



Molecular Investigation of Hepatozoon Species in Cats in Different Regions of Türkiye *

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Hepatozoon species are known important vector-borne pathogens and these species may infect various hosts. *Hepatozoon* species, *Hepatozoon felis*, *H. canis* and *H. silvestris*, infect domestic and wild cats, and these species may cause mild to severe clinical manifestations in cats. There have been numerous studies conducted to the determination of *Hepatozoon* species in cats from different countries. However, there are paucity of data on presence and prevalence of *Hepatozoon* species among cat population in Türkiye. In this study, 320 gDNA obtained from cat blood samples collected from Sivas, Ankara, Malatya, Denizli, and İstanbul provinces were investigated for *Hepatozoon* species using genus-specific PCR assay. All PCR positive samples were sent to DNA sequences analyses. In the current work, *Hepatozoon* spp. were detected in three (two samples in Sivas and one sample in Malatya) of 320 cat samples (0.93%, 3/320; 95% CI 0.19-2.72). According to the results of DNA sequence, all positive samples were identified as *H. felis*. However, *H. canis* and *H. silvestris* was not detected in cat samples. Determined consensus sequences were deposited in the GenBank with accession numbers: PV639385-PV639387. BLASTn analyses revealed that 97.72–100% nucleotide identities were determined between our *H. felis* isolates and *H. felis* isolates present in GenBank. The results of the study showed that *H. felis* is circulated among owned cats in Türkiye. Considering the *H. felis* may cause infection in cats, necessary precaution should be taken to protect stray or owned cats health by veterinarians and cat owners.

Key Words: Cat, *Hepatozoon felis*, DNA sequence, phylogenetic analyses

Türkiye'nin Farklı Bölgelerindeki Kedilerde Bulunan Hepatozoon Türlerinin Moleküler İncelenmesi

Hepatozoon türleri bilinen önemli vektör kaynaklı patojenlerdir ve bu türler çeşitli konakçıları enfekte etmektedir. *Hepatozoon* türleri; *Hepatozoon felis*, *H. canis* ve *H. silvestris*, evcil ve vahşi kedileri enfekte eder ve bu türler kedilerde hafif ila şiddetli klinik belirtilere neden olabilir. Farklı ülkelerde, kedilerde *Hepatozoon* türlerinin belirlenmesine yönelik çok sayıda çalışma yapılmıştır. Bununla birlikte, Türkiye'deki kedi popülasyonunda *Hepatozoon* türlerinin varlığı ve yaygınlığına ilişkin veriler yetersizdir. Bu çalışmada, Sivas, Ankara, Malatya, Denizli ve İstanbul illerinden toplanan kedi kanı örneklerinden elde edilen 320 gDNA, soy spesifik PCR testi kullanılarak *Hepatozoon* türleri açısından araştırılmıştır. Tüm PCR pozitif örnekler DNA dizi analizlerine gönderilmiştir. Bu çalışmada, 320 kedi örneğinin üçünde (Sivas'ta iki ve Malatya'da bir örnek) *Hepatozoon* spp. (%0.93, 3/320; 95% CI 0.19-2.72) tespit edilmiştir. DNA dizisi ve filogenetik analiz sonuçlarına göre, tüm pozitif örnekler *H. felis* olarak tanımlanmıştır. Bununla birlikte, kedi örneklerinde *H. canis* ve *H. silvestris* tespit edilememiştir. Belirlenen konsensüs dizileri GenBank'ta PV639385-PV639387 erişim numaraları ile depolanmıştır. BLASTn analizleri, *H. felis* izolatlarımız ile GenBank'ta bulunan *H. felis* izolatları arasında %97.72-100 nükleotid benzerliği tespit edildiğini ortaya koymuştur. Çalışmanın sonuçları, *H. felis*'in Türkiye'deki sahipli kediler arasında sirküle olduğunu göstermiştir. *Hepatozoon felis*'in kedilerde enfeksiyona neden olabileceği göz önünde bulundurularak, veteriner hekimler ve kedi sahipleri tarafından sahipli veya sahipsiz kedilerin sağlığını korumak için gerekli önlemler alınmalıdır.

Anahtar Kelimeler: Kedi, *Hepatozoon felis*, DNA sekans, filogenetik analiz

Introduction

Species in the genus *Hepatozoon* (Eucoccidiorida, Hepatozoidae) are apicomplexan parasitic pathogens. Recent studies have identified more than 340 *Hepatozoon* species affecting mammals, birds, reptiles, and amphibians (1-3). Transmission of *Hepatozoon* species occurs by ingestion of infected vectors, such as ticks, mites, mosquitoes, or fleas, by vertebrate hosts (4). In studies, it has been reported that especially tick species of the Ixodidae family are very important to the transmission of the pathogens (5-8). Sexual development and sporogony stages of pathogens occur in these arthropods (4, 7).

The first report of hepatozoonosis in domestic cats was made in India in 1908. Subsequently, the infection was reported in felids in many countries, including South Africa, Asia, Southern Europe, and the USA (3, 5-9). To date, three *Hepatozoon* species, *Hepatozoon felis*, *H. canis*, and *H. silvestris*, have been reported in domestic

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and wild cats (2, 7, 10-13). In addition, *H. felis* has been reported as the most frequently diagnosed species in feline hepatozoonosis cases in different countries of the world (14, 15). The infection is mostly subclinical in cats. More pathogenic effects can be seen in the case of under stress, reduced immune systems or with secondary infections (FIV, FeLV, and feline hemoplasma etc.) in cats (6). *Hepatozoon* species cause diseases characterized by clinical symptoms such as fever, diarrhoea, vomiting, weight loss, lethargy, anorexia, jaundice, lymphadenopathy, and gait disturbances (14, 16). *Hepatozoon* spp. in cats is particularly associated with infection of muscle tissues. The agents can be found in the skeletal muscles and myocardium of the hosts (6). Studies in Switzerland (2) and Italy (17) have shown that *H. silvestris* causes severe lymphoplasmacytic and histiocytic myocarditis in domestic cats and deaths due to intestinal invagination (2, 17).

It is very important to use rapid and reliable diagnostic methods to minimize the negative results caused by hepatozoonosis (18). In cases of hepatozoonosis, it has been reported that the level of parasitemia in cats is generally low, with fewer than 1% of neutrophils and monocytes containing gamonts. In addition, microscopic examination cannot detect low-level infections. Serological methods may cause cross-reactions and produce erroneous results (4, 14, 19). The fact that microscopic examination and serological diagnostic methods do not give sufficiently accurate results has led researchers to use molecular diagnostic methods such as PCR, which have both higher sensitivity and specificity (3, 20). Molecular methods are based on the principle of amplifying the DNA of the target pathogen in vitro and making it detectable. These methods allow the detection of very few amounts of DNA in the sample, the identification of pathogens causing infection in the hosts at the species level and the detection of mixed infections. In addition, by revealing the nucleotide sequences of pathogens with sequence analyses, it contributes to the investigation of the genetic diversity of pathogens circulating in hosts, the detection of new strains, genotypes or species, and the understanding of their taxonomic positions and phylogenetic relationships (20-24).

Hepatozoonosis is a disease that has been detected in different hosts, including domestic animals (such as cats, dogs) and wild animals (such as jackals, foxes) worldwide (3, 25). Although *Hepatozoon* spp. have been detected in many different species, it is seen that most of the studies have been carried out on dogs (26). Türkiye has a large number of vectors due to its geographical location, diverse climate, and vegetation. Several studies have been carried out in Türkiye to investigate the prevalence, distribution, and genetic diversity of vector-borne pathogens, and many pathogen species have been detected in different hosts (21, 23, 27). Moreover, several *Hepatozoon* species, *H. canis*, *Hepatozoon* spp., and *H. viperoi* sp. nov., have been identified in different hosts from several parts of Türkiye (28-34). According to the literature review, there is a

limited number of studies on the presence and prevalence of feline *Hepatozoon* species in Türkiye. The aims of this study was i) to investigate the molecular prevalence of *Hepatozoon* species in cat blood samples obtained from different parts of Türkiye by using conventional PCR, ii) to perform phylogenetic analyses of positive samples identified in the study.

Materials and Methods

Research and Publication Ethics: All procedures performed in studies involving animals were in accordance with the ethical standards approved by the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (Approval number: 05.03.2025-65202830-050.04.04-10).

Study Area and Samples: Türkiye is geographically located between Europe and Asia and has a subtropical climate. The country is divided into seven different geographical regions (Eastern Anatolia, Central Anatolia, the Black Sea, Southeastern Anatolia, Marmara, the Aegean, and the Mediterranean). This geographical structure and climatic characteristics allow various vector species to be live in the country. Therefore, vector-borne diseases are quite common in the country (35). In this study, a total of 320 cat blood samples from five regions of Türkiye, Central Anatolia [Sivas (n: 60), Ankara (n: 70)], Eastern Anatolia [Malatya (n: 60)], Aegean [Denizli (n: 60)], and Marmara [Istanbul (n: 70)] were collected in different studies (Figure 1). Genomic DNA was isolated from blood samples in accordance with the protocol outlined by Sambrook et al. (36), with minor modifications. Data about modifications during DNA extraction process were detailed in the study conducted by Erol et al. (27).



Figur 1. The locations of sampling area were indicated in the Türkiye maps

PCR Assay for the Research of *Hepatozoon* spp. in Cat Blood Samples: The obtained DNA samples were researched in terms of *Hepatozoon* species with conventional PCR using primers Hep-F (5'-ATACATATGAGCAAATCTCAAC-3') and Hep-R (5'-CTTTATTATTCCATGCTGCAG-3') primers (37). These primers were amplified partial parts of *18S SSU rRNA* gene region of *Hepatozoon* species.

The PCR assay was carried out in a total volume of 25 μ L, which included 10 \times PCR buffer (Invitrogen™, Carlsbad, USA), 2.5 μ L MgCl₂ (50 mM) (Invitrogen™, Carlsbad, USA), 200 μ M of each dNTP (Cat.No.: R0181, Thermo Scientific™, Lithuania), 1 μ L (10 pmol/ μ L) of each of the primers, 1.25 U of Taq DNA polymerase (5U/ μ L) (Ref.No.: 100021276, Invitrogen™, Carlsbad, USA),

2.5 µL template DNA, and DNase-RNase-free sterile water. To prevent false results, positive [*H. canis* (GenBank Accession Number MW350128, (33))] and negative (DNase-RNase-free sterile water, Cat. No.: 129114, Qiagen®, Hilden, Germany) control groups were added to each PCR mix.

The thermal cycling protocol utilized for the PCR comprised an initial denaturation step at 94°C for a duration of 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The protocol was concluded with a final extension step at 72°C for 5 min. The resulting PCR products were loaded onto a 1% agarose gel and then electrophoresed at 90 volts for 60 min. Subsequently, the agarose gel was subjected to staining with ethidium bromide for a period of 20 min. The results were then subjected to visualization using a UV transilluminator.

Sequencing and Phylogenetic Analysis:

Nucleotide sequence analysis of all positive samples was done with primers used PCR assay. Sequence analyses were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3730XL analyzer (Applied Biosystems, Foster City, CA) according to the standard instructions recommended by the manufacturer. Prior to the nucleotide sequencing, the purification of all PCR products was achieved through the utilization of the HighPrep™ PCR Clean-Up System (Cat. No.: AC-60005, MagBio), in accordance with the manufacturer's instructions. The resulting chromatograms were then subjected to a quality assessment using the FinchTV (version 1.4.0) program, developed by Geospiza Inc. (Seattle, Washington, USA). Nucleotides exhibiting substandard chromatogram quality scores were subjected, and nucleotides had low chromatogram quality scores were trimmed. The MEGA-11 software (38) was utilized to generate the consensus sequence. These consensus sequences were subsequently submitted to the GenBank database, and accession numbers were obtained.

Phylogenetic trees were constructed using a maximum likelihood (ML) analysis implemented in MEGA-11 software (38). The resulting trees were evaluated to reveal genetic variation between the *Hepatozoon* species identified in this study and those in GenBank and to determine phylogenetic relationships. Pre-construction of the phylogenetic tree involved the determination of the optimal algorithm for use in the phylogenetic tree of related pathogens. Prior to the construction of the phylogenetic tree, it was determined that the most appropriate algorithm to be employed in the phylogenetic tree of related pathogens was the Tamura-Nei (TN93) + G parameter model (39) using the Find Best-Fit Substitution Model in MEGA-11. This algorithm was subsequently utilized in the construction of the phylogenetic tree. The bootstrap analysis (1000 replications) was conducted.

Results

In the study, 320 cat blood samples collected from Sivas, Ankara, Malatya, Denizli, and İstanbul provinces were investigated by *Hepatozoon* spp. PCR. According to PCR results, three of 320 cat blood samples (0.93%, 3/320; 95% CI 0.19-2.72) were found to be *Hepatozoon* spp. (Figure 2). Two of the *Hepatozoon* spp. positive samples were detected in Sivas (3.33%, 2/60; 95% CI 0.41-11.53) and other was in Malatya (1.66%, 1/60; 95% CI 0.04-8.94). No positivity was detected in samples obtained from other provinces. According to Fisher's exact test, no statistically significant difference was detected in *Hepatozoon* spp. positivity among the regions ($p>0.05$).

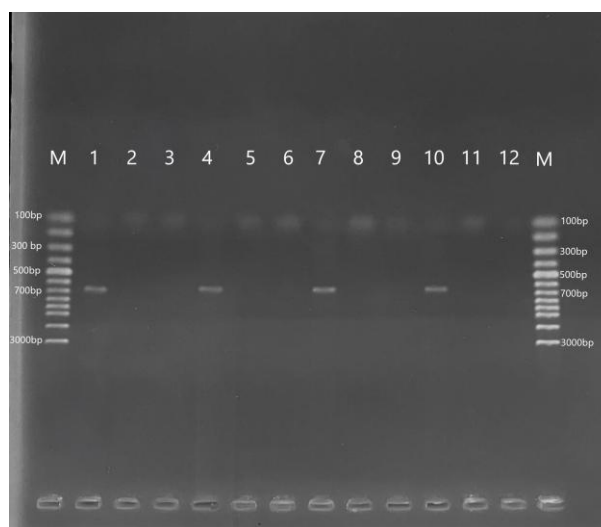


Figure 2. PCR products of *H. felis* species. M. Marker, 1. *Hepatozoon* spp. positive control, 2. Negative control, 4-7-10. *Hepatozoon* spp. positive cat samples. 3-5-6-8-9-11-12. Negative cat samples

The consensus sequence belonging to three PCR positive samples were uploaded to the GenBank under accession numbers PV639385-PV639387. These three sequence had 100% nucleotide identities each other.

The BLASTn analyses of sequence identified in this work revealed that 97.72-100% nucleotide similarities were seen with our sequence and *H. felis* sequence present in the GenBank reported from different regions of the world. *Hepatozoon felis* sequences detected in this study had 100% nucleotide identities with *H. felis* reported in China (PP528683, *Eurasian lynx*), India (ON075470, Asiatic lion), and Spain (OR263294, *Felis catus*). Furthermore, our sequences showed high levels of nucleotide similarity to those from Israel (99.84%, KC138533, *Felis catus*), China (99.84%, PP528682, *Hyalomma asiaticum*), Spain (99.83%, OR263293, *Felis catus*), Spain (99.67%, AY628681, AY620232, OR263291, and OR263276, *Felis catus*), India (99.67%, KX017290, Asiatic lion), Spain (99.51%, OR263292, OR263286, OR263288, and OR263280, *Felis catus*), India (99.00%, OK036954,

Panthera tigris tigris), Namibia (98.99%, MW872057, *Parahyaena brunnea*), Korea (98.99%, GQ377216, Korean leopard cat), Italy (98.99%, KY511259, cat), Japan (97.72%, AB771543, *Prionailurus iriomotensis*), and Japan (97.72%, AB771552 and AB771562 *Prionailurus bengalensis euptilurus*).

Phylogenetic analysis of the 18S SSU rRNA gene region was performed by aligning our *H. felis* sequences with different *H. felis* isolates present in GenBank. The sequences obtained from *H. felis* in this study share the same branch as *H. felis* from various countries (Figure 3).

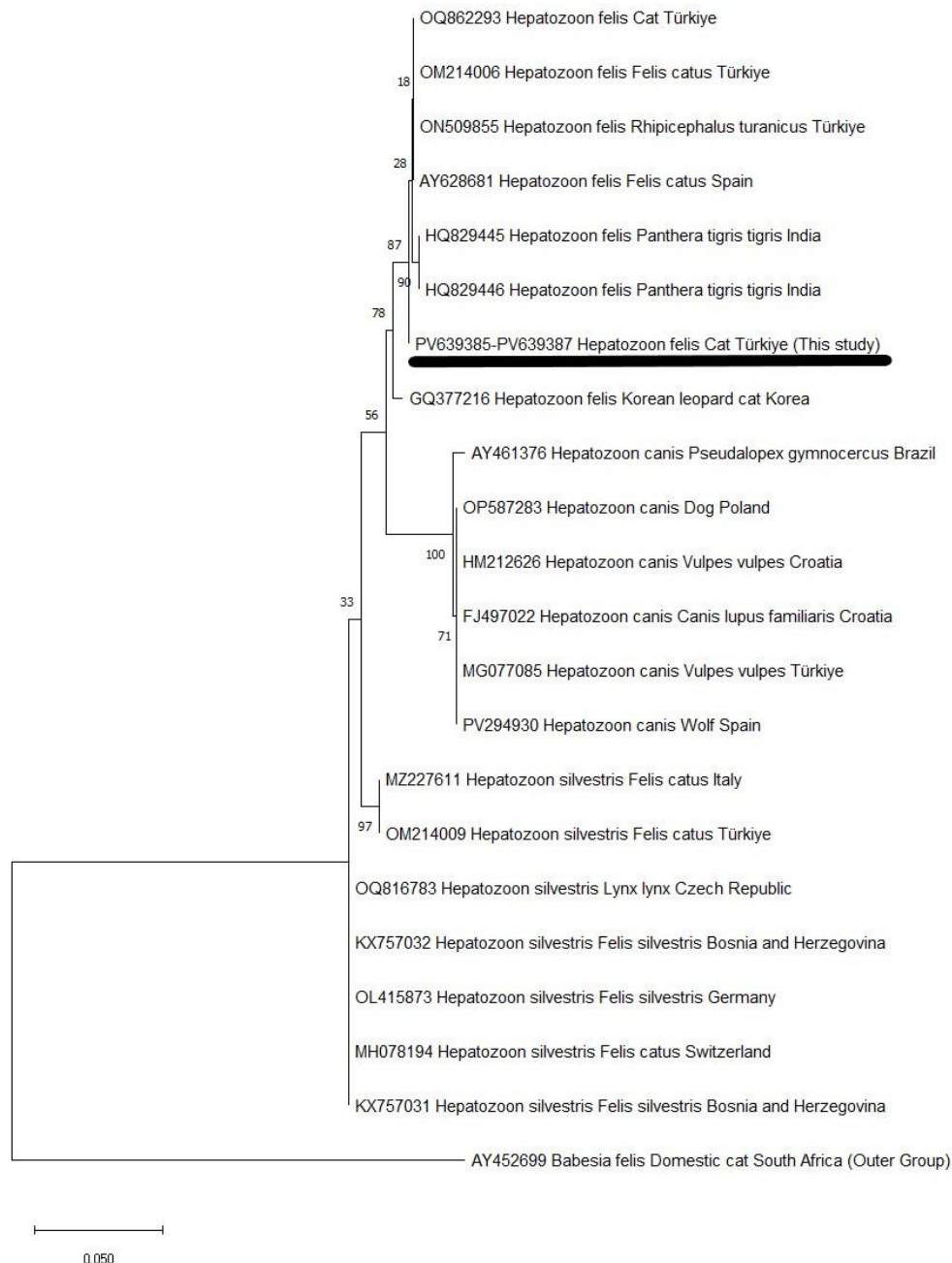


Figure 3. Phylogenetic tree of *H. felis* 18S SSU rRNA gene sequence. *Hepatozoon felis* isolates identified in the study are underlined. Numbers at the nodes represent the bootstrap values with 1,000 replicates. The evolutionary history was inferred by using the Maximum Likelihood method and TN93 + G model (39). The scale bar represents 0.050 substitutions per nucleotide position. Evolutionary analyses were conducted in MEGA-11 (38).

Discussion

Vector-borne and vector-borne diseases have become more important in recent years due to global warming and changing ecosystems (40, 41). Every year, billions of dollars are lost due to economic losses caused by infections and deaths resulting from vectors and vector-borne pathogens (41). It is known that *Hepatozoon* species may cause mild to severe infection in cats. According to the literature review, lots of studies have been conducted in the world for the determination of the presence and prevalence of *Hepatozoon* species in cats (2, 3, 7, 8, 10). However, limited data were present about the presence and prevalence of *Hepatozoon* species in cat population in Türkiye (9, 20, 42). In this study, *Hepatozoon* species were researched in cat blood samples collected from different climatic regions of Türkiye with molecular method. Moreover, phylogenetic analyses of positive samples were performed to reveal genetic relationship between *Hepatozoon* species.

Rapid and reliable detection of vector-borne diseases, such as *Hepatozoon* spp., in different hosts, is very important. In recent years, molecular identification techniques such as PCR have been used for the identification of *Hepatozoon* species due to their high sensitivity and specificity compared to microscopic methods (20). In recent years, various molecular studies have been carried out to the determination of *Hepatozoon* species in cats in different countries like Angola (43), Brazil (44-46), Cyprus (5), France (15), Greece (15, 47), Germany (48), Hungary (49), Israel (15, 50), Italy (12, 13, 15), Portugal (15, 51), Republic of Cape Verde (Africa) (19), Spain (6, 15, 52), and Thailand (10). In Türkiye, few studies have been conducted to determine the presence of *Hepatozoon* spp. in cats. In these studies, the prevalence was found as 10.8%, 2.37%, and 18.64% in Tekirdağ (42), İzmir (9) and Samsun (20), respectively. In the present study, *Hepatozoon* spp. was detected in 0.93% (95% CI 0.19-2.72) of cat blood samples obtained from diverse regions throughout Türkiye using a PCR test. Our prevalence value was lower than above-mentioned studies conducted in Türkiye. Studies performed in different parts of the world revealed that the prevalence of vector-borne pathogens, like *Hepatozoon* species, may be changed several factors, such as distribution and abundance of vector species in sampling areas, methods used in the studies, number of animals, genders and age of animals, health status of animals, predatory activities of animals, and characteristics of cats (i.e., shelter or owned cats) included studies (15, 20, 47, 48). It has also been reported that the low prevalence of *Hepatozoon* spp. infections in cats may vary depending on the seasonal time of sampling (as it may be affected by the seasonal variation in the activity of ticks and other vectors) (52). In this study, all animals researched in terms of *Hepatozoon* spp. were owned

cats. These animals are under the control of veterinarians. Therefore, it is thought that our prevalence value was lower than above-mentioned studies.

In recent years, DNA sequence analyses are used for several reasons, such as to verify PCR results, to perform phylogenetic analyses of pathogens, to detect novel genotypic variants, and to identify new species by researchers (13, 20, 34, 47). The *18S rRNA* gene region has been widely used in the detection of *Hepatozoon* species in hosts (11-13, 20, 50). In this study, the sequence of the partial region of the *18S rRNA* gene was performed in order to verify the genus-specific PCR results, to identify *Hepatozoon* species circulated in cat population, and to perform phylogenetic analyses of the positive samples. *Hepatozoon felis* consensus sequences obtained in this study were compared with other *H. felis* isolates uploaded to the GenBank, and high nucleotide similarities (97.72-100%) were seen. Despite the *18S rRNA* gene region has low genetic diversity among *H. felis* species, this gene is mostly used to identify *Hepatozoon* species in hosts from various parts of the world (2, 14, 20, 47, 50). However, the phylogenetic analyses were based on only three *Hepatozoon* spp. positive samples, which limits comprehensive inferences regarding the genetic diversity and phylogenetic relationships of *Hepatozoon* species circulating in Türkiye. Therefore, the phylogenetic findings should be interpreted with caution and supported by further molecular studies including a larger number of positive samples from different geographic regions.

As a result, in this study, *Hepatozoon* spp. were researched in cats using molecular and phylogenetic analysis. *Hepatozoon* spp. were found in cats (0.93%, 3/320) in different provinces of Türkiye. Furthermore, phylogenetic analyses indicated that the species belongs to *H. felis*. *Hepatozoon felis* was identified for the first time in Sivas and Malatya provinces with this study. This result will contribute to the understanding of the epidemiology of *H. felis* in Türkiye. The main limitation of this study is the low number of *Hepatozoon* spp. positive samples. The detection of only three positive cases among 320 samples limits the statistical power and reduces the reliability of regional comparisons. Therefore, the prevalence and regional distribution findings should be interpreted with caution, and further studies involving larger sample sizes, broader geographic coverage, and seasonal sampling are needed to confirm these results. Considering that *H. felis* can cause clinical symptoms in cats, it is thought that measures should be taken to protect cats from this pathogen. *H. canis* and *H. silvestris*, other *Hepatozoon* species seen in cats, were not detected in this study. However, it is thought that large-scale molecular studies are needed to understand the epidemiology of *Hepatozoon* species in cats.

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