University of Veterinary Medicine
Doctoral School of Veterinary Science

Molecular epidemiological investigation of unicellular parasites of companion animals

PhD dissertation

Barbara Tuska-Szalay

2025

Supervisor and consultants:

.....

Sándor Hornok DSc.
Department of Parasitology and Zoology
University of Veterinary Medicine Budapest, Hungary
supervisor

Róbert Farkas DSc
Department of Parasitology and Zoology
University of Veterinary Medicine Budapest, Hungary
consultant

Attila D. Sándor PhD
Department of Parasitology and Parasitic Diseases
University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania
consultant

Copy…of four

.....

Barbara Tuska-Szalay

Table of contents

Abbreviations

PCR	Polymerase Chain Reaction
CNS	Central Nervous System
MPS	Mononuclear Phagocytic System
s.s.	sensu stricto
Mhc	*Mycoplasma haemocanis*
CMhp	*Candidatus* Mycoplasma haematoparvum
AK	Acanthamoeba Keratitis
GAE	Granulomatous Amoebic Encephalitis
DIC	Disseminated Intravascular Coagulation
ATV	Atovaquone
°C	Celsius degree
IFAT	Indirect Fluorescent Antibody Test
ELISA	Enzyme-Linked Immunosorbent Assay
EPM	Equine Protozoal Myeloencephalitis
*cox*1	cytochrome c oxidase subunit 1
cytb	cytochrome b
BARF	Biologically Appropriate Raw Food/ Bone And Raw Food
bg	beta-giardin
gdh	glutamate dehydrogenase
tpi	triosephosphate isomerase
n.s.	not specified
min	minute
s	secundum
HCT	hematocrit
RBC	red blood cell count
HGB	hemoglobin concentration

1. Summary

Since the number of pet animals has increased worldwide in the last decades, and the diagnostic tools in veterinary parasitology also developed – ensuring access to more information on parasites –, the aim of the present study was to reveal the presence of different protozoa of various companion animals, and to examine their genetic diversity depending on the possible ways of their transmission. Therefore, altogether 1039 samples were collected from companion animals and examined for the presence of unicellular parasites with traditional parasitological methods and molecular biological methods including phylogenetic analysis.

First, 164 samples were collected from five rodent species and rabbits in five locations in Hungary, to examine the presence of *G. duodenalis*. Parasitological analysis revealed the presence of cysts in 58.3% of asymptomatic Norway rats and 27.6% of chinchillas. Three degus were also found *Giardia*-infected (prevalence: 16.7%) using flotation technique. With PCR targeting three genetic markers, 3.2% of the samples showed positivity, whereas a rate of 21.9% prevalence was detected with flotation. The PCR products of five samples could be DNA sequenced. Phylogenetic analysis based on the partial sequences of the beta-giardin gene revealed the presence of assemblages B and G in rats. In addition, assemblage E was detected in a beaver, while assemblage B was present in a chinchilla. The results show that synanthropic rodent species have different epidemiological roles in the study region, depending on the prevalence of shedding *Giardia* cysts or harboring zoonotic variants of *G. duodenalis*. Moreover, our findings confirm that pet rodents may pose a risk for zoonotic *Giardia*-transmission.

Oropharyngeal swab samples were collected in Hungary and Romania from 99 columbiform birds, including 76 feral pigeons (*Columba livia domestica*: 42 kept for racing, 32 with urban and two with rural habitat), four common wood pigeons (*C. palumbus*), 16 ring doves (*Streptopelia risoria*) and three Eurasian collared doves (*S. decaocto*). These samples were analyzed for the presence of *T. gallinae* using molecular methods. Racing feral pigeons had significantly higher prevalence of *T. gallinae* infection than urban feral pigeons. The rate of PCR-positivity was the highest among wood pigeons and ring doves. Based on 18S rRNA gene, *T. gallinae* was the most heterogenous among racing feral pigeons sampled in a trading-breeding place. Clinical signs were associated with only one 18S rRNA gene subtype. The most divergent 18S rRNA gene subtype, *Trichomonas* sp. Hu-TG37 clustered with *T. canistomae* and *T. tenax* and represents probably a new species. To our knowledge, this is the first report on the genetic diversity of *T. gallinae* in the southern central and southeastern European region. The results suggest that most detected *T. gallinae* 18S rRNA gene subtypes are not host-specific and do not cause clinical signs. The highest number of 18S rRNA gene subtypes was demonstrated among racing feral pigeons. Significantly more captive than free-

living columbiform birds had *T. gallinae* infection. These data highlight the importance of epizootic monitoring of the genetic diversity and presence of *T. gallinae* in trading-breeding places of pigeons and doves.

In a further investigation, four wildcats, 94 domestic cats, and 25 dogs, originating from 18 different locations in Hungary, were investigated for the presence of oral and large intestinal trichomonads based on the 18S rRNA gene and ITS2. All oral swabs were negative by PCR. However, *Tritrichomonas foetus* was detected in a high proportion among tested domestic cats (13.8%) and dogs (16%), and *Pentatrichomonas hominis* only in two domestic cats. In addition, a novel *Tritrichomonas* genotype was identified in one cat, probably representing a new species that was shown to be phylogenetically most closely related to *Tritrichomonas casperi* described recently from mice. All positive dogs and half of the positive cats showed symptoms, and among cats, the most frequently infected breed was the Ragdoll. With molecular methods, this study evaluated the prevalence of oral and intestinal trichomonads in clinical samples of dogs and cats from Hungary, providing the first evidence of *T. foetus* in dogs of this region. In contrast to literature data, *P. hominis* was more prevalent in cats than in dogs. Finally, a hitherto unknown large intestinal *Tritrichomonas* species (closely related to *T. casperi*) was shown to be present in a cat, raising two possibilities. First, this novel genotype might have been a rodent-associated pseudoparasite in the relevant cat. Otherwise, the cat was actually infected, thus suggesting the role of a predator-prey link in the evolution of this trichomonad.

At a reptile farm in Ireland, fecal samples were collected from 98 captive reptiles, representing 43 species of three orders (Squamata, Testudines, and Crocodylia). After DNA extraction, all samples were screened by conventional PCRs, targeting the ribosomal small subunit (SSU) RNA and alpha-tubulin genes of trichomonads and SSU RNA gene of *Acanthamoeba* spp. One leopard gecko (*Eublepharis macularius*) was positive for a not yet reported species/genotype of the genus *Monocercomonas*, different from *M. colubrorum*. Various *Acanthamoeba* genotypes were detected in six reptilian species, i.e., *Acanthamoeba* genotype T11 in *Eunectes notaeus* and *Heloderma suspectum*/*horridum*; genotype T4 in *Varanus exanthematicus*, *Chlamydosaurus kingii*, and *Macrochelys temminckii*; and the genotype T13 in *Iguana iguana*. Some of these amoeba species might have clinicopathological significance in both humans and animals. Our findings highlight the importance of monitoring pathogenic protozoa in pet as well as wildlife reptiles, as a source of possible infection for animals and humans living nearby.

Fecal samples of 89 pet animals kept on BARF diet were subjected to coprological examination followed by molecular analyses. Oocysts of *Cystoisospora canis*, a *Cystoisospora ohioensis*-like sp. and *Eimeria stiedai*, as well as sporocysts of a *Sarcocystis* sp. were detected. All samples were negative for *Neospora caninum* and *Toxoplasma gondii*. In conclusion, no evidence was found for the infection of BARF-fed dogs and cats with parasites

that are usually associated with this diet and considered as clinico-pathological risk factors for these pets themselves (e.g., *N. caninum*, *T. gondii*). However, BARF-feeding may contribute to the contamination of the environment with *E. stiedai* oocysts, thus increasing the risks of biliary coccidiosis in nearby living pet rabbits that would otherwise not have access to oocysts of *E. stiedai.*

Fecal samples of four American Staffordshire terrier dogs (used for illegal fighting) were analyzed by DNA extraction, molecular-phylogenetic and parasitological methods, in order to examine the occurrence of protozoan, apicomplexan parasites. In one sample, the DNA of *Sarcocystis morae* was shown to be present. This species was identified based on 100% identity with already reported sequences of *S. morae* from cervids in Lithuania and Spain. The result was also confirmed by phylogenetic analysis. The sporocysts of the canine *S. morae* isolate measured 14.95 × 9.75 µm on average. This is the first molecular evidence in support of the final host role of domestic dogs in the life cycle of *S. morae*. The most likely source of the infection was raw meat given to the examined dog to increase its physical achievement. In conclusion, under similar circumstances dogs may participate in the life cycle of *S. morae* in a 'natural way', shedding sporocysts/oocysts when used for hunting or taken to walks in forested areas.

To investigate the presence of *Hepatozoon felis* and *Cytauxzoon europaeus* 127 domestic cats and 4 wildcats were screened by PCRs targeting the 18S rRNA gene of *Hepatozoon* spp. and piroplasms, as well as the *cytb* gene of *Cytauxzoon* spp. The samples were collected inside and outside a region of Hungary, where both protozoan groups are endemic in wildcats. Among domestic cats, one proved to be infected with *H. felis*. Furthermore, spleen samples of four wildcats were also examined, among which three tested positive for *H. felis*, and one had co-infection with *C. europaeus*. Importantly, *H. felis* from the co-infected wildcat belonged to genogroup II, similarly to *H. felis* from the positive domestic cat. Based on phylogenetic evidence, this genogroup probably represents a separate species from genogroup I of *H. felis*, which was hitherto reported from Mediterranean countries in Europe. The two other wildcats also harbored *H. felis* from genogroup I. Neither *Hepatozoon* nor *Cytauxzoon* infections were detected outside the recently discovered endemic region. In conclusion, this study demonstrates for the first time in Europe that *H. felis* from genogroup II may emerge in free-roaming domestic cats in regions where this protozoan parasite is endemic in wildcats.

Concerning blood samples from 79 American Staffordshire Terrier dogs, confiscated for illegal dog fights, were molecularly analyzed for tick-borne pathogens. *Babesia gibsoni* was detected in 32 dogs, i.e. with a prevalence of 40.5%. In addition, *Babesia vulpes* was found in 8 samples (prevalence of 10.1%), for the first time in dogs in Hungary. Canine hemoplasmas were also identified in 49 samples (62%): only *Mycoplasma haemocanis* in 32 (40.5%) dogs,

only “*Candidatus* Mycoplasma haematoparvum” in 9 (11.4%) dogs, and both hemoplasmas in 8 (10.1%) dogs. Thus, hemoplasma infections also showed a particularly high prevalence in this dog population. Based on a partial fragment of the 18S rRNA gene, *B. gibsoni* from Hungary exhibited complete sequence identity with conspecific strains reported from Europe and Asia. The cytochrome c oxidase subunit 1 (*cox*1) gene sequence of this isolate showed the closest identity with *B. gibsoni* reported from Japan but had a nonsynonymous mutation (M33I). Furthermore, the 11 *B. gibsoni*-positive samples analyzed for sequence variants of the cytochrome b (*cytb*) gene showed the presence of a common mutation (P310S). Most importantly, *B. gibsoni* had two further nonsynonymous mutations, M121I and F258L, in a dog with severe and relapsing anemia following atovaquone treatment. Phylogenetically, both *cytb* sequence variants clustered together, with a clear geographical pattern showing the closest relationship of both haplotypes identified in Hungary with those from China and Japan. To the best of our knowledge, this is the first *cox*1 and *cytb* characterization of *B. gibsoni* in Europe, as well as the first report on the emergence of this piroplasm and hemoplasmas with high prevalence among “fighting dogs” north of the Mediterranean Basin.

Last but not least, the genetic diversity of protozoan parasites was analyzed according to their different transmission routes (life cycle strategies), focusing on those species which were recently discovered or molecularly analyzed for the first time in Hungary or its geographical region. The results showed that among four apicomplexan parasites (*B. gibsoni*, *C. europaeus*, *S. morae* and *H. felis*) the latter had the highest genetic diversity as reflected by its 18S rRNA gene sequences showing high (1.75%) maximum intraspecific pairwise distance, and also, based on its phylogenetic clustering. This is probably related to the long evolutionary history of *H. felis*, the absence of its intravascular division and other life cycle characteristics precluding direct transmission between hosts. On the other hand, among non-apicomplexan protozoa (*T. gallinae*, *P. hominis*, *T. foetus* and *A. castellanii*), the latter proved to have the highest genetic diversity (7.73%), most likely due to its long evolutionary history, lateral gene transfer, homologous recombination and the absence of direct host-to-host dispersal. Transmission mode had a significant impact on the genetic diversity among protozoan parasites, depending on life cycle strategies and consequent frequency/chance of sexual reproduction *vs* binary fission. In particular, the absence of direct transmission between hosts is a common trait of *H. felis* and *A. castellanii*, contributing to their high genetic diversity.

2. Introduction

2.1. Companion animals

Most animals that live in the companion of human beings serve emotional, and not economic purposes, since people do not slaughter and eat them, do not use their milk, egg or fur. Rather they give them personal names, treat them as a member of the family, cuddle and groom them. Moreover, they are eagerly trying to provide them healthy and happy life. Pet animals have been proven to have a positive effect on people's mental and physical health, the cause of which might be a decrease in cortisol levels [1, 2]. Pets can induce a better sense of well-being, encourage a more active lifestyle and play a role in forming social relationships [3]. That strong bond between humans and pets can also have a significant benefit in healthcare, mainly at palliative care [4]. Among domesticated animals not only dogs, cats and horses are considered as companion animals but also birds, reptiles, amphibians, rabbits, rodents and fishes [3, 5]. In addition to the increase in the number of pet dogs and cats, the number of these non-traditional companion animals is also growing in families, therefore, the investigation of the parasites of all has an increasing importance [3, 6].

2.2. Protozoa

Protozoa are unicellular parasites with variable shape and size (usually less than 300 μm). In their structure, they are like other eukaryotes, but species belonging to phylum Apicomplexa have their characteristic apical complex with which they can enter the host cells. The DNA of most protozoa is stored in their nucleus and mitochondria, however, there are species, e.g. *Giardia* and *Trichomonas*, from which mitochondria are absent. Moreover, in trypanosomes the DNA structure of mitochondrion is found in an organelle called kinetoplast. Some groups of protozoa multiply asexually (binary or multiple fissions), but the multiplication mode of the majority is an alternation between asexual and sexual reproduction. They are able to adapt to diverse habitats, such as sea water, polar ice, fresh water and soil. Some protozoa can survive unfavorable environmental conditions by forming cysts or oocysts. Parasitic protozoa are heterotrophic, their energy is mostly generated by anaerobic glycolysis but in some cases also by complete aerobic oxidation of carbohydrates [7].

Some protozoa are known for their zoonotic potential (e.g.: *Leishmania*, *Giardia*, *Toxoplasma* spp.) which may pose a threat to humans worldwide. Urbanization may imply potential spillovers of the protozoan infections between humans, domestic and wild animals, since wild and domestic carnivores are considered the first source of human infections with zoonotic agents [8].

Currently, the term 'Protozoa' is regarded as a collective name without phylogenetic or taxonomic significance, to which wide range of single celled parasites belong. The taxonomic classification of the ones of veterinary importance is shown in Table 1 [7, 9].

Table 1: Classification of protozoa of companion animals based on the website http://taxonomicon.taxonomy.nl/. Red color indicates groups targeted in the thesis.

Phylum	Class	Order	Family	Genus
Euglenozoa	Kinetoplastea	Trypanosomatida	Trypanosomatidae	***Trypanosoma***[1] ***Leishmania***[1]
Metamonada	Trepomonades	Diplomonadida	Hexamitidae	***Giardia***[2]
Parabasalia*	Trichomonadea	Trichomonadida	Trichomonadidae	***Trichomonas***[3] ***Tritrichomonas***[3] ***Pentatrichomonas***[3]
			Monocercomonadidae	***Monocercomonas***[3]
Amoebozoa	Archamoebea*	Entamoebida	Entamoebidae	***Entamoeba***[2]
	Discosea*	Centramoebida	Acanthamoebidae	***Acanthamoeba***[2]
Apicomplexa	Coccidea	Cryptosporida	Cryptosporidiidae	***Cryptosporidium***[2]
		Eimeriida	Eimeriidae	***Eimeria***[3] ***Isospora***[3] ***/Cystoisospora***[3]***/***
			Sarcocystidae	***Toxoplasma***[4] ***Neospora***[4] ***Sarcocystis***[4]
		Adeleida	Hepatozoidae	***Hepatozoon***[1]
	Haematozoea	Haemosporida	Plasmodiidae	***Plasmodium***[1]
		Piroplasmida	Babesiidae	***Babesia***[1]
			Theileriidae	***Theileria***[1] ***Cytauxzoon***[1]

[1] vector-borne protozoa, [2] waterborne protozoa, [3] non-vector-borne protozoa with homoxenous life cycle, [4] non-vector-borne protozoa with heteroxenous life cycle

2.3. Transmission routes of protozoa

All parasites need a host to survive and multiply, thus, to ensure their needs different strategies have evolved for efficient spread. Protozoa that can spread vertically may infect the offsprings transplacentally or galactogenically (e.g., *Toxoplasma gondii*), while others which spread horizontally between the hosts can use different strategies. On the basis of the latter there are protozoa which primarily spread indirectly and are transmitted by arthropods, e.g., *Leishmania*

spp. by sandflies, *Babesia* spp. by tick vectors, and they are called vector-borne protozoa. However, there are protozoa which do not need any vector to be spread and can infect the host directly, such as through fecal-oral route (e.g., *Giardia* spp., intestinal trichomonads, different coccidia) or sexually (e.g., *Trypanosoma brucei equiperdum*). In addition, some protozoa might be spread indirectly by water (as waterborne protozoa, e.g., *Giardia* spp., *Cryptosporidium* spp., *Entamoeba* and *Acanthamoeba* spp.), with contaminated food (all enteral protozoa) or with infected raw meat and viscera (as foodborne protozoa). Typical examples for the latter include *Sarcocystis* spp., *Toxoplasma gondii* and *Neospora caninum* [7, 10, 11]. Although specific transmission pathways of most protozoa can be difficult to determine, some routes are more frequent than others [10]. Therefore, in this study, the most common protozoa of companion animals are detailed along two main groups, i.e., vector-borne and non-vector-borne protozoa. The latter is further divided into three groups, such as waterborne protozoa, protozoa with homoxenous life cycle (developing without intermediate host) and protozoa with heteroxenous life cycle (developing with intermediate host).

2.4. Vector-borne protozoa

2.4.1. *Hepatozoon*

Canine and feline hepatozoonosis is caused by different apicomplexan protozoa from Hepatozoidae family. *Hepatozoon canis* and *Hepatozoon americanum* can infect dogs, while *Hepatozoon felis*, *Hepatozoon silvestris* and *H. canis* can cause symptoms in cats [12–14]. The main transmission route is the ingestion of ixodid tick vectors, however, in the absence of vectors, transplacental infection is also thought to be possible [12, 14]. The pathogenicity and clinical manifestation of the disease strongly depend on the species. Among species occurring in Europe, *H. canis* mostly invade to lymph nodes, spleen, bone marrow, liver, kidney and lungs, while in infection with *H. felis* and *H. silvestris* the skeletal and cardiac muscles are affected [18].

Hepatozoon canis is transmitted by the ingestion of brown dog tick *Rhipicephalus sanguineus* sensu lato, as a result of which two merogonies take place in the aforementioned organs of the host, then elliptic gamonts are formed in neutrophil granulocytes and monocytes [7]. Dogs infected with *H. canis* may remain asymptomatic carriers, or present mild disease accompanied with anemia and lethargy, however, dogs with high parasitemia frequently show severe clinical symptoms e.g., fever, severe lethargy, weight loss, enlargement of the lymph nodes and the spleen. Although *H. americanum* is primarily present in the USA and in Central and South America, and not in Europe, the infection is worth mentioning, since it usually leads to more severe, sometimes fatal disease. To establish a diagnosis, gamonts might be detected in the blood smear by light microscope, however, studies showed that PCR is up to 22 times

more sensitive [7, 14]. As treatment imidocarb diproprionate is recommended, however, the chance of complete elimination is limited. Therefore, tick control is strongly recommended [7]. *Hepatozoon canis* had been reported as autochthonous parasite in Southern European countries with a Mediterranean climate (e.g., Greece, Italy, Spain, Portugal) [14]. However, its presence in dogs was also reported in Romania, Poland, Serbia and Hungary [15–18].

In Europe, also cats can be threatened by *Hepatozoon* species among which *H. felis* is the most common. The vector of *H. felis* is still unknown, however, it is assumed that *Rhipicephalus sanguineus* might be involved in the transmission [12, 19]. The development of *H. felis* takes place in skeletal and cardiac muscles of the host, and the gamont forms can be observed in neutrophil granulocytes during parasitemia, though, only in less than 1% [14, 20]. Therefore, as in dogs it is recommended to use PCR for diagnosis [13]. Symptoms include anemia, increased levels of creatine-kinase and lactate-dehydrogenase, however, the infection often remains subclinical. Furthermore, there is no controlled treatment of feline hepatozoonosis, however, combination of imidocarb dipoprionate and doxycycline has proved to be effective [20]. In Europe the infection with *Hepatozoon* spp. can be considered emerging among domestic cats (*Felis catus*), since during the last decade several cases were reported in Western Europe [12] and in Mediterranean countries, e.g., in Italy, Spain and Greece [21–24]. In Central Europe, the first clinical case in a domestic cat infected with *H. felis* was recently described [25].

Beside cats and dogs, non-traditional pet can also be infected with *Hepatozoon* species, such as rats with *Hepatozoon muris* by oral transmission of *Laelaps* mites, and snakes with other *Hepatozoon* spp. by ingesting infected Diptera [7].

2.4.2. *Babesia*

Piroplasms (Apicomplexa: Piroplasmida), i.e. species of *Babesia*, *Theileria and Cytauxzoon*, are obligate intracellular protozoan parasites, transmitted cyclically to their vertebrate hosts by hard and soft ticks [26]. Among them, members of the group *Babesia* (sensu stricto) infect erythrocytes and thus can affect humans, wildlife, livestock and companion animals [26, 27]. *Babesia caballi* is worldwide distributed and causing equine piroplasmosis along with *Theileria equi*. Clinical disease is mostly attributed to intravascular hemolytic anemia causing varying degrees of symptoms, but most horses remain long-term carriers. The clinical manifestation of *B. caballi* infection in horses is usually less severe than *T. equi* infection [28]. Main vectors are ixodid ticks belonging to the genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus*. The distribution area in Europe extends from Portugal and Spain to France, Italy, Hungary, Romania, Ukraine and Russia [7]. *Babesia caballi* is endemic in Italy [29], however, in Hungary it seems less common, since hitherto only horses from Hortobágy region have been confirmed positive with serological test. Interestingly, 6.74% of horses

examined in that study were infected with *Babesia canis*, which is primarily common in dogs [30].

Canine babesiosis is caused by several *Babesia* spp., which are classified based on their size. Thus, large *Babesia* species of the dog include *B. canis*, *Babesia vogeli* and *Babesia rossi*, all from the group *Babesia* (s.s.) [26]. On the other hand, *Babesia gibsoni* (*Babesia* (s.s.)), *Babesia conradae* (Western *Babesia* group) and *Babesia vulpes* (*Babesia microti* group) belong to the category of small *Babesia* species [26, 31]. Among large *Babesia* spp. *B. canis* is a widely distributed protozoon of great clinical importance. The prevalence of *B. canis* depends largely on the occurrence of the *Dermacentor reticulatus* vector which occurs widely in Europe. In acute phase of the disease fever, apathy, anemia and CNS signs are characteristic. Disseminated intravascular coagulation (DIC) associated with acute renal failure is a common complication. Since parasitemia is usually low (1%), the diagnosis might be challenging. It is worth noting that if the patients stay untreated (without the application of imidocarb diproprionate), they may die within 24 hours. Chronic cases may show apathy, emaciation, anemia, icterus and/or hemoglobinuria [7]. *Babesia canis* has been causing severe disease in dogs for a long time in Hungary, and its presence was also confirmed molecularly in 2005 [32].

Among small *Babesia* spp., *B. gibsoni* is regarded as an emerging pathogen, with increasing significance in dogs both in North America and Europe [31, 33]. This species may induce severe clinical signs such as *B. canis*, although it may also cause subclinical infection depending on such factors, as the pathogenicity of its strain, the immune status or age of the host [34, 35]. Considering the epidemiology of *B. gibsoni* infection, this piroplasm can also be acquired transplacentally [36]. However, the predominant mode of its transmission appears to be through bite wounds [34], in which cases usually illegal dog “fighting” and relevant breeds (American Staffordshire Terrier and American Pit Bull Terrier) are involved [34, 37]. These dogs frequently have co-infections with other hemotropic pathogens which are also able to spread via dog bites, as exemplified by *B. vulpes* and canine hemotropic mycoplasmas (*Mycoplasma haemocanis* (Mhc) and “*Candidatus* Mycoplasma haematoparvum” (CMhp)) [38, 39]. In addition, like other babesiae, *B. gibsoni* can also be transmitted by hard ticks. *Babesia gibsoni* has a worldwide distribution, and is endemic to North America, Asia, Africa, Australia and Europe. Hard tick vectors of this piroplasm include *Haemaphysalis longicornis* and *H. bispinosa* in Asia, probably *H. leachi* and/or *Rhipicephalus sanguineus* (s.s.) in Africa [40], and the latter tick species in North America and Europe [33, 35, 41, 42].

In Europe, the prevalence of *B. gibsoni* tends to be higher in countries of the Mediterranean Basin and the Balkan Peninsula, where its biological vector, *R. sanguineus* occurs, ranging from 0.7–5.7% in random sample groups and 2.0–28.6% among symptomatic and “fighting dogs”. However, owing to the possibility of its spread by other means, the

sporadic, autochthonous occurrence of *B. gibsoni* has also been documented north of the Mediterranean Basin [31]. In particular, clinical cases have been reported from Mediterranean countries (Croatia, Italy and Spain), Serbia, Romania and, in addition, from Germany, Poland, Slovakia, and the UK [31, 43–45]. However, in Europe, data are incomplete in terms of geographical distribution and prevalence. In addition, the mutation (M121I) which is thought to be responsible for atovaquone (ATV) resistance [46] has not hitherto been reported on this continent. In Hungary, the first reports on the occurrence of small *Babesia* spp. in dogs were based on studying piroplasms in blood smears and splenic impression smears [47, 48]. However, their species status remains uncertain due to the lack of molecular identification, especially since *B. canis* can also exhibit small *Babesia* spp.-like morphology depending on the conditions of sampling [49] and *B. vulpes* is known to occur in the country [50]. Additionally, *B. gibsoni* was molecularly identified in a stray dog imported from Hungary to Germany [51].

It is worth noting that rodents can also become infected with a zoonotic small *Babesia* species, namely the *Babesia microti*. Its occurrence is rare in Europe, however, several *Ixodes* spp. can play a role in its life cycle. Disease in humans develops few weeks after inoculation, especially in immunosuppressed patients [7].

2.4.3. *Cytauxzoon*

Cytauxzoon spp. infect mammalian hosts by their tick vector during blood meal [52], however, transplacental route can also occur [53]. As belonging to Theileriidae family first they replicate in MPS cells, then in red blood cells of cats and wild cats [7]. Feline cytauxzoonosis caused by *Cytauxzoon felis* is a long-recognized disease of domestic cats in North America, where the bobcat (*Lynx rufus*) is the natural reservoir of the infection [52]. The vectors are two tick species of the genera *Amblyomma* and *Dermacentor* [52, 53]. The infection in domestic cats is often associated with high mortality and affected animals show fever, vomiting, anemia, icterus and hepatosplenomegaly. The detection of *C. felis* is frequently based on the examination of blood smears, however, molecular methods are more specific and sensitive in the identification [52–54]. Concerning the treatment of feline cytauxzoonosis the combination therapy of azithromycin and atovaquone (A&A) has proved to be the most effective, however, atovaquone targets the cytochrome b (*cytb*) and its efficacy is mutable [52]. In Europe, feline *Cytauxzoon* infection is caused by three recently described species, *Cytauxzoon europaeus*, *Cytauxzoon otrantorum* and *Cytauxzoon banethi* [55]. Their presence was reported in European wildcat (*Felis silvestris*) and lynx (*Lynx pardinus*, *Lynx lynx*), as well as in domestic cats from Switzerland, Germany, France, Italy, Spain and Portugal. Interestingly, European *Cytauxzoon* spp. seem less virulent than *C. felis* [56]. The vector has hitherto been unknown, but *Ixodes ricinus* or a *Dermacentor* sp. might play a role in the transmission [57]. In line with this, in Hungary *C. europaeus* was detected in *I. ricinus* removed from an infected wildcat [58].

2.5. Non-vector-borne protozoa

2.5.1. Waterborne protozoa

2.5.1.1. *Giardia*

Giardia species (Metamonada: Diplomonadida) are flagellate protozoan parasites that infect a broad range of vertebrate hosts and are ubiquitous in mammals worldwide [59, 60]. They have two developmental stages: (1) the cyst that is ingested by the host, usually with drinking water or food, and will give rise to (2) the trophozoite that will multiply epicellularly on the small intestinal mucosa. The latter way of parasitism may entail pathological consequences, such as villous atrophy, malabsorption and diarrhea, but a high ratio of *Giardia*-infections remain asymptomatic [60]. Transmission of giardiasis occurs through the fecal-oral route (between animals or as zoonotic agent) or indirect (i.e., waterborne or foodborne). Cysts play a major role in diagnostics. It is worth mentioning that finding them under the microscope might be challenging, particularly if it is performed by a non-trained professional. However, other techniques are also available, such as detection of coproantigens (cyst wall proteins) with rapid immunochromatographic assays, direct fluorescence assays or molecular techniques [61, 62]. Currently, there are eight *Giardia* species recognized [63]. Among these, from a veterinary-medical point of view, the most important is *Giardia duodenalis*, which is the only species infecting humans; although various mammalian hosts, including pets and livestock, are also susceptible [64]. In terms of treatment different drugs might be used for giardiosis, e.g., fenbendazole, metronidazole, tinidazole or secnidazole [65, 66].

For more than two decades, increasing amount of data attest that *G. duodenalis* should be considered as a species complex, members of which show little morphological variation but are genetically distinct enough to be assigned to nine distinct assemblages (A–I) [59, 67, 68]. These assemblages were proposed to deserve taxonomic revision as separate species [68]. Considering the hosts of the main assemblages within *G. duodenalis*, rodents appear to predominate [69]. They can harbor at least six assemblages, including among the others the zoonotic A and B, as well as the rodent-specific assemblage G and assemblage E from livestock [70]. Moreover, rodents are the only hosts of *G. duodenalis* that can be found close to humans in both rural and urban areas as synanthropic wild animals and pets [71, 72].

Despite this, studies on the prevalence and genetic diversity of *G. duodenalis* infecting various species of rodents are less frequently reported than research in this context involving single species of other synanthropic mammals, such as dogs or cattle. This is in part due to the fact that rodents tend to harbor another species, *Giardia muris* more frequently than *G. duodenalis* [73]. On the other hand, genotyping is not always successful in small mammals

[70]. Nevertheless, importance of this topic is well illustrated by the nomenclature of the disease caused by *G. duodenalis* in humans, as it is frequently referred to as “beaver fever” on account of beavers passing cysts of zoonotic genotypes into water [74].

In Europe, only a limited number of reports are available on the prevalence and genotypes of *G. duodenalis* in small mammals, some of them targeting wild rodents [73], others sampling pet rodents [75, 76]. At the same time, while beavers appear to be repeatedly investigated in this context in North America (e.g., [77]), there appears to be only one relevant report from Europe [78]. Regarding Hungary, data are available from a study in which dogs were tested for the presence of *Giardia*, and assemblage C and D were found [79]. Furthermore, assemblage A and B were also detected in aquatic birds and in different water sources [80, 81].

2.5.1.2. *Acanthamoeba*

Among species that belong to phylum Amoebozoa the opportunistic *Acanthamoeba* species phylogenetically belong to Acanthamoebidae family. They are free-living protozoa found on various environmental sources, as soil, air, water etc., and have also two life stages, a trophozoite stage and a cyst stage. The latter may spread through the airways and eyes (with contact lenses in humans) [82–84]. Based on the cyst stage they have been assigned into three separate groups (Group I-III) [82]. In addition, they have also allocated into genotype groups based on the complete sequence of 18S rRNA, which currently results in 23 described groups (T1-23), among which T4 is the most frequently found in clinical cases (e.g., *Acanthamoeba castellanii* and *Acanthamoeba lugdunensis*) [85]. Common host of these species might be the reptiles, since these ubiquitous protists have been detected in the gastrointestinal tract, feces, brain and skin lesions of them [86–88]. However, some of them can be pathogenic to other companion animals and also to humans [89], causing skin lesions, keratitis (Acanthamoeba Keratitis: AK), or encephalitis (Granulomatous Amoebic Encephalitis: GAE) [90, 91]. Although *Acanthamoeba* infections are not limited to the eyes, keratitis is the most common clinical consequence in humans, as well as in cats, as only keratitis has been reported in domesticated cats [92, 93]. In dogs the investigations have noted another variety of signs, including anorexia, pyrexia, secretions from the eyes and nose, limb stiffness and neurological manifestations [94–96]. Interestingly, in a horse amoebic placentitis was reported which was caused by *Acanthamoeba hatchetti* belonging to T11 [97].

Acanthamoeba Keratitis is treated topically in humans with various eye drops, such as chlorhexidine, polyhexamethylene biguanide and diamidines, while in animals is lack of data but topical dexamethasone was proved to worsen the symptoms in rabbits and hamsters [94].

2.5.2. Protozoa with homoxenous life cycle

2.5.2.1. Trichomonads

Trichomonads *sensu lato* (Parabasalia: Trichomonadidae) are anaerobic protozoan parasites that live on the mucosal surface of the gastrointestinal tract and reproductive system of both animals and humans. Their multiplication takes place by longitudinal binary division, and the transmission is direct between the hosts. They are highly active flagellates and have only trophozoite form [98, 99], however, in an unfavorable condition they are able to form pseudocyst [100]. Although they are thought to be vulnerable, some of them proved to be more resistant to environmental conditions, since they could survive for seven days in moist feces at room temperature (23-24ºC) [101].

In dogs and cats trichomonads might be found in the oral cavity, such as *Trichomonas tenax*, *Trichomonas canistomae* and *Trichomonas felistomae* [102–104], and also in the gut since two species are able to colonize their large intestinal tract. One is *Tritrichomonas foetus* which is able to cause chronic and recurrent diarrhea accompanied with mucus and/or fresh blood in both [98, 99, 105]. The other protozoon is *Pentatrichomonas hominis* which is considered commensal and opportunistic, thus its clinical importance has been contested, however, its presence has already been described in dogs and cats in connection with diarrheal symptoms [106, 107]. They are transmitted directly between hosts via fecal-oral route [98]. Based on microscopical examination these species are difficult to distinguish morphologically from each other, and also from *Giardia duodenalis* which often occurs in co-infection [98, 99, 107].

Many diagnostic methods are available such as direct examination of fresh feces, fecal culture and PCR, the latter of which is the most used and most sensitive procedure. Furthermore, there are various approaches in sample collection, i.e.: freshly voided stool, collection manually by fecal loop or colon flush technique. It is worth noting that the excretion of trophozoites might be intermittent and can be influenced by previous antibiotic therapy [98, 99]. Ronidazole is currently the only effective drug for the treatment of *T. foetus* infection in a dose of 30 mg/kg once daily for a period of 14 days in cats [98, 108]. However, both dogs and cats may be affected by neurotoxic side effects such as lethargy, ataxia and seizures [109]. The treatment of *P. hominis* is still in question since the infection has been successfully treated with metronidazole in puppies [110], but in kittens this has not been shown to be effective [111].

Concerning the occurrence of *Tritrichomonas foetus* which is worldwide distributed and the most common trichomonad in cats [98, 99], it has already been reported in several countries in Europe using direct examination, fecal culture or PCR. Based on the latter, the prevalence of *T. foetus* infection in cats with chronic diarrhea was the highest (38.7%) in Spain [112], followed by 24.4% in Switzerland [113]. In addition, in studies in Germany and Italy not

only symptomatic cats were examined and *T. foetus* occurred with 15.7% and 5.2% prevalence rates, respectively [71, 114]. Within Central and Eastern Europe, the highest prevalence (20.51%) was reported in Poland [115]. However, among neighboring countries of Hungary the occurrence of *T. foetus* in cats was only reported in Austria with a prevalence of 2.9%, with *P. hominis* also being detected in the study [116]. In comparison with cats, *T. foetus* occurs sporadically in dogs [105, 106, 117], and in Europe this was reported only in Italy [118, 119]. By contrast, *P. hominis* might be more common in dogs than cats, as indicated by its high prevalence of 31.4% in China [106]. Interestingly, relevant data in Europe are scarce, since *P. hominis* was reported only in breeding kennel dogs in France, with 12.1% prevalence and in a case in Slovenia [120, 121]. In addition, *P. hominis* in cats was detected by PCR only in the United States, Japan, Thailand, Austria and the Czech Republic [111, 116, 122–124]. Among oral trichomonads in pet animals, apart from *T. canistomae* and *T. felistomae* [103, 104], the zoonotic *T. tenax* has also been detected [102, 125, 126]. Furthermore, a new trichomonad species, *Trichomonas brixi* has recently been reported in dogs and cats in Czechia [125]. Overall, few data are available on the prevalence of trichomonads in dogs and cats in Central and Eastern Europe.

Considering avian trichomonads, *Trichomonas gallinae* is widespread in birds from various orders, including Columbiformes, Accipitriformes, Strigiformes, Psittaciformes, Falconiformes and Passeriformes [127–129]. Among columbiform birds, the rock pigeon (*Columba livia*) is the main reservoir of this parasite. The most important route of trophozoite transmission between birds is oral by saliva, through shared water and food sources [128, 130, 131]. In columbids the predominant way of spreading to nestlings is via crop milk [127]. Additionally, predatory birds can become infected by consuming a carrier prey item, since trichomonas trophozoites may survive in carcasses for at least 48 hours [128, 130, 131]. *Trichomonas gallinae* can persist for up to one hour in various water sources, e.g., in gutters and drinkers [132], but higher temperatures (30-35˚C) can further prolong its survival [133]. Although it is able to form pseudocyst in unfavorable conditions, the moist environment is essential to maintain its infectivity [128].

Trichomonas gallinae is often considered a normal inhabitant (commensal) of the mucosal surface in the upper gastrointestinal tract [128]. However, by eliciting inflammation in the underlying tissues or when entering more distally the digestive tract of birds, this protozoan parasite might cause mild to severe lesions depending on strain virulence and host susceptibility. Infection with highly pathogenic strains may lead to death. However, columbiform birds may also be asymptomatic carriers of *T. gallinae*, ensuring the carefree spread of this protozoon. In addition, if protective immunity develops, affected birds become resistant to a new infection [128, 130, 134, 135].

Regarding pathogenesis, *T. gallinae* trophozoites establish preferentially in the upper gastrointestinal tract (the oropharynx, esophagus and crop), where they can cause yellowish necrotic lesions. In severe cases trichomonosis can lead to starvation and suffocation. Furthermore, *T. gallinae* can spread to tissues of the cranium, thorax, and abdomen, as well as of the liver and air sacs causing similar lesions, with deepening tissue involvement, referred to as canker [127]. Recently, massive death of passeriform birds (the greenfinch, *Chloris chloris,* and the goldfinch, *Carduelis carduelis*) due to infection with a specific lineage of *T. gallinae* has been reported throughout Europe [129]. Nitroimidazoles are the most used drugs in birds, but resistant strains have been reported in domestic pigeons. Thus, novel treatments based on propolis or plant extracts are emerging [136, 137].

Avian trichomonosis has been reported to have a worldwide occurrence [128]. In Europe, as on other continents, columbiform birds play the most significant role in the maintenance of *T. gallinae* [128]. The prevalence in western and southern Europe is high among wild columbids (74%: [135]), unlike in northern central Europe where only a little more than one third of racing pigeons proved to be PCR positive [138].

Considering additional companion animals, members of the family Trichomonadidae also occur in reptiles, and can be found in the urogenital and gastrointestinal tract of their host. They have hitherto been detected in numerous reptile species, mostly based on morphological observations, e.g., in *Bothros jararaca*, *Eryx johnii*, *Phelsuma dubia*, and *Physignathus cocincinus* [139, 140].

2.5.2.2. *Monocercomonas*

Species of Monocercomonadidae are also mucosoflagellates known for their flagella and for their dwelling on the surface of the mucous membrane. All species of Parabasalia phylum have parabasal body that is connected to their flagellar apparatus [141]. Monocercomonadidae species differ from Trichomonadidae species by the absence of undulating membrane [142]. They live in the digestive tract of mammals, birds, reptiles, amphibians, fish and arthropods, and transmitted directly. *Monocercomonas ruminantium* occurs in rumen of cattle, while *Monocercomonas cuniculi* is found in caecum of rabbits, however, both are considered non-pathogenic protozoa [7]. In addition, *Monocercomonas colubrorum* is a facultatively pathogenic species often found in snakes and lizards caused depression, weight loss, abdominal pain, aggressiveness and diarrhea. Interestingly, this parasite is prone to burrow under the epithelium, as a result of which can be seen between the epithelium and lamina propria. Although *Monocercomonas* spp. mainly occur in the gastrointestinal tract, they can also be found in gall bladder, lungs and oviducts of snakes causing inflammation of the affected organ [142, 143].

2.5.2.3. Protozoa causing intestinal coccidiosis

Members of Coccidea class are the causative agents of coccidioses in a broader sense. However, the term “coccidiosis” is also used in a narrower sense to define the infections that are caused by species of the family Eimeriidae. Protozoa (*Eimeria* and *Isospora* spp.) belonging to the latter family are worldwide distributed and have strict host specificity. It is worth mentioning that the formerly uniform genus *Isospora* was divided into the genera *Isospora* infecting birds and *Cystoisospora* infecting mammals.

The most important species that can infect companion animals are summarized in the Table 2 [7, 144]. Three types of development alternate in their life cycle, merogony, gamogony and sporogony. The first two phases take place in the host cell intracellularly, while sporulation befalls in the environment, and the host gets infected by ingestion of sporulated oocysts. Most species develop in the epithelial cells of the host’s gut causing damage and inflammation, mainly enteritis with varying severity, e.g., in rabbits diarrhea, obstipation or tympany can be observed, while in carnivores the infection is often inapparent, or diarrhea, vomiting and anorexia occur. Hemorrhagic enteritis is rare in rabbits, however, they can frequently have more serious multifactorial disorders (enteritis complex), associated with pathogenic *Escherichia coli* strains, *Clostridium* spp., *Bacillus piliformis* and rotavirus infections. Furthermore, *Eimeria stiedai*-infected rabbits with liver coccidiosis (papillomatous proliferation of bile ducts) may show similar symptoms, which often accompanied with icterus [7]. With parasitological methods the oocysts of the coccidia can be routinely detected. In their oocysts the distribution of sporocysts and sporozoites are different depending on whether it is an *Eimeria* or an *Isospora*/*Cystoisospora*. The classification based on other features (size, shape etc.) of oocysts is possible in some cases e.g., for *E. stiedai*, *Cystoisospora canis*, *Cystoisospora felis*, *Cystoisospora rivolta* and the *Cystoisospora ohioensis*-complex. Into the latter “group” *Cystoisospora ohioensis*, *Cystoisospora neorivolta* and *Cystoisospora burrowsi* belong to and are difficult to be distinguished, therefore, they are described as *C. ohioensis*-complex [145, 146]. All species are transmitted through fecal-oral route, however, the infection might have been associated with raw meat consumption, since monozoic tissue cyst of *Cystoisospora* spp. can occur in paratenic hosts [147]. In course of the treatment of rabbits, dogs and cats toltrazuril and sulfonamides are commonly used drugs [7].

As a result of a survey in 2001 in Hungary, 490 dogs were screened for various parasites and *Cystoisospora* spp. occurred with a prevalence of 3.5% [148]. Moreover, considering coccidiosis of rabbits in Hungary also little data are available but in a recently published study 32% of rabbits from Hungarian and Slovakian farms proved to be infected with any *Eimeria* spp. [149].

Table 2. Species causing “coccidiosis” in companion animals.

Host	Species
pigeon	*Eimeria labbeana*, *E. columbarum*
horse	*Eimeria leuckarti*
rabbit	*Eimeria stiedai*, *E. intestinalis*, *E. flavescens*, *E. irresidua*, *E. magna*, *E. media*, *E. piriformis*
mice	*Eimeria falciformis*, *E. vermiformis*, *E. papillata*
rat	*Eimeria separata*, *E. nieschulzi*
guinea pig	*Eimeria caviae*
captive birds	*Isospora serini*, *Eimeria reichenowi*, *E. gruis*
dog	*Cystoisospora canis*, *C. ohioensis*, *C. burrowsi*
cat	*Cystoisospora felis*, *C. rivolta*
reptile	*Eimeria* spp., *Isospora* spp.

2.5.3. Protozoa with heteroxenous life cycle

2.5.3.1. Toxoplasma

Toxoplasmosis is caused by an obligate, intracellular parasite namely *Toxoplasma gondii*, which belongs to the Sarcocystidae family. It can infect almost all warm-blooded animals, including cats, dogs and humans, and is known as a major concern of public health worldwide. Globally about 60% people of population are reported to be infected with *T. gondii* [150].

Felines are the definitive hosts, shedding the oocysts into the environment for a period of 1–2 weeks. It is worth noting that not just the sporozoites (within the sporulated oocysts) are the infectious stages of *T. gondii* but also tachyzoites and bradyzoites (in tissue cysts) [150–152]. Tissue cysts of *T. gondii* may occur in the most common meat-producing animals, such as poultry, cattle, sheep and pig. Among pets while dogs are the intermediate hosts of this parasite, cats can shed more than 1 million oocysts/g with their feces, thus, the latter are of great epidemiological importance concerning both animals and humans [7, 150, 153]. The development of *T. gondii* depends on whether it happens in the final or intermediate host. In case of the previous one if the cat ingests the bradyzoites (from tissue cyst containing raw meat/viscera), an enteroepithelial phase takes please with merogony, gamogony and oocyst formation. In addition, if the cat is infected by a sporulated oocyst, usually an extraintestinal development, i.e., tachyzoite proliferation and bradyzoite formation in tissue cyst befall. Moreover, some extraintestinal parasite stages may return to the gut and complete the lifecycle and form oocysts. Concerning intermediate hosts, followed by the ingestion of oocyst or tissue cyst tachyzoites are rapidly produced within nucleated host cells in a parasitophorous vacuole. Tachyzoites due to their rapid proliferation, are released by rupture of the host cell and spread

throughout the body by bloodstream [7]. Transitions may occur between the tachyzoites and bradyzoites, some of which are thought to enable the bradyzoite to hide from the immune detection, thereby leading to persistent infection [154]. They reach various tissues such as skeletal muscle, cardiac muscle, or CNS (brain, spinal cord and retina), and consequently cause a life-long, latent infection. Regarding its effect on the CNS, the dopaminergic system is of great interest, as dopamine is essential for movements and various forms of learning, including fear [154]. Interestingly, the infected rodents are more active and attracted to the cat odor, which may lead to suicidal tendencies, as it might be presented in humans as well [155–157]. In addition, transplacental infection can also happen which may cause serious problems of the fetus in humans, such as abortion or congenital infection. Of which latter can entail with severe pathological defects (hydrocephalus, blindness or mental retardation) [158]. Small ruminants are highly susceptible to *T. gondii* infection, which often causes severe problems, such as abortion, absorbed or mummified fetus. In cats the infection is inapparent when it is limited to intestinal infection. Although extraintestinal infection occurs rarely, it may cause more serious symptoms in kittens, e.g., anorexia, apathy, pneumonia, fever, diarrhea, encephalitis, hepatitis or uveitis. Clinical manifestation is also rare in dogs, even though they are often infected. However, if pregnant bitches have primary infection with *T. gondii* they may abort, and their puppies presumably show CNS symptoms. Neosporosis and *Hammondia heydorni* infection must be considered in differential diagnosis. While *T. gondii* will not produce oocysts in the intestine of dogs [7], the DNA of *T. gondii* can be detected in dog's feces [152].

The oocysts are rarely found in the feces of cats, since the period of oocyst shedding is significantly short, in addition, oocysts of *T. gondii* cannot be morphologically differentiated from those of *Hammondia hammondi*. In diagnosis, out of PCR methods antibodies can also be detected in both cats and intermediate hosts, as it was performed in Hungary in 2008, when 330 cats were examined to assess the seroprevalence of *T. gondii* and *Neospora caninum* infection of cats with indirect fluorescent antibody test (IFAT). The prevalence of toxoplasmosis was 47.6%, interestingly, 22.4% among urban, 50% among suburban and 61.3% among rural cats. Furthermore, older female cats proved to be more predisposed to the infection with *T. gondii* [159]. The correlation between the older age and *T. gondii* positivity was also confirmed by a study in 2024. In this, fewer (31.7%) cats were found to be seropositive, among which there was a higher proportion of cats living outdoors [160].

Treatment of domestic carnivores is only recommended in case of acute disease. Cats are primarily treated with clindamycin, which may decrease the shedding of oocysts but it cannot eliminate bradyzoites hiding in tissue cysts. [7, 161].

2.5.3.2. *Neospora*

Neosporosis is a worldwide distributed parasitic infection caused by *Neospora caninum* from Sarcocystidae family. Primarily dogs and cattle are affected by this protozoon, however, wide range of domestic and wild animals can also be infected. In addition, *Neospora hughesi* may occur in horses and underlie of various neurological symptoms associated with equine protozoal myeloencephalitis (EPM).

The definitive host of *Neospora caninum* is the dog (or wolf (*Canis lupus*)/ coyote (*Canis latrans*)), by which the oocysts are excreted to the environment and survive for months to years. While in the intermediate host the bradyzoites within the thick-walled tissue cyst can survive at 4°C for a minimum of 14 days and are also resistant to pepsin and trypsin digestion. *Neospora caninum* can spread both horizontally and vertically. Considering the latter one, transplacental and galactogen transmission may occur, while during horizontal transmission *N. caninum* can spread with contaminated water and food, or with infected tissues of intermediate hosts. After the ingestion of sporulated oocysts or tissue cysts, the sporozoites and bradyzoites transform into tachyzoites in the intestinal epithelium, then reach uterus through the bloodstream, and invade many other cell types, mostly CNS cells, endothelial cells and myocytes. Thus, the formed tissue cysts are mostly found in nerve tissues (brain, spinal cord and retina) and in muscles [7, 162]. Dogs of any ages can have neosporosis, however, serious symptoms usually occur in young dogs, particularly in congenitally infected puppies. First hind limb paresis can be observed that might develop into a progressive paralysis. Interestingly, the neurological symptoms depend on which site is parasitized. The hind limbs are frequently affected and show rigid hyperextension. Furthermore, difficulties in swallowing, jaw paralysis, muscle atrophy and even heart failure can be detected. In cattle *N. caninum* causes abortion at 5–6-month of pregnancy. Fetus may die in the uterus then be resorbed, mummified, autolyzed, stillborn, or born alive with or without clinical signs. Neurological signs have only been reported in calves younger than 2 months of age. [163].

Diagnosis requires the detection of *N. caninum* oocysts in the dog's feces, but it is assumed that dogs showing clinical signs may not shed the oocysts. Histopathology and immunohistochemistry are highly recommended in reproductive problems, as molecular and serological tests also. The IFAT, the *Neospora* agglutination test and the enzyme-linked immunosorbent assay (ELISA) are the most used techniques for the detection of *N. caninum* antibodies [162]. The prevalence of *Neospora caninum* in dogs in Europe is relatively high (17.94%) compared to the data from other continents [164]. Regarding countries neighboring Hungary, the seroprevalence in Serbia was 15.4% for cows and 17.2% for dogs [165], while in Romania it was 32.7% for dogs [166]. In Hungary the presence of *N. caninum* was first described in 1998 and 2001 in association with bovine abortion [167, 168], then in 2006

seropositive cattle and dogs were also reported [169, 170]. Although cats were also screened for *N. caninum*, it occurred at a low prevalence compared to *T. gondii* [159].

Overall, the screening proved to be very important in that field, since the risk of abortion is two to three times higher in seropositive than in seronegative cows [171]. In order to decrease the economic loss of cattle farms and prevent the spread of *N. caninum*, dogs should never be fed with placenta or dead calves, fetuses or raw/undercooked meat, and should be protected from fecal materials of carnivores around livestock [163, 171]. Although dogs with systemic infections can be treated with clindamycin or pyrimethamine in combination with sulfonamides, the shedding of cysts cannot be stopped by chemotherapy [7].

2.5.3.3. *Sarcocystis*

Sarcocystis species are intracellular protozoan parasites, which are obligatorily heteroxenous, i.e., their development involves both an intermediate and a final host. While there are approximately 200 valid species in the genus *Sarcocystis*, until recently the complete life cycle was only known for 26 of them [172]. However, the life cycle of *Sarcocystis* species was described first by Rommel et al. in 1972 [173]. *Sarcocystis* species usually have herbivorous/omnivorous animals as intermediate hosts, in which they undergo asexual multiplication in endothelial cells of blood vessels and eventually establish in muscle or nerve cells [172]. After infected tissues of the intermediate host are consumed by the final host, sexual reproduction takes place in its small intestinal wall, entailing passage of infective oocysts/sporocysts to the environment [172]. In most cases, after intestinal sporulation the thin wall of oocysts ruptures releasing the sporocysts into the feces. Sporocysts measure 13-16x8-11 µm in dogs and 10-12x7-8 µm in cats, depending on the species. Consequently, the intermediate hosts are becoming infected by ingesting sporocysts with food or water [7].

Sarcocystis-infection may cause pathologic consequences in the intermediate host, while carnivorous final hosts usually remain unaffected even in case of copious sporocyst shedding [172]. However, occasionally they may show symptoms of diarrhea due to sarcocystiosis limited to their intestine. Regarding intermediate hosts the clinical symptoms are also rare, sometimes generalized myositis can be observed [7]. Twenty-one valid species of *Sarcocystis* are known to infect dogs as final host, most of which develop in domestic and wild ruminants as intermediate hosts [174]. In addition, several new *Sarcocystis* spp. have recently been described from cervids in Europe [175, 176], for most of which the final hosts are unknown but are suspected to be canids based on phylogenetic properties. Interestingly, carnivores might also be intermediate hosts, e.g., of *Sarcocystis arctica* and *Sarcocystis lutrae* in Europe [177]. Furthermore, *Sarcocystis neurona* uses dogs and cats as intermediate hosts, and can cause equine protozoal myeloencephalitis (EPM) in horses as aberrant hosts in North

America [7, 178]. In cases where myositis occurs in dogs, decoquinate treatment can be effective [179].

Out of fecal examination and necroscopy, serological and molecular techniques are also required to make diagnosis [7]. Zoonotic species (e.g., *Sarcocystis suihominis* or *Sarcocystis hominis*) also exist, and have particularly important public health significance. In Europe, the prevalence of human sarcocystiosis has been estimated between 1.1-10.4% [180], however, to the best of our knowledge, in Hungary molecularly identified *Sarcocystis* spp. have hitherto only been reported from cattle and mallards [181, 182]. Overall, carnivores have a significant role in the epidemiology of many *Sarcocystis* spp., therefore, it is important to avoid feeding dogs and cats with raw/undercooked meat unless it has been frozen (3 days at -20 °C) [7, 150, 153].

2.6. Genetic diversity of protozoa

Parasitic species exhibit a broad spectrum of population structures and life-history strategies, including different transmission modes, life cycle complexity, off-host survival mechanisms and dispersal ability [183]. Life history traits determine genetic diversity and thus the evolutionary potential of host–parasite interactions. In this way, genetic diversity is crucial for the parasites, ensuring higher chances of host-adaptation and survival in the course of their evolution [183]. On the other hand, genetic diversity is also important from taxonomic and diagnostic points of view when studying these parasites. Although there are mechanisms which decrease genetic variability among parasites, for instance the treatments of parasitic infections [184], numerous events in the life cycle act in the opposite way. In general, haplotype diversity is controlled by multiple processes, such as mutation, recombination, and demography [185].

Among most protozoan parasites, there is evidence for a role of frequent, although not obligate, genetic exchange, which enhances parasite diversity [186]. Genetic exchange is known to occur during the life cycle of many parasitic protozoa, most notably in association with their sexual reproduction in the final host or biological vector [187]. Sexual reproduction is an essential part of protozoan life cycles, but it may not be exactly known (in each and every taxon) where and how it takes place. It is considered a major source of genetic diversity in a population, and thus advantageous, because it accelerates adaptation to fluctuating environments or purges deleterious mutations [188]. On the other hand, unisexual reproduction introduces more limited genetic diversity through mother-daughter cell fusion, and has been found in both vector-borne and non-vector-borne protozoa [189]. Predominance of binary fission (unisexual reproduction) may act at the cost of processes ensuring genetic diversity. For instance, when occurring in the blood stream, it can ensure mechanical transmission of identical genotypes to multiple (a multitude of) hosts from a common source.

Population genetics theory predicts that clonally reproducing organisms show low genetic diversity, whereas sexually reproducing organisms show high genetic diversity as a result of recombination [190]. Homologous recombination is critical to maintain genome stability and to ensure genetic diversity during meiosis [191]. It is also known that genetic diversity in vector-borne-protozoan populations depends on transmission dynamics [192, 193].

Hungary is among the few European countries where in the past decade a high number (nearly twenty species) of protozoan parasites were either discovered to occur or were at least analysed with molecular biological tools for the first time, providing new molecular data in an international context [194]. Utilizing this opportunity, it was considered important to estimate the transmission route-dependent, 18S rRNA gene diversity of selected protozoan parasites, focusing on species that were reported recently in Hungary, including in the first place those that are being discussed here.

3. Aims of the studies

The aims of the study were:

Ad 1. to screen *Giardia*-infection in rodents and rabbits with traditional parasitological methods and further evaluation of positive samples with molecular biological tools.

Ad 2. to investigate the prevalence, genetic diversity and phylogenetic relationships of avian *Trichomonas* species in Hungary and Romania, where no similar data are available.

Ad 3. to determine the presence and prevalence of trichomonads infecting cats and dogs in Hungary.

Ad 4. to screen the feces of a broad range of captive reptiles for DNA of protozoan parasites with veterinary-medical significance from phylum Parabasalia.

Ad 5. to reveal if there is a correlation between the presence of *Acanthamoeba* and ocular illnesses in canine and feline patients.

Ad 6. to identify protozoa found in the feces of dogs and cats fed with raw meat, to ascertain the source of their food, and to assess the epidemiological and public health significance of detected parasites.

Ad 7. to examine *Sarcocystis* sporocysts from dog feces with molecular-phylogenetic and parasitological methods.

Ad 8. to screen for the presence of *Hepatozoon felis* and *Cytauxzoon europaeus* in outdoor domestic cats that live in a recently discovered endemic area in Hungary, where these infections were reported to emerge in wildcats [59]; furthermore, to examine domestic cats outside this endemic region in comparison.

Ad 9. to investigate (a) the prevalence of *Babesia gibsoni* in “fighting dogs” subjected to police confiscation in Hungary; (b) the molecular characteristics of *B. gibsoni* focusing on the cytochrome c oxidase subunit 1 (*cox*1) and cytochrome b (*cytb*) genes; (c) hematological parameters and co-infections in the dogs; and (d) the occurrence of any *cytb* mutations that were reported to be relevant in the context of ATV resistance.

Ad 10. to reveal whether the mode of transmission influences the genetic diversity of protozoa found in this study.

4. Materials and methods

4.1. Sample collections from different hosts

4.1.1. Samples to screen *Giardia* species

In this study, 27 intestinal contents and 137 fecal samples (164 samples in total) of five rodent species and rabbits were included (Table 5). Most samples were collected from small mammals that were rescued or found dead due to natural causes, as well as from patients of an exotic pet clinic, sampled during regular veterinary care between 2022 and 2023 in Budapest (sampling site “d”) and Komárom (sampling site “e”), Hungary (Figure 1). Fecal samples were stored for a maximum of four days at 2-6 °C before processing. All available relevant clinical data (i.e., anamnesis) were recorded. In addition, contents of the small (n=12) and large intestine (n=15) were removed from beavers that were caught as part of an official campaign, to reduce their populations [195]. Ethical permissions were issued by local county authorities: (a) Győr-Moson-Sopron (14178–10/2016, 88–4/2018, GY-02/TV/00293–7/2019); (b) Jász-Nagykun-Szolnok (JN 07/61/01703–2019, JN/07/61/00079–69/2018, PE/KTFO/5519–11/2019); (c) Zala (ZA/KTF/00092–7/2020); (d) Budapest (e) Komárom.

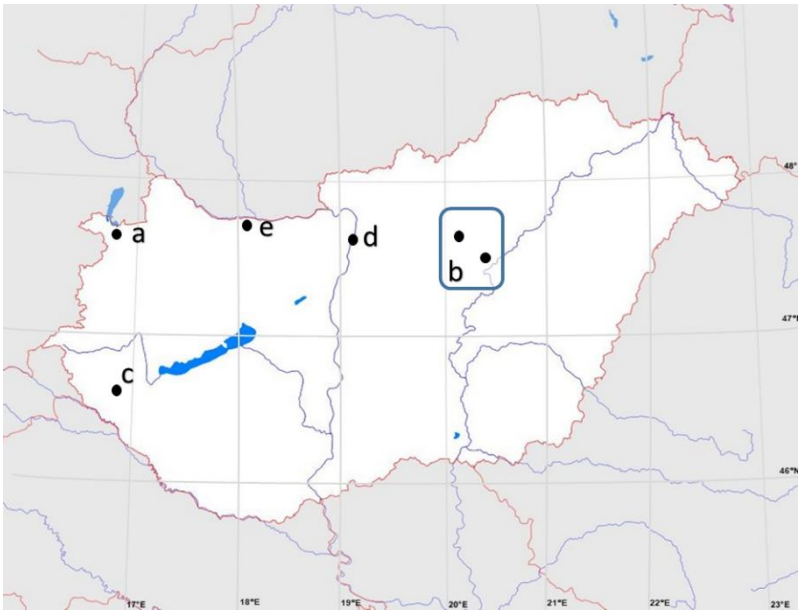

Figure 1. The origin of rodents and lagomorphs tested in the study. **(a)** Győr-Moson-Sopron county (Hegykő) 3 European beavers (*Castor fiber)* **(b)** Jász-Nagykun-Szolnok county (Jásztelek and Jászsági main channel) 6 European beavers (*Castor fiber*) **(c)** Zala county (Szentpéterfölde) 6 European beavers (*Castor fiber*) **(d)** Budapest [12 chinchillas (*Chinchilla lanigera*); 2 degus (*Octodon degus*); 22 rabbits (*Oryctolagus cuniculi*); 12 Norway rats (*Rattus norvegicus*); 10 guinea pigs (*Cavia porcellus*)] **(e)** Komárom-Esztergom county (Komárom) 11 chinchillas. 68 faecal samples (of 137 fecal samples in total) from 10 degus, 53 chinchillas and 5 domestic rabbits have no location data.

4.1.2. Samples to investigate *Trichomonas* species of avian hosts

Oropharyngeal mucosal samples were collected with sterile cotton swab applicators randomly from 99 columbiform birds that underwent routine veterinary examination in Hungary (n=77) and Romania (n=22) between May and August 2021. Four avian host species were sampled, each bird on one occasion, including feral pigeons (*Columba livia domestica*: 42 racing, 32 urban and 2 rural), wood pigeons (n=4), ring doves (n=16) and Eurasian collared doves (n=3). Racing pigeons were sampled at a trading-breeding place in Csepel (Budapest, Hungary). To assess the necessity of culturing *T. gallinae* prior to DNA extraction, the swab sampling was performed in duplicates from 20 racing pigeons, and one swab sample was placed into 8 ml CPLM culture medium with Trichomonas selective supplement (Biolab Diagnostics Laboratory Inc., Budapest Hungary), containing streptomycin, penicillin and sterile inactivated horse serum (pH adjusted to 6). These cultures (n=20) were kept at 37 ºC for two days. All other swab samples (n=99) were placed in 2 ml sterile Sarstedt tubes and frozen at -20 ºC.

4.1.3. Samples to investigate trichomonads infecting dogs and cats

From June 2021 to September 2023, 208 samples were collected from 25 dogs (*Canis lupus familiaris*), 94 domestic cats (*Felis catus*) and four wildcats (*Felis silvestris*) in Hungary. Domestic cat and dog samples originated from 18 locations, including the South-Central part of the country (n=31), the capital city Budapest and its surroundings (n=28), Lake Balaton and the surroundings (n=29) and Aggtelek National Park (n=30) (Figure 10). Wildcats were sampled at the latter location. Sampling of cats in and around the Aggtelek National Park was carried out as part of a targeted sampling campaign with nature conservation purposes.

Five collection methods were applied during the study: fecal swabbing (114), voided feces (45), InPouch® TF-Feline test from Biomed Diagnostics [DCN Dx, Carlsbad, CA, USA (9)], oral swabbing (35), and post-mortem sampling of the intestinal wall of the colon (5). For the evaluation of sensitivity in detecting intestinal trichomonads, different sampling methods were used simultaneously on a limited number of animals. Of the 114 animals which were sampled with fecal swabs, voided feces (n = 40) or samples for InPouch test (n = 5) were also collected. Only one cat underwent sampling was sampled with all three methods. During fecal swabbing, a cotton swab was introduced 3–4 cm deep into the rectum of the animal and gently rotated at least three times, connecting it with the rectal wall. A similar procedure was performed for oral swabbing. In addition, intestinal tissue samples were taken from the carcasses of four wildcats and one domestic cat. Swab samples and tissue samples were then placed in sterile Sarstedt tubes. During the collection of fresh feces, it was a prerequisite that the samples should be at least 1 g, free from litter or other contaminants. These freshly voided feces were obtained immediately after excretion and placed in sterile fecal collection containers. The tubes and fecal containers were stored in a freezer (−20 °C) until evaluation.

Lastly, the test with InPouch TF-Feline was performed according to the manufacturer's instructions.

During the investigation a total of seven different cat breeds were sampled: Ragdoll (41), Devon Rex (1), Maine coon (2), European shorthair (47), Persian- Himalayan (1), British shorthair (1), and Persian (1). Regarding dogs, a higher ratio of mixed breeds were included in the study.

4.1.4. Samples to screen parabasalids and acanthamoebae from captive reptiles

Fecal samples of 98 reptiles were collected at the National Reptile Zoo in Kilkenny City, Ireland, between March and July 2021. These captive animals represented 43 species and belonged to three orders (Squamata, Testudines, and Crocodylia; Supplementary Table 1). None of them have been purchased recently and they did not show any clinical symptoms. From all of them fecal samples were obtained in a non-invasive way, i.e., their fresh feces were collected from their artificial enclosure, attempting to exclude soil contamination. Fecal samples were placed inside pre-labelled Sarstedt tubes which were then stored at -20 °C until sample processing.

4.1.5. Samples to examine the presence of acanthamoebae in the eyes of dogs and cats

In conjunction with two veterinary ophthalmologists in Hungary, a total of 115 samples from dogs and 45 cat's samples were obtained and used in our investigation. Samples were taken between May and September of 2023 and came from locations distributed throughout Budapest and the northern part of Hungary. All 115 of the canine samples came from domestic, privately owned dogs that presented to veterinarians with clinical ocular signs. The majority (67%) of the feline samples were acquired from feral cats residing in Aggtelek National Park, with only 15 samples coming from privately owned cats. To acquire our conjunctival samples, we gently rubbed sterile cotton swabs along the conjunctiva of our subjects. The cotton swabs were then placed and sealed in Sarstedt tubes and stored frozen at -20℃ until processing.

4.1.6. Samples to investigate protozoa from the feces of dogs and cats kept on BARF diet

81 dogs and eight cats were involved in this study, all kept on BARF diet. From September 2021 to July 2022 fecal samples were collected from three different part of Hungary, the region of Budapest, Central Transdanubia and Northern Great Plain. The samples were stored at 2-6 °C before processing.

4.1.7. Samples to examine *Sarcocystis* sporocysts from dog feces

Fecal samples of four dogs were analyzed. All were American Staffordshire terriers (two males and two females, estimated age 5-6 years, weight 16.7-23.2 kg), originating from the region of Cegléd/Nagykőrös in Hungary. These dogs were confiscated during a police operation against illegal dog fighting in 2020, November. Their fecal samples were analyzed as part of a campaign to screen *Babesia*-, and *Giardia*-infection with parasitological and molecular methods.

4.1.8. Samples for screening the presence of *H. felis* and *C. europaeus* in wildcats and outdoor domestic cats

During this survey infections with *Hepatozoon* and *Cytauxzoon* spp. were investigated in 131 cats. In 2021–2022, anticoagulated (EDTA-containing) blood samples were collected from the vena cephalica antebrachii of 126 clinically normal domestic cats *(Felis catus*) with known history of outdoor activity: 88 were from non-endemic regions of eastern and southeastern Hungary (Debrecen: n = 73, Szeged: n = 15), and 38 from the recently discovered endemic region (north-eastern Hungary: Aggtelek National Park) (Figure 15). In addition, spleen samples of a domestic cat and four European wildcats (*Felis silvestris*) which were found as road-kills between 2015–2021 in the endemic area were also examined. For DNA extraction from tissues, samples were taken from the middle of organs to exclude surface contamination, using a sterile scalpel blade or scissors. All tissue was kept frozen at −20 °C until processing.

4.1.9. Samples to investigate *B. gibsoni* in fighting dogs in Hungary

In this study, samples of 86 American Staffordshire Terrier dogs were used. These dogs were confiscated by the police because they had been involved in illegal dog fights. The dogs came from the North Central region of Hungary. The mean age of dogs was 16.57 ± 16.73 months according to estimated data, and they included 30 puppies (mean age: 5.52 ± 2.87 months). The sexes were nearly evenly distributed, and no ticks were found on the dogs. From the confiscated dogs, EDTA blood samples were collected, drawn from the cephalic vein in December 2020 (prior to any treatment).

4.2. Data recording

All data were obtained from owners, veterinarians or collaborating partners (co-authors) at the time of sampling. Generally, all available and relevant clinical data (i.e., anamnesis) were recorded during the study. However, in three cases, additional data was collected, which are

detailed below. In addition, during the examination of genetic diversity the sequences of protozoa were synthesized (4.2.4.).

4.2.1. In the investigation of cats and dogs for the presence of trichomonads

Most of the samples were accompanied with a sample inquiry, to provide information on the location, date of birth, breed, sex, sampling method, collection time and symptoms (but not treatments). A map of Hungary was created to illustrate the geographic locations the sampled cats and dogs, using Google DrawingTM (Figure 10).

4.2.2. In the investigation of cats and dogs kept on BARF diet for the presence of protozoa

Questionnaires were filled out by the owners, to ascertain the source and type of raw meat or viscera given to the animals, whether the food contained nerve tissue, or if it was frozen before feeding. Additional questions referred to the usage of anthelmintics and signs of illness during the BARF diet.

4.2.3. In the investigation of dogs for the presence of *B. gibsoni*

The age and sex of all dogs tested were provided. Although circumstances did not allow a detailed physical examination to be carried out, at the time of confiscation most dogs were clinically normal, but in a poor condition and with fresh or healing wounds on their bodies. However, two dogs showed lethargy and signs of anemia: in these *B. gibsoni* was detected via PCR during preliminary testing in a commercial laboratory (Figure 2: during confiscation). A third dog had pica and gastrointestinal symptoms. Four dogs were found dead during the police operation, at their original keeping place.

4.2.4. In the investigation of genetic diversity of protozoa

Eight protozoan parasite species discovered recently in Hungary were chosen, with relatively long 18S rRNA gene sequences in GenBank, available from various hosts/countries. These included four heteroxenous, relatively stenoxenous apicomplexan parasites, i.e., *Babesia gibsoni* (representing Babesiidae; with transovarial, tick-borne transmission), *Cytauxzoon europaeus* (representing Theileriidae; with transstadial, tick-borne transmission), *Hepatozoon felis* (representing haemogregarines; with transstadial, tick-borne transmission) and *Sarcocystis morae* (representing cystogenic coccidia; with dogs as final and cervids as intermediate hosts). In addition, four homoxenous, more euryxenous non-apicomplexan parasites were analysed: *Trichomonas gallinae* (typically spreading between hosts by direct contact), *Pentatrichomonas hominis* and *Tritrichomonas foetus* (with the potential involvement

of transport hosts in the life cycle) and *Acanthamoeba castellanii*, a soil- and water-inhabiting, free-living amoeba that can become opportunistic parasite but is never shed from its host.

4.3. Hematology and treatment

Hematology was only performed during the examination of fighting dogs tested for the presence of *B. gibsoni*. Blood samples were available from 79 dogs for molecular biological analyses, and from 70 of them for hematological examination, as some samples could not be evaluated due to in vitro clotting caused by sampling error or other reasons. Hematological parameters were evaluated by ADVIA 2120 Hematology System (Siemens Healthineers, Erlangen, Germany). The follow-up of hematological parameters was conducted with URIT-3000 Vet Plus Hematology Analyzer (Medex Worldwide, Bucheon-si, Province, Gyeonggi-do, South Korea). Pre-treatment blood analyses were performed on the day of sampling (Figure 2: day 7 and 9), thereafter the remainder EDTA-anticoagulated blood samples were stored at -20 °C until further study**.**

All infected dogs which had anemia (hematocrit (HCT) < 30%) were treated with ATV at a dose of 17 mg/kg BID (bis in die) and azithromycin at a dose of 13 mg/kg SID (semil in die) for 10 days (Figure 2: day 3 and day 35). Moreover, one dog (named Bejgli) developed secondary immune-mediated hemolytic anemia. Therefore, this dog additionally received prednisolone and pantoprazole at a dose of 2 mg/kg SID and 1mg/kg SID, respectively (Figure 2: day 3). She responded well to the treatment but then developed recurrent anemia. Therefore, on day 33 another blood was taken from Bejgli for further molecular examination. Moreover, six months later 16 out of confiscated dogs (including Bejgli) were resampled for molecular analysis, though no data are available in terms of treatment after day 35 (Figure 2).

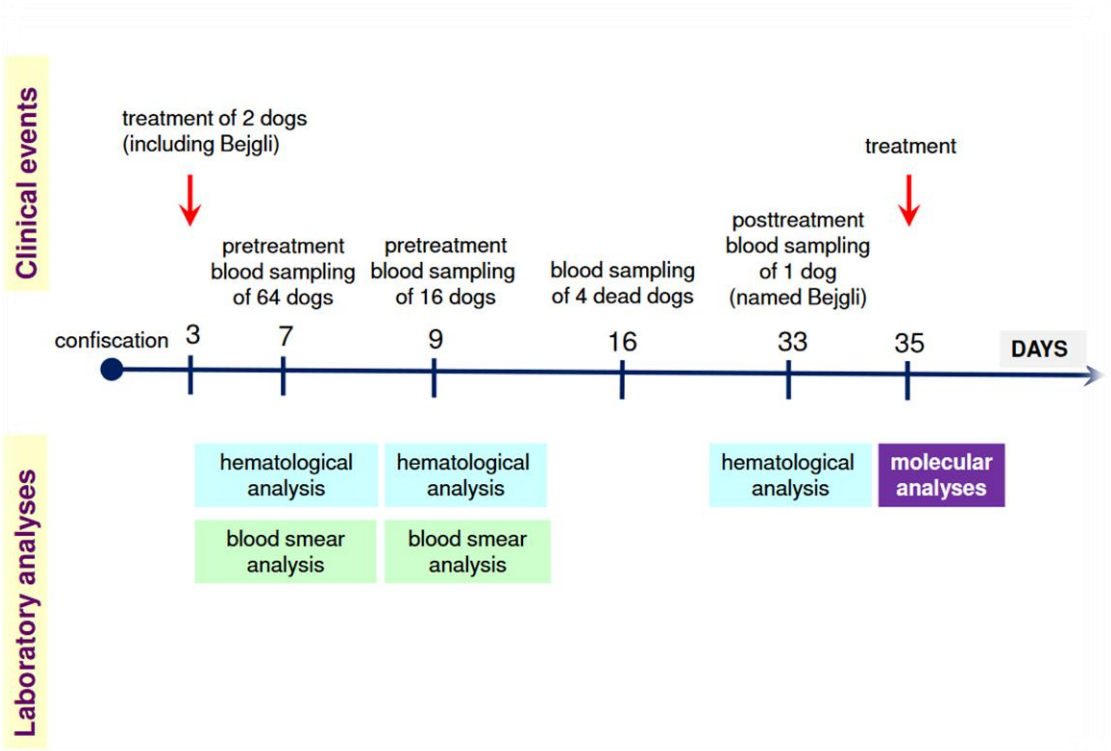

Figure 2. Timeline illustrating the sequence of events relevant to the study, including clinical observations/interventions, as well as laboratory analyses.

4.4. Parasitological examination

Traditional parasitological methods were performed in five different cases (4.4.1-4.4.5).

4.4.1. Flotation technique for the detection of *Giardia* spp. in rodents and rabbits

Screening for the presence of *Giardia* cysts was performed with flotation in the case of all samples, except for beaver gut contents (all of which were included in molecular analyses). This involved soaking of 3 g feces in 10 ml 0.9 % sodium chloride solution for 10-15 minutes, then flotation in 1200 g/l $ZnSO_4$ solution at 3000 rpm for 5 min with Megafuge 8 centrifuge (Thermo Fisher Scientific, Budapest Hungary). Evaluation of the presence of cysts was done using Leica DM 2000 light microscope (Leica Microsystems, Wetzlar, Germany) with 400× magnification.

4.4.2. Flotation technique for the detection of protozoa of dogs and cats kept on BARF diet

Flotation (using 1300 g/l zinc-sulfate solution) was carried out on 3-5 gram of the samples that arrived at the laboratory of Department of Parasitology and Zoology, University of Veterinary Medicine, Budapest. Diagnostic evaluation of samples and measurements were performed

with a calibrated light microscope (Leica Microsystems, Wetzlar, Germany). Then the fecal samples were stored at -20ºC until further investigation.

4.4.3. Flotation technique for the detection of *Sarcocystis* spp. in dog feces

First, molecular screening was carried out to detect piroplasm and other apicomplexan DNA in the feces. The *Sarcocystis*-positive fecal sample was further analyzed by flotation which was performed in Breza solution (specific gravity 1.3 g/l) and concentrated sporocysts were examined with light microscopy, including measurement of 50 sporocysts with morphometry.

4.4.4. Evaluation of blood smears for the presence of *Hepatozoon* and *Cytauxzoon* spp. in cats

Blood smears were prepared from freshly collected blood samples taken from cats, then fixed with ethanol, stained with May–Grünwald–Giemsa and examined under light microscope (Leica Microsystems, Wetzlar, Germany).

4.4.5. Evaluation of blood smear for the presence of *B. gibsoni* in dogs

After the blood was taken from the confiscated dogs, blood smears were prepared, fixed with ethanol, stained with May–Grünwald-Giemsa and examined by a Leica light microscope (Leica Microsystems, Wetzlar, Germany).

4.5. Molecular analysis

4.5.1. DNA extraction

4.5.1.1. Fecal samples

From all fecal samples examined in this study the DNA was extracted using the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions or with slight modifications, which are detailed below (4.5.1.1.1.-4.5.1.1.4.). During DNA extractions, each set of samples included an extraction control to monitor cross contamination. Furthermore, all DNA extracts were stored at -20 ºC until molecular analysis by conventional PCRs.

4.5.1.1.1. Examining the feces and intestinal content of rodents and rabbits for the presence of *Giardia* spp.

Prior to DNA extraction 1 g of samples were soaked in 0.9 % sodium chloride solution according to the method mentioned in 4.4.1., then filtered. DNA was extracted from all *Giardia* cyst-containing samples and all beaver samples using the kit according to the manufacturer’s

instructions with one modification. In particular, during the incubation with InhibitEX Buffer, fecal samples were subjected to three freeze-thaw cycles including freezing at -80 °C for overnight, followed by prolonged incubation at room temperature.

4.5.1.1.2. Examining the feces of cats and dogs for the presence of trichomonads, and of reptiles for parabasalids

In both investigations the DNA was extracted using the kit with some modifications, i.e., prior to adding Buffer AL, the solution was incubated at 56 °C for 60 min, and then the Buffer AW1 was used twice during the washing procedure.

4.5.1.1.3. Examining the feces of cats and dogs kept on BARF diet for the presence of protozoa

The DNA was extracted using the kit with slight modification. In particular, as the first step, mixtures of 300 mg fecal sample and InhibitEx solution were subjected to three freeze-thaw cycles, i.e., frozen overnight at -80 ºC and then kept in room temperature (20-25 ºC) for 12 hours. The latter procedure was included to increase the breaking up of cyst walls of protozoan parasites and thus the efficacy of DNA extraction.

4.5.1.1.4. Examining the feces of dogs for the presence of *Sarcocystis* spp.

DNA was extracted directly from the fecal sample using the kit according to the manufacturer's instructions without modification.

4.5.1.2. Oral/oropharyngeal/conjunctival swab samples

Oral swabs of domestic cats and wildcats, oropharyngeal swabs of columbiform birds and conjunctival swabs of dogs and cats were examined for the presence of trichomonads and *Acanthamoeba* spp., respectively. DNA from all samples were extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden Germany) according to the manufacturer’s blood protocol, with slight modifications. When DNA extraction was performed from 200 µl of culture medium in duplicates, after adding 200 µl AL buffer the process was continued with the blood DNA extraction protocol. On the other hand, thawed swabs were overlaid with 200 µl AL buffer and 200 µl sterile PBS, incubated for 10 min at 56 ºC prior to removal of cotton swab from the fluid, followed by adding proteinase-K and continuing the procedure according to the tissue protocol. In each group of 23 samples an extraction control (180 µl tissue lysis buffer) was included to monitor cross-contamination. All DNA extracts were stored at -20 ºC until molecular analysis by conventional PCRs.

4.5.1.3. Tissue samples

4.5.1.3.1. Examining intestinal tissue samples for the presence of trichomonads in a domestic cat and wildcats

The DNA from all intestinal tissue samples were extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's tissue protocol. Furthermore, all DNA extracts were stored at -20 ºC until molecular analysis by conventional PCRs.

4.5.1.3.2. Examining spleen tissue samples of felines for the presence of piroplasms and *Hepatozoon* sp.

The DNA was extracted individually from approximately 10 mg of spleen using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's tissue protocol. Prior to all DNA extracts were screened for piroplasms and *Hepatozoon* spp., they were stored at -20 ºC until molecular analysis by conventional PCRs.

4.5.1.4. Blood samples

4.5.1.4.1. Screening for the presence of piroplasms and *Hepatozoon* spp. in domestic cats

The DNA was extracted individually from 200 µl of collected blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's blood protocol. All DNA extracts were stored at -20 ºC until molecular analysis by conventional PCRs.

4.5.1.4.2. Screening for piroplasms and canine hemoplasmas in fighting dogs

DNA was extracted from 200 µl blood samples of 79 dogs using the blood protocol of NucleoSpin® Tissue Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. All DNA extracts were screened for piroplasms and canine hemoplasmas, but for molecular characterization of *B. gibsoni*, 10–11 samples were selected to represent dogs with mono- and co-infections, including all anemic dogs for which blood sample was available. All DNA extracts were stored at -20 ºC until molecular analysis by conventional PCRs.

4.5.2. PCR

4.5.2.1. Nested PCR for verifying the presence of *Giardia* spp.

All samples from beavers, and *Giardia*-positive samples from other species were investigated for the following three genetic markers: glutamate dehydrogenase (*gdh*), beta-giardin (*bg*) and triosephosphate isomerase (*tpi*) genes. The nPCRs were performed as reported [196, 197], using 1 µl of template DNA from the first-round PCR. In all cases, the amplification was done in a T100 Thermal Cycler (Bio-Rad, California, US) in a final volume of 25 µl using 2× Red PCR Master mix (Rovalab, Teltow, Germany) and 10 µM of each primer (GeneriBiotech,

Hradec Králové, Czech Republic). The amplification conditions, target genes and primers are shown in Table 3 [196, 197]. Positive and negative controls were included in each PCR reaction set. For the visualization of PCR products, 1.5% of agarose gel electrophoresis was prepared and stained with SYBR Safe DNA gel stain (Invitrogen, California, USA).

4.5.2.2. Conventional PCR for screening *T. gallinae* among columbiform birds

All DNA extracts and extraction controls were analyzed with three conventional PCRs: first with a screening assay amplifying a short, approx. 500-bp-long fragment of the 18S rRNA gene to detect the presence of Trichomonadea, followed by a primary and a secondary assay for sequencing approx. 1550-1600 and 1200-bp-long parts of two genetic markers (18S rRNA gene and alpha-tubulin genes, respectively). The reasons for selecting these two genetic markers were to include a conserved gene (18S rRNA) that is widely used in molecular characterization of *T. gallinae*, and a protein encoding gene (alpha-tubulin) for which sequences corresponding to 18S rRNA gene subtypes are available from North America [134] but not from Europe. The suitability of the screening assay was checked by sequencing PCR products of 13 samples which verified the presence of *T. gallinae* in all cases. PCR reaction components are described in Supplementary Table 2, while primers and cycling conditions of PCRs are summarized in Table 3 [134, 198–200].

4.5.2.3. Conventional PCR for testing trichomonads in dogs and cats

All samples were screened for the short fragment of 18S rRNA gene, then only the positive samples were examined further with PCRs for the long fragment of 18S rRNA gene and ITS2. Further details of the PCRs are summarized in Table 3 and Supplementary Table 2 [198, 199, 201].

4.5.2.4. Conventional PCRs for screening parabasalids and acanthamoebae in captive reptiles and the latter in dogs and cats

The details of primers and PCRs targeting trichomonads and *Acanthamoeba* spp. are summarized in Table 3 and Supplementary Table 2 [134, 198–200, 202].

4.5.2.5. Conventional PCR to identify protozoa found in the feces of dogs and cats fed with raw meat

DNA extracts were molecularly analyzed by conventional PCRs for *Neospora caninum*, *Toxoplasma gondii*, *Cystoisospora* spp., piroplasms and *Sarcocystis* spp. PCR components and cycling conditions are summarized in Table 3 and Supplementary Table 2 [203–211].

4.5.2.6. Conventional PCR for proving the presence of *Sarcocystis* spp. in dogs' feces

DNA extracts were molecularly analyzed with a conventional PCR modified from Casati et al. [209], amplifying an approx. 500-bp-long part of the 18S rDNA with the primers BJ1 and BN2, as it is shown in Table 3. This method is also suitable to detect *Babesia* DNA in fecal material [211], as well as to verify the presence of other apicomplexan genera including *Sarcocystis*, as reported [182]. The protocol of the PCR conducted is detailed in Supplementary Table 2.

4.5.2.7. Conventional PCR for testing *Hepatozoon* and *Cytauxzoon* spp. in domestic cats

The primers and cycling conditions of PCR analyses are summarized in Table 3 [212–214]. In the conventional PCR used to detect piroplasms (including *Cytauxzoon* spp.) 5 µL of extracted DNA was added to 20 µL of reaction mixture, while in the PCR amplifying a fragment of the 18S rRNA gene of *Hepatozoon* spp. the volume of reaction mixture was increased, since the protocol was modified by using 0.2 µL of each primer, 1 µL extra MgCl2 and 17.9 µL PCR grade water. All details are found in Supplementary Table 2.

4.5.2.8. Conventional PCR for screening piroplasms in fighting dogs

The components, primers and cycling conditions of the PCRs targeting 18S rRNA, *cox*1 and *cytb* genes of *Babesia* spp. are detailed in Table 3 and Supplementary Table 2 [37, 209, 215, 216].

In all PCRs sequence-verified positive controls were included, e.g.: in the investigation of *T. gallinae*: *Trichomonas vaginalis* DNA served as a control, while in the 18S rRNS PCR of piroplasms: *B. vogeli* DNA; in the *cox*1 and *cytb* PCRs: *B. gibsoni* DNA. In addition, non-template reaction mixture was also provided as negative control. As a last step, all PCR products mentioned in 4.5.2.2.- 4.5.2.8. were electrophoresed in 1.5% agarose gel (100V, 55-60 min), stained with ethidium-bromide and visualized under UV light.

4.5.2.9. Real-time PCR to detect canine hemoplasmas

DNA samples were screened for canine hemoplasmas via two species-specific TaqMan real-time qPCRs, which detect part of the 16S rRNA gene of either *Mycoplasma haemocanis* or "*Ca*. Mycoplasma haematoparvum" [217]. In these assays, plasmid DNA containing the cloned 16S rRNA gene of *M. haemocanis* or "*Ca*. M. haematoparvum" were used as positive controls.

Table 3: Primers and details for conventional PCR methods used in this study.

Target group	Target gene	Primer name	Primer sequence (5'-3')	Amplicon length (bp)	Thermocycling profile	Reference
Giardia spp.	***bg* (nested PCR)**	G7 G759	AAG CCC GAC GAC CTC ACC CGC AGT GC GAG GCC GCC CTG GAT CTT CGA GAC GAC	753	95 °C for 5 min; 40× (95 °C for 45 s, 50 °C for 30 s, 72 °C for 60 s); 72 °C for 7 min	[197]
		B-F B-R	GAA CGA ACG AGA TCG AGG TCC G CTC GAC GAG CTT CGT GTT	511	95 °C for 5 min; 35× (95 °C for 45 s, 55 °C for 30 s, 72 °C for 45 s); 72 °C for 7 min	
	***gdh* (nested PCR)**	GDHeF GDHeR	TCA ACG TYA AYC GYG GYT TCC GT GTT RTC CTT GCA CAT CTC C	n.s.	95 °C for 5 min; 40× (95 °C for 45 s, 50 °C for 30 s, 72 °C for 60 s); 72 °C for 7 min	
		GDHiF GDHiR	CAG TAC AAC TCY GCT CTC GG GTT RTC CTT GCA CAT CTC C	432	95 °C for 5 min; 40× (95 °C for 45 s, 60 °C for 30 s, 72 °C for 45 s); 72 °C for 7 min	
	tpi	ALA3542 ALA3542	AAA TIA TGC CTG CTC GTC G CAA ACC TTI TCC GCA AAC C	605	95 °C for 5 min; 40× (95 °C for 45 s, 50 °C for 30 s, 72 °C for 60 s); 72 °C for 7 min	[196]
Trichomonadida screening assay for short fragments	**18S rRNA**	1055F 16SR1	GGT GGT GCA TGG CCG TCA CCT ACC GTT ACC TTG	500	95 °C for 5 min; 40× (95 °C for 45 s; 50 °C for 45 s; 72 °C for 1,5 min); 72 °C for 10 min	[198]
Trichomonadida seminested PCR assay for long fragments	**18S rRNA**	16SL 16SR1	TAC TTG GTT GAT CCT GCC TCA CCT ACC GTT ACC TTG	1550	95 °C for 5 min; 45× (95 °C for 45 s; 48 °C for 45 s; 72 °C for 1,5 min); 72 °C for 10 min	[199]

	(nested PCR)					
		16SL 1385R	TAC TTG GTT GAT CCT GCC GAT CCT AAC ATT GTA GC	1450	95 °C for 5 min; 45× (95 °C for 45 s; 42 °C for 45 s; 72 °C for 1,5 min); 72 °C for 10 min	
Trichomonadida nested PCR assay for long fragments	**alpha-tubulin (nested PCR)**	AtubA AtubB	RGT NGG NAA YGC NTG YTG GGA CCA TNC CYT CNC CNA CRT ACC A	1200	95 °C for 5 min; 40× (95 °C for 45 s; 55 °C for 45 s; 72 °C for 1,5 min); 72 °C for 7 min;	[134, 200]
		AtubF1 AtubR1	TAY TGY YWN GAR CAY GGN AT ACR AAN GCN CGY TTN GMR WAC AT	1200	95 °C for 5 min; 40× (95 °C for 45 s; 45 °C for 45 s; 72 °C for 1,5 min); 72 °C for 7 min	
Trichomonadida	**ITS2**	TFR1 TFR2	TGC TTC AGT TCA GCG GGT CTT CC CGG TAG GTG AAC CTG CCG TTG G	330-380	95 °C for 5 min; 40× (95 °C for 30 s; 65 °C for 30 s; 72 °C for 50 s); 72 °C for 5 min	[201]
Acanthamoeba	**18S rRNA**	JDP1 JDP2	GGC CCA GAT CGT TTA CCG TGA A TCT CAC AAG CTG CTA GGG GAG TCA	480	95 °C for 5 min; 35× (95 °C for 35 s; 56 °C for 45 s; 72 °C for 1 min); 72 °C for 7 min	[202]
***Neospora* - *Toxoplasma* - *Cystoisospora* spp.**	**COI**	Toxo_COI_For Toxo_COI_Rev	GGA GGA GGT GTA GGT TGG AC CAT TTT GTA TTA TCT CTG GG	700	95 °C for 5 min; 40× (95 °C for 40 s; 55 °C for 30 s; 72 °C for 1,5 min); 72 °C for 10 min	[203]
***Neospora* sp.**	**NC5**	Np7 Np10	GGG TGA ACC GAG GGA GTT G TCG TCC GCT TGC TCC CTA TGA AT	200	95 °C for 5 min; 40× (95 °C for 40 s; 55 °C for 30 s; 72 °C for 1,5 min); 72 °C for 10 min	[204, 205]

***Toxoplasma* sp.**	**repeat region**	TOX-8 (fw) TOX5 (rev)	CCC AGC TGC GTC TGT CGG GAT CGC TGC AGA CAC AGT GCA TCT GGA TT	480	95 °C for 5 min; 35× (95 °C for 40 s; 60 °C for 1 min; 72 °C for 1 min); 72 °C for 10 min	[206–208]
***Piroplasms* *Sarcocystis* spp.**	**18S rRNA**	BJ1 BN2	GTC TTG TAA TTG GAA TGA TGG TAG TTT ATG GTT AGG ACT ACG	500	95 °C for 10 min; 40× (95 °C for 30 s; 54 °C for 30 s; 72 °C for 40 s); 72 °C for 5 min	[209]
***Hepatozoon* spp.**	**18S rRNA**	HepF HepR	ATA CAT GAG CAA AAT CTC AAC CTT ATT ATT CCA TGC TGC AG	650	95 °C for 5 min; 35× (95 °C for 40 s; 57 °C for 40 s; 72 °C for 60 s); 72 °C for 7 min	[212]
***Cytauxzoon* spp.**	***cytb***	Cytaux_cytb_Finn Cytaux_cytb_Rinn	ACC TAC TAA ACC TTA TTC AAG CRT T AGA CTC TTA GAT GYA AAC TTC CC	1333	95 °C for 5 min; 45× (95 °C for 20 s; 55 °C for 30 s; 68 °C for 1,5 min); 68 °C for 7 min	[213, 214]
Babesia gibsoni	***cox*1**	BgCOX1F MHR1	ATG CTT CAG AGT TAT AAT TCA G GCT GAT ACA ATA TAG GAT CTC C	700	95 °C for 5 min; 35× (95 °C for 40 s; 49 °C for 40 s; 72 °C for 90 s); 72 °C for 5 min	[215, 216]
Babesia gibsoni	***cytb***	427F CYTbR1	GCA TTC TTA GGT TAT GTT TTA CCA A ATA TGC AAA CTT CCC GGC TA	800	95 °C for 5 min; 35× (95 °C for 40 s; 53 °C for 40 s; 72 °C for 90 s); 72 °C for 7 min	[37, 216]

4.5.3. Sequencing

The sequencing in this study was conducted at two companies:

4.5.3.1. Macrogen Europe (Amsterdam, Netherlands)

PCR products of *Giardia*-positive samples were purified by using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and were further sequenced at Macrogen Europe (Amsterdam, Netherlands). Nucleotide sequences were analyzed using Basic Local Alignment Search Tool (BLAST) and aligned with homologous sequences available in GenBank. New sequences were submitted to GenBank (Table 4).

4.5.3.2. Biomi Ltd. (Gödöllő, Hungary)

Purification of selected positive PCR products and sequencing were done by Eurofins Biomi Ltd. (Gödöllő, Hungary). Quality control and trimming of sequences were performed with the BioEdit program (Informer Technologies, Inc.). Obtained sequences were compared to GenBank sequences by the BLASTN program (https://blast.ncbi.nlm.nih.gov). The sequences obtained were submitted to GenBank under accession numbers shown in Table 4.

Table 4: Accession numbers of the positive samples in GenBank

Species name	Host	Accession numbers
Giardia duodenalis	reptile	**PP481180-PP481181** (*bg* gene) **PP501010** (*gdh* gene) **PP501011** (*tpi* gene)
Trichomonas gallinae	pigeon	**ON631556-ON631566** (18S rRNA gene, long fragment) **ON808545-ON808550** (alpha-tubulin gene)
Tritrichomonas foetus	cat, dog	**PP227421-PP227425** (18S rRNA gene) **PP239334-PP239337** (ITS2)
***Monocercomonas* sp.**	reptile	**OM455397**
***Acanthamoeba* spp.**	reptile	**OM455398-OM455403**
Sarcocystis morae	dog	**MW579603**
Hepatozoon felis	cat	**OQ102981** (18S rRNA gene)
	wildcat	**OQ445870-OQ445872** (18S rRNA gene)
Cytauxzoon europaeus	wildcat	**OQ445869** (18S rRNA gene) **OQ455050** (*cytb* gene)
Babesia gibsoni	dog	**MW805762** (18S rRNA gene) **MW816918** (*cox*1 gene) **MW816919-MW816920** (*cytb* gene)
Babesia vulpes	dog	**MW805763** (18S rRNA gene)

4.5.4. Alignments and phylogenetic analyses

4.5.4.1. Comparison with GenBank data

New sequences obtained during our studies were compared to GenBank data by using the nucleotide BLASTn program (https://blast.ncbi.nlm.nih.gov).

4.5.4.2. Phylogenetic analyses

All sequences retrieved from GenBank and included in the phylogenetic analysis had 97-100% coverage with sequences from this study and were trimmed to the same length. The dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analysis was conducted by using the Maximum Likelihood method and the Jukes-Cantor model according to the best-fit selection with the program MEGA 7.0 [218]. However, some of the phylogenetic trees of Trichomonadea based on the 18S rRNA gene, were made with the Neighbor-Joining method and p-distance model (Figure 8, 9), and that for *bg* gene sequences of *Giardia* spp. with the Maximum Likelihood method and General Time Reversible (GTR) model using MEGA 11.0 [219]. The percentage of trees in which the associated taxa clustered together are shown next to the branches. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were also conducted in MEGA7. In course of the assessment of trichomonads of dogs and cats, the sequences in the phylogenetic analysis of the 18S rRNA gene (n=36) represented six orders of Trichomonadea. However, the availability of sequences in GenBank covering the same length of ITS2 as amplified in this study, limited the number of sequences to 19 that could be used in the relevant phylogenetic analysis. The sequence datasets were resampled 1,000 times to generate bootstrap values.

4.5.4.3. Assessing the genetic diversity of selected protozoa

The slowly evolving, 18S rRNA gene was chosen for assessing the genetic diversity of protozoan species focusing on those discovered during our studies in Hungary, because it is conservative enough to allow simultaneous comparison of phylogenetically distant species. Longer (>1200 bp) sequences of the 18S rRNA gene were retrieved from GenBank to the extent they were available, for each of the above eight protozoan parasite species. Sequences were compared with BlastN program (https://blast.ncbi.nlm.nih.gov) to obtain the maximum pairwise (p) distance within each species. The length of alignment used for phylogenetic analyses was 1360 bp for apicomplexan, and 1706 bp for non-apicomplexan protozoa. All positions containing gaps and missing data were eliminated. The evolutionary history was

inferred using the Neighbor-Joining method and p-distance model. This sequence dataset was resampled 1,000 times to generate bootstrap values. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA11 [200].

4.5.5. Statistical analysis

In the investigations we had sufficient data, a standard descriptive statistical analysis was used to review the acquired data such as prevalence (the result of which can be read in subsections 5.1.- 5.4.; 5.8.; 5.9.), mean and median ages (5.3.; 5.9.). Comparisons between different factors (sex, age, breed, symptom) (5.2.; 5.3.; 5.9.) were performed with the Fisher Exact Test (https://www.langsrud.com/fisher.htm). In some cases (5.3.; 5.9.) 95% confidence intervals (CI) were also calculated. During the investigation of fighting dogs (5.9.) normality was evaluated using Kolmogorov-Smirnov test, and mean values were compared with Student's t-test. Differences were regarded significant at $P < 0.05$.

5. Results

The results of each topic are described in the following subsections (5.1.-5.10.).

5.1. *Giardia*-infection in rodents and rabbits

The parasitological screening revealed the presence of cysts in 58.3% (7 of 12) asymptomatic Norway rats, as well as in 27.6% (21 of 76) of chinchillas, including two with diarrhea. Two degus were also *Giardia*-infected (prevalence: 16.7%), one showing diarrhea (Figure 3; Table 5). The flotation revealed only low number of cysts in the great majority of rodents with positive results, only one rat having relatively high numbers. On the other hand, no *Giardia* cysts were found in any samples of guinea pigs or domestic rabbits of this study (Table 5).

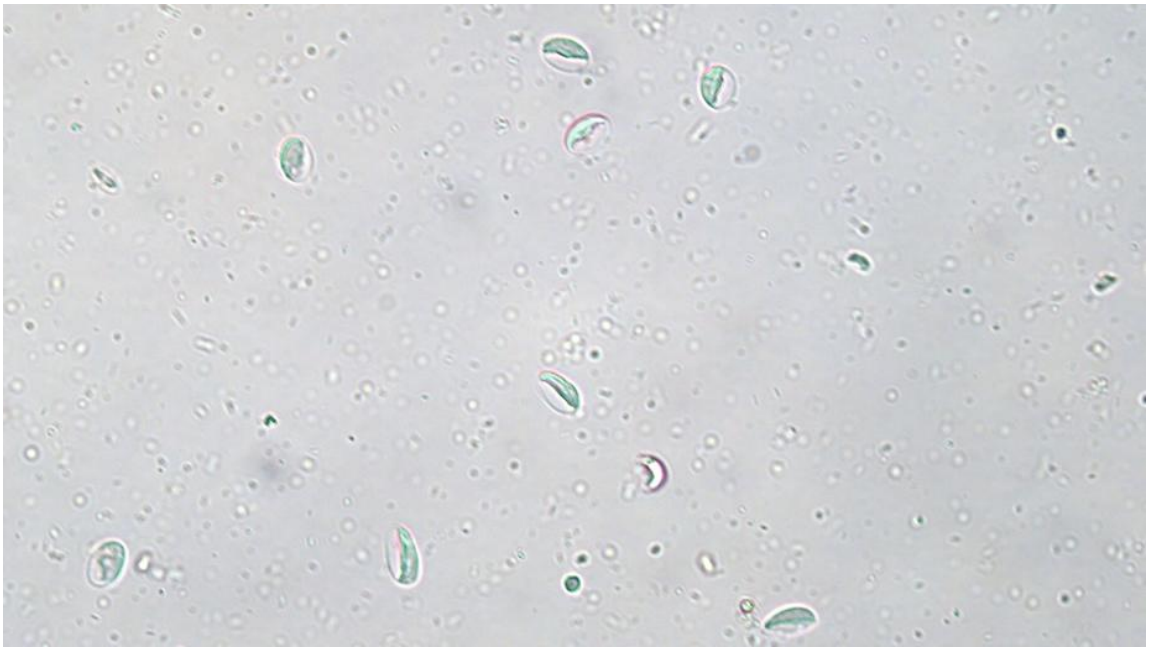

Figure 3: *Giardia duodenalis* cysts in fecal sample of a degu examined with flotation.

PCRs targeting three genetic markers yielded sufficient amount of PCR product for DNA sequencing, only in case of a few cyst-shedding rodents (Table 5). Based on the *bg* gene, assemblage B (NCBI acc. No. PP481180) was identified in one, and assemblage G (PP481181) in two rats (Table 5). This assignment was confirmed by their phylogenetic clustering (Figure 4). On the other hand, part of the *gdh* gene of assemblage E (PP501010) was successfully amplified from the content of a beaver's small intestine. This genotype was 100% (274/274 bp) identical only to *G. duodenalis* assemblage E (isolate 6L) reported form sheep in Spain (JF792403). In addition, the *tpi* gene sequence of *G. duodenalis* assemblage B (PP501011) was detected in one chinchilla. This had 100% (457/457 bp) identity with isolate IRU20 reported from a human being in Iran (MH310971).

Table 5. Host species and results of parasitological and molecular evaluation of their samples.

Host species	Total number evaluated (n=164 in total)	Number of *Giardia*-positive samples based on flotation (%)	Results of molecular analyses	
			Number of PCR-positives (gene)	Assemblage if successfully sequenced (accession number)
rat (*Rattus norvegicus*)	12	7 (58.3%)	3 (25.0%) (*bg*)	B (PP481180), 2×G (PP481181)
chinchilla (*Chinchilla lanigera*)	76	21 (27.6%)	1 (1.3%) (*tpi*)	B (PP501011)
guinea pig (*Cavia porcellus*)	10	0	ND	-
degu (*Octodon degus*)	12	2 (16.7%)	0	-
beaver (*Castor fiber*)	27	ND	1 (3.7%) (*gdh*)	E (PP501010)
rabbit (*Oryctolagus cuniculus var. domestica*)	27	0	ND	-

Abbreviations: ND – not done; *tpi* - triosephosphate isomerase; *bg* - beta-giardin; *gdh* - NADP-glutamate dehydrogenase

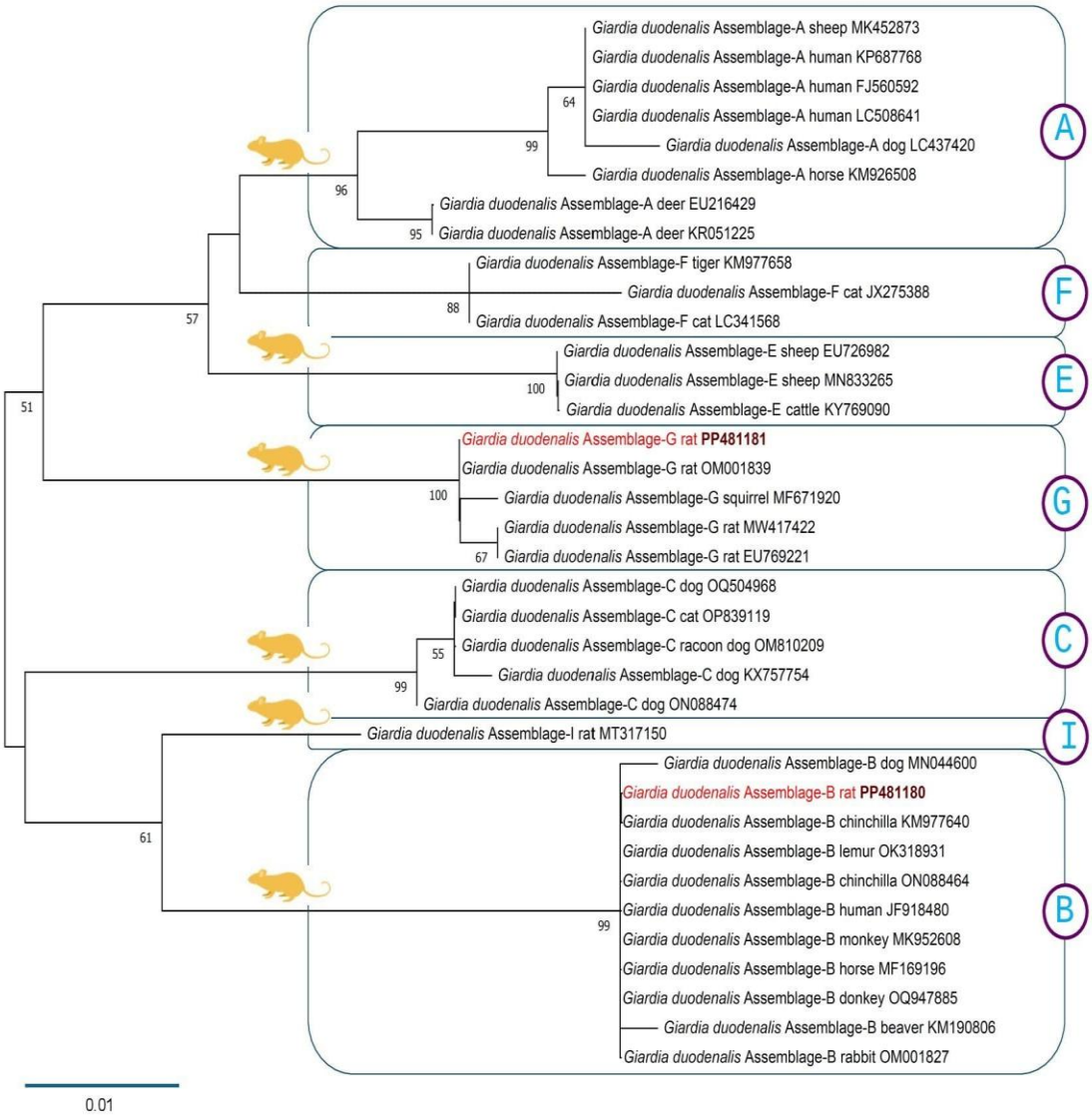

Figure 4: Phylogenetic tree of *Giardia duodenalis* sequences based on the beta-giardin *(bg)* gene, made with the Maximum Likelihood method and the General Time Reversible (GTR) model. In each row, after the species or genus name, the generic name of the isolation source and the GenBank accession number are shown. Sequences obtained in this study are in red and bold accession numbers. Mouse silhouette placed on the main branch of an assemblage indicates that it was reported to occur in rodents. There were a total of 413 positions in the final dataset. The scale-bar indicates the number of substitutions per site.

5.2. Avian *Trichomonas* species in Columbiformes

According to the preliminary comparison on the efficacy of molecular detection of *T. gallinae*, 19 out of 20 swab samples but only 18 out of 20 culture medium samples were PCR positive (i.e., one pigeon was positive only by its culture medium sample vs two pigeons diagnosed as infected only from their swab samples). Therefore, swab samples were used in the remaining part of the study.

Trichomonas gallinae was detected in all four studied bird species, with an overall prevalence of 73% (72 out of 99) (Table 6). Based on the screening assay, racing feral pigeons had a significantly ($P < 0.0001$) higher prevalence of *T. gallinae* infection (95%: 40 of 42) than urban feral pigeons (34%: 11 of 32). Among other columbiform bird species, the rate of PCR-positivity was the highest, 100% among wood pigeons (4 out of 4), followed in decreasing order by ring doves (94%: 15 out of 16) and collared doves (33%: 1 out of 3) (Table 6) These results also imply that the association of *T. gallinae* infection with artificially bred, captive columbiform birds (racing feral pigeons and ring doves) was highly significant ($P < 0.0001$) in comparison with free-living columbiform birds (urban and rural feral pigeons, wood pigeons and collared doves) (55/58 vs 17/41, respectively).

Based on the long fragment of the 18S rRNA gene, six *Trichomonas* subtypes were detected in 37 columbiform birds (Table 6). Compared to a reference sequence from North America (GenBank: EU215373), these had four to eight nucleotide differences (Table 7), i.e., 99.5-99.7% (1471-1475/1479 bp) identity. In a smaller geographical context, the 18S rRNA gene sequences obtained in this study from central and southeastern Europe had higher, up to 11 nucleotide differences with conspecific sequences from western and southwestern Europe: only 99.3-99.7% (1468-1474/1479 bp) sequence identity with *T. gallinae* from passeriform birds sampled in 2019 in France (e.g., MK172846), whereas 99.5-100% (1443/1450-1449/1449 bp) sequence identity with samples collected from columbiform birds in 2019 in Portugal (e.g., MK932772).

Only two genetic variants were detected among urban feral pigeons (subtypes B, D in Hungary; A, D in Romania), while four subtypes (B, C, D and the most divergent Hu-TG37) occurred in racing feral pigeons kept in one trading-breeding place (Table 6). Among racing feral pigeons, 18S rRNA gene subtype D was only found in birds that originated from Germany, while in Hungary subtypes A and E were exclusively found in wood pigeons and ring doves, respectively (Table 6). In the latter species, the occurrence of 18S rRNA gene subtypes was related to the origin (breeding place) of captive birds: subtype E was detected in two ring doves of one sampling locality, whereas subtype B in four birds at the other sampling locality. Clinical signs (lesions in the oropharyngeal cavity or the eyes, conjunctiva) were only associated with subtype D (Figure 7).

Table 6. Results of molecular analyses for the 18S rRNA gene of Trichomonadea according to sample types of columbiform birds.

Species or type of sample source	Country of origin	Prevalence** (positive/all)	Long 18S rRNA genotype (n)	GenBank accession number
Racing pigeon (*Columba livia domestica*)	Hungary	97% (29/30)	**B** (16), **C** (3), *Trichomonas* sp. Hu-TG37 (1)	ON631556, ON631557, ON631566
	Germany*	89% (8/9)	**D** (3)	ON631558
	Denmark*	100% (2/2)	**B** (1)	ON631556
	Belgium*	- (1/1)	**B** (1)	ON631556
Urban pigeon (*Columba livia domestica*)	Hungary	40% (4/10)	**B** (1), **D** (1)	ON631559, ON631560
	Romania	32% (7/22)	**A** (1), **D** (2)	ON631561, ON631562
Feral pigeon (*Columba livia domestica*)	Hungary	50% (1/2)	-	--
Common wood pigeon (*Columba palumbus*)	Hungary	100% (14/14)	**A** (1), **B** (4)	ON631563, ON631564
Ring dove (*Streptopelia risoria*)	Hungary	83% (5/6)	**E** (2)	ON631565
Eurasian collared dove (*Streptopelia decaocto*)	Hungary	33% (1/3)	-	-

*kept separately after arrival, but contact with Hungarian birds cannot be excluded
**based on the short 18S rRNA gene screening PCR

Table 7. Site-specific variations among 18S rRNA gene subtypes of *Trichomonas gallinae* and *Trichomonas* sp. Hu-TG37 compared to positions in EU215373 used as a reference sequence (identical with genotype A). Nucleotides that are identical to the reference are indicated with "-". Note that only the last three positions shown in this table are covered by the screening (short 16S rRNA gene) PCR, i.e., genotyping was possible from 37 of 72 *Trichomonas*-positive specimens yielding sequenceable product in the long 18S rRNA PCR.

18S rRNA genotype	Position (in which the nucleotide is indicated below)														
	33	215	384	386	408	418	590	592	648	825	837	850	1120	1143	1281
A	A	T	T	A	G	C	C	A	C	A	G	T	A	A	C
B	-	-	-	T	-	-	-	-	-	-	A	-	C	G	-
C	-	-	-	T	-	-	-	-	-	G	A	C	C	G	-
D	-	-	A	T	-	-	T	T	T	-	-	-	C	G	-
E	-	-	-	T	-	-	-	-	T	G	A	C	C	G	-
Hu-TG37	T	A	-	T	A	T	-	-	-	-	-	-	T	T	T

Amplification and sequencing of part of the alpha-tubulin gene were successful from at least one sample representing each 18S subtype. Alpha-tubulin gene sequences obtained in this study had a lower (98.5-98.6%: 1008-1009/1023 bp) or a higher level (99.6-99.7%: 1019-1020/1023 bp) of sequence identity to that of an isolate (EU215382) used as a reference. Based on the corresponding amino acid sequences, most mutations in this protein encoding gene were synonymous, but subtypes E and D had a single amino acid difference compared to the reference isolate (valine instead of alanine at position 149, and isoleucine instead of valine at position 34, respectively).

Considering the results of phylogenetic analyses, the topology of the 18S rRNA phylogenetic tree (Figure 5) did not show clear clustering of *T. gallinae* subtypes obtained in this study according to host species, living place (or keeping modes) of columbiform birds. However, subtype A (identified both in Hungary and Romania) formed a sister group to all other 18S rRNA gene subtypes (B, C, D, E): although with only moderate (65%) support (Figure 5) but confirmed by the alpha-tubulin phylogenetic tree (Figure 6). More importantly, *Trichomonas* sp. Hu-TG37 (detected in a racing feral pigeon in south Hungary) belonged to the phylogenetic group of *T. canistomae* and *T. tenax* with moderate (61%) support, implying, that this is a separate species (in other words: if this isolate would belong to *T. gallinae*, this species would not be monophyletic). The separation of *Trichomonas* sp. Hu-TG37 from *T. gallinae* was also confirmed by the alpha-tubulin phylogenetic tree (Figure 6).

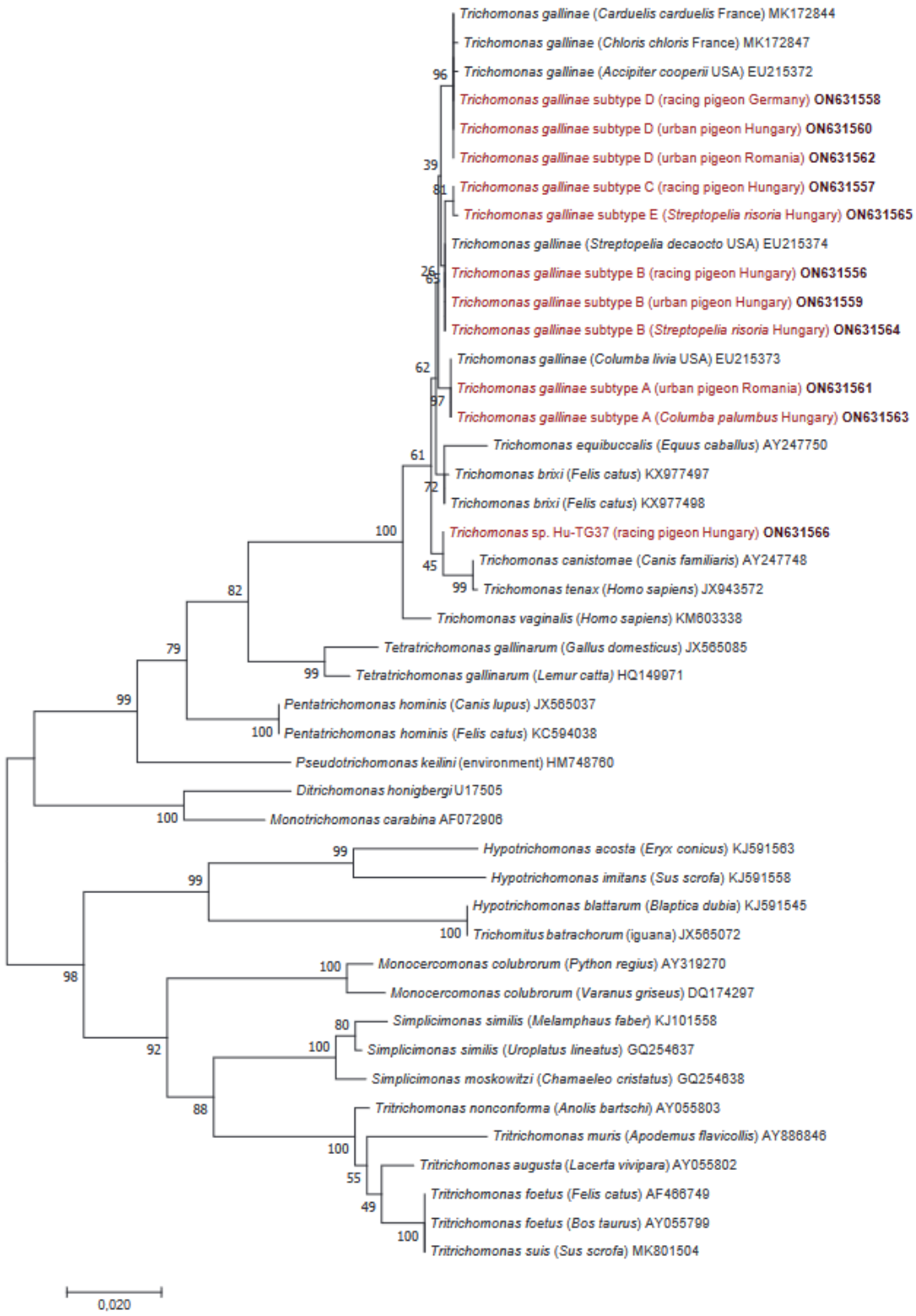

Figure 5. Phylogenetic tree of Trichomonadea based on the 18S rRNA gene, made with the Maximum Likelihood method and the Jukes-Cantor model. In each row, after the species or genus name, the isolation source, for *Trichomonas gallinae* the country of origin and the GenBank accession number are shown. Sequences obtained in this study and representing each subtype are in red and bold accession numbers. The analysis involved 44 nucleotide sequences and 1000 bootstrap replications. There were a total of 1121 positions in the final dataset. The scale-bar indicates the number of substitutions per site.

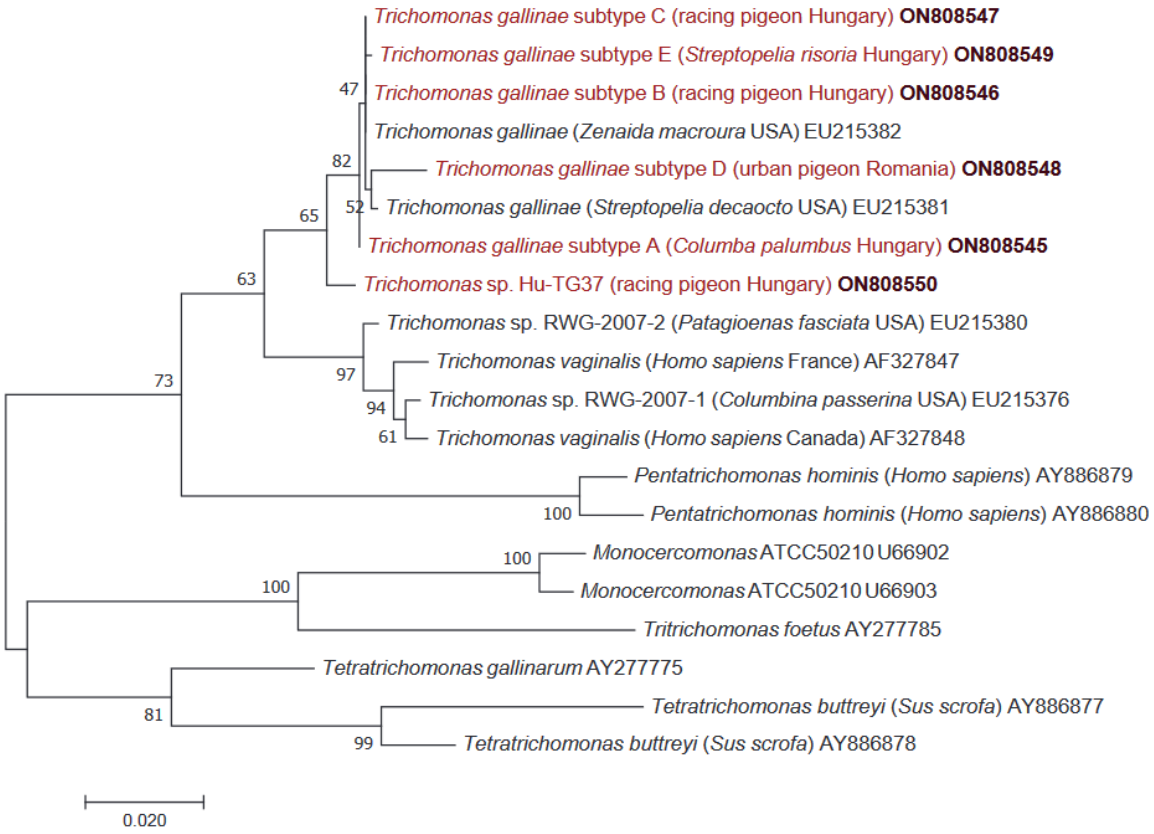

Figure 6. Phylogenetic tree of Trichomonadea based on the alpha-tubulin gene, made with the Maximum Likelihood method and the Jukes-Cantor model. In each row, after the species or genus name, the isolation source, for species closely related to *Trichomonas gallinae*, the country of origin and the GenBank accession number are shown. Sequences obtained in this study and representing each subtype are in red and bold accession numbers. The analysis involved 20 nucleotide sequences and 1000 bootstrap replications. There were a total of 977 positions in the final dataset. The scale-bar indicates the number of substitutions per site.

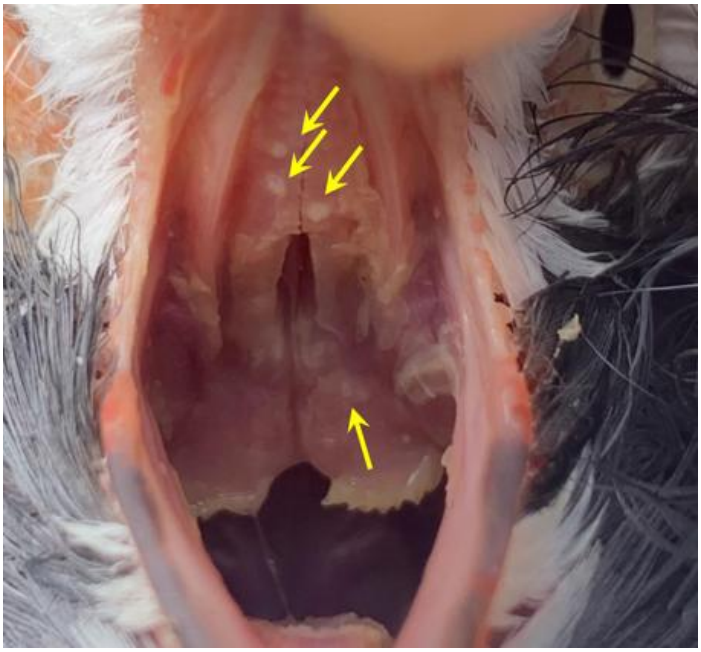

Figure 7. Small necrotic-inflammatory foci on the palate of a racing pigeon.

5.3. Trichomonads infecting cats and dogs in Hungary

5.3.1. Molecular identification and phylogenetic analyses of trichomonads

Altogether 123 animals were PCR-tested, among which 20 were positive for trichomonads (Table 8). No significant difference was found between the rate of PCR-positivity according to sampling methods. Thirteen (13.8%) of domestic cats were positive for *T. foetus* and two (2.1%) for *P. hominis*. In addition, one feline sample (1.1%) contained the DNA of a different *Tritrichomonas* species which in the sequenced, 337-bp-long part of its 18S rRNA gene (PP227424) was genetically most closely related to *Tritrichomonas casperi* (ON927245) isolated from mouse (*Mus musculus*), showing 96.44% identity to the latter. Regarding dogs, 4 out of 25 (16%) proved to be *T. foetus* positive. Wild cats did not harbor any trichomonads.

All six feline isolates of *T. foetus*, from which a longer part of the 18S rRNA gene was successfully amplified, had 100% sequence identity to each other (PP227421), and to sequences of *T. foetus* deposited in GenBank from cat (AF466749), cattle (AY055799) as well as to that of *T. suis* from pig (MK801504). The same can be said for the 17 *T. foetus* positive samples (PP227422, PP227423), in which the short part of 18S rRNA was examined. Based on the examination of ITS2, the sequence of *T. foetus* from this study (PP239334), 100% sequence identity was shown to *T. foetus* sequences of cats from China (OP866181 and OP856640, respectively) and the USA (AF466749).

Considering the short 18S rRNA sequence of the two *P. hominis* positive cat samples (PP227425) it showed 100% identity to a *P. hominis* isolate from a cat (KC594038) and 99.3% identity to *P. hominis* from a dog (AY758392). The longer part of the 18S rRNA gene could not be amplified from these samples. Regarding the corresponding ITS2 sequence (PP239337), it was 99,7% identical to *P. hominis* of a cat from Czechia (KC594038) and of a dog from the USA (AY758392). In addition, the ITS2 also showed 99.7% sequence identity to *P. hominis* of a human sample from Thailand (AF156964). All data are summarized in Table 8.

Table 8. PCR results based on targeted genes with related GenBank accession numbers

	PCR positive and sequenced			GenBank accession numbers		
	18S rRNA gene: short	**18S rRNA gene: long**	**ITS**	**18S rRNA gene: short**	**18S rRNA gene: long**	**ITS**
Tritrichomonas foetus	17	6	17	PP227422, PP227423	PP227421	PP239334, PP239335
Pentatrichomonas hominis	2	0	2	PP227425	0	PP239337
***Tritrichomonas* sp.**	1	0	1	PP227424	0	PP239336

Based on results of the short 18S rRNA gene and ITS2 phylogenetic analyses, all *T. foetus* sequences from cats and cattle clustered together, including those from this study (PP227422 and PP239334, respectively), with moderate to high support (Figure 8, Figure 9). Similarly, based on both genetic markers, *P. hominis* sequences from this study (PP227425 and PP239337, respectively) belonged to the phylogenetic group of *P. hominis* isolates from cats, dogs and human with high (100%) support (Figure 8, Figure 9). In line with the molecular comparisons described above, phylogenetic analyses of the 18S rRNA gene and ITS2 sequences of the novel *Tritrichomonas* genotype obtained from a cat in northeastern Hungary (PP227424: 337 bp long, and PP239336: 342 bp long, respectively) showed that it is a sister species of *T. casperi* from mice (*Mus musculus*) (Figure 8, Figure 9).

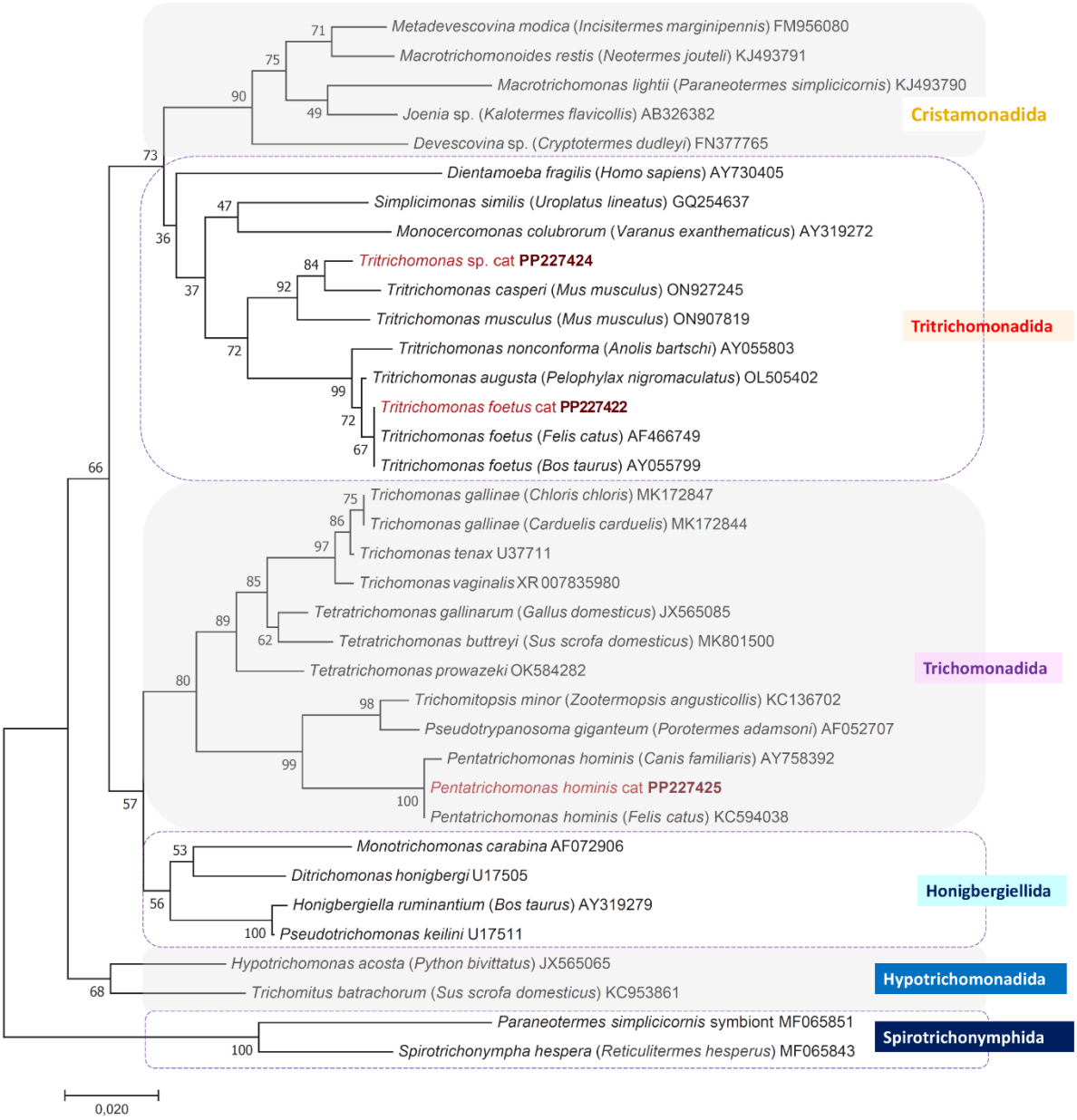

Figure 8: Phylogenetic tree of Trichomonadea based on the 18S rRNA gene, made with the Neighbor-Joining method and p-distance model. In each row, after the species or genus name, the isolation source of trichomonads and the GenBank accession number are shown. Sequences obtained in this study are in red and bold accession numbers. The analysis involved 36 nucleotide sequences. The final length of the alignment was 281 bp. Two species of Spirotrichonymphida were used as outgroup. The scale-bar indicates the number of substitutions per site.

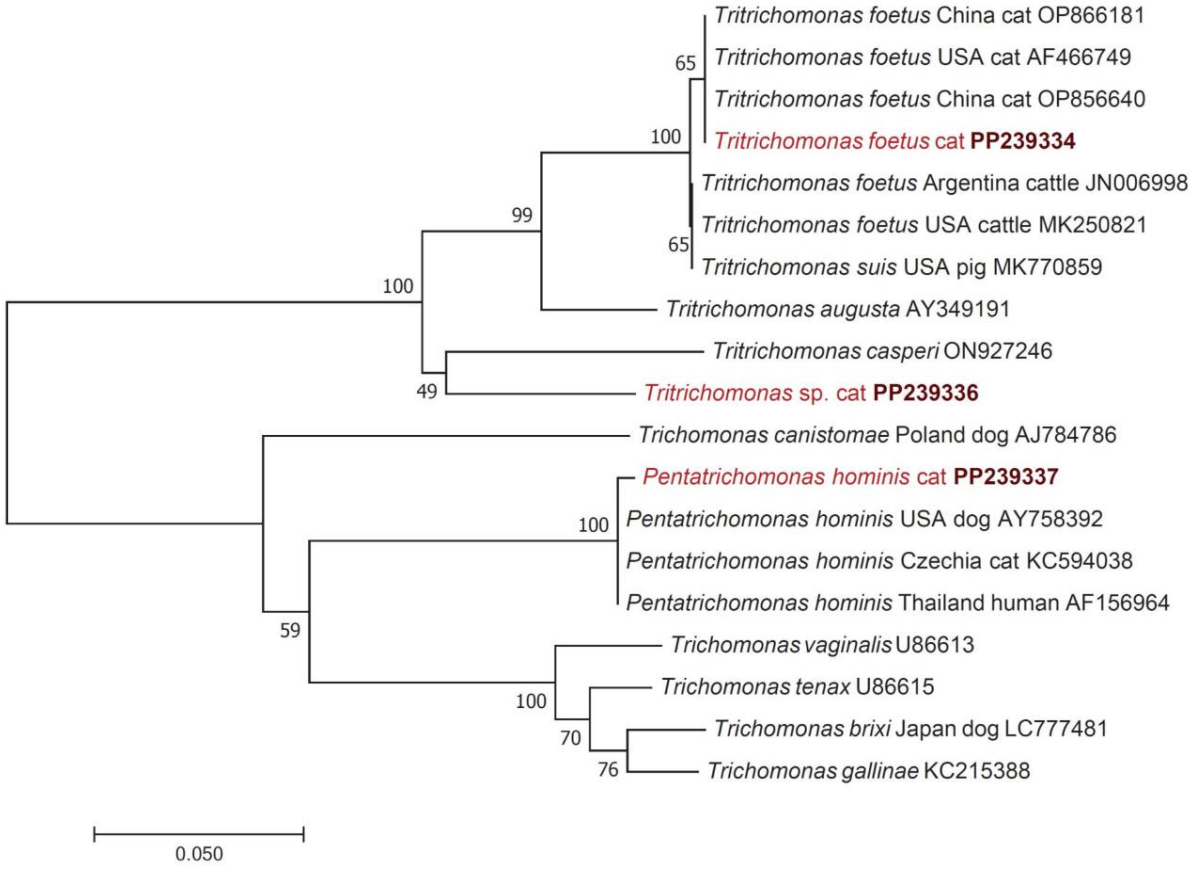

Figure 9: Phylogenetic tree of Trichomonadea based on the ITS gene made with the Neighbor-Joining method and p-distance model. In each row, after the species or genus name, the isolation source of trichomonads and the GenBank accession number are shown. Sequences obtained in this study are in red and bold accession numbers.

5.3.2. Geographical distribution of positive samples

All 13 *T. foetus* positive domestic cats and four dogs were from the South-Central region of Hungary. One of the *P. hominis* positive cats was from Budapest, and the other was from Aggtelek National Park, similar to the *Tritrichomonas* sp. infected cat (Table 9, Figure 10).

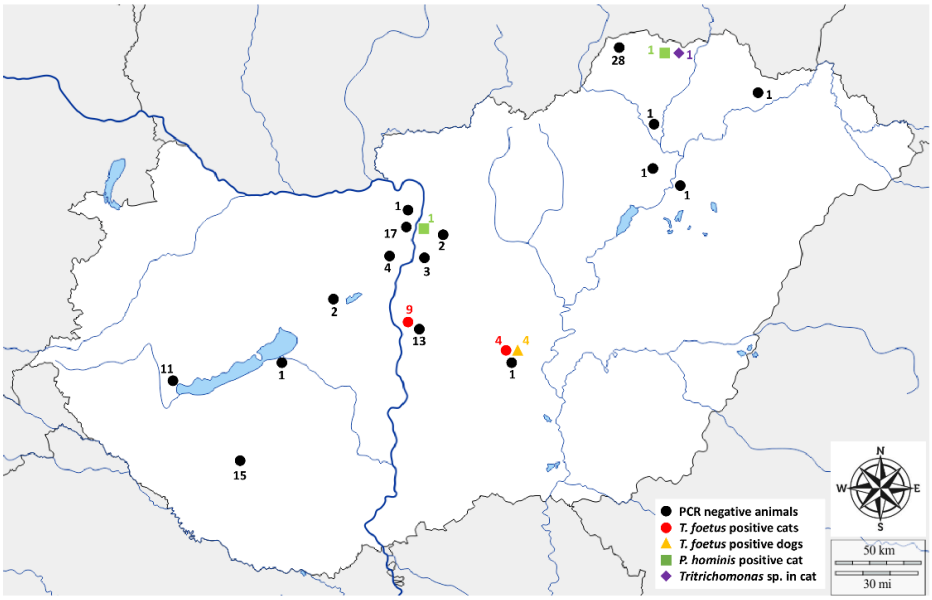

Figure 10: Geographical distribution of samples used in this study. The number next to a mark indicates the number of animals tested at that location.

5.3.3. Analysis of host data and morbidity

The sex of 90 cats (95.7% of all) and 21 dogs (84% of all) participating in the study was known. Based on their data, the sex ratio was 65.6% (n=59) females and 34.4% (n=31) males among cats, and 42.9% (n=9) females and 57.1% (n=12) males among dogs. Age was reported for 68 (72.3%) of the domestic cats and 22 (88%) of the dogs participating in the investigation. The mean age of the cats was 6.47 years (median 6 years), ranging from kittens to 14 years of age. The mean age of dogs was 6.3 years (median 6 years). In total, 17% (n= 16) of cats and 32% (n= 8) of dogs showed symptoms of gastrointestinal disorder.

Data of PCR-positive cats and dogs are summarized in Table 9. Among the *T. foetus* positive cats (n=13) there were more females (69.2%) than males (30.8%), while both *P. hominis* positive cats were females. Based on this, there was no significant correlation between PCR-positivity and the sex. On the other hand, the rate of PCR positivity was significantly (p=0.0011) higher among Ragdoll cats (13 of 41: 31.7%) than among European Shorthair cats (2 of 47: 4.3%). The mean age of *T. foetus*- and *P. hominis*-infected cats were 15.2 (median 12), and 6.5 (median 6.5) months, respectively. This implies that significantly (p=0.0273) more cats were PCR-positive below 1 year of age (9 of 21: 42.9%), than among older cats (7 of 47: 14.6%). Among *T. foetus* positive dogs, the age and sex were provided for only one dog (3-month-old male).

Out of 16 PCR positive cats, eight (50%) showed clinical symptoms, mainly diarrhea (Table 9). Thus, PCR-positive cats showed gastrointestinal symptoms significantly (p=0.0011) more frequently than negative cats (9 of 78: 11.5%). At the same time, all the positive dogs showed relevant clinical signs.

Table 9: Data of the 20 animals, found positive for Trichomonadidae by PCR.

Site	Animal	Breed	Age*	Sex*	Symptoms	Sampling	PCR result
Kunszentmiklós	Cat	Ragdoll	2 y	F	No	feces + swab + InPouch	*T. foetus*
Kunszentmiklós	Cat	Ragdoll	1.5 y	F	No	swab	*T. foetus*
Kunszentmiklós	Cat	Ragdoll	2 y	F	No	swab	*T. foetus*
Kunszentmiklós	Cat	Ragdoll	3 y	M	No	swab	*T. foetus*
Kunszentmiklós	Cat	Ragdoll	1.5 y	F	Diarrhea	swab	*T. foetus*
Kunszentmiklós	Cat	Ragdoll	4 y	F	No	swab	*T. foetus*
Kunszentmiklós	Cat	Ragdoll	1 y	M	No	swab	*T. foetus*
Kunszentmiklós	Cat	Ragdoll	2 m	F	Diarrhea	InPouch	*T. foetus*
Kunszentmiklós	Cat	Ragdoll	4 m	M	Diarrhea	InPouch	*T. foetus*
Budapest	Cat	Persian-Himalayan	7 m	F	Strong diarrhea	feces + swab	*P. hominis*
Komjáti	Cat	European shorthair	1y	F	Unknown	swab	*Tritrichomonas* sp.
Komjáti	Cat	European shorthair	6m	F	Unknown	swab	*P. hominis*
Kecskemét	Cat	Ragdoll	3 m	M	Diarrhea	swab	*T. foetus*
Kecskemét	Cat	Ragdoll	3 m	F	Diarrhea	swab	*T. foetus*
Kecskemét	Cat	Ragdoll	3 m	F	Diarrhea	swab	*T. foetus*
Kecskemét	Cat	Ragdoll	3 m	F	Diarrhea	swab	*T. foetus*
Kecskemét	Dog	Maltese Dog	3 m	M	Diarrhea	swab	*T. foetus*
Kecskemét	Dog	Unknown	Unknown	Unknown	Diarrhea	swab	*T. foetus*
Kecskemét	Dog	Unknown	Unknown	Unknown	Diarrhea	swab	*T. foetus*
Kecskemét	Dog	Unknown	Unknown	Unknown	Diarrhea	swab	*T. foetus*

*Abbreviations: m=month, y=year, M=male, F=female.

5.4. Protozoa found in the feces of captive reptiles

A single sample from a leopard gecko (*Eublepharis macularius*) was positive in the PCR targeting the ribosomal small subunit (SSU) RNA gene or 16S-like rRNA gene of trichomonads. Sequencing verified the presence of a not yet reported *Monocercomonas* genotype/species, with only up to 96.1% (1446/1505 bp) identity to the closest match available in GenBank (DQ174303) that represented *Monocercomonas colubrorum* [220] and 94.9-95.9% (1432/1509 to 1443/1504 bp) identity to further sequences reported from this species and its genus. The phylogenetic separation of the new genotype from *Monocercomonas* sequences retrieved from GenBank, including those of *M. colubrorum*, was highly (100%) supported (Figure 11). However, amplification of part of the alpha-tubulin gene was not successful from the *Monocercomonas*-positive sample.

Fecal samples of six reptile species showed positivity in the PCR targeting SSU rDNA, which one is specific for the genus *Acanthamoeba*, and all detected species/genotypes clustered with other *Acanthamoeba* sequences available in GenBank (Figure 12). In two samples, one from a yellow anaconda (*Eunectes notaeus*) and the other from a Gila monster (*Heloderma suspectum*) or a beaded lizard (*Heloderma horridum*) kept together, *Acanthamoeba hatchetti* was identified (OM455398 and OM455399, respectively), with 100% (408/408 bp) sequence identity to an isolate from compost in Switzerland (KC164235) [221]. Based on the phylogenetic examination they clustered with the strains of T11 genotype (Figure 12). From a bosc monitor (*Varanus exanthemicus*) the amplified sequence (OM455400) had 100% (414/414 bp) identity to strains of *A. castellanii* (accession numbers KX018029, KX018030) detected in conjunctival swabs of dogs reported from Turkey [222]. The sequence from a frilled dragon (*Chlamydosaurus kingii*: OM455401) and an alligator snapping turtle (*Macrochelys temminckii*: OM455402) showed 99.75% (406/407 bp) identity with *A. lugdunensis* (KY072781) from a human patient with keratitis in Spain [223]. Furthermore, the sample of a green iguana (*Iguana iguana*; OM455403) was 100% identical (404/404 bp) to *Acanthamoeba* T13 genotype (KF928948) from grassland soil, Italy [89]. In addition, phylogenetically it grouped together with the strains of T13 genotype (Figure 12).

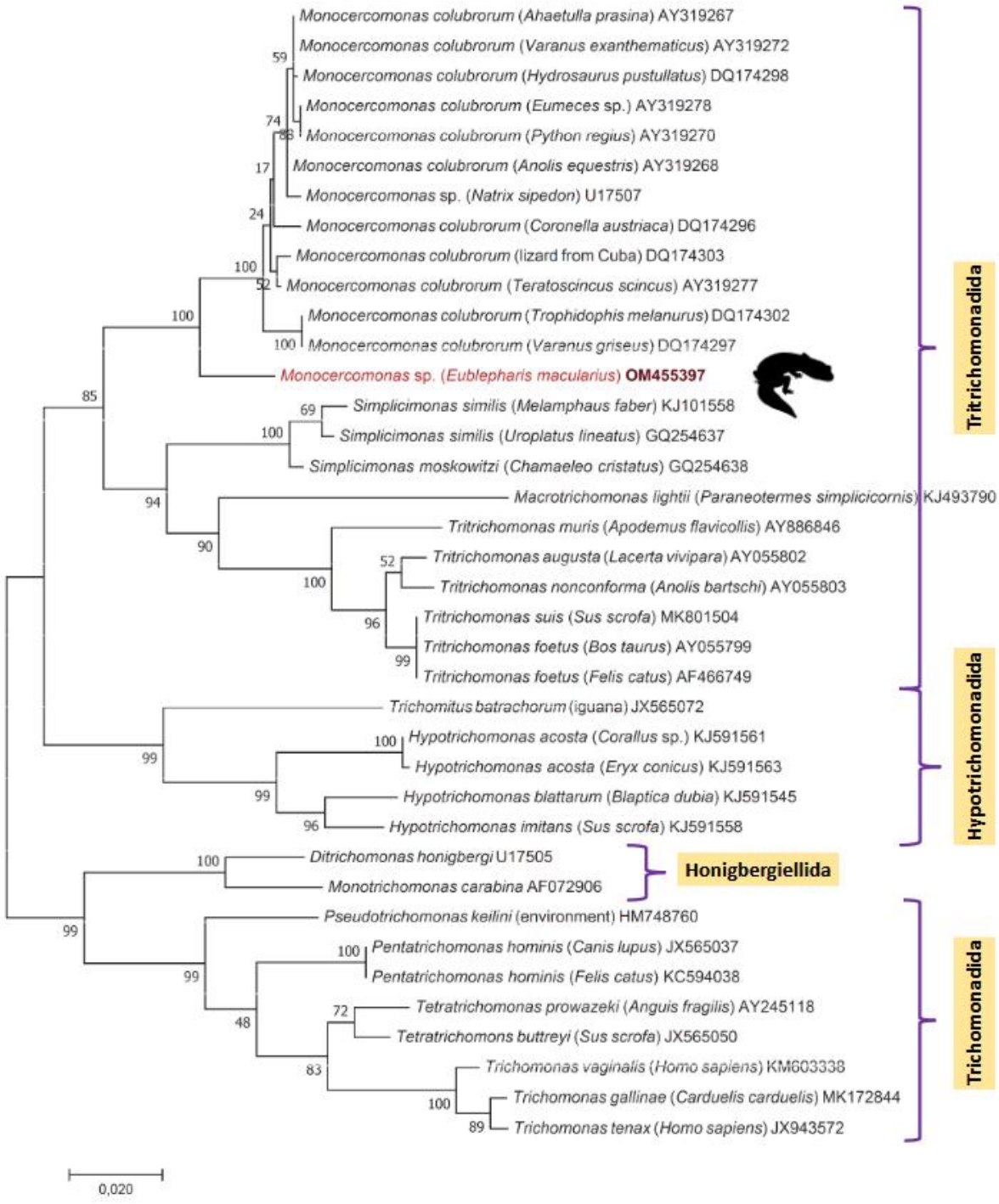

Figure 11. Phylogenetic tree of Trichomonadea. In each row, after the species or genus name, the isolation source and GenBank accession number are shown. Sequences obtained in this study are indicated by red fonts and bold accession numbers. The reptile species which had PCR positive sample is shown with its silhouette. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [1]. The tree with the highest log likelihood (-8331,32) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. 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 is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 38 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1402 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.0.

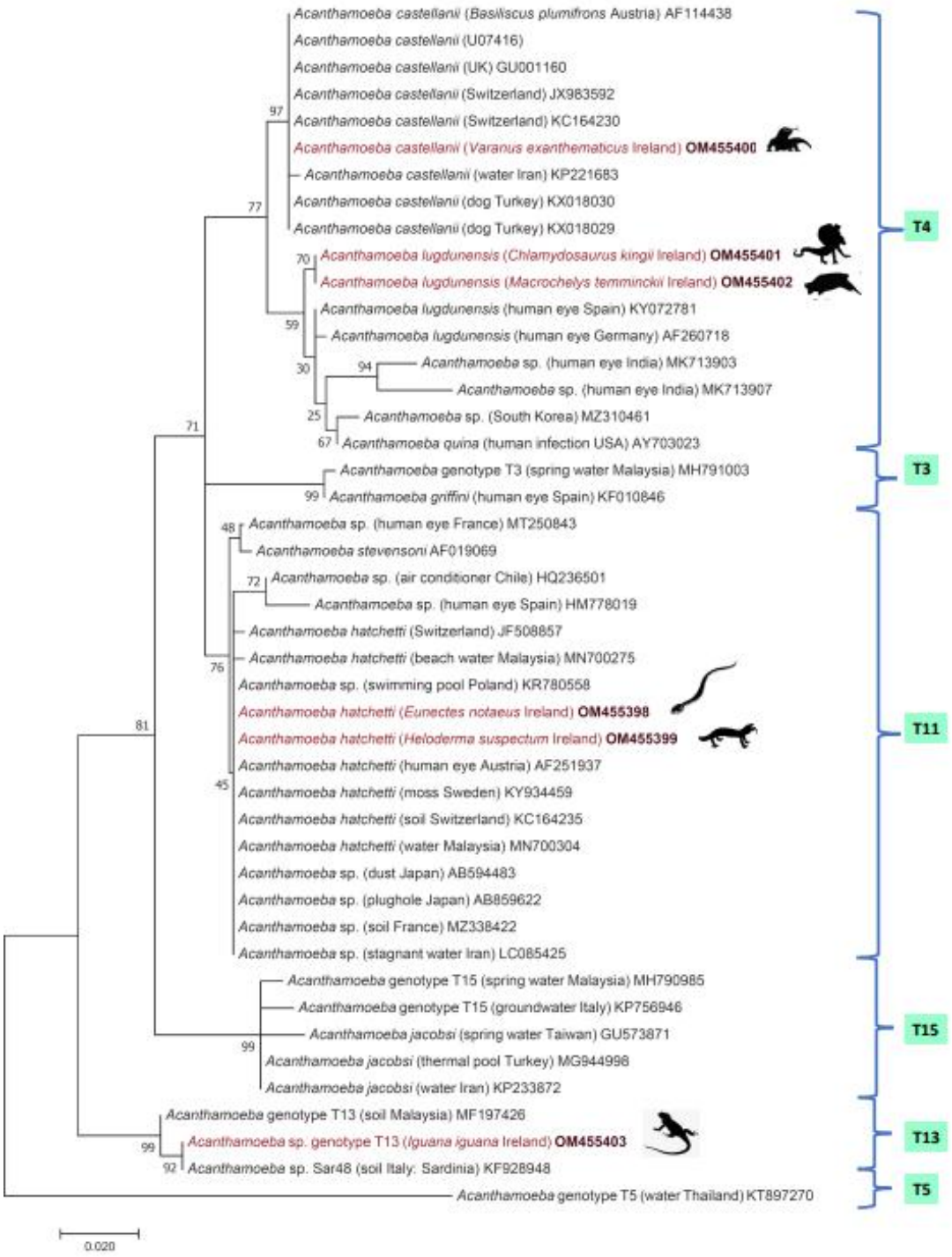

Figure 12. Phylogenetic tree of *Acanthamoeba* spp. In each row, after the species or genus name, the isolation source, the country of origin and GenBank accession number are shown. Sequences obtained in this study are indicated by red fonts and bold accession numbers. Reptiles which had PCR positive samples are shown with their silhouette. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The tree with the highest log likelihood (-8520,58) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. 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 is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 404 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.0.

5.5. Result of the screening of conjunctival swabs of dogs and cats for the presence of *Acanthamoeba* spp.

All DNA samples extracted from the conjunctival swabs returned negative results from PCR's targeting *Acanthamoeba* spp.

5.6. Protozoa found in the feces of dogs and cats fed with raw meat

5.6.1. Parasitological analysis

In the fecal samples of two dogs, oocysts of *Cystoisospora canis* (40×32 µm) (in BARF8), a *Cystoisospora ohioensis*-like sp. (25×16 µm) and *Eimeria stiedai* (32×20 µm) were detected (in BARF9) (Figure 13). In addition, in one sample (BARF76) sporocysts of a *Sarcocystis* sp. (13×7 µm) were seen.

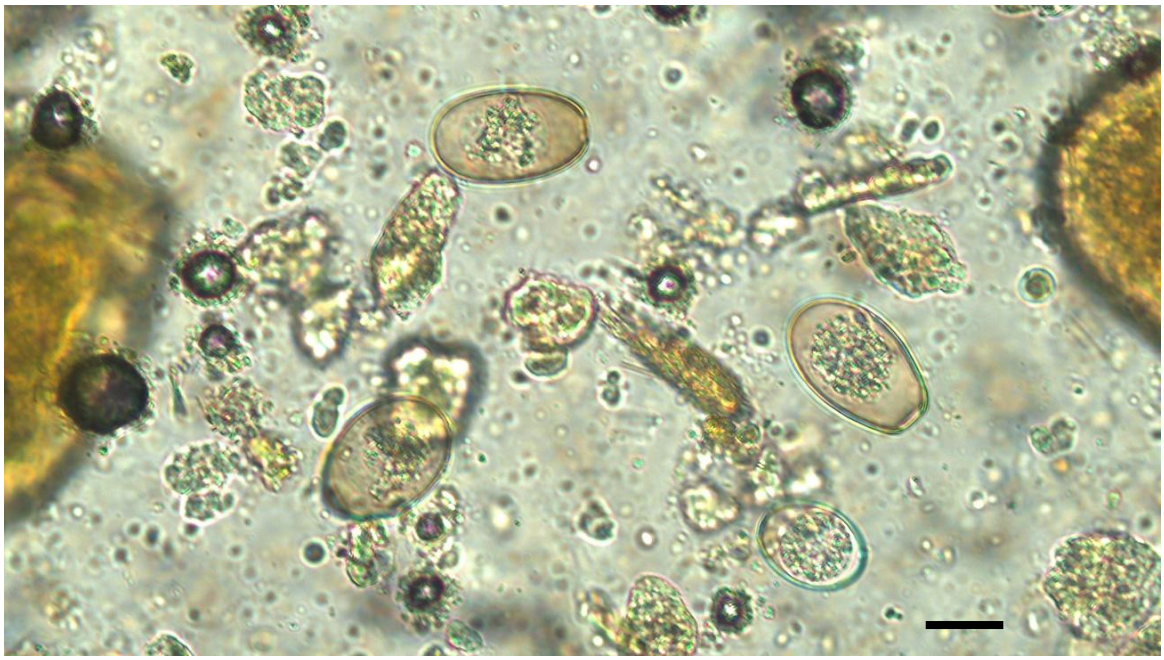

Figure 13. Pseudoparasites revealed from the feces of BARF-fed pets: oocysts of *Eimeria stiedai* (larger) and *Cystoisospora ohioensis*-like sp. Bar = 10 µm.

5.6.2. Molecular analysis

The samples examined for *Neospora caninum*, *Toxoplasma gondii*, *Cystoisospora* spp., *Babesia* spp. and *Sarcocystis* spp. were negative with molecular diagnostic methods.

5.6.3. Questionnaire-based data

Data of positive samples and some related information based on the questionnaire are summarized in Table 10. The most often fed contents were the meat/viscera of rabbit (66%),

then cattle and lamb with 61%, fish and chicken with 57%. In addition, the owners fed their pet with duck, turkey, wild animals and horse meat. Interestingly, seafood was given in the smallest percentage (1%). We received no usable information on whether any animals ate nerve tissue. Altogether 96% of the owners froze the raw meat before consumption, and 49% of them used anthelmintic treatment every three months (against ascarid roundworms, hookworms and tapeworms). The owners did not notice relevant symptoms during BARF diet.

Table 10. Detailing the results and the diet of the animals with positive samples.

Sample	Parasitological examination (flotation)	PCR	Fed raw food	Frozen (-20°C) before feeding	Regular worming	Species
BARF8	+ (*C. canis*)	-	Raw meat: lamb, chicken, rabbit, game, fish Viscera: liver, spleen, kidney of lamb, chicken	+	+	*Canis lupus familiaris*
BARF9	+ (*C. ohioensis*-like sp., *E. stiedai*)	-	Raw meat: beef, lamb, rabbit, horse, duck, game Viscera: liver, spleen, kidney of ruminants and rabbit	+	+	*Canis lupus familiaris*
BARF73	+ (*Sarcocystis* sp.)	-	Raw meat: beef, chicken Viscera: liver and brain stem of chicken	-	+	*Canis lupus familiaris*

5.7. Results of the examination of *Sarcocystis* sporocysts from dog feces

In one fecal sample, the DNA of *Sarcocystis morae* was present, with 100% (491/491 bp) identity to GenBank sequences from fallow deer (MN443755), red deer (KY973375) and red fox (KT873775), reported from Lithuania, Spain and Germany, respectively. The phylogenetic analysis also supported the species identity of this canine isolate, because it clustered within the phylogenetic group of *S. morae* sequences deposited in GenBank from various parts of Europe. These formed a sister group to *S. grueneri* (Figure 14).

Following concentration with flotation, the only protozoan parasites seen in the PCR-positive fecal sample were *S. morae* sporocysts. These sporocysts were oval in shape and measured (12.5–17.5) × (7.5–12.5) µm, with mean values of 14.95 × 9.75 µm (Figure 14, insert). This size range considerably overlaps with measurements of sporocysts of *Sarcocystis cervicanis* ([15.1–17.1] × [10.3–11.9] µm: [224], an unidentified *Sarcocystis* sp. (15.4 × 8.8 µm:

[225]) and *Sarcocystis gracilis* (15 × 10 µm: [226].The sporocysts of these species were all reported from dogs after consuming the meat of relevant intermediate hosts, i.e., red deer, fallow deer and roe deer, respectively. Thus, *Sarcocystis* species that have cervids as intermediate hosts and canids as final hosts cannot be distinguished according to their sporocyst size in dog feces.

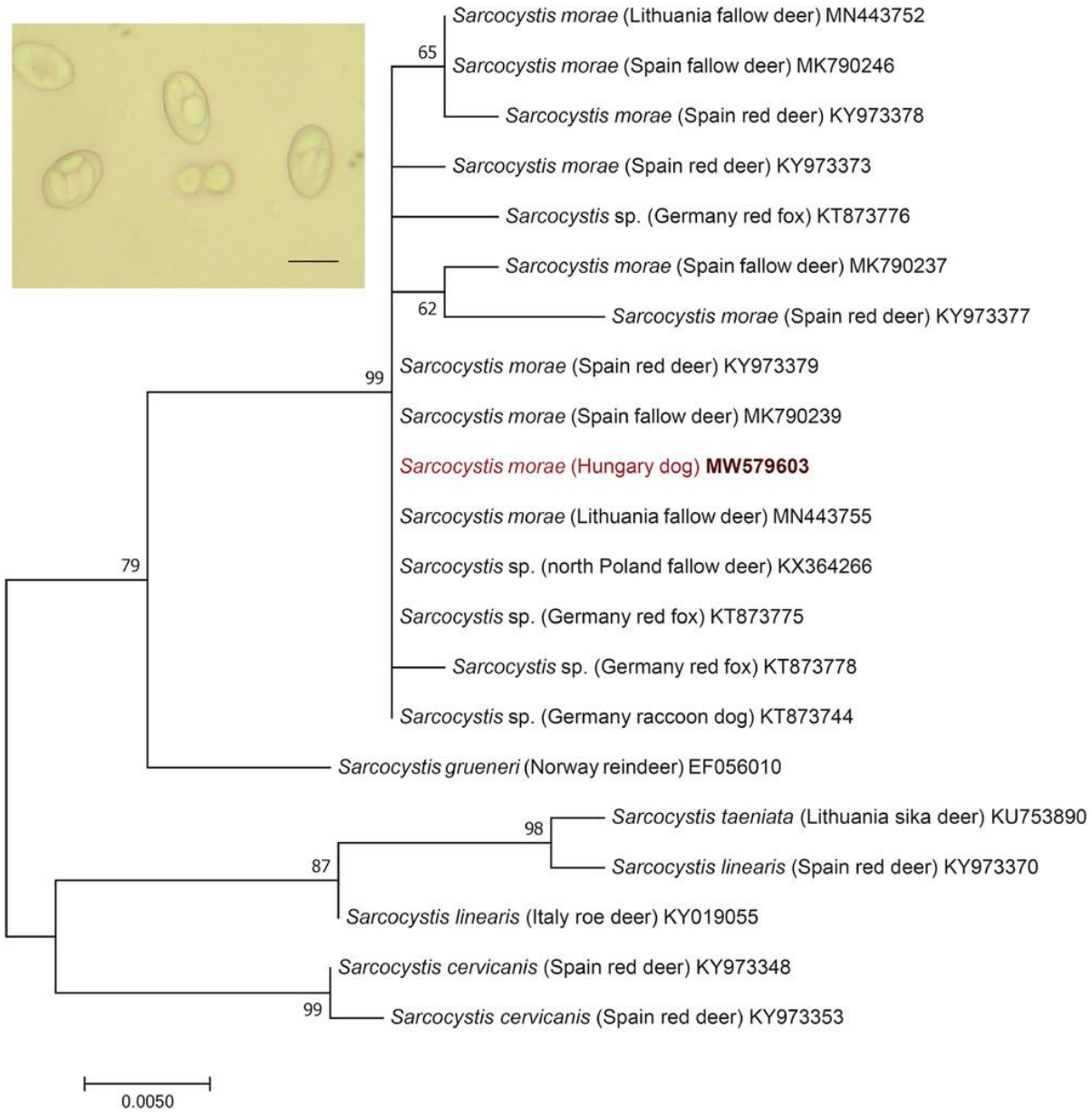

Figure 14. Phylogenetic tree of species closely related to *Sarcocystis morae* based on the 18S rRNA gene. The tree was generated with the Maximum Likelihood method and the Jukes–Cantor model. The sequence obtained in this study is indicated with red color and bold accession number. Branch lengths represent the number of substitutions per site inferred according to the scale shown. Insert: three sporocysts of S. morae from dog feces (bar = 10 µm)

5.8. *Hepatozoon felis* and *Cytauxzoon europaeus* in outdoor domestic cats

Concerning domestic cats in the study, only 1 out of 127 proved to be positive in the PCR detecting *Hepatozoon* spp., and this cat was kept in the region where *H. felis* is endemic among wildcats (Figure 15). The corresponding 18S rRNA sequence (OQ102981) showed 100% (596/596 bp) identity with *H. felis* from *Felis silvestris* in Hungary (OM422755) and 99.8% (622/623 bp) sequence identity to another isolate reported from a leopard in India (OL852083). Importantly, the *Hepatozoon* sequence identified for the first time in a domestic cat of the endemic region belonged to genogroup II of *H. felis* (Figure 16). However, in the blood smear of the affected cat, no gamonts were seen in neutrophil granulocytes. None of the domestic cats were PCR positive for piroplasms, including *Cytauxzoon* spp.

Three of the four wildcats examined in this study tested positive for *H. felis*, among which one had co-infection with *C. europaeus* (Table 11). In wildcats that had mono-infection with *H. felis* one sequence (OQ445871) showed 100% (593/593 bp) identity to *H. felis* from *F. silvestris* in Hungary (OM422755), i.e., from genogroup II as the sample from the domestic cat (see above). However, two further samples (OQ445870, OQ445872) were 100% (595/595 bp) identical to a sequence (OL960187) reported previously from wildcats in Hungary representing genogroup I of *H. felis*. The phylogenetic relationships of these sequences are shown in Figure 16.

Furthermore, one *H. felis*-infected wildcat was PCR-positive for piroplasms. The 18S rRNA gene sequence from this cat (OQ445869) showed 100% (452/452 bp) identity with *C. europaeus* from a wildcat in Germany (ON380465). At the same time, the corresponding *cytb* sequence had only 99.6% (1183/1188 bp) identity with *C. europaeus* from a wildcat also from Germany (ON856000), and was also different (only 1187/1188 bp identical) from the single conspecific sequence reported previously in Hungary.

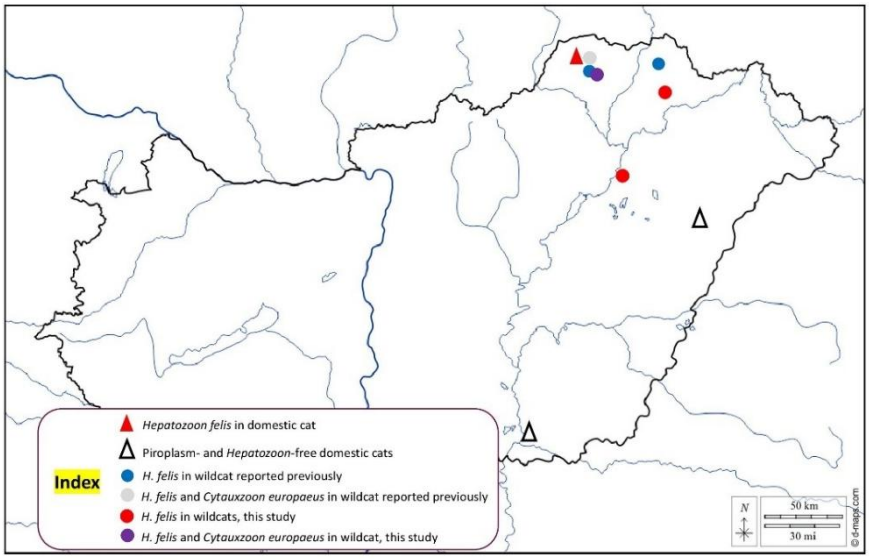

Figure 15. Map of Hungary showing the sampling sites and positive cases.

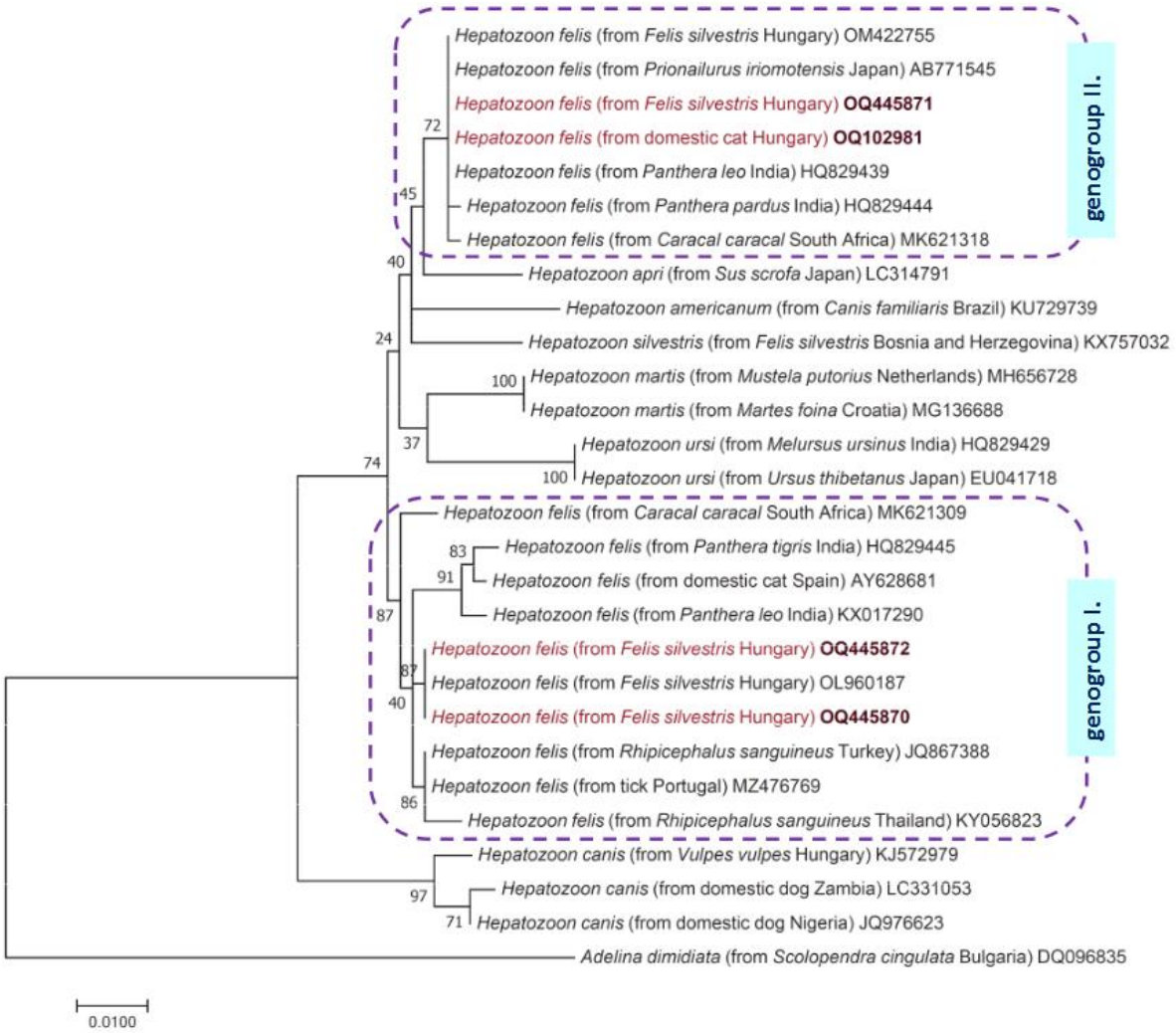

Figure 16. Phylogenetic tree of *Hepatozoon* species from carnivores based on 18S rRNA gene sequences. In each row, after the species name, the host species, country of origin, and the GenBank accession number are shown. New sequences (from this study) are marked with red fonts and maroon accession numbers. The evolutionary history was inferred by using the Maximum Likelihood method and the Jukes–Cantor model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences, and there were a total of 581 positions in the final dataset. *Adelina dimidiata* (Apicomplexa: Adeleidae) was used as outgroup. All positions containing gaps and missing data were eliminated.

Table 11. Data and PCR results of domestic and wild cats from the Aggtelek National Park.

Sample ID	Sample source	Sex	Location	PCR result *Hepatozoon*/*Cytauxzoon*
WC21	***Felis silvestris***	**male**	**Bodrogolaszi**	**+/-**
WC22	***Felis silvestris***	**male**	**Újszentmargita**	**+/-**
WC23	*Felis silvestris*	male	Múcsony	-/-
WC24	***Felis silvestris***	**male**	**Szendrő**	**+/+**
WC25	*Felis catus*	male	Ónod	-/-
AGCAT1	*Felis catus*	male	Jósvafő	-/-
AGCAT2	*Felis catus*	female	Jósvafő	-/-
AGCAT3	*Felis catus*	male	Jósvafő	-/-
AGCAT4	*Felis catus*	male	Jósvafő	-/-
AGCAT5	*Felis catus*	female	Jósvafő	-/-
AGCAT6	*Felis catus*	male	Jósvafő	-/-
AGCAT7	*Felis catus*	female	Jósvafő	-/-
AGCAT8	*Felis catus*	female	Jósvafő	-/-
AGCAT9	*Felis catus*	male	Jósvafő	-/-
AGCAT10	*Felis catus*	female	Jósvafő	-/-
AGCAT11	*Felis catus*	male	Jósvafő	-/-
AGCAT12	*Felis catus*	male	Jósvafő	-/-
AGCAT13	*Felis catus*	male	Jósvafő	-/-
AGCAT14	*Felis catus*	female	Komjáti	-/-
AGCAT15	*Felis catus*	female	Komjáti	-/-
AGCAT16	*Felis catus*	female	Komjáti	-/-
AGCAT17	*Felis catus*	female	Komjáti	-/-
AGCAT18	*Felis catus*	male	Komjáti	-/-
AGCAT19	*Felis catus*	female	Komjáti	-/-
AGCAT20	*Felis catus*	male	Szögliget	-/-
AGCAT21	***Felis catus***	**female**	**Szinpetri**	**+/-**
AGCAT22	*Felis catus*	female	Jósvafő	-/-
AGCAT23	*Felis catus*	female	Szinpetri	-/-
AGCAT25	*Felis catus*	female	Szinpetri	-/-
AGCAT26	*Felis catus*	female	Szinpetri	-/-
AGCAT27	*Felis catus*	male	Szinpetri	-/-
AGCAT28	*Felis catus*	male	Szendrő	-/-
AGCAT29	*Felis catus*	female	Szendrő	-/-
AGCAT30	*Felis catus*	female	Szendrő	-/-
AGCAT31	*Felis catus*	female	Komjáti	-/-
AGCAT32	*Felis catus*	female	Komjáti	-/-
AGCAT33	*Felis catus*	female	Komjáti	-/-
AGCAT34	*Felis catus*	male	Komjáti	-/-
AGCAT35	*Felis catus*	male	Komjáti	-/-
AGCAT36	*Felis catus*	female	Edelény	-/-
AGCAT37	*Felis catus*	female	Szendrő	-/-
AGCAT38	*Felis catus*	male	Felsőnyárád	-/-
AGCAT39	*Felis catus*	female	Felsőnyárád	-/-

5.9. Results of the investigation of the prevalence of *Babesia gibsoni* in "fighting dogs" and its molecular characteristics and co-infections

5.9.1. Phylogenetic analyses

In the PCR for detecting piroplasms, 40 of the 79 dogs were positive. DNA of *B. gibsoni* was identified by sequencing in 32 samples (prevalence of 40.5%; CI: 29.6–52.2%), whereas *B. vulpes* was present in 8 samples (prevalence of 10.1%; CI: 4.5–19.0%).

However, single or double co-infections with canine hemoplasmas were detected in 49 of the 79 dogs (62.03%, CI: 50.41–72.72%) (Table 12). Hemotropic mycoplasmas occurred with a prevalence of 62.45% (25 of 40 dogs, CI: 45.80–77.27%) in *Babesia*-infected dogs, and in 65.63% (21 of 32 dogs, CI: 46.81-81.43%) of *B. gibsoni*-infected dogs. Interestingly, out of the 30 puppies tested, 13 were PCR-negative, whereas 2 had *B. gibsoni*, 2 had *B. vulpes* and 10 had hemoplasma mono-infection. In addition, 3 of them had co-infections.

Babesia gibsoni from Hungary showed sequence identity of 100% (413/413 bp) with conspecific strains reported from various geographical regions, including Europe (Romania: KY433318), as well as eastern and southern Asia, i.e. genotype Asia-1 from Japan (AF175300) and genotype Asia-2 from Malaysia and Sri-Lanka (AF175301). Similarly, *B. vulpes* from dogs in Hungary showed identity of 100% (436/436 bp) with sequences previously reported from foxes in Hungary (KM232513) or other parts of Europe (e.g. Croatia: HM212628).

Two additional genetic markers were analyzed to reveal genetic characteristics and geographical relationships of *B. gibsoni* that emerged in Hungary. The *cox*1 gene sequence of this isolate (which was identical to all the 10 analyzed Hungarian samples) showed the closest, but only 99.5–99.7% (647/650 to 648/650 bp) identity with *B. gibsoni* reported on GenBank from Japan (AB685182-AB685185 and AB499087, respectively). The sequence homology was lower, 99.4% (646/650 bp) in comparison with further isolates from China (KP666169) and Japan (AB685188). Based on the closest match (AB685184), one synonymous and a nonsynonymous mutation were identified, the latter representing an exchange of methionine to isoleucine at position 33 in the amino-acid chain (M33I).

Regarding the *cytb* gene, *B. gibsoni* from Hungary had two sequence variants. The most prevalent haplotype (occurring in 10 out of 11 tested dogs) was 99.9% (732/733 bp) identical to the most closely related sequence available on GenBank, reported from Japan (AB685184). This single nucleotide polymorphism, characteristic of all haplotypes of *B. gibsoni* sequenced here (including the most divergent one outlined below) is a nonsynonymous mutation, i.e. exchange of proline to serine at position 310 in the amino-acid sequence (P310S). Despite ATV treatment, the most divergent *B. gibsoni cytb* haplotype was found in the dog Bejgli that had persistent anemia. This variant differed from the same reference sequence (AB685184) at three positions, amounting to only 99.6% (730/733) sequence identity. All differences represented nonsynonymous mutations, i.e. in addition to the above

(P310S) there were two further changes: methionine to isoleucine at position 121 (M121I) and phenylalanine to leucine at position 258 (F258L) (Figure 17).

Phylogenetically, the two *B. gibsoni cytb* haplotypes from Hungary were associated and most closely related to haplotypes from Japan (Fig. 3). Regarding the overall relationships of Eurasian *B. gibsoni cytb* sequences, the topology of the phylogenetic tree reflected that their clustering is coherent according to geographical region. However, although *B. gibsoni cytb* sequences reported from eastern Asia (Xi'an city and Wuhan in eastern China, as well as Japan) or southern Asia (India) formed two sister groups, their separation received low (52%) bootstrap support (Figure 18).

Last but not least, during posttreatment molecular analysis (6 months after treatment) two dogs out of the six available *B. gibsoni*-positives (most prevalent haplotype based on *cytb* gene), and also Beigli were shown to still harbor *B. gibsoni*.

```
AB685184  119 GQMSYWGATVMTNLFYWIPDFVIVLLGGYSVSVPTLQSFYMLHFILPFVLLGVVVVHIYY  178
              GQ SYWGATVMTNLFYWIPDFVIVLLGGYSVSVPTLQSFYMLHFILPFVLLGVVVVHIYY
Bejgli    119 GQISYWGATVMTNLFYWIPDFVIVLLGGYSVSVPTLQSFYMLHFILPFVLLGVVVVHIYY  178

AB685184  179 LHSSSSTNPLSGVDSWYVSSFYPVMMFSDLKMLTMLFAALGVQLTYGMMPLFQGDVDNSI  238
              LHSSSSTNPLSGVDSWYVSSFYPVMMFSDLKMLTMLFAALGVQLTYGMMPLFQGDVDNSI
Bejgli    179 LHSSSSTNPLSGVDSWYVSSFYPVMMFSDLKMLTMLFAALGVQLTYGMMPLFQGDVDNSI  238

AB685184  239 ESNPLQTPLHIVPEWYLLTFYATLKLFPSKLAGLIAMAALLESLILIVESSAMSPMMSCV  298
              ESNPLQTPLHIVPEWYLLT YATLKLFPSKLAGLIAMAALLESLILIVESSAMSPMMSCV
Bejgli    239 ESNPLQTPLHIVPEWYLLTLYATLKLFPSKLAGLIAMAALLESLILIVESSAMSPMMSCV  298

AB685184  299 HYHSMWTMISMPMIPALYMLGCLGSLSLNDGLMFMGMSAMFIMLVSVTKLLDCASMRL  356
              HYHSMWTMISM MIPALYMLGCLGSLSLNDGLMFMGMSAMFIMLVSVTKLLDCASMRL
Bejgli    299 HYHSMWTMISMSMIPALYMLGCLGSLSLNDGLMFMGMSAMFIMLVSVTKLLDCASMRL  356
```

Figure 17. Protein BLAST comparison of *Babesia gibsoni cytb* amino-acid sequences for the GenBank reference sequence AB685184 and the sequence from the dog named Bejgli. Yellow color indicates mutations.

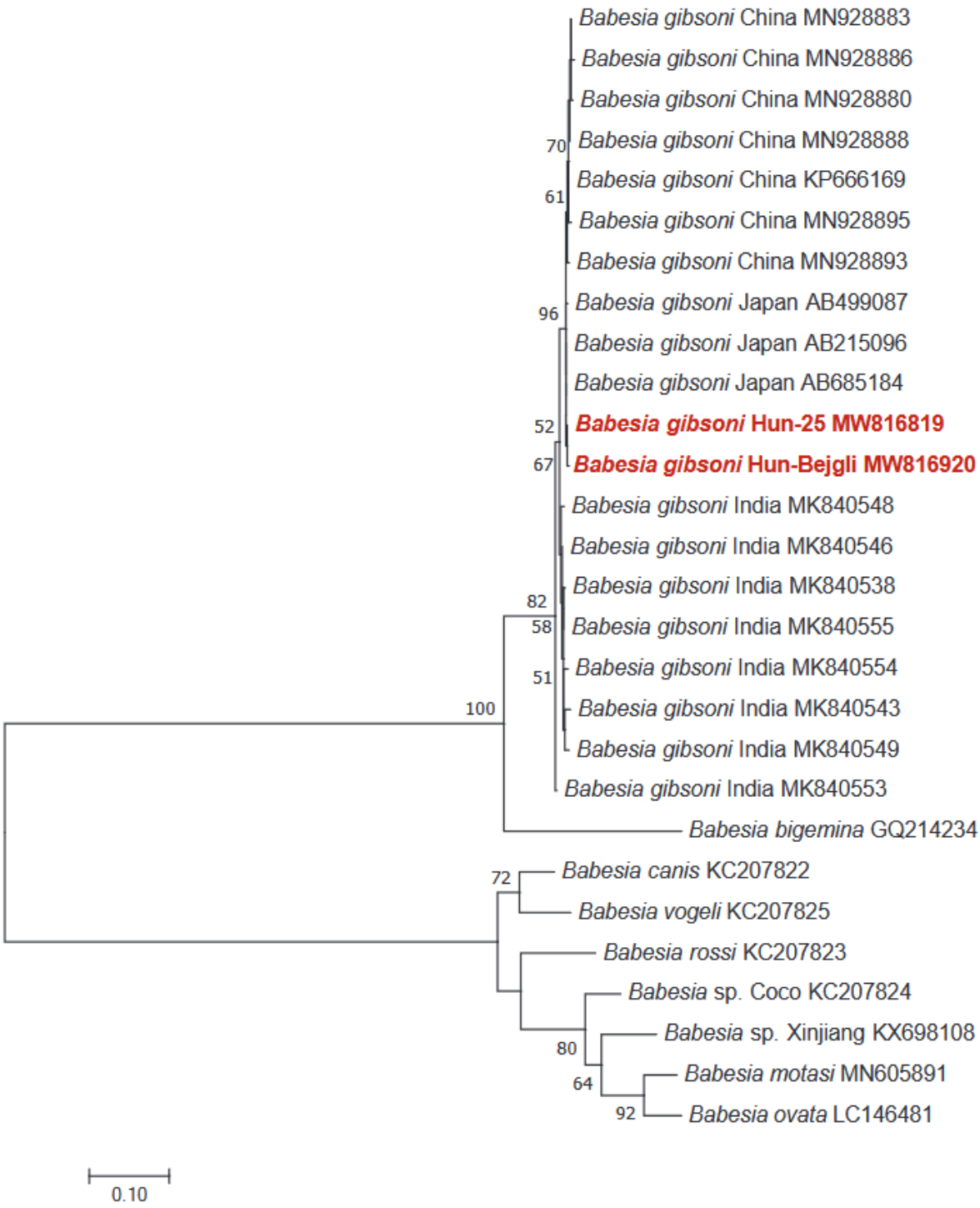

Figure 18. Phylogenetic tree for haplotypes of *Babesia gibsoni* and closely related piroplasms based on the *cytb* gene. The tree was generated with the Maximum Likelihood method and Jukes-Cantor model in MEGA 7.0. Nucleotide sequences obtained in this study are indicated in red. There were 648 positions in the final dataset. Branch lengths represent the number of substitutions per site inferred according to the scale shown.

5.9.2. Hematology

Intraerythrocytic merozoites of small *Babesia* spp. were microscopically detected in the blood smears of all dogs which were PCR-positive for piroplasms. Hemoplasmas were not seen in the blood smears. Prior to treatment of *B. gibsoni*-infected dogs, their mean hematocrit (HCT) was 38.50 ± 7.29%, the red blood cell count (RBC) was (5.34 ± 1.20) × 10^{12}/l, and the hemoglobin concentration (HGB) was 12.55 ± 2.68 g/dl. On the other hand, in *B. vulpes*-infected dogs the mean HCT was 43.80 ± 3.03%, the RBC was (6.10 ± 0.59) × 10^{12}/l and the HGB was 14.50 ± 1.38 g/dl. Comparisons with dogs PCR-negative for piroplasms (HCT: 44.43 ± 5.01%; RBC: (6.38 ± 0.72) × 10^{12}/l; HGB: 14.78 ± 1.75 g/dl), revealed that all these hematological parameters were significantly lower in *B. gibsoni*-infected dogs (Student's test, HCT: $t_{(63)}$ = 3.89, P = 0.0002; RBC: $t_{(63)}$ = 4.39, P < 0.0001; HGB; $t_{(63)}$ = 4.06, P = 0.0001) unlike in the case of *B. vulpes*-infected dogs (Student's test, HCT: $t_{(40)}$ = 0.27, P = 0.79; RBC: $t_{(40)}$ = 0.32, P = 0.75; HGB; $t_{(40)}$ = 0.67, P = 0.51).

In Table 12, pretreatment HCT values are summarized according to co-infections of dogs. Considering *B. gibsoni*-infected dogs, co-infection with both "*Ca.* Mycoplasma haematoparvum" (CMhp) and *Mycoplasma haemocanis* (Mhc) resulted in significantly lower HCT (33.00 ± 7.26%) than CMhp co-infection alone (40.86 ± 3.53%; Student's test, HCT: $t_{(9)}$ = 2.46, P = 0.036). Among *B. gibsoni*-infected dogs, 8 had mild anemia (30% < HCT < 38%), 3 had moderate anemia (HCT < 30%), and in one severe anemia developed (HCT < 20%). In the latter two groups, two dogs had co-infection with both hemoplasmas, and two had co-infection only with Mhc. The most severely affected dog (Bejgli) was concurrently infected with *B. gibsoni* and *M. haemocanis*: its HCT level was 12% and, based on follow-up, the hematological values of this dog declined despite ATV treatment. At this point, hemoplasma positivity was not yet known (Figure 2), therefore specific treatment against *M. haemocanis* was not given to this dog.

Table 12. Occurrence of vector-borne pathogens and pretreatment hematocrit (HCT) values in fighting dogs

Pathogen	No. of dogs positive by PCR				Mean HCT ± SD[a] in dogs with:			
	Mhc co-infection	CMhp co-infection	Mhc and CMhp co-infection	No co-infection	Mhc co-infection	CMhp co-infection	Mhc and CMhp co-infection	No coinfection
***Babesia gibsoni* (*n* = 32)**	9	8	4	11	38.13 ± 9.77	40.86 ± 3.53	33.00 ± 7.26	39.44 ± 6.75
***Babesia vulpes* (*n* = 8)**	1	1	2	4	44[b]	48[b]	42.50 ± 3.53	42[b]

[a] To assess this parameter, blood samples were available from 28 *B. gibsoni*-infected and from 5 *B. vulpes*-infected dogs.
[b] Blood sample from one dog was available for hematological evaluation.
Abbreviations: HCT, hematocrit; SD, standard deviation; Mhc, *Mycoplasma haemocanis*; CMhp, *Candidatus* Mycoplasma haematoparvum.

5.10. Results of phylogenetic analysis of selected protozoa

Among the four heteroxenous apicomplexan parasites phylogenetically analysed here (Figure 19A), *H. felis* had the highest rate of genetic diversity (maximum p-distance: 29/1654 bp = 1.75%), followed by *B. gibsoni* (21/1579 bp = 1.33%). The genetic diversity was low among 18S rRNA gene sequences of *S. morae* (3/1808 bp = 0.17%) and *C. europaeus* (1/1227 bp = 0.08%). This was confirmed by the evolutionary distance relative to the above diversity rates as reflected by the shape (width) of their cluster in the phylogenetic tree, with *H. felis* having the longest and *C. europaeus* the shortest horizontal distance within their group (Figure 19A).

Regarding homoxenous, non-apicomplexan protozoa (Figure 19B), the highest rate of genetic diversity belonged to the free-living amoeba, *A. castellanii* (maximum p-distance: 180/2328 bp = 7.73%), and this value was much lower in the case of *T. gallinae* (13/1570 bp = 0.83%) and *T. foetus* (5/1462 bp = 0.34%), the lowest belonging to *P. hominis* (2/1500 bp = 0,13%). This was confirmed by the evolutionary distances reflected by the shape (width) of their cluster in the phylogenetic tree, with *A. castellanii* sequences forming the broadest and *P. hominis* the narrowest group (Figure 19B).

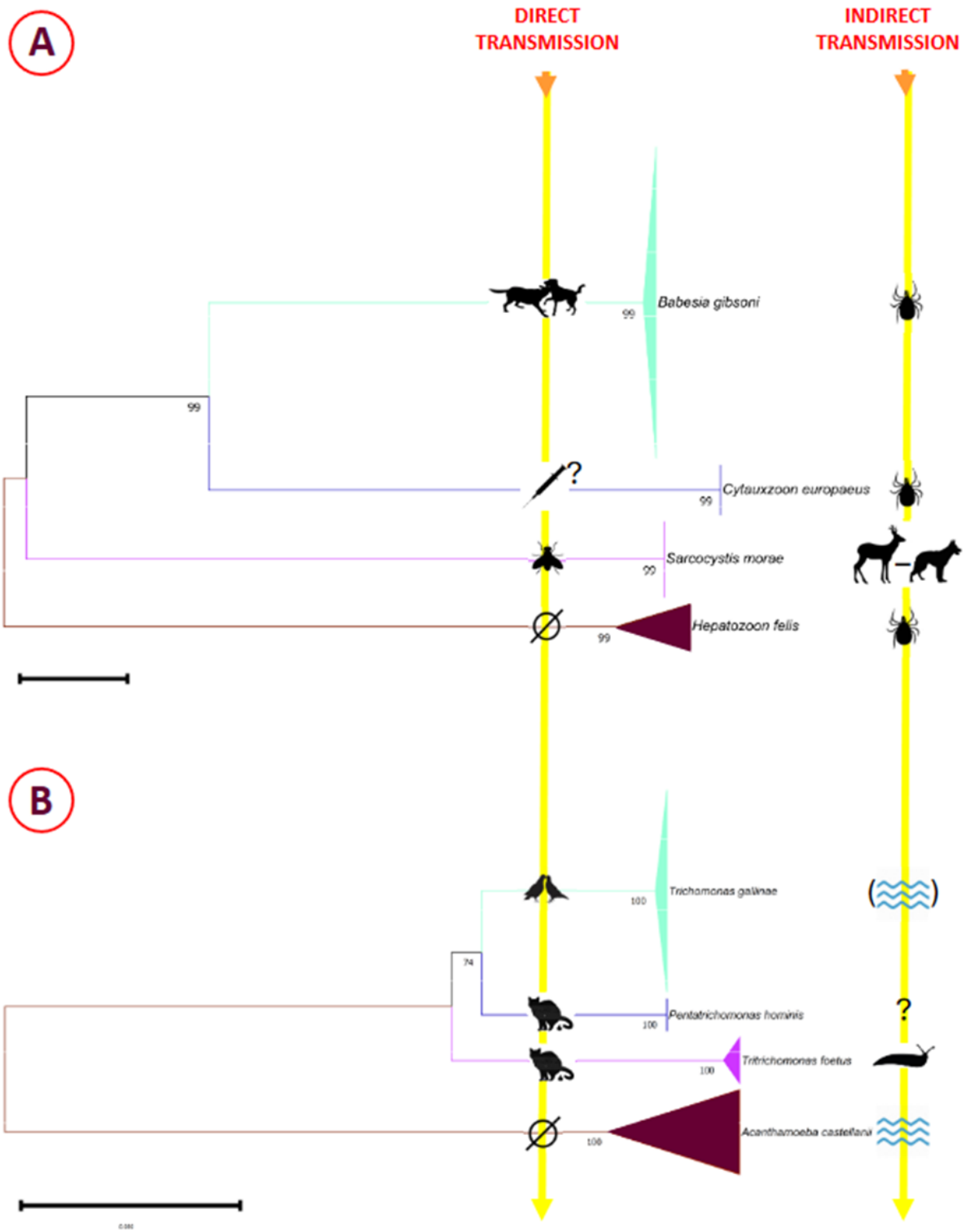

Figure 19. Phylogenetic trees of selected protozoan parasites (A. phylum Apicomplexa, B. phyla Parabasalia and Amoebozoa) based on the 18S rRNA gene. The evolutionary history was inferred using the Neighbor-Joining method and p-distance model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches. The clades are shown collapsed, with horizontal distances reflecting the numbers of substitutions per site according to the scale bar. Maximum pairwise distances within a clade (species) are included in the text. Pictograms of typical transmission routes are shown on the branches or next to the clade, depending on direct or indirect spreading between identical host types. The analyses involved (A) 76 sequences and 1360 positions, or (B) 56 sequences and 1706 positions. Evolutionary analyses were conducted in MEGA11.

6. Discussion

6.1. Pet and wild rodents as hosts of *Giardia duodenalis* in Central Europe, Hungary

This study investigated the presence and assemblages of *G. duodenalis* in six species of small mammals, some of which were targeted in this context for the first time in the Carpathian Basin. Emphasizing the importance of similar studies, previous data attest the high veterinary-medical significance of rodents in the epidemiology of giardiosis. These small mammals were reported to carry the highest diversity of assemblages (up to four in a single study: [227], and the zoonotic A and B were demonstrated in the highest ratio based on either the number of rodent species [69] or considering the overall prevalence of infection across different studies [228].

In this survey, the highest prevalence of infection with *G. duodenalis*, and two assemblages (B and G) were found in rats. These findings confirm that, apart from *G. muris* and assemblage G, zoonotic genotypes of *G. duodenalis* may also occur in this host. Previously, the following rat-associated assemblages were reported: in Southern Europe (Spain: up to 36% prevalence, assemblages B, G by [229]), Central Europe (Austria: 34% prevalence, assemblages A, G by [72]) and Northern Europe (Sweden: assemblage G by [230]) suggesting that urban and rural rat populations carry this protozoan parasite (including its zoonotic variants) with high prevalence. This is especially important in the era of urbanization when rat populations and associated disease risks show a rising tendency in several parts of Europe (e.g., [231]).

In Europe, pet chinchillas could carry a broad range of *G. duodenalis* genotypes, including the zoonotic assemblages A (in Belgium: [227]) and B (in Belgium: [227]; in Italy: [75]; Romania: [76]; Czechia: [232]). In addition, the following assemblages were reported from this host: D (in Romania: [76]), C (in Belgium: [227]; in Italy: [75]) and E (in Belgium: [227]; in Romania: [76]). In most studies assemblage B was demonstrated from chinchillas either solely [232], or as the predominant genotype [75, 76, 227]. In one of these studies, each *Giardia*-infected chinchilla was infected with at least one of the two zoonotic assemblages (A and/or B), underlining the potential risks associated with these pet rodents in human infection [227].

It was also shown here that pet degus may shed *Giardia* cysts. Unfortunately, the corresponding genotype could not be identified from this host species, probably because of the low number of cysts, which prevented the extraction of sufficient DNA, as also reported in other studies [75]. Nevertheless, to our knowledge, this is the first report on the *Giardia*-carrier status of pet degus based on a regular parasitological laboratory method. Previously, infection with this parasite was either not detected in pet degus [233], or only with highly sensitive immunological method [234]. These results suggest that (especially compared to chinchillas)

degus are less important hosts of *G. duodenalis* and play a subordinate role in its zoonotic transmission.

Assemblage E was also demonstrated for the first time in beavers, although reported previously in other rodent species including chinchillas [227]. Beavers frequently shed zoonotic A and B assemblages of *G. duodenalis* during their water-associated life, and they were found responsible for human infections in North America where people had access to (bathing in or drinking) water downstream [74, 77, 235]. This was also confirmed by a European study, where assemblages A and B were found in this host (Poland: [78]). Although, based on our preliminary results, a similar epidemiological role could not be established in the southern part of Central Europe, this situation deserves continuous monitoring, because of increasing beaver populations throughout Europe [236].

On the other hand, no rabbits and guinea pigs were found to carry *G. duodenalis* in this study. Similarly, previous reports on *Giardia*-positivity of guinea pigs could not confirm infection in these pet rodents (e.g., [237, 238], or the prevalence was low [239]. Thus, based on these results, the role of these hosts, from the point of view of zoonotic transmission, appears to be negligible in the evaluated population. This is in line with a previous large scale study involving samples from Germany and other European countries, where chinchillas had significantly higher prevalence of *Giardia*-infection than rabbits and guinea pigs [239].

In conclusion, findings of this research support that synanthropic rodent species are not equally important in their epidemiological role with respect to shedding *Giardia* cysts; especially when focusing on the question whether zoonotic assemblage is involved in their infection. The majority of animals tested here were asymptomatic carriers, further increasing the necessity of awareness that clinically normal pet rodents may pose a risk of shedding cysts of even zoonotic *Giardia* genotypes (most likely from assemblage B).

6.2. Molecular epidemiological study of *Trichomonas gallinae* focusing on Central and Southeastern Europe

To our knowledge, this is the first study on the genetic diversity of *T. gallinae* in Hungary, Romania and the whole southeastern European region, complementing previous reports from western, central and southern Europe (see below). Most infected birds in this study did not show clinical signs of trichomonosis except five pigeons (5%). A lower prevalence of clinical trichomonosis (0.37%) has been reported in a study involving 612 wild and domestic pigeons [240]. Since *T. gallinae* can cause the death of infected hosts [241], the rarity of symptoms can in part be explained by the death of severely affected birds, which can die before they are examined [240]. Furthermore, from an epidemiological point of view, subclinical cases might ensure easier spread of these protozoa. Therefore, screening pigeons for Trichomonas spp. should become an integral part of veterinary practice.

In this study, *T. gallinae* was detected in all four columbiform bird species examined. The infection rate was 73% which is similar to the 74% overall prevalence reported in wild columbids from western and southern Europe [135]. Within the Mediterranean region, in the Iberian Peninsula 44.8% of wild and domestic pigeons were shown to carry T. gallinae [240], but in another study from Spain the prevalence was much higher (79.4%: [242]). Furthermore, in the UK other columbiform species including *C. palumbus* and *S. decaocto* were also examined and a 60% incidence was reported [243]. In Germany, four species (*C. livia*, *C. oenas*, *C. palumbus*, and *S. decaocto*) were screened and 50% of the birds were infected with *T. gallinae* [244]. The prevalence was 37% among racing pigeons in Poland [138].

In Hungary, based on our screening assay, racing feral pigeons had a significantly higher prevalence of *T. gallinae* infection than urban feral pigeons (95% vs 33%). The possible reason for this difference might be that at trading-breeding places birds of different origin are housed close to each other, and the chances for infection are higher if naive racing pigeons can get into contact with carrier birds not only outside, but inside such enclosures, e.g., by sharing food and drinking water or by kissing. Therefore, we propose that similar places play a crucial role as hotspots in the transmission of *T. gallinae*. To our knowledge, there is no similar study in Europe that compares *T. gallinae* according to the place where pigeons are kept.

All four examined wood pigeons were infected. Although the sample size was limited, this apparently high (100%) infection rate is similar to what was reported among wood pigeons in Germany (70%: [135]) and the Iberian Peninsula (83.3%: [245]). This high prevalence is likely associated with urbanization of wood pigeons in Hungary, resulting in close contact of birds (e.g., via sharing common drinking sources) in green areas where the number of wood pigeons has recently increased significantly [246].

Concerning the other studied bird species, the prevalence was also high (94%) among ring doves in Hungary. There is a lack of literature data on trichomonosis of both collared dove and ring dove in Europe, despite the fact that ring doves were found to be susceptible to *T. gallinae* during experimental infection [247]. In the Caribbean an outbreak was reported [248], drawing the attention of veterinarians to the necessity to monitor *T. gallinae* in this bird species.

Based on the long fragment of the 18S rRNA gene, six *Trichomonas* subtypes were detected in columbiform birds in Hungary and Romania. Compared to a reference sequence, they had up to eight nucleotide differences, meaning that the maximum genetic difference was low (0.5%) compared to what was reported from North America (3.4%: [134]) and even from Austria, a country neighboring Hungary (2.9%: [249]). In Hungary, two genetic variants were detected among urban feral pigeons, vs four 18S rRNA gene subtypes occurred in racing feral pigeons kept in the same trading-breeding place, highlighting the epidemiological importance of similar facilities in general.

It is noteworthy that each bird from which *T. gallinae* was sequenced, carried a single 18S rRNA gene subtype, as also demonstrated repeatedly with different modes of detection. Since it was reported that *T. gallinae* triggers premunition [133] and immunity lasts until the loss of infection [127], the a priori presence of any variant probably protected the relevant birds during a heterologous challenge, which is likely to occur in the environment of a pigeon trading-breeding place. It is also highly relevant to note that among racing pigeons, 18S rRNA gene subtype D was only found in pigeons that originated from Germany. We suspect that subtype D was already present in these birds when imported to Hungary and probably premunition protected them from becoming infected in the pigeon trading place by other variants.

The clinical signs relevant to trichomonosis were only associated with 18S rRNA gene subtype D in Hungary. There are several reports indicating that certain haplotypes are highly correlated with more severe lesions in various bird species [240, 242, 250]. However, no close correlation has been found in terms of pathogenicity and geographical distribution when columbids were examined across Europe [135].

Considering the results in a phylogenetic context, the topology of the 18S rRNA phylogenetic tree did not show clear clustering of *T. gallinae* subtypes from this study according to host species, living place or keeping mode of columbiform birds. Clustering of *Trichomonas* sp. Hu-TG37 to the phylogenetic group of *T. canistomae* and *T. tenax* suggests that it might represent a new species. This separate position of *Trichomonas* sp. Hu-TG37 was confirmed also by the alpha-tubulin phylogenetic tree. It is known that some *Trichomonas* spp. show high genetic diversity depending on bird species, and few of these infections are caused by variants/species closely related to *T. vaginalis*, *T. tenax* [134, 249] or *T. canistomae* [242]. *Trichomonas* sp. Hu-TG37 identified for the first time in the present study belongs to the phylogenetic group of *Trichomonas* spp. infecting (among the others) domestic carnivores. This calls for further epidemiological studies on the possible contact between dogs and pigeons (e.g., via water deposited in gardens in drinking bowls) and its role in the transmission of these protozoan parasites.

In conclusion, this is the first report in Hungary and Romania on the prevalence and 18S rRNA gene subtypes of *T. gallinae* in various columbiform birds using molecular methods. The results suggest that most of these variants are not host-specific and do not cause clinical signs. The highest degree of genetic diversity and high prevalence of infection was observed among racing pigeons and captive ring doves, thereby highlighting the epidemiological importance of pigeon/dove trading-breeding places.

6.3. Molecular-phylogenetic investigation of trichomonads in dogs and cats reveals a novel *Tritrichomonas* species

Trichomonosis is a widespread parasitic infection in cats, and its most frequent causative agent is *T. foetus*, as reported in several countries [107]. However, relevant data were not available from Hungary, and some countries of its geographical region, justifying the need for a survey described in the present study. In addition, molecular-phylogenetic data on other trichomonads of dogs and cats are limited even in a worldwide context.

In the present study four wildcats from Aggtelek National Park were screened for oral and intestinal trichomonads. Although trichomonad DNA was not found, the opportunity to be infected was given, since in that region outdoor domestic cats and wildcats have been proven to share their living space with each other. This is also supported by recent reports of *Hepatozoon felis* in wildcats and domestic cats in the same region [58, 251].

On the other hand, domestic cats sampled in this study showed 13.8% prevalence of *T. foetus*. Although in Western Europe the occurrence of *T. foetus* seems to be more common, its presence in Central and Eastern Europe cannot be neglected. Based on the studies using PCR, the highest prevalence (38.7%) was reported in Spain, among 93 densely housed cats with chronic diarrhea [112]. In Switzerland, 10 out of 45 cats with diarrhea proved to be positive for *T. foetus* [113]. Furthermore, in Germany 15.7% of 230 purebred cats were PCR-positive but only 61% of them showed diarrheic symptoms [114]. In Italy 267 cats kept in different environments were screened and 14 of them (5.2%) were clinically *Tritrichomonas*-infected [71]. Among neighboring countries, similar studies were conducted. However, *T. foetus* positive cats were found only in Austria, with 2.9% prevalence [116, 252]. In addition, in the northern part of Central Europe (Poland) one clinical case was reported [115].

In dogs the occurrence of *T. foetus* is not as common as in cats. This is supported by data from different continents i.e., from East China and the United States where *T. foetus* was reported only with 0.6% and 2.6% prevalence in dogs, respectively [106, 117]. To the best of our knowledge, this is the first report of *T. foetus* in dogs in Europe north of the Mediterranean Basin, since previously this has only been reported in Italy: once in 2018 when one out of 100 shelter dogs proved to be infected [118], then in 2020 when *T. foetus* was found in an atypical location i.e., in a subcutaneous mass of a dog [119]. Among the 25 dogs in the present study, four were positive for *T. foetus* and all had diarrhea. This observation may contradict the statement that *P. hominis* is more frequent than *T. foetus* in dogs with diarrhea [117]. Since symptomatic trichomonosis appears between 7 weeks to 6 months of age [117] this corresponds well to that of the PCR-positive dog for which the age was known (3 months). In contrast, in a study two adult dogs were positive for *T. foetus* among 38 diarrheic dogs, with one of them being co-infected with *P. hominis* [117]. Similarly, in East China, two adult (>12

month-old) dogs out of 315 proved to be positive for *T. foetus*, and one of them had diarrhea [106].

While *T. foetus* is a protozoon with pathogenic potential, until recently *P. hominis* has been considered as a non-pathogenic opportunistic parasite in different mammalian hosts including dogs, cats, and human beings [253]. Hence all dogs and cats infected with *P. hominis* have the potential for zoonotic transmission [123]. Some recently published studies reported association between *P. hominis* infection and the occurrence of diarrhea [107, 110, 254], which can also be supported with our results, since one of the two *P. hominis* positive cats had strong diarrhea. Whether *P. hominis* can cause large bowel diarrhea by itself or only in co-infection with other parasites is still unknown [111, 122]. It is noteworthy that this protozoon is frequently misidentified as *T. foetus*, therefore its veterinary-medical significance is probably underestimated [122]. In the present study *P. hominis* was identified in 2.1% of the cats, one of which (a 7-month-old Persian-Himalayan cat) had diarrhea. This is in line with an American study that also revealed the presence of *P. hominis* in diarrheic young purebred cats [111]. *Pentatrichomonas hominis* is known to be a less frequently observed protozoon in cats than *T. foetus* [116, 122, 123]. Thus, not surprisingly, in Europe only a few reports have hitherto provided data on *P. hominis* in cats [116, 252]. Therefore, this is the third study in Europe showing potential pathogenic role of *P. hominis* in cats. In addition, based on the ITS2 phylogenetic tree, the feline *P. hominis* isolate clustered together with *P. hominis* from a human sampled in Thailand. This supports the theory that *P. hominis* is a zoonotic parasite, although its zoonotic transmission still has to be proved [123, 253].

In this study, the swab sample of a single female cat without any symptoms contained the DNA of a novel *Tritrichomonas* genotype or species which showed the highest, 96.44% identity to *T. casperi* and clustered as its sister species on both phylogenetic trees. *Tritrichomonas casperi* was reported to colonize the caecum of a laboratory mouse (*Mus musculus*) [255]. The relevant sample in this study was obtained from the rectum via mucosal swabbing; therefore, it was probably associated with epicellular parasitism and not with a digested prey item as a pseudoparasite. However, the latter cannot be completely excluded, as this finding can still be associated with mice eaten by the PCR-positive cat, thus originating from a rodent-associated trichomonad. Nevertheless, since felines are not known as natural hosts of *T. casperi* described from mouse, data should be interpreted with caution, especially considering the short size (337 bp) of the 18S rRNA sequence used in our phylogenetic analysis. If confirmed, the detection of DNA from a *T. casperi*-related species from cats could indicate a possible role of a predator-prey link in the evolution of this feline trichomonad, similar to what is known of avian trichomonosis [256]. Further studies are needed to confirm the identity of this *Tritrichomonas* sp. and its phylogenetic relationship with *T. casperi*.

Although *T. foetus* usually infects young (<12 months) animals [94, 102, 111], this could not be precisely confirmed in our study, as there were PCR-positive cats of different ages. The mean age was more than 12 months among positive cats, which can be explained by the fact that some positive Ragdoll cats were from the same cattery, including older cats which were certainly asymptomatic carriers. Concerning the breed of *T. foetus*-infected cats, only the Ragdoll and European shorthair breeds yielded positive results. However, no conclusion can be drawn from this, since more than 93% of the cats were from these two breeds in this study. In line with this, cats from catteries or shelters are at increased risk for becoming infected [98] e.g.: in the UK and Norway, *T. foetus* has also been reported in other breeds such as Siamese, Bengal and Burmese along with the Ragdoll [257, 258]. In addition, in Germany the Norwegian Forest cats were the most infected among other breeds [114].

In this survey, no significant association of trichomonad-infection was observed with the sex of animals, confirming the results of the above studies, because in the context of *T. foetus* infection the sex was not reported as a predisposing factor. However, significant association was found here between the presence of *T. foetus* DNA and gastrointestinal symptoms at the time of sampling, or with cats having a history of signs of enteritis. This is in line with previous studies, where symptoms played a key role in the sampling of *T. foetus* positive cats [114, 259]. This is particularly relevant at young age, because older infected cats may be asymptomatic carriers with long history of diarrhea in kittenhood [98], as also shown here. In addition, if cats are kept together in breeding facilities, it is more likely for them to contract *T. foetus* infection, as was found among Ragdoll cats of this study.

6.4. Molecular evidence of *Monocercomonas* and *Acanthamoeba* in the feces of captive reptiles

Trichomonads and related mucosoflagellates are considered as nonpathogenic commensalists [139], although in some cases they might cause loss of appetite, diarrhea, and weight loss of reptiles [260]. Occasionally, their establishment in the gallbladder results in cholangitis. The pathogenic role of some species from order Trichomonadida is supported by the fact that in a fecal sample of a viper (*Bothrops jararaca*) with diarrhea, high numbers of trichomonads were detected microscopically [139]. Furthermore, in a black throat monitor lizard (*Varanus albigularis ionidesi*), a coinfection with two protozoa, including *Trichomonas* and *Cryptosporidium* spp. caused diarrhea, salivation, vomiting, anorexia, and lethargy[261]. *Trichomonas* species have been described as frequently found protozoa in reptiles of Ceylon (*Vipera russeli*, *Calotes versicolor*) [262]; however, with molecular investigations, it has not since been clarified which *Trichomonas* species are involved. Furthermore, *Monocercomonas* spp. are known to live in the large intestine and pass with the feces of squamate reptiles [263]. They may cause moderate depression, loss of activity and weight [142].

In the present study, a novel *Monocercomonas*-like genotype was found in a leopard gecko. *Monocercomonas colubrorum* is considered a common species in a wide range of Squamata [220, 264], but, to the best of our knowledge, no other species of this genus has hitherto been described from reptiles. Therefore, based on the sequence data so far it cannot be assumed, but it is possible that our finding represents a new species, or perhaps even a new genus. Considering that phylogenetically it belonged to a sister clade of all other reported *Monocercomonas* sequences (Figure 11).

Since reptiles are frequently kept as pet animals, the parasites they carry can be a source of infection for other animals or humans. Reptiles that are sold as pets might not born in captivity but brought from the wild, thereby increasing the chances of carrying different pathogens [140]. Opportunistic *Acanthamoeba* spp. are geographically widespread and locally live in diverse environmental substances, including soil, water reservoirs, and even hospitals [89, 94]. Although *Acanthamoeba* spp. play an important role in maintaining bacterial biomass [89], they are of great public health significance because they can cause encephalitis (GAE), skin lesions in immunocompromised patients, and keratitis (AK) mainly in people wearing contact lenses [90, 91]. Furthermore, these species also have veterinary significance, as their presence is proven in animals, including dogs, cats, pigs, horses, rabbits, birds, amphibians, and reptiles [91]. They may also have clinical significance in some animals, since conjunctival swabs of birds, dogs and cats contained these protozoa [94], however in cats with keratitis screening their corneal scrapings also proved to be effective [265]. Interestingly, in a study *Acanthamoeba* sp. from the eyes of cats showed 100% identity to *A. castellanii* genotype 4 from human eyes [266]. In addition, *Acanthamoeba* sp. might also occur in the brain of animals, as it has been reported in a dog and in a rhesus macaque [267, 268].

Although *Acanthamoeba* spp. are ubiquitous protozoa, considering our carefully performed non-invasive sampling, molecular evidence is provided in this study, for the presence of four different genotypes of *Acanthamoeba* in the feces of reptiles. *Acanthamoeba hatchetti* is known to have clinicopathological significance in both humans and animals, e.g., *A. hatchetti* was detected in a horse with severe placentitis [97]. In addition, *Acanthamoeba* genotype T4 species are also opportunistic pathogens both in humans and animals causing keratitis and/or encephalitis [269].

Beside the classification based on cyst size and shape (Group I-III: [90]), *Acanthamoeba* spp. based on the SSU rDNA can be divided into 23 genotypes (T1-T23; [270]), however, most of the AK cases are caused by the T4 genotype [271]. In this study, an *Acanthamoeba* sp. clustering with several isolates reported as *A. castellanii* and two identical *A. lugdunensis* sequences belonged to the phylogenetic group of the T4 genotype that is frequently isolated form human clinical cases [272] (Figure 12). In previous reports, although *Acanthamoeba* species have been found in reptile feces [273] and in the gut contents of

reptiles [86] they have been molecularly detected only from a necrotic lesion of basilisk lizard [274]. To our knowledge, this is the first time that acanthamoebae are reported by molecular methods in the feces of several species of captive reptiles. These results could be important for human health since reptiles are frequently kept as pet animals. Furthermore, the vector role of *Acanthamoeba* spp. is also notable, since these amphizoic free-living amoebae can harbor different pathogens [91].

In conclusion, molecular evidence is provided here for the presence of *Acanthamoeba* DNA in the feces of captive reptiles. Although fecal samples analyzed here were collected in artificial enclosures, it cannot be completely ruled out that our PCR exceptionally could have amplified contaminating or air-borne acanthamoebae. Nevertheless, the above findings of opportunistic pathogens highlight the importance of monitoring protozoa and bacteria in the feces of pet reptiles as a source of infections for other animals and humans living nearby. Furthermore, these data could even have epidemiological relevance in natural ecosystems, e.g., when raw juice is made for human consumption from fruits that may have become contaminated with the feces of arboreal reptiles.

6.5. The presence of *Acanthamoeba* spp. in dogs and cats with ocular signs in Hungary

Protozoa are well documented as the causative agents of numerous diseases affecting both humans and animals. Studies suggest that certain protozoa are becoming more pathogenic. Along with *Acanthamoeba*, meningoencephalitis in humans has also been linked to free-living amoebae including *Balamuthia* and *Sappinia*. [275].

Since *Acanthamoeba* is a well-known cause of eye disease in humans and is increasingly being reported in Europe, we were encouraged to see if we could isolate it from small animal patients with ophthalmic diseases in Hungary. *Acanthamoeba* is described as an opportunistic parasite which mainly causes disease in immunosuppressed individuals. However, considering a dog from Spain and a Boxer dog from United States, both cases clearly demonstrate that dogs are susceptible to systemic infections caused by *Acanthamoeba* spp. including *A. castellanii* like those seen in cases of GAE in humans. However, neither of the aforementioned examples involved immunosuppressed dogs; both featured young animals, indicating that the *Acanthamoeba* can take advantage of hosts with underdeveloped immune systems also [276, 277]. Samples used in our study included samples taken from both juvenile puppies and geriatric dogs but still no *Acanthamoeba* was detected. Although *Acanthamoeba* is abundant in the environment, and there is an increase in reported cases, AK still remains rare in humans [278]. When discussing AK, *Acanthamoeba* may be considered as a non-opportunistic protozoa as the host does not need to be immunosuppressed, instead the leading

factor in the appearance of keratitis in humans in the presence of *Acanthamoeba* species is the use of contact lenses and the unhygienic keeping of them [279].

It is apparent that *Acanthamoeba* has the capacity to be the source of disease in humans in the form of GAE and AK and although the manifestation of diseases in animals as a result of the presence of that protozoon is not as well documented, cases of disease in animals have been described. *Acanthamoeba quina* has been recorded as the probable cause of meningoencephalitis in a Rhesus Macaque and *A. hatchettti* has been recorded causing severe placentitis in a horse [280, 281]. Studies have found that animals have may shed strains of *Acanthamoeba* that have been linked to AK cases in humans, in their feces, which suggests that disease in humans could potentially be caused by zoonosis. For example, *A. lugdunensis* has been isolated from reptile feces and the eyes of an AK sufferer [282, 283]. It has been proven that AK like disease can occur in cats, but this has only been proven to be the case when cats were exposed to *A. castellanii* contaminated contact lenses [284]. Naturally occurring ocular lesions due to an *Acanthamoeba* infection in animals is rare and very limited literature sources have it recorded, the case of sclerokeratitis in a cat is just one of very limited examples [285]. Perhaps as the mannose-binding abilities of *Acanthamoeba* differs among species and the unlikely nature of cats and dogs to use contact lenses, the risk of small animals presenting to veterinarians with ocular lesions due to the presence of *Acanthamoeba* is negligible, the result of this study supports this. However, to the best of our knowledge, our study did not utilize any patients who were immunosuppressed, and perhaps further studies need to be conducted on immunosuppressed cats and dogs with ocular lesions for further evidence.

6.6. Parasitological and molecular investigation of consequences of raw meat feeding (BARF) in dogs and cats: implications for other pets living nearby

In recent years, raw meat diet became more popular among pet owners, due to its potential beneficial effect on health. Consequently, research on the topic has increased and also reported the risks to parasitic infections related to raw meat feeding [286].

Although dogs consuming raw meat are potentially exposed to the infection with *Neospora caninum*, the samples in our study were negative for this protozoon. It is worth to mention that shedding of *N. caninum* oocysts is often limited, therefore, false negative results can also occur. Serology is a more sensitive method. For instance, in a German survey bitches were screened for *N. caninum* among which six seropositive dogs were fed with fresh raw meat [287]. Furthermore, a recently published study analyzed commercial frozen RMBDs and reported the presence of *Sarcocystis cruzi*, *Sarcocystis tenella* and *T. gondii* [153]. It is worth noting that the definitive host of *T. gondii* is the cat, however, interestingly, the DNA of this

zoonotic protozoon has also been reported in dog feces [152]. This phenomenon might be related to coprophagia of feline feces, or to the ingestion of meat harboring *T. gondii* [152], the latter relevant to BARF diet. The chance to detect its oocyst in cat feces is limited, since the oocysts are shed for relatively short periods of time (2-3 weeks) after infection, and the detection with microscope has low sensitivity. Although the presence of *Sarcocystis* spp. had been reported in Hungary [58, 181, 288], we could not prove the presence of *Sarcocystis* spp. with molecular method, even though, we detected sporocysts of *Sarcocystis* in one sample. This might be explained by the low number of sporocysts. Furthermore, this was the only positive sample which was from a dog fed with fresh raw meat. Dogs can be infected with several *Sarcocystis* species, among which the most common species are *Sarcocystis cruzi* and *Sarcocystis tenella* [153]. In this study the affected dog was fed with beef, hence, most probably it was infected by *S. cruzi*. Although sarcocystiosis is certainly associated with raw meat consumption considering the life cycle of this apicomplexan parasite, the clinic-pathological significance of this is low, because dogs as final hosts are usually symptomless [289].

Among coccidia, *Cystoisospora* spp. may infect dogs and cats, and may cause gastro-intestinal symptoms. As a result of a survey in 2001 in Hungary, where 490 dogs were screened to different parasites, the prevalence of *Cystoisospora* spp. was 3.5% [148]. During the microscopical examination in the present study two dogs (2.2%) were infected with *Cystoisospora* spp., i.e., one with *C. canis* and the other with a *C. ohioensis*-like species. Considering the latter, based on the morphological examination of the oocysts, it is difficult to distinguish *Cystoisospora burrowsi*, *Cystoisospora neorivolta* and *C.ohioensis*, since they are similar and their size ranges overlap [146]. The infection might have been associated with raw meat consumption, since monozoic tissue cyst of this protozoon can occur in paratenic hosts [147]. However, we postulate that in the present cases the occurrence of *Cystoisospora* spp. was not associated with BARF-diet, because their prevalence was higher in the era preceding the widespread application of raw meat feeding [148].

In addition, oocysts of *E. stiedai* were also observed in one of the samples in coinfection with *C. ohioensis*-like species. *Eimeria stiedai* is the causative agent of biliary or liver coccidiosis of rabbits. It is an exceptional species in the strictly host-specific genus *Eimeria*, because it can infect both European brown hares (*Lepus europaeus*) and domestic rabbits (*Oryctolagus cuniculus*) [290]. Contamination of grass and other plants with *E. stiedai* oocysts may lead to transfer of the agent from wild to pet rabbits where these share habitats in rural areas [291]. However, pet rabbits in cities are kept in gardens or other types of enclosures where hares are excluded. According to the present results, however, even in highly urbanized areas there is a chance to contract *E. stiedai* by pet rabbits, e.g., when these are kept together (in the same household) or in the proximity of dogs fed by raw meat including rabbit liver. These

dogs may pass *E. stiedai* from their food with their feces as pseudoparasites and may thus contaminate their environment of pet rabbits with sporulating *E. stiedai* oocysts. This may entail the consequences of biliary coccidiosis among pet rabbits, otherwise not having access to infectious oocysts of this *Eimeria* species. Furthermore, considering other pathogenic *Eimeria* species, rabbits can also be infected with *Eimeria* species causing intestinal coccidiosis, but as these species do not occur in viscera, they are unlikely to have relevance (as pseudoparasites) to BARF diet.

In summary, *E. stiedai* was detected as pseudoparasites in this study from dogs and cats kept on raw meat diet. Without analyzing the meat/viscera meant for consumption by dogs or cats, freezing for 2-3 days is strongly advised and this would kill protozoa potentially present [150]. This method also has the advantage that the meat remains raw, fulfilling the criterion of BARF, but void of living parasites that would risk the health status of BARF-fed pets or others living nearby.

6.7. Dogs are final hosts of *Sarcocystis morae* (Apicomplexa: Sarcocystidae) – first report of this species in Hungary and its region

This is the first molecular evidence of the final host role of domestic dogs in the life cycle of *S. morae*. Prior to this study, to the best of our knowledge, no experimental proof has been published in support of this, although sequences closely related to that of *S. morae* were reported from two wild living canids, the red fox (*Vulpes vulpes*) and the raccoon dog (*Nyctereutes procyonoides*) sampled in Germany [292].

Sarcocystis morae has recently been described as a new species, with red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) as typical intermediate hosts [176]. In a broader sense, *S. morae* belongs to the group of *Sarcocystis* species, of which canids are suspected to be the final hosts based on phylogenetic properties [176].

In particular, it has long been postulated that red foxes and hunting dogs may play the final host role in the life cycle of *Sarcocystis* species affecting cervids, but for several species the exact final hosts are still unknown or are only suspected [293]. In this context, based on molecular results and less consistent (98.8-99.1%) sequence identities, it was suggested that domestic dogs may be the final hosts of *S. linearis* and/or *S. taeniata* [293]. Similarly, while tissue cysts of a *Sarcocystis* species with band-like protrusions were reported to be infective for the dog during experimental feeding [225], later the relevant species could not be identified with certainty [176].

On the other hand, while the final host spectra of *Sarcocystis* species are frequently referred to at the family level, it is not necessarily true that all canids are natural hosts for the same *Sarcocystis* species. For instance, although hunting dogs may have regular access to

mallards infected with *S. rileyi*, they are not known to be susceptible to this species [294] , in contrast to red foxes [181].

In summary, *Sarcocystis* species which infect cervids as intermediate host and have sarcocyst wall with ribbon-like or band-like protrusions [176] cluster in the same phylogenetic group based on both the 18S rRNA [176] and the cytochrome *c* oxidase subunit 1 (*cox*1) genes [295]. Among these five species (Figure 14), only *S. cervicanis* [224, 296] and *S. grueneri* [226] have hitherto been known to infect dogs as final hosts. Based on the present results, *S. morae* is also added to this list.

In a geographical context, results of the molecular-phylogenetic analysis performed here are less conclusive, because identical or closely related 18S rRNA sequences of *S. morae* have been reported from northern, central and western Europe, as reflected by their clustering (Figure 14). Nevertheless, *S. morae* has not hitherto been reported in Hungary and its region. At the same time, the occurrence of a closely related species, *S. grueneri* was already demonstrated in red deer in the country [297], and *S. gracilis* (having dogs as final hosts) was originally described from roe deer in Hungary [298].

The *Sarcocystis*-infected dog in this study could probably ingest meat from cervids as part of the raw meat-based diet (including game animals as its source) which is practiced in Hungary among dog owners to increase physical achievement of their dog [170]. This mode of feeding is known as a potential source of transmission of *Sarcocystis* species [153]. Thus, giving raw meat of game animals to dogs allow the latter to participate in the life cycle of *S. morae* in a "natural way", shedding sporocysts/oocysts when used for hunting or taken to walks in forested areas.

6.8. Screening of domestic cats from North-Eastern Hungary for *Hepatozoon felis* and *Cytauxzoon europaeus* that cause infections in local wildcat populations

In this study, the occurrence of *H. felis* and *C. europaeus* was investigated primarily in domestic cats in an area, where both protozoan parasites are endemic in wildcats [58]. The European wildcat is distributed widely in Europe, from the Iberian Peninsula to the Caucasus Mountains. The spread and genetic diversity of the species are threatened by the closely related domestic cat, since free-range domestic cats might be in a much higher density in their natural habitat, which creates the conditions for hybridization and close contact as well as sharing pathogens [299]. This has been reported at a high level in Hungary [300]. In addition, those cats with outdoor lifestyle are exposed to different arthropods, such as fleas and ticks, which may transmit different vector-borne pathogens [301] including *Hepatozoon* spp. and piroplasms. Furthermore, with the increase of urbanization and common living space the role of different

reservoirs, such as lynx and wildcat in the sylvatic cycle increases the possibility of pathogen transmission to domestic cats [302].

Feline hepatozoonosis is assumed to be transmitted by ticks, e.g., *R. sanguineus*, presumably similarly to *H. canis* [19]. Feline cytauxzoonosis is known to be a tick-borne disease, although in Europe the tick vector needs clarification [57].

In a worldwide context, similarly to our result, the same 18S lineage of *H. felis* had been found in an Asiatic lion (*Panthera leo persica*) in India (HQ829439) [299]. Thereafter, the same "genogroup II" lineage of *H. felis* was also reported in an Iriomote cat *(Prionailurus iriomotensis*) in Japan (AB771519) [300]. In Europe, *H. felis* is indigenous almost exclusively to Mediterranean countries, where it has been emerging in domestic cats since 2017. In a study 7% of domestic cats imported from the Mediterranean or South-Eastern Europe to Germany were positive to *H. felis* [303]. In Southern Italy 5.1% of domestic cats were positive with PCR and they were infected mainly with *H. felis*, however, one of them was infected with *H. canis*, and another one with *H. silvestris* [9]. Interestingly, few years later in North-Eastern Italy, *H. silvestris* was found with a higher prevalence [22]. In other Mediterranean countries, Spain and Greece, *H. felis* was reported with 1.6% and 25.5% prevalence, respectively [21, 23]. As an exception among non-Mediterranean countries, in Austria an autochthonous case was reported, affecting a six-year-old tick-infested male cat which showed symptoms of anorexia, fever, icterus and was diagnosed with hepatozoonosis [25]. It is important to note that *H. felis* reported from Mediterranean countries in Europe represents genogroup I (e.g., under AY628681 from Spain or KY649442 from Italy) which is probably a different species than *H. felis* of genogroup II, detected in domestic cats in Central Europe in this study, as shown by the phylogenetic analysis (Figure 16).

During our investigation in Hungary, one domestic cat proved to be infected with *H. felis*. However, this cat did not show any clinical signs of the disease, and no gamonts were found in its neutrophil granulocytes. This might be due to the low parasitemia, i.e., usually only 1% of neutrophils are infected [20]. It is very important to note that the *H. felis*-infected domestic cat was kept outdoors in the Aggtelek National Park, where this protozoan parasite has recently been recognized to cause emerging infection among wildcats [58]. This is the only area in Europe north of the Mediterranean Basin where *H. felis* is known to be endemic. No cats were PCR positive outside this region in Hungary.

The present study describes the first evidence of the presence of *H. felis* in domestic cats in Hungary. In a broader context, this is the first evidence in Europe that domestic cats can become infected with *H. felis* from genogroup II where this species is endemic in wildcats. Although different wild rodents may also take part in its transmission [301], the infection most likely happened by ingesting infected ticks in the habitat (wooded area of Aggtelek National Park) shared with wildcats.

Feline cytauxzoonosis is considered a severe disease in the United States, with acute course and high mortality [52]. Nevertheless, in Europe *Cytauxzoon* spp. might be less virulent species, which mainly cause subclinical infection, or clinical manifestation associated with co-infection or immunodeficiency [57]. In Europe several countries (France, Italy, Germany and Switzerland) reported the presence of *Cytauxzoon* sp. in domestic cats without [22]or with clinical signs [56, 57, 302, 304]. Moreover, in Portugal a *Cytauxzoon* sp.-infected cat was described as showing clinical symptoms without co-infection with FIV or FeLV, and eventually the cat died [305]. Thus, feline cytauxzoonoosis in Europe may even have a serious outcome. Investigating the domestic cats in our study, none of them had PCR-positive result, but among wildcats one was infected with *C. europaeus* in co-infection with *H. felis*. *Cytauxzoon europaeus* has been reported in wildcats in different European countries, i.e., in Germany, the Czech Republic, Luxembourg, Bosnia and Herzegovina, Italy, Switzerland, France and Hungary [56, 58].

In conclusion, feline hepatozoonosis and cytauxzoonosis are emerging infections in the southern part of Central Europe. Hitherto *H. felis* and *C. europaeus* have only been found in wildcats in this endemic area, but according to the present results at least *H. felis* also emerged in domestic cat. This study suggests for the first time in Europe that *H. felis* from genogroup II may emerge in free-roaming domestic cats in such regions where this protozoan parasite is endemic in wildcats. The prevalence of *H. felis*-infection in this region was shown to be relatively high among wildcats, both in our previous and this study, but (based on the present results) is low among domestic cats. To monitor the possible emergence of *H. felis* with higher prevalence or in other populations of domestic cats and the transmission of *C. europaeus* from wild to domestic cats in the region, further investigations are needed.

6.9. *Babesia gibsoni* emerging with high prevalence and co-infections in “fighting dogs” in Hungary

To the best of our knowledge, this is the first report of *B. gibsoni* as an emerging pathogen with remarkably high prevalence among “fighting dogs” in Hungary. The results are also relevant in a broader geographical context, because in countries north of the Mediterranean Basin hitherto only sporadic occurrence of this piroplasm has been reported. In addition, to the best of our knowledge, no data on ATV resistance of *B. gibsoni* have been reported in Europe, i.e. the most relevant mutation has only been reported from North America and Asia [37, 46, 306].

It is a major advantage of this study that, apart from the availability of blood smears and hematological data, molecular identification of piroplasms was also possible, unlike in previous reports on small *Babesia* spp. in dogs in Hungary [47, 48]. Considering the results of hematological analyses, the mean levels of the most important parameters (HCT, RBC and HGB) were significantly lower in *B. gibsoni*-infected dogs than in dogs PCR-negative for

piroplasms. On the other hand, in the case of *B. vulpes* these were non-significant associations, implying that *B. gibsoni* caused anemia, whereas *B. vulpes* did not cause anemia in dogs of this study. Therefore, although the number of *B. vulpes*-infected dogs was too low to draw any general conclusions here, the clinical importance of this piroplasm appeared to be lower in Hungary compared to what was reported from Spain, where the majority of *B. vulpes*-infected dogs (79.6%) showed anemia [307]. Regarding dogs harboring multiple pathogens that infected their red blood cells concurrently, pretreatment HCT values revealed that *B. gibsoni* co-infection with both CMhp and Mhc induced more severe anemia than CMhp co-infection alone. However, the lowest HCT value was measured in a dog infected simultaneously with *B. gibsoni* and Mhc. While these results indicate that co-infections may exacerbate the manifestation of babesiosis-associated clinical signs, especially if three pathogens affect red blood cells simultaneously, anemia was still less frequently observed than in a similar study in North America (49.07%: [38]).

It is worth noting that hemotropic mycoplasmas may be transmitted via dog bites, similarly to *B. gibsoni* and probably also to *B. vulpes* [38, 39], thereby making the chance of co-infection more likely. It can be assumed that in the dogs studied here, the predominant mode of infection was through dog bites, since most of them took part in dog fights, presumably except for the puppies. This is confirmed by the scars found on their bodies as also described in the North American study [38]. Vertical transmission could also occur [36], because several puppies were found infected among the seized dogs, without a history of dog fight. Furthermore, tick-borne transmission cannot be completely ruled out here, since *R. sanguineus* (s.s.), the vector of *B. gibsoni* [33] and canine hemoplasmas [308] has been recently reported to emerge in Hungary [309]. However, the lack of any ticks on dogs of this study argues against a significant role of the latter.

Regarding molecular analyses, *B. gibsoni* was identified in the present study with a prevalence of 40.5%. This is considered remarkable for Europe, because similarly high prevalence (39%) has only been reported in the USA, where 269 confiscated “fighting dogs” were examined [38]. In Europe, a relatively high prevalence (28.6%) was reported in western Romania among “fighting dogs” [310]. However, prevalence rates of *B. gibsoni* infections are usually much lower among dogs kept as pet animals. In particular, *B. gibsoni* occurred in 0.7% of asymptomatic dogs in Croatia [311], in 2% of dogs in Spain [312] and in 3.3% of dogs in Serbia [313]. In case of dogs imported from Romania and Hungary to Germany, *B. gibsoni* was identified with a prevalence of only 1.9% [51]. The lowest prevalence (0.2%) of *B. gibsoni*-infected dogs was documented in the Czech Republic [314].

The present survey also confirmed *B. vulpes* infection with a prevalence of 10.1%. To the best of our knowledge, this is the first report on the occurrence of *B. vulpes* in dogs in Hungary, where previously this piroplasm has only been reported in red foxes (*Vulpes vulpes*)

[50]. *Babesia vulpes* has been reported in three dogs in Portugal [315], as well as in France (prevalence of 0.7%; [316]), Croatia (prevalence of 3%; [311]) and Serbia (prevalence of 10.1%; [317]). Among European countries, *B. vulpes* was identified with the highest prevalence (62.5%) in dogs in north-western Spain [307]. Regarding North American studies*, B. vulpes* was either not detected in "fighting dogs" infected with *B. gibsoni* [38], or these two species occurred simultaneously in some of the dogs [39]. However, in the present study no co-infection was found with both piroplasms, which is most likely a consequence of the low prevalence of *B. vulpes*.

Interestingly, hemoplasmas were detected with a high prevalence among *Babesia* spp.-infected (62.45%) and *B. gibsoni*-infected dogs (65.63%). Both CMhp and Mhc were already reported in non-pet dogs in the southern part of Hungary [318]. It is important to note that hemoplasma co-infection compared to *B. gibsoni* mono-infection occurred at a higher rate in this study (Table 12) than in a previous study from North America which also focused on confiscated "fighting dogs" (28%; [38]). Moreover, in the latter study, strong association was observed between *B. gibsoni* and CMhp infections. Therefore, based on the present study, it can be assumed that dogs involved in dog fights are at an increased risk of infection with *B. gibsoni*, *B. vulpes* and hemoplasmas in Europe, similarly to what was reported in North America [38, 39].

Three genetic markers were targeted for sequence analyses. Based on the 18S rRNA gene of piroplasms, all sequences of *B. gibsoni* (as well as those of *B. vulpes*) were identical to each other and to several conspecific sequences available on GenBank from Europe and Asia. However, the *cox*1 sequences of *B. gibsoni* obtained from 10 dogs had a single nucleotide polymorphism in comparison with Asian genotypes (from Japan and China), i.e. possessed a nonsynonymous mutation (methionine to isoleucine) at position 33 in the amino-acid chain (M33I). The significance of this finding remains unknown, although mutation in *cox*1 gene at position 310 has been examined in connection with diminazene aceturate resistance, with lack of evidence [216].

Cytochrome b gene and related mutations have been recently studied in Asia and in the USA. However, to the best of our knowledge, in Europe this is the first reported molecular study of the *B. gibsoni cytb* gene, in which three different mutations were detected. The mutation P310S was reported in a Japanese survey, in which it was assumed that this mutation may not contribute to the development of ATV resistance [319]. However, in our study two other nonsynonymous mutations were also found in the *cytb* gene in a dog which showed relapsing anemia despite treatment with ATV. The mutation M121I was found for the first time in Europe being reported so far only in Japan [46, 306] and in the USA [37]. This mutation is thought to be responsible for ATV resistance [46]. On the other hand, in this study a F258L mutation was also found, presumably for the first time in a worldwide context. Since ATV is a widely used

drug in combination with azithromycin against *B. gibsoni* [320], the above results suggest that the possibility of therapeutic resistance should also be expected in Europe.

Considering the suitability of various genetic markers to analyze the geographical origin of *Babesia* isolates, the 18S rRNA gene did not reveal a clear pattern when sequences were analyzed in a geographical context [215]. Similarly, according to differences in the *cox*1 gene among *Babesia* isolates, there was no phylogenetic grouping by geographical origin [321]. However, the *cytb* gene proved to be successful in showing geographically consistent clustering of *B. gibsoni* isolates from China and Japan [215]. Therefore, the latter genetic marker was chosen here in an attempt to trace back the origin (or at least the geographical relationships) of the two "Hungarian" *cytb* strains of *B. gibsoni*. The phylogenetic analysis including several *B. gibsoni* sequences from Japan, China and India available on GenBank showed that *B. gibsoni* from Hungary is most closely related to conspecific haplotypes from Japan (Figure 18). This is in line with previous, repeated findings of the Asian genotype of *B. gibsoni* in Europe (in Germany: [322]; in the UK: [43]) and argues for a single or a limited number of introduction event(s) from that direction. The similar phylogenetic affiliation of the Hungarian and other European *B. gibsoni* variants (with Chinese and Japanese, and other Asian isolates, respectively) also suggests that the typical mode of transmission in Europe is via dog bites, implying predominantly asexual reproduction of *Babesia* spp. in dogs (i.e. in the absence of sexual recombination in biological vector ticks, these isolates may show long-term genetic consistency in most loci).

Babesia gibsoni is an emerging pathogen in the southern part of central Europe, mainly in "fighting dogs" among which transmission probably takes place via dog bites. Co-infections with *B. vulpes* and hemotropic mycoplasmas may have exacerbated the symptoms. The *cytb* mutations found in the present study revealed the most likely geographical origin of these two *B. gibsoni* mitochondrial lineages in China and Japan. One of these mutations was reported to be relevant in the context of ATV resistance in North America and Asia.

6.10. Transmission route-dependent genetic diversity of selected protozoan parasites as reflected by the phylogenetic analysis of the 18S rRNA gene

Genetic diversity of unicellular parasites is not only important for the protozoa themselves, ensuring their adaptation to environmental challenges, but it is also crucial to consider when diagnostic methods are designed, or attempts are made to counteract genetic resistance to therapy. Although new methods have been developed to study this topic, such as amplified fragment length polymorphism [323], later nanopore sequencing [324] and single-cell genome sequencing [325], PCR amplification and Sanger sequencing of the highly conserved 18S rRNA gene across a broad taxonomic range of protozoa seems to be still unexplored.

Therefore, to serve as an initiative, this study aimed to focus on those unicellular parasites that have been recently discovered and analyzed in a phylogenetic context in Hungary, however without simultaneously considering their transmission routes. All the protozoan examples included here are associated with pet animals frequently transported due to human activity. Therefore, in agreement with former studies (e.g., [326]), we speculate that there is no large-scale geographical pattern that could have influenced this assessment. On the other hand, it has long been addressed how different modes of transmission, e.g., waterborne *vs* vector-borne, might affect genetic diversity [327].

Among the analysed apicomplexan protozoa, *H. felis* was found to be the genetically most diverse. Its developmental stages in the blood stream are gamonts that do not divide, precluding blood-borne mechanical transmission from one host to another. This may reduce opportunities for protozoan parasites to infect new hosts. When several host individuals become infected from a common source, this allows the spread and establishment of the same or very similar genotypes. In addition, *Hepatozoon canis* and *Hepatozoon americanum* are species associated with dogs which eat cadavers (of carnivores, prey items) potentially containing monozoic cysts, probably allowing short term, non-tick-borne transmission between dogs [328]. On the other hand, *H. felis* infects felids. These hosts usually do not engage in scavenging, and monozoic cysts are not known to form in their body. This may imply that in case of the latter species, infections from common infectious sources (that would promote the spread of identical genotypes) are rare or non-existent.

On the other hand, *H. felis* is probably a parasite with long evolutionary history: among haemogregarines, this species usually belongs to the most basal clade of *Hepatozoon* spp. [329, 330], as also shown here in a broader taxonomic context, i.e., *H. felis* had the longest evolutionary distance and broadest phylogenetic cluster among the studied apicomplexan parasites. This could have ensured the most important evolutionary prerequisite (i.e. sufficient time) for its long-term genetic diversification. However, as *H. felis* was shown here to have a high rate of genetic diversity, whereas this was low for *C. europaeus* (both with transstadial tick-borne transmission), transstadial transmission alone may not explain this difference between these two protozoan parasites. Last but not least, based on its high genetic diversity, it was also postulated that *H. felis* may be a complex of two species [58, 331, 332].

Genetic diversity has been found to be lower for *B. gibsoni* that has a dividing blood-form and it is well-known to spread directly (mechanically) between fighting dogs [333], in addition to a long-term biological, transovarial, tick-borne transmission. With the predominance of the direct transmission, identical genotypes are transmitted from the same donor animal to several recipient hosts relatively quickly (cf. founder effect, bottle neck effect: ingested/inoculated parasites that multiply in the new host represent only a minority of the original population). Accordingly, studies found that the overall nucleotide diversity of *B. gibsoni*

was only 0.22-0.23% in populations from Asia and three continents, respectively, revealing the low genetic variation among *B. gibsoni* populations [334]. As reported, this diversity was much lower than that of *H. canis* [335]. Then, in turn, the genetic variation in *H. canis* was reported to be lower, than in the case of *H. americanum* and *H. felis* [336]. Thus, high genetic diversity of *H. felis* in the latter study is in line with the present findings.

Low or lack of genetic diversity in *Cytauxzoon felis*, a close relative of *C. europaeus*, is also well-documented [337], as demonstrated here and in previous studies of the latter species in Europe [58, 214]. It is noteworthy in this context that clinical cytauxzoonosis can be induced through injection of tissue or blood harvested from cats with acute cytauxzoonosis [338], implying the potential role of mechanical (iatrogenic) spread, as these piroplasms multiply in the blood [55]. *Theileria* spp. (in the same family, Theileriidae, with *Cytauxzoon* spp.) also divide in the blood and can be easily transmitted mechanically between host individuals by blood-sucking flies [339], even iatrogenically, contributing to their low genetic diversity [340].

The restricted local genetic diversity of *Sarcocystis* spp. infecting ruminants is also well documented, as exemplified by *Sarcocystis cruzi* [341], and it is in line with the low genetic variation of *S. morae* shown here. Dogs were proved to be the final hosts of the latter cystogenic coccidium for the first time in Hungary [288]. Mechanical transport hosts of *Sarcocystis* sporocysts are insects [342], but these may spread the infection between different host types, from the final to the intermediate hosts, while still contributing to the distribution of identical protozoan genotypes from a common source (i.e. the final host).

Among non-apicomplexan protozoa, soil/water-inhabiting, free-living amoebae, which are only opportunistic parasites, had the highest rate of genetic diversity. High genetic diversity of *Acanthamoeba* spp., as reported in Hungary [343] and elsewhere, was explained by extensive lateral gene transfer and was also related to the long evolutionary history [344]. In addition, it has been observed that the expression of meiotic genes was increased in the cultures of polyploid *Acanthamoeba* spp. However, these are probably unrelated to sexual reproduction, but rather involved in homologous recombination [345]. Importantly, direct transmission of acanthamoebae between hosts is unlikely, therefore this cannot serve widespread genetic homogeneity.

On the contrary, based on literature data, in case of the directly transmitted *T. gallinae*, very low intraspecific sequence divergence has been detected, including samples from Hungary [346], and also in a broader geographical context [134]. Regionally occurring *P. hominis* also showed minimal differences in the 18S rRNA gene [347], which is in line with the present results. As the third example with low intraspecific genetic diversity, *T. foetus* isolates were reported to be identical, even when examined from different countries [106, 348]. Mechanical transport hosts are probably slugs [349]. In summary, the sexual reproduction in

trichomonads remains to be clarified, although for *Trichomonas vaginalis* it has been inferred from the possession of meiotic genes [350].

Conclusion

The results draw attention to emerging protozoa from a wide range of companion animals. The significance lines in that the number of companion animals has been increasing, and the possible diagnostic methods have also developed a lot in recent decades. In conclusion, the present study has some limitations, most notably the lack of relatively long 18S rRNA gene sequences in GenBank for most parasitic protozoa, thus not all apicomplexan taxa could be included here. Based on the eight analyzed apicomplexan and non-apicomplexan species, both direct and indirect (transstadial and transovarial tick-borne) transmission modes were represented. Although preliminarily, it was proved that the transmission mode has a significant impact on the genetic diversity among protozoan parasites, depending on various life cycle strategies and consequent frequency/chance of sexual reproduction vs binary fission. In particular, *H. felis* and *A. castellanii* were shown to have the highest rate of genetic diversities, and these two protozoa do not have direct transmission between their hosts, contributing to this phenomenon. However, the scope of this pilot study should be extended in the future to draw final conclusions in this context.

7. Overview of the new scientific results

Ad 1. First report on the *Giardia*-carrier status of pet degus, and on Assemblage E of *G. duodenalis* in beavers.

Ad 2. First report on the genetic diversity of *T. gallinae* in the southern central and southeastern European region.

Ad 3. First evidence of *T. foetus* in dogs of our region, and a hitherto unknown large intestinal *Tritrichomonas* sp. was shown to be present in a cat.

Ad 4. Not yet reported species of the genus *Monocercomonas*, and *Acanthamoeba* spp. with possible clinicopathological significance in reptiles, and including zoonotic species.

Ad 5. No correlation between the presence of *Acanthamoeba* and ocular illnesses in canine and feline patients.

Ad 6. BARF-feeding may contribute to the contamination of the environment with *E. stiedai* oocysts.

Ad 7. First molecular evidence in support of the final host role of domestic dogs in the life cycle of *S. morae*.

Ad 8. First report in Europe on the presence of *H. felis* from genogroup II in free-roaming domestic cats.

Ad 9. The first *cox*1 and *cytb* characterization of *B. gibsoni* in Europe, as well as the first report on the emergence of this piroplasm and hemoplasmas with high prevalence among “fighting dogs” north of the Mediterranean Basin.

Ad 10. The absence of direct transmission between hosts may play a role in the high genetic diversity of certain protozoan parasites, as exemplified by *Hepatozoon* and *Acanthamoeba* spp.

8. References

1. Cirulli F, Borgi M, Berry A, Francia N, Alleva E (2011) Animal-assisted interventions as innovative tools for mental health. Ann Ist Super Sanita 47:341–348. https://doi.org/10.4415/ANN_11_04_04

2. Serpell J (1998) In the company of animals: A study of human-animal relationships. Ethics and the Environment 3:105–110

3. Varela K, Brown JA, Lipton B, Dunn J, Stanek D, Behravesh CB, Chapman H, Conger TH, Vanover T, Edling T, Holzbauer S, Lennox AM, Lindquist S, Loerzel S, Mehlenbacher S, Mitchell M, Murphy M, Olsen CW, Yager CM (2022) A review of zoonotic disease threats to pet owners: A compendium of measures to prevent zoonotic diseases associated with non-traditional pets such as rodents and other small mammals, Reptiles, Amphibians, Backyard Poultry, and Other Selected Animals. Vector-Borne and Zoonotic Diseases 22:303–360. https://doi.org/10.1089/vbz.2022.0022

4. MacDonald JM, Barrett D (2016) Companion animals and well-being in palliative care nursing: a literature review. J Clin Nurs 25:300–310. https://doi.org/10.1111/jocn.13022

5. Macauley L, Chur-Hansen A (2023) Human health benefits of non-conventional companion animals: A narrative Review. Animals 13:28. https://doi.org/10.3390/ani13010028

6. Jekl V, Hauptman K, Knotek Z (2017) Evidence-based advances in rodent medicine. Veterinary Clinics of North America: Exotic Animal Practice 20:805–816. https://doi.org/10.1016/j.cvex.2017.04.012

7. DEPLAZES 2016 PARASITOLOGY IN VETERINARY MEDICINE - Entomopraxis. https://entomopraxis.com/tienda/ca/otras-tematicas-entomologicas-ecologia-evolucion-aplicada/2543-desplazes-2016-parasitology-in-veterinary-medicine.html. Accessed 26 Feb 2024

8. Otranto D, Cantacessi C, Pfeffer M, Dantas-Torres F, Brianti E, Deplazes P, Genchi C, Guberti V, Capelli G (2015) The role of wild canids and felids in spreading parasites to dogs and cats in Europe: Part I: Protozoa and tick-borne agents. Veterinary Parasitology 213:12–23. https://doi.org/10.1016/j.vetpar.2015.04.022

9. Protozoológia - egysejtű élősködők. http://www.a3shop.hu/allatorvosi-konyvek/magyar-nyelvu-konyvek--allatorvos-tudomany-11813/protozoologia-egysejtu-eloskodok/234733/. Accessed 27 Mar 2024

10. Thompson RCA, Smith A (2011) Zoonotic enteric protozoa. Veterinary Parasitology 182:70–78. https://doi.org/10.1016/j.vetpar.2011.07.016

11. Nichols GL (2000) Food-borne protozoa. Br Med Bull 56:209–235. https://doi.org/10.1258/0007142001902905

12. Schäfer I, Kohn B, Nijhof AM, Müller E (2022) Molecular detection of *Hepatozoon* species infections in domestic cats living in Germany. Journal of Feline Medicine and Surgery 24:994–1000. https://doi.org/10.1177/1098612X211055680

13. Lloret A, Addie DD, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hartmann K, Horzinek MC, Hosie MJ, Lutz H, Marsilio F, Pennisi MG, Radford AD, Thiry E, Truyen U, Möstl K, European Advisory Board on Cat Diseases (2015) Hepatozoonosis in cats: ABCD guidelines on prevention and management. J Feline Med Surg 17:642–644. https://doi.org/10.1177/1098612X15589879

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

15. Ciuca L, Martinescu G, Miron LD, Roman C, Acatrinei D, Cringoli G, Rinaldi L, Maurelli MP (2021) Occurrence of *Babesia* species and co-infection with *Hepatozoon canis* in symptomatic dogs and in their ticks in Eastern Romania. Pathogens 10:1339. https://doi.org/10.3390/pathogens10101339

16. Tołkacz K, Kretschmer M, Nowak S, Mysłajek RW, Alsarraf M, Wężyk D, Bajer A (2023) The first report on *Hepatozoon canis* in dogs and wolves in Poland: clinical and epidemiological features. Parasites & Vectors 16:313. https://doi.org/10.1186/s13071-023-05928-5

17. Sukara R, Andrić N, Andrić JF, Mihaljica D, Veinović G, Ranković V, Tomanović S (2023) Autochthonous infection with *Ehrlichia canis* and *Hepatozoon canis* in dogs from Serbia. Vet Med Sci 9:111–118. https://doi.org/10.1002/vms3.1061

18. Hornok S, Tánczos B, Fernández de Mera IG, de la Fuente J, Hofmann-Lehmann R, Farkas R (2013) High prevalence of *Hepatozoon*-infection among shepherd dogs in a region considered to be free of *Rhipicephalus sanguineus*. Vet Parasitol 196:189–193. https://doi.org/10.1016/j.vetpar.2013.02.009

19. 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

20. 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

21. Morelli S, Diakou A, Traversa D, Di Gennaro E, Simonato G, Colombo M, Dimzas D, Grillini M, Frangipane di Regalbono A, Beugnet F, Halos L, Paoletti B, Di Cesare A (2021) 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

22. Grillini M, Simonato G, Tessarin C, Dotto G, Traversa D, Cassini R, Marchiori E, Frangipane di Regalbono A (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

23. Díaz-Regañón D, Villaescusa A, Ayllón T, Rodríguez-Franco F, Baneth G, Calleja-Bueno L, García-Sancho M, Agulla B, Sainz Á (2017) Molecular detection of *Hepatozoon* spp. and *Cytauxzoon* sp. in domestic and stray cats from Madrid, Spain. Parasit Vectors 10:112. https://doi.org/10.1186/s13071-017-2056-1

24. Giannelli A, Latrofa MS, Nachum-Biala Y, Hodžić A, Greco G, Attanasi A, Annoscia G, Otranto D, Baneth G (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

25. 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

26. Schreeg ME, Marr HS, Tarigo JL, Cohn LA, Bird DM, Scholl EH, Levy MG, Wiegmann BM, Birkenheuer AJ (2016) Mitochondrial genome sequences and structures aid in the resolution of Piroplasmida phylogeny. PLOS ONE 11:e0165702. https://doi.org/10.1371/journal.pone.0165702

27. Homer MJ, Aguilar-Delfin I, Telford SR, Krause PJ, Persing DH (2000) Babesiosis. Clin Microbiol Rev 13:451–469. https://doi.org/10.1128/CMR.13.3.451

28. Tirosh-Levy S, Mazuz ML, Savitsky I, Pinkas D, Gottlieb Y, Steinman A (2021) Serological and molecular prevalence of *Babesia caballi* in apparently healthy horses in Israel. Pathogens 10:445. https://doi.org/10.3390/pathogens10040445

29. Romiti F, Magliano A, Antognetti V, Manna G, Cersini A, Scicluna MT, De Liberato C (2020) Investigation of Ixodid ticks as vectors of *Babesia caballi* and *Theileria equi* (Protozoa: Apicomplexa) in central Italy. Journal of Vector Ecology 45:25–31. https://doi.org/10.1111/jvec.12370

30. Hornok S, Edelhofer R, Földvári G, Joachim A, Farkas R (2007) Serological evidence for *Babesia canis* infection of horses and an endemic focus of *B. caballi* in Hungary. Acta Vet Hung 55:491–500. https://doi.org/10.1556/AVet.55.2007.4.8

31. Solano-Gallego L, Sainz Á, Roura X, Estrada-Peña A, Miró G (2016) A review of canine babesiosis: the European perspective. Parasites & Vectors 9:336. https://doi.org/10.1186/s13071-016-1596-0

32. Földvári G, Hell É, Farkas R (2005) *Babesia canis canis* in dogs from Hungary: detection by PCR and sequencing. Veterinary Parasitology 127:221–226. https://doi.org/10.1016/j.vetpar.2004.10.016

33. Boozer L, Macintire D (2005) *Babesia gibsoni*: An emerging pathogen in dogs

34. Kirk SK, Levy JK, Crawford PC (2017) Efficacy of azithromycin and compounded atovaquone for treatment of *Babesia gibsoni* in dogs. J Vet Intern Med 31:1108–1112. https://doi.org/10.1111/jvim.14777

35. Solano-Gallego L, Baneth G (2011) Babesiosis in dogs and cats--expanding parasitological and clinical spectra. Vet Parasitol 181:48–60. https://doi.org/10.1016/j.vetpar.2011.04.023

36. Fukumoto S, Suzuki H, Igarashi I, Xuan X (2005) Fatal experimental transplacental *Babesia gibsoni* infections in dogs. Int J Parasitol 35:1031–1035. https://doi.org/10.1016/j.ijpara.2005.03.018

37. Birkenheuer AJ, Marr HS, Wilson JM, Breitschwerdt EB, Qurollo BA (2018) *Babesia gibsoni* cytochrome b mutations in canine blood samples submitted to a US veterinary diagnostic laboratory. J Vet Intern Med 32:1965–1969. https://doi.org/10.1111/jvim.15300

38. Cannon SH, Levy JK, Kirk SK, Crawford PC, Leutenegger CM, Shuster JJ, Liu J, Chandrashekar R (2016) Infectious diseases in dogs rescued during dogfighting investigations. Vet J 211:64–69. https://doi.org/10.1016/j.tvjl.2016.02.012

39. Barash NR, Thomas B, Birkenheuer AJ, Breitschwerdt EB, Lemler E, Qurollo BA (2019) Prevalence of *Babesia* spp. and clinical characteristics of *Babesia vulpes* infections in North American dogs. J Vet Intern Med 33:2075–2081. https://doi.org/10.1111/jvim.15560

40. Matjila PT, Penzhorn BL, Leisewitz AL, Bhoora R, Barker R (2007) Molecular characterisation of *Babesia gibsoni* infection from a pit-bull terrier pup recently imported into South Africa. J S Afr Vet Assoc 78:2–5. https://doi.org/10.4102/jsava.v78i1.277

41. Prakash BK, Low VL, Vinnie-Siow WY, Tan TK, Lim YA-L, Morvarid AR, AbuBakar S, Sofian-Azirun M (2018) Detection of *Babesia* spp. in dogs and their ticks from Peninsular Malaysia: Emphasis on *Babesia gibsoni* and *Babesia vogeli* infections in *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae). J Med Entomol 55:1337–1340. https://doi.org/10.1093/jme/tjy072

42. Jain Jose K, Lakshmanan B, Wahlang L, Syamala K, Aravindakshan TV (2018) Molecular evidence of haemoparasites in ixodid ticks of dogs- first report in India. Vet Parasitol Reg Stud Reports 13:177–179. https://doi.org/10.1016/j.vprsr.2018.06.006

43. Smith FD, Wall LER (2013) Prevalence of *Babesia* and *Anaplasma* in ticks infesting dogs in Great Britain. Vet Parasitol 198:18–23. https://doi.org/10.1016/j.vetpar.2013.08.026

44. Víchová B, Horská M, Blaňarová L, Švihran M, Andersson M, Peťko B (2016) First molecular identification of *Babesia gibsoni* in dogs from Slovakia, central Europe. Ticks Tick Borne Dis 7:54–59. https://doi.org/10.1016/j.ttbdis.2015.08.004

45. Adaszek L, Lyp P, Poblocki P, Skrzypczak M, Mazurek L, Winiarczyk S (2018) The first case of *Babesia gibsoni* infection in a dog in Poland. Veterinární medicína 63:225–228. https://doi.org/10.17221/155/2017-VETMED

46. Iguchi A, Matsuu A, Ikadai H, Talukder MH, Hikasa Y (2012) Development of in vitro atovaquone-resistant *Babesia gibsoni* with a single-nucleotide polymorphism in cytb. Vet Parasitol 185:145–150. https://doi.org/10.1016/j.vetpar.2011.09.031

47. Farkas R, Földvfri G, Fenyves B, Kótai I, Szilágyi A, Hegedüs GyT (2004) First detection of small babesiae in two dogs in Hungary. Veterinary Record 154:176–178. https://doi.org/10.1136/vr.154.6.176

48. Dékay V (2013). A *Babesia gibsoni* infection in a pit bull terrier [In Hungarian]. Kisállatpraxis. 14, 214–218.

49. Demeter Z, Palade EA, Balogh E, Jakab C, Farkas R, Tánczos B, Hornok S (2011) Postmortem small babesia-like morphology of *Babesia canis* - short communication. Acta Vet Hung 59:427–432. https://doi.org/10.1556/AVet.2011.029

50. Farkas R, Takács N, Hornyák Á, Nachum-Biala Y, Hornok S, Baneth G (2015) First report on *Babesia cf. microti* infection of red foxes (*Vulpes vulpes*) from Hungary. Parasit Vectors 8:55. https://doi.org/10.1186/s13071-015-0660-5

51. Hamel D, Silaghi C, Lescai D, Pfister K (2012) Epidemiological aspects on vector-borne infections in stray and pet dogs from Romania and Hungary with focus on *Babesia* spp. Parasitol Res 110:1537–1545. https://doi.org/10.1007/s00436-011-2659-y

52. Wang J-L, Li T-T, Liu G-H, Zhu X-Q, Yao C (2017) Two tales of *Cytauxzoon felis* infections in domestic cats. Clin Microbiol Rev 30:861–885. https://doi.org/10.1128/CMR.00010-17

53. Reichard MV, Edwards AC, Meinkoth JH, Snider TA, Meinkoth KR, Heinz RE, Little SE (2010) Confirmation of *Amblyomma americanum* (Acari: Ixodidae) as a vector for *Cytauxzoon felis* (Piroplasmorida: Theileriidae) to domestic cats. J Med Entomol 47:890–896. https://doi.org/10.1603/me10013

54. Willi B, Meli ML, Cafarelli C, Gilli UO, Kipar A, Hubbuch A, Riond B, Howard J, Schaarschmidt D, Regli W, Hofmann-Lehmann R (2022) *Cytauxzoon europaeus* infections in domestic cats in Switzerland and in European wildcats in France: a tale that started more than two decades ago. Parasit Vectors 15:19. https://doi.org/10.1186/s13071-021-05111-8

55. Wikander YM, Reif KE (2023) *Cytauxzoon felis*: An overview. pathogens 12:133. https://doi.org/10.3390/pathogens12010133

56. Panait LC, Stock G, Globokar M, Balzer J, Groth B, Mihalca AD, Pantchev N (2020) First report of *Cytauxzoon* sp. infection in Germany: organism description and molecular confirmation in a domestic cat. Parasitol Res 119:3005–3011. https://doi.org/10.1007/s00436-020-06811-3

57. Antognoni MT, Rocconi F, Ravagnan S, Vascellari M, Capelli G, Miglio A, Tommaso MD (2022) *Cytauxzoon* sp. infection and coinfections in three domestic cats in Central Italy. Veterinary Sciences 9:. https://doi.org/10.3390/vetsci9020050

58. Hornok S, Boldogh SA, Takács N, Kontschán J, Szekeres S, Sós E, Sándor AD, Wang Y, Tuska-Szalay B (2022) Molecular epidemiological study on ticks and tick-borne protozoan parasites (Apicomplexa: *Cytauxzoon* and *Hepatozoon* spp.) from wild cats (*Felis silvestris*), Mustelidae and red squirrels (*Sciurus vulgaris*) in central Europe, Hungary. Parasit Vectors 15:174. https://doi.org/10.1186/s13071-022-05271-1

59. Monis PT, Andrews RH, Mayrhofer G, Ey PL (2003) Genetic diversity within the morphological species *Giardia intestinalis* and its relationship to host origin. Infect Genet Evol 3:29–38. https://doi.org/10.1016/s1567-1348(02)00149-1

60. Cacciò SM, Ryan U (2008) Molecular epidemiology of giardiasis. Mol Biochem Parasitol 160:75–80. https://doi.org/10.1016/j.molbiopara.2008.04.006

61. Dixon BR (2021) *Giardia duodenalis* in humans and animals – Transmission and disease. Research in Veterinary Science 135:283–289. https://doi.org/10.1016/j.rvsc.2020.09.034

62. Uiterwijk M, Nijsse R, Kooyman FNJ, Wagenaar JA, Mughini-Gras L, Koop G, Ploeger HW (2018) Comparing four diagnostic tests for *Giardia duodenalis* in dogs using latent class analysis. Parasites & Vectors 11:439. https://doi.org/10.1186/s13071-018-3014-2

63. Lyu Z, Shao J, Xue M, Ye Q, Chen B, Qin Y, Wen J (2018) A new species of *Giardia* Künstler, 1882 (Sarcomastigophora: Hexamitidae) in hamsters. Parasites & Vectors 11:202. https://doi.org/10.1186/s13071-018-2786-8

64. Cacciò SM, Lalle M, Svärd SG (2018) Host specificity in the *Giardia duodenalis* species complex. Infection, Genetics and Evolution 66:335–345. https://doi.org/10.1016/j.meegid.2017.12.001

65. Tangtrongsup S, Scorza V (2010) Update on the diagnosis and management of *Giardia* spp. infections in dogs and cats. Topics in Companion Animal Medicine 25:155–162. https://doi.org/10.1053/j.tcam.2010.07.003

66. Cheung W, Russo C, Maher S, Malik R, Šlapeta J (2019) Successful use of secnidazole to manage a giardiosis outbreak in a shelter. Veterinary Parasitology 274:108911. https://doi.org/10.1016/j.vetpar.2019.08.005

67. Baruch AC, Isaac-Renton J, Adam RD (1996) The molecular epidemiology of *Giardia lamblia*: a sequence-based approach. J Infect Dis 174:233–236. https://doi.org/10.1093/infdis/174.1.233

68. Wielinga C, Williams A, Monis P, Thompson RCA (2023) Proposed taxonomic revision of *Giardia duodenalis*. Infect Genet Evol 111:105430. https://doi.org/10.1016/j.meegid.2023.105430

69. Cai W, Ryan U, Xiao L, Feng Y (2021) Zoonotic giardiasis: an update. Parasitol Res 120:4199–4218. https://doi.org/10.1007/s00436-021-07325-2

70. Vioque F, Dashti A, Santín M, Ruiz-Fons F, Köster PC, Hernández-Castro C, García JT, Bailo B, Ortega S, Olea PP, Arce F, Chicharro C, Nieto J, González F, Viñuela J, Carmena D, González-Barrio D (2022) Wild

micromammal host spectrum of zoonotic eukaryotic parasites in Spain. Occurrence and genetic characterisation. Transboundary and Emerging Diseases 69:e2926–e2942. https://doi.org/10.1111/tbed.14643

71. Veronesi F, Gazzonis AL, Napoli E, Brianti E, Santoro A, Zanzani SA, Olivieri E, Diaferia M, Giannetto S, Pennisi MG, Manfredi MT (2016) Cross-sectional survey on *Tritrichomonas foetus* infection in Italian cats. Veterinary Parasitology: Regional Studies and Reports 6:14–19. https://doi.org/10.1016/j.vprsr.2016.11.004

72. Cervero-Aragó S, Desvars-Larrive A, Lindner G, Sommer R, Häfeli I, Walochnik J (2021) Surface waters and urban brown rats as potential sources of human-infective *Cryptosporidium* and *Giardia* in Vienna, Austria. Microorganisms 9:1596. https://doi.org/10.3390/microorganisms9081596

73. Helmy YA, Spierling NG, Schmidt S, Rosenfeld UM, Reil D, Imholt C, Jacob J, Ulrich RG, Aebischer T, Klotz C (2018) Occurrence and distribution of *Giardia* species in wild rodents in Germany. Parasites & Vectors 11:213. https://doi.org/10.1186/s13071-018-2802-z

74. Tsui CK-M, Miller R, Uyaguari-Diaz M, Tang P, Chauve C, Hsiao W, Isaac-Renton J, Prystajecky N (2018) Beaver Fever: Whole-genome characterization of waterborne outbreak and sporadic isolates to study the zoonotic transmission of giardiasis. mSphere 3:e00090-18. https://doi.org/10.1128/mSphere.00090-18

75. Veronesi F, Piergili Fioretti D, Morganti G, Bietta A, Moretta I, Moretti A, Traversa D (2012) Occurrence of *Giardia duodenalis* infection in chinchillas (*Chincilla lanigera*) from Italian breeding facilities. Res Vet Sci 93:807–810. https://doi.org/10.1016/j.rvsc.2011.12.019

76. Gherman CM, Kalmár Z, Györke A, Mircean V (2018) Occurrence of *Giardia duodenalis* assemblages in farmed long-tailed chinchillas *Chinchilla lanigera* (Rodentia) from Romania. Parasit Vectors 11:86. https://doi.org/10.1186/s13071-018-2652-8

77. Fayer R, Santín M, Trout JM, DeStefano S, Koenen K, Kaur T (2006) Prevalence of Microsporidia, *Cryptosporidium* spp., and *Giardia* spp. in beavers (*Castor canadensis*) in Massachusetts. J Zoo Wildl Med 37:492–497. https://doi.org/10.1638/06-013.1

78. Sroka J, Giżejewski Z, Wójcik-Fatla A, Stojecki K, Bilska-Zając E, Dutkiewicz J, Cencek T, Karamon J, Zając V, Kusyk P, Dąbrowska J, Kochanowski M (2015) Potential role of beavers (*Castor fiber*) in contamination of water in the Masurian Lake District (north-eastern Poland) with protozoan parasites *Cryptosporidium* spp. and *Giardia duodenalis*. Journal of Veterinary Research 59:219–228. https://doi.org/10.1515/bvip-2015-0033

79. Szénási Z, Marton S, Kucsera I, Tánczos B, Horváth K, Orosz E, Lukács Z, Szeidemann Z (2007) Preliminary investigation of the prevalence and genotype distribution of *Giardia intestinalis* in dogs in Hungary. Parasitol Res 101:145–152. https://doi.org/10.1007/s00436-007-0622-8

80. Plutzer J (2013) *Cryptosporidium* and *Giardia* as water contaminant pathogens in Hungary. Orvosi Hetilap 154:1836–1842. https://doi.org/10.1556/oh.2013.29749

81. Plutzer J, Tomor B (2009) The role of aquatic birds in the environmental dissemination of human pathogenic *Giardia duodenalis* cysts and *Cryptosporidium* oocysts in Hungary. Parasitol Int 58:227–231. https://doi.org/10.1016/j.parint.2009.05.004

82. Booton GC, Visvesvara GS, Byers TJ, Kelly DJ, Fuerst PA (2005) Identification and distribution of *Acanthamoeba* species genotypes associated with nonkeratitis infections. J Clin Microbiol 43:1689–1693. https://doi.org/10.1128/JCM.43.4.1689-1693.2005

83. Susanto IK, Wahdini S, Sari IP (2020) Potential transmission of *Acanthamoeba* spp. from contact lens solution and tap water in Jakarta, Indonesia. Open Access Macedonian Journal of Medical Sciences 8:333–337. https://doi.org/10.3889/oamjms.2020.4551

84. Mirabedini Z, Ahmed Khan N, Niyyati M, Javanmard E, Hamedanipour M, Arab-Mazar Z (2022) Can free living *Acanthamoeba* act as a Trojan Horse for SARS-Cov-2 on viral survival and transmission in the environment? A Narrative Review. Iran J Parasitol 17:138–144. https://doi.org/10.18502/ijpa.v17i2.9529

85. Otero-Ruiz A, Gonzalez-Zuñiga LD, Rodriguez-Anaya LZ, Lares-Jiménez LF, Gonzalez-Galaviz JR, Lares-Villa F (2022) Distribution and current state of molecular genetic characterization in pathogenic free-living amoebae. Pathogens 11:1199. https://doi.org/10.3390/pathogens11101199

86. Sesma MJ, Ramos LZ (1989) Isolation of free-living amoebas from the intestinal contents of reptiles. J Parasitol 75:322–324

87. Corsaro D (2020) Update on *Acanthamoeba* phylogeny. Parasitol Res 119:3327–3338. https://doi.org/10.1007/s00436-020-06843-9

88. Schuster FL, Visvesvara GS (2004) Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int J Parasitol 34:1001–1027. https://doi.org/10.1016/j.ijpara.2004.06.004

89. Geisen S, Fiore-Donno AM, Walochnik J, Bonkowski M (2014) *Acanthamoeba* everywhere: high diversity of *Acanthamoeba* in soils. Parasitol Res 113:3151–3158. https://doi.org/10.1007/s00436-014-3976-8

90. Marciano-Cabral F, Cabral G (2003) *Acanthamoeba* spp. as agents of disease in humans. Clin Microbiol Rev 16:273–307. https://doi.org/10.1128/CMR.16.2.273-307.2003

91. Siddiqui R, Khan NA (2012) Biology and pathogenesis of *Acanthamoeba*. Parasit Vectors 5:6. https://doi.org/10.1186/1756-3305-5-6

92. Ledbetter EC, McDonough SP, Dong L, Liotta JL, Bowman DD, Kim SG (2020) *Acanthamoeba* sclerokeratitis in a cat. J Am Vet Med Assoc 257:1280–1287. https://doi.org/10.2460/javma.257.12.1280

93. Ledbetter EC, Capistrano da Silva E, Dong L, McDonough SP (2023) Experimental induction of acute *Acanthamoeba castellanii* keratitis in cats. Transl Vis Sci Technol 12:10. https://doi.org/10.1167/tvst.12.8.10

94. Cooper E, Cowmeadow W, Elsheikha HM (2021) Should veterinary practitioners be concerned about *Acanthamoeba* keratitis? Parasitologia 1:12–19. https://doi.org/10.3390/parasitologia1010002

95. Valladares M, Reyes-Batlle M, Mora-Peces I, Martín-Navarro CM, López-Arencibia A, Dorta-Gorrín A, Comyn-Afonso E, Martínez-Carretero E, Maciver SK, Piñero JE, Valladares B, Lorenzo-Morales J (2014) A multisystemic *Acanthamoeba* infection in a dog in Tenerife, Canary Islands, Spain. Vet Parasitol 205:707–711. https://doi.org/10.1016/j.vetpar.2014.08.004

96. Kent M, Platt SR, Rech RR, Eagleson JS, Howerth EW, Shoff M, Fuerst PA, Booton G, Visvesvara GS, Schatzberg SJ (2011) Multisystemic infection with an *Acanthamoeba* sp in a dog. J Am Vet Med Assoc 238:1476–1481. https://doi.org/10.2460/javma.238.11.1476

97. Begg AP, Todhunter K, Donahoe SL, Krockenberger M, Šlapeta J (2014) Severe amoebic placentitis in a horse caused by an *Acanthamoeba hatchetti* isolate identified using next-generation sequencing. J Clin Microbiol 52:3101–3104. https://doi.org/10.1128/JCM.01071-14

98. Gookin JL, Hanrahan K, Levy MG (2017) The conundrum of feline trichomonosis. J Feline Med Surg 19:261–274. https://doi.org/10.1177/1098612X17693499

99. Bastos BF, Almeida FM de, Brener B (2019) What is known about *Tritrichomonas foetus* infection in cats? Rev Bras Parasitol Vet 28:1–11. https://doi.org/10.1590/S1984-29612019005

100. Shiratori M, Patel A, Gerhold RW, Sullivan SA, Carlton JM (2023) Persistent *Trichomonas vaginalis* infections and the pseudocyst form. Trends in Parasitology 39:1023–1031. https://doi.org/10.1016/j.pt.2023.09.009

101. Hale S, Norris JM, Šlapeta J (2009) Prolonged resilience of *Tritrichomonas foetus* in cat faeces at ambient temperature. Veterinary Parasitology 166:60–65. https://doi.org/10.1016/j.vetpar.2009.07.032

102. Dybicz M, Perkowski K, Baltaza W, Padzik M, Sędzikowska A, Chomicz L (2018) Molecular identification of *Trichomonas tenax* in the oral environment of domesticated animals in Poland – potential effects of host diversity for human health. Ann Agric Environ Med 25:464–468. https://doi.org/10.26444/aaem/92309

103. Hegner R, Ratcliffe H (1927) Trichomonads from the vagina of the monkey, from the mouth of the cat and man, and from the intestine of the monkey, opossum and Prairie-Dog. The Journal of Parasitology 14:27–35. https://doi.org/10.2307/3271399

104. Hegner R, Ratcliffe H (1927) Trichomonads from the mouth of the dog. The Journal of Parasitology 14:51–53. https://doi.org/10.2307/3271403

105. Gookin JL, Birkenheuer AJ, St. John V, Spector M, Levy MG (2005) Molecular characterization of trichomonads from feces of dogs with diarrhea. Journal of Parasitology 91:939–943. https://doi.org/10.1645/GE-474R.1

106. Li W-C, Wang K, Zhang W, Wu J, Gu Y-F, Zhang X-C (2016) Prevalence and molecular characterization of intestinal trichomonads in pet dogs in East China. Korean J Parasitol 54:703–710. https://doi.org/10.3347/kjp.2016.54.6.703

107. Bastos BF, Brener B, de Figueiredo MA, Leles D, Mendes-de-Almeida F (2018) *Pentatrichomonas hominis* infection in two domestic cats with chronic diarrhea. JFMS Open Rep 4:2055116918774959. https://doi.org/10.1177/2055116918774959

108. Gookin JL, Copple CN, Papich MG, Poore MF, Stauffer SH, Birkenheuer AJ, Twedt DC, Levy MG (2006) Efficacy of ronidazole for treatment of feline *Tritrichomonas foetus* infection. J Vet Intern Med 20:536–543. https://doi.org/10.1892/0891-6640(2006)20[536:eorfto]2.0.co;2

109. Rosado TW, Specht A, Marks SL (2007) Neurotoxicosis in 4 cats receiving ronidazole. J Vet Intern Med 21:328–331. https://doi.org/10.1111/j.1939-1676.2007.tb02968.x

110. Kim Y-A, Kim H-Y, Cho S-H, Cheun H-I, Yu J-R, Lee S-E (2010) PCR Detection and Molecular Characterization of *Pentatrichomonas hominis* from feces of dogs with diarrhea in the Republic of Korea. Korean J Parasito 48:9–13. https://doi.org/10.3347/kjp.2010.48.1.9

111. Romatowski J (2000) *Pentatrichomonas hominis* infection in four kittens. J Am Vet Med Assoc 216:1270–1272. https://doi.org/10.2460/javma.2000.216.1270

112. Arranz-Solís D, Pedraza-Díaz S, Miró G, Rojo-Montejo S, Hernández L, Ortega-Mora LM, Collantes-Fernández E (2016) *Tritrichomonas foetus* infection in cats with diarrhea from densely housed origins. Vet Parasitol 221:118–122. https://doi.org/10.1016/j.vetpar.2016.03.019

113. Frey CF, Schild M, Hemphill A, Stünzi P, Müller N, Gottstein B, Burgener IA (2009) Intestinal *Tritrichomonas foetus* infection in cats in Switzerland detected by in vitro cultivation and PCR. Parasitol Res 104:783–788. https://doi.org/10.1007/s00436-008-1255-2

114. Kuehner KA, Marks SL, Kass PH, Sauter-Louis C, Grahn RA, Barutzki D, Hartmann K (2011) *Tritrichomonas foetus* infection in purebred cats in Germany: prevalence of clinical signs and the role of co-infection with other enteroparasites. J Feline Med Surg 13:251–258. https://doi.org/10.1016/j.jfms.2010.12.002

115. Dąbrowska J, Karamon J, Kochanowski M, Jędryczko R, Cencek T (2015) *Tritrichomonas foetus* infection in cat - first detection in Poland. Acta Parasitol 60:605–608. https://doi.org/10.1515/ap-2015-0084

116. Mostegl MM, Wetscher A, Richter B, Nedorost N, Dinhopl N, Weissenböck H (2012) Detection of *Tritrichomonas foetus* and *Pentatrichomonas hominis* in intestinal tissue specimens of cats by chromogenic in situ hybridization. Vet Parasitol 183:209–214. https://doi.org/10.1016/j.vetpar.2011.07.050

117. Tolbert MK, Leutenegger CM, Lobetti R, Birrell J, Gookin JL (2012) Species identification of trichomonads and associated coinfections in dogs with diarrhea and suspected trichomonosis. Vet Parasitol 187:319–322. https://doi.org/10.1016/j.vetpar.2011.12.031

118. Iatta R, Buonfrate D, Paradies P, Cavalera MA, Capogna A, Iarussi F, Šlapeta J, Giorli G, Trerotoli P, Bisoffi Z, Otranto D (2019) Occurrence, diagnosis and follow-up of canine strongyloidiosis in naturally infected shelter dogs. Parasitology 146:246–252. https://doi.org/10.1017/S0031182018001312

119. Franchi R, Bertazzolo W, Marino M, De Marco B (2020) A case of “misplaced” *Tritrichomonas foetus* infection in a dog in Northern Italy. Veterinary Parasitology: Regional Studies and Reports 22:100451. https://doi.org/10.1016/j.vprsr.2020.100451

120. Grellet A, Brunopolack null, Feugier A, Boucraut-Baralon C, Grandjean D, Vandewynckel L, Cian A, Meloni D, Viscogliosi E (2013) Prevalence, risk factors of infection and molecular characterization of trichomonads in puppies from French breeding kennels. Vet Parasitol 197:418–426. https://doi.org/10.1016/j.vetpar.2013.07.030

121. Brložnik M, Faraguna S, Slavec B, Kostanjšek R, Rataj AV, Gruntar I (2016) *Pentatrichomonas hominis* coinfection in a puppy from a slovenian animal shelter. Slovenian Veterinary Research

122. Gookin JL, Stauffer SH, Levy MG (2007) Identification of *Pentatrichomonas hominis* in feline fecal samples by polymerase chain reaction assay. Vet Parasitol 145:11–15. https://doi.org/10.1016/j.vetpar.2006.10.020

123. Itoh N, Iijima Y, Ogura I, Yonekura N, Kameshima S, Kimura Y (2020) Molecular prevalence of trichomonad species from pet shop puppies and kittens in Japan. Rev Bras Parasitol Vet 29:e014820. https://doi.org/10.1590/S1984-29612020098

124. Mahittikorn A, Udonsom R, Koompapong K, Chiabchalard R, Sutthikornchai C, Sreepian PM, Mori H, Popruk S (2021) Molecular identification of *Pentatrichomonas hominis* in animals in central and western Thailand. BMC Vet Res 17:203. https://doi.org/10.1186/s12917-021-02904-y

125. Kellerová P, Tachezy J (2017) Zoonotic *Trichomonas tenax* and a new trichomonad species, *Trichomonas brixi* n. sp., from the oral cavities of dogs and cats. Int J Parasitol 47:247–255. https://doi.org/10.1016/j.ijpara.2016.12.006

126. Szczepaniak K, Łojszczyk-Szczepaniak A, Tomczuk K, Skrzypek T, Lisiak B, Abd-Al-Hammza Abbass Z (2016) Canine *Trichomonas tenax* mandibular gland infestation. Acta Veterinaria Scandinavica 58:15. https://doi.org/10.1186/s13028-016-0197-4

127. Stabler RM (1954) *Trichomonas gallinae*:a review. Exp Parasitol 3:368–402. https://doi.org/10.1016/0014-4894(54)90035-1

128. Amin A, Bilic I, Liebhart D, Hess M (2014) Trichomonads in birds--a review. Parasitology 141:733–747. https://doi.org/10.1017/S0031182013002096

129. Chavatte J-M, Giraud P, Esperet D, Place G, Cavalier F, Landau I An outbreak of trichomonosis in European greenfinches *Chloris chloris* and European goldfinches *Carduelis carduelis* wintering in Northern France. Parasite 26:21. https://doi.org/10.1051/parasite/2019022

130. Gerhold RW, Tate CM, Gibbs SE, Mead DG, Allison AB, Fischer JR (2007) Necropsy findings and arbovirus surveillance in mourning doves from the southeastern United States. J Wildl Dis 43:129–135. https://doi.org/10.7589/0090-3558-43.1.129

131. Villanúa D, Höfle U, Pérez-Rodríguez L, Gortázar C (2006) *Trichomonas gallinae* in wintering Common Wood Pigeons *Columba palumbus* in Spain. Ibis 148:641–648. https://doi.org/10.1111/j.1474-919X.2006.00561.x

132. Purple KE, Humm JM, Kirby RB, Saidak CG, Gerhold R (2015) *Trichomonas gallinae* persistence in four water treatments. J Wildl Dis 51:739–742. https://doi.org/10.7589/2014-05-137

133. Kocan RM (1969) Various grains and liquid as potential vehicles of transmission for *Trichomonas gallinae*. Wildl Dis 5:148–149. https://doi.org/10.7589/0090-3558-5.3.148

134. Gerhold RW, Yabsley MJ, Smith AJ, Ostergaard E, Mannan W, Cann JD, Fischer JR (2008) Molecular characterization of the *Trichomonas gallinae* morphologic complex in the United States. J Parasitol 94:1335–1341. https://doi.org/10.1645/GE-1585.1

135. Marx M, Reiner G, Willems H, Rocha G, Hillerich K, Masello JF, Mayr SL, Moussa S, Dunn JC, Thomas RC, Goodman SJ, Hamer KC, Metzger B, Cecere JG, Spina F, Koschkar S, Calderón L, Romeike T, Quillfeldt P (2017) High prevalence of *Trichomonas gallinae* in wild columbids across western and southern Europe. Parasit Vectors 10:242. https://doi.org/10.1186/s13071-017-2170-0

136. Kerek Á, Csanády P, Tuska-Szalay B, Kovács L, Jerzsele Á (2023) In vitro efficacy of Hungarian propolis against bacteria, yeast, and *Trichomonas gallinae* isolated from pigeons—A possible antibiotic alternative? Resources 12:101. https://doi.org/10.3390/resources12090101

137. Gómez-Muñoz MT, Gómez-Molinero MÁ, González F, Azami-Conesa I, Bailén M, García Piqueras M, Sansano-Maestre J (2022) Avian oropharyngeal trichomonosis: treatment, failures and alternatives, a systematic review. Microorganisms 10:2297. https://doi.org/10.3390/microorganisms10112297

138. Bobrek K, Urbanowicz J, Chorbiński P, Gaweł A (2017) Molecular analysis of *Trichomonas gallinae* in racing pigeons from Upper Silesia, Poland. Pol J Vet Sci 20:185–187. https://doi.org/10.1515/pjvs-2017-0023

139. Vilela FC, Silva MG da, Barrella TH, Silva RJ da (2003) Trichomoniasis in *Bothrops jararaca* (serpentes, viperidae). J Venom Anim Toxins incl Trop Dis 9:105–110. https://doi.org/10.1590/S1678-91992003000100007

140. Rataj AV, Lindtner-Knific R, Vlahović K, Mavri U, Dovč A (2011) Parasites in pet reptiles. Acta Vet Scand 53:33. https://doi.org/10.1186/1751-0147-53-33

141. Cepicka I, Dolan M, Gile G (2016) Parabasalia. pp 1–44

142. Zwart P, Teunis S, Cornelissen J (1984) Monocercomoniasis in Reptiles. The Journal of Zoo Animal Medicine 15:129–134. https://doi.org/10.2307/20094704

143. Richter B, Kübber-Heiss A, Weissenböck H, Schmidt P (2008) Detection of *Cryptosporidium* spp., *Entamoeba* spp. and *Monocercomonas* spp. in the gastrointestinal tract of snakes by In-situ Hybridization. Journal of Comparative Pathology 138:63–71. https://doi.org/10.1016/j.jcpa.2007.11.001

144. Raś-Noryńska M, Sokół R (2015) Internal parasites of reptiles. Ann Parasitol 61:115–117

145. Barrera J, Montoya A, Marino V, Sarquis J, Checa R, Miró G (2023) *Cystoisospora* spp. infection at a dog breeding facility in the Madrid region: Infection rate and clinical management based on toltrazuril metaphylaxis. Veterinary Parasitology Regional Studies and Reports 48:100971. https://doi.org/10.1016/j.vprsr.2023.100971

146. Dubey JP (2019) Re-evaluation of merogony of a *Cystoisospora ohioensis*-like coccidian and its distinction from gametogony in the intestine of a naturally infected dog. Parasitology 146:740–745. https://doi.org/10.1017/S0031182018002202

147. Lindsay DS, Houk AE, Mitchell SM, Dubey JP (2014) Developmental biology of *Cystoisospora* (Apicomplexa: Sarcocystidae) monozoic tissue cysts. J Parasitol 100:392–398. https://doi.org/10.1645/13-494.1

148. Fok E, Szatmári V, Busák K, Rozgonyi F (2001) Prevalence of intestinal parasites in dogs in some urban and rural areas of Hungary. Vet Q 23:96–98. https://doi.org/10.1080/01652176.2001.9695091

149. Demeter C, Matics Z, Demeter-Jeremiás A, Sándor F, Gerencsér Z, Német Z (2023) Survey of the seasonal dependency of *Eimeria* oocysts and *Passalurus ambiguus* infections in industrial rabbit farms. World Rabbit Science 31:277–283. https://doi.org/10.4995/wrs.2023.19396

150. Ahmed F, Cappai MG, Morrone S, Cavallo L, Berlinguer F, Dessì G, Tamponi C, Scala A, Varcasia A (2021) Raw meat based diet (RMBD) for household pets as potential door opener to parasitic load of domestic and urban environment. Revival of understated zoonotic hazards? A review. One Health 13:100327. https://doi.org/10.1016/j.onehlt.2021.100327

151. Dubey JP, Lindsay DS (2006) Neosporosis, toxoplasmosis, and sarcocystosis in ruminants. Veterinary Clinics of North America: Food Animal Practice 22:645–671. https://doi.org/10.1016/j.cvfa.2006.08.001

152. Zhu N, Yang L, Xin S, Huang W, Jiang Y, Yang Y (2022) Low prevalence of *Toxoplasma gondii* in dogs from Central China. Front Cell Infect Microbiol 12:885348. https://doi.org/10.3389/fcimb.2022.885348

153. van Bree FPJ, Bokken GCAM, Mineur R, Franssen F, Opsteegh M, van der Giessen JWB, Lipman LJA, Overgaauw PAM (2018) Zoonotic bacteria and parasites found in raw meat-based diets for cats and dogs. Vet Rec 182:50. https://doi.org/10.1136/vr.104535

154. Mendez OA, Koshy AA (2017) Toxoplasma gondii: Entry, association, and physiological influence on the central nervous system. PLoS Pathog 13:e1006351. https://doi.org/10.1371/journal.ppat.1006351

155. Flegr J, Havlícek J, Kodym P, Malý M, Smahel Z (2002) Increased risk of traffic accidents in subjects with latent toxoplasmosis: a retrospective case-control study. BMC Infect Dis 2:11. https://doi.org/10.1186/1471-2334-2-11

156. Webster JP (2007) The Effect of *Toxoplasma gondii* on animal behavior: Playing cat and mouse. Schizophr Bull 33:752–756. https://doi.org/10.1093/schbul/sbl073

157. Postolache TT, Wadhawan A, Rujescu D, Hoisington AJ, Dagdag A, Baca-Garcia E, Lowry CA, Okusaga OO, Brenner LA (2021) *Toxoplasma gondii*, suicidal behavior, and intermediate phenotypes for suicidal behavior. Front Psychiatry 12:665682. https://doi.org/10.3389/fpsyt.2021.665682

158. Tarekegn ZS, Dejene H, Addisu A, Dagnachew S (2020) Potential risk factors associated with seropositivity for *Toxoplasma gondii* among pregnant women and HIV infected individuals in Ethiopia: A

systematic review and meta-analysis. PLoS Negl Trop Dis 14:e0008944. https://doi.org/10.1371/journal.pntd.0008944

159. Hornok S, Edelhofer R, Joachim A, Farkas R, Berta K, Répási A, Lakatos B (2008) Seroprevalence of T*oxoplasma gondii* and *Neospora caninum* infection of cats in Hungary. Acta Vet Hung 56:81–88. https://doi.org/10.1556/AVet.56.2008.1.8

160. Dunay MP, Zólyomi D, Gulyás E, Dunay IR (2024) *Toxoplasma gondii* seroprevalence and risk factors of cats in the Budapest area. Eur J Microbiol Immunol (Bp) 14:340–345. https://doi.org/10.1556/1886.2024.00079

161. Malmasi A, Mosallanejad B, Mohebali M, Sharifian Fard M, Taheri M (2009) Prevention of shedding and re-shedding of *Toxoplasma gondii* oocysts in experimentally infected cats treated with oral Clindamycin: a preliminary study. Zoonoses Public Health 56:102–104. https://doi.org/10.1111/j.1863-2378.2008.01174.x

162. Silva RC, Machado GP (2016) Canine neosporosis: perspectives on pathogenesis and management. Vet Med (Auckl) 7:59–70. https://doi.org/10.2147/VMRR.S76969

163. Dubey JP (2003) Review of *Neospora caninum* and neosporosis in animals. Korean J Parasitol 41:1–16. https://doi.org/10.3347/kjp.2003.41.1.1

164. Anvari D, Saberi R, Sharif M, Sarvi S, Hosseini SA, Moosazadeh M, Hosseininejad Z, Chegeni TN, Daryani A (2020) Seroprevalence of *Neospora caninum* infection in dog population worldwide: A systematic review and meta-analysis. Acta Parasit 65:273–290. https://doi.org/10.2478/s11686-019-00163-4

165. Kuruca L, Spasojević-Kosić L, Simin S, Savović M, Lauš S, Lalošević V (2013) *Neospora caninum* antibodies in dairy cows and domestic dogs from Vojvodina, Serbia. Parasite 20:40. https://doi.org/10.1051/parasite/2013036

166. Gavrea R, Mircean V, Pastiu A, Cozma V (2012) Epidemiological survey of *Neospora caninum* infection in dogs from Romania. Vet Parasitol 188:382–385. https://doi.org/10.1016/j.vetpar.2012.03.044

167. Bacsadi Á, Bajmócy E, Matiz K, Kiss I (2001) Bovine abortion associated with *Neospora caninum* in Hungary. Acta Veterinaria Hungarica 49:185–189. https://doi.org/10.1556/004.49.2001.2.7

168. Hornok S, Näslund K, Hajtós I, Tanyi J, Tekes L, Varga I, Uggla A, Björkman C (1998) Detection of antibodies to *Neospora caninum* in bovine postabortion blood samples from Hungary. Acta Vet Hung 46:431–436

169. Hornok S, Edelhofer R, Hajtós I (2006) Seroprevalence of neosporosis in beef and dairy cattle breeds in Northeast Hungary. Acta Vet Hung 54:485–491. https://doi.org/10.1556/AVet.54.2006.4.6

170. Hornok S, Edelhofer R, Fok E, Berta K, Fejes P, Répási A, Farkas R (2006) Canine neosporosis in Hungary: screening for seroconversion of household, herding and stray dogs. Vet Parasitol 137:197–201. https://doi.org/10.1016/j.vetpar.2006.01.030

171. Haddad JPA, Dohoo IR, VanLeewen JA (2005) A review of *Neospora caninum* in dairy and beef cattle — a Canadian perspective. Can Vet J 46:230–243

172. Dubey JP, Calero-Bernal R, Rosenthal BM, Speer CA, Fayer R (2016a): General biology of *Sarcocystis* species. In: Sarcocystosis of animals and humans. CRC Press, Boca Raton, Florida, pp. 1–108.

173. Rommel M, Heydorn AO, Gruber F (1972) [Life cycle of Sarcosporidia. 1. The sporocyst of *S. tenella* in cat feces]. Berl Munch Tierarztl Wochenschr 85:101–105

174. Dubey JP, Calero-Bernal R, Rosenthal BM, Speer CA, Fayer R (2016b): Sarcocystosis in dogs (*Canis familiaris*). In: Sarcocystosis of animals and humans. CRC Press, Boca Raton, Florida, pp. 269–272.

176. Gjerde B, Luzón M, Alunda JM, de la Fuente C (2017) Morphological and molecular characteristics of six *Sarcocystis* spp. from red deer (*Cervus elaphus*) in Spain, including *Sarcocystis cervicanis* and three new species. Parasitol Res 116:2795–2811. https://doi.org/10.1007/s00436-017-5590-z

177. Kirillova V, Prakas P, Calero-Bernal R, Gavarāne I, Fernández-García JL, Martínez-González M, Rudaitytė-Lukošienė E, Martínez-Estéllez MÁH, Butkauskas D, Kirjušina M (2018) Identification and genetic

characterization of *Sarcocystis arctica* and *Sarcocystis lutrae* in red foxes (*Vulpes vulpes*) from Baltic States and Spain. Parasit Vectors 11:173. https://doi.org/10.1186/s13071-018-2694-y

178. Dubey JP, Benson J, Larson MA (2003) Clinical *Sarcocystis neurona* encephalomyelitis in a domestic cat following routine surgery. Vet Parasitol 112:261–267. https://doi.org/10.1016/s0304-4017(03)00019-0

179. Sykes J e., Dubey J p., Lindsay L l., Prato P, Lappin M r., Guo L t., Mizisin A p., Shelton G d. (2011) Severe myositis associated with *Sarcocystis* spp. Infection in 2 Dogs. Journal of Veterinary Internal Medicine 25:1277–1283. https://doi.org/10.1111/j.1939-1676.2011.00828.x

180. Poulsen CS, Stensvold CR (2014) Current Status of Epidemiology and Diagnosis of Human Sarcocystosis. J Clin Microbiol 52:3524–3530. https://doi.org/10.1128/JCM.00955-14

181. Szekeres S, Juhász A, Kondor M, Takács N, Sugár L, Hornok S (2019) *Sarcocystis rileyi* emerging in Hungary: is rice breast disease underreported in the region? Acta Vet Hung 67:401–406. https://doi.org/10.1556/004.2019.040

182. Hornok S, Mester A, Takács N, Baska F, Majoros G, Fok É, Biksi I, Német Z, Hornyák Á, Jánosi Sz, Farka R (2015) *Sarcocystis*-infection of cattle in Hungary. Parasites & Vectors 8:. https://doi.org/10.1186/s13071-015-0685-9

183. Barrett LG, Thrall PH, Burdon JJ, Linde CC (2008) Life history determines genetic structure and evolutionary potential of host-parasite interactions. Trends Ecol Evol 23:678–685. https://doi.org/10.1016/j.tree.2008.06.017

184. Franssen SU, Takele Y, Adem E, Sanders MJ, Müller I, Kropf P, Cotton JA (2021) Diversity and within-host evolution of *Leishmania donovani* from visceral leishmaniasis patients with and without HIV coinfection in Northern Ethiopia. mBio 12:e0097121. https://doi.org/10.1128/mBio.00971-21

185. Stumpf MPH (2004) Haplotype diversity and SNP frequency dependence in the description of genetic variation. Eur J Hum Genet 12:469–477. https://doi.org/10.1038/sj.ejhg.5201179

186. Ramírez JD, Llewellyn MS (2014) Reproductive clonality in protozoan pathogens--truth or artefact? Mol Ecol 23:4195–4202. https://doi.org/10.1111/mec.12872

187. Walliker D (1989) Implications of genetic exchange in the study of protozoan infections. Parasitology 99 Suppl:S49-58. https://doi.org/10.1017/s0031182000083414

188. Bradic M, Carlton JM (2018) Does the common sexually transmitted parasite *Trichomonas vaginalis* have sex? PLoS Pathog 14:e1006831. https://doi.org/10.1371/journal.ppat.1006831

189. Feretzaki M, Heitman J (2013) Unisexual reproduction drives evolution of eukaryotic microbial pathogens. PLoS Pathog 9:e1003674. https://doi.org/10.1371/journal.ppat.1003674

190. Schurko AM, Neiman M, Logsdon JM (2009) Signs of sex: what we know and how we know it. Trends Ecol Evol 24:208–217. https://doi.org/10.1016/j.tree.2008.11.010

191. Kelso AA, Waldvogel SM, Luthman AJ, Sehorn MG (2017) Homologous recombination in protozoan parasites and recombinase inhibitors. Front Microbiol 8:1716. https://doi.org/10.3389/fmicb.2017.01716

192. Gwarinda HB, Tessema SK, Raman J, Greenhouse B, Birkholtz L-M (2021) Parasite genetic diversity reflects continued residual malaria transmission in Vhembe District, a hotspot in the Limpopo Province of South Africa. Malar J 20:96. https://doi.org/10.1186/s12936-021-03635-z

193. Kwiatkowski D (2024) Modelling transmission dynamics and genomic diversity in a recombining parasite population. Wellcome Open Res 9:215. https://doi.org/10.12688/wellcomeopenres.19092.1

194. Tuska-Szalay B, Jerzsele Á, Hornok S (2024) Egysejtű élősködők ellen alkalmazható gyógyszerek az állatorvoslásban : Irodalmi összefoglaló. MAGYAR ÁLLATORVOSOK LAPJA 146:487–500

195. Szekeres S, Czabán D, Takács N, Széll Z, Gubányi A, Kontschán J, Hornok S, Sréter T (2022) First report of molecular taxonomic analyses of European beaver metazoan parasites from Hungary. Parasitol Res 121:1895–1902. https://doi.org/10.1007/s00436-022-07547-y

196. Cacciò SM, Beck R, Lalle M, Marinculic A, Pozio E (2008) Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. Int J Parasitol 38:1523–1531. https://doi.org/10.1016/j.ijpara.2008.04.008

197. Gillhuber J, Pallant L, Ash A, Thompson RCA, Pfister K, Scheuerle MC (2013) Molecular identification of zoonotic and livestock-specific *Giardia*-species in faecal samples of calves in Southern Germany. Parasit Vectors 6:346. https://doi.org/10.1186/1756-3305-6-346

198. Cepicka I, Hampl V, Kulda J, Flegr J (2006) New evolutionary lineages, unexpected diversity, and host specificity in the parabasalid genus *Tetratrichomonas*. Mol Phylogenet Evol 39:542–551. https://doi.org/10.1016/j.ympev.2006.01.005

199. Cepicka I, Kutisová K, Tachezy J, Kulda J, Flegr J (2005) Cryptic species within the *Tetratrichomonas gallinarum* species complex revealed by molecular polymorphism. Vet Parasitol 128:11–21. https://doi.org/10.1016/j.vetpar.2004.11.003

200. Edgcomb VP, Roger AJ, Simpson AGB, Kysela DT, Sogin ML (2001) Evolutionary relationships among “Jakobid” flagellates as indicated by alpha- and beta-tubulin phylogenies. Molecular Biology and Evolution 18:514–522. https://doi.org/10.1093/oxfordjournals.molbev.a003830

201. Felleisen RSJ (1997) Comparative sequence analysis of 5·8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. Parasitology 115:111–119. https://doi.org/10.1017/S0031182097001212

202. Schroeder JM, Booton GC, Hay J, Niszl IA, Seal DV, Markus MB, Fuerst PA, Byers TJ (2001) Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. J Clin Microbiol 39:1903–1911. https://doi.org/10.1128/JCM.39.5.1903-1911.2001

203. Ogedengbe JD, Hanner RH, Barta JR (2011) DNA barcoding identifies *Eimeria* species and contributes to the phylogenetics of coccidian parasites (Eimeriorina, Apicomplexa, Alveolata). Int J Parasitol 41:843–850. https://doi.org/10.1016/j.ijpara.2011.03.007

204. Yamage M, Flechtner O, Gottstein B (1996) *Neospora caninum*: specific oligonucleotide primers for the detection of brain “cyst” DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). J Parasitol 82:272–279

205. Wapenaar W, Jenkins MC, O’Handley RM, Barkema HW (2006) *Neospora caninum*-like oocysts observed in feces of free-ranging red foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*). J Parasitol 92:1270–1274. https://doi.org/10.1645/GE-913R.1

206. Homan WL, Vercammen M, De Braekeleer J, Verschueren H (2000) Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int J Parasitol 30:69–75. https://doi.org/10.1016/s0020-7519(99)00170-8

207. Reischl U, Bretagne S, Krüger D, Ernault P, Costa J-M (2003) Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. BMC Infect Dis 3:7. https://doi.org/10.1186/1471-2334-3-7

208. Schares G, Herrmann DC, Beckert A, Schares S, Hosseininejad M, Pantchev N, Globokar Vrhovec M, Conraths FJ (2008) Characterization of a repetitive DNA fragment in *Hammondia hammondi* and its utility for the specific differentiation of *H. hammondi* from *Toxoplasma gondii* by PCR. Mol Cell Probes 22:244–251. https://doi.org/10.1016/j.mcp.2008.04.003

209. Casati S, Sager H, Gern L, Piffaretti J-C (2006) Presence of potentially pathogenic *Babesia* sp. for human in *Ixodes ricinus* in Switzerland. Ann Agric Environ Med 13:65–70

210. Ho MS, Barr BC, Marsh AE, Anderson ML, Rowe JD, Tarantal AF, Hendrickx AG, Sverlow K, Dubey JP, Conrad PA (1996) Identification of bovine *Neospora* parasites by PCR amplification and specific small-subunit rRNA sequence probe hybridization. J Clin Microbiol 34:1203–1208. https://doi.org/10.1128/jcm.34.5.1203-1208.1996

211. Hornok S, Estók P, Kováts D, Flaisz B, Takács N, Szőke K, Krawczyk A, Kontschán J, Gyuranecz M, Fedák A, Farkas R, Haarsma A-J, Sprong H (2015) Screening of bat faeces for arthropod-borne apicomplexan

protozoa: *Babesia canis* and *Besnoitia besnoiti*-like sequences from Chiroptera. Parasites & Vectors 8:441. https://doi.org/10.1186/s13071-015-1052-6

212. Inokuma H, Okuda M, Ohno K, Shimoda K, Onishi T (2002) Analysis of the 18S rRNA gene sequence of a *Hepatozoon* detected in two Japanese dogs. Vet Parasitol 106:265–271. https://doi.org/10.1016/s0304-4017(02)00065-1

213. Schreeg ME, Marr HS, Tarigo J, Cohn LA, Levy MG, Birkenheuer AJ (2013) Pharmacogenomics of *Cytauxzoon felis* cytochrome b: implications for atovaquone and azithromycin therapy in domestic cats with cytauxzoonosis. J Clin Microbiol 51:3066–3069. https://doi.org/10.1128/JCM.01407-13

214. Panait LC, Mihalca AD, Modrý D, Juránková J, Ionică AM, Deak G, Gherman CM, Heddergott M, Hodžić A, Veronesi F, Reichard M, Zieman EA, Nielsen CK, Jiménez-Ruiz FA, Hrazdilová K (2021) Three new species of *Cytauxzoon* in European wild felids. Vet Parasitol 290:109344. https://doi.org/10.1016/j.vetpar.2021.109344

215. Guo J, Miao X, He P, Li M, Wang S, Cui J, Huang C, He L, Zhao J (2019) *Babesia gibsoni* endemic to Wuhan, China: mitochondrial genome sequencing, annotation, and comparison with apicomplexan parasites. Parasitol Res 118:235–243. https://doi.org/10.1007/s00436-018-6158-2

216. Wickramasekara Rajapakshage BK, Yamasaki M, Hwang S-J, Sasaki N, Murakami M, Tamura Y, Lim SY, Nakamura K, Ohta H, Takiguchi M (2012) Involvement of mitochondrial genes of *Babesia gibsoni* in resistance to diminazene aceturate. J Vet Med Sci 74:1139–1148. https://doi.org/10.1292/jvms.12-0056

217. Wengi N, Willi B, Boretti FS, Cattori V, Riond B, Meli ML, Reusch CE, Lutz H, Hofmann-Lehmann R (2008) Real-time PCR-based prevalence study, infection follow-up and molecular characterization of canine hemotropic mycoplasmas. Vet Microbiol 126:132–141. https://doi.org/10.1016/j.vetmic.2007.06.018

218. Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054

219. Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular evolutionary genetics analysis version 11. Mol Biol Evol 38:3022–3027. https://doi.org/10.1093/molbev/msab120

220. Hampl V, Cepicka I, Flegr J, Tachezy J, Kulda J (2007) Morphological and molecular diversity of the monocercomonadid genera *Monocercomonas*, *Hexamastix*, and *Honigbergiella* gen. nov. Protist 158:365–383. https://doi.org/10.1016/j.protis.2007.02.003

221. Conza L, Pagani SC, Gaia V (2013) Presence of *Legionella* and free-living *Amoebae* in composts and bioaerosols from composting facilities. PLoS One 8:e68244. https://doi.org/10.1371/journal.pone.0068244

222. Karakuş M, Aykur M, Özbel Y, Töz S, Dağcı H (2016) Molecular detection and genotyping of *Acanthamoeba* spp. among stray dogs using conjunctival swab sampling. Acta Trop 164:23–26. https://doi.org/10.1016/j.actatropica.2016.08.011

223. Martín-Pérez T, Criado-Fornelio A, Martínez J, Blanco MA, Fuentes I, Pérez-Serrano J (2017) Isolation and molecular characterization of *Acanthamoeba* from patients with keratitis in Spain. Eur J Protistol 61:244–252. https://doi.org/10.1016/j.ejop.2017.06.009

224. Hernández Rodriguez S, Martinez-Gómez F, Navarrete I, Acosta-Garcia I (1981a). Optical and electron microscopic study of the cysts of *Sarcocystis cervicanis* [in Spanish]. Rev. Iber. Parasitol. 41 ,351–361.

225. Poli A, Mancianti F, Marconcini A, Nigro M, Colagreco R (1988) Prevalence, ultrastructure of the cyst wall and infectivity for the dog and cat of *Sarcocystis* sp. from fallow deer (*Cervus dama*). Journal of Wildlife Diseases 24:97–104. https://doi.org/10.7589/0090-3558-24.1.97

226. Dubey, JP, Calero-Bernal R, Rosenthal BM, Speer CA, Fayer R (2016c). *Sarcocystis* infection in cervids. In: Sarcocystosis of animals and humans. CRC Press, Boca Raton, Florida. pp. 293–307.

227. Levecke B, Meulemans L, Dalemans T, Casaert S, Claerebout E, Geurden T (2011) Mixed *Giardia duodenalis* assemblage A, B, C and E infections in pet chinchillas (*Chinchilla lanigera*) in Flanders (Belgium). Vet Parasitol 177:166–170. https://doi.org/10.1016/j.vetpar.2010.11.027

228. Egan S, Barbosa AD, Feng Y, Xiao L, Ryan U (2024) Critters and contamination: Zoonotic protozoans in urban rodents and water quality. Water Res 251:121165. https://doi.org/10.1016/j.watres.2024.121165

229. Fernández-Álvarez Á, Martín-Alonso A, Abreu-Acosta N, Feliu C, Hugot J-P, Valladares B, Foronda P (2014) Identification of a novel assemblage G subgenotype and a zoonotic assemblage B in rodent isolates of *Giardia duodenalis* in the Canary Islands, Spain. Parasitology 141:206–215. https://doi.org/10.1017/S003118201300139X

230. Lebbad M, Mattsson JG, Christensson B, Ljungström B, Backhans A, Andersson JO, Svärd SG (2010) From mouse to moose: multilocus genotyping of *Giardia* isolates from various animal species. Vet Parasitol 168:231–239. https://doi.org/10.1016/j.vetpar.2009.11.003

231. de Cock MP, de Vries A, Fonville M, Esser HJ, Mehl C, Ulrich RG, Joeres M, Hoffmann D, Eisenberg T, Schmidt K, Hulst M, van der Poel WHM, Sprong H, Maas M (2023) Increased rat-borne zoonotic disease hazard in greener urban areas. Sci Total Environ 896:165069. https://doi.org/10.1016/j.scitotenv.2023.165069

232. Lecová L, Hammerbauerová I, Tůmová P, Nohýnková E (2020) Companion animals as a potential source of *Giardia intestinalis* infection in humans in the Czech Republic - A pilot study. Vet Parasitol Reg Stud Reports 21:100431. https://doi.org/10.1016/j.vprsr.2020.100431

233. Jekl V, Hauptman K, Knotek Z (2011) Diseases in pet degus: a retrospective study in 300 animals. J Small Anim Pract 52:107–112. https://doi.org/10.1111/j.1748-5827.2010.01028.x

234. Najecki, D., Tate, B. A. 1999. Husbandry and management of the degu (*Octodon degus*). Lab Animal 28(3), 54-57. | Animal Welfare Institute. https://awionline.org/lab-animal-search/najecki-d-tate-b-1999-husbandry-and-management-degu-octodon-degus-lab-animal-283. Accessed 12 Jun 2024

235. Heyworth MF (2016) *Giardia duodenalis* genetic assemblages and hosts. Parasite 23:13. https://doi.org/10.1051/parasite/2016013

236. Serva D, Biondi M, Iannella M (2023) The Eurasian beaver range expansion reveals uneven future trends and possible conservation issues: an European assessment. Biodivers Conserv 32:1999–2016. https://doi.org/10.1007/s10531-023-02587-x

237. d'Ovidio D, Noviello E, Ianniello D, Cringoli G, Rinaldi L (2015) Survey of endoparasites in pet guinea pigs in Italy. Parasitol Res 114:1213–1216. https://doi.org/10.1007/s00436-014-4289-7

238. Wang N, Wang K, Liu Y, Zhang X, Zhao J, Zhang S, Zhang L (2022) Molecular characterization of *Cryptosporidium* spp., *Enterocytozoon bieneusi* and *Giardia duodenalis* in laboratory rodents in China. Parasite 29:46. https://doi.org/10.1051/parasite/2022046

239. Pantchev N, Broglia A, Paoletti B, Globokar Vrhovec M, Bertram A, Nöckler K, Cacciò SM (2014) Occurrence and molecular typing of *Giardia* isolates in pet rabbits, chinchillas, guinea pigs and ferrets collected in Europe during 2006-2012. Vet Rec 175:18. https://doi.org/10.1136/vr.102236

240. Sansano-Maestre J, Garijo-Toledo MM, Gómez-Muñoz MT (2009) Prevalence and genotyping of *Trichomonas gallinae* in pigeons and birds of prey. Avian Pathol 38:201–207. https://doi.org/10.1080/03079450902912135

241. Stockdale JE, Dunn JC, Goodman SJ, Morris AJ, Sheehan DK, Grice PV, Hamer KC (2015) The protozoan parasite *Trichomonas gallinae* causes adult and nestling mortality in a declining population of European Turtle Doves, *Streptopelia turtur*. Parasitology 142:490–498. https://doi.org/10.1017/S0031182014001474

242. Martínez-Herrero MC, Sansano-Maestre J, López Márquez I, Obón E, Ponce C, González J, Garijo-Toledo MM, Gómez-Muñoz MT (2014) Genetic characterization of oropharyngeal trichomonad isolates from wild birds indicates that genotype is associated with host species, diet and presence of pathognomonic lesions. Avian Pathol 43:535–546. https://doi.org/10.1080/03079457.2014.967660

243. Lennon RJ, Dunn JC, Stockdale JE, Goodman SJ, Morris AJ, Hamer KC (2013) Trichomonad parasite infection in four species of Columbidae in the UK. Parasitology 140:1368–1376. https://doi.org/10.1017/S0031182013000887

244. Quillfeldt P, Schumm YR, Marek C, Mader V, Fischer D, Marx M (2018) Prevalence and genotyping of *Trichomonas* infections in wild birds in central Germany. PLoS One 13:e0200798. https://doi.org/10.1371/journal.pone.0200798

245. Santos N, Jambas J, Monteiro A, Amaral J, Martins N, Garcia J, Fernández AM, Tyler KM, Almeida T, Abrantes J, Esteves PJ (2019) *Trichomonas* infection in a community of free-ranging domestic and wild Columbiformes and Bonelli's eagle (*Aquila fasciata*). Frontiers in Veterinary Science 6:

246. Bankovics A (2001) Status of wild pigeons and doves in Hungary. Naturzale 61–70

247. Powell EC, Hollander WF (1982) *Trichomonas gallinae* infections in the ringdove (*Streptopelia risoria*). Journal of Wildlife Diseases 18:89–90. https://doi.org/10.7589/0090-3558-18.1.89

248. Stimmelmayr R, Stefani LM, Thrall MA, Landers K, Revan F, Miller A, Beckstead R, Gerhold R (2012) Trichomonosis in free-ranging Eurasian collared doves (*Streptopelia decaocto*) and African collared dove hybrids (*Streptopelia risoria*) in the Caribbean and description of ITS-1 region genotypes. Avian Dis 56:441–445. https://doi.org/10.1637/9905-082311-Case.1

249. Grabensteiner E, Bilic I, Kolbe T, Hess M (2010) Molecular analysis of clonal trichomonad isolates indicate the existence of heterogenic species present in different birds and within the same host. Vet Parasitol 172:53–64. https://doi.org/10.1016/j.vetpar.2010.04.015

250. Martínez-Herrero MC, Sansano-Maestre J, Azami-Conesa I, González-González F, Suárez Regalado L, Garijo-Toledo MM, Gómez-Muñoz MT (2021) Sequence subtyping of *Trichomonas gallinae* from Bonelli's eagle (*Aquila fasciata*) during four years (2014–2017) reveals that MLS type is associated with lesions. Avian Pathology 50:339–349. https://doi.org/10.1080/03079457.2021.1940099

251. Tuska-Szalay B, Boldogh SA, Farkas R, Rompos L, Takács N, Beresnyák V, Izsó Á, Kontschán J, Lanszki J, Hornok S (2023) Screening of domestic cats from North-Eastern Hungary for *Hepatozoon felis* and *Cytauxzoon europaeus* that cause infections in local wildcat populations. Pathogens 12:656. https://doi.org/10.3390/pathogens12050656

252. Ceplecha V, Svobodova V, Lendon C, Husnik R, Horackova K, Svoboda M (2017) A survey of feline trichomonosis suggests a low incidence of *Tritrichomonas blagburni* among cats in the Czech Republic. Vet Med 62:269–273. https://doi.org/10.17221/106/2016-VETMED

253. Maritz JM, Land KM, Carlton JM, Hirt RP (2014) What is the importance of zoonotic trichomonads for human health? Trends Parasitol 30:333–341. https://doi.org/10.1016/j.pt.2014.05.005

254. Zhang N, Zhang H, Yu Y, Gong P, Li J, Li Z, Li T, Cong Z, Tian C, Liu X, Yu X, Zhang X (2019) High prevalence of *Pentatrichomonas hominis* infection in gastrointestinal cancer patients. Parasites & Vectors 12:423. https://doi.org/10.1186/s13071-019-3684-4

255. Tuzlak L, Alves-Ferreira EVC, Schwartz CL, Kennard A, Leung JM, Shehata C, Grigg ME (2023) Fine structure and molecular characterization of two new parabasalid species that naturally colonize laboratory mice, *Tritrichomonas musculus* and *Tritrichomonas casperi*. Journal of Eukaryotic Microbiology 70:e12989. https://doi.org/10.1111/jeu.12989

256. Alrefaei AF, Gerhold RW, Nader JL, Bell DJ, Tyler KM (2019) Improved subtyping affords better discrimination of *Trichomonas gallinae* strains and suggests hybrid lineages. Infect Genet Evol 73:234–241. https://doi.org/10.1016/j.meegid.2019.05.007

257. Holliday M, Deni D, Gunn-Moore DA (2009) *Tritrichomonas foetus* infection in cats with diarrhoea in a rescue colony in Italy. J Feline Med Surg 11:131–134. https://doi.org/10.1016/j.jfms.2008.06.004

258. Gunn-Moore DA, McCann TM, Reed N, Simpson KE, Tennant B (2007) Prevalence of *Tritrichomonas foetus* infection in cats with diarrhoea in the UK. J Feline Med Surg 9:214–218. https://doi.org/10.1016/j.jfms.2007.01.003

259. Crisi PE, Paoletti B, Morelli S, Simonato G, Colombo M, Tiscar PG, Boari A (2021) *Tritrichomonas foetus* in cats from Central Italy: Clinical signs and risk factors. Vet Parasitol Reg Stud Reports 24:100577. https://doi.org/10.1016/j.vprsr.2021.100577

260. Machin R (2015) Common gastrointestinal parasites in reptiles. In Practice 37:. https://doi.org/10.1136/inp.h4914

261. Corriveau LA, Thompson SB (2013) *Trichomonas* and *Cryptosporidium* spp. in a black throat monitor (*Varanus albigularis ionidesi*). Proceedings Assoc of Rept and Amphib Veterinarians, p 56. https://cdn.ymaws.com/members.arav.org/resource/resmgr/Files/Proceedings.... Accessed 30.03.2025

262. Kannangara DW (1970) *Trichomonas* sp. from reptiles of Ceylon. Trans R Soc Trop Med Hyg 64:935. https://doi.org/10.1016/0035-9203(70)90115-x

263. Silva ACD, Carli GAD, Brasseur P, Tasca T, Castilhos D, Wendorff A (1998) Hemolytic activity of *Monocercomonas* spp. Parasite 5:79–82. https://doi.org/10.1051/parasite/1998051079

264. Moskowitz N (1951) Observations on some intestinal flagellates from reptilian host (Squamata). Journal of Morphology 89:257–321. https://doi.org/10.1002/jmor.1050890204

265. Ledbetter EC, Kim SG, Schaefer DM, Liotta JL, Bowman DD, Lejeune M (2021) Detection of free-living amoebae in domestic cats with and without naturally-acquired keratitis. The Veterinary Journal 274:105712. https://doi.org/10.1016/j.tvjl.2021.105712

266. Ithoi I, Mahmud R, Abdul Basher MH, Jali A, Abdulsalam AM, Ibrahim J, Mak JW (2013) *Acanthamoeba* genotype T4 detected in naturally-infected feline corneas found to be in homology with those causing human keratitis. Trop Biomed 30:131–140

267. Westmoreland SV, Rosen J, MacKey J, Romsey C, Xia D-L, Visvesvera GS, Mansfield KG (2004) Necrotizing meningoencephalitis and pneumonitis in a Simian Immunodeficiency Virus—infected rhesus macaque due to *Acanthamoeba*. Vet Pathol 41:398–404. https://doi.org/10.1354/vp.41-4-398

268. Dubey JP, Benson JE, Blakeley KT, Booton GC, Visvesvara GS (2005) Disseminated *Acanthamoeba* sp. infection in a dog. Veterinary Parasitology 128:183–187. https://doi.org/10.1016/j.vetpar.2004.11.022

269. Yu H-S, Kong H-H, Kim S-Y, Hahn Y-H, Hahn T-W, Chung D-I (2004) Laboratory investigation of *Acanthamoeba lugdunensis* from patients with keratitis. Invest Ophthalmol Vis Sci 45:1418–1426. https://doi.org/10.1167/iovs.03-0433

270. Norouzi M, Saberi R, Niyyati M, Lorenzo-Morales J, Mirjalali H, Fatemi M, Javanmard E, Karamati SA (2021) Molecular identification of pathogenic free-living amoeba from household biofilm samples in Iran: A risk factor for *Acanthamoeba* Keratitis. Microorganisms 9:2098. https://doi.org/10.3390/microorganisms9102098

271. Jercic MI, Aguayo C, Saldarriaga-Córdoba M, Muiño L, Chenet SM, Lagos J, Osuna A, Fernández J (2019) Genotypic diversity of *Acanthamoeba* strains isolated from Chilean patients with *Acanthamoeba* keratitis. Parasit Vectors 12:58. https://doi.org/10.1186/s13071-019-3302-5

272. Khan NA (2006) *Acanthamoeba*: biology and increasing importance in human health. FEMS Microbiol Rev 30:564–595. https://doi.org/10.1111/j.1574-6976.2006.00023.x

273. Frank W, Bosch I (1972) Isolierung von Amoeben des Typs „*Hartmannella-Acanthamoeba*“ und „*Naegleria*“ aus Kaltblütern. Z Parasitenk 40:139–150. https://doi.org/10.1007/BF00329149

274. Walochnik J, Hassl A, Simon K, Benyr G, Aspöck H (1999) Isolation and identification by partial sequencing of the 18S ribosomal gene of free-living amoebae from necrotic tissue of *Basilliscus plumifrons* (Sauria: Iguanidae). Parasitol Res 85:601–603. https://doi.org/10.1007/s004360050602

275. Parija S, Giri S (2012) Emerging protozoal pathogens in India: How prepared are we to face the threat? Trop Parasitol 2:13. https://doi.org/10.4103/2229-5070.97233

276. Valladares M, Reyes-Batlle M, Mora-Peces I, Martín-Navarro CM, López-Arencibia A, Dorta-Gorrín A, Comyn-Afonso E, Martínez-Carretero E, Maciver SK, Piñero JE, Valladares B, Lorenzo-Morales J (2014) A multisystemic *Acanthamoeba* infection in a dog in Tenerife, Canary Islands, Spain. Veterinary Parasitology 205:707–711. https://doi.org/10.1016/j.vetpar.2014.08.004

277. Kent M, Platt SR, Rech RR, Eagleson JS, Howerth EW, Shoff M, Fuerst PA, Booton G, Visvesvara GS, Schatzberg SJ (2011) Multisystemic infection with an *Acanthamoeba* sp. in a dog. javma 238:1476–1481. https://doi.org/10.2460/javma.238.11.1476

278. Randag AC, Van Rooij J, Van Goor AT, Verkerk S, Wisse RPL, Saelens IEY, Stoutenbeek R, Van Dooren BTH, Cheng YYY, Eggink CA (2019) The rising incidence of *Acanthamoeba* keratitis: A 7-year nationwide survey and clinical assessment of risk factors and functional outcomes. PLoS ONE 14:e0222092. https://doi.org/10.1371/journal.pone.0222092

279. Ibrahim YW, Boase DL, Cree IA (2009) How could contact lens wearers be at risk of *Acanthamoeba* infection? A Review. Journal of Optometry 2:60–66. https://doi.org/10.3921/joptom.2009.60

280. Westmoreland SV, Rosen J, MacKey J, Romsey C, Xia D-L, Visvesvera GS, Mansfield KG (2004) Necrotizing meningoencephalitis and pneumonitis in a Simian Immunodeficiency Virus—infected rhesus macaque due to *Acanthamoeba*. Vet Pathol 41:398–404. https://doi.org/10.1354/vp.41-4-398

281. Begg AP, Todhunter K, Donahoe SL, Krockenberger M, Šlapeta J (2014) Severe amoebic placentitis in a horse caused by an *Acanthamoeba hatchetti* isolate identified using next-generation sequencing. J Clin Microbiol 52:3101–3104. https://doi.org/10.1128/JCM.01071-14

282. Tuska-Szalay B, Kelly H, Takács N, Kontschán J, Votýpka J, Hornok S (2022) Molecular evidence of *Monocercomonas* and *Acanthamoeba* in the feces of captive reptiles. Parasitol Res 121:3681–3687. https://doi.org/10.1007/s00436-022-07677-3

283. Martín-Pérez T, Criado-Fornelio A, Martínez J, Blanco MA, Fuentes I, Pérez-Serrano J (2017) Isolation and molecular characterization of *Acanthamoeba* from patients with keratitis in Spain. European Journal of Protistology 61:244–252. https://doi.org/10.1016/j.ejop.2017.06.009

284. Ledbetter EC, Capistrano Da Silva E, Dong L, McDonough SP (2023) Experimental induction of acute *Acanthamoeba castellanii* keratitis in cats. Trans Vis Sci Tech 12:10. https://doi.org/10.1167/tvst.12.8.10

285. Ledbetter EC, McDonough SP, Dong L, Liotta JL, Bowman DD, Kim SG (2020) *Acanthamoeba* sclerokeratitis in a cat. javma 257:1280–1287. https://doi.org/10.2460/javma.257.12.1280

286. Freeman LM, Chandler ML, Hamper BA, Weeth LP (2013) Current knowledge about the risks and benefits of raw meat-based diets for dogs and cats. J Am Vet Med Assoc 243:1549–1558. https://doi.org/10.2460/javma.243.11.1549

287. Villagra-Blanco R, Angelova L, Conze T, Schares G, Bärwald A, Taubert A, Hermosilla C, Wehrend A (2018) Seroprevalence of *Neospora caninum*-specific antibodies in German breeding bitches. Parasit Vectors 11:96. https://doi.org/10.1186/s13071-018-2683-1

288. Tuska-Szalay B, Takács N, Kontschán J, Vizi Z, Hornok S (2021) Dogs are final hosts of *Sarcocystis morae* (Apicomplexa: Sarcocystidae): First report of this species in Hungary and its region - Short communication. Acta Vet Hung 69:157–160. https://doi.org/10.1556/004.2021.00017

289. Saari S, Näreaho A, Nikander S (2018) Canine parasites and parasitic diseases. Academic Press

290. Varga I (1976) Experimental transmission of *Eimeria stiedai* to the hare. Acta Vet Acad Sci Hung 26:105–112

291. Bochyńska D, Lloyd S, Restif O, Hughes K (2022) *Eimeria stiedae* causes most of the white-spotted liver lesions in wild European rabbits in Cambridgeshire, United Kingdom. J Vet Diagn Invest 34:199–205. https://doi.org/10.1177/10406387211066923

292. Moré G, Maksimov A, Conraths FJ, Schares G (2016) Molecular identification of *Sarcocystis* spp. in foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) from Germany. Veterinary Parasitology 220:9–14. https://doi.org/10.1016/j.vetpar.2016.02.011

293. Basso W, Alvarez Rojas CA, Buob D, Ruetten M, Deplazes P (2020) *Sarcocystis* infection in red deer *(Cervus elaphus)* with eosinophilic myositis/fasciitis in Switzerland and involvement of red foxes (*Vulpes vulpes*) and hunting dogs in the transmission. International Journal for Parasitology: Parasites and Wildlife 13:130–141. https://doi.org/10.1016/j.ijppaw.2020.09.005

294. Dubey JP, Cawthorn RJ, Speer CA, Wobeser GA (2003) Redescription of the Sarcocysts of *Sarcocystis rileyi* (Apicomplexa: Sarcocystidae). Journal of Eukaryotic Microbiology 50:476–482. https://doi.org/10.1111/j.1550-7408.2003.tb00274.x

295. Rudaitytė-Lukošienė E, Prakas P, Strazdaitė-Žielienė Ž, Servienė E, Januškevičius V, Butkauskas D (2020) Molecular identification of two *Sarcocystis* species in fallow deer (*Dama dama*) from Lithuania. Parasitology International 75:102044. https://doi.org/10.1016/j.parint.2019.102044

296. Hernández Rodriguez S, Navarrete I, Martinez-Gómez F (1981b). *Sarcocystis cervicanis*, nueva especie parasita del ciervo (*Cervus elaphus*) [in Spanish]. Rev. Iber. Parasitol. 41, 43–51.

297. Entzeroth R, Nemeséri L, Scholtyseck E (1983) Prevalence and ultrastructure of *Sarcocystis* sp. from red deer (*Cervus elaphus L.*) in Hungary. Parasit. Hung. 16, 47–52.

298. Rátz I (1909). Protozoa parasitising the muscles and species occurring in the Hungarian fauna [in Hungarian]. Állattani Közl. 8 ,1–37.

299. Beugin M, Salvador O, Leblanc G, Queney G, Natoli E, Pontier D (2019) Hybridization between *Felis silvestris silvestris* and *Felis silvestris catus* in two contrasted environments in France. Ecol Evol 10:263–276. https://doi.org/10.1002/ece3.5892

300. Pierpaoli M, Birò ZS, Herrmann M, Hupe K, Fernandes M, Ragni B, Szemethy L, Randi E (2003) Genetic distinction of wildcat (*Felis silvestris*) populations in Europe, and hybridization with domestic cats in Hungary. Mol Ecol 12:2585–2598. https://doi.org/10.1046/j.1365-294x.2003.01939.x

301. Otranto D, Napoli E, Latrofa MS, Annoscia G, Tarallo VD, Greco G, Lorusso E, Gulotta L, Falsone L, Basano FS, Pennisi MG, Deuster K, Capelli G, Dantas-Torres F, Brianti E (2017) Feline and canine leishmaniosis and other vector-borne diseases in the Aeolian Islands: Pathogen and vector circulation in a confined environment. Vet Parasitol 236:144–151. https://doi.org/10.1016/j.vetpar.2017.01.019

302. Morganti G, Veronesi F, Stefanetti V, Di Muccio T, Fiorentino E, Diaferia M, Santoro A, Passamonti F, Gramiccia M (2019) Emerging feline vector-borne pathogens in Italy. Parasites & Vectors 12:193. https://doi.org/10.1186/s13071-019-3409-8

303. Schäfer I, Kohn B, Nijhof AM, 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

304. Pawar RM, Poornachandar A, Srinivas P, Rao KR, Lakshmikantan U, Shivaji S (2012) Molecular characterization of *Hepatozoon* spp. infection in endangered Indian wild felids and canids. Vet Parasitol 186:475–479. https://doi.org/10.1016/j.vetpar.2011.11.036

305. Tateno M, Nishio T, Matsuo T, Sakuma M, Nakanishi N, Izawa M, Asari Y, Okamura M, Miyama TS, Setoguchi A, Endo Y (2013) Epidemiological survey of tick-borne protozoal infection in Iriomote cats and Tsushima leopard cats in Japan. Journal of Veterinary Medical Science 75:985–989. https://doi.org/10.1292/jvms.13-0015

306. Sakuma M, Setoguchi A, Endo Y (2009) Possible emergence of drug-resistant variants of *Babesia gibsoni* in clinical cases treated with atovaquone and azithromycin. J Vet Intern Med 23:493–498. https://doi.org/10.1111/j.1939-1676.2009.0300.x

307. Miró G, Checa R, Paparini A, Ortega N, González-Fraga JL, Gofton A, Bartolomé A, Montoya A, Gálvez R, Mayo PP, Irwin P (2015) *Theileria annae* (syn. *Babesia microti*-like) infection in dogs in NW Spain detected using direct and indirect diagnostic techniques: clinical report of 75 cases. Parasit Vectors 8:217. https://doi.org/10.1186/s13071-015-0825-2

308. Seneviratna P, Weerasinghe null, Ariyadasa S (1973) Transmission of *Haemobartonella canis* by the dog tick, *Rhipicephalus sanguineus*. Res Vet Sci 14:112–114

309. Hornok S, Kováts D, Horváth G, Kontschán J, Farkas R (2020) Checklist of the hard tick (Acari: Ixodidae) fauna of Hungary with emphasis on host-associations and the emergence of *Rhipicephalus sanguineus*. Exp Appl Acarol 80:311–328. https://doi.org/10.1007/s10493-019-00461-6

310. Imre M, Farkas R, Ilie MS, Imre K, Dărăbuş G (2013) Survey of babesiosis in symptomatic dogs from Romania: occurrence of *Babesia gibsoni* associated with breed. Ticks Tick Borne Dis 4:500–502. https://doi.org/10.1016/j.ttbdis.2013.06.006

311. Beck R, Vojta L, Mrljak V, Marinculić A, Beck A, Zivicnjak T, Cacciò SM (2009) Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. Int J Parasitol 39:843–848. https://doi.org/10.1016/j.ijpara.2008.12.005

312. Tabar M-D, Francino O, Altet L, Sánchez A, Ferrer L, Roura X (2009) PCR survey of vectorborne pathogens in dogs living in and around Barcelona, an area endemic for leishmaniasis. Vet Rec 164:112–116. https://doi.org/10.1136/vr.164.4.112

313. Davitkov D, Vucicevic M, Stevanovic J, Krstic V, Tomanovic S, Glavinic U, Stanimirovic Z (2015) Clinical babesiosis and molecular identification of *Babesia canis* and *Babesia gibsoni* infections in dogs from Serbia. Acta Vet Hung 63:199–208. https://doi.org/10.1556/AVet.2015.017

314. Mitkova B, Hrazdilova K, Novotna M, Jurankova J, Hofmannova L, Forejtek P, Modry D (2017) Autochthonous *Babesia canis*, *Hepatozoon canis* and imported *Babesia gibsoni* infection in dogs in the Czech Republic. Veterinární medicína 62:138–146. https://doi.org/10.17221/152/2016-VETMED

315. Simões PB, Cardoso L, Araújo M, Yisaschar-Mekuzas Y, Baneth G (2011) Babesiosis due to the canine *Babesia microti*-like small piroplasm in dogs-first report from Portugal and possible vertical transmission. Parasit Vectors 4:50. https://doi.org/10.1186/1756-3305-4-50

316. René-Martellet M, Moro CV, Chêne J, Bourdoiseau G, Chabanne L, Mavingui P (2015) Update on epidemiology of canine babesiosis in Southern France. BMC Vet Res 11:223. https://doi.org/10.1186/s12917-015-0525-3

317. Gabrielli S, Otašević S, Ignjatović A, Savić S, Fraulo M, Arsić-Arsenijević V, Momčilović S, Cancrini G (2015) Canine babesioses in noninvestigated areas of Serbia. Vector Borne Zoonotic Dis 15:535–538. https://doi.org/10.1089/vbz.2015.1797

318. Hornok S, Dénes B, Meli ML, Tánczos B, Fekete L, Gyuranecz M, de la Fuente J, de Mera IGF, Farkas R, Hofmann-Lehmann R (2013) Non-pet dogs as sentinels and potential synanthropic reservoirs of tick-borne and zoonotic bacteria. Vet Microbiol 167:700–703. https://doi.org/10.1016/j.vetmic.2013.08.011

319. Sakuma M, Fukuda K, Takayama K, Kobayashi Y, Shimokawa Miyama T, Setoguchi A, Endo Y (2012) Molecular epidemiological survey of the *Babesia gibsoni* cytochrome b gene in western Japan. J Vet Med Sci 74:1341–1344. https://doi.org/10.1292/jvms.12-0140

320. Birkenheuer AJ, Levy MG, Breitschwerdt EB (2004) Efficacy of combined atovaquone and azithromycin for therapy of chronic *Babesia gibsoni* (Asian genotype) infections in dogs. Journal of Veterinary Internal Medicine 18:494–498. https://doi.org/10.1111/j.1939-1676.2004.tb02573.x

321. Garrett KB, Hernandez SM, Balsamo G, Barron H, Beasley JC, Brown JD, Cloherty E, Farid H, Gabriel M, Groves B, Hamer S, Hill J, Lewis M, McManners K, Nemeth N, Oesterle P, Ortiz S, Peshock L, Schnellbacher R, Schott R, Straif-Bourgeois S, Yabsley MJ (2019) Prevalence, distribution, and diversity of cryptic piroplasm infections in raccoons from selected areas of the United States and Canada. International Journal for Parasitology: Parasites and Wildlife 9:224–233. https://doi.org/10.1016/j.ijppaw.2019.05.007

322. Hartelt K, Rieker T, Oehme RM, Brockmann SO, Müller W, Dorn N (2007) First evidence of *Babesia gibsoni* (Asian genotype) in dogs in Western Europe. Vector Borne Zoonotic Dis 7:163–166. https://doi.org/10.1089/vbz.2006.0580

323. Kumar A, Misra P, Dube A (2013) Amplified fragment length polymorphism: an adept technique for genome mapping, genetic differentiation, and intraspecific variation in protozoan parasites. Parasitol Res 112:457–466. https://doi.org/10.1007/s00436-012-3238-6

324. Díaz-Viraqué F, Pita S, Greif G, de Souza R de CM, Iraola G, Robello C (2019) Nanopore sequencing significantly improves genome assembly of the protozoan parasite *Trypanosoma cruzi*. Genome Biology and Evolution 11:1952–1957. https://doi.org/10.1093/gbe/evz129

325. Dia A, Cheeseman IH (2021) Single-cell genome sequencing of protozoan parasites. Trends Parasitol 37:803–814. https://doi.org/10.1016/j.pt.2021.05.013

326. Garcia–R JC, French N, Pita A, Velathanthiri N, Shrestha R, Hayman D (2017) Local and global genetic diversity of protozoan parasites: Spatial distribution of *Cryptosporidium* and *Giardia* genotypes. PLoS Negl Trop Dis 11:e0005736. https://doi.org/10.1371/journal.pntd.0005736

327. Barbosa A, Reiss A, Jackson B, Warren K, Paparini A, Gillespie G, Stokeld D, Irwin P, Ryan U (2017) Prevalence, genetic diversity and potential clinical impact of blood-borne and enteric protozoan parasites in native mammals from northern Australia. Vet Parasitol 238:94–105. https://doi.org/10.1016/j.vetpar.2017.04.007

328. Baneth G, Shkap V (2003) Monozoic cysts of *Hepatozoon canis*. J Parasitol 89:379–381. https://doi.org/10.1645/0022-3395(2003)089[0379:MCOHC]2.0.CO;2

329. Perles L, Roque ALR, D'Andrea PS, Lemos ERS, Santos AF, Morales AC, Machado RZ, André MR (2019) Genetic diversity of *Hepatozoon* spp. in rodents from Brazil. Sci Rep 9:10122. https://doi.org/10.1038/s41598-019-46662-2

330. Thomas R, Santodomingo A, Saboya-Acosta L, Quintero-Galvis JF, Moreno L, Uribe JE, Muñoz-Leal S (2024) *Hepatozoon* (Eucoccidiorida: Hepatozoidae) in wild mammals of the Americas: a systematic review. Parasit Vectors 17:108. https://doi.org/10.1186/s13071-024-06154-3

331. Harris DJ, Halajian A, Santos J, Rampedi KM, 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 90:1747. https://doi.org/10.4102/jsava.v90i0.1747

332. Traversa D, Di Cesare A, Morelli S, Paoletti B, Grillini M, di Regalbono AF, da Silva de Mattos Queiroz A, Beugnet F, Brustenga L, Milillo P, Antunes Barros L (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

333. Tuska-Szalay B, Vizi Z, Hofmann-Lehmann R, Vajdovich P, Takács N, Meli ML, Farkas R, Stummer-Knyihár V, Jerzsele Á, Kontschán J, Szekeres S, Hornok S (2021) *Babesia gibsoni* emerging with high prevalence and co-infections in "fighting dogs" in Hungary. Current Research in Parasitology & Vector-Borne Diseases 1:100048. https://doi.org/10.1016/j.crpvbd.2021.100048

334. Yin F, Guo C, Tian Z, Li D, Mu D, Liu H, Guan G, Yin H, Li F (2023) Analysis of genetic diversity and population structure of *Babesia gibsoni*. Front Vet Sci 10:1147958. https://doi.org/10.3389/fvets.2023.1147958

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

336. Margalit Levi M, Nachum-Biala Y, King R, Baneth G (2018) A survey of *Babesia* spp. and *Hepatozoon* spp. in wild canids in Israel. Parasites & Vectors 11:150. https://doi.org/10.1186/s13071-018-2715-x

337. Tarigo JL, Kelly LS, Brown HM, Peterson DS (2019) Limited genetic variability of *Cytauxzoon felis* apical membrane antigen-1 (ama1) from domestic cats and bobcats. Parasit Vectors 12:115. https://doi.org/10.1186/s13071-019-3347-5

338. Yang TS, Reichard MV, Thomas JE, Marr HS, Karounos M, Hyatt J, Miller C, Birkenheuer AJ (2023) Transmission of *Cytauxzoon felis* by injection of *Amblyomma americanum* salivary glands. Parasitol Int 95:102753. https://doi.org/10.1016/j.parint.2023.102753

339. Hornok S, Takács N, Szekeres S, Szőke K, Kontschán J, Horváth G, Sugár L (2020) DNA of *Theileria orientalis*, *T. equi* and *T. capreoli* in stable flies (*Stomoxys calcitrans*). Parasit Vectors 13:186. https://doi.org/10.1186/s13071-020-04041-1

340. Nguyen AHL, Tiawsirisup S, Kaewthamasorn M (2020) Low level of genetic diversity and high occurrence of vector-borne protozoa in water buffaloes in Thailand based on 18S ribosomal RNA and mitochondrial cytochrome b genes. Infect Genet Evol 82:104304. https://doi.org/10.1016/j.meegid.2020.104304

341. Rosenthal BM, Dunams DB, Pritt B (2008) Restricted genetic diversity in the ubiquitous cattle parasite, *Sarcocystis cruzi*. Infect Genet Evol 8:588–592. https://doi.org/10.1016/j.meegid.2008.04.004

342. Graczyk TK, Knight R, Tamang L (2005) Mechanical transmission of human protozoan parasites by insects. Clin Microbiol Rev 18:128–132. https://doi.org/10.1128/CMR.18.1.128-132.2005

343. Kiss C, Barna Z, Vargha M, Török JK (2014) Incidence and molecular diversity of *Acanthamoeba* species isolated from public baths in Hungary. Parasitol Res 113:2551–2557. https://doi.org/10.1007/s00436-014-3905-x

344. Clarke M, Lohan AJ, Liu B, Lagkouvardos I, Roy S, Zafar N, Bertelli C, Schilde C, Kianianmomeni A, Bürglin TR, Frech C, Turcotte B, Kopec KO, Synnott JM, Choo C, Paponov I, Finkler A, Heng Tan CS, Hutchins AP, Weinmeier T, Rattei T, Chu JSC, Gimenez G, Irimia M, Rigden DJ, Fitzpatrick DA, Lorenzo-Morales J, Bateman A, Chiu CH, Tang P, Hegemann P, Fromm H, Raoult D, Greub G, Miranda-Saavedra D, Chen N, Nash P, Ginger ML, Horn M, Schaap P, Caler L, Loftus BJ (2013) Genome of *Acanthamoeba castellanii*

highlights extensive lateral gene transfer and early evolution of tyrosine kinase signaling. Genome Biology 14:. https://doi.org/10.1186/gb-2013-14-2-r11

345. Maciver SK, Koutsogiannis Z, de Obeso Fernández del Valle A (2019) 'Meiotic genes' are constitutively expressed in an asexual amoeba and are not necessarily involved in sexual reproduction. Biol Lett 15:20180871. https://doi.org/10.1098/rsbl.2018.0871

346. Tuska-Szalay B, Sipos G, Takács N, Kontschán J, Sándor AD, Péter Á, Berta K, Kerek Á, Jerzsele Á, Votýpka J, Hornok S (2022) Molecular epidemiological study of *Trichomonas gallinae* focusing on central and southeastern Europe. Front Vet Sci 9:1050561. https://doi.org/10.3389/fvets.2022.1050561

347. Li WC, Wang K, Gu Y (2018) Occurrence of *Blastocystis* sp. and *Pentatrichomonas hominis* in sheep and goats in China. Parasites & Vectors 11:93. https://doi.org/10.1186/s13071-018-2671-5

348. Tuska-Szalay B, Gilbert J, Takács N, Boldogh SA, Fáy J, Sterczer Á, Psáder R, Kontschán J, Izsó Á, Hornok S (2024) Molecular-phylogenetic investigation of trichomonads in dogs and cats reveals a novel *Tritrichomonas* species. Parasites & Vectors 17:271. https://doi.org/10.1186/s13071-024-06343-0

349. Van der Saag M, McDonell D, Slapeta J (2011) Cat genotype *Tritrichomonas foetus* survives passage through the alimentary tract of two common slug species. Vet Parasitol 177:262–266. https://doi.org/10.1016/j.vetpar.2010.11.054

350. Malik S-B, Pightling AW, Stefaniak LM, Schurko AM, Logsdon JM (2007) An expanded inventory of conserved meiotic genes provides evidence for sex in T*richomonas vaginalis*. PLoS One 3:e2879. https://doi.org/10.1371/journal.pone.0002879

9. Scientific publications

9.1. First authored peer-reviewed publications with impact factor

Tuska-Szalay B, Sipos D, Czabán D, Kalmár Z, Keve G, Szekeres S, Kelemen BS, Sándor AD, Hornok S (2025) Pet and wild rodents as hosts of *Giardia duodenalis* in Central Europe, Hungary. Acta Vet Hung. https://doi: 10.1556/004.2024.01115.

Tuska-Szalay B, Jerzsele Á, Hornok S (2024) Antiprotozoal agents used in veterinary medicine: synopsis [In Hungarian]. MAGYAR ÁLLATORVOSOK LAPJA, 146 (8). pp. 487-500. ISSN 0025-004X. https://doi.org/10.56385/magyallorv.2024.08.487-500

Tuska-Szalay B, Gilbert J, Takács N. Boldogh SA, Fáy J, Sterczer Á, Psáder Á, Kontschán J, Izsó Á, Hornok S (2024) Molecular-phylogenetic investigation of trichomonads in dogs and cats reveals a novel *Tritrichomonas* species. Parasites Vectors 17, 271. https://doi.org/10.1186/s13071-024-06343-0

Tuska-Szalay B, Papdeák V, Vizi Z, Takács N, Hornok S (2024) Parasitological and molecular investigation of consequences of raw meat feeding (BARF) in dogs and cats: implications for other pets living nearby. Parasitol Res. 123(2):114. https://doi: 10.1007/s00436-024-08124-1.

Tuska-Szalay B, Boldogh SA, Farkas R, Rompos L, Takács N, Beresnyák V, Izsó Á, Kontschán J, Lanszki J, Hornok S (2023) Screening of Domestic Cats from North-Eastern Hungary for *Hepatozoon felis* and *Cytauxzoon europaeus* That Cause Infections in Local Wildcat Populations. Pathogens. 12(5):656. https://doi: 10.3390/pathogens12050656.

Tuska-Szalay B, Sipos G, Takács N, Kontschán J, Sándor AD, Péter Á, Berta K, Kerek Á, Jerzsele Á, Votýpka J, Hornok S (2022) Molecular epidemiological study of *Trichomonas gallinae* focusing on central and southeastern Europe. Front Vet Sci. 9:1050561. https://doi: 10.3389/fvets.2022.1050561.

Tuska-Szalay B, Kelly H, Takács N, Kontschán J, Votýpka J, Hornok S (2022) Molecular evidence of *Monocercomonas* and *Acanthamoeba* in the feces of captive reptiles. Parasitol Res. 121(12):3681-3687. https://doi: 10.1007/s00436-022-07677-3.

Tuska-Szalay B, Vizi Z, Hofmann-Lehmann R, Vajdovich P, Takács N, Meli ML, Farkas R, Stummer-Knyihár V, Jerzsele Á, Kontschán J, Szekeres S, Hornok S (2021) *Babesia gibsoni* emerging with high prevalence and co-infections in "fighting dogs" in Hungary. Curr Res Parasitol Vector Borne Dis. 1:100048. https://doi: 10.1016/j.crpvbd.2021.100048.

Tuska-Szalay B, Takács N, Kontschán J, Vizi Z, Hornok S (2021) Dogs are final hosts of *Sarcocystis morae* (Apicomplexa: Sarcocystidae): First report of this species in Hungary and its region - Short communication. Acta Vet Hung. 69(2):157-160. https://doi: 10.1556/004.2021.00017.

9.2. Last authored peer-reviewed publications with impact factor

Hornok S, Keve G, **Tuska-Szalay B** (2025) Transmission route-dependent genetic diversity of selected protozoan parasites as reflected by the phylogenetic analysis of the 18S rRNA gene. Acta Vet Hung. https://doi.org/10.1556/004.2025.01128

Hornok S, Boldogh SA, Takács N, Sándor AD, **Tuska-Szalay B** (2022) Zoonotic ecotype-I of *Anaplasma phagocytophilum* in sympatric wildcat, pine marten and red squirrel - Short communication. Acta Vet Hung. https://doi: 10.1556/004.2022.00021.

Hornok S, Boldogh SA, Takács N, Kontschán J, Szekeres S, Sós E, Sándor AD, Wang Y, **Tuska-Szalay B** (2022) Molecular epidemiological study on ticks and tick-borne protozoan parasites (Apicomplexa: *Cytauxzoon* and *Hepatozoon* spp.) from wild cats (*Felis silvestris*), Mustelidae and red squirrels (*Sciurus vulgaris*) in central Europe, Hungary. Parasit Vectors. 15(1):174. https://doi: 10.1186/s13071-022-05271-1.

9.3. Co-authored peer-reviewed publications with impact factor

Hornok S, Daccord J, Takács N, Kontschán J, **Tuska-Szalay B**, Sándor AD, Szekeres S, Meli ML, Hofmann-Lehmann R (2022) Investigation on haplotypes of ixodid ticks and retrospective finding of *Borrelia miyamotoi* in bank vole (*Myodes glareolus*) in Switzerland. Ticks Tick Borne Dis. ;13(1):101865. doi: 10.1016/j.ttbdis.2021.101865.

Kerek Á, Csanády P, **Tuska-Szalay B**, Kovács L, Jerzsele Á (2023) In Vitro Efficacy of Hungarian Propolis against Bacteria, Yeast, and *Trichomonas gallinae* Isolated from Pigeons—A Possible Antibiotic Alternative? Resources. 12(9):101. https://doi.org/10.3390/resources12090101

9.4. Co-authored publications under review

Szekeres S, Takács N, Ózsvári L, **Tuska-Szalay B**, Bárdos K, Kerek Á, Dobra P, Kovács L, Keve G, Molnár-Nagy V, Bata Zs, Hornok S (2025) Molecular investigation of hindgut flagellates from turkeys and pheasants in Hungary confirms the endemicity of a new species closely related to *Histomonas meleagridis.*

Kerek Á, Yurt A, Szabó Á, **Tuska-Szalay B**, Jerzsele Á (2025) In vitro sensitivity of *Tritrichomonas foetus* strains to propolis and nitroimidazoles.

10. Supplements

Supplementary Table 1: Various reptile (and one amphibian) species tested in this study.

order	suborder	(super)family	English name of species	Latin name of species	sample number
TESTUDINES	Cryptodira	Chelydridae	Alligator snapping turtle	*Macrochelys temminckii*	HK69
		Testudinidae	Leopard tortoise	*Stigmochelys pardalis*	HK2, HK10, HK17, HK53
			Red footed tortoise	*Chelonoidis carbonaria*	HK4, HK36, HK38, HK56, HK61, HK62, HK87
			Pancake tortoise	*Malacochersus tornieri*	HK7, HK96
			Yellow footed tortoise	*Chelonoidis denticulata*	HK66, HK81
	Pleurodira	Chelidae	Roti Island snakeneck turtle	*Chelonia mccordi*	HK32
SQUAMATA	Sauria	Gekkonidae	Leopard gecko	*Eublepharis macularius*	HK33, HK34, HK37, HK98, HK99
			Crested gecko	*Rhacodactylus ciliatus*	HK35, HK60, HK65, HK14
			Henkel's leaf-tailed gecko	*Uroplatus henkeli*	HK85
	Scincomorpha	(Scincoidea)	Blue tongued skink	*Tiliqua scincoides*	HK15, HK8
	Autarchoglossa	Helodermatidae	Gila monster	*Heloderma suspectum**	HK23, HK75, HK97 (*housed together)
			Beaded lizard	*Heloderma horridum**	
		Gerrhosauridae	Sudan plated lizard	*Gerrhosaurus major*	HK3, HK71, HK76
		Teiidae	Black and white tegu	*Salvator merianae*	HK40, HK49, HK88
		Varandiae	Bosc monitor	*Varanus exanthematicus*	HK24, HK45
			Rough-necked monitor	*Varanus rudicollis*	HK63
			Asian water monitor	*Varanus salvator*	HK11

	Iguania	Iguanidae	Rhinoceros iguana	*Cyclura coronuta*	HK13, HK16, HK48, HK70, HK79, HK89
			Fiji banded iguana	*Brachylophus bulabula*	HK19
			Spiny tailed iguana	*Ctenosaura pectinata*	HK22, HK77
			Green iguana	*Iguana iguana*	HK52, HK5, HK72, HK88, HK91
			Casquehead iguana	*Laemanctus serratus*	HK68
			Desert iguana	*Dipsosaurus dorsalis*	HK93
		Agamidae	Indian garden lizard	*Calotes versicolor*	HK39, HK55, HK27
			Bearded dragon	*Pogona vitticeps*	HK9
			Frilled dragon	*Chlamydosaurus kingii*	HK6, HK8, HK25, HK42
			Australian water dragon	*Intellagama lesururii*	HK18, HK57
			Striped water dragon	*Tropicagama temporalis*	HK54, HK50, HK44, HK59
			Asian water dragon	*Physignathus cocincinus*	HK94
			Sulawesi sailfin lizard	*Hydrosaurus celebensis*	HK73, HK90
	Serpentes	Boidae	Boa constrictor	*Boa constrictor*	HK30, HK12, HK31, HK86
			Yellow anaconda	*Eunectes notaeus*	HK51
			Colombian rainbow boa	*Epicrates maurus*	HK92
		Pythonidae	Royal python	*Python reguis*	HK21, HK41, HK64, HK74
			Burmese python	*Python bivittatus*	HK26, HK67, HK80
			Carpet python	*Morelia spilota*	HK28, HK43
			Spotted python	*Antaresia maculosa*	HK84
		Colubridae	Milk snake	*Lampropeltis triangulum*	HK1
			California kingsnake	*Lampropeltis californiae*	HK20, HK29, HK78
			Gopher snake	*Pituophis catenifer*	HK46, HK83
		Elapidae	Monocled cobra	*Naja kaouthia*	HK95
	Anguimorpha	Anguidae	Sheltopusik	*Pseudopus apodus*	HK82
CROCODYLIA	Alligatorinae	Alligatoridae	Chinese alligator	*Alligator sinensis*	HK58
ANURA		Pelodryadidae	White-lipped tree frog	*Litoria infrafrenata*	HK47

Supplementary Table 2. PCR reaction components in the study.

Parasite/target gene/	Reaction mixture						DNA
	1.0 U HotStar Taq Plus DNA Polymerase*	dNTP mix	Primers	10×Coral Load PCR buffer**	extra MgCl2	PCR grade water	
T. gallinae /18S rRNA/	0.2 µl	0.5 µl	1 µl	2.5 µl	1 µl	14.8 µl	5 µl
T. gallinae /alpha-tubulin/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Trichomonadida /short and long fragment of 18S rRNA/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Trichomonadida /ITS2/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Acanthamoeba /18S rRNA/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Neospora Toxoplasma Cystoisospora spp. (COI/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Neospora sp. /NC5/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Toxoplasma sp. /repeat region/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Piroplasms *Sarcocystis* spp. /18S rRNA/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Hepatozoon spp./18S rRNA/	0.2 µl	0.5 µl	0.4 µl	2.5 µl	1 µl	17.8 µl	
Cytauxzoon spp. /*cytb*/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Babesia gibsoni /*cox1*/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	
Babesia gibsoni /*cytb*/	0.2 µl	0.5 µl	1 µl	2.5 µl	-	15.8 µl	

*Qiagen, Hilden, Germany, **15 mM MgCl2 included

10. Acknowledgements

First, I would like to express my sincere gratitude to my supervisor, Prof. Sándor Hornok. I was able to complete my studies and write my dissertation because of his direction, assistance and patience. I would like to also express my gratitude to my colleagues in the Department of Parasitology and Zoology at the University of Veterinary Medicine Budapest for their assistance with this study, especially to Nóra Takács, whose precise work greatly contributed to achieving our goals. Additionally, I would want to express my gratitude to my family, my husband and my children for their unwavering support and patience as I conducted my research and wrote my dissertation. Last but not least, I would like to thank the indispensable contributions of those who, under my organization, participated in sample collections, i.e., Sándor Boldogh, Judit Kisgergely, Zsolt Szentgáli, Roland Psáder, Ágnes Sterczer, Zsuzsanna Vizi, József Fáy, Viktor Beresnyák, Dávid Czabán, Ditta Sipos, Gábor Sipos, Viktória Papdeák, Hannah Kelly, Julia Gilbert and all others who are acknowledged as co-authors or listed in the publications.