Culex pipiens as a potential vector for transmission of *Dirofilaria immitis* and other unclassified Filarioidea in Southwest Spain

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Highlights

- *Dirofilaria immitis* DNA detected in thorax of *Culex pipiens* f. *pipiens*.
- *Culex pipiens* f. *pipiens* as a potential vector of *Dirofilaria immitis* in Spain.
- Detection of unclassified Filarioidea, possibly of avian origin in Spanish mosquitoes.
- *Culex pipiens* biotypes can acts as a potential vector for other filarioid nematodes

Abstract

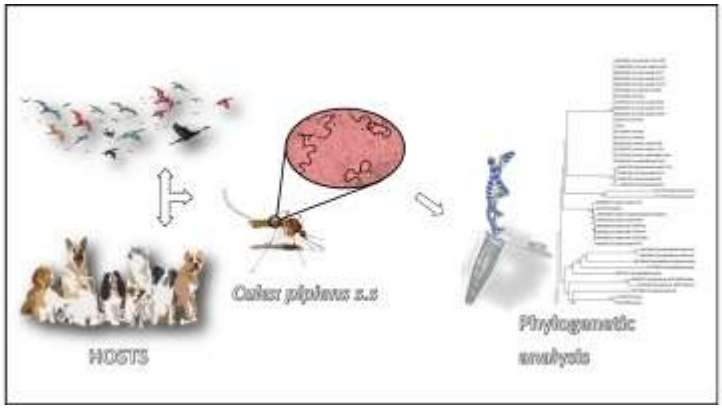
Dirofilaria immitis is one of the most frequently detected mosquito-transmitted zoonotic filarioid nematode in mammals in Europe, being canine dirofilariosis a major animal health problem, endemic in the Mediterranean area. This study, focused on Southwest Spain, in order to bring new insights into (i) the epidemiology of *Dirofilaria* spp., (ii) the species of Culicid vectors possibly involved in their transmission and (iii) the genetic variability of those potential vectors. A total of 881 adult female mosquitoes from 11 different species, were captured during 2012–2013, and detection of filarioid DNA was attempted by PCR using specific primers (ITS-2 and COI), followed by DNA sequencing. In a single *Culex pipiens* specimen *D. immitis* DNA was detected both in the head-thorax and abdomen sections. Filarioid nematode DNA was also detected in eight additional *Cx. pipiens* specimens also in both the thorax and the abdomen, but analysis of sequence data did not allow unambiguous assignment of any of the obtained sequences to a previously defined species. All *Cx. pipiens* with filarioid DNA were individually analysed by CQ11 to discriminate between *pipiens*, *molestus*, and hybrid forms. Besides, rDNA ITS-2 sequence analysis revealed the presence of haplotype H1 and H2 of *Cx. pipiens*. To our knowledge this study revealed, for the first time in Spain, the occurrence of likely mature infection of *D.*

immitis in *Cx. pipiens*, as well as with other yet uncharacterized nematodes, supporting its role as a potential vector of these filarids.

Keywords:

Dirofilaria immitis; Dirofilariosis; *Culex pipiens*; *pipiens* and *molestus* forms; Southwest Spain

Graphical abstract



1. Introduction

Mosquitoes are vectors of agents of infectious and parasitic diseases to humans and animals, such as malaria, arbovirosis and filariosis. Filarioid nematode parasites affect millions of people and animals worldwide, and represent a major health hazard with significant economic implications (Laaksonen et al., 2010). Canine dirofilariosis is a major veterinary health problem in tropical, subtropical and temperate regions of the world (Simón et al., 2012), with at least 70 species of mosquitoes being considered as potential vectors (Cancrini et al., 2006).

Dirofilariosis is endemic in the Mediterranean region, affecting southern European countries including Portugal, Spain, France and Italy (Genchi et al., 2005 and Morchón et al., 2012). In recent years, climate change, increased transport of animals (including dogs), and the dispersal of invertebrate vector species of filarioid nematodes, have led to the geographical expansion of dirofilariosis (Genchi et al., 2014), which is usually associated with *Dirofilaria repens* (Tappe et al., 2014). Humans are only accidental hosts as the parasite cannot develop to the adult stage (dead-end host), but the observed increase in the number of cases of human dirofilariosis in recent decades has led to its classification as an emerging zoonosis (Pampiglione and Rivasi, 2001).

D. immitis is present in Spain, with prevalences ranging from 0.8% to 36.7% in dogs along the Iberian Peninsula, and 19%-39% in the Balearic and Canaries islands (reviewed by Diosdado et al., 2016). In other vertebrate hosts, the detection of *D. immitis* ranged between 1.7% and 32% in the red fox (*Vulpes vulpes*) (Gortázar et al., 1998), and 2.1% in the wolf

(*Canis lupus*) and Eurasian otters (*Lutra lutra*) (Morchón et al., 2012). In cats, seroprevalence values of 11.4% and 18.1% were detected in Barcelona and the Canary Islands, respectively (Montoya-Alonso et al., 2014 and Montoya-Alonso et al., 2015). In neighbouring Portugal, canine dirofilariosis is endemic with an overall national seroprevalence of 2.1%- 15.1% (Alho et al., 2014 and Vieira et al., 2015).

The incrimination of vectors of *Dirofilaria* in Spain has led so far to the detection of *D. immitis* DNA in the abdomen of two *Culex pipiens* (haplotype H1) in Salamanca (western Spain) and in *Cx. theileri* in the Canary Islands, and no mature infections have been found, as no DNA was detected in the head-thorax of the analysed specimens (Morchón et al., 2007 and Morchón et al., 2011).

Studies that will turn preliminary results into definite evidence for irrefutable implication of *Cx. pipiens* as a potential vector for canine heartworm disease transmission are of great relevance (Cancrini et al., 2006, Morchón et al., 2007, Yildirim et al., 2011 and Latrofa et al., 2012), especially considering its abundance and wide geographic distribution, its genetic heterogeneity, having two bioforms with different behaviours (Gomes et al., 2015). The so-called pipiens form is characterized as being anautogenous, eurygamous, and preferably ornithophilic, while the *molestus* form is autogenous, stenogamous and preferably mamophilic. Therefore, disclosure of which form(s) of the vector may be implicated in *Dirofilaria* spp. transmission is undoubtedly relevant. Although Spain is an endemic country for canine heartworm disease (Simón et al., 2012), the implementation of appropriate control measures call for a more profound understanding of the epidemiology of this disease, of which the identification of the vector species involved, is of paramount importance to understand the dynamics of filarioid transmission to both animals and humans. Despite the high level of risk for dirofilariosis in the Extremadura region of Spain (Simón et al., 2014), there is no information regarding the distribution and prevalence of filarioid nematodes in mosquito vectors.

The objectives of this study were (i) to bring new insights on the distribution of these filarioid nematodes in field-collected mosquitoes from Extremadura (southwest Spain), (ii) to identify which species of mosquitoes could be involved in their transmission and (iii) assess the vector genetic variation.

2. Material and methods

2.1. Study region, mosquito collection and identification

The study was conducted in the Extremadura region in Southwest Spain (39°12′N 6°09′W). This region is characterized by a Mediterranean climate (Kottek et al., 2006), except to the north, where it is continental, and in the west, where due to the Atlantic Climate influence, displays milder characteristics. Most of the territory has an altitude between 200 and 600 m above sea level. The median annual rainfall is 605 mm (400–1.500 mm), with mean annual temperature ranging from 13 °C (in the North) to 18 °C (in the South), winter average temperatures slightly below 7.5 °C and summer average temperatures between 22 °C and 26 °C.

Mosquitoes were collected monthly from January of 2012 to December of 2013. CDC miniature light-traps (Model 512; John W. Hock Company, Gainesville, FL, USA) were placed outdoors in 21 stations distributed along 18 municipalities. Traps were placed at

approximately 1 m from the ground and run for 24 h, without any baiting. Captured adult specimens were initially stored at −80 °C and subsequently morphologically identified to species and/or species complex under a stereomicroscope, following Becker et al. (2010). Each mosquito was transferred to individual, sterilized 1.5 ml vials and stored at −20 °C before being processed for DNA extraction.

2.2. DNA extraction and molecular identification

Female mosquitoes were dissected into head-thorax and abdomen, under the stereomicroscope using sterile needles, to discriminate between *Dirofilaria* spp. infective/infected status, respectively, in case a positive amplification result for *Dirofilaria* DNA detection was obtained (Ferreira et al., 2015). Genomic DNA was extracted using the method described by Collins et al. (1988). After DNA precipitation and washing (70% ethanol), the sediment was resuspended in 100 μl of TE buffer (pH 7.0) and stored at −20 °C. Negative controls (no DNA template) were performed for each extraction procedure.

To detect and identify *Dirofilaria* at the species level, PCR amplification of the internal transcribed spacer-2 (ITS-2) of *Dirofilaria* rDNA was performed using primers DIDR-F1 (5′-AGTGCGAATTGCAGACGCATTGAG-3′) and DIDR-R1 (5′-AGCGGGTAATCACGACTGAGTTGA-3′), as described by Rishniw et al. (2006). Optimal conditions for PCR amplification were as follows: initial denaturation at 94 °C for 2 min, 32 cycles consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 7 min.

As a confirmation protocol (for *Dirofilaria* detection/identification), amplification and sequencing of the filarial mitochondrial DNA cytochrome oxidase subunit I (COI) gene was also performed in a subsample of mosquitoes that tested *Dirofilaria* DNA positive by ITS-2 PCR. This was carried out following conditions described by Casiraghi et al. (2001), using primers COIintF (5′-TGATTGGTGGTTTTGGTAA-3′) and COIintR (5′-ATAAGTACGAGTATCAATATC-3′), and the cycling profile we used was an initial denaturation at 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s, extension at 72 °C for 90 s, with a final extension of 74 °C for 7 min.

In order to identify the members of the *Cx. pipiens* complex a PCR assay targeting species-specific polymorphisms in the intron-2 of the acetylcholinesterase-2 (ACE-2) gene was performed (Smith and Fonseca, 2004). Specific primers ACEpip (5′-GGAAACAAC GACGTATGTACT-3′), ACEquin (5′-CCTTCTTGAATG GCTGTGGCA-3′), ACEtorr (5′-TGCCTGTGCTACCAGTGATGTT-3′) and the universal primer B1246s (5′-TGGAGCTCCTCT TCACGG-3′) were used to identify *Cx. pipiens*, *Cx. quinquefasciatus* and *Cx. torrentium*, respectively. Genomic DNA from homozygous *Cx. pipiens* and *Cx. quinquefasciatus* were used as positive controls. To differentiate between *molestus* and *pipiens* biological forms of *Cx. pipiens* PCR amplification of the flanking region of microsatellite CQ11 was performed according to Bahnck and Fonseca (2006) using primers pipCQ11R (5′-CATGTT GAGCTTCGGTGAA-3′), specific for the *pipiens* form (200 bp amplicon), and molCQ11R (5′-CCCTCCAGTAAGGTATCAAC-3′), specific for the *molestus* form (250 bp amplicon) along with the universal primer CQ11F2 (5′-GATCCTAGC AAGCGAGAAC-3′). This analysis was carried out in *Cx. pipiens* specimens for which a *Dirofilaria* spp. DNA amplification had been obtained and with a subsample of negative

mosquitoes. Positive controls (i.e. DNA from *molestus* and *pipiens* forms) were included in the PCR assays.

Finally, the haplotype of *Cx. pipiens* ITS-2 polymorphisms was assessed on the dirofilarial positive mosquitoes, by amplifying rDNA with primers 5.8S (5′-TGTGAACTGCAGGACACATG-3′) and 28S (5′-ATGCTTAAATTTAGGGGGTA-3′) (Bargues et al., 2006). The thermal cycler was set at 94 °C for 5 min, followed by 30 cycles with a denaturation step for 30 s at 94 °C, annealing for 30 s at 50 °C, and extension for 1 min at 72 °C, and a final extension for 7 min at 72 °C.

All the obtained amplicons by the different methods were analyzed by electrophoresis in 1.5% agarose gels stained with GreenSafe Premium (Nzytech®, Portugal), using a 100 bp DNA ladder as molecular weight marker (GeneRuler 100 bp DNA Ladder; ThermoFisher Scientific). All PCR products were sent to purify and sequence on both strands using kit BigDyeTerminator v1.1 Applied Biosystem, ABI PRISM 3700 DNA Analyzer (Stabvida® Sequence Service, Portugal).

2.3. Filarioidea DNA sequence analyses

Nucleotide (nt) sequence analyses were essentially carried out as previously described (Bravo-Barriga et al., 2016). Briefly, similarity searches were carried out through the NCBI web server using BLASTn, while multiple sequence alignments were performed using the iterative G-INS-i (COI) as implemented in MAFFT vs. 7. Editing of the COI alignments was done using GUIDANCE, selecting columns with confidence levels above 0.9. For Maximum Likelihood (ML) and Bayesian phylogenetic analyses (see below), the choice of the best fitting evolutionary model was based on those defined by JModeltest2.

Neighbor-Joining (NJ) and ML analyses were carried out using Mega 6.0 software and genetic distance matrixes corrected using the Tamura-Nei formula (NJ) or the GTR + Γ+I model (ML). Bayesian phylogenetic reconstruction was carried out with MrBayes v3.0b4 software, as previously described (Bravo-Barriga et al., 2016). A Maximum Clade Credibility COI tree was constructed using the GTR + Γ+I as implemented in the BEASTv1.7.5 software (Drummond et al., 2012), using as coalescent priors a Bayesian skyline plot, and a strict molecular clock. This analysis was run for 100×10^6 generations starting from a random tree and sampling every 1000th generation. Two separate runs were combined using LogCombiner and the first 10% discarded as burn-in. Convergence was monitored with Tracer v1.6, confirming that ESS values of all the estimated parameters confirmed to be above 200. The phylogenetic trees were manipulated for display using FigTree v.1.4.2. NeighborNet networks (NNn) were constructed using HKY-corrected distance matrix and the Splits Tree 4 software (Huson and Bryant, 2006).

2.4. rDNA ITS-2 sequence analysis

Cx. pipiens ITS-2 sequences were edited with BioEdit (Hall, 1999) aligned with known Culicidae sequences available in the GenBank-EMBL using CLUSTAL-W version 2.0 (Thompson et al., 1994). This served as the starting point for assignment to a known genetic haplotype, using an approach previously followed by Bargues et al. (2006) and Morchón et al. (2007), based on the construction of multiple sequence alignment between all most similar ITS-2 sequences of mosquitoes available from GenBank-EMBL (Supplementary data 1).

All the sequences obtained in the course of this work were deposited in the DDBJ database under accession numbers **LC107816**; **LC107949; LC107817; LC107818; LC107819** (COI) and **LC114271; LC114272; LC120317; LC120318** (rDNA ITS-2).

3. Results

3.1. Identification of mosquitoes and *Filarioidea* detection

The majority (97.96%) of female mosquitoes captured, totalling 881 adult females from 11 different species, were identified as *Cx. pipiens* (68.56%), followed by *Cx. theileri* (11.35%), *Culex* spp. (7.15%) which could not be identified further than the genus level due to bad conditions, *Culiseta longiareolata* (6.92%), *Aedes (Ochlerotatus) caspius* (2.27%) and *Cx. univittatus* (1.70%), while the remaining species were in abundances lower than 1% (Table 1). The seasonal distribution of the population of *Cx. pipiens* attracted to CDC light-traps is shown in Fig. 1. The data gained during the two consecutive seasons showed a strong influence of climate on the abundance of *Cx. pipiens*, with a clear monophasic distribution from May to August, which was coincident with the average monthly temperature (26 °C) and low relative humidity (40%), typically observed for this bioclimatic area.

Table 1. Relative abundance of culicid mosquito species captured in southwest Spain, and detection of *Dirofilaria immitis* and other Filarioid DNA.

Species	**Relative abundance (%) n = 881**	***D. immitis* Filarioid DNA**	
Culex pipiens s.l.	68.56	+1	+8
Culex theileri	11.35	–	–
Culex spp.	7.15	–	–
Culiseta longiareolata	6.92	–	–
Aedes (Ochlerotatus)caspius	2.27	–	–
Culex univittatus	1.70	–	–
Culiseta annulata	0.79	–	–
Culex hortensis hortensis	0.45	–	–
Anopheles maculipennis s.l.	0.34	–	–
Culiseta subochrea	0.23	–	–
Culex laticintus	0.11	–	–
Anopheles claviger s.l.	0.11	–	–

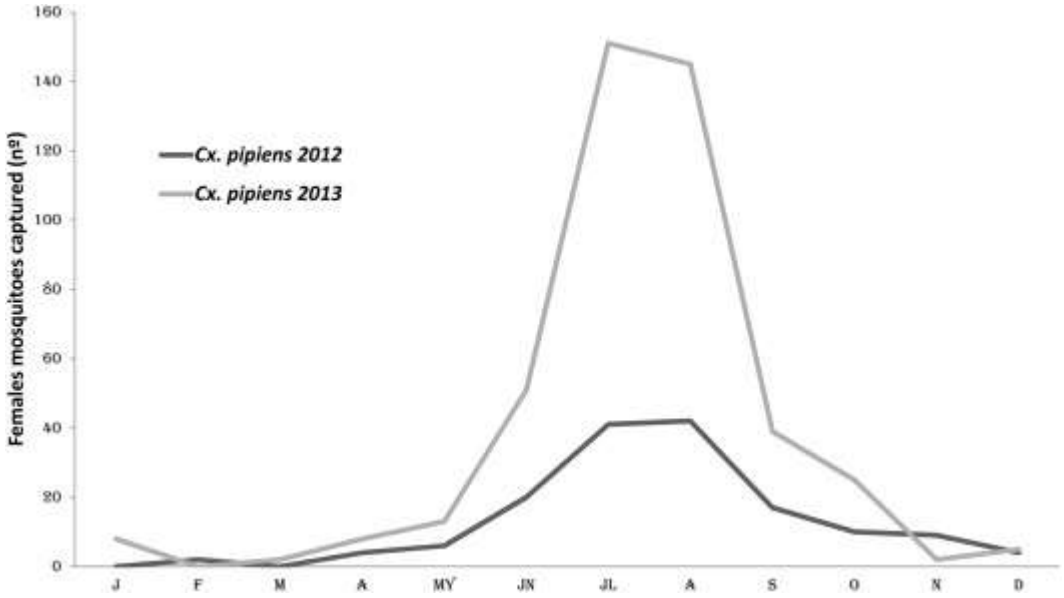


Fig. 1. Seasonal dynamics of *Culex pipiens* females collected in 2012 and 2013 in Extremadura (Spain).

All captured mosquitoes were analysed by PCR, and nine of them, all identified as *Cx. pipiens* s.l. by morphology, tested positive for filarioid DNA. These mosquitoes had been collected between June and August in 2012 and 2013 in five rural areas of Extremadura region (Table 2). For one specimen, corresponding to a gravid female captured in the north of the study area (Table 2), two ITS-2 amplicons were obtained (from the head-thorax and abdomen fractions, respectively), and Mega-BLAST analysis revealed 100% identity with *D. immitis* sequences previously deposited in GenBank™, (**U159111**) for both sequences. Hence, prevalence of *D. immitis* in *Cx. pipiens* s.l. was 0.16%.

For the remaining 8 positive specimens, only 4 ITS-2 amplicons, with good enough quality that would support further genetic study were obtained from mosquitoes head-thoraxes and abdomens (Table 2), the analysis of which did not unambiguously match with any homologous sequences deposited in the GenBank™. Only up to 71% identity with *Mansonella* sp. could be obtained in short good-quality segments (data not shown). This result suggested that the obtained sequences might include Filarioidea sequences of at least two types: *D. immitis* and *Mansonella*-like nematodes. Unfortunately, and despite multiple amplification/sequencing attempts, the overall low quality of the sequences obtained (possibly due to low amplification yields) precluded a more comprehensive analyses based on the ITS-2 marker.

As a result of the limited amount of information gained from the analysis of ITS-2 sequences, a choice was made to use COI sequencing to confirm the presence of nematode DNA (Table 2), also providing an opportunity to obtain unambiguous nucleotide sequence data for phylogenetic analyses, using for comparison reference sequences available from the public database [accession numbers are shown in maximum clade credibility tree (Fig. 2) and consensus tree (Fig. 3) and NNn-network presented as Supplementary data 2]. As expected, the analysis of the filarioid COI amplicon obtained from *Cx. pipiens* s.l. demonstrated that one of the sequences consistent clustering within the *D. immitis* radiation (**LC107816**).

Table 2. Details about the filarial species found, indicating the access number of COI and homology in GenBank™, as well as information of specimens of *Culex pipiens* positive (biologial form, access number of rDNA, positive body location by PCR, gonotrophic state and place and date of capture).

Filarial species	DDBJ* Accession n°.	Max.% identity of filarial species to GenBank entry (accession n°.)	Biological forms of *Cx. pipiens* CQ11	DDBJ Accession n°. Mosquito species rDNA ITS-2	PCR body location	Gonotrophic state	Collection district and region**	Collection date
Dirofilaria immitis (strain 11e2a)	LC107816	100% (U159111)	Culex pipiens f. pipiens	LC114271	Head-Torax/abdomen	Gravid	Coria (1)	August 2013
Filarioidea (strain 75)	LC107949	92% (HQ186250)	Culex pipiens f. pipiens	LC114272	Head-Torax	Gravid	Guadalupe (1)	August 2012
Filarioidea (strain 92)	LC107817	92% (JX870433)	Culex pipiens f. pipiens	LC120317	Head-Torax/abdomen	Gravid	Castuera (2)	June 2013
Filarioidea (strain 97)	LC107818	91% (JX870433)	Culex pipiens hybrid	–	Head-Torax	Gravid	Zafra (2)	August 2013
Filarioidea	–		Culex pipiens f. pipiens	–	Abdomen	Gravid	Berlanga (2)	July 2013
Filarioidea	–		Culex pipiens hybrid	–	Abdomen	Gravid	Berlanga (2)	July 2013
Filarioidea (strain 124)	LC107819	91% (HQ186250)	Culex pipiens hybrid	LC120318	Abdomen	Unfed	Berlanga (2)	August 2013
Filarioidea	–		Culex pipiens f. pipiens	–	Abdomen	Unfed	Berlanga (2)	July 2013
Filarioidea	–		Culex pipiens f. molestus	–	Abdomen	Unfed	Berlanga (2)	July 2013

* DNA Data Bank of Japan.

** Region: 1- Cáceres, 2- Badajoz.

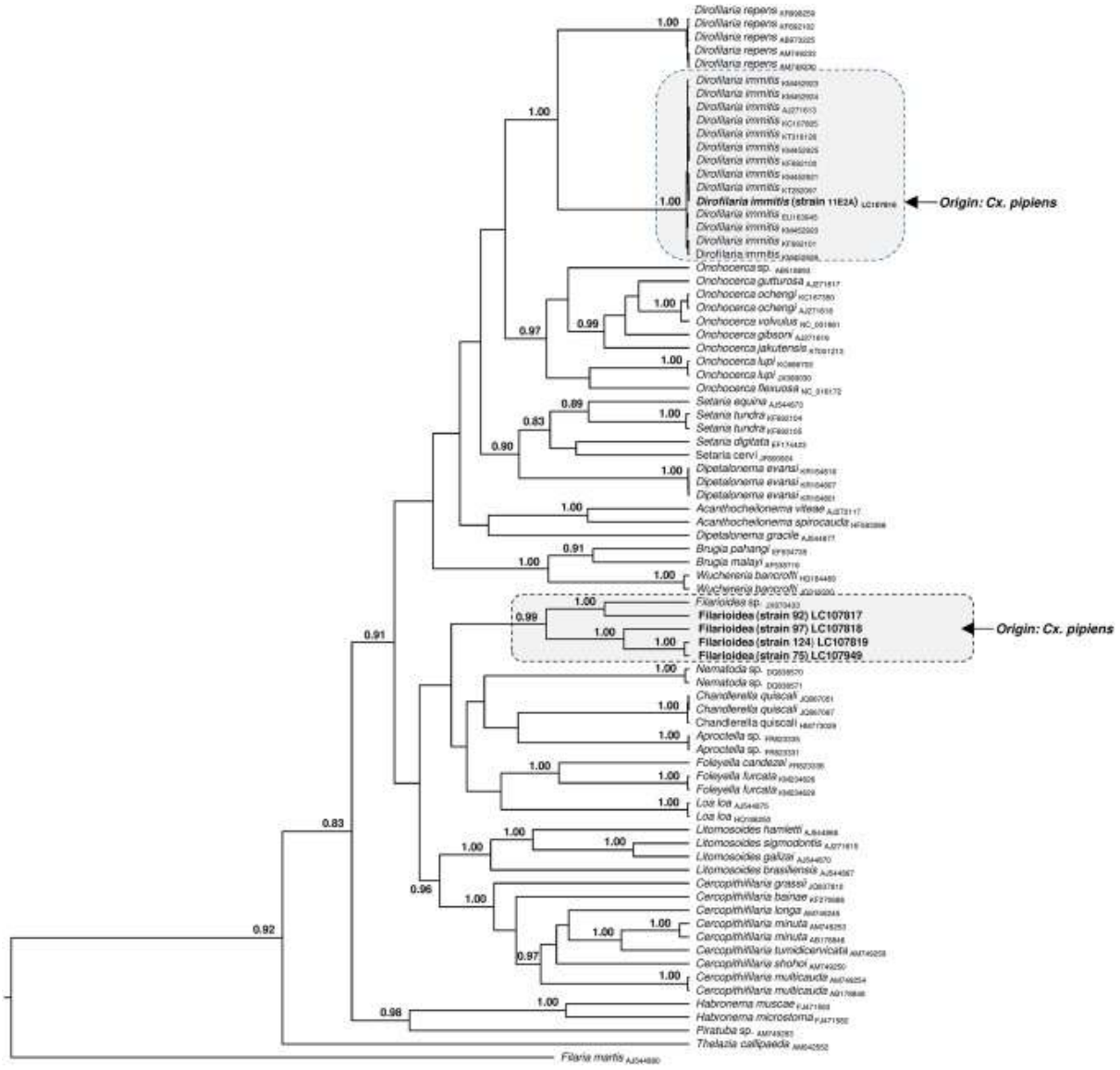


Fig. 2. Maximum Clade Probability Tree based on the analysis of COI sequences. At specific branch nodes posterior probabilities (pp) ≥0.80 are indicated. The clusters of sequences boxed in grey include those that include data obtained in the course of this work. The tree has been rooted using as outgroup *Filaria martis* (AJ544880).

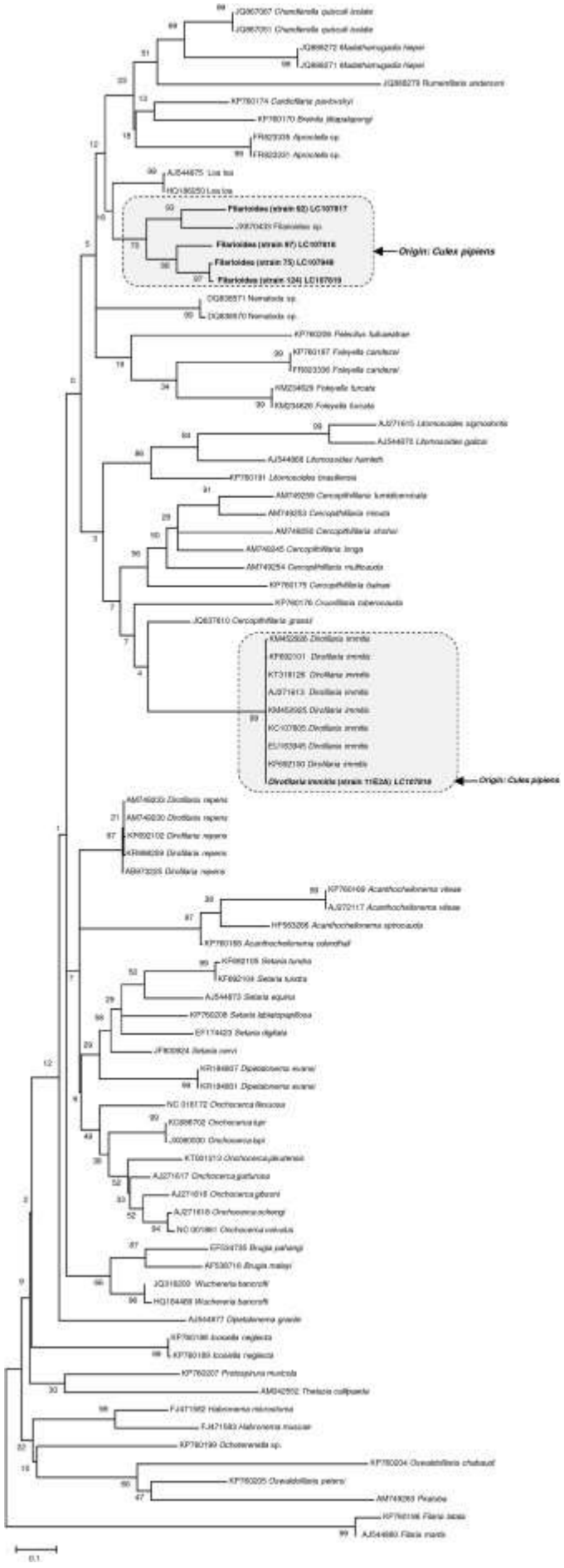

Filaroides (strain 62) LC107917
Filaroides (strain 97) LC107916
Filaroides (strain 75) LC107948
Filaroides (strain 124) LC107919
Origin: Culex pipiens
Dirofilaria immitis (strain 11E2A) LC107918
Origin: Culex pipiens
0.1

Fig. 3. Phylogenetic analysis of COI sequences. At specific branch nodes, either bootstrap values (b) ≥75% or posterior probabilities (pp) ≥0.85 are indicated, according to the following rationale: bNJ,bML,ppBayes. Bootstrap/posterior probability values below these limits are indicated by "—". The size bar indicates the number of nucleotide substitutions per site. The clusters of sequences limited by dotted lines are those that include sequences amplified in the course of this work. All the sequences used (most of which correspond to references downloaded from the public GenBank™ database) are indicated by species and accession number. The phylogenetic tree was rooted using *Filaria martis* (AJ544880) and *Filaria latala* (KP760186) as outgroup.

Unfortunately, unambiguous COI sequence data was only obtained from four additional amplicons. Database matches using Mega-BLAST revealed 91–92% identity with two sequences in particular, identified as Filarioidea sp. (**JX870433**; from a Canadian northern saw-whet owl) and *Loa loa* (**HQ186250**; human specimen) (Table 2), clearly suggesting a filarioid origin. Further characterization of COI sequences was carried out on the basis of phylogenetic analyses (Fig. 2 and Fig. 3), regardless of the strategy used for phylogenetic reconstruction (NJ, ML or Bayesian analysis), one of the sequences obtained shared direct common ancestry with Filarioidea sp. (**JX870433**), whereas the other three clustered together, but segregated away from the latter. Despite the fact that all the phylogenetic reconstruction approaches used produced trees with congruent topologies, only the maximum clade credibility tree and the Bayesian consensus tree gave statistical support to a cluster that included all the Spanish sequences and that also included Filarioidea sp. (**JX870433**) but did not allow clear assignment of four of the obtained sequences to any specific filarioid species. Similar conclusions could be drawn from the analysis of the NNn-network (Supplementary data 2).

3.2. Characterizing Filarioid DNA positive *Culex pipiens*

All mosquitoes positive for filaroid DNA were identified as *Cx. pipiens* s.s. by ACE-2 assay. The mosquito found infected with *D. immitis* was identified as *Cx. pipiens* form *pipiens* based on the analysis of the CQ11 locus, while the remaining eight mosquitoes were identified as *molestus*, *pipiens* or hybrids (Table 2). These specimens were further processed so as to obtain mosquito-specific rDNA ITS-2 sequences. Sequence analysis comparisons showed that the specimen infected with *D. immitis* (**LC114271**) was 100% identical to haplotype H1 of *Cx. pipiens* (Supplementary data 1). Among the remaining mosquitoes infected with other Filarioidea members, one mosquito specimen, classified as *Cx. pipiens* f. *pipiens* by CQ11 locus, had rDNA ITS-2 sequences 100% identical to haplotype H1 (**LC114272**), while another mosquito classified also as *Cx. pipiens* f. *pipiens* was characterized as haplotype H2 (**LC120317**). Another specimen, a hybrid between *Cx. pipiens* f. *pipiens*/*Cx. pipiens* f. *molestus*, did not conform to any given haplotype and appeared to be a hybrid form by the analysis of rDNA ITS-2 as well (**LC120318**). Its sequence differs from haplotype H2 in one indel (C) and from haplotype H1 in one trinucleotide microsatellite CGT (Supplementary data 1).

The other 5 filarioid positive mosquitoes did not produce good quality rDNA ITS-2 sequences.

4. Discussion

This study rested of the analysis of a total of 881 female adult mosquitoes, collected in Southwest Spain (Extremadura region) using CDC light-traps, and the majority of which

were classified into three species *Cx. pipiens*, *Cx. theileri*, and *Cs. longiareolata*. As expected from the a priori know density fluctuations of the Culicid mosquito fauna in the Iberian Peninsula (Almeida et al., 2010 and Roiz et al., 2014), the number of mosquitoes collected was highest from May to August. Detection of filarioid DNA in these mosquitoes revealed nematode-infections between June and August, coinciding with the time of average temperatures that are favourable for *Cx. pipiens* higher abundances and parasite development in the vector (Roiz et al., 2014 and Sassnau et al., 2014). Regardless of the observed monophasic distribution, mosquitoes were nevertheless captured throughout the year, as previously (Bargues et al., 2006 and Roiz et al., 2015). This probably reflects an outstanding environmental adaptation capacity of *Cx. pipiens*, allowing it to use almost any kind of water collection as breeding site, and having a very broad period of seasonal activity. Changes in seasonal vector densities are likely to impact the rate of infection as previously described by Simón et al. (2012) and several models for the transmission periods have been proposed based on DDU (*Dirofilaria* development units) (Sassnau et al., 2014). In fact, it has been shown that Spain is the European Union country with the longest transmission period for *Dirofilaria* (Simón et al., 2014).

The detection of *D. immitis* in a single *Cx. pipiens* specimen is indicative of the nematode low prevalence in this species (0.16%), in line with previous reports from mainland Spain, although with a higher infection rate (0.3%) (Morchón et al., 2007). In neighbouring Portugal, *Cx. pipiens* revealed a higher *D. immitis* infection rate (0.5%) (Ferreira et al., 2015), while in Italy, the prevalence rates for this species range between 0.05% and 0.54% (Cancrini et al., 2006 and Latrofa et al., 2012), being lower (0.12%) in Turkey (Yildirim et al., 2011) or in Germany (0.02%) (Kronefeld et al., 2014), but reaching as high as 1.49% in Hungary (Zittra et al., 2015).

Other mosquito species, have been found infected throughout Europe, such as *Cx. theileri* in the Canary islands (Morchón et al., 2011), Madeira island (Santa-Ana et al., 2006), and mainland Portugal (Ferreira et al., 2015), with infection rates that range from 0.17% to 1.13%. *Anopheles maculipennis* s.l. (3.12–5.26%) and *Ae. (Och.) caspius* (0.18–3.73%), have also been found infected, in Italy (Cancrini et al., 2007 and Latrofa et al., 2012), Portugal (Ferreira et al., 2015) and Hungary (Zittra et al., 2015). *Cx. theileri*, *Ae. (Och.) caspius* and *An. maculipennis* s.l have also been collected in this study, albeit not infected with *Dirofilaria* spp., demanding further studies to ascertain their role in its transmission in this region of Spain.

The fact that *D. immitis* was detected in a gravid *Cx. pipiens* f. *pipiens*, both in head-thorax and in the abdomen, holds the possibility that after blood meal digestion, larval development to the infective L3 stage in Malpighian tubules and migration to the head-thorax, may have occurred (Anderson, 2000). To our knowledge, this is the first time in Spain, that *D. immitis* DNA has been detected in the head-thorax of *Cx. pipiens* f. *pipiens*, suggesting it may play a role as a competent vector for their transmission. This hypothesis requires further confirmation, specially accounting the limited number of infected specimens. However, elsewhere, *Cx. pipiens* s.l. has been found as a competent vector for this parasite (Capelli et al., 2013). To the west of Extremadura, in neighbouring Portugal, conclusive implication of *Cx. pipiens* as a vector for *D. immitis* could not be ascertained either, since nematode DNA was detected only in abdominal macerates (Ferreira et al., 2015). Gouveia (2007) has suggested that the *molestus* population from Madeira (Portugal) is not able to support the full development of *D. immitis*. However, both Lai et al. (2000) and Carvalho et al. (2008) have

provided compelling evidence for the vector competence of *Cx. quinquefasciatus*, a member of the same complex, for the transmission of *D. immitis* by means of experimental infections.

According to Bargues et al. (2006) and Morchón et al. (2007), the analysis of nuclear rDNA ITS-2 sequences allows validation of the classification and haplotype characterization of mosquitoes. These studies suggested that haplotype H1 may be genetically ascribed to *Cx. pipiens* f. *pipiens* while haplotype H2 as an intermediate form or hybrid. In this study, the haplotype H1 was detected in two mosquitoes (infected with filarioid nematodes) identified as *Cx. pipiens* f. *pipiens* by CQ11. Moreover, the analysis of ITS-2 sequences also indicated the presence of haplotype H2 as well as hybrid forms in mosquitoes with filarioid DNA. Despite its usefulness, genetic analysis of mosquito populations solely based on such a strategy is not devoid of analytical limitations, which could be overcome via combination with microsatellite analyses in future studies (Gomes et al., 2013).

In four of the *Cx. pipiens* specimens analysed, filarioid ITS-2 and COI sequences were found, that neither database-searches nor phylogenetic analyses allowed their unambiguous assignment to any previously defined species. This suggests that previously unknown filarioid nematodes occur in mainland Spain. Furthermore, when taken into account along with the wide geographical distribution of the collection points and the time-frame dispersal of collection moments (Table 2), this observation supports the existence of an active and sustained transmission cycle for such uncharacterized nematodes. The phylogenetic analysis of the obtained sequence data suggested shared ancestry with unclassified worms belonging to the Filarioidea superfamily, previously detected in *Aegolius acadicus* (the northern saw-whet owl) from Canada. Hence, the worms hereby detected, could also be of avian origin. The detection of filarioid worms with a possible avian origin in *Cx. pipiens*, mainly of the *pipiens* form and hybrids is consistent with its ornithophilic habits (Gomes et al., 2013), considering also that it feeds mainly (83%) on a high diversity of avian species (Hamer et al., 2009), as well as with the abundance of migratory birds in this area, mainly from Africa (WysInfo Docuwebs, 2015).

Previous studies have already reported the detection of unknown species of filarioid nematodes in mosquitoes collected in Hungary (Kemenesi et al., 2015), Germany (Czajka et al., 2012), while Boothe et al. (2015) reported detection of filarioid nematodes in 3.8% of blood-fed *Cx. pipiens.* Kronefeld et al. (2014) suggested the presence of *Cardiofilaria pavlovskyi* on mosquitoes in Germany. Curiously, this species has also been previously reported in birds in Spain by López-Caballero (1982). Because filarioid nematodes are difficult to collect, their diversity is mostly unknown, with only a few relatively recent studies reporting new species such as *Pelecitus* nematides from the Tehuantepec jackrabbit (*Lepus flavigularis*) (Jiménez-Ruiz et al., 2004) and *Diplotriaena railliet* in birds such as *Passer domesticus* and *P. pyrhonotus* from Pakistan (Chandio et al., 2015). Genetic studies were conducted for *Micipsella numidica* in the hare *Lepus europaeus* from Italy (Gabrielli et al., 2015) and *Rumenfilaria andersoni* along with other 48 species of onchocercid subfamilies of filarial nematodes (Lefoulon et al., 2012 and Lefoulon et al., 2015). Regardless of our ability to assign the obtained filarioid DNA sequences to a given species or genus, reports such as these do suggest that diverse Filarioidea nematodes may be widely distributed in Europe, in a large range of hosts. Their genetic characterization and the identification of which are the vector/host system(s) they use for natural maintenance, ask for future clarification.

5. Conclusion

Our new findings, suggest that *Cx. pipiens* f. *pipiens* can support the development of the parasite and thus could act as vector of *D. immitis*, for first time in Spain. Being *Cx. pipiens* an endemic species and highly abundant in the surveyed area, its possible role as vector could have a clear impact on human and veterinary health that must be taken into consideration to establish control measures for dirofilariosis. On the other hand, the presence of unidentified Filarioidea nematodes in Spain, possibly of avian origin, calls for a deeper understanding in terms of a characterization of their life cycle, host-range, associated vectors and possible pathogenicity.

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Supplementary data 1 Nucleotide differences found by rDNA ITS-2 sequence comparing different Culex pipiens.
Sequences used for these analysis (Country and accessions) were as follows: AM084682-3 from Spain; AF305553 from China; U22111-12, U22113, U22115-6, U22119-20, U22129, U33043-4 from USA; AJ850084-6 from Russia.

Species	POSITIONS																																					
	9	9	9	9	9	9	9	9	9	9	9	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 2	1 2	1 2	1 2	1 2	1 2
	4	4	4	4	4	4	4	4	4	5	5	3	5	7	7	8	8	8	8	8	8	4	5	5	5	8	8	8	3	3	4	4	6	6
	0	1	2	3	5	6	7	8	9	0	1	0	9	1	2	0	1	2	3	4	5	4	7	8	9	6	7	8	6	7	1	3	1	2
Cx. pipiens H1(AM084682)	**A**	**C**	**G**	**C**	**-**	**-**	**-**	**-**	**-**	**G**	**T**	**G**	**C**	**C**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**G**	**T**	**-**	**A**	**A**	**C**	**A**	**C**	**C**
***Cx. pipiens* with *D. immitis* (strain 11E2A; LC114271)**	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	·	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	·	·	·	·	·
***Cx. pipiens* with Filarioidea DNA (strain 75; LC114272)**	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	·	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	·	·	·	·	·
Cx. pipiens H2 (AM084683)	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**G**	**T**	**C**	·	·	**-**	·	·	·	·	·	·
***Cx. pipiens* with Filarioidea DNA (strain 92; LC120317)**	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**G**	**T**	**C**	·	·	**-**	·	·	·	·	·	·
***Cx. p.* hybrid with Filarioidea DNA (strain 124; LC120318)**	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	·	·	·	·	**-**
Cx. p. molestus (AJ850085-6)	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	·	·	·	·	**-**
Cx. p. x Cx. q. hybrid (U33043-44)	**G**	·	**A**	·	**C**	**-**	**-**	**-**	**-**	**A**	**C**	·	**-**	·	**-**	**A**	**C**	**G**	**A**	**C**	**G**	**-**	**-**	**-**	**-**	·	·	**-**	·	**T**	·	·	·	·
Cx. pipiens (U22111-2)	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**T**	**C**	**G**	·	·	·	·	·	**-**
Cx. pipiens (U22113)	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	·	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	**T**	·	·	·	·
Cx. pipiens (U22115)	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	·	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**-**	**-**	**-**	·	·	**-**	·	·	·	·	·	**-**
Cx. pipiens (U22116)	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	·	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**-**	**-**	**-**	·	·	**-**	·	·	·	·	·	**-**
Cx. pipiens (U22119-20)	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	·	·	·	**-**	**A**	**C**	**G**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	**T**	·	·	·	·
Cx. pipiens quinquefasciatus (U22129)	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	·	·	·	**-**	**A**	**C**	**G**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	·	·	**-**	·	**T**	·	·	·	·
Cx. p. molestus (AJ850084)	·	·	·	·	**-**	**-**	**-**	**-**	**-**	·	·	·	·	·	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**G**	**T**	**C**	·	·	**-**	·	**T**	·	·	·	**-**
Cx. pipiens quinquefasciatus (AF305553)	**G**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**C**	**A**	**C**	**-**	·	·	**A**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	·	·	**-**	**-**	**-**	**T**	**-**	**-**	**-**

Numbers (to be read in vertical) refer to positions obtained in the alignment made with Mega 6.0 and CLUSTAL-W 2.0. ·=Identical, – = insertion/deletion.

Supplementary data 2 NeighborNet network constructed with SplitsTree software employing the matrix of genetic distances (corrected with the HKY formula) between individual nematode COI sequences amplified from mosquitoes collected in Spain, and reference sequences downloaded from the GenBank™ database. Filaria latala and Filaria martis were used as outgroups.