Morchón et al., J Clinic Experiment Pathol 2011, S3
DOI: 10.4172/2161-0681.S3-001

Journal of
Clinical & Experimental Pathology

Research Article Open Access

Molecular Characterization of *Culex Theileri* from Canary Islands, Spain, a Potential Vector of *Dirofilaria Immitis*

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Abstract

Dirofilaria immitis is the causal agent of heartworm diseases and of human pulmonary dirofilariosis. The infection is transmitted by several species of culicid mosquitoes that are frequently able to bite both animal reservoirs and humans. Canary Islands (Spain) constitute a well documented endemic area of canine dirofilariosis in which the mosquito species involved in the transmission of *D. immitis* are not known. The objectives of the present work were the identification of vectors of this parasite in Canary Islands and their molecular characterization. A total of 1219 female mosquitoes were captured. The most abundant species was *Culex theileri* (52.26%) followed by *Cx. pipiens* (35.44%), *Anopheles cinereus hispaniola* (6.23%), *Culiseta longiareolata* (5.74%), and *Culex laticintus* (0.33%). PCR was applied for the detection of larval *D. immitis* DNA in mosquitoes. *D. immitis* DNA was observed in the abdomen of one *Cx. theileri* female: 0.082% of the entire mosquito population and 0.17% in *Cx. theileri*. A molecular identification of *Cx. theileri*, the potential mosquito vector of dirofilariosis in this zoonotic focus in Canary Islands of Spain, has been made for first time based on sequences of the 18S rRNA gene, the second internal transcribed spacer (ITS2) of ribosomal DNA and the barcode region of the cytochrome c oxidase I (*cox*1) gene of mitochondrial DNA, allowing a broad mosquito molecular basis for future populations genetic analyses of this vector species. Parasitological and entomological molecular results suggest that *Cx. theileri* is a potential natural vector of *D. immitis* in Canary Islands.

Keywords: *Dirofilaria immitis; Culex theileri*; Canary Islands; Spain; 18S rRNA gene; ITS-2 rDNA; barcode region mtDNA.

Introduction

Dirofilaria immitis is the causal agent of canine and feline cardiopulmonary dirofilariosis (heartworm disease) and of human pulmonary dirofilariosis, worldwide [1]. In animal reservoirs the disease, that afects pulmonary arteries, lungs and heart is serious and potentially fatal [2], while in humans which are accidental hosts, an iatrogenic damage can be produced after the fortuitous discovery of a benign pulmonary nodule that is frequently confused with lung cancer [3].

Heartworm disease is a vector borne transmitted disease. Numerous species of culicid mosquitoes belonging to the genera *Aedes* spp., *Culex* spp. and *Anopheles* spp. have been related to the transmission of *D. immitis* in different endemic areas. Nevertheless, the real vectorial capacity based both on the captures with bait traps and the detection of parasite larvae in the cephalic region of the mosquitoes by PCR, carried out in Italy, has been demonstrated in *Ae. albopictus*, *Cx. pipiens* and *An. maculipennis* s.l.; abdomens of *Cx. modestus*, *Cx. torrentium*, *Ae. punctor*, *Ae. cinereus*, *Ae. detritus* and *Ae. geniculatus* were also positive to *D. immitis* [4]. Moreover, other species including *An. claviger*, *An. hyrcanus*, *An. maculipennis*, *An. superpictus*, *Cx. modestus*, *Cx. pipiens* and *Cx. tritaenorhynchus*, are assumed to be involved in the transmission of dirofilariosis in other endemic areas [5-8].

Recently *Cx. theileri* has been incriminated in the transmission of *D. immitis* in the island of Madeira [8] and in the Ardebil province of Iran [9]. In the latter study has shown that this mosquito is the most abundant species and the main vector of the parasite. *Cx. theileri* has a wide distribution in the Old World [10], being present in many endemic areas of animal and human dirofilariosis, considering its current geographical distribution [1]. In Spain the presence of *Cx. theileri* has been reported in the Canary Islands [11], Andalucía [12] and Salamanca [13], areas in which the existence of *D. immitis* is well documented [14-17].

The aim of the present work was to investigate the potential natural vectors of *D. immitis* in the Canary Islands, Spain and their molecular characterization. The classification of this vector species as well as of the filarial larvae stages found in a naturally infected specimen has been confirmed by molecular characterization. The molecular markers used for the characterisation of *Cx. theileri* were selected according to previous results having shown their usefulness for mosquitoes in general or Culicids in particular: small subunit or 18S rRNA gene [18-21] and the second internal transcribed spacer ITS-2 [22-25] within the nuclear rDNA, and the cytochrome c oxidase subunit I *cox*1 [9,22-19] within the mtDNA. This finding represents a new record for vector species not only for the Canary Island fauna but also for the whole Spanish country where the only mosquito species involved in *D. immitis* transmission yet identified is *Cx. pipiens*.

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Received October 20, 2011; **Accepted** December 03, 2011; **Published** December 06, 2011

Citation: Morchón R, Bargues MD, Latorre-Estivalis JM, Pou-Barreto C, Melero-Alcibar R, et al. (2011) Molecular Characterization of *Culex Theileri* from Canary Islands, Spain, a Potential Vector of *Dirofilaria Immitis*. J Clinic Experiment Pathol S3:001. doi:10.4172/2161-0681.S3-001

Materials and Methods

Study area

Tenerife and Gran Canaria islands are located in Canary Islands (Spain) at the 28th parallel on the African Atlantic coast, situated only 95 km from the Western Sahara. Tenerife is the largest and most populous of the Canary Islands with a land area of 2034 km^2, triangular island with 110 km in diameter. Gran Canaria is the second most populous island with 1530 km^2, circular island with 40 km in diameter. The climate of Canary Islands is very different from those of the European continent. A significant change in climate, vegetation, and mountain geography can be observed by travelling only a few kilometers in both islands [16,17] being Gran Canaria a drier climate than Tenerife. The captures were carried out in 4 places on Tenerife (San Cristóbal de La Laguna, San Andrés, Güímar, Guía de Isora and Granadilla) and 3 places on Gran Canaria (Jardín Botánico, Veneguera and Juncalillo).

Mosquito sampling

Female mosquito sampling was carried out from July to September of years 2005, 2006, 2007. Captures were made 19.00 to 24.00 h, employing the same two persons as bait inside the trap. The collection method was human baited landing captures. The trap dimensions were 170 x 120 x 170 cm. Collections were made by aspirating females that landed on bait and rested in the trap with a paper cup aspirator [25]. The female mosquitoes sampled were kept under controlled conditions (25-27ºC, 80% relative humidity, r.h.) for 5 days to allow the infective larvae to develop in the abdomen (if the last infective blood meal was taken at least 3 days before the capture, keeping the mosquitoes for 5 days should allow larval development in Malpighian tubules) or, in the case of an incompetent host, to assure that blocked microfilariae have been expelled. Mosquitoes were then identified according to the keys proposed by [30] and [13], killed and fixed in 70% ethanol.

Molecular diagnostics

DNA extraction and amplification for detection of *Dirofilaria* in mosquitoesL: The presence of filarial parasites in mosquitoes was evaluated by PCR examination in female mosquitoes previously described [25]. Since the different larval stages of filarial parasites cannot be distinguished by PCR-based method, DNA extraction was performed separately on the insect abdomen and thorax-head to discriminate between *Dirofilaria* infected/infective specimens [31]. Total genomic DNA from mosquitoes was extracted by the kit Instagene™ Matrix (Biorad) with some modifications: each mosquito part was suspended in 1ml of autoclaved water in a microfuge tube, disrupted and centrifuged for 1 min at 13400 xg in the Centrifuge 5415R (Eppendorf). The supernatant was removed and 200 µl of InstaGene matrix were added to the pellet before incubation at 56 ºC for 30 min. The tubes were first shaken in a vortex at high speed for 10 seconds and placed in a 100 ºC boiling waterbath for 8 min and afterwards at high speed for 10 sec plus spin at 13400 xg for 3 min. The supernatant was stored at -20 °C until use. To detect filarial parasites, the extracted DNA was analyzed with "filarial" specific ribosomal primers named S2-S16 [32]. Conditions for the detection of filarial DNA were those described for the amplification of the spacer 5S of the ribosomal gene [33]. The reactions give rise to amplification products of approximately 400 bp for most filarial species. Positive samples were checked with primers specific for *D. immitis* previously designed [31].

DNA extraction and amplification for mosquito 18S rRNA gene, ITS-2 rDNA, and *cox*-1 mtDNA sequencing: Mosquito molecular characterization was applied only to *Cx. theileri*, the most prevalent species and the only one harbouring filarial parasites. The thoracic part and legs of 27 adult specimens of *Cx. theileri* from Güimar and San Cristobal de La Laguna, Tenerife and Veneguera, Gran Canaria (Canary Islands, Spain) were used for DNA extraction, using the InstaGene™ Matrix kit using the same methodology described previously. One population of *Cx. theileri* from Sueca, Valencia (Spain) were also processed for comparative purposes. The volume used were 4-6 µl of the supernatant per 25 µl PCR reaction. Each one of the three DNA markers were PCR amplified independently for each mosquito specimen and each PCR product was sequenced for a bona-fide haplotype characterization. The 18S rRNA gene and ITS-2 rDNA were amplified using primers and PCR conditions previously described [21, 24, 25]. The barcode region of the *cox*1 gene was amplified using the LCO and HCO primers [34] and PCR conditions were 1min at 95° C, 35 cycles of 1min at 94° C, 1 min at 55° C, 1.5 min at 72° C , and a final cycle of 7 min at 72° C. In the case *Cx theileri* ITS-2, the PCR fragment obtained was subcloned in the pGEM-T Easy Vector (Promega, Madison, WI) and sequenced using vector primers (M13 forward and M13 reverse).

The sequencing of the complete 18S rRNA gene, ITS-2 rDNA and the barcode region of the mtDNA *cox*1 gene was performed on both strands by the dideoxy chain-termination method [35]. It was carried out with the Taq dye-terminator chemistry kit for ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), using PCR primers. The sequences were translated into amino acids using MAGA 5.0 version [36].

Sequence Alignment and Software programs used: Sequences were aligned using CLUSTAL-W version 1.8 [37] and MEGA 5.0 and assembly was made with the Staden Package version 1.5 [38]. Sequences of *Cx. theileri* from the Canary Island were compared with known Culicidae sequences available in the GenBank-EMBL. Homologies were performed using the BLASTN program from the National Centre for Biotechnology information website (http://www.ncbi.nlm.nih.gov/BLAST). Genetic distances were measured using parameters provided by PAUP v.4.0b10 [39].

Results

A total of 1219 female mosquitoes were captured. The most abundant species was *Culex theileri* (637 specimens captured, 52.26%) followed by *Cx. pipiens* (432 specimens, 35.44%), *Anopheles cinereus hispaniola* (76 specimens, 6.23%), *Culiseta longiareolata* (70 specimens, 5.74%), and *Culex laticintus* (4 specimens, 0.33%) (Table 1).

D. immitis DNA was observed in the abdomen of one *Cx. theileri* females captured in Güimar, Tenerife island. Prevalence of *D. immitis* was therefore 0.16% in *Cx. theileri*, 0.42% in Güimar, 0.12% in Tenerife island and 0.082% of the entire mosquito population.

18S rRNA gene: The 18S rDNA sequence of *Cx. theileri* from Tenerife, Gran Canaria and Valencia collections were identical base to base, with a length of 1935 bp, an AT content of 54.6 %, and base frequencies of: A = 0.281, G = 0.255, C = 0.199, and T = 0.265. This 18S sequence has been deposited in GenBank under the Accession No. JN051383. When comparing the 18S sequence of *Cx. theileri* with the 7 almost complete sequences of other *Culex* species available in the

	Tenerife					Gran Canaria				
	San Cristóbal de La Laguna	San Andrés	Güimar	Guía de Isora	Granadilla	Jardín Botánico	Veneguera	Juncalillo	TOTAL	Relative abundance
Culex theileri	79	71	159 (1+)	46	74	30	166	12	**637**	**52,26%**
Culex pipiens	63	72	66	69	39	92		31	**432**	**35,44%**
Anopheles cinereus hispaniola		19					36	21	**76**	**6,23%**
Culiseta langiareolata	9		13		42	6			**70**	**5,74%**
Culex laticintus							4		**4**	**0,33%**
TOTAL	**151**	**162**	**238**	**115**	**155**	**128**	**206**	**64**	**1219**	

Table 1: Number and relative abundance of culicid mosquito species captured in Tenerife and Gran Canaria, Canary Islands, Spain.

```
                                                                                  11
                                                            12255555777777788888844
                                                  111111111122222222223333333333444268113341222555701101101
                                   123456789012345678901234567890123456789012772784547234459190390367

Cx. theileri                       TCTGGTTGATCTGCCAGTAGTATACGCTTGTCTCAAAGGTTACCGCCGGAGCGC--CGGCCGCCTT
Cx. tritaeniorhynchus              ***********************************...................--..........
Cx. pipiens molestus               ******************************************............--..........
Cx. pipiens quinquefasciatus       ******************************************............--..........
Cx. pipiens                        ******************************************............--..........
Cx. restuans                       ******************************************............--..........
Cx. salinarius                     ******************************************............--..........
Cx. territans                      ******************************************ATATTAACTGCGTCATCT-CT-CG

                                   11111111111111111111111111111111111111111111111111111111111111111
                                   44444444444444444444444444444444444444444444444445558899999999999
                                   22222233333333334444444444555555555566666666777712244400001111111
                                   13678901234567890123456789012345678901234569124691225667890123456

Cx. theileri                       CGTTGGGTGTGTGGCCTCTCGGGGCG---------GTGCGCTTCCACGAAACGTGTAACAAGGTT
Cx. tritaeniorhynchus              .C.-...C...---........-------------.....T...AG.....-CC...........
Cx. pipiens molestus               ...-...C.C..C..GG.CGTTC...CGGTC--GT.C...T.CT.......TCC***********
Cx. pipiens quinquefasciatus       ...-...C.C..C.GGG.CGTTC...CGGTCCTGC.C...T.CT.......TCC***********
Cx. pipiens                        ...-...C.C..C..GG.CGTTC...CGGTC--GT.C...T.CT.......TCC***********
Cx. restuans                       ...-...C.C.CCT.GGT------------------...T.CT....C..TCC***********
Cx. salinarius                     ..C-.C.G.C..CCTTCGGG----------------...T.C........TCC***********
Cx. territans                      GT.-------------------------------------..ACTC.T--CC***********
```

Table 2: Variable positions found in the complete 18S rRNA gene sequence of *Culex theleri* and other *Culex* species. Numbers (to be read in vertical) refer to positions obtained in the alignment; . = Identical, - = insertion/deletion, * = not sequenced. *Cx. tritaeniorhynchus* (U48385; Miller et al. 1997); *Cx. pipiens, Cx. pipiens* form *molestus, Cx. quinquefasciatus, Cx. restuans, Cx. salinarius, Cx. territans* (AY988445- AY988450; Sephard et al. 2006).

GenBank: *Cx. pipiens, Cx. pipiens* form *molestus, Cx. quinquefasciatus, Cx. restuans, Cx. salinarius, Cx. territans* (AY988445-AY988450; [40]) and *Cx. tritaeniorhynchus* (U48385; [41]), a total of 79 variable positions appeared (12 p-info, 38 singleton sites and 29 indels) in a 1946 bp-long pairwise alignment. Positions 1423-1522 of this alignment concentrate the majority of nucleotide differences detected in the entire gene (Table 2).

ITS-2 rDNA: ITS-2 sequences were obtained from two cloned specimens of *Cx. theileri* from Tenerife and Valencia populations and deposited in the GenBank under the following accession numbers: JN051384-JN051386 and JN051387. The length and GC content of the ITS-2 sequences were 313-317 bp long (mean, 315.5 bp) and 58.60-59.62% (59.09%) respectively, for the different clones obtained. ITS-2 sequence heterogeinity was evaluated in a multiple sequence alignment (320 bp-long) showing a 7.50% of divergence, of which true mutations represent a 4.37% and indels a 3.12%. Polymorphic sites between sequences are concentrated between positions 225–245

```
                            112222222222222222333333
                            352222333333344444600011
                            405680236789013456345134

Cx. theileri, Tenerife      AA--CGTGTGTGCTCTGA---A--
Cx. theileri, Tenerife      GGTC.-.CCTG--.TGTGAAGGGA
Cx. theileri, Tenerife      ..TC.-.CCTG--.TGT.AAGGGA
Cx. theileri, Valencia      ..ACT.C..CG.TGTGT.---.--
```

Numbers (to be read in vertical) refer to positions obtained in the alignment . = Identical, - = insertion/deletion.

Table 3 : Polymorphic sites (n = 24), including parsimony informative, singleton sites and gapped or ambiguous characters, detected in the ITS-2 rDNA sequence alignment of the *Cx. theileri* populations compared according to MEGA 5.0.

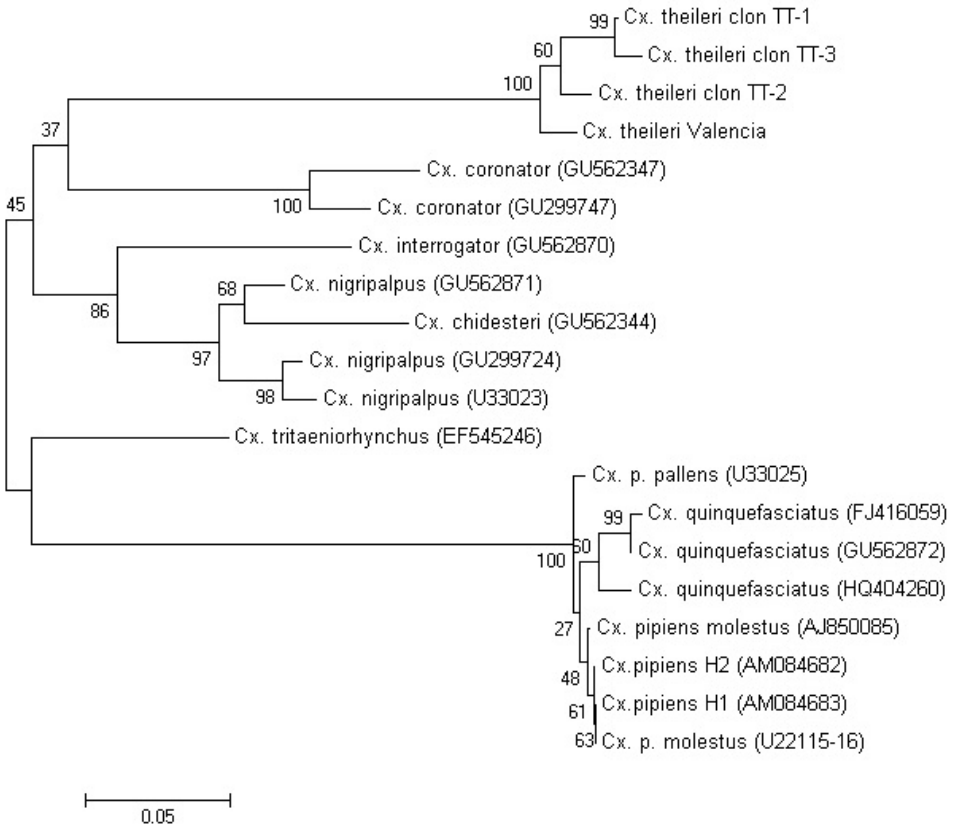

Figure 1: Phylogenetic tree using rDNA ITS-2 data set nucleotide sequences according to MEGA 5, including the *Cx. theileri* populations studied, together with other proximal *Culex* species available in GenBank. Evolutionary relationships of taxa were calculated using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

	Nt	Aa
	1134556	1
	7834275	7
	1001694	6
Cx. theileri cox1-a (Tenerife)	ATGTGTC	V
Cx. theileri cox1-b (Gran Canaria)	.C.....	.
Cx. theileri cox1-c (Valencia)	TT.A.CT	.
Cx. theileri isolates NHM11527-28 (Iran)*	.TAA..T	.
Cx. theileri isolate NHM11529 (Iran)**	.TAAA.T	M

* FJ210898, FJ210899; ** FJ210900

Table 4: Nucleotide (Nt) and amino acid (Aa) differences found in the mtDNA *cox*1 sequence of *Cx. theileri* from Spain (Canary Islands and Valencia) and *Cx. theileri* haplotypes available in GenBank. Position = numbers (to be read in vertical) refer to variable positions obtained in the alignment made with MEGA 5.0. Identical = .; haplotype codes correspond to barcode region of sequences of the gene.

and 302-311 of their respective alignment, in which ones, variable nucleotide tandem repeats are frequent (Table 3). Worth mentioning is the very high number of nucleotide differences detected in the pairwise comparisons of the ITS-2 sequences of *Cx. theileri* with other ITS-2 complete sequences of *Culex* species, available in GenBank. The ITS-2 dataset distance matrix obtained with PAUP shows that the number of total and mean character differences between *Cx. theileri* and the other species considered are considerably high in all cases (Figure 1).

mtDNA *cox*1: Three haplotypes were detected in specimens and populations analyzed, being identical in length (682 bp) and AT content (69.65%) The two populations from Tenerife present identical *cox*1 sequences, and were described as haplotype *Cx.theileri-cox*1a. This haplotype share a 99.85% of identity when compared with the *cox*1 haplotype from Gran Canaria (*Cx. theileri-cox*1b), as a result of only one mutation (C/T) in position 180 of their respective alignment. The haplotype sequence from Valencia (*Cx. theileri cox* 1-c) differ at

five variable positions when compared with the haplotypes (*cox*1-a and *cox*1-b) from Canary Islands (Table 4). All of these three new *Cx. theileri* haplotypes have been deposited in GenBank under the codes *Cx.theileri-cox*1a (JN051388), *Cx.theileri-cox*1b (JN051389) and *Cx.theileri-cox*1c (JN051390). When comparing the three *cox*1 haplotypes of *Cx. theileri* from Canary Island and Valencia with the other two *cox*1 haplotypes of *Cx. theileri* from Iran (FJ210898-FJ210900; [9], they proved to be very similar, differing in only 7 variable positions (1.03%) (Table 4). In the amino acidic sequence alignment (227 aa long) between the five *Cx. theileri cox*1 haplotypes compared, only one amino acid change (V/M) in position 127 is observed and originated by the isolate NHM11529 from Iran (Table 4).

Discussion

Heartworm disease is a vector borne transmitted parasitosis that affects dogs, cats and humans living in endemic area all over the world. Thus, the identification of the mosquito vector species involved in the transmission in such endemic area is a key feature to a correct understanding of the transmission dynamics in both animal reservoirs and human hosts. Tenerife and Gran Canaria (Canary Islands) have been reported as hyperendemic áreas for dirofilariosis with high canine prevalences [16,17]. Moreover the risks for humans have been demonstrated by the detection of specific anti-*D. immitis* antibodies in resident of the both islands [42,17].

In Spain there is only one previous work in which the DNA of *D. immitis* has been identified in a culicid mosquito species, *Cx. pipiens* haplotype H1 [25], which is not present in the Canary Islands, considering that its real vectorial competence has been recently demonstrated in other two endemic areas, the nearby island of Madeira [8] and Irán [9] we can argue that *Cx. theileri* is the, or one of the culicid vector species involved in the transmission of *D. immitis* in the canine population of the Canary Islands. Mosquitoes were captured in traps with human baits as attractant and maintained alive for 5 days in the laboratory. It has been demonstrated that insect defence mechanisms against *Dirofilaria* larvae are efficient only on recently ingested microfilariae or on those that have penetrated the primary cells of the Malpighian tubules (Vegni-Talluri & Cancrini, 1994).

Molecular techniques applied to different parts of the mosquito anatomy permit us to discriminate between species which act as potential vectors (those that contain DNA of *D. immitis* only in the abdomen) and those that really transmmit the parasite, whose DNA is detected in the cephalic region after the full development of the L3. The fact that only one infected mosquito has been identified is not a surprising fact, because most of the epidemiological findings on *D. immitis* vectors reveal very low prevalences [25,43,44]. Nevertheless we cannot ruled out that most abundant captures, both of *Cx. theileri* and other culicid species could reveal higher prevalences like other studies have demonstrated (to 8.6% have been cited in different mosquito species have demonstrated [8, 9,45-47].

Although *D. immitis* poses a problem for Canary Islands, there is no data related to potential mosquito vectors of this parasite until today. A molecular identification of *Cx. theileri*, the potential mosquito vector of dirofilariosis in this zoonotic focus in Canary Islands of Spain, has been made for first time based on sequences of the 18S rRNA gene, the second internal transcribed spacer (ITS2) of ribosomal DNA and the barcode region of the cytochrome c oxidase I (*cox*1) gene of mitochondrial DNA, allowing a broad mosquito molecular basis for future populations genetic analyses of this vector species. Nuclear rDNA and mtDNA sequences furnish appropriate markers to clarify the systematics and classify specimens and haplotypes even in particularly confusing insect groups. Recent analyses on the usefulness of the molecular markers offered by DNA in different organism groups have shown that (i) rDNA markers are the appropriate targets when dealing with systematic-taxonomic and phylogenetic aspects as well as for molecular characterization of species by haplotyping, (ii) mtDNA markers are more convenient for population and intraspecific variability studies, and (iii) both rDNA and mtDNA markers may be used for the classification and haplotyping of specimens [48,49].

The 18S rRNA gene lenght and AT content obtained for *Cx. theileri* (1935 bp and 54.6 %) are similar in length range (1821-1952 bp) and slight AT bias to other Culicinae [21,40,50] and Culicomorpha in general [40]. The lack of variation detected between geographically distant populations of *Cx. theileri* analyzed is in correspondence to a gene which evolves very slowly [48]. Its usefulness may be applied to the levels of subfamily, tribe, genera, subgenera, series, group, subgroup, complexes and species distinction [21,48]. Althought the complete sequence of this gene have been obtained, a region between positions 1423 and 1476, corresponding to the highly informative variable region V7, contained the majority of nucleotide differences, as previously detected in the complete sequences of other Culicid species [21,40].

The ITS-2 rDNA is even the usual key marker for the differentiation of problematic taxa, as in cryptic and sibling species of mosquitoes [22-25,51,52]. The length and GC content of this spacer for *Cx. thieleri* (315.5 bp and 59.09 %, in average) fit with the length and GC % obtained in other *Culex* species (271-339 bp and 52.8-57.5%) [24,51-53]. The intra-specific variability observed in the ITS-2 sequences of *Cx. theileri* in Canary Islands are similar to the heterogeneity observed between sequences of the same individual in different populations of the *Cx. pipiens* complex [54], as well as with the heterogeneity observed in individuals of the same species of *Culex* and *Lutzia* from Brazil [53].

The results obtained with the barcode region of the mtDNA sequenced corroborate their usefulness for mosquito species and population identification. Three *Cx. theileri cox*-1 haplotypes were reported for first time in Spain, two for Canary Island and one for Valencia. Haplotype *cox*1-a is present in the endemic zone of Tenerife, where positive mosquito female was detected. The two *cox*1a and *cox*1b haplotypes described from Canary Islands (Tenerife and Gran Canaria, respectively) were 99.85 % similar and in comparison with the haplotype *cox*1c detected in Valencia, differences increase only up to 0.73%. The sequence comparison analyses performed with the only sequences available of this species from Iran, allow us to identify specific mutations able to discriminate between Spanish and Iranian populations of this vector species. The nucleotide sequence divergence in insect mitochondrial DNA is approximately 2% per million years [55] which is mostly due to silent changes. Differences in mtDNA cox1 gene between our European and Asiatic populations of *Cx. theileri* are 1.03 %, which correspond to 515.000 years of divergence.

In conclusion, in spite of, like in other endemic areas, a very low number of individuals of *Cx. theileri* (a specimen) has been found infected with *D. immitis* larvae in the abdomen, some evidences support that *Cx. theileri* can acts as potential vector of *D. immitis* in the Canary Islands. These are:

1) The high molecular similarity observed between *Cx. theileri* from the Canary Islands and those of Iran (whose vectorial competence for *D. immitis* has been demonstrated)

2) The vectorial activity of this mosquito species in the nearby island of Madeira and

3) Its marked zooantropophilic behaviour.

The Canary Islands have a semitropical climate, which is influenced by the Atlantic trade winds and their proximity to the western Sahara. For this reason, this finding, together with the report in Madeira island [8], implies a dirofilariosis transmission complexity in Southern Europe markedly higher than that considered so far. However, higher vector potential of a mosquito species in a region or in different regions could be elucidated by ecological factors such as geographical features, season a climate, and also host preference of mosquito species [56].

Acknowledgements

Study supported by Projects Nos. GOCE-2003-010284 (EDEN, European Commission, Brussels), MOSQIMAC-MAC/2/M063 (Madeira-Azores-Canarias (MAC) Transnational Cooperation Program, FEDER), Red de Investigación Colaborativa en Enfermedades Tropicales RICET (RD06/0021/0017 of the RETICS Program), FIS, Spanish Ministry of Health, Madrid, and GVACOMP2007-294 (Generalitat Valenciana), Valencia, Spain.

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This article was originally published in a special issue, **Epidemiology and Ecology of Parasitic Diseases** handled by Editor(s). Dr. Joseph W. Camp, Purdue University, USA