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Filarial worm circulation by mosquitoes along an urbanization gradient in southern Spain

Josué Martínez-de la Puente^{1,2} Martina Ferraguti¹ Jéssica Jiménez-Peñuela¹

Santiago Ruiz^{3,2} Javier Martínez⁴ David Roiz⁵ Ramón Soriguer^{1,2} Jordi Figuerola^{1,2}

Abstract

Mosquitoes are the main vectors of pathogens affecting wild animals, livestock and humans. Here, we used molecular tools to assess the local circulation of filarial parasites in mosquitoes collected during 2013 from natural, rural and urban habitats from southern Spain. We screened parasites in 22,791 female mosquitoes of the genera *Aedes*, *Culex* and *Culiseta*. Filarial worms were only detected in two mosquito pools. An *Ae. caspius* pool was positive for *Setaria equina* and an unidentified worm related to *Onchocerca* was detected in a *Cx. pipiens* pool. None of the mosquito pools were positive for *Dirofilaria*. These results underlay the role of *Ae. caspius* in the transmission of *Setaria* parasites among livestock and/or wildlife to humans in southern Spain.

KEYWORDS: *Aedes*, *Culex*, *Onchocerca*, *Setaria equina*, vector-borne pathogen, zoonotic diseases

1 Estación Biológica de Doñana (EBD-CSIC), Seville, Spain

2 Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain

3 Diputación de Huelva, Área de Medio Ambiente, Servicio de Control de Mosquitos, Huelva, Spain

4 Departamento de Biomedicina y Biotecnología (área Parasitología), Universidad de Alcalá, Alcalá de Henares, Spain

5 Infectious Diseases and Vectors: Ecology, Genetics, Evolution and Control. IRD (Institut de Recherche pour le Développement), Montpellier, France

Correspondence: Josué Martínez de la Puente, Estación Biológica de Doñana (EBD-CSIC), Sevilla, Spain.

Email: jmp@ebd.csic.es

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INTRODUCTION

Helminths causing lymphatic filariasis affects over 120 million people worldwide (Taylor, Hoerauf, & Bockarie, 2010). Approximately 95% of the filarial species affecting humans have a zoonotic origin (Taylor, Latham, & Mark, 2001). In Spain, *Dirofilaria* parasites are considered endemic, reaching high prevalence in domestic animals (i.e. cats and dogs) (Morchón, Carretón, González Miguel, & Mellado Hernández, 2012) and occasionally affecting humans (Laynez-Roldán et al., 2018). The incidence of *Dirofilaria* has been largely studied in vertebrate hosts (Simón et al., 2017), recording the highest prevalence of *Dirofilaria immitis* in dogs from the province of Huelva (36.7%; Ortega-Mora, Gomez-Bautista, Rojo-Vazquez, Rodenas, & Guerrero, 1991). In spite of their importance for pathogen transmission, information on the potential role of mosquitoes in the local circulation of most filarial parasites is currently unknown. Molecular tools allow the screening of a high number of potential vectors identifying the presence of the parasites (Ionică et al., 2017; Latrofa, Dantas-Torres, et al., 2012; Latrofa, Montarsi, et al., 2012). Using molecular approaches, Morchón et al., (2007) and Bravo-Barriga et al., (2016) identified the presence of *D. immitis* and different strains of Filarioidea in *Culex pipiens* mosquitoes from Spain.

Human-related changes in landscape are considered a key factor modelling the epidemiology of human and zoonotic pathogens (Morse, 1995). Deforestation, agricultural intensification and urbanization, among others, affect the transmission rate of pathogens between animals and from animals to humans (Lindahl & Grace, 2015). In vector-borne pathogens, urbanization affects the availability of mosquitoes and hosts, including people, potentially determining the circulation of pathogens and the risk of outbreaks (Ferraguti et al., 2016; Martínez-de la Puente et al., 2018). Here, we obtained a general overview of the filarial parasites harboured by mosquitoes of the genera *Aedes*, *Culex* and *Culiseta* in an urbanization gradient from southern Spain.

MATERIAL AND METHODS

Mosquitoes were collected from April to December 2013 in 49 natural, rural and urban areas from southern Spain (Figure 1) using BG-sentinel traps and CDC incandescent light-traps both supplemented with dry ice. Additionally, resting female mosquitoes were captured with a CDC backpack aspirator, model 2846. Adult mosquitoes were preserved in dry ice and stored frozen until identification. Mosquitoes were separated over a filter paper on a chilled plate under a stereomicroscope and morphologically identified to species level (see Ferraguti et al., 2016 for further details). Female mosquitoes of the same species, locality and date of capture were grouped in pools containing between 1 and 53 individuals. Female mosquitoes showing the presence of blood in their abdomen were not included in this study to avoid the potential amplification of parasites contained in a recent blood meal.

The genomic DNA of mosquito pools was extracted using a QIAamp Viral RNA kit (Qiagen, Germany) according to the manufacturer's recommendations. The species included in this study were: *Ae. (Oc.) caspius*, *Ae. (Oc.) detritus*, *Cx. pipiens*, *Cx. perexiguus*, *Cx. modestus*, *Cx. theileri* and *Cs. annulata* (Table 1). Initially, samples were screened for the presence of parasite DNA following Bataille et al. (2012). Those samples providing positive amplifications, including unspecific ones, were reanalysed using the PCR primers COIintF (5'-TGATTGGTGGTTTTGGTAA-3') and COIintR (5'-ATAAGTACGAGTATCAATATC-3') designed by Casiraghi, Anderson, Bandi, Bazzocchi, and Genchi (2001) to amplify an approximately 650 bp fragment of the cytochrome oxidase subunit I (COI) gene. Reactions were conducted in 48- or 96-well plates including, at least, one negative control of the reaction and one positive control (i.e., DNA of *Dirofilaria*). Sequencing reactions were performed according to Big Dye 1.1 technology (Applied Biosystems) and labelled DNA fragments were resolved with an ABI 3,130 × 1 automated sequencer (Applied Biosystems). Sequences were edited using the software Sequencher™ v4.9 (Gene Codes Corp., © 1991–2009, Ann Arbor, MI 48,108) and deposited in GenBank (MK541847-48).

To assess the parasite identity, the two DNA sequences of the COI gene obtained in this study were compared with those deposited in GenBank and the Barcode of Life Data Systems (BOLD). Due to the low similitude value obtained for one of the sequences isolated here (see results), we conducted further phylogenetic analyses. The two DNA sequences were aligned together with other 76 sequences obtained from GenBank belonging to the superfamilies Filarioidea and Spiruroidea. The alignment was performed using ClustalW algorithm implement in BioEdit 7.0.5.3 (Hall, 1999). Flank position was manually established. The final alignment contained 600 positions and 78 sequences. The substitution model GTR + I+G was selected using Mega 7.0.26 software (Kumar, Stecher, & Tamura, 2016) to perform the Bayesian analysis with MrBayes 3.2.6 software (<http://nbisweden.github.io/MrBayes/download.html>). This analysis consisted of two runs of four chains each with 4,000,000 generations per run, a burn-in of 1,000,000 generations and a sampling interval of 100 generations. A consensus tree was built from 60,000 trees. The final standard deviation of the split frequencies was lower than 0.01. The alignment was also analysed using a Maximum Likelihood inference (PhyML program) (Guindon et al., 2010), using the same substitution model mentioned above. The subtree pruning and regrafting (SPR) tree rearrangement option was selected and a Bayesian-like transformation of aLRT (aBayes) was used to obtain the clade support (Anisimova, Gil, Dufayard, Dessimoz, & Gascuel, 2011). Both trees were rooted with the superfamily Spiruroidea, closely related to the superfamily Filarioidea.

RESULTS

Overall, 22,791 mosquitoes were collected and grouped in pools. Of them, two out of 1,282 mosquito pools were positive for the presence of filarial DNA (Figure 1; Table 1). The sequence recorded in an *Ae. caspius* pool from *Los Alamos*, Huelva, was 99% similar to *Setaria equina* sequences deposited in GenBank and BOLD system. In addition, worm isolated from a pool of *Cx. pipiens* collected in the Doñana National Park was $\leq 92\%$ similar to sequences corresponding to *Onchocerca*.

The phylogenetic analyses support the identification of both parasites amplified from mosquitoes (Figure 2). The sequence from *Ae. caspius* clustered together with other sequences of *S. equina* and the sequence from *Cx. pipiens* clustered with those from *Onchocerca* parasites, supporting that these sequences corresponded to an *Onchocerca* species non-previously characterized molecularly.

DISCUSSION

We provide strong evidence of the local circulation of *S. equina* and an unidentified worm likely belonging to the genus *Onchocerca* in southern Spain. By contrast, we did not find any evidence of the presence of *Dirofilaria* in mosquitoes. *Dirofilaria immitis* was previously recorded in *Cx. pipiens* mosquitoes from the Iberian Peninsula (Bravo-Barriga et al., 2016; Ferreira et al., 2015; Morchón et al., 2007). Although, the recorded prevalence was low, with only 0.16% (Bravo-Barriga et al., 2016) and 0.27% (Morchón et al., 2007) of the mosquitoes tested providing positive results. As other vector-borne pathogens, *Dirofilaria* distribution may have a heterogeneous spatial pattern, being detected in several studies, but not being detected in some cases in other European areas despite being tested in a large number of mosquitoes (Czajka et al., 2012). Additionally, other mosquito species present in the area that were underrepresented in this study (i.e. *Cx. theileri*) could be more relevant for *Dirofilaria* transmission than the three main species analysed here (*Cx. pipiens*, *Cx. perexiguus* and *Cx. modestus*).

We identified the presence of *S. equina* in *Ae. caspius*. Although the molecular detection of parasite DNA does not fully demonstrate that this species is the biological vector, the results target *Ae. caspius* as a potential vector for *S. equina*. Previous studies supported the role of *Ae. caspius* as vectors of *Setaria* parasites (Pietrobelli, Cancrini, Frangipane di Regalbono, Galuppi, & Tampieri, 1998) with parasite DNA molecularly identified in wild *Aedes* mosquitoes (Cancrini, Pietrobelli, Fangipane Di Regalbono, & Tampieri, 1997; Ionică et al., 2017; Kemenesi et al., 2015). *Setaria equina* is considered a widespread mosquito-borne parasite commonly found infecting equidae (Hornok, Genchi, Bazzocchi, Fok, & Farkas, 2007; Marzok & Desouky, 2009). This parasite courses apparently benign infections, although *S. equina* infections were associated to ocular disease and adnexa (Marzok & Desouky, 2009; van der Kolk & Kroeze, 2013). Furthermore, *S. equina* is considered a zoonotic parasite occasionally affecting humans (Nabie, Spotin, & Rouhani, 2017). The positive pool found in this study was collected in an industrialized peri-urban area of Huelva with human populations and a hospital in the surroundings. All these data, together with the mammophilic feeding pattern of *Ae. caspius* (Martínez-de la Puente, Ruiz, Soriguer, & Figuerola, 2013), support the role of this mosquito species in the transmission of *S. equina* between equids and potentially to humans.

Furthermore, an unidentified worm was detected in a *Cx. pipiens* pool. Phylogenetic analyses clustered this sequence isolated from mosquitoes with those previously recorded from *Onchocerca* parasites. Similarly, the presence of DNA from unidentified filarial worms was recorded in *Culex* mosquitoes, including *Cx. pipiens* (Czajka et al., 2012; Kemenesi et al., 2015). *Onchocerca* parasites are usually found infecting ungulates (Lefoulon et al., 2017) and they are transmitted by both blackflies (Diptera: Simuliidae) and *Culicoides* (Diptera: Ceratopogonidae) (Muller, 1979). Here, the *Onchocerca* was isolated from mosquitoes collected in the Doñana National Park, a conserved area hosting a high diversity of wild animals, including birds and mammals such as deer, cattle and horses. These animals are common hosts of *Cx. pipiens*, suggesting the possibility that the parasite found here could infect a mammal species present in the area. However, *Cx. pipiens* show an ornithophilic feeding behaviour (Martínez-de la Puente et al., 2016) suggesting the possibility that this parasite could also infect birds (see Kemenesi et al., 2015; Czajka et al., 2012).

In summary, we provide support for the local circulation of *S. equina* and likely, a non-previously molecularly characterized *Onchocerca* species in southern Spain, together with the apparent absence of *Dirofilaria* at least in the three species of mosquitoes with a higher number of samples (*Cx. pipiens*, *Cx. perexiguus* and *Cx. modestus*). Further studies should confirm the vector competence of these mosquitoes for the transmission of the parasites isolated here and link the morphological identifications of parasites to genetic sequences.

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CONFLICT OF INTEREST: The authors declare that they have no competing interests.

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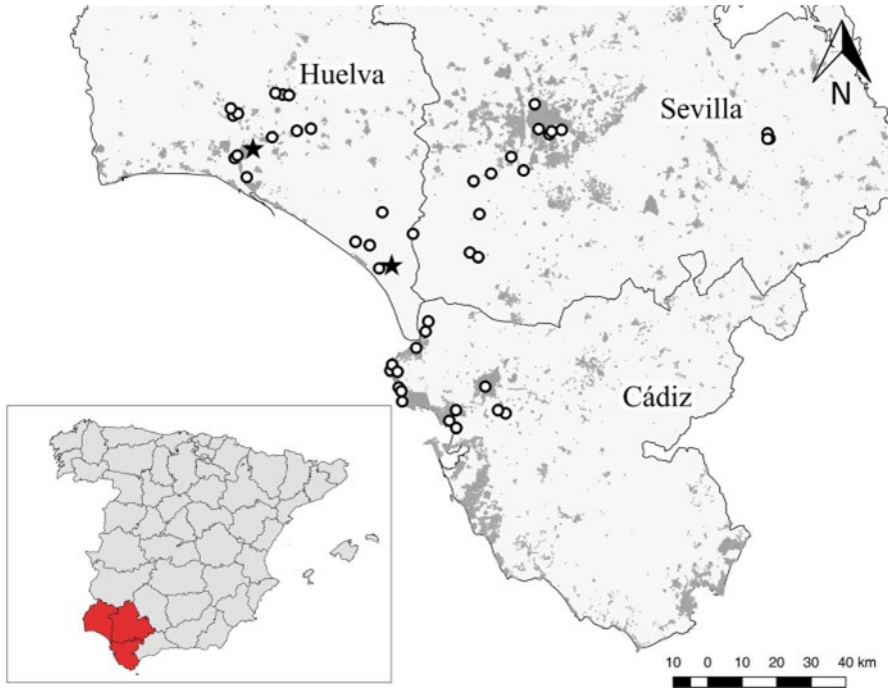


FIGURE 1 Sampling localities of mosquitoes in southern Spain, 2013. Stars and open circles represent sampling localities with positive and negative presence of parasite DNA respectively. Urbanized areas are shown in grey [Colour figure can be viewed at wileyonlinelibrary.com]

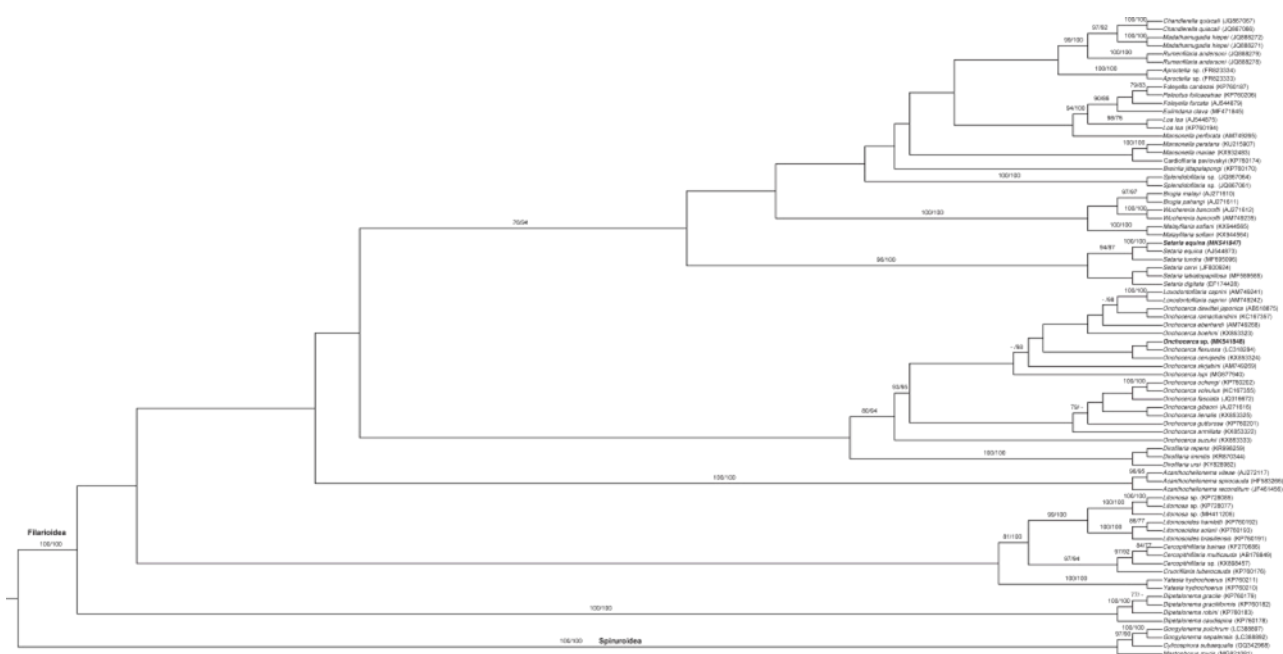


FIGURE 2 Phylogenetic tree derived from Bayesian inference using the GTR + I + G substitution model. The tree was rooted with Spiruroidea species. Nodal support values of Bayesian (before the slash) and maximum likelihood (after the slash) inference are given. Only support values higher than 75% are indicated. Sequences obtained in the present study are shown in bold

TABLE 1 Number of mosquito females grouped in pools tested for the presence of parasite DNA in this study. The parasites identified are shown in brackets

Mosquito species	Pools tested	Mosquito tested	Positive pools
<i>Aedes (Oc.) detritus</i>	1	1	0
<i>Aedes (Oc.) caspius</i>	2	52	1 (<i>Setaria equina</i>)
<i>Culex pipiens</i>	1,025	19,754	1 (<i>Onchocerca</i> sp.)
<i>Culex perexiguus</i>	200	2,490	0
<i>Culex modestus</i>	52	473	0
<i>Culex theileri</i>	1	19	0
<i>Culiseta annulata</i>	1	2	0
Total	1,282	22,791	2