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

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Acta Veterinaria Hungarica

73 (2025) 1, 64–72

DOI:
10.1556/004.2025.01128

Received: 11 September 2024 • Revised manuscript received: 23 January 2025 • Accepted: 23 January 2025
Published online: 27 March 2025

RESEARCH ARTICLE

ABSTRACT

In this pilot study, the genetic diversity of protozoan parasites was analysed according to their different transmission routes (life cycle strategies), focusing on those species which were recently discovered or molecularly analysed for the first time in Hungary or its geographical region. The results showed that among four apicomplexan parasites (*Babesia gibsoni*, *Cytauxzoon europaeus*, *Sarcocystis morae* and *Hepatozoon 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 (*Trichomonas gallinae*, *Pentatrichomonas hominis*, *Tritrichomonas foetus* and *Acanthamoeba 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.

KEYWORDS

Babesia, *Cytauxzoon*, *Sarcocystis*, *Hepatozoon*, trichomonads, *Acanthamoeba*, SSU rDNA

INTRODUCTION

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 (Barrett et al., 2008). Life history traits determine genetic diversity and thus the evolutionary potential of host–parasite interactions. Genetic diversity is crucial for the parasites, ensuring higher chances of host-adaptation and survival in the course of their evolution (Hughes, 1991; Barrett et al., 2008). 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 (Franssen et al., 2021), 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 (Stumpf, 2004).

Among most protozoan parasites, there is evidence for a role of frequent, although not obligate, genetic exchange, which enhances parasite diversity (Ramírez and Llewellyn, 2014).

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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 (Walliker, 1989). 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 (Bradic and Carlton, 2018). 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 (Feretzaki and Heitman, 2013). 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 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 (Schurko et al., 2009). Homologous recombination is critical to maintain genome stability and to ensure genetic diversity during meiosis (Kelso et al., 2017). It is also known that genetic diversity in vector-borneprotozoan populations depends on transmission dynamics (Gwarinda et al., 2021; Kwiatkowski, 2024).

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 analysed with molecular biological tools for the first time, providing new molecular data in an international context (Hornok et al., 2022; Tuska-Szalay et al., 2021a, 2021b, 2022, 2024a, 2024b). Utilizing this opportunity, we initiated this pilot study, to estimate the transmission route-dependent, 18S rRNA gene diversity of selected protozoan parasites, focusing on species that have been reported recently in Hungary. To test if this theory is valid in a broader context, it will be necessary to broaden the scope by including protozoa with not yet available genetic data in future analyses.

MATERIALS AND METHODS

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, generally stenoxenous apicomplexan parasites: *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 is an opportunistic parasite but is not shed from its host.

The slowly evolving 18S rRNA gene was chosen for assessing the genetic diversity of these species, because it is conservative enough to allow simultaneous comparison of phylogenetically distant species. Longer (>1,200 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. DNA 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 1,360 bp for apicomplexan, and 1,706 bp for non-apicomplexan protozoa. All positions containing gaps and missing data were eliminated. The evolutionary history was inferred using the neighbour-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 (Figs 1 and 2). Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

RESULTS

Among the four heteroxenous apicomplexan parasites phylogenetically analysed here (Figs 1 and 3A), *H. felis* had the highest rate of genetic diversity (maximum p-distance: 29/1,654 bp = 1.75%), followed by *B. gibsoni* (21/1,579 bp = 1.33%). The genetic diversity was low among 18S rRNA gene sequences of *S. morae* (3/1,808 bp = 0.17%) and *C. europaeus* (1/1,227 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 (Fig. 3A).

Regarding homoxenous, non-apicomplexan protozoa (Figs 2 and 3B), the highest rate of genetic diversity belonged to the free-living amoeba, *A. castellanii* (maximum p-distance: 180/2,328 bp = 7.73%), and this value was much lower in the case of *T. gallinae* (13/1,570 bp = 0.83%) and *T. foetus* (5/1,462 bp = 0.34%), the lowest belonging to *P. hominis* (2/1,500 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 (Fig. 3B).

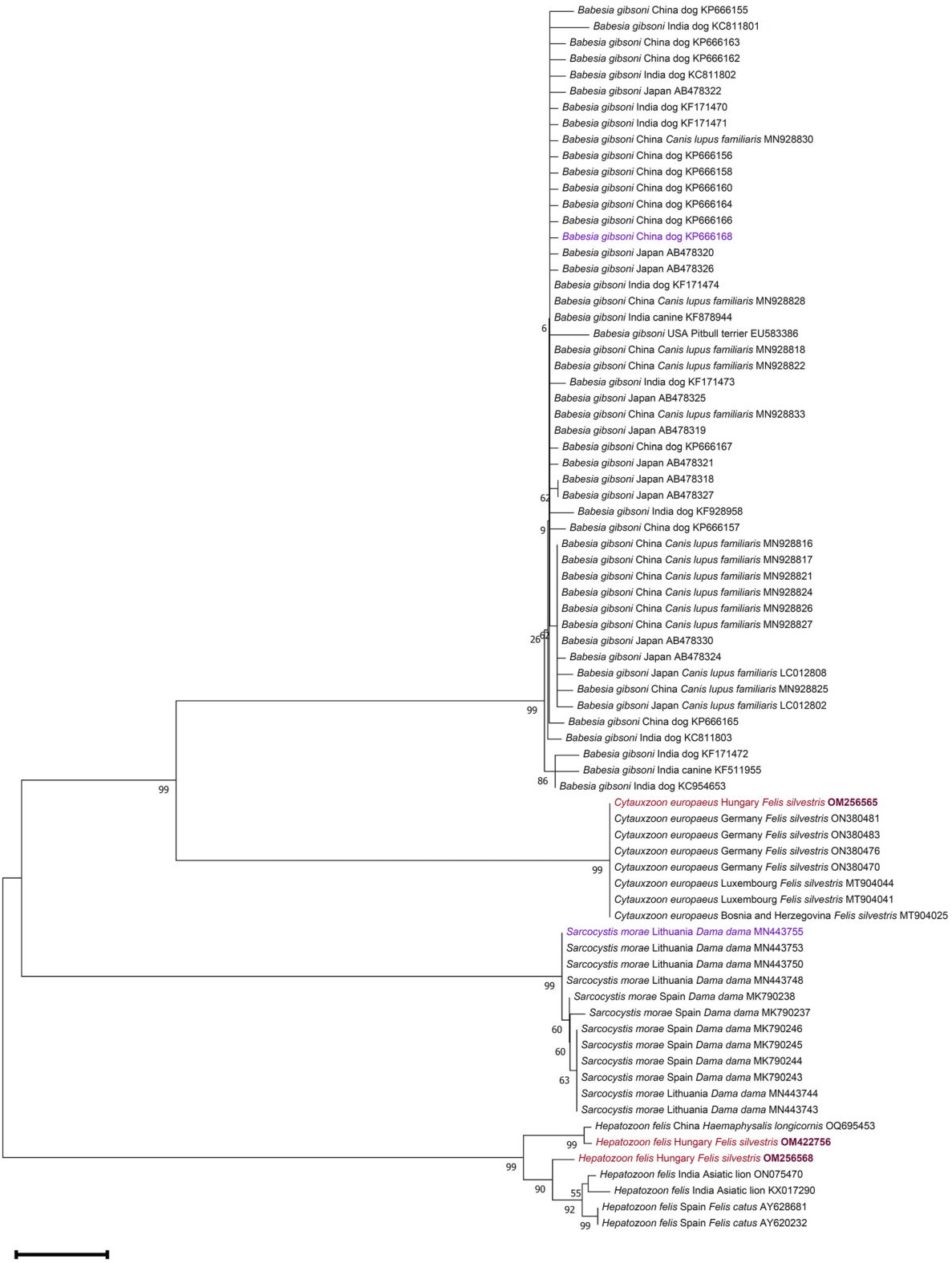

Fig. 1. Phylogenetic tree of selected species from the phylum Apicomplexa based on the 18S rRNA gene, made with the neighbour-joining method and the p-distance model. In each row, after the species name, the country of origin, the generic or Latin name of the isolation source and the GenBank accession number are shown. Sequences from Hungary are indicated with red fonts and maroon, bold accession numbers. When only relatively short (<500 bp) sequences were available from Hungary, the closest (100%) match from GenBank was included in purple. The analyses involved 76 sequences and 1,360 positions. Evolutionary analyses were conducted in MEGA11

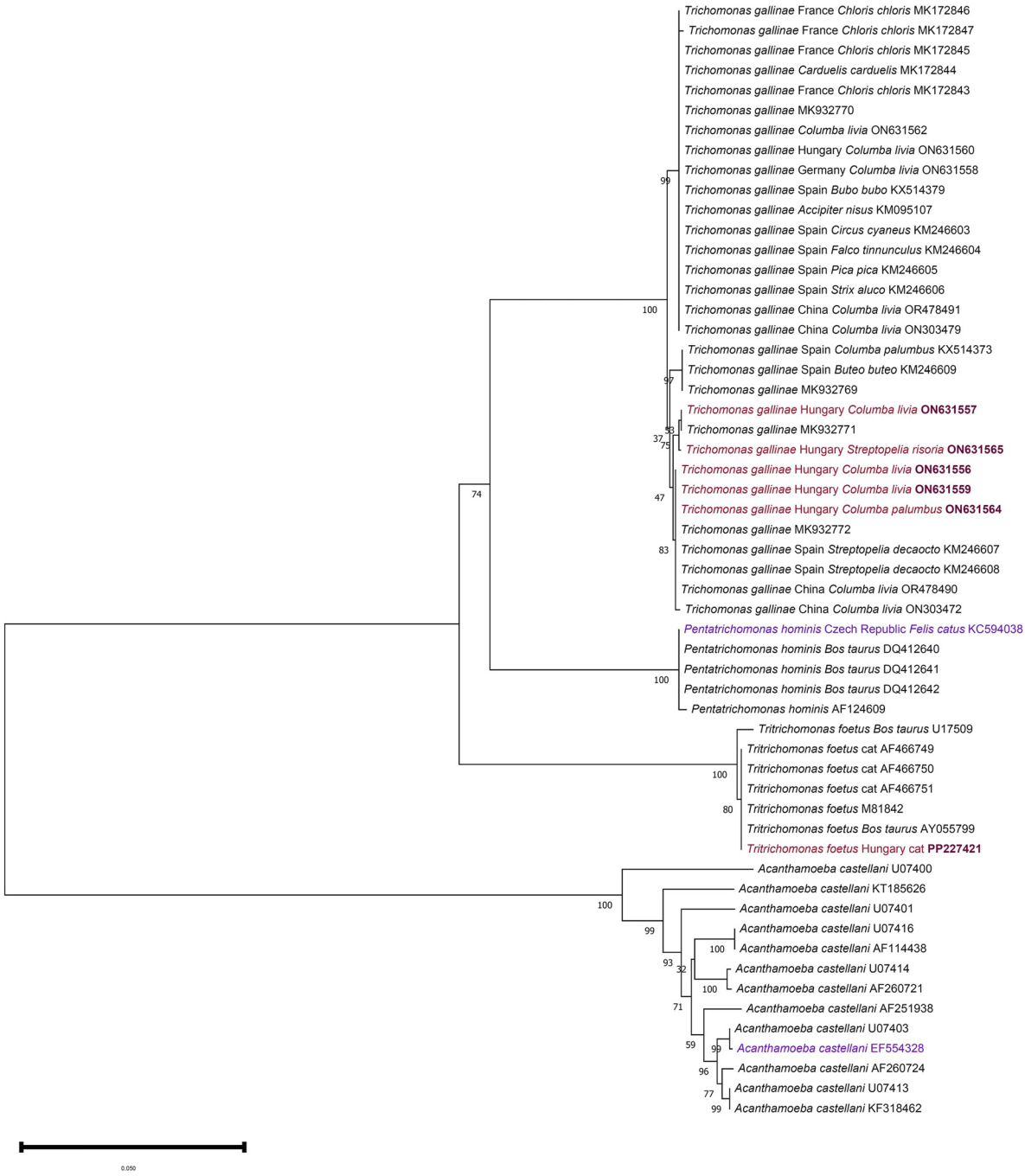

Fig. 2. Phylogenetic tree of selected species from the phyla Parabasalia and Amoebozoa based on the 18S rRNA gene, made with the neighbour-joining method and the p-distance model. In each row, after the species name, the country of origin, the generic or the Latin name of the isolation source and the GenBank accession number are shown. Sequences from Hungary are indicated with red fonts and maroon, bold accession numbers. When only relatively short (<500 bp) sequences were available from Hungary, the closest (99.5–100%) match from GenBank is shown in purple. The analyses involved 56 sequences and 1,706 positions. Evolutionary analyses were conducted in MEGA11

DISCUSSION

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 (Kumar et al., 2013), later nanopore sequencing (Díaz-Viraqué et al., 2019) and single-cell genome sequencing (Dia and Cheeseman, 2021), PCR amplification and Sanger sequencing of the highly conserved 18S rRNA gene across a broad taxonomic range of protozoa seems to be still unexplored. To serve as an initiative, this study focused on those unicellular parasites

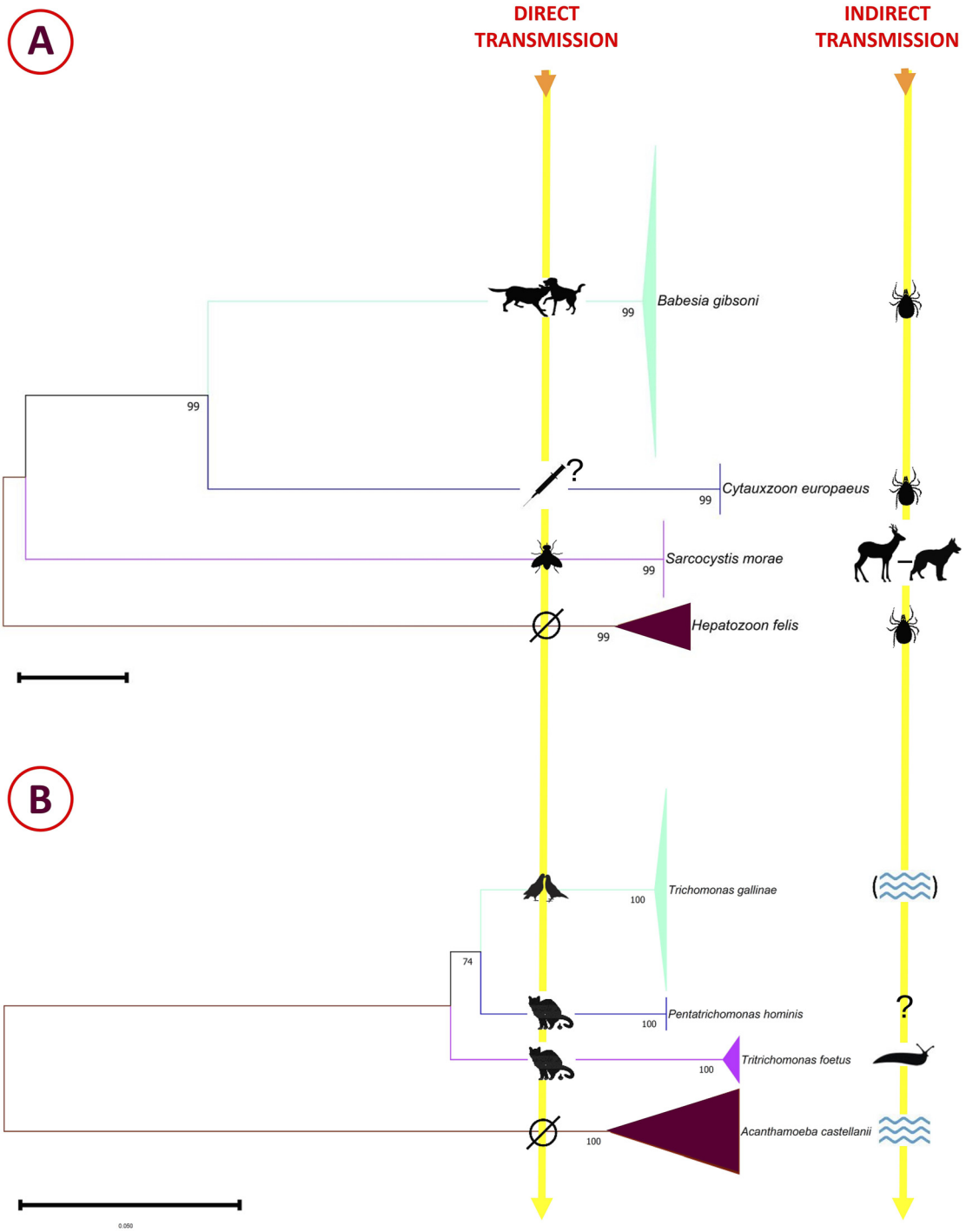

Fig. 3. Phylogenetic trees of selected protozoan parasites (A. phylum Apicomplexa, B. phyla Parabasalia and Amoebozoa) based on the 18S rRNA gene. The evolutionary history was inferred using the neighbour-joining method and the p-distance model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown below the branches. The clades are 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 1,360 positions, or (B) 56 sequences and 1,706 positions. Evolutionary analyses were conducted in MEGA11

that have been recently discovered and analysed 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. In agreement with former studies (e.g., Garcia-R et al., 2017), we speculated that there were 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 (Barbosa et al., 2017).

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 (Baneth and Shkap, 2003). *Hepatozoon 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 this species, infections from common infectious sources (that would promote the spread of identical genotypes) are rare or non-existent.

Hepatozoon felis is probably a parasite with long evolutionary history: among haemogregarines, this species usually belongs to the most basal clade of *Hepatozoon* spp. (Perles et al., 2019; Thomas et al., 2024), as was also shown here in a broader taxonomic context, i.e., *H. felis* had the longest evolutionary distance and the 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 had also been postulated that *H. felis* may be a complex of two species (Harris et al., 2019; Hornok et al., 2022; Traversa et al., 2024).

Genetic diversity has been found to be lower for *B. gibsoni* that has a dividing blood-form and is well-known to spread directly (mechanically) among fighting dogs (Tuska-Szalay et al., 2021a), in addition to the 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 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, overall nucleotide diversity of *B. gibsoni* was only 0.22–0.23% in populations from four continents, revealing the low genetic variation among *B. gibsoni* populations (Yin et al., 2023). As reported, this diversity was much lower than that of *H. canis* (Vasquez-Aguilar et al., 2021). In turn, the genetic variation in *H. canis* has been reported to be lower than in the cases of *H. americanum* and *H. felis* (Margalet Levi et al., 2018). 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 (Tarigo et al., 2019), as demonstrated here and in previous studies of the latter species in Europe (Panait et al., 2021; Hornok et al., 2022). Clinical cytauxzoonosis can be induced through injection of tissue or blood harvested from cats with acute cytauxzoonosis (Yang et al., 2023), implying the potential role of mechanical (iatrogenic) spread, as these piroplasms multiply in the blood (Wikander and Reif, 2023). *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 (Hornok et al., 2020), even iatrogenically, contributing to their low genetic diversity (Nguyen et al., 2020).

The restricted local genetic diversity of *Sarcocystis* spp. infecting ruminants is also well documented, as exemplified by *Sarcocystis cruzi* (Rosenthal et al., 2008) 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 (Tuska-Szalay et al., 2021b). Mechanical transport hosts of *Sarcocystis* sporocysts are insects (Graczyk et al., 2005), but these may spread the infection between different host types, from the final to the intermediate hosts, while 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 from Hungary (Kiss et al., 2014) and elsewhere, was explained by extensive lateral gene transfer and was also related to the long evolutionary history (Clarke et al., 2013). 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 (Maciver et al., 2019). Importantly, direct transmission of acanthamoebae between hosts is unlikely, therefore this cannot serve widespread genetic homogeneity.

On the contrary, based on published data, in case of the directly transmitted *T. gallinae*, very low intraspecific sequence divergence has been detected, including samples from Hungary (Tuska-Szalay et al., 2022), and also in a broader geographical context (Gerhold et al., 2008). Regionally occurring *P. hominis* also showed minimal differences in the 18S rRNA gene (Li et al., 2018), 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 (Li et al., 2016; Tuska-Szalay et al., 2024b). Mechanical transport hosts are probably slugs (Van der Saag et al., 2011). 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 (Malik et al., 2007).

Due to the lack of relatively long 18S rRNA gene sequences in GenBank for most parasitic protozoa, not all apicomplexan taxa could be included here. Based on the eight analysed apicomplexan and non-apicomplexan species, both direct and indirect (transstadial and transovarial tick-borne) transmission modes were represented. Although preliminarily, it was proved that 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.

ACKNOWLEDGEMENT

This study was conducted in the frame of research funded by the Office for Supported Research Groups, Hungarian Research Network (HUN-REN), Hungary (Project No. 1500107).

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