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RESEARCH ARTICLE

Preliminary Study on the Genetic Diversity of *Hepatozoon canis* in Dogs and *Rhipicephalus sanguineus* Sensu Lato Ticks

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Abstract

Hepatozoon canis, a protozoan parasite, is the primary cause of canine hepatozoonosis worldwide, typically causing subclinical infection in dogs but potentially leading to severe illness when accompanied by other pathogens. This study investigates the genetic diversity of *H. canis* in dogs and *Rhipicephalus sanguineus* sensu lato ticks using bioinformatics analysis. Archived DNA samples from dogs and ticks were analyzed through PCR amplification of the *18S rRNA gene*, followed by sequence comparison using BLAST analysis and phylogenetic analysis using bioinformatics tools. The results revealed genetic variability, identifying several single nucleotide polymorphisms (SNPs) critical for distinguishing between different haplotypes. Minimum Spanning Networks created in PopART identified 18 distinct haplotypes across a broad geographical distribution. The study highlights the extensive genetic diversity of *H. canis*, with implications for understanding its evolutionary dynamics, transmission, pathogenicity, and resistance. Future studies should employ more variable genomic regions to further elucidate the genetic landscape of *H. canis*, aiding in the development of targeted control strategies and enhancing epidemiological knowledge.

Key Words: *18S rRNA*, Bioinformatics, genetic diversity, haplotype, *Hepatozoon canis*, *Rhipicephalus sanguineus* sensu lato

Köpeklerde ve *Rhipicephalus sanguineus* Sensu Lato Kenelerinde *Hepatozoon canis*'in Genetik Çeşitliliği Üzerine Ön Çalışma

Öz

Hepatozoon canis, köpeklerde tipik olarak subklinik enfeksiyona neden olan ancak diğer patojenlerle birlikte olduğunda ciddi hastalıklara yol açabilen bir protozoon paraziti, dünya çapında köpeklerde hepatozoonozun birincil sebebidir. Bu çalışma, köpeklerde ve *Rhipicephalus sanguineus* sensu lato kenelerinde *H. canis*'in genetik çeşitliliğini biyoinformatik analiz kullanarak araştırmaktadır. Köpeklerden ve kenelerden alınan arşiv DNA örnekleri, *18S rRNA* geninin PCR amplifikasyonu ile analiz edilmiş ve ardından BLAST analizi ile dizi karşılaştırması ve biyoinformatik araçlar kullanılarak filogenetik analiz yapılmıştır. Sonuçlar, farklı haplotipleri ayırt etmek için kritik olan çeşitli tek nükleotid polimorfizmlerini (SNP'ler) tanımlayarak genetik değişkenliği ortaya koymuştur. PopART'da oluşturulan Minimum Yayılma Ağları, geniş bir coğrafi dağılıma sahip 18 farklı haplotipi belirlemiştir. Çalışma, *H. canis*'in geniş genetik çeşitliliğini vurgulamakta ve evrimsel dinamiklerini, bulaşma yollarını, patojenitesini ve direncini anlamak için önemli çıkarımlarda bulunmaktadır. Gelecekteki çalışmalar, *H. canis*'in genetik yapısını daha ayrıntılı bir şekilde açıklığa kavuşturmak, hedeflenmiş kontrol stratejilerinin geliştirilmesine ve epidemiyolojik bilginin artırılmasına yardımcı olmak için daha değişken genom bölgelerini kullanmalıdır.

Anahtar Kelimeler: *18S rRNA*, Biyoinformatik, genetik çeşitlilik, haplotip, *Hepatozoon canis*, *Rhipicephalus sanguineus* sensu lato

INTRODUCTION

Hepatozoon species, which are blood parasites belonging to the class Apicomplexa, infect a wide range of vertebrate hosts including amphibians, reptiles, birds, marsupials, and mammals (1). Among these, *Hepatozoon canis* is a protozoan that primarily infects dogs and other wild carnivores, and it is recognized as the most common cause of canine hepatozoonosis worldwide (2). While infections are typically subclinical, they can lead to moderate to severe illness characterized by cachexia and anemia, especially when accompanied by other pathogens (3). Though dogs are the primary intermediate hosts for *H. canis*, various wild canine species, foxes, and other carnivores have also been reported to be infected with *H. canis* or other *Hepatozoon* species (4).

Dogs become infected with *H. canis* by ingesting *Rhipicephalus sanguineus* sensu lato ticks that contain mature oocysts with infective sporozoites. Once ingested, these sporozoites are released in the intestine, entering the bloodstream and lymphatic system. They enter to tissues such as the liver, kidneys, spleen, bone marrow, and lymph nodes, where they undergo merogony, an asexual replication process, forming meronts. The micro and macromerozoites within these meronts are then released and invade neutrophils and monocytes in the bloodstream, where they develop into gamonts through sexual reproduction. When a tick feeds on the host's blood, these infected blood cells are ingested and broken down in the tick's gut. The free gamonts divide to form macrogametes and microgametes, which fuse to form a zygote. The zygote then develops into an oocyst, within which sporozoites are produced through sporogony (5,6).

Hepatozoon canis infection is predominantly reported in tropical, subtropical, and temperate regions where vector tick species are abundant. In Europe, the infection is mainly observed in areas near the Mediterranean basin. Molecular studies in Türkiye have reported high rates of *H. canis* infection in dogs and in *R. sanguineus* sensu lato ticks, the main known vector (7–11). *Hepatozoon canis* is transmitted transstadially among the developmental stages of ticks (larva, nymph, adult) (5,10). A study conducted in Chile analyzed haplotypes of *Hepatozoon* spp. *18S rRNA* sequences from rodents and their associated ticks, revealing significant genetic diversity in the haplotypes found in these hosts (12). While there are studies on the genetic diversity of *H. canis* in various vertebrate hosts, there is a lack of research on the genetic diversity of this parasite in infected dogs and vector ticks. This preliminary study aims to investigate the genetic diversity of *H. canis* in infected dogs and *R. sanguineus* s.l. ticks using bioinformatics analysis.

MATERIAL AND METHODS

Amplification of *H. canis 18S rRNA* Gene

In this study, archived DNA samples obtained from dogs and ticks in 2015 were used (10). DNA samples were extracted from 2 engorged nymphs (AYN1 and AYN8) and 2 engorged adults (AEG4-2 and AEG2) of *R. sanguineus* sensu lato, as well as from 3 dogs (Nimf2, Nimf4, Nimf5). To investigate the genetic diversity of *H. canis*, the *18S rRNA* gene was amplified by PCR. Primers HEPF and HEPR were used to amplify the *Hepatozoon* sp. *18S rRNA* gene fragment (13). PCR amplification was carried out using Phusion® High-Fidelity PCR Master Mix with GC Buffer (#M0532S; NEB). The PCR reaction was performed in a total volume of 20 µL, containing; 10 µL of 2X Phusion Master Mix, 1 µL of each forward and reverse primer, 1 µL of template DNA, 7 µL of nuclease-free water. All samples (n=7) were subjected to sequence analysis.

Bioinformatics Analyses

The obtained nucleotide sequences were compared to those in the NCBI database using BLAST analysis. Phylogenetic analysis was conducted using the MEGA X program (14). Sequence data from different geographic regions reported in GenBank, obtained from dogs and ticks infected with *H. canis*, were used for comparison. All sequences were aligned using the MEGA X and CLC Sequence Viewer 8.0 program and adjusted to equalize the ends of the sequences. For data analysis, sequences were converted to Nexus format for use in the PopART (Population Analysis with Reticulate Trees) software (15). Haplotypes were created using Minimum Spanning Networks in PopART, and relationships between haplotypes were analyzed. Nucleotide content, haplotype numbers, haplotype and nucleotide diversity values, and the amount of mutation among molecular haplotypes were determined using the DnaSP 6 program (16).

RESULTS

The sequence analysis of the *18S rRNA* gene from *H. canis* in both dogs and *R. sanguineus* sensu lato ticks revealed genetic diversity. Many regions of the sequences were highly conserved across all samples, which indicates a high degree of similarity in the *18S rRNA* gene among these samples. However, several single nucleotide polymorphisms (SNPs) were identified, which are critical for distinguishing between different haplotypes. Using BLAST analysis, the obtained nucleotide sequences were compared to those in the NCBI database, confirming the presence of *H. canis*. The phylogenetic analysis conducted using MEGA X and CLC Sequence Viewer 8.0 aligned the sequences from different geographic regions reported in GenBank. This alignment revealed the evolutionary relationships and geographical distribution of *H. canis*. The alignment results showed that while the majority of the *18S rRNA* gene sequences were conserved, the identified SNPs contributed to the genetic variability observed among the samples (Figure 1). In addition, phylogenetic analysis showed that *H. canis* sequences from various parts of the world formed different clades (Figure 2).

In PopART, haplotypes were created using Minimum Spanning Networks to analyze relationships between them. The analysis revealed 18 distinct haplotypes, indicating significant genetic variation within *H. canis* populations in both dogs and ticks (Table 1). Haplotype 3, which had the highest number of samples (8), suggests a widespread or common genetic variant. Haplotypes 1, 2, 10, 14, 17, and 18 also had multiple samples, though fewer than Haplotype 3. The samples originated from various regions, including Spain, India, Portugal, Croatia, Nigeria, Germany, Iran, Israel, Türkiye, Italy, Brazil, Hungary, Egypt, Japan, and Taiwan, indicating a broad geographical distribution of these haplotypes. The samples included both dogs and ticks (*R. sanguineus* sensu

lato*, Ixodes ricinus, Dermacentor marginatus, Amblyomma cajennense, Haemaphysalis longicornis, Haemaphysalis bispinosa*), highlighting potential host diversity or transmission dynamics. Among the 38 aligned nucleotide sites, 49 variable sites were detected (Figure 3). The analysis showed a notable degree of haplotype diversity, with several unique haplotypes identified in the samples.

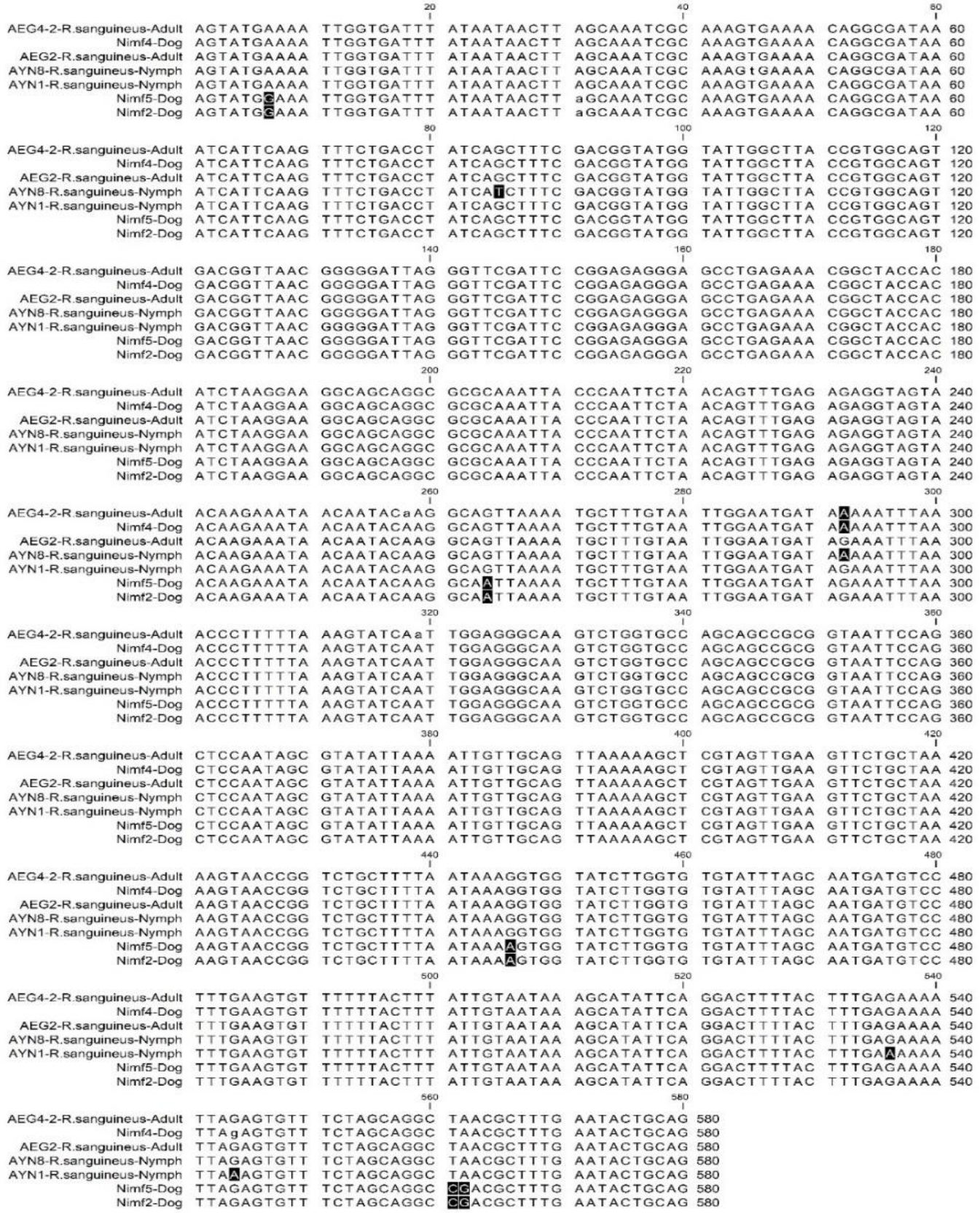

Figure 1. Sequence alignment of the *18S rRNA* gene from *H. canis* in dogs and *R. sanguineus* sensu lato

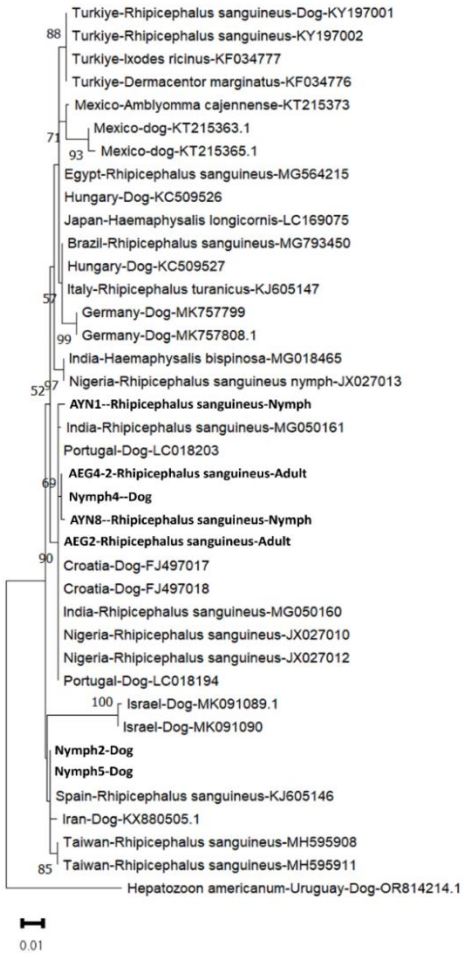

Figure 2. The phylogenetic tree created using the Mega X program shows the phylogenetic relationship of *H. canis* (in bold), identified in this study, with *H. canis* sequences reported in different regions in ticks and dogs obtained from GenBank. The evolutionary history was inferred based on the Tamura 3 model. Next to each branch is the percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (100 replicates). Only bootstrap values higher than 50 are displayed next to the branches. As an outgroup, *H. americanum* (OR814214) was utilized. The scale bar indicates the evolutionary distance in terms of nucleotide substitutions per site.

Table 1: Distribution of haplotype samples and geographic origins

Haplotype	Number of Samples	Sample Details
Hap_1	2	**AEG4-2- *Rhipicephalus_sanguineus*-Adult, Nimf4-Dog**
Hap_2	3	**Nimf5-Dog, Nimf2-Dog,** Spain-*Rhipicephalus_sanguineus*-KJ605146
Hap_3	8	AEG2-*Rhipicephalus_sanguineus*-Adult India-*Rhipicephalus_sanguineus*-MG050160, Portugal-Dog-LC018203, Portugal-Dog-LC018194, Croatia-Dog-FJ497017, Croatia-Dog-FJ497018, Nigeria-*Rhipicephalus_sanguineus*-JX027012, Nigeria-*Rhipicephalus_sanguineus*-JX027010
Hap_4	1	**AYN8-*Rhipicephalus_sanguineus*-Nymph**
Hap_5	1	**AYN1-*Rhipicephalus_sanguineus*-Nymph**
Hap_6	1	Mexico-dog-KT215365.1
Hap_7	1	Mexico-dog-KT215363.1
Hap_8	1	Mexico-*Amblyomma_cajennense*-KT215373
Hap_9	1	India-*Rhipicephalus_sanguineus*-MG050161
Hap_10	2	Germany-Dog-MK757799, Germany-Dog-MK757808.1
Hap_11	1	Iran-Dog-KX880505.1
Hap_12	1	Israel-Dog-MK091089.1
Hap_13	1	Israel-Dog-MK091090
Hap_14	4	Turkiye-*Rhipicephalus_sanguineus*-Dog-KY197001, Turkiye-*Rhipicephalus_sanguineus*-KY197002, Turkiye-*Ixodes_ricinus*-KF034777, Turkiye-*Dermacentor_marginatus*-KF034776
Hap_15	3	Italy-*Rhipicephalus_turanicus*-KJ605147, Brazil-*Rhipicephalus_sanguineus*-MG793450, Hungary-Dog-KC509527
Hap_16	3	Egypt-*Rhipicephalus_sanguineus*-MG564215, Hungary-Dog-KC509526, Japan-*Haemaphysalis_longicornis*-LC169075
Hap_17	2	India-*Haemaphysalis_bispinosa*-MG018465, Nigeria-*Rhipicephalus_sanguineus*_nymph-JX027013
Hap_18	2	Taiwan-*Rhipicephalus_sanguineus*-MH595911 Taiwan-*Rhipicephalus_sanguineus*-MH595908

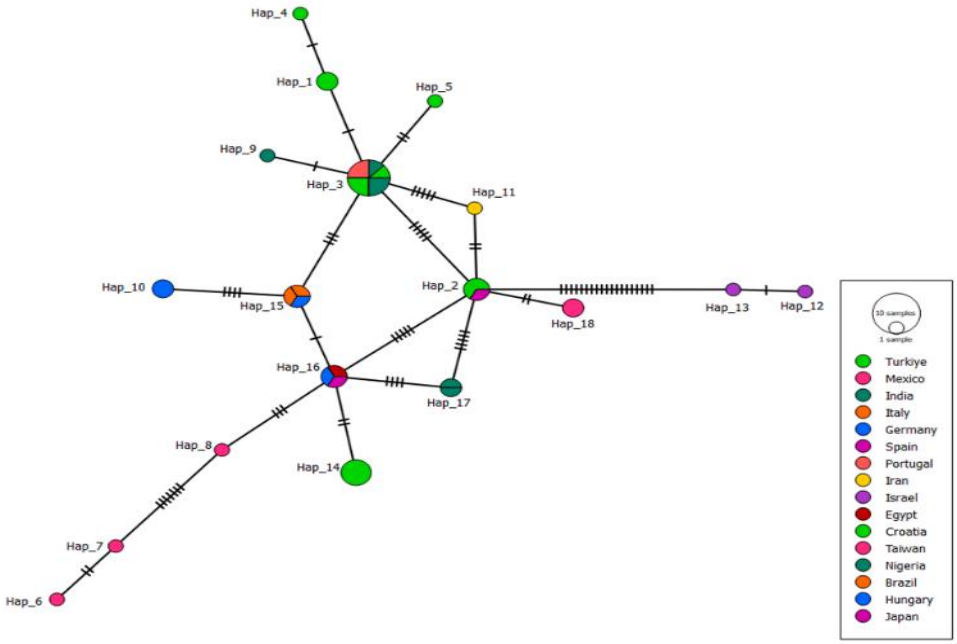

Figure 3: Haplotype network of *H. canis* dog and tick host. The size of the circle represents the frequency of each haplotype. The different colored dots represent haplotypes from the different populations.

DISCUSSION AND CONCLUSION

In this study the observation of multiple haplotypes with varying degrees of relatedness suggests that *H. canis* exhibits significant genetic diversity within the studied populations. This genetic variation, observed across different geographic regions, plays a crucial role in understanding the evolutionary dynamics of this parasite. The identified SNPs in the sequence alignment offer valuable insights into genetic variations that may be associated with geographical adaptations or host-specific interactions. Such diversity could impact the parasite's transmission dynamics, pathogenicity, and resistance to environmental pressures.

Studies on the genetic diversity of *Hepatozoon* spp. across various hosts and regions have revealed a complex and rich genetic landscape for these parasites. Research on snakes from North Africa and the Mediterranean Basin demonstrates significant patterns of genetic diversity, suggesting a complex evolutionary history in reptilian hosts (17). Similarly, substantial genetic diversity has been observed in *Hepatozoon* spp. infecting coyotes from the South-Central United States, indicating a high adaptability and local environmental influences on parasite genetics (18). In Chile, the genetic variability of *Hepatozoon* spp. in rodents emphasizes the importance of regional studies to understand their genetic structure (12). The first molecular detection and genetic analysis of *Hepatozoon* sp. in a crocodile monitor in Thailand provides new insights into the host range and genetic variability in reptilian species (19). In the eastern Amazon, studies on *Hydrochoerus hydrochaeris* and *Pecari tajacu* highlight the genetic richness and host-specific adaptations of these parasites (20). Investigations in South Africa and globally on domestic cats revealed significant genetic variations in *Hepatozoon felis*, indicating a broad host range and extensive diversity (21). Additionally, the molecular prevalence and genetic diversity of *Hepatozoon* spp. in stray cats of İzmir, Türkiye, underscore their widespread presence and variability in feline populations (22). Genetic studies of *H. canis* in golden jackals and grey wolves in Serbia show high degrees of genetic variation, reflecting the dynamic epidemiology of these parasites in wild canid populations (23). Furthermore, research on dogs and foxes in Brandenburg, Germany, identified identical *18S rRNA* haplotypes of *H. canis*, highlighting the genetic similarities and potential transmission pathways between domestic and wild canids in this region (24). These findings collectively illustrate the extensive genetic diversity and adaptability of *Hepatozoon* spp. across different hosts and regions.

This preliminary study highlights the genetic diversity of *H. canis* in both dogs and *R. sanguineus* sensu lato ticks, emphasizing the importance of understanding the evolutionary relationships and geographical distribution of this parasite. The presence of distinct haplotypes and SNPs within the *18S rRNA* gene underscores the genetic variability of *H. canis*, which is crucial for developing targeted control strategies and enhancing our understanding of the parasite's biology and epidemiology. To further refine our insights, haplotype networks using more variable target regions, such as recently published mitochondrial and apicoplast genomes, should be employed in future epidemiological studies (25). This approach could significantly improve resolution and provide deeper insights into the genetic landscape of *H. canis*.

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CONFLICTS OF INTEREST

There is no conflicts of interest.

AUTHOR CONTRIBUTIONS

All analyses and writing of the study and final checks were carried out by SO.

ETHICAL STATEMENT

Not applicable

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