J. Lucientes
Animal Health Department
The AgriFood Institute of Aragon (IA2)
Faculty of Veterinary Medicine
C/ Miguel Servet 177, 50013
Zaragoza, Spain
E-mail: jlucien@unizar.es

First Findings and Molecular Data of *Phlebotomus mascittii* (Diptera: Psychodidae) in the Cantabrian cornice (Northern Spain)

P. M. Alarcón-Elbal [1], M. A. González [2], S. Delacour-Estrella [1], D. Bravo-Barriga [3], R. Estrada Peña [1], F. Goiri [2], A. L. García- Pérez [2], and J. Lucientes [1]

[1] Animal Health Department, The AgriFood Institute of Aragon (IA2), Faculty of Veterinary Medicine, C/ Miguel Servet 177, 50013, Zaragoza, Spain

[2] Department of Animal Health. NEIKER-Basque Institute for Agricultural Research and Development, Basque Research and Technology Alliance (BRTA), 48160 Derio, Bizkaia, Spain

[3] Department of Animal Health, Veterinary Faculty, University of Extremadura, Av. de la Universidad s/n, 10003, Cáceres, Spain

Abstract

Phlebotomus (*Transphlebotomus*) *mascittii* Grassi, 1908 (Diptera: Psychodidae) has been found in several European countries. In Spain, sporadic records were reported in the early ‘80s in Catalonia (Northeast Spain), and it was never detected again. Recent entomological surveys carried out between 2004−2020 revealed the presence of several specimens of *P. mascittii* in Spain. The species identification was confirmed by both morphological and molecular analyses. The analysed specimens belonged to the haplotype (COI_2) defined by one polymorphic site compared to other European specimens. *Phlebotomus mascittii* was found in low population densities in rural areas associated with livestock farms and in an urban cemetery during the summer season. This study provides the first records of this species in various localities along the Cantabrian cornice (Northern Spain) and represents its westernmost observation in the Palearctic region. The implications of the finding of this uncommon species are discussed at different levels, with emphasis on its suspected role in the transmission of leishmaniosis.

Key words. Haplotype, leishmaniosis, new records, *Phlebotomus mascittii*, Spain

Resumen

Phlebotomus (*Transphlebotomus*) *mascittii* Grassi, 1908 (Diptera: Psychodidae) se ha encontrado en varios países europeos. En España, a principios de los años 80, se reportó de forma esporádica en Cataluña (noreste de España), y nunca se volvió a detectar. Estudios entomológicos realizados entre 2004 y 2020 revelaron la presencia de varios ejemplares de *P. mascittii* en España. La identificación de la especie se confirmó mediante análisis morfológico y molecular. Los ejemplares analizados pertenecían al haplotipo (COI_2) definido por un sitio polimórfico en comparación con otros ejemplares europeos. *Phlebotomus mascittii* se encontró en bajas densidades poblacionales en zonas rurales asociadas a explotaciones ganaderas y en un cementerio urbano durante la temporada de verano. Este estudio proporciona los primeros registros de esta especie en varias localidades de la cornisa cantábrica (norte de España) y representa además la observación más occidental en la región paleártica. Se discuten a diferentes niveles las implicaciones del hallazgo de esta especie poco común de flebótomo, con énfasis en su presunto rol en la transmisión de la leishmaniosis.

Palabras clave. Haplotipo, leishmaniosis, nueva cita, *Phlebotomus mascittii*, España

Introduction

Leishmaniosis is a tropical and subtropical vector-borne zoonotic disease caused by an intracellular parasite of the genus *Leishmania* Ross, 1903 (Kinetoplastida: Trypanosomatidae) that is transmitted to humans and other vertebrates by the bite of infected female sand flies (Diptera: Psychodidae). In southern Europe, most of the reported cases are due to zoonotic visceral leishmaniasis (VL), which is the most dangerous form and is lethal when untreated; however, cutaneous leishmaniasis (CL), which is more benign than VL, is also present (Dujardin *et al.,* 2008). On the other hand, dogs are considered the major reservoir of the parasite, and canine leishmaniosis (CanL) has exhibited an expansion to new locations due to the increase in dog relocation (Le Rutte *et al.,* 2018) and probably a vector transmission favoured by global warming. In this epidemiological context, *Leishmania infantum* is responsible for the majority of human CL and VL cases in the WHO European Region (ECDC, 2021a).

Among the over 800 sand fly species known worldwide, 12 have been identified in mainland Spain (Aransay *et al.,* 2004), but only *Phlebotomus perniciosus* Newstead, 1911 and *Phlebotomus ariasi* Tonnoir, 1921 are proven vectors of leishmaniosis in the territory (Rioux *et al.,* 1986). Of the phlebotomine species identified in the Spanish mainland, *Phlebotomus mascittii* Grasii, 1908 is probably the less known. However, in the central regions of Europe where *Leishmania* circulates, *P. mascittii*, the only *Phlebotomus* species reported so far (Obwaller *et al.,* 2016), has been considered its possible vector.

Phlebotomus mascittii has been recorded in several countries of the Mediterranean basin, especially in Southern and Western European countries, some countries of Western Asia, and occasionally in North Africa. It has also been reported in

Central European countries with colder climate (ECDC, 2021b) and it is considered the northernmost distributed sand fly species in the continent. In Spain, *P. mascittii* was first detected in the early '80s in the Catalonian provinces of Barcelona and Girona in Northeastern Spain (Rioux *et al.*, 1984). However, this species has not been found again despite the increasing number of studies on this family over the last years.

The aim of this study was to update the information on *P. mascittii* in Spain from published data (Rioux *et al.* 1984) and those obtained over the last 16 years of active entomological investigation.

Material and Methods

Phlebotomus specimens were collected in the frame of two research projects conducted between 2004 and 2020:

1) The National Program for the Surveillance, Control, and Eradication of Bluetongue in Spain is an entomological campaign initiated in 2004 and currently in progress focused on collecting data on the seasonal abundance, diversity, and geographical distribution of *Culicoides* biting midges (Diptera: Ceratopogonidae) in mainland Spain and the Balearic Islands. CDC miniature black-light (UV) traps (Model 1212; John W. Hock Company, Gainesville, FL) were used for surveillance. These traps collect not only *Culicoides* but also many other insects that exhibit a positive phototropism such as sand flies and mosquitoes, among others. Traps were placed on farms overnight once a week throughout the year in a variable number of animal holdings composed mainly of sheep, goats, and cattle.

2) A local research project aimed at revealing the diversity of blood-sucking Dipteran pests in urban and rural areas of the Basque Country (Northern Spain) along 2019-2020. CDC miniature light traps equipped with incandescent light bulb (Model

512; John W. Hock Company, Gainesville, FL) and baited with about 4 lb of dry ice were deployed bi-weekly during 24 h periods in different settings (cemeteries, animal protection centres, and livestock farms) over a period of 6 months (1-May to 31-October).

In both studies, phlebotomine sand flies were separated from other species of medical and veterinary interest at the laboratory, and preserved in 70% ethanol. Sand fly specimens were dissected by removing the head and mounted on microscope slides in Hoyer's mounting medium. The identification was done by morphological characteristics under a stereomicroscope (Gállego *et al.*, 1992), and only those identified as *P. mascittii* were selected for further analysis. One of the limitations of the study is that here, we combine data sets from two time periods with different trapping methods, however, this fact does not engage the objective of the study.

Morphological identification was confirmed by DNA sequencing of three randomly selected samples from the region of the Basque Country. DNA was extracted from the thorax and legs using QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The cytochrome c oxidase 1 (*cox*1) gene was partially amplified using the primer set LCO1490 and HCO2198 following the PCR protocol previously described by Folmer et al. (1994). The amplified fragment was cleaned and sent for sequencing to STABvida (https://www.stabvida.com/es). Sequences were then edited through Chromas Lite 2.1.1 (Technelysium Pty Ltd) and consensus sequences for each forward/reverse pair were created with BioEdit Sequence Alignment Editor (version 7.2.5, Carlsbad, CA. USA). Identification at the species level was based on BLASTn homology searches of the *cox*1 gene sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the Barcode of Life Data Systems-v4 (Bold Systems v4). Finally, the nucleotide sequences obtained between

630−655 pb were deposited at the DNA Data Bank of Japan (DDBJ: (accession numbers LC604871-3). For the analysis of haplotypes, multiple alignments of nucleotide sequences (n = 18) were performed using the iterative G-INS-I method as implemented in MAFFT vs. 7. Fifteen COI sequences from five European countries with a final length of 613 bp were included in the analysis. Haplotype networks were illustrated with a median-joining network (MJN) algorithm (ε = 0) using the software PopART v. 1.7 (http://popart.otago.ac.nz/index.shtml) to analyze haplotype genealogy.

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Results

Altogether, 699 sampling sites were surveyed, 690 sites between 2004 and 2020 within the Spanish National Program for the Surveillance, Control, and Eradication of Bluetongue and 9 sites in 2019 and 2020 in the Basque Country (Figure 1). A total of 14 *P. mascittii* specimens were collected (13 females and 1 male) along the Cantabrian cornice (Cantabria and the Basque Country Autonomous communities) in five municipalities (Table 1 and Figure 1a). Seven specimens of *P. mascittii* were collected in four settings located in rural areas associated with cattle farms, and seven specimens in two traps placed at an urban cemetery near a stonewall with vegetation (Figure 1a). The earliest capture was recorded on June 25 and the latest one on August 20, at altitudes between 61 to 440 m a.s.l. (Table 1).

All phlebotomine sand flies were identified by the morphology of the female spermathecae (without neck, almost tubular, with irregular segmentation and wide efferent ducts) and male genitalia (aedeagus not bifid, narrowed gradually, with rounded tip; at the tip half as wide as at the base).

The sequences obtained (accession numbers LC604871-3) were subjected to BLASTn analyses and revealed identity values ranging between 99.8−100% with *P.*

mascittii (accession numbers: KY848831.1 or MN812830.1 from Serbia and Austria, respectively). When submitted to the BOLD System identification tool, all sequences showed 100% similarity with our analized specimens. The analyses revealed two haplotypes (COI_1 and COI_2) defined by one polymorphic site (Pos: 106, A/G). Our specimens shared the haplotype COI_2 found in all the countries analysed (Figure 1b).

Discussion

This work provides the first findings of *P. mascittii* in two regions from the Cantabrian cornice (Northern Spain), a geographic strip along the Atlantic Coastline from the border with Portugal to the border with France. This region has a humid oceanic climate, with an annual precipitation around 1,200 mm at the coasts and higher in the mountains, and the mean temperature about 14 °C. This report represents also the westernmost detection of this species in the Palearctic region, beyond the records of Charente-Maritime, in the French Atlantic Coast (Callot, 1951).

Phlebotomus mascittii identification was based on both morphology and sequencing of the COI marker gene. All specimens were morphologically identified to the species level. However, most females had hardly visible spermathecae, which made morphological discrimination tricky, as noted by Kniha *et al.* (2020). This issue might have contributed to the species being underreported. It is therefore advisable to observe the spermathecae under different wavelengths of light (Kniha *et al.*, 2020) or dissect the abdomen, extract the spermathecae and add a staining medium. Besides, the use of the COI genetic marker for species identification confirmation has become more widely used (Praprotnik *et al.*, 2019; Kniha *et al.*, 2020). Here, we present the first sequences of this species from Spain. The study of the haplotype revealed its relation with specimens from Eastern and Central European countries, namely, Austria, Slovakia, Slovenia, and Serbia (Kniha *et al.*, 2020). Due to these close genetic relationships between the populations studied, it supports a possible fairly recent dispersal of this species in Europe already postulated by Kniha *et al.* (2020).

In the '80s, seven specimens of *P. mascittii* had been found in Northeastern Spain in the Catalonian provinces of Barcelona and Girona (Rioux *et al.*, 1984). Thus, the present findings represent the second record for the Iberian Peninsula, and the first detection of a sand fly species in the Autonomous Community of Cantabria. In addition, this is the fourth sand fly species identified in the Basque Country, where *P. perniciosus*, *P. ariasi* and *Sergentomyia minuta* (Rondani, 1843) had previously been recorded (Lucientes *et al.*, 2002; Aransay *et al.*, 2004). Based on the large-scale entomological survey carried out here during 16 years, and considering that this species has not been detected in Central and Southern Spain, it seems that *P. mascittii* distribution appears to be limited to areas of Northern and Northeastern Spain (Figure 1a).

The apparent low density of this species observed in our surveys is consistent with other European studies (Ready, 2010). However, this might be due to the fact that this sand fly species is presumed to be averse to light, so its by-catch capture in light traps might not represent the true density and distribution of the population (Naucke *et al.*, 2008). Kniha *et al.* (2020) trapped substantial numbers of *P. mascittii* in Austria using CDC miniature light traps with an additional source of CO_2. This is in accordance with our findings, where the highest number of specimens was collected in a cemetery by light traps baited with carbon dioxide. However, other hypothesis should not be excluded (i.e., it is a genuinely infrequent species).

In European countries, sand fly activity is mainly limited to the summer months. In our study, *P. mascittii* was caught between late June and mid-August, which would suggest an adult activity during the summer months, being also in agreement with the first captures in Spain in the '80s (Rioux *et al.,* 1984). However, Naucke *et al.* (2008) also proved the winter activity of this species in Corsica, specifically in a railway tunnel where the temperature was higher and the place was anthropized and sheltered. *Phlebotomus mascittii* is widely distributed along different elevation gradients, which was also observed in the Spanish catches, where it was described from nearly 800 m a.s.l. (Rioux *et al.,* 1984) to only 61 m a.s.l. in the present study.

Little is known about the feeding preferences of *P. mascittii,* but it appears to be an opportunistic feeder. The main hosts of this species are humans and dogs, but it has also been described feeding on horses (Bongiorno *et al.*, 2003). Previous field surveys gave evidence of its anthropophilic nature (Grimm *et al.,* 1993) since the species has been caught close to human dwellings as well as in a wild biotope (Depaquit *et al.,* 2005). In our study, the catches in the urban cemetery corroborate its adaption to anthropogenic environments, which has already been noted in Austria (Obwaller *et al.,* 2016). Regarding its behaviour, *P. mascittii* is the only European sand fly species that can be found in unusual ecological niches such as tunnels (Naucke *et al.,* 2008), and has been proposed to be cavernicolous. The specimens of the cemetery were captured near a stone wall, denoting some leaning to rest inside cracks of walls, as reported by Bongiorno *et al.* (2003).

Although *P. mascittii* has never been proven to be a vector of leishmaniosis, the recent detection of *Leishmania* spp. DNA in specimens collected from Austria (Obwaller *et al.*, 2016) and Italy (Zanet *et al.*, 2014) may support its possible role in the transmission. Furthermore, the species belongs to the subgenera *Adlerius* and *Larroussius*, which include all the potential vectors of Mediterranean VL, so further entomological and epidemiological studies are needed to elucidate its vector capacity. In most regions of central Europe, it is the only sand fly found in areas with confirmed autochthonous leishmaniosis cases (Obwaller *et al.,* 2016), reinforcing the hypothesis of the vectorial capacity of *P. mascittii* and its possible impact on both human and animal health. The scarcity of reports of this species both in the Iberian Peninsula and in most of the European countries where it is found, make it difficult to draw firm conclusions about its medical and veterinary interest. The geographic distribution of human leishmaniosis in Spain confirms that the disease is present in almost the whole territory, yet the risk is still low in the northwestern and Cantabrian coastline (Fernández Martínez *et al.,* 2019). Although, Northern Spain continues to be considered as a non-endemic area for CanL (Solano-Gallego *et al.,* 2011), the recent detection of *L. infantum* in wild and other canid species (Oleaga *et al.,* 2018), highlights the need for further studies focused on the distribution and the epidemiology of the parasite, and the potential role that sand flies pose as its vector.

Finally, this study emphasizes how collections from other entomological surveillance studies, focused on species of medical and veterinary interest (i.e. *Culicoides* and mosquitoes), can incidentally contribute in providing interesting data of other non-target species. We also highlighted the importance of encourage inter-institutional scientific collaboration and the One Health multi-disciplinary working groups.

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