

RESEARCH

Open Access



First molecular characterization of *Dirofilaria immitis* in Cuba

Lisset Roblejo-Arias¹, Cristian Díaz-Corona¹, Elianne Piloto-Sardiñas^{1,2}, Adrian A. Díaz-Sánchez³, Zbigniew Zajac⁴, Joanna Kulisz⁴, Aneta Woźniak⁴, Sara Moutailler², Dasiel Obregon⁵, Angélique Foucault-Simonin², Belkis Corona-González^{1*} and Alejandro Cabezas-Cruz^{2*}

Abstract

Background Dirofilarioses are widespread diseases caused by mosquito-borne nematodes of the family Onchocercidae, genus *Dirofilaria*. The major etiologic agent of canine dirofilariosis in the American continent is the zoonotic parasite *Dirofilaria immitis*. Existing reports of filarioid nematodes in Cuba are based solely on morphological and immunological analysis which do not allow unambiguous identification and/or direct detection of causal agents.

Results Here we present the molecular characterization of filarioid nematodes found in a dog in Cuba. Based on the molecular and phylogenetic analysis of the 5.8S-ITS2-28S region and *cox1* gene fragments, the worms were unambiguously classified as *D. immitis*. Sequence analysis showed high identity of the gene fragments in this study with others previously obtained from *D. immitis* found in dogs, wolves and jackals but also from mosquito vectors of *D. immitis*.

Conclusions Further studies are guarantee to better understand the epidemiological impact of canine dirofilariosis in Cuba as well as the competence of different species of culicid mosquitoes as vectors of *Dirofilaria* in the country.

Keywords Zoonosis, *Dirofilaria immitis*, Canine filariae, Dog, Cuba

Background

Dirofilariosis is a mosquito-borne disease with worldwide distribution caused by nematodes of the genus *Dirofilaria*, family Onchocercidae. Filariae of the *Dirofilaria* genus can infect wild and domestic animals of several orders including Rodentia, Artiodactyla, Perissodactyla, Carnivora, Lagomorpha, Edentata, and Primates [1]. About 60–70 mosquito species of the Culicidae family are considered as intermediate hosts and/or vectors of *Dirofilaria* worldwide [2].

Dirofilaria repens and *Dirofilaria immitis* are two *Dirofilaria* species of special interest due to their negative impact on company animals (i.e., dogs and cats) as well as their zoonotic potential [3, 4]. The final hosts of both parasite species are mainly canine predators including dogs, wolves (*Canis lupus*), foxes (*Vulpes vulpes*), and

*Correspondence:

Belkis Corona-González

bcorogonz@gmail.com

Alejandro Cabezas-Cruz

alejandrocabezas@vet-alfort.fr

¹Direction of Animal Health, National Center for Animal and Plant Health, Carretera de Tapaste y Autopista Nacional, Apartado Postal 10, San José de las Lajas, Mayabeque 32700, Cuba

²Laboratoire de Santé Animale, ANSES, INRAE, Ecole Nationale Vétérinaire d'Alfort, UMR BIPAR, Maisons-Alfort, France

³University of Saskatchewan, Saskatchewan, Canada

⁴Department of Biology and Parasitology, Medical University of Lublin, Radziwiłłowska 11st, Lublin 20-080, Poland

⁵School of Environmental Sciences, University of Guelph, Guelph, ON, Canada



© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

jackals (*Canis aureus*), but cats and weasels (*Mustela nivalis*), can also be infected [5]. Originally, dirofilariasis was considered a disease of strict veterinary importance [6]. However, it is currently recognized as an emerging zoonosis [6]. Clinical symptoms of dogs infected by *D. immitis* include respiratory distress, epistaxis, haemoptysis, ascites, exercise intolerance, and anorexia [2, 7]. However, most animals infected with *D. immitis* are asymptomatic or display no abnormalities in different laboratory tests [8]. Several parasitological, serological, and molecular tests are available for the detection of *D. immitis* with varying levels of sensitivity and specificity [9–11]. Microscopic analysis tests, such as blood smears from peripheral veins and capillaries [12], and the modified Knott test (concentration method) are used to detect circulating microfilariae [13] and considered quick and low-cost. However, the specificity and sensitivity of filarioid species identification with these tests are low sensitivity for people with no or poor experience in the diagnosis [14].

The diagnostic tests based on molecular methods have high sensitivity, and allow for filarioid differentiation [14, 15]. In addition, molecular methods can detect low parasitemia in infected animals given a more realistic picture of the parasite prevalence [14, 15]. The Polymerase Chain Reaction (PCR) is currently being recommended as a species-specific test in the detection of *D. immitis* [10, 16], however the combination of different diagnostic tests is an important element in epidemiological studies addressing the detection of *D. immitis* [17, 18].

Several species of *Dirofilaria* including *D. acutiuscula*, *D. striata*, *D. immitis* and *D. repens* have been reported as causing infection in dogs in the Americas [19–21], but *D. immitis* is the most important causative agent

of canine dirofilariasis in the continent [22]. In North America, the prevalence of *D. immitis* in domestic dogs has been estimated to range from 1 to 12% [19], while in Central and South America, the prevalence is much higher, reaching 42% in cities on the Gulf Coast of Mexico, 63.2% in the Caribbean, 45% in Brazil, and 74% in Argentina [20, 23].

Dirofilaria immitis was reported in Cuba in 1977 and in the 80's several reports were published on the presence of this parasite in the country [24–27]. In all these reports, the identification of the parasite was achieved by morphological and immunological analysis [24–27]. In addition, in 1992, the presence of *D. immitis* was reported in a dog using a coagglutination assay [28]. However, to the authors' knowledge, no molecular characterization of *D. immitis* strains circulating in Cuba is available. Therefore, the aim of our study was to describe the morphological and molecular characterization of filarioid nematode collected from an infected dog in Cuba. Sequence and phylogenetic analyses showed to the presence of a *D. immitis* strain similar to that reported in other canids and mosquito vectors of *D. immitis*.

Results

Clinical examination and microscopic evaluation

No clinical sign or symptoms indicative of diseases were observed in the dog. However, microscopic observation of Giemsa-stained blood smear revealed the presence of Microfilariae. The morphological examination of the microfilariae showed unsheathed microfilaria and structures such as the cephalic space, nerve ring, excretory pore, anal pore, and the terminal nucleus at the tail were identified (Fig. 1), which allowed assigning the worm to

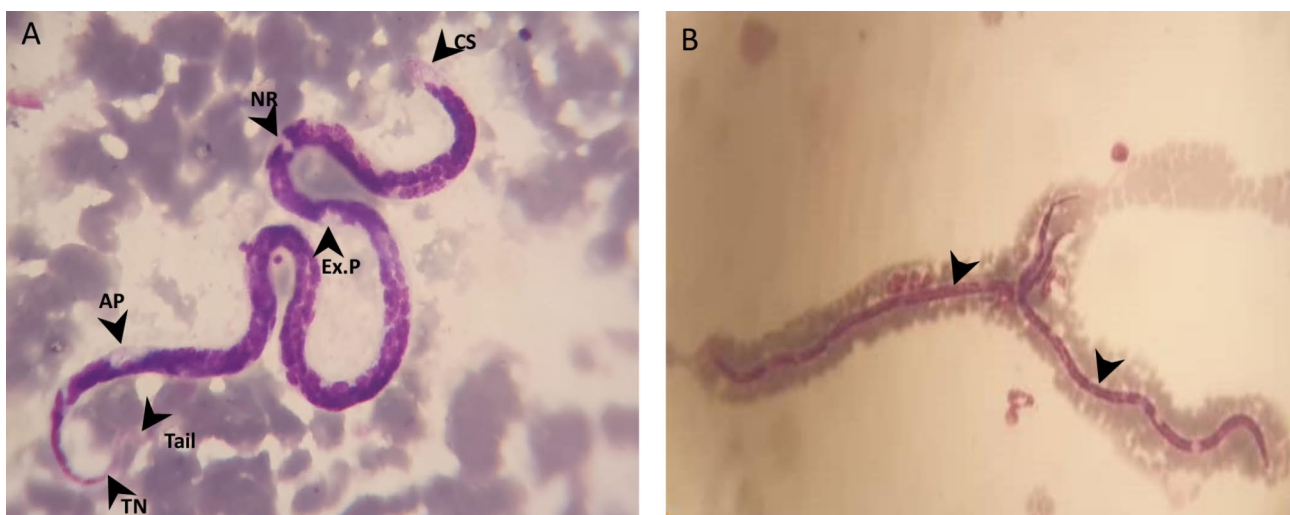


Fig. 1 Microscopic observation of microfilaria. **(A)** Microfilaria of the genus *Dirofilaria* identified on Giemsa-stained thin blood smears from a dog. The morphological marks show the position of several structures. CS: cephalic space; NR: nerve ring; Ex.P: excretory pore; AP: anal pore; TN: terminal nucleus. (1000x magnification). **(B)** Two distended microfilariae of the genus *Dirofilaria* (arrow) (100x magnification)

the genus *Dirofilaria*. The dog had never been tested for heartworm or administered heartworm prophylactics.

PCR results

The pan-filarioid primer pair targeting the 5.8 S ribosomal RNA gene and internal transcribed spacer 2 (5.8S-ITS2-28S) rRNA region amplified a PCR amplicon at size corresponding to *D. immitis* DNA (542 bp) (Fig. 2A). On the other hand, a 150 bp PCR product was amplified using the *D. immitis* *cox1*-specific primers (Fig. 2B). The nucleotide sequences originating from the 5.8S-ITS2-28S region obtained in the present study was submitted to GenBank (accession number OQ784647). Due to short fragment size, GenBank did not allow the deposition of the amplified *cox1* fragment, which can be provided upon request.

Phylogenetic analysis

Phylogenetic analysis of 5.8S-ITS2-28S rRNA region (Fig. 3A) and *cox1* gene fragment (Fig. 3B) placed sequences obtained in the current study together with other *D. immitis* sequences available in GenBank. Obtained fragment of 5.8S-ITS2-28S rRNA region clustered together with previously reported sequences of *D. immitis* collected from its primary hosts, i.e., canine *Canis lupus familiaris* (Malaysia MW019915, Brazil KX93211, Iran JX889636), and *Vulpes vulpes* (Bulgaria MN596213). Analysed sequence showed also similarity to microfilariae of this species collected from insects of Ctenocephalides family (MW019916). Similarly, sequence of *cox1* gene clustered with other *D. immitis* collected from canine, e.g., *C. lupus familiaris* (Thailand MT027229, Slovakia OQ726920), *Vulpes zerda* (USA

MN945948); its primary vector *Culex quinquefasciatus* (Myanmar OL721654) and accidental host, e.g., human (Iran MH920260).

Discussion

Dirofilariosis is an emerging parasitic infection of growing concern in the world [29]. Canine heartworm, caused by *D. immitis*, has a wide distribution in Latin America [30], and the Caribbean region in Turk and Caicos Islands [31], Curacao [32], Grenada [33], St Kitt [34], Haiti [35], Dominican Republic [36] and Puerto Rico [37] and in island environments outside America [34]. In Cuba, the first reports were made from 1977 to 1992 using microscopic examination and coagglutination assay [24–28], and as far as the authors know, no other reports in animals or humans have been published.

The gold standard test for the diagnosis of microfilariae is the microscopic examination of blood smear stained with Giemsa or hematoxylin and eosin [38]. However, microscopic examination has low sensitivity, cannot clearly discriminate among closely related species of filarioid nematodes (e.g., *D. immitis*, *D. repens*, and *D. reconditum* or *Brugia malayi* and *Brugia pahangi*), and requires considerable expertise [39]. Here, we provided morphological and molecular evidence of the presence of *D. immitis* in a dog from Cuba. Until now, no studies combining morphological and molecular diagnosis of canine microfilariae in Cuba have been published.

Sequence analysis of 5.8S-ITS2-28S rRNA and *cox1* gene fragments showed high identity with sequences obtained previously from *D. immitis* parasites found in dogs, wolfs and jackals [5, 6] as well as mosquito vectors of this nematode [2]. Low genetic diversity between

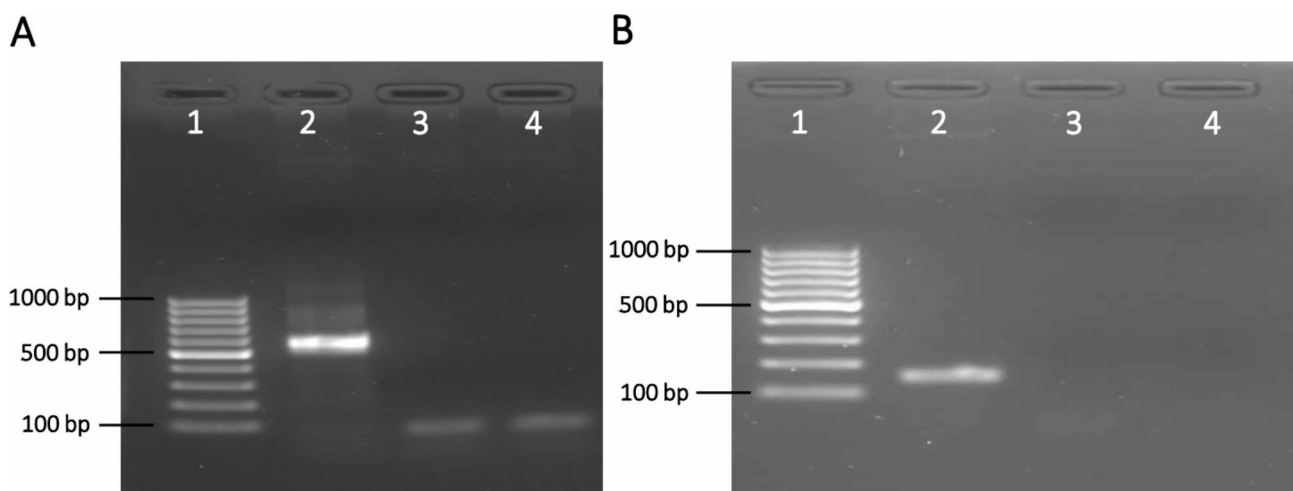


Fig. 2 Gel electrophoresis of PCR products amplified using filarioid-specific primers. **(A)** Amplification of PCR products using filarioid-specific 5.8S-ITS2-28S region primers on a 1.5% agarose gel. Lane 1: GeneRuler 100 bp Plus DNA Ladder; lane 2: dog sample DNA; lane 3: negative control; and lane 4: water control. **(B)** Amplification of PCR products using primers for the cytochrome oxidase subunit 1 (*cox1*) fragment specific to *D. immitis* on a 2% agarose gel. Lane 1: GeneRuler 100 bp Plus DNA Ladder; lane 2: dog sample DNA; lane 3: negative control; and lane 4: water control. The original photograph of the gel electrophoresis is available as Supplementary Figure S1

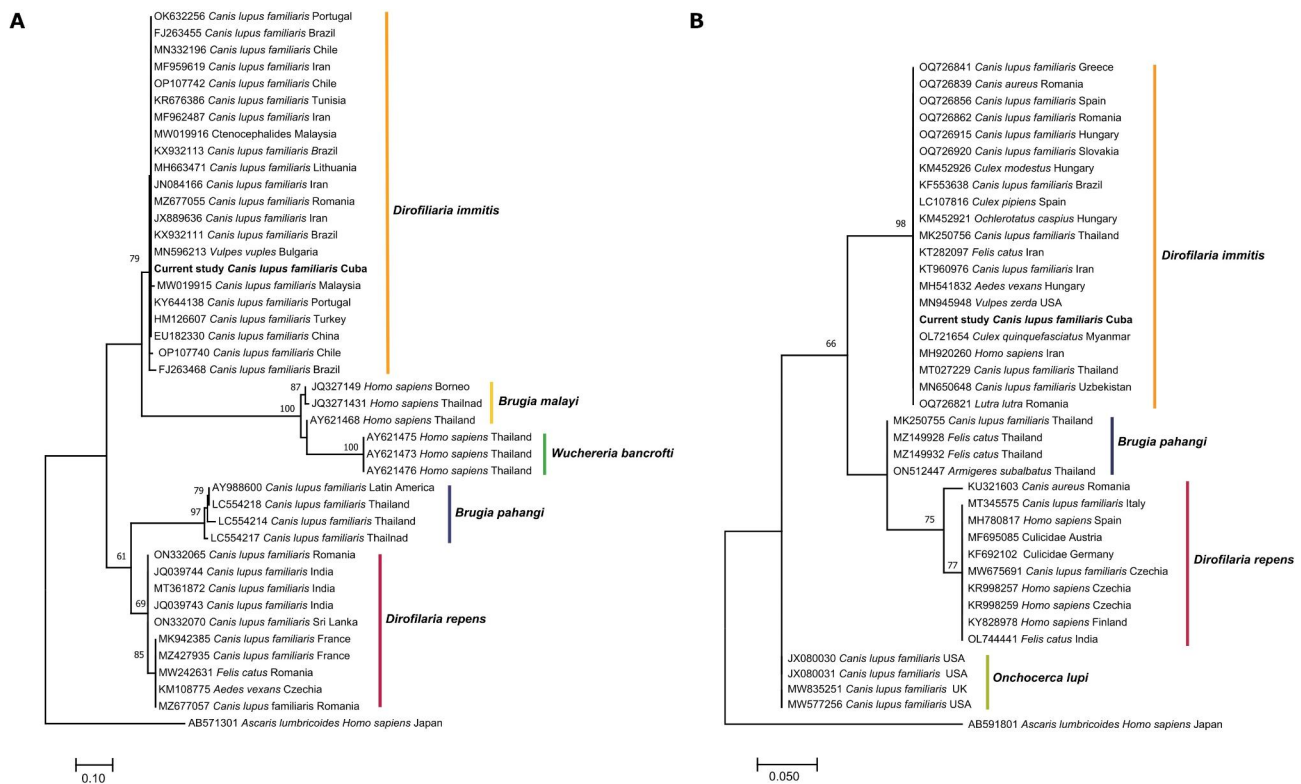


Fig. 3 Phylogenetic tree of selected representatives of Onchocercidae. **(A)** Phylogram representing analysis of the 5.8S-ITS2-28S rRNA region. The evolutionary history was inferred with maximum likelihood method and Tamura 3-parameter (T92) model. Analysis contains sequences uploaded from GenBank (with accession numbers and host) and obtained in the current study (in bold). Bootstrap values are represented as per cent of internal branches (1000 replicates), values lower than 60 are hidden. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Ascaris lumbricoides* (AB571301) was used to root the tree. **(B)** Phylogram representing analysis of *cox1* gene. The evolutionary history was inferred with maximum likelihood method and Tamura 3-parameter (T92) model. Analysis contains sequences uploaded from GenBank (with accession numbers and hosts) and obtained in the current study (in bold). Bootstrap values are represented as per cent of internal branches (1000 replicates), values lower than 60 are hidden. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Ascaris lumbricoides* (AB591801) was used to root the tree

sequences of *D. immitis* obtained in the current study and other sequences uploaded to GenBank data BLAST is supported by previously published reports [40–42] and may confirm stable maintenance and circulation of this parasite between its vectors and hosts in the environment. Moreover, this phenomenon can be also explained by potential infection of *Wolbachia* sp., common bacterial endosymbiont of *D. immitis* influencing its survival and reproduction rate [43] but additionally reducing genetic polymorphism in *Wolbachia*-infected species [44].

Conclusions

Our findings justify a better characterization of the epidemiology of *Dirofilaria* infection in dogs, wild animals, and humans in Cuba. The role of different species and/or strains of culicid mosquitoes as vectors of *D. immitis* in the country should also be evaluated. These studies are important due to the risk of introduction of non-endemic *Dirofilaria* species or strains in the country. Additionally, veterinarians should be aware of the possibility that *D.*

immitis infection might be an emerging condition with zoonotic potential in Cuba.

Methods

Clinical examination

A one-year-old intact male mixed-breed dog born in Cuba and that had not left the country, underwent a routine examination by a local veterinary. The clinical inspection included anamnesis and clinical evaluation. The veterinarian performed a thorough physical examination that included several aspects such as body temperature recording, evaluation of mucous membranes, pulse assessment, hydration status, capillary refilling time, and manually checking the animal for skin lesions and tick infestation. Moreover, the evaluation involved observing the dog's behavior, gait, and coordination, along with a neurologic examination the respiratory system was examined for symptoms. During the examination, the veterinarian searched for any signs or symptoms of dirofilariosis. The anamnesis was performed to gather information regarding the dog's medical history,

Table 1 Primer sequences used in this study

Primer names	Primer sequences	Targeted DNA	References
DIDR-F1	5' AGTGCGAATTGCAGACGCATTGAG 3'	5.8S-ITS2-28S rRNA region	[46]
DIDR-R1	5' AGCGGGTAATCACGACTGAGTTGA 3'		
DI-F1	5' ATGGGGTGCCCTGAAATGG 3'	<i>cox1</i> gene	[47]
DI-R1	5' CCCTCTACACTCAAAGGAGGA 3'		

including previous illnesses, surgeries, and treatments. Any abnormalities or concerns detected were documented and addressed accordingly.

Sample collection

A blood sample was aseptically drawn from the jugular vein using sterile Vacutainer needles and EDTA tubes (Becton-Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) and maintained at 4 °C within 24 h of blood collection until further analysis.

Microscopic evaluation

A thin blood smear was prepared, stained with Giemsa solution (Merck, Boston, MA, USA) and examined under a light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) at final magnifications of 100X and 1000X. Morphological identification was performed using keys previously reported [45].

DNA extraction and polymerase chain reaction

Total nucleic acid was extracted from 300 µL of EDTA-anticoagulated blood sample using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. A negative control was set in which DNA extraction was carried out using 300 µL phosphate-buffered saline (PBS) (Sigma, St. Louis, MO, USA) instead of blood. The quantitative and qualitative evaluation of nucleic acid extraction was determined using a Colibri Microvolume Spectrophotometer (Titertek-Berthold, Pforzheim, Germany). The extracted nucleic acid sample was stored at 20 °C until further use.

PCR reactions were carried out with species-specific primers to amplify fragments of 5.8S-ITS2-28S rRNA [46] and cytochrome c oxidase subunit 1 (*cox1*) [47] (Table 1). Each PCR reaction consisted of 1X Phusion HF Buffer (Thermo Scientific), 200 µM dNTPs, 0.5 µM each primer, 0.02 U/µL Phusion DNA polymerase (Thermo Scientific) and 5 µL of DNA solution in a total volume of 20 µL. The program for 5.8S-ITS2-28S rRNA fragment amplification consisted of a denaturing step at 98 °C for 30 s and 35 cycles of denaturing (10 s at 98 °C), annealing (30 s at 60 °C) and extension (30 s at 72 °C), a final extension (10 min at 72 °C) and a soak at 4 °C. The program for *cox1* gene fragment amplification consisted in a denaturing step at 95 °C for 60 s, 35 cycles of denaturing (20 s at 95 °C), annealing (20 s at 60 °C) and extension

(40 s at 72 °C), a final extension (10 min at 72 °C) and a soak at 4 °C. An Eppendorf Mastercycler Nexus Gradient (Eppendorf, Hamburg, Germany) was used for the PCR reactions. The molecular size of the PCR amplicons was examined on a 1.5% agarose gel for 5.8S-ITS2-28S rRNA and 2% agarose gel for *cox1* gene, using a GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific). Ethidium bromide was used as DNA-staining agent, visualized under UV light. Amplicon sequencing was commissioned to Eurofins MWG Operon (Ebersberg, Germany).

Phylogenetic analysis

In order to determine species identity and genetic diversity of filarioid nematodes collected in the current study, obtained sequences of 5.8S-ITS2-28S rRNA and *cox1* gene were trimmed manually in BioEdit software v.7.2 [48], analyzed in GenBank database through the National Center for Biotechnology Information (NCBI; Bethesda, MD) and searched against Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 17 April 2023). Next, sequences were aligned using MUSCLE algorithm available in MEGA X. Phylogenetic trees were constructed using maximum likelihood (ML) method and Tamura 3-parameter (T92) model, according to the lowest Bayesian Information Criterion (BIC) and Akaike information criterion corrected for small sample sizes (AICc) [49]. Reliability of internal branches was assessed using the bootstrapping method with 1000 replicates.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12917-023-03803-0>.

Supplementary Material 1: Supplementary Figure S1 original photograph of the gel electrophoresis of Filarioid PCR products. (A) Amplification of PCR products using filarioid-specific 5.8S-ITS2-28S region primers on a 1.5% agarose gel. Lane 1: GeneRuler 100 bp Plus DNA Ladder; lane 2: dog sample DNA; lane 3: negative control; and lane 4: water control. (B) Amplification of PCR products using primers for the cytochrome oxidase subunit 1 (*cox1*) fragment specific to *D. immitis* on a 2% agarose gel. Lane 1: GeneRuler 100 bp Plus DNA Ladder; lane 2: dog sample DNA; lane 3: negative control; and lane 4: water control. Lanes 5 to 10 in both panels (A and B) are not relevant to the current study.

Acknowledgements

Not applicable.

Authors' contributions

LRA: Investigation, Writing - Review & Editing; CDC, EPS, AADS: Writing - Review & Editing; ZZ, JK, AW, Visualization, Formal analysis, Writing - Original

Draft, Writing - Review & Editing; SM: Resources, Writing - Review & Editing; DO: Writing - Review & Editing; AFS: Investigation, Data Curation, Writing - Review & Editing; BCG: Conceptualization, Resources, Visualization, Writing - Original Draft, Writing - Review & Editing; ACC: Conceptualization, Resources, Writing - Original Draft, Supervision, Writing - Review & Editing.

Funding

The research received no specific funds.

Data Availability

All data generated or analysed during this study are included in the article.

Declarations

Ethics approval and consent to participate

Written informed consent was obtained from the owner for the participation of their dog in this study. Veterinarians examined dog with the assistance and acceptance of their owner. The committee on ethics and animal welfare at CENSA approved the experimental design of this research. The National Center of Animal Health of Cuba authorized the export of the DNA sample used in this work, under the Zoo-Sanitary Export Certification, Number R.S:04252023. All the methods used were in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

Received: 6 May 2023 / Accepted: 1 November 2023

Published online: 17 November 2023

References

- Pampiglione S, Rivasi F. The species of the genus *Dirofilaria*, Railliet and Henry, 1911. *Parassitologia*. 1997;39(4):369–74.
- McCall JW, Genchi C, Kramer LH, Guerrero J, Venco L. Heartworm Disease in animals and humans. *Adv Parasitol*. 2008;66:193–285.
- Orihel T, Eberhard M. Zoonotic filariasis. *Clin Microb Rev*. 1998;11:366–81.
- Genchi C, Simón F, Kramer L. *Dirofilariosis* in humans: is it a real zoonotic concern? Proceedings of the 30th World Congress of the World Small Animal Association (2005). <http://www.wsava2005.com>.
- Otranto D, Deplazes P. Zoonotic nematodes of wild carnivores. *Int J Parasitol Parasites Wildl*. 2019;9:370–83. <https://doi.org/10.1016/j.ijppaw.2018.12.011>.
- Pampiglione S, Rivasi F, *Dirofilariosis* MW, Service, editors. The Encyclopedia of Arthropod-transmitted Infections. CABI Publishing; 2001. pp. 143–50.
- Taylor MA, Coop RL, Wall RL. *Helmintologia veterinária. Parasitologia veterinária*. 4th ed. Rio de Janeiro: Editora Guanabara Koogan (2017):65–478.
- Dantas-Torres F, Otranto D. *Dirofilariosis* in the Americas: a more virulent *Dirofilaria immitis*? *Parasit Vectors*. 2013;6:288. <https://doi.org/10.1186/1756-3305-6-288>.
- Ferreira C, Afonso A, Calado M, Maurício I, Alho AM, Meireles J, Carvalho LM, Belo S. Molecular characterization of *Dirofilaria* spp. circulating in Portugal. *Parasites Vectors*. 2017;10(1):1–8.
- Moreira HR, Madeira EAO, Cunha DNL, Scofield A, Góes-Cavalcante G, Abel I, Guimaraes RJP, Fernandes JI. *Dirofilaria immitis* Infection in dogs in Algodão Island, Brazilian Amazon. *Pesqui Vet Bras*. 2019;39(7):510–5. <https://doi.org/10.1590/1678-6160-pvb-5916>.
- Trancoso TAL, Lima NC, Barbosa AS, Leles D, Fonseca ABM, Labarthe NV, Bastos OMP, Uchoa CMA. Detection of *Dirofilaria immitis* using microscopic, serological, molecular techniques among dogs in Cabo Frio, RJ. *Braz Journ Vet Parasitol*. 2020;29:1. <https://doi.org/10.1590/s1984-29612020009>. Brazil.
- Pastrav IR, Ionica AM, Pesteau C, Novakova E, Modry D, Mihalca AD. Peripheral venous vs. capillary microfilaraemia in a dog co-infected with *Dirofilaria repens* and *D. immitis*: a comparative approach using triatomine bugs for blood collection. *Vet Parasitol*. 2018;257:54–7. <https://doi.org/10.1016/j.vetpar.2018.05.017>.
- Newton MD, Wright LM. The occurrence of a dog filariid other than *Dirofilaria immitis* in the United States. *J Parasitol*. 1956;42:246–58.
- Kamyngkird K, Junsiri W, Witsanuwat C, Kengradomkij C, Saengow S, Sangchuto K, Kajeerum W, Pangjai D, Nimsuphan B, Inpankeaw T, Jittapalagong S. Prevalence and risk factors associated with *Dirofilaria immitis* infection in dogs e cats in Songkhla and Satun provinces, Thailand. *Agric Nat Res*. 2017;51:99302. <https://doi.org/10.1016/j.anres.2017.05.003>.
- Nuchprayoon S. DNA-based diagnosis of lymphatic filariasis. *South Asi J Trop Med Publ Heal*. 2009;40(5):904–13.
- Ramos RAN, Rego AGO, Firmino EDF, Ramos CAN, Carvalho GA, Dantas-Torres F, Otranto D, Alves LC. Filarioids Infection dogs in northeastern Brazil. *Vet Parasitol*. 2016;226:26–9. <https://doi.org/10.1016/j.vetpar.2016.06.025>.
- Genchi M, Rinaldi L, Venco L, Cringoli G, Vismarra A, Kramer L. *Dirofilaria immitis* and *D. repens* in dog and cat: a questionnaire study in Italy. *Vet Parasitol*. 2019;267:26–31. <https://doi.org/10.1016/j.vetpar.2019.01.014>.
- Paranese R, Iatta R, Mendoza-Roldan JA, Szlosek D, Braff J, Liu J, Beugnet F, Dantas-Torres F, Beall MJ, Otranto D. Comparison of diagnostic tools for the detection of *Dirofilaria immitis* Infection in dogs. *Pathogens*. 2020;9:499. <https://doi.org/10.3390/pathogens9060499>.
- Simón F, Siles-Lucas M, Morchón R, González-Miguel J, Mellado I, Carretón E, Montoya-Alonso JA. Human and animal dirofilariosis: the emergence of a zoonotic mosaic. *Clin Microbiol Rev*. 2012;25:507–44.
- Vezzani D, Carbajo AE, Fontanarrosa MF, Scodellaro CF, Basabe J, Cangiano G, Eiras DF. Epidemiology of canine heartworm in its southern distribution limit in South America: risk factors, inter-annual trend and spatial patterns. *Vet Parasitol*. 2011;176:240–9. <https://doi.org/10.1016/j.vetpar.2010.10.046>.
- Labarthe N, Guerrero J. Epidemiology of heartworm: what is happening in South America and Mexico? *Vet Parasitol*. 2005;133:149–56. <https://doi.org/10.1016/j.vetpar.2005.04.006>.
- Dantas-Torres F. Canine vector-borne Diseases in Brazil. *Parasites Vectors*. 2008;1(25):1–17. <https://doi.org/10.1186/1756-3305-1-25>.
- Lee AC, Montgomery SP, Theis JH, Blagburn BL, Eberhard ML. Public health issues concerning the widespread distribution of canine heartworm Disease. *Trends Parasitol*. 2010;26:68–173. <https://doi.org/10.1016/j.pt.2010.01.003>.
- Sotolongo GF. Incidencia De La *Dirofilaria immitis* en Los perros de la Ciudad De La Habana. *Rev Cubana Med Trop*. 1977;29(1):9–12.
- Duménigo RB, Aguiar PH, Galvez MD. Prevalencia De *Dirofilaria immitis* en perros de Ciudad De La Habana. *Rev Cubana Med Trop*. 1982;34(3):262–8.
- Pérez O, Lastre M, Aguiar PM, Gálvez M. Búsqueda De *Dirofilaria immitis* en perros en la provincia Ciudad De La Habana. *Rev Cubana Med Trop*. 1985;37(2):174–7.
- Duménigo RB, Espino AM, Bouza M. Prevalencia De Filariasis canina en la Isla De La Juventud. *Rev Salud Anim*. 1988;10:247–50.
- Rojas L, Dumenigo BE, Fonte L, Fachado A, Finlay CM. Identification of *Dirofilaria immitis*-infected dogs with a Coagglutination Assay. *J Parasitol*. 1992;78(1):166–8.
- Simón F, Morchón R, González-Miguel J, Marcos-Atxutegi C, Siles-Lucas M. What is new about animal and human dirofilariosis? *Trends Parasitol*. 2009;25(9):404–9.
- Maggi RG, Krämer F. A review on the occurrence of companion vector-borne Diseases in pet animals in Latin America. *Parasites & Vectors*. 2019;12(1):1–37.
- Hoff B, McEwen B, Peregrine AS. A survey for Infection with *Dirofilaria immitis*, *Ehrlichia canis*, *Borrelia burgdorferi*, and *Babesia canis* in feral and client-owned dogs in the turks and Caicos Islands, British West Indies. *Can Veterinary J*. 2008;49(6):593.
- Saleh FC, Kirkpatrick CE, de Haseth O, Lok JB. Occurrence of some blood and intestinal parasites in dogs in Curacao, Netherlands Antilles. *Trop Geogr Med*. 1988;40:318–21.
- Chikweto A, Bhaiyat MI, Lanza-Perea M, Veytsman S, Tiwari K, de Allie C, Sharma RN. Retrospective study of canine heartworm Disease with caval syndrome in Grenada, West Indies. *Vet Parasitol*. 2014;205:721–4.
- Conan A, Napier P, Shell L, Knobel DL, Dundas J, Scorpio D, Ketzis J. Heterogeneous distribution of *Dirofilaria immitis* in dogs in St. Kitts, West Indies, 2014–2015. *Veterinary Parasitology: Regional Studies and Reports*. 2017;10:139–42.
- Starkey LA, Newton K, Brunner J, Crowder K, Edourad EJP, Meneus P, Little SE. Prevalence of vector-borne pathogens in dogs from Haiti. *Vet Parasitol*. 2016;224:7–12.
- Duran-Struock R, Jost C, Hernandez AH. *Dirofilaria immitis* prevalence in a canine population in the Samana Peninsula (Dominican Republic)-June 2001. *Vet Parasitol*. 2005;133:323–7.

37. McCown ME, Opel T, Grzeszak B. Vector-borne Disease surveillance in Puerto Rico: pathogen prevalence rates in canines-implications for public health and the U.S. military-applying the One Health concept. *J Spec Oper Med*. 2013;13:59–63.
38. Ricciardi A, Ndao M. Diagnosis of parasitic Infections: what's going on? *J Biomol Screen*. 2015;20:6–21.
39. Nuchprayoon S, Junpee A, Poovorawan Y, Scott AL. Detection and differentiation of filarial parasites by universal primers and polymerase chain reaction-restriction fragment length polymorphism analysis. *Am J Trop Med Hyg*. 2005;73:895–900.
40. Sharifdini M, Karimi M, Ashrafi K, Soleimani M, Mirjalali H. Prevalence and molecular characterization of *Dirofilaria Immitis* in road killed canids of northern Iran. *BMC Vet Res*. 2022;18(1):161.
41. Godel C, Kumar S, Koutsovoulos G, Ludin P, Nilsson D, Comandatore F, et al. The genome of the heartworm, *Dirofilaria Immitis*, reveals drug and vaccine targets. *FASEB J*. 2012;26(11):4650–61. <https://doi.org/10.1096/fj.12-205096>.
42. Gomes-de-Sá S, Santos-Silva S, Moreira ADS, Barradas PF, Amorim I, Cardoso L, Mesquita JR. *Dirofilaria Immitis* antigenemia and microfilaremia in Iberian wolves and red foxes from Portugal. *Parasit Vectors*. 2022;15(1):119.
43. Belanger DH, Perkins SL. Wolbachia Infection and mitochondrial diversity in the canine heartworm (*Dirofilaria Immitis*). *Mitochondrial DNA*. 2010;21(6):227–33.
44. Cariou M, Duret L, Charlat S. The global impact of Wolbachia on mitochondrial diversity and evolution. *J Evol Biol*. 2017;30(12):2204–10.
45. Furtado AP, Melo FTV, Giese EG, dos Santos JN. Morphological redescription of *Dirofilaria Immitis*. *J Parasitol*. 2010;96(3):499–504.
46. Rishniw M, Barr SC, Simpson KW, Frongillo MF, Franz M, Dominguez JL. Discrimination between six species of canine microfilariiae by a single polymerase chain reaction. *Vet Parasitol*. 2006;135:303–14.
47. Oh IY, Kim KT, Sung HJ. Molecular detection of *Dirofilaria Immitis* specific gene from infected dog blood sample using polymerase chain reaction. *Iran J Parasitol*. 2017;12:433–40.
48. Hall T, Biosciences I, Carlsbad CJGGB. BioEdit: an important software for molecular biology. *GERF Bull Biosci*. 2011;2(1):60–1.
49. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol*. 2018;35(6):1547.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.