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RESEARCH

New insights on the phylogeography of *Hepatozoon canis* in Brazil

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Abstract

This study aimed to molecularly characterize the *Hepatozoon* spp. infecting domestic and wild dogs in Brazil. A total of 22 whole blood samples tested positive for *Hepatozoon* spp., and five samples were sequenced for the 18S rDNA gene from *H. canis* after PCR amplification with four primer sets. Phylogenetic analysis using Bayesian inference showed that the three *H. canis* isolates from domestic dogs were not monophyletic; however, they were more closely related to each other than to other *H. canis* sequences. The isolate from the hoary fox (*Lycalopex vetulus*) was phylogenetically more distant. Two haplotype networks were constructed, identifying 10 haplotypes of *H. canis* in Brazil, with H10 constituting the largest group. It contains nine isolates, including three from domestic dogs. The H5 haplotype grouped the sequence of *L. vetulus* with two additional sequences from hosts *Tapirus terrestris* and *L. vetulus*, representing the sole haplotype with wild hosts. Bayesian analysis suggested the possible existence of two genetic groups of *H. canis* in Brazil, indicating gene flow of this agent within the country. These findings contribute valuable insights for a more comprehensive understanding of the molecular diversity of *Hepatozoon* spp. in Brazil and may help in the development of effective control measures.

Keywords Phylogeny · Haplotypes · Domestic canids · Wild canids

Introduction

Hepatozoon spp. are hemoprotozoans that infect a range of animals, including domestic and wild carnivores. Among domestic dogs, two distinct species have been identified: *Hepatozoon canis* and *Hepatozoon americanum* (Mathew et al. 2000; Baneth et al. 2003). The transmission of these protozoa occurs when the intermediate hosts ingest the invertebrate definitive host carrying the agent's oocysts. *Rhipicephalus sanguineus* sensu lato, *Amblyomma ovale*, and *Rhipicephalus turanicus* are acknowledged biological vectors of *H. canis*, and *Amblyomma maculatum* is the vector for *H. americanum*. Intrauterine transmission, along with preying on or scavenging animals with infected ticks, is also a possible mode of infections (Baneth et al. 2007; Rubini et al. 2009; de Miranda et al. 2011; Baneth 2011; Giannelli et al. 2017; Baneth and Allen 2022). *H. canis* has also been reported in wild carnivore species including red foxes (*Vulpes vulpes*), gray wolves (*Canis lupus*), golden jackals (*Canis aureus*), black-backed jackals (*Canis mesomelas*), African wild dogs, coyotes (*Canis latrans*), tigers (*Panthera tigris*), raccoons (*Procyon lotor*), beech martens (*Martes foina*), and opossums (*Didelphis albiventris*) (Starkey et al. 2013; Baneth et al. 2013; Najm et al. 2014; Hodžić et al. 2015; da Silva et al. 2017; Battisti et al. 2020).

Infection with *H. canis* in dogs is usually subclinical or causes mild clinical signs; however, it can become severe and potentially fatal in some cases. Blood smear evaluation is an easy and practical diagnostic method, but molecular diagnosis is more appropriate due to its high sensitivity and specificity (Criado-Fornelio et al. 2006; Otranto et al. 2011; Modrý et al. 2017). In this study, we aimed to expand the fragments of the 18S rDNA gene of *H. canis* in Brazil using

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four pairs of oligonucleotides, to achieve a better molecular characterization of the agent in the country.

Despite the readily available information on the morphology and life cycle of *Hepatozoon* spp. and their respective hosts, the taxonomy of many species remains uncertain, primarily due to incongruent phylogenetic data. The molecular detection of *H. canis* has been documented in various studies worldwide, covering regions such as Africa, the Americas, Asia, and Europe (O'Dwyer et al. 2001; Allen et al. 2008; Li et al. 2008; Gabrielli et al. 2010; Kistler et al. 2014; Farkas et al. 2014). Nevertheless, the genetic diversity and phylogeography of these hemoparasites have received limited attention (Gabrielli et al. 2010; Najm et al. 2014). Current molecular data suggest that *H. canis* exhibits no discernible population genetic differentiation due to the extensive range of definitive hosts and the impact of human activities (Vásquez-Aguilar et al. 2021). Consequently, molecular characterization within the Brazilian context is of paramount importance for evaluating the genetic diversity of this pathogen and for elucidating the distinctions between species and genera within the country (Mathew et al. 2000; O'Donoghue 2017).

Materials and methods

We conducted testing on 72 samples of whole blood obtained from domestic dogs and two samples from hoary fox (*Lycalopex vetulus*) presenting suspected blood parasites or prior suspicions of *Hepatozoon* spp. The samples were sourced from the routine activities of the Clinical Pathology Laboratory of the Veterinary Hospital at the University of Brasilia, spanning the period from March 2020 to February 2022.

DNA extraction was performed on 200 µl of whole blood using commercial kits (Illustra Blood genomic Prep Mini Spin kit, GE Healthcare®, Piscataway, NJ). After extraction, all DNA samples were analyzed using a conventional PCR (cPCR) targeting the mammalian GAPDH gene. This step served to confirm DNA integrity and to verify the absence of PCR inhibitors, in accordance with the methodology outlined by (Birkenheuer et al. 2003).

Initially, we conducted a screening PCR on the suspected samples targeting the genera *Hepatozoon* spp. using the Hep-F and Hep-R oligonucleotides, as outlined by Inokuma et al. (2002). Positive samples from this screening were subsequently subjected to four different PCR protocols designed to amplify various overlapping regions of 18S rDNA from *Hepatozoon* spp. The selection of protocols was based on the studies of Criado-Fornelio et al. (2006) (targeting a 1760-bp fragment), Perkins and Keller (2001) (targeting a 1000-bp fragment), Spolidorio et al. (2009) (targeting a 660-bp fragment), and Ujvari et al. (2004) (targeting a 600-bp fragment). These regions were designated by numerical order for clarity in results: region 1 with Ham-1 and Hepf-2 oligonucleotides; region 2 with oligonucleotides Hemo-1 and Hemo-2; region 3 with Hep300 and Hep900 oligonucleotides; and region 4 with oligonucleotides Hep1 and Hep-4 (Fig. 1).

All samples underwent duplicate testing within the same thermocycler (Biorad® C1000TM Thermal Cycler, Hercules, CA). Autoclaved Milli-Q® ultrapure water served as the negative control, while DNA samples from animals naturally infected by *Hepatozoon* sp. were used as positive controls for all testing protocols. Subsequently, PCR products were subjected to electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination using a transilluminator (UV transilluminator®, UVP LLC, Upland, 32 CA).

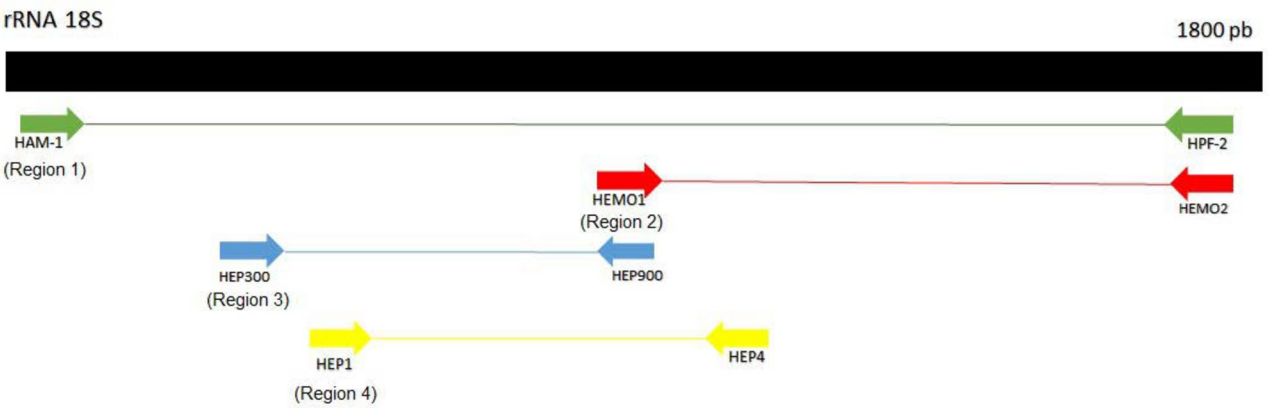

Fig. 1 Diagrammatic representation of the complete 18S rRNA gene and oligonucleotides. Green arrows show an almost complete gene sequence (Ham-1 and Hpf-2) (Criado-Fornelio et al. 2006). In red, partial gene sequence (Hemo-1 and Hemo-2) (Perkins and Keller 2001). In blue, partial sequence (Hep300 and Hep900) (Ujvari et al. 2004), and the yellow arrows, partial sequence (Hep1 and Hep4) (Spolidorio et al. 2009)

PCR products obtained from four dogs and one fox were purified using the PureLink™ Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen®, Carlsbad, CA) following the manufacturer's instructions. After trimming, the sequenced products from the four regions of the 18S rRNA gene were quality-checked and merged to produce a consensus sequence using Geneious version 9.0.5. The nucleotide sequences have been deposited in the GenBank database under the accession numbers (OR143354–OR143357). For further analysis, the sequences were subjected to the BLASTn tool (http://www.ncbi.nlm.nih.gov/BLAST) against the non-redundant (nr) database to facilitate phylogenetic investigations. Alignment for the construction of phylogenetic tree and haplotype networks was performed using the MUSCLE algorithm.

The phylogenetic tree was rooted with an outgroup and developed using MrBayes v3.2 software, applying the "General Time Reversible" (GTR) model with gamma distribution (+G) for the substitution matrix, as determined by the jModelTest v2.1.10 program. The Monte Carlo Markov Chain (MCMC) algorithm was run with four chains for 1,000,000 generations, with samples taken every 100 generations. The initial 25% of the generation data was discarded as "burn-in" to ensure the analysis stabilization. Haplotype networks were constructed with sequences from the current study ($n=4$) and all available Brazilian *H. canis* sequences in the GenBank database that meet the criteria of adequate length (at least approximately 500 nucleotides each) and correspond to the same nucleotide region ($n=32$). The haplotype networks were generated using PopART v.1.7 software with the "Median Joining" method. Two distinct networks were developed based on the geographic location of the isolates and the hosts from which they were identified. Genetic diversity among Brazilian isolates was computed using nucleotide diversity (π), number of haplotypes (h), haplotypic diversity (Dh), mean number of nucleotide differences (K), and number of segregating sites (S) using DnaSP v. 6.

The probable number of distinct genetic groups within Brazilian *H. canis* population, exhibiting genetic differentiation, was estimated through Bayesian analysis using the BAPS v.6.0 program. The algorithms "clustering with linked loci" and "codon" were used as a linked model. Multiple potential genetic groups ($K=2$ to $K=19$) were considered in three independent analyses. The selection of K was based on the most likely outcome, and sequences grouped according to Brazilian federative units.

Results

Twenty-two samples yielded positive results in the screening PCR for *Hepatozoon* spp. Among these, gametocytes were observed in eight blood smears. Notably, seven of these positive samples originated from domestic dogs (*Canis lupus familiaris*), while one was obtained from a hoary fox (*Lycalopex vetulus*). Subsequently, the positive samples were subjected to molecular characterization using all four sets of oligonucleotides. The results of these amplifications are presented in Supplementary Table 1. From the 22 tested samples, seven displayed positive PCR products in all regions of the 18S rDNA gene (Supplementary Table 1). Among these, five samples (designated as 2, 3, 4, 5, and 7) were selected for further analysis due to their superior amplification quality and were subsequently subjected to DNA sequencing.

Sequence analysis by BLASTn

The amplification of fragments of the 18S rDNA gene using different sets of primers resulted in the acquisition of five sequences, with four originating from domestic dogs and one from a wild canid. The sequences obtained in this study showed a high level of identity (99.2–99.7%) with *Hepatozoon canis* isolates from diverse geographic locations, as verified by BLASTn analysis (Table 1). Among the isolates, sample ID-3 (derived from a domestic dog) exhibited 99.63% identity with an isolate detected in a red fox (*Vulpes vulpes*) from the Czech Republic. Isolate ID-7 (from a domestic dog) displayed 99.77% identity with an isolate identified in a domestic dog from Zambia. The sequence ID-2 (from a hoary fox) exhibited 99.21% identity with an isolate from a domestic dog in Israel. Additionally, isolates ID-5 and ID-4 (both from domestic dogs) showed 99.73%

Table 1 Identity values shown from the BLASTn analysis of the tested isolates

Sequences	Sequence size (bp)	Identity (%)	Coverage (%)	*E*-value	Sequences with the highest identity	Countries
ID-2	1356	99.63%	100%	0.0	*Hepatozoon canis* KU893125.1	Czech Republic
ID-3	1313	99.77%	100%	0.0	*Hepatozoon canis* LC331054.1	Zambia
ID-4	1388	99.21%	100%	0.0	*Hepatozoon canis* MK091088.1	Israel
ID-5	1490	99.73%	100%	0.0	*Hepatozoon canis* KX712127.1	Romania
ID-7	1558	99.68%	100%	0.0	*Hepatozoon canis* KX712127.1	Romania

and 99.68% identity, respectively, with an isolate from a golden jackal (*Canis aureus)* in Romania (Table 1).

Isolate ID-7 (domestic dog) was excluded from the phylogenetic analyses, as indicated in Table 1. This decision was prompted by the identification of double peaks during the chromatogram analysis, particularly noteworthy in three sites (at positions 335 [A/G], 362 [G/A], and 376 [G/A]). The presence of these double peaks suggested a potential co-infection involving different isolates. It is important to note that although molecular cloning could have been employed to precisely characterize these sequences, this technique was not available for use in this study. The isolates subjected to sequencing displayed a notable degree of similarity among the analyzed samples, as outlined in Table 1.

Phylogenetic analyses

Four pairs of oligonucleotides were utilized to amplify fragments of the 18S rDNA gene from *H. canis* isolates in Brazil. The use of multiple primer sets enabled the generation of a more comprehensive consensus sequence, enhancing coverage and aiding in molecular characterization. Notably, three of the amplified sequences represented the largest fragments identified in *Canis lupus familiaris* from Brazilian isolates up to the present time. A phylogenetic analysis was performed on the extended fragments, each comprising at least 1300 nucleotides, from the 18S rDNA gene of *H. canis* isolates. The sequences derived from domestic dogs (ID-3, ID-4, and ID-5) and a wild canid (ID-2) clustered together within the same clade as other *H. canis* isolates with robust support (Fig. 2). However, these four sequences occupied distinct minor clades when compared to each other. Specifically, ID-3, ID-4, and ID-5 were not monophyletic but were phylogenetically closer, forming polytomies and occupying a more basal position relative to the other *H. canis* sequences. In contrast, the ID-2 isolate was phylogenetically more distant from the other three isolates identified in this study, grouping with strong support alongside sequences from various countries in the Middle East, South America, Europe, and Asia. Notably, this isolate, the sole Brazilian representative in this clade, had hosts that included both domestic and wild canids. Importantly, other Brazilian isolates obtained from GenBank did not form a monophyletic cluster.

Two networks were constructed to analyze the phylogeography of isolates and their correlations with host species. These networks revealed 10 haplotypes in Brazil. Notably, the H10 haplotype was the most extensive, exhibiting a broad geographic distribution and a diversity of host species. This group included nine isolates, three of which were from domestic dogs in this study (ID-3, ID-4, and ID-5). The H10 haplotype also comprised sequences obtained from *Canis lupus familiaris* and *Rhipicephalus sanguineus* s.l. The sequence ID-2, obtained from a wild canid, clustered with two additional sequences in the H5 haplotype, which included *Tapirus terrestris* and *Lycalopex vetulus* as their respective hosts (Fig. 3). The diversity indices for the Brazilian isolates were calculated as follows: nucleotide diversity ($\pi = 0.00684$), number of haplotypes ($h = 10$), haplotypic diversity (Hd = 0.651), mean number of nucleotide differences ($K = 2.62063$), and number of segregating sites ($S = 14$).

The Bayesian analysis, employing statistical methods, revealed two genetically differentiated groups ($K = 2$) among *H. canis* isolates in Brazil. The analysis exhibited a log marginal probability of –309.6008 and a posterior probability of 1.0, indicating high confidence in the result. Each color in the visualization represents a distinct genetic group. Genetic group 1 comprised isolates from the Federal District, Minas Gerais, and Rio Grande do Norte ($n = 10$). In contrast, genetic group 2 includes isolates from Bahia, Goiás, Mato Grosso do Sul, Pará, Pernambuco, Rio de Janeiro, Rio Grande do Sul, Santa Catarina, and São Paulo, constituting the group with the highest number of sequences ($n = 25$) (Fig. 4).

Discussion

Before this study, *Hepatozoon* isolates deposited in the database for domestic dogs in the country had small or intermediate sequences, hindering the molecular characterization and understanding of *H. canis*'s molecular diversity in Brazil (Paludo et al. 2005; André et al. 2010; Gomes et al. 2016; Modrý et al. 2017). Therefore, our study introduces a straightforward methodology to generate larger 18S rDNA sequences, eliminating the need for molecular cloning, which future investigations can readily replicate.

Despite their high identity values, domestic dog isolates ID-3, ID-4, and ID-5 are positioned more distantly in the phylogenetic tree compared to the wild canid isolate ID-2. This finding is corroborated by the haplotype network, which separates the isolates into two distinct groups: ID-3, ID-4, and ID-5 (H10) and ID-2 (H5). This observed distance implies that animals of the species *Canis lupus familiaris* and *Lycalopex vetulus* in the Federal District may not share the same epidemiological cycle of *H. canis* transmission. Additionally, the tree displays several polytomies where isolates are phylogenetically close. To resolve this, the use of new markers, such as mitochondrial cox1 and cytb for the agent, is suggested, coupled with larger fragments of the 18S rDNA gene, to reduce polytomies (Criado-Fornelio et al. 2006; Hrazdilová et al. 2021; Kolangath et al. 2022). Currently, only two other Brazilian sequences, both from Rio Grande do Sul, have extensive fragments of the 18S rDNA gene. Including more isolates in the analysis could lead to

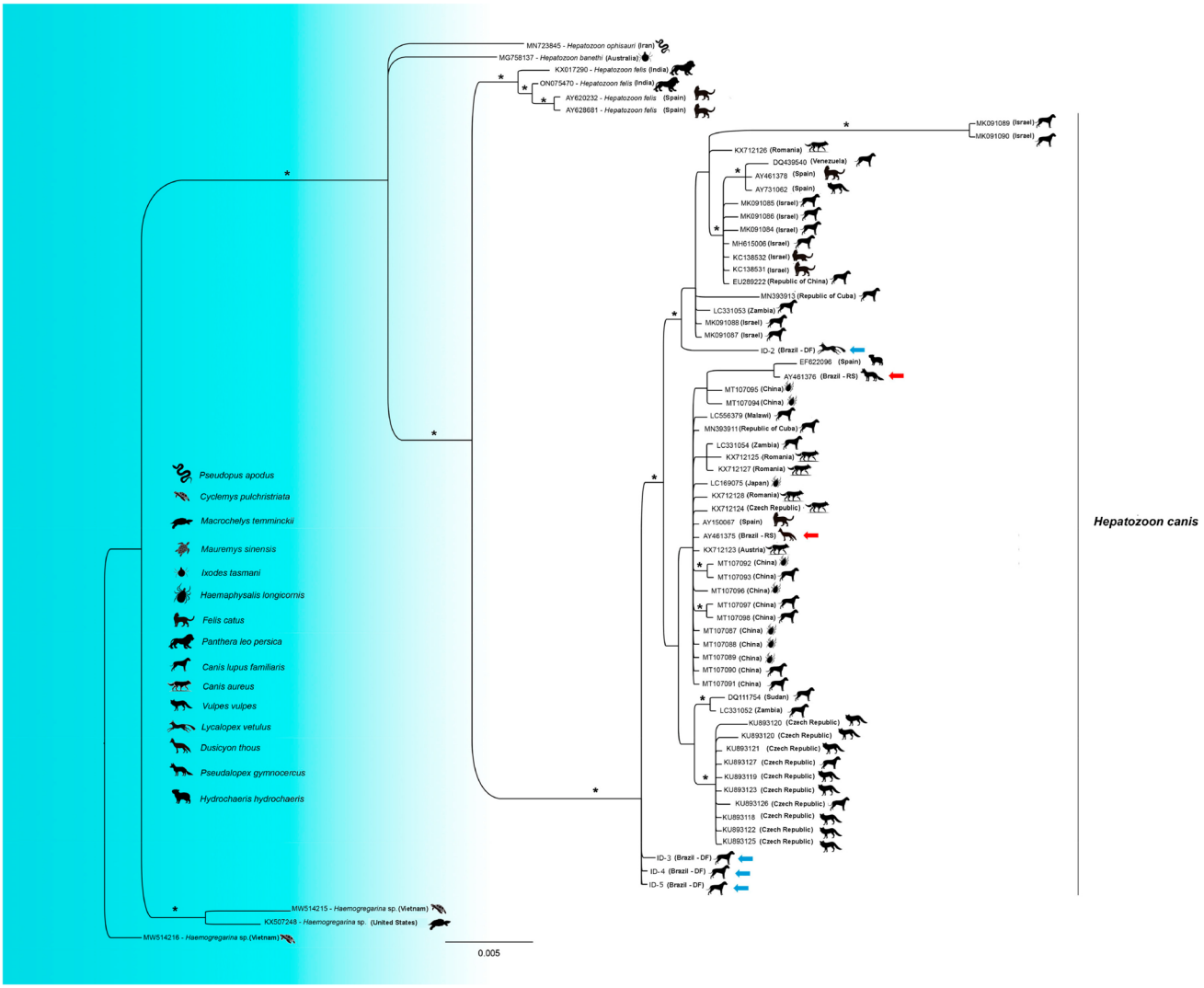

Fig. 2 Phylogenetic tree of 18S rRNA gene of four sequences obtained in the study—tree obtained by Bayesian analysis of partial fragments of 18S rRNA (1335 nt) from 58 *Hepatozoon canis* isolates. Subsequent probabilities of 0.90 or greater are represented by asterisks. The sequences obtained in the present study are highlighted with blue arrows. The other Brazilian sequences obtained from GenBank are marked with red arrows

a more comprehensive phylogenetic elucidation (Criado-Fornelio et al. 2006).

This current investigation represents the first comprehensive study in Brazil to explore the molecular characteristics of *H. canis* in the country. Previously, a study indicated that Brazilian haplotypes are shared by other countries suggesting a global genetic flow of the pathogen (Vásquez-Aguilar et al. 2021). *Hepatozoon canis* has a high degree of haplotypic diversity in Brazil, which is consistent with studies carried out in foxes and dogs in different locations around the world (Criado-Fornelio et al. 2006; Najm et al. 2014; Helm et al. 2020; Kolangath et al. 2022). This substantial diversity observed, with 10 haplotypes identified among 36 Brazilian sequences, contrasts with previous research that reported 12 Brazilian haplotypes (Vásquez-Aguilar et al. 2021). This disparity can potentially be attributed to the inclusion of different sequences and regions in the alignment. The high haplotype diversity in Brazil, coupled with low nucleotide diversity, suggests that the haplotypes differ by only a few base pairs and are genetically close. Thus, there are indications of a recent expansion of the populations of *H. canis* in Brazil as observed globally (Vásquez-Aguilar et al. 2021). However, further population studies and additional analyses, including neutrality tests, are essential to improve our understanding of this phenomenon.

Among the isolates in this study, the domestic dog was the predominant host related to the haplotype H10. However, it remains challenging to determine whether the involvement of other species in this haplotype is incidental or essential in the epidemiological cycle (Criado-Fornelio et al. 2006;

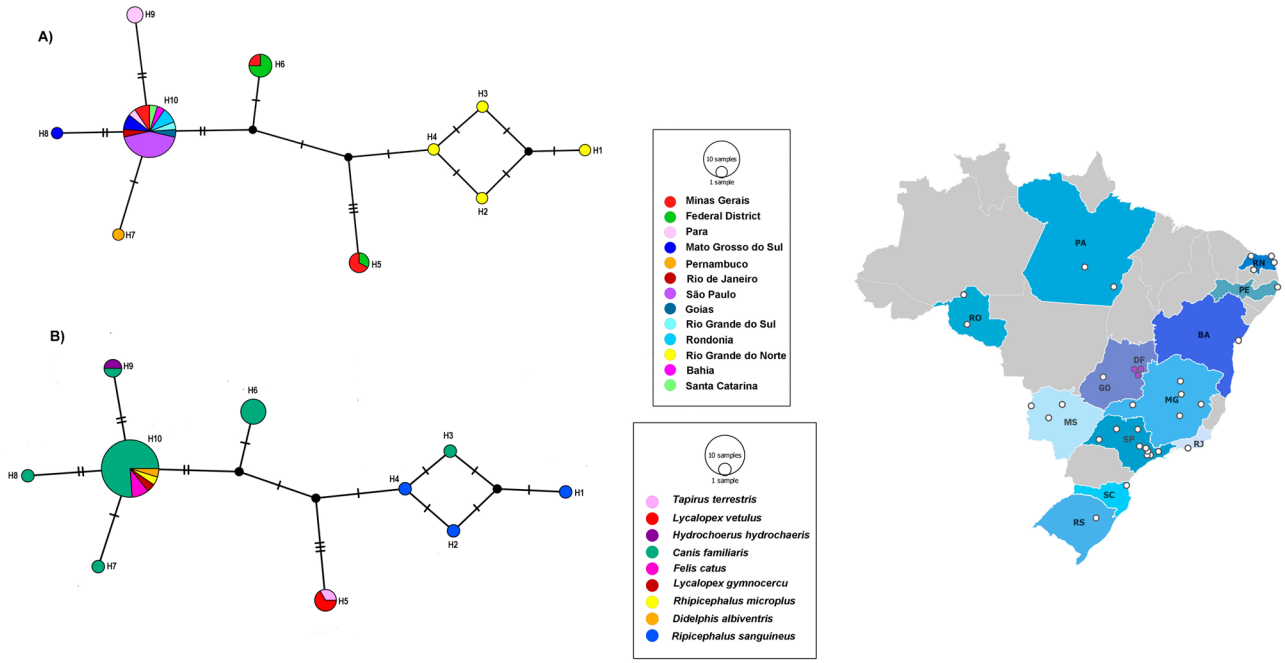

Fig. 3 Haplotype network of *H. canis*: **A** Isolates in Brazil distributed by states. **B** Representing hosts of *H. canis* isolates

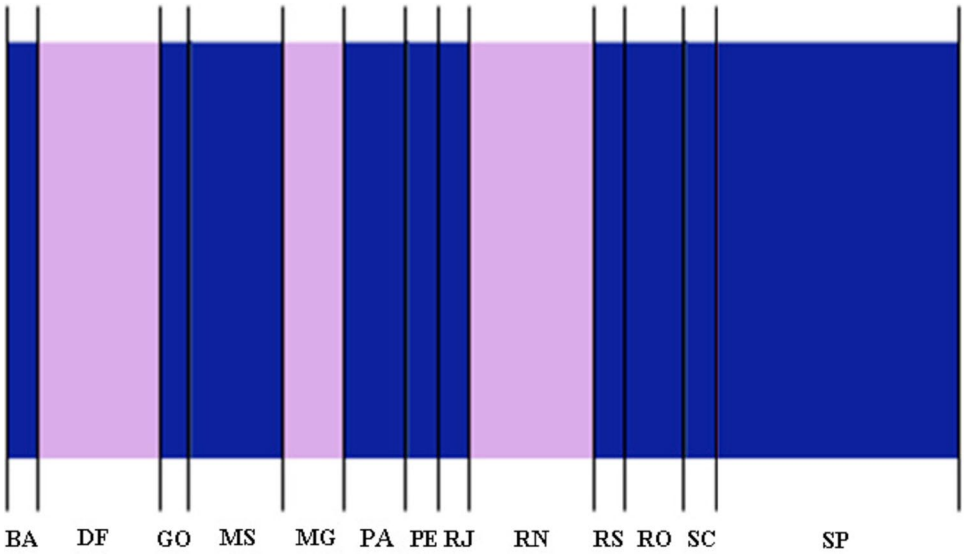

Fig. 4 Bayesian analysis of population structure of *Hepatozoon canis* in Brazil. The analysis indicates two genetic groups distributed by the states

Spolidorio et al. 2009; André et al. 2010; Dantas-Torres and Otranto 2015; Gomes et al. 2016; da Silva et al. 2017; Revathi et al. 2022). Its inclusion in the most extensive Brazilian haplotype may suggest a significant role in the epidemiological cycle or the probability of accidental infection. The haplotype H5 comprises the ID-2 sequence (*Lycalopex vetulus*) from the present study and two sequences from wild hosts in Minas Gerais, namely tapir (*Tapirus terrestris*) and hoary fox (*Lycalopex vetulus*). This haplotype is the only one with Brazilian isolates exclusively consisting of sequences from wild animals. This raises the hypothesis of a potential sylvatic cycle in which the domestic dog may not participate in Brazil, or alternatively, dog sequences in this group have not been sampled thus far. It remains uncertain whether these host species (tapir and hoary fox) are connected to the biological cycle of *H. canis* in dogs in Brazil. Further prevalence studies with molecular characterization are needed. However, it is notable that the animals from Minas Gerais (tapir and hoary fox) and the Federal District (hoary fox), sharing the same haplotype, have a certain geographic

proximity and involve the same host species, the hoary fox. Additionally, Bayesian analysis indicates with high support that *H. canis* isolates from the Federal District, Minas Gerais, and Rio Grande do Norte most likely belong to the same genetic group, pointing to the existence of gene flow between these populations.

Another Brazilian haplotype (H9) drew attention for presenting, from the same region (Brazil-Pará), a domestic canid and a capybara as hosts of the agent *H. canis*. It is possible to suggest that the two hosts either have some participation in the same biological cycle for the agent or that the capybara is an accidental host, since this animal can be found in urban areas. Also, further prevalence studies with molecular data are recommended to understand this participation in the biological cycle, reinforcing the possibility of infection by occasional ingestion of the invertebrate host (Criado-Fornelio et al. 2006; de Azevedo Gomes et al. 2018).

Upon analyzing the two genetic groups and their distribution across states in Brazil, it is evident that several geographically distant states, spanning different macroregions, share the same group, particularly the dark blue one. Additionally, three haplotypes consist of sequences from at least two states, with H10 encompassing sequences from 10 states. This pattern strongly suggests the existence of gene flow of the *H. canis* agent within the country, likely associated with the movement of wild animals and human activities, including the displacement of dogs as pets. To assess whether there is genetic differentiation and whether populations are genetically structured within and between states, detailed population genetic studies are warranted. These studies would provide a better understanding of the genetic dynamics and connectivity of *H. canis* populations in different regions of Brazil.

Conclusion

In summary, our investigation successfully expanded the fragments of the 18S rDNA gene of *H. canis* in Brazil, thereby contributing to a more comprehensive molecular characterization of the agent within the country without molecular cloning. Phylogenetic and phylogeographic analyses revealed that the Brazilian isolates of *H. canis* display elevated genetic diversity and do not form a monophyletic clade, likely due to substantial global gene flow of the agent. The proximity of marsupials and ungulates to wild or domestic canids implies their potential involvement in the biological cycle of *H. canis*, demanding further research for confirmation. Notably, our results delineated two primary genetic groups of *H. canis* in Brazil. These findings underscore the significance of ongoing surveillance and molecular characterization efforts to enhance our comprehension of *H. canis* epidemiology and its potential implications for both animal and public health.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00436-024-08147-8.

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Author contribution Thais de Oliveira Fernandes, Matheus Almeida Duarte, Adriana Pereira Furtado, and Marcela Corrêa Scalon developed the investigation, wrote the main manuscript draft, and prepared the figures. Thais de Oliveira Fernandes and Matheus Almeida Duarte made the molecular analysis. Giane Regina Paludo made the article conceptualization, funding acquisition, supervision, and review and editing of the main manuscript text. All the authors reviewed the manuscript.

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Data availability The representative DNA sequences in conclusions of this article have been deposited in the GenBank database under the accession numbers (OR143354–OR143357).

Declarations

Ethics approval The methodology of this research has been approved by the local Ethics Committee of University of Brasilia (CEUA – Comissão de Ética no Uso Animal), protocol number 40/2017.

Competing interests The authors declare no competing interests.

Consent to participate Not applicable.

Consent for publication Not applicable.

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