Current Research in Parasitology & Vector-Borne Diseases 6 (2024) 100202

Contents lists available at ScienceDirect

Current Research in Parasitology & Vector-Borne Diseases

journal homepage: www.sciencedirect.com/journal/current-research-in-parasitology-and-vector-borne-diseases

Geographical isolation and hyperendemicity of *Hepatozoon felis*: Epidemiological scenario in Skopelos, Greece, and phylogenetic analysis

Simone Morelli [a,*], Donato Traversa [a], Angela Di Cesare [a], Mariasole Colombo [a], Marika Grillini [b], Barbara Paoletti [a], Aurora Mondazzi [a], Antonio Frangipane di Regalbono [b], Raffaella Iorio [a], Chiara Astuti [a], Constantina N. Tsokana [c], Anastasia Diakou [c]

[a] Department of Veterinary Medicine, University of Teramo, Località Piano d'Accio snc, 64100, Teramo, Italy
[b] Department of Animal Medicine, Production and Health, University of Padova, 35020, Legnaro, Padova, Italy
[c] School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece

ARTICLE INFO

Keywords:
Hepatozoonosis
Cats
Molecular biology
Epizootiology

ABSTRACT

Feline hepatozoonosis is a vector-borne disease caused by different species of the genus *Hepatozoon*, i.e. *Hepatozoon felis*, *Hepatozoon silvestris* and *Hepatozoon canis*. Knowledge on the biology, epidemiology and taxonomy of *Hepatozoon* spp. is still limited, despite the fact that the number of documented *Hepatozoon* spp. infections in domestic cats increased in recent years in different countries. This study was carried out to evaluate the prevalence and the genetic profile of *Hepatozoon* spp. in cats living on the island of Skopelos, Greece. Individual blood samples were collected from 54 owned cats and were subjected to Giemsa-stained blood smear examination to investigate the presence of *Hepatozoon* spp. gamonts and to a specific PCR protocol targeting the 18S rRNA gene of *Hepatozoon*. A total of 45 cats (83.3%) were found infected by *Hepatozoon* spp. by at least one of the methods applied. In particular, 43 (79.6%) of the cats were PCR-positive, and in 6 (11.1%) cats gamonts of *Hepatozoon* spp. were found in the blood smears. A total of 26 *H. felis* sequences were obtained and the presence of three undescribed single nucleotide polymorphisms were detected. The present results indicate that *H. felis* species complex may be hyperendemic in isolated/confined areas. In such contexts, geographical isolation may favor the origin of new genotypes or haplotypes or even new species.

1. Introduction

Tick-borne diseases (TBDs) are spreading in new, previously non-endemic areas of the world due to various factors. These include climate and land use changes, increased movement of animals and humans, and occasionally, economic and humanitarian crises (Diakou, 2024a, 2024b). However, TBDs are often overlooked in feline veterinary medicine and are less frequently documented compared to dogs (Day, 2016; Morelli et al., 2021a). The infrequent records of TBDs in cats are attributed to several factors, e.g. more successful immune response, poorly known or unknown pathogenicity of many tick-borne pathogens (TBPs), subclinical infections that develop to clinical only in case of concurrent immunosuppression, or factual low prevalence of TBPs (Day, 2016; Richards et al., 2017; Pereira et al., 2019).

Nevertheless, protozoans of the genus *Hepatozoon* are an exception because they cause infections which are increasingly described in cat populations in several areas (Baneth et al., 2013; Day, 2016; Richards et al., 2017; Pereira et al., 2019; Morelli et al., 2021b; Baneth and Allen, 2022).

Cats may be infected with three species of *Hepatozoon*, i.e. *Hepatozoon felis*, *Hepatozoon silvestris*, and *Hepatozoon canis*. The most frequently found is *H. felis*, which has been reported in all the continents except Australia (Allen et al., 2011; Harris et al., 2019; Morelli et al., 2021b; Panda et al., 2024; Traversa et al., 2024). *Hepatozoon silvestris* has been detected primarily in European wildcats (*Felis silvestris silvestris*), but it is also described in domestic cats from southern and central Europe (Giannelli et al., 2017; Hodžić et al., 2017; Kegler et al., 2018; Simonato et al., 2022). Domestic cats and wildcats are sporadically infected with *H. canis* (Baneth et al., 2013; Baneth and Allen, 2022).

Infections with *H. felis* cause mild inflammatory responses in myocardial and skeletal muscles and are often subclinical. When present, clinical signs are non-specific and may include fever, jaundice,

* Corresponding author.
E-mail address: smorelli@unite.it (S. Morelli).

https://doi.org/10.1016/j.crpvbd.2024.100202
Received 30 May 2024; Received in revised form 9 July 2024; Accepted 18 July 2024
Available online 19 July 2024

anorexia, lethargy, and lymphadenomegaly. The main laboratory findings are anemia, lymphopenia, mild neutropenia, and thrombocytopenia (Vilhena et al., 2017; Basso et al., 2019; Qurollo, 2019). Severe clinical manifestations (e.g. hemolytic anemia, renal failure) have also been described (Baneth et al., 1998; Basso et al., 2019). Conversely, *H. silvestris* seems more pathogenic, as cases of fatal myocarditis with pulmonary edema and intestinal intussusception have been reported (Kegler et al., 2018; Simonato et al., 2022).

Hepatozoon spp. are transmitted to vertebrates *via* the ingestion of hematophagous arthropods, typically ticks, harboring mature oocysts that contain sporozoites (Smith, 1996; Baneth et al., 2007). These invade cells of the liver, spleen, lymph nodes, muscles, and bone marrow, develop into meronts and release micromerozoites (Baneth et al., 2007; Baneth, 2011). The latter mature into gamonts in monocytes and neutrophils and are then ingested by ticks that feed on an infected host (Baneth et al., 2007; Baneth, 2011). At present, the life-cycles of feline *Hepatozoon* spp. have not been ultimately clarified. Nonetheless, biological features of *H. felis* and *H. silvestris* overlap those of the well-known *H. canis*, although feline *Hepatozoon* spp. infect predominantly muscular tissues rather than liver and spleen (Baneth et al., 2013; Baneth and Allen, 2022). The DNA of feline *Hepatozoon* spp. has been found in various ticks, e.g. *Rhipicephalus sanguineus* (*s.l.*), *Ixodes ricinus*, *Ixodes hexagonus*, *Haemaphysalis erinacei* and *Haemaphysalis sulcata* (Aktas, 2014; Maia et al., 2014; Duplan et al., 2018; Karasartova et al., 2018; Diakou et al., 2020). Most probably, *H. felis* and *H. silvestris* are also transmitted with the ingestion of ticks, favored by self-grooming, and can likely also be transmitted transplacentally or *via* predation (Baneth et al., 2013; Hodzic et al., 2017).

Recent data have suggested that *H. felis* is a species-complex encompassing multiple variants, including a worldwide predominant and widespread genotype (Harris et al., 2019; Morelli et al., 2021b; Panda et al., 2024; Traversa et al., 2024). It has also been hypothesized that different species or genotypes may have different biology and pathogenic potential (Harris et al., 2019; Morelli et al., 2021b). Additionally, different species or genotypes may circulate within different cat populations of the same country or in the same cat population of a confined area (Hodzic et al., 2017; Harris et al., 2019; Morelli et al., 2021b; Panda et al., 2024). Surveys have shown that *Hepatozoon* spp. may infect cat populations living in confined areas with very high prevalences in spite of a very low presence or absence of tick infestations (Morelli et al., 2021b; Traversa et al., 2024). In one of these scenarios, a genotype with a high genetic distance from *H. felis*, possibly representing a new undescribed species, was found on Skopelos Island in Greece a few years ago (Morelli et al., 2021b). The apparent spreading of cat hepatozoonosis in cats, the increasing number of clinical cases, and the poor knowledge of the taxonomy of feline *Hepatozoon* spp. call for further studies aiming to fill the present gaps. This study aims at updating the epidemiological scenario of feline hepatozoonosis in the confined area of Skopelos, four years after the above-mentioned study (Morelli et al., 2021b).

2. Materials and methods

In July 2023, individual blood samples in EDTA were collected from 54 privately owned cats living in Skopelos Island in the framework of routine clinical examination with the consent of the owners. All samples examined in the present study were surplus material from the blood sample needed for the routine examination of the cats. Signalment data and any clinical signs observed were recorded for each cat. The owners of the cats reported absent, or inconsistent ectoparasite prophylaxis. Among the cats included in the study, 49 had constant outdoor access, while 5 were housed indoors, with occasional outdoor access. Regarding sex, 26 were male and 28 were female. The age of the sampled cats ranged from a minimum of 9 months to a maximum of 15 years, the median age was 2 years-old with 31 cats being less than 2 years-old and 23 being over 2 years-old.

All samples were subjected to Giemsa-stained blood smear examination to detect *Hepatozoon* spp. gamonts under the microscope at 1000× magnification (Baneth et al., 2013).

DNA extraction was performed for each blood sample (using 200 μl) with a commercial kit (Exgene Blood extraction kit, GeneAll Biotech, Seoul, Korea), following the manufacturer's instructions. A fragment of approximately 373 bp of the 18S rRNA gene of *Hepatozoon* spp. was amplified by PCR as previously described (primer pair 5′-GGG GAT GAT GTC AAR TCA GCA C-3′ and 5′-CAC CAG CTT CGA GTT AAG CCA AT-3′) (Tabar et al., 2008; Morelli et al., 2021b). Amplicons were purified using a QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany) and then sequenced by Macrogen Italia (Milano, Italy). Purified amplicons were bidirectionally sequenced, and the presence of heteroplasmy or wrong base calls was ruled out by visually checking electropherograms. All sequences were compared with each other and with those available in GenBank using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997).

An alignment was produced using MEGA X software (Kumar et al., 2018) including *H. felis* sequences herein obtained, sequences recently obtained in cats from countries of the Mediterranean basin (Carbonara et al., 2023; GenBank: OP144204, OP144205, OP144206) and all sequences involved in recent phylogenetic analyses on *Hepatozoon* (Panda et al., 2024; Traversa et al., 2024). JModelTest (Posada, 2008) was used to determine the best-fitting substitution model to perform a maximum likelihood phylogenetic analysis. The evolutionary history was inferred by using the maximum likelihood method and Hasegawa-Kishino-Yano model with discrete gamma distribution to model evolutionary rate differences among sites (4 categories (+G, parameter = 2.3153)) allowing some of them to be invariable (+I, 51.02% sites) (Hasegawa et al., 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. The tree with the highest log-likelihood (−867.07) is shown. Nodal support is inferred from 1000 bootstrap replicates. The phylogenetic tree was rooted using *Haemogregarina podocnemis* (GenBank: MF476205.1) as the outgroup (Traversa et al., 2024).

All statistical analyses were performed using GraphPad Prism 10.1.1 software. Fisher's exact test was used to evaluate significant associations between positivity to *Hepatozoon* spp. and possible risk factors, i.e. age, sex, and presence of clinical signs. The presence of significant associations with the mentioned risk factors was also evaluated with a multiple logistic regression with a strength measured using the odds ratio (OR) and a 95% confidence interval.

3. Results

Overall, 45 out of 54 (83.3%) cats were infected with *Hepatozoon*. DNA of *Hepatozoon* spp. was detected in the blood samples of 43 out of 54 (79.6%) cats, while gamonts of *Hepatozoon* spp. (Fig. 1) were observed microscopically in the blood smears of 6 cats (11.1%) of which 2 were PCR-negative.

During the clinical examination, one cat (1.8%) had one tick attached, identified as *R. sanguineus*. Clinical signs were observed in 12 out of 54 (22.2%) cats, i.e. oculo-nasal discharge ($n = 8$ cats), cough ($n = 3$), sneezing ($n = 1$), conjunctivitis ($n = 1$) and weight loss ($n = 1$). Among them, 10 were positive at PCR, while two cats with oculo-nasal discharge were negative.

Sequences were obtained from 26 amplicons. Twenty-two sequences, here indicated as 1Sk (GenBank: PP815050), had 100% identity with *H. felis* from domestic cats found in Italy (GenBank: KY649442.1) (Giannelli et al., 2017) and 100% and 94.06% identity, respectively, with H1 and H2 previously reported in cats from the same area (Island of Skopelos, Greece) (Morelli et al., 2021b). Three undescribed single nucleotide polymorphisms (SNPs) were found in the phylogenetic analysis, i.e. 6Sk (1 sequence, GenBank: PP815051), 24Sk (1 sequence,

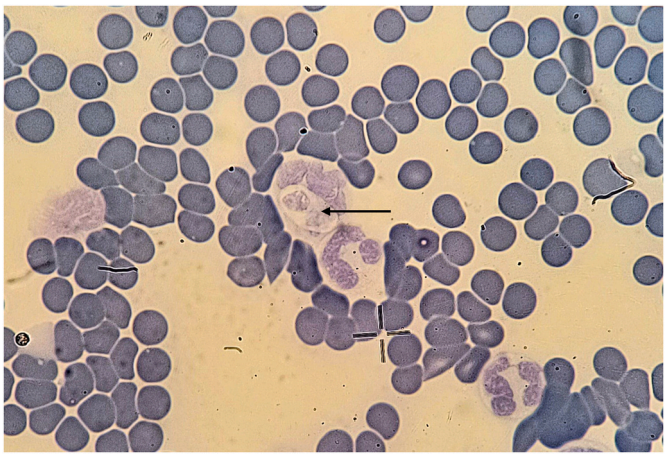

Fig. 1. *Hepatozoon felis* gamont (*arrow*) in a neutrophile (blood smear, Giemsa stain) in a cat from Skopelos Island.

GenBank: PP815052) and 49Sk (2 sequences, GenBank: PP815053) with 99.74% identity with the sequences KY649442.1 and H1, and 94.32%, 94.32% and 93.80%, respectively, with H2 (Morelli et al., 2021b). The sequence 1Sk showed 99.74% identity with 6Sk, 24Sk and 49Sk, respectively; 6Sk had 99.48% identity with 24Sk and 49Sk, that were also 99.48% identical between them.

The isolates 1Sk, 6Sk, 24Sk and 49Sk showed 97.93%, 97.67%, 97.67%, and 98.19% identity, respectively, with an isolate detected in a cat with severe clinical disease in Austria (Basso et al., 2019; GenBank: MK724001).

The phylogenetic analysis showed that all the isolates herein reported fall within the "Clade 1" described by Panda et al. (2024) and confirmed in Traversa et al. (2024) (Fig. 2).

No statistically significant associations were found between positivity to *Hepatozoon* spp. and the possible risk factors tested (Table 1).

4. Discussion

The results of the present study indicate that *H. felis* is hyperendemic on the Island of Skopelos. The positivity rate herein found exceeds the one recorded four years ago on the same island (25.5%) (Morelli et al., 2021b). The number of animals examined here is more than twice the number sampled in the previous study, confirming that the high prevalence of *H. felis* obtained in 2021 was not influenced by the low number of cats included, but rather represented a factual occurrence of the protozoan (Morelli et al., 2021b).

Large-scale or nationwide surveys in other countries on more than 100 cats have reported prevalences of *H. felis* infections ranging between 0.5% and 36% (Criado-Fornelio et al., 2006; Baneth et al., 2013; Grillini et al., 2021; Schäfer et al., 2022; Carbonara et al., 2023). Overall, infection rates higher than 25% have been detected in different areas of the world, e.g. South Africa, Thailand, and the Mediterranean basin (Jittapalapong et al., 2006; Baneth et al., 2013; Attipa et al., 2017; Harris et al., 2019; Morelli et al., 2021b). These may have been influenced by the detection of very high prevalence in certain sites or domestic cat populations. Data obtained in Cyprus (Attipa et al., 2017), Greece (Morelli et al., 2021b) and Brazil (Traversa et al., 2024) corroborate the present results, indicating that feline *H. felis* infections may occur with hyperendemic foci in confined areas. Infection rates may reach values from 37.9% up to ~83% in cat populations living in isolated geographies, even in the absence of arthropod infestations of the animals at the instant of sampling (Attipa et al., 2017; Morelli et al., 2021b; Traversa et al., 2024; present study). Although biological and epidemiological knowledge on *H. felis* is poor, outdoor access is a known risk factor for feline hepatozoonosis. This was not included in the present statistical analysis due to a significant imbalance between the number of cats with a predominant outdoor lifestyle and those without. Inconsistent or no administration of ectoparasiticides may have influenced the high positivity rate detected here. *Rhiphicephalus sanguineus* has been regarded as one of the potential main vectors of *H. felis* (Bhusri et al., 2017). Notably, this tick species is present on the Island of Skopelos, as documented in the present study, as well as on other Greek islands where the occurrence of *H. felis* has been previously documented at high rates, such as the Island of Crete (Morelli et al., 2021b). Nevertheless, alternative routes of transmission have been hypothesized for *H. felis*, e.g. vertically or through predation (i.e. ingesting preys/ticks attached on preys) which may intensify the presence of this protozoan in a specific area (Baneth et al., 2013; Schäfer et al., 2022), and explain the high positivity rate recorded here.

The absence of statistical correlation between the presence of clinical signs and positivity to *H. felis* supports previous data and confirms that most cats infected with *H. felis* remain asymptomatic (Baneth et al., 2013; Giannelli et al., 2017; Grillini et al., 2021). The fact that many of the PCR-positive cats displayed respiratory clinical signs should be interpreted with caution. Upper respiratory tract diseases are commonly found in outdoor cats (Gao et al., 2023) and, since comorbidities were not evaluated here, their involvement in causing respiratory clinical signs in the present cat population cannot be excluded.

The low number of cats positive for *Hepatozoon* spp. gamonts at blood smear microscopy is in line with previous studies (Jittapalapong et al., 2006; Baneth et al., 2013; Morelli et al., 2021b). Accordingly, PCR is the most sensitive method for detecting *Hepatozoon* spp., as infected cats typically have low parasitemia (Baneth, 2011; Basso et al., 2019; Pereira et al., 2019). Nevertheless, the sensitivity of PCR for *Hepatozoon* spp. is not 100%, and a combined use of both techniques is herein suggested as gamonts of *Hepatozoon* may also be present in PCR-negative cats. A higher number of samples positive for gamonts could have been probably found examining the buffy coat smear (Otranto et al., 2011). However, this was not possible in this study due to the low amounts of blood available for each cat, as all the samples were obtained from surplus material.

The results of the phylogenetic analysis suggest the presence of three new SNPs within the *H. felis* Clade 1 described by Panda et al. (2024) and recently confirmed in South America (Traversa et al., 2024). All sequences generated in the present study clustered together with sequence types recently obtained from cats living throughout the Mediterranean basin, including Greece (Carbonara et al., 2023). The high genetic variability of *H. felis* is ultimately confirmed, thus indicating that this species complex may undergo rapid and constant genetic variation in isolated/confined areas. The factual impact of the existence of a high number of genotypes is currently unknown. A similar scenario has been reported for the closely related *H. canis*, for which several haplotypes have been described (Vásquez-Aguilar et al., 2021). At present, there is still no evidence of a higher or lower pathogenicity of a given genotype of *H. canis*. Interestingly, the two 49Sk isolates herein found had a higher grade of identity with an isolate involved in a severe case of feline hepatozonoosis in a cat from Austria (Basso et al., 2019), if compared to previous isolates detected in Greece (Morelli et al., 2021b). However, none of the two cats had clinical signs. Hence, a possible higher pathogenicity linked to the different genotypes of *H. felis* (Morelli et al., 2021b; Traversa et al., 2024) should be further evaluated in future studies.

At present, five genotypes of *H. felis* have been detected on Skopelos Island and, among them, haplotype H2 (Morelli et al., 2021b) may represent a new species as it had high genetic distance from the other four genotypes described here and in Morelli et al. (2021b). Even though isolates identical to H2 have not been reported in the present study, the presence of multiple genotypes in an isolated area can have important epidemiological implications. In fact, geographical isolation, that hinders the process of gene flow, is regarded as one of the key factors leading to allopatric speciation (Coyne, 1992; Sobel et al., 2010; Matute

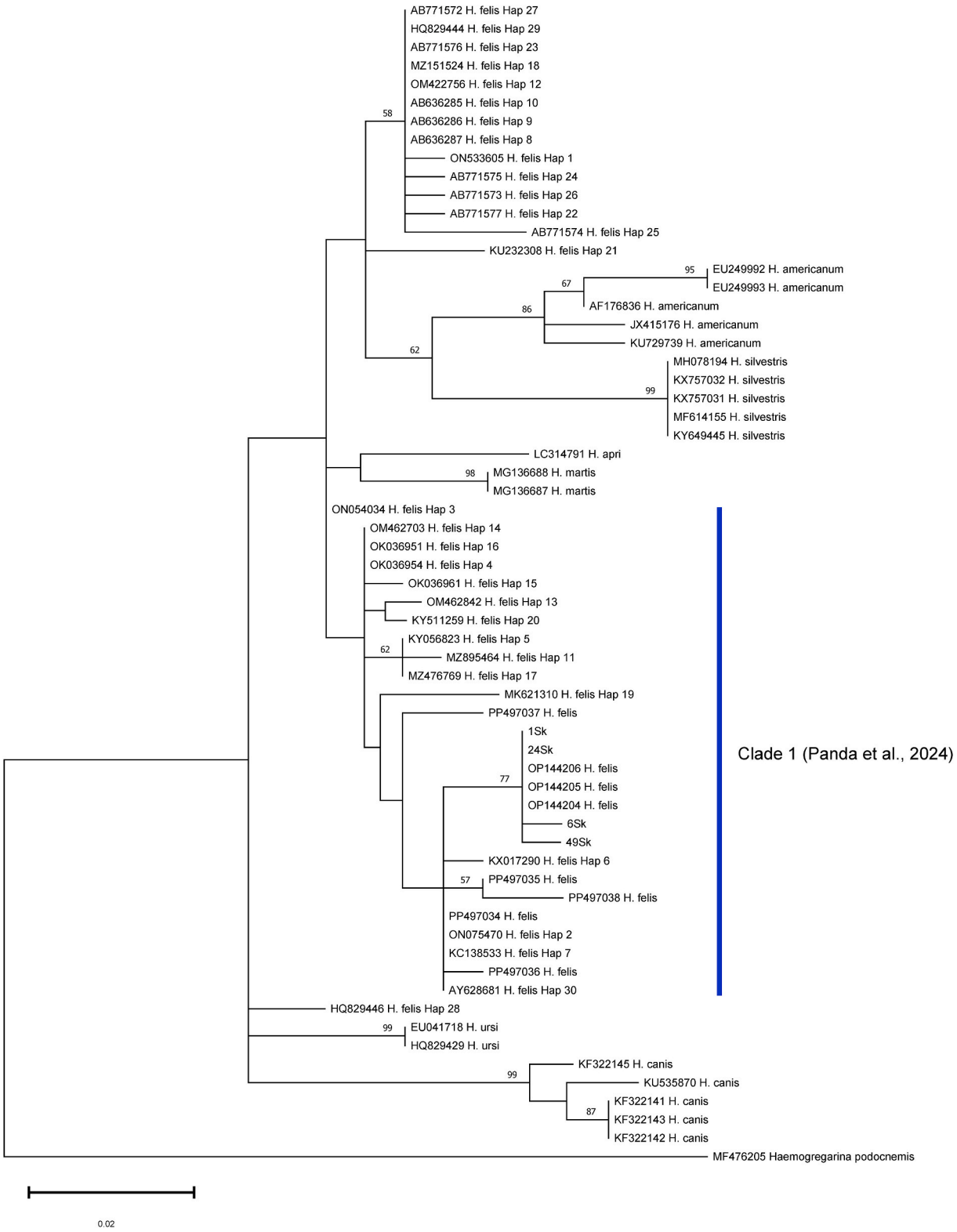

Fig. 2. Phylogenetic tree inferred by maximum likelihood showing relationships between sequences obtained in the present study and those included in recent phylogenetic analyses by Panda et al. (2024) and Traversa et al. (2024), and a recent epidemiological study (Carbonara et al., 2023). The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. This analysis involved 63 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 216 positions in the final dataset. Nodal support is shown above the branches. The newly generated sequences are labelled as follows: 1Sk (GenBank: PP815050); 6Sk (GenBank: PP815051); 24Sk (PP815052); 49Sk (PP815053).

Table 1
Results of the multiple logistic regression analysis. No significant statistical associations were found in this study.

Variable	*P*-value	OR	95% CI
Presence of clinical signs	0.8345	1.203	0.2382–8.976
Male sex	0.8667	0.8868	0.2115–3.695
> 2 years old	0.1309	2.995	0.7467–13.670

Abbreviations: OR, odds ratio; CI, confidence interval.

and Cooper, 2021). Therefore, it is likely that epidemiological scenarios as the one emerged in Skopelos and in other islands of the Mediterranean basin, e.g. Crete (Morelli et al., 2021b) and Cyprus (Attipa et al., 2017) may favor the origin of new *H. felis* genotypes, or even of new *Hepatozoon* species, with different biological features and pathogenicity. On the whole, further molecular studies, e.g. evaluating other genetic markers such as the ITS, are needed to clarify the phylogenetic status of *Hepatozoon* spp. infecting felines.

5. Conclusions

New insights on the epidemiology of *H. felis* have emerged from data of the present study confirming that this protozoan may occur with hyperendemic foci in cats living in isolated areas. In this context, it should be kept in mind that despite the predominant subclinical nature of *H. felis* infections in cats, moderate to severe clinical implications have been described (Lloret et al., 2015; Basso et al., 2019; Qurollo, 2019). Also, information on potential implications in exacerbating co-morbidities, or in fetal and/or peri-natal diseases as suggested for *H. canis* (De Bonis et al., 2021) is lacking. Therefore, further studies aiming at implementing biological and clinical knowledge on feline *Hepatozoon* species and/or genotypes are herein encouraged.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval

No ethical permission was necessary as all samples were obtained from surplus material deriving from routine clinical procedures.

Data availability

The data supporting the conclusions of this article are included within the article. The raw data are provided in Supplementary Table S1. The newly generated sequences were submitted to the GenBank database under the accession numbers PP815050-PP815053.

CRediT authorship contribution statement

Simone Morelli: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Donato Traversa:** Conceptualization, Investigation, Writing – review & editing, Supervision. **Angela Di Cesare:** Conceptualization, Investigation, Writing – review & editing, Supervision. **Mariasole Colombo:** Investigation. **Marika Grillini:** Investigation. **Barbara Paoletti:** Investigation. **Aurora Mondazzi:** Investigation. **Antonio Frangipane di Regalbono:** Conceptualization. **Raffaella Iorio:** Investigation. **Chiara Astuti:** Investigation. **Constantina N. Tsokana:** Investigation. **Anastasia Diakou:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are grateful to Leonardo Brustenga for his support in the phylogenetic analysis and to the veterinarians Niki Lemoni and Dimitris Papaioannou, owners of the clinic Vetcare in Skopelos for their invaluable help in collecting the samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2024.100202.

References

Aktas, M., 2014. A survey of ixodid tick species and molecular identification of tick-borne pathogens. Vet. Parasitol. 200, 276–283. https://doi.org/10.1016/j.vetpar.2013.12.008.

Allen, K.E., Yabsley, M.J., Johnson, E.M., Reichard, M.V., Panciera, R.J., Ewing, S.A., Little, S.E., 2011. Novel *Hepatozoon* in vertebrates from the southern United States. J. Parasitol. 97, 648–653. https://doi.org/10.1645/GE-2672.1.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402. https://doi.org/10.1093/nar/25.17.3389.

Attipa, C., Papasouliotis, K., Solano-Gallego, L., Baneth, G., Nachum-Biala, Y., Sarvani, E., et al., 2017. Prevalence study and risk factor analysis of selected bacterial, protozoal and viral, including vector-borne, pathogens in cats from Cyprus. Parasites Vectors 10, 130. https://doi.org/10.1186/s13071-017-2063-2.

Baneth, G., Aroch, I., Tal, N., Harrus, S., 1998. *Hepatozoon* species infection in domestic cats: a retrospective study. Vet. Parasitol. 79, 123–133. https://doi.org/10.1016/s0304-4017(98)00160-5.

Baneth, G., 2011. Perspectives on canine and feline hepatozoonosis. Vet. Parasitol. 181, 3–11. https://doi.org/10.1016/j.vetpar.2011.04.015.

Baneth, G., Allen, K., 2022. Hepatozoonosis of dogs and cats. Vet. Clin. North Am. Small Anim. Pract. 52, 1341–1358. https://doi.org/10.1016/j.cvsm.2022.06.011.

Baneth, G., Samish, M., Shkap, V., 2007. Life cycle of *Hepatozoon canis* (Apicomplexa: Adeleorina: Hepatozoidae) in the tick *Rhipicephalus sanguineus* and domestic dog (*Canis familiaris*). J. Parasitol. 93, 283–299. https://doi.org/10.1645/GE-494R.1.

Baneth, G., Sheiner, A., Eyal, O., Hahn, S., Beaufils, J.P., Anug, Y., Talmi-Frank, D., 2013. Redescription of *Hepatozoon felis* (Apicomplexa: Hepatozoidae) based on phylogenetic analysis, tissue and blood form morphology, and possible transplacental transmission. Parasites Vectors 6, 102. https://doi.org/10.1186/1756-3305-6-102.

Basso, W., Görner, D., Globokar, M., Keidel, A., Pantchev, N., 2019. First autochthonous case of clinical *Hepatozoon felis* infection in a domestic cat in Central Europe. Parasitol. Int. 72, 101945 https://doi.org/10.1016/j.parint.2019.101945.

Bhusri, B., Sariya, L., Mongkolphan, C., Suksai, P., Kaewchot, S., Changbunjong, T., 2017. Molecular characterization of *Hepatozoon felis* in *Rhipicephalus sanguineus* ticks infested on captive lions (*Panthera leo*). J. Parasit. Dis. 41, 903–907. https://doi.org/10.1007/s12639-017-0902-x.

Carbonara, M., Iatta, R., Sgroi, G., Papadopoulos, E., Lima, C., Bouhsira, E., et al., 2023. *Hepatozoon* species infecting domestic cats from countries of the Mediterranean basin. Ticks Tick Borne Dis. 14, 102192 https://doi.org/10.1016/j.ttbdis.2023.102192.

Coyne, J.A., 1992. Genetics and speciation. Nature 355, 511–515. https://doi.org/10.1038/355511a0.

Criado-Fornelio, A., Ruas, J.L., Casado, N., Farias, N.A., Soares, M.P., Müller, G., et al., 2006. New molecular data on mammalian *Hepatozoon* species (Apicomplexa: Adeleorina) from Brazil and Spain. J. Parasitol. 92, 93–99. https://doi.org/10.1645/GE-464R.1.

Day, M.J., 2016. Cats are not small dogs: Is there an immunological explanation for why cats are less affected by arthropod-borne disease than dogs? Parasites Vectors 9, 507. https://doi.org/10.1186/s13071-016-1798-5.

De Bonis, A., Colombo, M., Terragni, R., Bacci, B., Morelli, S., Grillini, M., Vignoli, M., 2021. Potential role of *Hepatozoon canis* in a fatal systemic disease in a puppy. Pathogens 10, 1193. https://doi.org/10.3390/pathogens10091193.

Diakou, A., 2024a. Biting back: advances in fighting ticks and understanding tick-borne pathogens. Pathogens 13, 73. https://doi.org/10.3390/pathogens13010073.

Diakou, A., 2024b. Pet travellers' unwanted companions: a parasitic voyage across Europe. Companion Animals 29, 50–61. https://doi.org/10.12968/coan.2023.0060.

Diakou, A., Dimzas, D., Astaras, C., Savvas, I., Di Cesare, A., Morelli, S., et al., 2020. Clinical investigations and treatment outcome in a European wildcat (*Felis silvestris silvestris*) infected by cardio-pulmonary nematodes. Vet. Parasitol. Reg. Stud. Reports 19, 100357. https://doi.org/10.1016/j.vprsr.2019.100357.

Duplan, F., Davies, S., Filler, S., Abdullah, S., Keyte, S., Newbury, H., et al., 2018. *Anaplasma phagocytophilum, Bartonella* spp., haemoplasma species and *Hepatozoon* spp. in ticks infesting cats: A large-scale survey. Parasites Vectors 11, 201. https://doi.org/10.1186/s13071-018-2789-5.

Gao, J., Li, Y., Xie, Q., Al-Zaban, M.I., Al-Saeed, F.A., Shati, A.A., et al., 2023. Epidemiological investigation of feline upper respiratory tract infection encourages a geographically specific FCV vaccine. Vet. Sci. 10, 46. https://doi.org/10.3390/vetsci10010046.

Giannelli, A., Latrofa, M.S., Nachum-Biala, Y., Hodžić, A., Greco, G., Attanasi, A., et al., 2017. Three different *Hepatozoon* species in domestic cats from southern Italy. Ticks Tick Borne Dis. 8, 721–724. https://doi.org/10.1016/j.ttbdis.2017.05.005.

Grillini, M., Simonato, G., Tessarin, C., Dotto, G., Traversa, D., Cassini, R., et al., 2021. *Cytauxzoon* sp. and *Hepatozoon* spp. in domestic cats: A preliminary study in north-eastern Italy. Pathogens 10, 1214. https://doi.org/10.3390/pathogens10091214.

Harris, D.J., Halajian, A., Santos, J., Rampedi, K.M., Xavier, R., 2019. Genetic diversity of *Hepatozoon* (Apicomplexa) from domestic cats in South Africa, with a global reassessment of *Hepatozoon felis* diversity. J. S. Afr. Vet. Assoc. 90, e1–e6. https://doi.org/10.4102/jsava.v90i0.1747.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174. https://doi.org/10.1007/BF02101694.

Hodžić, A., Alić, A., Prašović, S., Otranto, D., Baneth, G., Duscher, G.G., 2017. *Hepatozoon silvestris* sp. nov.: Morphological and molecular characterization of a new species of *Hepatozoon* (Adeleorina: Hepatozoidae) from the European wild cat (*Felis silvestris silvestris*). Parasitology 144, 650–661. https://doi.org/10.1017/S0031182016002316.

Jittapalapong, S., Rungphisutthipongse, O., Maruyama, S., Schaefer, J.J., Stich, R.W., 2006. Detection of *Hepatozoon canis* in stray dogs and cats in Bangkok, Thailand. Ann. N. Y. Acad. Sci. 1081, 479–488. https://doi.org/10.1196/annals.1373.071.

Karasartova, D., Gureser, A.S., Gokce, T., Celebi, B., Yapar, D., Keskin, A., et al., 2018. Bacterial and protozoal pathogens found in ticks collected from humans in Corum Province of Turkey. PLoS Negl. Trop. Dis. 12, e0006395 https://doi.org/10.1371/journal.pntd.0006395.

Kegler, K., Nufer, U., Alic, A., Posthaus, H., Olias, P., Basso, W., 2018. Fatal infection with emerging apicomplexan parasite *Hepatozoon silvestris* in a domestic cat. Parasites Vectors 11, 428. https://doi.org/10.1186/s13071-018-2992-4.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. Mega X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096.

Lloret, A., Addie, D.D., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., et al., 2015. Hepatozoonosis in cats: ABCD guidelines on prevention and management. J. Feline Med. Surg. 17, 642–644. https://doi.org/10.1177/1098612X15589879. PMID: 26101318.

Maia, C., Ferreira, A., Nunes, M., Vieira, M.L., Campino, L., Cardoso, L., 2014. Molecular detection of bacterial and parasitic pathogens in hard ticks from Portugal. Ticks Tick Borne Dis. 5, 409–414. https://doi.org/10.1016/j.ttbdis.2014.01.009.

Matute, D.R., Cooper, B.S., 2021. Comparative studies on speciation: 30 years since Coyne and Orr. Evolution 75, 764–778. https://doi.org/10.1111/evo.14181.

Morelli, S., Diakou, A., Di Cesare, A., Colombo, M., Traversa, D., 2021a. Canine and feline parasitology: analogies, differences, and relevance for human health. Clin. Microbiol. Rev. 34, e0026620 https://doi.org/10.1128/CMR.00266-20.

Morelli, S., Diakou, A., Traversa, D., Di Gennaro, E., Simonato, G., Colombo, M., et al., 2021b. First record of *Hepatozoon* spp. in domestic cats in Greece. Ticks Tick Borne Dis. 12, 101580 https://doi.org/10.1016/j.ttbdis.2020.101580.

Otranto, D., Dantas-Torres, F., Weigl, S., Latrofa, M.S., Stanneck, D., Decaprariis, D., et al., 2011. Diagnosis of *Hepatozoon canis* in young dogs by cytology and PCR. Parasites Vectors 4, 55. https://doi.org/10.1186/1756-3305-4-55.

Panda, R., Nehra, A.K., Ram, H., Karikalan, M., Garg, R., Nala, R.R., Pawde, A.M., 2024. Phylogenetic analysis and haplotype networking of *Hepatozoon felis* infecting wild animals in Gir National Park, Gujarat, India. Parasitol. Res. 123, 92. https://doi.org/10.1007/s00436-023-08109-6.

Pereira, C., Maia, J.P., Marcos, R., Luzzago, C., Puente-Payo, P., Dall'Ara, P., et al., 2019. Molecular detection of *Hepatozoon felis* in cats from Maio Island, Republic of Cape Verde and global distribution of feline hepatozoonosis. Parasites Vectors 12, 294. https://doi.org/10.1186/s13071-019-3551-3.

Posada, D., 2008. jModelTest: Phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256. https://doi.org/10.1093/molbev/msn083.

Qurollo, B., 2019. Feline vector-borne diseases in North America. Vet. Clin. North Am. Small Anim. Pract. 49, 687–702. https://doi.org/10.1016/j.cvsm.2019.02.012.

Richards, S.L., Langley, R., Apperson, C.S., Watson, E., 2017. Do tick attachment times vary between different tick-pathogen systems? Environments 4, 37.

Schäfer, I., Kohn, B., Nijhof, A.M., Müller, E., 2022. Molecular detection of *Hepatozoon* species infections in domestic cats living in Germany. J. Feline Med. Surg. 24, 994–1000. https://doi.org/10.1177/1098612X211055680.

Simonato, G., Franco, V., Salvatore, G., Manzocchi, S., Dotto, G., Morelli, S., et al., 2022. First autochthonous clinical case of *Hepatozoon silvestris* in a domestic cat in Italy with unusual presentation. Parasites Vectors 15, 440. https://doi.org/10.1186/s13071-022-05534-x.

Smith, T.G., 1996. The genus *Hepatozoon* (Apicomplexa: Adeleina). J. Parasitol. 82, 565–585.

Sobel, J.M., Chen, G.F., Watt, L.R., Schemske, D.W., 2010. The biology of speciation. Evolution 64, 295–315. https://doi.org/10.1111/j.1558-5646.2009.00877.x.

Tabar, M.D., Altet, L., Francino, O., Sánchez, A., Ferrer, L., Roura, X., 2008. Vector-borne infections in cats: Molecular study in Barcelona area (Spain). Vet. Parasitol. 151, 332–336. https://doi.org/10.1016/j.vetpar.2007.10.019.

Traversa, D., Di Cesare, A., Morelli, S., Paoletti, B., Grillini, M., Frangipane Di Regalbono, A., et al., 2024. *Hepatozoon* spp. in stray cats from the metropolitan area of Rio de Janeiro, Brazil. Parasite 31, 24. https://doi.org/10.1051/parasite/2024026.

Vásquez-Aguilar, A.A., Barbachano-Guerrero, A., Angulo, D.F., Jarquín-Díaz, V.H., 2021. Phylogeography and population differentiation in *Hepatozoon canis* (Apicomplexa: Hepatozoidae) reveal expansion and gene flow in world populations. Parasites Vectors 14, 467. https://doi.org/10.1186/s13071-021-04924-x.

Vilhena, H., Tvarijonaviciute, A., Cerón, J.J., Vieira, L., Pastor, J., Silvestre-Ferreira, A. C., 2017. Acute phase proteins response in cats naturally infected with *Hepatozoon felis* and *Babesia vogeli*. Vet. Clin. Pathol. 46, 72–76. https://doi.org/10.1111/vcp.12451.