prague, Czech Republic) equipped with bedding (Krmne smesi Kvidera, Spálené Poříčí, Czech Republic), breeding material (Woodwool, Ratiboř, Czech Republic) and hay (Krmne smesi Kvidera, Spálené Poříčí, Czech Republic), provided with a standard feed mixture ST-1 (SubliCZ.cz, Sojovice, Czech Republic) and water ad libitum, with a 12 h light/12 h dark photoperiod, temperature 22–25 °C and humidity 40–60%.

2.2. Experimental Infections of Sand Flies

Phlebotomus papatasi females were infected with *L. major* according to [17]. Briefly, parasites from log-phase cultures were resuspended in heat-inactivated defibrinated ram blood at a concentration of 2×10^6 promastigotes/mL. Sand flies were allowed to ingest this infectious suspension through a chick-skin membrane and after feeding were maintained in the same conditions as the colony. For experimental infections of rodents, female sand flies were dissected 8 days post-bloodmeal when mature infections had developed and metacyclic forms had accumulated in the thoracic midguts.

2.3. Experimental Infections of *M. shawi*

Rodents (at age 6–10 weeks) were anesthetized with a mixture of 50 mg/kg ketamine and 5 mg/kg xylazine and infected according to [18]. Parasites derived from thoracic midguts of infected sand flies were pooled in sterile saline and counted using a Bürker chamber. Dissected salivary glands (SG) of *P. papatasi* females were pooled (10 glands per 10 µL of saline) and stored at −20 °C. Before inoculating mice, SG were frozen and thawed three times in liquid nitrogen, and the resulting lysate was mixed into the inoculum. Finally, 5.5 µL of the mixture was intradermally injected into the left pinnae. The infection dose was either 7000 or 14,000 parasites corresponding to 1 or 2 infected sand fly guts with an addition of 0.5 SG. Animals were checked weekly for external signs of the disease. They were subjected to xenodiagnosis at 4–5-week intervals and sacrificed at various time intervals between weeks 15 and 38.

2.4. Xenodiagnoses

Rodents were anesthetized with ketamine/xylazine, and 30–40 *P. papatasi* females kept in small plastic tubes covered with fine nylon mesh were allowed to feed on pinnae of inoculated ears (Figure 1). On days 7–10 after feeding, the females were dissected, the guts were inspected by light microscopy, and the intensity and location of infections were assessed as previously described [17].

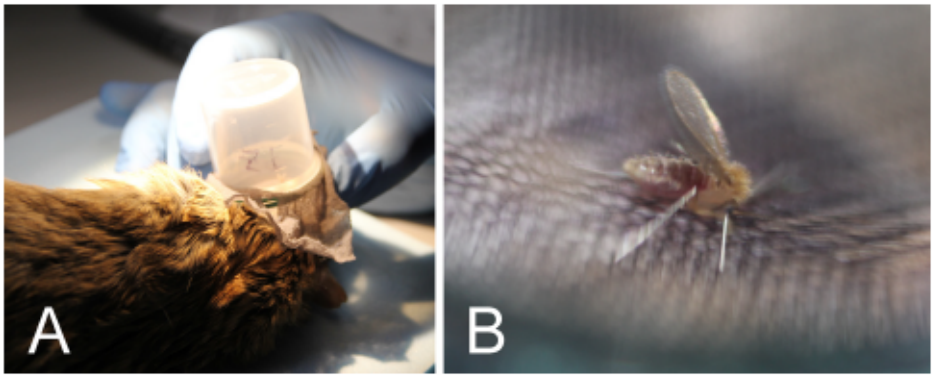


Figure 1. Xenodiagnosis. (**A**), Sand flies held in plastic tubes coated with nylon mesh on the pinna of *M. shawi*; (**B**), Detail of feeding female *P. papatasi*.

2.5. Tissue Sampling and Leishmania Detection Post-Mortem by qPCR

Rodents were sacrificed by cervical dislocation under anesthesia. Whole ears (inoculated and contralateral), whole ear-draining lymph nodes, whole spleen, liver (whole liver was homogenized and a quarter of the volume was used for analysis), paws (whole forepaws and skin and soft tissue from the bottoms of the hindpaws) and tail skin were removed and stored at −20 °C. For qPCR, extraction of total DNA from rodent tissues was performed using a DNA tissue isolation kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. Parasite quantification by quantitative PCR (qPCR) was performed in a Bio-Rad iCycler & iQ Real-Time PCR Systems using the SYBR Green detection method (SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad, Hercules, CA, USA). Primers targeting 116 bp long kinetoplast minicircle DNA sequence (forward primer (13A): 5′-GTGGGGGAGGGGCGTTCT-3′ and reverse primer (13B): 5′-ATTTTACACCAACCCCCAGTT-3′) were used [19]. One microliter of DNA was used per individual reaction. PCR amplifications were performed in triplicates using the following conditions: 3 min at 98 °C followed by 40 repetitive cycles: 10 s at 98 °C and 25 s at 61 °C. Tissue from a non-infected animal was used as a negative control. A series of 10-fold dilutions of *L. major* promastigote DNA, ranging from 5×10^{3} to 5×10^{-2} parasites per PCR reaction was used to prepare a standard curve. Quantitative results were expressed by interpolation with a standard curve. To monitor non-specific products or primer dimers, a

melting analysis was performed from 70 to 95 °C at the end of each run, with a slope of 0.5 °C/c, and 6 s at each temperature.

2.6. Statistical Analysis

To analyze the effects of symptoms and infection dose on the result of xenodiagnostic trials, we employed a marginal model accounting for the dependence of data collected from the same animal [20]. We set the actual presence/absence of symptoms and infection dose as fixed factors, animal identity as ID, binomial distribution, logit link function and ar1 correlation structure. Then, we ran a geeglm function as implemented in geepack (Generalized Estimating Equation Package; [21]). The calculations were performed in an R-environment (R Core Team, Vienna, Austria, 2021).

2.7. Animal Experimentation Guidelines

Animals were kept in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All experiments were approved by the Committee on the Ethics of Laboratory Experiments of Charles University in Prague and were carried out based on permission no. MSMT-31778/2019-6 of the Ministry of Education, Youth and Sports. The investigators are certified for animal experiments by the Ministry of Agriculture of the Czech Republic.

3. Results

In total, 32 animals were infected; 12 received an infectious dose of 14,000 promastigotes (derived from 2 sand fly guts) and 20 were infected with 7000 promastigotes (derived from 1 sand fly gut). Skin manifestations developed in 29 animals; the first skin changes usually appeared 9–15 weeks after infection, regardless of the infective dose, first as a swelling or non-ulcerative nodule or directly as an ulcerative skin lesion (Figure 2a,b). In most animals, the lesions then grew and persisted until the end of experiments, but the skin symptoms healed into the asymptomatic state in 2 animals (animals No. 3 and 28) and ulcerative lesions healed to a non-ulcerative swelling in 3 animals (animals No. 22, 29 and 31). Out of 32 infected jirds, two individuals were asymptomatic throughout the experiment; they were killed relatively early (15–16 weeks p.i.), and PCR confirmed the presence of parasites. One animal killed at 30 weeks p.i. was classified as uninfected because it never showed external signs of disease, was not infectious to sand flies and PCR analysis post-mortem was negative.

Xenodiagnostic experiments were conducted continuously at 4–5-week intervals throughout the experiment to capture both asymptomatic and symptomatic phases of the disease in individual animals (Figure 2a, Table S1). Overall, 20 of 30 animals (67%) were infectious to sand flies on at least one trial, and 14 of them were infectious repeatedly (in 2–5 trials). Six animals were infectious even 4–6 weeks before the appearance of skin symptoms, and in animals that healed lesions during the experiment, we observed infectivity even 14 and 17 weeks after the disappearance of skin manifestations. Analysis of all 113 xenodiagnostic trials with 2.189 sand flies showed no significant difference in the transmissibility of animals in the asymptomatic and symptomatic periods (geeglm: df = 1, Chi-square = 2.842, $p = 0.0918$). Moreover, the model revealed no significant difference in infectiousness between animals inoculated with different infective doses (df = 1, Chi-square = 0.7286, $p = 0.3933$).

At the end of the experiments, animals were sacrificed and their organs were analyzed by PCR. The intensity of infections in positive tissues was further quantified by qPCR. *Leishmania* were mainly localized in inoculated left ears and their draining lymph nodes, but also spread to other skin areas: right ears, paws and tail, and to a lesser extent, they were detected in the spleen and liver (Table 1). While the parasite load was very high in

inoculated ears, mostly only small numbers of leishmania (less than 100) were detected in other parts of the body.

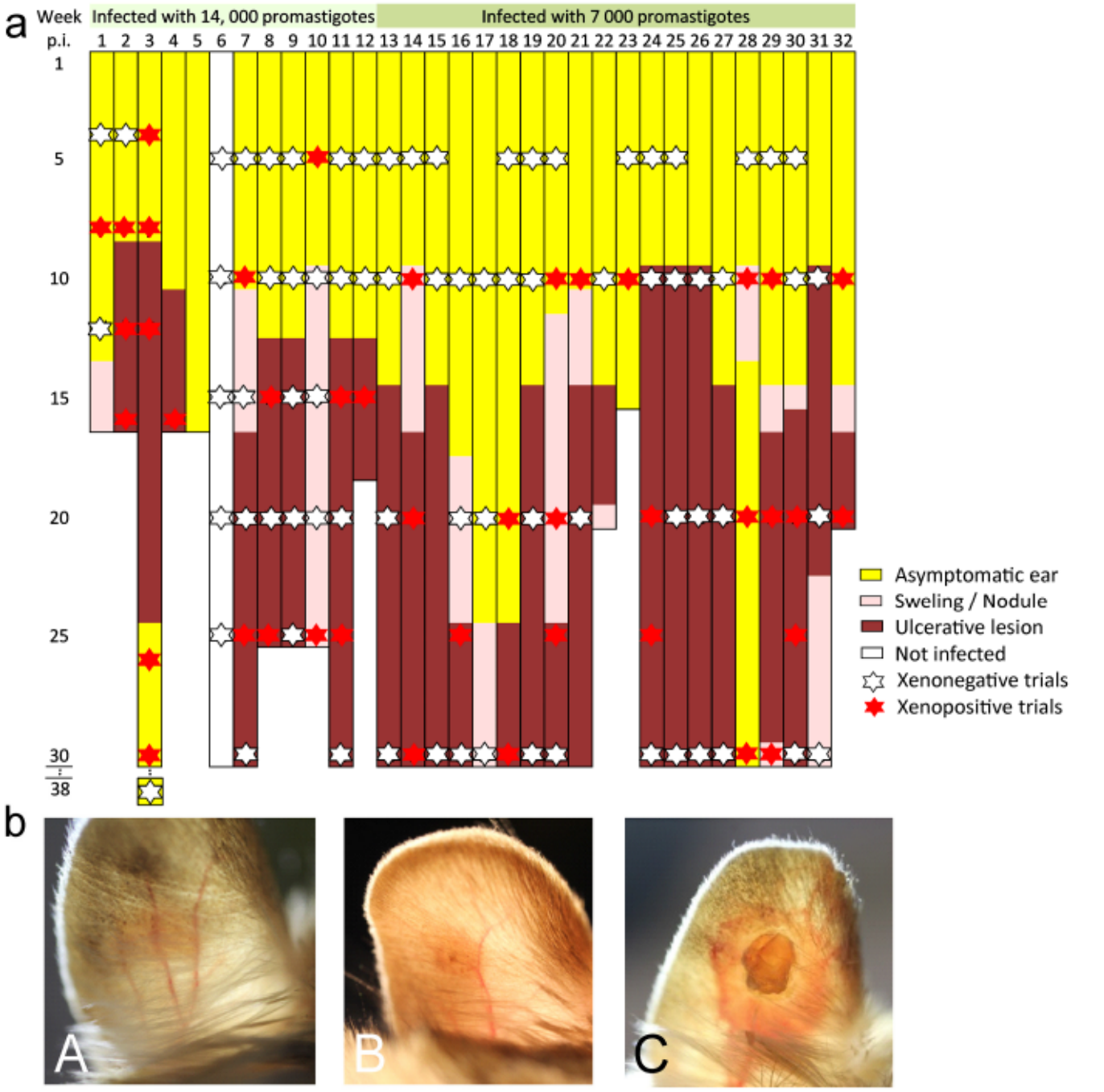


Figure 2. External manifestation and xenodiagnoses of *Leishmania major* in *Meriones shawi*. (**a**) Time course of external manifestations of infection on the pinnae of 32 animals. Xenodiagnostic experiments are labeled in the respective weeks after infection; positive trials are marked with a red asterisk, negative trials with a white asterisk. (**b**) Skin symptoms on pinnae. (**A**) Swelling; (**B**) Nodule; (**C**) Ulcerative lesion.

Table 1. Distribution of *Leishmania major* and parasite load in organs and tissues of *Meriones shawi* detected by PCR and qPCR post-mortem.

Infective Dose	Week p.i.	Meriones No.	IE	CE	IE-LN	CE-LN	FP	HP	Tail	Spleen	Liver
14×10^3	16	1	8.30×10^6	0	$<1 \times 10^2$	0	0	0	0	$<1 \times 10^2$	$<1 \times 10^2$
14×10^3	16	2	2.75×10^5	0	0	0	0	0	$<1 \times 10^2$	0	0
14×10^3	40	3 *	0	0	$<1 \times 10^2$	0	0	0	0	$<1 \times 10^2$	0
14×10^3	16	4	3.43×10^6	0	$<1 \times 10^2$	0	0	0	0	0	0
14×10^3	16	5	2.13×10^4	0	$<1 \times 10^2$	0	0	0	0	$<1 \times 10^2$	0
14×10^3	30	6 **	0	0	0	0	0	0	0	0	0
14×10^3	30	7	3.29×10^9	$<1 \times 10^2$	0	0	0	$<1 \times 10^2$	$<1 \times 10^2$	0	0
14×10^3	25	8	1.41×10^5	0	0	0	0	0	0	0	0
14×10^3	25	9	7.42×10^8	$<1 \times 10^2$	0	0	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$	0	$<1 \times 10^2$
14×10^3	25	10	1.08×10^4	0	0	0	0	0	0	0	0
14×10^3	30	11	4.78×10^7	$<1 \times 10^2$	0	0	0	$<1 \times 10^2$	$<1 \times 10^2$	0	0
14×10^3	18	12	3.94×10^5	0	$<1 \times 10^2$	0	$<1 \times 10^2$	0	0	0	0
7×10^3	30	13	2.19×10^7	$<1 \times 10^2$	0	0	0	$<1 \times 10^2$	$<1 \times 10^2$	0	$<1 \times 10^2$
7×10^3	30	14	5.86×10^5	$<1 \times 10^2$	0	0	0	0	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$
7×10^3	30	15	1.76×10^5	0	$<1 \times 10^2$	0	0	0	$<1 \times 10^2$	0	0
7×10^3	30	16	2.43×10^7	0	0	0	0	0	0	0	0
7×10^3	30	17	3.74×10^4	0	0	0	$<1 \times 10^2$	0	0	0	0
7×10^3	30	18	1.83×10^6	0	0	0	0	0	0	0	0
7×10^3	30	19	9.81×10^7	0	0	0	0	0	0	0	0
7×10^3	30	20	5.45×10^4	0	0	0	0	0	0	0	0
7×10^3	30	21	5.52×10^4	0	0	0	$<1 \times 10^2$	0	$<1 \times 10^2$	$<1 \times 10^2$	0
7×10^3	20	22	1.14×10^5	$<1 \times 10^2$	1.75×10^6	0	0	0	0	0	0
7×10^3	15	23	2.66×10^6	$<1 \times 10^2$	$<1 \times 10^2$	0	0	0	0	$<1 \times 10^2$	0
7×10^3	30	24	4.68×10^6	0	0	0	0	$<1 \times 10^2$	0	0	0
7×10^3	30	25	1.67×10^8	0	0	0	$<1 \times 10^2$	0	0	0	$<1 \times 10^2$
7×10^3	30	26	1.32×10^5	0	0	0	0	0	0	0	0
7×10^3	30	27	7.46×10^3	0	0	0	$<1 \times 10^2$	0	0	0	0
7×10^3	30	28	1.56×10^3	0	0	0	0	0	0	0	0
7×10^3	30	29	3.35×10^5	0	0	0	0	0	0	0	0
7×10^3	30	30	3.46×10	0	0	0	0	$<1 \times 10^2$	1.14×10^3	0	0
7×10^3	30	31	7.06×10^2	0	0	0	0	0	0	0	0

IE, inoculated ear; CE, contralateral ear; IE-LN, lymph node draining IE; CE-LN, lymph node draining CE; FP, forepaws; HP, hindpaws; *, healed ear infection; **, uninfected animal. Color differentiation of infection intensity: red, $>10^8$; dark blue, 10^6–10^8, light blue, 10^4–10^6, green, 10^2–10^4, pink, $<10^2$.

4. Discussion

Meriones shawi is a North African rodent species distributed from Morocco to Egypt, inhabiting clay and sandy deserts and dry steppes, and it is present often also in cereal crops and orchards. It is a colonial species living socially in underground burrows with nocturnal and diurnal activity [22]. Together with *Psammomys obesus*, these species play the main role in the transmission of *L. major* in North Africa [4,23]. Host competence of *M. shawi* for *L. major* was also confirmed by a laboratory study that demonstrated the infectiousness of the animals to the vectors five months after subcutaneous infection of ears with 10^6 promastigotes [24]. A thorough field study from central Tunisia, where both species live in sympatry, showed that the prevalence of the parasite is very high in both *M. shawi* and *P. obesus* (41% and 34%, respectively). Asymptomatic infections were observed in 40% of infected animals of both species, and among the symptomatic ones, skin lesions were localized mostly on the pinnae. The lesions appeared to be more destructive in *M. shawi* than in *P. obesus* [6]. In another study from Tunisia, only 35% of infected *P. obesus* had no visible ear lesions, but the ears may have been destroyed by another cause such as fighting, as 40% of those with ear lesions were negative for *L. major* when tested by footpad inoculation [7].

In reservoir hosts of *Leishmania*, it is generally assumed that the course of the disease is rather mild because it is also in the parasite's interest to survive in these hosts for long periods of winter or rainy seasons when no vectors are present [25]. Thus, an equilibrium between the reservoir hosts and their parasites has necessarily been established that allows both organisms to survive long enough. In this respect, an excellent coexistence has been observed between *L. major* and its main host in Central Asia and neighboring countries, the Great gerbil, *Rhombomys opimus*. Laboratory infections showed that although these animals were susceptible, ulceration and visceralization never developed [26]. In field-trapped animals in northeastern and central Iran, the proportion of asymptomatically infected

R. opimus reached 90% [8,9,27]. However, it should be noted that co-infections of *L. major* and *L. turanica* are common in endemic areas of Central Asia (reviewed in [2]), which may affect the immune response and overall course of *Leishmania* infections of rodents in this area.

In the field studies, it was not possible to distinguish whether asymptomatic animals were more resistant to leishmania than symptomatic ones, or have been infected with a less virulent variant of the parasite, or are pre- or post-symptomatic [6,7]. Our laboratory study showed that almost all *M. shawi* developed skin symptoms; however, symptoms developed relatively late, and some individuals can heal the lesions. Resolution of skin symptoms was observed in one-sixth of the animals, but the proportion of healed animals would probably be even higher if the experiments were extended for additional months.

We mimicked as closely as possible the natural course of initiation of infection. Sand fly salivary gland content has been added as salivary proteins and peptides with antihemostatic and immunomodulatory properties significantly affect the establishment of the parasite in the host (reviewed by [28,29]). Promastigotes were taken from thoracic midguts of infected sand flies: female sand flies were dissected 8 days post-bloodmeal, when mature *L. major* infection developed with a predominance of metacyclic stages. Infection dose was kept low, corresponding to parasite numbers in one or two infected thoracic midguts (7000 or 14,000 parasites, respectively). Intradermal needle inoculation allowed us to control the infectious dose, which is not possible with direct infection by sand fly bite, where different females inoculate the host with *Leishmania* numbers varying by several orders of magnitude [30,31]. Kimblin et al. (2008) [30] compared *L. major* infections initiated with different infection doses and showed that a low infection dose leads to less pathology but higher parasite number in the chronic phase, so that the host acts as an effective long-term reservoir of infection. With high dose (10^6) of parasites in inoculum, the growth of skin lesions would be unnaturally rapid as was described not only for *L major* but also for *L. amazonensis* in mice model [32,33].

In all infected animals used in our experiments, parasites were most abundant in the inoculated pinnae; in two-thirds of the individuals, they also spread to other areas of the body. Smaller numbers were detected in the collateral ear, the skin of the paws and tail, as well as in the lymph nodes, spleen and liver. This pattern of *L. major* dissemination with higher representation in the skin than in internal organs was also observed in other wild rodent species: *Arvicanthis niloticus*, *A. neumanni*, *Mastomys natalensis*, *Cricetulus griseus* and *Lagurus lagurus*, while extensive visceralization under the same experimental conditions was observed in *Phodopus sungorus*, with 71% of spleen samples infected [5,34].

Importantly, also, pre-symptomatic animals and animals with healed skin symptoms were infectious to the vector and statistically, based on the whole material of 113 xenodiagnostic trials, there was no significant difference between infectiousness in the symptomatic and asymptomatic periods. It is interesting to note that some individuals were not infectious for sand flies, although they had ulcerative lesions and a significant parasite load in the pinnae post-mortem. We attribute this phenomenon to the heterogeneous distribution of parasites in the pinnae, as has been reported for *L. donovani* in the skin of BALB/c mice [35]. Our next research goal will be to study the dissemination of *L. major* at the microscale in their natural host, *Meriones shawi*.

Until now, the infectiousness of asymptomatic hosts has been studied almost exclusively in visceral leishmaniasis. Xenodiagnostic experiments showed the transmissibility of *L. donovani* from asymptomatic BALB/c mice [18] and *L. infantum* from different mammalian hosts [36–41]. The infectiousness of asymptomatic dogs infected with *L. infantum* has been studied most intensively, and meta-analysis of data published up to 2009 by Quinnell and Courtenay [13] showed that the proportion of infectious dogs increased significantly with clinical severity, but asymptomatic dogs were also infectious and infected a similar proportions of flies as sick dogs. Canine studies also demonstrated that some dogs are "super-spreaders" while others contribute little to transmission (15% to 44% of dogs were responsible for >80% of all sand fly infections) [42]. The situation was different

for the natural hosts of *L. major* in this study. The majority (67%) of *Meriones shawi* were infectious, and 47% of the animals were infectious repeatedly in different time intervals post-infection.

In CL, it was previously assumed that the sand fly female becomes infected by feeding on the lesion, while asymptomatic individuals were not considered as a source of infection [43]. Only a few studies have described the infectiousness of asymptomatic hosts with CL, such as sloths *Choloepus hoffmanni* infected with *L. braziliensis* [44] or black rats infected with *L. tropica* [45]. Our study based on the natural model *Meriones shawi–L. major–P. papatasi* demonstrated the regular transmissibility of asymptomatic infected animals long before the appearance of skin lesions and long after their healing. Using a significant number of xenodiagnostic experiments, we have shown that skin lesions are not a prerequisite for vector infection, and their presence is not a good diagnostic marker for the presence of the parasite. These data are important for modeling the epidemiology of CL by *L. major* and for control management. These results also raise the question of the extent to which asymptomatic incidental hosts, including infected humans, may be involved in the transmission of CL. Only xenodiagnostic experiments covering both symptomatic and asymptomatic phases of infection can answer this question.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens12040614/s1, Table S1: Results of xenodiagnostic experiments performed with *Phlebotomus papatasi* on *Meriones shawi* infected with *Leishmania major*.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and the study protocol was approved by the Committee on the Ethics of Laboratory Experiments of Charles University in Prague (permission No. MSMT-31778/2019-6 of the Ministry of Education, Youth and Sports).

Data Availability Statement: Data are contained within the article or Supplementary Materials.

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Host competence of Algerian *Gerbillus amoenus* for *Leishmania major*

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Host competence of Algerian *Gerbillus amoenus* for *Leishmania major*

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ABSTRACT

Cutaneous leishmaniasis (CL) is the most important neglected disease reported in North Africa, Algeria ranks second in the world with more than 5000 cases per year. In Algeria, two rodent species *Psammomys obesus* and *Meriones shawi*, are so far known as proven reservoirs of *Leishmania major*, however, they are absent in several endemic localities. In this study, we experimentally infected *Gerbillus* rodents trapped around human dwellings in Illizi, Algeria to assess their susceptibility to *L. major*. Seven gerbils, morphologically and molecularly identified as *Gerbillus amoenus*, were intradermally inoculated with 10^4 parasites derived from culture, monitored for six months and their infectiousness for sand flies was tested by xenodiagnosis. The study revealed that *G. amoenus* was susceptible to *L. major* and was able to maintain and transmit the parasites to sand flies tested six months after infection, suggesting the role of this gerbil as a potential reservoir for *L. major*.

1. Introduction

Leishmaniases, regarded as neglected tropical diseases, are caused by protozoa belonging to the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) (Valero and Uriarte, 2020). The disease represents a major health problem in many countries, with approximately 2 million new cases reported yearly (WHO, 2020). *Leishmania* is a parasite with a digenetic life cycle, alternating between blood-feeding insects, phlebotomine sand flies (Diptera: Psychodidae), and mammalian hosts including humans. Sand flies comprise more than 950 different species distributed in the Old and New World, they are incriminated in the transmission of various pathogens to humans, including *Leishmania* parasites, they also transmit the bacteria *Bartonella bacilliformis* and viruses belonging mainly to the genus *Phlebovirus* of the family Bunyaviridae (Benallal et al., 2022).

In the Maghreb region, CL is caused by three different *Leishmania* species: *L. major*, *L. tropica*, and *L. infantum* with distinct eco-epidemiology and clinical manifestation (Aoun and Bouratbine, 2014). The most frequent species is *L. major* transmitted by *Ph. Papatasi* and maintained mostly in two rodent species *Psammomys obesus* (Fat Sand Rat) and *Meriones shawi* (Shaw's Jird) (Belazzoug, 1986; Ghawar et al., 2011). However, in addition to these two proven reservoirs, other rodent species are likely to be involved in parasite maintenance, as evidenced by infections in areas where neither *M. shawi* nor *P. obesus* lives. Algeria has 27 other rodent species, some of which may play an important role in maintaining pathogens but they are not considered reservoirs of *Leishmania* parasites yet (Ahmim, 2019), moreover, the hedgehogs *Atelerix algirus* and *Paraechinus aethiopicus* were found naturally infected with *L. major* (Tomás-Pérez et al., 2014). Here we describe the possible involvement of another rodent species *Gerbillus amoenus* in the life cycle of *L. major*.

The genus *Gerbillus* is one of the most diverse groups of rodents occurring in arid and semi-arid areas (Abiadh et al., 2010). Recently, *Gerbillus nanus*, a cryptic species of the African *G. amoenus* (Ahmim,

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2019; Ndiaye et al., 2016) was reported to be naturally infected by *L. major* in Iran (Azizi et al., 2011). In the central Sahara of Algeria, some sporadic outbreaks of CL have recently reemerged in the province of Illizi (INSP, 2017) where the proven reservoirs of *L. major* cited above do not occur (Ahmim, 2019) but rodents of the genus *Gerbillus* are abundant. Our study aimed to test the susceptibility of *Gerbillus* rodents to *Leishmania major* and to evaluate their role as reservoir hosts by assessing the maintenance and transmission of the parasites.

2. Material and methods

2.1. Study area

The province of Illizi is located about 2200 km from the capital of Algeria (26°30′18″ N, 8°28′56″ E, 567 m above sea level) in the southern-eastern part of the central Sahara in Algeria and covers an area of 204.675 km^2 (Fig. 1). The number of inhabitants is estimated to be 34.714 persons dispersed in 4 districts (Illizi, Debdeb, Bordj Omar Driss and In Amenas). Illizi is the gate to the Tassili N'Ajjer National Park and the environment is a mixture of rocky and sandy areas. Illizi has a hot desert climate with long, extremely hot summers and short cold winters (Beck et al., 2018). The average annual temperature is 33 °C. Illizi is virtually rainless throughout the year as the average annual rainfall is around 10 mm, the summers are especially dry and the relative humidity is very low with an annual average not exceeding 33%. The village of Indgidad-Imihrou; located about 180 km from the center of Illizi district; was chosen to conduct this study following the report in 2018 of two CL autochthonous cases. In this village, nomadic inhabitants live under huts made of stones and palm leaves.

2.2. Rodent trapping

In September 2019, 10 Sherman traps baited with fresh bread and roasted peanuts were set along dried rivers near active chicken coops, human inhabitants and animal shelters at sunset for three consecutive days. Traps were checked in the morning and the captured rodents were placed in cages for further analyses.

2.3. Breeding and molecular identification of the rodents

The trapped rodents were transferred to Algiers and handled at the Institut Pasteur of Algiers. As the institute's internal procedures recommended, all animals were left in quarantine for at least 2 months before entering the animal facility. Each animal was checked for the presence of ectoparasites (fleas and ticks) and the eventual presence of *Leishmania* lesions on the nose, ears, paws, and tail. Different body measurements (tail length, body length, hindfoot length, and ear height) were taken for morphological identification according to Granjon et al. and Ahmim (Ahmim, 2019; Granjon and Duplantier, 2009). One rodent was subjected to molecular identification targeting the mitochondrial gene Cyt B (Shenbrot et al., 2017).

The sequences were visualized and analyzed in BioEdit software (Hall, 1999) and then blasted against the GenBank database. The phylogenetic tree was drawn using the maximum likelihood method to estimate the evolutionary relationships among the sequences; General Time Reversible (GTR) and (G + I) was used as the best-fit model of nucleotide substitution according to the Bayesian Information Criterion (BIC) implemented in Mega 11 software (Tamura et al., 2021). The robustness of tree topologies in all the treatments was checked using 1000 bootstrap replicates and the Cyt B sequence of *M. shawi* was used as an outgroup. Pairwise Kimura 2-Parameters genetic distances were obtained for our Cyt B dataset under MEGA 11 (Tamura et al., 2021). The sequence of *Gerbillus amoenus* was submitted into the GenBank database under accession number OQ190727.



Fig. 1. Location map of the study area.

2.4. Parasite preparation

The virulent L. *major* strain MHOM/DZ/09/LIPA100/MON-25 isolated from a patient living in M'Sila province, Algeria was used. Promastigotes were cultured in a complete RPMI-1640 medium (Sigma-Aldrich) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Invitrogen Life Technologies, Villebon-Sur-Yvette, France). For experimental infection of the rodents, parasites in stationary phase culture were collected on day 6, washed three times in sterile saline, counted using a THOMA chamber, and re-suspended to a final density of 10^6 promastigotes/ml.

2.5. Rodent infection and monitoring

Five female BALB/c mice (6 weeks old, weight between 18 ± 2 grs) and seven *Gerbillus* were selected for experiment infection. Rodents were anaesthetized with a mixture of ketamine/xylazine (66 mg/kg ketamine and 26 mg/kg xylazine) ("Ketamine/Xylazine," 2006). 10 µL of the parasitic suspension corresponding to 10^4 parasites were injected intradermally using a 27.5-gauge sterile needle with a diameter of 27.5 mm to the left ear pinna. The proportion of metacyclic promastigotes present in the inoculum was calculated using a Giemsa-stained smear of the suspension. Inflammation (red papule) appearing around the inoculation site was defined as swelling and a visible difference in the appearance of the infected ear, and skin crusting was defined as a lesion. The evolution of infection was monitored weekly in both rodent species by measuring the lesion diameter using a digital caliper (Schuster et al., 2014). Food and water were provided *ad libitum*, and the animals were maintained in standard conditions (12 h dark/12 h light photoperiod, at a temperature of 22–25 °C and humidity of 40–60%).

2.6. Xenodiagnoses assay

Five to seven-day-old *Phlebotomus papatasi* sand fly females were used for xenodiagnosis experiments. The flies were maintained at 26 °C and 12/12 dark/light cycle as previously described (Volf and Volfova, 2011). The tests were performed six months post-infection only on *Gerbillus*, corresponding to the winter season in North Africa. Rodents were anaesthetized with a mixture of ketamine/xylazine (66 mg/kg ketamine and 26 mg/kg xylazine body weight, respectively), placed in small cages (20 × 20 × 20 cm) and exposed to 15–20 *Phlebotomus papatasi* females for 1 h. Engorged females were maintained at 26 °C on 50% aqueous sucrose solution (Volf and Volfova, 2011). On the 10th day post-bloodmeal (PBM) and after defecation, live females were dissected and their guts were examined under a light microscope. Intensities and locations of infections were evaluated as described previously (Sádlová and Volf, 2009). In dead females (engorged and gravid), *Leishmania*

parasites were detected using PCR targeting kDNA and ITS-1sequences (Rodgers et al., 1990; Schönian et al., 2003).

2.7. Tissue sampling and quantitative PCR

Six months post-infection, the gerbils were sacrificed by injecting an overdose of ketamine/xylazine. Both ears (inoculated and contralateral), both ears-draining lymph nodes, spleen, liver, paws, and tail were stored at −20 °C for standard and quantitative PCRs. Extraction of total DNA from gerbil tissues was performed using a QIAamp DNA blood mini kit (QiaGen, Germany) according to the manufacturer's instructions. A first screen for *Leishmania* parasites in the tissue was carried out using conventional PCR targeting kDNA and ITS-1 sequences (Rodgers et al., 1990; Schönian et al., 2003) to select the positive samples for parasite quantification. In the second step, only the positive samples revealed by the conventional PCR were considered for qPCR quantification of *Leishmania* parasites load, the reactions were performed in Mic Real-Time PCR Cycler Systems using SuperScript III Platinium SYBR Green One-Step qRT-PCR Kit detection method (Invitrogen, Life Technology) using minicircle kinetoplast DNA as the molecular target (Myskova et al., 2008). As reported previously, the qPCR was considered specific when the melting temperature was 84 °C for the positive controls (Mihoubi et al., 2006).

2.8. Statistical analysis

The lesion diameters in the two groups of rodents were compared using a one-way ANOVA test using Microsoft Excel version 2013. The differences were considered significant if $P < 0.05$.

2.9. Animal experiment guidelines

Parasite Eco-epidemiology and Population Genetics laboratory at Institut Pasteur of Algiers has been authorized by the Algerian Association for Animals experimentation Science to carry out studies on rodent reservoirs of cutaneous leishmaniasis (Agreement N°45/DGLPAG/DVA/SDA/14).

3. Results

During the three nights of trapping, a total of 9 rodents (seven females and two males of *Gerbillus*) were captured, the trap efficiency corresponds to 0.3 rodents/trap/night. Unfortunately, only females survived the trip to the laboratory located about 2000 km from Illizi and were not pregnant. The morphological identification of nine *Gerbillus amoenus* (2 males and 7 females) according to Ahmim (2019) (Suppl. information 1) was confirmed by sequencing of the Cyt B gene. Indeed, the maximum likelihood (ML) tree showed that the gerbil belonged to *G. amoenus* as it clustered within the monophyletic group of *G. amoenus* from Mali, Morocco, Mauritania, Egypt and Niger (Suppl. information 3) showing genetic distance ranging between 0.002 and 0.015, and differed from *G. nanus* which showed genetic distances ranging from 0.066 to 0.071 (Suppl. information 2).

Visual screening carried out on the nose, ears, paws, tail did not reveal presence of *Leishmania* lesions suggesting the absence of parasitic infection. In addition, no ectoparasites were found in the trapped rodents.

3.1. Infection lesion monitoring

The inoculum contained 25% metacyclic promastigote forms meaning that the infections were triggered by 2500 metacyclic promastigotes. At the fourth week post-infection, the rodents showed swelling on the inoculation sites which evolved to lesions from week 6 and week 7 in BALB/c mice and gerbils, respectively (Fig. 2). The lesion diameters increased to reach a peak of 2.31 mm in gerbils and 1.76 mm in mice at week 11. In both groups, monitoring was stopped at week 13 since it was impossible to measure the lesion diameters in the ears that started to necrotize. Comparison of lesion size between the two groups of rodents showed no statistically significant difference ($P = 0.51$, $F = 0.4595$) suggesting that in this respect, both groups of rodents have the same susceptibility to the *L. major* parasites.

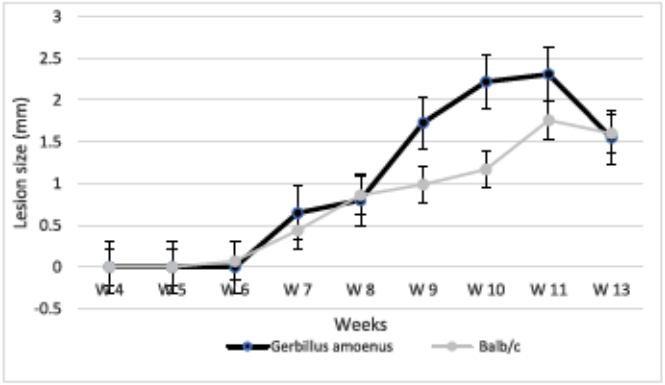


Fig. 2. Lesion growth in *Gerbillus amoenus* and Balb/c mice. Data are presented as the means ± standard errors of the means.

3.2. Xenodiagnoses

Three gerbils died before the end of the experiment, only four individuals served to the xenodiagnoses test. Six months post infection, the infected ears of surviving gerbils were already damaged by parasites (Fig. 3D). A total of 77 female sand flies were left to feed on four anaesthetized rodents (Each rodent was exposed separately to 22–25 sand fly females). Ten days PBM, only 10 female sand flies survived, one was positive for *Leishmania* parasites and showed a moderate parasite density in the thoracic midgut and the 67 dead females were tested by PCR, two of which were confirmed positive for *Leishmania* parasites. In total, 3.89% of 77 *Phlebotomus papatasi* females tested positive and infected on three different rodents.

3.3. Distribution of L. major in rodent bodies

In qPCR, the Ct of the negative samples was 31, all samples with a Ct ≥ 30 were considered negative. QPCR detected the parasites in all tested organs and tissues, but mostly in low numbers. Therefore, the positivity of the samples was double-checked using two PCR techniques (conventional and quantitative PCR) thus giving accuracy and insurance to our results. An exception was in gerbil N°4 which presented a high parasite load in the spleen, tail, and hind paws (Table 1).

4. Discussion

The genus *Gerbillus* is one of the most diverse rodent groups occurring in arid and semi-arid areas (Abiadh et al., 2010; Dahmana et al., 2020). *Gerbillus amoenus* (Blanford, 1875) has been reported from Morocco to Egypt, while its cryptic species *G. nanus* is widespread from the Middle East and the Arabian Peninsula to Asia (Ndiaye et al., 2016). So far, the rodent populations of Algeria have not been genetically studied in detail. Indeed, molecular identification of the gerbil in this study confirmed the occurrence of *G. amoenus* as the analyzed specimen was placed within the monophyletic group of *G. amoenus* from countries bordering Algeria such as Morocco, Libya, Mauritania, Mali and Niger as shown in Supplementary information 2. Thus, the molecular result confirms the deep molecular and karyotype analyses previously performed by Ndiaye (Ndiaye et al., 2016) and completes the range of this species in North Africa.

This study, to our knowledge, is the first to assess the susceptibility

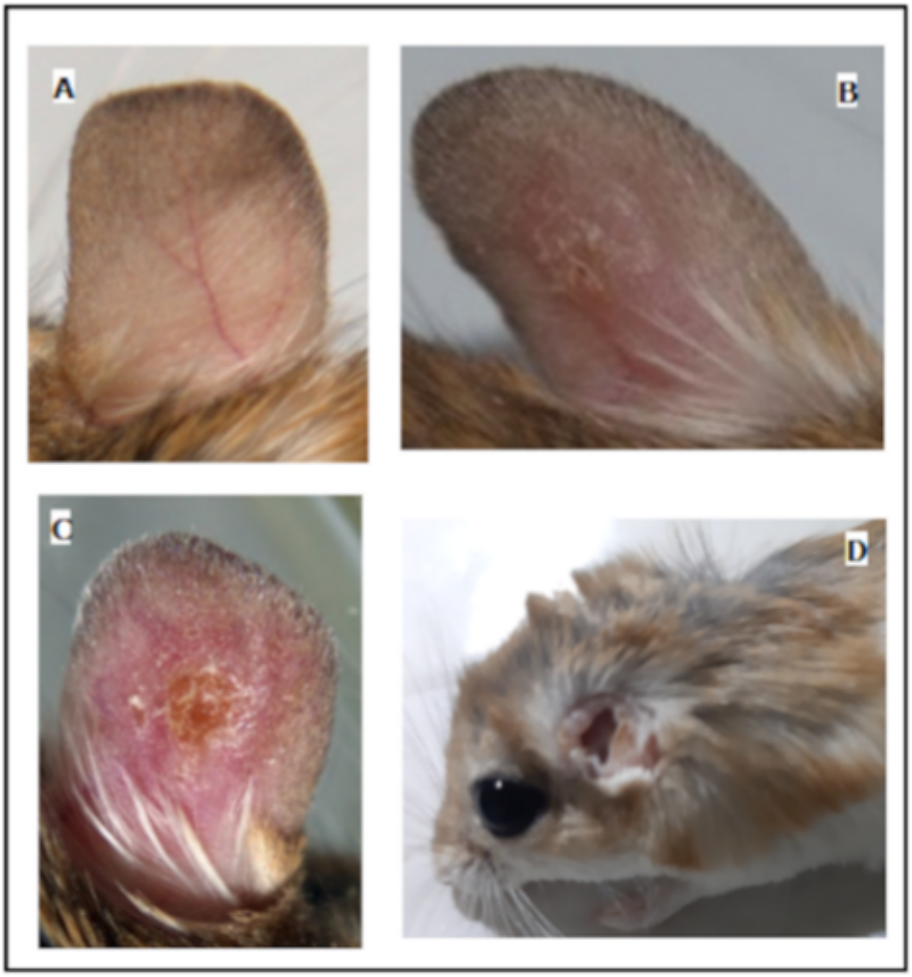


Fig. 3. The external manifestation of *L. major* infection in *Gerbillus amoenus*. A) non-infected ear, B) 8th-week post-infection, C) 11th-week post-infection, D) 6 months post-infection.

Table 1
qPCR detection of *Leishmania* parasites in gerbil tissues.

Rodent	Left inoculated ear (LE)	Right ear (RE)	LE draining LN	RE draining LN	Liver	Spleen	Tail	Hind paws
1	NA	<10	261	NA	12	33	107	98
2	NA	<10	34	NA	20	11	30	51
3	11	<10	NA	<10	<10	<10	105	38
4	NA	<10	84	<10	<10	2037	2751	7454

NA: not analyzed (left ears were mostly destroyed by infection), LN: lymph node.

and reservoir role of *G. amoenus* to *L. major*. The experimental infections performed showed that *G. amoenus* are susceptible to *L. major*, similar to BALB/c mice. In both species (BALB/c mouse and *G. amoenus*), parasites caused ear lesions and spread to different body parts including viscera. The spread of *Leishmania* throughout the gerbils' body has been described in other experimental infections of potential African reservoir hosts of *L. major* – rodents of the genera *Mastomys* and *Arvicanthis*, however, in these sub-Saharan species, *L. major* did not cause skin lesions and destruction of the pinnae, the parasites produced only slight skin changes such as hyperpigmentation (Sadlova et al., 2020). Importantly, *G. amoenus* maintained the parasites and were infectious to sand flies even after six months suggesting that this species meets the most important criteria of a reservoir host previously described (Chaves et al., 2007). *Gerbillus amoenus* is known to share burrows with the proven reservoir species of *L. major* such as *Meriones libycus* and *Psammomys obesus* (Ahmim, 2019; Tabbabi, 2019) which increases the risk of its natural infections with *Leishmania* parasites. In addition, this species can be used as a laboratory animal model for *L. major* infections. Similar to the Asian rodent species *Phodopus sungorus*, *Lagurus lagurus* and *Cricetulus griseus*, these wild rodents are genetically polymorphic and better mimic human infections than inbred mice strains (Vojtkova et al., 2020).

In conclusion, the current study confirmed the identity of *Gerbillus amoenus* in Algeria and demonstrated its potential reservoir role for *L. major* which is essential for control efforts against CL in Algeria and surrounding countries.

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Author's contributions

Kamal Eddine Benallal: designed, trapped, infected, analyzed data, wrote the manuscript and acquired funding, Ghania Mezai: cultivated parasites, Mohamed Meffisel and Yacine Dib: helped in rodent trapping, Karima Brahmi: helped in data analyses, Nadia Klari and Cylia Lardjane: rodent monitoring and data analyses, Ahmed-Faize Khardine: sequencing and qPCR analysis, Ihcen Kherachi: helped in experimental infection of rodents, Jovana Sadlova: reviewed and edited the manuscript, Petr Volf and Zoubir Harrat: reviewed, edited the manuscript and acquired funding.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2023.04.007.

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Effect of *Phlebotomus papatasi* on its Fitness, infectivity and antimony-resistant *Leishmania major* MON-25.

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Effect of *Phlebotomus papatasi* on the fitness, infectivity and antimony-resistance phenotype of antimony-resistant *Leishmania major* Mon-25

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ABSTRACT

Leishmania major is responsible for zoonotic cutaneous leishmaniasis. Therapy is mainly based on the use of antimony-based drugs; however, treatment failures and illness relapses were reported. Although studies were developed to understand mechanisms of drug resistance, the interactions of resistant parasites with their reservoir hosts and vectors remain poorly understood. Here we compared the development of two *L. major* MON-25 trivalent antimony-resistant lines, selected by a stepwise *in vitro* Sb(III)-drug pressure, to their wild-type parent line in the natural vector *Phlebotomus papatasi*. The intensity of infection, parasite location and morphological forms were compared by microscopy. Parasite growth curves and IC_{50} values have been determined before and after the passage in *Ph. papatasi*. qPCR was used to assess the amplification rates of some antimony-resistance gene markers. In the digestive tract of sand flies, Sb(III)-resistant lines developed similar infection rates as the wild-type lines during the early-stage infections, but significant differences were observed during the late-stage of the infections. Thus, on day 7 p. i., resistant lines showed lower representation of heavy infections with colonization of the stomodeal valve and lower percentage of metacyclic promastigote forms in comparison to wild-type strains. Observed differences between both resistant lines suggest that the level of Sb (III)-resistance negatively correlates with the quality of the development in the vector. Nevertheless, both resistant lines developed mature infections with the presence of infective metacyclic forms in almost half of infected sandflies. The passage of parasites through the sand fly guts does not significantly influence their capacity to multiply *in vitro*. The IC_{50} values and molecular analysis of antimony-resistance genes showed that the resistant phenotype of Sb(III)-resistant parasites is maintained after passage through the sand fly. Sb(III)-resistant lines of *L. major* MON-25 were able to produce mature infections in *Ph. papatasi* suggesting a possible circulation in the field using this vector.

1. Introduction

Leishmaniases are regarded as neglected vector-borne infectious diseases caused by flagellated protozoan belonging to the *Trypanosomatidae* family and *Leishmania* genus. According to the WHO, 200 countries and territories have reported cases of leishmaniasis (World

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Health Organization, 2020). Nowadays, more than 1 billion people live in areas considered endemic for leishmaniasis and are at risk of infection. Annually, 30,000 estimated new cases of visceral leishmaniasis (VL) are reported and more than 1 million new cases of cutaneous leishmaniasis (CL) occur (World Health Organization, 2023).

Algeria is ranked second endemic country to CL worldwide after Afghanistan (World Health Organization, 2022). The disease is caused by three *Leishmania* species with different vectors and reservoir hosts *i) L. major* MON-25 transmitted by *Ph. papatasi* (Izri et al., 1992) and wild rodents *Meriones shawi* and *Psammomys obesus* as the main and proven reservoirs (Belazzoug, 1983, 1986), *ii) L. tropica* (reported as *L. killicki* MON-301) transmitted by *Ph. sergenti* with the natural reservoir *Ctenodactylus gundi* (Boubidi et al., 2011; Jaouadi et al., 2011) and *iii) L. infantum* MON-24 transmitted by *Ph. (Laroussius) perfiliewi* and the dog as the main reservoir (Izri and Belazzoug, 1993; Benikhlef et al., 2004).

During the past 20 years, the emergence of clinical resistant *Leishmania* parasites against the available antileishmanial drugs such as pentavalent (Sb(V)) antimonials, miltefosine (MIL) and paromomycin has dangerously increased worldwide whereas clinical resistant cases to amphotericin B (AmB) remain rarely reported (Ponte-Sucre et al., 2017). In Algeria, meglumine antimoniate (Glucantime®) remains the first-line drug used for both VL and CL (Sereno et al., 2012). Unfortunately, treatment failures have been reported in several regions of Algeria, and drug resistance observed in patients treated with Glucantime® remains a major issue in antileishmanial chemotherapy (Ponte-Sucre et al., 2017; Eddaikra et al., 2018). Because antimonials are the primary antileishmanial drugs used in most developing countries, a comprehension of the fitness cost associated with the acquisition of drug resistance phenomenon is important to design efficient control tools and management of leishmaniasis (Ait-Oudhia et al., 2011). Moreover, various factors may play a role in the selection of Sb-resistant lines such as host genetic background and immunological responses to treatment, drug quality, the non-respect of therapy and recommended doses, and the intrinsic drug-susceptibility of *Leishmania* species (Ponte-Sucre et al., 2013; Eddaikra et al., 2018). In addition, *Leishmania* parasites are known to be essentially clonal with genetic exchanges taking place in invertebrate hosts (Akopyants et al., 2009) which may result in increasing the fitness of the offspring (Volf et al., 2007) and selection/elimination of some clones under the bottleneck phenomena (Bussotti et al., 2023). Thus, the spread of a drug-resistant phenotype in the field depends on the capacity of resistant parasites to survive inside the different hosts (reservoir and vector) and to be successfully transmitted between hosts during the parasite life-cycle. So far, the mechanisms by which resistant lines are maintained and transmitted between mammalian reservoirs and vectors remain poorly understood and relatively few studies focused on this important subject (Sebkova et al., 2014; Van Bockstal et al., 2019, 2020; Hendrickx et al., 2020a,b).

In this context with the objective to better understand the spreading of antimony-resistant *Leishmania* parasites in the field, this study aimed to assess the impact of Sb(III)-resistance on the development of *L. major* in the competent sand fly vector. For this purpose, females *Ph. papatasi* were experimentally infected with two susceptible wild-type strains (WT) and two Sb(III)-resistant lines differing in resistance levels. Parasite loads, morphological forms and location of parasites in various parts of the sand fly digestive tracts were compared *in vivo* and parasites isolated from infected guts of female sand flies were studied *in vitro* to evaluate several biological parameters: growth capacity, infection rate and parasite load in macrophages, parasites drug susceptibility and the expression rate of targeted genes as resistance markers.

2. Materials and methods

2.1. Parasite strains and cultures

Two virulent wild-type strains were used to run this study. The WT *Leishmania major* MON-25 strain MHOM/DZ/09/LIPA100/09/MON-25 (LIPA-WT) was isolated from a patient living in M'Sila province in Algeria and maintained in culture at Institut Pasteur d'Algérie MHOM/LY/87/CS3 (Liby) was isolated in Prague from a patient coming back from Libya. These two lines were cultured in complete M199 medium (Sigma) containing 10% heat-inactivated FCS (Gibco) supplemented with 1% BME vitamins (Basal Medium Eagle, Sigma), 2% sterile human urine and 250 µg/mL amikacin (Amikin, Bristol-Myers Squibb) for sand fly experiments in Prague.

2.2. Selection of Sb(III)-resistant lines

Leishmania-resistant lines were selected *in vitro* at PARACHEM laboratory (UMR CNRS 8076 BioCIS at Université Paris-Saclay) by discontinuous stepwise drug pressure on the LIPA-WT strain using antimony potassium tartrate (Sb(III)) (Sigma-Aldrich, Saint Quentin Fallavier, France) at different concentrations (0.1, 0.5, 1, 2, 5, 10 mM) according to Hendrickx et al. (2012) with slight modifications. Parasites (10^6 promastigotes/mL) were cultured in duplicate in M199 medium (ThermoFisher, Villebon sur Yvette, France) supplemented with 40 mM HEPES, 100 µM adenosine, 0.5 mg/mL haemin, and 10% heat-inactivated fetal calf serum (FCS) (Life Technologies-Gibco, Mexico origin, France). Drug-pressure selection was assessed in TPP® 24-wells flat-bottom plates at 26 °C in a dark environment. The assays were carried out in duplicate for each drug concentration and the parasites under drug pressure were incubated at 26 °C overnight until the appearance of clusters. The promastigotes which had survived the action of the first concentration (0.1 mM) of Sb(III) were transferred into 5 mL of free-drug complete M199 medium and then incubated at 26 °C. Once the promastigotes have reached their logarithmic phase of growth, the drug pressure resumed starting from the last value of the maximum concentration plateau obtained previously (0.1 mM), gradually increasing the concentrations (0.2, 0.25, 0.35, 0.4, 0.45 mM) until reaching a maximum concentration value at 0.5 mM, to get the intermediate resistant line (SbIIIR-0.5 mM). A second level of resistance was obtained by increasing the doses of Sb(III) starting from the last maximum dose of 0.5 mM until reaching a maximum dose of 5.5 mM (SbIIIR-5.5 mM). Parasite growth was determined using SYBR® Green I asymmetrical cyanine dye (Invitrogen, Villebon sur Yvette, France), the incorporation of SYBR® Green I in parasite DNA was measured using the Master epRealplex cycler® (Eppendorf, Montesson, France). The results were expressed as IC_{50} values that were determined by a nonlinear regression using ICEstimator website 1.2 version: ICE. Method Introduction (antimalarial-icestimator.net). Fluorescence data were compared to those acquired from the range with different parasite densities. Miltefosine (HePC: hexadecyl-phosphorylcholine) was used as a reference compound. The antileishmanial activity was expressed as IC_{50} in µM (concentration of drug inhibiting the parasite growth by 50%, in comparison to the controls treated with the excipient only).

2.3. Sand flies and sand fly infections

Phlebotomus papatasi colony was maintained in the Department of Parasitology at Charles University of Prague, under standard conditions at 26 °C, 50% sucrose and 14 h light/10 h dark photoperiod as previously described by Volf and Volfova (2011). Parasites (two WT strains (LIPA-WT and Liby) and two resistant lines derived from the LIPA-WT strain (SbIIIR-0.5 mM and SbIIIR-5.5 mM) at the logarithmic phase of culture were washed twice in sterile saline (NaCl) and re-suspended in heat-inactivated rabbit blood at a final concentration of 10^6 promastigotes/mL. 5–9 days old *Ph. papatasi* females were fed through a chicken-skin membrane containing a suspension of promastigote parasites in a dark room for 1 h at 26 °C according to Sádlová et al. (2017). Unfed females were removed, and the engorged ones were maintained under the same conditions as the colony. The experiments were performed in triplicate. On day 2 and 7 post-infection (p.i.), fifteen females from each cage were dissected in a drop of saline under

stereomicroscope. The intensity and location of infection in the digestive tracts of the sand flies were examined under the light microscope (Olympus DP70). Parasite load was considered light for <100 parasites per gut, moderate for 100–1000 parasites per gut, and heavy for more than 1000 parasites per gut, according to Myskova et al. (2008). The average number of parasites present in sand fly digestive tracts were estimated and represented graphically with Microsoft® Excel 2016 software. Differences in infection intensities, the percentage of morphological forms found in infected sand flies and parasite localization in digestive tracts were applied to Chi-square ($\chi 2$) tests. The statistical differences are considered significant if $^{*}P_{value}<0.05$ and highly significant if $^{**}P_{value}<0.01$.

2.4. Morphology of parasites in sand fly

Morphology of L. *major* parasites was evaluated from gut-smears of infected female sand flies dissected on day 7 p. i. Five gut-smears were used per each line and day of dissection. Ten random observation fields containing parasites were observed and photographed in each smear under the light microscope (Olympus DP70). The body length, body width and flagellum length were measured using the ImageJ® software (NIH, Bethesda, Maryland, USA). The morphological stages of the parasites were determined according to the established criteria of Sádlová et al. 2010; (2017) and Rogers et al. (2002): short promastigotes (SP) body length <14 μm; elongated nectomonads (EN) body length ⩾14 μm; metacyclic promastigotes (MC) flagellum two times > body length and a body length <14 μm.

2.5. Parasite recovery from sand flies

L. major LIPA-WT and Sb(III)-resistant lines were isolated from infected *Ph. papatasi* females dissected individually at day 7 p. i in sterile saline. The guts containing parasites were inoculated individually into SNB-9 blood agar slopes (Diamond and Herman, 1954) overlaid with M199 medium (the same used for cultivation of promastigotes, supplemented additionally with Fluorocytosine 100 μg/ml). After three days, if there was no contamination, the cultures were frozen.

2.6. L. major Mon-25 in vitro fitness

The fitness of the three *L. major* lines (LIPA-WT and both Sb(III)-resistant lines) was estimated by following up the growth curves of 2 × 10^6 parasites/mL promastigotes from the stationary phase of development inoculated in a suspension 10 mL of M199 complete medium and incubated at 26 °C. To avoid any bias, parasites were counted in duplicate (2 flasks of sub-cultures/parasites line) by two independent manipulators than the data were pooled. The parasite density was assessed every 24 h (24–120 h). Parasites growth curves were represented using GraphPad Prism software version 7.05 (GraphPad Software Inc.). Two-way ANOVA followed by *Dunnett's* multiple comparisons test was used for multigroups comparison. Results were expressed as mean ± SD and considered significant if $P_{value}< 0.05$.

2.7. Evaluation of Sb(III)-susceptibility

Sb(III)-susceptibility assays were performed on *L. major* MON-25 (LIPA-WT) and both antimony-resistant lines. Promastigotes (10^6 parasites/mL) isolated from the infected sand flies were cultured in sterile 96-Well flat-bottomed microplates (Thermo Scientific™) at 26 °C in a dark environment. The assays were carried out in duplicate for each *Leishmania* line. After 72 h of incubation, parasite growth was determined using SYBR® Green I asymmetrical cyanine dye (Invitrogen, Villebon sur Yvette, France). The incorporation of SYBR® Green I in the DNA of parasites was measured using the Master epRealplex cycler® (Eppendorf, Montesson, France). The results were expressed as IC_{50} values that were determined by a nonlinear regression using IC Estimator website 1.2 version as previously described (section *2.2. Selection of Sb(III)-resistant lines*).

2.8. Infection of macrophages by L. major LIPA-WT and Sb(III)-resistant lines

The murine monocytes/macrophages cell line RAW 264.7 was maintained on T75 sterile culture flasks with filter, in Dulbecco's Modified Eagle's liquid Medium (DMEM) (ThermoFisher, Villebon sur Yvette, France) supplemented with 10% heat-inactivated FCS and 1% Penicillin/Streptomycin antibiotics. The cultures were incubated in the dark at 37 °C under 5% CO2 and used for the experiments when cell's confluence reached 80%. To run the assay, sterile glass cover slips were placed at the bottom of 12-wells flat bottom plate (Thermo Scientific™). RAW 264.7 cell lines were seeded into the 12-wells plate at a density of 500,000 cells/mL in 100 μL of complete DMEM in each well and then incubated in a dark environment for 24 h at 37 °C under 5% CO2 to plate the cells onto the cover slips. In parallel, parasite suspensions of LIPA-WT, SbIIIR-0.5 mM and SbIIIR-5.5 mM were cultivated to yield a rate of 5x106 parasites/mL in complete DMEM liquid medium at 37 °C. After incubation of RAW 264.7 cells overnight, the DMEM was replaced with 100 μL of fresh DMEM containing a suspension of *L. major* parasites prepared at a concentration of 10^6 parasites/mL. The experiment was performed in triplicate for each parasite line. Infection kinetics was carried out at 6 h, 9 h, 24 h, 48 h and 72 h after infection; the infection rate (percentage of macrophage infected) as well as the parasite load per macrophage were assessed. For this, the medium was removed and the wells were washed 3 times with phosphate buffered saline solution (PBS), fixed with 4% glutaraldehyde and stained with 10% Giemsa. The cover slips were then mounted on a slide with Eukitt® embedding balm and observed under microscope. Images were acquired under Amscope T490B light microscope at X1,000 magnification.

2.9. Genes quantification and antimony-resistance markers expression rate

After sand flies infection, the parasites were isolated from the infected digestive tracts and maintained in culture on a free-drug medium to assess genes quantification and Sb(III)-resistance markers expression rate. Thus, 1 mL of each culture were centrifuged at 10,000 *g*, washed 3 times with 1X PBS and DNA was extracted from the pellet using Kit Isolate II genomic DNA®" (Bioline Meridian, USA) according to the supplier's recommendations then quantified with NanoDrop™ (ThermoFisher, France). In parallel, the same amount of parasite cultures was used for total RNA extraction. After centrifugation, the pellet was placed in TRIzol™ reagent (ThermoFisher, France) and stored at −80 °C. Total RNAs were extracted using RNA Purification Kit (ThermoFisher, France) according to the supplier's recommendations. Two real-time quantitative PCR (qPCR) experiments were performed, and the samples were analyzed in triplicates. To assess the copy number of specific resistance genes (Table 1), qPCR technique was applied on susceptible *L. major* (LIPA-WT) and its derivative resistant lines (SbIIIR-0.5 mM and SbIIIR-5.5 mM) before and after sand flies infection using the CFX OPUS 96® (BioRad, France). The experiment was carried out on a total volume of 20 μL. The final Mix included the SensiFAST® NO-ROX reagent (Bioline Meridian, France) at 1X, 400 nM of sense and antisense primers (Table 1) and 2 μL of the genomic DNA to be analyzed. The DNA of *Leishmania major* MHOM/PT/92/CRE26 was used as positive control. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene that codes for a glycolytic enzyme of *L. major*, was used as a reference gene to run the study (F): GAAGTACACGGTGGAGGCTG and (R): CGCTGATCACGACCTTCTTC).

To assess the gene expression, real-time quantitative reverse transcription PCR (qRT-PCR) assay was performed in 20 μL reactions volume using the SensiFAST® NO-ROX reagent (Bioline Meridian, France) as previously described using CFX OPUS 96® (BioRad, France). To run the

Table 1
Sequence of qPCR primers used for amplification assay and expression genes involved in *Leishmania* antimony resistance.

Symbol	Genes	Reference sequence	sense	Sequence 5'→3'
LmPRP1	pentamidine resistance protein 1 (PRP1)	LMJFC_230007600	F	GAT-GAT-GCT-GTG-CTG-GAC-TG
			R	TCA-GCC-ACA-TCA-GCA-GGT-AA
LmMRPA	ABC-thiol transporter	XM_001683297.1	F	CAA-GAT-GCA-CCT-CGT-ACT-GC
			R	CAG-CTC-ACA-AAC-TCG-AGA-CG
LmMDR1	p-glycoprotein (MDR1)	XM_001686140.1	F	AAT-CCT-CTG-TTC-AGT-GCC-GA
			R	GAG-ATC-CAT-TGC-GAT-ACG-GC
LmJBP1	DNA J-binding protein (JBP1)	XM_001681269.1	F	CCG-CGC-GAT-GTG-ATG-ATA-TT
			R	GGT-AAG-TCG-CTT-CCA-GTC-CT
LmAQP1	aquaglyceroporin (AQP1)	XM_001684934.1	F	ACT-CAG-CTG-TAC-GTG-GAC-AA
			R	TCG-CTT-ACT-CTC-GTG-TTG-GT
LmTDRX	thioredoxin - putative	LMJFC_010007800	F	AAG-GAT-CTG-GAC-AGG-CTC-AC
			R	ATG-TCC-GTG-TTG-TTG-TCT-GC
LmTXN1	tryparedoxin (TXN1)	XM_003722147.1	F	TGA-GAA-GCA-CCA-CGA-TTC-GA
			R	TTA-GCG-CCT-CCA-CAA-TGT-TG
LmTDR1	Thiol-dependent reductase 1	LmjF.33.0240	F	GAG-TCG-CAG-CTG-ATT-GTT-CA
			R	CCA-CCT-CGT-ACT-CCT-TCT-CC
LmPRX	Peroxiredoxin	XM_001683274.1	F	ACT-ACG-GTG-TGC-TGA-TCG-AA
			R	GTC-ATT-GAT-CGT-CGA-GTG-GC

experiment, 1 μg of isolated total RNA was treated with DNase I, Amplification Grade® (Life Technologies®) then reverse transcribed with Superscript® VILO™ (Life Technologies®) following the manufacturer's recommendations. The qPCR cycling conditions were 95 °C for 2 min, followed by 30 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 15 s. Primer amplification specificity was assessed by melting curve analysis. All samples were tested in triplicate, and the cycle threshold (Ct) was set at 0.1.

3. Results

3.1. Selection of antimony Sb(III)-resistant lines

After stepwise *in vitro* drug pressure, two antimony-resistant lines SbIIIR-0.5 mM and SbIIIR-5.5 mM were obtained from the *L. major* LIPA-WT strain, and were maintained *in vitro* under Sb(III)-drug pressure of 0.5 mM and 5.5 mM respectively to run the *in vivo* and *in vitro* assays with a maximum of two sub-passages to prevent parasites from losing their virulence as previously recommended (Bussotti et al., 2023).

3.2. L. major Mon-25 in vivo fitness

3.2.1. Sand fly infection rates and parasite loads

The development of the resistant and wild-type parasites in the infected sand flies was evaluated in a total of 90 dissected females per line to compare *i)* the infection rate and parasite loads, *ii)* the location of the parasites in the digestive tracts and *iii)* the morphological forms of the parasites. Two days post-infection (p.i.), the principal aim of the dissection was to assess the infection rate of blood-fed female sand flies and in this respect, there were no significant differences among the four strains and lines ($P_{value} = 0.2$, $\chi^2 = 1.420$, d. f. = 1); infection rates were above 95% in all groups (Fig. S1, Supplementary data). In late-stage infection on day 7 p. i (Fig. 1), infection rate of female sand flies remained high while the intensity of infection was different among the groups ($P_{value} = 0.0422$, $\chi^2 = 22.42$, d. f. = 3). In both wild-type strains (LIPA-WT and Liby), more than 70% of the infected females presented heavy parasite loads which were less represented in both resistant lines. Besides, the parasite load in females infected with SbIIIR-0.5 mM parasites was significantly higher than the females infected with SbIIIR-5.5 mM line ($P_{value} = 0.0098$, $\chi^2 = 34.62$, d. f. = 3), (Fig. 1).

3.2.2. Location of parasites in Ph. papatasi digestive tracts

Two days p. i., for each batch of infected sand flies (LIPA-WT, Liby, SbIIIR-0.5 mM and SbIIIR-5.5. mM), 100% of the parasites were localized in the abdominal midgut of sand flies vector, inside the peritrophic matrix that surrounds the blood meal. On day 7 p. i., the blood meal was completely digested, and the parasites moved from the abdominal midgut to the thoracic midgut (Fig. S2, Supplementary data). The stomodeal valve (SV) of infected female sand flies was heavily colonized with parasites, a sign of mature infections and a prerequisite for parasites transmission. The rate of SV colonization in infected females was significantly higher in the wild-type strains (LIPA-WT and Liby) compared to both resistant-lines ($P_{value} = 0.00325$, $\chi^2 = 28.65$, d. f. = 3), there was also a significant difference between the two resistant lines SbIIIR-0.5 mM and the SbIIIR-5.5 mM lines ($P_{value} = 0.0001$, $\chi^2 = 45.12$, d. f. = 2) (Fig. 2).

3.2.3. Morphology of parasites inside the sand fly digestive tracts

On day 7 p. i., four different morphological forms of parasites were observed: short promastigotes (SP), elongated nectomonads (EN), metacyclic promastigotes (MC) (Figure S3, Supplementary data) and rounded haptomonads. The relative representation of these morphological forms significantly differed between susceptible and resistant parasites ($P_{value} = 0.0008$, $\chi^2 = 38.56$, d. f. = 3). While the elongated nectomonads prevailed in both resistant lines, metacyclic forms were dominant in both-wild type strains (Fig. 3). The relative representation

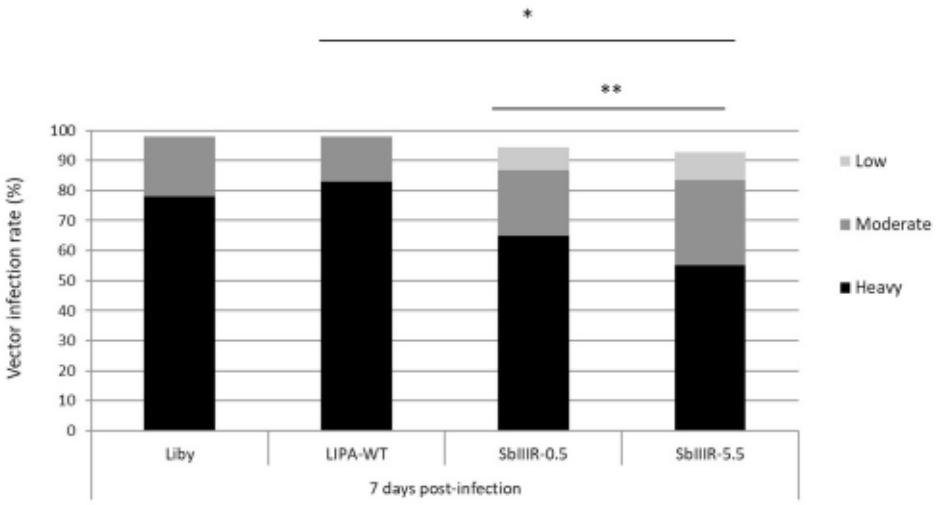


Fig. 1. Infection rates of females *Ph. papatasi* infected with *L. major* wild-type strains (Liby and LIPA-WT) and Sb(III)-resistant lines (SbIIIR-0.5 mM and SbIIIR-5.5 mM) on day 7 p. i. Parasite loads were estimated under the light microscope: low infection <100 parasites per gut, moderate infections 100–1000 parasites per gut, heavy infection >1000 parasites per gut. Differences between the groups (not infected/light infection/moderate infection/heavy infection) were evaluated using the Chi-square test ($\chi 2$). The statistical differences are considered significant if $^{*}P_{value}<0.05$ and highly significant if $^{**}P_{value}<0.01$.

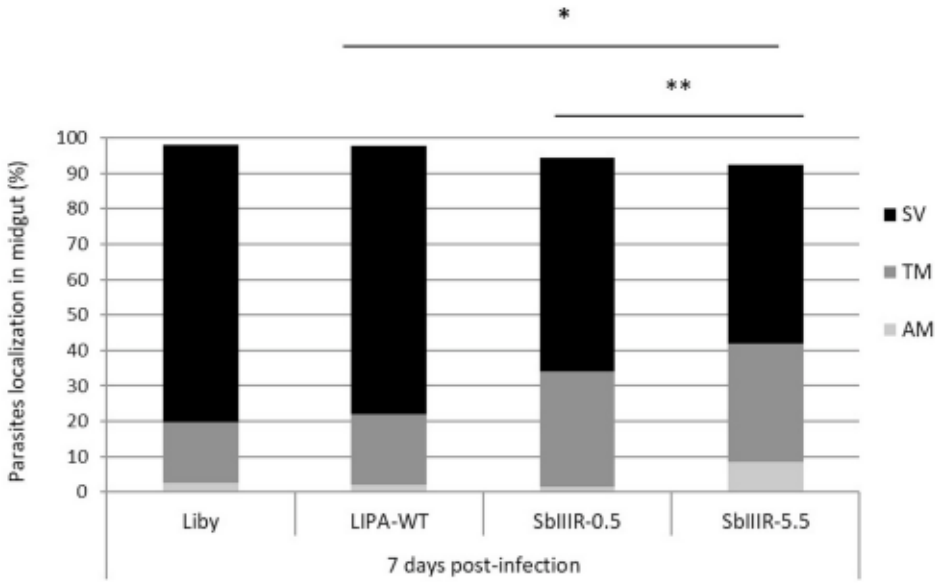


Fig. 2. Localization of L. *major* wild-type (Liby and LIPA-WT) and Sb(III)-resistant parasites (SbIIIR-0.5 mM and SbIIIR-5.5 mM) in guts of *Ph. papatasi* on day 7 p. i., **SV:** stomodeal valve, **TM:** thoracic midgut, **AM:** abdominal midgut. Differences between the groups (AM, TM, SV) were evaluated using the Chi-square ($\chi 2$) test. The statistical differences are considered significant if $^{*}P_{value}<0.05$ and highly significant if $^{**}P_{value}<0.01$.

of haptomonads was not evaluated because they remain attached to the stomodeal valve and are therefore underrepresented on the gut smears.

The results presented in this study showed clearly that the resistance to Sb(III) does not influence the early-stage development of *L. major* in sand flies as the infection rate was almost 100% in females infected with both susceptible or Sb(III)-resistant lines on day 2 p. i. In mature infections, heavy parasite loads were significantly more represented in female sand flies infected with both WT strains (LIPA-WT and Liby). In these groups, also the colonization of the SV was more often developed and metacyclic forms were more represented compared to females infected with Sb(III)-resistant lines (SbIIIR-0.5 mM and SbIIIR-5.5 mM). Therefore, a negative correlation was observed between the resistance level and the quality of parasite development in the vector's gut. The more *Leishmania* parasites were resistant, the less they colonized the SV and less heavy infections and metacyclic forms were observed.

3.3. *In vitro fitness of parasites isolated from infected sand flies*

The antimony-susceptibility, promastigote growth curves, infection rates and parasite loads in macrophages were studied in LIPA-WT, SbIIIR-0.5 and SbIIIR-5.5 mM parasites recovered from infected guts of *Ph. papatasi*. From 10 guts dissected per each *L. major* line, 2 guts free from any fungal contaminants were studied.

3.3.1. *Parasites Sb(III)-susceptibility*

Standard susceptibility assays were performed on promastigote forms to assess the Sb(III)-susceptibility of each parasite lines: LIPA- WT, SbIIIR-0.5 mM and SbIIIR-5.5 mM, before and after their passage through *Ph. papatasi* digestive tract (Table 2).

It should be noted that after passage of the parasites in sand fly guts, all three *Leishmania* lines were maintained in Sb(III)-free culture medium before calculation of the IC_{50} values. Results showed that the IC_{50}

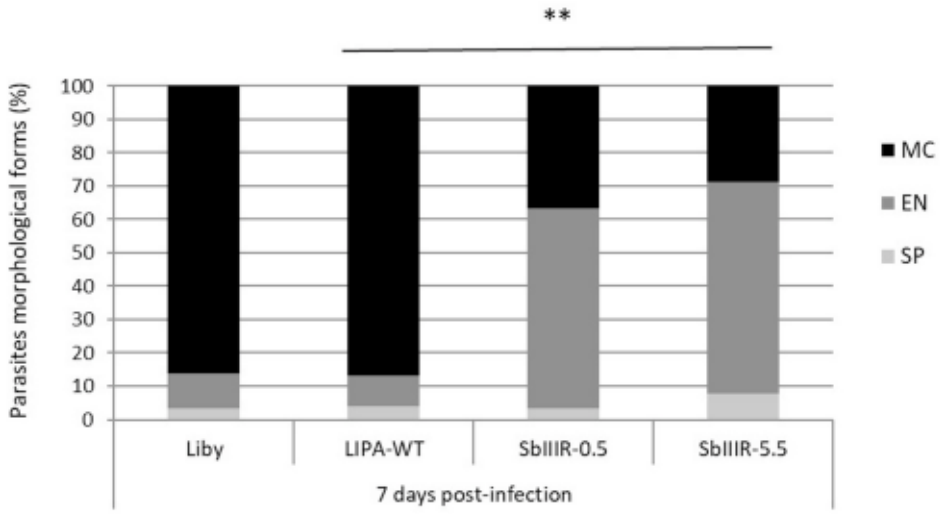


Fig. 3. Morphological forms of *L. major* LIPA-WT observed in infected *Ph. papatasi* at day 7 p. i. Parasites on gut smears of infected sand flies were fixed with methanol and stained with Giemsa. Scale bar = 10 μm. **SP:** short promastigote, **EN:** elongated nectomonad, **MC:** metacyclic promastigote.

Table 2
Susceptibility of L. *major* lines to Sb(III) drug before and after passage in females *Ph. papatasi*. Tests were running in duplicate and results were expressed as IC_{50} values that were determined by a nonlinear regression using ICEstimator website 1.2 version, and expressed as mean IC_{50} value (μM) ± standard error of the mean (SEM).

Leishmania promastigotes	IC_{50} ± SEM (μM)	
	Before infection	After infection
LIPA-WT	10.76 ± 2.38	2.55 ± 0.60
SbIIIR-0.5 mM	50.85 ± 4.65	1593 ± 100.08
SbIIIR-5.5 mM	250.8 ± 100.8	3342.31 ± 250.64

of the LIPA-WT strain decreased approximately 4-folds while the IC_{50} of both resistant lines increased 30-fold and 14-fold (SbIIIR-0.5 mM and SbIIIR-5.5 mM respectively) (Table 2). This would suggest that the environment inside the sand fly guts selected the highly resistant parasites under the vector bottle neck effect.

3.3.2. Promastigote growth curves

Growth curves of the LIPA-WT and the two selected resistant lines were compared before and after passage through sand fly guts (Fig. 4).

LIPA-WT line as well as both Sb(III)-resistant lines reached their peak of growth after 96 h in culture. Direct microscopic observation of the parasites in culture between 96 h and 120 h showed that the parasites reached their maximum capacity of multiplication and started to slowly lose their mobility which corresponds to the growth stationary phase. After 120 h in culture, the parasites started to transform into rounded forms corresponding to parasite apoptosis point. Although the multiplication *in vitro* was slightly enhanced post-passage through sand flies (LIPA-WT = from 17.01×10^6 to 19.15×10^6 parasites/mL, SbIIIR-0.5 mM = from 12.01×10^6 to 13.50×10^6 parasites/mL and SbIIIR-5.5 mM = from 11.02×10^6 to 14.25×10^6 parasites/mL), the differences were not significant ($P_{value} = 0.088$). Thus, these data suggest that the passage in the sand fly digestive tracts did not significantly change the *in vitro* multiplication and growth capacity of the parasites.

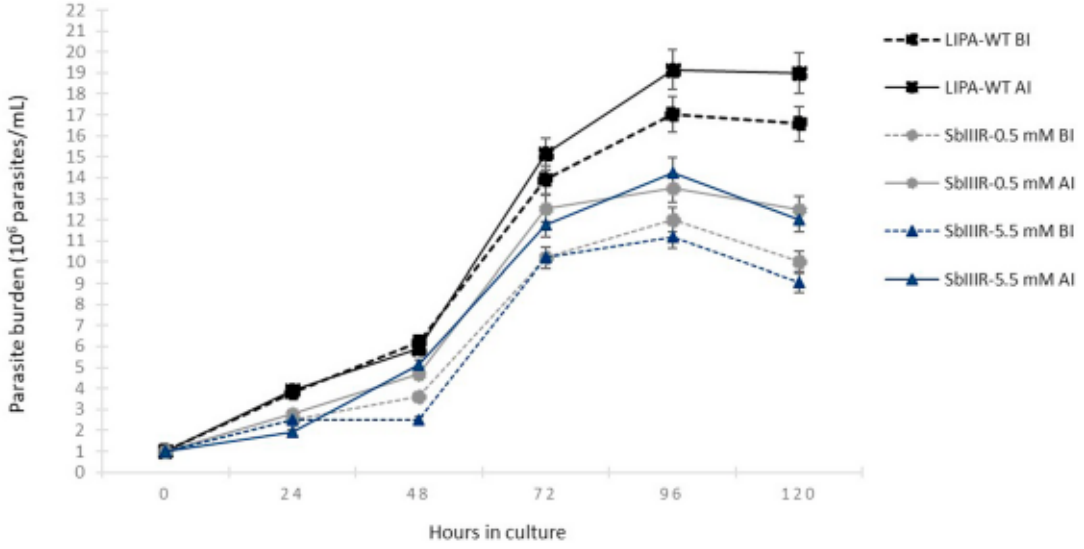


Fig. 4. Promastigote growth curves of *L. major* (LIPA-WT) and of its Sb(III)-resistant lines (SbIIIR-0.5 mM and SbIIIR-5.5 mM). **BI:** before infection, **AI:** after infection. Parasite density was determined in duplicate every 24 h (24 h–120 h). Data were analyzed with GraphPad Prism software version 7.05 (GraphPad Software Inc.). Two-way ANOVA followed by *Dunnett's* multiple comparisons test was used. Results were expressed as mean ± SD and considered significant if $P_{value} < 0.05$.

3.3.3. Development of L. major WT and Sb(III)-resistant derivative lines in macrophages RAW 264.7

The Sb(III)-resistant parasites (SbIIIR-0.5 mM and 5.5 mM) were compared to the LIPA-WT strain in i) the speed of entry into macrophages and morphology of intra-macrophage amastigote forms (Figure S4, Supplementary data), ii) the infection rate (the representation of infected macrophages) (Fig. 5. A), and iii) parasite loads in macrophages (Fig. 5. B).

In all three *L. major* lines, the promastigote parasites were able to differentiate into amastigote forms in RAW 264.7 macrophages. Under light microscopy, the amastigote forms showed an average diameter of 1.45 µm (LIPA-WT), 2.88 µm (SbIIR-0.5 mM) and 2.5 µm for SbIIIR-5.5 mM (data not shown). The LIPA-WT parasites and the resistant derivative SbIIIR-0.5 mM parasites were able to penetrate the cells after 6 h in contact while the SbIIIR-5.5 mM-resistant line spent an average of 10 h to infect the macrophages (LIPA-WT/SbIIIR-5.5 mM: $P_{value} < 0.01$; SbIIIR-0.5mM/SbIIIR-5.5 mM: $P_{value} < 0.05$).

The maximum infection rate of RAW 264.7 cells was reached after 48 h for LIPA-WT and SbIIIR-0.5 mM parasites whereas it required 24 h for SbIIIR-5.5 mM line to infect the macrophages (Fig. 5. B). However, the infection rate of SbIIIR-5.5 mM parasites remained significantly lower than in LIPA-WT and SbIIIR-0.5 mM lines.

In addition, SbIIIR-5.5 mM showed also the lowest number of amastigotes per macrophage: between 10 and 15 amastigotes were counted on RAW 264.7 macrophages for LIPA-WT and SbIIIR-0.5 mM lines and between 5 and 10 for SbIIIR-5.5 mM resistant parasites.

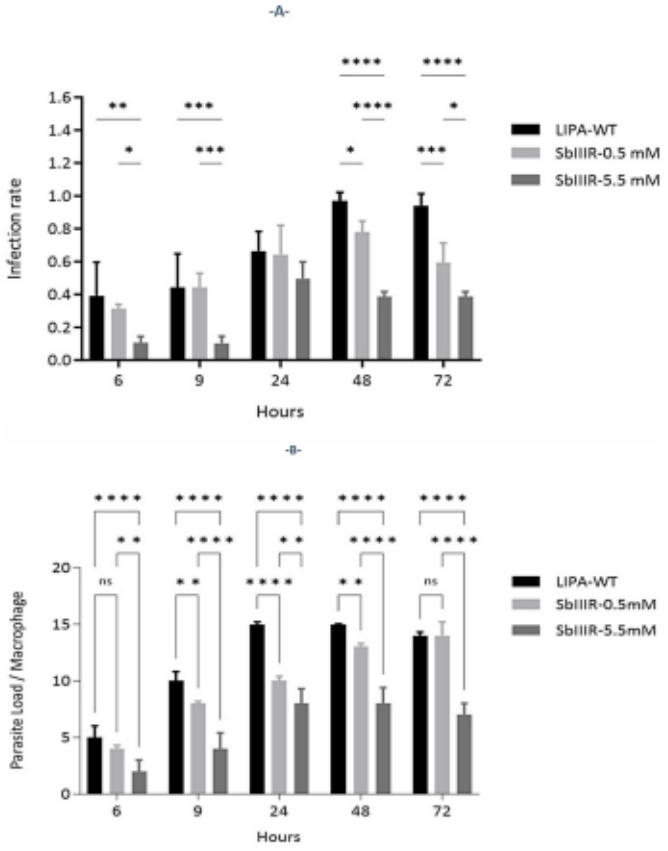


Fig. 5. Infection rate and parasite load of *L. major* LIPA-WT, SbIIIR-0.5 mM and SbIIIR-5.5 mM in murine macrophages RAW 264.7. (A) Infection rate = number of infected macrophages/number of total macrophages, (B) Parasite load/Macrophage = number of amastigotes/macrophages. The assay was assessed for 72 h. Parasites were fixed with paraformaldehyde (PFA), stained with 10% Giemsa then observed under light microscopy X1,000. Three experiments were carried out independently at 37 °C under 5% CO2 in a dark environment. Kinetics was carried at 6 h, 9 h, 24 h, 48 h and 72 h then (A) the number of infected macrophages and (B) the number of parasites that infected the macrophages were counted for each *Leishmania* lines. Two-way ANOVA followed by *Tukey's* test was used for multigroup comparisons. Results were expressed as mean ± SD and were based on three independent experiments (*$P_{value} < 0.05$, **$P_{value} < 0.01$, ***$P_{value} < 0.001$ and ****$P_{value} < 0.0001$).

3.4. Study of antimony-resistance biomarkers

The targeted resistance genes (Table 1) were analyzed using the quantitative real-time PCR (qPCR) to compare (*i*) the amplification rate (gene copy number) before and after the passage of the parasites in sand flies, (*ii*) the amplification rate within and between the three L. major lines LIPA-WT, SbIIIR-0.5 mM and SbIIIR-5.5 mM and (*iii*) the expression rate of each targeted resistance marker.

For this, we presented only the markers that showed noticeable changes in their copy numbers (Table S1, Supplementary data). For the susceptible LIPA-WT strain, either maintained in free-drug medium or after its passage through sand fly guts, we noticed that the copy number of three genes *LmMRPA*, *LmPRX* and *LmTDRX* did not change and have the same amplification level except for *LmTDR1* gene which presented a slight decrease of its copy number (Fig. 6; Table S1, Supplementary data). Regarding both Sb(III)-resistant lines, the copy number of *LmTDRX* gene decreased two fold after the passage of the resistant parasites in the sand fly guts. The copy number of *LmTDR1* slightly increased after infection of sand flies, *LmMRPA* was amplified only for the SbIIIR-0.5 mM line. The *LmPRX* gene copy number remained unchanged in the three parasite lines (Fig. 6; Table S1, Supplementary data).

When we compared the amplification rate (differences in copy numbers before and after the passage through sand fly guts) of four markers: *LmTDR1*, *LmTDRX*, *LmMRPA*, and *LmPRX* genes between LIPA-WT and Sb(III)-resistant lines, we noticed a significant difference in the relative copy number except for the couple LIPA-WT *vs* SbIIIR-0.5 mM for which *LmPRX* and *LmTDRX* genes respectively: before and after passage of the parasites in sand fly gut; did not show significant differences in their relative gene copy number (Fig. 6; Table S2, Supplementary data).

After the passage of the three *Leishmania* lines (WT and Sb(III)-resistant) in the sand fly digestive tract, the copy number of *LmTDRX*

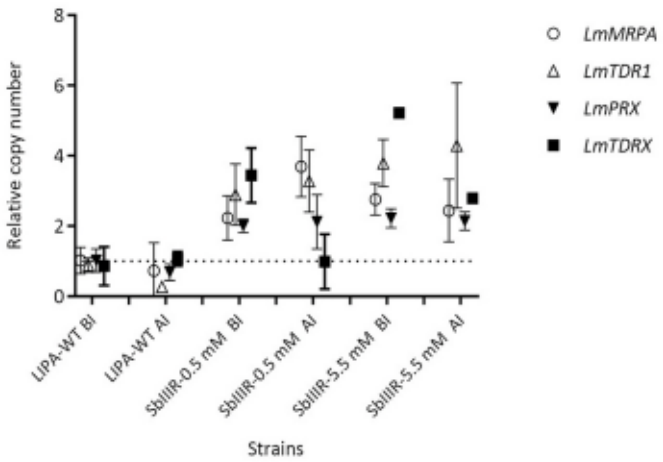


Fig. 6. Targeted gene copy number of *L. major* LIPA-WT, SbIIIR-0.5 mM and SbIIIR-5.5 mM lines after *in vitro* drug pressure and after passage in *Ph. papatasi* digestive tracts. The copy number was expressed as a ratio of *LmTDR1*, *LmTDRX*, *LmPRX* or *LmMRPA* gene to *GAPDH* gene and the data of reference strain *Leishmania major* to obtain the ΔΔCt. Data were expressed as the mean ± SD of three independent experiments. **BI:** before infection, **AI:** after infection. Two-way ANOVA followed by *Bonferonni's* test was used for multigroup comparisons. Results were expressed as mean ± SD and were based on three independent experiments.

gene decreased significantly in both resistant lines compared to LIPA-WT line while *LmMRPA* gene increased significantly its gene copy numbers in SbIIIR-0.5 mM resistant line. No differences in genes copy number were detected for the two other genes: *LmPRX* and *LmTDR1*. This suggests that *LmTDRX* gene would not be involved during the vector phase of *Leishmania* life-cycle. It is important to notice that *LmTDR1* and *LmTDRX* represent genes involved in the detoxification pathway in *Leishmania* parasites metabolism while *LmMRPA* gene code for ABC-thiol transporter involved in the sequestration of metal as thiol conjugate into a vacuole.

On the other hand, the evaluation of genes expression rate showed that three genes *LmMRPA, LmTDR1* and *LmPRX* were significantly expressed *in vivo* in both Sb(III)-resistant lines compared to the LIPA-WT strain despite the absence of drug. *LmPRX* gene is expressed 2 folds in both resistant lines while *LmMRPA* and *LmTDR1* genes were expressed 3 and 4 folds respectively in SbIIIR-0.5 and SbIIIR-5.5 mM lines compared to LIPA-WT (Fig. 7).

It appears that these genes retained the increased copy number during drug-pressure and after the passage in the sand fly digestive tracts, which seems normal given that they code for detoxifying proteins necessary to counter the action of drugs but also the hostile environment of the sand fly gut such as digestive enzymes and immune system.

4. Discussion

This study aimed to assess the impact of Sb(III)-resistance on the development of *L. major in vivo* in the sand fly and *in vitro* by comparing different biological parameters such as *i)* parasite load, location and metacyclogenesis in sand flies, *ii) in vitro* growth capacity, iii) infectiousness to macrophages, iv) drug susceptibility and *v)* the expression rate of the resistant markers.

During the vector phase of the life-cycle, *Leishmania* parasites must *i)* resist to the action of sand fly proteolytic digestive enzymes, *ii)* escape the peritrophic matrix and *iii)* attach to the midgut epithelium to prevent excretion via defecation by the sand fly, migrate anteriorly and colonize the stomodeal valve (Kamhawi, 2006; Dostálová and Volf, 2012). Here in our study, whatever *L. major* line, the parasites were able to survive, colonize the stomodeal valve and differentiate into infective forms (metacyclic promastigotes) inside the sand fly guts despite the detected differences in representation of observed parasite forms. In addition, the parasites did not lose their fitness and the resistance was stable after the passage of the two resistant-lines through sand flies. The same results were reported for the couples Sb(III)-resistant *L. infantum/Lutzomya (Lu.) longipalpis* and Sb(III)-resistant *L. infantum/Ph. perniciosus* (Seblova et al., 2014) as well as paramomycin-resistant *L. infantum/Lu. longipalpis* and *Ph. perniciosus* and paramomycin-resistant *L. donovani/Ph. argentipes* (Hendrickx et al., 2020a,b). These results suggest that there is no general link between the resistance and the loss of fitness in *Leishmania* parasites in sand flies so that many resistant lines are able to spread and circulate in nature and thus threatening human and animal health. Nevertheless, the development of resistant *Leishmania* lines in sand fly depends on the type of drug and parasite species, since the development of miltefosine-resistant *L. infantum* was significantly impaired in *Ph. perniciosus* and *Lu. longipalpis* (Van Bockstal et al., 2019).

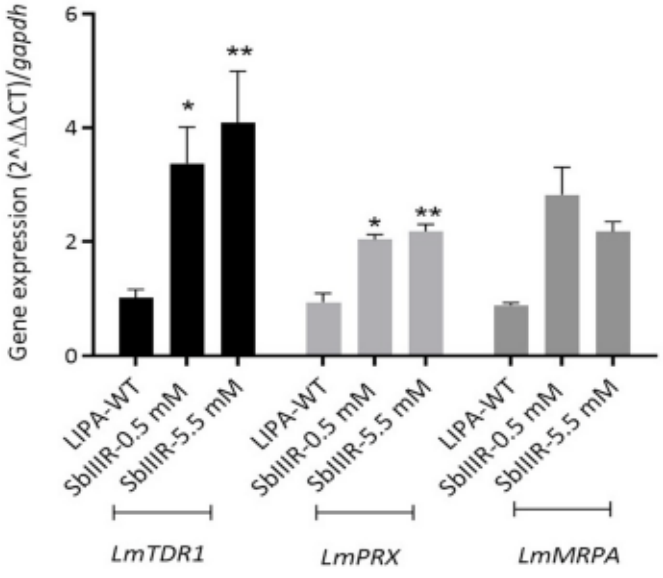


Fig. 7. Graphical representation of *LmTDR1*, *LmPRX* and *LmMRPA* gene expression normalized on *GAPDH* gene. The assay was ran on LIPA-WT, SbIIIR-0.5 mM and SbIIIR-5.5 mM lines after passage on infected *Ph. papatasi* digestive tracts. The gene expression level was expressed as a ratio of *LmTDR1*, *LmPRX* or *LmMRPA* gene, and mRNA level to *GAPDH* mRNA level. Data were expressed as the mean ± SD of three independent experiments. Two-way ANOVA followed by *Tukey's* test was used for multigroup comparisons. Results were expressed as mean ± SD and were based on three independent experiments ($*P_{value} < 0.05$, $**P_{value} < 0.01$).

Another important and amazing point was the inverse effect observed in *L. major* lines after their passage through the vector: the susceptible phenotype of WT line became more susceptible while both resistant lines became more resistant. This could be explained by the hostile environment existing in the vector gut which exerts pressure on the different *Leishmania* clones or sub-populations composing each of the three *L. major* lines, thus by eliminating some sub-populations under the phenomena of bottle neck, recently reported in *L. donovani* strain after its transition in *Ph. orientalis* (Bussotti et al., 2023). This phenomenon could be caused by different parameters *i)* the small number of parasites ingested during a sand fly blood meal, *ii)* the potential elimination of parasites by toxic products during blood meal digestion, or *iii)* their failure to attach to the sand fly midgut that leads to parasites excretion via defecation of the digested blood meal (Bussotti et al., 2023; Pruzinova et al., 2018).

Moreover, the ability of *Leishmania* parasites to amplify the copy number of specific genes and to use the advantage of these extra copies may increase the levels of gene products and their drug-resistance phenotype (Ponte-Sucre et al., 2013). Moreover, the amplification of the *LmMRPA* gene obtained during this study is in line with previous findings. Leprohon et al. (2009) demonstrated that *L. infantum* KOs lines for *MRPA* gene restored the susceptibility to antimonials, while Douanne et al. (2020) observed that in case of complete deletion of the gene, other transporters can be involved in drug resistance mechanism.

In addition, it was not surprising to see an increase in the copy number of some genes related to the detoxification pathway in the resistant lines since it is well known that one of the unique features of *Leishmania* parasites is "Gene plasticity" and their ability to alter the copy number of individual genes or in some cases; to alter a group of genes, chromosomes and even the entire genome (Ponte-Sucre et al., 2013). Indeed, the detoxification pathway is essential for *Leishmania* parasite to escape the host defense mechanism and to survive the drug treatment. Thus, many proteins are expressed to activate this pathway for the survival of the parasite within the host and already reported in other parasites and yeast species such as *Trypanosoma brucei brucei and Saccharomyces cerevisiae* in response to oxidative stress (Pedrajas et al., 1999; Reckenfelderbäumer et al., 2000). The thiol-dependent reductase 1 (TDR1) of *Leishmania* sp. has been implicated in parasite-host interaction. Like other proteins in the glutathione/trypanothione-based redox system, this gene allows the reduction of Sb(V) to Sb(III) and is over-expressed in antimony-resistant parasites (Denton et al., 2004; Nateghi-Rostami, Tasbihi & Darzi, 2022). However, these proteins have not yet been studied to understand their implication and specific role in the survival of *Leishmania* parasites during the vector phase of their life-cycle. For this, a complete genome sequencing of various *Leishmania* species could help to identify and validate new targets as resistance biomarkers to better understand the apparition of resistance

mechanisms as well as to identify new therapeutic targets against *Leishmania* parasite (Ponte-Sucre et al., 2013).

5. Conclusion & perspectives

Drug resistance becomes the major obstacle to the treatment, prevention and eradication of parasitic or bacterial diseases for humans in the absence of efficient vaccine (Sereno et al., 2012; Dujardin et Decuypere, 2013). Here, we established a laboratory experimental model *Ph. papatasi/L. major*, trying to be mimetic of parasite life cycle in the natural conditions which provided suitable indications about the potential transmission of parasite populations resistant to trivalent antimony by the sand fly in controlled conditions. The results presented in this study showed that *Ph. papatasi* could promote circulation of *L. major* Sb(III)- resistant parasites in the field. More disturbingly, our data suggest that the selective pressures from the vector may even enhance antimony resistance. Further experiments are needed to confirm this surprising finding. Moreover, studies using new molecular tools as WGS, NGS and CRISPR-cas9 will allow to better understand *i)* the role of *LmMRPA* and *LmTDR1* during the sand fly stage of parasites life-cycle *ii)* the risks of transmission of these drug-resistant parasites in the field to anticipate the evolution of the epidemiological situation in areas being endemic with zoonotic cutaneous leishmaniasis caused by *L. major* MON-25.

Authors contribution

Conceptualization: N.M, Z.H., S.C., P.M.L, K.E.B., J.S., B.V.; Parasites analyses: N.M., S.C., N.I., M.L., A.M.; Sand fly experiments: J.S., B.V., N. M., K.E.B.; Writing original draft: N.M., K.E.B., S.C., PM.L.; Editing: J.S., P.V., P.M.L., Z.H. All authors have read and agreed to the published version of the manuscript.

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Nalia Mekarnia: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kamal-Eddine Benallal:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jovana Sádlová:** Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Barbora Vojtková:** Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation. **Aurélie Mauras:** Methodology, Data curation. **Nicolas Imbert:** Methodology. **Maryline Longhitano:** Methodology. **Zoubir Harrat:** Writing – original draft, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Petr Volf:** Writing – original draft, Validation, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Philippe M. Loiseau:** Writing – original draft, Validation, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Sandrine Cojean:** Writing – original draft, Validation, Supervision, Resources, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2024.100554.

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Summary and Discussion:

This dissertation thesis sums the results of four sub-objectives constituting my Ph.D project. To achieve our objectives, various methods were deployed such as DNA-based tools, protein-based techniques, and experimental infections of sand flies and rodents. Rodent and sand fly samples were collected in Algeria in different provinces (TiziOuzou, Bejaïa, Setif and Tiaret in the north, Ghardaïa and Illizi in the central Sahara) where CL and VL are reported. The analyses were carried out at Charles University and the trapped rodents were reared at Institut Pasteur of Algeria before being sent to the Czech Republic.

The first sub-objective referred to the construction of DNA barcodes and MALDI-TOF protein profiling databases for proven and/or suspected sand fly and rodent species involved in the transmission cycle of *Leishmania* parasites. For these purposes, firstly, searching literature in several publication repositories and sand fly collection databases available at the Institut Pasteur of Algeria was carried out to compile a review of the sand fly species reported in the Maghreb region, focussing on the geographical distribution of each species to address distribution maps, proposing simplified morphological identification keys for beginners, and emphasising and highlighting the different gaps in sand fly studies to fulfil for the Maghreb region. Secondly, to construct DNA barcodes database, molecular tools (PCRs and sequencing technique) were used targeting two genes of the mitochondrial genome "Cox1 and CytB" to confirm species identification and carry out phylogeographic studies. Mitochondrial genome mtDNA was preferred to nuclear DNA due to the absence of recombination, uniparental inheritance, conserved structure and relatively high evolutionary rate. The analysis of intraspecific mtDNA variation can reveal information about the interconnectivity of populations and past demographic events such as population expansions. The cytochrome oxidase I (COI) gene or Cox1 is one of the most frequently employed mtDNA gene to investigate phylogeographic patterns and histories at the inter- and intraspecies level and has been extensively used for evolutionary studies in insects (Maaiki et al., 2011). Cox1 is known for its high discrimination efficiency between vertebrate species despite some reported limits in amplification success rate for some taxonomic groups such as Diptera, marine invertebrates, and nematodes [106], [107]. However, CytB was used for taxonomic identification of species that was impossible to amplify using Cox1. Besides DNA barcoding, the protein-based techniques were also deployed. While MALDI-TOF protein profiling enabled to construct sand fly and rodent databases, a MALDI-TOF peptide mass mapping allowed to analyse the source of female blood meals [79], [108], [109], [110], [111]. Analysis of the blood

meal sources of sand fly females trapped in different provinces of Algeria revealed that *Phlebotomus papatasi*, *Ph. perniciosus*, *Ph. bergeroti*, and *Ph. alexandri*, known to be anthropophilic were opportunists and fed, in addition to humans, on various animals such as cows, sheep, dogs, camels, donkeys, and chickens. The analysis of the *Paraphlebotomus* subgenus using the MALDI-TOF MS technique confirmed the previous suggestion to include *Ph. alexandri* into a new subgenus *Artemievus subg.* nov. *Depaquit* [52]. Recently, artificial intelligence such as machine learning has been integrated into MALDI-TOF analysis software to improve the discrimination level between cryptic species especially in *Larroussius* subgenus [82]. Recently, several new species and subgenera of sand flies were reported by combining morphology, morphometry, and molecular techniques (DNA and protein-based techniques) [64], [112]. In our study, combination of morphological and molecular tools allowed the description of a new species of sand fly belonging to the genus and subgenus *Sergentomyia*, this species updating the list of known sand flies and increasing the number of sand fly's species to 27 for Algeria (Benallal et al, 2024).

The second sub-objective focused on the putative reservoirs of *Leishmania* parasites. By definition, a reservoir host must maintain and transmit the pathogen to a new naïve host for at least a period corresponding to hibernation or the winter period when vector activity ceases [46], [86], [113]. In our study, *Gerbillus amoenus* were trapped in the central Sahara and after a quarantine period, 6 specimens were experimentally infected via intradermal route by 10^4 culture-derived *Leishmania major* [114] and subjected to xenodiagnostic experiments to assess their infectiousness to sand flies. The results showed that this rodent species was susceptible to *L. major* and was able to maintain and transmit again the parasites to naïve sand fly females after 6 months. Therefore, G. *amoenus* can play a role in the transmission cycle of *Leishmania* parasites in areas where the proven Algerian reservoirs of *L. major* such as *Meriones shawi* and *Psammomys obesus* are absent [95], [115].

The third sub-objective evaluated the interaction within the complex *Ph. papatasi-M. shawi* and *L. major*, it concerned the monitoring of the lesion kinetics and the role of asymptomatic rodents in the transmission of *L. major*. Previous studies reported that between 35 and 40 % of trapped rodents were naturally infected with *Leishmania* parasites but did not show any infection signs, *i.e.*, they were asymptomatic [116], [117], [118]. Our laboratory experiments built on these field studies to demonstrate whether such asymptomatic hosts contribute to parasite transmission, because infectiousness of asymptomatic hosts represents a great

challenge for disease control [119]. In this context, 32 *M. shawi* rodents were experimentally infected via intradermal route with a natural dose of *L. major* Mon-25 supplemented with salivary gland components. Xenodiagnostic experiments showed that asymptomatic animals were infectious to vectors several weeks before the appearance of skin lesions and several months after their healing in symptomatic once, and the difference in infectiousness between asymptomatic and symptomatic animals was not statistically significant. Therefore, the presence of lesions is not a condition for infectiousness, this finding suggested that it will be necessary to take into account the asymptomatic rodents in various modelling epidemiological studies when developing the risk map for good control strategies.

The last sub-objective was to assess of the impact of Sb(III)-resistance on the fitness of *L. major in vivo* (inside sand fly gut) and *in vitro* after recovering parasites from sand fly guts. During the last two decades, several studies reported an increase in clinical relapses and treatment failures among patients suffering from leishmaniasis who were treated with pentavalent antimonials (SbV), miltefosine (MIL) or paromomycin [40]. Algeria did not make the exception and reported the circulation of *Leishmania*-resistant strains against antimonials (SbV) that is the first-line drug given to patient for both VL and CL forms [43]. Most of the studies have addressed the various mechanisms of parasites resistance while few, if any, have focused on the transmission of these strains between hosts (vector and mammal) and the impact of resistance on the parasite fitness. The few previous studies concentrated on the impact of resistance on the development of parasites in sand fly only targeted *L. donovani* and *L. infantum* resistant strains to miltefosine (MIL) and paromomycin [44], [54], [120], [121]. For this purpose, two *Leishmania major* lines resistant to Sb(III) were generated in the laboratory at concentration of 0.5mM and 5.5mM by discontinuing drug pressure [122], then the fitness of the wild-type strain and both derived resistant lines was evaluated *i*) *in vivo* in *Ph. papatasi* by comparing parasite loads, morphological forms and location of parasites in the sand fly digestive tract and *ii*) *in vitro* by measuring and comparing different biological parameters such as growth capacity, parasites drug susceptibility, the expression rate of resistance genes, the percentage of infected macrophages as well as the parasite load per macrophage. The *in vivo* results showed that at day 7 post-infection, Sb(III) resistant *L. major* strains showed a lower representation of heavy infections compared to the wild type, migrated less to the stomodaeal valve and transformed less into metacyclic promastigote forms suggesting that the acquisition of resistance have a negative impact and could be a burden to the development of the resistant parasites in their natural vector. Nevertheless, these results also showed that despite

these constraints, nearly half of infected females developed a mature infection, which is sufficient to transmit parasites to naïve hosts [123] and allow circulation of resistant strains between hosts. Interestingly, *in vitro* results showed that the resistant strains became more resistant to Sb(III) after the transition of parasites through sand fly guts since their IC_{50} (corresponding to 50% of parasite mortalities) increased up to 30-folds while it decreased 5-folds in wild type parasites. This is probably a result of the bottleneck effect that occurs in sand fly guts with allelic selection as a key driver of genomic adaptation during *Leishmania* insect infection, as recently reported in the *L. donovani /Ph. orientalis* couple [124]. The *in vitro* experiments with murine macrophages RAW 264.7 showed that the resistant parasite lines were able to penetrate and multiply in murine macrophages, however, the most resistant line was delayed in penetration to macrophages and the infection rate and parasite load were lower compared to wild type. In addition, we studied the genes amplification and genes expression rate of the parasites. It is known that *Leishmania* parasites have the ability to amplify the copy number of specific genes and to use the advantage of these extra copies to increase the levels of gene products and, consequently, their drug-resistance phenotype [125]. In this study, four genes *LmMRPA*, *LmPRX*, *LmTDRX* and *LmTDR1* involved in the detoxification pathway underwent a significant change in their copy numbers after their passage in sand fly guts. Indeed, the detoxification pathway is essential for *Leishmania* parasite to escape the host defence mechanism and also to survive to drug treatment. In the context of antimony-resistance, this pathway is essential and has been often reported in other parasites and yeast species such as *Trypanosoma brucei brucei* and *Saccharomyces cerevisiae,* in response to oxidative stress [126], [127]. For the sensitive WT strain, the gene copy numbers did not change except for *LmTDR1* which showed a decrease in copy number. For the resistant strains, the gene coding *LmMRPA* and *LmTDR1* increased in copy numbers in contrast to *LmTDRX* that fell drastically suggesting that this gene is more involved in the presence of drug and the other genes are more likely involved in surviving of parasites inside sand fly guts where different barriers exist such as peritrophic matrix, digestive proteases and immune system.

Conclusions:

1. The review Benallal et al. 2022 documented the presence of 32 sand fly species in the Maghreb region of which 26 are reported in Algeria. In addition, it showed that leishmaniases remain a major public health problem in all countries of the region and more attention shall be also paid to presence and significance of sand fly-borne viruses in addition to the urgent need for fulfilling data gaps concerning leishmaniasis and sand fly vectors in Mauritania, and finally, combining different tools such as morphology, molecular (DNA and protein) and morphometrics approaches to describe new sand fly species;
2. The study Messahel et al. 2022 showed the efficiency of MALDI-TOF mass mapping peptide technique in the identification of blood meal sources dominated by cattle in the endemic focus of canine leishmaniasis in the north-eastern Algeria where three proven or suspected *Larroussius* vectors of *L. infantum* occur;
3. The study Benallal et al. 2024 (under review) described a new sand fly species belonging to genus and subgenus *Sergentomyia* named *Se. imihra* in the central Sahara and identified the blood meal sources using MALDI-TOF mass mapping peptide where camel, goat, sheep, dog, chicken and human were identified and demonstrating the opportunistic behaviour of *Phlebotomus papatasi*, *Ph. perniciosus*, *Ph. bergeroti,* and *Ph. alexandri* known as proven or suspected vector of leishmaniasis highlighting the risk of zoonotic disease transmission;
4. The study Benallal et al. 2023 demonstrated the susceptibility of *Gerbillus amoenus* to *L. major* and its ability to maintain and transmit parasites to naive sand flies after 6 months of infection. Thus, this rodent species can play a role in the maintenance of *L. major* in Algeria, particularly where the established reservoir hosts *Psammomys obesus* and *Meriones shawi* are absent.
5. The study by Sadlova et al. 2023 proved that asymptomatic *Meriones shawi,* a reservoir of *L. major* in the Maghreb region, are involved in parasite transmission to naïve sand flies and in the spread of parasites which should be considered when modelling the epidemiology of leishmaniasis in order to implement proper disease control strategies.
6. The study of Mekarnia et al. 2024 showed that despite the fact that the antimony resistant lines of *L. major* MON-25 showed reduced metacyclogenesis in the natural vector *Ph. papatasi*, they were able to produce mature infections and parasites recovered from sand fly guts showed

enhanced antimony resistance. This suggests that *Ph. papatasi* supports the development and circulation of *L. major* Sb(III)-resistant lines in the field.

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Appendix

List of publications:

1- Benallal KE, Garni R, Harrat Z, Volf P, Dvorak V. **Phlebotomine sand flies (Diptera: Psychodidae) of the Maghreb region: A systematic review of distribution, morphology, and role in the transmission of the pathogens**. PLoS Negl Trop Dis. 2022, 6;16(1):e0009952. doi: 10.1371/journal.pntd.0009952.

My contribution: data search, study design, writing original draft.

2- Messahel NE, Benallal KE, Halada P, Lafri I, Manseur H, Hakem A, Houali K, Harrat Z, Volf P, Dvorak V. **Identification of blood source preferences and *Leishmania* infection in sand flies (Diptera: Psychodidae) in north-eastern Algeria**. Vet Parasitol Reg Stud Reports. 2022, 31:100729. https://doi.org/10.1016/j.vprsr.2022.100729

My contribution: study design, molecular analysis, writing and editing manuscript.

3- Sadlova J, Vojtkova B, Lestinova T, Becvar T, Frynta D, Benallal KE, Mekarnia N, Harrat Z, Volf P. **Infectiousness of Asymptomatic *Meriones shawi*, Reservoir Host of *Leishmania major***. Pathogens. 2023, 12(4): 614. https://doi.org/10.3390/pathogens12040614.

My contribution: trapping, breeding and sending animal to Prague.

4- Benallal KE, Mezai G, Mefissel M, Klari N, Lardjane C, Khardine AF, Kherachi I, Dib Y, Brahmi K, Sadlova J, Volf P, Harrat Z. **Host competence of Algerian *Gerbillus amoenus* for *Leishmania major***. Int J Parasitol Parasites Wildl. 2023, 21:69-73. https://doi: 10.1016/j.ijppaw.2023.04.007

My contribution: sampling, experimental infections of animals, xenodiagnostic experiments, tissue isolations, molecular analysis, writing original draft.

5- Mekarnia N, Benallal KE, Sadlova J, Vojtkova B, Mauras A, Imbert N, Longhitano M, Harrat Z, Volf P, Loiseau PM, Cojean S. **Effect of the Passage of Antimony-resistant *Leishmania major* MON-25 in *Phlebotomus papatasi* on its Fitness, Virulence and Antimony Resistance Phenotype**. Int J Parasitol Drugs Drug Resist. 2024, 100554. doi: 10.1016/j.ijpddr.2024.100554.

My contribution: study design, experimental infections of sand flies, data analysis, writing original draft.

6- Benallal KE, Mefissel M, Dib Y, Depaquit J, Kavan D, Harrat Z, Dvorak V, Volf, P, Halada, P. **Phlebotomine sand fly survey, blood meal source identification and description of *Sergentomyia imihra* n. sp. in the Central Sahara of Algeria**. Parasites & Vectors. 2024, 17:449. https://doi.org/10.1186/s13071-024-06542-9

My contribution: sampling, study design, molecular analysis, writing original draft.

I hereby confirm the above described contribution of Kamal Eddine Benallal to the six listed scientific publications.



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RNDr. Vít Dvořák, Ph.D.

Curriculum vitae

Education

- **PhD**

Charles University: 2019 – present: « **Phlebotomine sand fly in the transmission cycles of leishmaniasis in Algeria** ».

- **Master**

University of Montpellier 2, France & Abomey Calavi, Benin, 2009: « **The factors controlling the susceptibility of Anopheles gambiae with Plasmodium berghei and development protocol for the in vitro culture ookinetes of Plasmodium falciparum** ».

- **Bachelor of Genetic**

University of Sciences and Technologies Houari Boumedienne, Algeria 2004: « **Lyme disease through the identification of spirochetes of the complex Borrelia burgdorferi sensu lato isolated from the gastrointestinal tract of ticks Ixodes ricinus in Algeria** ».

Committee Member

- Member of the National Committee for Malaria Elimination in Algeria as an entomology expert.
- Member of the National Committee for the National Multisectoral Integrated Leishmaniasis Control Plan.

Teaching Activities

- October 2015: « **Sensitivity Tests of Mosquitoes to Insecticides** » held in Institut Pasteur of Algiers**.**
- May 2011: «Insect Control in Aircraft » held in Institut Pasteur of Algiers.
- December 2010: « **The** Entomology of Malaria » held in Institut Pasteur of Algeria.
- October 2010: «La leishmaniose cutanée et changement climatique » held in the Social and Cultural Anthropology Research Center, Oran, Algeria.

Projects

- PI in **African Leishmaniasis Consortium: « Developing a Paradigm for Eliminating Neglected Tropical Diseases in Africa »** 2023-2026.
- **PI** in the project «**Transmission cycles of *Leishmania major* in natural reservoir hosts: local analyses with global implications »** Thesis project.
- Task leader in **LeishMati-Rise** (2018-2023),
- **PI** in **MediLabSecure, Entomology Package** (2014-2017 & 2018-2021, 2022-2024),
- **PI** in Acip-A-2-2014 project «**Bionomics, Receptivity to *Plasmodium falciparum* and Susceptibility to insecticides of *Anopheles sergentii* in the Maghreb » (2014-2016),**
- **Collaborator** in Acip-8-2009 project « **La région du Maghreb, une zone d'émèrgence ? L'exemple de la transmission des arbovirus par *Culex pipiens*** » (2009-2011),
- **Collaborator** in ACIP A-04-2007 «**Exploration éco-épidémiologique des foyers de leishmaniose cutannée à *Leishmania killicki*** » (2008-2010),

- Collaborator in EDEN-Malaria (2005-2010), **Collaborator** in the project « **The situation of Barbary apes in Algeria** » (2006-2007).

Conferences

1. Poster presentation in the Adapting to Change: Emerging Infectious Diseases in Shifting Climate conference held in France, Paris. October 16-18, 2024 « **Surveillance of Crimean-Congo Haemorrhagic Fever Virus in Central Sahara, Algeria » KE. Benallal,** A. Hachid, M. Tamani, FA. Khardine, NA. Gessoum, I. Lafri.
2. Oral communication in the International Symposium of Phlebotomine Sandflies held in Portoroze, Slovenia. 9-13 September 2024 « **Sand fly Fauna in Leishmaniasis Foci in Algeria: Species Composition and Blood Meal Sources Studied by Complementary Techniques » KE. Benallal**, P. Halada, J. Depaquit, H. Menseur, I. Lafri, M. Mefissel, Y. Dib, P. Volf, V. Dvorak.
3. Co-author in oral communication in the International Symposium of Phlebotomine Sandflies held in Portoroze, Slovenia. 9-13 September 2024 « **Infectiousness of Natural Host of *Leishmania major* to Sand flies at Micro- and Macro-scale** » B Vojtkova, T. Becvar, N. Mekarnia, D Frynta, **KE. Benallal**, Z. Harrat, P. Volf, J. Sadlova.
4. Shotgun presentation at the 3rd International Caparica Conference on Leishmaniasis held in the Hotel Tryp Lisboa Caparica Mar (Lisbon), Portugal, 24th to the 26th October 2022 « **Host competence of the Algerian *Gerbillus amoenus* for *Leishmania major* » Benallal KE**, Mezai G, Mefissel M, Klari N, Lardjane C, Kherachi I, Khardine AF, Dib Y, Jovana S, Z Harrat.
5. Online poster presentation in Ento 21' Event organized by Royal Entomological Society between 23rd and 27th August 2021 Genetic diversity of *Phlebotomus perniciosus* around the Mediterranean Basin Kamal Eddine BENALLAL*[1,2], Ida HANUSNIAKOVA[2], Hamza MANSOURI[3], Ismail LAFRI[3], Zoubir HARRAT[1], Vít DVORAK[2].
6. Oral presentation in the 20th Esove Conference held in Lisbon, Portugal, 3rd-7th October 2016 « **First report of Aedes (Stegomyia) albopictus (Diptera: Culicidae) and its establishment in Oran, West of Algeria** » KE. Benallal, A. Allal-Ikhlef, K. Benhammouda, K. Snouci, F. Schaffner, Z. Harrat.
7. Oral presentation in the 19th Esove Conference held in Thessaloniki, Greece, 13th-17th October 2014 « **Use of Morphometry for Discrimination of Atypical Form of *Phlebotomus perniciosus* in Algeria** » KE. Benallal, R. Benikhlef, R. Garni, B. Gassen, JP Dedet, Z. Harrat.
8. Poster presentation in the 18th Esove Conference held in Montpellier, France, 8th-11th October 2012 « **New Station of *Phlebotomus kazeruni* (Theodor and Mesghali; 1964) in the Hoggar (South Algeria)** » KE. Benallal, B. Gassen, L. Bouiba, J. Depaquit, Z. Harrat.

Courses & Training

- « **Mobile laboratory training** », May 2024, Institut Pasteur of Dakar, Senegal.
- « **Metagenomic for rapid viral surveillance using Nanopore technology** », May 2023, Institut Pasteur of Paris, France.

- « **Use of CRISPR-Cas9 genome editing technique in *Leishmania* parasites studies** », February-March, 2023, Parasite Signalisation Laboratory of Gérald Spaeth, Institut Pasteur of Paris, France.
- « ***Aedes albopictus* insecticide resistance assessment** », October 2022, IRD, Montpellier, France.
- « **Secure insectary experimental methods** », The Pirbright Institute, June 2022, England.
- « **Biosafety for containment and handling of mosquitoes and other biting insects** », Healthy Science e-Training Foundation, June 2022.
- « **Advanced Sequence Analysis and Phylogenetics**» Cairo, Egypt (2018),
- « **Entomological surveillance and vector control** » IRD & EID, France (2018),
- « **Wildlife Bats Capture and Sampling for Surveying Emerging Zoonotic Diseases** » the University of Njala, Sierra Leone (2017),
- « **Risk Assessment of Rift Valley Fever Introduction** » Institut Pasteur of Tunisia (2017),
- « **4th One Health Course**» Institute Pasteur of Paris (2016),
- « **Applications of Genomics in Public Health for Infectious Diseases of Poverty: Diagnostics and Vector Control** » Institute Pasteur of Tunisia (2014),
- « **Sand fly rearing and bio-essay tests** » University of Hacettepe, Ankara, Turkey (2012),
- « **Learning and Teaching Parasite Cell Biology, A Route to Successful Biomedical Research in Africa** » Malaria Research and Training Center, Mali (2012).

Publications

1- **Phlebotomine sand fly survey, blood meal source identification and description of *Sergentomyia imihra* n. sp. in the Central Sahara of Algeria**. **Benallal KE**, Mefissel M, Dib Y, Depaquit J, Kavan D, Harrat Z, Dvorak V, Volf, P, Halada, P. Parasites & Vectors. 2024, 17:449. https://doi.org/10.1186/s13071-024-06542-9

2- **Effect of *Phlebotomus papatasi* on the fitness, infectivity and antimony-resistance phenotype of antimony-resistant *Leishmania major* Mon-25.** Nalia Mekarnia, **Kamal-Eddine Benallal** , Jovana Sadlova, Barbora Vojtkova, Aurélie Mauras, Nicolas Imbert, Maryline Longhitano, Zoubir Harrat, Petr Volf, Philippe M. Loiseau, Sandrine Cojean. International Journal for Parasitology: Drugs and Drug Resistance 25 (2024) 100554. https://doi.org/10.1016/j.ijpddr.2024.100554

3- **Host competence of Algerian *Gerbillus amoenus* for *Leishmania major*. Kamal Eddine Benallal**, Ghania Mezai, Mohammed Mefissel, Nadia Klari, Cylia Lardjane, Ahmed-Fayez Khardine, Ihcen Kherachi, Yacine Dib, Karima Brahmi, Jovana Sadlova, Petr Volf, Zoubir Harrat. International Journal for Parasitology: Parasites and Wildlife 21 (2023) 69–73. https://doi: 10.1016/j.ijppaw.2023.04.007.

4- **Infectiousness of Asymptomatic *Meriones shawi*, Reservoir Host of *Leishmania major***. 2023. Jovana Sadlova, Barbora Vojtkova, Tereza Lestinova, Tomas Becvar,

Daniel Frynta, **Kamal Eddine Benallal**, Nalia Mekarnia, Zoubir Harrat and Petr Volf. Pathogens, 12, 614. https://doi.org/10.3390/pathogens12040614.

5- **Epidemiology, isolation and genetic characterization of Toscana virus in Algerian patients displaying neurological infection, 2016-2018**. Chahrazed Benbetka, Aissam Hachid, **Kamal Eddine Benallal**, Fayez Ahmed Khardine, Nazli Ayhan, Nesrine Bourdjoul, Wissam Meriem Boulehbal, Djamila Bellila, Aldjia Khaldi, Rémi Charrel. International Journal of Infectious Disease, 2023 (7) : 193-198.

6- **Assessment of expertise in morphological identification of mosquito species (Diptera, Culicidae) using photomicrographs**. Nil Rahola et al. Parasite. 2022; 29:45. Doi:10.1051/parasite/2022045.

7- **First Isolation of Punique Virus from Sand Flies Collected in Northern Algeria**. Manseur H, Hachid A, Khardine AF, **Benallal KE**, Bia T, Temani M, Hakem A, Sánchez-Seco MP, Bitam I, Vázquez A, Lafri I. Viruses. 2022 Aug 17;14(8):1796. doi: 10.3390/v14081796.

8- **Detection and Isolation of Sindbis Virus from Field Collected Mosquitoes in Timimoun, Algeria.** Nazli Ayhan, Aissam Hachid, Laurence Thirion, **Kamel Eddine Benallal**, Laura Pezzi, Fayez Ahmed Khardine, Chahrazed Benbetka, Sihem Benbetka, Zoubir Harrat and Remi Charrel. *Viruses* **2022**, *14*(5), 894; https://doi.org/10.3390/v14050894.

9- **Identification of blood source preferences and *Leishmania* infection in sand flies (Diptera: Psychodidae) in north-eastern Algeria.** Nacer EddineMessahel, **Kamel Eddine Benallal**, Petr Halada, Ismail Lafri, Hemza Manseur, Ahcene Hakem, Karim Houali, Zoubir Harrat, Petr Volf, Vit Dvorak. Veterinary Parasitology: Regional Studies and Reports. Volume 31, **2022**, 100729. https://doi.org/10.1016/j.vprsr.2022.100729

10- **Phlebotomine sand flies (Diptera: Psychodidae) of the Maghreb region: A systematic review of distribution, morphology, and role in the transmission of the pathogens**. **Benallal KE**, Garni R, Harrat Z, Volf P, Dvorak V. PLoS Negl Trop Dis. 2022 6;16(1):e0009952. doi: 10.1371/journal.pntd.0009952. eCollection 2022 Jan.

11- **Bat Capture Manual for Africa** Roland Suluku, Temidayo Adeyanju, **Benallal Kamal.** Sierra Leonean Writers Series edition, ISBN: 979-8454432041. August 11, 2021

12- **First detection of *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) in Algiers, the capital city of Algeria**. (2019). **K.E. Benallal**, R. Garni , L. Bouiba and Z. Harrat. **Journal of Arthropod-Borne Disease, 13(4):420-425**

13- **First field evidence infection of *Culex perexiguus* by West Nile Virus in Sahara Oasis of Algeria.** (2018). S. Benbetka, A. Hachid, **KE. Benallal**, C. Benbetka, A. Khaldi, I. Bitam, Z.Harrat. **Journal of Vector Borne Disease** 55(4):305-309.

14- **Sandflies's inventory in a leishmaniasis focus in Draa El Mizan, Kabylie.** (2017). A. B. Allal-Ikhlef, K. Senouci, **K. Benallal**, R. Triki-Yamani, Z. Harrat, L. Houti . J de la Faculté de Médecine d'Oran, N°0, 35-41.

15- **Morphological description of atypical *Phlebotomus perniciosus* Newstead, 1911 in Algeria.** (2017). **Benallal K**, Benikhlef R, Garni R, Gassen B, Dedet J.P, Z. Harrat. J Arthropod-Borne Diseases, 11(1): 139–146.

16- **First report of *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) in Oran, West of Algeria.** (2016). **Benallal K**, Allal-Ikhlef A, Benhamouda K, Schaffner F, Harrat Z. Acta Tropica. 164: 411-413. doi: 10.1016/j.actatropica.2016.09.027. Epub 2016 Sep 30.

17- **Molecular characterization of *Culex pipiens* (Diptera, Culicidae) in Reghaïa lake, Algeria.** (2015). **Benallal K**., Benbetka S., Tail G., Z. Harrat. Annals of Biological Sciences. 3 (1):20-24.

18- **Remote sensing, land cover changes and vector-borne diseases: Use of high spatial resolution satellite imagery to map the risk of occurrence of cutaneous leishmaniasis in Ghardaïa, Algeria.** (2014). Rafik Garni, Annelise Tran, Hélène Guis, Thierry Baldet, **Kamel Benallal**, Said Boubidi, Zoubir Harrat. Infect. Genet. Evol. http://dx.doi.org/10.1016/ j.meegid.2014.09.036.

19- **Entomological investigation following the resurgence of human visceral leishmaniasis in Southern Algeria**.(2013). **Benallal, K**., Gassen, B., Bouiba, L., Depaquit, J., Harrat., Z. Acta Tropica. 128 (3):518-21. doi: 10.1016/j.actatropica.2013.07.017.

20- **Nouvelles stations de *Sergentomyia schwetzi* (Adler, Theodor & Parrot ; 1929) dans le Tassili N'Ajjer.** (2012). **K. Benallal**, Y.Dib, SC. Boubidi, Z. Harrat. Archives Pasteur d'Algérie, Tome 68, 131-38.

21- ***Phlebotomus sergenti* (Parrot, 1917) identified as *Leishmania killicki* host in Ghardaïa, south Algeria.** (2011). Boubidi S.C., **Benallal K**., Boudrissa A., Bouiba L., Bouchareb B., Garni R., Bouratbine A., Ravel C., Pestova J, Dvorak V, Votypka J, Volf P & Harrat Z. Microbes and Infection. 13(7): 691-6.

Languages

1. English advanced
2. French advanced
3. Arabic advanced
4. Czech beginner
5. Berber Mother Tongue